Influence of oxygen addition on the phenolic composition of red wine

by

Lorraine Geldenhuys

Thesis presented in partial fulfilment of the requirements for the degree of
Master of Agricultural Sciences

at
Stellenbosch University
Department of Viticulture and Oenology, Faculty of AgriSciences

Supervisor: Dr WJ du Toit
Co-supervisor: Dr A Oberholster

December 2009

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 5 June 2009

Copyright © 2009 Stellenbosch University
All rights reserved

Summary

Tannins and colour components in red wine are important quality parameters. These factors can be manipulated in the vineyard by grape growing techniques or in the cellar by different wine-making practices. Grape seeds make a significant contribution to tannin concentration in wine when compared to those from the skins and pulp. Tannins contribute to the ageing potential, organoleptic properties and stabilisation of red wine colour. The colour of a red wine is also influenced by malolactic fermentation, the biological process that transforms malic acid into lactic acid which normally leads to an increase in pH. The subsequent change in pH alters the anthocyanin equilibrium, the primary colour components in red wine. Oxygen contributes to the polymerisation of anthocyanins over time to form more stable pigments that are less sensitive to pH fluctuations and sulphur dioxide bleaching. Limited research has been done on the use of oxygen after alcoholic fermentation and the impact it has on the phenolic composition of red wines. Similarly, only a few studies have examined the impact of either the addition or removal of seeds to the phenolic composition of a red wine in combination with oxygen addition. Additionally, little published data seems to exist on the effect of different pHs on red wine's phenolic and colour development after oxygen addition.

In our results we have shown that it is possible to stabilise wine colour by adding supplementary seeds before alcoholic fermentation. This led to an increase in colour intensity in certain cases. Some red wines produced without seeds had significantly lower colour intensities. This clearly suggests that catechins and proanthocyanidins are extracted from seeds and contribute to wine colour as they combine with other pigments to stabilise wine colour. Spectrophotometric and HPLC analyses have shown that the total phenolic content increased with seed concentration. However, we have observed that a wine may possibly become saturated with phenols when supplementary seeds are added. Anthocyanin concentrations often decreased when oxygen was added, while polymeric phenols and polymeric pigments sometimes increased.

When applying different oxygen dosages to a red wine on commercial scale with micro-oxygenation, it was found that monomeric anthocyanins decreased as more oxygen was added and this decrease in anthocyanins led to the formation of stable polymeric pigments. This was reflected in the significant increase in colour intensity for the wines receiving oxygen. Small differences were detected in the total phenol and tannin concentration for the control and oxygenated wines. However, some of these phenolic and colour differences disappeared during subsequent ageing of the wine.

When making wines of different initial pHs, we observed that the colour density decreased as the pH increased. The application of oxygen reduced the decrease in colour during MLF, especially at a lower pH. The addition of oxygen did not result in significantly different polymeric pigment concentrations in the various pH treatments, although the results could have been different if the wines were aged. However, pH differences in the range between 3.4 and 4.0 did not significantly influence the phenol composition of the wines under our conditions. This study led to a better understanding on the effect of oxygen additions under different conditions on red wine's phenolic and colour composition.

Opsomming

Tanniene en kleur komponente teenwoordig in rooiwyn is belangrike kwaliteit parameters. Hierdie faktore wat wynkwaliteit beïnvloed kan gemanipuleer word in die wingerd deur verskeie verbouingstegnieke toe te pas en in die kelder deur die toepassing van verskillende wynbereidingsmetodes. Die bydrae van sade tot die finale tannienkonsentrasie in rooiwyn is groot in vergelyking met dié van die pulp en doppe. Tanniene dra by tot die verouderings-potensiaal, organoleptiese eienskappe en die stabilisasie van die wynkleur. Die kleur van ‘n rooi wyn word ook beïnvloed deur appelmelksuurgisting (AMG), die biologiese proses wat appelsuur omskakel na melksuur en ‘n gevolglike toename in die pH van die wyn veroorsaak. Hierdie verandering in die pH van die wyn beïnvloed die antosianien ewewig, die primêre kleur komponente teenwoordig in rooiwyn. Suurstof dra by tot die polimerisasie van antosianiene oor tyd om meer stabiele kleur pigmente te vorm met ‘n hoër kleurintensiteit wat minder sensitief is teenoor pH veranderinge en die bleikingseffek van swaweldioksied. Beperkte navorsing is gedoen op die gebruik van suurstof na alkoholiese gisting en die impak daarvan op die fenoliese samestelling van ‘n rooiwyn. Slegs ‘n paar studies het die invloed van die verwydering of byvoeging van sade in kombinasie met suurstoftoediening op ‘n rooiwyn se fenoliese samestelling ondersoek. Dit wil voorkom of beperkte gepubliseerde data beskikbaar is oor die effek wat verskillende pH’s het op rooi wyn se fenoliese en kleurontwikkeling na suurstof byvoeging.

Ons resultate het aangedui dat dit wel moontlik is om in sekere gevalle die kleur van ‘n rooiwyn te stabiliseer deur addisionele sade by te voeg voor alkoholiese fermentasie. Hierdie byvoeging het ‘n toename in kleurintensiteit tot gevolg gehad. Sekere wyne wat gemaak is sonder sade het ‘n kenmerkend laer kleur intensiteit gehad. Hierdie bevinding is ‘n duidelike bewys dat katesjiene en prosianidiene geëkstraheer word vanuit die sade en bydra tot wynkleur deurdat hulle met ander pigmente verbind om die kleur sodoende te stabiliseer. Spektrofotometriese en hoë druk vloeistof chromatografie (HDVC) analises het gewys dat die totale fenoliese konsentrasie neem toe met ‘n toename in saad konsentrasie. Daar is egter waargeneem dat ‘n wyn moontlik versadig kan raak met fenole wanneer addisionele sade bygevoeg word. Antosianien konsentrasies het meestal afgeneem wanneer suurstof bygevoeg is, maar polimeriese fenole en polimeriese pigmente het partykeer toegeneem.

Met die toediening van verskillende suurstof dosisse tot ‘n rooiwyn op kommersiële skaal het ons bevind dat monomeriese antosianiene afneem wanneer meer suurstof bygevoeg word. Hierdie afname in antosianiene het egter gelei tot die vorming van stabiele polimeriese pigmente. Dié bevinding was gereflekteer in die toename in kleurintensiteit van wyne wat met suurstof behandel is. Klein verskille was waargeneem vir die totale fenol en tannien konsentrasies tussen die kontrole en wyne behandel met suurstof. Sekere van hierdie fenoliese kleur verskille het egter afgeneem tydens die daaropvolgende veroudering van die wyne.

Wyne wat gemaak is met verskillende aanvanklike pH’s se kleurintensiteit neem af soos die pH toeneem. Die toediening van suurstof het die kleurverlies tydens AMG verminder, veral by ‘n laer pH. Die toediening van suurstof het nie verskillende polimeriese pigment konsentrasies by verskillende pH’s veroorsaak nie, maar ‘n verskil kon moontlik waargeneem word indien die wyne verouder was. pH verskille tussen 3.4 en 4.0 het egter nie die fenoliese samestelling van die wyne onder ons omstandighede beduidend beïnvloed nie. Hierdie studie het gelei tot meer kennis oor die effek van suurstoftoedienings onder verskillende kondisies op rooiwyn se fenoliese en kleursamestelling.

This thesis is dedicated to my partner, mother and grandparents for their support

Biographical sketch

Lorraine Geldenhuys was born on 7 March 1984 in Alberton and matriculated at High School Gimnasium in Paarl in 2002. Lorraine obtained a Bachelors degree in Agricultural Science (Viticulture and Oenology) in 2006 at Stellenbosch University. In 2007 she enrolled for a Masters degree in Oenology at the same University.

Acknowledgements

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

- WJ DU TOIT and A OBERHOLSTER, my two supervisors for their guidance and support;
- WINETECH, THRIP and the NRF for financial support;
- THE STAFF of the Department of Viticulture and Oenology for their assistance;
- MY FRIENDS, for their help and support;
- THE MULLERS, for their interest, love and encouragement;
- MY MOTHER, Rachelle for her encouragement and support;
- MY GRANDPARENTS, Jerome and Lorraine van Staden, for their encouragement, interest, love and support;
- MY PARTNER and best friend, CM, without you I could never do this;
- THE ALMIGHTY, for teaching me more than I initially intended.

Preface

This thesis is presented as a compilation of 7 chapters.

Chapter 1 **General Introduction and project aims**

Chapter 2 **Literature review**
The influence of oxygen addition on red wines

Chapter 3 **Research results**
The effect of different oxygen dosages on the phenolic composition of wines made with different grape seed concentrations

Chapter 4 **Research results**
The influence of adding different oxygen dosages before malolactic fermentation on the colour and phenolic composition of Pinotage red wine

Chapter 5 **Research results**
The influence of oxygen additions on the composition of Cabernet Sauvignon wine with different pHs

Chapter 6 **General discussion and conclusions**

Chapter 7 **Addendum**

Contents

Chapter 1. Introduction and project aims 1

1.1 Introduction 2
1.2 Project aims 3
1.3 Literature Cited 4

Chapter 2. The influence of oxygen addition on red wines 5

2.1 Introduction 6
2.2 Phenols in wine 6
2.2.1 Anthocyanins 7
2.2.2 Tannins 8
2.2.2.1 Grape seed concentrations 9
2.2.3 Polymerisation reactions 10
2.3 Oxygen in wine 14
2.3.1 Must hyperoxidation 15
2.3.2 Micro-oxygenation 16
2.4 Ageing 18
2.5 Phenol analysis 18
2.5.1 Spectrometry methods 18
2.5.11 Colour analyses 18
2.5.1.2 Total phenol analyses 19
2.5.1.2.1 Folin-Ciocalteu 19
2.5.1.2.2 Total phenols as measured at 280 nm 19
2.5.1.3 Monomeric flavanols 19
2.5.1.4 Tannin assays 20
2.5.1.4.1 Protein precipitation assay 20
2.5.2 Chromatography methods 20
2.5.2.1 High Performance Liquid Chromatography (HPLC) 20
2.6 Conclusion 20
2.7 Literature cited 21

Chapter 3. The effect of different oxygen dosages on the phenolic composition of wines made with different grape seed concentrations 26

3.1 Introduction 27
3.2 Materials and methods 28
3.2.1 Preparation of wine 28
3.2.2 Malolactic fermentation 29
3.2.3 Ageing and bottling 29
3.2.4 Wine sampling 29
3.2.5 Spectrophotometric analyses 30

3.2.5.1 Colour intensity, optical densities and hue 30
3.2.5.2 Total anthocyanins 30
3.2.5.3 Total phenols and total red colour 30
3.2.5.4 DMAC index 30
3.2.5.5 Folin-Ciocalteu 31
3.2.5.6 Tannins 31
3.2.6 HPLC analysis 33
3.2.7 Statistical analysis 35
3.3 Results and discussion 35
3.3.1 Colour intensity and hue 35
3.3.2 Anthocyanin and polymeric pigment concentrations 36
3.3.3 Monomeric and dimeric flavanols 41
3.3.4 Total phenols 43
3.3.5 Polymeric phenol concentration 46
3.3.6 Tannins 46
3.3.7 Flavonol concentrations 48
3.4 Conclusions 50
3.5 Acknowledgements 50
3.6 References 50

Chapter 4. The influence of adding different oxygen dosages before malolactic fermentation on the colour and phenolic composition of Pinotage red wine 54

4.1 Introduction 55
4.2 Material and methods 55
4.2.1 Preparation of wine 55
4.2.2 Malolactic fermentation 56
4.2.3 Ageing and bottling 56
4.2.4 Wine sampling 56
4.2.5 Spectrophotometric analyses 56
4.2.6 Statistical analysis 56
4.3 Results and discussion 57
4.3.1 Position in tank 58
4.3.2 Colour intensity, optical density and hue 58
4.3.3 Total anthocyanins 60
4.3.4 Monomeric flavanols 63
4.3.5 Total phenols 64
4.3.6 Tannins 65
4.3.7 Polymeric phenols 66
4.3.8 Flavonols 67
4.4 Conclusion 67
4.5 Acknowledgements 68
4.6 References 68

Chapter 5. The influence of oxygen additions on the composition of Cabernet Sauvignon wine with different pHs **71**

5.1 Introduction 72
5.2 Materials and methods 73
5.2.1 Preparation of wine 73
5.2.2. Malolactic fermentation 73
5.2.3 Wine sampling 73
5.2.4 Spectrophotometric analyses 73
5.2.5 Statistical analysis 74
5.3 Results and discussion 74
5.3.1 Colour intensity, optical density and hue 74
5.3.2 Total red pigments 76
5.3.3 Total anthocyanins 76
5.3.4 Monomeric and dimeric flavanols 78
5.3.5 Total phenols 78
5.3.6 Polymeric phenols 79
5.3.7 Hydroxycinnamic acids 80
5.3.8 Flavonols 80
5.5 Conclusion 80
5.6 Acknowledgements 81
5.7 References 81

Chapter 6. General discussion and conclusion **83**

6.1 Concluding remarks and other perspectives 84
6.2 Literature cited 86

Chapter 7. Addendum **87**

Chapter 1

Introduction and project aims

Chapter 1: General introduction and project aims

1.1 Introduction

The first wine in South Africa was produced on a Sunday by the first commander of the Cape of Good Hope, Jan van Riebeeck on February 2, 1659. That day he wrote in his diary: “Today, praise be to God, wine was made for the first time from Cape grapes.” Three centuries later this date is again very significant in the history of South Africa when the president, F.W. de Klerk, announced the imminent release of Nelson Mandela after 27 years of incarceration in 1990 (Hughes and Hands, 1997). Over the years the development of wine production in South Africa evolved to such an extent that it is currently one of the biggest role players in the world wide wine industry. Since the establishment of the KWV South Africa (Pty) Ltd in 1918; the wine industry has seen a revolution in the production of wines. With increased consumption and consumer demand, the industry was forced to initiate greater scientific development through institutions like the University of Stellenbosch and Elsenburg Agricultural College. Competition between wine producers has led to the temptation to produce cheaper and larger volumes wine at the expense of quality. Fierce competition for market share has further increased innovation within the wine industry with some producers diverging from the somewhat traditional way of making wine in order to produce a different and unique product.

The making of red wine can be considered somewhat of an art in comparison with the making of white wines where results are more predictable. The reason for this is the composition and quality of red wine depends on a greater number of variables in the winemaking process than whites (Rankine, 2006). In general, the consumption of red wine has more health benefits than white wine due to the presence of more phenolic compounds that scavenges free radicals. These phenols are what separate white from red wines and are responsible for the colour, astringency and possible bitterness, oxidative characteristics and ageing potential of red wine (Joslyn and Goldstein., 1965; Robichaud *et al*., 1990; Gawel., 1998). Phenols, but more specifically tannins, can be extracted from different sources throughout the production of red wines. The greatest extraction happens during fermentation, with the skins contributing the most during the early stages of fermentation, but as the temperature and alcohol content increases the seeds contribute to a greater extent (Souquet *et al*., 1996; Prier *et al*., 1994, Adams and Harbertson, 1999). Grape seeds are richer in phenols (condensed tannins) than skins or pulp, in both red and white grapes and these concentrations increase with an in increase in grape seed concentration or the length of maceration (Lea *et al*., 1979; Canals *et al*., 2008). Seed tannins were found to be more astringent and bitter than tannins extracted from the skins (Meyer and Hernandez*l*., 1970; Kovac *et al*., 1992; Kovac *et al*., 1995; Gawel, 1998; Peyrot des Gachons and Kennedy, 2003).

The presence of tannins in red wine contributes to the stabilisation of wine colour over time as these compounds bind with anthocyanins to form red pigments in red wine. The colour of red wine also is greatly influenced by another important winemaking process, malolactic fermentation. This natural or induced biological process causes microbiological stabilisation, a reduction in malic acid and a subsequent increase in the pH (Brouillard and Delaporte, 1977). The change in pH causes a shift in the anthocyanin equilibrium, the molecules responsible for the colour of a red wine, producing more anthocyanins in the colourless form (Bousbouras and Kunkee., 1971).

Traditionally wine has been protected from oxygen for various reasons, but recent research showed that the controlled addition of oxygen early during the winemaking process can impart

benefits to the wine (Perez-Magarino *et al*., 2007). Oxygen plays a crucial role in some of the chemical reactions introducing colour stability and a reduction in the astringency of red wine (Castellari *et al*., 2000). The mouth feel characteristics, including astringency, can be changed by controlled aeration of the must or wine, as oxygen can be seen as the catalyst between the chemical transformation of these compounds to enhance quality and flavour (Gawel, 1998; McCord, 2003).

In this thesis our main aim was to evaluate the effect of oxygen addition soon after alcoholic fermentation on the colour and phenolic composition of red wines. The general effect of oxygen on red wine is known, but the effect that certain other parameters, such as additional tannins and changes in pH, have on this is not well understood. Micro-oxygenation is a fairly new technique and hence there is limited literature available on this technique. However, a few authors have studied the effect of micro-oxygenation; many applying the technique only later during the winemaking process and/or inducing only small amounts (Llaudy *et al*., 2006; Cano-Lopez *et al*., 2006; Cano-Lopez *et al*., 2008). Therefore, we have applied oxygen early (just after alcoholic fermentation) during the winemaking process and in varying concentrations to determine what effects it will have on the colour and phenolic composition of the subjected wines. Since malolactic fermentation is a process that involves all/most commercial wines, we have found it necessary to include malolactic fermentation as a winemaking process in our experiments in order for the results to be more representative and understandable for the industry. Also, oxygen plays a significant role in the interactions with tannins and therefore we have investigated not only the phenolic contribution of seeds to wine, but what effect the addition of oxygen has on the phenolic composition of wines made with different amounts of seeds. Very limited research is available on the effect of pH on the oxidation of phenolic composition of red wines. Colour components, tannins and hydroxycinnamic acids could be very pH dependent and greatly influenced by oxygen and therefore part of this study focussed on how the colour and phenolic composition of a red wine is influenced by oxygen addition after alcoholic fermentation at different pHs.

This research forms part of a larger research program conducted on oxygen, phenolic compounds and their evolution during ageing at the Department of Viticulture and Oenology, Stellenbosch University.

1.2 Project Aims

The specific aims of the study were as follows:

a) to determine the effect of different oxygen dosages on the phenolic and colour composition of red wines made with different grape seed concentrations;
b) to determine the influence of adding different oxygen dosages before malolactic fermentation on the colour and phenolic composition of Pinotage red wine;
c) to determine the influence of oxygen additions on the colour and phenolic composition of Cabernet Sauvignon wine with different pHs.

1.3 Literature Cited

Adams, D.O. and Harbertson, J.F. (1999). Use of alkaline phosphatase for the analysis of tannins in grapes and red wines. *American Journal of Enology and Viticulture.* **50**, 247-252.

Bousbouras, G.E. and Kunkee, R.E. (1971). Effect of pH on malolactic fermentation in wine. *American Journal of Enology and Viticulture*. **22**, 121-126.

Brouillard, R. and Delaporte, B. (1977). Chemistry of anthocyanin pigments. 2: kinetic and thermodynamic study of proton transfer, hydration and tautomeric reactions of malvidin-3-glucoside. *Journal of American Chemical Society*. **99**, 8461-8467.

Canals, R., Del Carmen-Llaudy, M., Canals, J.M. and Zamora, F. (2008). Influence of the elimination of seeds on the colour, phenolic composition and astringency of red wine. *European Food and Research Technology*. **226**, 1183-1190.

Cano-Lopez, M., Pardo-Minguez, F., Schmauch, G., Saucier, C., Teissedre, P.L., Lopez-Roca, J.M. and Gomez-Plaza, E. (2008). Effect of micro-oxygenation on color and anthocyanin-related compounds of wines with different phenolic contents. *Journal of Agicultural and Food Chemistry*. **56**, 5932-5941.

Cano-Lopez, M., Pardo-Minguez, F., Lopez-Roca, J.M. and Gomez-Plaza, E. (2006). Effect of microoxygenation on anthocyanin and derived pigment content and chromatic characteristics of red wines. *American Journal of Enology and Viticulture*. **57**, 325-331.

Castellari, M., Matricardi, L., Arfelli, G., Galassi, S. and Amati, A. (2000). Level of single bioactive phenolics in red wine as a function of the oxygen supplied during storage. *Food Chemistry*. **69**, 61-67.

Gawel, R. (1998). Red wine astringency: a review. *Australian Journal of Grape and Wine Research*. **4**, 74-95.

Hughes, D. and Hands, P. (1997). Wines and brandies of the cape of good hope. Stephan Phillps Ltd. Somerset-West, South Africa.

Joslyn, M.A. and Goldstein, J.L. (1965). Astringency of fruits and fruit products in relation to phenolic content. *Wallerstein Laboratories Communications*. **28**, 143-160.

Kovac, V.E., Alonso, E., Bourzeix, M. and Revilla, E. (1992). Effect of several enological practices on the content of catechins and proanthocyanidins of red wines. *Journal of Agricultural and Food Chemistry*. **40**, 1953-1957.

Kovac, V.E., Alonso, E. and Revilla, E. (1995). The effect of adding supplementary quantities of seeds during fermentation on the phenolic composition of wines. *American Journal of Enology and Viticulture*. **46**, 363-367.

Lea, A.G.H., Bridle, P., Timberlake, C.F. and Singleton, V.L. (1979). The procyanidins of white grapes and wines. *American Journal of Enology and Viticulture*. **30**, 289-300.

Llaudy. M., Canals, R., Gonzales-Manzano, S., Canals J.M., Santos-Buelga, C. and Zamora, F. (2006). Influence of micro-oxygenation treatment before oak ageing on phenolic compounds composition, astringency, and color of red wine. *Journal of Agricultural and Food Chemistry*. **54**, 4246-4252.

McCord, J. (2003). Application of toasted oak and micro-oxygenation to ageing of Cabernet Sauvignon wines. *Australian and New Zealand Grapegrower and Winemaker*. July: 43-53.

Meyer, J. and Hernandez, R. (1970). Seed tannin extraction in Cabernet Sauvignon. *American Journal of Enology and Viticulture*. **21**, 184-188.

Perez-Magarino, S., Sanchez-Iglesias, M., Ortega-Heras, M., Gonzales-Huerta, C. and Gonzales-Sanjose, M.L. (2007). Colour stabilisation of red wines by mircooxygenation treatment before malolactic fermentation. *Food Chemistry*. **101**, 881-893.

Peyrot Des Gachons, C. and Kennedy, J.A. (2003). Direct method for determining seed and skin proanthocyanidin extraction into red wine. *Journal of Agricultural and Food Chemistry*. **51**, 5877-5881.

Prieur, C., Rigaud, J., Cheynier, V. and Moutounet, M. (1994). Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry*. **36**, 781-784.

Rankine, B. (2006). Making good wine: a manual of winemaking practice for Australia and New Zealand. Pan Macmillan Pty Ltd. p 50-53.

Robichaud, J.L. and Noble, A.C. (1990). Astringency and bitterness of selected phenolics in wine. *Journal of the Science Food and Agriculture*. **53**, 343-353.

Souquet, J.M., Cheynier, V., Brossaud, F. and Moutounet, M. (1996). Polymeric proanthocyanidins from grape skins. *Phytochemistry*. **43**, 509-512.

Chapter 2

Literature review

The influence of oxygen addition on red wines

Chapter 2: The influence of oxygen on red wines

2.1 Introduction

Phenolic compounds in general have been studied intensively and their importance has long since been recognised in fruit, particularly in grapes (Joslyn and Goldstein, 1965). These compounds, specifically the anthocyanins, are what separate white from red wines. Phenols, and more specifically tannins, are known for their ability to oxidise and produce more stable compounds, their organoleptic properties and their effects on colour in wines. The colour of a red wine is greatly influenced by malolactic fermentation when a pH change causes a shift in the equilibrium of the anthocyanins to the colourless form (Brouillard and Delaporte, 1977). Oxygen contributes to the polymerisation of anthocyanins to form more stable pigments with a higher colour density that is less sensitive to pH fluctuations and SO_2 bleaching (Fell *et al*., 2007).

A range of analytical techniques are available to determine the total phenolic composition and tannin concentration in wines.

This review will give a short overview of the different phenols present in wine, especially those originating from grape seeds. It will discuss the influence of oxygen on polymerisation reactions in red wine. Different colour and phenolic analyses will be discussed as well as determining the tannin concentration in wine.

2.2 Phenols in wine

Phenols, the major substances contributing to colour, astringency and organoleptic properties in wine, can be divided into flavonoids and non-flavonoids (Nagel and Wulf, 1979; Robichaud and Noble, 1990). The latter is subdivided into benzoic and cinnamic acid derivatives with their concentration normally being much higher in red (100-200 mg/L) than white wines (10-20 mg/L) (Ribereau-Gayon *et al*., 2006). The cinnamic acids mainly occur esterified to tartaric acid and are highly oxidisable compounds. Measuring the loss of cinnamic acid concentration in wine could be an indication of how much a wine was exposed to oxygen (Cheynier *et al*., 1989a, 1989b; Cilliers and Singleton, 1989).

The flavonoids are more complex compounds and constitute most of the phenols present in a red wine. They can be separated into different groups depending on their structure. The first group, the flavonols, is mainly found in the skin, and can be identified at 360 nm (maximum absorbance) when analysed by HPLC (Peng *et al*., 2002). Quercetin and myricetin are among the most important compounds due to their involvement with oxygen (Price *et al*., 1995). The second group, flavan-3-ols (Fig. 2.1), consists mainly of (+)-catechin and (-)-epicatechin. An additional -OH group on the B ring leads to the formation of (+)-gallocatechin and (-)-epigallocatechin, whereas catechin-3-O-gallate or epicatechin-3-O-gallate is formed through the acylation of gallic acid on the C ring. A third group, flavan-3,4-diols, identified by an additional -OH group on the C ring at position 4 can be involved in polymerisation reactions together with flavanols to form condensed tannins. These compounds have a maximum absorbance at 280 nm. The last main group of flavonoids are the anthocyanins, which consist of different forms depending on pH, and are primarily responsible for the colour in red wine.

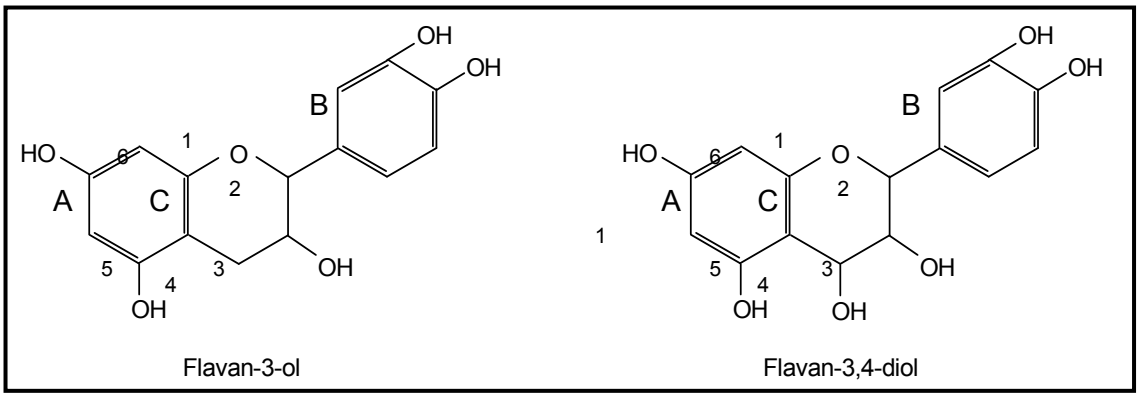


Figure 2.1: Basic structure of a flavan-3-ol and flavan-3,4-diol.

2.2.1 Anthocyanins

The colour of red grapes and wine plays an important part in its quality. This quality parameter can easily be measured from the vineyard through the winemaking and ageing processes (Iland, 1987; Gòmez-Cordovés *et al*., 1995; Kennedy *et al*., 2001). The red colour in wine is due to the extraction of anthocyanin pigments from the grape skins during fermentation, although anthocyanins can occur in the flesh of certain non *Vitis vinifera* species ('teinturier' grape varieties) (Ribereau-Gayon *et al*., 2006).

The anthocyanin equilibrium can be shifted depending on various factors, primarily the pH, SO_2 concentration and age of the wine (Fig. 2.2). Anthocyanins in the flavylium form have a positive (+) charge on the C-ring which is responsible for the colour reactivity of the pigment. The latter absorbs green light, and therefore transmits red light, with a maximum absorption at 520 nm.

Five different anthocyanin forms are found in wine: flavylium ion (red), carbinol base (colourless), chalcones (yellow), quinoidal base (violet) and the flavene sulphonase form (colourless). More than 50% of anthocyanins are in the flavylium ion form at a pH lower than 2.5 and more than 50% in the colourless form at pH higher than 2.5. Thus, given the normal pH range of red wines (pH 3-4), only 25% of the total anthocyanins are normally in the red form in a young wine (Brouillard and Delaporte, 1977; Glories, 1984a).

The colour of a young red wine is mainly due to monomeric anthocyanins. As wine ages, more colour is due to stable polymeric pigments (the result of polymerisation reactions) and copigmentation associations that are more resistant to pH fluctuations, sulphur dioxide bleaching and increases in alcohol concentrations. Romero-Cascales *et al.* (2005) found the anthocyanin concentration in wine after alcoholic fermentation varied between 225 and 361 mg/L in five different red cultivars. However, Moreno-Arribas *et al.* (2008) found higher concentrations in Tempranillo wines after alcoholic fermentation (409 mg/L), when using the same method for determining anthocyanins (HPLC). The authors showed wines stored in barrels have a lower anthocyanin concentration after malolactic fermentation (400 mg/L) than those kept in stainless steel tanks (590 mg/L). During barrel maturation small amounts of oxygen diffuse into the wine from the wood and the bung hole, causing the monomeric anthocyanins to decrease as they are involved in oxidation and condensation reactions that are favoured by the presence of oxygen (Morena-Arribas *et al*., 2008). The authors believe another possible explanation for these compounds being reduced during the ageing in barrels is due to their adsorption to yeast walls, as previously described during alcoholic fermentation when making red wine (Morata *et al*., 2003).

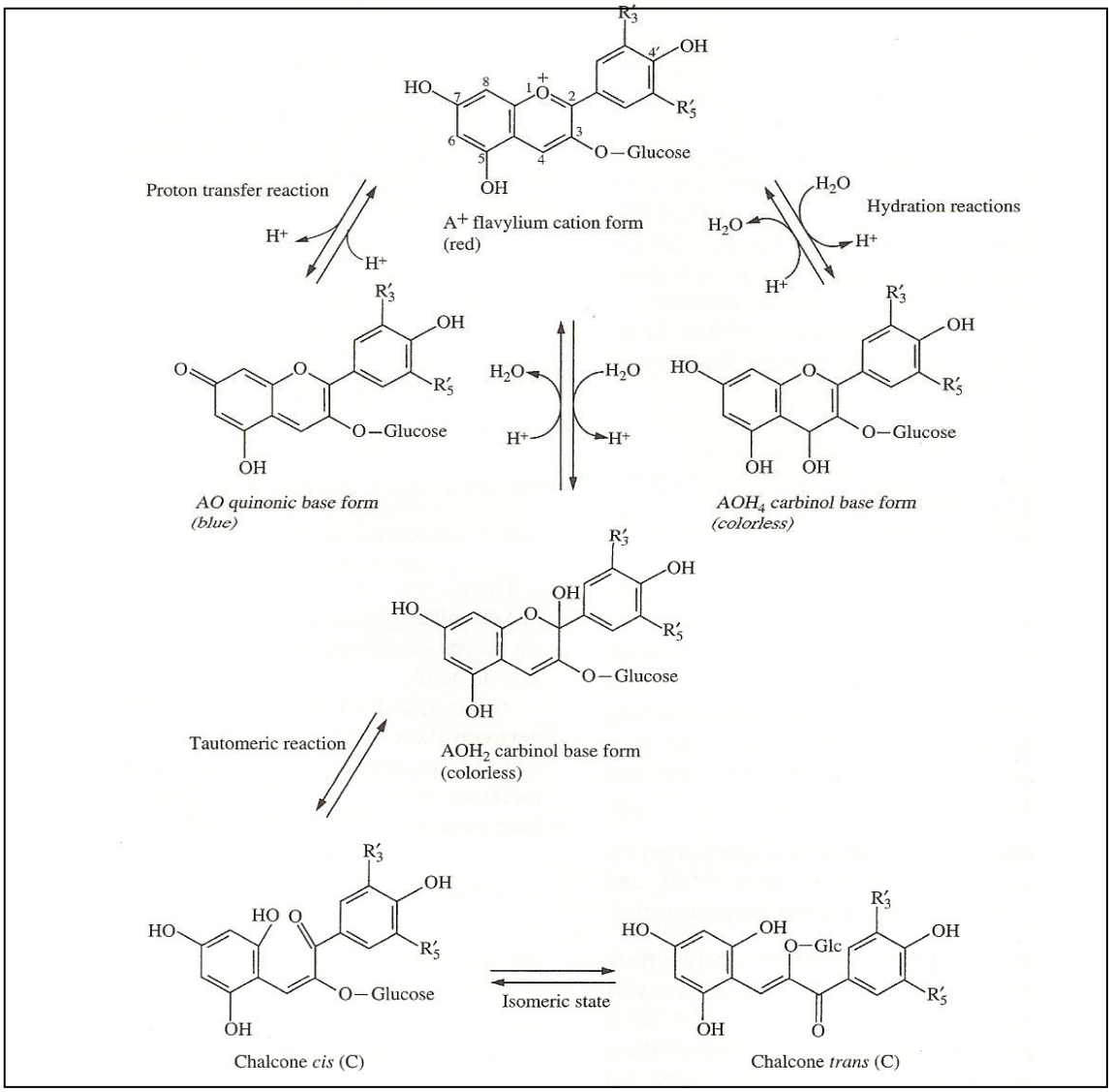


Figure 2.2: Anthocyanin equilibria illustrating the different forms in wine as affected by pH (Brouillard *et al*., 1978).

Copigmentation associations and polymerisation reactions stabilise wine colour (Boulton, 2001) and the latter is favoured by the presence of oxygen. Copigmentation is the term used to describe associations between pigments and other, usually non-coloured, phenol molecules in solution known as copigments or cofactors. The latter includes phenolic acids, flavan-3-ols and in particular, flavonols (Brouillard and Massa, 1989). Copigmentation accounts for between 30 and 50% of the colour of a young red wine and results in a shift of 5 to 20 nm of the maximum absorbance (hyperchromic shift), causing a blue-purple tone (Scheffieldt *et al*., 1978; Boulton, 2001). Anthocyanins involved in copigmentation are subjected to bleaching by sulphur dioxide (Levengood; 1996) and may be the first step towards the formation of stable polymeric pigments (Liao *et al*., 1992).

2.2.2 Tannins

Tannins play an important role in the organoleptic properties of wine and greatly influence wine quality (Glories 1988; Harbertson *et al*; 2002). Their concentrations in wine can vary greatly depending on wine style and winemaking techniques (Sacchi *et al*., 2005). Tannin is a broad term used in wine literature that includes polyphenolic compounds (polymeric phenols) of

flavan-3-ol monomers and oligomers (proanthocyanidins) and are mainly characterised by their ability to precipitate proteins. Tannins associated with enology can be broadly divided into hydrolysable and condensed tannins. Hydrolysable tannins originate from oak wood and consist of polygalloyl esters of glucose such as gallotannins and ellagitannins that release gallic acid after acid hydrolysis. These tannins do not occur naturally in the grapes, but are legally authorised to be added as wine tannins to a red wine or can be extracted from oak wood during barrel ageing. The term proanthocyanidins is used interchangeably with condensed tannins although proanthocyanidins should refer more to the oligomeric flavan-3-ols (<10 units) and condensed tannins to the larger polymers (>10 units). Proanthocyanidins can be subdivided into procyanidins and prodelphinidins. Procyanidins replace the previously used term 'leucocyanidin' and consist only of (+)-catechin or (-)-epicatechin units, while prodelphinidins also contains (+)-gallocatechin and (-)-epigallocatechin units (Hagerman, 2002).

2.2.2.1 Grape seed concentrations

Condensed tannins (proanthocyanidins) are the most abundant phenols present in grape skins (Souquet *et al*., 1996), seeds (Prier *et al*., 1994, Adams and Harbertson, 1999) and stems (Souquet *et al*., 2000; Singleton, 1992). The oligomeric and polymeric proanthocyanidins from grape seeds consists of the flavan-3-ol monomers (+)-catechin, (-)-epicatechin and (-)-epicatechingallate linked by C_4-C_8/C_4-C_6 interflavan bonds (Prieur 1994, Souquet, 1996, Harbertson *et al*., 2002).

These proanthocyanidins are mainly responsible for the astringency in red wines when extracted during fermentation. The perceived astringency varies with the degree of polymerisation (number of units in the polymer) of the flavan-3-ol units (Lea *et al*., 1979; Porter *et al*., 1984). These flavan-3-ols precipitate salivary proteins and creates a dry, astringent feeling as lubrication of the mouth is prevented (Noble, 1990; Gawel, 1998). The sensory threshold for grape seed tannin is approximately 0.02 g /100 ml in simple aqueous solutions (Berg and Akiyoshi, 1956). More tannin is present in grape seeds compared to that of the skins (Harbertson *et al*., 2002). Therefore, wines made from a higher seed percentage can be perceived as more astringent, but very little research has been performed on the topic (Kovac *et al.,* 1995). The contribution of monomers to astringency and bitterness differs from that of polymeric procyanidins (Arnold *et al*., 1980). The former was shown to be more bitter and less astringent than polymeric procyanidins (Gawel *et al*., 1998; Lea *et al*., 1990).

Skin and seed proanthocyanidins can be distinguished from each other by characterising the nature of constitutive extension and terminal flavan-3-ol units which ranges from 2 to 40 units in the polymer (Peng *et al*., 2001). Seed tannins have more (-)-epicatechin gallate in the terminal units and have a mean degree of polymerisation (mDP) of ± 5 compared to skins tannins which are characterised by the presence of (-)-epigallocatechin with a mDP of ± 40 (Downey *et al*., 2003; Lee *et al*., 2008). (-)-Epicatechin is the most abundant compound in the extended phenolic chains present in seeds and it is also considered the major component of tannin fractions, with (+)-catechin, (-)-epicatechin and (-)-epicatechin gallate commonly occurring in terminal units. (+)-Catechin is found to be the most abundant compound in terminal subunits of skin phenols/tannins (Downey *et al*., 2003).

Extensive work has been done by Downey *et al.* (2003) and Harbertson *et al.* (2002) on proanthocyanidin accumulation in the seed and skin during berry development. They found the bulk of tannin synthesis in the skin and seeds occur prior to variation and the accumulations are independent from each other. The concentration of proanthocyanidins increases during ripening of the berry (Kennedy *et al*., 2001).

A study conducted by Singleton and Esau (1969) showed 63% of the total phenols of red grapes are distributed in the seeds, 34% are present in the skins, 3% in the juice and 1% in the pulp. Thus it is evident that seeds contribute a considerable portion of the polyphenolic content of astringent red wines. The seeds may retain appreciable tannin concentrations after removal from fermentation (Singleton *et al*., 1964). The amount of total phenols increases with an increase in seed volume and seed contact time (Kovac *et al*., 1995). Thus, it is possible to reduce astringency if the seeds are removed earlier during fermentation. Meyer and Hernandez (1970) reported a 10% decrease in total phenolics, as determined by the Folin-Ciocalteu method, when seeds are removed early during the wine making process. The seeds are protected by a lipid layer and require a certain percentage of alcohol in solution to dissolve (Singleton and Esau, 1969). The increased fermentation temperature also increases the extraction of phenols from the seeds, as well as seeds that are cracked open (Meyer and Hernandez, 1970). Gonzàles-Manzano *et al.* (2004) reported that skins contribute significantly more flavan-3-ols than grape seeds during alcoholic fermentation. Amrani Joutei *et al.* (1994) also found that although the proanthocyanidin concentration is higher in the seeds, those in the skins are more readily extracted due to their apparent localisation. Peyrot des Gachons *et al.* (2003) confirmed earlier reports that during the early stages of fermentation the skins contribute most to the proanthocyanidins present, but as fermentation proceeds the proanthocyanidins in the seeds are more readily extracted as the ethanol concentration steadily increases (Thorngate *et al.,* 1994; Meyer *et al.,* 1970; Peng *et al.,* 2001).

Kovac and co-workers (1992) stated that the addition of seeds to a red wine leads to an increase in colour intensity. The concentration of total anthocyanins is higher in seed enriched wines, and this shows that the increase in the content of catechins and procyanidins caused by the addition of seeds may play an important role in the stabilisation of copigmentation of anthocyanins (Kovac *et al*., 1991; 1995). The same authors reported an increase in catechin and procyanidins concentration of 544 to 749 mg/L by adding supplementary seeds. In contrast, Lee *et al.* (2008) found the total proanthocyanidin content of wines, made from grapes where the seeds were removed from the grapes, was slightly higher than the conventional wines, although it did not differ statistically. They argue that the wines made without seeds, contained more proanthocyanidins, which could have been due to skin proanthocyanidins being more readily extracted during fermentation when compared to seed procyanidins.

Little investigation into the amount of tannin which fresh whole seeds could be expected to contribute to wine have been done or the influence of wine made with different seed concentrations.

2.2.3 Polymerisation reactions

Individual flavan-3-ols and/or flavan-3,4-diols (proanthocyanidins) can participate in polymerisation reactions to form condensed tannins with a molecular weight ranging from 600 to 3500. The disappearance of anthocyanins during ageing has been observed although the colour of a red wine remains stable or even intensifies (Jurd, 1972; Ribereau-Gayon *et al*., 2006). Somers (1971) suggested that certain non-enzymatic reactions lead to the formation of complexes stable to variations in pH and sulphur dioxide. Different mechanisms are involved in condensing anthocyanins with tannins. The characteristics of the formed complexes are dependant on the type of bonds. The colour of these complexes varies and three main types of reactions have been identified:

Direct condensation could involve either anthocyanin-tannin (A-T) or tannin-anthocyanin (T-A) condensation. These pathways are characterised by a direct linkage of the C_4-C_8 or C_4-C_6

carbon bonds of the flavonoids to form a polymer (Prieur *et al*., 1994). The type of linkage determines the type of dimeric procyanidin formed together with the three-dimensional shape of the tannin which in turn will affect the interaction with other compounds (Allen *et al*., 1997). These reaction products over time yield xanthylium salts, characterised by having an orange, tile-like colour (Mirabel *et al*., 1999). A cross bond is formed between flavanol units followed by cyclisation. The first condensation reaction, anthocyanins-tannin (A-T), involves the flavylium form (A^+) of anthocyanins reacting with the nucleophilic sites of procyanidins (P) to form a colourless flavene (A-P) (Fig 2.3). The presence of oxygen or an oxidising reagent is necessary for the flavene to recover its red colour (A^+-P). When no oxygen is present there is a decrease in colour. The second type of direct condensation, tannin-anthocyanin (T-A), does not involve oxygen and depends on temperature and the anthocyanin concentration present. Procyanidins forms carbocations after protonation of the molecule and react with nucleophilic sites of anthocyanin molecules (Fig 2.4). The complex formed (T-AOH) is colourless and turns red-orange (T-A complex) on dehydration (Ribereau-Gayon *et al*., 2006).

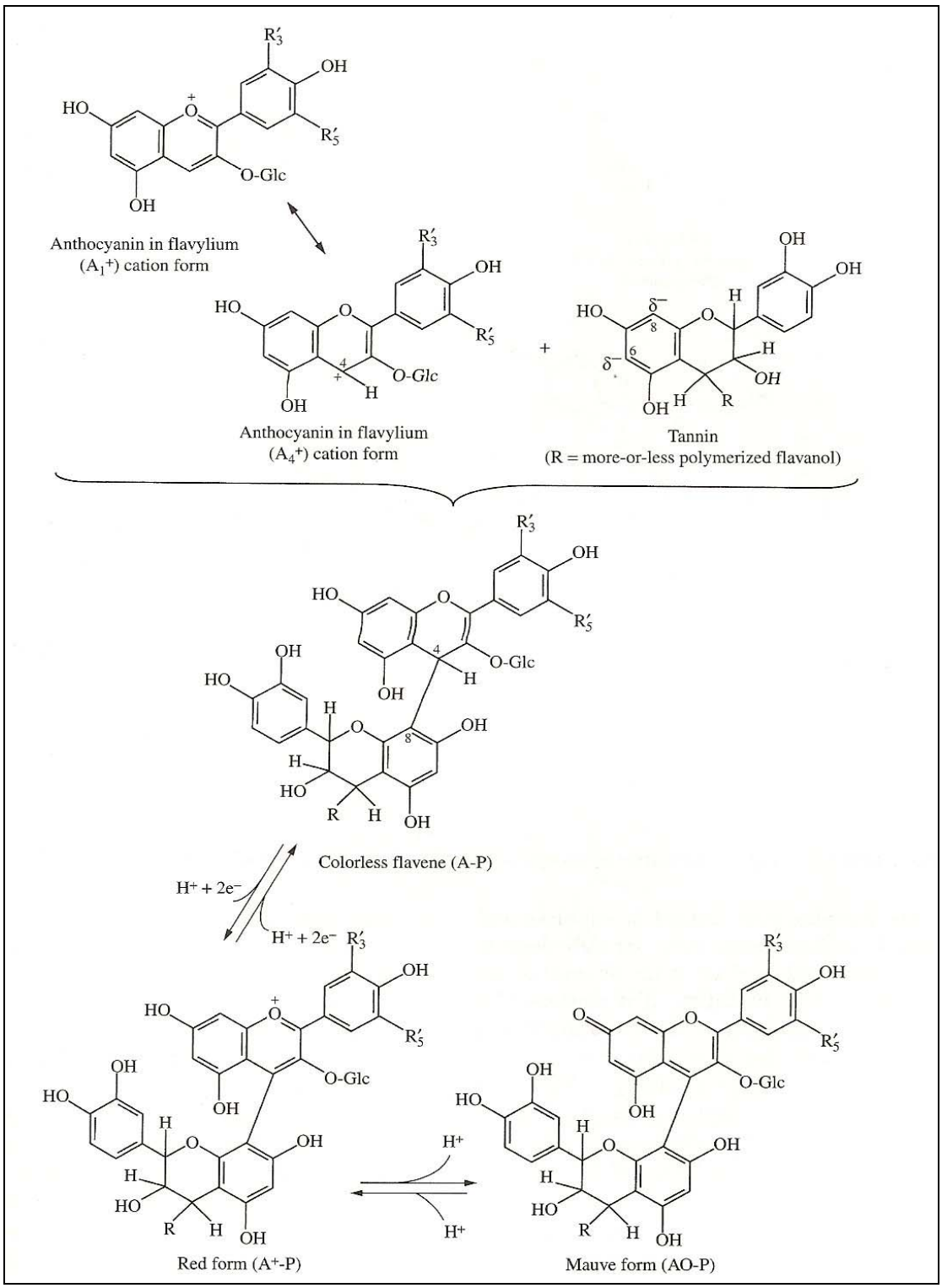


Figure 2.3: Direct A-T type condensation of anthocyanins and tannins (Galvin, 1993).

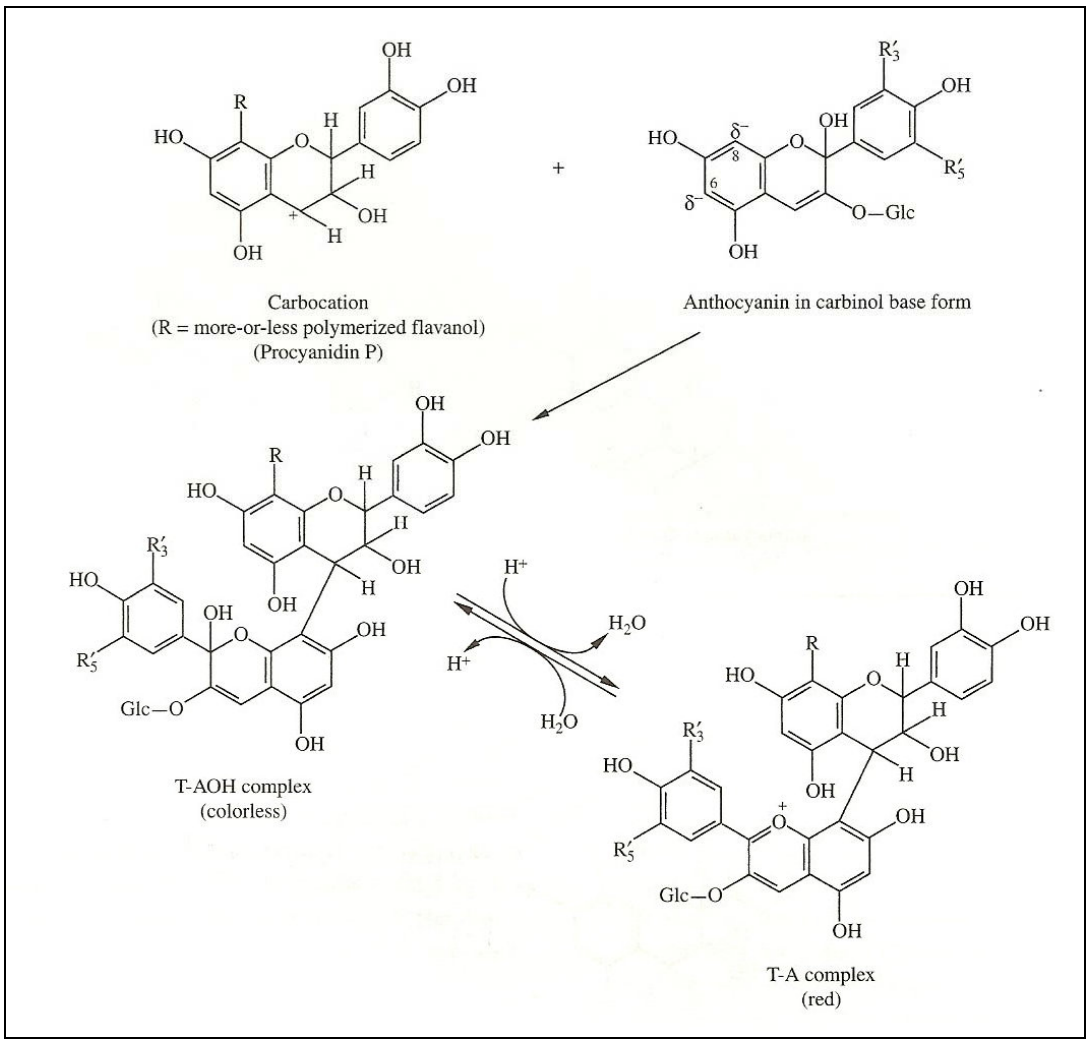


Figure 2.4: Direct T-A type condensation of procyanidins and anthocyanins (Galvin, 1993).

Thirdly, indirect condensation involves condensation with an ethyl cross bond (Fig 2.5) (Escribano-Bailon *et al*., 2001). With gentle and controlled aeration, combined oxidation of the procyanidins leads to the formation of H_2O_2, which in turn can oxidize ethanol to ethanal (acetaldehyde). Acetaldehyde is also secreted as a secondary metabolite by yeast (Morata *et al*., 2003; Timberlake and Bridle, 1977). The presence of acetaldehyde causes a shift in colour augmentation towards violet. This is attributed to the formation of new compounds linked by methyl methino (CH_3-CH) bridges that are detectable by HPLC (Dallas *et al*., 1996; Vidal *et al*., 2004). This acetaldehyde-mediated condensation is considerably faster than direct polymerisation and can occur between tannins and anthocyanins (Ribéreau-Gayon *et al*., 2006; Jurd *et al*., 1970). Es-Safi *et al.* (1999) reported that lower pH values lead to larger amounts of bridged compounds and faster condensation rates. With ring closure these reactions yield red-purple pigments stable to degradation and bleaching and make a considerable contribution to colour stabilisation (Cheynier *et al*., 1999).

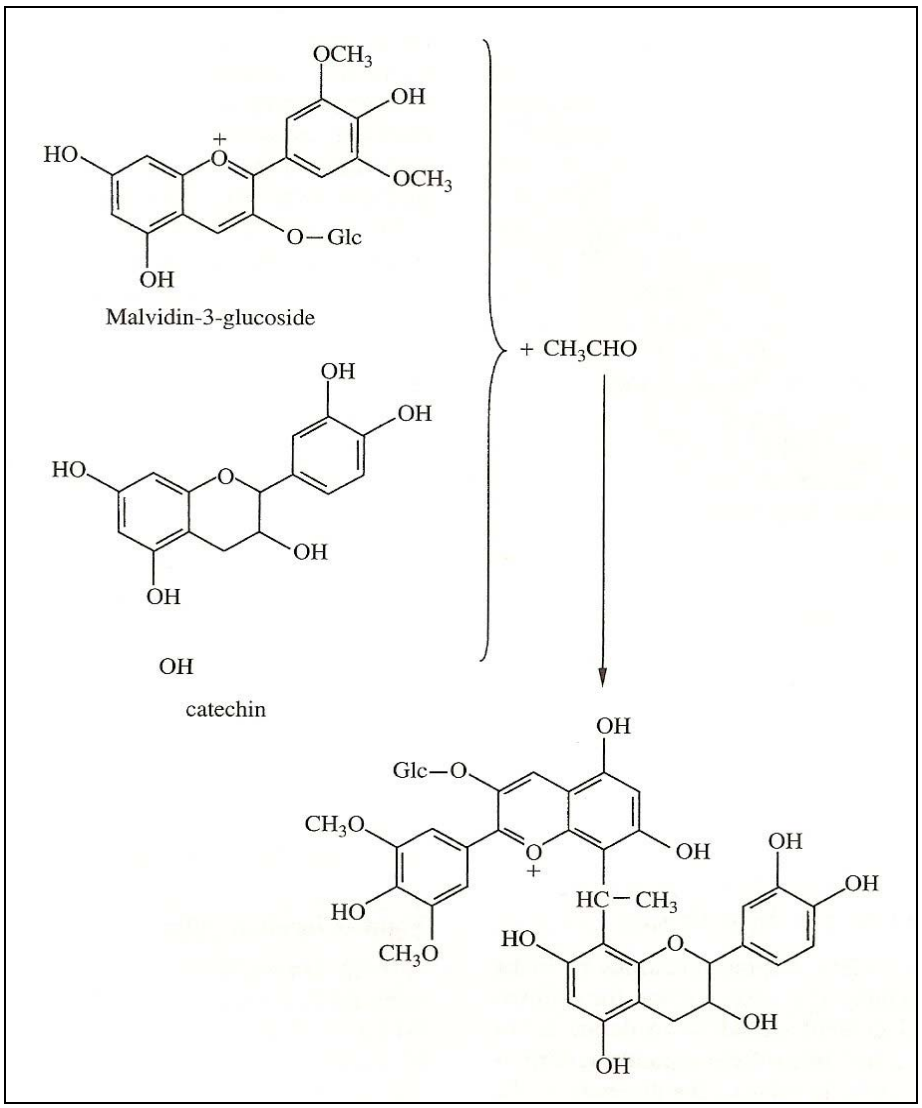


Figure 2.5: Reaction between (+)-catechin and malvidin-3-glucoside in the presence of acetaldehyde, illustrating indirect polymerisation (Timberlake and Bridle, 1976).

2.3 Oxygen in wine

Oxidation is a chemical process that involves the electron transfer from an atom, or group of atoms, through reactions that may or may not involve oxygen addition or hydrogen loss. This process either holds the key to the production of quality red wines or drastically reduces wine quality. The exposure of a white wine to oxygen is considered detrimental as it leads to the development of brown polymers (Waterhouse and Laurie, 2006). Oxygen influences the phenolic composition of wine as phenols are the primary substrates for oxidation. Under high pH conditions phenols can readily react with oxygen. Their weak acidic character (pKa 9-10) allows them to form phenolate anions that can more easily react with oxygen. The removal of one electron form the phenolate anion results in a semi-quinone that can disproportionate to yield a quinone and a phenol (Fig 2.6) (Singleton, 1987; Danilewicz, 2003).

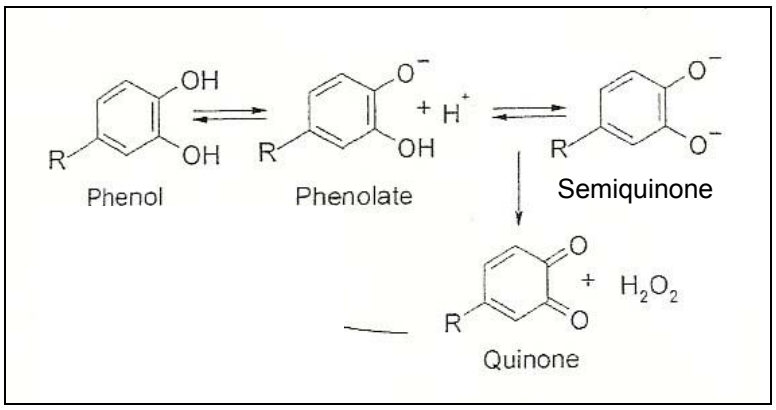


Figure 2.6: The formation of a phenolate anion from a phenol to yield a semiquinone and with disproportionation, a quinone (Waterhouse and Laurie, 2006).

The addition of oxygen contributes to polymerisation reactions which in turn affects wine colour. Many cases of sensory and/or microbiological spoilage can be traced back to wine oxidation, with the only exception being wines deliberately made under oxidative conditions such as madeiras, jerez or sherry to enhance the quality. Traditionally wine has always been protected from oxygen, but controlled constitutive amounts of oxygen applied to red wine can impart benefits to red wines by stabilising colour and reducing astringency (Castellari *et al*., 2000; Atanasova *et al*., 2002; Du Toit *et al*., 2006).

Phenolic compounds, particularly the flavan-3-ols present in grapes, contribute to astringency and bitterness in wine. These mouthfeel characteristics can be changed by controlled aeration of the must or wine, as oxygen can be seen as the catalyst between the chemical transformation of these compounds to enhance quality and flavour of the wine. Castellari and co-workers (2000) conducted a preliminary study to test the influence of oxygen addition to must before fermentation. Red pigments appeared to be influenced by the oxygen treatment after only 40 hours during maceration. There was a significant reduction in total phenolic and total anthocyanin concentration with aeration. After the wines were aged for six months the total phenolic and anthocyanin concentrations were still lower in the treated wines than in the control, together with colour density, tannins and monomeric flavanols. Polymeric pigments increased with oxygen addition, resulting in a lower monomeric anthocyanin content due to the conversion to more stable pigments.

2.3.1 Must hyperoxidation

Must has traditionally been carefully protected against oxidation to avoid browning. Schneider (1998) demonstrated that oxidation of must prior to fermentation was not as detrimental as expected. It is well known, and supported by popular data, that oxidation rarely improves the quality of white table wines, but generally does benefit red wines (Cheynier *et al*., 1989).

There is, however, a method used in the preparation of white wine to improve shelf life and typical cultivar aroma. This method is applied before alcoholic fermentation and is based on precipitating phenols that are responsible for bitterness and browning during ageing. This technique is called hyperoxidation (Cheynier *et al*., 1989). Polyphenols are the compounds most easily oxidised, and hyperoxidation reduces the amount of all phenolic compounds (Fell *et al*., 2007). Musts when oxidised have a faster transformation of phenolic precursors to form brown, insoluble polymers, leading to an increase in brown colour compounds. These can easily be removed by clarification. This in theory should produce wines that are more stable towards oxidation. However, the application of this technique could sometimes lead to a loss in aroma

and a general reduction in wine quality. Contradictory reports regarding this aspect exists, with certain authors finding an increase wine in quality for wines made from oxidised musts (Schneider, 1998).

2.3.2. Micro-oxygenation

A technique called micro-oxygenation was developed in France in the 1990's by Moutounet and Ducournau (1993) to counter colour loss in red wines after malolactic fermentation among several other benefits. This technique allows the controlled addition of small and continuous amounts of oxygen to diffuse into wine over time. The method can be applied after alcoholic fermentation, but is also used after malolactic fermentation or during ageing. The addition of oxygen in this way also improves the mouth feel and decreases astringency, herbaceous and vegetal characters. For this reason it is suggested to apply the technique to complement oak ageing and thus improve the quality of very astringent wines (Llaudy *et al.,* 2006; Sartini *et al.,* 2007).

Pure oxygen is dissolved in wine by placing a ceramic sparger close to the bottom of a stainless steel tank, or barrel in some cases. The diffuser is connected to equipment that controls the flow rate of oxygen in milligrams per litre of wine per day or month. The theory is that such a controlled, slow flow rate permits the phenols to consume the oxygen without acquiring oxidative characteristics (Du Toit *et al*., 2006). Two basic factors affects the micro-oxygenation technique, namely temperature and sulphur dioxide concentration, which can be controlled. The solubility of oxygen in wine increases as the temperature decreases. In literature this technique was mostly performed at around 16°C, to avoid the accumulation of dissolved oxygen in wine (Singleton, 1987 and 1999; Llaudy *et al*., 2006). Free sulphur dioxide concentration tends to decrease with an accumulation of dissolved oxygen. Sulphur dioxide is oxidised by oxygen to hydrogen peroxide due to the strong reductive nature of sulphur dioxide. Monitoring sulphur dioxide levels is important since it controls the wines' micro flora (Singleton, 1987).

A study was done by Perez-Magarino *et al.* (2007) on four Italian red wines to test the effect of micro-oxygenation. By applying micro-oxygenation the total phenol concentration decreased with a substantial loss in monomeric anthocyanins, which was also confirmed by Cano-Lopez *et al.* (2006). Similar results were obtained in the study done by Castellari *et al*. (2000) on must oxidation. The loss in phenolics could be due to polymerisation reactions, caused by oxygen as a catalyst. New colour pigment products more resistant to sulphur dioxide bleaching and changes in pH are also formed. Perez-Magarino *et al*. (2007) also found lower concentrations of catechins and proanthocyanidins in the oxygenated wines.

Llaudy and co-workers (2006) applied micro-oxygenation before barrel ageing and found a definite decrease in the astringency of the wines. No statistical differences were found between the control and oxygenated wine in proanthocyanidin concentrations, total phenol content or DMAC indexes (total monomeric flavan-3-ols). Fell *et al*. (2006) found similar results. In general, there seems to be little information available on the earlier application of oxygen during the winemaking process.

Micro-oxygenation has been previously used as an alternative to barrel ageing. It is generally well known that the ageing of red wine in oak barrels leads to the stabilisation of colour compounds, reduces astringency and also removes herbaceous notes (Fell *et al*., 2007). Small amounts of oxygen that are embedded in the pores of the wood, the interstices between staves, and the bunghole are released into the wine and are responsible for the abovementioned transformations (Vivas, 2000). The dissolved oxygen leads to the formation of ethanal from

ethanol. The ethanal can in turn react with flavanols to induce the formation of a very reactive carbocation that quickly reacts either with another flavanol molecule or with an anthocyanin, producing methyl methine flavanol-flavanol and flavanol-anthocyanin oligomers (Silva Ferreira *et al*., 2002). Ethanal also participates in the formation of new pigments such as vitisin B and other pyranoanthocyanins through cyclo-addition reactions (Fig 2.7) (Fulcrand *et al.,* 1997; Fulcrand *et al.,* 1998; Mateus *et al*., 2002; Llaudy *et al.*, 2006). Since micro-oxygenation releases small and controlled amounts of oxygen similar to barrel ageing, it can be seen as a possible alternative to oak ageing, which is becoming ever more expensive and can be a very labour intensive exercise.

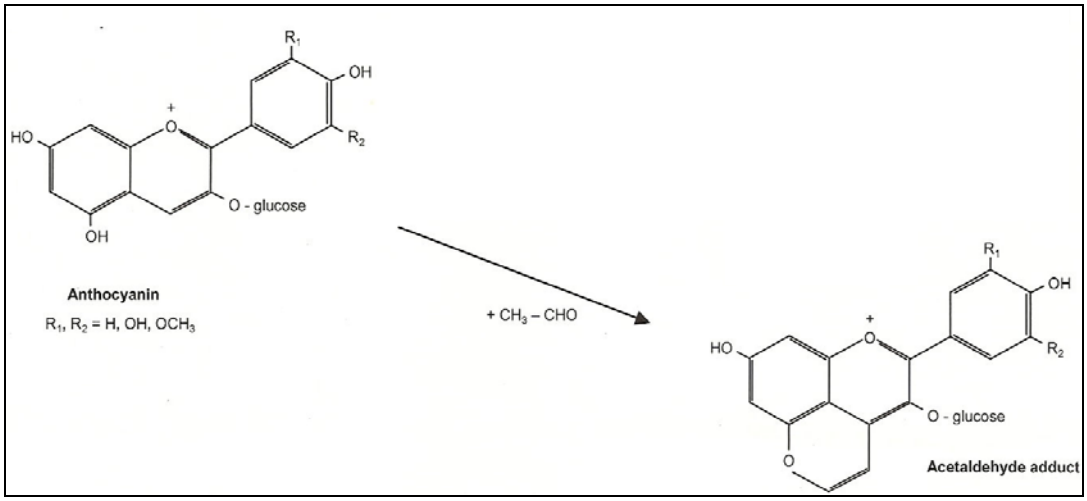


Figure 2.7: The formation of pyranoanthocyanins through cyclo addition reactions involving acetaldehyde and anthocyanins.

However, this method may also produce a wine with reduced colour (Cacho *et al*., 1995; Jordao *et al*., 2006). If too much oxygen is applied, large polymers with high molecular weight are formed and the polymeric pigments will precipitate. Also, if a wine is given too much oxygen for the phenolic content, it may lead to oxidation. Many oxidative reactions are irreversible and may contribute to the deterioration of wine quality. Controlled oxygen additions to red wine can lead to a decrease in astringency, but too long additions might also lead to wine tannins being perceived as dry and unpleasant (Du Toit *et al*., 2006).

It has been suggested that a wine is more capable of buffering large amounts of oxygen earlier during the winemaking process than later, such as micro-oxygenation during ageing. Observations by Rossi and Singleton (1965) suggested that a wine's oxygen capacity was related to its phenolic concentration. As more oxygen is readily available earlier, there should be a faster transformation of the anthocyanins extracted from the grapes to polymeric pigments which are more stable in terms of wine colour. They found that although a red wine can benefit from the addition of oxygen, there is no set recipe on how much oxygen must be applied. They believe these questions will stay unanswered until there is a better understanding of the chemical reactions involving oxygen. A value of approximately 8.6 mg/l oxygen is believed by Singleton (1987) to be air-saturated in a wine medium, although it could range from 6-9 mg/l depending on the temperature of the wine.

2.4 Ageing

Wines can be stored in either bottles or barrels. The way bottles are stored influences air diffusion and if stored upright more oxygen could come into contact with the wine and cause spoilage such as oxidation (Du Toit *et al*., 2006).

The porosity of oak wood allows a slow transference of oxygen which favours oxidation of wine compounds that could lead to changes in taste, astringency and colour that have been noted during the storage of red wine in barrels (Es-Safi *et al*., 1999). This phenomenon is associated with anthocyanin-flavanol pigment formation such as indirect condensation or acetaldehyde-mediated reactions (Bakker *et al*., 1993; Timberlake and Bridle, 1976; Gomez-Cordoves *et al*., 1995) which enhances the crimson colour of a wine. The main contribution of oak wood to wine is the ellagitannins which are able to dissolve into the ethanolic solution. These tannins greatly affect a wines phenolic composition and colour (Glories, 1993; Ribéreau-Gayon and Stonestreet, 1965). These compounds increase the rate of procyanidin condensation while at the same time limit degradation processes such as the precipitation of condensed tannin and destruction of anthocyanins (Moutounet *et al*., 1993).

Cano-Lopez *et al.* (2007) applied micro-oxygenation for five months to wine in a stainless steel tank and then either bottled or aged the wine in barrels. They found that the positive effect of the micro-oxygenation technique was maintained in bottles and barrels when tested months later. The oak-matured control wine had similar chromatic characteristics as the bottled micro-oxygenation wine. This suggests the micro-oxygenation technique leads to wines with similar chromatic characteristics to those aged in barrels.

2.5 Phenol analyses

Analysing the broad spectrum of phenolic molecules in wine is not always possible due to their diversity and specialised techniques required for identification. Even the most effective techniques are sometimes difficult to implement, and results can be incomplete and difficult to interpret. Most techniques are useful for research purposes, but cannot be routinely used in a commercial winery due to cost of instruments and specialised training needed. Methods of assessment have evolved to rapid, reproducible methods with the inclusion of most/all phenols present (Celotti and Cercereri de Prati, 2005; Riberaeu-Gayon *et al*., 2006). These methods can be divided into two broad categories namely spectroscopy/ spectrometry and chromatography.

2.5.1 Spectrophotometry methods

2.5.1.1 Colour analyses

Anthocyanins in the coloured form have a red colour with a maximum absorbance around 520 nm, while 420 nm is indicative of the brown colour of some polymers. Colour density is defined as the sum of the absorbance at 420 and 520 nm, including an additional reading at 620 nm representing the violet component in certain young wines (Glories, 1984a). The colour hue indicates the wine's development towards orange/brown and this value increases with ageing, reaching an upper limit of around 1.2-1.3 (Ribereau-Gayon *et al*., 2006). The hue is defined as the ratio of 420 nm to 520 nm (Somers and Evans, 1977).

The different fractions that contribute to red wine colour can be measured by spectrophotometry as described by Boulton (2001). These fractions can be classified as either copigmented, free anthocyanins or polymeric pigments. The addition of sulphur dioxide

bleaches monomeric anthocyanins, and so gives an indirect measurement of polymeric pigments. Using the same principle, free anthocyanins can also be determined. Red wine in an acidic medium (pH < 1.0) forces all anthocyanins, including those involved in copigmentation, into the red flavylium form to determine the total potential red colour of the wine (Boulton, 2001; Ribereau-Gayon *et al*., 2006).

2.5.1.2 Total phenol analysis

Various methods have been established to determine the phenol content of substances. Wine research highlighted the most frequently used methods, as obtained by Folin-Ciocalteu (Folin and Ciocalteu, 1927; Singleton and Rossi, 1965; Singleton *et al*., 1999) and a measurement at 280 nm (Ribereau-Gayon and Stonestreet, 1965; Somers and Evans, 1977). Other spectrophotometric assays that measure total phenol content include permanganate titration and reactions with iron salts (Slinkard and Singleton, 1977).

2.5.1.2.1 Folin-Ciocalteu

The Folin-Ciocalteu method relies on oxidation-reduction reactions, and measures the total phenolic content rather than tannin as this colourforming reaction is produced by monohydric phenols, polyphenols, flavonoids, tannins, and some other readily oxidised substances such as ascorbic acid. The reagent, phosphotungstic-phosphomolybdic acid oxidises phenols and is itself reduced to a blue molybdenum tungsten complex that is then measured at 765 nm. Phenols are more rapidly oxidised in solutions sufficiently alkaline to give appreciable concentrations of the phenolate ion. The final phenolic concentration is determined by obtaining a standard curve with gallic acid as reference. Gallic acid is a more satisfactory standard than tannic acid due to solubility, stability and is more affordable. The phenol content of a red wine can range between 180 and 3000 mg/L GAU (gallic acid units). All hydroxyl groups are oxidised in this method, which can lead to an over estimation of the phenols present (Singleton, 1965, Singleton *et al*., 1999).

2.5.1.2.2 Total phenols as measured at 280 nm

All phenols absorb light at 280 nm due to cyclic benzene ring A. Measuring the absorbance at 280 nm thus give an indication of the total phenol content of a wine. It is a fast and reproducible method with little preparation (Ribereau-Gayon *et al*., 2006; Somers and Evans, 1977).

2.5.1.3 Monomeric flavanols

Monomeric flavanols can be measured with the aldehydic reagent, 4-dimethylamino-cinnamaldehyde (DMAC) which reacts with free meta-hydroxyl groups on the A-ring (Nagel and Glories, 1991). Due to this mechanism, proanthocyanidins are also included in this measurement, but react with DMAC to a much lesser extent than monomeric flavan-3-ols. Anthocyanins and flavonols are excluded due to their electron-withdrawing functional groups. The reading is taken at 640 nm and the final concentration determined by a standard curve constructed with (+)-catechin and results are expressed in mg/L CE (catechin equivalents). The assay is mainly used for red wines, since the concentration of flavanols in white wines are very low (1.3-3.3 mg/L) or not detectable (McMurrough and McDowell, 1978; De Beer *et al*., 2004, Kennedy *et al*., 2006).

2.5.1.4 Tannin assay

The analysis of tannins in wine has been reported to be extremely difficult because the number of unique chemical structures could be vast in a pool of polymers having various lengths and four or more possible subunits (Harbertson *et al*., 2002). Therefore, one should look into the different type of analyses of tannins to evaluate the broad spectrum.

2.5.1.4.1 Protein precipitation assay

The tannin content of a wine can be quantified due to the nature of tannins precipitating with proteins. Such an assay was developed by Adams *et al.* (1999) to distinguish between monomeric anthocyanins and polymeric pigments. The latter can further be divided into small polymeric pigments (SPP), which do not precipitate with protein, and large polymeric pigments (LPP), that precipitate with protein as described by Hagerman and Butler (1978). This method was modified by Harbertson *et al*. (2002) to combine the assay with bisulphite bleaching to determine the monomeric pigments. The tannin-protein precipitate formed with bovine serum albumin (BSA) is redissolved and the amount of tannin is then determined by a reaction with ferric chloride. The procedure yields a coloured product with a maximum absorption at 510 nm. (+)-Catechin is used as a standard since it is found as one of the subunits and cannot precipitate protein by itself; it is prepared in the same buffer used to resuspend the tannin-protein complex. Work by Harbertson (2002) and Adams *et al.* (1999) confirmed that dimers and trimers do not respond in a protein-binding assay such as this method and is limited to those oligomeric proanthocyanidin with a degree of polymerisation greater than three units. Nonetheless, this is not a serious limitation as only a small portion of tannins in grape seeds are dimers and trimers.

The assay provides a rapid and reliable measurement of tannin in red wine, with the concentrations ranging from 20-830 mg/L CE (catechin equivalents). The assay is a direct measurement of chemical astringency as it only measures the phenolics that bind to protein.

2.5.2 Chromatography methods

2.5.2.1 High Performance Liquid Chromatography (HPLC)

The phenols in wine can be separated and quantified individually using reverse phase (RP) HPLC analysis. Phenol separation is achieved using a reverse phase column packed with spherical particles of silica bonded with octadecyl (C18) chains (Lamuela-Raventòs *et al*., 1994), or polymeric material such as polystyrene/divinylbenzene (Peng *et al*., 2002; Price *et al*., 1995). Compounds, such as monomeric pigments and non-pigmented phenols can be separated as individual peaks by using a slow gradient of the two solvents involved with an increase in the organic solvent component. After these have eluted, the gradient can be increased to yield the remaining phenolic material as a large peak that are defined as the non-pigmented (280 nm) and pigmented (520 nm) polymeric peak (Peng *et al*., 2002; Price *et al*., 1995). Individual phenols are quantified in comparison with a standard calibration curve.

2.6 Conclusion

Limited information is available on the effect of micro and/or macro-oxygenation on a red wine's phenolic composition. Oxygen is mainly involved with phenols in polymerisation reactions to form more stable polymeric compounds. Malolactic fermentation is an important winemaking practice in red wines, but the technique involves a decrease in red colour. By applying oxygen

earlier during the wine making process, ie, after fermentation, it could be possible to counter the colour loss during malolactic fermentation. Wines made with additional seeds could lead to a greater colour intensity and astringency.

Most methods for wine analyses are general assessments of the phenolic content. There is a lack of methods that can separate and quantify polymeric phenols and pigments rapidly and effectively. Although methods are available for determining the tannin concentration in red wine, they lack specificity.

2.7 Literature cited

Adams, D.O. and Harbertson, J.F. (1999). Use of alkaline phosphatase for the analysis of tannins in grapes and red wines. *American Journal of Enology and Viticulture*. **50**, 247-252.

Allen, M., Wall, G. and Bullied, N. (1997). Phenolics demystified. In: ASVO seminar proceedings. p. 4-11.

Atanasova, V., Fulcrand, H., Cheynier, V. and Moutounet, M. (2002). The effect of oxygenation on polyphenol changes occurring in the course of wine-making. *Analytica Chimica Acta*. **458**, 15-27.

Amrani Joutei, K., Glories, Y. and Mercier, M. (1994). Localisation des tannins dans la pellicule de baie de raisin. *Vitis*. **33**, 133-138.

Arnold, R.A., Noble, A.C. and Singleton, V.L. (1980). Bitterness and astringency of phenolic fractions in wine. *Journal of Agricultural and Food Chemistry.* **28**, 675-678.

Bakker, J., Picinelli, A. and Bridle, P. (1993). Model wine solutions: colour and composition changes during ageing. *Vitis*. **32**, 111-118.

Berg, H.W. and Akiyoshi, M. (1956). Some factors involved in browning of white wines. *American Journal of Enology and Viticulture*. **7**, 1-7.

Boulton, R. (2001). The copigmentation of anthocyanins and its role in the colour a red wine: a critical review. *American Journal of Enology and Viticulture.* **52**, 67-87.

Brouillard, R. and Delaporte, B. (1977). Chemistry of anthocyanin pigments. 2: kinetic and thermodynamic study of proton transfert, hydration and tautomeric reactions of malvidin-3-glucoside. *Journal of American Chemical Society*. **99**, 8461-8467.

Brouillard, R., Delaporte, B. and Dubois, J.E. (1978). Chemistry of anthocyanin pigments. 3. Relaxation amplitudes in pH-jump experiments. *Journal of American Chemical Society*. **100**, 6202-6205.

Brouillard, R. and Mazza, G. (1989). The copigmentation reaction of anthocyanins: a microprobe for the structural study of aqueous solutions. *Journal of American Chemical Society.* **111**, 2604-2610.

Cacho, J., Castells, J.E., Esteban, A., Laguna, B. and Sagrista, N. (1995). Iron, copper and manganese – influence on wine oxidation. *American Journal of Enology and Viticulture*. **46**, 380-384.

Castellari, M., Matricardi, L., Arfelli, G., Galassi, S. and Amati, A. (2000). Level of single bioactive phenolics in red wine as a function of the oxygen supplied during storage. *Food Chemistry*. **69**, 61-67.

Cano-López, M., Pardo-Minguez, F., López-Roca, M. and Gomez-Plaza, E. (2006). Effect of microoxygenation on anthocyanin and derived pigment content and chromatic characteristics of red wines. *American Journal of Enology and Viticulture*. **57**, 325-331.

Cano-Lopez, M., Pardo-Minguez, F., Lopez-Roca, J.M. and Gomez-Plaza, E. (2007). Chromatic characteristics and anthocyanin profile of a micro-oxygenated red wine after oak or bottle maturation. *European Food and Research Technology*. **225**, 127-132.

Celotti, E. and Carcereri De Prati, G. (2005). The phenolic quality of red grapes at delivery: Objective evaluation with colour measurements. *South African Journal of Enology and Viticulture.* **26**, 75-82.

Cheynier, V., Basire, N. and Rigaud, J. (1989a). Mechanism of trans-caffeoyltartaric acid and catechin oxidation in model solutions containing grape polyphenoloxidase. *Journal of Agricultural and Food Chemistry*. **37**: 1069-1071.

Cheynier, V., Rigaud, J. and Moutounet, M. (1989b). Mechanisms of anthocyanin degradation in grape must-like model solutions. *Journal of Chromatography*. **472**: 428-432.

Cheynier, V., Es Safi, N.E. and Fulcrand, H. (1999). Structure and colour properties of anthocyanins and related pigments. *In*: International Congress on pigments in Food and Technology. M.I.M. Mosquera *et al*. (Eds.), pp 23-25.

Cilliers, J.J.L. and Singleton, V.L. (1989). Characterization of the products of nonenzymatic autoxidative phenolic reactions in a caffeic acid model system. *Journal of Agricultural and Food Chemistry*. **39**, 1298-1303.

Dallas, C., Ricardo De Silva, J.M. and Laureano, O. (1996). Interactions of oligomeric procyanidins in model wine solutions containing malvidin-3-glucoside and acetaldehyde. *Journal of the Science of Food and Agriculture*. **70**, 493-500.

Danilewicz, J. (2003). Review of reaction mechanisms of oxygen and proposed intermediate reduction products in wine: central role of iron and copper. *American Journal of Enology and Viticulture*. **54**, 73-85.

De Beer, D., Harbertson, J.F., Kilmartin, P.A., Roginsky, V., Barsukova, T., Adams, D.O. and Waterhouse, A.L (2004). Phenolics: a Comparison of diverse analytical methods. *American Journal of Enology and Viticulture*. **55**, 389-400.

De Freitas, V.A.P. and Glories, Y. (1999). Concentration and compositional changes of procyanidins in grape seeds and skins of white *Vitis vinifera* varieties. *Journal of the Science of Food and Agriculture*. **79**: 1601-1606.

Downey, M.O., Harvey, J.S. and Robinson, S.P. (2003). Analysis of tannins in seeds and skins of Shiraz grapes throughout berry development. *Australian Journal for Grape and Wine Research*. **9**, 15-27.

Du Toit, W.J., Marais, J., Pretorius, I.S. and Du Toit, M. (2006). Oxygen in must and wine: a review. *South African Journal of Enology and Viticulture*. **27**, 76-94.

Escribano-Bailon, T., Alvarez-Garcia, M., Rivas-Gonzalo, J.C., Heredia, F.J. and Santos-Buelga, C. (2001). Colour and stability of pigments derived from the acetaldehyde-mediated condensation between malvidin-3-O-glucoside and (+)-catechin. *Journal of Agricultural and Food Chemistry*. **49**, 1213-1217.

Es-Safi, N., Fulcrand, H., Cheynier, V. and Moutounet, M., (1999). Competition between (+)-catechin and (-)-epicatechin in acetaldehyde-induced polymerization of flavanols. *Journal of Agricultural and Food Chemistry*. **47**, 2088-2095.

Fell, A.J., Dykes, S.I., Nicolau, L. and Kilmartin, P.A. (2007). Electrochemical microoxidation in red wine. *American Journal of Enology and Viticulture*. **58**, 443-450.

Folin, O. and Ciocalteu, V. (1927). On tyrosine and tryptophane determinations in proteins. *Journal of Biology and Chemistry*. **73**, 627-650.

Fulcrand, H., Cheynier, V., Oszmianski, J. and Moutounet, M. (1997). An oxidized tartaric acid residue as a new bridge potentially competing with acetaldehyde in flavan-3-ol condensation. *Phytochemistry*. **46**, 223-227.

Fulcrand, H., Benabdeljalil, C., Rigaud, J., Cheynier, V. and Moutounet, M. (1988). A new class of wine pigments generated by reaction between pyruvic acid and grape anthocyanins. *Phytochemistry*. **47**, 1401-1407.

Galvin, C. (1993). Thése de Doctorat Œnologie-Ampélologie, Université de Bordeux II, France.

Gawel, R. (1998). Red wine astringency: a review. *Australian Journal of Grape and Wine Research*. **4**, 74-96.

Glories, Y. (1984a). La coleur des vins rouges. 1° partie. Les equilibres des anthocyanes et des tanins. *Connais. Vigne Vin*. **18**, 195-217.

Glories, Y. and Galvin, C. (1988). Les complexes tannins-anthocyanes en presence d'ethanol. Conditions de leur formation*. Actualietes oenologiques*. Pp 403-413. Ed. Dupond, Paris.

Glories, Y. (1993). L'élevage des vins rouges en fût du chêne. In: Elevatge des vins en fûts de chêne. Y.Glories (Ed.). CEPS, Melun.

Gòmez-Cordovés, C., Gonzàles-San José, M.L., Junquera, B. and Estrella, I. (1995). Correlation between flavonoids and color in red wines aged in wood. *American Journal of Enology and Viticulture*. **46**, 295-298.

Gonzales-Manzano, S., Rivas-Gonzalo, J.C. and Santos-Buelga, C. (2004). Extraction of flavan-3-ols from grape seed and skin into wine using simulated maceration. *Analitica Chimica Acta*. **513**, 283-289.

Hagerman, A.E. (2002). Tannin Handbook. www.users.muohio.edu/hagermae/tannin/pdf.

Hagerman, A.E. and Butler, L.G (1978). Protein precipitation method for the quantitative determination of tannins. *Journal of Agricultural and Food Chemistry*. **26**, 809-812.

Harbertson, J.F., Kennedy, J.A. and Adams, D.O. (2002). Tannins in skins and seeds of Cabernet Sauvignon, Syrah, and Pinot noir berries during ripening. *American Journal of Enology and Viticulture*. **53**, 54-59.

Jordao, A.M., Ricardo-da-Silva, J.M. and Laureano, O. (2006). Effect of oak constituents and oxygen on the evolution of malvidin-3-glucoside and (+)-catechin in model wine. *American Journal of Enology and Viticulture*. **57**, 377-381.

Joslyn, M.A. and Goldstein, J.L. (1965). Astringency of fruits and fruit products in relation to phenolic content. *Wallerstein Laboratory Communications*. **28**: 143-160.

Jurd, L. and Somers, T.C. (1970). The formation of xanthylium salts from proanthocyanidins. *Phytochemistry.* **9**, 419-427.

Jurd, L. (1972). Anthocyanidins and related compounds. The dimerization of flavylium salts in aqueous solutions. *Tetrahedron*. **28**, 493-505.

Kennedy, J.A., Ferrier, J., Harbertson, J.F. and Peyrot Des Gachons, C. (2006). Analysis of tannins in red wine using multiple methods: Correlation with perceived astringency. *American Journal of Enology and Viticulture*. **57**, 481-485.

Kennedy, J.A., Hayasaka, Y., Vidal, S., Waters, E. and Jones, G.P. (2001). Composition of grape skin proanthocyanidins at different stages of berry development. *Journal of Agricultural and Food Chemistry*. **49**, 5348-5355.

Kovac, V.E., Bourzeix, M., Heredia, N. and Ramos, T.A. (1991). A study of catechins and proanthocyanidins of white grapes and wines. *Revue de French Oenologie.* **125**, 7-14.

Kovac, V.E., Alonso, E., Bourzeix, M. and Revilla, E. (1992). Effect of several enological practices on the content of catechins and proanthocyanidins of red wines. *Journal of Agricultural and Food Chemistry*. **40**, 1953-1957.

Kovac, V.E., Alonso, E. and Revilla, E. (1995). The effect of adding supplementary quantities of seeds during fermentation on the phenolic composition of wines. *American Journal of Enology and Viticulture*. **46**, 363-367.

Lamuela-Raventós, R.M. and Waterhouse, A.L. (1994). A direct HPLC separation of wine phenolics. *American Journal of Enology and Viticulture*. **45**, 1-5.

Lea, A.G.H., Bridle, P., Timberlake, C.F. and Singleton, V.L. (1979). The procyanidins of white grapes and wines. *American Journal of Enology and Viticulture*. **30**, 289-300.

Lea, A.G.H. (1990). Bitterness and astringency: the procyanidins of fermented apple ciders. *In* Bitterness in Foods and Beverages. Developments in Food Science. 25. R.L. Rouseff (Ed.), pp. 123-143. Elsevier, Amsterdam.

Lee, J., Kennedy, J.A., Devlin, C., Redhead, M. and Rennaker, C. (2008). Effect of early seed removal during fermentation on proanthocyanidin extraction in red wine: a commercial production example. *Food Chemistry*. **107**, 1270-1273.

Levengood, J.A. (1996). A survey of copigmentation in Cabernet Sauvignon wines. M.S. Thesis, University of California, Davis.

Liao, H., Cai, Y. and Haslam, E. (1992). Polyphenol interactions. Anthocyanins: Copigmentation and colour changes in red wines. *Journal of Agricultural and Food Chemistry*. **59**, 299-305.

Llaudy, M.D., Canals, R., Gonzales-Manzano, S., Canals, J.M., Santos-Buelga, C. and Zamora, F. (2006). Influence of micro-oxygenation treatment before oak aging on phenolic compounds composition, astringency, and color of red wine. *Journal of Agricultural and Food Chemistry*. **54**, 4246-4252.

Mateus, N., De Pascual-Teresa, S., Rivas-Gonzalo, J.C., Santos-Buelga, C. and De Freitas, V. (2002). Structural diversity of anthocyanin-derived pigments in port wines. *Food Chemistry*. **76**, 335-342.

McMurrough, I. and McDowell, J. (1978). Chromatographic separation and automated analysis of flavanols. *Analytical Biochemistry*. **91**,92-100.

Meyer, J. and Hernandez, R. (1970). Seed tannin extraction in Cabernet Sauvignon. *American Journal of Enology and Viticulture*. **21**, 184-188.

Mirabel, M., Saucier, C., Guerra, C. and Glories, Y. (1999). Copigmentation in model wine solutions: Occurrence and relation to wine ageing. *American Journal of Enology and Viticulture*. **50**, 211-218.

Morata, A., Gomez-Cordoves, C., Superviola, J., Bartolome, B., Colomo, B. and Suarez-Lepe, J.A. (2003). Adsorption of anthocyanins by yeast cell walls during the fermentation of red wines. *Journal of Agricultural and Food Chemistry*. **51**, 4084-4088.

Moreno-Arribas, M.V., Gomez-Cordoves, C. and Martin-Alvarez, P.J. (2008). Evolution of red wine anthocyanins during malolactic fermentation, postfermentative treatments and ageing with lees. *Food Chemistry*. **109**, 149-158.

Moutounet, M., Sarni, F. and Scalbert, A. (1993). L'élevage des vins rouges en fût du chêne. In: Elevatge des vins en fûts de chêne. Y.Glories (Ed.). CEPS, Melun.

Nagel, C.W. and Glories, Y. (1991). Use of a modified dimethylaminocinnamaldehyde reagent for analysis of flavanols. *American Journal of Enology and Viticulture*. **42**, 364-366.

Nagel, C.W. and Wulf, W. (1979). Changes in the anthocyanins, flavonoids and hydroxycinnamic acid esters during fermentation and aging of Merlot and Cabernet Sauvignon. *American Journal of Enology and Viticulture*. **30**, 111-116.

Noble, A.C. (1990). Bitterness and astringency in wine. *In* Bitterness in Foods and Beverages. R.L. Rouseff (Ed.), pp. 145-158. Elsevier Science, Amsterdam.

Peng, Z., Hayasaka, Y., Iland, P.G., Sefton, M., Hoj, P. and Walters, E.J. (2001). Quantitative analysis of polymeric procyanidins (tannins) from grape (Vitis vinifera) seeds by reverse phase high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry*. **49**, 26-31.

Peng, Z., Iland, P.G., Oberholster, A., Sefton, M.A. and Waters, E.J. (2002). Analysis of pigmented polymers in red wine by reverse phase HPLC. *Australian Journal for Grapegrowers and Winemakers Research*. **8**, 70-75.

Pérez-Magariño, S., Sánchez-Iglesias, M., Ortega-Heras, M., Gonzáles-Huerta, C. and Gonzáles-Sanjosé, M.L. (2007). Colour stabilization of red wines by microoxygenation treatment before malolactic fermentation. *Food Chemistry*. **101**, 881-893.

Peyrot Des Gachons, C. and Kennedy, J.A. (2003). Direct method for determining seed and skin proanthocyanidin extraction into red wine. *Journal of Agricultural and Food Chemistry*. **51**, 5877-5881.

Porter, L.J. and Woodruffe, J.(1984). Haemanalysis: the relative astringency of proanthocyanidin polymers. *Phytochemistry*. **23**, 1255-1256.

Price, S.F., Breen, P.J., Vallado, M. and Watson, B.T. (1995). Cluster sun exposure and quercetin in Pinot noir grapes and wine. *American Journal of Enology and Viticulture*. **46**, 187-194.

Prieur, C., Rigaud, J., Cheynier, V. and Moutounet, M. (1994). Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry*. **36**, 781-784.

Ribereau-Gayon, P., Glories, Y., Meujean, A and Dubourdieu, D. (2006). Handbook of Enology, Vol 2. The chemistry of wine stabilisation and treatments. John Wiley and Sons, Ltd. New York. p 141-203.

Ribereau-Gayon, P. and Stonestreet, E. (1965). Le dosage des anthocyanes dans les vins rouges. *Bulletin Society Chimica*. **9**, 2649-2652.

Robichaud, J.L. and Noble, A.C. (1990). Astringency and bitterness of selected phenolics in wine. *Journal of the Science Food and Agriculture*. **53**, 343-353.

Romero-Cascales, I., Ortega-Regules, A., Lopez-Roca, J.M., Fernandez-Fernandez, J.I. and Gomez-Plaza, E. (2005). Differences in anthocyanin extractability from grapes to wines according to variety. *American Journal of Enology and Viticulture*. **56**, 212-219.

Sacchi, K.L., Bisson, L.F. and Adams, D.O. (2005). A review of the effect of winemaking techniques on phenolic extraction in red wines. *American Journal of Enology and Viticulture*. **56**, 197-206.

Sartini, E., Arfelli, G., Fabiani, A. and Piva, A. (2007). Influence of chips, lees and micro-oxygenation during aging on the phenolic composition of a red Sangiovese wine. *Food Chemistry*. **104**, 1599-1604.

Scheffieldt, P. and Hrazdina, G. (1978). Co-pigmentation of anthocyanins under physiological conditions. *Journal of Food Science*. **43**, 517-520.

Schneider, V. (1998). Must hyperoxidation: a review. *American Journal of Enology and Viticultre*. **49**, 65-73.

Silva Ferreira, A.C., Barbe, J.C. and Bertrand, A. (2002). Kinetics of oxidative degradation of white wines and how they are affected by selected technological parameters. *Journal of Agricultural and Food Chemistry*. **50**, 5919-5924.

Singleton, V.L. and Draper, D.E. (1964). The transfer of polyphenolic compounds from grape seeds into wines. *American Journal of Enology and Viticulture.* **15**, 34-40.

Singleton, V.L. and Esau, P. (1969). Phenolic substances in grapes and wine and their significance. *American Journal of Enology and Viticulture.* **18**, 1-261.

Singleton, V.L. and Rossi, J.A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*. **16**, 144-158.

Singleton, V.L. (1987). Oxygen with phenols and related reactions n musts, wines, and model systems: Observations and practical implications. *American Journal of Enology and Viticulture*. **38**, 69-77.

Singleton, V.L. (1992). Tannins and the qualities of wines. *In*: Plant Polyphenols. R.W Hemingway and P. E. Laks (Eds.). pp. 859-880. Plenum Press, New York.

Singleton, V.L., Orthofer, R. and Lamuela-Raventos, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology*. **299**, 152-178.

Slinkard, K. and Singleton, V.L. (1977). Total phenol analysis: Automation and comparison with manual methods. *American Journal of Enology and Viticulture*. **28**, 49-55.

Somers, T.C. and Evans, M.E. (1977). Spectral evaluation of young red wines: anthocyanin equilibria, total phenolics, free and molecular SO2, “chemical age”. *Journal of the Science of Food and Agriculture*. **28**, 279-287.

Souquet, J.M., Cheynier, V., Brossaud, F. and Moutounet, M. (1996). Polymeric proanthocyanidins from grape skins. *Phytochemistry*. **43**, 509-512.

Souquet, J.M., Labarbe, B., Le Guerneve, C., Cheynier, V. and Moutounet, M. (2000). Phenolic composition of grape stems. *Journal of Agricultural and Food Chemistry*. **48**, 1076-1080.

Thorngate, J.H. and Singleton, V.L. (1994). Localization of procyanidins in grape seeds. *American Journal of Enology and Viticulture*. **45**, 259-262.

Timberlake, C.F. and Bridle, P. (1976). Interactions between anthocyanins, phenolic compounds and acetaldehyde. *American Journal of Enology and Viticulture*. **27**, 97-105.

Timberlake, C.F. and Bridle, P. (1977). Anthocyanins: colour augmentation with catechin and acetaldehyde. *Journal of the Science of Food and Agriculture*. **28**, 539-544.

Vidal, S., Francis, L., Noble, A., Kwiatkowski, M., Cheynier, V. and Waters, E. (2004). Taste and mouth-feel properties of different types of tannin-like polyphenolic compounds and anthocyanins in acid solution. *Journal of Agricultural and Food Chemistry*. **29**, 563-567.

Vivas, N. (2000). Apports récents à la connaissance du chêne de tonnellerie et à l’élevage des vins rouges en barriques. *Bulletin de l’OIV*, **73**, 79-108.

Vivas, N. and Glories, Y. (1996). Role of oak wood ellagitannins in the oxidation process of red wines during ageing. *American Journal of Enology and Viticulture*. **47**, 103-107.

Waterhouse, A.L. and Laurie, V.F. (2006). Oxidation of wine phenolics: a critical evaluation and hypothesis. *American Journal of Enology and Viticulture*. **57**, 306-313.

Yokotsuka, K., and Singleton, V.L. (2001). Effects of seed tannins on enzymatic decolorization of wine pigments in the presence of oxidizable phenols. *American Journal of Enology and Viticulture*. **52**, 93-100.

Chapter 3

Research results

The effect of different oxygen dosages on the phenolic composition of wines made with different grape seed concentrations

Chapter 3: The effect of different oxygen dosages on the phenolic composition of wines made with different grape seed concentrations

3.1 Introduction

Phenols, and more specifically tannins, are valued for their involvement in oxidation and polymerisation reactions to produce more stable compounds and improve wine colour. The latter is greatly influenced by malolactic fermentation where there is normally a colour decrease due to an increase in pH and a subsequent shift in the equilibrium of the anthocyanins to the colourless form (Bousbouras and Kunkee, 1971). Anthocyanins are the most significant colour components in red wine. Monomeric anthocyanins can participate in reactions during fermentation or ageing to form more complex polymeric pigments. The latter can arise from the interaction between anthocyanins and other phenolic compounds, primarily the flavan-3-ols. Several authors have confirmed the mechanisms that involve the formation of these more stable polymers (Alcalde-Eon *et al.,* 2006; Fulcrand *et al*., 2006; Remy *et al*., 2000 and Romero and Bakker, 2000). The proposed formations involves direct reactions between anthocyanins and flavanols, reactions involving acetaldehyde to form anthocyanin-tannin adducts linked by an ethyl-bridge and the formation of pyranoanthocyanins through the reaction between anthocyanins and other compounds such as vinylphenols in which acetaldehyde may also be involved (Ribereau-Gayon *et al*., 2006). These reactions have one contributing factor in common; they produce more stable compounds that stabilises wine colour and are more resistant to the effects of pH and decolouration by sulphur dioxide.

Oxygen plays a crucial role in some of these phenolic reactions and introducing oxygen to red wine has become increasingly popular, using a variety of means to simulate the advantages of barrel maturation (Waterhouse and Laurie, 2006). The technique developed by Moutounet and Ducournau in 1990 can vary in oxygen dosage and there have been experimented with the application thereof from after fermentation to before bottling (Fell *et al*., 2007). It is still unclear to what extent a wine is capable of consuming oxygen (Singleton, 2000). However, it seems that a wine is more buffered against larger oxygen dosages during the earlier winemaking stages. The various advantages of adding oxygen involves larger polymeric phenol content, stabilised wine colour and a reduction in the astringency of wines (Castellari *et al*., 2000; McCord, 2003).

Even though only a few studies have examined the impact of seed tannins in winemaking on the phenolic composition of red wine, all authors have reported the significant influence of either seed removal or the addition of seeds (Berg and Akiyoshi, 1956; Meyer and Hernandez, 1970; Kovac *et al*., 1992; Kovac *et al*., 1995; Peyrot des Gachons and Kennedy, 2003; Canals *et al*., 2008).

Grape seeds are richer in catechins (monomeric flavan-3-ols) and proanthocyanidins than skins or pulps, in both red and white grapes (Lea *et al*., 1979). The composition of grape seeds consists of condensed tannins which comprise of flavan-3-ol monomer subunits. Kovac et al. (1991) found that the condensed tannin concentration in the wine increased with an increase in grape seed concentration or the length of maceration. Seed and skin tannins can be distinguished by the presence of (-)-epicatechin gallate in the seeds and (-)-epigallocatechin in the skins (Lee *et al*., 2008). Additionally, recent work has shown that they differ in their potential to be oxidised (Peyrot des Gachons and Kennedy, 2003).

There is limited published literature regarding the contribution of grape seeds to wine and how oxygen affects these compounds originating from the seeds, especially over time (Singleton and Esau, 1969; Peynaud, 1971; Paronetto, 1977). Therefore, we have studied the effect of oxygen additions after alcoholic fermentation and malolactic fermentation on two different red wine cultivars' made with different grape seed concentrations on colour and phenolic composition.

3.2 Materials and Methods

3.2.1 Preparation of wine

In 2007, 54 kg of Cabernet Sauvignon grapes were harvested by hand from Tokara Wine Estate in Stellenbosch when the sugar concentration reached 23°B. The 54 kg grapes were crushed, the juice pressed and kept separately. The skins were then divided into three equal parts (container A, B and C). The seeds from part A was manually removed and added to the skins of part C. The juice was then equally divided between the three containers. The three containers thus had the same ratio of juice to skins, but one container did not contain any seeds (A), the other had normal amounts of seeds (B) and the last (C) double the normal amount of seeds. Sulphur dioxide (SO_2) was added (20ppm) and the SO_2 levels were confirmed by a Metrohm titration unit (Metrohm, Ltd., Switzerland). The three containers were stored at 4°C to allow cold maceration for two days. After the cold soaking period the must was left at room temperature overnight to reach ambient temperature for yeast inoculation. Total acidity was adjusted to 6 g/L using tartaric acid. Refer to Table 3.1 for analysis results of the juice for which a grapescan FT 120 instrument was used (Foss Electric, Denmark) (Niewoudt *et al*., 2004). The musts were inoculated with *Saccharomyces cerevisiae* yeast NT 50 at 0.25 g/L according to the supplier's recommendations (Anchor Yeast, Biotechnologies, South Africa) and left at 25°C to ferment. With the commencement of fermentation, the wines were kept at 15°C. A nitrogenous source, diammonium phosphate (DAP) (0.2 g/L), was added with the yeast and after 6 days of fermentation when the sugar concentration reached 14°B to each container. Alcoholic fermentation was considered completed when the sugar content of the wine was below 4 g/L. The FT 120 analyses showed that the wines were fermented dry (<4 g/L). The fermented skins in each container was pressed using a small scale basket press with a maximum pressure of 1.7 bars. Approximately 10 L of wine was obtained from each container.

The completion of alcoholic fermentation allowed us to start the addition of oxygen. In 2007 we only added one oxygen dosage, 4 mg/L per day, over four consecutive days (thus a total of 16 mg/L). The experiment with the control and oxygenated wines was performed in triplicate in 750 ml dark green glass bottles. The oxygen concentration was measured in all the wines before and after the application of oxygen using an Oxi 330i oxygen meter with a cell ox 325 probe (Wissenschaftlich-Technische Werkstatten). Oxygen was added by decanting the wine into a plastic bucket until the desired oxygen level was reached.

In the 2008 season 100 kg of Pinotage grapes, collected from the Wellington region, with a sugar concentration of 29.5°B was used. This was problematic, as we risked having an alcohol concentration of possibly more than 16 % and the wine may not have been able to complete malolactic fermentation. We thus diluted the juice with water to a sugar concentration of 25°B after the seeds were removed from the berries in the same manner as in 2007. The juice and skins were then equally divided into 20L containers. As the grapes were visually more rotten than in 2007 we added 30 ppm of sulphur dioxide. The seeds were divided into three parts as described in the 2007 Cabernet Sauvignon experiment. We allowed two days of cold

maceration for colour extraction at 4°C. Routine analyses (pH, TA, VA and sugar concentration) were done using a grapescan FT 120 instrument (Foss Electric., Denmark) and the TA was again adjusted to 6 g/L. The must was then kept at room temperature overnight and inoculated with *Saccharomyces cerevisiae* NT 50 yeast (0.25 g/L) the next morning and left to ferment at 25°C for one day to allow a trouble-free fermentation. It was then placed at 15°C to ferment dry. Fermentation was completed in eight days when the sugar concentration was below 4 g/L.

In 2008 we applied two oxygen additions, 16 mg/L and 32 mg/L, which were given over a time period of eight days. Thus, 4 mg/L and 8 mg/L oxygen were given every second day in the 16 mg/L dosage and 32 mg/L treatments, respectively. This allowed the wine to consume the oxygen over two days and not one, making it easier to apply the correct oxygen dosage.

The 2008 Cabernet Sauvignon was made in the same manner as the Cabernet Sauvignon in 2007, only double the amount of grapes was used and 30 ppm sulphur dioxide added. The TA of the juice measured after cold maceration was 3.50, 3.50 and 4.51 g/L for the no seed, normal seed and twice the normal seed wines respectively. We wanted to adjust it to 6 g/L, but made a calculation error and wrongly adjusted the TA to 8.27, 7.98 and 7.25 g/L respectively. However, after alcoholic fermentation the TA showed to be around 6 g/L (± 0.30 g/L). All experiments were performed in triplicate.

Table 3.1: Composition of the must before adjustment of the TA in 2007 and 2008.

Grapes	pH	°B (juice)	TA
2007 Cabernet Sauvignon	3.72	23.3	4.48
2008 Cabernet Sauvignon	3.82	23.2	3.80
2008 Pinotage	3.82	29.5	2.91

3.2.2 Malolactic fermentation

All the wines underwent malolactic fermentation (MLF) after the oxygen treatments. The wines were inoculated with CH16 (Christiaan Hansen) (*Oenococcus oeni*) at 1 g/hl. Malolactic fermentation was conducted at 20°C. Malic and lactic acid concentrations were monitored using a grapescan FT 120 instrument (Foss Electric, Denmark) (Nieuwoudt *et al*., 2004). MLF was considered to be completed when the malic acid concentration was lower than 0.3 g/L. Malolactic fermentation was completed within 34 days.

3.2.3 Ageing and bottling

The wine made in 2007 were not bottled or matured after malolactic fermentation. No SO_2 was added to the wine after alcoholic or malolactic fermentation. However, in 2008, the wine's free sulphur dioxide were adjusted to 40 ppm after malolactic fermentation, filtered through diatomaceous earth and sheet filter and bottled in 750 ml dark green bottles with screw caps. These wines were stored at 15°C for two months.

3.2.4 Wine sampling

Samples of the control and oxygenated wines were taken before and after oxygenation, after malolactic fermentation (before SO_2 addition) and after ageing. Samples were frozen immediately in 100 ml glass vials and stored at -20°C.

3.2.5 Spectrophotometric analyses

All spectrophotometric analyses were performed using an Analytic Jena Specord 50 UV/VIS spectrophotometer (Jena, Germany). Depending on the density of the wine or the required wavelength of the analysis the following cuvettes were used: 1 mm and 10 mm quartz cuvettes, 1 mm glass cuvettes or 10 mm plastic cuvettes.

In 2007, the measurements were conducted on control and treated samples taken after the addition of oxygen, and after MLF. In 2008, samples were taken before and after oxygen addition, after MLF and two months after storage in bottles. Samples were immediately stored at -20°C in 100 ml glass vials. All of the following analyses were done at the same time to quantify all samples under the same conditions or with the same calibration curve if necessary. Samples were centrifuged prior to analysis for 2 min at 2000 rpm to remove any solid particles that could influence the measurement. All analyses were performed in triplicate.

3.2.5.1 Colour intensity, optical densities and hue

Colour density was calculated as the sum of the measurements taken at 420 nm, 520 nm and 620 nm as described by Boulton (2001). Because of the density of the wine, the samples were measured in 1 mm glass cuvettes and the absorbance values multiplied with 10 to be in agreement with the Beer-Lambert Law. The 420 nm is the maximal absorbance for the yellow/brown colour, 520 nm measures red colour and 620 nm represents the purple/blue colour. The hue indicates the development of a brownish colour and is calculated by the absorbance at 420 nm divided by 520 nm (Somers and Evans, 1977).

3.2.5.2 Total anthocyanins

Total anthocyanin concentration was determined using the method described by Ribéreau-Gayon and Stonestreet (1965) that relies on the properties of anthocyanins to vary in colour according to the pH and to be bleached by sulphur dioxide. Six glass test tubes per individual sample were prepared, all containing 5 ml of 1 M hydrochloric acid, 250 µl of 0.1% hydrochloric acid made up with 96 % ethanol and 250 µl of the undiluted centrifuged wine. In three test tubes 2 ml water were added, while in the other three test tubes, 2 ml of freshly prepared 5% sodium bisulphite were added. The samples were vortexed and left to stand for 20 min after which the absorbance of samples were measured at 520 nm in 1 cm plastic cuvettes. The final absorbance reading is multiplied by a constant, 875, to obtain the anthocyanin concentration in mg/L. The method measures mainly anthocyanins, but a small percentage of polymeric pigments may also be included in this measurement.

3.2.5.3. Total phenols and total red colour

The method was developed by Somers and Evans (1977). The centrifuged wine (50 µL) was diluted a 100 times with the addition of 5 ml of 1 M hydrochloric acid that lowers the pH below 1.0 where all the anthocyanins are in the coloured flavylium form. The sample was left for 3 hours at room temperature to allow all anthocyanins and pigments to change into the red flavylium form. The analysis was performed in triplicate and the sample was measured at 280 nm and 520 nm in a 1 cm quartz cuvette. The absorbance values were multiplied by 101 to consider the dilution.

3.2.5.4 DMAC Index

Monomeric flavanols can be measured with the aldehydic reagent, 4-dimethyl-aminocinnamaldehyde (DMAC), which reacts with the aromatic ring on all free meta-hydroxyl

groups on the A-ring in an acidic medium to determine monomeric flavan-3-ols, as described by Nagel and Glories (1991). Due to this mechanism, proanthocyanidins are also included in this measurement, but react with DMAC to a much lesser extent than monomeric flavan-3-ols. Anthocyanins and flavonols are excluded due to their electron-withdrawing functional groups.

The DMAC reagent was prepared by dissolving 0.25 g of the *p*-dimethylamino-cinnamaldehyde (DMAC) in 500 ml of the HCl-MeOH (250 ml of concentrated hydrochloric acid (32%) made up to 1 L by using methanol). (+)-Catechin hydrate (Fluka) was used to construct a calibration curve ranging in concentration between 2.5 mg/L and 50 mg/L for the determination of the final concentration in mg/L CE (catechin equivalents). The absorbance was measured at 640 nm after exactly 2 minutes at room temperature. For further details refer to Table 3.2. The analysis was performed in triplicate for each sample. Due to the high proanthocyanidin content in grape seeds and its importance in the formation of wine tannins we found it necessary to also quantify these compounds with HPLC.

Table 3.2: Specific volumes of solvents used to perform the DMAC analysis.

Test tube	Catechin std	Samples	Solvent	DAC	HCl-MeOH	Total Volume
Cal. blank			500 µL	2.5 ml		3 ml
Std. curve	500 µL			2.5 ml		3 ml
Sample		500 µL		2.5 ml		3 ml
Sample blank		500 µL			2.5 ml	3 ml

3.2.5.5 Folin-Ciocalteu

The total phenol concentration was quantified using the method developed by Folin-Denis, but later improved by Folin-Ciocalteu (Singleton and Rossi, 1965). The reagent, phosphotungstic-phosphomolybdic acid oxidises phenols and is itself reduced to a blue molybdenum tungsten complex. All hydroxyl groups are oxidised in this method, thus it can be an over estimation of the phenols present (Singleton and Rossi, 1965; Singleton *et al*., 1999).

The standard samples and wine were prepared in the same way. The standard curve was constructed using gallic acid (Fluka) ranging in concentration from 0 to 2000 mg/L. Red wine samples were centrifuged for 2 min at 2000 rpm and diluted 10 times in a 10 ml volumetric flask. A 100 µL of each diluted sample was then added to a 10 ml volumetric flask with 6 ml of distilled water, 500 µL of undiluted Folin-Ciocalteu reagent (Fluka) and 1.5 ml of 20 % sodium bicarbonate. The volume of the flask was adjusted to exactly 10 ml and then vortexed. After 2 hours at room temperature, the absorbance of the sample was read at 765 nm.

3.2.5.6 Tannins

The tannin content of a wine can be quantified by using its capacity to bind protein and the precipitation of the resulting protein-tannin complex. The assay was developed by Hagerman and Butler (1978) to distinguish between monomeric anthocyanins and polymeric pigments. The method was later improved by Adams *et al.* (1999) and Harbertson *et al.* (2002) to combine the assay with bisulphate bleaching to determine the monomeric pigments.

When necessary, wines were diluted with a buffer of 12 % aqueous ethanol (v/v) containing 5 g/L potassium bitartrate adjusted to pH 3.3 with HCl. Refer to Table 3.3 for the detailed description of the solutions used. It is important to establish the dilution range for each specific wine, since an over estimation of the tannin concentration in a wine could occur. Tannin

precipitation was carried out using bovine serum albumin (BSA) (1 mg/ml) in a buffer containing 200 mM acetic acid and 170 mM NaCl adjusted to pH 4.9. A 2 ml microfuge tube contained 1 ml of the acetic acid/NaCl buffer with BSA (1 mg/ml) and 500 µL of the diluted/undiluted wine. The mixture was allowed to stand at room temperature for 15 min and then centrifuged for 5 min at 14 000 rpm to retain a pellet due to the formation of the tannin-protein complex. The supernatant was discarded, and the pellet washed twice with 1 ml of Buffer A and then centrifuged at 14 000 rpm for 1 min. The supernatant was once again discarded and 875 µL of Buffer C containing 5% triethanolamine (v/v) (TEA) and 5% sodium dodecyl sulphate (w/v) (SDS) were added to the pellet in the microfuge tube. The solution was incubated for 10 min at room temperature. After the incubation period, the sample was vortexed to completely dissolve the pellet and allowed to stand at room temperature for another 10 min. After the elapsed time, the absorbance of the sample was measured at 510 nm, and noted as the background reading. To the same cuvette, 125 µL of 10 mM ferric chloride in 10 mM HCl was added and left to incubate for 10 min. The final absorbance measurement was then taken at 510 nm.

The amount of protein precipitable tannin in the sample was calculated as the final absorbance minus the background and plotted onto a standard curve constructed with (+)-catechin hydrate (Fluka) to express the final concentration as mg/L catechin equivalents (CE). Catechin was used as a standard since it is found as one of the subunits and cannot precipitate protein by itself. Catechin concentrations ranging from 50 mg/L to 300 mg/L were used by combining aliquots of a 1 mg/ml solution of catechin in 10 % ethanol with the TEA/SDS buffer to give a final volume of 875 µL. Then 125 µL of the ferric chloride reagent was added and the mixture was vortexed and incubated for 10 min at room temperature, before the reading was taken at 510 nm. A zero catechin sample was prepared by adding 125 µL of the ferric chloride reagent to 875 µL of the TEA/SDS buffer and the absorbance of this solution was subtracted from each of the points on the standard curve.

Table 3.3: Reagents used for tannin determination by BSA precipitation.

Solution name/final solution concentration	Reagent and reagent volume (µL, ml) or mass (g,mg)	Final volume (ml)
Buffer A (200 mM Acetic acid and 170 mM NaCl)	Glacial acetic acid (6.0 ml) NaCl (4.97 g) 10 % NaOH (adjust to pH 4.9)	Fill to 500 ml with DI* H_20
Buffer B 12 % EtOH 5 g/L potassium bitartrate	Potassium bitartrate (2.5 g) 96 % Ethanol (60 ml) 2.0 N HCl (adjust to pH 3.3)	Fill to 500 ml with DI H_20
Buffer C 5 % triethanolamine (v/v) 5 % SDS	Sodium dodecyl sulphate (25 g) Triethanolamine (25 ml) 2.0 N HCl (adjust to pH 9.4)	Fill to 500 ml with DI H_20
Ferric chloride 0.01 N HCl 10 mM $FeCl_3$	Ferric chloride hexahydrate(0.27 g) 12.1 N HCl (80 µL)	Fill to 100 ml with DI H_20
Catechin 1 mg/ml (+)-catechin 10 % Ethanol (v/v)	(+)-Catechin (50 mg) 96% Ethanol (5.0 ml)	Fill to 50 ml with DI H_20
Protein 1 mg/ml BSA	Bovine serum albumin (50 mg)	Fill to 50 ml with DI H_20

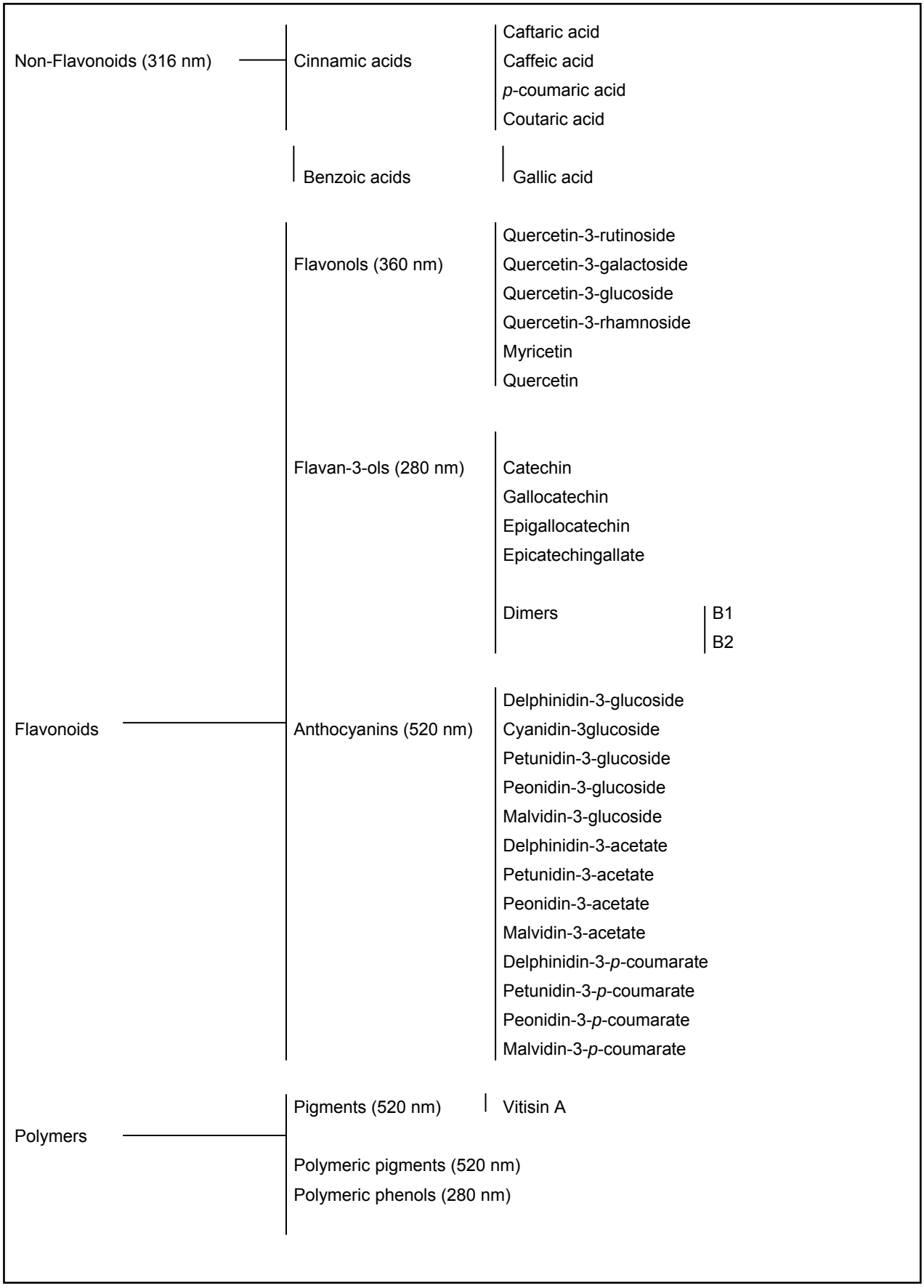
*DI represents de-ionized water

3.2.6 HPLC analysis

Reverse phase high performance liquid chromatography (RP-HPLC) was performed on a Hewlett Packard Agilent 1100 series HPLC system equipped with a diode array detector (Agilent Technologies, Palo Alto, CA, USA). Data processing was done with Chemstation software (Hewlett Packard, Waldbronn, Germany). Separations were carried out on a polystyrene/divinylbenzene reversed phase column (PLRP-S, 100Å, 250 × 4.6 mm, 5 µm) from Polymer Laboratories (Ltd) (Shropshire, UK) protected with a guard cartridge (PLRP-S, 10 × 4.6 mm) (Polymer Laboratories (Ltd), Shropshire, UK) with the same packing material. The following mobile phases were used: solvent A, containing de-ionised water with 1.5% v/v ortophosphoric acid (Reidel-de Haën) and solvent B consisting of 80% acetonitrile (Chromasolve, Reidel-de Haën) with 20% of solvent A. A linear gradient was used from 0 min, A 94%, B 6%; to 73 min, A 69%, B 31%; to 78 min, A 38%, B 62%, staying constant for 8 min to 86 min and then back to the starting conditions in 4 min to 90 min, A 94%, B 6%. A flow rate of 1 ml/min was used and a column temperature of 35^{o}C. This was adapted from the method of Peng *et al.* (2002).

Phenols were quantified using external standards: (+)-catechin hydrate (Fluka), (-)-epicatechin (Sigma), gallic acid (Fluka), caffeic acid (Sigma), *p*-coumaric acid (Sigma), malvidin-3-glucoside (Polyphenols Laboratories AS, Norway), quercetin-3-glucoside (Fluka) and quercetin (Extrasynthése, France). Monomeric and dimeric flavanols and polymeric phenols were quantified at 280 nm as mg/L catechin units with a quantification limit of 1.5 mg/L, and epicatechin as epicatechin with a quantification limit of 1.5 mg/L. Cinnamic acids have a maximal absorbance at 316 nm and caftaric acid and caffeic acid were quantified as mg/L caffeic acid, while coutaric acid and *p*-coumaric acid were expressed as mg/L *p*-coumaric units with a quantification limit of 0.275 mg/L. Flavonol-glycosides and flavonol aglycones were quantified at 360 nm as respectively mg/L quercetin-3-glucoside and mg/L quercetin with a quantification limit of 0.05 mg/L. Anthocyanins, pigments and polymeric pigments were quantified at 520 nm as mg/L malvidin-3-glucoside with a quantification limit of 1.25 mg/L. The quantification limit for gallic acid was 0.25 mg/L. The samples were unfrozen and centrifuged for 5 min at 14000 rpm before injection. Thereafter each sample was placed in a 1.5 ml dark coloured vial and protected with nitrogen gas from oxidation. Table 3.4 categorises the individual compounds as analysed by RP-HPLC. The limit of detection was defined as a signal to noise ratio of 3. The limit of quantification was determined as the smallest area that could be accurately integrated (<3% standard deviation).

Table 3.4: Division of compounds measured by HPLC. The cinnamic acids were combined to calculate the non-flavonoids.



3.2.7 Statistical Analysis

Mean values and standard deviations were calculated using one-way analysis of variance (ANOVA). The significance of differences between mean values obtained was determined using a student t-test at the 95% significance level, $p < 0.05$.

3.3 Results and discussion

3.3.1. Colour intensity, optical density and hue

The wines to which no seeds were added will be referred to as the 0x seeds, where the original amount of seeds were present, 1x seeds, and where double the amount of seeds were added, 2x seeds. For the 2007 Cabernet Sauvignon, there was a significant increase in colour intensity (CI) with an increase in seed concentration. This observation was more apparent between 0x and 1x seeds, with smaller differences between 1x and 2x seeds. Kovac *et al.* (1992) found that adding supplementary seeds lead to wines with higher colour intensities. The addition of oxygen had an immediate effect on the colour intensity, as these wines already showed higher colour intensity and optical density absorbance before MLF compared to the control (Addendum A, Table 7.1-7.5).

All the treatments, except the oxygenated wines of the 0x seeds, decreased significantly in colour intensity with MLF ($p < 0.05$). Atanasova *et al.* (2002) and Gomez-Plaza *et al.* (2004) postulated this occurrence to be due to the oxidation of anthocyanins and/or polymerisation and copigmentation of the anthocyanins with tannins. However, the completion of MLF also leads to an increase in wine pH and therefore, shifting the anthocyanin equilibrium to obtain higher concentrations in the colourless and purple anthocyanin form. In all treatments the oxygenated wines decreased more drastically in colour intensity with MLF than the control. After MLF the 1x and 2x seed treatments had similar colour intensities, but the 0x seeds were significantly lower. This observation indicated that the catechins and proanthocyanidins that were extracted from seeds contributed to wine colour as they combine with other pigments, such as anthocyanins to stabilise wine colour.

The control wines had a significantly lower colour hue (Addendum A, Fig 7.5) before MLF compared to the oxygenated wines, but this changed as the hue for the control of all seed treatments increased significantly to the end of MLF. This was especially true for the 0x and 1x seed wines. Colour hue for the oxygenated wines either remained similar (0x and 1x seed wines), or decreased (2x seed wines) with MLF. After MLF the control and oxygenated wines reached values not significantly different from each other for each seed treatment, except in the 2x seed treatment, although these differences are small in winemaking terms. Ribéreau-Gayon *et al.* (2006) indicated that the values for a young wine should be between 0.5 and 0.7, which corresponds with our wine data (0.54-0.67). However, the larger increase in hue for the control wines compared to the oxygenated wines in the 2x seed wine during MLF were unexpected. We would expect a higher hue due to the formation of polymeric pigments such as the pyranoanthocyanins in the oxygenated wines, which absorb relatively more light at 420 nm than at 520 nm (Singleton, 2000). However, a decrease in red colour (520 nm) can also lead to an increase in colour hue, which happened in most of these samples.

For the 2008 vintages we obtained lower general values for colour intensity when compared to the 2007 Cabernet Sauvignon wines (Addendum A, Table 7.1-7.5). It is evident that both oxygen and seed concentration had a more remarkable effect on the colour intensity for the 2007 vintage. De Freitas and Glories (1999) have shown that there can be considerable

variation in tannin composition of seeds when wines are made over different seasons. Another major contributing factor is that the Cabernet Sauvignon grapes of 2007 and 2008 came from different vineyards.

The wines in 2008 showed a different trend toward colour intensity. In the Cabernet Sauvignon there was a significant drop in CI from before MLF (±13.0 AU units) until after MLF (±7.0 AU units) in all treatments, but the colour intensity increased again with ageing in the 1x (±10.0 AU units) and 2x (±9.0 AU units) seed wines (Addendum A, Fig 7.1-7.5). However, the 0x seed wines decreased even further after two months of ageing. This could possibly be explained by the low proanthocyanidin concentration present in the 0x seed wine, which were insufficient to stabilise wine colour. Sufficient flavan-3-ol monomers and proanthocyanidins were present in the other treatments to polymerise and form copigments with pigments and thus stabilise wine colour. Colour intensity was also significantly higher in the 2x seed treatments after MLF where oxygen was added ($p = 0.002$).

In the 2008 Pinotage, such a sudden decrease in colour intensity after MLF was not observed (Addendum A, Table 7.1-7.5). All treatments increased significantly in colour intensity during ageing, especially in the oxygenated wines. This is in agreement with work done by other authors (Cano-Lopez *et al*., 2006; Sartini *et al*., 2007), as colour intensity is increased due to the formation of red polymers.

The colour hue did not follow the same trend in the 2008 wines as for the 2007 Cabernet Sauvignon. All the hues of the 2008 wines increased from before MLF until after MLF, and then decreased again after a two month ageing period. This fluctuation over time was more apparent in the 1x- and 2x-seeded wines. Singleton (2000) reported that colour hue was statistically lower in oxygenated wines, although other authors found the opposite to be true (Alcalde-Eon *et al*., 2006; Cano-Lopez *et al*., 2006).

3.3.2 Anthocyanin and polymeric pigment concentrations

After the completion of alcoholic fermentation, there was a significant higher monomeric anthocyanin concentration with an increase in seeds for the 2007 Cabernet Sauvignon wines when measured with spectrophotometry (Table 3.5). The anthocyanin concentration, when measured with spectrophotometry, decreased with MLF and was always significantly lower in the oxygenated wines after MLF. Kovac *et al.* (1995) showed that a wine's colour is stabilised when twice the normal amount of seeds are added and that the anthocyanin concentration increased with seed concentration, especially so regarding Cabernet Sauvignon. Our results are in agreement with work done by Canals *et al.* (2008) who added supplementary seeds to red wine and found an increase in anthocyanin concentration. Fulcrand *et al.* (2006) stipulated that higher procyanidin concentrations (as seen in section 3.3.3) exert a protective effect against oxidation of anthocyanins, as found in our study. The decrease in monomeric anthocyanins found in the oxygenated wines is in agreement with work done by other authors (Cano-Lopez *et al*., 2006; Fell *et al*., 2007; Jordao *et al*., 2006; Perez-Magarino *et al*., 2007 and Sartini *et al*., 2007).

Monomeric anthocyanin concentration determined by RP-HPLC were the lowest when wines were made without seeds (Table 3.5). This is due to the larger concentration of copigments extracted from the seeds which resulted in higher solubility of the anthocyanins in the treatments with seeds (Goto, 1987; Kovac *et al*., 1992). A study by Canals *et al*. (2008) showed that removing 80% of the seeds led to a significant decrease in anthocyanin concentration. In our study, the addition of 2x seeds did not contribute to a higher free anthocyanin concentration compared to the 1x seeds. The addition of oxygen decreased the free anthocyanin concentration significantly in all treatments before MLF, which were also found by other authors

(Cano-Lopez *et al*., 2006; Fell *et al*., 2007). After MLF all oxygenated treatments still had significantly lower monomeric anthocyanin concentrations, except for the 0x seed treatment. The losses in monomeric anthocyanins were probably due to their participation in direct and indirect polymerisation reactions that took place between proanthocyanidins and anthocyanins (Kennedy *et al*., 2004; Ribereau-Gayon *et al*., 2006). From Table 3.5 it is evident that as monomeric anthocyanins decreased, polymeric pigments increased.

The wines made without seeds had significantly lower polymeric pigment concentrations compared to the other seed treatments (Table 3.5). This could be explained by the insufficient amount of procyanidins present to react with anthocyanins and form stable polymeric pigments in the case of wines made without seeds. The other two treatments containing seeds had similar concentrations. The polymeric pigments increased with the application of oxgyen as well as during MLF. The exposure of wine to oxygen leads to the formation of acetaldehyde. This in turn leads to indirect polymerisation reactions with proanthocyanins and anthocyanins that could have contributed to the higher concentration of polymeric pigments observed in the oxygenated wines (Atanasova *et al*., 2002).

A very good correlation was obtained between anthocyanin concentrations measured by spectrometry and RP-HPLC (r^2 = 0.6498).

Table 3.5:. Monomeric anthocyanin and polymeric pigment concentrations (mg/L) as determined by spectrometry (Ribereau-Gayon and Stonestreet, 1965) and RP-HPLC (Peng *et al*., 2002) for the control and oxygenated wines made of different seed concentrations for the 2007 Cabernet Sauvignon.

SPECTROMETRY: MONOMERIC ANTHOCYANINS				**RP-HPLC: MONOMERIC ANTHOCYANINS**				**RP-HPLC: POLYMERIC PIGMENTS**			
*2007 Cabernet Sauvignon				*2007 Cabernet Sauvignon				*2007 Cabernet Sauvignon			
Treatment		Before MLF (mg/L)	After MLF (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)
0x	control	425 ±21^{bd}	362 ± 11^{a}	0x	control	302 ± 6^{c}	217 ± 6^{b}	0x	control	35.4 ± 1.8^{e}	35.4 ± 2.0^{e}
	16 mg/L	408 ± 25^{d}	329 ± 2^{c}		16 mg/L	286 ± 12^{f}	213 ± 3^{b}		16 mg/L	39.1 ± 1.8^{e}	38.5 ± 2.8^{e}
1x	control	457 ± 5^{e}	378 ± 6^{b}	1x	control	398 ± 19^{d}	261 ± 2^{e}	1x	control	61.0 ± 3.7^{ab}	62.9 ± 0.8^{ac}
	16 mg/L	434 ± 13^{b}	336 ± 7^{c}		16 mg/L	354 ± 4^{a}	218 ± 6^{b}		16 mg/L	65.6 ± 2.0^{cd}	71.2 ± 1.0^{f}
2x	control	540 ± 15^{gf}	407 ± 4^{d}	2x	control	359 ± 4^{a}	249 ± 3^{g}	2x	control	58.0 ± 0.9^{b}	62.7 ± 1.5^{ac}
	16 mg/L	512 ± 18^{g}	357 ± 12^{a}		16 mg/L	314 ± 11^{c}	209 ± 1^{b}		16 mg/L	62.2 ± 3.8^{abc}	67.1 ± 4.8^{d}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis. 0, 1 and 2 represents the wines made of different seed concentrations; 16 and 32 represents the 16 mg/L and 32 mg/L oxygen addition.

The 2008 wines (Table 3.6 and 3.7) showed similar trends to the 2007 wine with oxygen and MLF, but the increase in anthocyanin concentration with seed concentration was not as drastic as in the 2007 vintage. The 0x-seed wines still had the lowest concentration of anthocyanins throughout the experiment for the Pinotage (Table 3.7), but, unexpectedly, this was not true for the Cabernet Sauvignon (Table 3.6). This observation in the Cabernet Sauvignon was confirmed by results from RP-HPLC. Anthocyanin concentrations decreased with MLF in both cultivars and declined even further during the ageing period according to spectrometry and RP-HPLC analysis. However, the anthocyanin concentration in the Cabernet Sauvignon seemed to have stabilised, and was less affected by MLF and ageing than Pinotage. The lower anthocyanin concentrations observed after MLF in both vintages can be explained by the possible association of anthocyanins with yeast cells and bacteria cells (Vasserot *et al*., 1997; Medina *et al*., 2005) and polymerisation with tannins. The increase in anthocyanin concentration with seed concentration in some wines could be explained by the association with tannins and/or procyanidins extracted from the seeds, thus stabilising the resulting colour pigment. The increase in free anthocyanins concur with studies by Kovac *et al.* (1991 and 1995). Although the authors did not explain why the anthocyanin concentration increased as the seed concentration increased, they stated that by adding supplementary seeds, the red wine colour was stabilised. A possible explanation for the stabilisation of anthocyanins with higher seed content is the flavan-3-ols can protect the anthocyanins from oxidation due to their higher antioxidant capacity (Rice-Evans *et al*., 1997; Castillo *et al*., 2000). The decrease in anthocyanin concentration with oxygenated wines is most probably due to indirect polymerisation of anthocyanins with tannins, which is catalysed by the production of acetaldehyde (Gonzales-Neves *et al*., 2004 and Monagas *et al*., 2006). This indicates that phenols take part in polymerisation and condensation reactions, especially in the presence of oxygen (Perez-Magarino *et al*., 2007).

The addition of oxygen led to an increase in polymeric pigment concentration which were especially significant in the 2008 Pinotage (Table 3.8). However, unexpectedly, the wines made without seeds had the highest polymeric pigment concentrations in the Pinotage, and the 1x seed treatment had the lowest concentrations. Polymeric pigments can be formed by the reaction of both grape seed and skin tannins (Hayasaka and Kennedy, 2003). Thus, the higher concentrations of polymeric pigments observed in the wines made without seeds could have been due to the polymerisation of skin tannins. Polymeric pigments are expected to increase in the presence of oxygen due to direct and indirect polimerisation reactions favoured by the presence of oxygen (Atanasova *et al*., 2002). During MLF the polymeric pigment content increased, but decreased drastically during the two month ageing period. Polymeric pigments of the Cabernet Sauvignon wine also decreased during ageing, although the differences in concentrations were often small. This decrease indicate precipitation of the polymeric phenols and pigments. This is supported by a simultaneous decrease in the polymeric phenol concentration.

The 2008 Cabernet Sauvignon and Pinotage showed a very good correlation between anthocyanins as measured by spectrometry (Ribereau-Gayon and Stonestreet, 1965) and by RP-HPLC respectively (Peng *et al*., 2002) (r^2 = 0.7149 and r^2 = 0.8392). The anthocyanin concentrations determined by HPLC were much lower than values obtained by spectrophotometry. This is true for both vintages. The spectrophotometrical analysis is known to overestimate the total anthocyanin concentration due to the incorporation of polymerised pigments sensitive to the bleaching effect of bisulphite (Llaudy *et al*., 2006). The RP-HPLC method only measures monomeric anthocyanins, thus resulting in differences in the anthocyanin concentration determined (Rivas-Gonzalo *et al*., 1992; Santos-Buelga and Williamson (2003). Besides, the conversion of spectrophotometric data to anthocyanin

concentration is possibly imprecise due to the many different pigments showing different extinction coefficients that contribute to the absorbance (Canals *et al*., 2008).

Table 3.6: Monomeric anthocyanin concentration (mg/L) as determined by spectrometry (Ribereau-Gayon and Stonestreet, 1965) and RP-HPLC (Peng *et al*., 2002) for the control and oxygenated wines made of different seed concentrations for the 2008 Cabernet Sauvignon during the different stages of winemaking.

SPECTROMETRY: MONOMERIC ANTHOCYANINS					**RP-HPLC: MONOMERIC ANTHOCYANINS**				
*2008 Cabernet Sauvignon					*2008 Cabernet Sauvignon				
Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)
0x	control	646 ± 14^{a}	549 ± 8^{a}	464 ± 45^{bc}	0x	control	339 ± 4^{l}	285 ± 1^{b}	173 ± 27^{cf}
	16 mg/L	630 ± 17^{a}	545 ± 8^{de}	504 ± 14^{fg}		16 mg/L	321 ± 3^{a}	285 ± 2^{b}	187 ± 1^{cd}
	32 mg/L	617 ± 7^{ah}	551 ± 15^{dh}	477 ± 4^{bcf}		32 mg/L	312 ± 5^{a}	281 ± 3^{b}	179 ± 3^{cef}
1x	control	640 ± 16^{dei}	528 ± 6^{eij}	502 ± 22^{fk}	1x	control	312 ± 4^{a}	241 ± 7^{ij}	165 ± 3^{f}
	16 mg/L	579 ± 45^{dei}	535 ± 10^{ij}	496 ± 5^{bfk}		16 mg/L	281 ± 25^{bh}	254 ± 3^{gj}	172 ± 1^{cf}
	32 mg/L	592 ± 2^{jl}	542 ± 14^{eij}	498 ± 27^{fk}		32 mg/L	287 ± 6^{b}	243 ± 4^{ij}	168 ± 5^{fk}
2x	control	571 ± 4^{gjk}	521 ± 7^{gjkm}	498 ± 11^{fk}	2x	control	261 ± 3^{g}	244 ± 1^{ij}	183 ± 3^{cdk}
	16 mg/L	570 ± 40^{gj}	535 ± 20^{gj}	489 ± 9^{bfm}		16 mg/L	266 ± 24^{gh}	240 ± 1^{ij}	196 ± 9^{d}
	32 mg/L	537 ± 31^{ijl}	512 ± 6^{gklm}	452 ± 11^{c}		32 mg/L	231 ± 9^{i}	233 ± 4^{i}	190 ± 2^{de}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis. 0, 1 and 2 represents the wines made of different seed concentrations; 16 mg/L and 32 mg/L represents the different oxygen dosages applied.

Table 3.7: Monomeric anthocyanin concentration (mg/L) as determined by spectrometry (Ribereau-Gayon and Stonestreet, 1965) and RP-HPLC (Peng *et al*., 2002) for the control and oxygenated wines made of different seed concentrations for the 2008 Pinotage during the different stages of winemaking.

SPECTROMETRY: MONOMERIC ANTHOCYANINS					**RP-HPLC: MONOMERIC ANTHOCYANINS**				
*2008 Pinotage					*2008 Pinotage				
Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)
0x	control	538 ± 15^{a}	443 ± 11^{a}	346 ± 67^{bc}	0x	control	538 ± 15^{a}	443 ± 11^{b}	346 ± 67^{c}
	16 mg/L	577 ± 13^{a}	427 ± 4^{de}	387 ± 6^{fg}		16 mg/L	577 ± 13^{de}	427 ± 4^{f}	387 ± 6^{c}
	32 mg/L	533 ± 3^{ah}	437 ± 11^{dh}	364 ± 1^{bcf}		32 mg/L	533 ± 3^{d}	437 ± 11^{f}	364 ± 1^{c}
1x	control	671 ± 67^{dei}	575 ± 5^{eij}	496 ± 16^{fk}	1x	control	671 ± 67^{l}	575 ± 5^{eh}	496 ± 16^{f}
	16 mg/L	605 ± 62^{dei}	501 ± 9^{ij}	437 ± 18^{bfk}		16 mg/L	605 ± 62^{a}	501 ± 9^{m}	437 ± 18^{n}
	32 mg/L	483 ± 15^{jl}	480 ± 14^{eij}	405 ± 12^{fk}		32 mg/L	483 ± 15^{h}	480 ± 14^{o}	405 ± 12^{g}
2x	control	596 ± 1^{gjk}	525 ± 5^{gjkm}	448 ± 3^{fk}	2x	control	596 ± 1^{i}	525 ± 5^{j}	448 ± 3^{k}
	16 mg/L	504 ± 31^{gj}	452 ± 8^{gj}	382 ± 6^{bfm}		16 mg/L	504 ± 31^{de}	452 ± 8^{b}	382 ± 6^{g}
	32 mg/L	481 ± 5^{ijl}	456 ± 18^{gklm}	362 ± 2^{c}		32 mg/L	481 ± 5^{de}	456 ± 18^{f}	362 ± 2^{c}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis. 0, 1 and 2 represents the wines made of different seed concentrations; 16 mg/L and 32 mg/L represents the different oxygen dosages applied.

Table 3.8: Polymeric pigment concentration (mg/L) obtained from RP-HPLC (Peng *et al*., 2002) for the control and oxygenated wines made of different seed concentrations for the 2008 Pinotage and 2008 Cabernet Sauvignon during the different stages of winemaking.

RP-HPLC: POLYMERIC PIGMENTS					RP-HPLC: POLYMERIC PIGMENTS				
*2008 Pinotage					*2008 Cabernet Sauvignon				
Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)
0x	control	31.9 ± 0.1^{ab}	36.7 ± 0.7^{cd}	34 ± 0.4^{ace}	0x	control	26.1 ± 1.1^{acg}	25.2 ± 0.4^{acd}	24.9 ± 2.6^{acde}
	16 mg/L	33.5 ± 0.9^{ae}	47.8 ± 2.2^{f}	34.8 ± 2.3^{ac}		16 mg/L	23.5 ± 0.6^{ab}	25.1 ± 1.1^{acd}	23.2 ± 0.4^{ab}
	32 mg/L	33.8 ± 1.0^{ace}	52.6 ± 2.0^{p}	29.7 ± 1.0^{bgh}		32 mg/L	24.0 ± 1.1^{adef}	26.2 ± 1.3^{acg}	23.6 ± 1.4^{abf}
1x	control	28.5 ± 0.8^{gij}	33.3 ± 1.7^{ae}	25.0 ± 1.3^{k}	1x	control	26.1 ± 0.1^{acg}	22.3 ± 0.9^{b}	20.6 ± 0.7^{b}
	16 mg/L	31.8 ± 1.2^{bel}	38.3 ± 1.5^{dn}	25.7 ± 1.1^{ko}		16 mg/L	27.9 ± 1.9^{cghik}	22.3 ± 0.9^{cfgj}	23.6 ± 0.9^{ab}
	32 mg/L	32.3 ± 1.5^{aeh}	40.5 ± 0.7^{n}	27.8 ± 1.3^{gjo}		32 mg/L	27.9 ± 0.8^{cgh}	27.1 ± 1.3^{cghk}	24.9 ± 0.6^{adefk}
2x	control	31.1 ± 1.3^{bei}	34.8 ± 0.6^{ac}	26.3 ± 0.3^{jk}	2x	control	29.8 ± 1.4^{hi}	22.6 ± 0.3^{bd}	21.9 ± 0.4^{be}
	16 mg/L	34.5 ± 0.9^{acl}	43.4 ± 0.7^{m}	29.3 ± 0.3^{bg}		16 mg/L	29.5 ± 2.7^{hij}	27.9 ± 0.6^{cghk}	23.8 ± 4.0^{adef}
	32 mg/L	40.0 ± 1.5^{n}	45.5 ± 1.2^{fm}	31.9 ± 1.2^{bel}		32 mg/L	31.0 ± 3.4^{i}	28.4 ± 2.5^{ghi}	23.7 ± 1.1^{abf}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis. 0, 1 and 2 represents the wines made of different seed concentrations; 16 mg/L and 32 mg/L represents the different oxygen dosages applied.

3.3.3 Monomeric and dimeric flavanols

The DMAC assay is mainly used for red wines, since the monomeric flavanol concentration in white wines are very low (1.3-3.3 mg/L) or not detectable (McMurrough *et al*., 1978; De Beer *et al*, 2004, Kennedy *et al*., 2006). There was a significant difference in monomeric flavanol concentration with the DMAC index for the wines made with different seed concentrations in 2007, with smaller differences between the 1x- and 2x- seeds than with the 0x- seeds (Table 3.9). There were no significant differences observed between the 0x seed treatment for the control and oxygenated wines before and after MLF. For the 1x seed treatment, the oxygenated wines had lower concentrations ($p < 0.05$) than the control, but after MLF the concentration remained constant with no significant differences between the wines. The control of the 2x seed treatment had the highest concentration of all wines, with the oxygenated wines having significantly lower concentrations before MLF, but this was negated by MLF ($p>0.05$).

Table 3.9: Monomeric flavanol concentration (mg/L) determined by the DMAC assay (Nagel and Glories, 1991) for the control and oxygenated wines made of different seed concentrations for the 2007 Cabernet Sauvignon.

SPECTROMETRY: DMAC Index			
*2007 Cabernet Sauvignon			
Treatment		Before MLF (mg/L CE)	After MLF (mg/L CE)
0x	control	1.6 ± 0.3^{a}	1.8 ± 0.5^{a}
	16 mg/L	1.7 ± 0.4^{a}	3.3 ± 0.8^{a}
1x	control	54.3 ± 3.4^{b}	50.8 ± 4.2^{c}
	16 mg/L	48.0 ± 1.0^{c}	49.5 ± 1.9^{c}
2x	control	70.8 ± 2.0^{d}	65.7 ± 2.0^{e}
	16 mg/L	56.1 ± 4.1^{b}	63.9 ± 2.2^{e}

*Each value in the table indicates the average of three repeats and the value after '±' denotes the standard deviation of the repeats. The letter indicates the significant differences within each analysis. '16 mg/L' represents the oxygen dosage given after alcoholic fermentation. 0, 1 and 2 represents the wines made of different seed concentrations. Concentrations are given in mg/L catechin equivalents (CE).

Monomeric flavanol concentrations of the 2008 vintage for both cultivars proved to be much higher compared to the 2007 Cabernet Sauvignon (Table 3.10). The cultivar, the addition of oxygen and the onset of MLF had significant effects on the DMAC index. In this study it was obvious that more monomeric flavanols were extracted as the seed concentration was increased (Kovac *et al*., 1995; Canals *et al*., 2008). Both cultivars showed in most cases a definite and significant increase in concentration after ageing for two months. In the Cabernet Sauvignon, no significant differences could be found within seed treatments for the control and oxygenated wines after ageing for two months, but the higher seed treatments still had significantly higher concentrations of procyanidins. A different trend was observed in the Pinotage after two months. Where no seeds were present, the oxygenated wines had significant higher concentrations, with no significant differences observed within the 1x- seed treatments. However, in the 2x seeds the oxygenated wines had significantly lower concentrations of monomeric flavanols according to the DMAC index.

Cheynier *et al*. (1999) reported that the concentration of monomeric flavanols can vary over time due to the instability of ethyl-bridges formed through acetaldehyde-induced polymerisation. This causes cleavage and addition reactions and monomeric flavanols can be gradually converted to more stable derivatives. Nonetheless, the considerable increase in monomeric flavanols observed in our experiment can not fully be explained by cleavage and addition reactions. As explained by Canals *et al*. (2008), eliminating or adding seeds significantly changes the DMAC index. The DMAC reagent reacts with the catechin units at the ends of proanthocyanidins. Thus, variations in this index may be attributed not only to the changes in proanthocyanidin concentrations, but also to the changes in chain length, which can only be determined when analysing the mDP values for each wine which were not done in this study. Thus, we postulate that varying chain length could have partly contributed to the increasing flavanol concentration observed.

Table 3.10: Monomeric flavanol concentration (mg/L) determined by the DMAC assay (Nagel and Glories, 1991) for the control and oxygenated wines made of different seed concentrations for the 2008 Pinotage and 2008 Cabernet Sauvignon during the different stages of winemaking.

SPECTROMETRY: DMAC Index					**SPECTROMETRY: DMAC Index**				
*2008 Pinotage					*2008 Cabernet Sauvignon				
Treatment		Before MLF (mg/L CE)	After MLF (mg/L CE)	2 Months after (mg/L CE)	Treatment		Before MLF (mg/L CE)	After MLF (mg/L CE)	2 Months after (mg/L CE)
0x	control	204 ± 2^{ab}	182 ± 2^{a}	448 ± 63^{m}	0x	control	165 ± 23^{ab}	142 ± 3^{ac}	153 ± 10^{acd}
	16 mg/L	211 ± 17^{b}	185 ± 5^{a}	497 ± 29^{c}		16 mg/L	142 ± 1^{a}	146 ± 2^{be}	159 ± 2^{acd}
	32 mg/L	220 ± 11^{bd}	183 ± 2^{a}	480 ± 4^{c}		32 mg/L	138 ± 5^{a}	150 ± 2^{ef}	173 ± 7^{bc}
1x	control	281 ± 11^{ef}	252 ± 2^{gh}	666 ± 33^{i}	1x	control	148 ± 8^{bd}	163 ± 2^{ab}	205 ± 9^{eg}
	16 mg/L	267 ± 5^{eg}	245 ± 5^{dg}	669 ± 33^{i}		16 mg/L	185 ± 6^{hi}	173 ± 4^{bc}	230 ± 8^{fgj}
	32 mg/L	211 ± 17^{eg}	248 ± 7^{dg}	688 ± 7^{i}		32 mg/L	210 ± 3^{i}	170 ± 3^{bc}	230 ± 1^{fgj}
2x	control	335 ± 1^{j}	308 ± 4^{fk}	826 ± 12^{n}	2x	control	178 ± 1^{be}	240 ± 5^{j}	379 ± 20^{k}
	16 mg/L	308 ± 3^{fk}	275 ± 26^{eh}	787 ± 7^{l}		16 mg/L	277 ± 3^{h}	246 ± 1^{j}	394 ± 10^{k}
	32 mg/L	312 ± 5^{jk}	286 ± 4^{efk}	786 ± 7^{n}		32 mg/L	242 ± 23^{ij}	236 ± 8^{fj}	385 ± 20^{k}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis. 0, 1 and 2 represents the wines made of different seed concentrations; 16 mg/L and 32 mg/L represents the different oxygen dosages applied. Concentrations are given in mg/L catechin equivalents (CE).

Monomeric flavanol concentrations as determined by RP-HPLC, also presented increases in concentration with seed concentration (Addendum A, Table 7.6-7.10). However, trends within seed treatments and over time were not the same in the different cultivars and vintages. Monomeric flavanol concentrations determined by RP-HPLC were calculated as the sum of (+)-catechin, (-)-epicatechin, (+)-gallocatechin, (-)-epigallocatechin and (-)-epicatechingallate.

Significant increases in (+)-catechin and (-)-epicatechin concentrations were observed when additional seeds were added (Addendum A, Table 7.6 and 7.7) and the opposite was found when no seeds were added. Kovac *et al.* (1992; 1995) have repeatedly also found an increase in catechins (sum of (+)-catechin and (-)-epicatechin) with the addition of supplementary seeds during fermentation. This effect is not surprising, because grape seeds are rich in catechins and procyanidins (Gonzàles-Manzano *et al*., 2004). The (-)-epicatechin concentration found in the Cabernet Sauvignon (both vintages) was signficantly higher than that of the Pinotage, as was found by Goldberg *et al*. (1998). Our very high concentrations of (-)-epicatechin concur with a study by Santos-Buelga *et al.* (1995). Contradictory to our results and those of Canals *et al.* (2008), Lee *et al*. (2008) found wines made without seeds had the highest proanthocyanidin concentration and they attributed it to the more pronounced contribution of skin proanthocyanidins, which are more readily extracted during fermentation compared to the extractability of seed procyanidins. Oxygen addition led to significantly lower (-)-epicatechin concentrations in the 2007 Cabernet Sauvignon and 2008 Pinotage wines where 2x seeds were added.

Even though (-)-epigallocatechin is considered to mainly occur in the skins of grapes, Singleton (1980) reported that this compound can also be found in the seeds. Furthermore, in our study we have seen an increasing trend in (-)-epigallocatechin and (+)-gallocatechin (Addendum A, Table 7.8 and 7.9) with seed concentration during both vintages. (-)-Epicatechingallate is said to be a characteristic compound present in seeds (Prieur *et al*., 1994). However, only in 2007 did concentrations of (-)-epicatechingallate increase with the addition of seeds (Addendum A, Table 7.10).

Procyanidins B1 and B2 increased significantly when additional seeds were added and decreased significantly when seeds were removed in both vintages for both cultivars (Addendum A, Table 7.11 and 7.12). This is in agreement with a study by Kovac *et al.* (1992; 1995) and is due to the extraction of (+)-catechin, (-)-epicatechin and dimeric procyanidins from seeds. Oxygen addition only led to a signficant decrease in dimer B1 concentration in all three cultivars. The same tendency was also observed for the dimer B2 before MLF, but were often negated with MLF and further ageing. Du Toit *et al.* (2006) also found differences in dimer B1 and B2 concentrations in wines receiving micro-oxygenation, which is probably due to the difference in reactivity of these compounds towards oxygen.

3.3.4 Total phenols

Measuring total phenols at 280 nm using a spectrophotometer is a robust analysis method that showed an increase with seed concentration for the 2007 Cabernet Sauvignon wines (Table 3.11). Values for the 0x-seeded wines remained constant during the experiment with no significant differences between control and oxygenated wines after MLF. A significant increase was observed for the 1x seeded wines from before MLF until after MLF, but no significant differences were seen between control and oxygenated wines. In the 2x seeded wines, there was a significant decrease in total phenol values for the control and oxygenated wines after MLF, with the oxygenated wines showing significantly lower values than the control.

In 2008 it was evident that the total phenol values (280 nm) increased significantly in the 2x seeded wines in the Pinotage and 1x and 2x seeded wines in the Cabernet Sauvignon

compared to the 0x seed treatments (Table 3.12). Before MLF the control of the Pinotage had significantly higher values in most cases when compared to the oxygenated wines. However, as time progressed, these differences decreased and stabilised. No significant differences in the Pinotage were noted for the control and oxygenated wines after ageing. The same trend was observed for the Cabernet Sauvignon wines, but lower total phenol values were obtained for this cultivar.

Canals *et al.* (2008) confirmed that when seeds were removed from the must, the total phenolic content decreased and that the opposite were true when additional seeds were added. The losses in total phenol concentrations could be due to condensation, oxidation and later precipitation of phenols, especially when an excess of phenols are present, such as in the 2x seeds where we observed a decrease with MLF (Perez-Margarino *et al*., 2007).

Table 3.11: Total phenol values (AU) measured by spectrometry (Somers and Evans, 1977) for control and oxygenated wines made of different seed concentrations for the 2007 Cabernet Sauvignon before and after MLF.

SPECTROMETRY: TOTAL PHENOLS (280 nm)			
*2007 Cabernet Sauvignon			
Treatment		Before MLF (AU)	After MLF (AU)
0x	control	31.4 ± 0.8^{a}	30.0 ± 0.8^{a}
	16 mg/L	31.1 ± 1.3^{a}	29.1 ± 0.8^{a}
1x	control	43.8 ± 0.5^{b}	49.9 ± 0.9^{d}
	16 mg/L	42.4 ± 1.4^{b}	50.1 ± 1.5^{d}
2x	control	60.7 ± 4.4^{c}	55.2 ± 0.2^{e}
	16 mg/L	60.5 ± 2.2^{c}	50.6 ± 3.6^{d}

*Each value in table indicates the average of three repeats and the value after '±' denotes the standard deviation of the repeats. The letter indicates the significant differences within each analysis. 0, 1 and 2 represents the wines made of different seed concentrations; 16 mg/L represents the oxygen dosage applied.

Table 3.12: Total phenol values (AU) obtained by spectrometry (Somers and Evans, 1977) for the control and oxygenated wines made of different seed concentrations for the 2008 Pinotage and Cabernet Sauvignon during the different stages of winemaking.

SPECTROMETRY: TOTAL PHENOLS (280 nm)					**SPECTROMETRY: TOTAL PHENOLS (280 nm)**				
*2008 Pinotage					*2008 Cabernet Sauvignon				
Treatment		Before MLF (AU)	After MLF (AU)	2 Months after (AU)	Treatment		Before MLF (AU)	After MLF (AU)	2 Months after (AU)
0x	control	51 ± 1^{ab}	40 ± 2^{d}	41 ± 2^{d}	0x	control	33 ± 1^{ab}	32 ± 1^{ac}	30 ± 0^{c}
	16 mg/L	41 ± 4^{d}	43 ± 1^{de}	42 ± 1^{df}		16 mg/L	33 ± 0^{ab}	32 ± 1^{ade}	31 ± 1^{ade}
	32 mg/L	42 ± 6^{df}	43 ± 1^{de}	43 ± 0^{de}		32 mg/L	34 ± 0^{beg}	33 ± 0^{aef}	31 ± 0^{cd}
1x	control	51 ± 2^{ac}	51 ± 1^{ac}	50 ± 1^{abg}	1x	control	36 ± 1^{hi}	35 ± 0^{bgh}	32 ± 1^{aef}
	16 mg/L	46 ± 1^{bef}	50 ± 1^{abgh}	49 ± 2^{abi}		16 mg/L	37 ± 3^{ij}	35 ± 1^{ghj}	33 ± 1^{aef}
	32 mg/L	47 ± 2^{abe}	49 ± 1^{abi}	49 ± 1^{abij}		32 mg/L	35 ± 2^{ghj}	36 ± 1^{hi}	33 ± 0^{ab}
2x	control	60 ± 6^{k}	54 ± 3^{ch}	54 ± 1^{c}	2x	control	40 ± 0^{k}	37 ± 0^{il}	35 ± 0^{ghj}
	16 mg/L	51 ± 6^{ac}	53 ± 1^{cgj}	53 ± 0^{cgi}		16 mg/L	37 ± 3^{il}	39 ± 1^{kl}	36 ± 0^{ij}
	32 mg/L	53 ± 2^{cg}	54 ± 2^{ch}	54 ± 1^{cg}		32 mg/L	40 ± 2^{k}	39 ± 1^{kl}	36 ± 0^{hi}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis. 0, 1 and 2 represents the wines made of different seed concentrations; 16 mg/L and 32 mg/L represents the different oxygen dosages applied.

The significant contribution of seeds to the total phenol content of the 2007 Cabernet Sauvignon wines were confirmed when measured with the Folin-Ciocalteu (FC) assay (Table 3.13). It was also evident that in 2008 the amount of seeds present during fermentation contributed to the total phenol concentration (Table 3.14). Kovac *et al.* (1995), Meyer and Hernandez (1970) and Canals *et al*. (2008) found similar results with the addition or elimination of seeds. This was in agreement with the total phenol measurement (280 nm). Seed concentration, MLF, and the addition of oxygen influenced the phenol content significantly in 2007. The concentration differences were most significant between the 0x seed and other 2x seed treatments, with the latter increasing considerably. The completion of MLF only showed a significant decrease in total phenolics in the 0x seed treatment. Oxygen addition only had a significant effect on 2x seed wines where there was a significant increase in the FC value after MLF. Even though some concentrations differed significantly within seeds treatments, these were not extreme in winemaking terms. Llaudy *et al.* (2006) found only a slight decrease in total phenolic concentration during oxygen addition. Perez-Margarino *et al.* (2007) stated that in some cases oxygenated wines had lower phenolic concentrations. Losses in total phenols after malolactic fermentation are also in agreement with work done by Amati *et al.* (2002) and Ferranini *et al.* (2001).

Although the addition of oxygen caused significant increases and decreases in phenol concentration in 2008, no apparent trend could be recognised. This is in agreement with work done by Llaudy *et al.* (2006) and Fell *et al.* (2007).

Table 3.13 Total phenol concentration as determined by the Folin-Ciocalteu assay (Singleton and Rossi, 1965) for control and oxygenated wines made of different seed concentrations for the 2007 Cabernet Sauvignon before and after MLF.

SPECTROMETRY: FOLIN-CIOCALTEU			
*2007 Cabernet Sauvignon			
Treatment		Before MLF (mg/L GAU)	After MLF (mg/L GAU)
0x	control	1192 ± 106^{c}	978 ± 66^{f}
	16 mg/L	1335 ± 16^{g}	1132 ± 65^{c}
1x	control	2989 ± 41^{a}	2884 ± 60^{ab}
	16 mg/L	2959 ± 81^{a}	2798 ± 33^{a}
2x	control	3661 ± 60de	3627 ± 24^{d}
	16 mg/L	3395 ± 184^{h}	3771 ± 31^{e}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis. '16 mg/L' refers to the addition of oxygen after alcoholic fermentation. 0, 1 and 2 represents the wines made of different seed concentrations. Concentrations are given in mg/L gallic acid units (GAU).

Table 3.14: Total phenol concentration (Folin-Ciocalteu)measured by spectrometry for control and oxygenated wines made of different seed concentrations for the 2008 Pinotage and 2008 Cabernet Sauvignon during the different stages of winemaking.

SPECTROMETRY: FOLIN-CIOCALTEU					SPECTROMETRY: FOLIN-CIOCALTEU				
*2008 Pinotage					*2008 Cabernet Sauvignon				
Treatment		Before MLF (mg/L GAU)	After MLF (mg/L GAU)	2 Months after (mg/L GAU)	Treatment		Before MLF (mg/L GAU)	After MLF (mg/L GAU)	2 Months after (mg/L GAU)
0x	control	1785 ± 84^{a}	873 ± 22^{bc}	2140 ± 14de	0x	control	1320 ± 24^{ab}	1073 ± 48^{cd}	1069 ± 17^{cd}
	16 mg/L	1894 ± 125^{a}	848 ± 75^{bf}	2157 ± 19de		16 mg/L	1140 ± 37^{cde}	1050 ± 4^{c}	1133 ± 17^{cde}
	32 mg/L	1850 ± 32^{a}	800 ± 45^{b}	2149 ± 31^{d}		32 mg/L	1243 ± 45^{af}	1106 ± 13^{cde}	1150 ± 14^{def}
1x	control	2544 ± 61^{g}	1141 ± 59^{ef}	2583 ± 82^{gj}	1x	control	1679 ± 24^{j}	1173 ± 19^{ef}	1143 ± 7^{cdeg}
	16 mg/L	2063 ± 294de	1038 ± 10^{ch}	2516 ± 41^{gk}		16 mg/L	1564 ± 40^{h}	1176 ± 9^{ef}	1116 ± 17^{cde}
	32 mg/L	1749 ± 227^{a}	980 ± 4^{cfh}	2697 ± 166^{jl}		32 mg/L	1599 ± 2^{h}	1237 ± 17^{afg}	1134 ± 24^{cde}
2x	control	2348 ± 113^{ek}	1480 ± 3^{m}	2857 ± 7^{ln}	2x	control	1361 ± 35^{bi}	1359 ± 14^{bi}	1305 ± 45^{ab}
	16 mg/L	2233 ± 240de	1394 ± 46^{hi}	2946 ± 140^{n}		16 mg/L	1417 ± 111^{bi}	1438 ± 16^{hi}	1370 ± 33^{hi}
	32 mg/L	2283 ± 103de	1302 ± 8^{im}	2988 ± 96^{n}		32 mg/L	1371 ± 28^{bi}	1446 ± 5^{hi}	1416 ± 50^{hi}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis. 0, 1 and 2 represents the wines made of different seed concentrations; 16 mg/L and 32 mg/L represents the different oxygen dosages applied. Concentrations are given in mg/L gallic acid units (GAU).

3.3.5 Polymeric phenol concentration

The polymeric phenol concentration as determined by RP-HPLC (Addendum A, Table 7.13) increased with seed concentration in the 2007 Cabernet Sauvignon wines. This trend was less signficant in the 2008 vintage. The condensation of phenols with other phenols or pigments in polymerisation reactions is favoured when more phenols are present (Bourzeix *et al*., 1986; Revilla *et al*., 1991). However, no sigfnificant differences could be found between the control and oxygenated wines after MLF in the 2007 Cabernet Sauvignon. An increasing trend in the oxygenated wines of the 2008 Pinotage was observed after MLF, but this was not the case in the 2008 Cabernet Sauvignon. However, the concentration differences are small as polymerisation reactions takes place slowly over time (Cheynier *et al*., 1999). The 2008 Cabernet Sauvignon wines had the lowest polymeric phenol concentrations of all the wines, confirmed by the measurement of total phenols at 280 nm and the Folin-Ciocalteu assay (section 3.3.4). Even though the polymeric phenol concentrations of this cultivar was the lowest, it is still comparable to polymeric phenol data obtained by Rossouw and Marais (1994) who evaluted the polymeric phenol content of 84 Cabernet Sauvignon wines and found an average concentration of 295 mg/L. The higher phenol content in the 2007 Cabernet Sauvignon and 2008 Pinotage might have led to more phenolic compounds being available for the formation of polymeric phenols, which was also found by Cano-Lopez *et al*. (2008) when micro-oxygenation was applied to wines with different phenolic contents.

3.3.6 Tannins

In the 2007 experiment it seemed as if the seed elimination or addition had the most pronounced effect on the tannin concentration (Table 3.15), more so than in the 2008 vintage (Table 3.16). As was evident in the total phenol measurements, the tannin concentration increased with seed concentration during both vintages. During fermentation the increase in alcohol concentration and higher fermentation temperature contribute to the extraction of tannin from the seeds, and the more seeds present, the greater the extraction of procyanidins (Kovac

et al., 1995). No significant differences between the control and oxygenated wines could be seen after MLF in all the wines. Wines made with twice the normal seed concentration did not have twice the tannin concentration. The average concentration for the latter was approximately 520 mg/L higher than that of the 1x seed treatment.

The general tannin content in the 2008 wines proved to be lower compared to the 2007 vintage, especially in the case of Cabernet Sauvignon. Harbertson *et al*. (2008) confirmed that tannin concentration can vary drastically when measured with the BSA precipitation method. The two cultivars behaved differently towards the addition of oxygen. In the Cabernet Sauvignon the concentration fluctuated more with the different oxygen dosages, although no trend could be recognised. However, we did observe a general decrease in tannin concentration for both cultivars in 2008 after the two month ageing period. These changes can be due to oxidation of tannins or a change in the conformation of the tannin molecules, which could react differently with BSA. In the Pinotage, all the seed treatments remained unchanged and non-significant within each treatment after ageing, regardless of the oxygen dosage.

Tao *et al.* (2007) found an increase in tannin concentration as measured with bovine serum albumin during microoxygenation in wine with lower SO_2 additions. Where higher SO_2 levels were present this did not occur, which is probably due to larger tannin colour pigment moieties which can react with the BSA at lower SO_2 concentrations. These observations could possibly explain the difference in tannin concentration between the 2007 and 2008 Cabernet Sauvignon wines, but the 2008 Pinotage, with a relative high tannin concentration, also received 30 mg/L SO_2 at crushing. The increments in SO_2 concentrations used by Tao *et al.* (2007) were also much higher than in our study.

A dilution range was set up in our experiment, as recommended by Heredia *et al.* (2006), for each seed treatment of each cultivar prior to tannin analysis, to determine the maximum tannin concentration. Even after determining the dilution that ensured the highest tannin concentration, we found very low concentrations in the 2008 Cabernet Sauvignon and very high concentrations in the 2008 Pinotage. A recent study by Jensen *et al*. (2008) stated that until recently the effect of diluted/concentrated wine samples were underestimated or overestimated when analysing tannin concentration with the BSA precipitation method. Their results showed the tannin concentration could be underestimated when a wine with either very low or very high tannin concentration is used, such as in our study. They recommend dilutions should be carried out to give a tannin response factor between 0.3 and 0.75 absorbance units. Using this recently published information obtained from Jensen *et al*. (2008), the tannin response in the Pinotage (0.95) was more than 0.75 and less than 0.3 in the Cabernet Sauvignon (0.17), respectively. Thus, it is possible that overestimation and underestimation of the tannin concentration in the 2008 Pinotage and Cabernet Sauvignon occurred, respectively.

Table 3.15 Tannin concentration (mg/L) measured by protein (BSA) precipitation (Harbertson *et al.* 2002) for the control and oxygenated wines made of different seed concentrations for the 2007 Cabernet Sauvignon before and after MLF.

SPECTROMETRY: TANNINS (BSA PRECIPITATION)			
*2007 Cabernet Sauvignon			
Treatment		Before MLF (mg/L CE)	After MLF (mg/L CE)
0x	control	361 ± 13^{a}	284 ± 80^{a}
	16 mg/L	347 ± 61^{a}	236 ± 50^{a}
1x	control	810 ± 179^{b}	736 ± 41^{b}
	16 mg/L	1088 ± 206^{cd}	876 ± 70^{bc}
2x	control	1365 ± 91de	1440 ± 239^{e}
	16 mg/L	1410 ± 444^{e}	1391 ± 247^{e}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis. '16 mg/L' represents the oxygen dosage given after alcoholic fermentation. 0, 1 and 2 represents the wines made of different seed concentrations. Concentrations are given in mg/L catechin equivalents (CE).

Table 3.16 Tannin concentration (mg/L) measured by protein (BSA) precipitation (Harbertson *et al.* 2002) for the control and oxygenated wines made of different seed concentrations for the 2008 Pinotage and Cabernet Sauvignon wines during the different stages of winemaking.

SPECTROMETRY: TANNIN (BSA PRECIPITATION)					SPECTROMETRY: TANNIN (BSA PRECIPITATION)				
*2008 Pinotage					*2008 Cabernet Sauvignon				
Treatment		Before MLF (mg/L CE)	After MLF (mg/L CE)	2 Months after (mg/L CE)	Treatment		Before MLF (mg/L CE)	After MLF (mg/L CE)	2 Months after (mg/L CE)
0x	control	599 ± 16^{ab}	539 ± 37^{ac}	527 ± 20^{ad}	0x	control	23 ± 3^{a}	6 ± 0^{bc}	11 ± 1de
	16 mg/L	613 ± 63^{ab}	568 ± 27^{ace}	591 ± 8^{ace}		16 mg/L	6 ± 0^{bd}	6 ± 1^{bd}	11 ± 2^{ce}
	32 mg/L	654 ± 32^{a}	612 ± 17^{ab}	625 ± 11^{abf}		32 mg/L	6 ± 1^{bcd}	7 ± 2^{bcde}	15 ± 2^{e}
1x	control	904 ± 7^{g}	721 ± 5^{bfh}	645 ± 26^{abfi}	1x	control	33 ± 0^{f}	10 ± 1^{bcde}	14 ± 1^{e}
	16 mg/L	801 ± 28^{gh}	667 ± 66^{bcfij}	644 ± 59^{abfi}		16 mg/L	118 ± 5^{g}	28 ±1^{af}	27 ± 5^{af}
	32 mg/L	741 ± 111^{fh}	657 ±142^{bcdfik}	660±43^{bcdfij}		32 mg/L	30 ± 1^{f}	29 ± 3^{af}	30 ± 2^{f}
2x	control	878 ± 74^{gl}	625 ± 12^{abf}	716 ± 13^{bfh}	2x	control	123 ± 4^{gh}	42 ± 2^{i}	46 ± 7^{i}
	16 mg/L	777 ± 144^{hjl}	684 ± 2^{befh}	748 ± 17^{hi}		16 mg/L	124 ± 2^{h}	92 ± 6^{j}	104 ± 5^{k}
	32 mg/L	876 ± 64^{gl}	742 ± 77^{fh}	774 ± 11^{hjkl}		32 mg/L	122 ± 9^{gh}	86 ± 3^{j}	110 ± 4^{l}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis. 0, 1 and 2 represents the wines made of different seed concentrations; 16 mg/L and 32 mg/L represents the different oxygen dosages applied. Concentrations are given in mg/L catechin equivalents (CE).

3.3.7 Flavonol concentrations

In both vintages we observed an increase in flavonols with seed concentration, although there is no flavonols present in seeds (Ribereau-Gayon *et al.* 2006) (Table 3.17 and 3.18). We postulate that this is most probably due to copigmentation (Boulton, 2001). The larger amount of copigments extracted from the seeds increased the anthocyanin concentration. As mentioned by Rossouw and Marais (2004), flavonols are very effective copigments. Flavonols from the skins are the strongest copigments and with more anthocyanins present more of the flavonols will be removed from the solutions through copigmentation, resulting in larger solubility of the flavonols and higher extraction from the skins. However, in certain cases lower concentrations of flavonols were observed in the presence of oxygen. Quercetin has a high reactivity for

oxygen that could lead to the lower concentrations we observed (Rice-Evans *et al*., 1996; Fell *et al*., 2007). Some differences were observed in flavonol concentration before and after MLF for both vintages, but after an ageing period of two months, the flavonol concentration decreased drastically in the 2008 Pinotage. This is in accordance with a study done by Fang *et al.* (2007).

Table 3.17: Average flavonol concentration (mg/L) as determined by RP-HPLC for the control and oxygenated wines made of different seed concentrations for the 2007 Cabernet Sauvignon before and after MLF.

RP-HPLC: FLAVONOLS			
*2007 Cabernet Sauvignon			
Treatment		Before MLF (mg/L)	After MLF (mg/L)
0x	control	34.8 ± 1.0^{a}	31.5 ± 1.2^{a}
	16 mg/L	35.7 ± 2.1^{a}	31.4 ± 0.8^{a}
1x	control	50.1 ± 4.0^{b}	44.2 ± 3.3^{bc}
	16 mg/L	47.0 ± 1.4^{bc}	39.6 ± 0.9^{c}
2x	control	46.3 ± 0.6^{bc}	42.8 ± 0.8^{bc}
	16 mg/L	43.7 ± 1.8^{bc}	38.4 ± 1.4^{bc}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. Concentrations displayed in table are the average values for quercetin, myricetin, quercetin-3-rhamnoside, quercetin-3-glucoside and quercetin-3-rutinoside. The letter indicates the significant differences within each analysis. '16 mg/L' represents the addition of oxygen after alcoholic fermentation. 0, 1 and 2 represents the wines made of different seed concentrations.

Table 3.18: Average flavonol concentration (mg/L) as determined by RP-HPLC for the control and oxygenated wines made of different seed concentrations during the different stages of winemaking.

RP-HPLC: FLAVONOLS									
*2008 Pinotage					*2008 Cabernet Sauvignon				
Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)
0x	control	52 ± 3.8^{ab}	43.2 ± 1.3^{cd}	27.1 ± 2.8^{ef}	0x	control	12.9 ± 0.6^{abcd}	10.7 ± 0.2^{ehi}	12.6 ± 1.2^{acd}
	16 mg/L	49.3 ± 2.1^{ag}	45.4 ± 2^{ch}	27.2 ± 0.2^{ef}		16 mg/L	13.4 ± 0.4^{abc}	11.1 ± 0.3^{de}	13.9 ± 0.6^{ab}
	32 mg/L	47.6 ± 2.3^{ghi}	45.4 ± 0.8^{cj}	26.2 ± 0.7^{e}		32 mg/L	13.5 ± 0.2^{abf}	11.2 ± 0.1^{deg}	9.2 ± 0.6^{h}
1x	control	53 ± 0.3^{b}	47.2 ± 1.4^{ghj}	29.5 ± 1.6^{f}	1x	control	12.7 ± 0.1^{acd}	9.1 ± 0.2^{h}	11.4 ± 0.4^{dgijl}
	16 mg/L	47.5 ± 0.9^{ghj}	50.3 ± 1.4^{ak}	25.8 ± 1.6^{e}		16 mg/L	11.3 ± 0.6^{dgijl}	9.7 ± 0.1^{ehl}	11.7 ± 0.2^{cdfi}
	32 mg/L	45.6 ± 1.6^{chl}	50.2 ± 0.2^{akm}	25.5 ± 0.8^{e}		32 mg/L	11.5 ± 0.5^{dgij}	9.8 ± 0.7^{ehj}	12.0 ± 0.5^{cdfi}
2x	control	50 ± 1.8^{ai}	48.8 ± 0.8^{gik}	26.3 ± 0.5^{e}	2x	control	11.6 ± 0.1^{cdik}	9.5 ± 0.4^{eh}	19.5 ± 2.0^{m}
	16 mg/L	44.3 ± 1.1^{cd}	48.7 ± 0.7^{gik}	25.9 ± 0.2^{e}		16 mg/L	11.5 ± 0.5^{dgij}	10.5 ± 0.3^{ehi}	14.7 ± 4.8^{b}
	32 mg/L	47.9 ± 2.5^{gilm}	42.9 ± 1.2^{d}	26.9 ± 0.5^{e}		32 mg/L	10.5 ± 0.4^{ehi}	9.8 ± 0.5^{ehjk}	13.0 ± 0.1^{abcg}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. Concentrations displayed in table are the average values for quercetin, myricetin, quercetin-3-rhamnoside, quercetin-3-glucoside and quercetin-3-rutinoside. The letter indicates the significant differences within each analysis. 0, 1 and 2 represents the wines made of different seed concentrations; 16 and 32 represents the 16 mg/L and 32mg/L oxygen additions, respectively.

3.4 Conclusion

Removing seeds from the bottom of the tank during fermentation has been used in large scale commercial cellars to reduce astringency in red wines. Flavan-3-ols extracted from the seeds have been suggested to be better anthocyanin copigments than those extracted from the skins. Thus improved stabilisation of anthocyanin colour expression was expected in red wines made with higher seed contents. We have shown that it is possible to increase wine colour under certain circumstances by adding supplementary seeds before alcoholic fermentation, as it did increase the colour intensity. If no seeds are present it could produce a wine with significantly lower colour intensity. This indicated that catechins and procyanidins are extracted from seeds and contribute to wine colour as they combine with pigments to stabilise wine colour. The extraction of monomeric flavanols from the seeds was confirmed by DMAC and RP-HPLC (280 nm) analyses. Folin-Ciocalteu and BSA analyses confirmed that seeds contribute to the total phenolic content and tannin concentration in wines, respectively. However, wine may possibly become saturated with phenols when supplementary seeds are added, as in some instances only small differences between the wines made from 1x seeds and those made from 2x seeds were observed. In the latter this could lead to faster polymerisation and precipitation of tannin and/or pigments in the wine. Total monomeric anthocyanin concentrations in general decreased with oxygen additions, while polymeric phenol and polymeric pigments increased in general, even more so in the case of the 32 mg/L oxygen treatment.

This experiment demonstrated that seeds can have a considerable effect on the phenolic compounds extracted during red wine fermentation and hence influence the composition and the quality of wine. Little published information is available on wines made with altering grape seed concentrations. According to our knowledge it was the first time that the interaction between seed removal, oxygen addition and MLF was investigated to ascertain their combined effect on the phenolic composition. However, further studies should be performed to better understand the contribution of procyanidins originating from seeds to wine and their involvement in colour stabilisation. Future work should also include an expert tasting panel for the sensory evaluation of wines made with different grape seed concentrations.

3.5 Acknowledgements

The authors would like to thank the Tokara Winery for donating the grapes; Winetech, Thrip and the NRF for providing financial support and Prof Martin Kidd for analysing the data statistically.

3.6 References

Adams, D.O. and Harbertson, J.F. (1999). Use of alkaline phosphates for the analysis of tannins in grapes and red wines. *American Journal of Enology and Viticulture*. **50**, 247-252.

Alcalde-Eon, C., Escribano-Bailon, M.T., Santos-Buelga, C. and Rivas-Gonzalo, J.C. (2006). Changes in the detailed pigment composition of red wine during maturity and ageing. A comprehensive study. *Analytica Chimica Acta*. **563**, 238-254

Amati, A., Arfelli, G., Castellari, M and Simoni, M. (2002). Effetto dell'ossigenazione controllata sulla frazione fenolica dei vini rossi. *Industrie della Bevande*. **29**, 606-612

Atanasova, V., Fulcrand, H., Cheynier, V. and Moutounet, M. (2002). Effect of oxygenation on polyphenol changes occurring in the course of wine-making. *Analytica Chimica Acta*. **458**, 15-27.

Berg, H.W. and Akiyoshi, M. (1956). Some factors involved in browning of white wines. *American Journal of Enology and Viticulture*. **7**, 1-7.

Boulton, R. (2001). The copigmentation of anthocyanins and its role in the colour of red wine: a critical review. *The American Journal of Enology and Viticulture*. **52**, 67-87.

Bourzeix, M., Weyland, D. and Heredia, N. (1986). Etude des catechins et des procyanidols de la grappa de raisin, du vin et d'autres derives de la vigne. *Bulletin de la Societe Chimique de France.* 59, 1172-1254.

Bousbouras, G.E. and Kunkee, R.E. (1971). Effect of pH on malolactic fermentation in wine. *American Journal of Enology and Viticulture*. **22**, 121-126.

Canals, R., Del Carmen-Llaudy, M., Canals, J.M. and Zamora, F. (2008). Influence of the elimination of seeds on the colour, phenolic composition and astringency of red wine. *European Food and Research Technology*. **226**, 1183-1190.

Cano-Lopez, M., Pardo-Minguez, F., Lopez-Roca, J.M. and Gomez-Plaza, E. (2006). Effect of microoxygenation on anthocyanin and derived pigment content and chromatic characteristics of red wines. *American Journal of Enology and Viticulture*. **57**, 325-331

Cano-López, M., Pardo-Minguez, F.M., Schamauch. G., Saucier, C., Teissedre. P.L., López-Roca, J.M. and Gómez-Plaza, E. (2008). Effect of microoxygenation on color and anthocyanin-related compounds of wines with different phenolic contents. *Journal of Agricultural and Food Chemistry.* **56**, 5932–5941.

Castellari, M., Matricardi, L., Arfelli, G., Galassi, S. and Amati, A. (2000). Level of single bioactive phenolics in red wine as a function of the oxygen supplied during storage. *Food Chemistry*. **69**, 61-67.

Castillo, J., Benavente-Garcia, O., Lorente, M., Alcarez, M., Redondo, A., Ortuno, A. and Del Rio, J.A. (2000). Antioxidant activity and radioprotective effect against chromosomal damage induced in vivo by X-rays of flavan-3-ols (procyanidins) from grape seeds (Vitis vinifera): Comparative study versus other phenolic and organic compounds. *Journal of Agricultural and Food Chemistry*. **48**, 1738-1745.

Cheynier, V., Es Safi, N.E. and Fulcrand, H. (1999). Structure and colour properties of anthocyanins and related pigments. *In:* International congress on pigments in Food and Technology. M.I.M. Mosquera et al. (Eds.), pp 23-25.

De Beer, D., Harbertson, J.F., Kilmartin, P.A., Roginsky, V., Barsukova, T., Adams, D.O. and Waterhouse, A.L (2004). Phenolics: A comparison of diverse analytical methods. *American Journal of Enology and Viticulture*. **55**, 389-400.

De Freitas, V.A.P. and Glories, Y. (1999). Concentration and compositional changes of procyanidins in grape seeds and skins of white Vitis vinifera varieties. *Journal of the Science of Food and Agriculture*. **79**, 1601-1606.

Du Toit, W.J., Lisjak, K., Marais, J., Du Toit, M. (2006). The effect of microoxygenation on the phenolic composition, quality and aerobic wine-spoilage microorganisms of different South African red wines. *South African Journal of Enology and Viticulture.* **27**, 57-67.

Fang, F., Li, J.M., Pan, Q.H. and Huang, W.D. (2007). Determination of red wine flavonoids by HPLC and effect of ageing. *Food Chemistry*. **101**, 428-433.

Fell, A., Dykes, S.I., Nicolau, L. and Kilmartin, P.A. (2007). Electrochemical microoxidation of red wine. *American Journal of Enology and Viticulture*. **58**, 443-450.

Fulcrand, H., Duenas, M., Salas, E. and Cheynier, V. (2006). Phenolic reactions during winemaking and ageing. *American Journal of Enology and Viticulture*. **57**, 289-297.

Ferranini, R., Girardi, F., De Conti, D. and Castellari, M. (2001). Esperienze di applicazione della microossigenazione come tecnica d'affinamento dei vini. *Industrie della bevande*. **30**, 116-118.

Goldberg, D.M., Karumanchiri, A., Tsang, E. and Soleas, G.J. (1998). Catechin and epicatechin concentration of red wines: regional and cultivar-related differences. *American Journal of Enology and Viticulture*. **49**, 23-34.

Gomez-Plaza, E., Gil-Munoz, R., Lopez-Roca, J.M., Martinez-Cutillas, A. and Fernandez-Fernandez, J.I. (2004). Phenolic compounds and colour stability of red wines: effect of skin maceration time. *American Journal of Enology and Viticulture*. **52**, 266-270.

Gonzales-Neves, G., Charamelo, D., Balado, J., Barreiro, L., Bochicchio, R., Gatto, G., Gil, G., Tessore, A., Carbonneau, A. and Moutounet, M. (2004). Phenolic potential of Tannat, Cabernet Sauvignon and Merlot grapes and their correspondence with wine composition. *Analytica Chimica Acta*. **513**, 191-196.

Gonzàles-Manzano, S., Rivas-Gonzalo, J.C. and Santos-Buelga, C. (2004). Extraction of flavan-3-ols from grape seed and skin into wine using simulated maceration. *Analytica Chimica Acta*. **513**, 283-289.

Goto, T. (1987). Structure, stability and colour variation of natural anthocyanins. Progress in the chemistry of natural products. *Journal in the Science of Food and Agriculture*. **52**, 113-158.

Hagerman, A.E. and Butler, L.G. (1978). Protein precipitation method for the quantitative determination of tannins. *Journal of Agricultural and Food Chemistry*. **26**, 809-812.

Harbertson, J.F., Hodgins, R.E., Thurston, L.N., Schaffer, L.J., Reid, M.S., Landon, J.L., Ross, C.F. and Adams, D.O. (2008). Research Note: Variability of tannin concentration in red wines. *American Journal of Enology and Viticulture*. **59**, 210-214.

Harbertson, J.F., Kennedy, J.A. and Adams, D.O. (2002). Tannins in skins and seeds of Cabernet Sauvignon, Syrah, and Pinot noir berries during ripening. *American Journal of Enology and Viticulture.* **53**, 54-59.

Hayasaka, Y. and Kennedy, J.A. (2003). Mass spectrometric evidence for the formation of pigmented polymers in red wine. *Australian Journal of Grape and Wine Research*. **9**, 210-220.

Heredia, T.M., Adams, D.O., Fields, K.C., Held, P.G. and Harbertson, J.F. (2006). Evaluation of a comprehensive red wine phenolics assay using a microplate reader. *American Journal of Enology and Viticulture*. **57**, 497-502.

Jensen, J.S., Werge, H.H.M., Egebo, M. and Meyer, A.S. (2008). Effect of wine dilution on the reliability of tannin analysis by protein precipitation. *American Journal of Enology and Viticulture*. **59**, 103-105.

Jordao, A.M., Ricardo-da-Silva, J.M. and Laureano, O. (2006). Effect of oak constituents and oxygen on the evolution of malvidin-3-glucoside and (+)-catechin in model wine. *American Journal of Enology and Viticulture*. **57**, 377-381.

Kennedy, J.A. and Hayasaka, Y. (2004). Compositional investigation of pigmented tannin. In: Waterhouse, A.L and Kennedy, J.A. (eds). Red wine color: Revealing the mysteries. pp. 247-263.

Kennedy, J.A., Ferrier, J., Harbertson, J.F. and Peyrot des Gachons, C. (2006). Analysis of tannins in red wine using multiple methods: correlation with perceived astringency. *American Journal of Enology and Viticulture.* **57**, 481-485.

Kovac, V.E., Bourzeix, M., Heredia, N. and Ramos, T.A. (1991). A study of catechins and proanthocyanidins of white grapes and wines. *Revue de French Oenologie*. **125**, 7-14.

Kovac, V.E., Alonso, E., Bourzeix, M. and Revilla, E. (1992). Effect of several enological practices on the content of catechins and proanthocyanidins of red wines. *Journal of Agricultural and Food Chemistry*. **40**, 1953-1957.

Kovac, V.E., Alonso, E. and Revilla, E. (1995). The effect of adding supplementary quantities of seeds during fermentation on the phenolic composition of wines. *American Journal of Enology and Viticulture*. **46**, 363-367.

Lea, A.G.H., Bridle, P., Timerlake, C.F. and Singleton, V.L. (1979). The procyanidins of white grapes and wines. *American Journal of Enology and Viticulture*. **30**, 289-300.

Lee, J., Kennedy, J.A., Devlin, C., Redhead, M. and Renmaker, C. (2008). Effect of early seed removal during fermentation on proanthocyanidin extraction in red wine: a commercial production example. *Food Chemistry*. **107**, 1270-1273.

Llaudy. M., Canals, R., Gonzales-Manzano, S., Canals J.M., Santos-Buelga, C. and Zamora, F. (2006). Influence of micro-oxygenation treatment before oak ageing on phenolic compounds composition, astringency, and color of red wine. *Journal of Agricultural and Food Chemistry*. **54**, 4246-4252.

Mcmurrough, I. and Mcdowell, J. (1978). Chromatographic separation and automated analysis of flavanols. *Analytical Biochemistry*. **91**,92-100.

McCord, J. (2003). Application of toasted oak and micro-oxygenation to ageing of Cabernet Sauvignon wines. *Australian and New Zealand Grapegrower and Winemaker*. July: 43-53.

Medina, K., Boido, E., Dellacassa, E. and Carrau, F. (2005). Yeast interactions with anthocyanins during red wine fermentation. *American Journal of Enology and Viticulture*. **56**, 104-109.

Meyer, B.J. and Hernandez, R. (1970). Seed tannin extraction in Cabernet Sauvignon. *American Journal of Enology and Viticulture*. **21**, 184-188.

Monagas, M., Gomez-Cordoves, C. and Bartolome, B. (2006). Evolution of the phenolic content of red wines from *Vitis vinifera* L. during ageing in bottle. *Food Chemistry*. **95**, 405-412.

Nagel, C.W. and Glories, Y. (1991). Use of a modified dimethylaminocinnamaldehyde reagent for analysis of flavanols. *American Journal of Enology and Viticulture*. **42**, 364-366.

Nieuwoudt, H.H., Prior, B.A., Pretorius, I.S., Manley, M. and Bauer, F.F. (2004). Principle component analysis applied to fourier transform infrared spectroscopy for the design of calibration sets for glycerol prediction models in wine and for the detection and classification of outlier samples. *Journal of Agricultural and Food Chemistry*. **52**, 3728-3735.

Paranetto, L. (1977). Polifenoli e tecnica enologica; *Editroiale Selepress: Milano*, Spain.

Peng, Z., Iland, P.G., Oberholster, A., Sefton, M.A. and Waters, E.J. (2002). Analysis of pigmented polymers in red wine by reversed phase HPLC. *Australian Journal of Grape and Wine Research*.

Perez-Magarino, S., Sanchez-Iglesias, M., Ortega-Heras, M., Gonzales-Huerta, C. and Gonzales-Sanjose, M.L. (2007). Colour stabilisation of red wines by mircooxygenation treatment before malolactic fermentation. *Food Chemistry*. **101**, 881-893.

Peynaud, E. (1971). Connaisance et travail du vin; *Dunod: Paris*.

Peyrot des Gachons, C. and Kennedy, J.A. (2003). Direct method for determining seed and skin proanthocyanidin extraction into red wine. *Journal of Agricultural and Food Chemistry*. **51**, 5877-5881.

Prieur, C., Rigaud, J., Cheynier, V. and Moutounet, M. (1994). Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry*. **36**, 781-784.

Remy, S., Fulcrand, H., Labarbe, B., Cheynier, V. and Moutounet, M. (2000). First confirmation in red wine of products resulting from direct anthocyanin-tannin reactions. *Journal of the Science of Food and Agriculture*. **80**, 745-751.

Revilla, E., Bourzeix, M. and Alonso, E. (2001). Analysis of catechins and procyanidins in grape seeds by HPLC with photodiode array detection. *Chromotographia*. **31**, 465-468.

Ribereau-Gayon, P., Glories, Y., Meujean, A. and Dubourdieu, D. (2006). Handbook of enology, Vol. 2. The chemistry of wine stabilization and treatments. John Wiley and Sons, Ltd. New York.

Ribereau-Gayon, P. and Stonestreet, E. (1965). Le dosage des anthocyanes dans les vins rouges. *Bulletin de la Societe Chimique de France*. **9**, 2649-2652.

Rice-Evans, C.A., Miller, A.J. and Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine*. **20**, 933-956.

Rice-Evans, C., Miller, N. and Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trends in Plant Science*. **2**, 152-159.

Romero, C. and Bakker, J. (2000). Effect of acetaldehyde and several acids on the formation of vitisin A in model wine anthocyanin and colour evolution. *International Journal of Food Science and Technologies*. **35**, 129-140.

Rossouw, M. and Marais. J. (2004). The phenolic composition of South African Pinotage, Shiraz and Cabernet Sauvignon wines. *South African Journal of Enology and Viticulture*. **25**, 94-104.

Santos-Buelga, C. and Williamson, G. (2003). Methods in polyphenol analysis. Analysis of anthocyanins. The Royal Society of Chemistry, UK, 338-358.

Santos-Buelga, C., Francia-Aricha, E.M. and Escribano-Bailon, M.T. (1995). *Food Chemistry*. **53**, 197-205.

Sartini, E., Arfelli, G., Fabiani, A. and Piva, A. (2007). Influence of chips, lees, and micro-oxygenation during ageing on the phenolic composition of a red Sangiovese wine. *Food Chemistry*. **104**, 1599-1604.

Singleton, V.L. and Rossi, J.A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *The American Journal of Enology and Viticulture*. **16**, 144-158.

Singleton, V.L. and Esau, P. (1969). Phenolic substances in grapes and wines, and their significance, *Academic Press: New York*.

Singleton, V.L. (1980). Grape and wine phenolics; background and prospects. Centennial Davis University Report, 215-227.

Singleton, V.L., Orthofer, R. and Lamuela-Ruentos, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods of Enzymology*. **299**, 152-178.

Singleton V. L. (2000). A survey of wine ageing reactions, especially with oxygen. *In*: proceedings of the ASEV 50th Anniversary Annual Meeting. JM Rantz (Eds.). pp. 323-336. Davis, CA.

Somers, T.C. and Evans, M.E. (1977). Spectral evaluation of young red wines: anthocyanin equilibria, total phenolics, free and molecular SO2, “chemical age”. *Journal of the Science of Food and Agriculture*. **28**, 279-287.

Tao, J., Dykes, S.I. and Kilmartin, P.A. (2007).

Vasserot, Y., Caillet, S. and Maujean, A. (1997). Study of anthocyanin adsorption by yeast lees. Effect of some physicochemical parameters. *American Journal of Enology and Viticulture*. **48**, 433-437.

Waterhouse, A.L. and Laurie, V.F. (2006). Oxidation of wine phenolics: a critical evaluation and hypothesis. *American Journal of Enology and Viticulture*. **57**, 306-313.

Chapter 4

Research results

The influence of adding different oxygen dosages before malolactic fermentation on the colour and phenolic composition of Pinotage red wine

Chapter 4: The influence of adding different oxygen dosages before malolactic fermentation on the colour and phenolic composition of Pinotage red wine

4.1 Introduction

Introducing oxygen to red wine with micro-oxygenation has become increasingly popular, using a variety of means to simulate the advantages of oxygen exposure during barrel maturation. The technique varies in oxygen dosage addition and can be applied after fermentation and during maturation. Various effects of oxygen have been identified, largely involving the formation of polymeric phenols in the wine and the stabilisation of wine colour (Castellari *et al*., 2000; McCord, 2003, Llaudy *et al*., 2006 and Perez-Magarino *et al*., 2007).

Anthocyanins are responsible for the colour in red wine. They react with other phenolic compounds, primarily flavan-3-ols, during fermentation and ageing to form more complex and stable polymeric pigments. Several authors have elucidated the mechanisms that involve the formation of these more stable polymers (Saucier *et al*., 2004; Remy *et al*., 2000; Romero *et al*., 2000; Fulcrand *et al*., 1997). The proposed mechanisms involve direct condensation reactions between anthocyanins and flavanols, reactions involving acetaldehyde to form anthocyanin-tannin adducts linked by an ethyl-bridge and the formation of pyranoanthocyanins through the reaction between anthocyanins and yeast metabolites. These polymerisation reactions produce pigments that are less sensitive to the bleaching effect of sulphur dioxide and changes in pH.

Pinotage, which was developed in South Africa, is an important red wine cultivar of that country. To our knowledge there is still little published data on how oxygen additions before malolactic fermentation affect the colour and phenolic composition of Pinotage wine. The objective of this study was thus to investigate what influence different oxygen additions before malolactic fermentation have on the colour and phenolic composition of a Pinotage wine, as well as how these effects evolve during short term maturation. We also aimed to compare certain spectrometry analyses with that of the HPLC to build a knowledge basis for future researchers using these methods.

4.2 Materials and methods

4.2.1 Preparation of wine

The experiment was performed at the Distell Winery, Stellenbosch, South Africa on *cv.* Pinotage during the 2008 harvest season. The wine was prepared with standard winemaking techniques using the *Saccharomyces cerevisiae* yeast NT 50 at 0.25 g/L according to the supplier's recommendations (Anchor Yeast, Biotechnologies, South Africa). After the completion of alcoholic fermentation, the wine was divided into six stainless steel tanks of 200 L each. The length of each tank was 3.2 m to simulate those used in a commercial cellar.

Before the start of the experiment, the pH, titratable acidity (TA), volatile acidity (VA), sugar content, alcohol concentration and malic acid concentration were determined using a grapescan FT 120 instrument (Foss Electric, Denmark) (Nieuwoudt *et al*., 2004). The pH of the wine was 3.70 and confirmed using a Metrohm titration unit (Metrohm Ltd., Switzerland). Alcoholic fermentation was considered completed when the sugar content of the wine was below 4 g/L. The FT 120 instrument showed that the wines were fermented dry (a sugar content of 2.80 g/L).

The tanks were divided into two controls receiving no oxygen, two oxygenated tanks that received a total of 16 mg/L of oxygen, and another two oxygenated tanks which received a total of 32 mg/L of oxygen over a period of 8 days. The experiment was thus performed in duplicate. The oxygen was introduced to the wine by means of a micro-oxygenation unit, supplied by Agrovin DosiOx (Spain). The addition of oxygen was given just after the completion of alcoholic fermentation, before the onset of malolactic fermentation.

4.2.2 Malolactic fermentation

All the wines underwent malolactic fermentation (MLF) and were inoculated with CH16 (Christiaan Hansen) (*Oenococcus oeni*) at 1 g/hl. Malolactic fermentation was conducted at 20°C and took 34 days to complete. Malic and lactic acid concentrations were monitored with a grapescan FT 120 instrument (Foss Electric, Denmark) (Nieuwoudt *et al*., 2004). MLF was considered to be completed when the malic acid concentration was lower than 0.3 g/L.

4.2.3 Ageing and bottling

After the completion of malolactic fermentation the wine was aged in the tanks for a further two months. After the ageing period, the wine was discarded.

4.2.4 Wine sampling

Samples of each tank were taken at the bottom valve before the start of oxygen addition. After oxygenation samples were taken at the bottom, middle and top valve of each tank, including the control. After malolactic fermentation and ageing, samples were taken at the bottom valve. Fifty mg/L SO_2 was added to the wine after the samples after MLF were drawn. Samples were immediately frozen in 100 ml glass vials and stored at -20°C.

4.2.5 Spectrophotometric analyses

All spectrophotometric analyses were performed using an AnalyticJena Specord 50 UV/VIS spectrophotometer (Jena, Germany). Depending on the density of the wine or the required wavelength of the analysis the following cuvettes were used: 1 mm and 10 mm quartz cuvettes, 1 mm glass cuvettes or 10 mm plastic cuvettes.

All the preceding analyses were done at the same time to quantify all samples under the same conditions or with the same calibration curve if necessary. Samples were centrifuged for 2 min at 2000 rpm to remove any solid particles that could influence the reading. All analyses were performed in triplicate.

For a detailed description of all spectrophotometric and HPLC analyses, refer to Chapter 3, section 3.2.5 and 3.2.6.

4.2.6 Statistical analysis

Mean values and standard deviations were calculated using one-way analysis of variance (ANOVA). The significance of differences between mean values obtained was determined using a student t-test at the 95% significance level, $p < 0.05$.

4.3 Results and discussion

Table 4.1 shows the significance of the effect of time, the addition of oxygen and the interaction between these effects on a 5% confidence interval. It is clear that both time and oxygen influenced the majority of phenolic compounds and colour characteristics of the wine during the course of the experiment, which will be discussed in more detail in the following sections.

Table 4.1: Probability values for the effect of time, addition of oxygen and the interaction between these factors on wine characteristics. Values in red show factors significantly affecting the wine characteristics at a 5% significance level. Time includes the period before oxygen addition to the end of the two months ageing.

COMPOUND QUANTIFIED WITH RP-HPLC	TIME	O_2 TREATMENT	TIME*O_2TREATMENT
Gallic acid	0.000	0.020	0.395
Gallocatechin	0.000	0.132	0.206
Epigallocatechin	0.000	0.041	0.006
Catechin	0.000	0.026	0.001
B1	0.000	0.008	0.005
B2	0.000	0.014	0.018
Epicatechingallate	0.000	0.023	0.000
Monomeric Flavanols	0.000	0.013	0.003
Polyphenols	0.000	0.017	0.025
Caftaric acid	0.000	0.147	0.166
Coutaric acid	0.000	0.160	0.252
Caffeic acid	0.129	0.317	0.336
p-coumaric acid	0.000	0.500	0.941
Sum of Cinnamic Acids	0.003	0.183	0.126
Sum of Flavonols	0.000	0.291	0.918
Vitisin A	0.000	0.013	0.078
Delphinidin-3-glucoside	0.000	0.011	0.043
Cyanidin-3-glucoside	0.000	0.001	0.000
Petunidin-3-glucoside	0.000	0.017	0.075
Peonidin-3-glucoside	0.006	0.011	0.014
Malvidin-3-glucoside	0.000	0.004	0.001
Delphinidin-Acetgluc	0.001	0.014	0.043
Petunidin-Acetgluc	0.000	0.002	0.001
Peonidin-Acetgluc	0.000	0.020	0.026
Malvidin-Acetgluc	0.000	0.002	0.000
Delphinidin-p-coumgluc	0.000	0.011	0.269
Petunidin-p-coumgluc	0.000	0.102	0.339
Peonidin-p-coumgluc	0.000	0.008	0.015
Malvidin-p-coumgluc	0.013	0.007	0.003
Sum of monomeric anthocyanins	0.000	0.027	0.016
Polymeric Pigments	0.000	0.009	0.025

Table 4.1 (continue)

COMPOUNDS/CHARACTERISTICS QUANTIFIED WITH SPECTROMETRY	TIME	O_2 TREATMENT	TIME*O_2TREATMENT
Optical Density (420nm)	0.000	0.015	0.025
Optical Density (520nm)	0.000	0.087	0.257
Optical Density (620nm)	0.000	0.011	0.015
Colour Intensity	0.000	0.037	0.114
Hue/Tint	0.000	0.070	0.024
Sulphur Resistant Pigments	0.000	0.001	0.000
Monomeric Anthocyanins	0.000	0.011	0.012
Total Red Pigments	0.000	0.048	0.007
Total Phenols (Boulton)	0.000	0.161	0.000
Total Phenols (Folin Ciocalteu)	0.001	0.085	0.182
Tannin (BSA)	0.002	0.116	0.243
Monomeric Flavanols (DMACH)	0.000	0.020	0.004

4.3.1 Position in tank

All spectrophotometric and HPLC analyses showed no significant differences between the positions where the samples were taken after oxygenation. This indicates that the oxygen sparged at the bottom of the tank, which then diffused upwards, affected the different phenolic compounds throughout the length of the tank in the same manner; refer to Addendum, Table 7.14.

4.3.2 Colour intensity, optical density and hue

The addition of oxygen in both treatments (16 mg/L and 32 mg/L) quickly led to an increase in colour intensity (CI) (Table 4.2) which was significantly higher than the control after oxygen addition ($p = 0.037$), although no significant differences were found between oxygen treatments. All wines decreased in CI during MLF, but that of the oxygenated wines were still significantly higher than the control. However, there were no statistical differences between the control and oxygenated treatments after ageing for two months (also refer to Table 4.2 for detailed analyses on colour intensity and optical densities). This was also found in studies by Sartini *et al.* (2007), Perez-Magarino *et al.* (2007) and Cano-Lopez *et al.* (2006). Atanasova *et al.* (2002) performed a similar study and found no statistical differences between control and oxygenated wines after seven months of ageing.

The absorbance at 420 nm was significantly higher shorter after oxygen was applied as well as after MLF in both oxygenated wines when compared to the control, although no statistical differences could be detected between the oxygenated wines (Table 4.2). The 16 mg/L oxygen treatment still showed to be significantly higher than the control and 32 mg/L oxygen treatment after ageing. Ethyl bridges are formed though acetaldehyde-induced polymerisation which is favoured by the addition of oxygen, but the resulting complexes are unstable. Through cleavage and addition reactions these can be converted into more stable pyranoanthocyanin derivates which have a higher absorbance at 420 nm (Cheynier *et al*., 1999 and Bakker *et al*., 1993). These formations occur over time and will not have an effect shortly after oxygen addition, but only later during ageing. This could explain the early increases in CI and optical density at 420 nm.

Malolactic fermentation caused a definite decrease in the absorbance at 520 nm in all the treatments, which could probably be ascribed to the increase in pH normally associated with MLF, thus shifting the anthocyanin equilibrium to produce more anthocyanin molecules in the colourless form (Bousbouras and Kunkee, 1971). However, the addition of oxygen led to a significant higher red colour after MLF, which is due to stabilisation of the colour (Perez-Magarino et al., 2007) but these differences disappeared during ageing.

The percentage of blue (620 nm) colour in both oxygenated wines was significantly higher than that of the control wines (Table 4.2). The increase of blue colour could have contributed to the increase in colour intensity due to the formation of ethyl-linked pigments. These compounds contribute especially to the absorbance at 620 nm (Cano-Lopez *et al*., 2006; Cano-Lopez *et al*., 2008). Glories (1984) also found an increase in optical density (620 nm) in wines that received oxygen. He stipulated this increase to the formation of co-pigmented complexes and to the polymerisation phenomena with ethyl-bridges, which generate more red-violet compounds. Escribiano-Bailon *et al*. (2001) stated that red and violet polymeric compounds increase colour intensity and improve pigment stabilisation. The direct polymerisation reaction of (-)-epicatechin with malvidin monoglucoside also leads to a more purple colour (Ribereau-Gayon *et al*., 2006). This increasing trend continued until after MLF, but decreased during the ageing period of two months. Values for the 32 mg/L oxygen treatment were not significantly different from the control, but the oxygenated 16 mg/L had a higher absorbance at 620 nm ($p = 0.006$) after ageing for two months. However, the insignificance between the control and 32 mg/L was only 0.054 on 5% confidence interval. This is still a strong indication that when applying 32 mg/L of oxygen it still had an effect on the strength of absorbance at 620 nm.

The hue evolved in a very similar way in all the wines (Table 4.2). The colour hue was not significantly lower in oxygenated wines which are not in agreement with a study by Cano-Lopez *et al* (2006) who also applied oxygen before malolactic fermentation. Subsequent MLF and ageing led to no significant differences between the control and oxygenated wines. Our finding is in agreement with work done by Alcalde-Eon *et al.* (2006) who did not find any differences in colour hue for the control and oxygenated wines after ageing.

There was a significant increase in the modified hue for the oxygenated wines until after MLF, as was found by Singleton (2000) (Table 4.2). This increase in modified hue could be due to the formation of brown polyphenol oxidation products and to the formation of new polymeric pigments such as the pyranoanthocyanins, which absorb relatively more light at 420 nm than at 520 nm compared with the original monomeric anthocyanins (Alcalde-Eon *et al*., 2006). After two months no significant differences could be observed between the control and oxygen treated wines. The colour hue differs from the modified colour hue due to the possible bleaching of sulphur dioxide and changes in pH that could influence the reading. However, the small differences in hue obtained here, though sometimes significantly different, will not influence the visual perception of the wine.

Table 4.2: Colour parameters (colour intensities, optical densities and hues) for the control and oxygenated wines over time.

¥ Colour Intensity (AU)	Control	*MO 16	*MO 32
Before oxygen addition	12.28 ± 0.18^{ab}	12.35 ± 0.24^{ab}	12.125 ± 0.07^{ab}
After oxygen addition	11.56 ± 0.18^{ac}	12.66 ± 0.15^{b}	12.49 ± 0.39^{ab}
After MLF	8.47 ± 1.12^{d}	10.59 ± 0.26^{ce}	10.23 ± 0.62^{ef}
2 Months after MLF	8.87 ± 0.13^{dg}	9.73 ± 0.29^{efg}	9.31 ± 0.38^{df}
Optical Density - 420 nm (AU)	**Control**	***MO 16**	***MO 32**
Before oxygen addition	3.23 ± 0.06^{a}	3.24 ± 0.08^{a}	3.18 ± 0.03^{a}
After oxygen addition	3.14 ± 0.06^{ab}	3.66 ± 0.06^{c}	3.61 ± 0.11^{c}
After MLF	2.61 ± 0.27^{d}	3.26 ± 0.05^{a}	3.17 ± 0.17^{ab}
2 Months after MLF	2.65 ± 0.06^{d}	2.92 ± 0.10^{be}	2.80 ± 0.08^{de}
Optical Density - 520 nm (AU)	**Control**	***MO 16**	***MO 32**
Before oxygen addition	7.90 ± 0.09^{a}	7.95 ± 0.13^{a}	7.82 ± 0.05^{ab}
After oxygen addition	7.24 ± 0.15^{b}	7.61 ± 0.11^{ab}	7.51 ± 0.24^{ab}
After MLF	4.76 ± 0.72^{c}	5.84 ± 0.18^{d}	5.63 ± 0.37^{de}
2 Months after MLF	5.16 ± 0.11^{ce}	5.55 ± 0.16^{de}	5.32 ± 0.26^{cd}
Optical Density - 620 nm (AU)	**Control**	***MO 16**	***MO 32**
Before oxygen addition	1.15 ± 0.03^{ab}	1.16 ± 0.04^{ab}	1.13 ± 0.01^{a}
After oxygen addition	1.18 ± 0.02^{ab}	1.39 ± 0.03^{cde}	1.37 ± 0.07^{ce}
After MLF	1.10 ± 0.13^{a}	1.50 ± 0.04^{d}	1.43 ± 0.08^{cd}
2 Months after MLF	1.07 ± 0.01^{a}	1.27 ± 0.04^{be}	1.19 ± 0.04^{ab}
§Colour Hue (AU)	**Control**	***MO 16**	***MO 32**
Before oxygen addition	0.41 ± 0.06^{a}	0.41 ± 0.03^{a}	0.41 ± 0.05^{a}
After oxygen addition	0.43 ± 0.00^{e}	0.48 ± 0.00^{b}	0.48 ± 0.00^{b}
After MLF	0.55 ± 0.20^{c}	0.56 ± 0.26^{c}	0.56 ± 0.05^{c}
2 Months after MLF	0.51 ± 0.10^{d}	0.53 ± 0.05^{d}	0.53 ± 0.06^{d}
§Modified Colour Hue (AU)	**Control**	***MO 16**	***MO 32**
Before oxygen addition	0.46 ± 0.00^{a}	0.48 ± 0.01^{ab}	0.46 ± 0.00^{a}
After oxygen addition	0.48 ± 0.00^{bc}	0.52 ± 0.00^{d}	0.52 ± 0.00^{d}
After MLF	0.50 ± 0.01^{c}	0.53 ± 0.00^{d}	0.53 ± 0.00^{d}
2 Months after MLF	0.53 ± 0.01^{d}	0.53 ± 0.00^{d}	0.53 ± 0.02^{d}
¥Modified Colour Intensity (AU)	**Control**	***MO 16**	***MO 32**
Before oxygen addition	12.22 ± 0.58^{ab}	12.89 ± 0.02^{a}	12.78 ± 0.03^{ac}
After oxygen addition	11.98 ± 0.52^{bd}	11.78 ± 0.50^{bde}	12.07 ± 0.43^{bc}
After MLF	11.6 ± 0.33^{be}	12.45 ± 0.36^{acd}	12.36 ± 0.17^{acd}
2 Months after MLF	10.45 ± 0.07^{f}	11.21 ± 0.12^{ef}	11.20 ± 0.55^{e}

*MO 16 and MO 32 represent the oxygenated tanks that received 16 mg/L and 32 mg/L, respectively. ¥Colour intensity and modified colour intensity are calculated as the sum of optical densities at 420 nm, 520 nm and 620 nm. §Colour hue and modified colour hue are calculated as 420 nm/520 nm. Note: all values displayed in table given in absorbance values (AU) and are the average of duplicate treatments with the standard deviation of triplicate measurements displayed after the concentration. The letter after each concentration denotes the significant differences on a 5% confidence interval within each analysis.

4.3.3 Total anthocyanins

The total monomeric anthocyanin concentrations as determined by spectrophotometry and RP-HPLC (Table 4.3) decreased over time in all wines, but more significantly in the oxygen treated wines. The significant decrease in anthocyanins observed in the oxygenated wines is in agreement with work done by other authors (Cacho *et al*., 1995; Cano-Lopez *et al*., 2008; Fell *et al*., 2007; Llaudy *et al*., 2006; Perez-Magarino *et al*., 2007; Sartini *et al*., 2007; Tao *et al*., 2007;

Versari *et al*., 2008). The anthocyanin concentrations then remained stable during the two month ageing period.

In the control using both methods, the decrease in anthocyanin concentration continued until after MLF, but stabilised during the ageing period in most of the treatments. The control had approximately 30% more monomeric anthocyanins than the oxygenated wines after two months of ageing. The larger decrease in the oxygenated wines could be due to the partaking of anthocyanins in polymerisation reactions that involves acetaldehyde via the formation of ethyl bridges that is especially favoured by the presence of oxygen (Atanasova *et al*., 2002). This explains why the concentration of anthocyanins in the control decreased as well, but not as radically as in the oxygenated wines. Cheynier *et al.* (2006) and Salas *et al.* (2004) ascribed changes in colour during ageing to anthocyanin-tannin reactions. However, ethyl-bridges formed through acetaldehyde-induced polymerisation are unstable. Through cleavage and addition reactions they are gradually converted to more stable pyranoanthocyanin derivatives (Cheynier *et al*., 1999).

The anthocyanin concentrations determined by RP-HPLC were much lower than those obtained by spectrometry. The spectrometric analysis is known to overestimate the monomeric anthocyanin concentration, due to the inclusion of polymerised pigments sensitive to the bleaching effect of bisulphite (Llaudy *et al*., 2006). Remy *et al.* (2000) stated that pigments resistant to sulphite bleaching is not necessarily polymeric, and some polymeric pigments are not resistant to sulphite bleaching. On the contrary, the RP-HPLC method only measures monomeric anthocyanins, thus resulting in differences observed in the anthocyanin concentration determined with the two methods. This was proved in a study done by Rivas-Gonzalo *et al*. (1992). However, the two types of measurements showed the same tendency and correlated (r^2 = 0.9062).

Table 4.3: Total anthocyanin concentrations (mg/L) as determined by spectrometry (Ribereau-Gayon and Stonestreet, 1965) and RP-HPLC (Peng *et al*., 2002) for control and oxygenated tanks over time.

¢Spectrometry	Control (mg/L)	*MO 16 (mg/L)	*MO 32 (mg/L)
Before oxygen addition	459 ± 9.90^{a}	473 ± 0.8^{a}	461 ± 7.9^{a}
After oxygen addition	432 ± 21.6^{a}	326 ± 13.5^{b}	336 ± 8.1^{b}
After MLF	386 ± 17.0^{c}	272 ± 41.7^{d}	271 ± 9.1^{d}
2 Months after MLF	367 ± 26.2^{bc}	261 ± 37.5^{d}	266 ± 29.5^{d}
¢RP-HPLC	**Control (mg/L)**	***MO 16 (mg/L)**	***MO 32 (mg/L)**
Before oxygen addition	207 ± 0.04^{a}	198 ± 9.9^{a}	197 ± 1.8^{a}
After oxygen addition	193 ± 9.8^{a}	92 ± 5.5^{b}	95 ± 8.5^{b}
After MLF	76 ± 3.3^{bc}	39 ± 0.7^{d}	42 ± 4.6^{cd}
2 Months after MLF	76 ± 11.8^{bc}	83 ± 52.9^{be}	49 ± 11.3^{cde}

*MO 16 and MO 32 represent the oxygenated tanks that received 16 mg/L and 32 mg/L, respectively. ¢Note: all values displayed in table are the average of duplicate treatments with the standard deviation of triplicate measurements displayed after the concentration. The letter after each concentration denotes the significant difference on a 5% confidence interval within each analysis.

The large decrease in monomeric anthocyanin concentration in the oxygenated wines can be further explained when investigating the evolution of the polymeric pigments over time (Fig 4.1). The latter increased significantly with oxygen addition compared to the control, after which all wines stabilised in concentration. It is thus evident that monomeric anthocyanins are involved in polymerisation reactions, even more so in the presence of oxygen, since oxygen favours the formation of ethyl bridges (Atanasova *et al*., 2002; Llaudy *et al*., 2006). Bosso *et al.* (2000) and

Castel *et al.* (2001) also confirmed an increase in polymeric pigments, concluding that the addition of oxygen activated the reactions among free anthocyanins and flavanols, forming new coloured compounds stable to sulphur dioxide bleaching and pH changes. The addition of oxygen before MLF thus seems like a viable option to help stabilise the colour of Pinotage wine. Figure 4.2 shows the correlation between polymeric pigments and anthocyanin concentrations as obtained by spectrometry (Ribereau-Gayon and Stonestreet, 1965) and RP-HPLC (Peng *et al*., 2002).

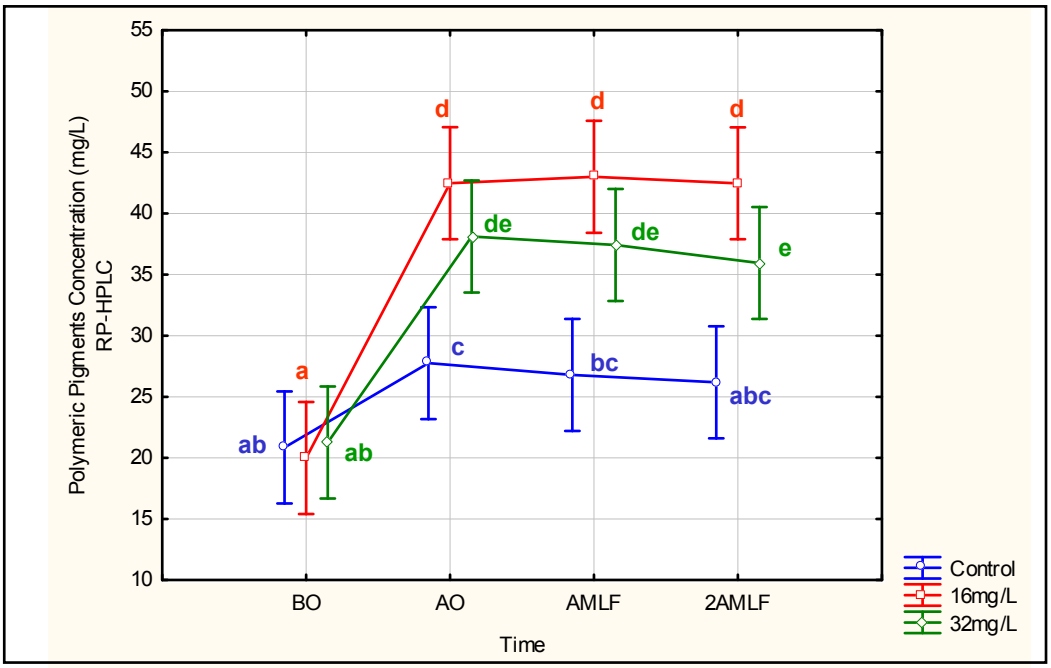


Figure 4.1: Polymeric pigment concentration (mg/L) as determined by RP-HPLC for the control and oxygenated wines over time (p = 0.025). Note: the lines represent the concentrations of the control and oxygenated wines (16 mg/L and 32 mg/L). BO represents samples analysed before oxygen addition; AO represents after oxygen was added; AMLF represents the completion of MLF and 2AMLF represents the end of a two month ageing period after MLF. Error bars indicate the standard deviation for two repeats and the letter at each data point indicates the significant difference.

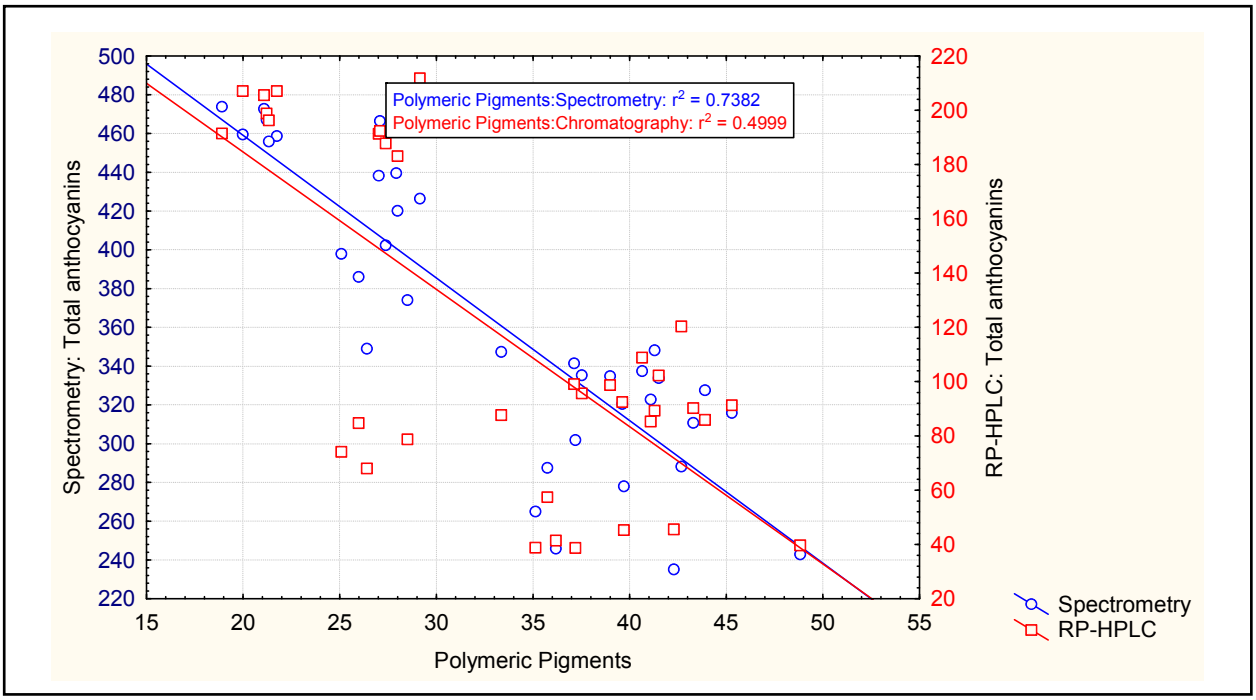


Figure 4.2 Correlation between polymeric pigments and anthocyanins as obtained by spectrometry and RP-HPLC. "r^2" represents the coefficient of determination for the correlation between the two methods.

4.3.4 Monomeric flavan-3-ols

Monomeric flavan3-ol concentrations decreased significantly in the oxygen treated tanks after oxygen addition, with no change in the control, as determined spectrophotometrically with the DMAC method (Table 4.4). The same decreasing trend was found for data obtained by the RP-HPLC for monomeric flavan-3-ols (Table 4.4), which was calculated as the sum of (+)-catechin, (+)-gallocatechin, (-)-epigallocatechin and (-)-epicatechingallate. An increase in the mean degree of polymerisation (mDP) could have led to this decrease (Llaudy *et al*., 2006), specifically the formation of larger polymers that is favoured by the addition of oxygen (Nikfardjam and Dykes, 2002). Another explanation could be due to the especially high reactivity of these molecules in an oxidative environment and their participation in polymerisation reactions that is favoured by the presence of oxygen (Somers, 1971). There was an increase in monomeric flavan-3-ol concentration after MLF for both analyses, with the controls having higher concentrations. Thereafter the control remained constant according to RP-HPLC analyses, but decreased in the DMAC index. In the latter the oxygenated treatments decreased even further with ageing, whereas the RP-HPLC data showed an increase in monomeric flavanol concentrations after two months of ageing. The increase in flavan-3-ol concentrations after MLF could be due to the cleavage of polymers and the resulting release in monomers (Cheynier *et al*., 1999). Although there was differences when comparing the DMAC index and HPLC results, the data still correlated well (r^2 = 0.8626). (+)-Catechin, the biggest contributing monomer in our wines, was found to be lower in oxygenated wines shortly after the addition of oxygen (Table 4.4). This was in agreement with work done by Ferranini *et al.* (2001) and Du Toit *et al*. (2006)

The RP-HPLC analysis show lower concentrations than the DMAC index which could be ascribed to the limited amount of available standards for the compounds quantified in our laboratory. Also, the DMAC index includes proanthocyanidins in its measurement, which could

affect the final concentration, although they react to a much lesser extent than the monomeric flavan-3-ols (Nagel and Glories, 1991).

Table 4.4: Monomeric flavanol concentrations (mg/L) as determined by spectrometry (Nagel and Glories, 1991) and RP-HPLC (Peng *et al*., 2002) for control and oxygenated wines over time. Individual (+)-catechin concentrations determined by RP-HPLC are also included in this table.

¢DMAC Index	Control (mg/L)	*MO 16 (mg/L)	*MO 32 (mg/L)
Before oxygen addition	387 ± 7.9^{ab}	415 ± 1.2^{a}	392 ± 12.7^{ab}
After oxygen addition	412 ± 15.2^{a}	326 ± 16.9^{cd}	328 ± 10.8^{cd}
After MLF	415 ± 11.7^{a}	372 ± 1.9^{be}	381 ± 1.6^{b}
2 Months after MLF	352 ± 10.9^{ce}	324 ± 4.7^{cd}	320 ± 6.1^{d}
¢RP-HPLC: All monomeric flavanols	**Control (mg/L)**	***MO 16 (mg/L)**	***MO 32 (mg/L)**
Before oxygen addition	102 ± 4.8^{m}	104 ± 1.1^{m}	103 ± 11.5^{m}
After oxygen addition	105 ± 12.0^{mn}	78 ± 10.7^{o}	78 ± 10.0^{o}
After MLF	132 ± 8.7^{p}	114 ± 1.5^{q}	112 ± 1.5^{nq}
2 Months after MLF	134 ± 7.9^{p}	129 ± 2.8^{p}	127 ± 5.1^{p}
RP-HPLC: (+)-Catechin	**Control (mg/L)**	***MO 16 (mg/L)**	***MO 32 (mg/L)**
Before oxygen addition	27.6 ± 3.5^{a}	29.1 ± 1.9^{ab}	27.6 ± 2.4^{a}
After oxygen addition	32.3 ± 3.4^{b}	17.0 ± 1.7^{c}	17.6 ± 2.3^{c}
After MLF	43.0 ± 1.8^{d}	38.6 ± 0.3^{e}	37.2 ± 0.8^{e}
2 Months after MLF	37.8 ± 1.6^{e}	40.9 ± 2.1^{de}	37.2 ± 2.2^{e}

*MO 16 and MO 32 represent the oxygenated tanks that received 16 mg/L and 32 mg/L, respectively. ¢Note: all values displayed in table are the average of duplicate treatments with the standard deviation of triplicate measurements displayed after the concentration. The letter after each concentration denotes the significant difference on a 5% confidence interval within each analysis.

4.3.5 Total phenols

Oxygen had a noticeable influence on the total phenol content of the oxygenated treatments (Fig 4.3). The addition of 16 mg/L and 32 mg/L of oxygen led to a significant decrease in total phenol concentration ($p < 0.05$) as determined spectrophotometrically by the Folin-Ciocalteu assay. The losses in total phenol concentrations could be due to condensation, oxidation and later precipitation. Llaudy *et al.* (2006) found only a slight decrease in total phenolic concentration during oxygen addition. Perez-Margarino *et al.* (2007) stated that in some cases oxygenated wines have lower phenolic concentrations. Losses in total phenols after malolactic fermentation are also in agreement with work done by Amati *et al.* (2002) and Ferranini *et al.* (2001).

After the completion of MLF the phenol concentration increased again for the oxygen treated wines. Fell *et al.* (2007) stipulated that even if there is little change in individual phenols and no phenols are added to the wine, changes in their structure as new compounds are formed can lead to a different response with the Folin-Ciocalteu assay. The total phenol concentrations stabilised over a two month period in our experiment and none of the wines differed significantly from each other or from the concentrations measured after MLF.

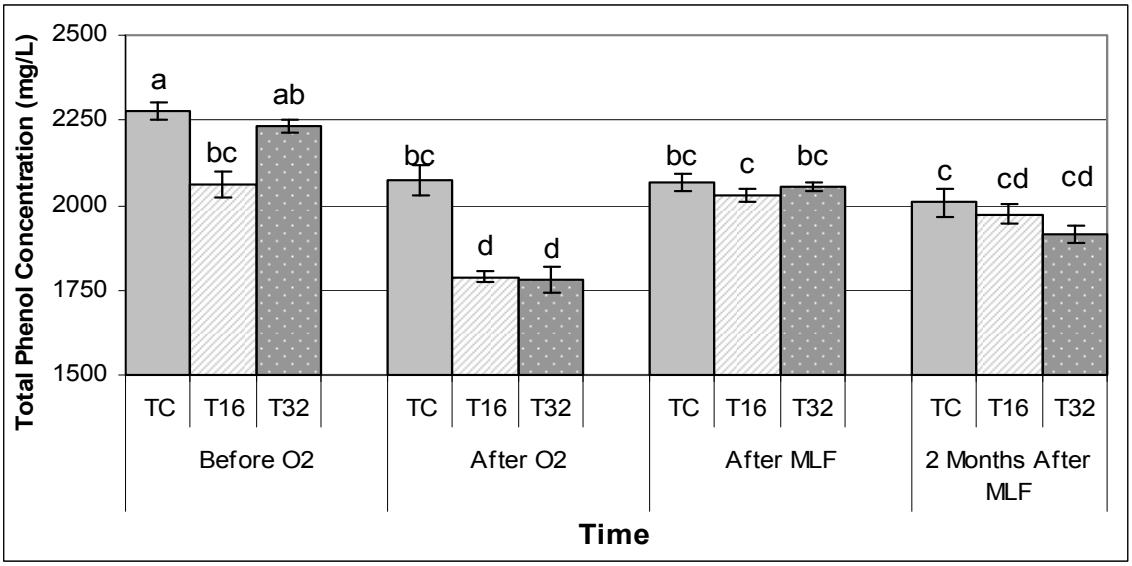


Figure 4.3: The graph indicates the total phenol concentration (mg/L gallic acid units) as measured with the Folin-Ciocalteu assay. The control is represented as 'TC', oxygenated tanks receiving 16 mg/L oxygen as 'T16' and tanks receiving 32 mg/L oxygen as 'T32'. The error bars denote the standard deviation of the mean between duplicate treatments. The letter above each bar represents the significant differences between tanks.

4.3.6 Tannins

The application of oxygen did not have a significant influence on the tannin concentration of the wines as measured with the BSA precipitation method. Small fluctuations in tannin concentration were noted for the control and treated wines, without any significant trends observed (Fig 4.4). The most apparent observation was the decreasing tendency in tannin concentration for oxygenated wines, although not significantly different from the control at any point of time. Work done by Llaudy *et al.* (2006) found a decreasing tendency in tannin concentration in both the control and oxygenated wines over time. In contrast with our results, Cano-Lopez and co-workers (2006) found a decrease in tannin concentration during MLF, with oxygenated wines having a slightly higher tannin content.

Work by Harbertson (2002) and Adams *et al*. (1999) confirmed that dimers and trimers do not respond in a protein-binding assay such as this method and it's effectiveness in determining tannin concentration is limited to those oligomeric proanthocyanidin with a degree of polymerisation greater than four units. Therefore the BSA tannin method should correlate well with the polymeric phenol peak which eluted at the end of a run as obtained from RP-HPLC. Contrary to this, we have found a very poor correlation (r^2 = 0.1713) between the tannin concentration and the polymeric phenol content determined by RP-HPLC for all samples. However, the control correlated better than oxygenated treatments and this correlation decreased with an increase in oxygen addition. When analysed individually, we found that the correlation of the oxygen treatments contributed most to the weak correlation ($R^2_{16mg/L}$ = 0.1716; $R^2_{32mg/L}$ = 0.0712) compared to the control ($R^2_{Control}$ = 0.4908). The lack of a good correlation could be due to the incorporation of pigments and ethyl bridges in the measurement of polymeric phenols determined by HPLC, thus disturbing the precipitation reaction with proteins to give an accurate indication of the amount of tannin present in a wine (Mercurio and Smith, 2008). Another speculation is that the larger oxidised tannin polymers may not precipitate protein as efficiently. The possibility exists that they are incorporated into soluble complexes with the protein and therefore gives an unexpected correlation (Harbertson, 2009). In contrast, De Beer *et al*. (2004) found a good correlation between the high molecular weight portion of

normal phase HPLC and the protein precipitable tannins, but this could vary between different wines.

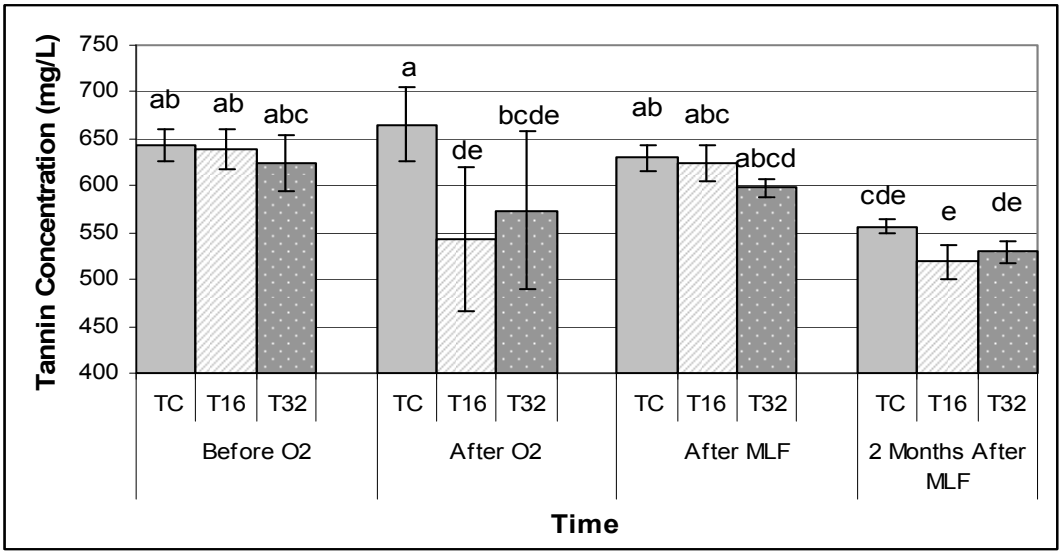


Figure 4.4: Tannin concentration (mg/L catechin equivalents) as determined by the BSA precipitation assay for the control and oxygenated wines. The control is represented as 'TC', oxygenated tanks receiving 16 mg/L oxygen as 'T16' and tanks receiving 32 mg/L oxygen as 'T32'. The error bars denote the standard deviation of the mean between duplicate treatments. The letter above each bar represents the significant differences between tanks.

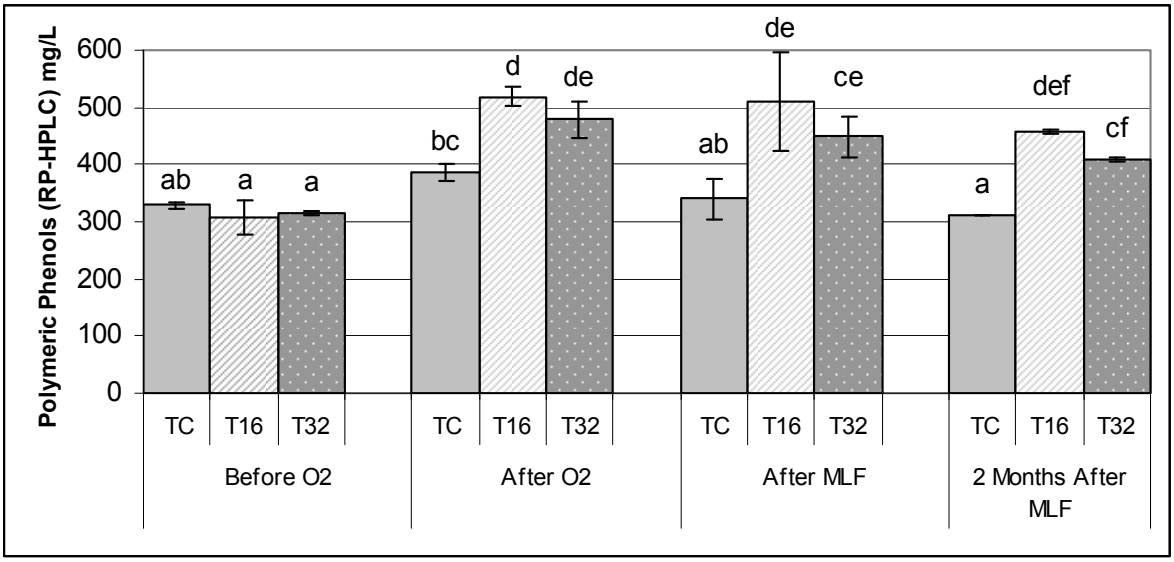


Figure 4.5: Polymeric phenol concentration (mg/L) as measured by RP-HPLC for the control and oxygenated wines over time. The control is represented as 'TC', oxygenated tanks receiving 16 mg/L oxygen as 'T16' and tanks receiving 32 mg/L oxygen as 'T32'. The error bars denote the standard deviation of the mean between duplicate treatments. The letter above each bar represents the significant differences between tanks.

4.3.7 Polymeric phenols

Polymeric phenols of all the wines increased significantly when analysed after the eight days of oxygen addition, except the control. However, the oxygenated wine's polymeric phenol content was always significantly higher than that of the control after oxygen was applied (Fig 4.5). The

initial significant increase in polymeric phenol concentration for the oxygenated wines is in agreement with the decrease in monomeric flavanol concentration determined by HPLC. It is thus evident that the reduction in monomeric flavanols was due to the participation in polymerisation reactions. The decrease in polymeric phenol concentration witnessed over time could be due to precipitation and build-up and especially breakdown reactions of the polymers, although they were not significant (Haslam *et al*., 1980; Vidal *et al*., 2002).

4.3.8 Flavonols

All individual flavonols decreased over time, except quercetin that steadily increased ($p = 0.00002$) with time (Fig 4.6). The latter is not in accord with studies done by Fang *et al.* (2007), who found a loss in quercetin as time proceeded. The increase in quercetin could be due to hydrolysis of quercetin-glucosides in the acidic wine medium (Price *et al*., 1995). However, there was a significantly lower quercetin concentration in the treated wines just after the oxygen treatment compared to the control. These differences disappeared after an ageing period of two months, our finding is in agreement with Sartini *et al.* (2007). The greater loss of quercetin in the oxygen treated wines could possibly be due to the high reactivity of this molecule with oxygen (Park *et al*., 2003). Perez-Magarino *et al.* (2007), however, found that the oxygenated wines had the highest concentration of quercetin and not the control.

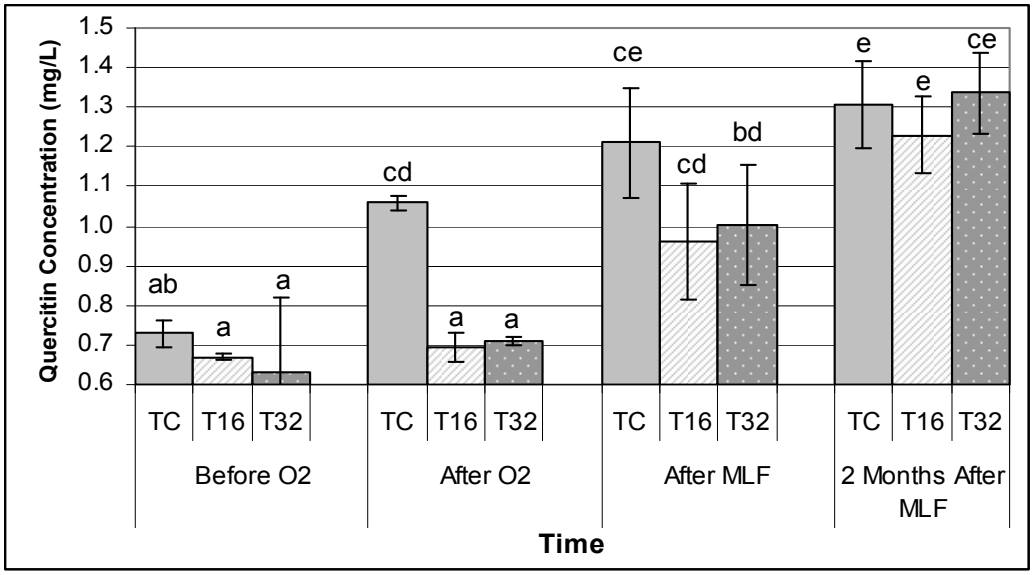


Figure 4.6: Quercetin concentrations (mg/L) as determined by RP-HPLC for the control and oxygenated wines. The control is represented as 'TC', oxygenated tanks receiving 16 mg/L oxygen as 'T16' and tanks receiving 32 mg/L oxygen as 'T32'. The error bars denote the standard deviation of the mean between duplicate treatments. The letter above each bar represents the significant differences between tanks.

4.4 Conclusion

We showed that oxygen sparged at the bottom of the tank does not influence the phenolic composition of the wine differently at different positions in the tank. This implicates that a winemaker using this technique on commercial scale would achieve the same effect of oxygenation throughout the tank if the tank is tall enough.

The application of microoxygenation is beneficial in terms of colour as we found significant differences between the controls and oxygen treated wines in terms of colour intensity, free anthocyanins and polymeric pigments and polymeric phenols just after MLF. This technique can thus be used to increase and stabilise colour of Pinotage wine before MLF, although it seems that these effects might disappear with further ageing. The treatment did not have a significant influence on the tannin concentration and small differences in total phenol concentrations were observed. However, differences observed between other authors and ourselves indicate the need for further research on the effect of micro-oxygenation on red wine's colour and phenolic composition.

The technique is easy to apply and can be used in most commercial wineries. Future work should include ageing the wine for longer periods of time and a tasting panel should also evaluate the wines.

4.5 Acknowledgements

The authors would like to thank Distell Winery for donating the wine, supplying the facilities to perform the experiment and the use of their equipment, Winetech, Thrip and the NRF for providing financial support and Prof Martin Kidd for analysing the data statistically.

4.6 Reference

Adams, D.O., and Harbertson, J.F. (1999). Use of alkaline phosphatase for the analysis of tannins in grapes and red wines. *American Journal of Enology and Viticulture*. **50**, 247-252.

Alcalde-Eon, C., Escribano-Bailon, M.T., Santos-Buelga, C. and Rivas-Gonzalo, J.C. (2006). Changes in the detailed pigment composition of red wine during maturity and ageing. A comprehensive study. *Analytica Chimica Acta*. **563**, 238-254

Amati, A., Arfelli, G., Castellari, M and Simoni, M. (2002). Effetto dell'ossigenazione controllata sulla frazione fenolica dei vini rossi. *Industrie della Bevande*. **29**, 606-612

Atanasova, V., Fulcrand, H., Cheynier, V. and Moutounet, M. (2002). Effect of oxygenation on polyphenol changes occurring in the course of wine-making. *Analytica Chimica Acta*. **458**, 15-27.

Bakker, J., Picinelli, A. and Bridle, P. (1993). Model wine solutions: colour and composition changes during ageing. *Vitis*. **32**, 111-118.

Bosso, A., Guaita, M., Vaudano, E. and Di-Stefano, R. (2000). Influenza dell' ossigeno sull' evoluzione dei composti fenolici durante la conservazione dei vini rossi. *Industrie delle Bevande*. **29**, 630-640.

Bousbouras, G.E. and Kunkee, R.E. (1971). Effect of pH on malolactic fermentation in wine. *American Journal of Enology and Viticulture*. **22**, 121-126.

Cacho, J., Castells, J.E., Esteban, A., Laguna, B. and Sagrista, N. 1995. Iron, copper and manganese, influence on wine oxidation. *American Journal of Enology and Viticulture*. **46**, 380-384.

Cano-Lopez, M., Pardo-Minguez, F., Lopez-Roca, J.M. and Gomez-Plaza, E. (2006). Effect of microoxygenation on anthocyanin and derived pigment content and chromatic characteristics of red wines. *American Journal of Enology and Viticulture*. **57**, 325-331

Cano-Lopez, M., Pardo-Minguez, F., Schmauch, G., Saucier, C., Teissedre, P.L., Lopez-Roca, J.M. and Gomez-Plaza, E. (2008). Effect of micro-oxygenation on color and anthocyanin-related compounds of wines with different phenolic contents. *Journal of Agicultural and Food Chemistry*. **56**, 5932-5941.

Castel, C., Morand, A., Pujol, G. and Naudin, R. (2001). Influence de la micro-oxygenation sur la composition phenolique et les caracteres sensoriels des vins rouges. *Industrie delle Bevande*. **30**, 271-276.

Castellari, M., Matricardi, L., Arfelli, G., Galassi, S. and Amati, A. (2000). Level of single bioactive phenolics in red wine as a function of the oxygen supplied during storage. *Food Chemistry*. **69**, 61-67.

Cheynier, V., Es Safi, N.E. and Fulcrand, H. (1999). Structure and colour properties of anthocyanins and related pigments. *In* International Congress on pigments in Food and Technology. M.I.M. Mosquera *et al*. (Eds.), pp 23-25.

Cheynier, V., Duenas-Paton, M., Salas, E., Maury, C., Souquet, J.M., Sarni-Manchado, P. and Fulcrand, H. (2006). Structure and properties of wine pigments and tannins. *American Journal of Enology and Viticulture*. **57**, 298-305.

De Beer, D., Harbertson, J.F., Kilmartin, P.A., Roginsky, V., Barsukova, T., Adams, D.O. and Waterhouse, A.L (2004). Phenolics: a Comparison of diverse analytical methods. *American Journal of Enology and Viticulture*. **55**, 389-400.

Du Toit, W.J., Lisjak, K., Marais. J. and Du Toit, M. (2006). The effect of microoxygenation on the phenolic composition, quality and aerobic wine-spoilage microorganisms of different *South African red wines. South African Journal of Enology and Viticulture*. **27**, 57-67.

Escribano-Bailon, T., Alvarez-Garcia, M., Rivas-Gonzalo, J.C., Heredia, F.J. and Santos-Buelga, C. (2001). Colour and stability of pigments derived from the acetaldehyde-mediated condensation between malvidin-3-O-glucoside and (+)-catechin. *Journal of Agricultural and Food Chemistry*. **49**, 1213-1217.

Fang, F. Li, J.M., Pan, Q.H. and Huang, W.D. (2007). Determination of red wine flavonoids by HPLC and effect of ageing. *Food Chemistry*. **101**, 428-433

Fell, A., Dykes, S.I., Nicolau, L. and Kilmartin, P.A. (2007). Electrochemical microoxidation of red wine. *American Journal of Enology and Viticulture*. **58**, 443-450

Ferranini, R., Girardi, F., De Conti, D. and Castellari, M. (2001). Esperienze di applicazione della microossigenazione come tecnica d'affinamento dei vini. *Industrie della bevande*. **30**, 116-118.

Fulcrand, H., Cheynier, V.. Oszmianski, J. and Moutounet, M. (1997). An oxidized tartaric acid residue as a new bridge potentially competing with acetaldehyde in flavan-3-ol condensation. *Phytochemistry*. **46**, 223-227.

Glories, Y. (1984). Anthocyanins and tannins from wine: Organoleptic properties. In: 'Plant flavonoids in biology and medicine II: Biochemical, cellular, and medicinal properties'. Eds. V.Cody et al. (Alan R. Liss, Inc., New York) pp 123-134.

Harbertson, J.F. (2009). Personal communication.

Harbertson, J.F., Kennedy, J.A., and Adams, D.O. (2002). Tannins in skins and seeds of Cabernet Sauvignon, Syrah, and Pinot noir berries during ripening. *American Journal of Enology and Viticulture.* **53**, 54-59.

Harbertson, J.F., Hodgins, R.E., Thurston, L.N., Schaffer, L.J., Reid, M.S., Landon, J.L., Ross, C.E. and Adams, D. (2008). Variability of tannin concentration in red wines. *American Journal of Enology and Viticulture*. **59**, 210-214.

Haslam E. (1980). In vino veritas: oligomeric procyanidins and the ageing of red wines. *Phytochemistry*. **19**, 2577-2582.

Llaudy. M., Canals, R., Gonzales-Manzano, S., Canals J.M., Santos-Buelga, C. and Zamora, F. (2006). Influence of micro-oxygenation treatment before oak ageing on phenolic compounds composition, astringency, and color of red wine. *Journal of Agricultural and Food Chemistry*. **54**, 4246-4252.

McCord, J. (2003). Application of toasted oak and micro-oxygenation to ageing of Cabernet Sauvignon wines. *Australian and New Zealand Grapegrower and Winemaker*. July: 43-53.

Mcmurrough, I. and Mcdowell, J. (1978). Chromatographic separation and automated analysis of flavanols. *Analytical Biochemistry*. **91**,92-100.

Mercurio, M.D. and Smith, P.A. (2008). Tannin Quantification in red grapes and wine: comparison of polysaccharide- and protein-based tannin precipitation techniques and their ability to model wine astringency. *Journal of Agricultural and Food Chemistry*. **56**, 5528-5537.

Nagel, C.W. and Glories, Y. (1991). Use of a modified dimethylaminocinnamaldehyde reagent for analysis of flavanols. *American Journal of Enology and Viticulture*. **42**, 364-366.

Nieuwoudt, H.H., Prior, B.A., Pretorius, I.S., Manley, M. and Bauer, F.F. (2004). Principle component analysis applied to fourier transform infrared spectroscopy for the design of calibration sets for glycerol prediction models in wine and for the detection and classification of outlier samples. *Journal of Agricultural and Food Chemistry*. **52**, 3728-3735.

Nikfardjam, M.P. and Dykes, S. (2002). Micro-oxygenation research at Lincoln University. *The Australian and New Zealand Grapegrower and Winemaker*. 66-68.

Park, C., So, H.S., Shin, C.H., Back, S.H., Moon, B.S., Lee, D.W. and Park, R. (2003). Quercetin protects the hydrogen peroxide-induces apoptosis via inhibition of mitochondrial dysfunction in H9c2 cardiomyoblast cells. *Biochemical Pharmocology*. **66**, 1278-1295.

Peng, Z., Iland, P.G., Oberholster, A., Sefton, M.A. and Waters, E.J. (2002). Analysis of pigmented polymers in red wine by reversed phase HPLC. *Australian Journal of Grape and Wine Research*.

Perez-Magarino, S., Sanchez-Iglesias, M., Ortega-Heras, M., Gonzales-Huerta, C. and Gonzales-Sanjose, M.L. (2007). Colour stabilisation of red wines by microoxygenation treatment before malolactic fermentation. *Food Chemistry*. **101**, 881-893.

Price, S., Breen, P., Valladao, M. and Watson, B. (1995) Cluster sun exposure and quercetin in Pinot noir grapes and wine. *American Journal of Enology and Viticulture* **46**, 187-194.

Remy, S., Fulcrand, H., Labarbe, B., Cheynier, V. and Moutounet, M. (2000). First confirmation in red wine of products resulting from direct anthocyanin-tannin reactions. *Journal of the Science of Food and Agriculture*. **80**, 745-751.

Ribereau-Gayon, P. and Stonestreet, E. (1965). Le dosage des anthocyanes dans les vins rouges. *Bulletin de la Societe Chimique de France*. **9**, 2649-2652.

Ribereau-Gayon, P., Glories, Y., Meujean, A. and Dubourdieu, D. (2006). "Handbook of Enology, Vol 2. The chemistry of wine stabilisation and treatments." (John Wiley and Sons, Ltd. New York).

Rivas-Gonzalo, JC., Gutierrez, Y., Hebrero, E. and Santos-Buelga, C. (1992). Comparison of methods for the determination of anthocyanins in red wines. *American Journal of Enology and Viticulture*. **43**,210-214.

Romero, C. and Bakker, J. (2000). Effect of acetaldehyde and several acids on the formation of vitisin A in model wine anthocyanin and colour evolution. *International Journal of Food Science and Technologies*. **35**, 129-140.

Salas, E., Atanasova, C., Poncet-Legrand, C., Meudec, E., Mazauric, J.P. and Cheynier, V. (2004). Demonstration of the occurrence of flavanol-anthocyanin adducts in wine and in model solutions. *Analytica Chimica Acta*. **513**, 325-332.

Sartini, E., Arfelli, G., Fabiani, A. and Piva, A. (2007). Influence of chips, lees, and micro-oxygenation during ageing on the phenolic composition of a red Sangiovese wine. *Food Chemistry*. **104**, 1599-1604.

Saucier, C., Mirabel, M., Guerra, C. and Glories, Y. (2004). Tannin-anthocyanin interactions: influence on wine colour. In: Red wine colour: Revealing the mysteries. Eds. Waterhouse, A.L. and Kennedy, J.A. (American Chemical Society/Oxford University Press, Washington, DC). 265-273.

Singleton V. (2000). A survey of wine ageing reactions, especially with oxygen. *In*: proceedings of the ASEV 50th Anniversary Annual Meeting. JM Rantz (Ed.). pp. 323-336. Davis, CA.

Somers, T.C. (1971). The polymeric nature of wine pigments. *Phytochemistry*. **10**, 2175-2186.

Tao, J., Dykes, S.I. and Kilmartin, P.A. (2007). Effect of SO2 concentration on polyphenol development during red wine micro-oxygenation. *Journal of Agricultural and Food Chemistry*. **55**, 6104-6109.

Versari, A., Boulton, R.B. and Parpinello, G.P. (2008). A comparison of analytical methods for measuring the color components of red wines. *Food Chemistry*. **106**, 397-402.

Vidal S, Cartalade, D.M., Souquet, J.M., Fulcrand, H. and Cheynier, V. (2002). Changes in proanthocyanidin chain-length in wine-like model solutions. *Journal of Agricultural and Food Chemistry*. **50**, 2261-2266.

Chapter 5

Research results

The influence of oxygen additions on the composition of Cabernet Sauvignon wine with different pHs

Chapter 5: The influence of oxygen additions on the composition of Cabernet Sauvignon wine with different pHs

5.1 Introduction

The anthocyanin concentration in wine decreases over time due to hydrolysis and/or polymerisation reactions. This disappearance of monomeric anthocyanins is due to a combination of reactions that affects wine colour stability. One of the most important factors influencing wine colour is pH, in addition to the type of anthocyanin molecule, the concentration in the solution, temperature and possible oxidative conditions occurring during winemaking. Phenolics react more readily with oxygen under high pH conditions (Waterhouse and Laurie, 2006).

The colour of a young red wine is mainly due to monomeric anthocyanins. As wine ages, a higher percentage of the colour is due to stable polymeric pigments (the result of polymerisation reactions) and copigmentation associations that are more resistant to pH fluctuations and sulphur dioxide bleaching (Boulton., 2001). During winemaking the pH has a tendency to change, especially during malolactic fermentation, where a general increase in pH is observed (Bousbouras and Kunkee, 1971).

Anthocyanin molecules occur in four different forms which are in equilibrium, with each form's concentration being primarily dependant on the pH. The red form (flavylium cation) has a nucleus with an electron deficit and therefore has a positive charged carbon ring (A^+), contributing to its red colour (Chapter 2, Fig 2.2). The blue quinoic base (AO) derives its name from having an aromatic ketone formed from a hydroxyl group on the A ring (Brouillard and Cheminat, 1986). The colourless carbinol base (AOH) is characterised by having an alcohol function. All these preceding structures form pale, yellow-coloured chalcones (C) when the heterocycle opens and have a ketone function at position C_2 or C_4. These two positions are causing the colourless form to have two isomers, *cis* or *trans* (Ribéreau-Gayon *et al*., 2006).

Glories (1984) conducted a study that showed that at a lower pH (3.4) more anthocyanins are present in the red form than at pH 4. More than 50% of anthocyanins are in the flavylium ion form at a pH lower than 2.5. Thus, given that the pH of a red wine is between pH 3 and 4, only around 25% of the anthocyanins are generally in the red form in a young wine (Brouillard and Mazza, 1989; Glories, 1984). Fulcrand *et al*. (2006) stated that the phenolic oxidation in wine is faster at a higher pH, as the removal of an electron is much easier from the phenolate anion than from the protonated phenols. According to Singleton (1987) there are approximately nine times as many phenolate anions present at pH 4 than at pH 3. Thus the auto-oxidation rate should therefore theoretically be nine times higher.

To the best of our knowledge, there is little published information available on the influence of pH and oxygen addition on changes in the colour and phenolic composition of red wine. Our study aimed to investigate the effects of both pH changes and oxygen additions when applied after alcoholic fermentation on red wines.

5.2 Material and Methods

5.2.1 Preparation of wine

Cabernet Sauvignon wine (2007 vintage) was obtained after the completion of alcoholic fermentation from Lourensford Wine Estate in the Somerset-West region in the Western Cape, South Africa.

The wine was analysed for pH, titratable acidity (TA), volatile acidity (VA), sugar content, alcohol concentration and malic acid concentration using a grapescan FT 120 instrument (Foss Electric, Denmark) (Nieuwoudt *et al*., 2004). The pH of the wine was 3.73 and confirmed by pH analysis using the Metrohm titration unit (Metrohm Ltd., Switzerland). Alcoholic fermentation was considered completed when the sugar content of the wine was below 4 g/L. The end of alcoholic fermentation was showed by FT 120 instrument (sugar content of 2.83 g/L).

The wine was divided into three 20 L canisters and the pH adjusted to 3.4, 3.7 and 4.0 with 10 M hydrochloric acid or potassium hydroxide. Hydrochloric acid was chosen for the adjustment in pH, because it was felt to be metabolically more inert than other mineral or organic acids.

Each specific pH treatment was then divided into 750 ml dark-coloured wine bottles to form a control and oxygenated group that was performed in triplicate. The control did not receive any oxygen at any point of time after collection at the wine estate. However, the oxygen treated wines received a total of 16 mg/L of oxygen over four consecutive days (4 mg/L/day) shortly after alcoholic fermentation and pH adjustment. The oxygen concentration was measured in all the wines before the application of oxygen using an Oxi 330i oxygen meter with a cell ox 325 probe (Wissenschaftlich-Technische Werkstatten). Thereafter, the treated wines were decanted by hand until 4 mg/L of oxygen was added and the headspace filled with nitrogen gas (Afrox, SA) and left until the next oxygen addition the following day.

5.2.2 Malolactic fermentation

All the wines underwent malolactic fermentation (MLF) after the oxygen treatment. The wines were inoculated with CH16 (Christiaan Hansen) (*Oenococcus oeni*) at 1 g/hl. Malolactic fermentation was conducted at 20°C. Malic and lactic acid concentrations were monitored on a grapescan FT 120 instrument (Foss Electric, Denmark) (Nieuwoudt *et al*., 2004). MLF was considered to be completed when the malic acid concentration was below 0.3 g/L. Malolactic fermentation was completed within 37 days. The wines were not further matured.

5.2.3 Wine sampling

Samples were taken after oxygen addition of both the control and treated wines. With the completion of MLF, samples of the control and oxygenated wines were taken. Samples were stored in 100 ml glass vials that were frozen immediately for spectrophotometric and HPLC analyses.

5.2.4 Spectrophotometric analyses

All spectrophotometric analyses were performed using an AnalyticJena Specord 50 UV/VIS spectrophotometer (Jena, Germany). Depending on the colour density of the wine or the required wavelength of the analysis the following cuvettes were used: 1 mm and 10 mm quartz cuvettes, 1 mm glass cuvettes or 10 mm plastic cuvettes.

The measurements were conducted on control and treated samples taken after the addition of oxygen, and after MLF. Samples were immediately frozen in 100 ml glass vials and stored at -20°C. All the preceding analyses were done at the same time to quantify all samples under the same conditions or with the same calibration curve if necessary. Samples were centrifuged for 2 min at 2000 rpm to remove any solid particles that could influence the reading. All analyses were performed in triplicate.

For a detailed description of all spectrophotometric and HPLC analyses, refer to Chapter 3, section 3.2.5 and 3.2.6, although the tannin concentration was not established in this experiment.

5.2.5 Statistical analysis

Mean values and standard deviations were calculated using one-way analysis of variance (ANOVA). The significance of differences between mean values obtained was determined using a student t-test at the 95% significance level, $p < 0.05$, using Statistica 8 (StatSoft Inc) software.

5.3 Results and discussion

5.3.1 Colour intensity, optical density and hue

All the wines showed a decrease in colour intensity with an increase in pH (Table 5.1). This can be explained by the increasing pH leading to a shift in the anthocyanin equilibrium, which leads to more anthocyanins in the colourless form (Brouillard and Delaporte, 1977; Ribereau-Gayon *et al.,* 2006). This is in agreement with work done by other authors (Van der Merwe, 2006). The application of oxygen did not influence the colour intensity significantly before or after MLF within the different pH treatments. The control wine of pH 3.4 before MLF had the highest colour intensity of all the wines, but the colour intensity decreased dramatically and significantly to values lower than the oxygenated wine after MLF.

The hue increased in the control and oxygenated wines during MLF for all pH treatments (Table 5.1). This increase in hue was due to the decrease in optical density at 520 nm. The formation of polymeric pigments such as the pyranoanthocyanins, which absorb relatively more light at 420 nm than at 520 nm could also have contributed to the higher colour hue (Fell *et al*., 2007).

The optical density (520 nm) decreased significantly as the pH increased (Table 5.2). The control wine for pH 3.4 before MLF had the highest 520 nm absorbance, but decreased drastically with MLF to absorbance values (7.78) lower than the oxygenated wine (8.66). Monomeric anthocyanins could have participated in polymerisation reactions due to the oxygen addition and this could have contributed to the higher colour density in the oxygenated wine (Gonzales-Neves *et al*., 2004; Monagas *et al*., 2006). Oxygen consumption could have induced colour stabilisation due to indirect polymerisation products such a ethyl-linked pigmented tannin (Sartini *et al*., 2007; Escribano-Bailon *et al*., 2001). All wines of all pH treatments decreased during MLF in absorbance values at 520 nm. The lower absorption at 520 nm observed after MLF in all pH's can be explained by the possible association of anthocyanins with yeast cells and bacteria cells (Medina *et al*., 2005) and due to direct association of anthocyanins with tannins (Fulcrand *et al*., 2006). The oxygenated wines seemed to have a higher 520 nm absorbance than the control after MLF, although it was shown to not be a significant difference. It is possible that this trend could have become significant if wines were aged for longer. We can only postulate that the increasing trend of higher 520 nm absorbance observed in the

oxygenated wines could be a result of polymerisation reactions that involves the anthocyanins and oxygen to produce more stable pigmented compounds with a higher absorbance at 520 nm. These reactions could include indirect condensation as well as oxidation of colourless direct condensation products (anthocyanins and tannin (A-T) to the red form (Ribereau-Gayon *et al*., 2006).

Optical density at 420 nm (Table 5.2) were not influenced significantly by MLF or the addition of oxygen, however, pH did cause significant differences between treatments. The fact that optical density at 420 nm did not increase over time might suggest that oxidation of the phenols did not take place to a large extent (Castellari *et al*., 2000; Perez-Prieto *et al*., 2003).

All wines increased in optical density at 620 nm during MLF (Table 5.2), although not always significant. Also, there was a trend in the oxygenated wines to have a higher absorbance at 620 nm, although only some wines proved to be significantly different. Glories (1984) also found an increase in optical density at 620 nm in wines that received oxygen. He stipulated this increase to the formation of co-pigmented complexes and to the polymerisation phenomena with ethyl-bridges, which generate more red-violet compounds. Escribiano-Bailon *et al*. (2001) stated that red and violet polymeric compounds increase colour intensity and improve pigment stabilisation.

Table 5.1 Colour intensity (the sum of 420 nm, 520 nm and 620 nm) and hue (420 nm/520 nm) for control and oxygenated wines before and after malolactic fermentation for wines made of different pHs

*COLOUR INTENSITY				*COLOUR HUE			
Treatment		Before MLF (AU)	After MLF (AU)	Treatment		Before MLF (AU)	After MLF (AU)
pH 3.4	control	17.09 ±0.81^{a}	14.52 ± 0.86^{bc}	pH 3.4	control	0.50 ± 0.00^{a}	0.62 ± 0.01^{bc}
	16 mg/L	15.80 ±0.82^{a}	15.91 ± 0.25^{abd}		16 mg/L	0.52 ± 0.01^{a}	0.60 ± 0.00^{bde}
pH 3.7	control	14.58±0.74^{bc}	13.48 ± 0.53^{ce}	pH 3.7	control	0.56 ± 0.01^{d}	0.63 ± 0.03^{bc}
	16 mg/L	15.17±0.60^{bd}	14.24 ± 0.51^{cd}		16 mg/L	0.57 ± 0.01^{df}	0.60 ± 0.01^{bf}
pH 4.0	control	12.28±1.99^{ef}	11.82 ± 0.43^{f}	pH 4.0	control	0.66 ± 0.04^{c}	0.76 ± 0.01^{g}
	16 mg/L	13.32±1.33^{ce}	12.49 ± 0.18^{ef}		16 mg/L	0.64 ± 0.01^{ce}	0.72 ± 0.01^{h}

*Values displayed in table are the average for four repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences between samples. '16 mg/L' represents the oxygen dosage given after alcoholic fermentation. '3.4', '3.7' and '4.0' represents wines made of different pHs. Values are given in absorbance units (AU).

Table 5.2: Optical densities (420 nm, 520 nm and 620 nm) for control and oxygenated wines before and after malolactic fermentation for wines made of different pHs

Treatment		*OPTICAL DENSITY (420 nm)		*OPTICAL DENSITY (520 nm)		*OPTICAL DENSITY (620 nm)	
		Before MLF (AU)	After MLF (AU)	Before MLF (AU)	After MLF (AU)	Before MLF (AU)	After MLF (AU)
pH 3.4	control	5.10 ± 0.22^{a}	4.86 ± 0.29^{ab}	10.19 ±0.48^{h}	7.78 ± 0.44^{ab}	1.80 ± 0.11^{ab}	1.89 ± 0.14^{ac}
	16 mg/L	4.83 ± 0.20^{ab}	5.19 ± 0.05^{a}	9.32 ± 0.55^{c}	8.66 ± 0.02^{acd}	1.65 ± 0.07^{b}	2.07 ± 0.00^{c}
pH 3.7	control	4.62 ± 0.20^{bc}	4.51 ± 0.21^{bcd}	8.22 ± 0.44^{ad}	7.15 ± 0.32^{be}	1.74 ± 0.10^{ab}	1.82 ± 0.15^{abc}
	16 mg/L	4.86 ± 0.14^{ab}	4.63 ± 0.17^{bc}	8.50 ± 0.39^{a}	7.74 ± 0.27^{bd}	1.81 ± 0.39^{abc}	1.87 ± 0.09^{acd}
pH 4.0	control	4.20 ± 0.56^{d}	4.28 ± 0.14^{cd}	6.41 ± 1.15^{ef}	5.65 ± 0.21^{g}	1.67 ± 0.29^{bd}	1.89 ± 0.08^{ac}
	16 mg/L	4.49 ± 0.40^{bcd}	4.40 ± 0.01^{cd}	7.07 ± 0.73^{be}	6.14 ± 0.02^{fg}	1.77 ± 0.20^{ab}	1.95 ± 0.00^{ac}

*Values displayed in table are the average for four repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences between samples. '16 mg/L' represents the oxygen dosage given after alcoholic fermentation. '3.4', '3.7' and '4.0' represents wines made of different pHs. Values are given in absorbance units (AU).

5.3.2 Total red pigments

None of the wines differed significantly from each other before MLF, including the control and oxygenated wines (Table 5.3). A significant decrease in absorbance units was observed in all wines during MLF (except for 16 mg/L O_2, pH 3.4). However, there was still no significant difference between any of the wines after MLF. The highest value was observed with the control at pH 3.4 before MLF (26.21 AU units), but it was also this wine that decreased most significantly during MLF.

The loss in red pigments during MLF could be a result of oxidation (Fell *et al*., 2007; Gomez-Plaza *et al*., 2001) and precipitation of the red pigments (Gil-Munoz *et al.* 1997; Perez-Prieto *et al.,* 2003 and Fourie., 2005) during ageing of wine. However, malolactic fermentation is not strictly regarded as an ageing process, even though these reactions may take place during MLF. Another explanation for the decrease in red colour could be due to polymerisation reactions that cause differences in the extinction coefficients of the newly formed pigments (Saucier *et al*., 2004; Salas *et al*., 2003; Boulton, 2001). However, in terms of total red pigments, the change in pH and addition of oxygen did not lead to significant changes.

Table 5.3: Total red pigments (Somers and Evans, 1977) measured by spectrometry for control and oxygenated wines before and after malolactic fermentation for wines made of different pHs

*SPECTROPHOTOMETRY: TOTAL RED PIGMENTS (520 nm)			
Treatment		Before MLF (AU)	After MLF (AU)
pH 3.4	control	26.21 ± 0.98^{a}	21.03 ± 0.69^{cd}
	16 mg/L	23.96 ± 0.66^{ab}	22.22 ± 0.04^{bd}
pH 3.7	control	23.84 ± 1.02^{ab}	21.70 ± 0.52^{cd}
	16 mg/L	24.68 ± 2.04^{ab}	20.59 ± 0.80^{cd}
pH 4.0	control	24.19 ± 2.44^{ab}	22.06 ± 0.76^{cd}
	16 mg/L	24.11 ± 1.69^{ab}	20.83 ± 0.49^{cd}

*Values displayed in table are the average for four repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences between samples. '16 mg/L' represents the oxygen dosage given after alcoholic fermentation. '3.4', '3.7' and '4.0' represents wines made of different pHs. Values are given in absorbance units (AU).

5.3.3 Total anthocyanins

Anthocyanin concentrations, as measured with spectrometry and RP-HPLC, decreased significantly during MLF in the control and oxygenated wines (except pH 3.7, 16 mg/L O2 treatment measured with RP-HPLC) for each individual pH treatment (Table 5.4). For both anthocyanin analyses, the control of pH 3.4 before MLF had the highest anthocyanin concentration of all the wines in the experiment; this was also true for the total red pigment measurement (section 5.3.2). However, during MLF the concentration decreased significantly to values that did not differ significantly from the oxygenated wines after MLF at pH 3.4. Polymerisation of the anthocyanins as well as association of the anthocyanins with bacteria cells could explain the lower anthocyanin concentrations observed after MLF (Gonzales-Neves *et al*., 2004; Monagas *et al*., 2006; Medina *et al*., 2005).

It seems as if the application of oxygen reduced the decrease in anthocyanins during MLF, especially at the lower pH value (Table 5.4). Phenols, such as anthocyanins, can react more readily with oxygen under higher pH conditions such as at pH 4.0 than at pH 3.4, leading to indirect condensation (Ribereau-Gayon *et al.* 2006) between molecules such as anthocyanin

and catechin moieties at pH 4. This could explain why only a significant difference in anthocyanin concentration after MLF was observed in the oxygenated wines compared to the control at pH 4, although it was not significantly different according to RP-HPLC (Waterhouse and Laurie, 2006). However, the larger percentage of flavylium ions at pH 3.4 and 3.7 might have led to more of these compounds associating with catechin moieties through direct condensation, where no oxygen was added (Ribereau-Gayon *et al.,* 2006). Anthocyanins measured with the spectrophotometer correlated with that of the HPLC (r^2 = 0.6574).

Table 5.4 Anthocyanin concentrations as obtained by spectrometry (Ribereau-Gayon and Stonestreet, 1965) and RP-HPLC (Peng *et al*., 2002) for control and oxygenated wines before and after malolactic fermentation for wines made of different pHs

*SPECTROPHOTOMETRY: ANTHOCYANINS				*RP-HPLC: ANTHOCYANINS			
Treatment		Before MLF (mg/L)	After MLF (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)
pH 3.4	control	308.6 ± 10.8^{a}	252.2 ± 2.2^{cd}	pH 3.4	control	387 ± 7^{a}	242 ± 7^{b}
	16mg/L	293.2 ± 1.3^{b}	244.5 ± 0.5^{cd}		16mg/L	338 ± 66^{a}	261 ± 75^{bc}
pH 3.7	control	256.3 ± 4.0^{ab}	230.2 ± 3.3^{cd}	pH 3.7	control	364 ± 2^{a}	237 ± 15^{b}
	16mg/L	256.9 ± 2.6^{ab}	222 ± 6.1^{cd}		16mg/L	327 ± 54^{ac}	264 ± 88^{bc}
pH 4.0	control	302.5 ± 15.5^{ab}	270.9 ± 3.9^{c}	pH 4.0	control	370 ± 14^{a}	262 ± 12^{bc}
	16mg/L	297.5 ± 4.3^{ab}	246.7 ± 8.5^{d}		16mg/L	332 ± 28^{a}	226 ± 8^{b}

*Values displayed in table are the average for four repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences between samples. '16 mg/L' represents the oxygen dosage given after alcoholic fermentation. '3.4', '3.7' and '4.0' represents wines made of different pHs. Concentrations are given in mg/L.

Monomeric anthocyanins are converted into polymeric pigments over time to produce more stable colour (Salas *et al*., 2004; Fell *et al*., 2007), which is supported by the increase in polymeric pigments observed with MLF (Table 5.5) although it was not significant. The addition of oxygen did not result in significantly different polymeric pigment formation compared to the controls and different pH treatments.

Table 5.5: Polymeric pigment concentrations determined by RP-HPLC (Peng *et al.,* 2002) for control and oxygenated wines before and after malolactic fermentation for wines made of different pHs

*RP-HPLC: POLYMERIC PIGMENTS			
Treatment		Before MLF (mg/L)	After MLF (mg/L)
pH 3.4	control	61.5 ± 5.1^{a}	75.8 ± 4.6^{a}
	16 mg/L	65.5 ± 5.0^{b}	73.3 ± 8.9^{ab}
pH 3.7	control	60.6 ± 0.9^{a}	63.6 ± 3.6^{a}
	16mg/L	61.9 ± 1.9^{a}	66.7 ± 1.8^{a}
pH 4.0	control	58.9 ± 2.4^{a}	66.1 ± 3.4^{a}
	16mg/L	62.9 ± 2.7^{a}	74.7 ± 3.8^{a}

*Values displayed in table are the average for four repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences between samples. '16 mg/L' represents the oxygen dosage given after alcoholic fermentation. '3.4', '3.7' and '4.0' represents wines made of different pHs. Concentrations are given in mg/L.

5.3.4 Monomeric and dimeric flavanols

The monomeric flavanol concentration as determined by the DMAC assay remained fairly constant and not significant for all the wines before and after MLF (Table 5.6), except for the oxygen treated wine at pH 3.4 before MLF, which was significantly higher.

Results obtained from the HPLC had smaller fluctuations in concentrations and some wines showed different trends compared to the DMAC index. No significant differences were observed between any of the wines after MLF. The monomeric flavanol concentrations determined by RP-HPLC were calculated as the sum of (+)-catechin, (-)-epicatechin, (+)-gallocatechin, (-)-epigallocatechin and (-)-epicatechingallate (see Addendum, Table 7.15 for individual concentrations of these compounds).

The small differences observed in the monomeric flavanol concentration could be due to build up and break down reactions (Vidal *et al*., 2002). A poor correlation was obtained between the DMAC index and the RP-HPLC analysis (r^2 = 0.0014).

Concentrations for dimers B1 and B2 as determined by RP-HPLC were not significantly influenced by the addition of oxygen, MLF or by the different pH treatments (Addendum, Table 7.16), except dimer B1 at pH 3.4 where oxygen was added.

Table 5.6 Monomeric flavanol concentrations, as obtained by spectrometry (Nagel and Glories, 1991) and RP-HPLC (Peng *et al.,* 2002), for control and oxygenated wines before and after malolactic fermentation for wines made of different pHs

*SPECTROPHOTOMETRY: MONOMERIC FLAVANOLS				*RP-HPLC: MONOMERIC FLAVANOLS			
Treatment		Before MLF (mg/L)	After MLF (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)
pH 3.4	control	350 ± 17.6^{a}	324 ± 7.4^{a}	pH 3.4	control	266 ± 22^{ab}	305 ± 39^{ab}
	16 mg/L	418 ± 45.9^{b}	324 ± 4.1^{a}		16 mg/L	286 ± 21^{ab}	275 ± 12^{ab}
pH 3.7	control	326 ± 0.5^{a}	323 ± 14.5^{a}	pH 3.7	control	275 ± 23^{ab}	267 ± 27^{ab}
	16 mg/L	306 ± 0.6^{a}	349 ± 16^{a}		16 mg/L	272 ± 24^{ab}	236 ± 61^{a}
pH 4.0	control	330 ± 15^{a}	325 ± 0.5^{a}	pH 4.0	control	279 ± 30^{ab}	255 ± 78^{ab}
	16 mg/L	327 ± 15.1^{a}	324 ± 9.1^{a}		16 mg/L	230 ± 67^{b}	264 ± 11^{ab}

*Values displayed in table are the average for four repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences between samples. '16 mg/L' represents the oxygen dosage given after alcoholic fermentation. '3.4', '3.7' and '4.0' represents wines made of different pHs. Concentrations are given in mg/L.

5.3.5 Total phenols

The majority of the different pH wines, their individual oxygen treatments and MLF did not show any statistical differences on a 5% confidence interval for total phenolics as measured by the Folin-Ciocalteu assay (Table 5.7). However, for the pH 3.4 treatments, the control and oxygenated wines already differed from each other by ±300 mg/L gallic acid units (GAU) shortly after the addition of oxygen, which was confirmed by the higher absorbance at 280 nm of the control (Table 5.8). However, after MLF no significant differences in phenolic concentrations were observed for any of the wines, as measured with both the Folin-Ciocalteu assay and the measurement at 280 nm. There seemed to have been a decreasing tendency in most wines with time, even though none were significant.

A study by De Beer *et al*. (2004) postulated that total phenols are degraded as ageing takes place. However, since the wines were not aged after MLF, we cannot explain the decrease in phenols due to degradation processes.

Table 5.7: Total phenolic concentration as measured with the Folin-Ciocalteu assay (Singleton and Rossi, 1965) for control and oxygenated wines before and after malolactic fermentation for wines made of different pHs.

*SPECTROMETRY: FOLIN-CIOCALTEU			
Treatment		Before MLF (mg/L GAU)	After MLF (mg/L GAU)
pH 3.4	control	3572 ± 98^{a}	3561 ± 30^{a}
	16 mg/L	3268 ± 220^{bd}	3376 ± 42^{ad}
pH 3.7	control	3372 ± 225^{a}	3379 ± 141^{a}
	16 mg/L	3374 ± 361^{a}	3263 ± 68^{a}
pH 4.0	control	3553 ± 200^{a}	3334 ± 163^{a}
	16 mg/L	3372 ± 67^{a}	3337 ± 21^{a}

*Values displayed in table are the average for four repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences between samples. '16 mg/L' represents the oxygen dosage given after alcoholic fermentation. '3.4', '3.7' and '4.0' represents wines made of different pHs. Concentrations are given in mg/L gallic acid units (GAU).

Table 5.8: Total phenols measured at 280 nm (Somers and Evans, 1977) for control and oxygenated wines before and after malolactic fermentation for wines made of different pHs

*SPECTROMETRY: TOTAL PHENOLS (280 nm)			
Treatment		Before MLF (AU)	After MLF (AU)
pH 3.4	control	56.10 ± 1.41^{a}	53.22 ± 1.86^{a}
	16 mg/L	53.37 ± 1.43^{bd}	55.47 ± 0.25^{ad}
pH 3.7	control	53.62 ± 1.89^{a}	53.81 ± 0.92^{a}
	16 mg/L	54.84 ± 3.38^{a}	53.24 ± 1.88^{a}
pH 4.0	control	52.62 ± 4.35^{a}	52.46 ± 1.16^{a}
	16 mg/L	53.82 ± 2.81^{a}	52.23 ± 1.20^{a}

*Values displayed in table are the average for four repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences between samples. '16 mg/L' represents the oxygen dosage given after alcoholic fermentation. '3.4', '3.7' and '4.0' represents wines made of different pHs. Concentrations are given in absorbance units (AU).

5.3.5 Polymeric phenols

The polymeric phenol content, as determined by RP-HPLC, mostly showed an increase in concentration from before until after MLF (Table 5.9). There was a significant increase in polymeric phenol concentration for both control and oxygenated wines at pH 3.4 after MLF, whereas at pH 3.7 no significant differences could be detected between the control and oxygenated wines before and after MLF. At pH 4.0 the control remained constant during MLF, but the oxygenated wine increased significantly during MLF. It is possible that oxygen contributed to the formation of more polyphenols at pH 4 due to higher reactivity (Waterhouse and Laurie, 2006).

Table 5.9: Polymeric phenol concentration as determined by RP-HPLC (Peng *et al.,* 2002) for control and oxygenated wines before and after malolactic fermentation for wines made of different pHs.

*RP-HPLC: POLYMERIC PHENOLS			
Treatment		Before MLF (mg/L)	After MLF (mg/L)
pH 3.4	control	864 ± 14^{ab}	890 ± 33^{c}
	16 mg/L	882 ± 51^{a}	910 ± 58^{c}
pH 3.7	control	868 ± 45^{ab}	823 ± 19^{ab}
	16 mg/L	838 ± 19^{ab}	849 ± 56^{a}
pH 4.0	control	830 ± 17^{b}	860 ± 23^{ab}
	16 mg/L	855 ± 7^{a}	899 ± 20^{c}

*Values displayed in table are the average for four repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences between samples. '16 mg/L' represents the oxygen dosage given after alcoholic fermentation. '3.4', '3.7' and '4.0' represents wines made of different pHs.

5.3.6 Hydroxycinnamic acids

Cilliers and Singleton (1989; 1990) conducted studies on the oxidation and subsequent decrease in hydroxycinnamic acid concentrations at higher pH values in model wine systems. We found non-significant decreases in concentrations of caftaric and caffeic acid (Addendum, Table 7.17) in the presence of oxygen at higher pH values. These acids become oxidised in the presence of oxygen through auto-oxidative reactions (Cilliers and Singleton, 1991). MLF also led to a significant decrease in the concentration of caftaric and caffeic acid, especially at a higher pH.

5.3.7 Flavonols

Most of the flavonols were often not significantly influenced by oxygen addition and pH differences and in most cases, MLF (Addendum, Table 7.18.1 and 7.18.2). The concentration of quercetin-3-glucoside decreased during MLF and this is in agreement with studies done by Fang *et al.* (2007), who found a loss in concentration as time proceeded. In most wines receiving oxygen there was a tendency for the flavonol concentration to decrease. Sartini *et al.* (2007) also found lower flavonol concentrations in wines treated with oxygen. The greater loss of quercetin in the oxygen treated wines could possibly be due to the high reactivity of this molecule with oxygen (Park *et al*., 2003). In contrast, Perez-Magarino *et al.* (2007) found that oxygenated wines had the highest concentration of quercetin and not the control.

5.5 Conclusion

From this work it is evident that producing wine with a lower pH would lead to an increase in colour intensity compared to a higher pH wine. Wines with a pH range of 3.4 and 4.0 could suffer a substantial loss in total red pigments and anthocyanins during MLF, although pH differences in the range between 3.4 and 4.0 did not significantly influence the total phenolic composition of the wines.

Monomeric anthocyanins decreased during the course of the experiment. This decrease seemed to be favoured by a lower pH when low oxygen concentrations were present, but the opposite was true to a certain extent where oxygen was added. From this we could conclude that the application of oxygen could limit the loss in colour during MLF, especially at a lower pH. The presence of oxygen did not result in significantly different polymeric pigment formation in

the pH range between 3.4 and 4.0. However, the results could have been different if the wines were aged longer.

The sensitivity of individual phenols must be investigated to better understand their reactivity and response to different pHs in both model wine systems and complex wine mediums. Managing the pH of a wine is important for other factors such as malolactic fermentation, colour and bacterial spoilage but could play a role in the colour and phenolic development to improve red wine quality.

5.6 Acknowledgements

The authors would like to thank Lourensford Wine Estate for donating the wine, the Winetech, Thrip and the NRF for providing financial support and Prof Martin Kidd for analysing the data statistically.

5.7 References

Boulton, R. (2001). The copigmentation of anthocyanins and its role in the colour of red wine: a critical review. *The American Journal of Enology and Viticulture*. **52**, 67-87.

Bousbouras, G.E. and Kunkee, R.E. (1971). Effect of pH on malolactic fermentation in wine. M.S Thesis, University of California, Davis, California.

Brouillard, R., and Mazza, G. (1989). The copigmentation reaction of anthocyanins: a microprobe for the structural study of aqueous solutions. *Journal of American Chemical Society*. **111**, 2604-2610.

Brouillard, R., and Cheminat, A. (1986). NMR spectrometry of anthocyanins. In: *Journees internationales d'Etude du Groupe Polyphenols*, Montpellier (France), 9-11 Jul.

Brouillard, R., Delaporte, B. and Dubois, J.E. (1978). Chemistry of anthocyanin pigments. 3. Relaxation amplitudes in pH-jump experiments. *Journal of American Chemical Society*. **100**, 6202-6205.

Brouillard, R. and Delaporte, B. (1977). Chemistry of anthocyanin pigments. 2: kinetic and thermodynamic study of proton transfert, hydration and tautomeric reactions of malvidin-3-glucoside. *Journal of American Chemical Society*. **99**, 8461-8467.

Castellari, M., Matricardi, L., Arfelli, G., Galassi, S and Amati, A. (2000). Level of single bioactive phenolics in red wine as a function of the oxygen supplied during storage. *Journal of Food Chemistry*. **69**, 61-67.

Cilliers, J.J.L. and Singleton, V.L. (1989). Characterization of the products of Nonenzymatic autoxidative phenolic reactions in a caffeic acid model system. *Journal of Agricultural and Food Chemistry*. **39**, 1298-1303.

Cilliers, J.J.L. and Singleton, V.L. (1990). Nonenzymatic autoxidative reactions of caffeic acid in wine. *American Journal of Enology and Viticulture*. **41**, 84-86.

Cilliers, J.J.L and Singleton, V.L. (1991). Characterisation of the products of nonenzymic auto-oxidative phenolic reactions in a caffeic acid model system*. Journal of Agricultural and Food Chemistry*, **39**, 1298−1303

De Beer, D., Harbertson, J.F., Kilmartin, P.A., Roginsky, V., Barsukova, T., Adams, D.O. and Waterhouse, A.L (2004). Phenolics: A comparison of diverse analytical methods. *American Journal of Enology and Viticulture*. **55**, 389-400.

Escribano-Bailon, T., Alvarez-Garcia, M., Rivas-Gonzalo, J.C., Heredia, F.J. and Santos-Buelga, C. (2001). Colour and stability of pigments derived from the acetaldehyde-mediated condensation between malvidin-3-O-glucoside and (+)-catechin. *Journal of Agricultural and Food Chemistry*. **49**, 1213-1217.

Fang, F. Li, J.M., Pan, Q.H. and Huang, W.D. (2007). Determination of red wine flavonoids by HPLC and effect of ageing. *Food Chemistry*. **101**, 428-433

Fell, A.J., Dykes, S.I., Nicolau, L. and Kilmartin, P.A. (2007). Electrochemical microoxidation in red wine. *American Journal of Enology and Viticulture*. *58*, 443-450.

Fourie, B.A. (2005). The influence of barrel type, oak blocks, oak tannins and oak powder on the composition and quality of red wines. MSc thesis in Oenology, Stellenbosch University, South Africa.

Fulcrand, H., Duenas, M., Salas, E. and Cheynier, V. (2006). Phenolic reactions during winemaking and ageing. *American Journal of Enology and Viticulture*. **57**, 289-297.

Glories, Y. (1984). Anthocyanins and tannins from wine: Organoleptic properties. In: 'Plant flavonoids in biology and medicine II: Biochemical, cellular, and medicinal properties'. Eds. V.Cody *et al*. (Alan R. Liss, Inc., New York) pp 123-134.

Gomez-Plaza, E., Gil-Munoz, R., Lopez-Roca, J.M., Martinez-Cutillas, A. and Fernandez-Fernandez, J.I. (2001). Phenolic compounds and colour stability of red wines: effect of skin maceration time. *American Journal of Enology and Viticulture*. **52**, 266-270.

Gonzales-Neves, G., Charamalo, D., Balado, J., Barreiro, L., Bochicchio, R., Gatto, G., Gil, G., Tessore, A., Carbonneau, A. and Moutounet, M. (2004). Phenolic potential of Tannat, Cabernet Sauvignon and Merlot grapes and their correspondence with wine composition. *Analitica Chimica Acta*. **513**, 191-196.

Gil-Munoz, R., Gomez-Plaza, E., Martinez, A., and Lopez-Roca, J.M. (1997). Evolution of the CIELAB and other spectrophotometric parameters during wine fermentation. Influence of some pre- and post fermentative factors. *Food Research International*. **30**, 699-705.

Monagas, M., Gomez-Cordoves, C., and Bartoome, B. (2006). Evolution of the phenolic content of red wines from *Vitis vinifera* L. during aging in bottle. *Food Chemistry*. **95**, 405-412.

Medina, K., Boido, E., Dellacassa, E. and Carrau, F. (2005). Yeast interactions with anthocyanins during red wine fermentation. *The American Journal of Enology and Viticulture*. **56**, 104-109.

Nagel, C.W. and Glories, Y. (1991). Use of a modified dimethylaminocinnamaldehyde reagent for analysis of flavanols. *American Journal of Enology and Viticulture*. **42**, 364-366.

Nieuwoudt, H.H., Prior, B.A., Pretorius, I.S., Manley, M., and Bauer, F.F. (2004). Principle component analysis applied to fourier transform infrared spectroscopy for the design of calibration sets for glycerol prediction models in wine and for the detection and classification of outlier samples. *Journal of Agricultural and Food Chemistry*. **52**, 3728-3735.

Park, C., So, H.S., Shin, C.H., Back, S.H., Moon, B.S., Lee, D.W. and Park, R. (2003). Quercetin protects the hydrogen peroxide-induces apoptosis via inhibition of mitochondrial dysfunction in H9c2 cardiomyoblast cells. *Biochemical Pharmocology*. **66**, 1278-1295.

Perez-Magarino, S., Sanchez-Iglesias, M., Ortega-Heras, M., Gonzales-Huerta, C. and Gonzales-Sanjose, M.L. (2007). Colour stabilisation of red wines by microoxygenation treatment before malolactic fermentation. *Food Chemistry*. **101**, 881-893.

Perez-Prieto, I.J., De la Hera-Orts, M.L., Lopea-Roca, J.M., Fernandez, J.I. and Gomez-Plaza, E. (2003). Oak-matured wines: influence of the characteristics of the barrel on wine colour and sensory characteristics. *Journal of the Science of Food and Agriculture*. **83**, 1445-1450.

Ribereau-Gayon, P., Glories, Y., Meujean, A. and Dubourdieu, D. (2006). "Handbook of Enology, Vol 2. The chemistry of wine stabilisation and treatments." (John Wiley and Sons, Ltd. New York).

Ribereau-Gayon, P., & Stonestreet, E. (1965). Le dosage des anthocyanes dans les vins rouges. *Bulletin de la Societe Chimique de France*. **9**, 2649-2652.

Salas E, H Fulcrand, E Meudec, V Cheynier, (2003). Reactions of anthocyanins and tannins in model solutions. *Journal of Agricultural and Food Chemistry*. **51**, 7951-7961.

Salas, E., Atanasova, C., Poncet-Legrand, C., Meudec, E., Mazauric, J.P. and Cheynier, V. (2004). Demonstration of the occurrence of flavanol-anthocyanin adducts in wine and in model solutions. *Analytica Chimica Acta*. **513**, 325-332.

Sartini, E., Arfelli, G., Fabiani, A. and Piva, A. (2007). Influence of chips, lees and micro-oxygenation during aging on the phenolic composition of a red Sangiovese wine. *Food Chemistry*. **104**, 1599-1604.

Saucier, C., Mirabel, M., Guerra, C. and Glories, Y. (2004). Tannin-anthocyanin interactions: influence on wine colour. In: Red wine colour: Revealing the mysteries. Eds. Waterhouse, A.L. and Kennedy, J.A. (American Chemical Society/Oxford University Press, Washington, DC). 265-273.

Singleton, V.L. (1987). Oxygen with phenols and related reactions n musts, wines, and model systems: Observations and practical implications. *American Journal of Enology and Viticulture*. **38**, 69-77.

Somers, T.C. and Evans, M.E. (1977). Spectral evaluation of young red wines: anthocyanin equilibria, total phenolics, free and molecular SO_2, "chemical age". *Journal of the Science of Food and Agriculture*. **28**, 279-287.

Van der Merwe, H. (2006). The evaluation of malolactic fermentation starter cultures under South African winemaking conditions. M.S Thesis, University of Stellenbosch, Stellenbosch.

Vidal S, Cartalade, D.M., Souquet, J.M., Fulcrand, H. and Cheynier, V. (2002). Changes in proanthocyanidin chain-length in wine-like model solutions. *Journal of Agricultural and Food Chemistry*. **50**, 2261-2266.

Waterhouse, A.L. and Laurie, V.F. (2006). Oxidation of wine phenolics: a critical evaluation and hypothesis. *American Journal of Enology and Viticulture*. **57**, 306-313.

Chapter 6

General discussion and conclusion

Chapter 6: General discussion and conclusions

6.1 Concluding remarks and other perspectives

In recent years the production of red wine has become increasingly sophisticated. Wine is considered one of the most complex beverages and the end product is subjected to several biological and physicochemical processes. Wine consists of a broad spectrum of flavours, aromas and other organoleptic properties. Phenolic compounds are important contributors to the chemical reactions that take place in wine and the resulting complexity (Fulcrand *et al*., 2006; Ribereau-Gayon *et al*., 2006; Thorngate and Singleton, 1994).

In most countries, high intake of saturated fat is positively related to high mortality from cholesterol. However, the situation in France is paradoxical (similar to Germany and the UK) where the mortality due to cholesterol is low, unlike other countries such as Spain or Italy. Researchers have partially attributed this to high wine consumption. Results have shown that a group of phenolic compounds, including the flavan-3-ols (catechins and proanthocyanidins), is responsible for that action (Renaud and De Lorgeril., 1992). Proanthocyanidins are oligomers derived from catechins and red wine is the beverage that contains the highest concentration of these compounds (Lee *et al*., 2008). The flavan-3-ol class of phenols is responsible for bitterness and astringency as well as providing colour stability and oxidative substrates (Singleton *et al*., 1992). The highest concentration of monomeric and polymeric flavan-3-ols is present in the grape seeds (Gonzales-Manzano *et al*., 2004).

The colour of a red wine is mainly due to the extraction of monomeric anthocyanins from grape skins during fermentation (Yokotsuka and Singleton, 2001). These pigments are influenced by malolactic fermentation and can participate in polymerisation reactions to form polymeric pigments, which are more stable to sulphur dioxide bleaching and changes in pH than monomeric anthocyanins (Morena-Arribas *et al*., 2008). The formation of these complexes can be enhanced by the presence of oxygen. However, it is still unclear how much oxygen a wine is capable to consume, but it is suggested that it is more beneficial if applied after alcoholic fermentation (Singleton, 1999).

Colour intensity was influenced differently by the addition of oxygen for the different experiments performed. In all cases oxygenation led to an increase in colour intensity and although not always significantly different from the control, an increasing tendency was observed. However, in all cases a decrease in colour intensity during MLF was seen. Total monomeric anthocyanins decreased in most wines when oxygen was applied, although not always significantly. The larger decrease in total monomeric anthocyanins in the oxygenated wines could be due to the partaking of anthocyanin compounds in polymerisation reactions that involves acetaldehyde via the formation of ethyl bridges that is especially favoured by the presence of oxygen. Such decreases in total monomeric anthocyanins (obtained by spectrophotometric methods or RP-HPLC) were in many cases followed by a subsequent increase in polymeric pigments, determined by RP-HPLC. In most cases the increases were significant, however when not, there was a strong increasing tendency for the formation of polymeric pigments in the oxygenated wines. Total monomeric anthocyanins measured by spectrophotometry and RP-HPLC correlated well in all experiments.

In most wines the addition of oxygen led to an initial decrease in monomeric flavan-3-ol concentration before MLF, but this often changed during MLF, with concentrations being the same or increasing after MLF. The monomeric flavanols determined by the DMAC assay did not always correlate well in all wines with the monomeric flavan-3-ols obtained by RP-HPLC. This

can be partly explained due to the inclusion of all flavanols and certain dimers to a lesser extent by the DMAC assay which was not included in the RP-HPLC determination.

Small fluctuations in the total phenolic content were observed when analysed with the Folin-Ciocalteu reagent. Most wines did not show any significant differences in the concentration of total phenol content after the completion of the experiments. Some significant differences were observed in the wines made of altering grape seed concentrations (2008).

Concentrations of polymeric phenols as determined by RP-HPLC were significantly increased in Pinotage wine receiving 16 mg/L or 32 mg/L of micro-oxygenation. However, only in the experiment with wines made of different grape seed concentrations (both vintages) were the results not as significant, even though all these wines showed an increase in polymeric phenol concentration with oxygen addition.

The tannin concentration remained non-significant in all wines analysed with the BSA precipitation method when treated with oxygen. None of the wines made of altering grape seed concentrations during both vintages were significantly influenced by the addition of oxygen at the end of the experiment. However, we did observe a general decrease in tannin concentration for both cultivars in 2008 after the two month ageing period. Wines made in commercial length tanks and subjected to micro-oxygenation also did not show any significant differences after ageing for two months. The small differences in tannin concentration could possibly change over time, but was not observed in our study due to the limited two month ageing period. However, the effect of adding oxygen to wine might influence the reactivity of BSA towards tannins and hence the final tannin concentration and need further investigation.

From the different methods used to determine the colour and phenolic composition of the wines in our study, certain methods seemed to have been more applicable when a wine has been subjected to the addition of oxygen. Total monomeric anthocyanins, determined by spectrophotometry and RP-HPLC, always showed a decrease in concentration in the oxygenated wines, together with a subsequent increase in polymeric pigments (RP-HPLC). By analysing these compounds, rather than tannins for example, one could get a clearer indication if a wine has been subjected to oxygenation.

From this study it is evident that making wine with altering grape seed concentrations would definitely affect the phenolic and colour composition of red wine. The addition of different grape seed concentrations had a more significant effect on the phenolic composition of red wine compared to the addition of oxygen after MLF. The technique of removing seeds from grapes could be labor intensive, depending on the scale of winemaking and equipment used. The style of wine should determine the use of this technique. More cultivars should be investigated in the future to determine whether certain trends may occur within cultivars when wines are made with altering seed concentrations. Also, the wines should be sensorially investigated to gain valuable information on the astringency and ageing potential of such wines. Future work should include an in-depth investigation of the different tannin methods available for the determination of tannin concentration in wine when working with wines made from altering grape seed concentrations. Recent studies showed that the present methods available for these analyses are yet to be precise and repeatable due to dilution problems and discrepancies exist regarding the total tannin concentration values generated using different methods.

Oxygen additions after alcoholic fermentation could lead to greater colour and polymeric phenol content in Pinotage wines. The application of 16 or 32 mg/L of oxygen seems to give similar results in Pinotage, but needs to be reaffirmed in a similar experiment. Future experiments could possibly include the same amount of oxygen given in our study, but testing the application thereof after MLF. We base this recommendation on the small (mostly insignificant) differences observed between the control and oxygenated wines when oxygen

was applied before MLF. Future work should include ageing the wines in tanks and possibly also barrels for longer periods of time.

pH also seems to influence the effect of oxygen on the phenolic composition of red wine to a very limited extent when applied before MLF. This was just a preliminary assessment that still needs to be investigated in more detail.

We have found that the addition of large amounts of oxygen applied after alcoholic fermentation does not always lead to distinguishable differences between the control and oxygenated wines. Also, applying double the amount of oxygen, does not necessarily lead to a two-fold outcome. This study improved our understanding on how colour and phenolic compounds are affected by oxygen addition when applied after alcoholic fermentation. However, uncertainty still exists regarding how much oxygen a wine is capable of consuming earlier during the winemaking process.

6.2 Literature cited

Fulcrand, H., Duenas, M., Salas, E. and Cheynier, V. (2006). Phenolic reactions during winemaking and ageing. *American Journal of Enology and Viticulture*. **57**, 289-297.

Gonzàles-Manzano, S., Rivas-Gonzalo, J.C. and Santos-Buelga, C. (2004). Extraction of flavan-3-ols from grape seed and skin into wine using simulated maceration. *Analytica Chimica Acta*. **513**, 283-289.

Lee, J., Kennedy, J.A., Devlin, C., Redhead, M. and Rennaker, C. (2008). Effect of early seed removal during fermentation on proanthocyanidin extraction in red wine: a commercial production example. *Food Chemistry*. **107**, 1270-1273.

Moreno-Arribas, M.V., Gomez-Cordoves, C. and Martin-Alvarez, P.J. (2008). Evolution of red wine anthocyanins during malolactic fermentation, postfermentative treatments and ageing with lees. *Food Chemistry*. **109**, 149-158.

Renaud, S. and De Lorgeril, M. (1992). Wine, alcohol, platelets and the French paradox for coronary heart disease. *Lancet*. **339**, 1523-1526.

Ribereau-Gayon, P., Glories, Y., Meujean, A. and Dubourdieu, D. (2006). Handbook of enology, Vol. 2. The chemistry of wine stabilization and treatments. John Wiley and Sons, Ltd. New York.

Singleton, V.L. (1992). Tannins and the qualities of wines. *In*: Plant polyphenols: synthesis, properties, significance. R. W. Hemingway and P. E Laks (Eds.). pp 859-880. Plenum Press, New York.

Singleton, V.L., Orthofer, R. and Lamuela-Raventos, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology*. **299**, 152-178.

Thorngate, J.H. and Singleton, V.L. (1994). Localisation of procyanidins in grape seeds. *American Journal of Enology and Viticulture*. **45**, 259-262.

Yokotsuka, K. and Singleton, V.L. (2001). Effects of seed tannin on enzymatic decoloration of wine pigments in the presence of oxidizable phenols. *American Journal of Enology and Viticulture*. **52**, 93-99.

Chapter 7

Addendum

Table 7.1 Colour Intensity (sum of 420 nm; 520 nm and 620 nm) average data for the 2007 Cabernet Sauvignon, 2008 Cabernet Sauvignon and 2008 Pinotage

COLOUR INTENSITY				COLOUR INTENSITY				COLOUR INTENSITY			
*2007 Cabernet Sauvignon				*2008 Cabernet Sauvignon				*2008 Pinotage			
Treatment		Before MLF (AU)	After MLF (AU)	Treatment		Before MLF (AU)	After MLF (AU)	2 Months after MLF (AU)	Treatment		Before MLF (AU)	After MLF (AU)	2 Months after MLF (AU)
0x	control	8.9 ± 0.1^{a}	8.29 ± 0.13^{a}	0x	control	13.29 ± 0.14^{ab}	7.58 ± 0.04^{cd}	9.88 ± 0.08^{e}	0x	control	11.44 ± 0.14^{ab}	9.84 ± 0.05^{cde}	12.53 ± 0.09^{a}
	16 mg/L	12.85 ± 0.26^{b}	8.66 ± 0.01^{a}		16 mg/L	12.75 ± 0.05^{ab}	7.64 ± 0.03^{c}	8.42 ± 0.01^{e}		16 mg/L	9.96 ± 0.07^{cf}	10.84 ± 0.04^{ceg}	12.27 ± 0.02^{a}
					32 mg/L	12.82 ± 0.10^{ab}	7.74 ± 0.05^{cf}	9.54 ± 0.04^{e}		32 mg/L	11.04 ± 0.08^{bhi}	7.84 ± 0.04^{bfgi}	12.27 ± 0.06^{a}
1x	control	17.35 ± 0.07^{c}	13.39 ± 0.05^{b}	1x	control	12.66 ± 0.01^{a}	6.49 ± 0.04^{gh}	9.77 ± 0.00^{l}	1x	control	10.29 ± 0.02^{fghjk}	7.84 ± 0.05^{dn}	9.69 ± 0.01^{cej}
	16 mg/L	19.25 ± 0.11^{d}	13.66 ± 0.16^{b}		16 mg/L	14.22 ± 0.64^{j}	7.39 ± 0.09^{cd}	6.94 ± 0.14^{ghi}		16 mg/L	11.96 ± 0.37^{ai}	8.36 ± 0.10^{dl}	10.87 ± 0.15^{bfm}
					32 mg/L	13.11 ± 0.02^{ab}	7.45 ± 0.08^{cd}	11.30 ± 0.36^{fi}		32 mg/L	11.93 ± 0.02^{ai}	8.88 ± 0.09^{eln}	11.18 ± 0.38^{bhio}
2x	control	20.87 ± 0.30^{e}	14.93 ± 0.20^{g}	2x	control	14.86 ± 0.24^{jk}	6.23 ± 0.02^{g}	10.77 ± 0.02^{f}	2x	control	10.69 ± 0.22^{bfgp}	7.93 ± 0.02^{p}	10.18 ± 0.02^{fghjq}
	16 mg/L	23.79 ± 0.27^{f}	13.80 ± 0.49^{b}		16 mg/L	13.41 ± 0.57^{b}	7.26 ± 0.01^{cd}	10.69 ± 0.08^{i}		16 mg/L	12.06 ± 0.38^{ao}	9.20 ± 0.02^{celg}	11.20 ± 0.08^{biko}
					32 mg/L	15.20 ± 0.29^{k}	9.92 ± 0.15^{dh}	9.25 ± 0.02^{i}		32 mg/L	11.71 ± 0.34^{aimp}	9.55 ± 0.18^{cej}	11.86 ± 0.02^{aim}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis for each cultivar. 0, 1 and 2 represents the wines made of different seed concentrations; 16 and 32 represents the 16 mg/L and 32 mg/L oxygen addition. Values are expressed in Absorbance Units (AU).

Table 7.2 Optical density (420 nm) average data for the 2007 Cabernet Sauvignon, 2008 Cabernet Sauvignon and 2008 Pinotage

OPTICAL DENSITY: 420 NM				OPTICAL DENSITY: 420 NM				OPTICAL DENSITY: 420 NM			
*2007 Cabernet Sauvignon				*2008 Cabernet Sauvignon				*2008 Pinotage			
Treatment		Before MLF (AU)	After MLF (AU)	Treatment		Before MLF (AU)	After MLF (AU)	2 Months after MLF (AU)	Treatment		Before MLF (AU)	After MLF (AU)	2 Months after MLF (AU)
0x	control	2.77 ± 0.08^{a}	2.78 ± 0.12^{a}	0x	control	3.90 ± 0.14^{a}	2.62 ± 0.04^{cd}	3.38 ± 0.08^{ae}	0x	control	4.00 ± 0.08^{ab}	3.58 ± 0.25^{cd}	4.47 ± 0.45^{e}
	16 mg/L	4.17 ± 0.26^{b}	2.91 ± 0.01^{a}		16 mg/L	3.82 ± 0.05^{ab}	2.63 ± 0.03^{cd}	3.02 ± 0.01^{ef}		16 mg/L	3.53 ± 0.68^{cfg}	3.76 ± 0.10^{cfh}	4.34 ± 0.13^{ae}
					32 mg/L	3.86 ± 0.10^{ab}	2.64 ± 0.05^{cd}	3.27 ± 0.04^{ef}		32 mg/L	3.89 ± 0.16^{bhi}	3.03 ± 0.04^{bfj}	4.33 ± 0.08^{ae}
1x	control	5.29 ± 0.09^{c}	4.30 ± 0.05^{bd}	1x	control	3.87 ± 0.01^{ab}	2.38 ± 0.04^{cd}	3.34 ± 0.00^{fg}	1x	control	3.62 ± 0.44^{bf}	3.03 ± 0.03^{d}	3.60 ± 0.06^{cfhk}
	16 mg/L	5.99 ± 0.15^{e}	4.33 ± 0.16^{bd}		16 mg/L	4.70 ± 0.64^{j}	2.63 ± 0.09^{cd}	2.51 ± 0.14^{efg}		16 mg/L	4.04 ± 0.18^{aij}	3.04 ± 0.09^{d}	3.76 ± 0.03^{bfj}
					32 mg/L	4.04 ± 0.02^{bh}	2.67 ± 0.08^{c}	3.65 ± 0.36^{ef}		32 mg/L	4.07 ± 0.21^{aij}	3.19 ± 0.07^{dg}	3.83 ± 0.11^{bfj}
2x	control	6.86 ± 0.38^{f}	5.02 ± 0.19^{c}	2x	control	5.07 ± 0.24^{i}	2.32 ± 0.02^{d}	3.48 ± 0.02^{g}	2x	control	3.92 ± 0.22^{bik}	3.11 ± 0.07^{d}	3.76 ± 0.03^{bfj}
	16 mg/L	7.91 ± 0.29^{g}	4.55 ± 0.51^{d}		16 mg/L	4.29 ± 0.57^{h}	2.60 ± 0.01^{cd}	3.45 ± 0.08^{fg}		16 mg/L	4.33 ± 0.15^{ae}	3.49 ± 0.07^{cfg}	4.05 ± 0.04^{aij}
					32 mg/L	5.32 ± 0.29^{i}	3.35 ± 0.15^{cd}	3.21 ± 0.02^{efg}		32 mg/L	4.22 ± 0.17^{aei}	3.64 ± 0.05^{bf}	4.31 ± 0.05^{ae}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis for each cultivar. 0, 1 and 2 represents the wines made of different seed concentrations; 16 and 32 represents the 16 mg/L and 32 mg/L oxygen addition. Values are expressed in Absorbance Units (AU).

Table 7.3 Optical density (520 nm) average data for the 2007 Cabernet Sauvignon, 2008 Cabernet Sauvignon and 2008 Pinotage

OPTICAL DENSITY: 520 NM				OPTICAL DENSITY: 520 NM				OPTICAL DENSITY: 520 NM			
*2007 Cabernet Sauvignon				*2008 Cabernet Sauvignon				*2008 Pinotage			
Treatment		Before MLF (AU)	After MLF (AU)	Treatment		Before MLF (AU)	After MLF (AU)	2 Months after MLF (AU)	Treatment		Before MLF (AU)	After MLF (AU)	2 Months after MLF (AU)
0x	control	5.11 ± 0.15^{a}	4.49 ± 0.18^{b}	0x	control	7.93 ± 0.20^{a}	3.95 ± 0.08^{bcd}	5.20 ± 0.16^{e}	0x	control	5.94 ± 0.18^{abc}	4.80 ± 0.31^{de}	6.24 ± 0.59^{ab}
	16 mg/L	6.58 ± 0.30^{e}	4.68 ± 0.01^{ab}		16 mg/L	7.47 ± 0.11^{f}	4.00 ± 0.06^{bcd}	4.20 ± 0.04^{e}		16 mg/L	5.13 ± 1.04^{fg}	5.16 ± 0.23^{dfh}	6.05 ± 0.25^{abi}
					32 mg/L	7.49 ± 0.10^{f}	4.10 ± 0.06^{bc}	4.94 ± 0.12^{e}		32 mg/L	5.70 ± 0.24^{acj}	3.57 ± 0.04^{fg}	6.06 ± 0.15^{abi}
1x	control	9.88 ± 0.06^{f}	7.34 ± 0.07^{cd}	1x	control	7.34 ± 0.03^{f}	3.24 ± 0.07^{gh}	5.06 ± 0.03^{i}	1x	control	5.21 ± 0.69^{fgj}	3.57 ± 0.08^{k}	4.57 ± 0.14^{dh}
	16 mg/L	10.44 ± 0.05^{g}	7.48 ± 0.25^{cd}		16 mg/L	7.67 ± 0.15^{af}	3.77 ± 0.18^{bdj}	3.44 ± 0.27^{k}		16 mg/L	6.34 ± 0.29^{b}	4.02 ± 0.11^{ek}	5.44 ± 0.22^{cg}
					32 mg/L	7.58 ± 0.02^{af}	3.79 ± 0.17^{bdj}	6.23 ± 0.64^{k}		32 mg/L	6.28 ± 0.38^{b}	4.32 ± 0.13^{de}	5.64 ± 0.16^{cgi}
2x	control	10.95 ± 0.48^{h}	7.78 ± 0.25^{c}	2x	control	7.66 ± 0.25^{af}	3.01 ± 0.02^{g}	5.90 ± 0.02^{c}	2x	control	5.21 ± 0.31^{fgj}	3.56 ± 0.12^{k}	4.83 ± 0.05^{df}
	16 mg/L	11.74 ± 0.34^{i}	7.31 ± 0.70^{d}		16 mg/L	7.46 ± 0.27^{f}	3.64 ± 0.04^{dj}	5.88 ± 0.13^{ik}		16 mg/L	6.10 ± 0.17^{abi}	4.28 ± 0.05^{eh}	5.43 ± 0.08^{cg}
					32 mg/L	7.67 ± 0.60^{af}	5.27 ± 0.30^{hj}	4.78 ± 0.05^{ik}		32 mg/L	5.93 ± 0.24^{abc}	4.42 ± 0.09^{de}	5.72 ± 0.09^{acj}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis for each cultivar. 0, 1 and 2 represents the wines made of different seed concentrations; 16 and 32 represents the 16 mg/L and 32 mg/L oxygen addition. Values are expressed in Absorbance Units (AU).

Table 7.4 Optical density (620 nm) average data for the 2007 Cabernet Sauvignon, 2008 Cabernet Sauvignon and 2008 Pinotage

OPTICAL DENSITY: 620 NM				OPTICAL DENSITY: 620 NM				OPTICAL DENSITY: 620 NM			
*2007 Cabernet Sauvignon				*2008 Cabernet Sauvignon				*2008 Pinotage			
Treatment		Before MLF (AU)	After MLF (AU)	Treatment		Before MLF (AU)	After MLF (AU)	2 Months after MLF (AU)	Treatment		Before MLF (AU)	After MLF (AU)	2 Months after MLF (AU)
0x	control	1.02 ± 0.04^{a}	1.02 ± 0.08^{a}	0x	control	1.46 ± 0.07^{ab}	1.01 ± 0.02^{c}	1.31 ± 0.04^{abd}	0x	control	1.5 ± 0.03^{abc}	1.47 ± 0.09^{d}	1.82 ± 0.20^{ef}
	16 mg/L	2.11 ± 0.21^{bc}	1.07 ± 0.00^{a}		16 mg/L	1.46 ± 0.03^{ab}	1.01 ± 0.02^{c}	1.20 ± 0.01^{abd}		16 mg/L	1.30 ± 0.28^{dg}	1.56 ± 0.05^{ab}	1.88 ± 0.08^{e}
					32 mg/L	1.47 ± 0.03^{ae}	1.01 ± 0.02^{c}	1.32 ± 0.03^{abdf}		32 mg/L	1.45 ± 0.07^{ab}	1.23 ± 0.02^{achi}	1.88 ± 0.04^{e}
1x	control	2.19 ± 0.06^{b}	1.75 ± 0.02^{d}	1x	control	1.46 ± 0.03^{ab}	0.88 ± 0.02^{c}	1.37 ± 0.00^{df}	1x	control	1.45 ± 0.19^{ab}	1.23 ± 0.01^{d}	1.51 ± 0.03^{aci}
	16 mg/L	2.82 ± 0.15^{e}	1.85 ± 0.07^{d}		16 mg/L	1.85 ± 0.32^{i}	0.99 ± 0.04^{c}	0.99 ± 0.05^{bdf}		16 mg/L	1.58 ± 0.07^{achj}	1.30 ± 0.03^{dg}	1.67 ± 0.03^{fh}
					32 mg/L	1.50 ± 0.01^{ag}	1.00 ± 0.04^{c}	1.42 ± 0.14^{bdef}		32 mg/L	1.58 ± 0.07^{achj}	1.37 ± 0.03^{bd}	1.71 ± 0.05^{fjk}
2x	control	3.06 ± 0.04^{f}	2.13 ± 0.15^{bc}	2x	control	2.12 ± 0.16^{h}	0.89 ± 0.01^{c}	1.39 ± 0.01^{f}	2x	control	1.56 ± 0.09^{achi}	1.27 ± 0.03^{d}	1.59 ± 0.02^{achj}
	16 mg/L	4.14 ± 0.18^{g}	1.94 ± 0.26^{cd}		16 mg/L	1.65 ± 0.31^{g}	1.02 ± 0.01^{c}	1.36 ± 0.02^{bdef}		16 mg/L	1.63 ± 0.06^{chj}	1.43 ± 0.03^{bgi}	1.72 ± 0.02^{fjk}
					32 mg/L	2.21 ± 0.13^{h}	1.30 ± 0.07^{c}	1.25 ± 0.01^{abd}		32 mg/L	1.56 ± 0.08^{achi}	1.48 ± 0.01^{ab}	1.83 ± 0.04^{ek}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis for each cultivar. 0, 1 and 2 represents the wines made of different seed concentrations; 16 and 32 represents the 16 mg/L and 32 mg/L oxygen addition. Values are expressed in Absorbance Units (AU).

Table 7.5 Colour hue (420 nm/520 nm) average data for the 2007 Cabernet Sauvignon, 2008 Cabernet Sauvignon and 2008 Pinotage

COLOUR HUE				COLOUR HUE					COLOUR HUE				
*2007 Cabernet Sauvignon				*2008 Cabernet Sauvignon					*2008 Pinotage				
Treatment		Before MLF (AU)	After MLF (AU)	Treatment		Before MLF (AU)	After MLF (AU)	2 Months after MLF (AU)	Treatment		Before MLF (AU)	After MLF (AU)	2 Months after MLF (AU)
0x	control	0.54 ± 0.00^{a}	0.62 ± 0.01^{b}	0x	control	0.49 ± 0.01^{a}	0.66 ± 0.00^{bc}	0.62 ± 0.06^{bd}	0x	control	0.67 ± 0.01^{a}	0.73 ± 0.01^{bcd}	0.72 ± 0.01^{ef}
	16 mg/L	0.63 ± 0.01^{bc}	0.62 ± 0.01^{b}		16 mg/L	0.51 ± 0.00^{a}	0.66 ± 0.00^{bc}	0.59 ± 0.00^{de}		16 mg/L	0.69 ± 0.01^{ag}	0.75 ± 0.02^{bdh}	0.72 ± 0.01^{cef}
					32 mg/L	0.52 ± 0.01^{a}	0.64 ± 0.00^{bfg}	0.59 ± 0.00^{de}		32 mg/L	0.68 ± 0.00^{ag}	0.73 ± 0.00^{bce}	0.72 ± 0.00^{f}
1x	control	0.54 ± 0.01^{a}	0.58 ± 0.00^{d}	1x	control	0.53 ± 0.00^{a}	0.73 ± 0.00^{hi}	0.67 ± 0.00^{cfj}	1x	control	0.70 ± 0.01^{gi}	0.85 ± 0.02^{m}	0.79 ± 0.01^{j}
	16 mg/L	0.57 ± 0.01^{d}	0.58 ± 0.00^{d}		16 mg/L	0.61 ± 0.07^{deg}	0.70 ± 0.01^{ch}	0.64 ± 0.01^{bfg}		16 mg/L	0.64 ± 0.00^{k}	0.76 ± 0.01^{h}	0.69 ± 0.03^{ag}
					32 mg/L	0.53 ± 0.00^{a}	0.67 ± 0.00^{hjk}	0.65 ± 0.00^{bfg}		32 mg/L	0.65 ± 0.01^{k}	0.74 ± 0.01^{bdh}	0.68 ± 0.00^{a}
2x	control	0.63 ± 0.01^{b}	0.65 ± 0.00^{c}	2x	control	0.66 ± 0.01^{bck}	0.77 ± 0.00^{i}	0.72 ± 0.01^{h}	2x	control	0.75 ± 0.00^{dh}	0.87 ± 0.01^{n}	0.78 ± 0.01^{j}
	16 mg/L	0.67 ± 0.01^{e}	0.62 ± 0.01^{b}		16 mg/L	0.57 ± 0.07^{e}	0.71 ± 0.01^{hj}	0.66 ± 0.01^{bc}		16 mg/L	0.71 ± 0.00^{fi}	0.81 ± 0.01^{l}	0.75 ± 0.01^{bdh}
					32 mg/L	0.70 ± 0.04^{ch}	0.73 ± 0.02^{h}	0.66 ± 0.00^{bc}		32 mg/L	0.71 ± 0.01^{fi}	0.82 ± 0.01^{l}	0.75 ± 0.01^{h}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis for each cultivar. 0, 1 and 2 represents the wines made of different seed concentrations; 16 and 32 represents the 16 mg/L and 32 mg/L oxygen addition. Values are expressed in Absorbance Units (AU).

Table 7.6 (+)-Catechin concentrations for the 2007 Cabernet Sauvignon, 2008 Cabernet Sauvignon and 2008 Pinotage

RP-HPLC: (+)-CATECHIN				RP-HPLC: (+)-CATECHIN					RP-HPLC: (+)-CATECHIN				
*2007 Cabernet Sauvignon				*2008 Pinotage					*2008 Cabernet Sauvignon				
Treatment		Before MLF (mg/L)	After MLF (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)
0x	control	5.1 ± 0.5^{c}	6.9 ± 0.2^{de}	0x	control	7.2 ± 0.7^{abc}	9.5 ± 0.4^{acd}	4.8 ± 0.3^{ab}	0x	control	$^{\S}< 1.5^{a}$	11.3 ± 0^{b}	9.1 ± 0.6^{bcd}
	16 mg/L	6.8 ± 2.6^{cd}	8.9 ± 0.8^{e}		16 mg/L	6.7 ± 0.5^{abc}	9.2 ± 0.5^{ace}	4.1 ± 0.8^{b}		16 mg/L	$^{\S}< 1.5^{a}$	10.5 ± 0.4^{bc}	8.6 ± 0.7^{bcd}
					32 mg/L	6.5 ± 0.8^{abc}	8.8 ± 0.5^{acf}	4.3 ± 0.2^{b}		32 mg/L	$^{\S}< 1.5^{a}$	11.7 ± 0.4^{b}	9.2 ± 0.2^{bcd}
1x	control	23.8 ± 0.6^{a}	29.1 ± 0.6^{b}	1x	control	13.7 ± 1.7^{dk}	33.1 ± 2.9^{j}	10.4 ± 1.0^{cd}	1x	control	6.7 ± 0.4^{d}	15.7 ± 0.3^{e}	7.3 ± 1.8^{cd}
	16 mg/L	22.6 ± 0.7^{a}	27.8 ± 1^{b}		16 mg/L	12.9 ± 1.7^{dek}	18.2 ± 0.4^{gl}	10.3 ± 4.5^{cd}		16 mg/L	10.3 ± 5.5^{bc}	16.3 ± 0.8^{e}	9.3 ± 1^{bcd}
					32 mg/L	11.8 ± 0.5^{defm}	18.2 ± 1.9^{gl}	9.7 ± 9^{cd}		32 mg/L	6.8 ± 0.3^{d}	15.9 ± 0.1^{e}	9.5 ± 0.6^{bcd}
2x	control	39.2 ± 0.8^{g}	42.5 ± 1.7^{f}	2x	control	20 ± 2.3^{g}	29.2 ± 2.4^{hij}	30.7 ± 2.4^{hj}	2x	control	25.4 ± 0.4^{fh}	27.1 ± 0.9^{gh}	23.2 ± 0.9^{f}
	16 mg/L	32.5 ± 1.3^{h}	41.6 ± 2.1^{f}		16 mg/L	15.9 ± 0.9^{klm}	26.4 ± 0.6^{i}	30.1 ± 0.5^{hij}		16 mg/L	18.4 ± 8.5^{e}	23.4 ± 0.7^{f}	29.6 ± 0.8^{g}
					32 mg/L	16 ± 2.8^{gk}	29.7 ± 0.6^{hij}	28.6 ± 0.5^{hi}		32 mg/L	23 ± 1.2^{f}	25.7 ± 0.8^{fh}	28.3 ± 1^{gh}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis for each cultivar. 0, 1 and 2 represents the wines made of different seed concentrations; 16 and 32 represents the 16 mg/L and 32 mg/L oxygen addition. Values are expressed in mg/L.

[§]Concentrations of this compound was below the limit of quantification

Table 7.7 (-)-Epicatechin concentrations for the 2007 Cabernet Sauvignon, 2008 Cabernet Sauvignon and 2008 Pinotage

RP-HPLC: (-)-EPICATECHIN				RP-HPLC: (-)-EPICATECHIN				RP-HPLC: (-)-EPICATECHIN			
*2007 Cabernet Sauvignon				*2008 Pinotage				*2008 Cabernet Sauvignon			
Treatment		Before MLF (mg/L)	After MLF (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)
0x	control	106.6 ± 0.6^{cd}	100.5 ± 0.5^{ef}	0x	control	18.1 ± 3.4^{ab}	15.4 ± 0.8^{a}	17.1 ± 2.5^{ac}	0x	control	98.9 ± 1.0^{aegh}	101 ± 0.4^{ab}	95 ± 2.4^{ci}
	16 mg/L	105.2 ± 4.3^{c}	98.4 ± 1.1^{f}		16 mg/L	19.7 ± 0.7^{bcd}	15.8 ± 0.1^{a}	18.9 ± 1.1^{bce}		16 mg/L	100.8 ± 0.5^{ab}	100.9 ± 0.6^{ab}	95.5 ± 1^{cd}
					32 mg/L	19.5 ± 1.6^{bc}	16.1 ± 0.1^{ae}	19.4 ± 1.3^{bc}		32 mg/L	100.7 ± 1.3^{aef}	99.4 ± 0.7^{aegh}	94.3 ± 0.1^{c}
1x	control	113.5 ± 2.7^{ab}	105.4 ± 0.5^{c}	1x	control	35.4 ± 1.1^{fk}	31.6 ± 2.1^{gj}	24.4 ± 1.3^{l}	1x	control	100 ± 0^{aeg}	99.7 ± 1.4^{aegh}	95.1 ± 0.5^{ci}
	16 mg/L	109.8 ± 2.4^{ad}	102.4 ± 1.8^{ce}		16 mg/L	32.4 ± 3.3^{gjk}	21.3 ± 1^{bd}	22.7 ± 0.6^{dl}		16 mg/L	102.7 ± 3.8^{bfi}	101.3 ± 0.9^{bfgj}	97.1 ± 0.2^{dhi}
					32 mg/L	30.6 ± 1.9^{j}	21.2 ± 1.9^{bd}	25.1 ± 1.5^{l}		32 mg/L	100 ± 1.7^{aeg}	99 ± 0.6^{aegh}	97.1 ± 0.4^{dhi}
2x	control	130.3 ± 0.2^{h}	124.8 ± 0.6^{g}	2x	control	54.2 ± 3.8^{m}	34.4 ± 3.9^{fg}	41.8 ± 2.6^{h}	2x	control	103.5 ± 1.1^{bj}	98 ± 0.9^{de}	100.9 ± 0.5^{ab}
	16 mg/L	121.3 ± 5.3^{g}	114.6 ± 5.5^{b}		16 mg/L	38.8 ± 2.2^{hi}	30.3 ± 1.1^{j}	35.5 ± 1.2^{fk}		16 mg/L	104.1 ± 1.7^{j}	99 ± 0.9^{aehk}	98.7 ± 4.4^{aegh}
					32 mg/L	37.1 ± 0.3^{fi}	31.2 ± 1^{j}	33.3 ± 0.3^{gjk}		32 mg/L	101.2 ± 2.1^{ab}	100 ± 0.9^{cdk}	99 ± 2.6^{aegh}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis for each cultivar. 0, 1 and 2 represents the wines made of different seed concentrations; 16 and 32 represents the 16 mg/L and 32 mg/L oxygen addition. Values are expressed in mg/L.

Table 7.8 (+)-Gallocatechin concentrations for the 2007 Cabernet Sauvignon, 2008 Cabernet Sauvignon and 2008 Pinotage

RP-HPLC: (+)-GALLOCATECHIN				RP-HPLC: (+)-GALLOCATECHIN				RP-HPLC: (+)-GALLOCATECHIN			
*2007 Cabernet Sauvignon				*2008 Pinotage				*2008 Cabernet Sauvignon			
Treatment		Before MLF (mg/L)	After MLF (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)
0x	control	3.23 ± 0.10^{e}	3.63 ± 0.13^{e}	0x	control	3.67 ± 0.1^{ab}	4.59 ± 0.1^{cd}	2.25 ± 0.2^{e}	0x	control	4.03 ± 0.4^{begh}	5.28 ± 0.9^{cfi}	3.98 ± 0.1^{begj}
	16 mg/L	3.48 ± 0.97^{e}	3.73 ± 0.21^{e}		16 mg/L	3.67 ± 0.1^{ab}	4.68 ± 0.1^{cd}	2.36 ± 0.1^{ef}		16 mg/L	4.18 ± 0.4^{ab}	4.92 ± 0.8^{ac}	2.84 ± 1.0^{d}
					32 mg/L	3.76 ± 0^{ag}	4.72 ± 0.1^{cdh}	2.32 ± 0^{e}		32 mg/L	3.81 ± 0.5^{be}	5.73 ± 0.7^{cf}	3.93 ± 0.4^{beg}
1x	control	5.53 ± 0.12^{ab}	5.73 ± 0.28^{ab}	1x	control	4.8 ± 0.1^{cdhl}	5.2 ± 0.1^{jlm}	4.04 ± 1.3^{a}	1x	control	5.23 ± 0.3^{cfi}	5.66 ± 0.3^{cfn}	4.83 ± 0.3^{ahijmn}
	16 mg/L	5.40 ± 0.28^{abc}	5.93 ± 0.75^{acd}		16 mg/L	4.63 ± 0.1^{cd}	5.24 ± 0^{il}	3.06 ± 0.4^{jkn}		16 mg/L	4.97 ± 0.2^{ac}	6.05 ± 0.1^{fl}	4.88 ± 0.1^{ach}
					32 mg/L	4.75 ± 0.1^{cdhm}	5.09 ± 0.1^{chi}	3.29 ± 0.1^{bgn}		32 mg/L	4.93 ± 0.1^{ac}	5.14 ± 0.6^{ci}	4.72 ± 0.3^{agim}
2x	control	6.03 ± 0.15^{acd}	6.30 ± 0.14^{b}	2x	control	5.03 ± 0.1^{cdhi}	5.1 ± 0.2^{chi}	3.28 ± 0^{bj}	2x	control	5.27 ± 0.2^{cfi}	5.71 ± 1.3^{cf}	4.54 ± 0.1^{aei}
	16 mg/L	5.15 ± 0.47^{cd}	6.43 ± 0.49^{b}		16 mg/L	4.95 ± 0.1^{cdhi}	5.18 ± 0^{hi}	2.81 ± 0^{fj}		16 mg/L	5.25 ± 0.2^{cfi}	6.91 ± 0.5^{kl}	2.92 ± 1.0^{d}
					32 mg/L	4.56 ± 0.1^{d}	5.39 ± 0^{i}	2.71 ± 0^{efk}		32 mg/L	5.51 ± 0.2^{cfm}	7.41 ± 0.2^{k}	3.32 ± 0.2^{bd}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis for each cultivar. 0, 1 and 2 represents the wines made of different seed concentrations; 16 and 32 represents the 16 mg/L and 32 mg/L oxygen addition. Values are expressed in mg/L.

Table 7.9 (-)-Epigallocatechin concentrations for the 2007 Cabernet Sauvignon, 2008 Cabernet Sauvignon and 2008 Pinotage

RP-HPLC: (-)-EPIGALLOCATECHIN				RP-HPLC: (-)-EPIGALLOCATECHIN				RP-HPLC: (-)-EPIGALLOCATECHIN			
*2007 Cabernet Sauvignon				*2008 Pinotage				*2008 Cabernet Sauvignon			
Treatment		Before MLF (mg/L)	After MLF (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)
0x	control	3.65 ± 0.17^{de}	3.60 ± 0.18^{e}	0x	control	6.52 ± 0.3^{ab}	5.87 ± 0.2^{c}	5.44 ± 0^{cd}	0x	control	1.86 ± 0^{ab}	1.71 ± 0.1^{ac}	1.57 ± 0.8^{af}
	16 mg/L	4.53 ± 1.80^{d}	4.20 ± 0.10^{de}		16 mg/L	5.81 ± 0.8^{c}	5.97 ± 0.3^{ac}	5.78 ± 0.4^{c}		16 mg/L	1.84 ± 0.1^{ab}	1.7 ± 0.1^{ac}	1.32 ± 0.1^{a}
					32 mg/L	5.43 ± 0.2^{cd}	5.93 ± 0.1^{ac}	5.04 ± 0.3^{b}		32 mg/L	1.8 ± 0.2^{ab}	1.76 ± 0.1^{ac}	2.75 ± 0.2^{de}
1x	control	6.23 ± 0.06^{ab}	7.00 ± 0.41^{ab}	1x	control	6.87 ± 0.3^{beh}	6.91 ± 0.1^{beh}	5.17 ± 0.1^{d}	1x	control	2.83 ± 0.3^{dej}	2.4 ± 0.3^{bcei}	1.57 ± 0.8^{af}
	16 mg/L	6.30 ± 0.38^{ac}	6.08 ± 0.22^{b}		16 mg/L	7.18 ± 0^{efj}	6.79 ± 0.2^{bei}	5.48 ± 0.2^{cd}		16 mg/L	3.72 ± 1.0^{gh}	2.44 ± 0.1^{bcei}	1.41 ± 0^{a}
					32 mg/L	6.9 ± 0.3^{beh}	7.12 ± 0.2^{bef}	7.64 ± 0.8^{gj}		32 mg/L	2.3 ± 0.1^{bcef}	2.56 ± 0.2^{beik}	1.53 ± 0.2^{a}
2x	control	8.43 ± 0.15^{f}	8.13 ± 0.51^{cg}	2x	control	6.67 ± 0.3^{be}	7.04 ± 0.2^{bef}	4.97 ± 0.5^{d}	2x	control	3.57 ± 0.1^{ghj}	2.34 ± 0.3^{bce}	3.2 ± 0.2^{dgk}
	16 mg/L	7.45 ± 0.13^{fg}	7.25 ± 0.31^{c}		16 mg/L	7.45 ± 0.1^{fgh}	7.30 ± 0.4^{fhij}	5.43 ± 0.7^{cd}		16 mg/L	3.49 ± 0.5^{dg}	3.15 ± 0.3^{dhi}	3.68 ± 1.6^{gh}
					32 mg/L	7.9 ± 0.1^{g}	7.59 ± 0.2^{fg}	5.16 ± 0.4^{d}		32 mg/L	3.85 ± 0^{gh}	3.11 ± 0.1^{dhi}	3.93 ± 0.9^{g}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis for each cultivar. 0, 1 and 2 represents the wines made of different seed concentrations; 16 and 32 represents the 16 mg/L and 32 mg/L oxygen addition. Values are expressed in mg/L.

Table 7.10 (-)-Epicatechingallate concentrations for the 2007 Cabernet Sauvignon, 2008 Cabernet Sauvignon and 2008 Pinotage

RP-HPLC: (-)-EPICATECHINGALLATE				RP-HPLC: (-)-EPICATECHINGALLATE				RP-HPLC: (-)-EPICATECHINGALLATE				
*2007 Cabernet Sauvignon				*2008 Pinotage					*2008 Cabernet Sauvignon			
Treatment		Before MLF (mg/L)	After MLF (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)
0x	control	2.70 ± 0.22^{ac}	1.90 ± 0.76^{a}	0x	control	1.53 ± 0^{ab}	§< 1.5^{ab}	1.56 ± 0^{abc}	0x	control	§< 1.5^{a}	§< 1.5^{a}	§< 1.5^{a}
	16 mg/L	3.37 ± 0.49^{ab}	3.03 ± 0.32^{ab}		16 mg/L	§< 1.5^{ab}	§< 1.5^{ab}	1.68 ± 0.2^{de}		16 mg/L	§< 1.5^{a}	§< 1.5^{a}	§< 1.5^{a}
					32 mg/L	§< 1.5^{ab}	1.55 ± 0.1^{a}	1.84 ± 0.1^{g}		32 mg/L	§< 1.5^{a}	§< 1.5^{a}	§< 1.5^{a}
1x	control	5.30 ± 1.06^{ab}	3.45 ± 0.10^{ab}	1x	control	§< 1.5^{ab}	1.53 ± 0^{ab}	1.58 ± 0.1^{acd}	1x	control	§< 1.5^{a}	§< 1.5^{a}	§< 1.5^{a}
	16 mg/L	4.13 ± 0.85^{ab}	3.03 ± 0.05^{ab}		16 mg/L	§< 1.5^{ab}	1.67 ± 0.1^{cde}	§< 1.5^{ab}		16 mg/L	§< 1.5^{a}	§< 1.5^{a}	§< 1.5^{a}
					32 mg/L	§< 1.5^{ab}	1.44 ± 0.1^{b}	§< 1.5^{ab}		32 mg/L	§< 1.5^{a}	§< 1.5^{a}	§< 1.5^{a}
2x	control	9.05 ± 0.21^{d}	8.25 ± 0.33^{b}	2x	control	§< 1.5^{ab}	2.32 ± 0.1^{f}	1.54 ± 0^{ab}	2x	control	§< 1.5^{a}	§< 1.5^{a}	§< 1.5^{a}
	16 mg/L	7.48 ± 0.19^{bc}	7.05 ± 0.25^{ab}		16 mg/L	§< 1.5^{ab}	2.42 ± 0.1^{f}	1.6 ± 0.1^{acd}		16 mg/L	§< 1.5^{a}	§< 1.5^{a}	1.6 ± 0.2^{b}
					32 mg/L	§< 1.5^{ab}	§< 1.5^{ab}	1.73 ± 0.1^{e}		32 mg/L	§< 1.5^{a}	§< 1.5^{a}	1.6 ± 0.2^{b}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis for each cultivar. 0, 1 and 2 represents the wines made of different seed concentrations; 16 and 32 represents the 16 mg/L and 32 mg/L oxygen addition. Values are expressed in mg/L.

§Concentrations of this compound was below the limit of quantification

Table 7.11 Concentrations of the dimer B1 for the 2007 Cabernet Sauvignon, 2008 Cabernet Sauvignon and 2008 Pinotage

RP-HPLC: DIMER B1				RP-HPLC: DIMER B1				RP-HPLC: DIMER B1			
*2007 Cabernet Sauvignon				*2008 Pinotage				*2008 Cabernet Sauvignon			
Treatment		Before MLF (mg/L)	After MLF (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)
0x	control	8.6 ± 0.2^{b}	9.4 ± 0.3^{c}	0x	control	7.6 ± 0.5^{a}	8.7 ± 0.5^{a}	6 ± 0.5^{a}	0x	control	§< 1.5^{a}	§< 1.5^{a}	§< 1.5^{a}
	16 mg/L	12.3 ± 5.3^{bc}	10.8 ± 0.6^{bc}		16 mg/L	8.0 ± 0.3^{a}	8.6 ± 0.7^{a}	6.9 ± 1.2^{a}		16 mg/L	§< 1.5^{a}	§< 1.5^{a}	§< 1.5^{a}
					32 mg/L	7.7 ± 0.2^{a}	8.1 ± 0.3^{a}	6.6 ± 0.1^{a}		32 mg/L	11.2 ± 0.5^{b}	§1.5 ± 0^{a}	4.3 ± 0.3^{a}
1x	control	29.3 ± 0.5^{a}	29.5 ± 0.6^{a}	1x	control	17.7 ± 0.6^{f}	17.8 ± 0.4^{f}	21.2 ± 1.9^{fg}	1x	control	16.1 ± 0.1^{g}	17.8 ± 0.5^{fg}	10.1 ± 0.6^{b}
	16 mg/L	28.9 ± 0.5^{a}	27.9 ± 1.1^{a}		16 mg/L	18.5 ± 1.3^{f}	29.4 ± 6.4^{bd}	20.2 ± 3.4^{fg}		16 mg/L	19.5 ± 7.0^{fg}	18.4 ± 0.3^{fg}	10.7 ± 0.8^{b}
					32 mg/L	19.6 ± 1.4^{f}	24.9 ± 5.6^{deg}	22.4 ± 8.3^{ef}		32 mg/L	19.9 ± 5.7^{f}	17.9 ± 0.5^{fg}	10.9 ± 0.2^{b}
2x	control	47.9 ± 0.7^{d}	46.0 ± 0.6^{d}	2x	control	30.9 ± 1.3^{b}	44.5 ± 3.3^{h}	37.9 ± 3.8^{c}	2x	control	28.3 ± 0.7^{e}	27.8 ± 1.4^{cde}	24.2 ± 0.5^{d}
	16 mg/L	41.6 ± 3.1^{e}	41.4 ± 1.8^{e}		16 mg/L	27.4 ± 0.9^{bd}	31.8 ± 5.9^{b}	37 ± 1.4^{c}		16 mg/L	24.6 ± 6.0^{cd}	27.7 ± 1.8^{cde}	20.5 ± 0.3^{f}
					32 mg/L	27.7 ± 0.5^{bd}	27.2 ± 0.9^{bde}	37.1 ± 0.8^{c}		32 mg/L	26 ± 1.3^{cde}	28.1 ± 1.0^{ce}	20.4 ± 1.5^{f}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis for each cultivar. 0, 1 and 2 represents the wines made of different seed concentrations; 16 and 32 represents the 16 mg/L and 32 mg/L oxygen addition. Values are expressed in mg/L.

§Concentrations of this compound was below the limit of quantification

Table 7.12 Concentrations of the dimer B2 for the 2007 Cabernet Sauvignon, 2008 Cabernet Sauvignon and 2008 Pinotage

RP-HPLC: DMER B2				RP-HPLC: DIMER B2				RP-HPLC: DIMER B2				
*2007 Cabernet Sauvignon				*2008 Pinotage				*2008 Cabernet Sauvignon				
Treatment		Before MLF (mg/L)	After MLF (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)
0x	control	6.1 ± 0.8^{c}	6.8 ± 0.5^{c}	0x	control	23.8 ± 4.6^{abc}	16.1 ± 0.2^{de}	18.8 ± 4.6^{def}	0x	control	§< 1.5^{a}	§< 1.5^{a}	§< 1.5^{a}
	16 mg/L	7.3 ± .0.5^{ac}	8.1 ± 0.7^{c}		16 mg/L	22.9 ± 2.5^{acf}	15.6 ± 1.1^{d}	15.7 ± 0.2^{d}		16 mg/L	§< 1.5^{a}	§< 1.5^{a}	§< 1.5^{a}
					32 mg/L	25.1 ± 2.2^{ab}	16.2 ± 1.9^{d}	17 ± 0.8^{de}		32 mg/L	§< 1.5^{a}	§< 1.5^{a}	§< 1.5^{a}
1x	control	26.7 ± 4.8^{ab}	26.5 ± 2.5^{ab}	1x	control	42.5 ± 3.9^{hl}	55.5 ± 2.0^{g}	24.4 ± 1.2^{abc}	1x	control	26.6 ± 0.5^{fg}	22.5 ± 2.6^{bf}	13.4 ± 2.7^{hi}
	16 mg/L	21.7 ± 1.6^{ac}	26.7 ± 2.2^{ab}		16 mg/L	39 ± 2.5^{lm}	31.1 ± 0.8^{k}	20.5 ± 1.1^{ce}		16 mg/L	25.3 ± 4.4^{bf}	17.3 ± 3.2^{ch}	12.8 ± 1.6^{a}
					32 mg/L	37.4 ± 0.3^{m}	29.8 ± 0.8^{k}	22.9 ± 3.1^{acf}		32 mg/L	17.9 ± 6.7^{ch}	17.5 ± 2.5^{ch}	14.3 ± 0.7^{hi}
2x	control	43.2 ± 4.3^{bd}	69.5 ± 5.3^{e}	2x	control	58.2 ± 1.1^{g}	46.2 ± 2.5^{hi}	25.8 ± 1.4^{ab}	2x	control	25.4 ± 1.4^{bf}	47.6 ± 1.8^{d}	31.1 ± 0.8^{eg}
	16 mg/L	34.1 ± 1.7^{df}	62.9 ± 6.9^{ef}		16 mg/L	51.2 ± 1.2^{j}	45.1 ± 2.4^{h}	27.2 ± 1.2^{bk}		16 mg/L	21.6 ± 1.6^{bc}	46.2 ± 1.2^{d}	33.9 ± 9.6^{e}
					32 mg/L	50 ± 0.2^{ij}	25.7 ± 7.4^{ab}	27.5 ± 0.7^{bk}		32 mg/L	21.8 ± 0.6^{bc}	45.5 ± 0.5^{d}	35.5 ± 1.6^{e}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis for each cultivar. 0, 1 and 2 represents the wines made of different seed concentrations; 16 and 32 represents the 16 mg/L and 32 mg/L oxygen addition. Values are expressed in mg/L.

§Concentrations of this compound was below the limit of quantification

Table 7.13 Polymeric phenol concentrations for the 2007 Cabernet Sauvignon, 2008 Cabernet Sauvignon and 2008 Pinotage

RP-HPLC: POLYMERIC PHENOLS				RP-HPLC: POLYMERIC PHENOLS				RP-HPLC: POLYMERIC PHENOLS			
*2007 Cabernet Sauvignon				*2008 Pinotage				*2008 Cabernet Sauvignon			
Treatment		Before MLF (mg/L)	After MLF (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)	Treatment		Before MLF (mg/L)	After MLF (mg/L)	2 Months after (mg/L)
0x	control	373 ± 20^{cd}	364 ± 8^{c}	0x	control	628 ± 9^{abc}	595 ± 20^{acd}	609 ± 31^{acd}	0x	control	241 ± 10^{abdg}	224 ± 6^{ae}	275 ± 20^{bcfh}
	16 mg/L	410 ± .17^{d}	391 ± 31^{cd}		16 mg/L	622 ± 9^{ac}	697 ± 23^{e}	634 ± 22^{ab}		16 mg/L	206 ± 0^{a}	222 ± 9^{ab}	252 ± 2^{abc}
					32 mg/L	621 ± 19^{ac}	744 ± 50^{f}	565 ± 13^{d}		32 mg/L	214 ± 12^{a}	231 ± 7^{abd}	268 ± 31^{bcef}
1x	control	823 ± 15^{ab}	806 ± 4^{a}	1x	control	632 ± 13^{ab}	586 ± 20^{cd}	578 ± 6^{d}	1x	control	248 ± 1^{abc}	217 ± 2^{ae}	250 ± 4^{abc}
	16 mg/L	857 ± 12^{b}	842 ± 21^{ab}		16 mg/L	624 ± 24^{ac}	637 ± 23^{abh}	578 ± 40^{d}		16 mg/L	279 ± 52^{cdfhj}	250 ± 6^{abc}	279 ± 3^{cdfhj}
					32 mg/L	631 ± 17^{ab}	667 ± 29^{be}	627 ± 19^{abc}		32 mg/L	251 ± 2^{abc}	257 ± 9^{abcf}	289 ± ^{acfghi}
2x	control	984 ± 10^{ef}	961 ± 18^{ef}	2x	control	754 ± 23^{fg}	668 ± 1^{be}	676 ± 16^{eh}	2x	control	326 ± 8^{hi}	239 ± 7^{abdg}	282 ± 3^{cdfhj}
	16 mg/L	987 ± 55^{e}	965 ± 62^{f}		16 mg/L	794 ± 13^{gi}	803 ± 19^{j}	747 ± 8^{f}		16 mg/L	306 ± 51^{fhi}	298 ± 8^{cfhi}	331 ± 131^{ij}
					32 mg/L	889 ± 63^{k}	847 ± 17^{j}	821 ± 30^{ij}		32 mg/L	340 ± 55^{i}	300 ± 28^{cfhi}	299 ± 9^{cfhi}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis for each cultivar. 0, 1 and 2 represents the wines made of different seed concentrations; 16 and 32 represents the 16 mg/L and 32 mg/L oxygen addition. Values are expressed in mg/L.

Table 7.14 All spectrophotometric and RP-HPLC analyses as analysed from samples taken at the bottom, middle and top valve of all tanks after oxygenation.

RP-HPLC	Bottom	Middle	Top
Gallic acid	15.87[a]	15.95[a]	15.84[a]
(+)-Gallocatechin	3.72[a]	3.88[a]	3.86[a]
(-)-Epigallocatechin	5.34[a]	5.31[a]	5.14[a]
(+)-Catechin	21.73[a]	23.82[a]	22.25[a]
(-)-Epicatechingallate	1.42[a]	1.45[a]	1.5[a]
Dimer B1	17.4[a]	17.92[a]	16.68[a]
Dimer B2	20.19[a]	20.15[a]	21.3[a]
Sum of Monomeric flavanols	85.66[a]	88.47[a]	86.56[a]
Polymeric phenols	461.08[a]	454.12[a]	469.52[a]
Caftaric acid	91.16[a]	91.97[a]	90.84a
Coutaric acid	1.3[a]	1.43[a]	1.37[a]
Caffeic acid	22.48[a]	22.64[a]	18.98[a]
p-Coumaric acid	1.28[a]	1.25[a]	1.32[a]
Sum of Hydroxycinnamic acids	116.22[a]	117.29[a]	112.29[a]
Quercetin-3-rutinoside	13.27[a]	13.35[a]	13.24[a]
Quercetin-3-glucoside	11.06[a]	10.76[a]	10.76[a]
Quercetin-3-rhamnoside	2[a]	2.01[a]	1.99[a]
Quercetin	0.86[a]	0.79[a]	0.82[a]
Sum of Flavonols	27.19[a]	26.91[a]	26.8[a]
Delphinidin-3-glucoside	3.71[a]	3.59[a]	3.48[a]
Cyanidin-3-glucoside	0.36[a]	0.36[a]	0.34[a]
Petudinin-3-glucoside	4.34[a]	3.48[a]	4.4[a]
Peonidin-3-glucoside	3.56[a]	3.67[a]	3.87[a]
Malvidin-3-glucoside	77.71[a]	76.84[a]	74.97[a]
Delphinidin-3-acetyl	1.21[a]	1.23[a]	1.21[a]
Petunidin-3-acetyl	2.7[a]	2.65[a]	2.59[a]
Peonidin-3-acetyl	2.54[a]	2.67[a]	2.68[a]
Malvidin-3-acetyl	24.8[a]	24.68[a]	24.08[a]
Delphinidin-3-p-coumaryl	0.95[a]	0.97[a]	0.92[a]
Petunidin-3-p-coumaryl	1.34[a]	1.34[a]	1.32[a]
Peonidin-3-p-coumaryl	0.7[a]	0.67[a]	0.69[a]
Malvidin-3-p-coumaryl	5.13[a]	4.88[a]	4.85[a]
Sum of monomeric anthocyanins	129.01[a]	126.98[a]	125.31[a]
Polymeric Pigments	36.11[a]	35.37[a]	36.89[a]

Table 7.14 (continue)

SPECTROMETRY			
Optical density (420 nm)*	3.48[a]	3.45[a]	3.48[a]
Optical density (520 nm)*	7.47[a]	7.42[a]	7.46[a]
Optical density (620 nm)*	1.33[a]	1.3[a]	1.31[a]
Colour Intensity*	12.29[a]	12.18[a]	12.25[a]
Colour hue*	0.47[a]	0.46[a]	0.47[a]
Modified colour intensity*	11.98[a]	12.06[a]	11.79[a]
Modified colour hue*	0.51[a]	0.51[a]	0.51[a]
Total phenols (Folin-Ciocalteu)	1861.68[a]	1919.73[a]	1865.08[a]
Tannin (BSA)	603.07[a]	589.15[a]	549.49[a]
Monomeric flavanols (DMAC Index)	363.93[a]	346.7[a]	354.76[a]
Total anthocyanins	368.07[a]	365.56[a]	361.31[a]

Note: all values displayed are the average of the control and oxygenated (16 mg/L and 32 mg/L) wines. The letter after each concentration/absorbency unit denotes the significant differences on a 5% confidence interval. Values are given in mg/L, except those indicated with (*) are given in absorbance units (AU).

Table 7.15 Monomeric flavanol concentrations (mg/L) determined by RP-HPLC for the 2007 Cabernet Sauvignon made of different pH treatments

Treatment		*(+)-CATECHIN		*(-)-EPICATECHIN		*(+)-GALLOCATECHIN		*(-)-EPIGALLOCATECHIN		*(-)-EPICATECHINGALLATE	
		Before MLF (mg/L)	After MLF (mg/L)	Before MLF (mg/L)	After MLF (mg/L)	Before MLF (mg/L)	After MLF (mg/L)	Before MLF (mg/L)	After MLF (mg/L)	Before MLF (mg/L)	After MLF (mg/L)
pH 3.4	control	*32.3 ± 0.8^{ab}	30.0 ± 5.7^{ab}	*145 ± 4.5^{ab}	163 ± 24.6^{a}	*14.7 ± 3.6^{ab}	15.2 ± 1.2^{ab}	*10.2 ± 5.2^{a}	6.9 ± 2.3^{b}	*9.2 ± 1.1^{a}	7.7 ± 3.0^{a}
	16mg/L	28.7 ± 4.7^{a}	28.6 ± 3.6^{a}	148 ± 14.1^{ab}	146 ± 9.4^{ab}	13.1 ± 2.8^{a}	16.0 ± 2.3^{ab}	8.6 ± 1.8^{ab}	5.6 ± 2.6^{ab}	8.5 ± 0.5^{a}	7.3 ± 2.8^{a}
pH 3.7	control	31.7 ± 0.4^{ab}	29.6 ± 1.6^{a}	153 ± 14.6^{ab}	136 ± 14.8^{ab}	14.6 ± 0.6^{ab}	16.3 ± 3.4^{ab}	4.9 ± 3.1^{b}	4.3 ± 4.6^{b}	8.2 ± 0.3^{a}	9.0 ± 1.2^{a}
	16mg/L	31.5 ± 1.2^{ab}	29.7 ± 1.8^{ab}	148 ± 18.4^{ab}	135 ± 18.8^{ab}	14.6 ± 0.1^{ab}	16.7 ± 2.6^{b}	4.7 ± 4.0^{b}	4.4 ± 4.2^{b}	8.6 ± 1.2^{a}	9.1 ± 1.4^{a}
pH 4.0	control	32.2 ± 1.3^{ab}	34.5 ± 6.1^{b}	150 ± 16.5^{ab}	142 ± 25.7^{ab}	14.2 ± 1.0^{ab}	13.9 ± 2.6^{ab}	7.5 ± 3.2^{ab}	8.8 ± 3.0^{ab}	9.1 ± 0.8^{a}	8.4 ± 0.2^{a}
	16mg/L	31.5 ± 0.7^{ab}	28.1 ± 1.1^{a}	142 ± 3.8^{b}	126 ± 4.5^{ab}	14.3 ± 1.0^{ab}	14.8 ± 1.0^{ab}	8.7 ± 1.2^{ab}	10.0 ± 1.9^{ab}	8.5 ± 0.7^{b}	9.6 ± 1.5^{ab}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis. 3.4, 3.7 and 4.0 represents wines made of different pHs. '16 mg/L' refers to the oxygenated wines.

Table 7.16 Dimeric flavanols determined by RP-HPLC for the 2007 Cabernet Sauvignon wines made of different pH treatments

RP-HPLC: DIMER B1				RP-HPLC: DIMER B2	
Treatment		Before MLF (mg/L)	After MLF (mg/L)	Before MLF (mg/L)	After MLF (mg/L)
pH 3.4	control	17.8 ± 5.9^{a}	24.5 ± 2.8^{ab}	20.8 ± 3.5^{ab}	23.9 ± 7.0^{ab}
	16mg/L	23.4 ± 4.1^{bc}	23.2 ± 2.0^{bc}	22.3 ± 4.4^{ab}	22.9 ± 3.4^{ab}
pH 3.7	control	21.3 ± 0.1^{ac}	23.7 ± 0.9^{bc}	19.6 ± 5.1^{b}	21.5 ± 4.2^{ab}
	16mg/L	21.1 ± 2.9^{ac}	22.9 ± 1.1^{bc}	20.7 ± 3.4^{ab}	18.9 ± 3.1^{b}
pH 4.0	control	21.0 ± 1.9^{ac}	24.3 ± 3.6^{bc}	19.7 ± 1.2^{ab}	29.1 ± 10.1^{ab}
	16mg/L	21.2 ± 1.7^{ac}	23.8 ± 1.6^{bc}	21.3 ± 2.1^{b}	23.1 ± 4.8^{ab}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis. 3.4, 3.7 and 4.0 represents wines made of different pHs. '16 mg/L' refers to the oxygenated wines.

Table 7.17 Hydroxycinnamic acids (mg/L) determined by RP-HPLC for the 2007 Cabernet Sauvignon wines made of different pH treatments.

*RP-HPLC: CAFTARIC ACID				*RP-HPLC: CAFFEIC ACID	
Treatment		Before MLF (mg/L)	After MLF (mg/L)	Before MLF (mg/L)	After MLF (mg/L)
pH 3.4	control	31.5 ± 0.3^{a}	26.3 ± 2.9^{ab}	16.1 ± 0.3^{a}	9.4 ± 3.1^{bc}
	16mg/L	30.4 ± 1.4^{a}	26.4 ± 3.8^{ab}	15.3 ± 0.9^{ad}	10.9 ± 5.7^{bd}
pH 3.7	control	31.4 ± 0.2^{a}	20.3 ± 3.4^{bc}	16.2 ± 0.3^{a}	10.4 ± 1.7^{be}
	16mg/L	29.5 ± 3.8^{a}	19.2 ± 10.1^{c}	14.8 ± 1.8^{ade}	9.7 ± 5.1^{bcf}
pH 4.0	control	31.3 ± 1.3^{a}	13.9 ± 5.1^{cd}	16.5 ± 0.6^{a}	5.8 ± 3.6^{cg}
	16mg/L	26.9 ± 6.3^{ab}	7.5 ± 3.2^{d}	13.9 ± 3.5^{adef}	3.7 ± 0.9^{g}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis. 3.4, 3.7 and 4.0 represents wines made of different pHs. '16 mg/L' refers to the oxygenated wines.

Table 7.18.1 Flavonol concentrations (mg/L) determined by RP-HPLC for the 2007 Cabernet Sauvignon wines made of different pH treatments.

*QUERCITIN-3-RUTINOSIDE				*QUERCITIN-3-GALACTOSIDE		QUERCITIN-3-GLUCOSIDE	
Treatment		Before MLF (mg/L)	After MLF (mg/L)	Before MLF (mg/L)	After MLF (mg/L)	Before MLF (mg/L)	After MLF (mg/L)
pH 3.4	control	2.08 ± 0.21^{ab}	1.18 ± 0.16^{ab}	8.37 ± 1.37^{abc}	6.78 ± 0.59^{ab}	18.2 ± 0.8^{ab}	14.0 ± 0.3^{c}
	16mg/L	2.13 ± 0.76^{ac}	1.43 ± 0.30^{ab}	8.06 ± 0.74^{abc}	7.37 ± 1.33^{ab}	16.6 ± 2.3^{abc}	14.7 ± 1.6^{cd}
pH 3.7	control	2.04 ± 0.24^{ad}	1.02 ± 0.11^{bc}	8.39 ± 0.92^{ac}	6.51 ± 0.27^{b}	17.2 ± 0.4^{abd}	14.5 ± 0.2^{c}
	16mg/L	1.95 ± 0.66^{ab}	1.50 ± 0.70^{ab}	8.09 ± 0.74^{abc}	7.17 ± 1.56^{ab}	16.9 ± 1.5^{abc}	15.4 ± 2.6^{ac}
pH 4.0	control	1.80 ± 0.26^{ab}	0.97 ± 0.10^{bd}	9.43 ± 0.83^{c}	6.93 ± 0.28^{ab}	18.4 ± 0.6^{b}	15.2 ± 0.5^{cd}
	16mg/L	1.90 ± 0.67^{ab}	1.25 ± 0.43^{ab}	8.36 ± 0.88^{abc}	6.30 ± 0.12^{ab}	17.4 ± 1.2^{abd}	14.3 ± 0.7^{cd}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis. 3.4, 3.7 and 4.0 represents wines made of different pHs. '16 mg/L' refers to the oxygenated wines.

Table 7.18.2 Flavonol concentrations (mg/L) determined by RP-HPLC for the 2007 Cabernet Sauvignon wines made of different pH treatments

*QUERCITIN-3-RHAMNOSIDE				*QUERCITIN		*MYRICITIN	
Treatment		Before MLF (mg/L)	After MLF (mg/L)	Before MLF (mg/L)	After MLF (mg/L)	Before MLF (mg/L)	After MLF (mg/L)
pH 3.4	control	8.8 ± 0.5^{ab}	8.5 ± 0.5^{ab}	15.4 ± 0.5^{ab}	15.7 ± 1.4^{a}	10.1 ± 4.9^{ab}	16.3 ± 0.5^{c}
	16mg/L	8.7 ± 0.5^{ab}	8.4 ± 0.5^{a}	14.7 ± 0.8^{ab}	13.8 ± 1.0^{ab}	10.8 ± 5.7^{ab}	14.2 ± 1.1^{ac}
pH 3.7	control	8.7 ± 0.5^{ab}	8.1 ± 0.6^{a}	14.0 ± 1.2^{ab}	13.5 ± 1.1^{ab}	12.5 ± 0.4^{abc}	14.2 ± 0.5^{ac}
	16mg/L	8.9 ± 0.6^{ab}	8.3 ± 0.6^{a}	13.1 ± 0.7^{abc}	12.8 ± 2.2^{abc}	13.0 ± 0.8^{ac}	13.4 ± 1.0^{ac}
pH 4.0	control	9.5 ± 0.5^{b}	8.5 ± 0.3^{a}	15.6 ± 1.1^{a}	11.7 ± 6.5^{abc}	13.9 ± 0.5^{ac}	15.8 ± 1.5^{c}
	16mg/L	9.0 ± 0.4^{ab}	8.6 ± 0.4^{ab}	10.8 ± 5.6^{bc}	7.6 ± 8.6^{c}	7.8 ± 7.1^{b}	14.6 ± 1.8^{ac}

*All values displayed in table are the average for three repeats, with the standard deviation expressed after '±'. The letter indicates the significant differences within each analysis. 3.4, 3.7 and 4.0 represents wines made of different pHs. '16 mg/L' refers to the oxygenated wines.