INVESTIGATION OF THE CHEMICAL AND SENSORY PROPERTIES OF RED WINE PIGMENTS

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TABLE OF CONTENTS

ABSTRACT

Phenolic compounds play an important role in red wine colour, bitterness, astringency, as well as a range of other tactile or 'mouth-feel' characteristics. Progressive changes of phenolic compounds, initially extracted from grapes, occur during the storage and aging of red wines. The decrease of astringency occurring during wine aging has been considered as a result of mainly anthocyanin-flavanol condensation either directly or mediated by aldehydes. The contribution of these polymeric pigments formed during wine aging to the unique properties of red wine is an important question still unanswered.

Experiments were conducted to synthesise polymeric pigments in model wine solutions under different conditions in the absence of acetaldehyde to provide material for chemical and sensory studies. Only small amounts of polymeric pigments were formed in these experiments confirming that direct polymerisation is a slow process. The low yield of polymers made it necessary to investigate the isolation of polymeric pigments directly from wine. A preparative fractionation protocol was developed to obtain fractions enriched in different red wine pigment combinations for further investigation. A HPLC method was also developed that separated the pigmented and non-pigmented polymers, as well as the monomeric anthocyanins, flavanols (monomeric to trimeric), flavonols and hydroxycinnamic acids from each other. A 6 month old and a 5 year old Shiraz wine were fractionated and further analysed by acid hydrolysis in the presence of a nucleophile, gel permeation chromatography (GPC) and by different mass spectrometry techniques. The various fractions isolated from the 6 month old wine contained combinations of pentameric to dimeric pigments, while those from the 5 year old wine contained pigments with an average degree of polymerisation (DP) of at least 11 but possibly up to 32.

Experiments were conducted to determine the sensorial contribution of anthocyanins to wine as well as the effect of anthocyanin-flavanol polymerisation reactions taking place during maturation. It was shown that significant changes occur in the polymeric phenol composition and in the mean degree of polymerisation of Shiraz wine during aging. With aging more skin tannin were incorporated in the pigmented polymers and the percentage of galloylation in these polymers decreased.

In order to describe the sensory attributes of the polymeric pigments a refined vocabulary, describing the astringent and other mouth-feel sensations elicited by dry red table wines representing different styles was developed and called the mouth-feel wheel. The developed mouth-feel wheel was used in a study to investigate the contribution of anthocyanins and anthocyanin-proanthocyanidin reaction products to the mouth-feel properties of red wine. Wines were made from both red and white grapes with and without pomace contact, as well as with and without anthocyanin addition to the white grapes. The white wine made like a red wine did not exhibit the same mouth-feel sensory attributes of a red wine: it was lower in viscosity, less particulate in nature and lower in intensity for the astringency descriptors fine emery, dry and grippy. The presence of anthocyanins during fermentation appeared to increase the intensity of astringency related terms. Treatments with added anthocyanins increased the amount of polymeric phenols to twice that when compared to treatments without added anthocyanins.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying.

Anita Oberholster Date

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PUBLICATIONS

Gawel R, Oberholster A, Francis IL (2000) Red wine mouth-feel terminology. *Australian Journal of Grape and Wine Research* 6, 203-207.

Peng Z, Iland PG, Oberholster A, Sefton MA, Waters EJ (2002) Analysis of pigmented polymers in red wine by reverse phase HPLC. *Australian Journal of Grape and Wine Research* 8, 70-75.

Oberholster A, Francis IL, Iland PG, Waters EJ (2008) Mouth-feel of white wines made with and without pomace contact and added anthocyanins. *Australian Journal of Grape and Wine Research* In Press.

Chapter 1

LITERATURE REVIEW

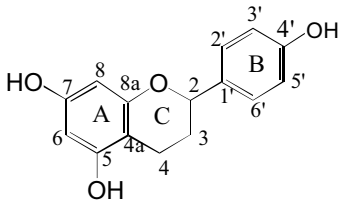
1.1 INTRODUCTION

The phenolic compounds in wine contribute significantly to wine character and quality. During aging, red wine changes in colour and other sensory characteristics. This results from slow processes that progressively incorporate anthocyanins, other phenolic monomers, and phenolic oligomers into polymeric material. Because of the complexity of grape and wine polyphenols, there are particular challenges associated with developing methods for their separation, fractionation and identification.

1.2 GRAPE PHENOLICS

Phenolic compounds exhibit a wide diversity of structures, but they can be divided into flavonoids and non-flavonoids. The flavonoid compounds in wine are mostly extracted from the skins and seeds of grapes during fermentation (Singleton and Esau 1969; Singleton and Noble 1973).

Flavonoids are characterised as molecules possessing two phenolic groups joined by a pyran (oxygen-containing) ring structure (see Figure 1.1). The main flavonoid species important to the chemical reactions and sensory properties of wine are the anthocyanins and flavanols (Singleton 1988; Singleton and Esau 1969; Singleton and Noble 1973). The non-flavonoids are represented by mostly phenolic acids (benzoic acid, hydroxycinnamic acids) and their esters and are present in the pulp of both red and white grapes at low levels.



Basic structure

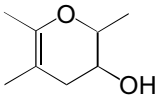
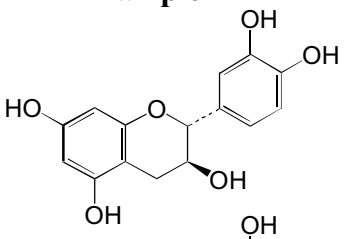
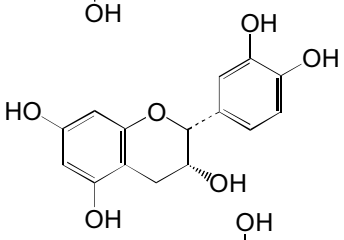
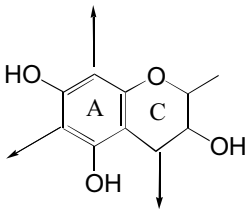
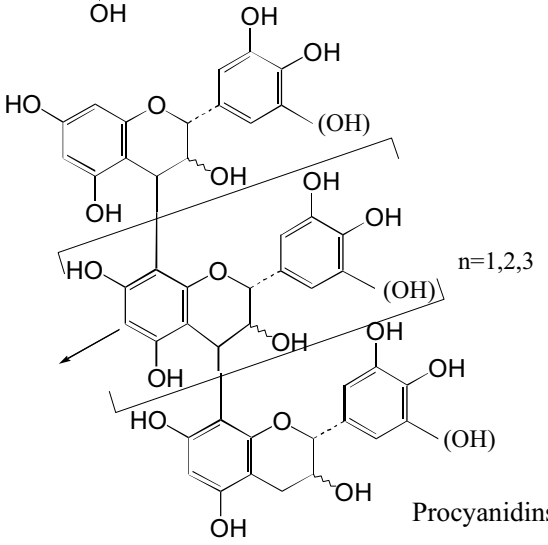
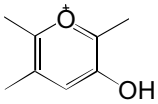
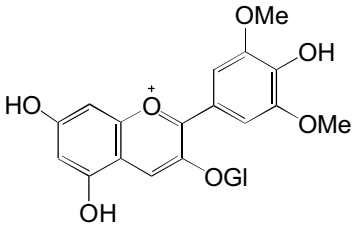
C ring substitution	**Class**	**Example**
	Flavan-3-ol	 (+)-Catechin
		 (-)-Epicatechin
	Proanthocyanidin	 n=1,2,3 Procyanidins
	Anthocyanin	 Malvidin-3-glucoside

Figure 1.1 Flavonoid nomenclature (Somers and Vérette 1988)

1.2.1 Anthocyanins

A number of anthocyanins are present in grapes. They vary in structure by the degree of hydroxylation or methoxylation of the B ring of the flavanoid structure (Figure 1.1). The main pigment is malvidin and its conjugates (60-80%) followed by delphinidin, cyanidin, petunidin and peonidin. Malvidin has been found in the form of 3-glucoside and, to a lesser extent, as esters of acetic, coumaric, and caffeic acids. The diglucosides are found only in non-vinifera grapes (Ribéreau-Gayon *et al.* 2000; Singleton and Esau 1969). Anthocyanins are the red pigments in grape skins which play an important role in the colour of wine. There are several forms of anthocyanins that exist in equilibrium at wine pH (Figure 1.2) (Somers and Vérette 1988). At wine pH, the percentage of total anthocyanins in the red, flavylium form is less than 25% (Singleton 1988). When sulfur dioxide is added to wine it combines with the flavylium form, resulting in an additional equilibrium between the coloured form and the uncoloured bisulfite adduct.

The colour of anthocyanin solutions is also influenced by the presence of other components in the medium that cause colour shifts towards violet (bathochromic effect) and an increase in intensity (hyperchromic effect). Co-pigmentation, an effect caused by pi-complexation of electron-poor and electron-rich aromatic compounds, is important in wine for several reasons. Principally, this effect increases the solubility of specific wine flavonoids, particularly condensed tannin and flavonols by interaction with anthocyanins; the converse is important as well. This will generally increase the absorbance and, in some cases, shift the wavelength of maximum absorbance. The magnitude of the co-pigmentation effect has been shown to be dependent on the structures and amounts of the anthocyanin and copigment molecules, and the pH, temperature and composition of the aqueous solvent solution (Brouillard and Dangles 1994).

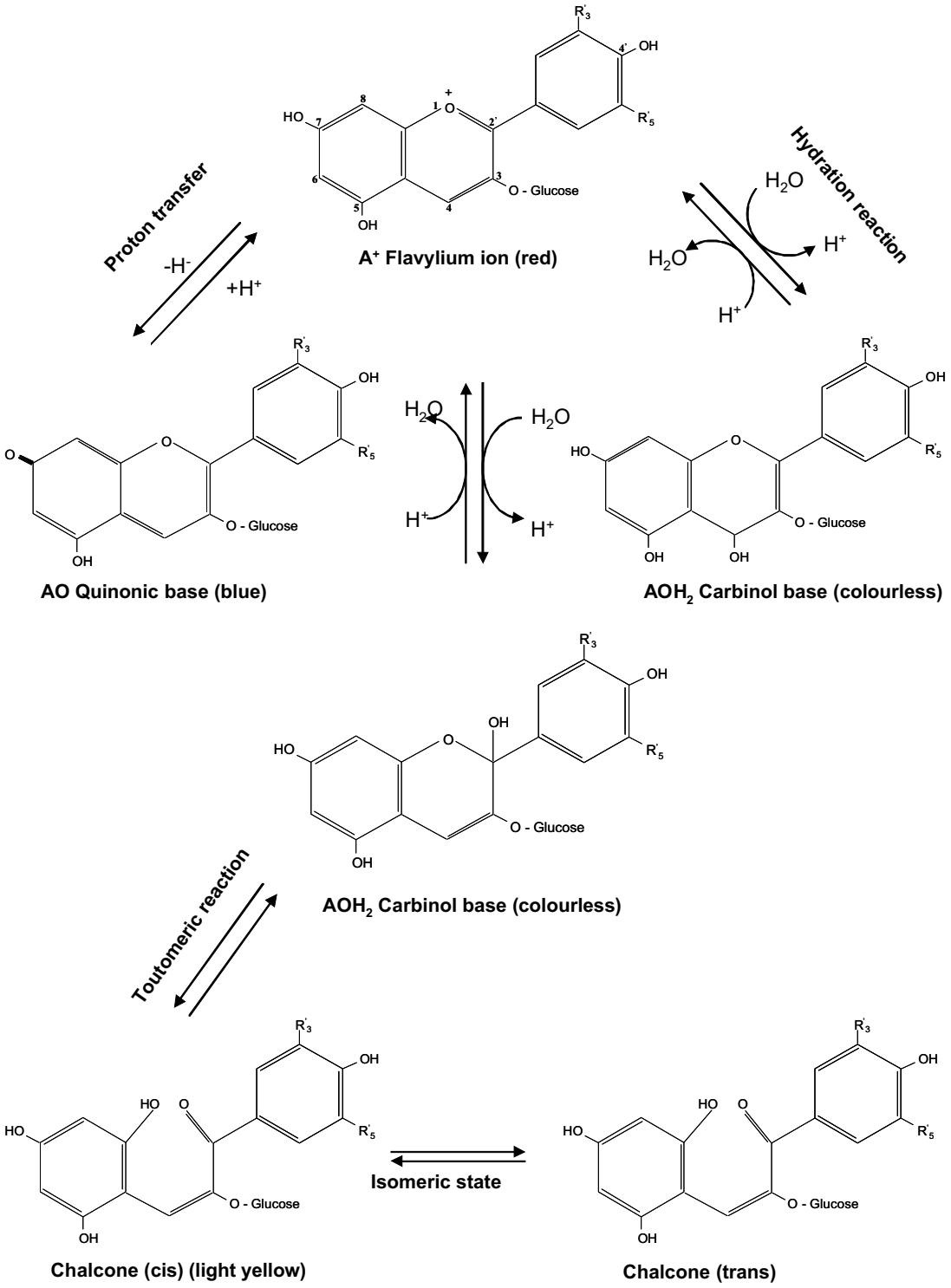
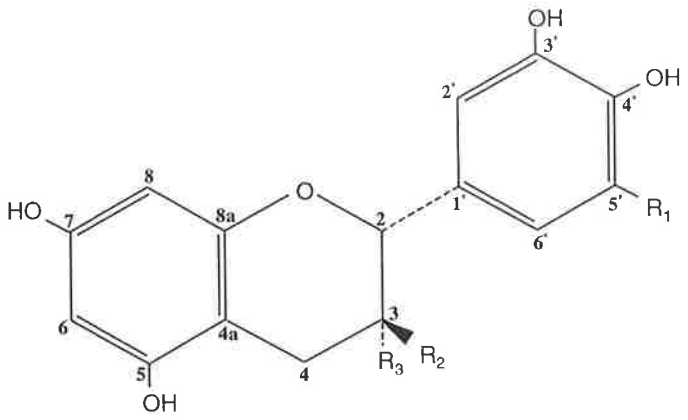


Figure 1.2 Anthocyanin equilibrium (Ribéreau-Gayon *et al.* 2000)

1.2.2 Flavan-3-ol monomers and oligomers

The two main types of flavan-3-ols found in wine are (+)-catechin and (-)-epicatechin. They form dimers, trimers and various higher oligomers (proanthocyanidins) and/or polymers through interflavan linkages. There are two types of proanthocyanidins, the type B proanthocyanidins are dimers formed through the condensation of two flavan-3-ols linked by a C_4-C_8 (B_1 to B_4) or C_4-C_6 (B_5 to B_8) bond. Type A procyanidins are dimers that, in addition to the C_4-C_8 or C_4-C_6 interflavan bond, also have an ether bond between the C_5 or C_7 carbons of the terminal units and the C_2 carbon of the upper unit. The procyanidins consist of (epi)catechin units (Figure 1.3, R_1 = H), while prodelphinidins are based on (epi)gallocatechin units (Figure 1.3, R_1 = OH), together they are referred to as the proanthocyanidins. Furthermore, some units are substituted by gallic acid on the 3-hydroxyl group.



	R_1	R_2	R_3
(+)-catechin	H	OH	H
(+)-gallocatechin	OH	OH	H
(-)-epicatechin	H	H	OH
(-)-epigallocatechin	OH	H	OH

Figure 1.3 Chemical structures of flavanols (Moutounet *et al.* 1996)

Souquet *et al.* (1996) showed that catechin, epicatechin, epicatechin gallate and epigallocatechin are the major constitutive units of grape skin tannins. Gallocatechin and epigallocatechin gallate has also been detected as units of tannins or proanthocyanidin chains.

For both grape seed and skin fractions, epicatechin was the major extension unit, with catechin the most abundant in the terminal units. In grape seeds, the proportion of gallolyation varied from 13 to 30% as the mDP increased from 2.3 to 15.1 (by thiolysis) and from 2.4 to 16.7 (by GPC) (Prieur *et al*. 1994), while Moutounet *et al.* (1996) determined that the average degree of polymerisation of grape skin tannins was 30 units with 15% galloylated and 30% being prodelphinidins. Skin tannins thus differ from seed tannins by their lower amounts of galloylated derivatives and higher average molecular weights.

1.2.3 Polymerisation reactions during wine making, maturation and aging

During the maturation and aging of wine, grape-derived anthocyanins and proanthocyanidins react with each other to form new wine pigments and proanthocyanidins and polymeric pigments and phenols.

1.2.3.1 Formation of pyranoanthocyanins

Monomeric pigments are produced in wine during fermentation and maturation by cycloaddition mechanisms between anthocyanins and various yeast derived metabolites formed during fermentation (Figure 1.4). Orange pigments have been identified in red wine (Cameira-dos-Santos *et al*. 1996; Francia-Aricha *et al*. 1997) which was shown to be condensation products between anthocyanin and vinyl phenol (Fulcrand *et al.* 1996b, 1998). These pigments were resistant to pH change and bisulphite bleaching (Sarni-Manchado *et al.* 1996). The two pigments, A and B, were formed by covalent binding of malvidin-3-*O*-glucoside and malvidin-3-*O*-glucoside-*p*-coumarate with 4-vinylphenols (Fulcrand *et al.* 1996b). Lately, the presence of new wine pigments formed through reaction of other vinylphenols such as 4-vinylcatechol and 4-vinylsyringol with anthocyanins, were shown to occur in wine (Håkansson *et al.* 2003). In addition, reaction products of anthocyanins and pyruvic acid (A-type vitisins) (Fulcrand *et al*. 1998; Mateus *et al.* 2002) and acetaldehyde (B-type vitisins) (Bakker *et al.* 1997; Bakker and Timberlake 1997) were found in red wine (Figure 1.4). The vitisins confer much colour at pH 4 to 6 where anthocyanins are colourless. Vitisin A is totally resistant to bleaching by sulfur dioxide, while vitisin B is partly bleached

(much less then malvidin-3-glucoside). Recently, these types of wine pigments have been referred to as pyranoanthocyanins (λ max 480 to 510 nm) (Benabdeljalil *et al.* 2000).

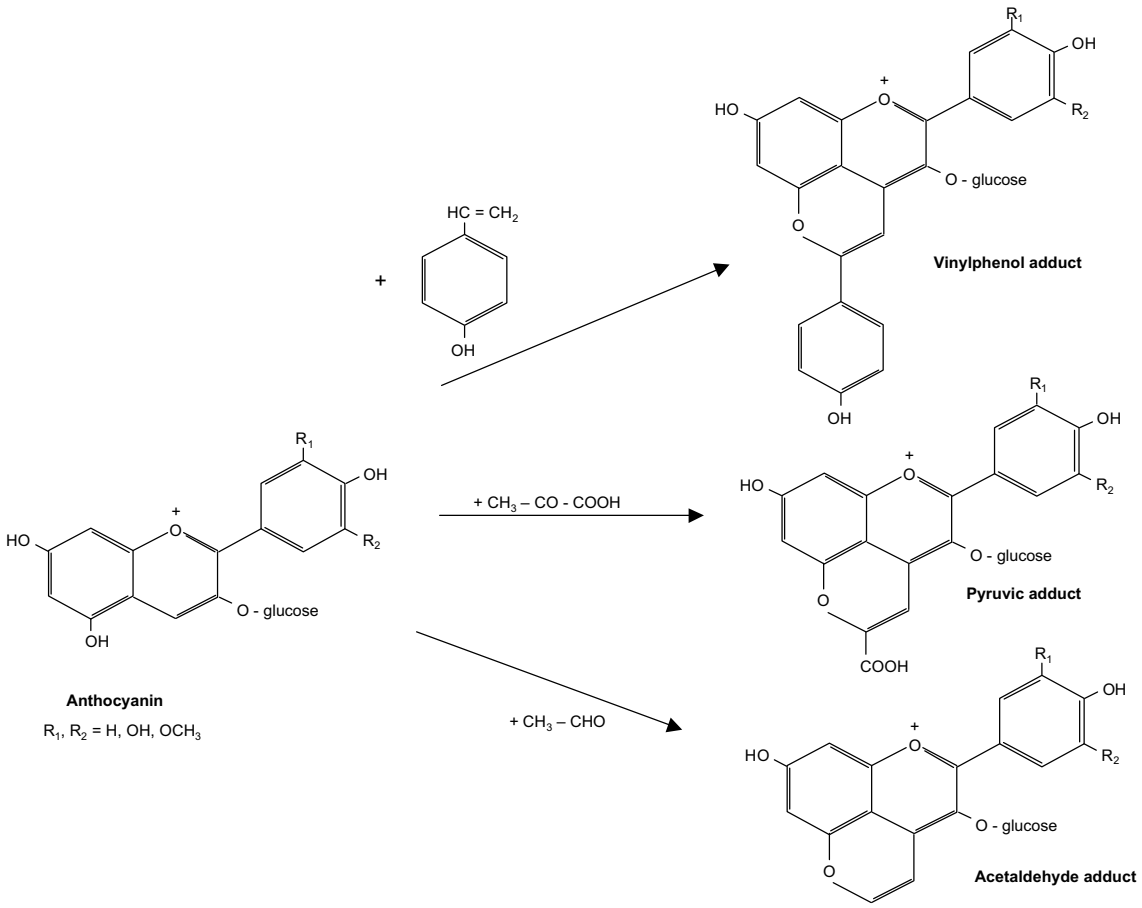


Figure 1.4 Anthocyanin reactions with yeast metabolites

1.2.3.2 Formation of wine polymeric phenols

There are several possible methods of polymerisation by which the pigments of young wines may be altered and stabilised during aging to form new polymeric pigments. These new pigment forms are, moreover, much less sensitive to changes in pH (Jurd 1969; Somers 1971; Somers and Evans 1977). Two main anthocyanin addition reactions form the polymeric species described. The first is direct condensation between anthocyanins (A) and flavan-3-ols or proanthocyanidins/tannins (T). Two related mechanisms involving nucleophilic addition have been proposed for this reaction. The first mechanism starts with a nucleophilic attack at the electrophilic C-4 of the anthocyanin flavylium form by a flavanol (in position C-8 or C-6),

yielding either a flavene, which can be oxidised to the corresponding red flavylium cation (Figure 1.5) and proceed further to a yellow xanthylium salt (Figure 1.6) (Baranowski and Nagel 1983; Jurd 1969; Liao *et al.* 1992; Santos-Buelga *et al.* 1995; Somers 1971), or a colourless bicyclic condensation product (A-(O)-T, structure similar to A-type proanthocyanidins) (Salas *et al.* 2003). In the second mechanism the electrophilic species is a carbocation by acidic cleavage of a proanthocyanidin interflavanic bond (Haslam 1980; Thorngate and Singleton 1994; Vidal *et al.* 2002), while the anthocyanin reacts, in its hydrated hemicetal form, as a nucleophile (Figure 1.7) (Remy *et al.* 2000). These two differ in the position of anthocyanin and flavanols in the resulting products, which are respectively denoted A-T and T-A. Direct condensation between anthocyanin and catechin has been shown to take place very slowly (Dallas *et al*. 1996b; Escribano-Bailòn *et al*. 1996; Rivas-Gonzalo *et al*. 1995; Santos-Buelga *et al*. 1995).

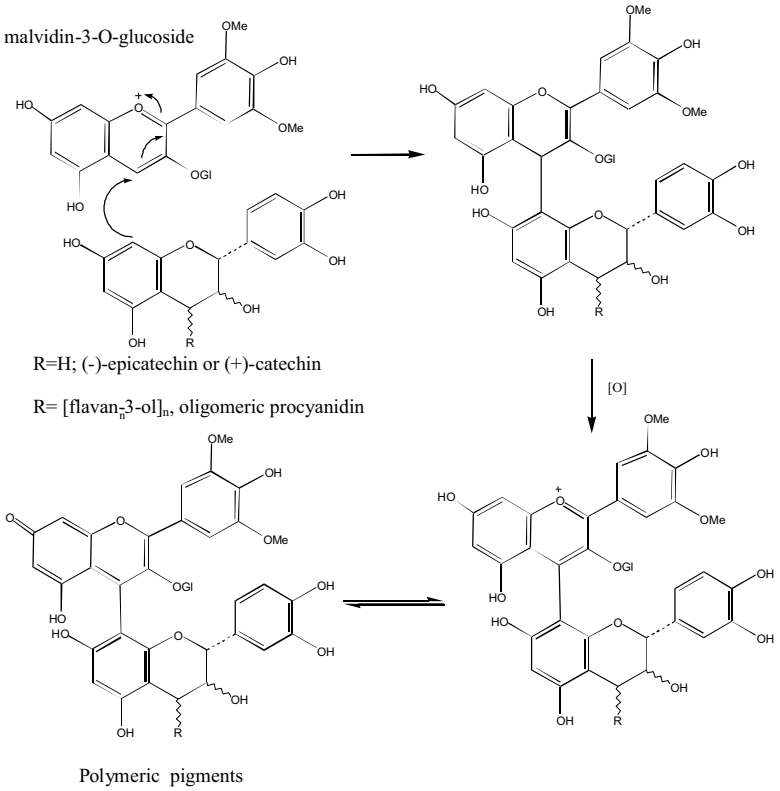


Figure 1.5 Generation of oligomeric/polymeric pigments in red wines (A^+-T) (Somers 1971)

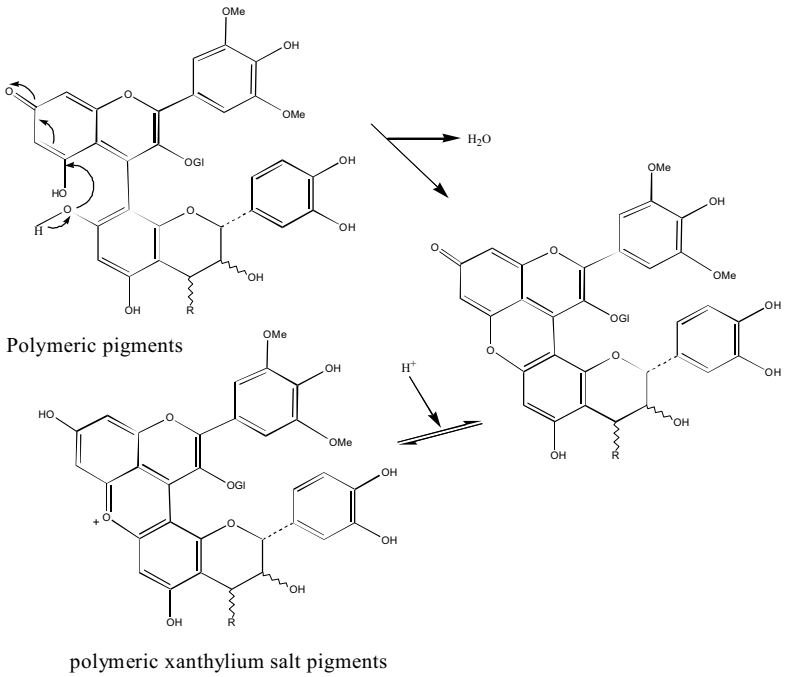


Figure 1.6 Formation of xanthylium salts during red wine maturation (Liao *et al*. 1992)

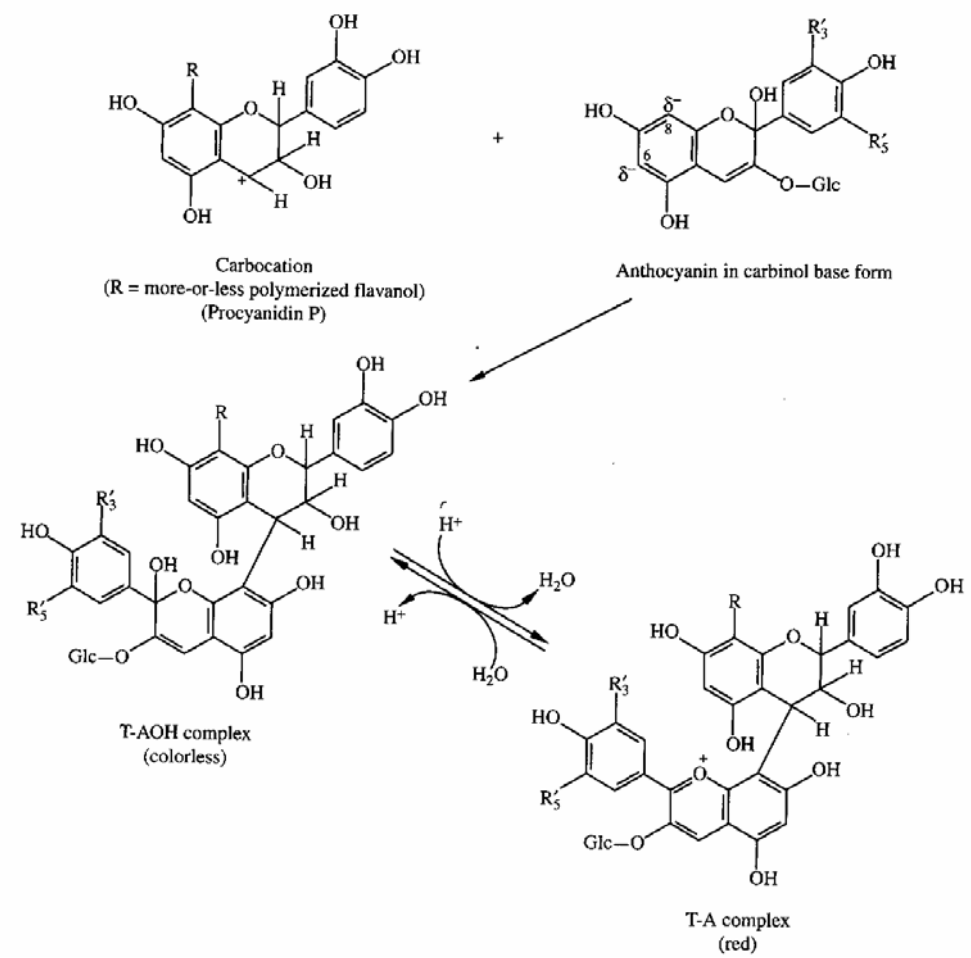


Figure 1.7 Direct T-A type condensation of proanthocyanidins and anthocyanins (Ribéreau-Gayon *et al.* 2000)

The second is an indirect condensation, with acetaldehyde (Bakker *et al*. 1993; Baranowski and Nagel 1983; Roggero *et al*. 1986; Timberlake and Bridle 1976) that could involve both anthocyanin-flavanol and flavanol-flavanol condensation (Es-Safi *et al.* 1996; Fulcrand *et al.* 1996a; Saucier *et al.* 1997a, b, c) by CH_3 - CH linkage (Figure 1.8). Acetaldehyde in wine is produced biochemically by yeast metabolism during fermentation (Romano *et al.* 1994). Another method of production of acetaldehyde is by oxidation of ethanol by phenolic compounds in the presence of oxygen (Wildenradt and Singleton 1974). Polymers formed through reactions with other aldehydes including glyoxylic acid and furfural have also been detected (Es-Safi *et al.* 2000; Fulcrand *et al.* 1997).

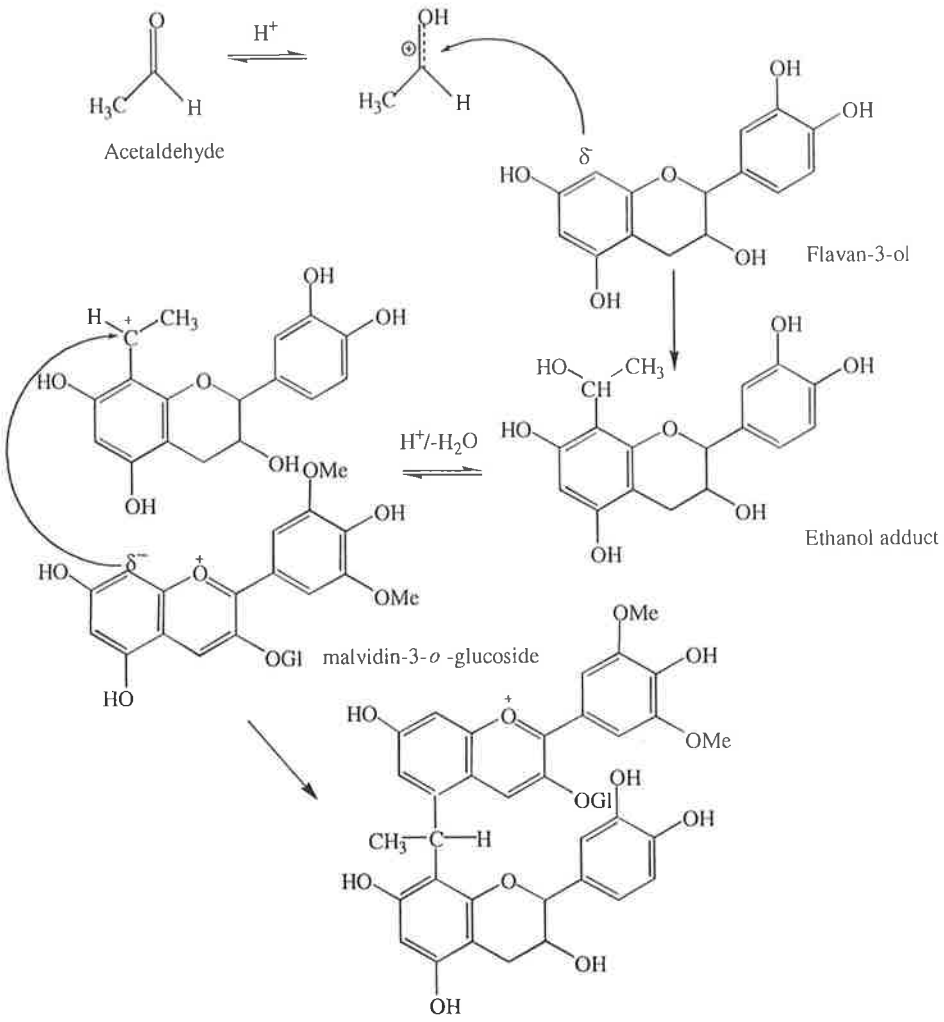


Figure 1.8 Mechanism of acetaldehyde-induced polymerisation (Timberlake and Bridle 1976)

The aldehyde-mediated polycondensation polymers are unstable and can rearrange into more stable pigments, namely pyranoanthocyanins and xanthyliums (Cheynier *et al.* 1999). Two reaction pathways have been postulated for the formation of flavanyl pyranoanthocyanins. It can form from the reaction of flavanol with a pyranoanthocyanin previously formed, or a vinyl-flavanol might react with an anthocyanin. Vinyl-flavanols result from the cleavage of ethyl-bridged polymers. Vinyl-linked flavanols and anthocyanin-flavanol products have been identified in wine (Alcalde-Eon *et al.* 2004). Another rearrangement can occur with polycondensated flavanol polymers. This was first observed with glyoxylic acid (Es-Safi *et al.* 1999c). Two flavanols linked by glyoxylic acid with a carboxy-methine bridge (colourless) formed yellowish compounds over time. These were identified as xanthylium salts formed by

dehydration and oxidation of the carboxy-methine bridged flavanol dimer (Es-Safi *et al.* 2000).

1.2.3.2.1 Model wine studies of polymeric pigment formation

Dallas *et al*. (1996a, b) found that the reaction rate of the procyanidins with malvidin-3-*O*-glucoside increased in the order monomers, dimers and trimers, while each procyanidin reacted much slower if esterified with gallic acid. A few authors did not see any reactions in model systems if acetaldehyde was not added (Bakker *et al*., 1993; Singleton and Trousdale, 1992; Thorngate and Singleton, 1994) supporting evidence that direct condensation reactions are slow. Remy-Tanneau *et al.* (2003) studied direct addition between malvidin-3-glucoside (2.2 g/L) and (-)-epicatechin (1.2 g/L) in a model system at 35ºC. Analyses of reaction products by high-performance liquid chromatography coupled to electrospray ionisation mass spectrometry (HPLC/ESI-MS) showed the formation of colorless dimers detected at m/z 781 in the negative ion mode. The signal at m/z 781 may correspond either to the malvidin-3-glucoside flavene-epicatechin dimer or to a derivative with an A type linkage as was earlier found by Remy *et al.* (2000) in a two year old Cabernet Sauvignon wine.

Acetaldehyde acts as an intermediate in the condensation between anthocyanins and flavanols or between two flavanol molecules by a Baeyer type reaction (Somers 1971; Timberlake and Bridle 1976). Numerous model studies have confirmed the formation of new pigments from mixtures of malvidin-3-*O*-glucoside and flavanols (catechin, epicatechin, procyanidin B2, procyanidin B3, B2-3'-*o*-gallate, C1) in the presence of acetaldehyde (Bakker *et al*. 1993; Cameira-dos-Santos *et al*. 1996; Dallas *et al*. 1996b; Escribano-Bailòn *et al*. 1996; Francia-Aricha *et al*. 1997; Rivas-Gonzalo *et al*. 1995). Mass spectrometry studies have demonstrated that the reaction starts with the protonation of acetaldehyde in an acidic medium, followed by addition of the resulting carbocation onto the flavanol (Figure 1.8) (Fulcrand *et al.* 1996a; Saucier *et al.* 1997b, c). This data is further supported by catechin-acetaldehyde condensations observed by Fulcrand *et al*. (1996b) and Rivas-Gonzalo *et al*. (1995). Saucier *et al.* (1997c) isolated a dimeric phenolic fraction from a two month old red wine positively identified by LC-ES-MS as two flavanol (catechin) monomer units linked by an ethyl bridge, which shows the presence of this type of condensation product in wine.

1.2.3.3 Polymerisation due to polyphenol oxidase (PPO)

Enzymatic oxidation takes place in the grape must, whereas catalytic oxidation (autoxidation) prevails in the later stages of wine-making and, further, in aging of wines. Enzymatic oxidation exerts very little influence in red wine, as the oxygen dissolved is almost entirely consumed by the fermenting yeasts (Cheynier *et al.* 1997b). In addition, enzymatic oxidation is restricted to polyphenol oxidase (PPO) (Fulcrand *et al*. 1997). The major substrates of PPO in grapes are thought to be caftaric acid (caffeoyl tartaric acid) and, to a lesser extent, coutaric acid (coumaroyl tartaric acid) which are both transformed to caftaric acid o-quinone with the production of glyoxylic acid. Glyoxylic acid can readily react with flavanols in a mechanism similar to acetaldehyde condensation (Fulcrand *et al*. 1997).

1.3 ISOLATION OF POLYMERIC PHENOLS

Separation of proanthocyanidins has been attained with HPLC (high performance liquid chromatography) (Lea 1979; Putman and Butler 1989; Revilla *et al*. 1991; Ricardo da Silva 1990). However, analysis of tannins becomes increasingly difficult as their molecular weight increase and separation only up to the tetramer have been achieved (Revilla *et al.* 1989).

Preparative methods based on the separation of oligomeric and polymeric proanthocyanidins on a molecular weight basis have been developed. These methods include counter-current chromatography, chromatography on Fractogel TSK HW-40 (Oszmianski and Bourzeix 1995; Ricardo da Silva *et al.* 1991b) and Sephadex LH-20 (Escribano-Bailón *et al*. 1992; Lea and Timberlake 1974; Lu and Foo 1997; Oszmianski and Sapis 1989; Thompson *et al.* 1972; Wilson 1981) columns and normal phase TLC or HPLC (Prieur *et al.* 1994; Rigaud *et al.* 1991).

Counter-current chromatography separates procyanidins according to their partition coefficients in two liquid phases. Separation is similar to that achieved by liquid chromatography (LC) (Lea *et al*. 1979; Putman and Butler 1985). Sephadex LH-20 has been successful in the partial separation of proanthocyanidins into monomer, dimer, trimer and

tetramer fractions. Dorner (1983) compared the separation efficiency of Sephadex LH-20 and Fractogel TSK HW-40 and concluded that Fractogel TSK HW-40 is better for the separation of polymeric proanthocyanidins because it can separate components with molecular weights in the range 100 to 10 000 Daltons. Derdelinckx and Jerumanis (1984) used Fractogel TSK HW-40 in conjunction with Sephadex LH-20 to obtain highly purified procyanidins. Rigaud *et al*. (1993) developed a HPLC method using a normal-phase silica column to separate procyanidins on a molecular weight basis, without derivatisation. The monomers and major dimers were resolved as discrete peaks. Oligomeric and polymeric components were eluted in order of increasing degree of polymerisation. This method successfully separated both grape seed (Prieur *et al*. 1994) and skin (Souquet *et al.* 1996) oligomeric and polymeric proanthocyanidins into several fractions. Although this method was found lacking for pigmented samples even after the modifications of Kennedy and Waterhouse (2000), it may still give insight into complex mixtures of samples.

Because of their complexity it is essential that wine pigments are separated from the monomeric anthocyanins and proanthocyanidins present in wines to be able to characterise and identify these products of wine aging.

Somers (1967; 1968) first demonstrated the presence of polymeric pigments in aged wines by gel permeation chromatography separation of the polymeric pigments from the anthocyanins. Sephadex LH-20 was the most popular hydrophobic resin used for the purification of polymeric phenols (Kantz and Singleton 1990, 1991). Kantz and Singleton (1990) reported that HPLC estimation of polymeric phenols was higher than the amount obtained from Sephadex LH-20, indicating a possible loss of material by irreversible absorption on the packing material. Oszmianski *et al*. (1988) developed a fractionation technique, which relies on the fact that various phenolic fractions have different affinities for the reverse-phase absorbent in a Sep-pak cartridge. Toyopearl HW-40 (s) has also become popular for the separation of wine pigments (Alcalde-Eon *et al.* 2004; Mateus *et al.* 2001). Anthocyanin pyruvic acid derivatives and vinyl-linked dimers were isolated with ethanol solutions while methanol resulted in the isolation of dimeric and trimeric pigments formed by direct condensation reactions with flavanols or through ethyl bridges. The individual fractions were eluted with specific solvents at particular pHs. Lin and Hilton (1980) tested a wide range of

chromatography resins and found strong sorption of polymeric pigments to ion-exchange, affinity and molecular sieve type resins, through possible hydrophobic interaction with aromatic rings as well as hydrogen bonding. Silica gel showed weaker absorption and the polymeric pigments could be eluted with 50% ethanol. Electrophoresis indicated that most of these polymers had isoelectric points below pH 1.7 and were thus neutral at wine pH. Spagna and Pifferi (1992) used sulphoxy ethyl cellulose cationic exchanger to purify monoglucoside anthocyanins from red polymers. Asenstorfer (2001) used sulphoxy ethyl cellulose in the absence and presence of bisulfite buffer to isolate a fraction containing bisulfite bleach resistant pigments. This fraction contained a number of oligomeric pigments identified as anthocyanin vinyl dimers and trimers. Vivar-Quintana *et al*. (2002) fractionated a nine month old wine using medium pressure liquid chromatography on a C18 reversed-phase column. A fraction containing anthocyanin-derived pigments such as pyranoanthocyanins was successfully isolated. Ultrafiltration and dialysis are sometimes used to obtain enriched wine fractions with less monomers and more polymers. Separation by ultrafiltration and dialysis are influenced by the association and aggregation of compounds present. Separation is thus not according to molecular size and an unknown amount of polymeric material is lost (Hsu and Heatherbell 1987).

HPLC separation of wine phenolics usually requires prior purification, which makes quantification difficult as some material is always lost. In most direct HPLC methods the polymeric phenols eluted as an increase in the baseline. However, the method of Price *et al*. (1995) enables the non-pigmented polymeric phenols and the polymeric pigments to elute as a single peak, at the end of the run.

1.4 CHARACTERISATION AND IDENTIFICATION OF POLYMERIC PHENOLS

1.4.1 Spectrophotometric analysis

Colour measurements have been widely used for the characterisation of grapes and wines since the 1970s. Wine density has been defined as the sum of the optical density (OD) at 420 nm and 520 nm where OD420 is defined as the yellow/brown coloured wine pigments/tannins and OD520 as the monomeric and polymeric red coloured pigments. These two values are suitable for studying wines with some age, but do not always cover the relatively deep colours

of young wines. According to Glories (1984) the blue component defined as 620 nm, attributed to the quinodal forms of free and combined anthocyanins must be taken into account in assessing these colours. The wine hue or tint, measured as the ratio of absorbances A_{420}/A_{520} typically increases from 0.4 - 0.5 in young wines to around 0.8 - 0.9 in mature red wines (Somers and Evans 1977). This indicates the increased contribution of polymeric pigments to the wine colour.

Several methods for the assessment of colour in red wines have generally recognised the ionisable and bleachable nature of the anthocyanins and have distinguished these from the polymeric forms that are generally less sensitive to pH and less bleached by bisulfite (Somers and Evans 1977). The addition of acetaldehyde eliminates the bleaching effect by the preferential binding of SO_2 to it. The contribution of colour due to copigmentation is also bleached with SO_2 (Levengood 1996). The SO_2 bleaching methods will attribute all the colour loss to pigments when as much as half of it could be from copigmented forms in young wines. Copigment complexes have extinction values several times those of the free anthocyanins. At high dilutions, typically 24:1, virtually all of the copigmented anthocyanins have returned to the ionisation equilibrium involving the flavylium cation, the pseudo base, and the chalcone forms. This loss in colour with dilution can be used in calculation of the fraction of colour that is due to copigmentation (Boulton 1996). An extensive review about copigmentation and its role in the colour of red wine by Boulton (2001) was published. These spectrophotometric measures can be applied to wine fractions to determine the total amount of pigments resistant to bisulphite bleaching. Somers and Evans (1977) also used a dilution of wine in 1M HCl to push the anthocyanin equilibrium to the flavylium form and break up any possible colour enhancement by copigmentation to measure the total amount of potential colour.

To express spectrophotometric absorbance measurements as amounts, the Beer-Lambert Law is used, which states that $\log_{10}\frac{I_0}{I} = \varepsilon c l$. I_0 and I are the intensities of the incident and transmitted light respectively, l is the path length of the absorbing solution in centimetres, and c is the concentration in moles per litre. $Log_{10}\frac{I_0}{I}$ is defined as the absorbance (A) or optical density (OD); ε is known as the molar extinction coefficient and has units of 1000 cm^2mol^{-1}.

Various spectrophotometric and spectroscopic methods have also been used to determine the total polyphenol content of wine. The Folin-Ciocalteu method has been the one of general preference (Singleton and Rossi 1965), usually with an arbitrary standard such as gallic acid. The Folin-Ciocalteu determines total phenols (and other easily oxidised substances) by producing a blue colour from reducing yellow heteropolyphosphomolybdate-tungstate anions. Somers and Evans (1977) used the expression $OD_{280} - 4$, as an index of total polyphenols in red wine. Bakker *et al*. (1986) compared the methods used by Singleton and Rossi (1965) and Somers and Evans (1977) and found a correlation coefficient of 0.956.

To obtain numerical data directly related to perceived colour; reflectance or transmittance values have to be converted into tristimulus values using the CIE method first published in 1931. A more recent development in colorimetry is the 1976 CIELAB uniform colour space and colour difference equation, which is the most accurate method of quantifying the perceived variables of colour. Almela and Javaloy (1996) found that CIELAB systems achieved better differentiation compared to the tristimulus system. Bakker *et al*. (1986) showed that there were many high correlations between the tristimulus parameters and the traditional analysis at two wavelengths determined on young ports, e.g. between a* (+a, redness) and A_{520nm} (r = 0.975), L* and colour density (r = -0.987) and total pigment (r = -0.906). Colour saturation, which has heavy weighting of the dominant colour redness in these young ports, also correlated well with colour density (r = 0.946). There was zero correlation between b* (+b, yellowness) and A_{420nm} (r = 0.002) and as a result the correlation between tint, the traditional measure of brownness, and hue angle was relatively weak (r = 0.711). Unlike redness, which has a clearly defined λ_{max}, brownness is more difficult to measure in young port wines since it has no clear spectral peak; the wavelength chosen for measurement (420 nm) is arbitrary. Hence, CIELAB 76 gives the more meaningful measure of brownness in wines. Lab hue angle tan b/a, correlates well with sensory perception of brownness while L correlates with intensity assessments (Bakker *et al.* 1993).

1.4.2 Chromatographic and acid hydrolysis analyses

Several methods have been developed to obtain molecular information about proanthocyanidins. Cleavage techniques rely on the conversion of proanthocyanidins into their

constitutive subunits via acid catalysis in the presence of a nucleophilic agent such as phloroglucinol (Czochanska *et al*. 1979; Koupai-Abyazani *et al*. 1992; Pérez-Ilzarbe *et al*. 1992) or benzyl mercaptan (Escribano-Bailón *et al*. 1992; Geiss *et al.* 1995; Guyot *et al.* 1997; Shen *et al.* 1986; Thompson *et al*. 1972). The degradation allows the distinction between terminal units (released as flavan-3-ols) and extension units (released as their benzylthioethers or phloroglucinol adducts) and thus, gives a measure of the mean degree of polymerisation (mDP). Also, with the additional knowledge of subunit composition, the mass-average molecular mass can be obtained. Matthews *et al*. (1997) showed that degradation with benzyl mercaptan (thiolysis) gave a four times higher yield than phloroglucinol, but benzyl mercaptan has a strong unpleasant odour and must be used in a fume hood, whereas phloroglucinol is odourless. A few years later Kennedy and Jones (2001) optimised the procedure using phloroglucinol before comparison with benzyl mercaptan as a nucleophile. They determined that the phloroglucinol-based assay were as effective as the less convenient benzyl mercaptan-based assay. The ester linkage of gallic acid are retained during hydrolysis and enzymatic hydrolysis with tannase are used to release gallic acid (Prieur *et al.* 1994; Ricardo da Silva *et al.* 1991b). Prieur *et al*. (1994) found that the galloylated moieties became less and less accessible to enzymatic hydrolysis as the polymer size of the procyanidin fraction increased. Unfortunately, Roux *et al*. (1998) found that thiolysis, failed to cleave A type proanthocyanidins, which means that their contribution to mDP will not be measured.

Pigment fractions obtained from model wine and wine have also been analysed by acid catalysis in the presence of a nucleophile. Es-Safi *et al*. (1999b) conducted thiolysis on an anthocyanin-ethyl-epicatechin dimer isolated from a model wine study. They found after complete thiolysis, free (-)-epicatechin and two thiol products. The spectra of these compounds have absorption maxima in the visible region, indicating that the flavylium chromophore was still present in both. This means that the cleavage took place between (-)-epicatechin and the bridge, which was also confirmed by the absence of free malvidin-3-*O*-glucoside and the fact that the obtained thiol derivatives were different from those released from ethyl-linked (-)-epicatechin dimers (Es-Safi *et al.* 1999a). This agreed with the findings of Oszmianski and Moutounet (1996) that the linkage between (-)-epicatechin and the bridge is more sensitive to thiolysis than that between the ethyl-group and malvidin-3-*O*-glucoside. It was also found that the anthocyanin-ethyl-flavanol adducts were more stable than their flavanol equivalents. The two thiol derivatives corresponded to R and S isomers caused by the

presence of an asymmetric carbon atom. Es-Safi (1999b) have determined that malvidin-3-*O*-glucoside was released by thiolysis in the underivatised form when it was present in a terminal position (linked through its C-6 or C-8 carbon) in the initial tannin structures (T-A). This implies that malvidin-3-glucoside reacted as a nucleophile, and thus as the hemicetal, which is known to be the predominant form at wine pH. Salas *et al*. (2004) showed that the covalent A-T structures did not react with benzyl mercaptan and remained intact. This is in agreement with the findings of Remy-Tanneau *et al*. (2003).

Complete acid hydrolysis of procyanidins with butanol and hydrochloric acid yield the resulting anthocyanidins and can also be used as an indication of chain length but is not as specific or sensitive as acid catalysis in the presence of excess nucleophile (Porter *et al.* 1986; Powell and Clifford 1995).

Gel permeation chromatography (GPC) methods have been developed for proanthocyanidin analysis and give information on their average molecular weight (M_w) while providing distribution information. Early methods have relied on pre-derivatisation of proanthocyanidins prior to analysis to eliminate interaction between phenolic functional groups and GPC packing material (Williams *et al.* 1983). Viriot *et al.* (1994) investigated the different derivatisation methods namely, underivatised (Bae *et al.* 1994), permethylated (Tanaka *et al.* 1985) and peracetylated (Williams *et al.* 1983) and found that the best results were obtained with the peracetylated derivatives. More, recently, direct analysis of proanthocyanidins has been shown to be successful as well (Kennedy and Taylor 2003). Pigmented phenols will however be more polar then the proanthocyanidins which may result in more interaction with the packing material. GPC gives somewhat higher degree of polymerisation (DP) values than acid catalysis in the presence of an nucleophile which can be attributed to the globular structures of the polymers opposed to linear structures, which results in larger molecular weight values (Koupai-Abyazani *et al.* 1992; Prieur *et al.* 1994). Kennedy and Taylor (2003) investigated the relationship between the average molecular mass of proanthocyanidins determined by acid-catalysed cleavage in the presence of excess phloroglucinol and the average molecular mass as determined by the GPC method using unmodified proanthocyanidins. Assuming a constant composition for the two samples, they determined that the elution time corresponding to 50% mass elution is in good agreement ($R^2 = 0.984$) with the mDP determined by acid catalysis.

1.4.2.1 Mass spectrometry analyses

Electrospray ionization (ESI) is the mass spectrometry method most commonly used for the identification of phenolic compounds. Polyphenols are weakly acidic compounds, indicating that dissociation is easier than protonation. Therefore negative ion mode should be preferred. The positive mode is most satisfactory for detection of anthocyanin compounds, probably because they take the form of the flavylium cation when the pH is low. ESI-MS is used either directly or on line after HPLC separation. A complete series of oligomers, from DP2 to DP9, as well as the corresponding monogalloylated and digalloylated species were detected by ESI-MS analysis of grape seed extract, cider apple, tea and litchi pericarp (Cheynier *et al.* 1997a; Friedrich *et al.* 2000; Hayasaka *et al.* 2003; Prieur *et al.* 1994). Application of ESI-MS made it possible to detect and partly characterise high molecular weight procyanidins in the extracts, which are eluted as unresolved peaks under current HPLC (LC) techniques. Hayasaka *et al.* (2003) found that the DP of the most abundant ion according to ESI-MS did not always correlate well with the DP estimated from acid hydrolysis. MS also does not allow to distinguish between catechin and epicatechin-based structures nor to determine the position of interflavan C-C linkages.

LC-ESI-MS analysis of solutions containing malvidin-3-glucoside, acetaldehyde and (epi)catechin showed the presence of both ethyl-linked flavanol oligomers and anthocyanin-ethyl-flavanol adducts (Fulcrand *et al.* 1996a), meaning that the anthocyanin competed with the flavanol in the addition process. Signals corresponding to dimeric, trimeric and tetrameric species containing one anthocyanin and one, two, or three flavanol units, were detected as the M^+ flavylium ions at m/z 809, 1125 and 1441, respectively. A tetrameric specie containing two anthocyanin and two flavanol units was also found as the doubly charged M^{2+} ion at m/z = 822. Equivalent dimeric and trimeric adducts were formed when malvidin-3-glucoside was replaced with cyanidin-3-glucoside (Saucier *et al.* 1997b). This suggests that a great diversity of products can be generated during wine ageing, their respective levels depending on the nature and relative amounts of flavanols and anthocyanins present.

A wine fraction was analysed by liquid chromatography coupled to electrospray ionization mass spectrometry in the negative ion mode. Various series of ion peaks containing a variable number of trihydroxylated units were detected as monocharged ions from dimers up to

pentamers. From pentamers, oligomers were mostly found as multicharged ions. Heptamer species corresponded to the highest mass detected. These results showed that wine condensed tannins consist of, besides procyanidins, mixed tri- and dihydroxylated flavanol units and also of pure trihydroxylated flavanol units (Fulcrand *et al.* 1999). The estimated mean degree of polymerisation (mDP) for the 3 to 4 year old Cabernet-Sauvignon was 6.4, which was similar to values reported earlier in wine by Sarni-Manchado *et al.* (1999b). The percentage of galloylated units was estimated at 3.3% and that of trihydroxylated units was found at 17.9%. In a general way, signal intensity decreased as the polymerization degree increased. Hayasaka and Kennedy (2003) performed ESI-MS analysis in the positive mode on wine tannin isolated with 50% acetone (0.1% TFA) from Toyoperal TSK HW 40-F size exclusion media. A tailing series of dominant ions separated by a mass of 288 was observed for the molecular ions of the procyanidin dimers to octamers. Other minor ions present appeared in two series, starting with m/z 781 (T-A type) and 783 (A-T) type. Within each of these series, and up to m/z 2509/2511 (T-A/A-T type), ions were separated by a mass of 288. The ions of m/z 781/783 agreed with the direct condensation products of malvidin-3-glucoside and (epi)catechin, as reported previously by Remy *et al*. (2000). To further characterise polymer structures, the product ion spectra of the respective ions were obtained by infusion ES-MS/MS. Procyanidins are predominantly fragmented by cleavage at the interflavonoid bonds to produce sequence ions separated by a mass of 288. To a lesser extent, Retro-Diels-Alder (RDA) fission also occurs on the C-ring of the flavan-3-ol subunit and produces a characteristic ion resulting from the elimination of mass of 152 (Karchesy *et al.* 1986). Anthocyanins are simply fragmented into anthocyanidins as a result of the elimination of a dehydrated sugar moiety with a mass of 162. The ions derived by the cleavage of the interflavonoid bonds, provided good evidence for the proposed structures of T-A and A-T type of anthocyanin-procyanidin polymers.

However, there are some limitations to the application of ESI-MS. Although the mass data obtained generally allow unambiguous molecular weight determination, the interpretation becomes increasingly difficult as the molecular weight increases due to the limited mass range of the quadrupole analyser and frequent overlapping of multiple ions generated from related molecules. The use of other techniques complementary to LC-MS is essential for total characterisation and identification of these complex flavanol polymers.

1.5. SENSORY PROPERTIES OF GRAPE AND WINE PHENOLICS

Phenols in wine contribute significantly to wine character. They play an important role in red wine colour, bitterness, astringency, as well as other tactile or 'mouthfeel' characteristics. The colour and taste changes occurring in the course of wine aging are believed to result from the conversion of grape anthocyanins and proanthocyanidins to other polymeric species. The impact of polymerisation reactions on wine sensory properties is largely unknown.

1.5.1 Contribution of flavonoids to wine bitterness and astringency

Monomeric and polymeric flavan-3-ols are the primary contributors to the astringency and bitterness of red wine (Singleton and Trousdale, 1992). Numerous sensory studies in white wine and model solutions have demonstrated that catechin elicits both bitterness and astringency (Arnold *et al*. 1980; Robichaud and Noble 1990). The intensity of bitterness increased notably faster than the intensity of astringency as the concentration of catechin was raised (Leach and Noble, 1986; Robichaud and Noble 1990).

Thorngate (1995) have showed that the bitterness and astringency of epicatechin and catechin differ, although the only difference between these two monomeric flavan-3-ols is the stereochemistry of the hydroxyl group at position 3 of the heterocyclic C ring. The total duration of astringency and bitterness of epicatechin was much longer than for catechin, while Kallithraka *et al*. (1997b) also found epicatechin more astringent and bitter in comparison with catechin at high concentrations in a model wine solution.

In contrast with catechin the rate of increase of astringency for grape seed fractions and ciders (primarily tetrameric to octameric polymers consisting of catechin and epicatechin units) was faster than for bitterness (Lea 1990; Lea and Arnold 1978). Generally the condensed tannins are more astringent than the smaller oligomers, while the monomers, dimers and trimers evoke more bitterness than the condensed tannins (Arnold *et al.* 1980). This concept has been used to explain the "hardness" of young wines and the "softness" of older wines, as a result of their oligomeric to polymeric procyanidin ratios (Haslam 1980; Lea 1990). Vidal *et al.* (2003a) isolated grape and apple proanthocyanidin fractions and evaluated their astringency properties

in a wine-like medium. Confirming previous results, it was found that astringency increased with degree of polymerisation, becoming more drying. An increases in galloylation of fractions resulted in fractions being rated higher in astringency related terms such as coarseness, while the presence of epigallocatechin units had the opposite effect.

The only apparent difference between pomace-fermented white wines and red wine are the anthocyanin content. When white wines are pomace fermented like red wines, it has been suggested that they result in wines coarser in flavour and less astringent, with different sensory profiles to those of red wine (Singleton and Trousdale 1992). They do not resemble red wine in flavour. Pure anthocyanins have been reported to have only a very mild indistinct taste (Singleton and Noble 1973; Singleton and Trousdale, 1992; Vidal *et al.* 2004a). Singleton and Trousdale (1992) suggested that the incorporation of anthocyanins in polymeric procyanidins is primarily responsible for the distinctive astringency of red wines. Anthocyanins seem to increase the amount of tannin retained in wine. Kantz and Singleton (1991) suggested that the sugar of the anthocyanin and perhaps the polarity of the flavylium cation both increase the solubility and decrease the precipitability of the resultant anthocyanin-bearing tannin molecule. Ribéreau-Gayon and Glories (1980) also reported that these pigmented tannins are less reactive towards proteins and accordingly less astringent. There is little robust evidence to support the role of anthocyanins in the mouthfeel properties of red wine.

1.5.2 Factors affecting perceived astringency and bitterness

Some wine components have astringent sub-qualities themselves or have separate taste qualities which indirectly affect astringency perception by altering the salivary flow rate and composition of the individual's saliva.

Ethanol enhances the intensity and duration of bitterness, whereas varying wine pH and sourness has little or no effect on the perceived bitterness (Fischer *et al.* 1994; DeMiglio *et al.* 2002). In addition Noble (1990) has shown that ethanol suppresses the astringency of phenols, by possibly making them less available for hydrogen bonding, while raising acidity (and perceived sourness) seems to increase the intensity (Guinard *et al*. 1986; Kallithraka *et al.* 1997a) and duration (Fischer *et al*. 1994) of astringency. Model studies have also

demonstrated that ethanol has a depressive effect on the precipitation of condensed tannins by proline-rich peptides, probably by increasing the solubility of the protein-polyphenol complex (Yokotsuka and Singleton 1987). Astringency was also reduced by an increase in viscosity, while bitterness was not affected (Ishikawa and Noble 1995; Lyman and Green 1990). It is speculated that the sensation of astringency decreases as viscosity is raised because it counteracts the reduction in salivary lubrication produced by tannins binding to the salivary proteins.

The lubricating properties of saliva primarily arise from the existence of mucoproteins (mucins) and glycosylated proline rich proteins (GPRP's). All PRP's have a strong affinity for polyphenols, as a result of their characteristic extensive proline rich repeat sequence (Hagerman and Butler 1980). The bulkiness of the N-methylene group of proline restricts the conformation of the amino acid residue preceding proline, resulting in an open and flexible protein structure which fosters access of phenyl residues to the peptide bonds (Luck *et al.* 1994). The association between polyphenols and salivary proteins result from hydrophobic interactions between the aromatic portion of the phenols and the non-polar amino acid side chains such as phenylalanine, as well as hydrogen bonding between the phenolic hydroxyl and the protein amide carbonyl (Hagerman and Butler 1980; Oh *et al.* 1980). Generally the capacity of polyphenols to bind proteins increase with the degree of polymerisation (Lea and Arnold, 1978) and galloylation (Ricardo da Silva *et al.* 1991a; Yokotsuka and Singleton 1995). The pattern of hydroxylation on either the A or B rings of condensed tannins influences protein interaction, explaining the higher affinity of prodelphinidins (3 hydroxyl groups) for proteins, than procyanidin (2 hydroxyl groups) (Cheynier *et al.* 1992).

The influence of procyanidin structure on their interactions with various proteins, including classical oenology fining agents, a grape arabinogalactan protein (Ricardo da Silva *et al.* 1991a) and poly(L-proline) was investigated in wine-like model solutions (Cheynier *et al.* 1992; Saulnier and Brillouet 1989). The affinity of procyanidins towards polyprolines, casein and gelatins appeared essentially determined by the number of *o*-substituents and also with the degree of polymerisation of the polyphenols. The influence of the interflavanic linkage position was also important, as oligomers containing a C4-C6 bond showed a higher affinity for proteins than those containing just C4-C8 linkages (Saulnier and Brillouet 1989). This indicates that the shape and flexibility of these molecules allowed easier access of the proteins

to the *o*-dihydroxyphenyl reactive sites. The affinity of proteins for phenols is also considerably influenced by the amino acid composition and structure of peptides and their interactions with phenols is not linear, which indicates multiple binding sites (Yokotsuka and Singleton 1995).

1.5.3 Sensory analysis of astringency attributes

Astringency has been shown to be a multiple perceptual phenomenon. Lawless *et al*. (1994) subdivided the astringent sensation into rough, dry and puckery notes. These attributes were defined as: 1. 'drying', the lack of lubrication or moistness resulting in increased friction between oral surfaces 2. 'roughening', unsmooth texture in the oral cavity marked by inequalities, ridges and/or projections felt when the oral surfaces come in contact with one another 3. 'puckery', the drawing, and/or hightening sensation felt in the mouth, lips and/or cheeks. The mouthfeel properties of beverages have received comparatively little attention. During descriptive analysis of wines, flavour intensity, body, bitterness and astringency are sometimes rated in addition to their aroma (Guinard and Cliff 1987; Noble *et al.* 1987; Reynolds *et al.* 1996a, b). The mouthfeel attributes of beer has been investigated and density, viscosity, oily mouthcoat, astringency and stickiness were some of the terms used (Langstaff *et al*. 1991).

Descriptive analysis (Meilgaard *et al*. 1991; Stone *et al*. 1974) is the preferred scoring technique for the sensory evaluation of wine attributes. It involves the scoring of the intensity of defined attributes by a trained panel. While this method has been applied mainly to studies on wine aroma, it is also suitable for scoring of palate attributes. An extension of this method is to continuously score an attribute after tasting a wine, to produce a time-intensity curve so that parameters such as the length and the maximum intensity of astringency can be measured (Noble 1995). In order to effectively study the impact of polyphenolic compounds on red wine sensory properties, it is necessary to carefully define attributes to be scored by a panel. These terms should preferably be defined by references or physical standards (Muñoz 1986; Rainey 1986). At the beginning of the study described in this thesis, there was little work published regarding the assessment of red wine palate or mouthfeel properties. However, as a result of research completed in collaboration with Gawel (2000), discussed in this thesis (chapter 5)

literature describing and defining the mouth-feel properties of wine has been published. Subsequent studies have used the developed mouth-feel wheel to evaluate the contribution of different phenolic fractions to the mouth-feel character of a wine, as well as a tool to differentiate between different wines and panellists (Pickering and Robert 2006; Vidal *et al.* 2003a, 2004b). The 'texture' of a wine influences the overall perception of quality.

1.6 CONCLUSION

Grape anthocyanins and flavanols are particularly important among wine phenolics, as they are responsible for red wine colour and the formation of polymeric pigments during the aging of wine. Considerable advances have been made in elucidating the structures and distribution of polyphenols in grapes. A number of reactions involving anthocyanins and tannins have also been demonstrated in model systems. The role of native and derived polyphenols on wine organoleptic properties is speculative. In addition, the relationship that exists between the structure and taste (e.g. astringency, bitterness) of various procyanidins and anthocyanin-procyanidin-derived molecules co-existing in wines remain to be established.

Chapter 2

PIGMENT TRANSFORMATIONS IN MODEL SYSTEMS

2.1 INTRODUCTION

During the storage and ageing of red wines, progressive changes occur in the phenolic composition of the wine. There have been many studies in model solutions on the mechanisms involved in these complex transformations, as well as on the structures of the resulting compounds (Bakker *et al.* 1993; Es-Safi *et al.* 1996, 1999b; García-Viguera *et al.* 1994; Pissarra *et al.* 2004; Romero and Bakker 1999; Salas *et al.* 2004; Santos-Buelga *et al.* 1995; Zimman and Waterhouse 2004). Among these transformations, the decrease of astringency is considered to be the result of anthocyanin-flavanol condensation either involving acetaldehyde or not.

The polymerisation reactions between anthocyanins and flavanol monomers and dimers have been extensively studied in model wine systems (Dallas *et al.* 1996a, b, 2003; Oszmianski and Moutounet 1996). The composition of a model wine mimicks that of a real wine and consists of a saturated potassium bitartrate solution containing 12% ethanol adjusted to pH 3.2 to 3.6. Dallas and co-workers (1996b) found that a procyanidin dimer alone, when added to a model wine, was partly transformed into a monomer and trimer and more complex compounds after two weeks, showing that carbon-carbon bond breaking and carbon-carbon bond forming of procyanidins can occur at wine pH. It was also shown that reactions between the carbocations resulting from cleavage of tannin interflavanic bonds and anthocyanins do occur in wine, even though catechin and tannin preferentially react with each other (Dallas *et al.* 1996b, 2003; Fulcrand *et al.* 1996a; Thorngate and Singleton 1994; Timberlake and Bridle 1976). Similar reactions may arise between flavanols and glyoxylic acid, which arose from oxidation of tartaric acid in the presence of air (Es-Safi *et al.* 2000; Fulcrand *et al.* 1997). Escribano-Bailon *et al.* (1996) detected the presence of 4-8 and 4-6 linked pigments only after 4 months in model systems at pH 2, which is in agreement with the findings of Dallas *et al.* (1996a, b). In solutions of malvidin-3-glucoside and catechin to which acetaldehyde was not added, direct condensation between anthocyanin and catechin occured very slowly and finally resulted in the formation of yellowish pigments whose spectra had maxima at wavelengths ranging between 425 to 450

nm (Dallas *et al.* 1996a; Escribano-Bailòn *et al.* 1996; Santos-Buelga *et al.* 1995; Thorngate and Singleton 1994). It should be noted that Santos-Buelga *et al.* (1995) found that the pigments formed, at least at the 30 days point of observation, did not seem to show a tendency to polymerise further but rather, once a maximum has been reached, remain stable.

Santos-Buelga and colleagues (1995) found a greater loss of malvidin-3-glucoside and catechin at pH 3.2 than pH 4, with the addition of air, in a model system. There was also a greater loss at 32ºC compared to 15ºC and new pigments only appeared after two weeks according to analysis by RP-HPLC. Dallas *et al.* (2003) studied the transformation of procyanidins in model wine solutions and determined, in agreement with Santos-Buelga *et al.* (1995) that a decrease in pH, an increase in temperature and the presence of acetaldehyde, increased the rate of polymerisation. When the procyanidin dimers (B1, B2, B3, B1 3-*O*-gallate, B2 3'-*O*-gallate) and trimers (C1, T1) were compared it was established that the length of the molecule determined transformation rate, with trimers reacting more rapidly than the dimers (Fulcrand *et al.* 1996a). For the same degree of polymerisation, the presence of catechin in the oligomer seemed to increase their stability when compared to the same molecule without catechin, while in the case of the monomers epicatechin reacted faster than catechin (Es-Safi *et al.* 1999a).

With the addition of acetaldehyde to model systems containing anthocyanins and flavanols, new pigments were observed within hours to days after addition and could be isolated after two weeks (Escribano-Bailòn *et al.* 1996; Francia-Aricha *et al.* 1997). Similar results were reported by others (Bakker *et al.* 1993; Dallas *et al.* 1996a, b; García-Viguera *et al.* 1994; Oszmianski and Moutounet 1996; Rivas-Gonzalo *et al.* 1995). Es-Safi *et al*. (1999b) characterised the derivatives formed during a model wine study where epicatechin, malvidin-3-*O*-glucoside and acetaldehyde was added and found ethyl-linked anthocyanin-flavanol as well as flavanol oligomers. This shows that epicatechin and malvidin-3-*O*-glucoside competed in the condensation process. Saucier *et al*. (1997c) isolated an ethyl-linked pigment from red wine, thus proving the occurrence of this condensation pathway.

Singleton and Trousdale (1992) conducted a study to elucidate the reasons for the difference between white wine made like a red wine and a red wine when the only difference between the wines is the anthocyanins. In their study, three white table wines were treated with additions of different amounts of purified anthocyanin and grape seed

tannin. They concluded that the anthocyanins quickly complexed with other phenolics to retain them in solution. The lack of such complexing explains why wines made from white grapes by red vinification methods contain less polymeric phenols or tannins and astringency.

The investigations of Singleton and Trousdale (1992) were repeated in this present study to ascertain that polymerisation reactions in model solution could form significant amounts of pigmented polymers within a relative short period of time through interaction between anthocyanins and tannin. This was a preliminary study to determine whether it would be possible to up-scale the experiment for the generation of larger quantities of pigmented polymers for sensory studies. The different parameters that influence the rate of polymerisation, such as temperature, pH, anthocyanin and flavanol concentration were also investigated.

2.2 MATERIALS AND METHODS

2.2.1 Preparation of grape seed extract

Grape seed extract (36.27 g) was isolated from Muscat Gordo Blanco grapes (40 kg). Seeds (100 g) were homogenised with 70% ethanol acidified with 0.2% acetic acid (700 mL). The resulting mixture extracted for one hour in the presence of 50 mg/L sulfur dioxide, to protect the extracted phenolics against possible oxidation. At the end of the hour the slurry was centrifuged (11000 *g*, 45 min, 4°C). The supernatant was concentrated on a rotary vacuum evaporator at 35°C and lyophilized. The concentration of total phenols in this grape seed extract was 420 mg gallic acid equivalents per gram determined by the Folin-Ciocalteu method as described in section 2.2.2.

2.2.2 Determination of the phenolic content of the extract by Folin-Ciocalteu

The phenolic content of the grape seed extract was determined by the Folin-Ciocalteu method, as modified by Singleton and Rossi (1965). The Folin-Ciocalteu reagent was obtained from Sigma-Aldrich, (Sydney, Australia). A 1:10 dilution of the sample (1 mL) was mixed with water (60 mL), Folin-Ciocalteu reagent (5 mL) and 20% aqueous sodium carbonate (15 mL) and made up to volume in a 100 mL volumetric flask. After 2 hours at

room temperature the absorbance was read at 765 nm in a 10 mm cell (Varian Techtron Model 635D spectrophotometer). The total phenolic content was calculated in gallic acid equivalents by comparison with a standard curve similarly prepared with 0, 50, 100, 150, 250 and 500 mg/L gallic acid (Fluka Chemie AG, Buchs, Switzerland) standard.

2.2.3 Isolation of anthocyanin extracts

2.2.3.1 Methanol extraction

Grape skins from Shiraz grapes (2.26 kg) were extracted with 50% methanol acidified with 0.2% acetic acid, containing 15 mg/L sulfur dioxide. The mixture was centrifuged (2700 *g*, 45 min, 4ºC) and the supernatant concentrated under rotary vacuum evaporation at 25ºC. The extract was loaded onto a Sephadex LH-20 column and washed with water to remove sugars and acids, followed with the elution of the anthocyanins with 60% methanol containing 0.2% acetic acid according to the method of Kantz and Singleton (1990). The column was further washed with 50% acetone to remove phenolic material still retained by the stationary phase. The retained colour consisted of mainly anthocyanin-*p*-coumarates and other unknown pigments. The amount of anthocyanin in the extract (6.43 g) was determined by RP-HPLC on a PLRP-S reverse phase column (section 2.2.4) as malvidin-3-*O*-glucoside equivalents.

2.2.3.2 Iso-amyl alcohol extraction

Shiraz berries were pressed and the skins covered with iso-amyl alcohol and left to extract for 24 hours. The iso-amyl alcohol was then decanted from the skins and concentrated by rotary evaporation at reduced pressure at 35ºC with water added to act as azeotrope. The dissolved anthocyanins were crystallised with the addition of ether and cooling of the mixture to 4ºC for at least 12 hours. The precipitated crystals were dissolved in methanol and re-crystallised with the addition of ether as before. The anthocyanin extract was re-crystallised 5 times and the purity determined by RP-HPLC analysis as described in section 2.2.4, using a PLRP-S column.

2.2.4 HPLC analysis

The HPLC apparatus were a Waters (Milford Massachusetts, USA) and Hewlett Packard Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) with binary pumps and diode array detectors. The method of Singleton and Trousdale (1992) with Solvent A (0.05 M NH_4HPO_4 in water, pH 2.6) and Solvent B (20% A mixed with 80% acetonitrile) used on a Licrosorb C_{18} column (250 × 4.6 mm, 5 µm; Merck, Darmstadt, Germany) was investigated. Elution conditions were as follows: flow-rate at 1 mL/min, isocratic for 5 min at 100% A, linear gradient to 85% A in 15 min, to 77.5% A in 15 min, 37.5% A in 10 min, 0% A in 10 min, and then back to the starting conditions. A newly developed HPLC method discussed in detail in section 3.3.2 was also used for analysis. It is only briefly described here. A binary solvent system with Solvent A as 1.5% H_4PO_4 in water, pH 1.4, and Solvent B as 20% A mixed with 80% acetonitrile, was used on a PLRP-S column (250 × 4.6 mm, 5 µm; Polymer labs, UK). Elution conditions were as follows: flow-rate at 1 mL/min, linear gradient from 93.8% A to 72.5% A in 70 min, isocratic at 72.5% A for 3 min, followed with a linear gradient to 37.5% A in 5 min, staying isocratically at 37.5% A for 6 min and back to 93.8% A in 6 min. In both methods, column temperatures were held at 35ºC, and the columns were re-equilibrated with the starting solvent conditions for 10 min between runs. Peak areas were determined and mostly used for comparison between the different treatments. The concentration of the anthocyanins and polymeric pigments were calculated as mg malvidin-3-glucoside equivalents per liter at 520 nm. Malvidin-3-*O*-glucoside from Roth (France) was used as authentic reference.

2.2.5 Ultrafiltration

Ultrafiltration was performed in 400 mL capacity stirring cells (Waite Campus Engineering Workshop, SA, Australia) at 4ºC under a nitrogen pressure of about 400 kPa. Cells were equipped with Diaflo YM 10 (10 k Molecular Weight Cut-off) membranes from Amicon Ltd (MA, USA). Filters were stored in 70% EtOH and soaked for 30 min in a 0.1 M NaOH solution and then flushed with water, before use.

2.2.6 Experiments

All experiments were done in duplicate.

2.2.6.1 Experiment A

A Semillon white wine (Nuriootpa, South Australia, two years old) with a pH of 3 was separated into three portions of 150 mL each. One fraction was prepared without addition of anthocyanin extract, another with the isolated anthocyanin extract (prepared as described in section 2.2.3.1) added at 250 mg/L and a third with the anthocyanin extract added at 500 mg/L. Portions 2 and 3 were mixed until the pigment was uniformly dissolved and the pH verified as unchanged. From each of these three samples, sub-portions were treated with 0, 500 and a 1000 mg GAE/L (gallic acid equivalents per liter) of grape seed tannin (section 2.2.1). The pH was unchanged. The final nine samples were placed in 50 mL Schott-bottles, the headspace replaced with nitrogen and the mixture constantly swirled on a shaker at 25ºC in the dark for 14 days, preceding analysis by RP-HPLC (section 2.2.4).

2.2.6.2 Experiment B

Four different white wine bases (Chardonnay, Sultana/Riesling blend, Semillon Pressings and Semillon – all Barossa valley, two years old) were ultrafiltrated through a 10 kDa membrane (see section 2.2.5) to remove high molecular weight compounds like proteins, polysaccharides and polymeric phenols. Of each white wine base, one portion was prepared without addition of pigment and another with the isolated anthocyanin extract (prepared as described in section 2.2.3.2) added at 625 mg/L malvidin-3-glucoside equivalents. Sub-portions were treated with a 1000 mg/L GAE/L seed tannin. Grape seed tannin was prepared as described in section 2.2.1, except that the extract was first loaded onto a C_{18} column and washed with water to remove proteins and polysaccharides before it was lyophilized. Samples of the respective mixtures were sealed in ampoules and heated at 45ºC for 1 month. The polymeric material formed was investigated by RP-HPLC (section 2.2.4).

2.2.6.3 Experiment C

Due to the variability in the yield of polymeric phenols in the use of different base wines, it was decided to investigate polymerisation in a model wine system. The slow reaction rate of direct polymerisation has also prompted us to additionally study polymerisation in the presence of acetaldehyde. A model wine solution (12% v/v aqueous EtOH, saturated with potassium bitartrate, adjusted to pH 3.4 with HCl) was divided into two parts. The anthocyanin extract (prepared as described in section 2.2.3.2) was added to one part at 625 mg/L malvidin-3-glucoside equivalents while no anthocyanin was added to the second part. Sub-portions were treated with a 1000 mg/L GAE/L seed tannin. Samples were prepared with and without the addition of 25 mg/L acetaldehyde and sealed in ampoules as described above, stored at 18ºC and analysed at two week intervals over 12 weeks by RP-HPLC. This experiment was repeated at pH 1 and 2 because the reaction between anthocyanins and flavanols mediated by acetaldehyde depends on the formation of a carbocation and should thus be influenced by pH.

2.3 RESULTS

2.3.1 Experiment A

The anthocyanin addition of 250 mg/L and 500 mg/L to the white wine is similar to the amount of anthocyanins present in a lighter style red wine and deeply coloured medium bodied red wine, respectively, while the 500 and 1000 GAE/L addition of grape seed tannin was comparable with wines containing 1.19 and 2.38 g/L phenolic material (Figure 1).

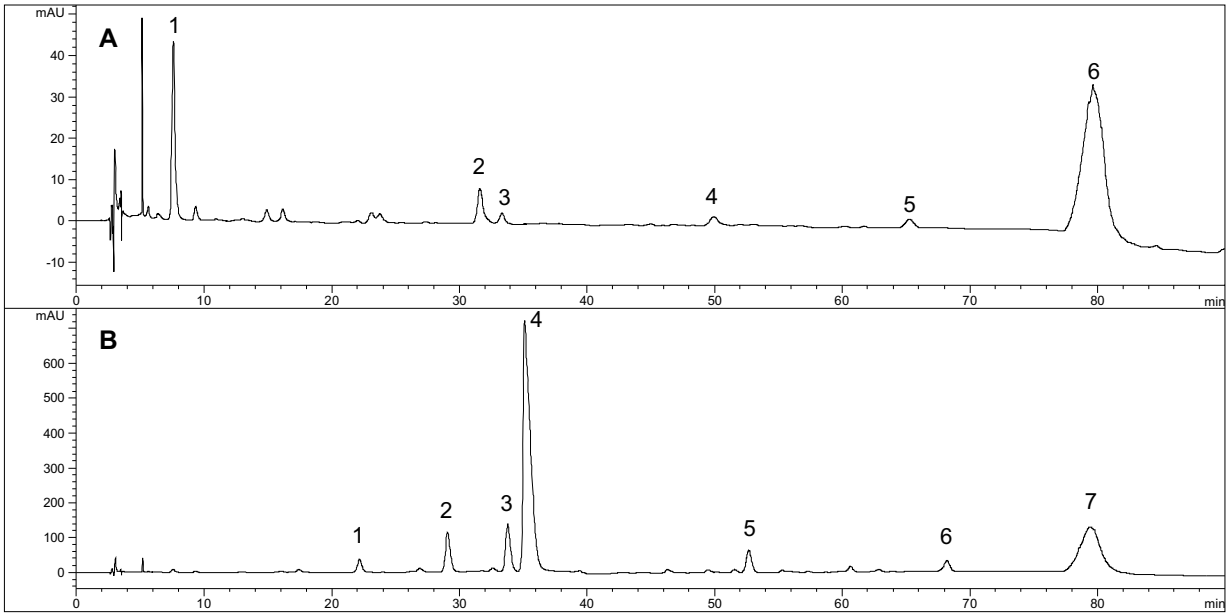


Figure 1. RP-HPLC chromatogram at 280 nm of (A) 1000 mg GAE/L grape seed tannin with peak 1 = gallic acid; 2 = epicatechin; 3 = procyanidin trimer; 4 = procyanidin trimer; 5 = monogalloyl procyanidin trimer; 6 = polymeric phenol and at 520 nm (B) 500 mg/L anthocyanin extract added with peak 1 = delphinidin-3-glucoside; 2 = petunidin-3-glucoside; 3 = peonidin-3-glucoside; 4 = malvidin-3-glucoside; 5 = malvidin-3-(6-acetyl)-glucoside; 6 = malvidin-3-(6-*p*-coumaroyl)-glucoside; 7 = polymeric pigment, to a model wine solution.

Table 1 and 2 show the polymeric phenol and pigment peak areas respectively, as determined by RP-HPLC for the samples after 14 days reaction time at 25°C between anthocyanins and grape seed tannin. The addition of anthocyanin did not increase the polymer concentration indicating a lack of solubility of the added anthocyanin and anthocyanin condensation products in the white wine. The addition of grape seed tannin (GST) did increase the polymer concentration. The addition of both anthocyanin and seed tannin increased the concentration of polymer over and above the expected increase. The lower amount of polymeric phenols observed when a 1000 and 500 mg/L of tannin and anthocyanins were respectively added, compared to when a 1000 and 250 mg/L of tannin and anthocyanin were added indicate precipitation of the polymeric phenols. The increase in the absorbance of the polymeric pigments for the 1000 and 500 mg/L tannin anthocyanin additions that are still observed are most probably the result of the stronger absorbance of the new pigments compared to the equivalent anthocyanins although the concentration is likely less.

Table 1. The polymeric phenols measured at 280 nm as a function of tannin and anthocyanin pigment addition to white wines as determined by RP-HPLC after 14 days.

Pigment added	Tannin added (mg GAE/L)		
(mg/L)	**0**	**500**	**1000**
0	0[†]	5174	13389
250	449	6949	15175
500	503	7970	13838

[†]The area (205 AU) of the polymeric phenol peak obtained for the control sample was subtracted from the values obtained for experimental samples with anthocyanin and/or grape seed tannin added.

Table 2. The polymeric pigments measured at 520 nm as a function of tannin and anthocyanin pigment addition to white wines as determined by RP-HPLC after 14 days.

Pigment added	Tannin added (mg GAE/L)		
(mg/L)	**0**	**500**	**1000**
0	0[†]	6	36
250	76	105	136
500	103	125	441

[†]The area (8 AU) of the polymeric pigment peak obtained for the control sample was subtracted from the values obtained for experimental samples with anthocyanin and/or grape seed tannin added.

The total phenol content calculated by Folin-Ciocalteu using a gallic acid standard (Table 3), for the model wine prepared with no added purified anthocyanin and 500 GAE/L tannin was 762 mg/L after 14 days, consisting of 391 mg/L from the base wine and the remaining 371 mg/L from the added tannin. This result indicates incomplete solubility and/or precipitation of the seed tannin.

Table 3. The total phenols (mg GAE/L) found as a function of tannin and anthocyanin pigment addition to white wines determined by Folin-Ciocalteu after 14 days.

Pigment added	Tannin added (mg GAE/L)		
(mg/L)	**0**	**500**	**1000**
0	0[†]	371	888
250	99	405	633

500	282	510	655

†The amount (391 mg GAE/L) determined for the control sample was subtracted from the values obtained for experimental samples with anthocyanin and/or grape seed tannin added.

2.3.2 Experiment B

Of the wines tested only the Sultana and Riesling blend white wine base did not result in the formation of a precipitate, while only a small amount of precipitate was formed when the Semillon pressings was used as a base (Table 6 and 7). Substantial differences were observed in the polymerisation results of the different white wine bases. Composition of the base wine thus had a significant effect on the polymerisation reactions.

Table 6. The polymeric phenols measured at 280 nm as a function of tannin and anthocyanin pigment addition to different white wines by RP-HPLC after 30 days.

White wine base	Pigment added	Tannin added (mg GAE/L)	
	(mg/L)	**0**	**1000**
Chardonnay††	**0**	0†	702
	625	547	1337
Sultana/Riesling	**0**	0†	460
	625	320	990
Semillon Pressings††	**0**	0†	1337
	625	837	2007
Semillon††	**0**	0†	289
	625	289	779

†The areas (103, 80, 93, 81 AU) of the polymeric phenol peaks obtained for the control samples (Chardonnay, Sultana/Riesling, Semillon Pressings, Semillon) were subtracted from the values obtained for experimental samples with anthocyanin and/or grape seed tannin added.

†† Precipitate observed.

Table 7. The polymeric pigments measured at 520 nm as a function of tannin and anthocyanin pigment addition to different white wines by RP-HPLC after 30 days.

White wine base	Pigment added	Tannin added (mg GAE/L)

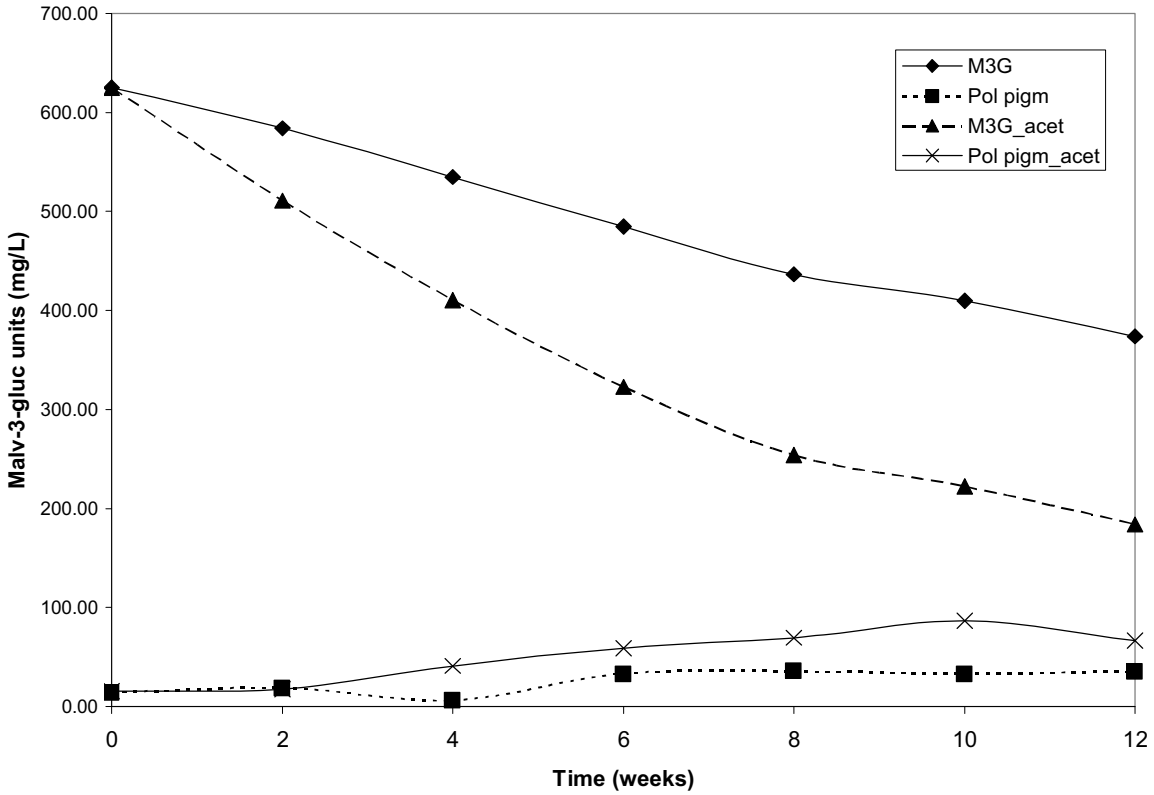
	(mg/L)	**0**	**1000**
Chardonnay††	**0**	0†	26
	625	164	255
Sultana/Riesling	**0**	0†	52
	625	108	194
Semillon Pressings††	**0**	0†	66
	625	256	386
Semillon††	**0**	0†	8
	625	95	189

†The areas (6, 6, 4, 1 AU) of the polymeric pigment peaks obtained for the control samples (Chardonnay, Sultana/Riesling, Semillon Pressings, Semillon) were subtracted from the values obtained for experimental samples with anthocyanin and/or grape seed tannin added.

† †Precipitate observed.

2.3.3 Experiment C

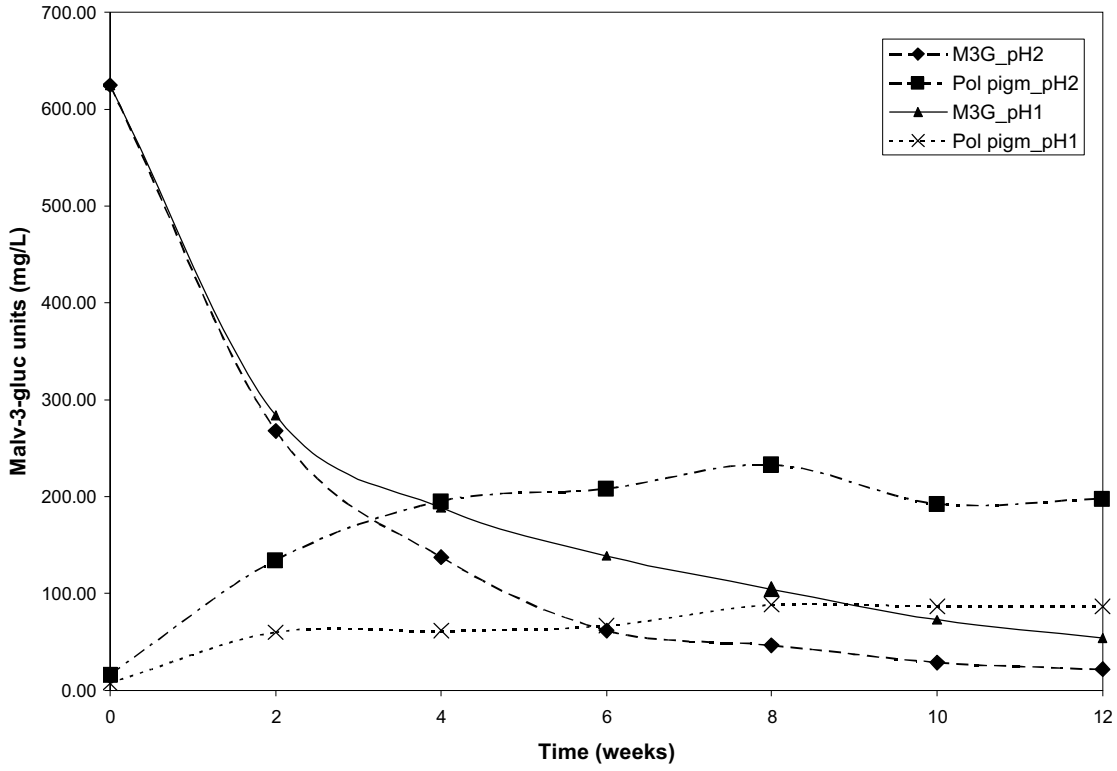
The malvidin-3-glucoside concentration decreased over time as expected with a simultaneous increase in polymeric pigment concentration (Figure 2). This decrease in malvidin-3-glucoside and increase in polymeric pigment concentration was greater in the presence of acetaldehyde.



M3G = malvidin-3-glucoside; Pol pigm = Polymeric pigments; M3G_acet = malvidin-3-glucoside in the presence of acetaldehyde; Pol pigm_acet = polymeric pigments in the presence of acetaldehyde

Figure 2. Changes in the concentration of malvidin-3-glucoside and polymeric pigments over time in model wine systems at pH 3.4, in the presence and absence of acetaldehyde.

From Figure 3 it is clear that polymerisation reactions were faster at pH 2 then at pH 1. The decrease in malvidin-3-glucoside in the presence of acetaldehyde at pH 2 was also greater then at pH 3.4 (Figure 2). This is also true for the increase in polymeric pigments.



M3G = malvidin-3-glucoside; Pol pigm = Polymeric pigments

Figure 3. Malvidin-3-glucoside and polymeric pigment changes in model wine systems containing malvidin-3-glucoside, seed tannin and acetaldehyde at pH 1 and pH 2 over time.

2.3. DISCUSSION

The purified anthocyanin extract added to the model wine systems were comparable with the anthocyanin extract used by Singleton and Trousdale (1992). When the polymeric phenol and pigment content of samples from experiment A (Table 1 and 2) were evaluated after two weeks, it was clear that the model system with only 500 mg/L anthocyanin extract added showed a decrease compared to only 250 mg/L anthocyanin extract added. Singleton and Trousdale (1992) did not see this. The results in Table 1 show that, up to a 1000 mg/L of grape seed tannin was soluble. The increase seen in polymeric pigment peak area when grape seed tannin was added (Table 2) is probably the result of polymerisation reactions that have taken place during the two week period and the formation of xanthylium salts that increase maximally at around 440 nm but would also result in an increase in the absorbance at 520 nm (Timberlake and Bridle 1976). The largest increase in

polymeric pigment concentration was observed for the 500 mg/L anthocyanin and 1000 mg/L grape seed tannin addition. Differences between the amount of polymeric pigments and phenols formed for the 250, 500 mg/L anthocyanin and 500, 1000 mg/L tannin combinations with a pigment to tannin ratio of 1:2 and a molar ratio closer to 1:3, indicated that it is not only the ratio but also the actual amounts that have an influence on polymerisation. The 250, 500 mg/L anthocyanin to tannin combination formed respectively 128 and 123% of the expected polymeric pigment and phenol, while the 500, 1000 mg/L combination formed 317 and 99.6 % of the expected amounts.

Generally, the total phenol determination according to Folin-Ciocalteu indicated a loss of phenols possibly due to precipitation (Table 3). This is in contrast with the results obtained by Singleton and Trousdale (1992). It has been speculated that the ratio of non-pigmented to pigmented phenols are important for the formation of stable pigmented polymers. The suggested ratios have been mostly four to two non-pigmented to one pigmented phenol. During this study it was found that the maximum and minimum amount of non-pigmented polymer was formed with approximately a 2 to 1 (500 to 250 mg/L or 1000 to 500 mg/L seed tannin to anthocyanin) and 4 to 1 (1000 to 250 mg/L seed tannin to anthocyanin) mass non-pigmented to pigmented phenol ratio. The total amount of added pigment and tannin also had an influence on the increase in concentration of polymeric pigments with the maximum amount obtained when 500 mg/L of anthocyanin and 1000 mg/L GAE of tannin was added. This was in contrast with the results of Singleton and Trousdale who found that the combination of 250 mg/L of anthocyanins and a 1000 mg/L GAE tannin resulted in the highest concentration of polymeric pigments formed (Singleton and Trousdale 1992). It was hypothesized that the incorporation of anthocyanin into the tannin polymer increased the solubility and content of polymeric phenols due to their higher polarity and hydrophilicity as a result of the incorporated anthocyanin and its attached sugar.

The determination of polymeric phenol and pigment amounts according to RP-HPLC in this study differed from that of Singleton and Trousdale (1992), due to differences in the separation profile. They determined polymeric content according to a broad hump or series of unresolved peaks that increased during aging as a result of new monomeric and oligomeric pigments co-eluting with it which increased the calculated peak area and thus the concentration. The HPLC method of Singleton and Trousdale (1992) was repeated and compared with the method used in this study. The new polymeric (non-monomeric)

pigments and phenols formed resulted in an increase in the baseline beneath the known monomeric pigment and phenol peaks, making accurate quantification impossible.

Samples were heated to 45°C to accelerate aging during experiment B, as has been the practice of other researchers (Santos-Buelga *et al.* 1995). However, the prediction of accelerated aging for the behaviour of phenolics during actual wine aging has not been well investigated. The accelerated aging resulted in the formation of a precipitate presumably due to increased polymerisation. It has been shown (Dallas *et al.* 1996a, b; Es-Safi *et al.* 1996) that when acetaldehyde is added to model solutions containing anthocyanins and flavanols, various oligomeric bridged compounds are formed and that the polymerisation rate accelerate. In experiment C, the polymeric pigment formation was 5.4 times higher in the model wine solutions containing acetaldehyde than in those not containing acetaldehyde after the first four weeks, but only 1.6 times after twelve weeks. The mechanism of acetaldehyde mediated polymerisation, demonstrated by Fulcrand *et al*. (1996a), starts with acetaldehyde condensation on flavanols leading to a carbocation intermediate, which reacts in turn with either another molecule of flavanol or the hydrated form of an anthocyanin. Both Dallas *et al*. (2003) and Santo-Buelga *et al*. (1995) found that a decrease in pH increased the rate of acetaldehyde mediated polymerisation. A lower pH will promote the formation of the carbocation intermediate. Only pHs between 2 and 4 have been investigated, with the highest polymerisation rate at pH 2. However, in this study it was found that the polymerisation rate at pH 2 was higher then at pH 1. This is because the lower pH will favour the flavylium form of the anthocyanin at the expense of the hydrated form which makes it unavailable for condensation with the carbocation intermediate.

2.4 CONCLUSION

Polymerisation is a slow process and only a small amount of material formed during the time of investigation. The highest concentration of polymeric pigments was formed with the addition of acetaldehyde at pH 2 in the model wine solutions. This supports the findings of other researchers. The slow formation of polymeric phenols and its lower solubility in model systems compared to wine complicates the possibility of up-scaling and isolation of the polymeric pigments from the anthocyanins and grape seed tannin present will also be necessary. The resulting low yield of polymers necessitated further investigation into the isolation of polymeric pigments directly from wine.

Chapter 3

METHOD DEVELOPMENT FOR THE SEPARATION OF PIGMENTED POLYMERS IN WINE

3.1 INTRODUCTION

In this chapter techniques to isolate polymeric pigments from wine will be investigated. Model wine studies showed that it would be difficult to relate the general composition of polymeric pigments formed in model solutions to those found in wine. Just using different white wine bases had a substantial effect on the amount of new pigments formed and an unknown effect on the composition of the pigments.

Chromatographic and spectroscopic methods important to the analysis of grape and wine polyphenols are discussed in this chapter and their possible application for the isolation and identification of polymeric pigments in wine is investigated.

3.1.1 Spectroscopic methods

The method used most commonly for the assessment of colour in red wines is the Somers' method (Somers and Evans 1977). As red wine ages, the absorbance maximum $\lambda \sim 520$ nm declines in intensity corresponding to an increase in the yellow colour at $\lambda \sim 420$ nm. The wine hue or tint, measured as the ratio of absorbances A_{420}/ A_{520} typically increases from 0.4-0.5 in young wines to around 0.8-0.9 in mature red wines. This indicates the increased contribution of polymeric pigments to the wine colour. Colour density is determined as the sum of the absorbance at 420 nm and 520 nm. Somers measurements use the ionisable and bleachable nature of the anthocyanins to distinguish these from the polymeric forms that are generally less sensitive to pH and less bleached by bisulphite (Somers and Evans 1977). The spectral measures of total anthocyanins are based upon the decolouring effect of bisulfite on the anthocyanin monomers and its lack of effect on the red polymers. It has been determined that bisulfite addition takes place at position 4 of the flavylium ion (Berke *et al.* 1998) and has been postulated that those pigments that have not been substituted at position 4 such as the acetaldehyde pigments (Fulcrand *et al.* 1996a), would be bleachable (Asenstorfer *et al.* 2001).

Folin-Ciocalteau has been the method of general preference for the determination of total phenols (Singleton and Rossi 1965). It determines total phenols (and other easily oxidised substances) by measuring the blue colour formed from the reduced yellow heteropolyphosphomolybdate-tungstate anions (Slinkard and Singleton 1977). When more sensitive methods like high performance liquid chromatography (HPLC) were developed it was found through comparison of the colour measurements and concentrations of the pigments determined, that they usually have a linear relationship with the Folin-Ciocalteu method (Rivas-Gonzalo *et al.* 1992). Spectrophotometric methods are more widely used by the industry and give a general indication of quality through colour density and indication of total anthocyanins and phenols as well as polymeric pigment content. It is a faster method then HPLC and cheaper but HPLC gives more detailed information, such as the contribution of the individual anthocyanins, and can also separate most of the monomeric phenols and thus enable individual quantification. As a result HPC analysis is more specific and small differences in composition can be determined. Reverse phase chromatography, in which components are separated by partition between a non-polar stationary phase (e.g. C_{18} alkyl groupings bonded to a silica support) and a polar mobile phase, is the general rule for phenol separation. Isocratic systems are useful for the separation of components having closely similar polarities, whereas gradient systems are more suitable for the range of phenolic compounds in grapes and wines.

3.1.2 Chromatographic methods

Separation of grape seed and skin proanthocyanidins has been achieved with the use of HPLC (high performance liquid chromatography) columns coupled with UV-visible detectors (Jaworski and Lee 1987; Oszmianski and Lee 1990; Revilla *et al.* 1991; Ricardo da Silva 1990; Roggero *et al.* 1990) up to the tetramer. Analysis of larger proanthocyanins (tannins) becomes increasingly difficult, as their molecular weight increase (Bartolomé *et al.* 1996; Lamuela-Raventós and Waterhouse 1994; Peng *et al.* 2001) and the elution order of oligomers does not correspond to the degree of polymerisation. Furthermore, the larger oligomers appear as unresolved peaks on the chromatogram because of the enormous variety of isomers and oligomers. The HPLC separation of wine phenolics usually requires prior purification, which makes quantification difficult (Bourzeix *et al.* 1986; Salogoïty-Auguste and Bertrand 1984). Artefacts such as oxidation, isomerisation or hydrolysis may result from these manipulations.

Most importantly the purification method may not be perfectly reproducible as a result of the associations between the different phenols. For instance, quercetin, whose solubility is zero in water and is very low in ethanol, may be detected in large quantities in some wines. Consequently, analysts who calculate the recovery ratios of various phenolics after extracting them from a synthetic wine (constituted by ethanol, water, and potassium hydrogen tartrate), which does not have the colloidal properties of a real wine, obtain very low results for many compounds (Salogoïty-Auguste and Bertrand 1984; Viseras *et al.* 1986). For analysis of anthocyanins satisfactory resolution occurs only with the use of strongly acidic solvents, pH close to 1.5 (Hebrero *et al.* 1988). Such a low pH imposes stress on the column packing material, as it may induce hydrolysis of the bonded alkyl groups from the silica support. Lamuela-Raventós and Waterhouse (1994) eluted anthocyanins without prior purification with a trinary solvent system that changed the pH from 2.6 to 1.5 with 0.2 M H_3PO_4 after elution of the main procyanidins. Polymeric phenols and pigments are increasingly the major phenolic fraction during wine maturation, and were not resolved by most RP-HPLC methods. In wine samples, the failure was due to the large number of species present of approximately the same polarity and size. Price (1994) used a low pH procedure on a polystyrene reversed-phase column, which has the advantage of being extremely stable under all pH conditions. A very slow gradient was used to separate the monomeric phenols while retaining the larger phenols which do not separate as individual species in individual peaks. The retained material was then pushed of the column at the end of the run by a sharp increase in organic solvent in the mobile phase. This resulted in a large peak at the end of the chromatogram which was referred to as the 'polymeric' peak.

3.1.3 Fractionation methods

Preparative methods based on the separation of procyanidins on a molecular weight basis have been developed, for example liquid chromatography on hydrophobic stationary phases such as Fractogel TSK HW-40 (Oszmianski and Bourzeix 1995; Revilla *et al.* 1992; Ricardo da Silva *et al.* 1991b; Shoji *et al.* 1999) and Sephadex LH-20 (Alcalde-Eon *et al.* 2004; Derdelinckx and Jerumanis 1984; Dorner 1983; Escribano-Bailón *et al.* 1992; Kantz and Singleton 1990; 1991; Lea and Timberlake 1974; Rigaud *et al.* 1991). These fractionation methods have also been useful for the separation of monomeric and higher molecular weight pigmented fractions. Ion exchange has also been successful in the separation of monomeric anthocyanins and

smaller pigments (Asenstorfer *et al.* 2001). Strong absorption of polymeric phenols to most types of resins, through possible hydrophobic interaction with aromatic rings, as well as hydrogen bonding, result in a loss of phenolic material (Lin and Hilton 1980). This is one of the reasons counter-current chromatography (CCC) has been seen as a possible solution for the quantitative fractionation and isolation of preparative amounts of phenolic material (Degenhardt *et al.* 2000; Lea 1980; Putman and Butler 1985). Chromatography on Sephadex LH-20 used either singly or in conjunction with Fractogel TSK ('Toyapearl") is the most widely used preparative technique for the separation of procyanidins (Bakker and Timberlake 1997; Derdelinckx and Jerumanis 1984; Lea and Timberlake 1974; Perez-Ilzarbe *et al.* 1992; Putman and Butler 1985; Ricardo da Silva *et al.* 1991a, b; Sun *et al.* 1999). Cameira-dos-Santos *et al*. (1996) purified pigment on Silica 60 with ethanol (EtOH), 90% methanol (MeOH) and then acidified MeOH (0.1% trifluoroacetic acid), while Fulcrand *et al*. (1996b) isolated Pigment A and B from a preparative silica gel HPLC column. Cation exchange chromatography in the absence and presence of excess bisulphite was used by Asenstorfer (2001) for the isolation of wine pigments from red wine. The charged pigments was retained by the cation exchange column, isolated and reacted with potassium metabisulfite. The negatively charged bisulphite addition products washed through the column in the 0.1 M potassium bisulphite solution and the retained pigments were eluted with 50% methanol. The separation of small phenol chains have been achieved with these separation techniques, but the polymeric phenols were often less resolved, giving broad peaks and complex elution patterns.

Modern counter-current chromatographs are available in two types, called 'CCC' for the planetary motioned coiled tube type developed by Ito and Oka (1990) and 'CPC' (centrifugal partition chromatography) for the type containing discrete partition cells inside a rotor (Okuda *et al.* 1994). CCC is a liquid-liquid partition technique that separates phenolics according to their partition coefficients in two liquid phases (Marston and Hostettmann 1994) and eliminates various complications that arise when using solid supports. Okuda's group in Japan has successfully used CCC in separating hydrolysable tannins (Okuda *et al.* 1989), while Putman and Butler (1989) have applied the technique to the fractionation of condensed tannins in sorghum grain. Optimisation of the solvent mixtures for high selectivity has proven difficult however, and the technique appears relegated to a preliminary purification role (Delaunay *et al.* 2002; Shibusawa *et al.* 2000). Centrifugal partition chromatography was successfully used to purify gram quantities of anthocyanins (Renault *et al.* 1997), while CCC has been used to

isolate the different classes of anthocyanins (Degenhardt *et al.* 2000; Vidal *et al.* 2004c) from plant extracts. Salas *et al*. (2005) analysed a blended red wine (four months of age) made from Cabernet Sauvignon (60%) and Tannat (40%) cultivars. The wine was first washed on a resin to eliminate proteins, residual sugars, organic acids and ions before fractionation by high speed counter-current chromatography (HSCCC). The different anthocyanins together with some anthocyanin-derived pigments were roughly separated into different fractions. The mobile phases used for CCC and CPC separation may also be used for thin layer chromatography (TLC) separations. Compounds will however, separate differently on TLC as separation is based on both partition and absorption mechanisms. Lea (1979) and Oszmianski and Sapis (1989) have successfully used thin layer chromatography (TLC) to identify up to the dimeric procyanidins from seeds and wines, while it also successfully separated anthocyanins (Liao *et al.* 1992; Oh and Hoff 1979; Timberlake and Bridle 1976).

Ultrafiltration is mostly used for the removal of polysaccharides, proteins and larger components from wine (Desportes *et al.* 2000; Hsu *et al.* 1987), but may also be used as a rough fractionation method for wine. The possibility of the preparative fractionation of red wine with ultrafiltration would by worthwhile investigating. Ultrafiltration (UF) is a process where a semi-permeable membrane separates the components of the solute mixture according to their molecular size.

Rigaud *et al*. (1993) adapted a normal-phase HPLC method from the TLC method described by Lea (1978) on silica. This method successfully separated both grape seed (Prieur *et al.* 1994) and skin (Souquet *et al.* 1996) oligomeric and polymeric proanthocyanidins in several fractions, it however fails when anthocyanins are present as in the case with wine. The increased polarity of the charged flavylium form of anthocyanins resulted in strong absorption of these compounds onto the silica packing material. Kennedy and Waterhouse (2000) reduced absorption by the addition of an ion-pair reagent to the mobile phase.

High voltage paper electrophoresis (HVPE) has been used for the determination of ionisation constants of low molecular weight species (Asenstorfer 2001; Donner 1997; Ryder *et al.* 1984; Tate 1981; Tate *et al.* 1982) and can also be used as a separation and characterisation technique of charged species. The method is capable of separating species as a result of their

different electrophoretic profiles as a function of pH and thus according to their unknown ionisation constants.

In this chapter the aim is to develop a fractionation protocol to obtain different polymeric pigment fractions from a red wine as well as analytical methods for purification and quantification. The investigation of the formation of polymeric pigments during maturation depended on the availability of an HPLC method that could separate and quantify the pigmented polymers without interference of the monomers.

3.2 MATERIALS AND METHODS

3.2.1 General material and methods

All water used during experimentation was purified by a Milli-Q reagent water system from Millipore Pty Ltd (NSW, Australia).

3.2.1.1 Experimental wines

The two wines used during development (1995 and 1997 vintage) were fermented to dryness using small-lot winemaking procedures followed immediately by storage in glass wine bottles under screw cap closures. Shiraz grapes came from the Barossa Valley, South Australia, and were harvested between 23 and 25º Brix. After crushing 250 mg/L diammonium phosphate (DAP) and, 250 mg/L of yeast (*Saccharomyces cerevisiae* strain EC1118; Lalvin) as a 10% solution in a 10% aqueous solution of glucose, were added and the pH adjusted to pH 3.6. The musts were fermented at approximately 18ºC and were plunged every 6 hours. The 1997 wine was stored at 4 to 6ºC after the completion of the wine for the duration of the study.

3.2.1.2 Somers measurements

Somers' method was applied to the wine samples according to the methodology described in Iland *et al*. (2000). Absorbance measurements of the samples were taken at 420 and 520 nm after which 30 µL of 20% $Na_2S_2O_5$ (sodium metabisulfite) and 20 µL of 10% acetaldehyde was added respectively to one of two lots of 2 mL samples, with the absorbance of the

foremost taken directly at 520 nm while the latter were taken at 420 and 520 nm after 45 minutes. In addition, a 100 μL of each sample was added to 10 mL of a 1 M HCl solution and the absorbance read at 280 and 520 nm after 3 hours. All readings were converted to a pathlength of 1 cm and multiplied by the dilution factor. Absorbance measures were performed using a Perkin Elmer (Lambda 5) (Perkin Elmer Life And Analytical Sciences, Inc., Massachusetts, USA) and Varian Techtron Model 635D spectrophotometer (Varian Techtron Pty. Ltd., Mulgrave, Australia).

3.2.1.3 Ultracentrifugation

Cellulose ultrafiltration membranes (Microcon 3, Amicon Inc., Beverly, MA) of 3000 nominal molecular weight cut-off (NMWCO) were used. The samples (0.5 mL) were placed in the cell and centrifuged at 10000 *g* for 75 min or until the retentate volume was reduced to ~ 0.05 mL. The volume of the retentate was adjusted to 0.5 mL by the addition of model wine (12% EtOH in saturated potassium hydrogen tartrate solution, pH adjusted to 3.4 with 1 M HCl). After gentle mixing, samples were further centrifuged as described above. Centrifugation was repeated twice, and after adjustment to its original volume, the final retentate was analysed by RP-HPLC.

3.2.2. RP-HPLC analysis

Based on the method used by Price *et al.* (1995), a method was developed for the separation of all classes of wine phenolics in a single run using a diode array detector. No sample preparation was needed. An article based on the developed method has already been published (Peng *et al.* 2002). It was later also used as a basis to develop a similar elution profile on silica based columns.

Standard Waters and Hewlett-Packard apparatus was used. The optimal elution gradient was developed on a 250 × 4.6 mm polystyrene divinylbenzene reverse phase column (PLRP-S 100Å 5 μm, Polymer labs, UK) with a guard cartridge (10 × 4.6 mm) packed with the same material. A gradient elution profile was used with Solvent A being 1.5% (v/v) phosphoric acid and Solvent B 80% acetonitrile with 20% (v/v) of Solvent A. Gradient conditions were: 0 min,

A 94%, B 6% with a flow-rate of 1 mL/min, linearly to A 69%, B 31% in 73 min, A 38%, B 62% in 5 min, isocratic at A 38%, B 62% for 8 min, back to starting conditions in 4 min, A 94 %, B 6%. The column temperature was held at 35ºC, and the column was re-equilibrated with the starting solvent conditions for 10 minutes between runs. The wines were centrifuged at 13000 *g* in a Mikro 12-24 centrifuge (Hettich) for 5 minutes before injections. A diode array detector recorded data in the range from 250 to 550 nm. Peaks were identified by spectral comparison to published spectra and retention times as well as by comparison to known standards. The standards used were of the highest purity commercially available. Gallic acid, catechin, quercetin-glucoside, quercetin-rutinoside, quercetin aglycone, kaempferol aglycone, myricetin aglycone and caffeic acid standards were obtained from Fluka Chemie AG (Buchs, Switzerland), and epicatechin and epicatechingallate from Sigma-Aldrich (Sydney, Australia). Caftaric acid and Vitisin A was a gift from Robert Asenstorfer, The University of Adelaide. Malvidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside were obtained from Roth (France) as well as PolyphenolsAS (Norway).

3.2.2.1 Calibration

Calibrations at the different wavelengths were achieved with the injection of three different concentrations of respectively, catechin at 280 nm, caffeic acid at 320 nm, quercetin-3-glucoside at 360 nm, and malvidin-3-*O*-glucoside at 520 nm. Phenols with absorbance maximums close to any of these wavelengths were then quantified as an equivalent of the specific standard calibrated at that wavelength. Flavan-3-ols were thus quantified as catechin equivalents and similarly the pigmented phenols and anthocyanins as malvidin-3-*O*-glucoside, flavonols as quercetin-3-glucoside and cinnamic acids as caffeic acid equivalents.

3.2.2.2 Recovery of 'polymeric' phenols

The recovery of the 'polymeric' phenols during HPLC analysis was investigated.

3.2.2.2.1 Preparation of polymer pigmented standard

A pigmented polymer standard was prepared from the 1997 experimental wine described in section 3.2.1.1. Wine was placed in dialysis tubing (approximately 12 kDa Molecular Weight

Cut-Off) purchased from Selby Scientific (SA, Australia) and was dialysed for 24 hours after which it was ultracentrifuged according to the procedure described in section 3.2.1.3.

3.2.2.2.2 Preparation of polymer seed standard

A polymeric procyanidin standard was prepared from grape seeds. Seeds were manually separated from ~ 100 berries and homogenized in a high-speed homogeniser (Ultra-turrax T25, Janke & Kunkel GmbH & Co.) with 20 mL of 70% (v/v) aqueous ethanol and extracted with occasional manual shaking at room temperature for 1 h. The slurry was centrifuged (12000 *g*, 5ºC, 40 min), and the precipitate was re-extracted twice with the same solvent for 30 min (2 × 20 mL). The supernatants were pooled, and the volume was adjusted such that 100 mL corresponded to the extract from 6 g of seeds. Sephadex LH-20 gel (Pharmacia Biotech AB, Uppsala, Sweden), pre-swollen in 60% (v/v) methanol containing 0.2% formic acid, was slurry-packed into a glass column (3.5 × 16 cm); prior to sample loading, the column was equilibrated with water. The ethanol extracts of grape seeds were loaded onto the column, and the column was washed sequentially with 2.3 L of water and 2.3 L of 60% (v/v) methanol containing 0.2% (v/v) formic acid at a flow rate of 0.3 – 0.5 mL/min. Polymeric procyanidins were eluted with 2.3 L of 60% (v/v) acetone containing 0.2% (v/v) formic acid. Acetone was evaporated with a rotary vacuum evaporator at 35ºC. The residual sample was lyophilised and further dried in a desiccator at room temperature for > 24 h. One hundred milligrams of the polymeric procyanidin standard was equivalent to 97 ± 8 mg of gallic acid, when assessed by the Folin-Ciocalteau method (Chapter 2, section 2.2.2).

The commercial quebracho tannin (Quebracho UNITAN ATO) that was also used during the investigation was a gift from Redox Chemicals Pty. Ltd., South Australia. The three standards, pigmented wine polymer, polymer seed tannin and the quebracho tannin, was injected onto the HPLC column, and samples were run either “through a bypass” (the column was replaced by a short piece of stainless steel tubing) or through the particular column (as described in section 3.2.2). The samples were run in triplicate each “through the bypass” or through the HPLC column. The eluting solvent for the polymeric standards running through the bypass was 50:50 (acetonitrile:Solvent A), the equivalent solvent under which the polymeric fraction elutes when run through the column. The ratio of the peak area obtained through the column to that

obtained through a bypass was used as an indication of the recovery of the polymeric sample when passed through the column.

3.2.2.3 Comparison with C_{18} column

The HPLC apparatus used were standard Waters instruments with diode array detectors.
A Licrosorb C_{18} column (250 mm × 4.6 mm, 5 μm; Merck, Darmstadt, Germany) with a C_{18} guard cartridge was employed. Solvent A and B was the same as described in section 3.2.2 and initially the same elution profile was used to investigate the difference between the polymer and C_{18} column. The method was then optimised for the C_{18} column. Gradient conditions with a flow-rate of 1 mL/min were from 6 to 28% of Solvent B in 78 min, to 32% B in 3 min, isocratic at 32% B for 5 min, to 49% B in 3 min, isocratic at 49% B for 7 min and then back to the starting conditions in 4 min. The recovery of 'polymeric' phenols was also investigated as described in 3.2.2.2.

3.2.2.4 Semi-preparative chromatography

The developed C_{18} method was adapted for a semi-preparative Econosil (250 mm × 7 mm, 10 μm; Alltech, Sydney, Australia) column. The gradient conditions with a flow rate of 2 mL/min was Solvent A (1.5% ortophosphoric acid) 87% and Solvent B (80% CH_3CN, 20% Solvent A) 13% at 0 min, with 28% B after 78 min, 32% B after 81 min for 5 min before increasing to 49% B at 89 min for 7 min and then back to the starting conditions at 100 min. Collected fractions were diluted with equal amounts of water before concentration on a rotary vacuum evaporator at 35ºC. The concentrated samples were washed with water on C_{18} (Sep-Pak Classic) cartridges and eluted with methanol.

3.2.3 Preparation of grape seed extract

Shiraz seeds (0.8g) were ground in 70% ethanol acidified with 0.2% acetic acid (10 mL) and sodium metabisulfite (3 to 4 mg), after which they were allowed to extract for 2 hours under nitrogen at 4°C. The seed mixture was centrifuged and the supernatant was concentrated on the rotary vacuum evaporator at 30ºC.

3.2.4 Ultrafiltration

Ultrafiltration was performed in 400 mL capacity stirring cells (Waite Campus Engineering Workshop, SA, Australia) at 4ºC under nitrogen pressure of about 400 kPa. Cells were equipped with a Diaflo YM 10 and YM 3 (10 k and 3 k Molecular Weight Cut-off) membranes from Amicon Ltd (MA, USA). Filters were stored in 70% EtOH and soaked for 30 min in a 0.1M NaOH solution and then flushed with water, before use.

One lot of 400 mL red wine was put through the new membrane as a washing step. A two year old Shiraz wine made experimentally as described in section 3.2.1.1 was ultrafiltrated and the permeate and retentate were collected. The unfiltered wine, permeate, retentate or concentrate and reconstructed wine (filtrate and concentrate back-added in ratio of retentate and permeate) were analysed by Somers measurements (as described in section 3.2.1.2) and Folin-Ciocalteu (Chapter 2, section 2.2.2). The duo trio test was performed with two controls (unfiltered wine) and the reconstructed wine. In the duo-trio test, the subjects were presented with three wines (samples); the first was identified as the reference (or control) and the other two were coded. The subject's task was to indicate which product is most similar to the reference. The control in this study was the original wine, which was compared with a coded sample of the control and reconstructed sample. Colour, aroma and palate were separately evaluated by 21 people (all working at the Australian Wine Research Institute with extensive tasting experience) in booths under sodium lights. The young Shiraz wine (section 3.2.1.1) was also repeatedly ultrafiltrated and every 100 mL of the ultrafiltrate was collected and analysed by Somers' and Folin-Ciocalteu measurements.

3.2.5 Fractionation with Sephadex LH-20

Sephadex LH-20 was swollen in 50% methanol for at least two hours and slurry packed in a glass chromatographic column (15 cm x 3.2 cm) at a solvent flow rate of 0.3 mL/min. The void volume was measured by passing a dilute aqueous solution of Blue Dextran through the gel bed. For storage, the column was washed with 25% methanol and left with solvent well above the top of the gel to prevent microbial growth and to prevent entrance of air into the gel, which causes cracking of the gel bed.

The column was equilibrated with acidified water (0.2% acetic acid) before sample loading. A young Shiraz wine (10 mL) (section 3.2.1.1) was loaded onto the column and washed with water (0.2% acetic acid). The more hydrophilic phenols or according to Kantz and Singleton (1990, 1991) the so-called non-polymerics were eluted with 60% MeOH (0.2% acetic acid) until no colour eluted, followed with 50% acetone (0.2% acetic acid) for the more hydrophobic or polymeric phenols. The collected fractions were concentrated on a rotary vacuum evaporator at 35°C and analysed by RP-HPLC (discussed in section 3.2.2). The fractionation was repeated with a two-year-old experimental Shiraz made from the 1995 vintage (section 3.2.1.1). The collected methanol and acetone fractions were ultracentrifuged with 3 and 10 kDa membranes according to the method described in section 3.2.1.3.

3.2.6 Investigation of sulphoxy ethyl cellulose (cation-exchange) column

The sulphoxy ethyl cellulose packing material was prepared by stirring it sequentially with 15 volumes 0.2 N HCl in MeOH, water, 0.5 N sodium hydroxide (NaOH), water and 0.002 N hydrochloric acid (HCl). A column was packed with a slurry of sulphoxy ethyl cellulose in 0.002 N HCl and flushed overnight with the same solvent to equilibrate. The 1995 Shiraz wine was loaded onto the column and washed with 10% MeOH, followed with 50% MeOH containing 2 M sodium chloride (NaCl) to displace the retained phenolics (positively charged pigments). The MeOH was removed from the collected fractions on a rotary vacuum evaporator at 35 ºC. The collected pigment sample was then washed on a C_{18} column with water to remove the NaCl. The sulphoxy ethyl cellulose column was equilibrated with 0.1 M sodium metabisulfite solution and it was added to the sample. After sample loading the column was washed with the sodium metabisulfite solution (fraction A, metabisulfite addition products), followed with 50% MeOH (2 M NaCl) to elute the retained phenolics (fraction B, positively charged pigments). After fractionation the column was regenerated by the sequential washing of 15 column volumes of 0.2 M HCl; water until the pH of the eluate was neutral; 0.5 M NaOH until basic and then once again with water until neutral. Fractionation was repeated with the 1997 Shiraz wine. The obtained fractions were analysed by RP-HPLC.

3.2.7 Investigation of Licrosorb Si-100 NP-HPLC column:

The normal-phase HPLC method developed by Rigaud *et al.* (1993) was tested with a grape seed extract (section 3.2.3). A LiChrospher Si 100 (particle size 5 µm; 250 × 4 mm I.D.) column purchased from Merck, protected with a guard column (20 × 4 I.D.) packed with the same material was used. The solvents were dichloromethane, methanol, formic acid and water with volume ratios of (A) 5:43:1:1 and (B) 41:7:1:1. The elution conditions were as follows: flow-rate 1 mL/min; oven temperature, 30 ºC; linear gradients from 0 to 20% A in 30 min, from 20 to 50% A in 30 min and from 50 to 100% A in 5 min, followed by isocratic elution with 100% A for 5 min and re-equilibration of the column. The method used by Prieur (1994) was also evaluated. The elution conditions were as follows: flow-rate, 1 mL/min; temperature 26 ºC; mobile phase A, dichloromethane-methanol-water-trifluoric acid (10:18:2:0.005), mobile phase B, dichloromethane-methanol-water-trifluoric acid (82:18:2:0.005); linear gradients from 0 to 40% A in 50 min, from 40 to 55% A in 5 min, and from 55 to 100% A in 5 min, followed by washing for 5 min and re-conditioning of the column. The same solvent system as with Prieur's method was used for method A with a 1 ml/min flow-rate and column temperature at 30 ºC (Prieur *et al.* 1994). Elutions conditions were optimised as follows; linear gradients from 0 to 40% A in 50 min, from 40 to 55% A in 10 min, from 55 to 100% A in 10 min, followed by isocratic elution with 100% A for 20 min and re-conditioning of the column. Method B with the same solvent system, used the following elution conditions; linear gradients from 0 to 40% A in 60 min, from 40 to 55% A in 10 min, followed by isocratic elution with 55% A for 5 min, a linear gradient from 55 to 100% A in 15 min, followed by isocratic elution with 100% A for 20 min and re-conditioning of the column.

Fractions obtained from low-pressure liquid chromatography (section 3.2.5 and 3.2.6) were injected on to the LiChrospher Si 100 column connected to a fraction collector. Fractions were concentrated on a rotary vacuum evaporator at 35 ºC, dissolved in methanol-water and either centrifuged (10000 *g* for 5 min) or loaded on C_{18} Sep-Pak cartridges, washed with water and eluted with methanol. These fractions were further analysed by RP-HPLC (section 3.2.2) and ESI-MS (section 3.2.11).

The NP-HPLC column was also tested with the solvent systems butanol-acetic acid-water (method C) and propanol-acetic acid-water (method D) in ratios of 6:1:2, developed in section 3.2.9. Isocratic elution with a flow-rate of 0.2 mL/min due to pressure concerns was used.

3.2.8 Investigation of combination of fractionation methods

The anthocyanin fraction A obtained with sodium metabisulfite elution from the sulphoxy ethyl cellulose column (section 3.2.6) was further fractionated on the NP-HPLC column (discussed in section 3.2.7, method A). Fractions were collected (A*i*, A*ii*, A*iii*) and analysed by RP-HPLC and electrospray-ionisation mass spectrometry (ESI-MS). The polymeric peak of the anthocyanin fraction was also collected from RP-HPLC (A1) and analysed by mass spectrometry. The mainly polymer fraction obtained from the 50% MeOH elution after sodium metabisulfite removal of bleachable pigments, fraction B, was also further fractionated on NP-HPLC (section 3.2.7, method A), collected (B*i*, B*ii*, B*iii*) and analysed by ESI-MS.

Fraction A1 was washed with 30% methanol on Sephadex LH-20 and the retained phenolics eluted with 60% acetone. When the acetone fraction was further washed with 40% MeOH on Sephadex LH-20, the anthocyanin-3-*p*-coumarylglucosides were removed to attain a polymer fraction with only small amounts of anthocyanin-3-glucosides (fraction A2), which was analysed by mass spectrometry.

3.2.9 Investigation of CCC solvent systems for normal-phase separation

Thin layer chromatography (TLC) separations on silica 60 F254 plates (Merck) with the mobile phase toluene-acetone-formic acid, 30:60:10, as well as the combination, 30:30:10, was investigated. Lea *et al*. (1979) used these mobile phase combinations respectively for the separation of the pigmented phenols and procyanidins. The dichloromethane-methanol-water-trifluoric acid solvent system used by Prieur *et al*. (1994) on NP-HPLC for the separation of procyanidins (section 3.2.7) at ratios of 21:27:1:1, 5:43:1:1 and 41:7:1:1 was also tested. Organic solvents such as diethylether, dichloromethane, acetone and methanol were in addition tested to determine the mobility of the different phenolics in these solvents on silica.

Different spray reagents were also investigated for illumination of the different phenols. An analytical quartz lamp with a wavelength maximum of 366 nm was used for inspection of chromatograms in long-wave UV light; the maximum wavelength of the short wave UV lamp is 254 nm. A 10% ferric chloride solution in ethanol form blue-greenish phenol spots, while silver nitrate-ammonium hydroxide (prepared by mixing 0.1 N silver nitrate and 5 N ammonium hydroxide solution in the proportion 1:5) reduce compounds such as procyanidins and form dark spots (Bate-Smith and Westall 1950). Iodine was used as a general detection reagent. The chromatogram was introduced into a closed vessel on the floor of which some crystals of iodine have been placed. Many organic compounds yield brown spots (Barret 1962). Hydrochloric acid (36%) vapour for glycals (Edward and Waldron 1952) was also used and turned all pigmented phenols pink. A 1% aluminium chloride solution in ethanol, which stains flavonoids yellow in long wavelength UV, was also tested (Gage *et al.* 1951). A 6.25% stannic chloride solution in a mixture of equal volumes chloroform and acetic acid turn light yellow flavonol-glycosides blue, although yellow kaempherol stay yellow; orange-brown aglycones turn purple and orange xanthylium salts stay orange, after heating for 5 to 10 min at 100 ºC.

Droplet counter-current chromatography (DCCC) solvent systems (both upper and lower layers) as seen in Table 3.1, were also investigated as possible mobile phases on silica support. A combination of n-butanol, acetic acid and water (Marston *et al.* 1988) was employed for anthocyanins, while different combinations of dichloromethane, methanol and water (Ito and Oka 1990; Slacanin *et al.* 1989, 1991; Zhang *et al.* 1988); n-butanol, n-propanol and water (Okuda *et al.* 1986, 1988) and n-butanol, acetic acid and water (Okuda *et al.* 1986) have been used for the separation of flavonoids and flavonoid-glycosides.

Table 3.1 DCCC solvent systems investigated

Solvent systems	Reference
polyphenols, chloroform-methanol-water (43:37:20)	(Marston *et al.* 1988)
polyphenols, chloroform-methanol-water (7:13:8)	(Itokawa *et al.* 1981)
butanol-propanol-water (2:1:3)	(Okuda *et al.* 1986)
butanol-acetic acid-water (4:1:5)	(Francis and Andersen 1984)
chloroform-methanol-water-butanol (10:10:6:1)	(Isobe *et al.* 1980)

A range of different butanol-acetic acid-water combinations with and without methanol to increase the polarity of the solvent systems were examined. The wine was additionally separated 2-dimensionally with butanol-acetic-acid-water, 6:1:2, in one direction and 2% acetic acid in another (Wang *et al.* 1978). Combinations of propanol, dichloromethane, butanol, methanol, acetic acid and water; including dichloromethane-propanol-acetic acid-water ratios 2:5:1:2 and 1:6:1:2, butanol-acetic acid-water, 6:1:3 (upper layer) and 50:15:35 (upper layer) systems were investigated.

3.2.10 Preparative normal-phase medium pressure liquid chromatography (NP-MPLC)

A Waters prepLC 25 mm chamber assembly and universal base were used. Two Waters preppak cartridges (Prep Nova Silica HR 60 Å, 6 μm, 25 × 100 mm) were used in series with a Waters extention tube. The elution at 280 nm was recorded with a LAMBDA-MAX Model 481 LC spectrophotometer (Waters) connected to a chartographer (chartspeed 15 cm/hr and flow speed of 4 mL/min). The column was stored in 100% isopropanol after use. The 3-month-old experimental Shiraz (see section 3.2.1.1) was fractionated on the preparative silica column using the solvent system butanol-acetic acid-water, 50:15:35, under radial compression pressure. The wine was concentrated on a rotary vacuum evaporator at 35 ºC and 400 mL wine was concentrated, dissolved in the mobile phase to a 50 mL sample and placed

on the preparative column. The column was connected to a fraction collector and every 5 mL was collected until no appreciable amount of colour was eluted from the column (1367 mL). Collected fractions were run on silica 60 TLC plates with the same mobile system and those found with similar Rf spots were accordingly pooled together. The combined fractions were analysed by RP-HPLC (section 3.2.2).

The fractionation on preparative Nova Silica column was repeated with the equivalent of 500 mL young Shiraz wine as described above, with the solvent system butanol-acetic acid-water, 6:1:2. Collected fractions were, as before, combined according to their separation by TLC using the same mobile system.

3.2.11 Combination of NP chromatography methods for the isolation of polymeric pigment from wine

The polymer fractions obtained preparatively in section 3.2.10 were further fractionated by TLC separation using propanol-acetic acid-water (6:1:2) as mobile phase on Silica 60 F546, 0.25 mm glass plates (Merck). TLC plates were placed in the mobile phase, the silica was extracted with methanol and the extract concentrated to test if binders will be an impurity. All fractions isolated from TLC with methanol and water were analysed with ESI-MS as described in section 3.2.12. Initial samples were concentrated on a rotary vacuum evaporator at 35 ºC and centrifuged for 5 min at 10000 *g*. After initial analyses, all samples were washed on C_{18} Sep-Paks (Waters). The Sep-Pak columns were equilibrated with 10 mL methanol followed with 10 mL water. After absorption of the samples, they were washed with 10 mL water for the removal of sugars and organic acids and then eluted with 2 mL methanol. Elution profiles of the analytical (0.2 mm), semi-preparative (0.25 mm) and preparative (1 mm) silica plates were also compared.

3.2.12 Investigation by electrospray ionisation mass spectrometry (ESI-MS) analysis

3.2.12.1 Direct injection ESI-MS analysis

The ionspray mass spectra of the samples were obtained using an API-300 mass spectrometer with an ionspray interface (PE Sciex, Thornhill, Ontario, Canada). The ionspray and orifice

potentials were respectively 5500 V and 30 V for the positive ion mode and –4500 V and –30 V for the negative ion mode. The curtain (nitrogen) and nebuliser (air) gases were set at 8 and 10 units, respectively. The isolated fractions were introduced into the mass spectrometer by a flow injector (8125, Rheodyn, Cotati, CA) with a 5 µL sample loop connected to the ion sprayer, The injected solution was delivered by 50% acetonitrile acidified with 2.5% acetic acid at a rate of 5 µL min^{-1}, using a syringe pump (Cole-Parmer, Niles, IL, USA).

3.2.12.2 Liquid chromatography (LC)/ESI-MS analysis

The sample was loaded through a 5 µL sample loop onto an HPLC C_{18} column (Nova-Pak, 60 Å, 4 µm, 2 × 150 mm, Waters) connected to a mass spectrometer (API-300, PE Sciex, Thornhill, Ontario, Canada). A range of different separation methods was tested. For method A, solvent A was 2.5% (v/v) formic acid and solvent B 90% (v/v) acetonitrile containing 2.5% (v/v) formic acid with a flow rate of 100 µL/min. A linear gradient of 0 to 15% B in 15 min, to 16% B in 25 min, to 17% B in 5 min, to 43% B in 3 min and to 52% B in 1 min, isocratically at 52 % B for 11 min, then linearly to 80% B in 10 min for 10 min. The column was equilibrated at the starting conditions for 10 min before the next injection. The eluant was split (1:4) post column by a tee, at a flow rate of 20 µL/min to the mass spectrometer and 80 µL/min to a UV detector (HP1100, Hewlett-Packard) monitoring at 280 nm.

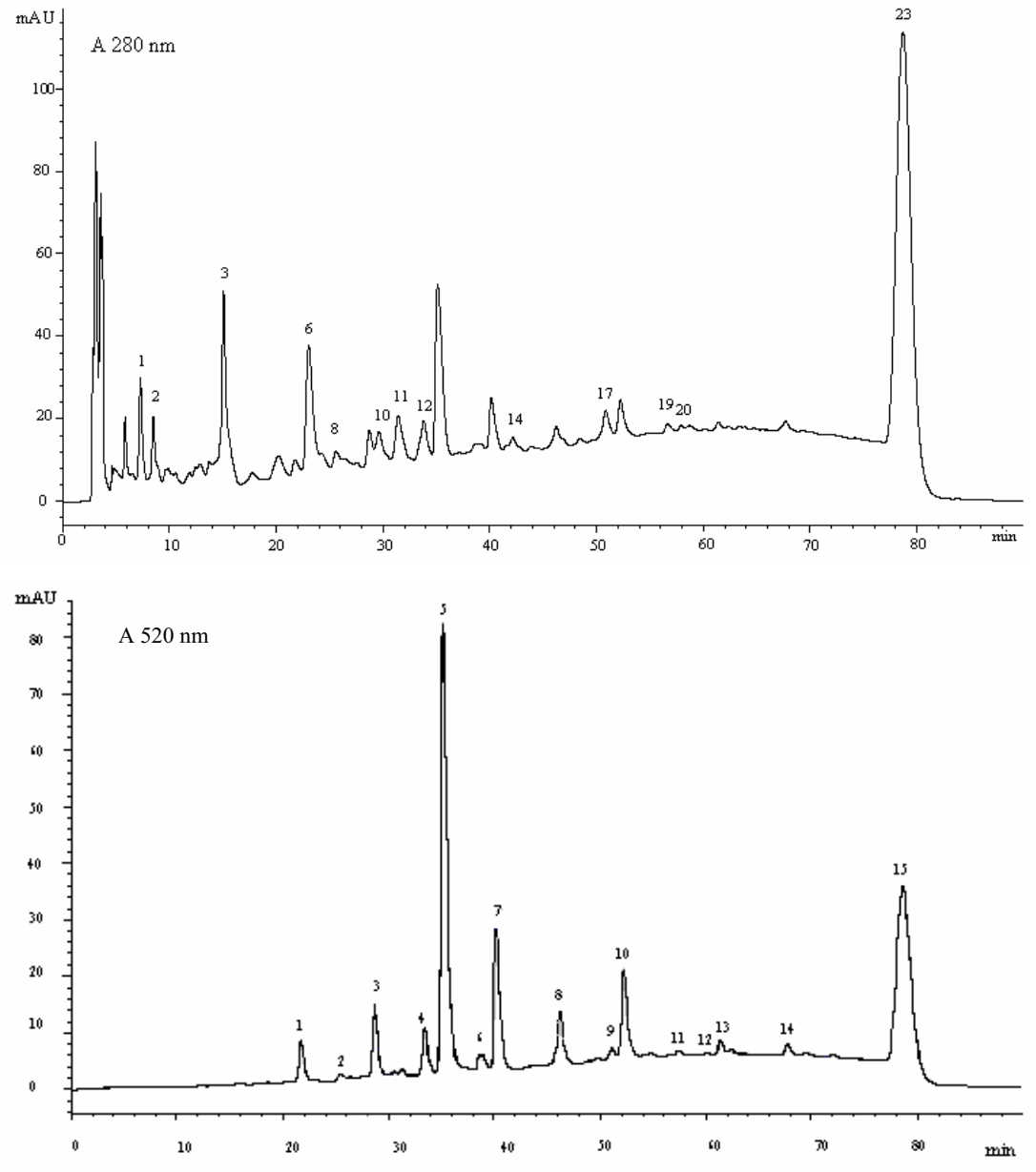
A new column (PLRP-S, 100 Å, 4 µm, 2 × 150 mm, Polymer labs, UK) was bought for LC-MS separations. Solvent A 4.5% (v/v) formic acid and solvent B 80% (v/v) acetonitrile containing 20% of solvent A was used with a flow rate of 100 µL/min. For method B the following linear gradient was used: 11 to 29% B in 50 min, isocratically at 29% B for 9 min, to 70% B in 5 min, isocratically at 70% B for 9 min, back to the starting conditions of 11% B in 2 min and staying at 11% B for another 15 min.

The mass spectrometer was operated under positive ion mode and scanned from m/z 250 to 3000 with a mass step size of 0.2 Da and a dwell time of 0.5 ms. The ion spray voltage was 5500 V, and the orifice potential was 40V. All mass spectral data were processed using Bio-Multiview software 1.2 v 3 (PE Sciex).

3.3 RESULTS

3.3.1 Quantitative separation of pigmented polymers

In the method used by Price *et al.* (1995) the anthocyanins, peonidin-3-glucoside-*p*-coumarate and malvidin-3-glucoside-*p*-coumarate co-eluted with the 'polymeric' peak at the end of the HPLC run. This method of Price was optimised to ensure the separation of the anthocyanin-*p*-coumarates and other monomeric pigments from the polymeric pigment peak (Figure 3.1).



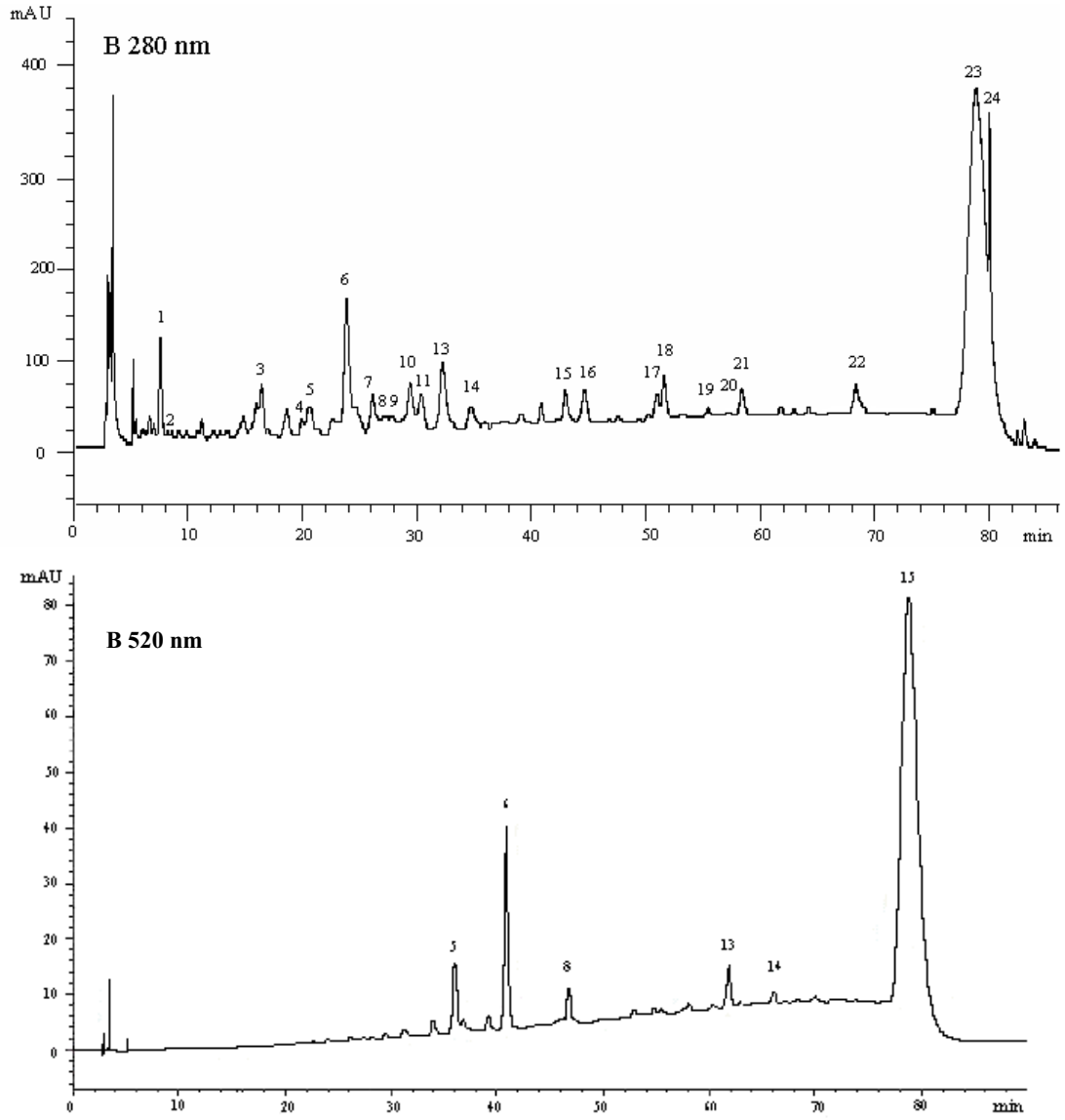


Figure 3.1 HPLC chromatogram of the phenolics of a young (A) and older (B) Shiraz wine at 280nm; Peak 1 = gallic acid, 2 = unknown benzoic acid, 3 = unknown hydroxycinnamic acid, 4 = unknown hydroxycinnamic acid, 5 = epigallocatechin, 6 = caftaric acid, 7 = unknown hydroxycinnamic acid, 8 = catechin, 9 = B_1 dimer, 10 = caffeic acid, 11 = coutaric acid, 12 = unknown hydroxycinnamic acid, 13 = epicatechin, 14 = B_2 dimer, 15 = unknown flavonol glycoside, 16 = p-coumaric acid, 17 = quercetin galactoside, 18 = quercetin glucoside, 19 = unknown flavonol, 20 = epicatechingallate, 21 = quercetin rhamnoside, 22 = myricetin aglycone, 23 = polymer peak, 24 = quercetin aglycone, and at 520 nm; Peak 1 = delphinidin-3-glucoside, 2 = cyanidin-3-glucoside, 3 = petunidin-3-glucoside, 4 = peonidin-3-glucoside, 5 = malvidin-3-glucoside, 6 = vitisin A, 7 = delphinidin-3-glucosylacetate, 8 = acetylvitisin A, 9 = peonidin-3-glucosylacetate, 10 = malvidin-3-glucosylacetate, 11 = delphinidin-3-

glucosylcoumarate, 12 = peonidin-3-glucosylcoumarate, 13 = petunidin-3-glucosylcoumarate, 14 = malvidin-3-glucosylcoumarate, 15 = pigmented polymer.

Different gradients in which pH, elution time and solvent composition were varied were investigated and tested on the polystyrene divinylbenzene (PLRP-S) column. The anthocyanin-glucosides retention times decreased and their resolution increased, with a decrease in pH. Different acids were also investigated, but it was found that even other strong acids such as trifluoro and perchloric acid was not as effective as ortho-phosphoric acid (pH 1.3) due to increased retention times for the anthocyanins, which was undesirable. A constant column temperature at e.g. 35 °C is a recommended practice. Under these conditions solvent viscosity is reduced, efficiency is improved and HPLC profiles are more reproducible, particularly when gradient systems are in use.

Although some compounds are quantified by calibration with standards, there are numerous instances where standards are not available, and compounds are quantified using the peak areas of a standard with similar spectral characteristics, and quantities are reported as equivalent amounts of that standard. Because there are a significant number of compounds in different phenolic classes, each of which has a different absorption maximum, it is common to quantify each class at their average maximum wavelength.

A number of treatments were applied to wines to confirm that the polymer peak was indeed polymeric in nature or at least contained predominantly polymeric material. A 3-month-old and two-year-old wine (section 3.2.1.1) were submitted to ultrafiltration through 3000 and 10000 NMWCO membranes according to the procedure described in section 3.2.1.3. The wines and the resulting retentates and filtrates were subjected to HPLC analysis. Most (80%) of the material eluting in the polymer peak was retained in the retentate and did not pass through the membrane in to the ultrafiltrate (Figure 3.2). Conversely, most (93%) of the monomeric pigments passed through the membrane and were present in the ultrafiltrate. Similarly, all the other 280 nm absorbing monomers are in the ultrafiltrate.

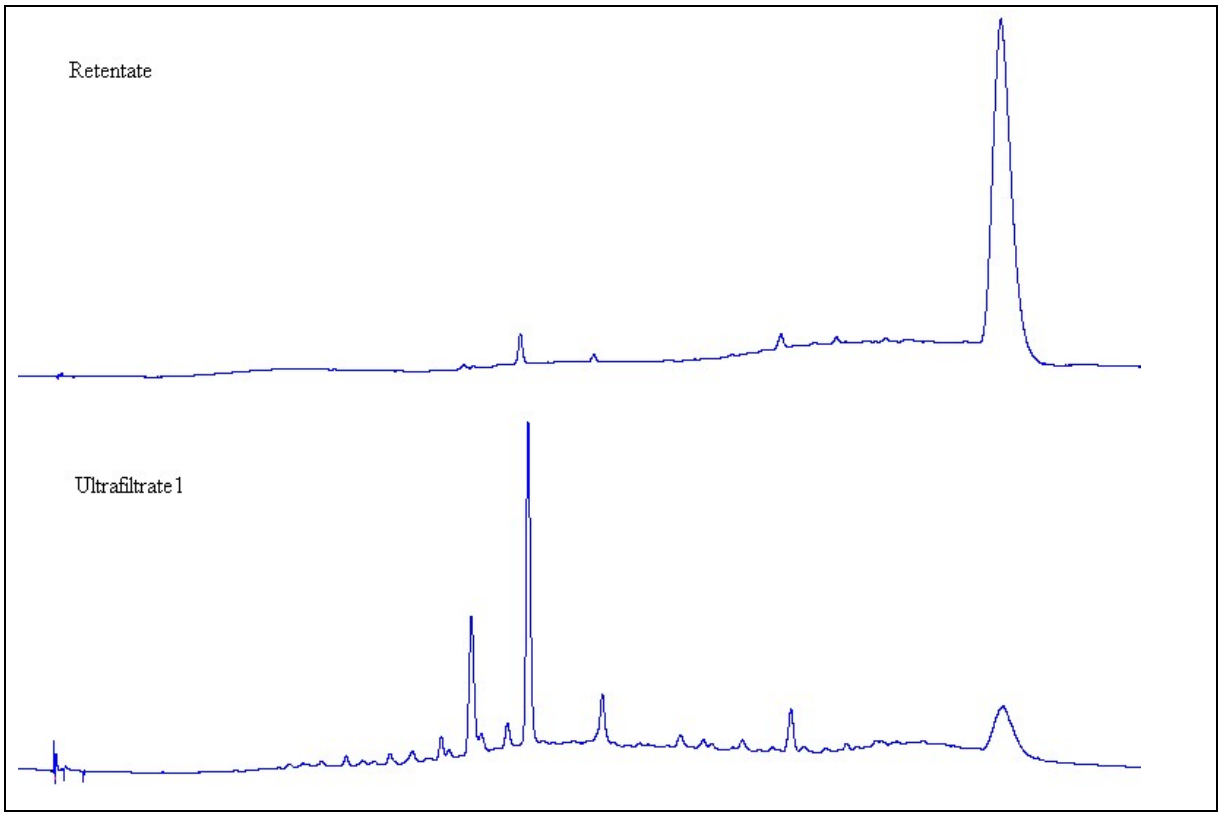


Figure 3.2 Ultracentrifugation of a 5 year old Shiraz wine with the HPLC chromatograms of the resulting retentate and the first ultrafiltrate before successive washing.

The recovery for pigmented wine polymer, polymer seed tannin and quebracho tannin from the PLRP-S column was determined as 88.7% (± 8.5% cv), 90.9% (± 2.3% cv) and 91% (± 4% cv), respectively.

A polymeric pigment sample was isolated through ultracentrifugation and subsequent separation on RP-HPLC. The increase in the baseline as well as the polymer peak (23) was collected (Figure 3.2, retentate). It was determined that 28% of the polymer was collected as part of the baseline. The collected baseline was injected onto the RP-HPLC column to show that the increase in the baseline beyond and above the solvent affect is actually part of the polymer fraction.

When the elution profile developed for the polymer column was used on a C_{18} column, the anthocyanin-*p*-coumarates did not separate from the polymer peak and a decrease in resolution were observed. A longer elution time was needed to obtain similar results on the C_{18} column

compared to the polymer column. The recovery of 'polymeric' material from the C_{18} column was also slightly less, 86.4 % and 89% for polymer seed tannin and quebracho tannin respectively (Peng *et al.* 2001).

The developed method for the C_{18} reverse phase HPLC column was also further adjusted for separation of wine on a semi-preparative Econosil (250 mm × 7 mm) column. All peaks eluted 10 min later compared to the analytical column, resulting in the co-elution of malvidin-3-glucoside-*p*-coumarate and the polymeric peak.

3.3.2 Investigation of the isolation and purification of pigmented polymers in wine

3.3.2.1 Ultrafiltration

Ultrafiltration was investigated for the possible preparative fractionation of polymeric pigments into different molecular weight ranges. These samples were investigated by duo-trio testing (Stone and Sidel 1993) to determine whether ultrafiltration affected the sensory characteristics of the wine in any way. The wines were evaluated by 21 panellists; of which 12 indicated the correct wine for difference in colour, 7 in aroma and 10 in flavour. These results indicated that ultrafiltration did not significantly influence the sensorial characteristics of aroma and flavour but did have a significant influence on the colour of a wine.

If ultrafiltration is to be used as a fractionation technique, it must be reproducible. Every 100 mL filtrate of a 3-month-old Shiraz wine was collected in triplicate and spectrophotometrically analysed until 800 mL was collected. The colour of the filtrate continually increased (data not shown) as a result of the raise in backpressure as the density of the retained wine increased. Back pressure also influenced the filtration speed (Alarcon-Mendez and Boulton 2001). This increase in filtrate colour was only observed after approximately 300 mL of wine passed through the membrane. Absorption of phenols onto the membrane may play a role in this observation. From here on all ultrafiltration membranes were 'washed' with 300 mL of the wine sample before samples were collected. This way it was assumed that the absorption of phenolic material onto the membrane itself was minimised or excluded. Flores *et al.* (1991) also found that regardless of the membrane MWCO the volume to concentration ratio

significantly affected the flux or speed of filtration. The changes in backpressure and filtration speed resulted in large deviation between ultrafiltration replicates (13%) in our study.

Fractions obtained from the ultrafiltration of a two year old experimental Shiraz with YM 10 membranes were analysed by Somers' measurements (section 3.2.1.2) and Folin-Ciocalteu (chapter 2, section 2.2.2), with results shown in Table 3.2.

Table 3.2 Somers measurements and Folin-Ciocalteu of the ultrafiltration fractions obtained with YM 10 membranes from a 2 year old Shiraz wine.

	Wine (0.45 µm filter)	Filtrate YM 10	Concentrate YM 10	% Retained[1]	Reconstructed[2]
[3]**Density**	8.23	0.75	6.42		7.33
[4]Hue	0.82	0.95	0.76		0.81
[5]$\mathbf{A}_{520}$	4.52	0.36	2.80	88	3.27
[6]$\mathbf{A}_{420}$	3.72	0.36	2.80	88	3.27
[7]**Tot anth**	59.0	21.1	33.1	61	51.8
[8]$\mathbf{A}_{520}^{HCl}$	59.0	21.1	33.1	82	54.2
[9]$\mathbf{A}_{520}^{SO_2}$	2.94	0.22	2.31	91	2.68
[10]$\mathbf{A}_{280}$	29.0	5.00	16.9	77	21.3
[11]**FC**	1281	269	812	75	1068

Sample dilution or concentration was taken into account.

[1](concentrate/(concentrate + filtrate)) * 100

[2]Filtrate and concentrate back-added in ratio of retentate and permeate

[3]520 nm + 420 nm

[4]420 nm/520 nm

[5]Absorbance at 520 nm

[6]Absorbance at 420 nm

[7]Total anthocyanins (mg/L) – $20(A_{520}^{HCl} - \frac{5}{3} A_{520}^{SO_2})$

[8]Absorbance at 520 nm at pH 1

[9]Absorbance at 520 nm in the presence of SO_2

[10]Absorbance at 280 nm

[11]Folin-Ciocalteu

A decrease in all the phenolic measurements were observed for the reconstructed wine compared to the control. According to the ultrafiltration results, 75% (FC) of the total phenolics and 61% (Tot anth) of the anthocyanins were retained by the YM 10 membranes (Table 3.2).

3.3.2.2 Sephadex LH-20

Kantz and Singleton (1990, 1991) used Sephadex LH-20 gel to develop a method to separate natural occurring phenolic compounds into two groups, a non-polymeric fraction and a polymeric fraction. The non-polymeric phenols were eluted with 60% methanol, while the polymeric phenols were eluted with 50% acetone. Acetone was chosen because the carbonyl oxygen serves as a strong H-bond acceptor and enables displacement of bound polymeric phenols from the Sephadex LH-20. According to the polymeric peaks in the RP-HPLC chromatograms generated at respectively 280 and 520 nm, recovery from Sephadex LH-20 of phenolic wine polymers was 99% and 74%. Recovery of malvidin-3-glucoside and malvidin-3-*p*-coumarylglucoside was only respectively 66% and 10%. After sample loading on the Sephadex LH-20 packing material, the column was washed with water, eluting the sugars and phenolic acids, as well as vitisin A and acetylvitisin A. This was followed with 60% methanol, eluting monomeric and oligomeric flavanols, as well as the anthocyanin-3-glucosides, anthocyanin-3-acetylglucosides and a small amount of anthocyanin-3-*p*-coumarylglucosides and pigmented polymers. When fractions were collected with a fraction collector and analysed by HPLC, it was clear that the percentage polymers eluting with the methanol fraction was increasing with continuous elution. In the methanol fraction the polymer peak contributed approximately to 10% of the eluted phenolics when analysed by RP-HPLC, while in the acetone fraction it contributed about 50%. The 2 year old Shiraz wine used for investigation had 10 times the amount of material eluting in the polymer peak compared to the young wine also used. In the young wine 37% of the polymeric peak eluted in the methanol fraction compared to 14% during fractionation of the older wine. The methanol fractions also had a higher 520 to 280 nm ratio (one to four) compared to the acetone fractions (one to eight), also indicating higher pigmentation and polarity (Singleton and Trousdale 1992). In the 50%

acetone fraction anthocyanin-3-*p*-coumarylglucosides, small wine pigments and pigmented polymers eluted at 520 nm, with flavonols, proanthocyanidins, and polymers eluting at 280 nm.

The methanol and acetone fractions were ultracentrifugated through both 3 and 10 kDa membranes, according to the procedure described in section 3.2.1.3. The 10 kDa membranes separately retained, in the case of the methanol and acetone fractions, 86% and 85% of the polymeric peak retained by the 3 kDa membrane even though the polymeric fractions probably do not have a molecular weight close to 10000.

Solutions with different methanol concentrations were investigated in an effort to remove most of the monomeric phenolic material with a minimum loss in material eluting in the polymer peak. But a 20% methanol solution only eluted malvidin-3-glucoside, vitisin A and phenolic acids from the Sephadex LH-20 column. The retained phenolics were removed with 60% acetone after no more colour was eluted with the methanol solution. The acetone fraction was further fractionated on the Sephadex LH-20 column with 40% MeOH (0.1% HCl), followed with the 60% acetone solution. The 40% MeOH fraction contained anthocyanin-3-glucosides, anthocyanin-3-acetylglucosides, vitisin B and its acetyl-derivative as well as a small amount of polymeric pigment, with some proanthocyanidins and polymeric phenols at 280 nm, but did not remove the anthocyanin-3-*p*-coumarylglucosides. The collected acetone fraction contained anthocyanin-3-*p*-coumarylglucosides, proanthocyanidins, polymeric pigments and phenols.

3.3.2.3 Sulphoxy ethyl cellulose fractionation

This cation exchange column is very useful for the isolation of vitisin A due to its polarity and neutral state, while anthocyanins can easily be separated, first as a result of its positive charge and secondly as a result of its reaction with sulfur dioxide to form a negative charged bisulfite complex (Asenstorfer *et al.* 2001). The 10% MeOH wash eluted vitisin A, acetyl vitisin A and a small amount of polymeric pigment. The sodium metabisulfite solution eluted the anthocyanins as well as a small amount of polymeric pigment (fraction A). Some flavonols was also present, which may indicate that some co-pigment associations still existed while eluting from the ion-exchange column. The 50% MeOH (0.1M NaCl) replaced the hydroxyl-

bonds to elute the proanthocyanidins, flavonols and polymers and will be referred to as the sulphoxy ethyl cellulose fraction B.

3.3.2.4 Investigation of Licrosorb Si-100 NP-HPLC column

Grape seed tannin was isolated (section 3.2.3) according to the method described by Peng *et al.* (2001) and injected on a Licrosorb Si-100 NP-HPLC column. The method used by Rigaud *et al.* (1993), as well as the improved method used by Prieur *et al.* (1994) were both investigated. The separation achieved was similar to those obtained by Rigaud *et al*. (1993) and Prieur *et al*. (1994) with grape seed samples. The method was then evaluated with a wine sample where a totally different elution profile was observed. The coloured compounds eluted in broad peaks. A two-year-old Shiraz wine (1995 vintage) was injected onto a semi-preparative HPLC C_{18} column (section 3.2.2.4) and the polymer peak (Figure 3.1) collected. This polymeric pigment sample was also injected onto the NP-HPLC column to determine separation of the polymeric pigments under current conditions without the interference of the anthocyanins (Kennedy and Waterhouse 2000). It was clear from the low resolution achieved that further optimisation was needed to obtain polymeric pigment fractions. The polymeric pigments eluted as low undefined lumps on the NP-HPLC column.

3.3.2.5 Identification of fractions obtained by multiple fractionation techniques

The polymer peak of fraction A resulting from RP-HPLC analysis was collected and will be referred to as fraction A1. This fraction was isolated to determine whether the material that eluted as a polymer peak in the sodium metabisulfite fraction obtained from sulphoxy ethyl cellulose when run on RP-HPLC, could be further fractionated on Sephadex LH-20. Fraction A (section 3.3.2.3) was loaded onto a Sephadex LH-20 column (3.3.2.2) and washed with 40% methanol to remove the anthocyanins before elution of the polymer fraction with 60% acetone (fraction A2). The metabisulfite fraction from sulphoxy ethyl cellulose (fraction A) was in addition further fractionated by NP-HPLC using method A (section 3.2.7) into fraction A*i* (0 – 55 min), A*ii* (56 – 70 min) and A*iii* (71 to 90 min) (not shown). Similarly fraction B (section 3.3.2.3) was also fractionated into fraction B*i*, B*ii* and B*iii* as seen in Figure 3.3. Fraction B*i* (0 to 25 min) mainly contained non-pigmented polymers, consisting of oligomeric proanthocyanidins, as well as most of the flavonols present in the original wine. Smaller

fractions were also collected Bi_1 (2 to 8 min), Bi_2 (8-10 min), Bi_3 (10 to 20 min) and Bi_4 (20 to 25 min). Identification according to RP-HPLC indicated that fractions Bi_1 contained the flavan-3-ol-gallates with fractions Bi_2, Bi_3 and Bi_4 containing proanthocyanidins while fraction Bi_3 also contained all the flavonols. Fraction B*ii* (25 to 50 min) and B*iii* (50 to 80 min) separated mainly in a polymer peak with RP-HPLC analysis. It is important to take the change in solvent composition into account as it resulted in a significant increase of 0.015 absorbance units in the baseline, due to the steep change in the linear gradient, after 60 min.

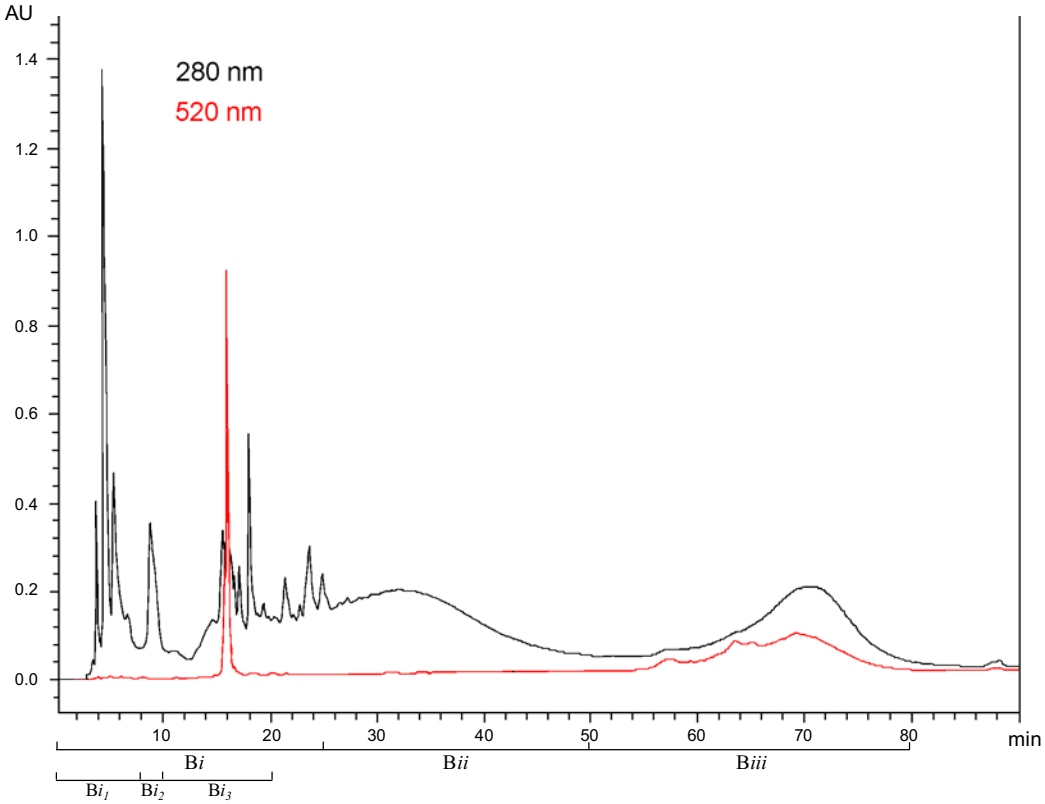


Figure 3.3 NP-HPLC chromatogram of fraction B according to method B (section 3.2.7).

3.3.2.5.1 ESI-MS analysis

The positive ion mass spectrum (3.2.11.1) of the metabisulfite fraction A, contained major ions for malvidin-3-glucoside (m/z 493.4) and the aglycone (m/z 331.2). Ions for peonidin-3-glucoside, the aglycone, as well as the acetyl and *p*-coumaryl derivatives of both peonidin-3-glucoside and malvidin-3-glucoside, were also visible. The ions m/z 809.6 and 956.2 corresponding to respectively malvidin-3-glucoside-ethyl-(epi)catechin (Es-Safi *et al.* 1999a; Rivas-Gonzalo *et al.* 1995) and malvidin-3-*p*-coumarylglucoside-ethyl-(epi)catechin was also

observed. The ESI-MS analysis of polymer fraction A1 contained ions corresponding to the *p*-coumaric and caffeic acid derivatives of the anthocyanin-3-glucosides as well as procyanidin oligomers from the monomer to the pentamer. Fraction A2 exhibited the ions 577.6, 805.4 and 1093.6 that could indicate the presence of a flavan-3-ol dimer and the pigments malvidin-3-glucoside-vinyl-flavan-3-ol monomer and malvidin-3-glucoside-vinyl-flavan-3-ol dimer. Fraction A*ii* and A*iii* collected from NP-HPLC were analysed by LC/ESI-MS (method A, section 3.2.11.2). The ions 809.6 and 655.4 corresponding to pigments malvidin-3-glucoside-ethyl-(epi)catechin and malvidin-3-caffeoylglucoside respectively, was seen with ESI-MS analysis of fraction A*ii*. In fraction A*iii* the ions 1069.6 and 781.4, referring to a possible malvidin-3-glucoside-flavan-3-ol dimer complex (Remy *et al.* 2000) and the resulting malvidin-3-glucoside-flavan-3-ol monomer complex after the loss of a flavan-3-ol monomer (m/z 289) was observed. The ions 809.6, 520.2 and 479.0 corresponding to respectively the pigments malvidin-3-glucoside-ethyl-(epi)catechin, petunidin-acetyl-3-glucoside and petunidin-3-glucoside were also present. The additional ions 655.2, 517.4 and 355.4, indicating the presence of malvidin-3-caffeoylglucoside and vitisin B (Bakker and Timberlake 1997) and its deglycosylated form, were seen with LC/ESI-MS (method B, section 3.2.11.2).

Polymer fraction B was fractionated by NP-HPLC and fraction B*ii* and B*iii* were collected and analysed by LC-ESI-MS (method B, section 3.2.11.2). The main ions for fraction B*ii* were mainly small with m/z 315.2 and 335.2 corresponding to vinylcatechin and ethylcatechin. This may indicate that the molecular ions are not visible due to instability of the compounds. The major ions in fraction B*iii* were m/z 1093.6, 805.4, 643.4 and 493.4 respectively, referring to possibly malvidin-3-glucoside-vinyl-dimer, malvidin-3-glucoside-vinyl-(epi)catechin, due to the loss of (epi)catechin, the deglycosylated malvidin-vinyl-monomer as well as malvidin-3-glucoside through the loss of the vinylmonomer.

3.3.2.6 Investigation of fractionation with preparative normal-phase chromatography

3.3.2.6.1 Investigation of Thin-Layer Chromatography (TLC) solvent systems

Initially the basis of the investigation was to determine the optimum solvent system for pigmented phenol separation on the NP-HPLC silica column. A range of different solvent systems used in the literature for phenolics and specifically, polymeric phenols were

evaluated. Toluene-acetone-formic acid (30:60:10) was tested for pigmented compounds but no clear separation was achieved, with the colour streaking. Another solvent ratio was investigated (30:30:10) which were used for the separation of procyanidins (Lea *et al.* 1979). The procyanidins separated well with some of the anthocyanins, while the rest of the coloured compounds stayed on the baseline. The dichloromethane-methanol-water-trifluoric acid solvent system used for NP-HPLC separation (described in section 3.3.2.4) at ratio of 21:27:1:1, resulted in the separation of the non-coloured compounds. When the ratio 5:43:1:1 was tested, the monomeric phenols were eluting with the front while the rest of the phenolics stayed on the baseline. Some coloured polymers separated with the 41:7:1:1 solvent ratio, but a large amount of material still remained on the baseline. Accordingly it was determined that a solvent system consisting of dichloromethane, methanol and water could not separate pigmented polymers due to too low solvent strengths and polarities. A variety of organic solvents were additionally investigated to determine their suitability for the elution of pigmented polymers on silica support. Dichloromethane and diethylether was both not polar enough, while methanol and 10% acetone moved most of the coloured species, but were not selective and did not separate into different fractions. This is usually the case when a large group of compounds with similar characteristics are present in a sample. Different reagents were also tested for the enhancement of absorbing spots on the silica plates. The most useful reagent was hydrochloric vapour, which enhanced the colour of the pigments separated.

None of the solvent systems investigated thus far were effective in separating non-monomeric pigments. It was consequently decided to investigate droplet counter-current chromatography (DCCC) solvent systems as possible mobile phases on silica support. In counter-current chromatography, the liquid phases are mutually saturated. The solvent system chloroform-methanol-water used for polyphenols (43:37:20) (Marston *et al.* 1988) and for phenolic-glycosides (7:13:8) (Itokawa *et al.* 1981) were tested. The top and bottom layers were investigated on TLC and were found either too polar with most of the phenols eluting close to the front or not polar enough with most of the material staying on the baseline. The lower layer of a solvent system used for the separation of flavonoid aglycones, chloroform-methanol-water-butanol (10:10:6:1) (Isobe *et al.* 1980), achieved limited separation, but was not polar enough. Next butanol-propanol-water (2:1:3) and butanol-acetic acid-water (4:1:5) solvent systems used respectively for tannins (Okuda *et al.* 1986) and anthocyanin or flavonol-glycosides (Francis and Andersen 1984), were studied. The upper layer of the latter separated

the anthocyanins well, while the lower layer was too polar and very little separation was achieved. The Rf's (reference to front) of respectively malvidin-3-glucosides, malvidin-3-acetylglucoside and malvidin-*p*-coumarylglucosides was 0.51, 0.65 and 0.77. Different butanol-acetic acid-water combinations with and without methanol, to increase the polarity of the solvent systems, as well as combinations of propanol, dichloromethane, butanol, methanol, acetic acid and water were tested. The lower layer of dichloromethane-propanol-acetic acid-water, 2:5:1:2, solvent system separated the polymeric sample although it did not move all the colour from the baseline, while the ratio 1:6:1:2 moved more colour, having higher polarity, but did not have enough selectivity.

From the results discussed above it was decided to explore the butanol-acetic acid-water, 50:15:35 (upper layer) and 6:1:3 (upper layer) mobile systems further. For the system butanol-acetic acid-water, 50:15:35, the reference to front (Rf) values for known standards were determined (Table 3.4).

Table 3.4 Rf values of phenolic standards separated in a butanol-acetic acid-water (50:15:35) solvent system on silica TLC plates (0.2 mm).

Compound	Rf
kaempherol	0.98
catechin	0.98
rutin	0.96
epigallocatechin	0.95
myrecetin	0.91
epicatechin	0.91
quercetin aglycone	0.80
caffeic acid	0.78
gallic acid	0.72
quercetin-3-glucoside	0.60
quercetin-3-galactoside	0.59
malvidin-3-glucoside	0.54
caftaric acid	0.35

Most of the flavonols and proanthocyanidins should not interfere with the fractionation of the coloured compounds on silica support as their mobility in solvent systems are significantly different to those of the pigments. Although cinnamic-tartaric acid derivatives and flavonol-glycosides acids elute late, many other support media (for example Sephadex LH-20 and sulphoxy ethyl cellulose) separate them from the anthocyanins.

3.3.2.6.2 Fractionation on normal-phase medium pressure liquid chromatography (NP-MPLC)

Fractionation on the preparative NP-MPLC silica column was tested with the 3-month-old experimental Shiraz (section 3.2.1.1) using the solvent system butanol-acetic acid-water, 50:15:35. The anthocyanin-3–*p*-coumarylglucosides, anthocyanin-3-acetylglucosides and anthocyanin-3-glucosides, eluted in this order and was the first coloured fractions, co-eluting with some flavonol-glycosides. It also contained a small amount of polymeric pigment and non-pigmented material. Collected samples were compared by TLC using the same mobile phase and similar samples were pooled. The combined collected samples resulted in three additional fractions after the anthocyanins and contained progressively less monomeric anthocyanins and a larger amount of polymeric phenols.

The fractions obtained through ion exchange (discussed in section 3.3.2.3) and normal phase chromatography were compared by TLC separation. Both fractions obtained from ion-exchange chromatography did not contain the low Rf pigmented spots seen in the normal phase fractions.

When a different combination of the solvent system butanol, acetic acid and water, 6:1:2, was examined it was found that the anthocyanin monomers were closer to the solvent front and more separated from the other unknown pigmented spots compared to the previous combination of butanol, acetic acid and water (50:15:35) used. Fractionation on the preparative NP silica column was repeated with the solvent system butanol, acetic acid and water (BAW), 6:1:2. Collected samples were pooled into seven fractions where the first three respectively contained large amounts of anthocyanin-3-*p*-coumarylglucosides, anthocyanin-3-acetylglucosides and anthocyanin-3-glucosides. The last four fractions contained progressively higher concentrations of the later eluting non-monomeric pigmented spots on TLC, as well as

vitisin A (Vit A). A slightly more polar mobile phase with higher mobility (propanol, acetic acid and water (PAW), 6:1:2) were also investigated. This resulted in the even earlier elution of the anthocyanins, and a lower concentration of material retained on the baseline (Table 3.5).

Table 3.5 Separation of wine fractions in the butanol-acetic acid-water and propanol-acetic acid-water mobile systems (0.2 mm TLC plates).

butanol-acetic acid-water (6-1-2)		propanol-acetic acid-water (6-1-2)	
Rf	*Compounds*	*Rf*	*Compounds*
0.7	flavanols, flavonols	0.67-0.64	malv-3-gluc
0.64	anth-p-coum	0.58-0.52	VitA, anth-gluc, polymer
0.6	anth-acet	0.45-0.37	VitAX, VitA, polymer
0.55-0.53	malv-3-gluc, peo-3-gluc	0.35-0.30	polymer
0.41-0.33	pet-3-gluc	0.29-0.23	polymer
0.34-0.31	delph-3-gluc, polymer	0.22-0.20	polymer
0.29-0.23	VitAX, polymer	0.19-0.17	polymer
0.24-0.18	VitA, polymer	0.16-0.13	polymer
0.18-0.0	polymer	0.10-0.03	polymer

Vit A = vitisin A; Vit AX = acetylated vitisin A.

The isocratic separation of wine with the mobile phases butanol-acetic acid-water (BAW), 6:1:2, and propanol, acetic acid and water (PAW), 6:1:2, were tested on the NP-HPLC column and samples collected. The results will be discussed further in chapter 4 with the characterisation of the isolated TLC pigment fractions.

3.3.2.6.3 Isolation of pigmented polymers by thin-layer chromatography (TLC)

The last fraction collected from preparative NP chromatography (PAW, 6:1:2) contained the highest concentration of polymeric pigments (fraction C) and was separated further by the more polar solvent system propanol, acetic acid and water, 6:1:2, on TLC silica plates (Figure 3.4*)*. The preparative isolation of pigmented polymers were investigated with the use of 1 mm silica TLC plates, but resolution decreased considerably compared to the use of semi-preparative 0.2 mm silica TLC plates, which was found the most suitable.

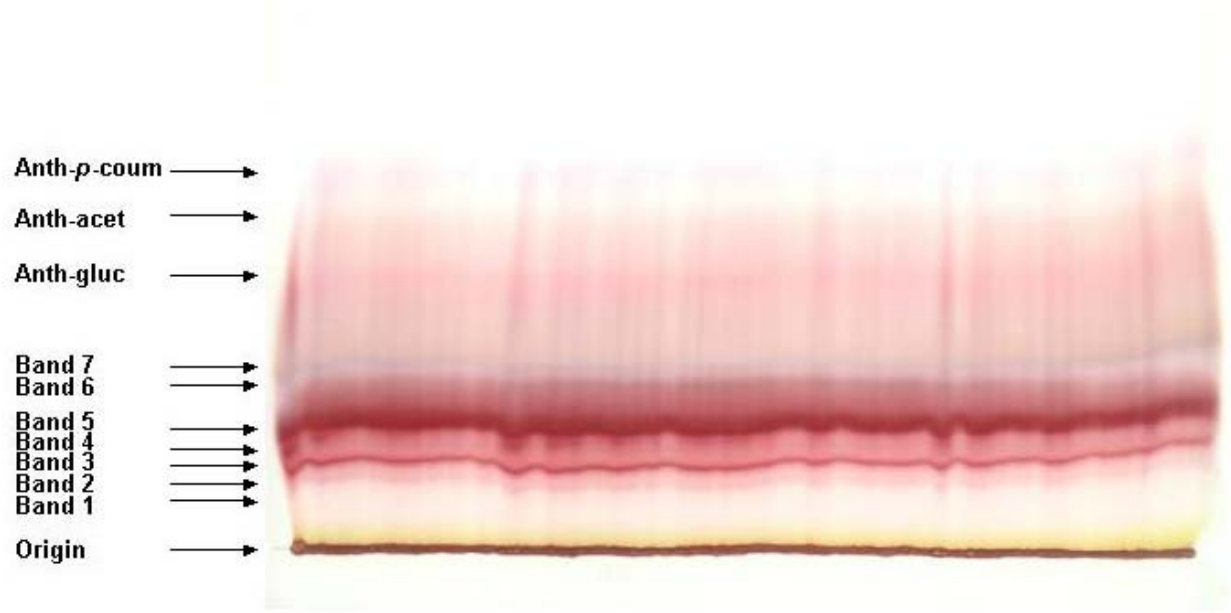


Figure 3.4 TLC separation of MPLC fraction C with the mobile phase propanol-acetic acid-water (6:1:2).

3.3.2.6.4 Mass spectrometry analysis of isolated TLC fractions

The TLC bands isolated from fraction C (section 3.2.10) were washed on C_{18} cartridges to remove acids and other impurities before analysis by LC-ESI-MS (method B, section 3.2.11.2). The absorbances of the eluted peaks were very low and this may have contributed to the low ionisation results. The same samples were analysed in a more concentrated solution and an isolated band containing mainly malvidin-3-glucoside (R_f 0.67) were also investigated. Even this band showed only low concentrations of the malvidin-3-glucoside (m/z 493.4) and the deglycosylated (m/z 331.2) ions. Both positive and negative electrospray mass spectrometry were tested with the positive mode giving the best results.

3.4 DISCUSSION

The RP-HPLC method of Price *et al.* (1995) was successfully optimised and the monomeric anthocyanins were separated from the polymeric peak and enabled quantification of the polymeric pigments. Improved recovery and shorter retention times were found on the

polystyrene vinylbensene column compared to a C_{18} column for the polymeric phenols and anthocyanins respectively. The polymeric nature of peak 15 and 23 was tested by ultracentrifugation (Figure 3.1). It was found that fractionation by ultracentrifugation was not only according to size as a 10000 NMWCO membrane removed more than 50% of the red colour of a red wine with only a small increase in retained colour when a 3000 NMWCO membrane was used (McKinnon 1996). Fractionation of the red pigments was in addition to size, a function of its dimensions, association with each other and the ultrafiltration membrane. For this reason the retentate was twice diluted back to the original wine volume and ultracentrifugation repeated. In the diluted retentate these associations would decrease and have a smaller influence on separation. Thus, as 86% of the anthocyanins were found in the ultrafiltrate, and only 7% of peak 15, we may conclude that more than 90% of peak 15 is not monomeric. Peak 23 also contained 80% non-pigmented material larger than the trimeric proanthocyanidins that are separated by RP-HPLC (Figure 3.2). In the future we will refer to this peak as the polymer peak. This 'polymeric' peak can be used to quantify polyphenolic material in the sample, but to discriminate among these larger molecules, other methods should be used to properly separate and analyse the higher molecular weight compounds, such as size exclusion or normal-phase liquid chromatography methods. Several methods were investigated to determine their ability to fractionate the polymeric phenols in wine into different polymeric fractions.

Ultrafiltration was the first fractionation method investigated. Sensory (duo-trio) analysis of the different fractions obtained showed that there were no significant differences between the reconstructed sample, where the filtrate and concentrate were added back together in the ratio it was separated during ultrafiltration, and the original wine before ultrafiltration. However, there were still losses of phenolic material due to absorption on the ultrafiltration membrane, even after pre-absorption of the membrane with the wine under investigation (Table 3.2). It is also clear from results obtained from the ultrafiltration of the two year experimental Shiraz with an YM 10 membrane (Table 3.2), that the molecular weight cut-off of the ultrafiltration membrane has a lesser influence on separation then association of the phenolics with each other and the membrane. This is in agreement with Shrikhande and Kupina (1993) who removed proanthocyanidins from pressed white wine and McKinnon (1996) who tested colour retention of red wine. Both found similar retention from a 1000, 2000 and 10000 molecular weight cut-off membrane. This is probably due to two reasons: (a) the intermolecular

association of proanthocyanidins due to weak hydrogen bonding creates a much larger molecular size; and (b) the branched structure of these compounds apparently inhibits the passage through the membrane pores. This shows that ultrafiltration is useful for the concentration of wine and/or samples but ineffective as a fractionation technique.

Different low pressure liquid chromatography methods were also evaluated for their ability to separate polymeric pigments into different fractions. RP-HPLC analyses of fractions obtained by Sephadex LH-20 separation showed that a larger fraction of polymeric phenols eluted in the methanol in a young wine when compared with an older wine. The hypothesis can be made that the chain length of polymeric pigments in the younger wine will be smaller and will thus be more polar than those in the older wine, which will explain the larger proportion of it eluting in the more polar methanol fraction in the young wine. The fact that the amount of polymeric pigments that eluted in the methanol fraction increased with elution time indicates that elution was not just according to the strength of interaction between the mobile phase and absorbed compounds, but also according to molecular sieve characteristics. The polymer peak of both the MeOH and acetone fractions were retained by a 10 kDa membrane during ultracentrifugation which indicate that it is not monomeric in nature. When the 280 nm versus 520 nm absorption of the polymeric peak obtained after 10 kDa membrane ultrafiltration, were compared, the methanol fraction has more colour. It can be postulated that the polymers with a higher percentage of pigmentation are more polar and will elute with the anthocyanins in the methanol fraction.

From investigations using mobile phases containing different percentages of methanol for the separation of monomeric and polymeric phenolics in wine on Sephadex LH-20, it is clear that the phenolics present in wine do not only separate according to the number of hydrogen molecules per phenol or consequently its interaction strength with the specific resin, but also according to its polarity, size and three-dimensional structure. Another complication is the association of the different phenolics present in a sample, with each other. Very little is known about these interactions and to what extent changes in sample composition may affect it.

It was also determined that sulphoxy ethyl cellulose resin did not fractionate the polymeric pigments but did separate pigments into those that form metabisulfite-complexes and those that do not. The exceptions were vitisn A and its acetyl derivative that eluted with the washing

step. Sulphoxy ethyl cellulose fractionation is useful for the isolation of low molecular weight pigments.

The metabisulfite fraction from sulphoxy ethyl cellulose separation (fraction A) and its resulting fractions obtained by further fractionation by RP-HPLC (A1), Sephadex LH-20 (A2) and NP-HPLC (A*ii* and A*iii*) contained pigments that were substituted in position 4 according to ESI-MS. These pigments have been described by Francia-Aricha *et al.* (1997), whereby an anthocyanin is linked in the C4 position via a vinyl linkage to either a (epi)catechin or proanthocyanidin. The C4 substituted pigments were not expected in the metabisulfite fraction and were not seen by Asenstorfer *et al.* (2001). Bakker and Timberlake (1997) found that while vitisin A was entirely resistant to sulfur dioxide bleaching vitisin B was partly bleached by sulfur dioxide. This will imply that vitisin B is able to form a sulfonate even though Berké *et al.* (1998) determined that addition only takes place in the C4 position of malvidin-3-glucoside. The acetaldehyde bridged pigments (Bakker *et al.* 1993) are unsubstituted in the C4 position and thus able to form bisulfite-addition products. The pigments observed were all of relatively low molecular weight due most probably to the young age of the wine investigated. The metabisulfite fraction (A) contained anthocyanins as well as direct condensation and ethyl linked malvidin-3-glucoside and (epi)catechin products, the methanol fraction (B) contained the vinyl linked products. The different polymer fractions collected from fraction A, gave different results, indicating that the individual techniques separated the polymers according to different characteristics. In fraction A2 similar compounds to those in fraction B was seen, they were just present in lower concentrations.

Polymeric pigments do not separate on NP-chromatography according to the same principles as the procyanidin polymers. This may be due to the higher polarity of the anthocyanin-glucosides, and their tendency to associate and/or interact with each other. Kennedy and Waterhouse (2000) came to the same conclusion when they investigated the separation of red wine by NP-HPLC. Kennedy and Waterhouse (2000) were able to separate the anthocyanins from the rest of the wine phenolics by the use of an ion-pair in the mobile system but there was very little fractionation of polymeric phenols. The flavonols and oligomeric proanthocyanidins were successfully separated from the polymeric pigments using NP-HPLC, by optimising the gradient conditions of the method used by Prieur *et al*. (1994). NP-HPLC may also be able to show differences in the different polymeric pigment fractions obtained by

the different fractionation methods being investigated, especially as the pigmented polymers all elute as a single peak at the end of the RP-HPLC run.

It was established that the polarity of the DCCC solvent systems tested to optimise separation of the pigmented phenols on silica, influence the elution position of phenolics but that selectivity is mainly influenced by the chemical properties of the solvent mixture used. It was also determined during the investigation discussed above, that isocratic systems, gave improved fractionation of the pigmented polymers compared to gradient systems on NP-HPLC columns. When butanol and propanol acetic acid and water solvent systems (6:1:2) for the separation of the different fractions on TLC silica plates were compared, it was determined that the propanol system was a better mobile phase for the polymeric 'rich' fractions. The different bands formed by TLC separation was isolated, washed and analysed by LC/ESI-MS. Only weak ionisation occurred. The fractions may have difficulty ionising or are unstable. These bands will be further investigated with other characterisation methods such as acid catalysis in the presence of a nucleophile and gel permeation chromatography which will be discussed in detail in chapter 4.

3.5 CONCLUSION

The developed HPLC method gives better separation of pigmented polymer and more accurate estimation of pigment polymerisation than any other method developed so far. This method was tested and investigated further by Peng *et al*. (2001) and published. Sephadex LH-20 was found to lack selectivity while sulphoxy ethyl cellulose will be a very effective isolation method for anthocyanins and other small pigments as shown by Asenstorfer *et al.* (2001) but neither will be effective in the fractionation of polymeric pigments present in red wine. MPLC of a Shiraz wine resulted in rough fractionation of flavonols, flavanols, phenolic acids and anthocyanins with an increasing concentration of non-pigmented and pigmented polyphenols with elution volume. Further fractionation by TLC showed promise and will be investigated further. The propanol-acetic acid-water solvent system will also be used on the NP-HPLC column and examined for identification and purification purposes. It was however determined that TLC separation gave higher resolution and consequently improved separation compared to the NP-HPLC system.

The MP-HPLC system together with subsequent TLC separation will now be employed as the new fractionation protocol. The different methodologies examined stress the difficulties faced when a complex sample such as wine is investigated.

Chapter 4

CHARACTERISATION AND IDENTIFICATION OF POLYMERIC PIGMENTS

4.1 INTRODUCTION

Different chromatographic techniques have been investigated in the purification of polymerised compounds. In an attempt to separate polymeric phenols according to their degree of polymerisation, size-exclusion (Shoji *et al.* 1999; Williams *et al.* 1983), normal-phase (Kennedy and Waterhouse 2000; Rigaud *et al.* 1993) and counter-current chromatographic methods (Okuda *et al.* 1988; Putman and Butler 1985; Salas *et al.* 2005; Shibusawa *et al.* 2000) have been developed and employed. Each of the above methods has both practical advantages as well as disadvantages, but using a combination of these methods allows researchers to separate individual oligomers based on their degree of polymerisation and determine the oligomeric composition of sample mixtures containing high-molecular-mass compounds. Spectrophotometric measurements, acid hydrolysis, acid hydrolysis in the presence of a nucleophile, gel permeation chromatography with reference standards and mass spectrometry are most commonly used for the characterisation of phenolic compounds (Fulcrand *et al.* 1999; Hayasaka and Kennedy 2003; Kennedy and Jones 2001; Porter *et al.* 1986; Remy-Tanneau *et al.* 2003; Williams *et al.* 1983). These methods have been shown to be successful in the case of non-pigmented phenols such as proanthocyanidins (Kennedy and Jones 2001; Prieur *et al.* 1994; Souquet *et al.* 1996). The characterisation of pigmented phenols is however more problematic.

Colour measurements used for the assessment of colour in red wines generally use the ionisable and bleachable nature of the anthocyanins to distinguish these from the polymeric forms that are generally less sensitive to pH and bleaching by bisulfite (Somers and Evans 1977). The addition of acetaldehyde eliminates the bleaching effect by the preferential binding of SO_2 to it. Various spectrophotometric and spectroscopic methods have also been used to determine the total polyphenol content of wine. Somers and Evans (1977) used the expression OD280 – 4, the subtraction to take the absorbance of non-phenolics into account, as an index of total phenols in red wine. However, the Folin-Ciocalteu method has been the one of general preference (Singleton and Rossi 1965), usually with an

arbitrary standard such as gallic acid used for quantification. To obtain numerical data directly related to perceived colour, reflectance or transmittance values have to be converted into tristimulus values using the CIE method first published in 1931 (1990). A more recent development in colorimetry is the 1976 CIELAB uniform colour space and colour difference equation, which is the most reliable method of quantifying the perceived variables of colour (Almela *et al.* 1996). Acid hydrolysis of procyanidins with butanol and hydrochloric acid yield the resulting anthocyanidins (Porter *et al.* 1986; Powell and Clifford 1995) for quantification.

Normal-phase high performance liquid chromatography (NP-HPLC) on silica has been used by Rigardo *et al*. (1993) and by subsequent authors for the fractionation, quantification and characterisation of procyanidin fractions according to molecular weight distribution (Prieur *et al.* 1994). Although this method was not successful in separating pigmented samples according to molecular weight, even after the modifications of Kennedy and Waterhouse (2000), it may still give insight into complex mixtures of samples.

Several methods have been developed to obtain molecular information about proanthocyanidins. Cleavage techniques rely on the conversion of proanthocyanidins into their constitutive subunits via acid catalysis in the presence of a nucleophilic agent such as benzyl mercaptan and phloroglucinol (Czochanska *et al.* 1979; Kennedy and Jones 2001; Koupai-Abyazani *et al.* 1992; Perez-Ilzarbe *et al.* 1992). The degradation allows the distinction between terminal units (released as flavan-3-ols) and extension units (released as their benzylthioethers or phloroglucinol adducts) and thus, gives a measure of the mean degree of polymerisation (mDP). Also, with the additional knowledge of subunit composition, the mass-average molecular mass can be obtained. Roux *et al*. (1998) found that thiolysis, failed to cleave A type proanthocyanidins. The ethyl-type linkage between epicatechin and the bridge is also more sensitive to thiolysis than that between the ethyl-type-group and malvidin-3-glucoside (Oszmianski and Moutounet 1996). Es-Safi *et al.* (1999b) have determined that malvidin-3-glucoside was released by thiolysis in the underivatised form when it was present in a terminal position (linked through it's C-6 or C-8 carbon) in the initial tannin structures (T-A), while Salas *et al*. (2004) showed that the covalent A-T structures do not react with benzyl mercaptan and remain intact. This is in agreement with the findings of Remy *et al*. (2003).

Gel permeation chromatography (GPC) methods are capable of providing information on compounds average molecular weight (M_w) while also providing information about their mass distribution (Bae *et al.* 1994; Kennedy *et al.* 2006; Kennedy and Taylor 2003; Viriot *et al.* 1994; Williams *et al.* 1983). Kennedy and Taylor (2003) investigated the relationship between the average molecular mass of proanthocyanidins determined by acid-catalysed cleavage in the presence of excess phloroglucinol and the average molecular mass as determined by the GPC method using unmodified proanthocyanidins. Assuming a constant composition for the two samples, they determined that the elution time corresponding to 50% mass elution is in good agreement ($R^2 = 0.984$) with the mDP determined by acid catalysis.

Electrospray ionisation mass spectrometry (ESI-MS) is mostly used for the identification of phenolic compounds. Detection in the positive mode is best for anthocyanin compounds, probably because they are in the flavylium cation form when pH is low. Fulcrand *et al.* (1999) showed that wine condensed tannins consist of mixed tri-((epi)gallocatechin) and dihydroxylated ((epi)catechin) flavanol units and also exclusively of (epi)catechin units. The estimated mean degree of polymerisation (mDP) for a 3 to 4 year old Cabernet-Sauvignon was 6.4, which was similar to values reported earlier in wine by Sarni-Manchado *et al.* (1999b). The percentage of galloylated units was estimated at 3.3% and that of (epi)gallocatechin units was found at 17.9%. Anthocyanins were simply fragmented into anthocyanidins as a result of the elimination of a dehydrated sugar moiety with a mass of 162, while the ions derived by the cleavage of the interflavonoid bonds, provide good evidence for the proposed structures of T-A and A-T type of anthocyanin-proanthocyanidin polymers (Hayasaka and Kennedy 2003; Remy *et al.* 2000).

Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) is another soft ionisation MS technique with a wider applicable mass range and has been successful in analysing apple, hop and grape seed procyanidins up to the decamer level as single charged species (Krueger *et al.* 2000; Ohnishi-Kameyama *et al.* 1997; Taylor *et al.* 2003; Vivas *et al.* 2004) as well as coffee pulp up to the hexameric level (Ramirez-Coronel *et al.* 2004).

Polysaccharides are one of the main groups of wine macromolecules associated with polymeric pigments through co-operative hydrogen bonding between the oxygen atom of the carbohydrate and the phenolic hydroxyl group as well as hydrophobic interactions (Vernhet *et al.* 1996). Doco *et al*. (1999) have found that the polysaccharide content of

wine is stable for 10 to 15 years after their production. The presence of polysaccharides could have a significant effect on the fractionation of the polymeric pigments and their characterisation. Grape-derived polysaccharides consist of acidic and neutral polysaccharides. The neutral sugar composition of these polysaccharides can be determined by gas chromatography mass spectrometry (GC-MS) analysis of their alditol acetate derivatives (Harris *et al.* 1984). Both the neutral and acidic glycosyl residues can also be determined by GC-MS of their per-*O*-trimethylsilylated (TMS) methyl glycosides obtained after acidic methanolysis of the polysaccharides as all monosaccharides are released during methanolysis (Sims and Bacic 1995). However, these procedures only allow quantification of the compositional monosaccharides but give no information of their linkage positions. The various types of sugars present in a polysaccharide and the position of linkages between monosaccharide residues can be determined by linkage analysis, also known as methylation analysis (Sims and Bacic 1995).

Different LC fractionation methods have been used together with spectroscopic measurements, acid hydrolysis methods and GPC to characterise the different TLC bands obtained as described in Chapter 3. In addition different MS methodologies have been employed to further identify and confirm the composition of these complex wine fractions.

4.2 MATERIALS AND METHODS

4.2.1 Red wine fractionation

4.2.1.1 NP-MPLC of six month and five year old Shiraz wine

Shiraz wines were prepared as described in chapter 3, section 3.2.1.1. The 6 month old and 5 year old wines were fractionated on preparative NP silica columns by MPLC as described in chapter 3, section 3.2.10, with mobile phase propanol-acetic-acid-water, 6:1:2. The wines were concentrated under reduced pressure at 35ºC and dissolved in the mobile phase (propanol-acetic acid-water, 6:1:2) resulting in a sample ten times the concentration of the original wine. For the 6 month old wine which contained a large concentration of monomeric pigment, the equivalent of 50 mL wine was loaded onto the column, while the equivalent of a 100 ml of the 5 year old wine was loaded. Separation was repeated for both the young and older wine. The chromatogram was calibrated with catechin standard and

0.090 AU and 0.064 AU were equal to 10 mm during fractionation of the 6 month old and 5 year old wine, respectively.

4.2.1.2 Separation and extraction on TLC plates

Separation of the collected NP-MPLC fractions (section 4.2.1.1) were performed on Silica 60 F546 as described in chapter 3, section 3.2.11. Bands were scraped from the plates and extracted overnight from the silica with a combination of water, methanol and propanol at 4ºC. The supernatants were removed with glass pasteur pipettes, centrifuged at 13400 *g* in a Mikro 12-24 centrifuge (Hettich) for 5 minutes and concentrated on a turbovap (Zymark, Massachusetts, USA) at 35 to 38ºC before analysis by RP-HPLC as described in chapter 3, section 3.2.2.

4.2.2 RP-HPLC

RP-HPLC was performed as described in chapter 3, section 3.2.2.

4.2.3 Paper-electrophoresis

The high voltage paper electrophoresis (HVPE) apparatus is based on the immersed strip method, where the paper is immersed in an inert liquid, which is used to dissipate the heat (Tate 1968). The coolant used in this case is tetrachloroethylene. A water-cooling coil provided additional cooling to maintain an operating temperature of approximately 25°C. An oxalate buffer (pH 1.5) was made by mixing 0.1 M oxalic acid and 0.1 M sodium oxalate, while a boric acid ammonia buffer (pH 9.2) was prepared by mixing 0.34 M boric acid, 0.4 M ammonium bicarbonate and 0.2 M ammonium hydroxide (all chemicals obtained from Sigma). A 0.1 M potassium metabisulfite buffer (pH 4.2) was also utilised. Chromatography paper (number 1; Whatman) was used and all relative mobilities were compared to Orange G (1-phenylazo-2-naphthol-3,5-disulphonate) as the anionic standard, fructose as the neutral standard and malvidin-3-*p*-coumaryl-glucoside as the positive standard. The position of fructose was revealed using a silver nitrate stain (Trevelyan *et al.* 1950). A polymeric pigment sample (isolated according to the protocol described in chapter 3, section 3.3.2.4) and FrDB4 (polymer fraction isolated as described in section 4.2.1.1 by MPLC and subsequent TLC separation) were investigated by paper electrophoresis.

4.2.4 UV-vis/CieLab analysis

The isolated bands of the 6 month old wine obtained by TLC (as described in section 4.2.1.2) were diluted to its original concentration in the wine with model wine (10% ethanol, saturated with potassium hydrogen tartrate and adjusted to pH 3.5 with concentrated hydrochloric acid). Absorbance measurements were performed according to the Somers method (described in chapter 3, section 3.2.1.2). The CieLab and tristimulus values were determined with the illuminant D65, 10 degree standard observer quarts and transmittance between 380 and 780 nm.

4.2.5 Infra-red analysis

The polymeric fraction isolated by NP-MPLC (section 4.2.1.1) from the 6 month old red wine was further separated by TLC into several bands. Band 4 was the most concentrated and will be referred to as FrDB4. The infra-red spectra of malvidin-3-*O*-glucoside, (+)-catechin and FrDB4 were obtained using diffuse reflectance. Methanolic solutions of the samples were dried in the presence of a few milligrams of potassium chloride. The samples were then finely ground and loaded into a Harrick sample holder (diameter of 11 mm and a depth of 1 mm; Model DRA-SX3; Harrick Scientific Corp., USA). To obtain the spectra, a double beam dispersive infra-red spectrometer (Model PE 983G; Perkin-Elmer, England) fitted with a Harrick praying mantis diffuse reflectance attachment (Model 3SP; Harrick Scientific Corp., USA) operating in double beam mode, was used. The spectrometer was linked to a personal computer and controlled through a modified PE983G emulator-controller (MDS engineering Associates, USA).

The sample compartment was continually purged throughout the duration of sample collection using a circulatory drier (Model CD3; Bodenseewerk Perkin-Elmer & Co GmbH, W. Germany). The diffuse reflectance data was recorded as % transmission using Grams/386 II software (Galactic Industries Corp, USA). The data was then converted to absorbance units.

4.2.6 Acid hydrolysis

Grape seed extract prepared as described in section 3.2.3, cyanidin-3-*O*-glucoside (PolyphenolAS, Norway) and isolated bands (FrDB2, FrDB 3, FrDB 5, FrDB6; section

4.2.1) were analysed according to the method described by Porter *et al.* (1986). All samples were at a concentration of 0.2 g/L in methanol. The initial absorbances of all samples were measured at 280 nm in methanol, in addition to RP-HPLC analysis (chapter 3, section 3.2.2). To each sample (1 mL), n-butanol with concentrated HCl (95:5 v/v) (6 mL) and 2% solution of ferric reagent ($NaFe(SO_4)_2.12H_2O$) in 2 M HCl (0.2 mL) were added. The samples were mixed and then heated for 40 min at 95 ± 2ºC. The absorbance of the solution was measured at 550 and 520 nm before and after hydrolysis, in addition to RP-HPLC analysis by direct injection after centrifugation (13400 *g*, 5 min).

4.2.7 Liquid chromatography electrospray ionisation mass spectrometry (LC/ESI-MS) analysis

LC/ESI-MS method B (described in chapter 3, section 3.2.12.2) were used to analyse the isolated fractions in section 4.2.1. The samples were washed according to the procedure described in section 4.2.8 and concentrated in methanol under reduced pressure at 35ºC. The LC gradient with Solvent A (aqueous 4.5% (w/w) formic acid) and Solvent B (20% (v/v) of Solvent A made up to 100% with CH_3CN) used for the isolated fractions was as follows: 0 min, A 94%, B 6%; 73 min, A 69%, B 31%; 78 to 86 min, A 38%, B 62%; 90 min, A 94%, B 6%. The flow rate was 1 mL/min.

4.2.8 Sample clean-up

C_{18} Sep-Paks (Waters) were connected to a vacuum manifold and prepared by the sequential addition of methanol (10 mL) and water (10 mL) at high suction. Samples were absorbed onto the Sep-Paks at low suction, after which it was washed with water (30 mL) at high suction to remove unbound sugars and organic acids. The absorbed phenolic compounds were eluted with 2 mL methanol at low suction and the eluate collected.

4.2.9 MALDI-TOF analysis

Grape seed tannin extract (chapter 3, section 3.2.3), retentate prepared from the 6 month old red wine (chapter 3, section 3.2.1.3) and FrDB1, FrDB 2, FrDB 4 and FrDB 5 (section 4.2.1) were combined with the matrix (saturated 3,5-dihydroxycinnamic acid in methanol) in a ratio of 40 mg/mL. Samples were analysed by linear Matrix Assisted Laser Desorption/Time-of-flight (MALDI-TOF) with PerSeptive Biosystems Voyager-De (PE-Biosystems, Framingham, MA, USA). Positive ion mode spectra were obtained with 20

kV acceleration voltage and 125 ns delay time. Each recorded spectrum was the sum of 128 consecutive pulses.

4.2.10 Separation of sugars and phenolics

When polysaccharides are isolated from red wine, various stationary phases such as Polyamide CC6 (Doco *et al.* 1996), Sephadex G-100 (Yokotsuka *et al.* 1994) and Sephadex LH-20 (Brillouet *et al.* 1990, 1991; Pellerin and Brillouet 1992) are used in combination with NaCl to remove the red pigments that adheres to it. Galetti *et al*. (1995) also used Sephadex LH-20 to isolate brown polymers for pyrolysis and there was no evidence of sugar dehydration products or other contaminants from the column.

Empty reservoirs (8 mL, polypropylene 20 µm polyethylen frits, Altech) were packed with dry Sephadex LH-20 (1.8 g). Packed reservoirs were washed with 10 void volume (V_o) of water and equilibrated with 15 V_o of 0.2 M NaCl before samples (0.5 – 1 mL), adjusted to a content of 0.2 M NaCl with a 1 M NaCl solution, were loaded. Samples were washed with water (15 mL, 16 V_o), followed with 60% (v/v) methanol (0.2% acetic acid) (25 mL) and 50% (v/v) acetone (0.2% acetic acid) (35 mL). Polysaccharides elute with the void volume and the polyphenols were washed off with 60% (v/v) acetone. The method was tested with and without the use of NaCl. Sample eluates were concentrated on a rotary vacuum evaporator at 35ºC, washed on C_{18} (section 4.2.8) and concentrated by a nitrogen stream at 30ºC, before analysis by RP-HPLC (section 4.2.2) and GC-MS analysis of the alditol acetates (section 4.2.11.4) as described in section 4.2.11.

4.2.11 Carbohydrate analysis

Methanol, chloroform, ethyl acetate, *t*-butyl alcohol, acetic acid (glacial) and ammonia (35% aqueous) were purchased from BDH laboratory chemicals (Victoria, Australia) and were Arista grade quality. Sodium hydroxide (minipearls), sulphuric acid, acetic anhydrid and perchloric acid (70% aqueous) were also purchased from BDH and were of AnalR grade quality. Dimethylsulphoxide (high purity Omnisolv grade) was sourced from Merck Chemicals (Victoria, Australia). Methyl iodide, sodium borodeuteride ($NaBD_4$) and trifluoroacetic acid (TFA) were supplied by Sigma-Aldrich Chemical Company (St Louis, MO, USA), while *myo*-inositol and 1-methylimidazole were sourced from Fluka Biochemika (Buchs, Switzerland). The water and dichloromethane used were re-distilled. Only chromic acid washed glassware were used. Gases were purchased from CIG

(Melbourne, Australia). Helium was of ultra-high purity grade and nitrogen was of CIG Industrial grade.

4.2.11.1 Carbohydrate hydrolysis

A 100 µg of each of the following standards were dissolved in 5 mL re-distilled water; α-L-rhamnose (Sigma R-3875), D-arabinose, D(+)xylose (Merck 8692), D(+)mannose (Sigma M-4625), D(+)galactose (Sigma G-0750) and α-D(+)glucose (Sigma G-5000). An internal standard of α-D-allose (10 µg) (Sigma A-6390) was added to the standards and all samples. The sample was dried in methanol with a stream of nitrogen at 30ºC and dissolved in 2.5 M TFA (500 µL) by vortex mixing for carbohydrate hydrolysis. The sample was placed in a 15 mL Kimax screw capped test tube (Kimble Kontes, New Jersey, USA), flushed with nitrogen, capped and sealed with Teflon tape and heated at 121 ± 2 ºC for 90 min. Next, the sample was cooled on ice and evaporated to dryness with nitrogen at 30ºC, to an oily smear.

4.2.11.2 Reduction of monosaccharides

Reduction of the resultant monosaccharides to their respective alditols was achieved as follows: sample was dissolved in freshly made 1 M sodium borodeuteride in 2 M NH_4OH (500 µL) by vortex mixing. The samples were then sonicated (2 min) before incubation (60ºC, 60 min). Excess reductant was destroyed with the addition of aliquots of acetic acid (50 µL) until the sample stopped fizzing (usually three additions). Then the sample was dried under nitrogen and 2.5 mL aliquots of fresh methanolic acetic acid (5% v/v acetic acid) was added and evaporated (usually two additions) to remove boric acid. The process was repeated until the sample formed an oily smear or a crystalline residue.

4.2.11.3 Acetylation

The above residue was dissolved in glacial acetic acid (200 µL) and acetylated by adding ethyl acetate (1 mL) and acetic anhydride (3 mL). The resulting suspension was made soluble with the addition of 70% aqueous perchloric acid (100 µL). The exothermic reaction was cooled on ice (5 min) and then treated with water (10 mL) followed by 1-methylimidizole (200 µL). After 5 min the alditol acetates were extracted with

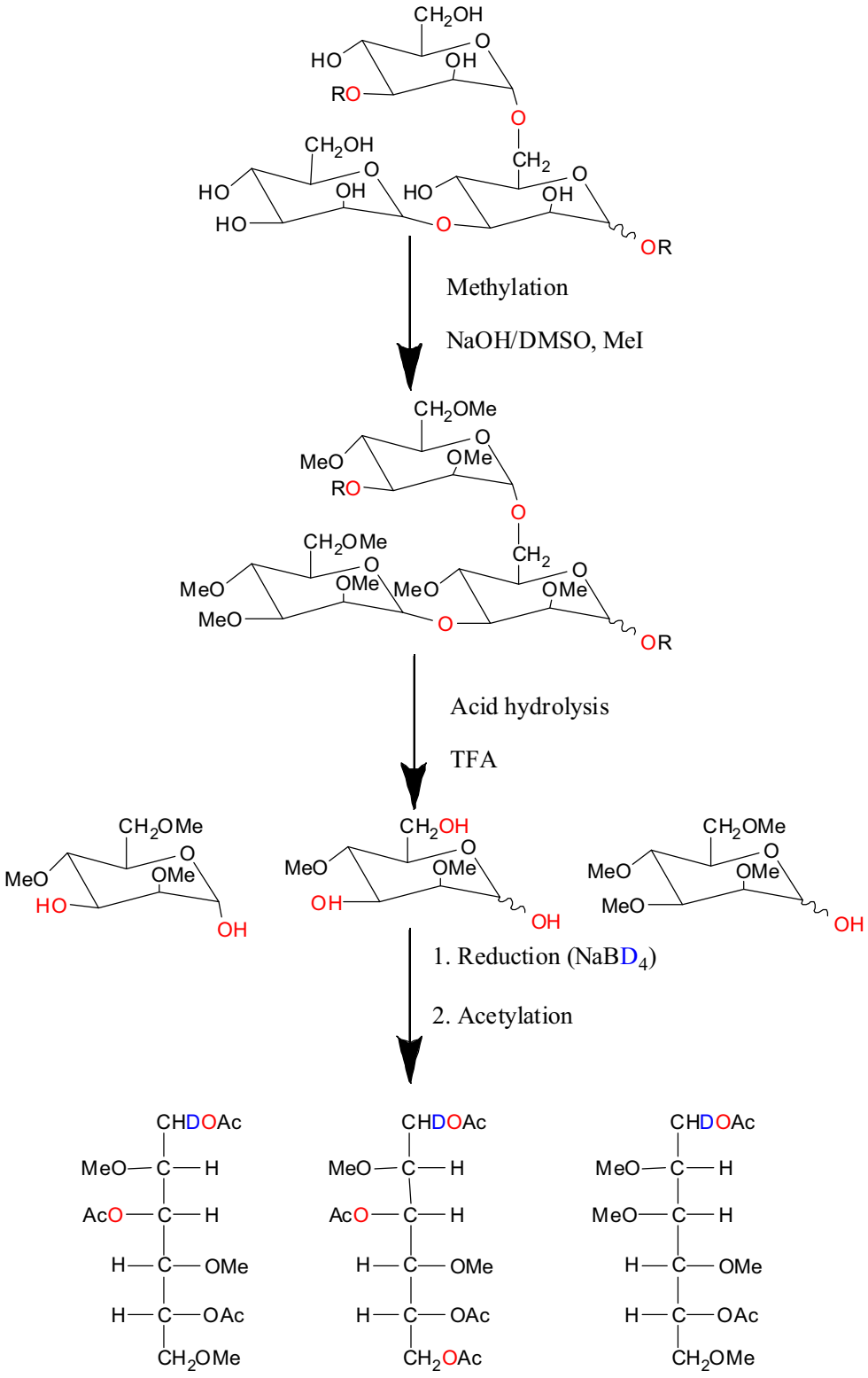
dichloromethane (2 × 1 mL). The organic phase was washed with water (2 × 5 mL), transferred to a glass vial and dried under nitrogen at 30ºC. The concentrated solution was analysed by GC-MS as described below.

4.2.11.4 GC-MS of alditol acetates

The alditol acetates from above were separated and identified by GC-MS using a Hewlett-Packard 593 GC-MS with a DB1701 low polarity column (30 m × 0.32 mm ID) (J & W Scientific, Inc., Folsom, CA). Injections were made with the injector at 240ºC operating in the split mode. The carrier gas was helium. The column was held at 110ºC for 2 min and then programmed at 3ºC/min to 240ºC. Electron impact ionisation at an ionisation potential of 70 eV was used for MS. Derivatives eluting from the gas chromatogram were detected and identified by comparison of their mass spectra with standard spectra by means of selected-ion monitoring mode for the ions of *m/z* 115, 129 and 145.

4.2.11.5 Glycosyl-residue composition (Methanolysis)

The neutral and acidic sugars were determined according to the method of Sims and Bacic (1995) and Lau and Bacic (1993) (see Figure 4.1). 1 M Methanolic-HCl (250 μL) (prepared by slowly adding acetyl chloride to methanol) were added to FrDOrig, FrDB1, FrDB2, FrDB3, FrDB5, FrDB6 and the retentate of the 6 month old Shiraz wine as well as malvidin-3-glucoside and the resulting solutions were heated (80ºC, 16 hr), which converts the polysaccharides into a mixture of methyl glycosides and methyl ester methyl glycosides of the glycosyluronic acids. The methanolic HCl was removed by adding *t*-butyl alcohol (100 μL) and then evaporating with a stream of filtered air at room temperature. The methyl glycosides and methyl ester methyl glycosides were silylated by adding Tri-Sil (650 μL) (Pierce Chemical Company) and heating the samples (80ºC, 20 min). The excess silylating reagent was removed by gentle evaporation at room temperature. The derivatives were redissolved in hexane (1 mL) and the insoluble salts were allowed to settle. The supernatant were transferred and evaporated with a stream of nitrogen. The residue was dissolved in hexane (100 μL).



where R = another sugar

Figure 4.1 Illustration of the series of reactions for linkage (methylation) analysis of a polysaccharide (section 4.2.11.5). The position of the glycosidic linkages and the reducing end hydroxyl group are outlined in red. After methylation (section 4.2.11.8) and hydrolysis (section 4.2.11.9), a hydroxyl group is present at the site where the sugar was once linked. Following reduction with sodium borodeuteride (section 4.2.11.9), C_1 is labelled with

deuterium (blue outline). After the subsequent acetylation step (4.2.11.9) an acetyl group is present where there was previously a site of linkage on the sugar. As the sugar which constitute the polysaccharide are in the pyranose form, position 5 is also acetylated.

4.2.11.6 Carboxyl reduction

Samples were dissolved in 500 mM imidazole-HCl (0.5 mL, pH 8), cooled in an ice bath, and the resulting esterified uronic acids were reduced by three additions of $NaBD_4$ (each 0.1 mL, 100 mg/mL in 500 mM imidazole-HCl, added at 5 min intervals) and left for 1 hr on ice. The use of $NaBD_4$ assists the MS analysis by labelling C_1 of the resulting alditol with a deuteride ion. Excess $NaBD_4$ was destroyed with acetic acid and the samples dialysed for 24 hr against deionized H_2O and freeze-dried. Samples were then dissolved in 50 mM MES-KOH (0.2 mL, pH 4.75) and free uronic acid residues were derivatised with 1-cyclo-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulphonate (0.04 mL, 500 mg/L, CMC, Sigma) for 2 to 3 hr at 30ºC. Afterwards, tris-HCl buffer (0.2 mL, pH 8) and a drop of *n*-octanol were added and samples were reduced (18 hr, 4ºC) with $NaBH_4$ (0.2 mL, 70 mg/mL in 0.05 M NaOH). Excess reductant was destroyed with acetic acid, after which samples were dialysed extensively against de-ionised H_2O and freeze-dried.

4.2.11.7 Preparation of the NaOH slurry in dimethoxysulfoxide (DMSO)

Preparation of the 50% NaOH solution was performed according to the method published by the American Society of Testing Material (E200 1997). The DMSO reagent was prepared immediately before use by mixing 50% w/v of NaOH (0.1 mL) with methanol (0.2 mL). The suspension was diluted with DMSO (3 mL) and vigorously shaken on a vortex mixer and then sonicated (3 - 5 min) until a fine dispersion of NaOH-DMSO was obtained. The NaOH precipitate was collected, washed with fresh DMSO (4 × 0.3 mL) and finally suspended in DMSO (0.2 mL) to obtain an essentially anhydrous suspension for use in the methylation reaction.

4.2.11.8 Methylation of polysaccharides

Methylation analysis were performed by the carbohydrate laboratory, Department of Botany, Melbourne University, by a modification of the sodium hydroxide methylation procedure as described by Sims and Bacic (1995) using methyl iodide. Samples, the origin

from TLC (Figure 4.3) isolated from fraction D (FrDOrig), FrDB1, FrDB2, FrDB4, FrDB5, FrDB6 and the 6 month old wine retentate and malvidin-3-*O*-glucoside standard (PhenolAS, Norway) were freeze-dried and analysed in duplicate. Samples were dissolved in dimethoxysulfoxide (50 μl) and methylated by sequential addition of a freshly prepared NaOH-DMSO slurry (120 mg/mL, 50 μL, 20 min) and three additions of methyl iodide (10, 10 and 20 μL) at 10 min intervals. After methylation, 1 mL freshly prepared aqueous sodium thiosulphate (100 mg/mL) was added, to remove the sodium iodide, along with 0.5 mL chloroform. The mixture was vortexed and centrifuged to separate the aqueous and organic phases. The upper aqueous phase was discarded. The lower chloroform phase which contained the methylated polysaccharides was collected and washed with water (4 × 0.5 mL). The polysaccharide samples were dried under a stream of nitrogen at 35ºC.

4.2.11.9 Hydrolysis, reduction and acetylation of the methylated polysaccharides

The dry methylated samples from section 4.2.11.8 were hydrolysed in 2.5 M TFA (100 μL) at 120ºC for 90 min. After cooling on ice, the samples were evaporated to dryness with a stream of nitrogen at 30ºC to produce an oily smear. The internal standard, *myo*-inositol, was added to the oily residue and the sample was once again evaporated to dryness under a stream of nitrogen at 30ºC. The methylated monosaccharides were reduced and then acetylated according the procedures discussed in sections 4.2.11.2 and 4.2.11.3.

4.2.11.10 Gas chromatography-mass spectrometry of monosaccharide derivatives

The partially methylated alditol acetates from section 4.2.11.9 were separated, identified and quantitated on a fused-silica capillary column (25 m × 0.22 mm i.d., film thickness 0.25 μm) with bonded-phase BPX70 (SGE, Melbourne, Australia) on a Finnigan MAT 1020B GC-MS. Identification were based on peak R_ts and by comparison of EI-MS with published spectra.

4.2.12 Determination of tannin mDP by phloroglucinolysis

The following standards were bought from Sigma-Aldrich (Sydney, Australia): catechin, epicatechin, epicatechin gallate and epigallocatechin. Epigallocatechin phloroglucinol adduct, epicatechin phloroglucinol adduct and catechin phloroglucinol adduct were gifts

from Dr J.A. Kennedy (University of Adelaide). The method described by Kennedy and Jones (2001) was tested on Shiraz grape seed and skin proanthocyanidin extracts. The seeds and skins were separated from the berry mesocarp, rinsed with distilled water and clad dry with tissue paper. The seed extract was prepared as described in chapter 3, section 3.2.3. The skins were extracted with methanol containing 0.1% HCl at room temperature for 1 hr. The slurry was centrifuged (12000 *g*, 5ºC, 40 min), and the supernatant concentrated under reduced pressure at 35ºC after which it was ultracentrifuged according to the procedure described in chapter 3, section 3.2.1.3. The seed and skin extracts were then lyophilized to a dry powder. Acid catalysis was also performed on catechin and epicatechin standards in addition to the isolated fractions, to investigate possible epimerization.

A 0.1 M HCl in methanol, containing 50 mg/mL phloroglucinol and 10 mg/mL ascorbic acid, solution (20 μL) was added to a sample (5 – 100 μg) and incubated at 50ºC. The solution was prepared by adding 0.1 ml 10 M HCl, 0.1 g ascorbic acid and 0.5 g of phloroglucinol together and making it up to 10 ml with methanol. The reaction was stopped after 20 min with the addition of a 40 mM aqueous sodium acetate solution (100 μL). The proanthocyanidin cleavage products were analysed by RP-HPLC. The column was a Wakosil SS C_{18} column (250 × 4.6 mm, 5 μm) purchased from SGE (Melbourne, Australia), protected by a guard column containing the same material. The HPLC conditions used were mobile phase A (1.5 % orthophosphoric acid) and B (80% acetonitrile and 20% solvent A) with starting conditions being isocractic elution for 10 min with 5% B, linear gradient to 18% B in 36.5 min, 35% B in 28.5 min and 100% B in 10 min. Return to 5% B in 5 min and equilibrate the system for 10 min at the starting conditions before the next injection.

The proanthocyanidin cleavage products were estimated by means of their response factor relative to catechin, which was used as the quantitave standard (Table 4.1). The molar absorbtivities determined by Kennedy and Jones (2001) were used. All samples were analysed in duplicate. To calculate the apparent mean degree of polymerisation (mDP), the sum of all subunits (flavan-3-ol monomers and phloroglucinol adducts, in moles) was divided by the sum of all flavan-3-ol monomers (in moles).

Table 4.1 Retention properties, molar absorptivities, and response factors for the major grape proanthocyanidin cleavage products.

Compound	Retention factor (k)	Molar absorbtivity (ε_{280})	Relative Molar Response[a,b]	Corrected Relative Mass Response[a,b]
Epigallocatechin phloroglucinol adduct	5.8	1344	0.35	0.33
Catechin phloroglucinol adduct	9.3	4218	1.1	1.1
Epicatechin phloroglucinol adduct	9.8	4218	1.1	1.1
Epigallocatechin	11.6	1344	0.35	0.33
Catechin	12.3	3862	1.0	1.0
Epicatechingallate phloroglucinol adduct	15.5	12889	3.3	2.2
Epicatechin	15.8	3862	1.0	1.0
Epicatechingallate	21.0	12889	3.3	2.2

[a]Relative to catechin.

[b]Relative mass response not including the phloroglucinol moiety.

4.2.13 Gel permeation chromatography

GPC was performed on acetylated phenols to remove possible interactions between the phenolic material and the GPC packing material. The acetylation procedure as described by Williams *et al*. (1983) was used with modifications. The samples were acetylated with pyridine-acetic anhydride (1:1), and after standing overnight the reaction mixtures were stopped by the addition of ice (10 mL). The peracetates were extracted with chloroform (500 µL × 3) and dried under nitrogen at 35 – 38ºC. For analysis, the acetylated samples were dissolved in freshly redistilled THF (tertrahydrofuran, Omnisolve, Merck).

Polystyrene standards (500, 1000, 2000, 3000, 5000, 10000, 20000, 30000 and 70000, all bought from Fluka), acetylated standards of malvidin-3-*O*-glucoside, catechin, epicatechin and apple and grape seed procyanidins with a mean degree of polymerisation of three, gifts from Dr S. Vidal, isolated as described in Vidal *et al*. (2003a) were used for the construction of the calibration curve of log(Mr) against time of elution. The acetylated samples (1 mg/mL in THF, injection volume 20 µL) were analysed on two columns of TSK-Gel Hxl, 500 and 1500 Å (300 mm × 7.8 mm I.D., Sigma Aldrich, NSW, Australia), connected in order of decreasing pore size.

4.3 RESULTS

4.3.1 Isolation and characterisation of the polymeric fraction of a 6 month old Shiraz wine

4.3.1.1 Isolation of different polymeric pigment fractions by preparative NP chromatography and TLC

The MPLC separation profile of the wine concentrate recorded at 280 nm is shown in Figure 4.2. The wine was generally separated into fractions (A and B) enriched in lower molecular weight (monomeric and oligomeric) phenols and those (C and D) enriched in higher molecular weight phenols (Table 4.2). Consecutive samples contained increasing percentages of polymeric phenols from 37 to 90%.

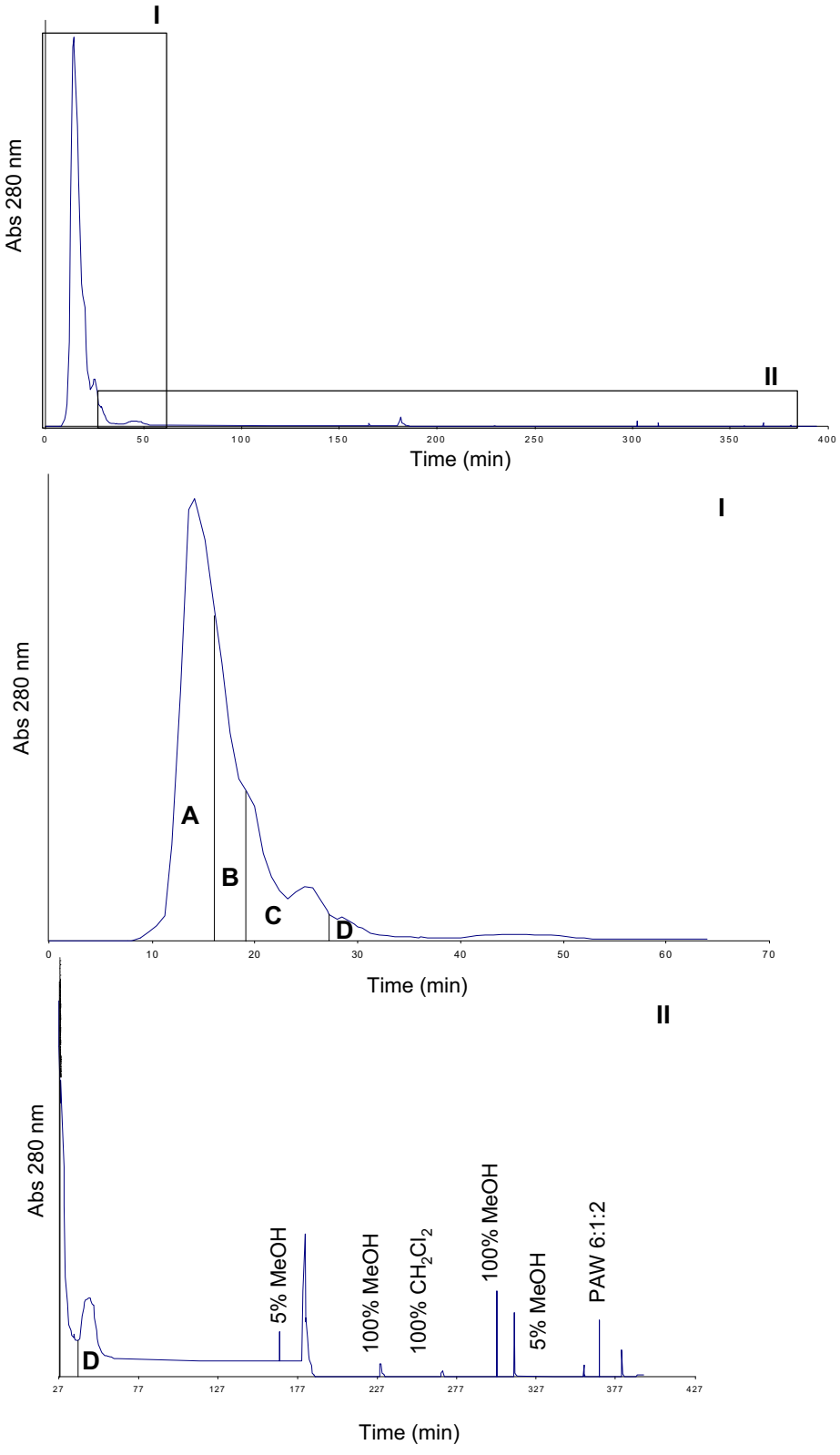


Figure 4.2 MPLC chromatogram of a 6 month old Shiraz wine with mobile phase propanol-acetic acid-water (6:1:2).

Table 4.2 Phenol composition (in mg) of fractions A to D obtained by MPLC fractionation.

Fraction*	Anth	Vitisin	Pigm	Pol pigm	Cinnmc	Flavo	Gallic	Flava	Proc	Pol ph
A	1617.2	775.67	635.15	5240.7	1532.3	nd	700.25	6531.3	85702	61676
B	985.01	723.51	373.18	1589.5	1840.2	508.79	204.46	4359.1	5467.2	13097
C	23.796	44.001	17.923	390.08	157.23	8.3676	10.321	115.46	28.694	3859.4
D	4.2666	1.6354	nd	22.575	9.7260	1.7185	4.7033	27.965	19.521	273.88

*Values in mg.

Compound abbreviations: Anth, monomeric anthocyanins; Vitisin, vitisin A and acetylated vitisin A; Pigm, low molecular weight pigments, Pol pigm, non-monomeric pigments; Cinnmc, cinnamic acids; Flavo, flavonols; Gallic, gallic acid; Flava, flavan-3-ol monomers; Proc, proanthocyanidins; Pol ph, polymeric phenols.

nd; not detected.

Fractions A to D were further fractionated by TLC (Figure 4.3). The separated TLC bands were isolated and analysed by RP-HPLC (Tables 4.2 to 4.5). The anthocyanins and proanthocyanidins eluted mostly in the early eluting bands with high mobility, while the late eluting bands consisted mainly of polymeric pigments and phenols. Fraction D contained the lowest amount of monomeric phenols and TLC separation gave different bands containing mostly polymeric phenols with different mobility in the mobile phase (*n*-propanol-acetic acid-water, 6:1:2) used.

Table 4.3 Phenol composition (in mg/L) of bands separated and isolated from fraction A by TLC.

Fraction A*	Anth	Vitisin	Pigm	Pol pigm	Cinn mc	Flavo	Gallic	Flava	Proc	Pol ph
Band 1	7.23	1.39	236	34.7	66.8	91.9	1.41	175	588	7020
Band 2	909	nd	51.1	6.69	2.31	8.97	152	122	756	458
Band 3	566	nd	22.6	26.4	9.72	34.2	60.4	26.0	107	471
Band 4	9464	nd	270	237	306	602	2.43	nd	522	3505
Band 5	550	nd	290	272	103	1098	nd	nd	249	3655
Band 6	758	6.76	180	279	86.7	834	1.88	610	172	3000
Band 7	89.6	61.5	10.8	134	366	26.8	0.33	nd	14.2	1608
Band 8	60.0	149	13.0	98.1	517	18.1	nd	nd	27.8	1179

*Values in mg/L.

Compound abbreviations as for Table 4.

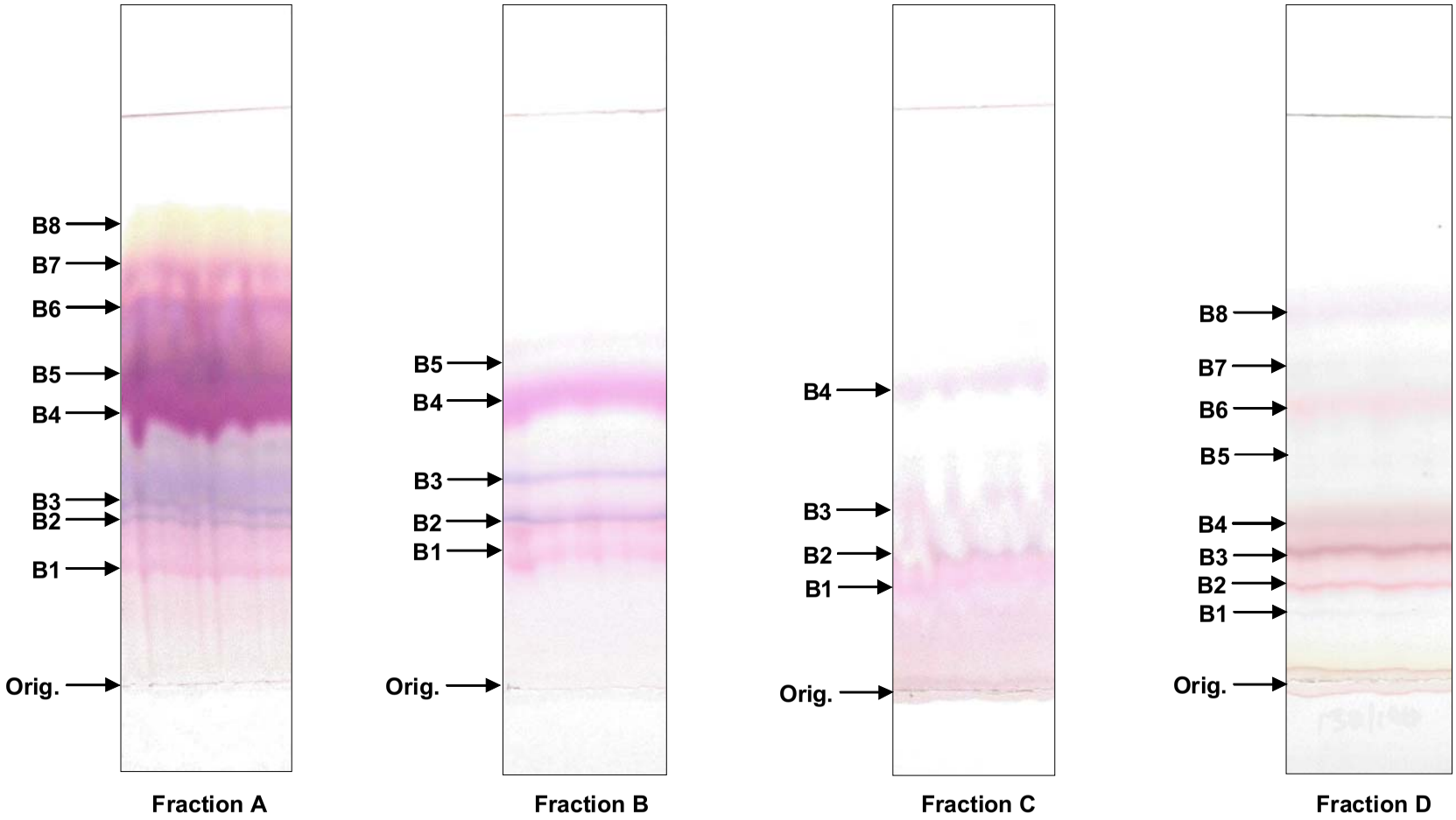


Figure 4.3 TLC plates showing the separation of fraction A (using mobile phase *n*-butanol-acetic acid-water (6:1:2)), and fraction B, C and D (using mobile phase *n*-propanol-acetic acid-water (6:1:2)) into individual bands.

Table 4.4 Phenol composition (in mg/L) of bands separated and isolated from fraction B by TLC.

Fraction B*	Anth	Vitisin	Pigm	Pol pigm	Cinn mc	Flavo	Gallic	Flava	Proc	Polyph
Band 1	9.76	334	29.4	37.7	8.65	nd	0.69	13.8	210.3	715
Band 2	24.5	45.7	56.8	22.7	20.3	2.67	nd	nd	12.5	444
Band 3	81.5	3.51	11.7	12.6	7.39	nd	nd	nd	48.1	254
Band 4	120	nd	13.7	9.54	nd	4.49	nd	nd	86.7	173
Band 5	945	nd	32.1	16.9	28.4	45.5	nd	nd	225	393

*Values in mg/L.

Compound abbreviations as for Table 4.2.

Table 4.5 Phenol composition (in mg/L) of bands separated and isolated from fraction C by TLC.

Fraction C*	Anth	Vitisin	Pigm	Pol pigm	Cinn mc	Flavo	Gallic	Flava	Proc	Pol ph
Band 1	0.86	0.14	0.35	15.1	nd	nd	nd	nd	3.32	321
Band 2	2.02	73.3	13.2	16.2	0.71	nd	6.47	nd	291	282
Band 3	22.5	50.8	25.0	24.4	1.23	4.44	2.12	19.6	320	483
Band 4	33.8	nd	0.06	1.92	nd	nd	nd	1.05	9.88	121

*Values in mg/L.

Compound abbreviations as for Table 4.2.

Table 4.6 Phenol composition (in mg/L) of bands separated and isolated from fraction D by TLC.

Fraction D*	Anth	Vitisin	Pigm	Pol pigm	Cinn mc	Flavo	Gallic	Flava	Proc	Pol ph
Origin	0.36	nd	nd	54.2	2.77	nd	nd	nd	1.26	342
Band 1	nd	nd	nd	13.7	2.37	nd	nd	nd	17.2	784
Band 2	2.36	nd	nd	73.6	6.50	nd	nd	28.4	1998	14356
Band 3	1.29	nd	nd	26.4	1.87	nd	nd	nd	3.84	554
Band 4	3.17	nd	nd	59.6	3.02	nd	nd	nd	7402	1454
Band 5	1.32	nd	nd	7.11	2.68	nd	nd	6.48	6.91	127
Band 6	nd	3.79	0.57	3.37	0.65	nd	nd	nd	33.4	103
Band 7	1.32	2.88	0.22	12.5	1.36	nd	nd	7.70	57.7	215
Band 8	15.1	nd	nd	8.35	5.90	1.40	nd	9.44	23.9	179

*Values in mg/L.

Compound abbreviations as for Table 4.2.

4.3.1.2 UV-vis/CieLab analysis

The absorbance characteristics of fractions A to D and the original wine were investigated with the Somers' method described in chapter 3, section 3.2.1.2 (Table 4.7). Fraction D collected from preparative NP chromatography has a high hue value, which confirms its

polymeric status. Fraction A also has a relatively high hue, but this is due to its high cinnamic acid and flavonol and low anthocyanin content. The percentage of wine colour (OD_{520}^{CH3CHO}) and total potential colour (OD_{520}^{HCl}) resistant to bleaching by sulphur dioxide generally increased from the early to later eluting peaks as would be expected if their polymeric content increased. The OD_{280}^{HCl} to OD_{520}^{HCl} ratio also increased from the first to the later eluting fractions as would be expected from larger polymer chains. This supported the higher polymeric pigment content of fraction D.

Table 4.7 Spectrophotometric analysis of isolated fractions.

Sample	OD_{520}	OD_{420}	**Hue**[a]	$\frac{OD_{520}^{SO2}}{OD_{520}^{CH3CHO}}$ ***100**	$\frac{OD_{520}^{SO2}}{OD_{520}^{HCl}}$ *** 100**	OD_{280}^{HCl}	OD_{520}^{HCl}	$\frac{OD_{280}^{HCl}}{OD_{520}^{HCl}}$
Fraction A	24.4	16.6	0.7	55.6	19.3	122	67.3	1.8
Fraction B	129	107	0.6	31.5	11.2	886	487	1.8
Fraction C	19.0	11.2	0.6	51.4	18.4	100	51.1	2.0
Fraction D	12.3	13.7	0.8	54.0	27.3	96.8	32.3	3.0
Original wine	3.26	2.08	0.7	49.5	10.0	21.1	15.8	1.3

[a] $OD_{420}^{CH3CHO} / OD_{520}^{CH3CHO}$, hue after abolishing the SO_2 effect.

TLC plates with the separated bands of fraction D were exposed respectively to HCl and SO_2 vapour and compared to the bands that were protected from air (Figure 4.4). All the bands became bright pink when exposed to HCl vapour, demonstrating the likely presence of anthocyanins in the separated phenol complexes. The CieLab and tristimulus values of the isolated TLC bands were determined (Table 4.8) and compared with the values obtained by the Somers' measurements (Somers and Evans 1977). The CieLab colour space chromaticity coordinates are more specific and can distinguish between smaller difference (Pérez-Caballero *et al.* 2003) then the standard colour measurements such as those by Somers and Evans (1977).

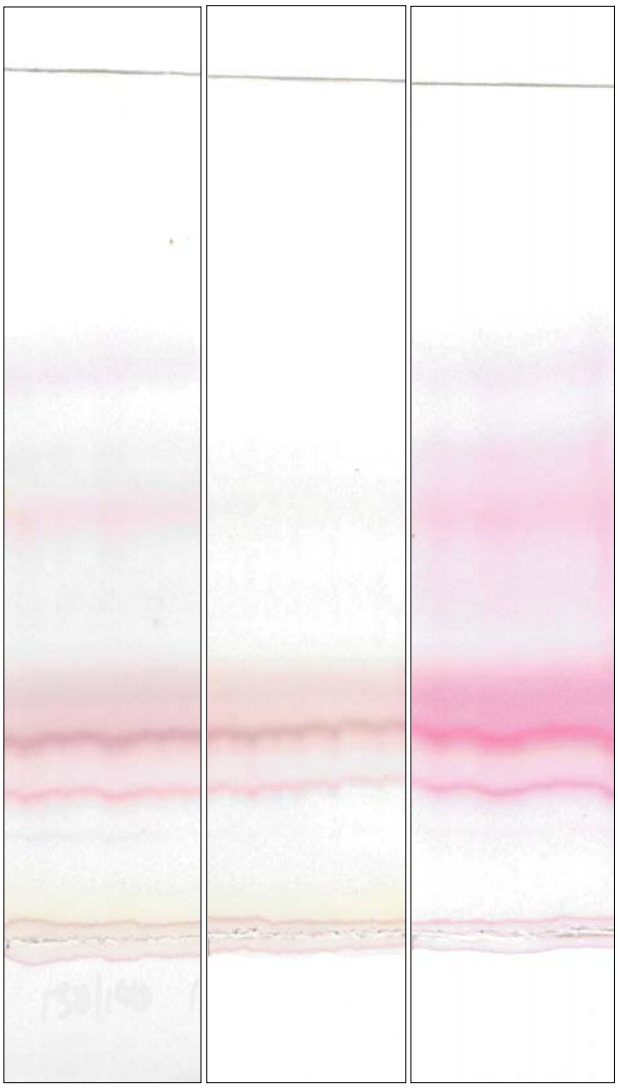


Figure 4.4 Comparison of bands separated from fraction D by TLC by respectively protecting plates from air or exposing it to HCl and SO_2 vapour.

Table 4.8 CieLab and Tristimulus values of the isolated bands of fraction D.

Samples	CieLab			Tristimulus		
	L	*a	*b	X	Y	Z
FrDOrig	84.84	7.38	6.45	65.67	65.69	63.86
FrDB1	97.59	0.63	1.97	89.60	93.90	99.21
FrDB2	90.06	5.58	4.25	75.34	76.42	77.56
FrDB3	94.33	3.54	3.09	83.62	86.04	89.20
FrDB4	72.93	13.38	9.99	47.47	45.05	40.08
FrDB5	93.84	3.03	3.30	82.25	84.89	87.70
FrDB6	93.94	2.54	3.38	82.23	85.14	87.85

4.3.1.3 Normal phase HPLC fractionation

Fraction D was analysed by NP-HPLC (chapter 3, section 3.2.7, method D) and compared with the unfractionated wine (Figure 4.5). It has previously been determined that the anthocyanins eluted between 20 and 50 min followed by the non-monomeric pigments. It was clear from the elution profile that fraction D was enriched in pigmented polymers present in the young wine.

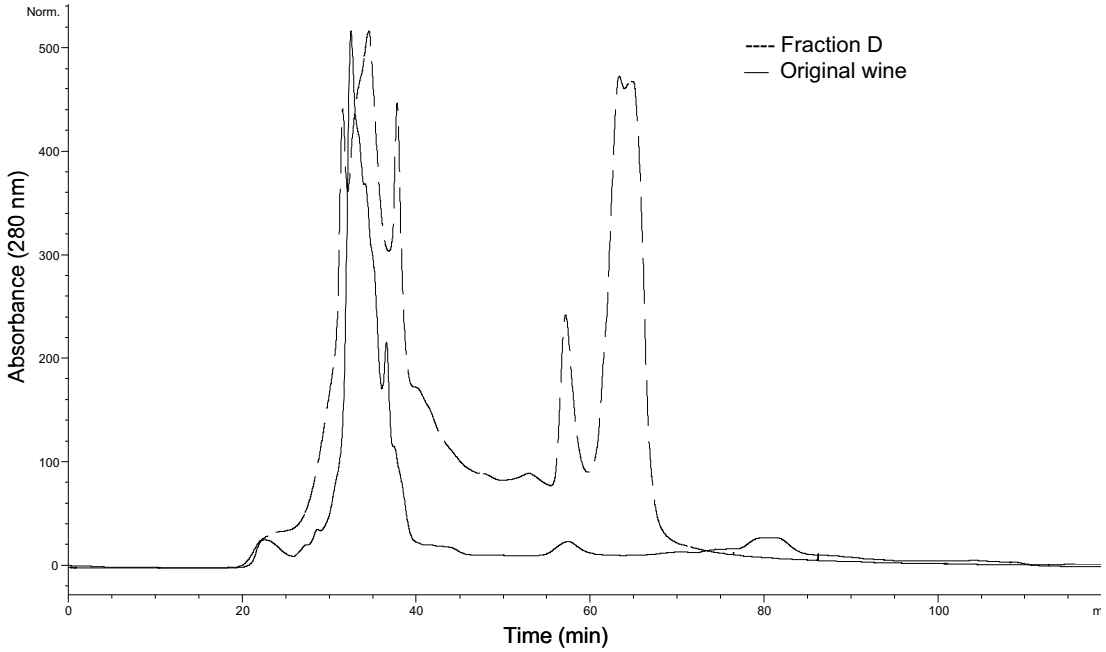


Figure 4.5 NP-HPLC of the 6 month old Shiraz wine and Fraction D.

4.3.1.4 Polymeric nature of the isolated TLC bands

4.3.1.4.1 Electrophoresis

The polymeric pigment (see chapter 3, section 3.3.2.4) formed two coloured spots that were more postive than the malvidin-3-*p*-coumaroylglucoside reference and a large streak of material that moved towards the negative electrode when separated in a boric acid ammonia buffer indicating the formation of a considerable amount of borate complexes (data not shown). Flavonols become yellow when exposed to ammonia vapour and can be seen overlapping one of the pigment spots.

Band 4 isolated from fraction D (FrDB4) by TLC (section 4.2.1.2) was also analysed by paper-electrophoresis in a borate buffer and stained with silvernitrate (Figure 4.6). Similar to the polymeric pigment sample investigated, FrDB4 streaked towards the negative electrode. Additionally the sample was also investigated with a metabisulfite buffer. It formed negative metabisulfite complexes with low mobility as would be expected from a polymer (Figure 4.5). The unknown compounds present in FrDB4 seem to consist mainly of malvidin-3-*O*-glucoside, with the characteristics of a non-monomeric compound.

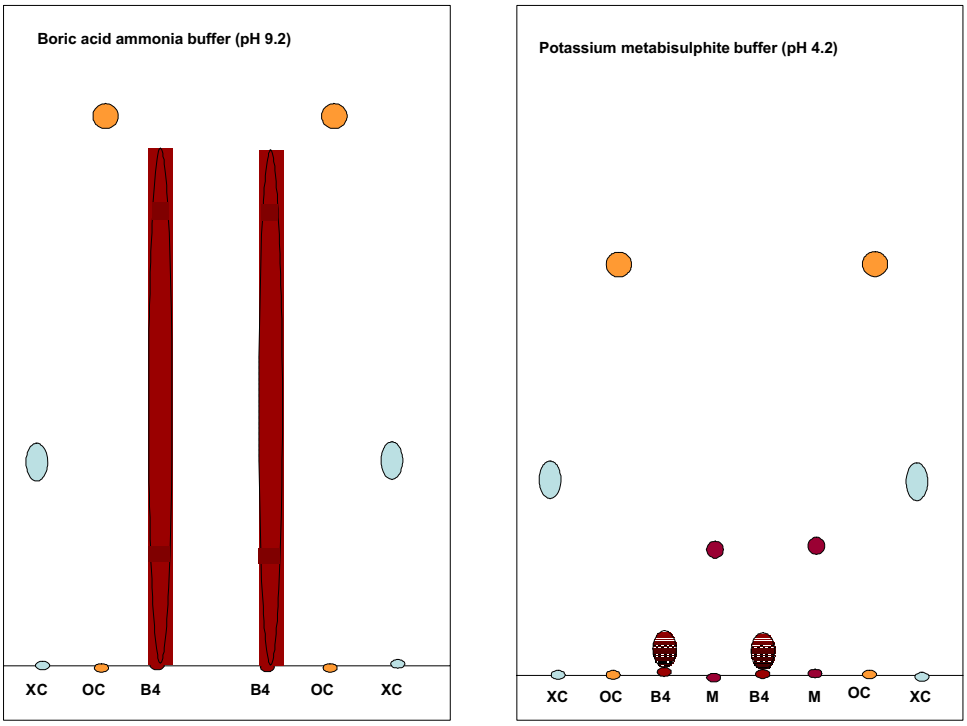


Figure 4.6 High voltage paper electrophoretogram of FrDB4.

4.3.1.4.2 Infra-red spectral data

A broad band due to the OH stretch is observed near 3350 cm^{-1} in all the analysed samples (Figure 4.7). Another band as a result of the aromatic ring quadrant stretch is observed at 1618 cm^{-1}, as well as one due to the aromatic semicircle stretch at 1520 cm^{-1} for catechin (Robb *et al.* 2002). Similarly, the infra-red spectrum of malvidin-3-*O*-glucoside has a strong band at 1641 cm^{-1} and a shoulder at 1720 cm^{-1} due to ring stretching. The 1641 cm^{-1} absorbance band has been attributed to arise from the aryl-conjugated heterocyclic atom of the flavylium ion (Ribéreau-Gayon and Josien 1960). The small to medium band at 1700-1720 cm^{-1} of malvidin-3-*O*-glucoside was also observed by Ribéreau-Gayon and Josien

(1960). The much stronger and broader band at 1649 cm^{-1} and the additional strong band at 1536 cm^{-1} of FrDB4 indicate a larger number of aromatic rings and further conjugation.

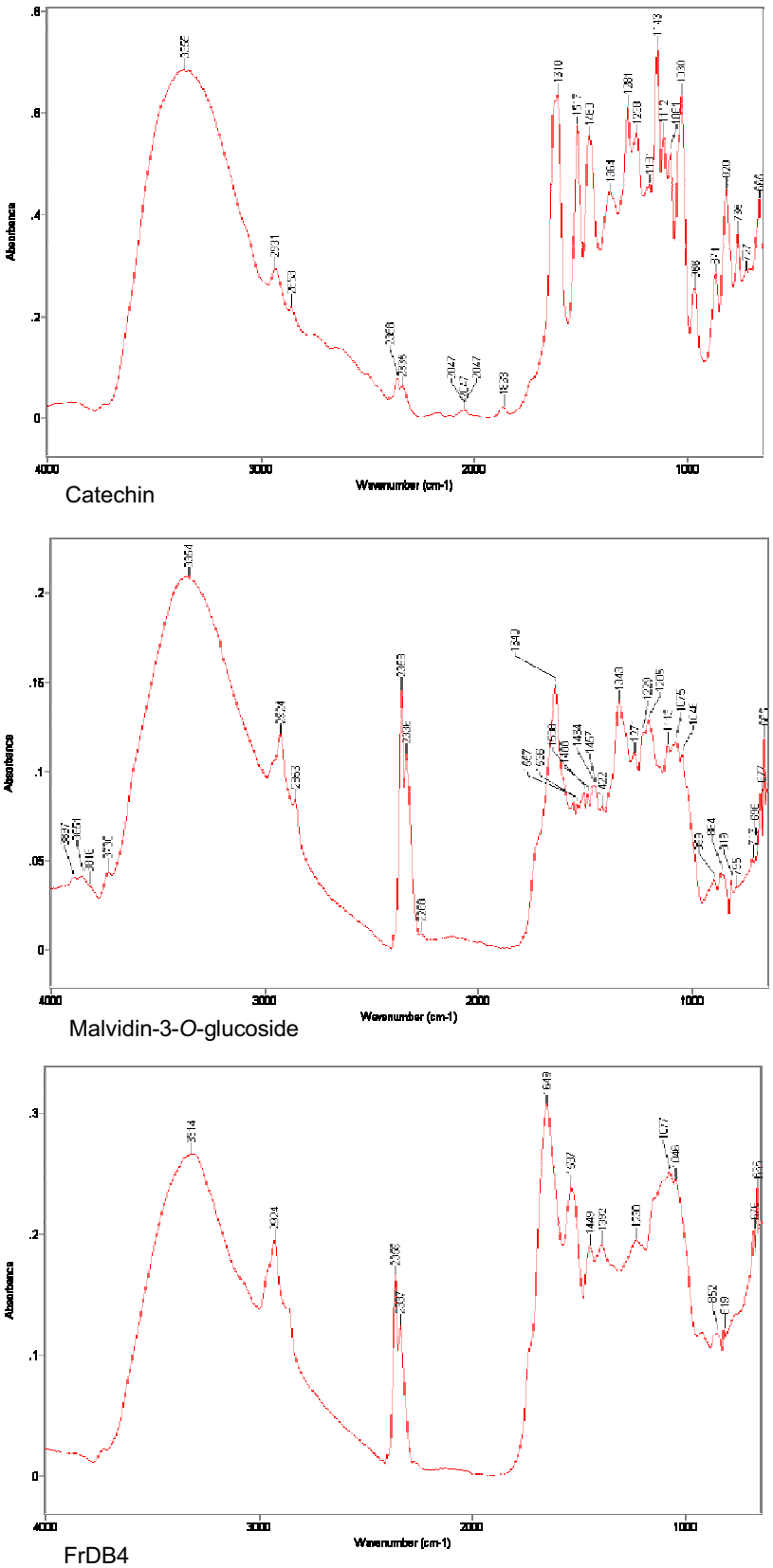


Figure 4.7 Infra-red spectra of malvidin-3-*O*-glucoside, catechin and FrDB4.

4.3.1.5 Acid hydrolysis in butanol and hydrochloric acid

During acid hydrolysis cyanidin or delphinidin is formed from the carbocation resulting from release of the 'upper' unit, in this case (epi)catechin or (epi)gallocatechin, respectively. The yield of cyanidin and/or delphinidin should increase as the chain length increases. Porter *et al.* (1986) has found that the maximum yield of cyanidin from a proanthocyanidin polymer is 58%. The ratio of OD_{550}/OD_{280} estimate the relative degree of polymerisation (Table 4.9) (Bate-Smith 1973; Stafford and Lester 1980).

Table 4.9 Results from acid hydrolysis

Sample	OD_{280}	OD_{550}	Ratio
GST*	1.64	3.89	2.37
FrDB4	6.02	5.26	0.87
FrDB3	5.17	3.62	0.70
FrDB2	0.73	4.42	6.05

*Grape Seed Tannin

4.3.1.6 LC-ESI-MS analysis

Identification of the individual molecular species contained within the various fractions and isolated bands were attempted using LC-ESI/MS (Table 4.10). All samples were washed on C_{18} Sep-Paks according to the method described in section 4.2.8, before analysis. All the fractions gave very weak mass signals with the most abundant ions being of low mass.

Table 4.10 LC-ESI/MS analysis of isolated TLC bands from 6 month old Shiraz wine.

Sample	Structure hypothesis	Expected *m/z*	Observed *m/z*
FrDOrig			
	malv-vinyl-cat (aglycone)	643.4	643.2
	cya-3-gluc-(epi)cat	765.0	765.2
	malv-3-gluc-(epi)cat-(epi)cat [T-A]	1069.0	1068.8
	malv-3-gluc-(epi)cat-(epi)cat-(epi)cat [A-T]	1359.0	1358.8
	malv-3-gluc-pyrano-(epi)cat-(epi)cat-(epi)cat-(epi)gallocat	1686.0	1686.8
FrDB1			
	cya-pyrano-(epi)cat (aglycone)	599.0	598.8
	malv-3-gluc-ethyl-(epi)cat	809.0	809.4

	malv-3-gluc-ethyl-(epi)cat-(epi)cat	1097.0	1096.2
	malv-3-gluc-ethyl-(epi)cat-(epi)cat-(epi)cat-pet-3-gluc	1890.0	1889.0
FrDB2			
	peo (aglycone)	301.0	301.2
	cya-3-gluc-ethyl-(epi)cat	765.0	765.2
	peo-(epi)cat-(epi)cat	879.0	879.2
FrDB3			
	delph (aglycone)	303.0	303.4
	pyrano-peo (aglycone)	325.0	325.4
	malv-3-gluc	493.0	493.2
	carboxyl-pyrano-peo-3-gluc	531.0	531.2
	cya-3-gluc-ethyl-(epi)cat	765.0	765.0
	cya-3-gluc-ethyl-(epi)cat-(epi)catgallate	1206.0	1207.0
FrDB4			
	pyrano-malv (aglycone)	355.0	355.0
	peo-3-gluc	463.0	463.0
	malv-3-gluc	493.0	493.0
	malv-3-(6-p-coum)-gluc	639.0	639.6
	malv-3-gluc-(epi)cat	809.0	809.6
	pet-3-gluc-(epi)cat-(epi)cat [A-T]	1057.0	1056.8
	malv-3-gluc-(epi)cat-(epi)cat-ethyl [A-T]	1099.0	1098.6
	malv-3-gluc-(epi)cat-(epi)cat-ethyl-(epi)cat [A-T]	1388.0	1387.8
FrDB5			
	pyrano-malv (aglycone)	355.0	355.4
	malv-3-gluc (hemiacetal)	511.0	511.2
	malv-pyrano-(epi)cat (aglycone)	644.0	643.2
	malv-3-(6-acetyl)-gluc(hemiacetal)-caftaric acid	863.0	863.2
	malv-3-gluc(hemiacetal)-ethyl-(epi)cat-(epi)cat	1116.0	1116.8
FrDB6			
	pyrano-peonidin (aglycone)	325.0	325.4
	Vit A (aglycone)	399.0	399.0

4.3.1.7 MALDI-TOF analysis

FrDB1, FrDB2, FrDB4 and FrDB5 were isolated according to the method described in section 4.2.1 and further investigated with MALDI-TOF analysis due to the low ionisation found with ESI-MS. In addition, a retentate sample of the original wine was prepared as described in section 3.2.1.3 and analysed with the isolated bands as a control. Good results have been obtained for seed tannin using MALDI-TOF MS analysis (Ohnishi-Kameyama *et al.* 1997; Taylor *et al.* 2003). Thus seed tannin was also analysed as a reference and similar results were obtained with procyanidin oligomer mass peaks up to the decamer being observed (data not shown).

Both FrDB1 and FrDB2 produced only low molecular weight peaks (Table 4.11). FrDB5 produced a range of pigment mass peaks consistent with the molecular ions of known wine pigments and anthocyanins as shown in Table 4.11. The mass peak with the highest intensity, m/z 1634.3, gave rise to a series of mass peaks consistent with the continuing loss of monosaccharides (see Figure 4.8). In contrast, in FrDB4 only low molecular weight mass peaks were observed. Additionally, there was however also a mass peak m/z 818.0 that produced mass ions m/z 637.4 after the loss of hexose minus water and m/z 461.2 after the loss of uronic acid minus water. The retentate of the 6 month old red wine gave rise to a range of mass peaks resulting from the loss of sugars in addition to the mass ions corresponding to wine pigments and anthocyanins shown in Table 4.11. Mass ions 1455.2 and 1293.5 resulted from the consecutive loss of hexose minus H_2O from mass peak m/z 1616.9, followed by mass ions 1149.6 and 1005.6 from the consecutive loss of deoxyhexose minus H_2O, and mass ions 842.6 and 681.4 after the losses of hexose minus H_2O.

Table 4.11 MALDI-TOF-MS analyses of isolated TLC bands and retentate from 6 month old Shiraz wine.

Sample	Structure hypothesis	Expected m/z	Observed m/z
FrDB1			
	pet-3-(6-*p*-coum)gluc-pyruvic acid	693.0	692.9
	malv-3-(6-*p*-coum)gluc	639.0	638.6
	peo-3-(6-acet)gluc	505.0	504.3
	peo-3-gluc	463.0	462.8
FrDB2			
	delph-3-(6-acet)gluc	507.0	505.1
	delph-3-gluc	465.0	464.8
FrDB4			
	peo-3-(6-*p*-coum)gluc-pyruvic acid	677.0	675.9
	acetyl Pigment A	651.0	651.8
	acetyl Vit B	559.0	559.1
	Vit B	517.0	516.2
	peo-3-(6-acetyl)glucoside	505.0	503.2
	acetyl Pigment A (aglycone) [651 – 162]	489.0	490.3
	peo-3-gluc	463.0	463.0
FrDB5			
	malv-3-(6-*p*-coum)gluc-4-vinyl-cat	953.0	951.0
	malv-3-(6-*p*-coum)-4-vinyl-cat [953 – 162]	791.0	790.0
	acetyl Pigment A	651.0	651.8
	acetyl Vitisin B	559.0	559.1
	Vit B	517.0	516.2
	peo-3-(6-acet)gluc	505.0	503.2
	acetyl Pigment A (aglycone) [651 – 162]	489.0	490.3

Retentate	peo-3-gluc	463.0	463.0
	malv-3-(6-*p*-coum)-gluc-pyrano-(epi)cat	951.0	948.8
	delph-3-(6-acet)gluc-pyrano-(epi)cat	819.0	817.6
	pet-3-gluc-(epi)cat (T-A)	767.0	767.3
	pet-3-(6-*p*-coum)gluc-pyruvic acid	693.0	692.9
	delph-3-(6-*p*-coum)gluc-acetaldehyde	635.0	635.2
	cya-3-(6-acet)gluc-acetaldehyde	515.0	514.9

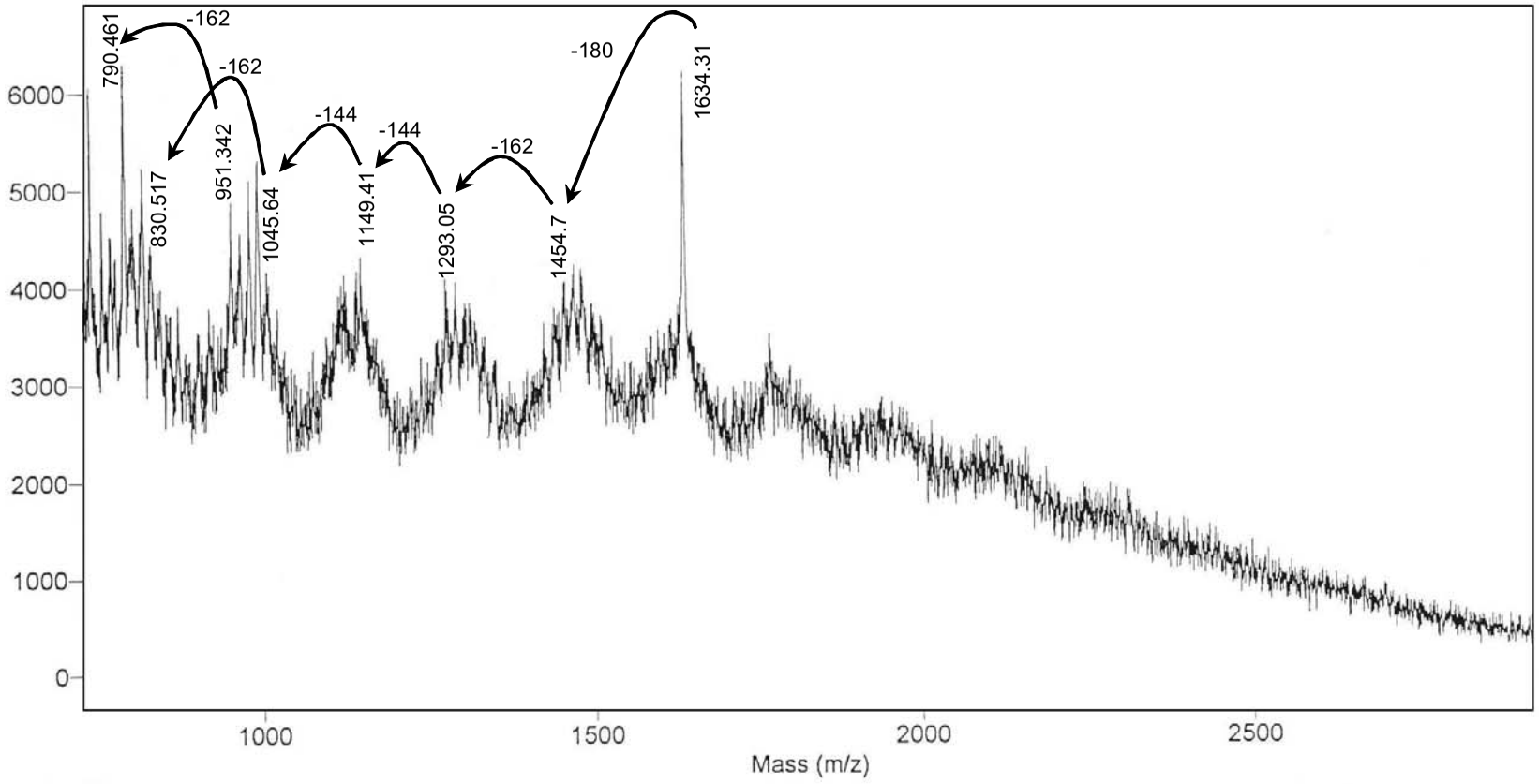


Figure 4.8 MALDI-TOF mass spectrum of FrDB5 isolated from the 6 month old Shiraz wine.

4.3.1.8 Carbohydrate analysis

To determine the monosaccharide composition of an oligosaccharide or glycoconjugate it is necessary to cleave the oligosaccharide into its components before analysis. All samples were washed on Sep-Paks to remove any monosaccharides before analysis. The neutral sugar composition of the fractions was then determined by the alditol acetate method (section 4.2.11). Samples were analysed in duplicate, except for FrDB1 where the duplicate were contaminated. The results show that all the fractions contained varying proportions of glucose as well as arabinose, galactose and mannose (Table 4.12).

Table 4.12 Amount of sugar measured for every 100 μg of polymer isolated according to HPLC quantification in μg catechin equivalents.

Samples	[1]Anth (nmol)	[2]Glu (nmol)	[3]Rha (nmol)	[4]Ara (nmol)	[5]Xyl (nmol)	[6]Man (nmol)	[7]Gal (nmol)
FrDOrig	56	102 ± 1	19 ± 6	185 ± 20	7 ± 148	29 ± 5	32 ± 4
FrDB1	27	60	3	88	4	6	2
FrDB2	86	189 ± 7	1 ± 1	36 ± 6	2 ± 3	4 ± 2	7 ± 2
FrDB3	72	113 ± 12	2 ± 1	17 ± 2	4 ± 3	6 ± 0	5 ± 2
FrDB4	600	833 ± 26	9 ± 1	24 ± 5	9 ± 1	20 ± 3	18 ± 0
FrDB5	339	356 ± 7	3 ± 1	7 ± 1	7 ± 0	8 ± 4	8 ± 4
FrDB6	8	22 ± 10	0.4 ± 0.6	3 ± 0	1 ± 0	2 ± 1	3 ± 1
Retentate	132	155 ± 6	24 ± 0	330 ± 25	12 ± 3	48 ± 0	106 ± 6

[1]Anthocyanin, [2]Glucose, [3]Rhamnose, [4]Arabinose, [5]Xylose, [6]Mannose, [7]Galactose.
Average of duplicate analyses ± standard deviation.

Methanolysis as a means of identifying neutral and acidic sugar residues were performed on the isolated TLC bands of fraction D as well as on a prepared wine retentate and commercial malvidin-3-*O*-glucoside standard to determine whether any uronic acids were present. Only the malvidin-3-*O*-glucoside standard contained significant amounts of glucuronic acid, while FrDOrig and the retentate had trace amounts of galacturonic acid (Table 4.13). A major unidentified component, which looked like a carbohydrate derivative, was detected during the methanolysis of FrDB2, but nothing unusual was present in the methylations of this sample (Table 4.14).

Table 4.13 Glycosyl-residue composition of isolated fractions and wine retentate.

	Ratio of anhydromoles								
Sample	**[1]Ara**	**[2]Rha**	**[3]Xyl**	**[4]Man**	**[5]Gal**	**[6]Glc**	**[7]GlcA**	**[8]GalA**	**Total carbo-hydrate (µg)**
malv3gluc	5.2	1.4	0.6	-	8.0	78	6.6	-	400
FrDOrig	92	2.2	-	0.4	2.0	1.9	-	1.4	1034
FrDB1	71	-	1.2	2.0	-	26	-	-	17
FrDB2	24	-	-	-	-	76	-	-	57
FrDB3	-	-	-	-	-	100	-	-	668
FrDB5	-	0.5	0.7	0.6	0.5	98	-	-	84
FrDB6	-	0.4	1.8	1.3	-	96	-	-	31
retentate	54	2.4	0.4	5.9	4.8	32	-	0.9	133

[1]Arabinose, [2]Rhamnose, [3]Xylose, [4]Mannose, [5]Galactose, [6]Glucose, [7]Glucuronic acid, [8]Galacturonic acid.
Average of duplicate analyses.

The polysaccharide types present were deduced from linkage composition (Table 4.14) based on the totals of individual glycosyl residues that are characteristic of well-defined polysaccharides as proposed previously by Nunan *et al*. (1997). In a pigmented sample, terminal and 1,2-Glc*p* from acylated anthocyanidin-glucosides would be expected. The retentate contained mostly arabinose and glucose with low levels of mannose and galactose and trace levels (< 1%) of rhamnose, galacturonic acid and xylose. Linkage analysis by methylation showed that there was a high proportion of 5-Ara*f* and 3,5-Ara*f*, estimating that *ca* 41% of the oligosaccharides present in this fraction was an arabinan (Vidal *et al.* 2000). The possible presence of xylans were indicated by 4-linked Xyl*p*, while terminal Ara*f*, 3-linked Gal*p* and 3,6-linked Gal*p* pointed towards type II arabinogalactans (Nunan *et al.* 1997, 1998; Sims and Bacic 1995; Vidal *et al.* 2000). A low concentration of galacturonans, which included both rhamnogalacturonan and homogalacturonan, and mannans could be present due to respectively the presence of 4-linked and 2,4-linked Rha*p* and terminal and 2-linked Man*p*.

Table 4.14 Glycosyl-linkage composition in mol % of isolated fractions and controls.

Monosaccharide[a]	Deduced Linkage	Linkage composition[b]							
		M3G	FrDOrig	FrDB1	FrDB2	FrDB3	FrDB5	FrDB6	Retentate
Ara(*f*)	terminal	nd[c]	22.5	7.8	1.4	nd	nd	nd	13.5
	2-	5.7	tr[d]	nd	nd	nd	nd	nd	0.5
	3-	nd	1.2	nd	nd	nd	nd	nd	0.4
	5-	nd	45.8	41.5	5.1	nd	nd	nd	31.5
	2,5-	nd	0.9	nd	nd	nd	nd	nd	0.4
	3,5-	nd	27.7	nd	nd	nd	nd	nd	9.2
Xyl(*p*)	4-	nd	tr	8.9	nd	nd	nd	nd	0.9
Rha(*p*)[e]	4-	0.3	tr	nd	nd	nd	nd	nd	0.5
	2,4-	nd	tr	nd	nd	nd	nd	nd	0.2
Gal(*p*)	terminal	nd	tr	nd	nd	nd	nd	nd	0.3
	2-	9.0	nd	nd	nd	nd	nd	nd	nd
	3-	nd	tr	nd	nd	nd	nd	nd	0.6
	3,6-	nd	tr	nd	nd	nd	nd	nd	0.5
Glc(*p*)	terminal	72.1	1.8	15.4	72.4	79.5	76.1	76.2	30.0
	2-	12.9	0.2	nd	13.5	17.1	17.5	14.3	3.4
	4-	nd	nd	26.4	5.9	0.9	2.5	5.1	1.7
	6-	nd	nd	nd	1.8	2.5	3.9	4.4	1.2
Man(*p*)	terminal	nd	tr	tr	nd	nd	nd	nd	2.1
	2-	nd	tr	tr	nd	nd	tr	tr	1.9
	3-	nd	tr	nd	nd	nd	nd	nd	0.8
	2,6-	nd	tr	nd	nd	nd	tr	nd	0.6
	3,4	nd	tr	nd	nd	nd	nd	nd	0.1

[a]*f* = furanose, *p* = pyranose.
[b]The molar ratios are the means of duplicate analyses.
[c]Not detected.
[d]Trace amount.
[e]1,4-di-*O*-acetyl-2,3-di-*O*-methyl-rhamnitol.

The prominence of terminal, 5-linked and 3,5-linked arabinose in FrDOrig indicated the presence of arabinans with side chains (Nunan *et al.* 1997, 1998; Vidal *et al.* 2000, 2003b). The presence of trace amounts of terminal, 2-, 3-, 2,6- and 3,4-linked mannose were probably due to mannan from yeast mannoproteins. The high level of 5-linked arabinose in FrDB1 indicates the presence of arabinan (Nunan *et al.* 1997, 1998), while terminal arabinose and xylose with 4-linked glucose are consistent with an arabinoxyloglucan (Sims and Bacic 1995). Trace amounts of terminal and 2-linked mannose were also present. FrDB2 contained some arabinan (terminal and 5-linked arabinose) and cellulose (4-linked glucose) (Nunan *et al.* 1997), while the terminal and 2-linked glucose most probably resulted from glycosylated phenolics, particularly the anthocyanins and flavonols. Similarly FrDB3, FrDB5 and FrDB6 contained high levels of terminal and 2-linked glucose with low levels of 4 and 6-linked glucose. In addition, FrDB5 and FrDB6 contained trace amounts of 2- and 2,6-linked mannose.

The polysaccharides present in the wine was expected to be excluded by the preparative silica chromatography (MPLC, section 4.2.1.1) during the first step of fractionation even though they would be slightly soluble in a mobile phase of propanol, acetic acid and water (6:1:2). Further separation of the polysaccharides by TLC would also be unexpected. Subsequently the mobility on TLC of grape polysaccharides was tested. Polysaccharides from Shiraz grapes were isolated by precipitation with five volumes of cold ethanol. Colour associated with the isolated polysaccharides were removed with size exclusion chromatography (SEC) using Sephadex LH-20 according to the protocol of Brillouet *et al*. (1990). The isolated polysaccharides were placed on a silica TLC plate, and eluted with the mobile phase propanol, acetic acid and water (60:1:2). The TLC plate was then stained with silver nitrate reagent (4g $AgNO_3$ and 40mL H_2O in 2L of acetone), to indicate the position of any polysaccharides. The only polysaccharides detected were those on the origin.

4.3.1.9 Determination of tannin mDP by phloroglucinol

The average mDP of the retentate of the 6 month old wine was only 1.9 (Table 4.15), which is in agreement with previous studies of young wines (Alcalde-Eon *et al.* 2004; Atanasova *et al.* 2002a; Mateus *et al.* 2001; Salas *et al.* 2005; Vivar-Quintana *et al.* 2002). The estimated average molecular weight was determined to be 575 g/mole for the proanthocyanidin part of the phenol compounds present (Table 4.14). Grape skin and seed tannin were also analysed to be able too compare results with known published data (Kennedy and Jones 2001).

Table 4.15 Terminal and extention unit composition of proanthocyanidins from the prepared wine retentate, grape skin and seed tannins.

Samples[a]	*Terminal units*[b]				*Extension units*[b]						
	EGC	**C**	**EC**	**ECG**	**EGC**	**C**	**EC**	**ECG**	**Yield**[c]	**mDP**[d]	**Est. M_r**[e]
Wine retentate	6.22	5.94	34.4	7.18	8.88	1.42	31.8	4.06	85.7	1.9	575
SkinTannin	0.27	7.10	1.85	-[f]	23.2	0.99	60.8	5.76	97.5	11.4	3418
SeedTannin	-	9.33	12.2	5.33	-	8.56	59.9	4.69	82.0	3.7	1138

[a]Average of duplicate measurements.

[b]Percentage composition of proanthocyanidins (in moles), with the following subunit abbreviations: EGC, epigallocatechin; C, catechin; EC, epicatechin; ECG, epicatechin gallate.

[c]Conversion yield (m/m) percentage.

[d]Mean degree of polymerisation.

[e]Estimated average molecular mass based upon proportional composition and mDP.

[f]Not detected.

The mean degree of polymerisation of the different bands isolated ranged from 1.4 to 4.8 (Table 4.16). The significant contribution of both epigallocatechin and epicatechingallate as terminal and extention units (Table 4.16), indicate that both skin and seed proanthocyanidins were incorporated into the wine polymeric phenols. The yields of some of the bands were very low. This could be a function of their resistance to acid hydrolysis or due to oxidative crosslinking which is known to reduce conversion yield (Kennedy *et al.* 2001).

Table 4.16 Results from acid catalysis in the presence of excess phloroglucinol for isolated TLC bands from fraction D.

Samples[a]	*Terminal units*[b]				*Extension units*[b]						
	EGC	**C**	**EC**	**ECG**	**EGC**	**C**	**EC**	**ECG**	**Yield**[c]	**mDP**[d]	**Est. M_r**[e]
FrDOrig	12.9	3.94	6.71	3.60	16.9	1.42	50.8	3.75	56.0	3.7	1127
FrDB1	-[f]	25.4	32.2	12.5	-	6.77	18.3	4.76	91.5	1.4	451
FrDB2	16.6	2.27	4.28	3.64	53.4	1.31	16.3	2.32	46.2	3.7	1159
FrDB3	12.3	2.85	3.14	2.32	49.7	2.37	23.8	3.50	38.2	4.8	1496
FrDB4	11.6	5.69	4.76	2.30	25.3	2.02	43.0	5.27	57.7	4.1	1261
FrDB5	27.7	3.87	8.01	5.51	40.1	1.00	11.9	1.93	17.6	2.2	692
FrDB6	42.8	4.63	3.98	8.39	19.4	1.71	16.0	3.08	26.6	1.7	531

[a]Average of duplicate measurements.

[b]Percentage composition of proanthocyanidins (in moles), and with the following subunit abbreviations: EGC, epigallocatechin; C, catechin; EC, epicatechin; ECG, epicatechin gallate.

[c]Conversion yield (m/m) percentage.

[d]Mean degree of polymerisation.

[e]Estimated average molecular mass based upon proportional composition and mDP.

[f]Not detected.

4.3.1.10 Gel permeation chromatography

All the bands isolated by TLC from fraction D exhibited a large mass distribution according to GPC. FrDOrig also revealed a small percentage (< 1%) of very high molecular weight (347639 to 24896) peaks. Similarly, high molecular weight peaks (< 1%) were also present in FrDB1 (22727 to 11957), FrDB2 (22471), FrDB3 (22350), FrDB5 (22251) and the retentate (22295) in addition to the average masses determined (Table 4.17) according to the method described by Kennedy and Taylor (2003). The presence of such high molecular weight peaks supports the fact that there are oligosaccharides present in the isolated bands.

Table 4.17 Molecular weight and resulting degree of polymerisation (DP) as determined by GPC compared to the DP according to acid-catalysis.

TLC bands	FrDOrig	FrDB1	FrDB2	FrDB3	FrDB4	FrDB5	FrDB6	Retentate
DP (acid-hydrolysis)	3.7	1.4	3.7	4.8	4.1	2.2	1.7	1.9
Mr (by GPC)*	4098	494	1480	1342	768	1092	1303	1857
DP (by GPC)	7.7	0.9	2.7	2.5	1.4	2	2.4	3.6

*Of the acetylated fractions.

4.3.2 Isolation and characterisation of the polymeric fractions of a 5 year old wine

4.3.2.1 Isolation of different polymeric pigment fractions by preparative NP chromatography and TLC

A 5 year old Shiraz wine prepared as described in chapter 3, section 3.2.1.1, was fractionated by preparative normal phase chromatography according to the protocol set out in section 4.2.1 (Table 4.18). The different fractions collected are indicated in Figure 4.9. Fraction E was more effectively separated by TLC using the butanol-acetic acid-water mobile phase, while fraction F, G and later eluting fractions showed improved separation with the propanol-acetic acid-water mobile phase (Figure 4.10). This is in agreement with findings for the 6 month old wine.

Table 4.18 Phenolic composition (in mg) of fractions collected by preparative normal phase fractionation of a 5 year old Shiraz wine.

Fraction*	Anth	Vitisin	Pigm	Pol pigm	Cinn mc	Flavo	Gallic	Flava	Proc	Pol ph
E	0.108	0.070	0.096	1.92	1.92	1.55	0.149	1.84	1.31	21.4
F	0.005	0.094	0.005	0.483	0.049	0.007	0.001	0.031	0.004	6.32
G	0.001	0.006	0.001	0.467	0.077	nd	0.001	0.008	0.083	6.20
H	nd	0.001	nd	0.041	0.001	nd	nd	nd	nd	0.487
I	nd	nd	nd	0.009	nd	nd	nd	nd	nd	0.101
J	0.000	0.000	nd	0.010	0.003	nd	nd	nd	nd	0.111
K	nd	0.001	nd	0.011	0.004	0.000	nd	nd	nd	0.150
L	nd	nd	nd	0.007	0.001	nd	nd	nd	nd	0.67
M	nd	0.000	nd	0.022	0.002	nd	nd	nd	nd	0.298
N	0.000	0.000	nd	0.016	0.019	nd	nd	0.003	2.20	0.161
O	nd	nd	nd	0.010	nd	nd	nd	nd	0.0	0.115
P	nd	nd	nd	0.005	nd	nd	nd	nd	0.0	0.050
Q	nd	nd	nd	0.009	nd	nd	nd	nd	0.0	0.086
R	nd	nd	nd	0.006	nd	nd	nd	nd	0.0	0.026
S	nd	nd	nd	0.006	nd	nd	nd	nd	22.9	0.030

*Values in mg.

Compound abbreviations: Anth, monomeric anthocyanins; Vitisin, vitisin A and acetylated vitisin A; Pigm, low molecular weight pigments, Pol pigm, non-monomeric pigments; Cinnmc, cinnamic acids; Flavo, flavonols; Gallic, gallic acid; Flava, flavan-3-ol monomers; Proc, proanthocyanidins; Pol ph, polymeric phenols.

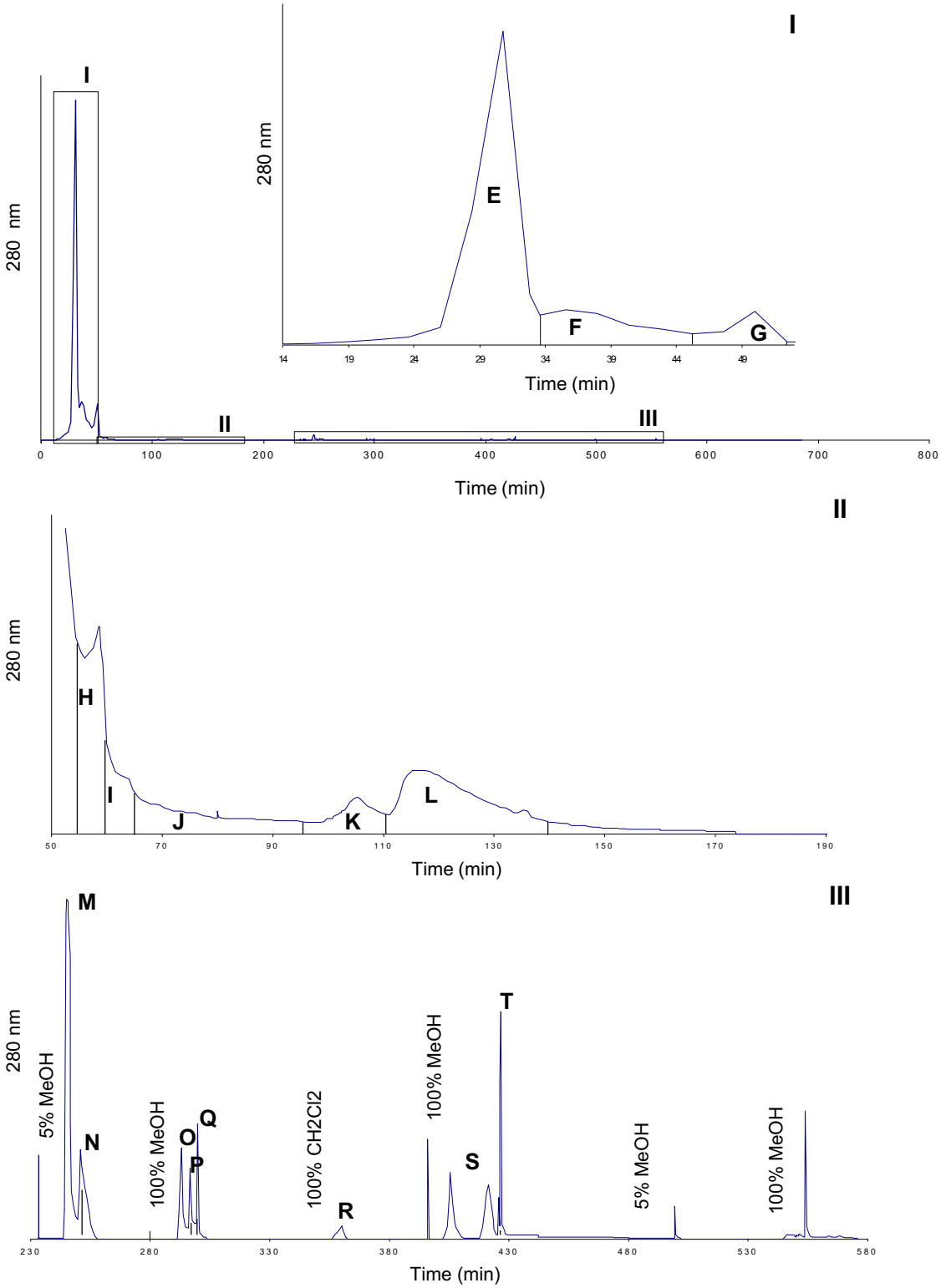


Figure 4.9 MPLC chromatogram of a five year old Shiraz wine with mobile phase propanol-acetic acid-water (6:1:2).

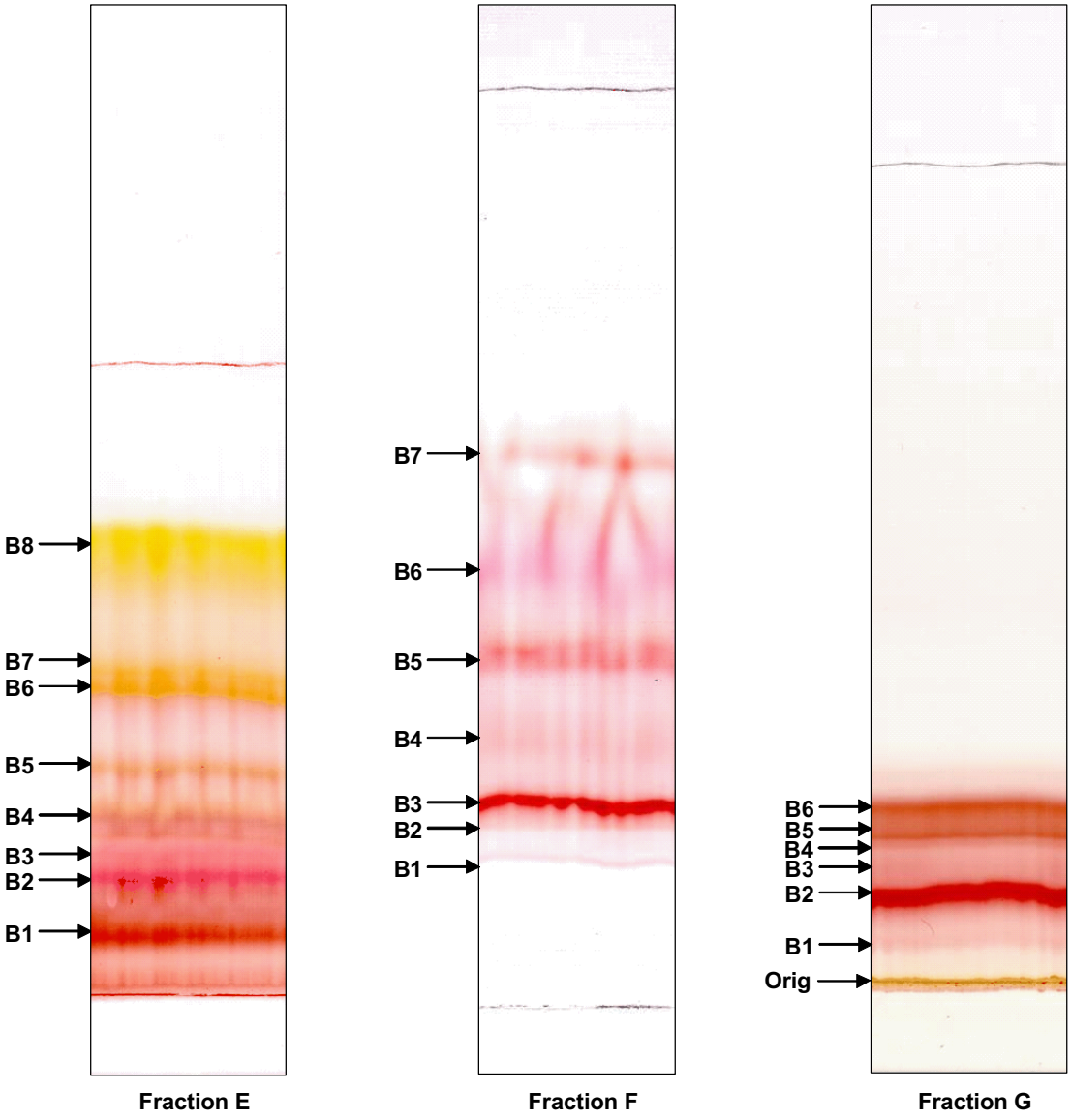


Figure 4.10 TLC plates showing the separation of fraction E (using mobile phase *n*-butanol-acetic acid-water (6:1:2)), and fraction F and G (using mobile phase *n*-propanol-acetic acid-water (6:1:2)) into individual bands.

Fractions E, F and G were further fractionated by TLC into separate bands shown in Figure 4.10. Individual bands were isolated and analysed by RP-HPLC (Table 4.19 to 4.21).

Table 4.19 Phenolic composition (in mg/L) of the isolated bands from fraction E on TLC with the mobile phase butanol-acetic acid-water (6:1:2).

Fraction E*	Anth	Vitisin	Pigm	Pol pigm	Cinnmc	Flavon	Gallic	Flava	Proc	Pol ph
Band1	18.9	nd	0.66	202	2.77	nd	nd	28.4	21.2	2843
Band2	24.0	1.44	3.02	411	2.74	nd	nd	25.4	29.9	4918
Band3	3.82	28.9	2.43	98.3	7.61	3.84	nd	6.14	24.2	1229
Band4	17.0	59.0	16.1	413	193	nd	nd	nd	179	5461
Band5	1.62	1.64	0.71	57.0	46.7	0.86	nd	nd	8.49	838
Band6	5.41	1.46	12.0	51.4	22.6	76.8	0.30	31.4	47.2	812
Band7	13.8	nd	nd	30.4	29.3	46.2	8.34	27.1	38.0	628
Band8	3.45	nd	nd	18.7	41.9	31.7	9.92	38.2	57.2	465

*Values in mg/L.

Compound abbreviations as for Table 4.18.

Table 4.20 Phenolic composition (in mg/L) of the isolated bands from fraction F on TLC with the mobile phase propanol-acetic acid-water (6:1:2).

Fraction F*	Anth	Vitisin	Pigm	Pol pigm	Cinnmc	Flavon	Gallic	Flava	Proc	Pol ph
Band1	nd	nd	nd	5.12	nd	nd	nd	nd	nd	66.2
Band2	nd	nd	nd	32.9	13.5	nd	nd	nd	10.3	361
Band3	nd	nd	nd	8.65	4.72	nd	nd	nd	2.56	97.2
Band4	nd	nd	nd	40.9	1.83	nd	nd	nd	4.30	535
Band5	nd	nd	nd	35.4	1.09	nd	nd	nd	nd	468
Band6	nd	nd	nd	37.3	8.15	nd	nd	nd	nd	406
Band7	3.19	nd	nd	29.8	18.0	nd	nd	nd	nd	345

*Values in mg/L.

Compound abbreviations as for Table 4.18.

Table 4.21 Phenolic composition (in mg/L) of the isolated bands from fraction G on TLC with the mobile phase propanol-acetic acid-water (6:1:2).

Fraction G*	Anth	Vitisin	Pigm	Pol pigm	Cinnmc	Flavon	Gallic	Flava	Proc	Pol ph
Origin	3.49	nd	nd	10.6	41.6	nd	nd	nd	nd	118
Band1	nd	nd	nd	20.4	13.8	nd	nd	nd	2.24	225
Band2	0.40	nd	nd	234	11.2	nd	nd	nd	6.31	2663
Band3	nd	nd	nd	43.5	3.87	nd	nd	nd	nd	526
Band4	nd	nd	nd	77.2	nd	nd	nd	nd	nd	939
Band5	nd	nd	nd	146	nd	nd	nd	nd	nd	1775
Band6	nd	nd	nd	21.3	nd	nd	nd	nd	49.3	259

*Values in mg/L.

Compound abbreviations as for Table 4.18.

All fractions collected as well as the isolated bands from fraction F and G and a prepared retentate were further characterised by acid-hydrolysis in the presence of excess phloroglucinol, GPC and LC-ESI-MS. Fraction E were not characterised further as it

contained large quantities of monomers and the focus of this study was to characterise the polymeric pigments. Except for fraction F and G the rest of the collected fractions were analysed without further fractionation due to the small quantities collected.

4.3.2.2 Analytical NP-HPLC analysis

Fractions E, F and G, isolated from preparative NP-HPLC, were analysed by analytical NP-HPLC (Figure 4.11). Fractions E and F were similar although fraction F contained less monomeric pigments and other phenols. The elution profile of fraction G indicated that it was enriched in early eluting and late eluting polymers. The assumed polymers were collected from fraction G and analysed by RP-HPLC where it eluted in the polymeric peak which confirmed its non-monomeric nature. Broad peaks were seen with NP-HPLC compared to the separate bands obtained with TLC. Thus, TLC gave superior resolution with the additional advantage that the amount of sample that could be separated by TLC was also significantly greater.

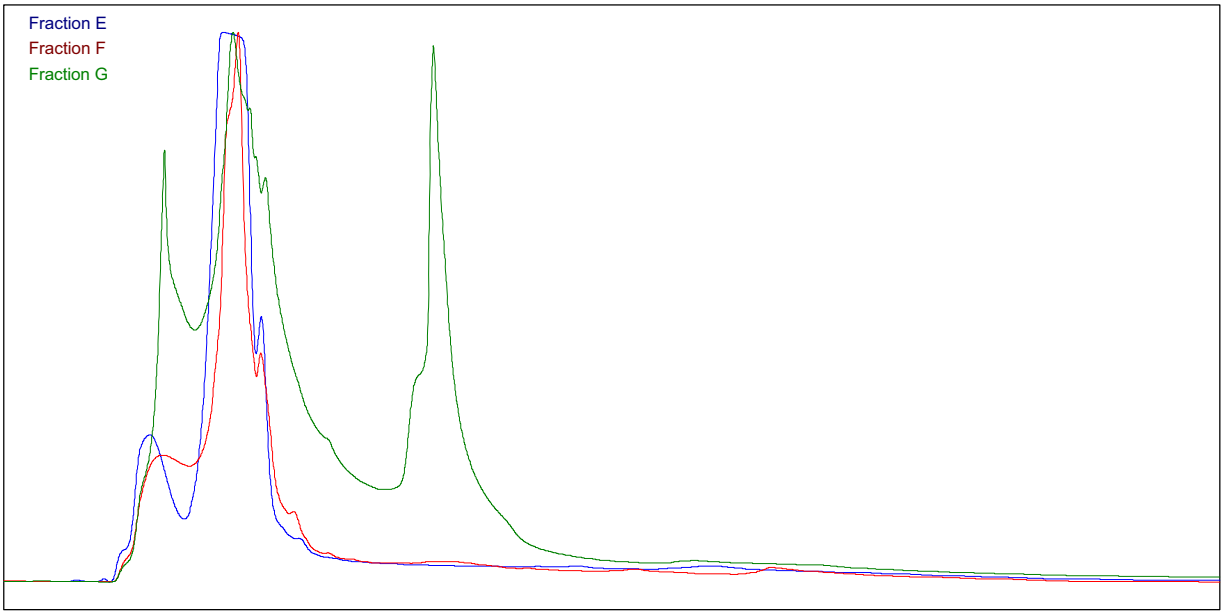


Figure 4.11 NP-HPLC chromatograms of fractions E, F and G obtained from the 5 year old Shiraz wine.

4.3.2.3 LC/ESI-MS analysis

From the LC/ESI-MS data the retentate contained pigmented proanthocyanidins of up to nine units (see Table 4.22). The direct condensation products of the A-T and T-A type as described by Hayasaka and Kennedy (2003) as well as pyruvic acid derivatives of malvidin-3-glucoside (Bakker and Timberlake 1997) were present.

Table 4.22 LC/ESI-MS analysis of the 5 year old wine retentate.

Sample	**Mass assignment**	**Expected *m/z***	**Observed *m/z***
Wine retentate			
	malv-3-(6-acet)-gluc-ethyl-(epi)cat-(epi)cat-ethyl-digalloylpentamer	2911.6	2912.6
	malv-3-gluc-trimer (A-T)	1521.0	1521.6
	malv-3-(6-p-coum)-gluc-ethyl-epi(cat)-malv-3-(6-acet)-gluc	1488.0	1487.8
	(epi)cat-(epi)gallocat-(epi)cat-(epi)catgallate	1323.0	1323.2
	malv-3-(6-acet)-gluc-ethyl-(epi)cat-(epi)cat	1139.0	1138.6
	unknown		861.4
	pigment A-*p*-coum	855.4	855.4
	malv-3-gluc-(epi)gallocat (A-T)	799.0	799.2
	malv-3-gluc-(epi)gallocat (T-A)	797.0	797.2
	malv-3-gluc-(epi)cat (T-A)	781.0	781.6
	malv-3,5-*O*-gluc or malv-3-(6-caffeoyl)-gluc	655.0	655.6
	malv-3-(6-p-coum)-gluc	639.0	639.2
	pigment A	609.0	609.2
	malv-3-(6-acet)-gluc	535.0	535.0
	malv-3-gluc	493.0	493.0
	vitisin B (-162, gluc)	355.0	355.2

The LC/ESI-MS results of the isolated bands from fraction F is shown in Table 4.23. Most of the bands displayed evidence of the presence of pigmented polymers formed through a combination of direct and indirect condensation. The anthocyanins were often linked to a proanthocyanidin with a vinyl linkage which in some cases have undergone ringclosure to form a pyrano link. The largest pigment observed was an octamer in FrFB5 and FrFB6, while FrFB1, FrFB2, FrFB3 and FrFB7 contain pigments with respectively two, three, five and six units. Several of the isolated bands exhibited mass ions which could be the result of polymers containing multiple pigments. Mass ion detected at *m/z* 1028.8 in FrFB1 and FrFB7 could be attributed to malv-3-gluc-ethyl-malv-3-gluc (hydrated/cationic) as found by Atanasova *et al.* (2002b).

Table 4.23 LC/ESI-MS results of the isolated TLC bands from fraction F.

Sample	Mass assignment	Expected *m/z*	Observed *m/z*
FrFB1			
	(epi)gallocat-(epi)cat-(epi)cat-(epi)catgallate	1323.0	1322.6
	malv-3-gluc-ethyl-malv-3-gluc (hydrated/cationic) (Atanasova, 2002)	1029.0	1028.8
	unknown		862.8
	malv-3-gluc-ethyl-cat	809.0	809.0
	monogalloyldimer-ethyl	759.0	759.0
	unknown		735.0
	cat-CHCOOH-cat	637.0	637.0
FrFB2			
	malv-3-gluc-vinyl-dimer [1093-162]	931.0	931.0
	cya-3-gluc-ethyl-(epi)cat	765.0	765.0
	unknown		598.6
	unknown		569.0
FrFB3			
	malv-3-gluc-pyrano-monogalloyltetramer	1821.0	1821.6
	(epi)cat-hexamer	1731.0	1731.6
	cya-3-gluc-ethyl-(epi)cat-ethyl-(epi)cat or peo-3-(6-acet)-gluc-epi(cat)-epi(cat)	1081.0	1081.0
FrFB4			
	monogalloyltrimer – H_2O – 2	999.0	999.0
	pet-3-gluc-2	623.0	623.0
	vitisin B aglycone [517–162]	355.0	355.0
	gallo(epi)cat	306.0	306.0
FrFB5			
	malv-3-(6-acet)-gluc-vinyl-(epi)cat-hexamer-vinyl-malv-3-(6-acet)-gluc	$[1425]^{2+}$	$[1425]^{2+}$
	malv-3-gluc-(epi)gallocat-(epi)cat-malv-3-gluc-hydrate	1593.0	1593.8
	(epi)cat-tetramer	1155.0	1154.2
	malv-3-gluc-(epi)gallocat-(epi)cat (A-T)	1087.0	1086.4
	pet-3-(6-p-coum)-gluc-pyruvic acid-2	691.0	690.8
	malv-3-gluc-vinyl-(epi)cat aglycone [807-162]	645.0	645.0
	unknown		555.0
	(epi)catgallate	443.0	441.8
FrFB6			
	digalloyl(epi)cat-decamer	$[1594.0]^{2+}$	$[1593.8]^{2+}$
	malv-3-(6-acet)-gluc-vinyl-(epi)cat-hexamer-vinyl-malv-3-(6-acet)-gluc	$[1425.0]^{2+}$	$[1425.0]^{2+}$
	(epi)cat-tetramer	1155.2	1154.2
	malv-3-gluc-(epi)gallocat-(epi)cat (A-T)	1087.0	1086.4
	malv-3-gluc-pyrano-(epi)catgallate	957.0	956.8
	unknown		833.6
	(epi)catgallate	443.0	442.0
FrFB7			
	malv-3-(6-*p*-coum)-gluc-pyrano-(epi)cat-(epi)cat-vinyl-gallo(epi)cat-(epi)cat-pyrano-malv-3-(6-*p*-coum)-gluc	$[1259.0]^{2+}$	$[1258.6]^{2+}$
	monogalloylpentamer	1595.0	1594.6
	malv-3(6-acet)-gluc-(epi)gallocat-(epi)cat-(epi)cat	1415.0	1415.8
	unknown		1316.6

	malv-3-(6-*p*-coum)-gluc-malv-3-(6-caffeoyl)-gluc	1293.0	1292.7
	malv-3-(6-*p*-coum)-gluc-pyrano-(epi)cat-gallo(epi)cat	1255.0	1254.6
	malv-3-(6-*p*-coum)-gluc-pyrano-(epi)cat-(epi)cat	1239.0	1237.8
	unknown		1228.4
	malv-3-gluc-ethyl-malv-3-gluc (hydrated/cationic)	1029.0	1028.8
	(epi)catgallate-ethyl	470.0	470.6

The isolated TLC bands of fraction G (Table 4.23) generally contained larger polymerised phenols then those isolated from fraction F. FrGB3 exhibited a mass ion (m/z = 1366.6^{2+}) corresponding to possibly the largest pigment observed, a malvidin-3-(6-*p*-coumaroyl)-glucoside condensed with a heptameric proanthocyanidin through an ethyl bond. FrGOrig, FrGB1 and FrGB2 and FrGB4 as well as FrGB5 and FrGB6 contained mass ions that corresponded to potential nonameric, pentameric, tetrameric and trimeric pigments.

Table 4.23 LC/ESI-MS analysis of TLC bands isolated from fraction G.

Sample	**Mass assignment**	**Expected *m/z***	**Observed *m/z***
FrGOrig	malv-3-(6-acet)-gluc-(epi)gallocatgallate-(epi)gallocat-(epi)cat-malv-3-gluc	$[1037.0]^{2+}$	$[1037.0]^{2+}$
	$[1037]^{2+}$ - glucose	$[956.0]^{2+}$	$[956.4]^{2+}$
	malv-3-gluc-vinyl-(epi)gallocat-(epi)cat-vinyl-(epi)gallocat-(epi)catgallate	1881.0	1880.8
	malv-3-gluc-(epi)gallocat-(epi)cat (T-A)	1085.0	1084.8
	(epi)cat-(epi)cat-(epi)cat – 2	865.0	864.8
	unknown		842.8
	vinyl-(epi)gallocat-(epi)catgallate	771.0	770.4
	vinyl-(epi)gallocat-(epi)cat	619.0	618.4
FrGB1			
	(epi)gallocat-trimer-(epi)cat-trimer	1779.0	1780.6
	malv-3-(6-acet)-gluc-pyrano-(epi)cat-trimer	1423.0	1422.4
	(epi)cat-(epi)gallocat-(epi)cat-(epi)catgallate	1323.0	1322.8
	unknown		1117.0
	malv-3-gluc-(epi)cat-(epi)cat (A-T)	1071.0	1071.2
	unknown		1052.8
	unknown		1022.0
	malv-3-gluc-pyrano-malv-3-gluc (cationic/quinodal)	1007.0	1006.4
	pet-3-(6-acet)-gluc-pyrano-(epi)cat	833.0	832.4
	Pigment A acetate	651.0	651.0
FrGB2			
	delph-3-(6-acet)-gluc-pyrano-(epi)gallocat-trimer-epi(cat)	1731.0	1731.2
	unknown		1586.0
	unknown		1252.2
	vinyl-(epi)gallocat-trimer-epi(cat)-2	1225.0	1225.0

	unknown		856.8
	unknown		788.4
	cya-3-gluc-ethyl-(epi)cat	765.0	764.8
	unknown		748.6
	unknown		691.2
	unknown		568.8
	pet-3-(6-acet)-gluc-acetaldehyde (pyrano)	545.0	545.0
	(epi)cat	291.0	291.6
FrGB3			
	malv-3-(6-*p*-coum)gluc-ethyl-(epi)cat-tetramer-(epi)gallocat-trimer	$[1366.0]^{2+}$	$[1366.6]^{2+}$
	malv-3-(6-*p*-coum)gluc-(epi)cat-pentamer-(epi)gallocat (T-A)	$[1192.0]^{2+}$	$[1191.4]^{2+}$
	(epi)cat-trimer-(epi)gallocat-trimer	$[889.5]^{2+}$	$[889.8]^{2+}$
	delph-3-gluc-pyrano-(epi)cat-tetramer or peo-3-gluc-vinyl-(epi)cat-tetramer	1641.0	1640.4
	unknown		1632
	malv-3-gluc-monogalloyl(epi)cat-trimer	1509.0	1509.4
	delph-3-(6-acet)-gluc-vinyl-(epi)cat-dimer or malv-3-gluc-pyrano-(epi)gallocat-(epi)cat	1109.0	1108.0
	unknown		796.8
	cya-3-gluc-pyrano-(epi)cat aglycone [761-162]	599.0	599.0
FrGB4			
	malv-3-(6-acet)-gluc-vinyl-(epi)cat-(epi)cat-vinyl-(epi)gallocat	1467.0	1466.4
	peo-3-gluc-(epi)cat-(epi)catgallate	1191.0	1190.2
	peo-3-(6-*p*-coum)-gluc-pyrano-(epi)catgallate	1073.0	1073.8
FrGB5			
	malv-3-(6-acet)-gluc-(epi)gallocat-(epi)cat-(epi)catgallate (A-T)	1569.0	1568.6
	digalloyl(epi)cat-dimer-ethyl	911.0	910.8
	unknown		886.8
	unknown		690.6
	(epi)cat-CHCOOH-(epi)cat	637.0	637.0
	unknown		569.2
	monogalloyl(epi)cat-ethyl	471.0	470.8
FrGB6			
	pet-3-gluc-ethyl-monogalloyl-(epi)cat-dimer	1235.0	1234.6
	malv-3-gluc-ethyl-(epi)gallocat-(epi)cat	1113.0	1112.8
	cya-3-(6-*p*-coum)-gluc-pyrano-(epi)catgallate	1059.0	1060.0
	peo-3-gluc-(epi)cat-(epi)cat (T-A)	1039.0	1038.8
	(epi)cat-CHCOOH-(epi)cat	637.0	636.8

FrG, analysed by LC-ESI/MS (Table 4.24) showed mass ions corresponding to malvidin-3-glucoside with eight to twelve units of proanthocyanidin monomers. This supports the MS analyses of the isolated TLC bands from FrG.

The fractions eluting after fraction G were not further separated due to the small amounts of material collected. They were however analysed by ESI-MS and the ions observed and

possible identifications are presented in Table 4.24. Possible pigmented polymers of up to seven units in length were observed, including tetramers containing two anthocyanins. The fractions ionised weakly however with the lower mass ions being more prominent.

Table 4.24 LC/ESI-MS analysis of fractions collected by preparative NP chromatography.

Sample	Mass assignment	Expected *m/z*	Observed *m/z*
FrG	malv-3-gluc-ethyl-monogalloyltrimer-ethyl-monogalloyloctamer	$[2011.0]^{2+}$	$[2011.2]^{2+}$
	malv-3-gluc-ethyl-trigalloyloctamer	$[1641.0]^{2+}$	$[1641.8]^{2+}$
	ethyl-monogalloyloctamer	$[1243.5]^{2+}$	$[1243.6]^{2+}$
	malv-3-gluc-pyrano-malv-3-gluc (cationic.quinodal)	1007.0	1006.8
FrH			
	unknown		2182.4
	unknown		2076.4
	peo-3-gluc-vinyl-monogalloyltrimer-ethyl-(epi)gallocat	1837.0	1836.8
	malv-3-gluc-vinyl-(epi)cat-tetramer	1669.0	1669.4
	monogalloyltrimer-ethyl-(epi)gallocat	1351.0	1350.4
	pet-3-gluc-pyruvic acid	547.0	546.4
FrI			
	peo-3-gluc-vinyl-(epi)gallocat-(epi)cat-tetramer	$[972.5]^{2+}$	$[972.0]^{2+}$
	malv-3-(6-*p*-coum)-gluc-ethyl-(epi)cat-tetramer	1819.0	1818.0
	malv-3-gluc-vinyl-(epi)cat-ethyl-(epi)cat-ethyl-malv-3-gluc	$[821.0]^{2+}$	$[820.8]^{2+}$
	malv-3-(6-*p*-coum)-gluc-ethyl-(epi)cat-trimer	1531.0	1531.6
	digalloyltetramer	1459.0	1457.8
	malv-3-(6-*p*-coum)-gluc-ethyl-(epi)cat-dimer	1243.0	1243.6
	pet-3-gluc-vinyl-(epi)cat-(epi)cat	1081.0	1079.8
	malv-3-(6-*p*-coum)-gluc-vinyl-(epi)cat	953.0	952.8
	unknown		885.0
	malv-3-gluc(hydrate)-ethyl-(epi)cat	827.0	826.8
	unknown		522.4
	(epi)catgallate	443.0	442.0
FrJ			
	malv-3-(6-acetyl)-gluc-pyrano-(epi)catgallate-vinyl-(epi)cat	1313.0	1311.8
	malv-3-(6-acetyl)-gluc-pyrano-(epi)catgallate-vinyl	1025.0	1024.4
FrK			
	malv-3-gluc-monogalloyltrimer-(epi)gallocat-malv-3-gluc (T-A/A-T)	2306.0	2306.0
	malv-3-gluc-vinyl-(epi)cat-trimer-malv-3-gluc	$[937.5]^{2+}$	$[937.8]^{2+}$
	malv-3-gluc-ethyl-(epi)cat-digalloyldimer	1689.0	1691.4
	monogalloyltrimer-(epi)gallocat	1323.0	1323.0
	malv-3-gluc-pyrano-malv-3-gluc (cationic/quinodal)	1007.0	1006.2
	(epi)cat-trimer	867.0	866.8
	(epi)cat-(epi)catgallate-ethyl	758.0	758.2
FrL			
	malv-3-gluc-tetragallodigalloylnonamer	$[1726.5]^{2+}$	$[1726.6]^{2+}$
	hexagallodigalloyldecamer or malv-3-(6-p-coum)-gluc-(epi)gallocat-trimer-digalloyltrimer-	$[1640.5]^{2+}$/ $[1641.0]^{2+}$	$[1640.8]^{2+}$

	ethyl-malv-3-(6-acet)-gluc (A-T)		
	malv-3-(6-p-coum)-gluc-(epi)cat-trimer (A-T)	1505.0	1505.6
	malv-3-(6-p-coum)-gluc-(epi)cat-dimer (T-A)	1215.0	1214.0
	unknown		869.8
	monogalloyldimer-vinyl	757.0	756.6
FrM			
	malv-3-gluc-vinyl-(epi)cat-(epi)cat-ethyl-malv-3-gluc	$[807.0]^{2+}$	$[807.6]^{2+}$
	(epi)cat-trimer-(epi)gallocat-dimer	1475.0	1475.0
	unknown		1303.4
	unknown		1260.4
	(epi)cat-dimer-(epi)gallocat-dimer-2	1185.0	1185.2
	malv-3-gluc-ethyl-(epi)cat-ethyl-(epi)gallocat	1141.0	1141.4
	malv-3-(6-acet)-gluc-pyrano-(epi)cat-(epi)cat	1135.0	1134.2
	unknown		916.6
	carboxy dicatechin xanthylium	617.0	617.4
	peo-3-gluc-pyruvic acid	531.0	531.0
FrN			
	malv-3-gluc-(epi)cat-trimer-(epi)gallocat-dimer-(epi)catgallate-ethyl-(epi)gallocat-vinyl-malv-3-gluc (T-A)	$[1627.0]^{2+}$	$[1626.8]^{2+}$
	malv-3-gluc-(epi)cat-trimer-(epi)gallocat-dimer-(epi)catgallate (T-A)	2405.0	2406.8
	malv-3-gluc-(epi)cat-trimer-(epi)gallocat (T-A)	1661.0	1661.0
	cya-3-gluc-ethyl-(epi)cat-dimer-ethyl-(epi)cat-dimer	1657.0	1656.8
	malv-3-gluc-(epi)cat-dimer-(epi)gallocat-ethyl (A-T)	1402.0	1402.8
	peo-3-(6-acet)-gluc-vinyl-(epi)cat	819.0	818.8
	peo-3-(6-acet)-gluc-acetaldehyde	529.0	529.2
FrO			
	malv-3-(6-p-coum)-gluc-(epi)gallocat-(epi)cat-hexamer-cya-3-gluc (T-A; T-A)	$[1559.0]^{2+}$	$[1558.8]^{2+}$
	cya-3-gluc-(epi)cat-hexamer-peo-3-gluc (T-A; A-T)	$[1320.0]^{2+}$	$[1320.8]^{2+}$
	pet-3-gluc-ethyl-(epi)gallocat-trimer-(epi)catgallate	1859.0	1859.2
	cya-3-gluc-(epi)cat-hexamer	$[1088.5]^{2+}$	$[1088.6]^{2+}$
	pet-3-gluc-pyrano-(epi)cat-tetramer	$[827.5]^{2+}$	$[827.2]^{2+}$
	unknown		$[447.2]^{2+}$
	unknown		733.0
	delph-3-(6-p-coum)-gluc-pyruvic acid	679.0	679.2
	delph-3-(6-p-coum)-gluc	611.0	612.4
	unknown		584.4
	unknown		529.2
	delph-3-(6-acet)-gluc	507.0	505.8
	unknown		409.2
	unknown		364.0
FrP			
	unknown		$[1331.2]^{2+}$
	delph-3-(6-p-coum)-gluc-(epi)cat-hexamer-ethyl-pet-3-(6-p-coum)-gluc	$[1443.0]^{2+}$	$[1443.6]^{2+}$
	malv-3-(6-p-coum)-gluc-pyrano-(epi)cat-tetramer-vinyl-(epi)cat-trimer-2	$[1351.5]^{2+}$	$[1351.6]^{2+}$
	delph-3-(6-p-coum)-gluc-vinyl-monogalloyl(epi)cat-heksamer	$[1258.0]^{2+}$	$[1258.0]^{2+}$
	malv-3-(6-acet)-gluc-ethyl-(epi)cat-tetramer-	$[1136.0]^{2+}$	$[1135.8]^{2+}$

	pyrano-malv-3-(6-acet)-gluc		
	unknown		$[1089.2]^{2+}$
	malv-3-(6-acetyl)-gluc-pyrano-monogalloyl-(epi)cat-pentamer	$[1076.0]^{2+}$	$[1075.8]^{2+}$
	malv-3-(6-p-coum)-gluc-pyrano-(epi)cat-(epi)cat-vinyl-malv-3-(6-p-coum)-gluc	$[951.0]^{2+}$	$[951.2]^{2+}$
	pet-3-(6-p-coum)-gluc-pyrano-(epi)cat-trimer	1514.0	1514.2
	(epi)cat-trimer	867.0	866.4
	pet-3-(6-p-coum)-gluc-pyruvic acid	693.0	693.4
	pet-3,5-digluc	641.0	640.8
	delph-3-(6-p-coum)-gluc	611.0	611.2
	(epi)cat-(epi)cat-2	577.0	577.6
FrQ			
	monogalloyl-(epi)cat-heptamer-H_2O	$[1076.5]^{2+}$	$[1076.4]^{2+}$
	unknown		2061.0
	malv-3-gluc-(epi)cat-trimer (A-T)	$[680.0]^{2+}$	$[679.8]^{2+}$
	monogalloyl-(epi)gallocat-(epi)cat-trimer-2	1321.0	1321.0
	monogalloyl(epi)cat-tetramer-2	1305.0	1305.0
	delph3-(6-p-coum)-gluc-pyrano-(epi)cat-dimer	$[606.0]^{2+}$	$[606.2]^{2+}$
	delph-3-gluc-(epi)cat-(epi)catgallate (A-T)	1195.0	1195.2
	unknown		1146.8
	poe-3-(6-acet)-gluc-ethyl-(epi)cat-dimer	1109.0	1108.4
	monogalloyltrimer-		1030.0
	unknown		$[363.8]^{2+}$
	delph-3-gluc-(epi)cat	753.0	753.2
	monogalloyl-(epi)cat-(epi)gallocat	747.0	747.2
	pet-3(6-acetyl),5-digluc	683.0	683.8
	malv-3-(6-caffeoyl)-gluc	655.0	655.2
	pet-3,5-digluc	641.0	641.6
	delph-3-(6-p-coum)-gluc	611.0	611.4
	unknown		563.6
	peo-3(6-acetyl)-gluc	505.0	505.4
FrR			
	digalloyl-digallo-(epi)cat-pentamer-2	$[889.0]^{2+}$	$[888.2]^{2+}$
	malv-3-(6-acetyl)-gluc-pyrano-(epi)cat-dimer-pyrano-malv-3-(6-acet)-gluc	$[846.0]^{2+}$	$[846.0]^{2+}$
	monogalloyl-digallo-(epi)cat-pentamer-2	$[813.0]^{2+}$	$[813.0]^{2+}$
	unknown		$[725.0]^{2+}$
	unknown		$[640.6]^{2+}$
	pet-3-(6-acetyl)-gluc-vinyl-pet-3-(6-acet)-gluc	$[533.0]^{2+}$	$[533.0]^{2+}$
	delph-3-gluc-(epi)cat-dimer (A-T)	$[521.5]^{2+}$	$[521.4]^{2+}$
	unknown		$[454.2]^{2+}$
	malv-3-gluc-(epi)cat	783.0	784.4
	malv-3-(6-acetyl),5-gluc	697.0	697.2
	pet-3-(6-acetyl),5-gluc	683.0	682.4
	pigment A or peo-3-(6-*p*-coum)-gluc	609.0	608.2
	unknown		563.4
	peo-3-(6-acetyl)-gluc-acetaldehyde	545.0	545.0
	peo-3-(6-acetyl)-gluc	505.0	505.4
FrS			
	malv-3-gluc-ethyl-(epi)cat-dimer-peo-3-(6-acet)-gluc (A-T)	$[801]^{2+}$	$[801]^{2+}$
	peo-3-(6-*p*-coum),5-gluc	771.0	771.2
	malv-3-(6-*p*-coum)-gluc-pyruvic acid (vitisin AX)	707.0	706.0
	delph-3-(6-*p*-coum)-gluc-pyruvic acid	679.0	679.8
	delph-3-(6-*p*-coum)-gluc	611.0	611.0
	malv-3-(6-acet)-gluc-acetaldehyde (vitisin B)	517.0	517.4

peo-3-(6-acetyl)-gluc	505.0	505.2
malv-3-gluc	493.0	493.0

4.3.2.4 Determination of tannin mDP by phloroglucinolysis

A mean degree of polymerisation of 10.4 was determined for the wine retentate (Table 4.25) in comparison to the 6 month old Shiraz wine which had a mDP of 1.9 for the proanthocyanidin part. Hayasaka and Kennedy (2003) found a mDP of 8.3 for wine tannin obtained from a 3 year old Pinot Noir red wine.

Table 4.25 Terminal and extention unit composition of proanthocyanidins from the 5 year old wine retentate.

Samples[a]	*Terminal units*[b]				*Extension units*[b]						
	EGC	**C**	**EC**	**ECG**	**EGC**	**C**	**EC**	**ECG**	**Yield %**[c]	**mDP**[d]	**Est. Mr**[e]
Wine retentate	-	7.02	2.59	-	26.1	-	60.1	4.20	35.5	10.4	3127

[a]Average of duplicate measurements.

[b]Percentage composition of proanthocyanidins (in moles), and with the following subunit abbreviations: EGC, epigallocatechin; C, catechin; EC, epicatechin; ECG, epicatechin gallate.

[c]Conversion yield (m/m) percentage.

[d]Mean degree of polymerisation.

[e]Estimated average molecular mass based upon proportional composition and mDP.

- Not detected.

Acid hydrolysis results of fraction F is tabulated in Table 4.26. FrFB7 only released epigallocatechin and a small amount of anthocyanins as well as other unknown pigments after acid hydrolysis. A substantial amount of new unknown procyanidins were also formed. In FrFB7 only a small percentage of the polymer was pigmented and thus most of the released products would be flavan-3-ol derivatives. Similarly, for both FrFB5 and FrFB6 only small amounts of terminal and extension units were identifiable, while both released a low concentration of anthocyanins, unknown pigments and unknown proanthocyanidins. No terminal units were detected for the other bands isolated.

Table 4.26 Terminal and extention unit composition of proanthocyanidins from part of the TLC bands isolated from fraction F.

Samples[a]	*Terminal units*[b]				*Extension units*[b]						
	EGC	**C**	**EC**	**ECG**	**EGC**	**C**	**EC**	**ECG**	**Yield %**[c]	**mDP**[d]	**Est. Mr**[e]
FrFB7	100	-	-	-	-	-	-	-	101	1.0	306
FrFB6	49.8	-	-	-	-	50.2	-	-	37.5	2.0	598
FrFB5	58.2	-	-	-	-	13.7	-	28.1	73.7	1.7	588
FrFB4	-	-	-	-	-	-	-	-	0.0		
FrFB3	-	-	-	-	-	100	-	-	10.9		
FrFB2	-	-	-	-	42.7	9.5	47.8	-	24.1		
FrFB1	-	-	-	-	99.8	-	0.2	-	38.6		

[a]Average of duplicate measurements.

[b]Percentage composition of proanthocyanidins (in moles), and with the following subunit abbreviations: EGC, epigallocatechin; C, catechin; EC, epicatechin; ECG, epicatechin gallate.

[c]Conversion yield (m/m) percentage.

[d]Mean degree of polymerisation.

[e]Estimated average molecular mass based upon proportional composition and mDP.

- Not detected.

If all the flavan-3-ols (monomers and other proanthocyanidins) seen after hydrolysis were to be taken into account, the yield would be significantly greater than 100% for some of the bands. This is due to absorbance differences as well as the fact that larger oligomers are not effectively quantified by RP-HPLC. The higher molecular weight compounds do not separate into individual peaks but causes an increase in the baseline with increasing organic solvent concentration in the mobile phase before it is pushed from the column as a broad peak by a steep increase in organic solvent at the end of the RP-HPLC run.

All the isolated bands from fraction G released low concentrations of anthocyanins, unknown pigments and proanthocyanidins. Fraction G clearly contained higher molecular weight proanthocyanidins compared to fraction F (Table 4.26 and 4.27). This would be expected due to its later elution from the preparative normal phase column. It is well known that phenolics do not just separate according to polarity but also according to molecular size and shape which influence association with the packing material. The isolated bands from fraction G contained a high percentage of (epi)gallocatechin, which indicate a large contribution from the skin proanthocyanidins, with approximately 2% galloylation (Table 4.27).

Table 4.27 Terminal and extention unit composition of proanthocyanidins from the TLC bands isolated from fraction G.

Samples[a]	*Terminal units*[b]				*Extension units*[b]						
	EGC	**C**	**EC**	**ECG**	**EGC**	**C**	**EC**	**ECG**	**Yield %**[c]	**mDP**[d]	**Est. Mr**[e]
FrGB6	-	23.8	-	-	-	-	76.2	-	19.7	4.2	1216
FrGB5	3.13	6.36	1.59	-	58.9	4.12	24.7	1.23	33.4	9	2723
FrGB4	3.92	2.46	1.18	-	79.2	1.60	10.9	0.78	97.9	13.2	4023
FrGB3	-	4.02	-	-	67.2	-	27.6	1.14	15.1	24.9	7531
FrGB2	-	5.54	2.04	-	45.6	-	44.7	2.12	11.9	13.2	3969
FrGB1	-	8.05	6.07	-	75.2	-	8.6	2.06	56.9	7.1	2160
FrGOrig	28.6	3.36	6.89	-	48.9	-	9.8	2.49	344	2.6	789

[a]Average of duplicate measurements.

[b]Percentage composition of proanthocyanidins (in moles), and with the following subunit abbreviations: EGC, epigallocatechin; C, catechin; EC, epicatechin; ECG, epicatechin gallate.

[c]Conversion yield (m/m) percentage.

[d]Mean degree of polymerisation.

[e]Estimated average molecular mass based upon proportional composition and mDP.

- Not detected.

Fractions H and I yielded no recognisable terminal or extension units. They did, however as was the case for all the fractions up to fraction O, release anthocyanins and unknown proanthocyanidins. Fraction L exhibited the highest mDP and contained a high percentage of (epi)gallocatechin extension units, indicating to the possibility that it may contain a large grape skin tannin. The amount of material available in all fractions were very low (< 5 μg in catechin units), which complicated the analyses. Hydrolysis of fraction Q was performed on less then one μg of sample. It is thus likely that the terminal and extension units could not be detected due to the low concentration of the hydrolysis products and question the invidividual reliabilities of the data.

Table 4.28 Terminal and extention unit composition of proanthocyanidins from fraction H to S isolated from MPLC.

Samples[a]	*Terminal units*[b]				*Extension units*[b]						
	EGC	**C**	**EC**	**ECG**	**EGC**	**C**	**EC**	**ECG**	**Yield %**[c]	**mDP**[d]	**Est. Mr**[e]
FrS	-	97.3	-	-	-	-	-	2.74	12.6	1.0	302
FrR	-	22.2	-	-	-	77.8	-	-	5.0	4.5	1309
FrQ	-	-	-	-	-	-	-	100	2.0	-	-
FrP	-	8.00	-	-	-	-	86.6	5.38	16.5	12.5	3727
FrO	-	4.83	-	-	-	-	89.0	6.13	4.1	20.7	6202
FrN	-	2.65	4.75	-	69.0	-	21.9	1.64	43.5	13.5	4102
FrM	-	-	17.5	-	39.3	-	38.6	4.59	5.4	5.7	1735
FrL	-	3.16	-	-	78.5	-	18.3	-	61.7	31.6	9567
FrK	-	5.20	-	-	68.1	-	25.5	1.21	120	19.2	5814
FrJ	-	-	-	-	-	-	100	-	1.0	-	-
FrI	-	-	-	-	-	-	-	-	0	-	-
FrH	-	-	-	-	-	-	-	-	0	-	-

[a]Average of duplicate measurements.

[b]Percentage composition of proanthocyanidins (in moles), and with the following subunit abbreviations: EGC, epigallocatechin; C, catechin; EC, epicatechin; ECG, epicatechingallate.

[c]Conversion yield (m/m) percentage.

[d]Mean degree of polymerisation.

[e]Estimated average molecular mass based upon proportional composition and mDP.

- Not detected.

4.3.2.5 Gel permeation chromatography

GPC results of the retentate, analysed according to the method described by Kennedy and Taylor (2003), showed a mDP of 10, while ESI-MS exhibited mass ions of polymers containing ten, four and three units. The GPC results of the isolated TLC bands of fraction F is tabulated in Table 4.29. Low molecular masses were generally observed by GPC corresponding to mDPs of below three. FrFB3 did however exhibit masses up to a DP of 6.3, while FrFB7 showed a mass distribution of polymers to a DP of 4.4.

Table 4.29 Terminal and extention unit composition of proanthocyanidins and GPC results for the TLC bands isolated from fraction F.

FrF	B1	B2	B3	B4	B5	B6	B7
DP (acid-hydrolysis)	-	-	-	-	1.7	2.0	1.0
Mr (by GPC)*	-	-	1405	919.2	873.9	1504	1346
DP (by GPC)	-	-	2.8	1.6	1.5	2.9	2.5

*Of the acetylated fractions.

The mDP's determined by GPC for the lower eluting bands of fraction G were larger than those of fraction F (Table 4.30). FrGB2 was the only band that showed good comparison between the mDP's determined by both methods. This was the main band isolated and consequently a larger amount of sample was available for the analyses. FrGB1 and FrGB3 gave no discernable results. This could in part be due to the small amount of sample that was available compounded by losses during the acetylation process.

Table 4.30 Terminal and extention unit composition of proanthocyanidins and GPC results for the TLC bands isolated from fraction G.

FrG	Orig	B1	B2	B3	B4	B5	B6
DP (acid-hydrolysis)	2.6	7.1	13.2	24.9	13.2	9.0	4.2
Mr (by GPC)*	2181	-	6025	-	779.9	852.0	2342
DP (by GPC)	4.0	-	11.4	-	1.4	1.6	4.7

*Of the acetylated fractions.

Only very small amounts of each of the fractions (H – P) were available for acetylation resulting in some samples giving no results, while in others, only very small peaks could be observed during GPC analysis (Table 4.31). The high acetylated mass for FrM by GPC indicate that oligosaccharides were associated with the phenolics of this fraction, while fraction Q, R and S gave no visible GPC results.

Table 4.31 Terminal and extention unit composition of proanthocyanidins and GPC results for fractions H to T.

Fr	H	I	J	K	L	M	N	O	P
DP (acid-hydrolysis)	-	-	-	19.2	31.6	5.7	13.5	20.7	12.5
Mr (by GPC)*	1498.8	786.4	623.2	1452.9	572.8	176887.6	-	-	1339.5
DP (by GPC)	3†	1.6†	1.2	3.7	1.4	-	-	-	2.6

*Mass of the acetylated fractions.

†Mass of acetylated (epi)cat was used as building unit where no acid-catalysis data were available.

4.4 DISCUSSION

4.4.1 Characterisation of the polymeric fraction of a 6 month old Shiraz wine.

Fraction D, and its isolated TLC bands, contained the highest concentration of polymeric phenols and were thus further investigated to determine the composition and properties of the main polymeric phenols in the young wine.

Somers' measurements (Somers and Evans 1977) indicated that all of the bands isolated form fraction D contained significant amounts of pigments. A comparison of the CieLab values (Table 4.7) and the Somers' measurements of the isolated bands, concluded that the absorbance at 520 nm and *a have a linear correlation as found before by Bakker *et al*. (1986), while colour density and lightness have an inverse relationship as expected. No correlation was found between hue and lightness (L), while there was a linear relationship between absorbance at 420 nm and *b.

The results from Porter's hydrolysis firstly told us that FrDB2, FrDB3 and FrDB4 and the GST contained catechin and/or epicatechin, as cyanidin was formed. From previous literature a dimer, trimer and tetramer procyanidin are expected to yield respectively a 550 to 280 nm ratio of 1.1, 2.5 and 2.6 to 2.9 (Porter *et al.* 1986; Stafford and Lester 1980). The low 550 to 280 nm ratio of FrDB3 and FrDB4 was possibly due to the fact that they contained the largest amount of material resistant to hydrolysis. According to the hydrolysis results, the GST fraction contained in average mostly dimers, while FrDB2 contained mainly polymers larger than tetramers (Table 4.9).

Cheynier and colleagues (Cheynier *et al.* 1997a; Fulcrand *et al.* 1997) determined during their investigations of procyanidins with LC-ESI/MS that detection was most effective when the mass spectrometer was run in the negative ion mode despite the acidic nature of the HPLC mobile phase. With our solvent systems as described in chapter 3, section 3.2.2, however, we found that the total ion counts were higher in the positive ion mode. In addition, the predominant occurrence of the procyanidins as singly charged species, rather than multiply charged species, as occurs in negative ion mode, simplified interpretation of the data. This is in agreement with the findings of Hayasaka and Kennedy (2003) and Es-Safi *et al*. (1999b).

Both the T-A and A-T type direct condensation polymeric pigments were observed in the TLC bands isolated from fraction D (see Table 4.10). Hayasaka and Kennedy (2003) identified polymers up to octamers in a 3 year old Pinot Noir wine. In the young (6 months) Shiraz wine analysed only polymers up to the tetramer could be detected and these were at very low signal intensity. Products resulting from ethanal cycloaddition between anthocyanins (pyrano-anthocyanins) and flavanols as well as ethyl-bridged pigments were identified. This is in agreement with Atanasova and co-workers (2002a) who detected dimer-pyrano-anthocyanins and ethyl-bridged pigments in a 7 month old red wine. It is proposed that the FrDOrig and FrDB1 fractions contained a malv-3-gluc-pyrano-monogallo(epi)cat-tetramer and malv-3-gluc-(epi)cat-trimer-pet-3-gluc respectively. Generally, the degree of polymerisation decreased with mobility of the bands, while the pigment content in the polymers increased. This is not surprising as separation have been shown to be as a result of polarity, size and association with the packing material. Pigments increase the polarity of fractions due to the relative high polarity of the anthocyanins compared to flavanols. There is also a decrease in polarity with increasing chain length. All the fractions gave very weak mass signals with the most abundant ions being of low mass. This could be as a result of low sample concentration, low ionisation of the components or the presence of impurities.

MALDI-TOF MS has advantages over other mass spectrometric systems in sensitivity and extended mass range. During MALDI-TOF/MS analyses the continuous loss of different sugars were observed in some of the fractions, indicating the presence of polysaccharides. This could have a significant influence on the ionisation potential of the samples and an important reason for the low ionisation seen during MS analysis.

The apparent presence and possible association of oligosaccharides with the pigmented phenols in the isolated fractions may have an important influence on their mouthfeel properties. Vidal *et al*. (2004b) found that two wine polysaccharide fractions (a mixture of arabinogalactan-proteins and mannoproteins as well as rhamnogalacturonan II) significantly increased the ‘fullness’ sensation of wine. The rhamnogalacturonan II fraction also significantly decreased the attribute ratings associated with astringency. The neutral sugar composition of the isolated bands and wine retentate were determined and some of the fractions contained far in excess of the amount of glucose that would be expected, estimated from the predicted anthocyanin concentration determined in malvidin-3-glucoside equivalents. Linkage analysis by methylation also showed that there was a high

proportion of 5-Ara*f* and 3,5-Ara*f*, estimating that *ca* 41% of the oligosaccharides present in the wine retentate was an arabinan (Vidal *et al.* 2000). Xylans, type II arabinogalactans, mannose and a low concentration of galacturonans was also present. FrDOrig, FrDB1 and FrDB2 also contained arabinans. FrDB1 additionally produced sugars indicating to the presence of arabinoxyloglucan and FrDB2 to cellulose. Other polysaccharides were only present in trace amounts. The polysaccharide to phenol ratio for the wine retentate was 34% (w/w) while FrDOrig contained 43% (w/w). In the rest of the bands the polysaccharide percentage varied from 33% in FrDB2 to 9% in FrDB4.

The polysaccharides present in the wine was expected to be excluded by the different packing materials during liquid chromatography and when the moveability of the isolated Shiraz polysaccharides were tested on TLC, it was found that the polysaccharides did not move with the mobile phase. This indicated that the oligosaccharides seen are most probably associated with the pigments in the different fractions. Evidence to suggest such a strong association between pigments and polysaccharides has not been shown before.

The different TLC bands isolated from fraction D and controls (wine retentate, grape seed tannin and grape skin tannin) were characterised during hydrolysis in the presence of a nucleophile. No anthocyanins were released by hydrolysis of the pigmented polymerised phenols. This is due to the resistance of direct condensation pigments to hydrolysis (Salas *et al.* 2004). Unknown pigments were however released during hydrolysis of some of the fractions in the presence of phloroglucinal. These could be due to the presence of indirect polymerised pigments such as those connected through an ethyl bridge. Es-Safi *et al.* (1999b) found that the linkage between the flavanol and ethyl bridge is more sensitive to acid hydrolysis than that between the ethyl group and malvidin-3-glucoside. The flavanol-ethyl linkages with anthocyanin were also less sensitive to hydrolysis then those involved in flavan-ethyl dimers (Es-Safi *et al.* 1999a). The hydrolysis results of the samples will as a result be influenced by the fact that flavanol units linked to anthocyanins and/or linked by ethyl, vinyl and other similar bridging compounds would be resistant to acid hydrolysis and would produce different hydrolysed flavanol and anthocyanin derivatives (Es-Safi *et al.* 1999a, b; Salas *et al.* 2004, 2005).

Large discrepancies were found between the mDP determined by acid-catalysis and GPC for the grape skin and seed samples. According to acid-catalysis and GPC respectively, the mDP of the grape skin tannin sample was 11.4 and 4 while that of the grape seed tannin

was 3.7 and 7. Although Kennedy and Taylor (2003) found good agreement between the determined mDP by acid-catalysis and GPC for grape skin tannin, they also found that the GPC results for grape seed tannin indicated that it was larger than grape skin tannin. Kennedy and Taylor (2003) concluded that it was likely due to the increase in C-3 galloylation which resulted in a more extended conformation. This is suggestive of the fact that GPC, like acid-catalysis in the presence of a nucleophile, is only indicative of the characteristics of an unknown sample. The higher DP according to GPC compared to acid-catalysis may also be as a result of the unidentified (ethyl-linked) proanthocyanidins and pigments released during acid-catalysis which is not taken into account when determining the DP from the released monomers and phloroglucinol-adducts. Prieur *et al*. (1994) also obtained higher DP values determined by GPC compared to thioacidolysis for grape seed tannin which they attributed to the globularity (spherical nature) of polymers. The presence of small percentages of very high molecular weights supports the fact that there are oligosaccharides present in the isolated bands.

The prepared retentate from the young Shiraz wine investigated contained a significant amount of oligosaccharides. Of these especially arabinan and mannan were able to move through the silica column and retain their association with pigmented polymers during fractionation. The oligosaccharides were more prevalent in the late eluting or less mobile TLC bands isolated. This severely compromised the ionisability of the polyphenols in the fractions. Acid-hydrolysis indicated a mean DP of 2 for the proanthocyanidin part whereas GPC determined a mDP closer to 4. This discrepancy in mDP was also exhibited in the analysis of each isolated band. The disparity would partly be due to the presence of pigments and other components such as ethyl-bridges in the polymers which are not taken into account during acid-hydrolysis. FrDOrig has according to spectrophotometric analysis the lowest amount of red colour ($OD_{280}^{HCl} / OD_{520}^{HCl} = 78$) per phenol of all the bands. ESI-MS results indicated the presence of dimeric to pentameric pigments of type A-T and T-A. This could point to the fact that the colourless forms (A-T) are dominant. Further fractionation of the wine did enable the identification of larger pigments then what were identified in the original wine. The presence of pentameric pigmented polymers in such a young red wine has not been shown before. Dimeric and pyrano pigments were prominent in the TLC bands with higher mobility. These pigments have also been found to be the most prominent in young wines by Atanasova *et al*. (2002a) and Salas *et al*. (2005).

4.4.2 Characterisation of the polymeric fraction of a 5 year old Shiraz wine

Analyses of the wine retentate determined a mDP of 10. Hayasaka and Kennedy (2003) calculated a mDP of 8.6 for a 3 year old Pinot Noir wine. ESI-MS showed mass ions corresponding to both T-A and A-T type pigmented polymers, in addition to ethyl linked pigments and proanthocyanidins up to the nonamer. This agrees with the findings of published literature (Alcalde-Eon *et al.* 2004; Hayasaka and Kennedy 2003; Remy *et al.* 2000; Sun *et al.* 2006). The presence of epigallocatechin and epicatechin gallate also indicate that both the grape skin and seed tannin contributed to the isolated wine tannin (Fulcrand *et al.* 1999; Prieur *et al.* 1994; Souquet *et al.* 1996).

In general, Fraction F only contained relatively low molecular weight pigments with an average DP of 2 except for FrFB3 where mass ions corresponding to pentameric and tertrameric pigments were present. The mean degree of polymerisation (mDP) determined by phloroglucinolysis for most of the TLC bands isolated from fraction G was higher then what was seen during ESI-MS analysis. This might be due to low ionisation and/or break up of the large proanthocyanidin chains. The larger mDP's determined by GPC compared to acid-catalysis could be the result of galloylation and pigmentation as discussed previously. In general the the TLC bands isolated from fraction G contained larger polymeric phenols then those isolated from fraction F. FrGB2 was the main band isolated from FrG and the mDP's determined with acid-catalysis and GPC were high (13.2 and 11.4). LC/ESI-MS data did not show mass ions of this size and delphinidin-3-(6-acetyl)-glucoside connected with a vinyl bond to a proanthocyanidin tetramer was the main polymeric phenol present. This may be as a result of the low ionisation potential of the larger pigments. Asenstorfer *et al.* (2001) and Sun *et al*. (2006) also observed vinyl adducts in respectively 4 and 1.5 year old red wines, but only pigmented trimers were observed. ESI-MS analysis of fraction G before TLC fractionation displayed ions corresponding to larger pigmented polymers then what was observed with the resulting isolated TLC bands. Malvidin-3-glucoside connected through ethyl type linkages to an eleven and eight unit proanthocyanidin was respectively seen. Ethyl linked anthocyanins to monomeric and dimeric proanthocyanidins has previously been identified (Alcalde-Eon *et al.* 2004; Salas *et al.* 2005; Sun *et al.* 2006). Pigments of this size have not been seen before and the next step would be to isolate this pigment for further positive identification. It is possible that these pigments were broken up or oxidised during subsequent fractionation.

Of the rest of the fractions obtained from MPLC only fractions K to P gave discernable results, indicating mDP's of 5.7 to 31.6. Both acid-catalysis in the presence of a nucleophile and GPC were not always effective due to too low sample concentrations leading to inaccurate determination.

4.5 CONCLUSION

Characterisation of the retentates of the 6 month and 5 year old Shiraz wines indicated large differences in their polymeric phenol composition. The older wine contained polymers of an average DP of 10 compared to the younger wine's DP of 4. LC/ESI-MS results exhibited ions of mostly small pigments for the young wine, while the 5 year old wine contained ions indicating anthocyanins connected to larger proanthocyanidin chains (up to nine units). In general, the characterisation of the isolated TLC bands of the young wine fraction was more successful then that of the fractions obtained from the older wine. Although bands from the young wine resulted in larger amounts of sample to work with, this was also due to the formation of more complex phenol polymers with aging resulting in smaller concentrations of a specific compound in fractions from the older wine. Some of the bands isolated from the young wine contained significant amount of polysaccharides which were shown to be associated with the polymeric pigments. Only one fraction of the 5 year old wine indicated the presence of oligosaccharides. The MS results of the different fractions for the two wines indicate that the presence of ethyl and vinyl linked proanthocyanidins and pigments also increased with aging. Acid-catalysis can not effectively characterise these types of polymers. The fractions containing the larger pigments did in general elute later from the normal phase resin. In fraction G of the 5 year old wine, TLC bands with proanthocyanidin chains with a mDP of 7 to 25 was calculated although GPC results did not always support this. FrGB2 did however have a mDP of 11.4 determined by GPC with a mDP of 13.2 according to acid-catalysis. This band contained the largest amount of material from fraction G. The next step would be to obtain enough of this band to isolate and positively identify the individual polymeric pigments within. The GPC results of fractions H to P that were obtained were inconsistent with that from acid-catalysis.

It is clear from the results obtained by the different characterisation methods that these methods are not ideal for the identification of pigmented polymers. This is specifically true if only small amounts of material are available. Isolation of the pigments from a resin was

also problematic. The use of CCC will improve not only the quantity of sample collected but may improve ESI-MS results, which is to date the most effective method used for the identification of pigmented polymers.

Chapter 5

DEVELOPMENT OF A VOCABULARY TO DESCRIBE THE MOUTH-FEEL PROPERTIES OF RED WINE

5.1 INTRODUCTION

The quality of red table wine is dependent on pleasing and complex mouth-feel sensations. A large vocabulary of descriptive terms is used by winemakers, wine judges and other experienced wine tasters to describe the sensory properties perceived in the mouth when tasting red wine. These perceptions encompass multiple and interacting sensations of acidity, sweetness, bitterness, retronasal aroma perception (flavour), viscosity, warmth and astringency.

Mouth-feel attributes such as density, viscosity, oily, mouthcoat and stickiness have been used in a beer sensory study (Langstaff *et al.* 1991), while Lee and Lawless (1991) generated the terms, 'drying', 'puckering', 'sour', 'astringent', 'bitter' and 'rough' to describe a range of chemically diverse astringents. Astringency perception is a tactile sensation (Breslin *et al.* 1993), while sourness and bitterness are tastes. The descriptive 'drying' was defined as the 'lack of lubrication or moistness resulting in friction between oral surfaces', 'roughing' as the 'un-smooth texture in the oral cavity when oral surfaces come in contact with one another', with 'puckery' as the 'drawing or tightening sensation felt in the mouth, lips and/or cheeks' and 'astringency as being the combination of the other three (Lawless *et al.* 1994). The main mechanism of phenolic astringency perception is believed to result from their binding to saliva proteins and subsequently precipitating them, stripping the mouth of its normal lubrication (Bate-Smith 1973). Some wine components such as acids have astringent sub-qualities themselves (Lawless *et al.* 1996; Rubico and McDaniel 1992; Thomas and Lawless 1995), while alcohol has been shown to reduce the astringent sensation (DeMiglio *et al.* 2002; Lea and Arnold 1978), which complicates matters even further. Fundamentally, the perception of astringency is a highly dynamic process, changing continuously during ingestion and especially following expectoration or swallowing (Noble 1995).

Descriptive analysis involve the detection and description of sensory attributes by a trained panel (Meilgaard *et al.* 1991; Stone *et al.* 1974). While this method has been applied mainly to studies on wine aroma, it is also suitable for the scoring of palate 'in-mouth' attributes. In order to effectively study the impact of polyphenolic compounds on red wine sensory properties, it is necessary to carefully define attributes to be scored by a panel. These terms should preferably be defined by references or physical standards (Muñoz 1986; Rainey 1986). There has been little work published regarding the assessment of red wine palate or mouth-feel properties. This was a significant void as the 'texture' of a wine will have an important influence on the overall perception of quality. Any discussion of the quality of red wine by experienced tasters usually includes some reference to astringent and other sensations experienced in the mouth. The lack of a defined and structured vocabulary to describe mouth-feel sensations is in contrast to that of wine aroma and flavour for which the aroma wheel (Noble *et al.* 1987) has been a valuable tool. It was seen as a necessity to develop a structured and well-defined vocabulary to assist wine tasters to better communicate the mouth-feel characteristics they perceive in red wines.

5.2 MATERIALS AND METHODS

5.2.1 Development of the descriptive analysis of mouth-feel properties

5.2.1.1 Deriving an astringency vocabulary

Thirteen volunteers (11 males and 2 females between the age of 24 and 50, all non-smokers), of whom all had extensive experience in wine tasting, were convened, three times a week, generally tasting five wines at a time. Sessions of 60 to 90 minutes were held in a tasting fasility. A list of terms was generated and discussed during which redundant terms were removed and others defined by panel consensus. Panel members were always encouraged to use and write any terms they felt inclined to use. In this way the list increased continually, encompassing attributes observed in the mouth. In total 144 red wines, including 13 experimental wines, were tasted during 47 sessions, on which the terminology to describe the

mouth-feel properties of wine was based. The wines were evaluated while in the mouth as well as after expectoration. The standards evaluated were analysed similarly to the wines.

Wines ranged from the current vintage to 33 years old and from commercial quality through to high quality examples of light, medium and full bodied wines (Table 5.1). Mostly wines were two to five years old and of Australian origin, covering all the major wine producing areas. Five Italian, one South African and two French wines were also evaluated. Cabernet Sauvignon, Shiraz, Merlot and Pinot Noir, including blends, were the varieties comprising the largest portion of tastings, with other varieties such as Grenache, Cabernet Franc, Cinsaut noir, Nebbiolo and Sangiovese also tasted. Wines were presented blind and in random order.

A range of possible astringency, drying and bitter standards was investigated (Table 5.2). The oenological commercially produced tannins Galacool (1.6 g/L; Tanin Galalcool, Vinkem, South Australia), and Tannin V.R. Supra (2.5 g/L; Tannin V.R. Supra, Vinkem, South Australia) and aluminium sulfate or Alum (0.8 g/L; Ace Chemical Co., South Australia) in water were subsequently used in assessments; these were the only standards considered appropriate for astringency related attributes by the panel members. The rest of the investigated compounds displayed a more complex array of attributes without exhibiting a main characteristic and would thus not have been a successful standard for a specific descriptor. Alum was used as a drying standard while the two tannins, Galacool and V.R. Supra, respectively covered foremost the fine grained and more particulate terms with dryness while V.R. Supra displayed more surface smoothness attributes.

Table 5.1 Wines used in development of mouth-feel wheel

Vintage	Producer/brand	Variety	Region and country
1992	Zema Estate	Cabernet Sauvignon	Coonawarra, Australia
1993	Primo Estate	Shiraz	South Australia, Australia
1992	Tuck's Ridge At Red Hill	Pinot Noir	Victoria, Australia
1994	Stonier Reserve	Pinot Noir	Victoria, Australia
1994	Peter Lehman Vine Vale	Shiraz	Barossa Valley, Australia
1994	Charles Melton	Shiraz	Barossa Valley, Australia
1996	Brokenwood	Shiraz	Barossa Valley, Australia
1997	Mountadam experimental	Pinot Noir	Barossa Valley, Australia
1997	Clare Estate	Cabernet Sauvignon	Clare Valley, Australia
1993	Joseph Moda Amarone, Primo Estate	Cabernet Sauvignon/Merlot	McLaren Vale, Australia
1992	The Angelus, Wirra Wirra	Cabernet Sauvignon	Adelaide Hills, Australia
1996	Saltram	Shiraz	Barossa Valley, Australia
1994	Leconfield	Cabernet Franc	Coonawarra, Australia
1993	Smithbrook	Pinot Noir	Coonawarra, Australia
1992	Mountadam experimental	Pinot Noir	Barossa Valley, Australia
1992	Mitchell	Cabernet Sauvignon	Clare Valley, Australia
1994	David Wynn	Cabernet Sauvignon	Coonawarra, Australia
1993	Richard Hamilton	Cabernet Sauvignon	Coonawarra, Australia
1996	Brokenwood experimental wine	Shiraz	Hunter Valley, Australia
1996	Waikerie, Minimal pruning	Shiraz	Riverland, Australia
	Waikerie, Minimal pruning 33% yield	Shiraz	Riverland, Australia
	Waikerie, Vertical shoot	Shiraz	Riverland, Australia

	position		
1996	Waikerie, Vertical shoot position, 33% yield	Shiraz	Riverland, Australia
1997	Nuriootpa experimental	Shiraz	Barossa Valley, Australia
1997	Henscke Mount Edelstone experimental	Shiraz	Barossa Valley, Australia
1997	Coonawarra experimental	Shiraz	Coonawarra, South Australia
1997	Lynedoch experimental	Shiraz	Barossa Valley, Australia
1997	Waikerie, minimal prune	Shiraz	Riverland, Australia
1995	Cassegrain	Cabernet Sauvignon/Merlot	New South Wales, Australia
1995	Stonier	Pinot Noir	Victoria, Australia
1994	Brokenwood Graveyard Shiraz	Shiraz	Hunter Valley, Australia
1994	Cape Mentelle	Shiraz	Margaret River, Australia
1995	Kingston Estate	Grenache	South Australia, Australia
1994	Prince Albert	Pinot Noir	Victoria, Australia
1994	Knappstein	Cabernet Franc	Victoria, Australia
1993	Barossa Valley Estate E & E	Shiraz	Barossa Valley, Australia
1996	Henschke Mount Edelstone	Shiraz	Eden Valley, Australia
1992	Katnook Estate	Shiraz	Coonawarra, Australia
1988	Bolla Amarone della	Corvino/Rondinella/Molinara	Veneto, Italy
1985	Chateau Leoville Barton	Cabernet Sauvignon/Cabernet Franc/Merlot/Petit Verdot	Bordeaux, France
1995	Sacred Hill	Shiraz/Cabernet Sauvignon	Australia
1994	Keystone Estate	Grenache/Shiraz	McLaren Vale, Australia
1994	Cape Mentelle	Cabernet Sauvignon/Merlot	Margaret River, Australia

1991	Cape Mentelle	Cabernet Sauvignon/Merlot	Margaret River, Australia
1993	Salisbury Estate	Cabernet Sauvignon	Victoria, Australia
1996	Mount Edelstone Henschke	Shiraz	Eden Valley, Australia
1986	The College	Cabernet Sauvignon/Cabernet Franc	Wagga Wagga, Australia
1988	Barbaresco Riserva	Nebbiolo	Piedmont, Italy
1994	Cassegrain	Chambourcin	New South Wales, Australia
1993	Leasingham Classic Clare	Shiraz	Clare Valley, Australia
1989	Yarra Yering Dry Red no. 1	Cabernet Sauvignon/Malbec/Merlot/Petit Verdot	Yarra Valley, Australia
1969	Bin 54 Stonyfell Burgundy	Shiraz	Barossa Valley, Australia
1992	Yeringberg	Pinot Noir	Yarra Valley, Australia
1992	James Irving Grand	Merlot	Australia
1994	Devils Lair	Pinot Noir	Margaret River, Australia
1993	Yeringberg	Cabernet Sauvignon/Cabernet Franc	Yarra Valley, Australia
	Brunello di Montalcino Reserve	Sangiovese	Florence, Italy
1993	Marienberg	Shiraz	McLaren Vale, Australia
1994	Richard Hamilton Old Vines Shiraz	Shiraz	McLaren Vale, Australia
1993	D'Arenberg Dead Arm	Cabernet Sauvignon/Shiraz	McLaren Vale, Australia
1993	Ebenezer	Cabernet Sauvignon/Malbec/Merlot	Barossa Valley, Australia
1993	St. Hallett Gamekeepers Reserve	Shiraz/Grenache	Barossa Valley, Australia
1994	Brokenwood	Hermitage	Hunter Valley, Australia

1994	Plantagenet	Pinot Noir	Mount Barker, Australia
	Leasingham Classic Clare	Cabernet Sauvignon	Clare Valley, Australia
1970	Kaiser Stahl Reserve Bin Claret 4147	Cabernet Sauvignon	Barossa Valley, Australia
1994	Saltram Metala	Shiraz/Cabernet Sauvignon	South Australia, Australia
1992	Grant Burge Meshach	Shiraz	Barossa Valley, Australia
1991	Yarra Yering Dry Red no. 1	Cabernet Sauvignon/Malbec/Merlot/Petit Verdot	Yarra Valley, Australia
1988	St. Huberts	Yarra Valley, Australia	Yarra Valley, Australia
1992	St. Huberts	Cabernet Sauvignon	Yarra Valley, Australia
1993	Evans & Tate	Cabernet Sauvignon	Margaret River, Australia
1994	Plantagenet Fine Red	Cabernet Sauvignon/Merlot/Pinot Noir	Western Australia, Australia
1993/1994	Evans & Tate	Shiraz/Cabernet Sauvignon	Margaret River, Australia
1994	Shottesbrook	Merlot	McLaren Vale, Australia
1994	D'Arenberg Wines Ironstone Pressing	Shiraz/Grenache	McLaren Vale, Australia
1993	Katnook Estate Riddock Shiraz	Shiraz	Coonawarra
1993	Clare Cabernet	Cabernet Sauvignon	Clare Valley
1996	Graveyard Shiraz Brokenwood	Shiraz	Hunter Valley, Australia
1991	Peter Lehman Mentor	Cabernet Sauvignon/Malbec/Shiraz	Barossa Valley, Australia
1990	Kalimna	Shiraz	South Australia, Australia
1993	Petuluma	Cabernet Sauvignon	Coonawarra, Australia
1994	Longhorn Creek	Cabernet Sauvignon	Barossa Valley, Australia
1993	Howard Park	Cabernet Sauvignon/Merlot	Margaret River, Australia
1993	Tyrell's Wine	Pinot Noir	Hunter Valley, Australia

1994	Rosemount Estate	Shiraz	South Australia, Australia
1990	Evans & Tate	Cabernet Sauvignon	Margaret River, Australia
1993	Katnook Estate	Merlot	Coonawarra, Australia
1995	Mount Hurtle	Grenache	South Australia, Australia
1994	Petuluma Shiraz	Shiraz	Lenswood, Australia
1994	Mount Langi Ghiran Vineyard	Shiraz	Victoria, Australia
1994	Jasper Hill	Shiraz	Victoria, Australia
1994	Tyrell's Long Flat Red	Shiraz/Cabernet Sauvignon	Hunter Valley, Australia
1994	Yarra Yering	Pinot Noir	Yarra Valley, Australia
1990	Katnook Estate	Merlot	Coonawarra, Australia
1979	Krandorf	Cabernet Sauvignon	McLaren Vale, Australia
1994	Inglewood Classic Red	Shiraz	Hunter Valley, Australia
1990	Grant Burge Meshach	Shiraz	Barossa Valley, Australia
1989	Ashton Hills Vineyard	Pinot Noir	South Australia, Australia
1988	Roseworthy Angaston Shiraz	Shiraz	South Australia, Australia
1988	Cullen	Cabernet Sauvignon/Merlot	Margaret River, Australia
1993	Scotchmans Hill	Pinot Noir	Victoria, Australia
1988	Peter Lehmans Stonewell	Shiraz	Barossa Valley, Australia
1994	Valiano Chianti Classico	Sangiovese	Chianti, Italy
1991	Miranda Rovalley Ridge	Cabernet Sauvignon/Shiraz	Barossa Valley, Australia
1990	Mount Mary Lilydale	Cabernet Sauvignon	Yarra Valley, Australia
1990	Mount Mary Lilydale	Pinot Noir	Yarra Valley, Australia
1992	Shottesbrooke	Cabernet Sauvignon/Merlot	McLaren Vale, Australia
1993	The Peppermint Paddock D'Arenberg	Chambourcin	McLaren Vale, Australia
1991	Lloyd Reserve	Shiraz	McLaren Vale, Australia
1991	Moorooduc Estate	Cabernet Sauvignon	Victoria, Australia
1991	St. Hallett Old Block Shiraz	Shiraz	Barossa Valley, Australia
1990	Yarra Yering	Pinot Noir	Yarra Valley, Australia

1992	Clarendon Hills	Cabernet Sauvignon	McLaren Vale, Australia
1988	Barbaresco Montestefano	Nebbiolo	Piedmont, Italy
1990	Moss Wood	Cabernet Sauvignon	Margaret River
1980	Morris	Durif	Rutherglen, Australia
1986	Bannockburn	Pinot Noir	Victoria, Australia
1982	Blaauklippen	Cabernet Sauvignon	Stellenbosch, South Africa
1993	Vasse Felix	Hermitage	Margaret River, Australia
1990	Chateau Langoa Barton Saint-Julien	Cabernet Sauvignon/Cabernet Franc/Merlot/Petit Verdot	Bordeaux, France
1993	Coldstream Hills	Pinot Noir	Victoria, Australia
1994	Best's Great Western	Dolcetto	Grampions, Australia
1994	Leconfield	Cabernet Sauvignon	Coonawarra, Australia

Table 5.2 Potential astringency and bitter standards investigated

Standard	Concentration	Summary of the description by panellists*
Tannin Oenologique	2.5 g/L in water	Bitter, fine emery, wet chamois, suede, drying, sawdust, chalky, grippy
	0.5 g/L in water	Bitter
	0.05 g/L in water	Like water
Quertannin	2.5 g/L in water	Bitter, silky, suede, wet chamois, furry, drying, dusty, chalky, grippy
	0.5 g/L in water	Bitter, watery
	0.05 g/L in water	Watery
Tannin V.R. Supra	2.5 g/L in water	Silky, wet chamois, suede, fine emery, dusty, drying (medium), bitter (medium)
	2.5 g/L in 10% EtOH	Viscous, silky, wet chamois, suede, fine emery, dusty, drying (medium), bitter (medium-high)
	2.5 g/L in model wine	Silky, wet chamois, suede, fine emery, talc, drying (medium-high), grippy, bitter (medium)
	0.5 g/L in water	Soft/body
	0.05 g/L in water	Watery
Tannin Galacool	2 g/L in water	Bitter, very drying, grippy
	1.6 g/L in water	Drying (high), fine
	1.6 g/L in 10% EtOH	Drying, hot
	1.6 g/L in model wine	Dry, viscous, wine-like
	0.5 g/L in water	Drying, bitter, grippy
	0.05 g/L in water	Drying, bitter
Grape seed tannin	0.05 g/L in water	Bitter, drying (low)
	2.5 g/L in water	Bitter, drying (high)
Tannic acid	1.25 g/L in water	Bitter (high), astringency (low)
Alum	0.8 g/L in water	Fine emery, sawdust, furry, drying (high), adhesive
	0.1 g/L in water	Weakly astringent
Quinine sulphate	0.015 g/L in water	Bitter (low – medium), silky
Caffeine	1.0 g/L in water	Bitter (medium)
Quinces	Tasted raw	Very drying, powdery
	Cooked and tasted	Just sour
Green tea	1 teabag in 250 mL water for 5 min	Prickle, bitter
	2 teabag in 250 mL water for 5 min	Bitter (high)
Orange pekoe tea	1 teabag in 250 mL water for 5 min	Bitter, drying
	2 teabag in 250 mL water for 5 min	Bitter (high), drying

Water was purified by a Milli-Q reagent water system from Millipore Pty Ltd (NSW, Australia).

*Terms used more then once in two trials by two subjets.

After 26 sessions determining and defining terms to describe astringency and the mouth-feel terms associated with it; other mouth-feel properties such as flavour, acidity and tactile sensations other then astringency were also observed and investigated. Possible standards for subcategories of acidity as well as mouth-feel properties such as weight and texture were evaluated for definition purposes. The standard was placed in the mouth and the tactile sensations exhibited on the mouth surfaces were noted (Table 5.3). Panel members decided that flavour intensity, concentration and length should also be considered to enhance possible differentiation of samples (Meilgaard *et al.* 1991). Consistency of the panel rating these parameters was tested.

Table 5.3 Possible texture, irritation and acidity standards investigated

Substance	Method	Description
Cream (Farmland reduced cream 25% fat)	Place very small amount in mouth	Gritty mouth-feel, but thick/smooth - mouthcoating
Olive oil (Baryes extra light 100%)	Place very small amount in mouth	Oily/slippery
Soda water (Schweppes)	Place small amount in mouth	Flat – spritz Fresh – prickle
Steel spoon	Place in mouth, suck	Metallic taste, feel of steel

Different finger touch standards were investigated as possible representations of the astringent sensation being experienced in the mouth (Table 5.4). The panel members found it very useful to define the astringent sensations being experienced in the mouth in this manner and decided by consensus which touch standards were appropriate. The touch standards were therefore used at subsequent tastings.

Table 5.4 Different touch standards used to represent tactile sensations observed in the mouth

Descriptor	Touch standard
Talc	Johnson baby powder
Satin	Satin cloth
Plaster	Gypsum powder
Chamois	Moistened chamois
Silk	High grade silk cloth
Velvet	Velvet felt in direction of the nap
Suede	Medium suede leather
Furry	Short velour cloth
Fine emery paper	1000 grade emery paper
Corduroy	Medium cord cloth
Abrasive	600 grade sandpaper
Hessian	Carpet backing

5.2.1.2 Panellist consistency and reliability in identifying astringent sub-qualities

Intermittently the consistency and progress of the panel were assessed according to the ability of tasters, with the utilization of the derived vocabulary, to apply the same terms to wines upon repeat presentations. The panellists were introduced to the Labelled Magnitude Scale (LMS; (Green *et al.* 1996)) and practised for eight sessions using the scale to rate the intensities of the astringent attributes of dry red wines. LMS was used because it gets around the problem that panellists avoid the ends of scales. Two commercial wines were presented with other wines and repeated on occasions separated by one or three days to make memorization of repeat wines difficult. Panel members identified astringent sub-qualities by referring to a list of astringent terms and referring to their verbal definition or touch standards. This exercise was repeated twice at fortnightly intervals using different commercial wines of similar style and age. The consistency of the judges in using astringent terms were analysed with chi-square analysis. The expected number of terms that would be correctly matched under chance performance was calculated as the mean of the relevant hypergeometric sampling distribution of each panellist, and the observed values were the actual number

correctly matched by the panellist (Mendenhall *et al.* 1986). Panellist reliability was evaluated by correlating the ratings given on the second to last and final sessions (Brien *et al.* 1987).

5.3 RESULTS

5.3.1 Development of mouth-feel terminology

After 47 sessions and after tasting 144 wines, 53 carefully defined mouth-feel descriptive terms comprising 33 astringent related terms and 20 other non-astringent mouth-feel terms, were generated. These terms were presented to both the original panellists and also nine experienced winemakers. They were asked to independently sort astringency terms according to similarity into different groups, using as many groups as necessary. The number of times each pair of terms was placed in the same group was assembled and served as a measure of similarity between terms. This data was analysed by hierachical agglomerative clustering (data not shown). The process was not repeated for the terms describing non-astringency mouth-feel terms as the panellists felt that natural and mutually exclusive categories existed for these terms. From this result and from subsequent panel discussions the mouth-feel attributes were separated into 13 classifications with the distinguishing properties for the astringency related terms tabulated below (Table 5.5).

The tasting of commercial tannin as possible astringency standards was considered as unsuccessful. The astringents trialled displayed complex profiles and most panellists felt that exposure to these standards interfered with their subsequent perception of the test samples. This belief is supported by the carry-over effect of astringency previously reported by Guinard *et al.* (1986). The touch standards, on the other hand, could be employed during tasting, and by their nature their use could not affect the astringency elicited by the wine. The panellists agreed that the touch standards produced cutaneous sensations similar to those experienced in the mouth and were therefore beneficial. The attributes that could not be represented by a physical standard were clearly defined (Table 5.6).

Table 5.5 Title and description of the groupings of astringency terms

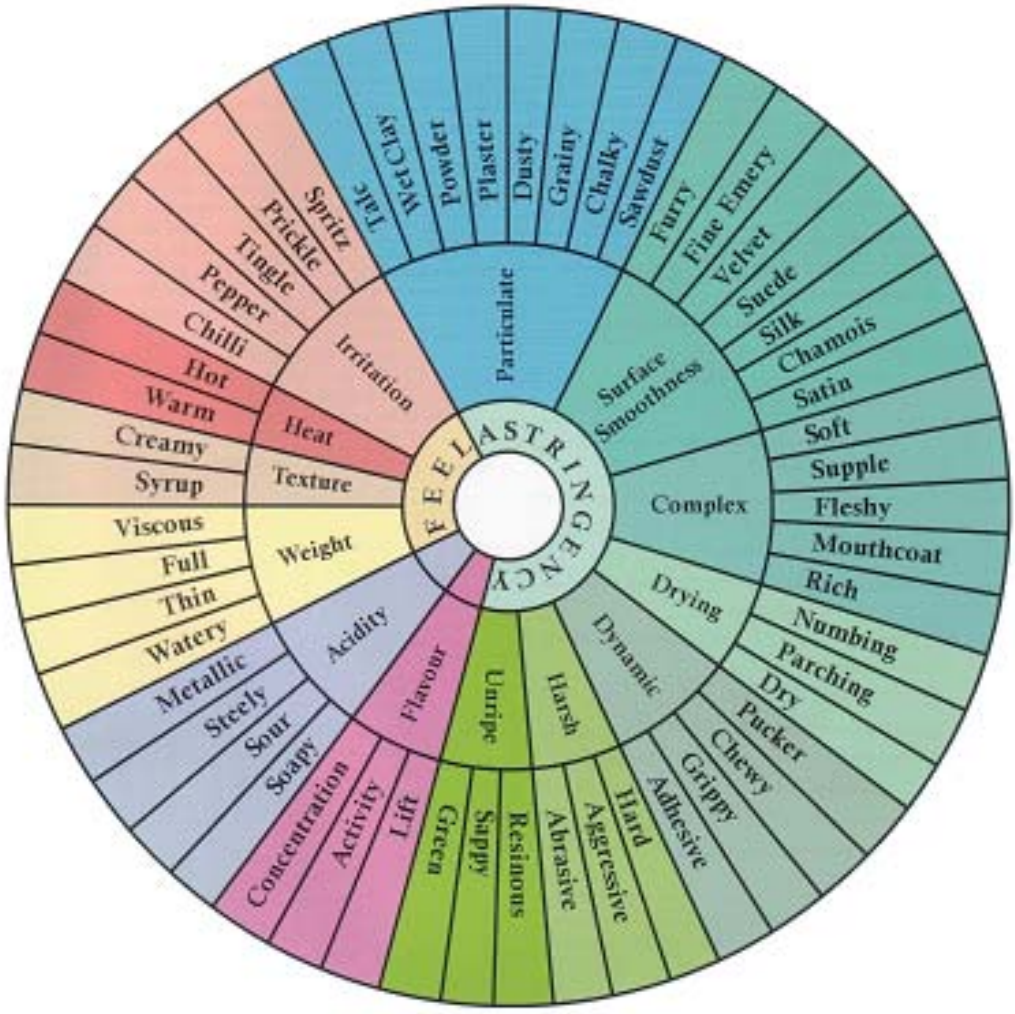
Grouping	Distinguishing feature of the group
Particulate	Feelings of particulate matter brushing against the surfaces of the mouth through the movement of the wine.
Surface smoothness	Textures felt on mouth surfaces when the different surfaces come in contact with each other.
Complex	A positive hedonic grouping consisting of an amalgam of pleasing astringency sensation, flavour and balanced acidity.
Drying	Dessicating
Dynamic	Sensations involving some form of mouth movement.
Harsh	A negative hedonic grouping suggesting aspects of excessive unbalanced astringency, excessive roughness and/or bitterness.
Unripe	A negative hedonic grouping consisting of an astringent feel associated with excessive acidity and associated green flavour notes.

Table 5.6 Definition of astringency terms

Term	Definition
Pucker	A reflex action of mouth surfaces being brought together and released in an attempt to lubricate mouth surfaces.
Chewy	Gives the feeling that mouth surfaces are sticking or adhering to one another, yet can be pulled away from each other with slight pressure.
Hard	Combined effect of bitterness and astringency. Synonym 'harsh'.
Aggressive	Balance term indicating excessive astringency.
Abrasive	Excessive astringency of a strongly roughing nature.
Soft	A light and finely textured astringency.
Supple	Balance term indicating excessive astringency.
Rich	High flavour concentration with balanced astringency.
Fleshy	High flavour concentration with suppleness.
Mouth-coat	Gives the impression of a coating film that adheres to mouth surfaces, and which falls from the mouth surfaces with time.
Parching	Drying with a background of alcohol hotness.
Green	Combined effect of excess acidity and astringency.
Sappy	Astringency with high acid and slightly bitter. Reminiscent of the astringency elicited by chewing on a green grape stalk.
Resinous	Astringency elicited as if chewing on piece of raw wood.

A mouth-feel wheel was thus established to assist in the description of a wine's mouth-feel characteristics (Figure 5.1). Another important outcome was the development of a highly trained group of tasters that could describe and quantify mouth-feel characteristics reproducibly: the mouth-feel panel.

Figure 5.1 Mouth-feel Wheel



5.3.2 Panel performance and consistency

The ability of the panel to identify astringent sub-qualities was significantly better than chance performance on each of the two occasions for both astringency in the mouth and after expectoration (Table. 5.7). The ability of panellists to consistently use astringency terms to describe the observed sensations in the mouth appeared to have improved with continued training. The percentage of times the same term was used to describe astringent sensations experienced in the mouth on repeat occasions increased from 17 to 44% by the end of training. Similarly, the matching of terms used after expectoration increased from 25 to 37%. These

increases in term matching performance were achieved in the context of a decline in the overall number of terms used, suggesting that it was not an artefact of an increased probability of matching terms due to a larger number of terms being selected.

Table 5.7 Results of significance test for above chance performance by the tasting panel in identifying the astringent sub-qualities of three commercial wines on a pair of repeat occasions

	In mouth			After expectoration		
	χ^2	d.f.	*P*	χ^2	d.f.	*P*
First pair of repeat occasions	17.87	9	0.037	33.41	9	<0.001
Second pair of repeat occasions	16.84	9	0.050	20.09	9	0.017

5.4 DISCUSSION

This work derived a refined vocabulary to describe the astringent and other mouth-feel sensations elicited by an extensive sample of dry red table wines representing different styles. The astringent terms were classified into categories representing; particulate, e.g. talc, dusty; surface smoothness, e.g. fine emery, silk; complex e.g. soft, mouthcoat; as well as harsh, e.g. hard and unripe, e.g. green. The terms describing drying and dynamic components of astringency were in many respects similar to the sub-qualities of 'roughing', 'puckering', 'drying', 'acidity' and bitterness' suggested by the panel of Lee and Lawless (1991). The astringent sub-quality 'acidity' reported by Lee and Lawless may be compared to the terms green, sappy and resinous suggested here. These terms have been used to describe the astringency of acidic wines such as those made from unripe fruit (Cheynier *et al.* 1999). The panellists in this study did not suggest bitterness as they were instructed to ignore taste qualities, however the term hard was reported by panellists to represent astringency with bitterness. The category of particulate or graininess has not been reported before and it is interesting to postulate whether this sensation is elicited by the insoluble complexes formed between the proline-rich saliva proteins and the phenols present in the wine or perhaps by those complexes that stay in solution (De Freitas and Mateus 2001; Kallithraka *et al.* 2001; Sarni-Manchado *et al.* 1999a). If the nature of these complexes could be understood, it may be possible to explain qualitative variation in astringency. At this time there is not even clarity on the association between the degree of interaction between phenols and proteins and overall

astringency. Rating astringency attributes using the mouth-feel wheel while the wine is in the mouth and after expectoration is an effective way to deal with the continuous change of astringency and it's sub-qualities.

5.5 CONCLUSION

This study has shown that astringency in red wine can manifest itself in many subtle yet complex forms, and that wine tasters can be trained to reproducibly discriminate and rate the intensities of astringent sub-qualities elicited by red wines (Gawel *et al.* 2001). The developed mouth-feel wheel will assist and stimulate the use of more defined descriptors for unambiguous communication. It will thus be a useful starting point for panel discussions regarding a set of samples under study. The sub-qualities of astringency may also enhance the possibility of understanding the relative role of wine phenol composition and also other wine components that affect the interaction between phenols and salivary proteins, in the mouth-feel properties of a red wine.

Chapter 6

MOUTH-FEEL OF WHITE WINES MADE WITH AND WITHOUT POMACE CONTACT AND ADDED ANTHOCYANINS

6.1 INTRODUCTION

Phenolic compounds contribute significantly to the sensory properties of red wine. They play an important role in red wine colour, bitterness, astringency, as well as a range of other tactile or 'mouth-feel' characteristics. They also most likely influence the overall flavour impression and retronasal aroma perception.

Monomeric and polymeric flavan-3-ols are the primary contributors to the astringency and bitterness of red wine and their role has been reviewed (Gawel 1998; Noble 1990; Singleton and Noble 1973). Generally the so-called condensed tannins or polymeric flavan-3-ols were believed to be more astringent than the smaller oligomers, while the monomers, dimers and trimers were reported to evoke more bitterness than the larger tannins (Arnold *et al.* 1980; Vidal *et al.* 2002). This concept has been used to explain the "hardness" of young wines and the "softness" of older wines, as a result of their different oligomeric to polymeric procyanidin ratios (Haslam 1980; Lea 1990). In more recent studies (Vidal *et al.* 2003a, 2004a), it was found that larger tannins extracted and fractionated from grape material were more astringent and drying than smaller tannins, in other words that increased degree of polymerisation gave rise to higher levels of astringency. In addition, it was found that seed tannins were more astringent (coarse, drying) than skin tannins of equivalent size, probably due to gallic acid derivatives, and that all the tannins studied had negligible bitterness (Vidal *et al.* 2003a). An ethyl-bridged flavanol fraction was rated by a sensory panel as more bitter than similar tannins, indicating that the insertion of an ethyl-bond in catechin units confers bitterness (Vidal *et al.* 2004a).

The sensory changes occurring in the course of wine aging are believed to result from the interactions of grape anthocyanins and proanthocyanidins (Singleton and Noble 1973), giving lower levels of astringency.

White grapes fermented like a typical red wine in the presence of skins and seeds result in wines with quite different astringent sensory profiles to those of red wines: they tend to be coarser and lower in astringency (Singleton and Trousdale 1992). The only apparent difference between the phenolic composition of pomace-fermented white wines and red wine is the anthocyanin content. Pure anthocyanins have been reported informally to have only a very mild indistinct taste (Singleton and Noble 1973; Singleton and Trousdale 1992). Singleton suggested that the incorporation of anthocyanins in polymeric procyanidins is primarily responsible for the distinctive astringency of red wines because anthocyanins seem to increase the amount of tannin retained in wine. Kantz and Singleton (1991) suggested that the sugar of the anthocyanin and perhaps the polarity of the flavylium cation both increase the solubility and decrease the precipitability of the resultant anthocyanin-bearing tannin molecule. It has been reported (Ribéreau-Gayon and Glories 1980) that these pigmented tannins are less reactive towards proteins and accordingly less astringent. Brossaud *et al*. (2001) found that the addition of an anthocyanin mixture to seed and skin tannin extracts increased the astringency of the solution over the astringency of either fraction alone, but had no effect on bitterness. Another study found that an anthocyanin fraction increased the perceived astringency and 'fullness' of a model wine (Vidal *et al.* 2004b). Vidal and co-workers concluded in a study where the anthocyanins used were of high purity that they did not contribute significantly to any mouth-feel properties (Vidal *et al.* 2004a), and that two fractions that were isolated from grape skins or wine that seemed to be predominantly derived anthocyanins had little or no effect on sensory attributes rated by a panel.

The model studies outlined above have suggested that the incorporation of anthocyanin pigments into proanthocyanidins may result in softening of a wine due to reduction in astringency. However, these studies were conducted in a model wine base and with isolates extracted under more severe conditions than those experienced during winemaking and from fractionation procedures that may have altered the composition of the tannins. The present study was conducted to further investigate the contribution of anthocyanins or tannin-anthocyanin reaction products to the mouth-feel properties of red wine, in an experiment complementing the model studies. White and red grape juices were fermented with and without pomace contact and anthocyanin addition and the subsequent young wines were characterised by sensory descriptive analysis.

6.2 MATERIALS AND METHODS

6.2.1 Fermentation of treated juices and processing of the resulting wines

Grapes were harvested in April during the 1999 season. Chardonnay grapes (23.5 °Brix, pH 3.81, titratable acidity (TA) 5.2 g/L as tartaric acid) were picked from a commercial vineyard in the Adelaide Hills (Lenswood), while the Shiraz grapes (24.5 °Brix, pH 3.94, TA 4.3 /L) were picked from an experimental block of irrigated vines at Nuriootpa Research Station. The grapes to be used in the winemaking experiments were stored at 0 ºC while the desired amount of anthocyanins was isolated from a portion of the batch of Shiraz grapes. The natural level of extraction of the anthocyanins from Shiraz skins into the wine was imitated by dispersing the pressed skins in model wine (pH 3.6, 12% ethanol saturated with potassium hydrate tartrate) under nitrogen at room temperature. The maximum amount and the composition of the anthocyanins extracted before deterioration was determined by analysing a sample of the extract daily.

6.2.1.1 Extraction and purification of anthocyanin extract

A portion of the Shiraz grapes (37.3 kg) was pressed in a small water bag press (2 × 20 psi). The pressed skins were extracted with isoamyl alcohol (1 L per kg of grapes) for 24 h at 4 ºC with intermittent agitation. The isoamyl alcohol extracts were decanted from the skins and seeds and concentrated under reduced pressure utilising water as an azeotrope. Cold diethyl ether was added to the concentrated isoamyl alcohol solution until it became cloudy (opaque). The solution was allowed to stand overnight at 4 ºC, during which time a precipitate formed. The precipitates were isolated by centrifugation (5400 *g*) for 20 min at 4 ºC re-solubilised in a minimal amount of acidified methanol (0.1% HCl) and analysed by RP-HPLC (section 6.2.2) to determine the purity of the material. The re-crystallisation steps were repeated 4 to 5 times to obtain the desired anthocyanin composition and required purity. According to RP-HPLC analysis phenolic peaks other than the anthocyanins identified contributed only 5% to the total

peak area at 520 nm. After 5 re-crystallisation steps, no polysaccharides and proteins were likelyto be present.

6.2.1.2 Winemaking experiments

All grapes were crushed and pressed in a research scale water bag press (2 × 20 psi), and the isolated red and white skins and seeds were respectively back added where needed in ratio of the free run juice and skins and seeds produced during pressing of the respective grapes (see Table 6.1). The amount of anthocyanin extract added was calculated according to the predetermined amount that would have been naturally extracted if red skins were present during fermentation (section 6.2.1). There were insufficient grapes to provide the desired amount of white juice needed for the five triplicate treatments. The 18 kg fresh Chardonnay grapes yielded 10 L of free run juice which was mixed with 16.2 L of a 1996 Chardonnay juice (24.9 °Brix, pH 3.81, TA 5.1 g/L) from the Riverland, which had been frozen since pressing. After the juice was divided into the separate fermentation replicates, there was enough juice for only two replicates for the control treatment but all other treatments were prepared in triplicate. The 18 kg of Shiraz and Chardonnay grapes yielded respectively 10.8 and 10 L of free run juice and 5.34 and 6.37 kg of skins and seeds.

Table 6.1 Composition of the different winemaking treatments.

Treatment Code	Treatment Description	Wine Sample Code
W	White free run juice (1.8 L)	W1, W2
WA	White free run juice (1.8 L) with added anthocyanins (1.44 g/L)	WA1, WA2, WA3
WS	White free run juice (1.67 L) with added white skins and seeds (0.635 kg/L)	WS1, WS2, WS3
WSA	White free run juice (1.67 L) with added white skins and seeds (0.635 kg/L) and anthocyanins (1.44 g/L)	WSA1, WSA2, WSA3
WRS	White free run juice (1.8 L) with added red skins and seeds (0.494 kg/L)	WRS1, WRS2, WRS3
RS	Red free run juice (1.8 L) with red skins and seeds (0.494 kg/L)	RS1, RS2, RS3

Individual grapes were picked from the Shiraz bunches for the winemaking process to exclude any damaged or mouldy grapes. A standardised winemaking procedure for small-lot ferments

was adapted to minimise the possible effect of laccase, with 60 mg/L SO_2 (sodium metabisulfite, UNIVAR) added after destemming to minimise oxidation. After crushing, 133 mg/L DAP (di-ammonium hydrogen orthophosphate, AnalaR BDH) was added and the pH of the must was adjusted to 3.6 with tartaric acid. The juice was inoculated with 840 mg/L of *Saccharomyces cerevisiae* EC1118 (Lalvin) as a 10% solution in a 10% aqueous solution of glucose (Iland *et al.* 2000).

All ferments were plunged three times a day to submerge and wet the cap. After 7 days of fermentation at 22 ºC the different ferments were pressed in a small water bag press (2 x 20 psi for 1 minute, the cake redistributed between pressings). The wines were fermented until dry in two-litre glass containers fitted with fermentation locks. Wine treatments were tested for fermentable sugars. All the treatments were dry (less than 2 g/L glucose and fructose) when inoculated with *Oenococcus oeni* DSM 7008 (Viniflora Oenos, Chr. Hansen) for malo-lactic fermentation (MLF). The depletion of malic acid was monitored and the W and WA treatments were re-inoculated with *Oenococcus oeni* Bitec *pro*Vino (Condimenta, Stuttgart, Germany), after a week to re-start MLF in these treatments. The wines took from 3 to 6 weeks to finish MLF and were racked off gross fermentation lees and stored at 2 ºC between 2 and 6 weeks, depending on when they finished MLF. Sodium metabisulfite was added to obtain similar free SO_2 levels (20-30 mg/L) in all the treatments. Copper(ll)sulfate ($CuSO_4$ Analytical UNIVAR reagent) was added after informal assessment, to treatments WA, WSA and WRS at levels between 0.05 mg/L and 0.1 mg/L. The wines were transferred to 375 mL clear bottles without filtration under inert gas (dry ice) cover and sealed with screw cap closures. The bottles were stored from bottling in August 1999 at 18 °C in an upright position to February 2000, when the wines were analysed.

6.2.2 Chemical analysis

The concentration of glucose and fructose was determined using commercial enzymatic assays (Boehringer Mannheim, Germany). Measurement of the pH, and the concentration of ethanol, SO_2, titratable acidity, and acetaldehyde as well as wine colour and phenolic measures were performed according to published methods (Amerine and Ough 1980; Rankine and Pocock

1970; Somers and Evans 1977). The wines were analysed at bottling and after 6 months of aging.

The wines were also analysed by high performance liquid chromatography (HPLC) using a reverse phase PLRP-S (100 Å, particle size 5 μm, 250 x 4.6 mm from Polymer Laboratories Ltd, UK) column equipped with a guard column containing the same material. A binary gradient was used with the mobile phases containing 1.5% (v/v) phosphoric acid (mobile phase A) and acetonitrile with 20% of mobile phase A (mobile phase B). Eluting peaks were monitored at 280 nm and 520 nm. The wine phenolics were eluted with a flow rate of 1 mL/min and a linear gradient from 6% B to 31% B in 73 min and from 31% B to 62% B in 5 min, remaining at 62% B for 8 min and then following a linear gradient back to the starting condition in 4 min. The column was then equilibrated at the starting condition for 10 min before the next injection. Peaks were identified by spectral comparison to published spectra and retention times as well as by comparison to known standards. Gallic acid, catechin, quercetin-glucoside, quercetin-rutinoside, quercetin aglycone, kaempferol aglycone, myricetin aglycone and caffeic acid standards were obtained from Fluka Chemie AG (Buchs, Switzerland), and epicatechin and epicatechingallate from Sigma-Aldrich (Sydney, Australia). Caftaric acid and Vitisin A was a gift from Robert Asenstorfer, The University of Adelaide. The flavan-3-ols (280 nm) and pigmented phenols (520 nm) were respectively quantified, according to the absorbance of a known concentration of catechin (Sigma-Aldrich, Sydney Australia) and malvidin-3-glucoside (Polyphenols AS).

6.2.3 Quantitative sensory descriptive analysis

Informal assessment showed that there were differences among the treatments and that all samples were suitable for descriptive analysis analysis showing no obvious wine faults chemically or sensorially. Descriptive analysis was carried out using ten judges, six of whom had participated in the development of the red wine mouth-feel wheel (Gawel *et. al.*, 2000) and all of whom have participated in wine sensory assessments previously. In the initial training period, the individual panellists, with reference to the developed mouth-feel wheel, generated terms to describe the mouth-feel attributes of the relevant samples. Then, after ten training sessions with evaluation of reference standards and discussion sessions, the terms

were refined by the panel so that by consensus, eleven mouth-feel terms were agreed upon as necessary to describe the mouth-feel sensations of the samples. It was decided to use broader particulate terms than those specified in the mouth-feel wheel. 'Fine' was used in the place of the finer particular terms such as 'talc' and 'dusty', while 'medium' and 'coarse' was representative of respectively 'chalky' and 'sawdust'. The list of terms and the composition of the reference standards are given in Table 6.2.

Table 6.2 Definitions and reference standards for the mouth-feel descriptors.

Attribute	Description	Standard
Fine grain	feeling of minute rough granules/particulate matter against the mouth surfaces through the movement of the wine	-
Medium grain		Bentonite powder (touch) [a]
Coarse grain		Celite S45filter aid (touch) [b]
Silk	texture felt on the mouth surfaces when different surfaces come in contact with each other	Silk material (touch)
Velvet		Velvet material (touch)
Fine emery		Sandpaper grade No. 1000 (touch)
Dry	lack of lubrication or moistness resulting in increased friction between oral surfaces	Aluminium sulfate 0.5 g/L[c]
Grippy	drawing or tightening sensation felt in the mouth, lips and/or cheeks, lack of slip between mouth surfaces resulting in the inability to easily move mouth surfaces across each other	-
Overall astringency	complex of drying, surface texture and dynamic sensations in the mouth	-
Viscosity	degree to which wine resists flow under an applied force in the mouth, amount of force required to manipulate the fluid in the mouth	Carboxymethylcellulose 4 g/L[d]
Bitter		Quinine sulfate 7 mg/L [e]

[a]Bentonite Volclay Pty Ltd., L-9913 powder, mean particle size 7.6 micrometer (min 65% passing)

[b]Celite 545, mean particle size 36.2 micrometer

[c]Aluminium sulfate from BDH Laboratory Supplies, 99.4%, 500 g, AnalaR, prod 10010

[d]Carboxymethylcellulose Sodium Salt from FLUKA, 9004-32-4, 100 g

[e]Quinine Sulfate, BDH Laboratory Supplies, prod 300102A, 25 g

The terms that did not have standards were agreed upon by written definition. Practice ratings in isolated booths were used to define the tasting procedure. The intensity of each of the eleven attributes was rated by making a mark on a line scale of 10 cm with the endpoints labelled "none" and "high", except the attribute viscosity, which was marked as "low, equal to water" and "high". All samples were served in coded black disposable plastic 215 mL cups at 20-22 ºC in isolated booths under sodium lights to mask the colour differences between the samples. Standards were also present in the booths if judges wanted to make use of them at any time during the session. A balanced incomplete block design (Plan 10.10, (Cochran and Cox 1957)) was used, with the 17 samples assessed in duplicate over 11 sessions. Three samples were presented at each session, with no sample presented with another more than once, except for one session where four samples were presented and consequently two pairs of samples were presented together twice. At every session each panel member received two cups with 10 mL of each of the three samples presented. They were requested to put all 10 mL in their mouth and to expectorate after 15 seconds, rinse with water and to wait for one minute, between every tasting. If another tasting of a sample was needed another 10 mL were provided. The particulate terms and viscosity were rated while the samples were in the mouth, while the other terms were rated after expectoration.

6.2.4 Data analysis

Statistical analyses were performed using SAS Institute Inc. JMP 3.1 (Cary, NC, USA). Analysis of variance was performed on each attribute rated by the judges for the effects of sample, judge, and replicate and their interactions. Judge was treated as a random effect in the model. A subsequent ANOVA was conducted for the effect of treatment and fermentation replicate nested within treatment. Principal component (PC) analysis was performed using the correlation matrix of the mean values, with no rotation. A contrast test was also carried out for those attributes where there was a significant difference between the different treatments, comparing each treatment with the control wine W.

6.3 RESULTS

6.3.1 Wine composition

The basic composition of the wines is shown in Table 6.3. The wines were similar in composition, although the RS treatment was high in alcohol compared to the other treatments, which were similar. Because the grapes were harvested towards the end of the season and stored for some time before winemaking, there was some concern about possible *Botrytis cinerea* infection of the grapes and acetaldehyde production during winemaking. However, no laccase was detected in any of the wines, and measured acetaldehyde was lower than 5 mg/L in all wines (data not shown), well below the reported aroma threshold of 50 mg/L (Ferreira *et al.* 2002). However, in preliminary informal sensory evaluation the wines were generally considered to be somewhat aldehydic in aroma, with a slight bruised apple/oxidised aroma. Attempts were made to have similar free sulfur dioxide concentrations for all the treatments before bottling, but this resulted in large differences in bound SO_2 contents while all treatments had low free sulfur dioxide concentrations.

Table 6.3 Basic chemical composition of wines from the different winemaking treatments. Each value is the mean of triplicates (except for Control W, where n=2) with standard deviation (SD) shown in brackets.

Treatment	pH	TA[a] (g/L)	VA[b] (g/L)	Ethanol (% w/v)	Glucose + Fructose (g/L)	Bound SO_2 mg/L)	Total SO_2 (mg/L)
W	3.53 (0.03)	5.9 (0.2)	0.36 (0.04)	13.3 (0.0)	0.3 (0.0)	60 (11)	63 (11)
WA	3.52 (0.02)	5.7 (0.2)	0.38 (0.02)	13.3 (0.1)	0.4 (0.1)	34 (3)	38 (3)
WS	3.49 (0.01)	6.1 (0.1)	0.44 (0.02)	12.9 (0.2)	0.2 (0.0)	25 (3)	29 (3)
WSA	3.47 (0.03)	6.1 (0.0)	0.43 (0.04)	13.0 (0.3)	0.3 (0.0)	20 (4)	25 (5)
WRS	3.48 (0.03)	6.0 (0.2)	0.42 (0.02)	13.0 (0.1)	0.3 (0.0)	3 (1)	6 (1)
RS	3.50 (0.01)	6.0 (0.1)	0.38 (0.04)	13.7 (0.1)	0.3 (0.1)	1 (1)	3 (1)

[a]TA, titratable acidity as tartaric acid.

[b]VA, volatile acidity as acetic acid.

6.3.2 Colour measurements

Spectral evaluations (Somers and Evans 1977) of the wines from the different treatments at bottling and after 6 months aging are shown in Table 6.4. The absorbances at 520 nm were significantly lower for treatments WA and WSA compared to treatments WRS and RS at bottling and 6 months later. This was also true for all the other measurements shown in Table 6.4 even after six months of bottle aging. For OD_{HCl}^{520nm} and OD_{HCl}^{280nm} treatments RS and WRS were not significantly different from each other. Similarly, treatments WA and WSA were also not significantly different from each other, except for the significantly higher colour expression (OD_{HCl}^{520nm}) of treatment WA compared to treatment WSA at bottling which disappeared after six months of aging. The absorbance at 520 nm and wine colour density decreased, while the colour resistant to metabisulfite bleaching and the wine colour hue increased in most of the treatments, over the six months of bottle aging. The decrease in colour density and wine colour at 520 nm was only significant for treatments RS and WRS, over the two time points. The increase in the hue of the wine over time was statistically significant for all treatments, while no significant increase in colour resistant to bisulfite bleaching was seen.

Table 6.4 Spectral evaluations of wines from the different wine treatments at bottling and after 6 months of aging. Each value is the mean of triplicates (except for Control W, where n=2) with standard deviation (SD) shown in brackets.

Treatments	Time (months)	OD^{520nm}	OD^{420nm}	$OD^{520nm}_{SO_2}$	$OD^{520nm}_{CH_3CHO}$	OD^{520nm}_{HCl}	OD^{280nm}_{HCl}	Colour density[1]	Hue[2]
W	0	0.45 (0.04)^{a}	0.54 (0.06)^{a}	0.39 (0.03)^{a}	0.40 (0.03)^{a}	3.38 (0.07)^{a}	18.79 (0.57)^{a}	0.94 (0.10)^{a}	1.32 (0.04)^{a}
W	*6*	*0.38 (0.01)^{a}*	*0.53 (0.01)^{a}*	*0.37 (0.01)^{a}*	*0.38 (0.01)^{a}*	*5.15 (2.29)$^{a, d}$*	*23.58 (7.07)$^{a, b, c}$*	*0.91 (0.03)^{a}*	*1.40 (0.01)^{b}*
WA	0	2.76 (0.04)^{b}	1.74 (0.04)^{b}	1.76 (0.10)$^{b, c}$	3.02 (0.06)^{b}	13.26 (0.56)^{b}	26.83 (0.81)$^{b, c}$	4.50 (0.08)^{b}	0.63 (0.01)^{c}
WA	*6*	*2.61 (0.20)^{b}*	*1.85 (0.11)^{b}*	*1.97 (0.22)^{c}*	*2.72 (0.14)^{b}*	*12.09 (2.22)$^{b, c, e}$*	*29.02 (5.24)^{c}*	*4.46 (0.31)^{b}*	*0.71 (0.01)*
WS	0	0.37 (0.01)^{a}	0.49 (0.01)^{a}	0.36 (0.01)^{a}	0.38 (0.01)^{a}	3.03 (0.00)^{a}	19.46 (1.07)$^{a, b}$	0.86 (0.02)^{a}	1.34 (0.01)^{a}
WS	*6*	*0.40 (0.01)^{a}*	*0.56 (0.02)^{a}*	*0.39 (0.02)^{a}*	*0.41(0.01)^{a}*	*4.44 (1.57)^{a}*	*21.75 (4.13)$^{a, b, c}$*	*0.96 (0.03)^{a}*	*1.41 (0.00)^{b}*
WSA	0	1.67 (0.04)^{c}	1.25 (0.04)^{e}	0.88 (0.03)$^{a, b}$	1.70 (0.06)^{c}	8.18 (0.36)$^{d, e}$	24.24 (0.73)$^{a, b, c}$	2.92 (0.08)^{c}	0.75 (0.01)
WSA	*6*	*1.34 (0.03)^{c}*	*1.19 (0.02)^{c}*	*0.96 (0.02)$^{a, b}$*	*1.46 (0.04)^{c}*	*9.43 (0.42)$^{c, d}$*	*27.57 (2.06)$^{b, c}$*	*2.53 (0.04)^{c}*	*0.89 (0.01)*
WRS	0	5.91 (0.08)^{d}	3.38 (0.02)^{d}	2.39 (0.03)^{e}	5.73 (0.11)^{d}	22.22 (0.79)$^{f, g}$	38.72 (0.58)^{d}	9.29 (0.06)^{d}	0.57 (0.01)^{d}
WRS	*6*	*4.70 (0.17)*	*3.03 (0.07)^{d}*	*2.53 (0.07)$^{b, c}$*	*4.87 (0.08)*	*22.05 (1.57)^{f}*	*41.41 (2.12)^{d}*	*7.73 (0.24)*	*0.65 (0.01)^{c}*
RS	0	7.62 (0.85)	4.48 (0.53)	3.54 (0.57)^{d}	7.34 (0.82)	25.28 (1.87)^{f}	43.90 (1.52)^{d}	12.10 (1.38)	0.59 (0.01)^{d}
RS	*6*	*6.06 (0.57)^{d}*	*3.89 (0.31)*	*3.66 (0.89)^{d}*	*6.05 (0.50)^{d}*	*21.41 (1.05)^{g}*	*40.50 (1.15)^{d}*	*9.95 (0.88)^{d}*	*0.64 (0.01)^{c}*

a, b, c, d, e, f and g represent different groups. Values in the same column with the same superscript letter were not significantly different at the 5% level.

[1]OD520nm + OD420nm

[2]OD420nm/OD520nm

6.3.3 Phenolic composition

The experimental samples were analysed for phenolic composition by RP-HPLC and the concentrations of the different phenolic compounds analysed at zero and six months are shown in Table 6.5. 'Polymeric phenols' and 'polymeric pigments' refer to the late eluting peak at the end of the RP-HPLC run detected at 280 and 520 nm respectively, as defined previously in section 3.3.1. 'Proanthocyanidins' refers to all peaks with a maximum absorbance at 272 to 280 nm other than catechin, epicatechin and epicatechin gallate eluting prior to the polymeric peak. 'Other pigments' refers to all peaks detected at 520 nm other than anthocyanins and vitisin A eluting prior to the polymeric peak.

Only treatments RS and WRS showed significant decreases in monomeric flavanols and anthocyanins and simultaneous increases in proanthocyanidins, polymeric phenols, other pigments and polymeric pigments during the six month aging period. This is in agreement with the findings of other researchers (Cheynier *et al.* 1997a; Mateus *et al.* 2001; Somers 1971). The rest of the treatments followed similar trends.

Table 6.5 The phenolic profile of wines from the different wine treatments at bottling and after 6 months of aging determined by high performance liquid chromatography (HPLC). Each value is the mean of triplicates (except for Control W, where n=2) with standard deviation (SD) shown in brackets.

Treatments	Time (months)	Catechin*	Epicatechin*	Proanthocyanidins*	Polymeric phenols*	Anthocyanins**	Vitisin A**	Other Pigments**	Polymeric pigments**
W	0	9.06 (3.68)^{a}	19.30 (4.23)$^{a, b}$	54.28 (8.54)$^{a, c}$	55.18 (9.35)$^{a, d}$	nd^{a}	nd^{a}	nd^{a}	nd^{a}
W	*6*	*11.38 (2.23)^{a}*	*8.87 (0.57)^{a}*	*125.49 (11.23)$^{a, b, c}$*	*55.97 (0.81)$^{a, d}$*	*nd^{a}*	*nd^{a}*	*nd^{a}*	*nd^{a}*
WA	0	13.16 (2.16)^{a}	21.02 (11.24)$^{a, b}$	42.06 (6.40)^{c}	218.95 (9.37)$^{a, b, d}$	937.45 (15.58)$^{b, c}$	19.29 (3.13)$^{b, c}$	25.65 (5.51)^{a}	38.74 (4.69)$^{a, b}$
WA	*6*	*13.33 (1.48)^{a}*	*11.60 (1.12)^{a}*	*122.83 (5.99)$^{a, c, d}$*	*198.13 (44.47)$^{a, c, d}$*	*527.84 (93.98)^{c}*	*22.5 (3.42)$^{b, c}$*	*70.49 (3.57)^{a}*	*84.98 (30.63)$^{a, b, c}$*
WS	0	35.13 (1.58)^{b}	58.03 (2.10)^{c}	180.35 (6.73)$^{a, e}$	31.24 (5.21)^{d}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
WS	*6*	*37.19 (1.52)^{b}*	*36.86 (2.21)$^{b, d}$*	*188.59 (16.65)$^{a, h}$*	*34.69 (9.11)^{d}*	*nd^{a}*	*nd^{a}*	*nd^{a}*	*nd^{a}*
WSA	0	35.95 (3.17)^{b}	49.08 (4.84)$^{c, d, e}$	109.14 (16.30)$^{a, c, f}$	111.01 (17.12)$^{a, d, e}$	649.19 (37.23)$^{c, d}$	3.55 (0.34)^{a}	11.59 (1.22)^{a}	14.09 (1.64)^{a}
WSA	*6*	*33.58 (8.22)^{b}*	*34.18 (5.30)$^{b, e}$*	*194.90 (16.63)$^{b, d, e, f, g, h}$*	*103.08 (6.03)$^{a, d, f}$*	*395.39 (16.21)$^{a, d}$*	*4.89 (0.60)^{a}*	*40.45 (3.29)^{a}*	*23.99 (2.21)$^{a, b}$*
WRS	0	42.61 (4.59)$^{b, c}$	99.29 (11.50)^{f}	263.36 (25.23)$^{e, i}$	308.70 (9.71)$^{a, g}$	2324.78 (64.87)^{e}	18.18 (0.38)^{c}	90.72 (6.07)^{a}	60.10 (3.09)$^{a, d, e}$
WRS	*6*	*19.85 (3.99)$^{a, b}$*	*81.98 (9.88)^{f}*	*452.91 (121.97)$^{j, k}$*	*344.28 (57.84)$^{b, c, e, f, g}$*	*1576.40 (51.28)^{f}*	*28.30 (9.61)^{b}*	*550.65 (185.02)^{b}*	*137.48 (13.34)$^{b, c, d}$*
RS	0	57.87 (19.12)$^{a, b}$	87.13 (6.06)$^{c, d, e}$	324.34 (46.85)$^{g, i, j}$	719.80 (173.93)^{h}	2481.81 (103.77)^{e}	38.50 (3.38)^{d}	217.00 (39.53)^{a}	157.80 (74.33)$^{c, e}$
RS	*6*	*23.20 (6.75)^{c}*	*47.56 (11.96)^{f}*	*470.59 (80.00)^{k}*	*669.02 (239.40)^{h}*	*1410.46 (538.92)$^{b, f}$*	*42.78 (0.70)^{d}*	*753.66 (274.98)^{b}*	*282.79 (108.89)*

a, b, c, d, e, f, g, h, i, j and k represent different groups. Values in the same column with the same superscript letter were not significantly different at the 5% level.

*Quantitated as catechin equivalents (mg/L); **Quantitated as malvidin-3-*O*-glucoside equivalents (mg/L)

nd = not detected

After six months of bottle aging the main differences between the treatment white free run juice (W) and the treatment with added white skins and seeds (WS) were expected to be due to skin and seed phenolic compounds extracted into the juice during fermentation and their subsequent reactions during aging. Table 6.5 shows that treatment WS had higher concentrations in comparison to treatment W for most phenolics, except polymeric phenols. Wines from treatments RS and WRS had significantly higher concentrations of epicatechin, proanthocyanidins and anthocyanins than all the other treatments, and as expected were not significantly different from each other. Treatment RS was also significantly higher than all other treatments and treatment WRS from WS for the concentration of pigmented and non-pigmented polymeric phenols. When the treatments with added anthocyanins (WA and WSA) are compared with their non-pigmented counterparts (W and WS), the main differences were the presence of monomeric and polymeric pigments, as well as the fact that their concentration of polymeric phenols is at least double the amount (although WSA was not significantly different to WS). Treatment WSA was expected to be comparable to treatment RS, but as Table 6.5 shows, WSA had a lower concentration of anthocyanins and pigments than treatment WA.

6.3.4 Sensory analysis

Informal, preliminary assessments with several experienced tasters confirmed that the samples generated from the experiment had different mouth-feel characteristics and that no sample was considered faulty, so accordingly all samples were included for the descriptive analysis study. An analysis of variance (Appendix I) of the descriptive analysis data for each attribute for the effects of judge, wine, and replicate and their interactions was performed for the 17 wines. This analysis showed that there was a significant judge-by-ferment interaction for the attributes ‘medium grain’ and ‘coarse’, as well as significant judge-by-replicate interactions for the attributes ‘viscosity’, ‘silk’ and ‘fine emery’. Examination of the raw data indicated that one judge had been rating the wines inconsistently and differently to the rest of the panel. Accordingly all scores from this judge were eliminated from the data set. From an ANOVA assessing the treatment effects, all attributes were significantly different across treatment, except for ‘silk’, and there were no significant differences between fermentation replicates,

except for the attribute velvet, where fermentation replicates of all treatments except W were variable. Data for this attribute are not discussed further.

To facilitate the interpretation of the differences and similarities of the samples, principal component analysis (PCA) was applied to the data.

The first two components accounted for 95% of the variance. The first principal component (PC1) differentiated the samples according to the relative intensity of 'bitter', 'dry', 'viscosity', 'astringency', 'grippy', 'medium grain' and 'fine emery' (Figure 6.1). All of these attributes were correlated. The second principal component (PC2) separated the samples on the basis of the 'fine grain' and 'coarse grain' attributes. The treatments without anthocyanins are situated at the far left of the PCA plot. The effect of fermenting the white juice with white skins and seeds was to increase the fine grain attribute compared to the white juice with no additions, but these samples were surprisingly similar in other attributes. The wine made with red grape skins and seeds added to the white juice (WRS) was rated similarly to the red juice with red skins and seeds (RS), being high in the astringent attributes, notably 'astringency', 'fine emery', 'grippy', and 'dry', as well as in 'viscosity'. The addition of anthocyanins to the white juice, whether with or without white skins (WSA and WA), increased the ratings for fine grain and also the attributes associated with PC1, but not sufficiently to make the samples similar to the red wine made conventionally (RS) or the white juice with red skins and seeds (WRS).

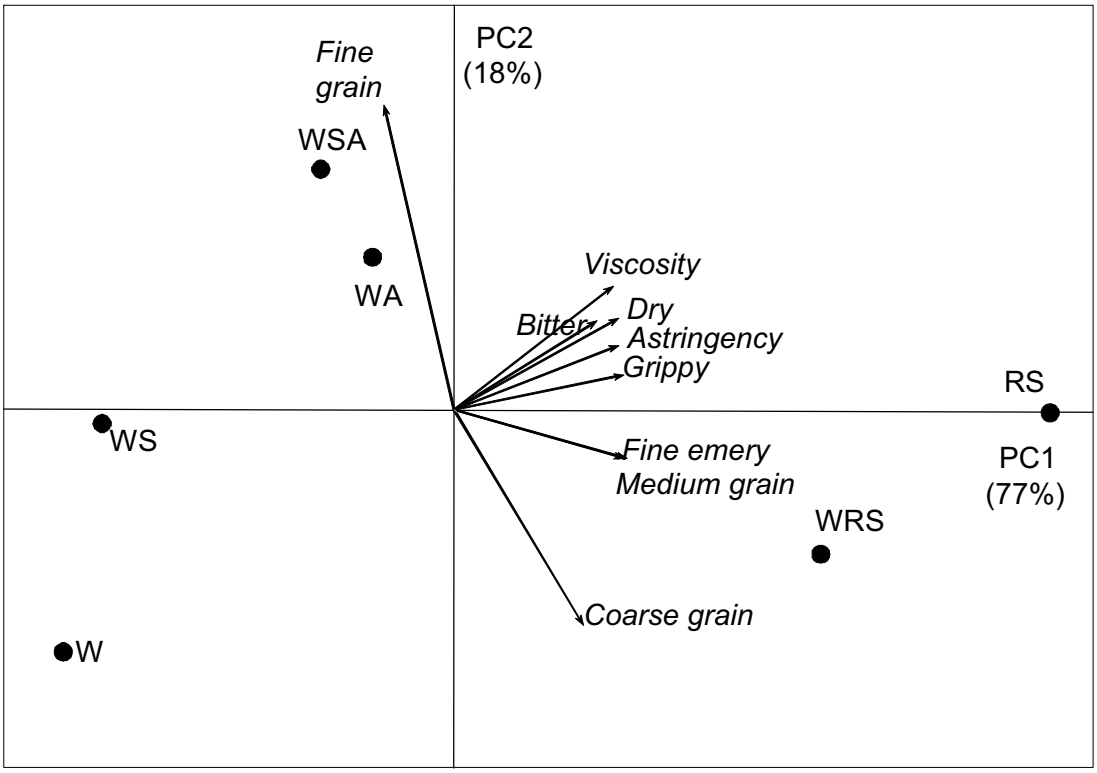


Figure 6.1 Principal component analysis (PCA) of the sensory profile of the six wine treatments. For details of sample codes see Table 6.1.

In order to assess the differences among the samples, Figure 6.2 shows the mean values for the six treatments for four of the attributes, selected as being representative of the sensory dimensions. 'Dry' was selected on the basis that the attributes 'dry', 'astringency', 'grippy', 'fine emery' and 'medium grain' were all correlated with each other ($r \geq 0.52$), 'Dry' was accordingly considered appropriate to represent this astringent set of attributes.

Figure 6.2 shows that the white juice with added anthocyanins and white skins/seeds was significantly higher in 'fine grain' attribute compared to the W treatment, with a trend that WA and WS were also rated relatively highly. The astringency, as represented by the 'dry' attribute, was significantly higher in all treatments compared to the control, W, treatment, while for 'bitterness' and 'viscosity', only the RS treatment was significantly different to the control.

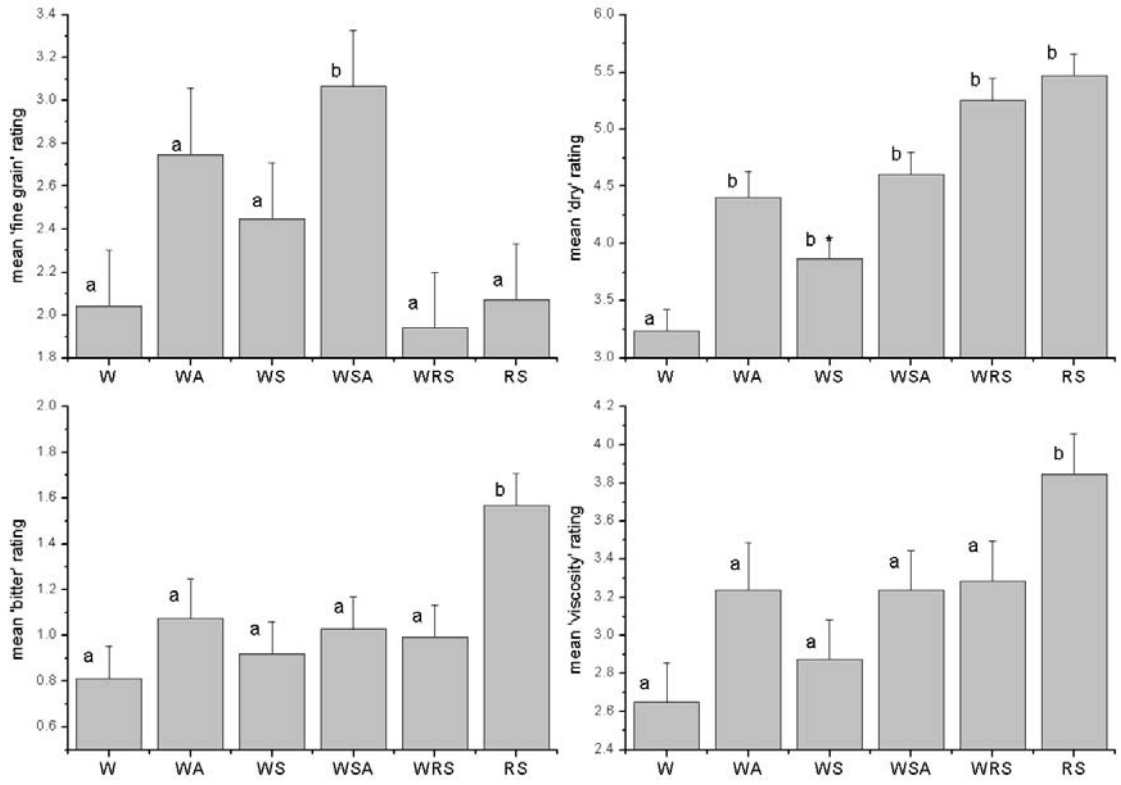


Figure 6.2 Mean sensory ratings for the six wine treatments for four of the statistical significant attributes. For details of sample codes see Table 6.1.

6.3.4.1 Correlation between sensory and chemical data

Table 6.6 shows the correlation matrix for the relationships between the sensory attribute scores and compositional measures for each of the fermentation replicates. The score for bitter correlated with the concentration of most phenolic compounds, but especially with proanthocyanidins (r=0.81, *p*=0.0001, n=17) and polymeric phenols (r=0.72, *p*=0.0010, n=17), which is in agreement with previous reports (Arnold *et al.* 1980; Leach and Noble 1986). In addition, bitterness was correlated with alcohol concentration (r=0.6489, *p*=0.01, n=17), also as previously reported by Noble (1990). The strong correlation between the astringency terms (fine emery, dry, grippy, astringent) are as a result of their close relation to each other and the wine treatments investigated.

Increasing 520 nm absorbance correlated with an increase in pigmented and non-pigmented polymers and an increasing intensity of the attributes correlating with ‘bitter’, ‘dry’, ‘viscosity’, ‘astringency’, ‘grippy’, ‘medium’ and ‘fine emery’ (PC1, Table 6.6). The treatments with added red skins and seeds, treatment WRS and RS, were scored the highest in the attributes ‘dry’, ‘astringency’, ‘viscosity’, ‘grippy’, ‘medium’ and ‘fine emery’ and contained the highest concentration of pigments and monomeric and polymeric phenols.

Table 6.6 Correlation matrix of the mean sensory mouth-feel attributes and the main phenolic compounds (HPLC) differentiated by PC1, across the samples.

	Medium grain	Viscosity	Fine emery	Dry	Grippy	Astringent	Bitter	Epicatechin	Proantho-cyanidins	Polymeric phenols	Antho-cyanins	Vitisin A	Other pigments	Polymeric pigments
Medium grain	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-
Viscosity	0.67**	1.00	-	-	-	-	-	-	-	-	-	-	-	-
Fine emery	0.79***	0.63**	1.00	-	-	-	-	-	-	-	-	-	-	-
Dry	0.80***	0.75***	0.80***	1.00	-	-	-	-	-	-	-	-	-	-
Grippy	0.83***	0.75***	0.92***	0.93***	1.00	-	-	-	-	-	-	-	-	-
Astringent	0.81***	0.69**	0.90***	0.94***	0.95***	1.00	-	-	-	-	-	-	-	-
Bitter	0.58*	0.64**	0.47	0.55*	0.60**	0.54*	1.00	-	-	-	-	-	-	-
Epicatechin	0.76***	0.45	0.64**	0.71**	0.70**	0.71***	0.49*	1.00	-	-	-	-	-	-
Proantho-cyanidins	0.71***	0.56*	0.50*	0.56*	0.62**	0.56*	0.81***	0.81***	1.00	-	-	-	-	-
Polymeric phenols	0.85***	0.72***	0.91***	0.81***	0.89***	0.88***	0.72***	0.71***	0.73***	1.00	-	-	-	-
Anthocyanins	0.85***	0.51*	0.83***	0.79***	0.87***	0.88***	0.54*	0.78***	0.65**	0.85***	1.00	-	-	-
Vitisin A	0.63**	0.58*	0.74***	0.66***	0.71***	0.79***	0.55*	0.42	0.40	0.83***	0.70**	1.00	-	-
Other pigments	0.78***	0.67**	0.86***	0.80***	0.82***	0.89***	0.46	0.61**	0.47	0.90***	0.77***	0.87***	1.00	-
Polymeric pigments	0.80***	0.75***	0.89***	0.80***	0.86***	0.85***	0.68**	0.65**	0.66**	0.98***	0.75***	0.82***	0.92***	1.00

*, **, *** Indicate significance at $p<0.05$, $p<0.01$, $p<0.001$, respectively.

6.4 DISCUSSION

White and red wine exhibit very different mouth-feel attributes with the only substantial difference being the presence of anthocyanins in the skins of red grapes. Anthocyanins have been said to have only a mild indistinct taste (Singleton and Noble 1973), while it has been reported to possibly increase the astringency of seed and skin extracts (Brossaud *et al.* 2001). However, this does not explain the complex mouth-feel differences between red and white wines. Anthocyanins were isolated and added to different white wine ferments to investigate the contribution of anthocyanins and their fermentation and aging products to sensory characteristics of wine.

Colour measurements of the different treatments showed increases in hue and resistance to metabisulfite bleaching over six months of bottle aging. This is due to the formation of new wine pigments. Pigments resistant to sulfite bleaching are not necessarily polymeric, and some polymeric pigments are not resistant to sulfite bleaching. Resistance in pigments to metabisulfite bleaching is due to substitution on the C ring at position 4 which impedes nucleophilic addition of sulfites (Timberlake and Bridle 1967). Conversion of anthocyanins to flavanol-anthocyanin polymers (Salas *et al.* 2004) has no effect on colour or colour intensity, but formation of pyranoanthocyanins (Bakker and Timberlake 1997; Fulcrand *et al.* 1996b, 1998) and ethyl-linked derivatives (Atanasova *et al.* 2002b; Mateus *et al.* 2003) shifts colour from red to orange or purple tints and enhances colour intensity and resistance to pH changes and sulfite bleaching. The decrease in colour intensity indicates the precipitation of coloured polymers, which was observed.

It would have been expected that there should have been larger differences between the control W and treatment WS according to HPLC analysis, but it was apparent that the control contained a higher concentration of phenolics then expected for a free run lightly pressed white wine (Somers and Ziemelis 1985), most likely due to the extended storage at 0 ºC prior to winemaking, allowing partial breakdown and extraction of skin phenolics into the berry. Treatments WSA and RS was expected to be comparable, but as a result of

some of the anthocyanins of the added anthocyanin extract being absorbed by grape skin components, precipitated or otherwise lost during fermentation, this was not true. In a future study it may be better to calculate the addition of anthocyanins according to the concentration of anthocyanins present in the red skins and not the extractable amount.

The similarity between mouthfeel attributes for the treatment pairs that differed only by the addition of white seeds and skins, WA *versus* WSA and W *versus* WS, suggests that the presence of skins and seeds during fermentation may not affect wine mouthfeel substantially. The presence of white skins and seeds mainly increased the rating of 'fine grain', and only slightly increased astringency attributes such as drying (Figure 6.2). This observation is of great interest: there have been few sensory studies involving skin contact of white grapes and in general, points to the key role of anthocyanins or other species from red skins in red wine astringency. It must be noted that the free run juice in the current study contained larger quantities of phenols than usual (Hernanz *et al.* 2007) as a result of damaged skins, and the small effect of skin contact during fermentation may be due to an already astringent white wine control. However, the amount of flavan-3-ols extracted into the white wine made like a red wine (WS) were up to four times more than what was extracted in 24 hr in studies investigating the effect of pre-fermentative maceration (Gomez-Miguez *et al.* 2007; Hernanz *et al.* 2007).

Mostly the treatments in which phenolic composition were not significantly different from each other were also not significantly different according to descriptive sensory analysis. The higher phenolic concentration of treatment RS may be related to a higher concentration of phenols released from the red skins into the free run juice compared to the white skins during pressing. The presence of anthocyanins may also increase retention in the wine as a result of higher solubility of the extracted phenols through increased polarity of anthocyanin associated complexes (Kantz and Singleton 1991; Singleton and Trousdale 1992).

It was clear that the addition of anthocyanins increased astringency, specifically the attributes 'dry' and 'grippy' and to a lesser extent resulted in an increase in 'viscosity'

and ‘fine emery’. This increase in astringency may have been related to low level phenolic impurities, as suggested by model studies where highly purified anthocyanin had no sensory effect (Vidal *et al.* 2004a) or by the formation of new polymeric pigments. Pigments other than monomeric anthocyanins made a 15 to 26% contribution to wine colour after six months of bottle aging in treatments WSA and WA, respectively. The treatments with added anthocyanin (WA and WSA) were rated as being more fine grained compared to the treatments with no added anthocyanin (W and WS). Vidal *et al.* (2004a) determined that polymeric pigments isolated from grape marc contributed to the ‘fine grain’ attribute, while those isolated from wine contributed more to the ‘medium grain’ attribute. The wines investigated in this study will compare more with the pigmented polymers isolated from the grape marc as these were all young wines, whereas the wine pigmented polymer was isolated from a 1999 Shiraz wine. Although Vidal and co-workers (2003a) concluded that anthocyanins did not contribute to astringency, these samples were evaluated in isolation and any possible synergistic effects in the wine would have been excluded.

6.5 CONCLUSION

In this study, a white wine made like a red wine did not exhibit the same mouth-feel sensory attributes of a red wine: it was lower in viscosity, less particulate in nature and lower in astringent intensity. It was found that differences in ratings of mouth-feel attributes could not be related closely to phenolic composition or structure. The reason for this was probably the relatively low concentration of phenolics in the wines investigated; it may have been possible to observe correlations with more phenolic red grapes and wines. Longer bottle-aging of the wines, which would result in development changes through different polymerisation reaction as a result of diverse phenolic compositions (Haslam 1993; Singleton and Trousdale 1992), might also have allowed larger differences in phenolic composition to be observed.

Sensory panellists were able to differentiate between the investigated treatments on the basis of relatively small compositional differences in some cases. This study has shown

that the presence of anthocyanins during fermentation increases the intensity of astringency related terms and can partly explain the differences perceived between the mouth-feel properties of a white and a red wine. The addition of anthocyanins mainly contributed to the fine grain sub-attribute of astringency. Further investigation of young *versus* aged wine treatments to determine the effect of anthocyanin polymerisation products, should be undertaken.

Chapter 7

SUMMARY

Phenolic compounds in wine contribute significantly to the sensory properties of wine, especially those of red wine. They play an important role in red wine colour, bitterness, astringency, as well as a range of other tactile or 'mouth-feel' characteristics. During the storage and aging of red wines, progressive changes of phenolic compounds, initially extracted from grapes, occur. There have been many studies on this complex phenomenon and the mechanisms involved in these transformations as well as on the structures of the resulting. Among these transformations, the decrease of astringency occurring during wine aging has been considered as a result of mainly anthocyanin-flavanol condensation either directly or mediated by aldehydes. The contribution of these polymeric pigments formed during wine aging to the unique properties of red wine is an important question still unanswered.

Singleton and Trousdale (1992) concluded from a model wine study that significant amounts of pigmented polymers could form within a relative short period of time through direct interaction between anthocyanins and flavanols in a model solution. This study was repeated to ascertain the findings and investigate the possibility to up-scale the experiment for the generation of larger quantities of pigmented polymers for sensory studies. Only a small amount of material formed, however during the time of investigation. This confirmed previous findings that direct polymerisation is a slow process. Polymerisation reactions are influenced by a large amount of variables such as pH, temperature, oxygen, sulphur dioxide, phenol composition and concentration and the presence of metabolites such as acetaldehyde. The slow formation of polymeric phenols and its lower solubility in model systems compared to wine complicates the possibility of up-scaling. The resulting low yield of polymers made it necessary to investigate the isolation of polymeric pigments directly from wine.

A preparative fractionation protocol was developed to obtain fractions enriched in different red wine pigment combinations for further investigation. NP chromatography was investigated as a result of success researchers such as Rigaud *et al.* (1993) and Prieur *et al*. (1994) had with the separation of procyanidin oligomers in grape seed tannin. It was quickly determined that

the solvent system used for the procyanidins were not optimal for the separation of pigmented wine phenolics. This is similar to the findings of Kennedy and Waterhouse (2000) for pigmented grape phenolics. Different CCC solvent systems were investigated by thin-layer chromatography (TLC) to develop a mobile phase which could effectively separate the wine pigments. 1-Propanol-acetic acid-water (6:1:2) was found to be the most successful mobile system. This mobile phase was used on a NP-MPLC system for the fractionation of a young (6 months) and older (5 years) Shiraz wine. Fractions of flavonols, flavanols, phenolic acids and anthocyanins with an increasing percentage of non-pigmented and pigmented polymeric phenols were obtained with elution volume. Using the same mobile phase on TLC enabled the separation of the collected polymeric pigments into different bands that were isolated. A HPLC method that could quantify the polymeric pigments present in the collected fractions was developed. The pigmented and non-pigmented polymers, as well as the monomeric anthocyanins and flavanols (monomeric to trimeric), flavonols and hydroxycinnamic acids (Peng *et al.* 2001) were successfully quantified.

The isolated bands were characterised by methods such as acid hydrolysis in the presence of a nucleophile, GPC and LC/ESI-MS. Characterisation of the retentates (wine concentrated through a 3kDa filter) of the 6 month and 5 year old wines indicated large differences in their polymeric phenol composition. The older wine contained polymers of an average DP of 10 compared to the younger wine's DP of 4. Mostly small pigments were identified in the young wine, while anthocyanins connected to larger proanthocyanidin chains of up to nine units were observed in the 5 year old Shiraz. Characterisation of the isolated TLC bands was difficult due to the formation of more complex polymeric phenols with aging resulting in smaller concentrations of a specific compound. Acid-catalysis in the presence of a nucleophile can only characterise the proanthocyanidin part of a polymeric pigment and the presence of ethyl and vinyl linked proanthocyanidins, which increase with aging, complicated matters further as they are resistant to hydrolysis. These problems were augmented by poor ionisation of the isolated TLC bands. The isolation of the different TLC bands did however still aid characterisation of the polymeric pigment fractions obtained from the Shiraz wines. The different bands isolated from the young wine contained combinations of pentameric to dimeric pigments, while those from the 5 year old wine contained pigments with an average DP of at least 11 but possibly up to 32. It is, however, difficult to interpret data from the different characterisation methods due to the varying results obtained.

The question remains what effect these changes in the polymeric profile of a red wine have on the quality of a wine. The quality of red table wine is dependent on pleasing and complex mouth-feel sensations. Any discussion of the quality of red wine by experienced tasters usually includes some reference to astringent and other sensations experienced in the mouth. The lack of a defined and structured vocabulary to describe mouth-feel sensations is in contrast to that of wine aroma and flavour where Noble presented the aroma wheel (Noble *et al.* 1987). It was seen as a necessity to develop a structured and well-defined vocabulary to assist wine tasters to better communicate the mouth-feel characteristics they perceive in red wines. Over 140 wines were tasted to obtain a refined vocabulary, describing the astringent and other mouth-feel sensations elicited by this extensive sample of dry red table wines representing different styles. This study showed that astringency in red wine can manifest itself in many subtle yet complex forms, and that wine tasters can be trained to reproducibly discriminate and rate the intensities of astringent sub-qualities elicited by red wines (Gawel *et al.* 2001). These sub-qualities of astringency may improve the possibility of understanding the relative role of wine phenol composition, and other wine components that affect the interaction between phenols and salivary proteins, and thus the mouth-feel properties of a red wine.

The mouth-feel wheel (Gawel *et al.* 2000) was used in a study to investigate the contribution of anthocyanins and anthocyanin-flavanol reaction products to the mouth-feel properties of red wine. Wines were made from both red and white fruit with and without pomace contact, with and without anthocyanin addition to the white fruit. The white wine made like a red wine did not exhibit the same mouth-feel sensory attributes of a red wine: it was lower in viscosity, less particulate in nature and lower in intensity for the astringency descriptors fine emery, dry and grippy. The presence of anthocyanins during fermentation did also appear to increase the intensity of astringency related terms. The differences in the ratings of the mouth-feel attributes could not be closely related to the phenolic composition of the wines investigated. This was partly due to the fact that the wines contained relatively low concentrations of phenols. Longer bottle-aging of the wines, which would result in development changes through different polymerisation reactions, would also have allowed larger differences in phenolic composition to be observed.

Positive steps have been taken to determine the sensorial contribution of anthocyanins to wine as well as the effect of polymerisation reactions during maturation. Significant changes in the polymeric pigment composition of the Shiraz wine occurred during aging. The next step would be to sensorially and chemically analyse a red wine during aging from bottling to determine the exact contribution of the different polymeric pigments formed.

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APPENDIX

403

***** Analysis of variance *****

Variate: Fine

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Judge2 stratum	8	397.309	49.664	13.65	
Replicat stratum	1	0.045	0.045	0.01	
Judge2.Replicat.*Units* stratum					
Treatmen	5	52.560	10.512	2.89	0.015
Treatmen.F_Rep	11	54.224	4.929	1.35	0.194
Residual	280	1018.846	3.639		
Total	305	1522.984			

* MESSAGE: the following units have large residuals.

Judge2 AP 2.93 s.e. 1.14

Judge2 EJW Replicat 2.00 *units* 11 5.66 s.e. 1.82

***** Tables of means *****

Variate: Fine

Grand mean 2.41

Treatmen	RS	W	WA	WRS	WS	WSA
	2.07	2.04	2.75	1.94	2.45	3.07
rep.	54	36	54	54	54	54

Treatmen	F_Rep	1.00	2.00	3.00
RS		2.19	1.87	2.15
W		2.34	1.74	
WA		2.57	3.37	2.31
WRS		1.76	2.37	1.69
WS		1.77	2.10	3.48
WSA		2.67	3.34	3.19

*** Standard errors of differences of means ***

Table	Treatmen	Treatmen F_Rep	
rep.	unequal	18	
d.f.	280	280	
s.e.d.	0.450X		min.rep
	0.410	0.636	max-min
	0.367		max.rep

(No comparisons in categories where s.e.d. marked with an X)

*** Least significant differences of means ***

Table	Treatmen	Treatmen F_Rep	
rep.	unequal	18	
d.f.	280	280	
l.s.d.	0.885X		min.rep
	0.808	1.252	max-min
	0.723		max.rep

(No comparisons in categories where s.e.d. marked with an X)

408

***** Analysis of variance *****

Variate: Coarse

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Judge2 stratum	8	232.443	29.055	18.28	
Replicat stratum	1	0.255	0.255	0.16	
Judge2.Replicat.*Units* stratum					
Treatmen	5	20.030	4.006	2.52	0.030
Treatmen.F_Rep	11	20.612	1.874	1.18	0.301
Residual	280	445.067	1.590		
Total	305	718.407			

* MESSAGE: the following units have large residuals.

Judge2 PI	1.907	s.e. 0.872		
Judge2 EJW	Replicat 1.00	*units* 15	3.902	s.e. 1.206
Judge2 HG	Replicat 2.00	*units* 17	4.301	s.e. 1.206
Judge2 MG	Replicat 1.00	*units* 11	5.502	s.e. 1.206
Judge2 MG	Replicat 1.00	*units* 13	4.481	s.e. 1.206
Judge2 MG	Replicat 2.00	*units* 2	3.778	s.e. 1.206
Judge2 MG	Replicat 2.00	*units* 7	4.836	s.e. 1.206
Judge2 PI	Replicat 1.00	*units* 13	4.226	s.e. 1.206
Judge2 PI	Replicat 1.00	*units* 17	3.737	s.e. 1.206
Judge2 PI	Replicat 2.00	*units* 3	3.691	s.e. 1.206
Judge2 PI	Replicat 2.00	*units* 14	3.872	s.e. 1.206
Judge2 PI	Replicat 2.00	*units* 15	3.553	s.e. 1.206

***** Tables of means *****

Variate: Coarse

Grand mean 0.567

Treatmen	RS	W	WA	WRS	WS	WSA
	0.919	0.620	0.322	0.860	0.395	0.303
rep.	54	36	54	54	54	54

Treatmen	F_Rep	1.00	2.00	3.00
RS		0.848	0.732	1.177
W		0.472	0.768	
WA		0.431	0.289	0.247
WRS		0.203	1.248	1.130
WS		0.619	0.550	0.016
WSA		0.176	0.167	0.566

*** Standard errors of differences of means ***

Table	Treatmen	Treatmen F_Rep	
rep.	unequal	18	
d.f.	280	280	
s.e.d.	0.2972X		min.rep
	0.2713	0.4203	max-min
	0.2426		max.rep

(No comparisons in categories where s.e.d. marked with an X)

*** Least significant differences of means ***

Table	Treatmen	Treatmen F_Rep	
rep.	unequal	18	
d.f.	280	280	
l.s.d.	0.5850X		min.rep
	0.5340	0.8273	max-min
	0.4776		max.rep

(No comparisons in categories where s.e.d. marked with an X)

413

***** Analysis of variance *****

Variate: Medium

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Judge2 stratum	8	569.604	71.200	20.32	
Replicat stratum	1	0.606	0.606	0.17	
Judge2.Replicat.*Units* stratum					
Treatmen	5	151.878	30.376	8.67	<.001
Treatmen.F_Rep	11	39.841	3.622	1.03	0.417
Residual	280	981.230	3.504		
Total	305	1743.159			

* MESSAGE: the following units have large residuals.

Judge2 EJW	Replicat 1.00	*units* 4	5.75	s.e. 1.79
Judge2 ILF	Replicat 1.00	*units* 15	5.53	s.e. 1.79
Judge2 ILF	Replicat 2.00	*units* 16	5.24	s.e. 1.79

***** Tables of means *****

Variate: Medium

Grand mean 1.90

Treatmen	RS	W	WA	WRS	WS	WSA
	2.92	1.25	1.67	2.72	1.14	1.47
rep.	54	36	54	54	54	54

Treatmen	F_Rep 1.00	2.00	3.00
RS	3.01	2.63	3.11
W	0.92	1.59	
WA	1.79	1.36	1.84
WRS	3.28	2.11	2.77
WS	0.96	1.61	0.83
WSA	2.13	0.99	1.30

*** Standard errors of differences of means ***

Table	Treatmen	Treatmen F_Rep	
rep.	unequal	18	
d.f.	280	280	
s.e.d.	0.441X		min.rep
	0.403	0.624	max-min
	0.360		max.rep

(No comparisons in categories where s.e.d. marked with an X)

*** Least significant differences of means ***

Table	Treatmen	Treatmen F_Rep	
rep.	unequal	18	
d.f.	280	280	
l.s.d.	0.869X		min.rep
	0.793	1.228	max-min
	0.709		max.rep

(No comparisons in categories where s.e.d. marked with an X)

418

***** Analysis of variance *****

Variate: Silk

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Judge2 stratum	8	734.291	91.786	24.43	
Replicat stratum	1	10.918	10.918	2.91	
Judge2.Replicat.*Units* stratum					
Treatmen	5	24.901	4.980	1.33	0.253
Treatmen.F_Rep	11	37.011	3.365	0.90	0.545
Residual	280	1051.835	3.757		
Total	305	1858.956			

* MESSAGE: the following units have large residuals.

Judge2 EJW	Replicat 2.00	*units* 17	6.64	s.e. 1.85
Judge2 PC	Replicat 1.00	*units* 1	6.31	s.e. 1.85
Judge2 PC	Replicat 1.00	*units* 8	7.79	s.e. 1.85
Judge2 PC	Replicat 1.00	*units* 13	5.93	s.e. 1.85
Judge2 PC	Replicat 2.00	*units* 11	7.79	s.e. 1.85
Judge2 RT	Replicat 2.00	*units* 15	5.80	s.e. 1.85

***** Tables of means *****

Variate: Silk

Grand mean 1.61

Treatmen	RS	W	WA	WRS	WS	WSA
	1.56	1.80	1.21	1.31	1.88	1.95
rep.	54	36	54	54	54	54

Treatmen	F_Rep 1.00	2.00	3.00
RS	2.19	0.72	1.78
W	1.74	1.85	
WA	1.51	0.80	1.30
WRS	1.23	1.53	1.19
WS	1.97	1.76	1.90
WSA	1.36	2.34	2.15

*** Standard errors of differences of means ***

Table	Treatmen	Treatmen F_Rep	
rep.	unequal	18	
d.f.	280	280	
s.e.d.	0.457X		min.rep
	0.417	0.646	max-min
	0.373		max.rep

(No comparisons in categories where s.e.d. marked with an X)

*** Least significant differences of means ***

Table	Treatmen	Treatmen F_Rep	
rep.	unequal	18	
d.f.	280	280	
l.s.d.	0.899X		min.rep
	0.821	1.272	max-min
	0.734		max.rep

(No comparisons in categories where s.e.d. marked with an X)

423.....

***** Analysis of variance *****

Variate: Velvet

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Judge2 stratum	8	583.116	72.889	15.80	
Replicat stratum	1	2.204	2.204	0.48	
Judge2.Replicat.*Units* stratum					
Treatmen	5	70.964	14.193	3.08	0.010
Treatmen.F_Rep	11	117.631	10.694	2.32	0.010
Residual	280	1291.345	4.612		
Total	305	2065.260			

* MESSAGE: the following units have large residuals.

Judge2 ILF Replicat 1.00 *units* 17 7.37 s.e. 2.05

***** Tables of means *****

Variate: Velvet

Grand mean 2.57

Treatmen	RS	W	WA	WRS	WS	WSA
	2.62	1.33	2.78	2.70	2.56	3.04
rep.	54	36	54	54	54	54

Treatmen	F_Rep	1.00	2.00	3.00
RS		3.01	2.73	2.13
W		1.37	1.29	.
WA		2.51	2.46	3.35
WRS		4.05	2.24	1.82
WS		1.55	2.83	3.29 --
WSA		3.03	2.28	3.82

*** Standard errors of differences of means ***

Table	Treatmen	Treatmen F_Rep	
rep.	unequal	18	
d.f.	280	280	
s.e.d.	0.506X		min.rep
	0.462	0.716	max-min
	0.413		max.rep

(No comparisons in categories where s.e.d. marked with an X)

*** Least significant differences of means ***

Table	Treatmen	Treatmen F_Rep	
rep.	unequal	18	
d.f.	280	280	
l.s.d.	0.996X		min.rep
	0.910	1.409	max-min
	0.814		max.rep

(No comparisons in categories where s.e.d. marked with an X)

428.....

***** Analysis of variance *****

Variate: Fine_eme

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Judge2 stratum	8	513.087	64.136	17.15	
Replicat stratum	1	1.912	1.912	0.51	
Judge2.Replicat.*Units* stratum					

Treatmen	5	254.395	50.879	13.61	<.001
Treatmen.F_Rep	11	44.345	4.031	1.08	0.379
Residual	280	1047.118	3.740		
Total	305	1860.857			

* MESSAGE: the following units have large residuals.

Judge2 ILF Replicat 2.00 *units* 12 5.47 s.e. 1.85
Judge2 ILF Replicat 2.00 *units* 15 6.61 s.e. 1.85

***** Tables of means *****

Variate: Fine_eme

Grand mean 1.81

Treatmen	RS	W	WA	WRS	WS	WSA
	3.02	1.02	1.66	2.95	0.72	1.23
rep.	54	36	54	54	54	54

Treatmen	F_Rep	1.00	2.00	3.00
RS		2.13	3.28	3.63
W		0.96	1.08	
WA		1.79	2.03	1.15
WRS		2.65	2.64	3.55
WS		0.95	0.80	0.42
WSA		1.02	1.49	1.18

*** Standard errors of differences of means ***

Table	Treatmen	Treatmen F_Rep	
rep.	unequal	18	
d.f.	280	280	
s.e.d.	0.456X		min.rep
	0.416	0.645	max-min
	0.372		max.rep

(No comparisons in categories where s.e.d. marked with an X)

*** Least significant differences of means ***

Table	Treatmen	Treatmen F_Rep	
rep.	unequal	18	
d.f.	280	280	
l.s.d.	0.897X		min.rep
	0.819	1.269	max-min
	0.733		max.rep

(No comparisons in categories where s.e.d. marked with an X)

433.....

***** Analysis of variance *****

Variate: Viscosit

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Judge2 stratum	8	256.843	32.105	11.87	
Replicat stratum	1	22.763	22.763	8.42	
Judge2.Replicat.*Units* stratum					
Treatmen	5	39.801	7.960	2.94	0.013
Treatmen.F_Rep	11	32.252	2.932	1.08	0.374
Residual	280	757.130	2.704		
Total	305	1108.790			

```
* MESSAGE: the following units have large residuals.

Judge2 EJW  Replicat 2.00  *units* 4          5.325   s.e. 1.573


***** Tables of means *****

Variate: Viscosit

Grand mean  3.219

 Treatmen        RS         W        WA       WRS        WS       WSA
            3.847     2.648     3.239     3.284     2.872     3.235
     rep.       54        36        54        54        54        54

 Treatmen    F_Rep      1.00      2.00      3.00
       RS                3.687     3.542     4.312
        W                2.581     2.716
       WA                3.552     3.171     2.994
      WRS                3.285     2.818     3.750
       WS                2.882     2.557     3.178
      WSA                3.852     3.137     2.717


*** Standard errors of differences of means ***

Table             Treatmen    Treatmen
                                 F_Rep
rep.                unequal         18
d.f.                    280        280
s.e.d.               0.3876X           min.rep
                     0.3538       0.5481  max-min
                     0.3165           max.rep

(No comparisons in categories where s.e.d. marked with an X)

*** Least significant differences of means ***

Table             Treatmen    Treatmen
                                 F_Rep
rep.                unequal         18
d.f.                    280        280
l.s.d.               0.7630X           min.rep
                     0.6965       1.0790  max-min
                     0.6230           max.rep

(No comparisons in categories where s.e.d. marked with an X)

438.....................................................................

***** Analysis of variance *****

Variate: Astringe

Source of variation      d.f.       s.s.       m.s.    v.r.  F pr.

Judge2 stratum              8    147.584     18.448    7.94

Replicat stratum            1      0.065      0.065    0.03

Judge2.Replicat.*Units* stratum
Treatmen                    5    311.492     62.298   26.82  <.001
Treatmen.F_Rep             11     25.985      2.362    1.02  0.431
Residual                  280    650.416      2.323

Total                     305   1135.542


* MESSAGE: the following units have large residuals.

Judge2 HG   Replicat 1.00  *units* 2           4.780   s.e. 1.458
Judge2 HG   Replicat 1.00  *units* 16         -4.570   s.e. 1.458
```

***** Tables of means *****

Variate: Astringe

Grand mean 4.445

Treatmen	RS	W	WA	WRS	WS	WSA
	5.592	2.878	4.493	5.597	3.229	4.358
rep.	54	36	54	54	54	54

Treatmen	F_Rep	1.00	2.00	3.00
RS		4.894	5.867	6.015
W		2.598	3.157	
WA		4.405	4.797	4.277
WRS		5.683	5.344	5.763
WS		2.828	3.390	3.470
WSA		4.347	4.529	4.198

*** Standard errors of differences of means ***

Table	Treatmen	Treatmen F_Rep	
rep.	unequal	18	
d.f.	280	280	
s.e.d.	0.3592X		min.rep
	0.3279	0.5080	max-min
	0.2933		max.rep

(No comparisons in categories where s.e.d. marked with an X)

*** Least significant differences of means ***

Table	Treatmen	Treatmen F_Rep	
rep.	unequal	18	
d.f.	280	280	
l.s.d.	0.7071X		min.rep
	0.6455	1.0001	max-min
	0.5774		max.rep

(No comparisons in categories where s.e.d. marked with an X)

443.....

***** Analysis of variance *****

Variate: Dry

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Judge2 stratum	8	141.799	17.725	8.72	
Replicat stratum	1	0.004	0.004	0.00	
Judge2.Replicat.*Units* stratum					
Treatmen	5	160.829	32.166	15.83	<.001
Treatmen.F_Rep	11	35.805	3.255	1.60	0.098
Residual	280	568.961	2.032		
Total	305	907.398			

* MESSAGE: the following units have large residuals.

Judge2 ILF Replicat 2.00 *units* 15 4.055 s.e. 1.364

***** Tables of means *****

Variate: Dry

Grand mean 4.544

Treatmen	RS	W	WA	WRS	WS	WSA

```
            5.465    3.233    4.401    5.253    3.868    4.605
rep.           54       36       54       54       54       54

Treatmen    F_Rep     1.00     2.00     3.00
      RS              4.813    5.674    5.909
       W              2.805    3.662
      WA              4.380    4.272    4.553
     WRS              5.171    5.012    5.576
      WS              3.253    4.076    4.274
     WSA              4.932    4.424    4.458

*** Standard errors of differences of means ***

Table             Treatmen    Treatmen
                                 F_Rep
rep.                unequal         18
d.f.                    280        280
s.e.d.               0.3360X            min.rep
                     0.3067     0.4752  max-min
                     0.2743             max.rep

(No comparisons in categories where s.e.d. marked with an X)

*** Least significant differences of means ***

Table             Treatmen    Treatmen
                                 F_Rep
rep.                unequal         18
d.f.                    280        280
l.s.d.               0.6614X            min.rep
                     0.6038     0.9353  max-min
                     0.5400             max.rep

(No comparisons in categories where s.e.d. marked with an X)

448.....................................................................

***** Analysis of variance *****

Variate: Grippy

Source of variation      d.f.       s.s.       m.s.    v.r.  F pr.

Judge2 stratum              8    775.236     96.904   40.53

Replicat stratum            1      2.356      2.356    0.99

Judge2.Replicat.*Units* stratum
Treatmen                    5    241.603     48.321   20.21  <.001
Treatmen.F_Rep             11     28.019      2.547    1.07  0.389
Residual                  280    669.399      2.391

Total                     305   1716.613

* MESSAGE: the following units have large residuals.

Judge2 AP          3.696   s.e. 1.592

Judge2 AP   Replicat 1.00  *units* 7          -4.646   s.e. 1.479


***** Tables of means *****

Variate: Grippy

Grand mean  3.779

Treatmen        RS        W       WA      WRS       WS      WSA
           5.007     2.516    3.549    4.739    2.830    3.614
     rep.     54        36       54       54       54       54

Treatmen    F_Rep     1.00     2.00     3.00
      RS              4.587    5.190    5.243
```

```
         W          2.365    2.667
        WA          3.661    3.504    3.484
       WRS          4.498    4.197    5.523
        WS          2.555    2.943    2.991
       WSA          3.587    3.896    3.359
```

*** Standard errors of differences of means ***

```
Table              Treatmen    Treatmen
                                 F_Rep
rep.                unequal        18
d.f.                    280       280
s.e.d.               0.3644X             min.rep
                     0.3327      0.5154  max-min
                     0.2976              max.rep
```

(No comparisons in categories where s.e.d. marked with an X)

*** Least significant differences of means ***

```
Table              Treatmen    Treatmen
                                 F_Rep
rep.                unequal        18
d.f.                    280       280
l.s.d.               0.7174X             min.rep
                     0.6549      1.0145  max-min
                     0.5857              max.rep
```

(No comparisons in categories where s.e.d. marked with an X)

453.....

***** Analysis of variance *****

Variate: Bitter

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Judge2 stratum	8	292.808	36.601	32.20	
Replicat stratum	1	2.498	2.498	2.20	
Judge2.Replicat.*Units* stratum					
Treatmen	5	17.374	3.475	3.06	0.011
Treatmen.F_Rep	11	6.311	0.574	0.50	0.900
Residual	280	318.258	1.137		
Total	305	637.248			

* MESSAGE: the following units have large residuals.

```
Judge2 EJW  Replicat 1.00  *units* 3          3.255   s.e. 1.020
Judge2 EJW  Replicat 1.00  *units* 15         7.170   s.e. 1.020
Judge2 ILF  Replicat 1.00  *units* 13         4.286   s.e. 1.020
Judge2 RT   Replicat 1.00  *units* 6          3.262   s.e. 1.020
```

***** Tables of means *****

Variate: Bitter

Grand mean 1.081

```
Treatmen        RS        W       WA      WRS       WS      WSA
           1.568     0.813    1.076    0.993    0.919    1.029
    rep.      54        36       54       54       54       54

Treatmen  F_Rep      1.00     2.00     3.00
      RS            1.862    1.389    1.453
       W            0.769    0.857
      WA            1.317    1.047    0.866
     WRS            0.957    1.073    0.949
      WS            0.694    1.058    1.004
```

WSA 1.080 1.108 0.899

*** Standard errors of differences of means ***

Table	Treatmen	Treatmen F_Rep	
rep.	unequal	18	
d.f.	280	280	
s.e.d.	0.2513X		min.rep
	0.2294	0.3554	max-min
	0.2052		max.rep

(No comparisons in categories where s.e.d. marked with an X)

*** Least significant differences of means ***

Table	Treatmen	Treatmen F_Rep	
rep.	unequal	18	
d.f.	280	280	
l.s.d.	0.4947X		min.rep
	0.4516	0.6995	max-min
	0.4039		max.rep

(No comparisons in categories where s.e.d. marked with an X)