EVALUATION OF PRESSURE- AND ELECTRODRIVEN SEPARATION TECHNIQUES FOR THE DETERMINATION OF PHENOLIC COMPOUNDS IN WINE

A.J. de Villiers

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Prof. Dr. P. J. F. Sandra (supervisor)
Prof. Dr. H. H. Lauer (co-supervisor)

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Summary

The phenolic content of wine is responsible for determining characteristics such as the organoleptic qualities, colour stability, ageing properties and health-beneficial effects associated with wine. The aim of this study was to investigate the possibilities offered by capillary electrophoresis (CE) as an alternative separation technique to high performance liquid chromatography (HPLC) for the analysis of polyphenols in wine. The complexity of wine samples was the cause that neither technique was capable of a satisfactory single-step analysis of wine. Suitable sample preparation techniques such as Sephadex- and Sep-Pak fractionation and ether extraction of wine polyphenols were investigated. These techniques did not, however, prove to be universal. A novel form of sample preparation namely a process analogous to lyophylization used to separate wine volatiles from non-volatiles was introduced.

The versatility of CE was further investigated in an attempt to eliminate the need for sample preparation. The use of polyvinylalcohol (PVA) coated capillaries, micellar electrokinetic chromatography (MEKC) and capillary gel electrophoresis (CGE) were investigated in this regard. Although none of these techniques could offer conclusive results, useful applications were forthcoming and routes for further investigation were outlined. Liquid chromatography coupled to electrospray ionisation mass spectroscopy (LC-ESI-MS) and capillary electrophoresis coupled to electrospray ionisation mass spectroscopy (CE-ESI-MS) were compared for the analysis of polyphenols in wine. While the latter technique could not produce sufficient separation compared to the former, future development of CE-ESI-MS should make it a powerful technique for these analyses.

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Opsomming

Die fenoliese komponente in wyn speel 'n bepalende rol by eienskappe soos die organoleptiese karakter, kleur stabiliteit, verouderingspotensiaal en gesondhieds-voordele wat met wyn geassosieër word. Die doel van hierdie projek was om ondersoek in te stel na die potensiaal wat kapillêre elektroforese (CE, "capillary electrophoresis") as 'n alternatiewe skeidingstegniek teenoor hoë druk vloeistof chromatografie (HDVC) vir die analise van die polifenole in wyn bied. Die kompleksiteit van wyn monsters is van so 'n aard dat 'n bevredigend enkelstap analise met geeneen van die tegnieke moontlik is nie. Gepaste monster-voorbereidingstappe soos Sephadex- en Sep-Pak fraksionering asook eter ekstraksie van die polifenole in wyn is ondersoek. Geeneen van die tegnieke was egter universeel toepaslik nie. 'n Nuwe metode van monster-voorbereiding, naamlik 'n proses analoog aan liofilisasie wat gebruik word om die wyn te skei in vlugtige en nie-vlugtige komponente is gedemonstreer.

Die veelsydigheid van CE was gevolglik ondersoek in 'n poging om monstervoorbereiding uit te skakel. Die gebruik van polyvinielalkohol-(PVA) bedekte kapillêre, missellêre elektrokinetiese chromatografie (MEKC) en kapillêre gel elektroforese (CGE, "capillary gel electrophoresis) is in hierdie verband ondersoek. Alhoewel geeneen van hierdie tegnieke onweerlegbare resultate gelewer het nie, het bruikbare toepassings hieruit voortgespruit en is die grondslag vir verdere navorsing gelê. Vloeistof chromatografie gekoppel aan elektrosproei ionisasie massaspektroskopie (LC-ESI-MS) en kapillêre elektroforese gekoppel aan elektrosproei ionisasie massa-spektroskopie (CE-ESI-MS) is vergelyk vir die analise van polifenole in wyn. Alhoewel laasgenoemde tegniek onvoldoende skeiding lewer vergeleke met eersgenoemde, behoort toekomstige ontwikkelinge op die gebied van CE-ESI-MS dit 'n kragtige tegniek vir die analise van hierdie monsters te maak.

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1. Introduction

The phenolic compounds are responsible for many of the important characteristics of wine. Polyphenols have long been known to be responsible for the colour of red wine, as well as colour changes occurring during ageing and oxidation of the wine. These compounds also determine the tannin content of red wines, while the tannins in turn play an important role in determining the organoleptic character of wines through bitterness and astringency. In addition, the polyphenols also play a crucial role in the wood maturation of wine and have been linked to many of the beneficial effects associated with drinking red wine.

It is clear that a reliable and efficient analytical method for the determination of these compounds is very important, not only in order to determine the phenolic pattern and content in wine, but also to study their changes taking place during the different winemaking and maturation steps.

Thus far, analysis of polyphenols in wine has primarily been performed using high performance liquid chromatography (HPLC). This separation technique suffers from long analysis times and often requires extensive sample preparation. In recent years, some studies have investigated the use of capillary electrophoresis (CE) for the analysis of these compounds. However, the possibilities offered by this technique have not completely been investigated. The aim of this study was to obtain a more comprehensive comparison in terms of advantages and disadvantages of each of these techniques with regard to the analysis of polyphenols in wine.

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2. Phenols in Wine

The significant role phenolic compounds play in determining the character of wine has been linked to a number of features associated with these compounds. Phenolic compounds are not only important in grapes and wine, but throughout the plant kingdom. It is known that polyphenols are responsible for the colour (and colour stability) of red wines. They also play an important role in determining the organoleptic characteristics of red wines in both their monomeric and polymeric forms (the tannins) [1,2,3]. They also play a crucial role in the wood maturation of wine [4].

In addition to the role these compounds play in determining the character of the wine, they are also responsible for many of the beneficial effects associated with drinking wine. Although at least 75% of the protective effect associated with drinking wine moderately can be attributed directly to the favourable effect of ethanol on circulating lipids and on avoiding blood clotting, thus reducing the risk of coronary heart disease (CHD), several studies have revealed that especially red wine was more potent than spirits or beer at reducing CHD [5]. The phenolic content of wine is widely held responsible for this feature associated with red wine since it is at least an order of magnitude lower in white wines and virtually absent in other alcoholic beverages. The flavonoids have been shown to be potent antioxidants due to their ability to act as free radical scavengers and in this way to protect cells against oxidative stress [6]. The ability of flavonoids to inhibit synthesis of pro-aggregatory eicosanoids thereby reducing the risk of heart attacks has also been demonstrated. Phenolic compounds in wine have been shown to inhibit oxidation of low-density lipoproteins (LDL) and also to prevent the cytotoxic effects of oxidised LDL [7]. Other anti-atherogenic effects include: inhibition of smooth muscle cell proliferation; decreasing cholesterol solubility and cholesterol absorption from the intestine and reducing the cytotoxicity of reactive oxygen species against primary cultures of 3T3 fibroblasts [5]. Resveratrol has attracted much attention because of its potent antifungal activity, and further investigation showed that this compound has many potentially beneficial biological uses [8]. The inhibition of cancer cell growth *in vitro*, as well as in human cancer patients, by polyphenols has also been reported [9]. The

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profound role played by the phenolic compounds in determining the characteristics of wine is in part due to the large diversity of the phenolic compounds present in wine.

2.1 Classification of Polyphenols

The phenolics in wine can principally be divided into two classes: flavonoids and non-flavonoids. The non-flavonoid group of polyphenols consists mainly of phenolic acids, i.e. the benzoic acid and hydroxybenzoic acid derivatives and cinnamic acid and hydroxycinnamic acid derivatives.

The benzoic acid derivatives differ from each other with regard to the number and pattern of substitution of hydroxyl- and methoxy groups at the aromatic ring: examples are 3,4,5-trihydroxybenzoic acid (gallic acid), shown in figure 1, and 4-hydroxy-3,5-dimethoxy-benzoic acid (syringic acid). In addition to the physiological effects associated with these acids, they also seem to influence the organoleptic character of wine, since gallic acid and its polymerised form, tannic acid, have been found to be bitter and astringent [3].

The hydroxycinnamic acids are important phenolic constituents of wine because of the role they play in enzymatically initiated oxidation reactions [10]. These compounds are found in the juice and the pulp of the grapes and, as such, are the main phenolic constituents of white wines prepared without pomace contact. Substitution of the aromatic ring by hydroxyl- and methoxy groups also provides variety within the group (figure 1). Furthermore, these acids can be esterified with for example tartaric acids as in caffeoyltartaric acid. The different substitution patterns of these acids play a profound role with regard to their reactivity, as will be discussed later. These acids may also be attached to flavonoids via the sugars bound to them [11]. The cinnamic acid derivatives are important from a metabolic point of view, since p-coumaric acid is a precursor in the synthesis of both the flavonoids and another class of phenolic compounds in wine, the stilbenes, which includes resveratrol (figure 1) [9].

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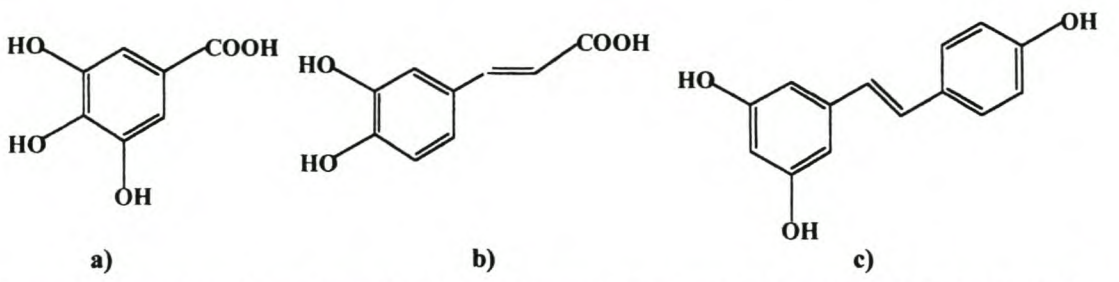

Figure 1: Examples of non-flavonoid phenolics present in wine: a) a benzoic acid derivative (gallic acid); b) a hydroxycinnamic acid (caffeic acid) and c) a stilbene (*trans*-resveratrol).

The flavonoid structure is based on a C6-C3-C6 skeleton, and this class contains several groups which differ in the oxidation level of the central heterocyclic ring (figure 2). Wine flavonoids can be divided into three groups in order of increasing oxidation state of ring C: flavanols, anthocyanidins and flavonols. Within these groups their diversity arises from the different substitution patterns at rings B and C by hydroxyl, methoxy, prenyl-, geranyl- and sugar residues, as well as their ability to exist in oligomeric or polymeric form.

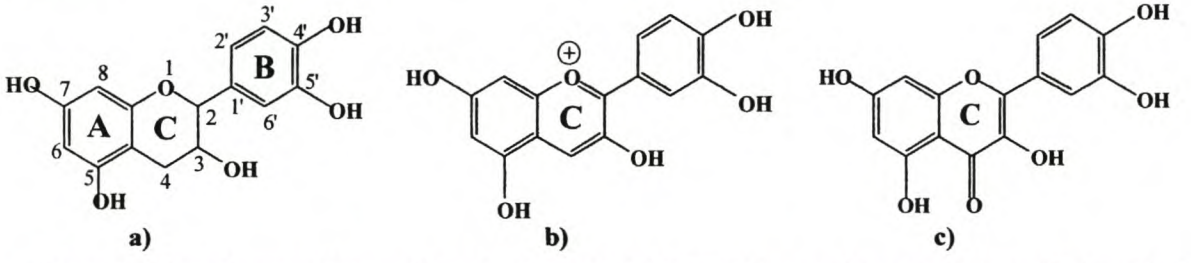

Figure 2: Three classes of flavonoids: a) a flavanol (catechin); b) an anthocyanidin (cyanidin); and c) a flavonol (quercetin).

2.2 Flavanols and Tannins

The flavanols are colourless compounds that are found in monomeric, oligomeric and polymeric form in wine. Grape tannins are generated by the polymerization of flavonoid phenolic units and since they are produced under strict biological control, only flavanols are incorporated in these tannins. The grape tannins can be divided into procyanidins, with a 1,2-dihydroxyphenyl system (ring B) of the flavanol unit, and prodelphinidins, with a 1,2,3-trihydroxyphenyl system (ring B). It is thought that flavonoid precursors,

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instead of flavonols themselves, are used in the synthesis of tannins and during this process the flavanol residues of tannins are generated.

Tannins are defined as components capable of cross-linking proteins, and there is evidence that a certain polymer size is required for the interaction with proteins to be effective. This phenomenon leads to coagulation of mucin in saliva by rendering the glyco-proteins therein insoluble. This effect of tannins, together with their ability to stop secretion by salivary glands and to harden mucous tissue, is responsible for the sensory perception known as astringency. Astringency is thus perceived as a dry, puckering sensation throughout the oral cavity, to be distinguished from taste sensation bitterness. Bitterness, also caused by tannins and other phenolic compounds, is registered by the circumvallate papillae located at the back of the tongue, only after swallowing in the case of drinking and as an aftertaste in the case of tasting. As wines age, the more bitter monomers and dimers are polymerized, and these polymerized tannins are more astringent and less bitter. Ultimately, further polymerization will lead to insoluble polymers, and their precipitation will lead to a decrease in astringency [1].

In contrast to the production of tannins in grapes, which is under enzymatic control, reactions involving tannins in wine are less controlled. Consequently these reactions generate modified tannins, degrade some existing ones and generate new ones. In this way tannins in wine can polymerise with anthocyanins to form pigmented tannins, which are more stable and soluble than non-pigmented tannins. Proposed mechanisms for this polymerisation between anthocyanins and tannins include acid catalysed fission of interflavan bonds, acetaldehyde induced addition reactions and physical entrapment where the pigments are physicochemically entrapped in a procyanidin matrix. Additionally, complex formation between tannins and polysaccharides can also take place, although the mechanism for this reaction is still not clear [12].

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2.3 Anthocyanidins and Anthocyanins

Anthocyanidins, when attached to a sugar molecule, form the more stable anthocyanins, the components that are responsible for the colour of red wine. This is a result of the aromatic character of ring C, which allows sideways overlap of the orbitals of all three rings and the resulting absorption of green light at approximately 520 nm, which gives wine the observed red colour. Further, acylation of the sugar group with a hydroxycinnamic acid such as p-coumaric acid may occur, and this is thought to stabilize the red coloured form of anthocyanins, the flavylium cation form (where ring C has aromatic character, figure 2 b). This cationic form can be destroyed by a number of reactions, all of which are detrimental to the colour of red wines. In the first place, loss of a proton may occur at a phenolic hydroxyl group, generating a quinoidal base. This quinoidal base is coloured blue, and its formation is favored at high pHs. Secondly, and more important, addition of water to ring C (the attack occurs at positions 2 or 4) and the subsequent loss of a proton can occur, generating the colourless hydrated form of the anthocyanin (figure 3). At normal wine pH (normally 3-4) less than half of the total anthocyanin pigment is in the coloured flavylium form because of this equilibrium. Here production of the colourless hydrated form is once again favored by an increase in pH.

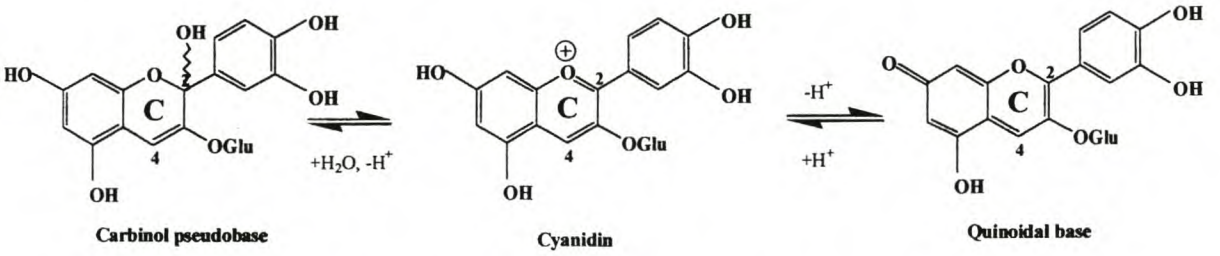

Figure 3: pH-dependent equilibria involving cyanidin in which the coloured flavylium cation form is destroyed.

A third reaction which destroys the flavylium cation and is detrimental to the colour of wine is the addition of bisulfite (this is the principle form of free sulfur dioxide at wine pH) by exclusive attack at position 4 of ring C, generating a colourless hydrogen sulfite addition product. This reaction is most important since the sulfite ion is much more reactive than water towards the positively charged flavylium cation [11]. The extent of

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formation of the hydrogen sulfite addition product depends upon the concentration of free sulfur dioxide, which is added to wine to prevent oxidation and to prevent microbial growth [13].

In wine, the incorporation of anthocyanins into polymeric pigments (as discussed above) provides colour stability by the binding of tannins to the 4-position on the heterocyclic ring and in this way protecting it from attack by water and/or bisulfite. A number of different pigments, such as pigment A and B and vitisin A and B (figure 4), which are derived from reactions between malvidin-3-glucoside and yeast-derived metabolites, have been found in wines. These pigments are resistant to pH affects and bisulfite bleaching. Their quantity increases as the wine matures, while the levels of grape-derived monomeric anthocyanins decline [14].

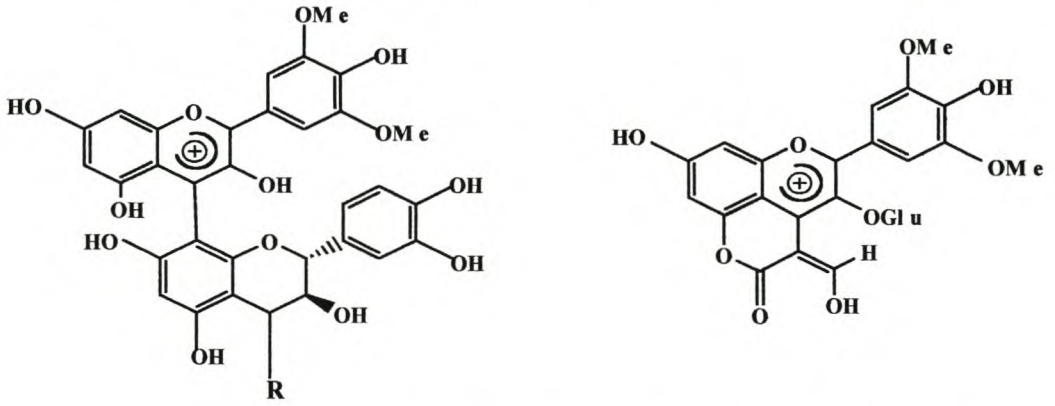

Figure 4: a) Postulated structure of a pigmented tannin (Somers, 1971), where a catechin-containing tannin (group R represents the rest of the tannin molecule) is joined to malvidin-3-glucoside through a C4-C8 linkage; and b) Structure of Vistisin A.

2.4 The Flavonols

The third class of flavonoids, the flavonols, are light yellow and are believed to protect plant tissues from damage by ultraviolet light. Among the flavonols, quercetin (figure 2) has gained considerable prominence because of its anti-tumour activity. It has also been proven to be a potent antioxidant, to surpress hydroperoxide induced cytotoxicity, and its modulation of eicosanoid synthesis has been described [9].

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2.5 Oxidation of Polyphenols

Following extraction from the grape, polyphenols are gradually degraded. However, the total amount of phenols in wine stays constant, and they are converted to other species [10]. One of the major groups of reactions involving polyphenols during the winemaking process, the addition reactions involving anthocyanins and/or flavanols has been discussed above. The remaining group involves the oxidation processes. Enzymatic oxidation initiated by polyphenoloxidase (PPO) is responsible for the discoloration process known as enzymatic browning, in which the first step is the enzymatically catalysed oxidation of mainly o-diphenolic substrates to o-quinones. The most important PPO substrates in wine musts are caffeoyltartaric acid and to a lesser extent p-coumaroyltartaric acid. Both are converted to caffeoyltartaric acid o-quinone, which can undergo oxidation by powerful reductants such as ascorbic acid or sulphite ions, or coupled oxidation of other o-diphenols. Both reactions result in the regeneration of caffeoyltartaric acid. In addition, however, primary and secondary o-quinones can undergo addition reactions involving nucleophilic phenolic compounds including *o*-diphenols as well as non *o*-diphenols such as malvidin-3-glucoside [10]. These enzymatically-catalysed oxidation reactions are prevalent during the early stages of winemaking, but as the amount of oxygen and enzymatic activity decrease, the addition reactions involving flavanols and anthocyanins become predominant.

Enzymatically-catalysed oxidation is, however, only responsible for the oxygen uptake in musts and in the very early stages of winemaking. The amount of oxygen a wine can take up precludes any auto-oxidizable compounds other than the polyphenols as the cause, since they are not present in sufficient amounts. Nonenzymatic oxidation of vicinal dihydroxyphenols also occurs in wine and this reaction produces an oxidant, presumed to be hydrogen peroxide, capable of converting ethanol to acetaldehyde through coupled oxidation.

Since the pKa values of most natural phenols is between 9 and 10, at or above this pH auto-oxidation is extremely fast (since 50% or more of the phenols are in the phenolate

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ionic form), reaching completion in 30 minutes at room temperature. While wine pH's are much lower than the pKa's of its phenols, there is only a small percentage of phenolate ions present. These phenolate ions readily undergo a single electron oxidation to form a semiquinone free radical. Although very reactive, this free radical is stabilised by resonance delocalization of the unpaired electron, and in the case of derivatives of 1,2- or 1,4-dihydroxybenzenes oxidative removal of a second electron leads to a relatively stable *ortho*- or *para*-quinone.

Polymerization of phenols as a result of oxidation is a well-known phenomenon for which several mechanisms have been proposed. A combination of these reactions is most likely what takes place during the oxidation of wine. The simplest form is the reaction between two semiquinone free radicals where the unpaired electrons combine to form a covalent bond, and the subsequent migration of the hydrogen atoms (in process analogues to keto-enol isomerism) would regenerate the re-oxidizable hydroquinone form. This product can then be re-oxidized and take part in further polymerization reactions. In addition, a quinone and a hydroquinone can interact to generate two semiquinone radicals, which can then polymerize in the same way (figure 5).

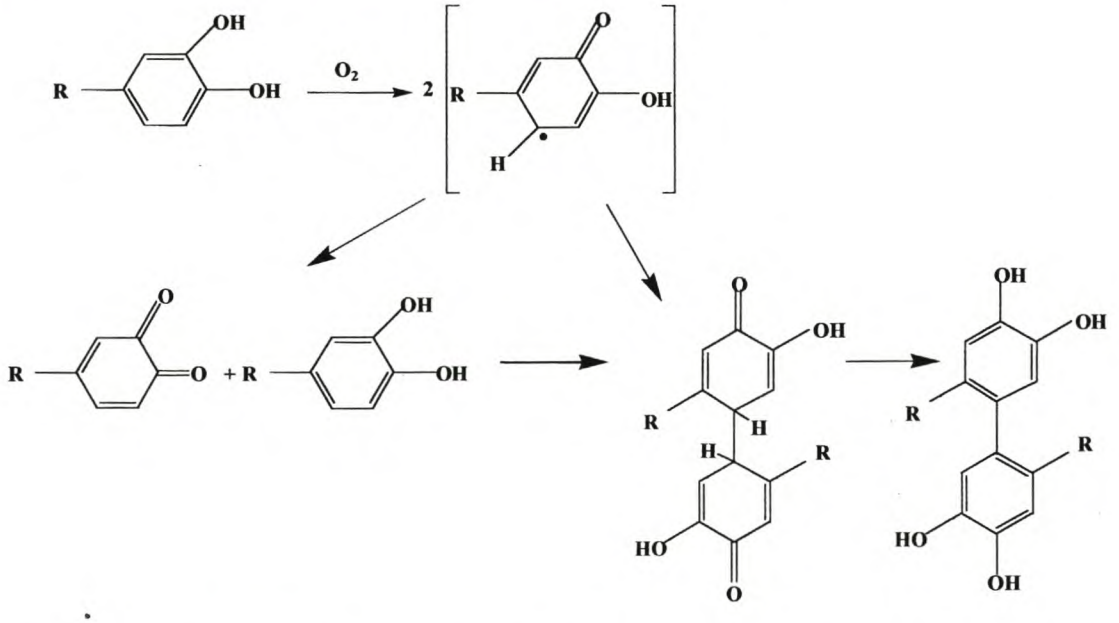

Figure 5: A simplified example of the regeneration of an oxidizable oligomeric substrate by reaction between two semi-quinones or a quinone and a phenol [15].

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A quinone can also act as an electrophile with nucleophilic centres such as oxygen free carbons in meta-polyphenols. In the case of the A ring of a flavonoid acting as a nucleophile, the result is the incorporation of a phenolic molecule originally not involved in oxidation, into a dimer which has a lower redox potential than the original hydroquinone. Since the product is more readily oxidized than the original phenol, further reactions of the products are favored, and in this way some of the original phenols remain unoxidized. This process is known as regenerative polymerization, and it explains why phenols not oxidized, even in alkaline conditions, increase oxygen consumption, and why oxygen uptake decreases with fast oxidation. This last phenomena is explained by the fact that regenerative oxidation is a slow process and the slower the oxidation takes place, the more this polymerization increases the pool of oxidizable substrates in the wine, and thus more oxidation takes place and more oxygen is needed [15].

2.6 Analysis of Polyphenols

Analysis of wine polyphenols has traditionally been accomplished by high-performance liquid chromatography (HPLC) [7,9,16-20] and thin layer chromatography (TLC) [21]. HPLC has also been used in the analysis of various phenolic compounds in a variety of plant-derived food samples such as tea (catechins)[22], apples [23], strawberries (anthocyanins)[24] and honey (flavonoids)[25]. Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) have been used, for example, in the analysis of monoterpene glycosides in grapes and wine [26]. The flavones, flavanones and flavonols present in *Canadian propolis* (a resinous hive product) were studied by GC-MS after derivatization with Bis-(trimethylsilyl) triflouroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) and with HPLC. The percentage occurrence of the higher molecular weight flavonoids such as quercetin were underestimated by the GC-MS analysis, and some of the compounds found in this analysis are thought to be produced during the derivatisation process. However, some of the minor flavonoids could only be detected by GC-MS analysis. It was concluded that although GC-MS is a more powerful technique for an overall view of the *propolis*

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composition, HPLC is more useful for the specific analysis of flavonoids in *propolis* [27]. GC-MS analysis of derivatised polyphenols with the aim of fingerprinting wines has also been reported, although once again the occurrence of the higher molecular weight flavonoids were lower than previously reported [28].

The application of capillary electrophoresis (CE) in the analysis of food samples has increased in recent years. The efficiency, versatility and rapidity of this method seem to be the major features in the analysis of these samples. In this regard, the successful separation of flavonoids [29-33] and phenolic carboxylic acids [34] has been demonstrated using capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) as well as capillary isotachophoresis (CITP) [35]. Most of this work has been done on standard solutions [30,34] or on simple extracts of the sample of interest [31-33,35]. The use of capillary electrophoresis in combination with electrospray mass spectrometry (CE-ESI-MS) in separating and identifying a standard solution of isoflavones has also been illustrated [36]. Collision-induced dissociation (CID) reactions were induced, which allows the recognition of certain functional groups and thus the identification of standard compounds.

The application of CE in the analysis of wine has also been reported. Prasongsidh *et al.* separated a standard solution of *cis*- and *trans* resveratrol, catechin, quercetin and gallic acid using sodium deoxycholate in MEKC, but injection of diluted wine provided a complex electropherogram in which the identification of some of the peaks is questionable [37]. Phenolic compounds in Spanish red wines were analysed using an ether extract as sample [38]. Solid phase extraction (SPE) on C18 has been used as sample preparation of Italian wines in the CZE analysis with a borate/phosphate buffer [39]. In a comparison between HPLC and CZE for the analysis of non-coloured phenolic compounds in red wines, García-Viguera *et al.* concluded that the major disadvantage of the latter technique is the lack of sensitivity, while advantages include shorter analysis time and better efficiency compared to HPLC [40].

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3. Techniques

3.1 High-Performance Liquid Chromatography

The name high-performance liquid chromatography (HPLC) is used to distinguish the sophisticated instrumentational form of liquid chromatography where high pressure is used to push a sample through a column packed with small particles (3-10 μm), from classical gravity-flow liquid chromatography. HPLC is the most widely used analytical separation technique because of its suitability for the analysis of non-volatile and thermally labile molecules. HPLC offers broad versatility because of the fact that in liquid chromatography (unlike in gas chromatography) the mobile phase is interactive, which adds another parameter for selectivity in addition to the stationary phase. Bonded phase partition chromatography has become the most widely used form of liquid chromatography, surpassing adsorption chromatography, ion exchange chromatography and size exclusion chromatography.

Reversed-phase HPLC refers to that form of bonded phase chromatography where apolar groups (primarily octadecyl, octyl and phenyl groups) are attached to the silanol groups on the surface of the silica gel used as support. In reversed-phase HPLC the mobile phase is relatively polar (normally aqueous mixtures of organic modifiers such as methanol, acetonitrile, and tetrahydrofuran) and an increase in organic modifier content results in a decrease in the polarity of the mobile phase. This means that the least polar compounds are retained longer on reversed-phase columns and thus elute later. In terms of the reversed-phase analysis of polyphenols in wine, this means that the most polar compounds such as gallic acid elute first, followed by other monomeric polyphenols in order of decreasing polarity. The polymeric polyphenols elute as a broad band after the monomers, due to their relatively apolar character [1,2].

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3.1.1 Efficiency in HPLC

In liquid chromatography, zone broadening in a packed column takes place as a result of the following phenomena:

- Eddy diffusion, which is a result of the multiple pathways a solute can follow through a packed bed,
- longitudinal diffusion which is directly proportional to the diffusion coefficient of the solute in the mobile phase, D_M, and inversely proportional to the mobile phase velocity,
- Mass transfer, which is a consequence of the time required for solute molecules to diffuse from the interior of one phase to the surface where transfer occurs, and is directly proportional to the mobile phase velocity.

Band broadening is summarized in the van Deemter equation:

$$H = A + \frac{B}{u_o} + Cu_0 \quad (1)$$

$$\sim 2\lambda d_p + \frac{2D_M}{u_0} + \frac{f(k')d_p^2}{D}u_0 \quad (2)$$

where H is the theoretical plate height (zone dispersion per unit length), u_0 the mobile phase velocity, d_p the particle diameter, λ the packing factor, f(k') a function of the capacity factor k' and D the diffusion coefficient representing diffusion in both the mobile and stationary phases[3].

Additional extra-column band broadening occurs in the dead volume outside the column itself. This volume includes the volume of tubing connecting the column to the injector and detector as well as in the injector and detector devices themselves.

The effect of the mobile phase flow rate can be deduced from an H-u curve. $H_{min} \approx 2d_p$ at the optimum flow velocity and the number of theoretical plates (N) in the case of liquid chromatography can be related to the particle diameter, d_p, and the length of the column (L) according to:

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$$N = \frac{L}{2d_p} \quad (3)$$

It seems obvious that the efficiency of an HPLC column can be dramatically increased by increasing the length of the column, or by decreasing the particle size of the packing material. The plate numbers in liquid chromatography are an order of magnitude smaller than those in gas chromatography, because of the pressure drop, which limits the overall column length and thus efficiency. In practice, the maximum column length for 5 μm particles is 25 to 30 cm.

3.1.2 Instrumental aspects of HPLC

In order to provide reasonable flow rates when working with column packings of 3- to 10 μm, high pressures are required, which places certain demands on HPLC equipment. In the first instance, the pumping system is required to generate pressures of up to 40 MPa with a pulse-free output while being able to produce flow rates ranging from 0.1 to 10 mL/min with sufficient reproducibility. There are a variety of pumping systems able to meet these requirements; the most widely used is the reciprocating pump. Advantages of this pump include small internal volume, high output pressure, constant flow rates and adaptability to gradient elution. However, this pump has the disadvantage of producing a pulsed flow, which requires a pulse damper normally in the form of a compressible fluid separated from the mobile phase by a diaphragm to eliminate baseline noise. Other pump systems are displacement pumps consisting of syringe-like chambers equipped with a plunger driven by a stepping motor and pneumatic pumps where the pressure from a gas cylinder delivered through a large piston drives the mobile phase. The latter pump is inconvenient for gradient elution [4].

A gradient programmer is needed to change the composition of the mobile phase (either continuously or stepwise) during gradient elution. Low-pressure gradient programmers where the gradient is formed ahead of the pump in use, consist of a proportioning valve with a micro-mixing vessel to promote complete mixing of different solvents. Alternatively, the output from two or more high-pressure pumps is programmed into a

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low-volume mixing chamber. To introduce the sample onto the pressurized column as a narrow plug, thereby avoiding peak broadening, the most widely used injection system is based on sampling loops in a six-way valve, which allow sample injection without significant interruption of the flow.

Liquid chromatographic columns are constructed from smooth-bore stainless steel tubes to withstand high pressures. Standard analytical columns range in length from 10 to 30 cm with an internal diameter of 4 to 5 mm. Short (3-7 cm) conventional-bore columns packed with 3 μm particles decrease analysis time as well as solvent consumption. A short guard column is often introduced before the analytical column to increase the life of the analytical column by removing particulate matter as well as sample components that interact irreversibly with the stationary phase. Finally, most commercial instruments are equipped with column heaters as well as equipment to control the temperature of the eluent. This makes it possible to control column temperatures to within a few tenths of a degree. Detection will be discussed in section 3.3.

3.2 Capillary Electrophoresis

3.2.1 Principles of Capillary Electrophoresis

3.2.1.1 Introduction

Electrophoresis can be defined as the differential migration of charged species in solution by applying an electric field (from the Greek words *elektron* = electron, and *phoresis* = carrying). Tiselius introduced electrophoresis as a separation technique in 1937 [5], but since thermal diffusion and convection in large diameter columns limit the efficiency in free solution, electrophoresis has traditionally been performed on glass plates coated with anti-convective media such as polyacrylamide or agarose gels. Although a widely used technique, especially in biochemical fields, slab gel electrophoresis suffers from the drawbacks of long analysis times because the magnitude of the applied electric field is limited, low efficiencies and difficulties in detection and automation. An alternative to

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the gel format is to perform the separation in narrow-bore capillaries, since these are themselves anti-convective. Hjertén described initial work in open tube electrophoresis in 1967 [6], but it wasn't until Jorgenson and Lukacs used 75 μm fused silica capillaries in the early 1980's [7] that the theory was fully developed, and that the potential for high performance capillary electrophoresis (HPCE) as an analytical technique was demonstrated.

Separation of solutes in an electric field is the result of their different velocities (v) in the presence of that field:

$$v = \mu_e E \qquad (4)$$

where μ_e is the electrophoretic mobility, and E the applied electric field. The electric field is a function of the applied voltage and the capillary length, while the mobility of a given ion in a given medium, is a constant for that ion. The mobility is determined by the electric force experienced by the molecule, balanced by its frictional drag through the medium. The mobility of a spherical ion can be calculated using the following simplified equation, in which the two forces are taken into account:

$$\mu_e = \frac{q}{6\pi\eta r} \qquad (5)$$

where q is the ion charge, η the viscosity of the solution and r the ion radius. From this equation it is evident that smaller, highly charged ions have the largest electrophoretic mobility. The electrophoretic mobility determined experimentally is dependent on the pH of the background electrolyte and thus the solute's pKa, and as such often differs from the values found in standard tables in which values are determined at the point of full solute charge and infinite dilution. This means that it is possible, by choosing the correct pH, to separate solutes with the same mobilities if their pKa values differ.

3.2.1.2 Electroosmotic Flow

Electroosmotic flow (EOF) is the other fundamental constituent of HPCE operation and is a consequence of the surface charge on the capillary wall and the effect of the applied voltage on the solution double layer at the wall (figure 6). By adding the effect of the

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EOF to the mobility of the solutes, the amount of time they remain in the capillary is altered. Under aqueous conditions the fused silica surface possesses an excess of negative charge because of deprotonation of the silanol groups, which becomes significant at a pH above 3. Adsorption of ionic species to the wall may also produce surface charge in non-ionic materials like Teflon®. Counterions build up a double layer near these charged surfaces to maintain a charge balance, and thus create a potential difference very close to the wall known as the zeta potential. When the voltage is applied the cations forming this diffuse double layer (in the case of fused silica capillaries) are attracted to the cathode, and their solvation causes them to drag the bulk solution in the capillary in this direction.

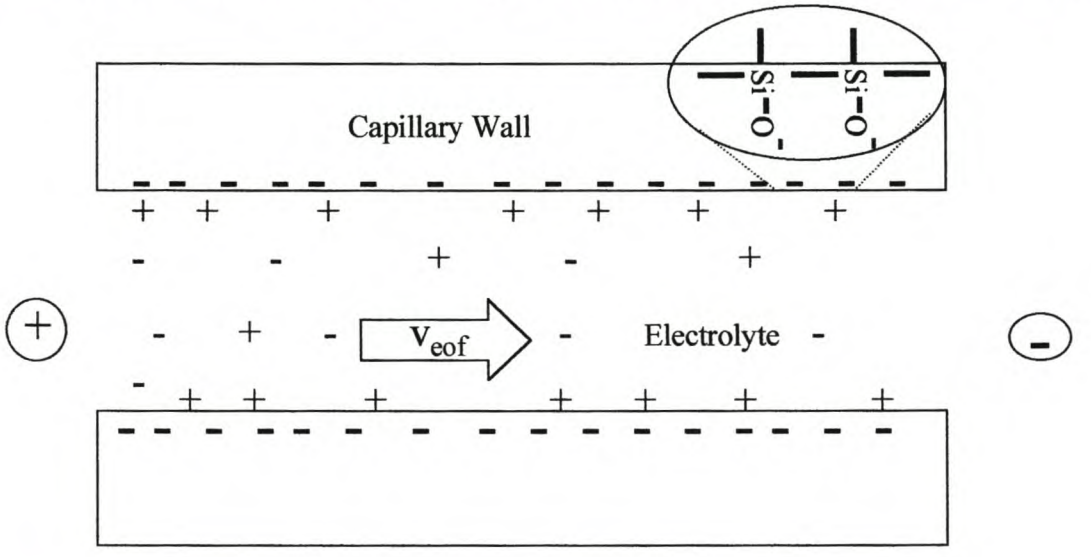

Figure 6: Schematic illustration of the generation of the EOF.

The magnitude of the EOF can be described in terms of velocity or mobility:

$$v_{eof} = \left(\frac{\varepsilon\zeta}{\eta}\right)E \text{, or} \tag{6}$$

$$\mu_{eof} = \left(\frac{\varepsilon\zeta}{\eta}\right) \tag{7}$$

where ζ is the zeta potential and ε the dielectric constant. Since the surface charge of the wall determines the zeta potential, and is in turn determined by the pH, the magnitude of the EOF can be varied by adjusting the pH. A higher pH increases the deprotonation of the silanol groups and thus increases the EOF.

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In the same way the zeta potential is dependent on the ionic strength of the medium. Increased ionic strength causes double layer compression that results in a decrease in the zeta potential and the EOF. As a consequence of the fact that the magnitude of the EOF can be more than an order of magnitude greater than the electrophoretic mobilities of the solutes, all the solutes can migrate toward the cathode. Thus it is possible to elute cations, anions and neutrals in one run, with the cations eluting first, the unseparated neutrals with the electroosmotic flow and the anions last. The EOF is, however, not always greater than the mobility of the solute. For small ions, and in cases where the wall charge is decreased while leaving the electrophoretic mobility unaffected, cations and anions will migrate in opposite directions.

The EOF can be altered using several methods. The most drastic of which is by changing the buffer pH, although this can also affect the solute charge and mobility. Changing the electric field on the other hand might reduce efficiency and resolution by Joule heating. Lastly, dynamic physical coating by using buffer additives or static chemical coating with for example polyvinylalcohol (PVA) of the capillary wall may be used to alter the EOF.

In the presence of EOF the measured mobility is called the apparent mobility (μ_a), which can be calculated using experimental parameters:

$$\mu_a = \frac{lL}{tV} \tag{8}$$

where μ_a is equal to the sum of the electrophoretic mobility of the ion (μ_e) and the electroosmotic mobility (μ_{eof}), l is the effective capillary length to the detector, L the total capillary length, V the applied voltage and t the migration time. The electrophoretic mobility can be calculated from the apparent mobility by using a neutral marker such as mesityl oxide or acetone to calculate μ_{eof}.

3.2.1.3 Efficiency in Capillary Electrophoresis

The EOF displays a flat flow velocity profile over the cross-section of the capillary. Although the flow rate decreases at the wall, it is relatively unimportant to the overall

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separation process. This is in contrast to the flow profile generated by pressure such as used in HPLC, which yields a laminar or parabolic flow profile (figure 7).

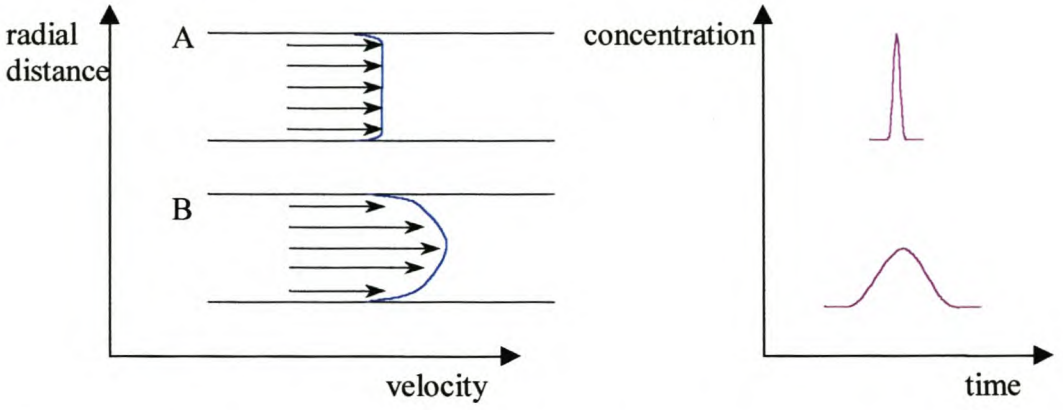

Figure 7: Comparison between the flow profiles and corresponding solute dispersion in CE (A) and in HPLC (B).

This flat profile is beneficial since it does not directly attribute to dispersion or solute zone broadening, and therefore amplifies the differences in solute mobility needed to separate solutes. Under ideal conditions (that is, small injection plug length, no solute-wall interactions and in the absence of Joule heating and conductivity perturbations) the sole contributor to zone broadening is longitudinal diffusion. This means that the efficiency can be related to the molecular diffusion term in liquid chromatography (equation 2):

$$\mathrm{H} = \frac{\sigma^2}{l} = \frac{2DL}{\mu_e V} = \frac{2D}{\mu_e E} \qquad \textbf{(9)}$$

where σ^2 is the variance of the peak and D is the diffusion coefficient of the solute in the electrolyte medium.

The electrophoretic expression for plate number can now be obtained with:

$$\mathrm{N} = \frac{l^2}{\sigma^2} = \frac{\mu_e E l}{2D} \qquad \textbf{(10)}$$

This equation explains why high fields are needed for high efficiencies. The equation also predicts that larger molecules, which have low diffusion coefficients, exhibit less dispersion than small molecules and thus have higher efficiencies.

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In practice, however, the measured efficiency is usually lower than the calculated efficiency. The reason for this is that longitudinal diffusion is in practice not the only dispersive process present. Although narrow bore capillaries were originally introduced to reduce the effects of Joule heating which is the heat generated by the passage of an electrical current and which has traditionally limited electrophoretic techniques, Joule heating ultimately limits the magnitude of the electric field which can be applied. Heating causes non-uniform radial temperature gradients, which result in local changes in viscosity and concomitant zone broadening. To reduce the effects of Joule heating, it is best to use very narrow bore capillaries so that less heat is generated. The high inner-surface to volume ratio helps to dissipate the generated heat faster. Additionally, the buffer ionic strength should be decreased to limit Joule heating but this may decrease buffering capacity and increase solute-wall interactions. Active temperature control is crucial, not only in dissipating the heat, but also to keep the temperature of the capillary lumen stable. This is because a change of 1 °C in temperature can result in viscosity (and thus mobility) changes of 2-3 % [8]. This alters not only the EOF and the velocities of the solutes, but also causes fluctuations in injected volumes, thereby detrimentally affecting the reproducibility.

3.2.1.4 Resolution in Capillary Electrophoresis

The resolution in HPCE is expressed by:

$$R = \left(\frac{1}{4\sqrt{2}}\right)(\Delta\mu)\left(\frac{V}{D(\bar{\mu} + \mu_{EOF})}\right)^{1/2} \qquad \textbf{(11)}$$

where $\Delta\mu = \mu_2 - \mu_1$, and $\bar{\mu} = (\mu_2 + \mu_1) / 2$. It is evident that an increase in the applied voltage does not result in a linear increase in resolution as it does in the case of efficiency because of the square root relationship. Thus high voltage increases are required to increase resolution by a substantial amount but this is often limited by Joule heating.

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3.2.2 Modes of Operation

The versatility of CE is due to its numerous operational modes, each of which relies on a different separation mechanism and thus has a different selectivity. Each mode can be used in a complementary manner with regard to other separation methods or other CE modes. The modes used in this study, namely capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC) and capillary gel electrophoresis (CGE) will briefly be discussed.

3.2.2.1 Capillary Zone Electrophoresis

Capillary zone electrophoresis (CZE) is the most widely used mode, mainly because of its simplicity and versatility. In this form, separation occurs in a capillary filled only with buffer. Discrete solute zones possess different velocities as a consequence of the interplay between electroosmosis and electrophoretic mobilities. Neutral molecules are not separated in this mode and elute with the EOF.

Of primary importance for a successful CZE separation is the choice of running buffer. The following requirements are important to consider when choosing a buffer:

- good buffering capacity in the pH range of choice,
- low absorbance at the wavelength of detection
- low mobility (lower conductivity than solutes) to minimise current generation
- the conductivity of the buffer should match that of the sample solvent as closely as possible to reduce peak shape distortion.

The pH of the buffer has to be chosen with care since it affects not only the charge of the solute molecules, but also the magnitude of the EOF. It is possible to perform chiral separations in CZE by simply adding the chiral selectors such as neutral or charged cyclodextrins, crown ethers or bile salts to the buffer.

Coating and modification of capillary surfaces are performed to manipulate the EOF and to surpress analyte-wall interactions. By dynamically or permanently coating of the wall

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with positive, neutral or negative coatings it is possible to reverse, nullify or increase the EOF. At present different permanently coated CE capillaries with reproducible properties have become commercially available.

Polyvinylalcohol (PVA) is a large linear molecule with a high content of hydroxyl groups for strong multipoint interaction *via* hydrogen bonding with silica surfaces. Immobilisation of PVA coating is achieved by thermal treatment [9]. PVA coatings are neutral and EOF is practically eliminated in these capillaries. This means that solutes are separated only based on their different electrophoretic mobilities.

3.2.2.2 Micellar Electrokinetic Chromatography

Micellar electrokinetic chromatography (MEKC) is a hybrid of electrophoresis and chromatography, and was introduced by Terabe in 1984 [10]. It has become one of the most widely used CE modes because of the possibility it offers to separate neutral molecules. This is accomplished by adding surfactants to the running buffer. At a concentration above the critical micelle concentration (CMC), aggregates are formed between individual surfactant molecules with the hydrophobic tails directed towards each other and the polar heads oriented towards the buffer. These aggregates are known as micelles, and can migrate either with or against the EOF, depending on the charge on the heads of the surfactant. During their migration, micelles interact with solute molecules in a chromatographic manner through hydrophobic interactions. In the case of anionic surfactants such as sodium dodecyl sulphate (SDS), the micelles migrate in the opposite direction to the EOF in other words to the anode. Since the EOF is faster than the electrophoretic velocity of the micelles, the net migration is in the direction of the EOF, in other words to the cathode. Neutral molecules are separated by partitioning in and out of the micelles on the grounds of their relative hydrophobicity. The more hydrophobic molecules interact more strongly with the micelles and are thus retained longer and elute later. Since the separation mechanism of neutral molecules in MEKC is essentially chromatographic, the capacity factor k' (the ratio of the moles of solute in the micelles to

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those in the mobile phase), has to be taken into account with respect to a moving stationary phase (pseudostationary), can be given by:

$$k' = \frac{(t_r - t_0)}{t_0\left(1 - \frac{t_r}{t_m}\right)} = K\left(\frac{V_S}{V_M}\right) \qquad (12)$$

where t_r is the migration time of the solute, t_0 the migration time of an unretained solute or EOF marker, t_m the micelle migration time, K the distribution coefficient, V_s the volume of micellar phase and V_m the volume of mobile phase or electrolyte. In general the capacity factor increases linearly with surfactant concentration, although in the case of ionic surfactants this leads to an increase in the current, which can detrimentally affect the separation. All neutral molecules elute in the time window between t_0 and t_m. It is therefore desirable to extend this window. This can be done by slowing down the EOF and using micelles with a high mobility. Selectivity in MEKC is easily adjusted by varying the physical nature of the micelles by using different surfactants. In every case the buffer concentration, pH, and temperature can also be adjusted to optimise selectivity. In addition, organic modifiers such as methanol or acetonitrile can be added to the buffer to modify solute-micelle interaction. For example, short-chain alcohols can enhance micelle formation (in other words reduce the CMC) when present at low concentration, but prevent micelle formation at higher concentrations, while organic solvents like acetone or acetonitrile will slightly inhibit micelle formation at low concentration and prevent it at higher concentrations. Addition of ionic species (*i.e.* electrolytes) will result in a decrease in the CMC and a slight increase in the aggregation numbers (the number of surfactant molecules comprising the micellar entity).

Another class of molecules able to form micellar aggregates in water, in addition to the long-chain alkyl surfactants most commonly used, are the bile salts. These molecules differ from the alkyl surfactants in that they possess a hydrophobic and a hydrophilic face (figure 8). As a consequence their aggregation behaviour differs from that of the alkyl surfactants. In this case initial primary micelles are formed, consisting of 2-8 monomers held together by hydrophobic interaction between the bile salt nonpolar parts. At higher

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bile salt concentrations, the primary micelles can further aggregate to form cylindrically shaped secondary micelles by intermolecular hydrogen bonding between their hydroxyl groups [11].

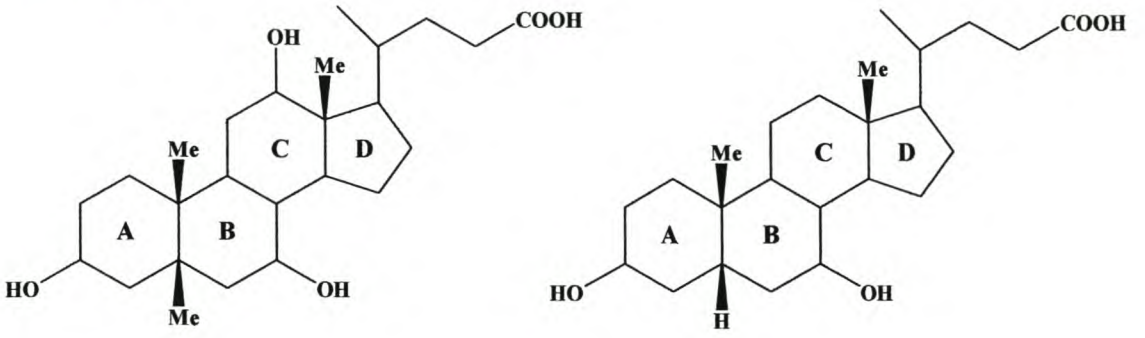

Figure 8: The structure of the bile salts cholic acid (a) and deoxycholic acid (b).

3.2.2.3 Capillary Gel Electrophoresis

Slab gel electrophoresis has been used primarily in the biological sciences for the separation of macromolecules such as proteins, DNA fragments and polysaccharides. This is achieved by electrophoresis through a suitable polymer that acts as a molecular sieve by hindering the larger solutes more than the smaller during their migration. The CE format of slab gel electrophoresis, capillary gel electrophoresis (CGE), offers certain advantages over the traditional slab gel electrophoresis, which can sometimes be a laborious and time-consuming technique. Advantages include the use of higher field strengths without the detrimental effect of Joule heating (although ultra-thin slab gels have been used to reduce Joule heating), on-capillary detection and automation (although DNA-sequencers are commercially available). The use of anti-convective gels such as agarose or polyacrylamide to eliminate conductive transport and reduce diffusion is a necessity for slab gel electrophoresis and places limits on the choice and concentration of material used. The anti-convective nature of capillaries relaxes these limitations. Since the publication of the first papers on the use of CGE in the early 1980s it has become evident that almost all methods developed for slab gel electrophoresis can easily be transferred to the capillary format, with the concomitant advantages of high resolution,

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automation and on-line data acquisition and storage. The multi-lane capabilities of the slab gel format can be matched in CGE using systems employing multiple capillaries (for example in DNA sequencing), although rapid analysis times in the case of CGE do not compensate for the preparative capabilities of the slab gel format.

In CGE two types of sieving matrices are used namely: the high-viscosity chemically cross-linked gels, and the non-cross-linked low-viscosity gels. In the first instance, the polymers are usually chemically bound to the capillary wall, while their pore structure is well defined due to the cross-linkage. Because of the high viscosity of these polymers, the polymerization has to be done within the capillary, which means that pore-size can not be adjusted after polymerization. These gels are also heat sensitive and bubble formation may occur as a result of any slight precipitation or shrinkage of the polymer, damaging the gel. On the other hand, non-cross-linked polymers have a flexible dynamic pore structure and are not attached to the capillary wall. Pre-polymerised polymer solutions can be hydrodynamically loaded into the capillary. Their pore size can be varied simply by changing the physical parameters such as the capillary temperature and polymer size or concentration. Replacing the matrix in the capillary solves problems caused by precipitation and bubble formation. Although the polymer structures differ radically from that of cross-linked gels, the mechanism of separation is identical. In order to get reproducible separations capillary wall coating is essential. This means that it has to be stable over a wide range of pH, while minimising EOF and preventing sample interactions with the wall surface silanols [12].

Recently Chang *et al.* [13] demonstrated a simple method for separating DNA fragments without having to fill the capillary with polymer solutions prior to analysis. In this method, the capillary is flushed beforehand with NaOH to ensure a high EOF, prior to being filled with the buffer not containing the polymer. The sample plug is then simply hydrodynamically injected as in CZE. A buffer vial containing the polymer solution is then placed on the inlet side of the capillary, and the voltage is applied. The polymer solution is then flushed into the capillary by the EOF. Thus this method eliminates the

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difficulties of filling the capillary and of injecting the sample in the case of highly viscous polymer solutions.

Since CGE is also a "zonal" electrophoretic technique, the resolution and efficiency obtained are often higher than those obtained by CZE and MEKC. Selectivity in CGE can also be altered by varying the physical characteristics of the polymer used, or by simple addition of chiral selectors and complexing agents to the running buffer.

3.2.3 Instrumental Aspects of Capillary Electrophoresis

The basic instrumental set-up of capillary electrophoresis instrumentation is depicted in figure 9. Each aspect will be discussed shortly.

- Fused silica capillaries are the most commonly used in CE, ranging in internal diameters from 10 to 200 μm, and in total length from 10 to 100 cm. The silica is coated with a layer of polyimide to make them strong and easy to handle. A small section of this coating has to be removed for detection purposes.

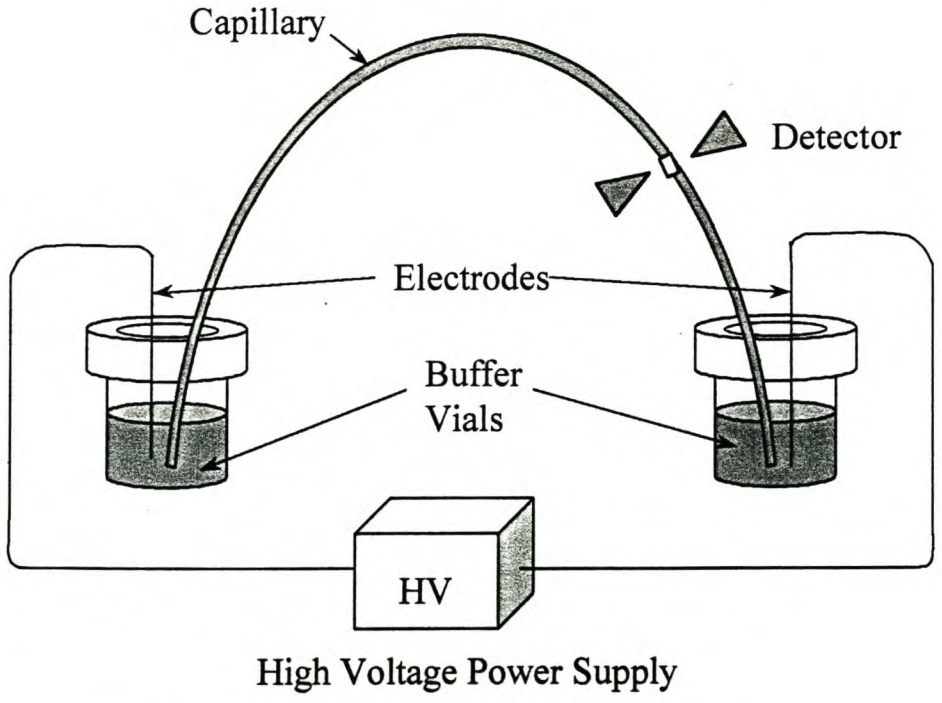

Figure 9: Schematic drawing of capillary electrophoresis instrumentation.

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- The capillary inlet– and outlet ends are placed in buffer vials during analyses. The inlet buffer vial is substituted by a sample vial during injection, after which the inlet buffer vial is replaced before the application of the separation voltage. These vials can also be replaced by vials containing cleaning solutions, for flushing purposes. The vials are placed in a vial carousel and substitution is performed automatically through the software.
- Because of the strong dependence of viscosity on the temperature, effective capillary thermostating is essential. This can be achieved by mounting the capillary in a high velocity air stream or in a liquid. Although the latter is more effective, the former is most often used because of simplicity, and is effective enough in the range of most CE experiments.
- A DC power supply capable of applying up to 30 kV and current levels of up to 300 μA is used in CE instruments. The power supply should be capable to switch the applied polarity for use in cases where the EOF is reduced or reversed. In addition, these power supplies have the ability to run voltage, current or power gradients (called field programming).

Important aspects in CE are sample introduction and detection. Sample injection in CE is described in section 3.2.4. The same detection principles are used in CE as in HPLC and this is described in Chapter 3.3.

3.2.4 Injection modes in Capillary Electrophoresis

There are two methods of sample introduction in CE namely: hydrodynamic injection and electrokinetic injection. The first is based on pressure differences between the inlet and outlet ends of the capillary. This can be produced using gravity, overpressure or vacuum mechanisms. In gravimetric or siphonic injection the sample vial at the inlet of the capillary is raised to a predetermined height for a defined time, and the resultant hydrostatic pressure forces the sample into the capillary. Alternatively, pressure can be

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applied to the sample vial on the inlet end of the capillary, or vacuum can be applied to the outlet to suck the sample into the capillary. This form of injection is universally applicable to all sample matrices without bias toward sample components, and in general delivers better reproducibility and greater control over the amount of sample injected compared to electrokinetic injection.

During electrokinetic injection, the sample vial is placed on the inlet side of the capillary and high voltage is applied for a short period of time. The solute molecules migrate into the capillary due to a combination of electrophoretic migration of the ions and of electroosmotic flow of the sample solution. This means that a difference in electrophoretic mobility between different solutes translates into a bias in injection amount between these solutes. An example is the size bias towards solutes with the same electrophoretic mobility, which is found when injecting onto a capillary filled with a sieving medium. This is a result of differences in mobilities in the sieving medium. Reproducible electrokinetic injection is, however, possible at much smaller volumes than in the case of hydrodynamic injection. Because the apparatus needed for electrokinetic injection has basically the same arrangement as the separation process, the ease of operation often makes this the preferred form of injection.

In CE techniques, sample plugs are commonly very small (nanoliters). This results in low sensitivity. A way to introduce larger sample sizes is called sample stacking [14].

3.2.5 Sample Stacking

Differences in sample zone and running buffer conductivities are responsible for a phenomenon known as electrodispersion. The correlation between the electric field (E), the current density (i), and the conductivity (κ) of the electrolyte is expressed as:

$$E = \frac{i}{\kappa} \qquad \textbf{(13)}$$

When, for example, the solute zone containing negative solutes has a lower conductivity than the running buffer, the solute molecules experience a greater electric field in this

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zone. The trailing edge of the solute zone diffuses into the buffer and on encountering the lower field, the solute molecules decelerate back into the sample zone (in the direction of voltage increase), thereby keeping the trailing edge sharp. The solutes at the leading edge, when diffusing into the buffer and encountering the voltage drop also slow down, but this results in a diffuse leading edge of the sample zone, in other words fronting occurs (figure 10). In the same manner, when the conductivity of the sample zone is higher than that of the running buffer, this difference translates into sharp leading and diffuse trailing edges and thus tailing. These distortions are often negligible when compared to other dispersive effects, but are particularly evident when samples containing solutes with a wide range of mobilities are analysed, while neutral molecules are unaffected by them.

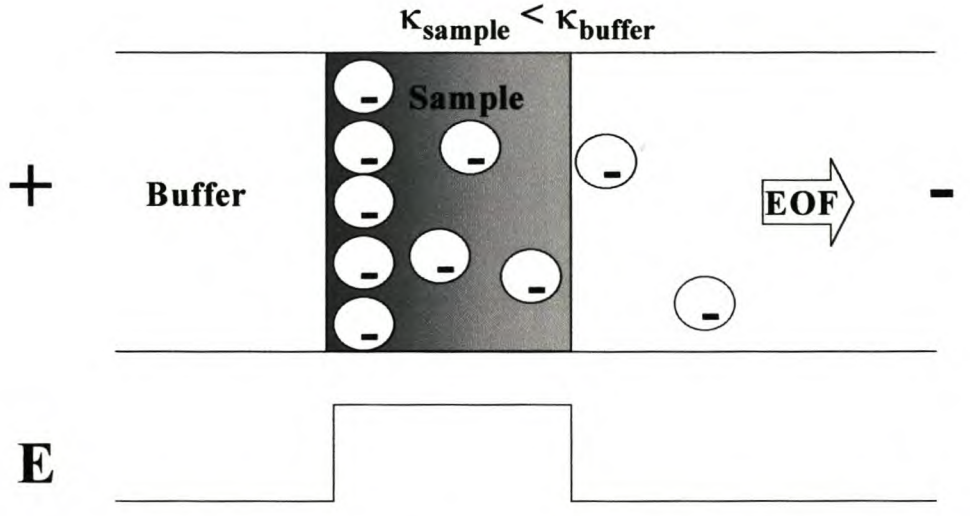

Figure 10: Electrodispersion. Due to the lower conductivity in the sample zone, the negative solute molecules experience a greater electric field and thus possess greater electrophoretic velocities, and stack at the back of the sample zone. This translates into peak fronting since detection takes place at the cathode.

This phenomenon can be used to translate a large volume of low-concentration analyte into a sharp zone of high concentration. This process is known as sample stacking. The many different techniques for on-column sample stacking can be divided into two groups in accordance with the movement of the boundary between buffers: moving boundary stacking and stationary boundary stacking.

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Field-amplified sample stacking is an example of the latter technique, wherein the sample is dissolved in a buffer of lower concentration, and hydrodynamically injected in the form of a long zone into a capillary containing a higher-concentration buffer. The electric field strength will be higher in the region of the sample buffer because of the differences in conductivity. The ions in the lower-concentration buffer will experience a larger electric field and thus have a larger electrophoretic velocity. As soon as these ions pass the concentration boundary, however, they will experience a lower field and accelerate back into the sample zone. The effective sample zone length is then decreased by a factor directly proportional to the difference in the electric field between the two zones. This stacking mechanism occurs for both positive species (who stack at the front of the sample zone), and negative species (who stack at the back), while the neutral species are left unaffected in the sample zone. There are, however, limitations with regard to the concentration differences between the buffers to be used and the length of the sample plug to be injected. This is because, as the conductivity of the sample zone decreases, the electric field in the supporting buffer (where the separation takes place) approaches zero. Broadening also occurs as a result of mismatches between local electroosmotic velocities and the bulk velocity, which causes a laminar flow [14]. Thus there is an optimal point as to the injection plug length and the conductivity differences to be used.

3.3 Detection

In both HPLC and CE, UV- and MS- detection are applied. The principles of these detection methods are described.

3.3.1 Ultraviolet Detection

The absorption of radiation in the ultraviolet and visible region of the electromagnetic spectrum is the result of transitions between electronic levels of the analyte, which are quantized. These transitions are the result of an electron being promoted from an occupied molecular orbital of lower energy, to an unoccupied one of higher energy.

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Certain transitions are, however, forbidden by selection rules, and these transitions, if visible, have a much lower intensity of absorption. For organic molecules the absorption occurs over a wide range of wavelengths because they have many excited modes of vibration and rotation at room temperature. The molecule can thus undergo electronic and vibrational-rotational excitation simultaneously, and since the wavelengths of these transitions differ only slightly (the difference between rotational- and vibrational energy levels are very small compared to those of electronic levels), the spectrophotometer is not able to resolve the closely spaced absorption lines, and the UV spectrum consists of a broad band centred around major transitions [15]. According to the Beer-Lambert Law the absorbance ($=\log(I_0/I)$ where I_0 and I are the intensities of the electromagnetic radiation entering and leaving the sample, respectively), is related to the molar absorptivity (ε), the concentration of the solute (c) and the path length of the sample cell (l) by:

$$A = \varepsilon c l \qquad \textbf{(14)}$$

UV spectroscopy has been an important technique for the structure analysis of in particular flavonoids in the past [16,17], mainly due to the amount of structural information gained by addition of reagents that react with one or more functional groups and induce shifts in the absorbance maxima. For example, the addition of boric acid and sodium acetate is used for the detection of *o*-dihydroxyl groups in all flavonoids except the anthocyanidins, since the complexation of borate to these functional groups causes bathochromic shifts [17]. The UV-spectra of the flavonoids consist of two major absorption maxima, one in the range of 240-285 nm (band II, originating from the A-ring benzoyl system), and one in the range of 300-400 nm (band I, originating from the B-ring cinnamoyl system). Anthocyanidins (and anthocyanins) are notable exceptions with band I absorption maxima in the range 465-550 nm, and band II being represented by a less intense peak in the 270-280 nm region. These absorption bands are due to $\pi \rightarrow \pi^*$ transitions, with the wavelength of maximum absorption varying according to the substitution pattern of the particular flavonoid.

The popularity of UV/Visible detection can be ascribed to its simplicity, ease of use, relatively low cost and its versatility. It is the most widely used detector in both liquid

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chromatography and in CE. There are several types of absorption detectors available that vary in their flexibility and their ability to provide single or multiple analysis wavelengths. Examples are the fixed single wavelength detectors where the atomic vapour lamp determines the wavelengths available for detection and the variable wavelength detector, which provides a continuum source, combined with a monochromator for wavelength selection. Of the multiple wavelength detectors, the photodiode array multiple wavelength absorbance detector is the most common. This detector employs an array of photodiodes placed across the plane of a polychromator. The light from the source (for example a deuterium lamp) passes through the flow cell and is subsequently dispersed by the polychromator for simultaneous measurement. This means that the complete spectral range is recorded continuously at microsecond intervals. The complete absorption spectrum of each component in the chromatogram is recorded and can be compared to a spectral library. To increase the signal-to-noise ratio, the output from a group of adjacent diodes (the associated effective bandwidth is specified by the analyst) can be averaged to produce one signal output [18].

In liquid chromatography a linear response range of 10^4 to 10^5 and detection limits in the most favourable cases in the range of 0.01 to 0.1 ppm are possible with UV detection. In the design of the detector flow cell a compromise has to be made between minimising the cell volume to reduce extracolumn band broadening, and maximizing the path length to increase sample detectability. For conventional columns cell volumes of 10 μL and a path length of ~10 mm are most often used.

The optical window in CE is made simply by removing a small sector of the outer polyimide coating near the outlet of the capillary. This means that since the window is on the capillary, there is no real postcolumn zone broadening as a result of dead volume. However, a too large window size with respect to the length of the eluting peak can cause an optical band broadening of the zone. This is as detrimental to the separation efficiency as the physical sources mentioned before. Because of the short period of time analytes spend in the detector (as short as 1-2 seconds), the detector response times must be fast. The largest drawback to UV-Vis absorbance detection in CE is its relatively weak

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sensitivity relative to that obtained by UV-Vis detection in HPLC. This is due to the short pathlength available, namely only the internal diameter of the capillary. Several techniques have been developed to increase the path length, including the use of a rectangular capillary where the width is much greater than the height in order to maximise the path length and heat dissipation. The Z-cell is another approach where illumination occurs down the bore of a section of the capillary. This is achieved either by bending a section of the capillary or, alternatively, by placing an inlet and an outlet capillary above one another in a detection cell. In this way a perpendicular flow along which the illumination can occur is produced. This is the case of the high sensitivity cell shown in figure 11. A third approach is the "bubble cell" where a section of the capillary is enlarged in the shape of a bubble, leading to a three to five times increase in path length and thus sensitivity [19].

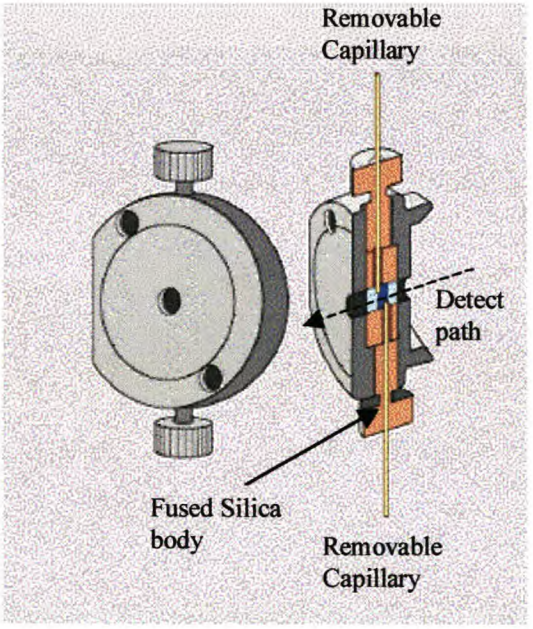

Figure 11: Schematics of the High Sensitivity Cell for UV-detection in CE.

3.3.2 Mass Spectroscopy

The coupling of mass spectroscopy (MS) to any separation technique provides the best combination of sensitivity and structural information. The ionisation of organic molecules for MS can be achieved in a variety of ways. The most important with regard

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to hyphenation with separation techniques include electron impact (EI), chemical ionisation (CI), atmospheric pressure chemical ionisation (APCI), and atmospheric pressure electrospray ionisation (APESI). It is impossible to describe HPLC-MS and CE-MS in detail in the framework of a Masters thesis. Moreover, the experiments with MS detection have been carried out in collaboration with Y. Zhao, G. Vanhoenacker and K. Lazou at the Department of Separation Sciences of the University of Gent, Belgium. For full details on LC-MS and CE-MS we refer the reader to the Ph.D. thesis of Y. Zhao available from our laboratory [20]. A brief description is given.

3.3.2.1 Liquid Chromatography-Mass Spectroscopy

APESI and APCI are the most widely used ionisation techniques for HPLC-MS. APESI is based on a liquid spray at atmospheric pressure passing through a needle held at a high potential (± 3000V) with respect to the cylindrical electrode which surrounds it, with the result that the liquid stream becomes charged. Evaporation of the solvent leads to highly charged droplets. These droplets undergo Coulombic explosions leading to smaller droplets that eventually form single, or multiple charged ions. The ions are analysed by a quadrupole mass spectrometer. In APCI, the nebulized mobile phase droplets are desolvated by a combination of temperature and gas flow, after which they are ionised by a corona discharge needle. The ions then enter the analyzer section of the mass spectrometer through a small 100 μm pinhole.

3.3.2.2 Capillary Electrophoresis-Mass Spectroscopy

In CE-APESI-MS the sheath-flow interface is used where the capillary extends all the way into the ionisation chamber. Because the flow rates associated with CE are in the range of nL/min and APESI sources require flow rates of ca. 2 to 10μL/min or more, a make-up flow is delivered. This sheath-flow capillary is made of stainless steel to provide electrical contact. One of the major disadvantages of MS detection in conjunction with CE is the small sample capacity of the latter. This leads to a high concentration detection limit even though the absolute amount detected can be quite low. A variety of solutions

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involving developments on both the CE and MS ends have been suggested to overcome this problem. These include methods for loading higher sample amounts from dilute solutions such as transient isotachophoresis (tITP) and membrane preconcentration, as well as new developments in MS detectors and interfaces [21].

3.4 Instrumentation

3.4.1 CE Instrumentation

The CE analyses were performed on a HP^{3D} CE capillary electrophoresis system equipped with diode array detection from Hewlett-Packard (Waldbronn, Germany). Data analysis was executed with ChemStation (Rev. A.06.01) software, also from Hewlett-Packard. The instrument is shown in figure 12.

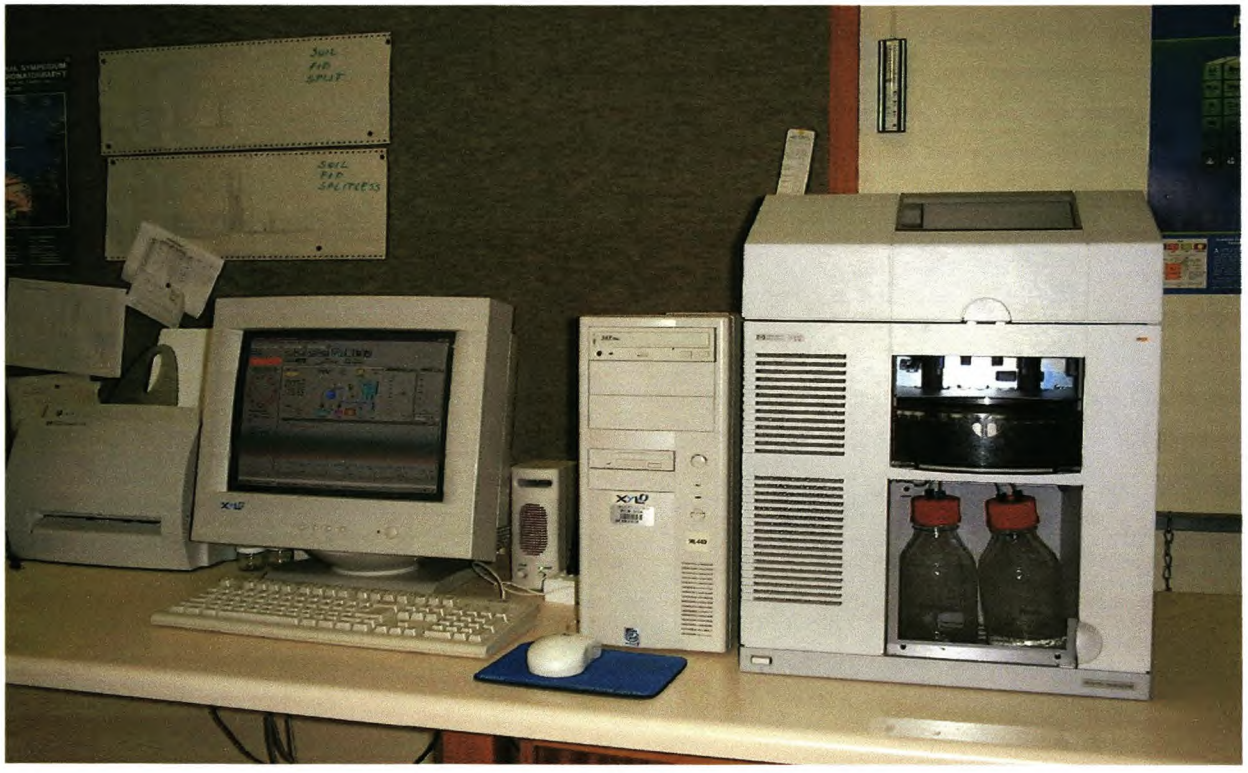

Figure 12: The HP^{3D} CE system.

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3.4.2 HPLC Instrumentation

The HPLC analyses were carried out on an Alliance 2690 Separations Module equipped with a Waters 996 Photodiode Array Detector, both from Waters Corp. (Milford, MA, U.S.A). The data analysis was done using Millenium^{32} Chromatography Manager software, also from Waters.

3.4.3 CE-MS Instrumentation

The CE-MS experiments were performed on a HP^{3D} CE capillary electrophoresis system coupled to a MSD mass spectrometer from Hewlett Packard. Data analysis was executed with ChemStation software, also from Hewlett Packard.

3.4.4 HPLC-MS Instrumentation

The LC-MS experiments were carried out on a system comprising of a HP1100 series liquid chromatograph coupled to the same MSD mass spectrometer as was used for the CE-MS experiments, both from Hewlett Packard. The former is a set of modules, consisting of a quaternary pump, a degassing unit, an injection unit, a column heater and a variable wavelength detector (VWD), which can be combined into an LC system.

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4. Results and discussion

4.1 Sample Preparation

Sample preparation is a necessary step in the analysis of phenolic compounds in wine. This is partly a consequence of the fact that many phenolics have similar absorption spectra with maxima in a narrow range of 280-320 nm.

4.1.1 Sephadex Fractionation

One of the main problems associated with direct injection of wine samples is the presence of polymeric polyphenols. These compounds elute together as a broad band in both HPLC and CE analysis, placing doubts on quantitative data. Attempts have been made to isolate these polymeric phenols in grape extracts and wine using Sephadex LH-20 [1,2] as well as size exclusion chromatography [3]. In the former studies, however, it was found by comparative quantitation of polymeric polyphenols using dialysis and Sephadex fractionation in both young and aged wines, that the latter technique produced consistently lower values for the percentage of polymeric polyphenols in aged wines. The evolution of phenolic polymers during ageing due to, amongst others, quinoid polymerisation and acetaldehyde-induced condensations is thought to be responsible for this inconsistency in chromatographic behaviour between the polymeric phenols in young and aged wines. The Sephadex fractionation procedure relies on the strong hydrogen-bonding character of the polymers relative to the non-polymeric phenols [2]. However, the phenolic polymers in aged red wines appeared to have lower adsorptive properties on Sephadex than the condensed tannin polymers found in solid grape tissues and young wines. This fractionation procedure is thus only suited for polymeric polyphenols in young wines and grape tissue extracts. This severely limits its applicability to wine analysis. This was also found to be the case in experiments done in this study, where the polymeric phenols could not be isolated in 1998 Simonsvlei Pinotage using this method. Cacho *et al.* [3] managed to separate polyphenols from grapes into two fractions using a Sephadex G column. One fraction contained polyphenols bigger than the pore size of the gel, the other those that are smaller. While this method might be used to study the

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polymerisation of phenolic compounds in grapes and wines, the two fractions were not successfully separated and this fractionation procedure could not be used for analytical purposes, especially when taking into account the variation of polymer size between wines of different vintages and cultivars.

4.1.2 Ether Extraction

As an alternative form of sample preparation the extraction of polyphenols with ether and consequent analysis by HPLC [4] and CE [5,6] has been reported. In this method the phenols are extracted and the ether evaporated under a stream of nitrogen, before being redissolved in methanol and injected. The main advantage of this procedure is the relative simplicity of the sample obtained, since the polymeric polyphenols are not extracted. The reason for this is not clear, since it is apparent from reversed-phase HPLC and SPE experiments that these compounds elute later than the more polar acids, suggesting that they are indeed more apolar. It should therefore be possible to extract these compounds using ether. A possible explanation is the incorporation of anthocyanidins into polymeric polyphenols, which would mean that these compounds possess a positive charge at the low pH's used for the ether extraction and are thus not extracted. In this study it was found that even those polyphenols recovered with this procedure, are not quantitatively extracted. The recovery was studied by extracting polyphenols from a standard solution, and comparing the corrected areas for each compound prior to and after extraction. In addition, it was found that by reducing the pH of the wine, the extraction efficiency could by increased (table 1).

Since the pKa values for most of the phenolic acids in wine are in the region of 3.5-4, the higher extraction efficiency of these compounds at a lower pH can easily be explained in terms of the smaller percentage of these compounds in the deprotonated ionic form at pH 2. The more polar polyphenols (such as gallic acid) also exhibit poor extraction efficiencies into the ether. Thus, while this form of sample preparation may be useful when qualitatively comparing wines, it is not of much use when a complete and quantitative analysis of wine polyphenols is the aim.

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Standards	Recovery (pH 2)	% rsd	Recovery (pH 3.5)	% rsd
resveratrol	112.3	5.6	72.0	26.5
epicatechin	22.1	12.4	19.2	22.9
catechin	26.7	22.5	21.8	35.7
o-coumaric acid	101.8	3.5	90.1	26.0
m-coumaric acid	102.0	5.1	88.5	25.8
p-coumaric acid	102.8	5.1	81.4	23.3
vanillic acid	88.3	4.9	80.8	27.4
quercetin	80.0	14.4	63.6	15.6
caffeic acid	91.0	3.9	78.7	22.4
gallic acid	39.8	10.7	31.1	20.4

Table 1: Recovery data for ether extraction of a polyphenol standard solution (n=3).

Ether extraction was performed by adding 20 ml of wine to the same volume of diethyl ether and the solution was then stirred for 5 minutes. The pH of the wine was adjusted with 1 M HCl when required. Following extraction, the ether was evaporated under a stream of nitrogen, and the sample dissolved in 5 ml of 40% methanol in water, giving a net concentration factor of 4.

4.1.3 Solid-Phase Extraction

As an alternative to fractionation procedures involving gel filtration chromatography, which can be laborious and time consuming, solid-phase extraction (SPE) has recently been used for sample preparation in a number of studies of polyphenols in a variety of samples [7-11]. SPE is a sample preparation technique developed as an alternative approach to liquid-liquid extraction. Analytes are concentrated from solution by adsorption onto a disposable solid-phase cartridge, followed by elution of the analyte with a solvent appropriate for instrumental analysis. The mechanisms of retention in SPE include reversed phase, normal phase and ion exchange. Reversed-phase SPE involves the partitioning of a (normally organic) solute molecule between water or an aqueous mobile phase and an apolar phase chemically bonded to silica, such as a C-18.

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For the polyphenols in wine this means that they are retained on the apolar phase because their solubility and affinity is much higher in this phase than in the polar aqueous mobile phase containing ~13 % ethanol. Polyphenols can then be eluted using a more hydrophobic solvent usually consisting of an aqueous solution of an organic modifier such as methanol or acetonitrile. Increasing the amount of organic modifier increases the solvent strength of the eluent. In certain cases it is possible to perform stepwise elution of different classes of analytes by using solvents of increasing strength, each of which is stronger than the preceding solvent and only capable of eluting a certain class of molecules. The concentration effect associated with SPE stems from the fact that the volume of the desorbing solvent can be much less than the sample volume introduced onto the cartridge. Purification is achieved when solutes are not retained into the "stationary" phase and elute with the "mobile" phase, or, alternatively, a suitable solvent that is too weak to desorb the impurities can be used to selectively desorb the compounds of interest.

Rossi *et al.* [7] demonstrated the use of SPE in a pre-concentration and clean-up step in the CZE analysis of wine polyphenols. Here the compounds of interest were retained on a preconditioned C18 Sep-Pak cartridge and eluted with ethyl acetate. This is an effective technique to get rid of other components in wine which are not of interest like proteins and sugars and to preconcentrate the sample, which is especially useful in the case of CE analysis. The obtained sample is still very complex and quantification is difficult. Jaworski and Lee [8] fractionated grape juice polyphenols into acidic and neutral fractions using a preconditioned C18 Sep-Pak cartridge. This was achieved by adjusting the pH of the sample to 7. In this way the acidic phenols are negatively charged and elute with the mobile phase, while the neutral phenolics are retained. The pH of the acidic fraction is then lowered to 2, to ensure that the acids are no longer charged, before it is passed through a second acidic Sep-Pak to retain the acidic phenolics. Both fractions are then eluted with methanol. While this technique is relatively simple and effective, the neutral fraction is still very complex and contains the polymeric phenols.

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The most complete fractionation procedure of wine polyphenols using SPE was described by Oszmianski *et al.* [9]. In this procedure the ethanol was removed from the wine by rotary evaporation, prior to the sample being loaded onto a neutral preconditioned C18 Sep-Pak cartridge. The acidic fraction was then separated in the same manner as discussed above. The neutral phenolic fraction was then further fractionated by stepwise elution of the procyanidins, catechins and anthocyanidin monomers (with 16 % acetonitrile at pH 2); the flavonols (with ethyl acetate) and the anthocyanidin polymers (with methanol). The acetonitrile and ethyl acetate was then removed by evaporation and the residue redissolved in methanol for HPLC analysis. A schematic representation of this procedure is provided in figure 13.

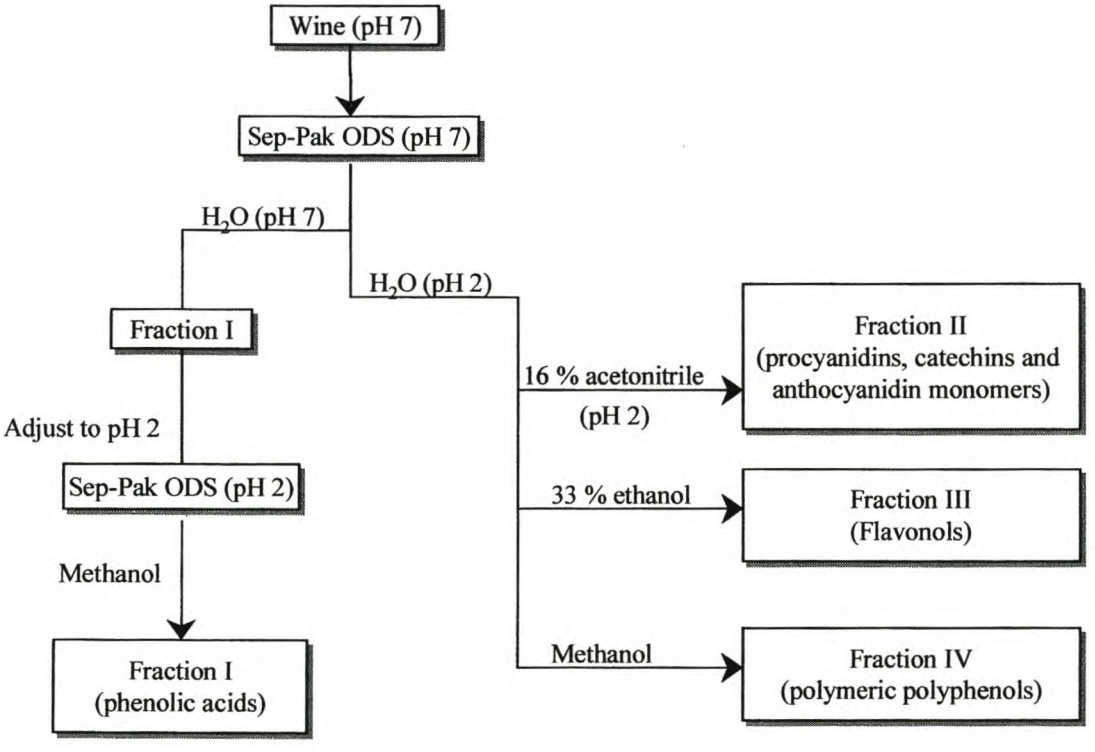

Figure 13: Schematic illustration of the Sep-pak procedure used to separate wine polyphenols into 4 fractions, based on the method employed by Oszmianski *et al.* [9].

In this study the fractions were analysed using CZE, and it was found that recoveries were consistently higher when the acetonitrile fraction was directly injected. The ethyl acetate fraction, however, caused current breakdown and therefore the use of a different

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eluent with the same solvent strength was investigated. THF and ethanol were investigated on a solution of standards dissolved in 20% methanol, containing standards of each class, except for the polymeric polyphenols.

The solvent optimisation on this standard solution indicated that 33% ethanol was strong enough to elute fraction III. In subsequent analyses of wine samples, however, it was found that this solvent also eluted the polymeric polyphenols (fraction IV).

The CZE patterns of the different fractions obtained using standard solutions (refer to figures for composition) are depicted in figures 14-16, while the 33% ethanol fraction in the case of a wine sample is depicted in figure 17.

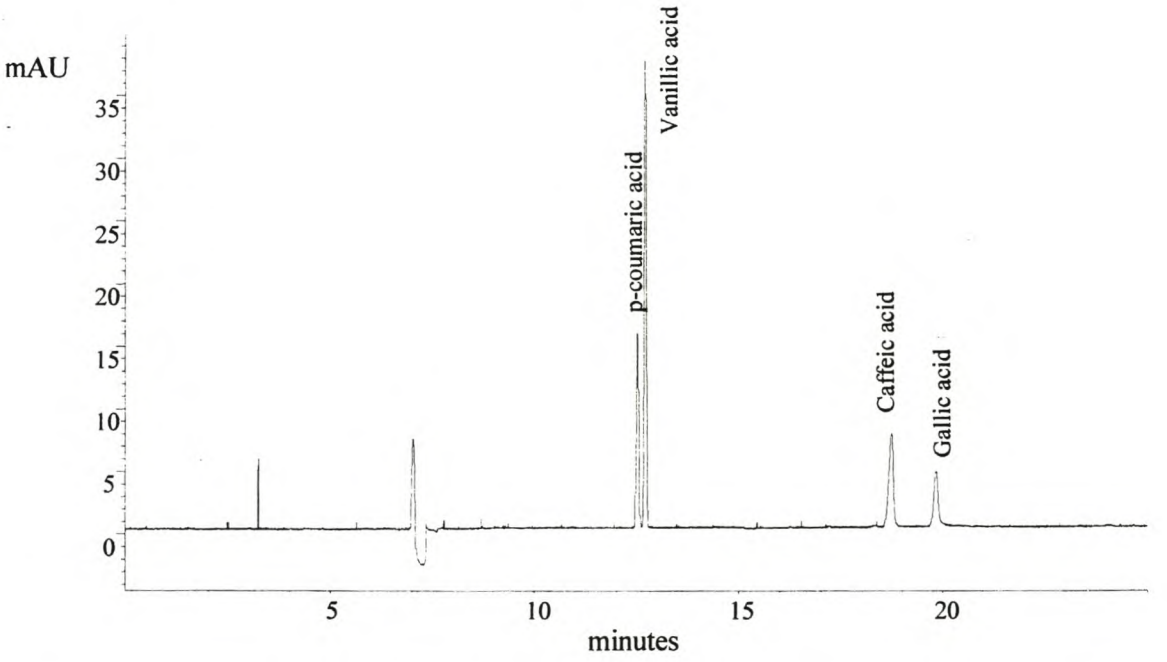

Figure 14: CZE separation of fraction I (the phenolic acids) obtained from the Sep-Pak fractionation of 0.5 ml of a standard solution (containing 250 ppm of catechin, rutin, and the identified acids), eluted with 3 ml methanol. Capillary: 72 cm effective length, 75 μm i.d. Buffer: 25 mM phosphate, 10mM borate, pH 8.8. Injection: hydrodynamic, 50 mbar for 4 seconds. Detection: 206 nm. Voltage: 25 kV. Temperature: 20°C.

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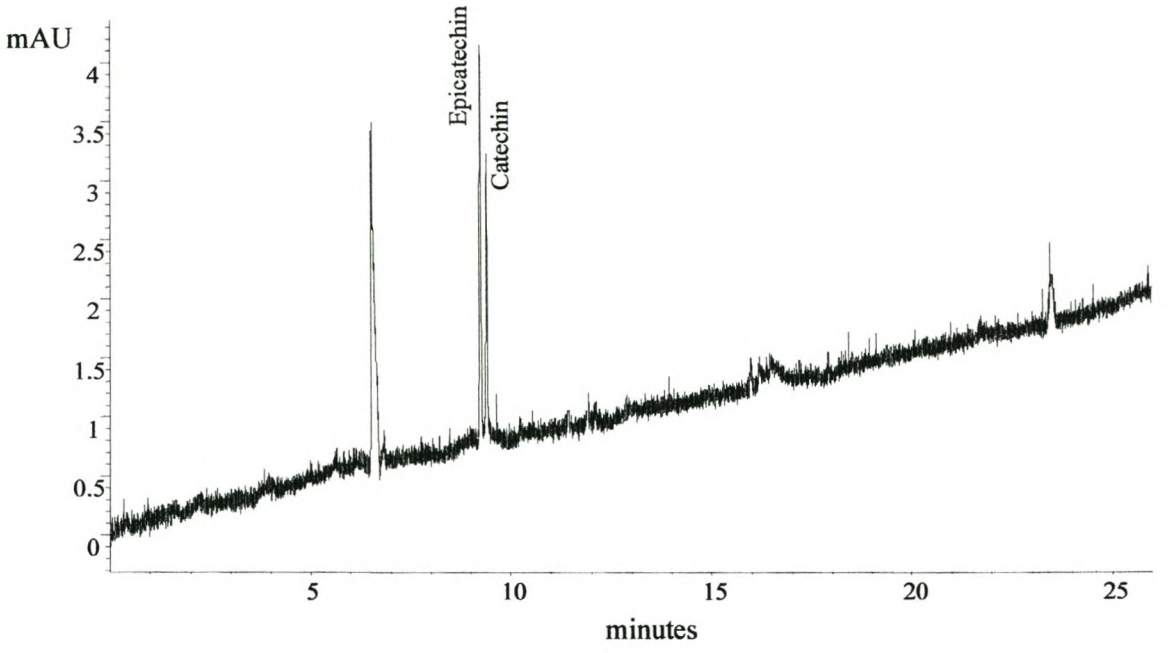

Figure 15: CZE separation of fraction II (the catechins, etc.) obtained from the Sep-Pak fractionation of 0.5 ml of a standard solution (containing 20 ppm catechin, epicatechin, resveratrol, o-coumaric acid and 15 ppm rutin), eluted with 5 ml 16% acetonitrile (pH 2). Other conditions as in figure 14.

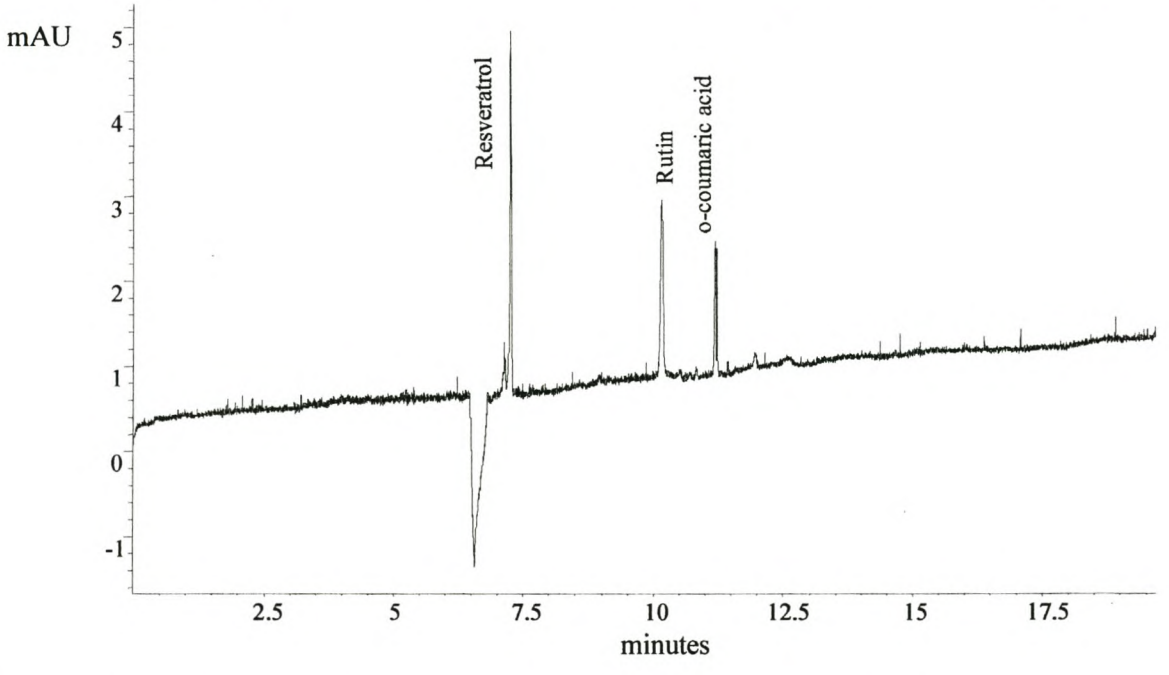

Figure 16: CZE separation of fraction III (the flavonols) obtained from the Sep-Pak fractionation of 0.5 ml of a standard solution (with the same composition as specified in figure 15), eluted with 3 ml 33% ethanol. Detection: 280 nm. Other conditions as in figure 14.

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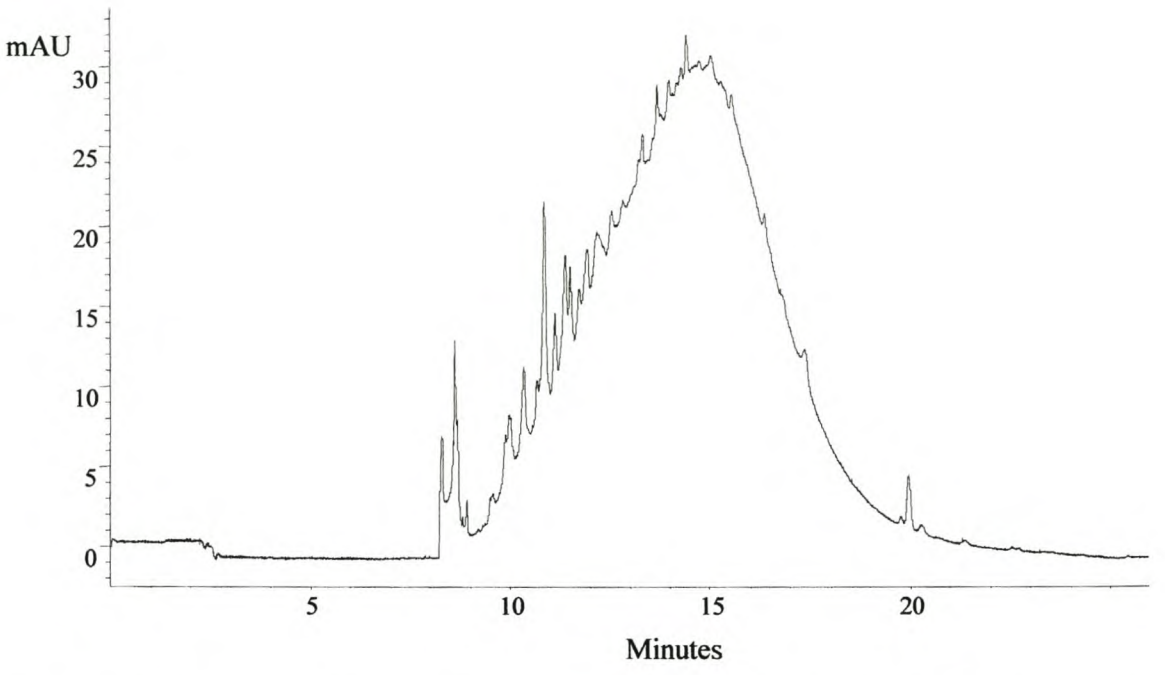

Figure 17: CZE separation of fraction III (the flavonols) obtained from the Sep-Pak fractionation of 0.5 ml of the non-volatile fraction (section 4.1.4) of the bitter Pinotage, eluted with 3 ml 33% ethanol. Other conditions as in figure 14.

From these experiments it was concluded that this procedure was not selective enough, since the eluents used to elute fraction II (the catechins, procyanidins and anthocyanidin monomers) and III (the flavonols) quantitatively, also co-eluted some of the polymeric polyphenols (IV). This may also be due to changes taking place in the polymeric polyphenol content of the wine during ageing. This means that the procedure might therefore be used successfully in the analysis of certain (for example young) wines, but is certainly not universal.

4.1.4 Separation of Volatiles and Non-volatiles

An additional form of sample preparation is fractionation of wine volatiles and non-volatiles in a process analogous to lyophylisation [12]. A schematic illustration of the experimental set-up is given in figure 18. In this procedure 40 ml of wine is placed in a round-bottom flask (1) and frozen with liquid nitrogen. Vacuum is then applied to the system for 10 minutes and the system is then closed (3), followed by bringing the wine to

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room temperature using a water bath. The right hand flask, which serves as a trap for the volatiles (2), is then cooled down slowly using liquid nitrogen, while stirring the wine continuously. The volatiles in the wine are gently transferred and frozen in the trap while the non-volatiles remain in the round-bottom flask. The trap is left in liquid nitrogen overnight to ensure complete transfer of the volatiles. Thereafter the vacuum is released using nitrogen gas and the system is then once again sealed. The volatile fraction, which is still frozen, is then melted in this inert atmosphere using a water bath and the non-volatile fraction (now in the form of a thick paste) is dissolved in 10 ml water.

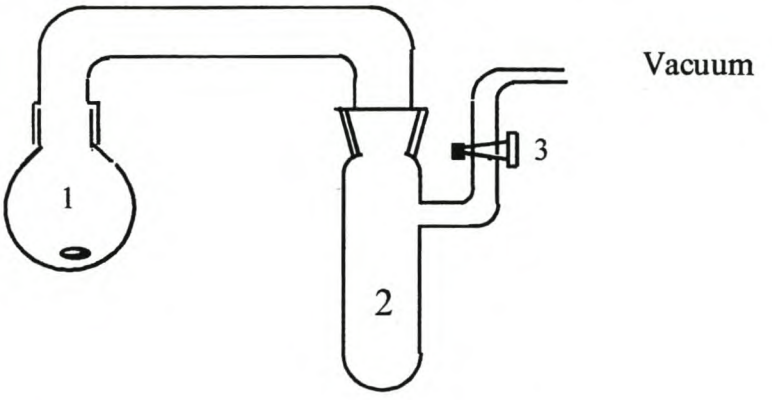

Figure 18: Experimental set-up for the separation of wine volatiles and non-volatiles.

The volatile fraction consists of water, ethanol and the volatile flavour components while all the polyphenols remain in the non-volatile fraction. The volatile fraction is ideally suited for analysis by GC, while the non-volatiles can be analysed by HPLC or CE. This sample preparation procedure is particularly useful when complete analysis of wine is the aim. However, when only interested in the polyphenols this rather time-consuming procedure may be considered unnecessary, since the non-volatile fraction virtually resembles un-extracted wine in this regard (figure 19).

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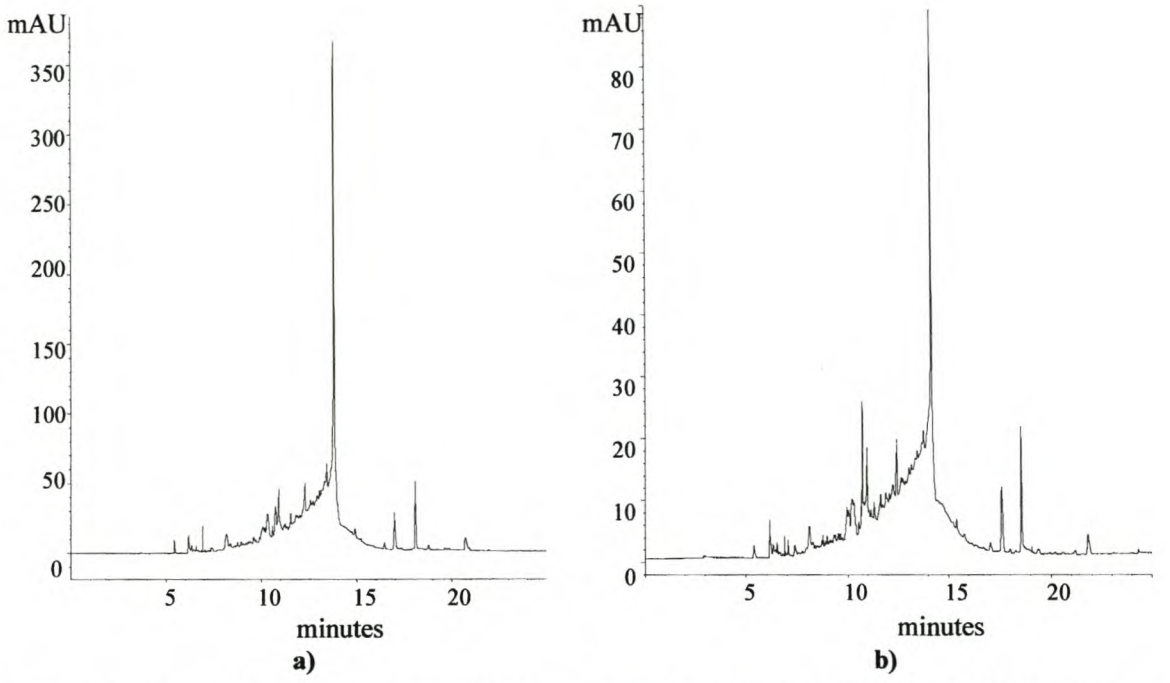

Figure 19: a) CZE injection of the non-volatile fraction of Simonsvlei Pinotage and b) direct injection of Simonsvlei Pinotage. Buffer: 100 mM borate, pH 9.3. Temperature: 30°C. Other conditions as in figure 14.

4.1.5 Storage of Wine Samples

The reactivity of the polyphenols necessitates that wine samples be stored in an inert atmosphere and that contact with atmospheric oxygen be kept at a minimum. This means that if a wine sample representative of a specific bottle is to be analysed, it either has to be taken out of a freshly opened bottle, or from well stored samples. To achieve this, opened wine bottles were transferred to 100 ml amber bottles by displacement of the wine using pressurised nitrogen gas. This also limits contact with oxygen. The bottles were completely filled so that as little air as possible was trapped. They were consequently stored at 4°C. The same constraints apply to the storage of standard solutions. Since oxidation of polyphenols occurs more rapidly at higher pH values, samples for analysis by CE can't be stored in running buffer. The alkaline oxidation of gallic acid has been discussed previously (section 2.5). It was found that the products formed were all highly negatively charged species such as a hexahydroxydiphenic dimer, which can undergo further oxidation resulting in a dihydroxyhexaphenic dimer [13]. This

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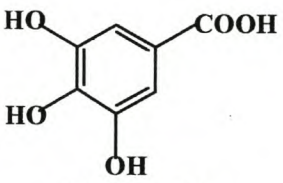
alters not only the electrophoretic mobility, but also the polar character of the molecule so that the amount of gallic acid detected by CE and HPLC would be misleading. This has been observed in the case of an oxidized gallic acid sample, where no peak was detected. Presumably this is because the electrophoretic mobility of the oxidation products is too great for these compounds to be detected at the cathodic end of the capillary.

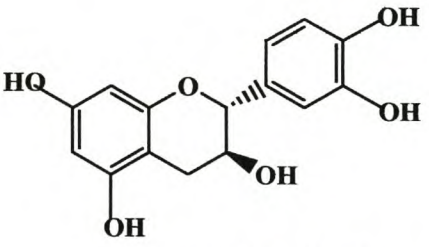
4.2 Capillary Electrophoresis Results

4.2.1 Introduction

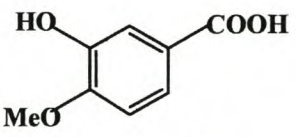
The experimental conditions in CZE entailed the use of a borate buffer prepared simply by dissolving the correct amount of sodium tetraborate in water. The pH of the resultant buffer was left unadjusted at 9.25, the pKa value of borate. Prior to each analysis, unless otherwise specified, the capillary was flushed with 0.1 M NaOH for 4 minutes, followed by deionised water for 4 minutes and finally by the running buffer for 4 minutes. All analyses, unless otherwise specified, were performed at 30 °C.



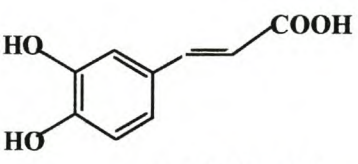
Gallic acid



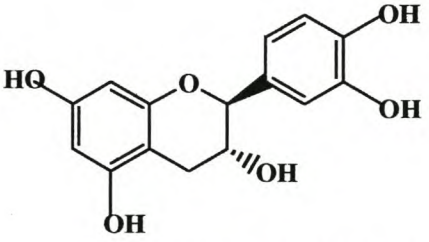
(+)-Catechin hydrate



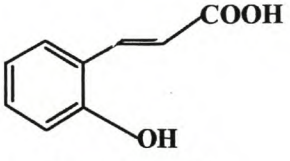
Vanillic acid



Caffeic acid



(-)-Epicatechin hydrate



o-coumaric acid

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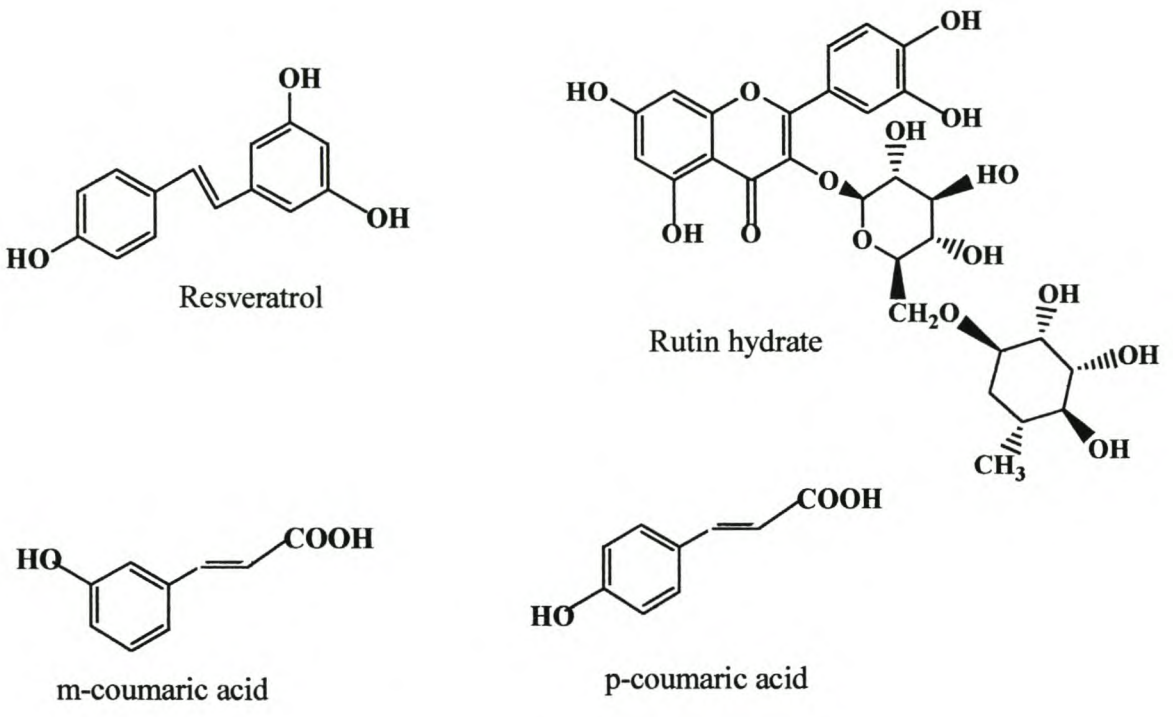

Figure 20: Structures of the phenolic compound used as standards.

Under these experimental conditions the phenolic acids, which possess moderately acidic carboxylic residues, are completely ionised, while the weakly acidic hydroxyl groups of the other phenolic compounds were only about 50% ionised because their pKa values vary between 9.5 and 10.5. Their electrophoretic migration is thus in the direction of the anode. However, the main driving force under these conditions is the EOF in the direction of the cathode, resulting in the migration of these compounds to the cathodic end of the capillary

At pH 9.3 tetrahydroxyborate ($B[OH]_4^-$) and related polyanion species form complexes with the *ortho*-dihydroxyl groups of the polyphenols. This increases the mass of the molecule, but more importantly, the charge is increased resulting in a net increase in the electrophoretic mobility. The concentrations of these complexes are directly proportional to the borate concentration, and increases with the pH since the concentration of alkaline borate anions is dependent on pH [14].

The effect of this complexation was studied in the first place by using the standards m-coumaric acid and caffeic acid (figure 20). Since *m*-coumaric acid lacks an *ortho*-

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dihydroxyl functional group compared to caffeic acid, it was expected that borate would only form a complex with the latter. To test the effect of the complexation on the separation, these samples were analysed using a borate at pH 9.2 and a non-complexing buffer, Bicine, at pH 8.9. The results are shown in figures 21 and 22.

It is evident that the electrophoretic mobility of caffeic acid is increased significantly in the borate buffer, when compared to its mobility in Bicine. The electrophoretic mobilities of these two acids in each of the buffers (table 2) were calculated using the following equations:

$$\mu_a = \mu_e + \mu_{eof}, \text{ and} \tag{15}$$

$$\mu_a = \frac{lL}{tV} \tag{16}$$

where μ_a is the apparent mobility, μ_{eof} the electroosmotic mobility and μ_e the electrophoretic mobility of each compound, while l is the effective and L the total length of the capillary, t is the migration time in seconds and V the applied voltage.

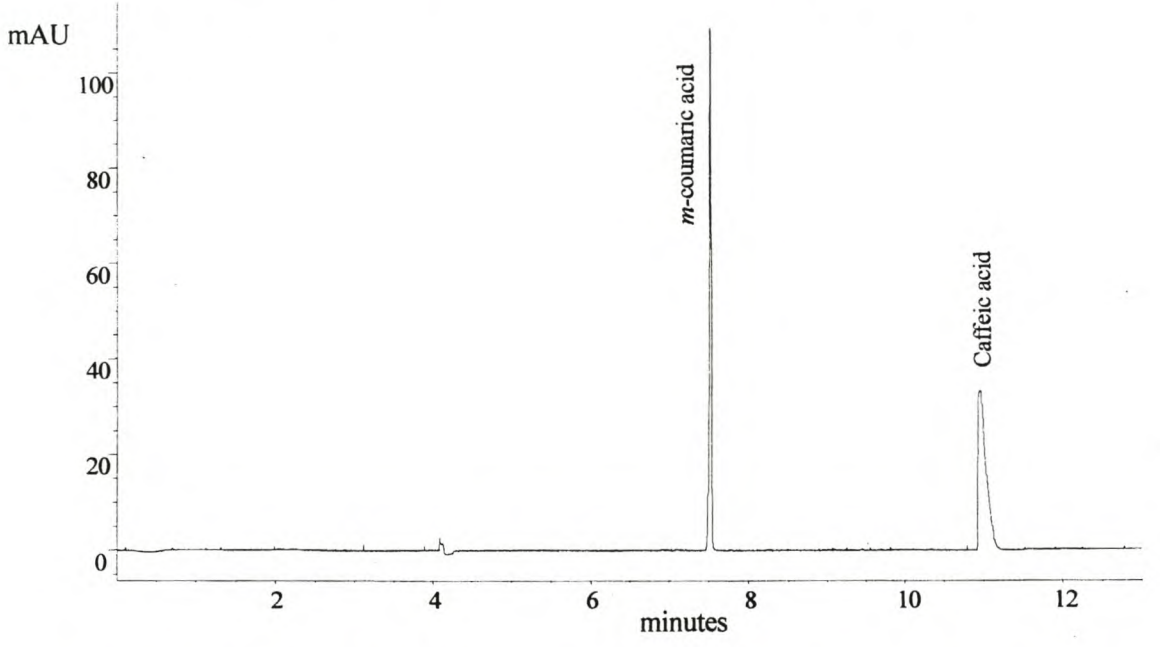

Figure 21: CZE separation of a 100ppm solution of *m*-coumaric acid and caffeic acid to study the effect of borate complexing. Capillary: 70.5 cm effective length, 75 μm i.d. Buffer: 100 mM borate, pH 9.3. Injection: hydrodynamic, 50 mbar for 3 seconds. Detection: 280 nm. Voltage: 30 kV. Temperature: 20°C.

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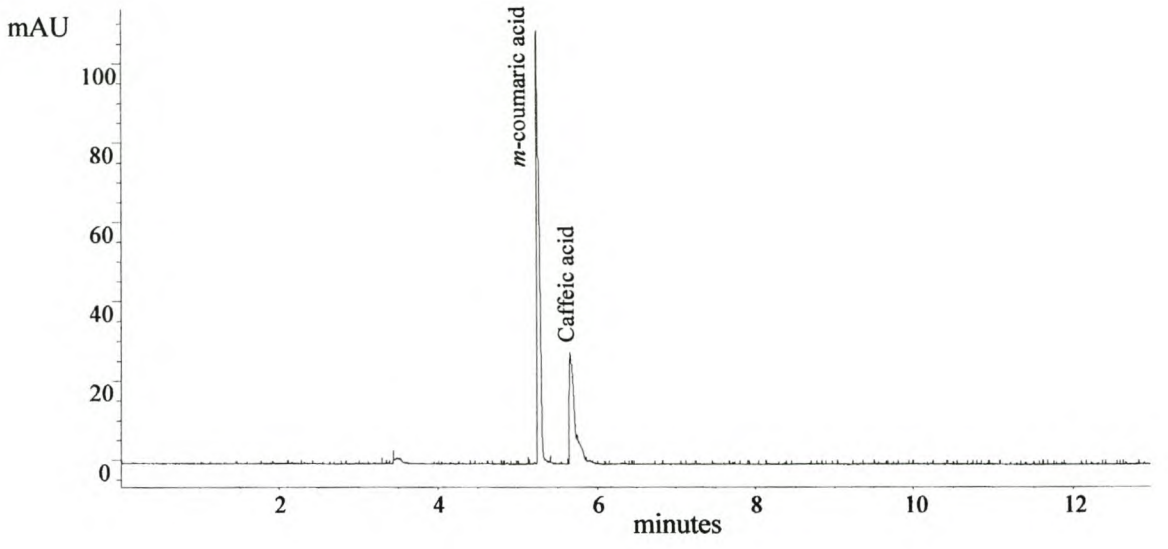

Figure 22: CZE separation of *m*-coumaric acid and caffeic acid to study the effect of borate complexing. Buffer: 20 mM Bicine, pH 8.9. Other conditions as in figure 21.

	Caffeic acid		*m*- coumaric acid	
Buffer	μ_a (cm^2/Vs)	μ_e (cm^2/Vs)	μ_a (cm^2/Vs)	μ_e (cm^2/Vs)
Bicine	5.46E-04	-3.55E-04	5.89E-04	-3.10E-04
Borate	2.82E-04	-4.73E-04	4.11E-04	-3.44E-04

Table 2: Comparison between the electrophoretic mobilities of *m*-coumaric- and caffeic acid in Bicine and borate buffers to illustrate the effect of borate complexation.

In these calculations, an EOF marker (MeOH) was used to determine μ_{eof}, while the migration time of each of the peaks was used to calculate μ_a according to equation 16. These values were substituted into equation 15 to determine the electrophoretic mobilities. The apparent mobilities are positive because the net migration of these molecules is in the direction of the EOF, while the electrophoretic mobilities are in the opposite direction. From table 2 it can be seen that the electrophoretic mobility of *m*-coumaric acid also increased slightly, contrary to what was expected. This shift appears large enough not to be caused by variable migration times. It seems then, as if some

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complexation occurs even in the case of *m*-coumaric acid (although the shift might also be due to the slight increase in the pH in the case of the borate buffer).

It was concluded that the effect of borate complexation on the electrophoretic mobilities of polyphenols is essential for their successful separation, especially since many of these molecules are structurally closely related and, without this added distinction, would not be separated. This proved an important factor in attempted CE-MS analysis of polyphenols, since the separation of the polyphenols would not be possible using a non-complexing volatile buffer such as ammonium acetate.

The effect of borate concentration on the complexation of these two standards was then investigated. Borate buffers of different concentrations (20, 50, 100 and 200 mM) were prepared and the effective mobilities calculated with the above-cited equations. The results are tabulated in table 3. The decrease in the apparent mobilities seems out of proportion relative to the increase in effective mobilities. This is, however, a consequence of the fact that an increase in the ionic strength leads to a compression of the double-layer at the capillary wall, a concomitant decrease in the zeta potential and thus the EOF. It is also apparent that the effective mobility of *m*-coumaric acid does in fact increase with an increase of the buffer concentration, indicating that some borate complexation may indeed occur.

	Caffeic acid		*m*-coumaric acid	
Borate concentration	μ_a (cm^2/Vs)	μ_e (cm^2/Vs)	μ_a (cm^2/Vs)	μ_e (cm^2/Vs)
200 mM	2.02E-04	-5.21E-04	3.04E-04	-4.19E-04
100 mM	2.91E-04	-4.72E-04	4.00E-04	-3.63E-04
50 mM	3.73E-04	-4.63E-04	4.85E-04	-3.51E-04
20 mM	4.91E-04	-4.70E-04	6.13E-04	-3.49E-04

Table 3: The effect of borate concentration on the mobilities of caffeic acid and *m*-coumaric acid.

Previous work done on borate-sugar complexes using cyclic and acyclic carbohydrates suggested that (a) polyols can form 1:1 and 1:2 complexes with borate, (b) hydroxyl

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groups on adjacent carbon atoms are mostly involved in complexation and (c) stabilisation of the complexes is increased with an increasing number of hydroxyl groups [14]. It seems obvious that borate can interact in a variety of ways with phenolic compounds. Additionally, in the pH range of these experiments, more highly condensed polyanions such as triborate $[B_3O_3(OH)_5]]^{2-}$ and tetraborate $[B_4O_5(OH)_4]^{3-}$ exist in the buffer solution, and may also take part in complexation reactions. In the light of especially point (a) it seems possible that two *m*-coumaric acid molecules might form complexes with any of these polyanions in the same manner as applies to caffeic acid, although most likely these complexes are less stable than those formed with molecules possessing *ortho*-dihydroxyl groups. This would explain the relative increase in the electrophoretic mobilities of *m*-coumaric acid in the presence of a borate buffer.

4.2.2 CZE Separation of Standards

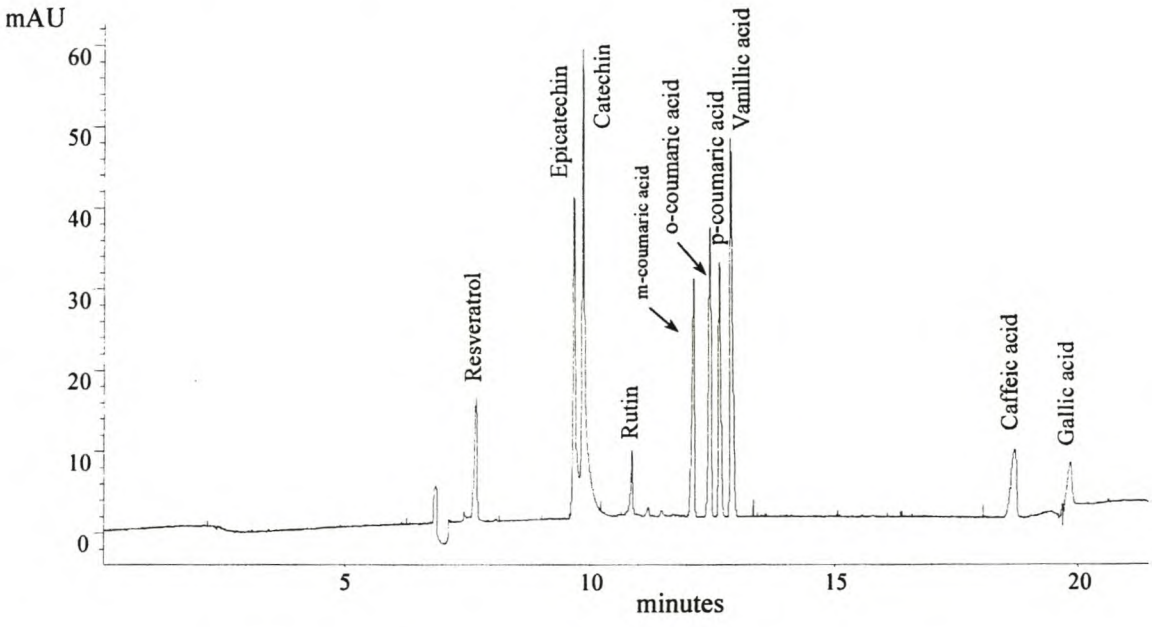

Figure 23: CZE separation of a standard solution of polyphenols (25 ppm in all cases, except Caffeic acid: 10 ppm and Gallic acid: 4 ppm). Capillary: 72 cm effective length, 75 μm i.d Buffer: 25 mM phosphate, 10 mM borate, pH 8.8. Injection: hydrodynamic, 50 mbar for 4 seconds. Detection: 206 nm. Voltage: 25 kV. Temperature: 20°C.

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In order to demonstrate the effective CZE separation of a relatively simple sample such as a standard solution (figure 20), the method described by Rossi *et al.* [7] for the separation of wine polyphenols was used. The result is depicted in figure 23. It is clear that this method is capable of successfully separating the standards within 20 minutes.

4.2.3 CZE Analysis of Wine and Ether Extract of Wine

In order to evaluate and compare the different operational modes of CE for the analysis of polyphenols it was decided to focus on two different samples of the same bottle of wine, namely an ether extract and direct injection of 1998 Simonsvlei Pinotage. Although the ether extract is not quantitatively representative of the wine, this sample was chosen for its relative simplicity, while direct injection was chosen to see which operational mode might resolve the bump caused by the polymeric polyphenols present in wine. To simplify experimental conditions, the buffer used henceforth was a 100 mM borate buffer, pH 9.3, and all experiments were performed at 30 °C.

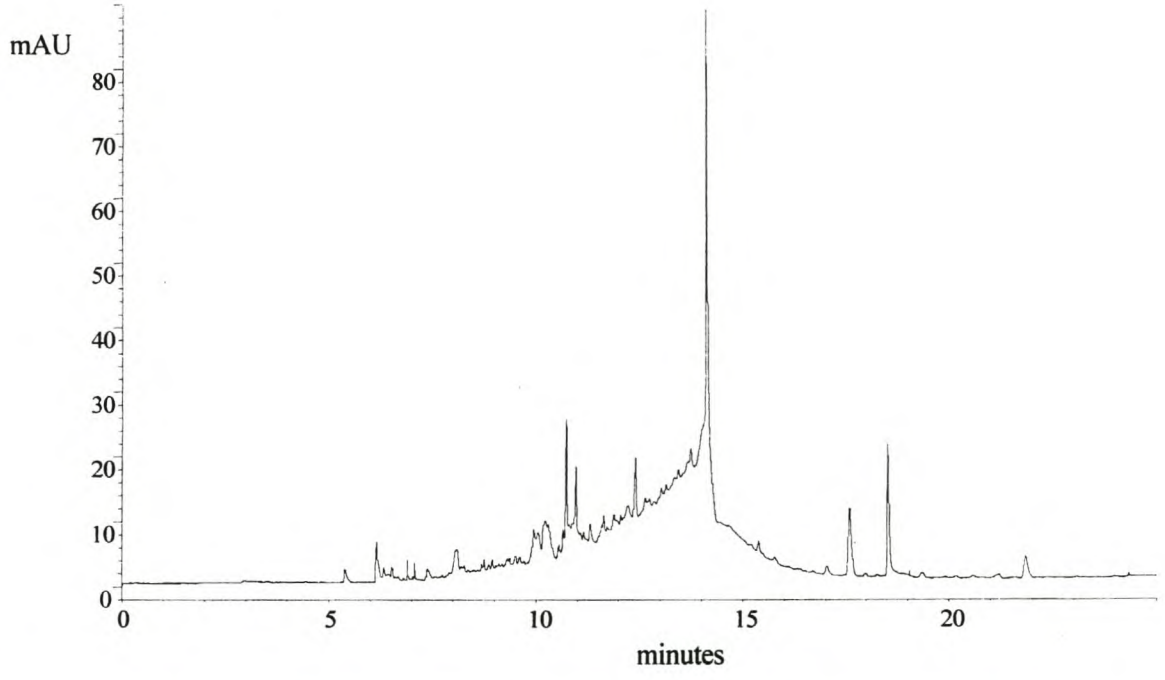

Figure 24: CZE analysis of a direct injection of Simonsvlei Pinotage. Capillary: 72 cm effective length, 75 μm i.d. Buffer: 100 mM Borate, pH 9.3. Injection: Hydrodynamic, 50 mbar for 4 seconds. Detection: 206 nm. Voltage: 25 kV. Temperature: 30°C.

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Direct wine injection (figure 24) clearly demonstrates the problems associated with the polymeric phenols in wine. The effect of sample stacking (section 3.2.5) was investigated by injecting a wine sample diluted in water (figure 25). While some sharpening due to the stacking effect can be observed, this did not solve the problem of the polymeric bump.

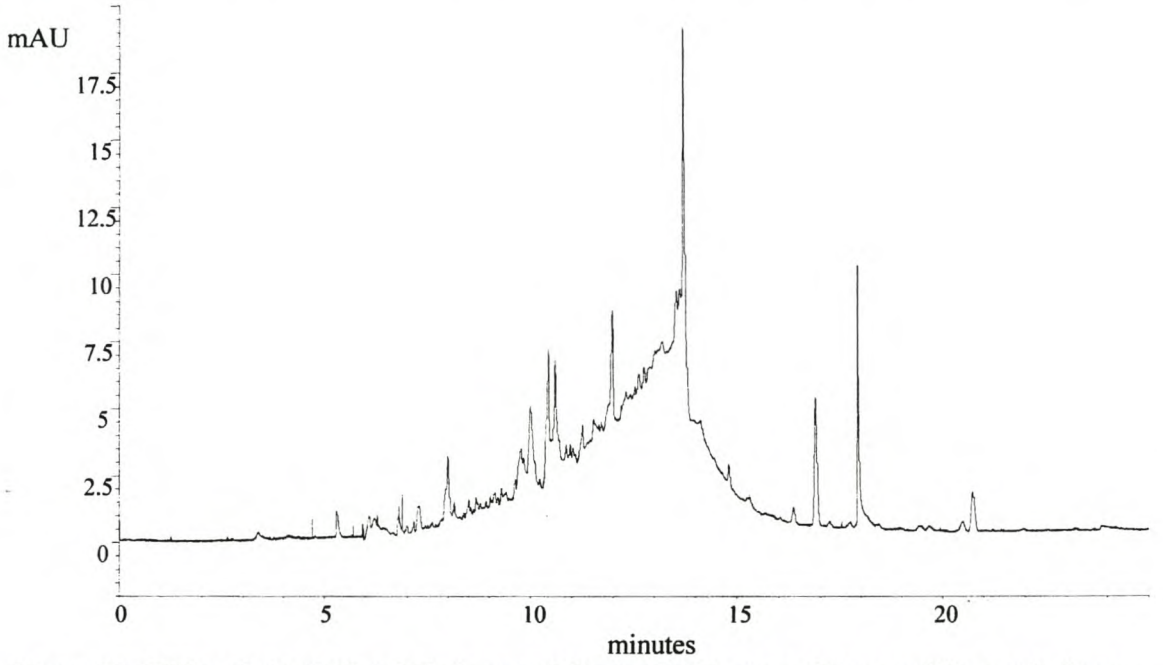

Figure 25: CZE analysis of diluted (5× in water) Simonsvlei Pinotage. Other conditions as in figure 24.

The results of the analysis of the ether extract together with the identified peaks can be seen in figure 26. Peaks were identified by spiking the sample with standards and comparison of the UV spectra. Some spectra representing the different classes are shown in figure 27. A default wavelength of 206 nm was chosen to detect all the different classes of phenolic compounds simultaneously. Catechin and epicatechin were not fully resolved under these conditions (10.8 min.), while *m*- and *o*-coumaric acid and rutin were either absent in the ether extract, or present in too low amounts to be identified.

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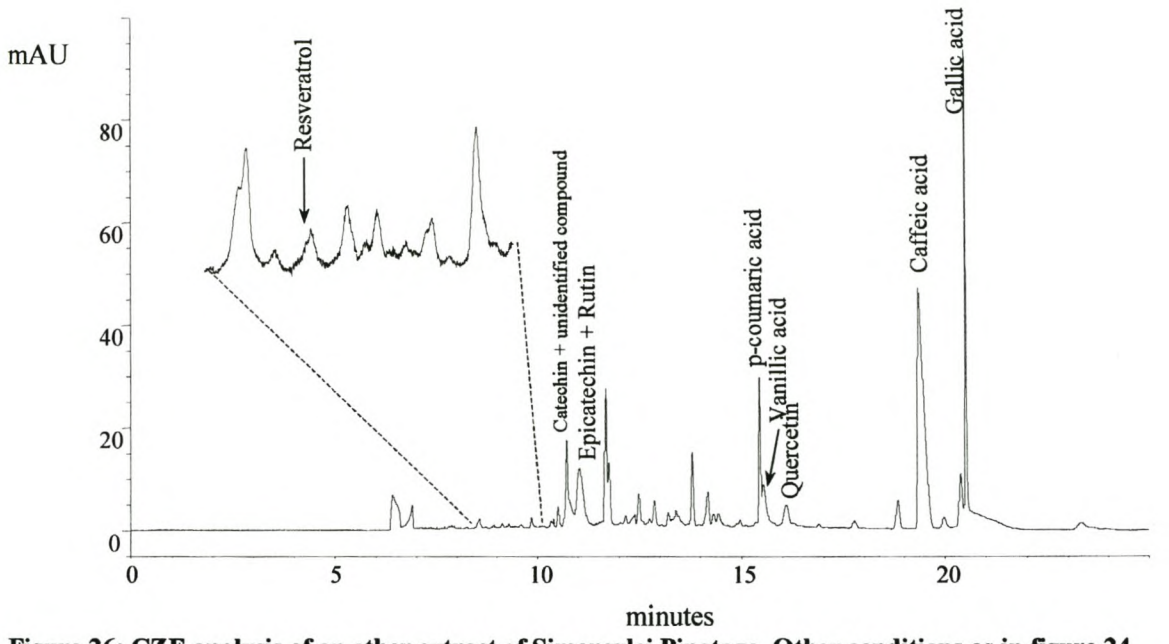

Figure 26: CZE analysis of an ether extract of Simonsvlei Pinotage. Other conditions as in figure 24.

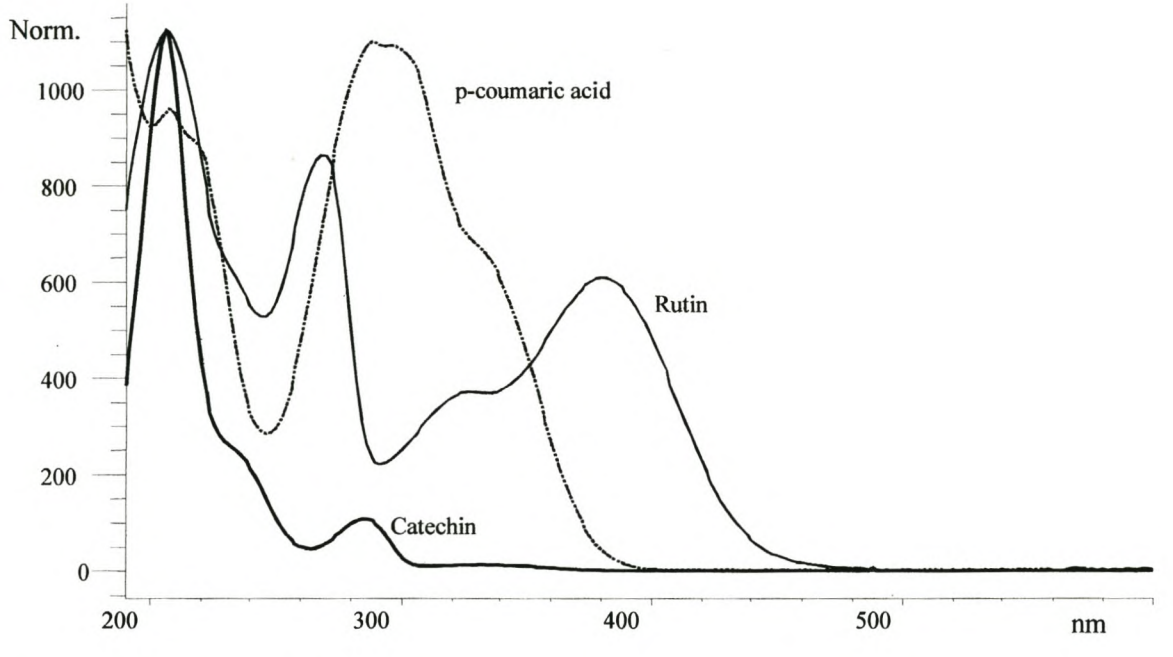

Figure 27: UV spectra of three classes of polyphenols present in wine: a flavanol (catechin), a flavonol (rutin) and a phenolic acid (p-coumaric acid).

Calibration curves for the identified peaks showed good linearity in the range 10-1000 ppm (figure 28). The results of the quantification of the identified standards, taking into

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account the recovery percentages mentioned in section 4.1.2, are presented in table 4, together with quantitative data reported in the literature for these compounds.

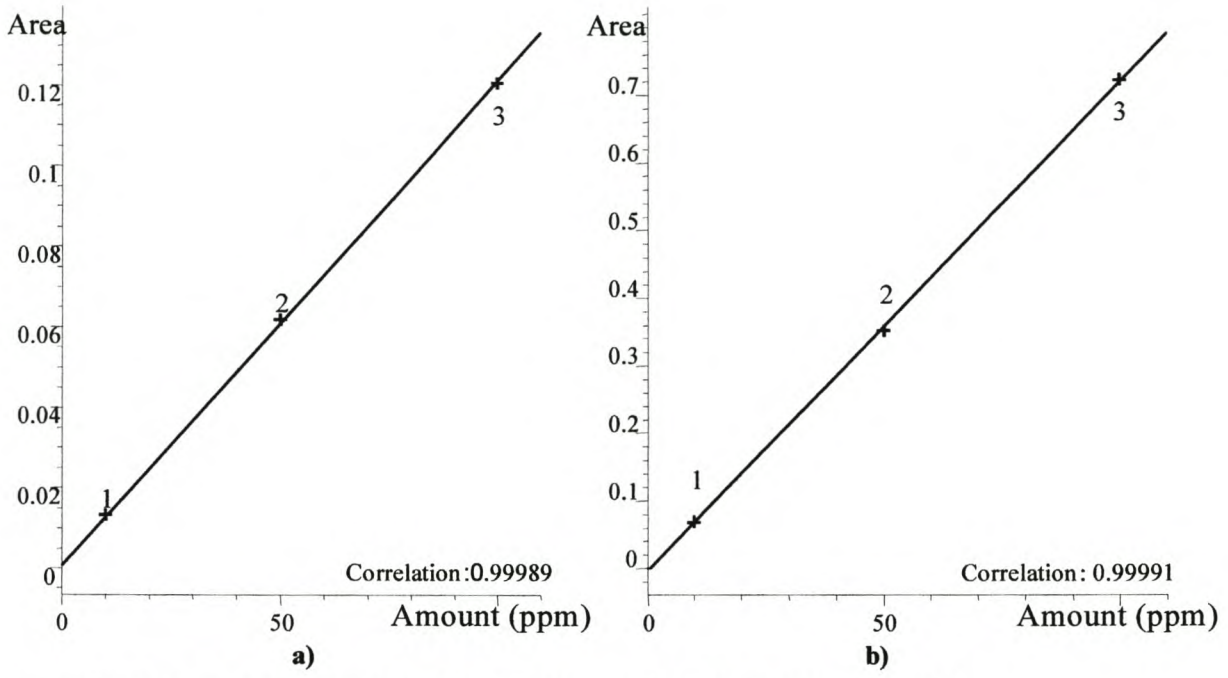

Figure 28: Calibration curves obtained by CE analysis of a) Rutin and b) Gallic acid.

Quantification data	Amount (ppm)	RSD%	n	Literature Amount(ppm) [ref.]		Correlation coefficient
resveratrol	1.63	3.17	4	1-12	[15]	0.9995
p-coumaric acid	10.21	5.17	4	1-8	[16,17]	0.9996
vanillic acid	6.84	8.26	4	2-5	[8,16]	0.9996
quercetin	7.89	6.68	4	0.5-20	[16,17]	0.9998
caffeic acid	55.49	4.46	4	2-12	[16]	0.9992
gallic acid	25.56	4.32	4	1-35	[8,16]	0.9999

Table 4: Quantitative results obtained from the ether extract of Simonsvlei Pinotage.

In conclusion, it has been shown that while some phenolic compounds present in wine can be separated and quantified using CZE, the complexity of the wine sample is such that extensive sample preparation is still needed for a satisfactory analysis. Moreover, an

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exhaustive qualitative and quantitative analysis of wine does not seem likely in a single-step method using CZE only.

4.2.4 Sensitivity in Capillary Electrophoresis

Sensitivity is a problem often associated with CE-UV analyses due to the limited pathlength provided by the internal diameter of the capillary. This phenomenon, together with the methods used to overcome it, have been discussed in section 3.3.1. In this study the sensitivities obtained in the case of a standard 75 μm capillary and a bubble cell capillary were compared in the analysis of an ether extract of wine (figure 29). The bubble cell capillary was purchased from Hewlett-Packard and was of the same length and internal diameter as the standard capillary. The bubble increases the pathlength of the detection cell by a factor of 2.7, and thus an increase in the sensitivity in the same order of magnitude was expected.

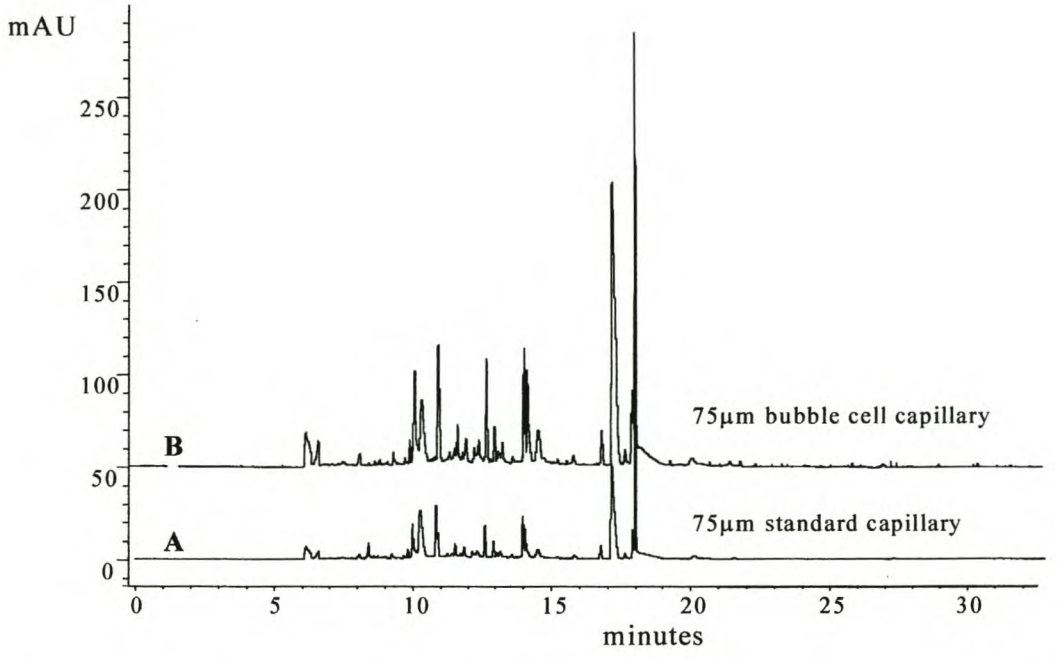

Figure 29: A comparison between the relative sensitivities obtained in the case of a standard capillary (A) and a bubble cell capillary (B). Sample: ether extract of Simonsvlei Pinotage. Capillary: 72 cm effective length, 75 μm i.d. in both cases. Other conditions as in figure 24.

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It is clear from figure 29 that an increase in sensitivity in the order of 2.5 times is obtained with the bubble cell capillary. Quantitative data reported in the literature indicates that even those polyphenols present in low concentrations, seldom occur at concentrations lower than 0.4 ppm. Rossi *et al.* [7] reported the limit of detection for a variety of wine phenols on a standard bare fused silica capillary as ranging between 0.015 and 0.3 ppm. In this study the limit of detection for a different set of standards was found to vary between 0.17 and 1.2 ppm when monitoring at the same wavelength (206 nm). Taking into account the increase in sensitivity afforded by the use of the bubble cell capillary, it seems as if the sensitivity of the CE analysis of wine polyphenols is sufficient to quantify these molecules.

An alternative approach to increase the sensitivity of CE analysis is to use a high sensitivity cell (section 3.3.1). In this device the path length of the detection cell is increased up to 1200 μm (a factor of 16). In this study we were unable to compare the sensitivity obtained with this cell to a standard capillary, since repeated leakage within the cell caused current breakdown. However, the use of these optical arrangements to increase sensitivity should dispel doubt about the sensitivity of CE-UV analysis of wine polyphenols.

4.2.5 Screening of Other CE Techniques Suitable for Wine Analysis

4.2.5.1 The Analysis of Wine and Ether Extract Using PVA Coated Capillaries

In PVA-coated capillaries the capillary wall is coated with neutral polyvinylalcohol, which eliminates the surface charge on the capillary wall and thus also the EOF (section 3.2.2.1). Under these conditions solute molecules are transported and separated due to their different electrophoretic mobilities. In the case of the analysis of wine polyphenols the solute molecules are negatively charged at the pH used. Under the electrophoretic conditions applied in the previous examples but now in the absence of an EOF these molecules will elute at the anode. Therefore the applied voltage has to be reversed in order to detect them.

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Qualitatively this means that the elution order on a PVA capillary should be the reverse of that observed on a standard bare fused silica capillary (figure 26). This is demonstrated in figure 30, where an ether extract of Simonsvlei Pinotage was hydrodynamically injected onto the PVA capillary. The identified peaks are indicated.

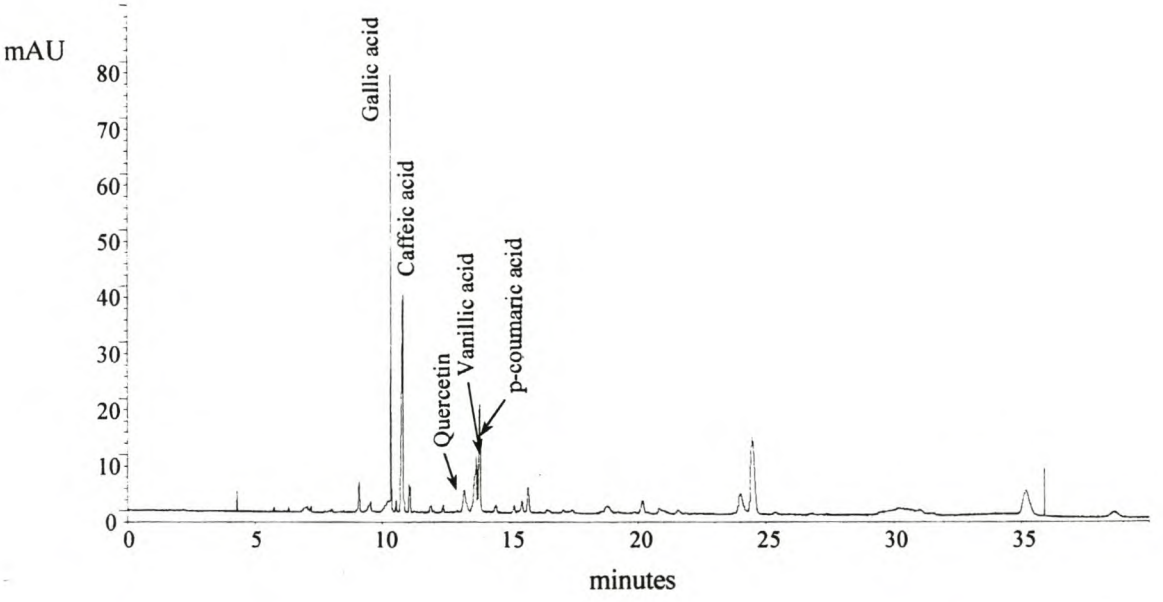

Figure 30: CZE analysis of an ether extract of Simonsvlei Pinotage on a PVA coated capillary. Capillary: PVA capillary, 56 cm effective length, 50 μm i.d. Buffer: 100 mM borate, pH 9.3. Injection: hydrodynamic, 50 mbar for 4 seconds. Detection: 206 nm. Voltage -25 kV. Temperature: 30°C.

This separation does not provide any new information when compared to the results obtained in the CZE run on a standard bare fused silica capillary. A disadvantage of this technique compared to the CZE analysis on a standard bare fused silica capillary is that, for the majority of the compounds, the migration times are longer. Besides the obvious drawback of longer analysis times, this also means that the later eluting peaks show more band broadening, resulting in a loss in detectability.

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In addition, direct hydrodynamic injection of wine results in an electropherogram complicated by the bump caused by polymeric phenols, as is the case on the bare fused silica capillary (figure 31a). However, when injecting electrokinetically (by applying a negative voltage for 12 seconds) the lack of EOF in the PVA-coated capillary results in loading of those solute molecules possessing negative charges. Thus more molecules with a large negative charge (such as the acids) are injected, compared to those molecules with little charge, such as the polymeric polyphenols. Figure 31 illustrates the differences between hydrodynamic- and electrokinetic injection.

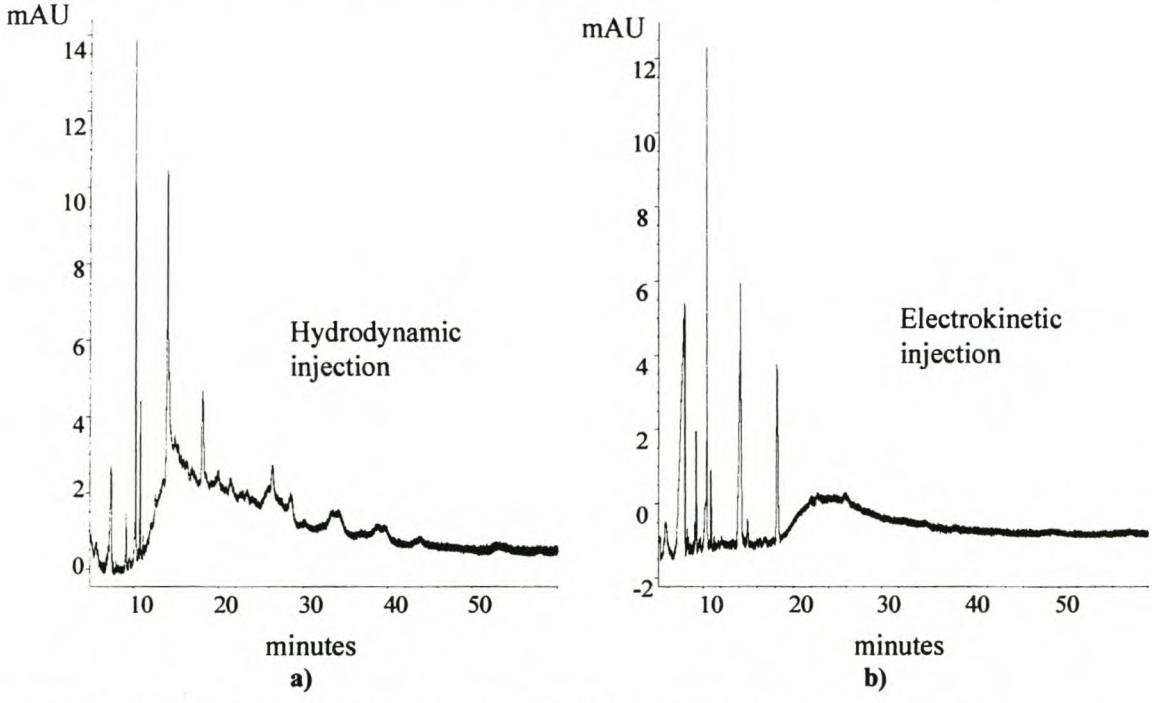

Figure 31: CZE analysis of Simonsvlei Pinotage on a PVA coated capillary. Injection: a) hydrodynamic, 50mbar for 4 seconds; b) electrokinetic: -25 kV for 12seconds. Other conditions as in figure 30.

In the case of electrokinetic injection the wine was diluted in buffer to provide the polyphenols with an additional charge through complexation and deprotonation. An alternative is to increase the pH of the wine sample prior to injection in order to ensure more negative charges on the analyte molecules. However, it seems as if the polymeric polyphenols do not form complexes to a significant degree, since they are not effectively injected. This is unexpected since it is obvious from the CZE analyses of wine that these polymers possess some charge because they elute some time after the EOF (figure 24).

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The fact that the amount of sample loaded was so small, especially in the case of the polymers, might also be due to degradation of the coating on the PVA capillary. This would result in a small negative charge on the uncoated capillary wall (at pH 9.3) and thus the development of an electroosmotic flow in the direction of the cathode.

In subsequent runs it was observed that the migration times of analytes did in fact increase, indicating the development of an EOF. This EOF would be in the opposite direction than the electrophoretic migration of the negative analyte molecules, thus preventing those with a small charge from being injected onto the capillary. The effect of electrokinetic injection of a wine sample will have to be investigated on a fresh PVA capillary.

4.2.5.2 The Application of Micellar Electrokinetic Chromatography to the Analysis of Wine

The aim of this study was to determine the influence of micelles on the separation of polyphenols in wine, especially since so much of the work done on the analysis of these compounds using CE has been done in this mode [18-22]. The two surfactants most commonly used in these studies, SDS and the bile salt sodium deoxycholate, were each dissolved in a 100 mM borate buffer and the analysis performed on a standard bare fused silica capillary. The application of SDS and of the bile salts to MEKC separations has been discussed in section 3.2.2.2. The electropherograms for the ether extract and non-volatile fraction using 50 mM SDS and of the ether extract using 50 mM sodium deoxycholate are presented in figures 32 and 33, and figure 34, respectively.

The surfactant was added at a concentration significantly higher than the critical micellar concentration (CMC), which is 6.4 mM in the case of sodium deoxycholate and 8.1 mM in the case of SDS. The magnitude of the EOF was slightly reduced relative to that of a standard borate buffer in both cases, resulting in extended migration times. This is caused by changes in the ionic strength and viscosity of the buffers due to the addition of the surfactants. It is clear that no significant changes in elution order occur in either case. In

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fact, the only significant shift observed in the case of the SDS buffer was that of resveratrol, the migration time of which changed from ~9 minutes in the case of a borate buffer to ~16 minutes with the use of an SDS buffer. This can be explained in terms of the relative apolar character of resveratrol compared to most other phenolic compounds (figure 20).

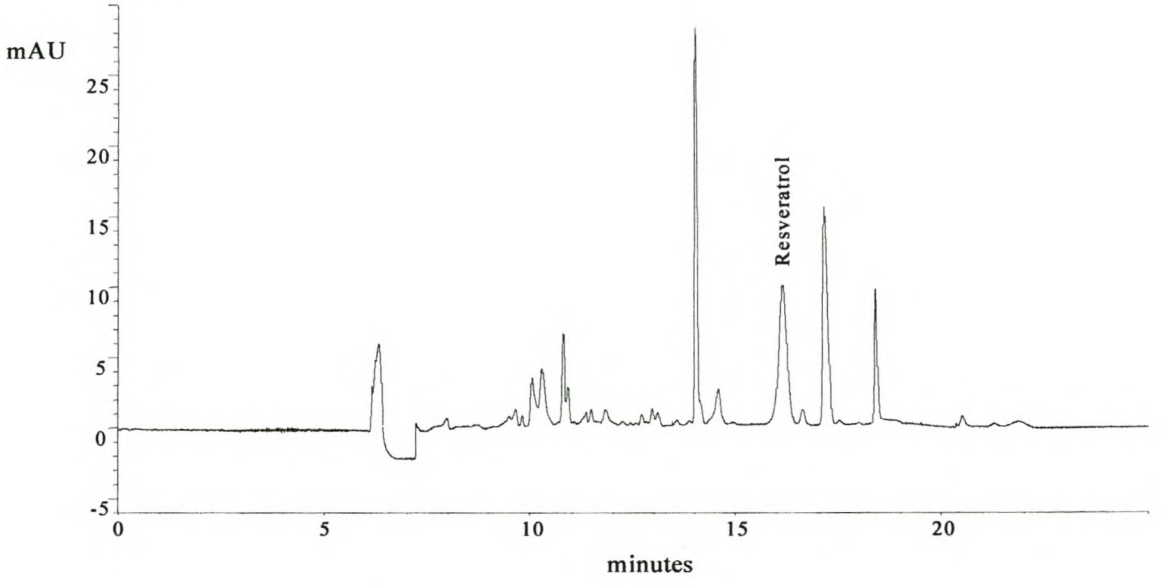

Figure 32: MEKC analysis of an ether extract of Simonsvlei Pinotage spiked with resveratrol. Capillary: bare fused silica, 72 cm effective length, 75 μm i.d. Buffer: 100 mM borate, 50 mM SDS, pH 9.3. Injection: hydrodynamic, 50 mbar for 4 seconds. Detection: 206 nm. Voltage: 25 kV. Temperature: 30°C.

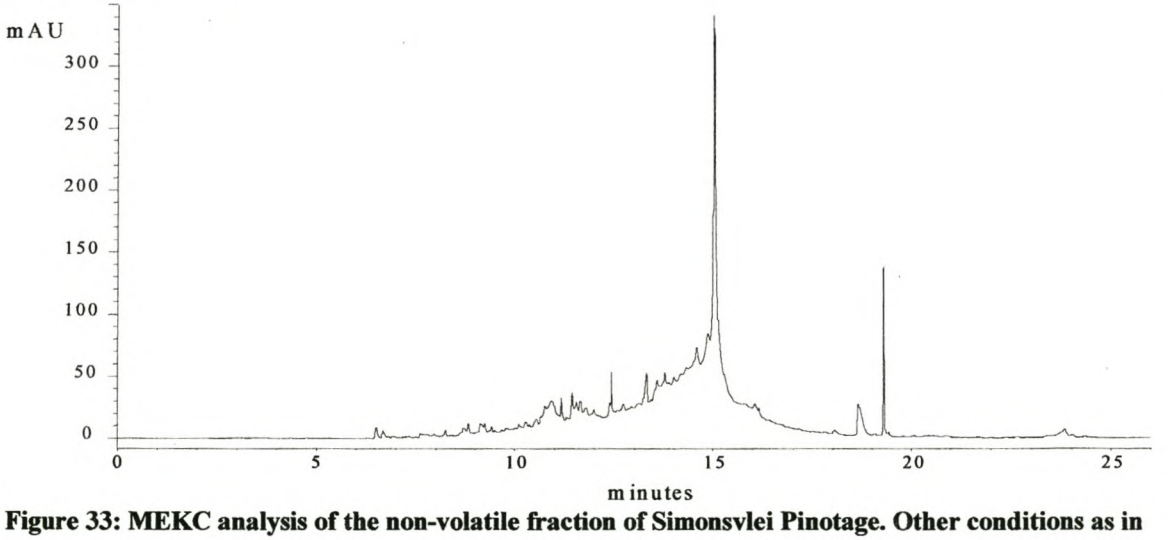

Figure 33: MEKC analysis of the non-volatile fraction of Simonsvlei Pinotage. Other conditions as in figure 32.

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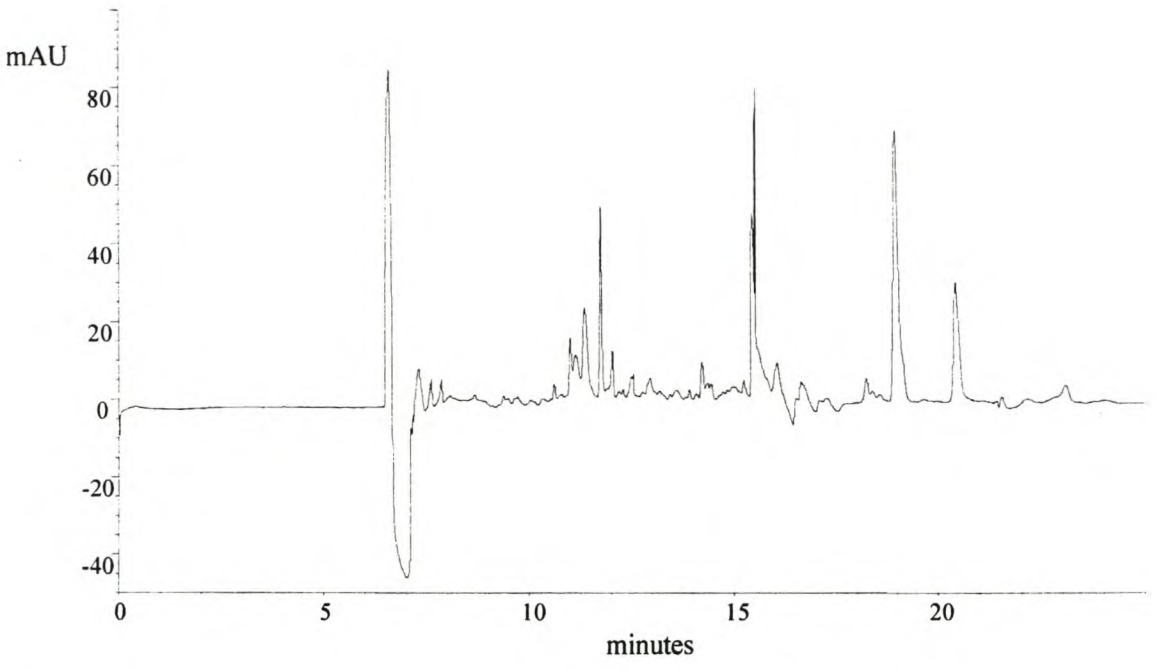

Figure 34: MEKC analysis of an ether extract of Simonsvlei Pinotage. Buffer: 100 mM borate, 50 mM sodium deoxycholate, pH 9.3. Other conditions as in figure 32.

It would seem that the other phenolic compounds are too polar to interact to any significant degree with the hydrophobic interiors of the micelles. This poses the question of why so much work on the analysis of polyphenols has been done using MEKC, since the elution order of most of these compounds is not altered by the use of surfactants, while their migration times do increase in the presence of the surfactants. The use of surfactants also does not provide a solution to the problem of the bump caused by the polymeric polyphenols. Moreover, the marriage of MEKC with MS is not realistic.

4.2.5.3 The Application of Capillary Gel Electrophoresis to the Analysis of Wine

The use of non-cross-linked polymers in capillary gel electrophoresis has been discussed in section 3.2.2.3. Additionally, the use of the EOF to fill the capillary with viscous polymer solutions according to the method introduced by Chang *et al.* [23] has been mentioned. CGE was chosen as a CE mode of operation that might resolve the bump caused by polymeric polyphenols in wine and because of the additional separation possibility it offers of separating molecules according to size. In this study, the use of

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polyethylene oxide (PEO) solutions for the analysis of wine samples was investigated. The polymer solutions (1.5 % and 1 % PEO in buffer) were prepared by dissolving the appropriate amount of PEO (MW 900,000) in a borate buffer on a stir heater at 80-90 °C, while stirring continuously to ensure a well-homogenised solution. Helium gas was then bubbled through the solutions, placed in an ultrasonic bath, to eliminate air bubbles. Prior to each analysis the capillary was flushed extensively with 1 M sodium hydroxide to remove polymer solutions and refresh the capillary wall, and thus to ensure a substantial EOF on the bare silica capillary. The sample was hydrodynamically injected by applying 50 mbar pressure for 6 seconds, or electrokinetically injected by applying 15 kV for 10 seconds, followed by a hydrodynamic injection of a plug of the borate buffer. The polymer solution was then placed at the inlet of the capillary, and a positive voltage of 25 kV was applied, ensuring the migration of the negatively charged polyphenols in the direction of the cathode due to polymer migration in that direction under the influence of the EOF. In this mode the larger polyphenols will be hindered to a greater extent in terms of their electrophoretic migration towards the anode by the polymer solution, and thus a decrease in their migration times is expected.

The effect of this sieving character can be seen in figure 35. The bump caused by the polymeric polyphenols, while still present, is spread out and at least partially resolved. The shift in the baseline at ~7.5 minutes can be ascribed to the polymer solution absorbing fractionally more than the borate buffer. The identified peaks are indicated in figure 35. A comparison between hydrodynamic and electrokinetic injection of wine samples illustrates that there is no significant bias in either case (figures 35 and 36). This is because the solute molecules are loaded by the substantial EOF in the case of electrokinetic injection, resulting in comparable fractions being loaded onto the capillary. The effect of an increase in the PEO concentration from 1.0 % to 1.5 % v/v (and thus also an increase in the viscosity of the polymer solution) can be seen in figures 37 a and b. As was expected, an increase in viscosity shortens the migration times of all the polyphenols. It appears as if a compromise between shorter analysis times (higher viscosity solutions) and better resolution (lower viscosity solutions) has to be found.

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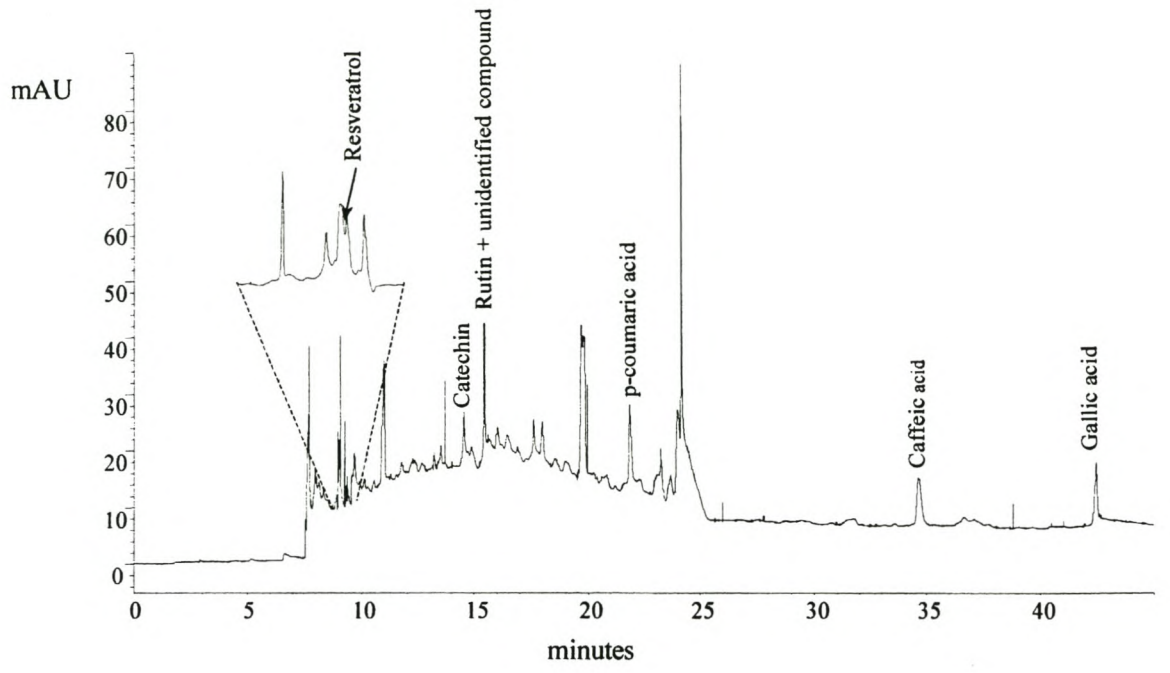

Figure 35: Direct CGE analysis of Simonsvlei Pinotage. Capillary: bare fused silica, 72 cm effective length, 75 μm i.d. Buffer: 100 mM borate, 1 % PEO, pH 9.3. Injection: hydrodynamic, 50 mbar for 6 seconds. Detection: 206 nm. Voltage: 25 kV. Temperature: 30°C.

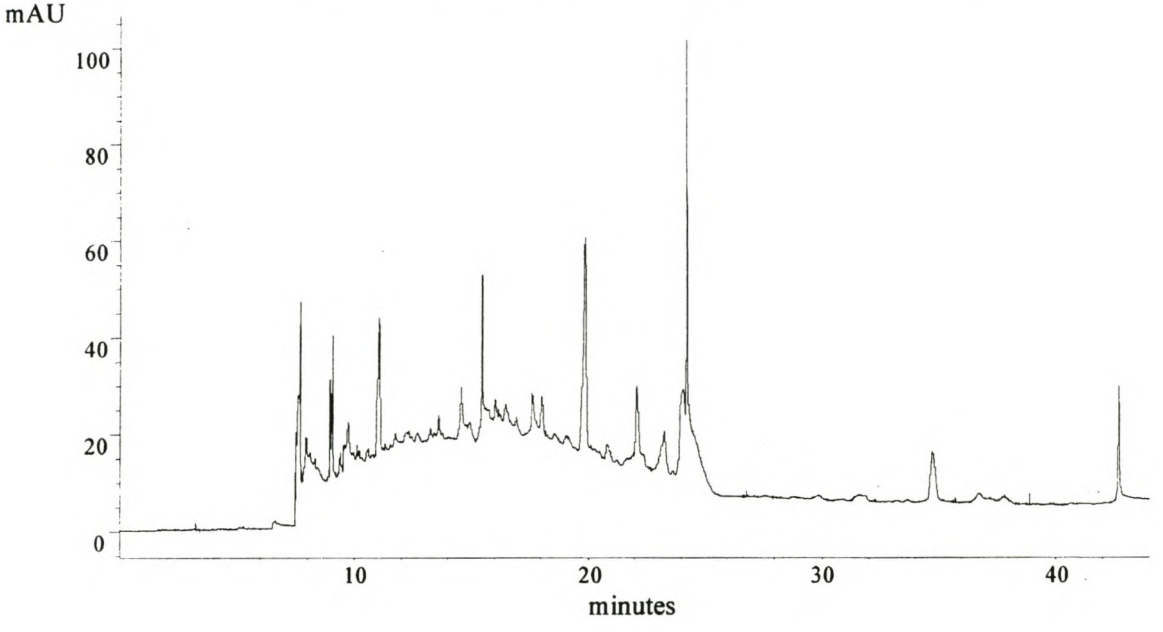

Figure 36: Direct CGE analysis of Simonsvlei Pinotage (pH adjusted to 9.5 to ensure that the polyphenols are charged). Injection: electrokinetic, 15 kV for 10 seconds. Other conditions as in figure 35.

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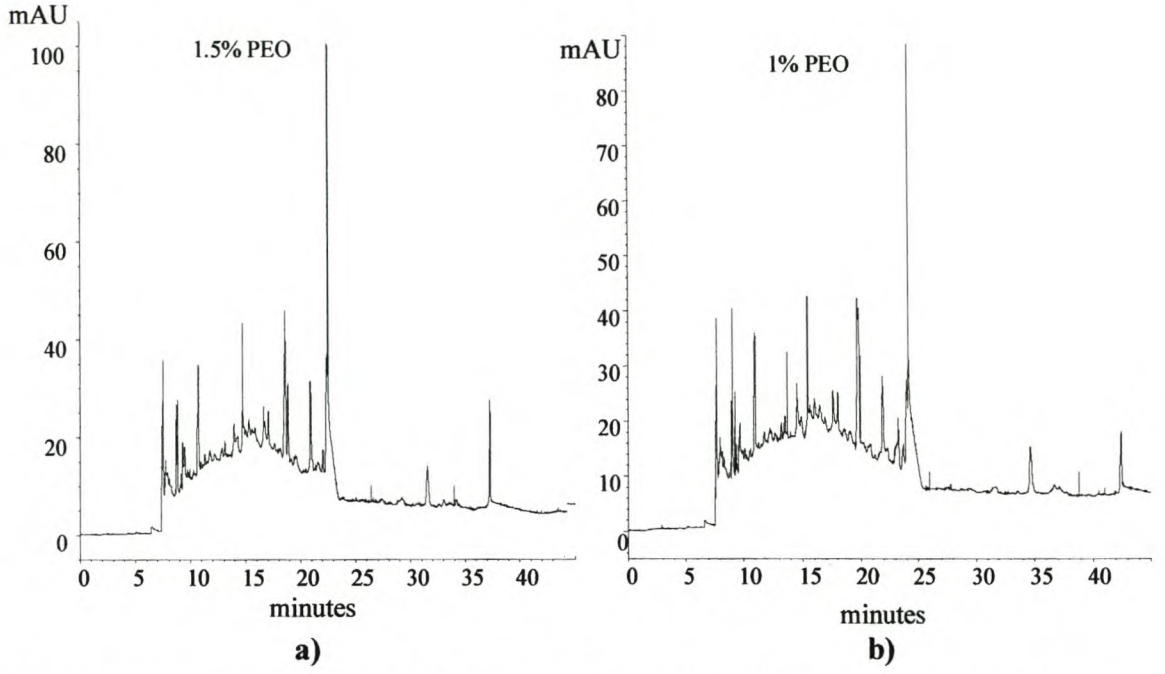

Figure 37: CGE analysis of Simonsvlei Pinotage. Buffer: a) 100 mM borate, 1.5 % PEO, pH 9.3; and b) 100 mM borate, 1 % PEO, pH 9.3. Other conditions as in figure 35.

Although the separation under these conditions is not optimal, adjustment of such parameters as the polymer type and concentration, buffer type and temperature may lead to better results. This study has only shown how a CGE method involving the use of polymers solutions might, when optimised, be used to analyse wine samples without a necessary sample preparation step.

3.3 High-Performance Liquid Chromatography Results

In the light of the amount of work done on the analysis of wine polyphenols using HPLC, the aim of this study was not an exhaustive investigation into the possibilities this technique offers, but rather a short comparative study to determine the advantages and shortcomings of HPLC when compared to CE techniques. Reversed-phase HPLC analysis of wine polyphenols has been discussed in section 3.1. Because this is such a well-established technique, a method for this analysis was selected on the basis of some studies described in the literature. The mobile phases used were acetonitrile (B) and

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water, both with 1 % trifluoro-acetic acid (TFA)(A). An adequate gradient was relatively easily obtained using these solvents (table 5).

Under these conditions, the chromatograms and identified peaks for a standard sample solution and for direct analysis of wine are depicted in figures 38 and 39, respectively. It is apparent that the standards are well separated using this relatively simple gradient, but also that the polymeric polyphenols complicate the chromatogram in the case of direct wine injection.

Time (minutes)	%B	Curve
0	5	-
15	15	6
40	45	6

Table 5: The gradient used for HPLC-UV analysis of wine polyphenols.

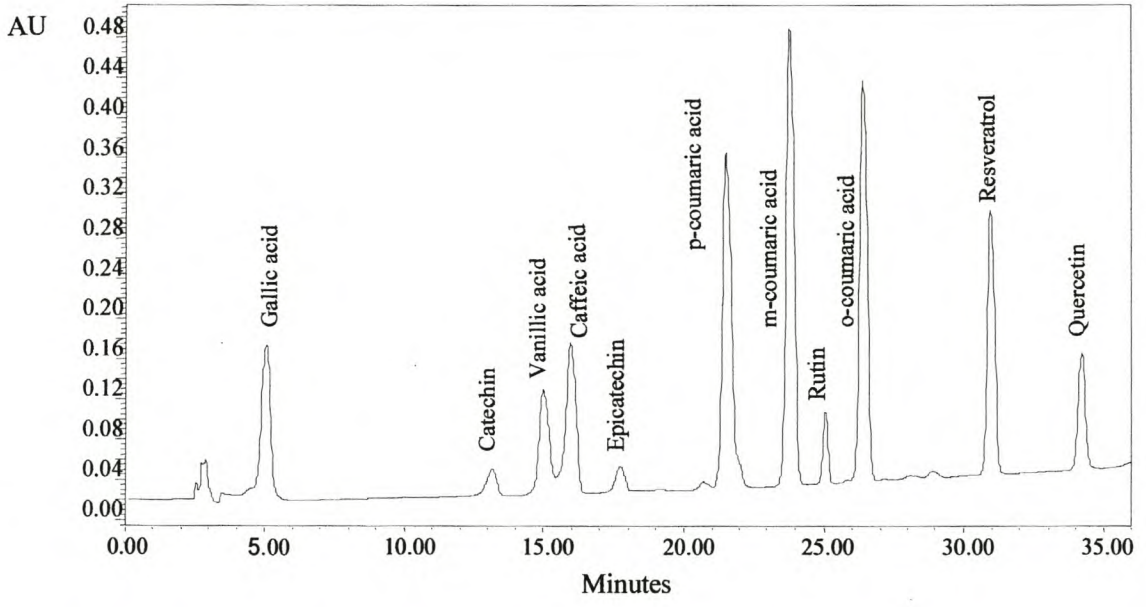

Figure 38: Reversed-phase HPLC (RPLC) separation of a standard solution of polyphenols (~50 ppm). Column: Macherey-Nagel C18, 250 L ×4 mm i.d., 5 μm d_p. Injection volume: 30 μL of standards dissolved in methanol. Gradient details are summarised in table 5. Detection: 280 nm. Temperature: 30°C.

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As was the case in the CZE analysis of wine, *o*- and *m*-coumaric acid are present in too low amounts to be identified. Catechin, epicatechin and rutin were easily separated in the HPLC analysis, in contrast to the CZE analysis although it has been shown that these compounds can be satisfactorily separated using CZE (section 4.2.2). Resveratrol, although also present in small amounts, seems to be better separated in the CZE analysis of wine.

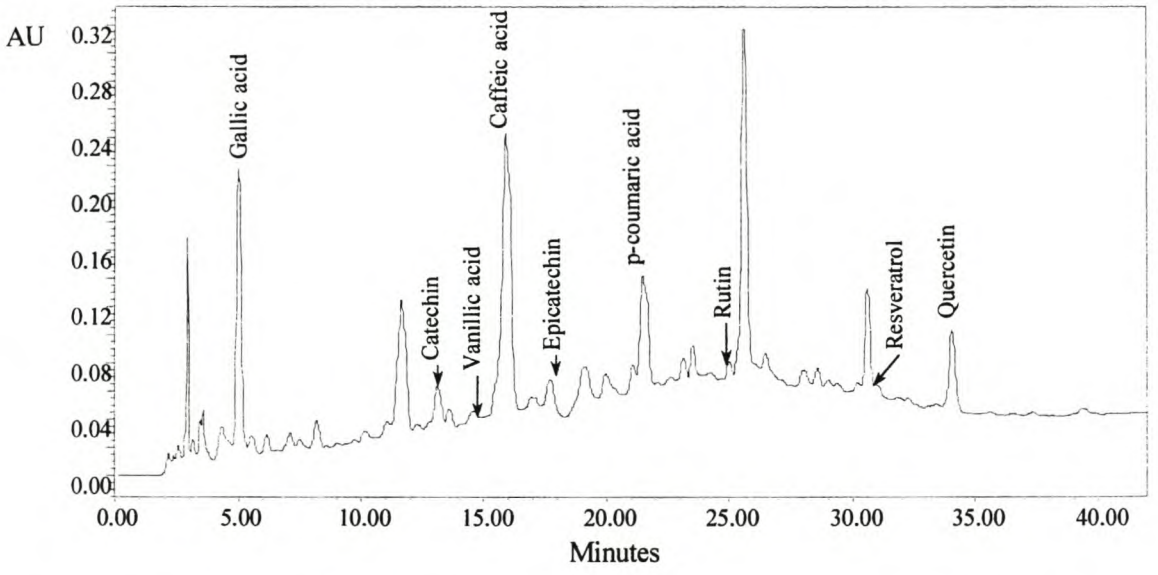

Figure 39: Direct RPLC analysis of Simonsvlei Pinotage. Other conditions as specified in figure 38.

One obvious advantage of the HPLC analysis of wine is that selective analysis of the anthocyanins is possible in the same run, by extracting a chromatogram at 520 nm. This is because the pH of the mobile phase is kept low by the addition of an acid, which means that the anthocyanins are present in the flavylium cationic form which absorbs at 520 nm (figure 40). In the case of CE analysis, the pH is such (9.3) that the anthocyanins are no longer present in the flavylium cationic form due to reactions discussed in section 2.3, and as a result, do not absorb at 520 nm. The analysis of anthocyanins using CE is possible and has been reported. However, a low-pH buffer is necessary, which means that a comprehensive CE analysis of wine polyphenols, including selective analysis of anthocyanins, probably requires two runs.

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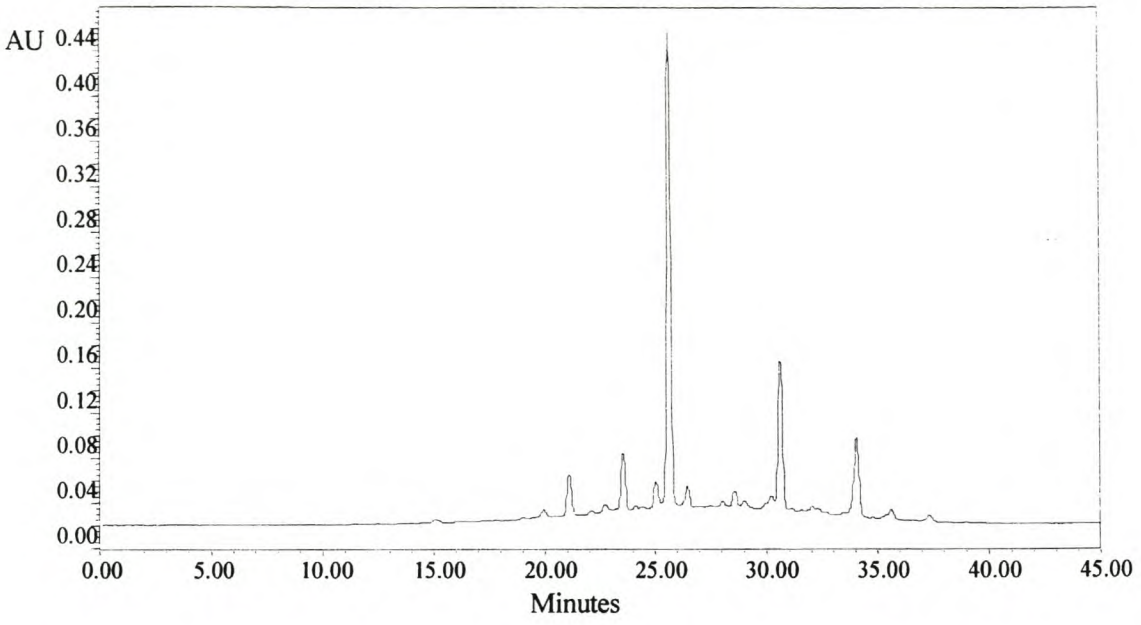

Figure 40: Direct RPLC analysis of Simonsvlei Pinotage, extracted at 520 nm for the selective detection of anthocyanidins. Other conditions same as figure 38.

3.4 High-Performance Liquid Chromatography-Mass Spectroscopy Results

The analyses in this study were performed in collaboration with Gerd Vanhoenacker at the Organic Chemistry Department of the University of Gent, Belgium. The aim of the LC-UV-ESI-MS study of polyphenols was to investigate the applicability of this technique for the analysis of wine. To accomplish this, a simple gradient based on the method of Guillén *et al.* [6] using 1 % acetic acid in water (A) and 1 % acetic acid in methanol (B) as eluents was used (table 6). A standard solution and an ether extract of the Simonsvlei Pinotage were analysed.

The ether extract was prepared by diluting 50 ml of the wine with 50 mL water. The solution was saturated with NaCl to minimise solubility of the compounds of interest in the aqueous phase. 40 mL of diethyl ether was then used to perform the extraction. After evaporation of the ether, the sample was dissolved in 2.5 ml of methanol/water (1/1). The standards used, together with the compounds identified through their mass spectra are listed in table 7.

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Time	%B
0	10
25	22
45	50
55	95
60	95
63	10
65	10

Table 6: The gradient used for the LC-UV-ESI-MS analysis of wine polyphenols, where eluent B is 1% acetic acid in methanol, and eluent A is 1% acetic acid in water.

No.	Standard	Mass	Identified compound	Mass
1	Gallic acid	170		
2	3,4-dihydroxybenzoic acid	154		
3	(+)-Catechin	290		
4	Caffeic acid	180		
5	(-)-Epicatechin	290		
6	p-coumaric acid	164		
7	Myricetin	318		
8	Quercetin	302		
9	Kaempferol	286		
10			5-hydroxy-methyl furfural	126
11			4-hydroxy-phenetyl alcohol	138
12			Vanillic acid	168
13			Syringic acid	198
14			Ferrulic acid	194

Table 7: The phenolic standards used in the LC-MS study (1-9), together with the compounds identified in the ether extract of Simonsvlei Pinotage (10-14).

The ESI-MS detection was performed in the negative mode and masses were detected over a range of 80-400 amu. The other MS parameters are summarised in table 8. The results of the analysis of the standard solution can be seen in figures 41 (UV detection at 280 nm) and in 42 (total ion MS chromatogram).

The standards are well separated using this gradient, and it is apparent that the relative sensitivities of the two detection techniques differ noticeably.

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Parameter	Value
Drying Gas Flow	12 L/min
Drying Gas Temperature	320°C
Fragmentor	60 V
Nebulizer Pressure	55 psig
Capillary Voltage	4000V

Table 8: Parameters used for ESI-MS detection of wine polyphenols.

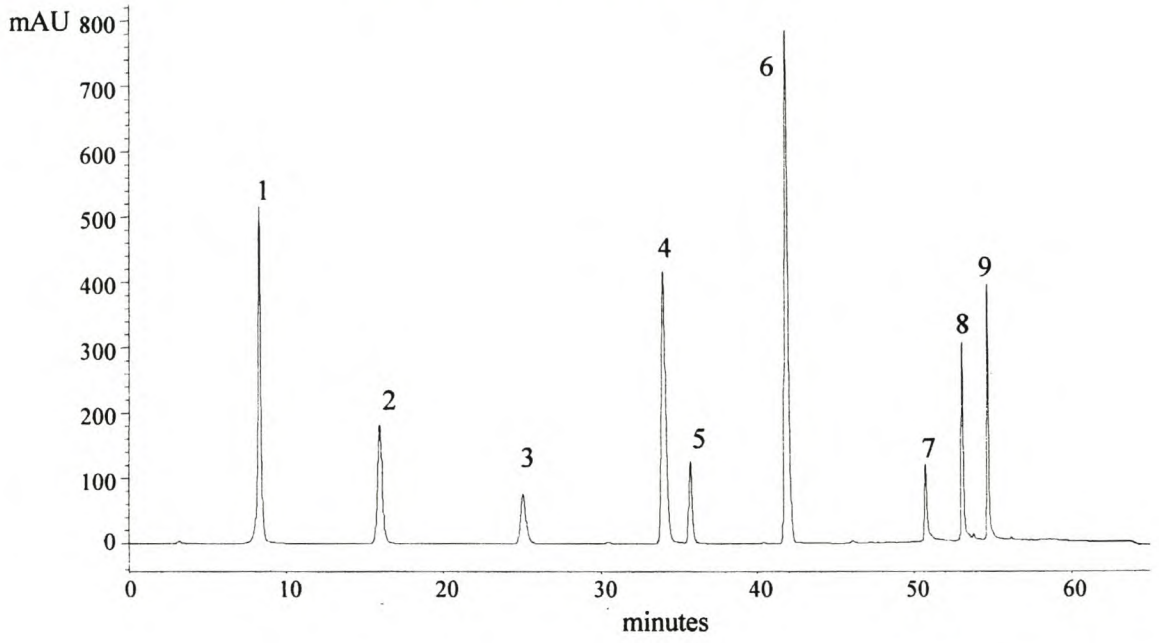

Figure 41: RPLC analysis of a standard solution (250 ppm each) of polyphenols with UV detection at 280 nm. Flow rate: 1 ml/min. Injection volume: 10 μL. Column: Alltima C18, 5 μm d_p, 250 mm L × 4.6 mm i.d. (Alltech). For gradient details and identification of peaks, tables 6 and 7. Temperature: ambient.

By comparing the relative sensitivity of the two detection techniques to the standards it appears that MS detection is more sensitive to the higher molecular weight polyphenols such as the flavanols (3,5) and flavonols (7-9), whereas UV detection is more sensitive to the phenolic acids (1,2,4,6). The relative sensitivities can be explained in terms of the UV spectra of these two classes of species, since the absorption maxima of the phenolic acids are close to 280 nm, while the maxima in the case of the flavanols and flavonols occur in the region of 210 and 400 nm. In the case of ESI-MS analysis of the polyphenols, the ions are predominantly formed by the loss of a proton. This is illustrated in figure 43 for

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some standard solutes. It appears as if the ionisation efficiency is slightly higher for the flavonoids than for the phenolic acids.

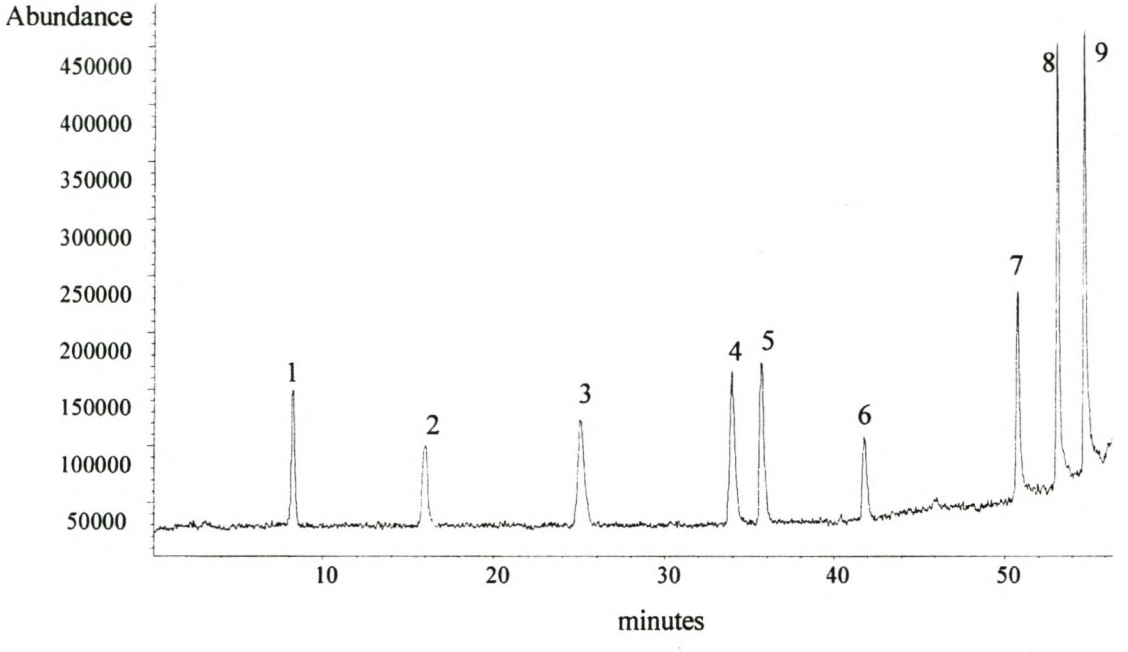

Figure 42: RPLC analysis of a standard solution (250 ppm each) of polyphenols with MS detection (TIC depicted). For MS interface parameters, table 8. Other conditions as in figure 41.

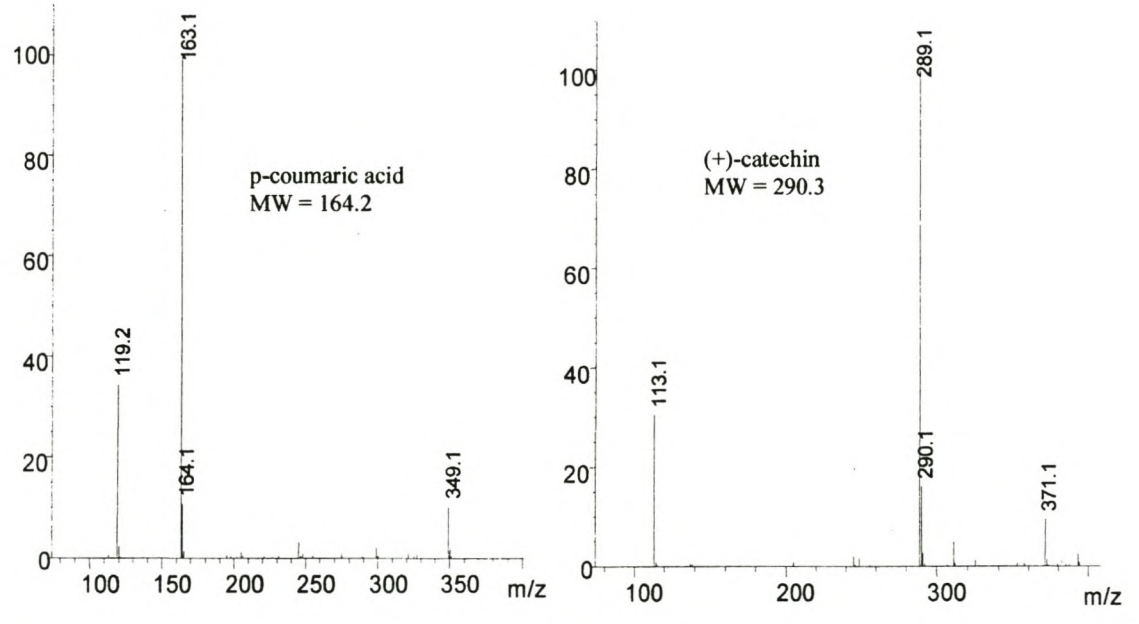

Figure 43: Mass spectra of p-coumaric acid and catechin obtained by ESI-MS detection in the negative mode. For operating conditions, table 8.

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The results of the analysis of an ether extract of Simonsvlei Pinotage are presented in figures 44 (UV detection at 280 nm) and 45 (total ion MS chromatogram). It is evident that the profiles obtained using the two detection techniques differ significantly due to the different response factors of each technique with regard to any specific compound. Qualitatively it can be seen that more compounds are detected by MS, which is a result of the fact that this form of detection is universal, while UV detection at 280 nm is a selective form of detection for the polyphenols. This may be advantageous if the aim of the study is analysis of only these compounds since the chromatogram is simpler. On the other hand, for complete analysis of wine samples MS detection should provide more information, although separation of all these compounds may prove difficult.

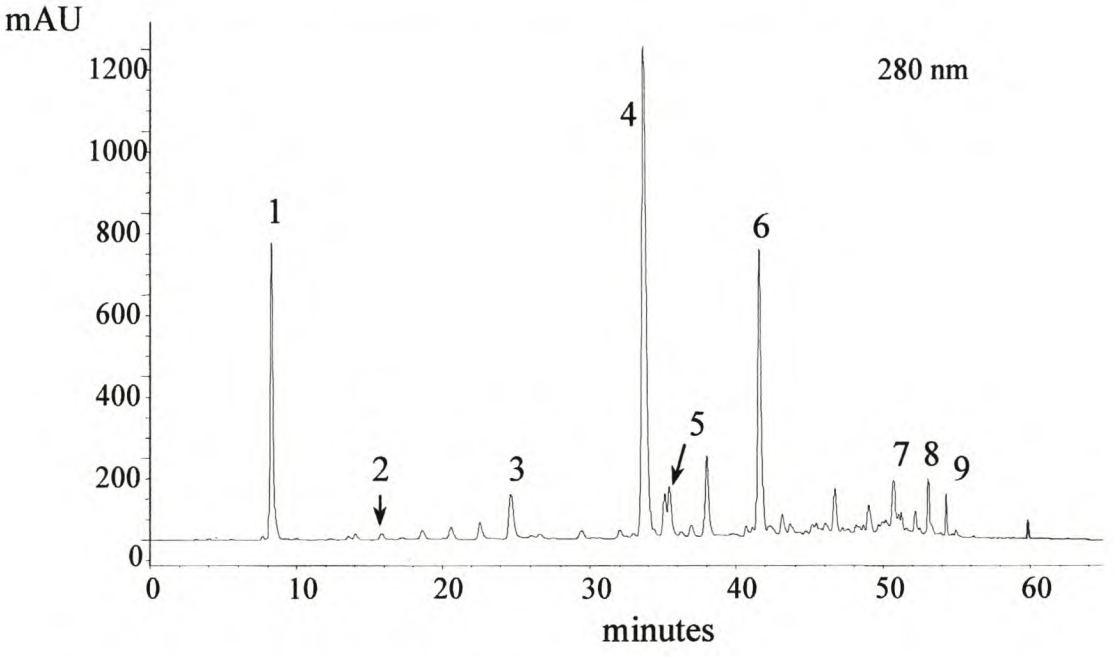

Figure 44: RPLC analysis of an ether extract of Simonsvlei Pinotage with UV detection at 280 nm. Other conditions as in figure 41.

This is clearly illustrated in the TIC (figure 45) where catechin (3) is not resolved from a neighbouring peak, which is not visible in the UV chromatogram, probably due to the lack of a chromophore absorbing at the selected wavelength. Similarly though, epicatechin (5) was not sufficiently separated from a closely eluting peak in the UV chromatogram, while clearly isolated in the TIC trace.

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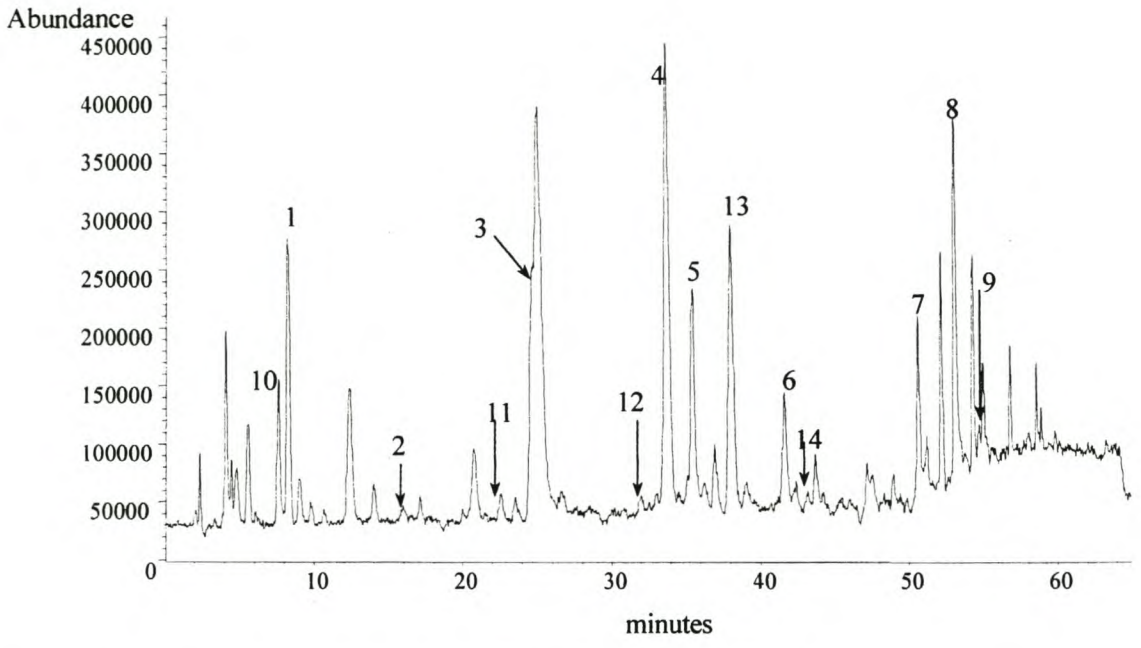

Figure 45: RPLC analysis of an ether extract of Simonsvlei Pinotage with MS detection (TIC depicted). Other conditions as in figure 42.

One obvious advantage of MS detection is the fact that this method of detection provides structural information of unknown peaks. Because the UV-spectra of the polyphenols are relatively similar, identification of these compounds by their UV-spectra is often not possible, and standards are needed for confirmation. This is not the case for MS detection, as can be seen in the identification of 5 non-standard peaks in the TIC of the ether extract.

3.5 Capillary Electrophoresis-Mass Spectroscopy Results

These analyses were also carried out in collaboration with Gerd Vanhoenacker. The aim in this case was to investigate the applicability of CE-ESI-MS for the analysis of polyphenols, also with the view to compare the performance of this relatively new technique to that of the well-established technique of LC-ESI-MS. To this end, a simple sample in the form of a standard solution was chosen. The standards are listed in table 9. The buffer used for the separation was a 75 mM borate buffer at pH 9.3, prepared by dissolving ammoniumtetraborate ($(NH_4)_2B_4O_7$) in water. Ionisation was performed in the

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negative mode and the mass range scanned was from 100 to 400 amu. The sheath flow consisted of a 80/20 solution of methanol and isopropanol. The other ESI operating parameters, are listed in table 10.

No.	Standard	Mass
1	(-)-Epicatechin	290
2	(+)-Catechin	290
3	Kaempferol	286
4	p-Coumaric acid	164
5	Myricetin	318
6	Quercetin	302
7	Caffeic acid	180
8	Gallic acid	170
9	3,4-Dihydroxy benzoic acid	154

Table 9: The standards used in the CE-ESI-MS analysis of polyphenols.

Parameter	Value
Sheath flow	5 µL/min
Nebulizer temperature	300 °C
Nebulizer pressure	10 psig
Drying gas	7 L/min
Capillary voltage	3500 V
Fragmentor	70 V

Table 10: The parameters used for CE-ESI-MS analysis of polyphenols.

The results obtained with UV- and MS detection are given in figures 46 and 47, respectively. These results were obtained in one run, with UV detection performed at 22 cm from the inlet side of the capillary, and MS detection at the outlet end of the capillary. Figure 48 shows a schematic drawing of the CE-UV-MS system used.

It is clear that the standards are not separated by the time that they reach the UV detection window. The separation has improved by the time these compounds reach the ionisation chamber. Although this is not an optimal separation, it appears that the separation of these compounds using the ammonium tetraborate buffer is feasible. The borate buffer

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causes few complications in CE-MS when present in low concentrations. When the concentration is increased though, reproducibility is compromised and current instabilities are experienced. This might be due to buffer precipitation occurring at the capillary tip.

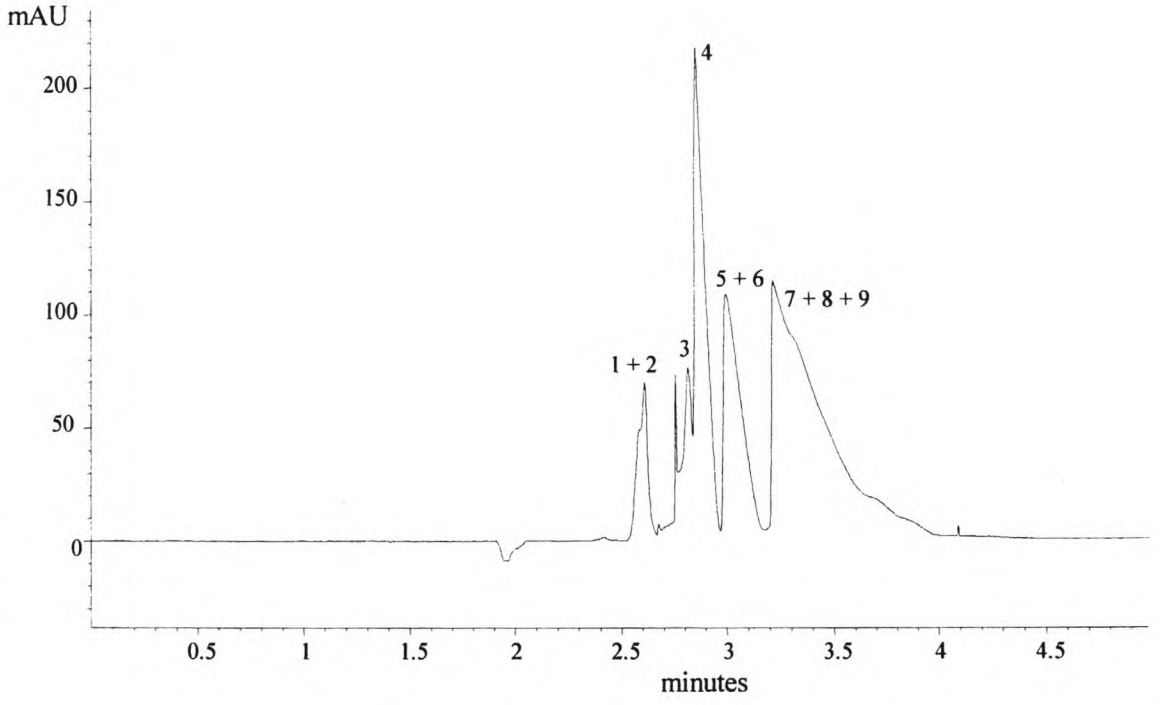

Figure 46: CZE-UV analysis of a standard solution of polyphenols (250 ppm each). Capillary: Bare fused silica, 50μm i.d., 22 cm effective length. Buffer: 75/4 mM $(NH_4)_2B_4O_7$(= 75 mM borate), pH 9.3. Voltage: 20 kV. Temperature: 25°C.

It can be seen that the borate buffer provides satisfactory results in the case of the flavonoids (1-3,5,6), but that the peak shape in the case of the phenolic acids eluting later (7-9) is distorted. The use of an ammonium acetate buffer (results not shown) improved the results in the case of the acids, but the peak shapes of the flavonoids deteriorated dramatically. It can also be seen that the sensitivity of this form of detection is not very high. This is especially obvious when comparing the signal-to-noise ratio to that obtained by the LC-UV-ESI-MS analysis of a standard solution of the same concentration. This is a result of the fact that the quantities used in CE analyses are so low, and that the make-up flow added in the interface further dilutes the sample. This leads to a high concentration detection limit, although the absolute amount detected is quite low.

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Another probable consequence of the addition of make-up flow, is the observed peak broadening and thus loss of efficiency.

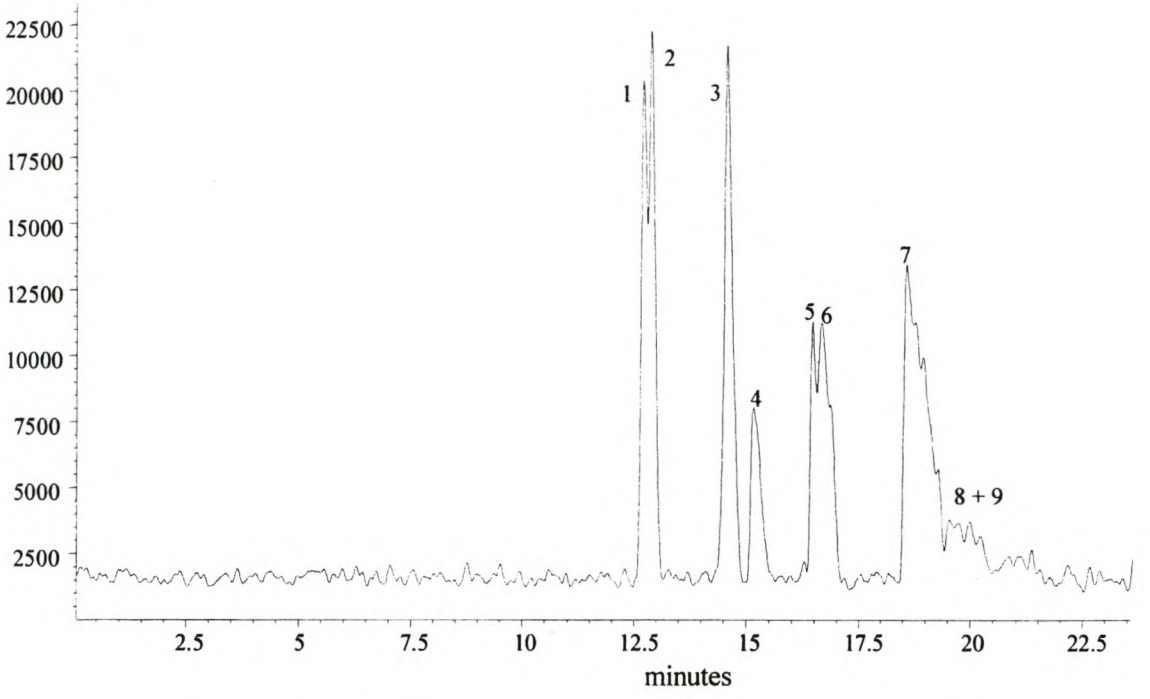

Figure 47: TIC obtained by CZE-ESI-MS analysis of a standard solution of polyphenols (250 ppm each). Capillary effective length: 89 cm. For ESI-MS parameters, table 12. Other conditions as in figure 46.

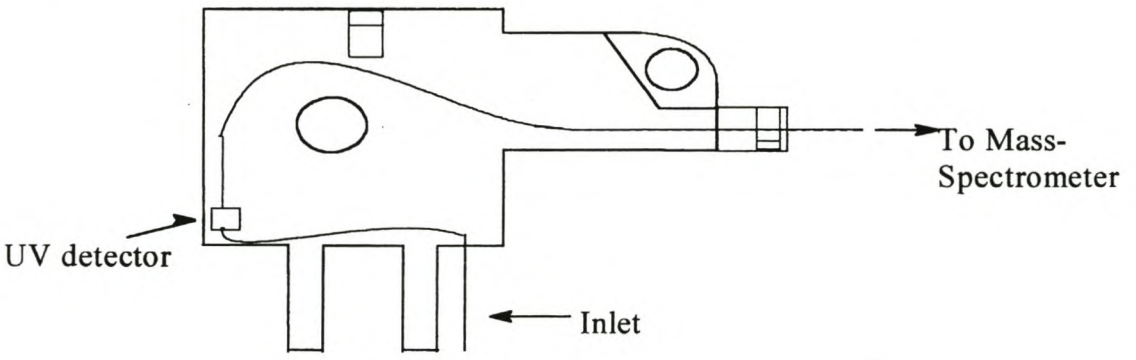

Figure 48: Schematic drawing of the cassette and capillary used for CE-UV-ESI-MS analysis of polyphenols. The total length of the capillary is 89 cm, with UV detection taking place after 22 cm.

The mass spectrum of p-coumaric acid obtained under these ionisation conditions is depicted in figure 49. It is clear that the negative ionisation in CE-ESI-MS occurs

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predominantly via the loss of a single proton, in other words in a manner similar to that observed in the LC-ESI-MS analysis.

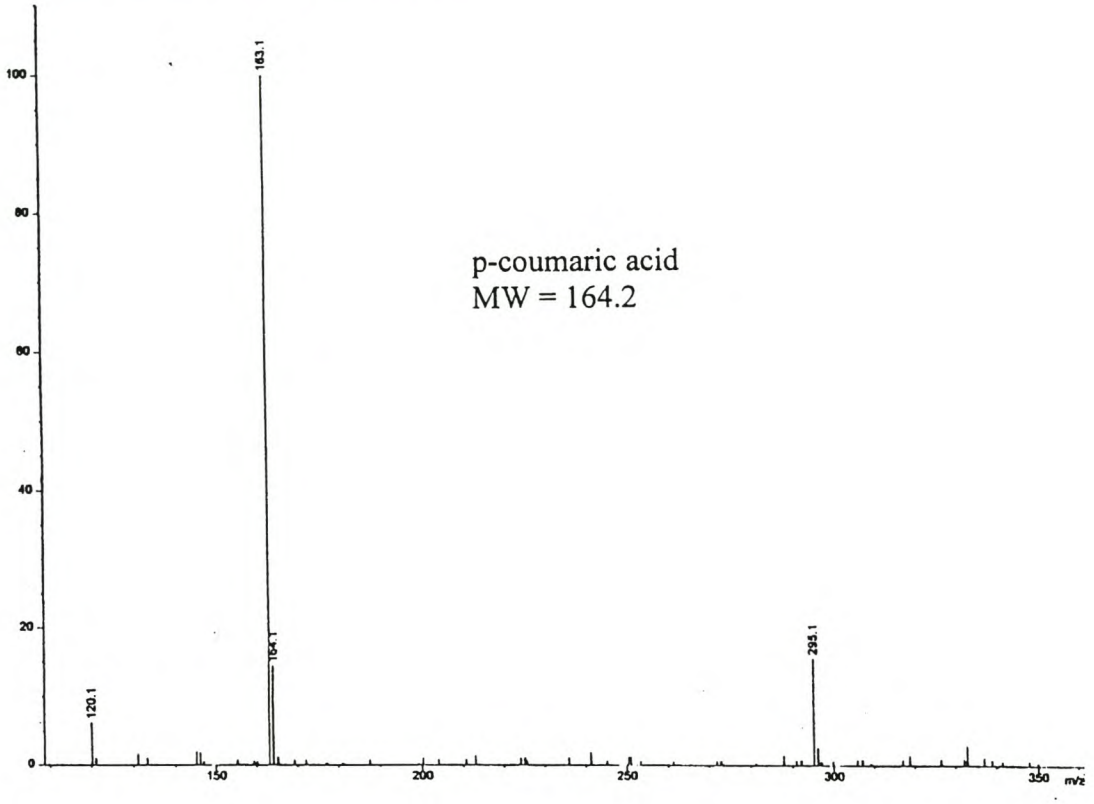

Figure 49: The mass spectrum of p-Coumaric acid obtained in the CE-ESI-MS analysis of polyphenols. Conditions as specified in figure 47.

Although it is obvious that CE-ESI-MS does not yet deliver results comparable to those obtained using LC-ESI-MS, it has been shown that it is possible to apply this technique to the analysis of polyphenols. It is expected that future improvements in the technique will close the gap currently existing between LC-and CE-MS, and will lead to better results, also in the case of the analysis of polyphenols.

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5. Conclusion

The aim of this Masters thesis was twofold. Firstly to study and master state-of the-art separation techniques like HPLC and CE, also in their hyphenation with MS, and secondly to compare both techniques to the analysis of polyphenols in wine. From this preliminary study the following conclusions can be drawn.

The complexity of wine is such that neither technique is able to present a completely satisfactory single-step analysis representative of the polyphenol content of wine. This complexity thus necessitates a comprehensive sample preparation step prior to analysis. An ideal sample preparation step would be simple (in terms of required equipment and time needed) and universally applicable (suitable for wines of different ages and cultivars). In this study Sephadex- and Sep-Pak fractionation of wine polyphenols were investigated. Neither technique was found to be universal. As another form of sample preparation, ether extraction of wine polyphenols was investigated. It was found that extraction was too selective and incomplete to deliver a sample representative of the complete polyphenol content in wine. However, this form of sample preparation proved useful in providing a relatively simple sample for comparison of the different separation techniques and might also be used to provide a "polyphenol fingerprint" of different wines. An additional form of sample preparation was performed by separating the wine into volatile and non-volatile fractions. While this method does not simplify the polyphenol content in the non-volatile fraction, it appears feasible that these fractions can be used for a comprehensive analysis of wine, e.g. by the analysis of the volatile fraction by GC. As an alternative method the direct analysis of wine was investigated by HPLC and CE. The efficiency and shorter analysis times of CE make this the preferred technique for this application. However, in direct analysis of wine the polymeric polyphenols complicated the electropherograms and thus any attempts at quantification. HPLC analysis resulted in a simpler and thus more useful chromatogram than in the case of the direct CZE analysis.

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The versatility offered by the different operational modes of CE was investigated in an attempt to obtain better results. The use of PVA coated capillaries did not result in any significant improvement, but rather in extended analysis times of the majority of compounds. This technique does, however, show promise in for example the analysis of phenolic acids, where efficient, rapid separations are possible. Additionally, direct electrokinetic injection of wine seems to result in less polymeric polyphenols being loaded due to the lack of EOF, although this phenomenon should be investigated further. The use of MEKC had no significant effect on the separation and did not simplify the electropherogram in the case of direct injection of wine. CGE proved to be a promising CE technique for the analysis of polyphenols in wine. Here the bump caused by the polymers was at least partially resolved, although at the cost of slightly extended analysis times. The use of different polymer solutions and buffers should be investigated and might lead to improvement in CGE analysis.

The advantages of CE over HPLC include short set-up times, simplicity of instrumentation, short analysis times and high efficiencies. However, the migration times are dependent on such factors as the age of the capillary, the depletion of the buffer and the nature of the treatment of the capillary wall. It can therefore be concluded that presently HPLC is the more rugged technique.

Another important factor that has to be considered is the coupling of these two separation techniques to MS detection. The fundamental difference here is that LC-MS is already an established technique, while CE-MS is still in its infancy. In this study, it was found that an ether extract of wine could be successfully analysed using LC-ESI-MS. The use of CE-ESI-MS, however, resulted in broad peaks and insufficient separation, even in the case of simple standard solutions. This is caused by the modification of the LC-MS interface for CE-MS, where a make-up flow has to be used to increase the flow rate from the capillary, which results in peak broadening. The main advantage of coupling MS detection to any separation method, is the possibility it offers to identify compounds. Future developments in the field of CE-MS should deliver results comparable to those obtained by LC-MS.

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The accuracy, sensitivity and ruggedness of HPLC still make this the method of choice for the analysis of polyphenols in wine samples. However, the versatility of CE has not fully been investigated in this regard. Furthermore, the use of Capillary Electrochromatography (CEC), a new CE method, in these analyses may provide an extra dimension to unravel the polyphenol complexity.

Further investigation into sample preparation and fractionation techniques may provide less complex samples. The higher efficiencies and rapid analysis times provided by CE, as well as the versatility offered by the different CE modes, could then make CE the separation technique of choice.

This thesis has broadened our insights into the applicability of CE into polyphenol analysis and opened new routes to further research in this respect.

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