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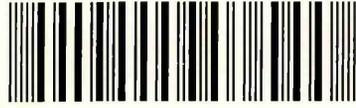
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# Development and Application of Capillary Electrochromatography using Modular Instrumentation.

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Thesis submitted in partial fulfilment of the requirements of Sheffield Hallam University for the degree of Doctor of Philosophy.

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# Acknowledgements

# Abstract.

Electrophoretic separations have been demonstrated for over a century resulting in methods being devised to separate a variety of compounds, mainly of biological origin. Only in the past twenty-five years has capillary electrophoresis (CE) emerged as a viable technique, with a variety of different separation methods being reported. One draw back of CE is its inability to separate neutral compounds, hence alternative methods have been developed to facilitate this.

This study investigated Capillary Electrochromatography (CEC), one of the techniques that can be used to separate neutral compounds, in which a capillary column is packed with a stationary phase designed for liquid chromatography. Separation is determined by interactions between the solutes and the stationary phase, with the flow being driven by electroosmosis.

Initial work involved the development of an in-house packing method for CEC columns. The method developed, which was a pressure driven system using a Shandon HPLC packer, proved to be successful. The reliability of the retaining frit and the nature of the packing material were major factors in column performance,

Once the column fabrication process had been developed, the experimental conditions for CEC in the Prince Technology CE instrument were optimised. The results showed that in many respects the system responded as a traditional LC system would, with changes in buffer compositions, stationary phase and, in this case, EOF etc. all producing definite and reproducible changes in the separation of the test mixture. Variations in sample loading technique were investigated and a simple method developed to improve the peak efficiencies and resolution of analytes, by focussing them on the head of the column.

Once the experimental conditions were established, a series of applications were undertaken with differing results. The applications included studies of a series of polyaromatic Hydrocarbons (PAH), a test mixture of small molecules, with different polarities, prostaglandins and nicotine metabolites. Separation of the PAH and test mixtures were successful and corroborated some of the observations made while studying the experimental conditions for CEC. Separation of the prostaglandin mixture was unsuccessful; this was not totally unexpected due to the similarity in their structures. Study of the nicotine metabolites allowed a comparison of CZE with CEC, however due to limited availability sample the work was not fully completed. Despite this, the study did indicate that both methods showed promise, but required further development

# Contents.

# Glossary

| | |
|---|---|
| $\mu_{ep}$ | Electrophoretic mobility |
| $\nu_{ep}$ | Electrophoretic velocity (mm s$^{-1}$) |
| $\nu_{EOF}$ | Electroosmotic velocity (cm s$^{-1}$) |
| $\mu_{EOF}$ | Electroosmotic mobility (cm$^{-2}$ V$^{-1}$ s$^{-1}$) |
| L | Total length of column (cm) |
| l | Effective capillary length, distance to detector (cm) |
| V | Applied voltage (V) |
| $\varepsilon_0$ | Permittivity of vacuum (8.85x10$^{-12}$ C$^2$ N$^{-1}$ $\mu^{-2}$) |
| $\varepsilon_r$ | Dielectric constant of the mobile phase |
| $\xi$ | Zeta potential (mV) |
| $\eta$ | Viscosity of the mobile phase ((water) 0.089 g cm$^{-1}$ s$^{-1}$ at 20°C) |
| E | Electric field strength (V cm$^{-1}$) |
| $\sigma$ | Charge density at the surface of the shear |
| R | Gas constant (8.315 J K$^{-1}$ mol$^{-1}$) |
| T | Temperature (K) |
| F | Faraday constant (9.65x10$^4$ C mol$^{-1}$) |
| q | Charge of particle |
| r | Stoke's radius of particle ($\mu$m) |
| c | Electrolyte concentration (mol L$^{-1}$) |
| $\delta$ | Thickness of the double layer (nm) |
| e | Charge per unit surface area |
| G | Gravitational constant (6.67 × 10$^{-11}$ Nm$^2$ Kg$^{-2}$) |
| K | Conductance ($\Omega^{-1}$) |

$\Lambda$    Molar conductance ($\Omega^{-1}\,m^2\,mol^{-1}$)

$R$    Resistance ($\Omega$ or $V\,A^{-1}$)

$I$    Current (A)

$W$    Watts ($J\,s^{-1}$)

# CHAPTER 1

## 1.1 Introduction.

Electrophoretic separations have been demonstrated for over a century (1). However, it was not until the work of Tiselius (2) in the 1930s, for which he obtained the 1948 Nobel Prize, that interest in electrophoretic separation began to gain momentum. Tiselius had developed moving boundary electrophoresis in free solution, thus allowing the separation of proteins in complex biomedical samples that by normal methods would be unresolvable.

One of the problems observed in electrophoretic separations was that of diffusion arising from convection in the bulk solution due to Joule heat, which reduced the efficiency of the separation. Therefore, support media were developed to reduce convection in the columns. Materials employed included cellulose powder, grains of starch or plastic, glass wool and various gels such as silica, agar, agarose, starch and polyacrylamide (3). Some of these are still routinely used today in many biomedical laboratories, in the form of slab gel electrophoresis, with the most commonly used support being that of a polyacrylamide gel. However, there are problems associated with the use of these support phases, principally that of adsorptive and steric interference which hinder reproducibility and sensitivity.

Another method that was developed to reduce unwanted convection movement was that of rotating the column during the separation. Kolin first described this in 1954 (4). It was not until the mid 1960s that Hjerten et al. (3, 5) reported the use of narrow bore columns (3mm ID).

In 1974 Virtenen (6) described the potential advantages of using capillary columns instead of the larger bore columns that had been used up to that date. This theory was later proven by Mikkers *et al.* in 1979 (7), who described electrophoresis in a 200 μm ID PTFE capillary. The initial results were poor as the sample was overloaded to overcome the poor detector sensitivity.

Jorgenson and Lukacs, in 1981 (8,9), were the first to demonstrate the true potential of Capillary Electrophoresis (CE). They used fused silica capillaries similar to those employed in GC, showing that high efficiencies could be obtained using columns with an internal diameter of less than 100μm.

Throughout the 1980s the development of CE was limited. However, towards the end of this decade interest in CE increased, which in part may be explained by the introduction of commercially available instruments. The availability of such instruments allowed CE to become more accessible to a wider range of groups. Prior to this CE had been developed on home-made instruments.

Since the initial demonstration of free solution electrophoresis as a viable analytical technique by Jorgenson and Lukacs, various methods have been developed to allow a greater range of solutes to be separated (Table 1). Many of the principles had been developed in the 1950s and 60s with the introduction of slab gel electrophoresis.

| Mode | Separation mechanisms |
|---|---|
| Capillary Zone Electrophoresis (CZE) | Size to charge ratio and shape. |
| Capillary Isoelectric Focusing (CIF) | Isoelectric point (pI) of solutes |
| Capillary Isotachophoresis | Mobility of solutes. |
| Capillary Gel Electrophoresis (CGE) | Size to charge ratio. Size. |
| Micellar ElectroKinetic Capillary Chromatography (MEKC) | Size to charge ratio. Partitioning between micelle and bulk solution. |
| Capillary ElectroChromatography (CEC) | Size to charge ratio. Partitioning between stationary phase and bulk solution. |

Table 1. Various Modes of Capillary Electrophoresis.

The majority of the separations result from differences in the size to charge ratio of the solutes, and hence differences in electrophoretic mobility, allowing them to be separated into discrete bands. The one potential disadvantage is that neutral species have no electrophoretic mobility and are consequently drawn through the column with the bulk flow without any separation. This resulted in new methods being developed to overcome the problem, for example MEKC and CEC. However, there have been other methods

developed and these will be discussed briefly to give an overview of possible

methods which can be employed.

## 1.2. Theory

### 1.2.1. Electroosmotic Flow.

The Electroosmotic Flow (EOF) leads to the bulk movement of solution through the capillary and is generated by a surface charge on the capillary wall. This is produced by ionisation at the inner wall of the capillary and/or the adsorption of ions onto the inner surface (10).

Capillary columns used in CE are made from fused silica and the inner surface is covered by silanol groups ( -Si-OH). The $pK_a$ of the silanol group is approximately 2.2 and dissociation occurs to give silanoate groups ( -Si-O⁻). Above pH 7-8, the silanol groups are totally dissociated.

When an electrolyte solution is introduced into the capillary, the surface becomes coated by counter ions, i.e. cations, to form two distinct layers at the capillary wall (Figure 1). The first layer is bound tightly to the surface and is referred to as the Stern or fixed layer. The second layer is formed due to the fixed layer being unable to completely neutralise the wall's charge. In this region the cations are more diffuse and can move between this layer and the bulk solution. This region is the Gouy-Chapman or mobile layer. At the interface of the two regions there is an electrical imbalance between the layers, referred to as the plane of shear.
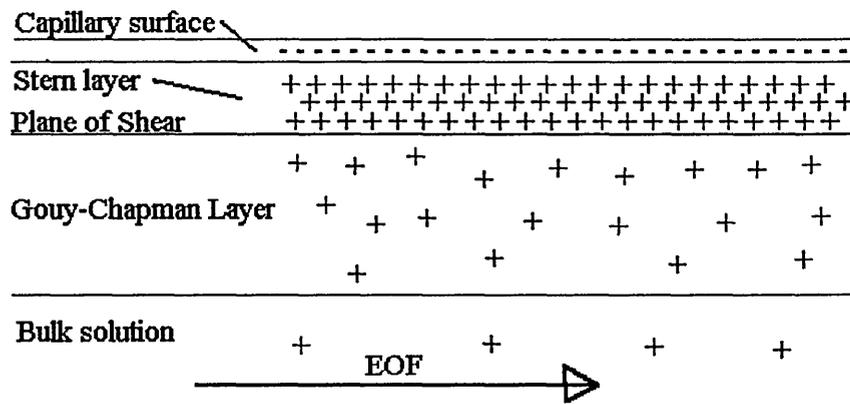
Figure 1. Electrical double layer at the surface of a capillary wall.

The magnitude of the flow is in part dependent on the electophoretic mobility of the electrolyte and also the formation of the double layer at the capillary surface, which can exert an additional force on the observed electroosmotic flow.

This is the result of the cations in the mobile layer being attracted towards the cathode. The movement of this layer causes bulk solution migration towards the cathode, as the ions in the diffuse region draw the ions in the bulk solution along, creating the electroosmotic flow.

The magnitude of the EOF is controlled by the zeta potential. The zeta potential is described as the potential difference between the fixed and mobile regions at the plane of shear, and can be calculated from:

$$\xi = \frac{4\pi\delta e}{\varepsilon_r}$$

$$\text{eq.} \quad 1.01$$

Where, $\delta$ is the double layer thickness, e is the charge per unit area and $\varepsilon_r$ is the dielectric constant.

The zeta potential is dependent on pH and electrolyte. A higher pH value will mean a greater number of silanoate groups (surface charge) to be present, so increasing pH increases the zeta potential and hence the EOF increases. However changing the electrolyte has only a small effect on the EOF, if all other parameters remain the same.

The thickness of the double layer ($\delta$) is proportional to the zeta potential. Therefore the size of the double layer can also affect the EOF. The double layer has a finite thickness, which falls away exponentially. (Figure 2).
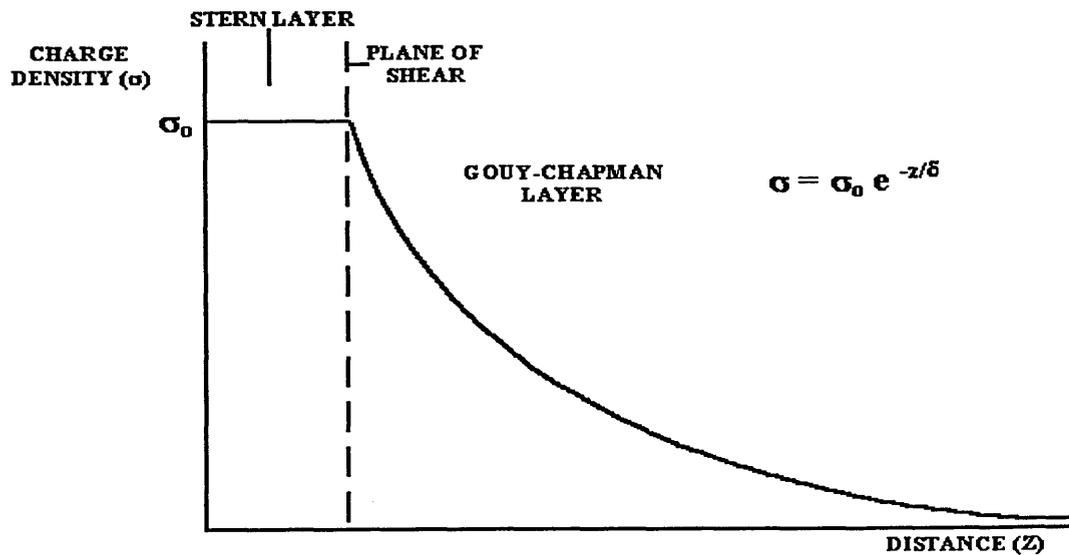


Figure. 2. Diagram showing how the charge density falls on moving away from the capillary wall.

The thickness of the double layer can be calculated by using the equation:

$$\delta = \left[ \frac{\varepsilon_0 \varepsilon_r RT}{2cF} \right]^{0.5}$$

eq. 1.02

Where, $\varepsilon_0$ is the permittivity of a vacuum, $\varepsilon_r$ is the dielectric constant, R is the gas constant, T is the temperature, c is the concentration of the electrolyte and F is the Faraday constant.

Therefore the concentration of the buffer also plays a part in determining the magnitude of the EOF, as well as the pH and type of electrolyte used. As the concentration of the electrolyte is reduced the double layer thickness is increased, thereby increasing the Zeta potential and hence the velocity of the EOF. This increase in EOF is actually dependent on the ionic strength of the electrolyte, so factors other than concentration (e.g. ionic charge and radius) are also important, which the dielectric constant of the electrolyte indicates.

In general, the thickness of the double layer is inversely proportional to the electrolyte concentration e.g. a buffer containing 10mM of electrolyte will give rise to a double layer of approximately 1nm thickness (11). The narrowness of the double layer formed means that, as an approximation, the EOF can be considered as being generated from the surface of the capillary.

## 1.2.2. Electrophoretic Mobility.

When a charged species is placed under the influence of an electric field, it will migrate towards the electrode of the opposite charge at a given rate i.e. its electrophoretic velocity ($v_{ep}$). The rate at which the solute travels through the solution is dependent on its charge and size and is known as its electrophoretic mobility. The electrophoretic mobility of an ion ($\mu_e$) can be defined as

$$\mu_e = \frac{q}{4\pi\eta r} \qquad\qquad \text{eq.} \quad 1.03$$

where, q is the charge on the ion, $\eta$ is the viscosity of the bulk solution and r is the radius of the ion.
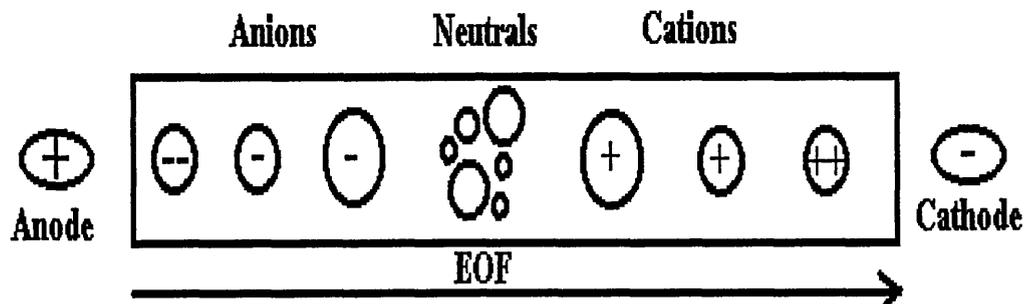


Figure 3. Various migration rates observed in CE.

Figure 3 shows that in CE the mobility of the solute particle is not only determined by its charge, but its size as well. This means that a small particle has a greater electrophoretic mobility than a larger particle of similar charge. Neutral particles cannot be separated, but are drawn through the capillary in a

discrete band at the same rate as the running buffer (as q is equal to zero, hence their electrophoretic mobility is zero).

The electrophoretic velocity of an ion is proportional to the electric field placed across the system. The electrophoretic velocity ($v_{ep}$) can be defined as

$$v_{ep} = \mu_e E \qquad\qquad \text{eq.} \quad 1.04$$

where, E is the electric field strength (E=voltage/length).

When placed in the buffer, individual ions are surrounded by counter ions, which form a double layer around them. Therefore the particles have individual zeta potentials, which are related to their electrophoretic mobilities. This relationship can be seen in the Helmholtz and Smoluchowski equation:

$$\mu_{eff} = \frac{\varepsilon \zeta_{eff}}{4\pi\eta} \qquad\qquad \text{eq. 1.05}$$

where, $\mu_{eff}$ is the effective electrophoretic mobility of the ion, $\varepsilon$ is the dielectric constant and $\zeta_{eff}$ is the effective Zeta potential.

It should be noted that the electroosmotic mobility of the bulk solution can be calculated using equation 1.05.

Equations 1.03 and 1.05 also show that the viscosity of the buffer affects the movement of the analyte in solution. The viscosity affects the movement by

increasing or decreasing frictional drag, depending on the relative viscosity of the solvent being used.

This means therefore that electrophoretic velocity is dependent on the mobility of the particle in the electrolyte used and the size of the electric field that is applied across the capillary. The observed velocity of a particle is thus dependent on two factors, its electrophoretic velocity and the velocity of the EOF, as shown in equation 1.06.

$$v_{obs} = v_e + v_{EOF} \qquad\qquad \text{eq.} \quad 1.06$$

This explains why simultaneous separations of cations and anions are possible. In the normal mode of operation, solutes are loaded at the anode and are detected at the cathode. The velocity of an ion through the column is dependent on its charge and size (Figure 3). Neutral compounds migrate at the same rate as the EOF, as they have no effective charge and are neither attracted nor repulsed by either electrode. The migration rates of cationic and anionic species vary according to their respective charge and size, therefore allowing separations to be achieved by differences in electrophoretic velocities. The velocities of cations are enhanced by the EOF, whilst anions can be drawn through the capillary if the velocity of the EOF is greater than the electrophoretic velocity of the anion.

## 1.2.3. Migration Time.

The linear velocity of the EOF through the capillary can be measured by use of a neutral marker added to the sample solution. Examples of such markers include thiourea, acetone and mesityl oxide. Migration times ($t_m$) may be calculated using equation 1.07.

$$t_m = \frac{l}{v_{obs}} \qquad \text{therefore,} \qquad v_{obs} = \frac{l}{t_m} \qquad \text{eq. 1.07}$$

Where, $l$ is the effective capillary length i.e. distance to the detector.

The migration time of a neutral marker will allow the determination of $v_{obs}$ for the EOF. The electrophoretic velocity of a charged solute can also be determined from this equation by substituting in the migration time. Additionally the observed electrophoretic velocity of an ion can be calculated by equation 1.08

$$v_{obs} = (\mu_e + \mu_{EOF}).E \qquad \text{eq. 1.08}$$

Substituting equation 1.07 into equation 1.08, when E is equal to $V/L$ will give

$$\left( \frac{\mu_e + \mu_{EOF}}{L} \right).V = \frac{l}{t_m} \qquad \text{eq. 1.09}$$

Where, V is the applied voltage and L is the total capillary length.

Rearrangement of eq. 1.09 leads to eqs. 1.10 & 1.11, from which electrophoretic mobility and migration time of solutes may be calculated.

$$\mu_e = \left(\frac{lL}{t_m V}\right) - \mu_{EOF} \qquad \text{eq. 1.10}$$

$$t_m = \frac{lL}{(\mu_e + \mu_{EOF})V} \qquad \text{eq. 1.11}$$

Equation 1.10 indicates that short columns, coupled with a high applied voltage, will give rise to reduced migration times.

## 1.2.4. Selectivity.

The selectivity of a separation can be defined as the degree of separation that is achieved between two consecutive solutes upon detection. Selectivity ($\alpha$), is calculated using equation 1.12. When there is no selectivity $\alpha = 1$, and as selectivity increases, $\alpha$ increases.

$$\alpha = \frac{t_2 - t_{nm}}{t_1 - t_{nm}} \qquad \text{eq. 1.12}$$

Where, $t_1$ and $t_2$ are the migration times of the solutes and $t_{nm}$ is the migration time of a neutral marker.

Selectivity can also be considered as a function of the ratio of the effective electrophoretic velocities of solutes. Electrophoretic velocity, $v_{ep}$, can be calculated using eq. 1.13, which in turn can be used in eq. 1.14 to determine selectivity. It is also possible to derive selectivity from the effective mobility of solutes (see equation 1.15).

$$v_{ep} = \frac{t}{l} - \frac{t_{nm}}{l} \qquad \text{eq. 1.13}$$

$$\alpha = \frac{v_2}{v_1}.Const. \qquad \text{eq. 1.14}$$

$$\alpha = \frac{\mu_2}{\mu_1}.Const. \qquad \text{eq. 1.15}$$

## 1.2.5. Resolution.

Resolution (R) can be defined as the degree of separation that is achieved between two peaks and can be calculated by equation 1.16, which is dependent on the migration times (t) and peak widths (w) of the two solutes.

$$R = \frac{2(t_2 - t_1)}{w_1 + w_2} = \frac{\Delta t}{w_{ave}} \qquad \text{eq. 1.16}$$

where $\Delta t$ is the difference in migration times of the solutes (where $t_1$ and $t_2$ are the migration times of the solutes), and $w_{ave}$ is the average peak width (where $w_1$ and $w_2$ are the peak widths of the solutes).

The peak widths, and hence peak efficiencies, of the solutes have a direct effect on the resolution, as do their electrophoretic mobility. Hence equation 1.17 can be used to determine resolution.

$$R = \frac{1}{4}\left(\frac{\Delta\mu_{app}}{\mu_{ave}}\right)N^{\frac{1}{2}}$$ eq. 1.17

Where, $\Delta\mu_{app}$ is the difference in the mobilities of the solutes, $\mu_{ave}$ is the average mobility of the solutes and N is the peak efficiency (see equations 1.18 and 1.19).

### 1.2.6. Efficiency.

In chromatography, efficiency is a measure of the number of theoretical plates produced by a column, where the greater the number of plates the more efficient is the separation. The increase in efficiency comes from a greater number of interactions of the solutes between the two phases. However, efficiency is reduced due to dispersive effects in the column, resulting in band broadening. Efficiency (N) can be calculated using either peak width at half height ($w_{1/2}$), or base width ($w_b$), shown in equations 1.18 and 1.19 respectively.

$$N = 5.54\left(\frac{t_m}{w_{\frac{1}{2}}}\right)^2$$ eq. 1.18

Where, $W_{1/2}$ is peak width at half height.

$$N = 16\left(\frac{t_m}{w_b}\right)^2$$ eq. 1.19

Where, $W_b$ is peak width at base.

N may be a useful term to compare efficiency between columns, however a more useful parameter is the height equivalent of a theoretical plate (HETP).

This allows the effect of individual factors to be assessed. HETP can be calculated using several equations, for example eqs. 1.20 and 1.21.

$$HETP = \frac{L}{N} = \frac{\sigma_T^2}{L}$$

<div align="right">eq. 1.20</div>

Where, L is the total capillary length and $\sigma_T^2$ is the total variances or the sum of all the dispersive effects in the system.

The alternative method would be to use the Van Deemter equation for plate height (eq. 1.21)

$$HETP = A + \frac{B}{v} + Cv$$

<div align="right">eq. 1.21</div>

Where, A, B and C are constants and $v$ is the flow velocity.

Each of the constants in equation 1.21 relates to one of the dispersive mechanisms and how it is affected by the flow velocity. Therefore the flow needs to be optimised to minimise the dispersive effects, which lead to a reduction in HETP.

The A term or eddy diffusion, takes into account the numerous different paths that a solute can travel as it passes through a column. It is independent of the velocity of the flow. $B/v$ or longitudinal or axial diffusion occurs as the solute diffuses into the surrounding solution, thereby increasing the width of the sample zone. This effect is greater the longer the solute is on the column, therefore use of a high velocity can minimise it. $Cv$ relates to the rate of

equilibration of the solute between the two phases. To reduce broadening due to this effect the flow velocity needs to be minimised.

CE is not a true chromatographic technique, as the analytes are separated by differences in their electrophoretic mobilities, instead of differing partition co-efficients, as in HPLC. Therefore, in CE efficiency is mainly a measure of peak shape and width. However, the electroosmotic flow generated in an open capillary column does have one major dispersive mechanism, that of longitudinal or axial diffusion (B/v term). Eddy diffusion and rate of equilibration do not apply to CE, hence the terms A and $C_V$ are eliminated.

In addition to the reduction in dispersive mechanisms, bulk solution movement through the capillary has a flat or plug profile. This is in contrast to a pressure driven system that has a parabolic flow profile. Therefore, an increase in peak efficiency can be observed in CEC due to the nature of the bulk flow (Figure 4).

The degree of axial diffusion over a given time can be determined using spatial variances ($\sigma^2$). Assuming that there are no other dispersive effects acting on the solute, equation 1.22 applies.

$$\sigma^2 = 2Dt \hspace{5cm} \text{eq. 1.22}$$

Where, D is the diffusion coefficient of the solutes and t is time.
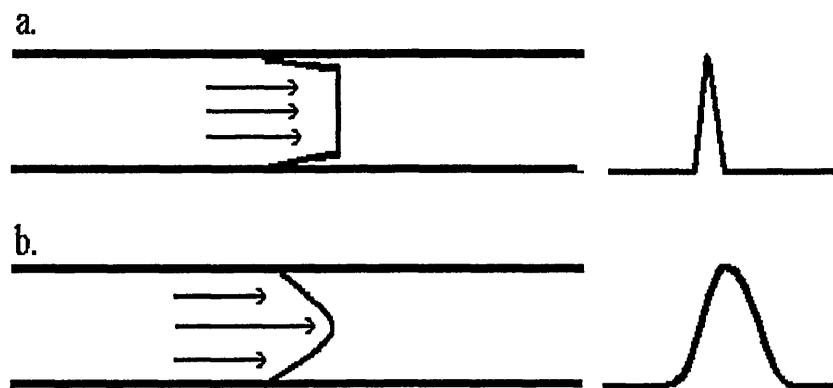
Figure 4. Flow profile in a column with (a) electro-drive and (b) pressure drive.

There are however several other dispersive forces acting on the zone as it migrates through the column, which can affect the total amount of axial diffusion that is observed. The total variance is the sum of several other variances that are found in the system (equation 1.23).

$$\sigma_T^2 = \sigma_D^2 + \sigma_{inj}^2 + \sigma_{det}^2 + \sigma_o^2 \qquad \text{eq. 1.23}$$

Where, $\sigma_D^2$ is the variance due to molecular diffusion, $\sigma_{inj}^2$ is variance arising from sample injection, $\sigma_{det}^2$ is variance due to the detector and $\sigma_o^2$ is variance due to other dispersive effects including solute adsorption, Joule heating, electromigration dispersion and nonuniform flow profile. These effects will be discussed in later sections.

## 1.2.7. Electric Field Strength.

As previously discussed, the electrophoretic velocity of a solute and the magnitude of the EOF are in part derived from the electric field strength (E) that is placed across a capillary column. The strength of the electric field is dependent on two factors, the applied voltage (V) and column length (L), (equation 1.24)

$$E = \frac{V}{L}$$ eq. 1.24

To decrease the analysis time the electric field needs to be maximised. The voltage is regulated in most commercial CE instruments to between ±30Kv. Variations in the capillary column length can be used to vary the strength of the electrical field. However, increasing the electric field strength can result in increased Joule heating.

In a CE instrument, conductivity can be related to the conductive medium (the electrolyte) and the dimensions of the capillary. This may be expressed by equation 1.25.

$$K = \frac{RA}{L} \quad \text{or} \quad R = \frac{KL}{A}$$ eq. 1.25

Where, R is resistance, A is cross sectional area of column and K is conductance.

When an electric potential is placed across a capillary column containing a solution of electrolyte a current is produced. Most of the energy that enters the system is converted into heat. The quantity of heat that is generated is proportional to the amount of power that is applied to the system. The heat that is generated is referred to as Joule heat. The rate of heat generation (P) is expressed in equation 1.26.

$$P = VI$$ 
eq. 1.26

Where, I is the current and V is the applied voltage.

Or in terms of V and R by substituting Ohm's law $(V = IR)$ into equation 1.26, to give equation 1.27.

$$P = \frac{V^2}{R}$$ 
eq. 1.27

This is then substituted into equation 1.25, to give the rate of heat generated in an enclosed system (see equation 1.28), given that $K = \Lambda C$ and $A = \pi d^2/4$

$$P = \frac{\pi d^2 V^2}{4 \Lambda C L}$$ 
eq. 1.28

Where $\Lambda$ is the molar conductivity of the solution, C is the concentration and d is the diameter of the column.

Equation 1.28 indicates therefore that the rate of Joule heating is dependent on the type and concentration of the buffer and the column diameter. As the

internal diameter of the column is reduced, so the current produced is also decreased. Therefore, if the internal diameter is halved then the current will decrease four fold (or the square of change in the I.D).

## 1.2.8. Buffer pH.

The pH of the buffer plays a vital role in CE, as it not only determines the magnitude of the EOF that is developed but also the effective mobility of the analytes.

As previously discussed, the EOF is derived in part from the surface charge, therefore as the pH rises so does the EOF. Variations in surface charge affect the zeta potential, which is one of the factors that affect the generation of the EOF and electrophoretic mobility of the solute. When an ion is placed in solution a double layer is formed around it. This double layer acts in a similar manner to that found at the surface of the capillary. Therefore changes in composition of the running buffer can affect the electrophoretic mobility of the ion, according to the Helmholz and Smoluchowski equation (see equation 1.05).

The electrophoretic mobility of an ion may also be varied with pH by altering its effective charge. The degree of dissociation ($\alpha$) of a solute is related to its pKa, therefore the effective mobility of the solute varies according to equation 1.29.

$$\mu_{eff} = \alpha \, \mu_e \qquad\qquad \text{eq. 1.29}$$

The pH is chosen to allow the optimal separation of the analytes, while not necessarily trying to optimise the velocity of the EOF. This is particularly useful when analytes have the same or similar pKa values, allowing the selection of a pH that will increase the electrophoretic mobility of one over the other.

1.2.9. Buffer Concentration or Ionic Strength.

As the concentration or the ionic strength of the electrolyte is decreased, the resultant EOF is increased. A high EOF is not always required, as good resolution is generally preferred over rapid analysis times. This requires a higher concentration of buffer or an increase in the ionic strength of the electrolyte to lower the zeta potential and hence to slow the EOF (13). Generally, an inorganic buffer has a higher ionic strength than an organic buffer of the same concentration. Therefore, when choosing a buffer, it is important to take into account both its conductive properties and its effective pH range (see Table 2).

If short analysis times are required low electrolyte concentrations can be used. However, problems can arise if the concentration used is excessively low, resulting in band broadening and asymmetric peaks. This is due to differences between the conductivity of the buffer and the sample plug, which can cause distortions in the electric field.

When the ionic strength of the buffer is significantly in excess of the sample plug's, a reduction in the peak distortions and an increase in sensitivity can be

observed. If a sample is dissolved in running buffer it can produce a significant lowering in the limit of detection, compared to that of the same sample being dissolved in either 10% buffer solution or in 100% water. This is due to the sample plug having a slightly different conductivity to that of the bulk solution, causing a stacking effect at the interface of the two zones. At the interface the electrophoretic velocity of the solute is decreased, due to the higher concentration of electrolyte in the bulk solution, which reduces the solute's Zeta potential thereby focussing the solute before it migrates into the bulk solution. Without this interface the solute will diffuse into the bulk solution, leading to band broadening and peak tailing or fronting, depending on the relative mobility of the solute to that of the electrolytes.

| Buffer | Useful pH range | Minimum useful $\lambda$ (nm) |
|---|---|---|
| Phosphate | 1.14-3.14 | 195 |
| Citrate | 3.06-5.40 | 260 |
| Acetate | 3.76-5.76 | 220 |
| MES[a] | 5.15-7.15 | 230 |
| PIPES[a] | 5.80-7.80 | 215 |
| Phosphate | 6.20-8.20 | 195 |
| HEPES[a] | 6.55-8.55 | 230 |
| Tricine[a] | 7.15-9.15 | 230 |
| Tris | 7.30-9.30 | 220 |
| Borate | 8.14-10.14 | 180 |
| CHES | 9.50 | <190 |

[a] Zwitterionic buffers.

Table 2. Commonly used buffers in capillary electrophoresis and their associated properties. (12).

1.2.10. Temperature.

Temperature control is important in CE as elevated temperatures can cause various problems. Increases in temperature are the result of joule heating; even a one degree rise increases the electrophoretic mobility of an ion by 2% (14). As the temperature rises so does the conductivity. This is due to the decreasing viscosity of the buffer, which leads to increased current. A thermal gradient is produced through the column, creating convection currents, which leads to a parabolic flow profile. The magnitude of these distortions is related to the quantity of heat that is produced and the rate at which it dissipates. This can be affected by the column's internal diameter, the thickness of the column

wall and the thermal properties of the column material. However, the upper limit for the column's I.D has been reported by Knox (15) as being about 200μm, as above this value the column cannot effectively dissipate the heat that is generated at its centre.

Elevated temperatures may also lead to band broadening and irreproducible migration times, due to convection currents and temperature gradients in the capillary altering the EOF that is being generated. Denaturation or decomposition of samples, especially those of biological origin, may also result from increases in temperature.

In extreme cases, for example if the buffer temperature rises, the CE instrument can automatically shut itself down, as the current generated would exceed the maximum operational limits of the instrument and if left would eventually lead to the buffer boiling.

However, when temperature is used in a controlled manner to aid separation there can be several potential advantages to be gained. The most apparent is the decrease in migration time due to the reduction in viscosity, which results in a greater EOF.

Variations in temperature can also enhance the resolution that can be obtained. This has been demonstrated by Guttman *et al.* (16), who showed that separation of a mixture of five proteins was optimised when the temperature was raised to 50°C. With the increase in temperature there was a

dramatic reduction in analysis time. This was only possible as a result of a structural reconfiguration of the proteins at elevated temperatures, which aided their movement through the gel.

It must be noted that with a change in the temperature there is also an effect on the volume of the sample loaded onto the capillary, which must also be taken into account. This is due to the changes in the density of the liquids with variations in temperatures.

1.2.11. Surface Modifiers.

Flow modifiers are used to alter the magnitude and the direction of the EOF. There are three potential effects that a modifier can have on the EOF. It can either reduce, eliminate, or reverse the EOF. This is achieved by blocking or altering the charge on the capillary wall. The use of modifiers can, therefore, be used to improve resolution by reducing the magnitude of the EOF or decrease the analysis time for anions by reversing the flow. An untreated fused silica column can act as a cation exchanger, due to the weakly acidic silanol groups present on the surface of the capillary. Therefore, the capillary surface needs to be modified to reduce any interactions between the cationic solutes and the capillary surface as these interactions lead to a loss of efficiency and irreproducible migration times. Capillaries can be coated in one of two ways, either by dynamically coating the surface with surfactants or by permanently altering the surface by covalently bonding different groups to it.

## 1.3. Instrumental Overview.

All capillary electrophoresis (CE) instruments are similar to each other in their basic design. A schematic is shown in figure 5. The basic configuration employs two electrolyte reservoirs bridged by a length of fused silica capillary, which can have an internal diameter of $10\mu m$ - $200\mu m$. Electrodes are placed in the electrolyte reservoirs and are connected to a high voltage power supply capable of providing up to 30Kv. A current limit of between $200\mu A$ and $300\mu A$ is usually found on most instruments. An electrical circuit can be established once the capillary is filled with the electrolyte. The application of a voltage across the capillary results in the production of an electroosmotic flow through the capillary.
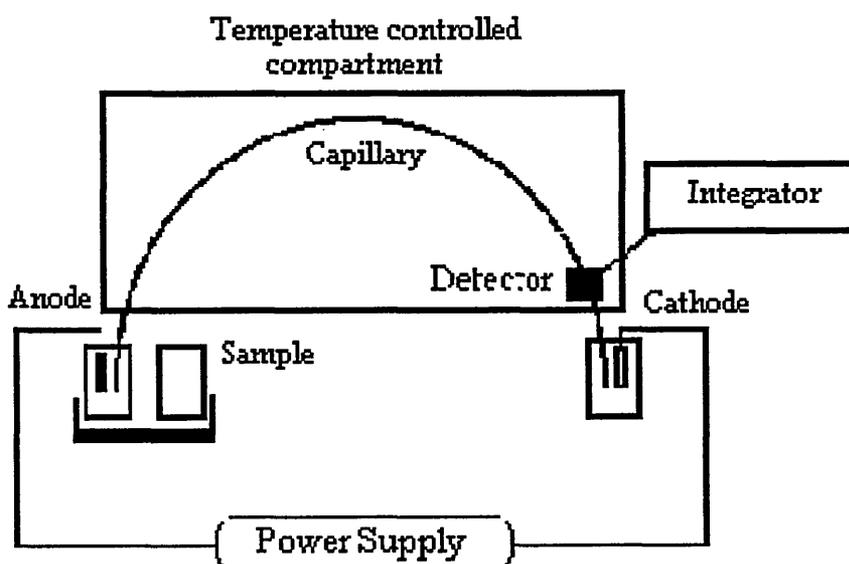
Figure 5. Block diagram of a CE instrument.

Many of the detectors that are being employed are also used in modern HPLC. The graphical representation of the data that is collected is referred to as an *electropherogram* instead of a *chromatogram* as in HPLC separations.

### 1.3.1. Injection Techniques.

The small sample volume requirements of the CE necessitate special injection methods and several different techniques have been reported that will deliver sample into the capillary. These include an electric sample splitter (17), rotary-type injector (18), freeze plug injections (19) and microinjection (20). However, commercially available instruments load samples by either electrokinetic or hydrodynamic methods.

### 1.3.1.1. Hydrodynamic Injection

### 1.3.1.1.1. Pressurisation.

The sample is introduced by the application of pressure to the sample vial, which forces the sample into the capillary. The volume of solution injected on column ($V_i$) is calculated by the Poiseulle equation, (equation 1.30).

$$V_i = \frac{\Delta P r^4 \pi t_i}{8 \eta L} \qquad \text{eq. 1.30}$$

Where, $\Delta P$ is the pressure drop across the capillary, r is the internal radius of the capillary, L is the total length of the capillary and $t_i$ is the injection time.

The plug length ($L_p$) can then be calculated as follows

$$L_p = \frac{V_i}{\pi r^2} = \frac{\Delta P r^2 t_i}{8 \eta L} \qquad \text{eq. 1.31}$$

Variations in the volume of sample loaded will affect peak area and height. This can occur due to siphoning if the ends of the capillary are not level with

each other. Changes in temperature can also affect the volume loaded by altering the hydrodynamic properties of the solutions.

## 1.3.1.1.2. Gravity Loading.

Samples introduced by gravity flow are siphoned into the capillary by lifting the sample vial above the outlet vial. The sample volume introduced can be calculated from

$$V_i = \frac{\rho G \pi r^4 \Delta h t_i}{8 \eta L}$$    eq. 1.32

Where, $\rho$ is the density of the buffer, G is the gravitational constant and $\Delta h$ is the difference in the heights of the liquids.

## 1.3.1.2. Electrokinetic Loading.

Electrokinetic loading can be used in CZE and when there is insufficient pressure available to load the sample, such as in gel electrophoresis or capillary electrochromatography. The sample is loaded by a combination of EOF and the electrophoretic mobility of the sample ions. The quantity of solute loaded ($Q_{inj}$) can be calculated by,

$$Q_{inj} = \frac{(\mu + \mu_{eof}) \pi r^2 V_i t_i}{L} . C$$    eq. 1.33

Where C is the concentration of solute, $\mu$ is the electrophoretic mobility of the solute and $\mu_{eof}$ is the electrophoretic mobility of the running buffer.

The quantity of each analyte that is injected is determined by its electrophoretic velocity, thus leading to sample bias on loading. Therefore,

peak area and height will increase proportionally with increasing electrophoretic mobility. Heung *et al.* (21) showed that sample bias could be corrected by the use of a bias factor b, as seen in equation 1.34. This ratio can then be used to correct the peak area or height of the solutes.

$$b = \frac{\left(\mu_1 + \mu_{eof}\right)}{\left(\mu_2 + \mu_{eof}\right)} = \frac{t_1}{t_2}$$
eq. 1.34

where, $\mu_1$ and $\mu_2$ are the electrophoretic mobilities of the solutes and $t_1$ and $t_2$ are the migration times of the solutes.

Differences in the ionic strength or the pH between the buffer and sample can affect the quantity of ions loaded and the efficiency of the separation. These effects can be minimised by dissolving the sample in the buffer, or by the addition of a non-detected ion to equalise the relative conductivities of the sample and buffer.

A side effect of the sample bias is that of sample depletion, especially with replicate injections from the same source. With multiple injections the concentration of the more mobile solutes will decrease disproportionally to that of the less mobile solutes. This results in variations in the amounts of solutes loaded and a decrease in ionic strength of the sample.

### 1.3.1.3. Sample Overload.

With the low sample volumes used in CE it is easy to overload the capillary column. By overloading the capillary, the maximum number of theoretical plates ($N_{max}$) that can be obtained is reduced, leading to a less efficient separation (see equation 1.35).

$$N_{max} = 12\left(\frac{q_c}{q_{inj}}\right)^2 \qquad \text{eq. 1.35}$$

Where $q_{inj}$ is the volume of the sample injected and $q_c$ is the volume of the column.

Aebersold and Morrision (22) suggest that as a rule of thumb, the injection plug should be 1 to 5% of the total volume of the capillary.

Another potential problem relates to differences in conductivity between the sample plug and the bulk solution. This can lead to an electric field variation along the capillary, which can result in either distorted peaks or focussing of the solutes.

### 1.3.1.4. Extraneous Injection.

Variations in the quantity of sample loaded may occur as a result of several different processes:

- The displacement of a small volume of sample into the capillary, as the capillary is inserted into the sample solution.

- Movement between the buffer and the sample solutions due to convection as a result of differing thermophysical properties of the solutions, such as viscosity, surface tension and density.

- The longitudinal diffusion of the solutes in the buffer solution, due to differences in the relative mobilities of the solutes and the electrolyte in the buffer.

## 1.3.2. Detectors.

CE has been coupled to a wide variety of detectors, many of which are already well established in HPLC. These detection methods include fluorescence (23-26), laser induced fluorescence (27,28), amperometry (29,30), conductivity (31,32), refractive index (33,34), laser Raman (35,36), radiometry (37,38), NMR (39,40) and MS (41). The most common detector is the UV/Vis absorbance detector, which has been utilised in this study. Table 3 lists approximate detection limits for some of the detectors used in CE (42).

| Detector | Approximate Detection Limits | |
|---|---|---|
| | Moles | Molarity[a] |
| UV/Vis absorbance | $10^{-13}$-$10^{-16}$ | $10^{-5}$-$10^{-7}$ |
| Indirect absorbance | $10^{-12}$-$10^{-15}$ | $10^{-4}$-$10^{-6}$ |
| Fluorescence | $10^{-15}$-$10^{-17}$ | $10^{-7}$-$10^{-9}$ |
| Indirect Fluorescence | $10^{-14}$-$10^{-16}$ | $10^{-6}$-$10^{-8}$ |
| Laser-induced Fluorescence | $10^{-18}$-$10^{-20}$ | $10^{-13}$-$10^{-16}$ |
| Mass Spectrometry | $10^{-16}$-$10^{-17}$ | $10^{-8}$-$10^{-10}$ |
| Amperometric | $10^{-18}$-$10^{-19}$ | $10^{-7}$-$10^{-10}$ |
| Conductivity | $10^{-15}$-$10^{-16}$ | $10^{-7}$-$10^{-9}$ |
| Refractive index | $10^{-14}$-$10^{-16}$ | $10^{-6}$-$10^{-8}$ |
| Radiometric | $10^{-17}$-$10^{-19}$ | $10^{-10}$-$10^{-12}$ |

[a] Depends upon volume of sample injected.

Table 3. Detectors which have been coupled to CE with their approximate detection limits (42).

## 1.3.2.1. UV/Vis detector.

The UV detector utilises changes in the quantity of light that is passed through a detection window or cell as a solute migrates past the detection window. The transmittance of light through the column is given by equation 1.37.

$$T = \frac{I}{I_o}$$
eg. 1.37

Where, $I_o$ is the light intensity of the initial beam of light and $I$ is the transmitted light intensity after passage through the capillary.

As a solute passes through the beam of light at the detection window, the solute will absorb a certain quantity of this light. Beer's Law, equation 1.38, defines the absorbance (A) of the solute.

$$A = \log \frac{1}{T} = \varepsilon bc$$
eq. 1.38

Where, $\varepsilon$ is the molar absorptivity, b is the path length and c is the concentration of the solute.

The detection wavelength chosen has to maximise the absorbance of the chromophores present in the solute, while minimising the background absorbance i.e. direct UV detection.  However, if the solutes do not absorb light the buffer needs to have a high absorbance, with the wavelength set to allow the maximum difference in absorbance between the solutes and the buffer i.e. indirect UV detection.

The detection cell path length is also an important factor to be considered, as the path length is directly proportional to the absorbance observed. To maximise the absorbance and hence sensitivity, the path length needs to be as large as possible (this will be discussed in section 1.3.2.2).

There are two types of UV detectors, single variable wavelength (monochromatic) detectors and multiple wavelength photodiode array detectors, with the latter being able to observe the entire spectrum simultaneously.

## 1.3.2.2. Detection Window.

In CE, detection is usually achieved on column. Therefore, the polyimide coating supporting the fused silica needs to be removed to produce a suitable detection window. There are several means of removing the polyimide. The first is to burn the polyimide off by using either a Bunsen flame or an electrically heated filament. The use of a Bunsen flame may be convenient but it produces a large and unstable detection window that is easily broken. The heated filament allows a great deal of control in the size of the detection window formed (1-3mm), allowing a more stable window to be produced, however, heating the fused silica can damage it. The second method would be to dissolve the polyimide by use of boiling concentrated sulphuric or nitric acid, which does not damage the fused silica and is useful when the inner surface of the capillary has been modified. The final method is to remove the polyimide with a sharp knife, however the silica column can be easily scratched, causing optical distortions and weak points.

The detection cell is part of the capillary. This means that the path-length is equivalent to the internal diameter of the capillary being used. A large internal diameter will give greater sensitivity, however, with larger internal diameter columns Joule heating can occur, as the current generated is proportional to the square of the internal diameter. A result of this several novel detection cells have been developed, which allow greater sensitivity to be achieved.

The Z-cell (43,44) works by forming a small section of capillary that is perpendicular to the rest of the column, as shown in figure 6, which is then used as the detection cell. A 3mm path length in the Z-cell will increase sensitivity by ten-fold (45) over that of a straight capillary.

Detector cell

Light source

Detector
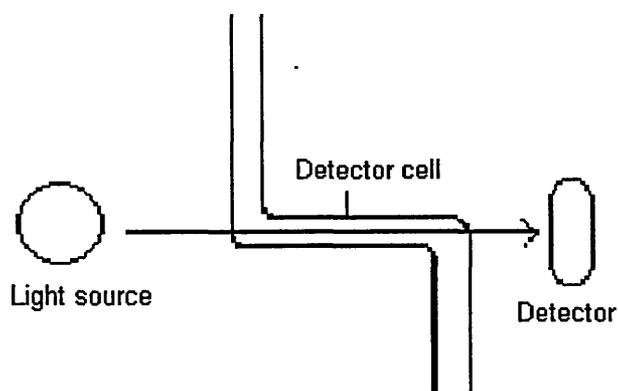
Figure 6. Diagram of a Z-cell.

The use of a rectangular column was reported by Tsuda *et al.* (46). The capillary was formed to give a path length of 1000µm along one axis. The increased sensitivity results from an increase in the path length and a reduction in optical distortion. The one drawback is the poor UV transmission exhibited by the borosilicate column.

The multireflection cell (47,48) works by removal of the polyimide to allow the surface to be coated with silver backed by an outer coating of black paint. The silver is used like a mirror to reflect light along a given path length inside the column, as shown in figure 7. The increase in sensitivity is not quite proportional of the increase in the cell path length, which is due to a small reduction in the intensity of light with each reflection.
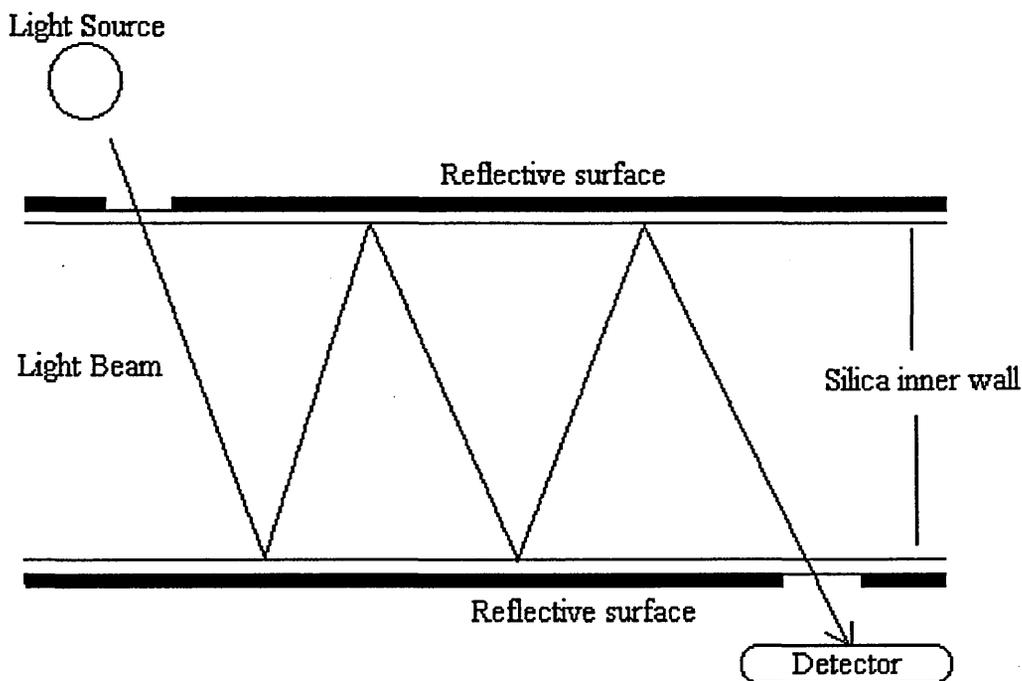
Figure 7. Diagram of a Multireflection cell.

The bubble cell is produced by either blowing or etching a bubble into a section of the capillary producing an increase in sensitivity proportional to the increase in the column internal diameter. This has been shown to provide a threefold increase in sensitivity when a $50\mu m$ column had a $150\mu m$ bubble cell fabricated within it (49).

The sleeve cell (50,51) works by carrying out the separation in a normal capillary column i.e. 50μm, which is then butted up against a wider bore column e.g. 200μm, to be used as the detection cell, (see figure 8). As the buffer flows into the cell, the flow is reduced to compensate for the increase in volume, thereby compressing the peak bandwidth. The compression of the bandwidth allows for sharper peaks and hence an increase in sensitivity in addition to the gain from the longer cell path-length.
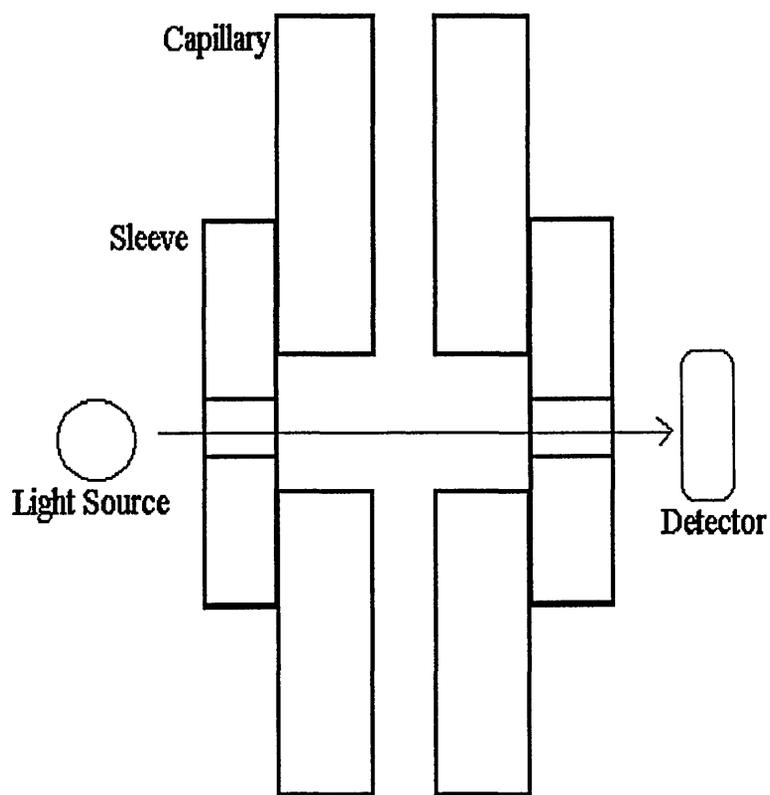


Figure 8. Diagram of a Sleeve cell

### 1.4. Capillary Electrochromatography (CEC).

#### 1.4.1. Introduction.

As the theory of CE would indicate, ions can be readily separated on the basis of their charge and size. However, if the compounds of interest are neutral, they cannot be separated under the same conditions, as all uncharged solutes irrespective of size migrate through the capillary at the same rate as the bulk solution. Several methods have been developed to address this problem.

In this study capillary electrochromatography (CEC) was investigated and developed as a possible routine method for separation of neutral compounds. The background to CEC will be discussed in the following section, along with a summary of the other electrophoretic techniques that could be utilised.

#### 1.4.2. Development of CEC

The development of CEC can be traced back to Strain in 1939 and Lecoq in 1944, who were the first to report the generation of an EOF in liquid chromatography (52). Prior to the work of Pretorious *et al.* (53) in the mid 70s and Jorgenson and Lukacs (9) in the early 80s, the electrically driven bulk movement of an eluent through a porous material had been discussed, but never demonstrated as a viable analytical technique.

In 1974, Pretorius *et al.* (53) reported the effect of using an applied voltage instead of pressure to move an unretained compound through a packed column. A narrow bore column was employed, on which both HPLC and

electrochromatography (EC) experiments were performed. The results supported the theory that band broadening commonly found in HPLC would be reduced, due to the flat flow profile generated by the EOF in EC.

In 1981 Jorgenson and Lukacs (9) reported data obtained on a crude non-aqueous CEC system using a 170$\mu$m I.D. column. Unlike the work of Pretorius, the compounds were partially retained on the column in their investigation. This work, as they freely admitted in the paper, was a crude attempt at CEC but did indicate that highly efficient separations were possible.

In 1983, Stevens and Cortes (13) cast doubt on the viability of CEC. Their investigation looked at various sizes of packing material. They concluded that if the packing material was smaller then 50$\mu$m in diameter there would be insufficient flow generated to allow effective separations to take place. Double layer overlap degrading the EOF was suggested as a possible explanation for their observations.

The conclusions of Cortes and Stevens were dismissed by Knox and Grant in 1987 (54), in their theoretical paper on miniaturisation of chromatography systems. This included a purely theoretical study of CEC. They determined the potential double layer thickness around particles in the stationary phase and then compared it to the mean channel size in the packed bed. They predicted the maximum electrolyte concentration (10mM) and the minimum particle size (0.4$\mu$m) that could be used before double layer overlap occurred.

Knox and Grant later confirmed their hypothesis in 1991 (55). It was at this time that renewed interest in CEC began.

The initial development of CEC led to a variety of terms being employed to described this technique. These included titles such as liquid chromatography with electroosmotic flow (56), electro-endosmotically driven liquid chromatography (54), electrically driven liquid chromatography (57), electroendosmotic capillary chromatography (58) and electrokinetic chromatography with packed capillaries (59). Tsuda (60) was the first to use the term capillary electrochromatography. However, Knox, in 1994 (61) proposed that CEC should be adopted as the official title and this term has become accepted.

The development of CEC following the work of Knox has been slow but steady. Several recent reviews have been published on the state of CEC. General reviews on the fundamentals of CEC have been published by Cikalo *et al.* (62), Colon *et al.* (63, 64), Crego *et al.* (65), Kowalczyk (66), Steiner *et al.* (67) and Angus *et al.* (68). Dittmann *et al.* (69) reviewed the theory of CEC, while Rathore and Horvath (70) compared the differences in LC, CE and CEC. Pursch and Sanders (71) reviewed the development of stationary phases. The steady development of CEC is reflected in the publications of Altria *et al.* (72) and Euerby *et al.* (73), which both review the current applications available for CEC and Krull *et al.* (74), which reviews progress in specific biological applications.

## 1.4.3. Theory.

### 1.4.3.1. Electroosmotic Flow in CEC.

As discussed in section 1.2.1 the EOF is generated near to the inner surface of the capillary. The same basic principle applies to CEC columns. Stationary phases in general are supported on silica particles, thus allowing a double layer to be formed around each particle, similar to that at the surface of the capillary (Figure 9). Once an electric field is applied, the cations in the diffuse layer around the packing materials and capillary wall are drawn towards the cathode, causing flow of the bulk solution thorough the packed column.
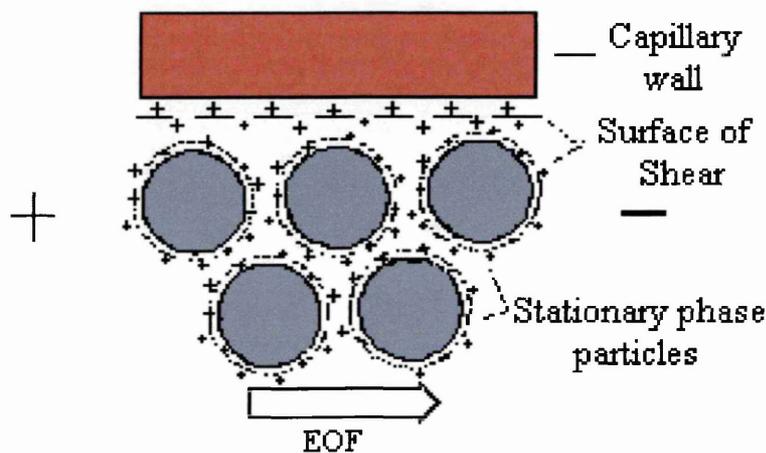


Figure. 9. Diagram showing how an electroosmotic flow is generated on a packed column.

To allow the generation of an EOF through a packed column, the channel size between the packed particles needs to be greater than twenty times the thickness of the double layer that is formed around each particle (75). When the channel size falls below this value double layer overlap can occur, which can affect the EOF that is generated and degrade the flat flow profile.

It must be noted that the observed velocity of the EOF is the averaged velocity of the flow rates in both the packed and unpacked sections of the capillary (76); the flow through the packed section is slower than in the open section. The electroosmotic conductivity contributes less than 5% of the conductivity through the packed section and the total conductivity of a good column is about one third the value from an unpacked column of identical dimensions (77, 78).

For practical purposes CEC is performed on columns having an I.D of 50-100µm. Yan *et al.* (79) demonstrated that Joule heat in larger bore columns cannot be efficiently dissipated, thus affecting the EOF. The packing material used is 1.5-5µm in diameter, with 3µm being the most commonly employed.

Once the EOF is established the CEC column acts like a conventional LC column. The analytes are adsorbed and desorbed to varying extents according to their different affinities for the two phases. CEC has several advantages over an LC system. These include a significant reduction in quantity of both packing material and eluent required and an increase in the potential efficiency of the CEC columns, when compared to the equivalent LC columns. This is the result of the flow being both uniform in its direction and independent of pore size, therefore the leading edge of the flow has an equal force acting on it as it moves through the packed column (see Figure 10). In LC columns, eddy diffusion and a pressure gradient across the face of the flow produces a parabolic flow profile, as shown in figure 11.
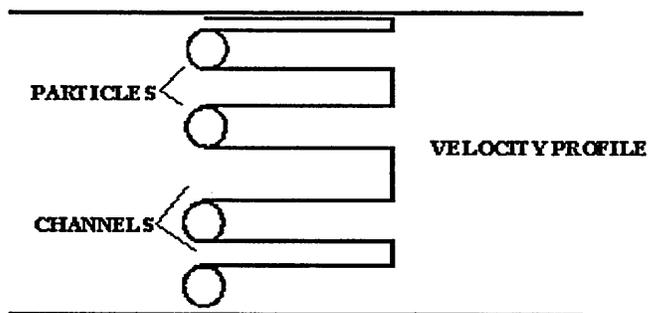
Figure. 10. Flat flow profile through a CEC column.
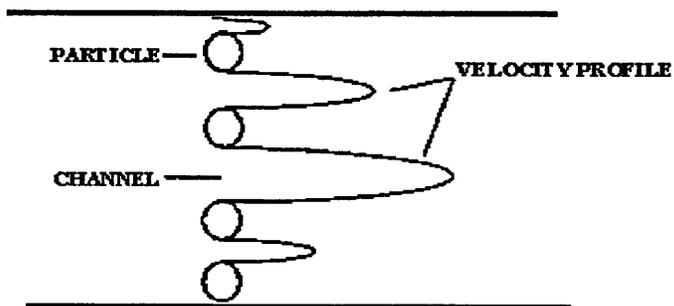


Figure. 11. Parabolic flow profile through a HPLC column.

Ross *et al.* (80) clearly demonstrated the increased efficiency of CEC over LC. They showed that eddy diffusion had been significantly reduced in CEC with consequent reduction in HETP. This led to an increase in the number of theoretical plates that could be potentially obtained, as shown by the van Deemter plot (Figure 12).
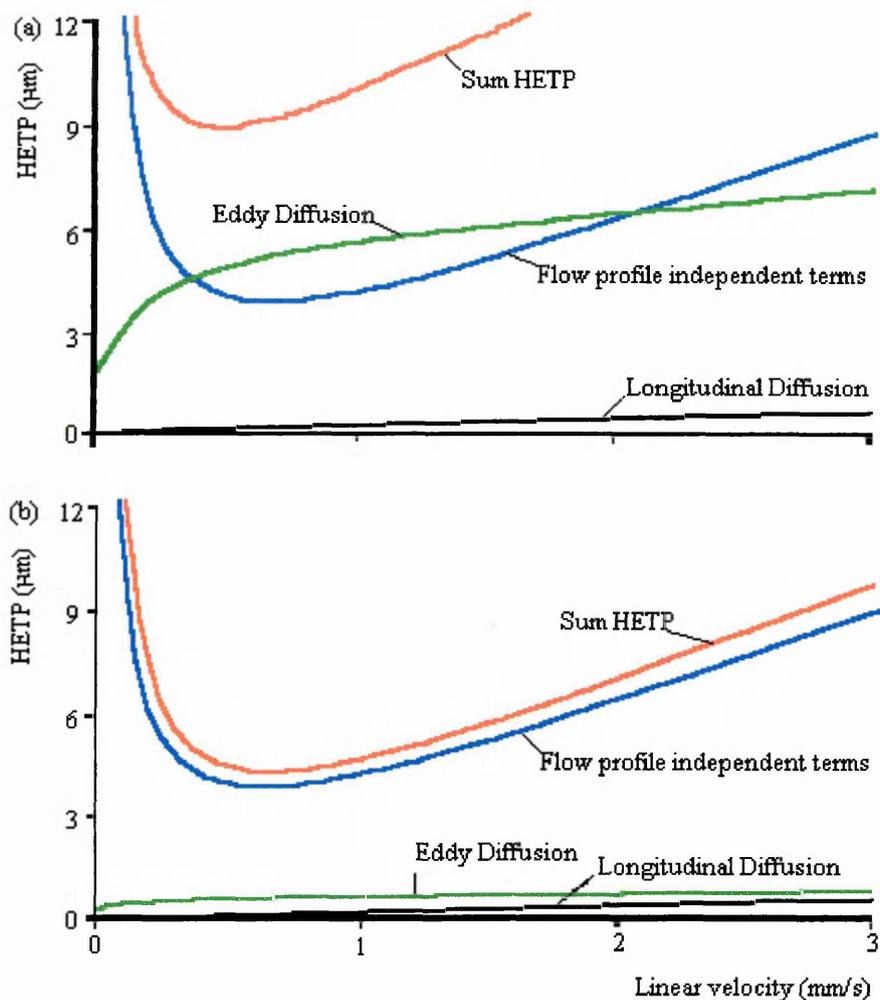
Figure. 12. Van Deemter plots for HPLC (a) and CEC (b), showing how the diffusion mechanisms seen in HPLC have been reduced and efficiency of the column is increased in CEC.

1.4.3.2. Mobile Phase.

In CZE, organic solvents are added to increase solubility and selectivity (81). Selectivity is improved by a combination of several factors. These include changes in the organic solvent, which affect the EOF generated and the electrophoretic mobility of the analytes as a result of changes in the zeta potential, viscosity and dielectric constant. Schwer and Kenndler (82) predicted the effect of varying the organic content using methanol and acetonitrile from 0-100%. They showed that as the organic content initially rises, the EOF is reduced to a minimum (at around 70 to 80% organic content)

- 49 -

before increasing again as the organic content is increased to 100%. In contrast, Kitagawa and Tsuda (83) demonstrated that there was no variation in EOF until the percentage of methanol was increased to over 90%.

The separation of the solutes in CEC is similar to LC, therefore the percentage organic solvent that is required is higher than in a CZE separation. Dittmann and Rozing (84) demonstrated the effects of three solvents, methanol, acetonitrile and THF. The results showed that, as well as variations in EOF, changes in selectivity were also observed with the changing solvents. However, the retention effects are similar to those observed in LC (85), indicating that LC methods may be transferable to CEC.

The majority of the methods that have been reported have used an organic content in the buffer of 70-80%. However, there have been several reports using 100% organic solvent (non-aqueous CEC) with organic electrolytes dissolved in the solvent (86, 87) to aid the flow. Wright *et al.* (88) have also developed a separation which does not require a supporting electrolyte. There are several claimed advantages to non-aqueous CEC compared to aqueous methods. Non-aqueous eluents increase the solubility of analytes as well as exploiting the chemical and physical properties of the organic solvents. A reduction in wall adsorption by hydrophobic compounds has also been observed (89).

The electrolytes that are used are the same as in CZE, however the concentrations that can be employed are restricted. The restrictions are due to

double layer overlap and to Joule heat, which can lead to bubble formation. This has meant that electrolytes, both organic and inorganic, are restricted to a total concentration below 10mM without pressurisation. However, Wan (90) has stated that the linear reduction in EOF with increased electrolyte concentration observed in CZE may not hold true in CEC due to thermal effects. Additives to the buffer include SDS, which helps stabilise and control the EOF (91) by altering the zeta potential. Flow reversal has been demonstrated in packed columns by the addition of a cationic surfactant to the running buffer by Li and Lloyd (92).

1.4.3.3. Stationary Phases.

The majority of stationary phases that have been utilised to date in CEC are HPLC packing materials. Because these phases are supported on a silica base, an EOF can be generated from residual silanols on the surface. The most commonly used stationary phase is octadecylsilane (ODS).

A number of different ODS phases from a variety of manufacturers have been studied (Table 4). Dittmann et al. (84, 93) have demonstrated that there are marked differences in the performances of different ODS phases when tested under the same conditions.

| Stationary phase | Efficiencies |
|---|---|
| 3μm Spherisorb ODS 1 | 200 000 – 240 000 |
| 3μm Nucleosil 100 $C_{18}$ | 91 000 – 147 000 |
| 3μm Spherisorb $C_{18}$ PAH | Up to 260 000 |
| 3μm Synchrom | 102 000 – 138 000 |
| 3μm Vydac $C_{18}$ | > 160 000 |
| 3μm CEC Hypersil | 240 000 – 280 000 |

Table 4. Efficiencies obtained for isocratic CEC using HPLC stationary phases. (From Ref. 62).

Most of the material used is 3μm to 5μm $d_p$, (particle diameter) with the 3μm material predominating. The size of the stationary phase particles that can be packed is limited by the packing method. However, columns have been packed with stationary phases in the region of 1.5μm $d_p$ (54, 94), with the possibility of packing submicron particles being put forward by Fermier et al. (95). The driving force behind the use of ever-smaller packing material is due to the increase in efficiency that can arise, as show in table 5.

| Particle size (μm) | Column length (cm) | Plates/column |
|---|---|---|
| 5 | 50 | 115 000 |
| 3 | 50 | 170 000 |
| 1.5 | 50 | 250 000 |

Table 5. Achievable plates in CEC. (from ref 63).

The 3μm to 5μm materials have pore sizes of between 8-10nm, which does not allow flow through the pores, due to double layer overlap degrading the EOF inside the pores (55). Increasing the pore size has been studied by Li *et al.* (96) who used material with pore sizes between 10 and 300nm, whilst Wei *et al.* (97) used pore sizes between 100 and 4000nm. The results showed that an increase in pore size increased efficiency. When pore size rose above 2000nm perfusive electroosmosis was able to be supported i.e. flow through the pores. This then allowed the use of larger diameter packing material (5-10μm), without any loss of efficiency. The use of nonporous 1.5μm particle material has also been investigated by Seifar *et al.* (94) and Engelhardt *et al.* (98), with both groups reporting highly efficient separations.

It must be noted that the reported particle size supplied by the manufacturers may be misleading, as this figure is an average representation of particle size. Additionally there are several different methods that can be used to determine particle size, each with a different bias. Packing material can have particles significantly smaller than the average and these can cause problems with both the column packing and double layer overlap. New monodisperse materials have now become available, which can reduce this problem (99).

In 1995 Smith *et al.* (100) demonstrated ion exchange electrochromatography to separate a set of basic compounds, with impressive results. The strong cation exchange phase not only allowed a more rapid separation of the tricyclic antidepressants than an ODS phase, but the efficiency calculated for one of the components was eight million plates per metre. This result is

significantly greater than the hundreds of thousands of plates normally observed with ODS phases. Wei *et al.* (101) and Li *et al.* (102) demonstrated that strong cation exchange columns can be used reproducibly for the separation of basic compounds. However, neither group has claimed the eight million plates per metre of Smith *et al.* (100).

There have been some other novel stationary phases used. These have including a cellulose based packing material developed by Maruska *et al.* (103, 104) which, when used in conjunction with a non-aqueous mobile phase, can separate polar and hydrophobic compounds. Van der Bosch *et al.* (105) investigated the used of a chiral stationary phase, with mixed results. The chiral phase showed greater selectivity, but lower efficiency, when compared to an ODS phase used in conjunction with a chiral additive in the buffer.

### 1.4.3.4. Column Fabrication.

Since the completion of this work several publications have taken an objective look at the packing of CEC columns (64,68,106), highlighting some of the causes of column failure in a concise manner.

### 1.4.3.4.1. Frits.

Before any column can be packed an initial retaining frit needs to be placed at one end of the column to allow the stationary phase to be packed up against it. There are several different methods that can be employed but they all rely on the formation of a porous silica frit. The end of the capillary is filled to approximately 1mm in depth with either a silica gel (107-109) or a sodium

silicate solution, which is reacted with formamide (55,59,85,110-112) and then fused into place by heating. An alternative method was described by Frame *et al.* (113), who demonstrated the use of a standard frit from a 1mm I.D. microbore LC column, with a short sleeve of PEEK tubing used to lock the capillary in place. This method allowed a more reproducible flow through the column.

Once the column has been packed, the initial retaining frit may be used as the permanent frit (59,108) or it can be replaced by one produced from the stationary phase. These frits can be manufactured by using either an electrically heated filament (114) or a micro torch (115) to fuse the packing material. The electrically heated filament is the preferred method as it allows a more controlled burn, resulting in a more reproducible frit. Smith *et al.* (100,111) and Boughtflower *et al.* (116) described the first purpose built electrical burner. Another novel method to produce the final frit was reported by Lelievre *et al.* (106), where a small plug of the stationary phase was removed and replaced with silica to form the final holding frit.

The formation of final retaining frits by heating the silica stationary phase can however cause several problems, which Poppe *et al.* (117,118) identified. Removal of the polyimide protective coating leaves a small fragile section of exposed silica column, which can be easily broken. Additionally, reproducibility in the formation of the final frit is difficult to achieve with successive columns. Heating the packing material also changes the characteristics of the material, which can lead to a non-homogeneous packed

bed around the frit. This can affect the EOF flow and can lead to bubble formation at the intersection of the packed and unpacked regions of a column (119-120).

A potential solution to this problem would be to have no frits present and there are two methods that utilise this approach, either by using a continous bed column (discussed in a later section) or by the use of tapered columns. There are two types of tapered columns; externally tapered (121), where the column is drawn out to produce a tapered end (fragile) and internally tapered (122), which are stronger and more robust.

1.4.3.4.2. Column Packing.

There have been several different processes developed to pack capillary columns. The most commonly utilised is that of a pressure driven system, where the stationary phased is slurried and loaded into the column under a pressure of 5000 to 10000 psi (~350 to 700bar). The pump used determines the pressure that can be used, with LC pumps (59,107,108,112,123) having a lower potential operating pressure than a Shandon HPLC packer (11,100, 123). An alternative system was reported by Boughtflower *et al.* (124), who developed a novel pressure vessel that packs multiple columns simultaneously and which also contains an ultrasonic probe to keep the packing material continuously agitated. Robson *et al.* (125) demonstrated the use of a supercritical fluid carrier ($CO_2$) to transport the stationary phase into the capillary. However, once the packing material was loaded, the column was still conditioned under pressure before the final frits were produced.

Yan (126) has patented an electrokinetic packing method. The stationary phase is slurried in a solution of running buffer instead of a solvent and is then loaded into the column under the influence of an EOF and the stationary phase's own electrophoretic mobility. Both the vial containing the slurry and the column are continuously agitated to avoid blockages. The advantage offered by this system is a homogeneously packed column, which can lead to an increase in efficiency. Efficiency can be further enhanced, through the ability to pack smaller diameter stationary phases, e.g. $1\mu m$ $d_p$, which cannot be readily packed using pressure driven systems.

Fermier *et al.* (95) employed centripetal forces to pack multiple columns. The columns are spun at approximately 1500rpm, allowing the packing material to be loaded at a uniform velocity. Consequently there are no large pressure drops across the capillary that can reduce the packing velocity. This produces columns with a homogeneous packed bed and uniform channels, which reduces the possibility of localised heating. They also claim to be able to pack submicron particles easily, which can potentially further increase the efficiency of CEC.

With the increased interest in CEC, commercially available columns have become available from a number of commercial suppliers (table 6). These companies can supply a variety of types of columns, depending on the stationary phase required and the instrumental configuration.

| Supplier | Stationary phases |
|---|---|
| Capital HPLC | Range of standard and customer specific phases to fit all instrument types. |
| Electropak | Capillary columns for CEC and micro –HPLC |
| Hewlett-Packard | ODS packed columns to fit HP instrumentation. |
| Hypersil | $C_{18}$ columns, diameters 50 or 100$\mu$m x 250mm. Compatible with most CE equipment |
| Innovatech | Range of standard and specialist phases to fit all instrument types. |
| LC Packings | Range of customer specific phases to fit all instrument types. |

Table 6. Commercial suppliers of CEC packed capillaries (from ref 70).


1.4.3.5. Pressurisation.

The majority of CEC separations are performed under atmospheric conditions. However, the application of pressure to a column can have potential advantages by increasing flow velocity, reducing the possibility of bubble formation and increasing the ionic strength of electrolyte that can be used. The application of pressure equalises the flow velocity between the packed and unpacked section of the column. These variations in flow can be the result of discontinuities in the EOF caused by the retaining frit, changes in electric field strengths and changes in zeta potential between the two regions in the column.


A dual system employing both pressure and electrophoresis was developed by Kitagawa and Tsuda (83,127) who used an applied electric field to fine tune separations on a narrow bore HPLC column (128-130). True pressurised

CEC systems were not reported until the early 1990s, when Behnke and Bayer (131) and Dekkers *et al.* (132) developed a system that allowed the inlet of the CEC column to be pressurised by an LC pump, which increased the flow velocity of the bulk solution. This dual system has been referred to as either pressure-assisted electrochromatography (PEC) (128) or pseudo-electrochromatography (133).

Pressurisation of the entire system has been reported by Smith and Evans (100,111) and Boughtflower *et al.* (116,124,134), with the capillary being pressurised to 15Bar. They used nitrogen to apply pressure to the entire column, in a home-made system for the research group at Glaxo UK. Dittman *et al.* (80,84) reported the development of the HP$^{3D}$CE instrument, which can pressurise the entire column to between 10-12Bar. This instrument can be used in a variety of modes from CEC to micro-LC.

1.4.3.6. Gradient CEC.

The simplest method to obtain a gradient is to alter the composition of the mobile phase by a stepwise approach. This is accomplished by changing the buffer vials during the run, thereby stepping the organic or electrolyte concentration from one level to another as the buffer in the vial is changed. Euerby *et al.* (73) reported the separation of six diuretic compounds and Ding *et al.* (135) separated isomeric polycyclic aromatic hydrocarbon-deoxyribonucleoside adducts. In both cases a step gradient allowed the reproducible separation of co-eluting solutes.

Behnke and Bayer (131) were the first to link a gradient HPLC to the inlet end of a CEC column. The LC pump now not only pressurised the system but also acted as the solvent delivery system. This allowed the solvent composition to be altered during the run, resulting in gradient CEC. The mobile phase flows across the entrance to the CEC column, with a small percentage of the buffer being drawn onto the column. This means that a large quantity of the buffer is wasted, however the precise composition of the buffer can be controlled, thereby reducing buffer depletion. Other reports of gradient CEC have utilised gradient LC pumps (136-140) with Taylor *et al.* (137,138) and Horvath *et al.* (139,140) demonstrating the effectiveness of this technique for the separation of biological compounds.

Another approach has been reported by Zhang *et al.* (141,142) and Wu *et al.* (143), who have described an alternative method for changing the eluent composition. Instead of premixing the eluent by the use of a gradient pump, the buffer vial is continually dosed with either a stronger electrolyte or solvent. This requires the content of the buffer vial to be continually agitated to allow mixing.

Yan *et al.* (144) described the separation of 16 EPA (Environmental Protection Agency) priority polycyclic aromatic hydrocarbons (PAH) by an alternative gradient elution method. Instead of the buffers being mixed by an LC system, the applied voltage was varied between different reservoirs, allowing different volumes of buffer to be placed at the head of the column where they were mixed forming a dynamic gradient. There is no wasted buffer with this method,

but the exact composition of the buffer at the head of the column cannot be calculated.

### 1.4.3.7. Detectors.

To date the UV/Vis absorbance detector has been utilised extensively in the development of CEC. Other detector systems have been investigated, with mass spectrometry being the most prominent.

The placement of the detection window has been studied when using both UV/Vis absorbance (145,146) and fluorescence (109) detectors. Such studies indicated that the signal is enhanced when detection is performed on column i.e. thorough the packed section, as opposed to in an unpacked section of the column. The degree of retention was also an important factor with unretained solute signals not being enhanced. Another feature of on column detection is that an increase in baseline noise is observed double that of off column detection, which can make quantification difficult. It was also observed that peak tailing only occurred when detection was off column, indicating that the holding frit could be responsible for peak tailing.

Fluorescence detectors (109) have been utilised to determine PAH and with laser induced fluorescence (133), to study the effects of varying column I.D. A novel detection system was developed by Pusecker *et al.* (147) who coupled CEC to an NMR instrument in order to identify metabolites of paracetamol in human urine.

From the early 1990s, there has been a steady interest in linking CEC to a mass spectrometer. This is, in part, due to the low flow rates (<1µl/min) that CEC columns offer, which allow direct coupling to various mass spectrometer ion sources. Verheij *et al.* (133) and Hugener *et al.* (148) were the first to couple a mass spectrometer to a pseudo electrochromatography system which employed both pressure and applied voltage. True CEC-MS was not achieved until Gordon *et al.* linked CEC with continuous-flow fast-atom bombardment mass spectrometry (149) and then electrospray (ESI) (123).

The interfacing of CEC to an MS instrument has meant that longer columns than normal are required; this increases dispersion of the solutes in the unpacked section of the capillary. Lane *et al.* (150) developed a novel interface which incorporated both the CEC column and the probe in the electrospray source, thus allowing shorter columns. Higher field strengths were possible, leading to reduced dispersive effects and decreased analysis times. Paterson *et al.* (134) demonstrated the viability of this method by analysing and quantifying a series of potential drug candidates. The concept was further developed by Schmeer *et al.* (99) who constructed a CEC column in the MS source, eliminating the need for any make up flow or capillary connections.

Smith *et al.* (100) reported that as peaks became sharper, with plates per metre above $10^6$, a full scan spectrum was impossible to obtain from each peak, as the overall scan time needed was greater than the peak width. This problem was overcome by Lazar *et al.* (151,152) and Wu *et al.* (153), who

both used a Time of Flight (TOF) spectrometer. This allowed greater sensitivity, due to its ability to scan the full spectrum of a compound in 100 to 200μs. The use of a tandem MS instrument was reported by Lane *et al.* (154) who used the tandem MS to decode beads used in combinatorial synthesis.

### 1.4.4. Applications.

CEC is still in its infancy, with the majority of the published work being concerned with the development of the general techniques required for CEC, including stationary phases and packing methods. However, there are an increasing number of applications being devised for CEC. These are similar to those already in use with both CZE and HPLC and are concerned with trace and impurity analysis, chiral compounds and main component assays.

One of the reasons for the development of CEC was to enable the separation of neutral compounds. The driving force behind this was to eliminate some of the associated problems that other CE based techniques had when dealing with neutral compounds. However, the range of target compounds has broadened to include separations based on ion suppression of both acids and bases. Acidic compounds are resolved at low pH, while basic analytes can be separated over a wider pH range. As a result, cationic species can be separated by either ion suppression at high pH or at low pH by a combination of their differing electrophoretic mobilities and retentive properties.

The most widely utilised class of compounds has been the aromatic hydrocarbons, especially the poly-aromatic hydrocarbons (PAH). These are

neutral solutes which are readily separated on ODS phases and a significant improvement over separation by LC, with an increase in efficiency of ca. 75%, has been reported (115).

The majority of the methods reported are similar (57,59,80,84,93,109,115,123,144), with the buffer being made from acetonitrile with 20-25% aqueous electrolyte. The electrolytes that are used also determine the pH, with borate ca. pH9, TRIS ca. pH8, MES ca. pH6 and phosphate ca. pH7.5. An interesting variation was the work of Whitaker and Sepaniak (85) who separated $C_{60}$ and $C_{70}$ fullerenes and various PAH by nonaqueous CEC.

One of the main driving forces behind the development of CEC has been the pharmaceutical industry. This is a result of the potential increases in efficiency and resolution coupled with the small sample requirements and rapid separations that CEC offers. Therefore, a large number of the applications that have been reported to date are pharmaceutical in origin. This can be seen from the range of compounds, which have been reported; antibiotics (111,121,155), barbiturates (156), prostaglandins (111), diuretics (100,105,137,157), steroids (99,111,113,121,123,138,139,150,158,159), macrocyclic lactones (150), phthalates (80), parabens (93), anti-depressants (100) and anti-inflammatories (160).

Steroids have been studied widely by CEC. This is the result of them being readily separated by CEC, whereas an equivalent HPLC method would

struggle to resolve the steroids even after optimising the separation. In the case of CEC, the methods used have required little or no optimisation. The general conditions that have been reported have all described separations on an ODS column with a buffer containing between 70 and 80% acetonitrile, at pHs above 6.

Another example of the versatility of CEC is the separation of diuretic compounds. Both Euerby *et al.* (73) and Taylor and Teale (137), demonstrated this separation at low pH using a gradient. Either a stepwise (73) or continuous flow (137) gradient had to be utilised to fully resolve the diuretics.

Basic compounds can be separated by two different approaches, either at high pH to suppress their charge or at lower pH using a combination of their differing electrophoretic mobilities and retentive properties. Taylor and Teale (137) demonstrated a gradient separation of two benzodiazepines at low pH with a buffer containing ammonium acetate in acetonitrile, in contrast to the separation of a group of tricyclic antidepressants by Smith and Evans (100) who utilised a high pH (9.8) to achieve separation.

Another application area for CEC is the separation of biological compounds, including amino acids (139,157,161-165), proteins (166-169), peptides (99,114,153,170-172), nucleic acids and oligonucleotides (131,135,173-176). The majority of these separations have been undertaken using continuous

bed and molecularly imprinted polymer phases instead of packed columns. Continuous beds are discussed in a later section.

As a result of the pharmaceutical and biological interest in CEC, chiral separations have been investigated. Several different methodologies have been put forward that can be utilised in chiral determinations. These are pre-derivatisation with a chiral derivatisation agent, the inclusion of a chiral additive in the mobile phase in conjuction with a reverse stationary phase (177), or various chiral stationary phases (178-182).

As well as the large number of publications from the pharmaceutical and biological sectors there has been a limited number of papers published relating to other areas. These include the forensic determinations of cannabinoids (183) and explosives (184) while Li *et al.* (185) reported ion exchange CEC for the environmental monitoring of small anions at a nuclear power station.

There is little variation in the composition of the buffers in many of the applications, with them typically containing 70 to 80% acetonitrile. The only variation is the buffer that is present, which is chosen in part as a result of the pH required, as most buffers only have a range of $\pm$ 1pH unit around their $pK_a$. On an equivalent LC system there would be a significant range of mobile phases required, so CEC can simplify method development considerably.

## 1.5. Alternative Methods.

### 1.5.1. Introduction.

The rationale for CEC is the separation of neutral compounds, which cannot be resolved under normal CZE conditions. There are however other approaches available to achieve the separation of neutral analytes. Two of these methods can be claimed to be extensions of the CEC technique that has been discussed previously (open tubular liquid chromatography and continuous bed), while the third is micellar electrokinetic capillary chromatography.

### 1.5.2. Open Tubular Liquid Chromatography (OT-LC).

Open tubular columns, or OTC, were first discussed for use with gas chromatography by Golay in 1958 (186). In chromatography, separation is achieved by a dynamic equilibrium of the solute between two phases, the stationary phase and the mobile phase. In OT-LC the packing material is removed and the functional groups are attached to the inner wall of the column. Columns used in OT-LC have an internal diameter of 3 to 5$\mu$m and lengths of up to 6 metres (187), as the phase ratio on the capillary is 350 times less than that found with normal LC packing material (188), and hence an excessive length of column is required to perform the separation.

Several problems (mainly instrumental) have held back the development of OTC in LC. Problems with preparation of the columns have also been encountered, as they require a high phase ratio to be coated onto the surface

of a narrow capillary, to allow sufficient resolution to be gained by the solutes which interact with the stationary phase on the capillary surface.

CE has overcome some of the instrumental problems that OTC-LC previously encountered. As with OTC-LC columns, the surface of the CE capillary is coated with a thin film of the stationary phase. However, the internal diameter of the columns has been increased to 10 - 50$\mu$m, with the length reduced to approximately 1m.

Initial attempts to modify CE capillaries involved etching the inner surface to increase surface area and reactivity before the stationary phase was bonded to the surface (189,190). These columns did not have a high enough phase ratio to allow efficient separations to be obtained.

Cross-linked polymeric stationary phases as the surface film have been investigated, with polysiloxanes (191-194) and polyacrylates (195,196) being attached to the surface by a variety of methods. These produced columns that were stable and which had a high phase loading. However, these films blocked the surface charge and hence suppressed the EOF.

To improve the efficiency of the columns both the thickness of the film placed on the surface and the diffusion coefficient needed to be increased. A further type of surface modification took into account the high diffusion coefficients of porous material. This resulted in a thin porous silica layer being placed on the surface of the capillary with the functional groups attached to the porous

surface coating. Several methods have been reported for the creation of this porous silica layer, for example Tock *et al.* (197) initially coated the surface dynamically by precipitating a layer of porous silica onto the capillary wall. This was later modified to statically load the layer (198), resulting in an increase in the phase ratio, but was unsuitable for columns with an internal diameter of less than 10μm.

A recent development has been to make use of sol-gel technology to produce the porous silica film (191,199,200). This method is simpler than the previous methods as the silica phase is pre-modified so the functional groups do not have to be added in a second reaction step once the silica layer has been deposited on the surface. This process can be controlled to produce films with the required properties e.g. pore size and surface area.

The main problem associated with OT-LC is a lack of sensitivity, due in part to the small capillary internal diameters and low sample volumes that are required to allow efficient separation.

## 1.5.3. Rigid Monoliths and Continuous Beds.

Hjerten *et al.* (201,202) were the first to adapt continuous beds from LC (203,204) to CE, producing a bed of soft hydrophilic polyacrylamide gel. Continuous beds are formed from a porous network, within the column, of either silica (205,206) or organic polymers (207).

A problem with these columns concerns the swelling and compressing of the gel on wetting. This results in the gel changing size. Wang *et al.* (208) proposed that this could be overcome by the use of a rigid monolith consisting of a macroporous poly(styrene-co-divinylbenzene) support structure, which could be prepared in-situ by a free radical polymerisation reaction. This lets the pore size be controlled, allowing the flow to pass through the column's support structure instead of over it, leading to an improvement in mass transfer.

Both continuous beds and rigid monoliths have been applied to CE, where the addition of charged groups to the polymer network has allowed an EOF to be generated. Fujimoto *et al.* (209-211) reported separations using a polyacrylamide gel, which was produced by silanizing the capillary wall prior to forming a continuous bed. Later Liao *et al.* in 1996 (213) formed a similar continuous bed in a one step reaction.

Peters *et al.* (214-216) demonstrated the formation of a rigid monolith in a one step polymerisation reaction. This allowed tight control over the formation of the monolith, thereby controlling pore size and chromatographic properties of the column.

A novel development from this is Molecular Imprinted Polymers, which allow the preparation of a stationary phase with a pre-determined selectivity (217). These columns are fabricated by a polymerisation reaction around an imprint species. The monomers used are selected for their ability to bond by a non-

covalent interaction with the imprint species, allowing them to be removed after the support structure has formed. When the imprint species are removed, the cavities that remained have a specific size, shape and chemical functionality, which allow highly selective separation and recognition of chiral compounds (218) and enantiomers (219).

These method offers the same potential separation ability that CEC does with LC stationary phases, but with the advantage of no frits being required.

### 1.5.4. Micellar ElectroKinetic Capillary Chromatography (MEKC).

MEKC was first discussed by Terabe *et al.* in 1984 (220). This technique allows the separation of both charged and uncharged compounds. The addition of a surfactant to the buffer, in concentrations greater than the critical micelle concentration of that surfactant, produces micelles that act as a pseudo stationary phase (Figure 13).
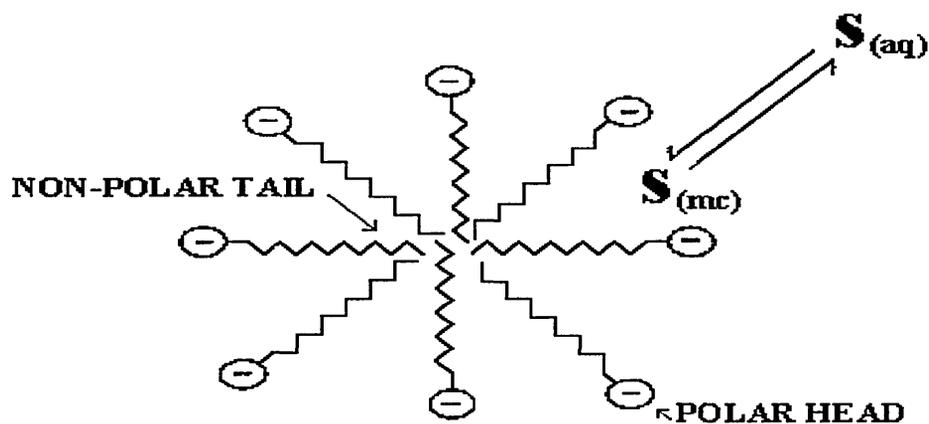


Figure 13. Diagram of an eight-membered micelle.

MEKC is considered to be a chromatographic technique, as the separation relies on the differential partitioning of the solutes between the micelles and the bulk solution. The separation mechanism is similar to reverse phase liquid chromatography in that the degree of hydrophobicity is related to the retentive properties. Hydrophilic compounds have little interaction with the micelles and are eluted first. The more hydrophobic compounds elute later because there is a greater degree of interaction between these and the micelle.

Micelles may lack some of the selectivity of a comparative LC stationary phase, but an increase in speed and efficiency of the separation balance this. This is partly a result of the dynamic equilibrium that is formed between the solute and micelle, allowing a greater number of interactions between the mobile and pseudo stationary phase than that observed in LC.

Micelles are approximately spheres made up of between 30 and 100 monomer units, with this configuration allowing the surfactant to be at its lowest possible energy state. They are short-lived entities with the monomers aggregating for about $10\mu s$ before dispersing. A dynamic equilibrium is created between the monomers in the bulk solution and the number of micelles. The internal region of the micelle has a unique microenvironment, which is distinctly different from that of the bulk solution. This is due to the amphiphilic nature of the surfactant, which has a hydrophobic tail and either a polar or ionic head.

Altering the head group (Table 7) of the surfactant can change the separation performance of the micelle. This can result in a change in the degree of solubility that is observed in the aqueous environment. Additionally, the length of the retention window can be altered (Figure 14), by changing the effective mobility of the surfactant and, therefore, its electrophoretic velocity.
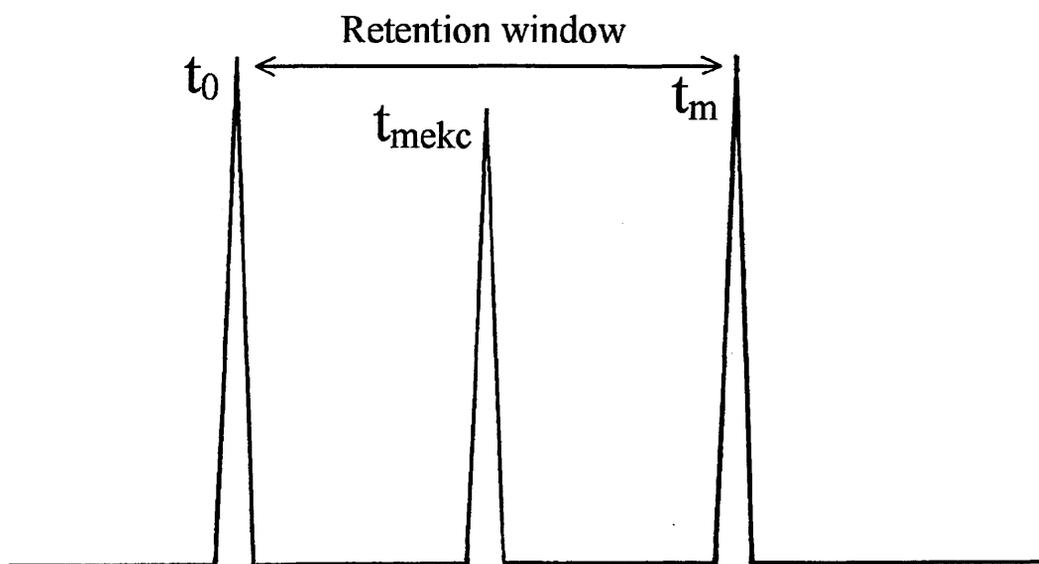


Figure 14. The retention window is the time period between $t_0$ and $t_m$, with $t_{mekc}$ being the elution time of the uncharged solute combined with the micelle and $t_m$ the surfactant migration time. $t_0$ is either the migration time of a solute that has no electrophoretic mobility and will not interact with the micelle (methanol or acetonitrile) or a disturbance in the baseline (which can be problematic due to reproducibility).

The hydrophobic tail (Table 7) can also have a significant effect on the separation, as this can alter the microenvironment that is present at the centre of the micelle. These variations can be achieved by altering the chain length or the nature of the tail.

| Head group | Examples |
|---|---|
| Anionic | Sodium Dodecylsulfate (SDS) |
| | Lithium Perfluoroctane Sulphide (LiPFOS) |
| Cationic | Dodecyltrimethylammonium Bromide (DTAB) |
| | Tetradecyltrimethylammonium Bromide (TTAB) |
| Zwitterionic | *N*-dodecylsultaine |
| Nonionic | Brij 35 |
| | Tween 20 |
| | |
| **Tail groups** | |
| Hydrocarbon | SDS – $C_{12}H_{25}OSO_3^-Na^+$ |
| Fluorocarbon | LiPFOS – $C_8F_{17}SO_3^-$ |
| Bile salts | Sodium Cholate |
| | Sodium deoxycholate |

Table 7. Different species of surfactants.

There are drawbacks to MEKC separations, namely the narrow retention window and a lack of sensitivity. The lack of sensitivity is due to the fact that as the solute passes the detector only a fraction of the solutes are detected, depending whether they are contained within the micelle or not. Also the surfactants preclude the use of certain detectors, most importantly the mass spectrometer.

## 1.6. Conclusion.

As this first chapter shows there is already a well established theoretical and practical knowledge of capillary electrophoresis (CE). However, the separation methods for neutral solutes are still in the early stages of development, with the exception of MEKC, which is well understood and has been successfully used in conjunction with various applications.

The aim of this investigation was to developed capillary electrochromatography (CEC), which is one of the methods that can be used to separate neutrals by CE, and which is currently experiencing interest from various research groups. To achieve this, various tasks were undertaken, firstly and most importantly the development of a suitable packing methodology, due to the costly of and limited commercially available CEC columns. Once a reliable column fabrication process had been developed the operational parameters of the columns and the Prince Technologies employed in these studies to determine the optimum conditions and a firm practical knowledge base. That knowledge would then be used when applications were undertaken, which was the final stage of the overall investigation. This process is reported and discussed in the following chapters.

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# CHAPTER 2, Materials and Methods.

## 2.1 Equipment

All electrochromatograms were generated using a Crystal CE 310 (Prince Technology, Netherlands) fitted with an ATI UNICAM 9200 UV/Vis detector (ATI Unicam, Cambridge, UK). Absorbance measurements were made at 254nm, with the signal being recorded on a Spectra - Physics 4290 integrator (Spectra - Physics, USA). Fused silica capillaries (Composite Metals Services, Worcs, UK) of 50µm I.D. and 375µm O.D. were employed throughout. The frits and detection window were formed using a capillary burner (Glaxo-Wellcome, UK), with the capillaries being packed using a Shandon HPLC Column Packer (Hypersil, Cheshire, UK).

## 2.2 Stationary Phases

All the packing materials were gratefully received as donations from various individuals who are listed below with the material that they provided;

| | |
|---|---|
| 5µm $d_p$ Spherisorb ODS-1 | Donated by G. Lord, UMIST, UK. |
| 5µm $d_p$ Spherisorb ODS-1 | Donated by P. Myers, Phase Sep., UK. |
| 5µm $d_p$ Spherisorb Silica | Donated by P. Myers, Phase Sep., UK. |
| 3µm $d_p$ Spherisorb ODS-1 | Donated by P. Myers, Phase Sep., UK. |
| 3µm $d_p$ Hypersil ODS | Donated by P. Cooper, Zeneca, UK. |
| 3µm $d_p$ Exsil 100 ODS | Donated by I. Chappel, Alltech, UK. |
| 3µm $d_p$ Exsil Pt C18 | Donated by I. Chappel, Alltech, UK. |

## 2.3 Reagents

Solvents used were acetonitrile and acetone (HPLC grade, Aldrich, Dorset, UK) and all the water used was distilled and then de-ionised (MilliQ water) in situ (Millipore, Herts, UK).

Chemicals used were di-sodium tetraborate and thiourea (BDH Chemicals Ltd., Dorset, UK) and TRIS, sodium phosphate, sodium hydroxide, hydrochloric acid, biphenyl and phenanthrene (Aldrich, Dorset, UK).

## 2.4 Solutions

0.1 molL$^{-1}$ NaOH 1g of sodium hydroxide was dissolved in 250mL H$_2$O.

0. 1 molL$^{-1}$ HCl. 21.4 mL of concentrated hydrochloric acid (11.65molL$^{-1}$) was diluted in 250mL H$_2$O.

1 molL$^{-1}$ borate (Stock Solution). 3.83g di-sodium tetraborate was dissolved in 500mL H$_2$O.

100 mmolL$^{-1}$ TRIS (Stock Solution). 0.21g TRIS was dissolved in 100mL of H$_2$O.

100 mmolL$^{-1}$ sodium phosphate (Stock Solution). 1.36g sodium phosphate was dissolved in 100mL H$_2$O.

100 mmolL$^{-1}$ ammonium acetate (Stock Solution). 0.77g ammonium acetate was dissolved in 100mL of H$_2$O.

From these stock solutions appropriate dilutions were made to obtain the final concentration required.

## 2.5 CEC Conditions

Column                Total length 60cm.

                      Effective Length 44cm.

                      Packed length 20cm.

                      I.D. 50μm

Conditioning          1hr on Shandon packer at 2000bar.

                      Ramped over 3hrs from 2Kv to 30Kv or 2Kv

                      overnight.

Loading               Electrokinetic – 30Kv for 0.2min.

Run Voltage           30Kv.


The test compounds (thiourea, biphenyl and phenanthrene) were dissolved in a mixture of acetonitrile (80%) and aqueous solution (20%) containing the appropriate electrolyte (unless otherwise stated). The pH of this solution was left unadjusted. The concentration of each of the analytes in solution was 50ppm (w/v).


The electrolytes used were borate or TRIS. The electrolyte was dissolved in water to give solutions of either 4mmolL$^{-1}$ or 20mmolL$^{-1}$ respectively. The aqueous solution was then added to acetonitrile in ratio of 80:20 organic to aqueous (unless otherwise stated). After mixing the aqueous and organic solutions, the pH was adjusted by the addition of 0.1 molL$^{-1}$ aqueous NaOH or HCl.

These are the general conditions used throughout this investigation unless otherwise stated. The packing procedure and column conditioning will be discussed in the following chapter.

# CHAPTER 3, Column Fabrication.

## 3.1 Column Fabrication, Introduction

The development of CEC as an analytical tool is still in its infancy, with various groups investigating its potential and few commercial columns being available. As with any new technique in its early development, there are no established procedures to follow. In this study of CEC, the packed columns had to be fabricated using the type of devices discussed in chapter 1. Therefore, before any investigation into the operational parameters and applications of CEC, a suitable method of column fabrication had to be devised with the equipment available. This chapter discusses the development of an in-house packing method and the associated problems that had to be overcome.

## 3.2 Packing Procedure

The chosen packing material was placed with 1ml of acetone in a 2ml glass sample vial. The vial was then placed in an ultrasonic bath and sonicated for approximately 5 minutes to form a well dispersed slurry.

The initial retaining frit was formed from dried silica (Spherisorb 5μm silica). A small plug, 0.5-1mm in length, was tapped into the capillary from a glass slide and then fused into place by heating the tip of the capillary in a Bunsen burner flame for 5 seconds.

The slurry was placed in a packing bomb (this vessel is described later in section 3.3.3), into which the capillary was positioned such that the capillary

end was at the mid-point of the bomb. The capillary was then locked into place.

The packing bomb was pressurised to 6000 psi with a Shandon HPLC packer for the duration of the packing. Once the desired length of packing was obtained the pressure was slowly decreased over a period of 5 minutes. The slurry was then replaced with water, prior to pressure being reapplied at 4000 psi for one hour.

Fusing the packing material together formed the final holding frits. These were formed using a modified Glaxo burner, with a 6 Volt power input. The first frit was formed at the inlet end and was heated for between 2 and 3 minutes, depending on the packing material. The initial retaining frit was then removed. The column was left for fifteen minutes under pressure with a water flow to allow the excess material at the inlet to be flushed out. The second frit was then formed, to give the desired packed length, by heating for 10 seconds. The system was then depressurised over a period of 5 minutes. Excess packing material from the outlet end was removed by reversing the capillary and flushing out with de-ionised water at 4000 psi.

Once the capillary had been flushed and slowly depressurised the detection window was formed. The polyimide coating was removed by a modified Glaxo burner (9 Volt power input, for 10 seconds), which burned off the outer coating. Any polyimide remaining in the window area was removed by gently wiping with acetone.

Before the column was placed in the CE it required an initial conditioning. The capillary was washed with the chosen running buffer for at least one hour at 4000 psi using the Shandon HPLC packer. The pressure was again removed slowly over a period of 5 minutes. The capillary was then placed in the CE to be finally conditioned by a 3 hour ramp, from 2Kv to 30Kv. An outline of this procedure is shown in Figure 1.



1. Formation of end frit
   3μm dry silica

2. Packing of column,
   high pressure.

3. Formation of 2nd end frit,
   from stationary phase.

4. Formation of retaining frit
   from stationary phase.

5. Removal of unwanted
   packing material.

6. Window formation.
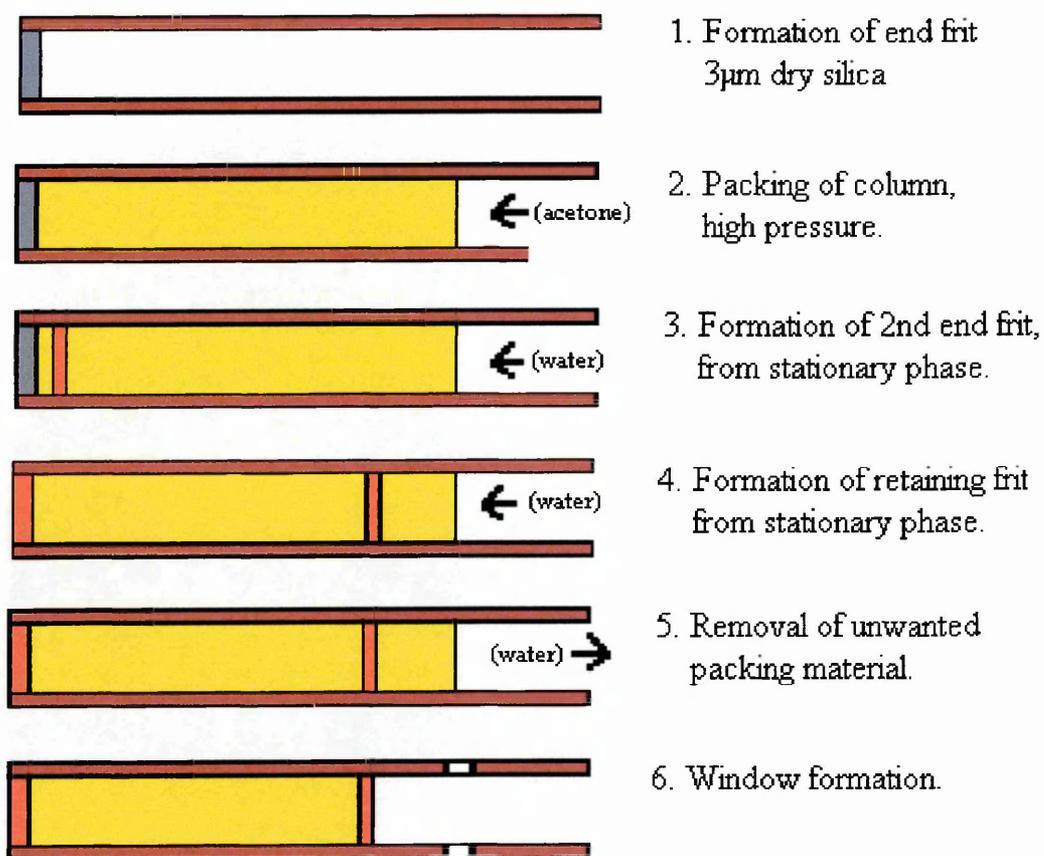
Figure 1. Schematic of capillary packing procedure

## 3.3 Discussion

### 3.3.1 Column Fabrication

Many of the ideas and concepts that were used in this development came from the work of other groups reported in the literature (1-10), which has already been discussed in chapter 1. The basic principles are from the method developed by G. Lord, who gave a practical demonstration of his technique.

### 3.3.2 Retaining Frit

The initial retaining frit is an important factor in the fabrication of the column. If it is too thick or impermeable then the packing process can fail due to low flow rates through the capillary. These can increase packing time and give an increased risk of the stationary phase aggregating and blocking the column. Another problem can be development of a high back pressure in the column once approximately 5-10cm of packing material has been loaded, resulting in a flow rate at which little or no material is forced into the column. Even if the desired length is packed a low flow rate can also affect the formation of the final holding frit, with an increased risk of the frit being impermeable.

The first method used was retained throughout the study because it was very simple and the dried silica could be stored over a long period. 5$\mu$m bare silica was slurried in acetone, then poured onto a glass slide where the acetone evaporated off, leaving a thin layer of dried silica. Tapping the end of the capillary into the silica then formed the frit; the end of the capillary had to be flat to obtain the best results. It was found, by trial and error, that a plug length

of 1mm, heated for *ca.* 5 seconds at the tip of the corona of the Bunsen flame, gave the best results.

### 3.3.3 The Slurry

To pack the capillary column a low viscosity solvent was required to draw the stationary phase thorough column, minimising back pressure as the packed length increased. The most commonly used solvents in the literature have been acetone and acetonitrile (5, 7, 11-13). In this study, acetone was used.

The stationary phase was mixed in a 1:10 ratio (w/v) with acetone before being sonicated for a minimum of 5 minutes. Other groups have used this ratio of packing material to solvent (1, 11). When the slurry was sonicated for less than 5 minutes there was found to be an increased probability of the material still being aggregated causing blockages or slow inefficient packing.

The packing bomb was adapted from an empty HPLC column, which was reduced in length to 2.5cm so that it held ~1.5ml of the slurry. Figure 2 shows the construction of the bomb and placement of the capillary.

Capillary inlet

Capillary tubing

Locking vespal
ferrule

50mm HPLC column
with 4.6mm I.D.

Capillary tubing placed
mid column

Frit

Solvent inlet

Solvent delivery
system
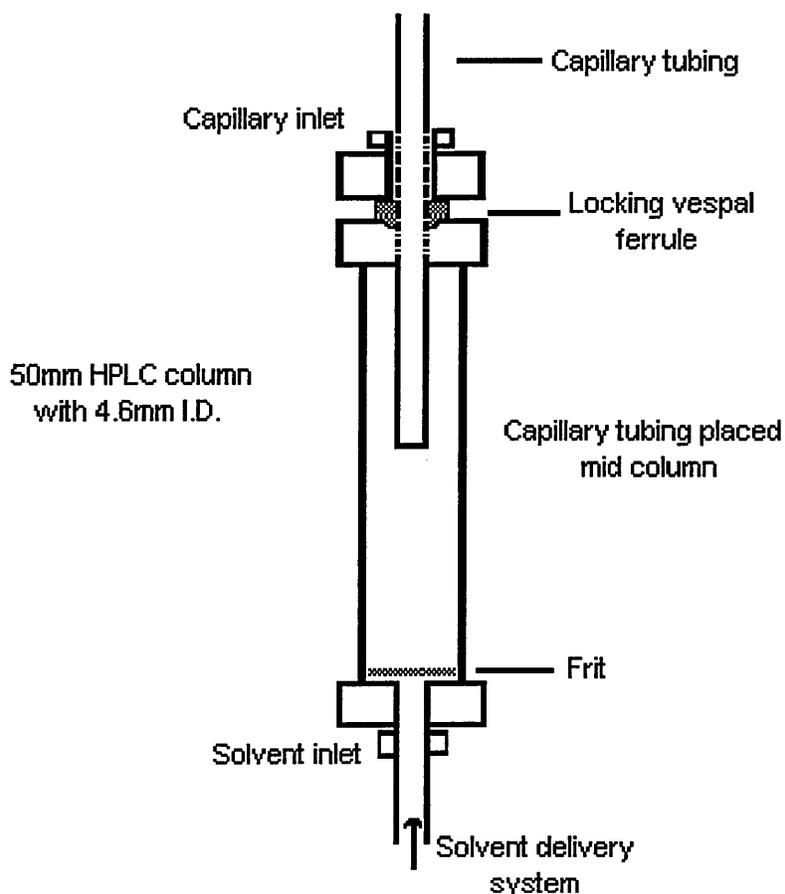
Figure 2. The packing bomb, used in the fabrication of CEC capillaries.

## 3.4 Column Packing

As discussed in chapter one, several methodologies (8,14,15) have been proposed for column fabrication, with early reports mainly concerning slurry packing under pressure with available equipment, e.g. HPLC pumps, to facilitate the packing. This approach was also the initial starting point in this work using various HPLC pumps. These pumps only regulated the flow rate, so as the packed length increased the back pressure rose, as a greater force was required to maintain the flow rate. This resulted in the pumps automatically shutting down as the pressure exceeded the maximum limit.

Even when the flow rate was reduced to counteract this the back pressure still built up.

In an attempt to overcome these problems a Shandon HPLC packer was modified to allow the packing bomb to be fitted. This offered two advantages over the HPLC pumps: higher pressures could be applied to the column and the pressure could be set and maintained by varying the flow. This new system worked well and allowed columns to be packed more readily than with the HPLC pumps.

There were occasions when the packing material aggregated in the packing bomb. It was found that if the packing bomb was placed in an ultrasonic bath once it had been pressurised, there was a reduction in the occurrence of this problem, presumably due to the slurry being continuously agitated.

The formation of large aggregates of packing material inside the capillary was also an occasional problem. This occurred even when the slurry had been sonicated for 5 or more minutes and the packing bomb was placed in an ultrasonic bath. It was found that if the capillary was coiled and placed in the ultrasonic bath the blockage was eliminated and packing continued.

3.4.1 Column Conditioning

Once the column had been packed, it needed to be conditioned before the final retaining frits were formed. Several conditioning procedures were studied during the development of the packing method.

Initially, the column was washed for half an hour with water at the same pressure as during the packing process, but this method did not produce frits that were consistent in nature. It was originally assumed that this inconsistency was due to impurities in the packing material and therefore an extra washing step was introduced.

10% acetonitrile in water was flushed through the column at a reduced pressure of 4000 psi for one hour, before being replaced with 100% water for a further hour. This method did not lead to any significant improvement in the success rate of the columns and neither did an extended flush with 100% water for one hour at 4000psi.

As none of these methods proved any more reliable than the others the simplest was used throughout the rest of the study i.e. conditioning with only water. This also had the added advantage of a shorter conditioning time.

## 3.4.2 Frit Formation

Fusion of the packing material formed the final holding frits. Initial attempts to form these frits used a coil of fuse wire and a 6V battery. The coil was placed onto the capillary and touching the wire to one of the terminals on the battery completed the circuit. This simple system was impractical, as it was difficult to obtain reproducible results.

A Glaxo burner was obtained, which allowed the time and temperature of heating to be controlled. The heating element was a coil of wire (Temco:

Pyromic 80/20 Nr-Cr. 38swg, 0.152mm) contained in a plastic case. This design was better because the capillary was supported at both sides of the heating element.

The design of the CE instrument posed a critical problem. On loading the capillary into the CE it is bent into a U-shape and the second frit is generally located at the top of the bend. On forming the frit, the supporting polyimide was burnt off hence when bent this area was liable to break.

To overcome this problem the burner was modified to allow a lower heat setting to be obtained. This setting was sufficient to fuse the silica but low enough that the polyimide was not blistered by the heat.

Modification of the burner meant that the original power supply had to be replaced with a variable voltage power supply, allowing greater control of the temperature that the burner operated at. The result was that the number of columns broken on loading decreased significantly. It was found that the optimum condition for the burner was to have the input voltage at 6 Volts. With this setting the outlet frit only required 10 seconds heating to form a stable outlet frit, while the inlet frit required between 2 and 3 minutes depending on the packing material.

## 3.5 Overview of Column Failures

Throughout this investigation the greatest problem was the reliability of the columns produced. Table 1 indicates the type and frequency of the column failures. The total number of columns packed was 215 and all eventually failed due to the various, stated below.

| Reason for failure | % of columns |
|---|---|
| Bubble formation | 8% |
| Localised heating | 17% |
| Disintegration of frit* | 51% |
| Column breakages | 20% |
| Unknown | 4% |

Table 1. Indication of the failure rate of columns for specific reasons. (n=215) The reasons for these failures are discussed in the following section. (*Break down of the inlet retain frit resulting in the stationary phase migrating towards the anode on the application of a current across the capillary)

### 3.5.1 Bubble Formation

Bubble formation is a major concern in CEC, resulting in deceased EOF or, in extreme cases, elimination of flow through the capillary. The formation of air bubbles can be detected by an increase in baseline noise, which is generally characterised by a series of very sharp peaks in the UV absorbance trace.

There are various potential causes of bubble formation. These include Joule heating, which can occur due to high electrolyte concentration and discontinuities in the flow velocity and electric field strengths in different

sections of the column, which limit the concentration of electrolytes to below 10mmolL$^{-1}$ (16). Incomplete degassing of the running buffer can also be a cause, as the rough surface of the retaining frit can result in a large number of possible nucleation sites for oxygen desolvation.

Poor quality frits at the outlet can also cause bubble formation because if the flow is restricted then variations in flow velocities between the packed and unpacked sections can be magnified. This results in voids in the bulk flow, which resemble air bubbles.

Limiting the electrolyte concentration and thoroughly degassing the bulk solution reduced bubble formation, with remaining problems due to restricted flows from poor frits. When bubble formation occurred, the column was flushed on the Shandon packer with freshly degassed buffer, before being reconditioned on the CE.

## 3.5.2 Localised Heating

Little has been reported in the literature about localised heating in CEC columns, which causes a small section of the packed bed to dry out, stopping the flow through the packed capillary. It has been claimed that this effect is caused by non-uniformities in the packed bed (15) when packing with a pressure driven system.

Every stationary phase used was affected, with the columns being unrecoverable by reconditioning. However, this effect was minimised by

gradual increase in the voltage applied when the column was being initially conditioned. If the column was not conditioned correctly with a gradual increase in the applied voltages, the risk of the column either disintegrating or showing localised heating was greatly increased.

3.5.3 Frit Failure

Disintegration of holding frits was the most serious problem encountered in the development of reliable CEC. In normal mode, the flow of the buffer is towards the cathode, but the stationary phase is constructed of silica particles, which are anionic in nature and thus flow towards the anode. This means that the inlet frit is susceptible to disintegration by movement of the stationary phase towards the anode, which is identified by a reduction in the expected current by approximately two thirds.

Breakdown of the inlet frit was unpredictable. However, observation indicated that if the column was conditioned over an extended period of time with the voltage being slowly increased there was a reduction in the number of columns that were lost before separations could be carried out on them. New columns were therefore conditioned in one of two ways, either by being left overnight at 2Kv or ramped from 2Kv to 30Kv over a three hour time period. In both cases this appeared to allow the packed bed to stabilise.

Another reason for the loss of the inlet frit was due to failure of the variable resistor in the burner, resulting in variable temperatures being produced during frit formation. There was no obvious indication that the resistor had

failed and this resulted in several weeks of failures before the problem was identified.

Even with the conditioning steps to stabilise the packed bed, many columns were lost and only a limited number of separations were made. Breakdown of the frit was probably the result of the frits not being stable/strong enough to cope with the rapid application and removal of the applied voltage on loading. Disintegration of the frits allowed the stationary phase to migrate towards the anode, disrupting the current flow through the column.

The majority of the frit failures occurred within the first ten injections, which indicated that the retaining frit was inherently weak. If a column survived ten injections then it generally lasted well, allowing up to a further fifty injections to be made, although there were still a significant number that failed earlier than this point. A limited number lasted for more than fifty injections. Generally these were the Exsil columns of both the ODS and Platinum C18 material, which were used to study variations in pH. It was felt that this was the result of the manufacturing process used to produce this material. This assumption was based on the fact that different batches of this material allowed strong frits to be burned, even if the surface chemistry varied between the two types of material supplied or between batches of the same phase (this will be discussed in section 3.5.5).

## 3.5.4 Column Breakage

As previously discussed in regard to frit formation, when the polyimide is removed the silica column is brittle and easily broken. Once the burner had been modified this became a minor problem. However, when the column was being inserted into the detector, the capillary was easily broken if not aligned properly.

## 3.5.5 Batch to Batch Variation in Packing Material

Variation between batches of stationary phase from the same manufacturer was of particular interest, because only a limited quantity of each packing material (~1g) was available. When the initial sample of packing material had been used, replacement material did not come from the same batch as the original. It was observed that different batches of material from the same supplier could display differences in their thermal and chromatographic properties.

As Buszewski et al. (17) state in a review of the preparation of stationary phase material for HPLC, variations in the manufacturing process can change the nature of the material. This results in changes in both the thermal and chemical properties of the material. Formation of new adsorption sites, which are energetically richer than the silanol groups, can alter the distribution of the stationary phase on the surface of the support material. Also the purity of the base silica material is important, as high levels of metal impurities can also affect the distribution of the stationary phase.

Variation in the properties of the packing material can affect the performance of the CEC columns produced. These variations can be observed in terms of stability of the EOF generated by the column and the robustness of the retaining frit. It must be noted that new stationary phases are being designed specifically for CEC.

Clear examples of problems with using LC materials were observed with the Exsil packing material and the Spherisorb material. The Exsil material allowed reproducible fabrication of columns from two batches, with no alteration to the heating regimes for the frits. The columns produced from both batches were robust and allowed extended use. However, the second batch could not hold a stable current, which affected the velocity of the EOF. This resulted in the velocity of the EOF being increased over a series of 30 injections from approximately 0.4 mms$^{-1}$ to 1.2 mms$^{-1}$. This was in contrast to the first batch, which had a stable EOF of *ca.* 1.4 mms$^{-1}$ under the same experimental conditions (20cm packed columns, with a running buffer of 4mmolL$^{-1}$ borate (aq) in acetonitrile at pH 9.2, electrokinetic loading at 30 Kv for 0.2 min). This phenomenon was observed in all five columns produced with the secon batch of material, which was then abandoned.

The Spherisorb material was the most commonly used packing in this investigation, with four batches being used. All but one of these batches produced working columns and the failure of one batch could be the result of the Glaxo burner not working. This would indicate that the Spherisorb material was the most reproducible from batch to batch, although it is not known if

these individual batches came from the same parent batch. Variations in column performance between batches will be discussed in the next section.

3.5.6 Column Stability

Column stability can be related to batch to batch reproducibility. The two batches of Exsil ODS gave significantly different performances in terms of stability of the EOF generated.

To test the columns that had been fabricated, ten replicate injections were made to check the stability of the column. Apart from the second batch of Exsil ODS that could not hold a stable flow, all other stationary phases exhibited the ability to hold a stable current. Generally, any observed instability in the flow was overcome by replacing the running buffer with freshly prepared buffer. In all cases the relative standard deviation of the EOF velocity was less than 5%, with the majority being less than 2%.

One experiment that clearly demonstrated the stability of the CEC columns involved a column receiving 30 repeat injections over a period of one day. (Table 2, Figure 3). These results are typical of those observed in later columns with packing material from different manufacturers.

|  | Thiourea | Biphenyl | Phenanthrene |
|---|---|---|---|
| **Mean Time (min)** | 4.68 | 6.38 | 6.97 |
| **Standard Deviation** | 0.08 | 0.09 | 0.09 |
| **%RSD** | 1.64 | 1.35 | 1.34 |

Table 2. Repeat injection (n=30) of a test mixture over a one day period. Using a 3μm Spherisorb ODS column packed to a length of 20cm with a running buffer of 4mmolL$^{-1}$ borate (aq) in acetonitrile at pH 9.2.
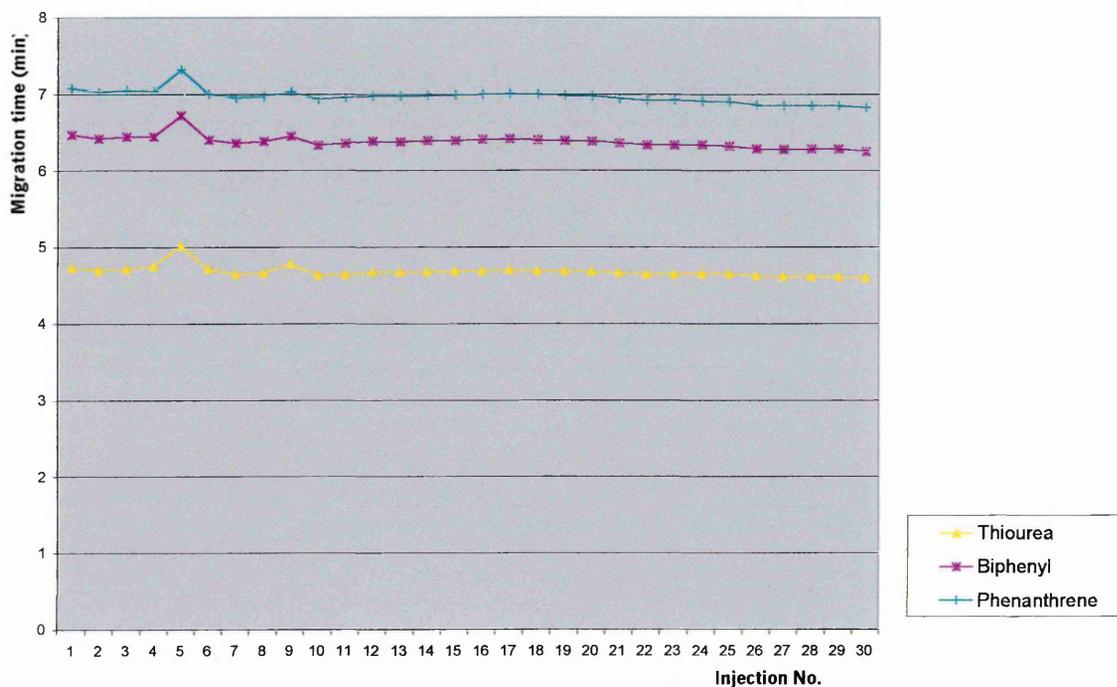


Figure 3. Variation in migration time of the test analytes over a one-day period. Using a 3μm Spherisorb ODS column packed to a length of 20cm with a running buffer of 4mmolL$^{-1}$ borate (aq) in acetonitrile at pH 9.2.

## 3.6 Conclusion

There are several areas that could be improved in the fabrication process but items required to make these improvements were not available at the time of the initial development of the packing method. These include micro-tight unions and in-line filters, developed for micro-HPLC. The in-line filter would provide an independent frit, which the stationary phase could pack up against, allowing more reproducible flows during packing and frit formation.

The micro-tight unions could be used to hold rigid any part of the capillary column that has had its polyimide coating burned away. This could strengthen the area around the second frit, as the capillary is easily snapped at this section of the column on loading into the CE instrument. This would also allow the use of a higher temperature over a much shorter period of time (~10s) in frit formation, as the original Glaxo burner was intended to be used.

The development of pressurised CEC has been documented (6,9,10,13,18-20). The experiences reported here have led to a feeling that pressurisation of the system could have distinct advantages, whether it be partial or total pressurisation of the system. Apart from the increase in electrolyte concentration that it would permit, its effect on the retaining frit(s) could be of greater importance. The pressure applied to the column could potentially increase the life span of the inlet frit, which proved unreliable throughout the development reported here.

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# CHAPTER 4, Optimisation of Operational Parameters.

## 4.1 Operational Parameters, Introduction.

The early stage of development of CEC has meant that there are, as yet, no defined and/or universally accepted procedures in the design of a CEC system. Therefore, each instrument manufacturer has design concepts that are slightly different, which has led to differences in the requirements and hence performance characteristics of each of the various instruments. Before any application work can be undertaken, therefore, an understanding of the basic instrumental and experimental conditions for the equipment used needs to be achieved.

The Prince Technology CE instrument utilised in this study is of modular design; this allows a variety of different detectors to be readily attached to the instrument. However, a minimum capillary length of 60cm is required. This is approximately 2 to 3 times longer than the minimum capillary length requirement of other instruments.

Given these differences and a lack of published data on the Prince Technology CE instrument, it was necessary to characterise how this system responds to variation in conditions, including composition of the running buffer and sample plug and the packed column. This then allowed informed decisions to be made when applications were undertaken, with the aim of improving selectivity and sensitivity.

## 4.2 Equipment

All the equipment and reagents that have been utilised in this chapter have been described previously in chapter 2.

## 4.3 Results and Discussion

### 4.3.1 Electrolyte.

Initially, borate was used as the supporting electrolyte, but the useable concentration was limited as precipitation occurred when acetonitrile, the major component of the buffer, was added. The concentration of aqueous borate was therefore restricted to below 6 mmolL$^{-1}$. A 4 mmolL$^{-1}$ borate solution was chosen to make up the running buffer, which led to a final concentration in the running buffer solution of 0.8 mmolL$^{-1}$, thereby reducing the risk of precipitation.

An attempt to increase the borate concentration was made by doubling the volume of the borate solution in the running buffer, from 20% to 40%, increasing the final concentration of borate in the buffer from 0.8 mmolL$^{-1}$ to 1.6 mmolL$^{-1}$. The result is shown in Figure 1.
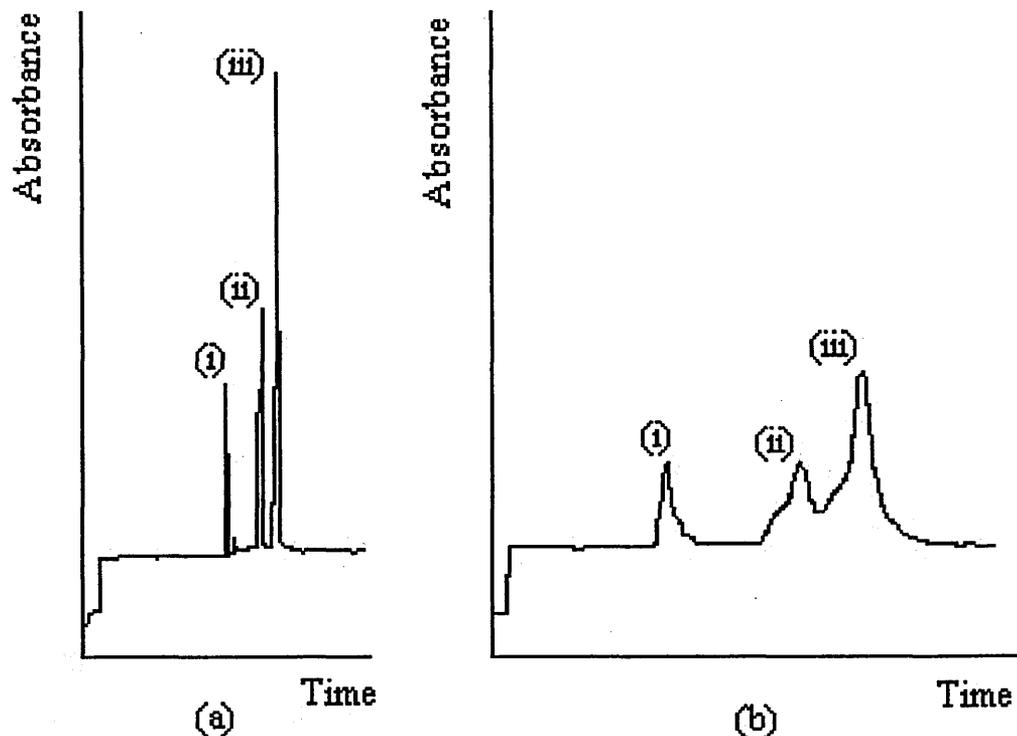
Figure 1. Representative electrochromatograms from a 20cm packed column of 5µm Spherisorb (n=3), (a) 80% acetonitrile with 20% 4 mmolL$^{-1}$ borate solution, pH 9.2. (b) 60% acetonitrile with 40% 4 mmolL$^{-1}$ borate solution, pH 9.2. Elution order (i) thiourea (flow marker), (ii) biphenyl and (iii) phenanthrene.

Significant band broadening of all the test analytes occurred when the borate solution volume was increased. There was an expectation that the migration times would be increased along with resolution as electrolyte concentration rose. Figure 1 demonstrates that while this prediction held there was also considerable band broadening with the increase in water content. Instead of further investigation, an alternative approach was attempted that allowed the electrolyte concentration to be varied while keeping the water content constant.

Due to the limited concentration that borate offers (<6 mmolL$^{-1}$) an alternative electrolyte was required. It was decided that instead of another inorganic

electrolyte, an organic electrolyte would be used. This was due to the lower conductivity offered by an organic electrolyte, hence greater concentrations could be used, thus enabling a greater control to be exerted on the velocity of the EOF. TRIS was chosen as the new electrolyte to investigate the effects of changing electrolyte concentration.

The investigation used a 3$\mu$m Spherisorb ODS column. The running buffer was comprised of 20% TRIS solution (10, 20, 30 or 40 mmolL$^{-1}$ of TRIS in an aqueous solution) in acetonitrile, adjusted to pH 8. This produced an effective concentration range of between 2 mmolL$^{-1}$ to 8 mmolL$^{-1}$ in the running buffer solution.

CE theory states that as the concentration of the electrolyte is reduced the observed EOF velocity is increased. The data contained in Table 1 confirm that this theory holds true for CEC systems as well.

| Conc$^n$ of Electrolyte (mmolL$^{-1}$) | Observed Velocity of EOF. (cm s$^{-1}$) | %RSD (n=10) |
|---|---|---|
| 30 | 0.107 | 0.74 |
| 20 | 0.112 | 0.61 |
| 10 | 0.123 | 1.93 |

Table 1. Observed velocity of the EOF as the electrolyte concentration is altered. (EOF measured by thiourea, which is unretained on the column). These were run on a 3$\mu$m Spherisorb column packed to a length of 20cm, with a running buffer of 20% TRIS (aq) in acetonitrile at pH 9.

With a decrease in the EOF velocity the observed migration times for the analytes increased as demonstrated in Tables 2 and 3. However, there

appeared to be narrowing of the peak widths, which could be an indication of a focussing effect on the analytes at higher electrolyte concentration. This could be explained by differences in the electrolyte levels between the sample plug (which remained constant and does not have its pH adjusted) and the bulk solution.

| Electrolyte concentration (mmolL$^{-1}$) | Migration time (min) | %RSD (n=10) | Base peak width (min) | Peak efficiency (N) |
|---|---|---|---|---|
| 30 | 7.65 | 0.33 | 0.081 | 142828 |
| 20 | 7.18 | 0.59 | 0.20 | 21033 |
| 10 | 6.57 | 1.97 | 0.19 | 18546 |

Table 2. Profile of biphenyl response to changes in the bulk electrolyte concentration. These were run on a 3μm Spherisorb column packed to a length of 20cm, with a running buffer of 20% TRIS (aq) in acetonitrile at pH 9. (Peak efficiencies calculated by equation 1.19)

| Electrolyte concentration (mmolL$^{-2}$) | Migration time (min) | %RSD (n=10) | Base peak width (min) | Peak efficiency (N) |
|---|---|---|---|---|
| 30 | 8.35 | 0.39 | 0.085 | 154439 |
| 20 | 7.74 | 0.63 | 0.14 | 45548 |
| 10 | 7.16 | 1.80 | 0.20 | 20477 |

Table 3. Profile of phenanthrene response to changes in the bulk electrolyte concentration. These were run on a 3μm Spherisorb column packed to a length of 20cm, with a running buffer of 20% TRIS (aq) in acetonitrile at pH 9. (Peak efficiencies calculated by equation 1.19)

The peak efficiency of phenanthrene at 20 mmolL$^{-1}$ electrolyte concentration was significantly higher then expected, if the trend follows that of biphenyl. This can be explained by the use of peak width at base instead of peak width at half height (equation 1.18) to determine peak efficiency. This was the result of the integrator only providing peak width at base data, which can be effected by interferences and peak tailing more readily then peak width at half height.

When the electrolyte concentration in the running buffer reached 8 mmolL$^{-1}$ air bubbles were observed. These air bubbles produced multiple sharp peaks on the electrochromatograms (Figure 2) and a variable EOF, and were confirmed by examination under a microscope. After reconditioning the capillary with a fresh buffer solution containing 40 mmolL$^{-1}$ TRIS solution, bubble formation was again observed. When the concentration was lowered bubble formation ceased.
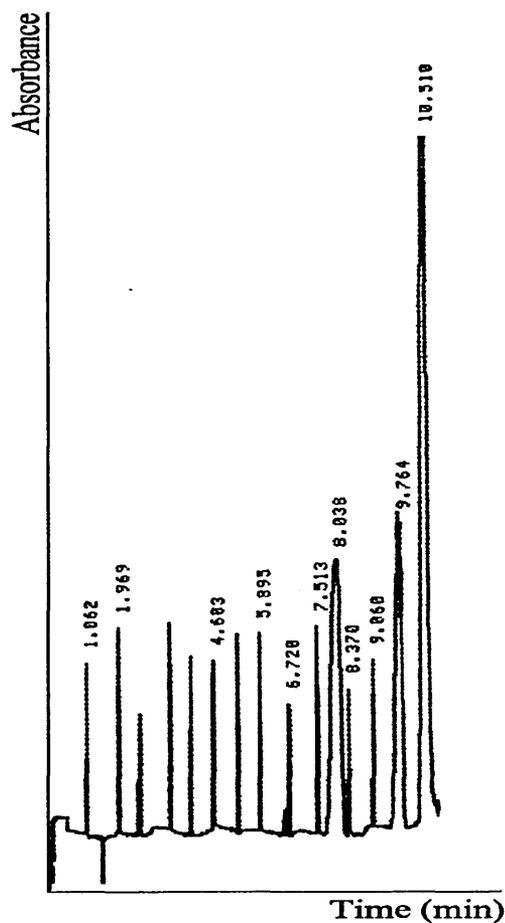


Figure 2. Example of an electrochromatogram that was produced when air bubbles were present in the CEC system.

## 4.3.2. Effect of pH

The choice of electrolyte can also be dictated by the pH requirements of the separation. Generally, acidic compounds require a low pH while basic compounds require a high pH, to suppress the charge present on the solute.

The running buffer used was 20% 20 mmolL$^{-1}$ TRIS (aq) in acetonitrile, with the pH adjusted with 0.1 molL$^{-1}$ HCl Other conditions were as stated previously and the stationary phases used were Exsil 100 ODS and Pt C18.

As expected, the linear velocity of the EOF decreased as the pH was reduced from pH 9 to pH 3 (Figure 3). In all cases the rate of decrease in the observed velocity was similar with both stationary phases (Tables 4-7). The velocity of the EOF observed with the ODS columns was approximately double that with the Pt C18 column. This was probably a result of end capping in the Pt C18 material reducing the magnitude of the EOF that could be developed in the packed section. End capping effectively reduces the number of silanol groups available to aid the formation of the double layer around the packing material particles, which helps to generate the EOF through the packed section.
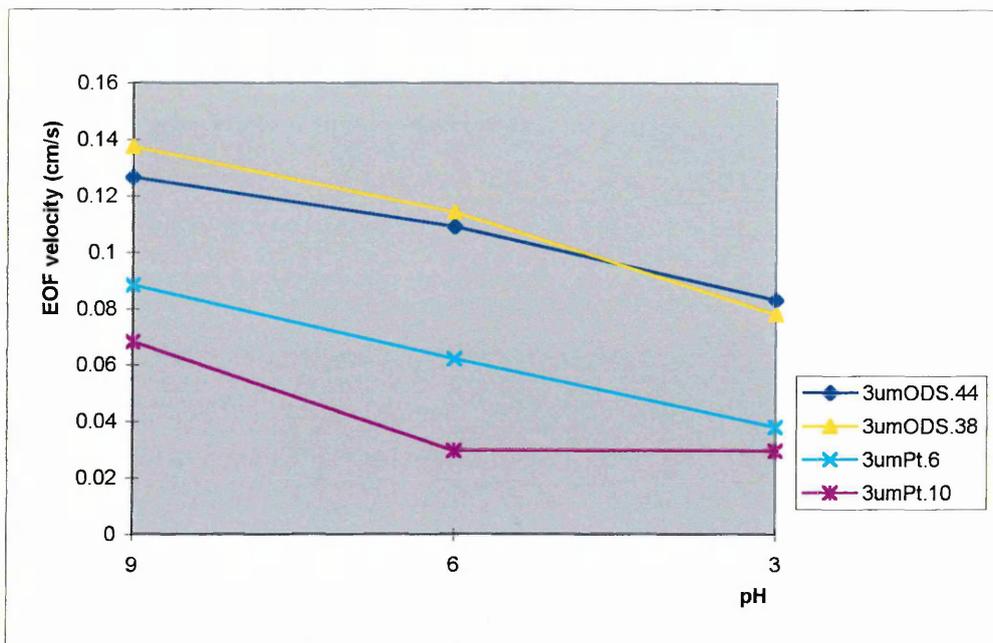
Figure 3. Variation in EOF velocity with differing pH on duplicate columns, using two different stationary phases: Exsil ODS and Pt C18 phases. The columns used were packed to a length of 20cm, with a running buffer of 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile with pH adjusted with 0.1molL$^{-1}$ HCl.

| pH | 9 | 6 | 3 |
|---|---|---|---|
| EOF Vel. (cm s$^{-1}$) | 0.14 | 0.11 | 0.08 |
| n | 10 | 7 | 5 |
| SD | 0.16 | 0.01 | 0.18 |
| %RSD | 3.07 | 0.18 | 1.89 |

Table 4. EOF reproducibility data for column 3$\mu$m ODS.38 at pH 3, 6 and 9. The column used was packed to a length of 20cm, with a running buffer of 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile with pH adjusted with 0.1molL$^{-1}$ HCl.

| pH | 9 | 6 | 3 |
|---|---|---|---|
| EOF Vel. (cm s$^{-1}$) | 0.13 | 0.11 | 0.08 |
| n | 10 | 17 | 12 |
| SD | 0.05 | 0.11 | 0.06 |
| %RSD | 0.83 | 1.70 | 0.63 |

Table 5. EOF reproducibility data for column 3$\mu$mODS.44 at pH 3, 6 and 9. The column used was packed to a length of 20cm, with a running buffer of 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile with pH adjusted with 0.1molL$^{-1}$ HCl.

| pH | 9 | 6 | 3 |
|---|---|---|---|
| EOF Vel. (cm s$^{-1}$) | 0.09 | 0.06 | 0.04 |
| n | 12 | 8 | 5 |
| SD | 0.34 | 4.71 | 0.92 |
| %RSD | 4.15 | 39.85 | 4.78 |

Table 6. EOF reproducibility data for column 3μmPt.6 at pH 3, 6 and 9. The column used was packed to a length of 20cm, with a running buffer of 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile with pH adjusted with 0.1molL$^{-1}$ HCl.

| pH | 9 | 6 | 3 |
|---|---|---|---|
| EOF Vel. (cm s$^{-1}$) | 0.07 | 0.04 | 0.03 |
| n | 11 | 5 | 5 |
| SD | 0.45 | 1.61 | 0.62 |
| %RSD | 4.18 | 7.72 | 2.50 |

Table 7. EOF reproducibility data for column 3μmPt.10 at pH 3, 6 and 9. The column used was packed to a length of 20cm, with a running buffer of 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile with pH adjusted with 0.1molL$^{-1}$ HCl.

A further observation was the stability of the EOF that was generated with the two types of material. The standard ODS material had a more stable EOF with the %RSD values being less than 3%, while the Pt C18 %RSD values were greater then 2.5% at each pH point studied. This could be the result of the Pt C18 material's inability to effectively maintain a constant EOF flow through the packed section, due to the smaller number of silanol groups present to form the double layer.

### 4.3.3 Mobile Phase

Change of the mobile phase composition in HPLC has an effect on the separation that can be achieved. In the following study the effect of decreasing the amount of organic solvent to aqueous phase was investigated.

This study was undertaken using a Spherisorb 3µm column. The investigation used a simple binary system of acetonitrile and water, where the volume of the organic solvent was reduced from 80% to 40% and was replaced by a corresponding volume of water. The final electrolyte concentration and pH were constant (0.8 mmolL$^{-1}$ of borate at pH 9.9).

The organic content was limited to a minimum of 50%, due to bubble formation when the organic content was reduced to 40%. However, the column was recoverable once it was reconditioned and the organic content increased.

No significant effects on the generation of the EOF were seen as the organic content was reduced (Figure 4). This indicates that the organic content has little effect on the generation of the EOF when the organic content is below 70% and that the choice of electrolyte is of greater importance.
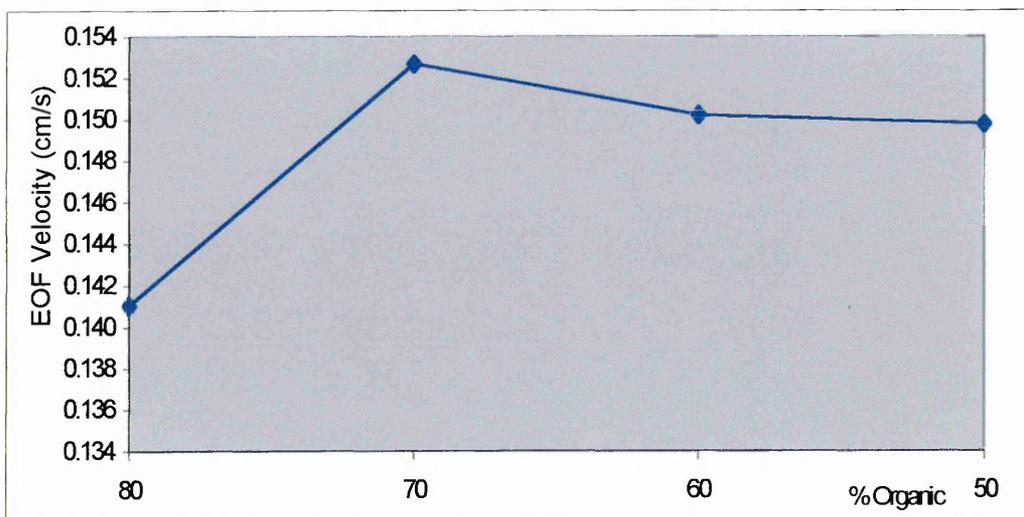
Figure 4. Variations in the EOF generated as the organic content is varied (n=5). The column used was a 3µm Spherisorb packed to a length of 20cm, with a running buffer of 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile (acetonitrile replaced with water) at pH 9.

An increase in the ratio of aqueous to organic solvent in the mobile phase led to an increase in migration time of the neutral marker, despite the increase in the EOF. This was probably due to the increased partitioning into the non-polar stationary phase as the percentage organic solvent in the mobile phase was reduced. This is clearly observed in Figure 5 and demonstrated in Figure 6, which shows how the migration times of the analytes increased with the reduction of the organic content.



(a)   Time (min)          (b)          Time (min)
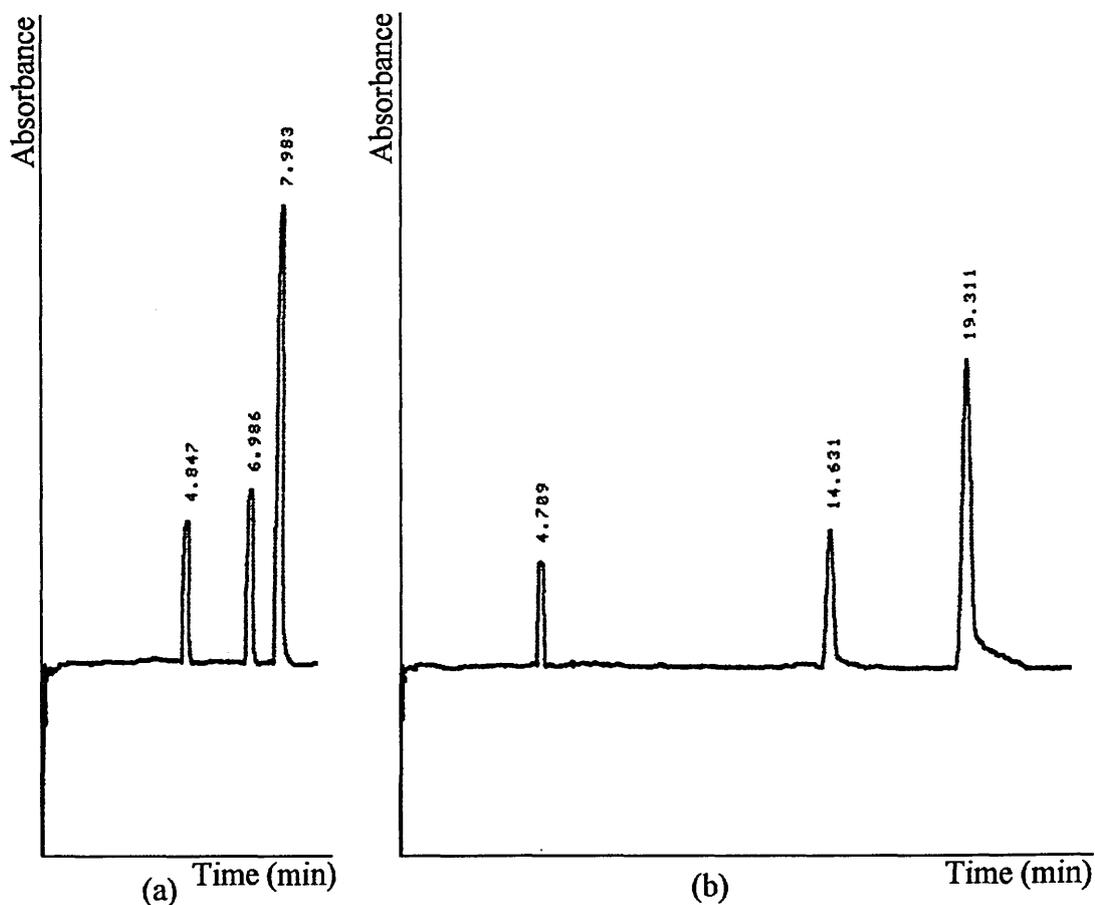
Figure 5. Representative electrochromatograms that illustrate the changes in the column performance as organic content is reduced from (a) 70% acetonitrile to (b) 50% acetonitrile content in the buffer (n=5).
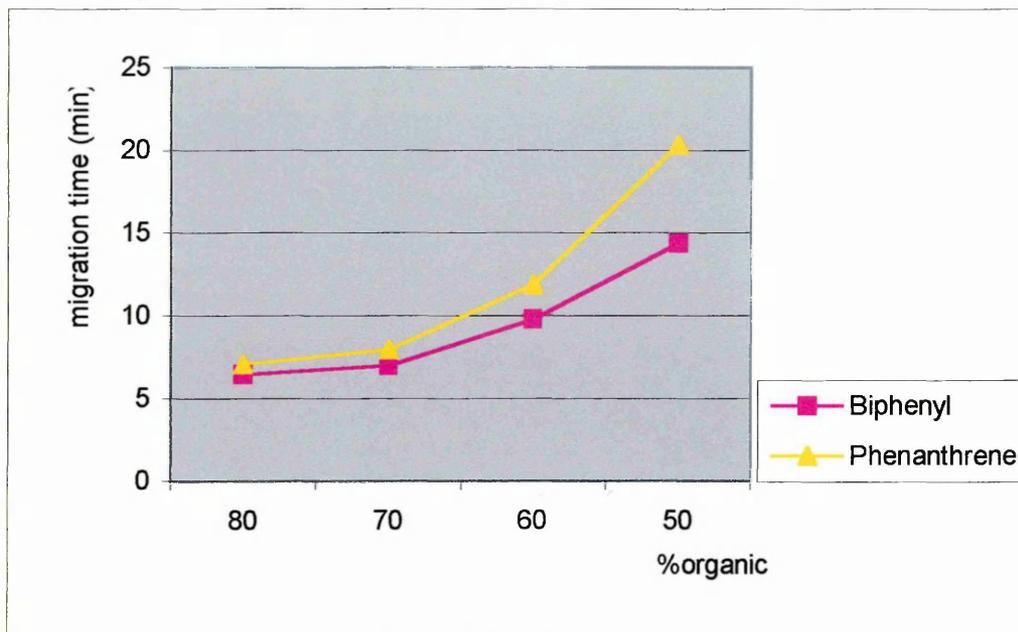
Figure 6. Migration time of the test compounds (biphenyl and phenanthrene), decreasing with increased levels of organic solvent (n=5).

With the increase in affinity for the stationary phase a corresponding increase in the resolution was also observed (Figure 7).
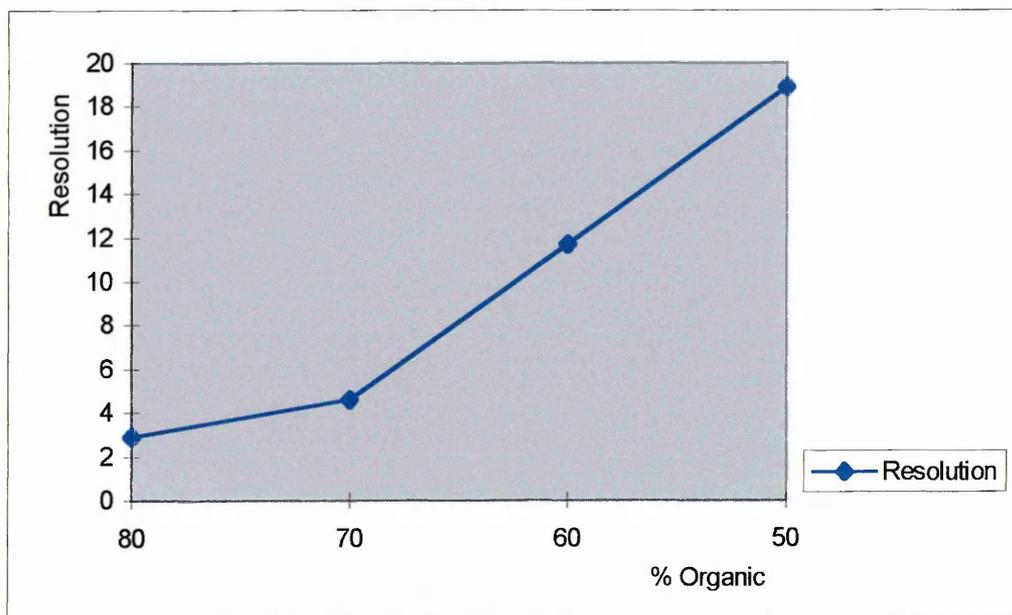


Figure 7. The change in the resolution between biphenyl and phenanthrene as the organic content was altered (n=5). Resolution was calculated from equation 1.16.

## 4.3.4 Variation in Applied Voltage

In CE the velocity of the EOF is directly proportional to the magnitude of the voltage applied to the column. Therefore, an investigation into how this variation in field strength would affect a CEC column was undertaken. The study was carried out on a 5μm Hypersil ODS column, with a mobile phase of 20% 4 mmolL$^{-1}$ borate in acetonitrile at pH 9.2.

Three voltages were utilised (10, 20 and 30Kv) and as clearly demonstrated in table 8, the velocity of the EOF decreased as the applied voltage was reduced.

| Voltage (Kv) | n | Linear Velocity (cms$^{-1}$) |
|:---:|:---:|:---:|
| 30 | 6 | 0.17 |
| 20 | 6 | 0.11 |
| 10 | 6 | 0.05 |

Table 8. Effect on the observed linear velocity of the EOF as the applied voltage is altered.

As a result of the reduced bulk flow there was significant band broadening (Figure 8), probably a result of increased longitudinal diffusion. However, the observed band broadening was of a similar magnitude to that of the increased migration time, so no significant effect on the peak efficiencies was observed. (Table 9).
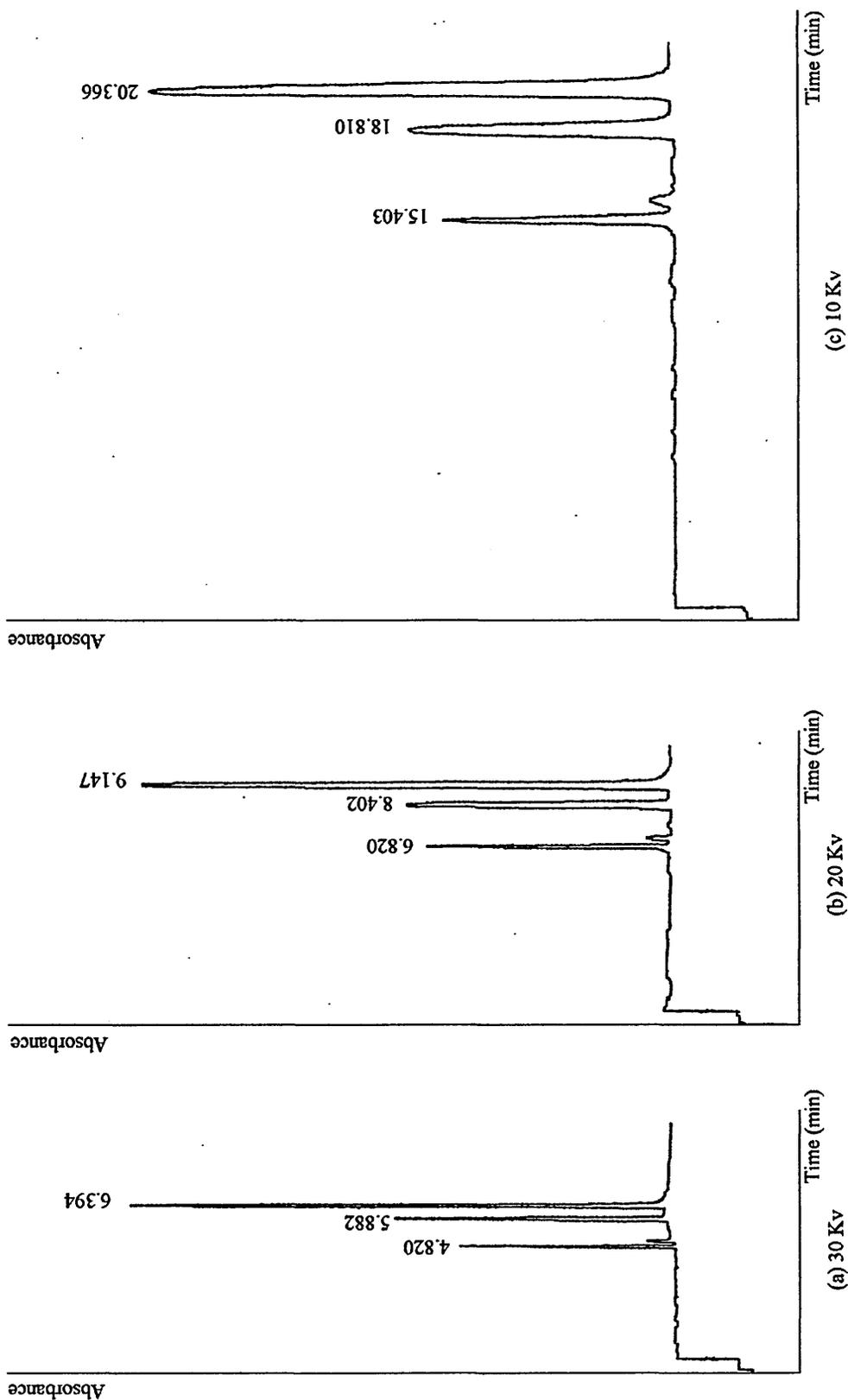
Figure 8. Variation in the electrochromatograms obtained as the applied voltage was altered (a) 30Kv (b) 20Kv and (c) 10Kv on a 5μm Spherisorb column packed to a length of 20cm with 20% 4 mmolL$^{-1}$ borate (aq) in acetonitrile at pH 9.2 (n=6).

**Biphenyl**

| Voltage (Kv) | Migration time (min) | Peak efficiency (N) |
|---|---|---|
| 30 | 5.367 | 22064 |
| 20 | 8.359 | 22155 |
| 10 | 18.360 | 17795 |

**Phenanthrene**

| Voltage (Kv) | Migration time (min) | Peak efficiency (N) |
|---|---|---|
| 30 | 5.865 | 24404 |
| 20 | 9.098 | 19414 |
| 10 | 19.920 | 17273 |

Table 9. Effect of variations in applied voltage on the migration times and peak efficiencies of biphenyl and phenanthrene. (Peak efficiencies calculated by equation 1.19)

This study indicates that packed capillary columns exhibit similar properties to open tubular columns with regard to the effects of changes in applied voltages. As a practical measure the voltage placed across the packed column is maximised (30Kv) to allow for rapid analysis times, however, reducing the applied voltage can aid resolution.

4.3.5 Packing Material

Over the period of this research various packing materials were used and variability between batches of stationary phase in terms of durability and repeatability were observed. As already discussed in the previous chapter, all the work was performed on ODS phases from a variety of manufacturers. Table 10 shows that the performances of all the ODS phases used were similar in terms of the velocity of the EOF observed and the resolution of the test compounds under similar conditions.

| Material | $d_p$ | n | EOF velocity (mm/s$^2$) | %RSD | Biphenyl | | Phenathrene | |
|---|---|---|---|---|---|---|---|---|
| | | | | | MT | Efficiency | MT | Efficiency |
| Hypersil ODS | 5µm | 34 | 1.71 (a) | 5.0 | 5.37 | 22064 | 5.83 | 14431 |
| | 3µm | 28 | 1.63 (a) | 1.5 | 6.02 | 76175 | 6.57 | 89631 |
| Exsil ODS | 3µm | 10 | 1.41 (a) | 3.0 | 7.49 | 19999 | 8.46 | 21102 |
| | 3µm | 10 | 1.35 (b) | 0.8 | 7.11 | 43764 | 8.03 | 55242 |
| Exsil Platinum C$_{18}$ | 3µm | 10 | 0.70 (b) | 3.8 | 11.94 | 15699 | 12.59 | 20254 |
| | 3µm | 10 | 0.90 (b) | 4.1 | 9.32 | 17789 | 9.80 | 21197 |
| Spherisorb ODS | 3µm | 10 | 1.41 (a) | 1.3 | 6.48 | 16796 | 6.99 | 19544 |
| | 3µm | 10 | 1.39 (b) | 0.6 | 6.50 | 17695 | 7.89 | 19694 |

Table 10. Relative performances of the stationary that were utilised, under similar conditions (a) 80% MeCN in 20% 4 mmolL$^{-1}$ Borate, pH 9.2, (b) 80% MeCN in 20% 20 mmolL$^{-1}$ TRIS, pH 9. (The Exsil ODS was from the initial batch of material supplied)

At the outset, effects of changes in stationary phase particle sizes, from 5μm to 3μm $d_p$ Spherisorb ODS, were examined. There were no significant changes in the EOF generated as the particle size was reduced. However, the stability of the observed flow velocity of the EOF was significantly improved with the 3μm packing. This could be as a result of more efficiently packed columns or of greater surface area being available to hold the double layer. The analytes were observed to have increased peak efficiencies as the particle size was reduced.

Exsil Platinum C18 demonstrated how the surface chemistry of the stationary phase could affect the performance of the packed capillary. The Platinum C18 stationary phase had a lower percentage of the ODS phase bound to its surface than the standard Exsil ODS phase. This was reflected in a reduction in resolution of the analytes when compared to the standard ODS phase (Figure 9). Also, as previously stated, the Platinum C18 is an end capped stationary phase. Consequently, when compared to ODS phases that are not end capped there was a significant reduction in the EOF that could be generated.
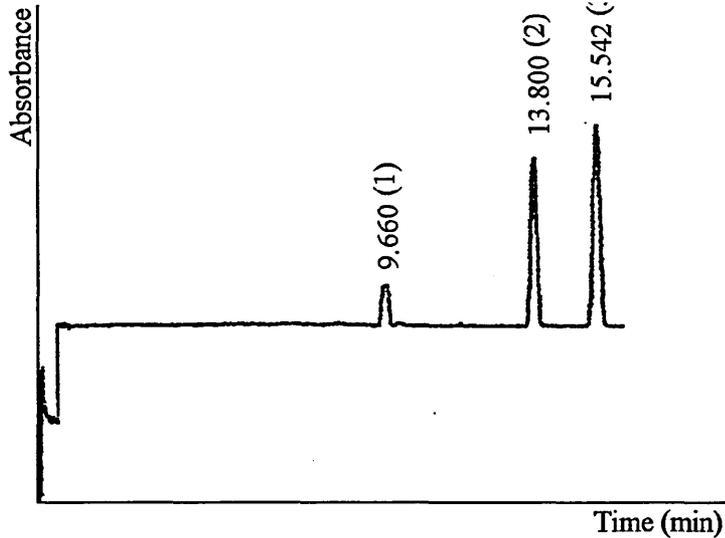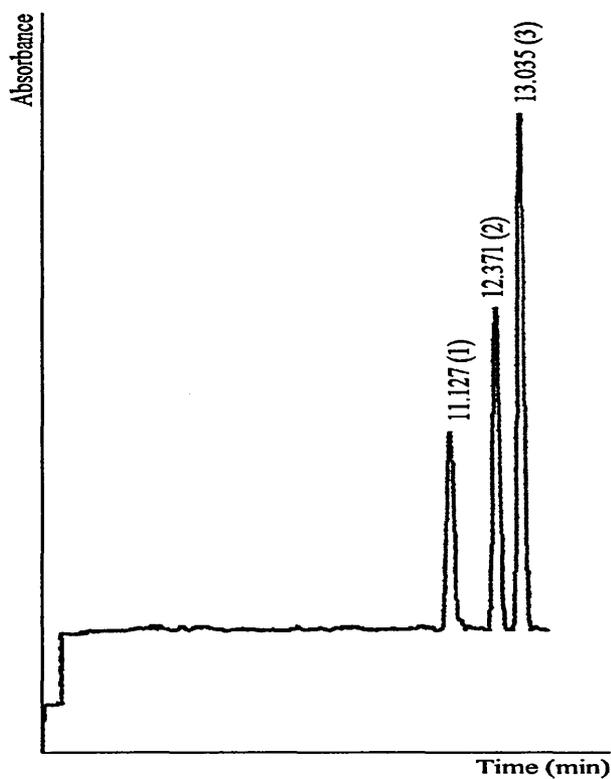
Figure 9. Differences in performance of the two Exsil stationary phases (a) ODS column (b) Platinum C18 packed to a length of 20cm. The running buffer was 20% 20 mmolL$^{-1}$ TRIS (aq) in acetonitrile at pH 8.9 (n=10). (1) thiourea (2) biphenyl (3) phenanthrene.

Dittmann *et al.* (1, 2) investigated differences in performance of a number of stationary phases with a greater number of analytes present and clearly demonstrated variations in the chromatographic performances of different

phases from various suppliers. When comparing phases from the same manufacturer the results showed a similar overall profile to those obtained in this study. However, variations in the different phases can be explained in terms of production techniques and purity of the packing material, which can affect both resolution and speed of analysis.

## 4.3.6 Column Length

As previously discussed, the Prince Technologies CE requires a minimum capillary length of 60cm and an effective length of 44cm (the length from the inlet to the detector). Therefore, the optimum packed length had to be determined to optimise speed of analysis and the resolution that could be obtained.

The initial work on packed length was carried out using Spherisorb 5μm ODS stationary phase. The objective was to determine the minimum length of packing required to effect the separation of a three component PAH test solution (thiourea, biphenyl and phenanthrene). Two packing lengths were investigated, 10cm and 20cm. It was observed that doubling the packed section from 10cm to 20cm had a marked effect, with all the analytes on a 20cm packed column being resolved, while there was no resolution of the PAH mixture on the 10cm column.

A more detailed study of packing length was undertaken later, using Spherisorb 3μm ODS. The capillaries were packed with sections ranging from 0cm to 40cm (Figure 10). The buffer was 20% 20 mmolL$^{-1}$ TRIS in acetonitrile at pH 8. Figure 11 shows there was a decline in the EOF generated in each of

the columns from 26mm s$^{-1}$ at 0cm to 8mm s$^{-1}$ at 40cm. Once the packed section increased above 30cm the rate of decrease in the velocity of the EOF was reduced. Rathore *et al.* (3,4) have stated that the flow velocity is the averaged flow through the column. The results obtained here also indicated that the flow is the averaged velocity of the two segments (packed and unpacked) within the capillary column.
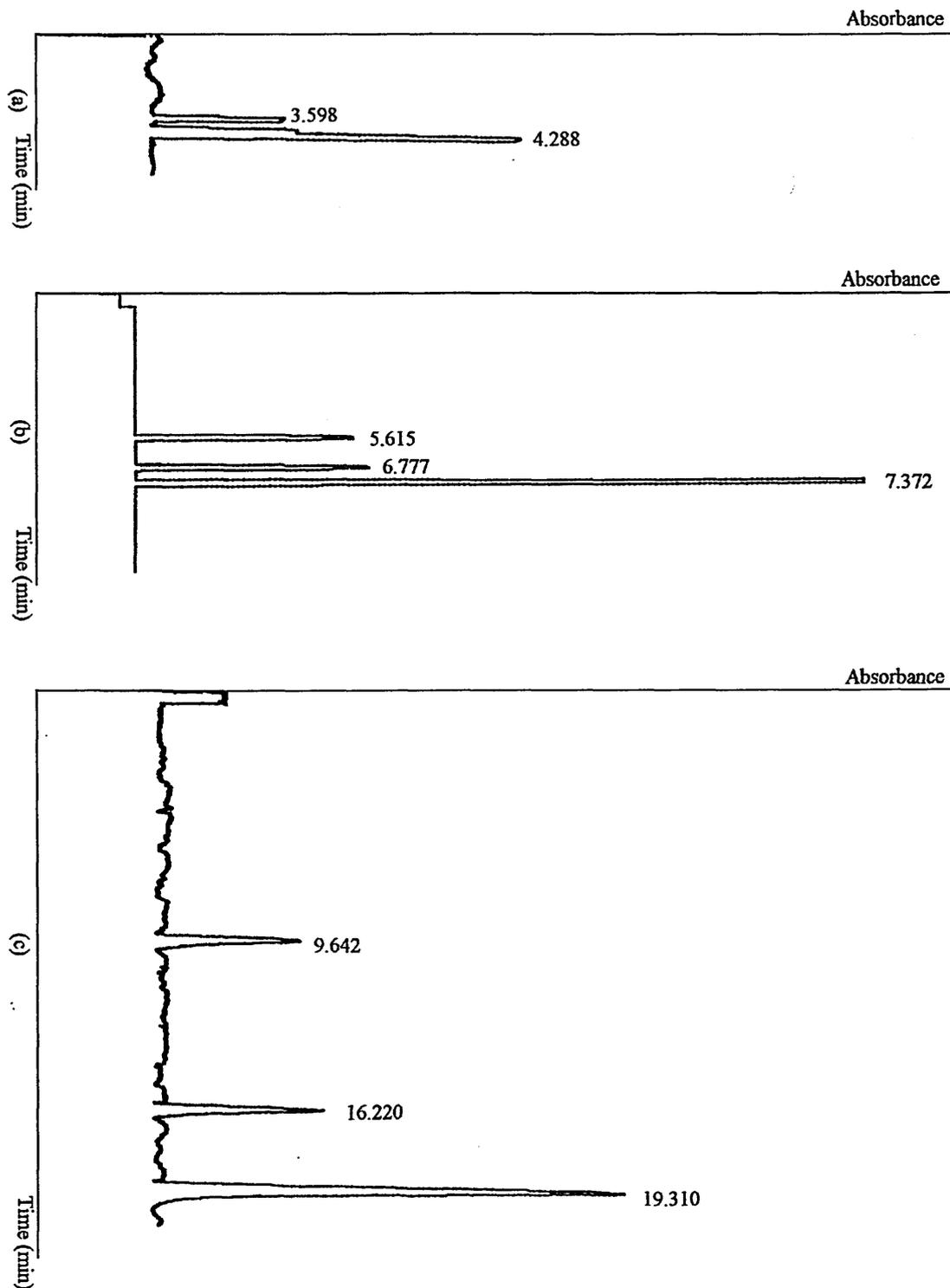
Figure 10. Representative electrochromatograms indicating the changes in performance as the packed length is altered (a) 10cm (b) 20cm and (c) 40cm in a column that has a total length of 60cm. Columns packed with $3\mu m$ Spherisorb and a running buffer of 20% 4 $mmolL^{-1}$ borate in acetonitrile at pH 9.2 (n=5).
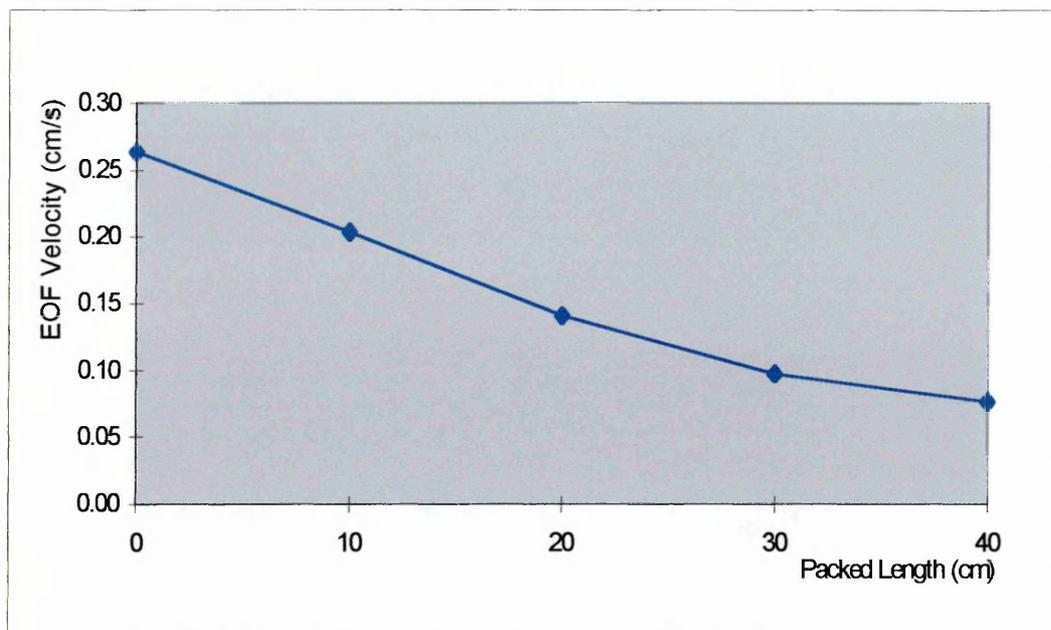
Figure 11. Variation in the EOF velocity with changing packed length. Columns packed with 3μm Spherisorb and a running buffer of 20% 4 mmolL$^{-1}$ borate in acetonitrile at pH 9.2 (n=5).

An increase in packed length was also associated with a corresponding increase in the resolution that is obtainable (Figure 12). Corresponding increases in peak efficiencies were calculated and are shown in table 11.
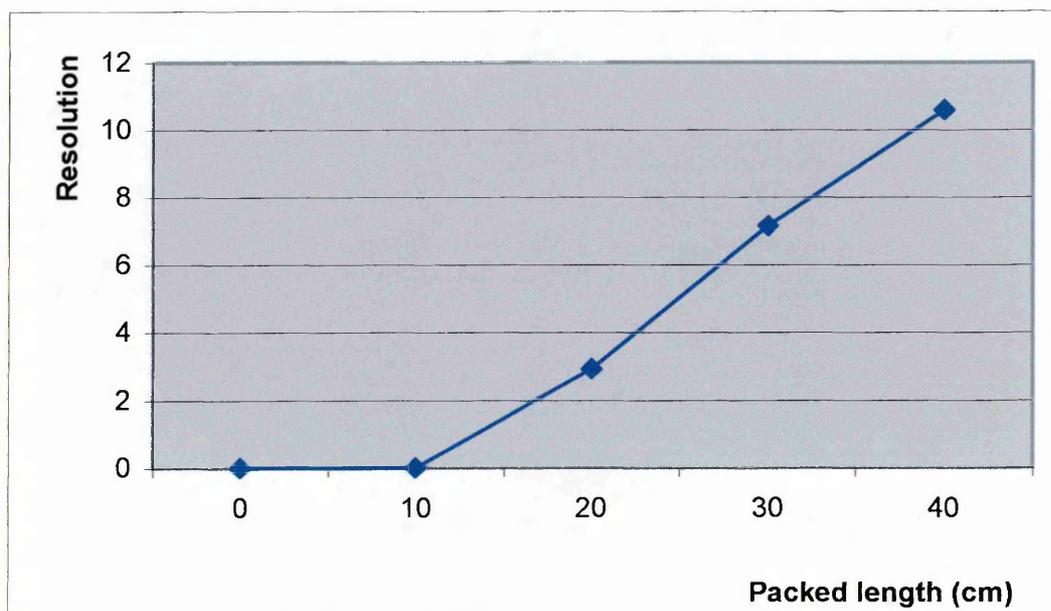


Figure 12. Increase in the resolution between biphenyl and phenanthrene with packed length. Columns packed with 3μm Spherisorb and a running buffer of 20% 4 mmolL$^{-1}$ borate in acetonitrile at pH 9.2 (n=5).

| Packed length (cm) | Peak Efficiency (N) | |
| --- | --- | --- |
| | Biphenyl | Phenanthrene |
| 10 | - | 9593 |
| 20 | 17840 | 19904 |
| 30 | 39476 | 49138 |
| 40 | 62108 | 58649 |

Table11.Increase in peak efficiencies with lengthening packed bed. Columns packed with 3μm Spherisorb and a running buffer of 20% 4 mmolL$^{-1}$ borate in acetonitrile at pH 9.2 (n=5).

### 4.4 Sample introduction

#### 4.4.1 Introduction

There has been little reported on the influence of the sample plug and how it can affect the performance of the separation, with the exception of Pyell *et al.* (5) and Stead *et al.* (6) who investigated plug width and sample plug composition respectively. It was therefore thought that an investigation into the effects of altering the properties of the sample plug, in terms of its conductivity, polarity and length, could lead to possible improvements in the chromatographic performance of the system.

This work was carried out using a Hypersil $3\mu m$ ODS stationary phase. The running buffer used was 20% 20 $mmolL^{-1}$ TRIS (aqueous) in acetonitrile, adjusted to pH 8 with 0.1 $molL^{-1}$ NaOH.

#### 4.4.2 Electrolyte Concentration of Sample

In the majority of cases the sample is loaded onto the column by placing it in running buffer, so there is little or no difference between the conductivity of the sample plug and the bulk solution. By altering the electrolyte concentration and, therefore, its conductivity in comparison to that of the bulk solution the analytes could possibly be focussed at the interface between the two regions. This has been used successfully in CZE to improve peak efficiency and is referred to as sample stacking.

The samples were prepared to resemble the bulk solution i.e. 20% TRIS in acetonitrile with varying electrolyte concentrations. The range used was 0

mmolL$^{-1}$ to 30 mmolL$^{-1}$ of TRIS, giving an effective concentration range of 0 mmolL$^{-1}$ to 6 mmolL$^{-1}$ in the sample matrix.

There were no detrimental effects on the EOF with the changes in the electrolyte concentration of the sample plug (Table 12). When there was no electrolyte present the EOF was slightly slower than at other concentrations.

| Electrolyte concentration in sample plug (mmolL$^{-1}$) | EOF Velocity (mms$^{-1}$) | SD (n=5) |
|---|---|---|
| 0 | 0.20 | 0.033 |
| 2 | 0.22 | 0.043 |
| 10 | 0.22 | 0.051 |
| 20 | 0.22 | 0.028 |
| 30 | 0.22 | 0.021 |

Table 12. Variation in the EOF velocity with changes in the electrolyte concentration in the sample plug.

By altering the electrolyte concentration in the sample plug it was hoped that a gain in chromatographic performance could be obtained by focussing the analytes at the head of the column. Table 13 indicates that there were no significant differences in the performance of the separation as a result of the difference in conductivity between the sample plug and the bulk solution.

| Electrolyte Conc$^n$ (mmolL$^{-1}$) | Biphenyl | | | Phenanthrene | | |
|---|---|---|---|---|---|---|
| | $M_t$ (min) | $P_w$ (min) | N | $M_t$ (min) | $P_w$ (min) | N |
| 0 | 6.498 | 0.20 | 17695 | 7.094 | 0.20 | 19694 |
| 2 | 7.087 | 0.20 | 20191 | 7.748 | 0.20 | 26369 |
| 10 | 7.063 | 0.20 | 19600 | 7.696 | 0.19 | 27872 |
| 20 | 7.000 | 0.20 | 18895 | 7.607 | 0.19 | 25486 |
| 30 | 6.991 | 0.20 | 20106 | 7.610 | 0.19 | 26955 |

Table 13. Effect on the test compounds of changes in the electrolyte concentration in the sample plug (n=5). $M_t$ is migration time (min), $P_w$ is peak width (min) and N is peak efficiency.

The same principles that have been studied in this section have already been successfully demonstrated in CZE. Differences in the relative electrolyte concentration between the sample plug and bulk solution can be used to focus solute ions as they migrate from the sample plug into the bulk solution. Focussing occurs due to reduction in the relative mobility of the ions and hence their velocity, as they migrate from a low electrolyte concentration to a high concentration region. Therefore, as the ions migrate into the high electrolyte concentration region the electrophoretic mobilities of the ions are consequently reduced and therefore the sample bands are compressed.

This can be used to explain why there were no significant advantages observed when this method was attempted for CEC. The focussing relies on changes in the solute's electrophoretic mobility, but in this study neutral analytes were used which have no inherent electrophoretic mobility. Therefore, as the sample plug is swept away with the bulk EOF the change in

electrolyte concentration has no effect on the solute's electrophoretic mobility and hence there is no focussing effect.

## 4.4.3 Organic Content of Sample Plug

The effect of changing the organic composition of the sample matrix was investigated following the work of Stead *et al.* (6) who used a similar approach for the on-line concentration of analytes. An investigation was undertaken to determine if similar enhancements in efficiencies and sensitivity could be achieved on the Prince instrument. By increasing the water content of the sample plug it was hoped that the solutes would have a greater affinity for the stationary phase on loading, thereby, concentrating the analytes into a narrower band at the head of the column.

The organic content (acetonitrile) of the sample matrix was replaced by water, while leaving the concentration of the electrolyte constant. Table 14 shows that variations in the organic content had no significant effect on the velocity of the EOF.

| %organic | EOF Velocity (mms$^{-1}$) | %RSD (n=10) |
|----------|---------------------------|-------------|
| 80       | 0.22                      | 0.27        |
| 70       | 0.22                      | 0.82        |
| 60       | 0.22                      | 0.60        |
| 50       | 0.22                      | 0.88        |
| 40       | 0.22                      | 0.47        |
| 30       | 0.22                      | 0.33        |

Table 14. Variation in the EOF with changes in the organic content of the sample plug. Running buffer 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile at pH 8.9 on a 20cm packed 3μm Spherisorb column (Sample plug: 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile (with the acetonitrile being replaced with water))

The effect of a decrease in the organic content of the analytes was an increase in migration time (Table 15), with a corresponding increase in peak efficiencies obtained as the peak widths were reduced (Figures 15 and 16).

| % organic content of sample plug | Biphenyl | | Phenanthrene | |
|---|---|---|---|---|
| | $M_t$ (min) | %RSD | $M_t$ (min) | %RSD |
| 80 | 7.146 | 0.41 | 7.802 | 0.38 |
| 70 | 7.231 | 0.85 | 7.901 | 0.87 |
| 60 | 7.287 | 0.70 | 7.969 | 0.64 |
| 50 | 7.401 | 0.41 | 8.114 | 0.39 |
| 40 | 7.431 | 0.42 | 8.159 | 0.50 |
| 30 | 7.416 | 0.29 | 8.145 | 0.30 |

Table 15. Test compound migration times with changing organic content in the sample plug (n=10). Running buffer 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile at pH 8.9 on a 20cm packed 3$\mu$m Spherisorb column (Sample plug: 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile (with the acetonitrile being replaced with water)).
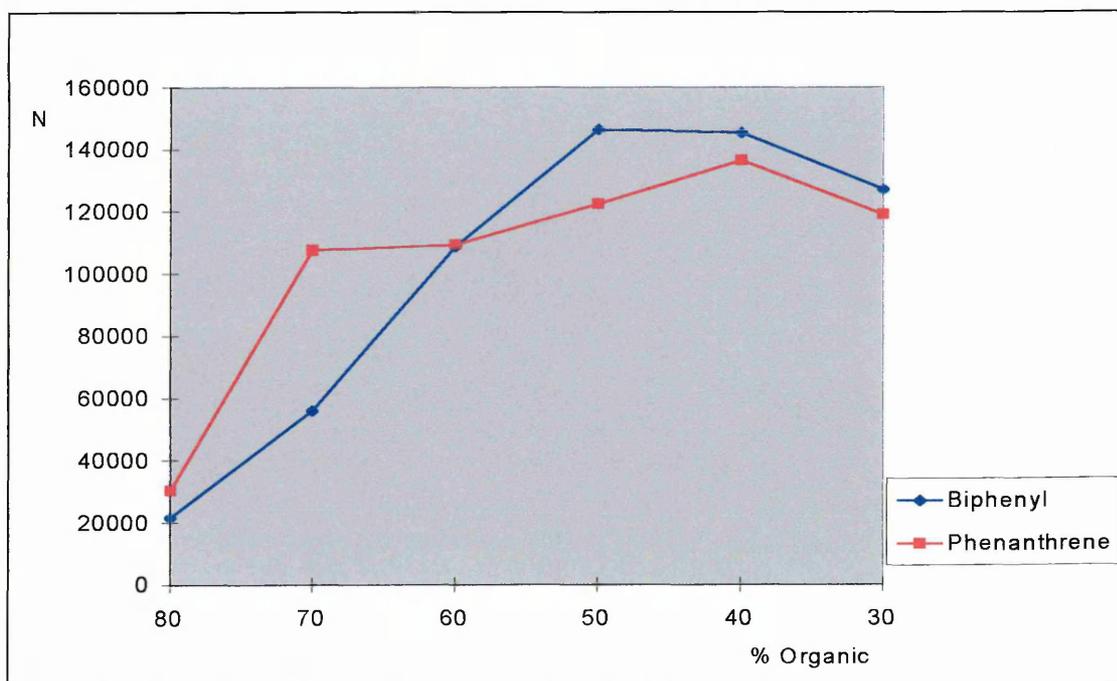


Figure 15 Increase in peak efficiencies with decrease in organic content in sample plug (n=10). Running buffer 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile at pH 8.9 on a 20cm packed 3$\mu$m Spherisorb column (Sample plug: 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile (with the acetonitrile being replaced with water)).
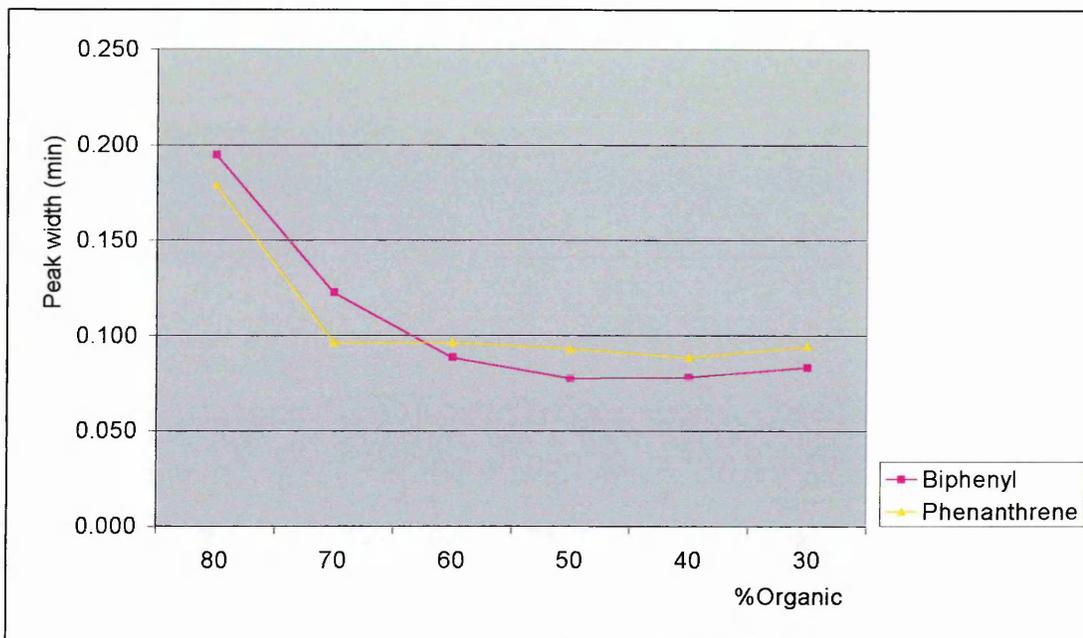
Figure 16. Reduction in the peak width with the increase in water content in the sample plug (n=10). Running buffer 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile at pH 8.9 on a 20cm packed 3μm Spherisorb column (Sample plug: 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile (with the acetonitrile being replaced with water))
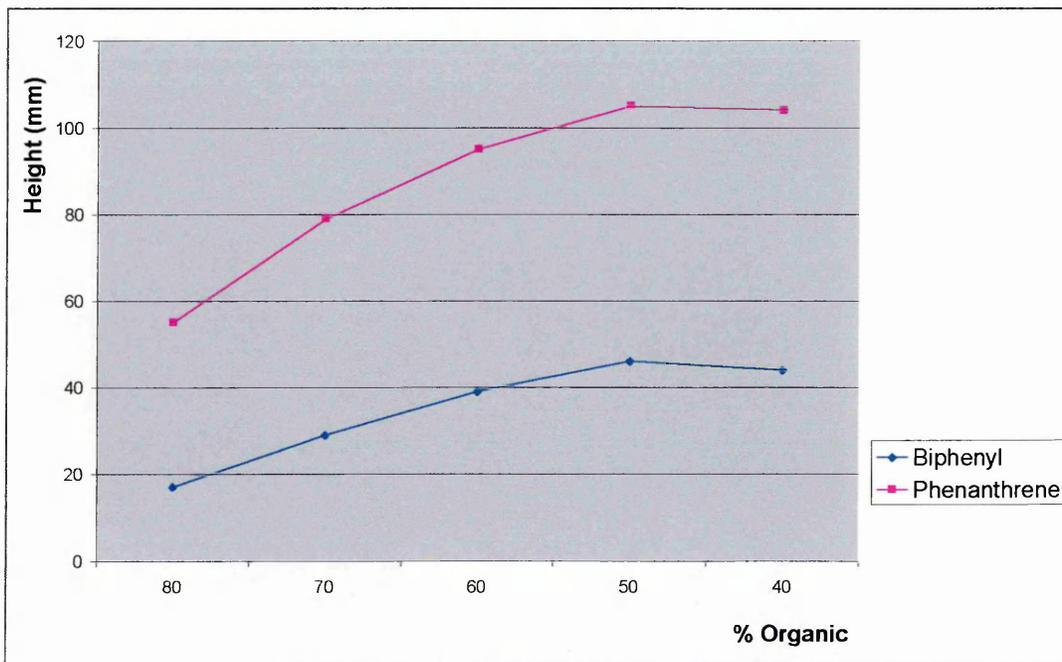


Figure 17. Variation in peak height with changes in the organic content of the sample plug (n=10). Running buffer 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile at pH 8.9 on a 20cm packed 3μm Spherisorb column (Sample plug: 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile (with the acetonitrile being replaced with water))

Along with the increase in peak efficiency, sensitivity was also improved. Figure 17 shows that peak heights were increased with the reduction in the organic content of the sample plug. This is likely to be the result of the high water content in the sample plug increasing the affinity of the solutes for the stationary phase, thereby focussing the analyte bands on the head of the column before the sample plug was swept away by the bulk solution.

This result confirmed the work of Stead *et al.* (6) and demonstrated that the technique could be utilized on the Prince instrument for non-polar solutes, however, further work is required to study how it could be modified for more polar solutes.

### 4.4.4 Sample Plug Length

The effect of varying the length of the sample plug loaded onto the column was investigated to determine if there was any advantage to be gained from increasing the plug length (and hence the quantity of analyte present on the column) with regard to peak efficiencies and sensitivity.

This study was undertaken by keeping the injection voltage constant but increasing the loading times from 0.1 minutes to 2 minutes. The results of the previous work indicated that the analytes had the greatest peak efficiencies when the organic content of the sample matrix was 50:50 organic:aqueous, so the test solution was made up to this specification.

As expected, with the increased volume of the test solution loaded, peak area increased steadily with the higher levels of analyte present (Figure 18). However, there was a dramatic increase in peak area observed for phenanthrene when the loading time rose above 1 minute.
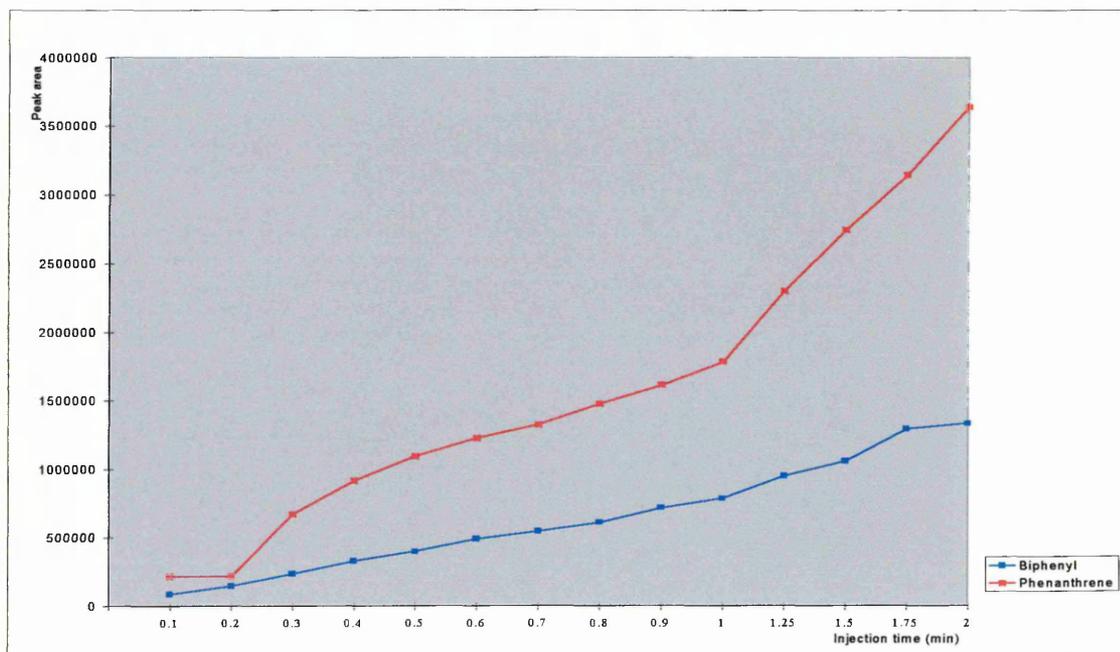


Figure 18. Increase in peak area with extended sample loading times (n=3). Running buffer 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile at pH 8.9 on a 20cm packed 3μm Spherisorb column (Sample plug: 20% 20mmolL$^{-1}$ TRIS (aq): 30% water: 50% acetonitrile).

The peak heights were also measured (Figure 19). A steady increase in peak height with increased loading time was observed until a plateau at around 0.7 minutes for biphenyl and 1.5 minutes for phenanthrene.
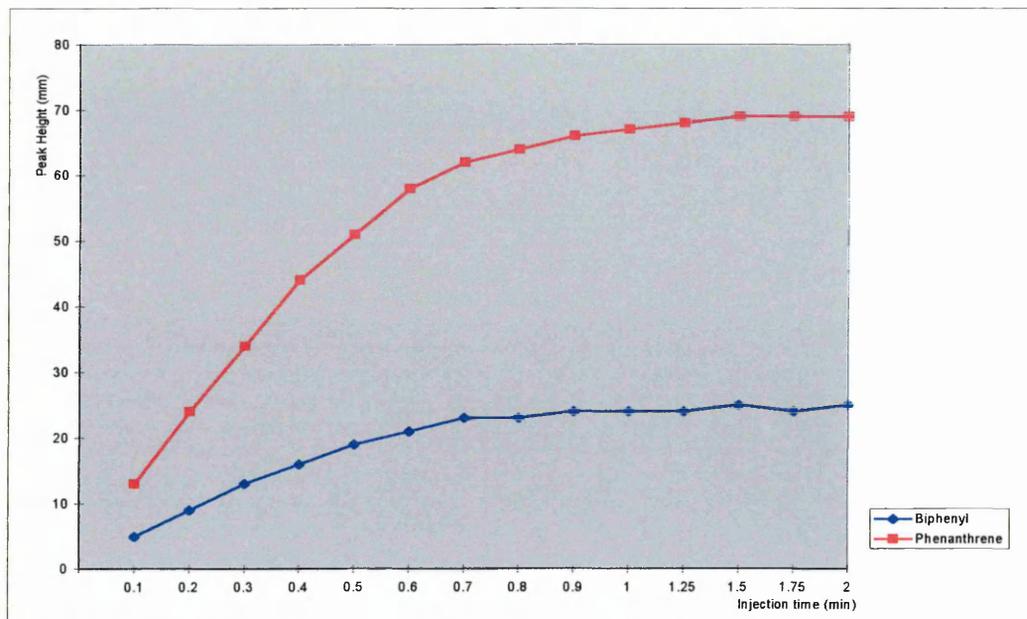
Figure 19. Variations in peak heights for the test compounds with changes in loading times (n=3). Running buffer 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile at pH 8.9 on a 20cm packed 3$\mu$m Spherisorb column (Sample plug: 20% 20mmolL$^{-1}$ TRIS (aq): 30% water: 50% acetonitrile).

As the loading times increased there was a corresponding decrease in the peak efficiency, due to the increase in migration times and peak width (Figure 20). This results from the increased length of the sample plug loaded on the head of the column, which allows the analytes to be more dispersed on loading, increasing peak width before any other factors involved with band broadening could contribute significantly. There is also the possibility of over loading the column with the increase in volume of sample solution loaded, which can lead to peak distortions and hence reduction in efficiency.
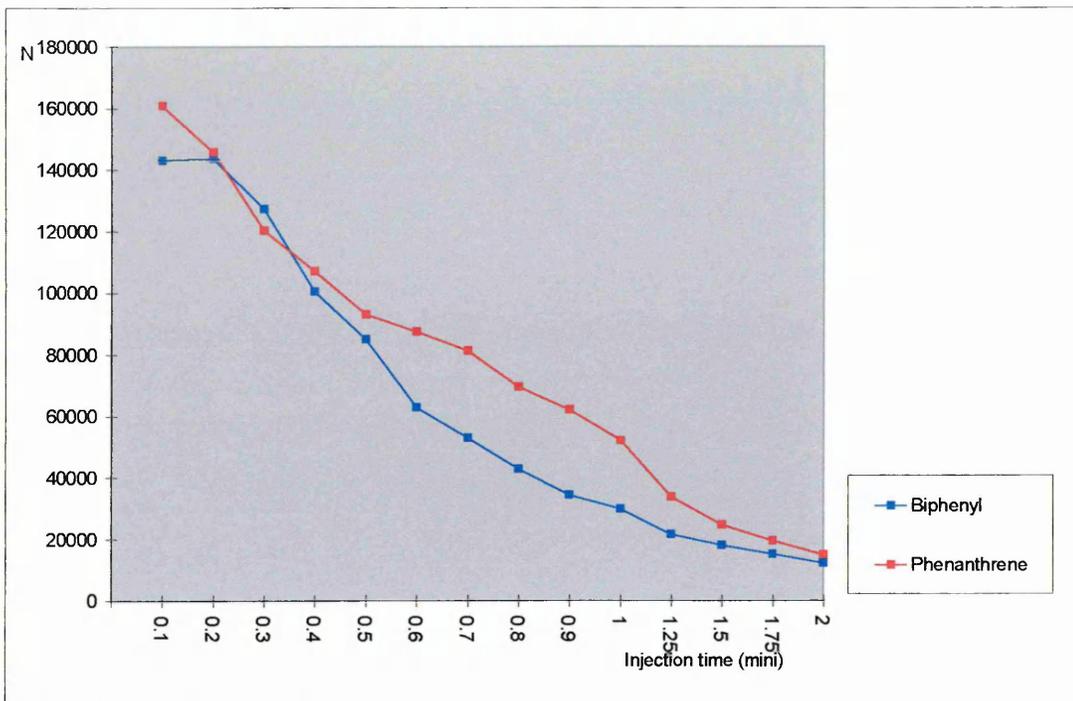
Figure 20. Variations in the peak efficiencies of the test compounds with increased sample loading times (n=3). Running buffer 20% 20mmolL$^{-1}$ TRIS (aq) in acetonitrile at pH 8.9 on a 20cm packed 3μm Spherisorb column (Sample plug: 20% 20mmolL$^{-1}$ TRIS (aq): 30% water: 50% acetonitrile).

## 4.5 Conclusion

The results obtained in these investigations have clearly demonstrated that a reduction in organic content in the sample plug in comparison to the bulk solution can have a significant effect on the chromatographic performance of the system, both in terms of sensitivity and peak efficiency. This is likely to be the result of the analytes having a greater affinity for the stationary phase when initially loaded onto the column. Therefore, a stacking effect can be observed on loading. Because of this stacking effect the sample plug can be increased allowing a larger volume of the sample matrix to be loaded and hence a higher amount of analyte, resulting in a significant increase in sensitivity. However the injection time should be around the time that the detector response plateaus, which in this instance would limit the injection time to below 0.6-0.7 minutes. Above this time there is no more gain in sensitivity while efficiency decreases. In all cases there were no effects on the velocity of the EOF generated, due to changes in the sample plug.

## 4.6. Optimal Conditions

Running buffer:               80% Acetonitrile
                                     20% Aqueous electrolyte

Sample plug:                  50% Acetonitrile
                                     30% Water
                                     20% Aqueous electrolyte

Column:                       20cm packed length

Applied voltage:           30Kv

The conditions set out above are those that would be used as the initial parameters for future application studies. The nature of the electrolyte has been left in general terms, as the selection is dependent on the pH at which the separation will be performed. However, as a preference an organic electrolyte would be chosen, due to their lower conductivity and hence the reduced risk of Joule heat.

A sample plug with a high water content allows focussing of the solutes at the head of the column, thus aiding resolution and peak efficiency of the analytes. A 20cm packed column is preferred, as this is the minimum length with which respectable resolution has been achieved. Improvements in resolution can either be achieved by altering the organic composition of the running buffer or by increasing the packed length of the column. It is felt that altering the organic content of the running buffer is more desirable as it is easier and faster to change the composition of the buffer than to fabricate a new column, which can be problematic.

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# CHAPTER 5, Applications of CEC

## 5.1. Introduction.

The true test of any chromatographic system is its ability to separate a variety of different classes of compounds. To date the majority of applications that have been published have displayed the potential of CEC as an emerging chromatographic technique, with few being used for routine analysis.

Therefore, to test the CEC system that has been developed a series of applications were undertaken. These were chosen to cover a range of analyte types i.e. acidic, basic and neutral. When developing the methodologies for the acidic and basic analytes a CZE method was also developed to allow a comparison of the different techniques.

In all cases, unless stated otherwise, the equipment, chemicals and operational parameters that are used in this chapter are the same as set out in chapter 2.

## 5.2. Polynuclear Aromatic Hydrocarbons (PAH)

PAH are a series of compounds that are constructed from two or more fused aromatic rings. This produces a series of non-polar compounds, which can be readily separated by either GC or gradient LC methods.

PAH have been a standard in the development of CEC since early reports which utilised this class of compounds to demonstrate the effectiveness of

their CEC systems. These reports include studies of the differences between manufacturers' stationary phases (1), detector configurations (2) and variations in mobile phases (1, 3, 4, 5).

The work undertaken with the PAH in this study was to evaluate the effectiveness of the columns fabricated in house compared to published results. This was also an opportunity to confirm some of the observations made when investigating the experimental parameters of the Prince Technology CE.

### 5.2.1. Chemicals.

The running buffers were as stated in chapter 2. The analytes included a neutral marker, thiourea, which was obtained from BDH Chemicals Ltd., Dorset, UK. The individual PAH, phenanthrene, anthracene, benzyl alcohol, fluorene and biphenyl, were obtained from Sigma-Aldrich, Dorset, UK, as was the 16 component EPA PAH standard solution.

### 5.2.2. Procedure.

This is described in chapter 2.

### 5.2.3. Results and Discussion.

The preliminary work undertaken to determine the experimental/instrumental parameters used a simple test solution. This test solution contained thiourea as the EOF marker and two PAH, biphenyl and phenanthrene, to indicate how changes in the system affected the analytes. The results indicated that a twenty centimetre packed column was sufficient and that the two favoured

buffer systems (borate and TRIS) allowed comparable results to be obtained.

This can be observed in figure 1.
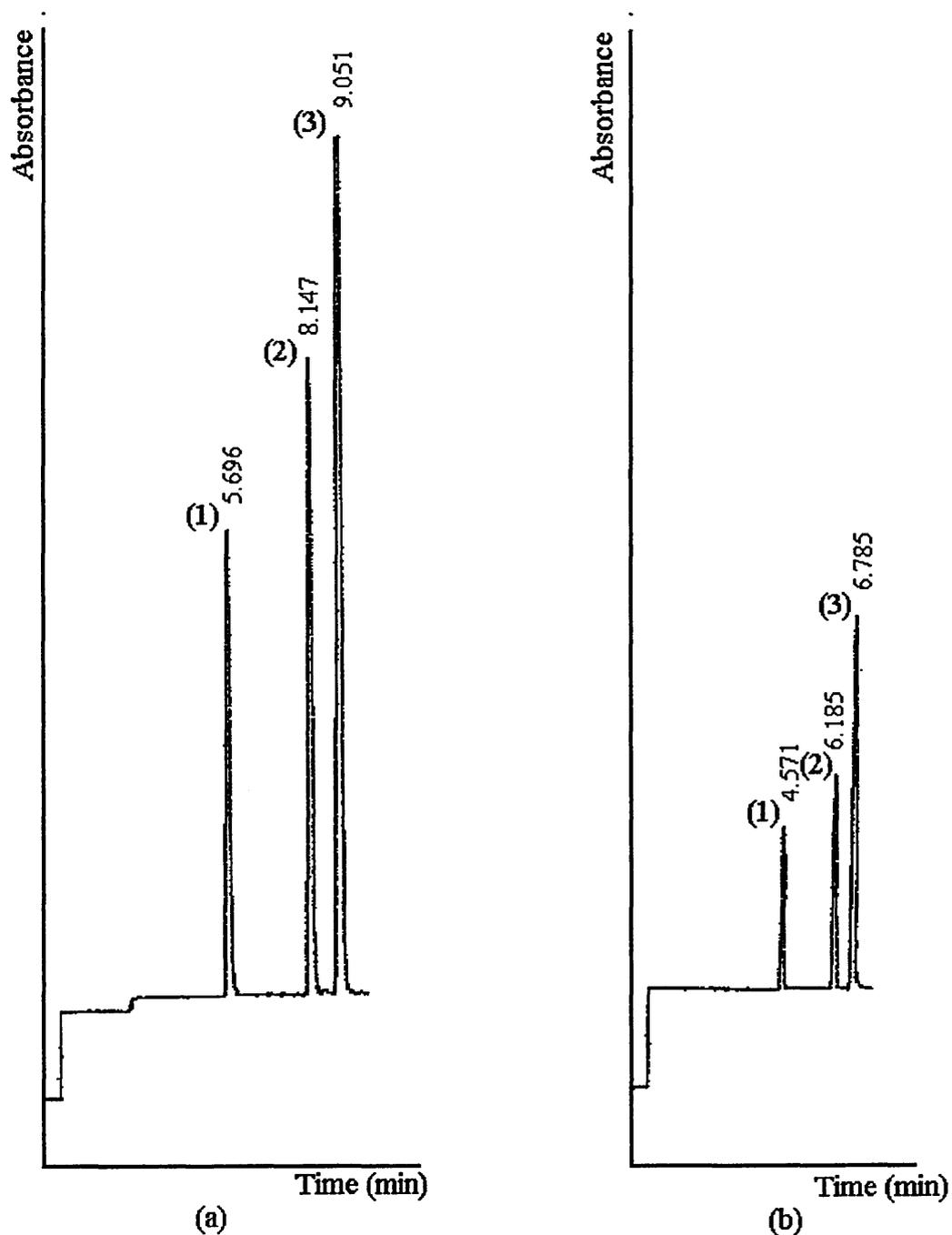


Figure 1. Representative electrochromatograms from a 3μm Spherisorb, 20cm packed column with an applied voltage of 30Kv (a) 20% 20 mmolL$^{-1}$ TRIS in acetonitrile at pH 10 (b) 20% 4 mmolL$^{-1}$ borate in acetonitrile at pH 10 (n=10). Peaks (1) thiourea (2) biphenyl (3) phenanthrene.

After the initial work with a simple three component system, the number of analytes was increased to six. When the sample was run at 30Kv there was poor resolution of the analytes. Therefore, the applied voltage was decreased to 20Kv. Figure 2 shows the resultant separation, which resulted in the near baseline separation of all the analytes
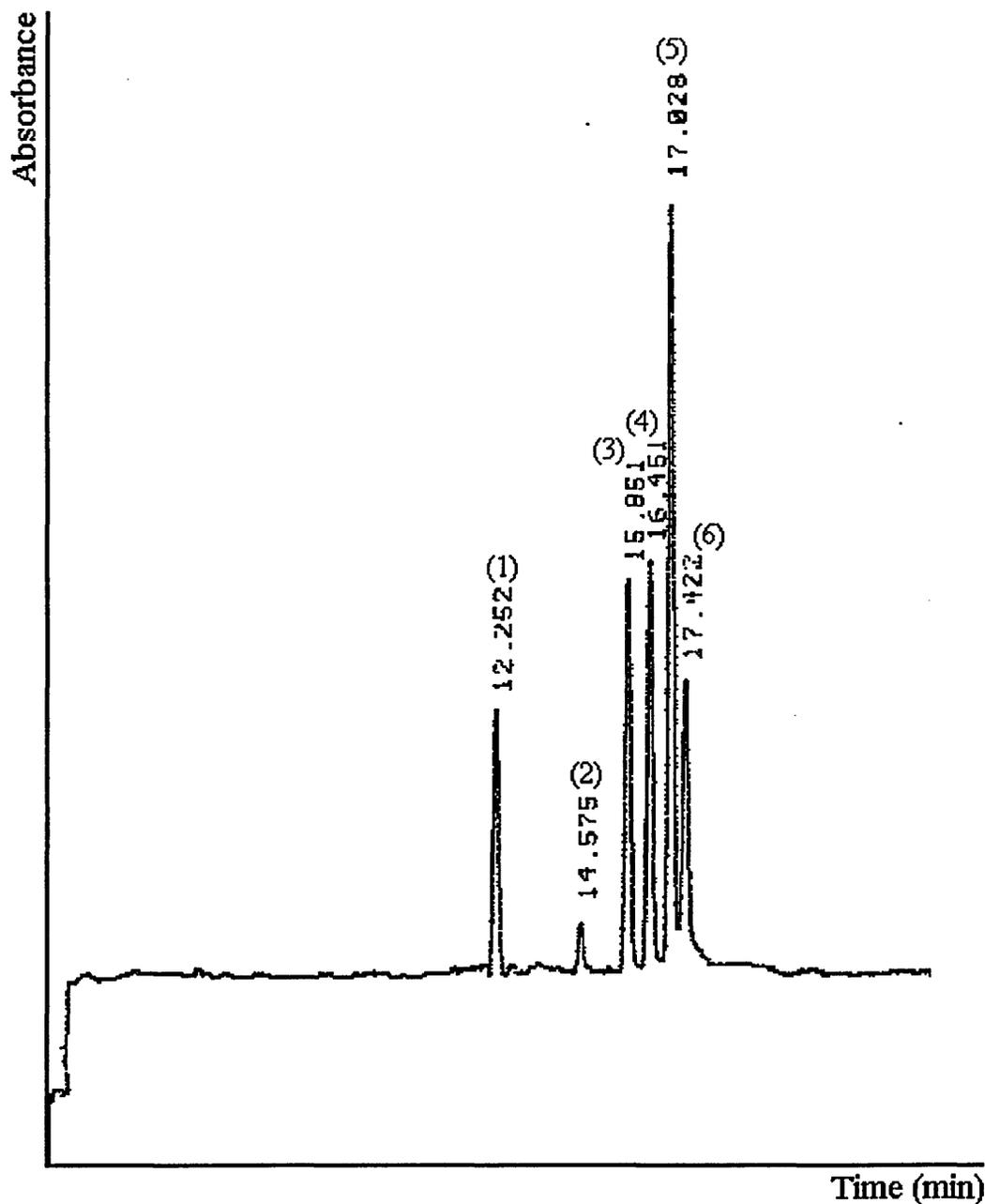


Figure 2. Representative electrochromatogram from a 3μm Spherisorb, 20cm packed column with an applied voltage of 20Kv, with a running buffer of 20% 4 mmolL$^{-1}$ borate in acetonitrile at pH 10 (n=5). Peaks (1) thiourea (2) benzyl alcohol (3) biphenyl (4) fluorene (5) phenanthrene (6) anthracene.

The next stage of the investigation was the separation of the 16 component EPA PAH standard. Separation of this complex mixture had already been demonstrated by other groups, so the result would indicate whether there were any significant differences between our system and those used by other groups.

The first study used a borate buffer system with a Hypersil ODS stationary phase. As could be predicted from earlier work, the resolution of the 16 components was poor when the operating voltage was at 30Kv, as can be observed in figure 3. Our earlier work on the six component solution and the work of Yan *et al.* (5) indicated that the optimal voltage for good resolution is around the 15-20Kv region, however this gave significantly increased analysis times.
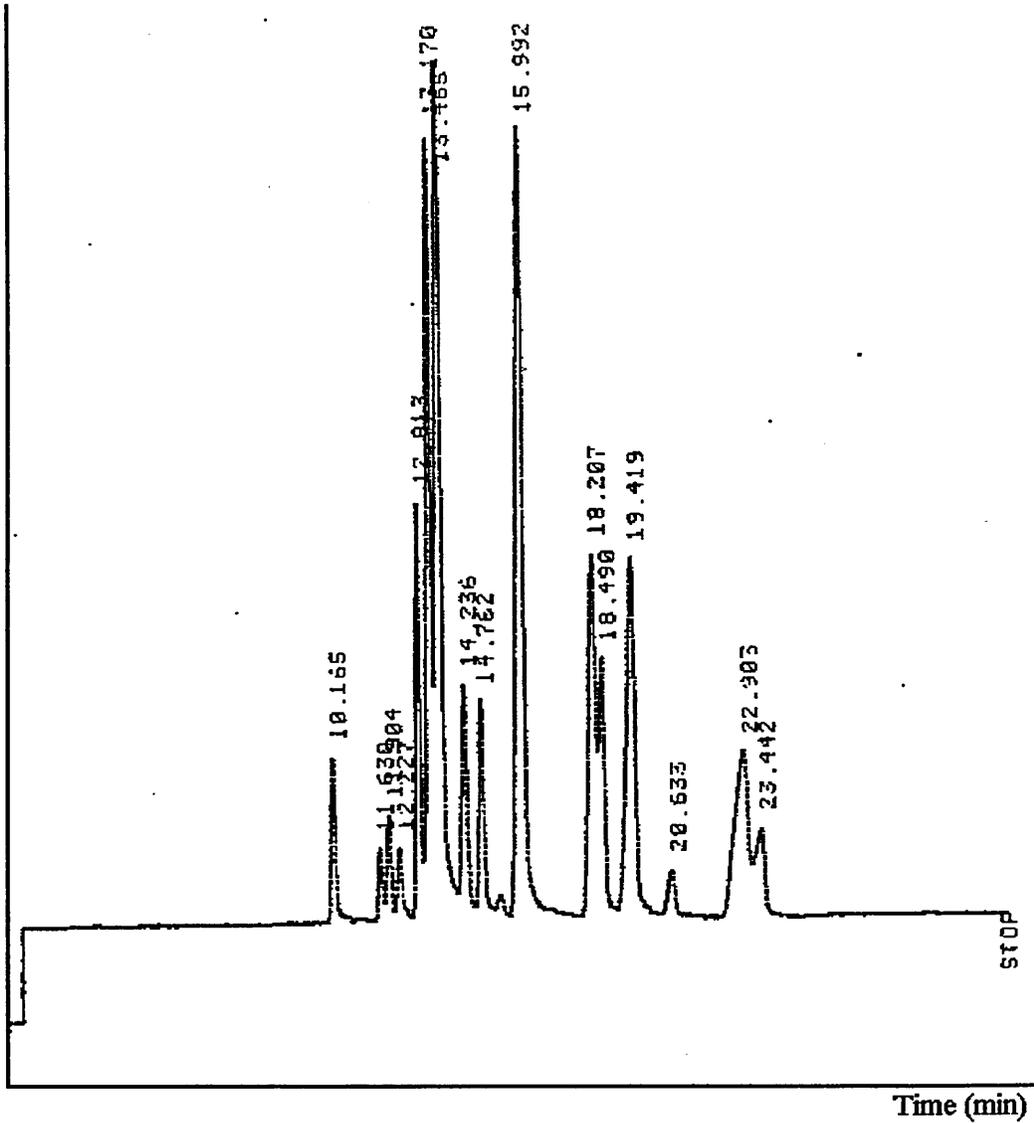
Figure 3. Representative electrochromatogram of a 16 component EPA standard PAH mixture separated on a 3μm Spherisorb, 20cm packed column with an applied voltage of 30Kv with a running buffer of 20% 4 mmolL$^{-1}$ borate in acetonitrile at pH 10 (n=5).
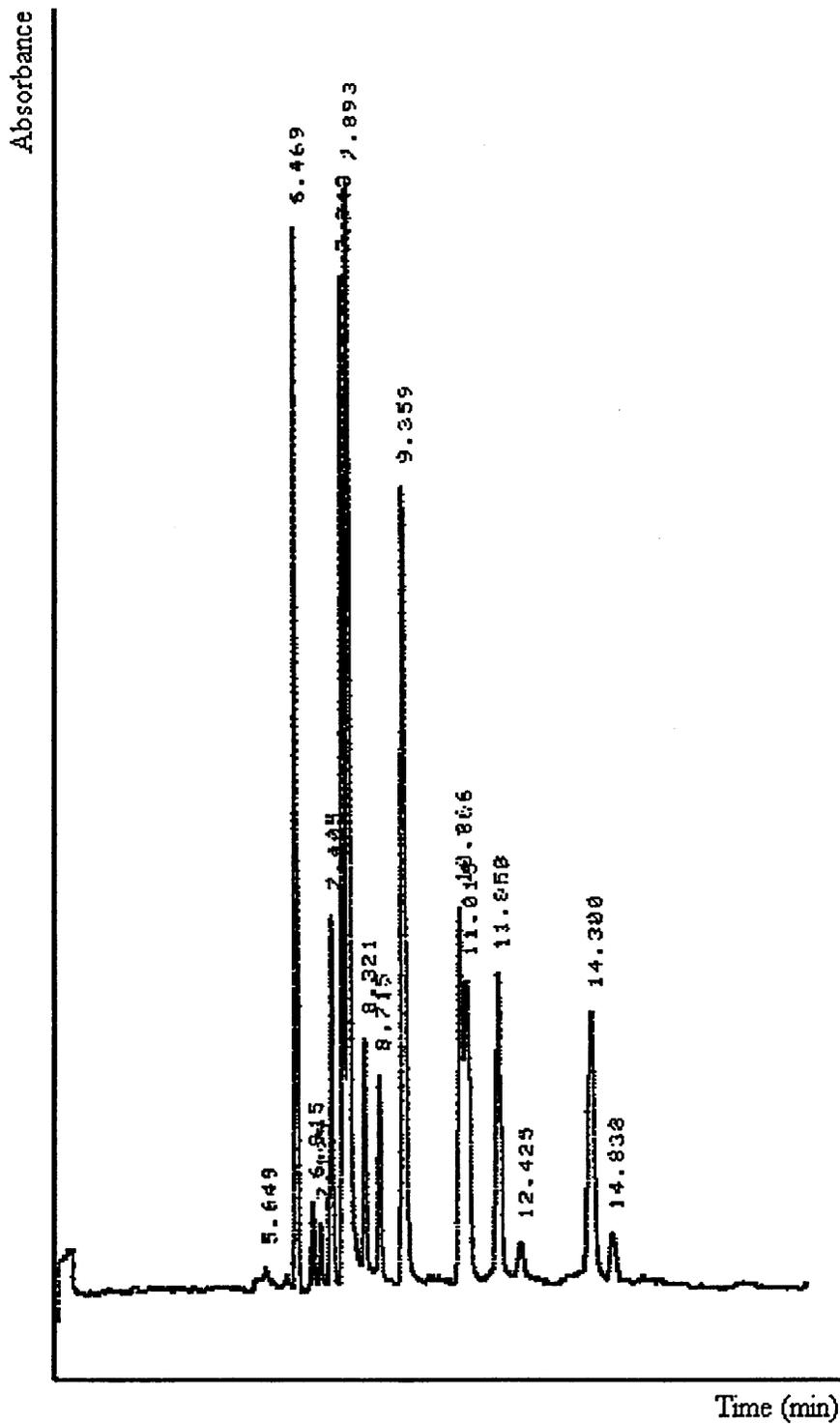
Figure 4. Representative electrochromatogram of a 16 component EPA standard PAH mixture separated on a 3μm Spherisorb, 20cm packed column with an applied voltage of 30Kv with a running buffer of 20% 20 mmolL$^{-1}$ TRIS in acetonitrile at pH 10 (n=5).

The comparison of a borate buffer to that of a TRIS buffer in the initial investigation of the instrumental parameters indicated that there could be a potential advantage, in terms of resolution and efficiency of the separations, through the use of TRIS. Hence, the next set of experiments was performed using a TRIS buffer system and at the same time the sample plug was also altered to a 50:50 organic aqueous solution. This was done to improve peak efficiency and because the previous work indicated there would be no adverse effects on the overall system.

These experiments indicated that there was an improvement in the chromatography that was obtained with the TRIS buffer, when compared to a similar separation performed with borate buffer (see figures 3 and 4) and this was not just the result of sample focussing on loading. The improvement was the noticeable difference in the migration time of the analytes, with those separated with the borate buffer having migration times approximately one and a half times greater than those of the TRIS separation.

To further improve the resolution, instead of reducing the applied voltage, the water content of the mobile phase was increased. The decision to use changes in buffer composition over increased packed length came as a result of the earlier work described in chapter 4, where significant improvements in resolution were obtained by varying the organic content and also the problematic nature of fabricating longer columns. The result was an increase in the run time of approximately 50%. However, this also improved the

resolution with near complete baseline resolution of the analytes being obtained, as shown in figure 5.
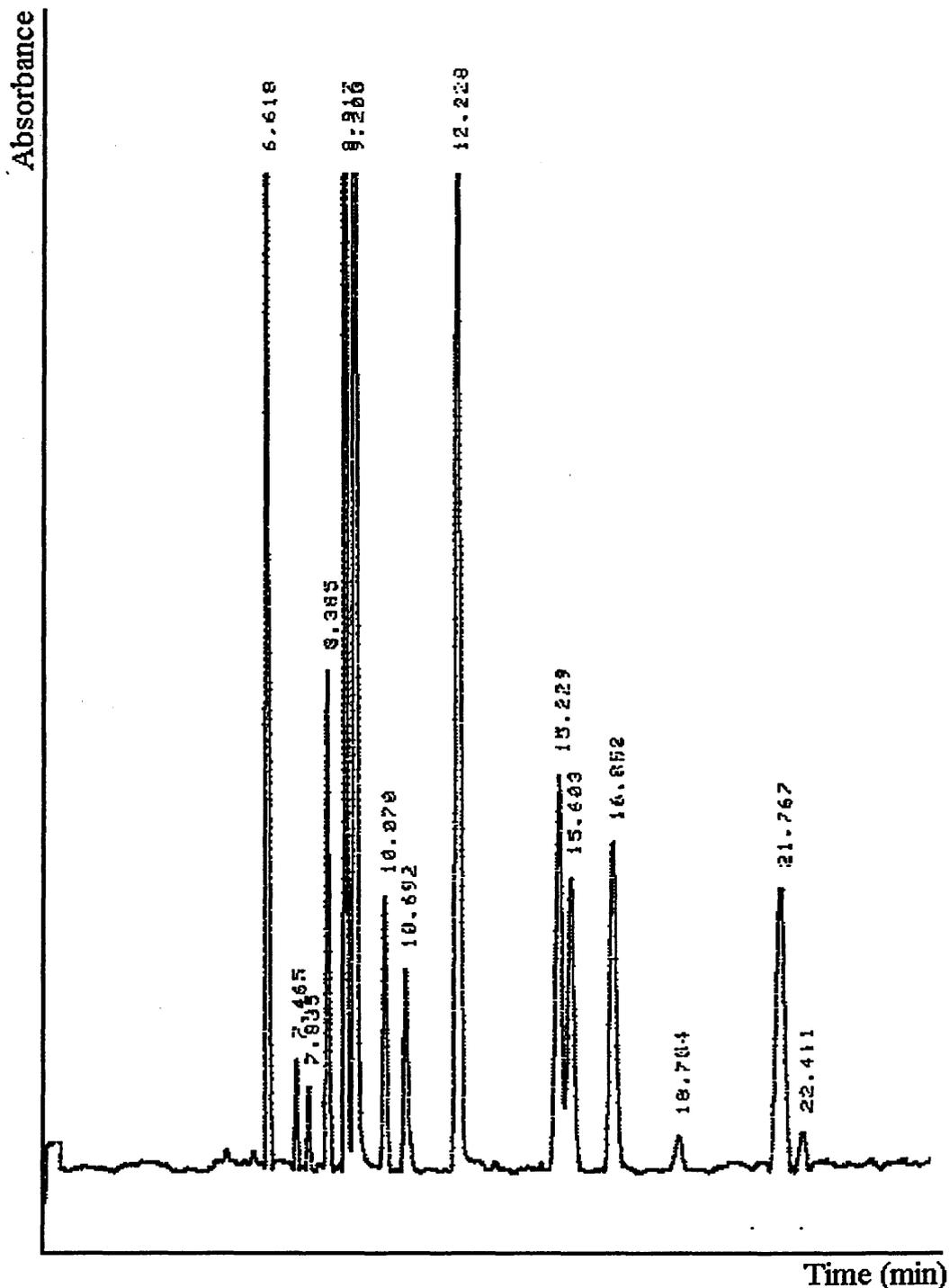


Figure 5. Representative electrochromatogram of a 16 component EPA standard PAH mixture separated on a 3μm Spherisorb, 20cm packed column with an applied voltage of 30Kv with a running buffer of 20% 20 mmolL$^{-1}$ TRIS in 70% acetonitrile and 10% water at pH 10 (n=5).

## 5.2.4. Conclusion.

The aim of this study was to evaluate how well our column fabrication procedure worked, through the separation of a 16 component PAH mixture. The results were compared to the work of Yan *et al.* (2,5) and Carter-Finch and Smith (6) who also analysed the same 16 component mixture. The separations that were achieved were comparable to their results, even taking into account experimental and instrumental differences. This indicates that the packing procedure that has been developed produces columns of a similar standard to those of other research groups.

This study also allowed some of the operational parameters that were investigated in the previous chapter to be used in a "real" application. These proved beneficial and in line with expectations based on the initial investigations presented in chapter 4. Enhancements to the separation were demonstrated when the electrolyte was changed from borate to TRIS, followed by the reduction in organic content of the running buffer and lastly alterations of the sample plug composition to focus the solutes at the head of the column. With each of the changes to the separation method additional improvements were observed, especially with respect to the resolution of the solutes.

To further enhance the separation of the PAH mixture, further work to investigate the effect of electrolyte concentration and pH of the running buffer could be performed.

### 5.3. Analysis of a Test Mixture.

This application was designed to test the ability of the system to separate a variety of compounds with a range of polarities. The effect of changing the organic composition of the running buffer was also studied.

### 5.3.1. Chemicals.

The running buffer was as stated in chapter 2. The test mixture contained pyridine and thiourea (BDH Chemicals Ltd., Dorset, UK), anisole, benzophenone and biphenyl (Sigma-Aldrich, Dorset, UK).

### 5.3.2. Procedure.

This is described in chapter 2.

### 5.3.3. Results and Discussion.

The analytes were chosen to represent a cross section of polarities, from non-polar (biphenyl) to basic (pyridine). The structures of the analytes are shown in table 1. The elution order was as expected, based on the structure of the analytes. This can be observed in figure 6. The most non-polar compounds have the greatest affinity for the stationary phase and hence the longer migration times.

Altering the percentage organic component of the running buffer did however seem to affect the elution of pyridine and anisole. As figure 6 indicates, with decreasing organic content the elution order of pyridine and anisole was reversed. This can also be clearly observed in figure 7. Pyridine reflects the

thiourea profile, which indicates that the EOF has a more dominant effect on the pyridine than changes to the organic content of the running buffer, which in turn has a dominant influence on the more non-polar solutes.

| Compound | Structure |
|---|---|
| Thiourea |  |
| Pyridine |  |
| Anisole |  |
| Benzophenone |  |
| Biphenyl |  |
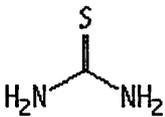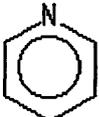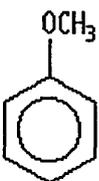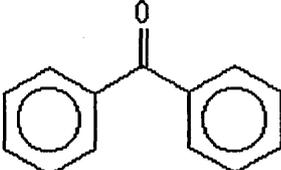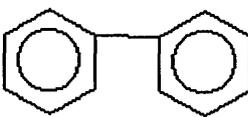
Table 1. The analytes used in this study and their respective structures.

Table 1 allows the differences in the structures of the analytes to be clearly seen, with the thiourea being the most polar and hence having no affinity for the reverse phase stationary phase. The remaining solutes display a range of polarities, with pyridine being the next in polarity, in part due to its size and the presence of the nitrogen atom. Both the ether and ketone are polar, due to the small dipole moment caused by the C-O-C bond in the ether not being linear, hence the dipoles of the C-O bonds not cancelling each other out, while the polar carbonyl group in the benzophenone creates its polarity. However, in this investigation the relative sizes of the analytes determine their elution

order with the smaller anisole molecule being eluted before the benzophenone. Biphenyl, which is non-polar, elutes latest as this has the greatest affinity for the stationary phase.
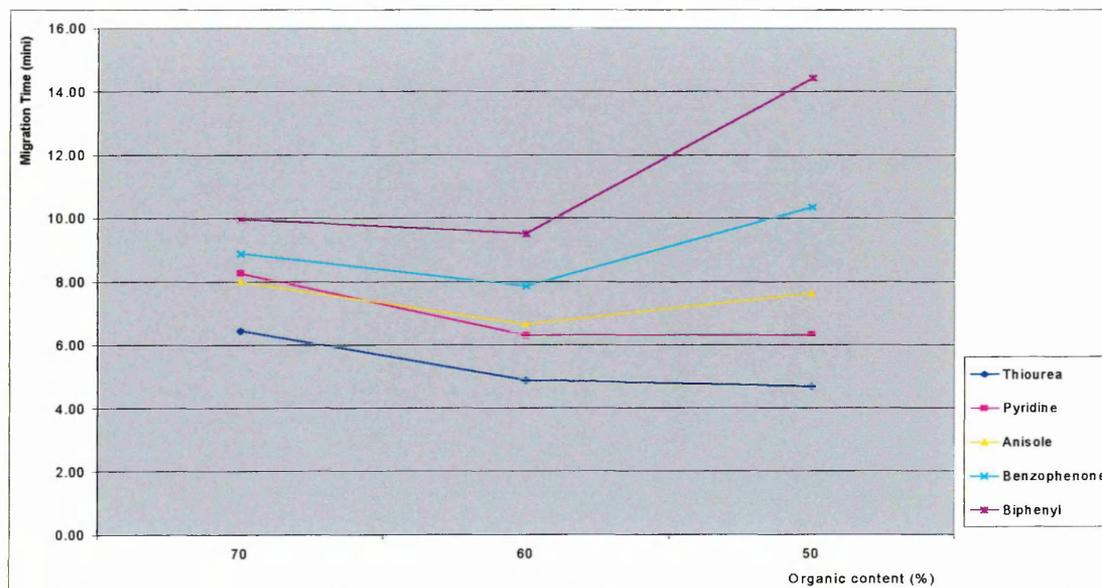


Figure 6. Variations in the migration times of the test compounds with changes in the organic content of the electrolyte (n=5). On a 3μm Spherisorb, 20cm packed column with an applied voltage of 30Kv and a running buffer of 20% 4 mmolL$^{-1}$ borate in acetonitrile (acetonitrile replaced with water as organic content is reduced)
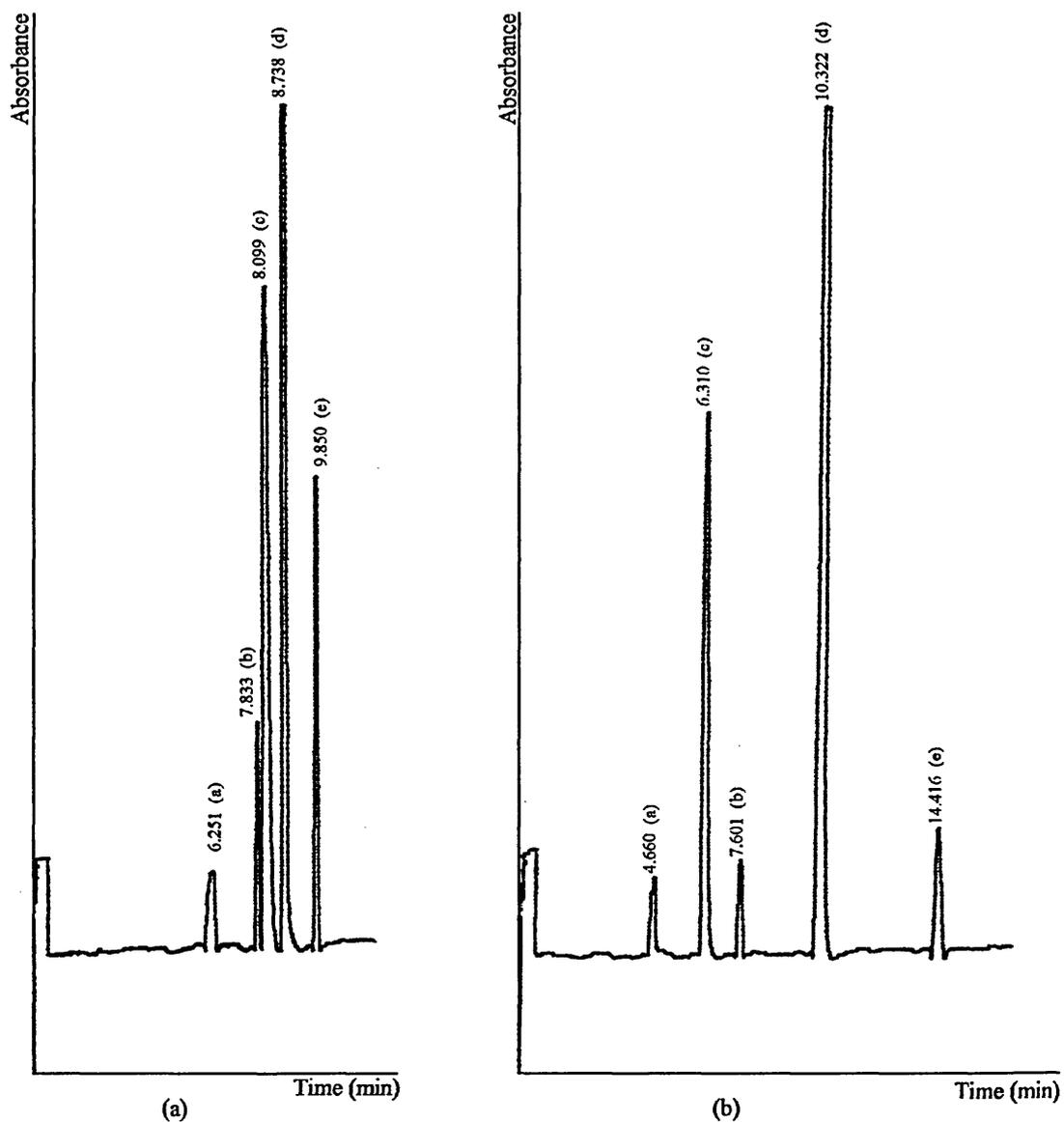
Figure 7. Electrochromatogram of the test solution on a 3μm Spherisorb, 20cm packed column with an applied voltage of 30Kv and a running buffer of 20% 4 mmolL$^{-1}$ borate in (a) 80% acetonitrile (b) 50% acetonitrile and 30% water at pH 9 (n=5). Peaks (a) thiourea, (b) pyridine, (c) anisole, (d) benzophenone, (e) biphenyl.

## 5.3.4. Conclusion.

Thiourea is used as a neutral marker and therefore it has no electrophoretic mobility. It is also polar and hence has no affinity for the stationary phase. Pyridine reflects the elution profile of thiourea, however the migration times are longer. If there were no interaction between pyridine and the stationary phase it would elute with the thiourea as at pH 9 pyridine is uncharged. However, as the migration times are greater than those of thiourea some interaction must occur but this is independent of the relative polarity of the mobile phase.

The remaining solutes are separated by interactions with the stationary phase. As expected, as the polarity of the solute is reduced the greater is the migration time observed.

As could be predicted from earlier work presented in chapter 4, the reduction in the organic content of the running buffer aided the resolution of the analytes. Overall this study shows that the system that has been developed is capable of simultaneously separating analytes which have different polarities. The order of elution, in the most part, is also as predicted based on knowledge of the stationary phase interactions with analytes of differing properties.

### 5.4. Prostaglandins.

### 5.4.1. Introduction.

Prostaglandins are metabolites of arachidonic acid, which is released from phospholipid stores (7,8). Arachidonic acid is a 20 carbon unsaturated fatty acid which contain four double bonds, the structure of which is shown in figure 8. Once released, the arachidonic acid forms a series of compounds referred to as eicosanoids. The dominant classes of eicosanoids that are produced are the prostaglandins, thromboxanes (figure 9) and the leukotrienes, with other derivatives also being produced.
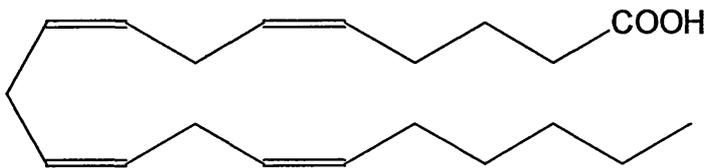


Figure 8. The structure of arachidonic acid.

The eicosanoids are an important class of compounds as they have been implicated in the control of a host of important biological functions. The most important of these functions is considered to be the mediation and modulation of inflammatory reactions (9-12).

Initially it was thought that these substances were produced by the prostate gland, hence prostaglandin. This proved an incorrect assumption, as over a period of time it was found that these substances covered a whole range of compounds (13,14). It is known that excess production of the eicosanoids can cause a host of physiological effects throughout the human body.

### 5.4.2. Available Methods for Analysis of Prostaglandins.

A variety of methods can be employed to determine levels of the eicosanoids. In most applications, these methods have to be able to deal with low concentration levels, typically below 1ng/ml. Their structures are all closely related, which means that resolution of closely related prostaglandins can be very difficult to obtain.

The main assay methods that have been used in studies of the effects of the eicosanoids on biological systems are immunoassays (both enzyme (EIA) and radio (RIA) methods (15)), gas chromatography – mass spectrometry (GC-MS) (16-18) and high performance liquid chromatography (HPLC) (19-22). These techniques have all been utilised in determining levels in biological tissues and fluids. There have also been reports of methods using CZE and MEKC to analyse samples containing prostaglandins (23,24).

Jin-Lau *et al.* (23) showed that separation of the weakly acidic prostaglandin and thromboxane was possible using CZE. This was achieved without reversing the direction of the EOF under basic conditions, which is the normal method of separating anionic groups. Hsieh and Kuo (24) separated a large series of eicosanoids (seven prostaglandins and a thromboxane) by utilising the pseudo-stationary phase offered by MEKC. This method provided a good separation of the analytes and also proved robust enough to handle samples extracted from biological materials.

There has been only one publication on the separation of prostaglandin from a number of intermediate compounds using CEC, by Smith and Evans (25). They employed an ODS column with a phosphate buffer at pH 7.3 to separate a single prostaglandin from five synthetic impurities.

## 5.4.3. Experimental.

### 5.4.3.1. Chemicals.

The running buffer was as stated in chapter 2. The prostaglandins and thromboxanes used were prostaglandin $E_1$, prostaglandin $E_2$, thromboxane, and thromboxane $F_{1\alpha}$ (Sigma-Aldrich, Dorset, UK).

### 5.4.3.2. Procedure.

This is described in chapter 2.

### 5.4.4. Results and Discussion.

Due to the structure of the eicosanoids, with a minimum of one double bond on the aliphatic chain, they have poor UV absorbing properties, their UV/vis spectra having maximum absorbance below 200nm (23). Another feature of these compounds is the similarity in structure (including structural isomers) and masses, as can be seen in table 2, which shows the analytes used in this investigation. These compounds are weakly acidic and are structurally similar, which suggests difficulty in resolving this series of eicosanoids.

The initial investigation was carried out at pH 7.3 (24) and it was felt that the additive effects of both the chromatographic and electrophoretic separation processes could have a beneficial effect on the separation. With the weakly

acidic solutes being retarded their natural tendency would be to migrate to the anode.

| | |
|---|---|
|  |  |
| Prostaglandin $E_1$ | Thromboxane |
|  |  |
| Prostaglandin $E_2$ | Thromboxane $F_{1\alpha}$ |

Table 2. The structures of the eicosanoid analytes that were investigated.

The initial results indicated that the analytes eluted around the same time (6.7min). This observation was confirmed when all the analytes were run together, as shown in figure 9.

It was thought that by suppressing the charge by reducing the pH of the running buffer and hence reducing the flow rate the analytes might be resolved. In this case, they would have no affinity to migrate to the anode and therefore be separated purely by a chromatographic method i.e. interaction with the stationary phase. When the analytes were analysed individually, they again all had approximately the same migration time of 9.5min. This result indicated that all the analytes again had the potential to co-elute when run together, which was confirmed when they were analysed simultaneously, as shown in figure 10.
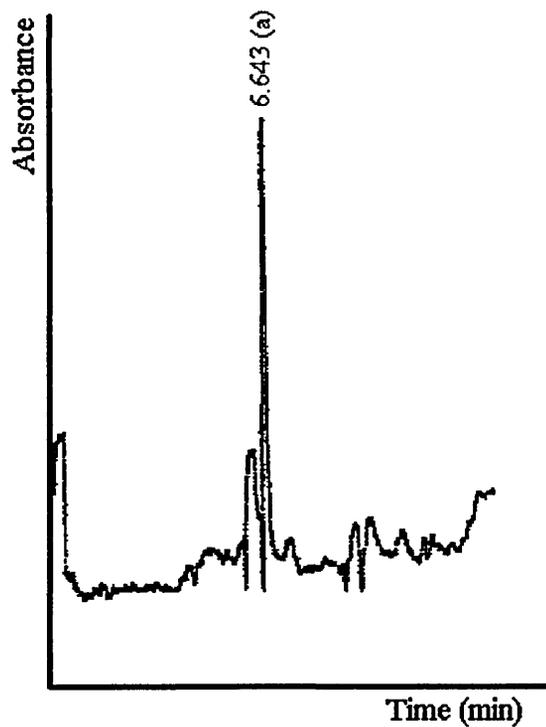
Figure 9. Electrochromatogram of the attempted separation of the eiconsanoid on a 3μm Spherisorb, 20cm packed column with an applied voltage of 30Kv and running buffer of 20% 20 mmolL⁻¹ TRIS in acetonitrile at pH 8. ((a) Prostaglandin $E_1$, $E_2$, thromboxane and thromboxane $F_{1\alpha}$)
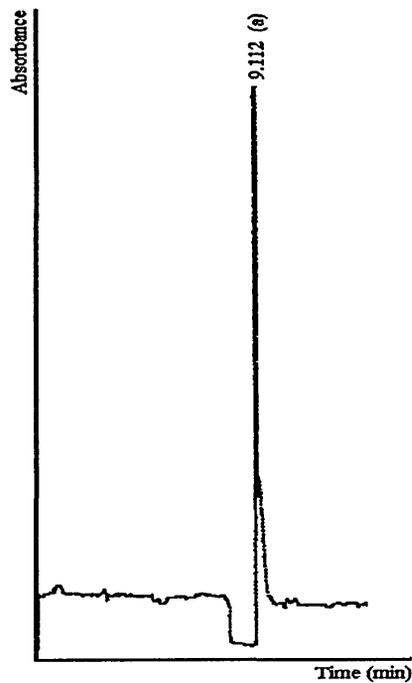


Figure 10. Electrochromatogram of the attempted separation of the eiconsanoids on a 3μm Spherisorb, 20cm packed column with an applied voltage of 30Kv running buffer of 20% 20 mmolL⁻¹ TRIS in acetonitrile at pH 3. ((a) Prostaglandin $E_1$, $E_2$, thromboxane and thromboxane $F_{1\alpha}$)

The organic composition of the buffer was then altered to see if this would improve resolution, as had been observed previously. The acetonitrile content was reduced and then replaced, initially with water and then methanol. The results of this were inconclusive, as the EOF that was generated was unstable throughout all the experiments that were attempted. However, such observations as were made suggested that there was no improvement in the resolution and peak tailing when methanol was used.

## 5.4.5. Conclusion

Due to the close structural similarities of the prostaglandins, as discussed at the beginning of this section, they could not be resolved under the conditions that were investigated. Further work is obviously needed to separate these compounds; this includes investigating increasing the packed length of the capillary, the pH of the buffer solution and the composition of the running buffer, including the type of organic phase i.e. use of a less polar solvent system.

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# CHAPTER 6, Applications of CEC – Nicotine Metabolities

## 6.1. Introduction.

Smoking is a major cause of preventable mortality and morbidity in the Western world and epidemiological studies of smoking-related diseases would be greatly enhanced if reliable methods could be established for identifying smokers and assessing their relative intakes of tobacco derived products. This is especially so for passive smoking. A number of methods, including HPLC (1,2), GC (3), colorimetry (4) and immunoassay (5) have been described for the determination of nicotine and its metabolites. Many of these methods target specific metabolites as useful markers for exposure to tobacco smoke. The separation of several metabolites from each other and from other chemicals commonly occurring in body fluids, e.g. caffeine, requires both high efficiency and selectivity because their structures are similar in size, as table 1 demonstrates.

Capillary electrophoresis (CE) methods have the capacity to provide both the efficiency and selectivity required for the separation of the nicotine metabolites. CZE can only separate charged groups, but MEKC and CEC allow separation of such compounds when their charge has been suppressed by pH control.
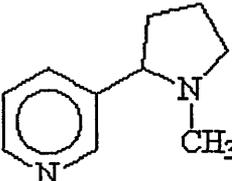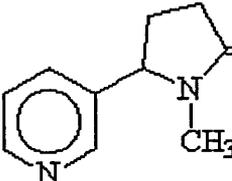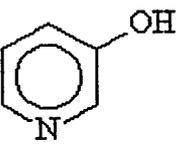
| | | |
|---|---|---|
| Nicotine | Cotinine | 3-Pyridyl carbinol |
| Nornicotine | Norcotinine | 3-Pyridyl-oxomethyl butyramide |
| Nicotine-1-N-oxide | 3-hydroxy cotinine | |
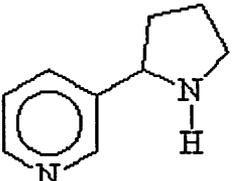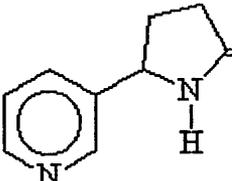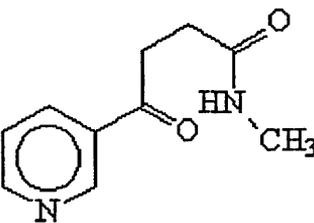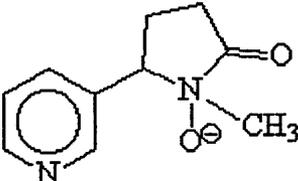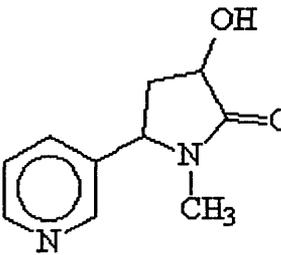
Table 1. The structures of nicotine and some of its metabolites.

## 6.2. Chemicals.

The running buffers are as stated in chapter 2. Pyridine and triethylamine (TEA) were purchased from Aldrich (Aldrich, Dorset, UK). The packing materials that were used were Xtec's ODS, Exsil's 100 ODS and Pt $C_{18}$.

## 6.3. Samples.

The nicotine and its metabolites, obtained from Dr. R. Smith of Sheffield Hallam University, were received as 10μg/ml aqueous solutions. These are listed below:

| nicotine | 5-hydroxycotinine |
|----------|-------------------|
| cotinine | 3-hydroxycotinine |
| norcotinine | nornicotine |
| nicotine-1-N-Oxide | cotinine-N-oxide |
| 3-pyridyl-oxomethyl butyramide | γ-3-pyridal γ-oxobutyric acid |
| 3-pyridylcarbinol | 3(R)-5(S)-trans-3-hydroxycotinine perchlorate |

The samples for CEC required blowing down under nitrogen before being reconstituted with the appropriate buffer. Nicotine, cotinine and pyridine were also obtained from Sigma-Aldrich, Dorset, UK.

## 6.4. Investigation utilising CZE.

### 6.4.1. Results and Discussion.

The study was undertaken at low pH (pH3), to ensure that the nicotine metabolites were charged. Being cationic, the analytes are drawn thorough the capillary without the need to alter the instrument's polarity. The analytes contain nitrogen heterocycles that absorb at 260nm.

### 6.4.2. The Effect of Electrolyte Concentration.

The concentration of electrolyte dictates the velocity of the EOF, as stated in chapter 1. With low concentrations of electrolyte the EOF velocity is high, whereas at high concentrations the velocity is reduced. This consequently affects the analytes in a similar manner, with low electrolyte concentrations resulting in high mobilities but reduced resolution and the reverse situation at

high concentration. Optimisation of the electrolyte concentration is therefore important.

Only three concentrations of electrolyte were investigated: 10, 50 and 100 mmolL$^{-1}$ phosphate. It was felt that this covered the range over which the analysis would be carried out and could be refined at a later point. As table 2 indicates, at low electrolyte concentration the electrophoretic mobility of the ions is such that the cotinine, nicotine and norcotinine co-elute, while at the higher concentrations separation is achieved and improved at 100 mmolL$^{-1}$. This is not a surprising result when considering how similar the structures of the analytes are, because reducing the electrophoretic mobility of the solute and the EOF velocity by increasing the electrolyte concentration potentially allows greater resolution to be achieved as the differences in the analytes mobilities are thus exaggerated.

| | Migration time (min) | | |
|---|---|---|---|
| Electrolyte Conc. (mmolL$^{-1}$) | 10mM | 50mM | 100mM |
| Cotinine | 4.2 | 4.4 | 4.5 |
| Nicotine | 4.2 | 4.6 | 4.7 |
| Norcontinine | 4.2 | 4.7 | 5.0 |
| Nornicotine | 7.5 | 8.0 | 8.3 |

Table 2. Migration times of nicotine and three of its metabolites with changes in the electrolyte concentration of the running buffer (n=5).

## 6.4.3. The Effect of Buffer Additives.

During the initial work undertaken to separate two of the compounds (nicotine and cotinine), peak tailing was observed. This was due to interactions of the analytes with the capillary wall, which at pH3 had not had all of its charge suppressed (anionic silanoate groups).

Therefore, two options were investigated to overcome this problem. The first was the addition of another base to the running buffer, which would interact with the silanol groups reducing the number of times that the analytes would interact with the capillary wall (6). The effect of adding triethylamine (TEA) to the buffer, whilst analysing nicotine, was investigated. This approach was to limit the interaction of the solute with the capillary wall and thus improve peak efficiency, with the effect on nicotine giving a representative view of how this additive would affect the overall separation. Differing volumes of TEA were added to the buffer, which was of 100 mmolL$^{-1}$ phosphate at pH 3. As figure 1 demonstrates, the exact concentration of additive is critical as too little can result in no peaks being resolved.

When this method was used in conjunction with the samples that were provided, however there was significant background noise and hence this method of suppressing the charge on the capillary wall was dismissed for this particular application.
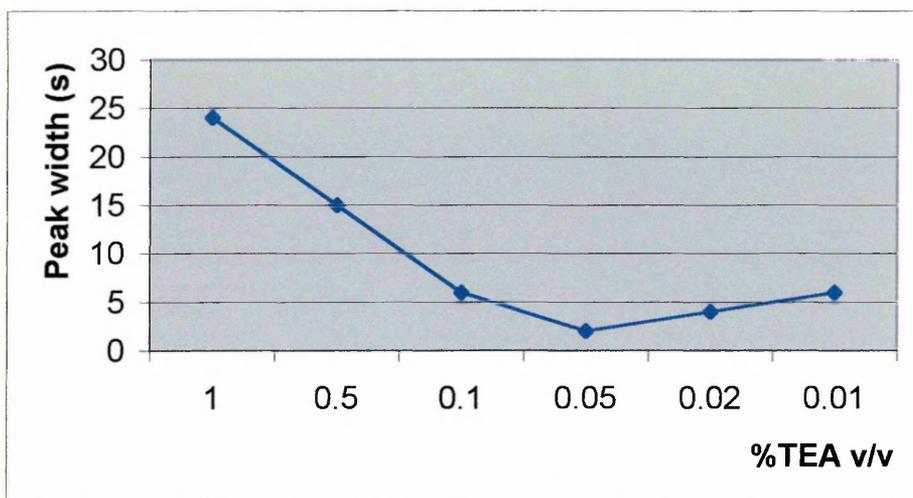
Figure 1. The effect on the peak width of nicotine of the addition of TEA to the running buffer (100mmolL$^{-1}$ phosphate, pH 3, n=3).

The second method be employed to reduce interaction of the analytes with the capillary wall was the introduction of an organic solvent to the aqueous buffer (7). This has been shown to have beneficial effects on the separation of analytes in CE. It works by altering the viscosity and dielectric constant of the buffer and hence the performance characteristics of the system. Addition of acetonitrile can potentially reduce the interactions between the basic nicotine metabolites and the acidic silanol groups on the surface of the capillary, reducing peak tailing and hence increasing the efficiency of the separation. Addition of 10% acetonitrile to the run buffer was seen to improve the peak shape when nicotine was analysed (see figure 2). This also resulted in an increase in sensitivity when compared to a similar analysis without the addition of acetonitrile.
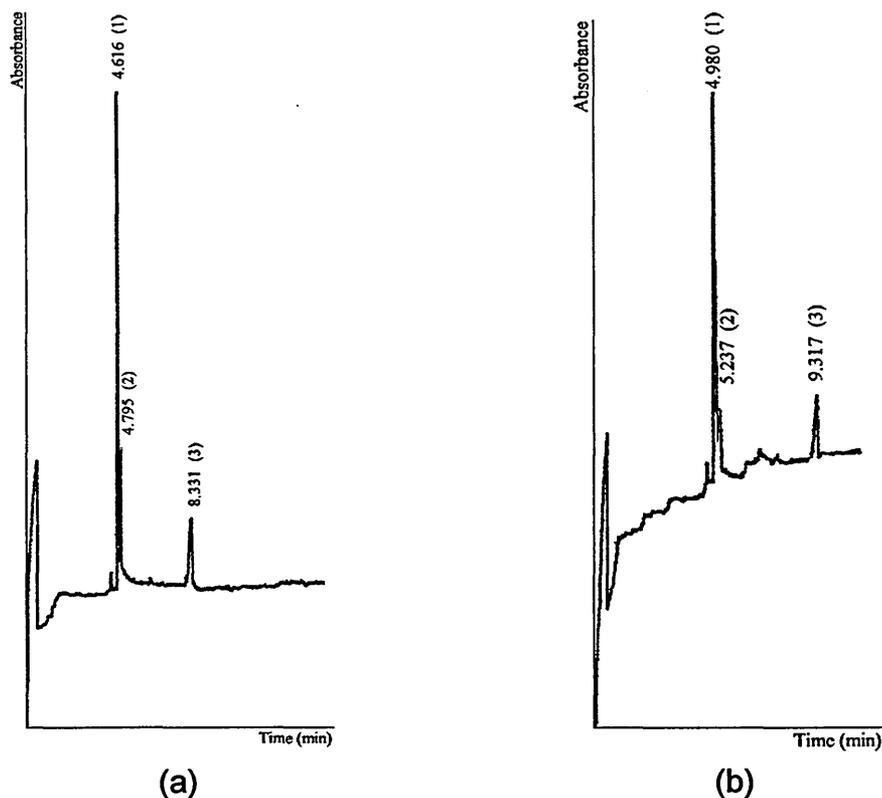
Figure 2. Electropherograms obtained with a running buffer of 100mmolL$^{-1}$ phosphate with the addition of (a) 10% acetonitrile (b) 0% acetonitrile (n=5). The analytes are (1) cotinine (2) nicotine (3) nornicotine.

## 6.4.4. Applied Voltage.

The effect of altering the applied voltage was studied. It was though that by decreasing the voltage, thus increasing differences in potential migration times, and hence an improvement in resolution might be observed. This was due to the applied voltage being one of the factors that controls the magnitude of the electrophoretic velocity of the analytes (equation 1.04). As figure 3 shows, an increase in migration time was observed with lower applied voltage.
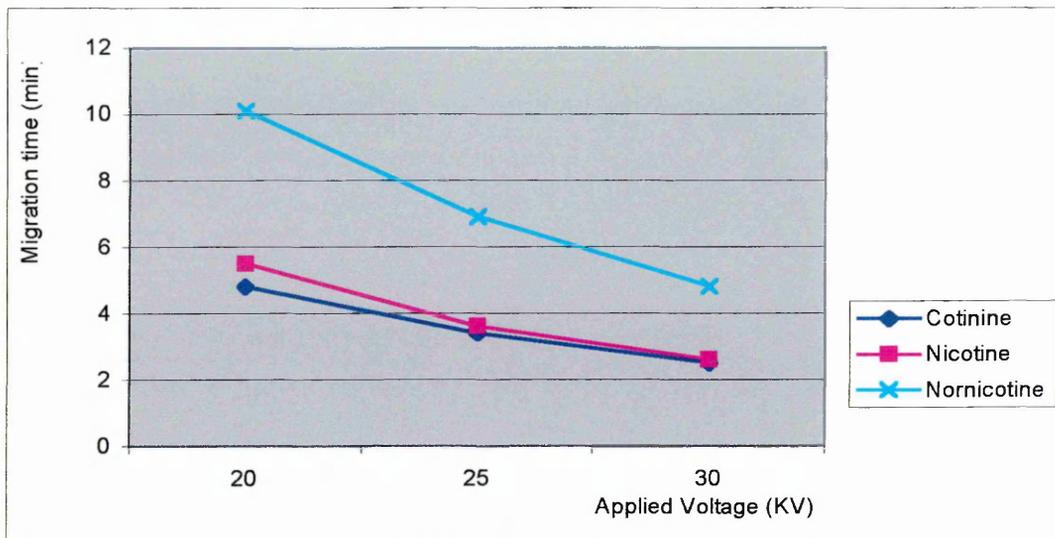
Figure 3. Changes in the migration time with voltage of nicotine and its metabolites (n=5).

Reduction of the applied voltage also resulted in an increased difference in the migration times observed. However, there was a corresponding increase in peak width, which did not allow an increase in resolution between cotinine and nicotine to be achieved as the voltage was reduced.

6.4.5. Conclusion.

The final separation that was developed can be seen in figure 4 and table 3. The conditions that were used for the separation were a running buffer of $100 mmoL^{-1}$ phosphate with 10% acetonitrile adjusted to pH 3 and an applied voltage of 25Kv. The conditions were chosen to give the maximum resolution combined with the greatest peak efficiency. An obvious problem is the poor sensitivity of the method and baseline stability, this meant that three separate runs had to be undertaken to determine the migration times of the metabolites.
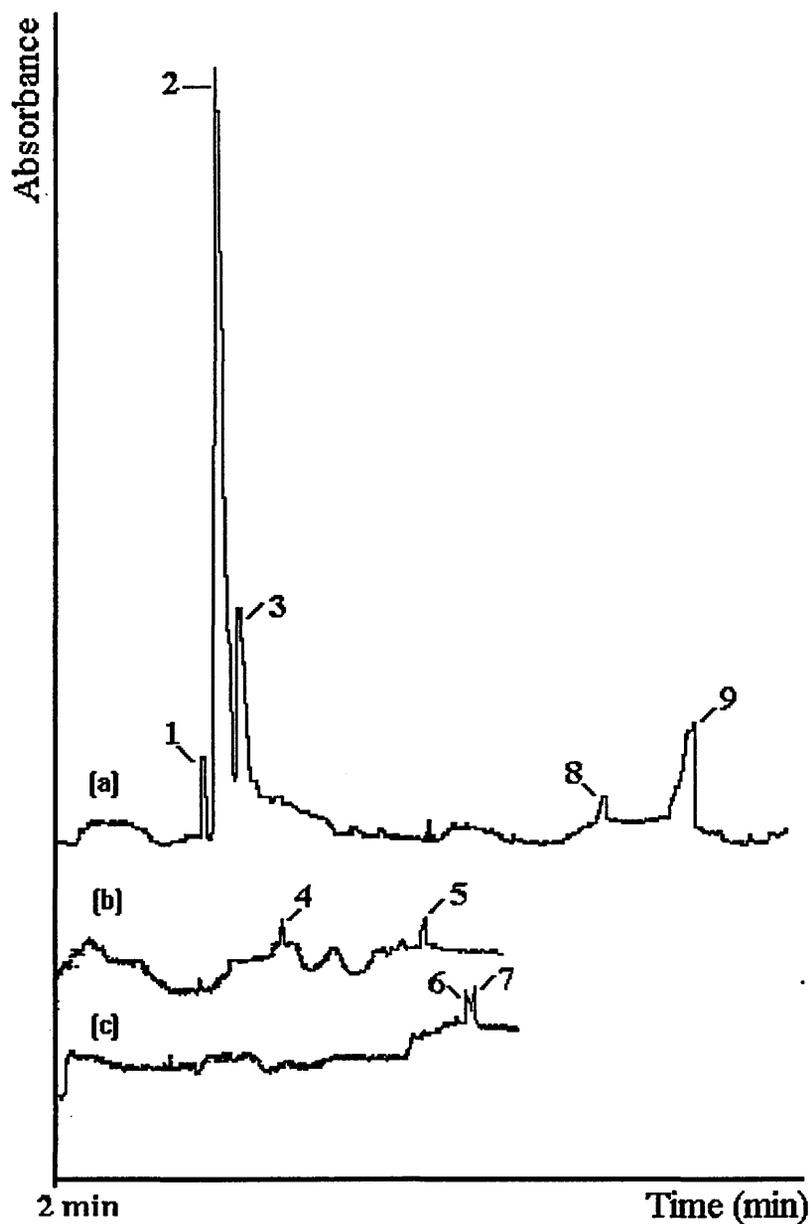
Figure 4. Three separate electrochromatograms are shown indicating the potential separation and relative positions of nicotine and its metabolites. The analytes are listed in table 3 with their respective migration times. The conditions used were 100mmolL$^{-1}$ phosphate (aq) with 10% acetonitrile at pH 3. This data was collected over three consecutive runs.

| Elution Order | Migration Time (min) | Concentration (ppm) | Nicotine Metabolites |
|---|---|---|---|
| 1 | 4.2 | 2 | Cotinine |
| 2 | 4.4 | 2 | Nicotine |
| 3 | 4.6 | 4 | Norcotinine |
| 4 | 4.8 | 5 | Nicotine-1-N-Oxide |
| 5 | 6.5 | 5 | 3-pyridyl-oxomethyl butyramide |
| 6 | 6.9 | 5 | 5-hydroxy cotinine |
| 7 | 7.0 | 5 | 3-hydroxy cotinine |
| 8 | 8.1 | 4 | Nornicotine |
| 9 | 8.9 | 2 | 3-pyridylcarbinol |
| N/D | ** | 10 | 3(R)-5(S)-trans 3-hydroxy cotinine perchlorate |
| N/D | ** | 10 | $\gamma$-3-pyridal $\gamma$-oxobutyric acid |
| N/D | ** | 10 | Cotinine-N-oxide |

Table 3. The migration times and elution order of the nicotine metabolites studied, from figure 14. N/D is not detected. This data was collected over three consecutive runs.

These results indicate that the separation of nicotine metabolites is possible using CZE, even with the limited investigation that was undertaken. To improve the separation alternative electrolytes should be investigated. This could improve the baseline stability and reduced the risk of Joule heating in the column as a result of the high electrolyte concentration. Because of the limited sample volumes and concentrations available sample loading also requires investigation to increase the sensitivity and improve resolution. Either a focussing or stacking technique at the head of the column could achieve this. Another potential way to improve sensitivity is optimisation of the wavelength used to monitor the analytes. A further important area to be considered in future investigations would be the electrolyte that is used, so instead of phosphate, ammonium acetate or formate could be utilised instead.

## 6.5. Investigation utilising CEC.

An initial investigation was undertaken using nicotine, cotinine and pyridine standards. As figure 5 shows, under standard conditions (20% 4 mmolL$^{-1}$ borate in acetonitrile, pH 9) the three analytes were readily resolved. Detection was carried out at 260nm.
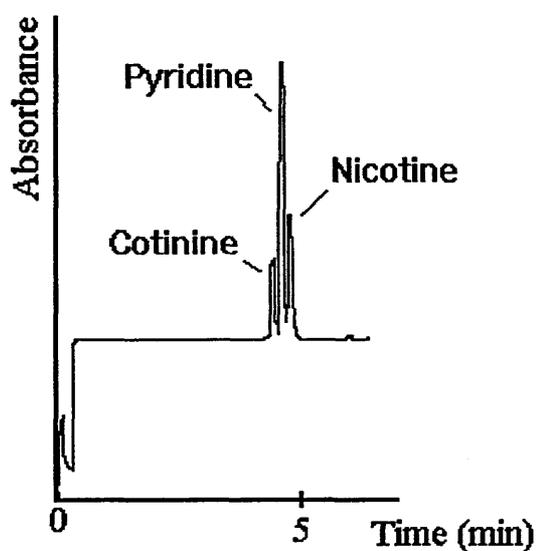


Figure 5. Separation of cotinine, nicotine and pyridine by CEC, using 3μm Spherisorb, 20cm packed length with an applied voltage of 30Kv and a running buffer of 20% 4mmolL$^{-1}$ borate in acetonitrile at pH9 (n=3).

Experiments were then performed using the nicotine and metabolites supplied by Dr. R. Smith. These samples were supplied as aqueous solutions. This meant that the solutions had to be blown down under nitrogen and reconstituted with acetonitrile. The low concentration of analyte in solution (10ppm) and the limited quantity of solution available necessitated this procedure.

The quantity of sample material available allowed only two experiments to be conducted. These were at low and high pH (3 and 9 respectively). The electrochromatograms obtained are shown in figure 6.

Figure 6 shows separations at both high and low pH, with neither separation having a clear advantage over the other. At high pH the basic analytes are neutral and hence would be separated by interaction with the stationary phase only. At lower pH, a combination of interaction with the stationary phase and electrophoretic mobility is expected to facilitate the separation. However, this does not appear to have happened, as the separation profiles of the two analyses are similar. In both cases there was peak tailing and fronting, which would indicate that there is interaction between the solutes and the silanol groups both on the stationary phase and capillary wall.

In a recent paper by Dittmann *et al.* (8) the separation of basic solutes by reverse phase CEC was discussed. Consideration was given to the interaction between the basic solutes and the ionizable silanol groups, which resulted in high retention and poor peak shapes; these effects were also observed in the work undertaken in this study. There were several suggested methods to overcome this problem, including to end cap the stationary phase (9), use a polar stationary phase (10,11) or add a competing amine to the running buffer (12,13), which has been shown to improve peak shape.
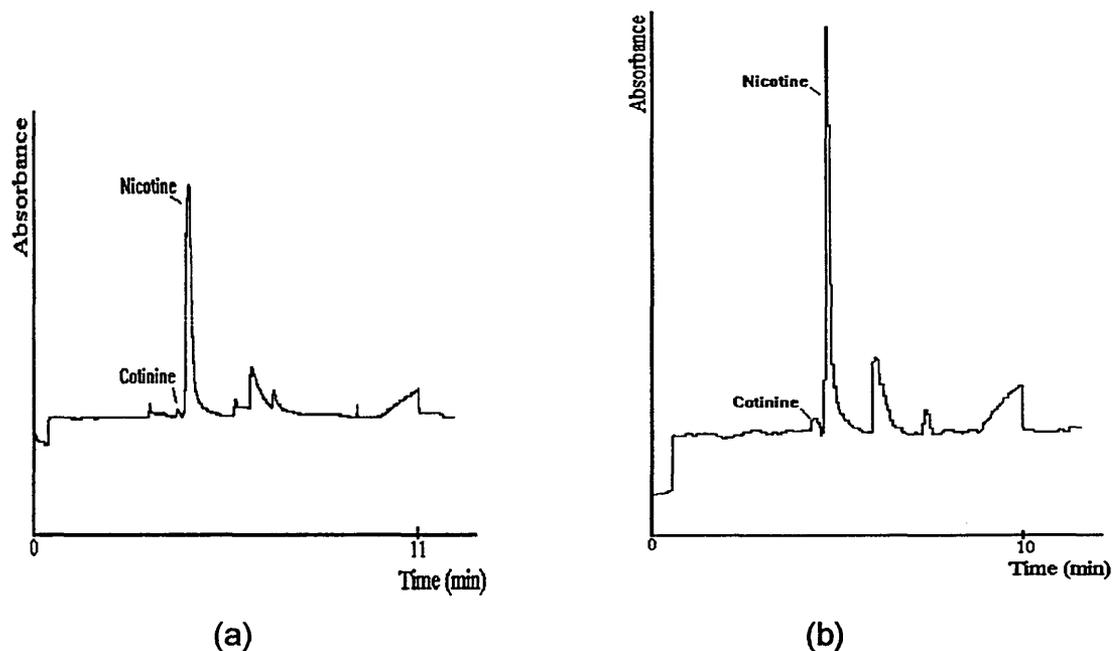
Figure 6. Separation of a mixture of nicotine and its metabolites by CEC using (a) 20% TRIS in acetonitrile pH 3 (b) 20% borate in acetonitrile pH 9 (n=3).

Therefore, further investigation was undertaken to improve peak shape and therefore sensitivity by reducing the interaction of the metabolites with the capillary and stationary phases surfaces. By changing the electrolyte to ammonium acetate, it was reasoned that the ammonium ions would interact with the silanoate groups on the capillary and stationary phases surfaces, thereby reducing the interactions between the basic nicotine metabolites and the active sites.

To determine the effect of altering the electrolyte, the same separation was performed on two different columns, Exsil Pt $C_{18}$ and Xtec's ODS phases packed to a length of 20cm. Electrochromatograms shown in figures 7-8.
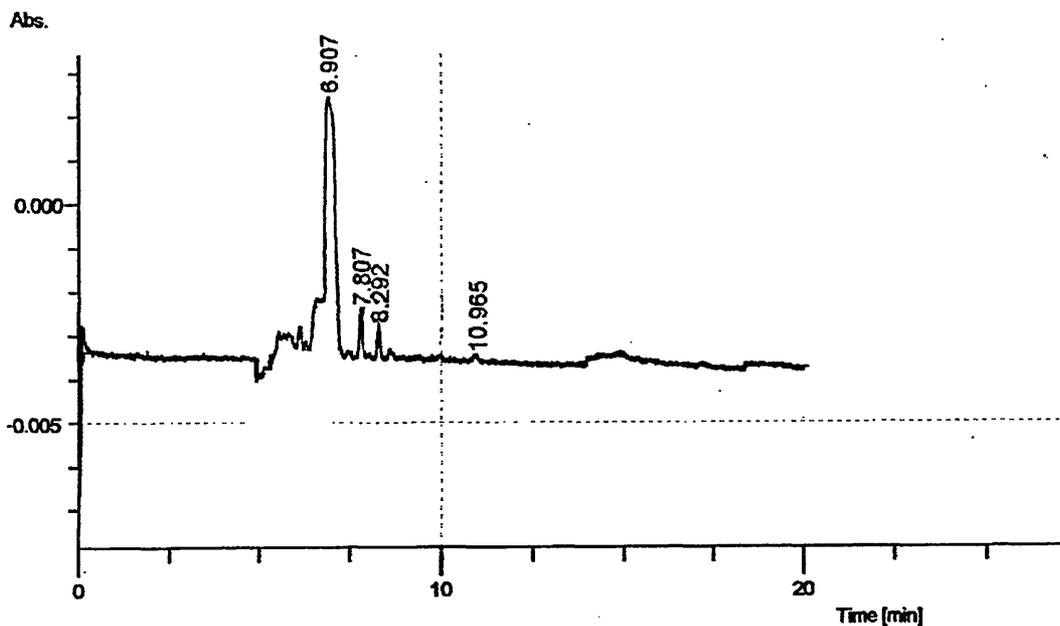
Figure 7. Separation of nicotine (6.91 min), nornicotine (6.91 min), cotinine (7.81 min) and norcotinine (8.29 min) on an Exsil Pt $C_{18}$ 20cm packed column, with a running buffer of 20% 20 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile (n=3).
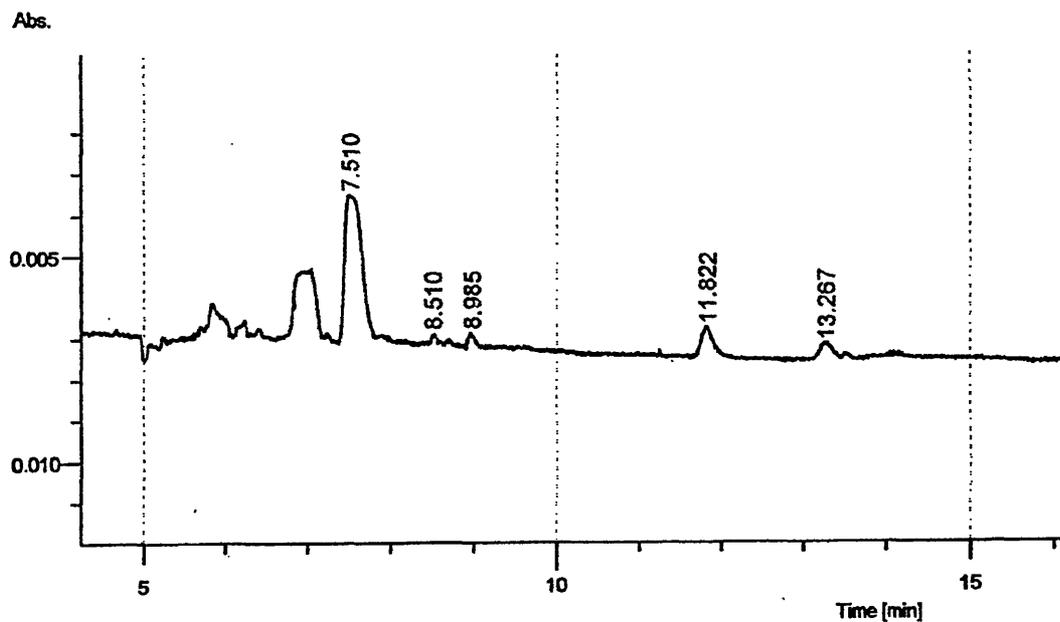


Figure 8. Separation of nicotine (8.52 min), nornicotine (8.99 min), cotinine (11.82 min) and norcotinine (13.29 min) on an Xtec ODS 20cm packed column, with a running buffer of 20% 20 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile (n=3).

The results do show an improvement in the peak shapes from those, which were previously achieved (see figure 15), which would indicate that the change of electrolyte has been beneficial. The Exsil packing material is end-capped which gave the best looking peak shapes (see figure 16), but it did not give sufficient resolution in comparison to the ODS stationary phase, which is a consideration when optimising the method.

6.5.1. Urine profile of smokers and non-smokers.

To further develop the separation of nicotine metabolites it was felt that the method should be able to distinguish between the urine of a smoker and a non-smoker. This would require a good separation of the nicotine metabolites from any other basic compounds found in urine.

Baidoo *et al* have developed an SPE method for the extraction of nicotine metabolites, and have reported 100% recovery of cotinine (14), which is one of the most important of the metabolites. The extraction method is as stated below and was used to extract samples reported in this section;

Cartridge       1g/6ml $C_{18}$ ODS (EC)
Conditioning 6 ml Methanol
                       6 ml Water
Sample        100ml pre-filtered urine
Wash            25 ml water
Extraction    6ml Methanol

The extracts were blown down under nitrogen and reconstituted into 0.5 ml of 70:30 methanol water.

As these samples had already been prepared previously they were adapted for CEC. A 100μl aliquot of each sample was taken and the methanol removed under nitrogen. The volume was then made back up to 100μl with the addition of 70μl of running buffer. This resulted in the sample having a higher water content than the running buffer allowing the sample to be stacked at the head of the column, as described in section 4.4.

The separation was performed on a 20cm packed column (Xtec ODS), effective length 56cm and total length 72cm. The running buffer was 40% of 10mM ammonium acetate in acetonitrile, pH unadjusted. The samples were loaded by electrokinetic loading, 0.15 min at 30 Kv, the separation was run at 30 Kv, with UV detection at 205 nm.

The CEC profiles that were obtained were from two non-smokers, one of whom did not consume caffeine either, and five smokers. The profiles are shown in figures 9-15.
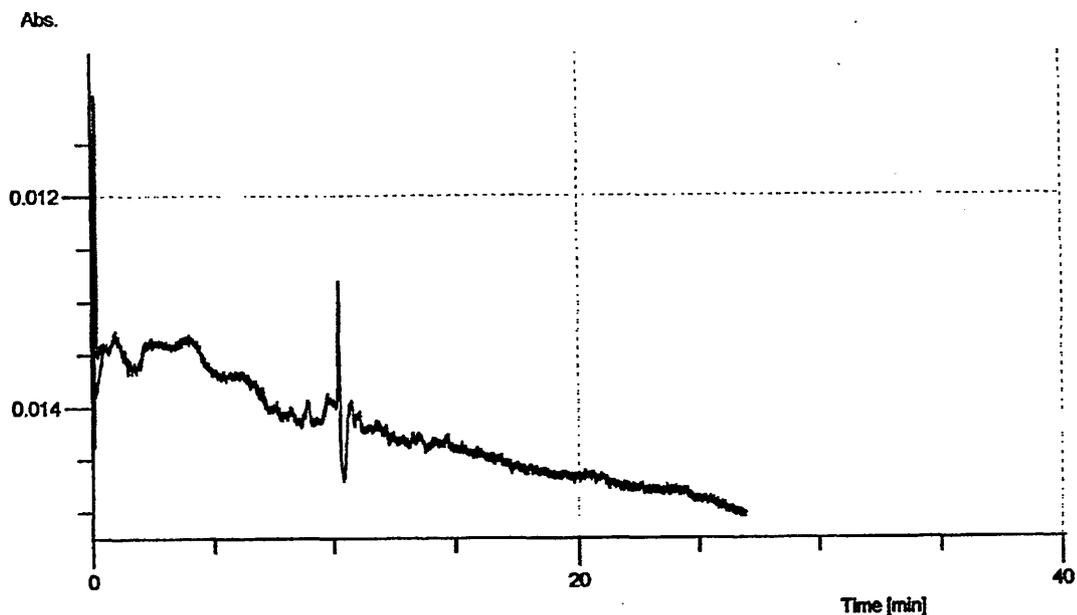
Figure 9. CEC Profile of a non-smoker's urine who does not consume caffeine products. The separation was carried out on a 20cm packed Xtec ODS column, with a running buffer of 40% 10 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile (n=3).
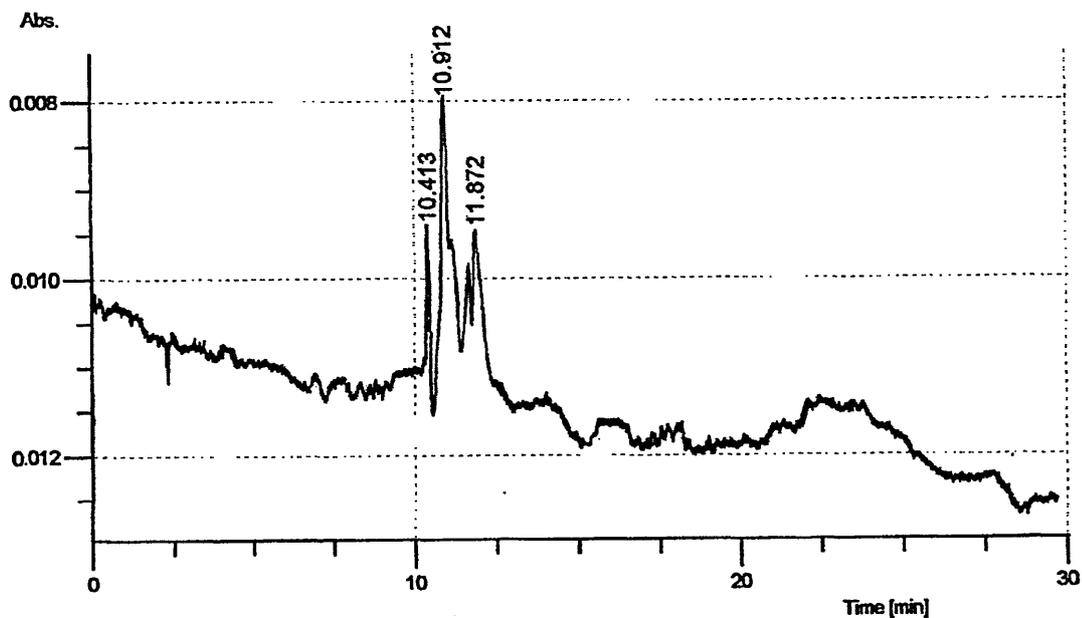


Figure 10. CEC Profile of a non-smoker's urine. The separation was carried out on a 20cm packed Xtec ODS column, with a running buffer of 40% 10 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile (n=3).
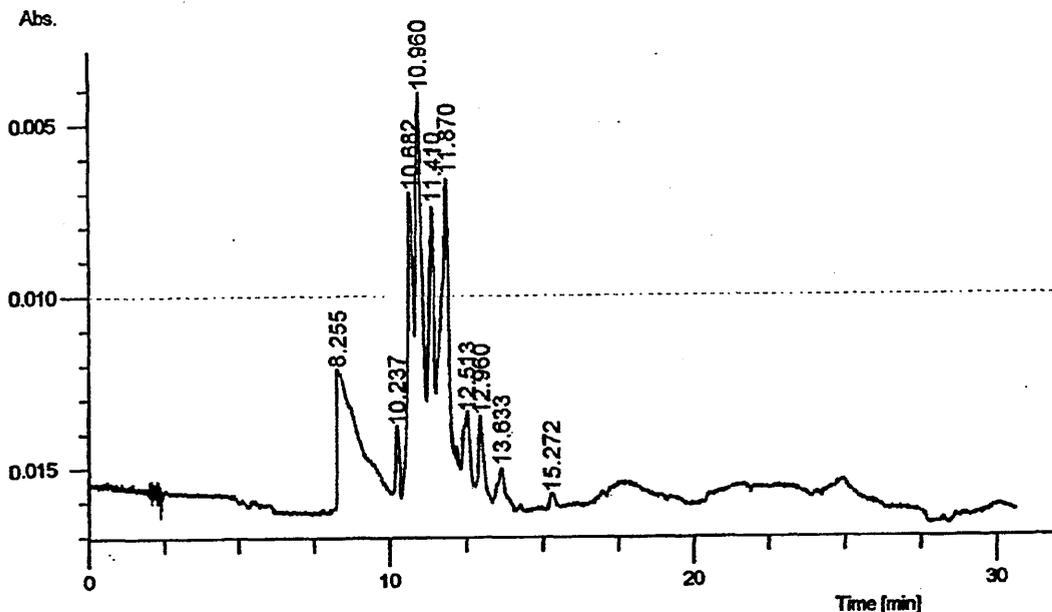
Figure 11. CEC Profile of smoker number one's urine (cotinine 12.96 min). The separation was carried out on a 20cm packed Xtec ODS column, with a running buffer of 40% 10 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile (n=3).
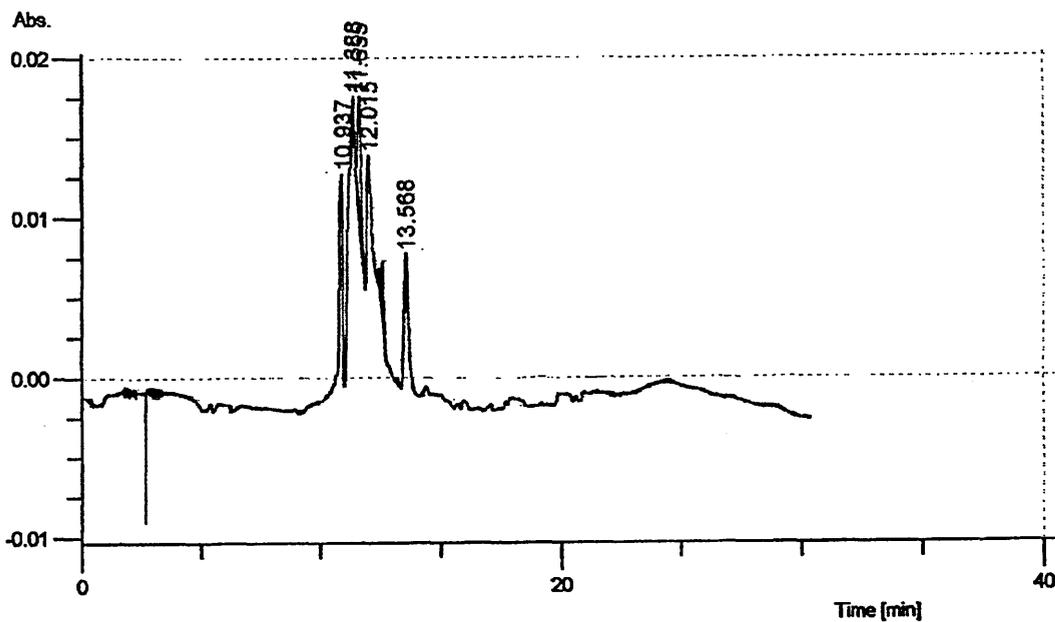


Figure 12. CEC Profile of smoker number two's urine (cotinine 13.57 min). The separation was carried out on a 20cm packed Xtec ODS column, with a running buffer of 40% 10 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile (n=3).
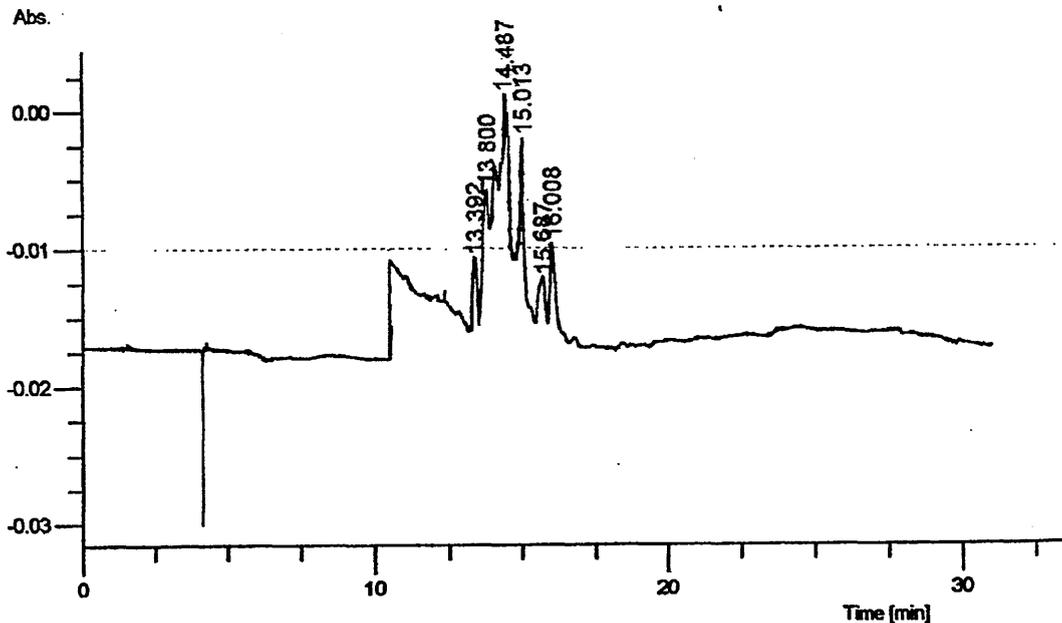
Figure 13. CEC Profile of smoker number three's urine (cotinine 16.01 min).
The separation was carried out on a 20cm packed Xtec ODS column, with a
running buffer of 40% 10 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile
(n=3).
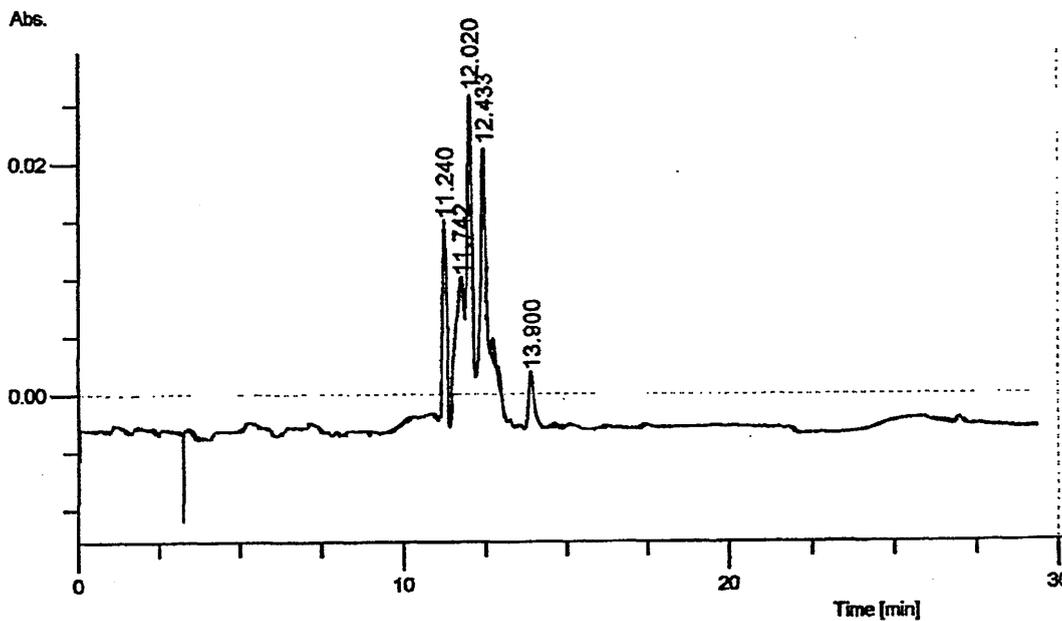


Figure 14. CEC Profile of smoker number four's urine (cotinine 13.90 min).
The separation was carried out on a 20cm packed Xtec ODS column, with a
running buffer of 40% 10 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile
(n=3).
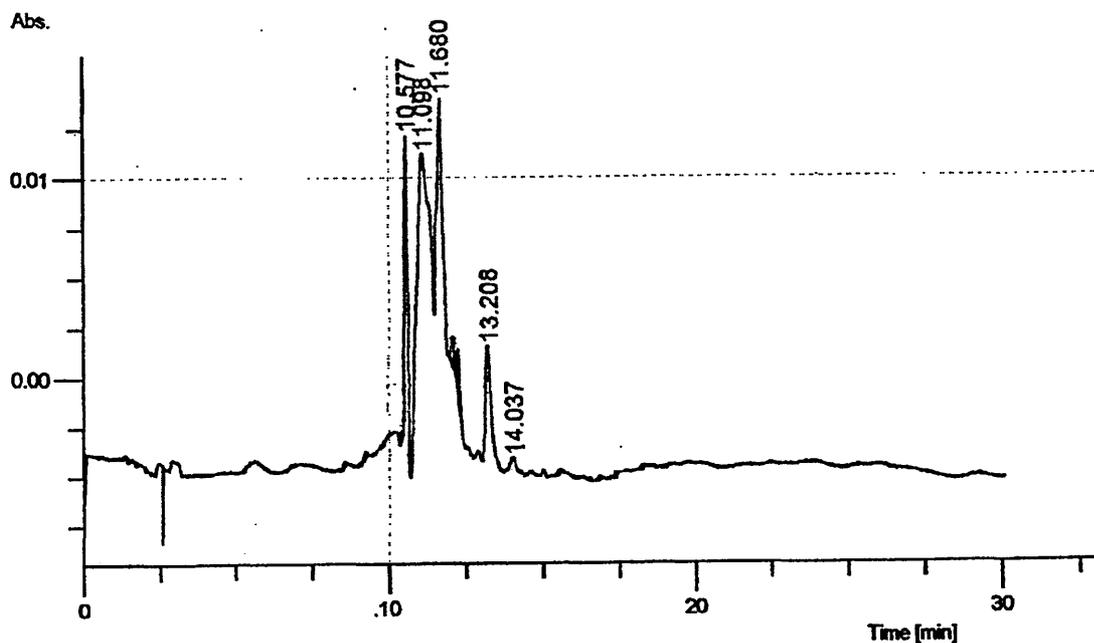
Figure 15. CEC Profile of smoker number five's urine (cotinine 13.21 min). The separation was carried out on a 20cm packed Xtec ODS column, with a running buffer of 40% 10 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile (n=3).

The results indicate that the non-smokers do have different profiles to those of smokers; this especially true for the later eluting compounds. However, the resolution at the front of the elution profile is poor with numerous compounds eluting in a tight group, which is true for both smokers and non-smokers. One significant observation is that cotinine (13.9 and 13.2 min, figures 23 and 24 respectively) can be readily detected eluting just after the mass of co-eluting compounds at the front of the separation profile and is not present in either of the non-smokers' urines.

The retention times for the profiles do varied slightly. This is due to the differing viscosity of the samples, caused by blood breakdown products from the kidneys, including bilirubin, urobilinoges and urobilin, which are also extracted with the nicotine metabolites. Even with the variation in migration

times of the separation they still show the same basic profiles. This effect was also observed when these samples were separated by CZE (14).

To improve the resolution at the front end of the separation the organic content was reduced to 50%, while maintaining the same effective electrolyte concentration. However, the 20cm column failed prior to starting, therefore the next planned experiment was undertaken which was to extend the packed length to 25cm, with a running buffer of 50% 8mM ammonium acetate (aq) in acetonitrile. This next experiment had been designed as there was concern that the lowering of the organic content would not provide significantly improved resolution at the front end of the separation and further lowering the organic content to 40% would increase the risk of bubble formation occurring. This was fortunate as this combination gave improved resolution of the clutter observed at the front end of the elution, as can be seen in figures 16-21.
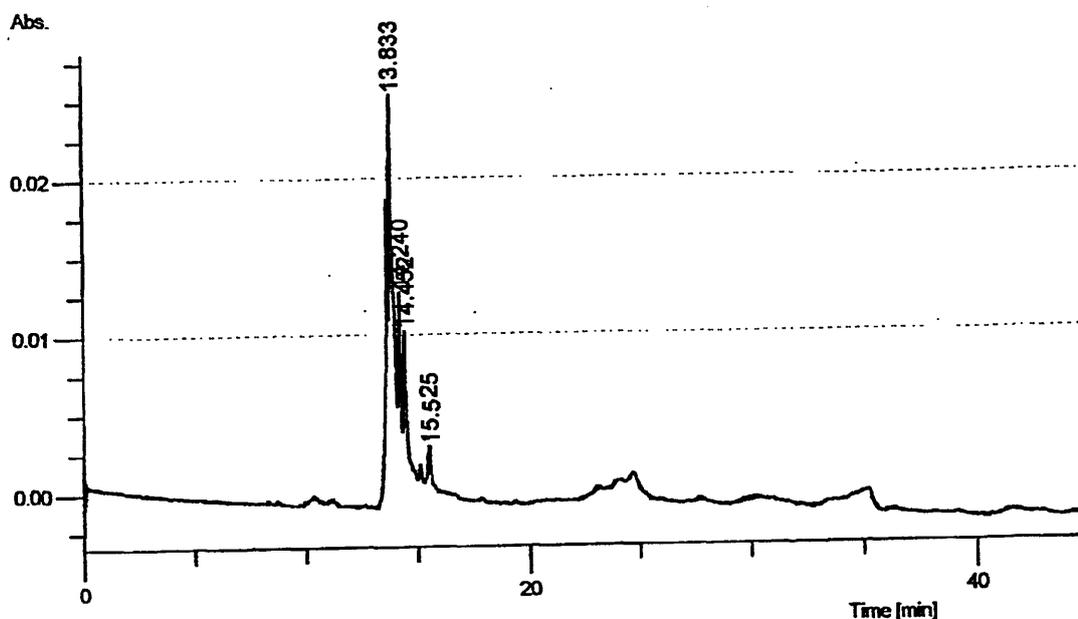


Figure 16. CEC Profile of a non-smoker's urine. The separation was carried out on a 25cm packed Xtec ODS column, with a running buffer of 50% 8 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile (n=3).
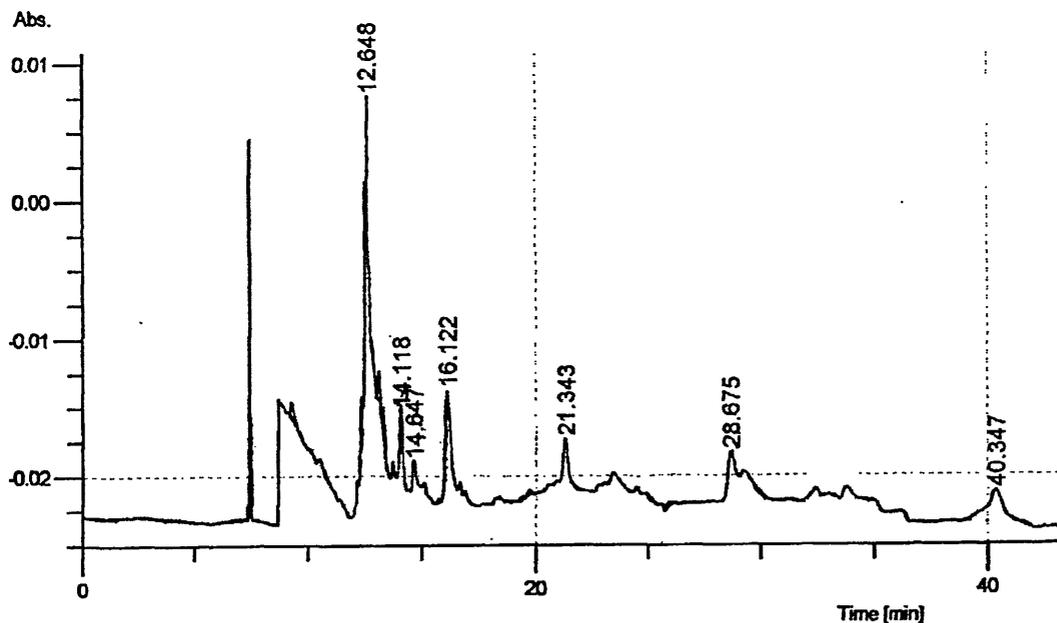
Figure 17. CEC Profile of smoker number one's urine (cotinine 16.12 min). The separation was carried out on a 25cm packed Xtec ODS column, with a running buffer of 50% 8 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile (n=3).
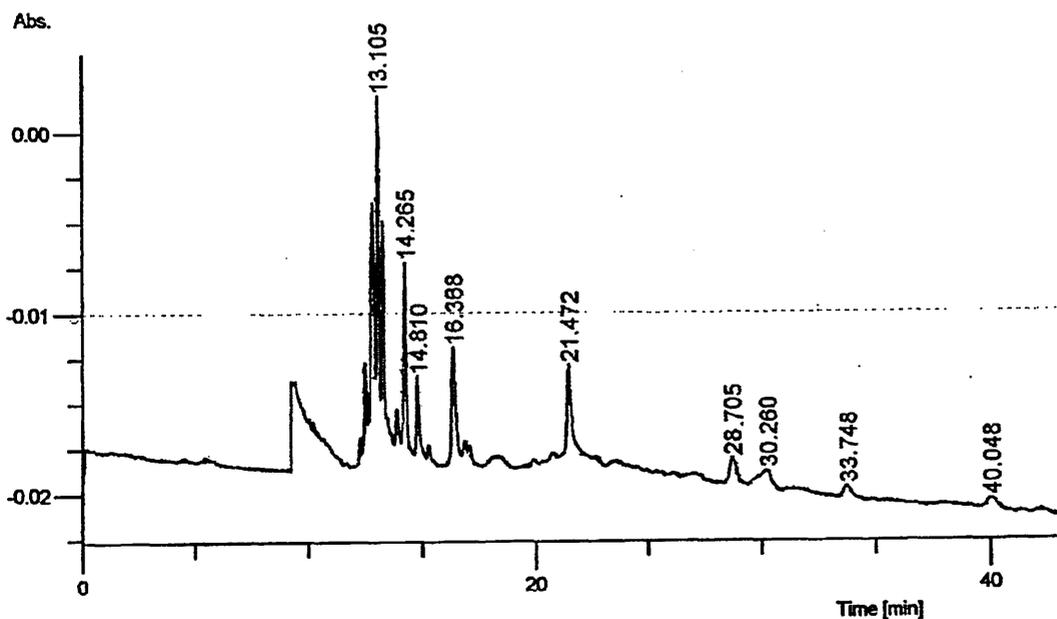


Figure 18. CEC Profile of smoker number two's urine (cotinine 16.37 min). The separation was carried out on a 25cm packed Xtec ODS column, with a running buffer of 50% 8 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile (n=3).
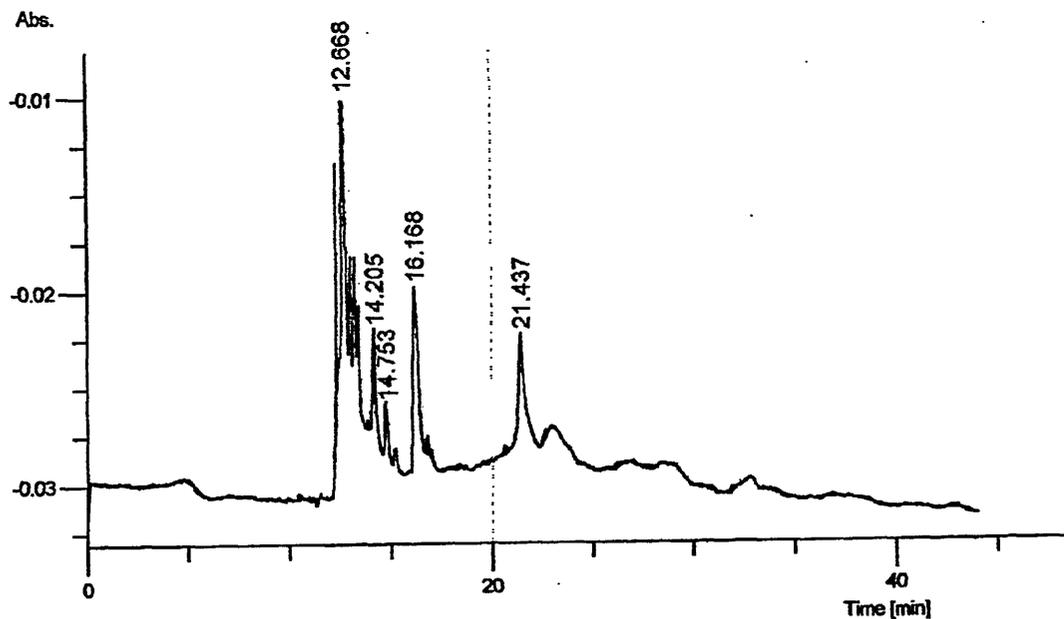
Figure 19. CEC Profile of smoker number three's urine (cotinine 16.16 min).
The separation was carried out on a 25cm packed Xtec ODS column, with a
running buffer of 50% 8 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile (n=3).
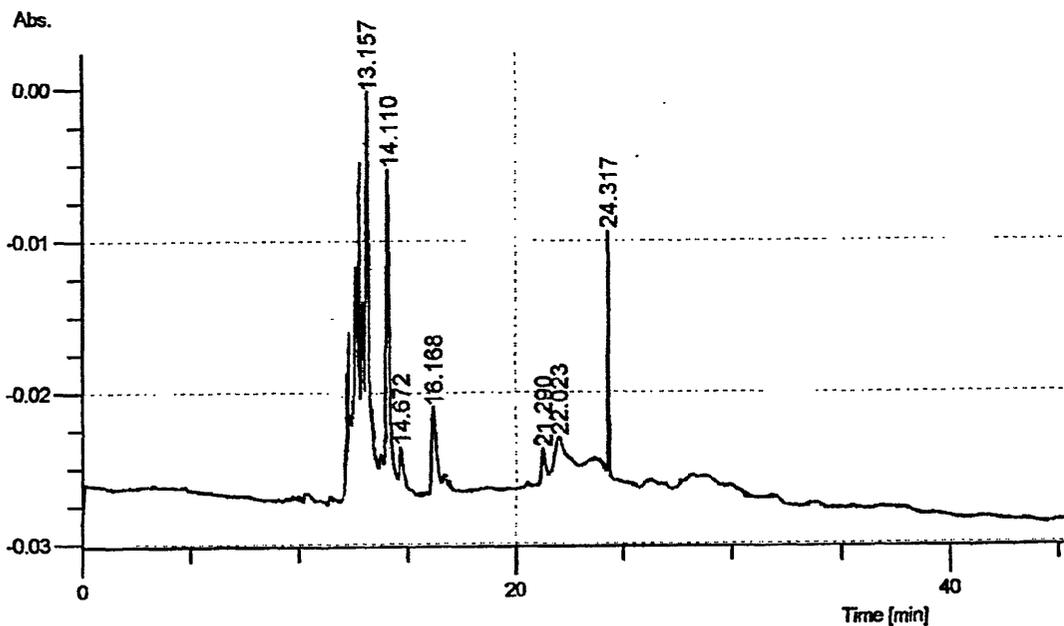


Figure 20. CEC Profile of smoker number four's urine (cotinine 16.17 min).
The separation was carried out on a 25cm packed Xtec ODS column, with a
running buffer of 50% 8 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile (n=3).
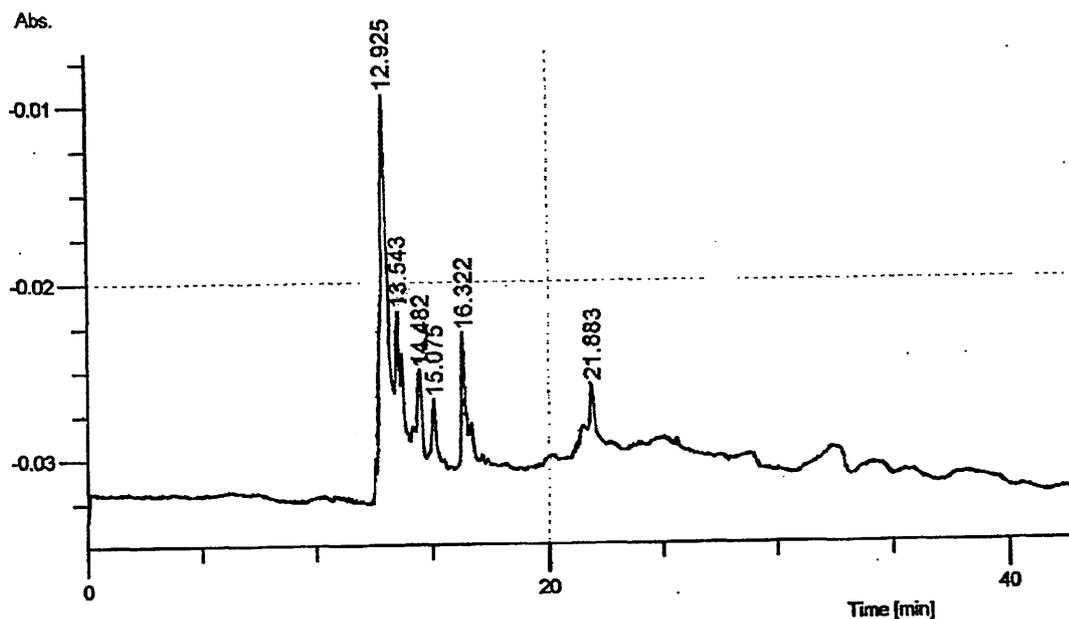
Figure 21. CEC Profile of smoker number five's urine (cotinine 16.32 min). The separation was carried out on a 25cm packed Xtec ODS column, with a running buffer of 50% 8 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile (n=3).

The change in conditions improved the resolution of the entire separation, in particular the mass of co-eluting peaks at the front of the separations. Of great interest was the baseline resolution of the cotinine (*ca.* 16.2 mins) as this can be used as a marker to distinguish between smokers and non-smokers. Another difference, which could be used to distinguish between smokers and non-smokers, is the unidentified peak at *ca.* 22 min, which is not observed in the non-smokers profile.

One other notably observation is the result of the non-smoker whom did consume caffeine product, the profile (figure 9) showed none of the earlier eluting compounds which are seen in all the other profiles. This could indicate that that caffeine and its metabolites form the bulk of the early eluting compounds observed.

## 6.5.2. Conclusion.

The initial work carried out with the TRIS and borate buffers are poor results with respect to resolution and peak shape, with significant peak tailing being observed. Further work changed the electrolyte to an ammonium salt (ammonium acetate) which led to an improvement in peak shape and resolution. This was the result of the ammonium ion interactions with silanoate groups on the capillary and stationary phase, thereby blocking adsorption of the basic metabolites. Further to this when comparing differing stationary phases the Exsil Platinum $C_{18}$ produced sharper peaks, but lacked the resolution that the Xtec ODS phase offered. This supports the idea that silanoate groups had a significant effect on the metabolites, as the $C_{18}$ material is end-capped hence reducing the number of active sites, which are present.

The profiling work carried out after the investigation showed the potential of this technique, allowing sufficient resolution of the mass of co-eluting compounds at the beginning of the separation, which highlighted the differences between smokers and non-smokers. An important factor in determining between a smoker and a non-smoker is the presence of cotinine as this is a major metabolite of nicotine, which was resolved and clearly identifiable.

### 6.6. Quantification of Cotinine in urine.

6.6.1 Introduction.

Cotinine is one of the most important nicotine metabolites as *ca.* 80% of ingested nicotine is metabolised to cotinine, this conversion taking a matter of hours to complete. Therefore, determining the levels of cotinine present will give a good indication of the degree of exposure to nicotine.

As section 6.5 has shown cotinine can be resolved from the other metabolites. Therefore, an investigation to determine the feasibility of measuring the level of cotinine in a smokers urine was undertaken. This required consideration of the dynamic range of the calibration curve, reproducibility of the injection as well as the analysis of urine from a smoker.

The initial conditions were taken from the work reported in section 6.5, which profiled the extracts from the urine of smokers and non-smokers. Both methods allowed cotinine to be resolved from the other metabolites, therefore an investigation into how the internal standard would perform with the differing conditions was undertaken. N,N–Diethylnicotinamide was used as the internal standard (IS). On the shorter 20cm packed column the IS elutes prior to cotinine (see figure 22), which would mean that the IS would co-elute with the earlier eluting compounds. However on the longer column this situation is reversed (see figure 23), with the IS eluting after the cotinine. Even with the changes in both organic content of the running buffer and column length it is the effect of the modification of the buffer composition which would have caused the movement of the IS.
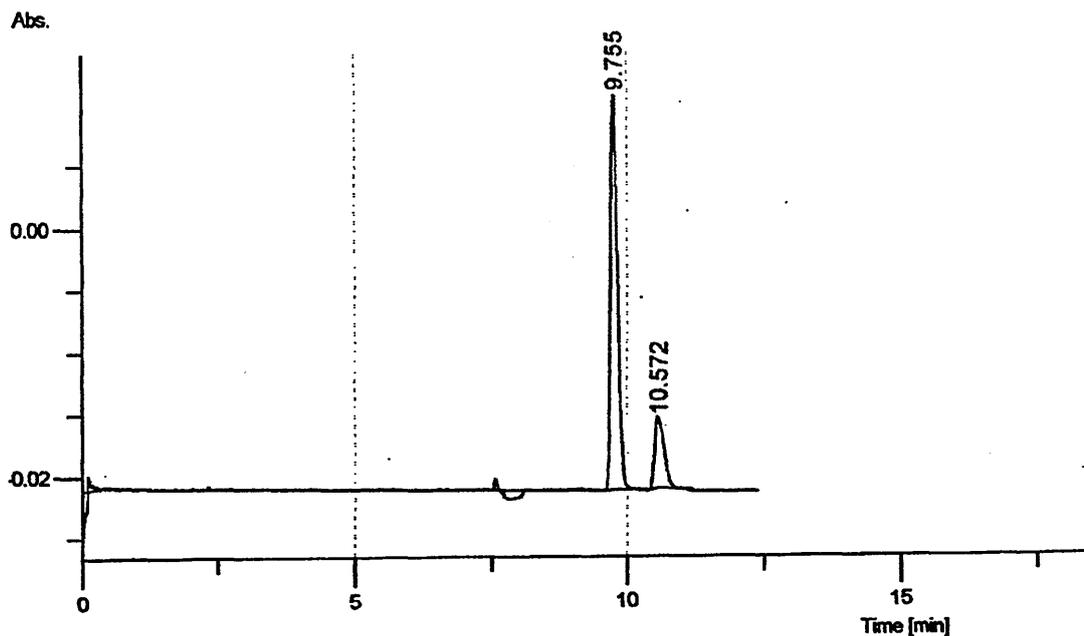
Figure 22. Separation of cotinine (10.572 min) and N,N–Diethylnicotinamide (9.755 min) on an Xtec ODS 20cm packed column, with a running buffer of 40% 10 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile (n=3).
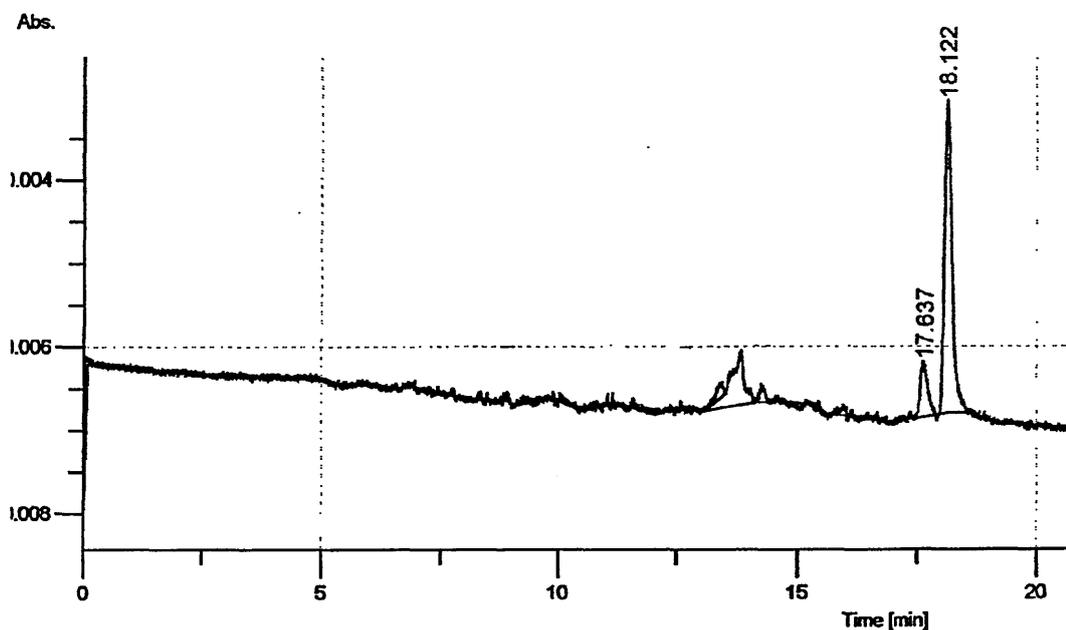


Figure 23. Separation of cotinine (17.637 min) and N,N–Diethylnicotinamide (18.122 min) on an Xtec ODS 25cm packed column, with a running buffer of 50% 8 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile (n=3).

## 6.6.2 Method.

### 6.6.2.1 Extraction.

The extraction method was scaled down from that described in section 6.5 from 100 ml of urine to either 5 or 10 ml of urine (calibration curve extracted from 5ml of urine), in order to reduce the risk of variations in viscosity, which large volumes urine could cause. The two volumes of urine were used to assess the degree of pre-concentration that was required, 50 or 100 times pre-concentration respectively. Once extracted the samples were reconstituted into 100 $\mu$l of running buffer. Also note the sorbent bed was also scaled down from 1g to 100mg.

### 6.6.2.2 Sample Preparation.

The internal standard (IS) used was N,N–Diethylnicotinamide. The working solution was made from 5$\mu$l neat IS diluted in 1ml of water to give 5000$\mu$g/ml stock solution of IS (using the approximation 1$\mu$l of IS is equal to 1 mg of IS). 50 $\mu$l of the stock solution was then diluted in 1ml of water to give 250$\mu$g/ml IS working solution.

Cotinine stock solution was prepared by diluting 5$\mu$l of neat cotinine in 1ml of water to give a stock solution of 5000$\mu$g/ml (using the approximation 1$\mu$l of cotinine is equal to 1 mg of cotinine). This solution was further diluted to give the spiking solutions, as listed below;

500 μg/ml solution, prepared from 100μl of stock solution (5000μg/ml) in 1ml water.

250 μg/ml solution, prepared from 50μl of stock solution (5000μg/ml) in 1ml water.

100 μg/ml solution, prepared from 20μl of stock solution (5000μg/ml) in 1ml water.

75 μg/ml solution, prepared from 150μl of 500 μg/ml solution in 1ml water.

50μg/ml solution, prepared from 100μl of 500 μg/ml solution in 1ml water.

20μg/ml solution, prepared from 200μl of 100 μg/ml solution in 1ml water.

10μg/ml solution, prepared from 100μl of 100 μg/ml solution in 1ml water.


The calibration curve was prepared by taking 5 ml of urine spiked with 100μl of the appropriate calibration solution and 10 μl of IS (250μg/ml). The blank was spiked with 110μl of water instead of calibration and IS, whilst the 0 μg/ml standard was spiked with 100μl of water and 10 μl of IS (250μg/ml). Both samples (5 and 10ml samples) were prepared with the addition of 10 μl of IS (250μg/ml) and 100μl of water.


6.6.2.3 Sample Analysis.

The samples were reconstituted into the running buffer, that was composed of 50% 8mM ammonium acetate (aqueous) in acetonitrile, pH unadjusted. The column had a packed length of 25cm (Xtec ODS) with an effective length of 56 cm and a total length of 72 cm. The samples were loaded by electrokinetic loading, 0.15 min at 30 Kv, the separation was run at 30 Kv with UV detection at 205 nm.

## 6.6.3 Results and Discussion.

Figure 24 shows two distinct dynamic ranges: below 100 ppm the gradient of the calibration curve is shallower than that above 100 ppm. The profile of the calibration curve is likely to be due to a concentration effect, with additional adsorption occurring once the concentration of the cotinine exceeds at specific level, which is around 100 ppm.
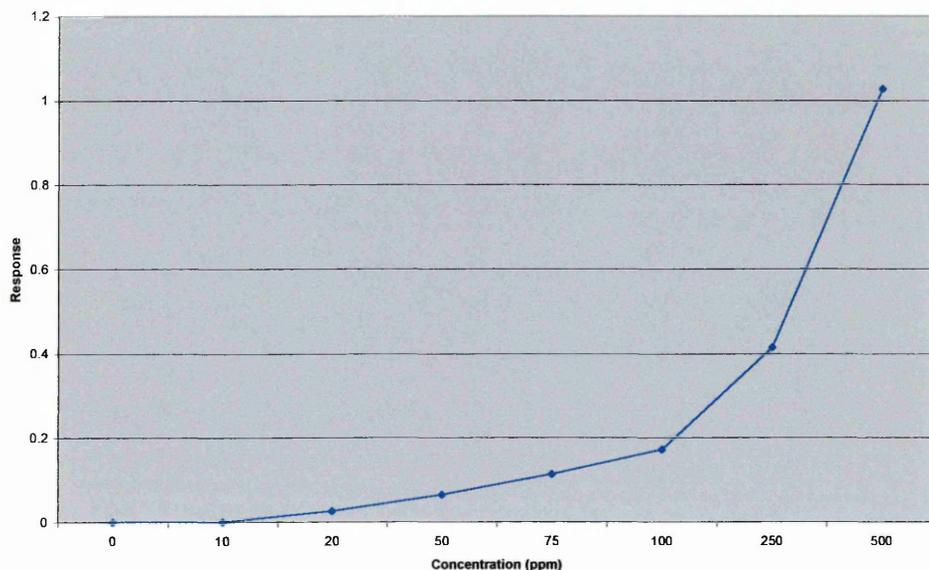


Figure 24. Plot of cotinine calibration curve from 0 $\mu$g/ml to 500 $\mu$g/ml, from a 25cm packed Xtec column, with a running buffer of 50% 8 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile.

The result expected from the smokers urine was expected to be below 100ppm, therefore this area was plotted to give the calibration curve (Figure 25). However, the 10$\mu$g/ml standard was below the limit of quantification, therefore only a four point calibration curve was constructed, from which the concentration of cotinine in the two samples was determined (listed in table 4).
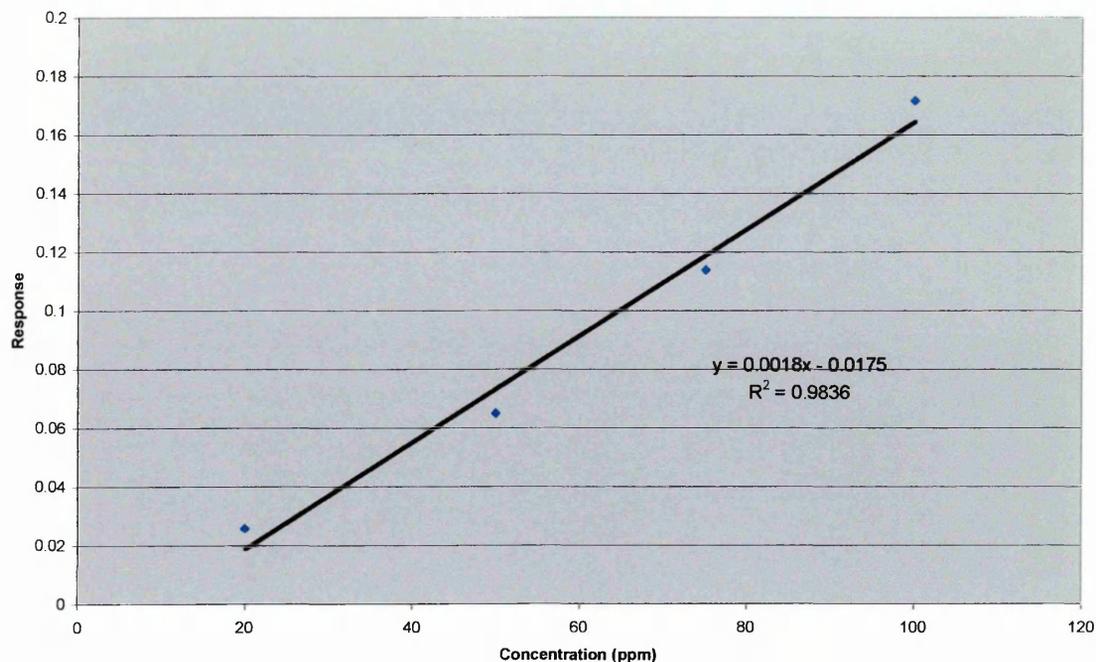
Figure 25. Plot of cotinine calibration curve from 20 µg/ml to 100 µg/ml, from a 25cm packed Xtec column, with a running buffer of 50% 8 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile.

| Sample | Cotinine (peak area) | IS (peak area) | Calculated Concentration | Actual concentration |
|--------|---------------------|----------------|--------------------------|----------------------|
| A | N/D | 0.000358 | N/A | N/A |
| B | 0.0000152 | 0.000484 | 27.2µg/ml | 0.272µg/ml |

Table 4. Quantification of the smoker's urine, Sample A 5ml sample volume (50 time pre-concentration) and Sample B 10ml sample volume (100 times pre-concentration). Calculated concentrations were determined from the calibration curve shown in figure 34. Actual concentration of cotinine is calculated by dividing the calculated concentration by its pre-concentration factor.

The level of cotinine found in the smokers urine which was pre-concentrated 100 times was calculated to be 0.27 µg/ml (figure 26). This would also explain why the 50 times concentrated sample failed to be quantified, as nominal concentration would have been below the lower limit of quantification (figure 27).
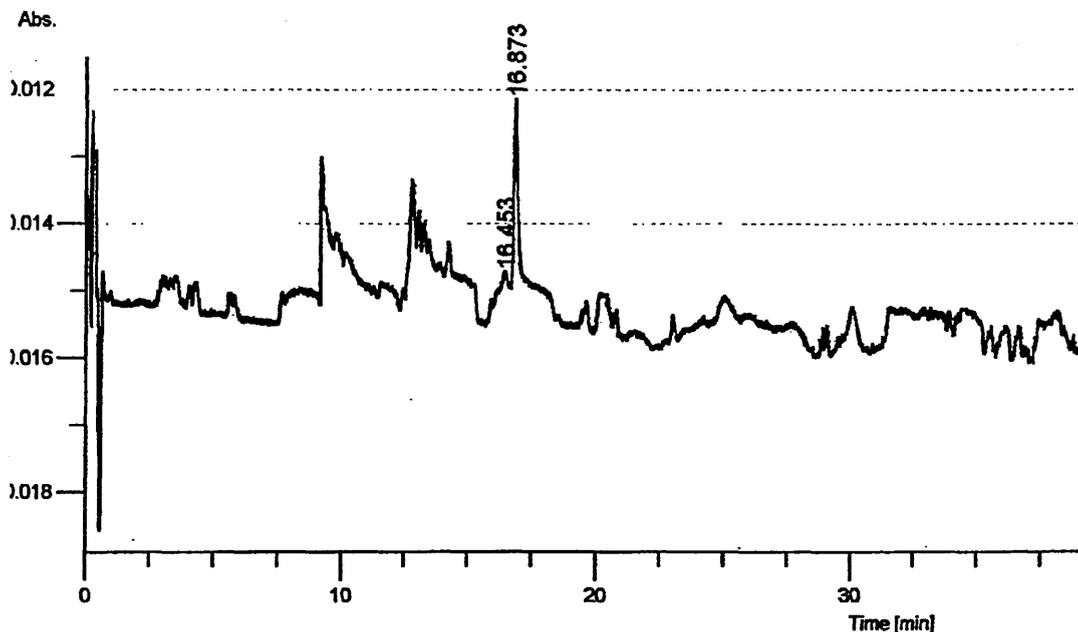
Figure 26. Electrochromatogram of Smokers urine, sample B, 100 times concentrated. Cotinine (16.453 min) and IS (16.873 min). Separation on a 25cm packed Xtec column, with a running buffer of 50% 8 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile.
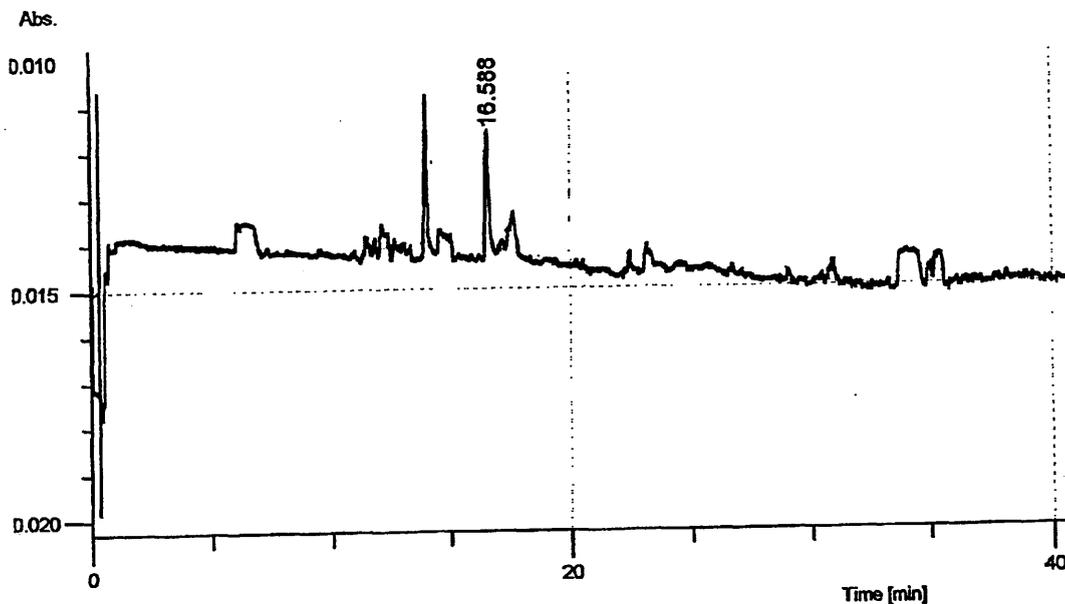


Figure 27. Electrochromatogram of Smokers urine, sample A, 50 times concentrated. IS (16.588 min). Separation on a 25cm packed Xtec column, with a running buffer of 50% 8 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile.

Six replicate injections were performed to determine the reproducibility of the methods over a series of injections (see Table 5). The results indicate the migration times are reproducible (*ca.* 0.75% RSD) but the peak areas have poor reproducible with % RSDs of 23% and 11% for cotinine and IS respectively.

| | Cotinine | | IS | |
|---|---|---|---|---|
| Injection | Peak area | Migration time (min) | Peak area | Migration time (min) |
| | | | | |
| 1 | 0.000149 | 16.0 | 0.000888 | 16.5 |
| 2 | 0.000103 | 15.9 | 0.000751 | 16.3 |
| 3* | 0.0000513 | 15.8 | 0.000710 | 16.3 |
| 4 | 0.000105 | 15.9 | 0.000704 | 16.4 |
| 5 | 0.000138 | 16.1 | 0.000893 | 16.6 |
| 6 | 0.000178 | 16.0 | 0.000898 | 16.5 |
| | | | | |
| Mean | 0.000135* (0.000121) | 16.0 | 0.000807 | 16.4 |
| SD | 3.15E-05* (4.41E-05) | 0.122 | 9.52E-05 | 0.121 |
| %RSD | 23.4* (36.6) | 0.766 | 11.8 | 0.739 |

Table 5. The reproducibility of the cotinine (n=5) and IS (n=6) determination in this analysis. This was performed by replicate injections of the 100 µg/ml calibration extract on a 25cm packed Xtec ODS column, with a running buffer of 50% 8 mmolL$^{-1}$ ammonium acetate (aq) in acetonitrile. (* Result excluded. Injection number three was excluded from the results, as it eluted with interfering peaks).

## 6.6.4 Conclusion

The results indicate that this is a viable method to quantify cotinine extracted from urine, even if it has not as yet been fully validated. The results show that cotinine in smokers urine can be extracted and quantified ($0.27\mu g/ml$ for test sample), this was pleasing as the exact levels of cotinine in the urine sample were unknown, hence the two pre-concentrations levels used.

However the reproducibility is poor for cotinine with respect to peak area with an %RSD of 23%, while reproducibility for the IS is better with an %RSD of 11%, from an extracted urine standard. Some of the variation in peak area could be accounted for due to the number of injections made on the column with no wash step and hence an increase in baseline noise (this effected injection number 3 significantly). This could indicate the need for an additional wash or gradient step to remove any unwanted artefacts from the column. As there are numerous basic compounds present, not only the nicotine metabolites but caffeine and its metabolites as well, which could interfere with the generation of the EOF and general condition of the column.

Further work that could be undertaken would be to investigate further pre-concentration of the urine. This could possibly be achieved by reducing the volume that the samples are reconstituted into, or by increasing the volume of urine sampled. However, if the volume of sample used was increased the sorbent bed of the SPE cartridge should also be increased. One method that has been used successfully (sections 4.4.3 and 6.5) and could be easily adapted to this would be to increase the water content of the sample plug.

This would result in the focussing of the analytes at the head of the column when loading and therefore producing sharper peaks.

The work undertaken in this chapter has not previously been reported anywhere else and has shown the viability of CEC to run actual biological samples, not just analysis of standard mixtures. Therefore, with the additional improvements suggested previously this method could become a useful tool in future studies investigating nicotine and it's metabolites. As this method has demonstrated the ability not only to discriminate between smokers and non-smokers, but also to be able to determine the level of nicotine via the quantification of cotinine.

## 6.7 Reference

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# 7. Conclusion.

The aims of this study were to develop a successful column fabrication method and hence understand the operational requirements needed for the Prince Technology CE instrument. Once these initial aims had been achieved, the findings were applied to the development of actual separations for specific classes of compounds i.e. PAH, nicotine metabolites and prostaglandins, which cover a range of polarities.

The results of this study have been mixed. The first requirement, that of a suitable packing method, was achieved, however, it was identified that one crucial element in the packing process is the packing material. This became evident over the period of this study, as a result of variation in the successful production of columns using different batches of packing material. Despite this, data obtained from the columns indicates that they were of a similar quality to those produced by other research groups.

The investigation into the operational parameters resulted in a detailed understanding of how the CEC system works and techniques that can be utilised to aid method development work.

The application work, while successful for the PAH and the test mixture, was disappointing with regard to the separation of the prostaglandins. This was off set by the work undertaken with nicotine and its metabolites, not only with the development of a basic CZE method but with the profiling and quantification of urine samples from smokers and non-smokers. The profiling of urine samples

ably demonstrated CEC's ability to resolve complex mixtures from actual biological samples. Additionally, quantification of the metabolite cotinine, which could be used to determine nicotine levels in urine, presents another useful tool to the analytical chemist.

Overall, CEC presents an exciting prospect for the future, which the nicotine metabolites applications clearly demonstrate. However, firstly the problems that are associated with this technique at the present time must be surmounted. In particular, these relate to the use of stationary phase, be it conventional packing material or a continuous bed formed in-situ.