The application of capillary electrophoresis and mass spectrometry to clinical and environmental problems

Thesis

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The application of capillary electrophoresis and mass spectrometry to clinical and environmental problems.

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This thesis is dedicated to my Father,

my friend and inspiration.

Abstract

Using capillary electrophoresis (CE) as a separation technique has allowed analytes, previously difficult to separate by standard methods because they did not conform to requirements for GC or HPLC, to be separated with speed and great efficiency. The only requirements for CE analysis are that the sample is soluble in a liquid matrix and that analytes are present as positive or negative ions whilst in this matrix. This technique has been used here to analyse both clinical and environmental samples, some as cations and others boron-containing complexes as anions. Samples were analysed using a combination of CE alone, mass spectrometry alone and also coupled capillary electrophoresis/electrospray mass spectrometry (CE/ES). Clinically orientated analytes, dipeptides in urine and acylcarnitines from blood spots were examined and peaks detected directly via uv absorbance. The environmental samples analysed included those which contained chromophoric or non-chromophoric herbicides as well as those containing diisocyanates. Analytes were either detected in their native form as with the dipeptides and chromophoric herbicides, or more typically after derivatisation to improve their absorbance characteristics. The exception was the non-chromophoric herbicides which were detected via indirect uv.

CE was an experimental technique for the analysis of all these compounds, except for the dipeptides, all the others having originally been analysed using HPLC or GC methods. In each case an evaluation of the CE method was performed to determine the suitability of the method. By analysing standards in each case, it was possible to confirm that the technique was suitable for qualitative and quantitative analysis of each class of compound.

CE proved to be a viable technique for the separation of all classes of compound dealt with in this thesis. However the method could not be relied upon to confirm the identity of these analytes by their migration time alone. To identify the analytes, experiments were carried out to couple CE with a mass spectrometer. Two techniques of mass spectrometry were used within this thesis, fast atom bombardment and electrospray but only electrospray ionisation mass spectrometry was used to couple to capillary electrophoresis and was the only mass spectrometric technique used to analyse clinical and environmental samples. CE instruments were successfully coupled to an electrospray mass spectrometer which then

became the detector. Mass/charge ratio measurements were obtained for each analyte used and these allowed the unambiguous identification of each analyte.

Other work involved using CE, ES and FAB mass spectrometry, to develop a new technique to detect diol containing compounds. This involved complexing the diol with a boron-containing acid to produce an anion which could then be detected using ES and FAB mass spectrometric methods. This work was viewed as a possible technique for the detection of diol containing lipids found within some body fluids.

Abbreviations used in this thesis.

ATP - Adenosine triphosphate.

BPI - Base peak intensity.

CE/ES - Capillary electrophoresis/electrospray mass spectrometry.

CE-MS - Capillary electrophoresis - Mass spectrometry.

CF-FAB - Continuous flow fast atom bombardment mass spectrometry.

CGE - Capillary Gel Electrophoresis.

CI - Chemical ionisation.

CITP-MS - Capillary isotachophoresis mass spectrometry.

CZE - Capillary Zone Electrophoresis.

DMSO - Dimethyl sulphoxide.

DNA - Deoxyribose nucleic acid.

DPBA - Diphenylborinic acid.

Eof - Electro-osmotic flow.

ESMS - Electrospray mass spectrometry.

FAB - Fast atom bombardment.

FABMS - Fast atom bombardment mass spectrometry.

FITC - Fluorescein isothiocyanate.

GC/MS - Gas chromatography/Mass spectrometry.

HDI - Hexamethylene diisocyanate.

HEGDME - Hexaethylene glycol dimethyl ether.

HPLC - High Performance Liquid Chromatography.

HSE - Health and Safety Executive.

IEF - Iso-electric focusing.

IPD - Indirect photometric detection.

IPDI - Isophorone diisocyanate.

ITP - Isotachophoresis.

LC-MS - Liquid Chromatography - Mass Spectrometry.

LOD - Limit of detection.

MCADD - Medium chain acyl-CoA dehydrogenase deficiency.

MDI - 4,4'- diphenylmethane diisocyanate.

MEKC - Micellar Electrokinetic Chromatography.

MPD - 3-methoxy-1,2-propandiol.

MPP - Methoxyphenyl piperazine.

MS/MS - Mass spectrometry/Mass spectrometry.

NDA - Naphthalenedicarboxylaldehyde.

NDI - 1,5-naphthylene diisocyanate.

NMI - N-methyl-1-naphthalenemethylamine.

NMR - Nuclear magnetic resonance.

PEG - Poly(ethyleneglycol).

QUATS - Quaternary ammonium species.

RSD - Relative standard deviation.

SDS - Sodium Dodecyl Sulphate.

TDI - Toluene diisocyanate.

TEGDEE - Tetraethyleneglycol diethyl ether.

TLC - Thin layer chromatography.

TMAHCl - Trimethyl ammonium hydrochloride.

TMCS - Trimethylchlorosilane.

TMVAH - Trimethyl vinyl ammonium hydroxide.

TOF - Time of flight.

TTAB - Tetradecyltrimethyl ammonium bromide.

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Chapter One

Introduction.

1. Electrophoresis - early work.

From its introduction to the present day the principle of electrophoresis has remained the same, that of the separation of charged molecules based on their differential migration in an applied electric field. The technique was first described in 1930 in the doctoral thesis of Arne Tiselius and published in the literature in 1937. The development and use of the electrophoretic technique has remained in the hands of biologists and biochemists until more recent times. Traditional electrophoresis proved to be a slow, labour-intensive technique, prone to poor reproducibility and limited quantitative capability and not easily amenable to automation[1]. This meant that high performance liquid chromatography (HPLC) was always the method chosen for quantitative analysis. Early electrophoresis work concentrated on the use of gels which, in the slab form, have been used primarily for the size-dependent separation of biological macromolecules, such as polynucleotides[2] and proteins. Gels act as size excluders which help to separate molecules in terms of their molecular size by a sieving action. Other important uses of gels are as anchoring points for the buffering ions, allowing pH gradients, as well as providing the user with a convenient method for analysis of the results in the form of slabs and strips of gel. Starch and cellulose acetate gels have been used but nowadays polyacrylamide gels, in conjunction with sodium dodecyl sulphate (SDS)[3] are more favourable especially for protein separations . As well as SDS, other stabilisers were originally added to the gels to suppress the thermal convection currents produced at the high voltages used.

Electrophoresis has now become of greater interest to the analytical chemist as the biotechnology industry has become more and more involved with larger biomolecules and mixtures of these. This interest has co-incided with the development of capillary techniques which have substantially improved the range and quantitative capabilities of electrophoresis. This has been the result of extensive work using open-tubular capillaries initially described by Hjerten[4] in 1967. Only millimetre-bore capillaries were available at this time which created problems with convection currents and joule heating. When silica capillaries of

75 μm internal diameter became available it was Jorgenson and Lukacs[5] who advanced the technique. They also clarified much of the theory and demonstrated the potential of capillary electrophoresis as an analytical technique[6]. Since their initial work various reviews have been published[7-11], each with their own slant on the latest developments in capillary electrophoresis which allow increasingly wide application in biochemical and chemical science. These developments mean that electrophoresis is no longer limited to the separation of macromolecules and can also be used to separate cations, anions, and neutral molecules in a single analysis.

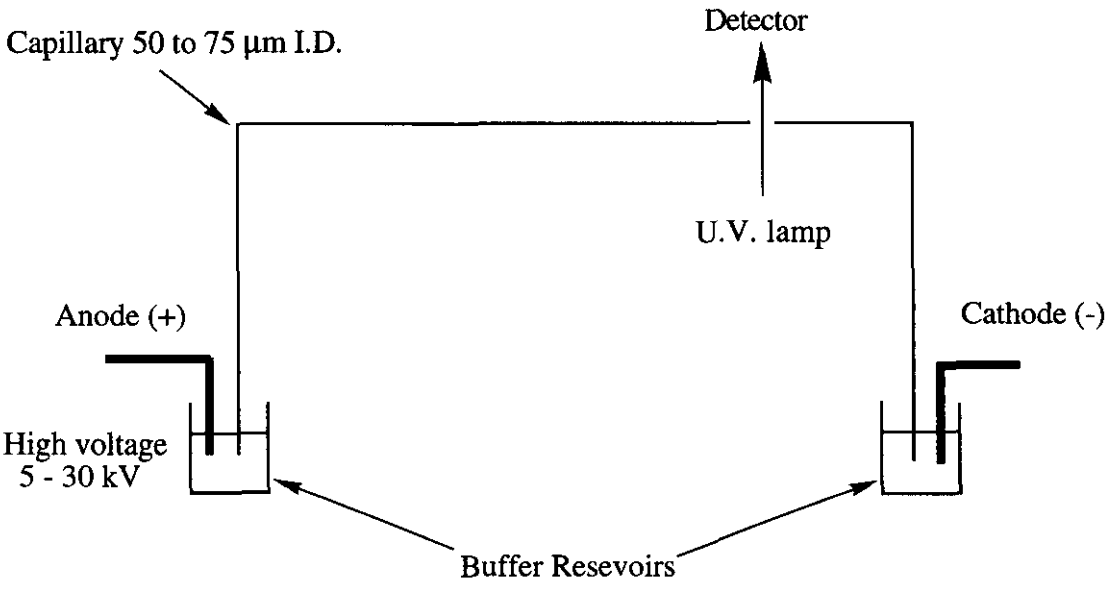
2. Capillary Electrophoresis.

Capillary electrophoresis as a term now encompasses more than one actual technique. The variety has sprung from the need to be able to separate a wider range of molecules, including neutral substances, at much lower concentrations than were originally amenable to electrophoresis. There are five primary electrophoretic techniques used: capillary zone electrophoresis (CZE) or free zone electrophoresis, micellar electrokinetic chromatography (MEKC), isotachophoresis (ITP), iso-electric focusing (IEF) and capillary gel electrophoresis (CGE). The only technique used in this thesis is that of CZE.

2. (1). Capillary Zone Electrophoresis.

A diagrammatical representation of the apparatus required for this technique is shown in Figure 1 below. The sample is introduced via one of a number of injection procedures into a polyimide-coated silica capillary, frequently having an internal diameter of 50 or 75 μm, which is filled with an aqueous buffer solution. This usually takes place at the anode (+) end of the capillary. Once introduced, both the anode and cathode ends of the capillary are placed in vials of buffer solution and a high potential, typically 5-30 kV is applied across the column.

Figure 1. Schematic of capillary electrophoresis apparatus.



Cationic molecules within the sample will then migrate towards the cathode (-), and the anions in the opposite direction towards the anode, the point at which they were initially injected. However, due to an overall flow in the direction of the cathode, all ions and neutral species within the capillary will eventually elute at the cathode end. This net flow towards the cathode is termed as electro-osmotic flow (Eof) and will be described briefly later. Separation is affected on the basis of the mass, charge, size, shape and hydrophobicity of each individual ion or molecule.

2. (1)a. Special features of capillary electrophoresis.

The use of such small bore capillaries, sizes of 75 - 20 µm i.d. and below have now been employed, is advantageous in performing electrophoresis due to the reductions possible in heating effects[12] which have long plagued traditional electrophoresis. Heating can cause non-uniform temperature gradients, local changes in viscosity and hence zone broadening. The application of the voltages used, (5-30 kV) to a liquid-filled capillary generates heat by the passage of an electrical current. This effect is called Joule heating. Micro-bore capillaries ensure that small volumes are accommodated which then limits the heat generated and also the high inner surface-area-to-volume ratio helps to dissipate the generated heat through the capillary wall. The outer diameter, typically 375 µm, is also advantageous because it is sufficiently large to negate the insulating properties of the polyimide coating, hence

improving heat dissipation to the surroundings. Manufacturers have further addressed this problem by introducing their own temperature controls, eg. Beckman Instruments have enclosed the capillary in a cartridge around which a cooling fluid can be circulated, the temperature of which can be strictly controlled between 20^{0} and 50^{o}C. Temperature regulation is usually performed to lengthen or shorten migration times by increasing or decreasing the viscosity of the buffers used. But some buffers change pH with temperature and, as they do so, effect the extent to which the analytes dissolved within them become ionised. Gradients of pH and temperature have been used to improve separation rather like gradient elution in HPLC[13]. The importance of temperature programming is emphasized in reports of separations of thermally labile microbiological nucleases[14] and large proteins[15].

2. (1)b. Buffers.

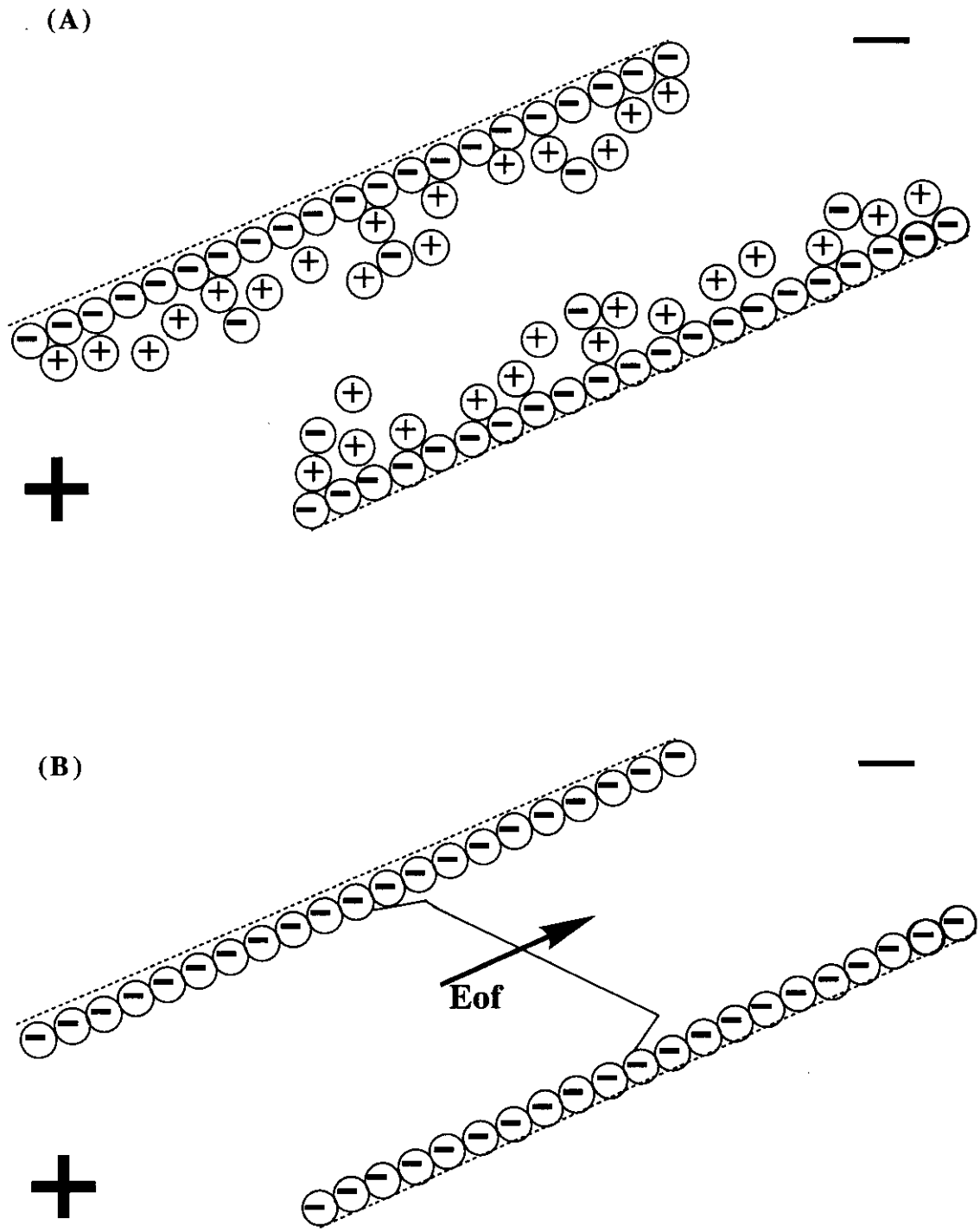
A convenient definition of a buffer is a substance which by its presence in solution increases the amount of acid or alkali that must be added to cause unit change in pH. Buffers are needed in CZE as the effectiveness of many chemical separations and the rates of many chemical reactions are governed by the pH of the solution. Buffers in CZE allow standardization of separation conditions and can be composed of various organic or inorganic compounds. Placing additives in these buffers can influence the type of detection used in CZE as well as the polarity of the analytes being examined and the polarity of the capillary being used. Additives can also be used to enhance difficult separations such as the resolution of the enantiomers of a chiral compound.

2. (1)c. Electro-osmotic flow (Eof)

Electromigration of ions due to charge attraction and repulsion within the capillary is not the only migratory effect. The other most significant effect is that of electro-osmotic flow. This is one of the most distinguishing properties of capillary electrophoresis. Eof is the bulk flow through the capillary which is due to the surface charge upon the capillary walls, usually originating from exposed negatively charged silanol groups. When in contact with an electrolyte solution, the silanol groups along the inner walls tend to ionize, creating an electrical double layer, or region of charge separation at the capillary wall / electrolyte interface as shown in Figure 2(A). The double layer is made up of the anionic silanols (SiO^{-}

) and the positive counterions. When voltage is applied, the counter-cations are attracted from the bulk solution to the walls. This leads to the cations at the wall being attracted to the cathode and as they are solvated they are said to drag the bulk solution (a large variety of charged and neutral species, including water) through the capillary to the negative electrode (Figure 2B).

Figure 2 . Schematic representation of the theory of electro-osmosis.



The potential across the double layer formed at the internal surface of the capillary is termed the zeta potential, denoted by ζ, and is given by :-

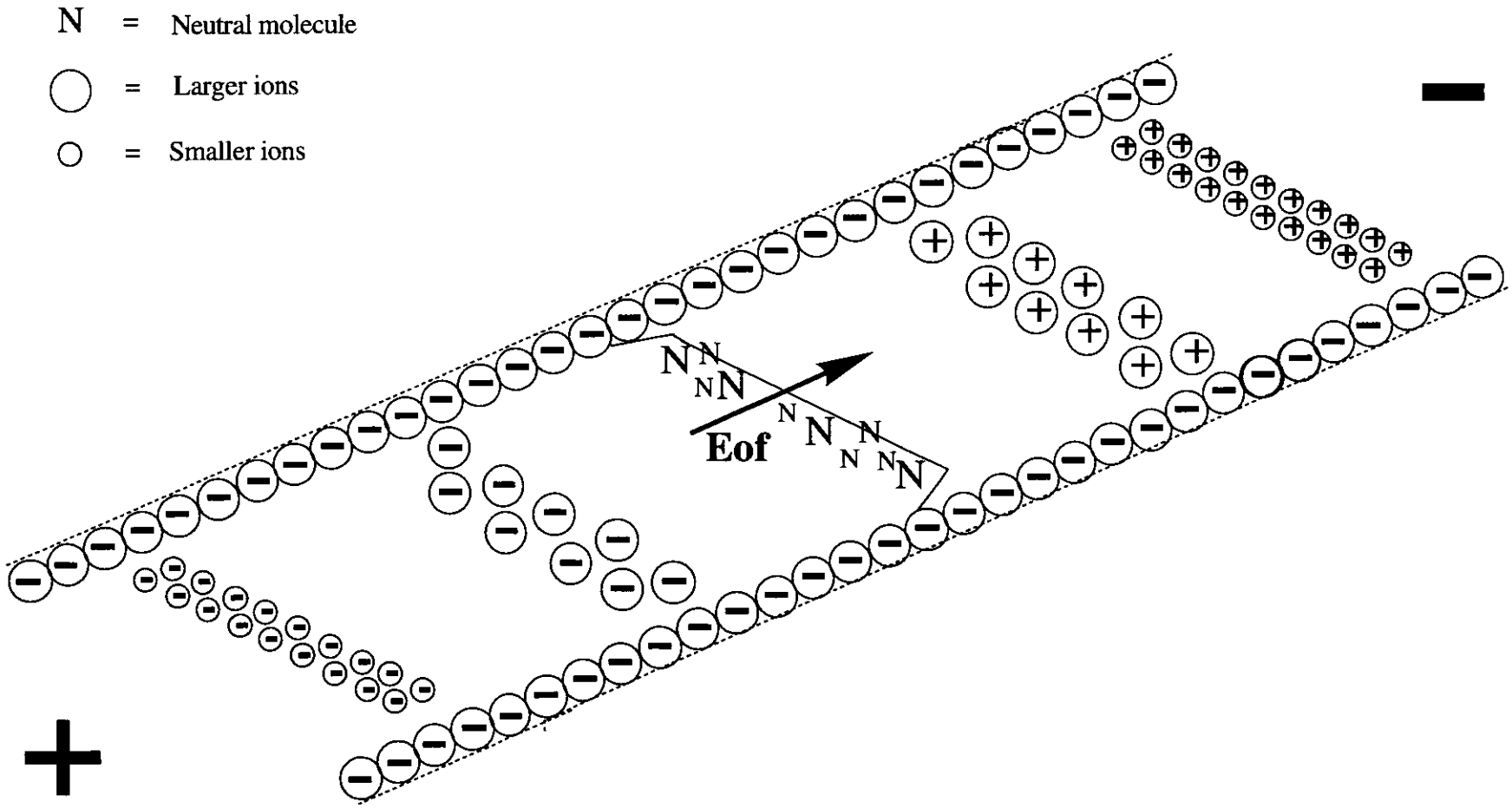
$$\zeta = \frac{4 \pi n u_{eo}}{\varepsilon}$$

Therefore :- $$u_{eo} = (\varepsilon \zeta / 4 \pi n)$$

where : ζ = zeta potential
π = 3.142
n = viscosity of the solution.
u_{eo}= electro-osmotic flow mobility.
ε = dielectric constant

The above explanation of Eof is a model which a number of researchers have used to rationalise it [4-5]. The extremely small size of the double layer leads to flow that for all practical purposes originates at the capillary walls. Flat flow profiles in capillaries are expected when the capillary radius is greater than seven times the double layer thickness[12]. The overall effect of electro-osmotic flow ensures that cations, anions and neutral species will elute at the cathode end of the capillary after passing and being detected by an on-line detector. Cations will migrate fastest due to their attraction to the cathode, followed by all neutral compounds in a discrete band and finally anions which are both pulled through the capillary by Eof and attracted back to the anode with the Eof pull dominating. Figure 3 is an illustrative model of this process. Eof has been studied by various groups by measuring the rate at which a neutral molecule, such as DMSO, mesityl oxide or acetone passes through the capillary either from its elution time or detection at intermediate points as it migrates through the capillary. This however does not account for possible wall adsorption effects which could slow the migration process. To counteract this Altria and Simpson[16-17] measured Eof by simply weighing the buffer solution where it emerged. The flow rate was found to be inversely proportional to ionic strength of the electrolyte and independent of the column diameter. Addition of methanol to the buffer reduced the flow rate and acetonitrile increased it[18]. The most dramatic changes in electro-osmotic flow can be made simply by altering the pH of the buffer system. At high, alkaline pH values the Eof is faster than at lower acidic pH values and below about pH 2 Eof is negligible. These effects are due to the amount of protonation or deprotonation of the silanol groups at the walls. High pH values cause deprotonation to SiO^- and low pH values cause protonation, whilst below pH 2 total saturation of the walls occurs. Whilst Eof is usually beneficial, it often needs to be

Figure 3. **A diagramatical representation of electro-osmotic flow and its effect on positive, negative and neutral molecules.**



controlled. At low or moderate pH values, compounds which exist as cations under these conditions will be adsorbed onto the capillary walls due to "Coulombic forces". This causes zone broadening and peak tailing. Adsorption has been especially problematic for basic protein separations[19]. One method of overcoming these problems is to conduct such separations at higher pH values above the iso-electric point of the protein molecules where they are negatively charged. This results in the proteins being repelled from the capillary walls so avoiding adsorbtion effects. Unfortunately this may introduce another problem as at higher pH values, Eof may be too rapid resulting in the elution of solutes before separation has occurred. In these cases Eof can be slowed without affecting the pH by the addition of salts like NaCl which decreases the thickness of the double layer [20] or by firstly coating the internal capillary walls with a non-ionic surfactant to reduce Eof pumping over a pH range of 4-11 as well as reducing protein adsorption[21]. Such modifications have dramatically improved protein separations. CZE separations have recently been improved by the addition of millimolar levels of phytic acid[22] to buffers which slows the Eof.

In some cases it is advantageous to reverse the electro-osmotic flow. This can be achieved by changing the polarity of the charge on the inner walls of the capillary from negative to positive by introducing additives to the buffer such as tetradecyltrimethyl ammonium bromide [TTAB]. Such a reversal was required during the separation of multiply charged negative carboxylic acids which migrated towards the anode at a faster rate than the electro-osmotic flow, so reversing the Eof and the polarity of the system swept them towards the cathode[23]. This group also had to reverse the polarity of the capillary in order to obtain an adequate separation. Buffer additives and permanently pre-coated capillaries can now be purchased from commercial sources. The latest method of manipulating electro-osmotic flow has been developed by Hayes et al[24] who have increased electro-osmotic flow by applying a radial voltage around the complete length of the capillary and also by applying voltage to a small portion of silver paint on the outside of the capillary.

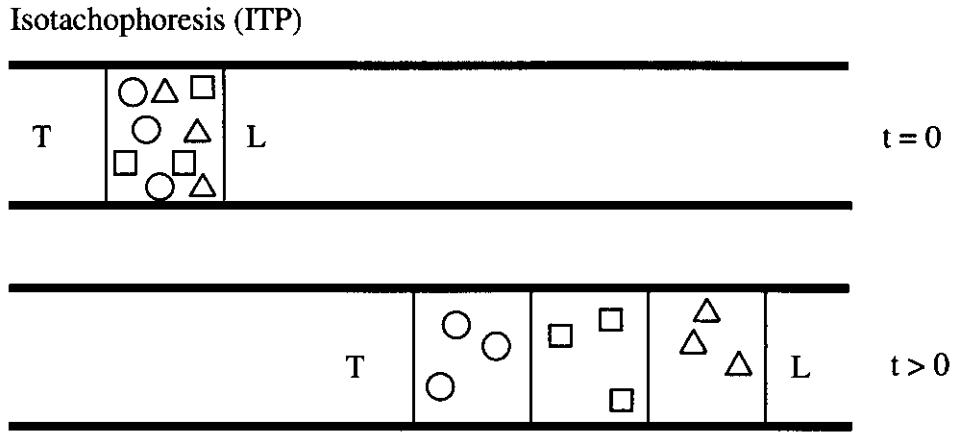
The newly available extent to which electro-osmotic flow can be manipulated has greatly increased the range and efficiency of CZE for many types of compound. Electro-osmotic

flow is particularly undesirable in other forms of capillary electrophoresis including isotachophoresis (ITP), isoelectric focusing (IEF) and capillary gel electrophoresis (CGE).

2. (2). Isotachophoresis.

Capillary isotachophoresis is a "moving boundary" electrophoretic technique. ITP combines two buffer systems to create a state in which the separated zones all move at the same velocity. The zones remain sandwiched between leading and terminating electrolytes (Figure 4).

Figure 4 - Illustration of Isotachophoresis.



T = Terminating Anion

L = Leading Anion

ITP only allows the analysis of either cations or anions during a single run. The nature of the ions to be addressed dictates the type of buffer to be used. For example for anion analysis the buffer must be selected so that the leading electrolyte contains an anion which has an effective mobility that is higher than that of the analytes being separated. Similarly, the terminating anion must have a lower mobility than that of the analytes. Upon application of the electric field the anions start to migrate towards the anode. The leading anions have the highest mobility and move fastest, followed by the anion with the next highest mobility and so on. The individual anions migrate in discrete zones, but they all move at the same velocity as that of the leading anion. The electro-osmotic flow rate varies strongly depending upon the choice of leading and terminating electrolyte used. Hence the reproducibility of ITP in quantitative analysis can be a serious problem unless Eof is suppressed or completely removed. This is again achieved by the use of additives such as

methylhydroxyethylcellulose[25]. The inherent concentrating effects and band sharpening that occur during ITP means that lower levels of analytes can be detected than with CZE. The improvement can be up to 3 orders of magnitude an improvement on CZE. Improved zone sharpening can be achieved by the addition of a high concentration of leading and/or trailing electrolytes to the sample. The concentration effect has been exploited by coupling ITP and CZE[26]. However some loss of resolution in the CZE separation can be observed after ITP pre-concentration. This can be avoided by increasing the buffer concentration in CZE, but higher buffer concentrations can cause heating problems in ITP so when coupling the two techniques subtle compromises have to be made[27-28].

2. (3). Iso-electric focusing. (IEF)

Capillary iso-electric focusing is used extensively to separate peptides and proteins on the basis of their isoelectric points, pI. The technique can be used to separate proteins that differ by 0.005 pI units and less. The isoelectric point of a peptide is the pH at which it exists as a neutral zwitterionic molecule. Above this pH the peptide exists as an anion and below this as a cation. The technique relies on the establishment of a pH gradient which is achieved using ampholyte molecules. Ampholytes are molecules that contain both an acidic and a basic moiety and can have isoelectric point values that span a desired pH range (pH 3- 9 for example). The capillary is filled with a mixture of ampholytes and solutes and the gradient is formed. A basic solution can be found at the cathode and an acidic solution at the anode. The electric field is applied and the ampholytes and proteins are focused. They migrate, due to electromigration, through the medium until they reach a region where they become uncharged (i.e. at their pI) and then electromigration stops.

The protein zones remain narrow since any protein which enters a zone of different pH will become charged once more and quickly migrate back to the point at which it has no net charge (its iso-electric point). Extremely sharp bands of sample result which are then mobilised, past an on-line detector by a technique called salt mobilisation. During focusing any electro-osmotic flow would be deleterious to the resolution so most IEF is performed using coated capillaries to eliminate this and wall adsorption effects. Compared to the focusing process which takes a few minutes, mobilisation can take up to 20 minutes plus.

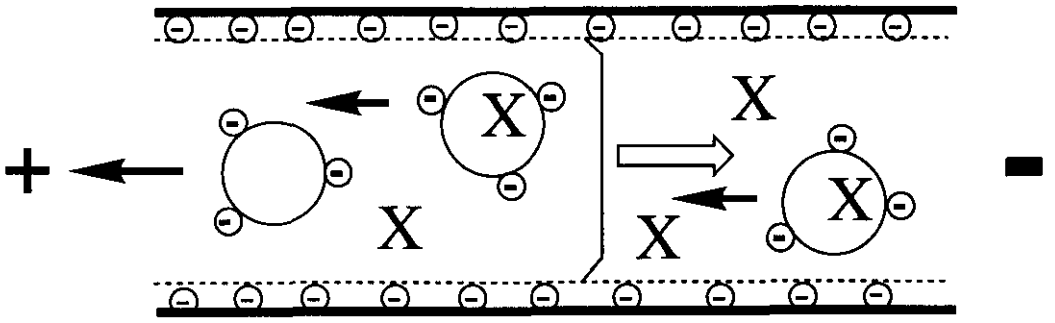
This makes IEF slower to perform than other CE techniques[29]. Mazzeo et al[30] proposed performing IEF in uncoated capillaries using polymeric additives which could act as dynamic coatings thereby covering many of the possible sites to which proteins may adsorb. In doing so they also demonstrated that by carefully choosing the type and concentration of these additives, electro-osmotic flow could be controlled but not eliminated, obviating the need for performing salt mobilisation, also speeding up the mobilisation process.

2. (4). Micellar Electrokinetic Chromatography (MEKC).

Perhaps the most important development in capillary electrophoretic methods is that of MEKC. This method was first introduced by Terabe et al [31-32] who has also recently published an extensive review of the technique[33]. MEKC is the only CE technique in which neutral molecules can be separated along with cations and anions.

Neutral species which are normally transported at the rate of the electro-osmotic flow in a descrete band are unseparated. In MEKC the separation of neutral species is accomplished by the use of surfactant carriers in the running buffer. When these surfactants are added at a concentration higher than their critical micellar concentration (CMC) the surfactant molecules aggregate together to form micelles. These micelles can be regarded as analogous to a stationary phase in HPLC, but in this case as a type of liquid-liquid chromatography. It is the interaction between the analytes and the micelles that brings about the separation. Both cationic and anionic micelles are possible and when the electric field is applied the anions migrate against the electro-osmotic flow towards the anode and the cations with the electro-osmotic flow towards the cathode. During this time, dependent upon their hydrophobicity, charged species can interact with the micelles due to electrostatic forces, whereas neutral species can only partition themselves in and out of the micelle. The extent to which molecules do interact with and remain within the miceller structures will dictate how long it takes them to migrate through the capillary. Using negatively charged micelles, the longer that neutral species spend included within the micelle the longer it will take for them to migrate through the capillary. They will act as if they are being detained by a stationary phase, since the micelles move against the Eof. As in CZE the Eof can be made faster than the rate at which the negatively charged ions and anionic micelles migrate to the anode. This ensures that all analytes eventually elute from the capillary, (Figure 5).

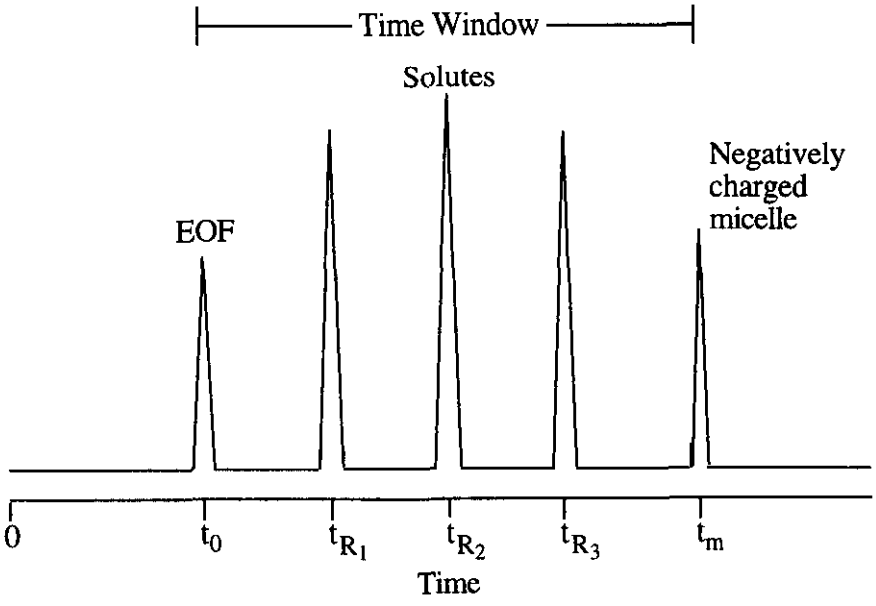
Figure 5 - Diagramatic representation of the principle of MEKC.



X = solute
Large open circle = carrier.
Large open arrow = electro-osmotic flow.
Black arrows = electrophoretic migration of the carrier.

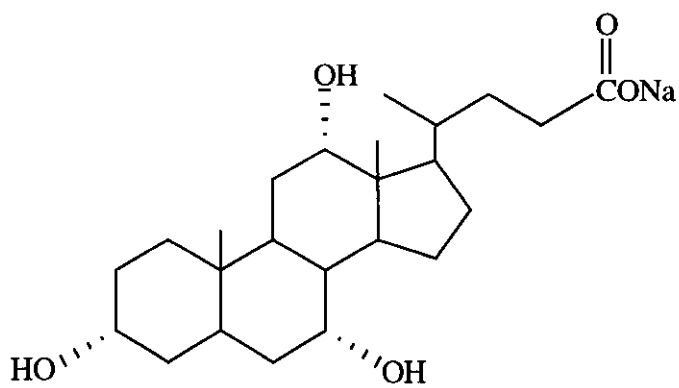
Eof is advantageous in this case. Generally separations can be improved by increasing the concentration of the surfactants, but this causes greater heat to be generated so low voltages are usually applied and capillary temperature control is very important. During the separation of neutral analytes, all are detected between t_0 and t_m, the time window, as portrayed in Figure 6. Sample resolution can be improved by extending this time window.

Figure 6 The elution time window for neutral solutes in MEKC.



Hydrophilic analytes which do not interact with the micelles are detected in the Eof band, whilst hydrophobic analytes are totally retained by the micelles. Analytes with intermediate solubilities including neutrals are detected between the two extremes. The limited detection

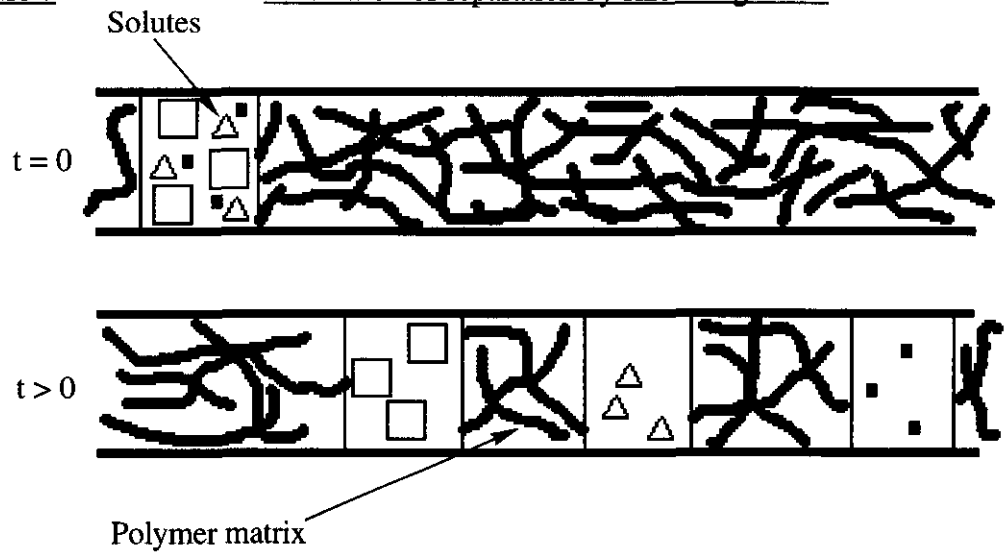
range has been an obstacle in the application of MEKC to complex sample analysis. The time window can be increased by employing conditions that open up the time window, that is, moderate Eof and micelles exhibiting high mobility. One method to increase the micellar velocity is to use small short-chain surfactants. These then form smaller micelles which have higher electrophoretic mobilities towards the anode. This then reduces the net micellar velocity towards the detector if a constant electro-osmotic flow is present. The problem with this approach is that very high concentrations of short chain surfactants need to be used which causes substantial heating and if too successful the micelles may leave the capillary at the anode or never pass the detector. The other approach to increase the time window, reducing Eof, has been achieved by coating the capillary with trimethylchlorosilane (TMCS) which increased the window from 5 minutes to 65 minutes[34]. Small percentages of organic modifiers, particularly propan-1-ol have been shown to improve separation efficiency in MEKC by lessening the hydrophobic interactions between the analyte and the micelle[35]. Urea has also been added to expand the time window and enhance sample resolution[36]. As all highly hydrophobic molecules are fully retained by the micelles separation by MEKC has proved difficult. This problem has been dealt with by adding cyclodextrins which compete for the analytes with the micelles[37,38]. These cyclodextrins add another phase to the separations, acting as neutral molecules. A great variety of anionic, cationic, non-ionic and zwitterionic surfactants, and mixtures of these can be used which means that conditions can be optimised for individual separations. An important application is that of the chiral separation of amino acids which has been achieved using chiral mixed micelles[39], and bile salt micelles [40] e.g. sodium cholate.



2. (5). Capillary Gel Electrophoresis. (CGE)

This technique is as near to traditional electrophoresis as can be incorporated into CE. CGE has been employed in the biological sciences for the size-based separation of macromolecules such as proteins, DNA [41] and nucleic acids[42] . The term gel is not strictly suitable as the material used is more of a cross-linked polymer such as poly(acrylamide). As charged analytes migrate through the polymer network they become hindered, with larger analytes being hindered more than smaller ones as illustrated in Figure 7.

Figure 7 Illustration of separation by size using CGE.

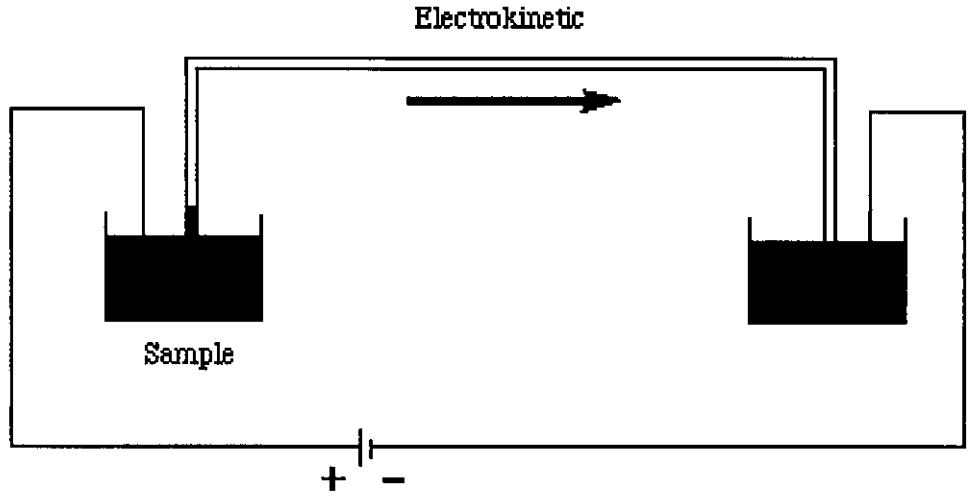


Large molecules such as DNA cannot be separated by normal CZE since they contain mass-to-charge ratios that do not vary with size. With DNA each nucleotide added to the chain adds an equivalent unit of mass and charge and does not affect the mobility in free solution. A variety of "gels" and pore sizes are available which can be used for particular separations e.g. agarose for proteins and cross-linked poly(acrylamide) for DNA. Whatever gels are used, the capillary wall has to be coated to eliminate electro-osmotic flow.

3. Sample injection procedures.

Samples for CZE must be introduced onto the column with minimum volume in a descrete band, so as to maintain the integrity of the technique and its separation efficiency. Tsuda et al. demonstrated that large sample volumes decreased separation efficiency in the separation of cations and anions in free solution CZE. Only 5µl of sample is required in order to

perform an analysis with nanolitre volumes being consumed during each injection. There are two main techniques by which injection can be performed, direct electrokinetic (voltage injection) and hydrodynamic (pressure injection) via the generation of a pressure difference between the inlet and outlet of the capillary. Electrokinetic injection can be used to introduce charged species onto the capillary and therefore can act as a sample clean up or concentration procedure, for example when using urine samples. This is due to ions of particular charge being preferentially injected dependent upon the polarity of the injection. This type of injection is achieved by placing electrodes in the inlet and outlet buffer reservoirs along with the capillary and applying the appropriate voltage for the appropriate length of time.



Upon application of a voltage ions will migrate towards the electrode of opposite charge. Cations will migrate preferentially when the outlet electrode is held at a negative potential with respect to the sample and anions when held at a positive potential.

However due to electroosmotic flow during the injection period some neutral and charged species with the same charge as that of the outlet electrode will also migrate onto the capillary. The quantity (Q) injected, (g or moles) can be calculated by:

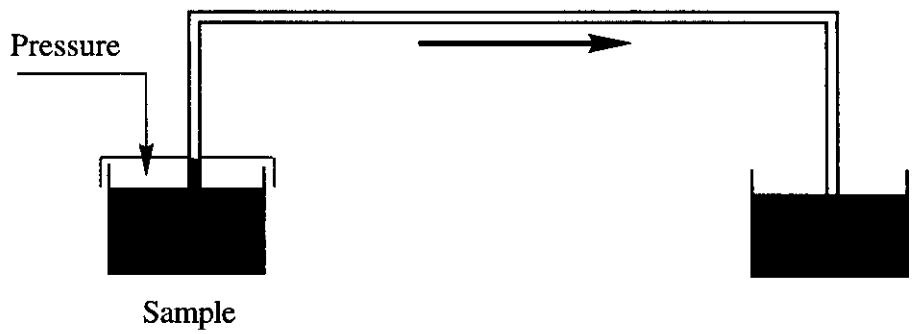
$$Q = \frac{(\mu_e + \mu_{EOF})\, V\pi r^2 C t}{L} \qquad \text{— equation 1.}$$

where
μ_e = electrophoretic mobility of the analyte
μ_{EOF} = EOF mobility
V = voltage
r = capillary radius
C = analyte concentration
t = time
L = capillary total length

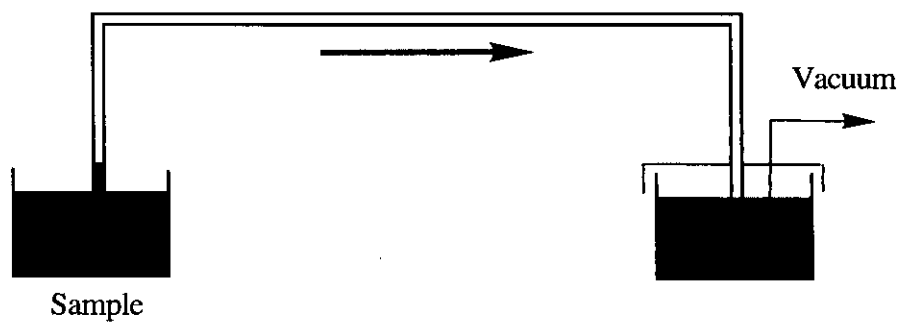
As described in equation 1, sample loading is dependant on the EOF, sample concentration and sample mobility.

Electrokinetic injection has been shown to introduce a bias to the injection process compared to hydrodynamic injection[43]. This is due to the difference in migration rates of various ions, so equal concentrations are not injected. This can be compensated for using peak area/retention time ratios. The other main injection method is hydrodynamic using pressure, vacuum and syphoning mechanisms. Injection is achieved via the formation of a small pressure gradient between inlet and outlet of the column. This is accomplished by:

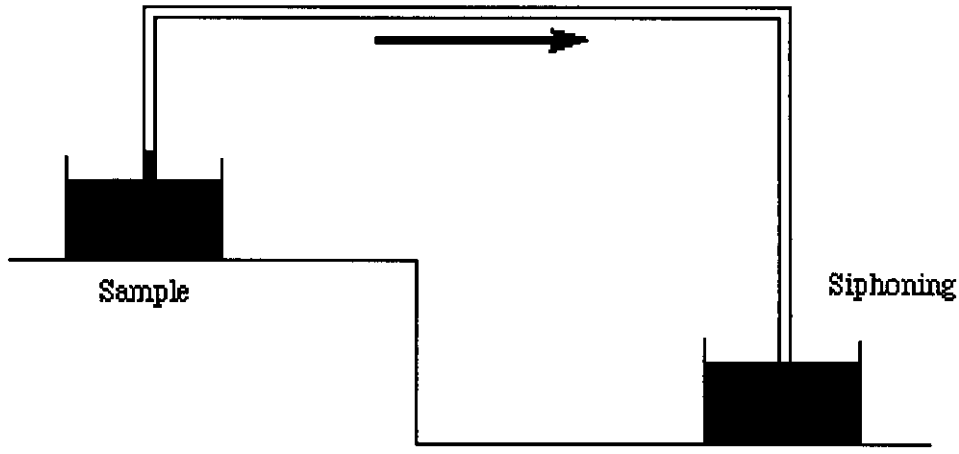
(a) the application of pressure at the injection end of the capillary,



(b) vacuum at the exit end of the capillary:



(c) by siphoning action obtained by elevating the injection reservoir relative to the exit reservoir.



The electrophoresis system used throughout this work used an automated pressure injection system at a pressure of 0.5psi. Injection volumes can be calculated using the Hagen-Poiseuille equation:

$$\text{Volume} = \Delta P\, d^4\, \pi\, t / 128\, nL \text{ (at 0.5psi} = 34475 \text{ dyne cm}^{-2}) \quad \text{- equation (2)}$$

where
- ΔP = pressure difference across the capillary
- d = capillary inside diameter
- t = time
- n = buffer viscosity
- L = total capillary length

The following Table, 1 illustrates the injection volumes in nanolitres (nl) for various column diameters and lengths for a hydrodynamic injection time of 1 sec, at 25^0C.

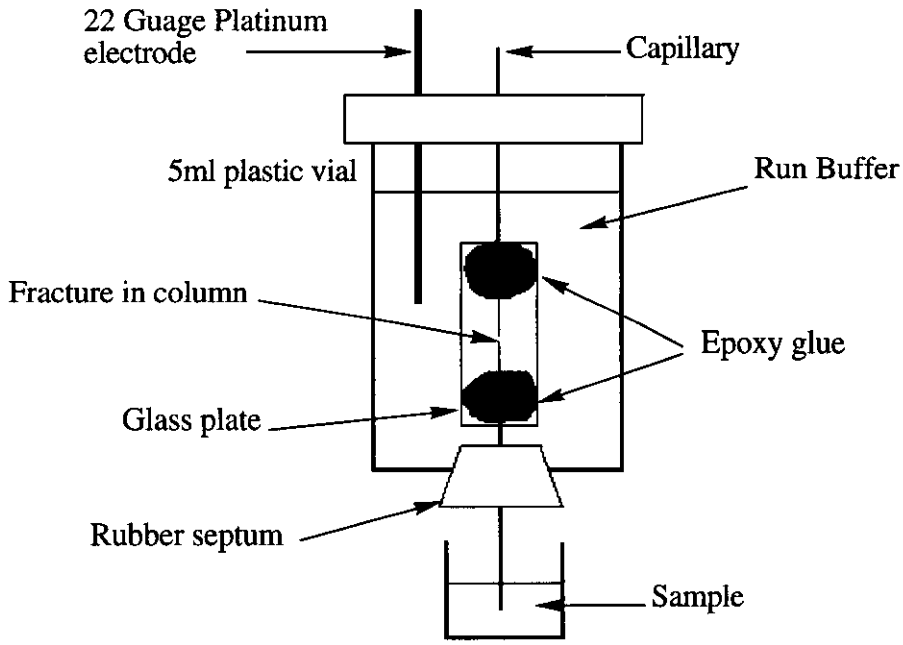
Table 1. **Volume injected in nanolitres / second for capillaries of different diameter.**

Length to detector (cm)

capillary i.d. µm	20	30	40	50	60	70	80	90
Volume injected (nl)								
50µm	2.2	1.6	1.3	1.0	0.9	0.8	0.7	0.6
75µm	11.2	8.1	6.4	5.3	4.5	3.9	3.5	3.1
100µm	35.3	25.8	20.3	16.7	14.2	12.4	10.9	9.8

Pressure injection does introduce a more representative sample into the capillary but computer-controlled pressure or height adjustment systems are required for adequate injection precision to be achieved. Also as already discussed electromigration introduces a biased injection[44] therefore the precise introduction of small sample volumes (1-10nl) into the capillary is a major problem in un-automated CZE. One alternative has been to use the electro-osmotic flow in the capillary to inject samples for quantitative analysis[45]. To accomplish this a potential was applied at a position after the inlet end at an electrical connection created through a fracture in the column. When a potential was applied between the fracture and the outlet end, sample is drawn into the column via electro-osmotic flow (see figure 8). The process can be thought of as being analogous to a syringe, drawing sample into the capillary. The result is the quantitative, reproducible introduction of small volumes of sample into the capillary.

Figure 8. Diagram of fracture injection apparatus.



With an automated system, injection reproducibility can be better than 1 to 2% RSD. Various phenomena can adversely effect injection reproducibility, these include sample viscosity changes via capillary temperature variation, siphoning if sample and buffer levels are not equal, diffusion and capillary action[46]. Sample injection can be used to enhance the sensitivity of the CZE technique. This is achieved by concentrating the sample in the capillary, a process called stacking. Stacking can be achieved either by the use of ITP

concentration, or by injecting the analytes dissolved in water or a buffer of lower concentration than the one used in the separation[47]. In the latter approach, the conductivity of the sample is significantly lower than that of the running buffer. Stacking can also be induced by injecting a short plug of water before the sample plug. Using this stacking technique more than a ten fold sample enrichment can be obtained[48].

4. Methods of Detection.

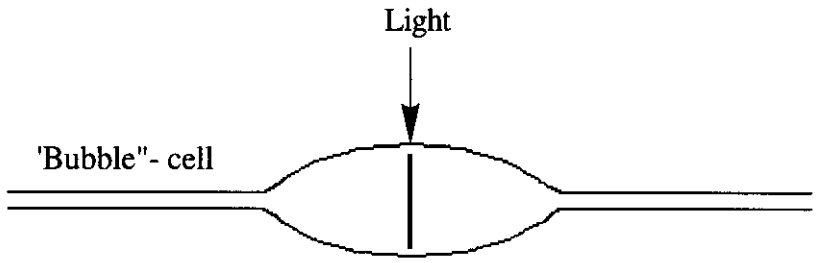
4. (1). U.V. absorbance detection methods.

Detector design in CE has proved to be a challenging aspect of the technique due to the minute capillary dimensions and constraints placed on sample loading[49]. Traditionally samples for CE have needed to be relatively concentrated due to the small volumes injected, hence the technique has not been used for trace analysis. In many cases a pre-concentration step or stacking procedure is performed before analysis. A number of mechanisms have been used to combat these inherent detection problems. As in HPLC UV-visible detection is by far the most common method used. This is a universal technique which relies on the analytes containing a UV-absorbing chromophoric group. By stripping a 5 mm section of the polyacrylamide coating from the outside of the capillary, the pre-focused rays of UV light pass through the capillary and are then detected by a photomultiplier. A range of absorption wavelengths from 200 - 340 nm can be used to supply spectral information. Detection can be optimised by firstly establishing each analytes' absorbtion maxima via the Beers Law as given in equation (3).

$$A = E\,c\,l \qquad \text{- equation (3)}$$

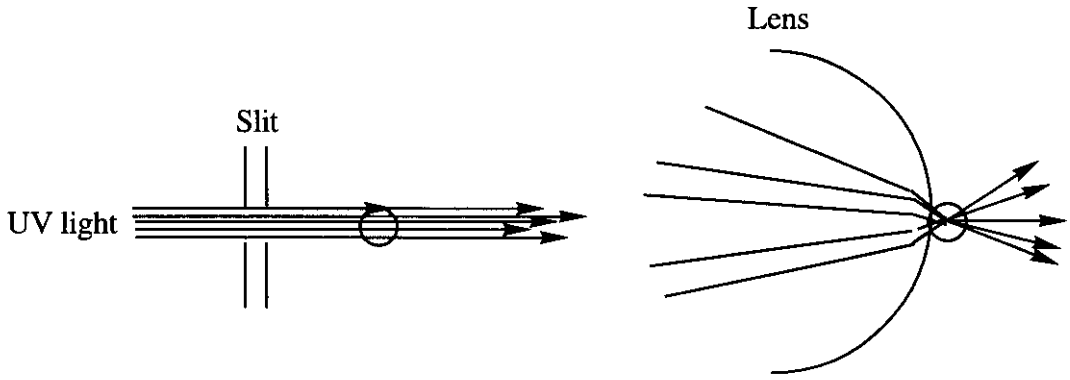
According to this law, where (E) is the extinction coefficient and (c) is the concentration of the analyte, the optical absorbance (A) of a sample is directly proportional to the optical path length (l) through which the absorbance measurement is performed. Thus an increase in the size of this optical pathway should also increase detection sensitivity. Increasing the pathway length using capillaries with larger internal diameters is not a viable option due to increased joule heating effects. However sensitivity and linear detection range can usually be improved by increasing the inner diameter of the capillary at the point of detection (e.g. Figure 9).

Figure 9. Diagram of "The Bubble - cell"



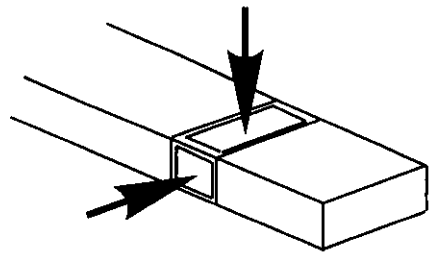
Although this approach is still somewhat limited due to increased heating effects, various methods of improving detection limits have been introduced. Firstly the amount of light focused through the capillary was increased using quartz or sapphire ball lenses (as shown in Figure 10) close to the capillary as opposed to directing light through a slit.

Figure 10. Diagram of Ball-lens scattering.



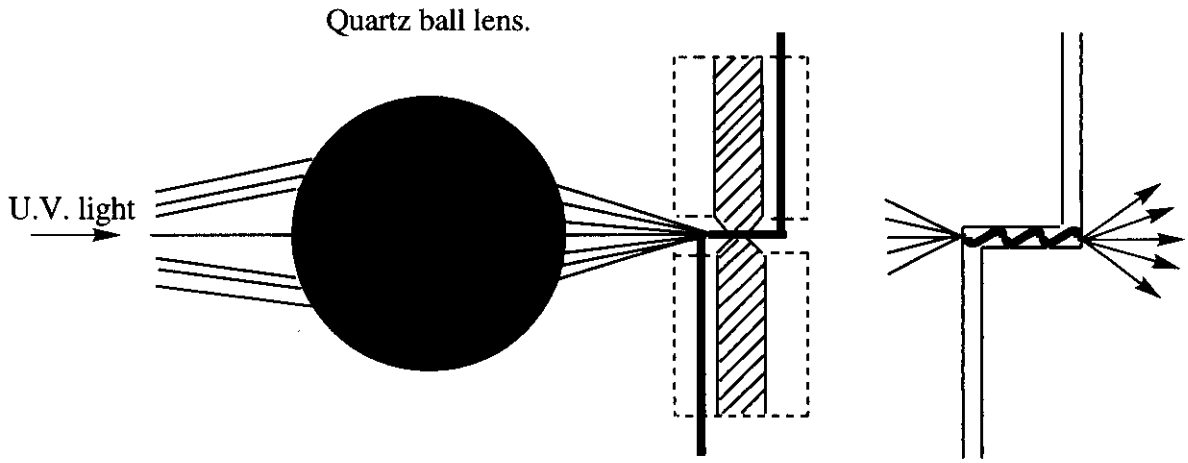
This set-up ensures as much light as possible is directed through the centre of the capillary and minimum light scattering occurs. The short path lengths associated with micro-capillaries can be extended by using flat capillaries. Rectangular and square capillaries of varying dimensions have also been used, (Figure 11). Narrow separation channels within these capillaries ensure efficient heat dissipation is maintained whilst increasing the path length and improving detector sensitivity[50].

Figure 11. Rectangular capillary to improve detection.



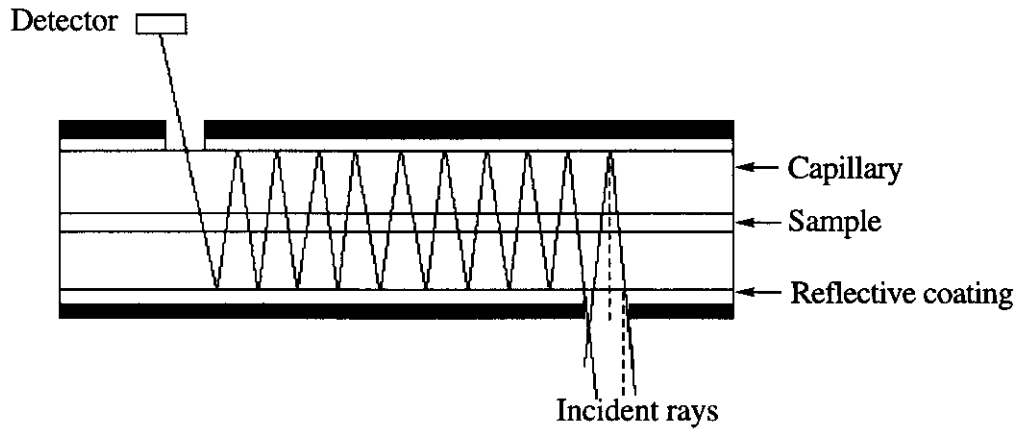
Light can be directed through both the 50μm and 100μm windows of the capillary. The main disadvantage of such shapes is their lack of flexibility when compared to circular capillaries. This means that it is possible to bend these circular capillaries into a "Z" shape, (Figure 12) which has been exploited by various groups to provide a path length of around 3mm[51]. Up to a 14 fold improvement on detection was obtained when proper optic lenses were used with this particular setup but there was also an associated loss of resolution.

Figure 12. Z-shaped capillary for improved detection.



Recent developments have further improved this situation by optimising the angle at which the light enters the capillary and greatly reducing the noise associated with the system[52]. An off-shoot of UV detection recently applied to CZE is multireflection cell absorbtion detection. The theory is that by making the light pass through the inner capillary diameter many times it is possible to increase the path length and improve detection. Multireflections can be obtained by either placing external mirrors around the capillary or making the inside of the capillary reflective using a silver coating. (Wang et al. demonstrated a forty fold increase in sensitivity for a cell construction with a forty-four fold increase in path length[53]). A critical parameter in such a cell construction is the incident light angle (Figure 13), which controls the number of reflections and the path length per reflection.

Figure 13. A multireflection cell capillary.



Diode Array Detectors.

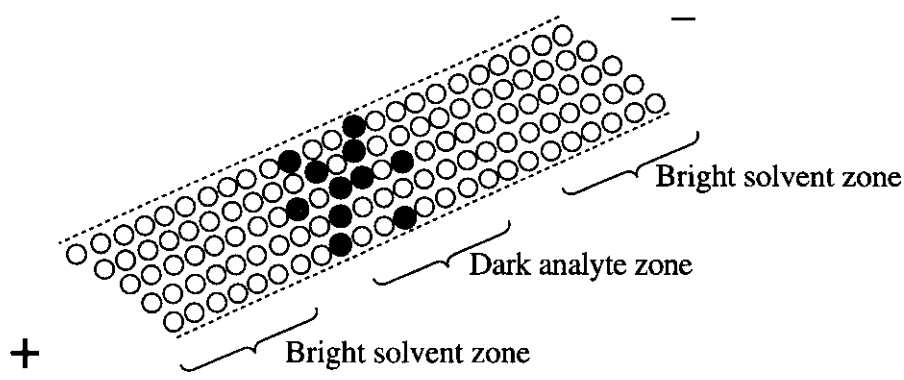
The use of diode array detectors is becoming more widespread and CE suppliers are producing instruments with this type of detector. This is a valuable alternative to single wavelength detectors. Previously, it was tedious to determine the optimum wavelength at which individual components within an analyte mixture should be measured. These could only be determined by injecting the sample repeatedly, changing the detector wavelength each time to make sure that all solutes were detected. With diode array detection it is possible to select a range of detection wavelength. For example from 190 to 520 nm with a band width of 330 nm. This means that within a single analysis, all solutes which absorb within this range can be detected and the optimum wavelength required for the detection of each can be determined. Also, whole spectra are made available by this method which aids identification of the molecules being analysed.

4. (2). Indirect detection methods.

Systems which classically detect via UV have been limited in terms of the number of types of molecule which could be detected as they were required to contain a chromophoric group or be derivatised. Indirect photometric methods have been introduced to CZE[54] which allow non-chromophoric compounds to be detected as negative peaks in a chromatogram. Conditions necessary for this technique to be viable include the use of a strongly absorbing molecule within the buffer which will then supply a high background so that when the non-absorbing sample molecules pass the detector a negative response will be seen, (see Figure

14). The key to these methods is the displacement of the highly absorbing mobile-phase additive in the buffer by the sample analytes.

Figure 14. Illustration of the bands formed during indirect uv detection.



The signal is derived from this mobile-phase additive rather than from the analyte itself as its concentration is lower in the eluted bands when compared with its steady state concentration[55]. It is also important that the compound used in the buffer has similar migration characteristics to the compounds being analysed. The resolution that can be obtained using indirect detection is similar to that using direct detection but the limits of detection (LOD) are reported to be higher by around two orders of magnitude.

4. (3). Detection involving fluorescence.

An increasingly popular detection medium for CZE is that of fluorescence. This is generally much more sensitive than UV detection. As with UV, an excitation source is used, usually tuneable helium-cadmium or argon lasers. Helium-cadmium lasers are relatively inexpensive and emit at 325 and 442 nm whereas argon lasers emit at 488 nm[56]. Laser power can be concentrated at these specific wavelengths and detection enhanced after reducing the background signal levels by ensuring the angle of the incident beam is at an optimum to avoid light scattering effects. Although fluorescence is the most sensitive detection method it is not the most commonly used as many solutes of interest do not exhibit native fluorescence and must be derivatised with some type of fluorophore. Various derivatising agents have been used for the analysis of compounds such as amino acids, proteins and peptides. These include naphthalenedicarboxylaldehyde (NDA)[57] and fluorescein isothiocyanate (FITC)[58]. Analogous to UV detection, indirect fluorescence detection has been shown to be an efficient means of visualising chromatographic samples that would normally be impossible to detect

without derivatisation[59]. Kuhr and Yeung compared direct and indirect fluorescence for the analysis of amino acids using salicylate as the background analyte[60]. Both methods proved viable for their analysis with the LOD for indirect fluorescence being about 3 orders of magnitude higher than that found for direct detection.

4. (4). Other detection methods.

Electrochemical detection is another detection method which has been examined extensively by Wallingford and Ewing for CZE with normal and micellar solutions[61]. They have also applied electrochemical detection to microbore capillary separations including those with 12 μm internal diameter which makes it possible to analyse samples from single cells[62]. Less popular on-column detection techniques include radioisotopic[63] methods and conductivity[64]. End column detection in which a sensing device is placed at the outlet of the fused-silica capillary has been the subject of greatest attention in recent years. This includes amperometric and conductimetric detection[65]. But the most exciting development which could eventually overcome the problems related with capillary electrophoresis detection is the coupling of CZE with mass spectrometry. Coupling CZE and MS provides mass specificity to the detection process, an advantage unrivaled by other analytical techniques. Three adapted LC-MS interfaces are currently in use for CE-MS: continuous-flow fast atom bombardment (CF-FAB), electrospray and ionspray.

5. Mass Spectrometric techniques.

5. (1). Fast atom bombardment mass spectrometry.

Classical mass spectrometric techniques like electron ionization and chemical ionization[66] require the sample to be presented to the ion source in the gas phase which is mainly achieved by heating the sample. This restricts the type of sample which can be analysed using mass spectrometry. Large thermally labile compounds will be degraded under such conditions and more polar, generally involatile compounds require excessive heat to get them into the gas phase for analysis. Fast Atom Bombardment Mass Spectrometry (FABMS) has now become an established method of ionizing materials directly from solution[67]. The FAB ionisation process[68-69] should give, in abundance, ions indicative of

the relative molar mass of the compound, and, additionally, structurally relatable fragmentation of the molecule should be in evidence. Neutral molecules (M) are ionised by protonation $[M + H]^+$ and proton abstraction $[M - H]^-$.

FAB ionization depends on the sputtering effect on a sample dissolved in a liquid matrix. The sample is bombarded with high velocity particles such as those of rare gas atoms Argon or Xenon of about 8 Kev energy. The energy of these particles is imparted via momentum transfer to the sample which then sets up a chain reaction within the analyte matrix. Mounting the sample on a stage or probe tip at a suitable angle, an approximate 70^0 angle of incidence (20^0 angle to the sample) appears optimal, allows efficient ionisation to take place and ions to be focused towards, and extracted by a split lens.

Dissolution within a matrix, like glycerol, ensures even distribution of the material so that maximum surface area can be exposed to the atom beam and to minimize evaporation, prolonging the liquid state of the sample in the high vacuum environment of the mass spectrometer. Unfortunately matrix (background) ions are also produced during ionization (e.g. glycerol gives rise to ions of protonated glycerol) and by mixing the sample with an organic compound the sample is somewhat "contaminated" leading to a loss of detection sensitivity compared to other mass spectrometric techniques.

5. (2). Electrospray Mass Spectrometry (ES).

Electrospray ionization sources coupled to mass spectrometers have now become well established as a method with great potential.[70-72] This technique has allowed the expansion of the range of peptides, proteins and oligonucleotides amenable to analysis[73]. The ability to analyse the large and labile molecules of biological importance had long been an important aim for mass spectroscopists. The main attraction of ESMS is that the mass/charge (m/z) range of the mass analyser need not be large because, as a result of extensive charging, ions above m/z 2000 are rarely observed (eg. a protein of mass 20,000Da with 18 charges has a m/z value of 1111.1). It is now possible to observe molecules with masses exceeding 500,000Da on a normal range mass spectrometer. This technique has allowed the examination of large biomolecular proteins which includes studies into heme binding in

myoglobin and haemoglobin [74]. Proteins are normally analysed as positive ions where the charges are produced by added protons. The extent of positive charging largely depends on the number of basic amino acid residues present within the protein (e.g. arginine, lysine). As well as protons, cationization can be produced by adding salts such as ammonium, sodium or potassium to the protein for analysis in the positive ion mode. In a similar fashion, anionic groups such as phosphates of nucleic acids produce negatively charged compounds for analysis in the negative ion mode. Most proteins produce a series of multiply charged ions, each adjacent ion in the series differs by one proton. This allows accurate measurement of the molecular mass from the mass/charge ratios measured by the spectrometer. These ions have the general form:- $[M + nH]^{n+}$.

where: M is the molecular mass
n is an integer number of protons (charges)
H is a proton (with mass of 1.00794)

Once (n) is known the molecular mass can be calculated from [M=n(m-nH)] where (m) is the observed mass in the spectrum.

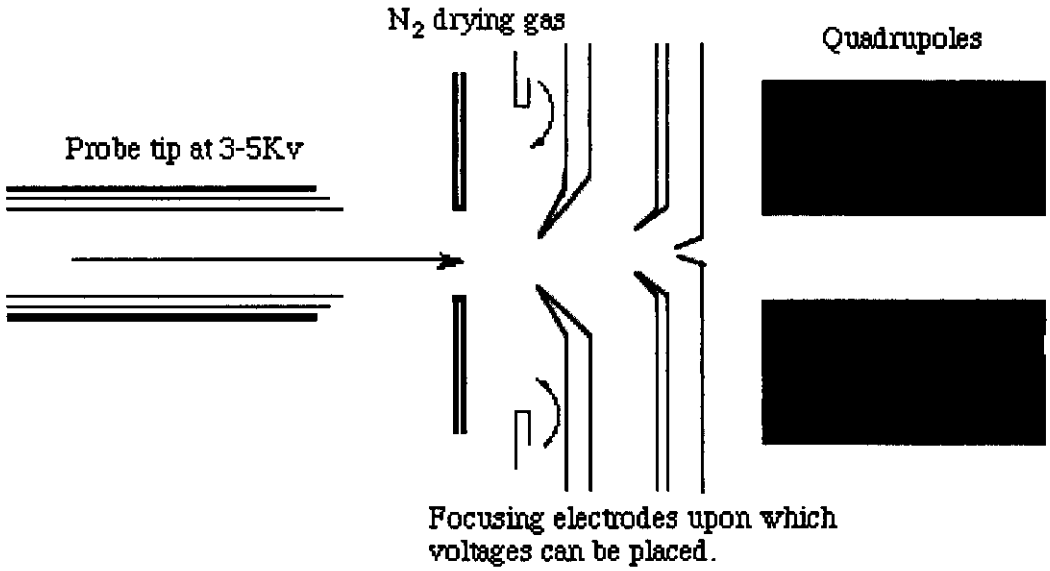
Historically ES-MS was initially reported by two groups, Yamashita and Fenn [75] and Aleksandrov et al [76] simultaneously, whilst Fenn and co-workers also demonstrated ES-MS in the negative ion mode. The technique is now known to take place in three consecutive stages: firstly, highly charged droplets are produced by a combination of spraying and a strong electric field, followed by ion desorption which produces an ion beam that is then sampled into a vacuum to create an ion beam to be focused by high potential skimmers before analysis in the mass spectrometer. In the area of analysis quadrupole spectrometers have been most extensively used which are relatively cheap and easy to use. Also structural analysis can be performed using triple quadrupole instruments for MS-MS studies.

5. (2).1. The ionization process.

Electrospray ionization relies on the application of a high potential field to the probe tip as the sample eluent emerges from it into an area of near atmospheric pressure which has a circulating drying gas within it. The evaporation of charged droplets to produce free gas-

phase ions from analyte species in solution was first proposed for MS by Dole et al [77]. The principal of applying a strong electrostatic field at the exit of a small tube supplying a solvent had previously been investigated by Zeleny in the teens of this century [78]. Dole's experimental results hold well today as another factor used in ES is that of a flow of drying bath gas (usually nitrogen) within the source (Figure 15), to encourage the evaporation of the solvent in which analyte molecules are suspended. This ensures the increase of the surface-charge density of the droplets at or near atmospheric pressure.

Figure 15. Illustrating the main components of the electrospray source.



As the charged droplets progress towards the counter electrode desolvation continues so they become smaller. As this occurs the charge density on the surface increases until the Rayleigh limit is reached. At this time Coulombic repulsion begins to match the surface tension of the droplet until a "Coulombic explosion" occurs tearing the droplet apart, producing charged daughter droplets which can then also evaporate. These events are repeated until the radius of the droplets become so small that the ions in the drop are desorbed into the ambient gas. Both cations and anions can be produced depending on the capillary bias. Those desorbing ions will still have solvent species attached which are not ions themselves, these are called "Quasi molecular" ions for mass analysis. This process is shown schematically in Figure 16 in Appendix 1. The electrospray is produced by application of potentials typically 3 - 6 kV between the probe tip and counter electrode located 0.3 - 2cm away. Typical flow rates of solvent are generally 1 - 20 µl/min. ES

requires volatile solvents to be used if efficient spraying is to be maintained which has somewhat restricted the use of the technique [79]. Methanol, acetonitrile and isopropyl alcohol have been used in 50 /50 mixtures with water and the addition of organic acids like acetic and formic acid ensures a good supply of protons for charging purposes. An off-shoot of ES termed Ionspray has allowed higher flow rates of up to 2ml/min to be achieved under assisted nebulisation and solvents of 100% water have been successfully used. A disadvantage of this method is that of loss of sensitivity but this is sometimes a worthwhile sacrifice for the higher flow rates which can make the technique more compatible with various HPLC methods. Although ES is termed as being a "soft ionization" technique, greater levels of fragmentation can be induced by varying and optimising internal source parameters. It is important that the bath gas used shouldn't undergo any reaction or charge exchange with the analyte ions and nitrogen is generally used for this purpose, which is also an inexpensive gas so ensuring the cheap, long running of the instrument. In the negative ion mode it is necessary to include an electron scavenger in the system eg. oxygen [80] to inhibit electrical discharge. Also reports of greater stability of ion beam is seen when solvents such as isopropyl alcohol are used in this mode. Negative ion formation by ES ionization has been demonstrated for a variety of small molecules with acidic functionalities such as carboxylic acids, herbicides and in this thesis esters of boron acids[81]. The efficiency of mass spectrometry and the variety of ionization processes/ flow rates used make the technique amenable to LC-MS and, more importantly for the work described herein, CE-MS.

6. Combined Mass Spectrometry and Capillary Electrophoresis.

6. (1). CE-MS using Continuous flow fast atom bombardment mass spectrometry (CF-FAB).

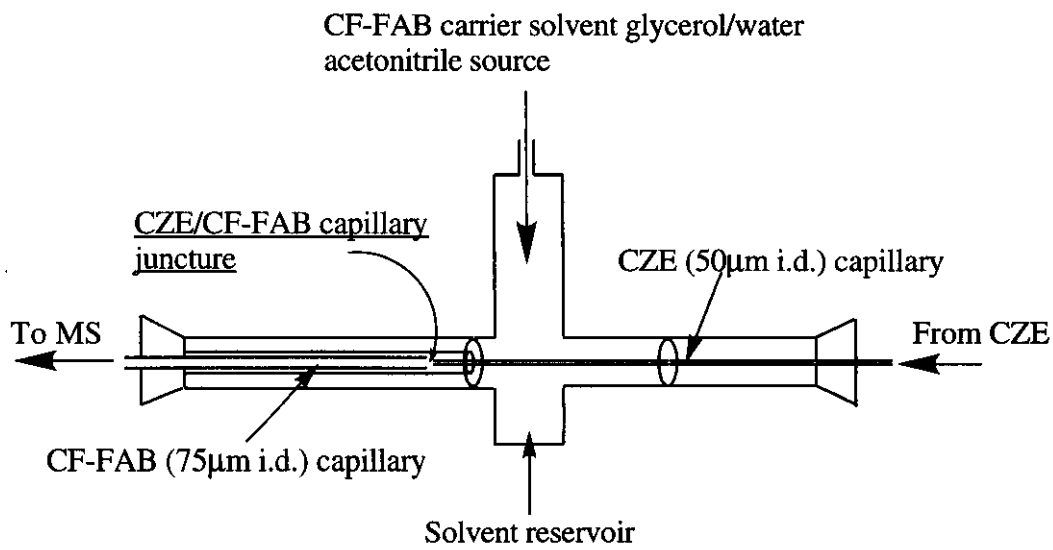
The coupling of liquid chromatography with mass spectrometry has allowed both structural and molecular information to be obtained as well as supplying the retention times of individual analytes. FAB has been used for on-line HPLC applications in various forms. One approach involved the use of a moving belt [82] onto which fractions of the HPLC eluant were deposited, and subsequently exposed to the ionizing beam of fast atoms. Initial reports

detailing continuous flow FAB interfaces using capillary inlet devices were published in the mid 1980's[83-84]. FAB and CZE are actually incompatible with each other in terms of their liquid composition and flow rates. FAB requires a solvent such as glycerol with 80-95% water. For CF-FAB this solvent / water mix is maintained at a steady flow rate of around 10 µl/min. Another characteristic of FAB is that the ion source is actually held under vacuum and is therefore at much lower pressure in comparison to the atmospheric pressure of CZE. This factor and the very low flow rates of CZE in nl/min which are essentially due to the electro-osmotic flow, necessitate that an interface be used between the two systems. Fast atom bombardment mass spectrometry coupling to CZE has been successfully achieved using two basic interfaces, these utilize coaxial flow[85] and liquid junction[86-87] systems. Both interfaces have their benefits and disadvantages.

6. (2). Liquid-junction interface.

This interface involves mixing the CE eluant in the FAB solvent base prior to the ionization chamber. The only critical dimension in this set up, Figure 17, is the junction distance between the CE and CF-FAB capillary which once optimised and maintained facilitates the use of the system for extended periods. The liquid mixture at this junction is then pulled into the mass spectrometer due to the pressure differential between the block and the FAB source.

Figure 17. Diagram of the liquid-junction CZE/MS interface.

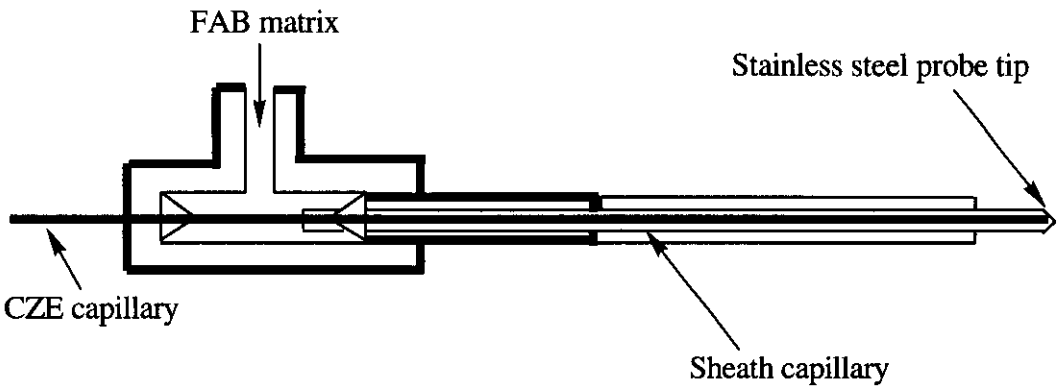


The main disadvantage of the LJ interface is the peak broadening effects which may be encountered due to the pre-source mixing and the dilution effects this causes. This CF-FAB eluant must then also traverse up to 10 cm of capillary within the probe before it reaches the source and this too accentuates band broadening and compromises the high resolution obtainable by CZE to some extent.

6. (3). Co-axial interfacing.

Using the co-axial approach (Figure 18), means that band broadening can be prevented and the integrity of the CZE separation is maintained. The system is shown in the figure below.

Figure 18. Diagram of a co-axial CZE/MS interface.



The different eluants don't actually meet and mix until they reach the probe tip within the source. To help combat the vacuum effect from the mass spectrometer the CE capillary ends a few mm before the CF-FAB probe tip so introducing a small dead volume mixing region but not to the same scale as with a LJ interface. The high voltages used in CZE mean that some form of insulation is required to prevent electrical shorting within the source. Having a coaxial arrangement also allows the capillary to be cooled by the FAB makeup matrix. In one case where a coaxial system was used to analyse bioactive peptides[88], helium gas was used as an extra cooling agent to help reduce joule heating effects. FAB itself doesn't involve the use of any heating filaments or hot surfaces and is therefore beneficial to the CZE process.

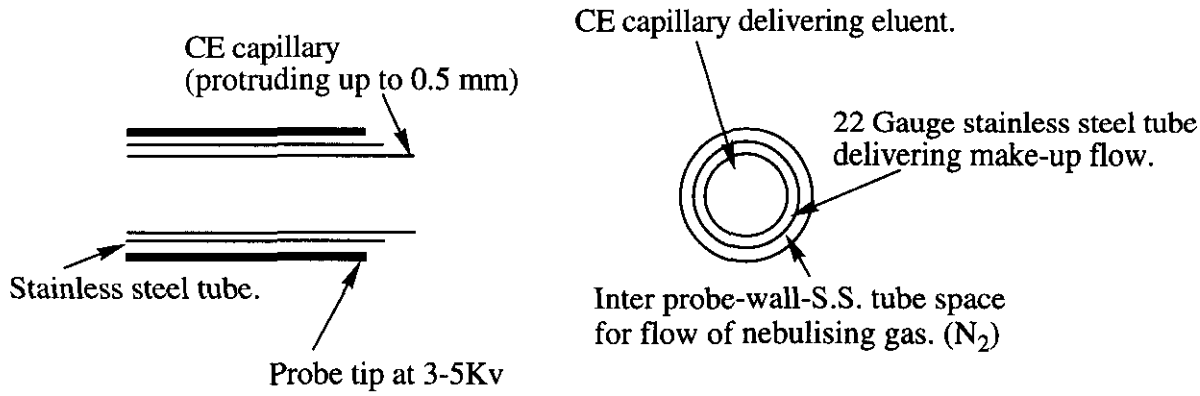
Although liquid junction interfaces introduce greater band broadening effects than coaxial interfaces, the mass flow to the mass spectrometer will be superior for the LJ type of interfaces so allowing higher loadability than with coaxial systems.

CF-FAB hasn't yet fulfilled its potential as a routine quantitative procedure which has been mostly due to ion beam instability and problems maintaining it. Some of the problems and suggestions as to how these have been solved, including the introduction of metal frits and wicks at the FAB probe tip have been reported[89].

6. (4). CE-MS using Electrospray mass spectrometry (ES).

Electrospray mass spectrometry offers many features which make it a compatible technique for routine coupling to capillary zone electrophoresis (CZE). Both can be used for peptide and protein work in particular. CE offers a fast, highly specific separation technique with a low sample requirement (10µl) and ES has the capability to measure compounds at the femtomole level. Both methods are capable of handling small polar molecules as well as extremely large molecules. The earliest reports of CE being coupled to electrospray mass spectrometry involved the use of a coaxial arrangement and highlighted the compatibility of the two techniques in terms of the flow rate requirements of ES and those displayed in CE using 100 µm i.d. capillaries[90]. An electrospay interface was seen as a viable alternative to FAB as ionisation occurs at atmospheric pressure therefore avoiding problems associated with coupling CE to a source under high vacuum[91]. Thermospray ionisation had also been considered but CE flow rates of around 1µl/min were known to be incompatible with this technique which isn't generally effective for liquid flows below a few tenths of a ml/min. Initial experiments with ES relied on an electrical contact being made at the exit of the capillary via a stainless steel sheath tube which facilitates an immediate electrical contact at the cathode end of the capillary as the liquid flow emerges. The premise was that the cathode need not be in a buffer reservoir but only biased negative with respect to the anode. It was found that efficient ES was hard to achieve using some of the higher molarity buffers above 10^{-2} M which were more frequently used in CZE. Also as smaller i.d capillaries, below 100 µm came into use CZE liquid flow rates were substantially less than 0.5µl/min into the nl/min range which made ES increasingly difficult to maintain. The same group then introduced further refinements to their coaxial interface to overcome these and other problems[92-93]. The flow rates could now be manipulated independently of the CZE liquid flow by introducing a second flow of make-up solvent via another stainless steel sheath tube (see Figure 19).

Figure 19. Diagram of the situation at the triaxial probe tip during CE/ES.



This meant not only that ES could be maintained and optimised at any particular flow but that higher molarity buffers could be used in CZE due to the inherent dilution effects of the make-up solvent upon the CE eluent. Another variable introduced was the ability to nebulise the liquid flow so allowing even greater flow rates to be accommodated. The introduction of these improvements meant that the electrical contact between the capillary - make-up flow and stainless steel sheath could be maintained. The CZE capillary protrudes a short distance (> 0.2mm) beyond the metal sheath to provide good performance. Thus ES is created at the capillary terminus which therefore avoids any post column region that would contribute to extra-column band spreading. These developments made the exploitation of a commercial interface a viable proposition[94]. This then lead the way for the interface to be utilized to analyse dynorphins[95], and other larger peptides. Conducting protein separations in CZE using low pH buffers ensures that they emerge from the capillary as cations therefore aiding initial ionization of the analytes. Unfortunately such buffer conditions also cause extreme tailing of the protein peaks and adsorption to the capillary walls. One attempt to solve these problems involved using buffers with pH's above the isoelectric (pI) point of the analytes, which meant the analytes migrated through the capillary as anions. However due to loss of protonation efficiency in the positive ES mode, one order of magnitude sensitivity is lost using the anion approach[96]. Hence protein work using CZE-ES continued with the use of acidic buffers to avoid the post column losses of sensitivity associated with separation of analytes as anions, on longer columns, with higher pH's. Recent advances in capillary technology greatly improved the resolution and peak shape of CE derived separations. Low pH buffers which previously caused peak deterioration, were used in conjunction with non-covalent coated capillaries[97] which possess an overall positive charge. This means that

cationic peptides and proteins will now be repelled by the charges at the capillary walls, so ensuring the elimination of adsorption effects and improving resolution. Reversing the charge on the walls also means that the Eof is reversed so the applied potential placed across the capillary must also be reversed to allow migration of analytes to the ES source. Sensitivity has been improved for protein analysis via the use of chemically modified 5 μm i.d. capillaries[98]. An approximate improvement in sensitivity of 25-50 fold led to the capability to detect attomole quantities of injected protein. Generally it was found that the smaller the capillary i.d. the better the sensitivity. This is related to the size of the CE currents generated and their compatibility with the ion currents developed by the electrospray. Larger capillaries commonly generate currents greater than 1 μA whereas ES currents are typically between 0.1 and 0.5 μA[99]. So smaller capillaries generate lower currents to match those from the ES source more closely to enhance efficient ionization and improve sensitivity. The major disadvantage of using such small capillaries is the problem of blocking and the care which needs to be taken to avoid it. Capillary isotachophoresis (CITP) has also shown great potential for coupling to electrospray mass spectrometry and improving sample detection limits using the CE-ES technique. Its feasibility was first demonstrated using quaternary phosphonium and ammonium salts, amino acids, catecholamides[100] and various polypeptides[101]. This method has been particularly useful as a concentrating technique using high volumes of low concentration samples[102]. The concentrating effect is due to the formation of tight analyte bands. Thus CITP-MS has the potential of allowing much greater sensitivities than are feasible with CZE-MS due to more efficient analyte ionization. Analyte pre-concentration by CITP has allowed a 200 fold detection improvement[103]. More detailed sensitivity considerations in terms of sample size, concentration and flow rate, overall detection efficiency and actual ionization efficiency have been examined for CE-ES by Smith et al[104]. CE-MS sensitivity has also been further improved using scanning array detectors[105]. Time of flight (TOF) and ion trap mass spectrometry have allowed the limits of detection of CE-MS to be improved to zeptomole (10^{-21}) levels. These and more recent developments in CE-ES are illustrated in various reviews[106-107] which fully explain the possible further improvements and potential of this promising technique.

The co-axial interface is now well developed for CE/ES and has become the most widely used method for coupling the two techniques, but as with CF-FAB, liquid junction interfaces have also been used with ion spray mass spectrometry for industrial applications[108]. The use of a liquid junction interface has in fact allowed some work to be performed with gel-filled capillaries which have previously proved very difficult[109]. It is apparent that both the LJ and coaxial interfaces have their place in coupling CE to MS and between them will eventually allow the full range of CE techniques to be utilized for CE/MS.

7. Aims and objectives of this thesis.

This thesis addresses various clinical and environmental problems using the analytical techniques of capillary electrophoresis and mass spectrometry. Capillary electrophoresis is used throughout the thesis as the main technique for analysis of all samples and standards of interest. CE is suggested and tried as an alternative to existing methods for the analysis of diisocyanates and herbicides from the environment as well as dipeptides and acylcarnitines. The development of a CE/MS interface using electrospray (ES) is also investigated and the intention is to apply the method to the analysis of real examples. This will also include using ES mass spectrometry as a stand-alone system for development before interfacing with CE. The other major technique to be used is FAB mass spectrometry which will be applied to the analysis of polyols and boron complexes. Each individual application is introduced at the beginning of the relevant chapter.

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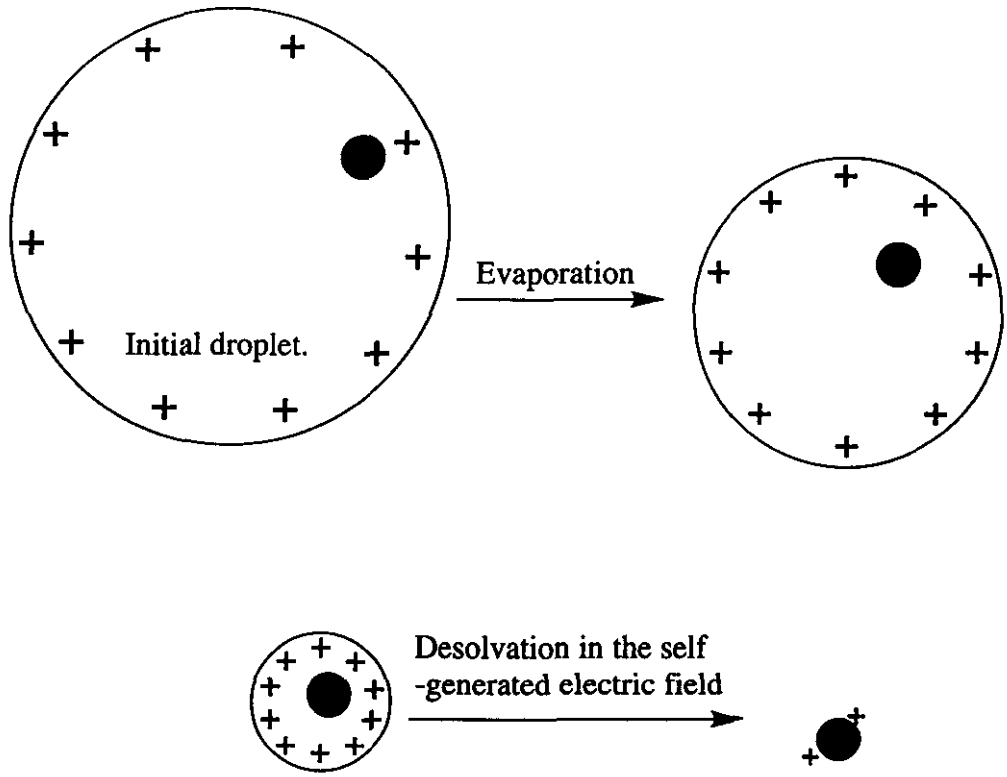
Appendix 1

Figures from Chapter 1.

Figure 16. Diagram to show the events occuring during electrospray ionisation. (see text for details.)



Chapter Two

Experimental.

1. Peptide and dipeptide work. (Chapter Three)

1. (1). Dipeptide sample preparation for CZE.

Dipeptide mixture separations involved making up 1 mg/ml stock solutions of each dipeptide in 10ml flasks of water or buffer dependent upon the type of experiment being performed. Measured volumes of these were then used and made up to 10 ml in graduated flasks. The volume used depended on what final concentration of dipeptide mixture was to be analysed. The final mixture would be equimolar if the relative molecular masses of the dipeptides were accounted for or more usually equal masses per ml of final solution.

1. (2). Workup procedure for urine for extraction of dipeptides and acylcarnitines.[110]

Bio-Rad AGI-X8, 100-200 mesh, formate form, anion-exchange resin (2ml) was used to pack a column of 1cm diameter. The resin was converted to the chloride form by eluting with 1M HCl (10ml). The column was then equilibrated with distilled water and the urine sample (3ml) applied to the head of the column. Dipeptides, other cationic and neutral species were eluted with distilled water (5ml). The eluent was then acidified with 0.5ml of 1M HCl. Bio-Rad AG50W-X8, 100-200 mesh, hydrogen form, cation-exchange resin (2ml) was used to pack a 1cm diameter column. The dipeptide-containing eluent from above was applied to the column. Neutral and loosely bound cationic species were washed off with 0.01M HCl (5ml) and distilled water (5ml). Dipeptides or acylcarnitines were eluted with 2M NH_4OH in 20% aqueous ethanol, the first 1ml being discarded and the following 9ml collected. The water was then removed by rotary evaporation using a GeneVac instrument. The resulting residue was dissolved in distilled water (0.5ml) and 100 µl of this solution used for CZE analysis.

1. (3) Typical buffer compositions and make up procedures. (Table 2.)

Buffer	Composition	Final concentration / pH
Citric acid	4.203g of citric acid was placed in a 500ml flask of D.I. water and pH adjusted with ammonium hydroxide.	40mM citrate at pH 3.6
Ammonium acetate	0.289g placed in 250ml flask and made to pH 2.5 with acetic acid.	15mM acetate at pH 2.5
β-Alanine	1.782g of β-Alanine was placed into a litre flask and 1.14ml of acetic acid added. The buffer was further acidified with 1M HCl.	20mM β-alanine at pH 2.8
Ammonium acetate / CH_3CN	0.771g ammonium acetate is placed in a 250ml flask. 25ml of acetonitrile is then added.	40mM ammonium acetate, 10% acetonitrile at pH 3.3

All solutions were made up to the mark of the respective volumetric flasks.

1. (4) Different rinsing procedures used for dipeptide analysis by CE.

Rinsing procedure (A) involved a 1 minute rinse with 1M NaOH, whilst (B) involved a 1 minute rinse with a highly concentrated 300mM buffer followed by a 2 minute rinse with the 30mM run buffer and no NaOH rinse. Procedure (C) involved a 1 minute rinse with 1M NaOH followed by rinse procedure (B).

2. Acylcarnitine analysis.

(Chapter Four)

2. (1). Development of buffers for the separation of acylcarnitines.

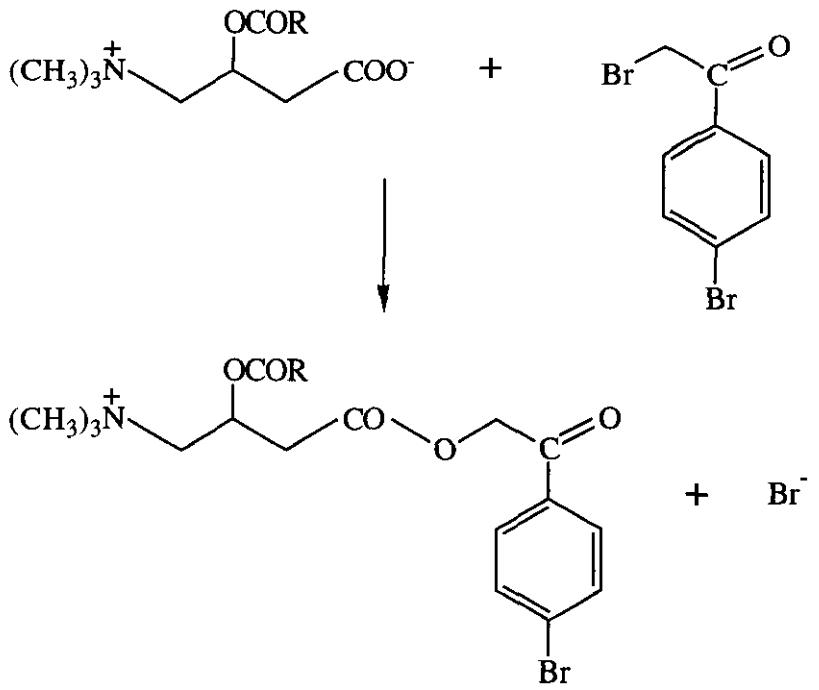
The first buffer used was a 30 mM phophate at pH 3.0 but adequate separation was not acheived. This led to the development of a 15 mM ammonium acetate buffer

adjusted to pH 4.3 with ethanoic acid. However when it came to separating three acylcarnitines there was a solubility problem so acetonitrile was added initially at 10% increased to 30 %. This resulting buffer was then used throughout the CE analysis except when 10 mM phytic acid was added in various experiments.

2. (2). Method for the bromophenacyl derivatisation of acylcarnitines also used for derivatization of glyphosate, (Chapter seven).

Sand was placed in a pyrex dish to a height sufficient to cover a 1ml Reactivial and the bed heated until a steady temperature of 80°C was achieved. The analyte (2mg) to be derivatised (either the glyphosate salt or acylcarnitine) was placed in a 1ml Reactivial, dissolved in dry acetonitrile and sonicated for 5 minutes. To this mixture was added 200µl of the *p* -bromo-phenacyl derivatising reagent. The mixture was then immediately placed in the sand bed up to the bottom of the Reactivial lid and stirred with a magnetic stirrer. It was then left to incubate at 80°C for 30 - 40 mins before being cooled. The diluted or neat sample was then analysed by CE using the 254nm absorbance filter. The expected reaction is detailed below.

Figure 20. Derivatisation of an acylcarnitine using *p* -bromo-phenacyl reagent.



The derivatising agent bonds to acylcarnitines at one site only i.e. the carboxyl group, whereas there are three potential bonding sites on glyphosate.

3. Work with boron-containing molecules. (Chapter Five)

3. (1). Initial experiments with boronic and borinic acids.

Experiments with 4-toluneboronic acid and 1,1,1-tris(hydroxymethyl)ethane were performed by either placing a drop of the FAB solvent onto the probe tip and mixing roughly equal amounts of the two compounds in the solvent or by making a paste of the two compounds in the FAB solvent and applying this paste to the probe tip before analysis[81]. These gave FAB mass spectra with significant molecular ions of the resulting complex. The same procedure was followed during early investigations using borinic acid and its ethanolamine complex.

3. (2) Production of diphenylborinic acid from its ethanolamine complex.

When the borinic acid was required from its ethanolamine complex, the necessary amount of the ethanolamine complex (0.5g) was dissolved in a minimum volume of methanol (approx 5ml) and hydrolysed by the addition of 15ml of 1M hydrochloric acid. The mixture was then agitated for 10 minutes and the gummy, water-insoluble diphenylborinic acid extracted with diethyl ether (10-20ml). This extraction procedure was repeated twice more to recover all acid formed. The ethereal solution was then dried over magnesium sulphate, but it was not allowed to stand, since the acid would begin to undergo degradation in aqueous or ethereal solution within about half an hour [112]. The ether solution was then filtered to remove the drying agent and the solvent removed under vacuum, finally being replaced by dichloromethane[81].

3. (3) Method for alkylation of alcohols.

This method[113] was used to produce dimethyl ethers from various ethylene glycols. KOH pellets (1.2g) were ground into a powder with a pestle and mortar. Approximately 0.9 g (16mmol) of this powder was then mixed with 4 ml of dimethyl sulphoxide (DMSO) and 2.7 mmoles of the ethylene glycol was added. To this solution was added bromoethane (872 mg, 0.59 ml, 8mmol). The contents of the flask were stirred for 30 minutes. Water (20ml) was poured into the flask and the product extracted with diethyl ether. The extraction was repeated to increase the yield of the reaction and the diethyl ether solutions pooled. After standing over $MgSO_4$ to remove any water present and filtering, the diethyl ether was

evaporated off and the remaining dimethyl ether kept for use. Some changes were made when both hexaethylene and pentaethylene glycol were used as the reactants. Iodomethane replaced bromoethane as the methylating agent and the number of extractions was increased. Firstly 3 extractions were performed with diethyl ether followed by 2 extractions with dichloromethane. Molecular sieve was then added to the resulting solution which was left overnight before filtering and evaporation of the solvent. The structures of all products were established by fast atom bombardment and electrospray mass spectrometry.

3. (4). Procedure for FAB of samples at St. Thomas' Hospital, London.

Boronate complexes using batyl alcohol and 1-monostearoylglycerol (between 0.1 and 0.01g) were dissolved in dichloromethane. The amount of the alcohol used was varied. The resultant samples were placed dropwise onto the FAB probe tip. The solvent was evaporated away using warm air leaving a residue of boronate complex on the probe tip. This procedure was repeated until enough sample had dried and accumulated to cover the probe tip. A small drop of the chosen matrix for that particular experiment was then mixed with the sample still on the probe tip and the analysis performed.

3. (5). Methods to enhance the complexation of diphenylborinic acid and various diols.

This experiment was designed to speed up the reaction whilst still keeping optimisation as the primary objective. Three bases were used as sinks for H^+ ions whilst maintaining the prescence of molecular sieve to remove water. Excesses (around 1-2g) of sodium hydrogen carbonate ($NaHCO_3$), pyridine and di-isopropylethylamine were all used at different times and in various combinations as detailed in Chapter 5. It is estimated that the rate of most reactions doubles for every 10^oC rise in temperature, so a refluxing apparatus was set-up for each reaction and the temperature was set at 80^oC. This ensured that the chloroform / dichloromethane solvent mixture boiled.

3. (6). Analysis of a spiroborate by capillary electrophoresis.

The spiroborate used (**5.8** in Chapter 5) was dissolved in 30mM boric acid buffer at pH 6.0 at a concentration of 100 μg/ml, and analysed using the same buffer. Results were obtained

by analysing the sample at regular intervals of 30 mins to investigate whether any hydrolysis of the spiroborate occurs and whether this could be measured using capillary electrophoresis.

4. Chromophoric herbicide analysis by CE and CE/ES.

4. (1). Buffer preparation for CE and CE/ES analysis.

In this instance the first buffer tried was the one used for all subsequent CE experiments on chromophoric herbicides. This was a 100 mM phosphoric acid solution at pH 3.2 which was used in conjunction with the Supelchem C8-unit capillaries. But when it came to the CE/ES experiments different buffers were used. This included a buffer of 10mM ammonium acetate in a solution of 50/50 water/methanol, adjusted to pH 3.2 with phosphoric acid and also a 30 mM phosphate buffer at pH 3.0 with 20% acetonitrile added to increase volatility.

5. Non-chromophoric herbicide analysis by CE.

5. (1). Initial attempts at visualising non-chromophoric chlormequat following CE. (Chapter seven)

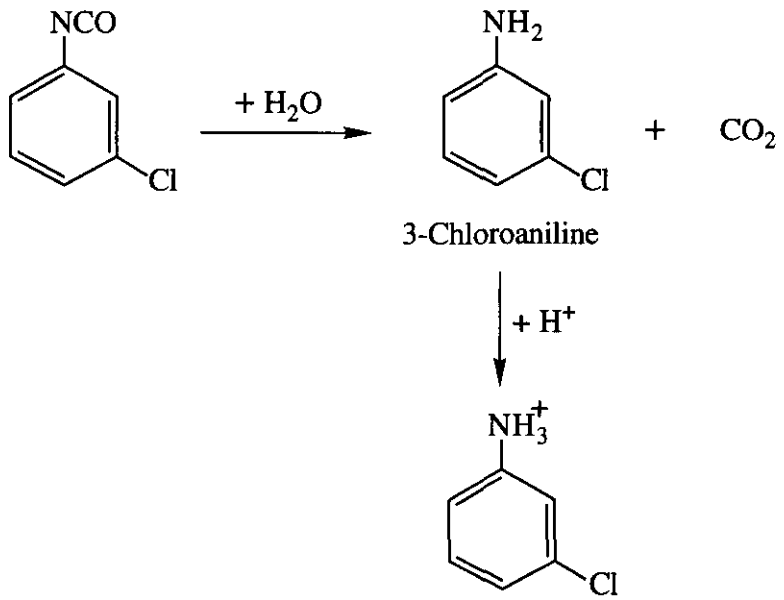
The first experiments to visualise chlormequat were performed using buffer solutions with additives of high absorbance. Firstly 30 mM benzoic acid was used and this was added to a 10mM ammonium acetate buffer at pH 3.5. This provided a high absorbance but did not allow detection of chlormequat. Another buffer was composed of 0.5 mM quinine sulphate dihydrate - 0.125 mM citric acid - 10% v/v methanol, pH 4.0 which is a standard buffer for indirect fluorescence detection[111]. The CE instrument used did not have a fluorescence detector but a filter of 300 nm wavelength was available. But again this buffer did not result in detection of the non-chromophoric chlormequat. Further experimentation involved using a buffer of creatinine at concentrations around 30mM and at pH 4.2. This buffer allowed the chlormequat to be visualised as an inverted peak as it passed the U.V. window. The creatinine buffer was then optimised in terms of concentration, (between 10 and 30mM) and pH, (between pH 3.6 and 4.2) for CE separation of six quaternary ammonium based compounds. Optimum conditions for CE separation were determined as 30mM creatinine and pH 3.6 but for CE/ES work the concentration of creatinine was reduced to 10 mM.

6. Diisocyanates. (Chapter Eight)

6. (1). Development of an internal standard for diisocyanate experiments.

A sample of 3-chlorophenylisocyanate was obtained and placed in water. The solution was then observed to see if any gaseous products, indicative of hydrolysis, were produced. It was thought that the sample may convert to 3-chloroaniline by adding it to water and in doing so would produce carbon dioxide as illustrated in Figure 21.

Figure 21.



To further test if 3-chloroaniline was produced, a sample of 3-chloroaniline and the 3-chlorophenylisocyanate in water were subjected to CZE analysis and the migration times of each compound measured.

6. (2). Optimising conditions for TDI isomer and internal standard separation.

The separation of two TDI isomers (2,4- and 2,6-toluene diisocyanate as their methoxyphenylpiperazine derivatives) was acheived using a 30mM phosphate buffer at pH 3.0 with added acetonitrile. Acetonitrile was added between 20 and 30% with 30% giving the best separation. However when an internal standard was added separation of the internal standard and TDI isomers was best at 20% acetonitrile. This meant that TDI isomer separation suffered so 25% acetonitrile was used.

7. Instrumentation.

7. (1) Fast Atom Bombardment mass spectrometry.

FAB mass spectrometry was carried out on two instruments. (a) For Xenon atom bombardment, a VG 20-250 instrument was used (at the Open University). An adapted saddle-field atom gun was operated with xenon (BDH Chemicals Ltd., 99.98%) at about 7 keV with a tube current of 1 mA. The instrument was calibrated with poly(ethylene glycol)[114] and operated with a gold-plated probe tip, negative-ion and positive-ion FAB spectra being recorded repetitively in alternate scans, each of 5 secs duration. (b) For caesium atom bombardment, a VG70-VSEQ was utilized (at St. Thomas' Hospital, London) with accelerating voltage of 8 kV. The VG caesium atom gun was operated at at 30 kV and, without ion source cooling, the ambient source temperature was about 50^0C. A stainless steel probe was used for FAB. The FAB ionization technique gives ions indicative of the relative molecular mass of the compound in abundance either in the positive ion mode as $[M + H]^+$ ions or in the negative ion mode as $[M - H]^-$ / M^- ions. Additionally, structurally diagnostic fragmentation of the molecule should be available with or without collision induced dissociation so leading towards MS-MS analysis. FAB also produces a spectrum with a relatively long lifetime. When solutions were measured it was sometimes unnecessary to use a matrix solvent but for other measurements it was necessary to carefully choose a suitable matrix. For example in the boron experiments (chapter 5) where glycerol frequently proved unsuitable a variety of other matrices were used. These included tetraethyleneglycol di-ethyl ether (TEGDEE), polyethyleneglycol (PEG 200), pentaethyleneglycol di-methyl ether and hexaethyleneglycol di-methyl ether.

7. (2). CE instrumentation used at the Open University.

All stand-alone CE work was performed on the Beckman P/ACE 200 instrument. This instrument allows a method or sequence to be set up which will suit the type of analysis being carried out. If required many different methods can be combined or the same one repeated in the form of a sequence. A typical method printout is shown in Figure 22, Appendix 2. All parameters can be set accordingly, whether they be voltage, UV absorbance wavelength, current, rinse times or separation and injection time. Once set these conditions are reproduced each time the instrument is operated. Temperature setting and regulation are

also easily controlled. Both injection options, those of voltage and pressure, are also fully automated for maximum reproducibility of injection volume. These instrument settings were controlled by and subsequent data handled by an IBM PC computer. An ultra-violet lamp was fitted to the instrument and various filters were available between 200 and 340nm. A variety of capillaries were utilized from a number of commercial sources, which included those with 50 and 75 μm internal diameters, both coated and uncoated.

7. (3) CE instrumentation used for CE/ES experiments.

Two CE instruments were used for CE/ESI work. The chromophoric pesticides were examined utilizing the ISCO 3850 manual CE system coupled to a VG Quattro instrument as were the dipeptide standards and urine samples. The non-chromophoric herbicides were analysed by a combination of the Beckman P/ACE 2100 CE instrument interfaced to a VG PLATFORM mass spectrometer. Diisocyanates were analysed using both systems. Standard polyacrylamide-coated silica capillaries of both 50 μm and 75 μm internal diameters and 90 cm in length were used in all cases. The VG PLATFORM is a benchtop single quadrupole system and the VG Quattro was used in the single quadrupole mode. Both instruments were equipped for CE/ES via a triaxial flow probe interface shown in Figure 23, Appendix 2. This probe supplies CE capillary flow, nebulising gas and electrospray make-up flow of 50/50 methanol or acetonitrile/water with 1% formic acid or acetic acid to the electrospray source. Throughout the length of the probe a 22 gauge stainless steel tube was used to deliver the makeup flow solvent whilst the nitrogen nebulising gas was also delivered coaxially. All flows converged at the probe tip where they were mixed and dispersed into droplets. CE/ESI mass spectra were obtained using make-up solution flow rates of 10 μl/min with the Isco system and 20 μl/min with the P/ACE 2100.

7. (3) a. P/ACE injection mechanisms.

The P/ACE instrument utilised a variable time length pressure injection to introduce fixed sample volumes illustrated in Table 3.

Table 3. Volume injected in nanolitres / second for capillaries of varying diameters.

Length of capillary (cm)

capillary i.d. µm	20	30	40	50	60	70	80	90
	Volume injected (nl/s)							
50µm	2.2	1.6	1.3	1.0	0.9	0.8	0.7	0.6
75µm	11.2	8.1	6.4	5.3	4.5	3.9	3.5	3.1
100µm	35.3	25.8	20.3	16.7	14.2	12.4	10.9	9.8

The P/ACE system also offered an electromigration injection system using applied voltage to introduce sample into the capillary, but this was not used during CE/ES studies.

During the analysis of chromophoric herbicides an automated method containing a multiple injection procedure was used and the method for this analysis is illustrated in Figure 24, Appendix 2.

7. (3) b. ISCO injection mechanism.

A 10 µl syringe was used to introduce samples onto the capillary with the ISCO CE. This was done via an injection splitter and calculations made as to the quantities of material introduced into the capillary. When using the ISCO system the amount of sample loaded can only be estimated by the following relationship:

$$V_c = \{\frac{d_c}{d_s}\}^4 \quad \{\frac{L_s}{L_c}\} \quad \{V_{syr}\}$$

Where:

V_c = Volume of sample injected into the capillary.

d_c = Internal diameter of the capillary column.

L_c = Length of the capillary column.

d_s = Internal diameter of the split-vent tube.

L_s = Length of the split-vent tube.

V_{syr} = Total volume of sample injected from the syringe.

7. (4) Experimental conditions using the ISCO CE system.

Buffers of 10mM ammonium acetate in a solution of 50/50 water/methanol, adjusted to pH 3.2 with phosphoric acid, were used for the diquat / paraquat analyses; all standards and

mixtures being prepared in buffer. Separation was carried out at 25 kV on a bare silica column of 75 μm i.d. at 25°C.

For the isocyanate experiments, a 30 mM phosphate / 30% CH_3CN solution (pH 3.0) was used with the bare silica column of 75 μm i.d. All derivatised MDI samples were made up in acetonitrile. Separation was carried out at 25 kV (25°C).

The dipeptide and urine analysis was performed on a 75 μm column utilizing a 30 mM phosphoric acid solution at pH 3.0 with 10% acetonitrile at a voltage of 25 kV (25°C). The CE/ES data were gained using standard solvents and a 10 μl/min flow rate.

7. (5) Experimental conditions using the P/ACE 2100 system.

The non-chromophoric herbicide mixture was separated using a 10 mM creatinine buffer at pH 3.6 with acetic acid at 25 kV and temperature of 25°C on a 50 μm i.d.x 90 cm capillary. The CE/ES data were gained using standard solvents and a 20 μl/min flow rate. Acquisition of data was initiated shortly after the peaks had passed the UV window. Oligopeptides were examined with a 10 mM β-alanine, 20% acetonitrile buffer at pH 4.5 with acetic acid for peptide work. A potential of 25 kV was applied across the capillary column. The CE/ES data were gained using standard solvents and a 20 μl/min flow rate. Acquisition of data was initiated shortly after the peaks had passed the UV window. The acquisition was not always begun at the same time after this had occurred so migration times of different experiments could not be compared. Acylcarnitines were analysed using a 20 mM ammonium acetate buffer at pH 3.8 with 20% acetonitrile added. To assist electrospray, a make-up flow of 50/50 methanol/water acidified with 1% formic acid was delivered to the probe tip at 10 μl/min and 20 μl/min, where it mixes with the CE buffer. This mixture is then nebulised using nitrogen gas which flows coaxially up the probe. A potential of +4 kV was applied to the probe tip for optimal electrospray performance. Mass spectral data were acquired using both selected ion recording (SIR; 0.2 secs dwell time, 0.2Da span) and full scan mode (300 - 750 Da in 2 secs).

Acylcarnitines were also analysed by CE/ES using the same arrangement as above but with a buffer composed of 15 mM ammomnium acetate, at pH 4.3 and 30% acetonitrile added.

8. CE/ES method development.

8. (1). Procedures used to combine Capillary Electrophoresis and Electrospray Mass Spectrometry.

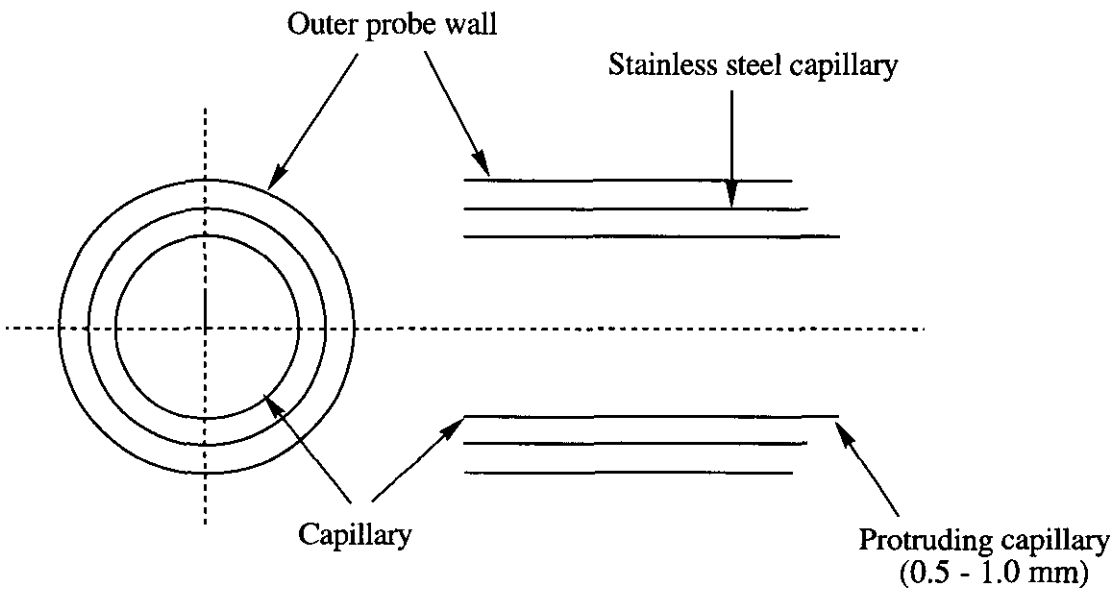
During this work two capillary electrophoresis systems (ISCO and Beckman 2100) and two mass spectrometers (Quattro and VG Platform) were used but in each case the coupling was achieved using the same triaxial probe as illustrated in Figure 23, Appendix 2. The probe was designed and produced at VG Biotech to be compatible with the mass spectrometers used. This probe allowed the capillary to reach the probe tip where the capillary flow, make-up flow and nebulising gas could mix in the source of the spectrometer. Probe tip voltage, nebulising gas and make-up flow could easily be removed by manipulation of the system. The probe itself is approx 30cm in length so 30cm of any capillary placed into it would be encased. Another 30cm of the capillary was encased in the CE cooling block. This meant that up to two thirds of the 1 metre capillary was not under any form of temperature control leading to heating effects within the capillary which in turn could cause non-uniform temperature gradients, local changes in viscosity and hence zone broadening. Some temperature regulating system could be integrated into the probe but this would still leave a third of the capillary uncooled. The ideal situation would be to combine the CE cooling unit and the triaxial probe into a single unit. This would mean that the length of the capillary could be changed within the CE cooling block where the capillary can be wrapped around a mandril and the entire length of the capillary could then be under uniform temperature control.

During the process of interfacing these two techniques many alterations had to be made to the experimental set-up. Most of these involved manipulation of the probe-tip set up and the extent to which the capillary and stainless steel delivery tube protruded was found to be critical to the stability of the electrospray and the efficiency of flow mixing. As was the concentricity of the tubes, all three tubes were kept as concentric as possible to avoid any flow disturbances. The situation at the probe tip is shown in Figure 25. The end of the capillary needed to be cut flush with no jagged edges to ensure an even flow of make-up solution and nebulising gas around the capillary tip where the buffer and analyte mixture elutes. Small adjustments were made to all these parameters until the best set of results was

obtained. The best results were obtained when each tube protruded between 0.5 and 1 mm from within each other. When the capillary protruded by more than 1mm the electrospray signal became unstable and the electrophoresis voltage collapsed. In the reverse situation when all the tubes are level or the capillary is actually inside the stainless steel tube, the ES signal is stable but the sensitivity of the system is up to a factor of 5 times poorer.

Figure 25.

Representations of the probe tip set-up during CE/ES.



The size of the stainless steel (S.S.) tube used to deliver the make-up flow and the rate of the make-up flow were also varied and it was concluded that a 22 guage S.S. tube and make-up flows of either 10 µl/min or 20 µl/min were used in subsequent analyses.

The CE injection process was tested whilst interfaced to the electrospray spectrometer. This was done using a mixture of quaternary ammonium herbicides. It was noticed that when the nebulising gas was on during the injection process, (pressure injection) three times more sample was placed into the capillary than when no gas was flowing, found by the intensity of the indirect absorbance traces after 20cm of capillary. Also, substantially less sample, around fifteen times less, was placed onto the capillary when the probe voltage (+4kV) was applied during pressure injection. These effects can be attributed to siphoning and reverse electromigration respectively.

When the nebulising gas was re-applied after injection it did however increase the migration speed of the sample through the capillary thought to be caused by siphoning effects. This also led to reduced separation of some mixtures as reported in the CE/ES separation results in chapters three and six.

Another effect investigated was the height of the injection port in relation to the electrospray source. It was found that the injection port should be level with the source to prevent excess sample being drawn into the capillary by siphoning. This was done by placing the CE system on an adjustable height trolley.

A further test of the injection procedure and the quantitative efficiency of this was to inject a series of peptide samples (15 - 640 fmol) in triplicate. Each successive sample was a dilution of the proceeding one. A sample was injected every two minutes and each time the nebulising gas and the probe-tip voltage were stopped and restarted after injection. The subsequent graph of the peak heights gave a correlation coefficient of 0.999 as explained in chapter 3, Section 2.(3).

8. (2). CE/ES experiments to improve quantitative viability of the method.

A sample mixture of six quaternary ammonium salts was injected into the CE capillary under different conditions. This was firstly injected whilst the nebulising nitrogen gas was flowing and a voltage was being applied at the tip of the triaxial flow probe. The procedure was then repeated but this time the nebulising gas was turned off before and during injection and only turned back on once separation had started. During this time the applied tip voltage of 5 Kv was still being applied. Finally both the tip voltage and the nebulising gas were removed during the injection procedure. These latter conditions were found to be best in order to place a reproducible sample in terms of volume into the capillary. This was established by comparing the U.V. traces after 20 cm of capillary.

8. (3). Other considerations for CE/ES.

(a) The use of buffer ions to monitor method performance.

A variety of buffers was used during the CE/ES work. Within two of these buffers were

molecules which supplied abundant background ions in the electrospray source. The presence of the $[M + H]^+$ ions (*m/z* 90) produced by the β-alanine (10 mM) in a buffer of pH 4.5 used for oligopeptide work and the ion at *m/z* 114 produced by the creatinine (10mM) in a buffer of pH 3.6 used to analyse herbicide mixtures, allowed the abundance and stability of the ion signal to be monitored. As the buffer was continually being pushed into the electrospray source it enabled electrospray performance and mixing of the flows at the probe tip to be assessed and optimised.

(b). Adjustment to the capillary electrophoresis separation voltage.

Separation voltages of 20 or 25 kV are standard for CE but to apply these voltages during CE/ES, voltages of 25 or 30 kV have to be applied. This is because the probe-tip voltage of +4 to 5kV is in anti-phase to that of the CE separation and so subtracts 4 to 5kV from the CE voltage. Similarly during negative electrospray the probe-tip voltage of -4 to 5kV is in phase and hence adds to the overall voltage across the system.

(c). Rate of make-up flow.

For the analyses in this thesis, make-up flow rates of 10 and 20 µl/min were used. Any attempts to decrease or increase the flow rate led to a deterioration in CE/ES performance or loss of CE separation voltage. Flows were also varied by the use of different stainless steel sheath tubes. A 22 guage sheath tube proved to give the best results, and when a 21 guage tube with a smaller internal diameter was used the electrospray became very unstable. This smaller tube restricted the flow of the make-up liquid as less space was available between the capillary and the inner sheath tube wall.

9. Reagents.

All the standard peptides examined during CE/ES studies were obtained from Sigma Chemical Co. as were the dipeptides. Spiroborates were kindly donated by Y. Okamoto (Kitasato University, Japan) and non-commercial boronic acids by P.D.G. Dean (Liverpool University). Diquat dibromide, paraquat dichloride and the internal standard (1,1'-diethyl-4,4'-bipyridyldiylium diiodide) were obtained from the Plant Protection Division of ICI, at Yalding. Derivatives of isocyanates were obtained from the Occupational Medicine and

Hygiene Laboratories of the HSE (David Bagon, John Groves and Peter Ellwood). Chlormequat, choline chloride, trimethylvinylammonium hydroxide, trimethylamine hydrochloride and the internal standards triethylamine and isopropylamine were obtained from Aldrich Ltd. The field samples containing chlormequat were supplied by the HSE. Other reagents, buffers and chemicals were purchased from commercial sources at the highest purity obtainable.

10. References.

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Appendix 2

Figures from Chapter 2.

Figure 22. A typical P/ACE 2000 capillary electrophoresis method printout.

Method: 24 Apr 94 14:47

Display Channel A with grid lines
Time: 0.0 to 20.0 Minutes
Channel A: 0.000 to 0.040 Absorbance
Print Method

STEP	PROCESS	DURATION	INLET	OUTLET	CONTROL SUMMARY
1	SET TEMP				Temp: 25 C Wait until reached
2	SET DETECTOR				UV: Filter: 3 - 280 nm Rate: 5 Hz Range: 0.200 -10% Normal Rise Auto Zero 0.0 min
3	RINSE	2.0 min	12	10	Forward: High Pressure
4	RINSE	1.0 min	33	10	Forward: High Pressure
5	INJECT	5.0 sec	11	1	Voltage: 5.0 kV
6	SEPARATE	10.0 min	34	1	Elect: Const Voltage: 25.0 kV Const Buffer

Figure 23. The VG BioTech triaxial flow probe for CE/ES.

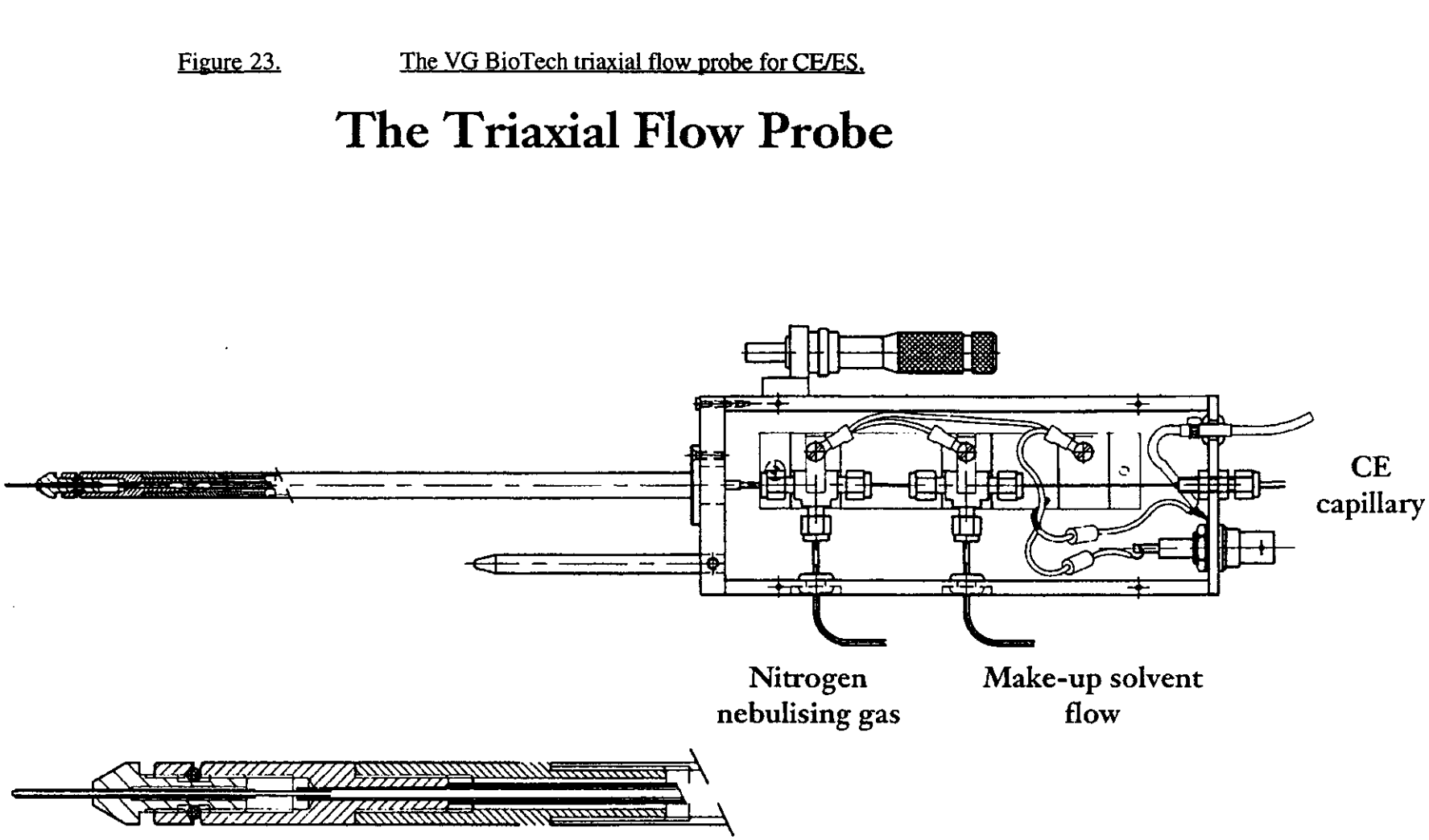


Figure 24. A P/ACE 2000 capillary electrophoresis method printout illustrating the multi-injection capability of the instrument.

Method: C:\MULIMETH.MTD 24 May 94 16:14

Display Channel A with grid lines
Time: 0.0 to 20.0 Minutes
Channel A: 0.000 to 0.040 Absorbance

STEP	PROCESS	DURATION	INLET	OUTLET	CONTROL SUMMARY
1	SET TEMP				Temp: 25 C
2	SET DETECTOR				UV: Filter: 1 - 214 nm Rate: 5 Hz Range: 0.200 -10% Normal Rise Auto Zero 0.0 min
3	RINSE	1.0 min	12	10	Forward: High Pressure
4	RINSE	2.0 min	33	10	Forward: High Pressure
5	INJECT	5.0 sec	11	1	Pressure
6	SEPARATE	4.0 min	34	1	Elect: Const Voltage: 25.0 kV Const Buffer
7	RINSE	1.0 min	12	10	Forward: High Pressure
8	RINSE	2.0 min	33	10	Forward: High Pressure
9	INJECT	5.0 sec	11	1	Pressure
10	SEPARATE	4.0 min	34	1	Elect: Const Voltage: 25.0 kV Const Buffer

Chapter Three - CE and CE/ES analysis of peptides with investigations into the presence of dipeptides in neonatal urine samples.

1. Introduction.

Dipeptides are excreted in urine of healthy individuals only in minute amounts. Strongly increased excretion of some dipeptides is observed in individuals suffering from some diseases, especially those concerning collagen metabolism and bone disorders[115,116]. Case studies of patients with peptiduria and especially glycylprolinuria have shown these conditions to be associated with a number of disorders. In one case a child suffering from chronic skin ulceration and edema was shown by mass spectrometry to excrete dipeptides containing proline/hydroxyproline. Other patients suffering from dermatological problems such as skin rashes and leg ulcerations showed massive iminodipeptiduria[117]. Most of the cases reported of iminodipeptiduria result due to defective collagen metabolism though urinary peptides could result from central nervous system disorders such as Huntingdons' chorea. Urinary dipeptides have also been found in patients suffering from McCune-Albright syndrome. This is the name that is used to describe a condition which includes polyostotic fibrosis dysplasia, pigmentation of certain areas of the skin and endocrine dysfunction with precocious puberty[118]. Another group has noticed dipeptides in urine obtained from babies with metabolic dysfunction concerning energy metabolism. Whether these two facts are coincidental or not is still to be investigated. During the course of work on urinary acylcarnitines by GC/MS it was noticed that several neonates excrete high levels of certain dipeptides (e.g. Ala-Leu or Ala-Ile, Pro-Ile or Ile-Pro.) The relevant samples have been studied by CE and CE/ES which has allowed speculative assignment of some of the peaks present.

2. Results and Discussion.

2. (1). Initial dipeptide separations.

Various buffers were used for these investigations as optimum conditions were sought for dipeptide separation. Standard phosphate or borate buffers were used as well as others

containing ammonium acetate, β-alanine or citrate typically made up as stated in Section 1 (3). of Chapter 2. This also meant exploiting a range of pH values and concentrations. Separation of a ten dipeptide mixture (Figure 26, Appendix 3) was easily attained within a separation time of 10 minutes, using a phosphate buffer at pH 2.5. The dipeptides used are listed in Table 4.

Table 4. - List of dipeptides used and their molecular weights.

Dipeptide	Abbreviation	Molecular Weight
Glycyl - glycine	Gly - gly	132.0
Alanyl - glycine	Ala - gly	146.1
Valyl - glycine	Val - gly	174.0
Leucyl - glycine	Leu - gly	188.2
Prolyl - glycine	Pro - gly	172.0
Glycyl - valine	Gly - val	174.0
Glycyl - tryptophan	Gly - trp	261.0
Alanyl - isoleucine	Ala - ile	202.3
Prolyl - isoleucine	Pro - ile	228.5
Prolyl - phenylalanine	Pro - phe	262.0

As can be seen from this table the dipeptide molecules separated are small molecules with very similar molecular weights yet the CE technique copes with this separation with a minimum of buffer preparation. Separation would depend upon the size, shape, isoelectric point and acid or alkaline nature of the various dipeptides. A similar separation was acheived using an ammonium acetate buffer also at pH 2.5. Upon repetition of the separation the peaks were seen to drift to longer migration times by up to 39 seconds after 4 repetitions. This was put down to changes at the inner capillary wall between subsequent analyses. Reproducibility of migration time for one dipeptide could easily be attained if buffers between pH 6 and 8 were used but under these conditions no separation of a mixture of dipeptides could be acheived. During these analyses a 0.1M sodium hydroxide rinse was

used between each analysis which meant that the capillary walls should be stripped and returned to their native state between each analysis. However it was thought that if the NaOH rinse was not done, reproducibility would eventually improve as the situation would exist where all the silanol sites would be bound by the positively charged hydrogen ions in the acidic buffers used which would not have been removed by an alkaline rinse. This meant that a stable unchanging environment would be maintained where electro-osmotic flow would also be stable. To test this a single dipeptide was analysed six times by repeated electrophoresis. Three rinsing procedures were tried (A - C in Chapter 2, Section 2 (4)). With the NaOH rinse (A) between analyses an upward drift of 64 seconds in migration time from first to last repetition was observed. Without this rinse (B) an upward drift of 25 seconds in migration time from first to last repetition was observed. And using rinse procedure (C) between analyses a downward drift of only 12 seconds in migration time from first to last repetition was seen. Between repetitions 3 and 5 there was a variation of only 5 seconds as the column seemed to be equilibrating. From this experiment it seemed that an NaOH rinse alone was detrimental to the reproducibility but it could be used if the capillary was re-equilibrated with a more concentrated buffer rinse and normal buffer rinse between analyses.

2. (2) Analysis of dipeptides and pre-treated urine samples using coated capillaries.

The analysis of dipeptides was continued using a Supelchem coated H150 capillary employing C8 units bonded to its walls. These capillaries are described as being mildly / moderately hydrophobic and were used to prevent silanol groups interacting with the dipeptides and hampering reproducibility. With these capillaries it wasn't necessary to have an intermediate rinse with a concentrated buffer. However for the first analyses a 1 minute NaOH rinse was still used. A six-component dipeptide mixture comprising, Prolyl-Isoleucine, Glycyl-Tryptophan, Alanyl-Glycine, Phenylalanyl-Proline, Alanyl-Isoleucine and Prolyl-Phenylalanine was used and they were separated using a buffer of 40mM phosphoric acid at pH 3.2. A chromatogram of the first two analyses of these dipeptides overlaid is given in Figure 27 (App. 3). As can be seen the reproducibility is within 3

seconds for the slowest migrating component. This was a great improvement upon results obtained using uncoated capillaries.

The next experiment involved using a urine sample suspected of containing either alanyl-leucine / isoleucine or prolyl-ilsoleucine / leucine. The samples analysed were obtained from patients at Sheffield hospital who had been found to have acylcarnitines in their urine and during the analysis by GC/MS, dipeptides were also found. The sample was analysed using the same coated capillary and buffer as for the previous dipeptide separations. Figure 28 (App. 3) illustrates the separation acheived and 5 major peaks can be observed. It was thought that the largest peak may be due to creatinine which is a major component of urine and would have passed through ion exchange chromatography along with any peptides or dipeptides if present. The next stage in these investigations was to couple CE with ES which would allow identification or at least further characterisation of the peaks observed from the urine sample.

2. (3) CE/ES of standard peptides.

A mixture of 5 standard peptides (Angiotensin I, II and III, Bradykin and Leu-Enkephalin-Arg) was analysed by CE/ES. The separation was acheived using a 10mM β-alanine buffer at pH 4.5. The β-alanine served 2 purposes, it acted as the buffering agent and it supplied an ion at *m/z* 90 which could be used to tune the electrospray initially and allowed constant monitoring of the electrospray signal during the procedure. The resultant base peak intensity (BPI) chromatogram (full scanning) obtained from 2.5 pmoles each of the standard peptides is shown in Figure 29 (App. 3). The mass spectra, of which Figure 30 (App. 3) is one example, illustrate that the doubly $[M + 2H]^{2+}$ or triply $[M + 3H]^{3+}$ charged species of each peptide is most prevalent. Figure 31 (App. 3) shows the BPI of the same five peptides obtained in SIR mode with 300 fmoles of each being injected onto the capillary over 5 seconds. Baseline separation of all the peptides is easily acheived and the components identified by the mass of their positive ions.The migration order was established as Angiotensin III (mass 883.1), Bradykinin (mass 1060.2), Angiotensin I (1296.5), Leu-Enkephalin-Arg (mass 711.8) and Angiotensin II (1046.2). Migration order was based mainly on charge, mass size and shape. Despite having the largest mass, angiotensin I

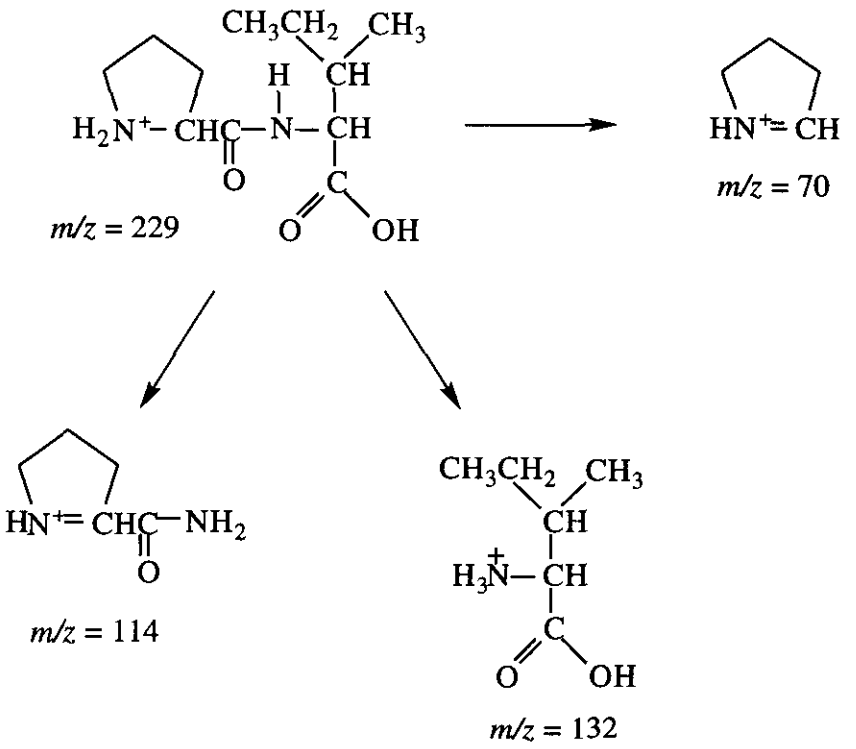
migrates rapidly through the capillary because it has the largest number of basic residues in its amino acid sequence. Consistent with this the mass spectrum (Figure 30, App. 3) displays a prominent $[M + 3H]^{3+}$ ion.

The combined CE/ES signal reproducibility and sensitivity was examined by introducing three 5-second hydrodynamic injections of angiotensin II at five different concentrations from 220pg/nl to 5pg/nl (630 fmol to 15 fmol) with the most concentrated sample being injected first. The SIR chromatogram of these injections is shown in Figure 32 (App. 3), illustrating a detection limit of less than 15fmol injected at which the signal to noise ratio is about eight. The signal reproducibility was found to be very good, less than 10% variation in the peak areas was observed. Constructing a calibration curve of peak heights compared to sample concentration gave a graph with a correlation coefficient of 0.999. This illustrates the fact that multiple injections can be performed without sample carry-over and even though both nebulising gas and probe tip voltage had to be disconnected before injection the system was stable enough to continue operation. Also no rinsing procedures were required between analyses.

2. (4). Electrospray mass spectrometry and CE/ES of dipeptides.

Firstly a single dipeptide was analysed using electrospray mass spectrometry to help understand the fragmentation pattern of these molecules. An electrospray spectrum was produced using a sample of prolyl-phenylalanine (5ng/µl). Peaks were observed for the protonated molecule at *m/z* 263 and a proline fragment at *m/z* 70. The two dipeptides observed by GC/MS (ala-leu and pro-ile or combinations of these) were then investigated by CE/ES. The $[M + H]^{+}$ ions at m/z 229 and 203 were observed and another major ion at m/z 132 was also seen as a fragment of the m/z 229 ion. The fragmentation proposed is illustrated in Figure 33.

Figure 33. - Typical fragmentation pattern of the dipeptide prolyl-isoleucine.



A standard mixture of 7 dipeptides all within the mass range (Mr = 140 to 368) was then examined by CE/ES using a 75μm capillary. Each dipeptide in this mixture was at a level of 40 μg/ml with an estimated 3 pmole of each being injected into the capillary. Figure 34 (App. 3) shows the separation achieved under CE/ES, using an ammonium acetate buffer adjusted to pH 3 with phosphoric acid. Its addition produces an involatile acidic buffer. This has been shown to cause little problem to the analysis due to dilution by the make-up solvent which dilutes the CE flow by 1000 times.

As the chromatogram in Figure 34 (App. 3) shows the mixture was separated and detected but only six dipeptide peaks out of the seven could be observed. Using CE/ES the migration order of the dipeptides could be determined as Ala-gly, Ala-ile, Leu-gly, Pro-ile, Gly-trp, Pro-phe, Phe-phe and Trp-tyr. Within the combined SIR spectra only six peaks are seen as the leucyl-glycine and prolyl-isoleucine are detected so close together that the two are superimposed to make one large peak at 23.18 minutes. Comparing the separation efficiency using both techniques shows average number of theoretical plates (N) for 6 peaks by CE alone at 55,500 and 30,800 using CE/ES.

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

where: t = migration time.

$w_{\frac{1}{2}}$ = peak width at half height.

Compared to the separation observed by CE alone CE/ES separation is relatively poor and resolution suffers. Using CE alone six dipeptides were completely separated in 13 minutes within a 3 minute period compared to 28 minutes and a 7 minute period to obtain a lesser separation by CE/ES. Capillaries with the same internal diameters were used for CE and CE/ES with the biggest difference being the connection to the ES source. Physically attaching the two systems together meant that a much longer capillary than normal had to be used (up-to a metre long compared to 50cm) and around two thirds of this was not under the temperature control of the CE system. This would cause some heating over this length of the capillary, leading to a subsequent degradation of resolution. Perhaps the largest effect which would result in the sample being pulled through the capillary faster than normal would be siphoning effects (see experimental, Section 6. (1)) from the nebulising gas and the levels at which the ES source and CE system are in relation to each other. These effects were examined in later analyses looking at other samples.

2. (5). Electrospray mass spectrometry and CE/ES of a urine sample.

Electrospray mass spectrometry was performed on the urine sample of interest. This was done in order to give some idea as to what peaks were present before CE/ES analysis. The full spectrum of the sample (m/z 0 - 300) is given in Figure 35 (App. 3). The base peak was observed at m/z 114 and other major peaks were observed at 132, 144 and 162. The dipeptides of interest would be expected at m/z 229 and 203. On investigation of the mass spectrum a small peak is apparent at these m/z ratios along with one at m/z 227. The peak at m/z 229 was then examined by MS/MS and the recorded daughter ions included ions, above 20% abundance compared to the "parent" ion, at m/z 70 and 142 as well as ions at m/z 132, 124 and 114 below 10% abundance. The structure of the prolyl-isoleucine dipeptide ion and probable fragment ions are illustrated in Figure 33. The same conditions used to analyse the dipeptide mixture were also used to analyse the urine sample by CE/ES. The urine sample

gave the SIR trace in Figure 36 (App. 3). Various peaks of interest were detected. For instance the peak corresponding to m/z 114 is most likely due to creatinine, and a peak of m/z 132 is possibly due to the amino acids leucine or isoleucine. An alternative source could be fragment ions (leucine or isoleucine) from the dipeptide, m/z 229 in Figure 35 (App. 3) though this was very small. Other peaks of major abundance were observed at m/z 121, 144 and 162 although no characteristic fragments or molecules could be determined which would lead to the identity of these ions.

3. Conclusions.

It is hoped that further investigations using the CE/ES technique would fully identify all molecules within the urine samples. With the separation power offered by capillary electrophoresis and the positive identification capabilities of electrospray mass spectrometry CE/ES may be an appropriate method for detecting small peptides in purified blood and urine samples. Further development of this method would include running the procedure on a routine basis if possible and exploring the reproducibility of the technique.

4. References.

(115). Jandyke, J. and Spiteller, G.; *J. Chromatogr.*, **382**, (1986), 39-45.

(116). Wagner, F.W., Kapleau, B.R. and Shepherd, S.L.; *Biochemical Medicine.*, **13**, (1975), 343-352.

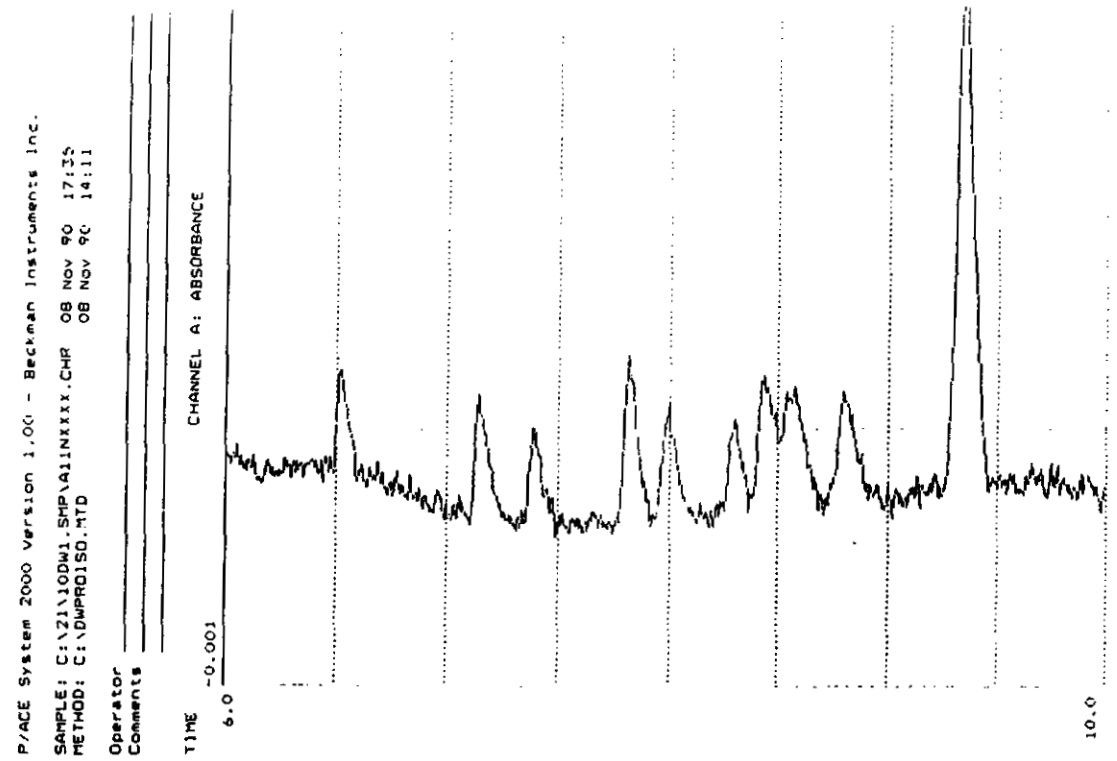
(117). Charpentier, C., Johnstone, R.A.W., Lemonnier, A., Myara, I., Rose, M.E. and Tuli, D.; *Clinica. Chem. Acta.*, **138**, (1984), 299-308.

(118). Gortatowski, J., Shaw, K.N.F. and Schroeder, W.A.; *Biochemical medicine.*, **5**, (1971), 348-370.

Appendix 3

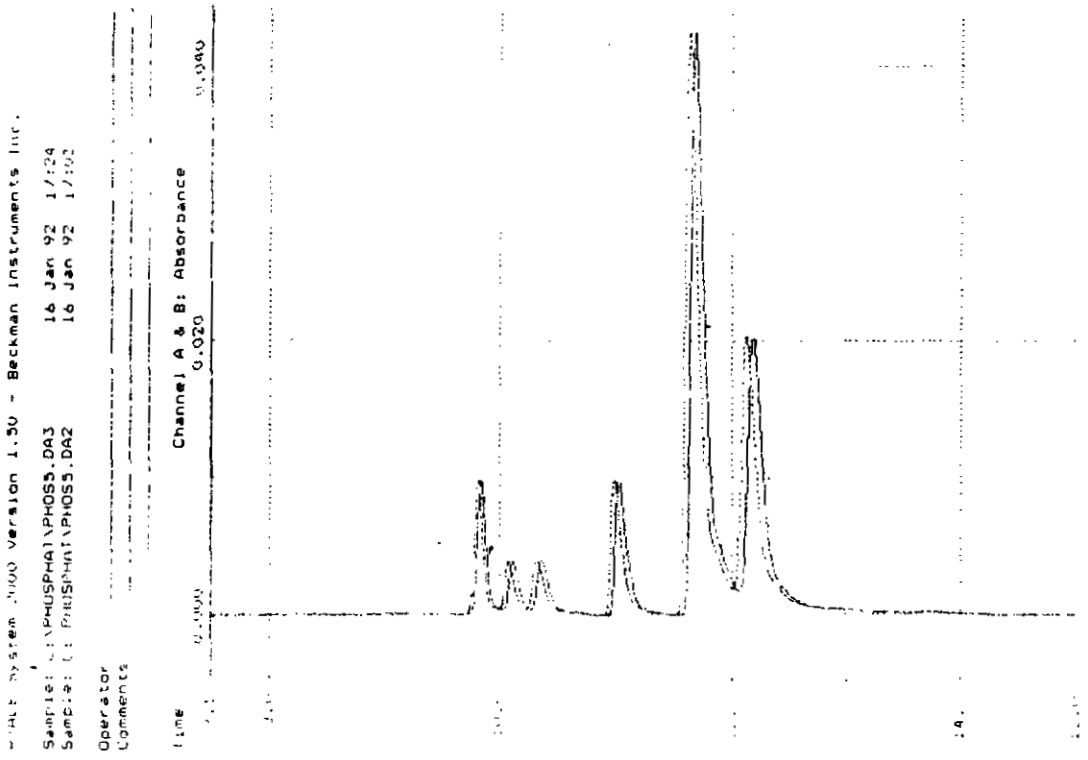
Figures from Chapter 3.

Figure 26. Chromatogram of the capillary electrophoresis separation of ten standard dipeptides listed in Table 4.



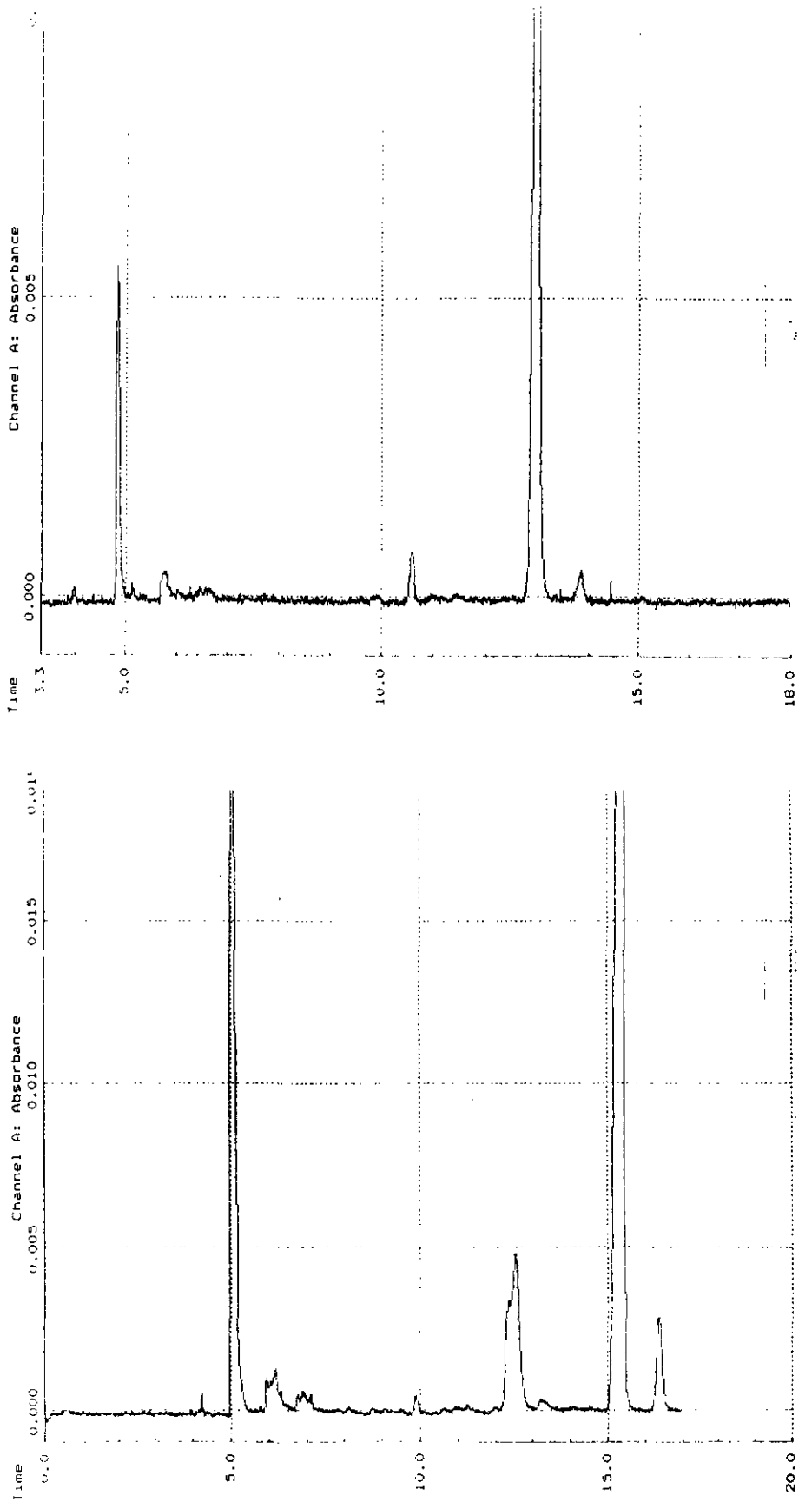
Buffer: 30 mM sodium hydrogen phosphate, adjusted to pH 2.5 with hydrochloric acid. Capillary: 75 µm i.d. x 50 cm; separation at 25 kV at 25°C; detection: uv absorption at 214 nm.

Figure 27. Overlaid chromatograms of the capillary electrophoresis separation of six standard dipeptides.



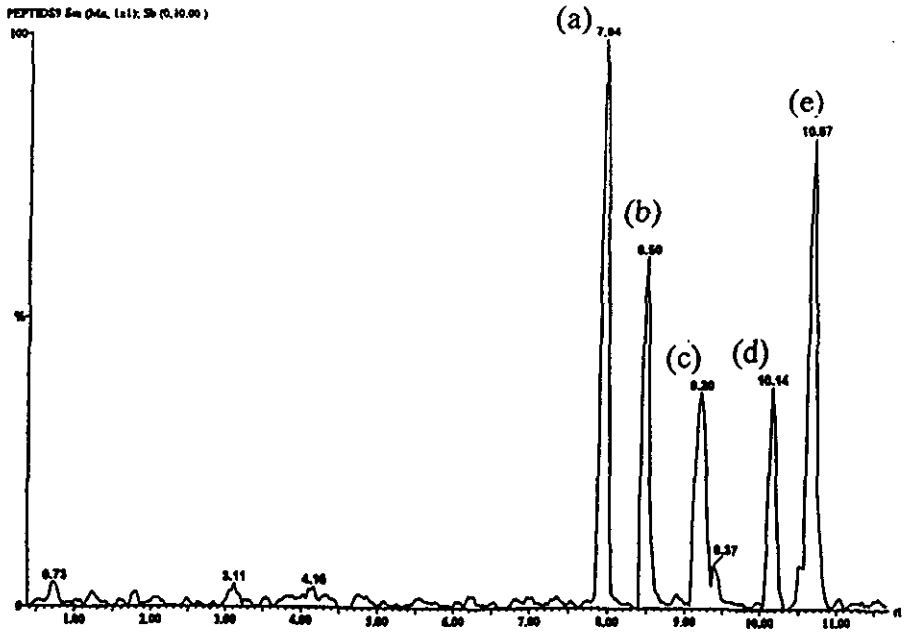
Buffer: 40 mM phosphoric acid solution at pH 3.2. Capillary: 50 µm i.d. x 50 cm; separation at 25 kV at 25°C; detection: uv absorption at 214 nm.

Figure 28. Chromatograms of the capillary electrophoresis separation of neonatal urine samples.



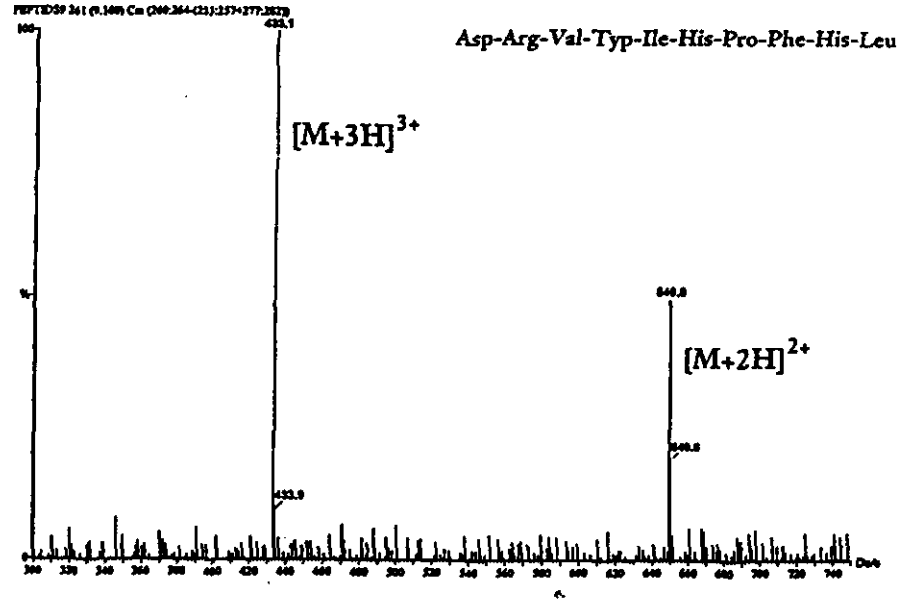
Buffer: 40 mM phosphoric acid solution at pH 3.2. Capillary: 50 µm i.d. x 50 cm; separation at 25 kV at 25°C; detection: uv absorption at 214 nm.

Figure 29. BPI chromatogram of the five peptide mix (2.5 pmole each) obtained by full scanning.



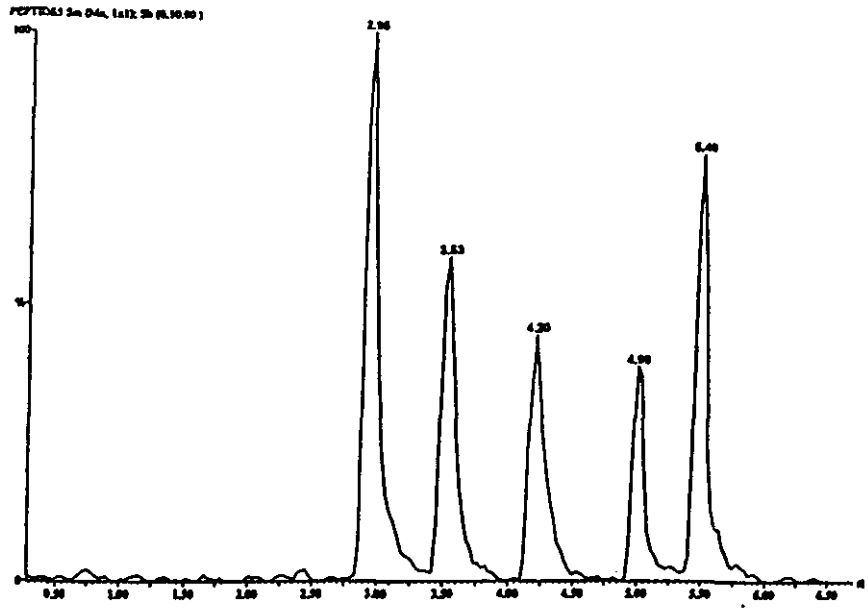
Migration order: (a). Angiotensin III, (b). Bradykinin, (c). Angiotensin I, (d). Leu-Enkephalin-Arg and (e). Angiotensin II.

Figure 30. Mass spectra of angiotensin I, illustrating the two types of ions formed under electrospray mass spectrometry.



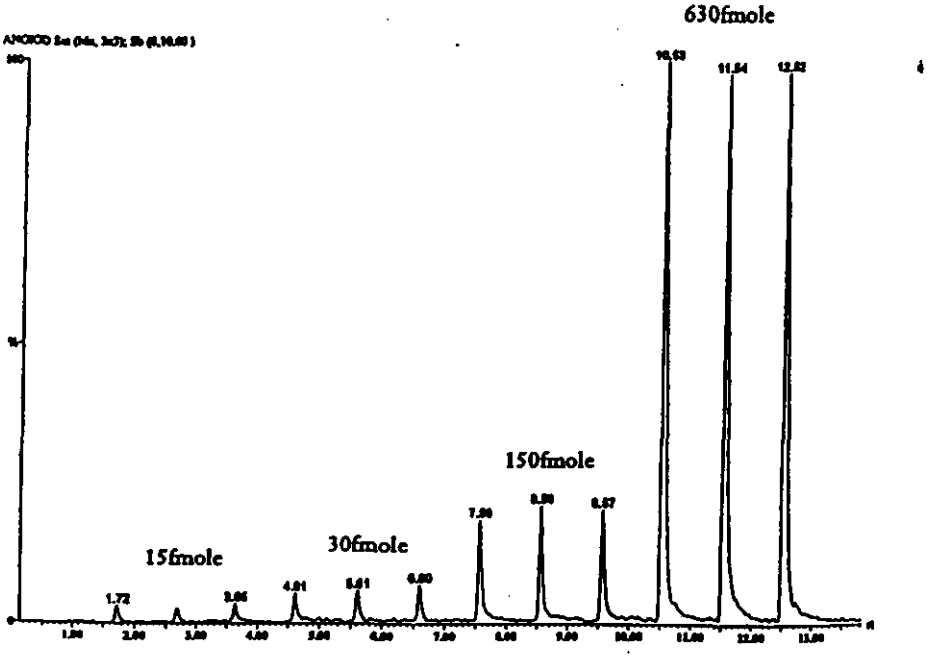
Buffer: 10 mM β-alanine / 20% acetonitrile adjusted to pH 4.5 with ethanoic acid.Capillary: 50 μm i.d. x 90 cm; separation at 21 kV at 25°C; detection: electrospray mass spectrometry.

Figure 31. BPI chromatogram of the five peptide mix (300 fmole each) obtained by SIR.



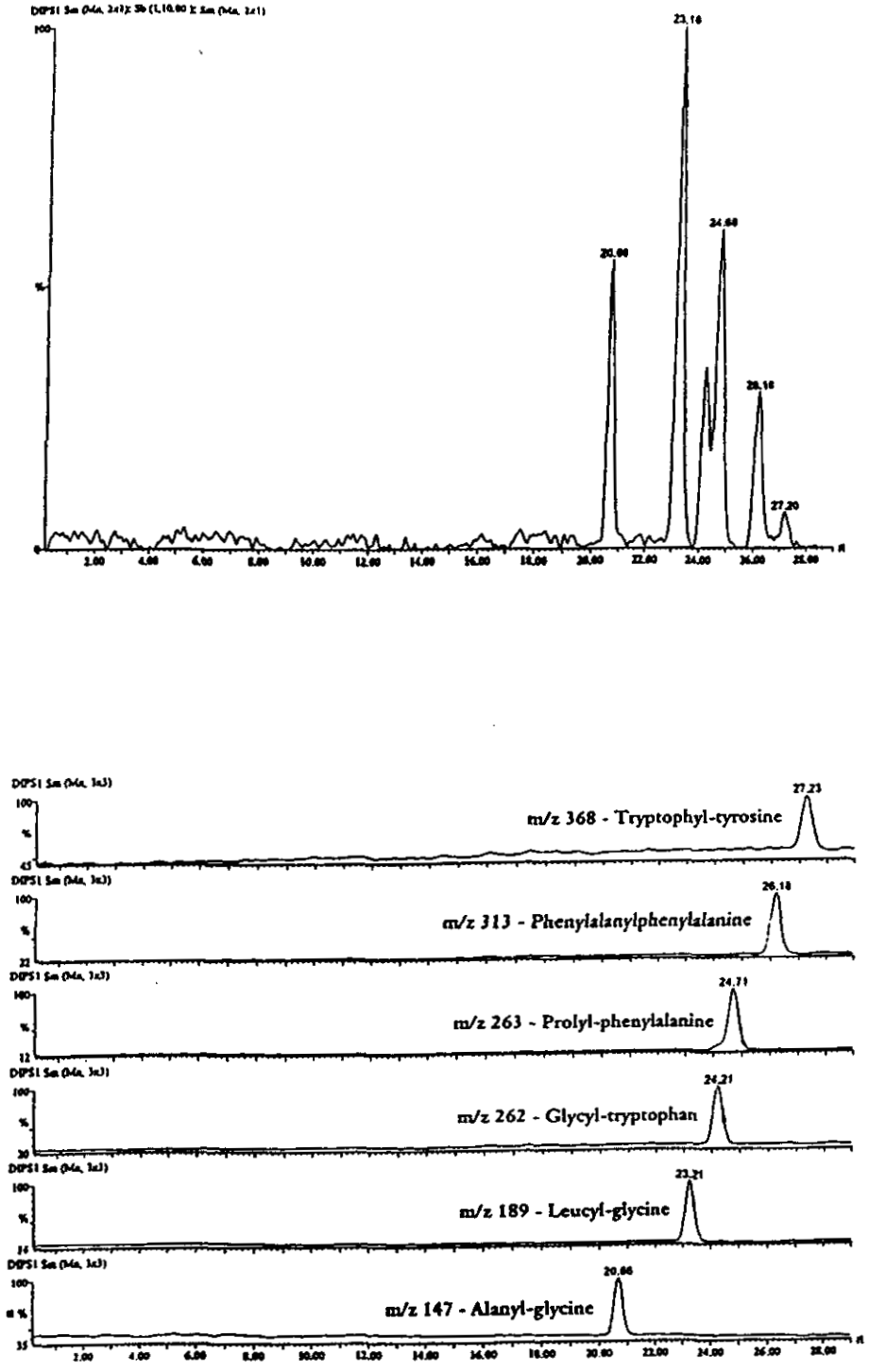
Buffer: 10 mM β-alanine / 20% acetonotrile adjusted to pH 4.5 with ethanoic acid.Capillary: 50 μm i.d. x 90 cm; separation at 21 kV at 25°C; detection: electrospray mass spectrometry.

Figure 32. System reproducibility and sensitivity chromatogram using angiotensin II.



Buffer: 10 mM β-alanine / 20% acetonotrile adjusted to pH 4.5 with ethanoic acid.Capillary: 50 μm i.d. x 90 cm; separation at 21 kV at 25°C; detection: electrospray mass spectrometry.

Figure 34. SIR chromatograms of the CE/ES of a mixture of seven standard dipeptides.



Buffer: 30 mM phosphoric acid / 10% acetonitrile at pH 3.0. Capillary: 75 µm i.d. x 90 cm; separation at 21 kV at 25°C; detection: electrospray mass spectrometry.

Figure 35. Electrospray analysis of a neonatal urine sample

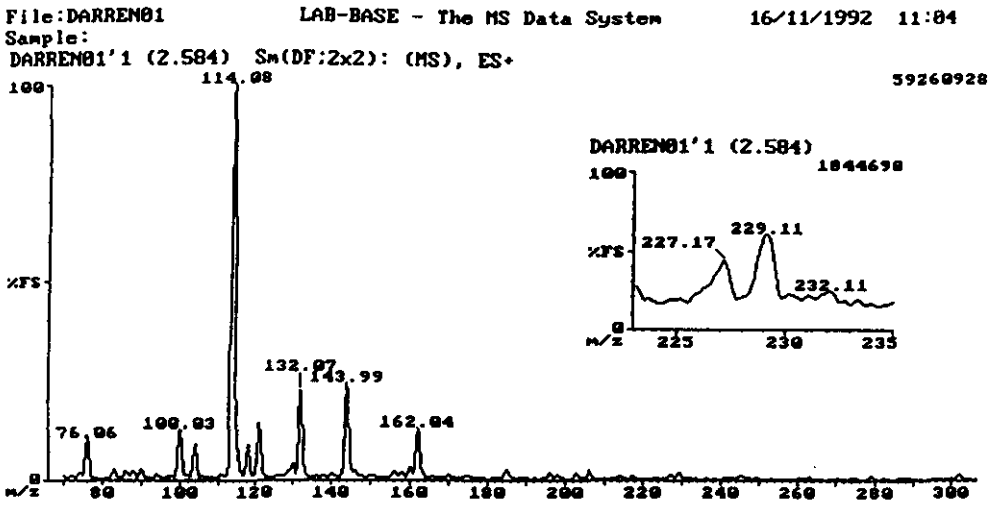
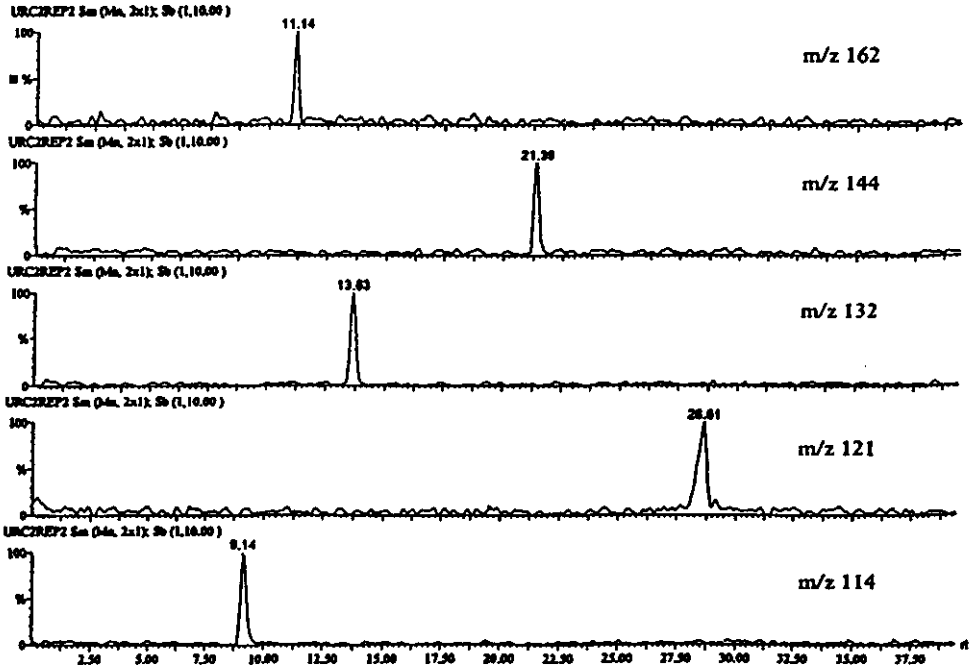


Figure 36. SIR chromatogram of the CE/ES of a neonatal urine sample.



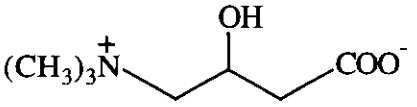
Buffer: 30 mM phosphoric acid / 10% acetonitrile at pH 3.0. Capillary: 75 µm i.d. x 90 cm; separation at 21 kV at 25°C; detection: electrospray mass spectrometry.

Chapter Four

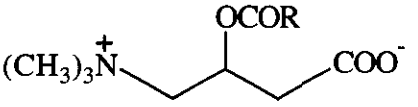
Exploring Acylcarnitines using CE and CE/ES.

(1). Acylcarnitines - action and interaction.

Carnitine (**4.1**), is a zwitterionic compound formed from lysine and is the molecule that facilitates around 10% of the body's energy requirements by allowing the transportation into mitochondria and metabolism of long-chain free fatty acids. This is achieved by the formation of acylcarnitines (**4.2**) when Acyl CoA complexes are combined with the carnitine as a transport mechanism. The fatty acids transported by this mechanism are an extremely valuable source of energy which is only accessible because of carnitine.



(4.1)



(4.2)

Fatty acids are metabolised via the β-oxidation pathway which provides a major part of the energy in some tissues when the animal is in the fed state and becomes a vital metabolic pathway during fasting. Most energy is formed when the fatty acids are oxidised in extrahepatic mitochondria where they are completely oxidised and the oxidation process is coupled to ATP synthesis. Fatty acids are transported around the body by the circulatory system bound to molecules of serum albumin or as triglycerides, due to their lack of solubility within the blood. Various mechanisms by which free fatty acids cross the plasma membrane when they enter cells have been proposed. Liver cell uptake has been linked to a fatty acid binding protein present in the plasma membrane. Other proposals include a link to active transport mechanisms via sodium, and hormones such as adrenalin and insulin have also been found to regulate fatty acid cellular intake. But there is still opposition to these mechanisms which argues that free fatty acids enter cells by no other means than by passive diffusion across the membrane. Fatty acids are activated by complexing them to coenzyme A during an acylation reaction to form acyl CoA compounds. This occurs on the outer mitochondrial membrane, whereas they are oxidised in the mitochondrial matrix. Mitochondria are decompartmentalised organelles which contain all the enzymes required for

the β-oxidation process. These enzymes have overlapping chain length specificities and act on particular chain-length substrates. Medium-chain acyl CoAs will permeate into the mitochondrial matrix directly but long-chain acyl CoA molecules do not readily traverse the inner mitochondrial membrane, and so a special transport mechanism is required. Activated long-chain fatty acids are carried across the inner mitochondrial membrane by carnitine. The acyl group is transferred from the sulphur atom of CoA to the hydroxyl group of the carnitine to form acyl carnitine (Figure 37).

Any deficiencies in the enzymes involved in β-oxidation will lead to an accumulation of a specific acyl Co-A complexes which can have toxic effects[119,120]. The carnitine responsible for transporting the acyl-CoA in the first place will also conjugate with excess acyl-CoA to form acylcarnitines in biological fluids at abnormally high levels. Detection of acylcarnitines and more specifically identification of the chain lengths of these will be indicative of a particular enzyme disorder. A defect in the translocase, or a deficiency of carnitine might be expected to impair the oxidation of long-chain fatty acids and so cause serious illness and possibly even death. Hence it was very important that some technique be introduced to analyse for compounds which would accurately show whether such an event had occurred, whether it be hereditary at birth or the result of a failure during later life. The role of acylcarnitines in some cases of Sudden Infant Death Syndrome (SIDS) has been extensively studied by Rose and co-workers who have used gas chromatography and mass spectrometry to analyse for these compounds. This research has confirmed that accumulation of various acylcarnitines within blood and urine is indicative of metabolic disease. Several methods are available for detection of acylcarnitines.[121] The most successful approaches involve mass spectrometry, especially the fast atom bombardment (FAB) method developed by Millington et al.[122-125] To date, the combination of continuous-flow FAB and tandem mass spectrometry (MS/MS) offers the most successful screening for acylcarnitines, despite the relatively expensive instrumentation. Less costly approaches are based on derivatisation of acylcarnitines to acyloxylactones [126,127] or to N-demethylated esters[128] followed by capillary-column gas chromatography / mass spectrometry. The experiments performed on acylcarnitines within this thesis primarily involve CZE and electrospray mass spectrometry

(ESMS) techniques which have not been, as yet, fully evaluated as methods of analysis for these analytes.

2. Methods for Analysis of Acylcarnitines.

As the acylcarnitine samples are generally extracts of blood or urine most analytical methods used to analyse for them require an extensive clean-up procedure to be carried out. Some methods of analysis require minimal sample preparation but for those used in this thesis ion-exchange chromatography was used to isolate the acylcarnitines (see Chapter 2, Section 1(2)). The only problem is that acylcarnitines as zwitterion molecules are not separated from other zwitterion molecules such as small peptides and amino acids.

2. (1) Nuclear Magnetic Resonance (NMR)

This method has been used to analyse for high concentrations of acylcarnitines but not for physiological trace levels.

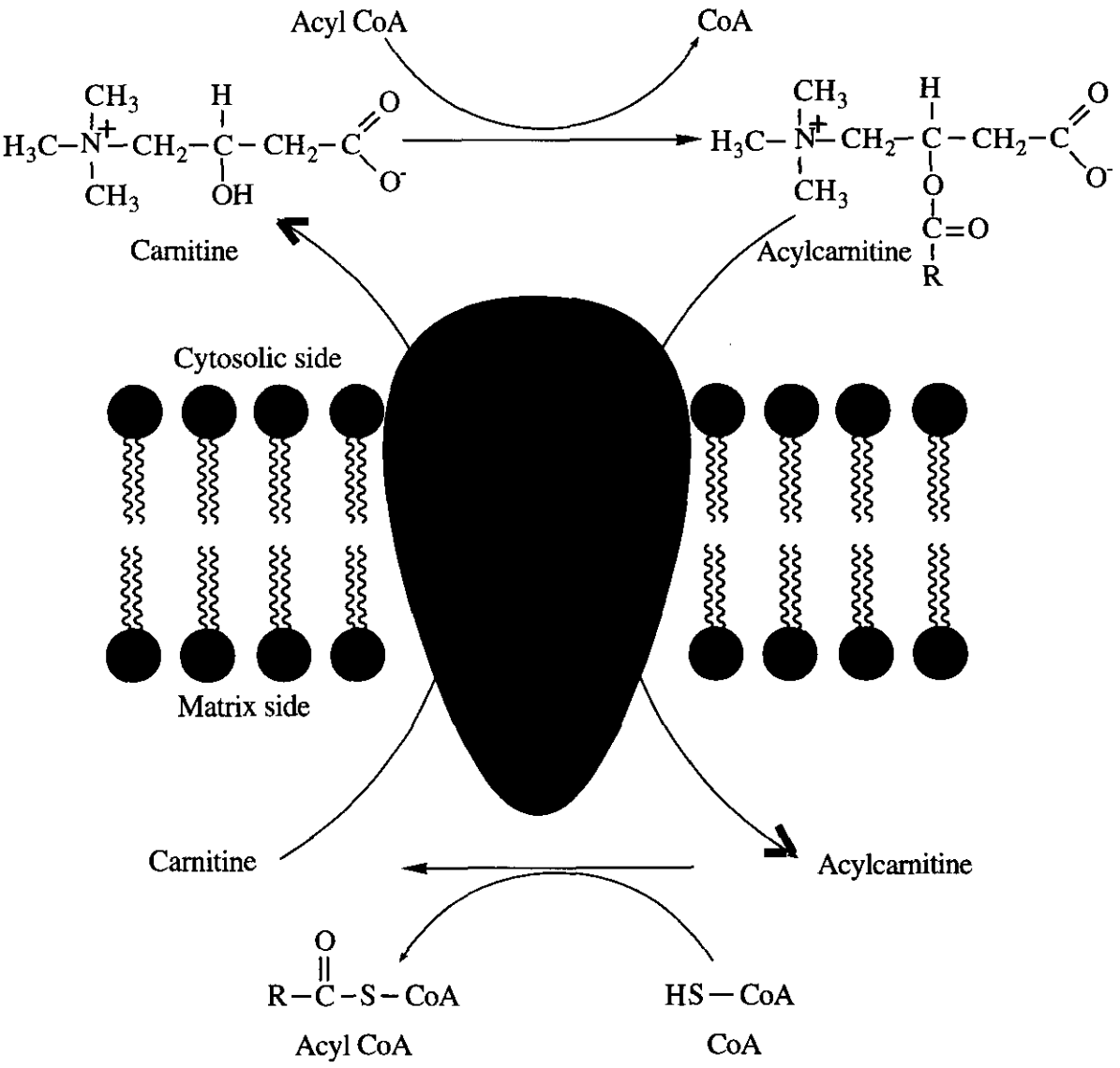
2. (2) Thin Layer Chromatography/HPLC.

Such methods are often used to fractionate / separate the sample before using another analytical technique to measure or quantify the analyte e.g. purification before CI mass spectrometry[129], although quantitative work has been done on specific trace acylcarnitines with radioisotopes using TLC and HPLC[130]. TLC/MS has been reported but is unlikely to become a routine for clinical analysis.

2. (3) Gas Chromatography

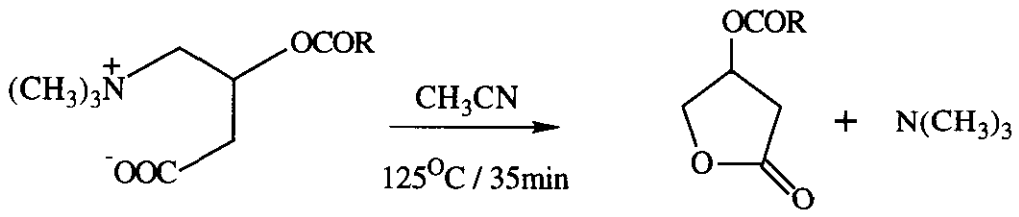
The main problem with this method is that acylcarnitines are involatile zwitterions and so will not pass unchanged through a GC column. This means various methods have had to be employed to convert them into volatile compounds that will undergo GC. One of these involves the cyclization of the acylcarnitine (Figure 38) which has allowed the analysis of a variety of acylcarnitines by GC.[126]

Figure 37. The entry of acylcarnitine into the mitochondrial matrix.



The entry of acylcarnitine into the mitochondrial matrix is mediated by a translocase. Carnitine returns to the cytosolic side of the inner mitochondrial membrane in exchange for acylcarnitine.

Figure 38. Procedure for lactone cyclization.



2. (4) Gas Chromatography/Mass Spectrometry (GC/MS)

This combination gives the best of both worlds. GC has been a powerful analytical tool for qualitative and quantitative characterization of volatile mixtures for nearly half a century. Its combination with MS has now made GC/MS into an established technique in analytical chemistry. As already described, acylcarnitines are charged and involatile and so cannot be determined directly by GC and hence GC/MS. The cyclization reaction shown above yields acylcarnitine derivatives that are amenable to GC/MS. The technique has been proven in the analysis of neonatal urine following extraction of acylcarnitines and cyclization. After GC the MS results showed unambiguously that octanoylcarnitine was present in the urine. This result is important because octanoylcarnitine is diagnostic of a life threatening disease called MCADD which manifests itself in a small proportion of cot death victims[131]. GC/MS is therefore an excellent analytical tool for diagnostic purposes though it is still too time-consuming to be used as a routine screening technique.

2. (5) Electrospray Mass Spectrometry.

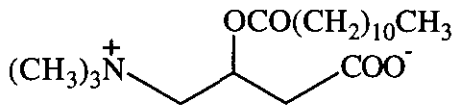
Many mass spectrometric methods have been used to analyse for acylcarnitines which have each yielded excellent results. The application of FAB mass spectrometry has been particularly fruitful. The advent of ES/MS has further added to the number of techniques available for the analysis of these naturally occurring compounds. A brief assessment of electrospray as an alternative to FAB mass spectroscopy[122-125] was attempted here.

3. Results.

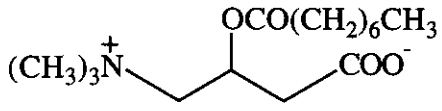
3. (1) Initial attempts to examine acylcarnitines by CZE.

As acylcarnitines universally contain carboxylic acid groups similar to peptides it could be expected that they would display similar absorbance characteristics. An ester group is also present in the side chain of each acylcarnitine. Acylcarnitines are also similar to peptides in their capacity to be zwitterions which makes the overall charge that they carry pH dependent.

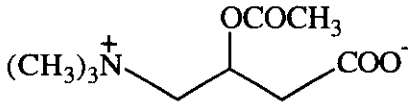
Only three acylcarnitines were used throughout these experiments:



Lauroylcarnitine



Octanoylcarnitine



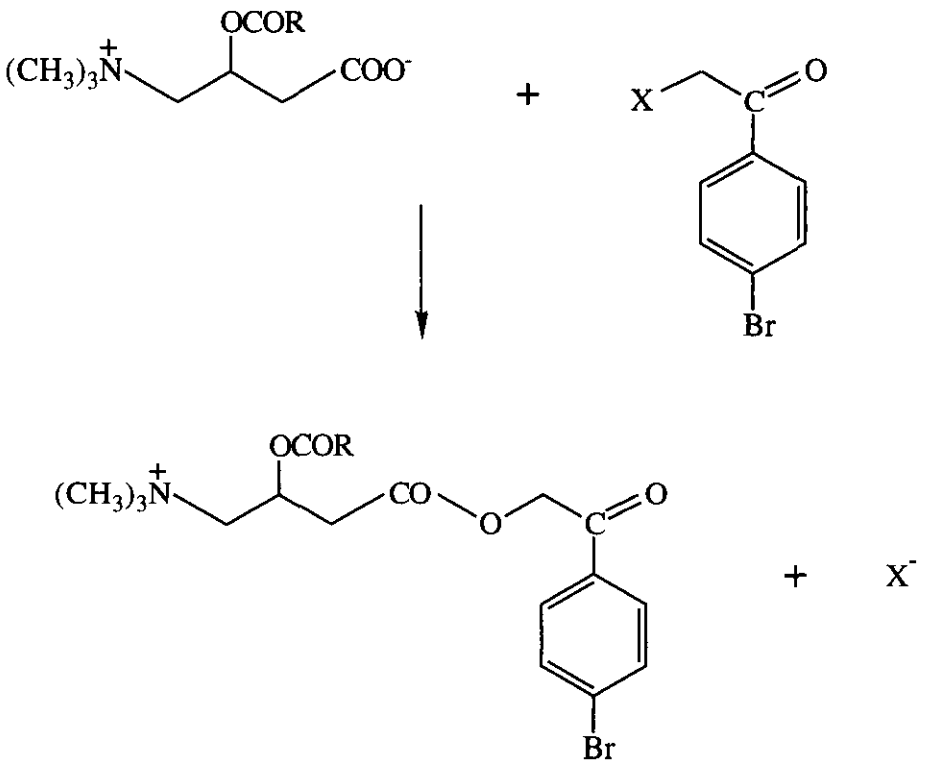
Acetylcarnitine

Each acylcarnitine varies only by the number of CH_2 units in its side chain. The approach based on inherent absorbance was tested by detecting them by direct U.V. at 200nm during separation by CZE. An acidic buffer at pH 3.0 was used for electrophoresis of acylcarnitines in their native form. Detection of octanoylcarnitine was possible under direct U.V. detection but even at 3 mg/ml an absorbance of only 0.0025 units was observed, which is an unacceptable detection efficiency for these particular analytes which would be present at sub - µg/ml levels in body fluids. A mixture of two acylcarnitines, lauroyl and octanoyl at 3 mg/ml each, gave a poorly resolved pair of peaks also at very low absorbance levels.

3. (2) Improving the detection limit of acylcarnitines by derivatisation.

To make the detection of acylcarnitines more feasible their derivatisation was attempted. This was done with a *p* -bromo-phenacyl derivatising agent in the presence of a crown ether. The derivatisation procedure is outlined in Section 2 (2) of the experimental procedures and is shown diagrammatically in Figure 39.

Figure 39. Derivatisation procedure for acylcarnitines. (X = Br or OSO_2CF_3)



After cooling the derivatised acylcarnitine was subjected to CE and detected at 254 nm using a phosphate buffer. A large peak was observed measuring 0.05 absorbance units which was more than an order of magnitude increase in absorbance for underivatised acylcarnitine and this was achieved using one third less sample. The method was then expanded inasmuch as two acylcarnitines, (acetyl- and octanoyl-) were individually derivatised and mixed before analysis. But the resulting electropherogram contained only one peak at around four minutes. The conclusion was that the conditions were not sufficiently selective to separate the two acylcarnitines.

3. (3) Optimisation of the separation buffer and preparation for CE/ES.

As with all CE work reported here, one aim was to create a method that is compatible with electrospray mass spectrometry as a detection mechanism. With this in mind a more volatile ammonium acetate buffer was used which was then optimised to ensure adequate separation of the derivatised acylcarnitines. The new buffer gave the result seen in Figure 40 (Appendix 4), as the two analytes were separated. The third peak visible in this electropherogram is due to an excess of the derivatising agent. The experiment was then repeated with three analytes, lauroyl-, acetyl- and octanoyl-carnitine. The level of acetonitrile in the buffer was then

increased in order to improve the resolution of peak three in the electropherogram by increasing the solubility of the lauroylcarnitine derivative. The individually derivatised acylcarnitines were mixed before electrophoresis was carried out. Three peaks were observed, as seen in the electopherogram in Figure 41 (App. 4) and each of these peaks corresponded to one of the acylcarnitines which could then be identified by its migration time when electrophoresed separately as illustrated with two acylcarnitines in Figure 42 (App. 4). The migration order determined by CE alone was established as being (1) acetylcarnitine, (2) octanoyl and (3) lauroylcarnitine. As expected, the order is determined by mass, given that charge is the same for each component. It was noticed that the absorbance level of the peaks due to the acylcarnitines in the mixture were the same size as those seen when the analytes were run individually. This was not expected because by mixing the three derivatized acylcarnitines, a 1 in 3 dilution factor had been introduced which suggests that the column was becoming saturated with the samples being injected onto it. Once separation had been achieved the experiment was repeated on a one metre column which would be used if CE/ES was performed. This was the final test before CE/ES could be attempted and results using the longer capillary are shown in Figure 43 (App. 4), where the migration time of the slowest migrating analyte (lauroylcarnitine) becomes 21 mins 50 secs compared to 4 mins 35 secs on the shorter capillary, Figure 41 (App. 4). Due to the longer migration times of all the analytes, separation between them is improved substantially, with 90 secs between acetyl- and octanoylcarnitine and 52 secs between octanoyl- and lauroylcarnitine.

3. (4) Quantification of acylcarnitine analysis.

This experiment was performed to confirm earlier observations that saturation occurs at a specific level of acylcarnitines injected into the capillary. This was also a chance to conduct quantification experiments and establish whether calibration curves were linear. A sequence of standard samples was analysed by electrophoresis after being injected for 8 seconds both by pressure and electromigration techniques. Octanoylcarnitine was used as the standard after it had been added to blood. The blood was then spotted onto Guthrie cards and allowed to dry. The blood spot was sonicated in a methanol /chloroform mixture and the extract used for analysis*.

* The sample work-up procedure was performed by B.M. Kelly, Open University.

This was done in order to simulate a real sample situation. This extract was then derivatised using the *p* -bromophenacyl ester. Results in Table 5.

Table 5. Results from the CE analysis of standard acylcarnitine and samples from patients with medium chain acyl-CoA dehydrogenase deficiency (MCADD).

	Peak Area.	
Approximate amount of Octanoylcarnitine (µg/blood spot)	8 Second pressure Injection	8 Second Electromigration Injection
62.5 (OCT 2)	0.23	1.37
31.2 (OCT 4)	0.30	3.18
6.25 (OCT 6)	0.21	3.85
3.12 (OCT 7)	0.16	2.85
1.25 (OCT 8)	0.09	2.59
0.125 (OCT 9)	0.05	0.72
Sample 7B	0.02	
Sample L5	0.07	
Sample P	0.08	

The real samples were obtained from patients with medium chain acyl-CoA dehydrogenase deficiency (MCADD) and octanoylcarnitine was expected in each one. It is only possible to report a result for octanoylcarnitine in the samples injected using pressure because electromigration did not prove to be reproducible enough, perhaps because of interference by the various components of blood. This meant that no reasonable migration time could be used as a reference point to try and identify the peak due to octanoylcarnitine in the samples. However even the peaks used to elucidate results from the samples injected by pressure are estimated as being due to that acylcarnitine analyte. Plotting graphs of the standard results pin-points the saturation of the capillary at the higher amounts of sample. The level at which this saturation occurs is also dependent upon which injection method is utilised. Under the conditions applied, electro-migration injection places more sample into the capillary which is illustrated by the point to which saturation persists utilising this method of injection, so

electromigration injection would be best if lower levels of acylcarnitine were to be analysed. At the lower end of the calibration curve for the pressure injections saturation does desist and a straight line can be drawn between the final three calibration points, though this does not go through the origin. Considering that the absorbance of the real samples falls within this range (using pressure injection) some form of quantification of these samples can be performed and estimates of the octanoylcarnitine present within these samples can be made. The estimated values (and the calibration curves themselves) are only equivalent to the amount of acylcarnitine that has been extracted from the blood spots and the efficiency of this extraction has not been fully assessed. Because of this and the calibration curve which does not have points through the origin, results were not extrapolated for this experiment.

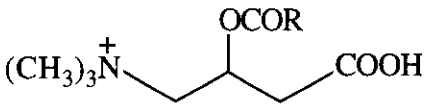
3. (5) Improved separation of the acylcarnitines by addition of Phytic acid.

The separation of the three derivatised acylcarnitines observed earlier was not adequate considering that between the acetyl- and octanoylcarnitine would elute several straight- and branched-chain analytes and similarly between octanoyl- and lauroylcarnitine. With the resolution obtained in the original separation these other analytes are unlikely to be resolved, so for subsequent analyses which might involve separating a wider range of acylcarnitines better separation efficiency would be required. This could be achieved by varying different experimental parameters or more simply by addition of a substance that binds to the walls of the capillary and causes a decrease in the rate of electro-osmotic flow. This in turn increases the time the analytes are in the capillary and improves the separation efficiency. This involved adding phytic acid (known to lower Eof)[132,133] at a level of 10 mM to the separation buffer. The only disadvantages are the increase in over-all separation time visible in Figure 44 (App. 4) even with a standard 50 cm long capillary and the appearance of an additional background peak in any resulting electrospray spectra for phytic acid (F.W. 923.28g). The real samples from earlier experiments were injected using both pressure and electromigration techniques and electrophoresed using buffers with and without phytic acid. Figure 45(a) (App. 4) shows two overlaid electropherograms of Sample P after electromigration injection. These were obtained using buffer without phytic acid added and show a substantial number of peaks. The improved separation of the same sample, achieved using a phytic acid buffer can be seen in Fig 45(b) (App. 4). The other samples (L5 and 7B)

also displayed similar but smaller peaks. However due to the behaviour of the CE method these could not be positively identified from their migration times alone. A more conclusive method of identification could be made using a mass spectrometric technique once CE separation was completed. It was decided to use electrospray mass spectrometry for this purpose.

3. (6) Analysis of underivatized acylcarnitines by electrospray mass spectrometry.

The initial analysis of underivatized acylcarnitines by electrospray mass spectrometry is fully detailed in a letter to the journal, *Organic Mass Spectrometry*[134]. In acid solution, the zwitterionic acylcarnitines exist as cations (**4.3**).



(**4.3**)

$[M + H]^+$ ions

Not having a strong basic site within their structure, these protonated species would be expected to yield single peaks for the singly charged cations. To ensure that the zwitterions exist in the cationic form for electrospray mass spectrometry, formic acid was added to the water/acetonitrile carrier solution. The anions present are thus formate and chloride (the standard acylcarnitines are used in the form of their HCl salts). Under these conditions each acylcarnitine examined exhibited $[M + H]^+$ ions (where M is defined as the zwitterion) as the only significant peak in their positive-ion electrospray mass spectra. Examples are shown in Figure 46 (a - c) (App. 4). Octanoylcarnitine is a key urinary metabolite for the diagnosis of medium-chain acyl-CoA dehydrogenase deficiency (MCADD). Its electrospray spectrum consists of $[M + H]^+$ ions at m/z 288 along with background ions only.

4-Phenylbutanoylcarnitine is not a natural product. It is used as an internal standard in gas chromatography / mass spectrometry studies[126,127]. It too provides a clear peak for the protonated molecule, at m/z 308. Figure 46 (c) (App. 4) also shows the largest acylcarnitine examined, hexadecanoylcarnitine (palmitoylcarnitine). Its protonated molecule at m/z 400 is accompanied by smaller peaks that are not considered to be background ions (m/z 415, 439,

221 and 204). These are thought to be due to impurities in the commercial sample. The peak at m/z 204 corresponds to the protonated molecule of acetylcarnitine which is thought to be an impurity rather than a fragment ion from $[M + H]^+$ ions of hexadecanoylcarnitine. Having established that underivatized acylcarnitines behave as expected under ES conditions, and predicting a similar behaviour for the derivatives, CE/ES was attempted next.

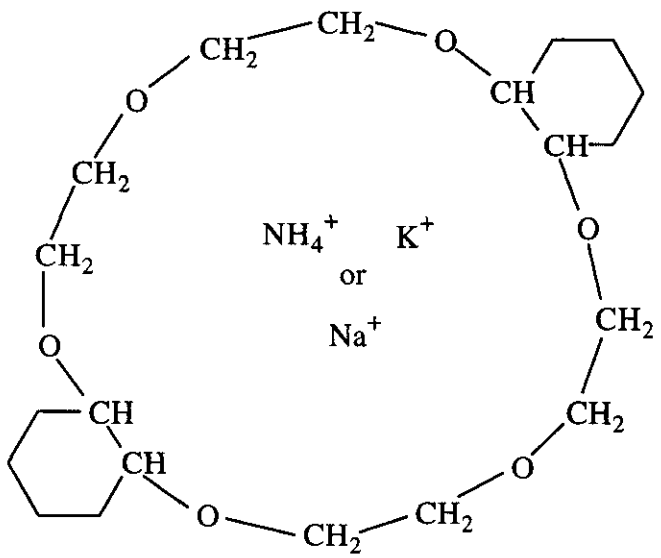
3. (7) CE/ES of acylcarnitines.

A mixture of three derivatised acylcarnitines was used in this experiment which was done in two stages. The first stage involved the sample being infused into the capillary which led into the ES source. The results of this can be seen in Figure 47 (App. 4). The expected masses for each derivatised analyte were observed along with their bromine isotope peaks in virtually a 1:1 ratio due to the 100% : 97.3% ratio of ^{79}Br : ^{81}Br.

Peak identification and m/z ions present:

Acetylcarnitine	400	Octanoylcarnitine	484
	402		486
Lauroylcarnitine	540		
	542		

Other peaks observed can be put down to the crown ether present in the original derivatising agent - dicyclohexyl-18-crown-6 ether shown below.



The crown ether has a molecular space within it which cations can bind and impart a positive charge to the molecule. The particular molecular ion formed is dependent upon which cation from a choice of sodium, potassium or ammonium ions becomes bound into the space.

Some of the molecular ions formed when cations bind with the crown ether.

$m/z = [Crown + NH_4]^+ = 390$ $m/z = [Crown + K]^+ = 411$

$m/z = [Crown + Na]^+ = 395$ $m/z = [Crown + (CH_3)_2NH_2]^+ = 418$

The ions at m/z 390 and 411 which originate from the crown ether can also be seen in Figure 47 (App. 4), the peak heights of which may be determined by the quantity of each cation available to bind with the crown ether and the binding efficiency. The second part of this experiment involved CE/ES of the 3 acylcarnitine mixture. The sample was injected as a discrete band and voltage applied so that electrophoresis proceeded to allow separation of the analytes. The results are shown in Figure 48 (App. 4) as an electropherogram. The extra information given by the electrospray spectrum allows the migration order through the capillary to be confirmed as can the identification of each analyte. The first three peaks are due to the derivatised acylcarnitines, highlighted in Figure 49 (App. 4). Other peaks in this chromatogram are due to excess derivatizing agent and underivatised acylcarnitines which actually give peaks of larger intensity than those due to the derivatised acylcarnitines.

4. Conclusions and Discussion.

It was expected that with the acylcarnitines having COO- and COOR groups, it would not be sufficient to visualise them with uv spectroscopy at the levels of interest. This view was confirmed. When derivatised the acylcarnitines actually saturate the capillary above certain levels but below this level a calibration graph can be produced with a correlation coefficient of 1.000. CE/ES as used here would not be a viable method. If a greater number of acylcarnitines within real samples could be efficiently separated by the CE process and the limit of detection of these was sufficient to detect them at physiologically significant levels then the interface to ES could be justified in order to identify these analytes unambiguously. The final status of the CE separation was that the potential for separation of a greater number

of acylcarnitines could be increased by combining the use of phytic acid and longer capillaries up to 1 metre in length. These two measures, one of which was brought about by necessity for CE/ES interfacing and the other facilitated by manipulation of the electro-osmotic flow, greatly improved the viability and potential of the CE method. The question of improved detection could be addressed by the use of either electromigration injection procedures or a different electrophoretic procedure such as isotachophoresis (ITP) which can be used to concentrate the samples in-situ within the capillary. Derivatisation of the acylcarnitines with fluorescing agents would also substantially improve the detection limits of the spectroscopic technique.

One of the outstanding questions to be adressed is that within the results of the CE/ES analysis the underivatised acylcarnitines produced peaks of greater size than those produced by those derivatised acylcarnitines. This is most likely to reflect the efficiency of the derivatisation procedure, the length of time between the derivatisation and the analysis or purely the way in which the ions are produced within the electrospray source. The fact that any underivatised acylcarnitines are visible at all does suggest an inefficient derivatisation procedure but the experiment must be investigated further to elucidate the problem. Detection of the underivatized acylcarnitines by uv is too insensitive to give observable peaks on the scale used. Even if it is the case that electrospray is more sensitive to underivatised acylcarnitines than to the derivatives it would be preferable to find a better derivatisation procedure that goes to completion. These are issues that would need to be clarified before proceeding with further method development in this area.

In conclusion, medium- and long-chain acylcarnitines are readily and directly compatible with electrospray mass spectrometry. To all intents and purposes, the mass spectra obtained comprise only protonated molecules. Fragmentation, if required, might be induced by increasing the cone voltage or by collisional activation in an MS/MS experiment. In these initial experiments, the electrospray mass spectra of any short-chain acylcarnitines were not recorded and the limit of detection of the method was not measured. Both of these aspects will have to be studied before the method can be applied to the analysis of biological fluids for a broad range of acylcarnitines. However, the initial results with medium- and long-chain

acylcarnitines suggest that it is worth investigating electrospray mass spectrometry as a new, direct approach to the determination of acylcarnitines in clinical samples.

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Appendix 4

Figures from Chapter 4.

Figure 40. Capillary electrophoresis chromatogram of the separation of acetyl - and octanoyl- carnitines.

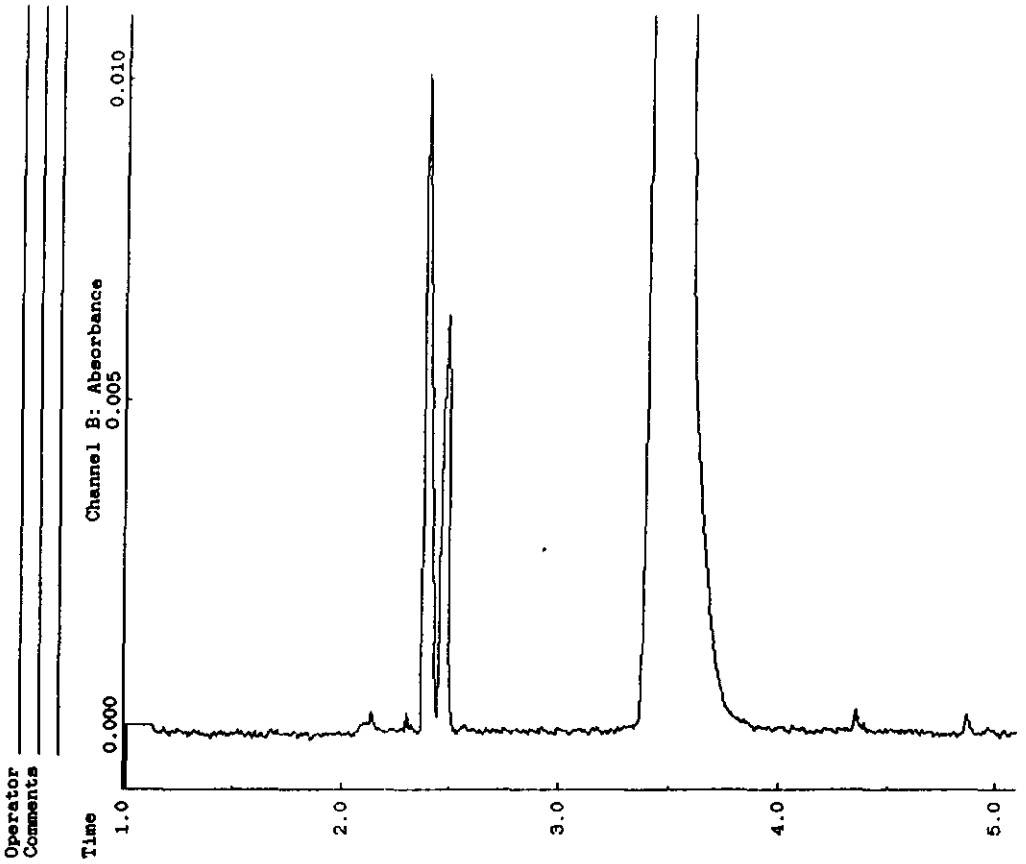
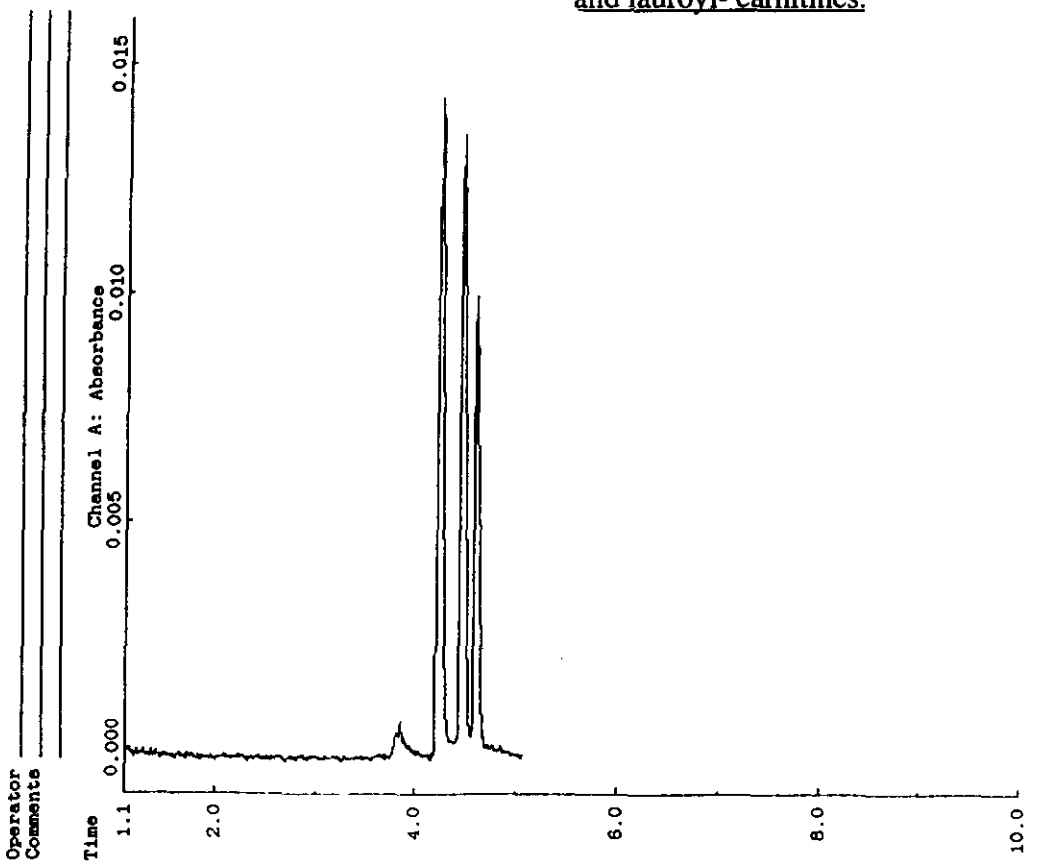
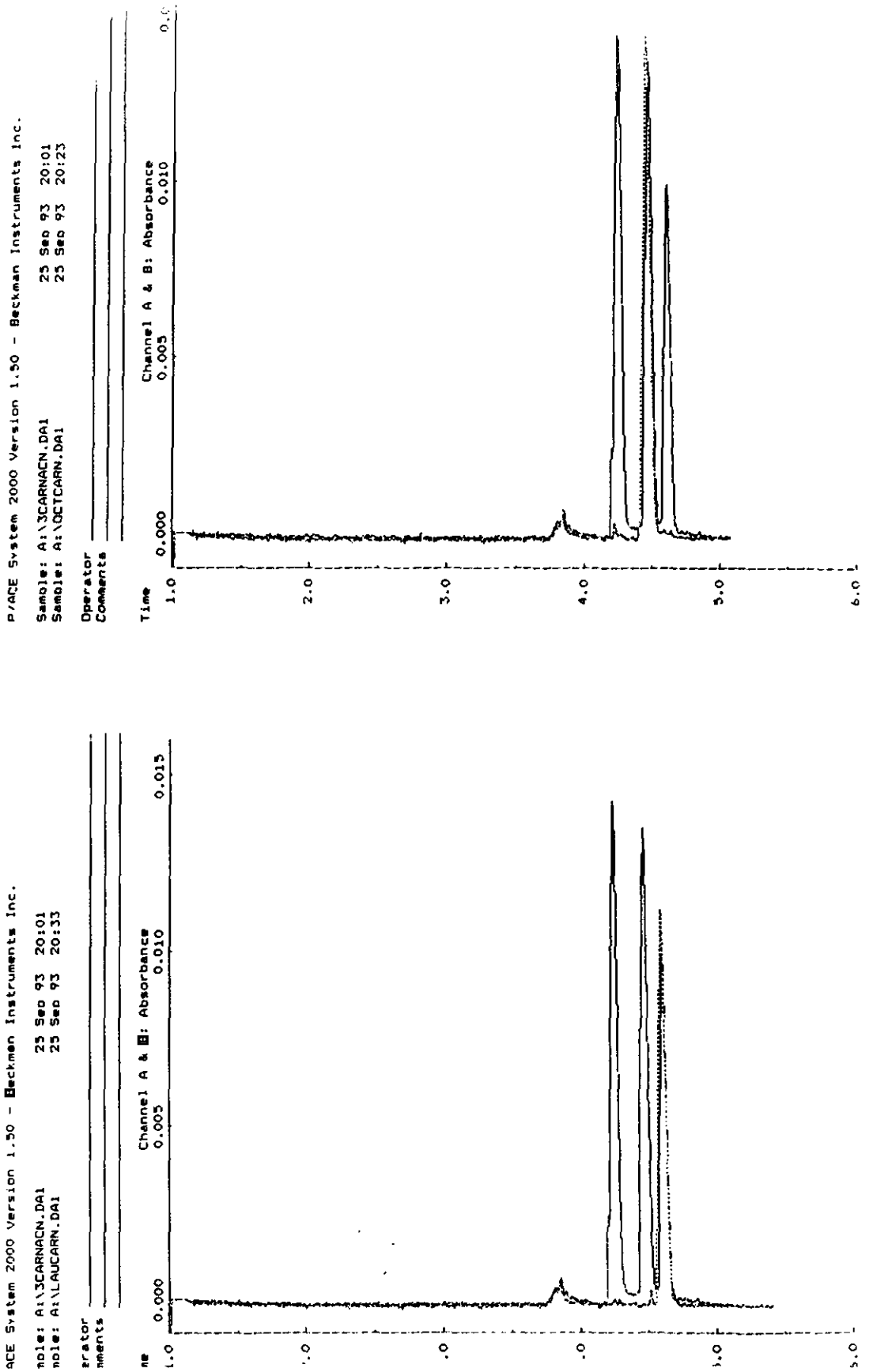


Figure 41. Capillary electrophoresis chromatogram of the separation of acetyl-, octanoyl- and lauroyl- carnitines.



Buffer: 15 mM ammonium acetate, 30% acetonitrile, pH 4.3 with ethanoic acid. Capillary: 50 µm i.d. x 50 cm; separation at 25 kV at 25°C; detection: uv detection at 254 nm.

Figure 42. Superimposed electropherograms which allow identification of octanoyl- and lauroyl- carnitine in the three acylcarnitine mixture.



Buffer: 20 mM ammonium acetate, 10% acetonitrile, pH 3.8. Capillary: 50 µm i.d. x 50 cm; separation at 25 kV at 25°C; detection: uv detection at 254 nm.

Figure 43. Capillary electrophoresis separation of the three acylcarnitines on a one metre capillary.

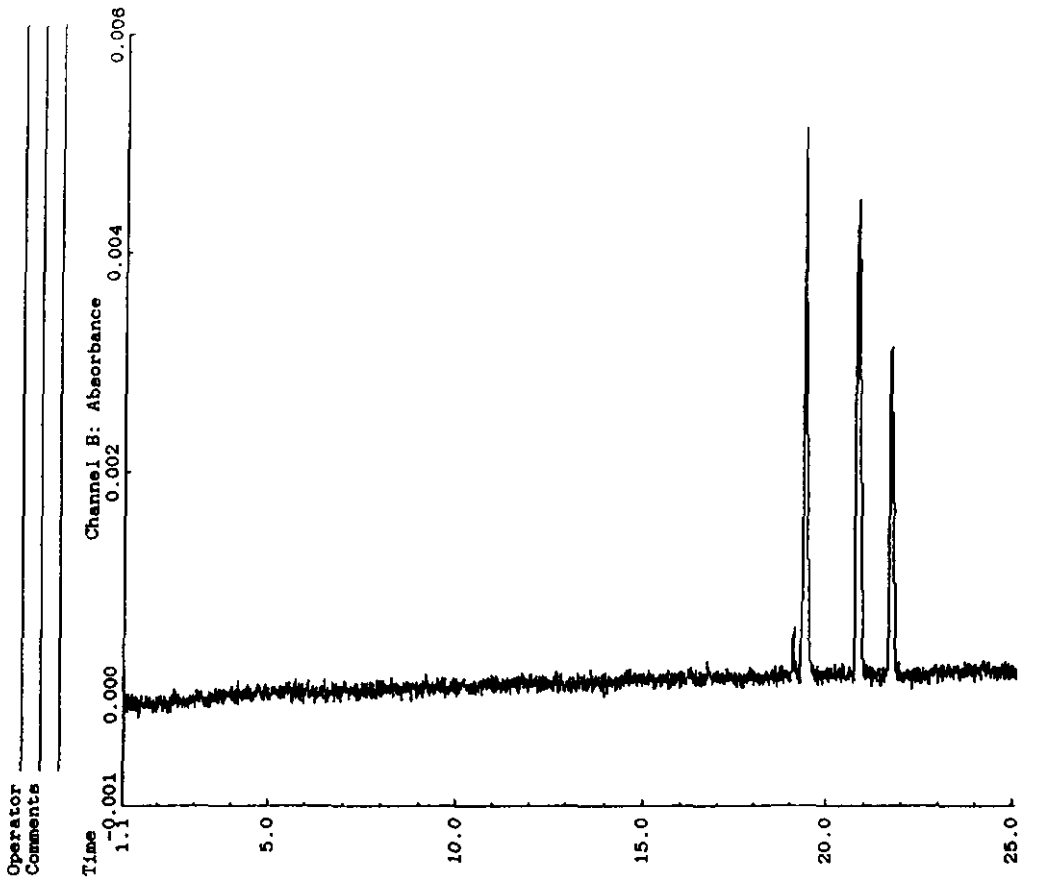
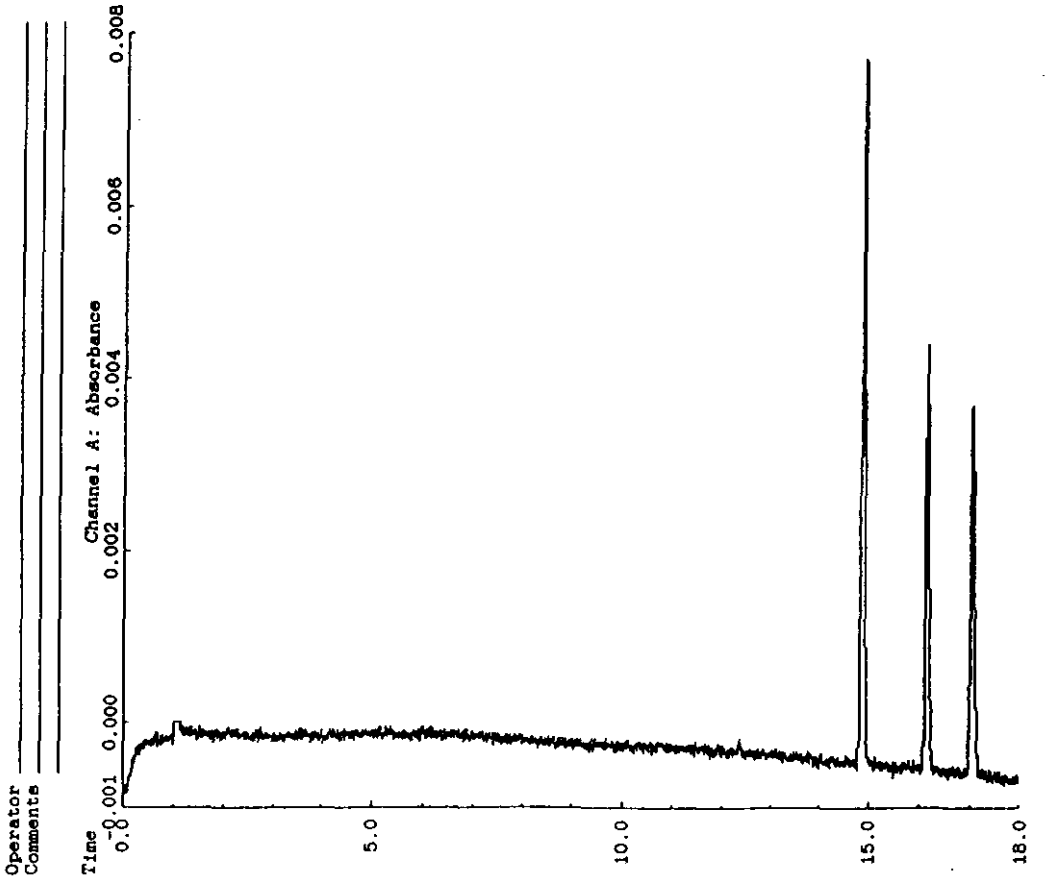


Figure 44. Capillary electrophoresis separation of three acylcarnitines using phytic acid to decrease electro-osmotic flow.



Buffer: 15 mM ammonium acetate, 30% acetonitrile, pH 4.3 with ethanoic acid, 10 mM phytic acid. Capillary: 50 µm i.d. x 50 cm; separation at 25 kV at 25°C; detection: uv detection at 254 nm.

Figure 45(a). Capillary electrophoresis of a sample from a patient with medium chain acyl CoA dehydrogenase deficiency (MCADD). Two chromatograms overlaid.

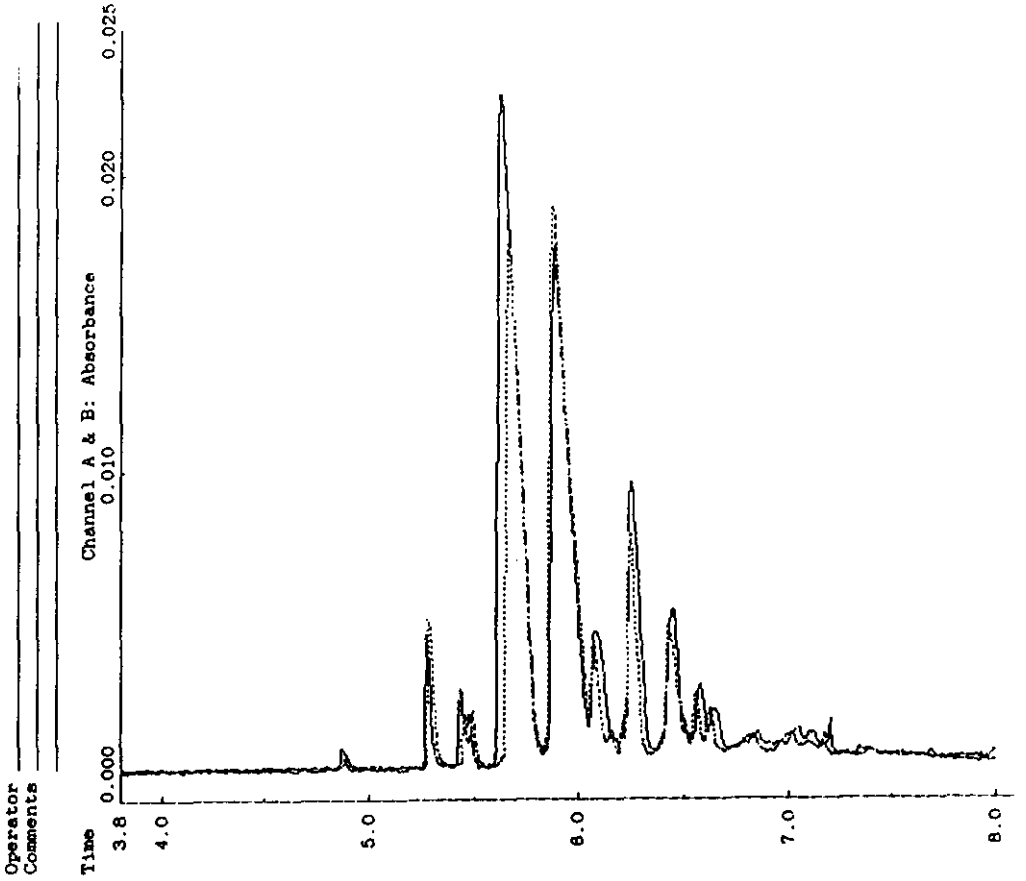
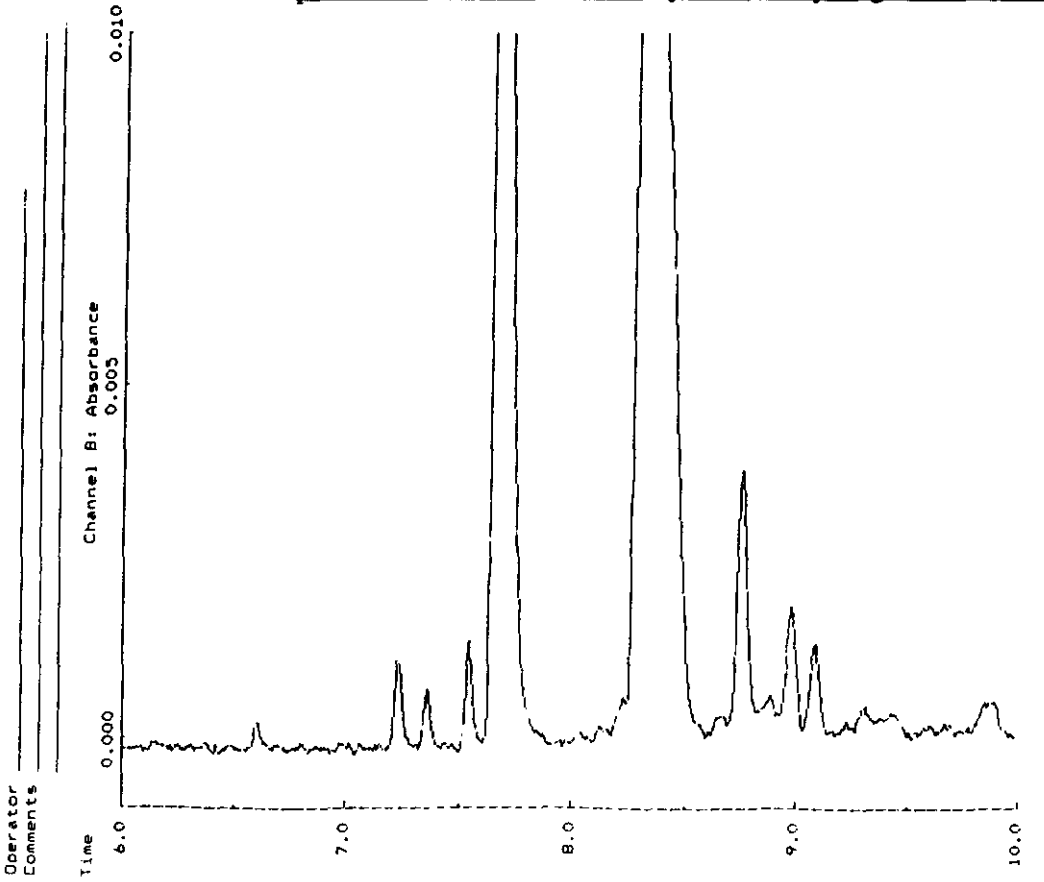
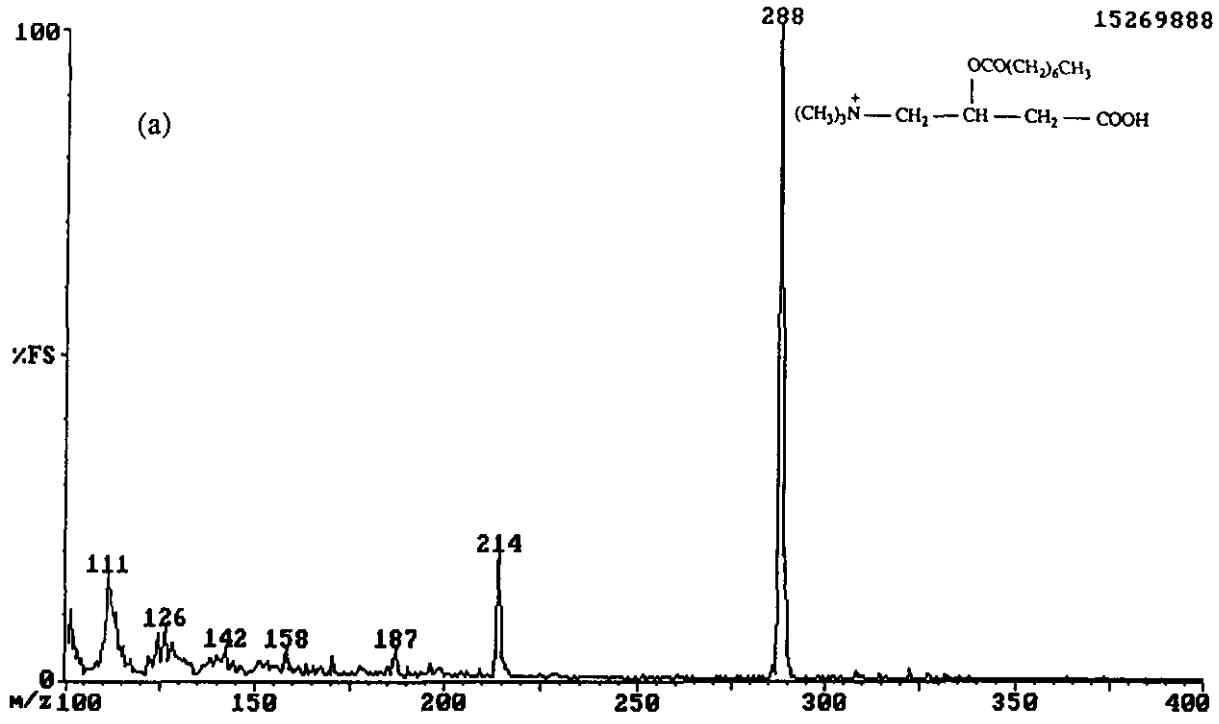


Figure 45(b). Capillary electrophoresis (using phytic acid to decrease Eof) of a sample from a patient with medium chain acyl CoA dehydrogenase deficiency (MCADD).



Buffer: 15 mM ammonium acetate, 30% acetonitrile, pH 4.3 with ethanoic acid, 10 mM phytic acid. Capillary: 50 μm i.d. x 50 cm; separation at 25 kV at 25°C; detection: uv detection at 254 nm.

Figure 46. Positive-ion electrospray mass spectra of (a) octanoylcarnitine, (b) 4-phenylbutanoylcarnitine, and (c) hexadecanoylcarnitine.



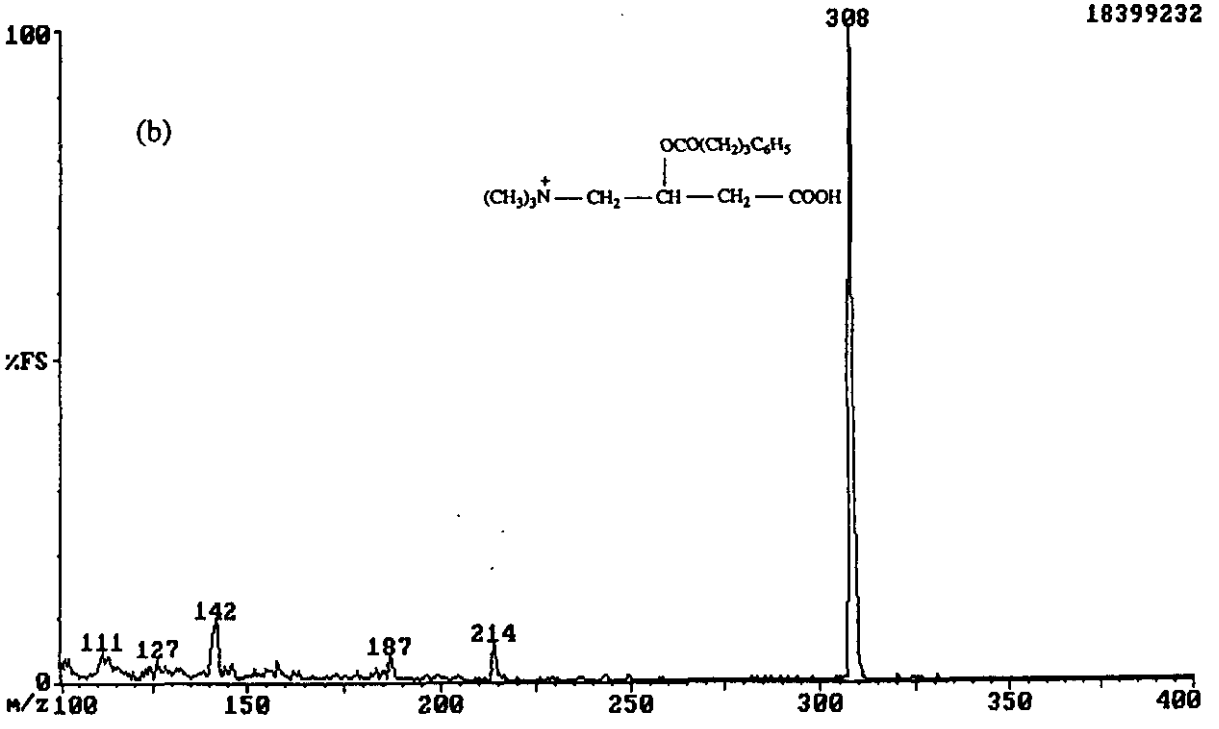
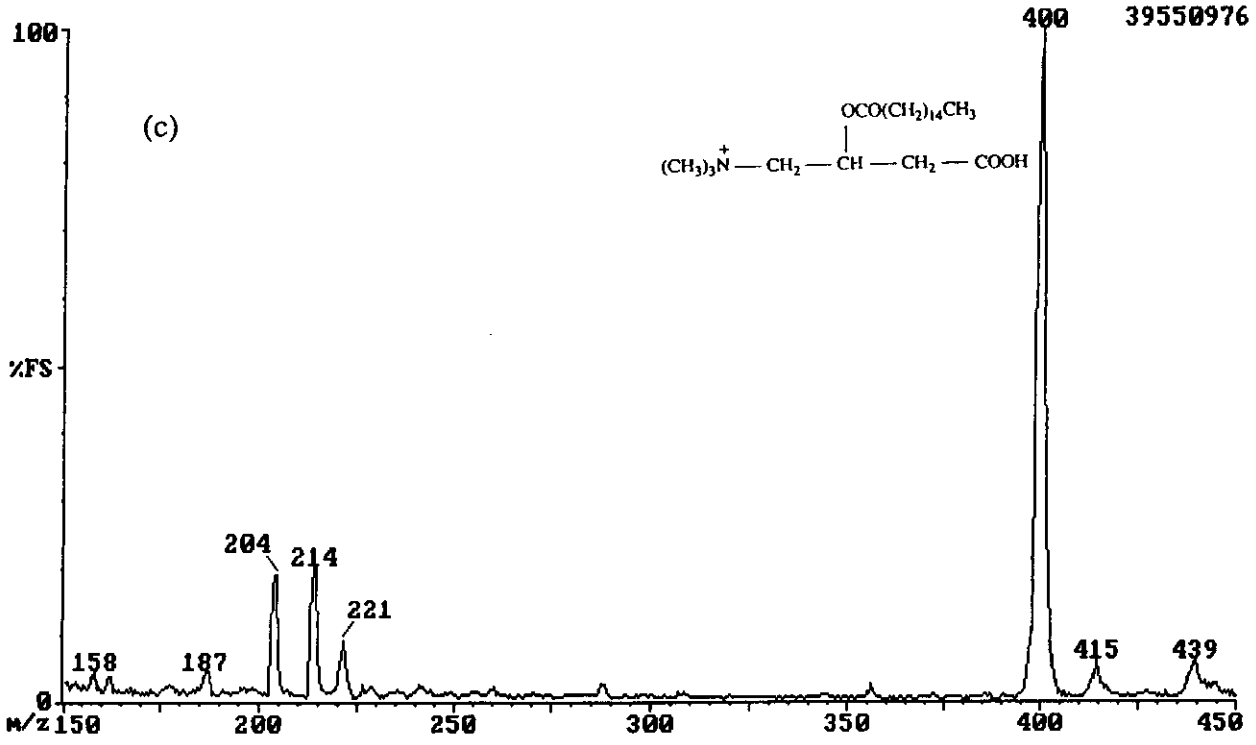


Figure 46. Positive-ion electrospray mass spectra of (a) octanoylcarnitine, (b) 4-phenylbutanoylcarnitine, and (c) hexadecanoylcarnitine.



The peaks at m/z 111, 126/7, 142, 158, 187 and 214 are background ions. Conditions: the carrier solution was water/acetonitrile (50:50) containing 1% formic acid; flow rate, 5µl min^{-1} Sample concentration, 100 ng μl^{-1} of acylcarnitine (10µl injected).

Figure 47. Capillary electrophoresis infusion of a mixture of three acylcarnitines into the electrospray source.

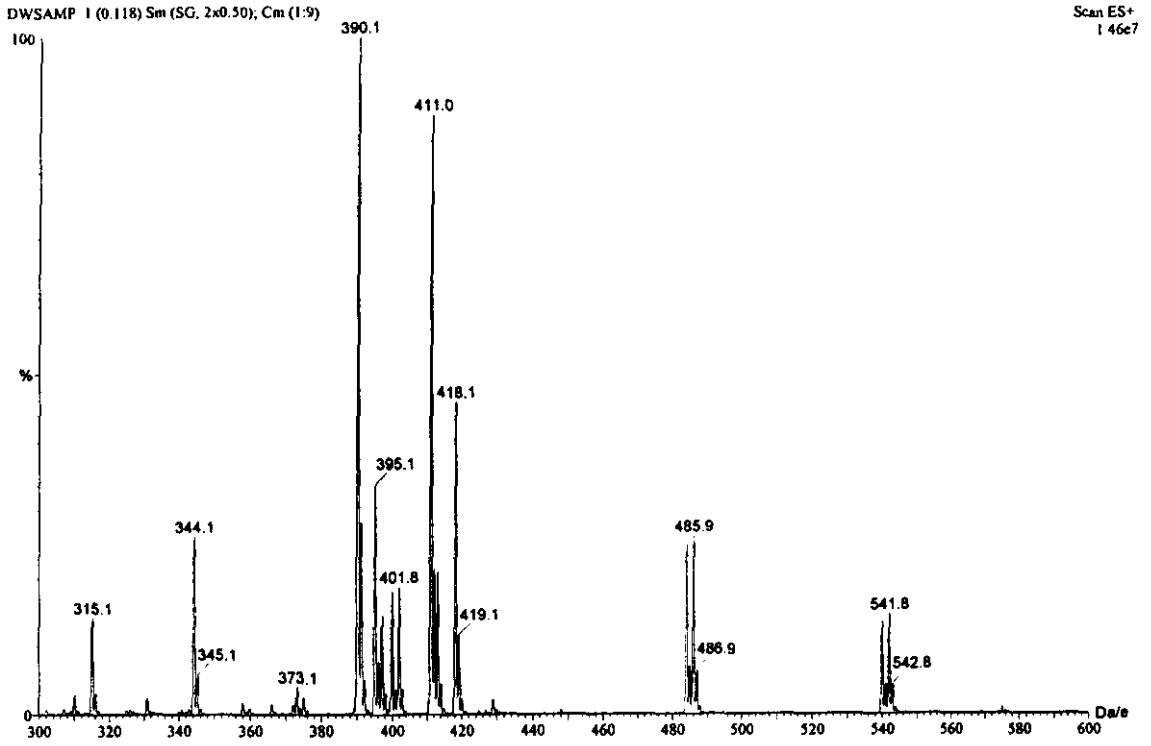
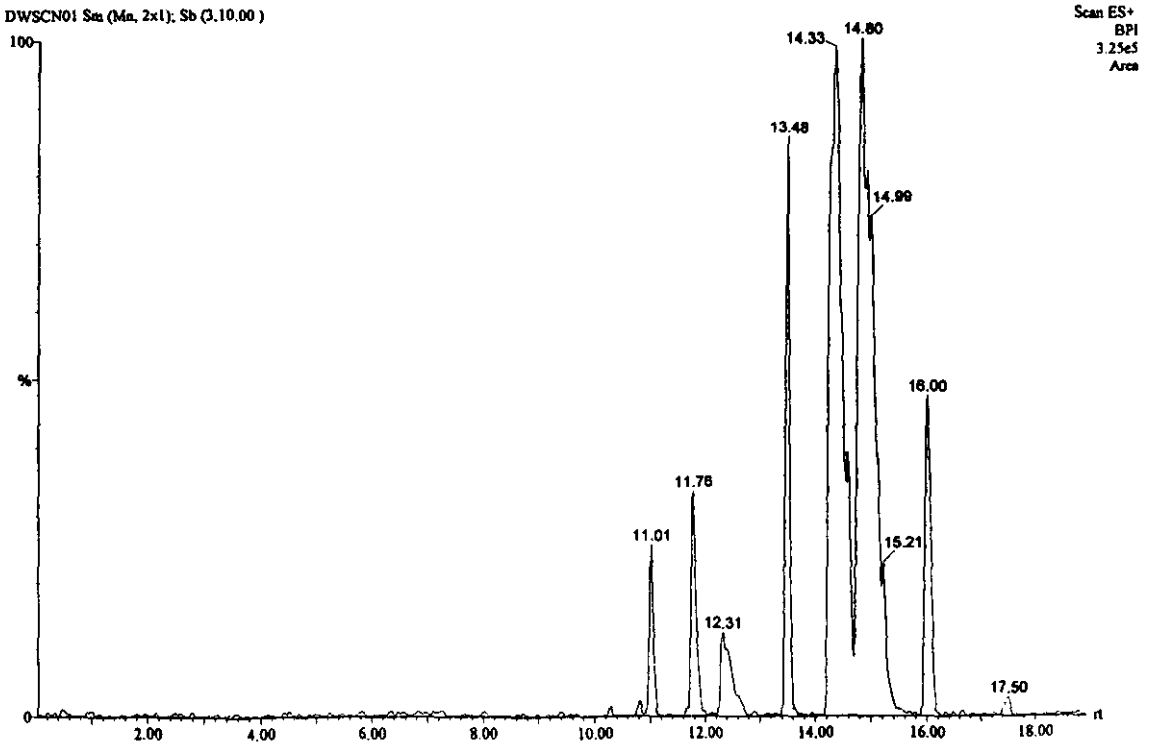
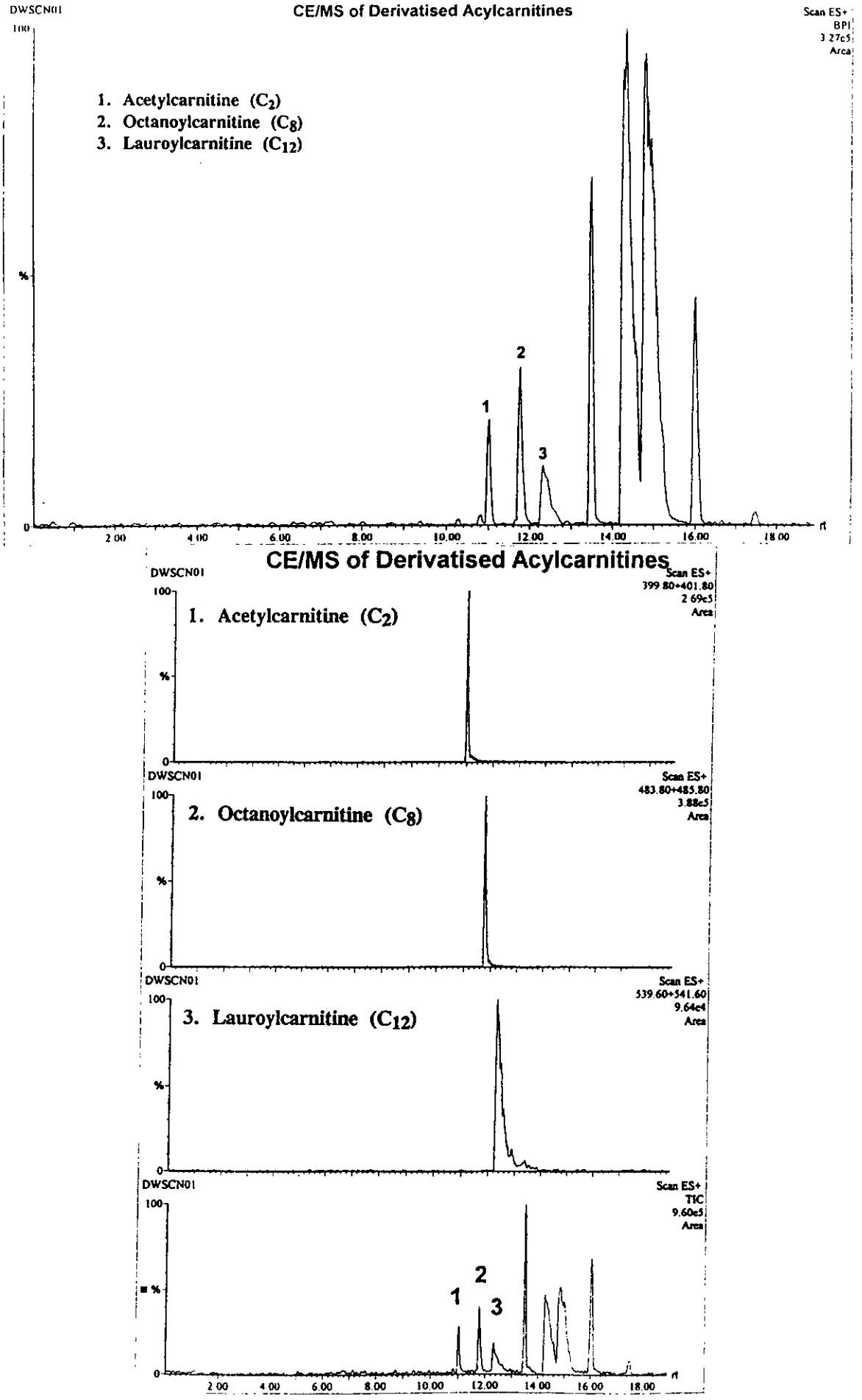


Figure 48. Capillary electrophoresis/electrospray mass spectrometry (CE/ES) spectra of a mixture of three acylcarnitines.



Buffer: 15 mM ammonium acetate, 30% acetonitrile, pH 4.3 with ethanoic acid. Capillary: 50 μm i.d. x 90 cm; separation at 21 kV at 25°C; detection: electrospray mass spectrometry.

Figure 49. Capillary electrophoresis/electrospray mass spectrometry (CE/ES) of a mixture of three acylcarnitines (SIR spectra).

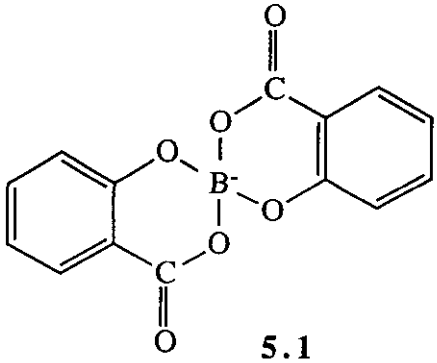


Chapter Five

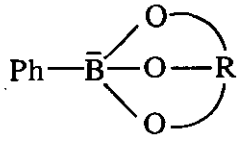
Studies into negative ion complexation using boron acids and diol compounds.

(1). Introduction.

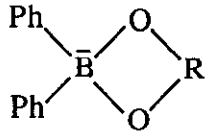
Boron is a trivalent atom but when in the tetravalent form it becomes a negatively charged entity so imparting an anionic charge to any molecule with which it forms such a complex. The boron compounds analysed in these studies range from those which have already been formed from previous reactions, (spiroborates **5.1**) and various boron-containing compounds formed from boronic acids (esters **5.2**) and borinic acids (esters **5.3**) used for "in-situ" derivatisation studies.



5.1



5.2



5.3

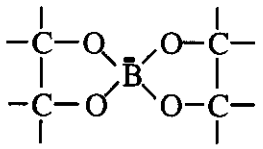
1. (1). Spiroborates.

Spiroborates are the cyclic esters of boric acid which can be synthesised using various methods, but those used in these studies were sythesised by Japanese workers[135,136] using 2-amino-4-methylpyridine borane. These reactions have directed chemists' attention to examine stereochemical and structural aspects of the diols, and to evaluate the equilibrium constants for a number of diol-boric acid reactions. The importance of the ratio of the two reacting molecules is illustrated below.

With excess boric acid.

Predominant products.

With excess diol.



Predominant product.

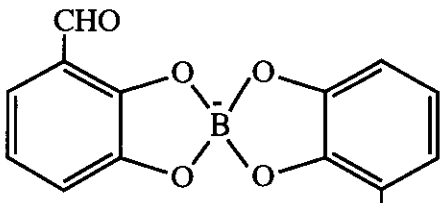
One major product of such reactions are spiroborate species examples of which are shown in Figure 50. Studies involving NMR and FAB mass spectrometry are reported in the negative ion mode. These have been extensively studied in a sequence of papers [135,136,137] using NMR spectroscopy and FAB mass spectrometry and now with electrospray mass spectrometry[138]. Using these techniques has allowed complete structural determination and confirmation of spiroborate complexes.

1. (2). Boron affinity chromatography.

The nature of boron chemistry and the ability to form complexes with boronic acid and *cis*-diols has been known since the 1920's. However this reaction has only recently been exploited for the purification of biomolecules[139]. The ability of boronic acids to form strongly bonded complexes with polyfunctional nucleophilic compounds has been exploited in affinity chromatography[140]. Applications of the phenylboronate complexation include the separation of many different kinds of molecule including nucleotides[141], oestrogen hormones[142], and in a clinical application it has been used to measure sugars and the level of glycosylated haemoglobin[143] which manifests itself in severe cases of diabetes.

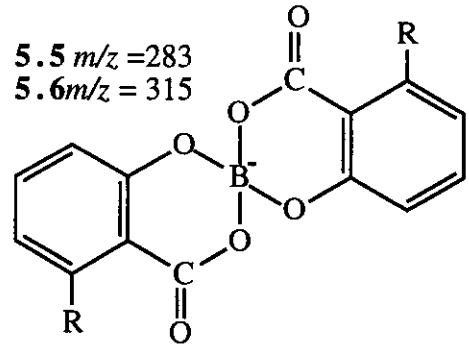
The interaction of a boronic acid with *cis*- diols is the basis for all separation techniques involving boronates. The hydroxyl groups must be close in their spatial orientation if the complexation is to be successful. The interaction between boron acids and poly-alcohols is more successful in simple poly-alcohols where the hydroxy groups can rotate, but the situation is very different in closed-ring carbohydrates where hydroxy groups are fixed. Two mechanisms have been proposed for the interaction of boronic acids with *cis*- diols. In one the trigonal boronic acid is ionised[144] to a tetrahedral boronate ion before reaction with a *cis*- diol to yield a cyclic boronate ester. In the other proposed mechanism, a sequential nucleophilic attack of the diol oxygen atoms on the boronic acid causes the formation of an

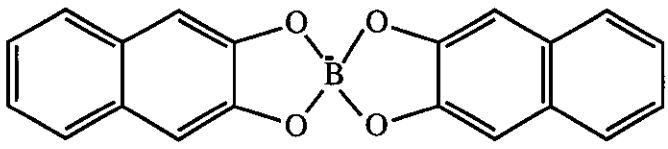
Figure 50. Examples of spiroborate species used in this chapter.



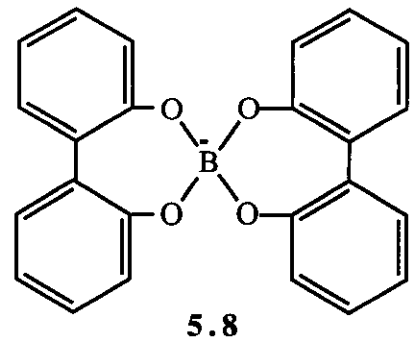
5.4
m/z = 283

Where R = H **5.5** *m/z* =283
R = OH **5.6***m/z* = 315

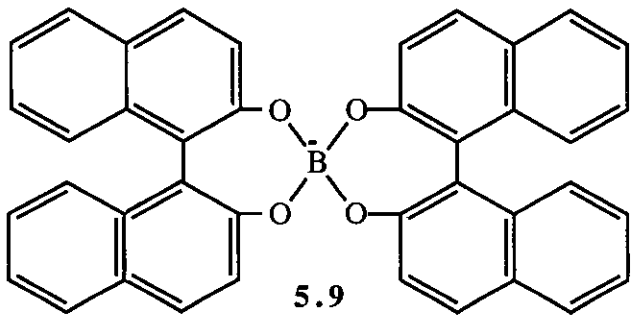




5.7
m/z = 327



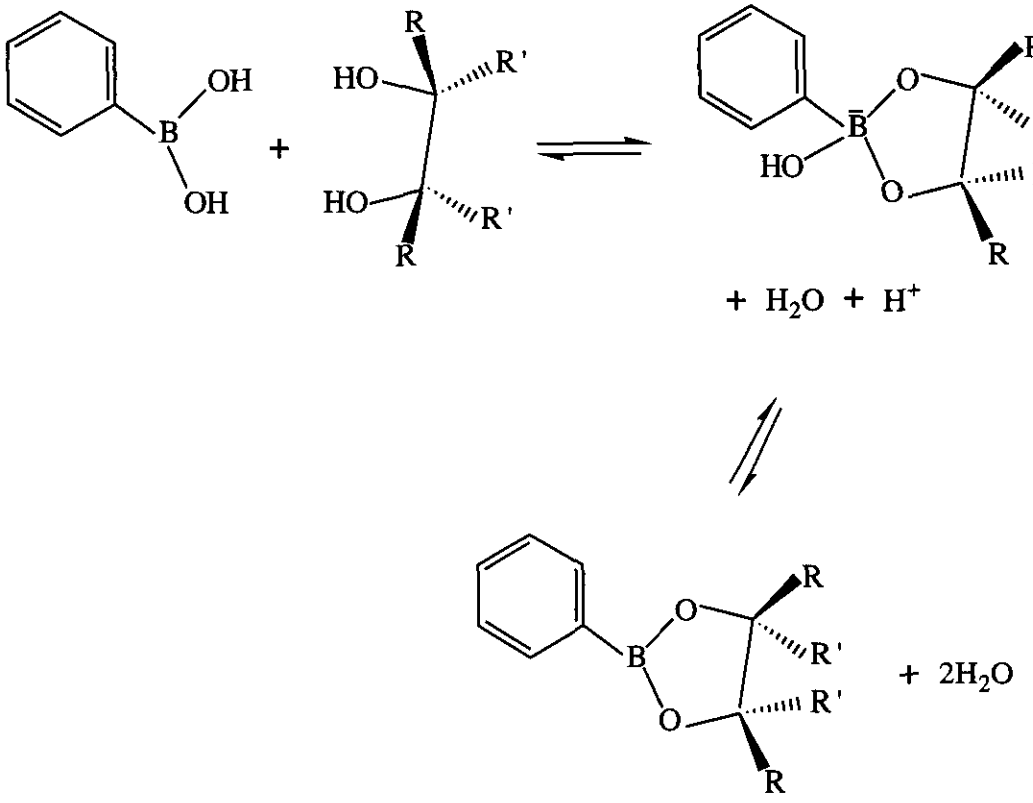
5.8
m/z = 355



5.9
m/z = 579

anionic and a neutral species in equilibrium. The basic, anionic esters can only exist in alkaline solution whereas the other form is found under acid conditions therefore providing a variable for chromatographic purposes[145] illustrated in Figure 51.

Figure 51.



A large number of boronic acid ligands have been synthesised, but only 3-aminophenylboronic acid (or its derivatives modified in various ways to improve its performance) has been extensively used for immobilisation in affinity chromatography. This somewhat restricts the technique because of the lack of satisfactory boronate ligands and ready made column matrices[146].

1. (3) Anion formation with Boronic acid.

A method has been described where boronic acid based compounds can be used to derivatise molecules which exhibit particular characteristics. When this derivatisation is complete, as in affinity chromatography, a negatively charged complex is created, which can then be analysed by fast atom bombardment mass spectrometry. Unlike in the chromatography case, however, the boronic acid has been successfully reacted with triols and analogous compounds[144]. Therefore the extent of the reaction is only dependent upon the spatial

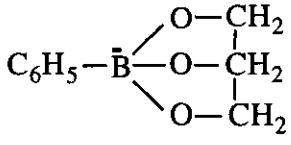
orientation of the functional groups to be complexed. The reaction is more preferable if these groups are in the *cis-* configuration as shown in the derivatisation of sugars with boric acid[140].

During this work various boronic acids have been mixed with both liquid and solid triol based compounds examples of which are given in Figure 52. Initially the liquids also acted as solvents for the reaction which could then be placed directly onto the FAB probe tip. The fact that solely by mixing the two compounds, an ionized product can be formed, means that the sensitivity of FAB and quality of the resulting mass spectra are very much improved. That is, having a pre-ionised molecule as a result of these reactions makes FAB an excellent tool for their analysis because it is a mass spectrometric technique suited to polar, involatile molecules and salts.

Figure 52.

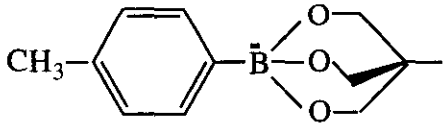
Some examples of boronic acid complexes with triols.

(a)



Benzene boronic acid + glycerol

(b)



4-tolueneboronic acid + 1,1,1 - tris(hydroxymethyl)ethane.

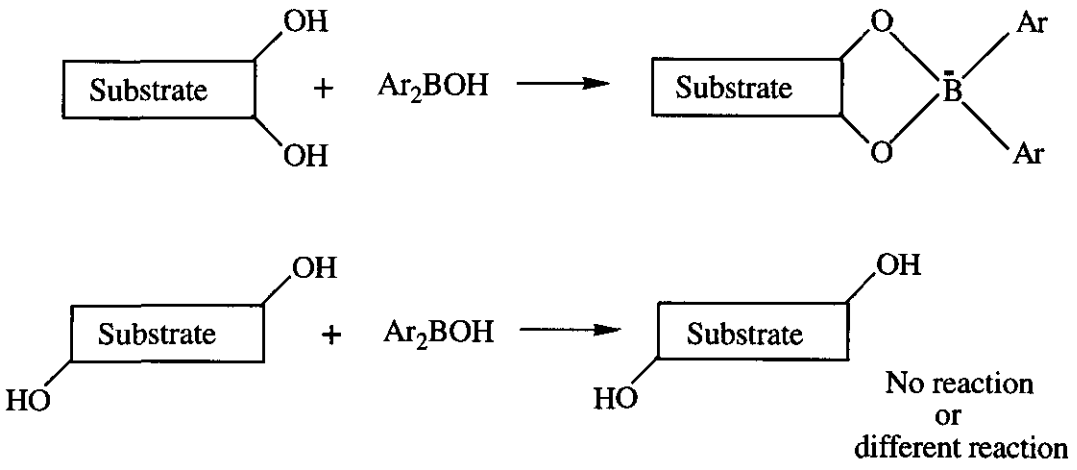
The above example using glycerol (a), gave very well defined, substantial peaks in a negative ion FAB spectrum. This was also the case with solids as in (b). Because in this case a solid reactant was used a solvent also had to be found for FAB work. This solvent had to have a low vapour pressure, high polarity and low or negligible affinity constant for complexation with boronic acids. PEG 200 was used. This method of derivatisation with boronic acids provides (1) a simple analysis of involatile and labile boronic acids, (2) insights into configuration and conformation of polyhydroxy compounds that are absent in

spectra of the neutral substrates, and (3) a measure of the affinity of the substrates for boronic acids as required for affinity chromatography[147].

1. (4). Anion complex formations with borinic acids.

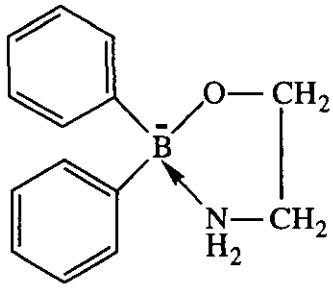
It was then proposed that in an analogous fashion borinic acids could also complex with diols of appropriate geometry (hydroxyl groups close in space) thereby distinguishing them from their isomeric counterparts with distant hydroxyl groups which could not undergo such a reaction (Figure 53).

Figure 53.



Reactions were carried out using diphenylborinic acid which due to its instability is used primarily as its ethanolamine complex shown below (Figure 54). The actual acid was later produced from this complex (as in Section 3. (2). of Chapter 2) and used directly as the derivatising agent.

Figure 54.



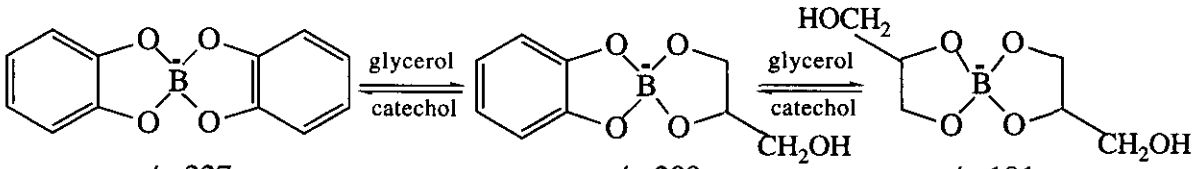
Diphenylborinic acid ethanolamine complex

1. (5). Solvent for FAB studies.

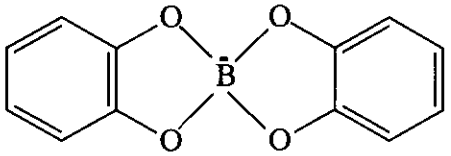
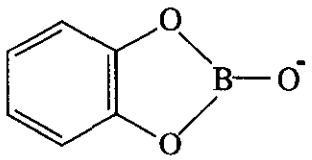
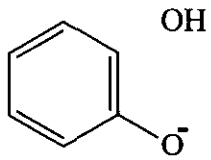
A major problem which had to be addressed was that of which solvent should be used for the FAB work in these studies. An earlier paper[137] found that during FAB mass spectrometry the borate complex formed with diol undergoes ligand exchange reactions with hydroxylic liquid matrices and with glycerol in particular. Glycerol is an unfortunate choice of liquid matrix for such complexes, as a liquid exchange with glycerol can be mistaken for sequential losses of two molecules of water as illustrated in Figure 55.

Figure 55.

Negative ions observed in the FAB spectrum of boron ester (**5.10**) using various matrix.

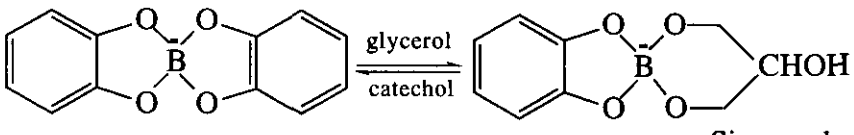


m/z 227 **5.10** *m/z* 209 *m/z* 191

	In Glycerol.	In TEGDEE.
 m/z 227	100%	100%
Apparent loss of water - (H_2O). m/z 209	22%	0%
Apparent loss of water - 2(H_2O). m/z 191	28%	0%
 m/z 135	24%	11%
 m/z 109	29%	4%

TEGDEE does not show the apparent losses of water molecules (minus 18 mass units) as are seen when glycerol is used. A case for the use of TEGDEE as the solvent matrix for this work is therefore established.

Glycerol can also ligand exchange with the borate complex to produce a six-membered ring structure as the two terminal hydroxyl oxygens bind with the boron. However this structure (shown below) is less stable than the five membered ring illustrated in Figure 55 and is rarely formed.



m/z 227

5.10

Six membered ring structure.

Because of the ligand exchange possibilities with glycerol, the solvent of choice for the continuence of the work was tetra-ethylene glycol diethyl ether (TEGDEE) as it provides longer-lasting FAB spectra than the more commmonly used dimethyl ether or thiolane. This is an essential requirement as any subsequent MS-MS experiments will take time to set up whilst the ions are being produced. There is no interference with the complex using this non-nucleophilic solvent and the borates complexes can be generated simply by mixing the two compounds on the FAB probe tip.

The use of this solvent was not however appropriate for all the studies performed. Whilst using another mass spectrometer at St Thomas' hospital TEGDEE itself proved to provide too high a vapour pressure for the particular source being used so that only very short-lived spectra were being obtained. This meant that other matrices had to be developed for use in this instrument.

1. (6). Fast atom bombardment mass spectrometry.

Fast atom bombardment mass spectrometry would be the detection method. FABMS usually acts by producing ions, both positively and negatively charged, within the FAB source. Material is removed into the gas-phase after ionization and some of it will be in the form of ions which can then be focused and analysed by the spectrometer. As well as straight forward FAB-MS, tandem MS-MS would also be able to provide us with further information. MS-MS will allow the analysis of any subsequent daughter ions which may be diagnostic of the complexes formed. In the FAB work reported here the analytes were pre-ionised by reaction with boron-containing acids prior to atom bombardment.

1. (7). Checking the progress of the reaction with NMR spectroscopy.

Ideally the borinic acid and the compounds used as reactants should form a negatively charged complex on the probe tip simply on mixing, that is, if a borinic acid is completely analogous with a boronic acid. This would then eliminate the need for ionisation by FAB. But there is a possibility that the energy of the fast atoms is required to complete the reaction. This can be confirmed using proton nuclear magnetic resonance spectroscopy (NMR) which will allow investigation of the completeness of the reaction in the absence of external factors.

1. (8). Capillary Electrophoresis of Boronate molecules.

As well as affinity chromatography, boron chemistry has now been exploited in capillary zone electrophoresis (CZE). Work has primarily centred on either neutral compounds or compounds which only exist in an ionised state under extreme conditions, for example, carbohydrates[148]. Such compounds would normally need to be analysed by micellar electrokinetic electrophoresis (MEKC). Again it is the complexation reaction with boron which is used to impart a negative charge upon the molecule which facilitates separation by CZE. Carbohydrates can be converted *in situ* to anionic borate complexes in a buffer solution containing the borate ion. Borate solutions are prepared by dissolving pellets of KOH in boric acid solutions and adjusting the pH to the indicated values. This approach has been exploited in the separation of reducing monosaccharides such as glucose, ribose, xylose, galactose and arabinose. The monosaccharides were firstly derivatised to N-pyridylglycylamines to enable detection by either U.V. or fluorimetric means. Separation of these negative species was achieved by generating a fast electro-osmotic flow rate at a high pH of 10.5. This ensures that all molecules elute at the cathode. The monosaccharides are separated dependent upon how fast they migrate against this rapid electro-osmotic flow. *Cis*-orientated hydroxyl groups at C3/C4 of the monosaccharide (arabinose and ribose) preferentially formed borate complexes compared to those with *trans*- disposed hydroxyls (lyxose and xylose). *Cis*- hydroxyl monosaccharides gave values of relative electrophoretic mobility greater than those of *trans*- diols.

1. (9). Applications of Boron work.

The current compounds of interest to be examined by this approach are monoalkyl- and monoacyl-glycerides which are compounds involved with β-oxidation of fatty acids. During the β-oxidation, triglycerols are progressively hydrolysed to produce diacyl- and monoacyl-glycerols when fatty acids are required for metabolic breakdown.

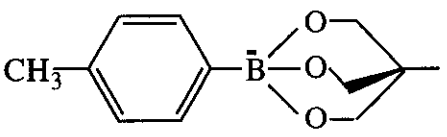
Using standard compounds of the same nature as these it is hypothesised that by derivatising these compounds with various boron-containing acids, charged molecules can be produced for examination by negative-ion mass spectrometry. It is hoped that a routine method, employing mass spectrometric detection can be developed for the eventual analysis of these compounds at physiologically significant levels.

2. Results and Discussion.

2. (1). Optimising Boronic acid complexes by choice of FAB matrix.

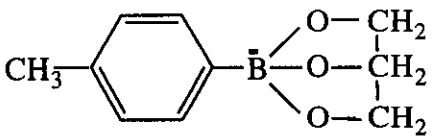
Initial work concentrated on forming boronic acid complexes which had been shown[149] to give useful FAB results. This work was repeated to confirm previous results, their reproducibility and also to illustrate how much difference the choice of solvent can make to the quality of results obtained. The reaction of boronic acids with triols and related compounds produces negatively charged caged compounds. For example, 1,1,1-tris(hydroxymethyl)ethane reacts with 4-toluneboronic acid to give the boronate **5.11**, which is highly compatible with FABMS. This experiment shows the reactions when glycerol and tetraethylene glycol diethyl ether (TEGDEE) are used as solvents. The acid and triol were mixed together on the FAB probe tip in either glycerol or TEGDEE solvent.

Species formed.



m/z 219

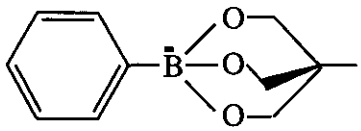
5.11



m/z 191

5.12

This experiment shows how much glycerol interferes with the formation of the complex negative ion required (i.e. m/z 219). With glycerol as the solvent, the $[M]^-$ at m/z 219 appears only at 20% abundance on the given mass spectrum along with $[M]^-$ **5.12**, at m/z 191 which is due to the boronic acid complexing with the glycerol. Negative background ions of glycerol are the most abundant species in this spectrum. When TEGDEE is used the required $[M]^-$ ion at m/z 219 appears at 100% relative abundance and the $[M]^-$ of the glycerol complex disappears. The only primary fragment ion at high mass in this spectrum corresponds to $[M - CH_2O]^-$ at m/z 189 (9%). Using non-nucleophilic TEGDEE as a solvent for these analyses, increases method sensitivity as no complexation with glycerol can occur. The same reaction, but using benzeneboronic acid, yields the anion **5.13**.

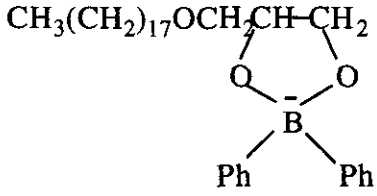


m/z 205

5.13

2. (2). Forming borinic acid complexes with diol molecules.

The next stage of these investigations was to use borinic acid in an analogous way to boronic acid and form complexes with diol compounds. This was done initially by mixing the diphenylborinic acid (DPBA) as its ethanolamine complex (Figure 54) with the chosen diol compound. Batyl alcohol was used as the diol and the species expected is an anion with m/z 507 as shown below.

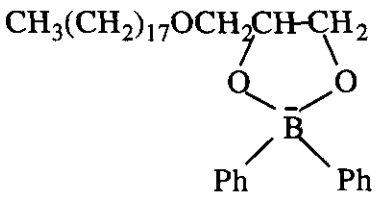
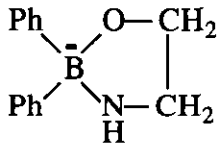
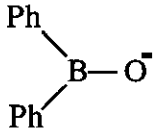


Diphenyl borinic acid and batyl alcohol complex.

It was also decided to use the diphenylborinic acid itself which was acheived by converting the DPBA ethanolamine complex into its acid form (as in section 3. (2) of Chapter 2) before complexing with the diol compound, so that the anionic complex was formed from the

borinic acid and batyl alcohol as the diol. The major ions observed in both spectra are shown in Table 6.

Table 6. Products from the reaction of diphenylborinic acid with batyl alcohol.

Observed m/z value of major ions.	Proposed ion structure.
m/z 507	**5.14** 
m/z 224 base peak when ethanolamine complex is used.	**5.15** 
m/z 181 base peak when borinic acid is used.	**5.16** 
m/z 43	**5.17** BO_2^-

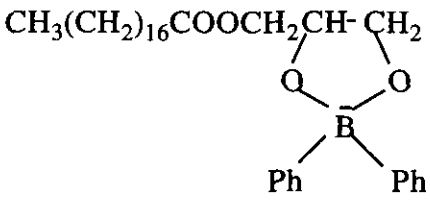
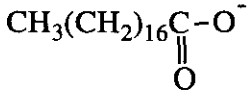
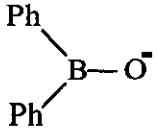
The first method of mixing relatively small diols, and related compounds, on the FAB probe tip with the ethanolamine complex of diphenylborinic acid, proved unsatisfactory with typical lipid metabolites such as batyl alchohol and 1-monostearoylglycerol. The main peaks observed were the $[M - H]^-$ ions of the reagent and substrate. For example, mixing batyl alcohol and the ethanolamine complex of diphenylborinic acid gave anion **5.15** from the reagent (100%) and the $[M - H]^-$ ion m/z 343, of batyl alcohol (12%); with the required complex at m/z 507, **5.14**, having a relative abundance of just 8%. The observation of both reagent and batyl alcohol remaining unreacted on the probe tip strongly suggested a sluggish reaction, which may be explained as a steric effect. The bulk of the long side-chain could

inhibit the approach of the reagent. A smaller, more active reagent, and greater control over the reaction conditions, were achieved by changing the reagent to diphenylborinic acid itself and carrying out the reaction conventionally "at the bench". To allow a greater reaction time, free diphenylborinic acid and the substrate diol were mixed in dichloromethane and left to stand for 3 hours or more. To encourage complete reaction, excess of diphenylborinic acid was used. The subsequent analysis by fast xenon or caesium atom bombardment yielded the required complex **5.14** at 80% of the base peak. The base peak was due to excess diphenylborinic acid at m/z 181, structure **5.16**. As the peak at m/z 507 is much larger when the acid is used it is also possible to see another peak next to it at 506 which is approximately 20% the abundance of the 507 peak. This is due to the presence of boron within the compound. The ^{10}B isotope occurs once for every four ^{11}B isotopes. Another peak which is characteristic of boron spectra is that at m/z 43 which corresponds to BO_2^- (**5.17**). A typical spectrum is shown in Figure 56 (Appendix 5), obtained by xenon atom bombardment of a TEGDEE solution. Such a spectrum is short-lived because, using the commercial xenon atom gun, the ion source is warm enough to volatize TEGDEE quickly.

2. (3) The formation of borinic acid complexes with other diol compounds.

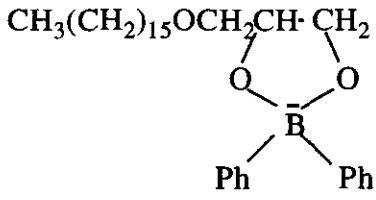
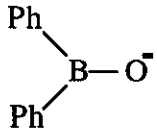
Once the method had shown to be viable with one diol, the next stage was to examine the possibilities of complexation with other similar compounds. This involved using two other diols, monostearoyl-*rac* -glycerol and 1-O-hexadecyl-*rac* -glycerol. Ions were observed for both of these compounds when complexed with diphenylborinic acid. The proposed structures of the main ions observed from the complexation of the borinic acid with monostearoyl-*rac* -glycerol (complex **5.18**) using TEGDEE as solvent are illustrated in Table 7.

Table 7. Major products from the reaction of monostearoyl-*rac* -glycerol with diphenylborinic acic.

Observed m/z value of major ions.	Proposed ion structure.
m/z 521 at 75% of base peak	**5.18** 
m/z 283 base peak (fragment of m/z 521)	**5.19** 
m/z 181 at 90% of base peak	
m/z 43 at 15% of base peak	BO_2^-

The example using 1-O-hexadecyl-*rac* -glycerol showed very low levels of complex (**5.20**) at an abundance of only 15% of the base peak at m/z 181. During this aquisition the ion current only reached 47mV which could explain the low response. Such a low ion current could be due to a lack of sample or an instrumental problem. The ions recorded were however those required at a reasonable ratio as in Table 8.

Table 8. Major products from the reaction of hexadecyl-*rac* -glycerol with diphenylborinic acic.

Observed m/z value of major ions.	Proposed ion structure.
m/z 479 due to the diol acid complex at 15% of base peak	**5.20** 
m/z 181 base peak 100%	
m/z 43 at 11% of base peak	BO_2^-

From the examples it appears that using DPBA as opposed to its ethanolamine complex helps to optimise the reaction and improve method sensitivity when used in conjunction with TEGDEE instead of glycerol.

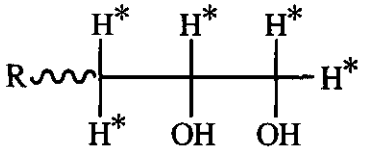
2. (4) To test where and when the borinic acid/diol reaction occurs.

All the previous experiments confirmed that negative ion complexes are formed when the diol of choice and the borinic acid are mixed together, placed into the FAB source and bombarded with xenon atoms. What these experiments do not show is whether the reaction would still occur without the energy supplied to it by the FAB source which possibly enhances the reaction. Proton NMR spectroscopy was the technique used to test the progress of the reaction without any external energy involvement.

The required diol and diphenylborinic acid (DPBA) were mixed and NMR solvent ($CDCl_3$) added followed by immediate examination by ^1H NMR spectroscopy. Another compound, 3-methoxy -1,2-propanediol (MPD) was introduced here as a model compound (NMR

spectra in Figure 57, App. 5) to react with the borinic acid. This has a very simple structure and its diol group makes it useful as an example of what should be expected with other more complex diols. This was mixed with DPBA and the spectra recorded in Figure 58 (App. 5). Batyl alcohol itself was then also mixed with DPBA and the results recorded in Figure 59 (App. 5). Both diol compounds showed the same result in terms of NMR peaks. By examining the splitting of peaks and the chemical shifts visible in these results, the extent of the reaction between these compounds could be measured.

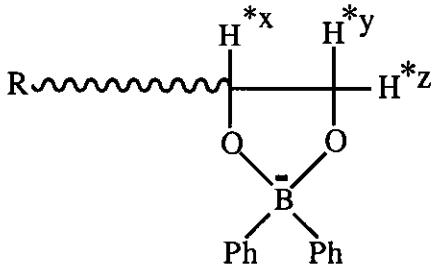
From examination of Figures 57 - 59 (App. 5) it could be deduced that the area of importance within the spectra was between 3 and 5 ppm. This is the area where both batyl alcohol and 3-methoxy-1,2-propanediol display three groups of peaks. The NMR of 3-methoxy-1,2-propanediol alone is given in Figure 57 (App. 5). There are four complex signals at chemical shifts of 3.24, 3.36, 3.46 and 3.67 ppm respectively. The peaks are due to protons found at the diol terminus. Definitive assignments of the non-equivalent protons in the two CH_2 groups would require further NMR investigations, but the CH proton was assigned to the multiplet at 3.67 ppm.



All protons shown are non-equivalent because of the chiral centre.

Chemical shifts of these peaks will be followed to check on the progress of the reaction. The diphenyl borinic acid (DPBA) shows no significant peaks in this area. Figure 58 (App. 5), shows the 3-methoxy -1,2- propandiol and DPBA mixture and it can be seen that the addition of DPBA does cause chemical shifts of the second order doublets along with a change in the splitting patterns of doublets to 4.05 and 4.31 and the multiplet from 3.67 to 4.62 ppm. Broad peaks between 3.2 and 3.8 could be due to water in the sample. Unfortunately, these signals prevent observations of any resonances from unreacted diol. Complexation with DPBA also effects the batyl alcohol in the same way by causing similar changes in chemical shifts. The structure below represents the final situation when the acid and diol are mixed and reacted.

Representation of the most affected hydrogens in the final acid/diol product.

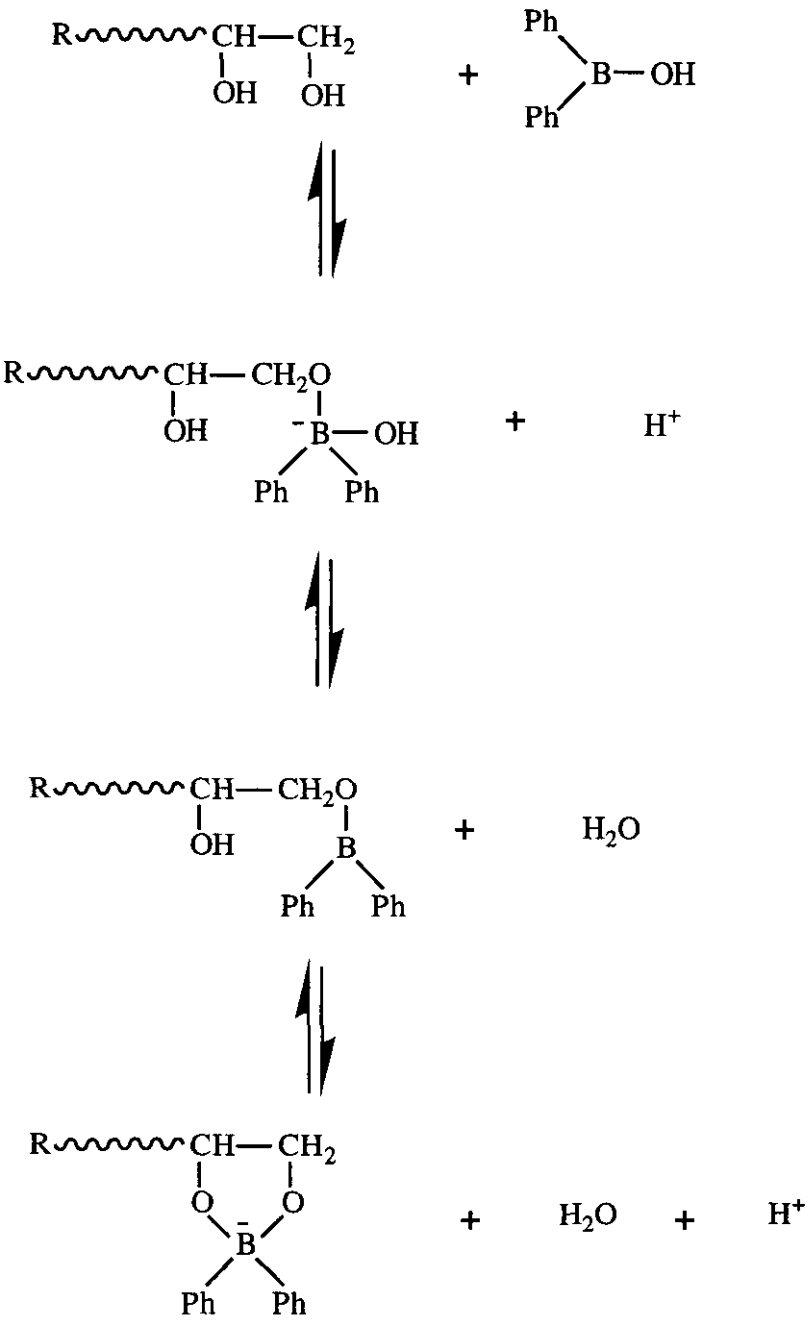


The hydrogen atoms marked by * are those most effected by bonding or partial bonding of the DPBA, probably corresponding to x, y and z on Figure 60 (App. 5) . The fact that the areas pinpointed as second-order doublets of doublets and a multiplet do undergo a chemical shift leads to the conclusion that the reaction between the acid and the diol is proceeding even without the energy input of FAB. However this does not tell us to what extent the reaction has proceeded, and whether the product is the fully formed five-membered ring.

2. (5) Optimisation of the complexation reaction.

The idea here was to facilitate the reaction by adding in various compounds, (Section 3(5) of Chapter 2). Looking more closely at the potential reaction mechanism it was apparent that it could take place in several stages, each being reversible as detailed in Figure 61.
Stage one sees the production of an ester and water as a by-product of the reaction. The complexation process is completed in stage two where hydrogen ions are formed. It was decide to try and drive the reaction in the forward direction by removing the by-product at each stage. Dehydrating agents like molecular sieve, and the basic compounds, sodium hydrocarbonate ($NaHCO_3$), pyridine and di-isopropyl-ethylamine were tried to remove the water and proton by-products. This should theoretically optimise the reaction to produce the required complex in larger amounts. Figure 62 (App. 5) shows the nmr spectra obtained when the molecular sieve which will remove water was added, Figure 63 (App. 5) when the sieve and $NaHCO_3$ were added and Figure 64 (App. 5) when a mixture containing both of these was refluxed for 24 hours. Addition of the molecular sieve resulted in the appearance of two new sets of peaks. One of these occurred at a chemical shift of 4.21 ppm and was assigned to another second-order signal from a CH_2 group with non-equivalent protons. Both vicinal and geminal couplings are apparent. The other second multiplet of peaks occured at 4.71 ppm.

Figure 61. Potential mechanism by which the acid and diol react.



Their appearance on addition of sieve, again suggests that the diol/acid complexation reaction may be proceeding further, as the environment of the hydrogen atoms at the diol terminus changes again. Figure 63 (App. 5) also shows the same set of peaks when both sieve and $NaHCO_3$ were added to the complexation mixture. The addition of the base to remove hydrogen atoms does not seem to further change the environment of the diol hydrogens. The other bases used, pyridine and di-isopropyl-ethylamine were added whilst maintaining the prescence of molecular sieve. Their addition did not produce any visible

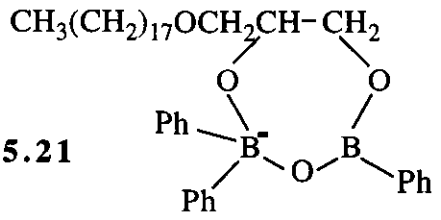
changes to the NMR spectra as all three sets of peaks originally identified in Figure 62 also appear when these bases were used. The bases used were chosen because they do not interfere with the NMR spectrum as well as being proton scavengers.

From these results it was decided to add molecular sieve to all the ensuing complexation mixtures. This was most convenient because the sieve seemed to have a significant effect on the reaction whilst being a solid it could easily be removed from the mixture before analysis. However, the complexity of the nmr spectra indicates that the reaction does not proceed fully to the five-membered ring. At least one intermediate is still present after prolonged refluxing. Therefore, the relatively "clean" FAB spectra observed suggest that fast atom bombardment is required to complete the reaction.

2. (6) Confirmation of the method on a more sensitive mass spectrometer.

This experiment involved performing the complexation reactions with batyl alcohol and monostearoyl-*rac* -glycerol as the diols and incorporating the lessons learnt during the NMR experiments. This meant adding molecular sieve to each reaction mixture before analysis on a more sensitive FAB mass spectrometer with a caesium atom gun at St. Thomas's hospital. It was hoped that this spectrometer would be more sensitive than the instrument used previously and some limit of detection studies were performed. The samples to be analysed were initially placed onto the probe tip in a matrix of TEGDEE but this again proved to be too volatile for this mass spectrometer which ran at a higher vacuum than the spectrometer at the Open University. This meant that it proved difficult to obtain a sufficient number of scans to establish viable spectra. Spectra obtained using TEGDEE are given in Figure 65(a) (App. 5) where 0.1g of batyl alcohol had been used during sample preparation. The major ions in this spectrum were again those at m/z 507 and 181. Structures could also be proposed for some of the minor peaks in this spectrum. The ion at m/z 611, which has an isotope pattern consistent with two boron atoms, is assigned to structure **5.21**. This anion would be a product of over-reaction between batyl alcohol and the excess of borinic acid but the mechanism of its formation is unclear.

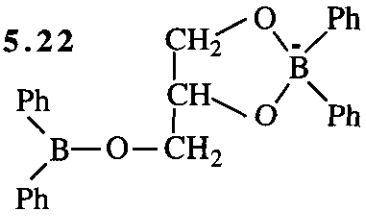
5.21



m/z = 611

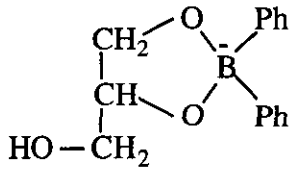
The peak at m/z 419 can be attributed to glycerol contamination in the source (ion **5.22**). This spectrum was obtained using TEGDEE as the solvent matrix but it was found that the longevity of the spectum was poor. An attempt to overcome the problem of spectral longevity involved using a matrix mixture of 50/50 TEGDEE and glycerol. This did improve the longevity of the spectra but also meant that the borinic acid reacted with the glycerol which competed with the diol to form a complex, which explains the increase in size of the extra peaks in figure 65(b) (App. 5) using batyl alcohol as the diol. The peak at m/z 255 was due to the complexation of borinic acid and glycerol **5.23** and the peak at m/z 419 is the result of two molecules of borinic acid complexing with a single glycerol molecule **5.22**.

5.22



m/z 419

5.23



m/z 255

The isotope patterns at m/z 255 and 419 are consistent with one and two boron atoms, respectively. The following table illustrates the abundance of the ions observed when the mixed matrix of 50/50 glycerol/TEGDEE was used.

Table 9. Major ions produced when DPBA and batyl alcohol are reacted in the presence of glycerol and TEGDEE.

Observed m/z value of major ions.	Percentage abundance compared to the base peak.
m/z 507	Base peak
m/z 419	52%
m/z 255	45%
m/z 181	39%

Some of the smaller peaks at m/z 343 and 363 are present irrespective of the identity of the diol substrate. For example, they also occur in the spectrum of 1-monostearoylglycerol which gives a large peak for its borinate complex at m/z 521 as in Table 10.

Table 10. Major ions produced when DPBA and 1-monostearoylglycerol are reacted in the presence of glycerol and TEGDEE.

Observed m/z value of major ions.	Percentage abundance compared to the base peak.
m/z 521	41%
m/z 419	77%
m/z 255	Base peak
m/z 181	68%

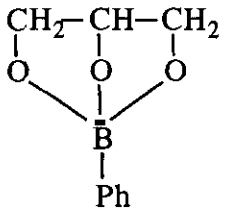
The longer lasting spectra obtained by using the mixed matrix meant that a spectrum of the batyl alcohol at a level of 0.01g could be obtained and the subsequent peak intensities are given in Table 11.

Table 11. Major ions produced when DPBA and batyl alcohol (0.01g) are reacted in the presence of glycerol and TEGDEE.

Observed m/z value of major ions.	Percentage abundance compared to the base peak.
m/z 507	31%
m/z 419	Base peak
m/z 255	84%
m/z 181	59%

On further decrease of the level of batyl alcohol to 0.001g the m/z at 507 became lost in the background. In this spectrum a new peak at m/z 177 is observed which may be due to the fragment **5.24**.

5.24



m/z 177

This ion was present at 34% abundance with m/z 419 being the base peak. The peaks at m/z 255 and m/z 181 were apparent at 88% and 32% respectively. An effort was made to deduce the daughter ions of the batyl alcohol/diol complex by conducting collision-induced dissociation of the anion. Product-ion scanning revealed peaks at m/z 181 (Ph_2BO^-) and 77 (Ph^-).

It was then decided to try poly(ethyleneglycol) (PEG) as the liquid matrix which allowed us to obtain even longer lasting spectra for batyl alcohol. However this introduced PEG background ions which could be seen along with the required ion complexes. These peaks can be seen 44 mass units apart (e.g. m/z 269, 313, 357, 401) throughout the range of the spectrum. The spectrometer used proved to be more sensitive than that used for previous experiments but it was thought that further improvement in spectra longevity could be acheived with an alternative matrix.

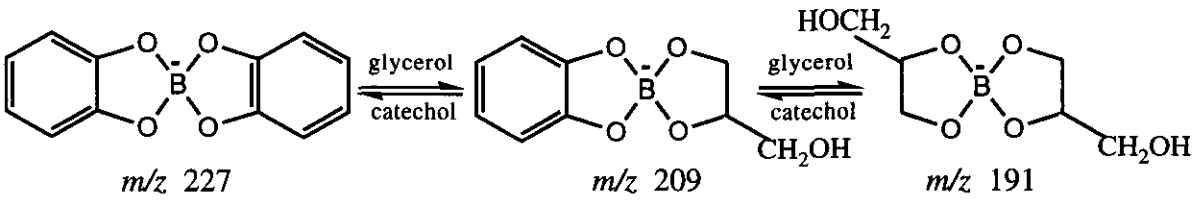
2. (7) Developing an alternative matrix for the analysis of the borinic acid derivatives.

Two different ethylene glycols were used to produce a liquid matrix that would be less volatile than TEGDEE so they could be used in a variety of ion sources, but also which wouldn't give a high level of background noise in the area of interest (i.e. m/z 507). This involved producing dimethyl ether derivatives of penta-ethylene and hexa-ethylene glycol, via the procedure detailed in Section 3.(3) of Chapter 2. The spectrum of hexa-ethylene glycol dimethyl ether (HEGDME) was obtained and is given in Figure 66 (App. 5) which illustrates fragment ions 44 mass units apart. When used as a matrix for the analysis of batyl alcohol complexes it proved to be a viable alternative to TEGDEE and glycerol, giving no noticeable background peaks in the spectrum, and longer lasting spectra as shown in Figure 67 (App. 5).

2. (8) Analysis of Boron esters using Electrospray mass spectroscopy.

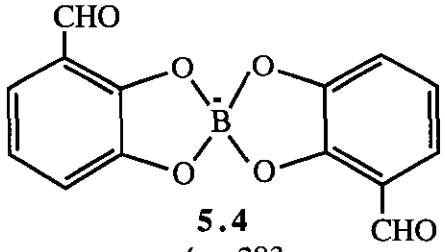
For the mass spectrometric analysis of spiroborates, negative-ion fast atom bombardment provides spectra with prominent molecular anions and some fragmentation[135,136]. However, the liquid matrix must be chosen with care. Nucleophilic solvents, particularly

those that are capable of a chelating effect, such as glycerol are not recommended because they exchange with the spiroborate ligands[135,136] as in scheme 1:



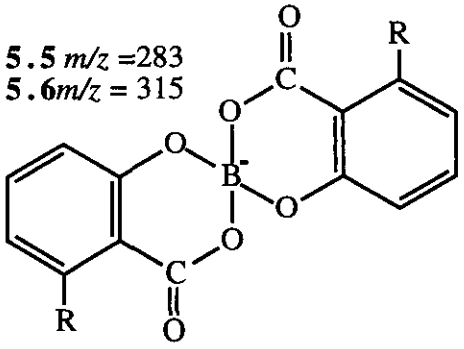
Scheme 1. Ligand exchange process that occurs during FABMS of a spiroborate in the presence of glycerol.

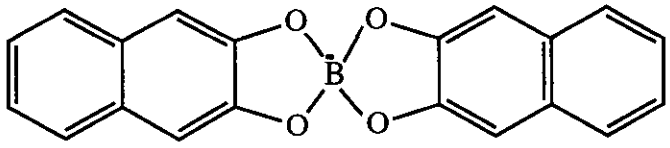
To avoid this problem, the solvent TEGDEE was used as liquid matrix. Another solution to the problem of ligand exchange would be the application of a different mass spectrometric method in which a potentially chelating solvent is not necessary. An appropriate method is negative-ion electrospray. It is common for substrates to be dissolved in water-acetonitrile mixtures for electrospray analysis. Borates are stable to acetonitrile but hydrolyse in water. To obtain electrospray spectra of the spiroborates, they were dissolved in water-acetonitrile and analysed within 1 hour. Even so, several spiroborates gave spectra consisting of the [M - H]$^-$ ions of the ligands only, indicating that hydrolysis was complete before analysis. The structures of spiroborates that produced electrospray spectra consisting of the intact anion are shown (**5.4 - 5.7**).



5.4
m/z = 283

Where R = H **5.5** *m/z* =283
R = OH **5.6***m/z* = 315

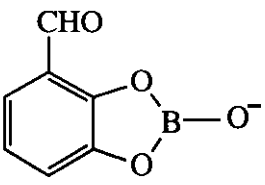
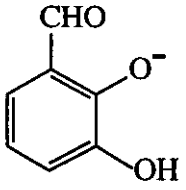
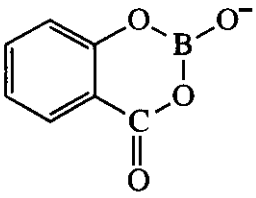
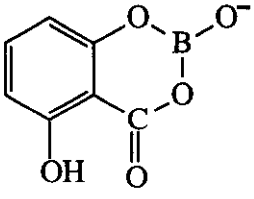




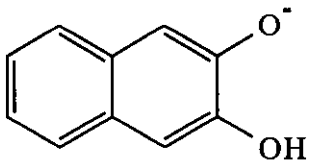
5.7
m/z = 327

At a cone voltage of -30 V, the spiroborates **5.4**, **5.5** and **5.6** showed no evidence of fragmentation, the only significant peaks being due to intact spiroborate M^- ions and background. Figure 68(a) and (b) (App. 5) shows the spectra of anions **5.4** and **5.5** respectively under these conditions. This behaviour should be contrasted with the negative-ion FAB mass spectra of the same spiroborates in which the intact anions produced the base peaks but some fragmentation also occurred, as shown in Table 12.

Table 12. Negative-ion FAB mass spectra of three spiroborates.

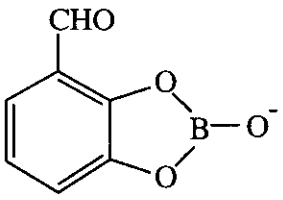
Spiroborate	Fragment ion(s) (percentage relative abundance)
5.4	 (11%)  (3%)
5.5	 (18%) -
5.6	 (9%) -

Compound **5.7** gave the intact anion at m/z 327 but also a large peak at m/z 159. Given that the other spiroborates did not fragment, this anion is ascribed to the [M - H]$^-$ ion of the free ligand produced by hydrolysis (structure **5.25**). Therefore, in the case of compound **5.7**, partial hydrolysis is proposed.



5.25

In an attempt to induce fragmentation, spiroborate **5.4** was re-examined with an increased cone voltage of -120 V. The spectrum obtained is shown in Figure 69 (App. 5). The key fragment ion produced occured at m/z 163. In common with the FAB mass spectrum, which also showed this peak, the ion was assigned to structure **5.26**.



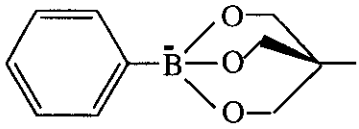
5.26

This aldehydic ion also appears to eject CO to give the small peak at m/z 135. The only other significant fragment ion occurs at m/z 254. It is difficult to propose a structure for this [M - 29]$^-$ ion without invoking a radical anion. The nature of this fragmentation requires further study.

In general for spiroborates, negative-ion electrospray provides a milder form of mass spectrometry than FAB. The former has the advantage of circumventing ligand exchange, but the potential for hydrolysis in the aqueous medium for electrospray is a considerable disadvantage.

2. (9) Analysis of further boron complexes using electrospray mass spectroscopy.

Electrospray can be used as a method of analysis to avoid the problem of ligand exchange which does occur when glycerol is used in FABMS. Negative-ion electrospray analysis was effected on boronate **5.27**.

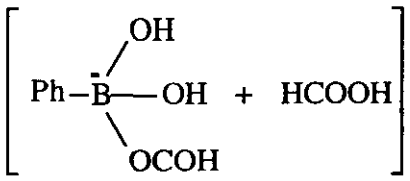
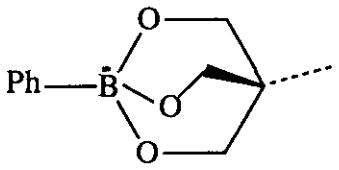
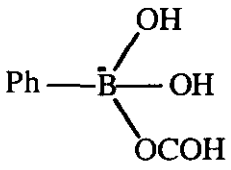


m/z 205

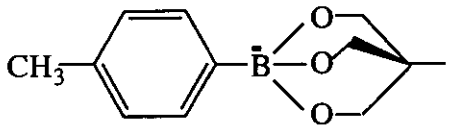
5.27

The sample was dissolved in water-acetonitrile and injected into the carrier of water-acetonitrile-formic acid (50:50:1) within 30 minutes to minimise hydrolysis. The main features of the spectrum, other than background ions from the carrier are given in Table 13.

Table 13. Main features of the negative-ion electrospray mass spectrum of anion 24 in $H_2O/CH_3CN/HCOOH$.

m/z value (relative abundance)	Proposed ion structure	Origin
213 (69%)		Reaction between formic acid and excess $PhB(OH)_2$
205 (93%)		**5.27**
175 (64%)	$[M - CH_2O]^-$	Fragment ion from **5.27**
167 (100%)		Reaction between formic acid and excess $PhB(OH)_2$

As with the analogous FAB mass spectrum of anion **5.28**, the electrospray spectrum of anion **5.27** exhibited a prominent peak for the intact anion (m/z 205) and a fragment ion corresponding to $[M - CH_2O]^-$ (m/z 175).

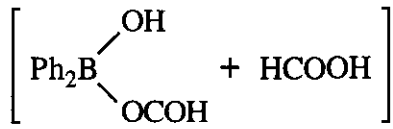


m/z 219

5.28

The crude reaction mixture contained benzeneboronic acid because it was used in excess to drive the reaction with the triol to completion. This excess of benzeneboronic acid reacted with formic acid in the carrier solution to give peaks at m/z 167 and 213 as indicated in Table 11. In the mass range m/z 50-240 there was no evidence for other boron-containing ions and/or fragment ions. Negative-ion electrospray mass spectrometry was also used to examine the diphenylborinate of batyl alcohol as shown in Figure 70 (App. 5). The intact molecular anion provided the only large peak in the high mass region (m/z 507). In the range

m/z 100-300 there were several other peaks including m/z 181 (98%) resulting from the excess borinic acid. The base peak, at m/z 273, is attributed to a reaction between excess of diphenylborinic acid and formic acid. Its proposed structure **5.29** is analogous to that observed with excess benzeneboronic acid and formic acid (Table 7, m/z 213).



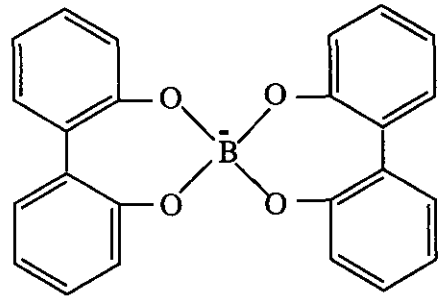
m/z 273

5.29

In summary, the use of a standard carrier solution containing formic acid (for enhancement of protonantion in the positive-ion mode) had no apparent effect on the observation of the molecular anions. However, it did react with excess derivatizing agent. Given that the formic acid additive is inappropriate for negative-ion work, it would be removed in any future work.

2. (10) Analysis of Boron species by CZE.

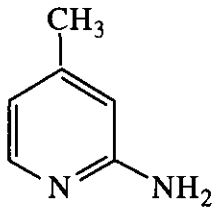
After successfully obtaining results for some boron species by electrospray mass spectrometry it was decided to expose them to CZE. Analytes similar to those that gave results by ES were used as they appeared to be least effected by the presence of water. The idea of this work being to explore the feasibility of eventually performing CE/ES on these molecules in an aqueous medium. A fully aqueous buffer of boric acid at alkaline pH was used. The spiroborate **5.8** was dissolved in the buffer and subjected to CZE.



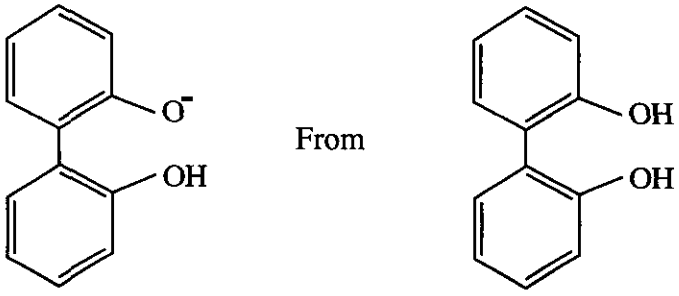
5.8

Electrophoresis of this sample resulted in the electropherograms in Figure 71 (App. 5). Figure 71(a) shows the first run of the sample and three peaks are displayed. It was thought that the first peak at 1.9 mins was due to the counter-cation which was present in the

spiroborate sample (**5.30**) but which under these conditions existed as a neutral species. This meant that it actually marked the electro-osmotic flow rate which approximated to 20 cm per minute using this particular buffer. At this high flow rate it was possible to cause the negatively charged spiroborate to migrate against the electrostatic flow and this was thought to be responsible for one of the remaining peaks. Figure 71(b) illustrates the result of running the same sample one half hour later. The peak at 3 minutes was observed to be almost twice as large as it was in Figure 71(a). It was postulated that this peak could be due to the hydrolysis of the spiroborate resulting in compound (**5.31**). Therefore spiroborate **5.8** was now believed to be the peak at 2.7 minutes which had decreased in size from that in Figure 71(a).



5.30



5.31

To further test the validity of this theory the biphenol compound from which the third peak was thought to be derived was also subjected to electrophoresis and its migration time noted as Figure 71(c). Under the same conditions as all the other analyses this compound gave a migration time which matched that of the unidentified peak at 3 minutes which confirmed the identity of this peak as the hydrolysis product of spiroborate **5.8**. Further evidence came in the migration order of the peaks. The spiroborate anion would not have as large a charge density as the biphenol anion and so would not be expected to migrate in opposition to the electro-osmotic flow as fast as the biphenol anion. As the electro-osmotic flow ensures that both anions will migrate past the U.V. window the spiroborate will migrate through the column at a faster rate than the biphenol hydrolysis product.

3. Conclusion.

The results obtained during these studies show that both boronic and borinic acid are extremely useful in providing a mechanism for the analysis of non-charged hydroxyl-containing compounds. This has been achieved by producing esters of the boron acids which provide excellent negative ion mass spectra. These pre-formed anions are stable enough to give lasting spectra of the intact molecular ion M^- as well as providing several fragment ions of low abundance during collision-induced dissociation experiments. All of the boron compounds are susceptible to ligand exchange during analysis by FABMS and hydrolysis during electrospray MS. In the former case the use of non-nucleophilic, non-chelating solvents was employed, these being methyl ethers of ethylene glycols, which overcame the stability problem. Most of the esters of boron acids subjected to negative-ion electrospray MS were successfully examined even though water was not actively excluded. Some of the spiroborates analysed did only provide $[M - H]^-$ ions of hydrolysis products but the susceptibility of the spiroborate to hydrolysis seemed to be structure dependent and upon further analysis of these under CZE the rate of hydrolysis could be estimated. The mass spectrometric analysis of monoalkylglycerols and monoglycerides was achieved using a relatively simple procedure and large, usually base, peaks were provided. Hence, the derivatisation and analysis of these by either FAB or electrospray may be a useful means of detecting them. Limits of detection, application to serum samples, quantification aspects and the value of tandem mass spectrometry for the analysis of mixtures containing such metabolites have yet to be addressed. The scope for further work in this area is therefore very wide but the results obtained so far do point to this being both worthwhile and beneficial to the scientific community.

4. References.

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Appendix 5

Figures from Chapter 5.

Figure 56. Negative-ion FAB mass spectrum of the diphenylborinate of batyl alcohol using a TEGDEE matrix.

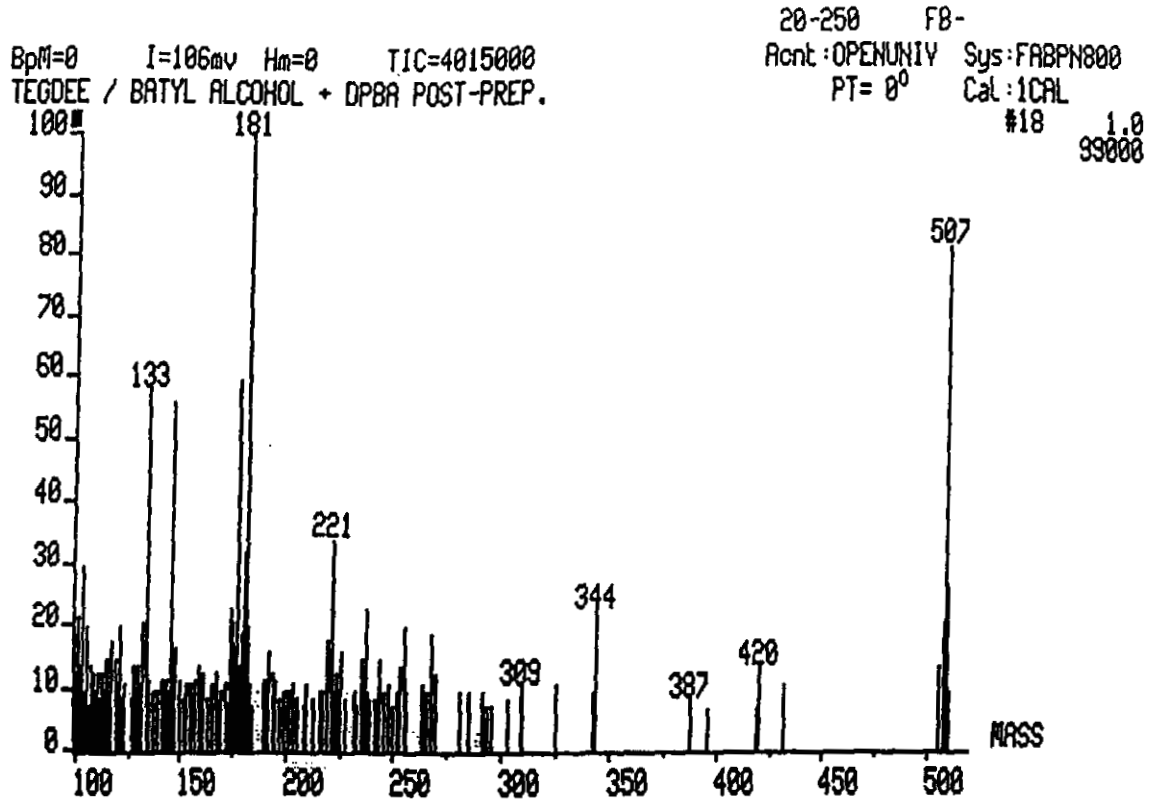


Figure 57. NMR spectrum of 3-methoxy-1,2-propandiol.

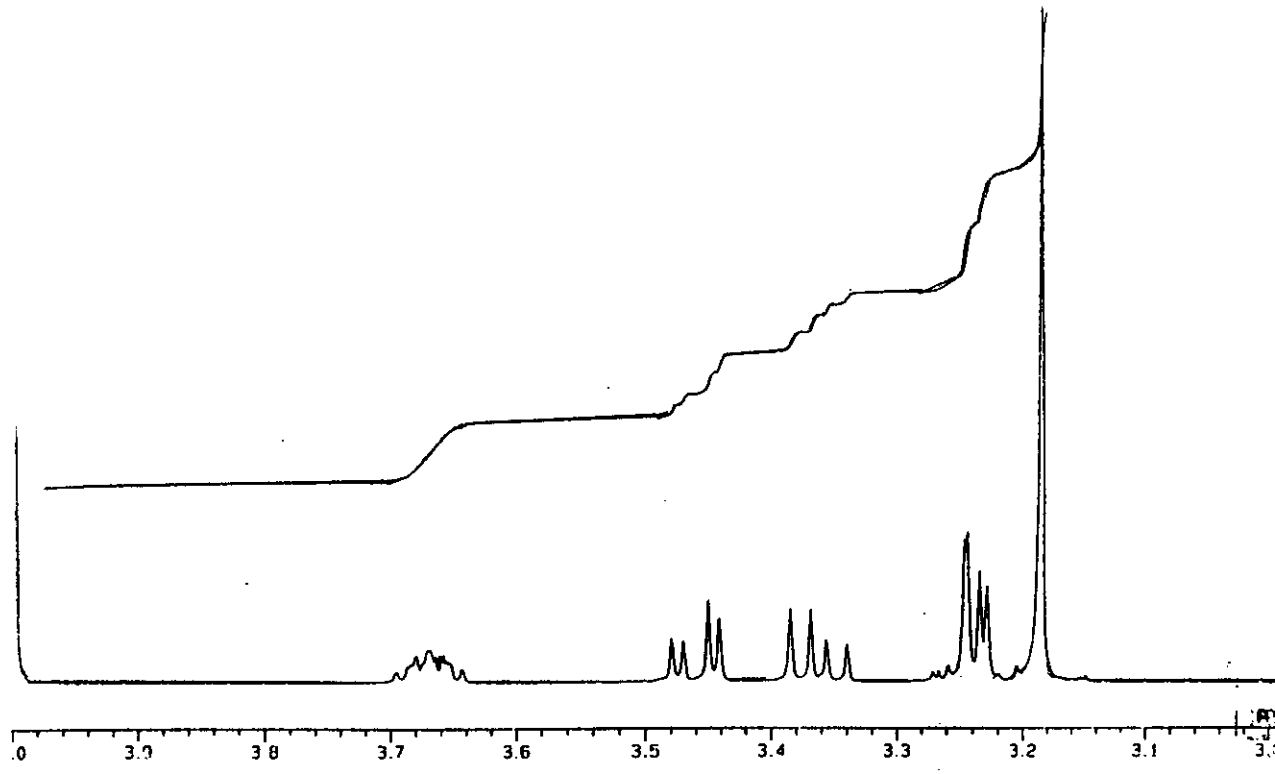


Figure 58. NMR spectrum of the 3-methoxy-1,2-propandiol and DPBA mixture.

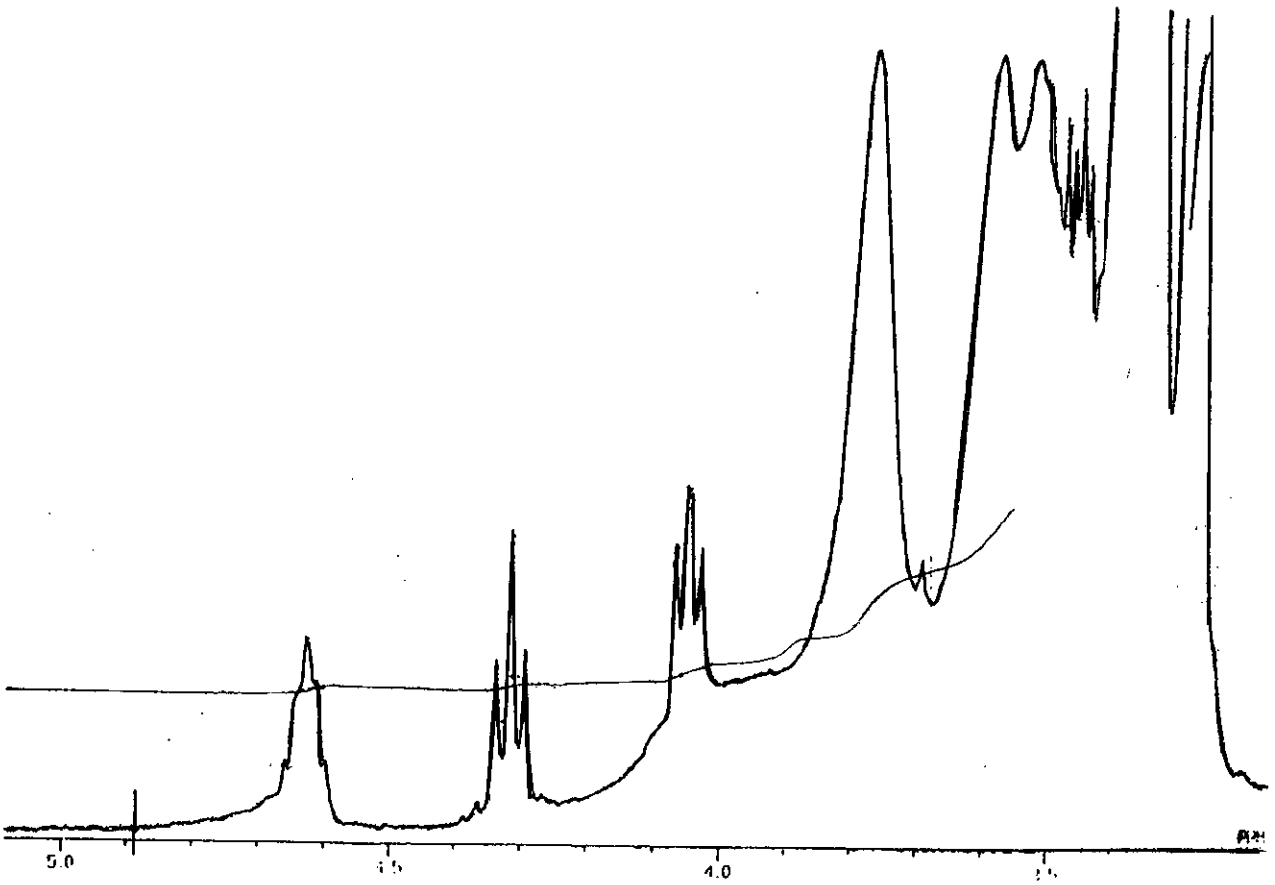


Figure 59. NMR spectrum of the batyl alcohol and DPBA mixture.

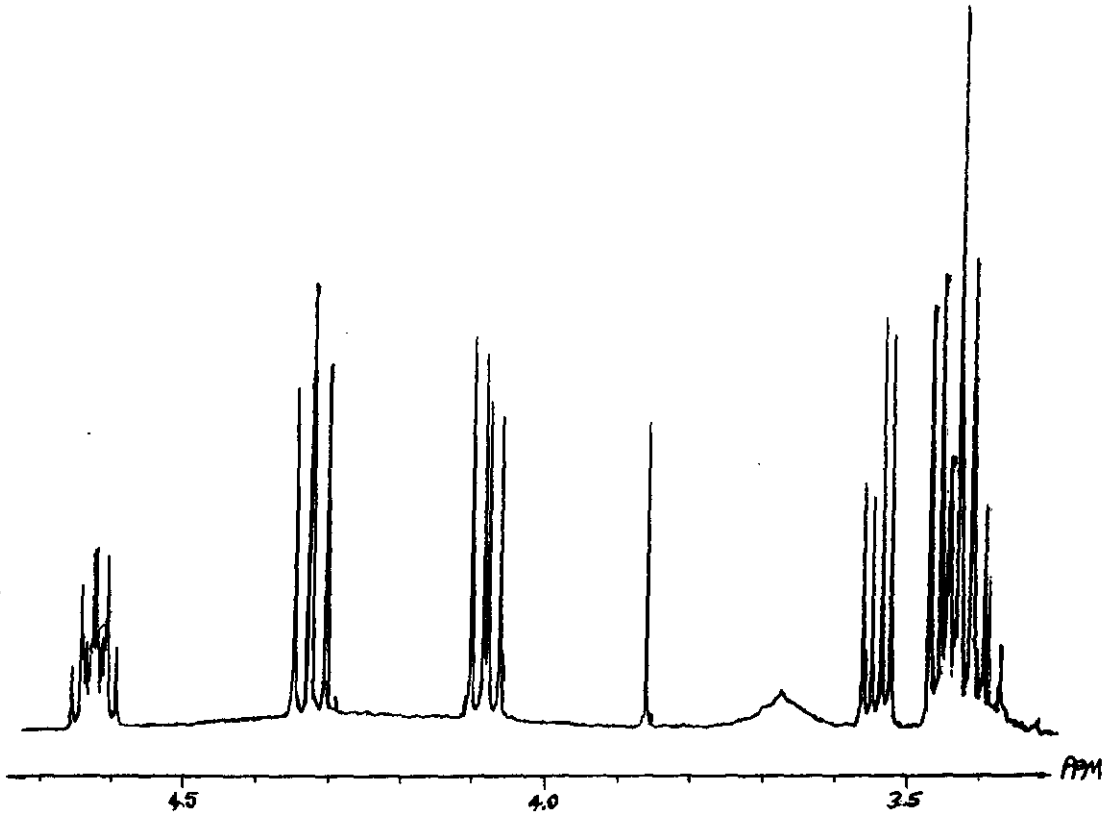


Figure 60. NMR spectrum of the batyl alcohol and DPBA mixture illustrating the areas of interest at X,Y and Z.

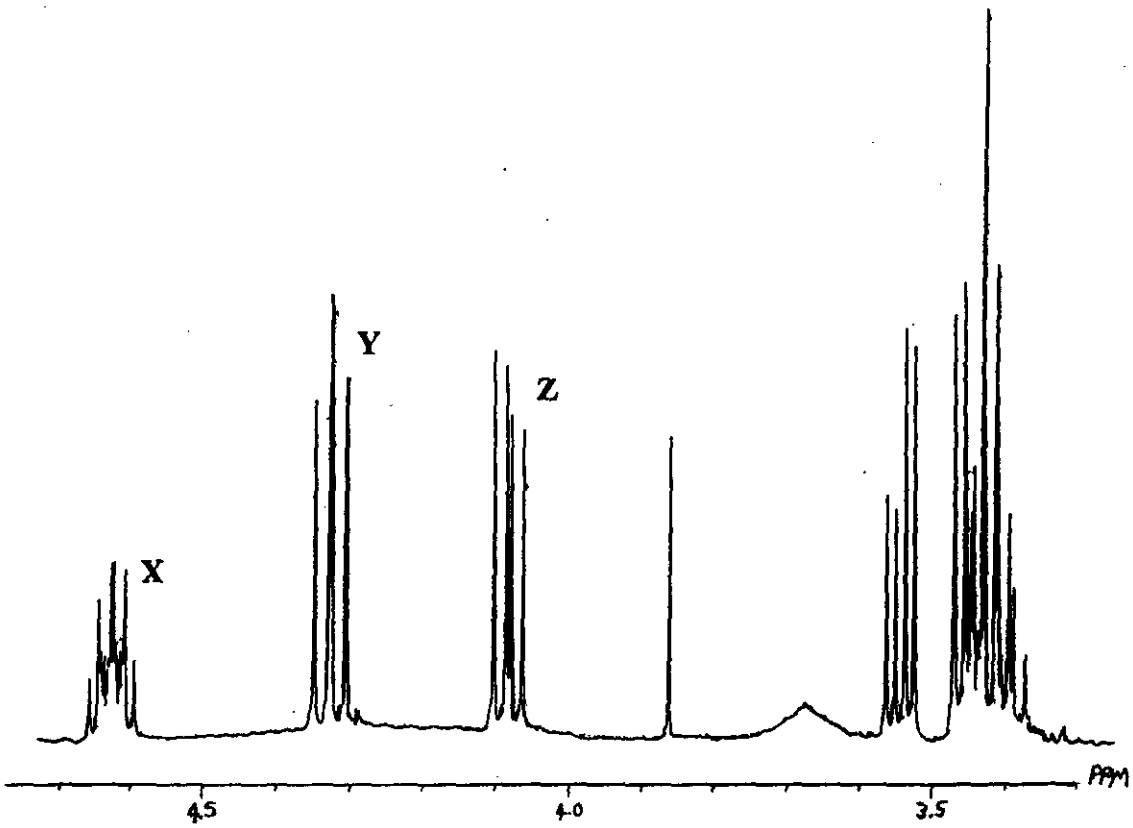


Figure 62. NMR spectrum of the batyl alcohol and DPBA mixture with the addition of molecular sieve.

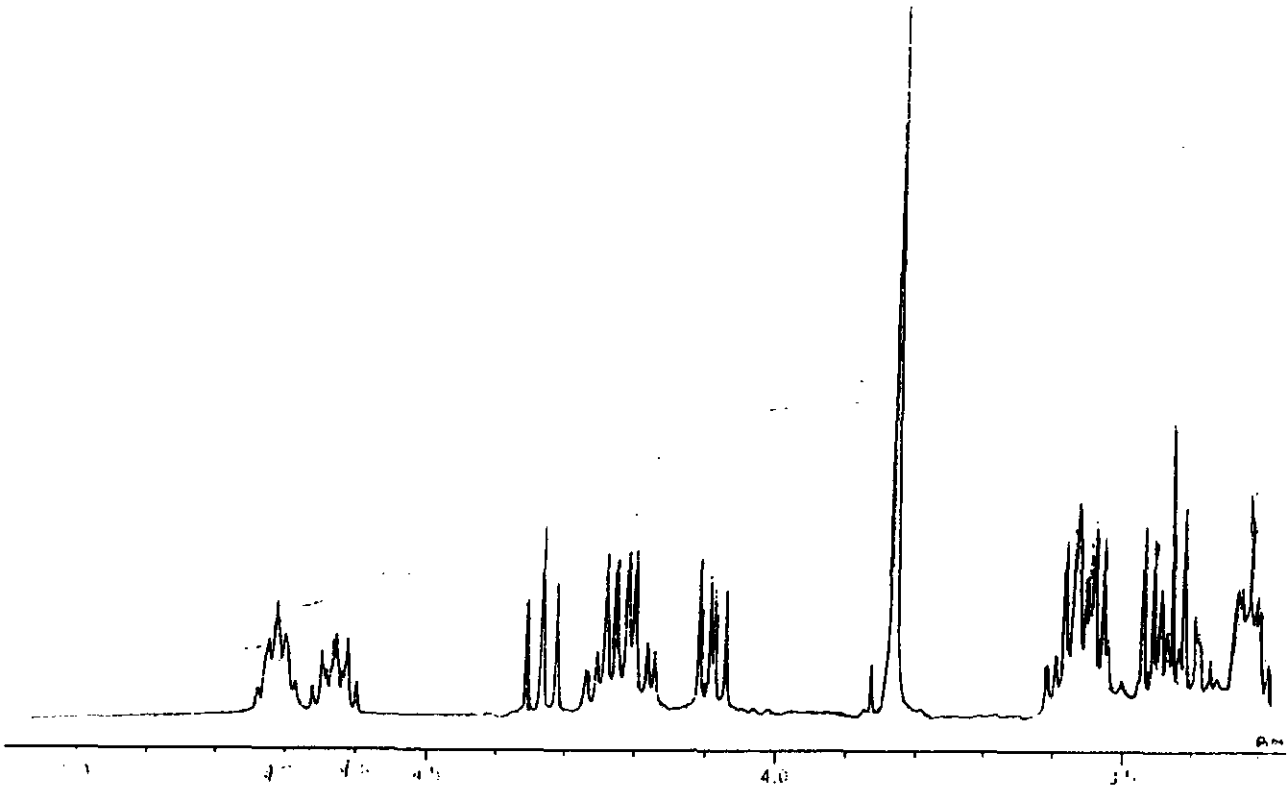


Figure 63. NMR spectrum of the batyl alcohol and DPBA mixture with the addition of molecular sieve and $NaHCO_3$

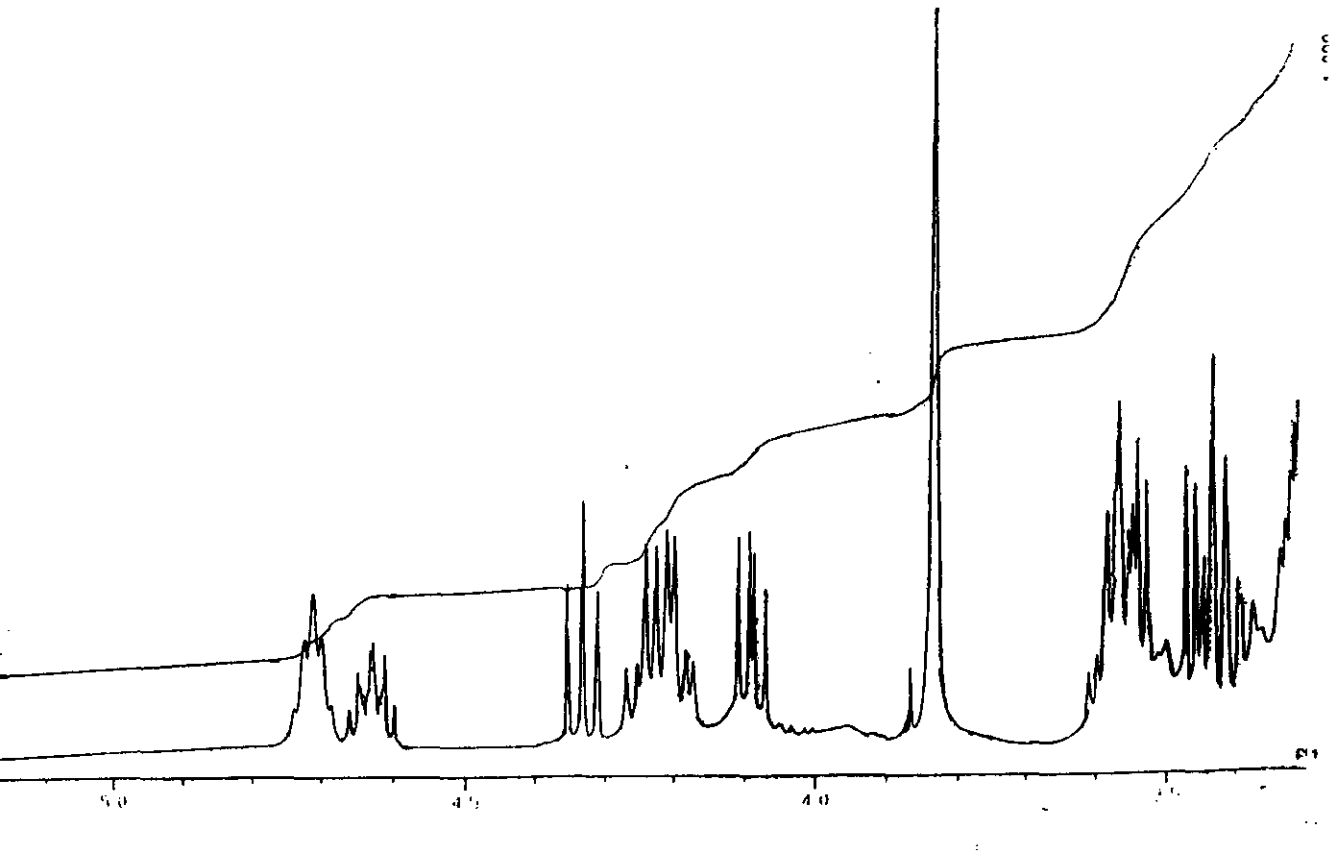


Figure 64. NMR spectrum of the batyl alcohol and DPBA mixture with the addition of molecular sieve and $NaHCO_3$ after reflux for 24 hours.

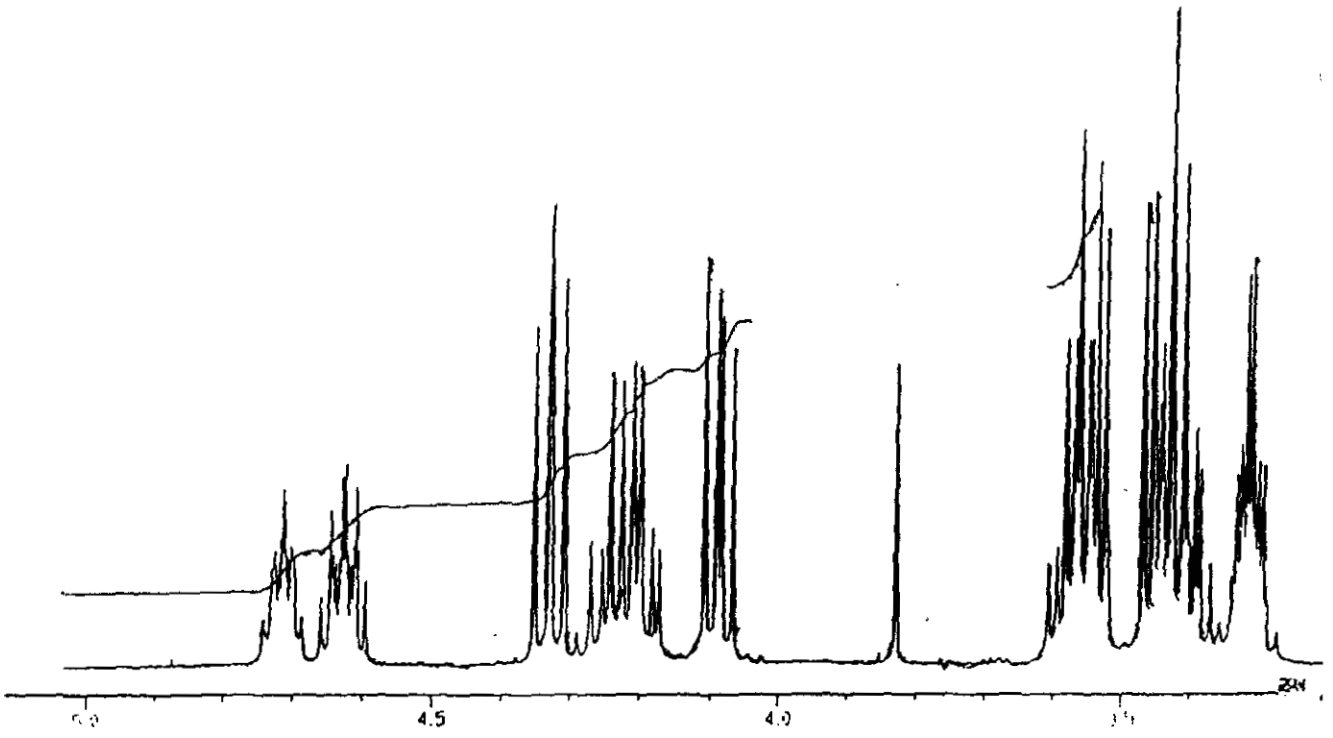


Figure 65(a). Negative-ion FAB mass spectra of the diphenylborinate of batyl alcohol using solvent (a) tetraethylene glycol diethyl ether (TEGDEE).

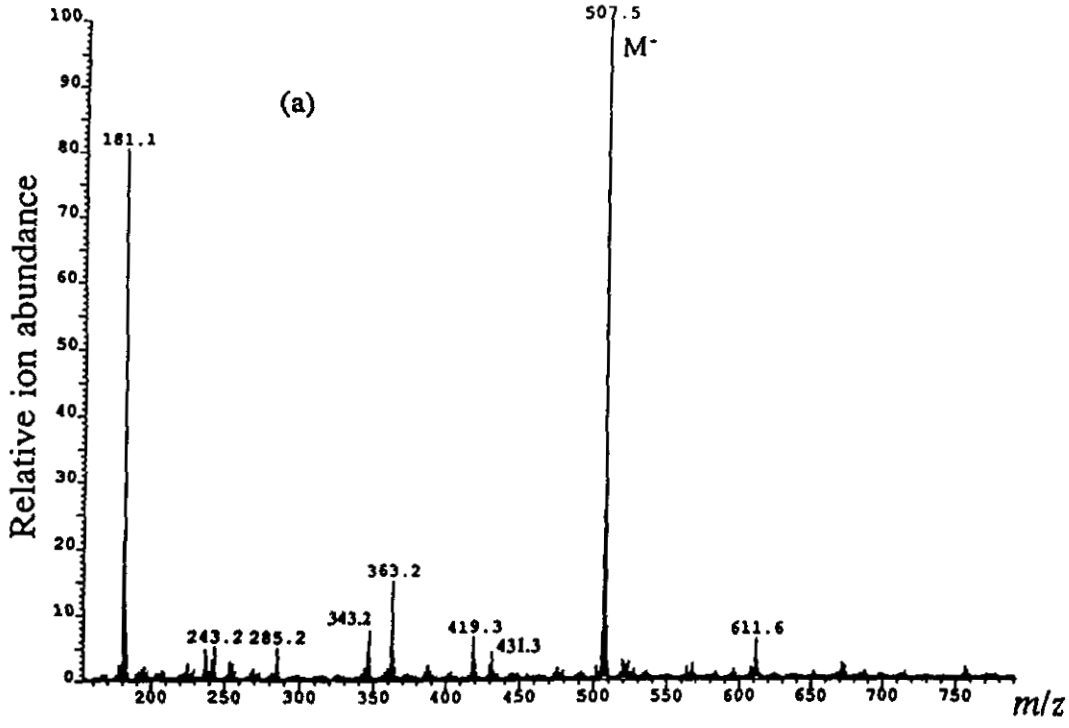


Figure 65(b). Negative-ion FAB mass spectra of the diphenylborinate of batyl alcohol using solvent (b) TEGDEE-glycerol.

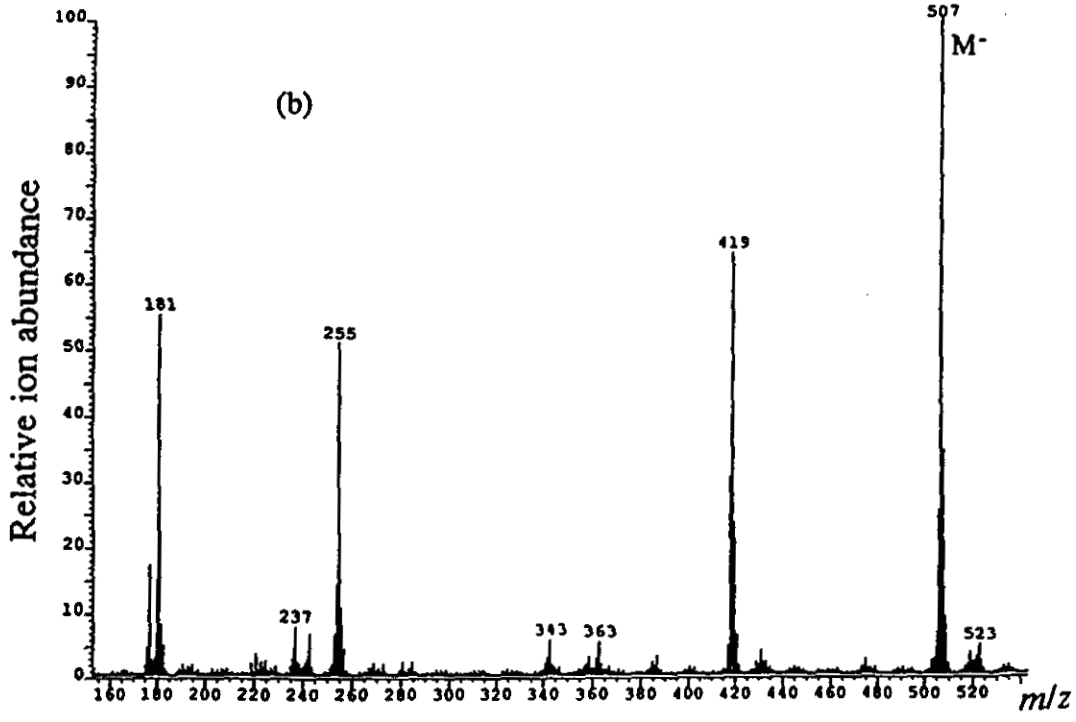


Figure 66. Negative-ion FAB mass spectra of hexaethyleneglycol dimethyl ether (HEGDMEE).

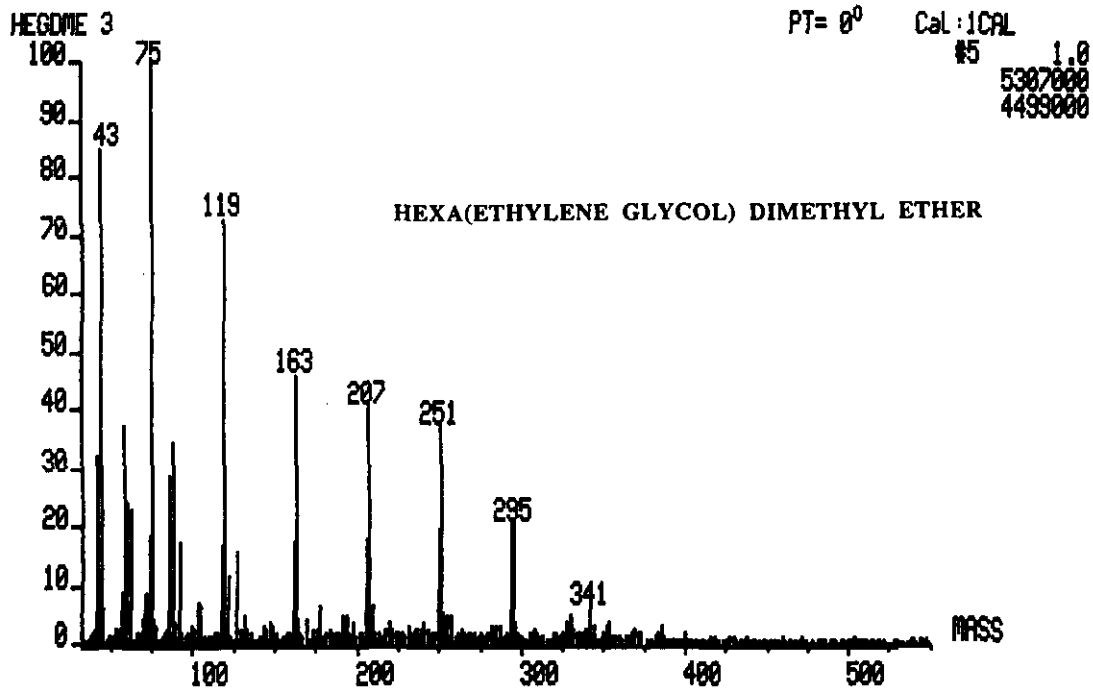


Figure 67. Negative-ion FAB mass spectrum of the diphenylborinate of batyl alcohol using a HEGDMEE matrix.

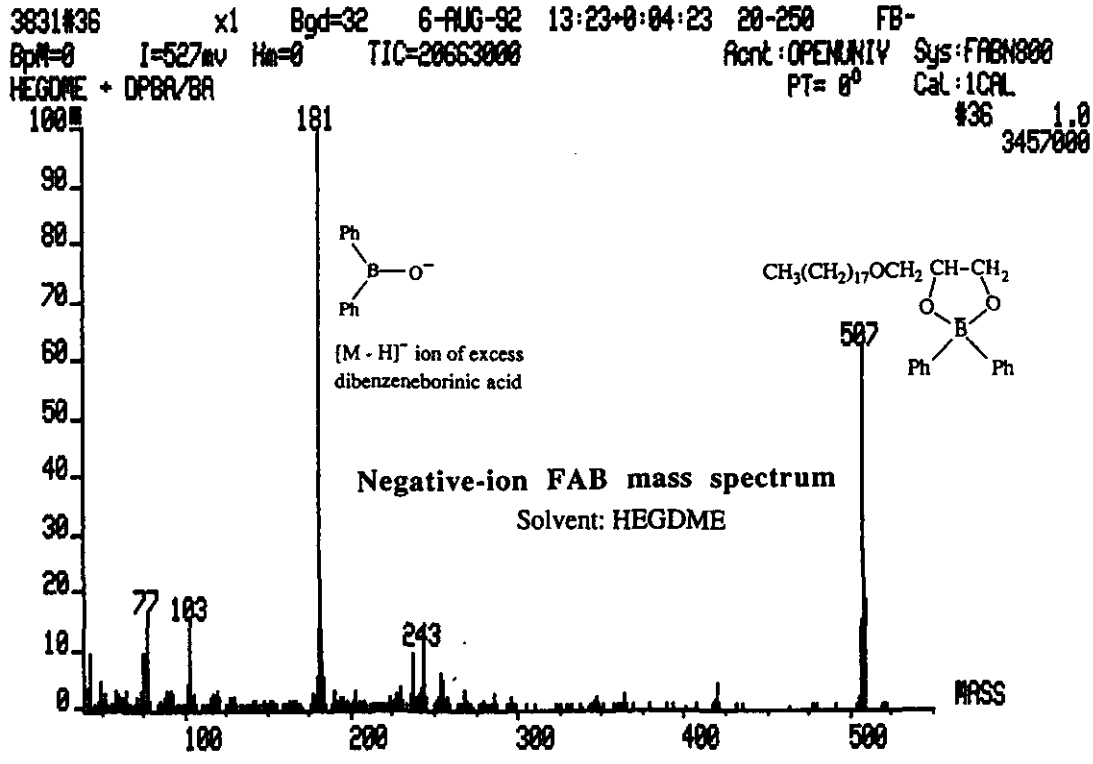
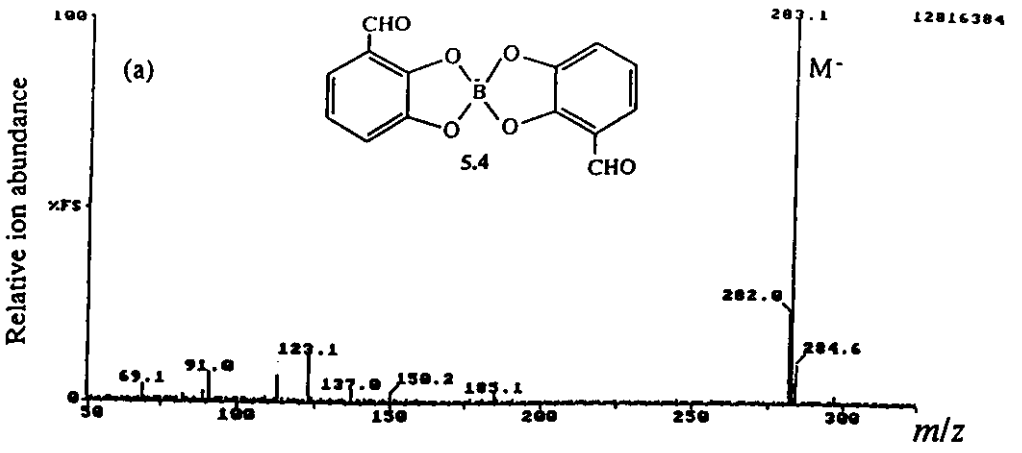
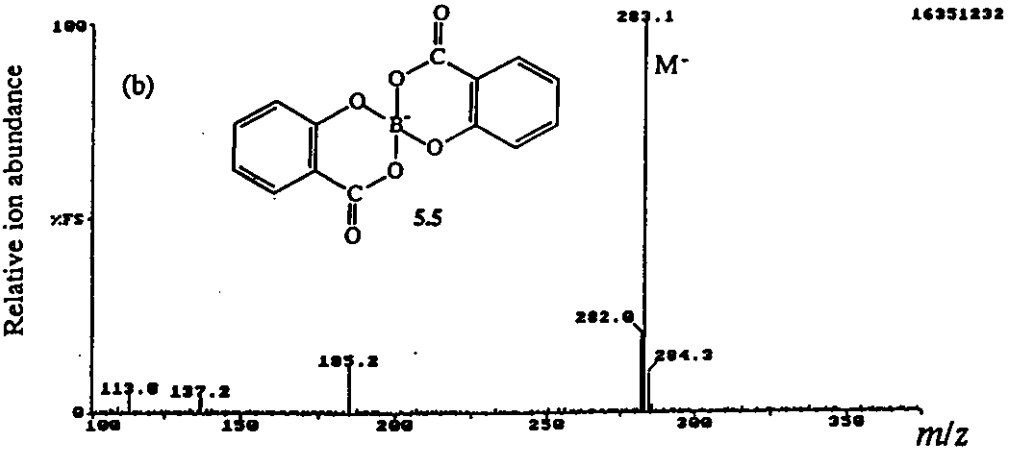


Figure 68(a). Negative-ion electrospray mass spectra of spiroborate 5.4.



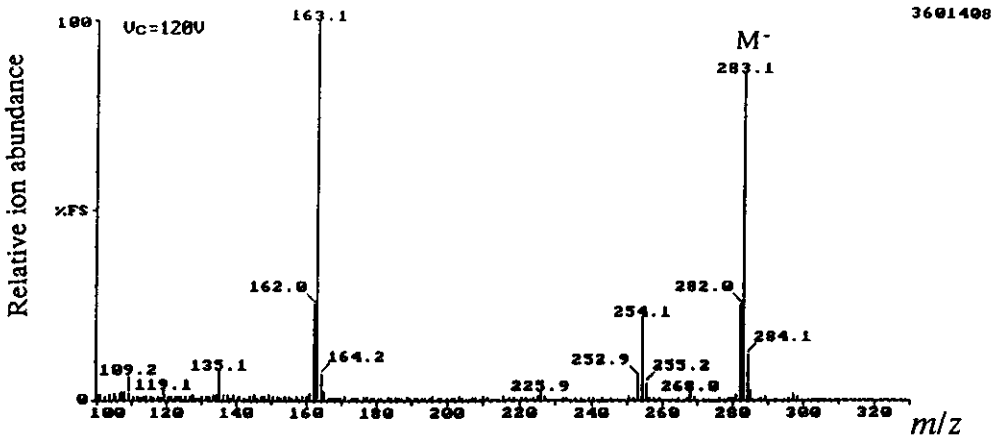
The samples were dissolved in water-acetonitrile (50:50) containing 0.5-1% ammonia and the cone voltage was - 30V

Figure 68(b). Negative-ion electrospray mass spectra of spiroborate 5.5.



The samples were dissolved in water-acetonitrile (50:50) containing 0.5-1% ammonia and the cone voltage was - 30V

Figure 69. Negative-ion electrospray mass spectra of spiroborate **5.4** as in Figure 68(a) but with a cone voltage of - 120V



The samples were dissolved in water-acetonitrile (50:50) containing 0.5-1% ammonia and the cone voltage was - 120V

Figure 70. Negative-ion electrospray mass spectrum of the diphenylborinate of batyl alcohol.

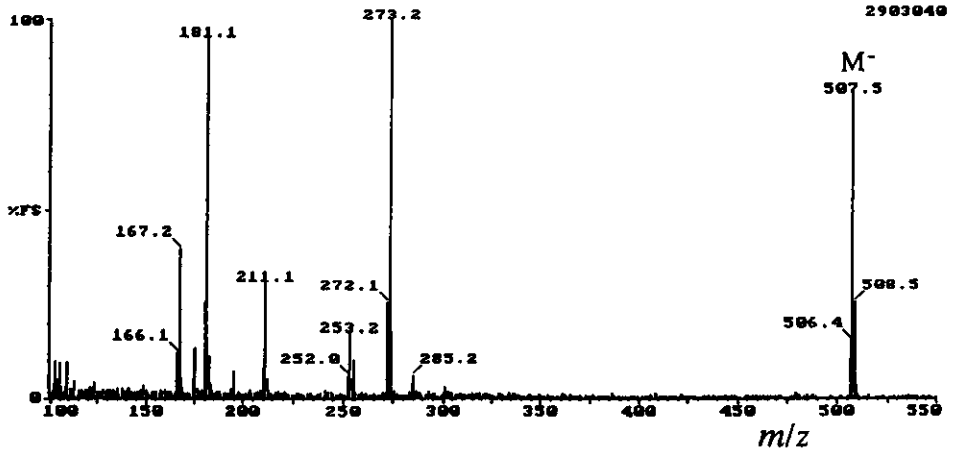
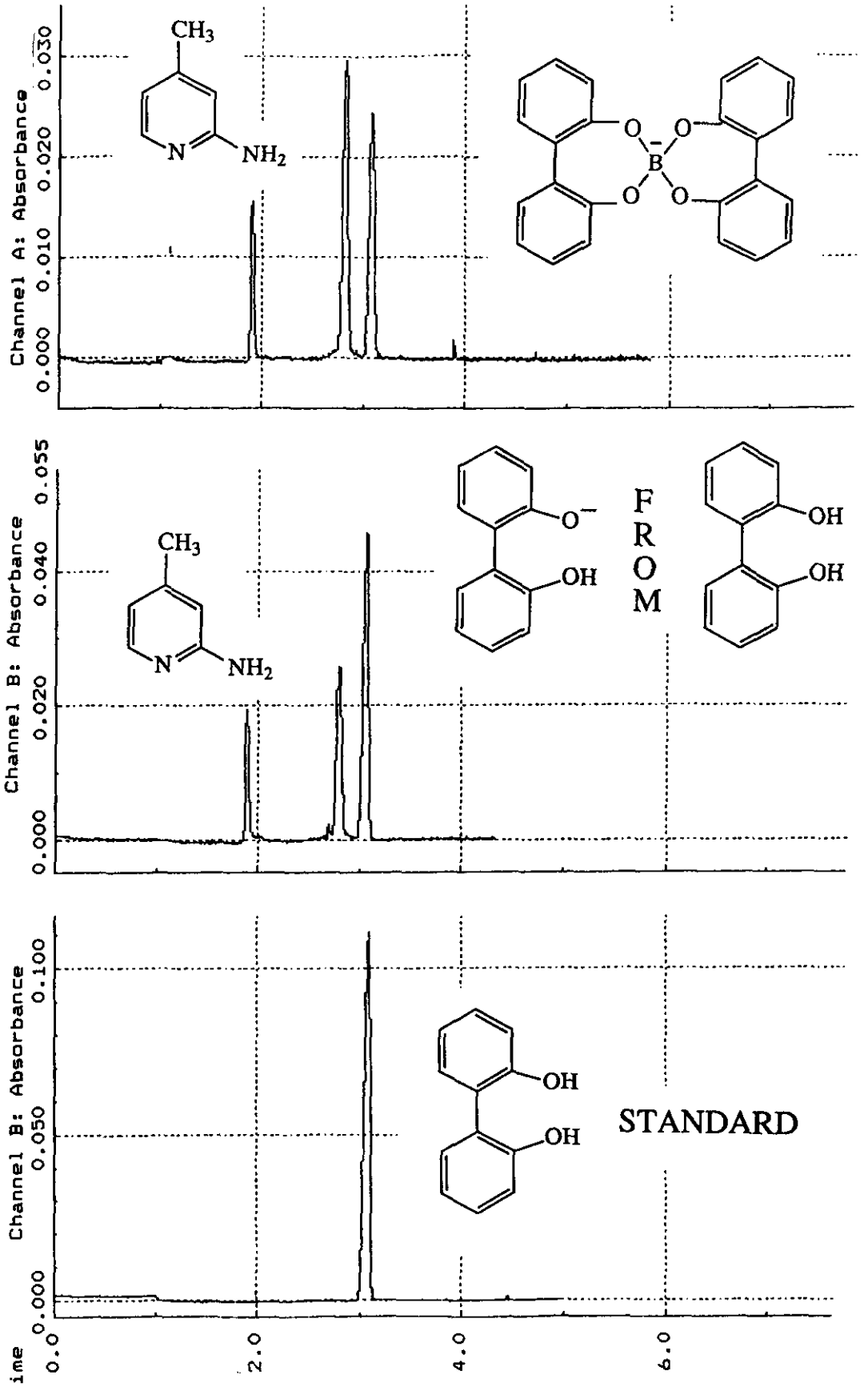


Figure 71(a) - (c). Capillary electrophoresis of spiroborate 5.8.



Buffer: 30mM boric acid at pH 6.0. Capillary: 50 µm i.d. x 50 cm; separation at 25 kV at 25°C; detection: uv absorption at 200 nm.

Chapter Six

Development of a CE procedure for the analysis of chromophoric herbicides.

1. Introduction: Herbicides, use and analysis.

Producing adequate amounts of food and fibre of high quality for the world's expanding population using environmentally acceptable farming practices remains an important challenge that is likely to make increasing demands on the skills of agricultural scientists. An agricultural crop is an unnatural plant community, composed essentially of a single species, which over time has been improved and selected to produce higher yields and better quality. In these circumstances, the natural balances which may control pest populations in natural plant communities are insufficient to prevent serious damage to the crop. While the search for alternative methods of controlling pests, weeds and diseases continues, the use of pesticides, herbicides and fungicides remains essential for the foreseeable future. Benefits from controlling weeds, diseases and pests in agriculture include increased volume of food and fibre production.

It is extremely important that the greatest care is taken to ensure that pesticides and their by-products are produced which pose minimal risks to the environment at large, crops and of course the people who'll eventually consume them. Some early pesticides such as arsenic and nicotine compounds, were poisonous substances and others remained active for too long in the environment, as in the case of the organo-chlorine insecticides. Nowadays much more selective pesticides which effect their target organism whilst leaving the surrounding ecosphere unharmed are being produced. For instance there are now pesticides that effectively control the pests but do little, if any, harm to bees or beneficial predatory insects. This also ensures that lower optimum amounts of these chemicals can now be used therefore reducing the chances of pollution occurring. As the populations of all countries continue to increase, greater strains are being placed on agriculture to produce more and more food. It is estimated that every year, 30% of the crops are destroyed by weeds, pests and diseases and without the use of pesticides, this loss would be doubled. But even so, there is an urgent need to reduce wastage still further.

To ensure that all new pesticides/herbicides are essentially safe both to the environment and to the public, agrochemical companies have developed various analytical procedures to test the toxicological significance of these products and their metabolites in the environment. Further to this, establishments such as the Health and Safety Executive (HSE) in Britain, and the Food and Drug Administration (FDA) in the U.S. have been formed to monitor these toxic compounds. An example of their importance was illustrated when the FDA found cyanide-laced grapes in a food shipment from Chile in 1989, which began what was called the "Great American Fruit Scares" and improved testing for all types of pesticide[150].

One of the classes of pesticides currently of interest to the HSE are the modern bipyridinium (quaternary ammonium) herbicides like paraquat and diquat which are widely used today to increase the yields of many modern crops. Bypyridyl describes a structure that contains two pyridine rings. In paraquat, a methyl group is attached to each nitrogen, giving a full chemical name as 1,1'-dimethyl-4,4'-dipyridylium ion (**6.1**) and in diquat the two nitrogen atoms are joined by an ethylene group to give 1,1'-ethylene-2,2'-dipyridylium ion (**6.2**) the structures of which are given in Figure 72. Paraquat is usually manufactured as a salt with chloride ion and diquat with bromine ion. These herbicides are extremely valuable because they are effective against a wide variety of plants and are completely deactivated on contact with soil. The result of this is that new crops can be planted as soon as the old weeds have been cleared. Paraquat and diquat, when sprayed as a composite, produce an effective contact herbicide (Gramoxone - Registered Trade Mark of Plant Protection, Ltd.) used for weed control and pre-harvest desiccation.

The main problem in the agricultural use of paraquat and diquat is that they are poisonous to several animal species, including fish, dog, cow and human! Levels of these herbicides have been found in ground water which could cause human health problems[151]. The majority of cases investigated by the HSE involve careless spraying of the pesticides by farmers or malpractice by the companies who distribute wrongly formulated pesticide concentrates. Most cases of pesticide poisoning are due to children drinking herbicides, carelessly put in unmarked soft-drink bottles, as well as criminal cases where paraquat has been intentionally added to canned juices, bottled drinks and paper packed milk[152]. Paraquat

has also been used in suicide attempts. Oral intake of paraquat first results in local effects - irritation of the mouth, throat, and oesophagus, and sometimes vomiting and diarrhoea. Paraquat causes widespread organ damage, but typically death results from effects on the lung[153]. Diquat-exposure leads to serious effects on the intestines which halts normal peristaltic movement[154]. Therefore it is important to determine these pesticides in blood and post-mortem samples as well as in environmental sources.

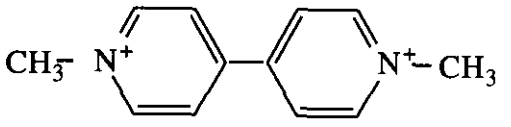
Gas chromatography has been the primary method used for pesticide residues analysis[155] but applications of HPLC, HPTLC and supercritical fluid chromatography (SFC) have steadily increased. Methods used to analyse such samples so far include gas chromatography and mass spectrometry[156], ion-exchange chromatography[157] and immunosorbent assay[158]. A preliminary spot test for paraquat has also been effective using an alkali-impregnated paper. The paper, first being spiked with the sample and then heated, gives a blue spot if paraquat is present. When combined with paper electrophoresis this test proved to be suitable for rapid preliminary detection of the compound in beverages in the field of forensic science[159]. FAB has successfully been utilized as an off-line method for specifically measuring both the doubly and singly charged ions of paraquat and diquat[160]. Comprehensive reviews of the types of chromatographic techniques currently being utilised for pesticide analysis have recently been published[161-162].

The CE work being performed on standard water samples has focused on developing a determination of diquat and paraquat formulations employing an internal standard. This particular example was chosen because of the laborious ion-pairing HPLC analysis (25 min per run) that were recently required to determine the diquat content of a concentrate sample. Capillary electrophoresis has only recently been used to measure the paraquat and diquat content of various samples. Isotachophoresis (ITP) was used initially as it enabled the detection of much lower concentrations of these compounds than CZE. With its sample-concentrating ability ITP has been used to measure ppb levels of these compounds. In parallel but independent experiments undertaken at the same time as those discussed in this thesis, other groups have used CZE to quickly measure paraquat and diquat in water[163] and

in serum[164] and these results as well as my own have shown the technique to be viable for the analysis of these pesticides.

Figure 72. Pesticide structures.

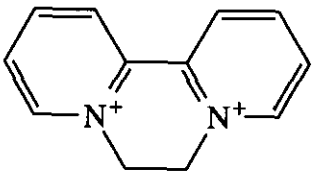
Paraquat dichloride



Mr = 186

6.1

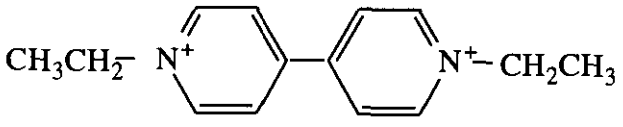
Diquat dibromide



Mr = 184

6.2

1,1'-Diethyl-4,4'-bipyridyldiylium diiodide



Mr = 214

6.3

Paraquat's herbicidal activity appears to depend on the ease of, the reversible one-electron reduction of the compound to form stable but air-sensitive cation radicals. A detailed review on the properties of this type of (bipyridinium) herbicides has been given by Summers[165].

2. Results and Discussion.

2.(1). Establishing separation conditions for CZE analysis.

As these compounds are chromophoric and charged, separation by CZE was feasible. Initial details to be investigated were the optimum absorption wavelengths of each compound, including an internal standard, and the best conditions necessary for adequate separation to take place. As the molecular masses of the two analytes of interest differ by only two mass units and their molecular conformation is so closely matched it was thought that their separation may be difficult.

Table 14. Optimum absorbance wavelengths.

Paraquat Dichloride	200 nm and 257 nm
Diquat Dibromide	200 nm and 310 nm
Paraquat Diethyl Diiodide (Int. Std.)	200 nm, 229 nm and 258 nm

All of the quaternary ammonium compounds had their lambda max absorbance at 200 nm. Both paraquat and diquat also had two other local maxima at around one third of the intensity of the true maximum. The internal standard compound displayed three absorbance maxima but again the true maximum was at 200 nm. As each analyte displayed different secondary, and in one case tertiary maxima, it was not possible to choose a more specific wavelength which would allow detection of all three selectively so 214 nm was chosen. Contrary to expectation, separation was achieved quickly and easily with a very simple buffer system. A 0.1M H_3PO_4 buffer made to pH 3.2 with 0.1M NaOH sufficed and peaks were separated to base line (Figure 73, Appendix 6). The theoretical plate number, which is a guide as to the efficiency of the separation, can be determined directly from these electropherograms using;

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

where: t = migration time.

$w_{\frac{1}{2}}$ = peak width at half height.

This equation could be used to determine plate number since both peaks were gaussian. Efficiency did not exceed 35,000 plates during this separation which would suggest the separation to be inefficient as 10^6 theoretical plates have been achieved using capillary electrophoresis. However, gains in resolution were not sought because separation achieved was adequate for the required analysis. These results were obtained using a Supelchem coated H150 capillary employing C8 units bonded to its walls. These capillaries are described as being mildly / moderately hydrophobic. Once separation had been achieved it was important to elucidate the exact migration order to establish which peak was due to which compound. Using this buffer and applying 15kV across the capillary at 25°C a current of 15.7μA was developed. All measurements were made at 214nm which, although not the optimum absorbance wavelength for detection of all the analytes, was the best compromise between sensitivity and selectivity.

The excellent reproducibility seen during these initial analyses allowed the unambiguous identification of each peak in the mixture and elucidation of migration order by superimposing chromatograms of each pesticide electrophoresed individually and as a mixture. The migration order was paraquat first, diquat second, followed by the internal standard, chosen due to its structural similarity to the other two.

2. (2). Evaluation of the method for quantitative analysis.

Having established, but not optimised separation conditions, the next step was to evaluate the method for quantitative analysis. One of the aspects to be considered when determining viability of quantitative analysis using CZE is that different solutes can have different migration velocities under the applied separation voltage. Different velocities must be corrected for since different residence times in the detection window artificially affect the peak area. The slower moving solutes will remain in the detection window longer than those of higher mobility, and so will have increased peak area. If this is the case the correction can easily be made by dividing integrated peak area by migration time. In this case the separation time between each peak is only 4 seconds so the correction is negligible to the final result.

To test the reproducibility of the system, one sample of the three-compound mixture was analysed five times, flushing the capillary between each analysis. The data for migration time, peak height and peak area are presented in Table 15. The table below contains the statistical processing of the reproducibility data obtained over five consecutive runs. These data were obtained using the Gold computer software integration package, and the relative standard deviations (RSD) were calculated.

Table 15. Reproducibility data for herbicide separation.

ANALYTE	MIGRATION TIME (Minutes)	PEAK HEIGHT (%)	PEAK AREA (%)
Paraquat (10ppm)	2.41	30.37	25.88
	2.40	30.07	26.67
	2.40	29.97	26.74
	2.40	30.25	27.33
	2.40	30.48	27.06
		(0.7% RSD)	**(2.04% RSD)**
Diquat (10ppm)	2.48	25.50	26.67
	2.47	26.07	27.65
	2.47	25.43	24.25
	2.47	24.82	24.79
	2.47	24.83	25.91
		(2.1% RSD)	**(5.3% RSD)**
Internal Standard (20ppm)	2.85	44.13	47.45
	2.84	43.86	45.68
	2.84	44.61	49.09
	2.84	44.92	47.88
	2.83	44.69	47.04
		(0.98% RSD)	**(2.6% RSD)**

The results of the RSD calculations are comparable to those obtained using standard HPLC or ion exchange techniques. This analysis was followed by the production of calibration curves using solutions containing a range of concentrations of the paraquat and diquat between 2.5 - 40 ppm, whilst the internal standard was kept constant to allow for any variations in sample injection. Peaks were obtained for these analyte standards at 1ppm and 0.5ppm and whilst still clearly visible were small and probably near to the limit of detection. The calibration curves based on peak areas (analyte / internal standard) for the two analytes gave correlation coefficients of 1.000 and 0.999 respectively using the equation of a straight line ($y = mx + c$). Reproducibility results, (Figure 74 (App. 6) shows two chromatograms superimposed) along with the calibration curves shown in Figure 75 indicated that the

method could be used for qualitative and quantitative analysis. But the obvious benefit of the method was the short time in which the analysis could be completed with the entire separation taking less than three minutes. Comparing this to ion-exchange methods which took up to 25 mins per run to perform, it was evident that around six analyses could be completed in the same time it previously took to do one.

2. (3). Optimal use of analysis time for a number of possible analyses.

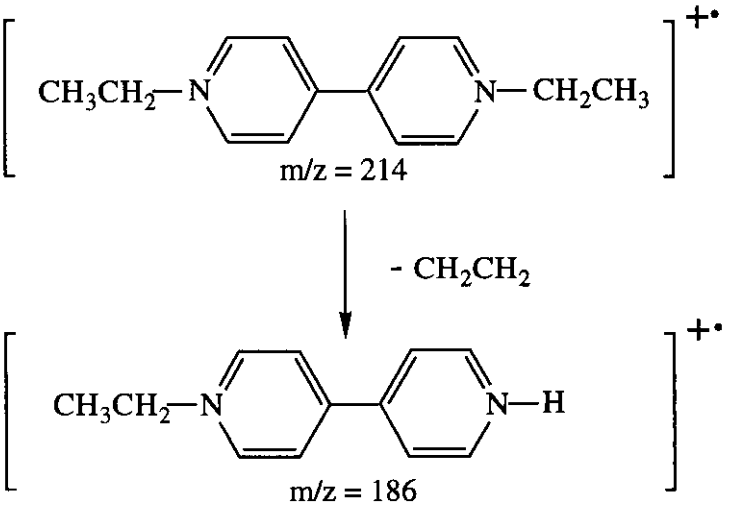
This involved setting up a method which contained multiple injections. This meant that 6 samples could be analysed within 24 minutes. The method shown in Figure 22 (App. 2), (Section 7(2) of Chapter 2, illustrates how, due to the automation of the technique, samples can be injected, separated and the next sample injected continuously so fully exploiting the potential of the CZE technique. Firstly a standard sample of the same composition was injected 6 times and then to show that carry over of sample was not a problem, samples of different concentrations were injected from 40 to 1.25 μg/ml (Figure 76 (App. 6)) and a graph of the corrected peak areas plotted. As with earlier experiments the resultant calibration curves gave correlation coefficients (diquat (0.998) and paraquat (0.999)) which confirmed the quantitative capabilities of the technique with these analytes. Carry over of sample was not a problem during this sequence of multiple injections as shown by the correlation coefficients obtained. This meant that the method could be used to analyse up to 6 different samples of any concentration within 24 minutes, with no loss of quantitative accuracy.

2. (4). Capillary electrophoresis/electrospray mass spectrometry CE/ES of the herbicides.

The combination of ISCO capillary electrophoresis system and electrospray was used to analyse these quaternary ammonium species (QUATS). As the actual instrumentation being used was also experimental in the process of coupling the two techniques, initial results were obtained using various buffers and non-optimised instrumental parameters. Some results were obtained whilst these parameters were being optimised but these were not of adequate resolution or reproducibility to be analytically sound. However they were constructively useful in determining what conditions should be useful in further

experiments. Early results involved obtaining separation, in 1 metre capillaries required for CE/ES coupling, similar to that achieved using shorter capillaries (Figure 73 (App. 6)). A mixture of the three compounds was firstly injected into the ion-source and the subsequent ions registered. Ions were seen at m/z values of 186, 184 and 214 corresponding to paraquat, diquat and the internal standard respectively. Whether the procedure ultimately worked depended upon the rate of make-up flow and the arrangement at the electrospray probe tip as discussed in Section 8 of Chapter 2. The successful buffer which gave the mass electropherogram shown in Figure 77, (App. 6) was a mixture of 30 mM phosphate and H_3PO_4 with 20% acetonitrile added to increase the volatility of the buffer system.

The detector in this case was the mass spectrometer alone as no in-line U.V. detection was used. Figure 77, (App. 6) shows the individual and combined selected ion-chromatogram obtained which illustrates the separation achieved whilst monitoring the ions noted above. This is the result of injecting diquat and paraquat at 40ng/µl but because there was a high background signal at m/z 214, the internal standard, was injected at ten times the level of the other two (QUATS). The increased level of this analyte meant that the eventual peak shape was very poor. The internal standard also ejects C_2H_4 to contribute to the occurrence of a second peak of ions at m/z 186 illustrated in structural terms below.



2. (5). CE/ES parameter variation and optimization.

Due to the nature of the QUAT compounds and the ionization method being used there was every expectation that doubly charged species of the pesticides would also be observed. Such ions were detected at m/z values of 93, 92 and 107 as in Figure 78 (App. 6), other ions observed included those at 185, 183 and the second 186 ion peak, again corresponding to each compound used. Their occurrence was directly dependent upon the size of the applied voltage placed on the cone skimmer. As this voltage was increased from 30V to 45V the intensity of the doubly charged ion signals also increased. A hypothesis to attempt to explain the occurrence of both doubly and singly charged ions is illustrated in Figure 79 (App. 6). The singly charged ions are produced by the addition of an electron to the doubly charged molecule within the ion source thereby creating a radical cation. The existence of doubly and singly charged ions of each of the bypyridyl herbicides can be explained by analogy with its mode of action within the treated plant. Work has shown that both paraquat and diquat cross the chloroplast envelope of their target plant with ease, and can accept electrons from various proteins within the plant. This leads to them becoming reduced to their radical forms[16].

$$BP^{2+} \xrightarrow[\text{chain (1 electron)}]{\text{electron-transport}} BP^{\bullet +}$$

The electron donor in CE/MS is possibly the acetic or formic acid within the makeup solution[166]. Figure 79 (App. 6) also illustrates how ions at m/z 185 and 186 could be produced by loss of an ethene and an ethyl group respectively from the internal standard ion at m/z 214.

2. (6). Detection limit studies using singly charged ions only.

This experiment was performed to compare the detection limits of CE/ES with those already established using CE alone. Sample mixtures of the three components at 40 ng/µl, 23 ng/µl, 5 ng/ul and 1 ng/µl were used to test detection limit capabilities of the system whilst in the selected ion monitoring mode for the singly charged ions only. As mentioned previously the internal standard had to be present at ten times the concentration of the other two herbicides due to a high background ion also being present at its m/z ratio. Resulting combined ion chromatograms from these experiments are shown in Figure 80 (App. 6)

which includes the 40 ng/µl sample and the 1 ng/µl sample. At this lower level both components could still be seen as ions at 184 and 186 but 1ng/µl is near the limit of detection. The peak due to paraquat at m/z 186 is 5 times the background and the diquat peak twice the background. As three times the background is regarded as a measure of the limit of detection (LOD) of analytes the LOD of both analytes has virtually been reached. The second peak at 186 is again due to the loss of 28 mass units from the internal standard peak which is also 3 times the background.

Comparing the peak widths in the CE/ES spectrum with those in Figure 73 (App. 6) of CE separation alone shows peak broadening during the extra length of the capillary used for CE/ES. CE separation gives peaks of 3 and 2.5 seconds in width at half height whereas peak widths using CE/ES are 9 and 8 seconds respectively. This may be due to heating effects within the extra length of capillary which was not being cooled during CE/ES using the ISCO CE system, whereas cooling was achieved using the P/ACE 2000 system. The CE capillary was one third the length of the CE/ES capillary used.

2. (7). Real standard (HSE) sample determination using CZE.

Diquat concentrates supplied by the Health and Safety Executive were analysed by CZE alone by firstly producing calibration curves corrected via the internal standard which again gave a correlation coefficient of 0.998. The samples were then run and the following results obtained.

Analyte		Sample MAFOJ141	Sample BX74A
	HSE result	22.6% +/- 0.4	23.5% +/- 0.5
Diquat Dibromide			
	Actual result	41.8% +/- 3	44.1% +/- 5

These differed by a factor of 2 from the results obtained by ion-exchange by the HSE. However the samples analysed had been stored in the laboratory for nearly a year. From earlier results using standards the CE method did prove viable as a quantitative method for the analysis of diquat which would implicate the samples as the suspect parameter in this experiment.

3. Conclusion and Discussion.

The results show that using ES as a detector does not significantly improve on the detection limit established by CZE using U.V. absorbance values. This may not be surprising as at the m/z range being measured there are also many background ions including those developed from the organic components of the makeup liquid which includes acetonitrile and acetic acid. The background ion at 214 illustrates this perfectly. Couple this with the fact that the technique was primarily developed to examine larger molecules with masses above 1000 and the possible losses of material on the capillary walls whilst migrating towards the ES source, this result may not be unexpected. In theory for detection limit studies all the ions which could possibly be formed within the source should have been measured which could also have improved the results obtained. This series of experiments was performed merely to establish the viability of CE/ES using buffer systems and analysing compounds the size of which would normally be regarded as rather small for electrospray mass spectrometry. The instruments used had not previously been coupled so the data were initial and utilised newly developed ideas.

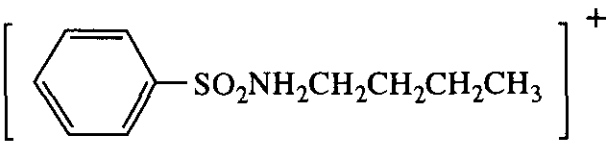
Throughout this CE/ES work, which was highly experimental, the time taken for the pesticides to migrate through the one metre capillary was never constant or reproducible. The application of the method was being illustrated and parameters were not optimised. Throughout the experiments the make-up flow rate, nebulising gas flow rate and make-up flow composition were being tested as well as capillary length and diameter. This set of results led to the conclusion that CE/ES had great potential for examining small molecules of an environmental nature but because of the lack of substantial improvement in detection of these molecules and the high cost of mass spectrometry it would not replace traditional methods of detection in this case. It must be stressed that the CE method demonstrated does have higher detection limits than the HPLC method used to analyse real samples from environmental sources. However if another CE technique, that of ITP, could be used which has the capability of pre-concentrating the sample before analysis, the improvement in detection limits could be significant.

The actual coupling of the instruments and obtaining results did not prove to be the main difficulty, but obtaining better quality results did take some time. The results did illustrate

the point that electrospray mass spectrometry and CE worked in a compatible way even when high molarity buffers using involatile salts were being used as long as the make-up flow was sufficiently large to dilute the capillary flow and a nebulising gas was used. Finally mass spectrometric detection supplied greater molecular specificity and allowed the identification of the molecular form in which compounds existed under different conditions. The appearance of the background ion at m/z 214 was at the time of the experimental work no more than an inconvenience. Since this time other groups have reported the same background ion in their spectra. It is now thought that this is actually a contaminant in the water used to make up solutions and matrices for spectrometric procedures. It is thought to be due to N-butylbenzenesulphonamide (Figure 81) which could be in the water as the breakdown product of an optical brightener *.

Figure 81.

N-butylbenzenesulphonamide $[M + H]^+$ ion



m/z = 214

How this contaminant became so widespread still isn't known. Some groups use it as a reference peak for tuning but when low mass work is to be performed it is still a peak which can severely interfere with results. If CE or CE/ES is to be used to analyse real samples from an environmental source these samples would also contain various impurities and how these would effect CE results is unknown.

* Footnote: Independent work by Dr. M.E. Rose and B.M Kelly suggests that the background ion at m/z 214 is the $[M + H]^+$ ion of N-butylbenzenesulphonamide, a contaminant of water supplies.

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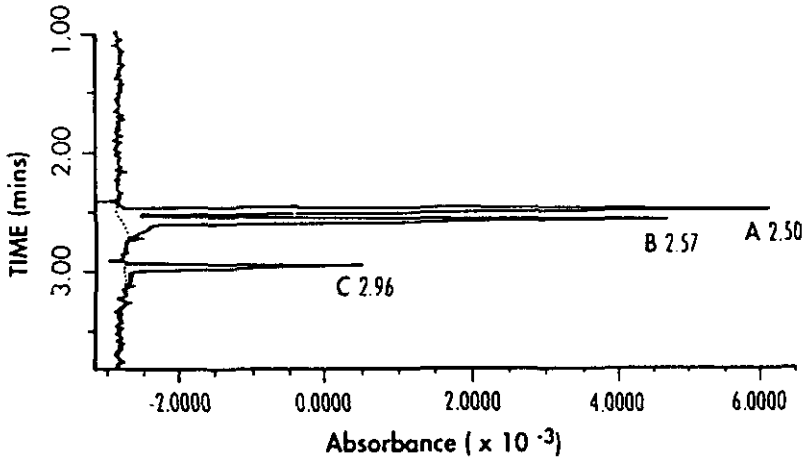
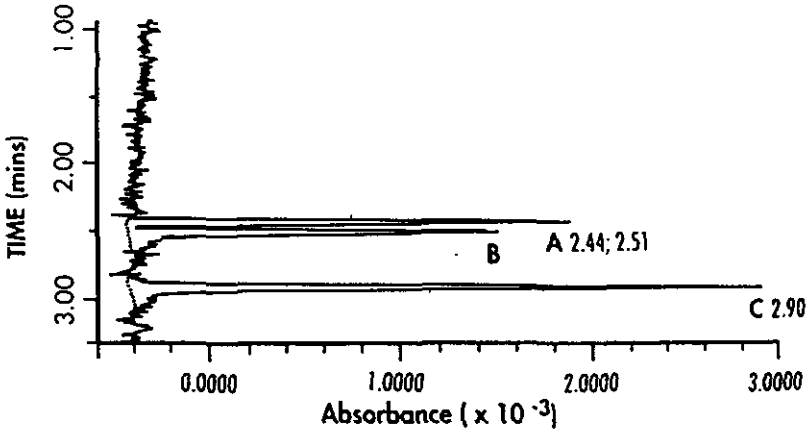
Appendix 6

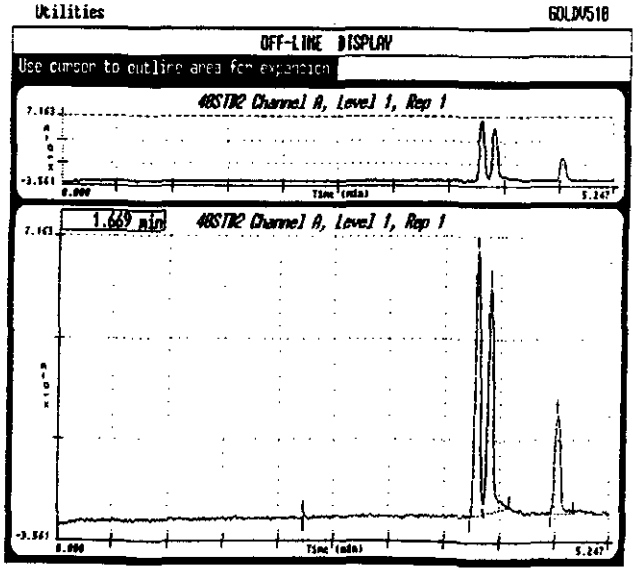
Figures from Chapter 6.

Figure 73. Capillary electrophoresis of the three chromophoric herbicide mixture, paraquat, diquat and the internal standard.

Examples of electropherograms obtained in the analysis of paraquat (A), diquat (B) and internal standard (C). The top trace is for concentrations of A and B at 40 ppm and C at 20 ppm. The lower trace is for concentration of A and B at 10 ppm and C at 20 ppm.

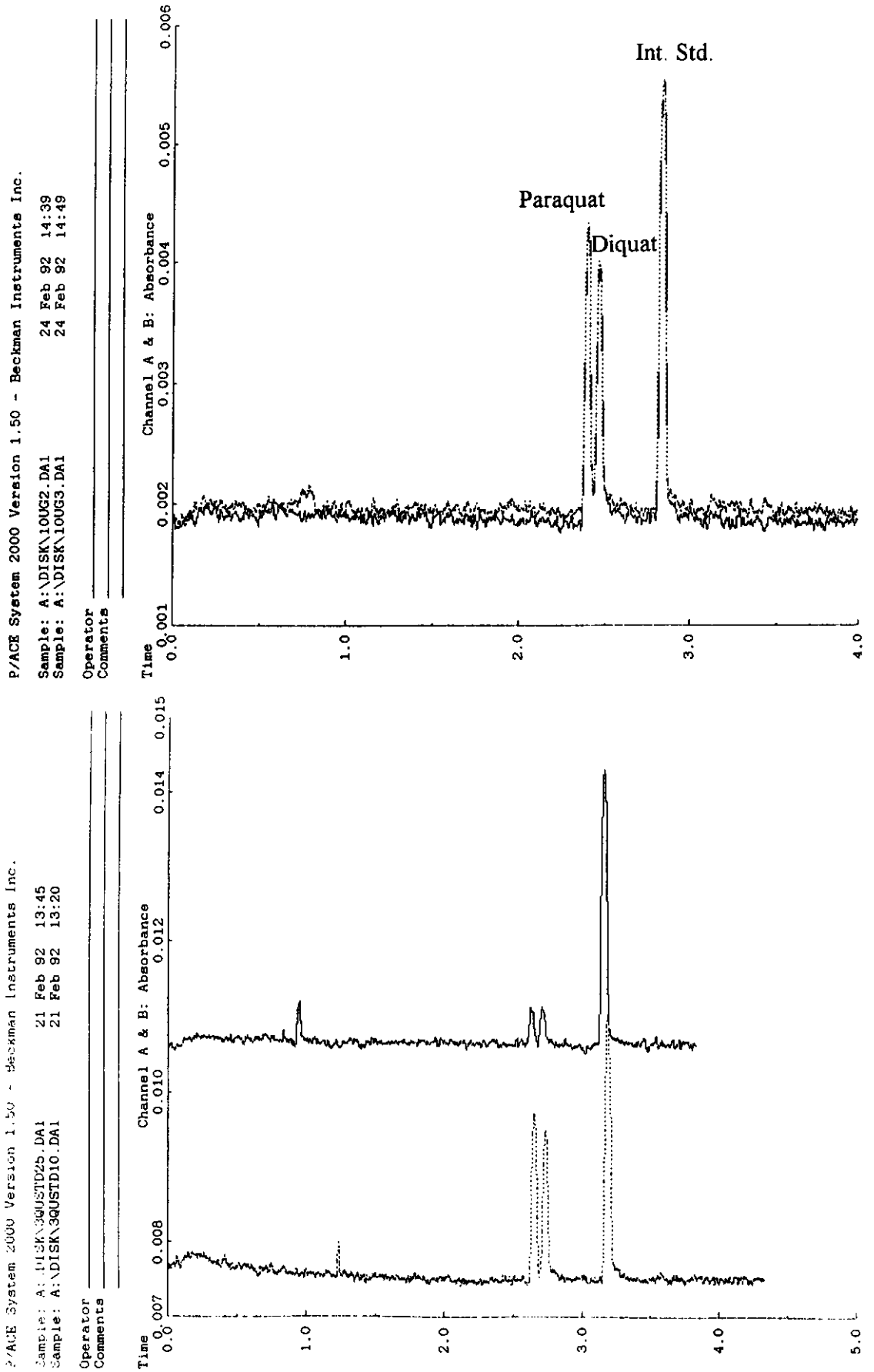






Buffer: 100 mM phosphoric acid solution at pH 3.2. Capillary: 50 µm i.d. x 50 cm Supelchem C8 - bonded capillary; separation at 15 kV at 25°C; detection: uv absorption at 214 nm.

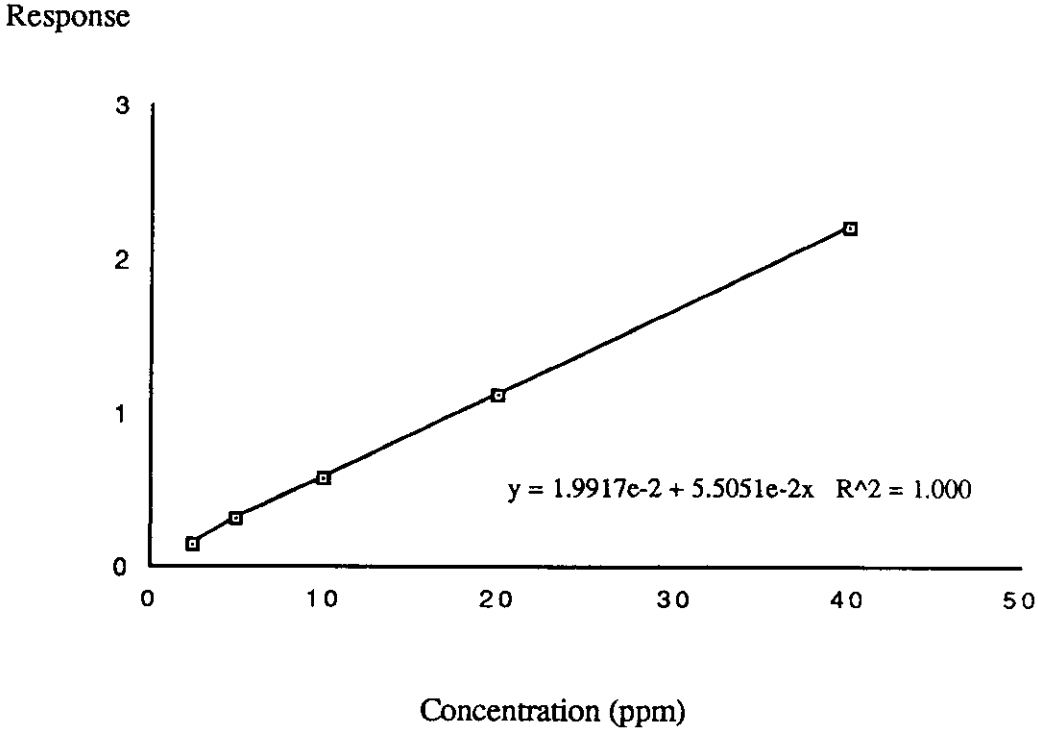
Figure 74. Two CE chromatograms of the three chromophoric herbicide mixture, overlaid to illustrate reproducibility.



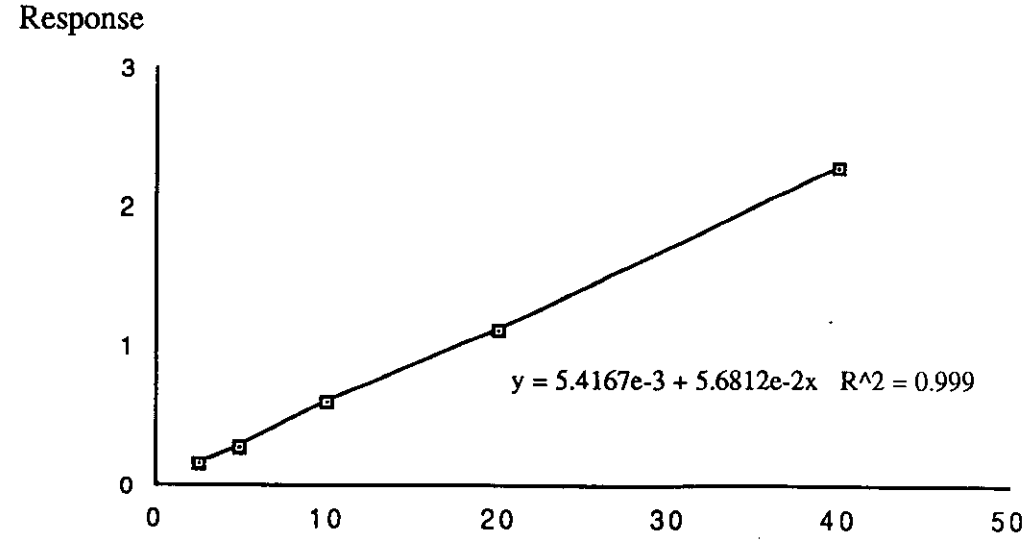
Buffer: 100 mM phosphoric acid solution at pH 3.2. Capillary: 50 µm i.d. x 50 cm Supelchem C8 - bonded capillary; separation at 15 kV at 25°C; detection: uv absorption at 214 nm.

Figure 75. Calibration curves from the CE analysis of paraquat and diquat from 0 to 40 ppm.

Linear fit curve for Diquat.

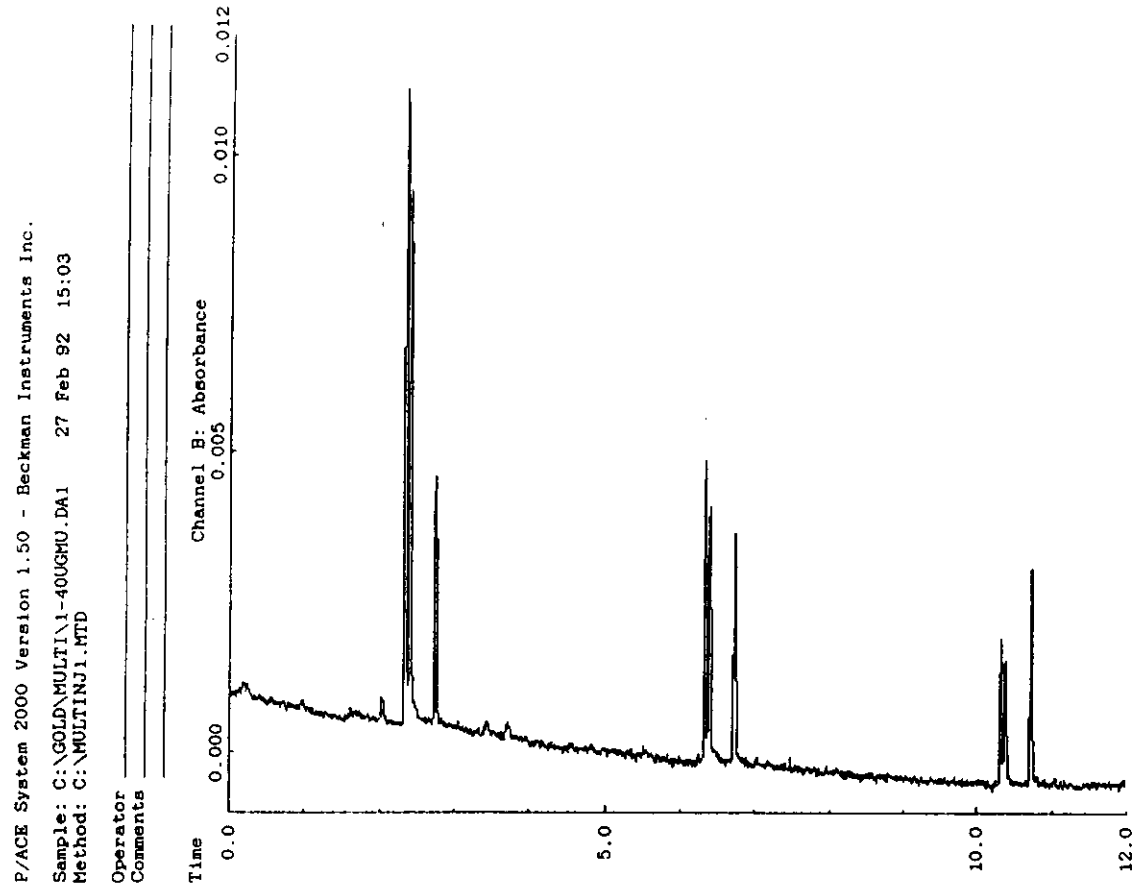


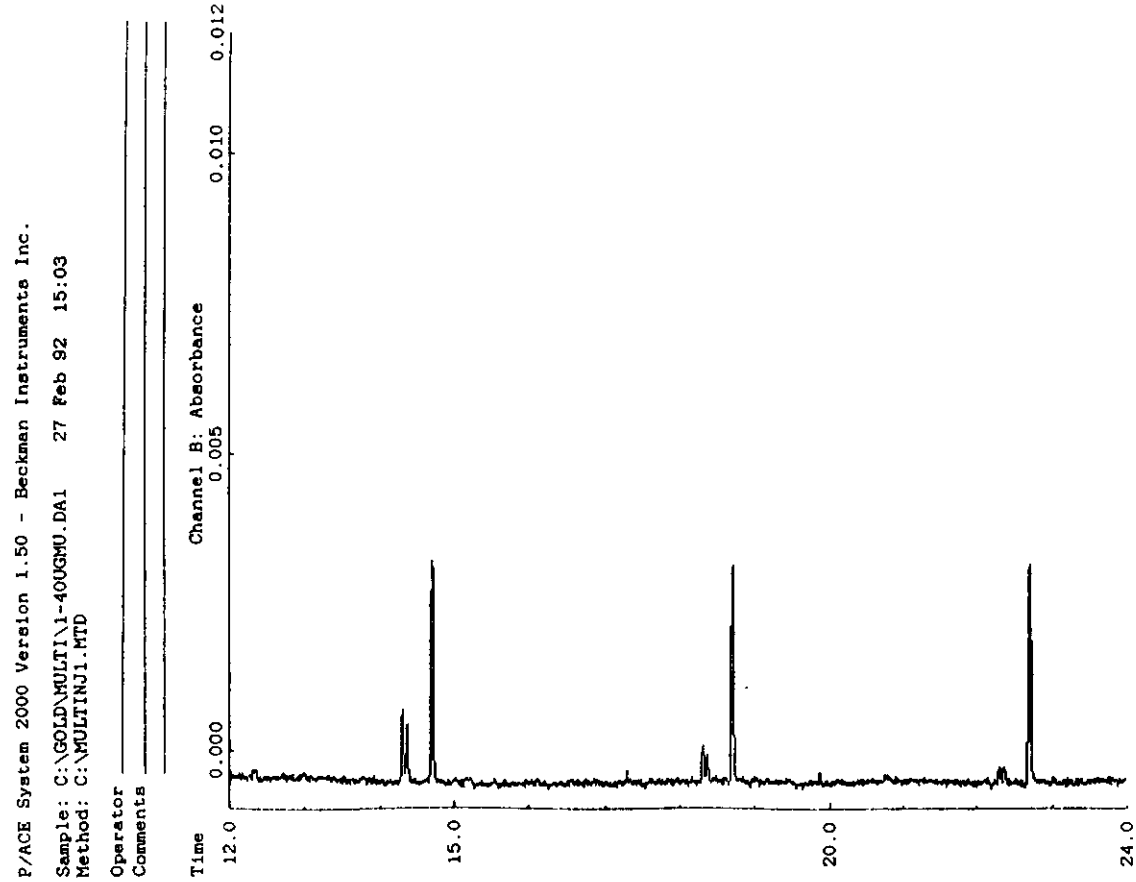
Linear fit curve for Paraquat.



Buffer: 100 mM phosphoric acid solution at pH 3.2. Capillary: 50 µm i.d. x 50 cm Supelchem C8 - bonded capillary; separation at 25 kV at 25°C; detection: uv absorption at 200 nm.

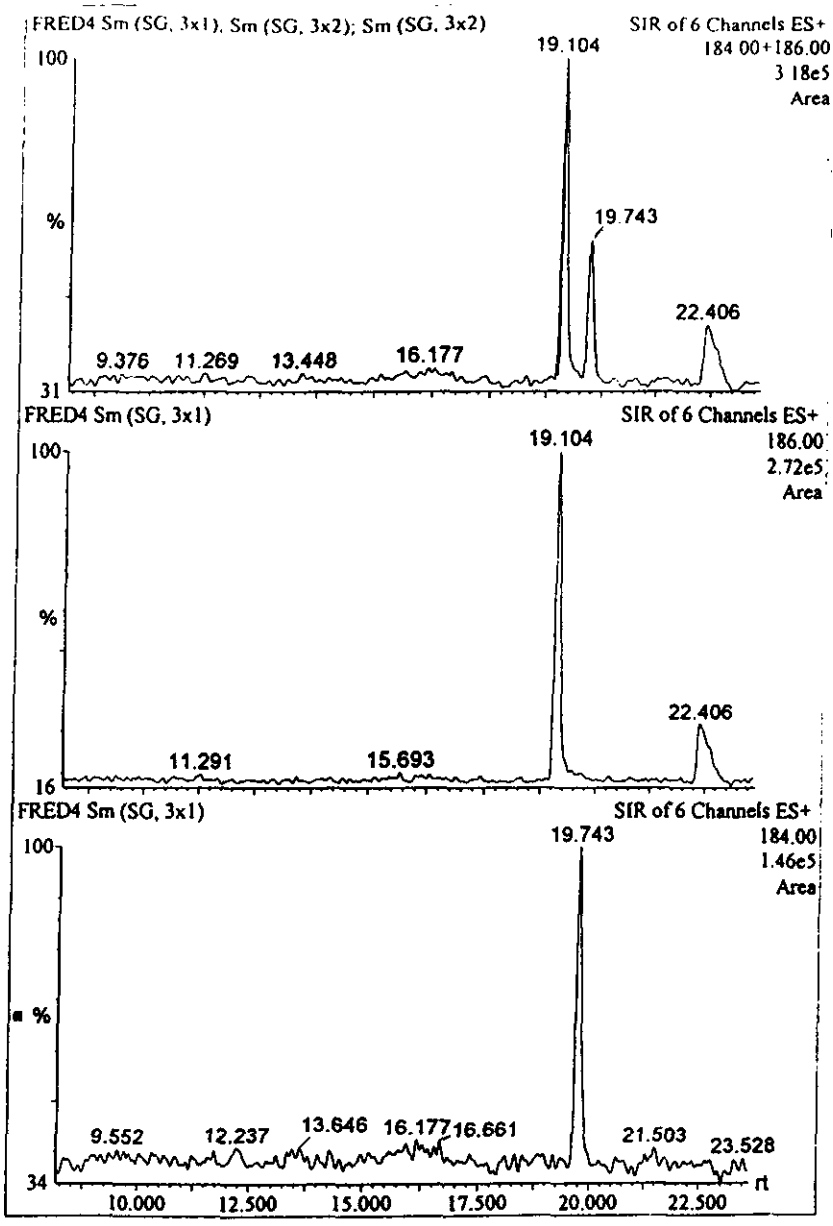
Figure 76. Capillary electrophoresis chromatograms of the multiple injection of samples of three chromophoric herbicides between 2.5 - 40 µg/ml.





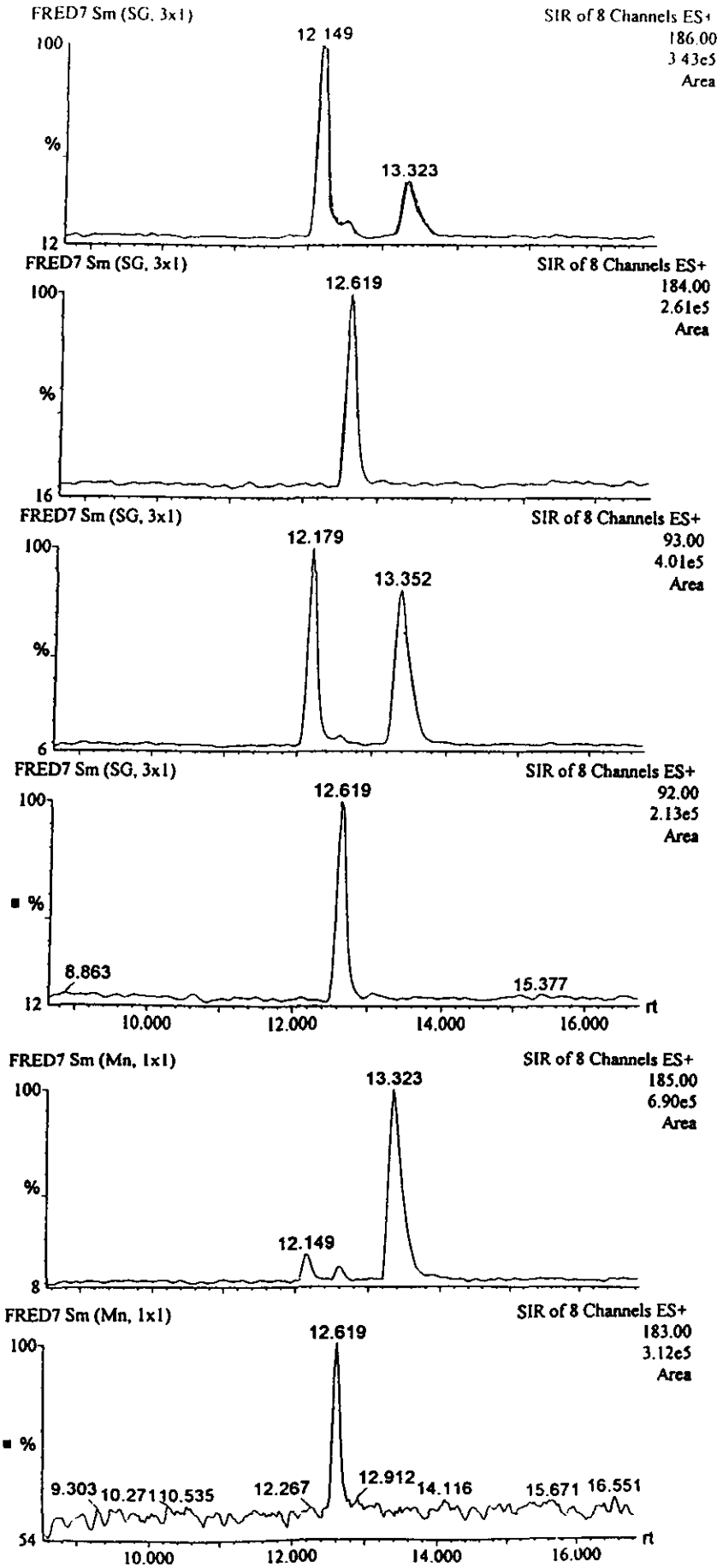
Buffer: 100 mM phosphoric acid solution at pH 3.2. Capillary: 50 µm i.d. x 50 cm Supelchem C8 - bonded capillary; separation at 25 kV at 25°C; detection: uv absorption at 200 nm.

Figure 77. Chromatogram of the ions produced by CE/ES of the three chromophoric herbicide mixture, combined and individual SIR chromatograms shown.



Buffer: 10 mM ammonium acetate in a solution of 50/50 water/methanol, adjusted to pH 3.2 with phosphoric acid. Capillary: 90 cm x 75 µm i.d. silica capillary. Separation at 21 kV, 25°C, detection by VG Quattro electrospray mass spectrometer.

Figure 78. Chromatograms of the ions produced when the three chromophoric herbicides are analysed by CE/ES. Doubly charged ions are shown.



Buffer: 10 mM ammonium acetate in a solution of 50/50 water/methanol, adjusted to pH 3.2 with phosphoric acid. Capillary: 90 cm x 75 µm i.d. silica capillary. Separation at 21 kV, 25°C, detection by VG Quattro electrospray mass spectrometer.

Figure 79. Hypothesis to explain the appearance of both singly and doubly charged ions during CE/ES analysis of chromophoric herbicides.

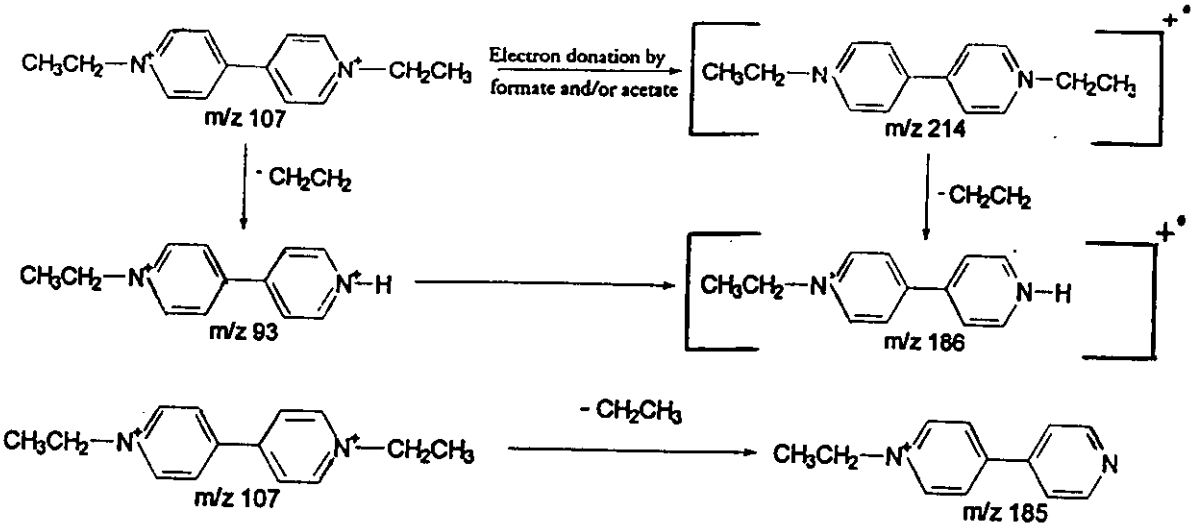
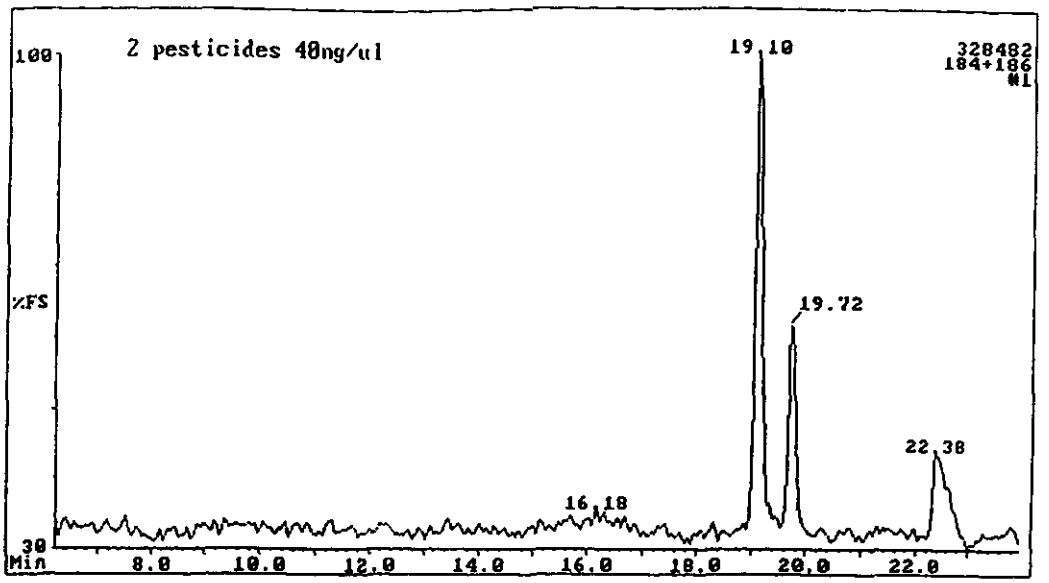
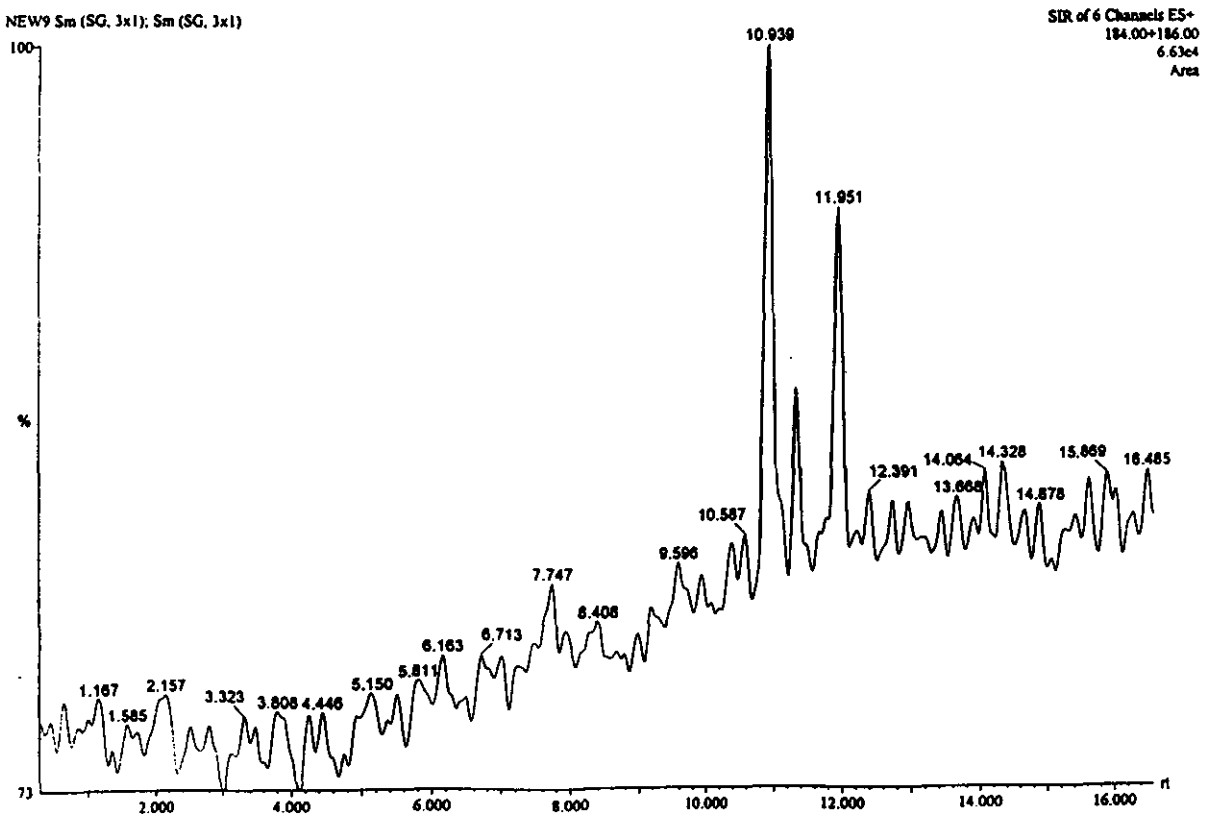


Figure 80. CE/ES chromatograms to illustrate the limit of detection of the three chromophoric herbicide mixture, (a) at 40 ng/µl and (b) at 1ng/µl.





Buffer: 10 mM ammonium acetate in a solution of 50/50 water/methanol, adjusted to pH 3.2 with phosphoric acid. Capillary: 90 cm x 75 µm i.d. silica capillary. Separation at 21 kV, 25°C, detection by VG Quattro electrospray mass spectrometer.

Chapter Seven

Capillary electrophoresis of quaternary ammonium herbicides with detection by indirect uv spectroscopy and electrospray mass spectrometry.

1. Introduction.

The standard visualization mechanism in CE is that of UV absorption although fluorescence detectors are becoming more widely used. However, these approaches limit the type of compounds which can be detected to those that contain a chromophore or fluorophore or can be derivatized so as to impart such properties upon them. Detection of non-chromophoric compounds was first facilitated by the advent of indirect detection methods. The key to this approach is the displacement of a highly absorbing mobile-phase additive in the buffer by the sample analytes. The signal is derived from this mobile-phase additive rather than from the analyte itself because the concentration of the chromophoric additive is lower in the eluted bands when compared with its steady state concentration[167]. This indirect photometric detection (IPD) has been applied to CE by Foret *et al.* [168] who observed the effect of ion mobility on the peak shape and found that higher sensitivity was obtained by selection of visualizing agents which had high molar absorptivity, and mobilities similar to those of the sample ions. The closer the two mobilities could be matched the better were the detection limits obtained, even if a compromise between the uv absorbance and the mobility of the visualising agent sometimes had to be made[169]. The main disadvantage of the indirect method of detection is that relatively poor limits of detection can be acheived e.g. there are often three orders of magnitude difference between direct and indirect fluorescence detection[170]. An important factor which has to be optimized before adequate sensitivity can be achieved is that of the noise coefficient which is the ratio of the concentration fluctuation to the concentration of the visualisation agent [171]. Although electromigration injection may provide an analysis with greater sensitivity it also introduces injection bias as the more highly charged molecules migrate onto the column much faster than those less highly charged[172]. Once the concentration of visualising agent and separation parameters have been

optimized the use of an internal standard during the analysis has been shown to validate IPD in CE as a fully quantitative method[173]. High-performance liquid chromatography has been successfully used to examine compounds using IPD[174,175], including one of the compounds in this study, chlormequat[176] thus showing the feasibility of IPD in a separative procedure. The technique of CE itself cannot provide low enough detection limits for real environmental samples but another form of CE, isotachophoresis, has successfully been used for the analysis of trace levels of such compounds[177]. In the present work, the testing of formulation products is paramount and therefore the required limits of detection are not stringent and in fact the samples have to be substantially diluted before analysis. CE provides a cheap, fast and accurate method of analysis for these compounds and has advantages over ion-chromatography in terms of speed and peak capacity. Other biologically important amines[178], inorganic cations[179-181] and anions[182] have also been analysed using IPD. This work has been applied to the examination of chlormequat (**7.1**) and choline chloride (**7.2**). Other molecules being examined in this class include their by-products, trimethylammonium chloride (TMAHCl) (**7.3**) and trimethylvinylammonium hydroxide (TMVAH) (**7.4**). The structures of these are shown in Figure 82. These quaternary ammonium species are of primary interest to the Health and Safety Executive (HSE) which requires a method for the determination of these compounds in order to calculate the purity of formulations sent to them in connection with the enforcement of Control of Pesticide Regulations (COPR).

Figure 82. Structures of the non-chromophoric herbicides under investigation.

	Structure	Chemical name
(7.1)	$ClCH_2-CH_2-\overset{+}{N}(CH_3)_3Cl^-$	Chlorocholine chloride (Chlormequat)
(7.2)	$HO-CH_2-CH_2-\overset{+}{N}(CH_3)_3Cl^-$	Choline chloride
(7.3)	$(CH_3)_3\overset{+}{N}-HCl^-$	Trimethylamine hydrochloride
(7.4)	$CH_2=CH-\overset{+}{N}(CH_3)_3OH^-$	Trimethylvinylammonium hydroxide
(7.5)	$(CH_3)_3\overset{+}{N}H$	Trimethylammonium
(7.6)	$C_3H_7\overset{+}{N}H_3$	Isopropylammonium

These aliphatic quaternary ammonium salts **7.1** and **7.2** are used to improve the resistance of plants to fungicidal infection as well as to the harmful effects of other herbicides. Chlormequat chloride is a known growth regulator and has been used to control excessive cotton growth and hence increase its yield[183]. This can be achieved in conjunction with other herbicides which would normally be detrimental to the plant targeted if used as a stand alone treatment. Such treatments include Terbutryne and Simazine which each have their beneficial properties regarding the growth of wheat crops but due to the lack of specificity of the former case and the effects on early wheat seedlings in the latter means that they are only useful if used in conjunction with chlormequat[184]. It was Caldicott[185], who in 1967 concluded that chlormequat itself was unlikely to have any direct fungicidal or insecticidal action but probably lead to physiological changes in the plant rendering it unsuitable for infection or infestation. Reports and trials confirm that the use of chlormequat and choline chloride do in fact help to increase the yields and quality of wheat crops[186].

Once visualization of these herbicides was achieved using CE, the technique of electrospray mass spectrometry (ES) was used as an on-line detection mechanism for the unambiguous identification of these compounds as they elute from the end of the column. Like CE, ES is

used to analyse a wide range of compounds from very small to very large polar analytes in aqueous solution. However the very low mass of the analyte cations being used here are normally thought of as too low for ES, given that the technique produces a high intensity of background ions at low m/z values.

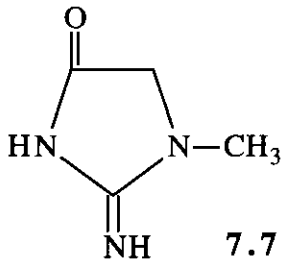
2. Results and Discussion.

2. (1) Initial attempt to visualise the non-chromophoric herbicides.

The most striking observation was that apart from one of these herbicides, no recognisable chromophore was present in these molecules. The double bond in trimethylvinylammonium hydroxide meant that this could be detected via direct U.V. with a true absorbance maximum at 193nm and a secondary maximum at 254 nm. Direct detection was achieved at 254 nm but only at a very high concentration (15% solution w/v). Initial experiments to detect chlormequat indirectly involved speculative use of a fluorescent compound with high absorbance using the 380nm wavelength filter. One approach involved adding quinine sulphate dihydrate to a standard citrate buffer. This was used as the analytes of interest were positively charged[187]. This did allow a very high background absorbance to be achieved but on running the sample no negative decline in the base-line was observed. Other additives tried were benzoic acid which was added to an ammonium acetate buffer. Using this buffer a large decline was observed in the base-line after 5 minutes and this was followed directly by a positive peak before the re-establishment of the base-line. Unfortunately this also occurred when a water blank was injected as the sample. This type of profile has been noted before and was reported as being due to H^+/OH^- ion migration from the sample plug. This means that the pH before the trough is slightly higher than that after it. The appearance of a negative peak with water alone could however be masking some effect due to the chlormequat in the sample. A mixed fluorescent buffer system containing salicylate[170,188] which had been reported for indirect fluorescent analysis was also tried but without success as it is usually used for detection of negatively charged ions.

2. (2) Selection of creatinine as a buffer additive for indirect detection.

In an attempt to mimic the conditions specified in the literature[167-169] for indirect detection it was decide to try creatinine as the additive in the buffer. Creatinine (Mr = 113) has a similar structure and relative molecular mass to the herbicide cations (Mr = 60 - 124) and standard amines being examined which, coupled with its high absorbance at 200 nm and excellent buffering capacity, makes it an ideal choice for this work. It was hoped that this compound would migrate at a similar rate to the analytes of interest, the similarity of these migration times being essential to ensure that the sample components migrate as symmetrical zones[189]. Creatinine, **7.7** also has an amine group and was eventually chosen due to its buffering capacity between pH 3.5 - 5.5 which meant no other compounds had to be added to the buffer which could cause interference. (At this pH creatinine would be cationic in nature, formally the charge residing on one of the amine groups).



7.7

The buffer originally comprised 30 mM creatinine at pH 4.8 and was used with chlormequat alone as the sample at a wavelength of 214 nm. This wavelength was used because the U.V. lamp was degrading and the instrument could not auto-zero at 200 nm. A negative peak was observed after 5 minutes on a 50 cm long column and on repeating the experiment with a water blank the peak did not appear. It was then decided to do a simple quantification experiment using chlormequat alone to further prove the origin of the peak. Samples of chlormequat from 1.2 mg/ml down to 12 µg/ml were injected and a steady decline in peak size observed. Peak area data were not recorded but as an estimate the depth of the peak in absorbance units was taken and using the peaks for chlormequat at 12, 36, 54, 72, 96 and 120 µg/ml a correlation coefficient of 0.98 was obtained. This gave enough evidence to proceed with development of this method. At this wavelength 12 ppm seemed to be the detection limit of chlormequat. It was hoped that this could be improved at 200 nm with a new lamp as this was the absorbance maximum of creatinine. Using this wavelength it was

later found that an analyte level of 5 ppm was the limit of detection. At this level the analyte peak / background ratio was 3.

Further development of the method required separation of the quaternary ammonium salts (QUATS) in a mixture, establishing an internal standard for use in quantitative work and full evaluation of the method for use with real formulation samples supplied by the HSE.

2. (3) Optimisation of pH and buffer concentration for separation of mixtures of the quaternary ammonium species.

An analyte mixture of QUATS **7.1** to **7.4** (approx equal amounts) was prepared and electrophoresed using a buffer at pH 4.2. Four negative peaks were observed as in Figure 83 (Appendix 7). A glyphosate solution, which also contained a counter cation of isopropylamine, was added to the mixture for the next procedure to see if it too could be separated using the same method. The separation was attempted at pH 4.2 and resolution was poor so pH was reduced further to 3.6. At this pH resolution was sufficient to allow visualisation of five peaks (Figure 84, App. 7). Each of the analytes, trimethylvinyl ammonium hydroxide (TMVAH), trimethylamine hydrochloride (TMAHCl), chlormequat, choline chloride and the glyphosate salt were then run separately and identification by migration time was possible to partially confirm the origin of each peak. Figure 85 (App. 7) shows the peak due to the glyphosate salt overlaid on the chromatogram of the five analyte mixture and can be used to identify which peak is due to the glyphosate salt. On further investigation it was found that this peak was actually due only to the counter-cation in the glyphosate salt. This cation was isopropylammmonium, identified when pure glyphosate and the glyphosate salt were analysed individually. Pure glyphosate did not produce any decline in the baseline and required another approach for its analysis. Separation of the QUATS had been achieved but any possibility of further optimisation had to be investigated. The first variable was buffer concentration, creatinine levels of 5 mM, 10 mM, 20 mM and 30 mM were tried.

Table 16. - Change in separation efficiency with creatinine concentration.

Concentration / mM	Time Window (secs) (between 1st and last peak)	Peak depth. (cm) (4th peak)
5 mM	37 seconds	1.21 cm
10 mM	44 seconds	1.25 cm
20 mM	53 seconds	1.28 cm
30 mM	60 seconds	1.29 cm

As the buffer concentration increased, analyte separation improved as did detection in terms of peak size. For subsequent CE analyses the 30 mM buffer was used but what these results showed was that a buffer of 10 mM creatinine could still provide adequate CE separation, so any possible complications that using such an involatile buffer may cause when coupling CE with ES are minimised.

It was intended that a quantification procedure be set-up for samples containing these analytes. For these studies another quaternary amine, triethylamine, was introduced into the sample mixture to act as the internal standard. This analyte gave a sixth peak at the end of the chromatogram (Figure 86, App. 7). The separation of the six peaks was completed to baseline, but a definite gradual improvement in peak width and symmetry is visible between the first peak (trimethylammonium; asymmetry ratio = 0.23; number of theoretical plates, n = 54,000) and the last peak (triethylammonium ions; asymmetry ratio = 1.0; n = 513,000). If the peaks were inverted some fronting could be observed especially on the earlier peaks. This phenomenon is explained by Foret et al[168] as an effect due to the different mobilities of the creatinine in the buffer and the ammonium ions being analysed. One way of suppressing this dispersion is by keeping the concentration of the analyte ions sufficiently below the concentration of the background electrolyte.

2. (4) Examination of standards and construction of calibration curves.

Although one of the compounds being analysed had been proven not to be glyphosate, the isopropylamine salt was kept in the sample mixture. This could be used to check the

resolution of each analysis and also to act as an internal standard. An equimolar mixture of the QUATS was made up to check if comparable peak areas were obtained for each analyte. Peak areas were measured by cutting them out and weighing them. More advanced Beckman system Gold software is capable of inverting the negative peaks to allow direct integration but this was not available at the time. Peak areas and reproducibility of the analyses were sufficient to proceed with the method and construct calibration curves from standards. The first step in these investigations involved reproducibility studies. The same mixture of six analytes (Figure 86, App. 7) was injected six times and the measurements of migration time of each peak recorded in Table 17.

Table 17. Reproducibility results : after six consecutive injections of the sample mixture.

	Peak i.d. and migration times. (mins)					
Repetition	A	B	C	D	E	F
1	5.612	6.041	6.347	6.549	6.464	7.129
2	5.603	6.036	6.342	6.535	6.455	7.115
3	5.603	6.031	6.337	6.535	6.455	7.114
4	5.603	6.031	6.333	6.535	6.450	7.105
5	5.603	6.031	6.333	6.530	6.450	7.103
6	5.603	6.026	6.328	6.526	6.446	7.098

Sample mixtures containing analytes A - F each at 100 µg/ml (100ppm) were used:

A - Trimethylamine Hydrochloride

B - Trimethylvinylammonium Hydroxide

C - Isopropylamine

D - Choline chloride

E - Chlormequat

F - Triethylamine

The relative standard deviation (RSD) for the reproducibility of migration times of all the peaks was less than 0.2%.

Relative standard deviation (RSD) for migration time reproducibility.	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6
	0.07%	0.09%	0.11%	0.10%	0.12%	0.16%

This result means that the identification of each of the peaks solely by migration time can be regarded as very reliable. Quantification also required the setting up of calibration curves using standard sample mixes of each amine of amounts from 5 to 100 μg/ml (5 to 100 ppm). The area of each peak at each amount is given in Table 18. These areas are firstly corrected by dividing by the area of the sixth peak, due to trimethylamine, the chosen internal standard. This was done to correct for any differences in the injection of each sample.

Table 18. Peak area data obtained for the construction of calibration curves for each analyte.

Standard Amounts (μg/ml)	Trimethyl-amine HCl.	Trimethyl-vinyl-ammonium Hydroxide.	Isopropyl amine.	Choline chloride.	Chlormequat.
100	1.213	1.144	1.361	0.800	0.733
90	1.027	1.020	1.252	0.716	0.678
80	0.898	0.902	1.090	0.611	0.563
70	0.771	0.814	0.963	0.580	0.548
60	0.665	0.679	0.760	0.460	0.434
50	0.616	0.573	0.668	0.379	0.369
40	0.459	0.486	0.579	0.310	0.314
30	0.331	0.342	0.456	0.270	0.255
20	0.256	0.250	0.282	0.189	0.146
10	0.133	0.128	0.143	0.058	0.079
5	0.078	0.069	0.083	0.0285	0.041

The correlation coefficients (R^2) for each of the curves, two of which are shown in Figure 87 (App. 7), was better than 0.99 in each case. This further confirmed the viability of the method for quantification purposes. Reproducibility data for peak area were aquired as the final stage of the evaluation of the procedure, results are shown in Table 19.

Table 19. Results for Peak Area reproducibility - (100μg/ml of each) for six consecutive injections.

Repetition number.	Trimethyl-amine HCl.	Trimethyl-vinyl-ammonium Hydroxide.	Isopropyl amine.	Choline chloride.	Chlorme - quat.
1	2.036	2.126	1.275	1.793	1.459
2	2.019	2.206	1.263	1.910	1.545
3	2.180	2.341	1.445	1.900	1.493
4	2.048	2.307	1.329	1.737	1.478
5	2.139	2.305	1.417	1.821	1.551
6	1.938	2.194	1.449	1.925	1.449
Relative Standard Deviations (RSD)	4.2%	3.7%	6.2%	4.06%	2.87%

CE is known to provide highly reproducible results in the direct detection mode and before proceeding with the current analysis, reproducibility data were obtained for six consecutive injections. An example of chromatogram reproducibility is shown in Figure 88 (App. 7) with two electropherograms being overlaid. From these results it is apparent that peak migration time is far more reproducible than peak area which is why the peak areas of the standards are divided by that of an internal standard. These results also show that the different analytes display variable absorbance characteristics when the same relative masses of each are injected as opposed to when the same molarities are used where peak areas are similar.

2. (5) Application of the CE method to determine herbicide values in real samples.

The CE method was applied to analyse concentrated formulation samples supplied by the HSE. These samples were thought to contain chlormequat and its by-products, trimethylamine and trimethylvinylammonium salts. The results were obtained by firstly producing new calibration curves for each of the analytes. The samples were then diluted and

injected, subsequently three inverted peaks were observed. The peak areas were measured and compared with the calibration curves to give a value for each cation. After considering the dilution factors (and, in the case of trimethylvinylammonium chloride, correcting for the difference in mass between the hydroxide standard used here (Mr = 103.2) and the chloride salt present in the formulation products (Mr = 121.6), a factor of 121.6/103.2), the concentrations of the formulation products were derived as in Table 20 below.

Table 20 - Capillary Electrophoresis of chlormequat formulation products: The concentrations were measured in grammes per 100 cm^3 (% w/v)

Sample number.	Chlormequat chloride/% w/v	Trimethyl-ammonium chloride/% w/v	Trimethylvinyl-ammonium chloride/% w/v
71692	39.0± 10%	4.8± 0.6	5.7± 0.5
30665	53.4± 10%	5.7± 0.6	6.7± 0.5
30660	57.6± 10%	7.0± 0.6	8.0± 0.5

These results were obtained from samples diluted with water, 2000 and 10000 times before analysis in order to be fitted to the calibration graphs. The method successfully identified the analytes in the real samples as chlormequat and its by-products as suspected thereby supplying the HSE with further important information on these samples for the enforcement of their regulations. As a comparison the results supplied by the HSE using ion exchange chromatography are shown in Table 21. This method was not optimised for chlormequat and so no direct comparisons can be made with the estimations made by CE. Not included in this Table are the estimations for choline chloride which were also measured for by the HSE though it was detected at only 1% or less in each sample. The dilution of the samples analysed by CE meant that choline chloride was not detected.

Table 21 - Ion chromatography of the chlormequat formulation products in Table 20: The concentrations were measured in grammes per 100 cm^3 (w/v)

Sample number.	Chlormequat chloride/% w/v	Trimethyl-ammonium chloride/% w/v	Trimethylvinyl ammonium chloride/% w/v.
71692	51± 5%	5.5± 0.35	5.4± 0.2
30665	48± 9%	5.8± 0.2	6.0± 0.2
30660	50± 1%	5.6± 0.1	5.0± 0.2

As can be seen by comparison there is favourable agreement between the two sets of results. Further experimentation using a variety of samples analysed using both techniques would be required before CE could be offered as a viable alternative to the current method of determination used by the HSE. CE does offer speed and ease of analysis each taking only 8 minutes for completion. At the concentrated level of the formulation samples there is no problem of detection limit being reached with either analysis.

2. (6) CE/ES of the quaternary ammonium herbicides.

The CE/ES apparatus (Section 8 of Chapter 2) was used to analyse the herbicide mixture with each component at 100 µg/ml. Figure 89(a) (App. 7) is a chromatogram of the separation of the herbicides after 20 cm of capillary. Full separation had not been achieved at this point. The selected ion recording (SIR) chromatogram (Fig 89(b)) (App. 7) shows the final separation of the mixture containing about 3 pmol of each compound achieved after 90 cm of capillary. This can be directly compared with the chromatogram of CE separation in Figure 86 (App. 7) obtained with 50cm length of capillary. It is assumed that CE separation had continued over the entire length of the 90cm capillary but there appears to be no difference in peak resolution, though this may be expected to be better using a 90cm capillary. It was noticed that the peak widths at half peak height were unaffected by the longer separation as illustrated in the Table 22. These deleterious effects could be due to the nebulising gas causing some siphoning and effectively pulling the analytes through the capillary at an increased rate than that of electrophoresis alone (Section 8.(1) of Chapter 2).

Table 22. - Peak width of the six analytes using CE and CE/ES.

Peak Number	1	2	3	4	5	6
Peak width by CE. (secs)	3.2	2.4	2.4	2.4	2.4	2.4
Peak width by CE/ES. (secs)	2.3	4.0	3.1	2.3	2.3	3.5

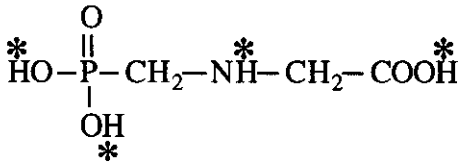
The components of this mixture are depicted in 89(c) (App. 7) after SIR monitoring for the cations listed in Figure 82. Both isopropylamine and trimethylamine gave the same cation m/z ratio of 60 whilst two peaks were observed from chlormequat at m/z 122 and 124 in a 3:1 ratio due to the presence of chlorine. The presence of creatinine in the buffer allowed the mass spectrometer to be tuned via the peak for its protonated molecule at m/z = 114. The elution order was established by mass spectral interpretation of these figures and known migration behaviour. The sample was transferred into the capillary in substantial amounts because the expected signals were observed readily despite the large solvent peaks in the mass range of the cations (60 - 124 mass units). Upon successful CE/ES of this mixture the next stage was to analyse one of the formulation samples using the same technique. As with CE alone, chlormequat and its by-products were observed further confirming the real sample to be impure. The CE/ES chromatogram of this analysis is given in Figure 90 (App. 7). Limit of detection studies were not undertaken but from examination of the spectra obtained using the 100 µg/ml sample it was thought that using ES in conjunction with CE would not improve the limits of detection for these analytes over that offered by CE alone. Experimental results have been reported in an application note[190] and in a *J. Chromatogr.* paper that is in press at the time of writing.

2. (7) Exploration of alternative methods for the analysis of non-chromophoric glyphosate.

From earlier experiments it was observed that the herbicide glyphosate could not be detected indirectly using U.V. with creatinine as the visualising agent. What was thought to be an inverse peak due to glyphosate salt was actually due to the counter isopropylammonium ion. Further attempts at detecting glyphosate included using a high absorbing buffer containing

glycyl-glycine which was chosen due to its structural similarity to glyphosate, but again a negative peak could not be attributed to glyphosate. As glyphosate actually displays reactions similar to those of an amino acid, with NH and COOH groups very apparent, glyphosate has been successfully visualised by other groups as dansyl derivatives which are also commonly used for amino acid analysis. It was decided to follow a different approach by derivatising glyphosate with a bromo-phenacyl reagent which would react with the COOH moiety as well as the exposed hydrogen on the nitrogen atom (reaction in Section 2.(2) of Chapter 2). This however meant that four possible derivatisation sites were available as shown in Figure 91, but it was hoped that the COOH would be derivatised primarily.

Figure 91 - The structure of glyphosate illustrating the possible sites for derivatisation.



* Other possible sites for derivatisation.

Once the derivatisation procedure had been completed the resulting mixture was cooled and analysed. The result was an electropherogram with two peaks which could be explained by the derivatising agent reacting at two of the possible four derivatisation sites. However on re-analysing the same sample three days later it was found that one of the peaks had disappeared, leaving only one peak . This peak can be superimposed onto one of the other peaks in the original electropherogram but is smaller. This suggests that both of the peaks begin to degrade but one persists longer than the other. This result does show that a reaction is occurring between the glyphosate and the derivatising agent but which sites are involved cannot be ascertained. It was concluded that this approach would not produce a robust analytical procedure.

2. (8) FAB mass spectrometry of the pre-derivatised glyphosate sample.

It was decided to perform FAB mass spectrometry on the derivatised sample of glyphosate to examine this further. The mass spectra obtained did not yield any further useful information

because in the positive ion mode the spectra were dominated by isopropylammonium ions, and in the negative ion mode by a prevelance of ions due to bromide and bromide with glycerol adducts.

3. Conclusions and summary.

A new method for the analysis of specific non-chromophoric herbicides was proposed and from the results obtained it was proved that the method was both qualitative and quantitative. The procedure has now been put forward as an alternative for use by the Health and Safety Executive for the analysis of such samples in the future, to enforce various government regulations. The use of CE/ES helped to confirm the migration order of the analytes and allowed the unambiguous identification of each herbicide and the accompanying internal standards. Using electrospray mass spectrometry as the detection mechanism did not however improve the detection limits of the procedure and as an expensive detector is not a viable alternative to CE alone. Mass spectrometry was used to further confirm the viability of the analysis. The purpose of the analysis was to establish the purity of the formulation sample and results show that the HSE samples contain trimethylammonium and trimethylvinylammonium cations as well as chlormequat. Through calibration and quantitative data the method was shown to be viable for the analysis of the compounds of interest at least in concentrated formulation samples (after appropriate dilution).

An alternative method for the analysis and detection of glyphosate has yet to be brought to fruition. However further optimisation of the derivatisation procedure which would include obtaining a greater understanding of the derivatization with bromophenacyl reagent would allow detection of the analyte by CE and possibly CE/ES.

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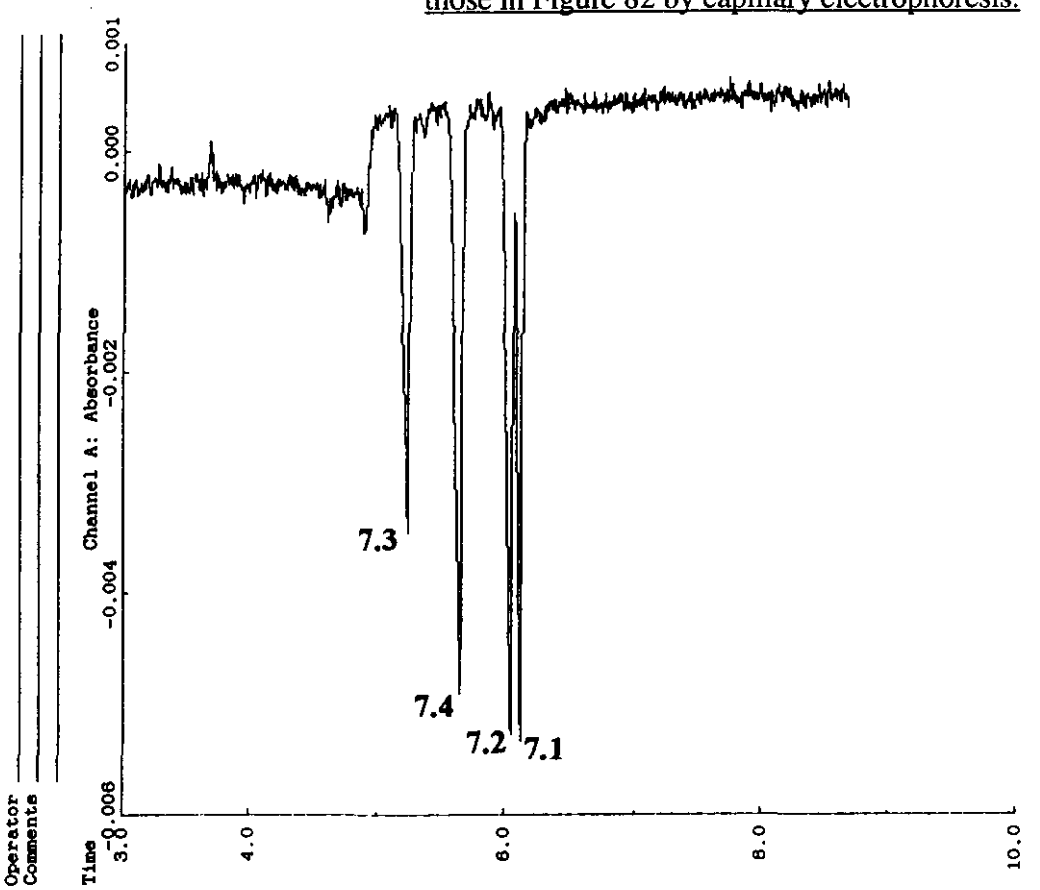
Appendix 7

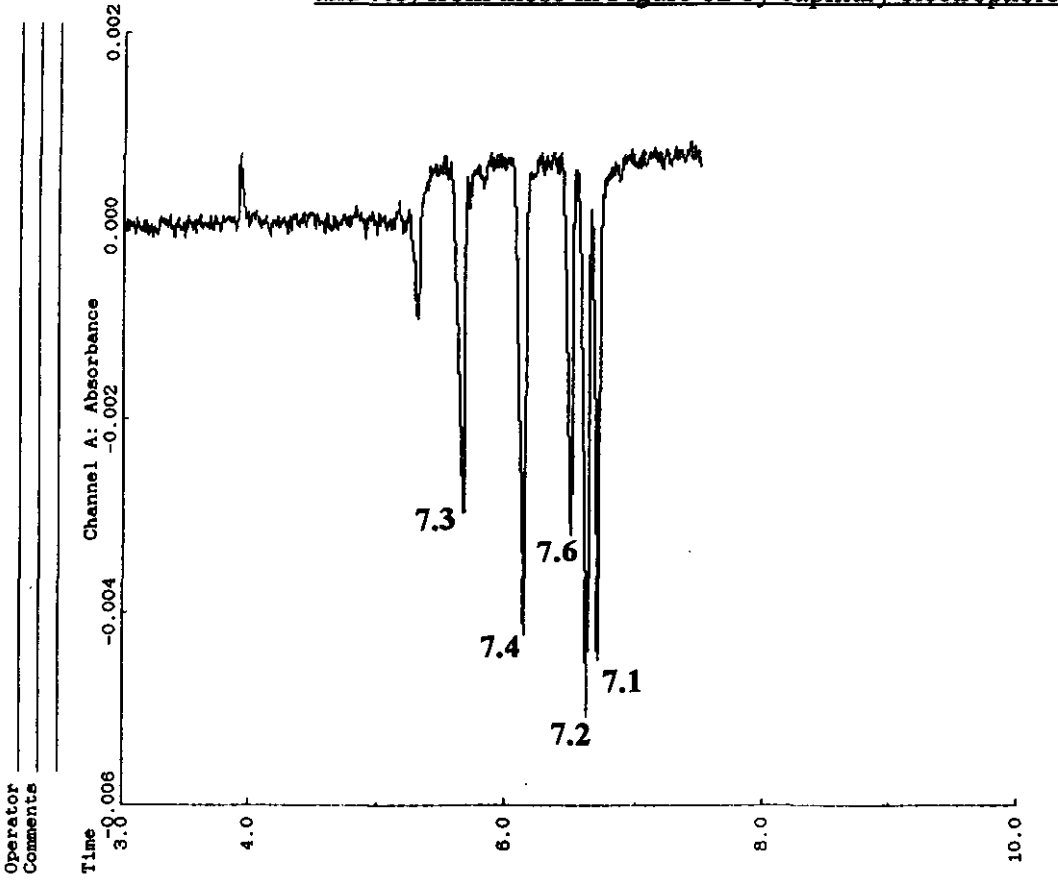
Figures from Chapter 7.

Figure 83. Electropherogram obtained by analysing four ammonium ions (**7.1 - 7.4**) from those in Figure 82 by capillary electrophoresis.



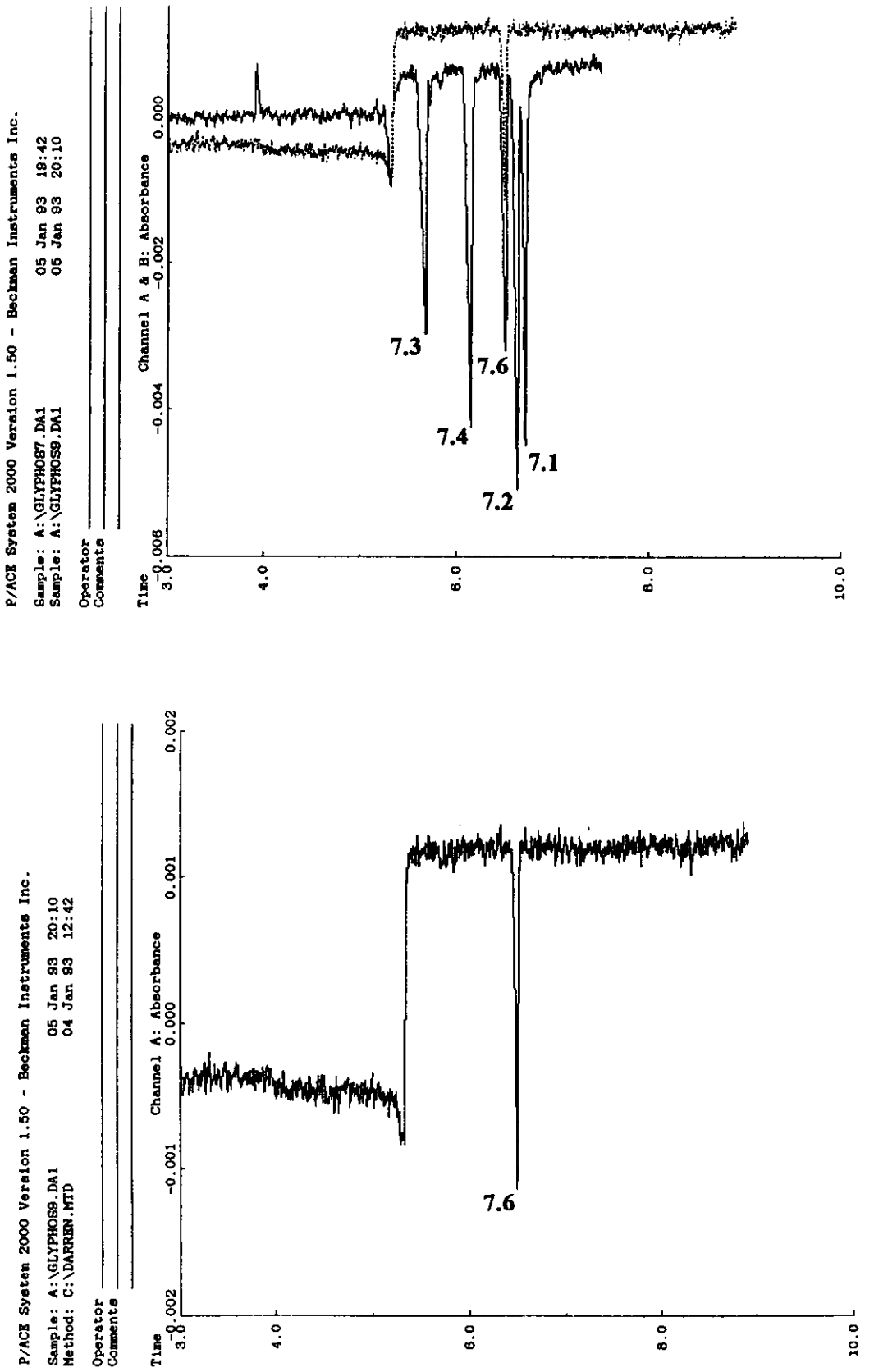
Buffer: 30mM creatinine adjusted to pH = 4.2 with ethanoic acid; capillary: 50 µm i.d. x 50 cm; separation at 25 kV at 25°C; detection: inverse uv absorption at 200 nm.

Figure 84. Electropherogram obtained by analysing five ammonium ions (**7.1 - 7.4** and **7.6**) from those in Figure 82 by capillary electrophoresis.



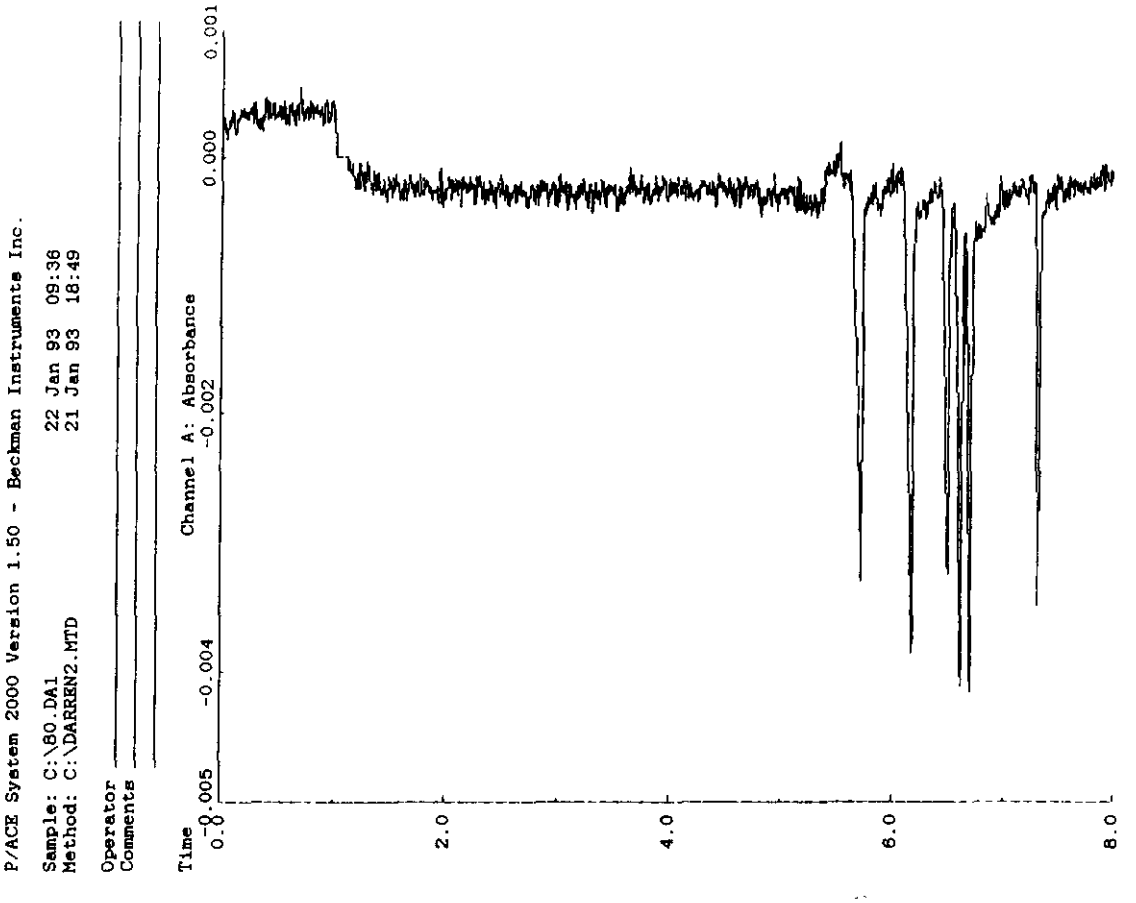
Buffer: 30mM creatinine adjusted to pH = 3.6 with ethanoic acid; column: 50 µm i.d. x 50 cm; separation at 25 kV at 25°C; detection: inverse uv absorption at 200 nm.

Figure 85. Two electropherograms overlaid, illustrating the reproducibility of the system which enables a single component, the isopropylammonium ion (from **7.6**, (Figure 82) to be identified within a mixture.



Buffer: 30mM creatinine made up to pH = 3.6 with ethanoic acid; column: 50 µm i.d. x 50 cm; separation at 25 kV at 25°C; detection: inverse uv absorption at 200 nm.

Figure 86. Electropherogram obtained by analysing the ammonium ions shown in Figure 82 by capillary electrophoresis.

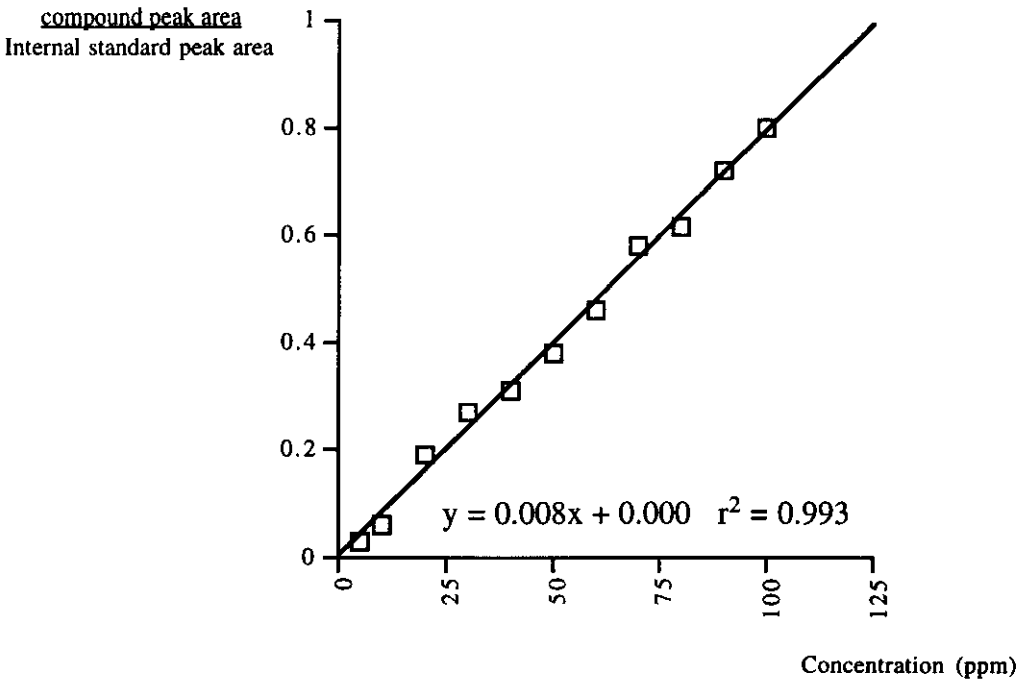


Buffer: 30mM creatinine adjusted to pH = 4.2 with ethanoic acid; column: 50 μm i.d. x 50 cm; separation at 25 kV at 25°C; detection: inverse uv absorption at 200 nm.

Figure 87. Calibration curves for CE analysis of choline chloride (a) and chlormequat ions (b) in the concentration range 5 -100 ppm.

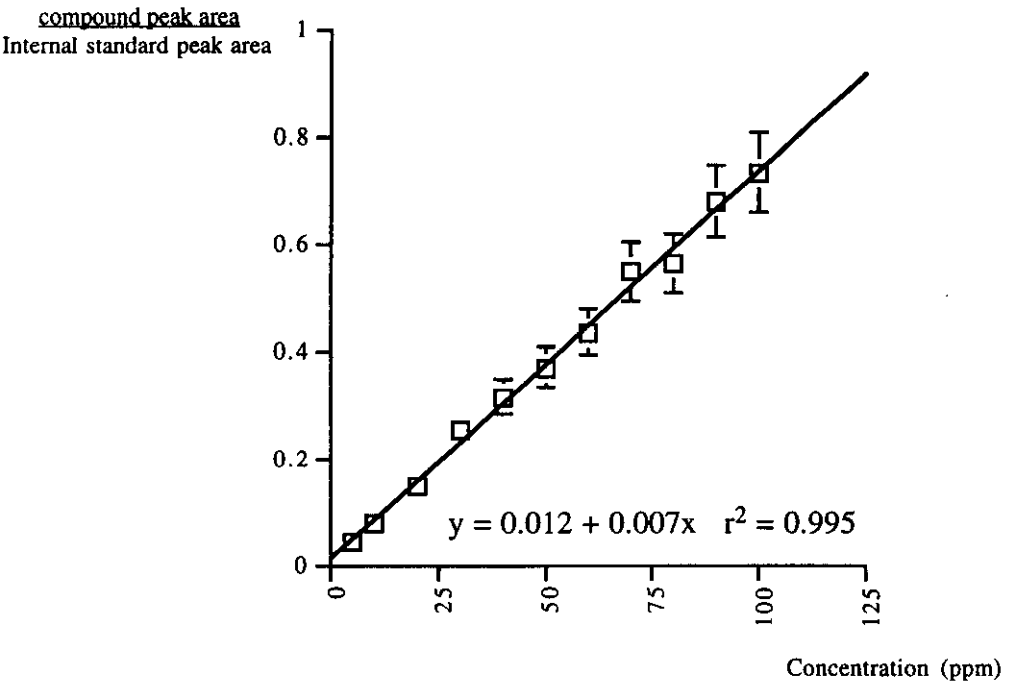
(a).

Choline chloride calibration curve using peak area.



(b).

Chlormequat calibration curve using peak area.



Buffer: 30mM creatinine adjusted to pH = 3.6 with ethanoic acid; column: 50 μm i.d. x 50 cm; separation at 25 kV at 25°C; detection: inverse uv absorption at 200 nm.

Figure 88. Two electropherograms of the CE analysis of the ammonium ions shown in Figure 82, overlaid to illustrate reproducibility.

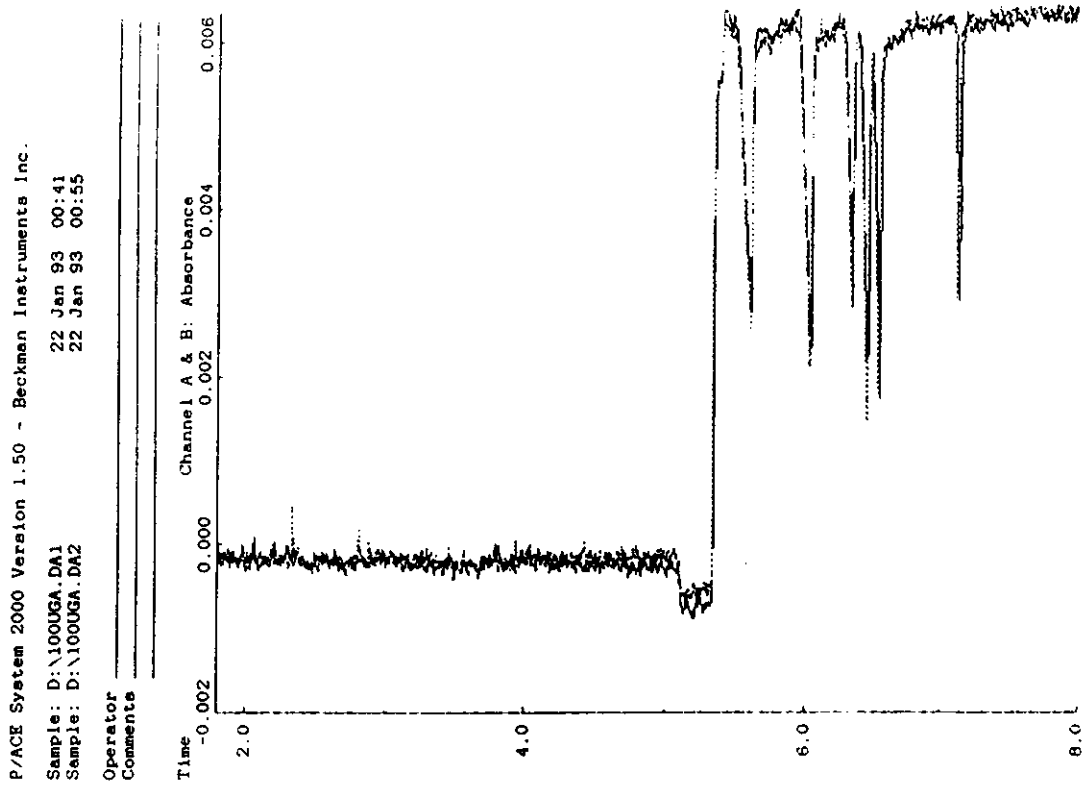
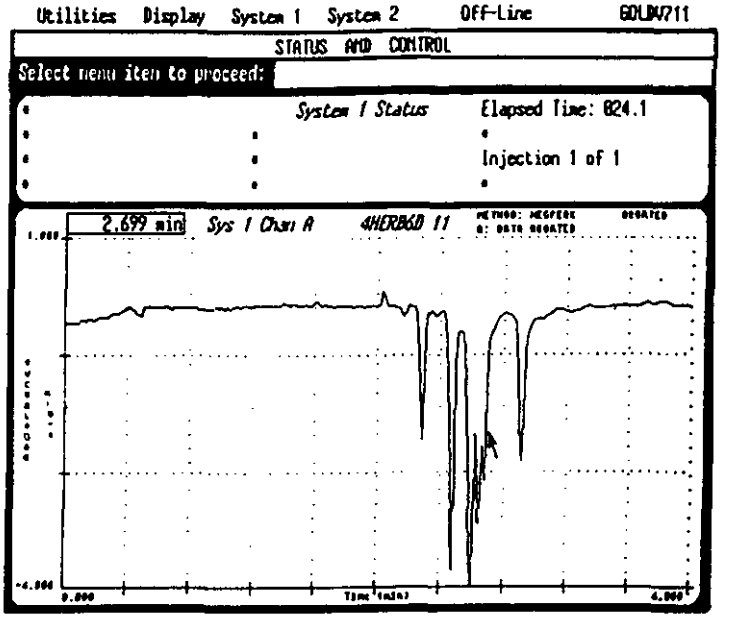
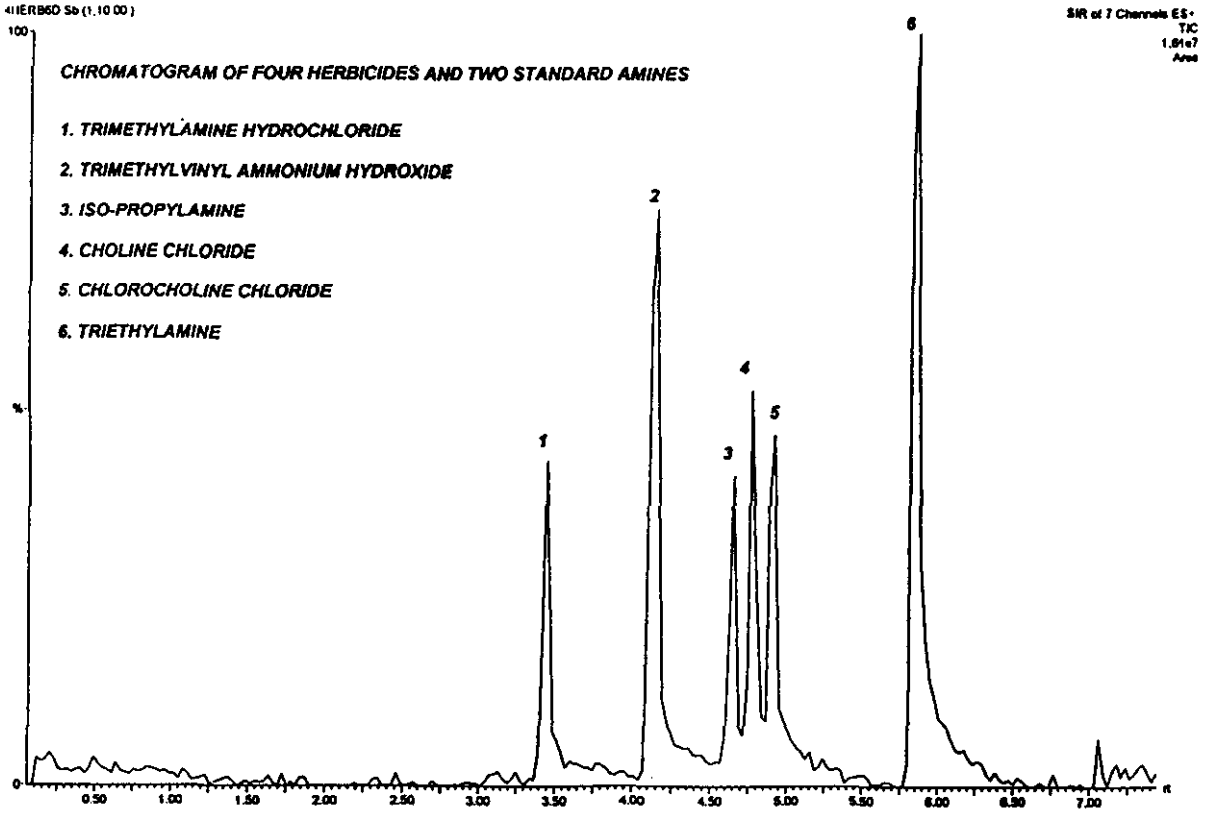


Figure 89(a). Inverse uv detection of the six ammonium ions shown in Figure 82, after migrating 20 cm in a coupled capillary electrophoresis/electrospray mass spectrometry system.



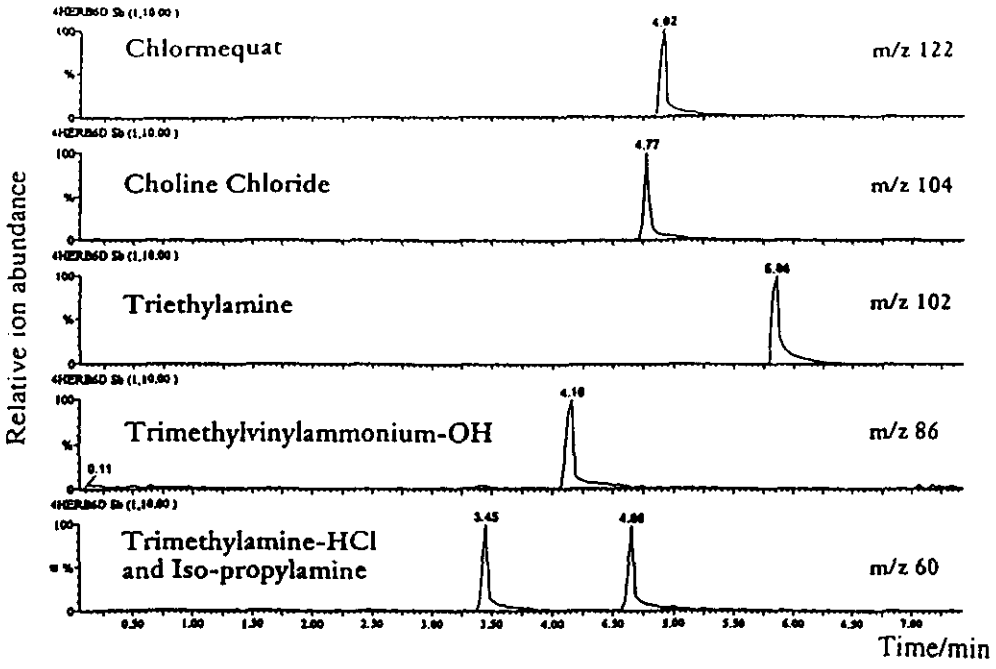
The CE conditions were those given in the legend to Figure 86 except that the complete length of the capillary was 90 cm and the potential difference across the capillary was 21 kV. Buffer: 10mM creatinine made up to pH = 3.6 with ethanoic acid.

Figure 89(b). The summed ion current trace for the analysis of the six ammonium ions shown in Figure 82, after migrating 90 cm in a coupled CE/ES system.



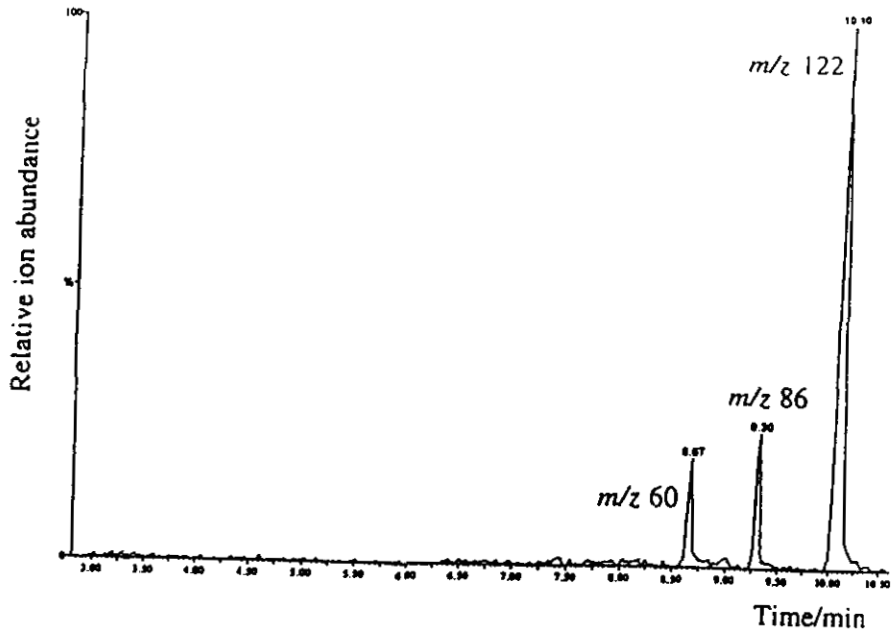
Peak identities: 1, trimethylammonium ion; 2, trimethylvinylammonium ion; 3, isopropylammonium ion; 4, choline; 5, chlormequat; 6, triethylammonium ion. [Acquisition of data was begun after the components had passed the uv window at 20 cm, so the migration times on this trace are not comparable with those given in other figures.] Buffer: 10mM creatinine made up to pH = 3.6 with ethanoic acid.

Figure 89(c). Selected -ion recording (SIR) of the components of the non-chromophoric QUAT mixture.



Buffer and conditions as detailed in figures 89(a) and 89(b).

Figure 90. Analysis by CE/ESMS of the chlormequat formulation which gave the quantitative CE results shown in Table 21.



Buffer: 10 mM creatinine at pH 3.6 with ethanoic acid. Capillary: 90 cm x 50 μm i.d. Separation at 21 kV, 25°C, detector: electrospray mass spectrometry.

Chapter Eight

Analysis of diisocyanates by capillary electrophoresis.

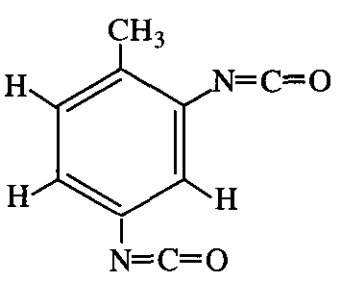
1. Introduction.

Diisocyanates comprise a family of compounds which contain the highly reactive N=C=O group. The reactivity of this grouping was first exploited early this century in the 1930s when the industrial interest in these compounds was established in Germany. They reacted the diisocyanates with polyhydroxyls to create new polymers called polyurethanes, a reaction that had been known since 1849[191]. By using various isocyanates for these reactions a wide variety of materials can be produced. This has lead to the development of many new materials such as flexible foams, solid elastomers, fibres, adhesives and polyurethane. Polyurethanes are now used in the manufacture of a wide range of products including upholstery, carpets, varnishes, printing inks, thermal insulation, adhesives and paints. Polyurethane paints are largely based on aliphatic isocyanates, particularly the two-pack paint systems used in car refinishing, which are usually applied by spraying. Commercially available diisocyanates used for polyurethane production are shown in Figure 92. Toluene diisocyanate (TDI) **8.1** (2 isomers), 4,4'- diphenylmethane diisocyanate (MDI) **8.2** and hexamethylene diisocyanate (HDI) **8.3** are the most commonly used.

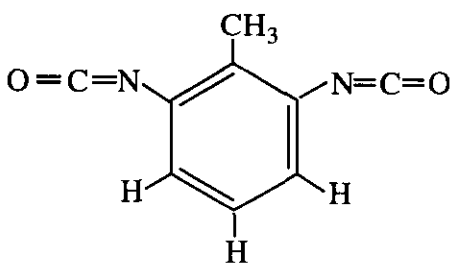
Figure 92.

Toluylene diisocyanate

8.1

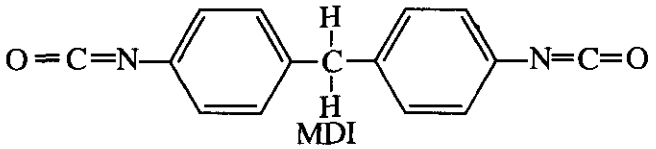


2,4 - TDI



2,6 - TDI

8.2 4,4-diphenylmethane diisocyanate



MDI

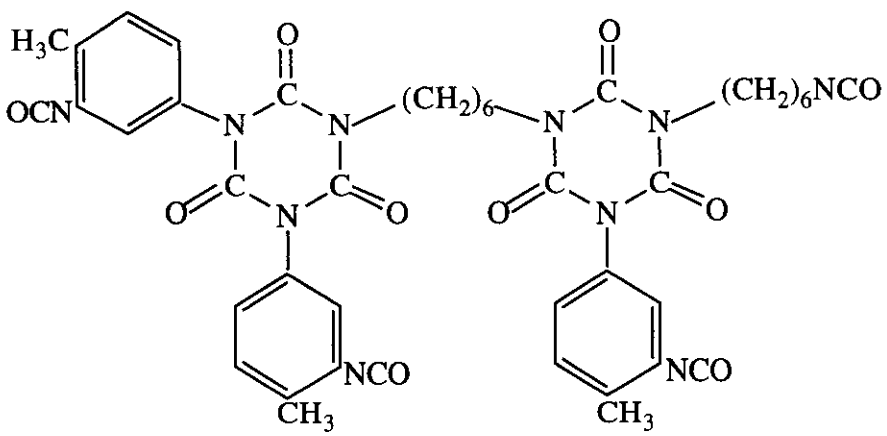
8.3 Hexamethane diisocyanate

HDI

TDI usually occurs on the market as an isomeric mixture containing 80 : 20 or 65 : 35% of the 2,4- and 2,6- isomer respectively[192] with greater reactivity of isocyanate group in the *para* - position. TDI is particularly used for flexible foams, but also for several other applications such as elastomers and coatings. Combinations of these isocyanates can be used to form prepolymers which can then be utilized to produce the wide variety of products already discussed[193].

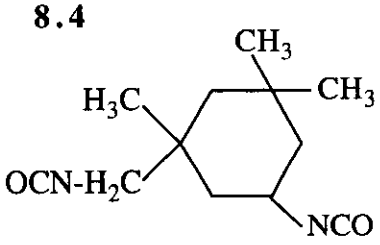
Figure 93.

A Prepolymer based on a mixture of 2,4-TDI and HDI isocyanates.



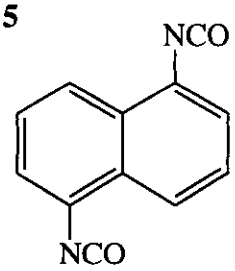
Other less common and less widely used diisocyanates include isophorone diisocyanate (IPDI) **8.4** and 1,5-naphthylene diisocyanate (NDI) **8.5**.

8.4



IPDI

8.5



NDI

Unfortunately due to their extreme reactivity, low levels of diisocyanates are known to act as sensitisers and cause asthmatic type reactions. If inhaled in the vapour or fine particle form the eyes, mucous membranes and the lungs are effected[194]. A study of workers producing polyurethane foam moulding[195] showed a definite correlation between this activity and the incidence of bronchitis and related illnesses. Exposure is most likely as the isocyanates evaporate during the exothermic polymerization reaction. The risk of atmospheric pollution is greater with TDI than with MDI as MDI has a higher boiling point and lower vapour pressure at 25^{o}C than TDI. Other responses to TDI and MDI vapours have been reported which include demonstrations of immunochemical effects, as circulating antibodies to both diisocyanates increase in those exposed[196,197]. Most illnesses develop in people who have been exposed to diisocyanates over a period of time but neurological complications have been known after a single severe exposure to TDI[198]. The high toxicity of the isocyanate group of compounds and the frequency of worker potential exposure to them make their monitoring of great concern to industrial hygienists and in particular the Health and Safety Executive (HSE).

1. (1) Collection methods.

A. Solvent collection methods.

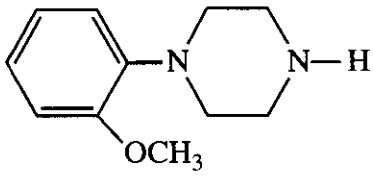
The sampling of airbourne diisocyanates is in most cases performed by using an impinger containing an absorber medium. The absorber medium used in the research described in this thesis was a reagent solution, in which the diisocyanates are reacted to form a derivative suitable for subsequent analysis. In this type of absorber media, the collection and derivatisation occur simultaneously. One reagent used is 1-(2-methoxyphenyl)piperazine (**8.6**) in Figure 94.

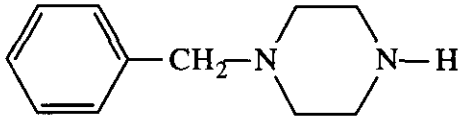
B. Solvent-free collection methods.

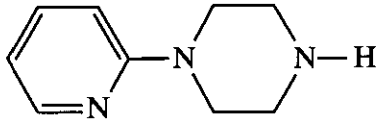
In 1982 only four methods were available for the solvent-free collection of diisocyanates. The first of these involved what was called the chemosorption method. This method relied upon the use of a sorbent, Amberlite XAD-2, which adsorbed the derivatising reagent by interaction of the non-polar surface of the sorbent with the hydrophobic part of the reagent[199]. Other methods include the use of glass wool or glass fibre filters impregnated

with derivatising agent. In another method silica gels have been used for this purpose. More recently specific products have been made available which utilise this type of procedure. One such product is the ORBO - 80 filter (Supelco). These filters utilised a glass fibre filter coated with 1-(2-pyridyl)piperazine (derivatisation agent **8.8** in Figure 94.)

Figure 94. - Commonly used derivatising agents for diisocyanate analysis.

8.6  1-(2-Methoxyphenyl)piperazine

8.7  1-Benzylpiperazine

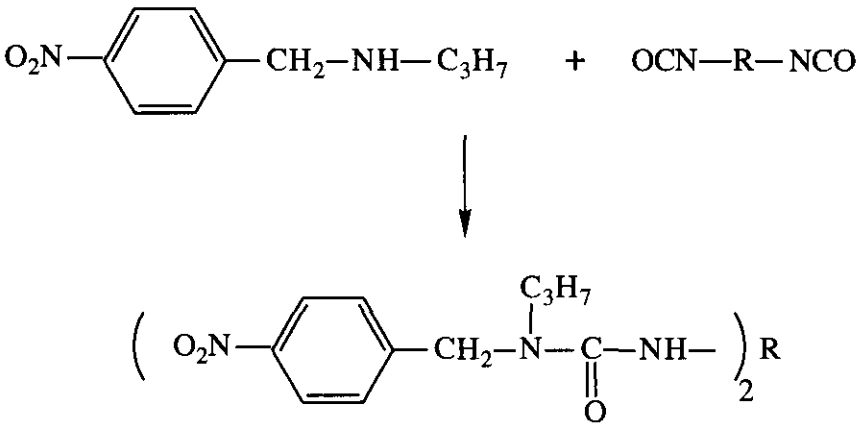
8.8  1-(2-Pyridyl)piperazine

1. (2) Determination methods for Diisocyanates.

Various methods have been formulated for the collection and measurement of isocyanates in air[200], each has its own benefits and disadvantages as well as a range of sampling times required to provide the sensitivity required. The first recognised method for the determination of isocyanates in air was colorimetric[201]. However, colorimetric methods are not specific; if mixtures of isocyanates are present, these methods indicate only the total concentration of the substances. Chromatographic techniques, including gas chromatography and thin-layer chromatography[202] separate out each analyte, allowing discrimination between different isocyanates. These methods are more specific and sensitive than the colorimetric methods as well as requiring less sampling time[203]. However HPLC is the technique which has been most widely used for the analysis of diisocyanates. During the development of HPLC techniques various derivatisation procedures have been utilized to improve detection limits and make the method as specific as possible for each diisocyanate to

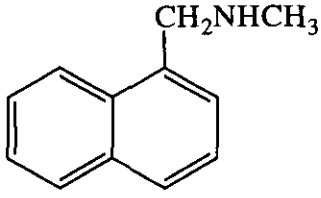
be detected. A high speed liquid chromatography method was described by Keller et al[204] in which a reagent containing N-4-nitrobenzyl-N-n-propylamine (nitro reagent) is used to convert the isocyanate into stable urea derivatives as below.

Figure 95.



Whilst keeping HPLC as the method of analysis various groups have investigated the use of different derivatising agents. An early example was ethyl urethane[205] which permitted the determination of both free monomeric TDI and MDI in prepolymers either separately or together. The use of other derivatising agents, some of which are shown in Figure 94 was described in another paper by members of the same group[206].

Other investigations in this area have centred on reacting the diisocyanates with fluorescent derivatives to improve the detection limits. A 50 fold increase has been achieved over standard UV detection using the nitro reagent. Both aromatic and aliphatic isocyanates react readily with N-methyl-1-naphthalenemethylamine (NMA)[207].

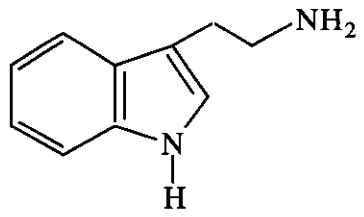


NMA

Another fluorescent derivatising agent, tryptamine (**8.9**) has been used for amperometric and fluorescence detection during HPLC. The development of fluorescence detection and the

subsequent introduction of amperometric detection is described in a sequence of papers from Wu et al[208-210].

8.9



All the derivatives so far have been used in conjunction with HPLC analysis but the aim of this study was to investigate the viability of utilizing capillary electrophoresis in place of HPLC. The use of capillary electrophoresis for the analysis of isocyanate derivatives was investigated. In the method currently employed for the determination of isocyanates, MDHS 25 (MDHS 25, Methods for the Determination of Hazardous Substances, March 1987) the isocyanate-containing species are derivatised with 1-(2-methoxyphenyl)- piperazine (MPP). The resulting compound is then analysed by HPLC with dual (UV and EC) detection as described by Bagon et al[211]. The species containing derivatised isocyanate functions are identified from their EC/UV detector response ratios and their isocyanate content is determined from their EC responses. This is done with the assumption that the EC detector response results solely from the MPP function of each derivatised species. Recent evidence from a NIOSH study has suggested that this may not be correct, and that in the case of derivatised isocyanate species containing aromatic functions (like those used in the present study) the EC response was found to increase with an increase in the aromatic content of the isocyanate. The MDHS 25 method employing EC responses for quantification purposes may thus not be accurate. The NIOSH study did however correctly identify the isocyanate derived species because the UV absorbance also increased with increasing aromatic content which meant a subsequent change in the EC/UV ratios. This method has formed the basis of interlaboratory quality control schemes for the HSE since 1981[212]. It was hoped that a new method of analysis involving capillary electrophoresis could be developed. UV detection was used during CE alone but during CE/ES some mass spectra were also obtained. The following results show just how far this objective was achieved and suggests further proposals for the attainment of this goal.

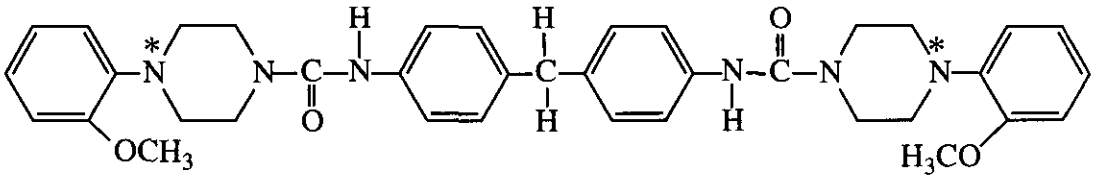
2. Results and Discussion.

2. (1) Initial separation of a mixture of the four derivatised diisocyanates.

The methoxyphenyl piperazine (MPP) derivatives of the diisocyanates were prepared at the HSE laboratories (see section 1. (1)B. Figure 94, structure **8.6**) according to the following reaction:

$$2R_2NH + OCN\text{-}R'\text{-}NCO \longrightarrow R_2NCONH\text{-}R'\text{-}NHCONR_2$$

The structure of the resultant MPP derivative of MDI is shown below:



Capillary electrophoresis was performed using derivatised isocyanates. This involved using an acidic buffer at pH 3.2 which would ensure that the amine nitrogen(s) * in the piperazine ring would become positively charged. Under these conditions the isocyanates would migrate through the capillary as cations or because there are two piperazine groups possibly as dications. Derivatising with 1-(2-methoxyphenyl)piperazine also improves the U.V. absorbance characteristics of each isocyanate.

The derivatised TDI (2 isomers), HDI and MDI were mixed for initial analysis by capillary electrophoresis. Whilst this situation would never actually arise within a real sample, the analysis and separation of the four analytes (Figure 96, Appendix 8), two of which are isomers, demonstrates the application of CE for this type of work. The sequence of these in terms of migration times was established from samples of each diisocyanate derivative analysed individually. The theoretical plates calculated using migration times and peak widths for the 2,4-TDI/MPP derivative was found to be 70,500 for the CE analysis compared to 1,360 for HPLC analysis. This illustrates a somewhat superior separation performance of CE over that known to be provided by HPLC (Figure 97, App.8)[206] even though CE separation had not been optimised.

2. (2) Identification of MDI within a real sample.

It was decided to concentrate initially on the derivatised MDI standard and to establish whether the CE method would be able to detect the analyte within a real sample as supplied by the HSE. CE analysis was performed on one of the MPP derivatised samples taken from an industrial atmosphere such as a car body paint spray shop where MDI was being used. The MDI would have been captured via an impinger containing a solvent and derivatising agent to react with isocyanate and prevent it from escaping back into the air. The set of chromatograms in Figure 98 (a-c) (App.8) are the results of this analysis and the analysis of the same sample spiked with MDI and of MDI standard (20µg/ml) alone. These results made it possible to confirm the identity of the peak due to MDI in the real sample. It was noticed that on spiking the real sample, one of the peaks increased in size whilst the other two decreased in size due to the dilution factor introduced by adding 700µl of the standard to 300µl of the sample. One of the other peaks was thought to be due to excess derivatising agent which would be present through natural wastage or from decomposition of the MPP/MDI derivative on storage in the CE buffer solution. The identity of the other peak in chromatograms (a) and (b) was and is still unknown. The analysis of the real sample collected from an industrial atmosphere indicated that CE was a viable alternative to HPLC for the successful analysis for diisocyanate derivatives present in concentrations at the ppm level. The level to which this method could detect these analytes had been determined earlier by limit of detection studies. For analysis of samples below these levels it would be possible to pre-concentrate the samples by a factor of ten or a hundred over HPLC because of the lower minimum sample volume requirement of CE compared to HPLC.

2. (3) Investigation of Toluylene Diisocyanate (TDI) standard.

The focus of the diisocyanate investigations moved to examine TDI standards. A sample of derivatised 2,4-TDI was supplied by the HSE which had been dried to a powder, having had the toluene solvent removed. As no separation could be performed with only one analyte the material was used to construct a calibration curve to partly assess quantitative aspects for further analysis when both TDI isomers were available. It was also assumed that the absorbance characteristics displayed by 2,4-TDI would be shared by 2,6-TDI. A number of calibration curves were constructed from integrated peak area data. An example of peak area

data for 2,4-TDI alone is given in the Table 23.

Table 23. - Calibration curve data for 2,4 - TDI standards.

µg/ml of 2,4-TDI standard	Peak Areas
35	0.628
30	0.525
25	0.459
20	0.368
15	0.258
10	0.201
5	0.105

Each curve gave a correlation coefficient of 0.99 or better e.g. from data in Table 23;

$$2,4\text{ - TDI} \qquad y = 1.8040\, e^{-2} + 1.7270\, e^{-2}\, x \qquad R\text{^}2 = 0.997$$

This suggested that CE would again be a viable alternative for the quantitative analysis of these compounds, provided that a mixture of 2,4- and 2,6-TDI could be separated and sufficient detection limits could be obtained to analyse real samples.

2. (4) Analysis of quality assessment samples of 2,4- and 2,6-TDI using 2,4-TDI standard calibration curve.

Still assuming that 2,4- and 2,6-TDI had the same absorbance, the 2,4-TDI calibration curve in Figure 99 (App.8) was used to determine the quantity of both isomers in quality assessment samples distributed to a number of national laboratories. The objective was to compare the accuracy of the results from various laboratories in the country which used a variety of analytical methods to establish their results. The results obtained using CE for the first time are illustrated in Table 24.

Table 24. - Initial results from the analysis of four quality control laboratory samples.

Sample	Area of 2,4-TDI	µg/ml 2,4-TDI	Area of 2,6-TDI	µg/ml 2,6-TDI
1E	0.4785	25.95	0.1870	10.15
2F	0.4282	23.77	0.3501	19.43
3H	0.2383	13.22	0.1934	10.73
4G	0.7196	39.90	0.5412	30.05

These results are obtained by interpolation and extrapolation from the original calibration graph. The dried samples which were previously dissolved in 1ml of toluene were reconstituted in 400µl of acetonitrile. This was done to increase the detection limit capability of the method whilst also incorporating acetonitrile into the sample to complement the buffer and sustain the solubility of the sample during injection. This concentration meant that the results for each sample (µg/ml) in Table 24 must be divided by 2.5 to allow for the concentration factor. Another factor that had to be recalculated for was the fact that the original results supplied by the HSE were calculated according to the NCO groups alone, whereas the above determination was for the total diisocyanate. The discrepancy results could be accounted for by division of the CE results by a further factor of 6.64, derived from;

$$\frac{\text{Mass of total diisocyanate}}{\text{Mass of 2 (NCO) groups}} = \frac{558}{84} = 6.64$$

Therefore, the total factor of division before parity between results could be obtained was 16.6. A final table of result comparison between those obtained by the HSE and those determined by CE is shown in Table 25.

Table 25. Comparison of the corrected results of capillary electrophoresis analysis against those obtained by the HSE.

	Expected results (µg/ml NCO)		Results obtained (µg/ml NCO)	
Sample	2,4-TDI	2,6-TDI	2,4-TDI	2,6-TDI
1E	0.81	0.25	1.56	0.61
2F	1.16	0.87	1.43	1.17
3H	0.58	0.44	0.80	0.65
4G	1.74	1.25	2.40	1.81

As an initial experiment the results were promising, but higher than those expected for every result by up to a factor of 2 in some cases. Examples of the chromatogram obtained from the analysis of these samples are supplied in Figure 100 (App. 8). The cause of this systematic error was not identified. If repeated, both 2,4- and 2,6-TDI standards should be used to generate calibration graphs and further evaluate the method.

2. (5) Construction of standard calibration curves using both TDI isomers.

A phosphate buffer with 30% acetonitrile was used to separate the two isomers when analysed as a mixture (Figure 101, App.8 illustrates the separation achieved). Calibration curves were then obtained for both analytes as they would be found in the same sample. Examples for 2,4- and 2,6-TDI are illustrated in Figure 102 (App. 8). It was noticed that the absorbance response was higher for 2,4 -TDI than for 2,6 -TDI which considering both analytes are isomers and were derivatised in the same way, needs further exploration by uv spectroscopy. Separation of the two isomers was adequate for calibration curves of each to be constructed which gave correlation coefficients of over 0.99.

2. (6) Second evaluation of unknown quality control samples supplied by the HSE.

Now that calibrations had been done using mixtures of the two isomers, a new set of quality control samples were obtained for "blind" analysis by CE. These samples were originally dissolved in 1 ml of toluene but the samples were blown dry and reconstituted in 250 µl of

50/50 buffer/acetonitrile before subsequent analysis. Again calibration curves with correlation coefficients of 0.99 or better were obtained (straight line equations below) for both TDI standards and used for extrapolation.

2,4 - TDI $y = 2.3431\ e^{-3} + 2.1441\ e^{-2}\ x$ R^2 = 0.998

2,6 - TDI $y = 1.6245\ e^{-2} + 1.6629\ e^{-2}\ x$ R^2 = 0.991

Table 26. Initial results (µg/ml) for the evaluation of quality control samples by capillary electrophoresis.

Sample	Area of 2,4-TDI	µg/ml 2,4-TDI	Area of 2,6-TDI	µg/ml 2,6-TDI
1	0.389	18.1	0.479	28.7
2	0.124	5.8	0.087	5.2
3	0.199	9.3	0.816	49.0
4	0.379	15.3	0.270	16.2

These samples were originally supplied as 1 ml solutions but the total volume of the reconstituted sample was 250 µl which introduced a concentration factor of 4 to the calculated values illustrated in Table 26 above. The final adjustment to these results was acheived by dividing the results in Table 26 by 26.6 (derived from 6.64 times 4) and the final estimations in terms of µg/ml of NCO groups displayed in Table 27.

Table 27. Corrected results (µg/ml) for the evaluation of quality control samples by capillary electrophoresis.

	Expected results (µg/ml NCO)		Results obtained (µg/ml NCO)	
Sample	2,4-TDI	2,6-TDI	2,4-TDI	2,6-TDI
1	0.45	0.52	0.67	1.08
2	0.13	0.11	0.22	0.19
3	0.23	0.73	0.35	1.83
4	0.45	0.37	0.57	0.61

Comparing the results obtained with those expected highlighted an over-estimation by the CE method. In terms of 2,4-TDI the results differed by an average factor of 1.5 whereas this increased to a factor of 2 for the 2,6-TDI estimations. The discrepancy between the HSE results and those by CE could be due to an under-estimation of the calibration standards and particularly the 2,6-TDI which was noticed as giving a lower absorbance response than 2,4-TDI throughout the CE experiments on the TDI isomers. There was not sufficient time to investigate these discrepancies further but some suggestions for future study include use of an internal standard that is a methoxyphenylpiperazine derivative of an isocyanate as well as investigating the use of different solvents by the HSE and for CE.

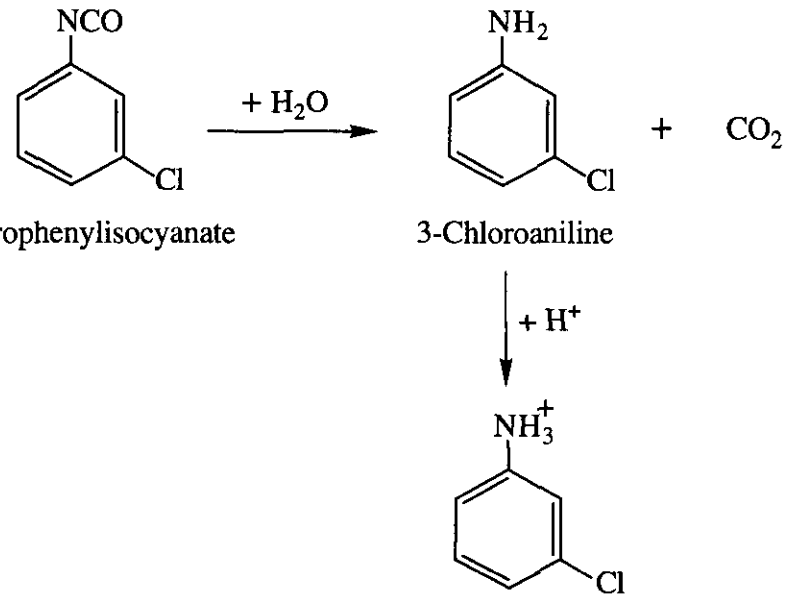
2. (7) Development of an internal standard for use in the CE analysis of TDI isomers.

TDI isomer calibration curves could be further validated by the use of an internal standard. The isocyanate, 3-chlorophenylisocyanate, was present in the laboratory but as an isocyanate would not become charged under the CE conditions used. However it was known that isocyanates react with water (according to equation **8.1**) to give an amine, especially when the isocyanate is aromatic, as in this case.

$$R-N=C=O + H_2O \longrightarrow R-NH_2 + CO_2 \quad \text{- Equ. 8.1.}$$

This reaction is demonstrated using the 3-chlorophenylisocyanate which upon reaction with water gave a gas, probably carbon dioxide, which bubbled from the solution. The 3-chloroaniline produced could now become charged in acid solution and was provisionally used as an internal standard for the TDI isomer separations (Figure 103).

Figure 103.



3-Chlorophenylisocyanate

3-Chloroaniline

Charged internal standard

The provisional internal standard was confirmed to be 3-chloroaniline when the 3-chlorophenolisocyanate and an actual sample of 3-chloroaniline were added to a mixture of the 2 TDI isomers and analysed. The two standard peaks were superimposed. Unfortunately the 3-chloroaniline had a migration time very similar to the 2 TDI isomers and a compromise over buffer composition had to be made to allow separation of the aniline from the isocyanates. This was acheived by lowering the percentage of acetonitrile in the buffer from 30% to 25% which increases the CE current. Further problems persisted as this change affected the separation of the isomers themselves. This lead to 3-chloroaniline being abandoned as the internal standard to be replaced by 4-chloroaniline which migrated faster and did not interfere with the two isomer peaks even using a buffer containing 30% acetonitrile. Chromatograms illustrating the difference in the migration times of 3- and 4-chloroaniline and their position in relation to the TDI isomers are shown in Figure 104 (App.8)

2. (8) TDI evaluation in terms of peak area reproducibility with and without internal standard correction.

This experiment was performed using a 20 μg/ml mixture of the two TDI analytes both with and without 4-chloroaniline added. Each sample was then analysed 5 times to establish peak area reproducibility and the value of using an internal standard. Results are tabulated below.

Table 28. - RSD results using the peak areas of the two TDI isomers with and without correction by division of an internal standard peak.

Repetition number.	2,4-TDI area	Internal Standard Area	2,6-TDI area
1	0.372	1.639	0.321
2	0.389	1.626	0.378
3	0.352	1.473	0.313
4	0.334	1.507	0.272
5	0.356	1.515	0.326
Uncorrected *Relative Standard Deviation (RSD)*	5.78%		11.8%
Corrected *Relative Standard Deviation (RSD)*	3.3%		9.5%

When corrected using an internal standard peak, the RSD is improved for each analyte. Calibration curves were constructed using the internal standard and once again the correlation coefficients bettered 0.99. The internal standard did not interfere with the isomer separation and it was thought that this could now be used within any subsequent quality control or "real" samples obtained from an industrial atmosphere.

2. (9) CE analysis of "real" TDI samples from industrial work-places.

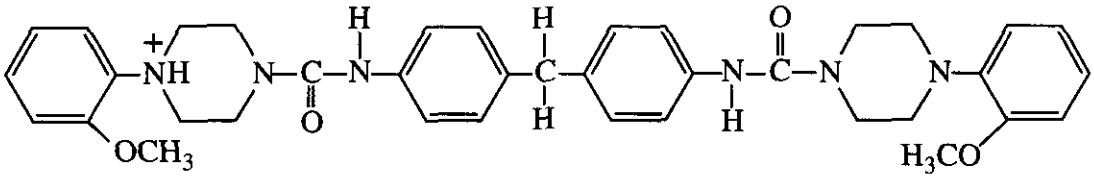
A number of samples obtained from actual industrial environments were also analysed by CE with the same phosphate buffer used throughout. Example chromatograms are given in Figure 105 (a-c) (App.8). These show that the method is able to detect both TDI isomers within actual samples without any form of pre-concentration or derivatisation with fluorescent agents. Where small or no peaks were observed for these analytes the samples were concentrated as with the three examples in Figure 106 (a-c) (App. 8). Samples were concentrated from 250 µl to 40 µl. In this way it was possible to distinguish between blanks, which were identified, and those samples which contained small traces of TDI. These samples, unlike those used for quality control experiments, were dissolved in methanol which did allow them to be directly analysed by CE without changing the solvent. The quality control sample chromatograms (Figure 100, App. 8) show a large tailed peak before the TDI isomer peaks which is put down to residual toluene in which the samples were originally dissolved. Whereas the chromatograms of the real industrial samples, both MDI (Section 2.(2), Figure 98 (a-c), Appendix 8) and TDI samples only show major peaks after the diisocyanate peaks. As with the MDI example one of these peaks is thought to be due to excess derivatising agent left over from impinger collection. This should not be present in the quality control samples as they are derivatised in a more controlled manner as the total diisocyanate to be derivatised is known before the procedure is carried out and the derivatised samples reconstituted in toluene after formation. The other peak in the "real" sample chromatograms is still unknown.

To finally investigate the practicality of the internal standard (4-chloroaniline) 10 µl of a 100 µg/ml solution was added to a real sample (40 µl) to test its migration time in relation to the TDI isomers. The resulting chromatograms (Figure 107 (a and b), App. 8) illustrate the migration time of the 4-chloroaniline which does not conflict with that of the TDI analytes. This means that it could be used both in calibrations and real analyses. One problem is that the internal standard could not be used with quality control samples as it was masked by the large peak seen before the TDI peaks (Figure 100, App.8). As the only difference between quality control and real samples is the final solvent they are dissolved in, it would seem to justify using one solvent, either methanol or acetonitrile to dissolve all samples, making CE

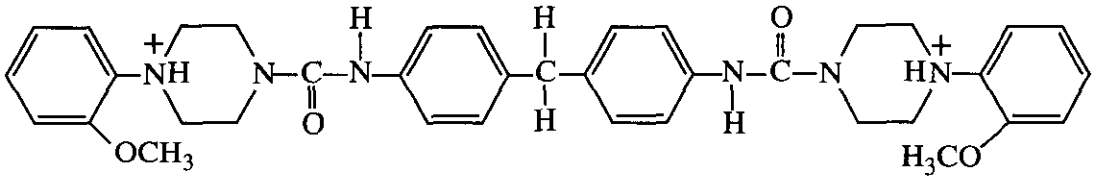
analysis possible without reconstituting any samples and allowing the use of 4-chloroaniline as an internal standard. As these samples had been stored for over a year quantification was not attempted at this time, results only being used for evaluation purposes.

2. (10) CE/ES of MDI and TDI diisocyanates.

Various other peaks were observed in the chromatograms of the "real" samples and although two of these were thought to be due to the TDI isomers this could not be confirmed absolutely using CE alone so CE/ES was investigated as a possible solution. Both MDI and TDI standards were subjected to CE/ES. When MDI was analysed alone it displayed two significant ions under electrospray, the singly charged $[M + H]^+$ ion at m/z 635 and doubly charged $[M + 2H]^{2+}$ ion at m/z 318 illustrated in Figure 108 (App. 8.)



Singly charged species *m/z* 635



Doubly charged species *m/z* 318

The doubly charged ion was of greater abundance than the singly charged ion and this was used for assessing detection limit. A signal three times the background level was obtained using the m/z 318 ion when just 3 femtomoles were injected into the capillary and this appeared to be the detection limit using this substance. A mixture of the three diisocyanates was then analysed and gave electrospray ions during CE/ES. TDI isomers were separated and each gave singly and doubly charged ions at m/z 559 and m/z 280 respectively, analogous to those for MDI. Again this technique was proven to be useful for the positive identification of the analytes of interest. The next stage in these investigations would be to improve the separation of real samples during CE/ES and to identify the unknown peaks within them.

3. Conclusions.

Detection of diisocyanates by CE was proved to be a viable alternative to HPLC in terms of improved separation efficiency. With further possibilities for improving detection limits still waiting to be explored CE should also at least match HPLC detection limit performance. Whilst HPLC consumes sample volumes of 10 µl per analysis, CE can require as little as 1µl for injection purposes. This allows a pre-concentration step to improve sensitivity of the technique for example with samples 80697, 80708 and 80712 which could have been further concentrated. There is also the possibility that the diisocyanates could be derivatised with fluorescent reagents, either those already investigated or one developed especially for the purpose. The CE analysis could then be performed using lamp or laser-induced fluorescence detection. This could produce a 100 fold improvement in the limits of detection. The potential of CE/ES in this area also provides a more specific detection mechanism which will also allow identification of all the analytes within diisocyanate samples.

Questions to be answered before further progress can be made must be that of the different absorbance characteristics observed between the 2,4- and 2,6-TDI at 254 nm and the systematic error observed during quantification. One area that could be addressed is to ensure that the same solvent is used throughout the procedure i.e. from the preparation to CE analysis. Currently both toluene and methanol are used to dissolve samples whereas the CE method detailed in this thesis utilized acetonitrile. By exploiting the same solvent throughout, possible errors during reconstitution could be reduced as could any other effect due to foreign solvents. An improved internal standard can be easily envisaged for the future. The work done so far was sufficiently promising for the HSE to sponsor a further studentship in this area.

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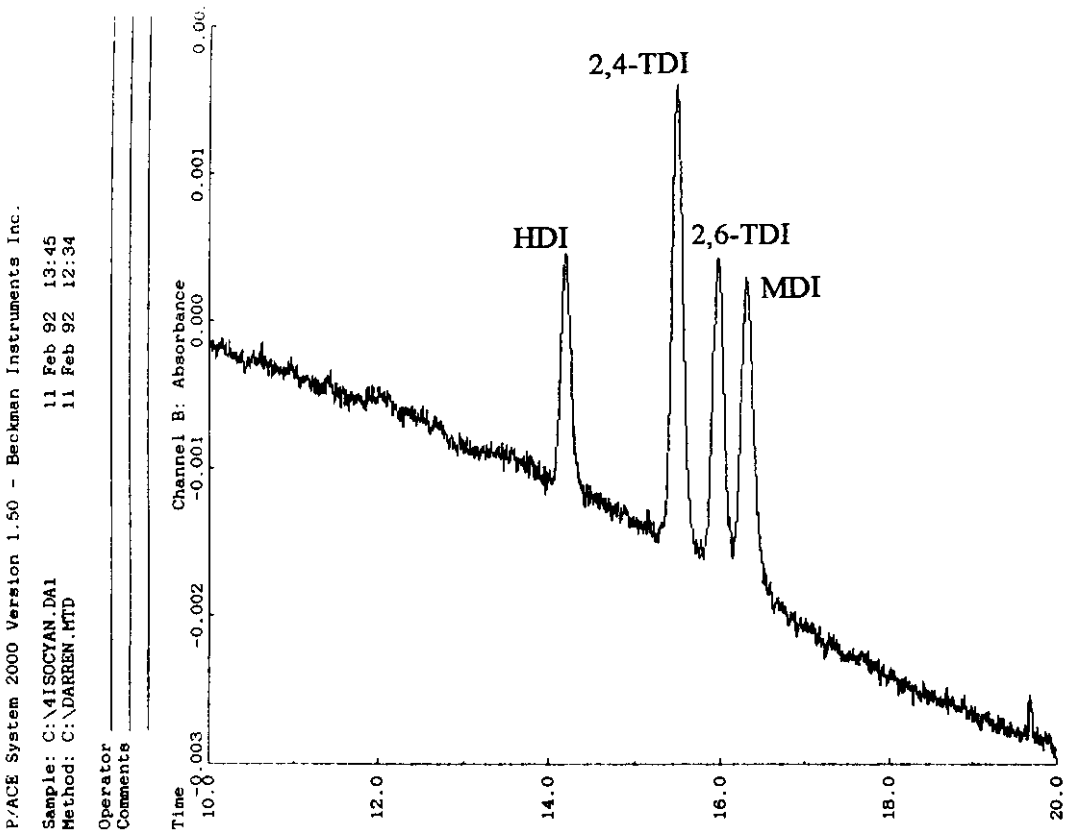
Appendix 8

Figures from Chapter 8.

Figure 96. Capillary electrophoresis chromatogram of the separation of 4 MPP derivatised diisocyanates, 2,4-TDI, 2,6-TDI, MDI and HDI.



Buffer: 300 mM phosphoric acid at pH 3.2 with 0.1M NaOH, 40% acetonitrile. Capillary: 50 cm x 50 µm i.d. SGE coated capillary. Separation: 15 kV, 25°C, detector uv absorbance at 200 nm.

Figure 97. Example of a chromatogram obtained in the HPLC analysis of MPP derivatives of 2,4-TDI, 2,6-TDI and phenyl isocyanate.

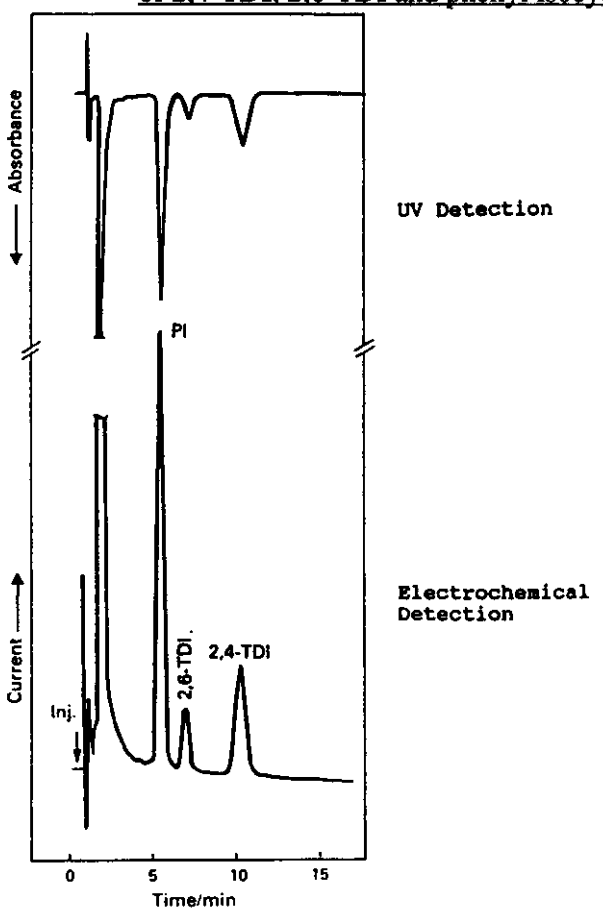
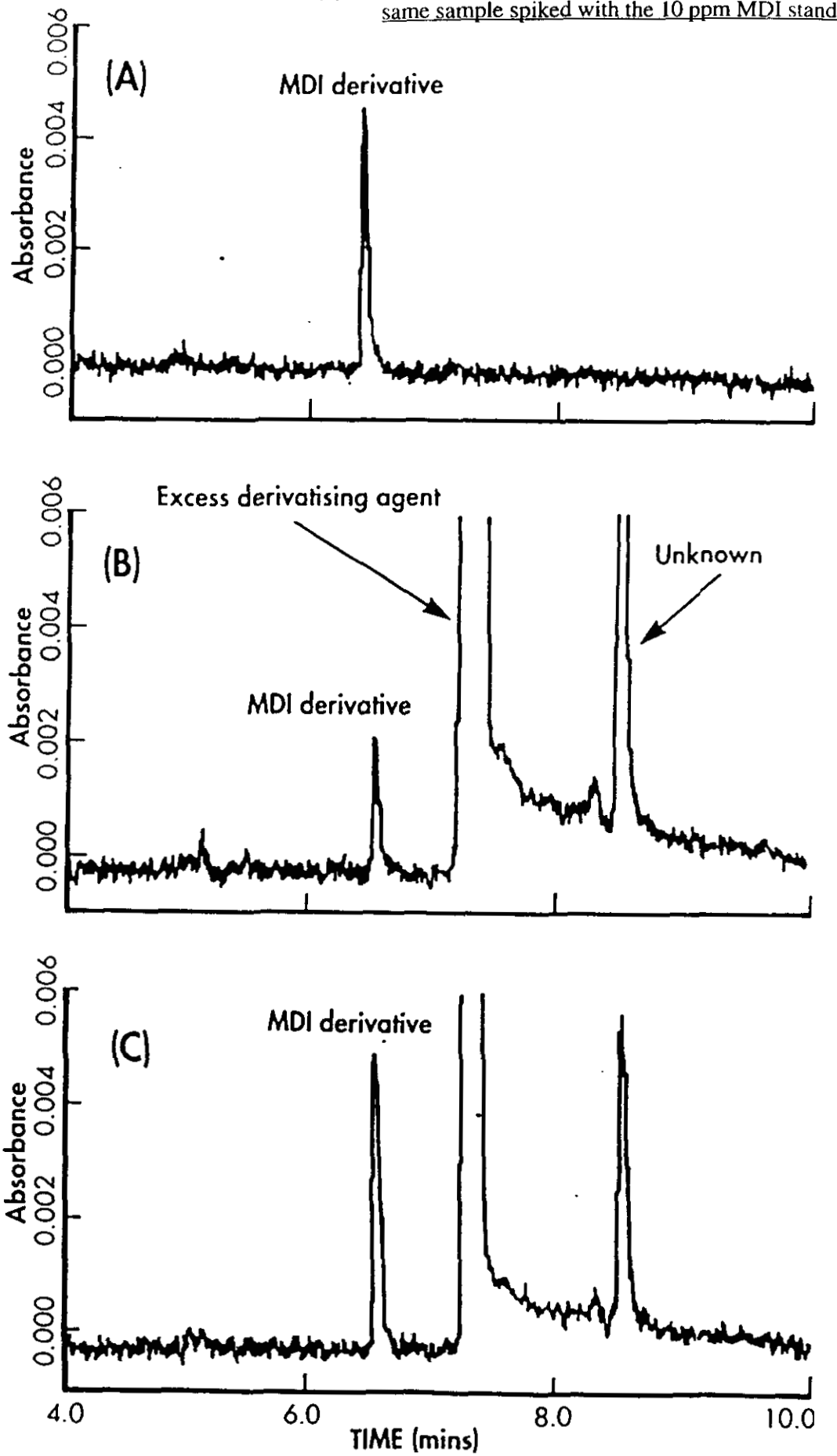


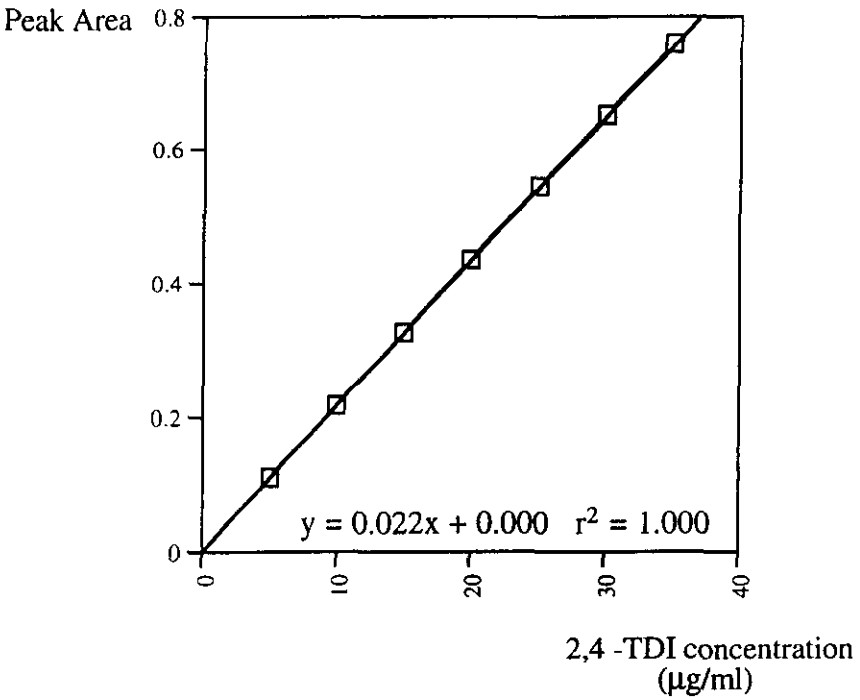
Figure 98(a - c). Capillary electrophoresis chromatograms of (a) 10 ppm MDI standard, (b) an industrial sample suspected of containing MDI and (c) the same sample spiked with the 10 ppm MDI standard.



Buffer: 30 mM phosphate at pH 3.0, 30% acetonitrile. Capillary: 50 cm x 50 μm i.d. SGE coated capillary, separation at 25 kV, 25°C, detector u.v. absorbance at 200 nm.

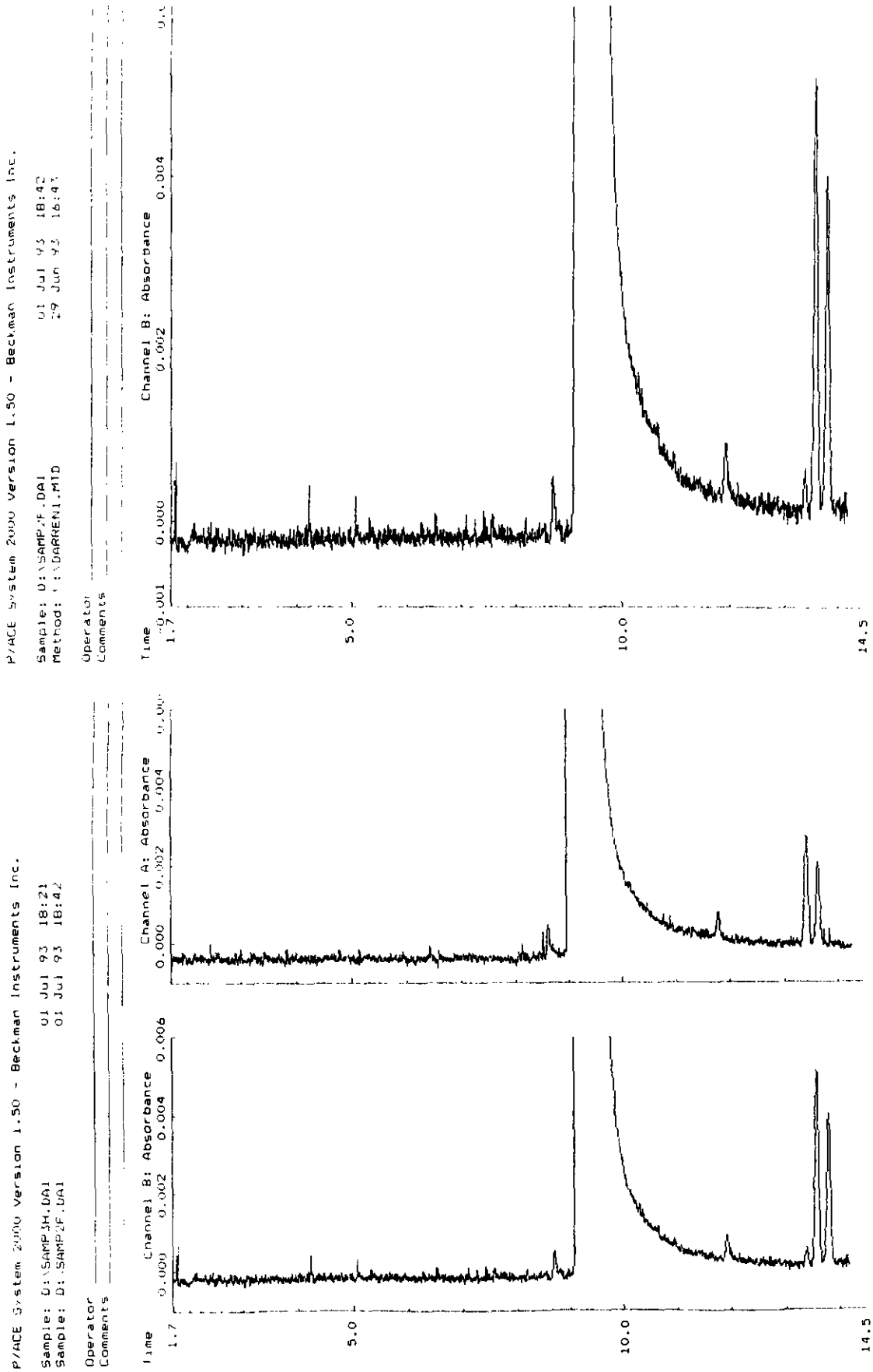
Figure 99. Calibration curve of 2,4,-TDI analysis.

2,4-TDI calibration curve of standards between 35 and 5 µg/ml.



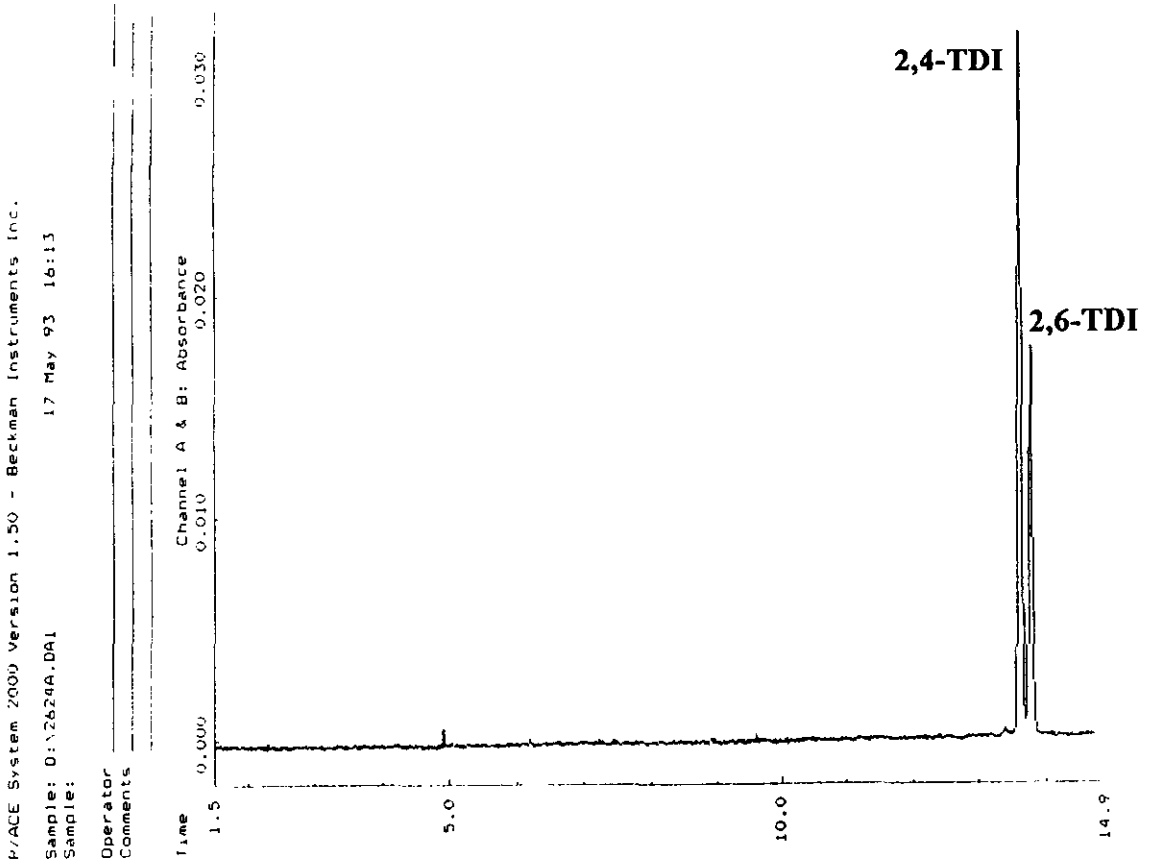
Buffer: 30 mM phosphate at pH 3.0, 30% acetonitrile. Capillary: 50 cm x 50 µm i.d. SGE coated capillary. Separation: 25 kV, 25°C, detector uv absorbance at 200 nm.

Figure 100. Examples of the CE analysis of quality control samples used in the blind" analysis studies.



Buffer: 30 mM phosphate at pH 3.0, 30% acetonitrile. Capillary: 50 cm x 50 µm i.d. SGE coated capillary. Separation: 25 kV, 25°C, detector uv absorbance at 200 nm.

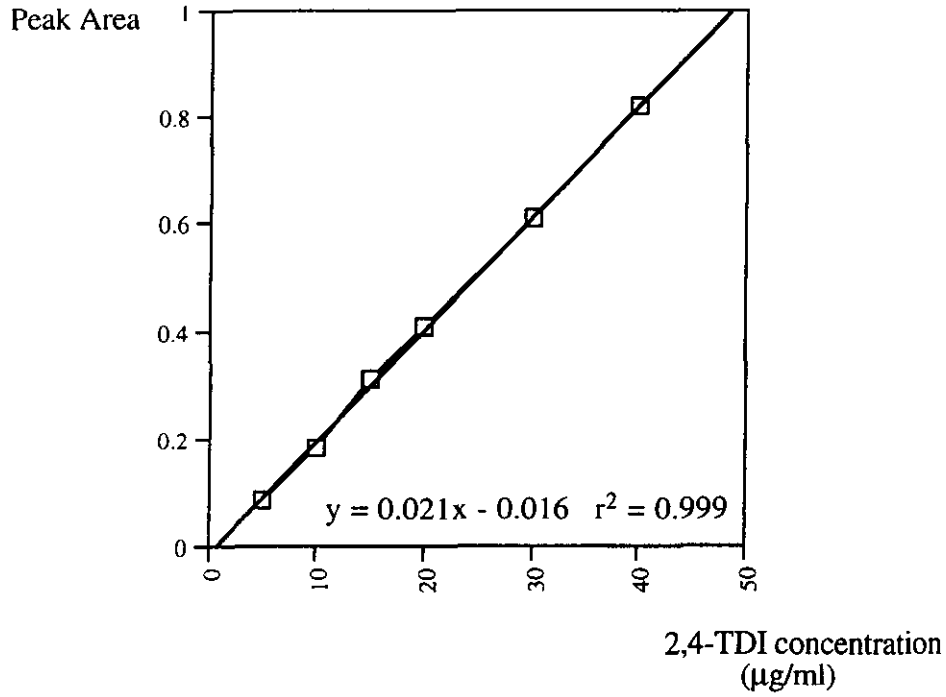
Figure 101. Capillary electrophoresis chromatogram of the separation of a mixture of 2,4- and 2,6-TDI isomers.



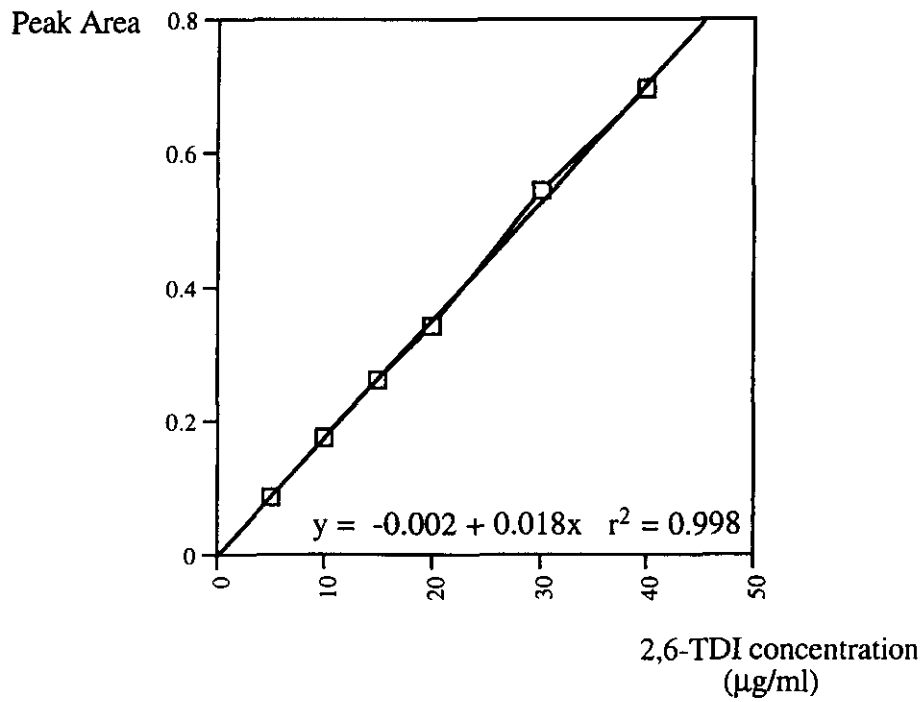
Buffer: 30 mM phosphate at pH 3.0, 30% acetonitrile. Capillary: 50 cm x 50 μm i.d. SGE coated capillary. Separation: 25 kV, 25°C, detector uv absorbance at 200 nm.

Figure 102. Calibration curves of 2,4 - and 2,6 - TDI obtained from the analysis of a mixture of both.

2,4-TDI calibration curve of standards between 40 and 5 ug/ml.

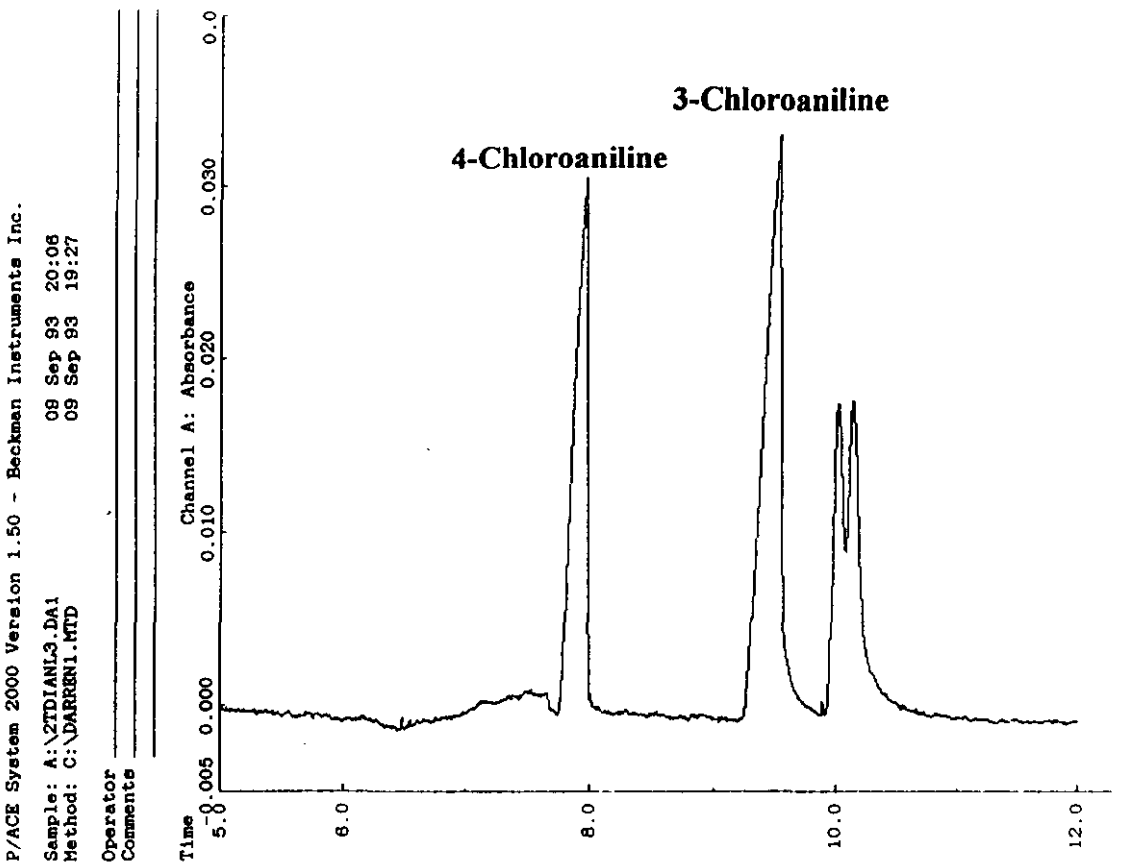


2,6-TDI calibration curve of standards between 40 and 5 ug/ml.



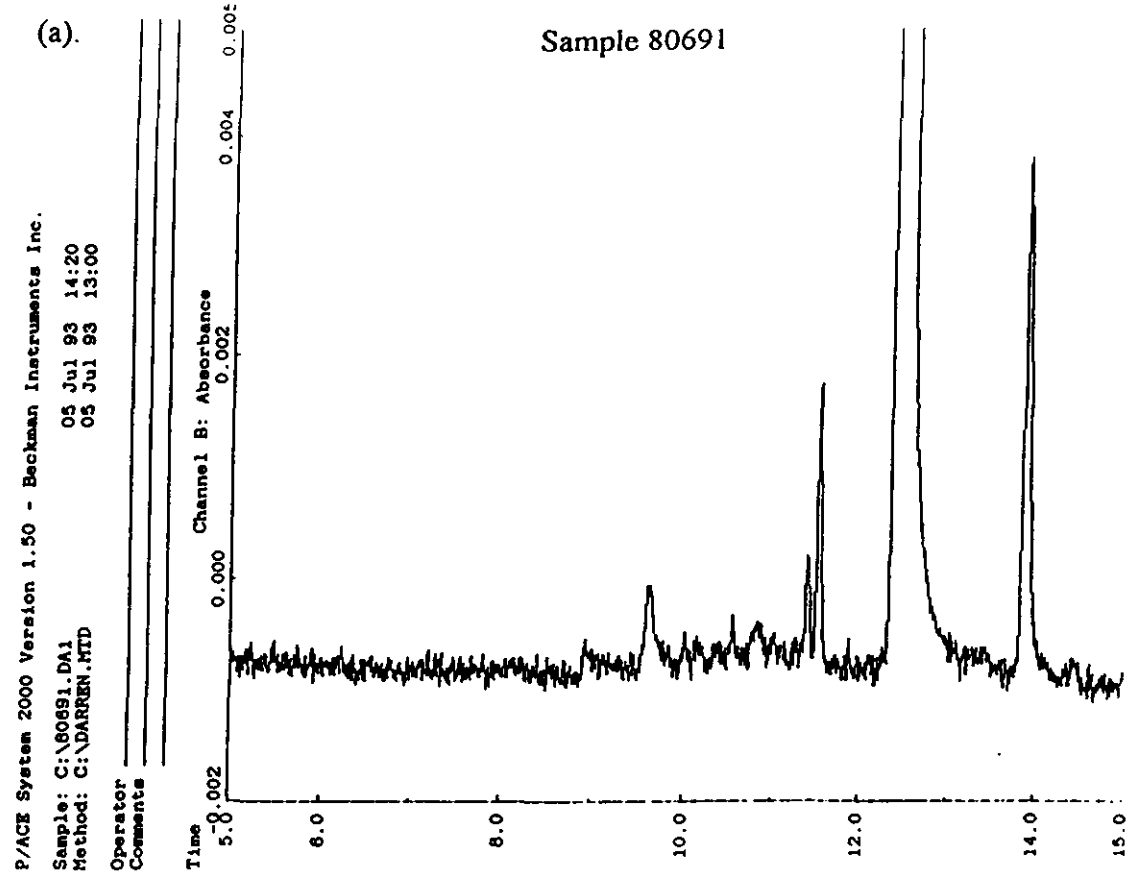
Buffer: 30 mM phosphate at pH 3.0, 30% acetonitrile. Capillary: 50 cm x 50 µm i.d. SGE coated capillary. Separation: 25 kV, 25°C, detector uv absorbance at 200 nm.

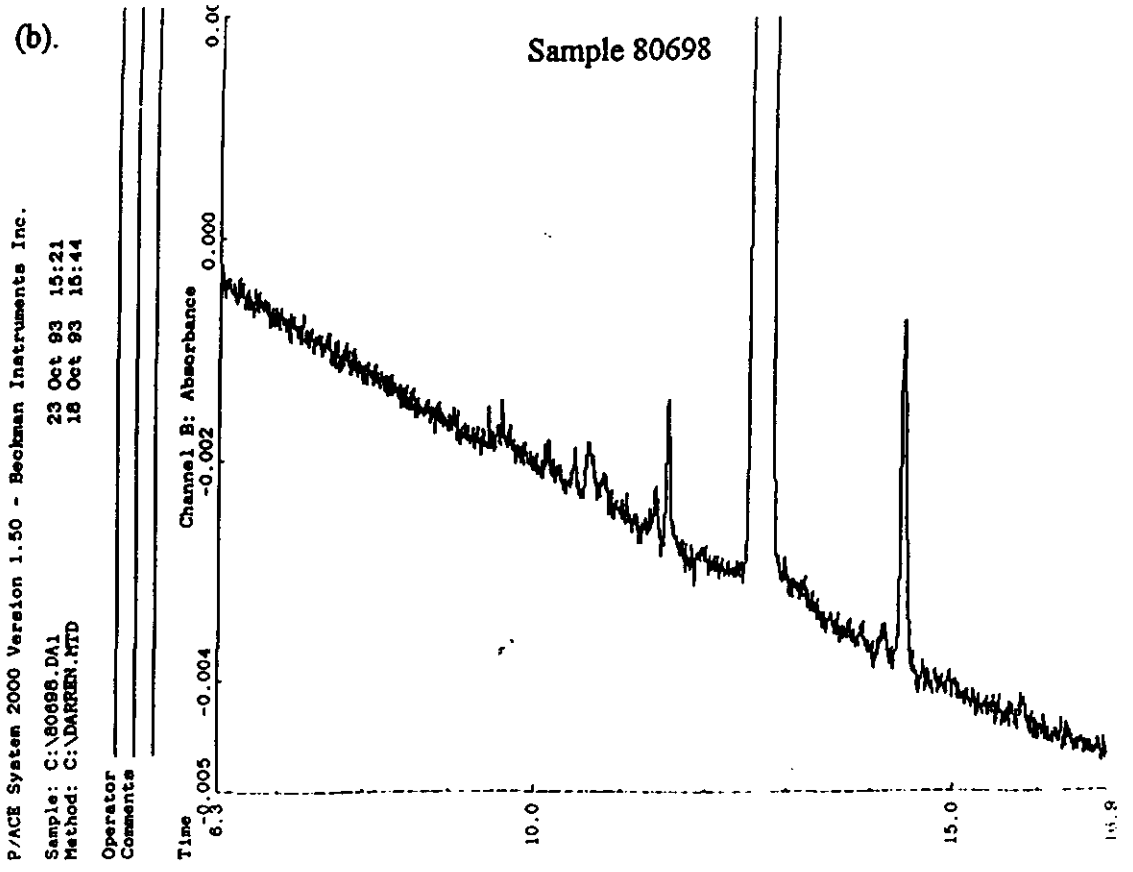
Figure 104. Chromatogram to show the relative position of 3- and 4-chloroaniline to that of the two TDI isomers when used as internal standards.



Buffer: 30 mM phosphate at pH 3.0, 25% acetonitrile. Capillary: 50 cm x 50 μm i.d. SGE coated capillary. Separation: 25 kV, 25°C, detector uv absorbance at 200 nm.

Figure 105. (a to c) Example chromatograms from the CE analysis of real isocyanate containing samples obtained from an industrial atmosphere.





Buffer: 30 mM phosphate at pH 3.0, 30% acetonitrile. Capillary: 50 cm x 50 µm i.d. SGE coated capillary. Separation: 25 kV, 25°C, detector uv absorbance at 200 nm.

(c).

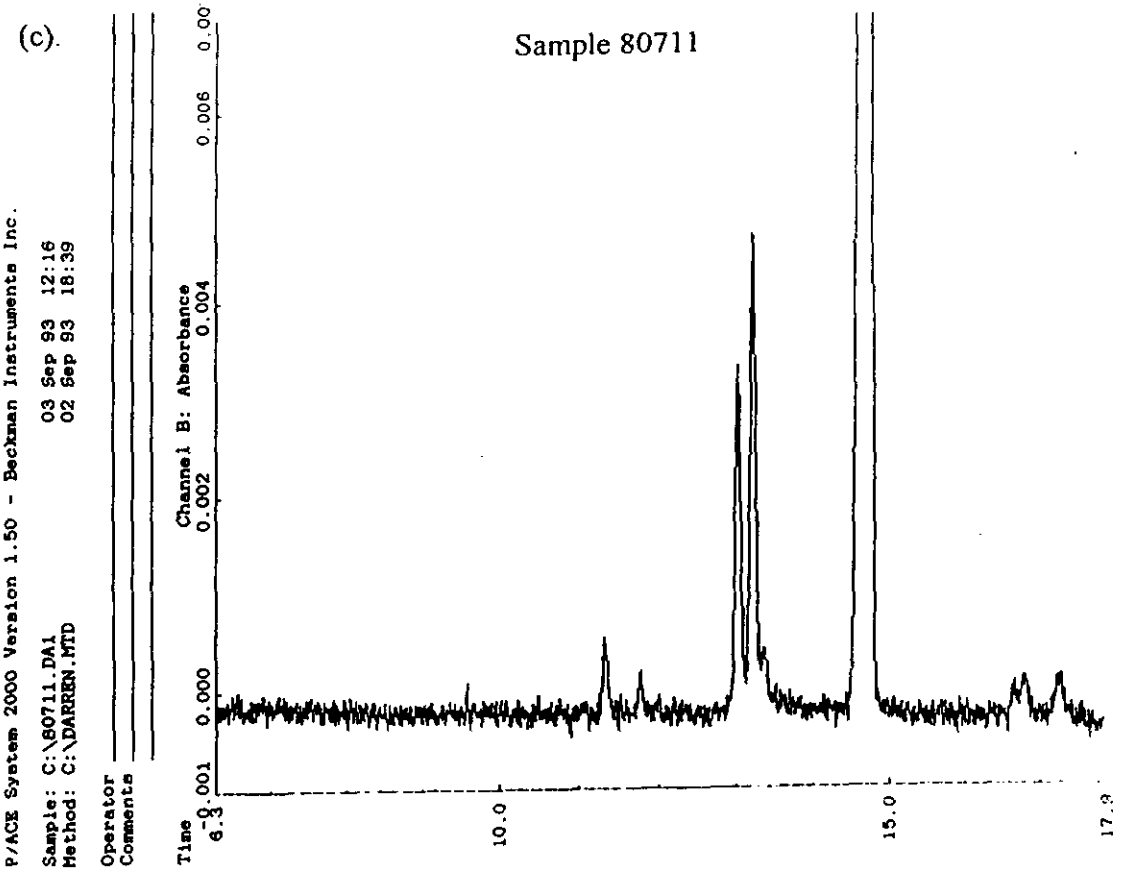
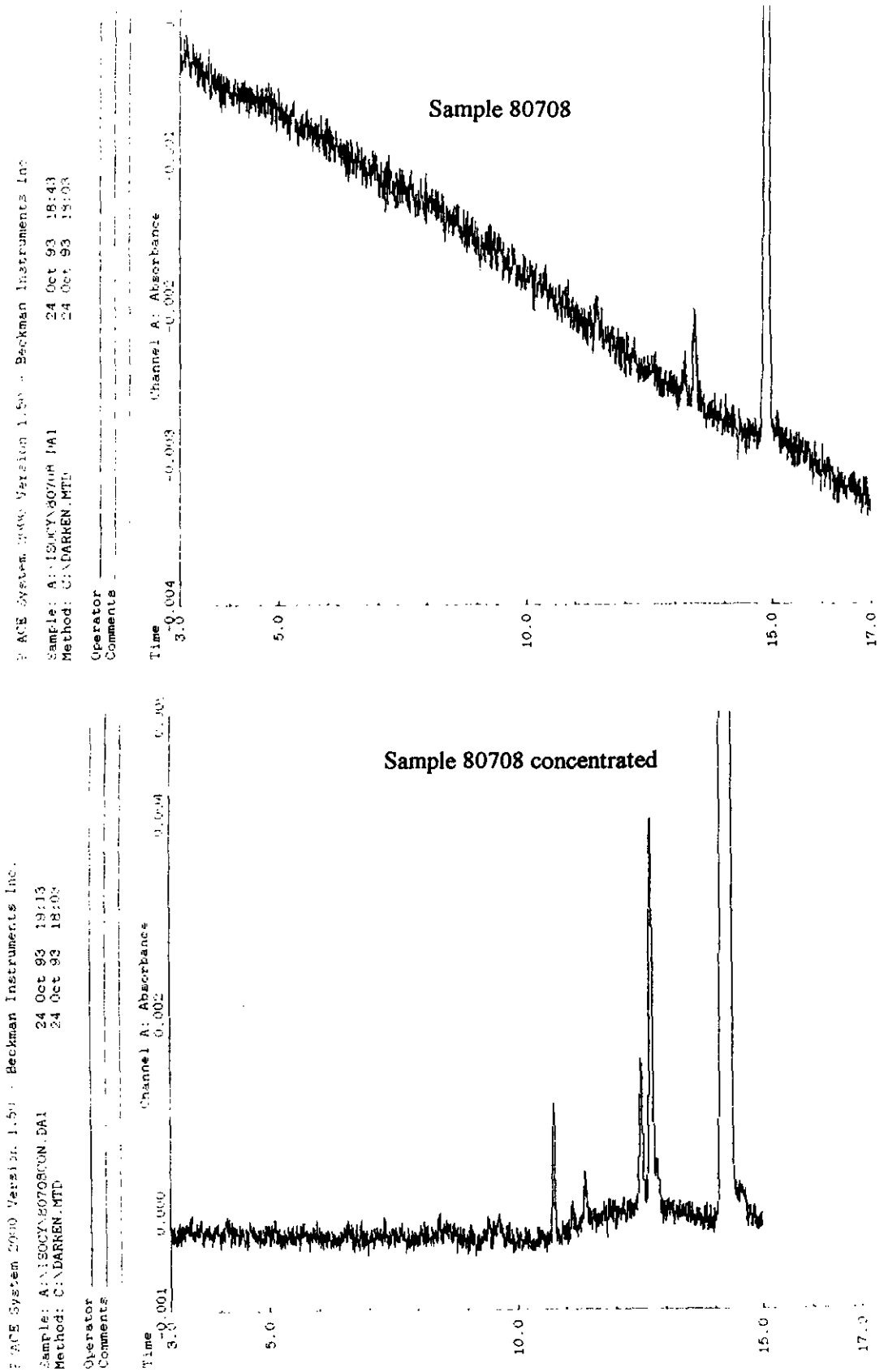
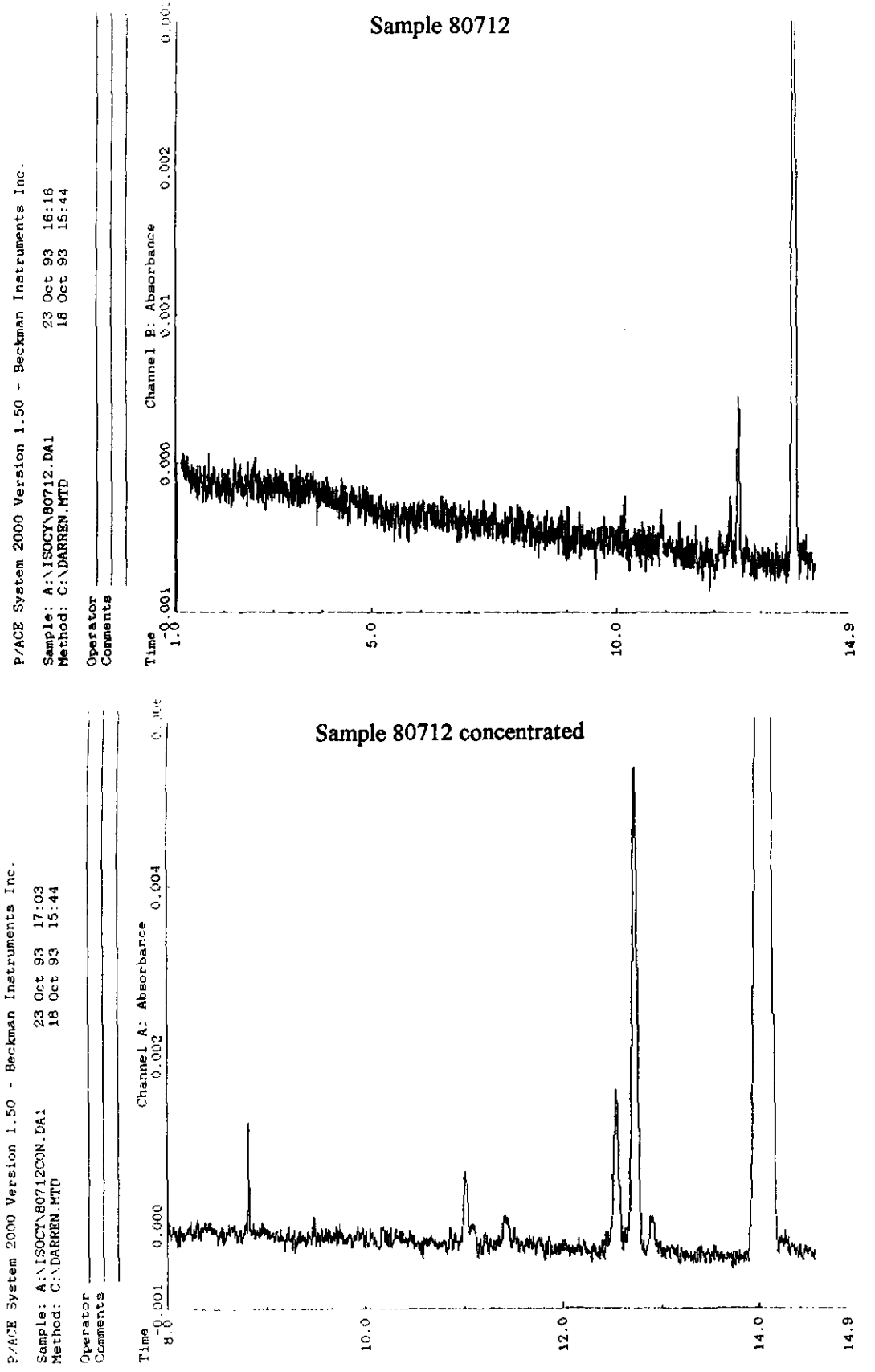

Sample 80711
P/ACE System 2000 Version 1.50 - Beckman Instruments Inc.
Sample: C:\80711.DA1
Method: C:\DARREN.MTD
03 Sep 93 12:16
02 Sep 93 18:39
Operator
Comments
Channel B: Absorbance
0.006
0.004
0.002
0.000
-0.001
Time
6.3
10.0
15.0
17.9

Figure 106 (a). CE chromatograms illustrating the effect of concentration of industrial sample before analysis.



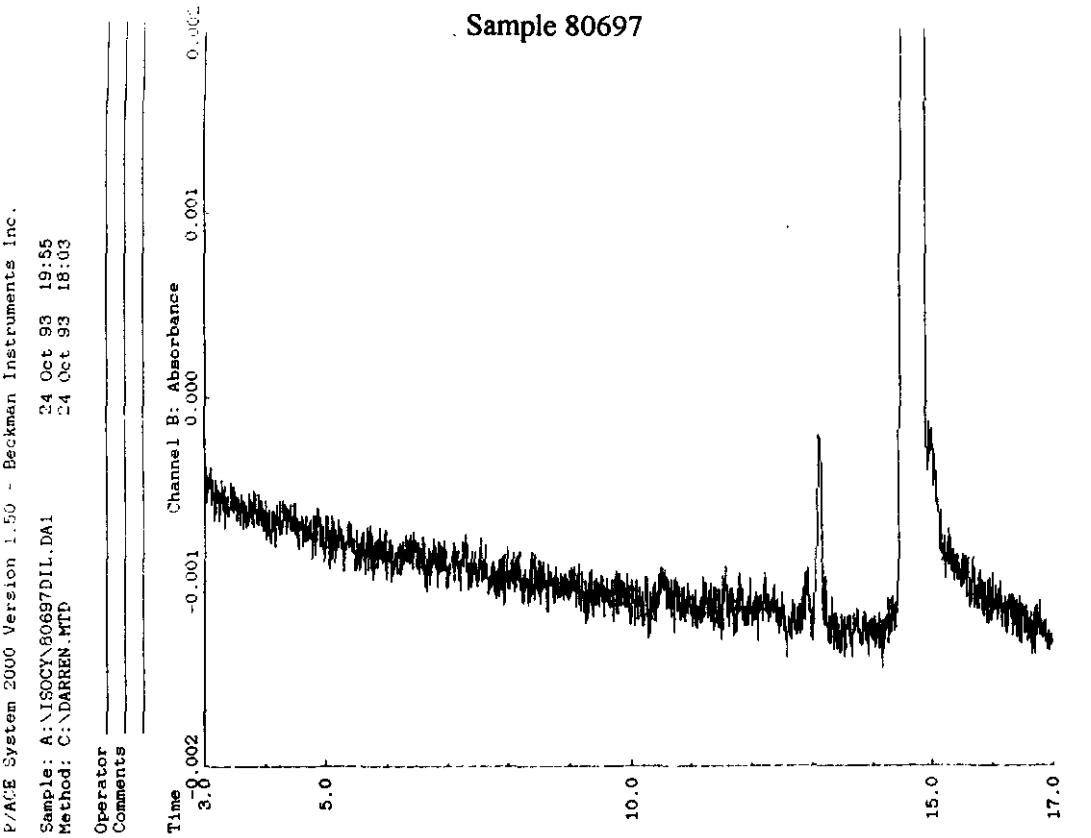
Buffer: 30 mM phosphate at pH 3.0, 25% acetonitrile. Capillary: 50 cm x 50 µm i.d. SGE coated capillary. Separation: 25 kV, 25°C, detector uv absorbance at 200 nm.

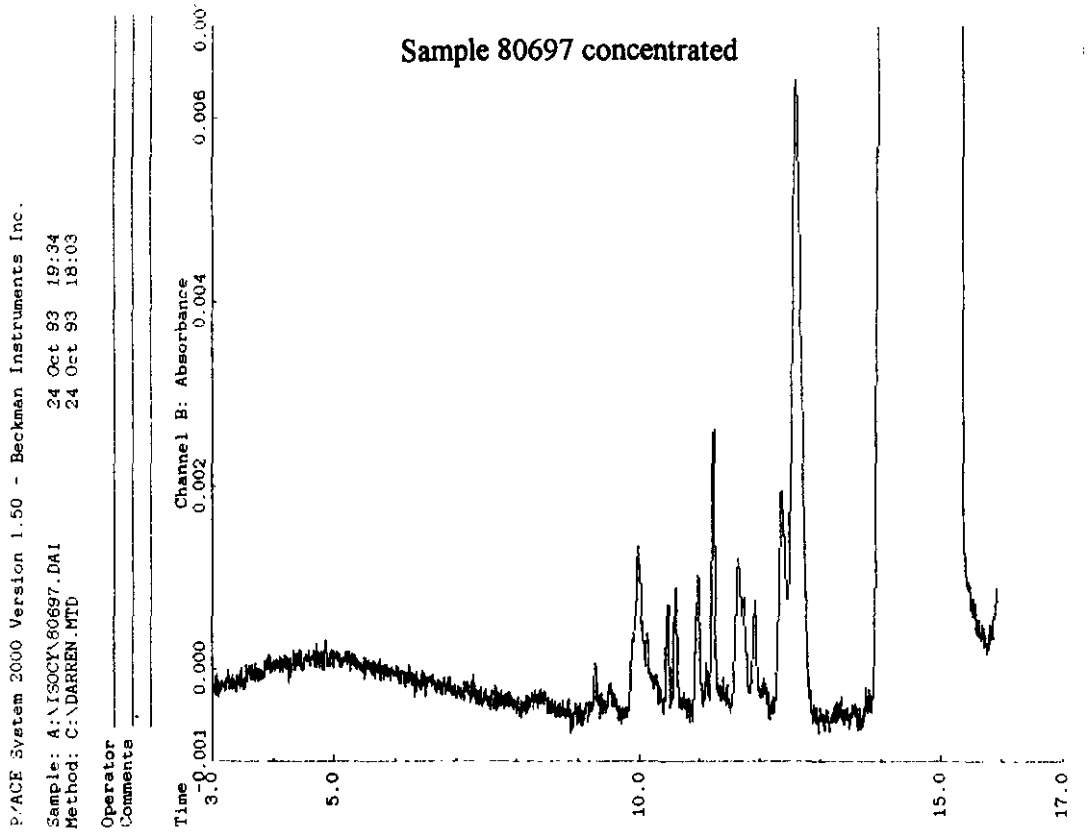
Figure 106(b). CE chromatograms illustrating the effect of concentration of industrial sample before analysis.



Buffer: 30 mM phosphate at pH 3.0, 30% acetonitrile. Capillary: 50 cm x 50 µm i.d. SGE coated capillary. Separation: 25 kV, 25°C, detector uv absorbance at 200 nm.

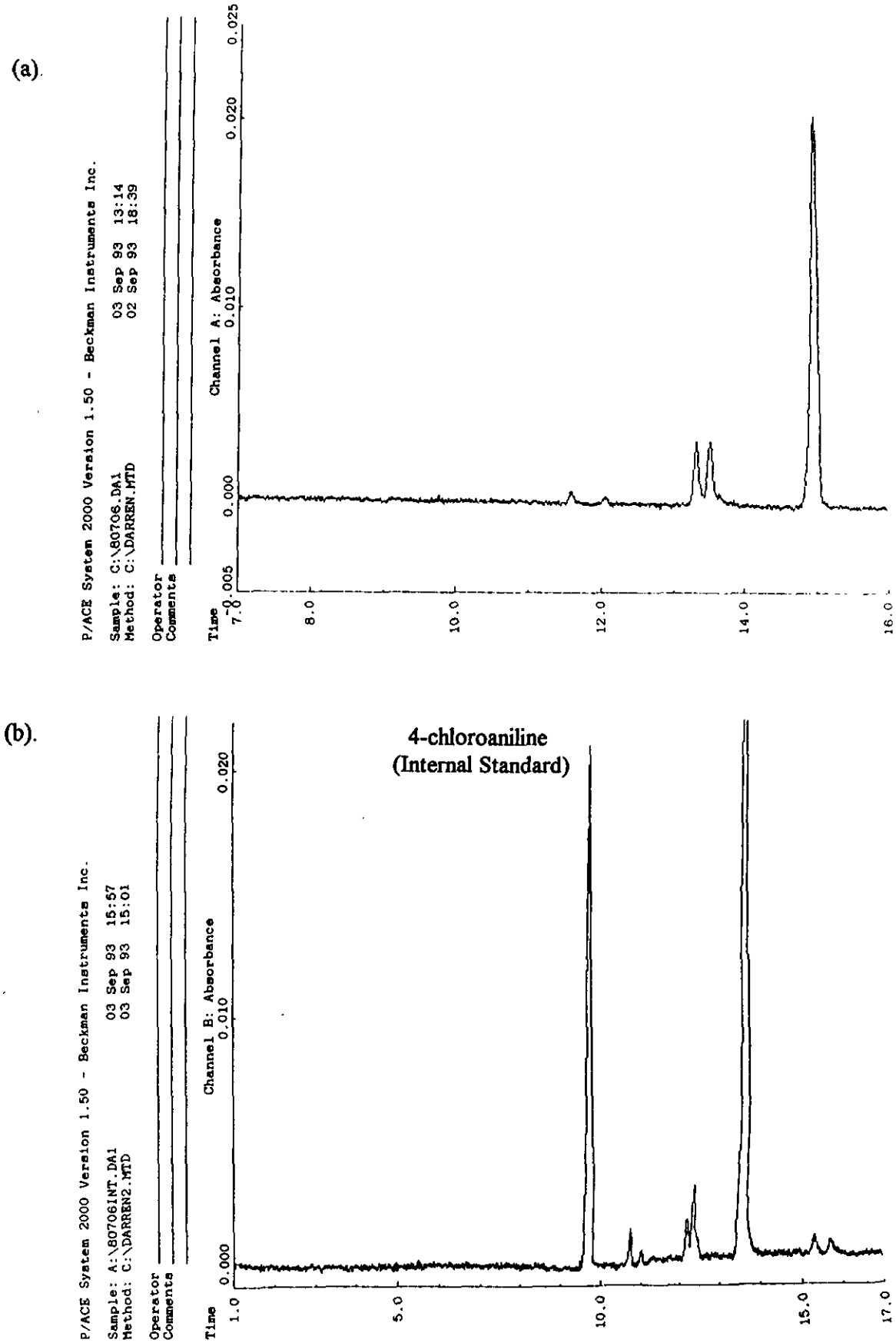
Figure 106(c). CE chromatograms illustrating the effect of concentration of industrial sample before analysis.





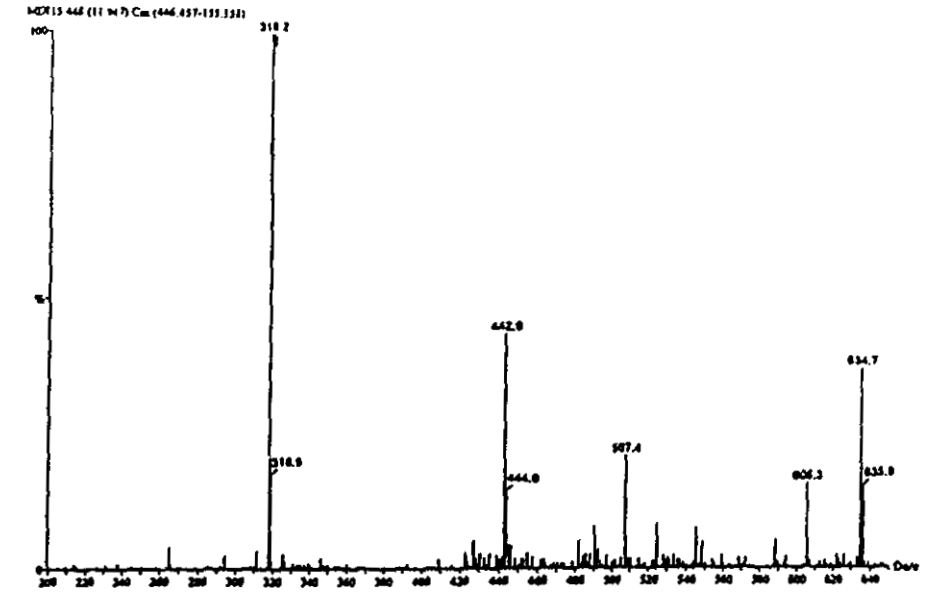
Buffer: 30 mM phosphate at pH 3.0, 30% acetonitrile. Capillary: 50 cm x 50 µm i.d. SGE coated capillary. Separation: 25 kV, 25°C, detector uv absorbance at 200 nm.

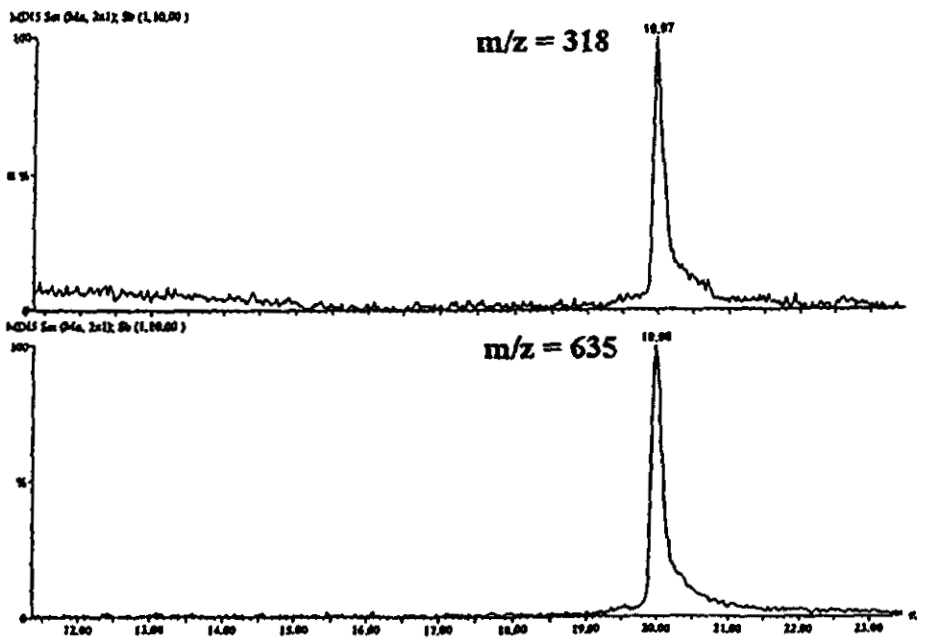
Figure 107. CE chromatograms of a real industrial sample (a) without internal standard and (b) with internal standard added.



Buffer: 30 mM phosphate at pH 3.0, 30% acetonitrile. Capillary: 50 cm x 50 µm i.d. SGE coated capillary. Separation: 25 kV, 25°C, detector uv absorbance at 200 nm.

Figure 108. Electrospray mass spectrum of MDI illustrating the two major ions formed using this technique.





Buffer: 30 mM phosphate at pH 3.0, 30% acetonitrile. Capillary: 90 cm x 75 µm i.d. Separation at 21 kV, 25°C, detector: electrospray mass spectrometer.