IN VITRO APPROACHES TO MODELLING DRUG METABOLISM USING CAPILLARY ELECTROPHORESIS

By

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of

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DECLARATION

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

Signed Einear Waad

ID No 99140411

Date 22/08/03

For my parents

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LIST OF ABBREVIATIONS

3-HC	3-Hydroxycoumarın
4-HC	4-Hydroxycoumarın
6,7-dıHC	6,7-Dıhydroxycoumarın
6-HC	6-Hydroxycoumarın
7-HC	7-Hydroxycoumarın
7-HC-G	7-Hydroxycoumarın-Glucuronıde
7-HC-S	7-Hydroxycoumarın-Sulphate
7-MC	7-Methoxycoumarın
BGE	Background Electrolyte
CE	Capıllary Electrophoresıs
CEC	Capıllary Electrochromatography
CE-EC	Capıllary Electrophoresıs-Electrochemıcal Detectıon
CF-FAB	Contınuous-Flow Fast Atom Bombardment
CGE	Capıllary Gel Electrophoresıs
CIEF	Capıllary Isoelectrıc Focusıng
CITP	Capıllary Isotachophoresıs
CL	Chemılumınescence
CMC	Crıtıcal Mıcelle Concentratıon
CYP 450	Cytochrome P450
CZE	Capıllary Zone Electrophoresıs
EOF	Electroosmotıc Flow
ER	Endoplasmıc Retıculum

ESI	Electrospray Ionization
FAD	Flavin Adenine Dinucleotide
FASS	Field Amplified Sample Stacking
FMN	Flavin Mononucleotide
HPA	Hydroxyphenyllactic Acid
HPAA	Hydroxyphenylacetic Acid
HPLC	High-Performance Liquid Chromatography
HPPA	Hydroxyphenylpropionic Acid
LC	Liquid chromatography
LIF	Laser-Induced Fluorescence
LOD	Limit of Detection
LOQ	Limit of Quantitation
LVSS	Large Volume Sample Stacking
MEKC	Micellar Electrokinetic Chromatography
NADPH	Nicotine Adenine Dinucleotide Phosphate
OHP	Outer Helmholtz Plane
PMT	Photomultiplier Tube
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TLC	Thin Layer Chromatography
UDP	Uridine diphosphate
UDPGT	Uridine Diphosphate Glucuronosyl Transferase
UTP	Uridine Triphosphate

ABSTRACT

The focus of this research was to develop improved methods for the analysis of drug metabolism by capillary electrophoresis Coumarin was initially chosen as the model for the study, which then broadened in its scope to include the analysis of amino acids Chapter one is a literature survey, which includes capillary electrophoresis techniques, with particular emphasis on sensitivity enhancement methods, *in vitro* models for drug metabolism, and coumarin analysis

Chapter two details the application of a sample enhancement strategy, pH-mediated sample stacking, to the analysis of three of the metabolites of coumarin 7-hydroxycoumarin, 4-hydroxycoumarin and o-hydroxyphenylacetic acid A suitable separation method for the three analytes was developed and, once optimised, was applied to the analysis of microsomal incubations of coumarin using Cynomologus monkey microsomes 7-hydroxycoumarin was identified in the incubation mixture by spiking of the incubation mix with a standard, and then formation of this metabolite was monitored over time

Chapter three deals with the development of an automated system for the analysis of microsomal incubation reactions A separation method, capable of separating seven of the potential metabolites of coumarin was initially developed and was then applied to the on-system technique The system allowed the metabolism of coumarin to be monitored over time with minimal sample handling

Chapter four is a detailed study of pH-mediated sample stacking with investigations carried out into the influence of injection type, injection length and sample base injection ratio Conclusions are drawn on the mode of action of this form of sample stacking under each of the injection conditions studied In addition, the use of an alternative sample matrix to the previously employed Ringer's solution is investigated

Chapter five details the application of pH-mediated sample stacking, with indirect detection, to the analysis of eight amino acids The developed method showed that pH-mediated sample stacking was compatible with indirect UV detection and the

eight amino acids could be detected sensitively with a run time of less than 13 minutes

Finally, chapter six summarizes the research carried out in each chapter and describes the future role of CE, as applied to the analysis of microsomal samples

CHAPTER ONE

LITERATURE SURVEY

1.1. CAPILLARY ELECTROPHORESIS OVERVIEW

1.1.1. Introduction

Electrophoresis is a separation method that is based upon the differential rates of migration of charged species under the influence of an electric field. Electrophoresis is typically performed in two formats: the traditional slab electrophoresis and capillary electrophoresis (CE). The idea of exploiting the anticonvective properties of capillaries to improve resolution had been formed in the early phases of development of electrophoresis, but it is only in the last 15 - 20 years that the full advantages of this technique have been realised (Issaq, 2000). Performing electrophoresis in narrow-bore capillaries leads to many advantages in electrophoretic separation. One such advantage is that the internal surface area of the capillary causes efficient heat dissipation, thus allowing increased voltages to be applied for separations. The applied voltage is usually in the range of 15 to 30 kV. The increased electric field results in more efficient separations, greater resolution and reduced analysis times (Oda and Landers, 1994; Shintani and Polonsky, 1996; Beckman Coulter Inc., 1994; Altria, 1995).

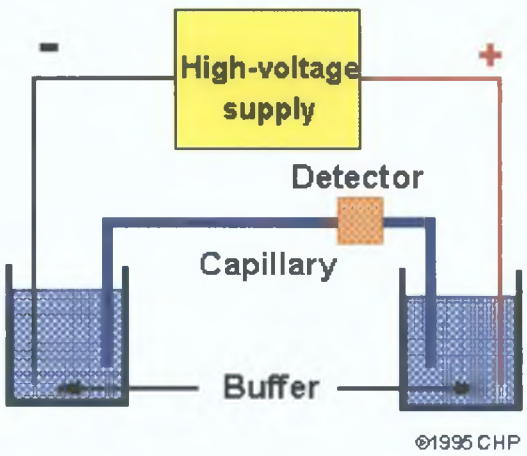

Figure 1.1: *Schematic of capillary electrophoresis instrumentation* (shown in reversed-polarity mode). The Chemistry Hypermedia Project. http://www.chem.vt.edu/chem-ed/sep/electrop/cap-el.html

The main components of a capillary electrophoresis system are illustrated in Figure 1 1 The instrumentation involves a high voltage power supply (typically 0 to 60 kV), a fused silica capillary (externally coated with polyimide to increase flexibility), two buffer reservoirs to accommodate the capillary and the electrodes, a cooling system (usually air or liquid) and the detector (Beckman Coulter Inc , 1994) The polyimide coating on the capillary absorbs UV light but can be easily removed by flame or acid to create a 'window' When the detector is aligned with this window, optical detection can be achieved on-capillary During a CE separation (normal polarity), the sample is injected at the positive end of the capillary Injection is typically performed either hydrodynamically or electrokinetically The ionic species migrate with electrophoretic mobility determined by their charge and mass (*Section 1 1 2 1*) A bulk flow can also exist within the capillary, termed electroosmotic flow (EOF) (*Section 1 1 2 2*) If sufficient, this EOF can ensure that all species (positive, negative and neutral) pass the on-line window and can be detected UV absorbance detection is most commonly used for CE analysis but alternative modes of detection are discussed in Section 1 2 2 3

1.1.2. Driving Forces Behind CE Separations

1 1 2 1. Electrophoretic Mobility

Analyte ions will migrate under the influence of an electric field with velocity (v_{ep}) This electrophoretic velocity (v_{ep}) is proportional to the field strength, E, when no electroosmotic flow is present

Eqn 1 1

$$v_{ep} = \mu_{ep} E$$

The electrophoretic mobility (μ_{ep}) is dependent on the size and charge of the ionic species, with smaller, more highly charged ions being detected first

The mobility is related to the charge/mass ratio of the ion (Equation 1 2)

Eqn.1.2

$$\mu_{ep} = (q / 6\pi) \eta r$$

where

μ_{ep} = electrophoretic mobility

q = number of charges on the ion

η = solution viscosity

r = radius of the ion

1.1.2 2 Electroosmotic Flow

Electroosmotic flow arises from the fact that nearly all surfaces are charged and attract oppositely charged ions (counter ions) The inner surface of the fused silica capillary utilised in capillary electrophoresis contains negatively charged silanol groups, which attract a cloud of counter ions into adjacent layers of liquid forming a double layer The pH of the background electrolyte (BGE) will determine the fraction of the silanol groups that will ionise The ionic layer that is formed has a positive charge density that decreases exponentially as the distance from the wall increases The Stern layer is the term given to the layer closest to the capillary surface whilst the outer Helmholtz plane (OHP), a more diffuse layer, is further from the surface Figure 1 2 is a representation of the ionic layer at the inner surface of the capillary

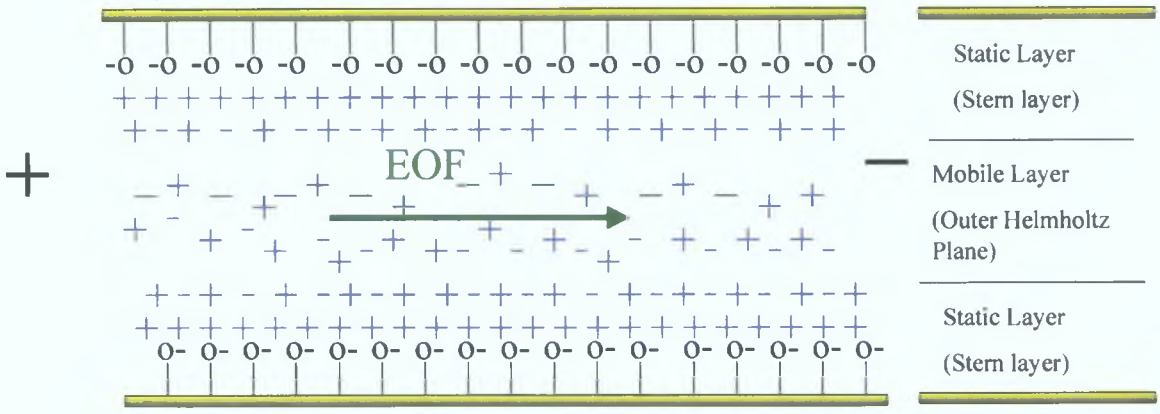

Figure 1.2: *Diagram of the capillary and the ionic layer.*

The positively charged ions migrate towards the negative electrode, and carry solvent molecules with them. Thus, EOF refers to the overall solvent movement. The magnitude of the EOF is dependent on such factors as buffer pH, viscosity and ionic strength and can be represented by equation 1.3. Uncharged molecules in the capillary move at the same rate as the EOF and are poorly separated, whilst positive ions move faster and negative ions, which are opposing the EOF, slower. If the EOF is sufficient, the differing electrophoretic mobilities of the analytes cause them to form zones by the time they reach the detector. If the EOF is too strong, however, the analytes will be swept too quickly through the capillary and will not be fully separated by the time they reach the detector. It is important therefore to ensure that the EOF is sufficient to avoid inconveniently long analysis times, but slow enough to allow adequate separation (Oda and Landers, 1994).

The mobility of the EOF, μ_{eo}, can be described by equation 1 3

Eqn 1 3

$$\mu_{eo} = (\varepsilon \zeta / 4 \pi \eta r_c)$$

where

μ_{eo} = mobility of EOF

ε = dielectric constant of solution

ζ = zeta potential

η = viscosity of solution

r_c = radius of capillary

The velocity of the EOF, v_{eo}, can hence be described by equation 1 4

Eqn.1 4

$$v_{eo} = \mu_{eo} E$$

The movement of ions through the capillary is dependent on both the EOF and the electrophoretic mobility of the ion The time, t, taken by the ion to migrate through the entire length of the capillary,L, can be represented by equation 1 5

Eqn 1 5

$$t = \frac{L}{v_{ep} + v_{eo}}$$

1.1.3. Efficiency and Resolution in Capillary Electrophoresis

1.1.3.1. Flow Profile

A distinct advantage of CE over more conventional pumped systems, such as HPLC, is the plug-like flow profile generated in the capillary. In pumped systems, the solution at the outer edges of the column is moving at a slower rate than the solution in the centre of the column. This parabolic flow means that the ions travel at different speeds across the column, resulting in peak-broadening (Figure 1.3). This problem does not arise in CE, however, as the EOF is generated along the length of the capillary itself, giving rise to a constant flow.

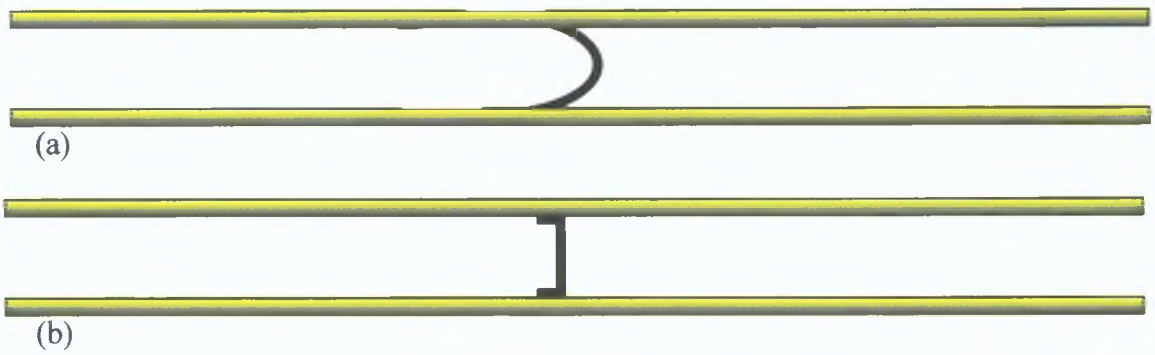

Figure 1.3: *Schematic of (a) hydrodynamic flow and (b) electroosmotic flow.*

1.1.3.2. Dispersion

As a result of the plug-like flow in capillary electrophoresis, dispersion can be assumed to be time-related molecular dispersion only. This peak dispersion, σ^2, can be calculated as:

Eqn.1.6.

$$\sigma^2 = 2\,D_m\,t$$

where:

D_m = diffusion coefficient of solute (cm^2/s).

1 1 3.3 Efficiency

Efficiency in capillary electrophoresis, expressed in terms of theoretical plates, N, can be determined by equation 1 7

Eqn.1 7

$$N = \frac{L^2}{\sigma^2} = \frac{\mu_{ep} V}{2 D_m}$$

Another, and more commonly used, expression for N is based on the width at half-height of a Gaussian peak and is represented by

Eqn.1 8

$$N = 5\ 54\ (L / w_{1/2})^2$$

where

5 54 = 8 ln 2

$w_{1/2}$ = width at half-height

Although it is not strictly correct to discuss theoretical plates in CE, given that the separation is based upon the relative mobilities of ions in an electric field and not the chromatographic partitioning of ions between two phases, N is a useful parameter to compare efficiency between CE systems

1.1 3 4. Resolution

The resolution of two components, R_s, in capillary electrophoresis can most easily be determined by dividing the difference in the migration distances of the components by the average peak width

Eqn 1 9

$$R_s = 2 (x_2 - x_1) / (w_1 + w_2)$$

where

x_1 = migration distance of faster moving analyte
x_2 = migration distance of slower moving analyte
w_1 = peak width of faster moving analyte
w_2 = peak width of slower moving analyte

Another expression for R_s is given in equation 1 10

Eqn 1 10

$$R_s = (0\ 177)\ \frac{\mu_{ep}\sqrt{V}}{\mu_{ep}\sqrt{D_m}}$$

1.1.4. Related Techniques

Capillary electrophoresis has diversified into a family of techniques, universally termed capillary electrophoresis This 'family' includes various modes of operation, often used for different applications Modes of CE, and their relevant applications are summarised in Table 1 1

Table 1.1 *Summary of modes of CE and their applications*

Mode	Principle of Separation	Small ions	Small molecules	peptides	Proteins	oligo-nucleotides	DNA
CZE	♦ Charge-to-mass ratio	√	√	√	√		
CIEF	♦ Isoelectric Point			√	√		
CITP	♦ Mobility – Separates analytes with the same mobility as the buffer ions, which differ at inlet and outlet	√	√	√	√		
MEKC	♦ Charged species Charge-to-mass ratio and partitioning into micelles based on hydrophobicity ♦ Neutral species partitioning into detergent micelles based on hydrophobicity		√	√		√	
CGE	♦ Charge-to-mass-ratio/mass			√	√	√	√
CEC[a]	♦ Partitioning of analyte between mobile & stationery phases		√	√	√	√	

Adapted from Oda and Landers, 1994

[a] Dermaux, 1999

1 1 4 1 Capillary Zone Electrophoresis (CZE)

Capillary Zone Electrophoresis is the simplest and most popular capillary electrophoresis technique and can separate both anionic and cationic components in a single analysis CZE employs a homogeneous buffer system and constant field strength exists throughout the length of the capillary Cations and anions migrate in opposite directions due to the force of electrophoretic mobility, but the stronger electroosmotic force carries all ions towards the cathode As electrophoresis occurs, the analytes are separated into 'zones', which migrate past the detector As cations are migrating in the same direction as the EOF they are detected first Neutral components are next in the migration order, as they have no electrophoretic mobility and are effected only by the EOF All these neutrals thus migrate with the EOF and cannot be separated using this mode of CE Negatively charged species are the last to migrate as their electrophoretic mobility is opposing the EOF By the addition of specialised reagents to the separation buffer, CZE can be easily adapted to perform other modes of capillary electrophoresis Some of these reagents are ampholines for Capillary Isoelectric Focusing (CIEF), a sieving matrix for Capillary Gel Electrophoresis (CGE) and surfactants for Micellar Electrokinetic Chromatography (MEKC)

1.1.4 2 Capillary Isoelectric Focusing

Capillary isoelectric focusing relies upon the separation of substances on the basis of their isoelectric points (Liu *et al* , 1996) CIEF separations can be performed in gel matrices or free solution Separation is achieved by filling the capillary with a mixture of carrier ampholytes and the sample The application of a voltage across the capillary generates a pH gradient, with a basic solution at the cathode and an acidic solution at the anode The ampholytes and solutes migrate until they reach their isoelectric pomts, where their charges are neutral If an analyte drifts towards the low pH side, for example, by thermal diffusion, it becomes positively charged

and will migrate back towards its isoelectric point Thus the analytes remain fixed or 'focused' at their isoelectric points Unlike other modes of capillary electrophoresis, isoelectric focusing can be carried out in the presence or absence of electroosmotic flow CIEF is most commonly used for the investigation of peptides (Shen *et al*, 2000) proteins (Hjerten and Zhu, 1985) and monoclonal antibodies (Wu and Pawliszyn, 1992)

1 1 4 3 Capillary Gel Electrophoresis

Capillary Gel Electrophoresis is a size-based separation technique employed for the separation of macromolecules such as proteins, DNA restriction fragments and oligonucleotides, which have similar or identical specific charge (i e same mass to charge ratio) The capillary is filled with a sieve-like gel matrix, which permits the size-based separation Two types of gel commonly used are the cross-linked (chemical gel) and the non-cross-linked, linear polyacrylamide (physical gel) Proteins can be separated by size using the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique, which involves denaturing the protein and then complexing it with SDS SDS-PAGE has been applied to the analysis of myoglobin (Cohen and Karger, 1987) amongst other analytes (Guttman, 1993) A review of CGE and its applications was published by Dolnik (Dolník, 1994)

1.1 4 4 Micellar Electrokinetic Chromatography

Micellar Electrokinetic Chromatography is a capillary electrophoresis technique that is based upon the differential partitioning of solutes into a micellar pseudophase MEKC was originally developed for the separation of small uncharged molecules, but is now widely used for the separation of both neutral and charged solutes (Otsuka and Terabe, 1998) In MEKC, ionic surfactants are added to the running electrolyte buffer These surfactants are amphiphilic molecules, each with a

hydrophobic tail and a polar or ionic head group If surfactant concentration reaches a sufficiently high point, known as the critical micelle concentration (CMC), the surfactants form micelles three-dimensional aggregates with the hydrophobic, non-polar moieties at the interior and the charged, polar groups at the exterior These micelles are roughly spherical and are composed of 30 to 100 monomer surfactant molecules This is known as the aggregation number, N These bubble-like micelles provide sites of interaction for the analyte in the capillary These sites are both in and on the micelle, the most important being the hydrophobic micelle core When the species to be separated is in the hydrophobic core it will migrate at the rate of the micelle When outside the micelle it will migrate at its own rate Each species will spend a specific amount of time in the micelle, dependent on its hydrophilicity, and thus they can be separated The selectivity of the electrophoretic technique can be controlled by the choice of surfactant, and by addition of organic modifiers to the buffer solution This technique is extremely useful in the separation of neutral analytes, as in the *in-vitro* study of phenol metabolism by human liver microsomes (phenol is a neutral analyte at the physiological pH of 7 4) (Davies *et al*, 1995) MEKC has also been applied to the analysis of proteins (Strege and Lagu, 1997) and metal ions (Haddad *et al*, 1997)

1 1.4.5 Capillary Isotachophoresis (CITP)

Isotachophoresis is based on the same principle as CZE with the separation of ionic species based on the differences in their effective mobility in an electric field CITP relies upon zero EOF and a heterogeneous buffer system In CITP for the separation of anions, the capillary and anode reservoir are filled with a leading electrolyte, the anions of which must have a higher mobility than any of the anions in the sample The counterion (cation) of this electrolyte must perform well as a buffer at the pH of the analysis The cathode reservoir is filled with a terminating electrolyte which contains anions of effective mobility lower than that of the sample anions The sample is then introduced between the two electrolytes, and a current is

passed through the system A uniform electric field occurs through the sample zone and separation occurs based on the individual mobilities of the analytes (Boček, 1988) CITP is particularly useful in the analysis of small ions (Valaskova and Havranek, 1999) and has been applied successfully to the separation of proteins (Smith *et al*, 1990), nucleosides (Bruchelt *et al*, 1993) and peptides (Schwer and Lottspeich, 1992) CITP can also be considered as a concentration technique as the analytes are 'stacked' on-column, into narrow zones

1 1 4 6 Capillary Electrochromatography (CEC)

This technique, which provides efficient separation of neutral solutes, involves the use of a capillary packed with a stationary phase, similar to the stationary phase employed in liquid chromatography (LC) The electroosmotic force causes the movement of the mobile phase through the column and, as in liquid chromatography, separation is based upon the partition of the solute between the stationary and the mobile phases CEC has been applied to a range of analytes including antibiotics (Smith and Evans, 1994), steroids (Smith and Evans, 1995, Euerby *et al*, 1997) and nucleotides (Helboe and Hansen, 1999) Some useful review articles on capillary electrochromatography have been published in recent years (Altria, 1999, Cikalo *et al*, 1998)

1.1.5. Conclusions

Capillary Electrophoresis is a versatile separations technique which has a number of distinct advantages over conventional separation methods One of these advantages is the small volume of sample required (typically nanolitres) for injection Aqueous buffers are primarily used, again in small volumes, reducing organic solvent waste On-capillary detection can be achieved in capillary electrophoresis and analysis times are typically short in comparison with chromatographic techniques Finally, commercial instrumentation available for capillary electrophoresis can be used to perform various modes of CE, making it suitable for the analysis of a wide variety of analytes Modes of CE, and their relevant applications are summarised in Table 1 1 Like all other separation techniques, CE does have its disadvantages Most modes of CE cannot be used to separate neutral species, and joule heating can also be a limitation The main disadvantage of CE, however, is its poor concentration sensitivity relative to other methods, when UV absorbance detection is used Methods of sensitivity enhancement to overcome this limitation are discussed in Section 1 2

1.2. SENSITIVITY ENHANCEMENT METHODS FOR CE

1.2.1. Introduction

CE is most commonly performed with UV absorbance detection CE with UV detection, however, suffers from poor concentration sensitivity when compared with other methods, with typical detection limits in the parts per million (ppm) range This poor sensitivity results from both the small sample volumes (picolitres to nanolitres) that can be introduced onto the capillary and the short optical path length (determined by the capillary diameter) for detection The low concentration sensitivity of CE-UV is of particular concern when performing analyses of

metabolic mixtures Minor metabolites are typically formed in low concentrations and may fall outside the limit of detection of a conventional CE-UV method (Thormann *et al* , 1998)

1.2.2. Modes of Sensitivity Enhancement

1 2 2 1 On-Capillary Sample Concentration

Off-column concentration techniques commonly employed for CE include liquid-liquid extraction (Kitahashi and Furuta, 2002, Pedersen-Bjergaard *et al* , 2000) and solid-phase extraction techniques (Zhou and Stewart, 2002 and Long *et al* , 2002) as well as on-line coupling of an LC to the CE instrumentation (Shihabi, 2000) These techniques generally provide much improved sensitivity but at the cost of increased analysis times In the past few years, on-column concentration techniques (sample stacking methods) have received a lot of interest in the field of CE Many of these techniques are simple to perform and can yield concentration factors of up to 100-fold, without significantly increased analysis times These techniques are particularly attractive as they eliminate the need for sample extraction and pre-treatment steps prior to analysis Some useful review articles on sample stacking for capillary electrophoresis have been published in recent years (Osbourn *et al* , 2000, Beckers and Boček, 2000, Hempel, 2000, Chien, 1998, Quirino and Terabe, 2000, Shihabi, 2000, Urbanek *et al* , 2003) On-column concentration techniques are discussed below and are summarised in Table 1 2

Table 1 2 *· Summary of on-column concentration techniques for CE*

Mode	Concentration Factor (approximates)	Advantages	Disadvantages
Field Amplified	10	♦ Very simple to perform	♦ Limited Injection volume ♦ Generally limited to samples of low ionic strength
Large Volume Sample Stacking	100	♦ No changes to instrumentation needed ♦ High concentration Factors can be achieved	♦ Complicated ♦ Polarity-switching methods not suited to automation
pH Focusing	5	♦ Simple to perform	♦ Limited to samples that will not degrade under altered pH ♦ Concentration factors lower than with other methods
Acetonitrile-Salt Stacking	10-20	♦ Useful for salt containing samples ♦ Simple to perform	♦ Not suitable for all samples ♦ Concentration factors are low ♦ for non-salt-containing samples
Sweeping MEKC	up-to 146,000	♦ With FASS, extremely high concentration factors can be achieved	♦ FASS generally limited to samples of low ionic strength ♦ Buffer constraints limit applicability of the technique
Isotachophoresis	10-1000	♦ Very high concentration factors can be achieved	♦ Most complicated method to perform
pH-mediated Sample Stacking	10	♦ Very simple to perform ♦ Very useful for high ionic strength samples	♦ Sample injection volume limited by capillary length
PH-mediated sample stacking with double capillary system	70	♦ High concentration factors can be achieved ♦ Very useful for high ionic strength samples	♦ Double capillary system required

1 2 2 1 1 Basic Principles of Sample Stacking

The velocity (v) of analyte ions is given by the following formulae (Shihabi, 2000)

Eqn. 1 11

$$v = \mu_{ep} E$$

Eqn. 1 12

$$v = q / 6 \pi \eta r$$

where

μ_{ep} = electrophoretic mobility

E = field strength

q = charge on the molecule

η = viscosity

r = ionic radius

Therefore, by changing one of these parameters, one can alter the velocity of the analyte ions Altering the velocity of the analyte ions allows one to control their migration through the capillary and force them into narrow zones The parameter that is most commonly manipulated to achieve sample stacking is the field strength This is typically carried out by employing a discontinuous buffer system in the capillary When there is a discontinuous buffer system in the capillary, the different buffer zones will experience different field strengths, based on their relative conductivities The analytes will migrate through these zones at different rates and thus their movement through the capillary can be controlled

1 2 2 1 2 Modes of On-Column Concentration

1 2 2 1 2 1 Field Amplified Sample Stacking (FASS)

This is the simplest and most commonly used mode of sample stacking Briefly, the sample is prepared in a low conductivity matrix relative to the BGE, such as water If a low and high conductivity solution are both present in the capillary, when a voltage is applied the low conductivity region (sample zone) will experience a higher electric field compared to the high conductivity region (separation buffer) The sample ions will thus have a greater velocity m the sample zone and will migrate rapidly through this zone, slowing down abruptly and 'stacking' into narrow bands when they reach the separation buffer interface This stacking mechanism occurs for both cations and anions with the positive species stacking up in front of the sample plug and the negative species at the back The analytes then undergo migration and separation as normal Figure 1 4 is a representation of the mechanism of FASS

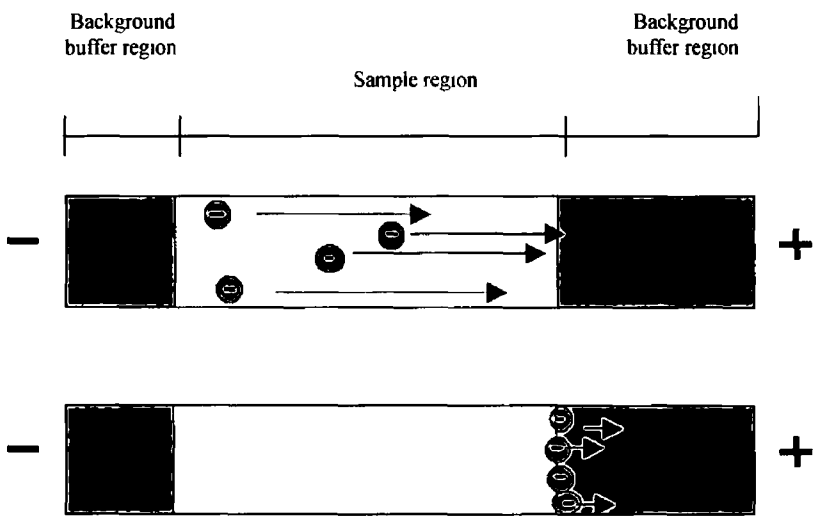

Figure 1 4 *Mechanism of field amplified sample stacking for anions*
Reproduced from Quirino and Terabe (2000)

This stacking mode can be used to achieve a concentration factor of up to 10 for hydrodynamic injection, and even greater effects can be achieved with electrokinetic injection (Chien and Burgi, 1992) A limitation of this mode is the relatively short sample plug length that can be injected before band-broadening occurs Differences between the flow rates of the sample and separation buffer can result in laminar flow within the capillary giving rise to peak width distortion when longer injection times are used (Quirino and Terabe, 2000) FASS is widely used and amongst other things has been applied to the analysis of species in plasma (Song *et al*, 1998, Wu *et al*, 2001, Wey *et al*, 1999) FASS has also been demonstrated in 'laboratory-on-a-chip" devices (Yang and Chien, 2001)

1 2 2 1 2 1 1 Destacking

When capillary electrophoresis is used for the analysis of high ionic strength samples, the opposite phenomenon occurs The analytes migrate more slowly in the sample zone than in the BGE When they reach the interface of the two zones (sample zone and BGE) they speed up, resulting in band broadening This can be a limitation for the analysis of physiological samples, which are typically highly conductive These samples must be significantly diluted, or extracted, prior to injection for field amplified stacking to occur Often this dilution is greater than the resulting concentration effect, resulting in an overall loss of sensitivity The use of very high concentration background electrolytes (>1 M) for the stacking of high ionic strength samples has recently been demonstrated (Ding *et al*, 1998) but it is unlikely that this method will achieve widespread use due to negative joule heating effects and long analysis times associated with such buffers FASS and its applications have been reviewed (Chien and Burgi, 1992)

1 2 2 1 2 2 Large Volume Sample Stacking of Anions (LVSS)

One means to overcome the limitation of short sample plug lengths associated with field amplified stacking is to remove the sample matrix after concentration This allows longer injection times to be used without significant band-broadening effects The sample matrix can be removed by pumping with external pressure or with EOF (Liu *et al*, 2002) LVSS is carried out in the same manner as FASS, with the sample being prepared in a low conductivity matrix prior to hydrodynamic injection, however, longer injection times are used The steps involved in LVSS with polarity switching are listed below and illustrated in Figure 1 5

1 A negative voltage is applied The neutral sample solvent is then pushed out of the injection end of the capillary by the EOF

2 The anions are subjected to a strong electric field strength, opposing the EOF, and so they move with high velocity to stack at the interface of the sample matrix and the separation buffer The electric current in the capillary is initially lower than current obtained when BGE only is in the capillary As the low conductivity plug is pumped out, the current steadily increases

3 This current is monitored and when it reaches approximately 90 to 99% of the BGE only value, the polarity is switched, from negative to positive polarity and separation occurs

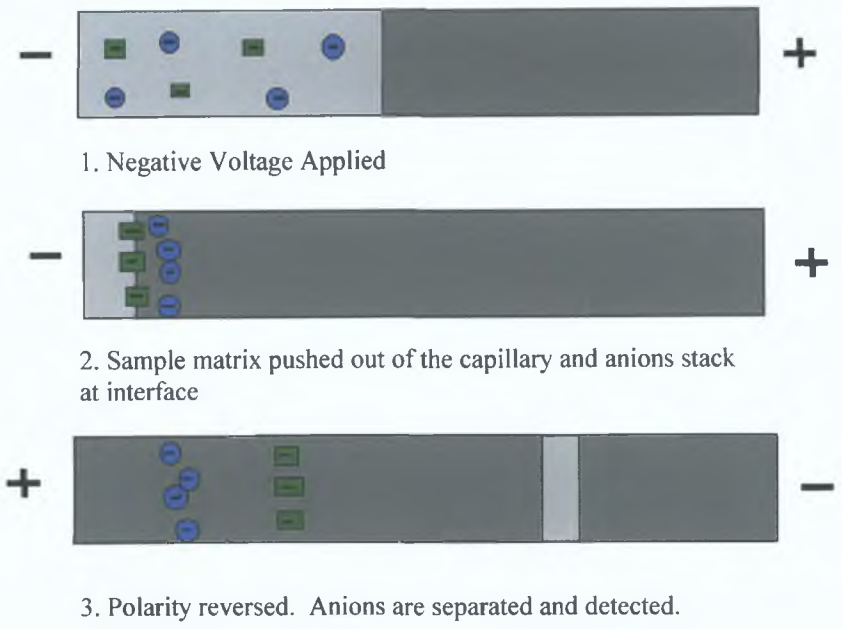

Figure 1.5: *LVSS of anions with polarity switching.*
Reproduced from: Quirino and Terabe (2000).

LVSS can be carried out without polarity switching (Quirino and Terabe, 1999). This can be advantageous, as the observed current does not have to be manually monitored and methods can be automated. To eliminate the polarity-switching step, the EOF has to be carefully manipulated. For the analysis of anions, the polarity is again reversed and the EOF is kept lower than the electrophoretic velocity of the anions. This can be achieved by using buffer additives or low pH buffers. The stacking proceeds as for the polarity switching method, but the anions move past the detector with electrophoretic velocity. A limitation of LVSS is that anions and cations cannot be detected in the same run, as is the case with other forms of CE. Concentration factors of more than 100 have been reported for LVSS (Burgi, 1993; Albert *et al.*, 1997; Núñez *et al.*, 2001; McGrath and Smyth, 1996).

1 2 2 1 2 3 pH Focusing

The sample stacking of peptides based on the pH of the sample matrix has been described by Aebersold and Morrison (1990) They dissolved the sample in buffer which was two units above the net isoelectric point, pI, so the peptides were negatively charged When a voltage was applied, the peptides originally migrated towards the anode until they reached the interface of the BGE, where they were stacked into a narrow zone After the pH gradient of the sample dissipated in the BGE, the peptides became positively charged and reversed their migration direction towards the cathode This method causes stacking to occur from both sides A five-fold concentration was achieved using this method (Figure 1 5)

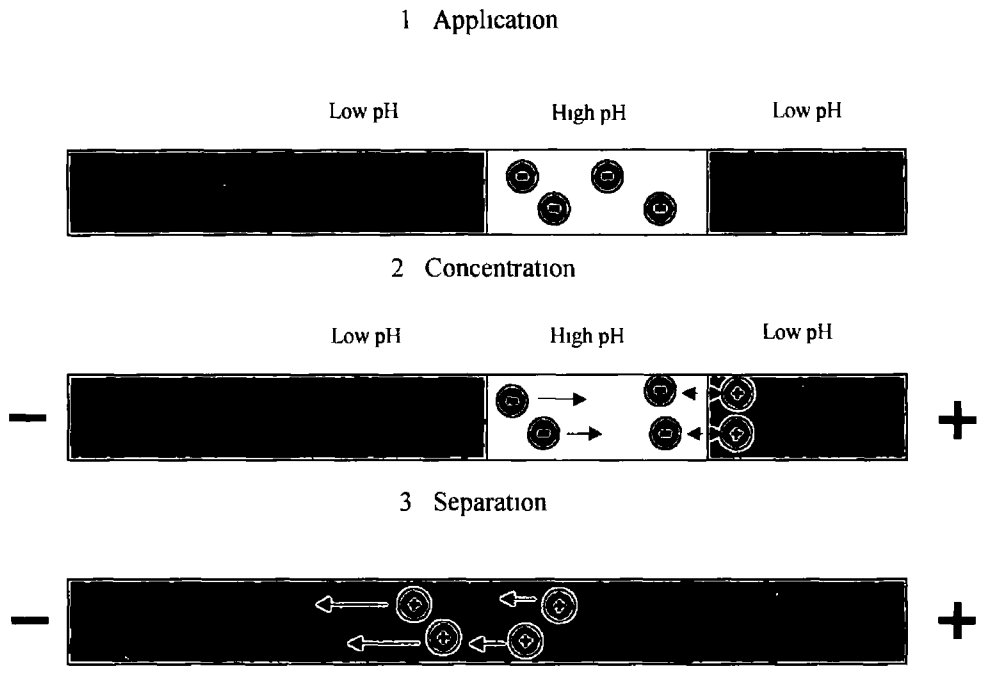

Figure 1 6 *Mechanism of pH focusing*
Reproduced from Aebersold and Morrison (1990)

The effect of sample pH and background electrolyte pH on separation in capillary electrophoresis has also been investigated (Friedberg *et al*, 1997, Britz-McKibben and Chen, 2000)

1 2 2 1 2 4 Acetonitrile – Salt Mixtures

Acetonitrile is often added to biological samples to remove proteins Due to its low conductivity, inclusion of acetonitrile in the sample can bring about a stacking effect, with a concentration factor of 10 to 20 times (Ban *et al*, 2001) In the presence of salts, however, (which are typically present at approximately 1% concentration in physiological samples), a further type of stacking occurs It is believed that the salts migrate rapidly in the acetonitrile, slowing down at the BGE interface, creating two zones a high conductivity zone (salt) and a low conductivity one (acetonitrile) As the boundary between the two zones moves in the sample, a sweeping effect is observed, concentrating the analytes into sharp bands The bands then enter the BGE where separation occurs This method is very useful for salt-containing samples (such as sodium chloride present in serum) as it removes proteins and counters the harmful effects of inorganic ions in the sample and can yield concentration effects of up to 30-fold (Friedberg *et al*, 1997, Shihabi, 1998, Shihabi, 1999)

1 2 2 1 2 5 Sweeping

Sweeping occurs when a pseudostationary stage is introduced into the capillary after separation has taken place This is typically performed by using a non-micellar BGE Micellar solutions are placed at the inlet and outlet ends of the capillary after the separation has been performed As the micelles move through the sample zone, they accumulate ions and stack them into narrow bands Sweeping is often used in conjunction with FASS, by preparing the sample in a low conductivity matrix relative to the BGE In this situation, FASS has already taken place and ions are stacked into bands before the addition of the micelle solution, which then further stacks the ions Quirino *et al* have demonstrated 10^{-4} to 10^{-5} –fold improvements in detector response for aromatic amines using this technique (Quirino and Terabe, 2000) Taylor *et al* (2001) have demonstrated the use of sweeping for samples in

complex biological matrices such as plasma and urine and Monton *et al* (2001) have applied sweeping to the analysis of phenol derivatives

1 2 2 1 2 6 Capillary Isotachophoretic Preconcentration

Capillary Isotachophoresis by its nature brings about preconcentration and is discussed in Section 1 3 5

1 2 2 1 2 7 pH-Mediated Sample Stacking

In this method, the background electrolyte is made from the salt of a weak base, such as ammonium The high ionic strength sample is electrokinetically injected into the capillary, resulting in the displacement of sample cations with ammonium ions The sample injection is immediately followed by the electrokinetic injection of a strong base, such as sodium hydroxide The hydroxide ions migrate through the sample zone due to their high mobility This leads to the neutralisation of the ammonium ions in the sample zone and a low conductivity region is formed Electrofocusing then occurs over the sample zone as in other stacking techniques A limitation of this technique is that a large portion of the capillary is taken up by the stacking process, reducing the length of capillary available for the electrophoretic separation Recent methods have employed double-capillaries to increase the capillary length available for separation (Zhao and Lunte, 1999) pH mediated sample stacking has been applied to the study of high-ionic strength physiological samples (Weiss *et al* , 2001, Park and Lunte, 1998, Hadwiger *et al* , 1996) Figure 1 7 illustrates the steps in the pH-mediated stacking technique

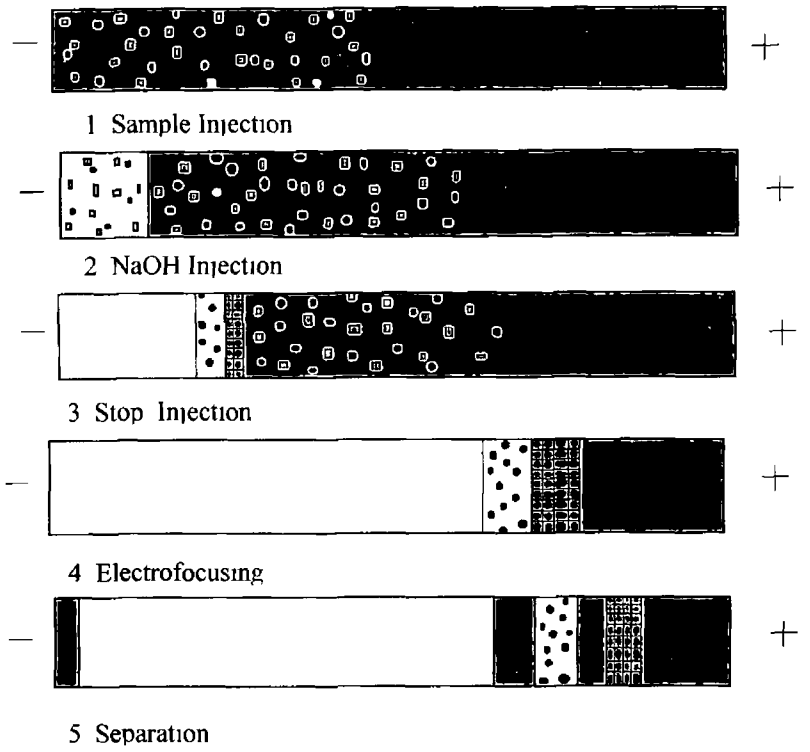

Figure 1 7 *pH-mediated sample stacking of anions*
Reproduced from Zhao (1997)

1 2 2 2 Alternative Capillary Geometries

According to the Beer-Lambert law (Equation 1 13), the absorbance of a sample is directly proportional to the optical path length through which the measurement is performed The short optical path length in CE therefore is disadvantageous for absorbance-based detection methods (Pentoney and Sweedler, 1994)

Eqn 1 13 Beer-Lambert Law $A = \varepsilon Cl_c$

where

A = Absorbance

ε = molar extinction coefficient

C = Concentration of analyte

l_c = optical path length

The transcolumn capillary arrangement which is most commonly used is illustrated in Figure 1 7 (Pentoney and Sweedler, 1994) This arrangement allows only a very short path length for detection Recent research has focused on altering the shape of the capillary with the aim of achieving an elongated path length for detection

Figure 1 8 *Transcolumn arrangement for absorbance detection*
Reproduced from Pentoney and Sweedler (1994)

1 2 2 2 1 Transcolumn

The optical path length for detection in CE is restricted by the inner diameter of the capillary, which is typically in the region of 25 μm to 100 μm UV absorbance detection is most commonly performed by passing a band of light through the capillary walls, orthogonal to the capillary axis The cylindrical shape of the capillary, however, means that the actual path length is less than the capillary diameter and may be approximated Equation 1 14

Eqn 1 14 $l_c = (\pi \times \text{Inner diameter}) / 4$

This would result in a path length of only 39 μm for a 50 μm I D capillary (Pentoney and Sweedler, 1994)

1 2 2 2 Rectangular Capillaries

Employing rectangular capillaries has been shown to increase the path length for detection to as much as 1 mm while providing improved heat dissipation capability (Tsuda *et al*, 1990) A disadvantage of this method, however, is the fragility of the elongated, borosilicate capillaries used Fused-silica polyimide capillaries in a rectangular configuration are not currently commercially available but their introduction could provide a simple, improved format for detection in CE

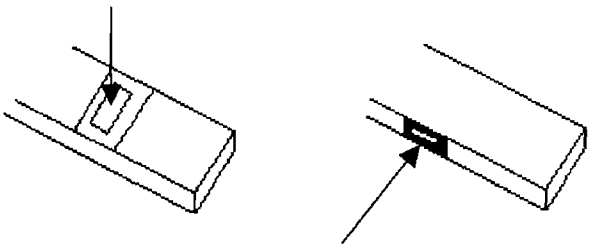

Figure 1 9 *Rectangular capillaries*
Reproduced from Tsuda *et al* (1990)

1 2 2 3 Extended Light Path Capillaries

Chervet *et al* (1991) have carried out CE separations on capillaries that have been bent into a Z-shape The flow cell was prepared by sandwiching an alumina shim alongside the capillary between two polyethylene discs They used cylindrical fused silica capillaries, that were bent into this z-shape, allowing axial detection through the bend Using this method, they achieved an optical pathlength of 3 mm This pathlength yielded a 40-fold improvement over a typical 75 μM capillary, but the

actual sensitivity enhancement was approximately six-fold, due to poor efficiency in light throughput A schematic of the configuration used is shown in Figure 1 10

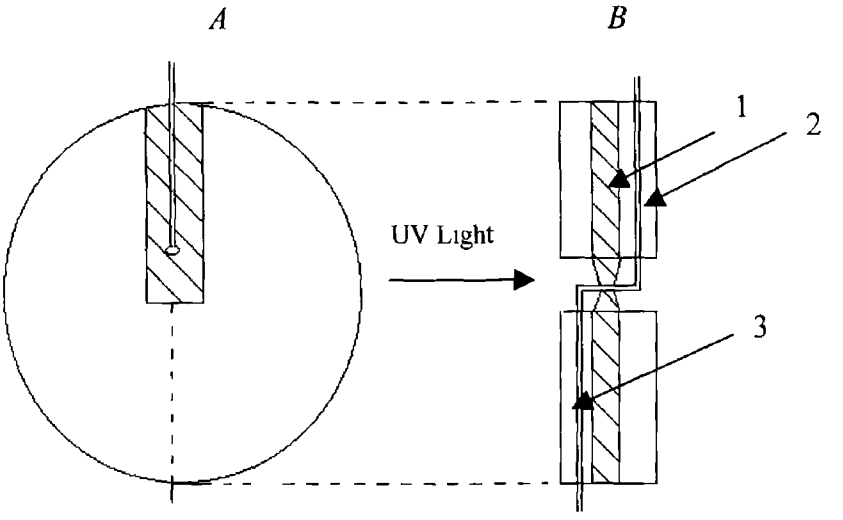

Figure 1 10 *Schematic of a Z-Cell*
Reproduced from Chervet *et al* (1991)

where

A = Front View
B = Cross-Sectional View
1 = Shim (alumina) with centred 300 μm i d hole
2 = plastic discs (polyethylene)
3=fused-silica capillary of 50 or 75μm i d , 280 μm o d

1 2 2 2 4 Multireflection Flow Cell

A nanolitre scale multireflection cell has been shown by Wang *et al*, (1991) to produce a 40-fold improvement in detection limits This cell was produced by placing a silver coating (mirror) on the internal surface of the detection portion of the capillary Two windows were left on either side of the capillary to serve as

entrance and exit sites for laser illumination This method utilised a 5-mW red HeNe laser as the light source and a photomultiplier tube (PMT) was utilised for detection A limitation of this method however, is the use of a laser as the light source, instead of the more common broadband source The multireflection flow cell is illustrated in Figure 1 11

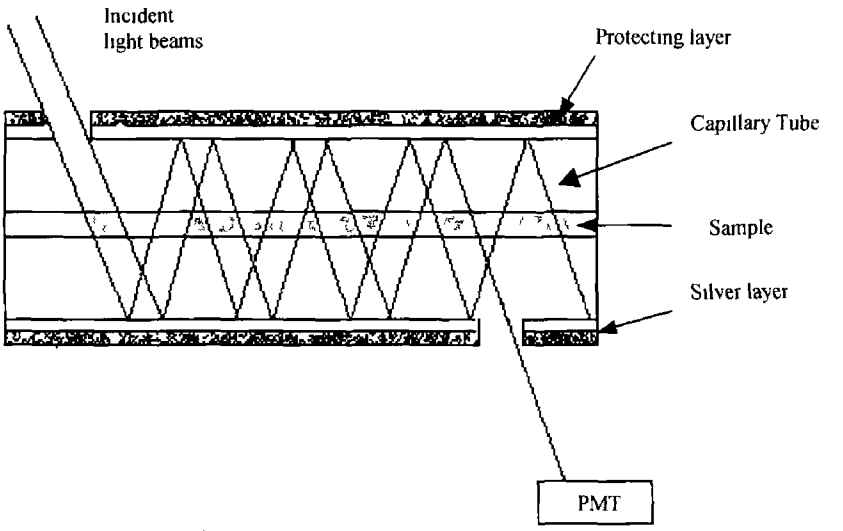

Figure 1 11 Multireflection flow cell
Reproduced from Wang *et al* (1991)

Table 1 3 *Comparison of some alternative capillary geometries*

Method	Enhancement	Pathlength (1 d)	Applicability to commercial systems	Comments
Z-Cell	3-5	3 mm (75 μm)	Direct	♦ Direct applicability of methods ♦ High noise levels ♦ Some resolution loss ♦ Commercially available ♦ Moderate cost
Z-Cell/Optics	10-20	3 mm (75 μm)	Direct	♦ Direct applicability of methods ♦ More than an order of magnitude gain ♦ Minimal resolution loss ♦ Commercially available ♦ Moderate cost
Rectangular	9 5	500 μm (50 μm)	Direct	♦ Some care in methods buffer choice, flow rates ♦ High sample loadings ♦ Questionable manufacturability
Multireflection	40	3 3 mm (75 μm)	No	♦ Questionable reproducible manufacturability ♦ Instruments require modifications

Adapted from Albin *et al* (1993)

1.2.2 3 Alternative Modes of Detection

UV absorbance detection is the most commonly used detection technique for capillary electrophoresis and practically all commercial CE systems currently come equipped with UV detectors There are other means of detection available, however, which can increase the concentration sensitivity of the technique These alternative detection modes are discussed below and are summarised in Table 1 4

Table 1.4 *Detection techniques for CE*

Method	Mass Detection Limit (moles)	Concentration Detection Limit (molar)	Advantages/Disadvantages
UV-Vis absorption	10^{-13}-10^{-16}	10^{-5}-10^{-8}	♦ Universal ♦ Diode array offers spectral information
Fluorescence	10^{-15}-10^{-17}	10^{-7}-10^{-9}	♦ Sensitive ♦ Usually requires sample derivatization
Laser-induced Fluorescence	10^{-18}-10^{-20}	10^{-14}-10^{-16}	♦ Extremely sensitive ♦ Usually requires sample derivatization ♦ Expensive
Chemiluminescence		10^{-7}-10^{-10}	♦ Relatively sensitive ♦ Low equipment cost ♦ Usually requires sample derivatization
Amperometry	10^{-18}-10^{-19}	10^{-10}-10^{-11}	♦ Sensitive ♦ Selective but useful only for electroactive analytes ♦ Requires special electronics and capillary modification
Conductivity	10^{-15}-10^{-16}	10^{-7}-10^{-8}	♦ Universal ♦ Requires special electronics and capillary modification
Mass Spectrometry	10^{-16}-10^{-17}	10^{-8}-10^{-9}	♦ Sensitive & offers structural information ♦ Interface between CE & MS complicated ♦ Expensive
Indirect U V , fluorescence, amperometry	10 – 100 times less than direct method	-	♦ Universal ♦ Lower sensitivity than direct methods

Reproduced from Marina and Torre (1994)

1 2 2 3 1 Fluorescence

Fluorescence detection for CE is typically achieved on-capillary with a lamp (deuterium, tungsten or xenon arc lamp) or a laser A grating monochromator is used to select the excitation wavelength and the resulting emission wavelengths are measured with a photomultiplier tube (Figure 1 11) Fluorescence detection is extremely sensitive and yoctomole detection limits for CE have been reported by Chen and Dovichi (1994) Native laser-induced fluorescence (LIF) detection has been reported for proteins (Timperman *et al* , 1995) and catecholamines (Chang and Yeung, 1995) amongst other analytes A limitation of the fluorescence detection method, however, is that analytes that do not exhibit native fluorescence must be derivatized either before injection, on-column or post-column to facilitate fluorescence detection Typically, derivatization is performed pre-column as larger volumes of the analyte and the derivatizing reagent can be used, before a small volume is injected onto the capillary On-column derivatization has been achieved, however, with a plug of the derivatizing reagent being injected onto the capillary before the sample injection (Fishman *et al* , 1994) Post-column reactors have been used to perform derivatization (Tsuda and Kobayashi, 1998), but despite the advantages afforded by a separation free of "labelled" analytes, post-column derivatization remains more complicated than other derivatization methods Derivatized samples analysed with LIF detection include dopamine (Robert *et al* , 1995), amino acids (Molina and Silva, 2002, Feng and Johnson, 1999) and porphyrins (Melanson and Lucy, 2002) An alternative to derivatization is to exploit the sensitive nature of fluorescence by combining it with either indirect fluorescence, or immunoassay Indirect fluorescence will be discussed in Section 1 2 2 3 4 2 A basic schematic of fluorescence detection is illustrated in Figure 1 12

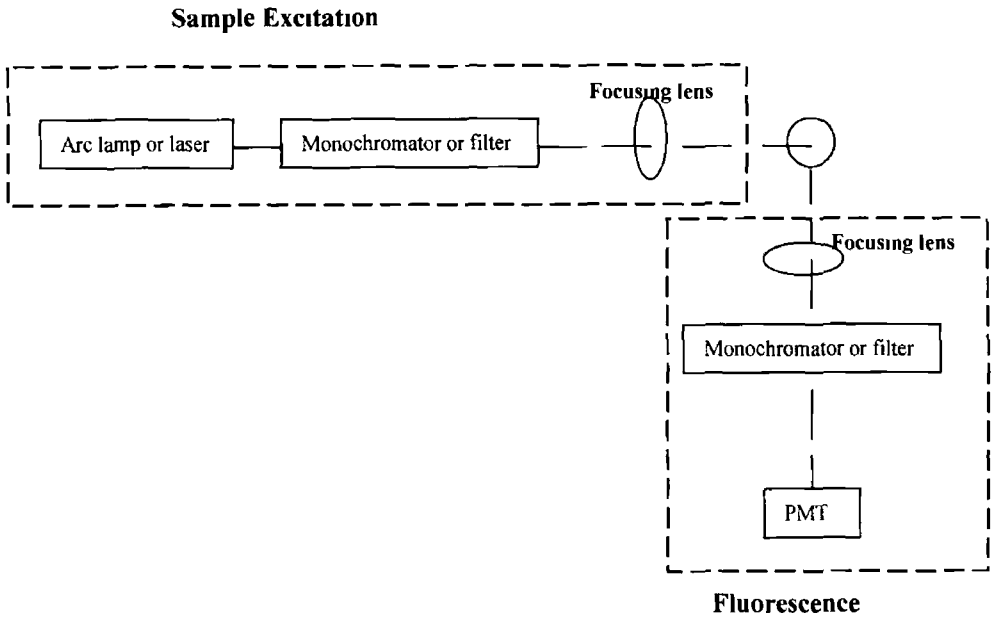

Figure 1.12 *Schematic of fluorescence detection*
Reproduced from Kuhn and Hoffstetter-Kuhn (1993)

1 2 2 3 2 Chemiluminescence

Chemiluminescence (CL) is one of the less-well developed detection methods for CE This is in part due to the need to derivatize almost all analytes before they can be detected Although bioluminescence is more common, very few chemical reactions exhibit CL, in which during a chemical reaction an excited species is produced, which emits light as it returns to the ground state An advantage to this, is the absence of the necessary light source for CL detection In fact the instrumental set-up for CL is relatively simple, with typically only a PMT being necessary CL is most commonly performed by joining a chemilummescent species to a derivitizing group which is reacted with the analyte of interest CL detection has been used by Dadoo *et al* (1994) and Hashimoto *et al* (1999), achieving limits of detection for luminol of 2×10^{-8} mol l^{-1} and 5×10^{-10} mol l^{-1} respectively

1 2 2 3 3 Electrochemical Detection

In more recent years, CE with electrochemical detection (CE-EC) has undergone huge advances, with the development of more complex electrode systems and the miniaturization of CE-EC for "lab-on-a-chip techniques" (Baldwin, 2000, O'Shea and Lunte, 1995, Ewing *et al*, 1994 and Yik and Li, 1992) The electrochemical techniques of amperometry, conductivity and potentiometry, and their applications as detectors for CE, are discussed below

1 2 2 3 3 1 Amperometric Detection

Amperometry is the most commonly used electrochemical detection method for capillary electrophoresis Amperometry is performed by measuring current at a working electrode as analytes are oxidised or reduced The working electrode is kept at a constant potential, relative to the reference electrode The current, which is proportional to the concentration of analyte present, is measured as a function of time as analytes migrate through the capillary The main drawback to CE with this mode of detection is the difficulty which arises in accurately aligning the working electrode with the capillary whilst isolating the electrodes from the separation voltage Amperometric detection is applicable only to electroactive compounds (those which are oxidizable, reducible, or both), but once performed, is a sensitive and relatively inexpensive detection technique Amperometric detection has been applied to L-Dopa in microdialysis samples (O'Shea *et al*, 1992) and the analysis of catecholamines (Wallingford and Ewing, 1987)

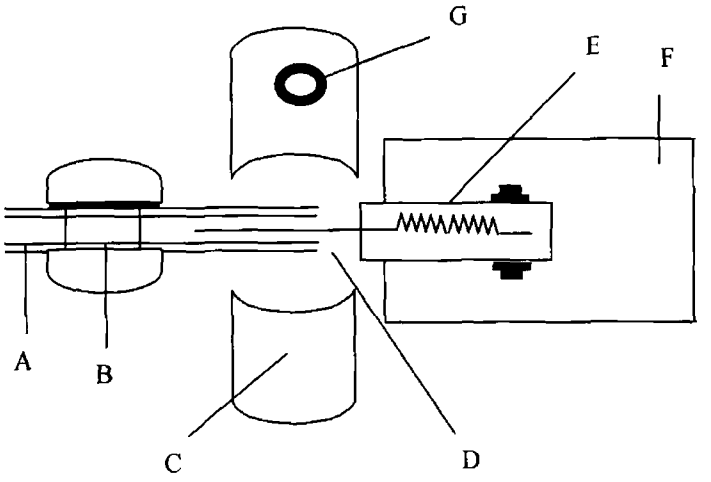

Figure 1.13. *Schematic of amperometric detector for CE*
Reproduced from Wallingford and Ewing (1988)

where

A = column

B = porous glass joint assembly

C = plexiglass block

D = carbon fiber working electrode

E = microscope slide

F = micromanipulator

G = reference electrode port

1 2 2 3 3 2 Conductimetric Detection

Conductimetric detection is achieved by measuring the potential between two electrodes while passing through a small constant current Analytes are detected because of the difference in the conductance of the electrolyte and the analyte ions As with amperometric detection, the main disadvantage of this technique is the difficulty in achieving correct positioning and decoupling of the electrochemical

detector It is, however, more universal than amperometric detection Many inorganic ions exhibit very poor optical absorption and thus conductivity detection has been applied to their analysis Huang *et al* (1987) have detected metal alkali ions and small ionic components in human serum using conductivity detection, with detection limits in the 10^{-7} M range

1 2 2 3 3 2 Potentiometric Detection

Potentiometric detection is the most straightforward of the three electrochemical detection methods employed for CE as it requires only the measurement of a potential by an ion selective microelectrode or at an electrode surface with respect to a reference electrode Potentiometric detection for CE has been illustrated for potassium in blood plasma (Nann *et al* , 1993)

1 2 2 3 3 Mass Spectrometry (MS)

The coupling of CE with MS detection provides a powerful tool for the separation of complex mixtures This highly sensitive detection method allows not only relative molecular mass determination but also structure elucidation of analytes (McCloskey, 1990) CE has been interfaced to MS by two main methods Electrospray Ionization (ESI) and Continuous-Flow Fast Atom Bombardment (CF-FAB) Currently, while CE-MS is an extremely promising technique, the high cost of the instrumentation is a barrier to its more widespread use CE-MS has been used, however, to analyse ammo acids (Soga and Heiger, 2000) and oligosaccharides (Kelly *et al* , 1994) amongst other species As MS is a complex analytical technique unto itself, it will not be discussed in further detail here Some useful review articles on MS include those by Tomer (2001) and by Burlingame *et al* (1998)

1 2 2 3 4 Indirect Methods of Detection

Almost all detection modes can be made to function in the indirect mode A UV active, fluorescent or electroactive compound is generally added to the background electrolyte, giving a constant signal Negative peaks are usually observed as the analytes displace the added component These negative peaks are typically converted to positive peaks by the CE software The sensitivity achieved by using indirect detection is represented by Equation 1 15 (Weinberger, 2000) Higher sensitivity is achieved when the concentration of the indirect reagent in the background electrolyte is minimal Indirect methods of detection typically have higher limits of detection than their direct counterparts (Yeung, 1989, Poppe and Xu, 1998)

Eqn 1 15.

$$C_{LOD} = \frac{C_R}{(DR)(TR)}$$

where

C_{LOD} = concentration limit of detection
C_R = concentration of the reagent
DR = Dynamic reserve
TR = Transfer Ratio

1 2 2 3 4 1 Indirect UV Absorbance Detection

UV absorbance is the most commonly used indirect detection mode and has been applied to the detection of such species as amino acids (Žunic *et al* , 2002, Wang *et*

al, 2000) and inorganic ions (Ackermans *et al*, 1991) which generally do not exhibit native absorbance In this detection mode, a highly absorbing species is added to the BGE, giving rise to a constant signal which is displaced by the analyte ions In indirect UV absorbance detection the sensitivity achieved is highly dependent upon the choice of an appropriate UV absorbing probe for the analyte of interest Ideally, the UV absorbing species should have an electrophoretic mobility similar to that of the ion of interest If the absorbing ions and the sample ions are of differing mobilities, the solute ions will displace UV absorbing ions as the solute migrates along the capillary, giving rise to poor peak shapes For example, if the mobility of the UV absorbing ion is too slow for the analyte, a fronted peak will be obtained Conversely, if the UV absorbing ion chosen is too fast, a tailing peak will be seen Figure 1 14 illustrates the relative mobilities of common UV absorbing ions and common solute ions for CE

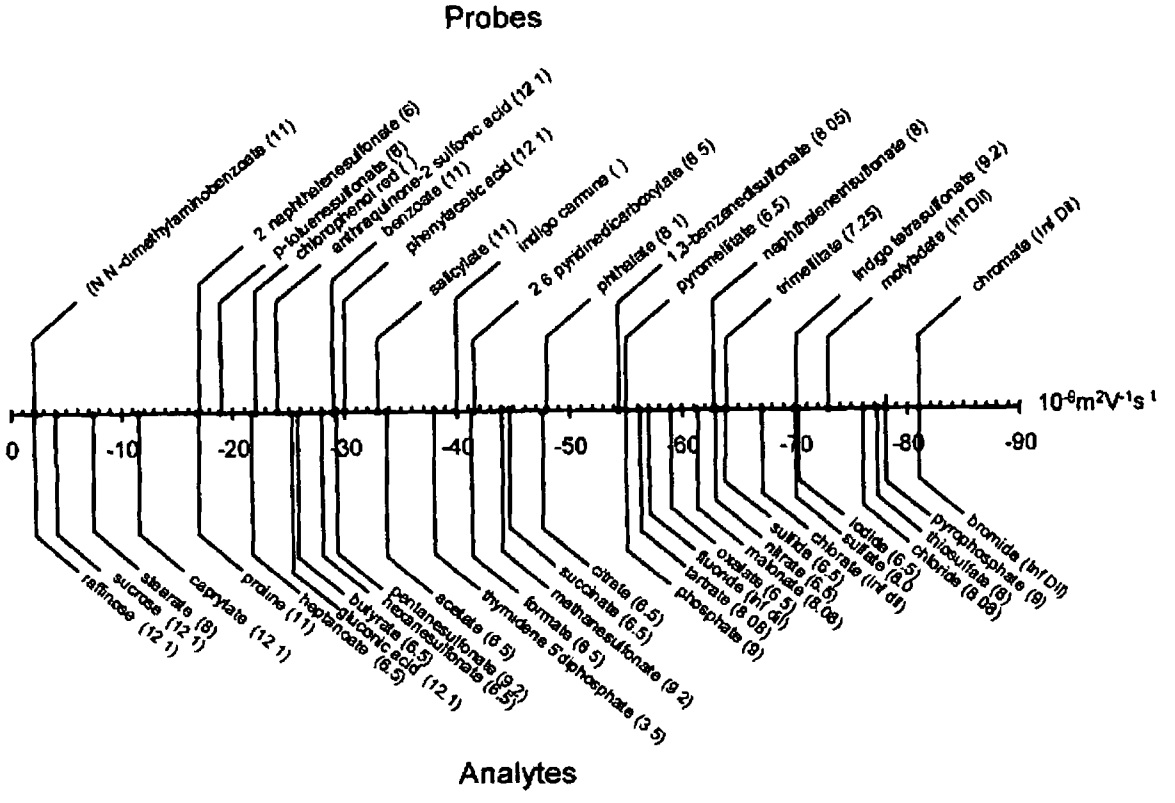

Figure 1 14 *Mobilities of some common indirect UV probes and analytes*
Reproduced from Doble *et al* (2000)

The concentration of the UV absorbing ion should typically be in the range from 1 mM to 10 mM If the concentration of the absorbing species is too high, the response change, as the ion is displaced, will be insufficient to give good limits of detection If, however, the concentration is too low, the linearity of the system will be compromised

1 2 2 3 4 2 Indirect Fluorescence Detection

Indirect fluorescence detection is similar in operation to indirect absorbance detection but is less widely used as most commercially available instruments are not compatible with this detection technique It offers, however, several distinct advantages over indirect absorbance detection Firstly, as direct fluorescence detection is very selective - limited to those compounds which exhibit native fluorescence- indirect fluorescence detection is more widely applicable Furthermore, it is possible to use very low concentrations of the indirect component in the BGE, giving high sensitivity based on equation 1 15 The excitation intensity can be increased to compensate if the concentration of the indirect component in the BGE or the pathlength are reduced Indeed minimum detectable concentrations of 10^{-7} M using capillaries with internal diameters as low as 5 μm have been reported (Pentoney and Sweedler, 1994) Indirect fluorescence detection has been applied to the analysis of a variety of analytes including glycosaminoglycan monosaccharides (Ruiz-Calero *et al*, 2000), amino acids (Kuhr and Yeung, 1988a) and nucleotides (Kuhr and Yeung, 1988b) Indirect UV absorption and indirect fluorescence were compared for the determination of isoprenyl pyrophosphates in 1995 (Andersson *et al*, 1995)

1.2.3.4.3 *Indirect Chemiluminescence Detection*

Indirect CL detection has more recently been used for capillary electrophoresis. In this technique, a stable background chemiluminescent signal is generated which is catalyzed by a probe ion present in the BGE. When this ion is displaced by a migrating sample ion or when complexation of the probe ion reduces the free probe ion concentration, a decrease in the background signal is observed. Indirect CL detection has been applied to the study of metal ions (Ren and Huang, 2001), catecholamines (Zhang *et al*., 1998) and monamines (Tsai and Whang, 1999) amongst other analytes.

1.2.3. Conclusions

On-column concentration techniques provide a simple and inexpensive means of improving concentration sensitivity in capillary electrophoresis. The method used must be suitable for the analyte involved, however, and the concentration factor achieved is dependent on the type of stacking employed. The curved bare-fused silica capillaries, which are most commonly used in capillary electrophoresis, are not optimal for UV absorbance detection. Their limited optical pathlengths are detrimental to CE-UV concentration sensitivity. Alternative capillaries are becoming commercially available and do provide increased sensitivity over conventional capillaries. (For example, Agilent Technologies[a] currently offer a high-sensitivity cell incorporating a fused-silica cell body and removable capillaries, while MicroSolv[b] offers square capillaries). These new capillaries are limited, however, by their increased price over conventional capillaries and by their often more fragile structures. A third way of enhancing concentration sensitivity is to use an alternative means of detection. These methods can yield excellent sensitivity but

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[a] Agilent Technologies. http://we.home.agilent.com

[b] MicroSolv Technology Corporation. http://home.skyweb.net/cori/microsolv

the current high cost of instrumentation can be a deterrent Strategies to improve concentration sensitivity for capillary electrophoresis have been reviewed by Hempel (2000)

1.3. *IN VITRO* METHODS FOR DRUG METABOLISM

The study of drug metabolism can provide valuable information about the biotransformation of drugs It is important that the biotransformation of drugs can be monitored as the metabolic pathway followed can determine whether a drug shows any pharmacological or toxicological activity (Pratt and Taylor, 1990) Information such as kinetic factors and inhibition constants, as well as information regarding the specific enzyme isoforms involved can also be determined from metabolic data (Gibson and Skett, 1994, Timbrell, 1991)

1.3.1. Biotransformation

The biotransformation of a toxic foreign compound is a major mechanism for drug elimination The metabolites produced by biotransformation are generally more polar and water soluble than the parent compound and are thus more readily excretable Their subsequent elimination prevents the build-up of these foreign compounds to toxic levels in the body Biotransformation does not necessarily detoxify the substance, however, as changing the structure of a compound to facilitate its removal from the body may produce a metabolite that is more toxic than the original parent compound Most drugs undergo several biotransformation reactions, which are catalysed by various enzymes The metabolic pathway of a compound is dependent on its structure, and these enzymes usually catalyse the metabolism of endogenous substances of similar structure such as steroids, fatty acids and prostaglandins for example (Pratt and Taylor, 1990) Drug metabolism can occur in most tissues and organs in the body, with the liver being the most

prolific (Remmer, 1970) Inside the cell itself, metabolic reactions occur in the endoplasmic reticulum (ER), the mitochondria and the cytosol The cytochrome P450 (CYP 450) monooxygenase system, which occurs in the ER, is the most important of the metabolic enzyme systems

Biotransformation reactions may be divided into two types

1.3.1.1 Phase I Reactions

The most important phase I reactions are oxidation, reduction and hydrolysis reactions Phase I reactions usually involve the addition of a functional group to the compound which can then undergo a phase II reaction The monooxygenase CYP 450 system, which is responsible for the hydroxylation of coumarin, is the most important system for phase I reactions (Gibson and Skett, 1994, Timbrell, 1991)

1 3 1 1 1 Monooxygenase System

A microsomal mixed-function oxidase reaction, catalysed by CYP 450, is responsible for the catalysis of the hydroxylation of hundreds of exogenous and endogenous substances in phase I metabolic reactions This monooxygenase system is located in the smooth ER of tissue cells, particularly the liver, and requires the presence of the nicotine adenine dinucleotide phosphate (NADPH) co-factor, magnesium ions and molecular oxygen for the reaction to occur The main steps of the reaction are illustrated schematically in Figure 1 15

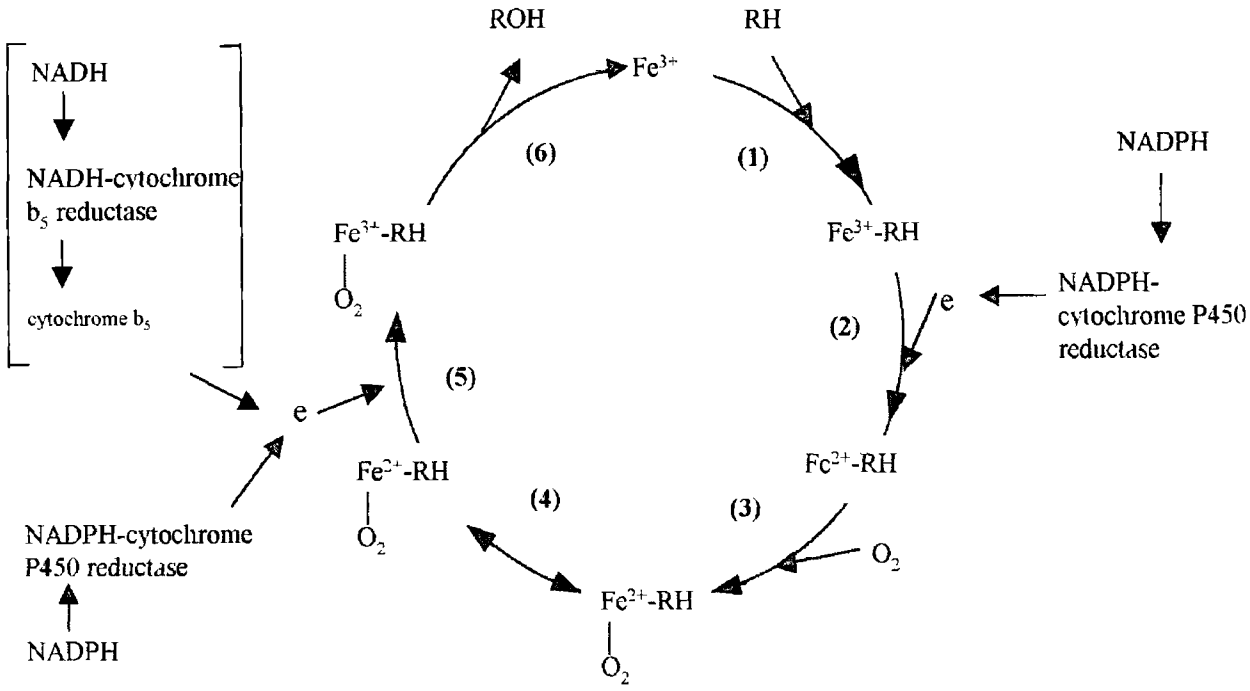

Figure 1 15 Mixed function oxidase system
Reproduced from Gibson and Skett (1994)

The principal stages of the reaction are as follows

1 The substrate, S, binds to the CYP 450, which has an iron atom at its active site in the oxidised Fe^{3+} form

2 This complex then undergoes reduction The incoming electron is provided by the NADPH, through the NADPH-Cytochrome-P450-reductase intermediary

3 Molecular Oxygen binds to the reduced Fe^{2+} enzyme substrate complex

4 The complex is the further reduced by the gain of a second electron, either via Cytochrome P450 reductase (a) or NADH-Cytochrome-b_5-reductase (b)

5 The resultant complex splits into water, oxidised substrate and the oxidised form of the enzyme

The overall stoichiometric reaction scheme can be written as

Eqn 1 6

$$NADPH + H^{+} + O_2 + SH \longrightarrow NADP^{+} + H_2O + SOH$$

where

SH = the substrate (steroid, fatty acid etc)
SOH = the oxidised substrate product

1 3 1 1 1 1 Components of the Monooxygenase System

The main components of the monooxygenase system are Cytochrome P450, NADPH-Cytochrome–P450-reductase and lipid

1 3 1 1 1 1 1 Cytochrome P450

Cytochrome P450 is a haemoprotein with iron protoporphyrin IX (Figure 1 13) as the prosthetic group The haem is non-covalently bound to the apoprotein The P450 enzyme consists of a family of isoenzymes, and occurs mainly in the membrane of the smooth ER although some are found in the mitochondrial inner membrane (Dixon and Webb, 1979) The P450 superfamily is composed of families and subfamilies of enzymes that are defined by their amino acid sequences The majority of human P450 enzymes have been identified (Nelson, 1999) and whilst all appear to be structurally similar, they are responsible for metabolising substrates of widely differing natures (Graham and Peterson, 1999) The expression of a

particular isoenzyme in a tissue may vary greatly and is related to such factors as species, genetics and environment (MacLeod *et al*, 2000) This can have serious consequences for drug metabolism, as it results in both interspecies and intraspecies variations in metabolic pathways In humans, the capacity of the cytochrome P450 enzyme system varies greatly from one person to another, leading to varying drug responses Genetic polymorphisms are one of the major causes of this interindivual variability, and their implication in drug metabolism has been studied in recent years (Meyer *et al*, 1990, Ritchie *et al*, 1980, Loriot *et al*, 2001, Çok *et al*, 2001, Xu *et al*, 2002, Raunio *et al*, 2001, Ujjin *et al*, 2002) In the monooxygenase system the CYP 450 haemoprotein binds both the oxygen, and the substrate in the mixed function oxidase system The CYP 450 name is derived from the fact that carbon monoxide, which binds with it in its reduced form, inhibits oxidation and exhibits a spectral absorbance maximum at 450 nm (Nelson, 2002) An abundance of review articles on the cytochrome P450 family have been published in recent years (Anzenbacher and Anzenbacherova, 2001, Anonymous, 2000, Snyder, 2000, Shewerta, 2000, Glue and Clement, 1999, Werck-Reichhart and Feyereisen, 2000, Hasler *et al*, 1999, Wrighton *et al*, 1996, Beaune, 1998)

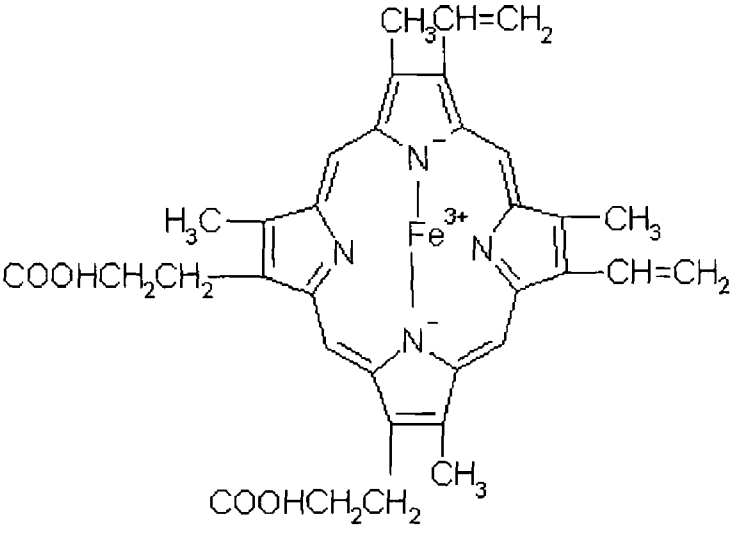

Figure 1 16. *Structure of iron protoporphyrin IX*
Reproduced from Gibson and Skett, 1994

1 3 1 1 1 2 NADPH-Cytochrome–P450-Reductase

NADPH-cytochrome-P450-reductase occurs alongside CYP 450 in the membrane of the smooth endoplasmic reticulum It is an unusual flavoprotein, in that it has both one mole of flavin adenine dinucleotide (FAD) and one mole of flavin mononucleotide (FMN) per mole of apoprotein, where most other flavoproteins have only one of these prosthetic groups NADPH-cytochrome P450 is so named as CYP 450 is the endogenous acceptor of reducing equivalents from the enzyme The flavoprotein is an essential component of the monooxygenase system as it acts as an intermediary electron transfer agent, accepting electrons from NADPH and transferring them sequentially to CYP 450 as required, to activate molecular oxygen for the various P450-catalysed oxidation reactions CYP 450, although in total a two electron acceptor, can only accept one electron at a time and thus the intermediary is necessary to transfer the two electrons from the $NADPH/H^+$ Although the mechanisms by which electrons are transferred is not fully understood, it is believed that FAD acts as the electron acceptor flavin, and FMN as the donor to cytochrome P450 (Gibson and Skett, 1994, Timbrell, 1991, Dixon and Webb, 1979, Nelson, 2002) [In mitochondria, ferrodoxin acts as the transfer agent]

1 3 1 1 1 3 Lipid

A lipid, originally identified as phosphatidylcholine, is also required for the mixed-monooxygenase system, although its role in the reaction remains unclear It is possible that the lipid is required for substrate binding, or in the electron transfer process (Gibson and Skett, 1994)

1.3.1.2. Phase II Reactions

Phase II reactions are generally conjugation reactions and involve the combination of a drug with an endogenous substance In order for this conjugation to proceed,

the drug, or metabolite, must have a functional group such as a hydroxyl group, to serve as an 'active site' for the conjugation This functional group may have been introduced in a phase I reaction (for example, the 7-hydroxylation of coumarin) Energy is also required to transform the endogenous substance to its activated form prior to its conjugation Conjugation is usually with a highly polar substance, such as glucuronic acid, rendering the whole compound more water-soluble If the compound entering the body is already functionalised, for example, phenol, a phase II conjugation reaction can occur immediately without the compound undergoing a phase I reaction Phase II reactions include sulphation, glucuronidation and methylation (Gibson and Skett, 1994, Timbrell, 1991)

1 3 1 2 1 Glucuronidation

Glucuronidation is the most common phase II conjugation reaction and is a major detoxication process in both humans and a large number of other species The glucuronidation reaction involves the formation of uridine diphosphate (UDP)-glucuronic acid from uridine triphosphate (UTP) and glucose-1-phosphate This activated glucuronide donor is then conjugated to the active centre of the drug, forming a glucuronide which is generally water soluble, less toxic than the parent compound, and easily excreted in either bile or urine Drug molecules which partake in the glucuronidation reaction can have a variety of suitable 'active site' functional groups, such as alcohols or phenols, carboxylic acids, thiols or aliphatic amines The conjugation reaction is catalysed by a family of membrane-bound enzymes, present in the endoplasmic reticulum of the cell, known as uridine diphosphate glucuronosyl transferases (UDPGTs) Currently, about 15 UDPGTs have been identified (Fisher *et al*, 2001) These enzymes are found most abundantly in the periportal regions of the liver (Gibson and Skett, 1994, Timbrell, 1991, Dixon and Webb, 1979, Nelson, 2002)

1.3.2. In Vitro Methods

In Vitro methods are a valuable tool in the study of drug metabolism These methods are used primarily as adjuncts, i e to provide information that can be used in conjunction with information gained from *in vivo* studies Information gathered from *in vitro* systems alone can be misleading due to species and strain differences in enzyme expression (Gonzalez, 1992) *In Vitro* methods, however, can provide us with valuable information on aspects such as drug metabolism, protein binding and drug clearance, and combining this information with existing knowledge of body systems can help us predict the effect of certain compounds *in vivo* (Castell and Gomez-Lechon, 1997) *In vitro* methods are also highly useful in that human tissues and organs can be used for carrying out tests that cannot be performed on living human subjects The same test can be performed on tissues or organs from a variety of animal species, providing us with important data on interspecies variations for such aspects as drug metabolism (Gibson and Skett, 1994) The application of *in vitro* models to the study of cytochromes P450 in humans was reviewed by Venkatakrishnan *et al* (2001)

1 3 2 1 In Vitro Systems

In Vitro methods involve the use of animal and human isolated cells, cellular components and tissues, which are maintained outside of the living body under conditions which support their stable existence *In Vitro* systems include cell culture systems, tissue slices and sub-cellular fractions, such as microsomes (Castell and Gomez-Lechon, 1997, Atterwill and Steele, 1987)

The liver is the largest visceral organ in the body, and is a major site of drug and xenobiotic metabolism and elimination (Gillette and Jollow, 1975) The major liver cells are the hepatocytes, comprising approximately 70% of liver cells CYP 450 enzymes are at their highest concentrations in hepatocyte cells, particularly in the

centrolobular region of the liver The remaining 30% of liver cells are sinusoidal cells, including fat-storage cells, Kupffer cells and endothelial cells Biotransformation can also occur in the Kupffer and endothelial cells as they contain CYP 450 proteins and peroxidases (Rudman, 1990) *In vitro* methods are generally highly simplified models of *in vivo* systems and thus the results obtained are not always directly comparable with living systems Differences between *in vitro* and *in vivo* systems can arise from factors such as administered dosages, which can be of unrealistically high concentrations in non-living models (Castell and Gomez-Lechon, 1997) Street *et al*, 1996, cloned the human CYP 450 2A6cDNA into Chinese hamster lung fibroplasts This type of cell system could overcome the difficulty of extrapolation of results obtained in animal studies to the human system and at the same time overcome the problem of limited availability of authentic human liver samples

1 3 2 2 Types of In Vitro System

1 3 2 2 1 Hepatocytes

Isolated liver parenchymal cells, also known as hepatocytes, are commercially available from a variety of animal species CYP 450 enzymes are present in high concentrations in hepatocytes and thus these isolated cells can be used for the *in vitro* study of metabolism by the CYP 450 enzyme system Hepatocytes are not the only P450-containing cells in the liver, however, and so studies carried out using hepatocytes will not be representative of the organ as a whole (Castell and Gomez-Lechon, 1997) The use of human hepatocyte systems for the *in vitro* study of cytochrome P450 has been reviewed (LeCluyse, 2001)

1 3 2 2 2 Subcellular Fractions

Subcellular fractions have been widely used for *in vitro* metabolism studies for decades and microsomes remain the most commonly used *in vitro* system today (Elkins *et al*, 2000) The microsomal fraction is composed mainly of vesicles formed from the endoplasmic reticulum during cell disruption The protein activity reflected in the microsomes is therefore that of the endoplasmic reticulum (Reid and Leech, 1980) Microsomes are routinely prepared in the laboratory from freshly sacrificed animal liver and can be easily incubated for *in vitro* analysis Microsomes are also readily commercially available from a wide variety of species and are relatively inexpensive in comparison with other *in vitro* systems

1 3 2 2 3 Liver Slices

Liver slices have been used for *in vitro* metabolism studies since the early 1900's and still remain a popular choice for metabolism studies today (Vickers, 1997) Liver slices are useful *in vitro* tools for analysis as they contain all of the cell types of the organ and so the functional capacity of the organ as a whole is retained Liver slices are also prepared in a similar manner, regardless of species- mechanical slicers can rapidly produce uniform slices for study Multiple studies can be carried out on one organ and so liver slices represent an efficient use of tissue (Castell and Gomez-Lechon, 1997)

1 3 2 2 4 Incubations

Incubations are typically carried out by maintaining the metabolism components, in a glass vial, (enzyme, substrate and necessary co-factors) at 37°C for the duration of the experiment The components are usually heated in the vial on a temperature-controlled water-bath or heating block Aliquots are taken from the metabolic mixture and analysed at timed intervals throughout the study (Burdon and Van Knippenberg, 1991)

1.3.3. Coumarin

Coumarm (1,2 benzopyrone) is an ideal probe drug for carrying out these metabolism studies as it is a simple organic substance that is metabolised at several sites, forming a variety of products Thus it serves as a model for the metabolism of more complicated structures (Williams, 1959) By monitoring the metabolism of coumarin, information on the activity of the CYP 450 isoform can be determined, along with toxicological information on coumarin itself

1 3 3.1 Structure and Occurrence

Coumarin is a naturally occurring constituent of many plant families and essential oils It is a typical secondary metabolite of higher plants and has only rarely been isolated from micro-organisms Coumarin itself is the parent compound of a large group of compounds known as benzopyrones These benzopyrones consist of a benzene ring joined to a pyrone ring and coumarins themselves are benzo-α-pyrones, with the alpha prefix referring to the position of the oxygen atom in the pyrone ring (Bogan, 1996) The structure of coumarin is shown in Figure 1 17

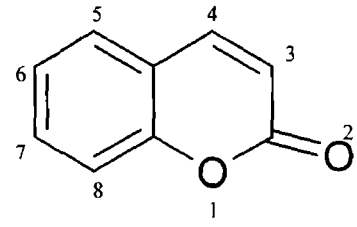

Figure 1.17 *The structure of coumarin*

1.3.3.2 Nomenclature

Coumarins usually have common names (generally related to their source) as well as IUPAC names, both of which are widely used Other names for coumarin include 1,2 benzopyrone, cis-o-coumaric acid lactone, 2H-chromen-2-one and tonka bean camphor (Egan *et al* , 1990)

1 3 3 3 Importance of Coumarin

Coumarin was widely used as a flavouring agent in foods until 1953, when a private pharmaceutical laboratory in the USA published the results of their studies revealing hepatotoxicity in rats and dogs (U S National Toxicology Program) Foodstuffs containing coumarin were legally termed 'adulterated' and food products containing coumarin were removed from the commercial market More recent studies conducted by the U S National Toxicology Program have also raised concerns about the safety of coumarin (Yourick and Bronough, 1997) UK authorities responded with the recommendation of a similar withdrawal in 1965 (Food Standards Committee, 1965) Coumarin is still widely used[c] however in the preparation of perfumes, soaps, toothpastes and cosmetics to neutralise disagreeable odours (Moran *et al*, 1987) Important derivatives of coumarin which have widespread industrial use include dicoumarol (prevents blood clotting), warfarin (a

[c] The use of coumarin in the perfume industry amounted to approximately 250,000 lb per annum in the 1970's (Opdyke, 1974)

rat poison) and umbelliferone (highly conjugated, used in sun-tan lotions) The potential risks of human exposure to coumarin were reviewed by Lake in 1999 (Lake, 1999), who concluded that the potential human risks associated with coumarin exposure were within acceptable limits In recent years much of the research on coumarin has centred on its use in the treatment of a wide variety of diseases These include high protein oedemas, brucellosis and certain types of cancer (Casley-Smith and Casley-Smith, 1997, Creagh *et al*, 1991) It has been shown *in vitro* that coumarin activates macrophages, giving rise to an increased immune response in the body It is believed that this action has an inhibitory effect on the induction of cancer

1.3.3 4 Metabolism

Coumarin is rapidly metabolised in the human liver via the CYP 450 system (specific P450 isoform – CYP2A6) The CYP 2A6 isoform has been well documented and was reviewed by Pelkonen *et al* in 2000 The primary urinary metabolite (approximately 60 – 90% of administered dosage) is 7-hydroxycoumarin (7-HC) (umbelliferone) (Williams, 1959) The majority of the 7-HC excreted is in the glucuronide form with only 2% excreted as free 7-HC (Sharifi, 1993) Hydroxylation at the C-3 position is also common 3-Hydroxycoumarin (3-HC) can then be further metabolised, non-enzymatically, to form ring-opened metabolites Figure 1 18 outlines the principal metabolic fate of coumarin in man Coumarin is largely metabolised to 7-HC in a phase I reaction via the monooxygenase CYP 450 system

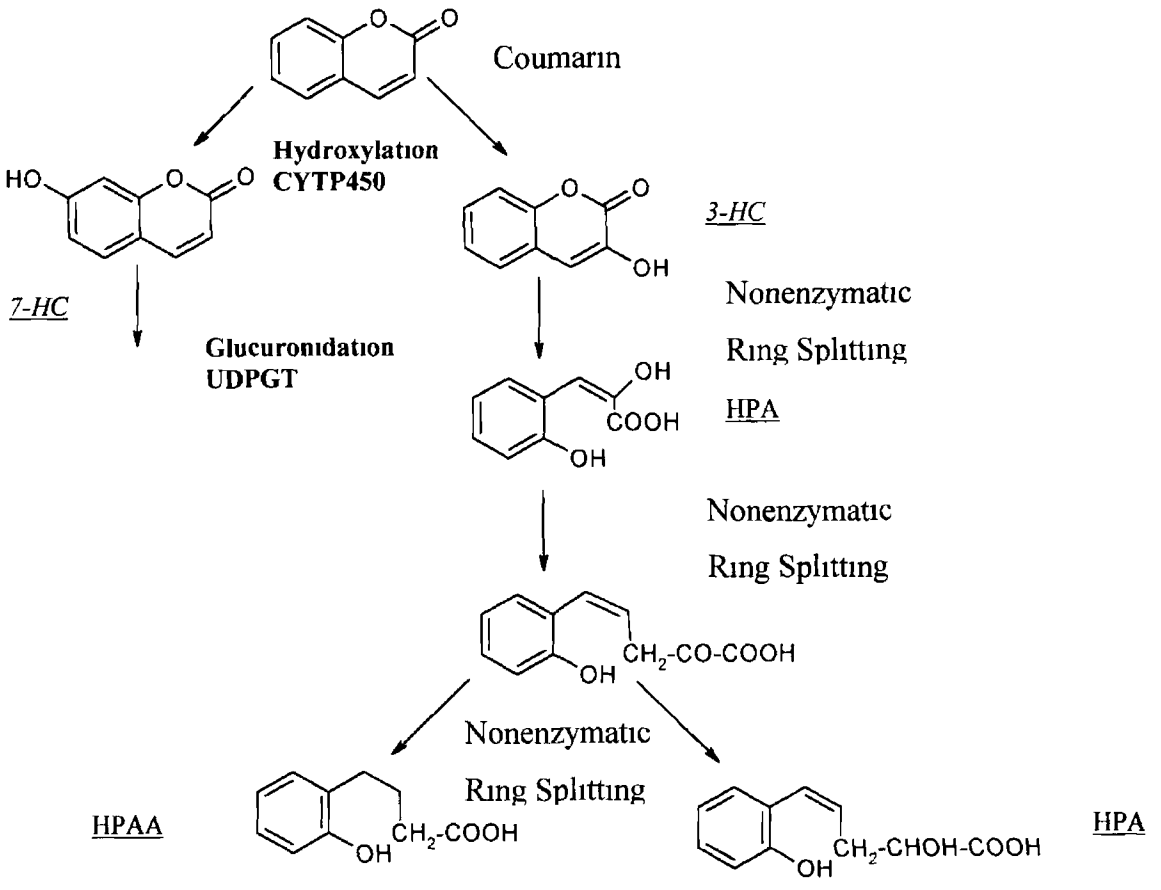

Figure 1 18. *The principal metabolic pathway of coumarin in humans*
Reproduced From Egan *et al* (1990)

This metabolite can then undergo a phase II conjugation reaction with UDP-glucuronic acid to yield 7-hydroxycoumarin-glucuronide (7-HC-G) Another phase I metabolite, 3-HC, undergoes ring-splitting to yield *o*-hydroxyphenylacetic acid (HPAA) and *o*-hydroxyphenyllactic acid (HPA) 4-Hydroxycoumarm (4-HC) and 6-Hydroxycoumarin (6-HC) are also formed to a lesser extent in humans

1 3 3 3 1 Interspecies Variations

The toxicology of coumarin first came to the fore in the mid-1950s, when hepatotoxic effects were demonstrated in both rats and dogs after the administration

of coumarin This evidence led to the ban imposed on coumarin for use in foodstuffs in the U S A It has been argued in more recent years, however, that as coumarin-induced hepatotoxicity is thought to be metabolism-mediated and that coumarin is metabolised quite differently in man (relative to rats and dogs) that a more suitable animal model for coumarin metabolism could yet be found

Toxicity, as a direct result of coumarin metabolism, has been attributed to the 3,4-epoxide intermediate formed during 3-hydroxylation of coumarin (Fentem and Fry, 1993) More recent studies have shown, however, that coumarin 3,4-epoxide and 3-HC are produced via distinct metabolic pathways (Born *et al*, 2002) and that it is the coumarin 3,4-epoxide species and its rearrangement product which give rise to the toxic effect Born *et al* (2000) have demonstrated that it is unlikely that humans would produce significant levels of this toxic epoxide species after low-level administration of coumarin In the rat, in which coumarin exhibits hepatotoxic effects, the 3-hydroxylation pathway is prominent with extensive degradation to HPAA

It is believed that the hepatotoxicity of coumarin is less marked in species that metabolise coumarin by a similar route to man (Cohen, 1979) In primates, as in humans, coumarin is predominantly hydroxylated at the carbon 7 position Large interindividual variability has been reported in the metabolism of coumarin, however, and in some cases the 3-HC pathway seemed to be of equal importance to the 7-HC pathway It has been postulated that 7-HC is the major metabolic fate of coumarin at low substrate concentrations but that the 3-HC pathway becomes increasingly prevalent as the coumarin concentration increases (Van Iersel *et al*, 1994) This is worrying in the light of increased use of coumarin for therapeutic purposes In the Cynomologus monkey, 3-HC and 4-HC are both minor metabolites of coumarin, as in humans Thus the Cynomologus monkey has been suggested as a suitable animal model for coumarin metabolism studies (Bogan *et al*, 1996a) Interspecies studies on coumarin metabolism, using microsomes, have compared metabolism for the human and monkey (Li *et al*, 1997), rat, gerbil and human

(Fentem and Fry, 1992), rat, Syrian hamster, gerbil and human (Lake *et al*, 1992) and human, dog, cat and horse (Chauret *et al*, 1997) The metabolites of coumarin that have been detected in various species are illustrated in Table 1 5

Table 1.5 *Oxidative metabolic pathways of coumarin in different species*

Species	*7-HC*	*Ring-Opening Metabolites*	*3-HC*	*4-HC*	*5-HC*	*6-HC*	*8-HC*	*6,7-DiHC*
Human	79	4	+	(+)	nd	(+)	nd	nd
Baboon	60							
Cynomologus Monkey	(++)	(+)	(+)					
Cat	19							
Dog	3							
Ferret	1		+		+	+	+	
Guinea-Pig	1	(+)	+	(+)	+	+	+	
Hamster	5							
Mouse	3	(+)	+	(+)	+	+		
Pig	12							
Rabbit	12	23	22	0 6	0 4	3	2	+
Rat	0 4	20	2	0 3	+	0 3	0 4	+
Squirrel Monkey	1	(++)						

Blank space indicates that no studies are available

Parentheses indicate that results have been obtained from studies on liver slices, hepatocytes or liver microsomes

Reproduced from Pelkonen *et al* (1997)

1.3.4. Conclusions

It is vital that the biotransformation of drugs, particularly those that are widely used for therapeutic purposes, can be monitored efficiently *In vitro* methods can be used to carry out a wide variety of metabolism studies, routinely and for a wide variety of species (Friedberg, 2000, Wrighton *et al*, 1993, Wrighton *et al*, 1995) Coumarin is an ideal probe for these studies as it serves as a simple model for more complicated metabolic processes

1.4. TECHNIQUES EMPLOYED IN THE ANALYSIS OF COUMARINS

1.4.1. Introduction

Although many techniques have been applied to the analysis of coumarin and its metabolites over the years, it is the chromatographic methods of analysis that have emerged as the most common means of coumarin analysis It is thus these chromatographic techniques that are discussed in detail in the section below and are summarised in Table 1 6 Other techniques that have been used include spectrofluorimetric (Izquierdo *et al*, 2000, Creaven *et al*, 1965, Gramond and Paris, 1971, Tan *et al*, 1976, Egan and O'Kennedy, 1993), immunoanalytical (Egan *et al*, 1991, Deasy *et al*,1994, Stocklein *et al*, 1995, Raunio *et al*, 1988, Dempsey *et al*, 1993) and electroanalytical methods (Dempsey *et al*, 1993, Carrazon *et al*, 1989, Wu and Dewald, 2001)

1 4 1 1 Thin-Layer Chromatography

Thin layer chromatography (TLC) is based upon the partitioning of solutes between a mobile carrier phase and a stationary phase (typically silica coated) Mobile phases for TLC are usually organic based The analyte is typically applied to the thin-layer plate and separation occurs as the components migrate along the solid surface, interacting with the mobile phase TLC has been applied to the analysis of *in vivo* metabolism of coumarin, 7-HC in urine and coumarin in flavourings The *in vivo* studies of 7-HC and 7-methoxycoumarin (7-MC) metabolism were based upon the separation of the principal metabolites of coumarin (7-HC, 7-MC, 6,7-diHC and 3,4-dihydro-7-hydroxycoumarin) after oral and intraperitoneal administration in rats (Indahl and Scheline, 1971) The metabolites were observed after separation by fluorescence at 254 nm 7-HC has also been detected by TLC after *in vivo* metabolism of coumarin (Cholerton *et al*, 1992) Urine samples were extracted into

chloroform after the metabolism reaction and spotted onto the TLC plates Results were detected using fluorescence densitometry TLC has also been applied to the analysis (both qualitative and quantitative) of coumarin in vanilla flavourings In this research coumarin was separated from other vanilla extracts and visualised under UV light after separation (Poole *et al* , 1993)

1 4 1 2 High-Performance Liquid Chromatography

HPLC is a commonly used separations technique, which is based on the partitioning of a solute between the mobile and stationary phases of a liquid chromatographic column Separation of analytes is due to the unique affinity of the analyte for the stationary phase Many of the metabolites of coumarin have been separated by HPLC analysis These methods have utilised both *in vivo* and *in vitro* sources and coumarin metabolites have been analysed in flavourings (Mazza, 1984), plant extracts (Nykolov *et al* , 1993), metabolic mixtures involving liver homogenates (Killard *et al* , 1996) and microsomes from various species (Lake *et al* , 1992, Fentem and Fry, 1992) as well as in urine and plasma (Bogan and O'Kennedy, 1996) Some of this research is outlined below

1 4 1 2 1 In vivo HPLC methods

Bogan *et al* (1996) employed HPLC for the simultaneous analysis of coumarin, 7-HC and 7-HC-G in human urine and plasma The three compounds were determined in serum after administration of 7-HC and in plasma after administration of coumarin to healthy volunteers The method developed involved reversed-phase HPLC on a C_{18} column 4-HC was employed as an internal standard and samples required a pre-treatment step prior to analysis Typical run times were of the order of twenty-one minutes The limits of detection (LOD) achieved were 50 ng/ml for

7-HC and 200 ng/ml for coumarin and 7-HC-G The linear range of the method was 0 5 – 100 μg/ml

1 4 1 2 2 In Vitro HPLC methods

In 1996, Killard *et al* analysed the phase II metabolism of coumarin by HPLC analysis of bovine liver homogenate incubations Once again, 4-HC was employed as the internal standard This method had a run time of twenty minutes and sample treatment was necessary before injection The limit of quantitation (LOQ) for this method was quoted as 1 47 μM Lake *et al* (1992) used a HPLC method capable of separating coumarin from fourteen of its known metabolites (not simultaneously) and applied it to the *in vitro* metabolism of coumarin by hepatic microsomes from the rat, Syrian hamster, gerbil and humans Retention times for this method were up to sixty minutes long and sample pre-treatment was required before analysis This method was subsequently applied by Van Iersel *et al* in 1994 to the metabolism of coumarin in a panel of twelve human liver microsomal samples Nine polar metabolites were detected in this study and interindividual differences in metabolism were reported Fentem and Fry (1992) suggested the gerbil as a suitable animal model after analysis of coumarin metabolism by rat, gerbil and human liver microsomes The reversed-phase HPLC assay was capable of separating coumarin from ten known metabolites Retention times were up to twenty-four minutes long and samples were treated prior to analysis

1.4.2. Capillary Electrophoresis

Capillary Electrophoresis has been discussed in detail in Section 1

1 4 2 1 CE and the Analysis of Coumarins

Capillary Electrophoresis has been applied to the determination of coumarin and its derivatives in a variety of matrices including plant extracts (Ochocka *et al*, 1995), urine and serum (Bogan *et al*, 1995, Bogan *et al*, 1996) and microsomal incubation mixtures (Duffy and O'Kennedy, 1998, Bogan *et al*, 1996b, Deasy *et al*, 1995)

1 4 2 1 1 In Vivo CE Studies

In vivo studies carried out involved the determination of coumarin metabolites after oral administration of coumarin Bogan *et al* (1995) determined free and total 7-HC in urine and serum An extraction step was necessary before injection to remove endogenous species that interfered with detection of the metabolites The 7-HC was then treated with β-glucuronidase to liberate it to its unconjugated form prior to analysis The LOQ for this CE method was 1 µg/ml, while the linear detection range was 0 to 50 µg/ml Analysis time was ninety seconds Urine was then analysed by HPLC for comparison No statistical difference was noted between the results but the HPLC method required a twelve minute run time Subsequently, Bogan *et al* (1996) described a method for the analysis of 7-HC and 7-HC-G in urine This method did not require the sample clean-up step and as both phase I and phase II metabolites could be separated in one analysis, the deconjugation step was no longer necessary The LOQ for 7-HC was 2 µg/ml and 5 µg/ml for 7-HC-G Typical run times were under eight minutes

1 4 2 1 2 In Vitro CE Studies

In vitro metabolism studies have been carried out using liver slices, microsomes, and tissue homogenates from various animal species Duffy and O'Kennedy in 1998 performed incubations of mouse liver slices, separating the major phase I and phase

II metabolites No sample pre-treatment step was required and 7-HC, 7-HC-G and 7-hydroxycoumarin sulphate (7-HC-S) were separated simultaneously in six minutes LODs were 2 μg/ml for 7-HC-G, 0 5 μg/ml for 7-HC-S and 1 μg/ml for 7-HC Bogan *et al* (1996b) in unpublished data, developed a method for the determination of 7-HC and 7-HC-G after incubation with rabbit tissue homogenates (liver, kidney, heart, lung, spleen, large intestine and fat) Separation with this method was achieved in four minutes, but an extraction step was required prior to injection to remove endogenous interferants The β-glucuronidase activity for each organ was then determined LODs for this method were 1 μg/ml for 7-HC and 2 μg/ml for 7-HC-G Deasy *et al* (1995) performed separation of 7-HC from its incubation cofactors after incubation with human liver microsomes This method did not require an extraction step and had a run time of only three and a half minutes The LOD for 7-HC was 1 μg/ml The $NADP^+$/NADPH cofactor reactions involved in coumarin metabolism were also investigated A similar method was later applied to microsomal incubations of seven other animal species (Van Iersel *et al* , 1994) Bovine, gerbil, mouse, rat, rabbit, porcine, Cynomologus monkey and human liver microsomes were incubated and the resultant 7-HC was separated by capillary electrophoresis Bogan *et al* (1996a) found that the Cynomologus monkey was a suitable animal model for coumarin metabolism in humans, with 7-HC being produced in similar concentrations in both species These two methods provided a rapid means of determining the major phase I metabolite of coumarin Other metabolites were not separated by these methods and were not investigated A separation method for seven potential metabolites of coumarin has been published (Tegtmeier, 2000) however this method was not applied to incubations of coumarin and did not include incubation components in the separation study Limits of detection of this separation method were 1 mg/ml

Table 1.6 *Chromatographic methods of analysis of coumarins*

Method and Application	*Mode of Detection*	*Limit of Detection*	*References*
Thin-Layer Chromatography			
In vivo 7-HC metabolism	Colorimetric Fluorescence Fluorescence	-------- 1 ng/ml 5 ng	Johansen (1965) Cholerton *et al* (1992) Indahl and Scheline (1971) Sherma *et al* (1987)
Vanilla flavouring	UV absorbance Radiochemical	500 ng (Coumarin)	Poole *et al*, (1993)
High-Performance Liquid Chromatography			
In vivo coumarin metabolism	UV absorbance	500 ng/ml (7-HC) 0 3ng/ml, 50 ng/ml (7-HC) 0 6 ng/ml, 200 ng/ml (7-HC-G)	Moran *et al* (1987) Egan and O'Kennedy, (1992) Sharifi *et al* (1993) Bogan and O'Kennedy (1997) Sharifi *et al* (1993) Bogan and O'Kennedy (1997)
In Vitro metabolism	Radiochemical		Fentem *et al* (1991) Lake *et al* (1992) Van Iersel *et al* (1994) Steensma *et al* (1994)
Coumarin in foodstuffs/ plant extracts	UV absorbance	0 6 ng/ml	Nykolov *et al* (1993) Archer (1988)
Gas Chromatography			
Coumarin	Mass Spec	7 7 ng/ml	Hawthorne *et al* (1988)
Coumarin in flavours/fragrances	FTIR		Grundschober
Tobacco	FID/mass spec		Christakopoulos *et al* (1992)
Capillary Electrophoresis			
In vivo metabolism of coumarin	UV absorbance	1 µg/ml	Bogan *et al* (1995) Bogan *et al* (1996)
In Vitro metabolism of coumarin and 7-hydroxycoumarin	UV absorbance	1 µg/ml	Bogan *et al* (1996a) Deasy *et al* (1995)

Reproduced from Bogan *et al* (1997)

1.4.3. Conclusions and Comparison of Methods

There are several advantages to choosing CE over HPLC methods for the analysis of coumarin metabolites HPLC columns are typically expensive and substances such as proteins, present in metabolic mixtures, must be removed before injection to avoid fouling the column The precipitation step is not necessary when employing CE for analysis The BGE can be changed easily between runs if necessary, preventing damage to the column, and so metabolic mixtures can be injected directly onto the capillary without the need for prior protein removal Another advantage of using CE for the analysis of metabolic data is that the high voltage applied across the capillary effectively separates the components of the incubation mix, terminating the reaction This eliminates the need to stop the reaction by a separate step, such as the addition of acid to the mixture Typical analysis times in CE are also shorter when compared with HPLC separations Two studies have analysed coumarin incubation mixtures by HPLC as well as by CE (Bogan *et al*, 1995, Deasy *et al*, 1995) In both studies, no statistical differences were found between the results obtained but the CE analysis times were considerably shorter in both instances when CE was performed A disadvantage of CE, however, is its poor concentration sensitivity This can lead to problems with the detection of minor metabolites, which are typically formed in low concentrations Sample concentration techniques could be employed to overcome this limitation, however, allowing for the rapid, sensitive determination of analytes in physiological samples The following research chapters will aim to develop improved methods for the analysis of drug metabolism Techniques investugated will include sample stacking techniques for improved sensitivity and on-capillary methods for automated analysis In addition, studies will be carried out into the mode of action of pH-mediated stacking and its application to amino acids

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CHAPTER TWO

APPLICATION OF PH-MEDIATED SAMPLE STACKING TO THE ANALYSIS OF COUMARIN METABOLITES IN MICROSOMAL INCUBATIONS

2.1. INTRODUCTION

Traditionally, HPLC has been used to monitor the metabolism of coumarin (Killard *et al*, 1996, Lake *et al*, 1992, Van Iersel *et al*, 1994 and Fentem and Fry, 1992), although more recent research has employed CE (Duffy *et al*, 1998, Deasy *et al*, 1995, Bogan *et al*, 1995) CE is a versatile analytical separations technique, which has gained popularity over the last decade There are several advantages to choosing CE over HPLC for the analysis of metabolic reactions Some of these advantages have been discussed previously in Chapter 1 CE does have limitations, however, and its poor concentration sensitivity when combined with UV absorbance detection is arguably its biggest disadvantage This limitation is particularly obvious when CE-UV is faced with the detection of low concentration analytes, such as metabolites in metabolic mixtures Sample stacking techniques have become increasingly popular over the last decade as a means to overcome this limitation and to improve limits of detection for CE The various modes of sample stacking have been discussed in detail in Chapter 1

On-column concentration techniques are an attractive means of concentration enhancement as they allow rapid separations to be carried out, conveniently, and without the need for costly instrumentation Samples can still be injected directly onto the capillary without the need for pre-treatment steps, and for many of the stacking techniques, analysis times are not significantly increased Not all concentration enhancement techniques are suitable for use with physiological samples, however These typically high ionic strength samples must often undergo extraction, or dilution before on-column concentration techniques can be applied This can seriously undermine the effectiveness of the method, as the concentration factor achieved is often inadequate compensation for the increased sample handling and is in any case often offset by the dilution factor required

pH–mediated sample stacking is an on-column concentration technique that can be used to overcome these limitations for physiological samples The theory behind

base-mediated stacking has been explained in Chapter 1 Briefly, the BGE is composed of the salt of a weak base, such as ammonia After the sample injection, a plug of strong base, such as sodium hydroxide, is injected Neutralization of the sample zone occurs, creating a region of low conductivity across which the analytes stack, leading to concentration factors of up to 300-fold (Zhao and Lunte, 1999) Previous research has utilised Ringer's solution as the sample matrix for pH-mediated techniques Ringer's solution is prepared from sodium, potassium and calcium chlorides at physiological levels and is commonly used in the preparation of metabolic samples as it mimics physiological conditions (Hadwiger *et al*, 1996) For this study it was desired that metabolites of coumarin could be detected in phosphate buffer, the matrix of the *in vitro* incubation mix This would allow metabolic mixtures to be injected directly onto the capillary and analysed, with sample stacking

In this chapter, pH-mediated sample stacking was performed with the samples prepared in phosphate buffer, and applied to the analysis of the metabolism of coumarin by the cytochrome P450 2A6 isoform, in Cynomologus monkey microsomes

2.2. EXPERIMENTAL

2.2.1. Instrumentation

Separations were carried out on a Beckman P/ACE System MDQ instrument (Fullerton, CA) The capillary employed was polyimide fused silica from Polymicro Technologies (Phoenix, AZ) purchased from Composite Metal supplies (Worcester, England) The incubation mixtures were maintained in a Grant Boeckel BBA heating block during the course of the incubations and centrifugation was performed on a Fisher microcentrifuge, Model 235 C fixed speed centrifuge The pH values of buffer solutions were determined using an EDT microprocessor pH meter, model RE 357

2.2.2. Reagents and Analytes

7-HC, 4-HC, coumarin, methylamine, tetradecyltrimethyl-ammonium bromide (TTAB), β-nicotinamide adenine dinucleotide phosphate ($NADP^+$) and D-glucose-6-phosphate (dipotassium salt) were purchased from Sigma (St Louis, MO) 2-hydroxyphenylacetic acid (HPAA), potassium dihydrogen phosphate and ammonium hydroxide were purchased from Aldrich Chemical Co (Milwaukee, WI) Glucose-6-phosphate dehydrogenase (from yeast) was purchased from ICN (Costa Mesa, CA) Male Cynomologus monkey microsomes were purchased from In Vitro Technologies Inc (Baltimore, MA) All other chemicals were purchased from Sigma (St Louis, MO)

2.2.3. Preparation of Solutions

The BGE was prepared from 50 mM methylamine and adjusted to pH 10 7 by addition of 1 M ammonium hydroxide In order to reverse the EOF, TTAB was added to the BGE at a concentration of 0 5 mM (Huang *et al*, 1989) 0 1 M stock solutions of 7-HC, 4-HC and HPAA were prepared in pure methanol These standards were then diluted to 10 μM in phosphate buffer (pH 7 5, 25 mM) and in the BGE to prepare standards for injection The standards were prepared in the BGE to provide a control, to compare the peaks of the stacked standard with a case where no band-broadening would be expected to occur $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase solutions were prepared in phosphate buffer (pH 7 5, 25 mM) Nanopure water (18 2 Ω) was used throughout the study

2 2 3 1 EOF Reversal

In a typical CE system, the direction of the EOF is from the anode to the cathode When the magnitude of the EOF is sufficient, this flow ensures that all analytes, regardless of charge, move to the cathode When reverse polarity is employed in the CE system, the intrinsic direction of the EOF must be reversed to ensure that all analytes pass the detector This EOF reversal is performed by the addition of a cationic surfactant, at an appropriate concentration, to the BGE This cationic surfactant adsorbs onto the capillary wall to form a dynamic layer (Figure 2 1) The capillary wall is thus cationic and this reverses the direction of the EOF within the capillary The order of migration is thus also reversed to anions, neutrals, cations Reversal of the EOF is often performed to allow for effective electrokinetic injection of anionic species onto the capillary It is also routinely performed for the analysis of anions However the preferential migration of anions means that anionic species can be detected in shorter analysis times

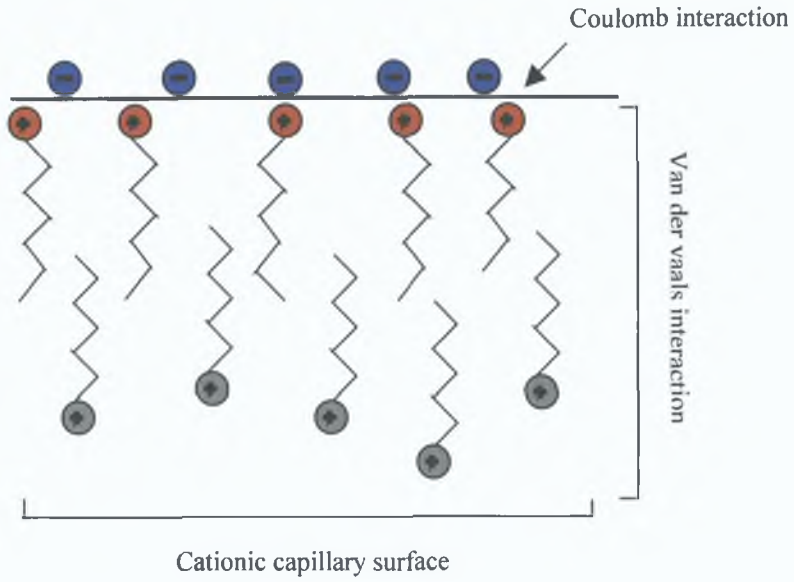


Figure 2.1: *Mechanism of EOF reversal using a cationic surfactant.*
Reproduced from: Khaledi (1998). The surfactant adsorbs onto the capillary wall, forming an outer cationic layer.

2.2.4. Handling of Microsomes

Microsomes were obtained in 500 µl vials from the supplier. These microsomes were thawed as rapidly as possible by holding the vial in water heated to 60 °C. Once defrosted, microsomes were divided into 30 µl aliquots in plastic vials. These aliquots were then frozen at –80 °C for use in the incubation studies. When an incubation was being performed, the aliquot of microsomes was defrosted quickly in water heated to 60°C and once defrosted was instantly added to the incubation mixture. The effects of freezing and thawing microsomes have been discussed by Pearce *et al.* (Pearce *et al.*, 1996).

2.2.5. *Microsomal Incubations*

The incubation procedure followed was similar to that described by Deasy *et al* (1995) The incubations were carried out by mixing 195 µL of coumarin standard (50 µg/mL), 15 µL of $NADP^+$ (20 mM), 10 µL of glucose-6-phosphate (50 mg/mL) and 5 µL of glucose-6-phosphate dehydrogenase (4 U/mL) in a glass vial The concentration of the microsomes used was 20 mg/mL and was indicated by the supplier These concentrations are higher than those required to maintain maximum metabolic rates under the conditions chosen The incubations were initiated by the addition of 25 µL of microsomes to the incubation vial Cynomologus monkey microsomes were chosen for the study for their similarity in metabolic profile of 7-HC to human microsomes (Bogan *et al*, 1996) Male monkey microsomes were used as data from the supplier indicated a higher level of coumarin 7-hydroxylation in the male of the species The vial was maintained at 37 °C in a heating block Aliquots were taken from the mixture at 0, 15, 30, 45, 60, 90 and 120 minute intervals over the period of the incubation and centrifuged for ten minutes at 9455 x g r c f to remove excess protein This protein should not harm the capillary but its removal reduces the risk of clogging of the capillary during the analysis and also allows for more sensitive detection of the analytes of interest A series of controls were carried out for the incubations by substituting, respectively, the coumarin, $NADP^+$ and microsomes in the incubation mixture with phosphate buffer (25 mM, pH 7 5) Metabolites were identified by comparison of the incubation mixtures with the control incubations and by the spiking of the incubation mix with a standard solution of the suspected metabolite

2.2.6. *Capillary Electrophoresis Separation*

The separation capillary was fused silica, 50 µM x 61 2 cm (50 cm to detector) The capillary was initially primed by sequential flushing at 30 °C with methanol (5 minutes), 1 M hydrochloric acid (5 minutes), H_2O, (2 minutes) 0 1 M sodium

hydroxide (NaOH) (5 minutes), H_2O (2 minutes) and the BGE (7 minutes) Between runs the capillary was flushed at 50 p s i for 1 minute with 5 mM sodium dodecyl sulphate (SDS) / 0 1 M NaOH (50 50, v/v), 1 minute with 0 1 M NaOH and 1 minute with the BGE Once the conditions were optimised, samples were introduced onto the capillary by a 45 second electrokinetic injection at –10 kV, followed by a 90 second electrokinetic injection of NaOH at –10 kV for sample stacking The concentration of the NaOH used was 50 mM, to match the concentration of the BGE This had previously been optimised by Zhao (Zhao, 1997) and so was not investigated further Separation was carried out at –20 kV with a rise time of 0 17 minutes

2.2.7. Optimisation of Sample Injection:Base Injection Ratio

A sample injection time of 45 seconds was chosen and base injection times were varied to find the optimal injection ratio 45 seconds was initially chosen as previous research had indicated that an injection ratio of approximately 2 1 tended to be optimal and the maximum injection time allowed by the instrument was 99 seconds Base injection times ranging from 45 seconds to 99 seconds were thus investigated

2.2.8. Optimisation of Sample Injection Time

Once the optimal injection ratio was determined, samples were injected for a range of sample injection times followed by injection of the base at the optimal ratio previously determined

2.2.9. Comparison of Stacked and Unstacked Samples

In order to provide a comparison by which to evaluate the stacking method, samples were prepared in phosphate buffer (25 mM, pH 7 5) and in the BGE In each case, samples were injected without stacking (i e no base injection was performed) Sample injection times were optimised for each of these conditions

2.2.10. Calculation of Peak Efficiency, N.

Peak efficiencies were calculated based on the formula

Eqn. 2 1

$$N = 5\ 54\ (T_i/W_{1/2})^2$$

where

N = efficiency

T_i = Migration time of peak

$W_{1/2}$ = Peak width at half-height

2.3. RESULTS AND DISCUSSION

2.3.1. Development of the Separation Conditions

CE has previously been used to monitor the *in vitro* metabolism of coumarin, both in humans and in other species The electrophoretic methods were based on the separation of the primary metabolite, 7-HC, from the metabolic mixture Limits of detection of 7-HC for these studies were 6 17 μM (1 μg/mL) 7-HC, however, is not the only known metabolite of coumarin in man Various other metabolites, such as

4-HC and ring-opened metabolites, were not included in these previous studies This research aimed to expand the separation of metabolites of coumarin in microsomal incubations with increased sensitivity pH-mediated sample stacking was chosen to perform this

2 3 1 1 Choice of Detection Wavelength

Detection wavelengths of either 214 nm or 320 nm are typically employed for the analysis of coumarin metabolites (Bogan *et al*, 1997) While 214 nm is more sensitive for these analytes, 320 nm is employed when endogenous species, present in urine and serum, co-migrate with the analytes and interfere with the detection As this was not an issue for this separation, 214 nm was chosen as the detection wavelength for this study

2 3 1 2 Structures of the Analytes

The structures of the three analytes of interest in this study are shown in Figures 2 2 to 2 4

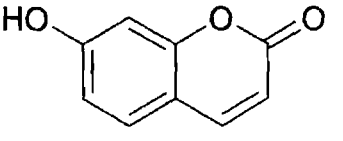


Figure 2 2. *Structure of 7-hydroxycoumarin*

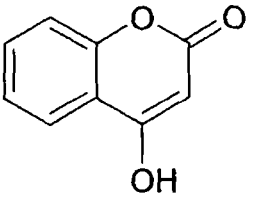


Figure 2 3 *Structure of 4-hydroxycoumarin*

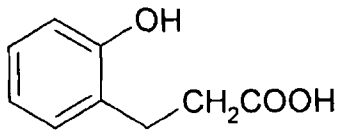


Figure 2.4 *Structure of o-hydroxyphenylacetic acid*

2 3.1.3. Choice of Background Electrolyte

For base-mediated sample stacking to occur the BGE must be composed of the salt of a weak base, such as ammonia (Osbourn *et al* , 2000) A BGE that could be used at a high pH was also necessary as it was considered that at an elevated pH, the hydroxyl groups of the analytes would be deprotonated, facilitating their separation Thus a suitable BGE would have to provide a buffering capacity at a relatively high pH, whilst being suitable for use with base-mediated stacking Three ammonium-based buffers were considered suitable for investigation in this study These buffers were methylamine, ethanolamine and ammonia/ammonium The pK_a values of these buffers are 10 6, 9 5 and 9 25, respectively The pH values studied were all within 1 unit of the pK_a value of the buffer being used Initial separations were performed for these three BGEs at a variety of concentrations and for methylamine at a variety of pH values These conditions are detailed in Table 2 1

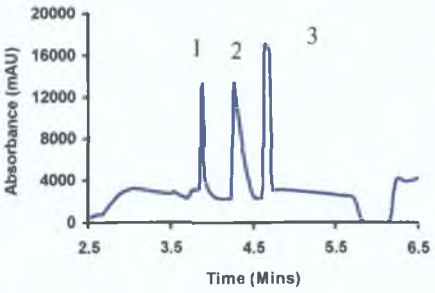
Table 2 1. *Ammonium-based buffers investigated as suitable BGEs*

BGE	pK_a	Concentrations (mM)	pH
Methylamine	10 6	50,100,150	10 5
Methylamine	10 6	50,100,150	10 7
Methylamine	10 6	50,100,150	11 0
NH_3/NH_4^+	9 25	100,300	10 0
Ethanolamine	9 5	50, 150	10 5

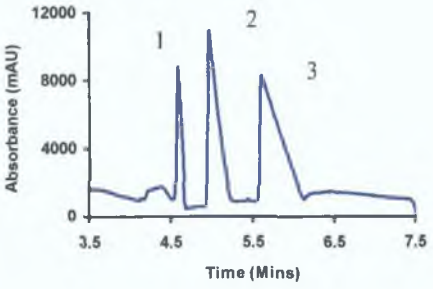
According to Zhao (1997), 'analytes can be stacked completely when the ratio of base to sample injection time is more than 2 1 and the sample injection time is less than 30 seconds' This was used as a starting point for the analysis and separations were carried out with a 25 second injection of the analyte mixture followed by a 50 second injection of base for each of the BGE conditions tabulated above Since the BGE was the condition under investigation, injection conditions were not optimised at this stage The mixture analysed was 100 μM 7-HC, 4-HC and HPAA in phosphate buffer (25 mM, pH 7 5) Samples were introduced electrokinetically at – 10 kV and separation was carried out for a range of voltages from -5 to -30 kV These methods were carried out for each of the buffers listed above In each case the concentration of base matched the concentration of the amine in the BGE Some of the resulting electropherograms can be seen in Figures 2 5 to 2 7 50 mM methylamine pH 10 7 was then chosen as the BGE on the basis of these results

The thirteen electropherograms represent an initial separation of the three analytes of interest under differing BGE conditions From these thirteen electropherograms, only five of the separation conditions resulted in resolution of the three analytes with acceptable peak shapes, namely those shown in Figure 2 5a, Figure 2 5b, Figure 2 5d, Figure 2 5e and Figure 2 6b These figures correspond to buffers 50 mM methylamine, pH 10 5, 50 mM methylamine, pH 10 7, 100 mM methylamine, pH 10 5, 100 mM methylamine, pH 10 7 and 300 mM NH_3/NH_4^+, pH 10 0 respectively 50 mM methylamine, pH 10 5 (Figure 2 5a) suffered from very poor peak shape for the 4-HC species although all three peaks exhibited high absorbance and were separated in under five minutes For the 50 mM methylamine pH 10 7 buffer (Figure 2 5b), peak shapes were slightly improved over the 50 mM methylamine pH 10 5 buffer (Figure 2 5a) although 4-HC peak shape was still poor 100 mM methylamine, pH 10 5 and 100 mM methylamine, pH 10 7 (Figures 2 5d and 2 5e respectively) both show fronting of the HPAA peak, and once again, poor peak shape The 300 mM NH_3/NH_4^+, pH 10 0 BGE (Figure 2 6b), although having improved peak shape for 4-HC, suffers fronting for the 7-HC peak Overall, methylamine at 50 mM and 100 mM and at pH 10 5 and 10 7 proved suitable, but at 150 mM or at pH 11 0 they did not Poor separation was achieved with NH_3/NH_4^+

and ethanolamine buffers under all conditions tested. From these results it was decided to choose the 50 mM methylamine pH 10.7 BGE (Figure 2.5b) for further investigation as all peaks were baseline resolved and of acceptable peak shape, within a reasonable analysis time.



(a)



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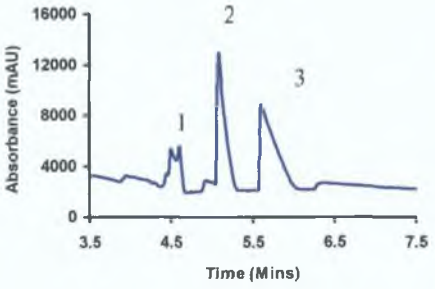

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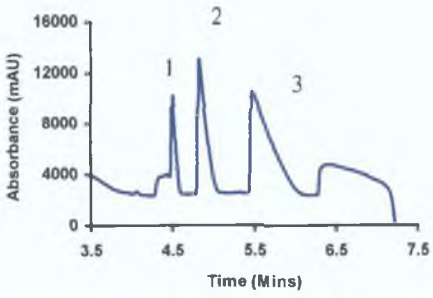
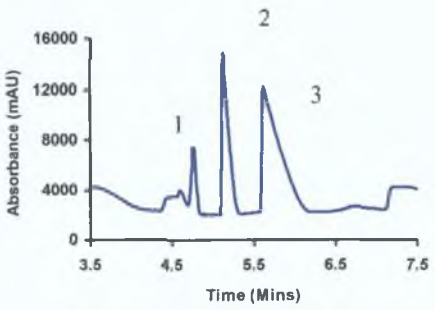


(d)



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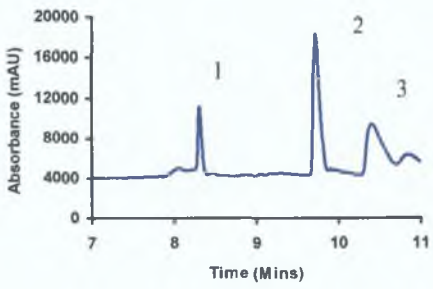


(f)

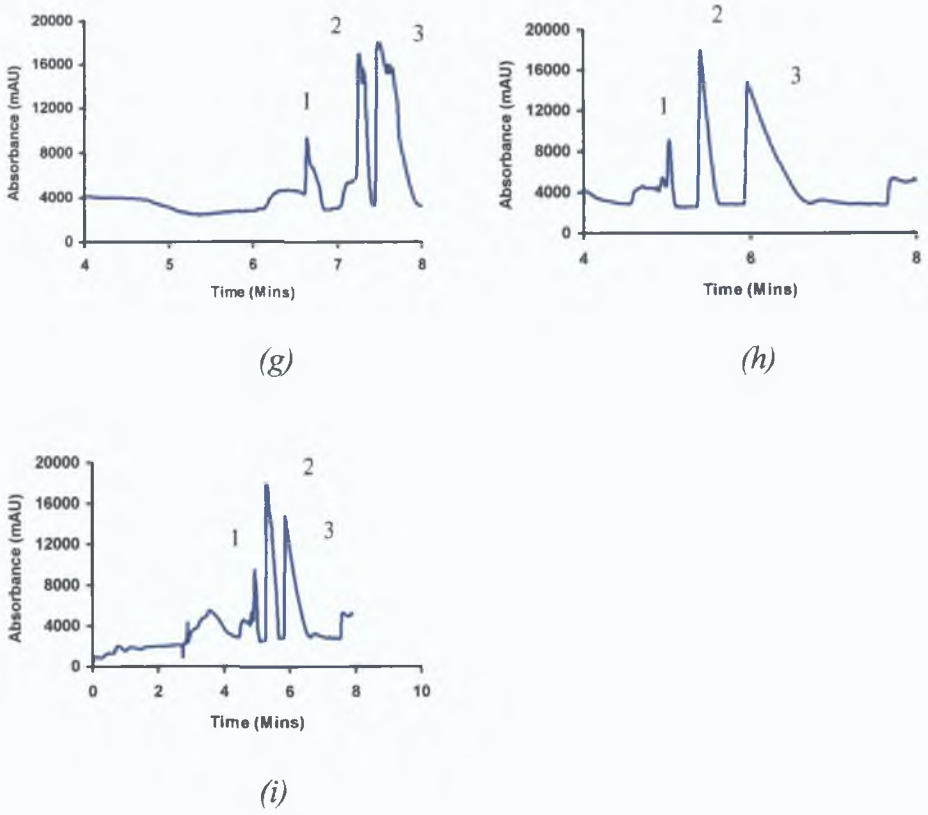
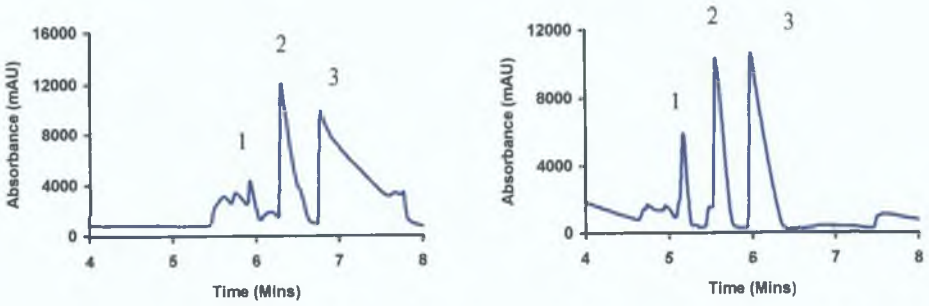
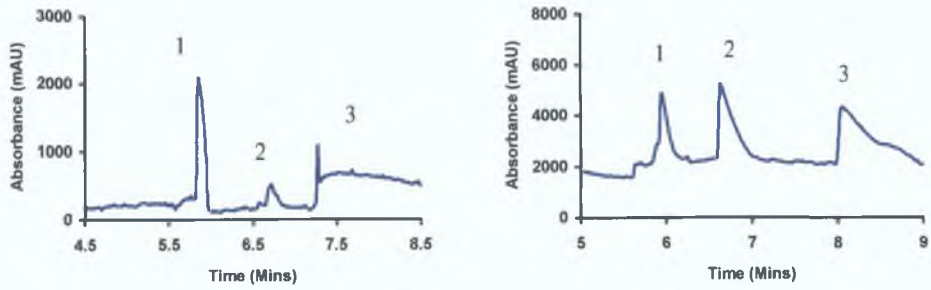


Figure 2.5: *Separation of coumarin metabolites in methylamine BGEs. BGEs are (a) 50 mM methylamine pH 10.5, (b) 50 mM methylamine pH 10.7, (c) 50 mM methylamine pH 11.0, (d) 100 mM methylamine pH 10.5, (e) 100 mM methylamine pH 10.7 and (f) 100 mM methylamine pH 11.0, (g) 150 mM methylamine pH 10.5, (h) 150 mM methylamine pH 10.7 and (i)150 mM methylamine pH 11.0. Analytes are [1] HPAA, [2] 7-HC and [3] 4-HC.*



(a) *(b)*

Figure 2.6: *Separation of coumarin metabolites in ammonium/ammonia BGEs. BGEs are (a) 100 mM NH_3/NH_4^+ pH 10.0. (b) 300 mM NH_3/NH_4^+ pH 10.0. Analytes are [1] HPAA, [2] 7-HC and [3] 4-HC.*



(a) *(b)*

Figure 2.7: *Separation of coumarin metabolites in ethanolamine BGEs. BGEs are (a) 50 mM ethanolamine pH 10.5 and (b) 200 mM ethanolamine pH 10.5. Analytes are [1] HPAA, [2] 7-HC and [3] 4-HC.*

2 3 1 2 1 Ohm's Plot

An Ohm's plot was then carried out to determine the working voltage range of the chosen buffer (Figure 2 18) This plot was linear in the voltage range of 0 to ~-23 kV, indicating that at these voltages, the generated heat is being efficiently dissipated Voltages from -5 to –25 kV were then investigated for the separation – 20 kV yielded the best compromise between resolution and analysis time and so was selected for the method

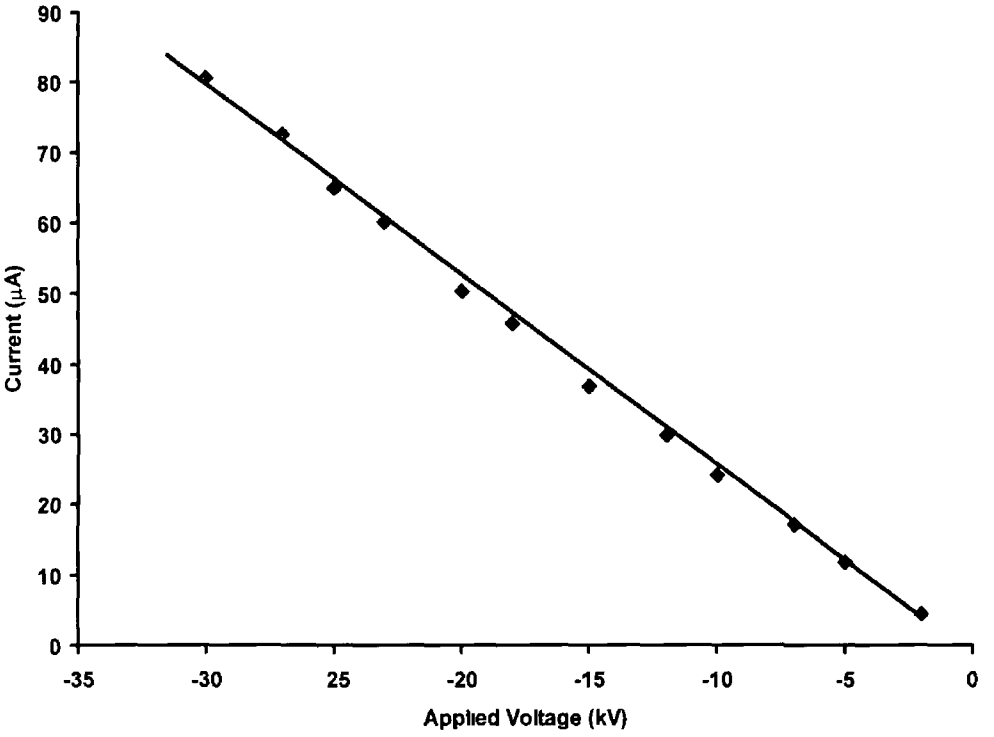


Figure 2 8 *Ohm's law plot for 50 mM methylamine pH 10 7 buffer*

2 3.1 4 Optimised Injection Times

For optimal base-mediated sample stacking to take place, it has been reported that electrokinetic injections must be used (Park and Lunte, 1998) Previous research had utilized injection voltages of –15 kV (on home-built CE systems) but as the available instrument had an upper limit of –10 kV for electrokinetic injection, this

was utilized instead In order to achieve the best possible results for pH-mediated sample stacking, it is essential to ensure that the ratio of the sample injection time to base injection time is fully optimised If the sample injection time is too long relative to the base injection time, the earlier injected portion of the sample zone may not be titrated by the base and will appear as a broad band If, however, the base injection time is too long, too much of the capillary will be taken up by the stacking process, leaving only a short portion of the capillary remaining in which separation can occur This will result in poor resolution for the separation Sample injection parameters were optimised by varying the length of both sample and base injection until optimum conditions were reached (Tables 2 1 and 2 2) As the aim of the research was to develop a more sensitive method for the determination of metabolites, the optimal conditions were taken to be those which yielded the greatest peak heights

As can be seen from the Table 2 2, optimal sensitivity was achieved for all three analytes when the base injection time was double the sample injection time (base injection time of 90 seconds) Peak heights of 1963, 2416 and 2607 mAU for HPAA, 7-HC and 4-HC, respectively were achieved when the 45 second injection of sample was followed by a 90 second injection of base The ratio of 2 1 was thus chosen to be optimal and was employed for further study Once the injection ratio had been determined it was necessary to optimise the sample injection time Using this ratio, samples were injected for a range of times from 5 to 50 seconds 50 seconds was the maximum sample injection time studied, as the maximum injection time allowed by the instrument is 99 seconds Thus for sample injection times above 50 seconds, a base injection at the optimal ratio could not be performed

Table 2 2. *Optimisation of sample base injection ratio Standards were 10 µM prepared in phosphate (pH 7 5, 25 mM) All sample injections were 45 seconds (n = 3)*

Base Inj Time (s)	HPAA Peak Height (mAU)	HPAA Efficiency N (/1000)	7-HC Peak Height (mAU)	7-HC Efficiency N (/1000)	4-HC Peak Height (mAU)	4-HC Efficiency N (/1000)
45	1058 ± 247	69 ± 26	1791 ± 323	79 ± 38	1980 ± 324	13 ± 2
55	1462 ± 33	112 ± 7	2073 ± 89	63 ± 15	2298 ± 87	13 ± 2
65	1655 ± 97	117 ± 12	2169 ± 74	62 ± 16	2331 ± 179	13 ± 2
75	1705 ± 185	107 ± 6	2068 ± 176	44 ± 0 3	2443 ± 139	13 ± 2
85	1673 ± 171	108 ± 18	2225 ± 84	51 ± 8	2523 ± 116	13 ± 2
90	1963 ± 127	106 ± 13	2416 ± 189	48 ± 5	2607 ± 444	11 ± 1
95	1865 ± 179	112 ± 8	2332 ± 92	49 ± 2	2560 ± 102	11 ± 1
99	1954 ± 216	114 ± 17	2232 ± 20	50 ± 3	2315 ± 243	10 ± 1

Table 2 3 *Optimisation of sample injection time Standards were 10 µM prepared in phosphate buffer (pH 7 5, 25 mM) All base injection times were double the respective sample injection time*

Sample Inj Time (s)	HPAA Peak Height (mAU)	HPAA Efficiency N (/1000)	7-HC Peak Height (mAU)	7-HC Efficiency N (/1000)	4-HC Peak Height (mAU)	4-HC Efficiency N (/1000)
5	276 ± 6	90 ± 1	378 ± 9	49 ± 2	338 ± 11	14 ± 1
10	462 ± 8	69 ± 2	578 ± 9	45 ± 0 2	638 ± 10	17 ± 1
15	558 ± 6	77 ± 2	729 ± 16	52 ± 3	697 ± 13	14 ± 3
20	811 ± 13	73 ± 3	1201 ± 30	49 ± 1	1166 ± 9	14 ± 2
25	1053 ± 56	72 ± 4	1399 ± 14	45 ± 3	1728 ± 43	16± 3
30	1194 ± 9	72 ± 5	1698 ± 17	43 ± 3	1957 ± 22	16± 3
35	1308 ± 18	71 ± 4	1819 ± 71	42 ± 2	2045 ± 70	17 ± 2
40	1449 ± 97	66 ± 2	2171 ± 166	39 ± 1	2248 ± 104	17 ± 1
45	1963 ± 128	106 ± 13	2416 ± 189	48 ± 5	2607 ± 444	11± 1
50	1641 ± 18	65 ± 2	2437 ± 15	40 ± 2	2482 ± 78	17 ± 1

From Table 2 3 the optimal injection conditions were determined to be a 45 second injection of sample followed by a 90 second injection of base The highest peak height for 7-HC was achieved for a sample injection time of 50 seconds However, the increase in peak height over the 45 second injection was very small (< 1 %) For the other two analytes, HPAA and 4-HC, the 45 second injection yielded the highest peak heights When the sample injection time was increased above this, i e to 50 seconds, there was a marked decrease in sample heights for these two analytes indicating that the capillary may have been overloaded

2.3.2. Evaluation of the Stacking Method

In order to evaluate the stacking method, as compared to a non-stacking method, a series of separations were carried out without the base injection All other separation conditions were the same as for the developed stacking method The injection time for these unstacked samples was also optimised (Table 2 4) Standards were also prepared in the BGE for comparison (Table 2 5) Samples prepared in the BGE provide a good control against which to compare separations as the mobilities of the sample matrix and the BGE are matched, removing any band-broadening effects which may ensue from a mismatch of these components

Table 2 4 shows the peak heights and efficiencies that were achieved when the developed method was performed without the base injection Samples were injected for 5, 10, 15, 20 and 45 seconds In general, the highest efficiency values were obtained for a sample injection time of 5 seconds For the 10 second injection the efficiency was significantly lower indicating that as more sample was injected on to the capillary it was becoming overloaded This was in contrast to the results for the stacking method for which acceptable efficiency values were obtained even for long injection times (i e > 30 seconds) This was because the sample zone is being compressed during the stacking process, allowing larger samples to be injected onto the capillary before overloading occurred

Results obtained for the samples prepared in the BGE showed an improvement (i e greater peak heights and increased efficiencies) over samples prepared in phosphate when no stacking was performed (Table 2 5) This was due to the removal of deleterious sample matrix effects which can cause band-broadening in the capillary The results obtained were thus as expected As for the samples prepared in phosphate buffer and injected without stacking (Table 2 4) there was a dramatic decrease in separation efficiency when injections are performed for longer than 5 seconds The improvement over the unstacked phosphate samples could be seen in the peak heights and efficiency values which are higher for each sample injection time performed For a 5 second injection, for example, once again using HPAA as the representative analyte, the samples prepared in the BGE showed over a three-fold increase in peak height and an almost three-fold increase in peak efficiency over unstacked phosphate samples

For clarity, comparisons will be drawn for one of the analytes, HPAA The results obtained for HPAA can be considered to be representative of the system as a whole One approach used to calculate the stacking effect was to compare the peak height of the samples at similar efficiency values (Weiss *et al*, 2001) The highest efficiency value obtained for unstacked samples was 29,300 plates, corresponding to a sample injection time of 5 seconds The peak height of HPAA for this injection was 29 mAU The closest efficiency value obtained for stacked samples was 65,000 plates, based on a 50 second injection In fact this was the worst efficiency value obtained for the stacked samples The average peak height m this case was 1,641 mAU This represents a 56-fold increase on the capillary

Another approach used to estimate the stacking effect was to compare the peak height of the samples achieved for the same injection times The peak height achieved for a 45 second sample injection with sample stacking was 1,963 mAU This is in comparison to a peak height of 143 mAU for samples prepared in BGE and of 116 mAU for unstacked samples, representing, respectively, a fourteen-fold and a seventeen-fold increase on-column Efficiencies were also much greater for the unstacked samples The peak efficiency for the stacked method was 106,000

plates for the 45 second injection, which represents a six-fold increase over the samples prepared in BGE and a 35-fold increase over the unstacked samples

From this data (Tables 2 1-2 5) it can be seen that the electropherograms obtained using pH-mediated sample stacking showed both greater peak heights and increased efficiency over the unstacked samples

Table 2 4: *Peak data obtained for samples in phosphate without stacking Standards were 10 μM prepared in phosphate buffer (pH 7 5, 25 mM) Separations were performed as for the stacking experiments with the exception that no base injection was performed (n = 3)*

Sample Inj Time (s)	HPAA Peak Height (mAU)	HPAA Efficiency N (/1000)	7-HC Peak Height (mAU)	7-HC Efficiency N (/1000)	4-HC Peak Height (mAU)	4-HC Efficiency N (/1000)
5	29 ± 24	29 ± 6	113 ± 8	32 ± 7	84 ± 25	22 ± 9
10	60 ± 7	8 ± 1	167 ± 11	11 ± 2	154 ± 13	6 ± 0 9
15	62 ± 1	4 ± 0 3	145 ± 11	7 ± 0 7	165 ± 22	3 ± 0 4
20	97 ± 32	5 ± 3	189 ± 34	3 ± 0 3	215 ± 7	2 ± 0 4
45	116 ± 26	3 ± 0 7	249 ± 33	3 ± 0 1	248 ± 17	2 ± 0 1

Table 2 5 *Peak data obtained for samples prepared in BGE Standards were 10 μM prepared in the BGE Separations were performed as for the stacking experiments with the exception that no base injection was performed (n = 3)*

Sample Inj Time (s)	HPAA Peak Height (mAU)	HPAA Efficiency N (/1000)	7-HC Peak Height (mAU)	7-HC Efficiency N (/1000)	4-HC Peak Height (mAU)	4-HC Efficiency N (/1000)
5	99 ± 7	84 ± 16	167 ± 23	65 ± 10	154 ± 20	37 ± 14
10	140 ± 8	33 ± 9	266 ± 7	38 ± 4	288 ± 52	22 ± 1
15	143 ± 13	14 ± 4	300 ± 10	19 ± 4	395 ± 16	18 ± 2
20	131 ± 9	16 ± 4	286 ± 19	25 ± 6	373 ± 25	19 ± 2
45	143 ± 25	19 ± 4	298 ± 18	26 ± 5	385 ± 27	18 ± 0 4

2.3.3. Optimised System

The optimised separation conditions for base-mediated stacking are outlined in Table 2 6

Table 2 6 *Optimised separation conditions for base-mediated stacking*

Parameter	**Optimum Value**
Capillary dimensions	61 cm X 50 μm
Capillary length to detector	50 cm
Background electrolyte	50 mM methylamine pH 10 7 with 0 5 mM TTAB
Sample matrix	25 mM phosphate pH 7 5 buffer
Applied Voltage	- 20 kV ramped for 0 17 min
Detection	U V absorbance at 214 nm
Sample injection time	45 seconds
Sample injection voltage	-10 kV
Base injection time	90 seconds
Base injection voltage	-10 kV

Figure 2.9 shows the electropherogram achieved for HPAA, 7-HC and 4-HC under separation conditions outlined in Table 2.6. The three analyte peaks are fully baseline resolved and separated with a run time of only 7.5 minutes.

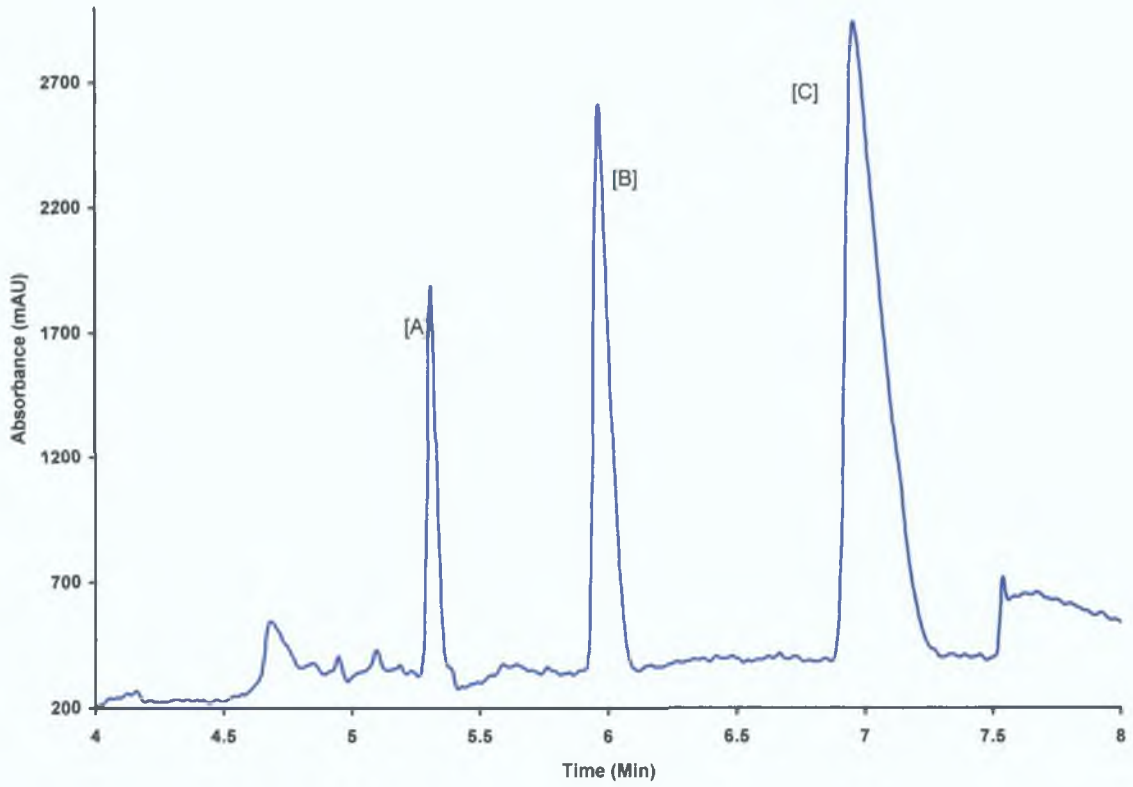


Figure 2.9: *Electropherogram showing the separation of a standard mixture of coumarin metabolites with sample stacking. Standard 10 µM mixture of [A] HPAA [B] 7-HC and [C] 4-HC. Separation was carried out in a 50 cm effective capillary, at – 20 kV, reverse polarity, using 50 mM methylamine pH 10.7 with 0.5 mM TTAB as BGE. Samples were introduced by a 45 second electrokinetic injection at –10kV followed by a 90 second injection of NaOH (-10 kV) and detection was by UV absorption at 214 nm.*

Figure 2.10 shows the separation achieved for samples prepared in phosphate buffer (25 mM; pH 7.5) without sample stacking. The electropherogram shown was

achieved by using the same conditions as for the sample stacking (Table 2.6) with the exceptions that there was no base injection, and that the sample injection was for 5 seconds. This was previously determined to be the optimum sample injection time for samples in phosphate without sample stacking (Table 2.4).

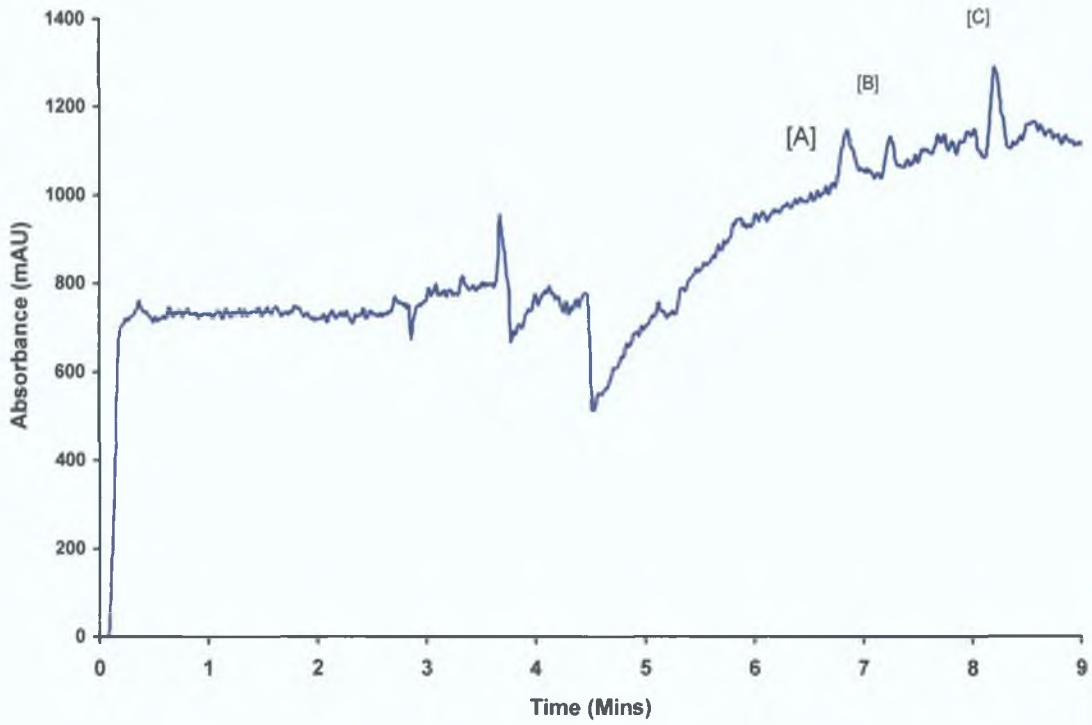


Figure 2.10: *Electropherogram showing the separation of a standard mixture of coumarin metabolites prepared in phosphate buffer without sample stacking. Separation of [A] HPAA, [B] 7-HC and [C] 4-HC without sample stacking. Separation was carried out in a 50 cm effective capillary, at –20 kV, reverse polarity, using 50 mM methylamine pH 10.7 with 0.5 mM TTAB as BGE. Samples were introduced by a 5 second electrokinetic injection at –10kV and detection was by UV absorption at 214 nm.*

By comparison of the electropherograms obtained both with sample stacking (Figure 2.9) and without sample stacking (Figure 2.10) it can be seen that sample stacking offers a vast improvement for the separation of these analytes. When the

method was performed without sample stacking, the analytes were barely detectable at the 10 μM concentration used for analysis and peak shape was very poor.

Figure 2.11 shows the separation achieved for samples prepared in the BGE. The electropherogram shown was achieved by using the same conditions as for the sample stacking (Table 2.6) with the exceptions that there was no base injection, and that the sample injection was for 5 seconds. This was previously determined to be the optimum sample injection time for samples prepared in BGE with no sample stacking (Table 2.5).

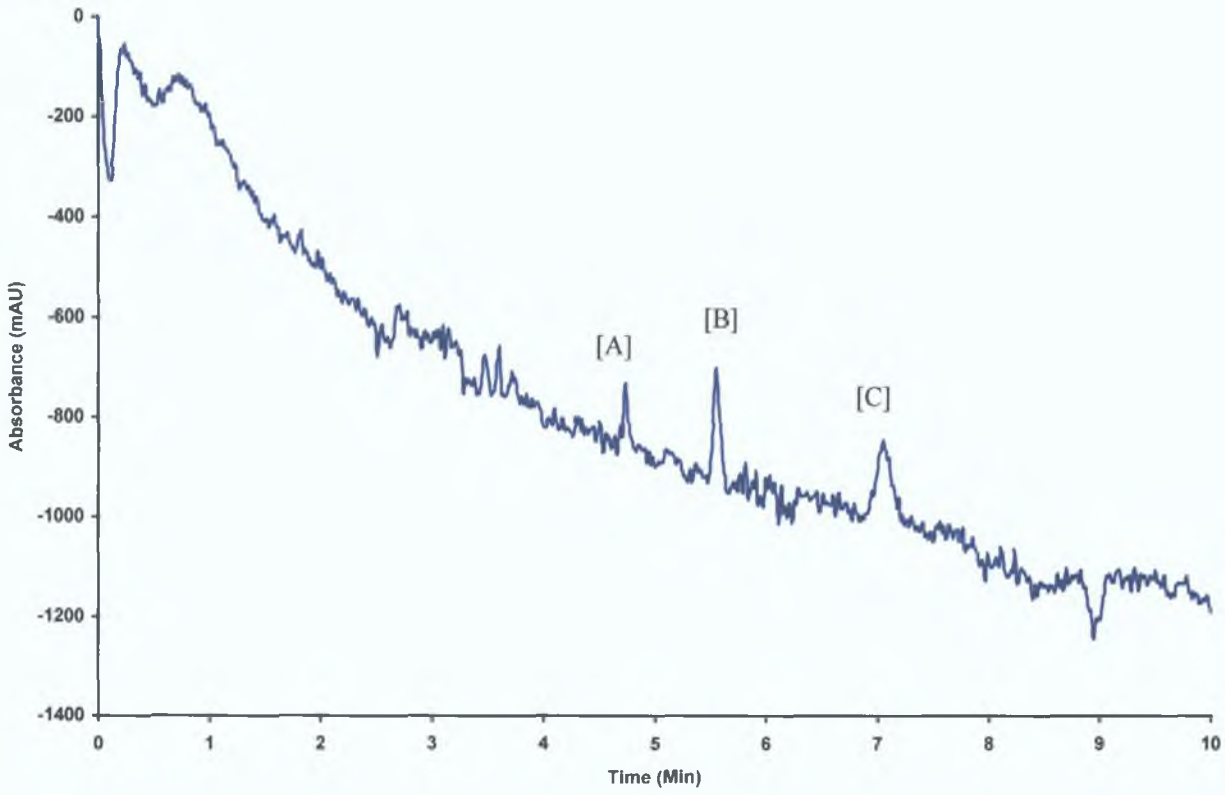


Figure 2.11: *Electropherogram showing the separation of a standard mixture of coumarin metabolites prepared in the BGE. Separation of [A] HPAA, [B] 7-HC and [C] 4-HC for samples prepared in BGE. Separation was carried out in a 50 cm capillary, at –20 kV, reverse polarity, using 50 mM methylamine pH 10.7 with 0.5*

mM TTAB as BGE Samples were introduced by a 5 second electrokinetic injection at –10 kV and detection was by UV absorption at 214 nm

By comparison with Figure 2 10 it can be seen that the peak shape was vastly improved when samples were prepared in the BGE, for cases where sample stacking was not performed This is due to the removal of negative effects arising from the incompatability of the sample matrix and the BGE as previously described The signal-to-noise ratio was also improved when the BGE is employed as the sample matrix, leading to improved limits of detection for the analytes, over unstacked phosphate samples These improvements are still vastly inferior to those obtained by performing sample stacking, as is evident from comparison with Figure 2 9

2.3.4. Limits of Detection

The limits of detection of the analytes, for the sample stacking method, based on a signal to noise ratio of 3 were 0 1 μM for 7-HC, 0 5 μM for HPAA and 0 3 μM for 4-HC This represents a 60-fold improvement in detection limits for 7-HC by CE over previously published methods (Duffy *et al*, 1998, Deasy *et al*, 1995) The linear ranges for the three analytes were 0 1 μM to 350 μM, 1μM to 250 μM and 0 5 μM to 350 μM respectively Results were based on mean values of three runs and the R^2 values of the three calibrations were 0 991 (7-HC), 0 992 (HPAA) and 0 965 (4-HC)

2.3.5. Microsomal Incubations

Once the optimal conditions had been established, the stacking method was applied to microsomal incubations of coumarin 7-HC is the primary metabolite of coumarin in Cynomologus monkeys, with ring-opening metabolites and 3-

hydroxycoumarin being formed to a lesser extent (Weaver *et al* , 1999) Indeed,7-hydroxylation accounts for over 50 % of coumarin metabolism in both monkeys and humans (Weinmann, 1997) In these microsomal incubations, 7-HC was detected The suspected metabolite peak was identified by spiking of the incubation mixture with standards of 7-HC The amount of 7-HC formed was then calculated by comparison with a calibration curve These calibration curves were performed on the day in which the assay was carried out in order to eliminate any day-to-day variations There was no 4-HC or HPAA detected Figure 2 12 shows an electropherogram after a 90 minute microsomal incubation of coumarin The electropherogram is overlaid with an incubation mixture spiked with 7-HC to show the migration of the 7-HC peak

2 3 5 1 Control Incubations

Control incubations were performed to ensure that the suspected 7-HC peak would not be present in incubations that were performed without one of the necessary incubation components Figures 2 13 and 2 14 are electropherograms of control incubations, after 90 minutes carried out without $NADP^+$ and without microsomes respectively Both of these incubations were then spiked with 7-HC and overlaid with the original incubation electropherogram In both cases, as expected, there was no 7-HC peak seen in the incubation mixture

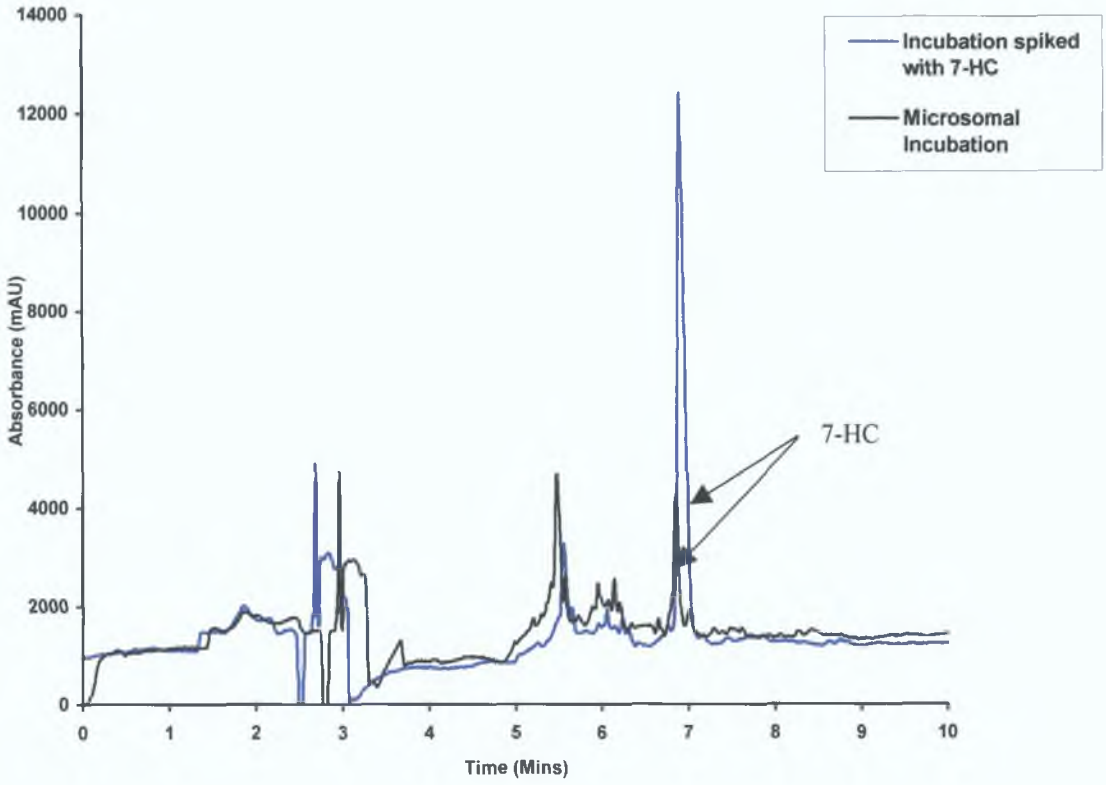


Figure 2.12: *Electropherogram of microsomal incubation of coumarin and incubation of coumarin spiked with 7-HC. Separation was carried out in a 50 cm effective capillary, at – 20 kV, reverse polarity, using 50 mM methylamine pH 10.7 with 0.5 mM TTAB as BGE. Samples were introduced by a 45 second electrokinetic injection at –10 kV followed by a 90 second injection of NaOH (-10 kV) and detection was by UV absorption at 214 nm. The 7-HC peak is indicated at a migration time of approximately 7 minutes.*

The electropherogram in Figure 2.12 shows the separation of the components of an incubation of coumarin after 90 minutes. The overlaid incubation was spiked with 7-HC to help identify the metabolite peak. It can clearly be seen that the peak at approximately 7 minutes corresponds to the 7-HC peak in the control incubation

mixture. Once identified, the 7-HC formed was quantified by analysis of this peak. Peak area values, compared against a calibration curve, were used for the quantification.

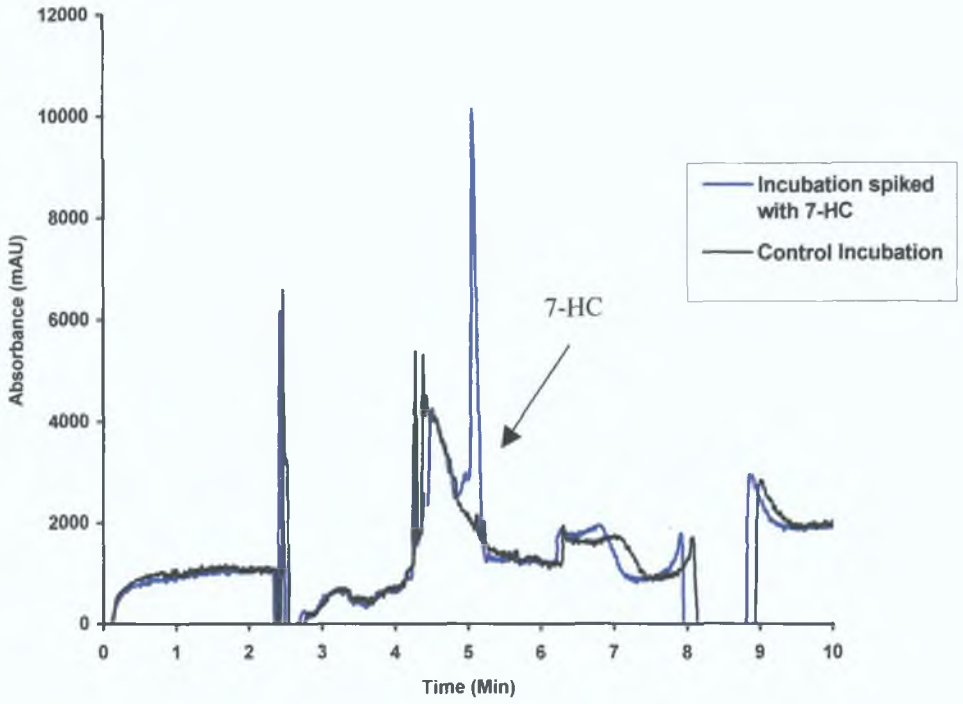


Figure 2.13: *Electropherogram of control incubation of coumarin carried out without $NADP^+$ and control incubation without $NADP^+$ spiked with 7-HC. Separation was carried out in a 50 cm effective capillary, at – 20 kV, reverse polarity, using 50 mM methylamine pH 10.7 with 0.5 mM TTAB as BGE. Samples were introduced by a 45 second electrokinetic injection at –10kV followed by a 90 second injection of NaOH (-10 kV) and detection was by UV absorption at 214 nm. The 7-HC peak is indicated at a migration time of approximately 5 minutes.*

As described previously (Section 2.1.3.4.) control incubations were performed to validate formation of 7-HC in the actual incubations. Figure 2.13 above shows overlays of electropherograms obtained for control incubations performed without $NADP^+$ and a similar incubation spiked with 7-HC. In this case there should be no 7-HC formation in the control incubation. In the spiked incubation, the 7-HC peak

was clearly visible at approximately 5 minutes. This peak was absent in the control incubation indicating that the metabolite was not produced when $NADP^+$ was omitted from the incubation mixture.

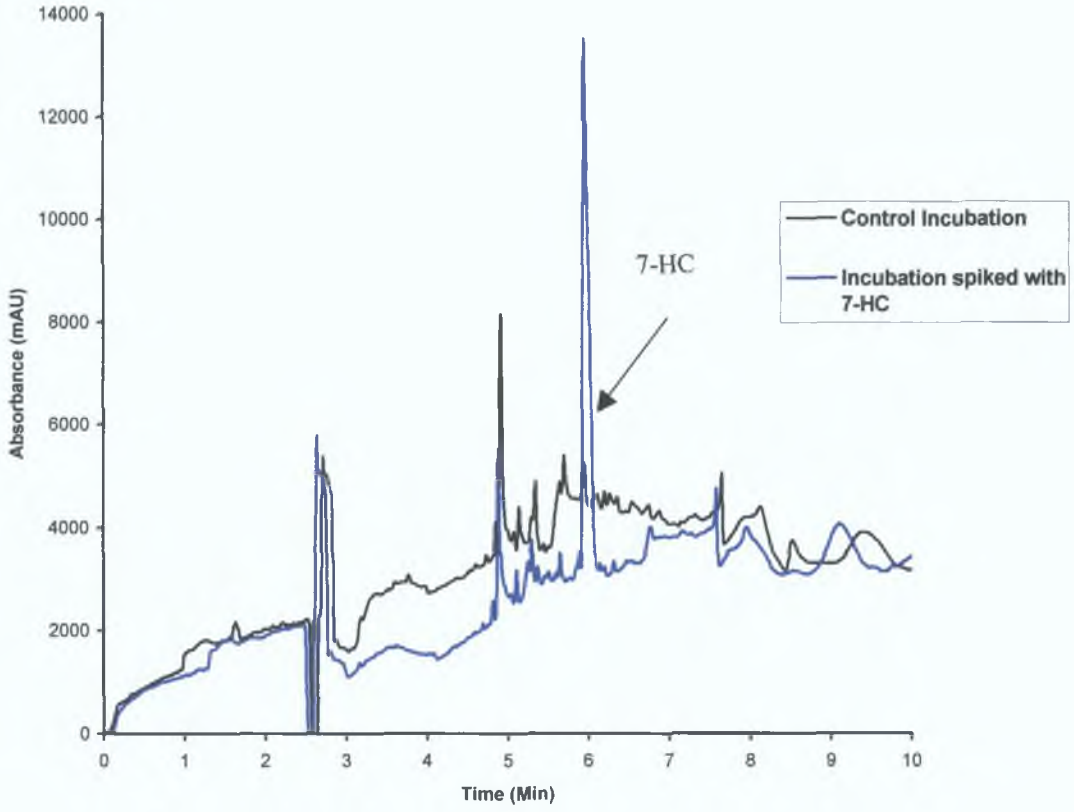


Figure 2.14: *Electropherogram of control incubation of coumarin carried out without microsomes and control incubation without microsomes spiked with 7-HC. Separation was carried out in a 50 cm effective capillary, at – 20 kV, reverse polarity, using 50 mM methylamine pH 10.7 with 0.5 mM TTAB as BGE. Samples were introduced by a 45 second electrokinetic injection at –10kV followed by a 90 second injection of NaOH (-10 kV) and detection was by UV absorption at 214 nm. The 7-HC peak is indicated at a migration time of approximately 6 minutes.*

A second control incubation was performed by omitting the microsomes from the incubation mixture. The 7-HC peak, clearly visible at 6 minutes in the spiked incubation mixture was once again absent in the control incubation, indicating that

the suspected 7-HC peak is only formed when microsomes were present in the incubation mixture For each control case studied (i e no microsomes, no NADP+) both the control incubation and the relative spiked incubation were performed on the same day Variations in migration times between different controls could be attributed to day-to-day variations

2.3.6. Production of 7-Hydroxycoumarin

A plot of 7-HC concentration produced (nmol per mg of protein) over time is shown in Figure 2 15 For clarity, the time is plotted as the time at which the aliquot was taken from the incubation vial, although the incubation would proceed whilst the sample was being transferred to the CE system This would account for the appearance of 7-HC in the sample taken at 0 minutes The formation of 7-HC proceeded in a fairly linear manner over the first 45 minutes, reaching a plateau as the activity of the microsomes decreased At 45 minutes the activity of the microsomes was 438 4 pmol/mg/minute Previous research has quoted microsomal activity for Cynomologus monkeys as 616 8 pmol/mg/minute (Bogan *et al* , 1996) and 556 9 pmol/mg/minute (Weaver *et al* , 1999) The activity of the microsomes at 90 minutes was calculated to be 223 pmol/mg protein/minute This is in agreement with characterization information provided by the microsome suppliers (Lot number NAE, male Cynomologus monkey microsomes, In Vitro Technologies, Inc) who quoted the 7-hydroxylation activity of this lot of enzymes as 217 pmol/mg protein/minute The represents a deviation of less than 3 % Figure 2 15 shows the formation of 7-HC (nmol/mg protein) over the period of the incubation As described previously, the profile of 7-HC formation proceeds in a fairly linear manner over the first 45 minutes, levelling off as the activity of the enzyme decreases

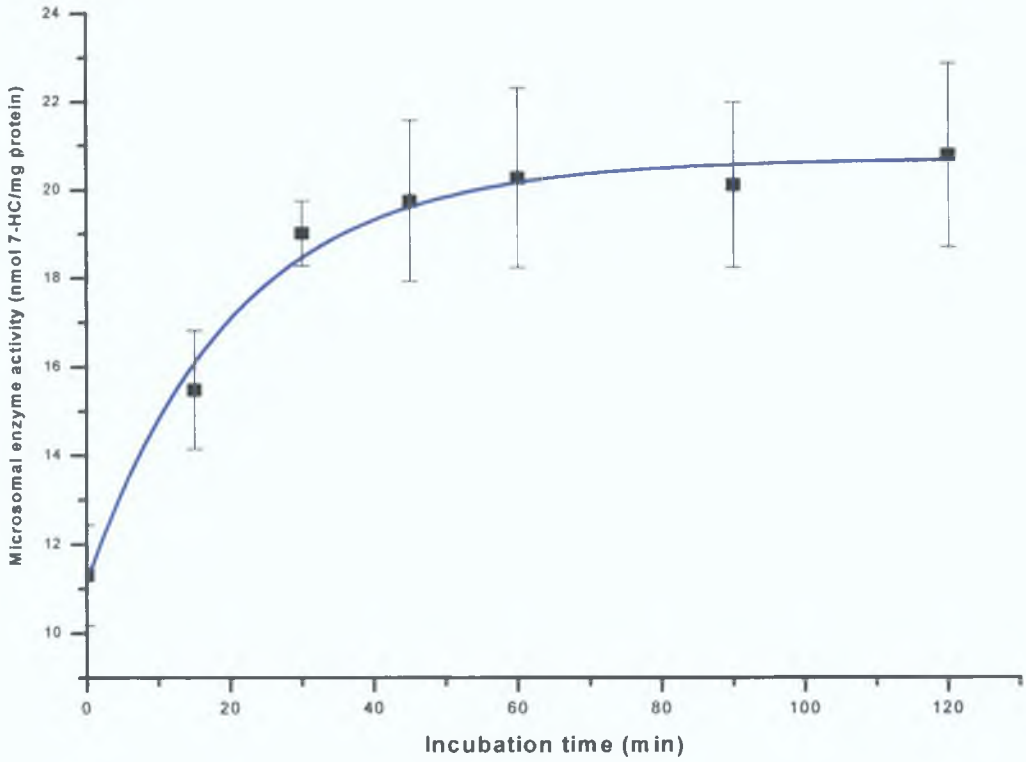


***Figure 2.15**: Plot of the rate of 7-hydroxycoumarin production per mg of microsomal protein versus time for Cynomologus monkey microsomes (n = 3).*

2.4. CONCLUSIONS AND FUTURE WORK

pH-mediated sample stacking was found to be easily applicable to the analysis of microsomal incubations of coumarin, providing a sensitive method for the determination of metabolites with minimal sample handling The metabolites could be detected directly in phosphate buffer, the matrix of the incubation mixture Previous research has detected analytes in Ringer's solution The extent of coumarin 7-hydroxylation determined for Cynomologus monkey microsomes was found to be in agreement with previous research and characterisation information provided with the microsomes The method developed allows three of the potential metabolites of coumarin to be separated with increased sensitivity with a run time of less than eight minutes Limits of detection for 7-HC were increased almost 60-fold over previously published methods 7-HC was determined in the incubation mixture However, neither 4-HC or HPAA were detected Applying this method to the analysis of microsomal incubations of different species could allow the other two metabolites to be determined in incubation mixtures 4-HC and HPAA are both potential metabolites of coumarin for humans Therefore, human microsomes would be a suitable choice for this further study

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CHAPTER THREE

DEVELOPMENT OF AN AUTOMATED TECHNIQUE FOR THE ANALYSIS OF MICROSOMAL INCUBATIONS

3.1. INTRODUCTION

Prediction of drug metabolism in humans based on *in vitro* models is of vital importance in the pharmaceutical industry By monitoring the metabolism of a model compound under mimicked physiological conditions, information such as kinetic factors, inhibition constants, potential metabolites and toxicity can be gathered These *in vitro* techniques offer a simple, cost-effective and rapid means by which to perform initial drug metabolism studies, and lessen the need for *in vivo* testing (Atterwill and Steele, 1987)

In vitro incubation reactions are routinely performed by maintaining the necessary reaction components in a water bath or heating block for the incubation period Aliquots are taken from the incubation mixture at timed intervals and transferred for analysis These incubation procedures are time-consuming and labour intensive, requiring the user to be present over the incubation period, and to sample at short intervals Larger sample volumes are also required, as sufficient sample must be available to allow aliquots to be taken at each interval for analysis Potential problems with this technique also include possible sampling errors and inconsistent timing of injections

CE is a versatile separations technique that has been used in recent years to study drug metabolism (Naylor *et al*, 1996) Capillary Electrophoresis is a convenient tool for the analysis of metabolic mixtures as it permits rapid separations without the need for sample clean up prior to analysis The separation of the individual components of the incubation mixture on the capillary terminates the reaction and so it is unnecessary to stop the reaction by other means, such as precipitation of the protein, before injection CE has been applied to the analysis of the phase I and phase II metabolism reactions for coumarin (Bogan *et al*, 1995, Bogan *et al*, 1996, Deasy *et al*, 1995, Killard *et al*, 1996) The importance of coumarin as a model compound for metabolism studies has previously been described in Chapter 1

Deasy *et al* (1995) have described an experimental procedure for monitoring the *in vitro* metabolism of coumarin In this method, incubations were performed as described previously At timed intervals, aliquots were taken from the incubation mixture and injected directly onto the CE system Under the separation conditions established in that study, coumarin and 7-HC were resolved from the other components of the incubation mixture in under three and a half minutes A limitation of this separation method, however, is that 7-HC was the only metabolite studied Other potential metabolites were not considered in that research

The aim of the present research was to develop a separation method capable of resolving coumarin and several of its potential metabolites from the incubation components within a reasonable analysis time Once developed, this separation method would be applied to the development of a technique for performing on-system incubations of coumarin Performing incubations on-system would provide a more automated approach, removing potential sources of human error, whilst allowing minimal sample volumes to be used The metabolism of coumarin was again studied using Cynomologus monkey microsomes due to their similarity in metabolic profile to human liver microsomes (Bogan *et al* , 1996)

3.2. EXPERIMENTAL

3.2.1. Instrumentation

Separations were carried out on a Beckman P/ACE System MDQ instrument (Fullerton, CA) The capillary employed was polyimide fused silica from Polymicro Technologies (Phoenix, AZ) purchased from Composite Metal Supplies (Worcester, England) The incubation mixtures were maintained in a Grant Boeckel BBA heating block during the course of the incubations The pH values of buffer solutions were determined using an EDT microprocessor pH meter, model RE 357

3.2.2. Reagents and Analytes

All chemicals were purchased from Sigma (St Louis, MO) unless otherwise stated Potassium dihydrogen phosphate was purchased from Aldrich Chemical Co (Milwaukee, WI) Glucose-6-phosphate dehydrogenase (from yeast) was purchased from ICN Biomedicals, (Irvine, CA) Male Cynomologus monkey microsomes were purchased from In Vitro Technologies (Baltimore, MA) 3-HC was purchased from ICC Chemicals (Somerville, NJ) 6-HC was synthesized by Dr Oliver Egan, BEST Centre, Dublin City University using a method supplied by Dr J R Fry, University of Nottingham, U K (Personal Communication) Details of synthesis and characterisation information are provided by Cooke (Cooke, 1999)

3.2.3. Preparation of Solutions

The CAPS (3-cyclohexylamino-1-propane-sulfonic acid) BGE was prepared from 0 075 M CAPS and adjusted to pH 10 5 by addition of 1 M NaOH The borate BGE was prepared by adjusting the pH of 0 05 M boric acid to the desired pH by the addition of 1 M NaOH The phosphate buffer for the incubations was prepared from 0 025 M potassium dihydrogen phosphate and adjusted to pH 7 5 by addition

of 0 1 M KOH Standards of coumarin, and coumarin metabolites were prepared from stock solutions of 10 mg/ml in methanol by dilution in phosphate buffer (25 mM, pH 7 5) 7-HC, 4-HC and 6,7-HC stock solutions required sonication to facilitate full dissolution β-Nicotinamide adenine dinucleotide phosphate ($NADP^+$), glucose-6-phosphate and glucose-6-phosphate dehydrogenase solutions were prepared in phosphate buffer (25 mM, pH 7 5) Nanopure water (18 2 MΩ) was used throughout the study Bare fused silica capillary was purchased from Polymicro (Phoenix, AZ) All prepared solutions were stored at 4°C to minimise sample degradation throughout the duration of the study Electrolyte solutions were degassed and filtered through 0 22 μm filters prior to use

3.2.4. Capillary Electrophoresis Separation

Separations were carried out on a Beckman P/ACE system MDQ instrument (Fullerton, CA) equipped with a UV detector module The separation capillary was fused silica, 61 2 cm x 50 μm (50 cm to detector) The capillary was initially primed by flushing sequentially with methanol (5 min), 1 M hydrochloric acid (5 min), deionised water (2 min), 1 M NaOH (5 min), deionised water (2 min) and the BGE (7 min) at 30 °C Between runs the capillary was flushed for 1 minute with 50/50 (v/v) 5 mM SDS / 1 M NaOH (20 p s i) When agitation was required, the capillary was then emptied of BGE, by flushing with air from an empty vial for 1 minute (20 p s i) Once empty, air was flushed gently (10 p s i) from an empty vial into the sample vial to agitate the sample during the incubation A 1 minute rinse with the BGE (20 p s i) was performed prior to injection Samples were introduced on to the capillary by a 5 second pressure injection (0 5 p s i) and separation was carried out at 20 kV (0 17 min rise time) at 25°C Typical running current for the CAPS BGE was ~18 μA The method was timed to ensure that the injections were performed at exactly fifteen minute intervals once the system was running on the autosampler Detection was performed by UV absorbance at 214 nm

3.2.5. Handling of Microsomes

Microsomes were obtained in 500 µL vials from the supplier These microsomes were thawed as rapidly as possible by holding the vial in water heated to 60 °C Once defrosted, microsomes were divided into 30 µL aliquots in plastic vials These aliquots were then frozen at –80 °C for use in the incubation studies When an incubation was being performed, the aliquot of microsomes was rapidly defrosted and immediately added to the incubation mixture The effects of freezing and thawing microsomes have been discussed by Pearce *et al* (1996)

3.2.6. Microsomal Incubations

The incubation procedure was similar to that described by Deasy *et al* (1995) Briefly, the incubations were performed by mixing 195 µL of coumarin standard (50 µg/mL), 15 µL of $NADP^+$ (20 mM), 10 µL of glucose-6-phosphate (50 µg/mL) and 8 µL of glucose-6-phosphate dehydrogenase (4 U/mL) in a vial These concentrations are higher than those required to maintain maximum metabolic rates under the conditions chosen The incubations were initiated by the addition of 25 µL of microsomes to the reaction vial, which was maintained at 37°C Off-system incubations were carried out in a heating block 15 µL aliquots were taken from the vial and injected on to the capillary at fifteen-minute intervals For the on-system reactions, the incubations were carried out in plastic CE micro-vials or 0 3 mL un-graduated glass screw-thread micro-vials A CE method was set up to heat the stored sample on the system to 37 °C and the autosampler was used to inject from the vial at fifteen-minute intervals Both on-system and off-system incubations were performed for 120 minutes A control was carried out for each of the incubations by substituting the coumarin in the incubation mixture with phosphate buffer (25 mM, pH 7 5)

3.2.7. Calibration

Calibration standards were prepared by serial dilution of the stock standards into a 10% microsomal solution Seven standards of 7-HC were prepared in the concentration range 0 5 μg/mL to 30 μg/mL The standards were each run three times, in random order and a curve was plotted using the mean value of the three runs In addition, each standard was run once between incubations to ensure correlation with the calibration The variation in peak area of each of the standards for the on-system incubations, calculated from the equation (standard deviation/average) x 100, was less than 10% During the course of this study, two calibration curves were obtained with R^2 values of 0 9911 and 0 9919, respectively

3.3. RESULTS AND DISCUSSION

3.3.1. Development of the Separation Conditions

In previous analyses of microsomal incubations of coumarin, phosphate buffer has typically been employed as the separation BGE This is because phosphate - a typically high-conductivity buffer - is also present in the incubation mixture and so by using it as the BGE, negative sample matrix/BGE effects could be avoided Phosphate is not considered to be an optimal CE buffer, however, generating high currents at relatively low concentrations Thus, a new BGE for the separation would be desirable, if negative sample matrix effects could be avoided or eliminated

When developing a CE method, other factors such as injection time, capillary length etc must be optimised in order to achieve the best possible separation The optimal injection time in CE (when sample stacking methods are not being employed) is considered to be that which allows a volume of 1 to 5 % of the total capillary volume to be injected Longer injection times typically lead to overloading of the capillary, resulting in poor peak shape, whilst shorter injection times, which

introduce only a small volume of sample, suffer from poor limits of detection and increased error due to ubiquitous injection The injection volume can be calculated as a percentage of total capillary volume but is typically determined by varying the injection times over several runs The length of capillary used is also important, as while longer capillaries lead to greater resolution and more efficient heat dissipation, this is at the cost of increased analysis times A compromise must thus be made by choosing a capillary length that is sufficient to provide a good separation but short enough to allow adequate separation to occur within a reasonable analysis time

Following optimisation of the separation conditions, blank incubations were successfully run to ensure that the components of the incubation mixture would not interfere with the detection of the metabolites of interest under the separation conditions established Standard curves, linear in the range of interest, were plotted for each of the metabolites of interest

3 3 1.1 Choice of Background Electrolyte

Several important points must be taken into consideration when the separation buffer for a CE method is being determined Firstly, for a buffer to be effective, the pH used must be within 1 unit of its pK_a value The separation buffer should also ideally have a pH close to the pK_a value of the analytes Matching buffer ion mobility with analyte mobility is important for maintaining good peak shape As the pH of the BGE is raised, more of the silanol groups of the capillary are ionised, and the resulting electrical layer is more dense Thus the EOF increases with increasing buffer pH Analysis times are therefore reduced when the pH of the BGE is raised It is also imperative that the components of the buffer system have a low UV absorbance at the wavelength utilized, so that they don't interfere with detection of the analytes The ionic strength of the buffer must also be taken into consideration High ionic strength buffers offer greater buffering capacity, and a decrease in analyte ion/ionised silanol group interactions at the capillary wall,

improving resolution. This improved resolution is at the expense of lowering the EOF and increasing analysis time, however. Under high electrical fields, the current associated with these high ionic strength buffers may also pose thermostatting problems for the instrument.

A phosphate buffer (25 mM; pH 7.5) was used as the BGE in previous studies of coumarin metabolism (Deasy *et al.*, 1995). As mentioned previously, however, phosphate is not an ideal BGE due to its highly conductive nature. It was decided to choose lower conductivity buffers for this study. Borate, and the zwittterionic CAPS buffers were chosen, being of lower conductivity and generating significantly lower currents at similar applied voltages. This is evident from the Ohm's law plot (Figure 3.1) which shows that the current generated at 20 kV was 87.5 μA for phosphate, but only 12.0 and 9.9 μA for borate and CAPS respectively. The Ohm's plot is linear from approximately 7 to 25 kV for phosphate and from 0 to 30 kV for the other two BGEs, indicating the range of voltages that can effectively be applied to a separation for that BGE before negative Joule heating effects occur.

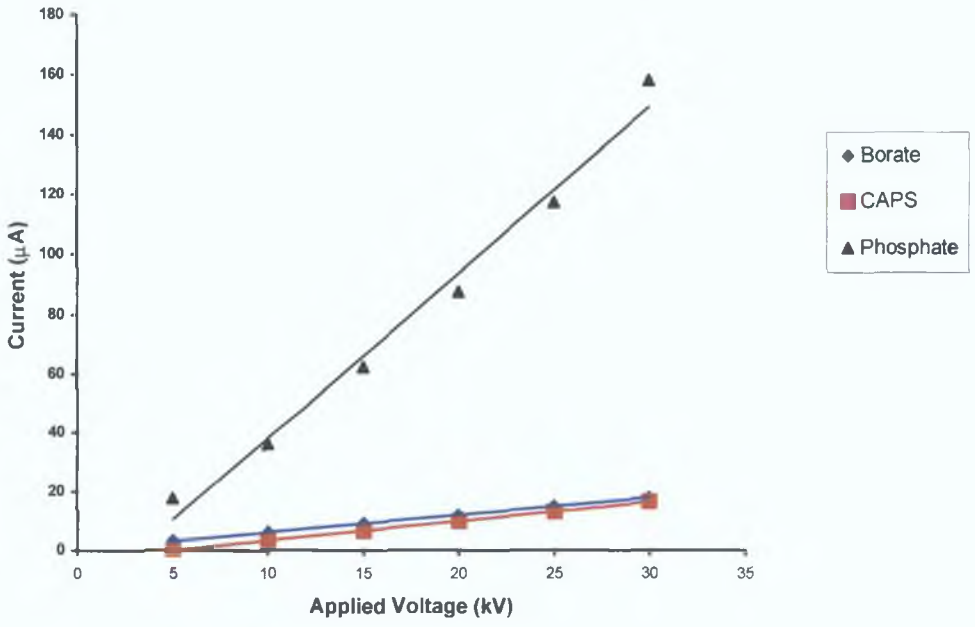
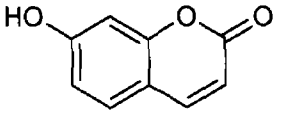
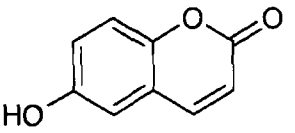


Figure 3.1: *Ohm's Law plot for phosphate (25 mM; pH 7.5), borate (50 mM; pH 9.5) and CAPS (50 mM; pH 10.5) buffers in a 50 cm effective capillary.*

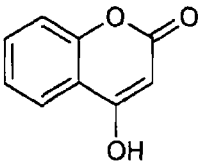
The separation developed would be required to resolve several of the metabolites of coumarin, of similar structure from each other The structures of the seven metabolites which were studied are illustrated in Figure 3 2



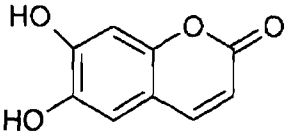
7-Hydroxycoumarin



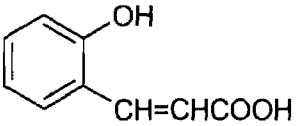
6-Hydroxycoumarin



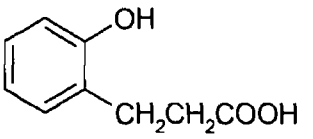
4-Hydroxycoumarin



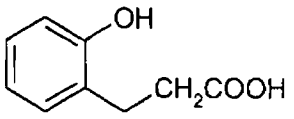
6,7-Dihydroxycoumarin



o-Coumaric acid



o-Hydroxyphenylpropionic acid



o-Hydroxyphenylacetic acid

Figure 3 2: *Structures of seven coumarin metabolites of interest in this study*

It was also considered that at the pH previously used (pH 7 5) these hydroxylated coumarins might not be sufficiently differentially charged to achieve a full separation For this reason buffers with a high useful pH, i e >8, were investigated Borate and CAPS both meet this criterion, with borate having a useful range of 8 1-10 1 and CAPS from 9 7-11 1

3 3.1 2 Sample Matrix Effects

Phosphate buffer is a relatively high conductivity buffer, as discussed previously Conversely, both borate and CAPS buffers generate relatively low currents at the same voltages This would indicate that injecting from phosphate buffer into borate or CAPS buffers could lead to disadvantages associated with destacking To investigate whether this would lead to problems in this separation method, mixtures of coumarin and 7-HC were prepared in three sample matrices and injected Samples were introduced by hydrodynamic injection in all cases The samples were prepared in phosphate, deionised water and the BGE (i e borate or CAPS) These two matrices (i e water and BGE) could then be compared with the phosphate pH 7 5, which is used in the incubation mixtures, to determine if problems with injecting from a higher conductivity buffer (phosphate) into a lower conductivity one (borate or CAPS) would be encountered 7-HC was chosen to be representative of the hydroxylated metabolites, whilst coumarin, being neutral, should not undergo stacking or destacking under any of the established conditions and so can act as a control against which to measure observed stacking or destacking The effect on peak width and peak height as injection time was increased was then monitored Deionised water was chosen as one of the controls as it has a very low ionic strength and therefore field-amplified stacking would be expected to occur (Chapter 1) If, however, dramatic differences exist between the flow rates of the sample matrix and the separation buffer, laminar flow within the capillary can result in band broadening Borate buffer (50 mM, pH 9 5) and CAPS buffer (75 mM, pH 10 5) were also utilised as control sample matrices when they were used as the BGE By matching the separation buffer and the sample matrix,

the buffer system is homogeneous and any stacking or destacking effect should be negligible.

Figures 3.3 to 3.8 show the effects of increasing sample injection time on peak width. The effects on peak height are not shown as these are effectively linked to the increase in peak width. In a case where stacking is occurring, the peak width should remain fairly constant as the sample injection time increases. This is because as sample stacking is compressing the sample into a narrow zone, the peak height increases rapidly as sample injection increases, while the peak width remains narrow. This continues until the system is overloaded, at which point the peak width also starts to significantly increase. Where no stacking is occurring, the peak width should increase proportionally as the injection time increases.

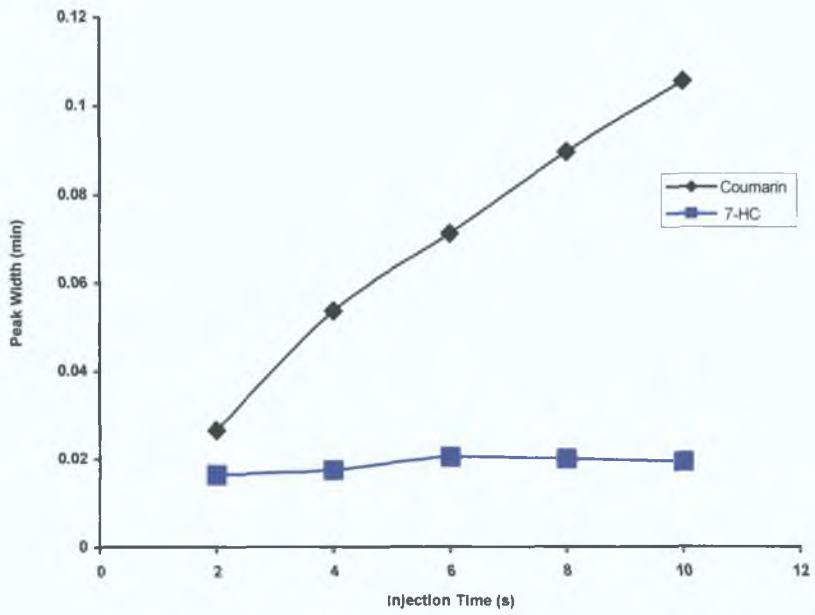


Figure 3.3: *Effect of increasing injection time on samples prepared in deionised water and injected into a borate (50 mM; pH 9.5) BGE.*

From Figure 3.3 it can be concluded that sample stacking was occurring for the 7-HC species, prepared in water, when it was injected into the borate BGE. This is to

be expected as field amplified sample stacking occurs when a sample in a low conductivity matrix, such as water, is injected into a relatively high conductivity matrix, i.e. borate. This is evident in the very slight increase in peak width as the sample injection time was increased. As increasing amount of analyte was being injected onto the capillary and only slight deviations in sample width were being noted, it can be inferred that the sample peak height must be increasing rapidly, as sample stacking is taking place. Conversely, there was no sample stacking noted for the neutral species, coumarin, under these conditions. This is also to be expected as uncharged species migrate with the EOF, and thus are not directly affected by the differing conductivities of the sample matrix and the BGE.

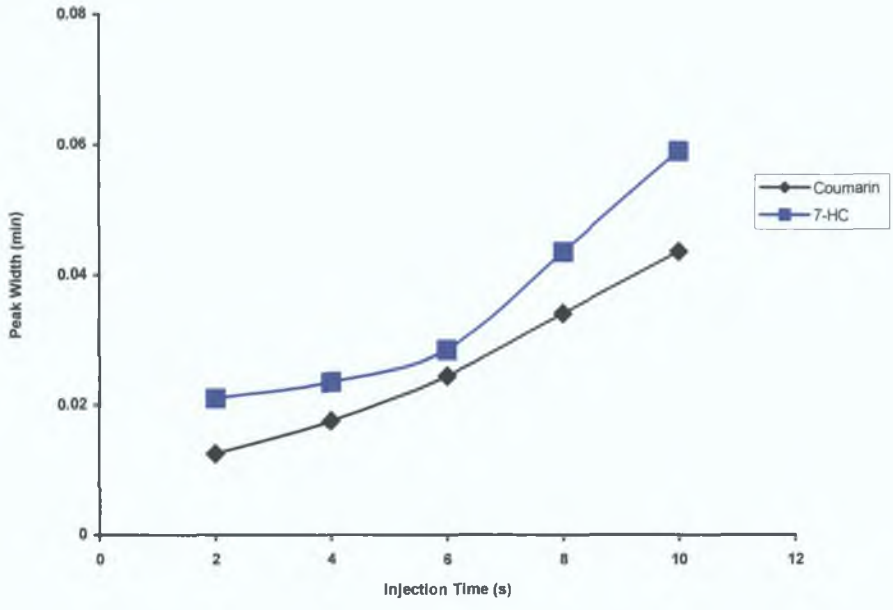


Figure 3.4: *Effect of increasing injection time on samples prepared in borate (50 mM; pH 9.5) and injected into a borate (50 mM; pH 9.5) BGE.*

Figure 3.4 shows the effect of increasing injection time on samples prepared in BGE. Once again, no sample stacking is noted for coumarin, the neutral species. For the 7-HC (anionic) species, there was no evident stacking effect. The 7-HC peak width increased fairly proportionally as the sample injection time was

increased, as would be expected. Under the conditions of this experiment, the sample was being injected from a matrix with a matching conductivity to the BGE. There was thus no discontinuous BGE system present in the capillary under which sample stacking could take place.

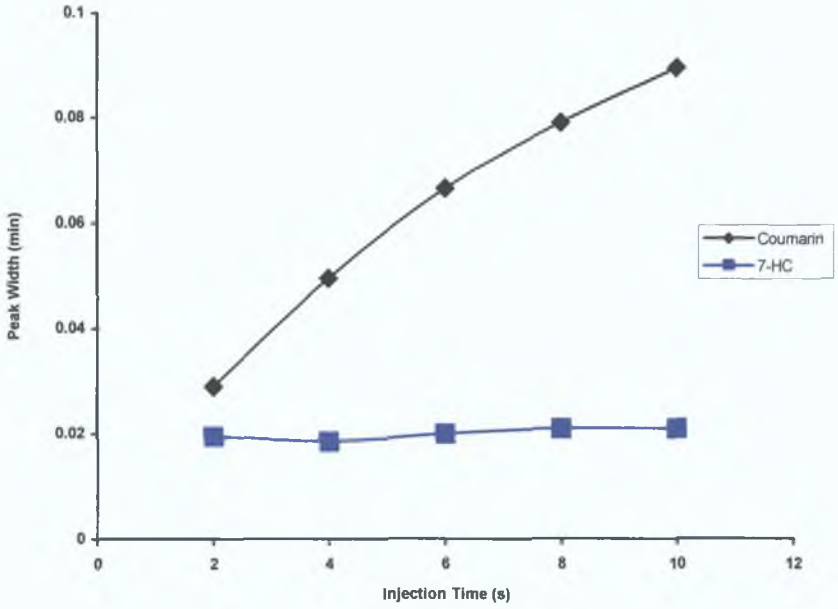


Figure 3.5: *Effect of increasing injection time on samples prepared in phosphate (25 mM; pH 7.5) and injected into a borate (50 mM; pH 9.5) BGE.*

The effect of sample injection time on samples prepared in phosphate and injected into the borate BGE is illustrated in Figure 3.5. It was this experiment which was of most interest, to determine if detrimental destacking effects would be observed when injecting from a high conductivity matrix (phosphate) into a lower conductivity one (borate). The reverse appears to be true, however, with sample stacking appearing to take place under these conditions. The slowly increasing peak width for 7-HC compared to the rapidly increasing coumarin one, would indicate that some stacking phenomenon was occurring for 7-HC. Whilst this result is unexpected, it is welcomed as it would indicate that destacking effects would not

undermine the development of a separation method for the metabolites of coumarin in a phosphate matrix, using borate as a BGE

The same three experiments (i e increasing sample injection time for samples prepared in deionised water, phosphate and BGE) were performed with CAPS as the BGE and the same results were noted For the sample prepared in deionised water (Figure 3 6), FASS appeared to take place for the 7-HC species whilst no stacking was observed for the coumarin As previously described for the borate BGE (Figure 3 4), under conditions where the conductivity of the sample matrix and the BGE are equivalent, no sample stacking would be expected to occur This was the case for the CAPS BGE, with no stacking observed for the neutral species or the anionic 7-HC

Once again it appears from Figure 3 8 that stacking was occurring for 7-HC when it was prepared m phosphate buffer and injected into a CAPS BGE CAPS is thus also suitable for consideration as a BGE for the separation of coumarin metabolites This result was again surprising, given the high conductivity of the phosphate buffer relative to the CAPS BGE

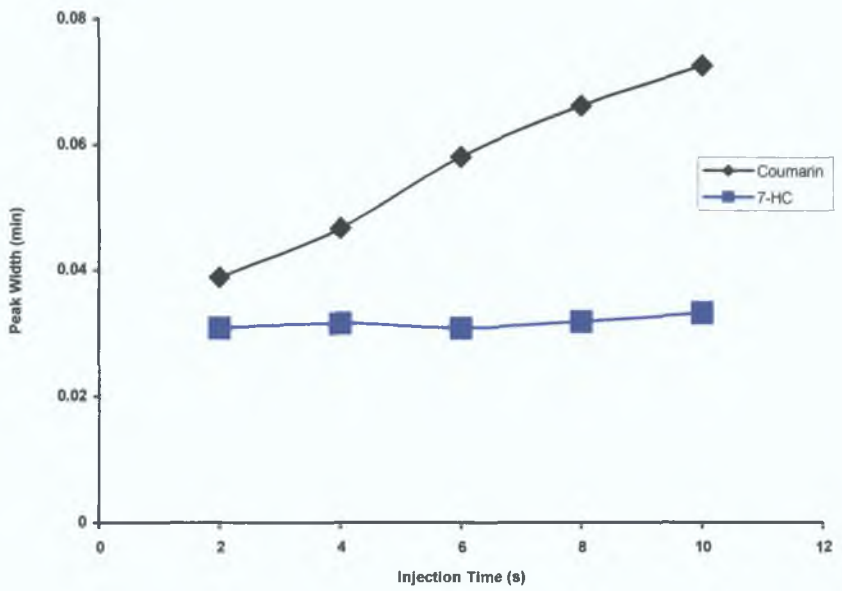


Figure 3.6: *Effect of increasing injection time on samples prepared in deionised water and injected into a CAPS (75 mM; pH 10.5) BGE.*

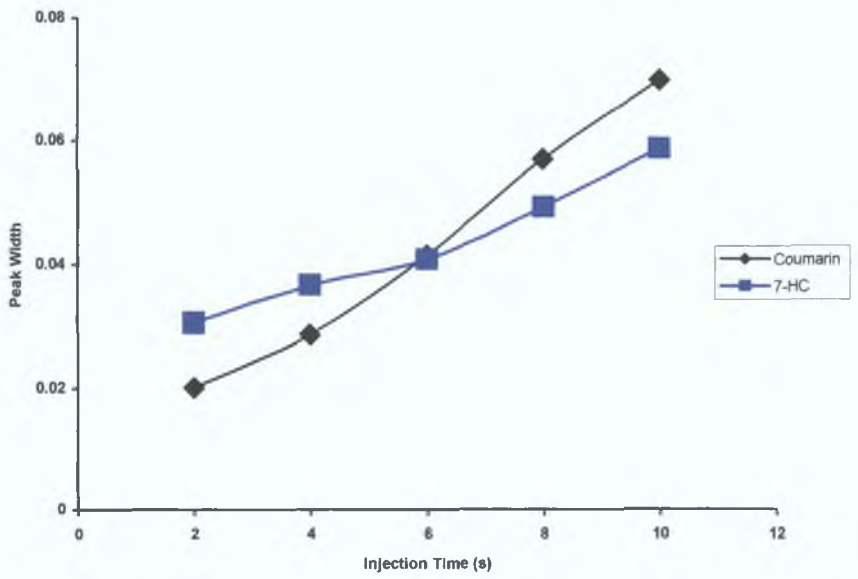


Figure 3.7: *Effect of increasing injection time on samples prepared in CAPS (75 mM; pH 10.5) and injected into CAPS (75 mM; pH 10.5) BGE.*

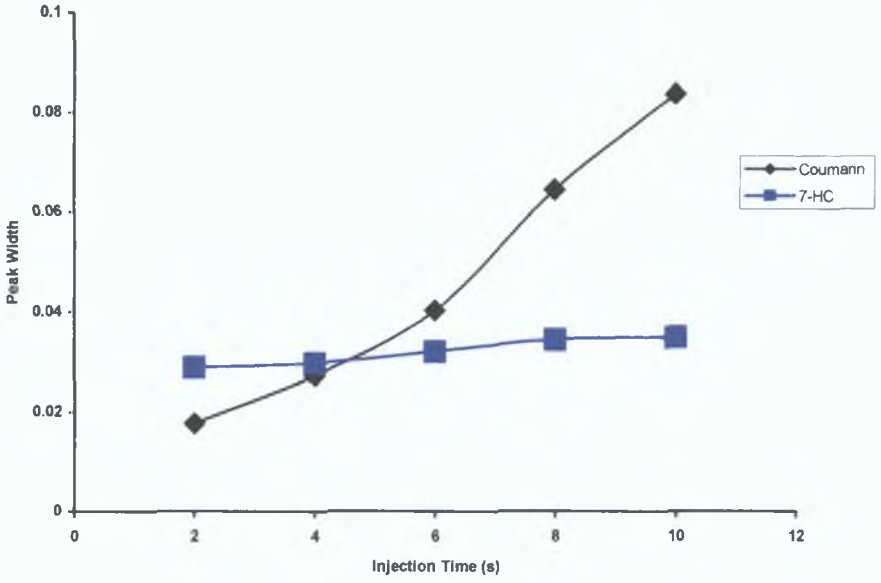


Figure 3.8: *Effect of increasing injection time on samples prepared in phosphate (50 mM; pH 7.5) and injected into CAPS (75 mM; pH 10.5) BGE.*

It is evident from Figures 3.3 to 3.8 that the peak width increased with injection time when there was no stacking involved. This was the case for the neutral species, coumarin, in all matrices, and for both species when the system within the capillary was homogeneous (i.e. the samples were prepared in the BGE). When stacking was involved, the peak width resulting from the charged species did not appear to increase dramatically with injection time.

These graphs would therefore indicate that stacking occurs in the injection of the phosphate sample matrix into the borate separation buffer (Figure 3.5) and into the CAPS buffer (Figure 3.8), as the peak width of the 7-HC species did not increase significantly as the injection time was lengthened. This result is unexpected considering the relatively high conductivity of the phosphate buffer, but does indicate that one would not experience problems due to destacking in a separation method involving borate or CAPS as the BGE.

3 3 1 3 Application of the Methods to Coumarin Metabolites

Once it had been determined that destacking effects would not be a barrier to using either borate or CAPS as BGEs for the separation, the method development continued by the preparation of a mixture of coumarin metabolites, in phosphate buffer (25 mM, pH 7 5) The metabolites used were 6-HC, 7-HC, 4-HC, HPAA, HPPA, CA, 6,7-HC at a concentration of 100 µg/ml each

Separation conditions were then optimised, using the borate and CAPS buffers at a variety of concentrations and pH values Various injection times and capillary lengths were also investigated The resulting optimised separations are shown in Figure 3 9 (borate) and 3 10 (CAPS)

It was desired that HPAA, HPPA and CA could be included in the separation These are possible metabolites of coumarin, resulting from a ring-opening reaction However, using the borate separation conditions, these metabolites could not be completely resolved from the other metabolites Thus, interest turned to the CAPS BGE Once it had been determined that the separation method could resolve all of the metabolites of interest within a reasonable analysis time, it was necessary to ensure that these metabolites did not co-migrate with the co-factors of the reaction ($NADP^+$, glucose-6-phosphate, glucose-6-phospate dehydrogenase, NADPH) Thus, these components were added to the separation For clarity, a separation of the incubation co-factors is overlaid with a separation of the metabolites (Figure 3 11) As the borate separation could detect five metabolites in the *in vitro* mix and the CAPS method could detect seven, it was decided that the CAPS separation, which resolved seven of the metabolites of coumarin in under 7 minutes, would be used for the on-system study

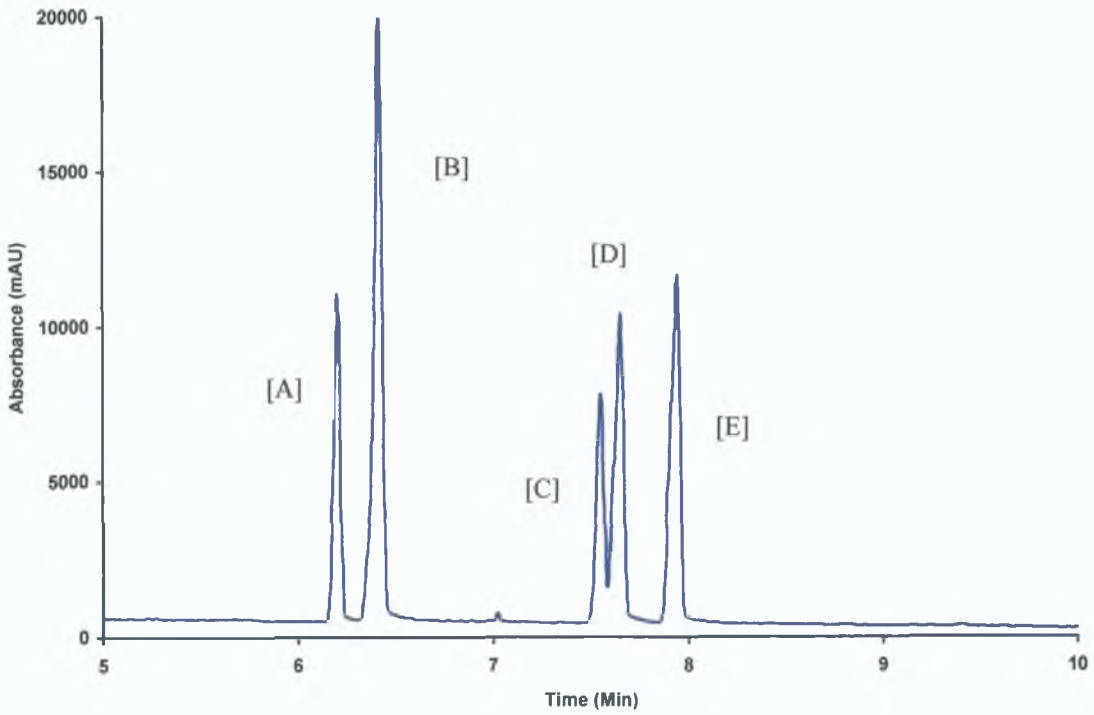


Figure 3.9: *Separation of coumarin metabolites in borate buffer (50 mM; pH 9.5) at 20 kV.*

Analytes were [A] 6,7-HC [B] 6-HC [C] 7-HC [D] 4-HC and [E] HPPA. Samples (100 μg/ml of each metabolite in phosphate buffer) were introduced by pressure (0.5 p.s.i.) for 5 seconds. The 50 μm i.d. capillary was 61.2 cm (50 cm effective) and detection was by UV absorbance at 214 nm.

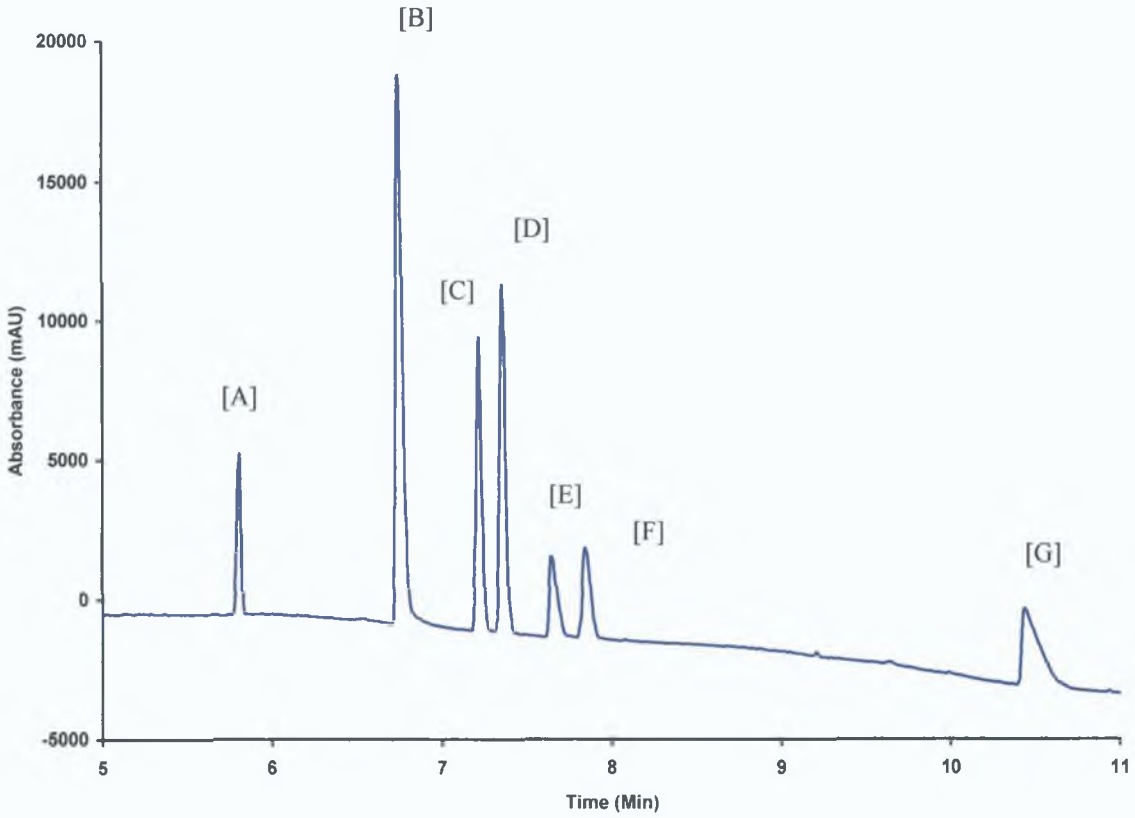


Figure 3.10: *Separation of coumarin metabolites in CAPS buffer (75 mM; pH 10.5) at 20 kV.*

Analytes are [A] 6,7HC [B] 6-HC [C] 7-HC [D] 4-HC [E] HPPA [F] o-HPAA and [G] CA. Samples (100 μg/ml of each metabolite in phosphate buffer) were introduced by pressure (0.5 p.s.i.) for 5 seconds. The 50 μm i.d. capillary was 61.2 cm (50 cm effective) and detection was by UV absorbance at 214 nm.

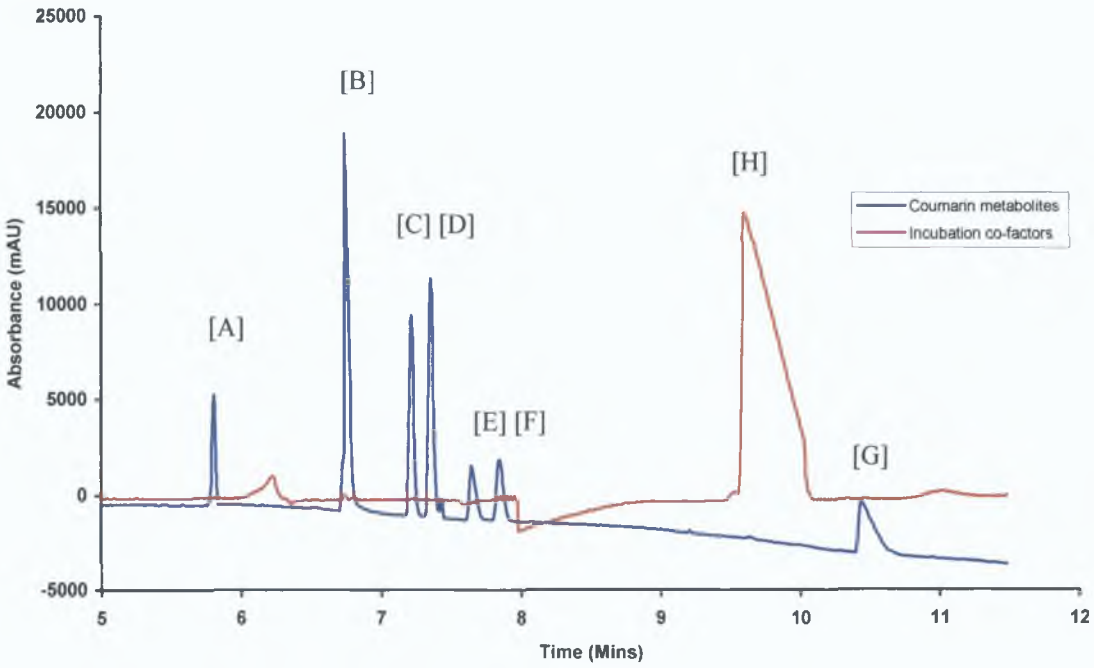


Figure 3.11: *Separation of incubation co-factors in optimised CAPS separation conditions. Electropherogram is overlaid with optimised separation to show the relative migration of the incubation components. Under these established conditions, all of the seven analytes of interest were resolved from the incubation components, making this method suitable for application to in vitro metabolism studies.*

Analytes are [A] 6,7HC [B] 6-HC [C] 7-HC [D] 4-HC [E] HPPA [F] o-HPAA and [G] CA. [H] represents $NADP^+$, a component of the incubation mixture. Samples (100 µg/ml of each metabolite in phosphate buffer) were introduced by pressure (0.5 p.s.i.) for 5 seconds. The 50 µm i.d. capillary was 61.2 cm (50 cm effective) and detection was by UV absorbance at 214 nm.

3.3.2. Development of the On-System Technique

In all cases, both on-system and off-system incubations were carried out in triplicate

3.3 2 1. Off-system Incubations

Off-system incubations were performed to provide a comparison with the new on-system technique As this off-system methodology was well established and commonly practiced, the results obtained for the off-system incubations were taken to be accurate The aim thus was to develop an on-system technique, which would yield results comparable to those obtained by this traditional off-system method

3.3.2.2 On-system Incubations with Plastic Vials

Incubations were initially performed on-system using the plastic CE micro vials supplied with the system 7-HC formation was momtored and plotted per mg microsomal protein versus time 7-HC formation was also plotted for the off-system incubations, to allow comparisons to be drawn (Figure 3 13) For the incubations performed with the plastic vials, the resulting formation of 7-HC appeared to be lower than for the off-system incubations Indeed, the mean activity for the on-system incubations was 114 pmol/mg protein compared to 189 for the off-system incubation, a significant difference of almost 40% Because of the temperature dependence of the microsomal system it was initially considered that the auto sampler temperature might not be correct, and that it might be providing a temperature of less than 37°C, leading to a lower rate of metabolism At this stage, the temperature of the P/ACE MDQ system was calibrated by a Beckman representative and determined to be accurate It was thus considered that the plastic CE vials were not providing adequate heat transfer for the reaction to occur To determine if this was the case, it was decided to perform on-system incubations in glass vials

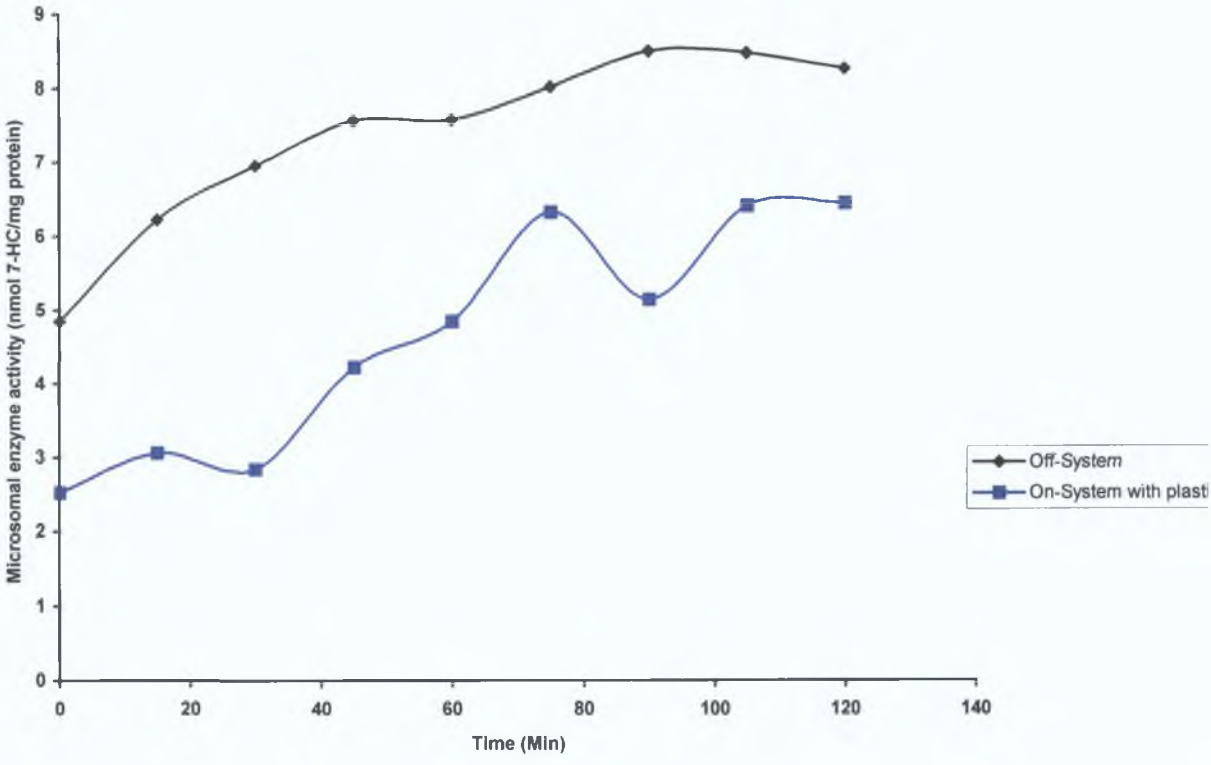


Figure 3.13: *Formation of 7-HC over time for on-system incubations performed in plastic vials and for off-system incubations.*

3.3.2.3. On-System Incubations with Glass Vials

The glass vials employed were 0.3 mL glass screw-thread micro vials. The centre of the vial cap was removed to allow air to reach the sample during the incubation and the vial cap was fitted securely to the vial before being placed on the system. As the vial cap needed to fit tightly to allow pressurisation of the sample during injection, the vial caps were replaced after each incubation. The results obtained for the incubations carried out in glass vials are shown in Figure 3.14. A higher rate of coumarin 7-hydroxylation was noted when the glass vials were used. Significant differences between the on-system and off-system results were still evident, however. Unlike in the off-system incubations, in which an almost linear plot until

45 minutes was obtained, tapering off as the enzyme activity decreased, the formation of 7-HC in the on-system incubations was not as smooth. It was considered that this result could be due to settling of the incubation components in the vial.

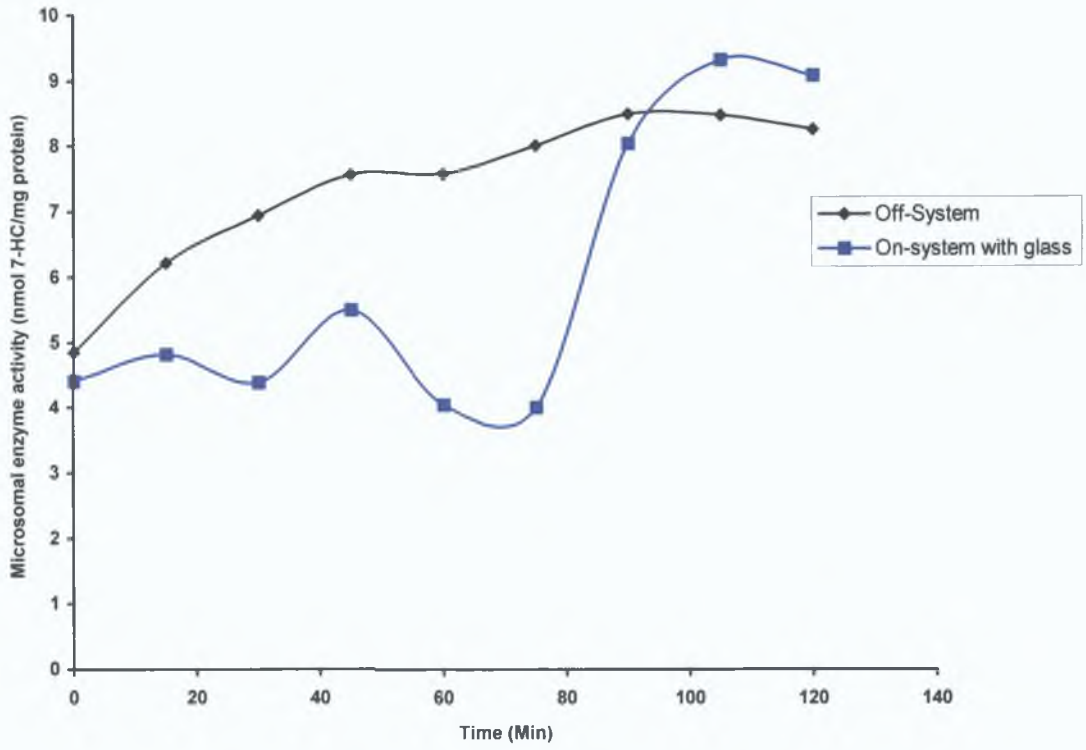


Figure 3.14: *Formation of 7-HC over time for on-system incubations performed in glass vials and for off-system incubations.*

3.3.2.4. On-system Incubations with Sample Agitation.

In the off-system incubation technique, the incubation mixture was agitated gently by vortex-mixing before each aliquot was removed. This sample agitation step was not being performed for the on-system incubations and it was believed that this omitted step was giving rise to the different results obtained for the two methods. To overcome this, an additional step was added to the on-system method. After the

NaOH/SDS rinse, air was flushed into an empty vial, to empty the capillary. Air was then gently flushed into the incubation vial for 0.5 minutes to agitate the incubation components. The resulting 7-HC formation for on-system incubations performed with sample agitation is shown in Figure 3.15. As can be seen from this data, incubations carried out on the CE system using glass vials with agitation of the sample resulted in comparable results to incubations performed by the traditional off-system technique.

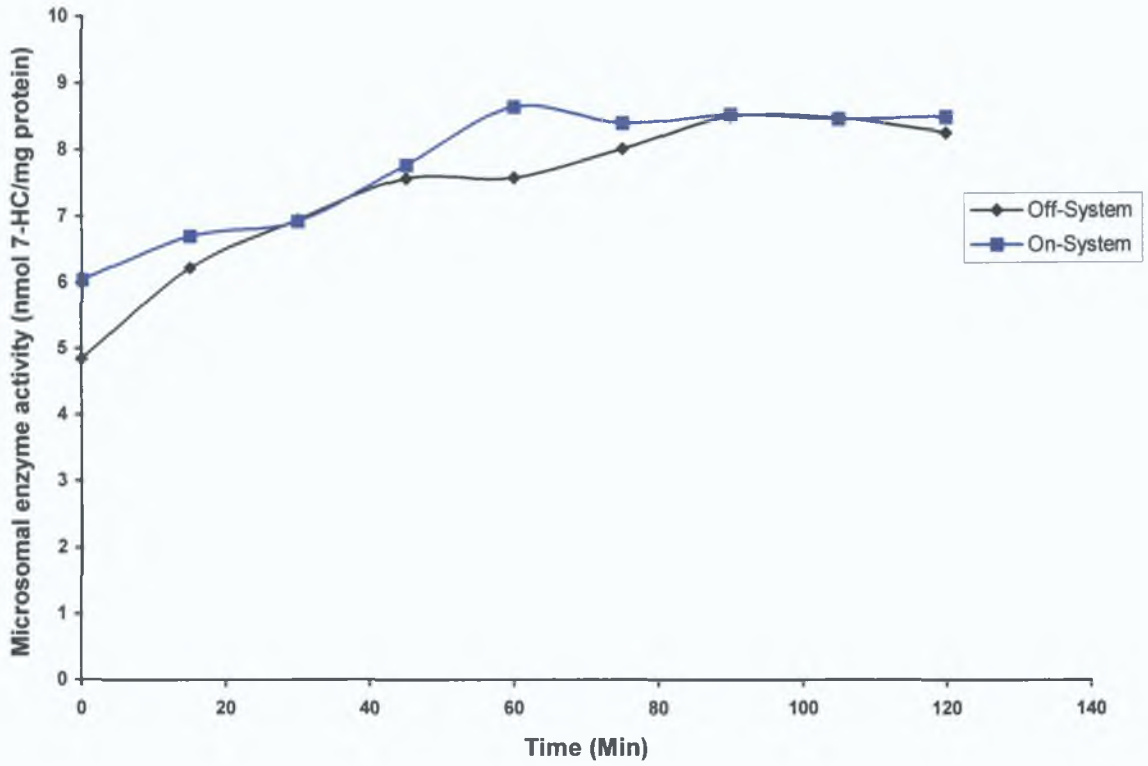


Figure 3.15: *Formation of 7-HC for the developed on-system method and for the off-system method.*

3.3.2.5. Control Incubations and Electropherograms

Figure 3.16 shows overlays of electropherograms obtained for the on-system and off-system incubation methods. A blank incubation and an incubation spiked with 7-HC are included for comparison.

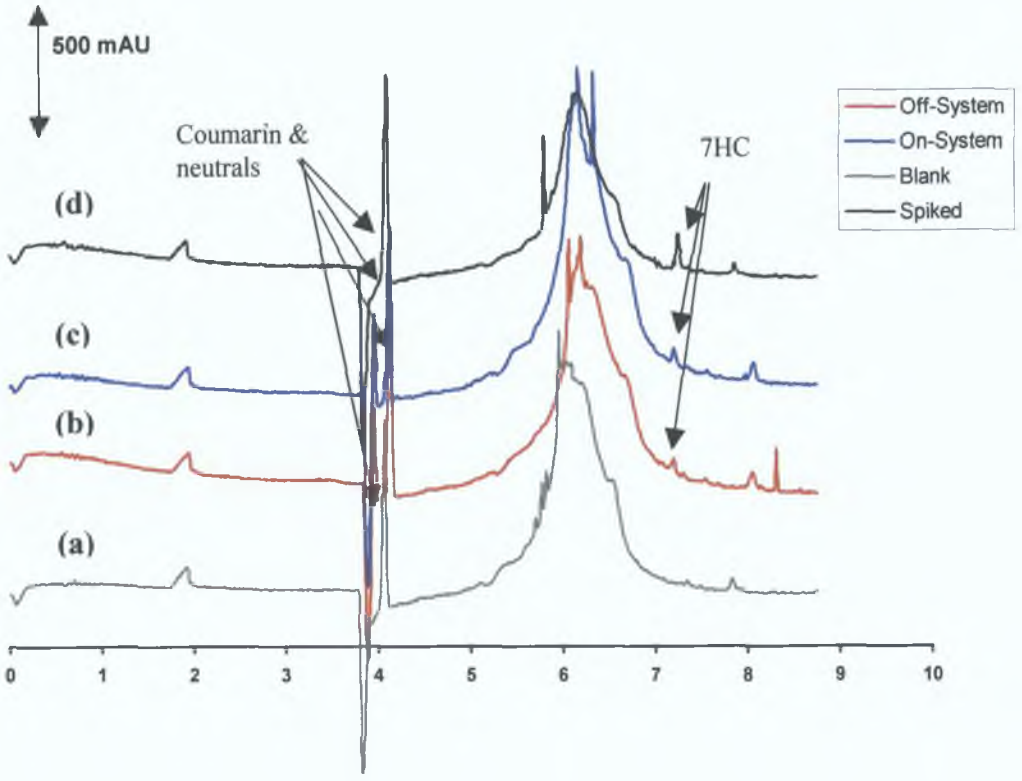


Figure 3.16: *Electropherograms obtained for the on-system and off-system methods.*

Electropherogram of a) blank incubation, b) incubation carried out off-system after 120 minutes, c) incubation carried out on-system after 120 minutes and d) incubation after 120 minutes spiked with 7-HC. The 7-HC peak is indicated on the spiked electropherogram.

The 7-HC peak was clearly visible at approximately 7.25 minutes in the incubation mixture spiked with 7-HC. As expected, this peak did not appear in the control incubation (a), which was performed by substituting the coumarin in the incubation

mixture with phosphate buffer, as described previously This peak was visible, however, in the incubations carried out both on- (c) and off-system (b), confirming the formation of 7-HC during the course of these incubations

3.3.3. Comparison of Techniques

After three incubations were performed using both the developed on-system technique and the traditional off-system technique, formation of 7-HC in both was compared For the off-system method, the overall formation of 7-HC, after 120 minutes, was 8,244 pmol/mg protein In comparison, the formation of 7-HC for the developed on-system method was 8,475 pmol/mg protein at the same time interval, representing a difference of less than 3 %

From Figure 3 15, it can be seen that the formation of 7-HC proceeds in roughly the same manner over the course of the incubations carried out for both methods, with the amount of 7-HC formed increasing rapidly over the first 45 minutes and then reaching a plateau as the activity of the enzyme decreases

3.4. CONCLUSIONS AND FUTURE WORK

Previously described methods for the analysis of microsomal incubations of coumarin have focused on the separation of the primary human metabolite, 7-HC, from the components of the incubation mixture In this chapter, development of a new separation method was described to expand the separation of coumarin metabolites to detect seven potential coumarin metabolites simultaneously Using this separation method, all seven metabolites were resolved from the metabolism assay, in under seven minutes Once this separation had been achieved, it was applied to the development of an automated system to perform microsomal incubations on-system Using the temperature-controlled sample compartment of the instrument, incubation mixtures could be heated to physiological temperature on the system, and injected onto the capillary at timed intervals over an incubation period This on-system method successfully reduced the sample handling and labour intensity associated with drug metabolism techniques whilst allowing minimal sample volumes to be used Results obtained using this automated technique proved to be consistent with those obtained using traditional off-system methods Whilst this method should prove useful in the study of microsomal incubations of coumarin, the poor concentration limits of detection might prove a drawback when the analysis of minor metabolites is required Future research could involve the application of sample stacking techniques to the on-system method in order to improve limits of detection, while at the same time performing the incubations with minimal sample handling

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CHAPTER FOUR

INVESTIGATIONS INTO THE MECHANISM OF pH-MEDIATED SAMPLE STACKING FOR ANIONS PREPARED IN PHOSPHATE BUFFER

4.1. INTRODUCTION

The limitations of CE when combined with UV absorbance detection have been well documented Approaches to overcome this limitation, including sample stacking techniques, have thus received great attention in recent years pH-mediated sample stacking is a relatively immature technique which has been applied to the analysis of both cations and anions in physiological solutions Whilst the stacking effect achieved with pH-mediated sample stacking is lower than that obtained with other stacking approaches, such as FASS and sweeping, its relative ease and direct applicability to high-ionic strength matrices has made it an extremely important technique for the analysis of biochemical samples Whilst this technique has been proven to greatly enhance sensititivity, leading to concentration factors of approximately 10-fold, the mechanism of this technique has been little investigated

Previous research has indicated that for successful pH-mediated sample stacking to occur that an electrokinetic (EK) injection of the sample must be followed by an EK injection of base The reality of this statement has not been fully evaluated, and pH-mediated sample stacking with hydrodynamic (HD) sample injection, or HD base injection has not been reported In addition, previous pH-mediated sample stacking methods have employed Ringer's solution as the sample matrix In this system, the sample cations (Na^+) are replaced by the ammonium ion during the EK injection of the sample This ammonium, which is now in the sample zone, is then titrated to ammonia by the hydroxide ions in the base This titration leads to the formation of a low conductivity sample zone across which sample stacking occurs The effect of performing pH-mediated sample stacking on samples prepared in alternative sample matrices has not yet been evaluated

The aim of this research was to study the effects of sample injection type and length on the stacking effect achieved In addition, the effect of applying pH-mediated sample stacking to the analysis of samples prepared in phosphate buffer, another commonly used physiological matrix, was also investigated

4.2. EXPERIMENTAL

4.2.1. Instrumentation

All separations were carried out on a Beckman P/ACE system MDQ instrument (Fullerton, CA) equipped with a fixed wavelength UV detector The capillary employed was polyimide fused silica of 50 μm internal diameter and 375 μm outer diameter from Polymicro Technologies (Phoenix, AZ) The pH values of buffer solutions were determined using an EDT microprocessor pH meter, model RE 357

4.2.2. Reagents and Analytes

HPAA, Potassium dihydrogenphospate and potassium hydroxide were purchased from Aldrich Chemical Co (Milwaukee, WI) All other chemicals were purchased from Sigma (St Louis, MO)

4.2.3. Preparation of Solutions

The background electrolyte was prepared from 50 mM methylamine and adjusted to pH 10 7 by addition of 1 M ammonium hydroxide In order to reverse the EOF, TTAB was added to the BGE at a concentration of 0 5 mM (Huang *et al* , 1989) EOF reversal has been discussed in detail in Chapter 2 Phosphate buffer was prepared from 25 mM potassium dihydrogen phosphate and adjusted to pH 7 5 by addition of 0 1 M potassium hydroxide 0 1 M stock solutions of 7-HC, 4-HC and HPAA were prepared in pure methanol These standards were then diluted in phosphate buffer (25 mM, pH 7 5) to prepare 10 μM standards for injection Standards were also prepared at 10 μM in the methylamine BGE for use in comparative studies

4.2.4. Capillary Electrophoresis Separation

The separation capillary was fused silica, 50 μM x 61 2 cm (50 cm to detector) The capillary was initially primed by sequential flushing at 30 °C with methanol (5 minutes), 1 M hydrochloric acid (5 minutes), H_2O, (2 minutes) 0 1 M sodium hydroxide (NaOH) (5 minutes), H_2O (2 minutes) and the BGE (7 minutes) Between runs the capillary was flushed at 50 p s i for 1 minute with 5 mM sodium dodecyl sulphate (SDS) / 0 1 M NaOH (50 50, v/v), 1 mmute with 0 1 M NaOH and 1 mmute with the BGE Samples were introduced onto the capillary by hydrodynamic or electrokinetic injections of 45 seconds The base was introduced by either a hydrodynamic or electrokinetic injection, of varying lengths The concentration of the NaOH used was 50 mM, to match the concentration of the BGE This had previously been optimised by Zhao (1997) and so was not investigated further Separation was carried out at –20 kV with a rise time of 0 17 minutes

4.2.5. Control Experiments

In order to evaluate the stacking effect achieved, samples were prepared in phosphate buffer (25 mM, pH 7 5) and in the BGE and injected without sample stacking (i e there was no proceeding base injection) Samples were introduced by both HD and EK injection for a range of injection times from 5 to 45 seconds

4.2.6. Optimisation of the sample injection:base injection ratio

For each of the injection types, a sample injection time of 45 seconds was initially chosen Base injection times were then varied in order to determine the optimal injection ratio 45 seconds was initially chosen as previous research had indicated that an injection ratio of approximately 2 1 tended to be optimal (Zhao, 1997) and

the maximum injection time allowed by the instrument was 99 seconds Base injection times ranging from 45 seconds to 99 seconds were thus investigated As increased sensitivity was desired for the analytes, the optimal ratio was taken to be that which yielded the greatest peak heights in each case

4.2.7. Optimisation of sample injection time

Once the optimal injection ratio was determined, samples were injected for a range of sample injection times followed by injection of the base at the optimal ratio previously determined

4.2.8. Calculation of peak efficiency, N.

Peak efficiencies were calculated based on the formula

Eqn 4 1

$$N = 5\,54\,(T_i/W_{1/2})^2$$

where

N = efficiency

T_i = Migration time of peak

$W_{1/2}$ = Peak width at half-height

4.3. RESULTS AND DISCUSSION

4.3.1. Control Separations

In order to effectively evaluate whether stacking was taking place, and if it was, to measure the extent of it, control separations were performed Standards were prepared in phosphate buffer, and in the BGE, and in both cases were introduced by either EK or HD injection No base injection was performed Sample injection times were varied in order to determine the optimal conditions, and also to determine at what injection length the capillary became overloaded Table 4 1 shows the results obtained for standards prepared in phosphate and introduced by HD injection

Table 4 1 *Hydrodynamic sample injections of varying lengths Standards were 10 μM prepared in phosphate (pH 7 5, 25 mM) (n = 3)*

Sample Inj Time (s)	HPAA Peak Height (mAU)	HPAA Peak Width at ½ height (min)	7-HC Peak Height (mAU)	7-HC Peak Width at ½ height (min)	4-HC Peak Height (mAU)	4-HC Peak Width at ½ height (min)
5	68 ± 15	0 054 ± 0 022	225 ± 72	0 098 ± 0 093	98 ± 39	0 417 ± 0 198
10	109 ± 12	0 073 ± 0 001	214 ± 17	0 083 ± 0 012	148 ± 26	0 206 ± 0 042
15	127 ± 9	0 133 ± 0 017	269 ± 29	0 102 ± 0 023	178 ± 17	0 186 ± 0 018
20	179 ± 34	0 226 ± 0 081	325 ± 40	0 090 ± 0 005	181 ± 37	0 205 ± 0 039
25	179 ± 12	0 226 ± 0 010	401 ± 52	0 096 ± 0 010	238 ± 31	0 232 ± 0 028
30	145 ± 18	0 355 ± 0 213	441 ± 188	0 280 ± 0 032	269 ± 10	0 352 ± 0 028
35	141 ± 7	0 375 ± 0 105	460 ± 28	0 258 ± 0 032	317 ± 35	0 307 ± 0 053
40	173 ± 20	0 320 ± 0 041	386 ± 38	0 302 ± 0 027	339 ± 22	0 353 ± 0 032
45	160 ± 7	0 534 ± 0 118	398 ± 12	0 347 ± 0 037	347 ± 22	0 389 ± 0 046

Standards were also prepared in the BGE, and injected for various injection lengths Control standards are routinely prepared in the BGE as they allow comparisons to be drawn against a so-called 'ideal' situation, i e one where no band-broadening or negative sample matrix effects would be observed Table 4 2 below shows the results obtained when samples were prepared in the BGE and introduced for a range of injection times from 5 to 45 seconds by HD injection

Table 4 2 *Hydrodynamic sample injections of varying lengths Standards were 10 µM prepared in methylamine BGE (pH 10 7, 50 mM) (n = 3)*

Sample Inj Time (s)	HPAA Peak Height (mAU)	HPAA Width (½ height)	7-HC Peak Height (mAU)	7-HC Width (½ height)	4-HC Peak Height (mAU)	4-HC Width (½ height)
5	139 ± 27	0 136 ± 0 022	99 ± 40	0 271 ± 0 230	135 ± 49	0 274 ± 0 121
10	170 ± 35	0 170 ± 0 028	158 ± 21	0 332 ± 0 242	179 ± 54	0 306 ± 0 084
15	140 ± 12	0 168 ± 0 069	190 ± 6	0 203 ± 0 085	289 ± 81	0 275 ± 0 068
20	185 ± 42	0 137 ± 0 014	230 ± 45	0 206 ± 0 114	346 ± 18	0 280 ± 0 069
25	149 ± 77	0 208 ± 0 042	232 ± 90	0 261 ± 0 060	302 ± 137	0 305 ± 0 024
30	187 ± 15	0 195 ± 0 027	305 ± 41	0 257 ± 0 057	427 ± 41	0 277 ± 0 016
35	210 ± 30	0 274 ± 0 030	307 ± 37	0 248 ± 0 046	481 ± 71	0 391 ± 0 133
40	226 ± 12	0 233 ± 0 046	353 ± 66	0 274 ± 0 060	518 ± 78	0 334 ± 0 054
45	196 ± 41	0 313 ± 0 087	443 ± 19	0 307 ± 0 036	540 ± 132	0 249 ± 0 114

The same standards (i e prepared in phosphate and prepared in BGE) were then introduced by EK injection, for the same range of injection times, in order to provide a control against future stacking experiments which involved EK sample injection Table 4 3 and Table 4 4 show the results obtained for samples prepared in phosphate buffer and in the BGE, respectively, and introduced by EK injection

Table 4 3 *Electrokinetic sample injections of varying lengths Standards were 10 µM prepared in phosphate (pH 7 5, 25 mM) (n = 3)*

Sample Inj Time (s)	HPAA Peak Height (mAU)	HPAA Peak Width at ½ height (min)	7-HC Peak Height (mAU)	7-HC Peak Width at ½ height (min)	4-HC Peak Height (mAU)	4-HC Peak Width at ½ height (min)
5	29 ± 24	0 292 ± 0 007	113 ± 8	0 279 ± 0 130	84 ± 25	0 263 ± 0 009
10	60 ± 7	0 424 ± 0 000	167 ± 11	0 054 ± 0 084	154 ± 13	0 166 ± 0 104
15	62 ± 1	0 430 ± 0 000	145 ± 11	0 139 ± 0 028	165 ± 22	0 223 ± 0 008
20	97 ± 32	0 490 ± 0 007	189 ± 34	0 078 ± 0 114	215 ± 7	0 254 ± 0 013
25	143 ± 13	0 445 ± 0 003	145 ± 12	0 167 ± 0 114	159 ± 14	0 316 ± 0 010
30	152 ± 29	0 495 ± 0 006	188 ± 24	0 271 ± 0 060	219 ± 18	0 420 ± 0 015
35	151 ± 31	0 581 ± 0 001	214 ± 31	0 112 ± 0 054	155 ± 21	0 311 ± 0 020
40	136 ± 22	0 698 ± 0 006	204 ± 22	0 109 ± 0 023	169 ± 14	0 306 ± 0 009
45	116 ± 26	0 621 ± 0 004	249 ± 33	0 421 ± 0 087	248 ± 17	0 294 ± 0 008

Table 4 4. *Electrokinetic sample injections of varying lengths Standards were 10 µM prepared in methylamine BGE (pH 10 7, 50 mM) (n = 3)*

Sample Inj Time (s)	HPAA Peak Height (mAU)	HPAA Peak Width at ½ height (min)	7-HC Peak Height (mAU)	7-HC Peak Width at ½ height (min)	4-HC Peak Height (mAU)	4-HC Peak Width at ½ height (min)
5	99 ± 7	0 049 ± 0 002	167 ± 23	0 067 ± 0 004	154 ± 20	0 128 ± 0 023
10	140 ± 8	0 158 ± 0 163	266 ± 7	0 107 ± 0 036	288 ± 52	0 130 ± 0 028
15	143 ± 13	0 112 ± 0 021	300 ± 10	0 102 ± 0 009	395 ± 16	0 146 ± 0 053
20	131 ± 9	0 153 ± 0 064	286 ± 18	0 181 ± 0 096	373 ± 25	0 184 ± 0 055
25	152 ± 26	0 184 ± 0 029	266 ± 48	0 158 ± 0 011	387 ± 98	0 146 ± 0 007
30	162 ± 7	0 195 ± 0 032	299 ± 17	0 173 ± 0 017	402 ± 48	0 179 ± 0 014
35	201 ± 53	0 319 ± 0 018	305 ± 66	0 209 ± 0 009	391 ± 147	0 213 ± 0 042
40	185 ± 3	0 203 ± 0 009	326 ± 54	0 222 ± 0 027	414 ± 76	0 208 ± 0 020
45	143 ± 25	2 488 ± 0 039	298 ± 18	0 262 ± 0 045	385 ± 27	0 280 ± 0 017

This data (Table 4 1 to Table 4 4) was then used to provide a control against which to measure sample stacking as achieved in future experiments

4.3.2 Effect of Injection Type on pH-Mediated Stacking

pH-mediated sample stacking has previously only been reported for EK sample injection and indeed it has been reported that pH-mediated stacking did not occur when HD injection was employed for either the sample or the acid/base introduction (Park, 1998) In this study the effect of performing pH-mediated sample stacking with HD injection for either the sample or base introduction, or for the introduction of both, has been investigated

4.3.2.1. HD/HD Injection Scheme

For the HD/HD injection scheme both sample and base injections were performed by applying pressure Table 4 5 below shows the results that were obtained under these conditions

Table 4 5 *Hydrodynamic sample injections of 45 seconds followed by hydrodynamic base injections of varying lengths Standards were 10 μM prepared in phosphate (pH 7 5, 25 mM) (n = 3)*

Base Inj Time (s)	HPAA Peak Height (mAU)	HPAA Peak Width at $^1/_2$ height (min)	7-HC Peak Height (mAU)	7-HC Peak Width at $^1/_2$ height (min)	4-HC Peak Height (mAU)	4-HC Peak Width at $^1/_2$ height (min)
45	822 ± 89	0 054 ± 0 002	1255 ± 90	0 088 ± 0 003	865 ± 49	0 201 ± 0 006
55	925 ± 27	0 052 ± 0 001	1096 ± 50	0 092 ± 0 002	584 ± 25	0 249 ± 0 019
65	1000 ± 19	0 051 ± 0 001	1104 ± 58	0 089 ± 0 001	481 ± 49	0 367 ± 0 066
75	989 ± 53	0 051 ± 0 001	1058 ± 39	0 086 ± 0 002	378 ± 18	0 576 ± 0 036
85	960 ± 132	0 052 ± 0 001	1024 ± 38	0 088 ± 0 005	328 ± 53	0 508 ± 0 028
90	1058 ± 11	0 054 ± 0 001	1037 ± 31	0 088 ± 0 002	253 ± 36	0 518 ± 0 031
95	1061 ± 18	0 056 ± 0 001	1177 ± 34	0 089 ± 0 001	273 ± 3	0 534 ± 0 040
99	1112 ± 37	0 035 ± 0 005	1615 ± 18	0 042 ± 0 001	296 ± 19	0 153 ± 0 035

By comparison of Table 4 5 with Table 4 1 it can be determined that base-mediated sample stacking is occurring for two of the species in the phosphate mixture, when HD sample injection is followed by a HD base injection HPAA and 7-HC both show greatly increased peak heights, and narrower peak widths when the proceeding base injection is employed For HPAA the increase in peak height is from 160 mAU to 1,112 mAU – an increase of almost 7-fold For 7-HC the increase in peak height is 4-fold from 398 mAU to 1,615 mAU This comparison is drawn from a 45 second injection of sample followed by a 99 second injection of base, both performed by HD injection Peak widths are also narrower for these two analytes when the base injection is employed As it can be assumed that in each case, the same amount of sample is being loaded on to the capillary (i e 45 second HD injection of sample in each case), then the improvement in peak height and decrease in peak width must be attributed to a narrowing of the sample zone on the capillary A different result is seen for the third analyte, 4-HC, however By comparison with Table 4 1, 4-HC shows an improvement in peak height when a short base injection

is employed (i e 45 seconds) but the peak height drops dramatically as the base injection time is increased To explain this, it is necessary to look at the proposed mechanism by which pH-mediated sample stacking takes place According to the literature (Park and Lunte, 1998) when the sample is introduced (typically by EK injection), cations in the sample matrix are replaced by cations from the BGE These ammonium ions are then titrated by the injected base anions, leading to the formation of the low conductivity sample zone

With HD injection of the sample, a different phenomenon is seen Cations in the sample matrix are not replaced during the sample injection As the sample zone enters the capillary, displacement of the sample matrix begins to occur at the interface between the sample matrix and the BGE, replacing the sample cations at the front of the sample zone with titrable BGE cations The base is then injected Once on the capillary the hydroxide ions move with high mobility through the sample zone

A possible explanation for the results obtained for the 4-HC species is that due to the HD injection of the sample, not all of the sample matrix ions have been replaced and the back of the sample zone still contains many of the initial sample matrix cations A short base injection is thus sufficient to provide adequate OH^- ions to titrate the small amount of replaced cations Any additional OH^- ions introduced into this region would cause an increase in conductivity in this zone, and subsequently 'destacking' would be the expected result This destacking would be evident in increased peak width and deteriorating peak heights for the slowest migrating sample anions as the base injection time is increased A schematic of this proposed mechanism is shown in Figure 4 1 , but the mechanism will be investigated further before any conclusions will be drawn

Step 1: Capillary filled with BGE

Step 2: HD injection of sample. Sample anions and cations introduced onto capillary. BGE cations begin to replace sample matrix cations at interface of sample zone and BGE.

Step 3: HD injection of base. Na^+ and OH^- introduced onto capillary. BGE cations continue to replace sample matrix cations but full replacement of sample zone has not taken place. OH^- ions titrate replaced portion of sample zone but excess ions in back of sample zone result in increased conductivity.

Step 4: Sample anions at titrated front of sample zone stack and move through capillary in narrow bands. Sample anions at higher conductivity rear of sample zone undergo destacking, and move through capillary in broader bands.

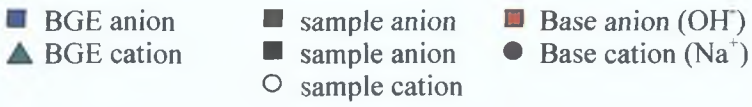

Figure 4.1: *Schematic of proposed mechanism for pH-mediated sample stacking performed with HD sample and HD base injection.*

4 3 2.2 HD/EK Injection Scheme

For the HD/EK injection scheme, HD introduction of the sample was followed by EK introduction of the base

Table 4 6 *Hydrodynamic sample injections of 45 seconds followed by electrokinetic base injections of varying lengths Standards were 10 µM prepared in phosphate (pH 7 5, 25 mM) (n = 3)*

Base Inj Time (s)	HPAA Peak Height (mAU)	HPAA Peak Width at $^1/_2$ height (min)	7-HC Peak Height (mAU)	7-HC Peak Width at $^1/_2$ height (min)	4-HC Peak Height (mAU)	4-HC Peak Width at $^1/_2$ height (min)
45	1917 ± 89	0 043 ± 0 002	2838 ± 505	0 074 ± 0 006	1806 ± 170	0 114 ± 0 004
55	2172 ± 74	0 041 ± 0 001	2840 ± 423	0 068 ± 0 006	1950 ± 245	0 111 ± 0 010
65	2089 ± 77	0 041 ± 0 001	3019 ± 215	0 061 ± 0 003	2091 ± 286	0 106 ± 0 119
75	2200 ± 16	0 044 ± 0 002	3130 ± 232	0 063 ± 0 002	2077 ± 279	0 113 ± 0 006
85	2270 ± 33	0 044 ± 0 001	3285 ± 280	0 065 ± 0 002	2344 ± 263	0 113 ± 0 007
90	2262 ± 101	0 044 ± 0 001	3278 ± 81	0 066 ± 0 001	2218 ± 25	0 119 ± 0 007
95	2218 ± 20	0 044 ± 0 001	3229 ± 102	0 070 ± 0 001	2298 ± 170	0 116 ± 0 001
99	2154 ± 131	0 044 ± 0 001	3108 ± 172	0 073 ± 0 001	2361 ± 268	0 117 ± 0 002

From Table 4 6 it can be seen that stacking also takes place when a HD sample injection is followed by an EK injection of base In this case, peak heights are improved by 13-fold, 8-fold and 7-fold (for HPAA, 7-HC and 4-HC respectively) when the base injection is performed for 85 seconds This is based on improvements in peak height from 160 mAU to 2,154 mAU for HPAA, from 398 mAU to 3,108 mAU for 7-HC and from 347 mAU to 2,361 mAU for 4-HC It does appear that efficient sample stacking is thus occurring for the 4-HC species, in contrast to the HD/HD injection scheme It can be argued that an EK injection of the base is more efficient, preferentially introducing the fast-moving OH^- ions, and so allowing more OH^- to be introduced in a smaller matrix volume This would

allow more room on the capillary for the separation to occur If, however, as was proposed earlier, the drawback with the pressure injection of the sample is that sufficient replacement of the sample matrix ions with the titrable BGE cations has not occurred for full titration of the sample zone to take place, more efficient OH^- injection would not overcome this It would appear that for this HD/EK case, that the replacement of the sample zone cations has occurred right through the sample zone before the OH^- ions pass through This argues against the scheme put forward in Section 4 3 2 1 *which proposed* that the use of a HD sample injection leads to incomplete replacement of the sample zone before the OH^- titration

Another explanation for the lack of apparent stacking seen with HD injection of the base could be that the long HD base injection time effectively overloads the capillary, leading to poor resolution and peak shape In support of this, the 4-HC peak width increases rapidly as the base injection time is increased If, however, too much of the capillary was being taking up by the stacking process, poor resolution of the peaks would be expected, and poor peak shape for all of the analytes, which is not the case Figure 4 2 shows an electropherogram obtained using the HD/HD injection process The electropherogram in Figure 4 2 shows good peak shape for both the HPAA and 7-HC species, while the 4-HC peak shape, conversely, is poor This discounts the theory that a long HD injection of base overloads the capillary In this case, poor peak shape and resolution would be expected for all of the analytes Figure 4 3 is a schematic of the proposed mechanism for sample stacking with a HD sample injection followed by an EK base injection

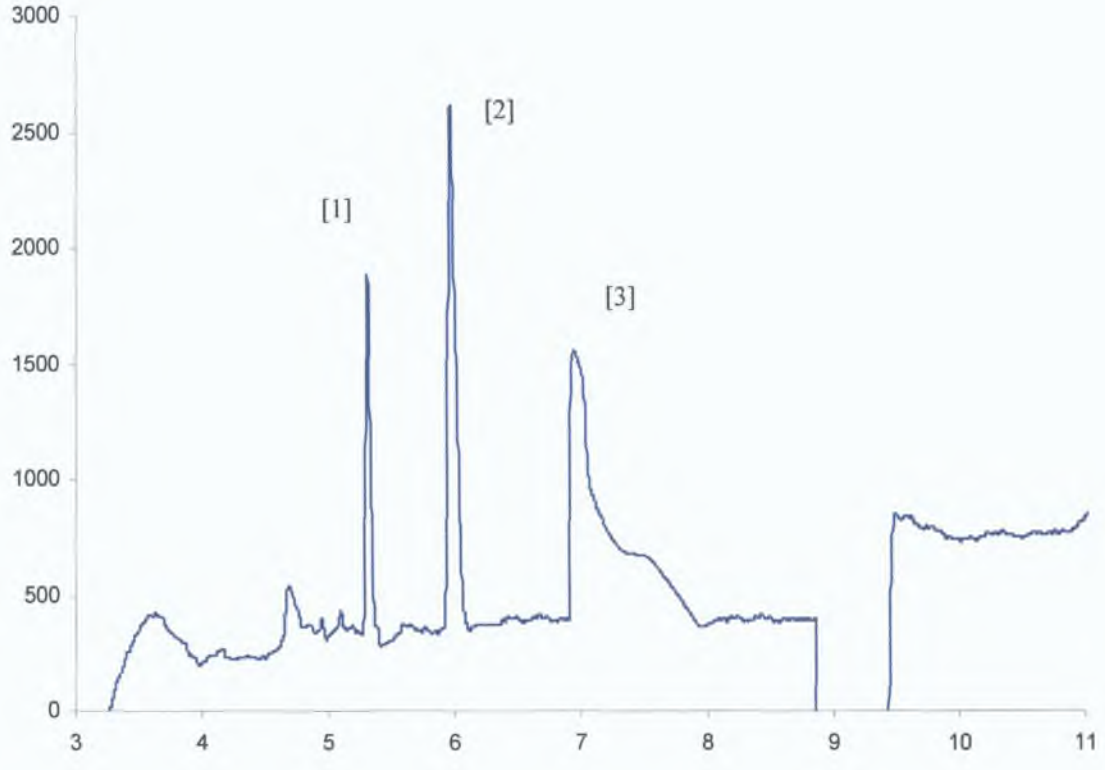

Figure 4.2: *Separation of a standard 10 μM mixture of [1] HPAA [2] 7-HC and [3] 4-HC using HD/HD injections. The electropherogram was obtained for a 45 second HD injection of sample followed by a 99 second HD injection of base.*

Step 1: Capillary filled with BGE

Step 2: HD injection of sample. Sample anions and cations introduced onto capillary. BGE cations begin to replace sample matrix cations at interface of sample zone and BGE.

Step 3: EK injection of base. Base anions (i.e. OH^-) preferentially introduced onto capillary, creating highly negative zone at rear of sample zone, which attracts BGE cations. Cations move through sample zone, which is then titrated by the faster moving OH^- ions.

Step 4: Sample anions stack in low conductivity zone and move through capillary in narrow bands.

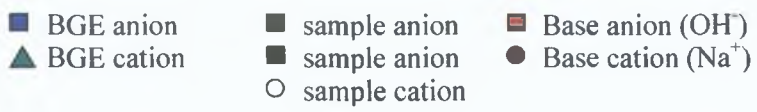

Figure 4.3: *Schematic of proposed mechanism for pH-mediated sample stacking performed with a HD sample injection and EK base injection.*

4 3 2 3 EK/HD Injection Scheme

The results obtained when an EK injection of sample is followed by a HD injection of base are shown in Table 4 7

Table 4 7 *Electrokinetic sample injections of 45 seconds followed by hydrodynamic base injections of varying lengths Standards were 10 μM prepared in phosphate (pH 7 5, 25 mM)*

Base Inj Time (s)	HPAA Peak Height (mAU)	HPAA Peak Width at $^1/_2$ height (min)	7-HC Peak Height (mAU)	7-HC Peak Width at $^1/_2$ height (min)	4-HC Peak Height (mAU)	4-HC Peak Width at $^1/_2$ height (min)
45	1081 ± 30	0 0297 ± 0 0007	1392 ± 95	0 0491 ± 0 0012	1110 ± 96	0 1063 ± 0 0172
55	1092 ± 31	0 0285 ± 0 001	1403 ± 68	0 0477 ± 0 0001	995 ± 78	0 0921 ± 0 0016
65	1207 ± 32	0 0295 ± 0 0007	1529 ± 110	0 0479 ± 0 001	1035 ± 153	0 0926 ± 0 0052
75	1265 ± 77	0 0293 ± 0 0005	1537 ± 102	0 0459 ± 0 0002	966 ± 120	0 0858 ± 0 0007
85	1435 ± 12	0 0304 ± 0 0008	1635 ± 50	0 0441 ± 0 0004	853 ± 80	0 0778 ± 0 0023
90	1501 ± 81	0 0307 ± 0 0003	1528 ± 88	0 0436 ± 0 0007	639 ± 24	0 0732 ± 0 0038
95	1460 ± 37	0 0304 ± 0 0004	1487 ± 20	0 0430 ± 0 0005	382 ± 117	0 0720 ± 0 0033
99	1543 ± 53	0 0315 ± 0 0004	1552 ± 47	0 0433 ± 0 0002	380 ± 48	0 0705 ± 0 0003

The results for an EK injection of sample followed by a HD base injection are shown in Table 4 7 For this combination of injections, peak heights are compared against Table 4 3, in which samples are introduced by EK injection, with no sample stacking The same amount of sample should therefore be introduced onto the capillary and so the stacking effect achieved can be directly determined Stacking appears again to occur for the HPAA and 7-HC species, with improvements in peak height of almost 5- and 7-fold, based on a 99 second base injection As with the HD/HD injections, however, peak height for the 4-HC species decreases

significantly as the base injection length is increased For a 99 second injection of base there is a slight improvement in peak height over the unstacked sample, but at less than 2 %, this can be considered negligible

These results would again indicate that the EK base injection is more efficient for the stacking process In each case where HD base injection has been employed, little or no stacking effect has been seen for the 4-HC species, at the base injection lengths which provide optimal stacking results for the other two analytes Figure 4 4 is a schematic of the proposed EK/HD stacking mechanism

Step 1: Capillary filled with BGE

Step 2: EK injection of sample. Sample anions preferentially introduced onto capillary. BGE cations begin to replace sample cations in sample zone.

Step 3: HD injection of base. Na^+ and OH^- introduced onto capillary. BGE cations continue to replace sample matrix cations but full replacement of sample zone has not taken place.OH^- ions titrate replaced portion of sample zone but excess ions in back of sample zone result in increased conductivity.

Step 4: Sample anions at titrated front of sample zone stack and move through capillary in narrow bands. Sample anions at higher conductivity rear of sample zone undergo destacking, and move through capillary in broader bands.

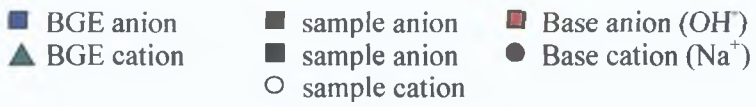

Figure 4.4: *Schematic of proposed mechanism for pH-mediated sample stacking performed with EK sample injection and HD base injection.*

4 3 2.4 EK/EK Injection Scheme

For the final injection combination, both sample and base introduction were performed by electrokinetic injection The results are shown in Table 4 8

Table 4 8 *Electrokinetic sample injections of 45 seconds followed by electrokinetic base injections of varying lengths Standards were 10 μM prepared in phosphate (pH 7 5, 25 mM)*

Base Inj Time (s)	HPAA Peak Height (mAU)	HPAA Peak Width at $^1/_2$ height (min)	7-HC Peak Height (mAU)	7-HC Peak Width at $^1/_2$ height (min)	4-HC Peak Height (mAU)	4-HC Peak Width at $^1/_2$ height (min)
45	1087 ± 25	0 036 ± 0 001	2485 ± 94	0 051 ± 0 005	2238 ± 80	0 132 ± 0 004
55	1185 ± 60	0 037 ± 0 000	2433 ± 78	0 055 ± 0 001	2284 ± 110	0 128 ± 0 009
65	1427 ± 231	0 039 ± 0 002	1746 ± 132	0 066 ± 0 004	2452 ± 181	0 150 ± 0 016
75	1728 ± 16	0 040 ± 0 000	2818 ± 281	0 063 ± 0 003	2490 ± 142	0 132 ± 0 004
85	1734 ± 138	0 040 ± 0 001	2300 ± 118	0 068 ± 0 004	2481 ± 203	0 136 ± 0 003
90	1775 ± 30	0 043 ± 0 001	2372 ± 156	0 081 ± 0 004	2923 ± 289	0 152 ± 0 014
95	1738 ± 56	0 040 ± 0 001	2495 ± 158	0 076 ± 0 005	2977 ± 349	0 143 ± 0 008
99	1502 ± 32	0 039 ± 0 003	2904 ± 354	0 049 ± 0 001	2638 ± 265	0 121 ± 0 021

The results of the EK/EK injections are shown in Table 4 8 As expected, stacking is seen for all three species Compared to EK injection alone, stacking factors of 5-fold, 10- fold and 9-fold are achieved for HPAA, 7-HC and 4-HC respectively (based on a 95 second base injection) It would appear from these results that whilst either HD or EK injection of the sample is conducive to sample stacking, EK injection of the base is required for efficient stacking of all the analytes This could be due to the fact that more of the capillary is taken up by a HD injection than by an EK one, thus reducing the length of capillary available for the separation It is evident that in both situations (i e either HD or EK injection of the base) that

sufficient OH ions are being introduced, as the front of the sample zone is fully titrated, resulting in efficient stacking of the faster migrating anions (HPAA and 7-HC) Figure 4 5 is a schematic of sample stacking performed with an EK injection of sample followed by an EK injection of base

Step 1: Capillary filled with BGE.

Step 2: EK injection of sample. Sample anions preferentially introduced into capillary. BGE cations begin to replace sample cations in sample zone.

Step 3: EK injection of base. Base anions (i.e. OH^-) preferentially introduced into capillary, creating highly negative zone at rear of sample zone, which attracts BGE cations. Cations move through sample zone, which is then titrated by the faster moving OH- ions.

Step 4: Sample ions stack in low conductivity zone and move through capillary in narrow bands.

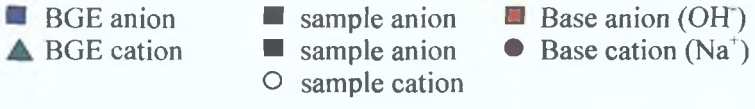

Figure 4.5: *Schematic of proposed mechanism for pH-mediated sample stacking performed with EK sample and EK base injection.*

4 3 2 5 Optimised Stacking Effect

As increased sensitivity was the main goal in this study, the optimised stacking effect was taken to be that which resulted in the greatest increase in peak heights for the analytes In cases where the greatest improvements in peak heights for the individual analytes did not occur at the same base injection lengths, the optimal base injection time was taken to be that which resulted in the greatest increase in peak heights for the majority of the analytes Table 4 9 shows the optimised stacking effect achieved for the three analytes under each of the injection combinations carried out The stacking effect was determined by comparing the peak height for the stacking technique with the peak height for the injection alone The peak height for the stacking technique was taken to be that obtained at the optimum ratio previously determined HD/HD and HD/EK injection combinations were compared with HD injection alone and EK/EK and EK/HD injection combinations were compared to EK injection alone In these cases the same amount of the sample should have been introduced onto the capillary and a direct comparison can be made

Table 4.9 *Optimised stacking effect for each injection combination Standards were 10 μM prepared in phosphate (pH 7 5, 25 mM) Figures have been rounded to the nearest whole number*

Mode	Base In Length (s)	HPAA Stacking effect (Peak height with stacking, without stacking)	7-HC Stacking effect (Peak height with stacking, without stacking)	4-HC Stacking effect (Peak height with stacking, without stacking)
HD/HD	99	7 (1,112 mAU, 160 mAU)	4 (1,615 mAU, 398 mAU)	1* (296 mAU, 347 mAU)
HD/EK	85	13 (2,270 mAU, 160 mAU)	8 (3,285 mAU, 398 mAU)	7 (2,344 mAU, 347 mAU)
EK/HD	99	5 (1,543 mAU, 116 mAU)	7 (1,552 mAU, 249 mAU)	2 (380 mAU, 248 mAU)
EK/EK	95	5(1,738 mAU, 116 mAU)	10 (2,495 mAU, 249 mAU)	9 (2,638 mA, 248 mAU)

* An actual decrease in peak height was observed for 4-HC when HD/HD injection was performed

From Table 4 9 it is evident that sample stacking occurs for all three species when an EK base injection is performed, regardless of the mode of introduction of the sample Both HD/EK and EK/EK schemes achieved sample stacking to some extent for all three of the analytes In contrast, however, the peak heights of the slowest migrating analyte, the 4-HC, decreased rapidly as the base injection time was increased when a HD base injection was performed Thus, for a HD base injection, the optimal base injection ratio for the first two analytes, HPAA and 7-HC, resulted in a negligible stacking effect for the 4-HC species

A possible explanation for this could lie in the biased introduction that takes place when an EK injection is performed Overall it can be conceded that base-mediated sample stacking is dependent on EK injection of the base for full stacking to be achieved In each of the schemes listed and performed above, it was the HD/EK and

EK/EK injection schemes which resulted in sample stacking for analytes throughout the entire sample zone

When an EK injection is performed, preferential introduction occurs for the faster-moving, more highly charged ions In the case of reversed polarity (as in the base-stacking performed in this chapter), it is the anions which are preferentially introduced Thus, when EK injection of the NaOH is performed, it is mainly OH^- ions which are injected onto the capillary Figure 4 3 is a schematic of the proposed mechanism that takes places when the base is introduced by EK injection The preferential introduction of OH ions results in a highly negatively charged zone at the back of the capillary Whilst the base introduction is taking place, NH_4^+ cations, which are opposing the EOF, are moving into the sample zone and replacing the sample cations The result of the large negative charge at the back of the sample zone, is to attract the NH_4^+ cations towards this negative zone, as they tend to electroneutrality, causing complete replacement of the sample zone to occur Thus the highly negative OH^- zone effectively gives the ammonium cations the momentum they need to complete the replacement of the sample zone When a HD injection of base is used, the zone introduced contains both Na^+ cations and OH anions, and thus does not have this overall net negative charge which attracts the cations of the BGE As a result, the movement of the BGE cations through the sample zone is slower, and thus the replacement of the cations in the sample zone is incomplete by the time the OH^- ions migrate through it, resulting in incomplete titration of the back of the sample zone This would lead to stacking of the analytes at the front of the zone, i e the faster moving 7-HC and HPAA, whilst stacking would not take place for the slower moving 4-HC, which is in the untitrated portion at the back of the sample zone

Where the base introduction was performed by HD injection, full titration of the sample zone did not occur, resulting in a lack of stacking for the slowest migrating analyte, in this case 4-HC It can thus be concluded that for successful base-mediated sample stacking to occur, an EK base injection is required Contrary to

previous reports, however, sample introduction can be performed either by HD or EK injection This is important factor for this technique, as whilst the preferential introduction of certain ions from the sample is sometimes desirable, it does lead to discrimination in the amount of each sample component introduced Where a quantitative representation of the sample components is required, HD injection must be used in order to ensure an unbiased introduction of each of the sample components

Whilst both HD/EK and EK/EK injection schemes achieve sample stacking for all three of the analytes, it is to varying extents Whilst the HD/EK injection combination achieves a much greater stacking effect for the HPAA species, greater effects for the other two analytes are achieved with the EK/EK injection combination Thus it would appear that injection conditions should be optimised for the analytes of interest during the method development procedure

4.3.3. Current Profiles

Previous discussions of the mechanism for pH-mediated stacking have focused on the monitoring of the current during the electrophoretic process During the sample injection, the current reaches its highest level, due to introduction of the high conductivity sample matrix into the capillary Conversely, while the base injection is performed, the current decreases dramatically, in response to the low conductivity zone created by the titration of the sample zone During the separation phase, the current finally levels out at the current which reflects BGE only in the capillary The current profile during the experimental process can thus provide valuable information on the process that is taking place inside the capillary

With reverse polarity, as in this study, a decrease in the recorded current should be observed at the start of the separation, before the current levels out at the current recorded with BGE only in the capillary This profile would prove that there is a discontinuous buffer system within the capillary and that a low conductivity region has been created in which sample stacking can take place Thus, monitoring of the current during the stacking process can give a further strong indication of whether sample stacking is taking place or not The current as monitored for the injection combinations are shown in Figures 4 6 to 4 9 respectively In each figure, the current obtained for BGE in the capillary alone is indicated

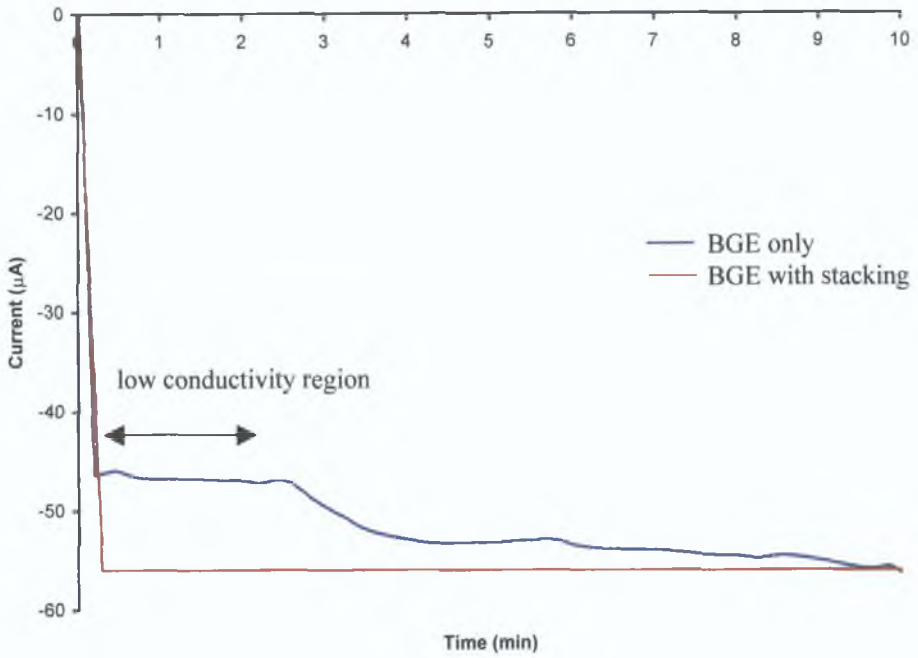

Figure 4.6: *Current profile recorded for pH-mediated sample stacking performed with a hydrodynamic sample injected followed by a hydrodynamic base injection.*

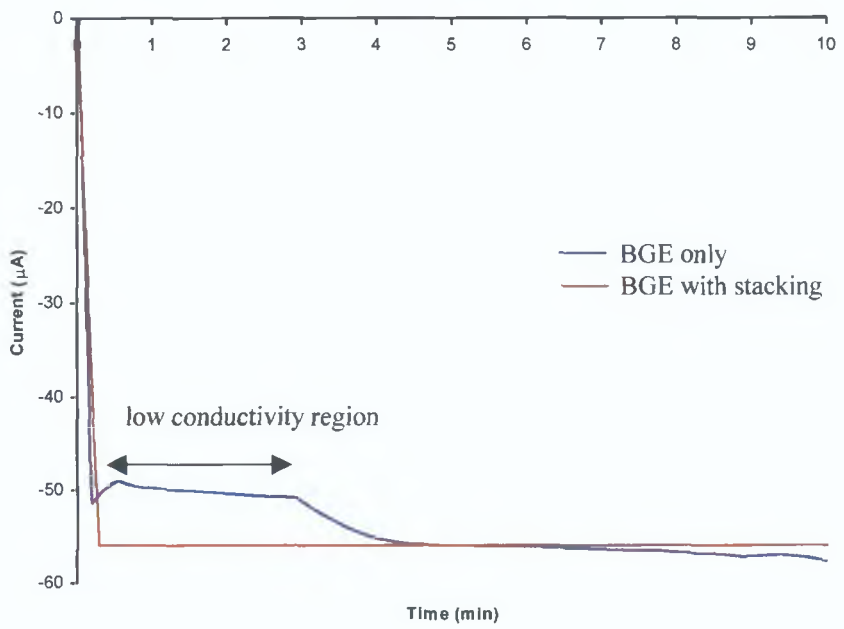

Figure 4.7: *Current profile recorded for pH-mediated sample stacking performed with a hydrodynamic sample injection followed by an electrokinetic base injection.*

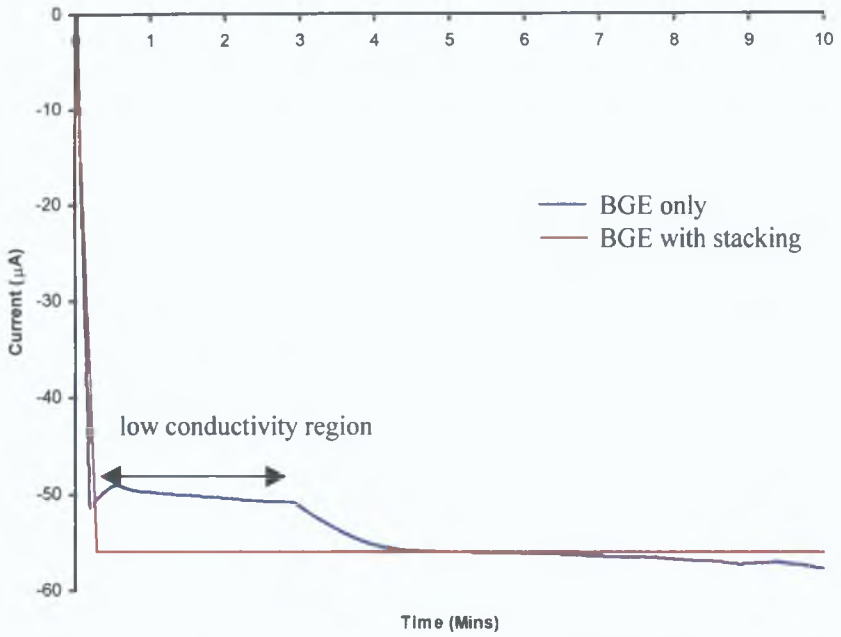

Figure 4.8: *Current profile recorded for pH-mediated sample stacking performed with an electrokinetic sample injection followed by a hydrodynamic base injection.*

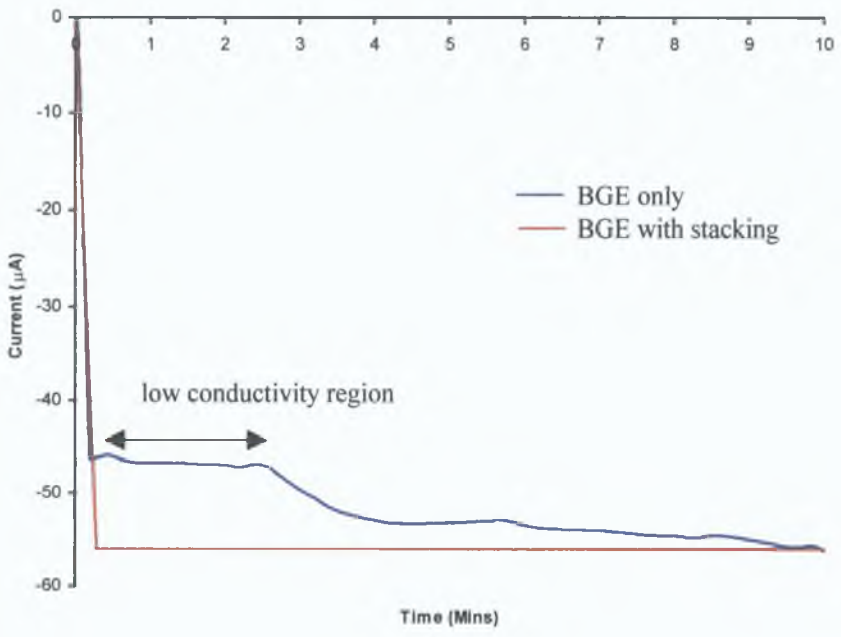

Figure 4.9: *Current profile recorded for pH-mediated sample stacking performed with an electrokinetic sample injection followed by an electrokinetic base injection.*

It can be determined from these current profiles that sample stacking is taking place to some extent on the capillary for all of these injection combinations The decrease in current as the base is injected, which eventually increases to the current achieved with the BGE alone, indicates a region of low conductivity has been formed in the capillary It was not possible to elucidate from the current profiles, however, if the HD base injection did result in increased conductivity at the rear of the sample zone, resulting in the adverse stacking effect seen for the 4-HC species when the HD base injection was employed

4.3.4. Effect of Injection Length

One means of determining the efficiency of the stacking process occurring is to compare the length of sample injection that can be performed before overloading of the capillary occurs, for each of the injection combinations Overloading is evident typically in significantly increased peak widths To compare the stacking effect achieved for the different modes of injection, the peak width for one of the analytes, HPAA, is plotted against injection time The base injection length for each of the injection combinations is that which proved to be optimal in Section 4 3 2 (See Table 4 9)

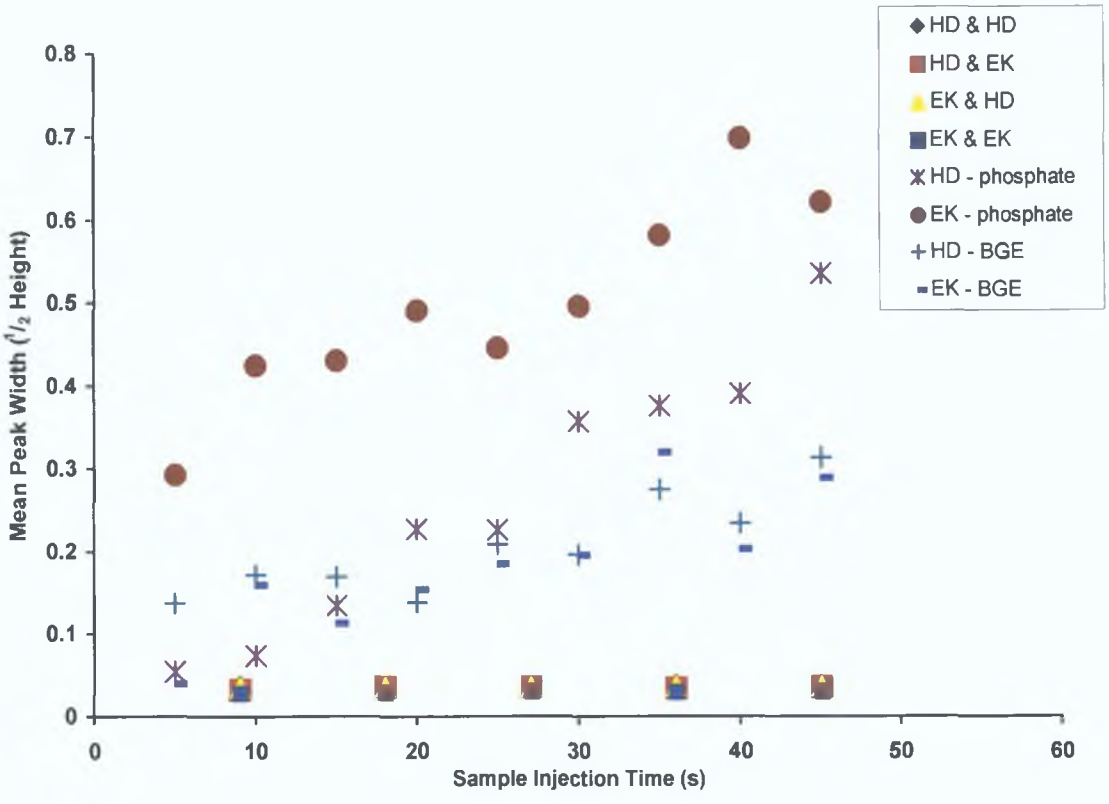

Figure 4.10: *Peak width versus injection time for different injection combinations.*

It can clearly be seen from Figure 4.10 that the length of injection that is possible with sample stacking before overloading of the capillary occurs is greatly superior to that allowed when stacking is not performed. Indeed, for each of the modes of stacking investigated, the peak width obtained for a 45 second sample injection was still narrower than the peak width obtained for a 5 second sample injection without sample stacking. It is evident also that longer injection lengths can be performed when the sample is prepared in the BGE, when sample stacking is not performed. This is to be expected as the removal of any negative matrix effects limits band-broadening and effectively results in improved peak shapes and narrower peak width than for samples prepared in alternative matrices. In order to compare the various modes of sample stacking against each other, however, the stacking data was replotted and is shown in Figure 4.11.

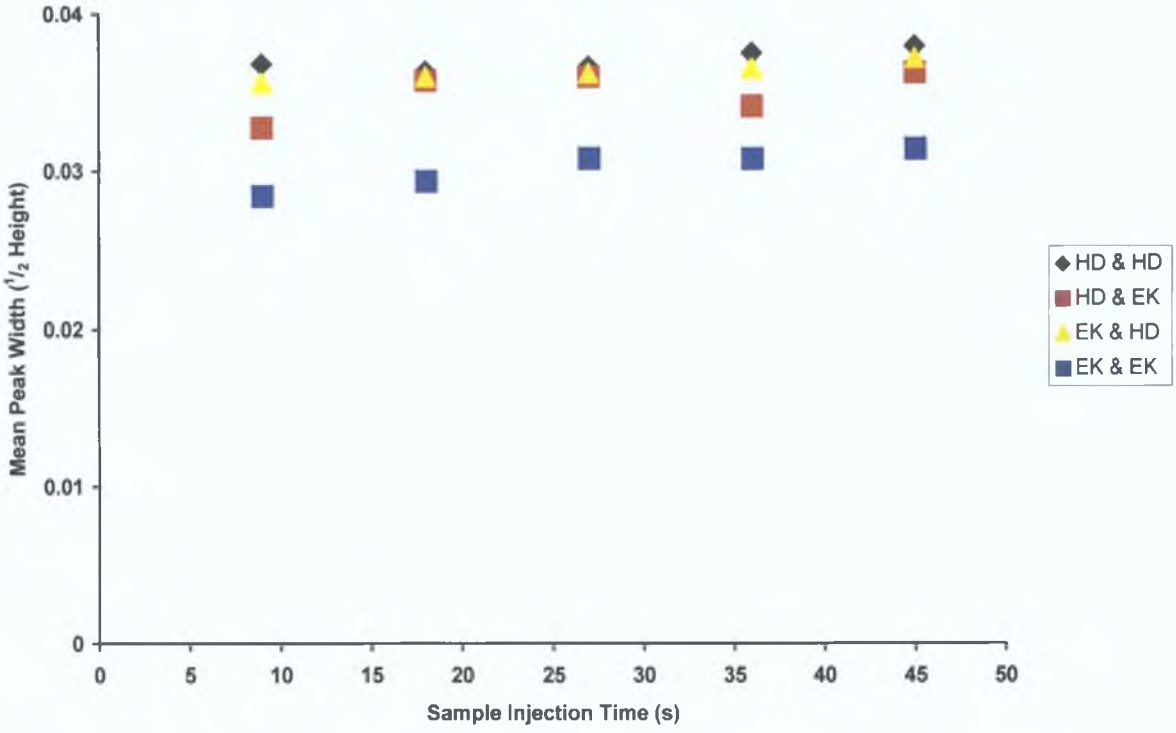

Figure 4.11: *Peak width versus injection time for sample stacking performed with different injection combinations.*

From Figure 4.11 it is clear that sample stacking is efficient for this analyte using all four of the injection combinations. The peak widths for the analyte increase only very slightly as the sample injection time is increased, indicating that the peak height is increasing dramatically as the sample is stacked into a narrow band on the capillary. By comparison, however, it can be determined that the EK/EK injection scheme is the most effective resulting in the narrowest peak heights for the analyte in all cases. In a situation where the injection type was not dictated by any other factors, it would appear to be preferential to employ EK injections for both the sample and the base introductions. At the highest injection length investigated, a 45 second injection for the sample followed by optimal base injection, the peak width has not begun to increase significantly, indicating that the capacity of the stacking system has not yet been reached at this stage. This would lead us to conclude that even longer sample injection times could be performed, without deleterious band-

broadening effects, if the injection length allowed by the CE instrumentation was not a limiting factor

4.3.5. Sample/Base Injection Ratio

It would appear from data illustrated in Section 4 3 2 that similar injection ratios are required to perform sample stacking, regardless of injection type In each case where sample stacking occurred, a base injection of between 85 to 99 seconds was required to achieve the maximum stacking effect The only exception to this was the length of HD base injection required to achieve optimal stacking for the 4-HC species An explanation for this was put forward in Section 4 3 2 5 Where sample stacking was observed for all of the analytes, the same base injection length did not necessarily result in the highest peak heights for all three of the analytes In these cases the optimal base injection chosen was that which yielded good peak heights for all of the analytes Thus it seems that the optimal base injection length must be determined experimentally for each new system employed but that a base sample injection ratio of 2 1 could prove a good starting point for the investigation

4.3.6. Effect of employing phosphate buffer as the sample matrix

From the results previously discussed it would appear that phosphate buffer is suitable as a sample matrix for pH-mediated sample stacking Replacement of the sample matrix cations with cations from the BGE does occur, leading to stacking effects, as evidenced by the results previously detailed This is an extremely important conclusion, as it would indicate that pH-mediated sample stacking is not restricted to samples prepared in Ringer's solution, but that potentially a wide range of biological sample matrices could be compatible with this technique

4.4. CONCLUSIONS AND FUTURE WORK

From the results presented in this chapter it can be concluded that sample stacking can be performed with HD injection, despite previous reports to the contrary (Park and Lunte, 1998) In addition, pH-mediated sample stacking can be performed successfully in matrices other than the previously reported Ringer's solution (Weiss *et al*, 2001, Park and Lunte, 1998) The advantages of these developments are extremely significant

HD sample injection is often preferential to EK injection as a representative volume of each component of the sample is introduced onto the capillary when HD injection is performed This is in contrast to EK injection, in which the sample components with the highest electrophoretic mobility are preferentially introduced Sample components with low electrophoretic mobility can thus be difficult to introduce by EK injection This means that the fact that HD injection can be employed for pH-mediated sample stacking has the potential to greatly increase the usefulness of this technique In addition, the use of phosphate buffer as a sample matrix for this technique indicates that this technique could be more widely applicable to the analysis of physiological samples then previously thought The possibility now exists that pH-mediated stacking could be applicable to a wide variety of sample matrices, in particular those of biological origin Further research in this area should be focused on the application of this technique to a range of physiological sample matrices, in order to fully evaluate the potential of this technique for the analysis of many biochemical samples

4.5. BIBLIOGRAPHY

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CHAPTER FIVE

APPLICATION OF pH-MEDIATED SAMPLE STACKING TO THE ANALYSIS OF SOME ALIPHATIC AMINO ACIDS WITH INDIRECT UV ABSORBANCE DETECTION

5.1. INTRODUCTION

Amino acids, the basic structural units of proteins, play an important role as indicators of metabolic processes (Stryer, 1995) The analysis of amino acids can be difficult for several reasons, however As ammo acids are, for the most part, structurally similar, sensitive bioanalytical methods are required to adequately separate them In addition, the lack of a strong chromophore for most native amino acids has meant that amino acids cannot be detected by direct UV absorbance without prior derivitization Combine this with the sensitive detection limits required for their analysis in physiological matrices, and the analysis of amino acids becomes a real challenge

CE has been extensively applied to the study of amino acids in recent years (Prata *et al*, 2001, Smith, 1999) The high separation efficiency of CE, combined with its speed of analysis and low volume sample requirements has made it an obvious choice for this type of analysis As UV absorbance detection is the most widely employed detection mode for CE, alternative detection strategies or sample derivitization have to be considered when the analysis of amino acids is required, however Some of the alternative detection strategies typically involve derivitization also Mass spectrometry (Martin-Girardeau and Renou-Gonnord, 2000), L I F (Ummadi and Weimer, 2002) and electrochemical detection (Weng and Jin, 2003, Dong *et al*, 2002) are some of the detection techniques that have previously been employed

Indirect UV absorbance detection is another approach that has been applied to the detection of amino acids This technique involves adding a highly absorbing UV probe to the background electrolyte (BGE) Displacement of the BGE ions by the sample ions results in negative peaks being recorded for the analytes The advantage of indirect UV absorbance detection for the analysis of amino acids, is that no derivitization is required However, as with direct UV detection, indirect detection suffers from poor concentration sensitivity

One approach to overcome this poor concentration sensitivity is to incorporate sample stacking into the separation (Osbourn *et al*, 2000) Most stacking

techniques rely on the creation of a discontinuous buffer zone within the capillary, to control the mobility of the ions as they travel towards the detector Commonly used sample stacking techniques include field amplified sample stacking (FASS) and large volume sample stacking which have been discussed in detail in Chapter 1

FASS has been successfully applied to the analysis of aromatic amino acids with direct UV detection (Lee *et al*, 2000) Stacking factors achieved in this study were of the order of 10-fold Large volume sample stacking (LVSS) is another stacking technique that has been applied, with varying levels of success, to the analysis of amino acids In 2001, LVSS was carried out in combination with derivitization for the analysis of amino acids with direct detection (Latorre *et al*, 2001) Limits of detection for the amino acids in this study ranged from 0 42 to 2 09 μM LVSS was also applied to the analysis of aromatic amino acids without derivitization Direct UV detection was employed for this analysis (Lee *et al*, 2000) In this study, whilst the stacking factor achieved was impressive (250 to 500-fold), only five of the fourteen amino acids studied responded to the stacking technique In addition, LVSS is one of the more complicated stacking techniques, typically involving monitoring of the current and polarity switching during each analysis

pH-mediated sample stacking is a simple stacking technique that can be applied to the analysis of high ionic strength and physiological samples The application of such a technique to the analysis of amino acids, in conjunction with indirect UV absorbance, could allow for increased sensitivity of detection without the requirement of any sample pre-treatment or derivitization steps

The aim of this study was to develop a rapid and sensitive method for the analysis of a range of aliphatic amino acids, and to investigate the effect of applying indirect UV absorbance detection with the pH-mediated sample stacking technique

5.2. EXPERIMENTAL

5.2.1. Instrumentation

Separations were carried out on a Beckman P/ACE System MDQ instrument (Fullerton, CA) The capillary employed was polyimide fused silica from Polymicro Technologies (Phoenix, AZ) purchased from Composite Metal supplies (Worcester, England) The pH values of buffer solutions were determined using an EDT microprocessor pH meter, model RE 357

5.2.2. Reagents and Analytes

Ammonium hydroxide was purchased from Aldrich Chemical Co (Milwaukee, WI) Methylamine hydrochloride, p-aminosalicylic acid (PAS) and tetradecyltrimethyl-ammonium bromide (TTAB) were purchased from Sigma (St Louis, MO) along with an amino acid kit, which contained a variety of L-amino acids Sodium chloride (NaCl), calcium chloride ($CaCl_2$) and potassium chloride (KCl) were purchased from Fisher Chemical Co (Pittsburgh, PA) Cetyltrimethyl ammonium bromide (CTAB) was purchased from BDH Ltd (Poole, England) CTAOH was prepared from the CTAB All other chemicals were purchased from Sigma (St Louis, MO)

5 2.3. Preparation of Solutions

The BGE was prepared from 25 mM methylamine and 10 mM PAS and adjusted to pH 11 0 by addition of 1 M ammonium hydroxide In order to reverse the EOF, CTAOH was added to the BGE at a concentration of 0 5 mM CTAOH was prepared by passing the CTAB through an ion exchange column in order to replace the bromine ion with hydroxide Ringer's solution was prepared from 155 mM

NaCl, 2 3 mM $CaCl_2$ and 5 5 mM KCl in deionised water 1 mM stock solutions of eight amino acids were prepared in Ringers solution or in the BGE These standards were then diluted to 100 µM before injection The amino acids studied were glycine, L-serine, L-glutamine, L-proline, L-lysine, L-cysteine, L-leucine and L-valine Nanopure water (18 2 Ω) was used throughout the study CTAOH was prepared by passing CTAB through the ion-exchange resin AG1-X8 in the hydroxide form (Bio-rad, Hercules, CA)

5.2.4. Capillary Electrophoresis Separation

The separation capillary was fused silica, 50 µM x 61 2 cm (50 cm to detector) The capillary was initially primed by sequential flushing at 30 °C with methanol (5 minutes), 1 M hydrochloric acid (5 minutes), H_2O (2 minutes), 0 1 M sodium hydroxide (NaOH) (5 minutes), H_2O (2 minutes) and the BGE (7 minutes) Between runs the capillary was flushed at 20 p s i for 1 minute with 0 1 M NaOH and 1 minute with the BGE Once the conditions were optimised, samples were introduced onto the capillary by a 30 second EK injection at –10 kV, followed by a 60 second EK injection of NaOH at –10 kV for sample stacking The concentration of the NaOH used was 50 mM, to match the concentration of the BGE This had previously been optimised by Zhao (Zhao, 1997) and so was not investigated further Separation was carried out at –20 kV with a rise time of 0 17 minutes

5.2.5. Control Experiments

In order to provide a control against which to measure the stacking effect achieved, samples were prepared in Ringer's solution and in the BGE In each case, samples were injected without sample stacking (i e no base injection was performed)

5.2.6. Optimisation of stacking conditions

Optimisation of injection conditions for sample stacking was initially carried out by injecting the sample for a fixed length of time (i e 45 seconds) and varying the base injection time until the optimum sample base injection time ratio was determined Once the optimum ratio had been determined, the sample injection time was varied, with the base injection being performed at the optimum ratio, until the optimum sample injection time was determined As increasing sensitivity was the aim of the study, optimum conditions were taken to be those which yielded the greatest peak heights for the majority of the analytes

5.2.7. Calculation of Peak Efficiency, N.

Peak efficiencies were calculated based on the formula

Eqn. 5.1

$$N = 5\ 54\ (T_i/W_{1/2})^2$$

where

N = efficiency

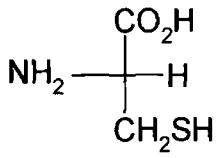
T_i = Migration time of peak

$W_{1/2}$ = Peak width at half-height

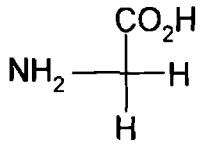
5.3. RESULTS AND DISCUSSION

5.3.1. Structures of the analytes

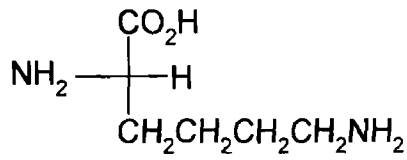
The structures of the amino acids of interest in this study are shown in Figure 5 1



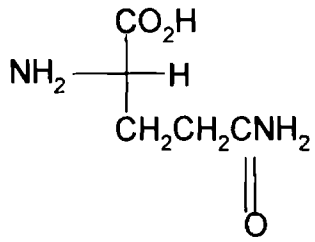
Cysteine



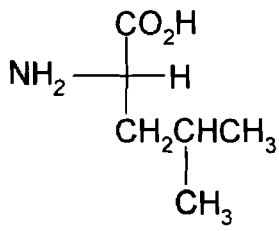
Glycine



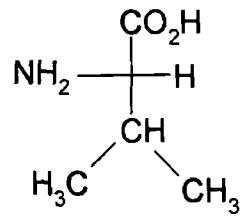
Lysine



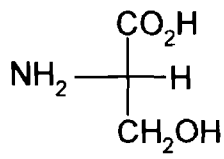
Glutamine



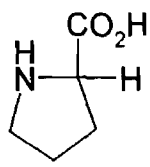
Leucine



Valine



Serine



Proline

Figure 5 1: Structures of the eight amino acids of interest in this study

5.3.2. Development of the separation conditions

In order to effectively separate the amino acids of interest, several factors had to be taken into consideration when choosing a suitable BGE As the amino acids are, for the most part, similar in structure and size, an elevated pH for the separation would be desirable At an elevated pH, the amino acids are more likely to be sufficiently differentially charged, thus allowing them to be successfully resolved from each other The UV absorbing probe for the indirect detection of the amino acids, was a large consideration In many cases, the UV absorbing probe acts as the BGE, thus avoiding the need to add an additional probe to the BGE For amino acids, this has been demonstrated using benzoate (Chen *et al*, 2000) and salicylate (Žunic *et al*, 2002) As it was wished to demonstrate the use of pH-mediated sample stacking with indirect detection, it was necessary to use a BGE which had available amine groups This would allow the pH-mediated stacking procedure to take place Thus the use of a separate BGE with the addition of a UV probe (i e a co-anionic BGE) was considered Finally, EOF reversal was necessary Although amino acids have been detected in normal polarity mode, for the pH-mediated stacking to occur, it is essential that the OH^- ions of the base are not opposing the BGE, but in fact have higher mobility than the analyte ions in the capillary This enables them to migrate through the sample zone, causing titration If normal polarity was to be applied, the OH ions would in fact be opposing the EOF, and pH-mediated stacking would not take place Some EOF modifiers were thus also investigated in the method development procedures

5.3.3. Choice of UV absorbing probe for indirect detection

According to a useful review on the design of BGEs for indirect UV detection (Doble *et al*, 2000) the three parameters that must be taken into account when choosing a probe for indirect detection are (i) the mobility of the probe, (ii) the mobilities of the analytes under investigation and (iii) the sensitivity of detection

Ideally, the mobility of the probe and the mobility of the analytes should be matched, to achieve the best possible peak shapes When the analytes have a range of mobilities, a probe which has mobility somewhere in the middle of the mobility range of the analytes will yield the best results As the analytes under investigation in this research are considered moderately mobile, some of the most suitable probes for their investigation would be benzoate, phenylacetic acid or salicylate (Doble *et al*, 2000) The relative mobilities of some common probes and solute ions are shown in Figure 1 14 It is of particular importance to match the mobility of the probe to the mobility of the analytes when co-anionic buffers are being used, as is the case in this research Co-anionic buffers are those which do not contain only one co-anion In this case, the analyte could potentially displace either the probe anion or the buffer co-anion The risk of this is reduced as the mobilities of the probe and the analytes are more closely matched Salicylic acid was the first UV probe chosen for this study as much previous research has indicated that it is suitable for the detection of amino acids, and compatible with high pH separations (Žunic *et al*, 2002, Brum *et al*, 1992) Salicylic acid thus satisfies the requirements of a UV absorbance probe with regard to this study

5.3.4. Choice of co-anion for the BGE

For base-mediated sample stacking to occur the BGE must be composed of the salt of a weak base, such as ammonia (Osbourn *et al*, 2000) A BGE that could be used at a high pH was also necessary as has been previously discussed Thus a suitable BGE would have to provide a buffering capacity at a relatively high pH, whilst being suitable for use with base-mediated stacking The ammonium-based buffers that were considered suitable for investigation in this study for the reasons outlined above are methylamine, diethanolamine (DEA) and ammonia/ammonium The pK_a values of these buffers are 10 6, 8 90 and 9 25 respectively The pH values studied were all within 1 unit of the pK_a value of the buffer being used Initial separations were performed for these three BGEs at 25 mM and 50 mM concentrations, each

with 10 mM PAS and with 0.5 mM surfactant. At this stage, a mixture containing 1 mM each of four amino acids (L-glutamine, L-serine, L-glycine and L-proline) in Ringers solution was injected in order to assess the potential of the BGE under study. The injection conditions for stacking were not optimised at this stage and an injection ratio of 45:90 seconds was taken as the starting point for analysis. Some of the electropherograms obtained are shown in Figures 5.2 to 5.5. From the four electropherograms shown, it is clearly evident that the methylamine BGE, at 25 mM, was the most suitable choice for further study. The four amino acid peaks were clearly resolved in under nine minutes on the capillary. All of the other BGEs investigated exhibited far less potential as the BGE, and indeed for the DEA and ammonium/ammonia BGEs, no peak was observed at all for the slowest migrating species, L-proline.

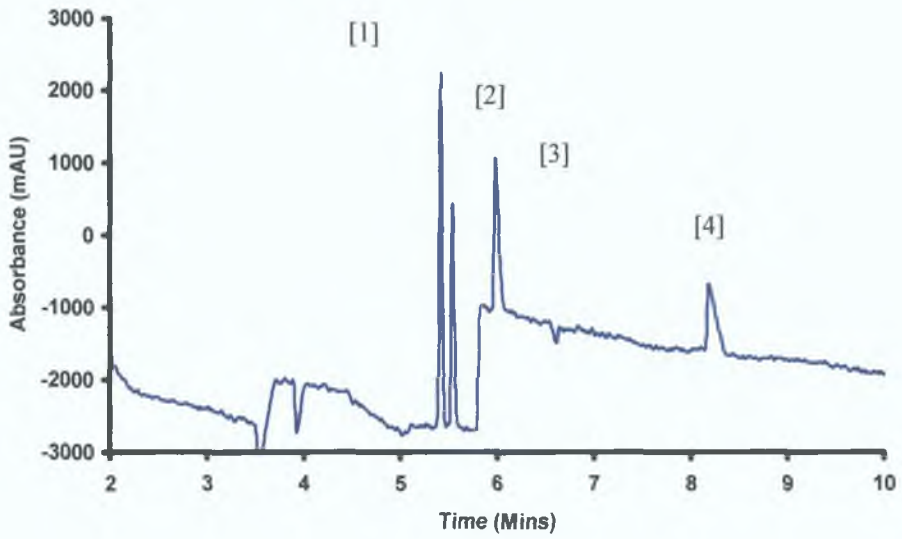


Figure 5.2: *Standard 1mM mixture of [1] glycine, [2] L-serine, [3] L-glutamine and [4] L-proline in Ringer's solution.*
The BGE was 25 mM methylamine with 10 mM salicylic acid and 0.5 mM TTAB, at pH 11.0. Separation was carried out in a 50 cm effective capillary at –20 kV, reverse polarity, with indirect UV absorbance detection at 254 nm.

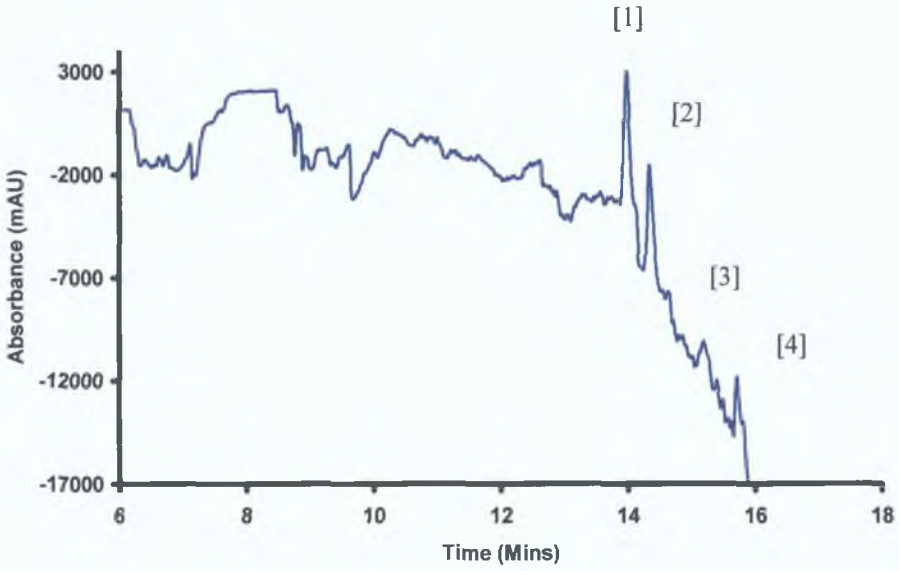


Figure 5.3: *Standard 1 mM mixture of [1] glycine, [2] L-serine, [3] L-glutamine and [4] L-proline in Ringer's solution.*
The BGE was 50 mM methylamine with 10 mM salicylic acid and 0.5 mM TTAB, at pH 11.0. Separation was carried out in a 50 cm effective capillary at –20 kV, reverse polarity, with indirect UV absorbance detection at 254 nm.

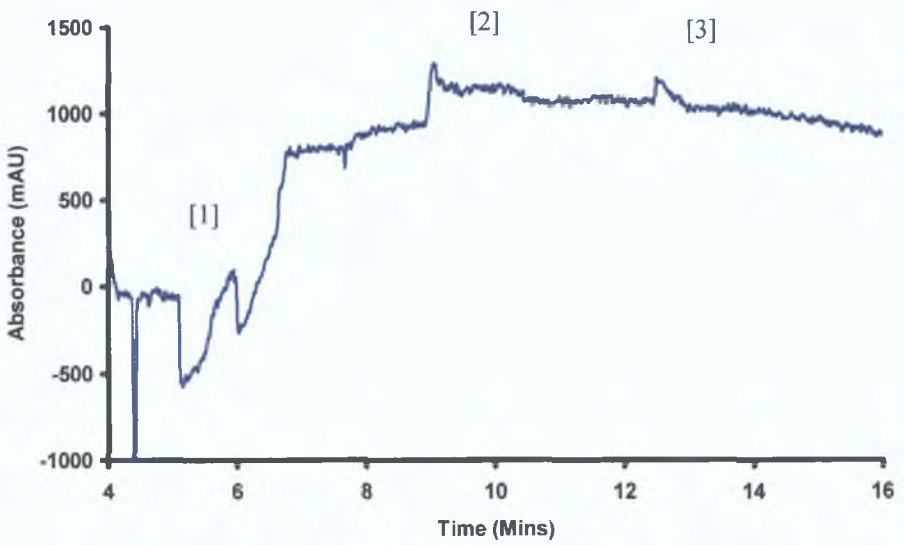


Figure 5.4: *Standard 1 mM mixture of [1] glycine, [2] L-serine, [3] L-glutamine and L-proline in Ringer's solution.*

No peak was observed for L-proline. The BGE was 25 mM DEA and 10 mM salicylic acid with 0.5 mM TTAB at pH 9.6. Separation was carried out in a 50 cm effective capillary at – 20 kV, reverse polarity, with indirect UV absorbance detection at 254 nm.

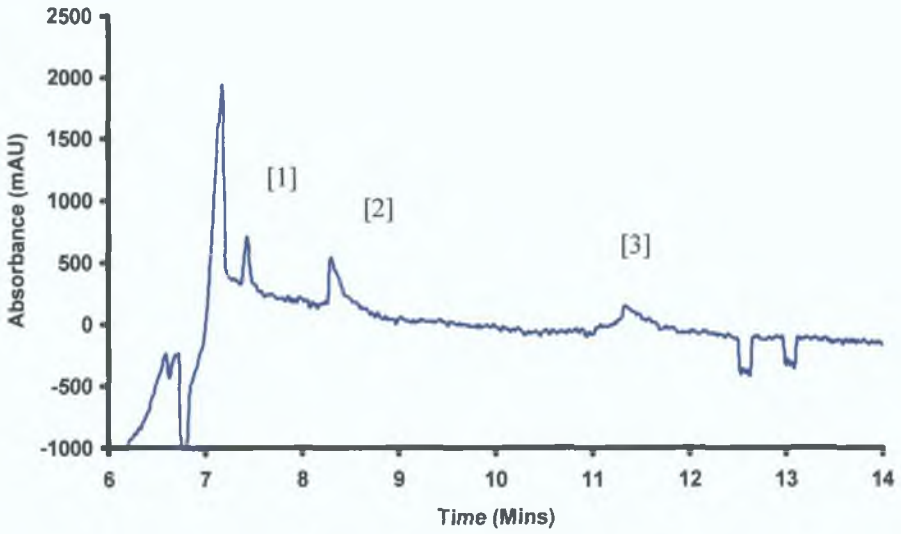


Figure 5.5: *Standard 1 mM mixture of [1] glycine, [2] L-serine, {3] L-glutamine and L-proline in Ringer's solution.*
No peak was observed for L-proline. The BGE was 25 mM NH_3/NH_4^+ and 10 mM salicylic acid with 0.5 mM TTAB at pH 9.6. Separation was carried out in a 50 cm effective capillary at –20 kV, reverse polarity, with indirect UV absorbance detection at 254 nm.

5.3.5. Choice of cationic surfactant for reversal of the EOF

In order to reverse the EOF, it was necessary to add a cationic surfactant to the BGE. TTAB and CTAB are some of the most commonly used cationic surfactants in CE analysis today (Khaledi, 1998). CTAOH is also commonly used as an EOF modifier when indirect detection is involved as it removes the Br^- ion which may lead to interfering system peaks (Choy *et al.*, 2002). Once the BGE was chosen i.e. 25 mM methylamine with 10 mM salicylic acid at pH 11.0, the effect of the three of these cationic surfactants was investigated. Figures 5.6, 5.7 and 5.8 show the separation that was obtained for the BGE with each of the three cationic surfactants. All three cationic surfactants investigated appeared to be suitable for use in this analysis. In all three cases, full resolution of the four analytes was achieved within

a reasonable analysis time. In Figure 5.6, in which the cationic surfactant is TTAB, a rise in the baseline is observed at approximately 5.8 minutes. Further preparations of this BGE yielded consistent results and so, as this artefact did not appear for either of the other two surfactants, it was decided not to use TTAB in the BGE. Good baselines were observed for both the CTAOH and CTAB surfactants (Figures 5.7 and Figures 5.8 respectively) although migration times were slightly improved for the analytes when CTAOH was employed. For this reason it was decided to use CTAOH as the EOF modifier in this study.

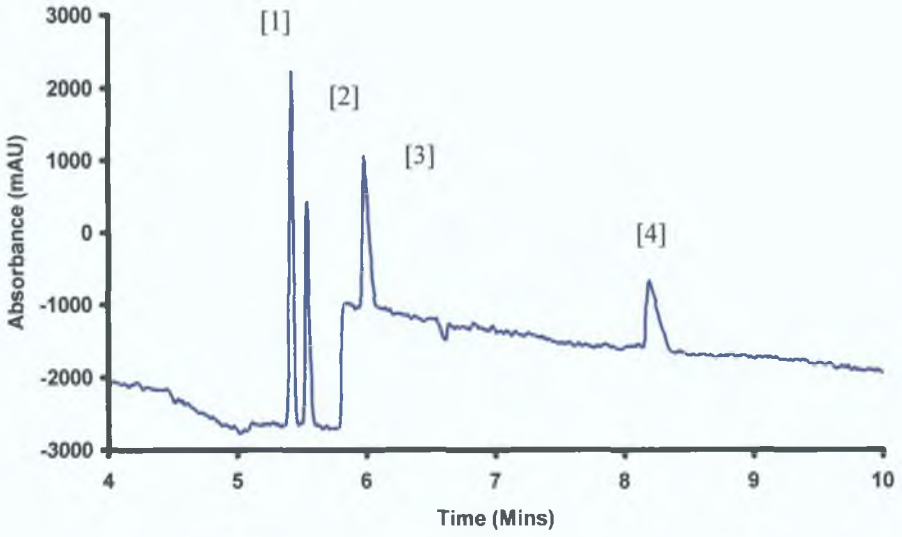


Figure 5.6: *Standard 1 mM mixture of [1] glycine, [2] L-serine, [3] L-glutamine and [4] L-proline in Ringer's solution.*
The BGE was 25 mM methylamine with 10 mM salicylic acid and 0.5 mM TTAB as the EOF modifier, at pH 11.0. Separation was carried out in a 50 cm effective capillary at –20 kV, reverse polarity, with indirect UV absorbance detection at 254 nm.

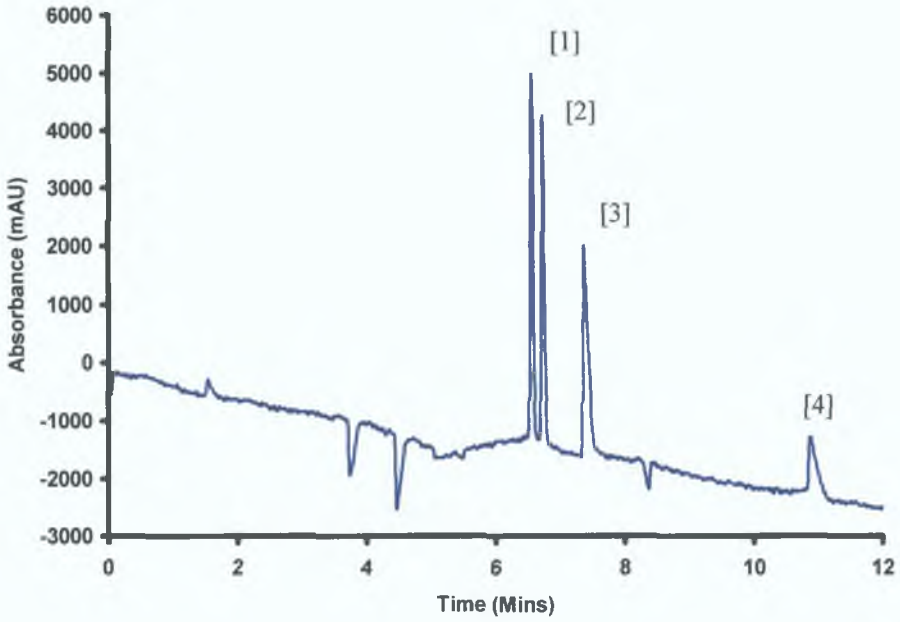


Figure 5.7: *Standard 1 mM mixture of [1] glycine, [2] L-serine, [3] L-glutamine and [4] L-proline in Ringer's solution. The BGE was 25 mM methylamine with 10 mM salicylic acid and 0.5 mM CTAOH as the EOF modifier, at pH 11.0. Separation was carried out in a 50 cm effective capillary at –20 kV, reverse polarity, with indirect UV absorbance detection at 254 nm.*

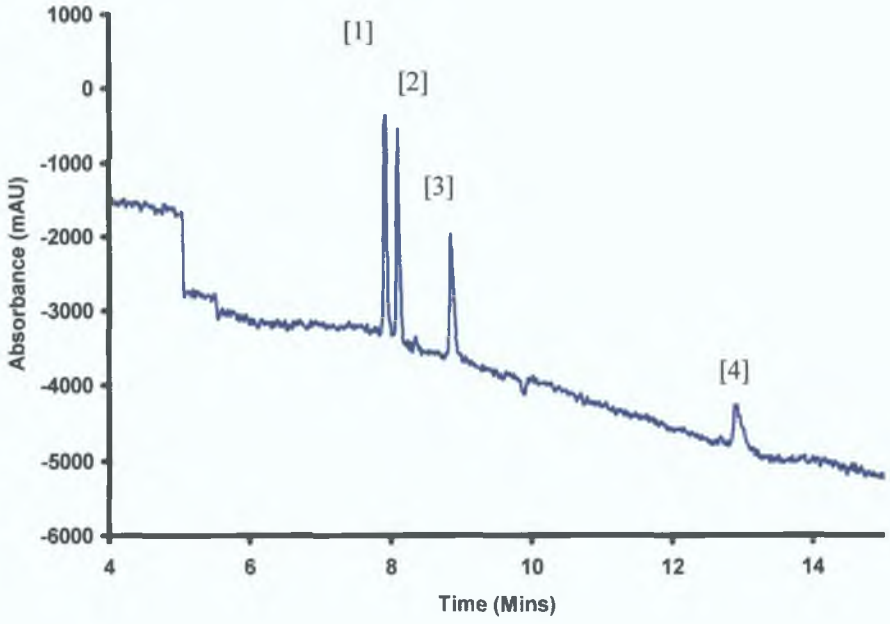


Figure 5.8: *Standard 1 mM mixture of [1] glycine, [2] L-serine, [3] L-glutamine and [4] L-proline in Ringer's solution.*
The BGE was 25 mM methylamine with 10 mM salicylic acid and 0.5 mM CTAB as the EOF modifier, at pH 11.0. Separation was carried out in a 50 cm effective capillary at –20 kV, reverse polarity, with indirect UV absorbance detection at 254 nm.

5.3.6. Optimisation of separation conditions

The detection wavelength of 254 nm was chosen as this has previously been reported to be the optimum wavelength for the salicylic acid probe (Bruin *et al.*, 1992; Žunić *et al.*, 2002). Applied voltage and the voltage of injection were optimised by following standard method development procedures.

5.3.7. Optimised stacking conditions

Once the BGE had been selected, pH-mediated sample stacking was performed Optimisation of the stacking process was carried out as detailed in Section 5 2 6 A standard mixture of eight amino acids at a concentration of 100 μM was employed for the stacking study Table 5 1 lists the data obtained for sample injections carried out for a fixed length (45 seconds) followed by base injections of varying lengths For clarity, the data presented is for one of the analytes, L-glutamine

Table 5 1 *Optimisation of sample base injection ratio All sample injections were 45 seconds Standards were 100 μM prepared in Ringers solution Data is given for one of the analytes, L-glutamine (n = 3)*

Sample/Base Injection length (s)	Peak Height (mAU)	Width at ½ height (min)	Efficiency N (/1000)
45/45	279 ± 13	0 054 ± 0 001	46 ± 1
45/50	273 ± 12	0 056 ± 0 0010	44 ± 1
45/55	286 ± 2	0 054 ± 0 001	46 ± 1
45/60	306 ± 5	0 052 ± 0 001	50 ± 1
45/65	293 ± 5	0 054 ± 0 001	45 ± 1
45/70	318± 4	0 050 ± 0 001	52 ± 2
45/75	322 ± 5	0 050 ± 0 001	53 ± 2
45/80	376 ± 10	0 048 ± 0 001	56 ± 3
45/85	404 ± 8	0 046 ± 0 001	64 ± 1
45/90	412 ± 1	0 045 ± 0 001	65 ± 1
45/95	407 ± 3	0 045 ± 0 001	60 ± 9
45/99	392 ± 10	0 046 ± 0 001	64 ± 1

From Table 5 1 it can be sumised that the level of sample stacking achieved increases as the base injection length is increased, until the optimum stacking conditions are achieved Increasing the base injection beyond this point results in

overloading of the capillary For the data presented in Table 5 1, optimum sample stacking would appear to be achieved at a sample base injection ratio of 1 2 At a base injection of 90 seconds, the highest peak heights and narrowest peak widths are achieved For the 95 and 99 second base injections, the peak height falls slightly, indicating that the stacking capacity of the system has been reached A ratio of 1 2 for the sample base injection lengths was thus chosen to be optimum and used in further optimization studies This result is in agreement with previously reported stacking conditions (Zhao, 1997) Once the optimum ratio had been determined, it was necessary to optimise the sample injection length Table 5 2 illustrates the data obtained when the sample injection times where varied whilst the sample base injection ratio was held constant

Table 5 2 *Optimisation of sample injection time Standards were 100 µM prepared in Ringer's solution Data is given for one of the analytes, L-glutamine (n = 3)*

Sample/Base Injection length (s)	Peak Height (mAU)	Width at ½ height (min)	Efficiency (/1000)
5/10	62 ± 3	0 0319 ± 0 0006	132 ± 7
10/20	141 ± 4	0 0318 ± 0 0003	133 ± 3
15/30	208 ± 6	0 0338 ± 0 0008	118 ± 8
20/40	275 ± 4	0 0336 ± 0 0004	119 ± 5
25/50	364 ± 7	0 0355 ± 0 0006	105 ± 4
30/60	441 ± 5	0 0367 ± 0 0006	100 ± 7
35/70	437 ± 5	0 0398 ± 0 0008	85 ± 5
40/80	425 ± 5	0 0414 ± 0 0004	77 ± 3
45/90	412 ± 1	0 0458 ± 0 0004	65 ± 1
50/99*	406 ± 7	0 0498 ± 0 0007	53 ± 2

* The longest injection length allowed by the instrument is 99 seconds

From Table 5 2, it is evident that increasing the sample injection length increases peak height rapidly, while the peak width increases more slowly, up to an injection

length of 30 seconds This is an indicator that sample stacking is taking place The highest peak height for the analyte is recorded at 30 seconds As the sample injection length is increased further, peak heights begin to fall slowly for the analyte, indicating that the stacking capacity of the system has been exceeded Although a greater amount of sample is being introduced onto the capillary, band-broadening resulting from overloading of the capillary leads to decreased peak heights and increased peak widths for the analyte From this data it was thus concluded that a sample injection of 30 seconds followed by a 60 second base injection were the optimal injection conditions for sample stacking for this study It must be noted that the results obtained for L-glutamine during optimisation of the separation conditions are representative of those obtained for the eight amino acids and that optimum conditions were determined based on examination of the data for all of the analytes

5.3.8. Evaluation of the stacking technique

In order to evaluate the stacking method, as compared to a non-stacking method, further separations were carried out without the base injection Standards were also prepared in the BGE for comparison Samples prepared in the BGE provide a good control against which to compare separations as the mobilities of the sample matrix and the BGE are matched, removing any band-broadening effects, which may ensue from a mismatch of these components Results for the non-stacking and BGE methods were performed as for the stacking method with the exceptions that no base injection was performed Tables 5 3 and 5 4 show some of the results obtained for these comparative studies

Table 5.3 *Peak data obtained for 100 μM standards in Ringer's solution without stacking Separations were performed as for the stacking experiments with the exception that no base injection was performed Data is presented for one of the analytes, L-glutamine (n = 3)*

Sample Inj Time (s)	Peak Height (mAU)	Width at ½ height (min)	Efficiency N (/1000)
5	53 ± 13	0 0369 ± 0 0012	71 ± 11
10	102 ± 23	0 0439 ± 0 0014	42 ± 5
15	142 ± 4	0 0491 ± 0 0003	36 ± 2
20	175 ± 9	0 0538 ± 0 0009	38 ± 1
45	188 ± 13	0 0986 ± 0 0042	10 ± 3

Data illustrated in table 5 3 would indicate that the optimum injection length for samples prepared in Ringer's solution and introduced without sample stacking is 5 seconds At injections above this length, peak widths become unacceptably large and peak efficiency is greatly compromised A similar trend is seen for samples prepared in the BGE (Table 5 4) Although peak heights are increased over samples prepared in Ringer's solution, deleterious band-broadening effects are still noted at injection lengths above 5 seconds

Table 5 4 *Peak data obtained for 100 μM standards in the BGE without stacking Separations were performed as for the stacking experiments with the exception that no base injection was performed Data is presented for one of the analytes, L-glutamine (n = 3)*

Sample Inj Time (s)	Peak Height (mAU)	Width at ½ height (min)	Efficiency N (/1000)
5	152 ± 20	0 0326 ± 0 0017	76 ± 11
10	214 ± 7	0 0404 ± 0 0006	53 ± 5
15	258 ± 27	0 0427 ± 0 0019	48 ± 2
20	233 ± 20	0 0431 ± 0 0021	42 ± 1
45	200 ± 24	0 0769 ± 0 0043	15 ± 3

There are several approaches that can be taken to evaluate the stacking effect achieved (Weiss *et al*, 2001) For this study, the results obtained for L-glutamine will be used for the comparisons The results obtained for L-glutamine were consistent with results obtained for the other amino acids and can be considered to be representative of the system as a whole

One approach used to calculate the stacking effect is to compare the peak height of the samples at similar efficiency values (Weiss *et al*, 2001) The highest efficiency value obtained for unstacked samples prepared in Ringer's solution was 71,000 plates, corresponding to a sample injection time of 5 seconds The peak height of L-glutamine for this injection was 53 mAU The closest efficiency value obtained for stacked samples was 77,000 plates, based on a 40 second sample injection The mean peak height in this case was 425 mAU This represents an eight-fold increase on the capillary When a similar comparison is drawn against sample prepared in the BGE without stacking, an increase in peak height from 152 mAU to 425 mAU is achieved, representing an almost three-fold increase

Another approach used to estimate the stacking effect is to compare the peak height of the samples achieved for the same injection times The peak height achieved for

a 45 second sample injection with sample stacking was 412 mAU This is in comparison with a peak height of 200 mAU for samples prepared in BGE and of 188 mAU for unstacked samples, representing approximately a two-fold increase on-column in each case Efficiencies are also improved when sample stacking is performed By comparison, the efficiency for the stacked method is 53,000 plates for the 45 second injection, which represents an almost four-fold increase over the standards prepared in BGE (15,000 plates) and a five-fold increase over the Ringer's standards (10,000 plates)

A final evaluation of the stacking method is obtained by the visual comparison of an electropherogram obtained from the stacking and from the non-stacking method Figure 5 9 is an overlay of electropherograms obtained both with and without sample stacking for samples prepared in Ringers solution Peak shape is vastly improved when pH-mediated sample stacking is performed and indeed some of the analyte peaks are not fully resolved from each other when sample stacking is not employed

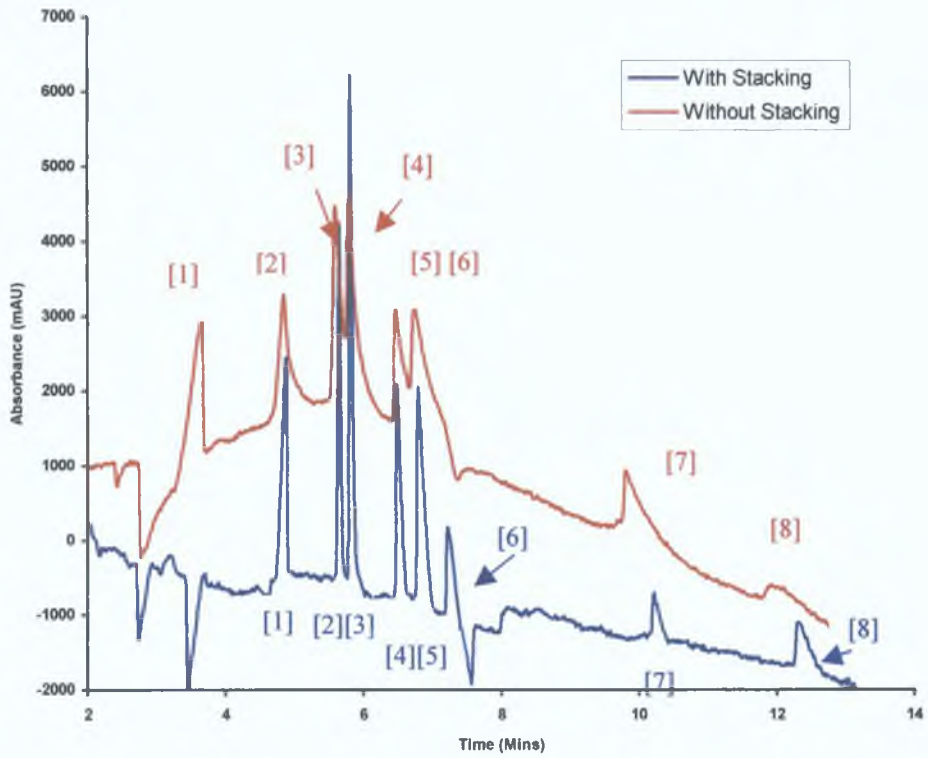


Figure 5.9: *Overlay of electropherograms obtained for the separation of amino acids with and without sample stacking.*

Standard 1 mM mixture of [1] L-cysteine, [2] glycine, [3] L-serine, [4] L-glutamine, [5] L-valine, [6] L-leucine, [7] L-proline and [8] L-lysine in Ringer's solution. The BGE was 25 mM methylamine with 10 mM salicylic acid and 0.5 mM CTAOH, at pH 11.0. Separations were carried out in a 50 cm effective capillary at –20 kV, reverse polarity, with indirect UV absorbance detection at 254 nm. Sample injections were 30 seconds at –10 kV. A base injection of 60 seconds at – 10 kV was included in the sample stacking method.

5.3.9. Optimised conditions

The optimised separation conditions for base-mediated stacking are outlined in Table 2 6

Table 5 5 *Optimised separation conditions for base-mediated stacking*

Parameter	Optimal Value
Capillary dimensions	61 cm X 50 µm
Capillary length to detector	50 cm
Background electrolyte	25 methylamine with 10 mM PAS and 0 5 mM CTAOH at pH 11 0
Sample matrix	Ringers solution
Applied Voltage	- 20 kV ramped for 0 17 min
Detection	Indirect UV absorbance at 254 nm
Sample injection time	30 seconds
Sample injection voltage	-10 kV
Base injection time	60 seconds
Base injection voltage	-10 kV
Running Current	~ 45 µA (for BGE)

5.3.10. Limits of detection

In order to put the result obtained in this study into perspective, it was necessary to compare the limits of detection achieved with those previously reported for a conventional CE method i e a method in which no sample stacking was performed The conventional CE method chosen for the comparison was that of Žunic *et al* (2002), as this recent method incorporated all of the amino acids employed in our study Table 5 6 lists the limits of detection achieved in our study both with and without sample stacking, as well as the limits obtained by the conventional method (Žunic *et al , 2002*)

Table 5 6 *Limits of detection for the amino acids in this study Results are given for the samples prepared in Ringer's solution and injected with sample stacking, and for samples prepared in the BGE without stacking For comparison, limits of detection achieved for these amino acids using a conventional CE method with indirect UV absorbance detection are also given*

Analyte	Limit of detection achieved with stacking (Standards prepared in Ringer's solution) (μM)	Limit of detection achieved without stacking (Standards prepared in BGE) (μM)	Limit of detection as per the literature [a] (μM)
L-cysteine	2 39	5 24	4 12
glycine	2 10	4 64	8 78
L-serine	1 34	4 84	9 18
L-glutamine	1 78	3 61	1 93
L-valine	1 47	5 22	2 76
L-leucine	4 17	7 40	n q
L-proline	8 91	12 50	6 88
L-lysine	9 15	12 96	5 22

Limits of detection for the sample stacking technique range from 1 34 μM to 9 15 μM for the eight amino acids studied From Table 5 6 it is evident that pH-mediated sample stacking does lead to improved limits of detection for the ammo acids, as compared to samples injected in the BGE In most cases, i e for five of the analytes, improvements in detection limits are also noted over the conventional CE method The limits of detection for L-leucine were not determined in the Žunic study and so no comparison can be made against which to determine if an improved limit of detection was achieved for this analyte For L-lysine and L-proline, whilst the stacking method achieved improved limits of detection as compared with the

[a] As per Žunic *et al*, 2002

samples prepared in the BGE, limits of detection were poor in comparison with the Žunić method.

5.3.11. Final Separation

A separation of the eight amino acids injected with sample stacking and incorporating all optimised conditions is shown in Figure 5.10.

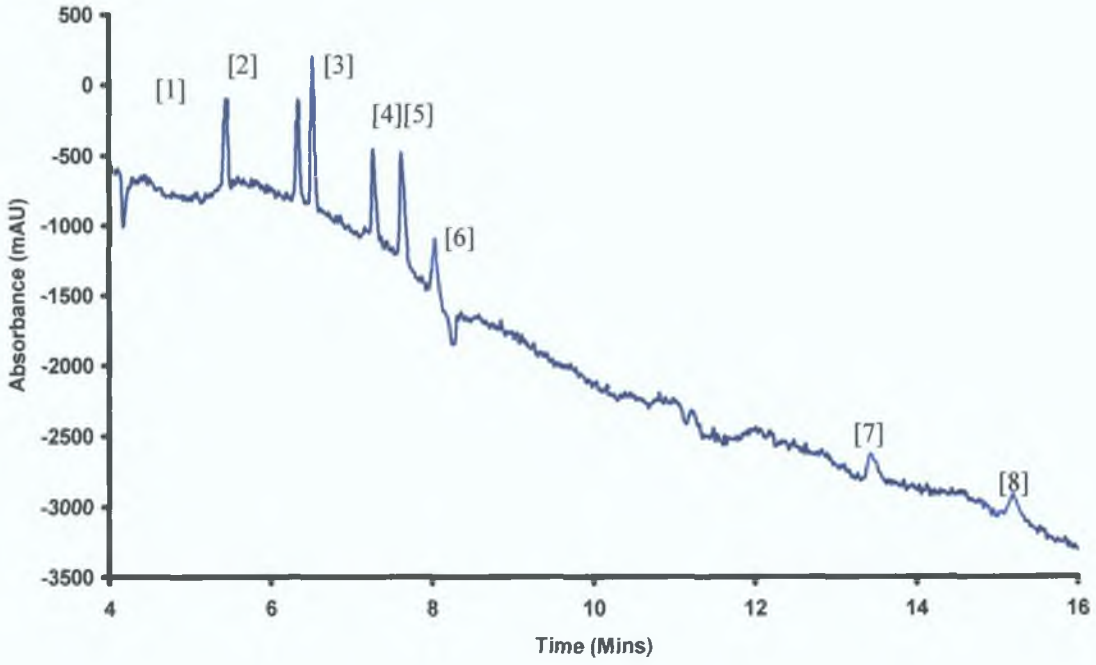


***Figure 5.10**: Standard 100 μM mixture of [1] L-cysteine, [2] glycine, [3] L-serine, [4] L-glutamine, [5] L-valine, [6] L-leucine, [7] L-proline and [8] L-lysine in Ringer's solution.*

The BGE was 25 mM methylamine with 10 mM salicylic acid and 0.5 mM CTAOH, at pH 11.0. Separations were carried out in a 50 cm effective capillary at –20 kV, reverse polarity, with indirect UV absorbance detection at 254 nm. Injections were 30 seconds for the sample and 60 seconds for the base at –10 kV.

5.4. CONCLUSIONS AND FUTURE WORK

pH-mediated sample stacking was found to be easily applicable to the analysis of eight amino acids using indirect UV absorbance detection The eight amino acids were fully resolved in under 16 minutes Limits of detection for the analytes ranged from 1 34 μM to 9 15 μM when sample stacking was employed Stacking was noted for all of the eight analytes when compared with samples prepared in BGE, although for two of the analytes, a conventional CE method with indirect detection (Žunić *et al* , 2002) results in better limits of detection An advantage of the stacking method, however, is that the samples could be detected sensitively in Ringer's solution, a highly conductive physiological solution No sample pre-treatment or derivitization was required resulting in a quick and simple, one-step analysis Further research in this area could focus on extending the separation to include more amino acids, and to investigate the stacking technique for amino acids prepared in other physiological matrices

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CHAPTER SIX

CONCLUSIONS AND FUTURE DEVELOPMENTS

6.1. GENERAL CONCLUSIONS

This thesis comprises a number of studies concerned with the analysis of metabolites of coumarin, and with the technique of pH-mediated sample stacking The first experimental chapter aims to apply a sample stacking technique to the capillary electrophoresis (CE) separation of coumarin metabolites in order to increase detection limits for low concentration metabolites In the second experimental chapter, a novel method for performing the microsomal incubation of coumarin on-system is developed The third experimental chapter has as a goal the elucidation of the mechanism of pH-mediated sample stacking using different injection combinations The final experimental chapter involves the application of this stacking technique to some amino acids, using indirect UV absorbance detection

6.1.1. Analysis of Coumarins

The analysis of coumarin is of vital importance due to the widespread use of coumarin as a flavouring in foodstuffs, as a therapeutic drug, and as an additive in cosmetics Coumarin is also widely studied as a model compound, particularly for metabolism reactions (Egan *et al* , 1990)

In the early years of coumarin analysis, techniques such as paper chromatography and thin-layer chromatography were most widely used, mainly for the qualitative analysis of coumarin in natural products such as clover and plant extracts With the emergence of more sophisticated separation technology, however, gas chromatography, high-performance liquid chromatography and CE have all usurped these earlier methods for the analysis of coumarins

CE has emerged as a promising technique for the analysis of coumarin in recent years CE has been applied to both *in vivo* and *in vitro* studies of coumarin metabolism reactions, as well as to the analysis of coumarins in natural products A limitation of CE for coumarin metabolism analysis has been its poor concentration

sensitivity, however In chapter two of this research we have shown that sample concentration factors of up to 60-fold can be achieved by the application of a simple stacking process to the CE separation This increased sensitivity has the potential to greatly increase the usefulness of CE for the analysis of coumarin, particularly for the low concentration metabolites Whilst this study is useful to illustrate the potential usefulness of the pH-mediated sample stacking technique for this form of analysis, a separation which includes only three of the potential metabolites of coumarin is limited in its scope Future studies could aim to include further metabolites in the separation

In chapter three a separation method capable of separating coumarin from seven of its metabolites, and from the incubation components was developed While this separation was useful in that it could be used for a wider range of coumarin metabolites, the method did not involve sample stacking and detection limits were poor, in line with previously published methods In the future, a combination of the sensitivity of the sample stacking method with the range of analytes of the chapter three method could achieve a powerful tool for the analysis of a wide variety of coumarin metabolites with greatly improved sensitivity

6.1.1. Analysis of In Vitro Metabolism Reactions

In vitro metabolism reactions are typically time-consuming and labour-intensive, requiring manual sampling at short periods over an incubation time of several hours These reactions are routinely performed by maintaining the necessary reaction components in a water bath or heating block for the incubation period, taking sample aliquots at timed intervals, and transferring for analysis Larger sample volumes are also required, as sufficient sample must be available to allow aliquots to be taken at each interval for analysis

In chapter three an alternative method for the analysis of metabolism reaction has been developed The incubation components are heated and maintained on the CE system over the incubation period, and agitated and injected at timed intervals In

this work only 7-hydroxycoumarin was identified in the incubation mixture, and detection limits were poor The combination of a sample stacking technique with this on-system method could potentially provide a very simple, sensitive and automated approach to this sort of analysis The use of an internal standard with this separation would also be of value as it would help to eliminate inconsistensies which can arise when using protein in the capillaries Future work could focus on applying sample stacking methods to this on-system technique, to allow the sensitive detection of low concentration metabolites with the requirements of minimal labour-intensity and minimal sample volumes

6.1.2. Analysis of Amino Acids

The analysis of amino acids at the low concentration levels often required for biochemical processes can be a challenge CE has been employed in recent years for amino acid analysis (Prata et al , 2001, Smith, 1999) but the lack of native absorbance for the aliphatic amino acids has meant that detection of these amino acids can be difficult In most cases, derivitization of the amino acids, followed by direct detection, is used

Indirect UV absorbance detection is also commonly used, however In this case no derivitization of the analyte is required, although an indirect probe must be added to the background electrolyte in order to generate a constant background signal Detection limits using indirect absorbance are typically of the order of low μM for aliphatic amino acids

Sample stacking has previously been applied to the analysis of amino acids by CE These studies either required sample derivitization or were applied to the direct detection of aromatic amino acids, however

In chapter five the research has aimed to apply pH-mediated sample stacking to the analysis of amino acids with indirect detection No sample derivtzation was required pH-mediated sample stacking was shown to be compatible with indirect UV absorbance detection Improved limits of detection were seen for some of the

analytes, but the stacking effect achieved was typically only 2 – to 3- fold Eight amino acids were successfully resolved with a run time of less than 13 minutes The pH-mediated sample stacking technique was not necessarily optimal for the study of these amino acids as whilst it yielded improved detection limits for some of the analytes, some of the analytes actually exhibitied higher limits of detection when the stacking method was employed The stacking effects that were achieved for some of the analytes were also quite poor when compared with stacking effects typically achieved for the pH-mediated stacking technique This would indicate that alternative stacking techniques should be investigated in order to find a method which yielded improved sensitivity for all of the ammo acids studied, whilst still being compatible with UV absorbance detection

6.2. BIBLIOGRAPHY

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