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Development and optimisation of a micellar electrokinetic capillary chromatography method for the separation and identification of phenolics from eucalypt species

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DEVELOPMENT AND OPTIMISATION OF A MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY METHOD FOR THE SEPARATION AND IDENTIFICATION OF PHENOLICS FROM EUCALYPT SPECIES

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ABSTRACT

Several buffers were examined for their ability to separate a complex mixture of phenolic compounds using micellar electrokinetic capillary chromatography (MEKC). The phenolic mixture included simple phenols, phenolic acids and coumarins. Of the different buffers examined, 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 gave the best separation.

The ability of organic modifiers, complexation agents such as α cyclodextrin, β cyclodextrin and mixed SDS / Brij 35 micelles to improve the separation and peak retention time reproducibility of the buffer system was investigated. Baseline separation of the 18 phenolics was achieved by the 6 mM borate, 10 mM phosphate, 100 mM SDS, 15 mM Brij 35, 10% acetonitrile, pH 8.3 buffer. However this buffer system had poor peak retention time reproducibility.

The MEKC system was used to analyse the phenolic content of a range of eucalypt species. Through spiking, several phenolic and related compounds were identified. These compounds included gallic acid, ferulic acid, p-coumaric acid and shikimic acid. GC-MS and HPLC supported the results obtained by CE.
I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Andrew John Tromans

15th November 1996
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CHAPTER 1. INTRODUCTION

1.1 INTRODUCTION

Chromatography is perhaps the most widely used analytical techniques for the study of organic compounds. One of the more recent techniques is capillary electrophoresis (CE). CE uses a thin capillary with both ends placed in a buffer solution to separate charged compounds through electrostatic interactions with the oppositely charged electrodes placed at both ends of the capillary.

In recent years several related techniques have been developed including MEKC and Capillary Isotachophoresis. MEKC is particularly useful in the separation of organic compounds. In MEKC a surfactant is added to the buffer system, allowing the uncharged organic compounds to be analysed by partitioning between the micelles and the buffer solution. As the charged exteriors of the micelles allow them to be subjected to the electrostatic interactions required to achieve separation, the organic compounds will be separated according to the time they spend in the micelle.

The purpose of this study is to examine MEKC as a possible alternative to HPLC in separating and identifying plant phenolics from a range of eucalypts. In particular a buffer system which gave adequate separation of the complex mixture of phenolics and was reproducible was sought.

In order to achieve optimal resolution of the phenolics, the effect of variations in ionic strength, pH and the surfactant type and concentration were tested. The use of buffer
additives such as organic modifiers and complexation agents were also studied as well as the effects of using combined cationic and neutral surfactants. Finally the effectiveness of buffer systems using organic modifiers and mixed micelles were examined.

The buffer system was used to analyse a range of eucalypts including *Eucalyptus marginata* (jarrah), *Eucalyptus calophylla* (marri), *Eucalyptus todtiana*, *Eucalyptus erythrocorys* (illyarrie) and *Eucalyptus rudis*. These species of eucalyptus were analysed for both bound phenolics and free phenolics.

Other chromatographic techniques such as GC-MS and HPLC was used to compliment MEKC in identifying the phenolics.

1.2 CHROMATOGRAPHY

Chromatography is perhaps the most effective method for the separation and quantification of organic compounds. The principle behind all types of chromatography is one where the sample is distributed between two phases. The extent to which the components in a mixture are distributed effects the degree of separation and the time taken for the sample components to elute (Smith, 1988).

There are generally two phases in chromatography, the mobile phase and the stationary phase. The mobile phase moves across the stationary phase carrying the components in the sample with it. The stationary phase is usually packed within a column or coated on a plate or column wall and retains the compounds by different amounts.
The two most common modes of separation in chromatography are partition and adsorption. Partition chromatography involves the partitioning of the sample between two immiscible phases. The mobile phase can be a liquid or a gas and the stationary phase a liquid. Adsorption chromatography involves gas-solid or liquid-solid interactions where the sample is adsorbed onto the solid stationary phase (Smith, 1988).

1.2.1 THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is designed almost exclusively for separating organic molecules. It has the advantages of being simple, quick and requires no expensive equipment.

A thin layer of stationary phase, often alumina or silica, is spread evenly on a flat, inert surface (usually glass or aluminium foil). The liquid samples are applied to the base of the plate whose bottom edge is then placed in a tank containing the liquid mobile phase. The mobile phase is drawn up the stationary phase by capillary action. The extent to which the compounds in the sample will move up the plate with the mobile phase is dependent upon their differing interactions with the two phases. The distance the sample has migrated is then measured visually (Grinberg, 1990).

TLC is suitable for the analysis of phenolics. Reproducibility is usually quoted as between 5% and 15%. Recently TLC has been performed using a relatively new technique called high pressure thin layer chromatography (HPTLC) (Knop, 1985).
1.2.2 GAS LIQUID CHROMATOGRAPHY

In gas liquid capillary chromatography (GC) the sample is carried through an open tubular column by a gaseous mobile phase. The retention time of an analyte depends upon its interaction with the stationary phase, which is coated on the wall of the capillary, and its volatility (Smith, 1988). The gas acts as a carrier for the analyte but has no separating capabilities.

GC, with its speed and excellent resolving capabilities, is ideal for separating complex mixtures such as plant phenolics. However, as GC requires volatile samples, it is unable to determine most substituted, non-volatile or thermally unstable phenolics (Vande Casteele et al., 1976).

Non-volatile compounds can be chemically modified to increase their volatility and make them suitable for GC. This modification process is called derivitisation. Derivitisation can also be used to increase the thermal stability of some heat sensitive compounds, to change the separating properties of compounds by adjusting their volatility and to introduce a detector orientated tag into the molecule that is to analysed.

One of the most common methods of derivitisation is to use a silylating agent. The silylating agent substitutes an active hydrogen in a polar functional group with a
trimethylsilyl group. For example, polar groups such as -OH, -COOH, -NH₂, =NH and -SH can be converted to an -O-Si(CH₃)₃ group (Figure 1.1) (Skoog et al., 1992).

Gas chromatographs are commonly linked to a mass spectrometer detector which allows the structure of the sample compounds to be determined. This method has been used to identify phenolics in plants (Greenaway et al., 1990; Greenaway et al., 1988; Chouchi et al., 1993).

1.2.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography (HPLC) involves pumping the mobile phase through a column which contains a liquid stationary phase coated onto a tightly packed granular support. The sample is carried through the column with the mobile phase which is pumped through the column under high pressure. Separation is achieved by the analyte partitioning between the mobile phase and the liquid stationary phase (Smith, 1988).

Numerous modes of separation can be carried out by HPLC. The most common modes of separation are partition, adsorption and ion exchange (Skoog et al., 1992). Over half of all chromatographic separations in HPLC employ a non polar liquid stationary phase (reverse phase chromatography). The most commonly used reverse phase is the octodecyl, C18 phase (Villeneuve et al., 1982).
HPLC has been the most successful method for separating plant phenolics (Burtscher et al., 1982; Vanhaelen et al., 1980). HPLC has the advantages of being quantitative and reasonably fast, however samples generally require considerable preparation and treatment before being passed through the column (Koster et al., 1983).

1.2.4 CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE) is a recent chromatographic technique that has been used to determine and quantify a large range of organic compounds and inorganic ions.

Capillary zone electrophoresis (CZE) functions by passing the sample through a fused silica capillary with a large potential difference applied across the ends of the capillary. The sample is injected, either hydrostatically or by electromigration, at one end of the capillary and is carried through to the detector end by the flow of a buffer solution. The flow of the buffer solution is called the electro-osmotic flow (EOF). The EOF is formed when the SiOH molecules on the inner wall of the capillary dissociate into SiO⁻ and H⁺ ions. The extent of the dissociation depends on the pH of the electrolyte solution (Kuhn et al., 1993). The resulting charged silica wall causes a rigid double layer of counterions (Helmholtz layer), under a diffuse double layer (Debye-Hückle layer), to be adsorbed onto the capillary wall. These layers of counterions cause an electrical potential between the silica wall and the electrolyte solution which decreases exponentially with the distance from the capillary wall (Kuhn et al., 1993). This electric potential is called the zeta potential. When the silica wall is negatively charged, the excess cationic species
at the wall migrate towards the cathode carrying the bulk solution and creating the EOF (Figure 1.2). The size of the EOF will depend on the zeta potential, which in turn depends on the pH and the ionic strength of the buffer.

The analyte molecules, travelling in the mobile phase, will be attracted or repelled from the electrodes at each end of the capillary depending on the molecules charge. The resulting difference in migration speeds of the molecules allows the compounds to be separated (Nielsen et al., 1993).

A major advantage of CE over GC is the minimal amount of sample preparation required as derivitisation of the sample is not necessary.

The primary advantage of CE over HPLC is its separating capabilities. The column efficiency in separating the sample components is measured by the number of theoretical plates. The number of theoretical plates is calculated by:

\[ N = 5.21 \left( \frac{t}{w_{1/2}} \right)^2 \]

where \( N \) is the number of theoretical plates, \( t \) is the retention time of the peak and \( w_{1/2} \) is the width of the peak at half peak height (Kuhn et al., 1993). Under average conditions CE can have between 50 000 to 600 000 theoretical plates whereas HPLC has between 5 000 and 20 000 theoretical plates (Nielsen et al., 1993).
**Figure 1.1** Example of derivitisation using a silylating agent.

**Figure 1.2** Electro-osmotic flow in a typical CZE system.
Despite this separating power, neutral species cannot be separated by CZE. To overcome this limitation, Terabe et al. (1985) developed micellar electrokinetic capillary chromatography (MEKC).

MEKC includes a surfactant in the buffer solution which, at high concentrations, form micelles with hydrophobic centres that can trap analyte molecules. As the outside of the micelles are usually polar they are affected by the potential difference applied across the capillary. Consequently, the micelles motion will either assist or oppose the EOF, depending on the charge on the micelle. The micelles are travelling at a different overall speed to the EOF and the sample components will elute at different times according to their affinity for the micelles.

Anionic, catonic and non-ionic surfactants can be used in a MEKC system. The choice of surfactant will affect the elution order of the components. Consider a MEKC system using an anionic surfactant and a negatively charged electrode at the detector end of the capillary. The negatively charged electrode will cause the micelles to be repelled and move against the EOF towards the positive end of the capillary. However, the speed at which the micelles migrate is always less than the EOF. This ensures the micelles elute at the detector end (Figure 1.3). The polar molecules in the sample will elute early while the non-polar molecules will spend more time in the slower moving micelles and will elute later (Terabe et al., 1985).
**Figure 1.3** Example of net micellar movement in a MEKC system.
Another factor that is considered in comparing chromatographic techniques is the elution window. The elution window is the time between the elution of the component that flows with the EOF, \( t_0 \), and the elution of a component which is retained almost completely in the micelles \( t_{mc} \). The elution window is calculated by:

\[
\text{elution window} = \frac{t_0}{t_{mc}}
\]

In HPLC there is no limit for the time taken for a component to elute, but in MEKC all the components will elute between \( t_0 \) and \( t_{mc} \).

MEKC has been used to separate a range of complex organic mixtures that have included phenolics (Cartoni et al., 1995; Bjerregaard et al., 1992) and related organic compounds such as tannins (Cork et al., 1991), flavonoids (Ferreres et al., 1994) and even deoxyribonucleic acid (Heiger et al., 1990).

1.2.4.1 REPRODUCIBILITY OF CE

A fundamental problem with MEKC is its poorer reproducibility of peak retention times and peak areas when compared to HPLC. Reproducibility refers to the amount by which the peak retention times or area differ between separate runs. The reproducibility of a buffer system depends on its ionic strength, pH and operating temperature. The reproducibility of a MEKC system also increases when a well conditioned column is used.
1.2.4.2 INCREASING SELECTIVITY AND RESOLUTION IN CE

CE has excellent resolving capabilities, however these capabilities can be further improved by modifying the buffer system using organic modifiers, complexation agents or by altering the type and concentration of surfactant.

Different surfactants that have been reported include; sodium dodecyl sulfate (SDS) (Sainthorant et al., 1995), cetyl trimethyl ammonium bromide (CTAB) (Bjergegaard et al., 1992), sodium tetradecyl sulfate (STS) (Ozaki et al., 1995) and dodecyl trimethyl ammonium bromide (DTAB) (Otsuka et al., 1985).

Buffer additives such as acetonitrile (Ozaki et al., 1995), isopropanol, methanol (Bretnall et al., 1995) and urea (Tomas-Barberan, 1995) have been reported to decrease the EOF and hence increase the size of the elution window. Some researchers have also reported success when mixtures of surfactants have been used. An example of a surfactant mixture is polyoxyethylene 23 lauryl ether (Brij 35) and SDS (Rasmussen et al., 1990).

Another type of buffer additive is a complexation agent. Complexation agents work by adding another molecule to the buffer that can complex with a non-polar component molecule. This effectively means that there are two pseudo-stationary phases for the component molecules to partition between. Examples of these are borate complexes (Morin et al., 1992), cyclodextrins (Szolar et al., 1995) and crown ethers (Kuhn et al., 1993). Cyclodextrins are particularly efficient in resolving enantiomers (Lurie et al., 1994).
1.4 PHENOLS

Phenols are molecules with at least one hydroxyl group attached to an aromatic ring (Figure 1.4a). The occurrence of phenolics varies considerably between different plant species. Phenolics such as gallic acid and caffeic acid are widespread while most other phenolics are less common (Harborne, 1980). Phenols are believed to play a major role in the deterrence of insect attack as well as being strongly associated with disease resistance (Waterman and Mole, 1994). In particular, phenolic production in jarrah has been linked to the tree’s resistance to *Phytophthora cinnamomi* (Cahill et al., 1992).

1.4.1 PHENOLIC STRUCTURE

Different classes of phenolics are grouped according to their structural configuration. The simplest class has a single aromatic ring with a carbon side chain. These compounds can be divided into subclasses according to the number of carbon atoms on the side chain. Phenolic structures are written in the form C₆Cₙ where C₆ refers to the aromatic group and the Cₙ refers to the number of carbon atoms on the side chain (Waterman and Mole, 1994). In general, most phenolics have between 0 and 3 carbon atoms on the side chain although phenolics with larger carbon side chains have been found.
C₆C₀ compounds make up the simplest subclass and are commonly derived from the trihydroxy compounds pyrogallol, phloroglucinol and catechol (Figure 1.4b). These compounds are often found as part of more complex molecules. C₆C₁ molecules are also found as part of more complex molecules, most commonly as esters where the phenolic compound bonds with another phenolic or alcohol compound (Figure 1.4c). This subclass includes gallic acid, salicylic acid, salicylaldehyde and protocatechuic acid (Knop, 1985).

C₆C₂ compounds are far less common than compounds from the other subclasses. C₆C₂ compounds are characterised by the acetophenones.

C₆C₃ compounds are collectively known as phenylpropanes or phenylpropenes depending on whether there is a double bond on the carbon side chain. Examples include para-coumaric acid, caffeic acid, ferulic acid and sinapic acid (Figure 1.4d).

There are many complex phenols that are based on the simple C₆C₃ structure. These include lignans, which are dimers of two C₆C₃ parent molecules. Another subclass, called coumarins, have a cyclised C₆C₃ structure (Figure 1.5). Examples include umbelliferone and aesculetin. Similar in structure to coumarins are chromones which exist mainly as benzopyran-4-one.

C₆C₃C₆ phenolics have a more complex structure. This class of phenolics have two aromatic groups joined by a three member carbon chain. These compounds are the most common phenolics encountered in plant analysis and form the basis for a class of compounds called flavonoids (Figure 1.6). Approximately 4,000 different flavonoid structures have been identified and more are continually being found.
Figure 1.4 Examples of phenolics with different basic structures.

a. - standard phenolic structure
b. - example of C₆C₀ structure
c. - example of C₆C₁ structure
d. - example of C₆C₃ structure

R = OH, R₁ = H  caffeic acid
R = OCH, R₁ = H  ferulic acid

Figure 1.5 Structure of two coumarin derivatives.

R = H  umbelliferone
R = OH  aesculetin
Flavonoids are compounds that have a benzopyran-4-one nucleus with an aromatic substituent at C₂. Another common characteristic of flavonoids is that nearly all contain oxygen atoms. They commonly react with other organic compounds to form different classes of compounds such as condensed tannins, neoflavonoids and isoflavonoids. Neoflavonoids are flavonoids with an additional aromatic group attached to C₄ and a carbon chain attached to C₂. Isoflavonoids are flavonoids that have reacted to become phenylbenzopyran-4-one (Waterman and Mole, 1994).

Another large and important class of phenolic derivatives are tannins (Figure 1.7). Tannins are important because of their perceived use as a feeding deterrent and appear to be produced from the basic structures described above. There are three distinct subclasses of tannins. The simplest subclass are called phlorotannins which are polymers made up of phloroglucinol held together by C-C and C-O bonds.

The second subclass are called hydrolysable tannins. Like phlorotannins, hydrolysable tannins are made from a single building block, in this case gallic acid. Another recognisable feature of these tannins is the cyclic sugar core to which gallic acid bonds through esterification. When the sugar is in its cyclic conformation a total of five gallic acid molecules can bond with it.
**Figure 1.6** Typical flavonoid skeleton. Individual flavonoids differ with positioning of OH and H groups.

**Figure 1.7** Ellagic acid. An example of a basic tannin structure.
The final subclass, the condensed tannins, are the most widely distributed. These are formed by the linking of a series of monomers which are usually related to flavon-3-ol.

Phenolics are commonly present in plants as "masked" phenolics. Masked or hidden phenolics are phenolics that are present in a glucoside, formed from the addition of a sugar molecule (Waterman and Mole, 1991).

1.4.2 PHENOLIC PRODUCTION IN PLANTS

Phenolic production in plants can occur through three pathways; the polyketide pathway, the shikimic acid pathway and by a combination of both pathways. Most simple phenols are derived from the polyketide pathway while almost all higher plant phenolics are produced from the shikimic acid pathway.

1.4.2.1 The polyketide pathway

The polyketide pathway is dependent upon acetyl co-enzyme A and its activated form, malonyl co-enzyme A (Harborne, 1980). Acetyl co-enzyme A is important because its ability to polymerise and produce carbon chains with keto functional groups on every second carbon atom. This process requires considerable energy and is achieved by
reacting Acetyl co-enzyme A with carbon dioxide to produce malonyl co-enzyme A. This activated compound does not require as much energy to polymerise into a carbon chain (Mann, 1994). The product of the polymerisation is a long carbon chain with a terminal acid. Eventually the carbon chain will cyclize into an aromatic to form a phenolic compound (Figure 1.8) (Waterman and Mole, 1994).

1.4.2.2 The shikimic acid pathway

The shikimic acid pathway is more complex than the polyketide pathway. Its progress depends upon the presence of crythrose-4-phosphate and phosphoenol pyruvate. These two chemicals are bonded through a series of reactions to form shikimic acid which, in turn, reacts with another molecule of phosphoenol pyruvate to form chorismic acid (Mann, 1994). At this point the pathway branches. Chorismic acid can either undergo a series of reactions to give the amino acid tryptophan, or react to form the amino acid phenylalanine. Catalysed by the enzyme phenylalanine ammonia lyase (PAL), phenylalanine is converted to produce trans-cinnamic acid (Figure 1.9) (Haslam, 1974). Trans-cinnamic acid subsequently undergoes several hydroxylation and methylation reactions to form simple phenolics such as p-coumaric acid, caffeic acid and ferulic acid.
Figure 1.8 Production of simple phenols via the polyketide pathway.
**Figure 1.9** Production of complex phenols via the shikimate acid pathway.
1.4.3 THE IMPORTANCE OF PLANT PHENOLICS

It has been proposed that one of the functions of phenolics, and of tannins in particular, is a role in the food selection of many herbivores (Waterman and Mole, 1994; Harborne, 1980). Of particular interest to the present study is that some phenolics have been linked to either stimulating or deterring oviposition of insect larvae (Waterman and Mole, 1994). Phenolic compounds may also act as deterrents to herbivores by being exuded in sticky or reactive substances onto the leaves or glandular hairs.

Phenolics have also been linked to the resistance of plants to certain pathogens such as fungal, bacterial and viral infections. There are generally two types of anti-infectional defence chemicals in plants. Those that are present in plants constantly and those that are produced by the plant in response to an infection. Phenols are believed to be present as both types (Harborne, 1980). In some reports condensed tannins have been shown to have an anti-fungal role, although this has not always been confirmed (Waterman and Mole, 1994).

1.4.4 EXTRACTION OF PHENOLICS FROM PLANTS

The manner in which phenols are extracted from plant material is extremely important as changes in the plants environment can alter the chemical nature of the extracted compounds. This will affect accurate quantification of the compounds within the sample. Extraction techniques deal with the processes of solvation and diffusion.
Phenols will leach out of the plant tissues into a suitable solvent provided there is a sufficient concentration gradient. It is best that samples are processed fresh as the chemicals in dried samples have to be rewetted and then “unstuck” from the tissue before they are able to diffuse into the solvent (Cork et al., 1991).

Different phenolics have varying degrees of polarity and hence will dissolve in polar solvents to different extents. Methanol and acetone are two commonly used solvents although their abilities to extract certain phenols have not been thoroughly studied. The use of water as a solvent in the extraction may increase the amount of phenols extracted, but it may also influence their breakdown after extraction (Cork et al., 1991).

There are two ways that the phenolic extraction can be maximised. The first is by repeatedly extracting the phenols with fresh solvent and the second is by using large volumes of liquid compared to the sample. This approach has drawbacks as it is labour intensive and a small mass of phenolics will be dissolved in a large volume of solvent (Waterman and Mole, 1994).

Extraction time is also an important variable. Short extraction times are beneficial because the phenolic compounds have less time to degrade, however short extractions are rarely quantitative (Cork et al., 1991).

Temperature control is important throughout the extraction. Liquid nitrogen is often used to freeze the plant material and extractions are generally performed at low temperature. Heating the sample will speed up the extraction but this may cause the
chemistry of the analytes to change. Samples being collected some distance from the laboratory should be transported in ice (Waterman and Mole, 1994).

1.4 AIMS

The purpose of this project is to develop and optimise a MEKC method for the separation of phenolics from eucalypts. A method was developed using a standard solution containing phenolics from different classes. Optimisation of the method was required to achieve the maximum resolution and reproducibility and involved studies into the effects of different variables including:

- buffer type
- buffer concentration
- surfactant type and concentration
- use of organic modifiers
- use of mixed micelles
- complexation by different classes of compounds

This method was then applied to phenolics extracted from eucalypt leaves. Adapting the method to plant samples required research into the effect of other non-phenolic compounds present in the sample that may interfere with separation and identification.
Other chromatographic techniques such as GC-MS and HPLC were required to verify the results obtained by MEKC.

1.5 SIGNIFICANCE OF THE STUDY

The high resolving power of CE suggests that this new chromatographic method may be suitable for separating plant phenolics. Extracts from several species of eucalypt were studied to determine if CE can be used to analyse plant phenolics. Phenolics are of interest because of their possible role in disease resistance in plants. For example, vast areas of jarrah forest in south western Australia are effected by the jarrah leafminer and Phytophthora cinnamomi. Both cause widespread damage and destruction in jarrah forests.

1.7 RESEARCH QUESTIONS

Questions that this project addressed included;

- What are the separation capabilities of different buffer systems?
- Can the use of additives improve resolution and reproducibility?
- Can MEKC be used to separate phenolic compounds from eucalypts?
- Do the types of phenolics vary between eucalypt species?
CHAPTER 2. EXPERIMENTAL

2.1 REAGENTS

All reagents were of analytical reagent grade. α and β cyclodextrins, polyoxyethelene 23 lauryl ether (Brij 35), cetyl trimethyl ammonium bromide (CTAB), tetradecyl trimethyl ammonium bromide (TTAB), sodium dodecyl sulfate (SDS), pyridine, butylated hydroxy toluene (BHT), ethyl acetate and phenolic compounds were purchased from Sigma Chemical Company (Australia) and used as received. Methanol, di-sodium tetraborate, sodium di-hydrogen orthophosphate, Sudan III, formic acid, hydrochloric acid, sodium hydroxide and potassium hydroxide were purchased from BHD chemicals (England) and used as received. The 0.45 µm filter units and bis-(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from Alltech (Australia). The fused silica capillary was obtained from Polymicro Technology (Phoenix, Az, USA).

2.2 BUFFER PREPARATION

2.2.1 PREPARATION OF BORATE / PHOSPHATE - SDS BUFFER

Separate 0.0625 M borate and 0.25 M phosphate stock solutions were prepared by dissolving 5.959 g di-sodium tetraborate and 9.75 g sodium di-hydrogen orthophosphate in 250 mL. The stock solutions were mixed and diluted appropriately to obtain a number
of borate, phosphate buffers of varying concentrations (Table 2.1). Each buffer system was prepared at a number of pHs between 7 and 10 (Table 2.1). The pH was adjusted with HCl or NaOH. SDS was added to each buffer in varying amounts (Table 2.1).

2.2.2 PREPARATION OF BORATE / PHOSPHATE - CTAB BUFFER

An 18 mM borate and 30 mM phosphate buffer was prepared by combining 14.5 mL of 0.0625 M di-sodium tetraborate and 6 mL of 0.25 M sodium di-hydrogen orthophosphate stock solutions. The buffer was adjusted to the required pH with NaOH or HCl and 0.911 g CTAB added to a 50 mL aliquot (Table 2.2).

2.2.3 PREPARATION OF SODIUM HYDROGEN CARBONATE - SDS BUFFER

A 50 mM sodium hydrogen carbonate buffer was prepared by dissolving 0.42 g NaHCO₃ in 100 mL Milli-Q water. The pH was adjusted to 8.3 with NaOH or HCl and 0.721 g SDS added to a 50 mL aliquot.

2.2.4 PREPARATION OF BORATE / PHOSPHATE - CTAB / TTAB BUFFERS

A buffer solution containing 6 mM borate and 10 mM phosphate buffer solution was prepared as described in Section 2.2.1 and adjusted to pH 8.5. TTAB (0.841 g) and CTAB (1.822 g) were added to separate 50 mL aliquots of this buffer.
2.2.5 PREPARATION OF BUFFERS CONTAINING ORGANIC MODIFIERS

Several 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffers were prepared as in Section 2.2.1. Before the buffers were diluted to volume, aliquots of organic modifiers were added (Table 2.3).

2.2.6 PREPARATION OF BUFFERS CONTAINING COMPLEXATION AGENTS

Several 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffers were prepared as in Section 2.2.1. SDS (1.442 g) and different amounts of α cyclodextrin and β cyclodextrin were added to separate 50 mL aliquots (Table 2.4).

2.2.7 PREPARATION OF MIXED MICELLE BUFFERS

A 6 mM borate, 10 mM phosphate solution was prepared by mixing aliquots of 0.0625 M di-sodium tetraborate and 0.25 M sodium di-hydrogen orthophosphate stock solutions. SDS (1.442 g) and different amounts of Brij 35 were added (Table 2.5).
2.2.8 PREPARATION OF MIXED MICELLE / ORGANIC MODIFIER BUFFERS

A 6 mM borate, 10 mM phosphate solution was prepared from 0.0625 M di-sodium tetraborate and 0.25 M sodium di-hydrogen orthophosphate stock solutions. Brij 35 surfactant and the organic modifiers were added, the resulting solution made up to volume and the pH was adjusted to 8.3. The surfactant types and concentrations are shown in Table 2.6, 1.442 g SDS was added to each buffer solution.
Table 2.1 Concentration and pH of borate, phosphate, SDS buffer

<table>
<thead>
<tr>
<th>borate concentration (mM)</th>
<th>phosphate concentration (mM)</th>
<th>pH</th>
<th>SDS concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>5</td>
<td>7, 8, 8.3, 8.5, 9, 10</td>
<td>50, 100, 150, 200</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>7, 8, 8.3, 8.5, 9, 10</td>
<td>50, 100, 150, 200</td>
</tr>
<tr>
<td>12</td>
<td>20</td>
<td>7, 8, 8.3, 8.5, 9, 10</td>
<td>50, 100, 150, 200</td>
</tr>
<tr>
<td>24</td>
<td>40</td>
<td>7, 8, 8.3, 8.5, 9, 10</td>
<td>50, 100, 150, 200</td>
</tr>
</tbody>
</table>

Table 2.2 Concentration and pH of borate, phosphate, CTAB buffer

<table>
<thead>
<tr>
<th>Additive</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>5%, 10%, 15%</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>5%, 10%, 15%</td>
</tr>
<tr>
<td>Urea</td>
<td>5 mM, 10 mM, 15 mM</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>5%, 10%, 15%</td>
</tr>
</tbody>
</table>

Table 2.3 Type and concentration of organic modifier added to the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer.
Additive Concentration

<table>
<thead>
<tr>
<th>Additive</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>α cyclodextrin</td>
<td>5 mM, 10 mM, 15 mM</td>
</tr>
<tr>
<td>β cyclodextrin</td>
<td>5 mM, 10 mM, 15 mM</td>
</tr>
</tbody>
</table>

*Table 2.4* Type and concentration of complexation agent added to the 6 mM borate 10 mM phosphate, 100 mM SDS, pH 8.3 buffer

<table>
<thead>
<tr>
<th>Borate concentration (mM)</th>
<th>Phosphate concentration (mM)</th>
<th>SDS concentration (mM)</th>
<th>Brij 35 concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>10</td>
<td>100</td>
<td>5, 10, 15</td>
</tr>
</tbody>
</table>

*Table 2.5* Surfactant concentration and type in the mixed SDS / Brij 35 buffer.

<table>
<thead>
<tr>
<th>SDS concentration (mM)</th>
<th>Brij 35 concentration (mM)</th>
<th>Additive concentration (% v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>5, 10, 15</td>
<td>5, 7, 10 methanol</td>
</tr>
<tr>
<td>100</td>
<td>5, 10, 15</td>
<td>5, 10 acetonitrile</td>
</tr>
</tbody>
</table>

*Table 2.6* Concentration and type of surfactant and additive used in 6 mM borate, 10 mM phosphate buffer, pH 8.3.
2.3 SAMPLE PREPARATION

A standard solution of 18 phenols, phenolic acids and coumarins was prepared (Table 2.7). Exactly 0.01 g of each compound was dissolved in 50% aqueous methanol solution before being made up to 100 mL volume. The samples were degassed with a 0.45 μm filter unit before being applied to the CE column.

2.4 EXTRACTION OF PHENOLICS FROM EUCALYPTS

2.4.1 EXTRACTION OF TOTAL PHENOLICS (INCLUDING GLYCOSIDES)

Leaves from species *E. marginata* (jarrah), *E. calophylla* (marri), *E. todtiana*, *E. erythrocorys* (illyarrie) and *E. rudis* were used in this study. The following extraction procedure was used for all species:

Approximately 2 g of fresh leaf material was frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. The sample was quantitatively transferred to a 50 mL centrifuge tube using 15 mL of 50% aqueous methanol which contained 5 mM butylated hydroxy toluene (BHT). The phenolics were extracted with 3 x 15 mL aliquots of 50% aqueous methanol at 4°C with minimum exposure to light. The combined extract was evaporated under reduced pressure to dryness and the residue taken up in
approximately 4 mL 50% aqueous methanol. The sample was defatted by passing the sample through a C18 solid phase extraction cartridge and degassed using a 0.45 μm filter unit.

2.4.2 EXTRACTION OF PHENOLICS BOUND AS GLYCOSIDES

The free phenolic extract (Section 2.4.1), was adjusted to pH 1 - 2 with 6 M HCl. This sample was heated to near boiling for 45 minutes, then allowed to cool before the phenolics were repeatedly extracted into ethyl acetate. The ethyl acetate fractions were evaporated to dryness using a rotary evaporator. The residue was dissolved in 2.5 mL 50% aqueous methanol and degassed prior to CE analysis.

2.4.3 EXTRACTION OF BOUND PHENOLICS FROM THE CELL WALL

The leaf tissue from which the free phenolics had been extracted (Section 2.4.1), was washed with water, dried and broken down further. The sample was then washed repeatedly with acetone. In a three necked round bottom flask, 50 mL of 1 M KOH was degassed for 20 minutes using N₂ gas. Exposure to light was minimised. The leaf sample was added and the N₂ gas bubbled through the mixture for 1 h. The pH was then adjusted to ~ 2 using 6 M HCl. The contents of the flask were passed through a sintered glass filter (porosity 3) and the effluent was collected. The phenolics in the effluent were extracted into ethyl acetate. The ethyl acetate fraction was evaporated to dryness in a
rotary evaporator. The residue was taken up in approximately 2.5 mL methanol and
degassed before being applied to the CE.

<table>
<thead>
<tr>
<th>Analyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
<tr>
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<tr>
<td>16</td>
</tr>
<tr>
<td>17</td>
</tr>
<tr>
<td>18</td>
</tr>
</tbody>
</table>

Table 2.7 Standard mix of phenols, phenolic acids and coumarins.
2.5 DERIVITISATION OF SAMPLES FOR USE IN GC-MS

The phenolics obtained from the procedures described in sections 2.4.2 and 2.4.3 were derivitised before being run on the GC-MS. The phenolic extract was evaporated to dryness and the residue taken up in ethyl acetate. An aliquot of phenolic extract (500 µL) was evaporated to dryness under a stream of nitrogen. Pyridine (200 µL) was added to the residue, followed by 100 µL BSTFA, under a stream of N₂. The mixture was then left to derivitise in the vial for 45 minutes at room temperature before the excess BSTFA was evaporated off, again under a stream of nitrogen. The residue was taken up in 50 µL of dry ethyl acetate. The sample (1 µL) was then taken up in a 10 µL Hamilton syringe and injected into the GC-MS.

2.6 INSTRUMENTATION

2.6.1 CAPILLARY ELECTROPHORESIS CONDITIONS

A Waters Quanta 4000 Capillary electrophoresis system was used for this study. The capillary was 60 cm long (effective length 52.5 cm) with OD 360 µm and ID 75 µm. Detection was by UV-VIS detector set at 214 nm. Unless otherwise stated the voltage applied was 18 kV and the injection was performed hydrostatically for 1 second. All work was carried out at room temperature (23 - 25 °C). Methanol was used to determine the
retention time of the unretained neutral solute \( (t_o) \) and sudan III for the retention time of the micelle \( (t_{mc}) \).

The capillary was conditioned daily by purging with Milli-Q grade water for 5 minutes followed by the buffer for 30 minutes. Between each run the column was washed with water for 2 minutes and buffer for 2 minutes. New columns were purged with water for 30 minutes and then buffer for 1 hour. KOH (1 M) was used for cleaning the column.

2.6.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY CONDITIONS

HPLC analysis was performed using a Varian 9010 gradient pump, a Varian 9050 variable wavelength UV-VIS detector and a Varian autosampler fitted with a 10 μL Rheodyne loop. Separation was achieved on an Altima C18, 5 μm column (250 mm x 4.6 mm) from Alltech, Australia. A gradient elution method was used comprising two solvents. Solvent A was methanol while solvent B was a formic acid/water solution (5:95 v/v). Gradient range was 0-2 minutes, isocratic 7%A in B; 2-8 minutes, 7-25%A in B; 8-25 minutes, 25-75%A in B, 25-30 minutes, 75-100% A in B. The flow rate was 1.5 mL min\(^{-1}\) and the wavelength of detection was 280 nm.
2.6.3 GAS CHROMATOGRAPH-MASS SPECTROMETER CONDITIONS

The derivitised plant samples were analysed using a Varian Saturn gas chromatograph-mass spectrometer. The sample was injected into the GC with an injector temperature of 150 °C and the column set at 100 °C. The column temperature was increased at 5.0 °C minute⁻¹ to 250 °C over 30 minutes. A 30 m SE54 column from Alltech, Australia was used, ID 0.25 mm and with a film thickness of 0.25 μm. The mass spectrometer detected over a mass range of 60 to 650 m/z at 1 sec.scan⁻¹ and the transfer line was kept at a constant 240 °C.

2.7 DATA ANALYSIS

Peak retention time was measured in minutes and peak height in microvolts. Reproducibility of peak retention time was measured in percentage relative standard deviation. % RSTDV is calculated by:

\[
\% \text{ RSTDV} = \frac{m \cdot 100}{S}
\]

Where \( m \) is the mean of the peak retention time and \( S \) is the standard deviation of the peak retention time. For the purpose of this study, acceptable peak retention time reproducibility shall mean a % RSTDV value of less than 1% (Cartoni et al., 1995; Bretnall et al., 1995).
The resolution of the peaks is calculated by:

\[ R = \frac{2 \cdot Z}{W_a + W_b} \]

where \( Z \) is the difference in retention time between the peaks of interest and \( W \) is the width of the two peaks. In this project acceptable resolution shall mean \( R \geq 1 \). This value corresponds to baseline resolution on a chromatogram. Co-elution refers to \( R < 0.5 \) while partial co-elution corresponds to \( 1 > R > 0.5 \) (Szolar et al., 1995).

The efficiency of a buffer solution will be determined by calculating the number of theoretical plates. The number of theoretical plates is calculated by:

\[ N = 16 \left( \frac{t_r}{w} \right)^2 \]

where \( N \) is the number of theoretical plates, \( t_r \) is the retention time of the peak and \( w \) is the peak width. The number of theoretical plates was calculated for several peaks with differing retention times (Szolar et al., 1995).
CHAPTER 3 DEVELOPMENT OF A SUITABLE BUFFER SYSTEM

3.1 INTRODUCTION

3.1.1 BUFFER SYSTEMS

MEKC requires electrically conducting solutions to act as running electrolytes in the capillary. As pH has a critical effect on the electro-osmotic flow and the electrophoretic mobilities of compounds, buffer solutions are commonly used as electrolytes (Kuhn et al., 1993). A wide range of buffer solutions has been employed in MEKC. For example the use of borate, phosphate buffers with SDS (Pietta et al., 1994) and CTAB (Suzuki et al., 1994), as well as NaHCO₃ buffers with SDS (Cartoni et al., 1995) have been reported for the separation of a wide variety of organic compounds including vitamins, organic acids and simple phenols. The ability of these buffers to separate the complex mixture of phenolics (including phenolic acids, simple phenols and coumarins) was investigated.

3.1.2 BUFFER CONCENTRATION

Changes in the buffer concentration can increase or decrease the elution window of the buffer. Decreasing the concentration of the buffer will reduce its ionic strength of the buffer and hence decrease the zeta potential in the capillary. The reduced zeta potential decreases the size of the EOF, leading to slower elution rates. It is critical that the buffer concentration is sufficient to ensure that the EOF is large enough to overcome the opposing micellar movement and cause the components to elute at the detector end of the capillary. Several
borate, phosphate, SDS buffers were prepared with different concentrations according to Table 2.1. These buffers were run on the CE to determine the effects of buffer concentration on the separation of the phenolic mixture.

3.1.3 pH EFFECTS

Changes in the pH of the buffer system can also affect the elution window of the buffer by altering the EOF. Varying the pH will cause the forward reaction in the hydrolysis equilibrium of the capillary wall (Figure 1.2) to predominate. The increased charge on the capillary wall will create a greater zeta potential and hence increase the EOF.

pH changes will also affect the electrophoretic mobilities of certain groups of compounds such as phenolic acids. With increase in pH, the ionisation of the phenolic acids increases. Consequently the charged phenolic acid molecules are repelled from the negative electrode as well as being subject to partitioning between the micelles and the buffer solution. Hence the pH can alter the elution order and elution speed of analyte compounds.

3.1.4 ALTERNATIVE SURFACTANTS

The surfactant in the buffer solution facilitates the separation of the components by forming micelles that act as a pseudo stationary phase. Using anionic surfactants, the resulting micelles migrate in the direction that opposes the EOF. However, the EOF is always stronger than the micellar movement and the components elute at the detector end of the capillary. Consequently, the net movement of the micelles is towards the detector, albeit at a slower rate than the EOF. As hydrophobic analyte compounds partition between the buffer solution and
the hydrophobic centre of the micelles, the retention time of the individual components will be slightly different, depending on the amount of time spent in the slower moving micelle.

In this study, the different separating capabilities of anionic surfactants (SDS) and cationic surfactants (TTAB and CTAB) was examined. As the CTAB and TTAB micelles have different charge densities when compared to the SDS micelle, they will have different electrophoretic mobilities. This will cause the retention times of the sample components to change and the elution window to vary. The use of surfactants with different hydrophobic chains will influence the hydrophobic nature of the micelle. Consequently, the affinity of the analytes for the different micelles will vary, potentially changing the elution order and selectivity of the buffer.

3.1.5 EFFECT OF ALTERING THE SDS CONCENTRATION

The concentration of the micelles in the buffer solution must be greater than the critical micelle concentration (CMC) if stable micelles are to form and separation of the phenolics is to be achieved. The critical micelle concentration is the minimum concentration at which the surfactant molecules aggregate into micelles. The average number of surfactant molecules that aggregate to form a single micelle is called the aggregation number (AN). The AN is altered by variables such as the ionic strength of the buffer, the presence of organic modifiers and the temperature (Nielsen et al., 1993). Excessively large concentrations of surfactant in a buffer system can degrade separation of components due to changes in the shape, size and conformation of the micelles (Kuhn et al., 1993). The concentration of the SDS surfactant was varied to determine the concentration for optimal separation of the 18 phenolics. The SDS concentrations are listed in Table 2.1
3.2 RESULTS AND DISCUSSION

3.2.1 BUFFER SYSTEMS

The different buffer systems prepared in order to test their capacity to separate a complex standard mix are listed in Table 2.1 and Table 2.2. The 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.5 buffer system, operating at room temperature, achieved the best separation of the complex phenolic mixture. Using this buffer system the majority of the components were resolved. However, o-coumaric acid and vanillic acid as well as ferulic acid and 4-methylesculetin co-eluted and m-coumaric acid and catechol partially co-eluted (Figure 3.1). The numbers in the electropherograms correspond to the compounds listed in Table 2.7. In contrast, the 18 mM borate, 30 mM phosphate, 50 mM CTAB buffer at pH 7 and 8.5 could not resolve any of the components. The poor separating capability of this buffer is due to the surfactant. CTAB form positively charged micelles that move with the EOF. Consequently, the hydrophobic phenolic compounds will elute before they have had time to separate. The 50 mM NaHCO3, 100 mM SDS, pH 8.3 buffer only resolved 13 broad peaks, clearly indicating significant amounts of co-elution and the buffers lack of selectivity for some closely related components (Figure 3.2).

Comparison of the number of theoretical plates for the buffer systems also clearly illustrates the different separating capabilities of these buffers for the phenolic mixture. The number of theoretical plates for the 50 mM NaHCO3, 100 mM SDS, pH 8.5 buffer ranged between 36 700 and 62 387. However, for components separated by the 6 mM borate, 10 mM
borate, 100 mM SDS, pH 8.5 buffer, theoretical plates in the range of 90 489 to 234 065 were easily achievable.

Further study of the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.5 buffer was undertaken to achieve optimal separation of the complex phenolic mixture.

3.2.2 BUFFER CONCENTRATION

Employing the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.5 buffer, the concentration of borate and phosphate was varied according to Table 2.1. The elution order of the components was determined through spiking.

The 3 mM borate, 5 mM phosphate, 100 mM SDS, pH 8.5 buffer gave poor separation, with only 14 peaks being resolved. Band broadening was also evident for the later eluting peaks. The 12 mM borate, 20 mM phosphate, 100 mM SDS, pH 8.5 buffer had similar separating capabilities to the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.5 buffer (Table 3.1). This is also highlighted by a study of the theoretical plates (Table 3.2). The theoretical plates did not change appreciably with increased concentration of borate and phosphate. The 24 mM borate, 40 mM phosphate, 100 mM SDS, pH 8.5 buffer gave poor separation and only resolved 12 components.

The effect of increasing the concentration of borate and phosphate on the retention time is clearly demonstrated in Figure 3.3. The 12 mM borate, 20 mM phosphate, 100 mM SDS, pH 8.5 buffer gave shorter retention times for the components compared to the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.5 buffer. The retention times decreased regardless of the type of component. The study of retention times was restricted to the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.5 and the 12 mM borate, 20 mM phosphate,
100 mM SDS, pH 8.5 buffer. Accurate identification of component peaks and measurement of the peak widths for the remaining buffers was impossible due to the large amount of co-elution.

Peak retention time reproducibility for each buffer is given in Figure 3.4. The 12 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.5 buffer has \%RSTDV values for peak retention times that exceeded the desired level of 1%. All reproducibility calculations were made using at least four consecutive runs.

The differences in the separating capabilities of the different buffer concentrations is primarily due to changes in the EOF. The EOF is created by the zeta potential which is itself dependent upon the concentration of the electrolyte solution. By increasing the concentration of the buffer solution, the EOF is enlarged, giving shorter retention times and decreasing the elution window.

The strong EOF created by the 24 mM borate, 40 mM phosphate, 100 mM SDS, pH 8.5 buffer reduces the time in which separation can be achieved and may not have allowed the components to partition fully between the micelle and the buffer. This resulted in co-elution between similar compounds. The poor separation achieved for the 3 mM borate, 5 mM phosphate, 100 mM SDS, pH 8.5 buffer is most likely due to a combination of co-elution and band broadening. Band broadening occurred as a result of the long retention times.

The ionic strength of the 12 mM borate, 20 mM phosphate, 100 mM SDS, pH 8.5 buffer and the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.5 buffer produce an EOF which allows for the adequate separation of the phenolics. However the differences in the peak retention time reproducibility (Figure 3.4) of the two systems suggest that the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.5 buffer is more suitable for the separation of the complex phenolic mixture.
Figure 3.1  Elution order of 18 phenolics using the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.5, buffer at 18 kV. The numbers correspond to the compounds listed in Table 2.7.

Figure 3.2  Electropherogram of the 50 mM NaHCO₃, 100 mM SDS, pH 8.3, 15 kV buffer system
<table>
<thead>
<tr>
<th>Buffer concentration (mM)</th>
<th>Co-eluting peaks</th>
<th>Partially resolved peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>(borate / phosphate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 / 10</td>
<td>• <em>o</em>-coumaric acid, <em>vanillic acid</em></td>
<td>• <em>m</em>-coumaric acid,</td>
</tr>
<tr>
<td></td>
<td>• ferulic acid, 4-methylcatechol</td>
<td>catechol</td>
</tr>
<tr>
<td>12 / 10</td>
<td>• protocatechuic acid,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>gallic acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• caffeic acid, coumarin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• 4-methylumbelliferone,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>salicylic acid</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.1** Separating capabilities of buffer with different concentrations of borate and phosphate (100 mM SDS, pH 8.5)

<table>
<thead>
<tr>
<th>Buffer concentration (mM)</th>
<th>chlorogenic acid (N)</th>
<th>umbelliferone (N)</th>
<th>salicylic acid (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(borate / phosphate)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 / 10</td>
<td>146 399</td>
<td>105 441</td>
<td>214 921</td>
</tr>
<tr>
<td>12 / 20</td>
<td>137 840</td>
<td>210 166</td>
<td>223 020</td>
</tr>
</tbody>
</table>

**Table 3.2** Number of theoretical plates (N) with change in buffer concentration (100 mM SDS, pH 8.5)
Figure 3.3  Retention time of components using different borate, phosphate buffer concentrations (100 mM SDS, pH 8.5)

Figure 3.4  Peak retention time reproducibility for different borate, phosphate buffer concentrations with 100 mM SDS, pH 8.5.
3.2.3 pH AND VOLTAGE EFFECTS

The pH of the 6 mM borate, 10 mM phosphate, 100 mM SDS buffer was varied according to the values listed in Table 2.1.

The 6 mM borate, 10 mM phosphate, 100 mM SDS buffer at pH 7 and 10 could only resolve 14 of the 18 peaks. In both cases the later eluting peaks had broad bases. In contrast, buffers with pHs ranging from 8 to 9 inclusive achieved good resolution with at most six components co-eluting (Table 3.3). The best separation was achieved with pH 8.3. The change in pH did not effect the efficiency of the buffer (Table 3.4).

The effect of altering pH on the peak retention times is shown in Figure 3.5. The retention times for components separated using buffers with pH 7 and 10 were not calculated as the excessive co-elution prevented accurate identification of the peaks. The retention times of the components did not change appreciably between pH 8 and 9. However, buffers with pH between these two values showed a slight increase in the retention times of the components. There was no major change in the elution window of the buffer when the pH was increased from 7 to 10 (Table 3.5).

In addition to optimising the pH, the voltage applied to the electrodes was also varied between 14 kV and 20 kV. Increasing the voltage had the effect of reducing retention times of the components (Figure 3.6).

With the exception of some individual components, the values for the peak retention time reproducibility remained below 1% RSTDV for the buffers over the pH range studied (Figure 3.7).

In all cases at least 7 runs were recorded for each pH.
Initially only the pH values of 7, 8, 8.5, 9 and 10 were tested. pH 8.5 gave the best separation of these five buffers and further studies were undertaken to find the exact pH for optimal resolution. The separation improved slightly for pH 8.3. The poor separation achieved by the buffer at pH 7 is not surprising. The separation of the non ionisable coumarins is largely unaffected by pH and relies predominantly on the hydrophobic interactions with the micelle (Morin et al., 1993). However the phenolic acids at pH 7 are only weakly ionised and the hydrophobic interactions are not sufficient to separate them. These acids at pH 7 are only crudely separated (Boyce, 1996).

The minimal change in elution window with large changes in pH (7 to 10) was not expected (Table 3.5). The increased pH should increase hydrolysis at the silica wall, increasing the zeta potential and hence the EOF. It is unclear as to why such small changes in the elution window were observed.

Increasing the voltage, or increasing the potential difference between the electrodes, increased the EOF as the positive ions carrying the bulk solution are more strongly attracted to the negative electrode. The migration times of the phenolic acids will be affected by increases in voltage. However, as there was no change in the elution order of the components, these effects were slight.

The best separation of the phenolic mixture was achieved with a 6 mM borate, 10 mM phosphate, 100 mM SDS buffer at pH 8.3 and an applied voltage of 19 kV (Figure 3.8).
<table>
<thead>
<tr>
<th>pH</th>
<th>Co-eluting peaks</th>
<th>Partially resolved peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>• chlorogenic acid, cinammic acid</td>
<td>• m-coumaric acid, catechol</td>
</tr>
<tr>
<td>8.3</td>
<td>• chlorogenic acid, 4-hydroxycoumarin</td>
<td>• catechol, m-coumaric acid</td>
</tr>
<tr>
<td>8.5</td>
<td>• o-coumaric, vanillic acid</td>
<td>• m-coumaric acid, catechol</td>
</tr>
<tr>
<td></td>
<td>• ferulic acid, 4-methylesculetin</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>• m-coumaric acid, catechol, chlorogenic acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• vanillic acid, cinammic acid 4-hydroxycoumarin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• protocatechuic acid, gallic acid</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.3** Separating capabilities of the 6 mM borate, 10 mM phosphate, 100 mM SDS buffer with changes in pH.

<table>
<thead>
<tr>
<th>pH</th>
<th>ferulic acid (N)</th>
<th>salicylic acid (N)</th>
<th>gallic acid (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>22 207</td>
<td>124 511</td>
<td>106 289</td>
</tr>
<tr>
<td>8.5</td>
<td>24 567</td>
<td>105 441</td>
<td>214 791</td>
</tr>
<tr>
<td>9</td>
<td>134 110</td>
<td>124 342</td>
<td>107 318</td>
</tr>
</tbody>
</table>

**Table 3.4** Separating efficiency of 6 mM borate, 10 mM phosphate, 100 mM SDS buffer with change in pH
Figure 3.5  Peak retention time with change in pH in the 6 mM borate, 10 mM phosphate 100 mM SDS buffer.

<table>
<thead>
<tr>
<th>pH</th>
<th>$t_0/t_{mc}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0.317</td>
</tr>
<tr>
<td>8</td>
<td>0.294</td>
</tr>
<tr>
<td>9</td>
<td>0.311</td>
</tr>
<tr>
<td>10</td>
<td>0.287</td>
</tr>
</tbody>
</table>

Table 3.5  Size of the elution window with changes in pH of the 6 mM borate, 10 mM phosphate 100 mM SDS, pH 8.5
**Figure 3.6** Retention times of components with change in applied voltage (6 mM borate 10 mM phosphate, 100 mM SDS, pH 8.5 buffer)

**Figure 3.7** Peak retention time reproducibility with change in pH in the 6 mM borate 10 mM phosphate, 100 mM SDS buffer.
Figure 3.8 Elution order of the 18 phenolics with the 6 mM borate 10 mM phosphate 100 mM SDS, pH 8.3, 19 kV buffer. The numbers correspond to the compounds listed in Table 2.7.
3.2.4 ALTERNATIVE SURFACTANTS

Cationic surfactants, CTAB and TTAB, were added to the 6 mM borate, 10 mM phosphate, pH 8.5 buffer to test their ability to separate the phenolic mixture.

The cationic surfactants decreased the separating capabilities of the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.5 buffer. TTAB gave an electropherogram with a very unstable baseline and could only resolve 8 peaks, while the CTAB buffer produced a slightly more stable baseline, but only resolved 11 peaks.

The poor separating capabilities of these buffers is due to the charge on the micelles. The cationic surfactants travel with the EOF. Consequently the elution window of the buffer will be small and the components will not have sufficient time to separate before they elute from the column.

3.2.5 EFFECT OF ALTERING THE SDS CONCENTRATION

The concentration of SDS in the 6 mM borate, 10 mM phosphate, pH 8.5 buffer was varied, according to Table 2.1, to test the effectiveness of different surfactant concentrations in separating the complex phenolic mixture.

The separating capabilities of the 6 mM borate, 10 mM phosphate, pH 8.5 buffer with different concentrations of SDS is shown in Table 3.6. 100 mM SDS clearly gives the best separation. The concentration of the surfactant does not change the efficiency of the buffer (Table 3.7).
The increased concentrations of surfactant caused a gradual increase in the retention times of the components (Figure 3.8), as well as an increase in the elution window of the buffer (Table 3.8).

The surfactant concentration had no effect on the peak retention time reproducibility of the buffer. Figure 3.9 shows that with the exception of ferulic acid in the 150 mM SDS buffer, all of the peaks have %RSTDV values of less than 1% for all SDS concentrations.

From the above results, it is clear that there is an optimal surfactant concentration at which the best separation of the 18 phenolics mix is achieved. The separating capability of the 6 mM borate, 10 mM phosphate buffer improved when the SDS concentration was increased to 100 mM SDS. However, further increases led to a drop in the separating capabilities. The poorer resolution at higher SDS concentrations could be due to increased electrostatic repulsion between the micelles and hence changes in the shape of the micelles. Also, joule heating, which accompanies higher SDS concentrations, has also been reported to contribute to the decreased separating power of buffers (Tomas-Barberan, 1995). Increased operating currents observed when using higher surfactant concentrations supports this theory.

The increase in surfactant concentration led to a slight rise in the retention times of the components and an increase in the size of the elution window. This may be due to the high concentrations of surfactant changing the size of the micelles and reducing the overall micelle charge density. This will result in reduced electrostatic repulsion between the micelle and the electrode and hence shorter elution windows. Decreased resolution with high surfactant concentrations has been reported by Tomas-Barberan. (1995).
Table 3.6 Separating capability of the 6 mM borate, 10 mM phosphate, pH 8.5 buffer with change in surfactant concentration.

<table>
<thead>
<tr>
<th>SDS concentration (mM)</th>
<th>co-eluting peaks</th>
<th>partially resolved peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>• vanillic acid, cinamic acid</td>
<td>• m-coumaric acid, catehol</td>
</tr>
<tr>
<td></td>
<td>• umbelliferone, salicylic acid</td>
<td>• caffeic acid, gallic acid</td>
</tr>
<tr>
<td>100</td>
<td>• chorogenic acid, 4-hydroxycoumarin</td>
<td>• catehol, m-coumaric acid</td>
</tr>
<tr>
<td>150</td>
<td>• orcinol, chlorogenic acid</td>
<td>• m-coumaric acid, catehol</td>
</tr>
<tr>
<td></td>
<td>• ferulic acid, 4-hydroxycoumarin</td>
<td>• o-coumaric acid, vanillic acid, cinamic acid</td>
</tr>
<tr>
<td>200</td>
<td>• orcinol, ferulic acid</td>
<td>• 4-methylesculetin, m-coumaric acid</td>
</tr>
<tr>
<td></td>
<td>• o-coumaric acid, vanillic acid</td>
<td>• catehol, cinamic acid</td>
</tr>
</tbody>
</table>

Table 3.7 Efficiency (theoretical plates) of 6 mM borate, 10 mM phosphate, pH 8.5 buffer with a change in SDS concentration.

<table>
<thead>
<tr>
<th>SDS concentration (mM)</th>
<th>ferulic acid</th>
<th>4-hydroxycoumarin</th>
<th>coumarin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N)</td>
<td>(N)</td>
<td>(N)</td>
</tr>
<tr>
<td>50</td>
<td>129 600</td>
<td>137 300</td>
<td>128 717</td>
</tr>
<tr>
<td>100</td>
<td>90 508</td>
<td>113 739</td>
<td>122 288</td>
</tr>
<tr>
<td>150</td>
<td>79 571</td>
<td>140 925</td>
<td>144 684</td>
</tr>
<tr>
<td>200</td>
<td>122 978</td>
<td>105 868</td>
<td>106 434</td>
</tr>
</tbody>
</table>
Figure 3.8  Peak retention time with changes in SDS concentration in the 6 mM borate, 10 mM phosphate, pH 8.5 buffer.

Figure 3.9  Peak retention time reproducibility with change in surfactant concentration in the 6 mM borate, 10 mM phosphate, pH 8.5 buffer.
<table>
<thead>
<tr>
<th>SDS concentration (mM)</th>
<th>$t_o / t_{mc}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.385</td>
</tr>
<tr>
<td>100</td>
<td>0.342</td>
</tr>
<tr>
<td>150</td>
<td>0.284</td>
</tr>
<tr>
<td>200</td>
<td>0.263</td>
</tr>
</tbody>
</table>

Table 3.8  Size of the elution window with increased concentration of SDS (6 mM borate, 10 mM phosphate, pH 8.5)
CHAPTER 4. THE ROLE OF ADDITIVES AND MIXED MICELLES IN MEKC.

4.1 INTRODUCTION

4.1.1 ORGANIC MODIFIERS

The use of organic modifiers to increase the retention times and separating capabilities of buffer systems has been reported (Bretnall et al., 1995; Pietta et al., 1994). Organic modifiers are believed to increase the elution window of a buffer by increasing the viscosity of the buffer solution which causes a reduction in the EOF (Kuhn et al., 1993). In addition, organic modifiers can increase the separating capabilities of the buffer by changing the polarity of the buffer solution or by altering the properties of the micelles (Kuhn et al., 1993). The effects of a range of organic modifiers including acetonitrile, methanol, urea and isopropanol were investigated.

4.1.2 COMPLEXATION AGENTS

Usually a sample component partitions between two phases, the buffer solution and the micelle. By adding a complexation agent, a third phase is provided, potentially achieving greater resolution of the components. Some of the most commonly used complexation agents are cyclodextrins. Cyclodextrins are neutral, water soluble, toroidal oligosaccharides with hydrophobic cavities within which a hydrophobic molecule can complex (Baker, 1995). Due to their neutral charge, cyclodextrins are
carried along by the EOF. This results in shorter retention times for any compound that complexes with the hydrophobic cavity. α, β and γ cyclodextrins are distinguishable by their different cavity sizes. Analyte molecules that are too large to complex with the hydrophobic cavity will be unaffected by the presence of the cyclodextrin in the buffer system. Two complexation agents were tested, α and β cyclodextrin.

4.1.3 MIXED MICELLES

The use of buffer systems utilising two different surfactant agents has been reported (Rasmussen et al., 1990). The resulting mixed micelles are likely to differ in size, shape, charge density and hydrophobicity when compared to single surfactant micelles. This allows for manipulation of retention times and elution orders as well as enhancing separation. The resolving capabilities of a mixed micelle system was investigated. The mixed micelles comprised anionic SDS surfactant molecules and neutral Brij 35 surfactant molecules.

4.1.4 MIXED MICELLES AND ORGANIC MODIFIERS

The improved selectivity achievable by mixed micelles, when combined with a larger elution window provided by an appropriate organic modifier, has the potential to achieve even higher separating capabilities. The separating capabilities of a borate, phosphate, SDS, Brij 35 buffer system with small amounts of methanol or acetonitrile added was investigated.
4.2 RESULTS AND DISCUSSION

4.2.1 ORGANIC MODIFIERS

The addition of methanol (Figure 4.1), isopropanol (Figure 4.2) and acetonitrile (Figure 4.3) to the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer increased the peak retention times of the components. Increasing the concentration of the organic modifier caused further increases in retention times. The addition of urea did not change the retention times of the components significantly (Figure 4.4).

The effect of the organic modifiers on retention times was not uniform for all sample components. Increasing the concentrations of methanol, isopropanol and acetonitrile caused the retention times of the later eluting peaks to increase by a greater degree compared to the early eluting peaks. Organic modifier concentrations of 15% or higher often gave runtimes that were unacceptably long. For example, when using 15% isopropanol, gallic acid eluted after 43 minutes.

Calculation of the elution window indicates that the EOF is reduced by the addition of organic modifiers. For example, the addition of 5% isopropanol caused the \( t_{0}/t_{mc} \) value to decrease from 0.329 to 0.296. Similar changes in the elution window were observed with acetonitrile and methanol. The addition of urea did not alter the size of the elution window.

The addition of organic modifiers decreases the stability of the buffer system. The retention times for the peaks in the 6 mM borate, 10 mM phosphate, 100 mM
SDS, pH 8.3 buffer have percentage relative standard deviations (% RSTDV) of less than 0.5%. With the addition of the organic modifiers, the % RSTDV of the components increased to over 1% (Figure 4.5, 4.6, 4.7). This loss in reproducibility of retention times was not noticed for 5% isopropanol (Figure 4.8).

The separating capabilities of the 6 mM borate 10 mM phosphate, 100 mM SDS, pH 8.3 buffer deteriorated with the addition of the organic modifiers. This is shown by the number of co-eluting and partially co-eluting peaks in the chromatograms (Table 4.1).

The addition of organic modifiers reduced the efficiency of the buffer systems (Table 4.2). The efficiency decreased further with increased organic modifier concentration. The efficiency of isopropanol was not calculated due to the poor resolution.

The elution order of the 18 phenolics remained unchanged with the addition of urea to the buffer solution. However, the elution order changes considerably with the addition of organic modifiers such as acetonitrile and methanol. Although the majority of the components in these buffer systems have increased retention times due to the smaller EOF, the retention times of some components decreased. For example, the electropherograms of the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer (Figure 4.9a) and the 6 mM borate, 10 mM phosphate, 100 mM SDS, 10% acetonitrile, pH 8.3 buffer (Figure 4.9b) show that the addition of acetonitrile causes coumarin and umbelliferone to elute earlier. Also, other components such as vanillic acid undergo smaller, localised changes. Similar changes in elution order are also evident with methanol and isopropanol.
With the exception of urea, all of the organic modifiers caused the elution window to increase. This is due to the organic modifiers increasing the viscosity of the buffer system, resulting in a reduced EOF. Similar results have been reported by Pietta et al. (1994) and Bretnall et al. (1995).

The fall in efficiency of the buffer with the addition of organic modifiers (illustrated in Table 4.2), is due to the increase in joule heating. The rise in temperature within the column due to joule heating increased the amount of longitudinal diffusion of the components. Joule heating is characterised by increased operating currents. Increases in the operating current of up to 20% were observed with the addition of organic modifiers. Similar results have been reported by Masselter et al., (1995) with the addition of acetonitrile.

The peak retention times of the majority of the components increased with the addition of organic modifiers, such as methanol and acetonitrile, due to the reduced EOF. However, non-polar molecules such as coumarin and umbelliferone elute much earlier with respect to other compounds (Figure 4.9b). In the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer with no organic modifiers added, hydrophobic compounds such as coumarin and umbelliferone elute late because of their strong affinity for the hydrophobic interior of the micelle. Under these conditions the partition equilibrium for the components lies very much to the right.

\[ \text{Buffer} \xrightarrow{A} \text{Micelle} \]
The addition of acetonitrile or methanol, to the aqueous buffer solution decreases the buffer's polarity and the partition equilibrium moves to the left. Tomas-Barberan (1995), reported similar changes in elution order.

The addition of these organic modifiers not only reduced the polarity of the buffer phase but is also likely to have affected micelle size and charge density. This would also contribute to the early elution of coumarin and umbelliferone.
Figure 4.1  Effect on peak retention time using a 6 mM borate, 10 mM phosphate 100 mM SDS, pH 8.3 buffer with the addition of methanol

Figure 4.2  Effect on peak retention time using a 6 mM borate, 10 mM phosphate 100 mM SDS, pH 8.3 buffer with the addition of isopropanol
Figure 4.3  Effect on peak retention time using a 6 mM borate, 10 mM phosphate 100 mM SDS, pH 8.3 buffer with the addition of acetonitrile.

Figure 4.4  Effect on peak retention time using a 6 mM borate, 10 mM phosphate 100 mM SDS, pH 8.3 buffer with the addition of urea.
Figure 4.5  Peak retention time reproducibility with addition of methanol to the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer

Figure 4.6  Peak retention time reproducibility with the addition of acetonitrile to the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer
Figure 4.7  Peak retention time reproducibility with the addition of urea to the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer

Figure 4.8  Peak retention time reproducibility with the addition of isopropanol to the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer
<table>
<thead>
<tr>
<th>Additive</th>
<th>Co-eluting peaks</th>
<th>Partially resolved peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additive</td>
<td>- chlorogenic acid, 4-hydroxycoumarin</td>
<td>- m-coumaric acid, catechol</td>
</tr>
<tr>
<td>5% Methanol</td>
<td>- o-coumaric acid, catechol</td>
<td>- chlorogenic acid, umbelliferone</td>
</tr>
<tr>
<td></td>
<td>- salicylic acid, 4-methylumbelliferone</td>
<td></td>
</tr>
<tr>
<td>5% Isopropanol</td>
<td>- cinamnic acid, chlorogenic acid</td>
<td>- vanillic acid, catechol, o-coumaric acid</td>
</tr>
<tr>
<td></td>
<td>- umbelliferone, salicylic acid</td>
<td>- gallic acid, caffeic acid</td>
</tr>
<tr>
<td>5 mM Urea</td>
<td>- m-coumaric acid, catechol</td>
<td>- m-coumaric acid, catechol</td>
</tr>
<tr>
<td></td>
<td>- o-coumaric acid, vanillic acid</td>
<td>- chlorogenic acid, 4-hydroxycoumarin</td>
</tr>
<tr>
<td>5% Acetonitrile</td>
<td>- m-coumaric acid, catechol</td>
<td>- ferulic acid, coumarin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- o-coumaric acid, vanillic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- cinnamic acid, 4-hydroxycoumarin</td>
</tr>
</tbody>
</table>

Table 4.1 Separating capabilities of 6 mM borate, 10 mM phosphate, 100 mM SDS pI 8.3 buffer with the addition of organic modifiers. Results are consistent over at least five runs.
<table>
<thead>
<tr>
<th>Additive concentration</th>
<th>$m$-coumaric acid (N)</th>
<th>salicylic acid (N)</th>
<th>protocatechuic acid (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>no additive</td>
<td>90 489</td>
<td>152 573</td>
<td>234 065</td>
</tr>
<tr>
<td>5% methanol</td>
<td>56 451</td>
<td>137 017</td>
<td>112 649</td>
</tr>
<tr>
<td>5% acetonitrile</td>
<td>55 650</td>
<td>43 104</td>
<td>94 195</td>
</tr>
<tr>
<td>5 mM urea</td>
<td>68 274</td>
<td>111 146</td>
<td>68 566</td>
</tr>
</tbody>
</table>

Table 4.2  Separating efficiencies (theoretical plates, N) of 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer with the addition of organic modifiers.
Figure 4.9a  Elution order of phenolics using a 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer. The numbers correspond to the compounds listed in Table 2.7.

Figure 4.9b  Elution order of phenolics using a 6 mM borate, 10 mM phosphate, 100 mM SDS, 10% acetonitrile, pH 8.3 buffer. The numbers correspond to the compounds listed in Table 2.7.
4.2.2 COMPLEXATION AGENTS

The addition of $\alpha$ cyclodextrin and $\beta$ cyclodextrin decreased the separating capabilities of the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer (Table 4.3). $\alpha$ cyclodextrin achieved particularly poor resolution with six compounds co-eluting.

The addition of $\alpha$ and $\beta$ cyclodextrin decreased the efficiency of the buffer system. The standard 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer had typical values of 62, 711 theoretical plates for early eluting peaks and 236,881 theoretical plates for later eluting peaks. The buffer containing 15 mM $\beta$ cyclodextrin had typical values of 24 406 for early eluting peaks and 138 354 for late eluting peaks. The efficiency of the $\alpha$ cyclodextrin buffer was not calculated because it was not considered a viable buffer additive due to its poor resolving capabilities for the phenolics. The addition of $\beta$ cyclodextrin did not change the elution order of the components.

$\alpha$ cyclodextrin (Figure 4.10) and $\beta$ cyclodextrin (Figure 4.11) had no significant effect on the peak retention times of early eluting components. However, later eluting peaks had shorter retention times with the addition of either cyclodextrin. Increased cyclodextrin concentration failed to further reduce the retention times of the components. $\alpha$ and $\beta$ cyclodextrin also had no effect on the size of the elution window.

$\beta$ cyclodextrin caused the peak retention time reproducibility of the buffer system to decrease. Figure 4.12 shows that the % RSTDV values for the peak
Retention time reproducibility of several components were greater than the desired 1% when β cyclodextrin was added. There is no discernible relationship between the concentration of β cyclodextrin and the increase in % RSTDV. α cyclodextrin had no significant effect on the reproducibility of the peak retention times for the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer (Figure 4.13).

The only affect the addition of α and β cyclodextrins had was to reduce the efficiency and separating capabilities of the buffer solution. The addition of α cyclodextrin and β cyclodextrin had minimal effect on retention times, elution orders and elution windows. This suggests that the phenolics have minimal interaction with α cyclodextrin and β cyclodextrin. The marked reduction in separating capabilities and theoretical plates indicates that addition of cyclodextrins has an affect on the partition equilibrium between the micelles and buffer.
Figure 4.10  Effect on peak retention time of 6 mM borate, 10 mM phosphate 100 mM SDS, pH 8.3 buffer with the addition of α cyclodextrin.

Figure 4.11  Effect on peak retention time of 6 mM borate, 10 mM phosphate 100 mM SDS, pH 8.3 buffer with the addition of β cyclodextrin.
Figure 4.12  Peak retention time reproducibility with the addition of β cyclodextrin to the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer

Graph 4.13  Peak retention time reproducibility with the addition of α cyclodextrin to the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer
<table>
<thead>
<tr>
<th>Additive</th>
<th>co-eluting peaks</th>
<th>partially resolved peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>no additive</td>
<td>• chlorogenic acid, 4-hydroxycoumarin</td>
<td>• m-coumaric acid, catechol</td>
</tr>
<tr>
<td>15 mM β cyclodextrin</td>
<td>• cinamnic acid, vanillic acid, coumarin, 4-methumbelliferone</td>
<td>• m-coumaric acid, catechol</td>
</tr>
<tr>
<td>15 mM α cyclodextrin</td>
<td>• ferulic acid, catechol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• m-coumaric acid, cinamnic acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• vanillic acid, o-coumaric acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• chlorogenic acid, 4-hydroxycoumarin</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3 Resolution of 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer with the addition of cyclodextrins
4.2.3 MIXED MICELLE BUFFER SYSTEM

The separating capability of a buffer utilising two surfactants of differing polarity was studied. The two surfactants employed were SDS (anionic) and Brij 35 (neutral).

The addition of Brij 35 to the 6 mM borate, 10 mM phosphate, 100 mM SDS buffer resulted in a slight decrease in the retention times of the components in the phenolic mix (Figure 4.16). The mixed micelle buffer also produced a slightly shorter elution window.

The peak retention time reproducibility of the peaks in the mixed micelle buffer did not change significantly from the single surfactant buffer system (Figure 4.17).

The concentration of Brij 35 was varied from 5 mM to 15 mM. The 15 mM Brij 35 and 100 mM SDS buffer system gave poorer separation compared to the single surfactant buffer (Table 4.4). Separation efficiency was seen to rise with the addition of the second buffer (Table 4.5).

The mixed micelles alter the selectivity of the buffer and change the elution order of the components. From the electropherogram (Figure 4.18) it is clear that the mixed micelle buffer system caused several components, including umbelliferone, coumarin and 4-methylumbelliferone, to decrease their retention times quite significantly. Localised changes were also apparent, such as chlorogenic acid and cinamnic acid in addition to orcinol and 4-methyleuscelatin reversing their elution order.
Although the change was small, the reduction in the elution window and peak retention times indicates a decrease in the electrophoretic mobility of the micelles. This decrease in electrophoretic mobility can be attributed to a smaller charge density on the mixed micelle. The reduced charge density is most likely due to the anionic SDS surfactant molecules in the micelles. Changes in the size and shape of the micelle are likely contributors to the reduced charge density. The hydrocarbon chain of the Brij 35 molecule is much longer than that of SDS, and may distort the shape of the micelle as well as possibly changing the A.N.

The late eluting, non-polar compounds are the best indicators of change in the properties of the micelle. The significant decrease in the retention times of compounds such as umbelliferone and coumarin, suggests the hydrophobic nature of the micelles was reduced. This is not surprising as Brij 35 molecules contain eleven oxyethylene units which increases the polar nature of the hydrocarbon portion of the surfactant molecule. This will cause the affinity of the non-polar coumarins for the micelle to be reduced and the partition equilibrium to shift away from the micellar phase.
Figure 4.16 Effect of mixed micelle system on peak retention time of 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer.

Figure 4.17 Peak retention time reproducibility with a mixed micelle buffer system (6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3)
<table>
<thead>
<tr>
<th>Surfactant concentration</th>
<th>co-eluting peaks</th>
<th>partially resolved peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM SDS</td>
<td>• chlorogenic acid, 4-hydroxycoumarin</td>
<td>• m-coumaric acid, catechol</td>
</tr>
<tr>
<td>100 mM SDS, 15 mM Brij 35</td>
<td>• cinnamic acid, vanillic acid, chlorogenic acid</td>
<td>• 4-methylumbelliferone, 4-hydroxycoumarin</td>
</tr>
<tr>
<td></td>
<td>• m-coumaric acid, o-coumaric acid, coumarin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• ferulic acid, umbelliferone</td>
<td></td>
</tr>
</tbody>
</table>

*Table 4.4.* Resolving capabilities of 6 mM borate, 10 mM phosphate, 100 mM SDS 15 mM Brij 35, pH 8.3 buffer system.

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>orcinol (N)</th>
<th>salicylic acid (N)</th>
<th>caffeic acid (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM SDS</td>
<td>33 931</td>
<td>51 898</td>
<td>52 003</td>
</tr>
<tr>
<td>100 mM SDS, 15 mM Brij 35</td>
<td>35 643</td>
<td>80 787</td>
<td>78 821</td>
</tr>
</tbody>
</table>

*Table 4.5.* Separating efficiency (theoretical plates, N) of 6 mM borate, 10 mM phosphate, pH 8.3 buffer with mixed surfactant.
**Figure 4.18** Elution order of 18 phenolics with 6 mM borate, 10 mM phosphate, 100 mM SDS, 15 mM Brij 35, pH 8.3 buffer. The numbers correspond to the compounds listed in Table 2.7.
4.2.4 MIXED SURFACTANT AND ORGANIC MODIFIER BUFFER SYSTEM

The effect of adding methanol or acetonitrile to a 6 mM borate, 10 mM phosphate, 100 mM SDS, 15 mM Brij 35, pH 8.3 buffer was investigated. The buffer additive, mixed micelle buffer system resulted in a significant increase in the retention time of the analytes (Figure 4.19, 4.20). Increasing the concentration of the organic modifiers further increased the retention times.

Buffers containing mixed micelles and organic modifiers provided excellent resolving capabilities (Table 4.5). In particular, the 6 mM borate, 10 mM phosphate, 10% acetonitrile, 15 mM Brij 35, 100 mM SDS pH 8.3 buffer provided baseline resolution for all 18 phenolics.

The number of theoretical plates in the mixed micelle, organic modifier buffer was lower compared to the single surfactant buffer with no organic modifiers (Table 4.6).

The 6 mM borate, 10 mM phosphate, 100 mM SDS, 15 mM Brij 35, pH 8.3 buffer system gave % RSTDV values for the peak retention times between 1.5% and 4% (Figure 4.21). The addition of methanol to the mixed surfactant buffer had a similar effect.

The electropherogram of the 6 mM borate, 10 mM phosphate, 10% acetonitrile, 15 mM Brij 35, 100 mM SDS, pH 8.3 buffer system (Figure 4.22) shows that the mixed micelles and the organic modifiers changed the elution order of the components. The greatest changes were seen for coumarin, umbelliferone and 4-methylumbelliferone which had greatly reduced retention times. Similar changes in
elution order are seen with the 6 mM borate, 10 mM phosphate, 5% methanol, 15 mM Brij 35, 100 mM SDS, pH 8.3 buffer system.

Baseline resolution of the 18 phenolic components was achieved using the 6 mM borate, 10 mM phosphate, 100 mM SDS, 15 mM Brij 35, 10% acetonitrile, pH 8.3 buffer. However, this was achieved at the expense of short retention times and peak retention time reproducibility.

The increase in the peak retention time of the components is due to the presence of the organic modifier which increases the viscosity of the buffer solution, reducing the size of the EOF. Previous experiments with organic modifiers indicated that the acetonitrile is responsible for the longer retention times.

The change in elution order of the 18 phenolic components was due to the combined effects of the organic modifier and the mixed micelle. As described earlier, organic modifiers decreased the polar nature of the buffer solution and may change the properties of the micelle, hence decreasing the partitioning of the hydrophobic analyte molecule between the micelle and the buffer. At the same time, the presence of the neutral surfactant decreases the hydrophobic nature of the micelle. These effects combined to cause the strongly hydrophobic coumarin molecules to elute after just 9 minutes.
Figure 4.19  Peak retention time of the 6 mM borate, 10 mM phosphate, 100 mM SDS, 15 mM Brij 35, pH 8.3 with addition of acetonitrile.

Figure 4.20  Peak retention time for 6 mM borate, 10 mM phosphate, 100 mM SDS, 15 mM Brij 35, pH 8.3 with addition of methanol.
<table>
<thead>
<tr>
<th>Buffer</th>
<th>co-eluting peaks</th>
<th>partially resolved peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM SDS</td>
<td>• chlorogenic acid,</td>
<td>• m-coumaric acid,</td>
</tr>
<tr>
<td></td>
<td>4-hydroxycoumarin</td>
<td>catechol</td>
</tr>
<tr>
<td>10% acetonitrile,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mM Brij 35,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mM SDS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.5 Resolving capabilities of mixed micelle, organic modifier buffers.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>coumarin (N)</th>
<th>cinnamic acid (N)</th>
<th>protocatachui acid (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM SDS</td>
<td>38 376</td>
<td>64 058</td>
<td>27 803</td>
</tr>
<tr>
<td>100 mM SDS,</td>
<td>78 164</td>
<td>23 995</td>
<td>22 803</td>
</tr>
<tr>
<td>15 mM Brij 35,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% acetonitrile</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.6 Separating efficiency (Theoretical plates, N) of 6 mM borate, 10 mM phosphate, 100 mM SDS, 15 mM Brij 35, 10% acetonitrile, pH 8.3 buffer.
Figure 4.21  Retention time reproducibility with mixed micelles and acetonitrile (6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3)

Figure 4.23 Elution order of 13 phenolics with 6 mM borate, 10 mM phosphate 100 mM SDS, 15 mM brij 35, 10% acetonitrile, pH 8.3 buffer. The numbers correspond to the compounds listed in Table 2.7.
CHAPTER 5. ANALYSIS OF PHENOLICS FROM EUCALYPTS BY CE

5.1 INTRODUCTION

In the previous chapters it was shown that the best buffer system to use for the separation of phenolics was the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.3 buffer. When run at 19 kV, this buffer gave satisfactory separation and good peak retention time reproducibility. This buffer system was used to analyse the phenolic extracts from eucalypt species including; *Eucalyptus marginata* (jarrah), *Eucalyptus calophylla* (marri), *Eucalyptus erythrocorys* (illyarrie), *Eucalyptus todiana* and *Eucalyptus rudis*. Both free and bound phenolics were extracted from the eucalypt leaves. Free phenolics are phenolics that are contained within the cytosol of the cell and include glycosides, which are phenolics bonded to sugar molecules. Bound phenolics are those that are bound to the cell wall.

Free phenolic extracts from all five eucalypts and bound phenolic extracts from jarrah and marri were run on the CE to determine if the MEKC method was suitable for separating and identifying phenolics from eucalypts. Identification of the compounds was achieved by spiking with standards. Tentative identifications of compounds using CE were confirmed by HPLC and GC-MS analysis.

Although the phenolics from all five species of eucalypts were examined, time constraints did not allow in depth studies of all the species. This study therefore concentrated on the jarrah and marri samples.
The reproducibility of the electropherograms recorded for the bound phenolic and free phenolic extracts was tested by performing repeated extractions and running the samples through the CE at least five times.

5.2 RESULTS AND DISCUSSION

5.2.1 CELL WALL BOUND PHENOLICS

The bound phenolics from jarrah and marri were extracted using the method described in Section 2.6. The electropherograms recorded for these extracts were surprisingly simple and clean. Only five peaks were recorded in the electropherogram for the jarrah extract (Figure 5.1). One component, with a retention time of 10.12 minutes was present in very large quantities relative to the other components.

The jarrah extract was spiked with standards that have approximately the same retention times as the recorded peaks. Three peaks in the jarrah sample were tentatively identified as p-coumaric acid, ferulic acid and gallic acid.

The electropherogram for the marri extract also indicated the presence of gallic acid (Figure 5.2). However, p-coumaric acid and ferulic acid were absent or were present at levels too low to be detected. Two other unidentified peaks were also present in the cell wall of marri.

The bound phenolic extracts from jarrah (Figure 5.3) and marri (Figure 5.4) were separated by HPLC. Gallic acid, ferulic acid and p-coumaric acid were again identified in the jarrah sample through spiking. HPLC also confirmed the presence of gallic acid in the marri sample.
GC-MS confirmed the presence of these phenolics in both *eucalypt* species (Figure 5.5, 5.6, 5.7). GC-MS failed to detect ferulic acid and *p*-coumaric acid in marri.

CE can be employed successfully for the separation and identification of phenolics extracted from the cell wall of eucalypts. However, the use of internal standards is recommended when identifying peaks to overcome variations in peak retention times due to sample matrix effects.

HPLC was used to substantiate the identification of phenolics made by CE. HPLC was employed because of its different separating mechanism compared to CE. Separation in HPLC using a reverse phase stationary phase is based primarily on the hydrophobic nature of the analyte compounds. However, in CE, the phenolic acids are separated by a combination of electrophoretic mobility and hydrophobic interactions with the micelles. Consequently, the elution order for gallic acid, ferulic acid and *p*-coumaric acid will change for HPLC. Using a different mode of separation and obtaining the same results substantiates the identification. Mass spectrometry was also employed as, unlike UV detection, it provides structural information and further evidence of the presence of a particular component.

The large quantity of gallic acid has implications for the production mechanism of secondary metabolites in eucalypts. The shikimic acid pathway is one of two major pathways for the production of secondary metabolites such as phenolics. One of the compounds used at the beginning of this pathway is shikimic acid. Gallic acid is the fully aromatised form of shikimic acid and is also one of the many phenolic co-"""""""""""""""""""""""""""""""""""""""""""""""""""

### References


### Acknowledgments

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acid pathway. It is also possible that gallic acid is produced directly from shikimic acid (Waterman and Mole, 1994).

Gallic acid is also the basis for more complex structures in plants such as hydrolysable tannins (Waterman and Mole, 1994). Gallic acid in the cell wall of the jarrah samples suggests that such tannins are possibly present in the cell walls of both jarrah and marri. Tannins are a very common group of compounds that are found in all classes of vascular plants and are of great importance due to their possible roles in disease resistance (Swain, 1979).

The identification of ferulic acid and $p$-coumaric acid in the bound jarrah sample was not surprising as these compounds are commonly found in cell walls while esterified to the hydroxyls of sugar molecules (Waterman and Mole, 1994).
**Figure 5.1** Electropherogram of bound phenolics from *E. marginata*. The numbers correspond to the compounds listed in Table 2.7. (19 corresponds to *p*-coumaric acid)

**Figure 5.2** Electropherogram of bound phenolics from *E. calophylla*. The numbers correspond to the compounds listed in Table 2.7.
Figure 5.3  HPLC chromatogram of bound phenolics from *E. marginata*. The numbers correspond to the compounds listed in Table 2.7. (19 corresponds to *p*-coumaric acid)

Figure 5.4  HPLC chromatogram of bound phenolics from *E. calophylla*. The numbers correspond to the compounds listed in Table 2.7
Figure 5.5  MS electropherogram of ferulic acid from *E. marginata*.

Figure 5.6  MS electropherogram of *p*-coumaric acid from *E. marginata*.

Figure 5.7  MS electropherogram of gallic acid from *E. marginata* and *E. calophylla*. 
5.2.2 FREE PHENOLICS

The free phenolics in *E. marginata*, *E. calophylla*, *E. erythrocorys*, *E. todtiana*, and *E. rudis* were extracted from the leaves by the method described in Section 2.4 and applied to the CE column.

The free phenolics, including phenolics and glycosides, when applied to the CE column, gave complex electropherograms (Figure 5.8, 5.9, 5.10, 5.11, 5.12). The electropherograms all have in common a large absorption band extending from approximately 9.5 minutes to 14 minutes.

GC-MS analysis indicated the presence of large levels of sugars and glycosides.

The free phenolic extract was acidified, heated and then extracted into ethyl acetate according to the method outlined in Section 2.5. The electropherograms of this acid-hydrolysed sample showed the absence of the large absorption band (Figure 5.13 and Figure 5.14). A comparison of the GC chromatograms of the free phenolic extracts before and after acid hydrolysis clearly illustrates the large falls in the levels of sugars and glycosides (compare Figure 5.15 and Figure 5.16).

Using spiking, another component in the free phenolics extract was tentatively identified as catechol.

GC-MS analysis also identified large amounts of shikimic acid in the jarrah extract (Figure 5.17). A similar analysis showed that only trace amounts of shikimic acid were present in the marri extract.
The free phenolic extracts gave complex electropherograms with a large number of the early eluting peaks being well resolved. The large absorption band extending from 9 minutes to 14 minutes is clearly due to large amounts of unresolved sugars and glycosides. Glycosides are "masked" phenolics, or phenolics that are bound to a sugar molecule (Waterman and Mole, 1994). Acidifying the phenolic sample hydrolyses the glycosidic linkage and the existing phenolics, as well as the resulting aglycones, can be extracted into ethyl acetate. The sugars remain in the water phase.

Shikimic acid is one of the precursors of phenolic compound synthesis through the shikimic acid pathway. Unfortunately, time constraints did not allow the purchase and identification of this component by CE.

Although no attempt was made to quantify the levels of phenolics or shikimic acid, it is evident that shikimic acid is present in large amounts in jarrah when compared to marri. Sample mass and extraction procedures were identical for jarrah and marri.

It is unclear whether the presence or levels of shikimic acid have any implications on the dominant secondary metabolite pathway. However, it is certainly an area of research worth pursuing.
Figure 5.8  Electropherogram of free phenolics from *E. marginata*.

Figure 5.9  Electropherogram free phenolics from *E. calophylla*. 
Figure 5.10  Electropherogram of free phenolics from *E. todtiana*.

Figure 5.11  Electropherogram of free phenolics from *E. erythrocorys*. 
Figure 5.12  Electropherogram of free phenolics from *E. rudis*
Figure 5.13 Electropherogram of free phenolics from *E. marginata* (acidified and heated). The numbers correspond to the compounds listed in Table 2.7

Figure 5.14 Electropherogram of free phenolics from *E. calophylla* (acidified and heated)
Figure 5.15 GC chromatogram of free phenolics from *E. marginata*.

Figure 5.16 GC chromatogram of free phenolics from *E. marginata* after acidification and heating.
Figure 5.17  MS electropherogram of shikimic acid from jarrah
CHAPTER 6 SUMMARY

6.1 SEPARATING CAPABILITIES OF DIFFERENT BUFFERS

Satisfactory separation of the 18 phenolics mix was only achieved by the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.5 buffer. The 50 mM NaHCO₃, 100 mM SDS, pH 8.5 buffer had very poor separating capabilities and the 18 mM borate, 30 mM phosphate, 50 mM CTAB buffer could not resolve any of the peaks. The poor resolution of the CTAB buffer is most likely due to the short elution window as the positively charged micelles travel with the EOF. The compounds clute before they have had time to partition between the micellar and buffer phase. These results also suggest that the separating capabilities of different buffer systems depend on the sample components that are being analysed. The different buffer systems have clearly different separating capabilities for the phenolics. This is highlighted by a study of the theoretical plates.

Increasing the ionic strength of the 6 mM borate, 10 mM phosphate, 100 mM SDS, pH 8.5 buffer decreases the peak retention times of the components and reduces the elution window. This was achieved by increasing the zeta potential at the silica wall-buffer solution interface resulting in an increased EOF.

The pH of the buffer solution did not have any noticeable effect on the elution window of the buffer or the peak retention times. Theoretically, higher pH should increase hydrolysis of the fused silica capillary, increasing the size of the EOF. It is unclear why this was not observed.
6.2 BUFFER ADDITIVES

The ability of buffer additives to improve resolution and their effect on reproducibility varied with the type of additive. The use of organic modifiers such as methanol, acetonitrile, urea and isopropanol decreased the separating capabilities and peak retention time reproducibility in the buffer system. However, most of the organic modifiers did succeed in increasing the retention time of most of the components as well as increasing the size of the elution window. The organic modifiers increased the elution window and reduced the peak retention times of the components by reducing the zeta potential in the capillary, and increasing the viscosity of the buffer. This resulted in a decrease in the EOF. The peak retention time reproducibility of the buffer was decreased with the addition of organic modifiers.

The use of mixed micelles (SDS and Brij 35) altered the elution order and peak retention times of the 18 phenolic mixture due to the altered hydrophobic properties of the micelles. The decreased overall charge on the micelles also reduced the peak retention times of the phenolic components. Despite these changes, the separation of the components did not improve. One of the limitations of MEKC is its narrow selectivity which is governed by the range of suitable surfactants. In this study, the use of mixed micelles to effectively manipulate the selectivity of the buffer system without any loss in efficiency or reproducibility has been demonstrated. The potential of mixed micelles has not been fully explored and is certainly worthy of further investigation.

Combining the increased elution window provided by organic modifiers and the improved efficiency of mixed micelles dramatically improved the separation of the 18
phenolics mixture. The 6 mM borate, 10 mM phosphate, 100 mM SDS, 15 mM Brij 35, 10% acetonitrile, pH 8.3 buffer achieved baseline resolution for all of the phenolic components, however, the peak retention time reproducibility of this system was significantly reduced. The increase in the % RSTDV for the peak retention times was considered to be too large to allow accurate analysis of phenolics extracted from eucalypts, and hence it was decided that the best buffer system to use was the original borate, phosphate buffer with pH 8.3, run at 19 kV.

6.3 SEPARATION OF PHENOLICS FROM EUCLYPTS USING MEKC

MEKC was shown to be suitable for the separation and identification of phenolics extracted from a range of eucalypt species.

The free phenolic samples produced very complex electropherograms with large numbers of peaks. Consequently only one compound, catechol, was identified by spiking with CE. However, GC-MS analysis of the free phenolics showed the presence of a large amount of shikimic acid present in *E. marginata* samples that were not present in the free phenolic samples from *E. calophylla*. The large amounts of sugars and glycosides in the free phenolic extracts can be minimised by heating and acidifying the sample. The removal of the sugars and glycosides can improve the clarity of the electropherograms and yield more information on the phenolic content in the eucalypts.

The bound phenolic samples were much simpler and three compounds were identified from the *E. marginata* and *E. calophylla* samples. Gallic acid was present in both samples, however, *p*-coumaric acid and ferulic acid were present in only *E. marginata*. 
The identity of these compounds was verified using other chromatographic techniques such as GC-MS and HPLC. These results helped to verify that accurate identification of phenolics can be achieved by using MEKC.

The use of folin reagent and spectrophotometry is a common method for quantifying levels of total phenolics. However, it is becoming increasingly evident that information regarding total levels of phenolics is limiting. A more in depth study of individual phenolics and their concentrations is necessary to gain an understanding of the biochemical and physiological processes operating in plants.

Chromatography can provide improved analysis due to its ability to separate, quantify and identify individual compounds within a sample. HPLC and GC have been used, however, CE, with its high separating efficiency, short runtimes, low solvent concentrations, cheap operating costs and its ease of use, is clearly a far more effective chromatographic technique to use in the study of plant phenolics. This study has shown MEKC to be a suitable technique when studying phenolics and has extended our understanding of MEKC by analysing aspects such as mixed micelles and combined mixed micelle / organic modifier systems.
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