THE COLOUR OF RED WINE

MARIA JOSEPHINE BIRSE

THE UNIVERSITY OF ADELAIDE

School of Agriculture, Food & Wine

Faculty of Sciences

**A THESIS SUBMITTED FOR THE FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY**

APRIL 2007

Abstract

The behaviour of pigments in red wine, namely anthocyanins and anthocyanin-derived pigments, was investigated at natural wine pH, at low pH and after addition of SO_2, namely SO_2 bleaching. An examination of current literature demonstrated absences in wine pigment research. Firstly, few researchers have published the colour properties of a particular wine pigment at different pH values and post-SO_2 bleaching. This was demonstrated using the CIELab colours of two individual anthocyanin-derived wine pigments (4-vinylcatechol and 4-vinylsyringol adducts to malvidin 3-glucoside), and an anthocyanin, malvidin 3-glucoside. The colours of the anthocyanin-derived pigments and their resistance to pH change and SO_2 bleaching were compared to malvidin 3-glucoside which was affected by media.

Generally, in the literature, wine pigments are characterized as individual components. But many pigments contribute to wine colour. So, two novel methods were created and demonstrated using red wines: Shiraz wines from four regions in Australia, and Cabernet Sauvignon wines made using two different strains, Saccharomyces cerevisiae (SC) or Saccharomyces bayanus (SB). The first method can be used to determine the CIELab colour of chromatographically separated wine pigments and allows their colours to be re-created, regardless of their identity. Thus objective measurement of pigment colour at its natural concentration in wine is now possible.

An additional method, the "post-column adjustment method" to pH-adjust and SO_2 bleach HPLC-separated wine pigments was created. The concentration and colour of HPLC-separated wine pigments at low pH, at wine pH and post-SO_2 bleaching can be measured. The method has highlighted the importance of the pH value when quantifying a wine pigment. For example, from low pH to wine pH, the apparent anthocyanin and pigmented polymer concentration was reduced, but the Vitisin A concentration was unchanged. SO_2 bleaching resulted in negligible anthocyanin concentration and a further reduction in pigmented polymer concentration, with Vitisin A unaffected. Relative quantities of wine pigments in both SC and SB Cabernet Sauvignon wines were not affected by pH change or SO_2 bleaching.

Also, using the Shiraz wines and Cabernet Sauvignon red wines, existing and improved colour measurement techniques were discussed. For the Australian Shiraz wines, grape origin was found to influence red wine colour, CIELab values provided enhanced colour measurements, and high wine colour (at natural wine pH) cannot be

attributed to individual monomeric anthocyanins (measured by HPLC analysis at low pH). Vitisin A was not responsible for differences in wine colour. SO_2-stable wine colour was related to regional differences. The percentage of SO_2 non-bleachable pigments was independent of wine region. Chemical index (ii) values indicated that the colour at 520 nm was attributable to pH-dependent wine pigments. Vitisin A and pigmented polymer concentrations correlated well with SO_2-stable wine colour. Pigmented polymer concentration may be the driving force behind wine colour density. Copigmentation was of no importance in the young red wine samples studied.

With the Cabernet Sauvignon red wines, the yeast strains used for fermentation affected wine colour and SO_2-stable wine colour. The change in wine colour density was not related to change in total red pigment colour or anthocyanin concentration. Pigmented polymer concentration, SO_2-stable wine colour and the percentage of SO_2 non-bleachable pigments were consistently higher in the SB wines.

The pH value was important when determining the colour of a wine or pigment. At low pH, the SC wines were more coloured than the SB wines. However, at real wine pH, the converse was true. For both wines, at low pH, the anthocyanin concentration was greater than the pigmented polymer concentration, indicating the importance of anthocyanins to wine colour only at low pH. But, at wine pH, the apparent anthocyanin concentration was much lower in both wines (for example, malvidin 3-glucoside provided more colour at low pH than at wine pH) than the apparent pigmented polymer concentration. Therefore, at wine pH, anthocyanins were less important to wine colour than pigmented polymers.

The concentrations of Vitisin A were similar in all three media, but colour losses were observed at wine pH and post-SO_2 bleaching. SB Vitisin A was more coloured. At low pH and at wine pH, Vitisin A was more coloured than malvidin 3-glucoside in both wines, even though the apparent Vitisin A concentration was lower. Differences in the colours of the SC and SB pigmented polymers peaks were observed at low pH, at wine pH and following SO_2 bleaching. The SB pigmented polymers were darker and more colourful, exhibited more colour absorbance and a slight bathochromic shift of λ_{max} value. From low pH to wine pH and following SO_2 bleaching, pigmented polymers become lighter, whilst retaining orange-red hues.

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A	Absorbance value
a* value	CIELab colour value. On the hue-circle in the horizontal plane, a* is a measure of redness and –a* of greenness.
A^+	Ionized anthocyanin
a.u.	Absorbance units
b* value	CIELab colour value. On the hue-circle in the horizontal plane, b* is a measure of yellowness and – b* of blueness.
AWRI	The Australian Wine Research Institute
c	Concentration
CAV	Colour activity value
C* value	Chroma derived from a* and b* values. Equivalent to $(a^{*2} + b^{*2})^{\frac{1}{2}}$
CIELab	Combination of CIE and L*, a*, b* values
CIE	Commission Internationale d'Eclairage
DAD	Diode array detector
DAP	Diammonium phosphate
ΔE* value	ΔE* is the Euclidean distance between the two points in three-dimensional space and is used to compare two colours (represented by the subscripts 0 and 1) : $\Delta E^* = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{\frac{1}{2}}$ where $\Delta L^* = L_0 - L_1$; $\Delta a^* = a_0 - a_1$; $\Delta b^* = b_0 - b_1$.
ESI-MS	Electrospray ionization mass spectrometry
F	Flavanol
H* value	Hue value derived from a* and b* values. Equivalent to $\tan^{-1} (a^*/b^*)$
l	Length
L* value	CIELab colour value. L* is a measure of lightness from completely dark (0) to completely white (100).
MLF	Malolactic fermentation
NWGIC	National Wine and Grape Industry Centre
PCC	Percentage colour contribution
RP	Reversed phase
s value	Saturation derived from a* and b* values. Equivalent to (C^*/L^*)
SB	*Saccharomyces bayanus* yeast strain
SC	*Saccharomyces cerevisiae* yeast strain
SO_2	Sulfur dioxide
SO_2-stable	"Stable" or not bleachable in the presence of the sulfite ion
^s_wpH value	Apparent pH values where *s* and *w* are the organic solvent system and calibration of the pH electrode using aqueous standards respectively.
WCD	Wine colour density
WCH	Wine colour hue

Acknowledgements

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, contains 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 being made available in the University of Adelaide Library.

The author acknowledges that copyright of published works cited within this thesis resides with the copyright holder of those works.

Maria Josephine Birse   Date

Publications

Refereed journals:

1. "Structures and colour properties of new red wine pigments": Hakansson, A.E., Pardon, K., Hayasaka, Y., <u>de Sa, M.,</u> and Herderich, M. (2003) Tetrahedron Lett. 44: 4887-4891. (Written under maiden name).

2. "Grape and wine tannins - an overview on current research, emerging applications, and future challenges": Herderich, M.J., <u>Birse, M.</u>, Dambergs, R.G., Holt, H., Iland, P., Lattey, K.A. and Smith, P.A., Advances in Tannin and Tannin Management, Proceedings of an ASVO seminar held at the Adelaide Convention Centre on 6 October 2005.

3. "Are the colour properties and pigment profiles of wines affected by the use of the different yeast strains; *Saccharomyces cerevisiae and Saccharomyces bayanus*?" Hayasaka, Y., <u>Birse, M.</u>, Eglinton, J., and Herderich, M. *prep.*

4. "The effect of grape-derived tannin on red wine sensory properties and phenolic composition" Parker, M., Smith, P.A., <u>Birse, M.</u>, Francis, I.F., Kwiatkowski, M.J., Lattey, K.A., Liebich, B., Herderich, M. (2007) *Aust. J. Grape and Wine Res., 13 (1): 30-37.*

Other publications:

1. "Anthocyanins, anthocyanin-derived pigments and the colour of red wine" Birse, M., Pollnitz, A., Kwiatkowski, M., Gockowiak, H., Parker, M., Eglinton, J., and Herderich, M. (2005). Proceedings of the Twelfth Australian Wine Industry Technical Conference held at Melbourne Convention Centre, Melbourne, Victoria on 24-29 July 2004, 303 – 304.

2. "CIELab colour values: enhanced wine colour measurement for use by the wine industry and in research applications" Birse, M., Pollnitz, A., and Herderich, M. (2005). Proceedings of the Twelfth Australian Wine Industry Technical Conference held at Melbourne Convention Centre, Melbourne, Victoria on 24-29 July 2004, 312 – 313.

3. "The effects on red wine of pre- and post-fermentation additions of grape-derived tannin" Parker, M., Smith, P., Birse, M., Kwiatkowski, M., Gockowiak, H., Lattey, K., Liebich, B., Francis, I.L., and Herderich, M. (2005). Proceedings of the Twelfth Australian Wine Industry Technical Conference held at Melbourne Convention Centre, Melbourne, Victoria on 24-29 July 2004, 310 – 311.

4. "Yeast and red wine colour: impact beyond fermentation" Hayasaka, Y., Birse, M., Eglinton, J., and Herderich, M. (2005). Proceedings of the Twelfth Australian Wine Industry Technical Conference held at Melbourne Convention Centre, Melbourne, Victoria on 24-29 July 2004, 317 – 318.

5. Article entitled Quality advances for grape and wine industry, Aust. N.Z. Wine Ind. J. (March/April 2006, Volume 21, Issue 3, 107 - 108) detailing my research outcomes.

Workshops:

"An introduction to colour analysis of red wine, 2006" – A half day workshop written by Maria Birse, edited by Ashley Ratcliffe and desktop published by River Murray Training Pty Ltd, Adelaide. Presentations written on behalf of the Cooperative Research Centre for Viticulture. Workshop includes four one hour seminars introducing and discussing colour, pigments in red wine, HPLC analysis and post column treatment of HPLC-separated data.

Software:

"Colour Burst, 2006" - HPLC-CIELab software to determine the colour of HPLC-separated wine pigments developed by Maria Birse and software written by Future Solutions Media, Adelaide. Software was written on behalf of the Cooperative Research Centre for Viticulture.

Chapter 1: Literature review

1.1.   Introduction

One of the primary sensory perceptions of a food or beverage is its colour. An initial judgement about a wine's quality is influenced by its appearance, notably its colour (Lawless and Heymann 1998). Beyond the simple dichotomy of "red" or "white", quantitative and qualitative aspects of colour serve as a clue to the variety (Mazza *et al.* 1999; Chicon *et al.* 2002; González-Neves *et al.* 2004b), viticultural practice (Keller *et al.* 1999; Mateus *et al.* 2002; Gambelli and Santaroni 2004; Van Leeuwen *et al.* 2004; Gonzalez-Neves *et al.* 2004a; Rolley 2004a; González-Neves *et al.* 2004b; Revilla *et al.* 2005), winemaking style (Revilla and González-SanJosé 2001b; Bosso *et al.* 2002; Ducruet and Glories 2002; Lurton 2002; Eglinton and Henschke 2003; Eglinton *et al.* 2003; Mollah and Reynolds 2003; Okubo *et al.* 2003; Bautista-Ortín *et al.* 2005; Mazza and Ford 2005; Garcia-Puente Rivas *et al.* 2006) or age of a wine (Somers 1971; Somers and Evans 1977; Ribereau-Gayon *et al.* 1983; Heredia *et al.* 1997; Revilla and González-SanJosé 2001b; Arnous *et al.* 2002; Gómez-Plaza *et al.* 2002; Crespy 2003; Alamo-Sanza *et al.* 2004; Esparza *et al.* 2004; Gonzalez-Martinez *et al.* 2004; Vivas *et al.* 2004; Hart and Kleinig 2005; Alcalde-Eon *et al.* 2006; Esparza *et al.* 2006; Garcia-Puente Rivas *et al.* 2006; Recamales *et al.* 2006). Thus, it is considered that colour is correlated with aroma, taste and textural qualities of a wine (Clydesdale 1984; Bakker *et al.* 1998; Parr *et al.* 2003; Vidal *et al.* 2004a; Bautista-Ortín *et al.* 2005; Rodríguez *et al.* 2005). In addition, colour can serve as an indicator of perceived quality (Somers and Evans 1974; Somers 1975; Ribereau-Gayon *et al.* 1983; Cruz-Ortiz *et al.* 1995; Marais *et al.* 2001; Perez-Prieto *et al.* 2003a; Rolley 2004a). Whilst colour provides information regarding the characteristics of a wine, it can potentially have a disruptive effect. For example, colour can influence the perception of expected odour and taste. Brochet stated that influenced by wine colour, a wine taster can have a preconceived idea about the profile of a wine (Brochet 1999a; Brochet 1999b). The wine colour can bias a taster to smell or taste something

that is not present in a wine. For example, Pangborn and co-workers added food colouring to white wine and wine tasters assessed sweetness based on the wine colour (Pangborn *et al.* 1963). The colour was found to influence the judgements made by the participants.

In grapes and young red wine, the red colour mainly results from the pigments that are present, the anthocyanins (Ribereau-Gayon 1982; Mayen *et al.* 1994; Mazza 1995; Mazza *et al.* 1999; Garcia-Beneytez *et al.* 2002; Mateus *et al.* 2002a; Mateus *et al.* 2002b; García-Beneytez *et al.* 2003; Wang *et al.* 2003a; Wang *et al.* 2003b; Bautista-Ortín *et al.* 2004; Corona *et al.* 2004; Hermosin-Gutierrez and Garcia-Romero 2004; Kammerer *et al.* 2004; Muòoz-Espada 2004; Núnez *et al.* 2004; Gonzalez-Neves *et al.* 2004a; González-Neves *et al.* 2004b; Bautista-Ortín *et al.* 2005; Hermosín Gutiérrez *et al.* 2005; Lorenzo *et al.* 2005; Romero-Cascales *et al.* 2005; Pérez-Magarino and González-San José 2006; Vian *et al.* 2006). During maturation and ageing of wine, anthocyanins undergo changes, becoming more complex and higher in molecular weight than the anthocyanin monomers from which they were formed (Somers 1971; Haslam 1980; Ribereau-Gayon *et al.* 1983; Mayen *et al.* 1994; Jones *et al.* 1999; Rivas-Gonzalo 1999; Escribano-Bailón *et al.* 2001; Morais *et al.* 2002; Atanasova *et al.* 2002a; Harbertson *et al.* 2003; Harbertson *et al.* 2003; Wang *et al.* 2003; Monagas *et al.* 2005b; Alcalde-Eon *et al.* 2006; Boido *et al.* 2006; Garcia-Puente Rivas *et al.* 2006). Vineyard management can alter the proportion of pigments in red grapes; whilst, winemaking techniques and wine ageing influence the development of subsequent pigments in the wine (Nagel and Wulf 1979; Sims and Morris 1984; Cameira-dos-Santos *et al.* 1996; Bakker *et al.* 1998; Romero and Bakker 2000b; Revilla and González-SanJosé 2001; Arnous *et al.* 2002; Gómez-Plaza *et al.* 2002; Tsanova-Savova *et al.* 2002; Zafrilla *et al.* 2003). The proportion of anthocyanins and other pigments will enhance the colour of a wine and may affect its sensory perception (Es-Safi *et al.* 2003b; Vidal *et al.* 2004a).

In current literature, there is a wealth of research regarding the measurement of wine colour and characterization of wine pigments. But in some cases, the

information does not reflect real wine conditions (Bakker *et al.* 1993; Dallas *et al.* 1996a; Francia-Aricha *et al.* 1997; Mirabel *et al.* 1999; Remy *et al.* 2000; Romero and Bakker 2000a; Es-Safi *et al.* 2000c; Lopez-Toledano 2002; Vidal *et al.* 2002; Mateus *et al.* 2003; Pissarra *et al.* 2003; Vidal *et al.* 2003; Pissarra *et al.* 2003a; Clark 2004; Eglinton *et al.* 2004). Pigments have been isolated from wine or synthesized and their colour properties discussed at concentrations higher than would be expected in red wine (Fulcrand *et al.* 1998a; Mateus *et al.* 2002; Atanasova *et al.* 2002b; de Freitas *et al.* 2004; Salas *et al.* 2004; Salas *et al.* 2004b; Salas *et al.* 2005; Oliveira *et al.* 2006), or there is a tentative implication that certain pigments are important to wine colour (Fulcrand *et al.* 1998a; Vivar-Quintana *et al.* 2002; Mateus *et al.* 2002a; Mateus *et al.* 2002c; Lee *et al.* 2004b; Mateus *et al.* 2005a). Also, some researchers have modified existing spectroscopic techniques to measure wine colour by measuring the absorbance values at three or four wavelengths (Juarez *et al.* 1997; Pérez-Caballero *et al.* 2003).

In this thesis, current literature will be reviewed with regard to red wine colour and the pigments that contribute to the colour. Techniques for wine colour measurements will be discussed and improved techniques to characterise wine pigments will be introduced and demonstrated. The results of this thesis will be highly applicable to a researcher, a viticulturist or a winemaker as the methods and information can be applied to various viticultural and winemaking scenerios in order to achieve the optimum red wine colour.

1.2. Measurement techniques used to characterize red wine colour

Wine is one of the oldest alcoholic beverages and has been produced using traditional viticultural and winemaking methods passed down through many generations. Consequently, the characteristics of a wine have been improved mostly by trial and error. In particular, early research regarding red wine colour chemistry has relied mainly on speculation. With the development of new analytical techniques such as UV-Vis spectrophotometry, HPLC analysis,

mass spectrometry, NMR spectroscopy and recently countercurrent coil chromatography (CCC) (summarized by Zimman and his co-authors), the application of science is increasing our knowledge of wine composition (Zimman *et al.* 2004). The complexity of the reactions that occur during the various stages of wine production and ageing are being uncovered daily and published accordingly. This has led to the identification and isolation of new compounds found in wine, particularly pigments in red wine.

UV-Vis spectrophotometry is used to measure the colour of grapes and intact red wines, but does not provide detailed information regarding individual pigments in a red wine. The introduction of high performance liquid chromatography (HPLC) using reversed phase columns in the late 1970s has made analysis of pigment composition plausible. Importantly, the application of HPLC-DAD systems, where a diode array detector (DAD) is attached to the HPLC column, enables the UV-visible spectra of HPLC-separated wine pigments to be visualized and compared. Mass spectrometry (MS) techniques such as fast atom bombardment mass spectrometry (FAB-MS), electrospray ionization mass spectrometry (ESI-MS) and electrospray ionization tandem mass spectrometry (ESI-MS/MS), have been successfully applied to assess the composition of pigments in grapes and wines (Hayasaka *et al.* 2005). For example, mass spectrometric techniques have enabled the detection and identification of high molecular weight and oligomeric pigments derived from anthocyanins that are present in low quantities in red wine (Vivas *et al.* 1996; Fulcrand *et al.* 1999; Revilla *et al.* 1999; Sugui *et al.* 1999; Benabdeljalil *et al.* 2000; da Costa *et al.* 2000; Favretto and Flamini 2000; Pezet *et al.* 2001; Vanhoenacker *et al.* 2001; Heier *et al.* 2002; Hayasaka and Asenstorfer 2002a; Mateus *et al.* 2002c; Wu *et al.* 2003; Hayasaka and Kennedy 2003a; Hayasaka *et al.* 2003; Mazzuca *et al.* 2004; Vidal *et al.* 2004c; Alcalde-Eon *et al.* 2006; Garcia-Puente Rivas *et al.* 2006). Powerful analytical techniques such as nuclear magnetic resonance (NMR) spectroscopy has become increasingly more sensitive, and the technique provides structural confirmation of many anthocyanin-derived pigments found in red wine (Fulcrand *et al.* 1996; Bakker and Timberlake 1997; Fulcrand *et al.* 1998a; Degenhardt *et al.* 2000; Mateus *et al.* 2001a; Atanasova *et al.* 2002b; Mateus

et al. 2002c; Håkansson *et al.* 2003; Mateus *et al.* 2003; Mateus *et al.* 2003; Pissarra *et al.* 2004; Mateus *et al.* 2004a; Lee *et al.* 2004b; Salas *et al.* 2004b; Pissarra *et al.* 2005; Mateus *et al.* 2006).

The introduction of preparative HPLC analysis, countercurrent coil chromatography (CCC) and multi-layer countercurrent coil chromatography (MLCCC) (Ito and Ying 1996; Ma and Ito 1996; Berthod *et al.* 1999; Mirabel *et al.* 1999; Degenhardt *et al.* 2000; Schwarz and Winterhalter 2003b; Schwarz *et al.* 2003c; Schwarz *et al.* 2003d; Schwarz and Winterhalter 2003g; Vidal *et al.* 2004; Vidal *et al.* 2004b; Salas *et al.* 2005a) have been used to isolate larger quantities of pigments from wine, prior to pigment characterization. However, these techniques are expensive to maintain, and the methods themselves are time-consuming.

In this thesis, two main techniques will be applied: UV/Vis spectroscopy to assay and measure red wine and pigment colour, and HPLC analysis (HPLC-UV/Vis and HPLC-MS) to detect and quantify pigments separated from a red wine.

1.2.1.   Standard spectroscopic measurement of red wine colour

Standard spectroscopic methods are commonly used in Australian wineries and research laboratories to measure the colour of grapes and wine. These methods are based on the original work by Chris Somers from the Australian Wine Research Institute (AWRI) (Somers 1975; Somers and Evans 1977; Somers and Evans 1979; Somers and Verette 1988; Somers 1998a). Additional methods used worldwide to measure wine colour have been developed by Glories (Glories 1984a; Glories 1984b) and L'Office International de la Vigne et du Vin (OIV) (OIV 1990). These methods employ absorbance measurements at two or more wavelengths to assay grape or wine colour.

Colour measurements are made on intact wine using a UV/Vis spectrophotometer. To obtain absorption information, a sample is placed in a cuvette in the spectrophotometer. The cuvettes can be made of either glass or quartz, where the latter is an ultraviolet transparent material. Ultraviolet or visible light at a certain wavelength, or from a range of wavelengths, is transmitted through the sample. The spectrophotometer measures how much light is transmitted through the sample, and calculates its absorbance. The absorbance values recorded are then adjusted accordingly for cuvette size (to equate the values to a path length of 10 mm) or for dilution.

In this thesis, four types of spectral measurements will be made on intact wine. Colour measurements will be made at two different pH values, and after addition of excess metabisulfite solution. Wine colour measurements are made at the natural pH of the wine and at low pH (the total red pigment colour). This is based on the premise that the pH environment of a pigment or a wine is a major influence to the colour expression. The third colour measurement is after addition of excess bisulfite solution and will be termed "post-SO_2 bleaching". Addition of excess SO_2 to a wine will enable identification of the colour provided by pigments that are stable to SO_2 bleaching. The colour of the wine remaining post-SO_2 bleaching shall be termed "SO_2 stable wine colour". Evaluating SO_2 stable wine colour is of particular importance in aged wines as the concentration of anthocyanins is minimal. SO_2 stable wine colour is a measure of the anthocyanin-derived pigments that are stable to SO_2 bleaching. It is believed that SO_2 stable wine colour is a major contributor to aged wine colour (Somers 1971; Bakker *et al.* 1998; Fulcrand *et al.* 1998; Lee 2004; Garcia-Puente Rivas *et al.* 2006).

In addition to the wine colour density and hue measurements, the spectral measurements can be used to calculate the "percentage of SO_2 non-bleachable pigments" and "chemical age" of a red wine. The percentage of SO_2 non-bleachable pigments is a comparison of the wine colour before and after addition of bisulfite solution (Eglinton and Henschke 2003; Iland *et al.* 2004). The percentage measurement is an indicator of how much colour is provided by pigments that are stable to SO_2 bleaching. The chemical age

defined the relationship between the so-called "polymeric pigments" and "wine anthocyanins" (Somers and Evans 1977). The chemical age assesses the "variation in the ageing characteristics" of a red wine.

Another spectral measurement presented in this thesis involves diluting a wine in a model wine solution at a given wine pH and measuring the colour absorbance. By diluting the wines in model wine, the pH value can be adjusted to achieve an almost constant value for a sample set of red wines. A graphical comparison of the colour values of the intact and diluted wines can be used to investigate the phenomenon of copigmentation (Boulton 2001).

1.2.1.1.   Wine colour density

The Australian wine industry employs the Sudrand method, the sum of the absorbance at 420 nm and 520 nm ($A_{420\ nm} + A_{520\ nm}$), (Sudrand 1958) to determine wine colour density (WCD). The wine colour density measurement is one of the many measurements which in Australia is referred to as "the Somers measurements". For example, Somers found that for red wines differing in age by 2 years, the wine colour density values ranged between 3 a.u. and 25 a.u. (Somers 1998b). Glories and Wrolstad have published modifications of the original Somers' method to include an additional absorbance measurement at 620 nm (Glories 1984a; Glories 1984b; Wrolstad 1993). While Mazza and co-workers have included an absorbance measurement at 700 nm to account for problems due to turbidity (Mazza *et al.* 1999). In the experimental section of this thesis, the wines will be centrifuged prior to analysis to reduce the necessity of absorbance measurements at high wavelengths.

1.2.1.2.   Wine colour hue

Wine colour hue (or tint) (WCH) is the ratio of $A_{420\ nm}$ to $A_{520\ nm}$. An increase in the hue value is expected for a red wine as it ages describes a shift from purple red via brick red to brown tones of the wine colour. For example,

Somers demonstrated that in young wines, the hue was 0.4 to 0.5 which increased to 0.8 to 0.9 in aged red wines (Somers and Verette 1988).

Although WCD and WCH measurements can be made quickly, the usefulness of these colour values are limited. WCD and WCH values are assumed to represent the colour of all the pigments in the wine even though absorbance measurements are made at only two wavelengths. Wine colour density values favour the absorbance maxima of anthocyanins which exhibit λ_{max} values at around 520 nm. But the wine colour density measurement does not account for the colour provided by anthocyanin-derived pigments such as Vitisin A and the ethyl-linked anthocyanin-flavanols whose λ_{max} values are at 512 nm and 540 nm respectively. More precise methods are needed to evaluate the colour over a wider spectral range, and it is CIELab colour values that are most applicable. CIELab colour values are calculated from absorbance values for every wavelength from 400 to 700 nm across the visible spectrum (Section 1.2.2.5 below). This allows for all the individual components or pigment colours that contribute to red wine colour to be taken into account.

1.2.1.3.   Total red pigment colour

Total red pigment colour is measured at 520 nm, and is determined on acidification of a red wine with hydrochloric acid to pH 1. At low pH, acidification enhances the colour intensity of a red wine and particularly favours the colours of anthocyanins through the formation of the flavylium cation (Section 1.4.1.). But in current literature there is little information regarding the pH effects on the colour of anthocyanin-derived pigments, and it is not known how much of the total red pigment colour is actually due ato pH-dependent anthocyanin-derived pigments.

1.2.1.4. SO$_2$ stable wine colour and percentage of SO$_2$ non-bleachable pigments

Sodium or potassium metabisulfite decomposes in water to form the cation and bisulfite ions. The bisulfite ion exists in equilibrium with molecular sulfur dioxide, water, sulfite and hydronium ions. Hence in winemaking, the term "SO$_2$" is used to denote an aqueous bisulfite solution.

Treatment of red wine with excess metabisulfite solution bleaches the colour of anthocyanins, and results in the formation of colourless sulfite adducts (Section 1.4.2.). Whilst "post-SO$_2$ bleaching", it is considered that C4-substituted anthocyanin-derived pigments do not experience a colour loss and would be resistant to SO$_2$ bleaching. This SO$_2$ measurement provides information regarding the colour of "SO$_2$ stable wine pigments" and their contribution to "SO$_2$ stable wine colour" (Eglinton and Henschke 2003). A comparison of wine colour absorbance at 520 nm before and after addition of excess bisulfite solution will determine the "percentage of SO$_2$ non-bleachable pigments" (Somers and Evans 1977; Iland *et al.* 2004). But as some anthocyanin-derived pigments are considered to exhibit yellow-red or orange-red hues with some colour absorbance maxima around 420 nm (such as some directly-linked anthocyanin-derived pigments which have λ_{max} values in the range 425 to 460 nm), an additional absorbance measurement will be made at 420 nm in this thesis. The wine colour density values before and after addition of excess bisulfite solution will be used to calculate the percentage of SO$_2$ non-bleachable pigments. Similarly, the SO$_2$ stable wine colour will be derived from absorbance measurements at 420 and 520 nm post bisulfite addition.

Absorbance values to measure wine colour post-SO$_2$ bleaching at only two wavelengths provides limited colour information. Therefore, on addition of bisulfite solution, CIELab colour values are a more precise colour measurement.

1.2.1.5.   Chemical age index (ii)

Red wines vary in their ageing characteristics: some wines appear to age quite rapidly reaching a superior quality within the first year, whilst others require several years of storage before reaching their optimum quality (Somers and Evans 1977). The chemical age index (ii) is used to interpret the relationship between the "polymeric pigments" and "wine anthocyanins" measured in their flavylium form (A_{SO2}/A_{HCl}) at 520 nm (Somers and Evans 1977). But it must be noted that A_{SO2} and A_{HCl} are not strictly representative of the absorbance values of polymeric pigments and wine anthocyanins. Importantly, A_{SO2} does not necessarily imply the colour of polymeric pigments, the value is representative of the colour absorbance post-SO_2 bleaching (Section 1.2.1.4). A_{HCl} value is the absorbance measurement following acidification of a wine sample at low pH (Section 1.2.1.3). The colour measurements may include the colour of anthocyanins in their flavylium form, but does not account for the colour of pH-dependent anthocyanin-derived pigments.

Thus, as the ratio suggests the chemical age (ii) provides an indication of how much of the total red pigment at low pH is provided by the "SO_2-stable wine pigments". At fermentation, there are few pigments stable to SO_2 bleaching, and so the chemical age index (ii) will approximate to zero. With ageing, there is a decrease in the concentration of anthocyanins and through the reaction of anthocyanins with other wine components there is a progressive increase in the SO_2 stable pigments, particularly anthocyanin-tannin polymeric pigments. As the wine ages, the chemical age index (ii) will increase accordingly.

The other chemical age measurement is the chemical age (i) index. The chemical age (i) measurement accounts for bleaching of colour after addition of excess bisulfite and the recovery of "SO_2 bleached" colour from the presence of bisulfite already in the wine by measuring the colour absorbance after addition of acetaldehyde (CH_3CHO) (Somers and Evans 1977). The measurement is based on the premise that the SO_2 bleaching of the colour of

anthocyanins is reversed by adding excess acetaldehyde. Acetaldehyde preferentially reacts with the bisulfite ion and the loss of colour from anthocyanins is retrieved. However, the chemical age (i) of red wines will not be measured in this thesis.

1.2.1.6.   Measurement of copigmentation

Copigmentation was first reported by Robinson and Robinson in 1931 (Robinson and Robinson 1931). It is a widespread phenomenon observed in plant tissues, particularly flowers that contain anthocyanin molecules (Bloor 2001; Rein *et al.* 2004). In wine, anthocyanins react with cofactors, to result in copigmentation and enhanced wine colour. Cofactors, some of which may be colourless, can include hydroxycinnamic acid esters, hydroxybenzoic acids, hydrolysable tannins, and flavo4nols such as quercetins and kaempferols (Somers and Evans 1979; Osawa 1982; Brouillard *et al.* 1989; Dangles *et al.* 1993a; Baranac *et al.* 1996; Figueiredo *et al.* 1996a; Mirabel *et al.* 1999; George 2001; Darias-Martin *et al.* 2002; Malien-Aubert *et al.* 2002; Bloomfield 2003; Somers 2003; Brenes *et al.* 2005; Schwarz *et al.* 2005; Stefano *et al.* 2005; Gomez-Miguez *et al.* 2006).

In young red wines made from *Vitis vinifera* grapes, copigmentation has been reported to result in both a higher pigment concentration and enhanced pigment colour (Boulton 1996; Boulton and Levengood 1997; Boulton 2000; Boulton 2001; Hermosín Gutiérrez *et al.* 2003; Boselli *et al.* 2004; Hermosin-Gutierrez and Garcia-Romero 2004; Pérez-Magariño and González-SanJosé 2004; Hermosín Gutiérrez *et al.* 2005; Lorenzo *et al.* 2005; Schwarz *et al.* 2005). In 2001, Boulton proposed that copigmentation can account for between 30 and 50% of the colour in young wines and is primarily influenced by the levels of several cofactors (Boulton 2001). Copigmentation effects are largely dependent on the molar ratio of cofactor to pigment in aged wines. As wine ageing progresses, the level of free copigmentation cofactors is considered to decreased (Mirabel *et al.* 1999; Boulton 2001; Somers 2003; Hermosín Gutiérrez *et al.* 2005). The reduction in cofactor concentration could explain the lack of copigmentation effects in matured wines. The colour

exhibited by the anthocyanins when they are in copigmented complexes can be severalfold greater than that of the free form (Boulton 2001). Consequently, copigmentation is thought to cause a simultaneous increase in colour intensity, known as the "hyperchromic effect", and a shift from reddish to bluish hues, "a bathochromic effect" (Boulton 2001).

The copigmentation complex is easily disrupted by diluting the wine in a model wine buffer. Dilution causes the components of the complex to return to free pigment and cofactor forms as equilibrium is reached. In this thesis, copigmentation is determined from diluted wine colour density measurements made at pH 3.6, at two absorbance values, at 420 nm and 520 nm. A graphical comparison of the wine colour of the intact wine (on the x-axis) plotted against the diluted wine colour (on the y-axis) is used to investigate copigmentation. Copigmentation is considered to exist where there is a non-linear deviation from Beer's Law (Equation 1.1 below) between the two colour values (Boulton 2001). At higher wine colour density values, a deviation from linearity may be observed which can indicate that copigmentation plays a role in the enhanced wine colour.

1.2.2. Spectroscopic measurement of wine pigments

In the literature, researchers have characterized the colour of wine pigments by different spectroscopic methods. For example, the extinction coefficient values of some wine pigments at a given pH value have been presented (Giusti *et al.* 1999; Håkansson *et al.* 2003; Oliveira *et al.* 2006), or the colour of a pigment has been compared to wine colour as a percentage colour contribution (Degenhardt *et al.* 2000; Kong *et al.* 2003; Schwarz *et al.* 2003d) or the colour of a wine pigment has been illustrated as an absorbance profile and wavelength with a value indicating maximum colour absorbance, its λ_{max} value (Bakker *et al.* 1993; Francia-Aricha *et al.* 1997; Es-Safi *et al.* 2000c; Escribano-Bailón *et al.* 2001; Mateus *et al.* 2001a; Schwarz and Winterhalter 2003b; Alcalde-Eon *et al.* 2004; Pissarra *et al.* 2004; Mateus *et al.* 2005a; Salas *et al.* 2005a; Duenas *et al.* 2006; Mateus *et al.* 2006). Other spectral

colour measurements of wine pigments is CIELab colour measurements. CIELab colour values can also be used to define the colour of red wines also.

1.2.2.1.   Extinction coefficient values

The extinction coefficient or molar absorptivity, ε, of a coloured substance is a measure of how well the substance absorbs light at a particular wavelength. The extinction coefficient (L cm^{-1} mol^{-1}) is the measure of the amount of light absorbed at a given wavelength by a 1 mol L^{-1} solution in a pathlength of 1 cm. Essentially, it is the proportionality constant derived from Beer's Law where the absorbance, A, is directly proportional to its concentration, c (mol L^{-1}) and pathlength, l (cm) (Equation 1.1).

$$A \propto cl$$
<div align="right">Equation 1.1</div>

The extinction coefficient of a pigment is entirely dependent on the pH value and the media used to dissolve the pigment. The value provides information regarding the colour absorbance of a pigment, and may be used to indicate its colour contribution to red wine colour. For example, a low extinction coefficient indicates that the pigment will have a relatively low contribution to wine colour. The extinction coefficient value of an anthocyanin-derived pigment such as a vinyladduct to malvidin 3-glucoside at low pH (pH 1) can be 3 to 5 times smaller than that of malvidin 3-glucoside (Håkansson *et al.* 2003). But, researchers are limited to comparing extinction coefficient values of the many anthocyanin-derived pigments that are found in wine and their possible influence on wine colour. The reason being that there is very little extinction coefficient information of anthocyanin-derived pigments recorded in current literature (Figueiredo *et al.* 1999; Escribano-Bailón *et al.* 2001; George *et al.* 2001; Mateus and de Freitas 2001; Håkansson *et al.* 2003; Oliveira *et al.* 2006). Instead researchers have focussed on the extinction coefficients of anthocyanins, particularly malvidin 3-glucoside. As anthocyanins exist in highly coloured forms at low pH, researchers have mainly published the extinction coefficients of anthocyanins, particularly malvidin 3-glucoside (Koeppen and Basson 1966; Somers 1966b; Niketic-Aleksic and Hrazdina 1972; Brouillard and Delaporte 1977; Metivier *et al.* 1980; Santos *et al.* 1993;

Heredia *et al.* 1998; Houbiers *et al.* 1998; Giusti *et al.* 1999; Skrede *et al.* 2000; Escribano-Bailón *et al.* 2001; Asenstorfer 2001; Lima *et al.* 2002; Håkansson *et al.* 2003; Turker *et al.* 2004; Wrolstad *et al.* 2005; Oliveira *et al.* 2006).

1.2.2.2. Colour dilution analysis and the percentage colour contribution of pigments to wine colour

The introduction of the technique known as countercurrent coil chromatographic (CCC) separation has enabled large quantities of some wine pigments to be isolated. Following separation, the colour contribution of wine pigments to red wine colour has been made possible by the application of colour dilution analysis (CDA). The CDA concept, which originated as a tool to identify the key chromophores in a complex mixture of Maillard reaction products (Hofmann 1998a; Hofmann 1998b), has been used to characterize the colour of individually purified wine pigments (Ito and Ying 1996; Ma and Ito 1996; Berthod *et al.* 1999; Mirabel *et al.* 1999; Degenhardt *et al.* 2000; Schwarz and Winterhalter 2003b; Schwarz *et al.* 2003c; Schwarz *et al.* 2003d; Vidal *et al.* 2004; Salas *et al.* 2005a). The method involves determination of the visual detection threshold of the pigments by inspecting a series of diluted pigments in daylight conditions against a white background. The lowest concentration of the colorant that is visually detectable is known as the visual detection threshold. The ratio of the concentration of the colorant divided by its detection threshold is the colour dilution value (CDV), and the pigment's colour contribution to wine colour is the percentage colour contribution (PCC). But the CDA technique has two drawbacks. Firstly, relatively large amounts of pure pigment are needed to make an assessment of the visual detection threshold, and the technique requires pure wine pigments. Isolation or synthesis of pure wine pigments can take several weeks to achieve. Hence, only a small number of wine pigments can be determined at a time. Secondly, in any wine of any age, there are many different combinations of anthocyanins and anthocyanin-derived pigments present. To assess the important contributors to wine colour, ideally it is necessary to study as many wine pigments as possible. But separation, isolation or synthesis of the many

pigments that could be present in a wine would essentially be a very cumbersome process. A more facile method is needed to assess the colours of the many anthocyanins and anthocyanin-derived pigments from a wine.

1.2.2.3.   Absorbance profile and associated λ_{max} values

In the literature, some researchers have defined the colour of a pigment by its absorbance profile and the wavelength where there is maximum peak absorbance, its λ_{max} value (Bakker and Timberlake 1997; Giusti *et al.* 1999; Santos-Buelga *et al.* 1999; Lu and Foo 2001; Mateus *et al.* 2002a; del Alamo-Sanza *et al.* 2004b; Rein *et al.* 2005; Wrolstad *et al.* 2005). Many researchers have presented λ_{max} values and associated absorbance profiles of anthocyanins and anthocyanin-derived pigments (Bakker and Timberlake 1997; Santos-Buelga *et al.* 1999; Lu and Foo 2001; Mateus *et al.* 2002a; del Alamo-Sanza *et al.* 2004b; Rein *et al.* 2005; Wrolstad *et al.* 2005). Use of absorbance profiles and λ_{max} values are non-intuitive and can be difficult for an inexperienced person to understand. In particular, the λ_{max} value requires an understanding of absorbance values, wavelengths and colours before making an adequate judgement. In addition, presenting an absorbance profile may not be straightforward; varying degrees of absorbance at different wavelengths may imply that the colour observed is not simply red or orange. For example, in addition to high red absorbance, different proportions of absorbance in the yellow, green and violet regions of the visible spectrum may indicate that a red-brown colour is observed.

Or researchers have simply stated the colours of anthocyanin-derived pigments (Bakker and Timberlake 1997; Gonnet 1998; Degenhardt *et al.* 2000; Romero and Bakker 2000a; Gonnet 2001; Burns *et al.* 2002; Pissarra *et al.* 2003a; Mateus *et al.* 2005a). Instead of referring to a pigment's λ_{max} value, researchers described the colour of a pigment using ambiguous descriptors of colour. For instance, Vitisin A is described as "brick-red" or is said to possess a "reddish-hue", whilst the glycosides of delphinidin and petunidin are described as "pink" and "purple" respectively (Gonnet 1998; Romero and

Bakker 2000a; Gonnet 2001; Burns *et al.* 2002; Pissarra *et al.* 2003a). These colour definitions are not exact and are merely subjective terminologies to describe colour.

In most cases, the implication is such that a particular pigment influences the colour of a particular age of wine with no reference to the concentration of the pigment present (Bakker and Timberlake 1997; Romero and Bakker 2000a; Romero and Bakker 2000b; Romero and Bakker 2001; Boido *et al.* 2006). Importantly, the colour of a pigment or wine will appear different if it is concentrated or in a dilute medium. A more precise definition of the colour of wine and wine pigments is necessary; the application of CIELab colour values fulfils this role.

1.2.2.4.   CIELab colour measurement

Wine researchers and the wine industry extensively use wine colour density and wine colour hue values determined from colour absorbance values as the measurements are quick and easy (Somers and Evans 1974; Somers 1975; Timberlake and Bridle 1976b; Somers and Evans 1979; Bakker *et al.* 1986a; Dallas *et al.* 1994; Almela *et al.* 1999; Ho *et al.* 2001; Gómez-Plaza *et al.* 2002; Perez-Prieto *et al.* 2003a; Bartowsky *et al.* 2004; Bautista-Ortín *et al.* 2004; Klenar *et al.* 2004). But it can be difficult to estimate colour perceived by the human eye from wine colour density and hue values. Colours can be precisely described using CIELab colour coordinates.

CIELab colour values are a more appropriate measurement for the colour of wine and wine pigments, as the system can be used to describe all the colours visible to the human eye. It has been specifically developed by the International Commission on Illumination (Commission Internationale d'Éclairage) (CIE 1986), hence "CIE" in the acronym. With CIELab, colour is described in a uniform three dimensional space: L*, a* and b*. Along the vertical axis, L* is a measure of lightness from completely opaque (0) to completely white (100). Simply, the L* value can be used to describe how dark or light a colour is. The hue circle, used to describe the colour in the horizontal

plane (Figure 1.1) where a* is a measure of redness (or –a* of greenness) and b* is a measure of yellowness (or –b* of blueness) (Figure 1.1).
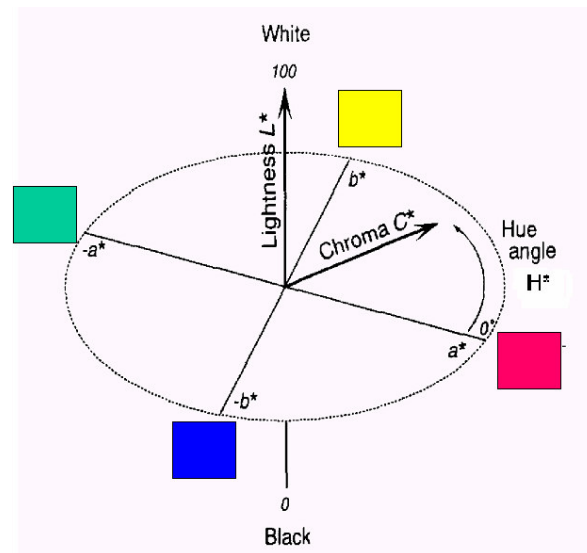
Figure 1.1: CIELab colour space describing colour in three dimensions, luminance, L*, the red-green axis, a*, and the blue-yellow axis, b* (Gonnet 1998).

As a result CIELab colour values are gaining popularity in application to wine research. For example, as will be demonstrated later, CIELab colour values can be used to reproduce wine and pigment colour at a later time (Chapters 5, 6, 7 and 8).

Although it is widely used within the food industry, use of CIELab colour values is a relatively new method to wine colour chemistry. For example, CIELab values has enabled discrimination of distinctive characteristics such as the influences of variety, viticultural practice and fermentation on wine colour (Bakker *et al.* 1986a; Almela *et al.* 1995; Carreño *et al.* 1995; Cruz-Ortiz *et al.* 1995; Almela *et al.* 1996; Carreño *et al.* 1996; Ayala *et al.* 1997; Gil-Munoz *et al.* 1997; Juarez *et al.* 1997; Almela *et al.* 1999; Ayala *et al.* 1999; Gonnet 1999; Gonnet 2001; Martinez *et al.* 2001; Meléndez *et al.* 2001; Chicon *et al.* 2002; McCaig 2002; Pérez-Magariño and Gonzàlez-San José 2002b; Gonnet 2003; Hermosín Gutiérrez 2003; Pérez-Caballero *et al.* 2003; Hermosin-Gutierrez and Garcia-Romero 2004; Hermosín Gutiérrez *et al.*

2005; Pérez-Magarino and González-San José 2006). Hence, correlations and equations relating CIELab colour values with absorbance measurements of wines and pigments have been published. For example, some CIELab values will correlate with absorbance measurements made at 420 nm and 520 nm, but the results provide no additional useful information to a wine researcher. It also seems that many researchers have reported such findings although there is no published data regarding application to wine colour (Bakker *et al.* 1986a; Almela *et al.* 1995; Almela *et al.* 1996; Bakker *et al.* 1998). In addition, some researchers have used CIELab colour values to create colour charts to measure the colour of Australian red grapes (Leamon *et al.* 2002; Mollah and Reynolds 2002; Mollah and Reynolds 2003). Although this has proven useful to compare the colours of grapes, it is difficult for an observer to imagine that a piece of uniformly coloured card or plastic looks like a wine in a glass. Thus, colour atlases created using Pantone® colours, cannot be easily applied as a specification for food, and particularly for red wine colour.

Whilst the CIELab colour values of some wine pigments have been recorded in the literature, the values L*, a* and b* provide limited information to a colour novice. It is difficult to make sense of a colour when it is described on a scale of "yellowness" or "redness" values. It is more meaningful to describe colour as it is perceived by the human eye, that is, by how light or dark it is, by its L* value, its intensity or brightness, its C* value (Equation 1.2), and the initial colour observed (e.g. violet-red, red or red-orange) or by its hue, H* value (Equation 1.2). In short, a colour is best described by its LCH values.

Luminance, represented by the L* value, is derived from the Latin, *luminare* "to illuminate", and defines how light or dark a colour is. The lightness or darkness observed in a particular wine colour can be from one of many intensity steps from black to white, where there is no colour to pure hue (Figure 1.2). In practice, there can be hundreds of minute steps from black to white, but has research shown that seven steps are the maximum number a human can retain in visual memory (Edwards 2004).
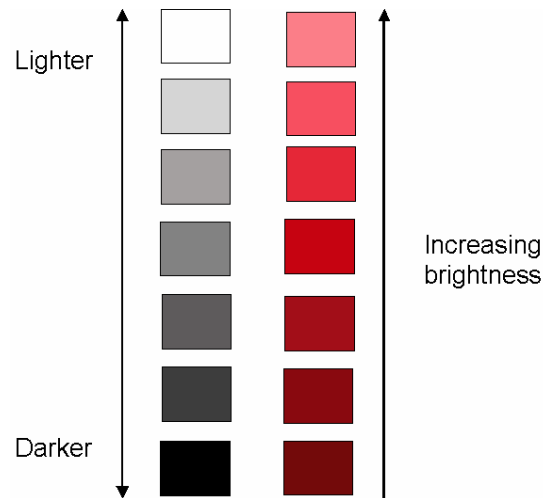
Figure 1.2: Graduated change in lightness value (from light to dark) and the change in saturation (from bright to dull).

The saturation or chroma corresponds to the brightness of a colour, and is generally observed by how intense the colour is. The chroma, C*, is derived from the a* and b* coordinates, and is calculated using Pythagoras' theorem (Equation 1.3):

$$C^* = \sqrt{a^{*2} + b^{*2}}$$ Equation 1.2

The C* value is the perpendicular distance from the lightness axis: the greater the distance from the L* axis, the greater the C* value (Figure 1.1).

Hue or H * is used to describe the first attribute of a colour that is observed. For example, the colours red and yellow are simply the hue. H* is the hue angle in degrees: 0° is located on the +a* axis (red), 90° is on the +b axis (yellow), 180° is on the −a* axis (green) and 270° is on the −b* axis (blue) (See Figure 1.1 above). Hue angle is calculated from the a* and b* values using trigonometric ratios (Figure 1.3):

$$H^* = \tan^{-1}\left[\frac{a^*}{b^*}\right]$$ Equation 1.3

The trigonometric function, tan^{-1}, is the angle whose tangent is between the opposite (a*) and adjacent sides (b*).
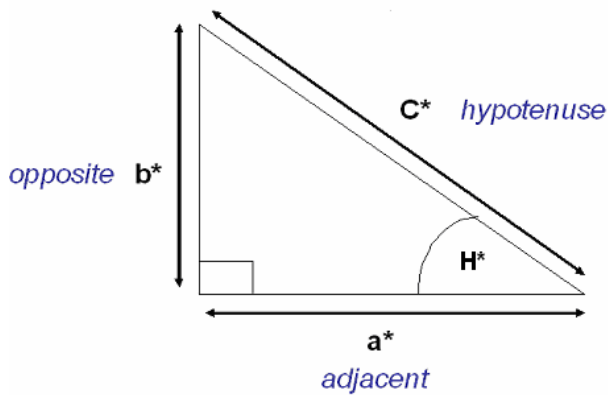
Figure 1.3: Trigonometric relationship involving the known sides a* and b* used to derive the chroma, C* and the hue angle, H* respectively.

For example, hue values that correspond to red-violet and red-orange (which can be representative of young and aged wines respectively) will exhibit hue angles that correspond to -45° and +45° respectively (Figure 1.1 above).

Alternatively, a hue colour wheel can be used to identify the initial colour recognised by an observer (Figure 1.4). The colours include the primary, secondary and tertiary colours. With respect to wine and pigments, the colours most applicable are red (a primary colour), violet and orange (secondary colours) and red-violet, red-orange and yellow-orange (tertiary colours) (Figure 1.4). Thus, rather than describing the colour of young or matured wines by red-violet or brick-red, CIELab colour values and in particular, H* values, are more accurate descriptors of red wine colour.

Figure 1.4: The hue wheel illustrating the primary, secondary and tertiary colours (Edwards 2004). With respect to wine and pigment colour, the primary colours (bold, large font) are red and yellow, the secondary colours (bold only), are violet and orange, while the tertiary colours (italics) are red-violet, red-orange and yellow-orange.

Two additional values presented in this thesis are derived from CIELab colour coordinates are colour difference and saturation values, denoted by ΔE^* and s respectively. ΔE^* values are generally used to compare two colours (represented by the subscripts, 0 and 1) (Equation 1.4). ΔE^* is essentially the Euclidean distance between the two points in three-dimensional space for two colours.

$$\Delta E^* = \left(\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}\right)^{\frac{1}{2}} \quad \text{Equation 1.4}$$

where, $\Delta L^* = L_0 - L_1$; $\Delta a^* = a_0 - a_1$; $\Delta b^* = b_0 - b_1$ (Almela *et al.* 1995; Almela *et al.* 1996; Juarez *et al.* 1997; Heredia *et al.* 1998; Gonnet 1999; Guan and Luo 1999; Gonnet 2001; Martinez *et al.* 2001; Mattivi *et al.* 2001). Originally, the colour difference equation was introduced to calculate "small" differences between colours. For example, a threshold value of $\Delta E^* \approx 1$ assumes a noticeable colour difference between two coloured solutions under ideal viewing conditions (CMC 1996), while a colour difference of around 3.0 CIELab units is an acceptable tolerance for observing red wines in a glass (Gonnet 1999; Gonnet 2001; Martinez *et al.* 2001).

The saturation or *s* is the colourfulness of an area visualized by an observer (Equation 1.5). Saturation is a scalar value derived from the standard CIELab colour values. It is determined as the proportion of chromaticity to lightness:

$$s = C^*/ L^*$$
Equation 1.5

where C* was described above in Equation 1.2.

There are many advantages to using CIELab values over conventional absorbance measurements. Firstly, CIELab values are calculated from measurements made across the entire visible spectrum from 400 to 700 nm. This will ensure that minor absorption values of pigments that contribute to red wine colour at additional wavelengths are included. A more precise definition of colour results and the colour is similar to that perceived by the human eye. Another advantage to employing CIELab colour values is that colour can be recreated at a later time using relevant colour software (e.g. Adobe® Photoshop® 6.0 for Windows®). An observer can express the luminosity, chroma and hue (LCH values) of a wine or a pigment more appropriately rather than relying on its absorption profile and a λ_{max} value as its characterization (Section 1.2.2.2.). Colour information is made easier to convey e.g. electronically via e-mail or the internet. Thus, it is necessary to demonstrate that CIELab values provide enhanced colour measurements over current wine colour measurements.

1.2.2.5.   Standard conditions to measure the CIELab colour values

To ensure repeatability and reproducibility of any colour measurement, it is necessary to employ standard conditions. The important factors in any CIELab colour measurement are the illumination, the object (the red wine sample in a cuvette) and an observer to sense the illuminated object.

Visible light corresponds to a wavelength range from 360 to 830 nm or 380 to 780 nm. A generally accepted visible wavelength range by wine researchers

is 400 to 700 nm (Davies and Mazza 1993; Gilchrist and Nobbs 1999; McCaig 2002; Cozzolino *et al.* 2003; Gonnet 2003; Håkansson *et al.* 2003; Kim 2003; Amico *et al.* 2004; Cozzolino *et al.* 2004; Hernández *et al.* 2004; Pinto and Paz 2004; Bao *et al.* 2005; Morata *et al.* 2007). As visible colour is influenced by the illumination of the sample, accepted conditions by wine researchers is colour measurement under daylight conditions. Daylight conditions can be re-created using the illuminant, D65. The D65 illuminant has a spectral energy distribution that closely matches that of a black-body at 6500K. The illuminant is representative of north-sky daylight, the colour specification used in northern Europe.

The object is the red wine sample. The sample is transferred to a suitable cuvette that is placed into the spectrophotometer. The internal dimension between opposite sides of the cuvette (or its pathlength) where the light passes through may either be 1 or 2 mm (for concentrated or intact red wine samples) or 10 mm (for diluted red wine samples).

Viewing the colour of an object is as a 10° standard observer (CIE 1964 supplementary colorimetry) (Gonnet 1999; Gonnet 2001; Pérez-Magariño and Gonzàlez-San José 2002b; Gonnet 2003; Pérez-Magariño and González-Sanjosé 2003). This is equivalent to placing an observer at a distance from the object so that it covers 10° of the field of vision. The angle approximates to viewing an object at an arm's length.

Expressing CIELab colour values, presenting an absorbance profile or quoting λ_{max} values can be used to characterize a wine pigment, but there are limitations. For example, in current literature, CIELab values and λ_{max} values have been used to evaluate the chromatic characteristics of anthocyanin-derived pigments in model systems (Bakker *et al.* 1986a; Almela *et al.* 1995; Cruz-Ortiz *et al.* 1995; Bakker and Timberlake 1997; Gil-Munoz *et al.* 1997; Juarez *et al.* 1997; Heredia *et al.* 1998; Almela *et al.* 1999; Martinez *et al.* 2001; Mattivi *et al.* 2001; Meléndez *et al.* 2001; Chicon *et al.* 2002; Pérez-Magariño and Gonzàlez-San José 2002; Gutiérrez 2003; Huertas *et al.* 2003;

Pérez-Caballero *et al.* 2003; Pérez-Magariño and González-Sanjosé 2003; Pissarra *et al.* 2003a; Pérez-Magariño and González-SanJosé 2004; Rodriguez-Mendez *et al.* 2004). But, it must be noted that in most cases, the concentration of the pigment has been omitted. Presentation of only a colour value provides a false characterization of a wine pigment. It is essential that both the colour and the concentration of the wine pigment be reported as some pigments have been investigated at higher concentrations than expected in wine. Beer's Law (Equation 1.1 above) states that the absorbance of a pigment is directly related to its concentration. So varying the concentration of pigment will vary the amount of light absorbed which will translate into a variation of the lightness, intensity and hue of a pigment's colour. Simply, at different concentrations, the colour will be different. Thus, a facile method is needed to determine both the natural concentration of a pigment and its respective colour from a red wine. As there are many anthocyanins and anthocyanin-derived pigments in a wine, ideally the new method must not involve lengthy and expensive isolation or separation techniques as is the current practice.

1.3.   Pigmented compounds in red wine

In grapes and young red wine, the pigments which provide most colour are the anthocyanins. Anthocyanins together with flavanols, the monomeric units of condensed tannins, belong to the family of polyphenols. It is during fermentation and ageing that anthocyanins will react with by-products of yeast metabolism and polymeric flavanols to form anthocyanin derived pigments.

Depending on the age of a red wine, the different amounts of anthocyanins and anthocyanin-derived pigments present will contribute to the colour of the wine. The colours provided by anthocyanins and anthocyanin-derived pigments are affected by the medium in which they exist. There are two important factors that can affect the colour of a pigment, and consequently the colour of the wine: the pH of the medium and the effect of SO_2 bleaching. Whilst it is known that the colour of anthocyanins are affected by the pH value

and by excess bisulfite bleaching, very little is presented in current literature regarding the stability of anthocyanin derived pigments to pH change and SO_2 bleaching.

Whilst UV-Vis spectrophotometry enables the measurement of the colour of grape juice and intact red wine, the technique does not provide detailed information regarding the individual pigments present in the sample. Thus, wine is separated by a chromatographic technique known as high performance liquid chromatography (HPLC). Following chromatographic separation, it is common practice to identify and quantify wine pigments by either UV/Vis spectroscopy, mass spectrometry or NMR spectroscopy. In this thesis, wine pigments separated by either HPLC-UV/Vis or HPLC-MS analyses will be presented.

1.3.1.   Polyphenols found in red wine

Polyphenols are a group of plant chemical substances, characterized by the presence of more than one phenol group per molecule. Research suggests that polyphenols are powerful types of antioxidants that can provide potential health benefits (Bakker and Timberlake 1997; Sun *et al.* 1998; del Alamo *et al.* 2000; Fischer *et al.* 2000; Annika-Nyman and Kumpulainen 2001; Malien-Aubert *et al.* 2001; Cren-Olivé *et al.* 2002; Doka and Bicanic 2002; Es-Safi *et al.* 2002a; Es-Safi *et al.* 2002b; Brouillard *et al.* 2003; Gu *et al.* 2003; Ju and Howard 2003; Kilmartin *et al.* 2003; Netzel *et al.* 2003; Peng *et al.* 2003; Sakakibara *et al.* 2003; Amico *et al.* 2004; Gambelli and Santaroni 2004; Jorgensen *et al.* 2004; Kerem *et al.* 2004; Bao *et al.* 2005; Brenes *et al.* 2005; de Beer *et al.* 2005; Cheynier *et al.* 2005; Gómez-Plaza *et al.* 2006). Sources of polyphenols in food include wine, tea, olive oil and chocolate. Common polyphenols found in red grapes and young red wine (Figure 1.5) include the coloured anthocyanins (which are extracted from the skin), and the colourless flavonols (mainly derived from seeds). In addition, anthocyanins are known to participate with other polyphenols in copigmentation and self-association reactions, particularly in young red wine. With time, there is a reduction in anthocyanin concentration as anthocyanin-derived pigments are formed by

reaction with yeast metabolites. Additional anthocyanin-derived pigments are considered to be formed by reactions with monomeric flavanols and polymeric flavanols known as the condensed tannins. Additional polyphenols, flavanols and non-flavonoids, hydroxycinnamic and hydroxybenzoic acids, are found in red wine, but shall not be discussed in this thesis.
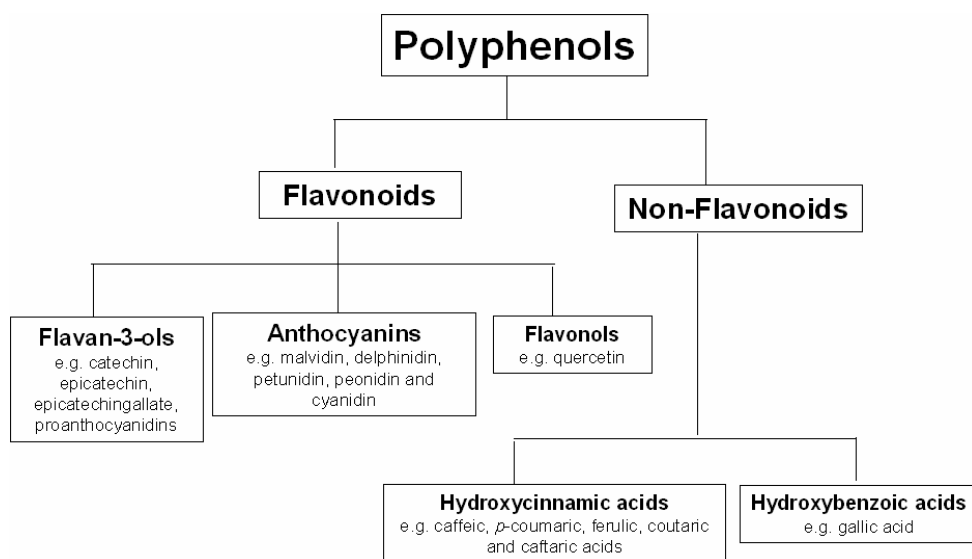
Figure 1.5: Polyphenols found in red wine.

1.3.2.   Anthocyanins

Anthocyanins (from the Greek, *anthos* = flower, *kyáneos* = purple) are water soluble pigments that reflect light in the red to blue range of the visible spectrum. Anthocyanin pigments are often observed in the plant kingdom, and serve to colour a wide range of fruits and flowers. The colour of young red wines mostly results from anthocyanins extracted from grapes during fermentation and winemaking.

1.3.2.1.   Structure of anthocyanins

The anthocyanidin molecule consists of three rings designated by A, B and C rings. When there is a single glucose molecule attached at the C ring, the

molecule is termed an anthocyanin (mono)glucoside. Numbering of the anthocyanin molecule starts at the oxygen molecule of the C ring, and moves clockwise back to the oxygen molecule (Figure 1.6).

In *Vitis vinifera* grapes, commonly found anthocyanidins are malvidin, delphinidin, petunidin, peonidin and cyanidin. These aglycones are linked to a glucose molecule at the 3-position of the C ring. The structures of the five anthocyanins with a glycoside linkage are summarized in Figure 1.6. In addition, the sugar molecule may be acylated with acetic, *p*-coumaric or caffeic acid. The 4-position of the C ring is highlighted (Figure 1.6). This is the position where the bisulfite ion (resulting from a decomposition of metabisulfite solution) combines with the anthocyanin molecule. The C4 position of the anthocyanin molecule is also where it is considered there is an attachment of a substituent that results in the formation of an "anthocyanin-derived pigment".
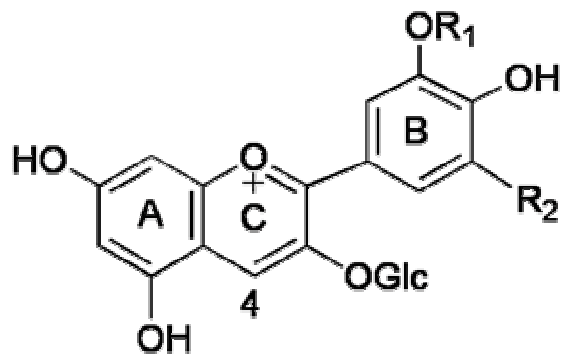
Figure 1.6: Structures of anthocyanin 3-glucosides [a] (as flavylium cations, 1a to 1e) commonly found in *Vitis vinifera* red grapes and wines.

1a: R_1 = Methyl, R_2 = OMethyl   **(Malvidin)**
1b: R_1 = H, R_2 = OH   **(Delphinidin)**
1c: R_1 = Methyl, R_2 = OH   **(Petunidin)**
1d: R_1 = Methyl, R_2 = H   **(Peonidin)**
1e: R_1 = H, R_2 = H   **(Cyanidin)**

[a] The 4-position of the anthocyanin molecule is highlighted. This is the position where the bisulfite ion combines with the anthocyanin. The C4-position is where there may be an attachment of a substituent resulting in the formation of an "anthocyanin-derived pigment".

The type and concentration of anthocyanins in red grapes and red wines are dependent on many factors, and can include grape variety, viticultural practice, temperature and climatic variation (Mazza 1995; Brossaud *et al.* 1999; Mazza *et al.* 1999). As an example, Pinot Noir grapes contain the above-mentioned anthocyanin glucosides, but lack the acylated forms (Mazza and Miniati 1993).

In any red wine, there are a numerous combination of anthocyanins, anthocyanins copigmented with other phenolic wine compounds (Gómez-Cordovés *et al.* 2004), and pigments derived from anthocyanins termed the "anthocyanin-derived pigments".

Major influences on the colour of anthocyanins are the pH value and sulfur dioxide bleaching. As anthocyanins are present in sufficient quantities in a red wine, the anthocyanin concentration will influence the colour of the wine. Appendices 1.1 to 1.2 illustrate examples of the colour properties of anthocyanins described in current literature. The colour properties presented include CIELab colour values, and the λ_{max} value and extinction coefficients of anthocyanins in different pH media.

In the laboratory, anthocyanins are routinely used to compare between red wine samples in terms of varietal differences, vineyard characteristics, vinification techniques and storage conditions (Wenzel *et al.* 1987; Dallas *et al.* 1994; Gao *et al.* 1997; Wightman *et al.* 1997; Bakker *et al.* 1998; Annika-Nyman and Kumpulainen 2001; Landrault *et al.* 2001; Mateus and de Freitas 2001; Mateus *et al.* 2001; Romero and Bakker 2001; Arnous *et al.* 2002; Mateus *et al.* 2002; Morais *et al.* 2002; Peng *et al.* 2002; Wang *et al.* 2003; Amico *et al.* 2004; Gambelli and Santaroni 2004; Gomez-Miguez and Heredia 2004; Núnez *et al.* 2004; del Alamo-Sanza *et al.* 2004b; Gómez-Míguez *et al.* 2005; Revilla *et al.* 2005; Wu and Prior 2005a; Wu and Prior 2005b). Thus, in most literature sources, the implication is such that anthocyanins are important to young wine colour since many studies have compared red wine colour with anthocyanin content. The association is probably justified;

anthocyanins are the major pigments present in *Vitis vinifera* red grapes, and anthocyanins are present in high concentrations during alcoholic fermentation.

In young red wine, some researchers believe the enhanced perception of wine colour is considered to be influenced by copigmentation and self-association reactions of anthocyanins with other polyphenols (Asen *et al.* 1972). In aged red wines, the concentration of malvidin 3-glucoside and other anthocyanins decrease to almost negligible amounts (Herderich *et al.* 2006) and so anthocyanins are considered to be minor contributors to aged red wine colour (Somers and Evans 1979). For example, Schwarz and co-workers demonstrated that malvidin 3-glucoside and the acylated anthocyanins contributed approximately 2 to 7% and approximately 3% respectively to wine colour in matured wines (Schwarz *et al.* 2003c). Also, as there are less anthocyanin molecules available in aged wines, copigmentation effects are considered minimal (Mirabel *et al.* 1999; Boulton 2001; Somers 2003; Hermosín Gutiérrez *et al.* 2005). Thus, in a bottle or barrel, anthocyanins will react with wine constituents such as flavanols to form complex pigment than the anthocyanins from which they were formed. These pigments, termed "anthocyanin-derived pigments", can be monomeric, oligomeric (consisting of a relatively low number of monomer units) or polymeric in nature. Some anthocyanin-derived pigments have anthocyanin and flavanol attachments, the anthocyanins in these structures will be denoted by the letter, A, and the flavanols by the letter, F.

1.3.3.   Flavan-3-ols and condensed tannins

Flavan-3-ols or flavanols are the monomeric units of tannins. Examples of flavanols found in red wine include the stereoisomers, (+)-catechin and (-)-epicatechin (Figure 1.7). Flavanols have a three ringed structure similar to anthocyanins, but flavanols are colourless grape and wine polyphenols. Polymerization of catechins occurs through the formation of interflavan bonds. The result are proanthocyanidins or condensed tannins where the unit size can be as many as 50 proanthocyanidin units exist. Consequently, tannins are

polymeric, high molecular weight structures. Condensed tannins are colourless in visible light, but are detected at 280 nm in the ultra-violet range.
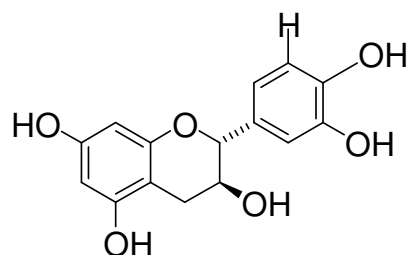
a) (+)-catechin
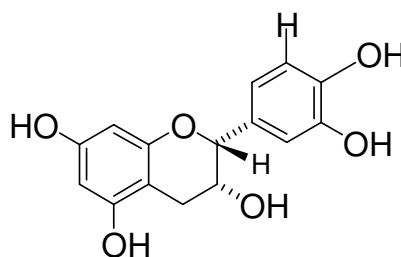
b) (-)-epicatechin

Figure 1.7: Flavan-3-ols (+)-catechin and (-)-epicatechin.

In general, the focus of many studies concerning tannins and the degree of polymerization in wine has been with regard in their contribution to "mouthfeel" and astringency of red wines (Ribereau-Gayon *et al.* 1983; Singleton and Trousdale 1992; Charlton *et al.* 2002; Vidal *et al.* 2002; Crespy 2003; Vidal *et al.* 2003; Boselli *et al.* 2004; Llaudy *et al.* 2004; Vidal *et al.* 2004a; Cheynier 2005; Herderich and Smith 2005).

In red wine, flavan-3-ols and tannins are considered to be involved in reactions with anthocyanins to form tannin-anthocyanin linkages that result in the formation of anthocyanin-derived pigments (Escribano-Bailon *et al.* 1996). Vidal and co-workers investigated the relationship between astringency and anthocyanin-derived pigments in red wine (Vidal *et al.* 2004a). The study concluded that purified anthocyanins were not astringent or bitter. Also, Vidal and co-workers speculated that during winemaking and upon storage, the

formation of anthocyanin-tannin pigments may lead to a decrease in astringency in red wine (Vidal *et al.* 2004a). To demonstrate the structures of anthocyanin-derived pigments with flavanol attachments later (Sections 1.3.4.2. and 1.3.4.3), flavanols will be denoted by the letter, F.

1.3.4. Anthocyanin-derived wine pigments

In aged red wine, anthocyanins have reacted with chemical components present in the wine to form anthocyanin-derived wine pigments. The reactions include condensation, polymerisation and oxidation reactions (Ribereau-Gayon *et al.* 1983; Fulcrand *et al.* 2004). In much of the literature, researchers have termed these pigments "polymeric pigments" (Somers 1971; Bakker *et al.* 1986b; Mayen *et al.* 1994; Escribano-Bailón *et al.* 1996; Mazza *et al.* 1999; Degenhardt *et al.* 2001; Ho *et al.* 2001; Harbertson *et al.* 2003; Mansfield and Zoecklein 2003; Adams *et al.* 2004; Sáenz-López *et al.* 2004; Zimman and Waterhouse 2004a; Cheynier *et al.* 2006; Guadalupe *et al.* 2006). Although some pigments detected in red wine may be polymeric, it cannot be assumed that all anthocyanin-derived pigments are polymeric. The structures of the three types of anthocyanin-derived pigments will be presented to demonstrate polymeric and monomeric anthocyanin-derived pigments (Sections 1.3.4.1. to 1.3.4.3. inclusive).

1.3.4.1. Polymeric pigments

It is evident from current literature that, like the term "tannin", there is some confusion and inconsistency in the use of the terms "polymeric pigments", "polymeric proanthocyanidins", "polymeric fraction", "polymeric anthocyanins" and "pigmented polymers" (Somers 1971; Timberlake and Bridle 1976b; Bakker *et al.* 1986b; Escribano-Bailon 1996; Dallas *et al.* 1996b; Mazza *et al.* 1999; Degenhardt *et al.* 2001; Ho *et al.* 2001; Mattivi *et al.* 2001; Parley *et al.* 2001; Romero and Bakker 2001; Harbertson *et al.* 2003; Mansfield and Zoecklein 2003; Schwarz and Winterhalter 2003b; Schwarz *et al.* 2003d; Adams *et al.* 2004; Sáenz-López *et al.* 2004; Zimman and Waterhouse

2004a). Also, when Somers demonstrated the presence of "pigmented polymers" in Shiraz red wine (Somers 1966a), using Gel Permeation Chromatography, he termed the spectroscopic measurement after adding excess bisulfite to a wine and measuring the remaining colour at 520 nm as the "amount of pigmented polymers" in the wine (Somers and Evans 1977; Glories 1978). But this measurement actually refers to the SO_2-stable wine colour. Nevertheless, following this publication, many researchers have referred to the spectroscopic measurement on addition of metabisulfite solution as the "polymeric pigment colour" or "polymeric anthocyanins" (Nagel and Wulf 1979; Sims and Morris 1984; Sims and Morris 1985; Sims and Morris 1986; Bakker *et al.* 1986b; Gómez-Plaza *et al.* 1999; Mazza *et al.* 1999; Shoji *et al.* 1999). One must be aware that this is not strictly correct. Thus, in current literature, the terms used to describe a "pigmented polymer" and "SO_2-stable wine colour" is rather ambiguous. For example, the term "polymeric pigments" is used to describe wine pigments when the pigments are not monomeric anthocyanins. Figures 1.8 to 1.11 below will illustrate that not all anthocyanin-derived pigments are polymeric, some are monomeric. Anthocyanin-derived pigments with high molecular weight tannin molecules attached to the anthocyanin molecule are polymeric and will fall into this category. Whilst pyranoanthocyanins, such as Vitisin A and Vitisin B, are monomeric anthocyanin-derived pigments.

The ambiguous use of the terms "polymeric pigment colour" or "polymeric anthocyanins" regarding colour measurements after SO_2 bleaching has led researchers to create an equation to determine the degree of anthocyanin polymerization, "%Polymerization". The equation has evolved from the belief that during ageing there is a reduction the number of copigmentation complexes as the monomeric anthocyanins transform into polymeric pigments (Hermosín Gutiérrez *et al.* 2005; Schwarz *et al.* 2005). Thus, a method to determine the %Polymerization was proposed by Boulton (Boulton 1996) and modified by Hermosín Gutiérrez and co-workers (Hermosín Gutiérrez *et al.* 2003). The %Polymerization, determined at 520 nm, equates to (A_{SO2}/A_{dil}) x 100 where A_{SO2} is termed the "polymeric pigment wine colour" after addition of SO_2 to the wine sample and A_{dil} is the absorbance following a 1:20 dilution of

the wine sample in model wine. In actual fact, the %Polymerization is strictly the ratio of the colour of SO_2 non-bleachable pigments to diluted wine colour. Although the equation may probably correct for potential copigmentation effects, it does not describe the degree of anthocyanin polymerization.

Similarly, SO_2-stable anthocyanin-derived pigments for example anthocyanin-tannin conjugates such as F-A type pigments (Figure 1.11) are considered susceptible to bleaching by the sulfite ion as the sulfite ion can attack at the 4-position of the anthocyanin molecule. The structure of Vitisin A is such that there is a substituent (the pyran ring) at the 4-position of the anthocyanin molecule (Figure 1.8). Therefore the colour of this pyranoanthocyanin would be considered stable to SO_2 bleaching. So a more appropriate term to describe the colour remaining after adding excess bisulfite to a wine is "SO_2-stable wine colour". This stable wine colour is the result of the colour provided by the "SO_2-stable wine pigments".

Since Somers demonstration of the presence of "pigmented polymers", researchers have quantified the polymeric pigments by this method (Somers 1966a). Many researchers have confirmed that the polymeric pigments contribute to wine colour (Somers 1971; Somers 1975; Bakker *et al.* 1986b; Dallas *et al.* 1994; Ho *et al.* 2001; Atanasova *et al.* 2002a; Perez-Prieto *et al.* 2003; Perez-Prieto *et al.* 2003a). For example, Somers found that the "polymeric pigments" contributed as much as 50% and 90% in one-year-old and 10-year-old red wines respectively (Somers 1971), and Schwarz and his co-workers found that the "pigmented polymers" contributed 70 % to 90 % to red wine colour (Schwarz *et al.* 2003c). It has also been reported that the percentage of polymeric pigments in wine increases with storage time (Somers 1971; Schwarz *et al.* 2003c). In experimental red wines, the percentage contribution of polymeric pigments to colour was reported to increase from approximately 60 % to over 90 % within 2 years (Bakker *et al.* 1998). Also, Romero and co-workers found that the percentage of "polymeric pigments" strongly determined the wine colour hue (Romero and Bakker 2001). So, in this thesis, it was deemed necessary to present correct definitions for "anthocyanin-derived pigments", "pigmented polymers", "SO_2-

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stable wine colour", and "SO_2-stable wine pigments", the pigments that contribute to SO_2-stable wine colour.

1.3.4.2. Anthocyanin-derived pigments and their structures

The common feature of anthocyanin-derived pigments is that there is functional group substitution at the C4-position of the anthocyanin molecule (Figures 1.8 to 1.11). Assessing the current literature, anthocyanin-derived pigments can be classified into three groups: pigments resulting from cycloadditions with yeast metabolites, pigments resulting from reactions with flavanols as direct linkages through the formation of interflavan-bonds, or pigments resulting from reactions with flavanols as indirect linkages via acetaldehyde derived linkages.

For all anthocyanin-derived pigments, additional functional groups attached to the parent anthocyanin will result in increased conjugation within the molecule. Conjugation affects the maximum absorbance of the pigment as the λ_{max} value will shift to different wavelengths. For example, the λ_{max} value of Vitisin A is hypsochromically shifted to a lower wavelength (Revilla *et al.* 1999; Mateus *et al.* 2001a; Mateus and de Freitas 2001b; Vivar-Quintana *et al.* 2002; Atanasova *et al.* 2002a; Morata *et al.* 2003; Wang *et al.* 2003b; Schwarz *et al.* 2003c; Amico *et al.* 2004) compared to the monomeric anthocyanins such as malvidin 3-glucoside (Koeppen and Basson 1966; Somers 1966; Niketic-Aleksic and Hrazdina 1972; Metivier *et al.* 1980; Heredia *et al.* 1998; Mazzaracchio *et al.* 2004; del Alamo Sanza *et al.* 2004b). Since the polyphenolic structure of the anthocyanin moiety is maintained, all anthocyanin-derived pigments will absorb at 280 nm in the ultra-violet spectral range.

1.3.4.2.1.   Pyranoanthocyanins   (including   Vitisin   A)   and pyranoanthocyanin-type pigments

The first group of anthocyanin-derived wine pigments results from a cycloaddition reaction with anthocyanins and yeast metabolites. Yeast metabolites released during fermentation react with anthocyanins to yield pyranoanthocyanins and pyranoanthocyanin-type pigments (Timberlake and Bridle 1976; Haslam 1980; Bakker and Timberlake 1997; Fulcrand *et al.* 1998a; Mirabel *et al.* 1999; Revilla *et al.* 1999; Romero and Bakker 1999; Amic 2000; Degenhardt *et al.* 2000; Kennedy and Waterhouse 2000; Romero and Bakker 2000a; Romero and Bakker 2000b; Asenstorfer *et al.* 2001; Escribano-Bailon 2001; Mateus and de Freitas 2001; Mateus *et al.* 2001; Roehri-Stoeckel *et al.* 2001; Romero and Bakker 2001; Revilla and González-SanJosé 2001b; Hayasaka and Asenstorfer 2002; Heier *et al.* 2002; Malien-Aubert *et al.* 2002; Shoji *et al.* 2002; Vivar-Quintana *et al.* 2002; Atanasova *et al.* 2002a; Mateus *et al.* 2002a; Atanasova *et al.* 2002b; Mateus *et al.* 2002b; Håkansson *et al.* 2003; Mateus *et al.* 2003; Mateus *et al.* 2003; Monagas *et al.* 2003; Morata *et al.* 2003; Schwarz *et al.* 2003a; Wang *et al.* 2003a; Wang *et al.* 2003b; Schwarz *et al.* 2003c; Alcalde-Eon *et al.* 2004; Amico *et al.* 2004; de Freitas 2004; de Villiers *et al.* 2004; Mateus *et al.* 2004; Mazzuca *et al.* 2004; Pozo-Bayón *et al.* 2004; Schwarz *et al.* 2004; Spranger *et al.* 2004; Wang 2004; Mateus *et al.* 2005; Schwarz *et al.* 2005; Monagas *et al.* 2005a) (Figures 1.8 to Figure 1.10). For example, pyruvic acid leads to the formation of Vitisin A where R_2 = COOH (Figure 1.8, Pigment 2a), while Vitisin B results from a reaction with acetaldehyde where R_2 = H (Figure 1.8, Pigment 3a). These two pigments are two examples of the many pyranoanthocyanins that have either been detected in red wine or have been synthesized by researchers (Figures 1.8 and 1.9). Reactions of anthocyanins in the presence of flavanols and acetaldehyde result in the formation of flavanyl-pyranoanthocyanins (Figure 1.9, Pigments 5a and 5b). These types of flavanyl-pyranoanthocyanin anthocyanin-derived pigments are termed "pyranoanthocyanin-type" pigments (Figures 1.8 to 1.10). Of all the pyranoanthocyanins and pyranoanthocyanin-type pigments detected in red

wine, Vitisin A has been found in high concentrations (Figure 1.8, Pigment 2a).
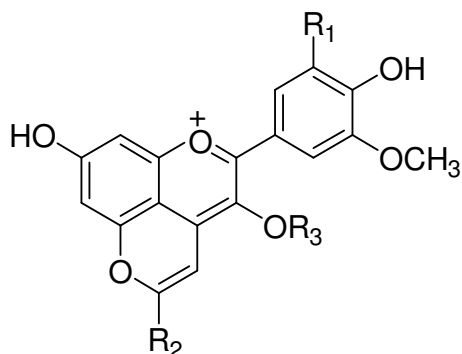
2a:	R_1 = OMe, R_2 = COOH, R_3 = Glc **(Vitisin A)**
2b:	R_1 = OMe, R_2 = COOH, R_3 = Glc-6'acetyl **(acetyl Vitisin A)**
2c:	R_1 = OMe, R_2 = COOH, R_3 = Glc-6'*p*-coumaroyl **(coumaroyl Vitisin A)**
2d:	R_1 = OH, R_2 = COOH, R_3 = Glc-6'*p*-coumaroyl
2e:	R_1 = OH, R_2 = H, R_3 = Glc-6'*p*-coumaroyl
2f:	R_1 = OMe, R_2 = Me, R_3 = Glc
2g:	R_1 = OMe, R_2 = COOMe, R_3 = Glc **(methyl Vitisin A)**
2h:	R_1 = OMe, R_2 = vinylcatechin, R_3 = Glc **(Portisin)**
2i:	R_1 = OMe, R_2 = vinylcatechin, R_3 = Glc-6'*p*-coumaroyl **(coumaroyl Portisin)**
2j:	R_1 = OMe, R_2 = (vinylcatechin)$_n$, R_3 = Glc **(vinyl pyranoflavanol)**
2k:	R_1 = OMe, R_2 = (vinylcatechin)$_n$, R_3 = Glc-6'*p*-coumaroyl
2l:	R_1 = OMe, R_2 = vinylphenol, R_3 = Glc
3a:	R_1 = OMe, R_2 = H, R_3 = Glc **(Vitisin B)**
3b:	R_1 = OMe, R_2 = H, R_3 = Glc-6'acetyl **(acetyl Vitisin B)**
3c:	R_1 = OMe, R_2 = H, R_3 = Glc-6'*p*-coumaroyl **(coumaroyl Vitisin B)**
3d:	R_1 = OMe, R_2 = OH, R_3 = H
3e:	R_1 = OMe, R_2 = (epi)catechin, R_3 = Glc

Figure 1.8: Anthocyanin-derived pigments: Pigments resulting from cycloadditions with yeast metabolites, the pyranoanthocyanins.
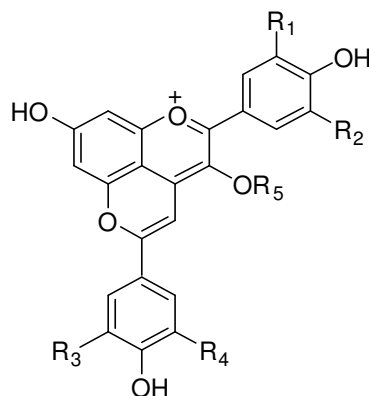
4a: R_1 = OMe, R_2 = OMe, R_3 = H, R_4 = H, R_5 = Glc

(Pigment A) (malvidin 3-glucoside 4-vinylphenol)

4b: R_1 = OMe, R_2 = OMe, R_3 = H, R_4 = H, R_5 = Glc-6'acetyl

(acetyl Pigment A) (malvidin 3-acetylglucoside 4-vinylphenol)

4c: R_1 = OMe, R_2 = OMe, R_3 = H, R_4 = H, R_5 = Glc-6'-*p*-coumaroyl

(coumaroyl Pigment A)

4d: R_1 = OMe, R_2 = OMe, R_3 = H, R_4 = H, R_5 = Glc-6'-caffeoyl

4e: R_1 = H, R_2 = OMe, R_3 =H, R_4 = H, R_5 = Glc-6'-*p*-coumaroyl **(Pigment B)**

4f: R_1 = OMe, R_2 = OH, R_3 = H, R_4 = H, R_5 = Glc

4g: R_1 = OMe, R_2 = H, R_3 = H, R_4 = H, R_5 = Glc

4h R_1 = OMe, R_2 = OMe, R_3 = H, R_4 = OH, R_5 = Glc **(Pinotin A)**

4i: R_1 = OMe, R_2 = OMe, R_3 = H, R_4 = OMe, R_5 = Glc

4j: R_1= OMe, R_2 = OMe, R_3 =OMe, R_4 = OMe, R_5 = Glc

4k: R_1 = OMe, R_2 = OMe, R_3 = H, R_4 = OH, R_5 = Glc-6'acetyl **(acetyl Pinotin A)**

4l: R_1 = OMe, R_2 = OMe, R_3 = H, R_4 = OH, R_5 = Glc-6'-*p*-coumaroyl **(coumaroyl Pinotin A)**

4m: R_1 = OMe, R_2 = OMe, R_3 = H, R_4 = OH, R_5 = Glc-6'acetyl

4n: R_1 = OMe, R_2 = OMe, R_3 = H, R_4 = OMe, R_5 = Glc-6'-*p*-coumaroyl

4o: R_1 = OMe, R_2 = OMe, R_3 = OH, R_4 = OMe, R_5 = Glc

4p: R_1 = OMe, R_2 = OH, R_3 = H, R_4 = H, R_5 = Glc

4q: R_1 = OMe, R_2 = OH, R_3 = H, R_4 = H, R_5 = Glc-6'acetyl

4r: R_1 = OMe, R_2 = OH, R_3 = H, R_4 = H, R_5 = Glc-6'-*p*-coumaroyl

Figure 1.9: Anthocyanin-derived pigments: Pigments resulting from cycloadditions with yeast metabolites, the pyranoanthocyanins (continued).
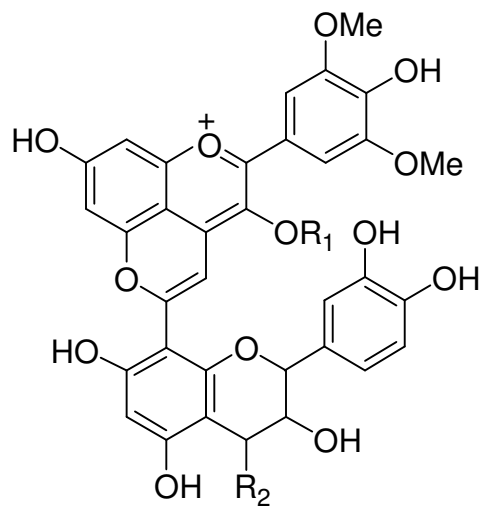
5a: R_1 = Glc, R_2 = H

5b: R_1 = Glc, R_2 = ((epi)catechin)$_n$

5c: R_1 = Glc-6'-acetyl, R_2 = ((epi)catechin)$_n$

5d: R_1 = Glc-6'-coumaroyl, R_2 = ((epi)catechin)$_n$

Note: **5b-d** are tannin derived pigments with n = 1, 2,…
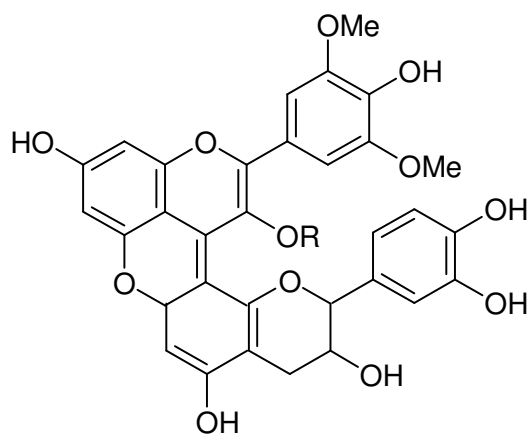
6a: R = H

6b: R = (catechin)$_n$ are tannin derived pigments with n = 1, 2,….

Figure 1.10: Anthocyanin-derived pigments: Pigments resulting from cycloadditions with yeast metabolites, the pyranoanthocyanins (continued) and pyranoanthocyanin-type pigments.

1.3.4.2.1.a. Vitisin A

Vitisin A results from a reaction between malvidin 3-glucoside and pyruvic acid. Since its discovery and identification (Bakker and Timberlake 1997), the structure of Vitisin A has been revised (Fulcrand *et al.* 1998a) and confirmed (Mateus and de Freitas 2001; Mateus *et al.* 2001) (Figure 1.8, Pigment 2a). Vitisin A represents the most abundant of the known chromatographically resolved oligomeric pigments. By HPLC analysis, it is estimated that Vitisin A, together with its acetyl and *p*-coumaryl derivatives, the pigment typically contributes between 1 and 4%, and as much as 10% of total red wine pigments in wine (Asenstorfer 2001b).

Vitisin A is found in greater abundance in Port wine than in red table wine (Bakker and Timberlake 1997). The concentration of Vitisin A is believed to increase with time. For example, Bakker and co-workers reported that the percentage of Vitisin A increases from 3.5% in young wines to 4.7% in older wines (Bakker *et al.* 1998). Researchers have demonstrated that the colour of Vitisin A is considered to be affected by the pH value. For example, Vitisin A contributes approximately 11 times more colour at pH 3, and 14 times more colour at pH 2, than the normal anthocyanins (Romero and Bakker 1999). Vitisin A is considered to show a large amount of colour expression up to neutral pH values (Schwarz *et al.* 2003d; Schwarz *et al.* 2003f). But in spite of its clear pH dependence, the colour of Vitisin A was considered less sensitive to pH change than malvidin 3-glucoside (Bakker and Timberlake 1997)

The hypsochromic spectral shift of Vitisin A (λ_{max} at 501 nm) from malvidin 3-glucoside (λ_{max} at 528 nm) is considered to contribute an orange-red hue in red wine (Bakker and Timberlake 1997). The same research group concluded that although the concentration of Vitisin A was small, the pigment expressed a strong red colour which they believed to be a contributory factor in the redness of older wines (Bakker and Timberlake 1997).

The presence of a functional group attached at the 4-position of the Vitisin A molecule is believed to protect Vitisin A against sulfite attack. This effect has

been demonstrated by several researchers, but the experiments have been mostly performed using model systems (Bakker and Timberlake 1997; Romero and Bakker 2000a; Asenstorfer *et al.* 2001; Vivar-Quintana *et al.* 2002; Atanasova *et al.* 2002a).

1.3.4.2.2. Directly linked anthocyanin-derived pigments

The second group of anthocyanin-derived pigments found in red wine are pigments with direct linkages of anthocyanins with flavanols (Timberlake and Bridle 1976; Rivas-Gonzalo *et al.* 1995; Escribano-Bailon 1996; Dallas *et al.* 1996a; Francia-Aricha *et al.* 1997; Es-Safi *et al.* 1999; Mirabel *et al.* 1999; Es-Safi *et al.* 2000; Asenstorfer *et al.* 2001; Escribano-Bailon *et al.* 2001; Hayasaka and Asenstorfer 2002; Heier *et al.* 2002; Atanasova *et al.* 2002a; Es-Safi *et al.* 2002a; Atanasova *et al.* 2002b; Es-Safi *et al.* 2002b; Mateus *et al.* 2002b; Monagas *et al.* 2003; Pissarra *et al.* 2003; Remy-Tanneau *et al.* 2003; Wang *et al.* 2003b; de Freitas *et al.* 2004; Pissarra *et al.* 2004; Wang 2004; Wang *et al.* 2004; Lee *et al.* 2004b; Pissarra *et al.* 2005; González-Paramás *et al.* 2006) (Figures 1.11 and 1.12). An interflavan bond is formed between the C4 of the anthocyanin molecule and C8 of the flavanol molecule, or vice versa. Directly linked anthocyanin-derived pigments include A$^+$-F and F-A$^+$ type pigments (Fulcrand *et al.* 2004) and the 4-8 linked polymeric anthocyanins, A-A$^+$ type pigments (Pigments 9a – 9c) (Vidal *et al.* 2003; Vidal *et al.* 2004; Vidal *et al.* 2004a; Vidal *et al.* 2004b; Vidal *et al.* 2004c). The A(-O-)F compounds are considered to exist, but no examples are presented in the figures as this type of pigment is considered to be colourless (Remy *et al.* 2000).
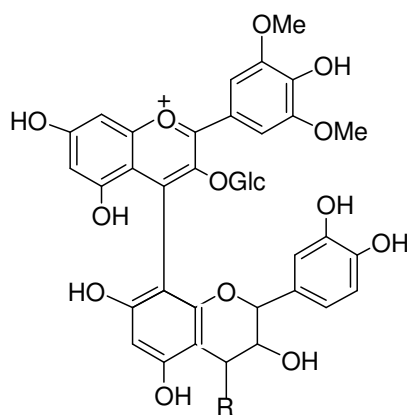
A⁺-F type anthocyanin-derived pigments

7a: R = H

7b: R = (catechin)$_n$ are tannin derived pigments with n = 1, 2,....
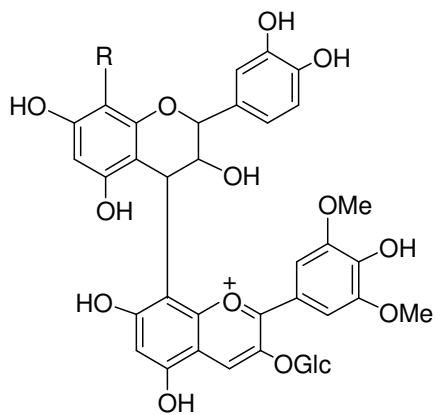
F-A⁺ type anthocyanin-derived pigments

8a: R = (catechin)$_n$ are tannin derived pigments with n = 1, 2,....

Figure 1.11: Anthocyanin-derived pigments: Directly linked anthocyanin-anthocyanin pigments.
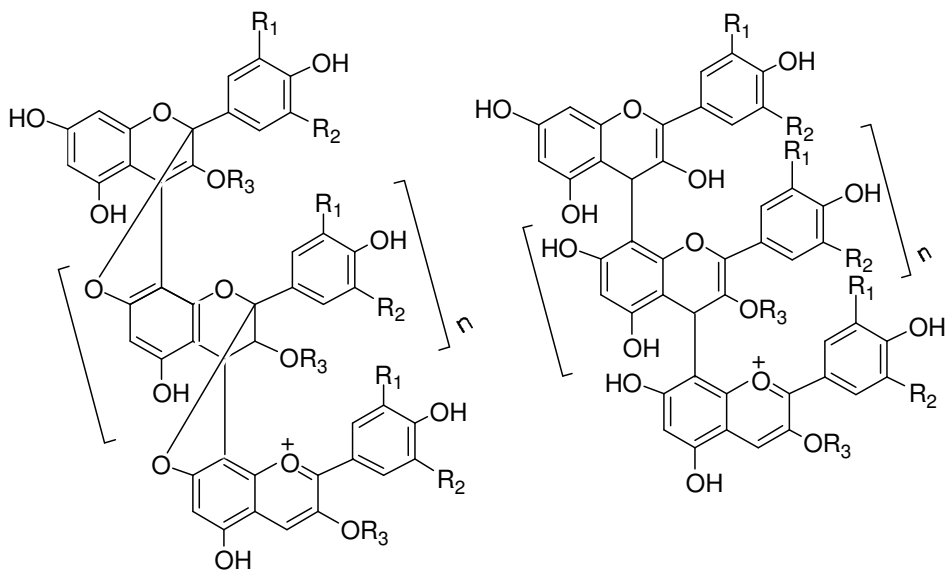
A-A⁺ type anthocyanin-derived pigments

9a: R_1 = OMe, R_2 = OMe, R_3 = Glc where n = 1, 2,….

9b: R_1 = OMe, R_2 = OMe, R_3 = Glc-6'acetyl where n = 1, 2,….

9c: R_1 = OMe, R_2 = OMe, R_3 = Glc-6'*p*-coumaroyl where n = 1, 2,….

Figure 1.12: Anthocyanin-derived pigments: Directly linked anthocyanin-anthocyanin pigments.

1.3.4.2.3.   Indirectly linked anthocyanin-derived pigments

The third type of reaction leading to the formation of anthocyanin-derived pigments are condensation reactions with flavanols mediated by acetaldehyde (Rivas-Gonzalo *et al.* 1995; Escribano-Bailon 1996; Francia-Aricha *et al.* 1998; Revilla *et al.* 1999; Asenstorfer *et al.* 2001; Heier *et al.* 2002; Malien-Aubert *et al.* 2002; Mateus *et al.* 2002b; Pissarra *et al.* 2003; de Villiers *et al.* 2004; Mazzuca *et al.* 2004; Salas *et al.* 2004; Vidal *et al.* 2004a; Salas *et al.* 2004b). In this case, the anthocyanin and flavanol are indirectly joined by an ethyl linked bridge. Instead of acetaldehyde, some researchers have considered reactions with other aldehyde groups in the formation of indirectly linked anthocyanin-derived pigments. Examples of indirectly linked

anthocyanin-derived pigments include A$^+$-Et-F and A$^+$-Et-AOH type pigments which are illustrated in Figures 1.13 and 1.14.
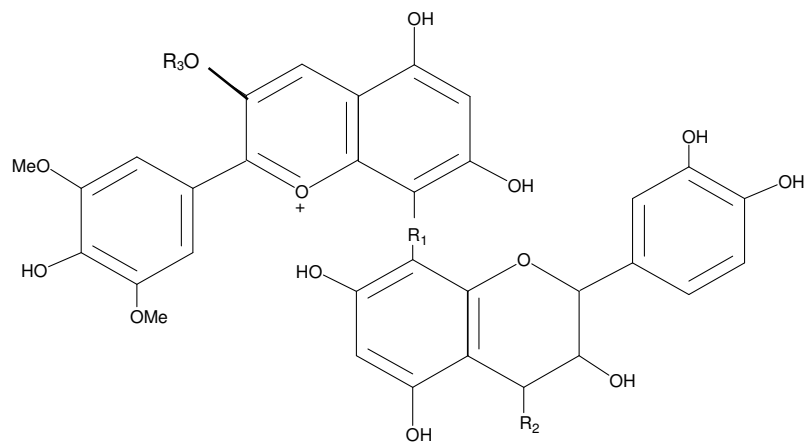
A+-Et-F type anthocyanin-derived pigments

10a: R_1 = Ethyl, R_2 = H, R_3 = Glc

10b: R_1 = Ethyl, R_2 = H, R_3 = Glc-6'-p-coumaroyl

10c: R_1 = Me, R_2 = (catechin)$_n$ are tannin derived pigments with n=1,2,….

10d: R_1 = Me, R_2 = furfural, R_3 = Glc

10e: R_1 = Me, R_2 = 5-(hydroxymethyl)furfural, R_3 = Glc

10f: R_1 = Me, R_2 = furfural, R_3 = Glc-6'acetyl

10g: R_1 = Benzyl, R_2 = H, R_3 = Glc

10h: R_1 = 3-Methylbutyl, R_2 = H, R_3 = Glc

10i: R_1 = Propyl, R_2 = H, R_3 = Glc
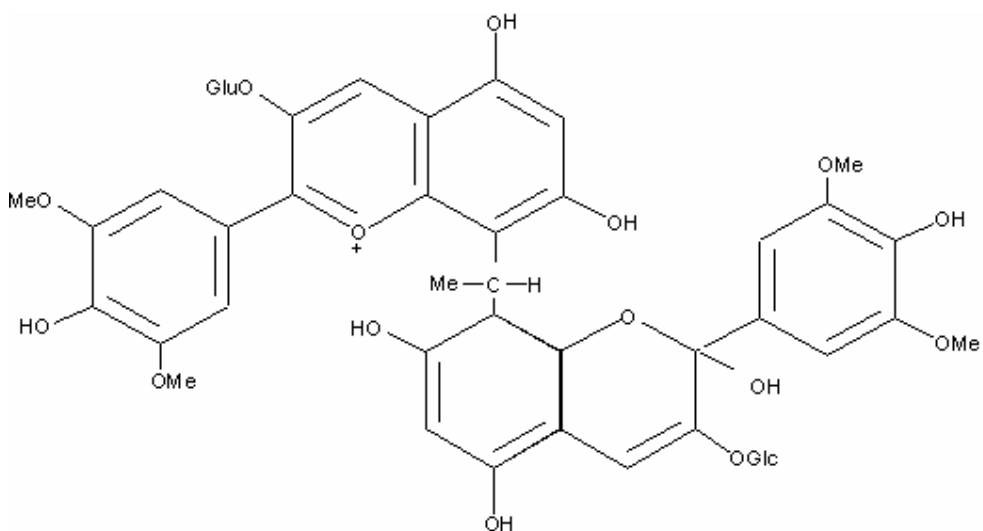
A+-Et-A type anthocyanin-derived pigments

Figure 1.13: Anthocyanin-derived pigments: Indirectly linked anthocyanin-flavanol pigments.

1.3.5.   **Summary of anthocyanin-derived pigments described in current literature**

An overview of anthocyanin-derived pigments is presented in Appendices 1.3 to 1.27. The tables include examples of the occurrence, isolation and extraction procedures of all three types of anthocyanin-derived pigments. The anthocyanin-derived pigments have been detected or isolated from grapes, grape marc and wine samples such as table, Port and model wine systems. The techniques used to identify and/or characterise the pigments are by HPLC/UV-Vis spectroscopy, mass spectrometry (including Electrospray Ionization-Mass spectrometry and Fast Atom Bombardment-Mass spectrometry), and NMR spectroscopy. For instance, UV/Vis spectroscopy has been used to present the colours of the pigments as CIELab colour values, λ_{max} values or their colour contribution to total wine colour using colour dilution analysis.

Thus, from the tables in Appendices 1.3 to 1.27, it can be summarized:

1. The behaviour of many individual wine pigments has been investigated at concentrations greater than would be expected in wine.
2. As malvidin-3-glucoside is the major anthocyanin in *Vitis vinifera* grapes, many researchers have concentrated on malvidin 3-glucoside derived pigments. It is highly likely that pigments can be formed from related anthocyanins such as the glycosides of delphinidin, petunidin, peonidin and cyanidin, and their acylated forms. But the concentration of anthocyanin-derived pigments other than malvidin 3-glucoside derived pigments have been quantified at lower concentrations (Heier *et al.* 2002).
3. For studies on the structure of wine pigments, researchers have favoured Port wines and model wine solutions, identification of anthocyanin-derived pigments needs to be assessed on more actual red wines.

4. Quantitative information on pigment concentration in wine, and the time course of anthocyanin-derived pigments formed during fermentation and ageing is minimal (Boido *et al.* 2006; Esparza *et al.* 2006; Herderich *et al.* 2006; Monagas *et al.* 2006d).

5. Some researchers have attempted to predict the presence of anthocyanin-derived pigments in a red wine on the basis of the polyphenols present in the wine. For example, the formation of some anthocyanin-catechin linked products have been speculated (Francia-Aricha *et al.* 1997; Es-Safi *et al.* 2000a). However, the presence of such compounds in wine (other than Port wine) and their contribution to red wine colour remains to be demonstrated. Another example is the detection of anthocyanin-tannin conjugates on the basis of molecular ions (Heier *et al.* 2002).

6. Little information exists regarding confirmation of the structures of many anthocyanin-derived pigments found in red wine by NMR spectroscopy (Bakker and Timberlake 1997; Atanasova *et al.* 2002b; Mateus *et al.* 2002c; Mateus *et al.* 2003; Lee *et al.* 2004b).

7. Some researchers have proposed that wine colour, particularly aged wine, is attributed to one pigment, the pigment a research group has chosen to investigate (Mateus *et al.* 2004a; Salas *et al.* 2004a; Mateus *et al.* 2006). The presumption is that this single, individual pigment has a major influence on wine colour. This theory ignores that wine colour is the result of a mixture of many colours from an infinite combination of anthocyanins and anthocyanin-derived pigments. In aged wines, where the anthocyanin concentration has decreased (Arnous *et al.* 2002; Perez-Prieto *et al.* 2003b; Alamo-Sanza *et al.* 2004; Pérez-Magariño and González-SanJosé 2004; Herderich *et al.* 2006), the colour provided by anthocyanin-derived pigments will be more important to red wine colour, but this needs to be demonstrated.

1.3.6.   Other pigments present in wine

In addition to anthocyanins and anthocyanin-derived pigments, there are other pigments in wine that do not have an anthocyanin moiety. These pigments

may well contribute to the colour of a red wine. For example, based on the chemical components present in a red wine, the formation of the following pigments has only been predicted using model wine solutions: yellow xanthylium salts (λ_{max} at 500 nm) (Timberlake and Bridle 1976a; Santos-Buelga *et al.* 1996; Dallas *et al.* 1996b; Mirabel *et al.* 1999; Es-Safi *et al.* 2000; Es-Safi *et al.* 2000b; Es-Safi *et al.* 2000c; Malien-Aubert *et al.* 2002; Es-Safi *et al.* 2002a; Es-Safi *et al.* 2003; Es-Safi and Cheynier 2003a; Es-Safi 2004a; Es-Safi and Cheynier 2004b), orange carotenoid and chlorophyll-derived compounds (λ_{max} at 398 - 486 nm) (Mendes-Pinto *et al.* 2004; Mendes-Pinto *et al.* 2005) and brick-red (λ_{max} at 500 nm) catechin-pyrylium derived pigments (de Freitas *et al.* 2004) have been synthesized in model wine solutions, but these pigments have not been detected in wine.

1.4. Factors that affect pigment and red wine colour

There are two major characteristics of the wine medium that affect the colour of wine pigments and consequently influence red wine colour. These factors are the pH value of the medium and the susceptibility to colour bleaching in the presence of the sulfite ion.

1.4.1. pH effects on anthocyanin colour

Here, malvidin 3-glucoside, the major anthocyanin found in *Vitis vinifera,* is used to represent the effect of pH media on the structure and colour of anthocyanins. At low pH (pH < 2), malvidin 3-glucoside exists as a cationic species known as a flavylium cation (Brouillard and Delaporte 1977) (Figure 1.4). At low pH, the flavylium cation is the major species. The flavylium cation is highly coloured and appears red. At pH values greater than pH 2, the cation is converted into other forms through deprotonation or hydration of the flavylium cation. The loss of the H^+ ion from flavylium cation results in the formation of three quinoidal base forms which are less red in colour than the flavylium cation, whilst hydration leads to the formation of hemiacetal structures. The hemiacetal forms can convert to an *E*-chalcone upon opening

of the B or pyrilium ring, which on further deprotonation forms the *Z*-chalcone (Figure 1.4). The structures of *E*- and *Z*-chalcone isomers have been proven by NMR spectroscopy (Santos *et al.* 1993; Houbiers *et al.* 1998). Current literature has stated that the hemiacetal and chalcones are colourless (Houbiers *et al.* 1998) (Figure 1.14).

quinoidal base isomers

flavylium cation (AH⁺)

Z-chalcone   *E*-chalcone   hemiacetal
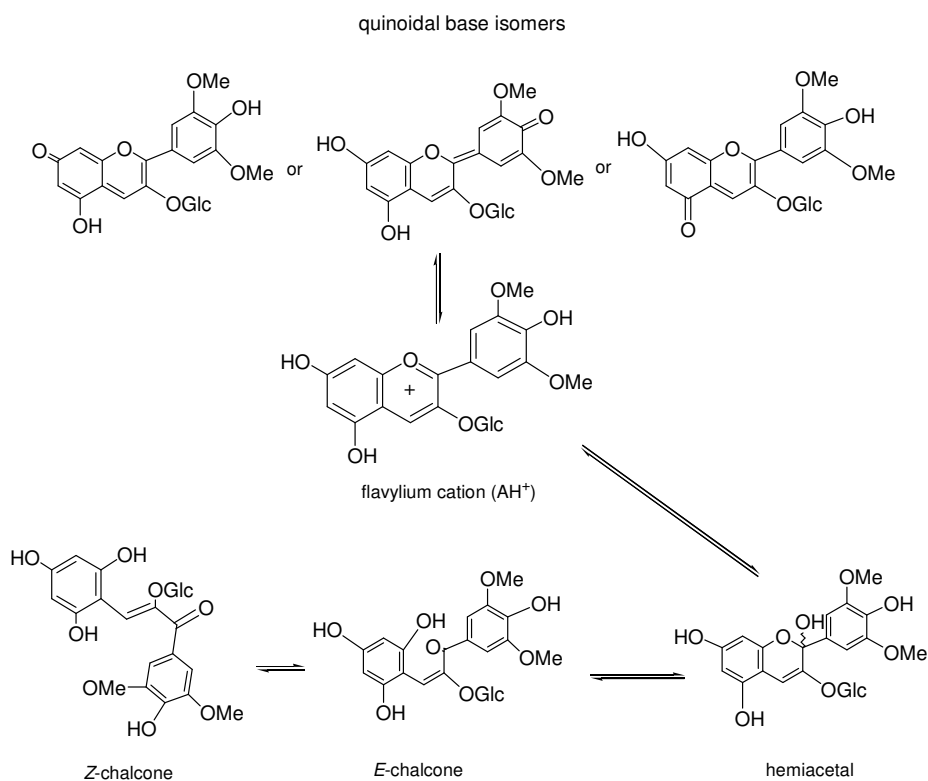
Figure 1.14: Isomers of malvidin-3-glucoside at different pH values. The figure has been adapted from Houbiers 1998 (Houbiers *et al.* 1998).

In the laboratory, it is general practice to chromatographically separate wine pigments from wine by HPLC analysis at low pH. At low pH, the highly coloured flavylium cations exist (Figure 1.14). At wine pH (pH 3.5), the less coloured quinoidal based forms and a complex mixture of the neutral and anionic species of malvidin 3-glucoside are the predominant forms (Asenstorfer 2001b; Asenstorfer and Jones 2002; Asenstorfer *et al.* 2003a; Asenstorfer *et al.* 2006). It is highly likely that the flavylium cations do not contribute to wine colour at natural wine pH. However, it is easier to quantify

the anthocyanins at low pH, because the flavylium cations are in their highly coloured forms. But, as the wine is no longer at its natural wine pH, it is likely that anthocyanin quantification may be over-estimated. This can be explained further in terms of how anthocyanin concentration is quantified at low pH and at wine pH by considering the absorbance maxima and extinction coefficient values of anthocyanins. The absorbance maxima and extinction coefficient values of anthocyanins will differ with different pH values. At low pH, the peak maxima of anthocyanins are hypsochromically shifted in the UV-visible spectrum by approximately 2 to 4 nm towards absorbance in the blue region. In addition, at low pH, anthocyanins have a high molar absorptivity (Appendices 1.1 and 1.2). Thus, at low pH, anthocyanins will appear to be intensely coloured and possess purple-red hues. In comparison, at wine pH, a bathochromic shift to lower wavelengths or red absorbance is observed (Giusti *et al.* 1999). The colour absorbance of anthocyanins decreases as the extinction coefficients of anthocyanins decreases (Appendices 1.1 and 1.2). This is best demonstrated in the extinction coefficients of malvidin 3-glucoside and cyanidin 3-glucoside which are reduced by 64 to 74% from low pH to wine pH (Håkansson *et al.* 2003; Torskangerpoll and Andersen 2005). So, at wine pH, high concentrations of anthocyanins will appear reduced in colour, and it is possible that a red hue is observed. Thus, as the colour of anthocyanins at low pH and at wine pH differs, so it follows that the concentration of anthocyanins determined by HPLC analysis at low pH will differ to anthocyanin quantification at wine pH. Later in Chapters 7 and 8, the colours and concentrations of anthocyanins will be studied at wine pH even though there is a reduction in absorbance and quantification of anthocyanins at the increased pH value (at wine pH), the anthocyanin (and anthocyanin-derived pigment) concentrations in Chapters 7 and 8 shall be termed "apparent". This is because the actual concentration of the anthocyanins remains unchanged whilst there is a change in the anthocyanin isoform with change in pH value. Studying anthocyanins at wine pH will enable a comparison of anthocyanin concentration to red wine colour at wine pH.

Quantification of anthocyanins and anthocyanin-derived pigments is important as to date, no one has published chromatographic separation of wine pigments at wine pH.

Appendices 1.1 and 1.2 provide a summary of the colours of anthocyanins in different pH media as described in current literature. The colour values are for the major anthocyanins found in *Vitis vinifera*, and include the CIELab colour values, and λ_{max} values and extinction coefficient values at low pH and at approximately wine pH.

1.4.2. SO_2 bleaching effects on anthocyanin colour

It is general practice to add sodium or potassium metabisulfite to grapes and wine to act as an antioxidant and an antimicrobial agent. The metabisulfite molecule decomposes in water to form the cation and bisulfite ions. The bisulfite ion exists in equilibrium with molecular sulfur dioxide, water, sulfite and hydronium ions. Hence, in winemaking, the term "SO_2" is used to denote an aqueous bisulfite solution. When excess bisulfite is added to a wine, there is a loss of the anthocyanin colour as the anthocyanin pigment is rendered colourless. Bisulfite ions react with anthocyanins at position 4 of the C ring (Figure 1.6) to form a bisulfite complex, which is colourless (Berké *et al.* 1998). In this thesis, additions of bisulfite to a wine shall be known as "SO_2 bleaching" or "bisulfite bleaching".

Addition of excess bisulfite to a wine will not only remove the colour of anthocyanins, but will highlight those pigments that are stable to bisulfite bleaching. This is of particular importance in aged wines where the concentration of anthocyanins is almost negligible (Herderich *et al.* 2006), whilst the colour of the wines remains red. For example, Herderich and co-workers compared the change in malvidin-3-glucoside concentration with wine colour density over 50 consecutive vintages of a red wine (Herderich *et al.* 2006). They demonstrated that as the wine aged, the concentration of anthocyanins decreased to almost negligible quantities, whilst the wine colour

density decreased, the wine colour remained red. Over the time period, the wine colour density decreased, but the change was not as dramatic as the change in anthocyanin concentration. Thus aged wine colour was not solely attributed to anthocyanins, but to other pigments that were present in the wine (Herderich *et al.* 2006). It is highly likely that pigments that contribute to aged wine colour would be those that are stable to SO_2 bleaching.

SO_2 bleaching of anthocyanin colour can be reversed by adding excess acetaldehyde. This is because acetaldehyde preferentially reacts with the bisulfite ion, and the colour of the anthocyanin is retrieved. But the effect of acetaldehyde addition to red wines will not be discussed in this thesis.

1.4.3. pH effects on anthocyanin-derived pigment colour

Although it is acknowledged that structure and colour of anthocyanins are affected by pH (Brouillard and Delaporte 1977), the effects of pH value on the colour of anthocyanin-derived pigments have been largely ignored. Instead the focus of pH value has been with regard to the formation of anthocyanin-derived pigments (Appendices 1.3 to 1.27). It is highly probable that some anthocyanin-derived pigments, such as the pyranoanthocyanin Vitisin A, exists as pH dependent forms (Asenstorfer *et al.* 2006), particularly as the anthocyanin moiety, malvidin 3-glucoside is pH-dependent. The pH dependence of Vitisin A has been demonstrated by its change of colour with pH value by Asenstorfer and co-workers (Asenstorfer *et al.* 2006).

1.4.4. SO_2 bleaching effects on anthocyanin-derived pigment colour

Pigments which are stable in the presence of excess bisulfite are termed "SO_2 stable wine pigments". Addition of excess metabisulfite to a wine can be used to differentiate between anthocyanins (whose colour is bleached by SO_2) and C4-substituted anthocyanin-derived pigments (whose colour can be considered resistant to SO_2 bleaching) (Somers 1971; Somers and Evans 1977). In current literature, much effort has been made towards identifying

and reporting the structures of anthocyanin-derived wine pigments. But research regarding the colour properties of most pigments in the presence of SO_2 is lacking (Appendices 1.3 to 1.27). Some examples of anthocyanin-derived pigments whose colour is resistant to SO_2 bleaching have been published (Sarni-Manchado *et al.* 1996; Francia-Aricha *et al.* 1997; Escribano-Bailón *et al.* 2001; Oliveira *et al.* 2006). It is highly likely that in any red wine, particularly matured wine, there are many SO_2 stable wine pigments which could contribute towards SO_2 stable wine colour, and would in turn influence aged red wine colour.

As stated above in Section 1.2.1.4, SO_2 stable wine colour is important in aged red wines where the concentration of anthocyanins is almost negligible whilst the colour of the wines remains red (Herderich *et al.* 2006). So, in any wine, by eliminating the colour of anthocyanins by the addition of excess bisulfite, it is possible to study the contribution of SO_2 stable anthocyanin derived wine pigments to wine colour and to SO_2 stable wine colour. In current literature, some researchers have investigated the colour stability to SO_2 bleaching of individual isolated anthocyanin-derived pigments (Ribereau-Gayon *et al.* 1983; Francia-Aricha *et al.* 1997; Bakker *et al.* 1998; Shoji *et al.* 1999; Escribano-Bailon 2001; Escribano-Bailón *et al.* 2001; Parley *et al.* 2001). Alternatively, other researchers have employed various methods to investigate all the SO_2 stable wine pigments present in a given red wine sample. But, the methods are limited because the individual SO_2 stable pigments cannot be differentiated. Examples in the literature include:

1. Additions of bisulfite solution made to a wine followed by cation exchange chromatography (Asenstorfer *et al.* 2001; Asenstorfer 2001).
2. Protein precipitation with bisulfite bleaching to differentiate between large and small polymeric pigments. The researchers hypothesized that large polymeric pigments precipitated with proteins, but the smaller pigments did not (Harbertson *et al.* 2003).
3. Additions of bisulfite solution were made to an acidified wine followed by gel filtration with ethanol (Asenstorfer *et al.* 2001; Alcalde-Eon *et al.* 2004). But the technique was not feasible as the sulfite ion is incapable of bleaching at low pH (Rotter 2006).

Thus, Appendices 1.1 to 1.27 summarize wine pigments described in the current literature. The tables include the colour properties of anthocyanins and anthocyanin-derived pigments in different pH media and anthocyanin-derived pigments in the presence of the sulfite ion.

1.5. Measurement of wine pigments by chromatographic techniques

It is general practice to evaluate the colour of an intact wine or an individual pigment by spectroscopic means. But to quantify individual wine pigments, it is necessary to separate a red wine using chromatographic techniques. Reversed phase HPLC analysis has become the standard method for separating and quantifying anthocyanins and related compounds (Figure 1.15 below). The technique separates the coloured anthocyanins and acylated anthocyanins in the visible spectrum, and the non-coloured phenolic grape and wine constituents such as the flavan-3-ols in the ultra-violet spectral range. But, chromatographic separation of polymeric and complex polyphenols has proven difficult. Recently specialized acid stable PLRP columns have been applied to determine "pigmented polymers" and anthocyanins in wine samples (Price *et al.* 1995; Peng *et al.* 2002).

In the HPLC analysis, acetonitrile and methanol are commonly used solvent systems. Gradient variation during the HPLC experiment using buffers such as aqueous phosphoric acid, trifluoroacetic acid or formic acid in combination with acetonitrile or methanol solvents to separate wine pigments according to polarity. Following HPLC separation, pigments can be detected using diode array systems or structurally characterized by mass spectrometry. In the former case, diode array dectection is used to create UV-visible spectra of HPLC-separated peaks. It is important to note that for every HPLC analysis, the spectral information obtained is not fully utilized.

HPLC analysis is a powerful separation tool, performed under highly acidic or low pH conditions (pH 1). The reason for the low pH value lies in the use of a buffer solution. The buffer solution maintains a constant pH value and resists changes in pH due to dilution or the addition of small amounts of acid or base. Even a small addition of a wine to the HPLC solvent system can alter the pH value. The most commonly used HPLC buffer is phosphoric acid. Phosphoric acid is ideal as it is non-volatile. But to ensure maximum performance of the phosphoric acid buffer it is necessary that a low pH value is maintained. In a dilute aqueous solution, phosphoric acid dissociates into four forms. In strongly basic conditions, the phosphate ion (PO_4^{3-}) predominates, while in weakly basic conditions, the hydrogen phosphate ion (HPO_4^{2-}) is prevalent. In weakly acid conditions, the dihydrogen phosphate ion ($H_2PO_4^-$) is most common, while in strongly acid conditions, aqueous phosphoric acid (H_3PO_4) is the main form. Thus, the buffering capacity of phosphoric acid is important. For example, at pH 4, phosphate is a poor buffer and will change to one of its other forms if a more acidic or basic sample were introduced. Thus, phosphoric acid acts as an ideal buffer at a low pH, pH ~ 1.0. Application of the gradients during an HPLC analysis means that the experiment is performed at low pH. At low pH, the pH of the medium has changed and any wine injected into the HPLC column is no longer separated at its natural wine pH.

At low pH, malvidin 3-glucoside and the other anthocyanins exists as highly coloured flavylium forms (Brouillard and Delaporte 1977; Houbiers *et al.* 1998). Consequently, the absorbance values and peak areas of highly coloured flavylium cations are high. Thus, conventional HPLC methods are sufficient for determining quantities of anthocyanins found in red grapes and young red wine to compare differences in variety, vineyard location, vinification, maceration techniques and storage conditions. The focus of such experiments is mostly the determination and quantification of anthocyanins in the red wine (Timberlake and Bridle 1976; Ribereau-Gayon *et al.* 1983; Wenzel 1987; Dallas *et al.* 1994; Almela *et al.* 1996; Wightman *et al.* 1997; Almela *et al.* 1999; Gómez-Plaza *et al.* 1999; Mazza *et al.* 1999; Parley *et al.* 2001; Arnous *et al.* 2002; Chicon *et al.* 2002; Gonzalez-Hernandez *et al.*

2002; Heier *et al.* 2002; Morais *et al.* 2002; Rodríguez-Delgado *et al.* 2002; Pérez-Magariño and González-San José 2002a; Monagas *et al.* 2003; Gómez-Míguez 2004; Gomez-Miguez and Heredia 2004; Hermosin-Gutierrez and Garcia-Romero 2004; Pérez-Magariño and González-SanJosé 2004; del Alamo-Sanza *et al.* 2004b; Hermosin-Gutiérrez *et al.* 2005; Monagas *et al.* 2005b). In the chromatogram, anthocyanins including malvidin 3-glucoside and the acylated derivatives of the anthocyanins are generally identified by their elution order and spectral profile (Figure 1.15). Further confirmation of malvidin 3-glucoside is usually in the form of a standard. This is not the case with anthocyanin-derived pigments as many anthocyanin-derived pigments are present in lower concentrations, and many remain hidden within most HPLC chromatograms. But some pigments such as Vitisin A and the pigmented polymers have been identified by the use of a standard or its position in the HPLC chromatogram (Figure 1.15). There are unidentified peaks in the HPLC chromatogram which may well be further examples of anthocyanin-derived wine pigments (Figure 1.15).
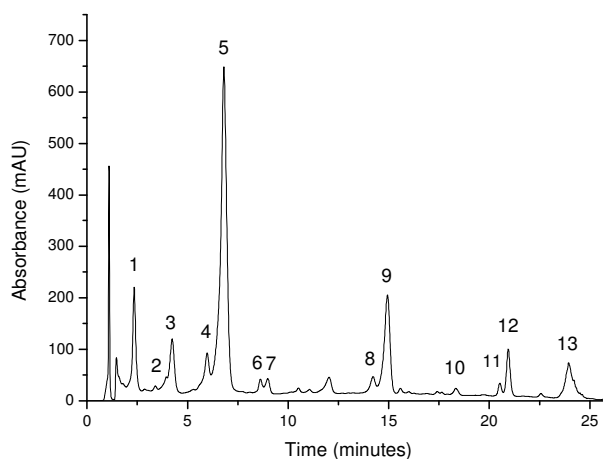
Figure 1.15: HPLC-UV/Vis chromatogram at 520 nm illustrating the separated anthocyanins and anthocyanin-derived pigments for a typical Australian Shiraz red wine. The following pigments have been identified: peak 1, delphinidin 3-glucoside; peak 2, cyanidin 3-glucoside; peak 3, petunidin 3-glucoside; peak 4, peonidin 3-glucoside; peak 5, malvidin 3-glucoside; peak 6, Vitisin A; peak 7, delphinidin-3-acetylglucoside; peak 8, peonidin-3-acetylglucoside; peak 9, malvidin 3-acetylglucoside; peak 10, petunidin 3-(*p*-coumaroyl) glucoside; peak 11, peonidin 3-(*p*-coumaroyl) glucoside; peak 12, malvidin 3-(*p*-coumaroyl) glucoside; peak 13, pigmented polymers. The HPLC analysis was performed using the gradient elution method described in Chapter 2, Table 2.3 (below).

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As there is little information regarding the pH dependence of anthocyanin-derived pigments is detailed in current literature, it is generally assumed that anthocyanin-derived pigments are not affected by the pH value. Although it is possible that some anthocyanin-derived pigments may not be pH dependent, there are some anthocyanin-derived pigments that are affected by pH change. At low pH, the peak areas of anthocyanin-derived pigments that are not pH dependent will remain unchanged at low pH. These pigments will be quantified in lower concentrations than anthocyanins.

In addition, for aged red wines, there is a reduction in the concentrations of malvidin 3-glucoside and other anthocyanins to negligible amounts (Somers 1971; Somers and Evans 1977; Dallas *et al.* 1994; Cliff *et al.* 2002; Hayasaka and Kennedy 2003a; Hayasaka *et al.* 2004). There is a simultaneous increase in the quantity of anthocyanin-derived pigments present. The chromatogram in Figure 1.15 illustrates two examples of anthocyanin-derived pigments, Vitisin A and the pigmented polymers. Vitisin A is an example of a pyranoanthocyanin described in Section 1.3.4.2.2.a above. The pigmented polymer peak (described Section 1.5.1. below) consists of many anthocyanin-derived pigments that have eluted last in the HPLC chromatogram (Figure 1.15).

As stated above, chromatographic separation of wine pigments by HPLC analysis has been performed at low pH, not at typical wine pH. It is not uncommon to find instances in current literature where the concentration of anthocyanins and anthocyanin-derived pigments separated at low pH, but have been compared with wine colour at its natural wine pH (McCloskey and Yengoyan 1981; Dallas *et al.* 1994; Fulcrand *et al.* 1996; Gómez-Plaza *et al.* 1999; Mazza *et al.* 1999; Arozarena *et al.* 2000; Parley *et al.* 2001; Chicon *et al.* 2002; Garcia-Beneytez *et al.* 2002; Gómez-Plaza *et al.* 2002; Tsanova-Savova *et al.* 2002; Mateus *et al.* 2002a; Mateus *et al.* 2002b; Fernández de Simón *et al.* 2003; Gonzalez-Martinez *et al.* 2004; Pérez-Magariño and González-SanJosé 2004; Gómez-Míguez *et al.* 2005; Lorenzo *et al.* 2005; Revilla *et al.* 2005; Alcalde-Eon *et al.* 2006; Boido *et al.* 2006; Cano-López *et*

al. 2006; Garcia-Puente Rivas *et al.* 2006). Thus, it could be mistakenly inferred that anthocyanins provide a larger than expected contribution to wine colour, particularly where some anthocyanin-derived pigments appeared to have minor peak areas in the HPLC chromatogram.

1.5.1. Pigmented polymers

All HPLC analyses will depend on the analytical HPLC method employed. In the literature, various HPLC analyses have been employed and consequently, terms such as "polymeric fraction" (Atanasova *et al.* 2002b; Monagas *et al.* 2003b; Schwarz *et al.* 2003d; Pinelo *et al.* 2006), "polymeric anthocyanins" (Price *et al.* 1995; Gao *et al.* 1997; Shoji *et al.* 1999; Tsanova-Savova *et al.* 2002; Harbertson *et al.* 2003; Perez-Prieto *et al.* 2003a; Obradovic *et al.* 2005), "polymeric pigments" (Somers 1971; Timberlake and Bridle 1976b; Bakker *et al.* 1986b; Mayen *et al.* 1994; Dallas *et al.* 1996b; Mazza *et al.* 1999; Degenhardt *et al.* 2001; Ho *et al.* 2001; Parley *et al.* 2001; Romero and Bakker 2001; Harbertson *et al.* 2003; Mansfield and Zoecklein 2003; Schwarz *et al.* 2003d; Schwarz and Winterhalter 2003g; Adams *et al.* 2004; Sáenz-López 2004; Zimman and Waterhouse 2004a; Cheynier *et al.* 2006; Guadalupe *et al.* 2006) and "pigmented polymers" (Jones *et al.* 1999; Landmann 2002; Peng *et al.* 2002; Hayasaka and Kennedy 2003; Bartowsky *et al.* 2004; Cozzolino *et al.* 2004; Eglinton *et al.* 2004; Hayasaka *et al.* 2004; Herderich and Smith 2005) have been used to describe the various collections of anthocyanin-derived pigments identified in red wines. For example, Schwarz and co-workers termed the largest highly polar peak in the HPLC chromatogram as the "polymeric fraction" (Schwarz *et al.* 2003c), whilst others have stated that the largest peak (not including the anthocyanins) was an elution of "polymeric anthocyanins" (Price *et al.* 1995; Waterhouse *et al.* 1999; Fischer *et al.* 2000). Some researchers have found that "polymeric anthocyanins" coeluted with monomeric acylated anthocyanins (McCloskey and Yengoyan 1981; Bakker *et al.* 1986b; Roggero *et al.* 1992). While Gao and co-workers established that after the monomeric anthocyanins had eluted, the remaining peaks were considered "polymeric anthocyanins" (Gao *et al.* 1997). "Polymeric anthocyanins" is not strictly a correct terminology for a

collection of anthocyanin-derived pigments appearing in an HPLC chromatogram. For example, some polymeric pigments may have a tannin subunit and would be classified as an anthocyanin-tannin conjugate (Pigments 5a – 5d, Figure 1.10; Pigments 7a – 7b Figure 1.11; Pigment 8a, Figure 1.9 Figure 1.12 and Pigments 10a – 10i, Figure 1.13). It must be emphasized that although there are exceptions to classification of wine pigments, the terms "polymeric pigments", "polymeric proanthocyanidins", "polymeric fraction", "polymeric anthocyanins" and "pigmented polymers" are not mutually exclusive. In this thesis, "pigmented polymers" shall be the term given to the convex "hump" observed as the last peak, at approximately 24 minutes in the HPLC chromatogram, from a red wine separated by RP-HPLC analysis (Peng *et al.* 2002; Hayasaka and Kennedy 2003a; Bartowsky *et al.* 2004; Cozzolino *et al.* 2004; Eglinton *et al.* 2004; Hayasaka *et al.* 2004; Herderich and Smith 2005). The term, pigmented polymers, has already been encountered with black tea polymers or "thearubigins" (Bruschi *et al.* 1999) and is now being applied to HPLC-separated red wine. As the pigmented polymers are coloured, the peak can be quantified at 520 nm like the anthocyanins. The peak is a result of a coelution of very many high molecular weight anthocyanin-derived pigments, and as a result this highly non-polar fraction is heterogeneous. The majority of these eluted coloured anthocyanin-derived compounds are complex and unknown in structure. The pigmented polymer concentration is large and for a young red wine, the pigmented polymers concentration can be similar to malvidin 3-glucoside at low pH (see Chapter 7 later). Thus, in some papers it is assumed that the pigmented polymers contribute significantly to wine colour. But to date, as no pigments from the pigmented polymer peak have been isolated from wine and characterized, no standard is available for this peak.

1.6.   Aims of the study

The literature review has highlighted a need to gain a greater knowledge and understanding of wine colour measurements and pigment characterization in red wine. The techniques such as HPLC analysis currently employed have

limitations. For example, HPLC analysis is performed at low pH, and improved analytical methods are needed to measure the colour and concentration of wine pigments under wine-like conditions and to study SO_2 stable wine colour. In order to achieve this, the thesis will investigate the following objectives using red wine samples. Two sets of red wines were used to investigate wine pigment characterization and enhanced wine colour measurements: Shiraz wine from four regions in Australia and South Australian Cabernet Sauvignon wine made using two different yeast strains.

1.7.   Objectives of thesis

1. *To demonstrate that the extinction coefficient values of wine pigments at different pH values and in the presence of SO_2 bleaching is important to the colour observed.*
2. *To demonstrate whether winemaking region influences wine colour and pigment concentration.*
3. *To demonstrate that CIELab values provide enhanced colour measurements over current wine colour measurements.*
4. *To demonstrate that some CIELab values will correlate with absorbance measurements at 420 nm and 520 nm.*
5. *To develop a method to determine the colour of a wine pigment without a lengthy, expensive isolation or separation technique at a given concentration in a red wine. That is, to create CIELab colour of a wine pigment and to associate the pigment's colour with its natural concentration in a red wine separated by HPLC analysis.*
6. *To demonstrate that the concentration and colour are important factors in the characterization of a wine pigment.*
7. *To investigate wine pigments as major influences to wine colour using malvidin 3-glucoside, Vitisin A and the pigmented polymers as examples.*
8. *To demonstrate that the concentration of pigments at low pH and the colour of a wine at its natural pH is an inaccurate representation, particularly as it is possible that some anthocyanin-derived pigments may be affected by the pH media.*

9. *To demonstrate that wine pigments of equivalent concentrations have different colours.*

10. *To investigate the importance of SO_2 stable wine colour to wine colour. Also to consider the percentage of SO_2 non-bleachable pigments and the chemical index (ii) as a further characterization of the colour of red wines.*

11. *To demonstrate that some anthocyanin-derived pigments have an influence on SO_2 stable wine colour.*

12. *To investigate the phenomenon of copigmentation and its affect on red wine colour.*

13. *To investigate the choice of yeast strain and its influence on red wine colour.*

14. *To investigate the contribution of anthocyanins and anthocyanin-derived pigments to wine colour at low pH and at natural wine pH.*

15. *To demonstrate that any wine pigment (whether an anthocyanin or anthocyanin-derived) chosen from many in a wine sample will not necessarily be a major contributor to red wine colour.*

16. *To develop a method for post-HPLC modification of separated wine pigments at wine pH and post-SO_2 bleaching to achieve pigment quantification and characterization in these two media.*

Once these objectives have been assessed and demonstrated satisfactorily, the further aims of this thesis will include:

1. *To investigate the differences in red wine colour resulting from the use of different yeast strains in terms of quantification of wine pigments in different pH media and post-SO_2 bleaching.*

2. *To determine the concentration of anthocyanins and anthocyanin-derived wine pigments at low pH (pH ~1.5), at wine pH (pH ~3.4) and post-SO_2 bleaching.*

3. *At wine pH, to demonstrate that anthocyanins play a minor role, whilst anthocyanin-derived pigments such as Vitisin A and the pigmented polymers provide a greater contribution to the colour than anthocyanins.*

4. In the case of SO_2 bleaching, to demonstrate that those anthocyanin-derived pigments that are stable in the presence of SO_2 play a major role towards SO_2 stable wine colour.

5. By considering malvidin 3-glucoside as an example of an anthocyanin, and Vitisin A and the pigmented polymers as examples of anthocyanin-derived pigments:

    a. To determine the CIELab colours of the pigments at low pH, at wine pH and post-SO_2 bleaching.

    b. To investigate whether different yeast strains have influenced the colours of the pigments.

    c. To investigate whether the pigments were responsible for the differences in wine colour at low pH, at wine pH and post-SO_2 bleaching.

Chapter 2: General materials and methods

This chapter describes the materials and methods that were common to most of the experiments conducted in this thesis. Materials and methods that were specific to a particular experiment will be described in the relevant chapters.

2.1. Materials

Investigations of red wine colour and wine pigments present were performed on wines made from two varieties of grapes, Shiraz and Cabernet Sauvignon. The grapes were sourced from different regions in Australia. The winemaking procedures and ageing characteristics were the parameters of the individual experimental design. The Shiraz and Cabernet Sauvignon red wines were part of a larger investigation into various aspects of winemaking techniques, and consequently, excess wines were available to study the influences to red wine colour.

In addition to the investigations using red wines, the characteristics of an individual anthocyanin and three individual anthocyanin-derived wine pigments, at low pH, at wine pH and in the presence of SO_2, were studied.

2.1.1. 2003 Wine trial using Australian Shiraz red wines

The Shiraz wines investigated were originally part of a trial used to benchmark a vineyard's performance (Rolley 2004). Approximately 50 kg of Australian Shiraz red grapes were obtained from four sampling locations during the 2003 vintage. The locations of the vineyards were Gundagai in New South Wales, and Bendigo, Echuca and Swan Hill in Victoria. The grapes were sourced from a total of 38 vineyard sites. In addition to the variation in the location of the grapes, the climate varied between the four regions (data not shown). The wines were made at the National Wine and Grape Industry Centre (NWGIC) experimental winery. The same winemaking conditions were applied to the grapes. The grapes were destemmed and crushed into 100 L fermenting

tanks. The composition of the must in each fermenter was adjusted with tartaric acid to give a pH of 3.6 and with potassium metabisulfite (PMS) to give 50 mg (total) SO_2/L. The must was inoculated with *S. cerevisiae* AWRI 796 at a concentration of 0.2 g/L after rehydration. Diammonium phosphate (DAP) was added at a concentration of 0.2 g/L. Fermentation was conducted at 25 to 30 °C. During fermentation, the cap was plunged at 4 to 5 hourly intervals using a stainless steel plunger. When the Baumé was less than 4, the ferment was inoculated with Viniflora® for malolactic fermentation (MLF) according to the supplier's recommendation. After 6 days on skin, where ferments were less than 1 Baumé, a pressing protocol was applied. An airbag press at 2 kPa was used to ensure reproducible colour extraction and uniformity of the red wines. The wines were checked for residual sugar using the Rebelein method (Iland *et al.* 2004) and hydrogen sulfide and aerated. On completion of MLF, the wines were transferred to clean 100 L tanks and cold stabilized at 4 °C. Prior to bottling and after approximately three weeks in cold stabilization, adjustments were made so that the levels of free SO_2 and tartaric acid were 25 to 30 mg/L and 6.5 g/L respectively. 750mL Screw cap bottles were washed with 5% PMS solution and flushed with carbon dioxide gas prior to filling. The wine was not filtered. Triplicate samples (Replicate 1, 2 and 3) from each site were prepared. The bottles were sealed and stored at approximately 14°C prior to transportation to the AWRI. Only replicates 1 and 2 were analyzed. The third replicate was kept for future studies.

2.1.2. 2002 Wine trial using South Australian Cabernet Sauvignon red wines

Cabernet Sauvignon red grapes (12 bins of approximately 400 kg each) were mechanically harvested during the 2002 Australian vintage from a Padthaway vineyard. The grapes from two randomly selected bins were destemmed and crushed into three rotary fermenting tanks each of 900 L capacity. The Cabernet Sauvignon wines were made by Stephen Clarke and Chris Day at Hickinbotham Roseworthy experimental winery science laboratory at the University of Adelaide. The composition of the must in each fermenter was

adjusted with tartaric acid to give pH 3.6 and with potassium metabisulfite to give 20 mg (total) SO_2/L. The must was inoculated with *Saccharomyces cerevisiae* AWRI 838 (SC, 3 fermenters) or S. *bayanus* AWRI 1375 (SB, 3 fermenters) wine yeast at 5 x 10^6 cells/mL. Fermentations were conducted at 20 °C. The ferments were pressed when the total soluble solids (TSS) data was between 0 and 2.6 °Be (mean of 0.9 °Be) after 5 to 6 days of fermentation. Fermentation was completed 2 days after pressing (residual sugar < 5 g/L) in upright stainless steel tanks of 760 L capacity. The wines were racked from gross lees and inoculated for malolactic fermentation (MLF) with Oenococcus oeni Lalvin® EQ54. After MLF was complete, the concentration of SO_2 (total) was adjusted to 60 mg/L. The wines were cold stabilised at 0 °C for 6 weeks, filtered (0.45 µm), bottled in 750 mL roll on tamper evident (ROTE)-sealed bottles. Storage of the bottles were at 15 °C until analysis of the wine at given time intervals. The time intervals were 0 (two days after pressing, termed "post-pressing"), 7, 13, 22 and 25 months after inoculation with wine yeast.

2.1.3. Colour properties of four wine pigments

The colour properties of an anthocyanin, malvidin 3-glucoside and three anthocyanin-derived pigments, Vitisin A, and 4-vinylcatechol and 4-vinylsyringol adducts to malvidin 3-glucoside were investigated at low pH, at wine pH and post-SO_2 bleaching (Table 2.1).

2.1.3.1.   Malvidin 3-glucoside

The anthocyanin, malvidin 3-glucoside, was obtained from Polyphenols Laboratories, Sandnes, Norway. The pigment was employed in four ways in this thesis:
1. As a standard to confirm the identity of malvidin 3-glucoside in the HPLC chromatogram using the standard HPLC method.
2. As an external calibration to quantify the concentration of wine pigments separated by HPLC analysis.

3. To confirm that post-HPLC column pH adjustment and SO$_2$ bleaching was successful.

    a. Following HPLC separation and pH adjustment, a lower absorbance of malvidin 3-glucoside was observed. The change in pH value resulted in a shift of equilibrium as the less coloured isoforms of malvidin 3-glucoside were observed.

    b. The colour of malvidin 3-glucoside was bleached by SO$_2$, hence no absorbance and no peak area was recorded at the standard elution time of the pigment.

4. To compare the spectroscopic colour properties of malvidin 3-glucoside with two anthocyanin-derived pigments, vinylpyranoanthocyanins (Section 2.1.3.2 below) at low pH, at wine pH and post-SO$_2$ bleaching (Table 2.1).

2.1.3.2. Anthocyanin-derived pigments

The colour properties of three anthocyanin-derived pigments, Vitisin A, and 4-vinylcatechol and 4-vinylsyringol adducts to malvidin 3-glucoside were investigated. The three pigments were examples of the first type of anthocyanin-derived pigments described earlier, the pyranoanthocyanins. The pyranoanthocyanins were synthesized in the laboratory at the AWRI (Håkansson *et al.* 2003; Schwarz *et al.* 2003d).

Vitisin A was used as a standard to confirm its identity in the HPLC chromatogram using the standard HPLC method (conventional HPLC analysis) (Section 2.2.2.1.) and following post-HPLC column pH adjustment and SO$_2$ bleaching (Chapter 7).

The pigments, 4-vinylcatechol and 4-vinylsyringol adducts to malvidin 3-glucoside, were prepared in three different media solutions, and spectroscopic colour properties evaluated (Table 2.1). The colour properties of the two vinyl adducts were compared to malvidin 3-glucoside at low pH, at wine pH and in the presence of excess bisulfite solution (Table 2.1).

Table 2.1: Media conditions subjected to individual isolated wine pigments.

Media condition	Model solution
At low pH, at pH 1.5	Model wine [a] containing 50 mM/L HCl
At wine pH, at pH 3.6	Model wine [a]
Excess bisulfite solution	2 g/L $Na_2S_2O_3$ in model wine [a]

[a] Model wine prepared using 0.25 % w/v potassium bitartrate, 12 % v/v ethanol (aq) adjusted to pH 3.6 using NaOH (aq).

2.2. Methods

Spectroscopic measurements were used to determine the colour properties of all the pigments in the red wines. Chromatographic analysis, which included UV/Vis and MS analyses, of separated wine pigments were also performed. As the pH media and the presence of SO_2 in the wines affected wine colour, the chemistry of the wines (e.g. pH, titratable acidity, concentration of organic acids and free and total SO_2) were determined using standard procedures by the Analytical Service at the AWRI.

2.2.1. Standard colour measurement of red wine by UV-Vis spectrometry

Prior to any colour measurement, the wine was centrifuged at 4000 rpm for 5 minutes at 20 ^{o}C in a Universal 32R centrifuge (Hettich). Colour measurements were made using a Cary 300 UV-Vis spectrophotometer (Varian, Victoria, Australia) at 25 ^{o}C with appropriate quartz cuvettes. In addition to the standard absorbance measurements made on the red wine, the CIELab colours and hues of wines at their natural pH, the wine colour post-addition of excess sodium metabisulfite solution, and the diluted wine colour at pH 1 and at pH 3.6 were made simultaneously with the standard absorbance measurements.

Spectral absorbance profiles of the red wines across the visible spectrum from 400 to 700 nm were determined using the Cary WinUV Scan Application 02.00 (Varian, Victoria, Australia). The absorbance values at two wavelengths (Table 2.2) were adjusted for cuvette size and dilution.

Table 2.2: Spectral measurements made on wine samples at the AWRI using the Varian UV/Vis spectrophotometer.

Measurement	Colour measurement	Cuvette dimensions	Multiplication factor [d]
Wine colour density, WCD (a.u.)	$A_{420\ (colour)} + A_{520\ (colour)}$	1 mm	10
Wine colour hue, WCH	$A_{420\ (colour)} / A_{520\ (colour)}$	1 mm	Not applicable
SO$_2$ stable wine colour (a.u.) [a]	$A_{420\ (SO2)} + A_{520\ (SO2)}$	1 mm	10
SO$_2$ stable pigments (%) [a]	$\dfrac{A_{420\ (SO2)} + A_{520\ (SO2)}}{(A_{420\ (colour)} + A_{520\ (colour)})}$	1 mm	Not applicable
Total red pigment colour [b]	$A_{520\ (HCl)}$	10 mm	101
Wine colour in model wine [c] at pH 3.6 [d] (a.u)	$A_{420\ (buffered\ colour)} + A_{520\ (buffered\ colour)}$	10 mm	10

[a] Addition of 30 μL of 20 % w/v Na$_2$S$_2$O$_3$ (aq) to 2 mL of sample followed by measurement after 30 minutes.

[b] Addition of 100 μL sample to 10 mL 1 M HCl (aq) followed by spectroscopic measurement after 4 hours.

[c] Model wine prepared using 0.25 % w/v potassium bitartrate, 12 % v/v ethanol (aq) adjusted to pH 3.6 using NaOH. It is assumed that the pH change of a wine on addition of the metabisulfite is negligible.

[d] Addition of 200 μL wine to 1800 μL model wine solution was used to account for the differences in pH between individual wines and to investigate the phenomenon known as copigmentation.

All the wine colour measurements were made using the cuvette dimensions described in Table 2.2. But as a methodology had yet not been established at 0 months ("post-pressing") for the wine colour measurement of the Cabernet Sauvignon red wines, spectral measurements were made using a Foss NIRSystem 6500 spectrophotometer (FOSS NIRSystems, Silver Spring, MD, USA) in 1 mm cuvettes at 33 °C, the standard instrument operating temperature.

Simultaneously to the absorbance colour values, CIELab colour measurements were made. These measurements included wine colour at its natural wine pH, at low pH and the SO_2 stable wine colour. CIELab colour values (L*, a* and b*) were obtained using the Cary WinUV Color Application 3.00 (Beta) (Startek Technology Pty, Victoria, Australia) across a wavelength range of 400 to 700 nm at 1 nm increments. Wine samples were viewed as a 10° standard observer and the lighting conditions were representative of a D65 illuminant. The CIELab colour coordinates were used to calculate chroma, C* and hue, H* values where C* = $\sqrt{(a^{*2} + b^{*2})}$ (Equation 1.2) and H* = $\tan^{-1}(a*/b*)$ (Equation 1.3) above.

A baseline correction was applied prior to each colour measurement. Water was used as a blank for wine colour at wine pH and SO_2-stable wine colour, and 1 M HCl for the acid-diluted wine, at low pH. The CIELab colour values were not adjusted for dilution or optical pathlength. In addition, the percentage of SO_2 stable pigments was not determined from CIELab colour coordinates as the calculation required a ratio of absorbance profiles before and after addition of excess bisulfite solution (Table 2.2).

The program, Adobe® Photoshop® 6.0 for Windows® (Adobe Systems Incorporated, California, USA) was employed to reproduce the CIELab colours of the wines following spectroscopic colour measurement. The program was also employed to determine the peaks from an HPLC-separated wine using the HPLC-CIELab method (Chapter 3) and on application of the post-HPLC column adjustment method (Chapter 8). The colours of HPLC-separated wine pigments at low pH, at wine pH and post-SO_2 bleaching using the HPLC-CIELab method was achieved (Chapter 8). The reproduced CIELab colour values were used to illustrate the colour of the wine or pigment to the reader. These colours enabled the reader to visualize the colour of the wine, the SO_2-stable wine colour or highly acidified diluted wine colour. But, it must be noted that the CIELab colours viewed in this thesis, either on the computer monitor or in the printed page, are dependent on the hardware (and any calibration methods employed), the software of the computer, and ambient

lighting used to view the colours. As an example, the colours of the pigmented polymers determined using the HPLC-CIELab method (Chapter 6) was viewed under two different lighting conditions. First, the colours of the pigmented polymers were viewed using a halogen light (equivalent to black body radiation of 3130 to 3430 K) in a darkened room. The colours of the pigmented polymers appeared brown. However, when the colours of the pigmented polymers were viewed under normal daylight conditions (equivalent to black body radiation equivalent to approximately 5000 K), the colours were very different; the pigmented polymers appeared red-violet instead.

2.2.2. Chromatographic analysis of wines

The red wines were subjected to chromatographic separation and, where appropriate, the pigments were characterized in terms of absorbance values by UV/Vis or by mass spectrometry. No attempt was made to structurally characterize the pigments in the wines.

2.2.2.1. Analysis of wine pigments by HPLC-UV/Vis spectroscopy

In this thesis, all wine samples were analysed using the standard HPLC method called the "Hydro 6 method" (Eglinton *et al.* 2004). Separation was at 25 °C by RP-HPLC analysis using an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) (Table 2.3). A sample volume of 20 µL was injected into the Phenomenex Synergi Hydro-RP column (150 mm x 2 mm) and the flow rate of the solvent system was 0.4 mL/min.

Table 2.3: Gradient elution of the standard HPLC method.

Time (minutes)	Solvent A [a]	Solvent B [b]
0.00	85.5	14.5
18.00	72.5	27.5
20.00	72.5	27.5
21.00	49.5	50.5
22.00	49.5	50.5
26.00	0	100.0
28.00	0	100.0
28.01	85.5	14.5
38.00	85.5	14.5

[a] Solvent A: 1 % acetonitrile, 1.5 % phosphoric acid in water.
[b] Solvent B: 20 % solvent A [a], 80 % acetonitrile.

Using the standard HPLC method, the highly polar anthocyanins and their associated acylated derivatives eluted first in the HPLC chromatogram and appeared as well-separated peaks (Figure 1.15). Anthocyanins and their acylated derivatives were identified in terms of their expected elution order and their spectral characteristics. In addition, the major anthocyanin, malvidin 3-glucoside, was established using a standard. Vitisin A, the monomeric anthocyanin-derived pigment, eluted at approximately 10 minutes. Similarly, Vitisin A was confirmed using a Vitisin A reference standard. Pigments that eluted as the last "hump" at the end of the chromatogram were known as the pigmented polymers. These pigments were of low polarity and eluted at approximately 24 minutes in the HPLC chromatogram.

As the maximum absorption of anthocyanins is considered to be in the range from 520 to 530 nm, the wine pigments were detected by photodiode array detection at 520 nm. Quantification of the wine pigments was in mg/L malvidin-3-glucoside equivalents using an external calibration function. The calibration function was calculated using replicated spiked standard additions of malvidin-3-glucoside hydrochloride (Polyphenols Laboratories, Sandnes, Norway) in the HPLC method developed by Eglinton and co-workers at the

AWRI (Eglinton *et al.* 2004). The calibration equation used to calculate the concentration in mg/L malvidin 3-glucoside equivalents:

Pigment concentration = 0.00915 x peak area + 0.79060

2.2.2.2. Analysis of wine pigments by mass spectrometry

HPLC-MS analysis was carried out on the Cabernet Sauvignon red wines from the 2002 AWRI wine trial at 0 months ("post-pressing") and 13 months post-fermentation. The wine samples at 0 months were stored at –20 °C until analysis at 13 months.

2.2.2.2.a. Analysis of wine pigments by electrospray ionization - tandem mass spectrometry

Mass spectrometric analysis was carried out with an API-300 triple quadrupole mass spectrometer equipped with an electrospray (ES) ion source (MDS-Sciex, Concord, Canada). A 3 mL aliquot of the wine sample was diluted with an equal volume of water, followed by the addition of a few drops of 5 M HCl. The diluted wine was loaded onto a C18 SepPak cartridge (Waters Australia, Rydalmere, NSW). The cartridge was washed with 30 mL of water. Anthocyanin and related pigments were eluted from the cartridge with 2 mL of methanol. The methanol extract was concentrated to 0.1 mL under nitrogen, diluted with 0.1 mL of formic acid: water: acetonitrile solution (5:15:80, v/v/v) and was infused (5 µL/min) into the ES source with a syringe pump (Cole-Parmer, Vernon, USA). The ES needle, orifice and ring potentials were set to 5000 V, 30 V and 250 V, respectively. The curtain (nitrogen) and nebulizer (air) gases were set at 8 and 12 units, respectively. Nitrogen gas was used as the collision gas (set at 2 units) and the collision energy was varied in the range of 30 to 45 eV. Neutral loss spectra and product ion spectra were recorded with a step mass size of 0.1 and dwell time of 0.5 msec. The mass spectra were consecutively accumulated until an appropriate ion intensity was obtained using the multi-channel-acquisition (MCA) mode within the Sample Control software version 1.3 (MDS Sciex).

2.2.2.2.b. Analysis of wine pigments by infusion electrospray ionization - tandem mass spectrometry

A 20 μL aliquot of the wine sample was injected by an autosampler (HP1100, Agilent) and separated on a Phenomenex Synergi Hydro-RP (150 mm x 2 mm) HPLC column. For HPLC-MS, a binary gradient with mobile phases containing formic acid: water: acetonitrile: (solvent A) 5:95:0 and (solvent B) 5:15:80 (v/v/v) was used. The linear gradient elution conditions were as follows: flow rate 180 μL/min, 0 min (10 % solvent B), 35 min (35 % solvent B), 60 min (60 % solvent B), 61 min (60 % solvent B). The eluent from the HPLC was split using a T-piece with 22.5 % of the total flow to the mass spectrometer and 77.5 % to the UV detector (HP1100, Agilent). The wavelengths monitored were 280, 450 and 520 nm.

ES mass spectra were recorded from m/z 200 to m/z 2000 with a step mass size of 0.2 and dwell time of 0.4 msec. In HPLC-MS/MS experiments, the product ion scan was carried out with a dwell time of 0.5 msec and a step mass size of 0.2 and the dwell time for multiple ion reaction (MRM) was set at 200 msec for each reaction. The ions selected for MRM experiments were *m/z* 561 and *m/z* 399 for Vitisin A, *m/z* 517 and *m/z* 355 for Vitisin B, *m/z* 625 and *m/z* 463 for Pinotin A and *m/z* 609 and *m/z* 447 for Pigment A.

2.2.3. Wine chemistry determination

Wine chemistry was measured by the Analytical Service at the AWRI. The measurements included the pH, titratable acidity, organic acid quantification, and free and total SO_2. The pH of the wines was analyzed using a calibrated pH meter and combination Orion electrode. The titratable acidity, expressed as g/L tartaric acid, was measured at an endpoint pH of 8.2 using a degassed wine sample. Organic acid (malic, lactic, acetic, succinic and tartaric acid) quantification was by HPLC analysis. HPLC analysis of malic and lactic acids in the wines was made in conjunction with enzymatic analysis. As colour measurement of wine post-acetaldehyde addition was not applied, only the

free and total sulfur dioxide (expressed as mg/L) were measured using the Rankine and Pocock aspiration method (Rankine and Pocock 1970).

2.2.4. Statistical applications

Several software programs were employed in this thesis:
1. The JMP[TM] package version 5.0.1a (Statistical Discovery Software[TM], SAS Institute Inc., USA) was used to create box plots of the Shiraz red wine colour data.
2. The Microsoft ® Excel 2002 software was used in the development of the HPLC-CIELab method to calculate the mean spectra of the intact wine. The JMP[TM] package version 5.0.1a was used to calculate the mean spectra of the HPLC-separated wine across the entire chromatogram (from zero to 26 minutes).
3. GraphPad Prism 4 for Windows (GraphPad Software, San Diego, USA) was used as a statistical and graph-plotting program. The program was used to estimate the correlations at various confidence intervals and perform 2-way Student's t-tests.

Chapter 3: Concentration and colour properties of an anthocyanin and two anthocyanin-derived pigments

3.1.   Introduction

In the literature, some researchers have quantified some anthocyanin-derived pigments in negligible amounts, or have stated that some wine pigments were only barely detectable in a red wine sample (Revilla *et al.* 1999; Degenhardt *et al.* 2000; Asenstorfer *et al.* 2001; Mateus *et al.* 2001a; Mateus and de Freitas 2001b; Revilla and González-SanJosé 2001; Heier *et al.* 2002; Atanasova *et al.* 2002b; Mateus *et al.* 2002c; Monagas *et al.* 2003a; Morata *et al.* 2003; Schwarz *et al.* 2003d; Alcalde-Eon *et al.* 2004; de Villiers *et al.* 2004; Pozo-Bayón *et al.* 2004; Spranger *et al.* 2004; Schwarz *et al.* 2005; Oliveira *et al.* 2006). Since some researchers have invested a great deal of time and effort to isolate or synthesize a pigment, this has led to the claim that a given pigment is responsible for the colour of a red wine (for example, Lee and co-workers have suggested that because the ethyl-linked anthocyanin-derived pigments are purple in colour, the purple hues of young red wines, particularly immediately after the completion of fermentation, are a result of the high concentrations of these pigments (Lee *et al.* 2004). In most cases, the researchers have neglected to state the extinction coefficient of the wine pigment. The extinction coefficient is an important parameter to the colour observed. For example, in a given pH medium, a large extinction coefficient value will indicate a high colour absorbance. On the other hand, it is likely that a barely detectable pigment will have a low extinction coefficient value. There are, however, limited instances of extinction coefficient values of wine pigments at wine pH in the current literature (Lima *et al.* 2002; Håkansson *et al.* 2003). But, there is even less information regarding extinction coefficient values of wine pigments in the presence of SO_2 bleaching (Håkansson *et al.* 2003; Oliveira *et al.* 2006). Thus, it is necessary to demonstrate the influence of extinction coefficient value to the colour of wine pigments in different pH media and in the presence of the bisulfite bleaching.

3.2.　Objective

To demonstrate that extinction coefficient values at different pH values and in the presence of SO$_2$ bleaching is important to the resulting colour of the pigment observed.

3.3.　Materials and methods

The concentration and colour properties of an anthocyanin and two anthocyanin-derived pigments were evaluated at low pH, at wine pH and after bleaching with excess bisulfite solution. The anthocyanin was malvidin 3-glucoside, whilst the pyranoanthocyanins, 4-vinylcatechol and 4-vinylsyringol adducts to malvidin 3-glucoside represented anthocyanin-derived pigments. A concentration of 9.9 mg/L malvidin 3-glucoside, and 43.6 mg/L and 44.4 mg/L of the 4-vinylcatechol and 4-vinylsyringol adducts to malvidin 3-glucoside respectively were prepared in the following three media:

a.　Low pH conditions: Model wine solution containing 50 mM HCl to achieve pH 1.5.

b.　Wine pH conditions: Model wine with 0.25% w/v potassium bitartrate, 12% v/v ethanol (aq), adjusted to pH 3.6 with 1 M NaOH.

c.　Excess bisulfite solution: 2 g/L Na$_2$S$_2$O$_3$ in model wine solution at pH 3.6.

The colours of the wine pigments in the three media were determined as CIELab colour values, whilst the extinction coefficient values were determined from absorption measurements at 520 nm by standard UV-Vis spectroscopy.

3.4.　Results and discussion

Figure 3.1 illustrate the L* and C* values of the wine pigments at low pH, at wine pH and post-SO$_2$ bleaching.

At low pH, malvidin 3-glucoside appeared highly coloured (low L* and high C* values) compared to the two pyranoanthocyanins (Figure 3.1). The higher

colour of malvidin 3-glucoside was confirmed in terms of Beer's Law (Equation 1.1). That is, the concentration of malvidin 3-glucoside was 9.9 mg/L and its high extinction coefficient (27600 L cm^{-1} mol^{-1}) at 520 nm (Håkansson *et al.* 2003) resulted in a highly coloured pigment at low pH. Although the concentration of both pyranoanthocyanins were 4.5 times greater than the malvidin 3-glucoside concentration (approximately 44 mg/L), less colour was observed (higher L* and lower C* values) in the two pyranoanthocyanins compared to malvidin 3-glucoside. This was attributed to the low extinction coefficient values (10000 and 7800 L cm^{-1} mol^{-1} at 520 nm for both 4-vinylcatechol and 4-vinylsyringol adducts to malvidin 3-glucoside respectively (Håkansson *et al.* 2003).
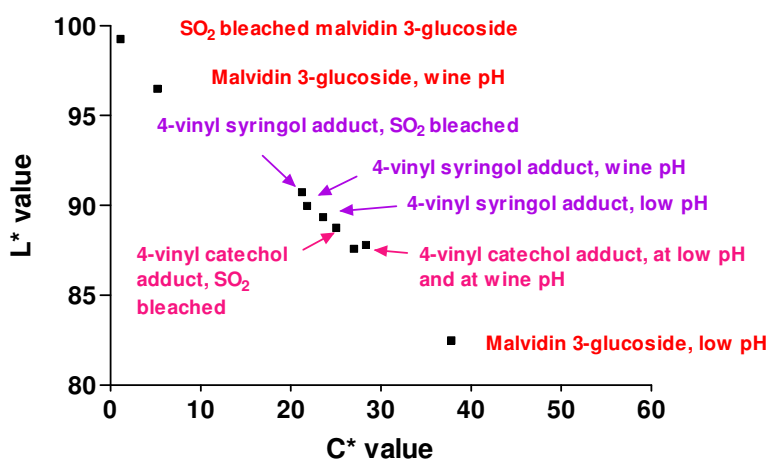
Figure 3.1: CIELab colour of malvidin 3-glucoside [a], and the 4-vinylcatechol [b] and 4-vinylsyringol [c] adducts to malvidin 3-glucoside at low pH [d], at wine pH [e] and in the presence of excess bisulfite [f] measured using a 1 mm cuvette.

[a] 9.9 mg/L of malvidin 3-glucoside.

[b] 43.6 mg/L of 4-vinylcatechol adduct to malvidin 3-glucoside.

[c] 44.4 mg/L of 4-vinylsyringol adduct to malvidin 3-glucoside.

[d] At pH 1.5, in model wine [e] containing 50 mM/L HCl.

[e] At pH 3.6, in model wine prepared using 0.25% w/v potassium bitartrate, 12% v/v ethanol and adjusted to pH 3.6 using 1 M NaOH.

[f] In the presence of excess bisulfite, 2 g/L $Na_2S_2O_3$ in model wine [e] at pH 3.6.

At wine pH, although the malvidin 3-glucoside concentration was unchanged (9.9 mg/L), the anthocyanin appeared lighter in colour (higher L* and lower C*

values) than the two vinyl adducts to malvidin 3-glucoside (Figure 3.1). In terms of Beers' Law (Equation 1.1), the reduction in colour was attributed to 74 % reduction in the extinction coefficient value of malvidin 3-glucoside at wine pH (7100 L cm^{-1} mol^{-1} at 520 nm) (Håkansson *et al.* 2003). There was a 16% to 26% reduction in the extinction coefficient values from low pH to wine pH for both pyranoanthocyanins (Håkansson *et al.* 2003). Extinction coefficients of 8400 and 5800 L cm^{-1} mol^{-1} at 520 nm were recorded for the 4-vinylcatechol and 4-vinylsyringol adducts to malvidin 3-glucoside respectively (Håkansson *et al.* 2003). In terms of Beer's Law (Equation 1.1), the higher concentrations of the pyranoanthocyanins (approximately 44 mg/L) and the extinction coefficients values resulted in more colour exhibited (low L* and C* values) for the two anthocyanin-derived pigments than malvidin 3-glucoside. It is most likely that for equivalent concentrations of the three pigments at wine pH, similar colour absorptions would have been observed as the extinction coefficient values were similar for all three pigments. In addition, the minor reduction in the extinction coefficient values of the pyranoanthocyanins implies the pH stability of the two anthocyanin-derived wine pigments.

The anthocyanin and anthocyanin-derived pigment colours in the presence of excess bisulfite solution were compared. The colour of malvidin 3-glucoside was bleached by the sulfite ion as CIELab colour values of L* = 99.5 and C* = 0 (indicating "white") were observed (Figure 3.1). The extinction coefficient value of malvidin 3-glucoside in the presence of the sulfite ion was not verified as it was assumed that the value for malvidin 3-glucoside in this medium would be negligible. Conversely, the colours of the pyranonanthocyanins were not bleached by the sulfite ion. The pigments were considered stable to SO$_2$-bleaching as only a minor reduction was observed (0 to 7% reduction) in the extinction coefficient values of the pyranoanthocyanins in the presence of the sulfite ion compared to the extinction coefficient values at wine pH. The extinction coefficient values in the presence of the sulfite ion were 7800 and 5800 L cm^{-1} mol^{-1} at 520 nm for the 4-vinylcatechol and 4-vinylsyringol adducts to malvidin 3-glucoside respectively. Almost equivalent CIELab colour values at wine pH and in the presence of SO$_2$ were observed for the 4-

vinylcatechol adduct (Figure 3.1) confirmed by the ΔE* values which tended to zero. This further indicated the SO_2 stability of the two pyranoanthocyanins and it was inferred that the two vinyl adducts to malvidin 3-glucoside would probably contribute to SO_2-stable wine colour.

Thus, the concentration, the extinction coefficient value and the pH value or presence of the sulfite ion will influence the colour of a wine pigment that is observed. When making an assessment of the colour contribution of a pigment to wine colour, it is important to acknowledge the concentration and extinction coefficient of a pigment at a given pH value or SO_2 bleaching medium. But, it can be difficult to determine the extinction coefficient values of the many wine pigments in a typical red wine without isolation or synthesis of each individual pigment. An HPLC-CIELab method will be developed in Chapter 4 which will provide an alternative and more appropriate method of pigment characterization. The HPLC-CIELab method can be used in conjunction with the post-HPLC column adjustment method can be used to determine the colour of a pigment at wine pH or an SO_2-stable wine pigment at its natural concentration in a red wine. The post-HPLC column adjustment method will be described in later in Chapters 7 and 8.

3.5.   Conclusions

1. *The colour of malvidin 3-glucoside at low pH, at wine pH and after bleaching with excess SO_2 was demonstrated.*
2. *The two pyranoanthocyanins, 4-vinylcatechol and 4-vinylsyringol adducts to malvidin 3-glucoside, were found to be approximately stable to pH change and in the presence of excess bisulfite solution. The vinyl adducts to malvidin 3-glucoside could be classed as examples of SO_2 stable wine pigments.*
3. *The concentration, extinction coefficient and pH value or presence of the sulfite ion will influence the colour of a wine pigment that is observed. But it can be difficult to determine the extinction coefficient of many wine pigments from a typical red wine without isolation or synthesis of each*

individual pigment. The HPLC-CIELab method developed in Chapter 4 and when used with the post HPLC-column pH adjustment method (which shall be developed in Chapter 7) can be used to determine the colour of a pigment at wine pH or an SO$_2$-stable wine pigment at its natural concentration in a red wine (Chapter 8).

Chapter 4: A method to determine the CIELab colour of HPLC separated peaks

This chapter describes the development, validation and application of a method termed the "HPLC-CIELab" method which can be used to determine the CIELab colour values of pigments separated from red wine by HPLC analysis.

4.1. Introduction

Appendices 1.1 to 1.24 illustrates the many wine pigments which have been either isolated from a wine or have been synthesized prior to an estimation of their colour properties. But most pigments have been characterized at concentrations greater than expected in wine. It is far more appropriate to determine the colour of a pigment at its natural concentration from an actual red wine. To determine the amount of colour a pigment contributes to a wine, an ideal scenario would involve the creation of a colour mixture. As an example, some researchers have mixed known quantities of food colours and obtained spectral profiles for each mixture (Hofer and Jenewein 1997; Berzas Nevado *et al.* 1999). They have then applied regression analysis to determine the presence and proportion of food colours from a certain foodstuff solely from its spectra. But, this method is not feasible with wine colour as there are a large number of pigments that could present in any given red wine. Pigments, including anthocyanins, and known and unknown anthocyanin-derived pigments, would be present in varying concentrations, and the method would require isolation of the major pigments before any colour mixture could be created.

Countercurrent coil chromatography has been used to isolate pigments from a red wine. The combination of countercurrent chromatography and colour dilution analysis (Section 1.2.3.3.) has enabled the colour properties of individual pigments from a red wine to be determined (Degenhardt *et al.* 2000; Garcia-Alonso *et al.* 2003; Schwarz *et al.* 2003a). Not only is the entire

process lengthy and requires expensive apparatus, but the methods are limited to assessing a small number of pigment samples at a time. Thus, a cost-effective, facile technique, that separates many pigments from a wine, is desperately needed.

In addition, the technique of colour dilution analysis is not entirely accurate. Colour blindness and judgement of the visualization detection threshold, may differ between observers and influence perceived colour contribution of a pigment. Thus, the results may not be totally reproducible and so an objective method is needed to assess pigment colour. To fulfil this requirement, colour must be represented by values that are independent of any observer. CIELab colour values are ideal for this purpose. Using CIELab values, colour can be reproduced and visually perceived by anybody. In fact, CIELab colour values have been successfully applied to compare the colours of different anthocyanins (Appendices 1.1 and 1.2). But, in many cases, the CIELab values of anthocyanins have been presented in the literature without stating the associated pigment concentration (Bakker and Timberlake 1997; Gonnet 1998; Heredia *et al.* 1998; Stintzing *et al.* 2002; Pissarra *et al.* 2003a). In terms of Beers' Law (Equation 1.1), the two variables, concentration and actual colour impact on the observation made by the human eye. Therefore, an objective method is needed to determine not only the colour but the concentration of a pigment from a red wine.

A pigment at its natural concentration in a wine is best determined by HPLC analysis. But, the technique has not been fully exploited. Currently, it remains general practice to only quantify the concentrations of pigments in wine, usually in terms of a reference. The spectral data accumulated during every HPLC-UV/Vis analysis is generally ignored. At every time point, two dimensional spectral data (wavelength versus absorabance data) is recorded. The spectral profile includes the absorbance value and associated wavelength range from 220 to 750 nm. Essentially, the three-dimensional database (which also includes the time axis) is occasionally used to confirm peak purity in HPLC chromatograms, but generally serves no other purpose.

Thus, a new and novel method which involves HPLC separation of a red wine followed by spectral data extraction and CIELab value calculation of wine pigments shall be presented in this chapter. The new and novel method, which has not been previously published anywhere, enables the CIELab colour of individual peaks from an HPLC chromatogram to be determined. The technique shall be termed the "HPLC-CIELab method".

4.2. Objective

To determine the CIELab colour of pigments separated from a wine by HPLC analysis allowing the pigment to be quantified at its natural concentration from a red wine.

4.3. Challenges to developing the HPLC-CIELab method

Throughout the stages of method development, various factors were considered and where necessary, experiments were applied to certain steps to validate the method.

Step	Procedure	Explanation
1	To demonstrate that colour is an intensive quantity.	*There are mixtures of pigments in a red wine. Consequently, a colour mixture is the weighted mean of all the component spectral profiles.*
2	To equate the colour of an HPLC-separated wine with an intact wine at low pH.	*Comparison of coloured systems must be performed at the same pH value. For example the absorption profiles of a wine at low pH and a wine separated by HPLC analysis at low pH would be similar. Essentially, the two profiles would differ in terms of their intensities. To associate the two absorption profiles, it is necessary to employ a normalization factor.*
3	To determine optimum colour values for any HPLC-separated peak using an appropriate multiplication factor.	*The normalization factor determined in Step 2 cannot be used for an individually separated peak. An appropriate multiplication factor will achieve CIELab colour values that are appropriate to red wine colour.*

4.4.   Materials and methods

4.4.1.   To demonstrate that colour is an intensive property *(Step 1)*

Colour is an intensive property. Understanding this concept is important as there are mixtures of pigments in a wine, and there can be some confusion regarding the definitions of colour mixing and colour additivity. For example, when two different red wine samples are mixed together, the solution does not appear darker or redder. Instead a change in the intensity or chromaticity of the colour occurs. The colour mixture is essentially the weighted mean of all the component spectral profiles.

To demonstrate that the colour observed is the weighted mean of its individual components, the spectral profile of four red wine samples A, B, C and D were created using the spectrophotometer. The vintage and varietal characteristics of the four wines were inconsequential to the experiment. The CIELab values of each wine were determined for a D65 illuminant, a 10° standard observer and by measuring the transmittance at 1 nm steps over the visible spectrum. Various colour mixtures were created (Table 4.1) and their corresponding CIELab colours measured. The colour values of the mixtures were denoted by the subscript 0. Using the spectral profiles of the individual wines, the weighted mean spectra for each mixture were calculated. The CIELab colour values of the calculated weighted means were denoted by the subscript 1.

The differences between the measured and calculated CIELab colour values spectra were compared in terms of ΔE^{*} values where
$\Delta E^{*} = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{\frac{1}{2}}$ and $\Delta L^{*} = L_{0} - L_{1}$; $\Delta a^{*} = a_{0} - a_{1}$; $\Delta b^{*} = b_{0} - b_{1}$ (Equation 1.4). An ΔE^{*} value that approximates to zero indicates there was no difference between the created and calculated colour mixtures. This would essentially confirm that colour is an intensive property.

4.4.2. Equating the colour of an HPLC-separated wine with the colour of an intact wine by means of a normalization factor *(Step 2)*

Any comparison of coloured systems must be performed at the same pH value. For example, as HPLC analysis is performed at low pH, it is essential to measure the colour of wine at the same pH value, that is, at low pH also. For example, the absorption profile of 100 μL of wine diluted in 1M HCl is similar to the spectral profile of a wine separated by HPLC analysis (Figure 4.1). The difference will be in the intensities of the absorption profiles as can be seen by the scale of the absorbance axes. The HPLC-separated wine will have a lower intensity as it has been diluted in HPLC solvents. For example, assuming negligible losses due to pigment adhesion in the HPLC column, (when a wine is separated by HPLC analysis) the spectral profile of the wine is equivalent to the weighted mean of the individual components observed. Based on the intensive property of colour, the spectral profile of the total colour of an HPLC-separated wine (which includes the colour absorbance of ALL the peaks in the chromatogram) will equate to the spectral profile of an intact wine. But as only a small volume of wine (20 μL) is injected into an HPLC column, the two spectral profiles will only differ in their absolute peak heights (Figure 4.1).
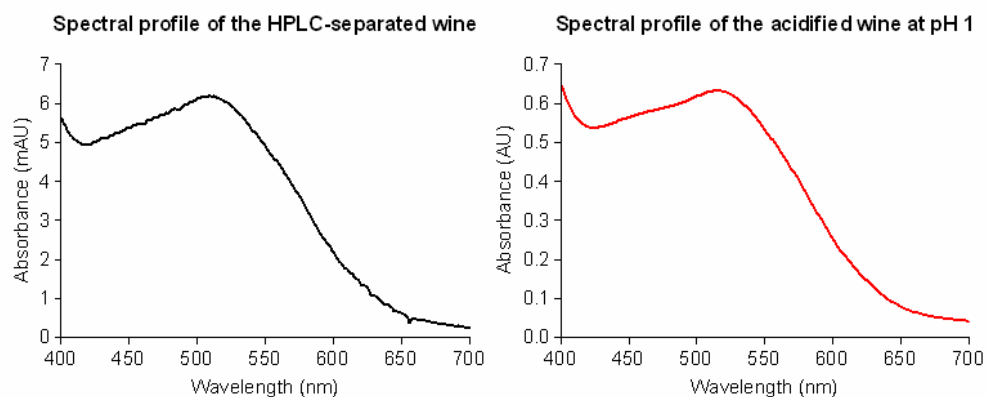
Figure 4.1: Approximately equivalent spectral profiles of an actual red wine sample separated by HPLC analysis (left) and the same intact, acidified wine at low pH (right). The vintage and varietal characteristics of the wine were of no consequence to the experiment.

The colour of an HPLC-separated wine is determined from an HPLC chromatogram. But, it must be noted that colour absorbance is not observed across the entire chromatogram. At the start and at the end (once all the pigments have eluted) there is no detection of wine pigments, and consequently, no colour absorbance. For example, in Figure 4.2, no pigments are detected before 0.9 minutes and after 26.0 minutes. Colour absorbance is observed between 0.9 and 26.0 minutes only. Even though the absorbance may be minimal at some time points, there will be some contribution to colour within this range.
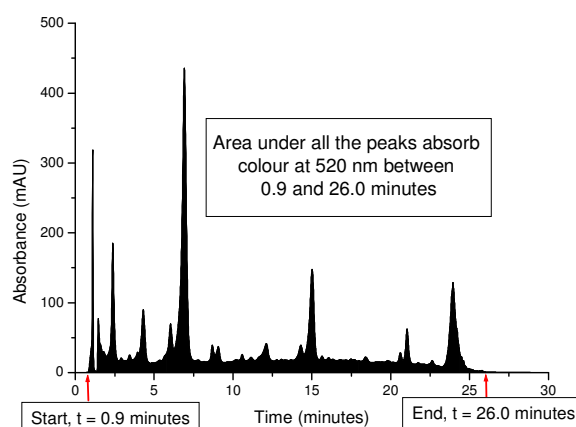
Figure 4.2: HPLC chromatogram at 520 nm for a typical red wine sample illustrating colour absorbance at 520 nm. The vintage and varietal characteristics of the wine were of no consequence to the experiment.

The spectral characteristics of the HPLC-separated wine and the spectra of the intact wine in a cuvette both measured at low pH are associated by a normalization factor. The normalization factor is the ratio of the spectra of the wine measured in a cuvette to the spectra of the HPLC-separated wine at the λ_{max} value of the wines. Multiplication of the normalization factor with the spectra of the HPLC-separated wine equates the two spectra. The advantage of applying a normalization factor is that errors such as dilution and pigment adhesion to the HPLC column, and additional unknown errors are minimized.

HPLC separation and colour at low pH of four red wine samples, chosen at random from the Shiraz red wine study (Chapter 2: Materials), were determined. HPLC-UV/Vis chromatographic analyses of the wines were performed using the standard HPLC method (Chapter 2: Methods). The absorption profiles of 100 μL of wine diluted in 10 mL of 1M HCl was measured after four hours using the standard spectrophotometer (Chapter 2: Methods).

Initially, a reference chromatogram at 520 nm was chosen. Note: A chromatogram at any absorbance value in the visible spectrum can be selected. As stated above, the time interval chosen for the data extraction does not need to be in the time frame of the entire HPLC analysis. For example, although HPLC separation of wine pigments using the standard HPLC method was performed over a period of 38 minutes, the time interval where colour absorbance was observed the sample was between 0.9 and 26.0 minutes (Figure 4.1). It was assumed that any absorbance within this time range, however minimal contributed to colour absorbance.

Data extraction was performed using an HPLC macro, Export3D.mac. This macro is part of the User Contributed Library supplied with Agilent LC Chemstation software (Agilent Technologies, Victoria, Australia). By specifying a wavelength range, one can obtain datasets over the entire recordable HPLC spectrum, from 220 to 750 nm. However, for the purpose of the CIELab calculation, 400 to 700 nm was chosen to represent the visible spectral range.

The exported absorbance data was saved in compatible Comma Separated Value (*.CSV) format. As the data sets were in excess of 20 MB with a mean spectrum determined from ~12000 columns, data extracted using Export3D.mac was limited to 5-minute intervals. Applying the concept that colour is an intensive property, the mean absorbance values were calculated using the JMP^{TM} package.

To equate the spectra of the wine at low pH and the HPLC-separated wine, a normalization factor was determined. The normalization factor was the ratio of the spectra of the wine at low pH measured in a cuvette to the spectra of the HPLC-separated wine at the λ_{max} value of the wines. The normalization factor was applied to the entire spectra of the HPLC-separated wine and the normalized spectra generated. To confirm that the normalization factor equated the two spectra, the CIELab colours of the diluted wine colour (denoted by 0) and the normalized HPLC wine colour (denoted by 1) were calculated. CIELab values (measured or calculated) were determined for a D65 illuminant, a 10° standard observer and by measuring the transmittance at 1 nm steps over the visible spectrum. The differences in the spectra were compared in terms of ΔE^* values where $\Delta E^* = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{\frac{1}{2}}$ and $\Delta L^* = L_0 - L_1$; $\Delta a^* = a_0 - a_1$; $\Delta b^* = b_0 - b_1$ (Equation 1.4).

4.4.3.   To determine optimum colour values for any HPLC-separated peak using an appropriate normalization factor *(Step 3)*

The normalization factor that equates an HPLC-separated wine and an intact wine is only applicable to wine, but the factor cannot be used for individual peaks. A factor that will produce optimum CIELab colour values for HPLC-separated peaks is necessary. Using the normalization factor for wine, no account is taken for the injection volume of wine and its dilution in the total volume of HPLC solvent. The dilution will depend upon the width of an individual peak. But, in any chromatogram there is a variation of peak widths and areas. To avoid discrimination against wider peaks, especially those that are more concentrated, a standard peak width needs to be chosen. For example, the elution peak for malvidin 3-glucoside is tall and narrow, whilst the peak representing the pigmented polymers is shorter and broader than malvidin 3-glucoside (Figure 1.15). Thus, a constant peak width of 0.5 minutes was chosen about the peak maximum. Acknowledging the intensive property of colour, the weighted mean spectra of any peak must be calculated about this peak maximum.

To determine an appropriate multiplication factor for HPLC-separated peaks, various dilution and normalization factors were estimated. The values were based on the standard HPLC method where the injection volume of wine and flow rate was 20 µL (= 0.02 mL) and 0.4 mL/min respectively:

a. The normalization factor of 0.008 equated the spectra of an intact wine at low pH with an HPLC-separated wine over a 25-minute interval as determined from the results in Section 4.4.2.

b. Colour absorbance in the HPLC chromatogram was noted between 0.9 and 26 minutes, over a 25-minute period (Figure 4.2). For a flow rate of 0.4 mL/min over 25 minutes, the total volume of HPLC eluent was 10 mL. The standard HPLC method used an injection volume of 0.02 mL wine, and so the dilution factor was 1:500 (= 0.002).

c. The total volume eluted for a peak of standard width of 0.5 minutes resulting from a flow rate of eluent pumped at a flow rate of 0.4 mL/min was 0.2 mL. For an injection volume of 0.02 mL, the dilution factor was 1:10 (= 0.1).

d. The normalization (0.008) and dilution factors (0.002) in (a) and (b) respectively differed by a factor of 4, therefore the dilution factor (0.1) in (c) was multiplied by a factor of 4. The estimated multiplication factor was 0.4.

e. Any peak with a constant width of 0.5 minutes where there is absorbance of colour over a time period of 25 minutes, the estimated dilution factor is given by 1:50 (= 0.02).

The standard HPLC method was used to separate a randomly selected Shiraz red wine sample (Chapter 2: Materials and methods). Malvidin 3-glucoside and the pigmented polymer peaks were chosen from the HPLC chromatogram (Figure 1.15) because in general by the standard HPLC method, these two peaks are quantified in the highest concentrations. The spectral profile of each peak was extracted over a time interval of 0.5 minutes either side of the peak maximum. The different multiplication factors (a) to (e) were applied to the malvidin 3-glucoside and the pigmented polymer peaks, and the CIELab colour values calculated. The most appropriate multiplication

factor that resulted in CIELab colour values that equated closely to wine colour was chosen.

4.5. Results

Steps 1 to 3 from Sections 4.4.1 to 4.4.3 inclusive have described the development of the HPLC-CIELab method. The results of the experiments are detailed below.

4.5.1. To demonstrate that colour is an intensive quantity *(Step 1)*

All four wine samples, the wine colours differed. For example, wine A was a light coloured red wine (high L* and low C* values) whilst wine C was a darker red wine (lower L* and high C* values).

Five combinations of the wine mixtures were prepared (Table 4.1. below). The spectral profile and CIELab values of each combination were measured. At the same time, the weighted mean spectra for each combination were calculated and the associated CIELab colour values determined. For all the mixtures, a range of ΔE^* values between the measured and calculated was observed. The ΔE^* values were low and in the range from 0.18 to 0.68, and indicated that there was minimal error, most likely due to sample preparation. Importantly, the ΔE^* values were less than 1 which indicated that there was no observable difference in the measured and calculated CIELab colour values (CMC 1996; Ayala *et al.* 1997; Gonnet 1998; Gonnet 1999; Pissarra *et al.* 2003a). Thus, the colours observed are the weighted means of the individual colours, and consequently, it is concluded that colour is an intensive property.

Table 4.1: CIELab colour values for the measured [b] and calculated [c] combinations of four red wine samples A, B, C and D.

Wine samples [a]		CIELab value			
		L*	C*	H*	ΔE*
Control wines	A	90.86	9.12	0.31	-
	B	89.08	9.92	0.41	-
	C	72.47	26.59	0.28	-
	D	74.60	26.46	0.19	-
A + B	Measured [b]	89.55	9.69	0.38	0.50
	Calculated [c]	89.96	9.51	0.36	
A + B + C	Measured [b]	83.64	15.91	0.31	0.18
	Calculated [c]	83.61	15.84	0.30	
A + B + C + D	Measured [b]	81.59	18.26	0.27	0.59
	Calculated [c]	81.22	18.64	0.25	
A + B + 2C + 4D	Measured [b]	77.96	22.20	0.23	0.68
	Calculated [c]	77.53	22.70	0.22	
2A + B + 3C + 2D	Measured [b]	79.43	20.42	0.26	0.47
	Calculated [c]	79.12	20.72	0.25	

[a] Typical Australian red wines of whose vintage and varietal characteristics were not important to the experiment.

[b] Measured CIELab colour values from the mixtures.

[c] Calculated CIELab colour values from the mean spectra of the control wines.

4.5.2. To equate the colour of an HPLC-separated wine with an intact wine by means of a normalization factor *(Step 2)*

A normalization factor is calculated from the ratio of the spectra of the wine at low pH measured in a cuvette to the spectra of the HPLC-separated wine at the λ_{max} value of the intact wine. For this sample set of wines, the λ_{max} value was 528 nm, and the normalization factor applicable to associate the HPLC-separated and intact wines was 0.008. The normalization factor was applied to the HPLC-separated spectra and the CIELab colour values determined. The colour values were compared to the CIELab colour values of the HCl-

diluted wine colour. An ΔE* value that approximates to 0 indicated that there is no difference between the measured and calculated colour values. The ΔE* values in the range 0.61 to 0.90 (Table 4.2), and confirmed that the colour difference was minimal.

Table 4.2: CIELab colour values of the diluted wine colour and the normalized HPLC-separated wine colour using five Shiraz red wine samples. The colour of the HPLC-separated wine has been determined on application of a normalization factor of 0.008.

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Sample	Diluted wine colour			Normalized HPLC wine colour			ΔE*
	L*	C*	H*	L*	C*	H*	
1	89.18	17.93	-0.13	89.02	17.35	-0.12	0.61
2	90.17	17.91	-0.14	89.48	17.64	-0.15	0.77
3	91.77	14.10	-0.11	91.19	13.52	-0.08	0.89
4	90.77	16.87	-0.15	90.00	16.44	-0.14	0.90
5	91.84	14.98	-0.15	91.16	14.80	-0.15	0.71

4.5.3. To determine optimum colour values for any HPLC-separated peak using an appropriate multiplication factor *(Step 3)*

A normalization factor of 0.008 applied to the HPLC-separated malvidin 3-glucoside and pigmented polymer peaks resulted in both pigments exhibiting red-violet hues (negative H* value) and fairly dark (moderately high L* and C* values) colours. A normalization factor of 0.4 resulted in orange-red hues (positive H* values) for both malvidin 3-glucoside and the pigmented polymer peaks, but the overall colour of the peaks were dark, almost black attributed to very low L* and C* values. A multiplication factor of 0.02 facilitates effective comparisons of any peak in an HPLC chromatogram as the colours are similar to wine colour observed in a 1 mm cuvette (See Chapters 5 and 6) . The CIELab colour values of malvidin 3-glucoside and pigmented polymer peaks had red to red-orange hues (neutral to positive H* values) and were fairly colourful (Table 4.3).

Table 4.3: Application of various multiplication factors to create CIELab colour values of malvidin 3-glucoside and the pigmented polymer peak for a random Shiraz red wine sample (See Chapter 2: Materials).

	Factor	L*	C*	H*	CIELab colour [a]
Malvidin 3-glucoside	0.008 [b]	72.43	49.81	-0.12	
	0.40 [c]	0.78	5.07	0.27	
	0.02 [d]	54.33	68.78	0.11	
Pigmented polymer peak	0.008 [b]	71.64	29.23	-0.13	
	0.40 [c]	0.45	3.39	0.23	
	0.02 [d]	46.10	50.87	0.02	

[a]The CIELab colours observed, either on the computer monitor or in the printed page, are dependent on the hardware (with associated calibration methods) and software of the computer, and ambient lighting used for viewing.

[b] Normalization factor of 0.008 determined by comparing the mean spectra of all the peaks in the HPLC chromatogram where there was colour (in this case over the time period of 25 minutes) and the diluted wine colour at low pH.

[c] Predicted normalization factor for a single peak.

[d] Optimum multiplication factor.

4.6.   Conclusions to method development

1. There was no noticeable colour difference between the measured and calculated spectra (Table 4.2), since $\Delta E^* < 1$. This experiment confirmed that colour is an intensive property.

2. The spectra of an HPLC-separated wine can be equated to the spectra of diluted wine colour at low pH by employing a normalization factor.

3. For any peak, a time interval of 0.5 minutes about the peak maximum should be chosen, and the weighted mean of the spectral data calculated. A multiplication factor of 0.02 should be applied to the spectral data to calculate the CIELab colour values that are meaningful to wine colour values.

4.7. Application of the HPLC-CIELab method to determine the CIELab colour of an HPLC-separated peak

The HPLC-CIELab method was also validated by applying the multiplication factor determined in Section 4.5 to malvidin 3-glucoside from four random Shiraz red wine samples. As stated earlier, any colour comparison must be performed under the same pH conditions, that is, the colour of HPLC-separated wine must be compared with acidified wine at low pH.

4.8. Objective

To demonstrate that the colour of malvidin 3-glucoside is responsible for the colour observed in a red wine at low pH.

4.9. Materials and methods

The standard HPLC method was used to separate four Shiraz red wine samples at low pH (Chapter 2: Materials and methods). The concentrations of malvidin 3-glucoside was determined using the malvidin 3-glucoside reference standard and calibration equation. The spectral data for malvidin 3-glucoside was extracted over 0.5 minutes about the peak maxima using the Export3d.mac macro (Section 4.4.2.). The spectra were averaged and a multiplication factor of 0.02 was applied to the mean spectral profile of the peaks. The CIELab colour values were determined for a D65 illuminant, a 10° standard observer and using transmittance values at 1 nm increments over the visible spectrum from 400 to 700 nm.

Simultaneously, the wines were diluted in HCl (100 μL of wine diluted in 10 mL of 1M HCl (aq)), and the CIELab colours of the wines were determined at low pH.

4.10.　　Results

The concentration of malvidin 3-glucoside varied in the four wines, and were in the range 54.0 to 100.6 mg/L (Table 4.4). Visually, the colours of malvidin 3-glucoside in the four wine samples appeared similar, but the CIELab colour values varied (Table 4.4).

Table 4.4: Concentration and CIELab colour values of malvidin 3-glucoside in four random Shiraz red wine samples at low pH. A time interval of 0.5 about the peak maxima was chosen and the mean spectral data calculated. A multiplication factor of 0.02 was applied to the mean spectral data prior to CIELab colour calculation.

Sample	Concentration (mg/L)	CIELab value			Colour of the pigment [a]
		L*	C*	H*	
1	100.6	54.55	79.57	0.32	
2	54.0	61.92	69.45	0.09	
3	99.2	56.96	76.11	0.22	
4	84.4	59.44	73.46	0.14	

[a] CIELab colours observed, either on the computer monitor or in the printed page, are dependent on the hardware (with associated calibration methods) and software of the computer, and ambient lighting used for viewing.

According to Beers' Law (Equation 1.1), the colour of the peak will be dependent upon quantities present in wine (at a given pH value). For example, dilute CIELab colour of a pigment (high L* and low C* values) will be observed when there is a low concentration of pigment. From Table 4.4, it is clear that the CIELab colour of malvidin 3-glucoside was a function of concentration. For example, Sample 1 had the highest concentration of malvidin 3-glucoside, 100.6 mg/L and the most colour (lowest L* and highest C* values) compared to the colour of malvidin 3-glucoside from the other wine samples (Table 4.4).

At low pH, the colour of malvidin 3-glucoside influenced the diluted wine colour in 1M hydrochloric acid (Figure 4.3). For example, the CIELab colour of

malvidin 3-glucoside in Sample 1 was the darkest and the most colourful (lowest L* and highest C* value) of the four samples. Similarly, Sample 1 had the darkest and the most colourful wine colour (lowest L* and highest C* value) at low pH.
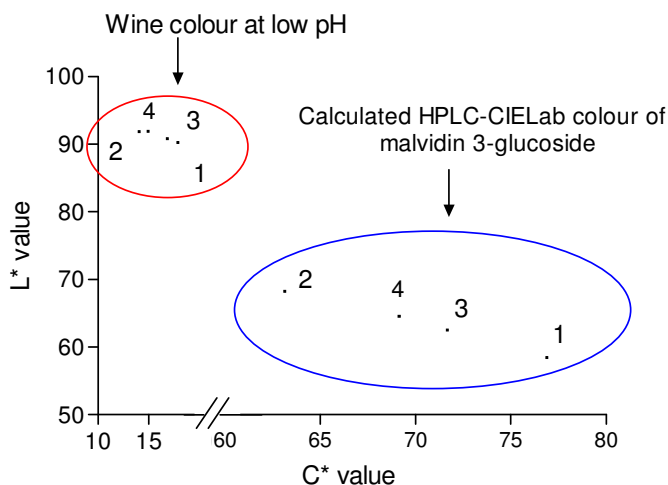
Figure 4.3: CIELab colour values represented by L* and C* values, of HPLC-separated malvidin 3-glucoside [a] compared to wine diluted in HCl [b] at low pH for four random Shiraz red wine samples.

[a] A time interval of 0.5 minutes about the peak maximum was chosen and the mean spectral data calculated. A multiplication factor of 0.02 was applied to the spectral data prior to CIELab colour calculation.

[b] 100 µL wine diluted in 1M HCl measured after 3 hours using a 10 mm cuvette.

Figure 4.3 illustrated a comparison of malvidin 3-glucoside only with wine colour at low pH. It is not of prime importance to investigate which pigments contributed the most to total red pigment colour. It is more relevant to consider the CIELab colour of HPLC-separated pigments with wine colour, at wine pH. Later, Chapter 7 will introduce a method of post-HPLC column adjustment of separated wine pigments. The method can be applied to conventional HPLC analysis and allows a better understanding wine pigments at wine pH, and after removal of the colour of anthocyanins by bleaching with SO_2 to reveal those pigments that contribute to SO_2 stable wine colour (Chapter 8). The HPLC-CIELab method can be applied to HPLC-separated peaks to determine the CIELab colours at wine pH and post-SO_2 bleaching (Chapter 8) provided that similar pH values or SO_2-bleached colour conditions are applied.

4.11.   Conclusions

1. *There are many advantages to using the HPLC-CIELab method: The calculation can be applied to one or many peaks from a chromatographically separated wine. The method also allows the colours of peaks to be re-created and consequently compared by anyone, regardless of the identity of the HPLC-separated compound.*

2. *Provided the HPLC analysis has been performed using similar media conditions, the HPLC-CIELab method is applicable to wine colour and SO_2 stable wine colour. The method will enable an observer to relate to the colour expression of wine pigment(s) at wine pH or post-SO_2 bleaching (See Chapter 8 later).*

3. *Expensive or time-consuming separation methods are not required to investigate the colour of many HPLC-separated peaks from a wine sample. If the peak is confirmed using an appropriate standard, the method enables the colour of a wine pigment at a given pH value to be achieved. In addition, the colours of many peaks can be achieved from the same HPLC chromatogram.*

4. *Separating a wine by HPLC analysis and employing the HPLC-CIELab method, an objective measurement of the concentration and colour of a pigment at its natural concentration in a wine can be made. The colour of the peaks will be dependent upon the quantities present in a wine at a given pH value.*

5. *Malvidin 3-glucoside is responsible for the colour observed in a red wine at low pH.*

Chapter 5: Colour measurement and wine pigment characterization in the Shiraz red wines

5.1. Objectives

1. *To evaluate whether winemaking region influences wine colour.*
   a. *To employ CIELab colour coordinates:*
   b. *To demonstrate that CIELab values provide enhanced colour measurements over current wine colour measurements.*
   c. *To correlate CIELab values with colour absorbance measurements.*
2. *To demonstrate that the CIELab colour and the concentration of a wine pigment is important to its characterization.*
3. *To investigate wine pigments as major influences to wine colour using an anthocyanin, malvidin 3-glucoside, and two anthocyanin-derived pigments, Vitisin A and the pigmented polymers as examples.*
4. *To demonstrate that the concentration of pigments at low pH*
   a. *Does not provide an accurate quantification of wine colour at its natural pH*
   b. *It ignores the possibility that some pigments may be affected by the pH of the media.*
5. *To demonstrate that at equivalent concentrations, different pigments have different colours.*
6. *To investigate the importance of SO_2 stable wine colour to wine colour, and the percentage of SO_2 non-bleachable pigments and chemical index (ii) in one-year old wines.*
7. *To demonstrate that some anthocyanin-derived pigments will have an influence on SO_2 stable wine colour.*
8. *To investigate the phenomenon of copigmentation and its effects on wine colour.*

5.2.　　　Materials and methods

Shiraz red wines from the 2003 wine trial were evaluated at approximately 18 months post-pressing (Chapter 2 - Materials). A total of 38 vineyards from four regions, Gundagai (8 vineyards), Bendigo (11 vineyards), Echuca (9 vineyards) and Swan Hill (10 vineyards) were considered. Two replicates from each vineyard were chosen. The following analyses were performed on two replicate red wine samples:

1. Spectroscopic absorbance and CIELab colour measurements at low pH, at wine pH, and the SO_2 stable wine colour were measured. The actual wine colour and SO_2-stable wine colour, as typically viewed in a cuvette, were recreated using CIELab colour coordinates and were illustrated in the tables below. The colours enable the reader to visually compare the wine colours and SO_2 stable wine colours. The analyses were performed in triplicate, and the mean ± standard error of the colour values was calculated. Statistical analysis of selected colour data was presented as box plots. Student's t-tests at the 90 to 95 % confidence intervals were applied to other data.

2. Chromatographic analysis of the wines was performed using the standard HPLC UV/Vis method (Chapter 2 – Methods). Identification of the anthocyanin peaks (the monoglucosides and their corresponding acylated derivatives) were based on their expected elution time in the HPLC chromatogram and associated spectral profile. The identity of malvidin 3-glucoside was confirmed using a standard. Confirmation of Vitisin A was achieved using a standard and co-injection. The pigmented polymer peak was the largest and last peak in the HPLC chromatogram, identified according to published data (Peng *et al.* 2002; Cozzolino *et al.* 2004; Eglinton *et al.* 2004). The pigments in the HPLC chromatogram were quantified as malvidin 3-glucoside equivalents (mg/L) using an external calibration function. The calibration equation was

Pigment concentration = 0.00915 x peak area + 0.79060.

The analyses were performed in duplicate, and the mean ± standard error of the colour values was calculated.

3. The CIELab colours of pigments separated by HPLC analysis were determined using the HPLC-CIELab method (described in Chapter 3). The CIELab colour values of Vitisin A and the pigmented polymers were compared at their natural concentrations in the red wines. The spectral data for HPLC-separated wine pigments were extracted across a time interval of 0.5 minutes about the peak maximum and the mean spectra calculated. A multiplication factor of 0.02 was applied to the mean spectral data prior to CIELab colour calculation (described in Chapter 3). The HPLC-CIELab colour calculations were performed in duplicate, and the mean ± standard errors of the colour values were calculated.

4. The colours of equivalent concentrations of pigments were also determined using the HPLC-CIELab method (described in Chapter 3). Six wine pigments were measured at three different concentrations (in mg/L as malvidin 3-glucoside equivalents) by conventional HPLC analysis at low pH from five random Shiraz red wine samples. The wine pigments were petunidin 3-glucoside and peonidin 3-acetylglucoside determined at a concentration of 10 mg/L, malvidin 3-acetylglucoside and malvidin 3-glucoside determined at a concentration of 20 mg/L, and delphinin 3-glucoside and Vitisin A determined at a concentration of 30 mg/L. The spectral data for the anthocyanin and Vitisin A concentrations were extracted across a time interval of 0.5 minutes about the peak maximum and the mean spectra was calculated. A multiplication factor of 0.02 was applied to the mean data prior to CIELab colour calculation (described in Chapter 3). The HPLC-CIELab colour calculations were performed in duplicate, and the mean ± standard errors of the colour values were calculated.

5. The chemistry of the wines was conducted by the Analytical Service at the AWRI. Wine chemistry measured included the pH values, titratable acidity, organic acid quantification, and free and total SO_2. The wine chemistry data were performed in duplicate and were presented in the tables as mean $\pm$ standard error values.

6. The phenomenon of copigmentation in the fairly young red wines was investigated. The intact wine colour was measured and compared with wine diluted 1:10 in model wine solution (Chapter 2 – Methods).

5.3. Results and discussion

5.3.1. Colour of the Shiraz red wines

The Shiraz red grapes were sourced from a range of vineyard locations in four regions in Australia: Gundagai, Bendigo, Swan Hill and Echuca. Environmental factors such as the climate, soil type, soil structure, fertilizer used, pruning practice and aspect of the vineyard would be expected to impact on the physiology of the vine, and consequently affected the resulting red wine colour (Keller *et al.* 1999; Mateus *et al.* 2002; Gambelli and Santaroni 2004; Van Leeuwen *et al.* 2004; Gonzalez-Neves *et al.* 2004a; Rolley 2004a; González-Neves *et al.* 2004b; Revilla *et al.* 2005). The winemaking conditions were practically identical. Nevertheless, the Gundagai region produced the darkest coloured wines with high wine colour density (13.4 $\pm$ 0.4 a.u.) (Table 5.1). The Gundagai wine colour differed significantly from the wines from the three remaining regions, Bendigo (11.6 $\pm$ 0.4 a.u.), Swan Hill (9.1 $\pm$ 0.4 a.u.) and Echuca (9.3 $\pm$ 0.4 a.u.) ($p < 0.05$). The results are in agreement with research regarding the impacts of wine region on red wine colour (Arozarena *et al.* 2000; Chicon *et al.* 2002; Cliff *et al.* 2002; Gonzalez-Hernandez *et al.* 2002; Mabrouk 2002; Giaccio and Del Signore 2004; Hermosin-Gutierrez and Garcia-Romero 2004; Klenar *et al.* 2004; Van Leeuwen *et al.* 2004; Gonzalez-Neves *et al.* 2004a; Hermosín Gutiérrez *et al.*

2005; Kontkanen *et al.* 2005) including some studies considering Australian winemaking regions (Somers 1975; Rolley 2004a).

Table 5.1: Wine colour density, wine chemistry and absorption at 420 nm for the Shiraz red wines [1].

Region	Gundagai	Bendigo	Swan Hill	Echuca
Number of vineyards per region	8	11	10	9
Wine colour density (a.u.)	13.4 ± 0.4 [a]	11.6 ± 0.4 [b]	9.1 ± 0.4 [c]	9.3 ± 0.4 [c]
A420 (a.u.)	5.5 ± 0.2 [a]	4.9 ± 0.1 [b]	3.7 ± 0.1 [c]	3.9 ± 0.2 [c]
Wine colour hue	0.7 ± 0.0 [a]	0.7 ± 0.0 [a]	0.7 ± 0.0 [a]	0.7 ± 0.0 [a]
Titratable acidity (g/L) at pH 8.4 [2]	7.1 ± 0.2 [a]	6.9 ± 0.1 [a]	7.1 ± 0.1 [a]	7.1 ± 0.1 [a]
pH value [2]	3.63 ± 0.03 [a]	3.66 ± 0.03 [a]	3.63 ± 0.03 [a]	3.59 ± 0.03 [a]
Baumé of grapes [3]	14 ± 1 [a]	14 ± 0 [a]	14 ± 1 [a]	14 ± 0 [a]
Free SO_2 (mg/L) [2]	12 ± 1 [a]	14 ± 1 [ab]	14 ± 1 [ab]	15 ± 1 [b]
Total SO_2 (mg/L) [2]	40 ± 2 [ab]	37 ± 2 [b]	43 ± 2 [a]	37 ± 2 [b]

[1] Mean ± standard error per region. Colour measurements were repeated in triplicate on two replicates from each region.

[2] Determination of titratable acidity, pH, free and total SO_2 described in Chapter 2: Materials and methods were conducted by the Analytical Service at the AWRI.

[3] Baumé data for the Shiraz red grapes (Rolley 2004b).

[a–c] Values with different subscript letters are significantly different at *P < 0.05*.

From the table, it is clear that the absorption values at 420 nm were low and differed significantly, while the wine hue value (0.7) was identical for all the wines (Table 5.1). To exclude colour contribution of oxidation reactions further, the level of free sulfur dioxide, which is used to identify an oxidised wine, was measured. For example, maximum and minimum values for free SO_2 values were 22 and 5 mg/L, recorded for a Swan Hill wine and a Bendigo wine respectively. Both wines exhibited similar 420 nm absorbance values of 4.65 a.u. This indicated that the low SO_2 value of the Bendigo wine did not compromise the colour of the wine. Therefore, it was concluded that the wines were not oxidised or spoilt.

Consistent values were observed for pH, titratable acidity, and free and total SO_2, and demonstrated sound winemaking practice. Thus, additional factors

in the red wines, such as grape origin and pigment concentration, were considered attributable to the colour differences in the wines (Table 5.1).

5.3.2. CIELab colour values as enhanced wine colour measurements

CIELab measurements are being extensively used by the food industry (McLaren 1980; MacDougall and Granov 1998; Nieto-Sandoval *et al.* 1999; Lee 2001; Sinnecker *et al.* 2002; Meléndez-Martínez *et al.* 2003; Hernández *et al.* 2004; Alonso-Salces *et al.* 2005). Now there is a progressive trend towards using CIELab colour values to represent the colour of grapes, wines and wine pigments (Bakker *et al.* 1986a; Almela *et al.* 1995; Carreño *et al.* 1995; Cruz-Ortiz *et al.* 1995; Almela *et al.* 1996; Carreño *et al.* 1996; Ayala *et al.* 1997; Gil-Munoz *et al.* 1997; Juarez *et al.* 1997; Almela *et al.* 1999; Ayala *et al.* 1999; Gonnet 1999; Gonnet 2001; Martinez *et al.* 2001; Meléndez *et al.* 2001; Chicon *et al.* 2002; McCaig 2002; Pérez-Magariño and Gonzàlez-San José 2002b; Gonnet 2003; Hermosín Gutiérrez *et al.* 2003; Pérez-Caballero *et al.* 2003; Hermosin-Gutierrez and Garcia-Romero 2004; Hermosín Gutiérrez *et al.* 2005; Pérez-Magarino and González-San José 2006). For example, CIELab values have been used to perform a statistical summary (e.g. mean, maximum and minimum) of the colour values of the Shiraz red wines (Table 5.2). LCH values were used to describe the wine colour where L* is the lightness, C* is the chroma and H* is the hue angle (Section 1.2.2.1: CIELab colour measurement earlier). By using LCH values, the reader can actually perceive the colours of the wines as viewed in a 1 mm cuvette. This makes the wine colour easier to describe, rather than attempting to express the wine colour by its red-green or yellow-blue characteristics. Thus, a large difference was observed in the lightness, chromaticity and hue values of the maximum and minimum red wine colour. For example, the least coloured wine was a Swan Hill red wine which had a red-orange hue (positive H* value) whilst the darkest colour wine was a Gundagai wine exhibited a purple-red hue (negative H* value) (Table 5.2). As the colour measurements were made on fairly young wines (at approximately 18 months post-pressing), the average

wine colour had a red hue (neutral H* values) and was fairly dark and bright (Table 5.2).

Table 5.2: Statistical summary of CIELab colour data for the Shiraz red wines at wine pH ranging from 3.00 to pH 3.85.

CIELab value	Mean ± s.e. [1]	Maximum colour	Median colour	Minimum colour
L*	66.6 ± 1.0	55.1	65.4	76.5
C*	31.5 ± 0.9	40.9	31.4	22.9
H*	0.0 ± 0.0	-0.1	0	0.1
Mean wine colour [2]				

[1] Mean ± standard error calculated from colour measurements repeated in triplicate on two replicates of 38 red wine samples.

[2] The CIELab colours shown are representative of the actual wine colours visible in a 1 mm cuvette. Importantly, the CIELab colour observed, either on the computer monitor or in the printed page, is dependent on the hardware and software of the computer, and ambient lighting used to view the colours.

CIELab colour values can also be used to compare the average wine colour for each wine region in this study (Table 5.3). For example, high wine colour density values were observed for the Gundagai wines. Accordingly, the Gundagai wine colours were darker (lower L* values), and more brightly coloured (higher C* values) with orange-red hues (positive H*). The Gundagai red wine colours were significantly different to the wines from Swan Hill and Echuca. For example, the Swan Hill and Echuca wines had the lowest wine colour density, were lighter (higher L* values), slightly duller (low C* values) and possessed only red hues (neutral H* values) (Table 5.3).

Table 5.3: Wine colour and CIELab colour values for the Shiraz red wines at wine pH (approximately pH 3.6).

Region	Gundagai [1]	Bendigo [1]	Swan Hill [1]	Echuca [1]
Wine colour density (a.u.)	13.4 ± 0.4 [a]	11.6 ± 0.4 [b]	9.1 ± 0.4 [c]	9.3 ± 0.4 [c]
Wine colour hue	0.7 ± 0.0 [b]	0.7 ± 0.0 [a]	0.7 ± 0.0 [b]	0.7 ± 0.0 [ab]
L*	59.6 ± 1.2 [c]	64.2 ± 1.0 [b]	70.5 ± 1.0 [a]	70.0 ± 1.1 [a]
C*	36.4 ± 1.1 [a]	32.9 ± 0.9 [b]	28.8 ± 0.9 [c]	29.3 ± 1.0 [c]
H*	0.05 ± 0.01 [a]	0.04 ± 0.01 [a]	0.00 ± 0.01 [b]	-0.01 ± 0.01 [b]

Wine colour [2]				

[1] Mean ± standard error is calculated from colour measurements repeated twice on the replicates from each region.

[2] The CIELab colours shown are representative of the actual wine colours visible in a 1 mm cuvette. Importantly, the CIELab colours observed, either on the computer monitor or in the printed page, are dependent on the hardware and software of the computer, and ambient lighting used to view the colours.

[a–c] Values with different subscript letters are significantly different at $P < 0.05$.

In the current literature, some researchers have attempted to calculate CIELab colour values of red wine using absorbance values at 420, 520 and 620 nm only (Glories 1984a; Glories 1984b; OIV 1990; Juarez *et al.* 1997; Pérez-Magariño and Gonzàlez-San José 2002b; Pérez-Magariño and González-Sanjosé 2003), or CIELab colour coordinates have been measured for table and fortified wines, and then the colour values have been associated with conventional absorbance measurements (Bakker *et al.* 1986a; Almela *et al.* 1995; Almela *et al.* 1996; Bakker *et al.* 1998). Table 5.4 confirms that some CIELab colour values were highly correlated positively or negatively with absorbance values. For example, wines with high wine colour density were darker (low L* values) and highly coloured (high C* values). Similarly, a high absorbance at 520 nm indicated the red characteristics (high a* values) observed in the red wines. But, some colour values showed little or no correlation at all with the absorbance values. For example, the H* and hue values showed low correlation with each other and other CIELab colour values. The H* value was not correlated with wine colour and absorbance at

420 and 520 nm. Overall, the results demonstrated that red wine colour can be described in terms of L*, a* and C* values, and less so by b* and H* values. Thus, Table 5.4 shows repeatability of previous work attempted by researchers such as Esparza and co-workers (Esparza *et al.* 2006), but no further factual colour information can be gained by this approach.

Table 5.4: Correlation coefficients (r) of the relationships between absorbance measurements and CIELab colour values of the 76 Shiraz red wines (including replicates) at wine pH (approximately pH 3.6). Signficance of the correlation coefficient is determined by *, **, *** significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.

Colour value	L*	a*	b*	C*	H*
A420 nm (a.u.)	-0.98 ***	0.90 ***	0.42 ***	0.90 ***	0.39 ***
A520 nm (a.u.)	-0.99 ***	0.97 ***	-0.28 ***	0.97 ***	0.24 *
Wine colour density (a.u.)	-1.00 ***	0.95 ***	0.34 **	0.95 ***	0.30 *
Wine colour hue	0.27 *	-0.50 ***	0.44 ***	-0.50 ***	0.49 ***

The box plots of the red wines from the four regions provide more valuable colour information (Figure 5.1) than the correlation table (Table 5.4). Despite sample variation within each region, Figure 5.1 illustrates that L*, C* and in particular H* were closely related to the wine colour density. For example, the Gundagai wines had the highest wine colour density. The high wine colour density values were mirrored in the lower L*, and higher C* and H* values. The opposite was observed for the lighter coloured Swan Hill and Echuca red wines which exhibited higher L*, and lower C* and H* values. It must be noted that Table 5.4 above illustrated the poor correlation of H* value with wine colour density. Converse to this, the box plots in Figure 5.1 showed that the H* value appears related to wine colour density. Similar to Table 5.4 above, there was no relationship between wine colour hue and any of the CIELab colour values for wines from the four regions in this study (Figure 5.1). Thus, the box plots clearly show that wine colour density is best described in terms of LCH values, and consequently it is these CIELab values that have provided the most valuable information regarding regional variation.
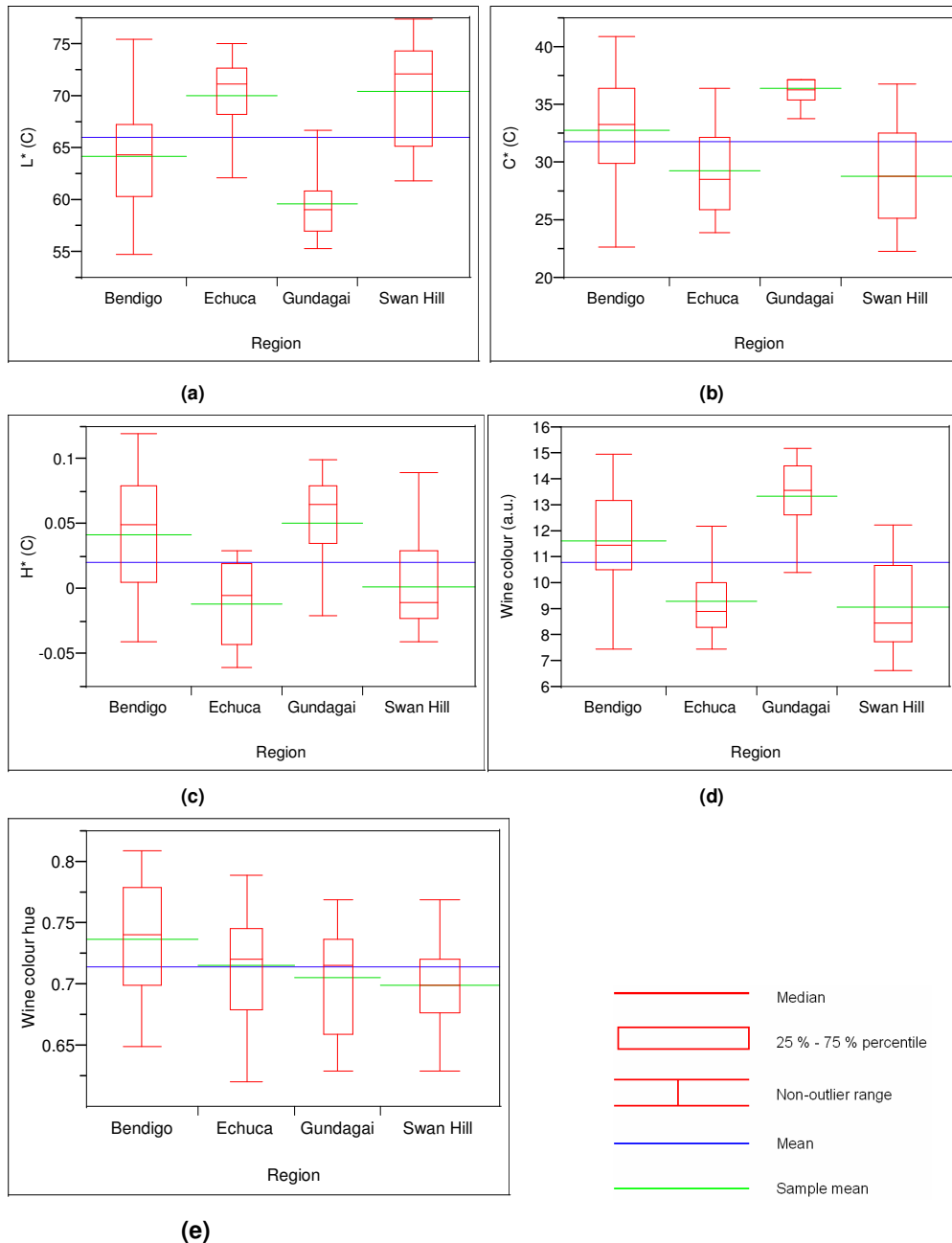
Figure 5.1: Descriptive statistics of the CIELab colour coordinates, L*, C*, and H* values (a), (b) and (c) respectively, and wine colour density (d) and wine hue (e) for the Shiraz red wines (at approximately pH 3.6) from all four regions in this study presented as box plots.

Additional uses of CIELab colour coordinates in the current literature have been where some researchers have created equations to describe wine colour density in terms of CIELab values, particularly using L*, a* and b* values (Almela *et al.* 1999; Pérez-Magariño and Gonzàlez-San José 2002b;

Pérez-Magariño and González-Sanjosé 2003; Gomez-Miguez and Heredia 2004). Thus, for this sample set of Shiraz red wines, a representation of wine colour density (WCD) in terms of L*, a* and b* values is given by the equations, L* = 94.31 − 2.60*WCD, a* = 10.39 + 1.98*WCD and
b* = -1.74 + 0.22*WCD (Figure 5.2).
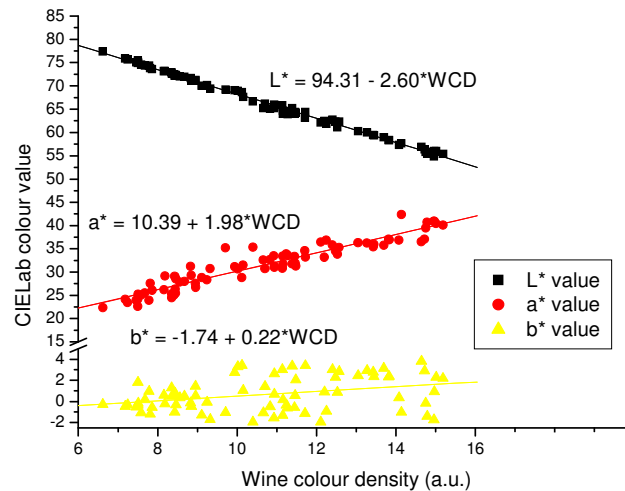
Figure 5.2: Relating wine colour density with individual CIELab colour values for the Shiraz red wines at wine pH values ranging from pH 3.00 to pH 3.85.

But, it is clear that these equations are applicable only to this dataset and the CIELab colour values cannot be employed to generalize wine colour.

CIELab values can be used to aid an observer to perceive the colour of a red wine, particularly when there are certain limitations regarding absorbance measurement. For example, when absorbance values at the two wavelengths are recorded, the wine colour density and wine colour hue values provide limited information regarding the nuances of colour observed. The use of such absorbance values may leave the reader unable to visualize the actual colour of the wine based on its wine colour density and hue values. For example, a lightly coloured wine with absorbance values, $A_{420\ nm}$ = 2 a.u. and $A_{520\ nm}$ = 5 a.u. will have a wine colour density of 7 a.u. This lightly coloured wine will have the same hue value (0.4), and will appear to have a red-orange hue as a

highly coloured wine with absorbance values $A_{420\ nm}$ = 8 a.u. and $A_{520\ nm}$ = 20 a.u. But, the overall wine colour of the latter wine will appear much darker as the wine colour density will be 28 a.u.

Although CIELab values provide enhanced colour information, CIELab colour values will not replace existing wine colour measurements. For example, wine colour absorbance measurements will always remain useful in laboratories with spectrophotometers capable of single wavelength absorbance measurements. CIELab colour values facilitate colour expression to an observer. But CIELab values have limitations, for example, CIELab colour coordinates colour can be represented in three dimensions, it can be difficult for an observer to compare the colour of a range of wines without plotting the CIELab data or using appropriate software to view the colour. So, the saturation value, s, can be calculated. The saturation value is the ratio of C^* to L^*, and effectively reduces the CIELab coordinates from three values to a single value. The saturation and wine colour density values essentially provide the same colour information (Figure 5.3). For example, for this sample set of Shiraz red wines, there is a high correlation between the saturation (calculated using the entire visible spectra) and wine colour density (calculated from two absorbance values, $A_{420\ nm}$ and $A_{520\ nm}$), r = 0.98 ($P < 0.05$) for 76 samples (including replicates) (Figure 5.3).
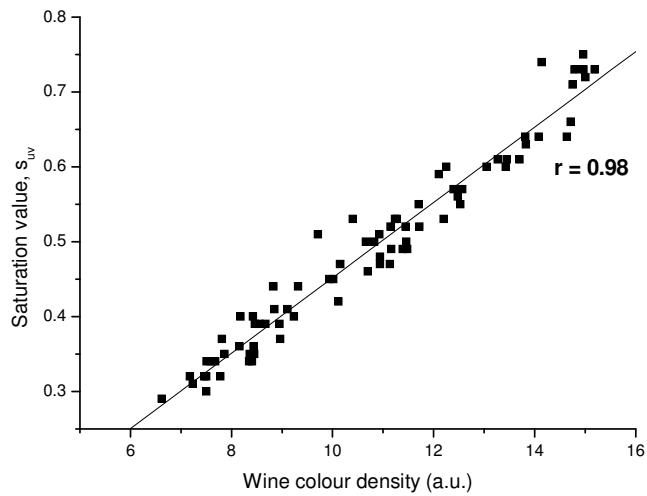
Figure 5.3: Relationship between wine colour density values and CIELab saturation, *s* values for the Shiraz red wines at wine pH (approximately pH 3.6) for correlation coefficient significant at *P* < 0.05.

5.3.3. Pigment composition of the Shiraz red wines

Whilst CIELab values can be used to illustrate and differentiate between intact wine colours, it remains necessary to investigate the actual wine pigments that are responsible for the colour differences. In this study, several wine pigments were elucidated: the anthocyanins (e.g. malvidin 3-glucoside, its acylated derivatives and additional monomeric anthocyanin glucosides) and anthocyanin-derived pigments (e.g. Vitisin A and the pigmented polymers).

5.3.3.1. Contribution of anthocyanins to the Shiraz red wine colour

In this sample set, the Shiraz red grapes may have had a similar complement of anthocyanins and polyphenolics, but the region were the grapes were sourced influenced the relative amounts of the individual anthocyanin monoglucosides. The anthocyanins in the wines varied markedly between regions (Table 5.5). For example, the Gundagai wines had the highest wine colour density (13.4 ± 0.4 a.u.), but the lowest concentration of malvidin 3-glucoside (63 ± 17 mg/L) and the other monomeric anthocyanins (Table 5.5).

This was in contrast to the lower wine colour density (11.6 ± 0.4 a.u.) and the higher concentration of monomeric anthocyanins (e.g. 95 ± 23 mg/L of malvidin 3-glucoside) observed in the Bendigo wines. Based on these observations, one cannot assume that wine colour density (measured at its natural wine pH) is related to anthocyanin concentration (determined at low pH). For example, the wine colour density of the Echuca wines (9.3 ± 0.4 a.u.) was significantly lower than the Bendigo wines (11.6 ± 0.4 a.u.), but there was no significant difference in the concentration of malvidin 3-glucoside between the two regions (approximately 96 mg/L (Table 5.5)). The concentration of the other monomeric anthocyanin glucosides of delphindin, petunidin and peonidin were higher in the Bendigo wines than in the Echuca wines. So, one could infer that the higher concentration of total anthocyanins in the Bendigo wines may have contributed to its higher wine colour density. However, this assumes that it is acceptable to compare anthocyanin concentration, determined at low pH, with wine colour measured at its natural pH.

Table 5.5: Wine colour density at approximately pH 3.6 and associated anthocyanin concentration in the Shiraz red wines determined by HPLC analysis [a].

Measured parameter [1]	Region			
	Gundagai	Bendigo	Swan Hill	Echuca
Wine colour density (a.u.) [2]	13.4 ± 0.4 [a]	11.6 ± 0.4 [b]	9.1 ± 0.4 [c]	9.3 ± 0.4 [c]
Delphinidin 3-glucoside (mg/L) [2]	10 ± 1 [b]	14 ± 1 [a]	9 ± 1 [b]	8 ± 0 [b]
Petunidin 3-glucoside (mg/L) [2]	13 ± 1 [b]	20 ± 1 [a]	13 ± 1 [b]	12 ± 1 [b]
Peonidin 3-glucoside (mg/L) [2]	11 ± 1 [b]	14 ± 1 [a]	10 ± 1 [b]	11 ± 1 [b]
Malvidin 3-glucoside (mg/L) [2]	63 ± 17 [c]	95 ± 23 [a]	79 ± 18 [b]	96 ± 20 [a]
Malvidin 3-acetylglucoside (mg/L) [2]	30 ± 2 [b]	35 ± 1 [ab]	32 ± 2 [b]	37 ± 2 [a]
Malvidin 3-*p*-coumaroylglucoside (mg/L) [2]	15 ± 1 [a]	16 ± 1 [a]	15 ± 1 [a]	15 ± 0 [a]
Total anthocyanins (mg/L) [2]	227 ± 41 [c]	254 ± 56 [a]	221 ± 39 [bc]	224 ± 42 [ab]

[1] Mean ± standard error was calculated from colour measurements repeated in triplicate on two replicates from each region.

[2] Concentration in terms of malvidin 3-glucoside chloride equivalents (mg/L) determined using the standard HPLC method at pH 1.5.

[a–c] Values with different subscript letters are significantly different at $P < 0.05$.

Anthocyanins are assumed to influence young wine colour as anthocyanins are extracted from red grapes during fermentation (Ribereau-Gayon 1982; Mayen *et al.* 1994; Mazza 1995; Mazza *et al.* 1999; Garcia-Beneytez *et al.* 2002; Mateus *et al.* 2002a; Mateus *et al.* 2002b; García-Beneytez *et al.* 2003; Wang *et al.* 2003a; Wang *et al.* 2003b; Bautista-Ortín *et al.* 2004; Corona *et al.* 2004; Hermosin-Gutierrez and Garcia-Romero 2004; Kammerer *et al.* 2004; Munoz-Espada *et al.* 2004; Núnez *et al.* 2004; Gonzalez-Neves *et al.* 2004a; González-Neves *et al.* 2004b; Bautista-Ortín *et al.* 2005; Hermosín Gutiérrez *et al.* 2005; Lorenzo *et al.* 2005; Romero-Cascales *et al.* 2005; Pérez-Magarino and González-San José 2006; Vian *et al.* 2006). But, the same is not applicable to the colour of aged wines. In matured wines, the concentration of anthocyanins such as malvidin 3-glucoside will have diminished to negligible amounts, while other wine pigments, namely stable red wine pigments, provide the colour to aged red wine (Somers 1971; Somers and Evans 1977; Haslam 1980; Ribereau-Gayon *et al.* 1983; Dallas *et al.* 1994; Mayen *et al.* 1994; Jones *et al.* 1999; Rivas-Gonzalo 1999; Escribano-Bailón *et al.* 2001; Cliff *et al.* 2002; Morais *et al.* 2002; Atanasova *et al.* 2002a; Harbertson *et al.* 2003; Hayasaka and Kennedy 2003a; Wang *et al.* 2003b; Hayasaka *et al.* 2004; Monagas *et al.* 2005a; Monagas *et al.* 2005b; Alcalde-Eon *et al.* 2006; Boido *et al.* 2006; Garcia-Puente Rivas *et al.* 2006; Monagas *et al.* 2006b; Monagas *et al.* 2006c; Monagas *et al.* 2006d). The correlation matrix in Table 5.6 illustrates there was little or no correlation between the concentration of the monomeric anthocyanins determined at low pH, and wine colour density and wine colour hue measured at natural wine pH. But, there appeared to be a moderately significant correlation between the anthocyanins such as monomeric glucosides of delphinidin, petunidin and peonidin, and malvidin 3-*p*-coumaroyl glucoside with wine colour density (Table 5.6). Even though malvidin 3-glucoside was the major anthocyanin in the wines, this pigment and its acylated glucoside showed no relationship with wine colour density or hue at all (Table 5.6).

Table 5.6: Range of anthocyanin monoglucoside concentrations [a] in the Shiraz red wine and correlation coefficients (r) of the relationships between anthocyanin concentration and wine colour density, wine colour hue at wine pH (approximately pH 3.6) and total red pigment colour at pH 1. Signficance of the correlation coefficient is determined by * and ** significant at $P < 0.05$ and $P < 0.01$ respectively, while n.s. denotes no significance.

Anthocyanins	Concentration (mg/L) [1]	Correlation of anthocyanin concentration with colour value [2]		
		Wine colour density (a.u.)	Hue value	Total red pigment colour (a.u.)
Delphinidin 3-glucoside	4 – 23	0.56 *	-0.09 n.s.	0.72 *
Petunidin 3-glucoside	12 – 31	0.42 *	0.06 n.s.	0.65 *
Peonidin 3-glucoside	9 – 17	0.48 *	-0.16 n.s.	0.61 *
Malvidin 3-glucoside	31 – 132	- 0.09 n.s.	0.16 n.s.	0.48 *
Malvidin 3-acetylglucoside	16 – 51	-0.01 n.s.	0.31 **	0.48 *
Malvidin 3-p-coumaroyl-glucoside	7 – 24	0.41 *	-0.15 n.s.	0.62 *

[1] Malvidin 3-glucoside chloride equivalents determined using the standard HPLC method at pH 1.5.

[2] Correlation coefficients of anthocyanin concentration with colour value for 76 wine samples.

It accepted practice by some researchers to present the concentration of wine pigments separated by HPLC analysis (at low pH) with wine colour density (at wine pH), even though the measurements have been made in two different pH media (McCloskey and Yengoyan 1981; Dallas *et al.* 1994; Fulcrand *et al.* 1996; Gómez-Plaza *et al.* 1999; Mazza *et al.* 1999; Arozarena *et al.* 2000; Parley *et al.* 2001; Chicon *et al.* 2002; Garcia-Beneytez *et al.* 2002; Gómez-Plaza *et al.* 2002; Tsanova-Savova *et al.* 2002; Mateus *et al.* 2002a; Mateus *et al.* 2002b; Fernández de Simón *et al.* 2003; Gonzalez-Martinez *et al.* 2004; Pérez-Magariño and González-SanJosé 2004; Gómez-Míguez *et al.* 2005; Lorenzo *et al.* 2005; Revilla *et al.* 2005; Alcalde-Eon *et al.* 2006; Boido *et al.* 2006; Cano-López *et al.* 2006; Garcia-Puente Rivas *et al.* 2006). From Table 5.6, it is clear that the concentration of anthocyanins at low pH showed very little or no correlation with wine colour density at its natural wine pH. It is more appropriate to associate anthocyanin concentration at low pH with total red pigment colour rather than comparing anthocyanin concentration at low pH with wine colour at its natural pH. Table 5.6 above illustrated that there was a

high significant correlation between all the anthocyanins and the total red pigment colour. This is because both quantites were determined at low pH (Table 5.5 above). The total red pigment colour reflected the presence of the highly coloured flavylium cations at low pH. This was also confirmed in Chapter 3 where the colour of malvidin 3-glucoside was the major influence to wine colour at low pH. However, the comparison of anthocyanin concentration at low pH with total red pigment colour does not provide a researcher or a winemaker with a better understanding of the contribution of pigments in wine at their natural wine pH. It is more valuable to compare the concentration of anthocyanins with wine colour where both have been measured at the same pH value, at wine pH. Later, in Chapter 8, the concentration and colours of anthocyanins (namely malvidin 3-glucoside) at wine pH will be presented and discussed.

5.3.3.2.　　Contribution of Vitisin A to Shiraz red wine colour

Vitisin A is an example of an anthocyanin-derived pigment that is considered stable to SO_2 bleaching as there is a functional group at the 4-position of the anthocyanin moiety. An investigation into the influence of Vitisin A to wine colour, and the colour properties of Vitisin A at low pH was made. For this sample set of Shiraz red wines, the minimum and maximum concentrations of Vitisin A were 5 mg/L (recorded for a Swan Hill wine) and 18 mg/L (recorded for a Bendigo wine) respectively. A similar trend was observed in the box plots of Vitisin A and wine colour density for the four regions (Figure 5.4). Minor differences and large spread of the data was observed and were probably attributed to the low concentration of Vitisin A (particularly compared to concentration of 31 to 132 mg/L of malvidin 3-glucoside). Consequently, it was difficult to assess the significant impact of Vitisin A on wine colour.
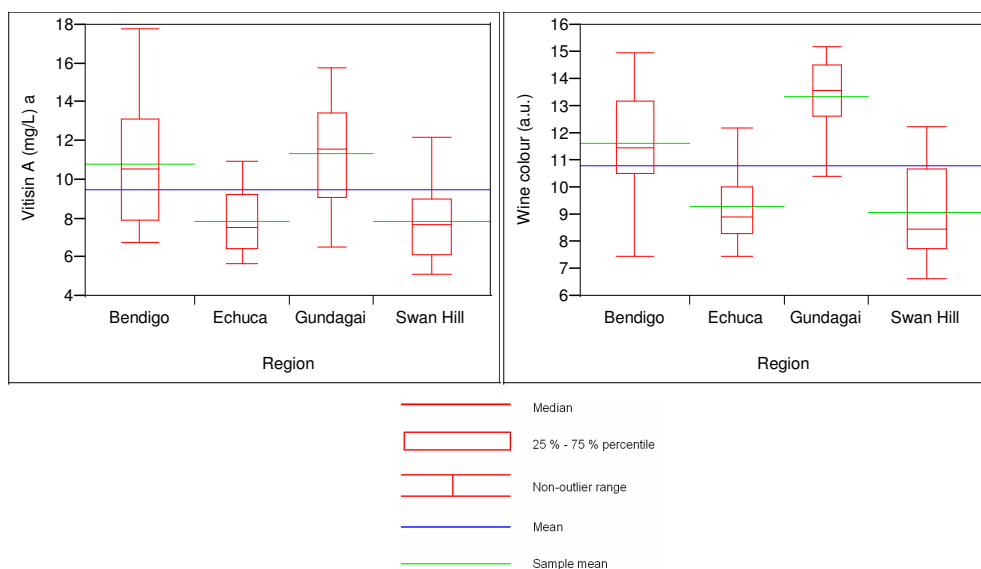
Figure 5.4: Descriptive statistics of Vitisin A [1] and wine colour density for the Shiraz red wines (at approximately pH 3.6) from all four regions presented as box plots.

[1] Malvidin 3-glucoside chloride equivalents determined using the standard HPLC method at pH 1.5.

For the entire sample set of 76 Shiraz wines (including replicates), Vitisin A showed a good correlation to wine colour (r = 0.77) ($P < 0.05$) (Figure 5.5). This result was in contrast to the findings by Schwarz and co-workers who proposed that Vitisin A only contributed a minimal amount, approximately 5 %, to total wine colour (Schwarz *et al.* 2003c). In addition, Vitisin A is considered to influence the hue value during the ageing of model wine solutions (Bakker *et al.* 1998; Romero and Bakker 1999). But, on application to the actual red wines, there was no evidence of its influence as a negligible correlation value was observed (r = -0.19) (Figure 5.5).
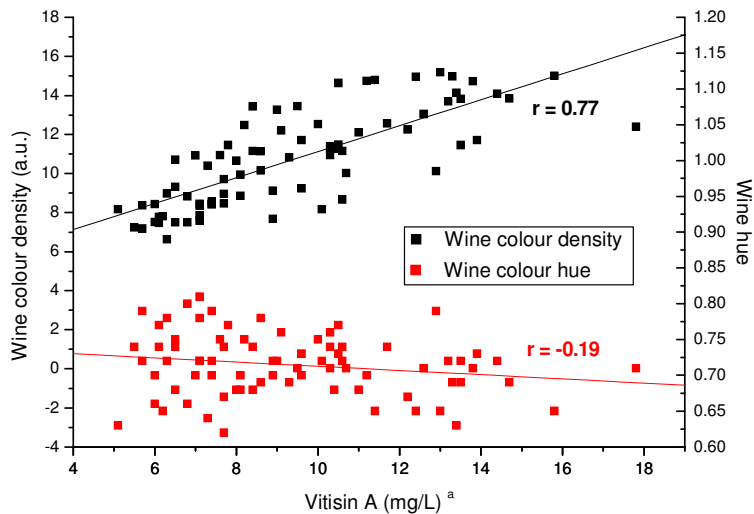
Figure 5.5: Relationship between Vitisin A concentration [a] with wine colour density (at approximately pH 3.6) for the Shiraz red wines for correlation coefficient significant at $P < 0.05$.

[1] Malvidin 3-glucoside chloride equivalents determined using the standard HPLC method at approximately pH 1.5.

The minimal difference observed in the box plots (Figure 5.4) and the moderate correlation between Vitisin A concentration and wine colour density (r = 0.77) in Figure 5.5 could be attributed to the measurements which were made in two different pH media. The low pH value may have affected quantification of Vitisin A. For example, Asenstorfer and co-workers have proposed that Vitisin A behaves in a similar fashion to malvidin 3-glucoside, and as the pyranoanthocyanin is believed to transform into different canonical forms at different pH values (Asenstorfer *et al.* 2006). A consequence of the formation of pH-dependent canonical isoforms is that Vitisin A will exhibit slightly different colours at low pH compared to at wine pH (Asenstorfer 2001). The pH of the media will affect Vitisin A quantification, and its subsequent correlation with wine colour. Later in Chapter 8, examples will be provided illustrating the change in the colour of Vitisin A with change in pH media (and SO_2 bleaching).

In the literature, several researchers have proposed that Vitisin A is red-brown, orange-red or brick-red (Romero and Bakker 1999; Romero and Bakker 2000b; Schwarz *et al.* 2003d). Unfortunately, the colours of Vitisin A have been presented without reporting its associated concentrations in red wine. To demonstrate the colour of Vitisin A separated by HPLC analysis, the CIELab colour of Vitisin A at low pH was determined at two concentrations. The colour of the minimum and maximum concentrations, 5 mg/L and 18 mg/L respectively, of Vitisin A in the Shiraz red wines were determined using the HPLC-CIELab method (described in Chapter 4) (Table 5.7). Although the concentrations of Vitisin A in the wines were low compared to the the range of malvidin 3-glucoside (31 to 132 mg/L) (Table 5.6 above), much colour was observed (Table 5.7). The large amount of colour was probably attributed to the high molar absorptivity coefficient of Vitisin A at low pH (24863 ± 1807 L cm^{-1} mol^{-1} at pH 0.0) (Asenstorfer 2001). At the higher concentration of 18 mg/L, Vitisin A appeared dark and bright (low L^* and high C^* values) and the H^* value indicated a red hue (neutral H^* value). The colour changed at the lower concentration. At 5 mg/L, the colour of Vitisin A was lighter and duller (increased L^* and decreased C^* values) with a red hue (neutral H^* values). Visually comparing the two colours in Table 5.7, the higher concentration of Vitisin A looked darker, and appeared to be a different colour than 5 mg/L of Vitisin A. Thus, Table 5.7 illustrates that the colour of a pigment is dependent not only its colour, but its associated concentration in a red wine. As the pH of the media affects Vitisin A quantification and its CIELab colour, it is more important to assess the impact of Vitisin A colour on wine colour at its natural wine pH. The concentration and CIELab colour of Vitisin A determined at wine pH will be discussed later in Chapter 8.

Table 5.7: CIELab colours for the maximum and minimum concentrations, 5 and 18 mg/L, of Vitisin A in the Shiraz red wines.

Concentration of Vitisin A (mg/L) [1]	CIELab colour values [2]			
	L*	C*	H*	Colour of Vitisin A [3,4]
5	56.3 ± 0.0	47.1 ± 0.1	0.0 ± 0.0	
18	44.0 ± 0.1	58.5 ± 0.1	0.0 ± 0.0	

[1] Malvidin 3-glucoside chloride equivalents determined using the standard HPLC method at approximately pH 1.5.

[2] Mean $\pm$ standard error values calculated from colour measurements repeated twice at each concentration.

[3] CIELab colour values of Vitisin A at low pH from absorbance values averaged over a 0.5 minute interval about the peak maximum followed by application of a multiplication factor of 0.02.

[4] CIELab colours observed, either on the computer monitor or in the printed page, are dependent on the hardware and software of the computer, and ambient lighting used to view the colours.

5.3.3.3. Contribution of the pigmented polymers to the Shiraz red wine colour

The differences in the red wine colours were investigated in terms of the influence of the pigmented polymer concentrations (Table 5.8). For example, the Gundagai wines had the highest wine colour density, (13.4 ± 0.4 a.u.) compared to the wine colour density values of the Bendigo, Swan Hill and Echuca wines. Similarly, the Gundagai pigmented polymers had the highest concentration (61 ± 8 mg/L) and the highest CIELab colour (low L* and high C* values). The pigmented polymers of the Gundagai wines were significantly different to the concentration and colour of the pigmented polymers from the three other regions (Table 5.8).

Table 5.8: Wine colour density, and CIELab colour and concentration of the pigmented polymer peaks of the Shiraz red wines determined at low pH [a].

Colour value [1]		Region			
		Gundagai	Bendigo	Swan Hill	Echuca
Wine colour density (a.u.)		13.4 ± 0.4 [a]	11.6 ± 0.4 [b]	9.1 ± 0.4 [c]	9.3 ± 0.4 [c]
CIELab value [2]	L*	43.6 ± 1.1 [c]	49.8 ± 1.1 [b]	55.4 ± 1.3 [a]	56.4 ± 0.9 [a]
	C*	53.6 ± 0.8 [a]	48.8 ± 0.9 [b]	48.4 ± 1.2 [b]	47.5 ± 0.7 [b]
	H*	0.0 ± 0.0 [c]	0.1 ± 0.0 [bc]	0.1 ± 0.0 [a]	0.1 ± 0.0 [ab]
Pigmented polymer concentration (mg/L) [3]		61 ± 8 [a]	54 ± 10 [b]	46 ± 10 [c]	45 ± 6 [c]

Colour of the pigmented polymer peak [4]				

[1] Mean ± standard error was calculated from colour measurements repeated in triplicate on each replicate from each region.

[2] CIELab colour values of the pigmented polymers from absorbance values averaged over a 0.5 minute interval about the peak maximum followed by application of a multiplication factor of 0.02.

[3] Malvidin 3-glucoside chloride equivalents using the standard HPLC method at approximately pH 1.5.

[4] CIELab colours observed, either on the computer monitor or in the printed page, are dependent on the hardware and software of the computer, and ambient lighting used to view the colours.

[a – c] Values with different subscript letters are significantly different at $P < 0.05$.

Visually, the colours of the pigmented polymer peaks appeared similar (Table 5.8), but the CIELab colour values demonstrated subtle differences. The Gundagai pigmented polymers were darker and more colourful (low L* and higher C* values) than the colours of the pigmented polymers from Bendigo, Swan Hill and Echuca. In addition, similar to the wine colour density, the concentration and CIELab colour values of the pigmented polymers differed significantly between the four regions (Table 5.8).

Similar to the observations made for Vitisin A, the CIELab colours of the pigmented polymers were a function of concentration. For example, the Gundagai pigmented polymers had the highest concentration (61 ± 8 mg/L) and the highest colour (low L* and high C*) values. Conversely, the Swan Hill

and Echuca pigmented polymers had the lowest concentration (approximately 46 mg/L) and lowest colour (high L* and low C*) values (Table 5.8).

Further to the data in Table 5.8, the box plots for the wines from the four regions illustrated that pigmented polymer concentration and wine colour density showed a consistent relationship (Figure 5.6). For example, the Echuca wines had the lowest concentration of pigmented polymers and the lowest wine colour density (Figure 5.6). Despite the large outlier range, similar trends across the four wine regions in this study were observed.
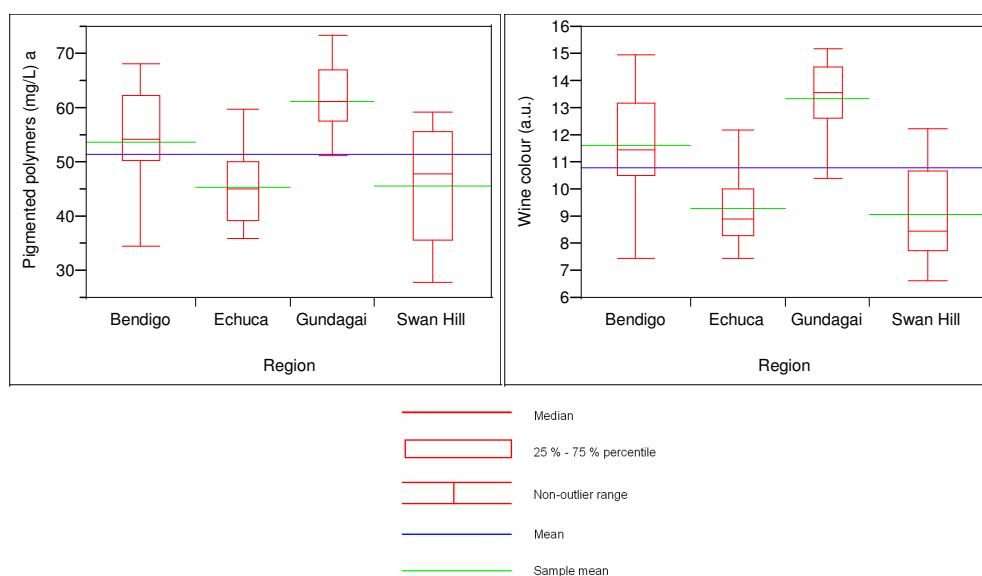
Figure 5.6: Descriptive statistics of pigmented polymer concentration [a] and wine colour density for the Shiraz red wines (at approximately pH 3.6) from all four regions presented as box plots.

[a] Malvidin 3-glucoside chloride equivalents determined using the standard HPLC method at approximately pH 1.5.

For the entire 76 wine samples (including replicates), the relationship betweent the pigmented polymer concentration (quantified at low pH by HPLC analysis) with wine colour (measured at its natural wine pH) were highly correlated (r = 0.80) ($P < 0.001$) (Figure 5.7). Based on this evidence alone, it could be inferred that the pigmented polymers influenced red wine colour.
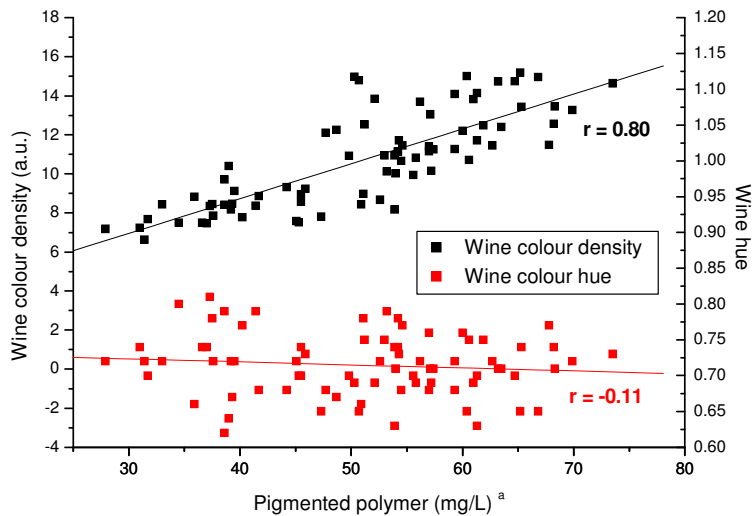
Figure 5.7: Relationship between pigmented polymer concentration [a] and wine colour density values (at approximately pH 3.6) for the Shiraz red wines for correlation coefficient significant at $P < 0.05$.

[a] Malvidin 3-glucoside chloride equivalents determined using the standard HPLC method at approximately pH 1.5.

But the correlation in Figure 5.7 assumes that the concentration and consequently the colour of the pigments that eluted in the pigmented polymer peak were unaffected by the low pH conditions. In fact,

1. There may be some pigments that have eluted in the pigmented polymer peak that were stable to pH change.
2. There may have been some pH-dependent pigments that eluted in the pigmented polymer peak. Such pH-dependent pigments would behave in a similar manner to the pH dependent anthocyanins and exhibit canonical isoforms such as the highly coloured flavylium cations at low pH.

But, it is not possible to confirm the pH stability of wine pigments by measuring concentrations determined using conventional HPLC analysis. So, this approach of comparing pigmented polymers quantified at low pH with wine colour at its natural pH could give misleading information.

Unlike the wine colour density, the wine colour hue was independent of pigmented polymer concentration as demonstrated by a negligible correlation (r = -0.19) (Figure 5.7).

In current literature, polymeric pigments are considered to be red-brown or brown in colour (Somers 1971; Harbertson *et al.* 2003; Schwarz *et al.* 2003d). In addition, it is also believed that polymerisation reactions give soluble brown compounds that express more colour than the red anthocyanins (Romero and Bakker 2001). It must be noted that Table 5.8 above illustrated the colours of pigmented polymers (which was the last peak in the HPLC chromatogram) (Jones *et al.* 1999; Peng *et al.* 2002; Hayasaka and Kennedy 2003; Bartowsky *et al.* 2004; Cozzolino *et al.* 2004; Eglinton *et al.* 2004; Hayasaka *et al.* 2004; Herderich and Smith 2005), and not the colours of polymeric pigments (which are large complex high molecular weight pigments isolated by either gel permeation or centrifugation chromatography) (Kantz and Singleton 1990; Shoji *et al.* 1999; Degenhardt *et al.* 2001; Guadalupe *et al.* 2006). From the table it is highly evident that the pigmented polymers had red (neutral H* values) or red-orange hues (positive H* values) and were not red-brown or brown in colour (Table 5.8 above).

5.3.4. Colours of equivalent concentrations of pigments from the Shiraz red wines

Although the concentration of a wine pigment provides useful quantitative information, it is often assumed that equivalent concentrations of two different pigments provide the same colour contribution to wine colour. This assumption is flawed as no account is taken for the extinction coefficient value of the pigment in a given pH medium. As stated in Chapter 3, the extinction coefficient value will affect the absorbance and colour of the wine pigment. Thus, it is likely that two different wine pigments present in a red wine at the same concentration could have vastly different colour values.

In this study, the colours of equivalent concentrations of some anthocyanins and some anthocyanin-derived pigments were compared. Six wine pigments were measured at three different concentrations (as malvidin 3-glucoside equivalents) from five random Shiraz red wine samples. The wine pigments were petunidin 3-glucoside and peonidin 3-acetylglucoside determined at a concentration of 10 mg/L, malvidin 3-acetylglucoside and malvidin 3-glucoside determined at a concentration of 20 mg/L, and delphinin 3-glucoside and Vitisin A determined at a concentration of 30 mg/L (Table 5.9).

It is clear from Table 5.9 that the colour difference, ΔE^* between pigments of the same concentration were in the range 5 to 28 CIELab units. Visually, the colours of the wine pigments were noticeably different. Thus, one cannot assume that equivalent concentrations of wine pigments will exhibit the same colour.

At a concentration of 10 mg/L, peonidin 3-acetylglucoside was darker and brighter (lower L* and higher C* values), and exhibited a red hue (neutral H* value) compared to petunidin 3-glucoside. At the higher concentration of 20 mg/L, malvidin 3-glucoside was lighter (higher L* value) than malvidin 3-acetylglucoside, although the two pigments had similar C* and H* values. At the highest concentration, 30 mg/L, Vitisin A was negligibly darker and brighter (lower L* and higher C* values) and possessed a slight red-orange hue (positive H* value) compared to delphinidin 3-glucoside. Thus, the colours of the pigments observed were dependent upon extinction coefficient values of the pigments. For example, although the concentration of Vitisin A was greater (30 mg/L) than malvidin 3-glucoside (20 mg/L), the reduced colour exhibited by Vitisin A (higher L* and lower C* values) is attributed to its extinction coefficient value. For example, the extinction coefficient value of Vitisin A is 24863 ± 1807 $Lcm^{-1}mol^{-1}$ at pH 0.0 (Asenstorfer 2001) compared to the extinction coefficient value of malvidin 3-glucoside of 27600 $Lcm^{-1}mol^{-1}$ at pH 1 (Håkansson *et al.* 2003). But it was not possible to compare the extinction coefficient values of the acylated derivatives of malvidin and peonidin with other wine pigments as there was an absence of these values from current literature.

Table 5.9: CIELab colours [d] of equivalent concentrations of wine pigments from the Shiraz red wines determined using the HPLC-CIELab method at low pH.

	Petunidin 3-glucoside (Sample 1) [b,c]			Peonidin 3-acetylglucoside (Sample 1) [b,c]			ΔE*
Concentration (mg/L) [a]	L*	C*	H*	L*	C*	H*	
10	82.6 ± 0.0	28.7 ± 0.1	-0.1 ± 0.0	67.7 ± 0.2	52.1 ± 0.2	0.0 ± 0.0	28

	Malvidin 3-acetylglucoside (Sample 2) [b,c]			Malvidin 3-glucoside (Sample 3) [b,c]			ΔE*
Concentration (mg/L) [a]	L*	C*	H*	L*	C*	H*	
20	60.0 ± 0.0	66.7 ± 0.1	0.0 ± 0.0	65.1 ± 0.1	65.5 ± 0.1	0.0 ± 0.0	5

	Delphinidin 3-glucoside (Sample 4) [b,c]			Vitisin A (Sample 5) [b,c]			ΔE*
Concentration (mg/L) [a]	L*	C*	H*	L*	C*	H*	
30	76.9 ± 0.1	40.3 ± 0.1	0.0 ± 0.0	76.0 ± 0.1	41.5 ± 0.1	0.1 ± 0.0	5

[a] Malvidin 3-glucoside chloride equivalents determined using the standard HPLC method at approximately pH 1.5.

[b] Mean ± standard error calculated from colour measurements repeated twice at each concentration.

[c] CIELab colour values calculated from absorbance values averaged over a 0.5 minute interval about the peak maximum followed by application of a multiplication factor of 0.02.

[d] CIELab colours observed, either on the computer monitor or in the printed page, are dependent on the hardware and software of the computer, and ambient lighting used to view the colours.

5.3.5. Contribution of SO_2-stable wine colour and the influence of SO_2-stable pigments to the Shiraz red wine colour

In addition to the known pigments separated and identified by HPLC analysis, the anthocyanins, Vitisin A and the pigmented polymers, a group of pigments that would contribute to wine colour are the SO_2 stable wine pigments. The SO_2-stable pigments (Somers 1971; Sarni-Manchado *et al.* 1996; Bakker and Timberlake 1997; Francia-Aricha *et al.* 1997; Gonnet 1998; Jones *et al.* 1999; Cheynier *et al.* 2006; Garcia-Puente Rivas *et al.* 2006) would contribute to SO_2 stable wine colour are probably anthocyanin-derived pigments with a functional group at the 4-position of the anthocyanin moiety (Figure 1.6). The presence of the functional group will ensure colour stability of the pigment in the presence of excess bisulfite. An investigation into the contribution of SO_2-stable wine pigments to wine colour and SO_2-stable wine pigments as a percentage of wine colour was made. Earlier, Table 5.2 above illustrated that the average Shiraz red wine colour was violet-red (negative H* value) and deeply coloured (low L* and high C* values). Addition of excess bisulfite to the wines bleached the colour of any anthocyanins. It is believed that the bisulfite ion also bleached the colour of any pigments with the 4-position of the anthocyanin moiety available to bisulfite attack. This resulted in a loss of colour (increased L* and decreased C* values) as the colour remaining or SO_2-stable wine colour exhibited orange-red hues (positive H* values) (Table 5.10).

Table 5.10: Wine colour density, SO_2-stable wine colour and percentage of SO_2-stable pigments in the wines at approximately pH 3.6 for the Shiraz red wines.

Measured parameter [1]		Region			
		Gundagai	Bendigo	Swan Hill	Echuca
Wine colour density (a.u.)		13.4 ± 0.4 [a]	11.6 ± 0.4 [b]	9.1 ± 0.4 [c]	9.3 ± 0.4 [c]
SO_2-stable wine colour	SO_2-stable wine colour (a.u.)	8.7 ± 0.3 [a]	7.3 ± 0.3 [b]	5.7 ± 0.2 [c]	5.7 ± 0.2 [c]
	L*	74.6 ± 3.2 [c]	78.8 ± 3.8 [b]	83.0 ± 3.0 [a]	83.6 ± 2.2 [a]
	C*	23.2 ± 3.1 [a]	19.6 ± 3.3 [b]	16.6 ± 2.8 [c]	15.9 ± 1.7 [c]
	H*	0.4 ± 0.1 [b]	0.5 ± 0.1 [a]	0.4 ± 0.1 [a]	0.4 ± 0.1 [a]
SO_2-stable pigments (%)		65 ± 3 [a]	63 ± 3 [a]	63 ± 2 [a]	61 ± 3 [b]

| SO_2-stable wine colour [2] | | | | |
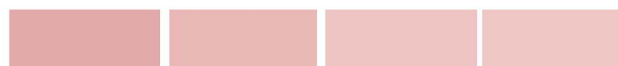
[1] Mean $\pm$ standard error calculated from triplicate colour measurements on the replicates from each region.

[2] The CIELab colours shown are representative of the actual SO_2 stable wine colours visible in a 1 mm cuvette. Importantly, the CIELab colours observed, either on the computer monitor or in the printed page, are dependent on the hardware and software of the computer, and ambient lighting used to view the colours.

[a–c] Values with different subscript letters are significantly different at P < 0.05.

For all four wine regions in this study, it was highly evident that the wine colour density values were related to SO_2-stable wine colour (Table 5.10). For example, the Gundagai wines had the highest wine colour density (13.4 ± 0.4 a.u.) and highest SO_2-stable wine colour (8.7 ± 0.3 a.u.). Similarly, the Swan Hill wines had the lowest wine colour density (9.1 ± 0.4 a.u.) and the lowest SO_2-stable wine colour (5.7 ± 0.2 a.u.). Figure 5.8 confirms that SO_2-stable wine colour was a major influence to wine colour (r = 0.97, P < 0.001) for the 76 Shiraz wine samples (including replicates). For this sample set of Shiraz wines, the two variables were related by the equation, y = 0.64 *x − 0.09, where y = SO_2 stable or SO_2 non-bleachable colour (a.u.) and x = wine colour density (a.u.).

There was no significant difference between the four wine regions in terms of the percentages of pigments that did not lose their colour upon SO_2 bleaching

(Table 5.10). Consequently, the percentage of SO_2 non-bleachable was independent of region (Table 5.10). This observation suggests that it is the not the quantity of SO_2-stable pigments. Rather it is the colour provided by SO_2-stable pigments that contributed the most to the overall red wine colour. In addition, pigments that were immune to SO_2 bleaching accounted for an average of 64 % of total wine colour for this sample set. Similarly, a high proportion of SO_2-stable pigments was observed in one year old red wine (Somers 1971; Schwarz *et al.* 2003a).
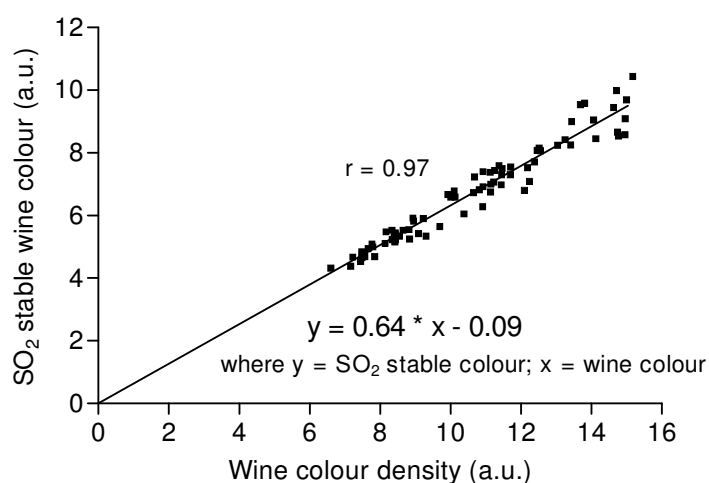
Figure 5.8: Relationship between wine colour density and SO_2-stable wine colour for the Shiraz red wines for correlation coefficient significant at *P* < 0.05.

The chemical age index (ii) of the red wines was determined (Somers and Evans 1977). The chemical age index (ii) provides an indication of the colour contribution of pH-dependent wine pigments to SO_2-stable wine colour. The chemical age index (ii) is used to interprete the relationship between the absorbance values of the SO_2-stable wine pigments and anthocyanin ions in their flavylium form (A_{SO2}/A_{HCl}) at 520 nm. Since the colour measurements were performed on the wines at approximately 18 months post-pressing, it can be assumed the anthocyanins have reacted with other polyphenols to form anthocyanin-derived wine pigments. For this sample set of Shiraz red wines, the chemical index (ii) values or A_{SO2}/A_{HCl} values were low, and ranged from 0.14 to 0.37 (raw data not shown). These values indicated that most of

the absorbance at 520 nm resulted from pH-dependent wine pigments, while the SO_2-stable, pH-stable pigments provided only a minor contribution. But Table 5.10 above showed that approximately 64 % of the pigments were SO_2 non-bleachable and importantly that SO_2-stable wine colour was a major contributor to wine colour. Thus, one must be aware that the calculation of the chemical index (ii) values uses colour measurements made at two different pH values: at low pH (to measure anthocyanins in their flavylium form) and at wine pH (to measure SO_2-stable wine pigments). In comparison, wine colour, SO_2-stable wine colour and the percentage of SO_2-non-bleachable pigments are always made at approximately wine pH. This latter measurement provides a more accurate comparison of colour values.

As examples of two anthocyanin-derived pigments, Vitisin A and the pigmented polymers compared well with SO_2-stable wine colour. This was even though the concentrations by HPLC analysis at low pH. Correlation values of 0.77 and 0.91 ($P < 0.05$ for 76 Shiraz wine samples including replicates) for Vitisin A and pigmented polymer concentration respectively resulted (Figure 5.9). The former value is consistent with published data regarding the stability of Vitisin A in the presence of the sulfite ion (Bakker and Timberlake 1997; Asenstorfer *et al.* 2001a). But, it must be acknowledged that the stability of Vitisin A to SO_2 bleaching was conducted using a model wine solution (Bakker and Timberlake 1997).
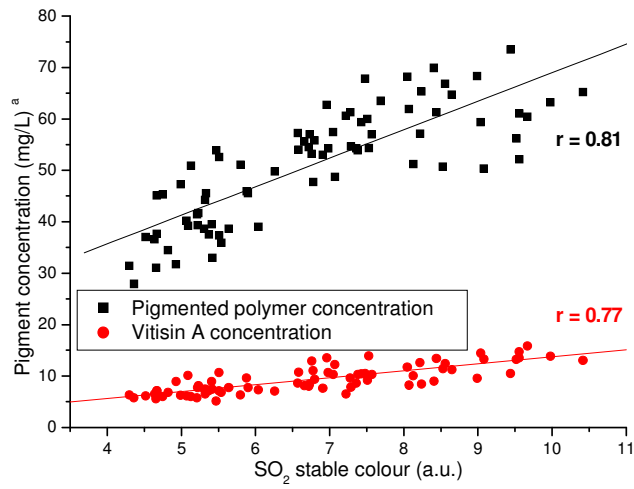
Figure 5.9: Relationship between Vitisin A and pigmented polymer concentration [a] and SO$_2$-stable wine colour for the Shiraz red wines for correlation coefficient significant at $P < 0.05$.

[a] Malvidin 3-glucoside chloride equivalents determined using the standard HPLC method at approximately pH 1.5.

Thus, it is highly probable that the colour differences of the SO$_2$-stable wine colour (Table 5.10) were a function of pigmented polymer concentration (Table 5.8). For example, the Gundagai red wines had the highest concentration of pigmented polymers (61 $\pm$ 8 mg/L) and the highest SO$_2$-stable wine colour (8.7 $\pm$ 0.3 a.u). On the other hand, the Swan Hill and Echuca red wines had the the lowest concentration of pigmented polymers (approximately 46 mg/L) and the lowest SO$_2$-stable wine colour (5.7 $\pm$ 0.2). Similiarly, the pigmented polymer concentration correlated well with SO$_2$-stable wine colour (r = 0.81) ($P < 0.05$ for 76 Shiraz wine samples including replicates) (Figure 5.9). A very good relationship between concentration of pigmented polymers determined by HPLC analysis and the SO$_2$-stable wine colour has already been observed by several researchers (Peng *et al.* 2002; Bartowsky *et al.* 2004; Eglinton *et al.* 2004). As there was a good correlation between SO$_2$-stable wine colour and wine colour density (r = 0.97) (Figure 5.8

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above), it could be inferred that the pigmented polymers contributed greatly to wine colour. But this inferrance relies on the assumption that all the oligomeric and polymeric anthocyanin-derived pigments that have eluted in the pigmented polymer peak were SO_2-stable and the pigments was unaffected by the low pH conditions. It is highly possible that some of the pigments that eluted in the pigmented polymer peak were stable to pH change and were stable to SO_2 bleaching. At the same time, there may also be a proportion of pigments that eluted in the pigmented polymer peak that were affected by the pH media and whose colour was bleached by the sulfite ion. These pH-dependent and SO_2 bleachable pigments would behave in a similar manner to anthocyanins and exhibit highly coloured isoforms at low pH, and whose colour would be bleached by the sulfite ion. Thus, it must be acknowledged that the pigmented polymer concentration was determined at approximately pH 1, whilst SO_2-stable wine colour was measured at approximately wine pH. So, despite the good correlation observed in Figure 5.9 above, one must be cautious before making any assumptions regarding the contribution of pigmented polymers to wine colour.

5.3.6.   Effect of copigmentation on the Shiraz red wine colour

The phenomenon of copigmentation on wine colour was investigated using these fairly young Shiraz red wines. The 18 month old wines were diluted with a model wine solution at pH 3.6, and the colour of the intact wines were compared with the diluted wine colour (Figure 5.10).
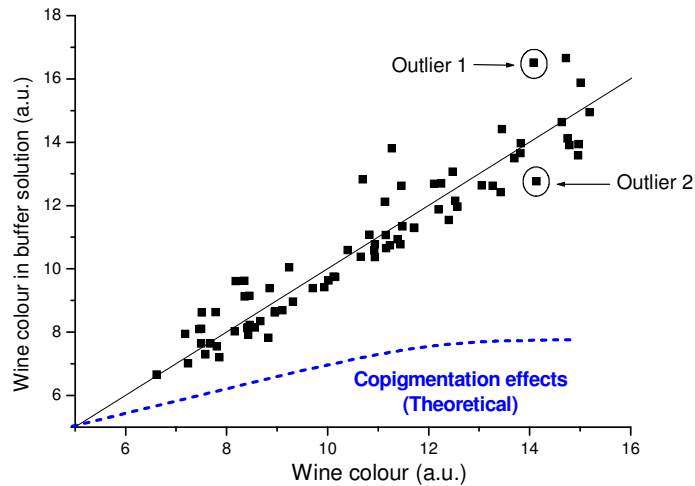
Figure 5.10: Copigmentation and its lack of influence on wine colour [1,2].

[1] Outliers 1 and 2 are replicate samples of wines made in the same vineyard and location

[2] Copigmentation would be observed if the data followed a trend similar to the dotted blue line. The line illustrates a deviation from Beer's Law.

Despite the presence of sufficiently high concentrations of anthocyanins (Table 5.5. above), Figure 5.10 confirms that copigmentation did not play a role within this sample set of wines. That is, Beer's Law has been obeyed (Equation 1.1) and wine colour density was directly proportional to diluted wine colour density (r = 0.95 for P < 0.001 for 76 Shiraz wine samples including replicates). An example of the theoretical effect of copigmentation is demonstrated by the dotted blue line (Figure 5.10). Figure 5.10 illustrates some outliers were observed below and above the regression line (intact black line). For example, outliers below the regression line were a function of wine composition. This resulted in an enhancement of the diluted wine colour in this area. Outliers above the regression line were influenced by factors such SO_2, pH and ethanol. Alternatively, in addition to copigmentation, the outliers could have been a result of variation and dilution error. For example, the outliers 1 and 2 highlighted were replicates of wines from the same block in the same vineyard in Gundagai: outlier 1 had a pH value of 3.28 and total SO_2 of 43 mg/L, while the outlier 2 had a pH value of 3.73 and total SO_2 of 54 mg/L. As both samples had the same free SO_2 levels, 14 mg/L, it was more

likely that the variations and scatter observed in Figure 5.10 reflected differences in experimental sampling and winemaking, rather than any copigmentation effects.

5.4.   Conclusions

1. *The region where grapes were sourced influenced wine colour.*
2. *CIELab values provide enhanced colour measurements over current wine colour measurements*
   a. *Colour was represented in three dimensions as LCH values, which allows colour to be reproduced and visually observed at a later time.*
   b. *Some CIELab values correlated with absorbance measurements at 420 nm and 520 nm e.g. L* and a* values, whilst others showed little or no correlation e.g. H* values and wine colour density were not related.*
   c. *Equations can be created to relate wine colour density values with CIELab colour coordinates. But, it must be acknowledged that any such equations are only relevant to the particular sample set employed.*
3. *Following quantification of anthocyanins and anthocyanin-derived wine pigments by HPLC analysis at low pH:*
   a. *High wine colour cannot be attributed to individual monomeric anthocyanins such as malvidin 3-glucoside or its acylated derivatives present in a wine.*
   b. *Vitisin A concentration was not responsible for the colour differences in the wines. Much colour was observed in the highest and lowest concentrations of Vitisin A in the Shiraz wines when expressed as CIELab colour values.*
   c. *Presenting the concentration of a wine pigment that has been determined at low pH provides only limited information. The colour of a wine pigment in a given media is also necessary when characterizing a wine pigment.*

d. The pigmented polymer concentration may be the driving force behind high wine colour density values. But this statement is flawed as pigmented polymers were quantified at low pH, not at wine pH. The inferrence that high pigmented polymer concentration results in high wine colour density relies on the assumption that most pigments that have eluted in the pigmented polymer peak were relatively unaffected by the pH of the media.

e. It is important to establish the colour of a pigment at wine pH

    i. Even though lower apparent concentrations of some pigments, such as anthocyanins, will be recorded.

    ii. The pH dependence of some anthocyanin-derived pigments is unclear from current literature.

    This will be investigated further in Chapter 8.

4. Similar to wine colour, the SO_2-stable wine colour was related to regional differences. For example, wines with high wine colour density had high SO_2-stable wine colour values. Similarly, wines with low wine colour density had consistently low SO_2-stable wine colour readings. The percentage of SO_2 non-bleachable pigments was consistent and independent of wine region. The chemical index (ii) values indicated that the major contributor at 520 nm was from the pH-dependent wine pigments, while the SO_2-stable wine pigments provided only a minor contribution. The SO_2-stable wine colour was found to be highly correlated and was a major influence to wine colour. Thus, the pH media of a colour measurement is important as it is possible that conflicting results may be achieved.

5. High correlations of Vitisin A and pigmented polymer concentrations determined at low pH with SO_2-stable wine colour were observed. The high correlations of pigmented polymers with SO_2-stable wine colour, and SO_2-stable wine colour with wine colour density (Conclusion 4) could infer that the pigmented polymers contributed greatly to wine colour. However, one must be aware that the pigmented polymer (and Vitisin A) concentrations were determined at low pH by HPLC analysis, and wine colour and SO_2-stable wine colour were made at the natural wine pH of the wines.

6. *Copigmentation was not important in the real wine samples studied and played no role in these fairly young red wines.*

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Chapter 6:   Colour measurement and wine pigment characterization in the Cabernet Sauvignon red wines

6.1.   Objectives

1. *To investigate that the differences in wine colour achieved during winemaking is a consequence of the choice of yeast.*
2. *To investigate the contribution of anthocyanins and anthocyanin-derived wine pigments to wine colour measured at low pH (total red pigment colour) and at wine pH.*
3. *To further investigate the importance of SO_2 stable wine colour to wine colour, and to demonstrate that some anthocyanin-derived pigments have an influence on SO_2 stable wine colour.*
4. *To demonstrate that any wine pigment (whether an anthocyanin or anthocyanin-derived) chosen from many pigments in a wine sample may not necessarily be a major contributor to wine colour.*

6.2.   Materials and methods

South Australian Cabernet Sauvignon red wines from the 2002 wine trial were evaluated at 0, 7, 13, 22 and 25 months post-pressing (Chapter 2 - Materials). The wines were made using two different yeast strains, *Saccharomyces cerevisiae* and *S. bayanus* respectively, abbreviated to SC and SB respectively. Spectroscopic absorbance and CIELab colour measurements of the wine were made at low pH, and at wine pH, and the SO_2 stable wine colour (Chapter 2 - Methods). Chromatographic analysis of the wines was performed using the standard HPLC method (Chapter 2 - Methods). The concentration of the anthocyanin monoglucosides, some acylated anthocyanin derivatives, and two anthocyanin-derived pigments, Vitisin A and the pigmented polymers were determined by HPLC-UV/Vis analysis. The pigments in the HPLC chromatogram were quantified as malvidin 3-glucoside equivalents (mg/L) using an external calibration function. The calibration equation was

Pigment concentration = 0.00915 x peak area + 0.79060.

As verification and confirmation of additional minor peaks in the HPLC chromatogram was not possible by UV/Vis analysis. So, HPLC-MS analysis was conducted by Yoji Hayasaka. The HPLC-MS analyses enabled screening of additional anthocyanin-derived pigments. The analyses were performed on the red wine samples at 0 and 13 months post-pressing. The HPLC-MS method confirmed the relative proportions of the anthocyanins and Vitisin A already observed by HPLC-UV/Vis analysis in the wines (data not shown), and enabled the identification of additional pigments including Vitisin B, two vinylphenol-derived pyranoanthocyanins, Pigment A and Pinotin A, and a flavanol-linked anthocyanin conjugate, an ethyl-bridged malvidin 3-glucoside-(epi) catechin dimer. The λ_{max} values of the anthocyanins and above mentioned anthocyanin-derived wine pigments were extracted from current published literature and were used to investigate the colour contribution of the pigments to red wine colour.

The colour measurements were made at 0, 7, 13, 22 and 25 months post-pressing enabled an investigation into the change in wine colour, SO_2-stable wine colour and the percentage of SO_2 non-bleachable pigments with time. The absorption spectra of the wine colour at 0 and 13 months post-pressing was presented. But a methodology had not yet been established at 0 months post-pressing for the SO_2-stable wine colour and percentage of SO_2 non-bleachable pigments, so only the absorption spectra of the SO_2-stable wine colour at 13 months post-pressing was presented. The second derivative absorption spectrum for each wine colour spectrum was calculated using the Savitzky-Golay filter method using the Origin ® Scientific Graphing and Analysis software (OriginLab Corporation, Northampton, USA). The second derivative absorption spectra enabled indentification of the important λ_{max} values and minor absorption regions (indicated by shoulders) that would have contributed to the wine colours and SO_2-stable wine colours.

An additional factor that influences the colour of a wine is its wine chemistry. The pH value, titratable acidity, organic acids present and the amount of free and total SO_2 in the red wine will affect its colour. The wine chemistry was

measured at 0 and 7 months post-pressing by the Analytical Service at the AWRI.

All analyses (except the wine chemistry measurements) were performed in triplicate on three replicate tanks for each SC and SB wine. The wine chemistry data was obtained from analyses performed on three replicate tanks. Statistical analysis of the data was presented as mean ± standard error of the replicated measurements.

6.3.   Results and discussion

6.3.1.   Chemistry of the Cabernet Sauvignon red wines

There was no significant difference in the colours of the wines made with SC or SB yeast strains (Table 6.1) at 0 months post-pressing. But the wine chemistry differed between the wines; that is, the titratable acidity, alcohol content, pH value and free and total SO_2 were significantly different between the wines. For example, the titratable acidity (8.2 mg/L) and total SO_2
(35 mg/L) were greater in the SB wines than in the SC wines. Although the colour was the same for the SC and SB wines, the pH value (pH 3.52), alcohol (14.7 %) and free SO_2 (24 mg/L) were significantly higher in the SC wines.

But at 7-months post-pressing, the colours of the SC and SB wines differed. The wine colour density of the SC wine was significantly lower (8.57 ± 0.25 a.u.) than the wine colour density of the SB wine (9.84 ± 0.21 a.u.). But there was negligible difference in the pH values, and the free and total SO_2 of the wines. Although there was a significant difference in the titratable acidity and ethanol, it is considered more probable that the greatest influence to the colours was the composition or the wine pigments present in the red wines.

Table 6.1: Wine colour density and wine chemistry of the SC and SB wines after 0 and 7 months 1. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively.

Time (months)	Treatment	SC wines	SB wines
0	Wine colour density (a.u.)	11.41 ± 0.25 [a]	11.45 ± 0.13 [a]
	Titratable acidity (mg/L) at pH 8.4	7.2 ± 0.2 [a]	8.2 ± 0.1 [b]
	pH	3.52 ± 0.0 [a]	3.50 ± 0.0 [a]
	Alcohol (%)	14.7 ± 0.1 [a]	13.2 ± 0.2 [b]
	Free SO_2 (mg/L)	3 ± 0 [a]	1 ± 1 [a]
	Total SO_2 (mg/L)	24 ± 1 [a]	35 ± 3 [b]
7	Wine colour density (a.u.)	8.57 ± 0.25 [a]	9.84 ± 0.21 [b]
	Titratable acidity (mg/L) at pH 8.4	5.1 ± 0.1 [a]	5.7 ± 0.0 [b]
	pH	3.67 ± 0.03 [a]	3.67 ± 0.00 [a]
	Alcohol (%)	14.7 ± 0.1 [a]	14.3 ± 0.1 [b]
	Free SO_2 (mg/L)	34 ± 1 [a]	29 ± 2 [a]
	Total SO_2 (mg/L)	52 ± 2 [a]	56 ± 1 [a]

[1] Mean ± standard error was calculated from measurements on the three replicate tanks for each SC and SB wine. Triplicate measurements were made to determine the wine colour density.

[a or b] Values with different subscript letters are significantly different at $P < 0.05$.

6.3.2. Colour and pigment composition of the wines

At 0 months (post-pressing), there was no difference between the wine colour density values of the wines (Figure 6.1 and Table 6.1). A rapid decrease in wine colour density values of both SC and SB wines was observed within the first 7 months. The overall SB wine colour remained significantly greater than the SC wine colour, and this colour difference between the wines remained unchanged until the final measurement at 25 months (Figure 6.1). For example, lower wine colour density values were observed in the SC wines (8.54 ± 0.14 a.u.) compared to the SB wines (10.09 ± 0.24 a.u.) at 25 months (Figure 6.1). For both wines, after 7 months post-pressing, a plateau was observed in the wine colour density values. It is possible that the wine colour

density may have experienced an exponential decrease between 0 and 7 months, but data within this time period was absent.
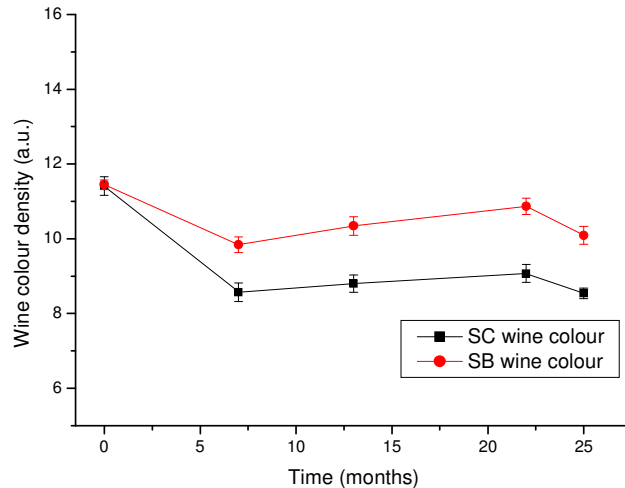
Figure 6.1: Change in wine colour density with time for the SC and SB wines [a]. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively.

[a] Measurements repeated in triplicate on the three replicate tanks for each SC and SB wine presented as mean ± standard error.

The colour differences observed were attributable to the choice of yeast as there was negligible difference between the replicated ferments (as demonstrated by the low standard error). Thus, choice of yeast can affect red wine colour which is in agreement with some researchers (Miltenberger *et al.* 1998; Girard *et al.* 2001; Caridi *et al.* 2002).

Comparing the wine colours in the different pH media, it is clear that the change in wine colour, at approximately pH 3.7, (Figure 6.1) did not mirror the change in total red pigment colour at pH 1 (Figure 6.2). In fact, an exponential decrease in the total red pigment colour was observed over time period of the measurement (Figure 6.2). In addition, converse to the wine colour measurements (Figure 6.1), throughout the measurement period the SC total red pigment colour (or colour at low pH) remained greater than in the SB total

red pigment colour (Figure 6.2). This observation confirms that the pH value of a colour measurement is important.
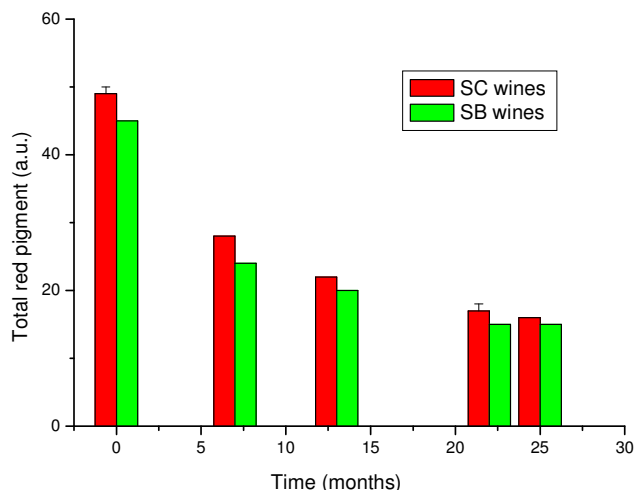
Figure 6.2: Change in total red pigment colour at pH 1 with time for the SC and SB wines [a]. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively.

[a] Measurements repeated in triplicate on the three replicate tanks for each SC and SB wine presented as mean ± standard error.

6.3.3. Composition of pigments in the Cabernet Sauvignon red wines

To evaluate the differences in the colour measurements at the two pH values, it was necessary to investigate the pigment composition in the wines. The composition of wine pigments in the SC and SB Cabernet Sauvignon red wines was considered by quantifying the anthocyanins and anthocyanin-derived pigments such as Vitisin A and the pigmented polymers detected in the wines by HPLC-UV/Vis analysis were used to verify their respective relationships to wine colour. In addition, five anthocyanin-derived pigments were identified in the red wines by HPLC-MS analysis, and their probable influence to SC and SB wine colour and SO_2 stable wine colour were also assessed.

6.3.4. Contribution of anthocyanins to the Cabernet Sauvignon red wine colour

For both wines, there was a decrease in the anthocyanin concentration in the Cabernet Sauvignon red wines, as observed by the reduction in the malvidin 3-glucoside concentration (Figure 6.3). The decrease in anthocyanin concentration, determined by HPLC analysis at low pH, mirrored the change in the total red pigment colour at low pH (Figure 6.2 above). It is possible that the total red pigment colour reflected the presence of the highly coloured flavylium forms of anthocyanins and their corresponding acylated derivatives (data not shown) quantified at low pH. Thus, one can speculate that pH-dependent pigments, such as malvidin 3-glucoside and the other anthocyanins in the wines, were the driving force behind the higher total red pigment colour observed in the SC wines. Additional evidence was demonstrated in Chapter 3. The change in malvidin 3-glucoside concentration (Figure 6.3) was related to the change in total red pigment colour (Figure 6.2). This was probably attributed to the approximately similar pH conditions of the two measurements. As there was no similarity between the change in the malvidin 3-glucoside (Figure 6.3) with the wine colour density (at approximately pH 3.7) (Figure 6.1), it could be misleading to infer any association with malvidin 3-glucoside determined at low pH with wine colour at its natural pH.

Similar to the higher SC total red pigment colour measured over the 25 month period (Figure 6.2), the concentration of malvidin 3-glucoside was greater in the SC wines than in the SB wines (Figure 6.3). For example, a high concentration of malvidin 3-glucoside, but lower wine colour density values were recorded in the SC wines than in the SB wines after 7 months post-pressing.
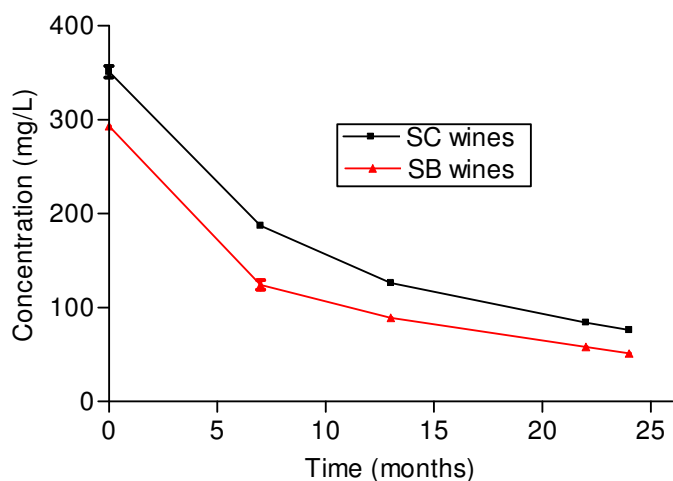
Figure 6.3: Change in malvidin 3-glucoside concentration with time for the SC and SB wines [a]. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively.

[a] Measurements repeated in triplicate on the three replicate tanks for each SC and SB wine presented as mean ± standard error. Unfortunately due to scale of the ordinate axis, the error bars are not visible.

In terms of concentration, the quantity of anthocyanins decreased from 0 to 13 months post-pressing for both SC and SB wines (Table 6.2). For example, the malvidin 3-glucoside concentration in the SC wines was 351 mg/L at 0 months and decreased to 126 mg/L after 13 months post-pressing. In addition, the monomeric anthocyanin concentration was consistently higher in the SC wines than in the SB wines over the entire measurement period (Table 6.2). For example, at 13 months post-pressing, the concentration of delphinidin 3-glucoside was 29 mg/L in the SC wines but only 24 mg/L of delphinidin 3-glucoside was recorded in the SB wines. The overall reduction in anthocyanin concentration ranged from 60 to 70 % over the period of the 13 month measurement (Table 6.2).

Table 6.2: Quantification of monomeric anthocyanins from the SC and SB wines at low pH. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively.

Pigment		Yeast strain	Time (months)		Figure	λ_{max} (nm)	Colour
			0	13			
Anthocyanins [1,2]	Delphinidin 3-glucoside	SC	72 ± 2 [a]	29 ± 1 [a]	Pigment 1b [3]	517 [4]	Red
		SB	63 ± 1 [b]	24 ± 1 [b]			
	Cyanidin 3-glucoside	SC	5 ± 0 [a]	6 ± 0 [a]	Pigment 1e [3]	511 [4]	Red
		SB	4 ± 0 [a]	5 ± 0 [a]			
	Petunidin 3-glucoside	SC	64 ± 1 [a]	19 ± 0 [a]	Pigment 1c [3]	519 [4]	Red
		SB	55 ± 0 [b]	15 ± 1 [b]			
	Malvidin 3-glucoside	SC	351 ± 6 [a]	126 ± 3 [a]	Pigment 1a [3]	528 [5]	Red
		SB	293 ± 3 [b]	89 ± 3 [b]			

[1] Concentration in mg/L malvidin 3-glucoside equivalents determined using standard HPLC method at pH 1.5.

[2] Mean ± standard error calculated from triplicate measurements on the three replicate tanks for each SC and SB wine.

[3] Anthocyanin structures described in Figure 1.6.

[4] λ_{max} values of the anthocyanin monoglucosides at pH 3.0 in model wine solution (Mazzaracchio *et al.* 2004)

[5] Measured λ_{max} value of malvidin 3-glucoside in model wine (0.25% w/v potassium bitartrate and 12% v/v ethanol, adjusted to pH 3.6 using 1 M NaOH).

[a or b] Values with different subscript letters are significantly different at $P < 0.05$.

6.3.5. Contribution of Vitisin A to the Cabernet Sauvignon red wine colour

Contrary to the exponential decay of malvidin 3-glucoside in both wines (Figure 6.3), Vitisin A concentration experienced an initial increase (between 0 and 7 months), and remained unchanged in the latter measurements (13 to 25 months) (Figure 6.4). Vitisin A is considered to be formed during fermentation and its concentration can slightly increase or decrease with maturation time. The variation in Vitisin A concentration is considered to depend upon on the availability of malvidin 3-glucoside and pyruvic acid (Schwarz *et al.* 2003d; Morata *et al.* 2006) or the presence of oxygen (Asenstorfer *et al.* 2003b).

At 0 and 13 months post-pressing, there was no significant difference between the peak areas of Vitisin A in the SC and SB wines (Table 6.3). So, although Vitisin A will have contributed to wine colour, the pigment was not responsible, in particular, for the higher SB wine colour at 13 months post-pressing. Figure 6.4 clearly demonstrates that the change in Vitisin A concentration was different to the change in wine colour density over the measurement time period (Figure 6.1). This observation further confirms that Vitisin A was not a major contributor to wine colour.
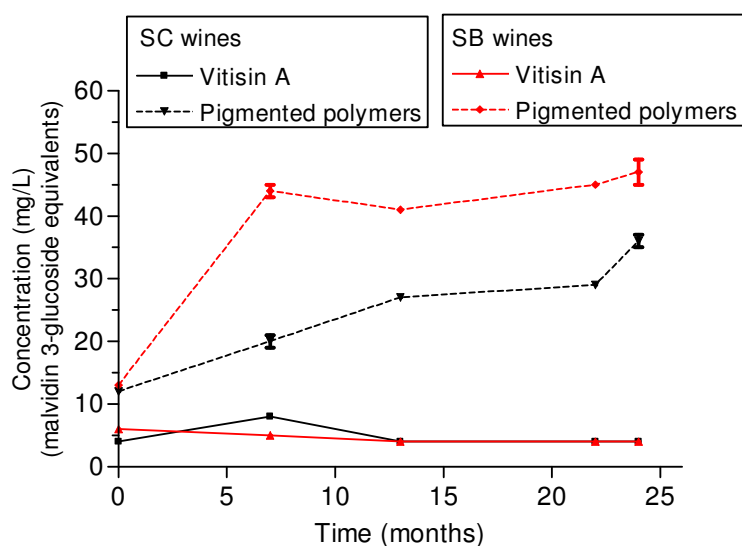
Figure 6.4: Change in Vitisin A and pigmented polymer peak concentration with time for the SC and SB wines [a]. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively.

[a] Measurements repeated in triplicate on the three replicate tanks for each SC and SB wine presented as mean $\pm$ standard error.

6.3.6.   Contribution of the pigmented polymers to Cabernet Sauvignon red wine colour

Initially, there was no significant difference between the pigmented polymer concentrations of both wines at 0 months post-pressing (Figure 6.4). But, a constant increase in the pigmented polymer concentration was apparent for

both SC and SB wines between 7 and 25 months (Figure 6.4). The higher concentration of SB pigmented polymers (Figure 6.4) appeared to reflect the higher SB wine colour density values (Figure 6.1), particularly from 7 months onwards. A similar observation was made earlier in the study using Shiraz red wines (Table 5.8). The results of the Shiraz red wine study demonstrated that pigmented polymer concentration and wine colour density values were highly correlated ($r = 0.80$) at the given analysis time period (Figure 5.7 above). Thus, it could be inferred that there is further evidence of the influence of the pigmented polymer concentration to red wine colour. But, similar to Section 5.3.3., one must be aware that the two measurements (pigmented polymer quantification at low pH and wine colour at natural wine pH) were made at different pH values, and the subsequent results could be misleading.

6.3.7. Contribution of additional anthocyanin-derived pigments detected in the Cabernet Sauvignon red wine

Verification and confirmation of additional minor peaks (excluding the anthocyanins, the acylated anthocyanins and Vitisin A) in the HPLC chromatogram was not possible by UV/Vis analysis. So, HPLC-MS analysis was used to screen for additional anthocyanin-derived pigments in the wine samples at 0 and 13 months post-pressing. From Table 6.3, it is apparent that in the time period from 0 to 13 months post-pressing, some pigments accumulated in the red wine (e.g. Pinotin A and Pigment A), whilst others disappeared or negligible amounts were detected (e.g. Vitisin B and the ethyl-linked pigments) as the pigments degraded to form other stable pigments (Lee *et al.* 2004). More Vitisin B was detected in the SB wines. The SB yeast is known to produce more acetaldehyde during fermentation (Eglinton and Henschke 2003), hence the higher concentration of Vitisin B in the SB wines (Morata *et al.* 2003a; Morata *et al.* 2007). But, the presence of Vitisin B did not have a major impact on the SB wine colour at 0 months post-pressing as the wine colour was equated to the SC wine colour. Also, the excess acetaldehyde during fermentation did not influence the production of Vitisin A

as there was no significant difference in the detection of this pyranoanthocynanin in both SC and SB wines at 0 months post-fermentation (Table 6.3). No Pinotin A was detected at 0 months. It is quite possible that the ratio of caffeic acid/malvidin 3-glucoside had decreased as a small amount of the Pinotin A was recorded after 13 months (Schwarz *et al.* 2003b; Schwarz *et al.* 2003c; Schwarz and Winterhalter 2003g; Schwarz *et al.* 2004a). There was also an increase in the detection of Pigment A in both wines consistent with the literature (Fulcrand *et al.* 1996; Asenstorfer *et al.* 2001a). At 13 months post-pressing, the levels of Pinotin A and Pigment A were approximately equivalent in both wines (Table 6.3). Therefore, the presence of these two anthocyanin-derived pigments was not responsible for the higher SB wine colour at the latter measurement time, at 13 months post-pressing (Figure 6.1).

Table 6.3: Quantification of five anthocyanin-derived wine pigments from the SC and SB wines at low pH. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively.

Pigment		Yeast strain	Time (months)		Figure	λ_{max} (nm)	Colour
			0	13			
Anthocyanin-derived pigments [1]	Vitisin A	SC	69 (26)	77 (30)	Pigment 2a [2]	500 – 511 [3]	Orange-red to red
		SB	90 (11)	65 (18)			
	Vitisin B	SC	388 (5)	8 (20)	Pigment 3a [2]	486 – 500 [3]	Orange-red to red
		SB	708 (15)	26 (29)			
	Pinotin A	SC	n.d. [4]	4 (25)	Pigment 4h [2]	503 – 512 [3]	Orange-red to red
		SB	n.d. [4]	4 (16)			
	Pigment A	SC	11 (45)	24 (25)	Pigment 4a [2]	508 – 510 [3]	Orange-red to red
		SB	7 (9)	17 (3)			
	Ethyl-bridged dimers [5]	SC	Data illustrated in		Pigment 10 [2]	540 – 545 [3]	Red-violet
		SB	Figure 6.6				

[1] Average peak area (x 10^3) and coefficient of variation (%) in brackets determined by HPLC-MS analysis performed on the three replicate tanks for each SC and SB wine.

[2] Anthocyanin-derived pigment structures referred to in Figures 1.8, 1.9 and 1.13.

[3] λ_{max} values referred to in Appendices 1.2 – 1.24.

[4] n.d.: not detected.

[5] Ethyl-bridged malvidin-3-glucoside (epi)catechin dimers (Figure 6.5 below).
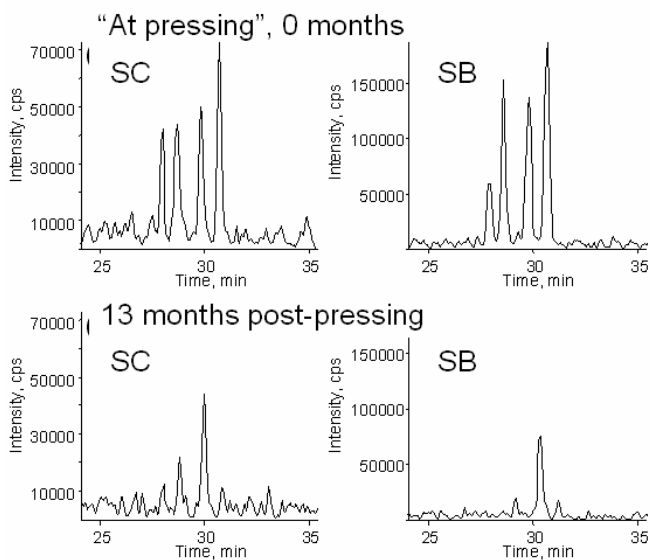
Figure 6.5: HPLC-MS analysis of ethyl-bridged malvidin-3-glucoside-(epi) catechin dimers of the SC and SB wines. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively.

6.3.8.   Contribution of SO$_2$-stable colour and the influence of SO$_2$ stable pigments to Cabernet Sauvignon red wine colour

From the results with the Shiraz red wine study (Section 5.3.5), it was demonstrated that SO$_2$-stable wine colour was a major influence to wine colour (r = 0.97) (Figure 5.8). In this study of Cabernet Sauvignon red wines, the change in SO$_2$-stable wine colour (Figure 6.6) also appeared related to the change in wine colour with time (Figure 6.1 above). For example, from 7 months post-pressing onwards, the SO$_2$-stable wine colour and wine colour density were consistently higher for the SB wines than for the SC wines. Thus, it could be assumed that the pigments that contributed to higher SB SO$_2$-stable wine colour influenced higher SB wine colour.

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Note: No data available for post-pressing samples at 0 months
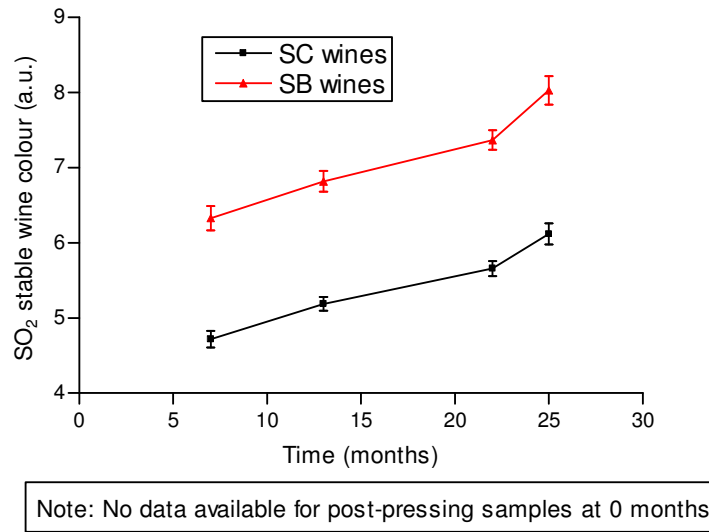
Figure 6.6: Change in the SO_2-stable wine colour with time in the SC and SB wines [a,b]. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively.

[a] Measurements repeated in triplicate on the three replicate tanks for each SC and SB wine presented as mean ± standard error.

[b] Data from 7 months to 25 months is shown. No data was available at 0 months ("at pressing") as the methodology had not yet been established.

Similarly, the percentage of SO_2 non-bleachable pigments was consistently higher in the SB wines than in the SC wines over the measurement time period (Figure 6.7). Similarly, it could be assumed that the percentage change of SO_2 non-bleachable pigments (Figure 6.7) exhibited similar trends to the wine colour density of the Cabernet Sauvignon red wines (Figure 6.1) over the measurement time period. But, as SO_2-stable wine colour and the percentage of SO_2 non-bleachable pigments were not made at 0 months post-pressing, it is difficult to make adequate statements regarding the change in colour values between 0 and 7 months post-pressing.

Figure 6.7: Change in the percentage of SO_2 non-bleachable pigments with time in the SC and SB wines [a,b]. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively.

[a] Measurements repeated in triplicate on the three replicate tanks for each SC and SB wine presented as mean ± standard error.

[b] Data from 7 months to 25 months is shown. No data was available at 0 months ("at pressing") as a methodology had yet not been established.

Both wine colour and SO_2-stable wine colour values were derived from spectral measurements made on intact wines. It is clear that information regarding characterization of <u>individual</u> wine pigments at wine pH that contributed to the Cabernet Sauvignon red wine colour at its natural wine pH is absent. Similarly, characterization of SO_2-stable pigments that contribute to SO_2-stable wine colour is also absent. A method is needed to quantify and determine the colour properties of individual red wine pigments at wine pH and SO_2-stable wine pigments. Later in Chapters 7 and 8, two instances of wine pigments will be considered: HPLC-separated pigments at wine pH and HPLC-separated pigments that are stable to SO_2-bleaching. In these chapters, the pigments will be quantified and their colour properties determined both at wine pH and post-SO_2 bleaching.

6.3.9. Detection of pigments present in a red wine and their apparent influence to wine colour

In the literature, the characteristics and colour properties of many anthocyanin-derived pigments have been investigated for aged wine samples and using model wine systems (Appendices 1.3. to 1.24.). But in all cases, the anthocyanin-derived pigment has been isolated before any characterisation. In several instances, researchers have chosen an individual anthocyanin-derived pigment and published its colour properties that has been mostly represented by its λ_{max} value at low pH (Revilla *et al.* 1999; Mateus and de Freitas 2001; Vivar-Quintana *et al.* 2002; Mateus *et al.* 2002b; Mateus *et al.* 2003; Mateus *et al.* 2003; Monagas *et al.* 2003; Morata *et al.* 2003; Wang *et al.* 2003a; Wang *et al.* 2003b; Schwarz *et al.* 2003c; Schwarz *et al.* 2003d; Alcalde-Eon *et al.* 2004; de Freitas *et al.* 2004a; de Freitas *et al.* 2004b; de Villiers *et al.* 2004; Pozo-Bayón *et al.* 2004; Wang *et al.* 2004). The actual concentration of the wine pigment is generally absent. It appears that, since much time and effort has been devoted into isolating the pigment, some researchers have speculated that the pigment is mostly responsible for the colour of a particular age of wine. For example, Romero and co-workers have stated that Vitisin A may contribute to an orange-red colour (Romero and Bakker 2000b). But, one must be aware that the colour of a wine, whether it is a young wine or an aged wine, is derived from many pigments, namely anthocyanins and anthocyanin-derived pigments. Importantly, there would be varying amounts of these pigments present in any red wine. For example, in young red wines, there is a high concentration of anthocyanins present resulting from colour extraction of red grapes compared to low concentrations of Vitisin A.

When the colour of a red wine is measured at its natural pH, accordingly the colour of the red wine pigments such as the anthocyanins present are also measured at wine pH. At wine pH, there is a hypsochromic shift of the λ_{max} values of anthocyanins towards purple-red absorbance. Thus, it could be inferred that the purple-red hues observed in young red wines are only the

result of the colour provided by monomeric anthocyanins. But, in addition to the anthocyanins, Vitisin A and the pigmented polymers, and various anthocyanin-derived wine pigments can be detected in wines (Table 6.3 above). For example, in the Cabernet Sauvignon red wines, ethyl-linked pigments were detected in the wines at 0 months post-pressing. It has been suggested that sufficiently high concentrations of ethyl-linked pigments provide the purple hues observed in young red wines immediately after the completion of fermentation (Lee *et al.* 2004). Therefore, it could be inferred that only the anthocyanins and the ethyl-linked pigments contribute to the wine colour.

But, identifying the presence of a pigment in a wine does not necessarily imply that the pigment contributes greatly to wine colour. This is important especially if no regard is given to the concentration of the pigments, or to the extinction coefficient of the pigment, or to the pH media. The same applies to the ethyl-linked wine pigments. It is difficult to assess important individual pigments without quantifying their concentration and colour properties with respect to wine colour. The use of λ_{max} values from published literature must be treated with caution; particularly as in most cases the pH media of a wine pigment has not been recorded. Importantly, pigments considered to be major contributors to red wine colour cannot be assumed especially when the pigment is considered in isolation. The choice of a pigment, at given measurement time periods, and the pigment's supposed influence on the colour of the Cabernet Sauvignon red wine shall be demonstrated in the following examples. Pigments identified in the Cabernet Sauvignon wines will be compared to wine colour at 0 and 13 months post-pressing, and SO_2 stable wine colour at 13 months post-pressing.

6.3.9.1. Example 1: Pigments that influence wine colour at pressing, 0 months

At 0 months post-pressing, there was no significant colour differences between wines made with either SC or SB yeast strain (Table 6.4), and as the ΔE^* value approximated to zero. Both SC and SB wine colours were dark and

brightly coloured, which was apparent from the low L* and high C* values (Table 6.4). Negative H* values were observed which indicated red-violet hues in both wines.

Table 6.4: Change in CIELab values of the wine colour and SO_2-stable wine colour of the SC and SB wines with time. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively.

CIELab colour value [1]		Yeast strain strain	Time (months)	
			0	13
Wine colour	L*	SC	63.64 ± 0.63 [a]	72.19 ± 0.77 [a]
		SB	63.64 ± 0.32 [a]	69.31 ± 0.68 [b]
	C*	SC	43.54 ± 0.52 [a]	28.10 ± 0.65 [a]
		SB	43.00 ± 0.32 [b]	31.42 ± 0.57 [b]
	H*	SC	-0.12 ± 0.01 [a]	0.19 ± 0.01 [a]
		SB	-0.08 ± 0.00 [b]	0.34 ± 0.01 [b]
SO_2 stable wine colour	L*	SC		85.99 ± 0.39 [a]
		SB		81.72 ± 0.47 [b]
	C*	SC	No data available [2]	17.04 ± 0.11 [a]
		SB		16.87 ± 0.41 [b]
	H*	SC		0.83 ± 0.01 [a]
		SB		0.80 ± 0.01 [b]

[1] Colour measurements repeated in triplicate on the three replicate tanks for each SC and SB wine and presented as mean ± standard error.

[2] SO_2 stable wine colour data is shown only at 13 months. No data was available at post-pressing, 0 months as methodology had not been established.

[a or b] Values with different subscript letters are significantly different at *P* < 0.05.

Similarly, the equivalence of the SC and SB wine colours was demonstrated in the spectral profile of the red wine samples at pressing (0 months) revealed high absorbance across the visible region and a broad absorption peak maximum was apparent (Figure 6.8 (a)). The red-violet hues of both red wine colours were attributed to the λ_{max} values of 547 and 544 nm for the SC and SB wines respectively. The λ_{max} values were identified following application of the second derivative to the absorption spectra (Figure 6.8 (b)). No shoulders were observed in the spectral profile, which indicated that there were no additional absorption values that had a major influence on wine colour.
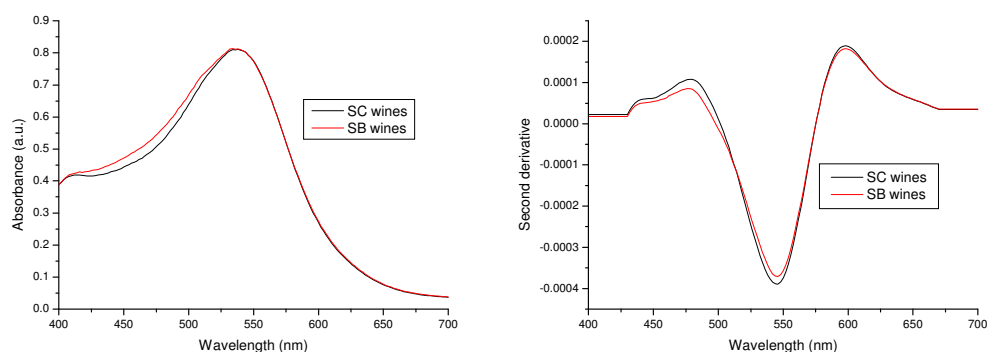
	(a) Visible spectral profile	(b) Second derivative spectra

Yeast	Peak (nm) at wine pH
SC wines	547
SB wines	544
Complementary colour [a]	Red-violet

[a] Complementary colour at the associated λ_{max} value.

Figure 6.8: Visible spectral profile (a) and second derivative spectra (b) of the SC and SB wines at 0 months ("at pressing") at natural wine pH. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively.

At 0 months, anthocyanins were extracted from red grapes during fermentation. As the anthocyanins were present in the highest concentrations (Table 6.2 above), it could be assumed that the young red wine colour (Table 6.4) was attributed only to the anthocyanins, and thus, anthocyanin-derived pigments may have contributed a minor amount. However, the λ_{max} values for the anthocyanins were much lower and were in the range from 511 to 528 nm at wine pH (Table 6.2) than the λ_{max} values of 544 and 547 nm observed for the SC and SB wines (Figure 6.8 (a) and (b)). Thus, based solely on the λ_{max} values of the anthocyanins and the red wine samples, one cannot conclude that anthocyanins alone were the major contributors to the young SC and SB red wine colour. In addition to the anthocyanins, substantial quantities of the ethyl-bridged malvidin 3-glucoside-(epi)catechin dimers were also detected at pressing (Table 6.3 above). As the λ_{max} value of the ethyl-bridged dimers ranged from 540 to 545 nm (Lee *et al.* 2004) one could assume these ethyl-

bridged dimers contributed to the colour to the young SC and SB wines. That is, the λ_{max} value of the ethyl-bridged dimers was similar to the λ_{max} values of 547 and 544 nm for the SC and SB wines respectively. An estimate of the quantity of ethyl-bridged dimers was illustrated in Figure 6.6 above. But, without the exact concentration of these dimers, one cannot fully justify the contribution of these anthocyanin-derived pigments to young wine colour. In addition, it is evident from Table 6.3 that not only were ethyl-bridged dimers present, but other anthocyanin-derived pigments such as Vitisin A and Vitisin B were detected in sufficient quantities at 0 months. The λ_{max} values for Vitisin A and Vitisin B were in the range 500 to 511 nm and 486 to 500 nm respectively (Table 6.3). But as the λ_{max} values of Vitisin A and Vitisin B were 50 to 60 nm less than the peak maximum of the wine. Thus, based on the λ_{max} values of these two pyranoanthocyanins, one cannot infer that Vitisin A and Vitisin B were major contributors to young wine colour.

Further examples of possible interpretation of λ_{max} values and pigments in the one year old Cabernet Sauvignon red wines are demonstrated.

6.3.9.2.   Example 2: Pigments that influence wine colour at 13 months

After ageing for 13 months, the colours of both the SC and SB wines were lighter and duller, as evident from their higher L* and reduced C* values (Table 6.4 above). Maturation of the wines has resulted in a change in the red wine colour from red-violet wine hues (low negative H* values) to more orange-red hues (low positive H* values) (Table 6.3). The colour change was obvious from the reduced absorbance values of both wines from colour measurement across the visible spectrum at 13 months (Figure 6.9 (a)) compared to at 0 months (Figure 6.8 (a) above). A comparison of spectral profiles at the two time measurements revealed a hypsochromic shift of the λ_{max} values of the wine colours to lower wavelengths. For example, at 0 months post-pressing, the λ_{max} value of the SC and SB wines were 544 and 547 nm respectively (Figure 6.8 (b)). Whilst at 13 months post-pressing, the

λ_{max} values shifted to lower wavelengths of 522 and 532 nm respectively for both SC and SB wines (Figure 6.9 (b)). In addition, shoulders were observed in the second derivative absorption spectra at 13 months post-pressing. The shoulders indicated a range of many absorbance maxima at approximately 439 to 488 nm, approximately 558 to 581 nm and approximately 619 to 640 nm for both SC and SB wines indicating the formation of additional different pigments over the 13 months. The colours associated with these wavelength maxima indicated that the pigments contributed orange, red-violet and blue to blue-green hues to the red wines. But compared to absorbance profile at 0 months post-pressing (Figure 6.8), the overall absorbance values for the year old wines in these three colour regions were much lower (Figure 6.9).
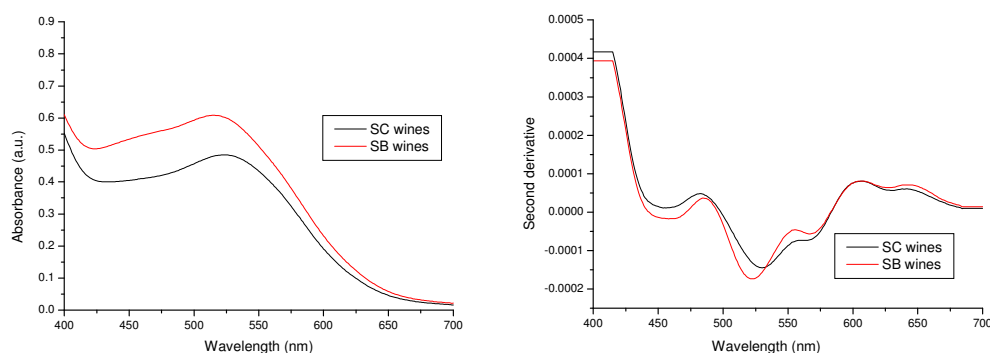
| (a) Visible spectral profile | (b) Second derivative spectra |

Yeast	Peaks and shoulders (nm) at wine pH			
SC wines	439 – 481	532	563 – 581	619 – 640
SB wines	447 – 488	522	558 – 581	619 – 640
Complementary colour [a]	Orange	Red/red-violet	Red-violet	Blue to blue-green

[a] Complementary colour at the associated λ_{max} value.

Figure 6.9: Visible spectral profile (a) and second derivative spectra (b) of the SC and SB wines at 13 months post pressing, at wine pH. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively.

At 13 months post-pressing, the SB wine colour was greater than the SC wine colour (lower L* and higher C* values) (Table 6.4 above). This is confirmed in the greater SB absorbance values across the visible spectrum (Figure 6.9). There was a greater concentration of anthocyanins quantified in the SC wines

at 13 months measurement time (Table 6.2). Based on the λ_{max} values 522 and 532 nm of the SC and SB wine colour respectively, it could be assumed that the anthocyanins were responsible for the colour of the one year old wines (Table 6.2 above). But, Table 6.2 revealed that the anthocyanin concentration had been reduced by 60 to 70 % since pressing. Instead of the anthocyanins, it was probably the anthocyanin-derived pigments detected in the SB wines that influenced the higher SB wine colour (Table 6.4 and Figure 6.9). The wine colour at 13 months could be attributed to Vitisin A, Pinotin A and Pigment A (Table 6.3). But the λ_{max} values of these anthocyanin-derived pigments were lower (Table 6.3) than the absorbance maxima of 522 and 532 nm observed for both SC and SB wines. For example, the λ_{max} values of Pinotin A and Pigment A are 503 to 512 nm and 508 to 510 nm respectively (Table 6.3 above). Consequently, based on the λ_{max} values, one could infer that these three anthocyanin-derived pigments did not contribute to the absorbance maxima. These anthocyanin-derived pigments may have contributed to the orange hue observed in the wine colour (Table 6.4 above). Similar to the inference based on the λ_{max} value that the anthocyanins contributed to wine colour at 13 months post-pressing, it could be inferred that the ethyl-linked pigments contributed to the one year old wine colour. But, at 13 months post-pressing, there was approximately a 67 % reduction in the ethyl-linked dimer concentration (Figure 6.6) as a result of degradation and formation to other stable red pigments (Es-Safi *et al.* 1999; Atanasova *et al.* 2002b; Lee *et al.* 2004). As a result, it could be speculated that the reduction in the anthocyanin concentration and detection of the ethyl-linked pigments could be responsible for the loss of colour in the purple-red region of the visible spectrum from 0 to 13 months post-pressing.

6.3.9.3.   Example 3: Pigments that influence SO$_2$-stable wine colour at 13 months

As a methodology had not yet been established for samples taken at pressing (0 months), no SO$_2$-stable wine colour data was available at this time period. At 13 months post-pressing, the SO$_2$-stable wine colour was lighter and duller

(higher L* and lower C* values) for both wines than the wine colour at the same time measurement (Table 6.4 above). There was a large increase in the H* values or orange-red hues observed in the wines. For example, the H* values increased from (0.19 to 0.34) for the SC and SB wines respectively to (0.80 to 0.83) for the SC and SB SO_2-stable wine colours respectively (Table 6.4).

Thus, the yeasts used to ferment the wines differed and would have influenced the formation of different types and concentrations of anthocyanins and anthocyanin-derived pigments detected in the SC and SB wines. The SO_2-stable wine pigments at 13 months post-pressing were approximately the same for both wines as demonstrated by the equivalent λ_{max} values of 516 nm for both SC and SB SO_2-stable wine colours (Figure 6.10). This could indicate that similar SO_2 stable pigments were present in the wines, but as the absorbance values at 516 nm differed, the SO_2 stable pigments present in the SB wines contributed greater colour than in the SC wines.

There was a reduction in absorbance values across the visible spectrum for the SO_2-stable wine colour (Figure 6.10) compared to the wine colour (Figure 6.9 above). The loss of wine colour was attributed to SO_2-bleaching of the colour of the anthocyanins in the wines. SO_2 bleaching effectively reduced the number of pigments that contributed to the SO_2-stable wine colour. Thus, those pigments with a functional group at the 4-position of the anthocyanin moiety that rendered the pigments stable to SO_2 bleaching would contribute to SO_2-stable wine colour. Also, the SO_2 bleaching of the anthocyanin colour resulted in the λ_{max} values of the SO_2-stable wine colour being bathochromatically shifted to lower wavelengths. For example, the λ_{max} values of the SC and SB wine colours, 532 and 522 nm respectively (Figure 6.9 above) were bathochromically shifted on SO_2-bleaching to λ_{max} values of 516 nm for both SC and SB SO_2-stable wine colours (Figure 6.10).
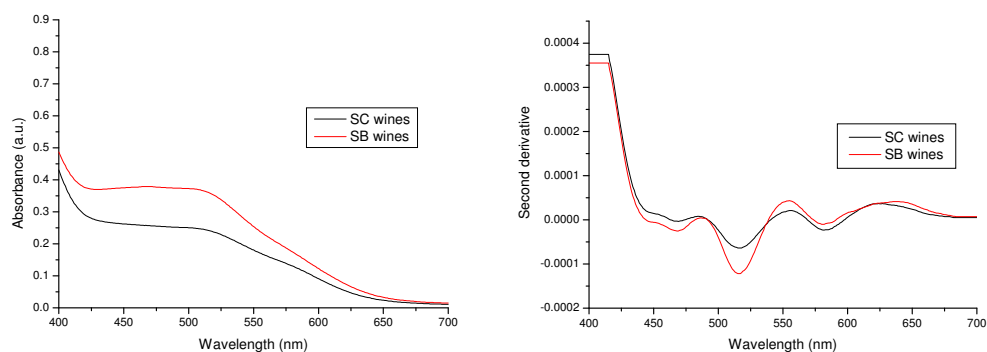
	(a) Visible spectral profile		(b) Second derivative spectra

Yeast	Peaks and shoulders (nm) of the SO_2-stable wine colour		
SC wines	458 – 478	516	567 – 609
SB wines	458 – 478	516	567 – 609
Complementary colour [a]	Orange-red	Red	Red-violet to violet

[a] Complementary colour at the associated λ_{max} value.

Figure 6.10: Visible spectral profile (a) and second derivative spectra of the SC and SB SO_2-stable wine colour at 13 months post-pressing. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively.

The anthocyanin-derived pigments with a substituent at the 4-position of the anthocyanin moiety were considered with respect to their λ_{max} values. The λ_{max} value for both the SC and SB SO_2-stable wine colour was 516 nm. Based on the λ_{max} values for Vitisin A, Pinotin A and Pigment A, it could be speculated that these three pigments were responsible for the SO_2-stable wine colour. The respective λ_{max} values of Vitisin A, Pinotin A and Pigment A were similar to the λ_{max} values of 516 nm of the SO_2-stable wine colour for both SC and SB wines (Table 6.3). The shoulders in the second derivative spectra indicated there were additional but unknown SO_2-stable pigments that provided orange-red colour in the 458 to 478 nm region, and red-violet to violet colour in the 567 to 609 nm regions.

Using the Cabernet Sauvignon red wines at 0 and 13 months to investigate pigment contribution to colour, it is easy to argue that any anthocyanin or

anthocyanin-derived pigment contributes to red wine colour by choosing which data to present and which data to ignore. For example, at 0 months post-pressing, in the assessments made above, the colour contribution of Vitisin B was ignored, even though large amounts of Vitisin B were detected in the SB wines (Table 6.4).

In the case of assessing pigment contribution to SO_2-stable wine colour, SO_2 bleaching removed the colour of the anthocyanins and effectively reduced the colour of pigments observed. The reduction in the number of pigments probably acted to facilitate speculation of possible SO_2 stable pigments which would have contributed to SO_2 stable wine colour. In this study, only a few anthocyanins and a chosen number of anthocyanin-derived pigments were identified by HPLC/UV-Vis and HPLC/MS analysis. There are numerous pigments of varying proportions present in the wine that were not identified. Some of these unknown pigments would have influenced wine colour and SO_2-stable wine colour. For example, in the SO_2 stable wine colour at 13 months post-pressing, there were unknown SO_2 stable wine pigments which would have provided orange-red colour in the 458 to 478 nm region, and red–violet colour in the 567 to 609 nm region (Figure 6.9).

Importantly, some anthocyanin-derived pigments in this study were only qualitatively identified at low pH by HPLC-MS, but not quantified. Without the concentration of a wine pigment, there is little evidence to support the influence of a particular wine pigment to wine colour and to SO_2 stable wine colour. As demonstrated earlier with regard to anthocyanin and pigmented polymers quantification, the pH media is an important factor to the colour observed. To illustrate the inferred contribution of the wine pigments in terms of their λ_{max} values, the anthocyanin-derived pigments identified in this study were assumed to be pH-dependent and in the case of SO_2 stable wine colour, stable to SO_2 bleaching. Thus, it is necessary to have more information regarding the pH-dependence and SO_2 stability of wine pigments before making assessments regarding its contribution to wine colour or SO_2 stable wine colour.

It must be noted that the mechanisms for the creation and development of wine pigments mentioned in this chapter were beyond the scope of this thesis, in particular with regard to the different yeasts used to ferment the red wines.

Sadly, there are many publications in the literature where scientists have devoted much time and effort to investigating and characterizing a particular anthocyanin or anthocyanin-derived pigment. In fact, it seems that the main purpose is to publish a paper, without focus on the relevance of the research. Unfortunately, the importance of some wine pigments to wine colour has been vastly over-stated, and selected methods have been used to accumulate data accumulated to support their original aim: that a given pigment contributes to wine colour.

6.4.　　Conclusions

1. *Winemaking conditions such as the yeast strain affected wine colour and SO_2-stable wine colour. This was demonstrated by the following observations:*
   a. *Although there was no significant difference in the colours of the wines made with SC or SB yeast strains at 0 months post-pressing, at 7-months post-pressing, the wine colour density of the SB wine was higher than the SC wine. This remained unchanged until the final measurement at 25 months post-pressing.*
   b. *From 7 months post-pressing onwards, the SC total red pigment colour was greater than the SB total red pigment colour (converse to the observation for wine colour). Similarly, the concentration of malvidin 3-glucoside was greater in the SC wines than in the SB wines. It was inferred that the higher SC anthocyanin concentration contributed to the higher SC total red pigment colour.*
   c. *Throughout the entire measurement period there was no significant difference between Vitisin A in both SC and SB wines. Vitisin A may have contributed to wine colour, but was*

not responsible for the higher SB wine colour particularly at 13 months post-pressing.

d. *There was a higher concentration of SB pigmented polymers than SC pigmented polymers after 7 months post-pressing. This probably contributed to the higher SB wine colour density values from this time period onwards.*

e. *More Vitisin B was detected in the SB wines at 0 months post-pressing, but the presence of Vitisin B did not have a major impact on the SB wine colour at the initial colour measurement as the wine colour was equivalent in both SC and SB wines.*

f. *At 13 months post-pressing, approximately equivalent amounts of Pinotin A and Pigment A were detected in both wines. These two anthocyanin-derived pigments were not responsible for the higher SB wine colour at this time point.*

g. *From 7 months onwards, the SO_2-stable wine colour and the percentage of SO_2 non-bleachable pigments was consistently higher in the SB wines than in the SC wines.*

2. *It is important to consider the pH media when assessing the relationship of anthocyanins or anthocyanin-derived pigments to wine colour:*

a. *From equivalent wine colour density values, a rapid decrease in wine colour density values was followed by a plateau at 7 months post-pressing was observed in both SC and SB wines. Conversely, an exponential decrease in the total red pigment colour measured at low pH for both wines over time.*

b. *The change in wine colour density at its natural pH with time was not related to the change in anthocyanin concentration (determined by HPLC analysis at low pH). This is probably because the concentration and colour measurements were made in different pH media. But, the decrease in the anthocyanin concentration, in particular malvidin 3-glucoside (measured at low pH) mirrored the change the total red pigment colour at low pH.*

c. There was an initial increase in Vitisin A concentration followed by a plateau in the latter measurements (from 13 to 25 months post-pressing) for both SC and SB wines.

d. Initially, there was no significant difference between the pigmented polymer concentrations of both wines at 0 months post-pressing. The concentration of pigmented polymers increased in both SC and SB wines from 7 months post-pressing onwards. This implies that the pigmented polymer concentration influenced the wine colour.

e. In the time period from 0 to 13 months post-pressing, some anthocyanin-derived pigments evolved (e.g. Pinotin A and Pigment A) whilst others disappeared or were detected in only negligible amounts (e.g. Vitisin B and the ethyl-linked pigments). The colour of a wine cannot be established based on the absence or detection of a wine pigment (see conclusion 4 below).

3. The change in SO_2-stable wine colour and the change in the percentage of SO_2-stable wine pigments were related to the change in wine colour with time.

4. All pigments may contribute to wine colour, but it is not possible to choose a pigment and correctly define its contribution to wine colour or SO_2-stable wine colour solely based on its characteristic λ_{max} value. Current literature must be interpreted with care, particularly as the λ_{max} values of anthocyanins and anthocyanin-derived pigments may have been presented in the literature without making its pH dependence or SO_2 stability clear. In addition, there will always be unknown pigments that contribute to wine colour. To establish the influence of a pigment to wine colour, not only must the pigment be identified in a wine, but its concentration and colour properties must be determined under wine-like conditions.

Chapter 7: Development, validation and application of a method to pH adjust and SO$_2$ bleach HPLC-separated wine pigments

7.1. Introduction

Chapters 4, 5 and 6 have highlighted the importance of characterising wine pigments at wine pH. As SO$_2$ stable wine colour is important to wine colour (Chapter 5.3.5 and 6.3.8), it is also necessary to characterise the individual SO$_2$ stable wine pigments. So it is important to separate pigments from a red wine. In fact, there are many separation techniques in practice used to identify and quantify wine pigments, HPLC analysis is one example. But HPLC analysis has not been fully exploited, mainly because HPLC experiments are performed under highly acidic conditions (pH 1). At low pH, phosphoric acid is an ideal buffer and facilitates good separation of wine pigments. But using the phosphoric acid buffer, the wine is no longer at its natural pH. However, changing the pH of the medium to wine pH will affect not only the buffering capacity of the phosphoric acid, but the absorbance and quantification of some wine pigments. In current literature, only a few examples of pH effects on wine pigments have been demonstrated (See Appendices 1.1 to 1.24). For example, the colour intensity of Vitisin A and the ethyl-linked malvidin 3-glucoside catechin II dimmers are known to change with pH value (Lee *et al.* 2004; Asenstorfer *et al.* 2006).

As discussed in Chapter 1, the colour stability of a red wine to SO$_2$ bleaching is an important indicator of the SO$_2$ stable wine pigments in that wine. The removal of the colour of anthocyanins will enable identification of those anthocyanin-derived pigments that are stable to bisulfite bleaching. SO$_2$ bleaching of wine pigments is important in aged wines where the anthocyanin pigment concentration has decreased to minimal amounts. The resulting SO$_2$ stable wine colour is considered to be provided by anthocyanin-derived pigments that are stable to SO$_2$ bleaching. So, in order to investigate to SO$_2$ stable wine pigments, neutral pH conditions are necessary to facilitate the

colour bleaching capacity of the bisulfite ion, particularly of anthocyanins. At low pH values, the bisulfite ion is unstable at low pH (Rotter 2006).

To study HPLC-separated wine pigments at natural wine pH or post-SO_2 bleaching or to maintain a low pH (as a control experiment) whilst retaining good peak resolution, any adjustment of pH or media conditions must be performed once the pigments have left the HPLC column. Thus, it is necessary to introduce the concept of post HPLC-column adjustment. The post-column adjustment technique involves application of conventional HPLC techniques to separate the pigments, with a slight modification. Once the separated wine pigments have left the HPLC column, the eluent is mixed with a secondary solvent system to either increase the pH value to wine pH, or to increase the pH value and to SO_2-bleach the wine pigments, or to act as a control by simply diluting the wine pigments at low pH values (Figure 7.1). Post-mixing, the eluents enter a diode array detector. Spectroscopic detection in the UV-visible range provides spectral information, whilst the HPLC chromatogram illustrates the peak areas of HPLC-separated pigments in one of the three secondary solvent media.
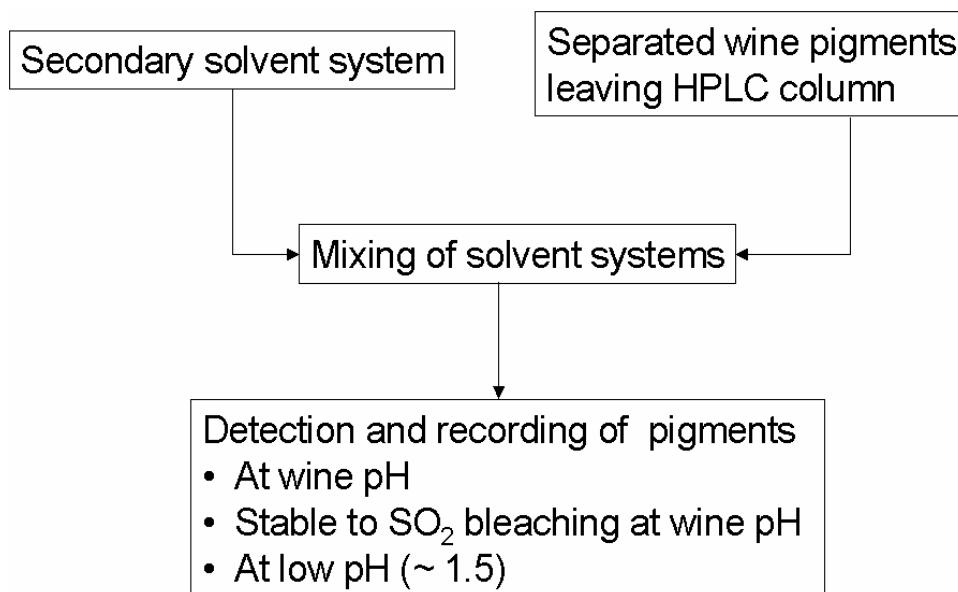
Figure 7.1: Flow chart describing the HPLC post-column adjustment method: The HPLC separated wine pigments leave the column and mix with appropriate secondary solvent system before entering the diode array detector prior to quantification.

This chapter describes the development, validation and application of an HPLC post-column adjustment technique for pH modification, and pH modification and SO_2 bleaching of wine pigments. The technique will facilitate investigations targeting individual HPLC-separated wine pigments at wine pH, rather than characterisation only at low pH as is current practice. In addition, the technique can be used to modify the pH value and SO_2 bleach the colour of wine pigments. The validity of the post column adjustment method with conventional analytical HPLC methods will be assessed. After this comparison, the post column adjustment method will be applied to analyse the anthocyanins and anthocyanin-derived pigments using red wines from the Cabernet Sauvignon study to investigate the stability of wine pigments to pH change and to SO_2 bleaching.

7.2. Objective

To develop and validate a post-column adjustment technique for HPLC-separated wine pigments which will allows modification of the pH conditions and/or SO_2 bleaching.

7.3. Materials and methods

The secondary solvent systems in the HPLC post-column adjustment method were pumped using an HPLC pump (GBC Scientific Equipment, Victoria, Australia) (Figure 7.1). High-pressure turbulence at the vortex of a Tee (Alltech, Australia Pty Ltd., New South Wales, Australia) (Figure 7.2) ensured thorough mixing of the HPLC eluent and the appropriate secondary solvent system.
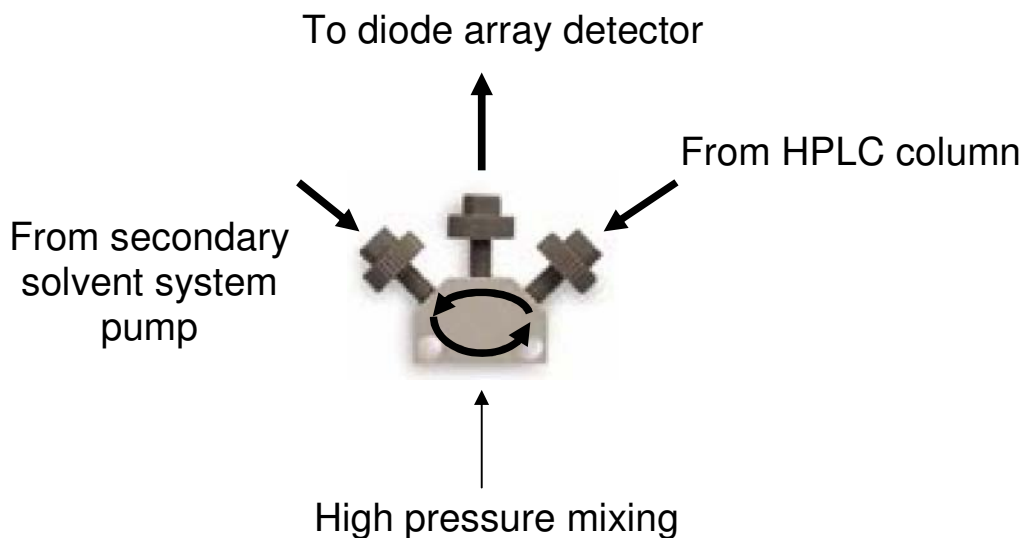
Figure 7.2: PEEK Tee piece illustrating the direction of solvent flow before and after mixing.

To ensure sample consistency throughout development of the post-column adjustment method, a stable pigment extract dissolved in model wine was used. Grape skin extract powder (code 5Z10019, Quest International Australia Pty Ltd., Australia) was dissolved at 0.1 g/L in model wine solution (0.02% sodium bitartrate and 12% v/v ethanol, adjusted to pH 3.6 at $21^{\circ}C$ using 1 M NaOH (aq)). A malvidin 3-glucoside standard acted as a reference for the anthocyanin in the HPLC chromatogram. The concentrations of wine pigments were determined in terms of malvidin 3-glucoside equivalents using the calibration function described in Chapter 2.2. The calibration function calculated the pigment concentration by conventional HPLC analysis without any instrument modification. Using the post-HPLC column adjustment method, quantification of wine pigments was as "apparent concentrations".

7.4. Challenges to developing the post-column adjustment technique for HPLC-separated wine pigments

Through stages of method development, various factors were considered and where necessary, experiments were applied to certain steps to assess viability (Table 7.1).

Table 7.1: Summary of challenges and protocol to develop the post-column adjustment technique for HPLC-separated wine pigments.

Step	Procedure	Explanation
1	pH measurement of HPLC solvents.	*HPLC solvents were mixtures of acetonitrile-water. It cannot be assumed that the pH of organic HPLC solvents is identical to aqueous pH values. For pH measurement at wine pH and post-SO$_2$ bleaching, the term "apparent pH values" was introduced.*
2	A constant concentration of H$_3$PO$_4$ in the HPLC solvents was applied to ensure constant pH values during the analysis.	*Gradients in the HPLC method resulted in a variation in the concentration of the buffer, phosphoric acid.*
3	Use of post-column secondary solvents at appropriate concentrations and flow rates were necessary to adjust pH value to wine pH and to SO$_2$ bleach HPLC-separated the wine pigments.	*Secondary solvents were necessary:* ** To adjust the pH values from low pH to wine pH.* ** To modify the pH values to enable SO$_2$ bleaching of HPLC-separated wine pigments.*
4	Use of a suitable reference solvent.	*A solvent to ensure that pH adjustment and SO$_2$ bleaching reactions were not attributable to dilution, and that a low pH value was maintained.*
5	Apparent pH measurement of a mixture of varying proportions of acetonitrile/water with aqueous ammonium acetate.	*Varying proportions of acetonitrile/water mixed with ammonium acetate may have resulted in a wide range of apparent pH values.*

Table 7.2: Summary of challenges and protocol to develop the post-column adjustment technique for HPLC-separated wine pigments (continued).

Step	Procedure	Explanation
6	Determination of the kinetics of the pH adjustment and SO$_2$ bleaching reactions.	*Confirmation of the end-point of the pH adjustment and SO$_2$ bleaching reactions.*
7	Calculation of a delay time between post-column mixing and diode array detection.	*Appropriate capillary tubing between the mixing tee piece and the diode array detector ensured sufficient time for reaction completion of both pH adjustment and SO$_2$ bleaching reactions.*

7.5. Materials and methods

A summary of the protocol is given in Table 7.1 and in the following text.

7.5.1. pH measurement of HPLC solvents *(Step 1)*

Appropriate measurement of the pH values of the HPLC solvent systems was necessary. The HPLC solvents, used in the standard HPLC method, were mixtures of organic and aqueous media, namely acetonitrile-water mixtures. One cannot assume that the pH value of organic HPLC solvents is identical to aqueous pH values as the pH value and acid-base equilibria of solutes are influenced by the nature of the solvent. Hence, the pK$_a$ values of acids depend on solvent composition. Ideally, it would be best to calibrate the pH electrode using the same mixed organic solvent as the mobile phase. But this was impossible to achieve as the organic solvent ratio in aqueous media would have varied during a typical gradient HPLC analysis.

It is current practice to calibrate pH meters using aqueous standards. So, in this thesis, it was acknowledged that any measured pH values were not true pH values. Instead the pH values were considered "apparent" values. The apparent pH values were quoted as ^s_wpH values where *s* and *w* were the organic solvent system and calibration of the pH electrode (as recommended by the manufacturer) using aqueous standards respectively (Barbosa *et al.* 1999a; Barbosa *et al.* 1999b; Canals *et al.* 2001; Beltrán *et al.* 2003). After

collection of the HPLC eluents and before the ^s_wpH values were measured, the solvent systems were left to equilibrate for 10 minutes.

7.5.2. Adjustment of HPLC solvents to ensure constant pH during gradient elution *(Step 2)*

In a typical HPLC experiment, gradients are applied and will result in minor variation in the concentration of the buffer, phosphoric acid. This will result in a variation in the apparent pH value of the HPLC eluent. To maintain a constant pH value, it was necessary to modify the concentration of phosphoric acid in the HPLC solvents to achieve a constant concentration of the buffer throughout the analysis. Both HPLC solvents A (1.0 % acetonitrile, 97.5 % water) and B (80.0 % acetonitrile and 18.5 % water) were modified so that a constant phosphoric acid concentration of 1.5 % was maintained throughout an HPLC experiment.

Confirmation that a constant pH value was achieved was demonstrated by standard (conventional) HPLC analysis without instrument modification. The HPLC experiment was performed with no sample injection to obtain an estimate of the likely variation in ^s_wpH measurement of the HPLC eluent. Sampling of the eluent was at 10 minute intervals to coincide with the gradient elution of anthocyanins, acylated anthocyanins and the pigmented polymers.

7.5.3. Use of secondary solvents at appropriate concentrations and flow rates to allow post-column pH adjustment *(Step 3)*

To achieve pH adjustment, a suitable solvent was necessary. The solvent, when pumped at an appropriate concentration and flow rate, would essentially adjust the pH value of the HPLC-separated wine pigments from low pH to wine pH. A solvent considered appropriate to increase the pH value of the system to wine pH was aqueous ammonium acetate. Ammonium acetate is a salt that dissociates in water to form the weak acidic ion, NH_4^+ and the weak

alkaline ion, CH_3COO^-. The salt gives a neutral pH in water ($K_a \approx K_b$), is soluble in organic solvents and is not likely to react with the constituents of the HPLC solvents.

The post-column apparatus was connected to the HPLC instrument. The standard HPLC method was applied using the HPLC solvents with a constant phosphoric acid concentration. The HPLC apparatus was operated in isocratic mode at a constant acetonitrile-water proportion of 20 %. No sample was injected to focus on the ^s_wpH values of the pH-adjusted HPLC eluent. Different concentrations and flow rates of ammonium acetate were used and the pH value verified. The values for the concentration and flow rates were optimized on a trial and error basis. When the ^s_wpH values reached values that approximated wine pH, the experiment was repeated in triplicate.

7.5.4. Use of a secondary solvent at an appropriate concentration and flow rate to allow post-column SO_2 bleaching *(Step 3 continued)*

To achieve SO_2 bleaching a suitable solvent was necessary to simultaneously modify the pH conditions and to SO_2 bleach HPLC-separated wine pigments. Sodium metabisulfite was used to bleach the colour from anthocyanins (Somers and Verette 1988), and was used to identify the SO_2-stable pigments in the HPLC-separated wine. But for a bleaching effect by the sulfite ion to occur, a pH value not less than 2.8 is preferable (Rotter 2006). An appropriate solvent was sodium metabisulfite in aqueous ammonium acetate. The flow rate of this secondary solvent was to be the same as the solvent used for pH adjustment. An appropriate concentration of sodium metabisulfite in aqueous aqueous ammonium acetate was necessary. In the spectroscopic determination of SO_2-stable pigments, 25% w/v $Na_2S_2O_3$ (aq) is added to a wine in a 1 mm cuvette (Somers and Verette 1988). This amount equates to a concentration of 1.31 M of bisulfite solution. As the sample volume separated by HPLC analysis was 20 µL, it was unnecessary to use a high concentration of bisulfite solution. The concentration was reduced by 10% to 0.1 M $Na_2S_2O_3$ and dissolved in 0.1 M aqueous ammonium acetate.

The standard HPLC experiment was performed using the phosphoric acid modified HPLC solvents and the pigment sample. To verify post column SO_2 bleaching of the wine pigments 0.1 M $Na_2S_2O_3$ in 0.1 M aqueous ammonium acetate was used.

7.5.5. Use of a reference solvent to ensure that pH adjustment and SO_2 bleaching reactions are not dilution effects *(Step 4)*

The secondary solvents used to pH adjust or to SO_2 bleach the wine pigments post-column may have acted to dilute the HPLC-separated wine pigments. A comparison using a reference or control solvent to maintain a low pH and to dilute the wine pigments was necessary. The aim of the reference solvent was to ensure that the wine pigments maintained similar low pH values as by conventional HPLC analysis. HPLC solvent A (1.5 % H_3PO_4, 1.0 % acetonitrile and 97.5 % water) was an appropriate reference solvent.

7.5.6. Verification of $^s_w pH$ values when varying proportions of acetonitrile/water are mixed with aqueous ammonium acetate *(Step 5)*

Earlier, to determine appropriate concentrations and flow rates of ammonium acetate to achieve wine pH, the HPLC experiment was performed in isocratic mode. But, in the standard HPLC method, there are gradients resulting from a variation (from 20.2 % to 80 %) in the acetonitrile concentration. Importantly, in a study using an acetate buffer and varied proportions of acetonitrile/water mixtures, Barbosa and co-workers recorded a wide range of $^s_w pH$ values (from $^s_w pH$ 4.66 to $^s_w pH$ 6.28) at $25^o C$ (Barbosa *et al.* 1999a; Barbosa *et al.* 1999b). In their experiments, the acetate buffer was composed of 0.1 M sodium acetate in 0.1 M acetic acid. As the acetonitrile/water proportions varied using the standard HPLC method, it was essential to confirm the stability of the $^s_w pH$ values following pH adjustment.

To assess the variation in ^s_wpH values on mixing aqueous ammonium acetate with different acetonitrile/water mixtures, two experiments were performed. The first experiment involved mixing the solvents (proportions of HPLC solvents for pH adjustment) and measuring the ^s_wpH value for a range of acetonitrile/water proportions. The amounts of each solvent were adjusted to correlate with HPLC conditions when 0.1 M aqueous ammonium acetate, pumped at a flow rate of 0.88 mL/min, is used to achieve a ^s_wpH value of 3.45 $\pm$ 0.03. The second experiment was an HPLC analysis with the post-column pH adjustment. The standard HPLC method with constant phosphoric acid and the secondary solvent, 0.1 M aqueous ammonium acetate pumped at a flow rate of 0.88 mL/min was applied.

7.5.6.1.   Experiment 1: Mixing experiment

No HPLC experiment was performed, the solvent mixtures were considered as an isolated system. Varied acetonitrile/water proportions were mixed with a constant concentration of aqueous ammonium acetate to replicate ^s_wpH values approximating to wine pH. The solvent mixtures were adjusted to equate to the volumes of ammonium acetate and phosphoric acid/aqueous acetonitrile solution following post column adjustment. To achieve a concentration of 1.5% phosphoric acid, 0.8 mL of 15% phosphoric acid was mixed with different acetonitrile/water proportions. The total acetonitrile/water volume was 7.2 mL. To the 8 mL phosphoric acid/acetonitrile (aq) solution, 18 mL of 0.1 M ammonium acetate (aq) was added. No pigment sample was employed as the focus was on the ^s_wpH values of the pH-adjusted system.

7.5.6.2. Experiment 2: HPLC experiment with post-column adjustment using aqueous ammonium acetate

The standard HPLC method was used with post-column adjustment. The HPLC solvents were modified so that there was a constant concentration of 1.5 % phosphoric acid. The secondary solvent was 0.1 M aqueous ammonium acetate pumped at a flow rate of 0.88 mL/min. As the aim was to verify ^s_wpH values of the pH-adjusted system, no pigment sample was used. To consider whether the ^s_wpH values were affected during elution of the anthocyanins, acylated anthocyanins, Vitisin A and the pigmented polymers, the HPLC eluent was sampled at three-minute intervals. The experiment was repeated in triplicate.

7.5.7. It is essential that the end-points of the pH adjustment and SO$_2$ bleaching reactions are known (Step 6)

The pH adjustment reaction involves increasing the pH value of a highly acidified system at low pH to wine pH. This is the reverse procedure which is used to determine the colour of a wine at low pH or the total red pigment colour. Measurement of the total red pigment colour was developed by Somers (Somers and Verette 1988). The measurement involves dilution of a wine sample in concentrated hydrochloric acid. The acidified wine is allowed to equilibrate for approximately 3 to 4 hours before any spectral measurements are made. It was possible that the contact time prior following post-column adjustment to diode array detection was in the millisecond range. But an approximate time was uncertain. Thus, it was essential to ensure that the HPLC-separated wine pigments were detected in the DAD at wine pH or post-SO$_2$ bleaching, but not before. Determination of the end-point of the pH adjustment and SO$_2$ bleaching reactions was necessary. The end-point of any reaction can be approximated to its $t_{90\%}$ value. Attempts to determine the $t_{90\%}$ value of the pH adjustment and SO$_2$ bleaching reactions using a standard spectrophotometer were not successful due to the fast speed of the reactions. For example, manual addition of the reactants, mixing by diffusion in the

cuvette, initiation of the software and scanning across the visible spectral range by the spectrophotometer contributed to a loss of reaction time (initiating and monitoring the reactions). In this time, the reactions would have finished and no observable change in the spectra would have been recorded. Thus, a spectrophotometer with a stopped-flow accessory, the π^*-180 spectrometer (Applied Photophysics Ltd, Surrey, United Kingdom) was used (Figure 7.3 below). The change in absorbance was monitored as decay curves, and the $t_{90\%}$ values (in seconds) for both pH adjustment and SO_2 bleaching reactions were estimated.
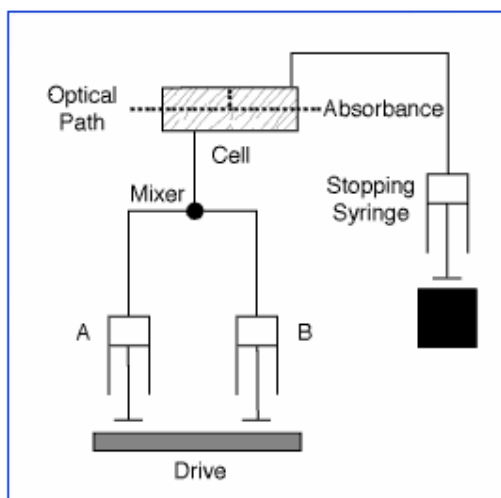
Figure 7.3: Schematics of the stopped-flow apparatus [a] for determining the rapid mixing and recording of spectral data on a millisecond scale (Scientific 2001).

[a] In the stopped-flow apparatus, small but equal volumes of solutions are driven from high performance syringes, denoted by A and B. The volumes are mixed and pass through a measurement flow cell into a stopping syringe. Prior to stopping, a steady state flow is achieved. As the solution fills the stopping syringe, the plunger hits a block causing the flow to stop instantaneously. This mechanism eliminates any delay caused by a manual start. Absorbance is measured in a 2 mm cell using a photodiode array accessory. Any reacted solution is ejected into a waste syringe.

7.5.8. Verification that wine pH can still be achieved by post-column adjustment when the concentration and flow rate of ammonium acetate is further modified *(Step 6 continued)*

In the stopped flow apparatus (Figure 7.3), the syringes held equal volumes of solvent. But in the post-column adjustment experiments earlier, to determine ^s_wpH values approximated to wine pH, different volumes of HPLC eluent were mixed with aqueous ammonium acetate. For example, in one minute, 0.4 mL of HPLC eluent was mixed with 0.8 mL of 0.1 M aqueous ammonium acetate. But using the stopped flow apparatus to study the reaction kinetics, it was necessary to equate the flow rates of both the HPLC eluent and aqueous ammonium acetate. A flow rate of 0.40 mL/min was chosen for both solvents. Simultaneously, the ammonium acetate concentration was increased to 0.25 M. The standard HPLC experiment was performed using the phosphoric acid modified HPLC solvents, the pigment sample and 0.25 M aqueous ammonium acetate at a flow rate of 0.40 mL/min.

7.5.9. Application of the stopped flow technique to assess reaction kinetics of pH adjustment and SO_2 bleaching *(Step 6 continued)*

The stopped flow apparatus (Figure 7.3 above) was used to investigate the reaction kinetics of the post-column adjustment reactions. Syringe A held the diluted pigment solution (1.516 g/L pigment in HPLC solvent$_{M3G}$). Solvent$_{M3G}$ was the solvent ratio where malvidin 3-glucoside elutes in the chromatogram using the standard HPLC method. The solvent ratio of Solvent$_{M3G}$ was 85.5% Solvent A and 14.5% Solvent B and equated to an acetonitrile proportion of 20.2 % v/v. Syringe B contained equal volumes of either

a. HPLC solvent$_{M3G}$ to act as the reference solvent, or

b. 0.25 M ammonium acetate (aq) for pH adjustment, or

c. 0.1 M sodium metabisulphite in 0.25 M ammonium acetate (aq) for SO_2 bleaching.

The kinetics measurements were performed in triplicate at 520 nm. Subsequently, the $t_{90\%}$ values were calculated. To confirm pH adjustment had taken place, the ^s_wpH values of the solvent systems were measured before and after the stopped flow measurement.

The stopped flow experiments were repeated for the pigment dissolved in HPLC solvent$_{PP}$. HPLC solvent$_{PP}$ was the solvent ratio where the pigmented polymer peak elutes in the chromatogram (49.5% Solvent A and 50.5% Solvent B at 80 % v/v acetonitrile) using the standard HPLC method.

7.5.10. Calculation of the delay time between post column mixing and diode array detection *(Step 7)*

Estimation of reaction times of the pH adjustment and SO_2 bleaching reactions was used to determine the dimensions of capillary tubing attached between the Tee piece and the diode array detector. Appropriate sized tubing would give the required dead volume and a delay to ensure that the reactions had finished. Completion of the pH-adjustment and SO_2 bleaching reactions was represented by the $t_{90\%}$ values. The $t_{90\%}$ values for the reactions were determined by performing kinetics measurements using the pigment sample and the three secondary solvents quantified in Step 6.

7.6. Results and discussion

Steps 1 to 7 have described the development of the post HPLC column adjustment method (Section 7.4). The results of the experiments are detailed below.

7.6.1. pH measurement of HPLC solvents *(Step 1)*

The pH values of organic HPLC solvents were termed apparent and were represented as ^s_wpH values.

7.6.2. Adjustment of HPLC solvents to ensure constant pH during gradient elution *(Step 2)*

In the standard HPLC method, the proportion of phosphoric acid in the HPLC solvents was 1.5 %. For this constant concentration of phosphoric acid, instead of pH values of ^s_wpH of 1.5, a range of ^s_wpH values from 1.60 to 1.71 was observed (Table 7.3). Table 7.3 illustrates that the apparent pH variation was minimal over the 40-minute time period and was considered not to impact on the pH adjustment and SO_2 bleaching reactions.

Table 7.3: Confirmation that approximately constant ^s_wpH values were achieved by conventional HPLC analysis (standard HPLC method) using modified HPLC solvents with constant phosphoric acid concentration [a].

Time (minutes)	Pigments eluted at given time point	^s_wpH value at 23°C
0 – 10	Anthocyanins	1.71
10 – 20	Acetylated anthocyanins and Vitisin A	1.61
20 – 30	Coumaroylated anthocyanins Pigmented polymers	1.60
30 – 38	-	1.63

[a] Solvent ratios were solvent A: 1.5 % phosphoric acid, 1.0% acetonitrile, 97.5% water, and solvent B: 1.5 % phosphoric acid, 80.0% acetonitrile and 18.5% water.

7.6.3. Use of secondary solvents at appropriate concentrations and flow rates to allow post-column pH adjustment and SO_2 bleaching *(Step 3)*

Application of low concentration and high flow rates of ammonium acetate resulted in low ^s_wpH values (Table 7.4). For example, 0.05 M ammonium acetate pumped at a flow rate of 0.4 mL/min resulted in a ^s_wpH value of 1.93. Table 7.4 illustrates that a target ^s_wpH value of 3.45 ± 0.03 was achieved using 0.1 M aqueous ammonium acetate pumped at a flow rate of 0.88 mL/min.

Table 7.4: Concentrations and flow rates of aqueous ammonium acetate post-HPLC column mixed with HPLC solvents in an attempt to achieve wine pH.

Concentration (M)	Flow rate (mL/min)	^s_wpH value
0.10	0.40	2.31
0.10	0.25	2.05
0.05	0.40	1.93
0.05	0.25	1.74
0.10	0.90	3.86
0.10	0.95	4.14
0.10	0.85	3.35 ± 0.03
0.10	0.88	3.45 ± 0.03

7.6.4. Use of sodium metabisulfite in aqueous ammonium acetate to SO$_2$ bleach HPLC-separated wine pigments *(Step 3)*

The secondary solvent used to SO$_2$ bleach HPLC-separate wine pigments was 0.1 M Na$_2$S$_2$O$_3$ dissolved in 0.1 M aqueous ammonium acetate. No monomeric anthocyanins and acylated anthocyanins were detected in the HPLC chromatograms for three repetitions of the post-column experiment (data not shown). It was concluded that successful SO$_2$ bleaching of the colour of the anthocyanins was achieved.

7.6.5. Use of a reference solvent to ensure that pH adjustment and SO$_2$ bleaching reactions are not dilution effects *(Step 4)*

The HPLC solvent A (1.5 % H$_3$PO$_4$, 1.0 % acetonitrile and 97.5 % water) was used as the reference solvent. In the standard HPLC method, there was a variation in the acetonitrile/water proportion. When the reference solvent was mixed with the HPLC eluent, this resulted in an acetonitrile proportion increase of 1 %. For example, the acetonitrile solvent ratios were in the range 20.2 % to 80.0 %, and an addition of HPLC solvent following post-HPLC column adjustment resulted in acetonitrile solvent ratios in the range 21.2 % to

81.0 %. This increment was considered not to adversely affect the solvent ratios and maintained a low pH value.

7.6.6. Verification that wine pH can still be achieved by post column adjustment using a modified concentration and flow rate of ammonium acetate *(Step 5)*

The results of three replicated HPLC analyses using 0.25 M aqueous ammonium acetate at a flow rate of 0.40 mL/min resulted in apparent pH values of ^s_wpH 3.72 ± 0.12 (data not shown).

7.6.7. Verification that apparent pH values approximate to wine pH when varying proportions of acetonitrile/water are mixed with aqueous ammonium acetate *(Step 5)*

7.6.7.1. Experiment 1: Mixing experiment

The acetonitrile solvent ratios in the standard HPLC method were in the range 20.2 % to 80 %. Table 7.5 illustrates that, for this range of acetonitrile proportions, the ^s_wpH values were in the range 3.29 to 3.60. These pH values are similar to range of pH values from 3.4 to 3.6 which are commonly found in wine (Somers 1975).

Table 7.5: ^s_wpH values in an aqueous system of constant phosphoric acid concentration and varied acetonitrile proportions following an addition of ammonium acetate at 24°C. The ^s_wpH values were adjusted to wine pH using 18 mL of 0.1 M aqueous ammonium acetate and 8 mL of acetonitrile/water/phosphoric acid proportions.

1.5 % Phosphoric acid	Acetonitrile (mL)	Water (mL)	Acetonitrile (v/v %)	^s_wpH value
0.8	0.0	7.2	0	2.93
0.8	0.8	6.4	10	3.23
0.8	1.6	5.6	20	3.29 [a]
0.8	2.4	4.8	30	3.31 [a]
0.8	3.2	4.0	40	3.36 [a]
0.8	4.0	3.2	50	3.45 [a]
0.8	4.8	2.4	60	3.50 [a]
0.8	5.6	1.6	70	3.50 [a]
0.8	6.4	0.8	80	3.60 [a]
0.8	7.2	0.0	90	3.75

[a] Range of acetonitrile (%) values applicable to the standard HPLC method.

7.6.7.2.   Experiment 2: HPLC experiment with post-column adjustment using aqueous ammonium acetate

The apparent pH values observed following elution of the anthocyanins, Vitisin A and the acylated anthocyanins were in the range from ^s_wpH values of approximately ^s_wpH 3.22 to ^s_wpH 3.32 (Table 7.6). The largest variation in ^s_wpH values was observed for the pigmented polymers, which eluted at ^s_wpH 3.50 $\pm$ 0.13. A wide range of apparent ^s_wpH values was expected within the time period of the pigmented polymer elution as the gradients were rapidly changing up to 38 minutes (the end of the HPLC experiment).

Table 7.6: ^s_wpH values using the standard HPLC method with post-column adjustment. The HPLC solvents contained a constant concentration of phosphoric acid, whilst pH adjustment was achieved using 0.1 M aqueous ammonium acetate pumped at a flow rate of 0.88 mL/min.

Time (minutes)	Pigments eluted	Acetonitrile (v/v %)	^s_wpH value [a]
0.0 – 2.9	Delphinidin 3-glucoside	20.2	3.22 ± 0.05
3.0 – 5.9	Cyanidin 3-glucoside, Petunidin 3-glucoside	20.2	3.22 ± 0.05
6.0 – 8.9	Peonidin 3-glucoside, Malvidin 3-glucoside	20.2	3.25 ± 0.04
9.0 – 11.9	Vitisin A, Delphinidin 3-acetylglucoside	20.2	3.27 ± 0.04
12.0 – 14.9	-	20.2	3.27 ± 0.02
15.0 – 17.9	Peonidin 3-acetylglucoside, Malvidin 3-acetylglucoside	20.2	3.28 ± 0.02
18.0 – 20.9	Petunidin 3-(*p*-coumaroyl) glucoside, Peonidin 3-(*p*-coumaroyl) glucoside	29.3	3.31 ± 0.02
21.0 – 23.9	Malvidin 3-(*p*-coumaroyl) glucoside	45.4	3.32 ± 0.05
24.0 – 26.9	Pigmented polymers	45.4 → 80	3.50 ± 0.13

[a] Standard HPLC method, using modified HPLC solvents with constant phosphoric acid concentration, was performed in triplicate and presented as mean ± standard error.

7.6.8. Spectral evaluation with stopped flow to assess reaction kinetics of pH adjustment and SO$_2$ bleaching *(Step 6)*

The π^*-180 spectrometer with a stopped flow accessory (Figure 7.3 above) was used to determine the reaction kinetics. The experiment involved mixing the contents of syringe A (pigment dissolved in solvent HPLC$_{M3G}$) with the contents of syringe B (the reference solvent, the pH adjustment solvent or the pH adjustment and SO$_2$ bleaching solvent). In the control experiment, the solvent HPLC$_{M3G}$ effectively diluted the pigment further in HPLC$_{M3G}$ solvent. Constant absorbance values of 0.28 ± 0.00 a.u. were observed (Figure 7.4). Exponential decay curves were observed for the pH adjustment and SO$_2$ bleaching reactions (Figure 7.4). The SO$_2$ bleaching reaction was fast and was almost complete at approximately 5 seconds. After 5 seconds, constant absorbance values were observed 0.05 ± 0.03 a.u. The absorbance change

following pH adjustment reaction was much slower, and was completed after approximately 30 seconds.

It was noted from Figure 7.4 that the exponential curves for pH adjustment and SO_2 bleaching did not have initial absorbance values of 0.28 a.u. of the reference solvent. Instead, the initial absorbance values for the pH adjustment and SO_2 bleaching reactions were 0.225 a.u. and 0.075 a.u. respectively (Figure 7.4). The low initial absorbance values (compared to 0.28 a.u.) were considered to be a consequence of a delay between mixing and measurement. It is considered the delay time was an artefact of the instrument and could not be resolved. The $t_{90\%}$ values for both pH adjustment and SO_2 bleaching reactions were calculated assuming an initial absorbance value of 0.28 a.u., the constant absorbance value of the pigment in HPLC solvent$_{M3G}$ in the control experiments.
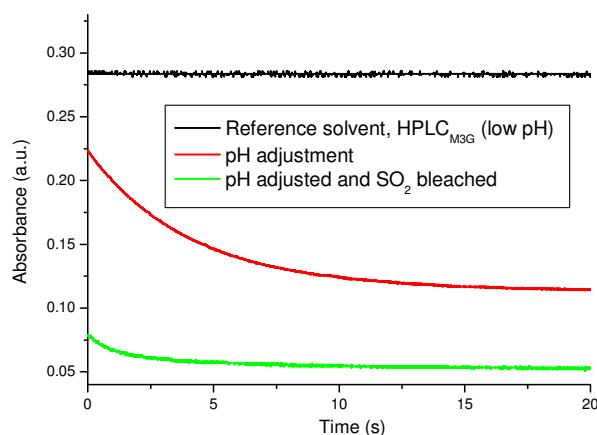
Figure 7.4: Reaction kinetics using a pigment sample [a] to investigate pH adjustment and post-SO_2 bleaching [b] monitored using stopped flow techniques.

[a] Pigment sample dissolved in HPLC$_{M3G}$ solvent.

[b] Secondary solvent systems:

- To act as a reference solvent: HPLC$_{M3G}$ was the solvent ratio at which malvidin 3-glucoside elutes in the chromatogram using the standard HPLC method. At this solvent ratio, 85.5% Solvent A and 14.5% Solvent B, equates to an acetonitrile proportion of 20.2 % v/v.
- For pH adjustment: 0.25 M ammonium acetate (aq).
- For SO_2 bleaching pigments: 0.1 M sodium metabisulfite in 0.25 M ammonium acetate (aq).

The $t_{90\%}$ values for the ammonium acetate (aq) and sodium metabisulfite in ammonium acetate (aq) were 30.50 ± 4.03 s and 0.14 ± 0.03 s respectively (Table 7.7). The $t_{90\%}$ values of the pH adjustment reaction indicated that, unlike the Somers method (Somers and Verette 1988) where a wine is subjected to a decrease from wine pH to pH 1, increasing from a low pH to wine pH did not require a waiting period of 3 to 4 hours.

Table 7.7: Absorbance values at time zero and after 20 seconds and the estimated $t_{90\%}$ values for the pH adjustment and SO_2 bleaching reactions using the stopped flow spectrophotometer

	Start (Time = 0 s)	Stop (Time = 20 s)	Difference (s)	Abs $T_{90\%}$ (a.u.)	Reaction completion ($T_{90\%}$ (s))
Control	0.28	0.28	0	-	-
pH adjustment	0.28	~ 0.12	~ 0.16	~ 0.136	30.50 ± 4.03 [a]
pH adjustment and SO_2 bleaching	0.28	~ 0.06	~ 0.22	~ 0.082	0.14 ± 0.03 [a]

[a] Experiments repeated in triplicate and presented as mean ± standard error.

The ^s_wpH values of the HPLC-diluted pigment and secondary solvent systems, prior to and post-mixing, confirmed that pH adjustment using the solvent systems had been achieved (Table 7.8).

Table 7.8: ^s_wpH values of the solvent systems before and after the stopped flow reaction.

Prior to stopped flow experiment				After stopped flow experiment
Syringe A		Syringe B		Mixed solvent systems
Solvent	^s_wpH value	Secondary solvent	^s_wpH value	^s_wpH value
Sample [a] dissolved in solvent$_{M3G}$[b]	1.62	Solvent$_{M3G}$[b]	1.62	1.62
		0.25 M ammonium acetate (aq)	6.96	3.52 ± 0.33 [c]
		0.1 M sodium metabisulfite in 0.25 M ammonium acetate (aq)	4.96	2.95 ± 0.22 [c]

[a] Pigment sample.

[b] Reference solvent, HPLC$_{M3G}$. HPLC$_{M3G}$ is the solvent proportion at which malvidin 3-glucoside elutes in the chromatogram using the standard HPLC method. The ratio was 85.5% Solvent A and 14.5% Solvent B and equated to an acetonitrile proportion of 20.2 % v/v.

[c] Measured ^s_wpH values at 23°C repeated in triplicate presented as mean ± standard error.

The ^s_wpH value of the SO$_2$ bleaching reaction (^s_wpH 2.95 ± 0.22) was much lower than the ^s_wpH value of the pH adjustment reaction (^s_wpH 3.52 ± 0.33) which was probably due to a fault in the pumping system. The ^s_wpH value was greater than 2.8 and successful SO$_2$ bleaching was evident from Figure 7.4 above.

The stopped flow experiments were repeated for the pigment dissolved in HPLC solvent$_{PP}$. HPLC solvent$_{PP}$ is the solvent ratio where the pigmented polymer peak elutes in the chromatogram (49.5% Solvent A and 50.5% Solvent B at 80 % v/v acetonitrile) using the standard HPLC method. Similar reaction kinetics to Figure 7.4 above was observed (data not shown).

7.6.9. Calculation of the delay time between post column mixing and diode array detection *(Step 7)*

Table 7.7 (above) has shown that the t$_{90\%}$ values for the ammonium acetate (aq) and sodium metabisulphite in ammonium acetate (aq) reactions on mixing with HPLC solvent were 30.50 ± 4.03 s and 0.14 ± 0.03 s respectively. As the SO$_2$ bleaching reaction was very fast, pH adjustment was the rate determining step. Thus, the t$_{90\%}$ value for the aqueous ammonium acetate reaction was used to calculate the dimensions of the capillary tubing attached between the Tee piece and diode array detector.

In approximately 30 seconds, the combined flow rate of 0.8 mL/min (0.4 mL/min for the HPLC eluent and 0.4 mL/min for the secondary solvent systems) resulted in approximately 0.4 mL or 400 µL of eluent flowing to the diode array detector. Capillary tubing with an internal diameter of 1 mm was used between the HPLC column and the DAD. A low internal diameter was preferred as peak broadening through diffusion would not be excessive. To obtain a delay of 30 seconds using a flow rate of 0.8 mL/min and a dead volume of 400 µL, the length of tubing required to ensure reaction completion and pH adjustment was $400 / (\pi \times 0.5^2) = 510$ mm.

Tables 7.9 and 7.10 below illustrates a brief summary for each step of method development.

Table 7.9: Summary of results.

Step	Summary
1	Apparent pH or ^s_wpH values are quoted for pH measurement of organic HPLC solvents.
2	A minor variation in ^s_wpH values of the eluent was observed for a constant concentration of phosphoric acid in the HPLC solvents. The variation was considered not to impact on the post-column adjusted ^s_wpH values.
3	A target ^s_wpH value of 3.45 ± 0.03 was achieved using 0.1 M aqueous ammonium acetate pumped at a high flow rate of 0.88 mL/min. SO_2 bleaching of HPLC-separated wine pigments, particularly the monomeric anthocyanins and acylated anthocyanins, was achieved using 0.1 M $Na_2S_2O_3$ in 0.1 M aqueous ammonium acetate.
4	HPLC solvent A (1.5 % H_3PO_4, 1.0 % acetonitrile and 97.5 % water) was used as the reference solvent.
5	For varied acetonitrile/water proportions and a concentration of 0.1 M aqueous ammonium acetate pumped at a flow rate of 0.88 mL/min was used to achieve an approximate wine pH: Experiment 1: No HPLC analysis was performed, the solvents were just mixed. A range of ^s_wpH values from ^s_wpH 3.29 to ^s_wpH 3.60 was observed. Experiment 2: HPLC analysis with post-column adjustment resulted in a range of ^s_wpH values ^s_wpH 3.22 to ^s_wpH 3.50.

Table 7.10: Summary of results (continued).

Step	Summary
6	For the purpose of measurement of reaction kinetics by stopped flow spectroscopy, an increment in the concentration and a reduction in the ammonium acetate flow rate were necessary. HPLC analysis with post-column adjustment using modified secondary solvents resulted in apparent pH values that approximated to wine pH. A ^s_wpH value of 3.72 ± 0.12 was achieved using 0.25 M aqueous ammonium acetate pumped at a flow rate of 0.40 mL/min.
	The $t_{90\%}$ values for the 0.25 M ammonium acetate (aq) and 0.1 M sodium metabisulphite in 0.25 M ammonium acetate (aq) reactions on mixing with HPLC solvent were 30.50 ± 4.03 s and 0.14 ± 0.03 s respectively.
7	To ensure the pH adjustment and SO_2 bleaching reactions had finished prior to detection, a capillary tubing of approximately 510 mm with an internal diameter of 1 mm created the necessary dead-volume between the mixing Tee piece and the diode array detector.

7.7.   Conclusions

1. *The post-column adjustment method is fit for the purpose of assessing the concentration and colour properties of assessing HPLC-separated wine pigments at wine pH and post-SO_2 bleaching. To achieve pH adjustment and SO_2-bleaching, the following secondary solvents must be pumped at a flow rate of 0.4 mL/min:*

    a. *HPLC solvent A (1.5 % H_3PO_4, 1.0 % acetonitrile and 97.5 % water) to act as the reference solvent,*

    b. *0.25 M aqueous ammonium acetate to pH adjust to wine pH,*

    c. *0.1 M sodium metabisulfite in 0.25 M aqueous ammonium acetate to pH adjust and to SO_2 bleach HPLC-separated wine pigments.*

2. *To ensure the pH adjustment and SO_2 bleaching reactions had finished, a capillary tubing of approximately 510 mm with an internal diameter of 1 mm must be attached between the mixing Tee piece and the diode array detector.*

7.8.   Application of the post-column adjustment method to Cabernet Sauvignon red wines

It is necessary to apply the post-HPLC column adjustment method to real wines, particularly as the preliminary experiments used grape pigment samples. Development of the technique focused on pH adjustment and confirmation of SO_2 bleaching. Quantification of wine pigments was not assessed. In addition, a comparison of the concentration of wine pigments by conventional HPLC and using the post column method is necessary to assess the influence of the secondary solvents on the peak shapes e.g. peak broadening, and quantification of HPLC-separated wine pigments.

7.9.   Objectives

1. *To show the post-HPLC column adjustment method, where there is no adjustment in pH values and a low pH is maintained, provides similar quantification results to conventional HPLC analysis.*

2. *To confirm that pH adjustment and SO_2 bleaching reactions are proven successful using the post-column adjustment method.*

3. *To apply the post column adjustment method to wine and investigate the effects of HPLC-separated pigments at low pH, at wine pH and post-SO_2 bleaching.*

7.10.   Materials and methods

Cabernet Sauvignon red wines from the 2002 winemaking trial were analysed at 22 months post-pressing. Conventional HPLC analysis was performed using the standard HPLC method without any modifications. The standard HPLC method (using a constant phosphoric acid concentration of 1.5 %) with post column adjustment (using modified secondary HPLC solvents) was applied. Secondary solvents either acted as a reference to maintain a low pH, to adjust the pH to wine pH or to pH adjust and SO_2 bleach the HPLC-separated wine pigments.

The solvents used were:

    a. The reference solvent: HPLC solvent A (1.5 % H_3PO_4, 1.0 % acetonitrile and 97.5 % water),

    b. 0.25 M ammonium acetate (aq) to increase the pH to wine pH,

    c. 0.1 M sodium metabisulfite in 0.25 M ammonium acetate (aq) to increase the pH and SO_2 bleach the wine pigments.

The secondary solvents were pumped at a flow rate of 0.40 mL/min. Capillary tubing, 510 mm in length and with an internal diameter 1 mm created the necessary dead-volume between the mixing Tee and the diode array detector. To ensure contamination from the previous secondary solvent was kept to a minimum, the subsequent solvent was flushed through the capillary tubing for a few minutes prior to the start of the HPLC experiment.

Three replicate tanks of each SC (*Saccharomyces cerevisiae*) and SB (*S. bayanus*) Cabernet Sauvignon red wines were analyzed. Conventional HPLC analysis of the wines was performed once. The post-column pH adjustment HPLC experiment using each secondary solvent was repeated twice for each wine.

The calibration equation used to represent wine pigments separated by HPLC analysis as malvidin 3-glucoside chloride equivalents has been determined at low pH by conventional HPLC analysis. At wine pH, pH dependent pigments such as malvidin 3-glucoside will convert to a different coloured form. Consequently, the peak representing the malvidin 3-glucoside will appear smaller, but the actual concentration of pigment will not have changed. Thus, pigments quantified at wine pH and post-SO_2 bleaching shall be considered "apparent concentrations". In addition, as equal flow rates of HPLC eluent and secondary solvent systems were applied in the post-column method, this resulted in a 1:1 dilution of the HPLC-separated wine. The "apparent" concentrations were adjusted accordingly to account for dilution. The apparent concentrations were presented in terms of malvidin 3-glucoside equivalents (in mg/L).

7.11. Results and discussion

7.11.1. Assessment of the validity of the post-HPLC column adjustment method

The validity of new post-HPLC column adjustment method was assessed by comparing the peak areas determined by conventional HPLC analysis and using the post-HPLC column adjustment method. For example, connection of the capillary tubing between the mixing Tee piece and the diode array detector resulted in a loss of resolution, peak broadening, a slight shift in the retention times and potential co-elution of some pigments (Figure 7.1). Therefore, the pigments were identified either in terms of their expected elution order and spectral characteristics or established using a standard (e.g. malvidin 3-glucoside and Vitisin A standards were employed). In addition, using the post-column adjustment method, negligible concentrations (approximating to zero) of some pigments were recorded. For example, the pigments, cyanidin 3-glucoside and petunidin 3-(p-coumaroyl) glucoside were considered "not reported" and were not represented in Table 7.11 below.

Dilution of the HPLC eluent using the post-column method resulted in reduced peak area and broader peaks of some pigments. The peaks were more difficult to integrate and consequently, the small areas resulted in an under-estimated calculation of the concentration of some pigments, due to the threshold effect. The calibration equation used to determine pigment concentration in mg/L malvidin 3-glucoside equivalents was obtained by conventional HPLC analysis using a reference method:

Pigment concentration = 0.00915 x peak area + 0.79060

The equation may have contributed to some of the differences in concentration observed. For some pigments, there was no significant differences in the concentration e.g. malvidin 3-glucoside, but not in all cases. For example, concentrations of 45 mg/L and 39 mg/L of SB pigmented polymers were recorded using conventional HPLC analysis and the post-

column adjustment methods respectively (Table 7.11). Despite the underestimation in pigment concentrations, the post-HPLC column pH adjustment experiment highlighted differences in the relative concentrations of the SB and SC wine pigments (Table 7.11 below).

Table 7.11: Concentration of anthocyanin and anthocyanin-derived pigments for a Cabernet Sauvignon red wine made with *Saccharomyces bayanus* yeast strain. The measurements, using the standard HPLC method, were made at 22 months post-fermentation.

Pigment	Concentration by conventional HPLC analysis (mg/L) [a,c,d]	"Apparent concentration" using post-column adjustment method (mg/L) [b,c,e]
Delphinidin 3-glucoside	13 ± 0	12 ± 0
Petunidin 3-glucoside	10 ± 0	7 ± 0
Peonidin 3-glucoside	7 ± 0	5 ± 0
Malvidin 3-glucoside	58 ± 2	59 ± 1
Vitisin A	4 ± 0	3 ± 0
Delphinidin 3-acetylglucoside	3 ± 0	3 ± 0
Peonidin 3-acetylglucoside	3 ± 0	2 ± 0
Malvidin 3-acetylglucoside	17 ± 1	13 ± 0
Peonidin 3-(*p*-coumaroyl)glucoside	2 ± 0	2 ± 0
Malvidin 3-(*p*-coumaroyl)glucoside	5 ± 0	4 ± 0
Pigmented polymers	45 ± 0	39 ± 1

[a] No modification to the HPLC apparatus.

[b] Post-column adjustment method using constant phosphoric acid concentration and HPLC Solvent A as the secondary solvent.

[c] Concentration in terms of malvidin 3-glucoside chloride equivalents (mg/L) determined using the standard HPLC method at pH 1.5. Data presented as mean ± standard error.

[d] Results of three replicate tanks at approximately ^s_wpH 1.5.

[e] Results of three replicate tanks where measurements were repeated twice at ^s_wpH 1.68.

Figure 7.5 below illustrates the change in peak areas on application of the post-column method using secondary solvent systems applied to the SB wines. Similar changes in the peak areas were observed in the chromatogram for the SC wines by conventional HPLC analysis and using the post-column adjustment method (data not shown).

Anthocyanins and anthocyanin-derived pigments were identified (Peaks 1 to 11 in the HPLC chromatogram (Figure 7.5)). There were some additional peaks observed in the HPLC chromatogram which were not characterized

(Figure 7.5). Although these uncharacterized peaks were not included in this study, it must be noted that some of these peaks were also affected by the change in pH and bleached by the sulfite ion.
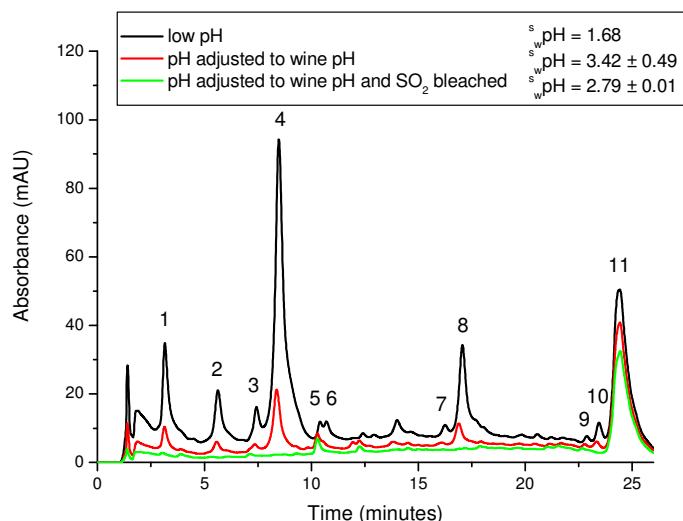
Figure 7.5 : HPLC chromatogram for a Cabernet Sauvignon red wine made with *Saccharomyces bayanus* yeast strain at 520 nm on application of the post-HPLC column pH adjustment method. The peak areas were solvent dependent and varied according to ^s_wpH value. The ^s_wpH values in the legend are pH measurements repeated for six tanks represented as mean ± standard error values.

The major pigments separated by HPLC analysis were peak 1, delphinidin 3-glucoside (D-3-G); peak 2, petunidin 3-glucoside (Pet-3-G); peak 3, peonidin 3-glucoside (Peo-3-G); peak 4, malvidin 3-glucoside (M-3-G); peak 5, Vitisin A; peak 6, delphinidin-3-acetylglucoside (Del-AG); peak 7, peonidin-3-acetylglucoside (Peo-AG); peak 8, malvidin 3-acetylglucoside (M-AG); peak 9, peonidin 3-(*p*-coumaroyl) glucoside (Peo-CG); peak 10, malvidin 3-(*p*-coumaroyl) glucoside (M-CG); peak 11, pigmented polymers.

7.11.2.   Confirmation of the success of post HPLC-column pH adjustment and SO$_2$-bleaching reactions

The ^s_wpH values of the HPLC eluent changed with secondary solvent system. On application of 0.25 M ammonium acetate (aq) and 0.1 M sodium metabisulfite in 0.25 M ammonium acetate (aq), ^s_wpH values were ^s_wpH 3.42 ± 0.49 and ^s_wpH 2.79 ± 0.01 respectively resulted.

The success of the pH adjustment reaction from low pH to wine pH was confirmed by comparing the spectral profile of malvidin 3-glucoside in the two pH media. A bathochromic shift in the spectral profile of malvidin 3-glucoside from low pH to wine pH was observed. At low pH, the λ_{max} value of malvidin 3-glucoside was 528 nm (Figure 7.6). At wine pH, a shift in the λ_{max} value of malvidin 3-glucoside towards the blue region to 532 nm was observed (Figure 7.6). This confirms findings by several researchers who have noted that malvidin 3-glucoside experiences a bathochromic shift to higher wavelengths on increasing from low pH to wine pH (Bakker and Timberlake 1997; Heredia *et al.* 1998; Stintzing *et al.* 2002).

In addition, there was reduced overall absorbance of malvidin 3-glucoside at wine pH compared to at low pH (Figure 7.6). A conversion of the highly coloured flavylium cation to a less coloured isoform resulted. This reduction in absorbance coincides with the reduction the extinction coefficients of malvidin 3-glucoside. For example, when measured at 520 nm, the extinction coefficient of malvidin 3-glucoside at pH 1 is 27600 L cm^{-1} mol^{-1} and reduces to 7100 L cm^{-1} mol^{-1} at wine pH (Håkansson *et al.* 2003).
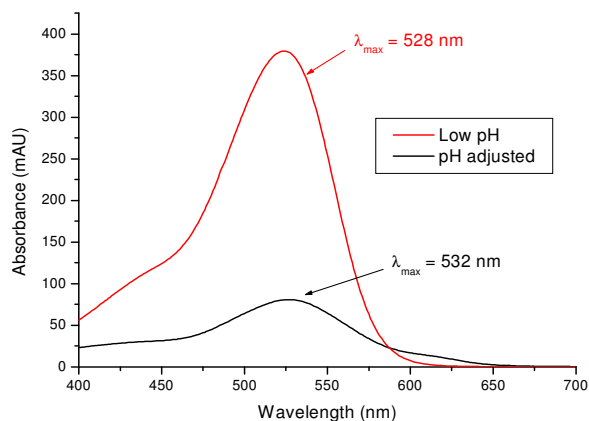
Figure 7.6: Spectral profile of the malvidin 3-glucoside standard at low pH (^s_wpH = 1.68) and at wine pH (^s_wpH = 3.70) on application of the post-column pH adjustment of the HPLC-separated pigment.

Lower than expected ^s_wpH values (^s_wpH 2.79 ± 0.01) were achieved using the sodium metabisulfite in ammonium acetate solution probably owing to a minor

fault in solvent pumping. In spite of this problem, the $^{s}_{w}pH$ value of the bisulfite solution was greater than 2.8 (Rotter 2006) and the SO_2 bleaching of the colour of all the anthocyanins proved successful. For example, at approximately 7 minutes in the HPLC chromatogram, the colour of malvidin 3-glucoside was bleached by the sulfite ion and consequently no peak was detected (Figure 7.5).

7.11.3. Application of the post HPLC-column method to investigate the effects of HPLC-separated wine pigments at low pH, at wine pH and post SO_2-bleaching

In addition to anthocyanins (and some of their acylated derivatives), the concentrations of two anthocyanin-derived pigments, Vitisin A, and the pigmented polymers were quantified (Figure 7.7). The same scale was used to compare the pigment concentrations. Figure 7.7 illustrates that the apparent concentrations were dependent upon solvent media. Similar observations were made for the change in the concentrations of the anthocyanins and anthocyanin-derived pigments for both wines made with SC and SB yeast strains (Figure 7.7 below):
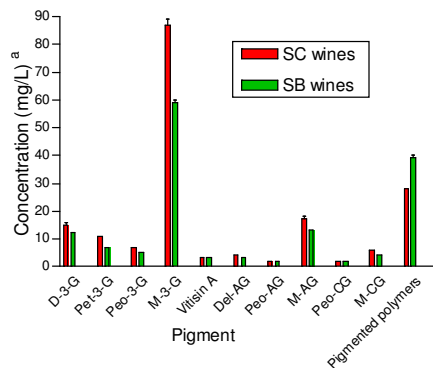
(a) Anthocyanins and anthocyanin-derived pigments at low pH.
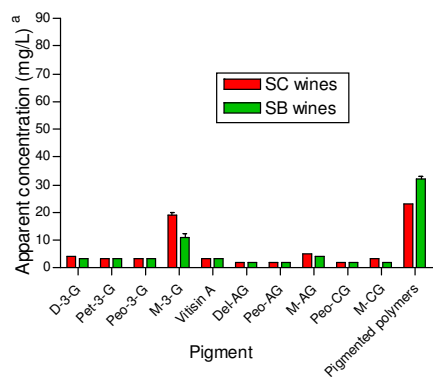
(b) Anthocyanins and anthocyanin-derived pigments at wine pH.
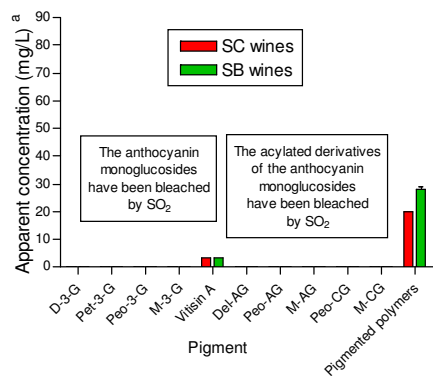
(c) Pigments which remain stable in the presence of SO_2.

Figure 7.7: Apparent anthocyanin and anthocyanin-derived pigment concentration [a] (a) at low pH [b] (^s_wpH = 1.68), (b) at wine pH [b] (^s_wpH = 3.42 $\pm$ 0.49) and (c) post-SO_2 bleaching [b] (^s_wpH = 2.79 $\pm$ 0.01) on application of the post-column adjustment method for the SC and SB wines. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively.

[a] Apparent concentrations expressed as malvidin 3-glucoside chloride equivalents determined from measurements made using the standard HPLC method. Abbreviations were referred to in Figure 7.5.

[b] HPLC measurements were repeated twice for the three replicate tanks of SC and SB wine, and presented as mean $\pm$ standard error.

At low pH, the two highest pigment concentrations werer recorded for malvidin 3-glucoside and the pigmented polymer peak (Figure 7.7 a). In the SC wines, the malvidin 3-glucoside concentration was greater (87 mg/L) than the concentration in the SB wines (59 mg/L). Similarly, the concentration of anthocyanins and acylated anthocyanins were greater in the SC wines than in the SB wines (Figure 7.7 a). However, the SC pigmented polymers were lower (28 mg/L) than the SB pigmented polymers (39 mg/L).

At wine pH, there was a significant reduction in the absorbance and concentration of all the anthocyanins (50 to 83 %) except for Vitisin A, which remained unchanged (Figure 7.7b). Upon pH adjustment to wine pH, the reduction was smaller (approximately 20 %) for the pigmented polymers of both SB and SC wines. The reduction in the pigmented polymer concentration demonstrates there were pigments that eluted in the peak which were pH-dependent.

In the wine pH-adjusted medium, the concentration of malvidin 3-glucoside was lower than the pigmented polymer peak concentration (Figure 7.7 b). For example, in the SC wines at wine pH, the apparent concentrations of malvidin 3-glucoside and the pigmented polymers were 19 mg/L and 23 mg/L respectively. This observation confirms that conventional HPLC analysis over-estimates the quantification of anthocyanins at low pH.

For both SC and SB wines, the bisulfite ion resulted in bleaching of the colour of the anthocyanins and the acylated derivatives of the anthocyanins. Consequently, no anthocyanins were detected (Figure 7.7c). The apparent concentration of Vitisin A remained unchanged (3 mg/L). Surprisingly, the apparent pigmented polymer concentration decreased further in the presence of sulfite ion. There was a further 8 to 15 % reduction in the pigmented polymer concentration post-SO_2 bleaching compared to at wine pH. Therefore there were pigments that eluted in the pigmented polymer peak whose colour was susceptible to bleaching by the sulfite ion. SO_2 bleaching did not affect the relative amounts of pigmented polymers between the SC and SB wines. Similar to the results at low pH, the concentration of the pigmented polymers

post-SO$_2$ bleaching was greater in the SB wines (28 mg/L) than in the SC wines (20 mg/L). But a comparison of pH effects and SO$_2$ bleaching on the pigmented polymers with current literature was not made as this work has not been done anywhere previously.

In addition, the pH conditions and SO$_2$ bleaching did not affect the relative amounts of pigments in the SC and SB wines. For example, similar to at low pH, the concentration of malvidin 3-glucoside at wine pH was greater in the SC wines (19 mg/L) than in the SB wines (11 mg/L).

Thus, development and validation of a post-column adjustment method has been demonstrated. Although the solvents used to pH adjust and to SO$_2$ bleach at wine pH were not identical to wine, the adjustment method will enable a comparison of relevant anthocyanins and anthocyanin-derived wine pigments and their associated concentrations at low pH, at wine pH and (excluding the anthocyanins), post-SO$_2$ bleaching. The post-column adjustment method is also suitable for targeted identification of individual pigments with specific properties, such as pigments that are resistant to SO$_2$ bleaching, and thus important to stable wine colour. More detail regarding the concentration and colour of the anthocyanins and anthocyanin-derived pigments at low pH, at wine-adjusted pH and post-SO$_2$ bleaching shall be discussed in the next chapter (Chapter 8).

7.12.   Conclusions

1. *Comparing pigment quantification by conventional HPLC analysis and applying the post-HPLC column adjustment method, negligible concentrations of some pigments were recorded, and consequently were considered "not reported". Dilution of the HPLC eluent resulted in reduced peak area and broader peaks of some pigments. As the peaks were difficult to integrate, the small peak areas resulted in an under-estimated calculation of the pigment concentration due to the threshold effect.*

2. The pH adjustment and SO_2 bleaching reactions were successful as confirmed by the effect of pH change on the absorbance profile of malvidin 3-glucoside, and the complete SO_2 bleaching of the colour of malvidin 3-glucoside.

3. The post column adjustment method enabled investigation of HPLC-separated wine pigments at low pH, at wine pH and post-SO_2 bleaching. In particular:

   a. Conventional HPLC analysis over-estimates the concentration of anthocyanins at low pH. Less coloured forms of anthocyanins were formed when HPLC conditions were adjusted to wine pH and lower "apparent" concentrations of anthocyanins were recorded. A reduction of 50 to 83 % in anthocyanin concentration was observed from low pH values to wine pH-adjusted values.

   b. Bleaching the colour of HPLC-separated anthocyanins allows focus to be made on SO_2-stable wine pigments and thus stable wine colour. The effect of SO_2 bleaching on anthocyanin-derived pigments (Vitisin A and the pigmented polymers) was considered.

   c. Vitisin A concentration was not affected by the change in pH values or following bleaching in the presence of excess SO_2.

   d. The apparent pigmented polymer concentration decreased by 20 % from low pH to wine pH. The apparent concentration of pigmented polymers decreased further by 8 to 15 % after bleaching with the bisulfite solution.

   e. Additional "uncharacterized" peaks in the HPLC chromatogram were pH-dependent and SO_2-bleachable.

3. At low pH and at wine pH, the concentration of malvidin 3-glucoside in the SC wines were greater than in the SB wines. The converse was

apparent for the pigmented polymers in all three media; there was a lower concentration of SC pigmented polymers than SB pigmented polymers.

Chapter 8: Colour measurement and wine pigment characterization in the Cabernet Sauvignon red wines

8.1. Introduction

From the information gathered and demonstrated in the earlier chapters, it has been highlighted the need for wine researchers and the wine industry to better understand the colour properties of wine pigments at wine pH. Knowledge of the colour contribution of pigments to wine colour will enable viticulturists and winemakers to manipulate various techniques and factors to achieve optimized wine colour. Wine pigments will contribute different amounts to wine colour depending not only on the age, style or variety of a wine, but the proportion and presence of anthocyanins and anthocyanin-derived pigments. For example, in aged wines, the concentration of anthocyanins has diminished to minimal amounts (Herderich *et al.* 2006). Thus, it is important to study the anthocyanin-derived pigments that are stable to SO_2 bleaching and contribute to SO_2-stable wine colour. But, characterization of wine pigments cannot be achieved by comparing the concentrations of pigments determined at low pH with wine colour at natural wine pH or with SO_2-stable wine colour (Chapters 5 and 6). The pH conditions will influence the quantification of pigments (as demonstrated in Chapter 7). In particular, the coloured forms of anthocyanins will be over-estimated at low pH (Chapter 7). But the problem of quantification of HPLC-separated wine pigments at low pH can be overcome by application of the post-column adjustment to HPLC-separated wine samples (Chapter 7). The post-HPLC column adjustment method enables the concentration of wine pigments to be determined at low pH, at wine pH and post-SO_2 bleaching. In particular, in the latter case, excess bisulfite will remove the colour of anthocyanins to enable quantification of SO_2-stable wine pigments. As the term "post-HPLC column adjustment suggests", adjustment of the pH or SO_2 bleaching conditions is achieved after the pigments have been separated in the HPLC column. Not only can wine pigments be quantified, but the colours of the HPLC-separated wine pigments in each of the three media can be determined using the HPLC-CIELab method (Chapter

3). Subsequently, the concentration and colours of HPLC-separated wine pigments at wine pH and post-SO$_2$ bleaching can be used to assess their contribution to wine colour and SO$_2$-stable wine colour respectively. This is all possible without lengthy separation or isolation techniques. For example, to date, the colours of malvidin 3-glucoside and Vitisin A have been studied as isolated systems at different pH values or in the presence of the sulfite ion. But these pigments were isolated or synthesized first. The post-HPLC column adjustment and HPLC-CIELab methods were used to determine the concentration and colours of anthocyanins and anthocyanin-derived pigments separated from a real wine by HPLC analysis at low pH, at wine pH, and excluding the anthocyanins, post-SO$_2$ bleaching. Following chromatographic separation using the standard HPLC method, several anthocyanins and Vitisin A were identified. In addition to peaks of the monomeric anthocyanins, the acylated derivatives of anthocyanins and Vitisin A, the pigmented polymer peak, the last peak in the HPLC chromatogram, was also characterized. The pigmented polymer peak is a co-elution of many anthocyanin-derived pigments and is the largest peak (except for malvidin 3-glucoside) identified in the HPLC chromatogram. But to date, only the concentration of this peak at low pH has been published (Peng *et al.* 2002; Hayasaka and Kennedy 2003; Bartowsky *et al.* 2004; Eglinton *et al.* 2004; Hayasaka *et al.* 2004; Herderich and Smith 2005). Little is known about the colour properties of this peak. For example, no one has presented the spectral profile or verified the colour of the pigmented polymer peak with its associated concentration in wine, even at low pH. Consequently, the concentration, colour and the spectral profile of of the pigmented polymers at low pH, at wine pH and and after bleaching with excess bisulfite is absent.

8.2.　　Objectives

1. *To investigate the differences in wine colour resulting from the use of different yeast strains in terms of quantification of wine pigments in different pH media and post-SO_2 bleaching:*

   a. *To determine the concentration of anthocyanins and anthocyanin-derived from HPLC-separated wine pigments <u>at low pH</u>.*

   b. *To determine the concentration of anthocyanins and anthocyanin-derived from HPLC-separated wine pigments <u>at wine pH</u>.*

   c. *At wine pH, to demonstrate that anthocyanins play a minor role whilst anthocyanin-derived pigments such as the pigmented polymers provide a greater contribution than anthocyanins to wine colour.*

   d. *To determine the concentration of anthocyanin-derived pigments from HPLC-separated wine pigments <u>post-SO_2 bleaching</u>. (The colour of anthocyanins is bleached by the sulfite ion). Earlier (Chapters 5 and 6), it was established that SO_2 stable wine colour is important to wine colour. Thus, to demonstrate that those anthocyanin-derived pigments that are stable in the presence of SO_2 play a major role towards SO_2 stable wine colour.*

   e. *To investigate colour differences on changing the pH value and in the presence of SO_2 bleaching in terms of anthocyanins and anthocyanin-derived pigments, Vitisin A and the pigmented polymers.*

2. *Using malvidin 3-glucoside as an example of an anthocyanin,*

   a. *To determine the CIELab colour of malvidin 3-glucoside at low pH and at wine pH, and to confirm that the colour properties of malvidin 3-glucoside is affected by the pH medium.*

   b. *To investigate whether different yeast strains have influenced the colour of malvidin 3-glucoside.*

3. *Using Vitisin A as an example of an anthocyanin-derived pigment,*

a. To determine the CIELab colour of Vitisin A at low pH, at wine pH and post-SO_2 bleaching.

b. To investigate whether different yeast strains have influenced the colour of Vitisin A

c. To investigate whether Vitisin A was responsible for the differences in wine colour in the different media.

4. Using the pigmented polymers as examples of anthocyanin-derived pigments, to investigate whether different yeast strains have influenced the colour of pigmented polymers

a. To determine the CIELab colour of pigmented polymers at low pH, at wine pH and post-SO_2 bleaching.

b. To demonstrate that some pigments that have eluted in the pigmented polymer peak are affected by the pH values, while some pigments in the pigmented polymers are stable in the presence of the bisulfite ion.

c. To investigate whether the pigmented polymers were responsible for the differences in wine colour in the different media.

8.3. Materials and methods

South Australian Cabernet Sauvignon red wines from the 2002 wine trial were evaluated at 22 months post-pressing (Chapter 2 – Materials). The wines were made with two different yeast strains, *Saccharomyces cerevisiae* and *S. bayanus* yeasts respectively, and were abbreviated to SC and SB. Spectroscopic absorbance and CIELab colour measurements were made at low pH and at wine pH, and the SO_2 stable wine colour was measured (Chapter 2 – Methods).

Chromatographic analysis of the wines was performed using the standard HPLC method with post-column media adjustment (Chapter 7). HPLC solvent A acted as the reference solvent and maintained a low pH value. The pH value was increased from low pH to wine pH using 0.25 M aqueous ammonium acetate. While 0.10 M sodium metabisulfite in 0.25 M aqueous

ammonium acetate was used to SO_2 bleach the colour of anthocyanins and highlight the SO_2-stable wine pigments in the red wines. Identification of the anthocyanin peaks (the monoglucosides and their corresponding acylated derivatives) were based on their expected elution time in the HPLC chromatogram and associated spectral profile. The identity of malvidin 3-glucoside was confirmed using a standard. Confirmation of Vitisin A was achieved using a standard and co-injection. The pigmented polymer peak was the largest and last peak in the HPLC chromatogram, identified according to published data (Peng *et al.* 2002; Cozzolino *et al.* 2004; Eglinton *et al.* 2004). The concentrations of the post-column adjusted pigments were quantified as malvidin 3-glucoside equivalents (mg/L) using an external calibration function at low pH by conventional HPLC analysis (Chapter 2) where

$$\text{Pigment concentration} = 0.00915 \times \text{peak area} + 0.79060.$$

As the actual amount of the pigments did not change in the different media and were probably represented by a less coloured isomer at wine pH or post-SO_2 bleaching, the concentrations of the pigments at wine pH and post-SO_2 bleaching were termed "apparent concentrations". The flow rates of the HPLC eluent and secondary solvent systems were 0.4 mL/min resulting in a 1:1 dilution of the HPLC-separated wine. The "apparent" concentrations were adjusted to account for this dilution. The post column adjustment HPLC analyses were performed in duplicate on three replicate tanks for each SC and SB wine.

It was not possible to identify the anthocyanin-derived pigments that eluted in the pigmented polymer peak using current literature. The reason for this that no one has published pH-dependent isoforms of anthocyanin-derived pigments, other than Vitisin A (See Appendices 1.3 to 1.5). Only HPLC-UV/Vis analysis was performed. Identification of the pigments that eluted in the pigmented polymer peak was impossible without accurate confirmation (e.g. by NMR or mass spectrometry).

The CIELab colours of the malvidin 3-glucoside, Vitisin A and the pigmented polymers at low pH, at wine pH and post-SO_2 bleaching were determined

using the HPLC-CIELab method (described in Chapter 4). The spectral data for malvidin 3-glucoside and Vitisin A were extracted across a time interval of 0.5 minutes about the peak maximum and the weighted mean spectral data calculated (Chapter 4). As the pigmented polymer peak had a large peak width, the peak was divided into three equal peak widths, PP1, PP2 and PP3 about the peak maximum. PP1 to PP3 had equal widths of 0.5 minutes (Figure 8.1) and the mean spectra were calculated for each interval. Prior to the CIELab colour calculation, a multiplication factor of 0.02 was applied to the mean spectra of malvidin 3-glucoside, Vitisin A and to each of the three peak widths, PP1, PP2 and PP3, of the pigmented polymer peak. The CIELab colour calculations were performed in duplicate on three replicate tanks for each SC and SB wine (Chapter 4). The mean $\pm$ standard error of the apparent concentrations was determined.
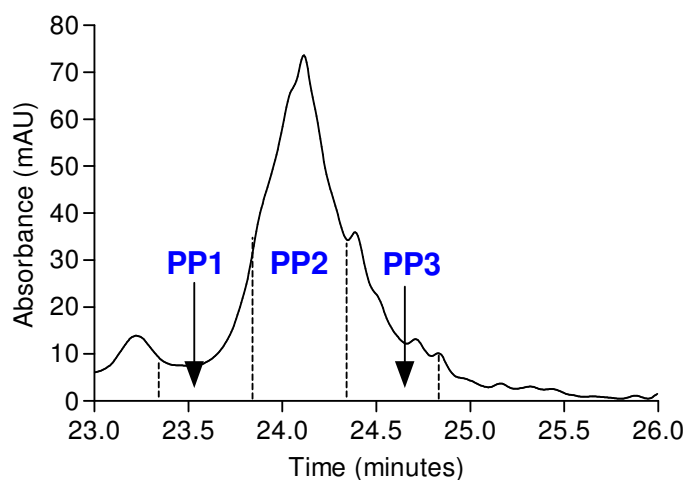
Figure 8.1: Pigmented polymer peak of the SC wine divided into three equal peak widths, PP1, PP2 and PP3 of equal width of 0.5 minutes. The peak was extracted from the chromatogram using the standard HPLC method. SC is Cabernet Sauvignon red wine made with *Saccharomyces cerevisiae* yeast strain.

In addition, the colours that contributed to the pigmented polymer peak were investigated in greater detail. The spectral profile of the pigmented polymers at 24 minutes at low pH, in the wine-pH adjusted medium, and post-SO_2 bleaching were extracted. The Savitzky-Golay filter method was applied to the absorbance spectra in the visible range 400 to 700 nm. The filter method calculated the second derivative absorbance spectra to identify the λ_{max}

values and shoulders in the absorption profile of the pigmented polymers. Accordingly, the λ_{max} values represented the maximum absorbance, while the shoulders illustrated many absorbance values across a particular visible colour region. All analyses were performed in triplicate on three replicate tanks for each SC and SB wine.

8.4.   Results and discussion

At 22 months post-pressing, wine colour measurement at wine pH and post-SO_2 bleaching was compared to the wine colour measured at low pH (Figure 8.2). The results in Figure 8.2 provide further evidence that colour comparison should only be made in the relevant and appropriate media. For example, at low pH, the SC wine colour was darker and more colourful (lower L* and higher C* values) than the SB wine colour (Figure 8.2). The converse was apparent for the wine colour at wine pH and post-SO_2 bleaching. That is, the SB wine colour was darker and bright (low L* and high C* values) than the SC wine colour. Similarly, even though bleaching of the anthocyanins had occurred, the SB SO_2-stable wine colour was darker and brighter (low L* and high C* values) than the SC SO_2-stable wine colour. The data in Figure 8.2 further supports the results in Chapters 5 and 6 that wine colour at low pH is not indicative of wine colour at wine pH. Also, Chapters 5 and 6 demonstrated that SO_2-stable wine colour correlated well with wine colour. From Figure 8.2, it can be seen that high SB SO_2 stable wine colour (low L* and high C* values) influenced high SB wine colour (low L* and high C* values). Similarly, low SC SO_2 stable wine colour influenced low SC wine colour (Figure 8.2). The error bars are not obviously noticeable in Figure 8.2, which clearly demonstrates there was negligible error in the CIELab colour measurements and winemaking replicates.
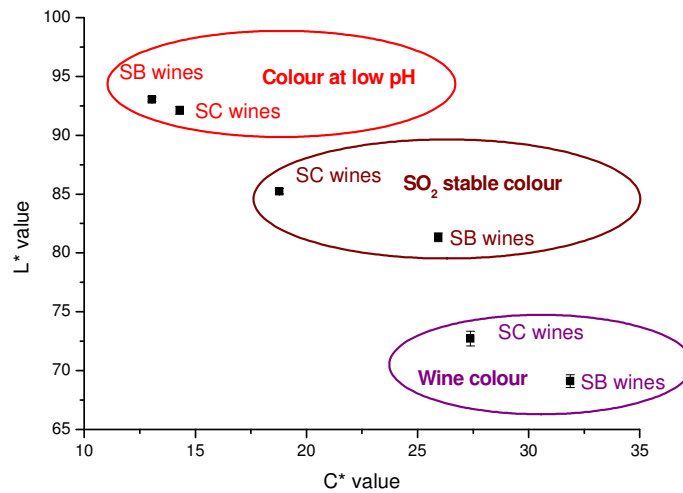
Figure 8.2: CIELab L* and C* values [a] for the wine colour at pH 1 [b], at natural wine pH [c] and SO$_2$ stable colour [c] of the SC and SB wines measured at 22 months post-pressing. SC and SB are Cabernet Sauvignon wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively

[a] Mean ± standard error calculated from triplicate colour measurements on the three replicate tanks for each SC and SB wine.

[b] Colour determined at low pH using a 10 mm cuvette.

[c] Colour measurements determined at wine pH (approximately pH 3.75) and post-SO$_2$ bleaching using a 1 mm cuvette.

The wine colour at low pH, at wine pH and SO$_2$-stable wine colour was investigated further by comparing the HPLC-separated pigments in similar pH or SO$_2$-bleaching media. It must be acknowledged that on application of the post column adjustment method, the solvents used to pH adjust or SO$_2$ bleach the wine pigments were not those found in wine. But, approximately wine-equivalent pH values were achieved using the post column adjustment method. The ^s_wpH values in the wine pH-adjusted medium and on bleaching with excess SO$_2$ were ^s_wpH 3.42 ± 0.49 and ^s_wpH 2.79 ± 0.01 respectively. The approximately wine equivalent pH values enabled the concentration and HPLC-CIELab colour of the anthocyanins and anthocyanin-derived pigments, and the spectral profile of the pigmented polymer peak to be compared at low pH or at wine pH, or post-SO$_2$ bleaching.

8.4.1. Wine colour and quantification of wine pigments at low pH

At low pH (pH 1.0), the SC wine colour was slightly darker and slightly more colourful (lower L* and higher C* values) than the SB wine colour (Table 8.1 and Figure 8.2). Both wines exhibited a red hue as the H* values approximated to zero. The increased colour of the SC wine could be attributed to the greater concentration of anthocyanins present, but not to the concentration of Vitisin A and the pigmented polymers (Table 8.1). For example, at ^s_wpH 1.68, the concentration of malvidin 3-glucoside in the SC wine was 87 mg/L compared to only 59 mg/L in the SB wine. There was no difference in the Vitisin A concentration for the SC and SB wines, 3 mg/L. So, Vitisin A may have contributed to the colour of both wines, but its presence in the wines was not responsible for the colour differences. There was a lower concentration of pigmented polymers in the SC wine, only 28 mg/L compared to 39 mg/L in the SB wine. The higher concentration of the pigmented polymers in the SB wines did not appear to influence the SB wine colour, which was significantly lighter (higher L* and lower C* values) than the SC wine colour (Figure 8.2 and Table 8.1). It could be inferred that at low pH, the pigmented polymers played a minor role in its contribution to SB wine colour. Similarly, the concentration of SC pigmented polymers could have been overpowered by the concentration of anthocyanins in the SC wines.

Table 8.1: Concentration of anthocyanins and anthocyanin-derived pigments (mg/L) a in the SC and SB red wines determined by HPLC-UV/Vis analysis at low pH ($^{s}_{w}$pH = 1.68) on application of post-column adjustment method. The concentrations are compared with diluted wine colour at pH 1 c. SC and SB are Cabernet Sauvignon wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively measured at 22 months post-pressing.

	Pigment	SC wines	SB wines
	Delphinidin 3-glucoside	15 ± 1 [a]	12 ± 0 [b]
	Petunidin 3-glucoside	11 ± 0 [a]	7 ± 0 [b]
	Peonidin 3-glucoside	7 ± 0 [a]	5 ± 0 [b]
	Malvidin 3-glucoside	87 ± 2 [a]	59 ± 1 [b]
Concentration of wine pigments at low pH (mg/L) [1,2]	**Vitisin A**	3 ± 0 [a]	3 ± 0 [a]
	Delphinidin 3-acetylglucoside	4 ± 0 [a]	3 ± 0 [a]
	Peonidin 3-acetylglucoside	2 ± 0 [a]	2 ± 0 [a]
	Malvidin 3-acetylglucoside	17 ± 1 [a]	13 ± 0 [b]
	Peonidin 3-(*p*-coumaroyl)glucoside	2 ± 0 [a]	2 ± 0 [b]
	Malvidin 3-(*p*-coumaroyl)glucoside	6 ± 0 [a]	4 ± 0 [b]
	Pigmented polymers	28 ± 0 [a]	39 ± 1 [b]

	CIELab value	SC wines	SB wines
Wine colour at pH 1 [3]	**L***	92.13 ± 0.33 [a]	93.05 ± 0.19 [b]
	C*	14.30 ± 0.24 [a]	13.05 ± 0.31 [a]
	H*	0.03 ± 0.02 [a]	0.06 ± 0.00 [a]
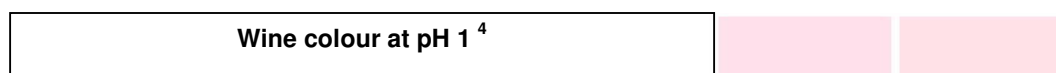
Wine colour at pH 1 [4]		

[1] Concentration in terms of malvidin 3-glucoside chloride equivalents (mg/L) determined using the standard HPLC method at pH 1.5.

[2] Mean ± standard error values calculated from HPLC measurements repeated twice on three replicate tanks for each SC and SB wine.

[3] SC and SB wines were diluted in 1M HCl. After 4 hours, CIELab measurements were made using a 10 mm cuvette. Mean ± standard error values were calculated from colour measurements repeated in triplicate on three replicate tanks for each SC and SB wine.

[4] The CIELab colours shown are representative of the actual wine colour at low pH visible in a 10 mm cuvette. Importantly, the CIELab colours observed, either on the computer monitor or in the printed page, were dependent on the hardware and software of the computer, and ambient lighting used to view the colours.

[a] or [b] Values with different subscript letters are significantly different at $P < 0.05$ when comparing between the SC and SB wines.

8.4.2. Wine colour and quantification of wine pigments at wine pH

At wine pH (pH 3.75), the SB wines were darker and more colourful (lower L^* and higher C^* values) compared to the SC wines (Figure 8.2 and Table 8.2). There was an increase in the hue of both wines (positive H^* values) as a minor red-orange hue was observed. At wine pH, the hue value of SB wine ($H^* = 0.48$) was almost twice the value of the SC wine ($H^* = 0.28$). This was converse to the wine colour at low pH (pH 1.0). At low pH, both wines exhibited red hues as the H^* values approximated to zero, while the SB wine colour was lighter (higher L^* and lower C^* values) than the SC wine colour (Figure 8.2 and Table 8.2) at low pH.

The relative amounts of the anthocyanins were unaffected by the increase in the pH value. There were more anthocyanins quantified in the SC wines than in the SB wines at low pH (Table 8.1) and at wine pH (Table 8.2). For example, at low pH (^s_wpH 1.68), 87 mg/L and 59 mg/L of malvidin 3-glucoside was quantified in the SC and SB wines respectively. Whilst the increase to wine pH (^s_wpH 3.42) resulted in apparent concentrations of 19 mg/L and 11 mg/L of malvidin 3-glucoside in the SC and SB wines respectively (Table 8.2).

On increasing the pH value from low pH (Table 8.1) to wine pH (Table 8.2), there was an overall decrease in the apparent concentration of the anthocyanins and the pigmented polymers, whilst the concentration of Vitisin A was unchanged. The percentage change in the concentrations of anthocyanins and their acylated derivatives ranged from 50 to 83 % in both SC and SB wines. For example, at low pH, malvidin 3-glucoside was predominately in its flavylium form. This was reflected in the large peak area of the anthocyanin, and consequently high concentration of the malvidin 3-glucoside reference standard (237 mg/L). At the adjusted pH, the actual amount of the malvidin 3-glucoside standard remained unchanged, but a shift in equilibrium had occurred. The anthocyanin converted to less coloured, pH dependent isoform (Brouillard and Delaporte 1977; Asenstorfer *et al.* 2003a).

Accordingly, the apparent concentration of the malvidin 3-glucoside reference standard decreased to 44 mg/L. Thus, adjusting the pH value from low pH to wine pH resulted in approximate 81 % decrease in apparent concentration of malvidin 3-glucoside. Similarly, observations regarding the change in anthocyanin colour with pH change have been made by Mattivi and co-workers (Mattivi *et al.* 2001).

The increase in the pH value did not affect the Vitisin A concentration, which was unchanged. There was 3 mg/L of Vitisin A quantified in both SC and SB wines at low pH and at wine pH (Tables 8.1 and 8.2). Thus, it could be implied that pH value did not affect quantification of the Vitisin A. In addition, as the Vitisin A concentration was similar in both wines, the presence of Vitisin A did not contribute to the colour differences observed in the SC and SB wine colours (Table 8.2). But it may have been difficult to determine an exact change in Vitisin A peak area with pH change due to a threshold effect and the low Vitisin A peak area which resulted in an approximate quantification of the pyranoanthocyanin.

However, the differences in the two wine colours could be attributed to the pigmented polymer concentration quantified in the wines. For example, at wine pH, the higher colour of the SB wines (low L* and high C* values) (Figure 8.2) could be the result of the higher concentration of SB pigmented polymers (32 mg/L) (Table 8.2). Similarly, the lighter and duller colour (higher L* and lower C* values) of the SC wines (Figure 8.2) could be a result of the lower SC pigmented polymer concentration (23 mg/L) (Table 8.2).

Table 8.2: Apparent concentration of anthocyanins and anthocyanin-derived pigments (mg/L)[1] in the SC and SB red wines determined by HPLC-UV/Vis analysis at wine pH, (^s_wpH = 3.42 ± 0.49) on application of post-column adjustment method. SC and SB are Cabernet Sauvignon wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively, measured at 22 months post-pressing.

	Pigment	SC wines	SB wines
Apparent concentration of pigments at wine pH (mg/L) [1,2]	**Delphinidin 3-glucoside**	4 ± 0 [a]	3 ± 0 [b]
	Petunidin 3-glucoside	3 ± 0 [a]	3 ± 0 [a]
	Peonidin 3-glucoside	3 ± 0 [a]	3 ± 0 [a]
	Malvidin 3-glucoside	19 ± 1 [a]	11 ± 1 [b]
	Vitisin A	3 ± 0 [a]	3 ± 0 [a]
	Delphinidin 3-acetylglucoside	2 ± 0 [a]	2 ± 0 [a]
	Peonidin 3-acetylglucoside	2 ± 0 [a]	2 ± 0 [a]
	Malvidin 3-acetylglucoside	5 ± 0 [a]	4 ± 0 [b]
	Peonidin 3-(p-coumaroyl) glucoside	2 ± 0 [a]	2 ± 0 [a]
	Malvidin 3-(*p*-coumaroyl)glucoside	3 ± 0 [a]	2 ± 0 [b]
	Pigmented polymers	23 ± 0 [a]	32 ± 1 [b]

	CIELab value	SC wines	SB wines
Wine colour [3]	**L***	72.72 ± 0.63 [a]	69.09 ± 0.55 [b]
	C*	27.38 ± 0.58 [a]	31.87 ± 0.41 [b]
	H*	0.28 ± 0.02 [a]	0.42 ± 0.00 [b]
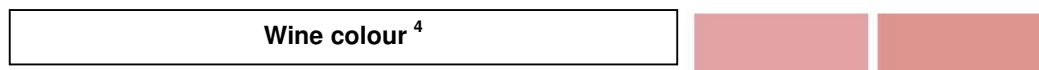
Wine colour [4]		

[1] Concentration in terms of malvidin 3-glucoside chloride equivalents (mg/L) determined using the standard HPLC method at pH 1.5.

[2] Mean ± standard error values calculated from HPLC measurements repeated twice on three replicate tanks for each SC and SB wine.

[3] CIELab colour of the SC and SB wines at natural pH (3.75 ± 0.02) using a 1 mm cuvette. Mean ± standard error calculated from colour measurements repeated in triplicate on three replicate tanks for each SC and SB wine.

[4] The CIELab colours shown are representative of the actual wine colour visible in a 1 mm cuvette. Importantly, the CIELab colours observed, either on the computer monitor or in the printed page, were dependent on the hardware and software of the computer, and ambient lighting used to view the colours.

[a or b] Values with different subscript letters are significantly different at $P < 0.05$ when comparing between the SC and SB wines.

Interestingly, on increasing the pH value to wine pH, there was a decrease in the apparent concentration of the pigmented polymers for both wines (Tables 8.1 and 8.2). For example, the SB pigmented polymer concentration was

39 mg/L at low pH, and decreased to 32 mg/L in the wine pH-adjusted medium. A 20 % reduction in the apparent pigmented polymer concentration was observed for both SC and SB wines. This was in contrast to the 50 – 83 % reduction of anthocyanin concentration in both wines (Tables 8.1 and 8.2).

Thus, the lower apparent concentration of malvidin 3-glucoside and the other anthocyanins at wine pH indicated that the anthocyanins were not important pigments at wine pH (Table 8.2). Rather, it appeared from the higher apparent concentrations of the pigmented polymers at wine pH, that it was the pigments that eluted in the pigmented polymer peak that had a greater influence on wine colour (Table 8.2). In addition, the apparent concentration of Vitisin A was not responsible for the colour differences, but its presence would have contributed to the colours of the red wines (Table 8.2).

8.4.3. Wine colour and quantification of wine pigments post-SO_2 bleaching

Post-SO_2 bleaching, the wines made with either SC or SB yeast strains exhibited the same red-orange hues (positive equivalent H* values) (Table 8.3). Similar to the wine colour measured at its natural wine pH (pH 3.75) (Figure 8.2 and Table 8.2), the SB SO_2 stable wine colour was darker and more colourful (lower L* and higher C* values) than the SC SO_2 stable wine colour (Figure 8.2 and Table 8.3). As a result of the bleaching of the colour of the anthocyanins, the SO_2 stable wine colour of both SC and SB wines was lighter (higher L* and lower C* values) than the wine colour (Figure 8.2).

At the increased pH value, in the presence of SO_2 (^s_wpH 2.79), the peaks of monomeric anthocyanins and their acylated derivatives were not detected as the colours of these pigments had been bleached by SO_2. For example, at the elution time for malvidin 3-glucoside (at approximately 8 minutes), no peak was detected. This confirmed that the colour of malvidin 3-glucoside was not stable to SO_2 bleaching. The colour of all the monomeric anthocyanins was similarly bleached.

Table 8.3: Apparent concentration of anthocyanin-derived pigments (mg/L) [1] in the SC and SB red wines determined by HPLC-UV/Vis analysis post-SO$_2$ bleaching, at wine pH ^s_wpH 2.79 on application of the post-column adjustment method. SC and SB are Cabernet Sauvignon wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively, measured 22 months post-pressing.

Concentration of pigments post-SO$_2$ bleaching (at wine pH) (mg/L) [1,2]	Pigment	SC wines	SB wines
	Vitisin A	3 ± 0 [a]	3 ± 0 [a]
	Pigmented polymers	20 ± 0 [a]	28 ± 1 [b]

	CIELab value	SC wines	SB wines
SO$_2$ stable wine colour [3]	L*	85.24 ± 0.23 [a]	81.33 ± 0.36 [b]
	C*	18.78 ± 0.31 [a]	25.93 ± 0.34 [b]
	H*	0.83 ± 0.02 [a]	0.83 ± 0.01 [a]
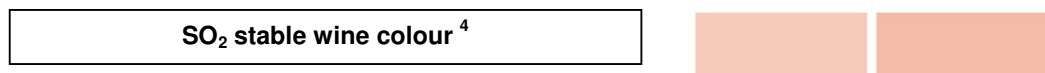
SO$_2$ stable wine colour [4]	

[1] Concentration in terms of malvidin 3-glucoside chloride equivalents (mg/L) determined using the standard HPLC method at pH 1.5.

[2] Mean ± standard error values calculated from HPLC measurements repeated twice on three replicate tanks for each SC and SB wine.

[3] CIELab colour of the SC and SB wines post bleaching with SO$_2$ using a 1 mm cuvette. Mean ± standard error calculated from colour measurements repeated in triplicate on three replicate tanks for each SC and SB wine.

[4] The CIELab colours shown are representative of the actual SO$_2$-stable wine colour visible in a 1 mm cuvette. Importantly, CIELab colours observed, either on the computer monitor or in the printed page, were dependent on the hardware and software of the computer, and ambient lighting used to view the colours.

[a or b] Values with different subscript letters are significantly different at $P < 0.05$ when comparing between the SC and SB wines.

Similar to the observations made with Vitisin A at low pH (Table 8.1) and at wine pH (Table 8.2), the apparent Vitisin A concentration of both wines was 3 mg/L post-SO$_2$ bleaching (Table 8.3). As stated earlier, it may have been difficult to determine a small change in Vitisin A concentration with pH value due to a threshold effect and the low peak area of Vitisin A. The results implied that the sulfite ion did not affect the absorbance of Vitisin A and that this pyranoanthocyanin was stable to SO$_2$ bleaching (Bakker *et al.* 1998; Asenstorfer *et al.* 2001). Thus, as the apparent Vitisin A concentration was

equivalent in both wines, Vitisin A would have contributed to the wine colour, but the pigment was not responsible for the SO_2 stable wine colour differences observed in the SC and SB SO_2 stable wine colour (Table 8.3).

Similar to the apparent concentrations of the pigments at low pH (Table 8.1) and in the wine pH-adjusted medium (Table 8.2), the SO_2 bleaching did not affect the relative amounts of pigmented polymers in the SC and SB wines (Table 8.3). For example, the apparent concentrations at low pH, in the wine-pH adjusted medium and post-SO_2 bleaching, the concentration of the SB SO_2 stable pigmented polymers remained higher (28 mg/L) than the SC SO_2 stable pigmented polymers (20 mg/L) (Table 8.3). The higher apparent concentration of the SB pigmented polymers may have contributed to the higher SB SO_2 stable wine colour (Table 8.3). Since the concentration of Vitisin A was equivalent in both SC and SB wines post-SO_2 bleaching, it could be inferred that the SO_2-stable wine colour was mostly attributable to concentrations of the SO_2-stable pigmented polymers. Importantly, compared to the apparent concentration of pigmented polymers at wine pH, approximately 87 % of the pigmented polymers were stable to SO_2 bleaching (Tables 8.2 and 8.3).

8.4.4.   Colour of malvidin 3-glucoside at low pH, in the wine pH-adjusted medium and post-SO_2 bleaching

At low pH, the hue of malvidin 3-glucoside was violet-red (negative H* value) in both wines. Malvidin 3-glucoside was darker and brighter in the SC wines (lower L* and higher C* value) than in the SB wines (Table 8.4). This correlated well with the higher malvidin 3-glucoside concentration in the SC wines (87 mg/L) than in the SB wines (59 mg/L) (Table 8.1).

Table 8.4: CIELab colour values of malvidin 3-glucoside at low pH (^s_wpH = 1.68), at wine pH (^s_wpH = 3.42) and post-SO$_2$ bleaching (^s_wpH = 2.79) for the SC and SB wines on application of post-column adjustment method. SC and SB are Cabernet Sauvignon wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively, measured at 22 months post-pressing.

CIELab value [1,2]	Malvidin 3-glucoside in the SC wines		
	Low pH	Wine adjusted pH	SO$_2$ bleaching
L*	85.45 ± 0.14 [a]	94.26 ± 0.38 [a]	100
C*	28.77 ± 0.39 [a]	6.81 ± 0.76 [a]	0
H*	-1.51 ± 0.00 [a]	1.46 ± 0.03 [a]	0
Perceived colour of pigments [3]			

CIELab value [1,2]	Malvidin 3-glucoside in the SB wines		
	Low pH	Wine adjusted pH	SO$_2$ bleaching
L*	88.33 ± 0.15 [b]	98.19 ± 0.02 [b]	100
C*	22.00 ± 0.34 [b]	2.78 ± 0.03 [b]	0
H*	-1.54 ± 0.00 [b]	-1.37 ± 0.00 [b]	0
Perceived colour of pigments [3]			

[1] HPLC-CIELab values determined applying a multiplication factor of 0.02 to the raw spectral data of each peak.

[2] Mean ± standard error values of the HPLC-CIELab colour values repeated twice on three replicate tanks for each SC and SB wine.

[3] CIELab colours observed, either on the computer monitor or in the printed page, were dependent on the hardware and software of the computer, and ambient lighting used to view the colours.

[a or b] Values with different subscript letters are significantly different at $P < 0.05$ when comparing between the SC and SB wines.

At the wine-adjusted pH values, the hue of malvidin 3-glucoside in the SC wines was orange-red (positive H* value), whilst the hue of malvidin 3-glucoside in the SB wines was red-violet (negative H* value). At wine pH, more colour was provided by malvidin 3-glucoside in the SC wines (lower L* and higher C* values) than in the SB wines (Table 8.4). This associated well

with the higher concentration of malvidin 3-glucoside in the SC wines at wine pH (Table 8.2). For example, at wine pH, the concentration of malvidin 3-glucoside was greater in the SC wines (19 mg/L) than in the SB wines (11 mg/L) (Table 8.2).

Importantly, the CIELab colour values illustrated in Table 8.4 demonstrates that, at wine pH, malvidin 3-glucoside is not an important contributor to wine colour. The change in the colour of malvidin 3-glucoside from low pH (^s_wpH 1.68) to wine pH (^s_wpH 3.42) was a result of a shift in equilibrium. At low pH, the colour of the highly coloured flavylium cation was observed (Table 8.4). Whilst the less coloured isoforms of malvidin 3-glucoside were apparent at wine pH (Table 8.4). This resulted in a reduction in peak area (data not shown) and lower anthocyanin quantification at wine pH and the colour of the pigment appeared almost white (Table 8.4).

Bleaching with the bisulfite ion resulted in the peak associated with malvidin 3-glucoside not being detected. The colour of malvidin 3-glucoside was considered bleached by the sulfite ion as no absorbance at the elution time of malvidin 3-glucoside was detected in the HPLC chromatogram. Consequently, the colour of malvidin 3-glucoside was represented by the CIELab values, $L^* = 100$, $C^* = 0$, and $H^* = 0$ (Table 8.4).

8.4.5. Colour of Vitisin A at low pH, in the wine pH-adjusted medium and post-SO_2 bleaching

Identical concentrations of Vitisin A (3 mg/L) in both SC and SB wines were recorded at low pH (Table 8.1), at wine pH (Table 8.2) and post-SO_2 bleaching (Table 8.3). But, the colours of Vitisin A changed in the different pH media, and on SO_2 bleaching (Table 8.5).

Table 8.5: CIELab colour values of Vitisin A at low pH (^s_wpH = 1.68), at wine pH (^s_wpH = 3.42) and post-SO$_2$ bleaching (^s_wpH = 2.79) for the SC and SB wines on application of post-column adjustment method. SC and SB are Cabernet Sauvignon wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively measured at 22 months post-pressing.

CIELab value [1,2]	Vitisin A in the SC wines		
	Low pH	Wine adjusted pH	SO$_2$ bleaching
L*	90.04 ± 0.12 [a]	93.17 ± 0.09 [a]	96.11 ± 0.08 [a]
C*	17.79 ± 0.22 [a]	9.58 ± 0.18 [a]	7.06 ± 0.11 [a]
H*	1.55 ± 0.00 [a]	1.37 ± 0.00 [a]	1.14 ± 0.01 [a]

Perceived colour of pigments [3]			

CIELab value [1,2]	Vitisin A in the SB wines		
	Low pH	Wine adjusted pH	SO$_2$ bleaching
L*	89.98 ± 0.22 [a]	93.23 ± 0.06 [a]	95.36 ± 0.19 [b]
C*	17.70 ± 0.50 [a]	9.57 ± 0.14 [a]	8.51 ± 0.46 [b]
H*	1.49 ± 0.01 [b]	1.30 ± 0.01 [b]	1.15 ± 0.01 [a]

Perceived colour of pigments [3]			

[1] HPLC-CIELab values determined applying a multiplication factor of 0.02 to the raw spectral data of each peak.

[2] Mean ± standard error values of the HPLC-CIELab colour values repeated twice on three replicate tanks for each SC and SB wine.

[3] CIELab colours observed, either on the computer monitor or in the printed page, were dependent on the hardware and software of the computer, and ambient lighting used to view the colours.

[a or b] Values with different subscript letters are significantly different at $P < 0.05$ when comparing between the SC and SB wines.

At low pH, approximately equivalent values for the apparent Vitisin A concentration (3 mg/L) were measured for both SC and SB wines (Table 8.1). The hue of Vitisin A was orange-red (positive H* values) in both wines (Table 8.5). There was a significant difference in the H* values of Vitisin A at low pH in both wines. This resulted in a visible difference in the colour of Vitisin A at ^s_wpH 1.68 between the SC and SB wines (Table 8.5) and further confirmed by $\Delta E^* > 1$.

At low pH (^s_wpH 1.68), compared to the colour of malvidin 3-glucoside (Table 8.4), the colour of Vitisin A (Table 8.5) was only slightly weaker (higher L* and lower C* values), despite the higher concentration of malvidin 3-glucoside in both SC and SB wines (Table 8.1). For example, at low pH, the apparent concentration of malvidin 3-glucoside (87 mg/L) was 29 times greater than the apparent concentration of Vitisin A (3 mg/L) in the SC wines (Table 8.1 above). From Table 8.1, it could have been assumed that the high concentration of malvidin 3-glucoside contributed more than Vitisin A to wine colour at low pH. But the actual CIELab colour values illustrate that actually Vitisin A, at a lower concentration, contributed a significant amount of colour at low pH (Table 8.5).

At wine pH, the hue of Vitisin A was also orange-red in both wines (positive H* values) (Table 8.5). This resulted in a difference in the colour of Vitisin A between the SC and SB wines as ∆E* values > 2 (Table 8.5). In addition, the H* value of the SC and SB wines differed significantly. One would expect to observe similar colours of Vitisin A at low pH and at wine pH given that similar apparent concentrations for both wines (3 mg/L) had been observed in both media (Tables 8.1 and 8.2 above). But, the colour of Vitisin A differed at ^s_wpH 1.68 to the colour of Vitisin A at ^s_wpH 3.42 (Table 8.5). The colour of Vitisin A at wine pH was lighter and duller (higher L* and lower C* values) than at low pH. From the results in Table 8.5, it is clear that at wine pH, the reduced Vitisin A colour is in agreement to the observations regarding the pH dependence of Vitisin A made by Asenstorfer (Asenstorfer 2001). Asenstorfer proposed that Vitisin A exhibits similar pH dependent canonical structures to malvidin 3-glucoside (Bakker and Timberlake 1997) and consequently, the colour of Vitisin A would vary with pH value.

In the wine pH-adjusted medium, for both SC and SB wines, the colour of Vitisin A (Table 8.5) was darker (lower L*) and more colourful (higher C* values) compared to malvidin 3-glucoside in the wine pH-adjusted medium (Table 8.4). The colour difference between the Vitisin A and malvidin

3-glucoside, the ΔE^* value, was approximately 6. As the ΔE^* value > 1, this demonstrated that the colour differences were very noticeable. The colours of the two pigments observed were in contrast to the lower apparent concentration of Vitisin A compared to the higher apparent concentration of malvidin 3-glucoside at wine pH. For example, in the SC wines, at wine pH, the apparent concentrations of Vitisin A and malvidin 3-glucoside were 3 mg/L and 19 mg/L respectively (Table 8.1 above). But Vitisin A had more colour (lower L* and higher C* values) than malvidin 3-glucoside (Table 8.4). Thus, from this observation (Table 8.5), it is evident that the concentration alone is not a factor in determining the colour expression of a wine pigment.

In the presence of the bisulfite ion, the hue of Vitisin A was orange-red (positive H* values) (Table 8.5). The change in media from at wine pH to post-SO_2 bleaching resulted in an overall decrease in the Vitisin A colour (Table 8.5). The colour of Vitisin A became lighter (L* increased), duller (C* reduced) and possessed a less orange-red hue (H* reduced) in both wines. The reduction in colour could be attributable to bleaching of some of the colour from Vitisin A in the presence of the bisulfite ion. This observation could imply that Vitisin A is not entirely stable to SO_2 bleaching. Although this observation is converse to the literature which has stated that Vitisin A is SO_2 stable, it must be noted that the experiments were performed in a model wine solution using an isolated Vitisin A pigment (Romero and Bakker 1999; Romero and Bakker 2000a; Romero and Bakker 2000b; Schwarz *et al.* 2003d). In contrast, here Vitisin A was separated by HPLC analysis from an actual wine.

In addition, there was a difference in the colour of Vitisin A between the SC and SB wines (Table 8.5). The significant differences in the L* and C* values and the ΔE^* values > 1 between SC Vitisin A and SB Vitisin A post-SO_2 bleaching indicated that the colour difference was noticeable. Thus, the threshold effect may have been responsible for the equivalent concentrations of Vitisin A (3 mg/L for both SC and SB wines) at low pH, at wine pH and post-SO_2 bleaching (Table 8.1 above). It is evident that the CIELab colours of Vitisin A were dependent on the pH value and in the presence of the sulfite

ion. Importantly, the colours of Vitisin A became increasingly lighter (higher L* and lower C* values) as the pH value was increased to wine pH and then finally post-SO$_2$ bleaching.

To a lesser effect, the colour of Vitisin A was less dependent on the type of yeast strain used to ferment the SC and SB wines (Table 8.5). For example, the colour of SB Vitisin A was slightly darker (lower L* and higher C* values) than the colour of SC Vitisin A in all three media (Table 8.5). This observation highlights the importance of using the HPLC-CIELab method in conjunction with pigment quantification using appropriate pH or SO$_2$-bleaching media to define the colours of pH-dependent pigments, and pigments whose colour may be bleached by excess bisulfite solution.

8.4.6. Colour of the pigmented polymers at low pH, in the wine pH-adjusted medium and post-SO$_2$ bleaching

At low pH (^s_wpH 1.68), orange-red hues (positive H* values) were observed in the equal widths of PP1, PP2 and PP3, representing parts of the pigmented polymer peak for both SC and SB wines (Table 8.6). In addition, the colours of PP1 to PP3 were darker (lower L*) and more colourful (larger C* values) (Table 8.6) than malvidin 3-glucoside (Table 8.4) and Vitisin A (Table 8.5) at low pH. At low pH, greater colour was observed for the pigmented polymers (Table 8.6) even though a higher concentration of malvidin 3-glucoside was recorded compared to the pigmented polymer and Vitisin A concentrations (Table 8.1 above). For example, even though the concentration of the SC pigmented polymers was 28 mg/L, and PP1 to PP3 were more colourful (lower L* and higher C*) than the higher concentration of malvidin 3-glucoside (87 mg/L) in the SC wines (Table 8.1 above).

Table 8.6: CIELab colour values of the pigmented polymers [1] at low pH (^s_wpH = 1.68) of the SC and SB wines on application of post-column adjustment method. SC and SB are Cabernet Sauvignon wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively, measured at 22 months post-pressing.

CIELab value [1,2]	SC Pigmented polymers		
	PP1	PP2	PP3
L*	86.94 ± 0.36 [a]	69.88 ± 0.43 [a]	81.64 ± 0.28 [a]
C*	18.48 ± 0.75 [a]	35.01 ± 0.40 [a]	19.86 ± 0.25 [a]
H*	1.53 ± 0.01 [a]	1.29 ± 0.00 [a]	1.18 ± 0.01 [a]
Perceived colour of pigments [4]			

CIELab value [1,2]	SB Pigmented polymers		
	PP1	PP2	PP3
L*	85.30 ± 0.11 [b]	61.00 ± 0.43 [b]	75.90 ± 0.26 [b]
C*	19.29 ± 0.14 [a]	44.91 ± 0.47 [b]	27.01 ± 0.32 [b]
H*	1.42 ± 0.00 [b]	1.21 ± 0.00 [b]	1.11 ± 0.00 [b]
Perceived colour of pigments [4]			

[1] The pigmented polymer peak was divided into three equal peak widths, PP1, PP2 and PP3 about the peak maximum where PP1 to PP3 had equal widths of 0.5 minutes.

[2] HPLC-CIELab values determined applying a multiplication factor of 0.02 to the raw spectral data of each peak.

[3] Mean ± standard error values of the HPLC-CIELab colour values for each peak repeated twice on three replicate tanks for each SC and SB wine.

[4] CIELab colours observed, either on the computer monitor or in the printed page, were dependent on the hardware and software of the computer, and ambient lighting used to view the colours.

[a or b] Values with different subscript letters are significantly different at $P < 0.05$ when comparing between the SC and SB wines.

At low pH, all the SB pigmented polymers had more colour (lower L* and higher C* values) than the SC pigmented polymers, which appeared lighter and duller in comparison (Table 8.6). Thus, the colour observed was a function of concentration. For example, a higher concentration of pigmented polymers quantified in the SB wines (39 mg/L) compared to the SC wines (28 mg/L) at low pH resulted in highly coloured SB pigmented polymers (Table 8.1 above).

Similarly, the colours observed for the pigmented polymers were associated to the spectral profile across the visible spectrum of the pigmented polymer at 24 minutes (Figure 8.3). The spectral profile illustrated greater absorbance of the SB pigmented polymer peak than for the SC pigmented polymer peak (Figure 8.3). The λ_{max} values of the SB pigmented polymer peak, 434 nm and 509 nm, were hypsochromically shifted slightly to lower wavelengths from the SC pigmented polymer peak, where the λ_{max} values were 430 nm and 506 nm respectively (Figure 8.3). These dominant λ_{max} values indicated that most of the colour of both pigmented polymer peaks was derived from pigments which contributed orange-red to red hues (Figure 8.3).

Table 8.6 above illustrated that the orange-red hues (positive H* values) observed for the pigmented polymer peaks (PP1 to PP3) at low pH. In addition to the λ_{max} values, both SC and SB pigmented polymer peaks exhibited similar shoulders in the 466 to 472 nm (orange-red absorbance) and 559 to 569 nm (red-violet absorbance) regions and similar λ_{max} values at 644 nm (blue absorbance) and at 675 nm (blue absorbance). These additional absorbance values were lower in value than the absorbance at 434 nm and 509 nm for the SB wines, and 430 nm and 506 nm for the SC wines. The additional absorbance values (including other λ_{max} values and the shoulders) in the spectral profile would have contributed to the overall colours observed in the pigmented polymers at low pH (Table 8.6).

At low pH, the λ_{max} values differed between the SC and SB wines by 2 to 4 nm, but the other λ_{max} values and shoulders in the spectral profile were similar. The overall absorbance was greater for the SB pigmented polymers than the SC pigmented polymers. The greater concentration (Table 8.1) and colour (Table 8.6) provided by the SB pigments indicated that there probably was a difference in the composition of the pigmented polymers between the wines.
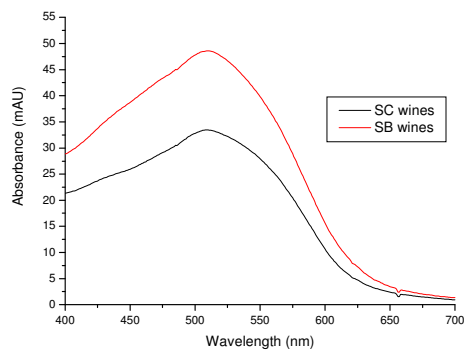
| | (a) Visual absorbance spectra | (b) Second derivative absorbance spectra |
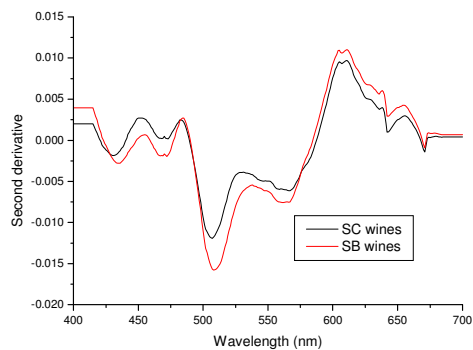
Yeast	Maxima and shoulders (nm) at low pH					
SC wines	430	466 – 472	507	559 – 569	644	675
SB wines	434	466 – 472	509	559 – 569	644	675
Complementary colour [a]	Orange-red	Orange-red	Red	Red-violet	Blue	Blue

Figure 8.3: Absorbance spectral profile (a) and associated second derivative spectra (b) of the pigmented polymer peak at 24 minutes in the HPLC chromatogram at low pH (^s_wpH = 1.68) for the SC and SB wines on application of post-column adjustment method. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively measured at 22 months post-pressing.

[a] Complementary colour at the associated λ_{max} value.

At wine pH, similar to at low pH, orange-red hues (positive H* values) were observed in PP1, PP2 and PP3, representing parts of the pigmented polymer peak for both SC and SB wines (Table 8.7).

Table 8.7: CIELab colour values of the pigmented polymers [1] at wine pH (^s_wpH = 3.42 ± 0.49) of the SC and SB wines on application of post-column adjustment method. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively measured at 22 months post-pressing.

CIELab value	Pigmented polymers in the SC wines [2,3]		
	PP1	PP2	PP3
L*	90.95 ± 0.61 [a]	73.31 ± 0.28 [a]	83.16 ± 0.25 [a]
C*	9.20 ± 0.64 [a]	26.01 ± 0.19 [a]	15.93 ± 0.29 [a]
H*	1.27 ± 0.01 [a]	1.11 ± 0.01 [a]	0.98 ± 0.01 [a]
Perceived colour of pigments [4]			

CIELab value	Pigmented polymers in the SB wines [2,3]		
	PP1	PP2	PP3
L*	87.00 ± 0.51 [b]	63.70 ± 0.81 [b]	78.53 ± 0.34 [b]
C*	13.53 ± 0.51 [b]	35.83 ± 0.73 [b]	21.68 ± 0.35 [b]
H*	1.20 ± 0.01 [b]	1.06 ± 0.01 [b]	0.91 ± 0.01 [b]
Perceived colour of pigments [4]			

[1] The pigmented polymer peak was divided into three equal peak widths, PP1, PP2 and PP3 about the peak maximum where PP1 to PP3 had equal widths of 0.5 minutes.

[2] HPLC-CIELab values determined applying a multiplication factor of 0.02 to the raw spectral data of each peak.

[3] Mean ± standard error values of the HPLC-CIELab colour values for each pigment repeated twice on three replicate tanks for each SC and SB wine.

[4] CIELab colours observed, either on the computer monitor or in the printed page, were dependent on the hardware and software of the computer, and ambient lighting used to view the colours.

[a or b] Values with different subscript letters are significantly different at $P < 0.05$ when comparing between the SC and SB wines.

(a) Visual absorbance spectra		(b) Second derivative absorbance spectra			
Yeast	**Maxima and shoulders (nm) at wine pH**				
SC wines	431	466 – 472	506	575 – 581	645	672
SB wines	434	466 – 472	509	575 – 581	645	672
Complementary colour [a]	Orange-red	Orange-red	Red	Violet	Blue	Blue

Figure 8.4: Absorbance spectral profile (a) and associated second derivative spectra (b) of the pigmented polymer peak at 24 minutes in the HPLC chromatogram at wine pH ($^{s}_{w}$pH = 3.42 ± 0.49) for the SC and SB wines on application of post-column adjustment method. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively measured at 22 months post-pressing.

[a] Complementary colour at the associated λ_{max} value.

Similar to the change in colour observed for malvidin 3-glucoside (Table 8.4) and Vitisin A (Table 8.5) from low pH to wine pH, there was a change in the colours of pigmented polymers peaks, PP1, PP2 and PP3 (Table 8.6 to Table 8.7) on changing the pH value. Increasing the pH value to wine pH, the colours of PP1 to PP3 became lighter in colour (increased L* and decreased C* value) and exhibited less orange-red hues (decreased H* values). The absorbance of the pigmented polymer peaks of both wines were reduced by 20 to 26 % from low pH (Figure 8.3) to wine pH (Figure 8.4). This observation indicates that the pigmented polymer peak was composed of anthocyanin-derived pigments that were affected by the change in pH value. These pigments may possess structures similar to monomeric anthocyanins (e.g. malvidin 3-glucoside) and exhibit pH-dependent isoforms. Similar to the observations at low pH, in the wine pH-adjusted medium there was more

colour absorbance provided by the SB pigmented polymers than by the SC pigmented polymers.

The reduction in the absorbance profile and in the intensity of the second derivative from low pH to wine pH did not affect the λ_{max} values which remained unchanged. For example, the λ_{max} values for the SC and SB pigmented polymers were 431 and 434 nm respectively at low pH and in the wine-pH adjusted medium. But a bathochromic shift to higher wavelengths was observed in one of the shoulders in the absorbance profile increasing the pH value to wine pH (Figure 8.4). For example, for both wines at low pH (Figure 8.3), the shoulder in the region 559 to 569 nm (red-violet absorbance) was replaced by a shoulder in the 575 to 581 nm region (violet absorbance) at wine pH (Figure 8.4). The bathochromic shift in the shoulders observed on increasing from low pH to wine pH could be attributed to high molecular weight anthocyanins or anthocyanin-derived wine pigments with anthocyanin moieties that were capable of forming pH dependent isomers. Such pigments could behave in a similar fashion to malvidin 3-glucoside as the pH value was changed. For example, the monomeric anthocyanin, malvidin 3-glucoside experienced a bathochromic shift to higher wavelengths on increasing from low pH (λ_{max} 528 nm) (Figure 8.3) to wine pH (λ_{max} 532 nm) (Figure 8.4). The same effect could have occurred for high molecular weight anthocyanins or pH-dependent anthocyanin-derived wine pigments. Whilst there was a bathochromic shift in one of the shoulders on increasing the pH value, the other shoulders at 466 to 472 nm (orange-red absorbance) and the minor λ_{max} values indicating minor orange-red, red and blue absorbance (Figure 8.4) remained unchanged.

At wine pH, the colours of PP1 to PP3 were darker (lower L* values) and more colourful (larger C* values) than malvidin 3-glucoside (Table 8.4 above) and Vitisin A at wine pH (Table 8.5 above). Similarly, the apparent concentration of the pigmented polymers was greater than the apparent concentrations of malvidin 3-glucoside and Vitisin A (Table 8.2 above). For example, at wine pH, the apparent concentration of the SB pigmented polymers was 32 mg/L,

and the peaks, PP1 to PP3, were more colourful (lower L* and higher C* values) than the lower apparent concentrations of malvidin 3-glucoside (11 mg/L) and Vitisin A (3 mg/L) in the SB wines (Table 8.2).

Table 8.8: CIELab colour values of the pigmented polymers [1] at post-SO_2 bleaching (^s_wpH = 2.79 ± 0.01) of the SC and SB wines (on application of post-column adjustment method). SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively, measured at 22 months post-pressing.

CIELab value [2,3]	Pigmented polymers in the SC wines		
	PP1	PP2	PP3
L*	93.40 ± 0.33 [a]	79.61 ± 0.18 [a]	87.15 ± 0.46 [a]
C*	8.55 ± 0.31 [a]	24.85 ± 0.21 [a]	15.75 ± 0.68 [a]
H*	0.98 ± 0.01 [a]	0.92 ± 0.01 [a]	0.79 ± 0.01 [a]

Perceived colour of pigments [4]
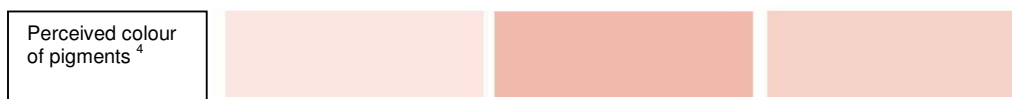
CIELab value [2,3]	Pigmented polymers in the SB wines		
	PP1	PP2	PP3
L*	90.64 ± 0.24 [b]	72.59 ± 0.49 [b]	83.44 ± 0.49 [b]
C*	12.39 ± 0.32 [b]	34.06 ± 0.46 [b]	21.55 ± 0.53 [b]
H*	0.99 ± 0.01 [a]	0.90 ± 0.01 [a]	0.77 ± 0.01 [a]

Perceived colour of pigments [4]

[1] The pigmented polymer peak was divided into three equal peak widths, PP1, PP2 and PP3 about the peak maximum where PP1 to PP3 had equal widths of 0.5 minutes.

[2] HPLC-CIELab values determined applying a multiplication factor of 0.02 to the raw spectral data of each peak.

[3] Mean ± standard error values of the HPLC-CIELab colour values for each pigment repeated twice on three replicate tanks for each SC and SB wine.

[4] CIELab colours observed, either on the computer monitor or in the printed page, were dependent on the hardware and software of the computer, and ambient lighting used to view the colours.

[a or b] Values with different subscript letters are significantly different at $P < 0.05$ when comparing between the SC and SB wines.

Similar to the change in colour of the pigmented polymer peak widths, PP1 to PP3, from low pH (Table 8.6) to wine pH (Table 8.7), there was a change in the colours of pigmented polymers in the presence of SO_2 (Table 8.8). Post-SO_2 bleaching, PP1 to PP3 were slightly lighter in colour (increased L* and decreased C* values) and reduced orange-red hues (decrease in H* values) (Table 8.8) compared to the colours of PP1 to PP3 at wine pH (Table 8.7). Similarly, the absorbance profiles of both pigmented polymer peaks were further reduced from wine pH to bleaching with SO_2 (Figure 8.3).

Post-SO_2 bleaching, the spectral profile across the visible spectrum illustrated greater absorbance of the SB pigmented polymer peak than for the pigmented polymer peak of the SC wines (Table 8.8). This was related to the reduction in the apparent pigmented polymer concentration (Table 8.3), and the lighter colours observed for the peaks, PP1 to PP3 (Figure 8.3) after SO_2 bleaching. The pH dependent and SO_2 stable pigments that eluted in the pigmented polymer had constant λ_{max} values. For example, the λ_{max} values of the SB pigmented polymer peak in the presence of SO_2 were similar to the λ_{max} values at low pH and at wine pH, 434 nm and 509 nm (Figure 8.5). The colour differences between the SB and SC pigmented polymers was apparent by the λ_{max} values of the SB pigmented polymers which were bathochromically shifted by approximately 3 nm from the λ_{max} values of the SC pigmented polymer peak (λ_{max} values of 431 nm and 506 nm) (Figure 8.3). These dominant λ_{max} values indicated that the pigments with absorbance maxima at this wavelength contributed to the orange-red colour observed in the pigmented polymers not only post-SO_2 bleaching, but at low pH and at wine pH as well. The SC and SB pigmented polymer peaks exhibited similar shoulders in the 466 to 473 nm and 575 to 581 nm regions and similar peak maxima at 645 nm and at 672 nm to the pigmented polymers at low pH and in the wine pH-adjusted media. Absorbance in the region 466 to 473 nm indicated that the pigments also contributed to the orange-red hue observed. A small proportion of the colour was from absorbance in the red-violet and blue regions represented by 575 to 581 nm and 645 to 672 nm respectively (Figure 8.6).
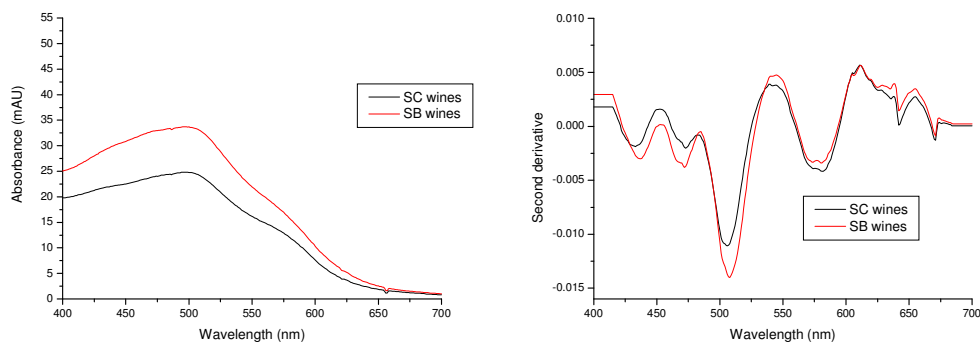
(a) Visual absorbance spectra   (b) Second derivative absorbance spectra

Yeast	Maxima and shoulders (nm) post-SO_2 bleaching					
SC wines	431	466 – 473	506	575 – 581	645	672
SB wines	434	466 – 473	509	575 – 581	645	672
Complementary colour [a]	Orange-red	Orange-red	Red	Red-violet	Blue	Blue

Figure 8.5: Absorbance spectral profile (a) and associated second derivative spectra (b) of the pigmented polymer peak at 24 minutes in the HPLC chromatogram post-SO_2 bleaching (at approximately wine pH) (^s_wpH = 2.79 ± 0.01) for the SC and SB wines on application of post-column adjustment method. SC and SB are Cabernet Sauvignon red wines made with *Saccharomyces cerevisiae* or *S. bayanus* yeast strains respectively measured at 22 months post-pressing.

[a] Complementary colour at the associated λ_{max} value.

Similar to the trend from low pH to wine pH, there was a further 8 to 15 % reduction in the overall absorbance of the pigmented polymer peaks of both wines from wine pH (Figure 8.4) to SO_2 bleaching (Figure 8.5). Post-SO_2 bleaching, the absorbance maximum and intensity of the second derivative were reduced compared to the same absorbance profile at wine pH. The peak maxima at 431 nm and 506 nm for the SC pigmented polymers and 434 nm and 509 nm for the SB pigmented polymers, and additional shoulders observed in the 575 to 581 nm region was the same as at wine pH. This indicated that there were a significant proportion of pigments that eluted in the pigmented polymer peak that were stable to pH change and in the presence of SO_2.

To date, the concentration of the pigmented polymer peak (determined by conventional HPLC analysis at low pH) is considered to be highly correlated with wine colour and SO_2-stable wine colour (measured at the wine's natural pH) (Peng *et al.* 2002; Hayasaka and Kennedy 2003; Bartowsky *et al.* 2004; Eglinton *et al.* 2004; Hayasaka *et al.* 2004; Herderich and Smith 2005). It could be considered unnecessary to determine the concentration and colour of pigmented polymers at wine pH and post-SO_2 bleaching based on "if it ain't broke, don't fix it" principle. However, a greater characterization of the pigmented polymer peak has been achieved using the post-column adjustment method and the HPLC-CIELab method, not only at wine pH and post-SO_2 bleaching, but at low pH as well. Nevertheless, the work in this chapter has shown that the colours of the pigmented polymers were different (for different yeast strain), and that they vary from low pH to wine pH. In addition, the colours of the pigmented polymers depend on the yeast used to ferment the SC and SB wines.

Importantly, the study has considered pigments that contribute to wine colour at the wine's natural pH, not at low pH. The methodologies employed in current literature may provide the wrong message to researchers and the wine industry. Here treatments using two different yeast strains were employed. By comparing the relative treatments, it could be implied that as there was more malvidin 3-glucoside in the SC wines, the anthocyanins were important to the wine colour. However, the SB wines had significantly more colour and SO_2 stable wine colour than the SC wines. The post column and HPLC-CIELab methods were used to generate detailed information regarding the concentration and colours of wine pigments that contributed to the higher SB wine colour and SO_2 stable wine colour. Thus, the important pigments are not malvidin 3-glucoside or the anthocyanins, but Vitisin A and the pigmented polymers. Vitisin A contributed to the wine colour, particularly to SO_2 stable wine colour even at low concentrations. Tables 8.6 to 8.8 have highlighted that there is a great need to focus on the pigmented polymers, and the colour differences and therefore less on malvidin 3-glucoside and the anthocyanins.

8.5.   Conclusions

8.5.1.   Wine colour and quantification of wine pigments at low pH, at wine pH and post-SO$_2$ bleaching

1. *The pH of the media is important when **quantifying** wine pigments. For example, at low pH, for both wines made with different yeast strains, the anthocyanin concentration was greater than the pigmented polymer concentration. While at wine pH, the converse was true: the apparent anthocyanin concentrations was very much reduced and lower than the apparent concentration of the pigmented polymers for both SC and SB wines.*

2. *The relative concentrations of anthocyanins and the pigmented polymers in both wines were not affected by the change in pH value. For example, the SC wines had a lower concentration of pigmented polymers than the SB wines at low pH, at wine pH and post-SO$_2$ bleaching.*

3. *When determining the **colour** of a wine, the pH of the sample media is important. For example, at low pH, the SC wines had more colour (low L^* and high C^* values) compared to the SB wine colour. However, the converse was apparent at wine pH as the SC wines had less colour (high L^* and low C^* values) compared to the SB wine colour due to the formation of less colour isoforms. The anthocyanins were significantly less important to wine colour at wine pH than the pigmented polymers.*

4. *The colour of the anthocyanins in both SC and SB wines was bleached by the sulfite ion. Both SC and SB wines exhibited red-violet hues (negative H^* values) at wine pH. In the presence of excess bisulfite solution, orange-red hues (positive H^* values) were observed. Compared to the colour of the wine at wine pH, SO$_2$ bleaching of the wines resulted in increased L^* and H^* values, and lower C^* values for both wines.*

5. *For both SC and SB wines, on adjusting from low pH to wine pH:*

a. There was a 50 to 80 % reduction in the apparent anthocyanin concentration. In the presence of excess bisulfite, the colour of the anthocyanins was bleached by the sulfite ion hence, no anthocyanin concentration was determined.

b. The presence of Vitisin A in the wines influenced wine colour, but as the concentration was equivalent in both wines in all three media, Vitisin A was not responsible for the colour differences observed in the SC and SB wines at low pH, at wine pH and post-SO_2 bleaching.

c. The change in pH from low pH to wine pH resulted in a 20% decrease in the apparent concentration of the pigmented polymers. The apparent concentration of the pigmented polymers decreased further as approximately 87 % of the pigments that eluted in the peak were considered stable to SO_2 bleaching.

8.5.2. Colour of malvidin 3-glucoside at low pH, in the wine pH-adjusted medium and post-SO_2 bleaching

1. At low pH, malvidin 3-glucoside was highly coloured (low L* and high C* values), but a reduction in the colour of malvidin 3-glucoside (increased L* and decreased C* values) was noted on changing the pH value from low pH to wine pH. The hue of malvidin 3-glucoside changed from red-violet at low pH to orange-red at wine pH. The change in malvidin 3-glucoside colour was attributed to a shift of equilibrium. Loss of malvidin 3-glucoside colour was noticeable as the highly coloured flavylium cation transformed to less coloured isoforms of malvidin 3-glucoside.

2. At low pH and at wine pH, malvidin 3-glucoside in the SC wines had more colour (low L* and high C* values) than malvidin 3-glucoside in the SB wines. At low pH, the colour contributed by anthocyanins would have been responsible for the higher SC wine colour. However, malvidin 3-glucoside was not responsible for wine colour at wine pH as transformation to the less coloured isoforms of malvidin 3-glucoside

have occurred. The SB wine colour at its natural wine pH was greater (lower L* and higher C* values) than the SC wine colour, but the colour provided by malvidin 3-glucoside in the SB wines was much less (higher L* and lower C* values) than malvidin 3-glucoside in the SC wines. Hence, the colour of malvidin 3-glucoside was not responsible for the colour differences at wine pH.

Note: The colour of malvidin 3-glucoside was bleached by the sulfite ion. No peak was detected at the elution time of malvidin 3-glucoside in the HPLC chromatogram. The colour of malvidin 3-glucoside was represented as having CIELab values of L* = 100, C* = 0 and H* = 0.

8.5.3. Colour of Vitisin A at low pH, in the wine pH-adjusted medium and post-SO$_2$ bleaching

1. *The colours of Vitisin A were affected by the pH media and in the presence of the sulfite ion. A loss of Vitisin A colour (increased L* and decreased C* values) became increasingly apparent as the media was changed from low pH to wine pH and finally in the presence of SO$_2$. In all three media, the hue of Vitisin A was orange-red. The loss of Vitisin A colour, on increasing the pH value to wine pH, could be attributed to the change in isoforms of Vitisin A. This demonstrates that there are pH-dependent isoforms of Vitisin A similar to malvidin 3-glucoside (Asenstorfer et al. 2006). The loss of Vitisin A colour on from wine pH to post-SO$_2$ bleaching may be because the pigment is not entirely stable in the presence of excess SO$_2$.*

2. *At wine pH, more colour was observed for Vitisin A in both wines, even though the apparent Vitisin A concentration was much lower than the apparent malvidin 3-glucoside concentration in both wines. For both SC and SB wines, at low pH, the colour of Vitisin A was slightly weaker (higher L* and lower C* values) than the colour of malvidin 3-glucoside. The converse was apparent at wine pH, that is there was more colour observed for Vitisin A (lower L* and higher C* values) than for malvidin 3-glucoside at wine pH, even though the concentration of Vitisin A was much smaller than the malvidin 3-glucoside concentration.*

3. There was a difference in the colours of Vitisin A from the SC and SB wines in the different media. At low pH, at wine pH and post-SO$_2$ bleaching, the ΔE^* values between SC Vitisin A and SB Vitisin A were greater than 1, which indicated an observable colour difference.

4. At low pH, at wine pH and post-SO$_2$ bleaching, the SB Vitisin A concentration and colour was greater than the SC Vitisin A concentration and colour. This could be a result of a greater amount of pyruvate available during fermentation using the SB yeast strain.

8.5.4. Colour of the pigmented polymers at low pH, in the wine pH-adjusted medium and post-SO$_2$ bleaching

1. The colour differences in the SC and SB pigmented polymers at low pH, at wine pH and post-SO$_2$ bleaching was a result of the composition of the pigments that eluted in the pigmented polymer peaks. The composition affected the colour absorbance of the pigmented polymers. Thus, it can be concluded that not all pigmented polymers have the same colour as there was a difference in the pigmented polymer composition:

    a. At low pH, at wine pH and post-SO$_2$ bleaching, the SB pigmented polymers were more colourful (lower L* and higher C* values) than the SC pigmented polymers.

    b. There was more colour absorbance across the visible spectrum for the SB pigmented polymers than for the SC pigmented polymers.

    c. In all three media, the λ_{max} value of the SB pigmented polymers (λ_{max} 434 nm) was bathochromically shifted from the λ_{max} value of SC pigmented polymers (λ_{max} 431 nm).

    d. The pigmented polymers of both SC and SB wines exhibited similar shoulders and minor absorbance maxima that corresponded to orange-red, red-violet and blue colour absorbances. At low pH, the shoulders of the SC and SB pigmented polymers were in the wavelength range 559 to 569 nm. However, at wine pH and post-SO$_2$ bleaching, there was a

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bathochromic shift to absorbance in the range 575 to 581 nm or reduced red absorbance.

2. The pH of the media and the presence of the sulfite ion affected the colours of the equal widths of PP1, PP2 and PP3, representing fractions of the pigmented polymer peak.

   a. Orange-red hues were observed for PP1 to PP3 for both wines at low pH, at wine pH and post-SO_2 bleaching. The colours of PP1 to PP3 became increasingly lighter (higher L^* and lower C^* values) as the pH value increased to wine pH and then finally to post-SO_2 bleaching.

   b. Some pigments that eluted in the pigmented polymer peak were not stable to pH change. Pigments that were pH-dependent were probably high molecular weight anthocyanin polymers or anthocyanin-derived pigments that may have exhibited isoforms similar to malvidin 3-glucoside at low pH and at wine pH.

   c. In addition, there were some of the pigments that eluted in the pigmented polymer peak were bleached by the sulfite ion, and some were SO_2-stable. The SO_2-stable pigments probably had a functional group attached at the 4-position of the anthocyanin moiety rendering it stable in the presence of the bisulfite ion.

   d. Subtle but significant differences in the visual colour, and obvious differences in the λ_{max} values and absorbance bands of the pigmented polymers at low pH, in the wine pH-adjusted medium and post-SO_2 bleaching were observed for both wines. Identification of anthocyanin-derived pigments that could have eluted in the pigmented polymer peak cannot be determined using current literature. It is difficult to speculate as to the types of anthocyanin-derived pigments that could elute in the pigmented polymer peak because no one has published the pH-dependent isoforms of anthocyanin-derived pigments other than Vitisin A. Further work is necessary to identify the differently coloured pigments that have eluted in the pigmented polymer peak (e.g. using NMR or mass spectrometry).

Chapter 9: Further work

1. The HPLC-CIELab software is now a commercially available product, as the "ColourBurst" software. The software is low in cost and easy to operate particularly for those who do not have an in-depth knowledge of computer software. No modification of spectrophotometer or HPLC instrumentation is necessary for the operation of the software. The software only requires a basic understanding of the HPLC technique and the ability to extract the spectral data from an HPLC analysis.

   Any winery or research laboratory can use this software to assess the colour of HPLC-separated wine pigments. Although the HPLC instrumentation operation is at low pH values, the colours of anthocyanins can be determined in grape juice or young red wines. In aged wines, the technique can be used to assess the colours of influential peaks to wine colour such as the colours of the pigmented polymer peaks.

2. There is a need to apply the HPLC-CIELab and post-HPLC column adjustment methods to additional wines and wine trials to assess the colours of wine pigments at low pH, at wine pH and to study stable wine colour, the colour of wine derived from SO_2 stable wine pigments. For example, in this thesis, two sets of wines were used: the first study included wines made using grapes grown in different regions in Australia (2003 Shiraz red wines) and the second study involved wines made using different yeast strains (2002 Cabernet Sauvignon red wines). There are a multitude of viticultural and oeneological factors that could be investigated. For example, as it is considered that red wine colour develops with time, the post-column adjustment and HPLC-CIELab methods can be applied to the variables of conservation e.g. temperature regime, oxygen access, wine pH, and the levels of free and total SO_2 in wines.

3. The anthocyanins contributed a minor amount to colour at wine pH, whilst Vitisin A contributed to colour at low pH, at wine pH and to SO_2-stable wine colour. It is the pigmented polymer where much wine colour and SO_2-stable wine colour is derived from. Therefore, the

structures and spectral properties of pigmented polymer peak needs further investigation.

4. Vitisin A and the pigmented polymer peak were only two examples of anthocyanin-derived pigments identified in the HPLC chromatogram by UV/Vis analysis. There are many unidentified pigments (including polymeric anthocyanins and additional anthocyanin-derived pigments in illustrated in Figures 1.8 to 1.13) responsible for the colour of red wine. Further investigation into additional pigments is warranted to identify, characterize and associate the colour properties of "unidentified" wine pigments to wine colour. This can be achieved by employing the HPLC-UV/Vis post-column adjustment method coupled with mass spectrometry or NMR spectroscopy. Where HPLC-UV/Vis anlaysis has been employed, application of the HPLC-CIELab method can be used to characterize the colour properties of the identified pigments.

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APPENDIX 1.1: Colour properties of the five major anthocyanins found in *Vitis vinifera* at low pH and at approximately wine pH.

Anthocyanin	pH value	CIELab colour value			λ_{max} (nm)	ε (M^{-1}cm^{-1})	Reference
		L*	C*	H*			
1a	Low pH	-	-	-	538	29500	(Koeppen and Basson 1966)
1a	Low pH	-	-	-	546	13900	(Somers 1966)
1a	Low pH	-	-	-	520	28000	(Niketic-Aleksic and Hrazdina 1972)
1a	Low pH	-	-	-	-	27000	(Brouillard and Delaporte 1977)
1a	Low pH	-	-	-	535	36400	(Metivier *et al.* 1980)
1a	Low pH	57.0	-	63.0	-	-	(Bakker and Timberlake 1997) [a]
1a	pH 3.0	20.0	-	10.0	-	-	
1a	Low pH	67.0	66.0	9.9	520	20200	(Heredia *et al.* 1998)
	pH 3.0	93.0	12.0	- 6.9	-	-	
1a	Low pH	-	-	-	-	27000	(Houbiers *et al.* 1998)
1a	Low pH	-	-	-	-	17100	(Escribano-Bailon *et al.* 2001)
1a	Low pH	53.0	90.0	37.6	-	-	(Mattivi *et al.* 2001)
1a	pH 3.0	70.1	53.0	9.8	-	-	(Mattivi *et al.* 2001)
1a	Flavylium cation (low pH)	-	-	-	-	27000	(Lima *et al.* 2002)
1a	Hemiacetal (pH 2.64)	-	-	-	-	19600	
1a	E-chalcone (pH ≥ 3)	-	-	-	-	13000	
1a	Z-chalcone (pH ≥ 3)	-	-	-	-	13000	
1a	Low pH	-	-	-	-	27600	(Håkansson *et al.* 2003)
1a	pH 3.6	-	-	-	-	7100	
1a	Low pH	-	-	-	-	26500	(Santos *et al.* 1993)
1a	Low pH	-	-	-	530	-	(del Alamo Sanza *et al.* 2004b)
1a	pH 3	-	-	-	521	-	(Mazzaracchio *et al.* 2004)
1a	pH 4	-	-	-	526	-	
1b	Low pH	-	-	-	543	29000	(Asen *et al.* 1959)
1b	Low pH	-	-	-	520	23700	(Heredia *et al.* 1998)
1b	Low pH	-	-	-	526	-	(del Alamo Sanza *et al.* 2004b)

[a] No C* values were recorded

APPENDIX 1.2: Colour properties of the five major anthocyanins found in *Vitis vinifera* at low pH and at approximately wine pH.

Anthocyanin	pH value	CIELab colour value			λ_{max} (nm)	ε (M^{-1}cm^{-1})	Reference
		L*	**C***	**H***			
1b	pH 3	-	-	-	-	-	(Mazzaracchio *et al.* 2004)
1b	pH 4	-	-	-	-	-	(Mazzaracchio *et al.* 2004)
1c	Low pH	-	-	-	546	12900	(Somers 1966)
1c	Low pH	-	-	-	520	18900	(Heredia *et al.* 1998)
1c	Low pH	-	-	-	528	-	(del Alamo Sanza *et al.* 2004b)
1c	pH 3	-	-	-	519	-	(Mazzaracchio *et al.* 2004)
1c	pH 4	-	-	-	524	-	(Mazzaracchio *et al.* 2004)
1d	Low pH	-	-	-	536	11300	(Somers 1966)
1d	Low pH	-	-	-	512	14100	(Heredia *et al.* 1998)
1d	Low pH	-	-	-	518	-	(del Alamo Sanza *et al.* 2004b)
1e	Low pH	-	-	-	530	34300	(Siegelman and Hendricks 1958)
1e	Low pH	-	-	-	510	26900	(Jurd and Asen 1966)
1e	Low pH	-	-	-	520	25740	(McClure 1967)
1e	Low pH	70.4	69.0	29.5	512	18800	(Heredia *et al.* 1998)
1e	pH 3	88.0	23.0	8.4	-	-	(Heredia *et al.* 1998)
1e	Low pH	-	-	-	-	26900	(Skrede *et al.* 2000)
1e	Low pH	66.0	97.0	52.2	-	-	(Mattivi *et al.* 2001)
1e	pH 3	73.0	57.0	35.1	-	-	(Mattivi *et al.* 2001)
1e	Low pH	92.9	8.9	11.9	-	-	(Stintzing *et al.* 2002)
1e	pH 3	92.5	9.1	8.0	-	-	(Stintzing *et al.* 2002)
1e	Low pH	-	-	-	516	-	(del Alamo Sanza *et al.* 2004b)
1e	pH 3	-	-	-	511	-	(Mazzaracchio *et al.* 2004)
1e	pH 4	-	-	-	515	-	(Mazzaracchio *et al.* 2004)
1e	Low pH	-	-	-	-	26900	(Turker *et al.* 2004)

APPENDIX 1.3: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments.

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement; (e) by the Colour Dilution Analysis method.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
2a	Isolation from Port wine	(a), (c), (d), (e)	Red		(Bakker and Timberlake 1997)
2a	Isolation from grape marc extracts	(a), (b), (d)	Orange	-	(Fulcrand *et al.* 1998)
2a	Detected in wine	(a), (b)	λ_{max} 512 nm	-	(Revilla *et al.* 1999)
2a	Isolation from Port wine	(a), (c), (e)	Orange-brown in solution (pH 2 - 4.5)	0.4 – 4.55 mg/L	(Romero and Bakker 1999)
2a	Detection in wine	(a), (b), (d)	-	7 mg/L	(Degenhardt *et al.* 2000)
2a	Synthesis	(a), (b), (c), (d)	-	-	(Kennedy and Waterhouse 2000)
2a	Synthesis in model wine using grape extract	(a), (d)	Brown-red and SO_2 bleaching	0.50 – 1.64 mg/L and 2.86 mg/L	(Romero and Bakker 2000a)
2a	Formation in model systems	(a), (d)	Orange-red/ brownish red	0.67 mg/L	(Romero and Bakker 2000b)
2a	Formation in wine	(a), (b)	Stable to SO_2 bleaching	2.54 – 6.65 mg/L	(Asenstorfer *et al.* 2001)
2a	Isolated from wine	(a), (b), (c)	λ_{max} 511 nm	19.3 – 51.2 mg/L	(Mateus *et al.* 2001)
2a	Isolation from Port wine	(a), (b), (c), (d)	λ_{max} 538 nm, $\varepsilon = 12900$ L cm^{-1} mol^{-1}	7 – 15.4 mg/L	(Mateus and de Freitas 2001)
2a	Detection in wine	(a), (b), (c)	-	0.6 – 1.8 mg/L	(Revilla and González-SanJosé 2001)

APPENDIX 1.4: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments.

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (d) by UV-Vis or CIELab measurement; (e) by the Colour Dilution Analysis method.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
2a	Isolation from wine	(a), (b)	λ_{max} ~ 500 nm and absorbance at 400 to 520 nm, stable to SO_2 bleaching	-	(Atanasova *et al.* 2002a)
2a	Isolation from Port wine	(a), (d)	Orange-red (stable to SO_2 bleaching)	5.3 – 12.5 mg/L	(Romero and Bakker 2001)
2a	Detection in wine	(a), (b)	-	16.45 – 21.49 mg/L (total vitisins)	(Atanasova *et al.* 2002b)
2a	Isolation from grape marc extracts	(b)	-	-	(Hayasaka and Asenstorfer 2002)
2a	Detection in wine	(a), (b)	-	-	(Heier *et al.* 2002)
2a	Isolation from Port wine	(a), (b)	-	-	(Mateus *et al.* 2002a)
2a	Detection in wine	(a), (b)	Orange red (with other pigments) and stable to SO_2 bleaching, λ_{max} 518 nm	-	(Vivar-Quintana *et al.* 2002)
2a	Detected in wine	(a), (b), (d)	λ_{max} 518 nm	0.60 – 1.89 mg/L	(Morata *et al.* 2003)
2a	Synthesis and isolation by CCC and preparative HPLC	(b), (e)	λ_{max} 512 nm, brick red with additional absorbance 350 – 400 nm	5.5 ± 2.9 mg/L	(Schwarz *et al.* 2003c)

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APPENDIX 1.5: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments.

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
2a	From grapes and wine in model system	(a), (b)	-	-	(Wang *et al.* 2003b)
2a	Detection in wine, from pomace	(a), (b)	-	-	(Amico *et al.* 2004)
2a	Synthesis	(a), (b), (c), (d)	λ_{max} 511 nm	-	(Mateus *et al.* 2004)
2a	Detection in wine	(a), (b)	λ_{max} 509 nm	1.5 – 2.4 mg/L	(de Villiers *et al.* 2004)
2a	Isolated from wine	(a), (b), (d)	λ_{max} 512 nm	10.6 – 59.6 mg/L	(Pozo-Bayón *et al.* 2004)
2a	Formation in wine	(b), (d)	-	1.3 – 3.7 mg/L	(Spranger *et al.* 2004)
2a	From wine and grapes	(a), (b)			(Wang *et al.* 2004)
2a (aglycone)	Synthesis	(d)	λ_{max} 509 nm in acidic to pH 5, orange hue of red coloration	-	(Amic´ *et al.* 2000)
2a (aglycone)	Formation in model systems	(b), (c), (d)	Purple in 0.1 M HCl or methanolic solution	470 mg	(Roehri-Stoeckel *et al.* 2001)
2a*	Isolation from grape marc extracts	(a), (b), (d)	-	-	(Fulcrand *et al.* 1998)
2a*	Detected in wine	(a), (b)	-	-	(Revilla *et al.* 1999)
2a*	From grapeskin extract and wine	(b)	-	-	(Hayasaka and Asenstorfer 2002)

APPENDIX 1.6: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside).

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
2a*	Formation in model systems	(a), (c)	-	-	(Heier et al. 2002)
2a*	Formation in model systems	(a), (b)	-	-	(Vivar-Quintana et al. 2002)
2a*	From grapes and wine in model system	(a), (b)	-	-	(Wang et al. 2003b)
2a*	Detection in wine	(a), (b)	Bleached with $NaHSO_3$, λ_{max} 503 – 507 nm, orange pink	-	(Alcalde-Eon et al. 2004)
2a*	Detection in wine, from pomace	(a), (b)	-	-	(Amico et al. 2004)
2a*	Detection in wine	(a), (b)	λ_{max} ~ 510 nm	-	(de Villiers et al. 2004)
2a*	From grapes	(a), (b)	-	-	(Mazzuca et al. 2004)
2a*	From wine and grapes	(a), (b)			(Wang et al. 2004)
2b	Isolation from Port wine	(a), (b), (c), (d)	-	-	(Bakker and Timberlake 1997)
2b	Isolation from grape marc extracts	(a), (b), (c)	-	-	(Fulcrand et al. 1998)

APPENDIX 1.7: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside).

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis/CIELab measurement; (e) by Colour Dilution Analysis

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
2b	Formation in model systems	(a), (d)	Red-brown colour	2.30 mg/L	(Romero and Bakker 2000a)
2b	Detection in wine	(a), (b), (c), (e)	-	-	(Degenhardt *et al.* 2000)
2b	Detection in wine	(a), (b), (c), (d)	-	0.2 – 7.5 mg/L	(Mateus and de Freitas 2001)
2b	Isolated from wine	(a), (b), (c)	-	4.0 – 11.4 mg/L	(Mateus *et al.* 2001)
2b	Isolation from Port wine	(a), (d)	Orange-red (stable to SO_2 bleaching)	0.6 – 3.8 mg/L	(Romero and Bakker 2001)
2b	Detection in wine	(a), (b)	-	16.45 – 21.49 mg/L (in total)	(Atanasova *et al.* 2002b)
2b	Isolation from grape marc extracts	(b)	-	-	(Hayasaka and Asenstorfer 2002)
2b	Detection in wine	(a), (b)	-	-	(Heier *et al.* 2002)
2b	Detection in wine	(a), (b)	-	-	(Mateus *et al.* 2002a)
2b	Detection in wine	(b), (e)	λ_{max} 532 nm	-	(Schwarz *et al.* 2003c)
2b	Detection in wine	(a), (b)	-	-	(de Villiers *et al.* 2004)
2b	From grapes and wine in model system	(a), (b)	-	-	(Wang *et al.* 2003b)
2b	Detected in Port wine	(a), (b), (c)	λ_{max} 511 nm	-	(de Freitas and Mateus 2004)
2b	From wine and grapes	(a), (b)			(Wang *et al.* 2004)

APPENDIX 1.8: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside).

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
2b*	Detection in wine	(a), (b)	-	-	(Heier *et al.* 2002)
2b*	Detected in Port wine	(b)	-	-	(de Freitas and Mateus 2004)
2b*	From wine and grapes	(a), (b)			(Wang *et al.* 2004)
2c	Isolation from grape marc extracts	(a), (b), (c)	-	-	(Fulcrand *et al.* 1998)
2c	Isolation from Port wine	(a), (b), (d)	-	1.39 – 3.75 mg/L	(Romero and Bakker 1999)
2c	Formation in model systems	(a), (d)	Brown-red and SO_2 bleaching	0.25 mg/L	(Romero and Bakker 2000a)
2c	Formation in model systems	(a), (d)	Orange-red/ brownish red	0.40 mg/L	(Romero and Bakker 2000b)
2c	Isolation from Port wine	(a), (b), (c)	-	4.8 – 10.8 mg/L	(Mateus *et al.* 2001)
2c	Isolated from wine	(a), (b), (c)	λ_{max} 503 nm	4.3 – 11.9 mg/L	(Mateus *et al.* 2001)
2c	Detection in wine	(a), (b), (c), (d)	λ_{max} 532 nm	4.8 – 10.8 mg/L	(Mateus and de Freitas 2001)
2c	Isolation from Port wine	(a), (d)	Orange-red (stable to SO_2 bleaching)	2.3 – 6.2 mg/L	(Romero and Bakker 2001)
2c	Detection in wine	(a), (b)	-	16.45 – 21.49 mg/L (in total)	(Atanasova *et al.* 2002b)

APPENDIX 1.9: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside).

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
2c	Isolation from grape marc extracts	(b)	-	-	(Hayasaka and Asenstorfer 2002)
2c	Detection in wine	(a), (b)	-	-	(Heier *et al.* 2002)
2c	Isolation from Port wine	(a), (b)	-	-	(Mateus *et al.* 2002a)
2b	From grape skins, in model wine	(a), (d)	Brown-red and SO_2 bleaching	0.19 – 1.19 mg/L and 2.30 mg/L	(Romero and Bakker 2000b)
2c	From grapes and wine in model system	(a), (b)	-	-	(Wang *et al.* 2003b)
2c	Detection in wine	(a), (b)	λ_{max} 516 nm, purple band	-	(Alcalde-Eon *et al.* 2004)
2c	Detection in wine	(a), (b)	-	-	(de Villiers *et al.* 2004)
2c	Detected in Port wine	(a), (b), (c)	λ_{max} 511 nm	-	(de Freitas and Mateus 2004)
2c*	Isolation from grape marc	(b)	-	-	(Hayasaka and Asenstorfer 2002)
2c*	Detection in wine	(a), (b)	-	-	(Heier *et al.* 2002)
2c*	Isolation from Port wine	(a), (b)	-	-	(Mateus *et al.* 2002a)
2c*	Detection in wine	(a), (b)	λ_{max} 513 nm, purple band	-	(Alcalde-Eon *et al.* 2004)
2c*	Detected in Port wine	(b)	-	-	(de Freitas and Mateus 2004)
2c*	From grapes	(a), (b)	-	-	(Mazzuca *et al.* 2004)

APPENDIX 1.10: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside).

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement; (e) by the Colour Dilution Analysis method.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
2e	Detection in wine	(a), (b)	Orange red; stable to SO_2 bleaching	-	(Vivar-Quintana *et al.* 2002)
2f	From grapeskin extract and wine	(b)	-	-	(Hayasaka and Asenstorfer 2002)
2g	Synthesis in model wine and formation in wine	(a), (b), (c), (e)	λ_{max} 532 nm	-	(Schwarz *et al.* 2003c)
2h	Detection in wine and synthesis	(a), (b), (c), (d)	λ_{max} 575 nm	-	(Mateus *et al.* 2003)
2h	Detected in Port wine	(a), (b), (c)	λ_{max} 583 nm	-	(de Freitas and Mateus 2004)
2h	Synthesis	(a), (b), (c), (d)	λ_{max} 572 nm	-	(Mateus *et al.* 2004)
2h	From wine and grapes	(a), (b)			(Wang *et al.* 2004)
2i	Detection in wine and synthesis	(a), (b), (c), (d)	λ_{max} 575 nm	-	(Mateus *et al.* 2003)
2i	Detected in Port wine	(a), (b), (c)	λ_{max} 583 nm	-	(de Freitas and Mateus 2004)
2l	Isolated from wine	(a), (b), (c), (d)	λ_{max} 538 nm	-	(Mateus *et al.* 2005)

APPENDIX 1.11: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside).

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
3	Detection in wine	(a), (b)	-	0.01 – 0.3 mg/L (in total)	(Revilla and González-SanJosé 2001)
3a (aglycone)	Synthesis	(d)	λ_{max} 486 nm in pH ~1 $\rightarrow$ pH 5, orange hue of red coloration	-	(Amic´ et al. 2000)
3a (aglycone)	Formation in model systems	(b), (c), (d)	Purple in 0.1 M HCl or methanolic solution	750 mg	(Roehri-Stoeckel et al. 2001)
3a	Detection in wine	(a), (b)	-	-	(Heier et al. 2002)
3a	Isolation from Port wine	(a), (b), (c), (d)	Orange	-	(Bakker and Timberlake 1997)
3a	Detection in wine	(a), (b)	Stable to SO_2 bleaching	-	(Asenstorfer et al. 2001)
3a	Isolation from wine	(a), (b)	λ_{max} ~ 500 nm with additional absorbance 400–520 nm, stable to SO_2 bleaching	-	(Atanasova et al. 2002a)
3a	Detection in wine	(a), (b)	Orange red (with other pigments) and stable to SO_2 bleaching, λ_{max} 498 nm	-	(Vivar-Quintana et al. 2002)
3a	Detection in wine	(a), (b)	λ_{max} 513 nm	-	(Monagas et al. 2003)
3a	Detected in wine	(a), (b)	-	-	(Wang et al. 2003a)

APPENDIX 1.12: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments.

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
3a	Detection in wine	(a), (b)	-	-	(de Villiers $et\ al.$ 2004)
3a	Detection in wine, from pomace	(a), (b)	-	-	(Amico $et\ al.$ 2004)
3a	From grapeskin extract and wine	(b)	-	-	(Hayasaka and Asenstorfer 2002)
3a	Detected in wine	(a), (b), (d)	λ_{max} 498 nm	0.72 – 1.84 mg/L	(Morata $et\ al.$ 2003)
3a	Detected in wine	(a), (b)	λ_{max} 496 nm	-	(Revilla $et\ al.$ 1999)
3a	Detected in Port wine	(a), (b), (c)	λ_{max} 511 nm	-	(de Freitas and Mateus 2004)
3a	Isolated from wine	(a), (b), (d)	-	-	(Pozo-Bayón $et\ al.$ 2004)
3a	From wine and grapes	(a), (b)			(Wang $et\ al.$ 2004)
3a*	Detected in Port wine	(b)	-	-	(de Freitas and Mateus 2004)
3a*	Detection in wine	(a), (b)	-	-	(Heier $et\ al.$ 2002)
3a*	Detection in wine	(a), (b)	λ_{max} 509 nm	-	(Monagas $et\ al.$ 2003)
3b	Isolation from Port wine	(a), (b), (c), (d)	-	-	(Bakker and Timberlake 1997)
3b	Detection in wine	(a), (b)	Orange red (with other pigments) and stable to SO_2 bleaching	-	(Vivar-Quintana $et\ al.$ 2002)
3b	Detection in wine	(a), (b)	-	-	(Heier $et\ al.$ 2002)

APPENDIX 1.13: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside.

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
3b	Isolation from wine	(a), (b), (d)	λ_{max} 500 nm, stable to SO_2 bleaching	-	(Vivar-Quintana *et al.* 2002)
3b	Detection in wine	(a), (b)	λ_{max} 518 nm	-	(Monagas *et al.* 2003)
3b	Detection in wine	(a), (b)	-	-	(de Villiers *et al.* 2004)
3b	Detected in Port wine	(b)	-	-	(de Freitas and Mateus 2004)
3b*	Detection in wine	(a), (b)	-	-	(Heier *et al.* 2002)
3b*	Detected in Port wine	(b)	-	-	(de Freitas and Mateus 2004)
3c	Isolation from Port wine	(a), (b)	-	-	(Heier *et al.* 2002)
3c	Detection in wine	(a), (b)	Orange red (with other pigments) and stable to SO_2 bleaching, λ_{max} 500 nm	-	(Vivar-Quintana *et al.* 2002)
3c	Detection in wine	(a), (b)	λ_{max} 513 nm	-	(Monagas *et al.* 2003)
3c	Detection in wine	(a), (b)	-	-	(de Villiers *et al.* 2004)
3c*	Isolation from Port wine	(a), (b)	-	-	(Heier *et al.* 2002)
3d	From grapeskin extract and wine	(b)	-	-	(Hayasaka and Asenstorfer 2002)
3e	Detection in wine	(a), (b)	λ_{max} 499, 507, 512 nm	-	(Alcalde-Eon *et al.* 2004)
3e	Isolation from wine	(a), (b)	λ_{max} 495 nm	-	(Atanasova *et al.* 2002a)

APPENDIX 1.14: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside).

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
3e	From grapeskin extract and wine	(b)	-	-	(Hayasaka and Asenstorfer 2002)
3e	Detection in wine	(a), (b)	λ_{max} 503, 508 nm	-	(Monagas *et al.* 2003)
3e	Detected in Port wine	(a), (b), (c)	λ_{max} 511 nm	-	(de Freitas and Mateus 2004)
3e*	Detection in wine	(a), (b)	λ_{max} 506 nm	-	(Alcalde-Eon *et al.* 2004)
3f	Isolation from wine	(a), (b)	λ_{max} 495 nm	-	(Atanasova *et al.* 2002a)
3f	Detection in wine	(a), (b)	λ_{max} 514 nm	-	(Monagas *et al.* 2003)
3f	Detected in Port wine	(b)	-	-	(de Freitas and Mateus 2004)
3 – derived products	Detected in wine	(a)	-	-	(Monagas *et al.* 2005a)

APPENDIX 1.15: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside).

(b) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
4a – 4e	Isolation from Port wine	(a), (b)	-		(Mateus *et al.* 2002a)
4a	Detection in wine	(a), (b)	Stable to SO_2 bleaching	-	(Asenstorfer *et al.* 2001)
4a	From grapeskin extract and wine	(b)	-	-	(Hayasaka and Asenstorfer 2002)
4a	Isolation from wine	(a), (b), (d)	λ_{max} 505 nm, stable to SO_2 bleaching	-	(Vivar-Quintana *et al.* 2002)
4a	Synthesis	(a), (b), (c)	-	-	(Schwarz *et al.* 2003a)
4a	Detection in wine	(a), (b)	λ_{max} 512 nm	-	(Alcalde-Eon *et al.* 2004)
4a	Detection in wine	(a), (b)	λ_{max} 503 nm	0.3 – 1.1 mg/L	(de Villiers *et al.* 2004)
4a	Detected in Port wine	(b)	-	-	(de Freitas and Mateus 2004)
4a	Isolated from wine	(a), (b), (d)	λ_{max} 504 nm	24.9 – 77.6 mg/L	(Pozo-Bayón *et al.* 2004)
4a	From wine and grapes	(a), (b)			(Wang *et al.* 2004)
4a	Detection in wine	(a), (b), (d)	-	0.15 – 0.42 mg/L	(Schwarz *et al.* 2005)
4a*	From grapeskin extract and wine	(b)	-	-	(Hayasaka and Asenstorfer 2002)
4a*	Detection in wine	(a), (b)	λ_{max} 503, 507 nm	-	(Alcalde-Eon *et al.* 2004)
4a*	Isolated from wine	(a), (b), (d)	λ_{max} 503 nm	12.1 – 12.5 mg/L	(Pozo-Bayón *et al.* 2004)

APPENDIX 1.16: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside.

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
4b	Detection in wine	(a), (b)	λ_{max} 504 – 509 nm	-	(Monagas *et al.* 2003)
4b	From grapes and wine in model system	(a), (b)	-	-	(Wang *et al.* 2003b)
4b	Detection in wine	(a), (b)	λ_{max} ~ 510 nm	-	(de Villiers *et al.* 2004)
4b	Detected in Port wine	(b)	-	-	(de Freitas and Mateus 2004)
4b	Isolated from wine	(a), (b), (d)	-	15.2 – 15.5 mg/L	(Pozo-Bayón *et al.* 2004)
4b	Detection in wine	(a), (b), (d)	-	0.04 – 2.32 mg/L	(Schwarz *et al.* 2005)
4c	Detection in wine	(a), (b)	λ_{max} 504 nm	-	(Monagas *et al.* 2003)
4c	From grapes and wine in model system	(a), (b)	-	-	(Wang *et al.* 2003b)
4c	Detection in wine	(a), (b)	λ_{max} 506 nm	-	(Alcalde-Eon *et al.* 2004)
4c	Detected in Port wine	(b)	-	-	(de Freitas and Mateus 2004)
4c	Detection in wine	(a), (b)	-	-	(de Villiers *et al.* 2004)
4c	Isolated from wine	(a), (b), (d)	-	13.7 – 15.5 mg/L	(Pozo-Bayón *et al.* 2004)
4c	Detection in wine	(a), (b), (d)	-	0.12 – 0.84 mg/L	(Schwarz *et al.* 2005)
4c*	Detected in Port wine	(b)	-	-	(de Freitas and Mateus 2004)

APPENDIX 1.17: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside).

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
4g (aglycone)	Synthesis	(d)	λ_{max} 508 nm in pH ~ 1 → pH 5	-	(Amic´ et al. 2000)
4g	Detection in wine	(a), (b)	Orange red (with other pigments) and stable to SO_2 bleaching	-	(Vivar-Quintana et al. 2002)
4g	From grapeskin extract and wine	(b)	-	-	(Hayasaka and Asenstorfer 2002)
4h	From grapeskin extract and wine	(b)	-	-	(Hayasaka and Asenstorfer 2002)
4h	Synthesis	(a), (b), (c)	-	-	(Schwarz et al. 2003a)
4h	Isolation from wine	(a), (b), (c)	-	-	(Schwarz et al. 2003b)
4h	Synthesis in model wine	(b), (c), (d)	λ_{max} 508 pH 1 – 5 orange	-	(Schwarz and Winterhalter 2003a)
4h	From grapes and wine in model system	(a), (b)	-	-	(Wang et al. 2003b)
4h	Detection in wine	(a), (b)	-	-	(de Villiers et al. 2004)
4h	Isolation and formation in model wine	(b)	λ_{max} 510 nm	0.26 – 11.78 mg/L	(Schwarz et al. 2004)
4h	From wine and grapes	(a), (b)			(Wang 2004)

APPENDIX 1.18: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside).

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
4i	Detection in wine	(a), (b)	Stable to SO_2 bleaching	-	(Asenstorfer $et\,al.$ 2001)
4i	Detection in wine	(a), (b)	λ_{max} 504 nm	-	(Monagas $et\,al.$ 2003)
4i	Synthesis	(a), (b), (c)	-	-	(Schwarz $et\,al.$ 2003a)
4i	Detection in wine	(a), (b)	Bleached with $NaHSO_3$, λ_{max} 511 nm, orange pink	-	(Alcalde-Eon $et\,al.$ 2004)
4i	Detection in wine	(a), (b)	-	-	(de Villiers $et\,al.$ 2004)
4i	Isolated from wine	(a), (b), (d)	λ_{max} 508 nm	16.7 – 25.2 mg/L	(Pozo-Bayón $et\,al.$ 2004)
4i	Detection in wine	(a), (b), (d)	-	0.10 – 0.17 mg/L	(Schwarz $et\,al.$ 2005)
4i*	Detection in wine	(a), (b)	λ_{max} 510 nm	-	(Alcalde-Eon $et\,al.$ 2004)
4j	Synthesis	(a), (b), (c)	-	-	(Schwarz $et\,al.$ 2003a)
4j	Synthesis	(b), (c), (d)	λ_{max} 512 nm at pH 1.5, pH 3.6 and pH 3.6 + SO_2 bleaching $\varepsilon = 8300$ L cm^{-1} mol^{-1} at pH 1.5 and $\varepsilon = 6200$ L cm^{-1} mol^{-1} at pH 3.6	-	(Håkansson $et\,al.$ 2003)
4k	Detection in wine	(a), (b)	-	-	(de Villiers $et\,al.$ 2004)
4k	From wine and grapes	(a), (b)			(Wang $et\,al.$ 2004)
4k	Detection in wine	(a), (b), (d)	-	0.09 – 0.24 mg/L	(Schwarz $et\,al.$ 2005)

APPENDIX 1.19: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside).

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
4l	Detection in wine	(a), (b)	-	-	(de Villiers *et al.* 2004)
4l	Detection in wine	(a), (b), (d)	-	0.18 – 0.22 mg/L	(Schwarz *et al.* 2005)
4l	From wine and grapes	(a), (b)			(Wang *et al.* 2004)
4m	Detection in wine	(a), (b)	-	-	(de Villiers *et al.* 2004)
4m	Detection in wine	(a), (b), (d)	-	0.06 mg/L	(Schwarz *et al.* 2005)
4n	Detection in wine	(a), (b)	-	-	(de Villiers *et al.* 2004)
4o	Detection in wine	(a), (b)	-	-	(de Villiers *et al.* 2004)
4o	Synthesis	(b), (c), (d)	λ_{max} 506 nm at pH 1.5, pH 3.6 and pH 3.6 + SO_2 bleaching, $\varepsilon = 12200$ L cm^{-1} mol^{-1} at pH 1.5 and $\varepsilon = 10000$ L cm^{-1} mol^{-1} at pH 3.6	-	(Håkansson *et al.* 2003)
4o	Detection in wine	(a), (b)	λ_{max} 504 nm	-	(Monagas *et al.* 2003)
4o	Isolated from wine	(a), (b), (d)	λ_{max} 510 nm	22.4 – 44.5 mg/L	(Pozo-Bayón *et al.* 2004)
4p	From grapes and wine in model system	(a), (b)	-	-	(Wang *et al.* 2003b)
4p	Detection in wine	(a), (b)	-	-	(de Villiers *et al.* 2004)
4p	Detection in wine	(a), (b)	λ_{max} 512 nm	-	(Alcalde-Eon *et al.* 2004)

APPENDIX 1.20: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside).

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
4p*	Detection in wine	(a), (b)	λ_{max} 498, 508 nm	-	(Alcalde-Eon *et al.* 2004)
4q	Detection in wine	(a), (b)	-	-	(de Villiers *et al.* 2004)
4r	Detection in wine	(a), (b)	λ_{max} 512 nm	-	(Alcalde-Eon *et al.* 2004)
4r	Detection in wine	(a), (b)	-	-	(de Villiers *et al.* 2004)
4s	From grapes and wine in model system	(a), (b)	-	-	(Wang *et al.* 2003b)
4 – derived products	Detected in wine	(a)	-	-	(Monagas *et al.* 2005a)
2 + 3 + 4	Isolation from grape marc extracts	(b)	-	-	(Hayasaka and Asenstorfer 2002)

APPENDIX 1.21: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside).

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
5	Formation in model systems	(a), (b), (d)	Violet in pH 1 – 5, CIELab values and stable to SO_2 bleaching	-	(Escribano-Bailon *et al.* 2001)
5	Isolation in wine	(a), (b)	-	0 – 0.5 mg/L	(Revilla and González-SanJosé 2001)
5	Formation in model systems	(a), (b), (c)	Blueish powder	-	(Shoji *et al.* 2002)
5a	Formation in model systems	(a), (b)	Colourless	-	(Mirabel *et al.* 1999)
5a	Detection in wine	(a), (b)	Stable to SO_2 bleaching	-	(Asenstorfer *et al.* 2001)
5a	Isolation from Port wine	(a), (b), (c)	-	-	(Mateus *et al.* 2002b)
5a	Detection in wine	(a), (b), (c), (d)	λ_{max} 512 nm	-	(Mateus *et al.* 2003)
5a	From grapes and wine in model system	(a), (b)	-	-	(Wang *et al.* 2003b)
5a	Detection in wine	(a), (b)	λ_{max} 510 nm	-	(Alcalde-Eon *et al.* 2004)
5b	Detection in wine	(a), (b), (c), (d)	λ_{max} 503 nm	-	(Mateus *et al.* 2003)
5b	Detected in Port wine	(a), (b), (c)	λ_{max} 520 nm	-	(de Freitas and Mateus 2004)
5b	Detection in wine	(a), (b)	-	-	(de Villiers *et al.* 2004)

APPENDIX 1.22: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside).

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
5b*	Detection in wine	(a), (b)	-	-	(de Villiers *et al.* 2004)
5c	Detected in Port wine	(b)	-	-	(de Freitas and Mateus 2004)
5d	Detected in Port wine	(a), (b), (c)	λ_{max} 511 nm	-	(de Freitas and Mateus 2004)
5b, 5c and 5d (n=2)	Isolation from Port wine	(a), (b)	-	-	(Mateus *et al.* 2002b)
6a	Formation in model systems	(a)	Yellow in pH 2.75 – 4	-	(Timberlake and Bridle 1976)
6a	Isolation from grapes	(a), (d)	-	-	(Haslam 1980)
6a	Isolation in model (extracts)	(a), (b)	Yellow-orange	-	(Mirabel *et al.* 1999)
6a	Formation in model systems	(a), (b)	Yellow	-	(Malien-Aubert *et al.* 2002)
6a	From grapes and wine in model system	(a), (b)	-	-	(Wang *et al.* 2003b)
6a	Detection in wine	(a), (b)	λ_{max} 538 nm	-	(Alcalde-Eon *et al.* 2004)
6a*	Detection in wine	(a), (b)	λ_{max} 530, 533 nm	-	(Alcalde-Eon *et al.* 2004)
6b	Formation in model systems	(a), (b)	Red	-	(Malien-Aubert *et al.* 2002)
6b	Isolation from grapes	(a), (d)	-	-	(Haslam 1980)

APPENDIX 1.23: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside).

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
7	Formation in model systems	(a), (c)	Yellow pigment	-	(Escribano-Bailon *et al.* 1996)
7a	Synthesis in model wine	(a), (d)	λ_{max} 425 – 450 nm	-	(Rivas-Gonzalo *et al.* 1995)
7a	Formation in model system	(a), (d)	λ_{max} 440 – 460 nm	-	(Francia-Aricha *et al.* 1998)
7a	Detected in wine	(a), (b)	-	-	(Revilla *et al.* 1999)
7a	Detection in wine	(a), (b)	-	-	(Asenstorfer *et al.* 2001)
7a	Detection in wine	(a), (b), (c), (d)	λ_{max} 503 nm and λ_{max} 512 nm	-	(Mateus *et al.* 2002b)
7a	Synthesis in model wine	(a), (b)	-	-	(Pissarra *et al.* 2003)
7a	Detection in wine	(a), (b)	-	-	(de Villiers *et al.* 2004)
7a	From grapes	(a), (b)	-	-	(Mazzuca *et al.* 2004)
7b	Formation in model system	(a), (d)	λ_{max} 440 – 460 nm	-	(Francia-Aricha *et al.* 1998)
7b	Detected in wine	(a), (b)	-	-	(Revilla *et al.* 1999)
7b	Detection in wine	(a), (b)	-	-	(Asenstorfer *et al.* 2001)
7a + 7b	Formation in model systems	(a), (b)	-	-	(Malien-Aubert *et al.* 2002)
8a	Detection in wine	(a), (b)	-	-	(Heier *et al.* 2002)

APPENDIX 1.24: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside).

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurements.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
8a*	Detection in wine	(a), (b)	-	-	(Heier *et al.* 2002)
8c	Synthesis in model wine	(b), (c)	λ_{max} ~ 520 nm and is pH-dependent	-	(Salas *et al.* 2004)
9a	From grape extract	(a), (b), (d)	-	-	(Vidal *et al.* 2004a)
9a*	From grape extract	(a), (b), (d)	-	-	(Vidal *et al.* 2004a)
9b	From grape extract	(a), (b), (d)	-	-	(Vidal *et al.* 2004a)
9b*	From grape extract	(a), (b), (d)	-	-	(Vidal *et al.* 2004a)
10a	Synthesis in model wine	(a), (d)	λ_{max} 505 and 540 nm	-	(Rivas-Gonzalo *et al.* 1995)
10a	Formation in model system	(a), (b)	λ_{max} 511 nm	-	(Francia-Aricha *et al.* 1997)
10a	Formation in model system	(a), (b)	Shoulder at 450 nm, λ_{max} 545 nm	-	(Es-Safi *et al.* 1999)
10a	Formation in model systems	(a), (b)	Yellow-orange	-	(Mirabel *et al.* 1999)
10a	Formation in model system	(a), (b), (d)	λ_{max} 545 nm at pH 2.2	-	(Es-Safi *et al.* 2000)

APPENDIX 1.25: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside).

(b) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurements.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
10a	Formation in model system	(a), (b), (d)	λ_{max} 560 nm at pH 5.5 and λ_{max} 535 nm at pH 0.5 and stable to SO_2 bleaching, violet, $\varepsilon = 17100$ L cm^{-1} mol^{-1}	-	(Escribano-Bailon *et al.* 2001)
10a	Detection in wine	(a), (b)	-	-	(Asenstorfer *et al.* 2001)
10a	Isolation from wine	(a), (b)	λ_{max} 545 nm	-	(Atanasova *et al.* 2002a)
10a	Detection in wine	(a), (b)	-	16.45 – 21.49 mg/L (in total)	(Atanasova *et al.* 2002b)
10a	Synthesis in model wine	(a), (b)	λ_{max} 530 – 544 nm	-	(Es-Safi *et al.* 2002a)
10a	Isolation from grape marc extracts	(b)	-		(Hayasaka and Asenstorfer 2002)
10a	Isolation from wine	(a), (b)	-	-	(Heier *et al.* 2002)
10a	Isolation from Port wine	(a), (b), (c)	-	-	(Mateus *et al.* 2002b)
10a	Detection in wine	(a), (b)	λ_{max} 543 nm	-	(Monagas *et al.* 2003)
10a	Synthesis in model wine	(a), (b)	-	-	(Pissarra *et al.* 2003)
10a	From grape extract and synthesis	(b), (d)	λmax 544 nm	-	(Remy-Tanneau et al. 2003)

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APPENDIX 1.26: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside.

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
10a	From grapes and wine in model system	(a), (b)	-	-	(Wang et al. 2003b)
10a	Detected in Port wine	(b)	-	-	(de Freitas and Mateus 2004)
10a	Formation in model systems	(a), (b), (c), (d)	λmax 540 nm	44 – 125 mg	(Lee et al. 2004b)
10a	Synthesis in model wine	(a), (b), (c), (d)	λ_{max} 540 nm	-	(Pissarra *et al.* 2004)
10a	From wine and grapes	(a), (b)			(Wang *et al.* 2004)
10a	In model wine	(a), (b), (c)	-	~ 15 mg/L	(Pissarra *et al.* 2005)
10a	Isolation from grape skins	(a), (b)	λ_{max} 530 nm	-	(González-Paramás *et al.* 2006)
10a*	Synthesis in model wine	(a), (d)	λ_{max} 523 - 525 nm and λ_{max} 530 – 544 nm (violet coloration)	1.7 – 19.4 mg/L	(Dallas *et al.* 1996a)
10a*	Isolation from wine	(a), (b)	-	-	(Heier *et al.* 2002)
10a*	Isolation from grape skins	(a), (b)	λ_{max} 534 nm	-	(González-Paramás *et al.* 2006)
10b	Formation in model systems	(a)	Red-brown in pH 2.75 – 4	-	(Timberlake and Bridle 1976)
10b	Formation in model systems	(a), (b), (c)	Yellow pigment	-	(Escribano-Bailon *et al.* 1996)

APPENDIX 1.27: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside.

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
10b	Formation in model system	(a), (b)	λ_{max} 490 nm	-	(Francia-Aricha *et al.* 1997)
10b	Isolation in model (extracts)	(a), (b)	Yellow-orange	-	(Mirabel *et al.* 1999)
10b	In model wine	(a), (b), (c), (d)	λ_{max} 518 nm	-	(Atanasova *et al.* 2002b)
10b	Synthesis in model wine	(a), (b)	-	-	(Es-Safi *et al.* 2002a)
10b	Isolation from Port wine	(a), (b), (c)	-	-	(Mateus *et al.* 2002b)
10b	Detection in wine	(a), (b)	λ_{max} 508 nm	-	(Monagas *et al.* 2003)
10b	Synthesis in model wine	(a), (b)	-	-	(Pissarra *et al.* 2003)
10c	In model wine	(a), (b), (c), (d)	λ_{max} 450 and 528 nm	-	(Atanasova *et al.* 2002b)
10c	Synthesis in model wine	(a), (b)	λ_{max} 545 nm	-	(Es-Safi *et al.* 2002a)
10c*	Formation in model system	(a), (b), (d)	Shoulder at 450 nm, λ_{max} 535 nm, reddish and yellowish colour	-	(Es-Safi *et al.* 2002b)
10d	Synthesis in model wine	(a), (b)	λ_{max} 530 – 544 nm	-	(Es-Safi *et al.* 2002a)

APPENDIX 1.28: Occurrence, isolation, colour properties and concentration of anthocyanin-derived pigments (* refers to homologue pigments formed from anthocyanins other than malvidin 3-glucoside.

(a) by HPLC/UV-Vis spectroscopy; (b) by ESI-MS or FAB-MS; (c) by NMR spectroscopy; (d) by UV-Vis or CIELab measurement.

Pigment	Study	Structural elucidation	Colour properties	Quantity	Reference
10d*	Formation in model system	(a), (b), (d)	Shoulder at 450 nm, λ_{max} 535 nm, reddish and yellowish colour	-	(Es-Safi et al. 2002b)
10e	From grapes and wine in model system	(a), (b)	-	-	(Wang et al. 2003b)
10f	Synthesis in model wine	(a), (b)	-	-	(Pissarra et al. 2003)
10f	Synthesis in model wine	(a), (b), (c), (d)	λ_{max} 540 nm	-	(Pissarra et al. 2004)
10b (diglc)	Formation in model systems	(a)	Yellow in pH 2.75 – 4	-	(Timberlake and Bridle 1976)
10a + 10b	Isolation from Port wine	(a), (b), (c)	-		(Mateus et al. 2002b)
10a + 10b	Formation in model systems	(a), (c)	Yellow at pH 2 and colourless pH 2-4	-	(Escribano-Bailon et al. 1996)
10a + 10b	Isolation from wine	(a), (b)	-	1.69 – 2.42 mg/L (in total)	(Atanasova et al. 2002b)
10i	In model wine	(a), (b), (c)	-	~ 7 mg/L	(Pissarra et al. 2005)