The effect of oxygen on the composition and microbiology of red wine

by

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DECLARATION

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

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SUMMARY

The winemaking process involves different complex chemical and biochemical reactions, which include those of oxygen (O_2). Oxygen can come into contact with the wine through various winemaking procedures and can be used by the winemaker to enhance the quality of red wine. In wine, the main substrates for oxidation are phenolic molecules, which form quinones. These can influence the sensory characteristics of the wine. O_2 can be used in fresh must to remove oxidisable phenolic molecules through a process called hyper-oxidation and can also be added to fermenting must to enhance the fermentation performance of yeast. Controlled O_2 additions during ageing can lead to the wine's colour being increased and the astringency of the wine decreased. This is due to the formation of acetaldehyde from the oxidation of ethanol, which induces the polymerisation of tannin and anthocyanin molecules. The addition of too much O_2 to wine can, however, lead to unwanted over-oxidation, with certain off-odours being formed. It can also enhance the growth of unwanted spoilage microorganisms, such as *Brettanomyces* and acetic acid bacteria. Although research on O_2 in wine was started many years ago, many questions still remain. These include the general effect of O_2 on the sensory and phenolic profile of red wine especially and the microbiology of wine during ageing. An effective way of measuring oxidation, especially in red wine must also be developed.

In the first part of this study, the effects of O_2 and sulfur dioxide (SO_2) additions on a strain of *Brettanomyces bruxellensis* (also known as *Dekkera bruxellensis*) and *Acetobacter pasteurianus* were investigated. Epifluorescence microscopy and plating revealed that the *A. pasteurianus* strain went into a viable but non-culturable state in the wine after prolonged storage under relative anaerobic conditions. This state, however, could be negated with successive increases in culturability by the addition of O_2, as would happen during the transfer of wine when air is introduced. The *A. pasteurianus* strain was also relatively resistant to SO_2, but the *B. bruxellensis* strain was more sensitive to SO_2. A short exposure time to molecular SO_2 drastically decreased the culturability of the *B. bruxellensis* strain, but bound SO_2 had no effect on the culturability or viability of either of the two types of microorganisms. Oxygen addition to the *B. bruxellensis* strain also led to a drastic increase in viability and culturability. It is thus clear that SO_2 and O_2 management in the cellar is of critical importance for the winemaker to produce wines that have not been spoiled by *Brettanomyces* or acetic acid bacteria. This study should contribute to the understanding of the factors responsible for the growth and survival of *Brettanomyces* and acetic acid bacteria in wine, but it should be kept in mind that only one strain of each microorganism was used. This should be expanded in future to include more strains that occur in wine.

The second part of this study investigated the effect of micro-oxygenation on four different South African red wines. It was found that the micro-oxygenation led to an increase in the colour density and SO_2 resistant pigments of the two wines in which

micro-oxygenation was started just after the completion of malolactic fermentation. In one of these wines, a tasting panel preferred the micro-oxygenation treated wines to the control. In the other two red wines, in which the micro-oxygenation was started seven months after the completion of malolactic fermentation, very little colour increase was observed. One of these two wines was also matured in an oak barrel, where the change in phenolic composition was on par with the treated wines. A prolonged period of micro-oxygenation, however, led to this wine obtaining an oxidised, over-aged character. Micro-oxygenation and maturation in an oak barrel also enhanced the survival of acetic acid bacteria and *Brettanomyces* in this wine. Micro-oxygenation can hence be used by the wine producer on young red wines to enhance the quality of the wine, but should be applied with care in older red wines. Future research into micro-oxygenation should focus on whether it can simulate an oak barrel. More research into the effect of micro-oxygenation on the sensory profile of the wine is needed.

As mentioned, the addition of O_2 can lead to oxidative degradation of wine. The brown colour in wine is often used as an indication of oxidation, but oxidative aromas can be perceived before a drastic increase in the brown colour has been observed in red wine.

The third part of this study was to assess the possible use of Fourier Transform Infrared Spectroscopy (FTIR) to measure the progression of oxidation in Pinotage red wines. Three wines were used in this study and clear separation between the control and aerated wines was observed by using Principle Component Analysis (PCA). Sensory analysis of these wines confirmed this observation, with a reduction especially in berry fruit and coffee characters and an increase first in potato skin and then acetaldehyde aroma characters as the oxidation progressed. PCA analysis also revealed that in certain wines the visible spectrum of light did not indicate the progression of oxidation as sensitively as with the use of FTIR. This also correlated with the inability of the panel to observe a drastic colour change. FTIR should be further investigated as a possible means of monitoring oxidation in wine and this study should be expanded to wines made from other cultivars as well.

OPSOMMING

Die wynbereidingsproses behels verskillende komplekse chemiese en biochemiese prosesse insluitende reaksie waarby suurstof (O_2) betrokke is. Suurstof kan deur verskillende wynbereidingprosesse met die wyn in kontak kom en dit kan deur die wynmaker gebruik word om die kwaliteit van rooiwyn te verbeter. In wyn is die hoofsubstrate vir oksidasie fenoliese molekules wat kinone vorm, wat die sensoriese eienskappe van die wyn kan beïnvloed. Suurstof kan in die druiwemos gebruik word om oksideerbare fenoliese komponente te verwyder deur ‘n proses genaamd hiper-oksidasie en suurstof kan ook tydens gisting bygevoeg word om die fermentasievermoë van die gis te verbeter. Gedurende veroudering van wyn kan gekontroleerde O_2-byvoegings tot ‘n kleurtoename en ‘n afname in vrankheid lei. Dit is as gevolg van die vorming van asetaldehied tydens die oksidasie van etanol, wat tot die polimerisasie van tannien- en antosanien-molekules lei. Die byvoeging van te veel O_2 kan egter tot ongewenste oor-oksidasie lei, wat tot die vorming van wangeur aanleiding kan gee. Dit kan ook die ongewenste groei van bederfmikroörganismes soos *Brettanomyces* en asynsuurbakterieë stimuleer. Alhoewel navorsing op O_2 in wyn al baie jare gelede begin is, is daar nog baie vrae wat beantwoord moet word. Dit sluit in die algemeen die effek wat O_2 het op die fenoliese en sensoriese profiele van veral rooiwyn en die mikrobiologiese samestelling van die wyn tydens veroudering. ‘n Effektiewe metode om oksidasie in veral rooiwyn te meet, moet ook nog ontwikkel word.

In die eerste gedeelte van hierdie studie is die effek wat O_2 en swaweldioksied (SO_2) toevoegings op arbitrêr gekose rasse van *Brettanomyces bruxellensis* (ook bekend as *Dekkera bruxellensis*) en *Acetobacter pasteurianus* het, ondersoek. Epifluoressensiemikroskopie en plaattelings het aangedui dat die *A. pasteurianus*-ras in ‘n lewensvatbare, maar nie-kultiveerbare staat ingaan na ‘n verlengde periode van storing onder relatiewe anaerobiese kondisies. Hierdie staat kan egter opgehef word deur die byvoeging van O_2 soos wat sou gebeur tydens die oortap van wyn wanneer lug tot die wyn toegevoeg word. Die *A. pasteurianus*-ras was ook relatief weerstandbiedend teen SO_2, maar die *B. bruxellensis*-ras was egter meer sensitief. ‘n Kort blootstellingstyd aan molekulêre SO_2 het die kultiveerbaarheid van die *B. bruxellensis*-ras drasties verlaag, maar gebonde SO_2 het geen effek op die kultiveerbaarheid of lewensvatbaarheid van beide organismes gehad nie. Suurstoftoevoeging tot die *B. bruxellensis*-ras het ook tot ‘n drastiese toename in kultiveerbaarheid en lewensvatbaarheid gelei. Dit is duidelik dat die korrekte bestuur van O_2 en SO_2 in die kelder van kritiese belang vir die wynmaker is om wyn te produseer wat nie deur *Brettanomyces* of asynsuurbakterieë bederf is nie. Hierdie studie behoort by te dra tot die kennis van die faktore wat die groei en oorlewing van *Brettanomyces* en asynsuurbakterieë beïnvloed. Daar moet egter ingedagte gehou word dat slegs ‘n enkele ras van *B. bruxellensis* en *A. pasterurianus* in hierdie studie

gebruik is; om tot ’n klinkklare gevolgtrekking rakende die mikrobiologiese effek van O_2 en SO_2 te kom, sal ‘n groter diversiteit van bederforganismes in toekomstige studies ingesluit moet word.

Die tweede gedeelte van die studie het gefokus op die effek van mikro-oksigenase op die kwaliteit en samestelling van vier Suid-Afrikaanse rooiwyne. Daar is gevind dat mikro-oksigenase gelei het tot ‘n toename in die kleurdigtheid en SO_2-weerstandbiedende pigmente in die wyne waar die proses begin is net na die voltooing van appelmelksuurgisting. In een van hierdie wyne het ‘n proepaneel die behandelde wyne bo die kontrolewyne verkies. In twee wyne waar die mikro-oksigenase eers sewe maande na die voltooing van appelmelksuurgisting ‘n aanvang geneem het, is baie min toename in kleurdightheid waargeneem. Een van hierdie wyne is in ’n houtvat verouder, waar die fenoliese ontwikkeling basies dieselfde was as dié in die behandelde wyne. Die hout geassosieerde geure was egter ook meer in die vatverouderde wyn as in die mikro-oksigenase behandelde wyne. ‘n Verlengde behandeling van die wyn het egter tot ‘n oorverouderde, geoksideerde aroma in die wyn gelei. Mikro-oksigenase en vatveroudering het ook tot die verlengde oorlewewing van asynsuurbakterieë en *Brettanomyces* in hierdie wyn gelei. Mikro-oksigenase kan dus op ‘n jong rooiwyn deur die wynmaker gebruik word om kwaliteit te verbeter, maar dit moet met sorg in ouer rooiwyne gebruik word. Toekomstige navorsing op mikro-oksigenase behoort daarop gemik te wees om vas te stel of dit ‘n houtvat kan simuleer. Verdere navorsing is ook nodig om die volle omvang van die effek van mikro-oksigenase op die sensoriese eienskappe van die wyn te bepaal.

Soos reeds genoem, kan die toevoeging van O_2 egter ook tot oksidatiewe bederf van wyn lei. Die bruinkleur van wyn word baie keer as ‘n aanduiding van oksidasie gebruik, maar oksidatiewe aromas kan dikwels waargeneem word in rooiwyn voor ‘n drastiese toename in die bruinkleur voorkom.

Die derde gedeelte van hierdie studie het gefokus op die moontlike gebruik van Fourier Transformasie Infrarooispektroskopie (FTIR) om die verloop van oksidasie in Pinotage-rooiwyn te monitor. Drie wyne is in hierdie studie gebruik en duidelike skeiding tussen die geoksideerde en kontrole wyne is waargeneem deur die FTIR spektrum te verwerk met Hoofkomponentanalise (Principle Component Analysis oftewel PCA). Sensoriese analises op hierdie wyne het hierdie waaneming bevestig, naamlik ‘n afname in veral die bessievrug- en koffie-geure en ‘n toename in eers die aartappelskil- en later asetaldehied-aromas met die verloop van oksidasie. PCA-ontledings het ook getoon dat die sigbare spektrum lig nie altyd die verloop van oksidasie so sensitief aangetoon het soos met die gebruik van FTIR nie. Dit het ook met die onvermoë van die paneel om ‘n drastiese kleurverandering in die wyn waar te neem ooreengestem. FTIR moet verder ondersoek word as ‘n moontlike manier om oksidasie in wyn op te monitor en hierdie studie moet uitgebrei word na wyne gemaak van ander kultivars.

This dissertation is dedicated to my family.
Hierdie proefskrif is aan my gesin opgedra.

BIOGRAPHICAL SKETCH

Wessel du Toit was born on 15 December 1973 in Worcester. After matriculating in 1992 he enrolled for a BSc degree at Stellenbosch University, which he obtained in 1996. He obtained an honours degree in Wine Biotechnology and MScAgric (both *cum laude*) in 1997 and 2000, respectively, at Stellenbosch University.

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PREFACE

This dissertation is presented as a compilation of six chapters. Each chapter is introduced separately. Chapters 3, 4 and 5 are written according to the style of the *Journal of Applied Microbiology*, *South African Journal of Enology and Viticulture* and *Journal of Agricultural and Food Chemistry*, respectively. Chapter 3 has been published in the *Journal of Applied Microbiology*.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**
Oxygen in wine

Chapter 3 **Research Results**
The effect of sulfur dioxide and oxygen on the viability and culturability of a strain of *Acetobacter pasteurianus* and a strain of *Brettanomyces bruxellensis* isolated from wine

Chapter 4 **Research Results**
The effect of micro-oxygenation on the phenolic composition, quality and certain wine micro-organisms of different South African red wines

Chapter 5 **Research Results**
Evaluating fourier transform infrared spectroscopy as a means to follow oxidation in Pinotage wines

Chapter 6 **General conclusions and future prospects**

CONTENTS

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 OXYGEN IN WINE

Wine has been made since before biblical times and is today a major source of revenue in countries such as Argentina, Australia, France, Germany, New Zealand, South Africa, Spain and the United States of America. In South Africa, the wine industry contributes to a large part of the agricultural activity in the Western Cape and many families have members who work in the wine industry. Due to the world becoming a global village and the subsequent wider availability of information, more wine producers are emerging. This not only increases the choice for the consumer, but also the competition between producers to place on the market a product with a quality that is better and more consistent than their competitors. Any defect in wine quality should thus be identified rapidly and rectified if possible. During the wine production process oxygen (O_2), can come into contact with the wine through various winemaking operations. These include racking, fining, filtration, centrifugation, barrel ageing and bottling of the wine (Vivas et al., 2003). It is not surprising that the wine producer should want to know the reactions involved when O_2 comes into contact with the wine. These reactions can increase the aroma, taste and colour of certain wines (Vivas and Glories, 1996) but, at excessive O_2 concentrations, can lead to a loss of flavour and colour, with certain off-odours being formed as well (Ribéreau-Gayon et al., 2000; Silva-Ferreira et al., 2002; 2003a; 2003b; Monagas et al., 2005). O_2 concentrations that are too high might also lead to the growth of spoilage microorganisms such as acetic acid bacteria and *Brettanomyces/Dekkera yeasts* (the asexual, non-sporulating form is known as *Brettanomyces* and the sexual sporulating form as *Dekkera*; for simplicity and due to the widespread use of the term ‘Brett character’ by the wine community, the generic name *Brettanomyces* will be used throughout this dissertation) (Du Toit and Pretorius, 2002). The chemical reactions of O_2 in wine are very complex partly due to the complex nature of wine itself, which contains different alcohols, acids, sugars, phenolic molecules and many other types of chemical compounds. The microbiology of wine is complex and the effects of O_2 have not been completely elucidated. New techniques such as micro-oxygenation, which add small amounts of O_2 into red wine, are now available but the effect on the wine quality and composition is not clear (Parish et al., 2000; Nikfardjam and Dykes 2003). At this stage, the progression of oxidation in wine is being measured using the

visible spectrum of light and the oxidation/reduction potential of the wine (Ribéreau-Gayon et al., 2000). New, innovative ways of measuring oxidation in wine should be developed.

1.2 PROJECT AIMS

Although previous studies have been undertaken on the effect of O_2 on wine quality and composition, many questions still remain unanswered. This study focussed on the effect of O_2 on certain aspects of the wine production process. The main aims of the study were:

(i) to elucidate the effect of O_2 additions on the survival, culturability and growth of a strain of *Acetobacter pasteurianus* and a strain of *Brettanomyces bruxellensis* in a relatively anaerobic wine; to ascertain the effect of different concentrations of molecular sulfur dioxide (SO_2) and bound SO_2 on these two micro-organisms; to investigate the period of time within molecular SO_2 inhibits *Brettanomyces;*

(ii) to investigate the effect of micro-oxygenation on the phenolic, microbial and sensory profile of different South African red wines; to investigate the effect of micro-oxygenation on the phenolic profile of red wines of different ages; and

(iii) to elucidate whether Fourier transform infrared spectroscopy (FTIR) can be used as a means to discriminate between Pinotage wines receiving O_2 from those that do not; to investigate the effect of oxidation on the sensory composition of a Pinotage wine; to compare the use of FTIR data against use of the change in colour in the visible spectrum of light as a means of measuring the oxidative development in a Pinotage red wine.

1.3. LITERATURE CITED

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2. OXYGEN IN WINE

2.1 INTRODUCTION

The atmosphere consists of approximately 21% oxygen (O_2). It plays a pivotal role in many metabolic and chemical reactions on earth, thus it is of little surprise that it plays a very important role in the winemaking process. Wine can never be completely protected from it. The general use of sulfur dioxde as an anti-oxidant dates back to the early 18th century and the protection of wine from unwanted oxidative spoilage has been recognised (Ribéreau-Gayon et al., 2000b) Oxygen can influence the composition and quality of wine drastically, either positively or negatively, and will be the focus of this review. This review will also focus on the basic steps involved in oxidation, substrates for oxidation in wine and the evolution of wine constituents during the wine production process when in contact with different concentrations of O_2.

2.2 BASIC REACTIONS OF OXYGEN IN WINE

Oxidation is the process where electron transfer takes place between reductive and oxidative partners. In wine, O_2 is predominantly responsible for this, with it being reduced to certain intermediates and eventually to hydrogen peroxide and then water. Molecular O_2 consists as a diradical and is thus in a triplet ground state. This limits the reactivity of O_2 and it cannot form bonds by accepting electron pairs. However, the addition of a single electron, originating from reduced transitional metal ions can overcome this limitation. This leads to an unpaired electron in the resulting negatively-charged superoxide radical, with a second electron transfer resulting in a peroxide anion (Miller et al., 1990, Danilewicz, 2003). This phenomenon results in O_2 being involved with different reactions in wine.

2.2.1 SUBSTRATES FOR OXIDATION IN WINE

Phenolic molecules originating from grapes can basically be divided into the non-flavonoids and flavonoids. The non-flavonoids, which are hydroxybenzoic and hydroxycinnamic derivatives, originate from the grape juice and are normally the principal phenolic molecule in white wines at concentrations ranging from 50-250 mg/L, depending on the cultivar, winemaking techniques, etc. Examples are the tartaric esters of caffeic acid, p-coutaric acid and furanic acids. These molecules have been shown to be the main phenolic molecules in white wine that did not receive prolonged periods of skin contact, because they occur at higher concentrations in the grape juice (Margalit, 1997, Monagas et al., 2005).

The second main group of grape derived phenolics are the flavonoids. This group of molecules basically consists of two phenolic rings attached to a pyran ring and has a more complex structure than non-flavonoids. In a young wine, they are normally in a more unpolymirised state, but as wine matures they undergo different polymirisation reactions in which O_2 can play an important role. The most important flavonoids in wine are the anthocyanins, flavanols and flavonols. Anthocyanins occur mainly in the skins of red grape cultivars and are responsible for the colour of red wine. In young red wines their concentrations can differ from 250 mg/L to more than 1000 mg/L. Different types occur in wine, depending on the OH and OCH_3 constitution of the B-ring of the molecule, and are esterified with glucose at the C3

position of the molecule. This leads to the occurrence in wine of cyanidin, peonidin, delphinidin, petunidin and malvidin-3-monoglucoside, which can also be acylated with a cinnamic acid derivate (Ribéreau-Gayon et al., 2000b, Monagas et al., 2005). Anthocyanins are amphotheric and pH influences their structure in wine. The positively-charged flavylium ion is mainly responsible for the red colour in a young red wine and is in equilibrium with the chalcone (colourless to yellow), quinodal base (violet), carbinol pseudo-base (colourless) and bisulphate addition product (colourless) (Hrazdina and Franzese, 1974).

The other important group of flavonoids in grapes and wine are the flavanols. These consist of flavan-3-ols (catechins) and flavan-3,4-diols. Different H and OH group substitutions on the C and B rings lead to different stereo-isomers being found viz. (+)-gallocatehin, (-)-epigallocatechin, (+)-catechin and (-)-epicatechin with the latter two occurring up to 200 mg/L in red wine. These molecules can associate through C4/C6 and C4/C8 bonds to form dimers, trimers and oligomers and thus form procyanidins. Dimeric procyanidins can be divided into type A, with interflavan C4/C6 and C4/C8 bonds, with ether bonds between the C5 or C7 carbon units of the terminal unit, and the C2 carbon of the upper unit. Type B dimeric procyanidins are characterised by C4/C6 and C4/C8 interflavan bonds. Trimeric procyanidins are divided into type C, with two type B interflavan bonds and type D, with a type A and a type B bond. These molecules can polymerise further to form so-called grape tannins or condensed tannins, which can be classified according to the mean degree of polymirisation (mDP). These molecules are considered oligomers when the mDP is five to 10 and polymers when larger than ten. The mDP for stems and pips is about 10, but about 30 for skins, indicating that the flavanoid molecules of skins are more polymerised than that of pips and stems (Ribéreau-Gayon et al., 2000b, Herderich and Smith, 2005). Flavan-3,4-diols can also polymerise in a similar fashion (Monagas et al., 2005). These condensed tannins normally exist at 1-3 g/L in red wine and their concentration depends on the cultivar and winemaking techniques such as skin maceration time, ageing procedures, etc. Other flavonoids that also exist in grapes and wine at lower concentrations are flavonols, such as kaempherol, quercitin and myricetin, which normally occur in white wine at 1-3 mg/L and in red wine at about 100 mg/L, as well as flavanonols (mainly taxifolin) (Ribéreau-Gayon et al., 2000b).

Phenolics also originate from the oak when wine comes in contact with it, mainly during ageing. This is the other main source of phenolics. These are mainly oak or hydrolysable tannins that contain a polyhydric alcohol of which the hydroxyl-groups have been esterified with gallic acid or hexahydroxydiphenic acid. Hydrolysable tannins can easily be hydrolysed by acid, base or enzymatically to form gallic or elligic acid. Ellagitannins can make up to 10% of the dry weight of the heartwood of oak. The most common ellagitannins are castalagin (isolated at up to 21 mg/L from oak aged wine) and vescalagin (up to 7 mg/L). Additional ellagitannins identified in oak are roburins A-E and grandinin (Puech et al., 1999). These tannins normally exist in much lower concentrations in wine compared to their concentrations in oak, but this could be due to their involvement in oxidation processes during the ageing of wine that contribute to their breakdown (Vivas and Glories, 1996b).

The other main substrates for oxidation in wine are ascorbic acid, ethanol and tartaric acid. Ascorbic acid occurs naturally in grapes and it can also be added to wine. Tartaric acid normally occurs at 1-6 g/L in grapes and wine and ethanol normally at 9-15% v/v (Boulton et al., 1996).

2.2.2 THE OXIDATION PROCESS

It is clear that phenolic molecules are quantitatively and qualitatively important constituents of wine, especially reds. During oxidation molecular O_2 is reduced in a stepwise manner to $2H_2O_2$ which requires the addition of four electrons, which can be illustrated as $O_2 + e^-, H^+ \rightarrow HO_2^{\cdot} + e^-, H^+ \rightarrow H_2O_2 + e^-, H^+ \rightarrow {}^{\cdot}OH (+H_2O) + e^-, H^+ \rightarrow (2)H_2O$. This leads to the formation of free superoxide ($O_2^{\cdot -}$) and peroxide (O_2^{2-}) radicals, which can be directly reduced by phenolic molecules and are a better oxidants than O_2 (Singleton, 1987; Danilewicz, 2003). Wine phenols, however, exist in either the phenol or phenolate anion form due to its acidic nature. Electron transfer takes place from the phenolate, leaving a free radical of semiquinone, which is further oxidised to the corresponding quinone. The quinone can thus be formed either from phenolate by molecular O_2 or ionic free O_2 (the intermediate between molecular O_2 and H_2O_2), or from the phenol. The semiquinone can partake in further radical reactions, due to the resonance stabilisation of the delocalised electron in the ortho- and para- positions of the aromatic ring (Singleton, 1987, Margalit, 1997).

The constitution of the phenolic molecule will also determine its reduction potential. The phenoxyl radical will more commonly reside on the B ring of catechin than the A ring. The reduction power of a phenolic molecule is determined mainly by ring constituents, with lower reduction potential leading to greater reducing power of the reduced component. Electron donating groups (-OMe, -Me, vicinal -OH groups) lower reduction potential, but electron-withdrawing groups (-CO_2Et, -COMe) have the opposite effect. Methyl gallate is thus a weaker reductant than -(-) epi-gallocatechin, due to its electron-withdrawing carboxylic-ester group. pH also influences this, due to the protonated carboxylic group being electron withdrawing at wine pH levels. This effect is negated at pH 7, where deprotonation takes place and where the reduction potential of methyl gallate becomes comparable to that of -(-)epi-gallocatechin. Malvidin-3-monoglucoside, with two -OMe groups on the B ring, exists mainly in equilibrium in wine between the positively-charged flavylium ion and the carbinol pseudobase, which does not have a charge on the C ring. In the carbinol pseudobase, the electron-withdrawing –OMe groups on the B ring should make it a strong reductant, but a rise in the reduction potential in the flavylium ion is observed. This is due to the positive charge on the C ring of the flavylium ion, which acts as electron withdrawing system, which lowers the sensitivity of anthocyanins in the red form to oxidative degradation (Cheminat and Brouillard,1986, Danilewicz, 2003). The amount of O_2 atoms consumed per mole of phenol in white wine is about 5.5 of that in red wine. This is mainly due to malvidine derivatives which occur in high concentrations in red wine and which are not directly oxidisable with O_2 (Boulton et al., 1996). Quinones, being electrophiles, can also readily react with nucleophilic centras such as phenols, phloroglucinol, SO_3^{2-}, RSH groups, etc. In the same manner, two semiquinone free radicals can also bind by sharing the unpaired electrons in a shared-pair covalent bond. This process, called regenerative polymerisation, leads to the generation of a reoxidisable hydroquinone. This has a lower reduction potential than its original constituents that increases the O_2 capacity of wine (Singleton, 1987). It is thus no surprise that phenolics act as the principal oxidation substrate for O_2 in wine, with especially the vicinal-1,2-dihydroxyphenyl units that readily react with O_2. These are found in abundance in hydrolysable and condensed tannins, for example. The total phenolic content of a wine can thus be an indication of its ability to consume O_2 (Boulton et al., 1996).

Ascorbic acid, which occurs naturally in grapes or is added during the wine production process, can also act as a substrate for oxidation in wine. In the process it reduces quinones back to the corresponding phenol (Peng et al., 1998, Bradshaw, et

al., 2001). It also undergoes two-electron oxidation. The ascorbate radical exists at wine pH mostly in the anion form, which loses a second electron to the quinone with dehydroascorbic acid being formed. The oxidation rate decreases at lower pH levels, becoming very low below pH 2 (Danilewicz, 2003). Ethanol can also be oxidised in wine by the resulting H_2O_2 to form acetaldehyde. This can happen in the presence of SO_2 because ethanol occurs at relatively high concentrations in wine (Boulton et al., 1996). Acetaldehyde plays an important role in the polymirisation of different phenolic molecules during ageing of wine (Dallas et al., 1996).

Iron, occurring normally at a few mg/L in wine, plays an important role in these oxidation reactions. Fe^{3+} is reduced to Fe^{2+} by phenols during oxidation, but oxidised back to Fe^{3+} in the presence of O_2 until all the phenolic substrates have been consumed (Powell and Taylor, 1982). The addition of ferrous sulphate increased the oxidation rate of (+)-catechin, as found by Oszmianski et al. (1996). It is thought that Fe^{3+} acts as a catalyst to overcome the high activation energy in the initial thermodynamically unfavourable electron reduction step of the oxidation process (Miller et al., 1990). Fe^{3+} also catalyses the oxidation of ascorbic acid, with two moles of Fe^{2+} being produced from one mole ascorbic acid (Hsieh and Hsieh, 1997). It has also been observed that Fe^{3+} also plays an important role in the oxidation of tartaric acid in wine. The overall oxidative process and the role of Fe^{3+}/Fe^{2+} in this can be seen in Fig. 2.1. For the oxidation of the phenolic molecule Fe^{3+} ions are thus required and Fe^{2+} for the reduction of H_2O_2, which leads to the oxidation of ethanol to acetaldehyde. Cupric ions can catalyse the aerial oxidation of Fe^{2+}, with the resulting cuprous ions being re-oxidised by O_2. The main anti-oxidative activity of sulfur dioxide in wine is due to the bisulfite ion, which reacts with H_2O_2 to produce sulfuric acid, thereby limiting further oxidation of phenolic molecules or ethanol (Danilewicz, 2003). The use of sulfur dioxide in conjunction with ascorbic acid has been recommended in order to react with the H_2O_2 generated by the oxidation of ascorbic acid (Peng et al., 1998).

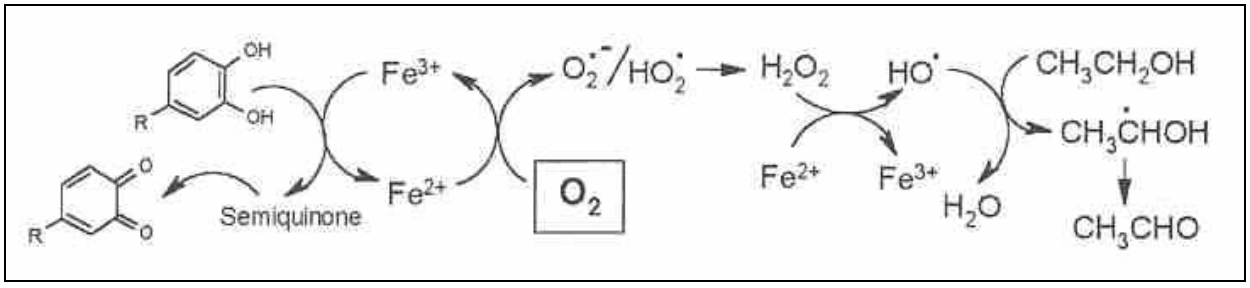

Fig. 2.1 The overall oxidative process in wine and the central role of iron (Danilewicz, 2003).

2.3 FACTORS AFFECTING OXYGEN PICK-UP AND CONSUMPTION IN WINE

2.3.1 WINEMAKING OPERATIONS

When wine is saturated with O_2 it contains about 6-8 mg/L O_2 at cellar temperatures. During the normal wine production process wine comes into contact with air, which can result in different O_2 concentrations dissolving in the wine. Must can be almost saturated with O_2 during the crushing and pressing of fresh grapes (Schneider, 1998). How much O_2 dissolves into the wine during fermentation when a pumping

over is applied, is debatable because the evaporating CO_2 probably sparge O_2 out of the wine (Boulton et al., 1996). Subsequent winemaking operations such as pumping (about 2 mg/L), transfer from tank to tank (up to 6 mg/L), filtration (4-7 mg/L), racking (3-5 mg/L), centrifugation (up to 8 mg/L), bottling (0.5-3 mg/L) and barrel ageing (20-45 mg/L/year) add more O_2 to the wine. During barrel ageing the humidity of the wood and the thickness and the grain of the staves all play a role. Lower humidity, tight grain and thinner staves all allow for more O_2 to permeate into the wine. In very dry wood of 20 mm thickness, it can be up to 0.1 ppm/h, which can lead to oxidation (Vivas et al., 2003). Wine is, however, seldom saturated with O_2, due to insufficient contact or the exclusion of air during the production process. The temperature of the wine also influences the dissolved O_2 saturation level, with higher concentrations dissolving at lower temperatures. Between 5 and 35°C the amount of O_2 necessary to saturate wine drops from 10.5 mg/L to 5.6 mg/L. The rate of quinone formation, however, increases with an increase in temperature, although the kintecs of this reaction is temperature independent (Margalit, 1997, Ribéreau-Gayon et al., 2000b; Vivas de Gaulejac et al., 2001). Oxygen can also be introduced in a controlled manner to wine by a process called micro-oxygenation, which will be discussed later in more detail. The contact of wine with O_2 can be minimised by the use of inert gasses, such as N_2, CO_2 and even argon gas, which can displace the air in a tank or barrel.

The addition of SO_2 can also influence the rate of O_2 consumption. The free sulfur dioxide in wine consists of the molecular, bisulfite and sulfite forms. The O_2 consumption rate in must declines drastically with the addition of SO_2. This is because the fact that SO_2 does not have an anti-oxidative effect in must, but rather inhibits oxidation enzymes. In wine, however, chemical oxidation occurs and mainly the sulfite form of SO_2 can react with O_2, but it is still slow under winemaking conditions such as low pH and high ethanol levels. The ascorbate-oxygen reaction is almost 1700 times faster than that between SO_2 and O_2. First order kinetics suggests that 4 mg/L SO_2 reacts with 1 mg/L O_2. The molecular form of SO_2 can also react with H_2O_2 that is formed from the oxidation of phenolic molecules. There seem to be surprisingly few kinetic studies on the interaction between O_2, H_2O_2 and SO_2 (Boulton et al., 1996; Ribéreau-Gayon, 2000a).

2.3.2 pH

Wine phenols exist in either the phenol or phenolate anion form. The negative charge of the phenolate anion is delocalised via the benzene ring from the oxygen atom to the ortho and para positions, lending 8 kcal resonans stabilisation to the phenolate anion compared to the phenol. However, at wine pH (pH 3-4), very little of the phenolic molecules, with a pK_a value of 9-10, are in the phenolate form, but the major influence pH has on this is clear, with 10 times more phenolate existing at pH 4 than 3. During oxidation removal of the phenolate anion will lead to its replacement due to equilibrium. Oxidation, when an electron is removed, is much easier from the phenolate anion than from the protonated phenol.

Phenolic molecules also differ in their susceptibility to high pH, with caffeic acid and gallic acid becoming less stable towards degradation at high pH, with (-)-epicatechin and (+)-catechin being much more resistant. The structures of the latter two molecules are not planar and the π electrons of the two benzene rings cannot interact with one another due to conjugation. The spatial arrangements of the OH groups and the π electrons influence the extent of π orbital overlap and consequently its susceptibility to chemical change. Care should therefore be taken especially when handling white wines high in pH because these are more susceptible to oxidation,

containing caffeic acid derivates as the main phenolic molecules (Cilliers and Singleton, 1990b, Cilliers and Singleton, 1990c, Boulton et al., 1996, Friedman and Jürgens, 2000). Cilliers and Singleton (1989) found that the amount of phenol consumed per phenol unit at wine pH was about 1.4 to 18 times higher than in alkaline conditions. The rate of the non-enzymatic auto-oxidation of caffeic acid is also enhanced by increasing pH and temperature. Although the oxidation of ascorbic acid by O_2, which is catalysed by Fe^{3+}, increases with a pH increase, the reduction of Fe^{3+} by ascorbic acid decreases, with the reaction ceasing at neutral pH (Danilewicz, 2003). Wine thus consumes much more O_2 under slow, acidic conditions than under fast alkaline conditions.

2.3.3 PHENOLIC CONCENTRATION AND COMPOSITION

The phenolic concentration of wine is an indication of its capacity for O_2, with higher phenol wines being able to accommodate higher concentrations of O_2. The removal of phenolic compounds from wine with fining reduces the wines capability to react with O_2. It is calculated that a young, full-bodied red wine can consume 2.4 g or more O_2 under slow acidic conditions (as would happen during barrel ageing when O_2 is added over a long period of time), which is more than its own volume in O_2 or 5-10 L of air (Singleton, 1987, Boulton et al., 1996). Winemaking practices that would lead to higher phenolic concentrations, such as skin contact, hard pressing and barrel ageing of wine should lead to a higher capacity of this wine for O_2.

Numerous studies have reported on the autocatalytic effect of forced oxidation in a wine based media, with O_2 consumption increasing when two different types of phenolic molecules are involved. The process of regenerative polymerisation, where slow oxidation leads to previously non-oxidisable moieties being incorporated into a re-oxidisable hydroquinone, also leads to the increase of the oxidisable substrates of a wine. However, this seems to be a relatively slow process. The resulting dimeric product has a lower redox potential than its original constituents and thus buffer the latter against oxidation. At lower pH levels the lower concentrations of phenolate anions will all have time to participate in the regenerative polymerisation reaction to form re-oxidisable hydroquinones with quinones. At high pH levels when O_2 is added at a fast rate, it is not long before no phenols remain to react. This is reflected in the fact that when forced oxidation of wine takes place, browning of the wine follows an autocatalytic pattern with an initial lag phase. This is due to the dimeric product having a lower redox potential, as mentioned earlier, with two dimeric oxidised semi-quinones reacting with each other to form a tetramer, etc. This process can take place until the molecule becomes too large and precipitates (Singleton, 1987, Boulton et al., 1996). Cilliers and Singleton (1990a) reported that one molecule of caffeic acid consumed 3.4 atoms of O, which increased to 4.9, 5.5 and 8.5 when phloroglucinol, cysteine and glutathione were added respectively. The association between catechin and caffeic acid and the addition of cysteine and glutathione increased this further to 13.2 and 19.2 after 9 h. Both cysteine and glutathione act in generating a re-oxidisable product by reducing the quinone back to a caffeic acid and by substituting in the quinone to regenerate the hydroquinone form of 2-S-cysteinyl caffeic acid or 2-S-glutathionyl-caftaric acid (Bassil et al., 2005). Depletion of glutathione and cysteine leads to quinone formation and browning. The addition of ferrous sulfate and Fe^{2+} to a model wine solution and wine increased the oxidation of (+)-catechin by O_2 and the rate of O_2 consumption (Vivas et al., 1993, Oszmianski et al., 1996). Ellagic tannins have a much higher capacity for O_2 consumption than condensed tannins. The rate of O_2 consumption is also faster in the case of ellagitannins, due to more vicinal ortho OH groups. When ellagic tannins and

condensed tannins are added together, the O_2 consumption rate dramatically increases initially, possibly indicating a competition for the O_2 (Vivas and Glories, 1996b).

2.3.4 DESIRABLE LEVELS OF O_2 IN DIFFERENT WINES

Boulton et al. (1996) reported on different levels of O_2 required for certain wine styles. In white wine, about 10 saturations lead to the wine becoming oxidised, but it is well known that even lower additions may lead to a reduction in the fruitiness of wine. Ten saturations led to the minimum concentration necessary to obtain a standard sherry. Red wines differ a lot in their capacity, but normally improve with up to 10 saturations (60 mL/L), with others showing improvement even up to 25 saturations (150 mL/L). A value of 10 saturations (or about 60-70 mg/L) is in line with the total amount of O_2 that a red wine can receive in a year because a few rackings and other winemaking procedures could contribute about 20 mg/L and the barrel ageing regime about 40 mg/L O_2 per year (Vivas et al., 1999a; 1999b; 2003).

2.4 OXYGEN ADDITION IN MUST, ENZYMATIC OXIDATION AND HYPEROXIDATION

During the crushing, pressing and other processing steps, O_2 comes into contact with the grape juice. This leads to enzymatic oxidation of phenolic molecules and for this to occur the oxidation enzyme, O_2 and the phenolic substrate must be present. The polyphenol-opixdases of healthy grapes are known as tyrosinase, cresolase and catechol oxidase, with laccase occurring in *Botrytis* infected grapes. The latter enzyme is considered a more dangerous enzyme by the winemaker because it is more resistant to SO_2 and has a wider substrate oxidation spectrum. It is not inhibited to the same degree by its oxidation products, as is tyrosinase. Laccase is more active at low pH must values and alcohol levels in wine than tyrosinase. The rate of browning and O_2 consumption is, however, not much different in juices prepared from healthy or rotten grapes (Ribéreau-Gayon et al., 2000a, Schneider, 1998).

The main substrates for these oxidization enzymes are the cinnamic acid derivatives, with caftaric acid and coutaric acid occurring at an average of 106 and 10 mg/L respectively in protected white juices (Singleton et al. 1984, Cheynier et al., 1989b). Caftaric acid concentration can also differ dramatically between cultivars, ranging from 40 to 400 mg/L (Singleton et al., 1986). These derivatives occur mainly in the liquid part of the grapes, with flavanoid based phenolics (mainly catechin and condensed tannins) being dominant in the skins, stems and pips. When juice and wine contain higher concentrations of these flavanoids, they also become more susceptible to oxidation and subsequent browning (Schneider, 1998). During oxidation, caftaric acid is oxidised to its corresponding quinone by tyrosinase. Glutathione, with a mercapto group, has a nucleophilic centre to substitute into the electrophilic ring of the quinone, leading to regeneration of the vicinal dihydroxy ring of the caffeic acid (Singleton et al., 1985). The product, 2-S-glutathionyl-caftaric acid or Grape Reaction Product (GRP) is no longer a substrate for further oxidation by tyrosinase. Laccase can, however, due to its wider substrate specificity, further oxidise the GRP, with a second addition of glutathione, if available, leading to formation of GRP2. It does not seem as if laccase can further oxidise the GRP2 under winemaking conditions (Singleton et al., 1985, Cheynier et al., 1986, Cheynier and Van Hulst 1988, Boulton et al., 1996). Depletion of glutathione and other nucleophiles, which can serve the same role, leads to browning and the use of cysteine to protect against oxidation should be investigated further. The glutathione

to caffeic acid ratio should give an indication of the susceptibility of a certain cultivar to oxidation. This ranges from 1.3 to 12.7 and 0.6 to 10.5 in berries and musts, respectively. Musts can also be divided into three groups according to their hydroxycinnamic acid content, with higher concentrations leading to browner colour. A hydroxycinnamic acid to glutathione ratio of 0.9 to 2.2, which leads to lightly coloured oxidised must, causes to the rapid formation of GRP and high levels of GRP, due to the availability of sufficient glutathione. In medium coloured juices (with a ratio of 1.1 to 3.6) GRP is formed with caftaric acid and GRP-o-quinone reacting further when glutathione exhaustion has taken place. Small amounts of GRP2 are also formed here. A ratio of 3.8 to 5.9 lead to dark coloured musts, which are due to glutathione being depleted by the high caftaric acid o-quinone concentration before GRP2 can be formed. This could explain the difference in sensitivity of different musts to oxidation. No correlation between sugar concentration and the ratio could be found (Singleton et al., 1985, Cheynier et al., 1989a, Boulton et al., 1996, Margalit, 1997). After depletion of the glutathione, the caftaric acid quinone can oxidise GRP and flavanols, and be reduced back to caftaric acid. It can also polymirise with caftaric acid to regenerate a re-oxidisable phenol. The kinetics of degradation differ between flavanoids, with procyanidin B2 disappearing relatively quickly compared to catechin, but the rate of oxidation between laccase and catechol oxidase did not differ significantly (Oszmanski et al. 1985, Schneider, 1998). Cheynier et al. (1988) found caftaric acid, catechin, epicatechin and epicatechin gallate undergo 70, 50, 46 and 46% decrease after 2 h of oxidation by grape polyphenoloxidase. When the flavanoids were oxidised with caffeic acid their oxidation rate increased, but the condensation reaction of catechin with caftaric acid was still slower than when trapped by glutathione. Catechin also increases the oxidation rate of procyanidin dimers and GRP, but not to the same degree as caftaric acid. Caftaric acid is thus enzymatically oxidised to its quinone with the consumption of half an atom of O. Catechin is either being oxidised to its corresponding quinone in the same manner, with the consumption of one O atom or by coupled oxidation by reducing the caftaric acid quinone. The caftaric acid o-quinone with catechin or the catechin o-quinone with caftaric acid can then form a condensation product with a lower redox potential than its monomer constituents and can hence be further oxidised (Cheynier et al., 1988). In a subsequent study, Cheynier and Ricardo da Silva (1991a), however, found polyphenoloxidase did not to degrade procyanidins alone but, in the presence of caftaric acid, the oxidative condensation of the galloylated procyanidins proceeded more quickly than the oxidative condensation of non-galloylated procyanidins. This degradation was also influenced by pH, with the nucleophilic addition of a phenolic ring on a quinone occurring between (+)-catechin and its oxidation products occurring at high pH, and semi-quinone radical coupling occurring at low pH. The colour of these products differed, being colourless at a pH lower than 4 and yellow at a high pH. Their interflavanic bonds also differ from the original monomer (Guyot et al., 1995, Monagas et al., 2005).

During red winemaking when an oxidative environment may prevail and under low glutathione and high hydroxycinnamic acid concentrations anthocyanins can react with caftaric acid quinones, leading to oxidation of the latter through coupled oxidation or condensation reactions. The latter reaction takes place when the nucleophilic C6 or C8 carbon forms a condensation reaction with the electrophilic quinone. O-Diphenolic anthocyanins, like delphinidin and petunidin-3-glucoside usually react rapidly, but malvidin-3-glucoside reacts more slowly due to its condensation with quinones (Monagas et al., 2005).

These phenomena of regenerative polymirisation contribute to the ability of the must to accommodate higher concentrations of O_2 than expected, but the O_2

accommodation of different musts can differ drastically, ranging from 0.5 to 5 mg/L/min. The consumption of O_2 by tyronase is very fast, ranging from 30 to 200 mg/L, with 10-15 mg/L being taken up during whole bunch crushing. The uptake is also faster initially, but decreases as the phenolic substrate is depleted, with laccase, if present, increasing the total uptake further (Cheynier et al., 1993, Schneider, 1998, Ribéreau-Gayon et al., 2000a).

The winemaker must apply certain winemaking techniques to prevent oxidation of must during the production process. Oxygen can be excluded by using inert gasses such as N_2 or CO_2 in presses, pipes and tanks. Oxidation enzymes can also be inhibited by the addition of SO_2. Up to 90% decrease in the activity of tyrosinase has been observed with the addition of 50 mg/L SO_2, but higher dosages are necessary to effectively inhibit laccase. SO_2 also reduces caftaric acid quinone and enhances the solubility of phenolic molecules. Settling of juice decreases the activity of tyrosinasebecause it is largely associated with the solid parts of the grape berry. Bentonite fining has also been found to do this, with 100g/hL leading to a 30% loss in activity, but it also removes glutathione. Heating of the must to 45 and 65ºC will destroy tyrosinase and laccase respectively (Schneider, 1998, Ribéreau-Gayon et al., 2000a).

Another strategy to prevent oxidation is to limit the phenolic substrate for oxidation, especially the flavanoid content, with soft pressing, no skin contact and removal of stems. A process called hyperoxidation, where large quantities of O_2 are added to the must, can also achieve this (Schneider, 1998). It leads to the oxidation of phenolic molecules, which settle and the juice can then be removed from the precipitate by racking with no SO_2 added to the must at crushing. To achieve this, O_2 is pumped either in line, while the juice is circulated in the same tank, pumped from tank to tank, added with a diffuser in the juice or used instead of N_2 when using flotation. Juice that did not receive any skin contact can thus be treated with one saturation, but up to three saturations are necessary for those that did receive skin contact to remove sufficient flavanoid molecules. It is imperative that the subsequent clarification before fermentation starts is done efficiently because the precipitate can dissolve again in alcohol. The reductive conditions during alcoholic fermentation and adsorption to yeast cells reduce the brown colour further (Schneider, 1991; Schneider, 1998).

It is unkown whether must hyperoxidation contribute to quality of wine. It is clear that bitterness and astringency decrease markedly with the O_2 addition and this difference becomes greater during ageing of the wine. These wines are obviously also less susceptible to unwanted browning. In different studies the aromas of Chardonnay, Riesling, Faberrebe and Parellada were considered more intense in the treated wines. This was more pronounced in the juices that received skin contact before the treatment. This could be due to an increase in fatty acids and esters. Other studies, however, showed a decrease in aroma quality, with more vegetative aromas being formed, possibly due to C6-aldehydes and alcohols being formed under these conditions. The addition of even H_2O_2 to wine did not decrease the methoxypyrazine level of white wine (Singleton et al., 1980, Cheynier et al., 1991b, Marais, 1998, Schneider, 1998). Non-volatile flavonoids can, however, indirectly influence the aroma of wine, by yielding acetaldehyde from ethanol during coupled oxidation (Schneider, 1998).

Phenolic molecules can also be removed with fining agents such as PVPP, gelatine and activated charcoal. Charcoal treated juice made from very rotten Sauvignon blanc grapes had a less intense brown colour than the control (Du Toit, 2003).

2.5 OXYGEN ADDITION DURING ALCOHOLIC FERMENTATION AND MALOLACTIC FERMENTATION

Completion of the alcoholic fermentation is a crucial step in the winemaking process. During this process yeast transforms sugar into alcohol, CO_2 and energy, and produces flavour compounds such as fatty acids, esters, higher alcohols etc. If this fermentation is not completed successfully, spoilage microorganisms can use residual sugar to spoil it. Numerous causes for stuck/sluggish alcoholic fermentation have been identified, which include high sugar, low nitrogen, thiamin depletion, excessive clarification, pesticides and a lack of O_2 (Bisson,1999). Yeast cells need O_2 to produce sterols and unsaturated fatty acids that play a key role in the fluidity and activity of membrane associated enzymes, which influence ethanol tolerance, fermentative capability and viability of yeast (Valero et al., 2001). A dosage of 5 mg/L O_2 is optimal to achieve this when added at the end of the cell growth phase but, when 1 mg/L was added, the relative increase in CO_2 production ranged from 10 to 41% between strains. By combining this addition with ammonia addition at the halfway mark of fermentation, it reduced the fermentation with up to 50% in problem fermentations. The maximum O_2 consumption rate was also found to take place at this time (Sablayrolles et al., 1996, Julien et al., 2000). Yeast also assimilates more nitrogen when it is supplied with O_2, but strain differences in fermentation efficiency exist in the absence of O_2. When added before the halfway mark of fermentation, O_2 is assumed to be used in mitochondrial development, ring cleavage of proline and respiration, despite the high sugar content of must. Salmon et al. (1998), however, found that the superfluous O_2 consumption rate during the growth phase of yeast was probably due to mitochondrial alternative respiratory pathways and that O_2 dependent ergosterol biosynthesis accounted for less than 15% of the total O_2 consumption at the beginning of the stationary phase. Blateryon et al. (2003) found that the addition of 5 mg/L O_2 to fermenting must did not affect the sensory characteristics of the wine compared to the control, but the addition of an excess (50 mg/L) did decrease the quality, with an increase in brown colour. In the absence of O_2 medium chain fatty acids, especially hexanoic, octanoic and decanoic acids accumulate in the yeast and can be secreted into the wine, contributing to sluggish/stuck fermentations (Bardi et al., 1999). Oxygen has also been found to be depleted from different musts within 2.75 to 4.25 h from the start of fermentation. Its addition may in future serve as a means of proline utilisation by yeast under fermentative conditions (Poole et al., 2002). Buescher et al. (2001) were able to induce *S. cerevisiae* strain L2226 to produce up to 20.96% alcohol when the yeast fermentation was supplied with O_2 during the first 48 h and nutrients were added together at the start of fermentation. Only 17.89% alcohol was produced when no O_2 was added. Non-*Saccharomyces* yeast strains can also contribute to the complexity of the wine, by producing certain metabolites. *Torulaspora delbrueckii* and *Kluyveromyces thermotolerans* survived longer during fermentation with *S. cerevisiae* in O_2 rich must (Holm Hansen et al., 2001). The addition of O_2 could be utilised by the winemaker to ensure a complete fermentation, especially in countries such as South Africa where grapes have relatively high sugar concentrations. The addition of O_2 to the must also leads to the production of higher concentrations of esters and higher alcohols by *S. cerevisiae* and *S. capensis* (Valero et al., 2002). Oxygen can be supplied in large scale fermentations by sparging air through the tank. This will also help to keep the yeast in suspension.

Certain wines, especially certain white varieties from the Loire valley, Burgundy and Champagne in France and other wine producing countries, are often matured on the yeast lees after fermentation. During this period of time the

inactivated yeast undergoes a process called autolysis, which is defined as the hydrolysis of intracellular endohydrolases activated upon cell death. During autolysis the yeast releases different nitrogenous compounds, lipids and polysaccharides into the wine. This process is believed to contribute to the fuller mouth feel and aroma of these wines while absorbing volatile thiols and anthocyanins. Autolysis can also contribute to a wine's protein and tartaric stability by releasing mannoproteins. O_2 can be introduced during this period by opening the barrels, transfer of the wine and through a process called battonage, where the lees is stirred periodically in order to mix it uniformly (Fornairon-Bonnefond et al., 2003). It has been observed that yeast lees has a capacity to consume this O_2, with rates ranging from 3 to 11 $\mu g\ O_2\ h^{-1}\ 10^{-9}$ from the second month the sixth month of lees contact. Specific uptake rates also differ between strains, with 100, 50, 42 and 11% of initial O_2 concentrations remaining in white wines for strains Su6, Uvaferm, L2898 and VL1, respectively after 3000 h of yeast lees contact. Production of biomass peroxydes is directly linked with O_2 consumption by yeast lees, with Cu^{2+} additions, which serves as auto-oxidation catalysts, increasing this rate. Cell viability of yeast lees decreased faster in the presence of O_2, but it did not affect the release of amino acids. These reactions lead to ergosterol levels being reduced in the yeast cell walls, with the formation of 9(11)-dehydro-ergosterol, 5α,6α-epoxy(22E)-ergosta-8,22-diene-3β,7α-diol or ergosterol epidioxide (Salmon et al., 2000, Fornairon-Bonnefond and Salmon, 2003). Yeast has a stronger capacity for absorbing O_2 than polyphenols, in the same order as 9 g/L of polyphenols, which is higher than the polyphenol concentrations normally found in wine. However, yeast lees and polyphenols in combination had a much lower capacity of O_2 consumption than the theoretical sum of this capacity when tested alone. This is due to the capacity of the yeast lees being reduced drastically after contact with polyphenols. This is probably because of a collapse of cytoplasmic intermembrane space, which lowers the accessibility and reactivity of O_2 towards the sterols and unsaturated fatty acids of the membranes. The initial slight decrease and later increase in the capacity of the polyphenols could be due to adsorption on the lees yeast with gradual release from the lees. The adsorption by the lees of polyphenols follows biphasic kinetics, with no preference for low or high polymeric size tannins, although epigallocatechin units were adsorbed more by the yeast (Salmon et al., 2002, Mazauric and Salmon, 2005). Therefore yeast lees plays a very important role in the reduction/oxidative potential of wine.

During red wine production the effective mixture of skins with the must is required for extraction of anthocyanin and tannins from the skins. Pre-fermentative O_2 addition to red must during skin contact resulted in lower concentrations of red pigments, anthocyanins, caftaric acid and total phenols. The concentrations of total tannins and anthocyanins after six months' storage were 1220 and192 mg/L, respectively, in the control compared to 679 and 150 mg/L in the must to which most O_2 was added. Wines made from the treated musts had more aged characteristics, such as more polymirised colour and a higher colour hue (Castellari et al., 1998). Pumping over in comparison to punch down and rotor tanks may also lead to lower extraction of polyphenols (Marais, 2003), although this could be due purely to this being a softer extraction technique, as Italian researchers did not find any significant difference in polyphenol concentrations after O_2 addition during fermentation. It's not known how much O_2 is taken up by the yeast, reacts with polyphenols or simply evaporates with the CO_2 during a red wine fermentation. More research on this is clearly necessary.

The addition of O_2 during fermentation has also been found to affect the subsequent malolactic fermentation. Aeration led to a hundred fold lower level of lactic acid bacteria than in the anaerobic control after alcohol fermentation, but the

former lactic acid bacteria numbers increased more rapidly to 10^8 cfu/mL, compared to 10^7 cfu/mL in the anaerobic treatment. In the aerated treatment where no temperature control was induced during alcoholic fermentation malic acid was consumed the fastest. This could be ascribed to differences in alcohol levels after fermentation (12 and 13% for the aerobic and anaerobic treatments, respectively) that led to different cell counts (Reguant et al., 2005). Such a significant difference in alcohol levels is uncommon because high concentrations of O_2 should be sparged off during fermentation by the resulting CO_2 release. Oxygen during malolactic fermentation can also influence the sensory characteristics of wine, especially in Chardonnay where diacetyl contributes to the typical buttery aroma of these wines. Oxygen enhances the conversion of α-acetolactate to diacetyl, with 12 mg/L being produced under semi-aerobic conditions compared to 2 mg/L in anaerobic conditions, however, this was consumed again by the bacteria. Cell growth, malic and citric acid degradation did, however, differ significantly between the semi-aerobic and anaerobic conditions. Limited exposure to air during malolactic fermentation could thus enhance diacetyl production, but this should be followed by SO_2 addition and filtration to avoid subsequent consumption by yeast and lactic acid bacteria (Nielsen and Richelieu, 2000; Bartowsky and Henschke, 2004). The general effect of O_2 on lactic acid bacteria during commercial winemaking is, however, not well understood and should be investigated further.

2.6 EFFECT OF OXYGEN DURING AGEING OF WINE

2.6.1 EFFECT OF OXYGEN ON WHITE WINE COLOUR

The colour of white wine is an important quality parameter. The colour of a young white wine has normally a slight yellow or greenish tint, with white wine that has been aged in barrels achieving a deeper yellow. A brown colour is normally unwanted because this indicates oxidation in white table wine and is normally measured at 420nm in white wine. As previously discussed, brown colouration can be induced by enzymatic oxidation, but these enzymes are normally not very active in wine because their precipitation during alcoholic fermentation occurs and alcohol inhibition of these enzymes takes place in wine. Hence, browning in white wine is a chemical process that is slower than enzymatic-induced oxidation. Browning in white wine can be due to three mechanisms. The first is the oxidation of phenolic molecules to their corresponding quinones in varying degrees of polymirisation and it is also influenced by the copper and iron concentrations. It produces a yellow-brown colouration. The second mechanism is the oxidation of tartaric acid to glyoxylic acid which leads to the condensation of phenolic molecules due to the glyoxylic acid acting as a bridge between phenolic molecules. Varying degrees of polymirisation of the latter can also contribute to the yellow-brown spectrum. Acetaldehyde, produced during coupled oxidation or fermentation can also enhance the yellow colour by inducing the condensation of phenolic molecules (Es-Safi et al., 1999c, Lopez-Toledano et al., 2004, Monagas et al., 2005).

The chemical mechanisms involved in the oxidation of phenolic molecules to quinones have been discussed earlier, and only those involved in the oxidation of white wine *per se* will be mentioned. The main phenolic molecules occurring in white wine that did not receive extensive skin contact or was not aged in oak barrels are the hydroxycinnamic acid derivatives. Caftaric, coutaric, ferulic and caffeic acid do not; however, seem to play a major role in the browning of white wine because little correlation could be found between their concentration in white wine and

susceptibility to browning. A good correlation, however, exists between flavanols and browning sensitivity, especially with (+)-catechin, (-)-epicatechin and dimeric procyanidins B1-B4. The hydroxycinnamic acids may, however, contribute to the browning by being involved in coupled oxidation reactions with these flavanols, as discussed earlier (Simpson, 1982, Fernández-Zurbano et al., 1995). Flavanols also differ in their sensitivity to oxidative degradation. Jorgensen et al. (2004) found that skin procyanidins degraded faster than those from seeds, with flavan-3-ol monomers slowing the degradation of seed procyanidins. After 21 h of oxidation under mildly basic conditions, skin procyanidins, seed procyanidins alone, and seed procyanidins with the added monomers declined to 11.8%, 25.1% and 28.2% respectively. This was also reflected in the rate of degradation of these three substrates. The degradation rates of individual subunits also differ with (-)-epigallocatechin being degraded faster than (-)-epicatechin, the former constituting the major part of skin procyanidins, which explains the faster degradation of the skin fraction. It is clear that winemaking techniques which influence the procyanidin concentration in wine would also affect its sensitivity to browning. Pressing method, skin contact, skin contact time, pasteurization of the juice and cultivar affect the procyanidin concentration of grape juice, with Elvira and Chardonnay found to have high concentrations of catechins and Seyval and Niagara to have high procyanidin concentrations, especially of B1 and B2 (Fuleki and Ricardo-da-Silva, 2003).

The second pathway of browning, relatively recently described, is that induced by the oxidation of tartaric acid, which yields glyoxylic acid. This acts as a bridging mechanism between flavanol molecules. The oxidation takes place in the presence of catechin and either Fe^{3+} or Cu^{2+}. The resulting colourless or yellow products absorb at a maximum between 440 and 460 nm. In these reactions (+)-catechin reacts with glyoxylic acid to produce a (+)-catechin/glyoxylic acid adduct, which reacts with a further (+)-catechin molecule to form a carboxymethine-linked (+)-catechin dimer. This carboxymethine bridge can form between C8-C8, C8-C6 or C6-C6 of the (+)-catechin units. Dehydration of the dimers forms xanthenes, which can undergo oxidation to form xanthylium salts. These salts have a yellow colour and a maximum absorption at 460 and 440 nm for the esterified and non-esterified salts, respectively. Copper and Fe^{3+} catalyse this reaction. Copper enhances the condensation reaction between (+)-catechin and glyoxylic acid and/or the reaction of a (+)-catechin with the (+)-catechin/glyoxylic acid adduct. However, the acid moiety of glyoxylic acid seems crucial for this reaction because Cu^{2+} did not enhance the acetaldehyde induced addition when it was used instead of glyoxylic acid. Iron probably exerts the same type of mechanism. Tartaric acid can be added to wine as an acid supplement in many wine countries and it can contain trace amounts of glyoxylic acid, which can influence the colour of white wine in the presence of especially higher concentrations of Cu^{2+} (Es-Safi et al., 2000, Clark et al., 2003 and Monagas et al., 2005).

The oxidation of a phenolic molecule produces H_2O_2, which in turn oxidises ethanol to form acetaldehyde. This can also be produced by yeast during alcoholic fermentation. Acetaldehyde can form ethyl bridges between two (+)-catechin molecules, with carboxymethine-bridged dimers being formed due to the oxidation of tartaric acid, as mentioned earlier. This reaction takes place faster in the case of (-)-epicatechin than with (+)-catechin when each is added alone with acetaldehyde. The degradation product of (+)-catechin has a more reddish hue than that of (-)-epicatechin. When the two favanols occur together, (-)-epicatechin also disappears faster than (+)-catechin, with both homo- and heterogeneous ethyl-linked oligomers being formed. The reaction is also faster at lower pH levels, due to more acetaldehyde carbocation, but the faster reaction of (-)-epicatechin compared to (+)-catechin is enhanced by a pH increase (Es-Safi et al., 1999b, Lopez-Toledano et al.,

2002a). Glyoxylic acid or acetaldehyde can be protonated to form an electrophilic C+ carbocation (R1), which undergoes a nucleophilic attack by the C6 or C8 of (+)-catechin to form the corresponding benzylic alcohol (Fig 2.2). Subsequent protonation, with the loss of H_2O, leads to an electrophilic benzylic carbocation being formed, which can undergo nucleophilic attack from (+)-catechin to form a dimer. This leads to C6-C6, C6-C8 or C8-C8 interactions between two (+)-catechin molecules, with the latter forming at the highest concentrations and the C6-C6 forming at very low concentrations, probably due to steric hindrance. Drinkine et al. (2005) investigated the effect of adding glyoxylic acid and acetaldehyde with (+)-catechin. They found that glyoxylic acid alone led to a three times faster disappearance of (+)-catechin than acetaldehyde alone ($t_{1/2}$= 2.3+/-0.2 h for glyoxylic acid and $t_{1/2}$= 6.7+/-0.2 h for acetaldehyde). This was due to structural differences, with glyoxylic acid having both an aldehyde and carboxylic acid group, which has some conjugation associated with its structure, leading to higher aldehyde polarisability. Acetaldehyde, with aldehyde and methyl functional groups, has no conjugation, which thus favours the faster reactions R1 and R2 in glyoxylic acid. However, the rate of the dimer formation was similar, implying that the reaction rate of R3 and R4 is faster with acetaldehyde. For RI_1(G) as indicated in Fig. 2.2, the intramolecular hydrogen bonds between the carboxyl functional group and the OH group of the benzylic alcohol may not favour its protonation and dehydration. The same applies to the carboxylic group and the OH group of C7, which may hinder nucleophilic addition of the second (+)-catechin. When mixed together, ethyl-bridged dimers appeared and disappeared sooner than carboxymethine-bridged dimers. Polymirisation proceeded further up to tetramer units, with polymers containing both ethyl and carboxymethine-bridges (Saucier et al., 1997, Drinkine et al., 2005). These reactions were, however, executed in the absence of metal catalysts, which would have induced the formation of xanthylium salts. The brown colour also increased linearly with an increase in polymirisation (Lopez-Toledano et al., 2004). These interactions between acetaldehyde and glyoxylic acid might influence the colour, state of polymirisation of flavanoids and ultimately the taste of wine and they should be investigated further.

An interesting observation made by Bonilla et al. (2001) is that yeast reduces the brown colour of oxidised white wine. The brown colour of an oxidised white wine, was reduced with an increase of a higher yeast dosage (ranging from 1-5 g/L), which was compatible to PVPP or activated charcoal fining. HPLC analysis revealed that vanillic, syringic, coutaric acids and especially flavan-3-ol derivatives were significantly reduced by the yeast addition. Yeast prevents the degradation of (-)-epicatechin and (+)-catechin, exhibiting a stronger inhibition of the degradation of the latter compound. This explains the prevention of brown colouration of sherry with flor yeast. The yeast prevents flavanol degradation rather than protecting the wine from air by growing on its surface. An increase in polymirisation leads to the resulting oxidative degradation product absorbing more in the brown spectrum. Yeast also seems to prefer association with the browner, more polymirised flavanols, compared to monomeric flavanols. This is reflected in 7.7, 36.4, 53.7 and 64.6% of the (+)-catechin, dimer, trimer and oligomers respectively being removed by the yeast in a model solution. The yeast thus seems to have a preference for these compounds, which absorb in the yellow/brown spectrum, with the cell walls being the active absorbing area. These reactions proceed very slowly under the acidic conditions in wine and probably play more of a role in browning if the wine contains higher levels of flavanols, and after it has been racked from the yeast lees (Bonilla et al., 2001, Lopez-Toledano et al., 2002b, Lopez-Toledano et al., 2004). The addition of yeast at lower concentrations also improved the aroma of the oxidised wine.

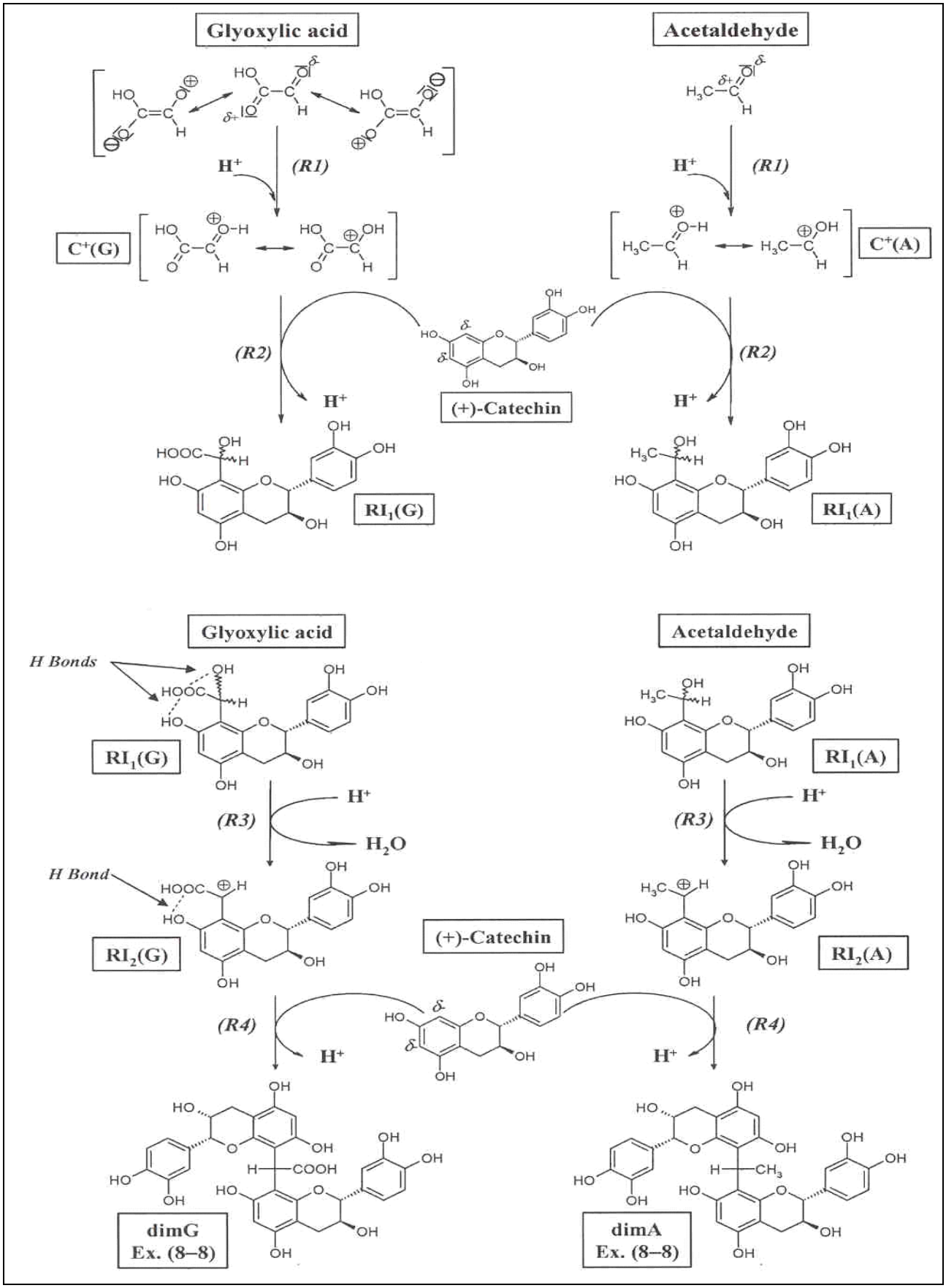

Fig. 2.2 The formation of dimers from glyoxylic and acetaldehyde (Drinkine et al., 2005)

Pinking of certain white wines remains a problem in the wine industry. This happens when wine is made reductively, i.e. when O_2 is kept to a large degree from coming into contact with it by use of inert gases, such as N_2 and CO_2. These wines can then become pink when exposed to small amounts of air, often during bottling and will become brown when further exposed to O_2 later. Although only aesthetically unacceptable, these bottled wines often have to be opened and treated. The compound responsible for pinking has not been identified, but is thought to be a phenolic chromophore. Wines made from Sauvignon blanc, Albarino, Garnatxa blanc and Verdejo were found to be pinking sensitive, although this did not happen in successive vintage years. An assay where H_2O_2 is added to wine has been developed to test for potential pinking. PVPP alone, PVPP plus bentonite and PVPP plus ascorbic acid were found to be 74%, 90% and 98% effective respectively in reducing tendencies of pinking These combinations having the same effectiveness in removing already developed pinking. Ascorbic acid alone was also found to be effective in preventing pinking, but new evidence suggests that it can enhance the formation of brown colouration under certain circumstances (Simpson, 1977, Lamuela-Raventó et al., 2001).

Ascorbic acid can also serve as a substrate for oxidation in wine. In the past this anti-oxidant has been used in many wineries for this purpose, especially in white wine, due to its O_2 scavenging ability. The products of its oxidation, dehydro-ascorbic acid and H_2O_2, necessitate the use of SO_2 in combination with ascorbic acid in order to prevent further oxidation by H_2O_2. In recent years, evidence has accumulated showing that ascorbic acid can be a pro-oxidant rather than an anti-oxidant in wines under certain conditions, with white wine becoming browner when an air headspace is left in combination with ascorbic acid. Also, sulfur dioxide does not seem to minimise this browning effect (Peng et al., 1998, Bradshaw et al., 2001). When Bradshaw et al. (2001 and 2003) oxidised ascorbic acid alone, two phases were observed. The first observed was the complete oxidation of ascorbic acid with species being generated that absorb in the visible spectra. The second generated species with a lighter or no colour. They also found that ascorbic acid enhanced the extent of browning in a model wine-like solution containing (+)-catechin. The onset of browning was, however, first preceded by a ‘lag phase’ when a decrease in browning was observed in comparison with the control containing no ascorbic acid. Pre-oxidised ascorbic acid did not exhibit this lag phase, but O_2 is required only for the initiation of the oxidation of ascorbic acid, with higher concentrations of initial O_2 shortening the lag phase. Higher concentrations of ascorbic acid enhanced the brown colour observed (0.06 for 1000 mg/L and 0.015 for 500 mg/L at 440nm), as well as extending the lag phase and time to reach maximum brown colour. Oxidation of tartaric acid yields glyoxylic acid, generating a colourless xanthene specie, which can undergo oxidation to form coloured xanthylium salts. The effective anti-oxidant activity of ascorbic acid initially prevents formation of the coloured xanthylium salts, explaining the initial decrease in colour during the lag phase. As mentioned previously, H_2O_2 is one of the oxidation products of ascorbic acid, but yields were only 21% as one would expect from a 1:1 production ratio from ascorbic acid. Furthermore, the addition of H_2O_2 to the model solution did not elicit the same extend of browning as ascorbic acid, suggesting that other oxidation products induce the browning. Other oxidation products of ascorbic acid under wine conditions include acetaldehyde, diketo-L-gulonic acid, L-threonic acid, oxalic acid, L-threo-2-pentulosonic acid, 4,5,5,6-tetrahydro-2,3-diketohexanoic acid and furfural. Dehydroascorbic acid is one of the initial oxidation products. After depletion of ascorbic acid during oxidation its oxidation products can then accelarate the formation of the coloured xanthylium salts, explaining the rapid increase in colour

during this period. These oxidation products are not oxidative enough to elicit this in the presence of ascorbic acid. This “cross over” of ascorbic acid as anti-oxidant to pro-oxidant thus depends on the concentration present.

Addition of SO_2 increases the lag phase mentioned above. A decrease was illustrated in the absence SO_2, the absence of ascorbic acid and formation of the brown xanthylium salts. In an ascorbic acid to SO_2 molar ratio of 0.8:1 the SO_2 increased the lag period to 4 days, but with a considerable loss to the SO_2 concentration; 100% loss in the SO_2, ascorbic acid, (+)-catechin combination compared to a 43% loss when the ascorbic acid was omitted. Increasing the SO_2 to 3:1 inhibited this over the 14-day time period tested. This ratio, which is 200 mg/L for ascorbic acid and SO_2 in the presence of 100 mg/L (+)-catechin, is quite high in winemaking terms. The ratio of SO_2 consumed to ascorbic acid was 1.7:1, which is higher than the expected 1:1. This is even more surprising considering that the oxidation of ascorbic acid yields only 21% of the expected H_2O_2. Sulfur dioxide seems to bleach the coloured xanthylium salt, but does not, contrary to popular belief; reduce dehydro-ascorbic acid back to ascorbic acid (Bradshaw et al., 2001; 2004). Flamini and Dalla Vedova (2003) found that *Oenococcus oeni* reduces glyoxal to glycolaldehyde, which has a 10 times higher browning capacity than ascorbic acid. In light of these findings wine producers should reconsider the use of ascorbic acid during the wine production process. This is especially true for white wines that have higher concentrations of flavanoids and to which tartaric acid, possibly containing glyoxylic acid impurities, has been added. Ascorbic acid has been hailed as a replacement for SO_2, but when it is added to wine it necessitates higher SO_2 additions that can prevent it from turning into a pro-oxidant after exposure to O_2.

2.6.2 EFFECT OF OXYGEN ON RED WINE COLOUR

Red wine obtains its colour from anthocyanins, which are normally extracted from the skins during the alcoholic fermentation. Different anthocyanins exist in grapes and wine, as mentioned earlier. The red colour can also be an indication of quality, with deep red wines normally judged as being of superior quality, depending on the other characteristics of the wine. In a young red wine up to 50% of anthocyanins can exist in the colourless carbinol pseudobase. During red wine ageing, the colour of red wine changes from red, in a young red wine, to mauve to brown/red, in the barrel, to eventually brown/orange after prolonged ageing in the bottle (Ribéreau-Gayon et al., 2000b). Different chemical reactions induce these changes in colour. These are:

1. Direct anthocyanin-tannin condensation reactions (A-T product). These reactions take place between the nucleophilic C6 or C8 carbons of (+)-catechin, (-)-epicatechin or procyanidins and the electrophilic C4 carbon of the anthocyanin molecule. This forms a colourless flavene, which can be oxidised to the corresponding flavylium ion, finally developing into a yellow xanthylium salt. These reactions take place during fermentation, with subsequent racking from the yeast or lactic acid bacteria lees introducing O_2. This increases the wine’s colour density when the flavene is oxidised (Liao et al., 1992, Santos-Buelga et al., 1999, Ribéreau-Gayon et al., 2000b).

2. Electrophilic carbocations, formed from procyanidins in a low pH medium such as wine, can react with nucleophilic C6 or C8 carbons of the anthocyanin in its hydrated hemiacetal form (T-A product). The product is colourless, but is rapidly dehydrated into a reddish-orange form. This reaction is stimulated by higher temperatures and O_2 is not required. It occurs predominantly during bottle ageing. Although the addition of oligomeric procyanidins with the anthocyanins in both A-T and T-A products seems to

occur more in wine than anthocyanin polymer additions, A-T and T-A polymers of up to octamer have been detected (Remy et al., 2000, Ribéreau-Gayon et al., 2000b, Hayaska and Kennedy, 2003).

3. Vinylphenols, normally associated with *Brettanomyces* spoilage, can also associate with anthocyanins. This is due to an electrophilic cyclo-addition of the ethylenic bond of the 4-vinylphenol molecule with C4 and C5 carbons of the anthocyanin, with subsequent oxidation leading to a pyrane ring. In aged Pinotage wines the pigment Pinotin A has been discovered. This is formed between the anthocyanin and a hydroxycinnamic acid moiety, especially caffeic acid in Pinotage, with oxidation leading to its formation. Anthocyanin-vinylcatechin products have also been identified, which possibly form from the reaction between a flavylium ion and a catechin molecule with a vinyl group on its C8 carbon, with oxidation leading to pigments having a red-orange colour. These molecules are also more resistant to SO_2 bleaching and pH changes, and they also contribute to the red to tawny change in colour of an older red wine. They can then act as a co-pigment, resulting in higher colour stability (Schwarz et al., 2003; Monagas et al., 2005).

4. The origin of acetaldehyde in wine has been discussed in earlier. In wine, which is an acid medium, acetaldehyde can be carbocated by the addition of a proton. This electrophilic moiety will then react with the C6 or C8 positions on a flavanol molecule, which, after the loss of H_2O, undergoes nucleophilic attack of the electrophilic C8 position of a colourless carbinol pseudobase anthocyanin molecule. The resulting product, with an ethyl bond, can be protonated to form a coloured compound. This reaction has been confirmed for malvidin-3-glucoside with different procyanidins and evidence suggests that the same reactions take place with cyanidin, delphinidin, peonidin and petunidin (Alcade-Eon et al., 2004; Monagas et al., 2005). (+)-Catechin, (-)-epicatechin and epigallocatechin have all been shown to react in this way with malvidin-3-monoglucoside. Trimeric and tetrameric pigments have been identified, but only position C8 of the anthocyanin molecule can be involved in this reaction, with the polymirisation ceasing when the anthocyanin forms the two terminal products of the chain. However, recent evidence suggests that the C6 position of the anthocyanin can also be reactive, as anthocyanins in the absence of flavanols formed dimers, trimers and tetramers via ethyl bonds with each other when acetaldehyde was added (Es-Safi et al., 1999a, Atanosova et al., 2002b). This reaction, which is faster than the previous two, takes place during barrel ageing when controlled oxygenation takes place. Oxygen can come into contact with the wine at this stage through winemaking actions, such as racking or topping up barrels. Oxygen also permeates through the staves of the barrel, with tight grain oak wood allowing higher O_2 concentrations in the wine. Anthocyanins involved in these polymirisation reactions are less prone to SO_2 bleaching and colour changes due to pH changes. The bisulfite ion, which decolourises the anthocyanin molecule, cannot associate that easily with the polymirisation product due to steric hindrance. In model solutions containing (+)-catechin, malvidin-3-glucoside, glyoxylic acid and the colourless (+)-catechin dimer with a carboxymethine bridge in coloured carboxymethine-bridged dimmers resulted, although model solutions containing the anthocyanin, (+)-catechin, tartaric acid and ethanol yielded only the flavanol dimer. Clearly, additional research is needed to evaluate the contribution of this to the changes observed in the evolution of red wine colour during ageing (Santos-Buelga et al., 1999, Monagas et al., 2005).

During barrel ageing, the colour intensity (the sum of the brown, red and violet colour) increases. In South African Pinotage and Shiraz wines it was found that the

origin of the barrel, American, French or Russian, did not affect the difference in colour intensity, colour hue or total red pigments. The colour density increased from 8-10 to 12-16 between 3-6 months after barrelling. Such a difference in colour density would be observed visually. During this period, of time the total red pigments decreased, but the percentage of pigments in the red form increased from 15 to 45%. A drop in free and total anthocyanins was thus observed, with the concentration of anthocyanins dropping from about 850 mg/L to 400 mg/L within six months. This transformation of colourless anthocyanins into the coloured form compensates for their loss and leads to the increase in colour density. Oxygen does not seem to influence the total concentration of pigment colour,, but does increase the proportion in the red form, as well as increase pigments resistant to SO_2 bleaching (Atanosova et al., 2002a, Fourie, 2005). Colour density can also decrease during ageing in a steel tank over a few months, but O_2 addition prevents this. The storage of red wine in non-aerated vats also leads to lower concentrations of coloured anthocyanins. Temperature plays a central role in these reactions, as high storage temperature in combination with high O_2 concentration can lead to anthocyanin and tannin breakdown reactions, which can increase the yellow hue of the wine. The oxygen addition should be in a controlled manner and not in excessive amounts because this can lead to excess acetaldehyde formation, excessive polymirisation and precipitation of colour matter. Favourable tannin to anthocyanin ratio is apparently also required, namely in the order of 4:1. Too low a ratio may lead to anthocyanin breakdown reactions and a too high ratio to over-polymirisation and precipitation. These ratios need to be investigated further under different winemaking conditions (Singleton, 1987; Ribéreau-Gayon et al., 2000b, Atanosova et al., 2002a).

During oak ageing, ellagitannins, such as vescalagin and castalagin, are extracted from the wood. Vivas and Glories (1996b) and Vivas (1999b) found that when these tannins oxidise, due to their greater oxidizing capacity, they produce larger amounts of acetaldehyde than condensed tannins. This leads to higher levels of polymirisation, which induces tannin-anthocyanin polymirisation, leading to higher colour density. This does not happen in all red wines and tannin addition can lead to certain wines becoming too astringent. These tannins also seem to buffer catechins from oxidation, by being oxidised themselves and thus preventing formation of a brick-yellow colour. In these experiments the ellagic tannins were, however, added at 300 to 1000 mg/L to the model solution or wine, and it is not certain if they will react during oak ageing to the same degree because they were isolated at only a few mg/L from oak-aged wines. More of these tannins could probably end up in the wine during ageing because they are easily hydrolysed (Puech et al., 1999). In our own laboratory we found that exposure of a young red wine to O_2 *per se* had a larger influence on colour development than the addition of commercial tannins according to the supplier's recommendations (unpublished data).

2.6.3 EFFECT OF OXYGEN ON RED WINE TASTE

Different phenolic molecules are involved with the bitterness, astringency and fullness of red wine, but it is mainly the flavanols that are responsible for these tastes and flavours. A very young red wine might be harsh, course, very astringent and even bitter. During ageing of red wine in barrels the wine becomes softer and less astringent. The different methods of polymirisation of (+)-catechin and (-)-epicatechin were discussed in the previous section under white wine oxidation, and basically the same reaction mechanisms occur for these compounds in red wine. Polymirisation leads to these molecules becoming less reactive towards mouth proteins, with the wine being perceived less astringent (Nikfardjam and Dykes, 2003). During ageing, it

is mainly the acetaldehyde induced polymirisation that contributes to the polymirisation of flavanols. The resulting products are not as reactive towards proteins as their constituents. However, direct C4-C8 and C4-C6 polymirisation reactions between procyanidin molecules produce products that are more reactive towards proteins and are hence more astringent than those formed from acetaldehyde-induced condensation reactions. In the case of flavanols, where the C6 and C8 positions can be occupied, polymers larger than trimers have been isolated. Both types of reactions produce procyanidins with a limit of 8 or 10 flavan units. The interaction of anthocyanin molecules with procyanidins can also influence the taste of wine because they can form the terminal subunits, thus preventing further polymirisation (Ribéreau-Gayon et al., 2000b, Monagas et al., 2005).

2.6.4 EFFECT OF OXYGEN ON WINE AROMA

The addition of O_2 to white wine is normally unwanted. Even small additions of O_2 to white wine can lead to loss of aroma, especially fruitiness, although the quality of some white wines may increase with a little O_2 contact. Periodical O_2 addition led to decreasing fruitiness and general quality, with a correlating increase in oxidation character in Sauvignon blanc and Chardonnay wines. In the Sauvignon blanc however, low initial concentrations of O_2 actually enhanced the quality score, by contributing to the complexity of the wine. The addition of H_2O_2 to a neutral Chenin blanc spiked with 2-methoxy-3-isobutylpyrazine did not lead to degradation of this important flavour compound of Sauvignon blanc over a three month period (Marais, 1998). It thus seems that this compound is resistant to oxidative degradation in wine. At high O_2 additions the formation of unwanted off-flavours will take place, with oxidised white wines being described as caramel, overripe fruit, crushed apple, acetaldehyde, woody, rancid, farm-feed, honey-like and cooked vegetables (Escudero et al., 2002, Silva Ferreira et al., 2003b). After 10 O_2 saturations white table wine normally becomes completely brown and maderised, and aromatic degradation can occur before it can be identified by colour change (Singleton et al., 1979, Boulton et al., 1996, Silva Ferreira et al., 2003a).

Simpson (1978) reported on the effect of O_2 addition and enhanced ageing at 50°C for 28 days on the composition of Riesling. The concentrations of ethyl n-hexanoate, hexyl acetate, acetic acid, ethyl n-octanoate, vitispirane, 1-hexanol, ethyl furonate and ethyl lactate did not differ significantly between the treatments. Benzaldehyde, diethyl succinate and 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), however, increased from 0, 3.8, 0.066 mg/L to 0.18, 4.4 and 0.09 mg/L, respectively. The concentration of 2-phenylethanol was, however, lower in the oxidised wine. Enhanced ageing under anaerobic conditions increased ethyl n-octanoate, vitispirane, ethyl furonate, ethyl n-decanoate, TDN and 2-phenethanol concentrations. Marais et al. (1992) found that TDN, trans-vitispirane, 2,6-dimethyl-7-octen-2,6-diol and trans-1,8-terpin concentrations and the intensity of the bottle-aged kerosene-like character increased significantly with ageing in Weisser Riesling wines. However, decreases were observed in diendiol-1, linalool, i-amyl acetate, ethyl caproate, hexyl acetate, 2-phenethyl acetate, hexanol, 2-phenyl ethanol and in the intensity of young wine character, with higher storage temperatures accelerating these changes. This study clearly showed that lower storage temperatures (15°C) were more favourable for the sensory development of Weisser Riesling wines during ageing.

Ferreira et al. (1998) developed a GCMS method to simultaneously measure compounds in wine, which was oxidised, that included t-2-hexenal, t-2-octenal, t-2-nonenal, furfural, 5-methyl-furfural, hexenal, benzaldehyde, furfurol and eugenol. In

subsequent studies phenylacetaldehyde, TDN, 3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolon), methional (Table 2.1) and other compounds were also isolated from oxidised white wines. The cooked vegetable aroma could especially be predicted from the t-2-nonenal, eugenol, benzaldehyde and furfural concentrations. The honey-like and boiled potatoe aromas could be predicted from the phenylacetaldehyde and methional concentrations. The woody aroma of oxidised wines, even in those that did not receive any wood contact, could be attributed to an increase in eugenol. Surprisingly enough, acetaldehyde did not seem to play a role in oxidised white wine aroma because it did not vary significantly during the oxidation process, but other unidentified compounds probably influence the aroma (Escudero et al., 2002, Silva Ferreira et al., 2002).

The oxidative aroma formation of white wine is dependent on several parameters, which include O_2 concentration, pH, storage conditions, SO_2 concentration, phenolic composition and ascorbic acid concentration. The floral aroma of white wine seems to degrade faster under higher temperatures, with O_2 additions with lower pH values increasing this trend. Sulfur dioxide additions decrease this degradation. At lower temperatures (15°C), however, degradation proceeds faster at pH 4 than pH3 and the addition of O_2 has an even more dramatic effect, with the floral aroma almost disappearing after a single saturation. This correlated with the high concentration of phenylacetaldehyde and methional (about 6 and 9 times higher) being found in the oxygenated wines stored at 45°C compared to

Table 2.1 Certain compounds isolated from oxidised white wine (Compiled from Simpson, 1978, Margalit, 1997, Silva Ferreira et al., 2002, Silva Ferreira et al., 2003a)

Compound	Associated flavours	Concentration (µg/L)
Furfural	Woody, cooked vegetables	90.2
t-2-nonenal	Cooked vegetables, woody	62.6
Eugenol	Cooked vegetables, woody	6.2
5-M-furfural	Woody	40.5
Benzaldehyde	Cooked vegetables, woody	165
Hexenal	Pungent	62.3
Phenylacetaldehyde	Honey-like	80-120
Methional	Farm-feed	30-40
TDN	Spicy, kerosene-like	90
Vitispirane	Camphor, eucalyptus	360

other treatments. The formation of linalool oxide and especially 2,2-dimethyl-5 -(1-methylpropenyl)-tetrahydrofuran) is enhanced by high temperatures and low pH values. Evidence suggests that the latter compound could impart a rotten food aroma to the wine. TDN and vitispirane are also greatly affected by lower pH values (Silva Ferreira et al., 2002). As mentioned earlier, non-volatile flavanoids have also been suggested to indirectly influence the aroma of wine by yielding acetaldehyde from ethanol during coupled oxidation (Schneider, 1998). But, in light of the mentioned findings by Escudero et al. (2002) that acetaldehyde does not seem to influence the oxidised aroma this should be investigated further. Hoenicke et al. (2002) showed that 2-amino-acetophenone, which imparts an untypical aged off-flavour (acacia, soapy) to wine, is formed via the intermediates 2-fromamidoacetophenone and 3-(2-formylaminophenyl)-3-oxopropionic acid after oxidative degradation of indole-3-acetic acid. More research is necessary on wine parameters that affect white wine aroma in combination with O_2, especially under South African conditions. This should include

reductive and oxidative treatment of juice, and O_2 specifications during different steps of the winemaking process, especially for different cultivars and white wine types.

The effect of O_2 on red wine aroma has not been investigated in detail. According to Blanchard et al. (2004) 3-mercaptohexanol, a fermentation product, is an important flavour compound of certain red and Rosé wines made from Cabernet Sauvignon, Merlot and Grenache grapes, imparting fruity aromas to these wines. Dissolved O_2 added to a red wine at 5 mg/L decreased within 48 h to 0.5 mg/L. This also led to a 30% decrease in 3-mercaptohexanol levels, but this decrease only started after 48 h. The addition of anthocyanins and especially catechins led to an even bigger decrease, with H_2O_2 also enhancing this decrease compared to O_2. The quinones generated can easily react with thiols according to a Michael addition reaction or generate H_2O_2, which can further oxidise 3-mercaptohexanol. In a model solution containing (+)-catechin, the 3-mercaptohexanol decreased from 1000 ng/L to 238 ng/L. When 30 g/L SO_2 was added to the sample, the 3-mercatohexanol level fell to only 647 ng/L. Anthocyanins and SO_2, as observed with 3-mercaptohexan-1-ol, seem to play a synergetic role in reducing the concentration of 3-mercaptohexanol. It is thus clear that the transport of wine can also lead to the reduction of the concentration of these odourous thiol compounds and that SO_2 plays a critical role in protecting them from oxidation (Murat et al., 2003, Blanchard et al., 2004). The oxidative environment inside an oak barrel also stimulates the formation of sotolon, which forms due to the oxidation of threonine. The oxidative degradation of phenylalanine and β-phenylethanol in a barrel also leads to higher concentrations of phenylacetaldehyde, reminiscent of an old oak oxidation flavour (Jarauta et al., 2005). In a study conducted by Cerdàn et al. (2004), the concentrations of ethyl butyrate and ethyl octanoate decreased in red wine during an 18 months period in barrels, while those of ethyl hexanoate and ethyl decanoate increased. It is clear that the effect of O_2 on the aroma composition of red wine should be investigated further.

Port sometimes acquires aromas reminiscent of truffles, quince or metals during ageing, which can be ascribed to dimethyl sulfide. This is due to the formation of this compound with oxidation, with low pH levels accentuating this phenomenon. Dimethyl sulphone follows the same pattern. Methional also decreases in concentration in the presence of O_2, but no methionol is formed. Another off-flavour, 2-mercapto-ethanol, also decreased; being oxidised to bis(2-hydroxydiethyl) disulfide, which does not have an odour. This explains why older ports never have sulfur off-flavours associated with cauliflower (methionol), rubber/burnt (2-mercapto-ethanol) or cooked potato (methional) (Silva Ferreira et al., 2003c). Low pH and high storage temperature are two parameters that increase the formation of TDN in port during ageing, with O_2 additions initially simulating its formation. High O_2 additions leading to its breakdown. Vitispirane, with a camphor or eucalyptus aroma, follows basically the same trend as the other parameters mentioned. Excessive O_2 also leads to the degradation of β-ionone and β-damascenone, which have a flavourful violet and ripe fruit aroma respectively. The degradation of these molecules can be effectively prevented by the use of SO_2, illustrating this preservatives anti-oxidative effect in even a product such as port, in which concentrations are normally kept low during ageing. Concentrations of SO_2 that are too high can also lead to the degredation of β-damascenone (Daniel et al., 2004; Silva Ferreira and De Pinho, 2004). Oxygen exposure in sweet fortified wines leads to the formation of sotolon and 5-(ethoxymethyl)-furfural that impart the characteristic aromas to mature sweet white and red wines, respectively. The presence of polyphenols in fortified red wines also leads to less oxidation of aroma compounds than in sweet white wines, due to the polyphenols' anti-oxidative characteristics and their capability to react with aldehydes (Cutzach et al., 1999).

2.6.5 MICRO-OXYGENATION

Micro-oxygenation is the process whereby O_2 is added with an apparatus to wine in a controlled manner. This is achieved by filling a known volume with gas at a high pressure. The volume is then transferred via a low-pressure circuit to the diffuser and into the wine. The latter normally consists of a ceramic or stainless steel sparger that produces small bubbles which can dissolve in the wine. The aim of micro-oxygenation is to introduce O_2 into the wine at a rate equal to or slightly less than the wine's ability to consume that O_2. It has to be managed in such a way that, after addition, all O_2 has been used up, while sufficient SO_2 is still left to protect the wine against oxidation. Different reasons for the application of micro-oxygenation are advocated by the producers of the machines. These are higher colour density, decreases in astringency, sulfur off-odours, green, vegetative aromas and production costs. Stainless steel tanks in conjunction with micro-oxygenation and alternative wood products can be used to simulate an oak barrel. Wine is also supposed to become ready for market sooner.

Oxygen can be supplied during different stages of the winemaking process. It can be supplied at 1-4 mg/L/day just after malolactic fermentation, especially to press wine fractions which are rich in polyphenols. The stage when micro-oxygenation is normally applied is during the ageing period after malolactic fermentation, when between 1-6 mg/L/month is introduced into the wine (Parish et al., 2000, Sullivan, 2002). The wine's temperature must be around 15°C because temperatures that are too high will lead to poor solubility of O_2 and temperatures that are too low to possible accumulation of O_2 in the headspace of the tank. Chemical reactions will also take place too slowly. With certain systems, which dose the O_2 according to mL/L, a tank of at least 2.2 m is required for sufficient pressure on the sparger to operate correctly.

Ribéreau-Gayon et al. (2000b) reported on a wine of which the colour intensity increased from 0.82 to 1.67 OD units in 5 months during micro-oxygenation. Anthocyanins and tannins also decreased in concentration, with an increase in the HCl index (polymirisation index), although a control receiving no O_2 was not included in this report. McCord (2003) found slight increases in the red and brown colour intensity of a Cabernet Sauvignon that received micro-oxygenation with a concurrent increase in polymirised anthocyanin level and a decrease in the free anthocyanin level in the wine that received O_2 compared to the control. The decrease in anthocyanin level was also higher where oak was added to the wine, possibly due to ellagitannin induced condensation. No conclusive results could be found concerning the effect of micro-oxygenation on oak compounds. The micro-oxygenation did, however, significantly reduce the unwanted sulfur compounds methyl and ethyl mercaptane to below their aroma threshold of 1 part per billion. Dimethyl sulfide concentration did not increase with the micro-oxygenation.

The effect of micro-oxygenation on the taste of wine has not been elucidated completely. Wine receiving micro-oxygenation can apparently go through structuring and harmonisation phases. The former leads to the wine actually becoming more astringent and harsh and can last from one to six months. During the subsequent harmonisation phase a decrease in astringency is observed, with the wine becoming more complex. This is then the ideal time to terminate the micro-oxygenation because as over-oxidation might lead to a mean degree of polymirisation of procyanidins that is too high, with a resulting increase in bitterness and with tannins becoming too dry (Parish et al., 2000, Nikfardjam and Dykes, 2003). Micro-oxygenation also leads to vegetative, green wines becoming fruitier (Sullivan, 2002). Taste, however, is still the main parameter to measure the progression of micro-oxygenation, but since it is a subjective procedure, it should be supplemented with other techniques. Oxygen can also be introduced by a sparger into barrels, which

introduces higher concentrations into the wine. This can lead to savings on racking costs (Vivas, 1999a). It is clear that additional research is needed on micro-oxygenation because little has been published on this process. This is probably due to the relatively large volumes needed for research on micro-oxygenation.

2.7 EFFECT OF O_2 ON ACETIC ACID BACTERIA AND *BRETTANOMYCES*

Acetic acid bacteria are Gram (+), catalase negative aerobic micro-organisms that occur in wine. They are notorious for wine spoilage due to the conversion of ethanol to acetic acid, which leads to acidification of the wine and an increase in the volatile acidity. Ethanol is transformed into acetaldehyde and the latter to acetic acid by alcohol dehydrogenase and acetaldehyde dehydrogenase, respectively. Low O_2 concentrations lead to the inhibition of acetaldehyde dehydrogenase, resulting in acetaldehyde accumulation in the wine. *Gluconobacter oxydans* normally dominates on healthy grapes, with *Acetobacter aceti* and *A. pasteurianus* dominating in wine. Recently, interest has been renewed in the metabolism and survival of acetic acid bacteria in wine, due to the fact that they survive under the relative anaerobic conditions often found in wine. Acetic acid bacteria have been isolated at high numbers from fermenting must and have been proven to be able to cause sluggish alcoholic fermentation (Du Toit and Lambrechts, 2002, Du Toit and Pretorius, 2002). The transfer and racking of wine after alcoholic fermentation and during ageing introduces O_2 into the wine that can lead to a 10^2-10^3 cfu/mL increase in acetic acid bacterial numbers. A 30-40-fold increase in acetic acid bacterial numbers was also noticed after the introduction of 7.5 mg/L O_2. Drysdale and Fleet (1989) found rapid growth of *A. pasteurianus* and *A. aceti* from 10^4 to 10^8 cells/mL in wine that was saturated with O_2. At 70% saturation, cell counts increased to 10^6-10^7 cells/mL, with 50% saturation contributing to the survival of the strains. Du Toit et al. (2005) found that acetic acid bacteria go into a viable, but non-culturable state in wine, especially under limited O_2 conditions. This can be negated by the addition of O_2, with plate and epifluorescence microscopy counts being the same after aeration. The O_2 permeating through oak staves during maturation probably also supports the survival of acetic acid bacteria in wine. Certain acetic acid bacteria strains also seem to be resistant to high concentrations of SO_2.

Brettanomyces are yeast able to spoil wine by production of 4-vinylphenol and 4-vinylguaiacol by the enzyme cinnamate decarboxylase from p-coumaric and p-ferulic acids, respectively. The vinylgroups can then be further reduced to 4-ethylphenol and 4-ethylguaiacol by vinylphenol reductase. These volatile phenols have flavours reminiscent of horse sweat, farmyard and medicine. At lower concentrations they can contribute to the complexity of the wine, but become unwanted at excessive concentrations. *Brettanomyces* have been found to survive especially in oak barrels, probably due to the β-glycosidase activity these microorganisms have, that can release some glucose from the oak for their growth. Oxygen has been found to contribute to the rapid growth of *Brettanomyces* in wine. Oxygen also stimulates the growth of *Brettanomyces* in must and induces acetic acid production. Faster production of 4-ethylphenol in the presence of O_2 has been observed, correlating with cell growth in red wine. The addition of SO_2, however, rapidly kills off *Brettanomyces* cells, with a 45 min exposure of cells to 0.64 mg/L molecular SO_2 being sufficient to achieve this. *Brettanomyces* also seem to be more sensitive to SO_2 than acetic acid bacteria, with only 0.25 mg/L molecular SO_2 inducing rapid cell death in certain strains. Bound SO_2 does not seem to affect *Brettanomyces* (Ciani and Ferraro, 1997, Du Toit et al., 2005).

The effect winemaking practices such as micro-oxygenation have on acetic acid bacteria and *Brettanomyces* numbers should be investigated. It is clear that the wine producer should avoid O_2 pickup and use SO_2 effectively when suspecting possible infection with either acetic acid bacteria or *Brettanomyces*.

2.8 ROLE OF OXYGEN IN BOTTLED WINE

2.8.1 OXYGEN PICK-UP DURING BOTTLING

The final step in the winemaking process is that of bottling. During this process the wine must be transferred into the bottles with minimum exposure to micro-organisms and O_2. The choice of bottles as a storage container is probably due to the inert characteristics of glass and it not being permeable to air. Before bottling, the wine should also be in the right state. This includes protein, temperature and SO_2 stability as well as a low dissolved O_2 level (below 0.5 mg/L, but ideally near 0.3 mg/L). White wine especially should be transferred into the pre-bottling tank with an inert gas. Nitrogen or argon gas can be used, with the latter having a higher specific gravity. Pipes and the filler also need to be filled with an inert gas before bottling starts. The headspace in tanks that is not completely full must be replaced with an inert gas (Allen, 1994). During the bottling process the filler should ideally not add much more than 1 mg/L of O_2 to the wine, but this can go up to 3 mg/L. During filling, a vacuum is often drawn inside the bottles, the wine flows inside and the headspace is filled with CO_2 (Lewis, 1991, Vivas and Glories, 1996a).

2.8.2 OXYGEN DIFFUSION AS INFLUENCED BY THE BOTTLE CLOSURE

The type of closure can play a critical role during the ageing of wine. Oxidative spoilage of bottled wine has been observed in both white and red wine. Corks differ in their permeability to air. Initial studies indicate that compounds within the cork are responsible for this phenomenon (Waters et al., 1996, Caloghiris et al., 1997). In an in-depth investigation, Godden et al. (2001) looked at the performance of a screw cap, two natural corks, two technical corks and 9 synthetic corks used to bottle the same Semillon wine. The wines bottled with the screw cap retained the most SO_2 and exhibited the least browning over the 20 months ageing period. Compared to this, the SO_2 loss was high in most of the synthetic corks, intermediate in the natural corks and least evident in the technical corks. Sulfur dioxide concentrations actually decreased from 7 and 61 mg/L free and total for a synthetic closure, compared to 20 and 84 mg/L and 24 and 90 mg/L for a technical cork and the screw cap respectively wines after six months bottle ageing. This was also reflected in the colour of the wines, with wines closed with synthetic corks generally becoming browner sooner. These wines also had an oxidised aroma and lost a high percentage of fruity character. The wine bottled with the screw cap however developed a higher reduced aroma, reminiscent of struck flint or rubber after 36 months, compared with the other treatments. At this stage, only the screw cap and cork based products had an acceptable aroma (Francis et al., 2003).

Lopes et al. (2005) measured the amount of O_2 permeating through different types of corks. Technical corks had very low O_2 diffusion after the first month (1.4-2.8 mg/L). Natural corks had rates between 2.3 and 3.8 mg/L and artificial corks 3.6-4.3 mg/L in the fist month, respectively. The diffusion rates then dropped, with technical

corks having between 0.01 and 0.10 mg/L O_2 diffusion per month after the first month. Natural corks had 0.24 to 0.5 mg/L per month and synthetic corks 0.85-1.5 mg/L O_2 diffusion/month during this period. Synthetic corks do not absorb wine as cork based products do, which probably contributes to the sealing capability of the latter. The exact method of O_2 diffusion into the bottles is unknown, but it could be that O_2 inside the corks initially permeates into the wine, with atmospheric O_2 permeating later. This may explain the high initial rate of O_2 diffusion in the first month. The large variability observed between natural corks might also explain the random oxidation phenomena of individual bottles. The way bottles are stored also influences air diffusion, with upright bottles, especially those sealed with agglomerated cork stoppers, permitting more O_2 to come into contact with the wine (Godden et al., 2001, Mas et al., 2002, Lopes et al., 2005).

2.9 MEASURING AND EVOLUTION OF OXIDATION OR REDOX POTENTIAL IN WINE

The measurement of O_2 in wine can be conducted with an O_2 meter. It consists of two electrodes with a membrane permeable to O_2. The O_2 causes a change in potential between the two electrodes that can be measured. The oxidation-reduction potential of wine can also be measured with a similar electrode. The potential is especially influenced by the O_2 concentration, metals, ethanol, phenolic compounds, type of container and temperature, with glycerol having little effect. Winemaking operations, especially those that introduce O_2 such as racking, topping up of barrels, filtration and tannin addition also influence this. The effect of different winemaking operations on this potential can be seen in Fig. 2. 3. It is clear that during alcoholic fermentation the potential drops due to the reductive conditions. It increases again with O_2 pickup at racking after malolactic fermentation and racking of the wine (Vivas and Glories, 1996a, Ribéreau-Gayon et al., 2000b).

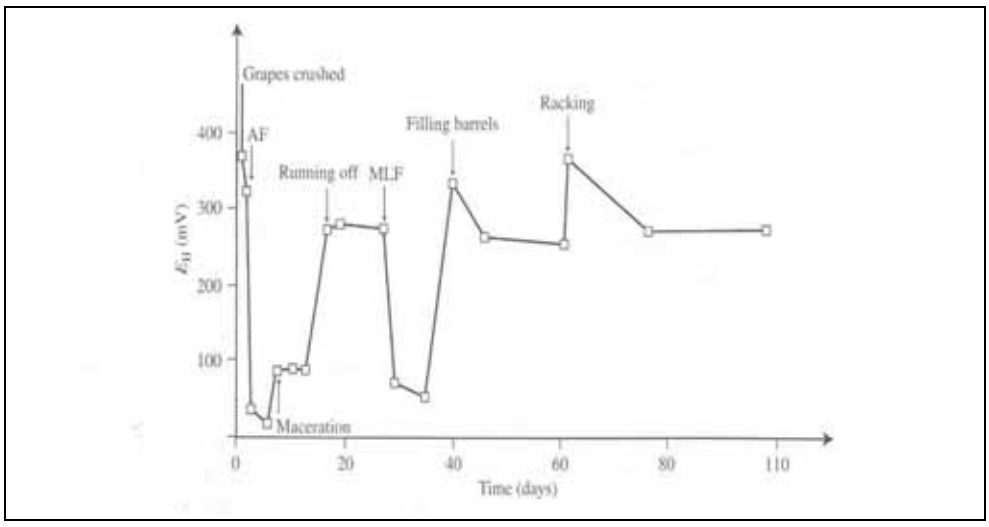

Fig. 2.3 The evolution of the oxidation-reduction potential of a red wine during the production process. AF: alcoholic fermentation, MLF: malolactic fermentation (Ribéreau-Gayon et al., 2000b).

The progression of oxidation can be measured especially in white wine with a spectrophotometer. Absorbency at 420 nm measures the brown spectrum of light and can serve as an indicator of oxidation. However, aromatic degradation can occur

before it can be identified by an intense yellow-brown colour (Silva Ferreira et al., 2003b). Other ways of measuring oxidation in wine should thus be developed. This is especially true for red wine, where a colour change is even less indicative of oxidation than in white wine.

2.1O CONCLUSIONS

It is clear that O_2 can play an important role during the winemaking process. The main substrates for oxidation in wine are phenolic compounds, but other compounds such as ascorbic acid and tartaric acid can also be oxidised. These reactions produce quinones, acetaldehyde, H_2O_2, glyoxylic acid etc, which can induce polymirisation reactions in the wine. Oxygen can be introduced by the winemaker in the must, during fermantation and especially during ageing of red wine to induce favourable chemical changes in the wine. This should, however, be done with care as different off-flavours can also be produced in the wine when too high O_2 concentrations are introduced. There are still many aspects regarding the reactions of O_2 in wine that remains unknown. These include the effect of O_2 on red wine aroma and sensory development and should be investigated in the future.

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3. THE EFFECT OF SULFUR DIOXIDE AND OXYGEN ON THE VIABILITY AND CULTURABILITY OF A STRAIN OF *ACETOBACTER PASTEURIANUS* AND A STRAIN OF *BRETTANOMYCES BRUXELLENSIS* ISOLATED FROM WINE

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Running headline: Effect of sulfur dioxide and oxygen on *Acetobacter* and *Brettanomyces*

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ABSTRACT

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Aims: The objective of this study was to investigate the effects of free molecular and bound forms of sulfur dioxide and oxygen on the viability and culturability of a selected strain of *Acetobacter pasteurianus* and a selected strain of *Brettanomyces bruxellensis* in wine.

Methods and Results: Acetic acid bacteria and *Brettanomyces*/*Dekkera* yeasts associated with wine spoilage were isolated from bottled commercial red wines. One bacterium, *A. pasteurianus* strain A8, and one yeast, *B. bruxellensis* strain B3a, were selected for further study. The resistance to sulfur dioxide and the effect of oxygen addition on these two selected strains were determined by using plating and epifluorescence techniques for monitoring cell viability in wine. *A. pasteurianus* A8 was more resistant to sulfur dioxide than *B. bruxellensis* B3a, with the latter being rapidly affected by a short exposure time to free molecular form of sulfur dioxide. As

expected, neither of these microbial strains was affected by the bound form of sulfur dioxide. The addition of oxygen negated the difference observed between plate and epifluorescence counts for *A. pasteurianus* A8 during storage, while it stimulated growth of *B. bruxellensis* B3a.

Conclusions: *A. pasteurianus* A8 can survive under anaerobic conditions in wine in the presence of sulfur dioxide. *B. bruxellensis* B3a is more sensitive to sulfur dioxide than *A. pasteurianus* A8, but can grow in the presence of oxygen. Care should be taken to exclude oxygen from contact with wine when it is being transferred or moved.

Significance and Impact of Study: Wine spoilage can be avoided by preventing growth of undesirable acetic acid bacteria and *Brettanomyces*/*Dekkera* yeasts through the effective use of sulfur dioxide and the management of oxygen throughout the winemaking process.

Keywords: Acetic acid bacteria, *Acetobacter*, *Brettanomyces*, *Dekkera*, sulfur dioxide, oxygen, wine

3.1 INTRODUCTION

Due to the high initial sugar content, low pH, anaerobic fermentation conditions and high alcohol concentrations at the end of fermentation, only a few yeasts and bacteria able to spoil wine can survive the strong selective pressures in fermenting grape must and in wine (Du Toit and Pretorius 2000). If uncontrolled, the metabolic activity of these spoiler microorganisms before, during or after fermentation can alter the chemical composition of wine, thereby adversely affecting the sensory properties (appearance, aroma and flavour) of the end product. Two of the primary and omnipresent groups of spoiler microorganisms that can persist throughout the harsh winemaking process are acetic acid bacteria and *Brettanomyces*/*Dekkera* yeasts. High volatile acidity (VA) and a vinegary taint in wine are often associated with the activity of acetic acid bacteria such as *Acetobacter aceti*, *Acetobacter pasteurianus* and *Gluconobacter oxydans*, while terms such as ‘Band-aid’, ‘medicinal’, ‘pharmaceutical’, ‘barnyard-like’, ‘horsey’, ‘sweaty’, ‘leathery’, ‘mouse urine’, ‘wet dog’, ‘smoky’, ‘spicy’, ‘cheesy’, ‘rancid’ and ‘metallic’ have been used to describe the aroma and taste of wine affected by *Brettanomyces*/*Dekkera* species, such as *Brettanomyces bruxellensis* and *Dekkera bruxellensis* (Heresztyn 1986; Chatonnet *et al.* 1992; Chatonnet *et al.* 1995; Du Toit and Pretorius 2000, 2002, Coulter *et al*.

2004; Kramer and Noonan 2004). While there are several research groups around the world who study these two major groups of microorganisms involved in wine spoilage, knowledge of their occurrence, metabolism, interactions with other microorganisms, and methods of inhibition in the winemaking process in particular are limited and need further investigation.

Acetic acid bacteria are Gram-negative, catalase-positive rods. They are classified into the genera *Acetobacter, Acidomonas, Gluconobacter* and *Gluconacetobacter* (Yamada *et al.* 1997; Ruiz *et al.* 2000). Of these, *Gluconobacter oxydans*, *Acetobacter aceti*, *Acetobacter pasteurianus*, *Gluconacetobacter liquefaciens* and *Gluconacetobacter hansenii* are normally associated with grapes and wine (Du Toit and Lambrechts 2002; Du Toit and Pretorius 2002). The oxidation of ethanol to acetic acid is the best-known characteristic of acetic acid bacteria. In this reaction, a membrane-bound alcohol dehydrogenase oxidises ethanol to acetaldehyde. This is further oxidised to acetate by a membrane-bound aldehyde dehydrogenase (Matsushita *et al.* 1994; Saeki *et al.* 1997b). Acetic acid bacteria can produce acetaldehyde at concentrations of up to 250 mg l^{-1}, which exceeds the threshold of 100-125 mg $l^{-1,}$ beyond which sensory perception is affected (Lambrechts and Pretorius, 2000). Acetaldehyde can give the wine an oxidised character. It also binds sulfur dioxide (SO_2) very effectively, rendering it ineffective as an antioxidant and antimicrobial agent. Other SO_2-binding compounds produced by acetic acid bacteria include gluconic acid from glucose and 5-oxo-fructose from fructose (Barbe *et al.* 2001). Acetic acid bacteria are also able to produce high concentrations of acetic acid, which is considered to be detrimental to wine quality at concentrations ranging from 0.7-1.2 g l^{-1} and higher, depending on the wine style, although it can be noticeable at lower concentrations (Drysdale and Fleet 1988; Lambrechts and Pretorius 2000).

Another product of the metabolism of acetic acid bacteria that could affect wine quality is ethyl acetate. This ester of acetic acid can contribute positively to wine aroma at low concentrations, but is considered undesirable at higher concentrations due to the low threshold of 12.3 mg l^{-1} (Lambrechts and Pretorius 2000) at which it affects flavour. Acetic acid bacteria can also oxidise higher alcohols, such as isoamyl alcohol, 1-propanol and 2-phenylethanol, to the corresponding aldehyde and carboxylic acid (Molinari *et al.* 1999). Another concern for winemakers is that some acetic acid bacteria, such as *Gluconobacter oxygens,* can use *p*-hydroxyquinone as a

terminal electron acceptor instead of oxygen (O_2) to grow on glycerol under anaerobic conditions (Adlercreutz and Mattiasson 1984).

Brettanomyces/*Dekkera* yeasts exist in two forms: *Brettanomyces*, the asexual, non-sporulating form, and *Dekkera*, the sexual sporulating form. For simplicity and due to the widespread use of the term 'Brett character' by the wine community, the generic name *Brettanomyces* will be used in this chapter. The principal spoiler compounds associated with *Brettanomyces* are reported to be two volatile phenols, 4-ethylphenol and 4-ethylguaiacol (Chatonnet *et al*. 1992, 1995; Singleton 1995), isovaleric (3-methylbutyric) acid (Licker *et al*. 1998) and certain tetrahydropyridines (Heresztyn, 1986). In a recent survey, Coulter *et al*. (2004) found that 4-ethylphenol and 4-ethylguaiacol (both compounds associated with 'Band-aid', 'medicinal', 'barnyard' and 'stable' aroma characters and 'metallic' taste attributes) were the main compounds derived from *Brettanomyces* that were associated with off-odours in red wines. They found that the concentration of isovaleric acid (associated with 'sweaty', 'cheesy' and 'rancid' characters) was independent of the concentrations of these two key spoiler compounds. It therefore appears that isovaleric acid may be involved in additional sensory effects with other *Brettanomyces*-derived compounds, thereby enhancing the apparent aroma intensity of those other compounds. It was also postulated that there could be other compounds, not yet identified, that might also contribute to the characters associated with *Brettanomyces* spoilage (Coulter *et al*. 2004). Furthermore, the survey concluded that the extent to which the sensory properties of a wine may be affected by 4-ethylphenol depended on the style and structure of the wine, *i.e.* the concentration and intensity of other wine compounds that could mask (*e.g*., volatile oak compounds) or accentuate (*e.g*., 4-ethylguaiacol) the aroma of 4-ethylphenol (Coulter *et al*. 2004). For example, in a light-bodied red wine with little oak influence, the sensory perception threshold of 4-ethylphenol may be as low as approximately 350 $\mu g\ l^{-1}$, compared with 1000 $\mu g\ l^{-1}$ in a full-bodied red wine with intense fruit and considerable oak influence.

The spoilage of wine by compounds produced by acetic acid bacteria and *Brettanomyces* yeast is a worldwide problem if the apparent recent increase in the number of wine press articles that refer to this issue is any indication. The widespread nature of this problem, and the seemingly increasing awareness of it by the wine trade and consuming public, presents an opportunity to gain a competitive

advantage to wineries able to manage and control the proliferation of acetic acid bacteria and *Brettanomyces* (Coulter *et al*. 2004) yeast.

This has compelled many winemakers to review their practices carefully and meticulously apply best practice winemaking measures to control microbiological instabilities. Best practice includes measures such as the use of healthy grapes; the application of general cleaning and sanitation practices in wineries; the use of appropriate clarification methods; the control and management of wine temperature, pH, SO_2 concentration, oxygen exposure and residual nutrient (glucose, fructose, nitrogen, *etc*.) concentrations in finished wines; and good barrel management (e.g., the use of new rather than old barrels, barrel sanitation, etc.) (Coulter *et al*. 2004). These factors are interdependent and a holistic approach is required to address them concurrently if winemakers are to achieve the best outcomes in controlling acetic acid bacteria and *Brettanomyces* yeast.

Traditionally, the enumeration of acetic acid bacteria and *Brettanomyces* yeast from wine for quality control purposes had been conducted by plating on selective media, which measures the culturability of the microorganisms. However, new evidence has come to the fore that acetic acid bacteria can exist in a viable, but non-culturable state in the wine, which means that under certain stress conditions these cells will not grow on selective media. This might obscure the ‘microbiological status’ of a wine (Millet and Lonvaud-Funel 2000).

The purpose of this study was to investigate the inhibitory effect of SO_2 and the stimulatory effect of O_2 on the viability and culturability of acetic acid bacteria (selected strain *A. pasteurianus* A8) and *Brettanomyces* yeast (selected strain *B. bruxellensis* B3a) in wine. SO_2 exists either in a bound or a free form in wine, with the free form consisting of molecular SO_2, bisulfite (HSO_3^-) and the sulfite ion (SO_3^{2-}) (Ribéreau-Gayon *et al.* 2000).

For the effective control of wine spoilage by acetic acid bacteria and *Brettanomyces* yeast, it is essential to establish the exact SO_2 resistance levels of these two organisms as well as the relationship between O_2 levels in wine and their proliferation.

3.2 MATERIALS AND METHODS

3.2.1 MICROBIOLOGICAL CULTURES AND MEDIA

Acetic acid bacteria and *Brettanomyces* yeasts were isolated from bottled commercial red wines by plating out the wine on selective media. For the isolation of acetic acid bacteria, the culture medium consisted of 57 g l^{-1} Man Rogosa Sharp (MRS) medium (pH 5 with HCl, 20 g l^{-1} agar) to which 2% v/v ethanol was added after sterilisation. This medium was supplemented with 7 mg l^{-1} penicillin and 50 mg l^{-1} pimaricin (Delvocid) to eliminate lactic acid bacteria and yeast, respectively. *Brettanomyces* strains were isolated on a rich medium (YPD) containing 10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} glucose (pH 4.6 with HCl, 20 g l^{-1} agar), supplemented with 50 mg l^{-1} chloramphenicol and 500 mg l^{-1} actidione to eliminate bacteria and *Saccharomyces* yeasts, respectively. Pure cultures were obtained. An acetic acid bacterium (A8), identified (Du Toit and Lambrechts 2002) as *Acetobacter pasteurianus*, and a *Brettanomyces* yeast (B3a), identified as *Brettanomyces bruxellensis*, (Ibeas *et al.* 1996) were selected from spoiled wines for further investigation. Both these selected microbial strains exhibited good aerobic growth in the fined wine. For each experiment, a single colony of the selected *A. pasteurianus* and *B. bruxellensis* strains were picked from agar plates and inoculated into a liquid medium consisting of 50% MRS or YPD, respectively, and 50% red wine, which was previously fined with 30 g l^{-1} activated charcoal to remove phenolic compounds. This inoculum was then grown aerobically at 25°C for two days until a high cell density was obtained. It was then used to inoculate undiluted, fined red wine. This pre-culture was grown again until an optical density (OD at 600 nm) of 1 was reached and then inoculated into a wine chosen for the experiments. This procedure was repeated for all the experiments, except for the one in which the SO_2 sensitivity of *B. bruxellensis* was tested in the late stationary phase. For this experiment, the OD_{600} of the inoculum was 3.3.

3.2.2 WINE AND EXPERIMENTAL PROCEDURES USED IN THE SO_2 AND O_2 EXPERIMENTS

Wine used for the experiments consisted of a Bordeaux red wine with the following chemical constitution: alcohol 11.25% v/v, total acidity 3.1 g l^{-1} (as H_2SO_4), volatile acidity 0.39 g l^{-1}, malic acid 0.1 g l^{-1}, pH 3.63, total SO_2 59 mg l^{-1} and free SO_2 14

mg l^{-1}. In most of the experiments, including all the SO_2 experiments, the wine was fined with 30 g l^{-1} activated charcoal, centrifuged at 9000 rpm for 10 min to remove excess charcoal; the pH was adjusted to the desired value with HCl or KOH and it was sterile filtered through a 0.45 µm filter. At this stage, the wine contained no measurable SO_2 according to the Ripper method (Iland *et al.* 1993). One hundred ml of the wine was then placed into glass vials and hermetically sealed with a rubber cap. In all the SO_2 experiments, except the one where the molecular SO_2 sensitivity of *B. bruxellensis* was tested under semi-anaerobic wine conditions, the wine in each vial was then sparged vigorously with sterile nitrogen to remove excess oxygen. This was done by inserting a sterile syringe through the rubber cap to the bottom of the glass vial. This served as an inflow for the nitrogen gas and a second sterile syringe, also inserted through the rubber cap but not in contact with the wine, served as an outflow for the oxygen and nitrogen. Sterile high purity nitrogen gas (filtered through a 0.22 µm filter) was used to sparge the wine for 7 min (at 20ºC) in each vial to remove excess oxygen. The free SO_2 concentration was measured after a few hours; no change in the SO_2 concentration indicated that very little, if any, oxygen remained in the wine.

In the experiment to investigate the effect of different concentrations of molecular SO_2 on the *A. pasteurianus* and *Brettanomyces*, SO_2 (2% stock solution, prepared from metabisulfite) was added with a sterile syringe to obtain the desired concentration in fined wine with a pH of 3.6. The wine was incubated for 2 h at 25ºC for the free and bound forms of SO_2 to reach equilibrium, although the reaction of SO_2 with acetaldehyde, which is a main SO_2 binding component in wine, occurs at a rapid rate. The free SO_2 concentration of the wine was determined before inoculation with the *A. pasteurianus* or *B. bruxellensis* strains. The wine containing the *A. pasteurianus* or *B. bruxellensis* strains was incubated for five days and samples were withdrawn by extraction with a`sterile syringe at days two, three and five for analysis by extraction with a sterile syringe. After a sample was extracted, the resulting vacuum was filled with sterile nitrogen gas.

The 2% SO_2 stock solution was first bound with an equimolar concentration of acetaldehyde before addition to the wine in the experiment to test the effect of bound SO_2 on *A. pasteurianus* and *B. bruxellensis*. This wine had a pH of 4.2 (at such a high pH, very little free molecular SO_2 exists in the molecular SO_2 form). The free molecular SO_2 concentration of the wine and the stock solution, to which acetaldehyde was added, were measured before the stock solution was added to the

wine to verify that no free SO_2 was present in the SO_2 stock solution or the wine. Samples were analysed at two and seven days after inoculation with *A. pasteurianus* A8 and *B. bruxellensis* B3a, respectively. The short-term effect of molecular SO_2 on the selected B3a strain of *B. bruxellensis* was tested by withdrawing samples immediately after 30, 120 and 330 min after yeast addition to wine containing 0.64 mg l^{-1} molecular SO_2.

An excessive concentration of acetaldehyde (twice the equimolar concentration) was also added to wine containing 0.64 mg l^{-1} molecular SO_2 5 min before inoculation with *B. bruxellensis* B3a. The same concentration of acetaldehyde was also added to the same wine 5 and 45 min after inoculation with *B. bruxellensis* B3a to investigate the effect of different exposure times of molecular SO_2 on *B. bruxellensis* strain B3a.

Examination of the effect of long-term storage (71 days) and oxygen addition on *A. pasteurianus* strain A8 was done in the original sterile red wine (pH adjusted to 3.9 with KOH) by extracting 30 ml of the original 100 ml of wine with a sterile syringe 17 days after inoculation. The wine was then squirted into another sterile 30 ml Petri glass container (under aseptic conditions in a laminar flow cabinet) to enhance O_2 pickup and sealed again hermetically. In this case, the control was the remaining wine in the original Petri glass container. This wine did not receive any O_2 and where the vacuum created by extraction of the wine was filled with sterile nitrogen gas. After 48 days, the wine that received O_2 after 17 days, as well as the control wine, was again aerated (in the same manner as at day 17). The same procedure was followed to aerate the wine to test the effect of oxygen on *B. bruxellensis* B3a. The aforementioned wine where the molecular SO_2 sensitivity of *B. bruxellensis* was tested under semi-anaerobic wine conditions was used for the latter experiment. This wine was aerated at 9 and 15 days after inoculation with *B. bruxellensis* B3a. All wines used for the different experiments were incubated at 25ºC for the duration of each experiment.

3.2.3 EXTRACTION OF PHENOLICS COMPOUNDS

Phenolic compounds were obtained from the original red wine (1.5 litres) by evaporating the ethanol in the wine, followed by fixation on a XAD 16 column. The phenolic compounds were then isolated by washing the column with 2 litres of distilled water containing 4% acetic acid, followed by elution with 2 litres of methanol containing 4% acetic acid. The eluate was then divided into two equal parts. In one

part, the methanol was removed by evaporation, 12% v/v ethanol added, and it was vigorously stirred to enhance O_2 pickup. It was kept overnight at 90ºC to encourage oxidation of the phenolic compounds. The ethanol and water were again removed with a rotary vacuum evaporator at 35ºC and the phenolic compounds re-dissolved in distilled water and lyophilised. Distilled water was also added to the non-oxidised phenols, which were also lyophilised. These phenolic compounds were then added to the fined wine at the same mass to volume ratio as in the original wine. *A. pasteurianus* was then inoculated into the original red wine, the fined wine, the fined wine to which the original phenols were added back, and the fined wine containing the oxidised phenols.

3.2.4 MONITORING OF VIABILITY AND CULTURABILITY

At regular intervals, the viability and culturability of the *A. pasteurianus* A8 and *B. bruxellensis* B3a were monitored in the wine by plating the wine on MRS and YPD media (without any antibiotic additions), respectively. Epifluorescence microscopy, as described by Millet and Lonvaud-Funel (2000), was also used to determine total cell numbers. In all experiments, cell counting was performed simultaneously by both methods in the same wine samples. By comparing the intensity of the epifluorescence of the cells with the background, an indication of the degree of viability could also be determined. For each experiment, five microscopic fields (each field containing at least 12 cells) were analysed to determine the epifluorescence intensity. All experiments were conducted in triplicate and results given are the average of the triplicates. Data reported here did not differ with more than 10% from the relative standard deviation.

3.3 RESULTS

The viability and culturability of *A. pasteurianus* strain A8 in non-fined red wine was monitored for 71 days (Fig. 3.1). Initially, the plate and epifluorescence counts were similar, with the latter decreasing after 5 days. Later, a 100-fold difference was observed between the plate and epifluorescence counts, but this difference decreased by exposing the wine to O_2 after 17 days. This exposure also led to an increase in the plate counts and, to a lesser extent, in the epifluorescence counts. At day 48, the wine was aerated again, which led to no observable difference between the two methods of enumeration. The difference was again prominent by day

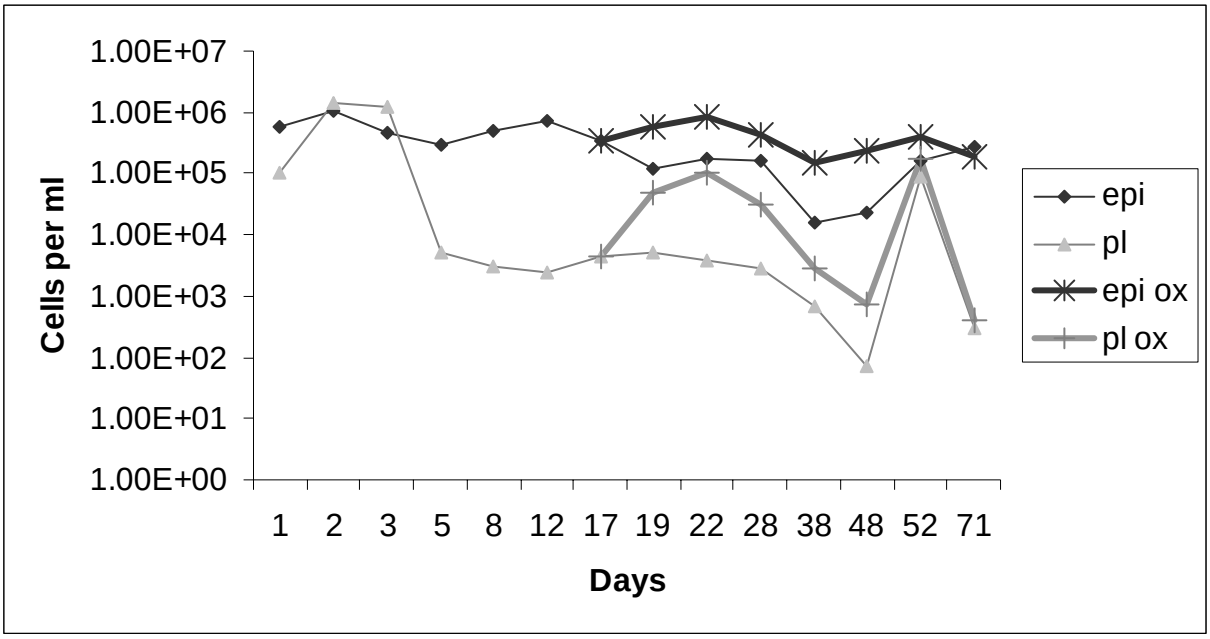

Fig. 3.1 The cell viability (microscopic epifluorescence enumeration) and culturability (plating on MRS agar medium) of *Acetobacter pasteurianus* A8 in non-fined red wine. Sub-samples of the flasks/cultures were aerated on day 17 and 48. Key: epi - epifluorescence count; epi ox - epifluorescence count after aeration; pl - plate count; pl ox – plate count after aeration. Values are the mean of triplicate cultures.

71. Fig. 3.2 presents the viability and culturability of *A. pasteurianus* A8 in the red wine that was previously fined with charcoal. Here, the epifluorescence counts were lower than in the original red wine 12 days after inoculation, with no colony-forming units (cfu) counted on the plates after day 19. Even the addition of O_2 at day 17 did not enhance the viability and culturability of the cells.

The effect of phenolic compounds and oxidised phenolic compounds on the viability and culturability of *A. pasteurianus* A8 was investigated to examine whether phenolic compounds or quinones can be used as alternative terminal electron acceptors to O_2. However, minimal difference was observed between the viability of *A. pasteurianus* A8 in the fined red wine and the fined red wine to which phenolic compounds and oxidised phenolic compounds were added (results not shown). In the original non-fined red wine, the viability and culturability were slightly higher than that observed in the fined red wine.

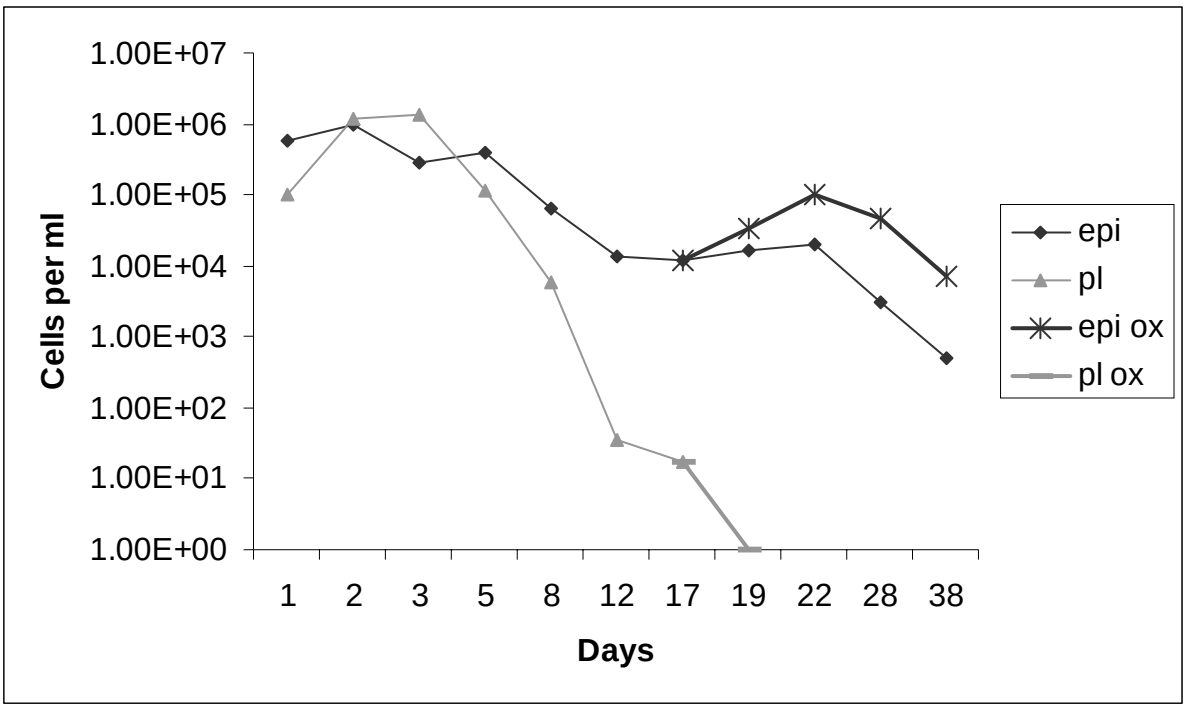

Fig. 3.2 The viability (microscopic epifluorescence enumeration) and culturability (plating on MRS agar medium) of *Acetobacter pasteurianus* A8 in red wine fined with activated charcoal. Sub-samples of the flasks/cultures were aerated on day 17. Key: epi - epifluorescence count; epi ox - epifluorescence count after aeration; pl - plate count; pl ox – plate count after aeration. No colonies were observed at day 19, 22, 28 and 38 for pl and pl ox. Values are the mean of triplicate cultures.

The effect of free molecular SO_2 on *A. pasteurianus* A8 is depicted in Fig. 3.3. The culturability and epifluorescence counts were not greatly affected by the low free molecular SO_2 concentration (0.35 mg l^{-1}). The epifluorescence counts did not differ significantly between the different SO_2 concentrations tested one day after addition of the SO_2. This was even true at the highest molecular SO_2 concentration tested (1.2 mg l^{-1}, which constitutes a free molecular SO_2 concentration of 75 mg l^{-1} at pH 3.6). However, this concentration of free molecular SO_2 caused a decrease in the viable cell numbers after 5 days of SO_2 exposure to $4.6x10^3$ cfu ml^{-1} when compared with the control, which showed viable cell numbers of $1.7x10^5$ cfu ml^{-1}. The molecular SO_2 did decrease the culturability of the cells, with no cells growing on the agar plates at 0.8 mg l^{-1} molecular SO_2 by day 2. The intensity of the epifluorescence also declined as the molecular SO_2 increased (Table 3.1). However, the bound form of SO_2 had no effect on the culturability and viability of the *A. pasteurianus* A8 (Table 3.1). Epifluorescence and plate counts ranged between $1.2x10^5$ and $2.3x10^5$ cfu ml^{-1}, and

$1.1x10^3$ and $3.3x10^3$ cfu ml^{-1} respectively, for 0, 80 and 160 mg l^{-1} bound SO_2 added after 7 days (results not shown).

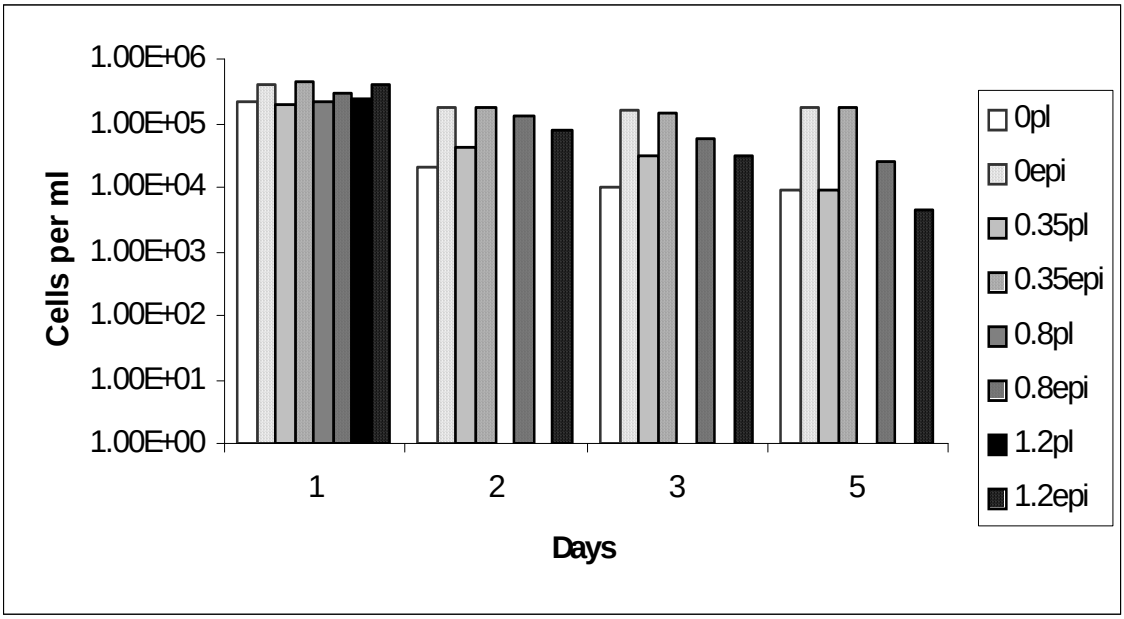

Fig. 3.3 Effect of different molecular SO_2 concentrations on the viability (microscopic epifluorescence enumeration) and culturability (plating on MRS agar medium) of *Acetobacter pasteurianus* A8 in fined red wine over time. Key: epi - epifluorescence count; pl - plate count. Values are the mean of triplicate cultures.

B. bruxellensis B3a was also tested for its sensitivity to different SO_2 forms. It is clear from Fig. 3.4 that the culturability of *B. bruxellensis* B3a was more sensitive to lower concentrations of molecular SO_2 (between 0.25 and 0.35 mg l^{-1}) than *A. pasteurianus* A8. However, low concentrations of molecular SO_2 (0.1 mg l^{-1}) did not affect the viability and culturability of the cells, nor the intensity of the epifluorescence (Table 3.1). At higher molecular SO_2 concentrations (0.8 mg l^{-1}), no cells grew on the agar plates, but the results obtained with epifluorescence microscopy showed the total number of cells did not decrease drastically from the control, which did not receive any molecular SO_2. It thus seems that the *B. bruxellensis* B3a cells were still viable but not culturable. Lower concentrations of molecular SO_2 (0.25 to 0.35 mg l^{-1}) also had a faster effect on the culturability of *B. bruxellensis* B3a, where growth on agar plates was inhibited after only one day. With *A. pasteurianus* A8, the inhibition took longer to occur. The bound SO_2, as in the case of *A. pasteurianus* A8, did not have any effect on the epifluorescence count and the plate count (results not shown), nor on the intensity of the epifluorescence (Table 3.1) at the highest concentration of total SO_2 tested (160 mg l^{-1}). The highest total (free plus bound) SO_2 concentration

used in these experiments was below the allowable limit set by most wine producing countries.

Table 3.1 The effect of different SO_2 concentrations, forms and exposure times on *Acetobacter pasteurianus* A8 and *B. bruxellensis* B3a in red wine (determined after 2, 5, or 7 days).

Microorganism	SO_2 form tested against microorganism	SO_2 concentration (mg l^{-1})	Average epifluorescence intensity
A. pasteurianus A8	Molecular SO_2 (after 5 days)	0	4.3
		0.35	3
		0.8	1.4
		1.2	1.2
	Bound SO_2 (after 7 days)	0	3.8
		80	3.8
		160	3.7
B. bruxellensis B3a	Molecular SO_2 (after 7 days)	0	12
		0.1	10.4
		0.25	1.2
		0.8	1.3
	Bound SO_2 (after 2 days)	0	12.6
		160	12.3

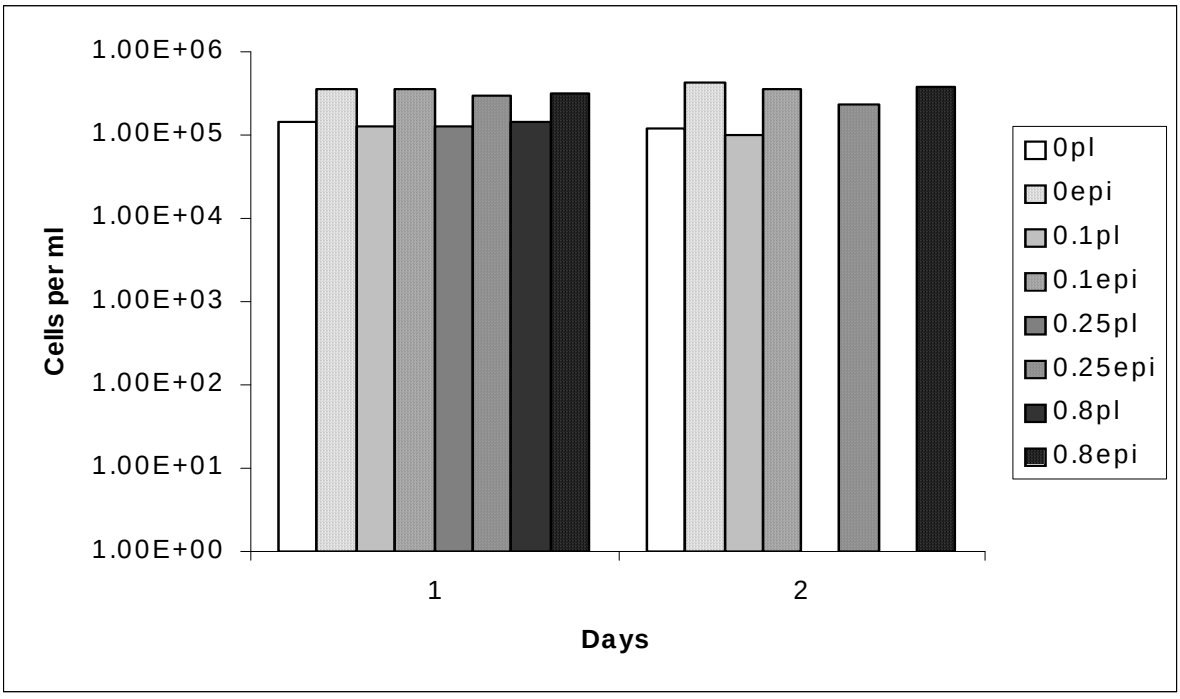

Fig. 3.4 Effect of different molecular SO_2 concentrations on the viability (microscopic epifluorescence enumeration) and culturability (plating on YPD agar medium) of *B. bruxellensis* B3a in fined red wine. Key: epi - epifluorescence count; pl - plate count. Values are the mean of triplicate cultures.

Fig. 3.5 shows the short-term effect of molecular SO_2 on *B. bruxellensis* B3a. Plate counts decreased almost 100-fold within 30 min, and 1000-fold within 2 h, in wine to which 0.64 mg l^{-1} molecular SO_2 was added. It was decided to differentiate between cells with a high epifluorescence intensity of 5 to 10 from those with an intensity lower than 5. The intensity of the control was 10. Bright cells with an epifluorescence intensity exceeding 5 decreased after 120 min and, after 330 min, no additional cells were counted on the plates and none showed an epifluorescence of greater than 5.

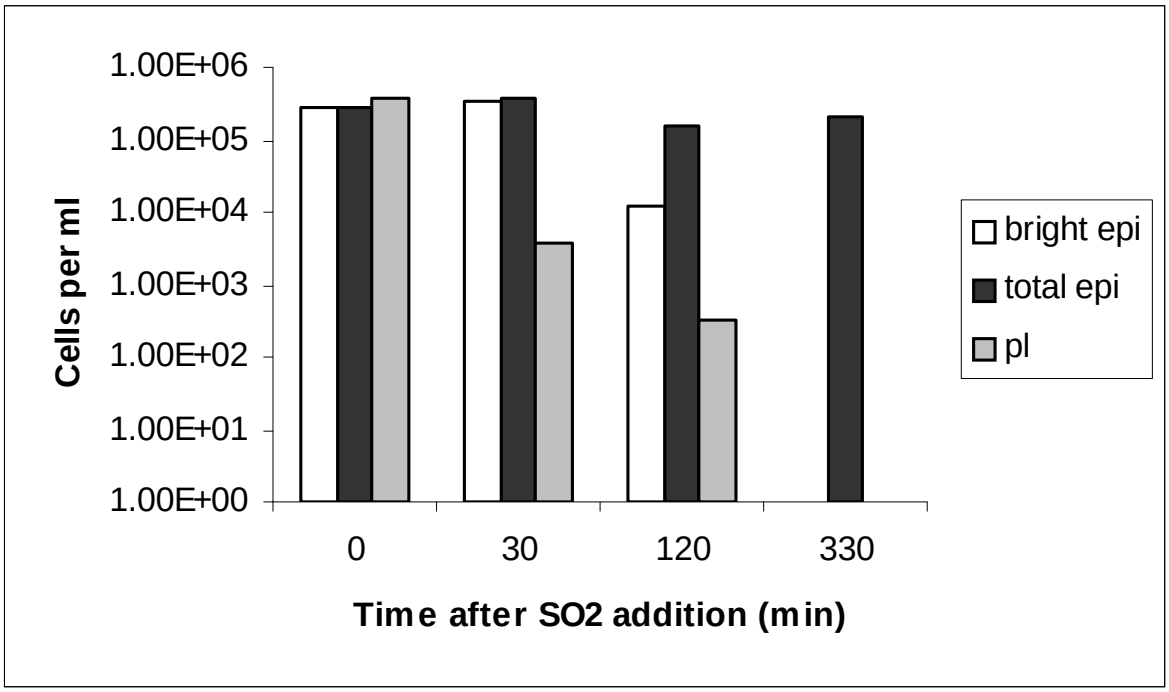

Fig. 3.5 The short-term effect of molecular SO_2 (0.64 mg l^{-1}) on the viability (microscopic epifluorescence enumeration) and culturability (plating on YPD agar medium) of *B. bruxellensis* B3a in red wine. Key: total epi - total epifluorescence counts; bright epi - number of cells which had an epifluorescence intensity higher than 5; pl - total plate count. Values are the mean of triplicate cultures.

In Fig. 3.6, the effect of different exposure times of *B. bruxellensis* to molecular SO_2 can be seen. Exposure of the cells to molecular SO_2 for only 5 min prior to acetaldehyde addition reduced the cell culturability by 100-fold and also the number of cells with an epifluorescence intensity higher than 5. A prior exposure time of 45 min to molecular SO_2, however, completely inhibited the cells from growing on the plates and no cells with an epifluorescence intensity higher than 5 were observed

(Fig. 3.6). It is also clear from Table 3.2 that the epifluorescence intensity of individual cells differed after the addition of SO_2.

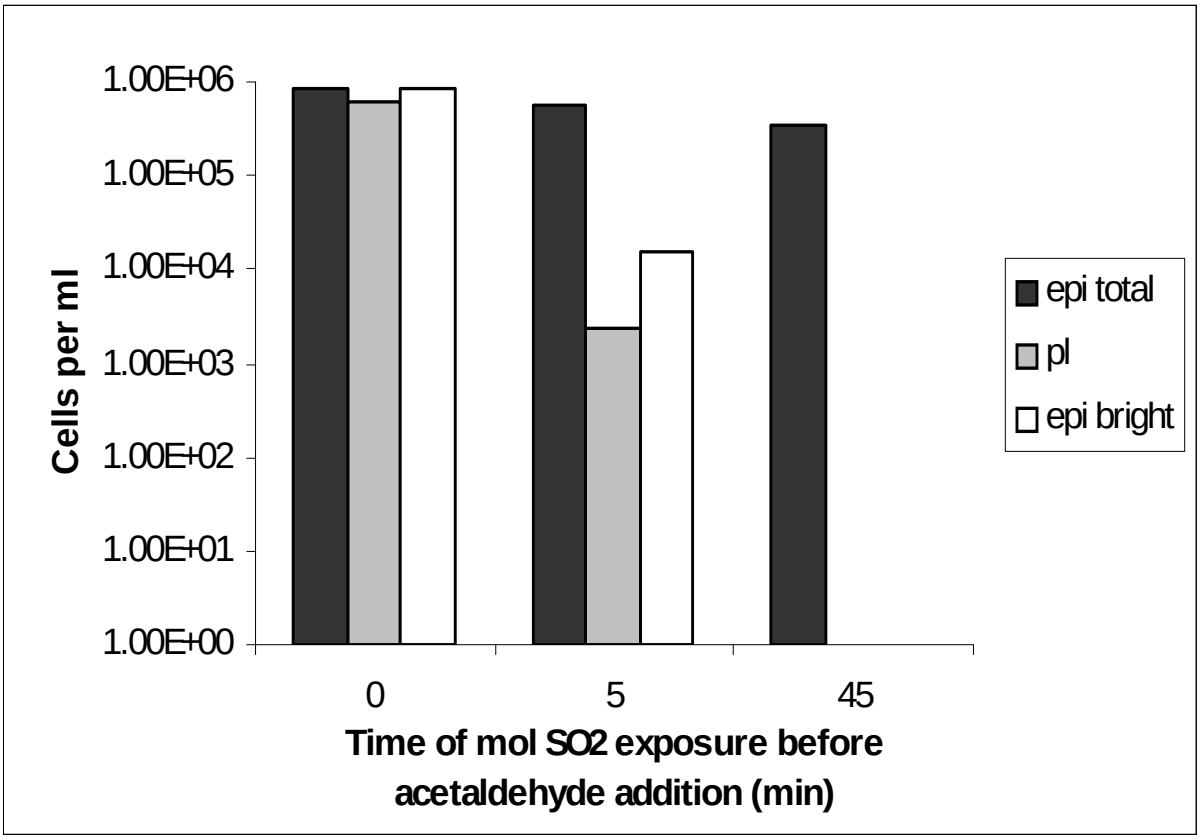

Fig. 3.6 Effect of different exposure times (0, 5 and 45 min) of molecular SO_2 (0.64 mg l^{-1}) on the viability (microscopic epifluorescence enumeration) and culturability (plating on YPD agar medium) of *B. bruxellensis* B3a in red wine. In the wine where 0 min of exposure was tested, the molecular SO_2 was bound with acetaldehyde 5 min before inoculation with *B. bruxellensis* B3a. The plating and epifluorescence counts were conducted two days after the SO_2 exposure. Key: total epi - total epifluorescence counts; bright epi - number of cells that had an epifluorescence intensity higher than 5; pl - total plate count. Values are the mean of triplicate cultures.

B. bruxellensis B3a cells were also exposed to molecular SO_2 in the late stationary phase (OD_{600}=3). At this growth stage, the yeast cells retained their sensitivity to molecular SO_2, with a concentration of 0.25 mg l^{-1} preventing the cells from being culturable (results not shown).

Wine with low concentrations of O_2 before SO_2 addition (this wine was not sparged with N_2 before the experiment) was also inoculated with *B. bruxellensis* B3a to test its sensitivity under more aerobic conditions (Fig. 3.7). *B. bruxellensis* B3a was

quite sensitive to molecular SO_2 as reflected in its viability and culturability; after 2 days no colonies were detected for any samples from sulfited wine. After 9 days however, about 10^2 cfu ml^{-1} started to grow on the plates where 0.25 mg l^{-1} molecular SO_2 was added. There was no growth at higher molecular SO_2 concentrations. At this stage, the wine was aerated and transferred to new vials, which were then filled completely. As a result, the *B. bruxellensis* B3a plate counts increased to $3.7x10^5$ cfu ml^- at day 14. The control for this experiment did not receive any O_2 at day 9 (at this stage the plate counts were $7.5x10^4$ cfu ml^{-1} and the epifluorescence intensity 3.6). Fourteen days later, at a stage when the epifluorescence intensity was 2.5 and the plate count was 16 cfu ml^{-1}, the wine was aerated again. This resulted in an increase in the epifluorescence intensity to 6.7 and the plate count to $7.8x10^5$ cfu ml^{-1} 10 days later.

Table 3.2 The effect of different SO_2 exposure times on *B. bruxellensis* B3a in wine (determined after 2 days).

SO_2 treatment	SO_2 exposure time (min)	Intensity of some individual cells at time of measurement	Average Intensity
Molecular SO_2 (0.64 mg l^{-1}) tested on short-term viability	0	8.8, 10.4, 10.2, 11.8, 12.5, 13.5, 9.8, 8.2, 15.2, 8.0, 12.9, 10.4, 4.9, 8.0	10
	30	1.2, 1.4, 9.1, 8.7, 8.7, 11.6, 7.2, 7.1, 9.1, 6.8, 8.4, 7.1, 8.2, 8.8, 5.0, 7.5, 7.7, 8.9	7.4
	120	2.8, 4.5, 5.0, 1.9, 1.4, 1.3, 1.3, 1.2, 1.4, 1.1, 1.1, 1.0	2
	330	1.1, 1.1, 1.0, 1.2, 1.1, 1.1, 1.2, 1.2, 1.2, 1.2, 1.2, 1.1, 1.1, 1.1, 1.2, 1.2, 1.1, 1.2,1.2	1.1
Molecular SO_2 (0.64 mg l^{-1}) exposure before bound with acetaldehyde	0	12.1, 11.1, 10.1,10.2, 9.8, 10.2, 9.9, 10.2, 10.8, 10.5, 10.2	10.5
	5	1.3, 2.0, 5.7, 5.1, 5.7, 1.4, 1.3, 1.2, 3.0. 1.6, 1.5, 5.8	3.0
	45	1.4, 1.4, 1.6, 1.4, 1.3, 1.2, 1.2, 1.1, 1.4, 1.3, 1.3,1.3, 1.1	1.3

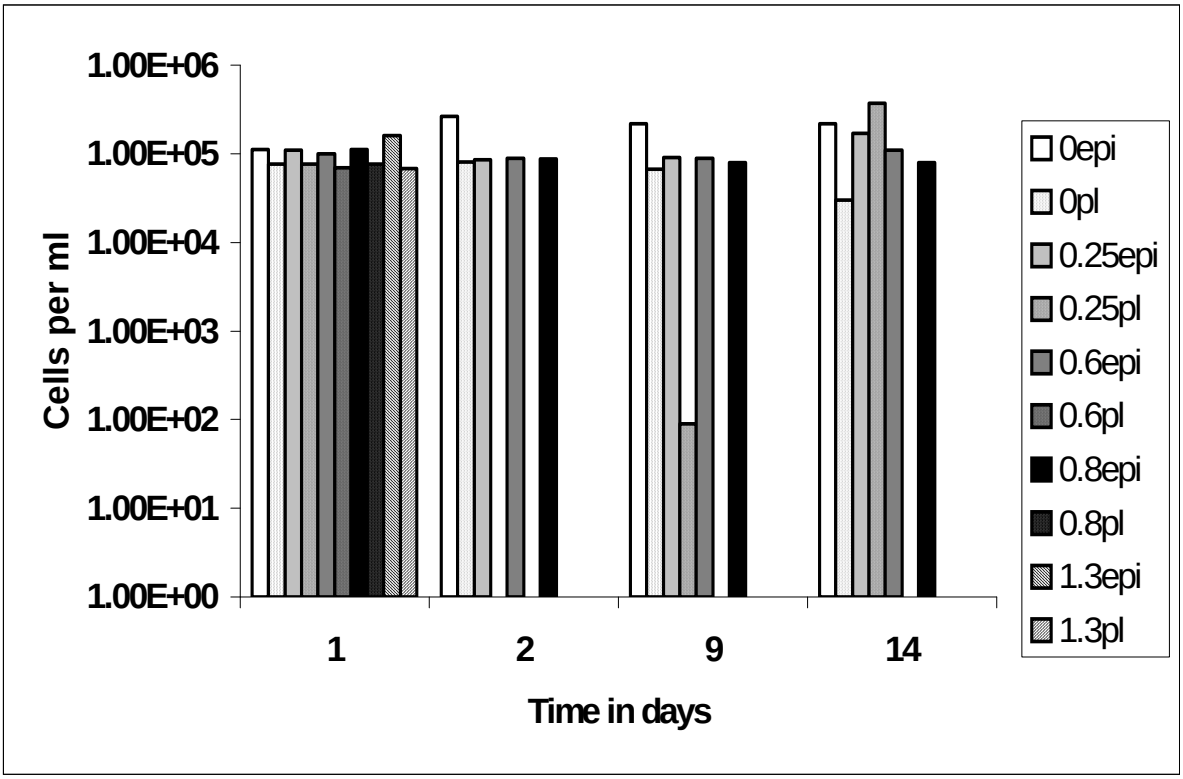

Fig. 3.7 Effect of different molecular SO_2 concentrations on the viability (microscopic epifluorescence enumeration) and culturability (plating on YPD agar medium) of *B. bruxellensis* B3a in fined red wine over time under semi-aerobic conditions. O_2 was added at day 9 to all the samples except the control. Values are the mean of triplicate cultures.

3.4 DISCUSSION

A major aspiration of the wine industry is to reduce the risk of spoiled wine caused by unintended microbial activity that leads to faults such as volatile acidic and phenolic aromas (vinegary and 'Brett' taints). While the problems associated with two of the main wine spoilage microbial groups, acetic acid bacteria (*e.g.*, *Acetobacter*) and *Brettanomyces* yeasts, have generally been considered controllable by winemakers, recent evidence suggests that wine acetification (high volatile acidity or VA) and 'Brett' spoilage are recurring problems that require further analysis and implementation of control measures (Du Toit and Pretorius 2002, Coulter *et al.* 2004, Kramer and Noonan 2004). To understand fully how to prevent and control the formation of spoiler compounds such as volatile acids (acetic acid) and volatile phenols (4-ethylphenol and 4-etylguaiacol), it is important to undertake critical analysis of the many factors associated with their development. Such investigations should include a review of the various types of spoiler bacteria and yeasts, as well as

the winemaking conditions that increase the ability of these microorganisms to flourish (Kramer and Noonan 2004). The present study focussed on the effects of different SO_2 and O_2 concentrations on the viability and culturability of *Acetobacter* and *Brettanomyces* in wine. Two strains isolated from bottled commercial red wines, *A. pasteurianus* A8 and *B. bruxellensis* B3a, were selected for our investigation.

Oxygen is considered necessary for the growth of acetic acid bacteria, as they are classified as strictly aerobic microorganisms. *A. pasteurianus* A8, however, had the ability to survive for a prolonged period (71 days) under relatively anaerobic conditions in wine. During this time, the plate counts for *A. pasteurianus* A8 were found to be up to a 100-fold lower than the epifluorescence counts. This viable but non-culturable state of the surviving *A. pasteurianus* A8 cells was negated by the addition of O_2 after 17 and 48 days of incubation, and this finding correlates well with results reported by Millet *et al*. (1995). Furthermore, the notion that dissolved O_2 in wine normally takes about 10 days to disappear through oxidation reactions (Ribéreau-Gayon *et al.* 2000) was confirmed by the relatively small differences observed between the plate and epifluorescence counts at similar time points in our results. Therefore, it appears that wine has the potential to support successive increases in the culturability of acetic acid bacteria when aerated, even after a prolonged period of time.

For winemaking, the practical significance of this conclusion is that care should be taken when wine is being transferred to exclude O_2 when acetic acid bacteria are present in the wine. Failure to exclude O_2 will probably result in the proliferation of acetic acid bacteria and a concomitant increase in the volatile acidity (VA) of the wine (Du Toit and Pretorius 2002). It therefore seems that O_2 is necessary to induce acetic acid bacteria to be completely culturable in wine, but the survival of bacteria in wine deprived of dissolved O_2 is still uncertain.

The activated charcoal that was used in some of our experiments to remove phenolic compounds probably also removed other components from the wine that also enabled *A. pasteurianus* A8 to survive. This might explain the difference in bacterial cell numbers found between the fined and non-fined wines. The interaction between phenolic compounds and surviving acetic acid bacteria in wine is at present unclear but presents a worthwhile proposition for further investigation.

Few studies have focused on the SO_2 resistance of wine-related acetic acid bacteria, with the work mostly being done on grape juice (Du Toit 2000). In the

present study, it was found that lower concentrations of SO_2 did not drastically affect the culturability and total cell numbers of *A. pasteurianus* A8, but the viability, as measured by the epifluorescence, was slightly decreased. At higher SO_2 concentrations, however, both the total cell number and viability of *A. pasteurianus* A8 were more affected, with the cells being unable to grow on agar plates. It has been reported that the levels of molecular SO_2 needed to prevent the growth of *Acetobacter* were dependent on the strain and ranged from 0.05 to 0.6 mg l^{-1}, whereas 0.8 mg l^{-1} molecular SO_2 was needed to prevent the growth of *Gluconoacetobacter hansenii* (Du Toit 2000; Du Toit and Pretorius, 2002). The latter SO_2 concentration correlated well with the value that we found necessary to curtail culturability of *A. pasteurianus* A8. The practical implication of these results is that winemakers can suppress the unwanted growth of acetic acid bacteria in wine by maintaining a relatively high concentration of SO_2 throughout the process of winemaking. But it is important to note that the SO_2 did not completely eliminate *A. pasteurianus* A8 at the concentrations tested in this study, and winemakers should therefore always use SO_2 in conjunction with other best practice winemaking procedures (e.g., use of healthy grapes as starting material, high standard cellar hygiene, appropriate pH, temperature, oxygen levels and other fermentation conditions, etc.) in order to limit the risk of bacterially-induced faults in wine.

B. bruxellensis B3a was found to be more sensitive than *A. pasteurianus* A8 to molecular SO_2, with 0.25 mg l^{-1} molecular SO_2 preventing it from growing on media as well as drastically reducing its viability. The action of SO_2 on *Brettanomyces* seemed to be rapid, with cells having their viability greatly reduced and losing their culturability completely within 330 min of exposure. The uptake of free molecular SO_2 by *Brettanomyces* is fast, as exposure of the cells to molecular SO_2 for only a short period of time showed. In *Saccharomyces cerevisiae*, the uptake of SO_2 has been proved to be a rapid process as well, with most SO_2 being taken up within 2 min (Macris and Markakis 1974). In this study, higher epifluorescence counts for some *B. bruxellensis* B3a cells than for others were observed after they had been exposed to molecular SO_2. This could be due to some cells being more resistant to SO_2 or some taking up more SO_2 than others were able to. Further research is required to explain whether *Brettanomyces* is able to produce volatile phenols in wines containing appropriate levels of molecular SO_2. The bound form of SO_2 did not affect *A. pasteurianus* A8 and *B. bruxellensis* B3a. The maintenance of a lower pH in wine is

therefore of critical importance to the winemaker due to the fact that a higher percentage of free SO_2 exists in the molecular form at a lower pH.

It was also clear from this study that the addition of O_2 (which might occur during racking and other transfer or transport) to wine that contains low concentrations of SO_2, can support the survival and growth of *Brettanomyces*. When it is suspected that wine has been contaminated by *Brettanomyces*, care should be taken to avoid excessive O_2 exposure and molecular SO_2 concentrations should be checked and regularly adjusted to 25 to 35 mg/L free SO_2. At excessive concentrations, SO_2 might affect the aroma and colour of red wine; the reaction of SO_2 with the red form of anthocyanins leads to the bleaching of red wine colour (Ribéreau-Gayon *et al.* 2000). Since low numbers of *Brettanomyces* contaminants in wine cannot be detected by traditional plating methods, wineries should, as part of their quality control system, consider monitoring the level of microbial contamination and proliferation by the use of epifluorescence.

In conclusion, this study has reiterated the importance of maintaining appropriate concentrations of molecular SO_2 and limiting O_2 exposure as tools to limit wine spoilage by acetic acid bacteria and *Brettannomyces* yeasts. However, this study was conducted by using a single strain of *A. pasteurianus* and *B. bruxellensis* in only one type of wine. It cannot be ruled out that strain differences and wine composition have an effect on these type of results. It is therefore important that future research includes other strains of acetic acid bacteria and *Brettanomyces,* and that the fermentations be conducted in wine derived from different grape varieties and sources. However, it is important to note that other factors apart from SO_2 and O_2 can play important roles in the spoilage of wine by acetic acid bacteria and *Brettanomyces* yeasts (Dias *et al.* 2003). For instance, the low pH, low temperature and the fining of wine with certain protein-based fining agents can also lead to a reduction in the number of unwanted bacterial and yeast cells surviving in wine (Murat and Dumeau 2003). A holistic approach is therefore required to control wine spoilage by acetic acid bacteria and *Brettanomyces* yeast.

3.5 ACKNOWLEDGEMENTS

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4. THE EFFECT OF MICRO-OXYGENATION ON THE PHENOLIC COMPOSITION, QUALITY AND CERTAIN WINE MICROORGANISMS OF DIFFERENT SOUTH AFRICAN RED WINES

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The effect of micro-oxygenation treatments on the composition of different South African red wines was evaluated. In some wines, micro-oxygenation led to an increase in colour density, with a corresponding change in colour, due to the formation of polymeric pigments. This increase also led to the red colour becoming more resistant to the bleaching effect of SO_2. Micro-oxygenation also seems to be more effective in increasing the colour densities of younger than older red wines, although total red pigments were found to be highest in an older red wine that had received micro-oxygenation, with differences in the gelatine index also observed over time. The micro-oxygenated and barrel-matured wines also had lower concentrations of catechin and the procyanidin B1, with a corresponding increase in polymeric pigment and polymeric phenols. Micro-oxygenation led to higher acetic acid bacteria counts, although no increase in volatile acidity was observed in these wines. Younger red wines that had undergone micro-oxygenation were preferred by a tasting panel. In an older red wine prolonged treatment with micro-oxygenation led to the wine becoming over-aged, with an increase in the barnyard/medicinal character, which corresponded with an increase in *Brettanomyces* counts. Micro-oxygenation can be used to increase the quality of young red wines, but further research is needed in this area.

Micro-oxygenation is a process during which measured amounts of oxygen (O_2) are introduced to wines with the aim of bringing about desirable changes. Some of these include enhanced colour stability and intensity, softening of astringent tannins and decreased reductive and vegetative aromas (Parish *et al.*, 2000). Certain lactic acid bacteria strains, acetic acid bacteria and *Brettanomyces* yeasts are considered spoilage microorganims of wine. Of these, acetic acid bacteria and *Brettanomyces* are aerobic microorganims and the introduction of O_2 into wine can lead to their growth and subsequent spoilage of the wine (Du Toit *et al.,* 2005) Micro-oxygenation has also been suggested as a means of replacing expensive oak barrels, by combining the process with alternative oak products in a stainless steel tank. During micro-oxygenation O_2 is supplied in the form of compressed gas via a micron-size diffuser positioned close to the bottom of the tank. The technique of micro-oxygenation has been developed largely due to the work of Patrick Ducournau and Thierry Lemaire (Parish *et al.*, 2000). However, during micro-oxygenation O_2 should be supplied at a slower rate than its rate of consumption by the wine to prevent unwanted accumulation in the headspace of the tank (Nikfardjam & Dykes, 2003).

Although this technique has been in use for several years, limited scientific publications are available. This is probably due to the large-scale experimental set-up necessary for the scientific investigation of its effect on wine, because most of the systems need a path length of 2.2 m for the O_2 bubbles to dissolve in the wine. The effect of micro-oxygenation on the colour development of South African red wines and the effect it has on younger red wines compared to older red wines is not clear. How a micro-oxygenation treated wine compares to the same wine being matured in an oak barrel is also not clear. The effect of micro-oxygenation on the microbial population of red wine is also not understood. This study was undertaken to ascertain the effect of micro-oxygenation after malolactic fermentation on the quality and composition of different commercial South African red wines.

4.1 MATERIALS AND METHODS

4.1.1 MICRO-OXYGENATION

Different commercial cellars participated in this project with different red wines. These are listed in Table 4.1 with the dosage of O_2 added to each tank. In all the wines, a control tank of similar size was kept that received no micro-oxygenation. For

wines A, B and D, the dosage and duration of the treatment were determined by the specific winemaker. All the wines were made according to standard red wine production methods, to the preference of the winemaker, with micro-oxygenation starting just after completion of malolactic fermentation in wines A and B. In wines C and D the treatment started seven months after the completion of malolactic fermentation. In wines A, B and D, the same oak staves (all French oak) at the same dosage were used in both the control and micro-oxygenation tanks. In wine C, American oak staves (medium toast plus) were added to 70% of the internal surface of a 300 L oak barrel, which followed the supplier's recommendations to simulate an oak barrel. The same Pinotage wine was also matured in a 300 L American oak barrel (medium toast plus). All wines were treated with micro-oxygenation equipment supplied by Parsec from Italy, which is distributed in South Africa by Tonneleria Nacional. This equipment doses the required O_2 dosage in mg/L/month. During the different treatments, the free SO_2 levels were kept between 25 and 35 mg/L, and the treatments were all conducted at cellar temperatures (15-20°C).

TABLE 4.1

Different commercial wines with their origins and treatments.

No	Cultivar and year	Origin of wine	Treatment
A	Cabernet Sauvignon 2002	Paarl	0, 1.5 and 3 mg O_2/L/month with oak staves starting just after the completion of malolactic fermentation.
B	Red blend 2003	Stellenbosch	0 and 4 mg O_2/L/month with oak staves starting just after the completion of malolactic fermentation.
C	Pinotage 2004	Paarl (University's cellar)	0, 1.5 and 3 mg O_2/L/month with oak staves starting seven months after the completion of malolactic fermentation. The same wine was also matured in an oak barrel of the same wood as the staves used (USA MT+).
D	Shiraz 2003	Worcester	0 and 3 mg O_2/L/month with oak staves starting seven months after the completion of malolactic fermentation.

4.1.2 PHENOLIC ANALYSIS

Different spectrophotometric analyses were conducted with a spectrophotometer according to Iland *et al*. (2000). These included wine colour density, modified wine colour density, wine colour hue, modified wine colour hue, total red pigments, total phenolics, degree of red pigment colouration, estimate of SO_2 resistant pigments and modified degree of red pigment colouration (the modified version of the analysis negates the effect of pH and SO_2 on the analysis). The fractions of co-pigmented

anthocyanin, free anthocyanins and polymeric colour pigment content in the red wines were also determined according to the method of Boulton (2001). The estimate of SO_2 resistant pigments and modified colour density were used to analyse these fractions. Total anthocyanin concentrations, total tannin concentrations, HCl index value (index of polymerisation of procyanidins) and gelatine index values (index of reactivity of phenolic molecules in wine towards gelatine) were conducted according to Ribéreau-Gayon *et al*. (2000).

Reverse Phase High Performance Liquid chromatography was performed on a Angilent 1100 series HPLC system equipped with a diode array detector (Angilent Technologies, Palo Alto, CA, USA). Data processing was done with Chemstation software (Hewlett-Packard, Waldbronn, Germany). A 100 mm x 4.6 mm Chromolith Peformance RP-18e column and pre-column was used (Merck). The mobile phases used were: Solvent A containing de-ionized water adjusted to a pH of 2.04 with Ortho-phosporic acid (Reidel-de Haën), and Solvent B, consisting of Acetonitrile (Chromasolve, Reidel-de Haën) with 20% of Solvent A. A flow rate of 2ml/min was used and column temperature was maintained at 35ºC. The Gradient profile used can be seen in Table 4.2.

TABLE 4.2.
Gradient profile of the HPLC analysis.

Time (min)	% Solvent A	% Solvent B
0	99	1
2	99	1
17	96	4
31	90	10
55	84	16
75	75	25
80	20	80
84	20	80
85	99	1

Quantification was done using external standards: (+)-Catechin Hydrate (Fluka), gallic acid (Fluka), vanillic acid (Fluka), *p*-coumaric acid (Sigma), malvidin-3-glucoside (Fluka), ellagic acid (Fluka), quercetin-3-glucoside (Fluka) and quercitin (Extrasynthèse).

Flavan-3-ols were quantified at 280 nm as mg/L catechin units, benzoic acids at 280 nm as mg/L vanillic acid units, cinnamic acids at 320 nm as mg/L *p*-coumaric

acid units, anthocyanins at 520 nm as mg/L malvidin-3-glucoside, flavonol-glucoside units at 360 nm as mg/L quercetin-3-glucosides and flavonol aglycones at 360 nm as mg/L quercetin units.

4.1.3 SENSORY EVALUATION

Two tastings of wine A were held during the course of the experiment. One was held eight weeks after the treatment started and the other after 12 weeks. The panel consisted of 10 experienced judges and the wines were tasted blindly in random order. At each tasting the panel had to conduct three triangle tests, and one rank tasting where they had to rank the wines from least acceptable to most acceptable.

Wine C was evaluated after 12 and 24 weeks of micro-oxygenation. For the 3 mg O_2/L/month treatment after 24 weeks, samples were also included that were drawn after 16 weeks of treatment. In samples of the 3 mg O_2/L/month treatments, the SO_2 concentration was also increased from 17 mg/L free to 30 mg/L free 12 h before the tasting. This was done to ascertain whether raising the SO_2 concentration had an effect on the sensory profile of the wine. The panel thus had to evaluate a control sample, a 1.5 mg O_2/L/month treated sample, a 3 mg O_2/L/month treated sample (after 16 and 24 weeks) as well as a 3 mg O_2/L/month sample after 24 weeks in which the SO_2 concentration had been increased. At these tastings members of the South African Pinotage Association, all experienced tasters of Pinotage, were used as a panel. The members had to indicate on an unstructured 10 cm line scale the intensities of fruitiness, oak associated flavours, astringency and bitterness at the initial tasting. It was then decided to change these characteristics to fruitiness, spiciness, vanilla/butterscotch, oak wood/coconut, barnyard/medicinal, oxidized/aged, bitterness and astringency for the 24 week tasting. At this tasting the each panel member also had to rank the wines according to preference. Wines B and D did not undergo sensory evaluation.

4.1.4 MICROBIOLOGICAL ANALYSIS

Acetic acid bacteria and *Brettanomyces* yeasts in wine C were enumerated by plating out the wine on selective media at the beginning, after four, 14 and 20 weeks of micro-oxygenation. For the enumeration of acetic acid bacteria, the culture medium consisted of 57 g/L Man Rogosa Sharp (MRS) medium (pH to 5 with HCl, 20 g/L agar) to which 20% sterile red wine C was added after sterilisation. This medium was supplemented with 7 mg/L penicillin and 50 mg/L pimaricin (Actistab, Gistbrocades,

Anchor Yeast Biotechnologies) to eliminate lactic acid bacteria and yeast, respectively. *Brettanomyces* strains were isolated on a YPD medium [containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose (pH 4.8 adjusted with HCl, 20 g/L agar)], supplemented with 50 mg/L chloramphenicol (Sigma) and 300 mg/L cyclohexamide (Sigma) to eliminate bacteria and non-*Brettanomyces* yeasts, respectively. Plates were incubated at 30°C for 10 days before counting.

4.1.5 STANDARD WINE ANALYSES

Vinlab Pty (Ltd), an accredited laboratory, conducted certain standard analyses in wine A. Analyses included pH, total acidity, alcohol, residual sugar, volatile acidity, extract, free and total SO_2. These analyses were done every three weeks according to the methods described by Iland *et al.* (2000). After 2002, analyses, except the free and total SO_2, were conducted in wines B, C and D with the GrapeScan FT 120 instrument (Foss Electric, Denmark) (Nieuwoudt *et al*., 2004). The instrument utilises Fourier transform infrared spectroscopy (FT-IR). All samples were degassed by filtration before the analysis, using the Filtration Unit (type 70500, Foss Electric, Denmark) with filter paper circles graded at 20-25 μm and with a diameter of 185 mm. Sulphur dioxide analyses were done with the Metrohm titration unit (Metrohm Ltd., Switzerland).

4.1.6 STATISTICAL ANALYSIS

The sensory results of wine A were statistically analysed as follows: The triangle tests were tested at the 0.05 and 0.01 probability level using the significance in triangle test (p=⅓). The ranking tasting was analysed at the probability level of 0.01 and 0.05 according to Basker (1998). The ranking data of wine C was statistically analysed as with wine A and, on the intensities data, bootstraps analysis was performed according to Efron & Tibshirani (1993).

4.2. RESULTS AND DISCUSSION

4.2.1 COLOUR AND PHENOLIC DEVELOPMENT

As mentioned, normally one of the main obstacles to micro-oxygenation research is the large scale of experiments required, which makes it difficult to repeat. This research was conducted on only one sample per treatment and thus one should not

consider absolute values, but rather the tendencies observed. In wine A, micro-oxygenation led to a decrease in total phenolic concentrations (which was high in this wine) after seven to nine weeks and were lower in the treated wines after 15 weeks (results not shown). A slight increase in total phenolic levels in the control tank was probably due to the contact of the wine with the oak staves, which can impart hydrolysable tannins to the wine (Peuch *et al*., 1999). The colour density (Fig. 4.1) increased in the treated wines and was also reflected in the polymeric colour increasing in the same wines (results not shown).

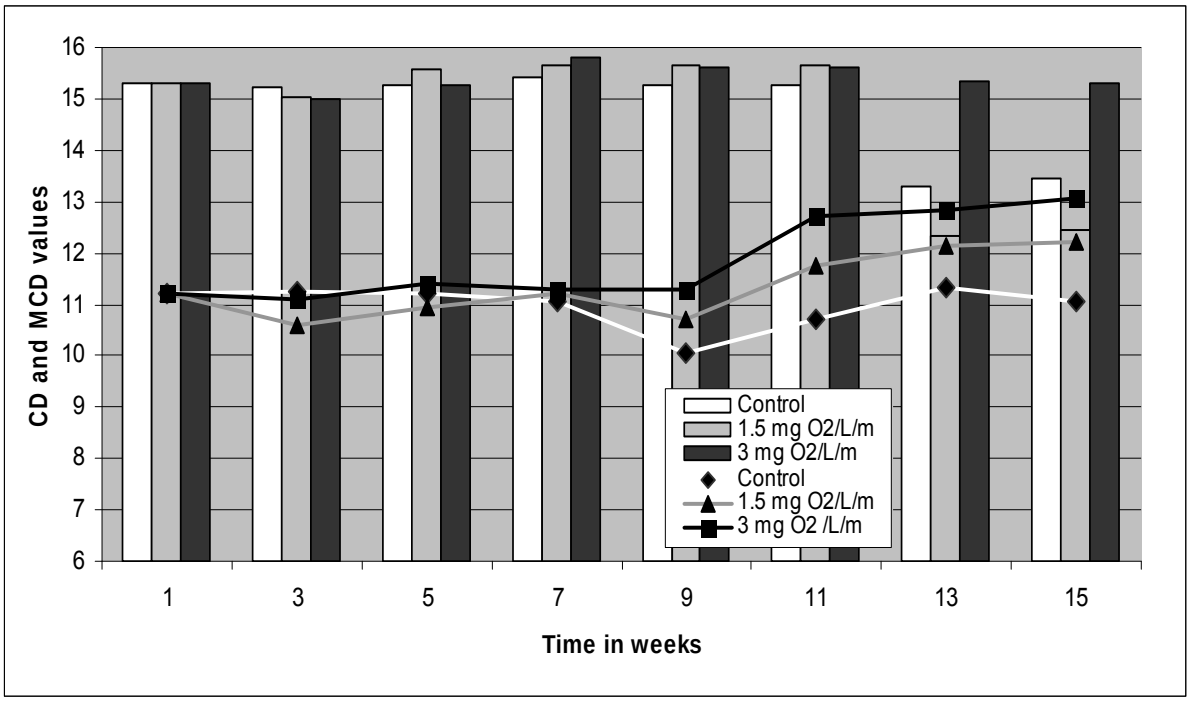


FIGURE 4.1

Colour (CD) (lines) and modified colour (bars) density (MCD) of wine A (see Table 4.1) during micro-oxygenation treatment.

The difference between the colour densities and modified colour densities was also smaller in the treated wines compared to the control for up to 11 weeks of the treatment, with the modified colour density decreasing in the control and the 1.5 mg O_2/L/month sample afterwards. The anthocyanin fraction also decreased more in the treated wines (results not shown). The increase in colour was due to a bigger proportion of colour pigments being in the red form, when incorporated in the polymeric colour form. However, after five weeks the percentage of pigments in the red form decreased, probably due to further polymerisation and precipitation (Fig. 4.2). In wine B an increase in the colour density during 10 weeks of treatment was observed, with the polymeric pigment fraction being the dominant part of this

wine's colour fraction at this stage (Figs. 4.3 & 4.4). These changes are due to colourless anthocyanins being transformed to red pigments due to interactions between nucleophilic C6 or C8 carbons of procyanidins with the electrophilic C4 of an anthocyanin molecule, which forms a colourless flavene. Subsequent aeration, as with racking or the addition of O_2 by micro-oxygenation, leads to the formation of the red form. Oxidation of phenolic molecules also leads to the formation of H_2O_2, which oxidises ethanol to acetaldehyde. The latter can form a bridge between an anthocyanin molecule and procyanidin moiety. The resulting molecule is also coloured and this all leads to the increase in colour density during ageing, as observed in red wine in the barrel. Colour pigments also become more resistant to the bleaching effect of SO_2, because the polymerised fraction of colour is less sensitive to SO_2 bleaching due to steric hindrance. One of the alleged uses of micro-oxygenation is to simulate an oak barrel, leading to the observed colour changes (Es-Safi *et al*., 1999, Santos-Buelga *et al.*, 1999; Fourie, 2005; Monagas *et al*., 2005).

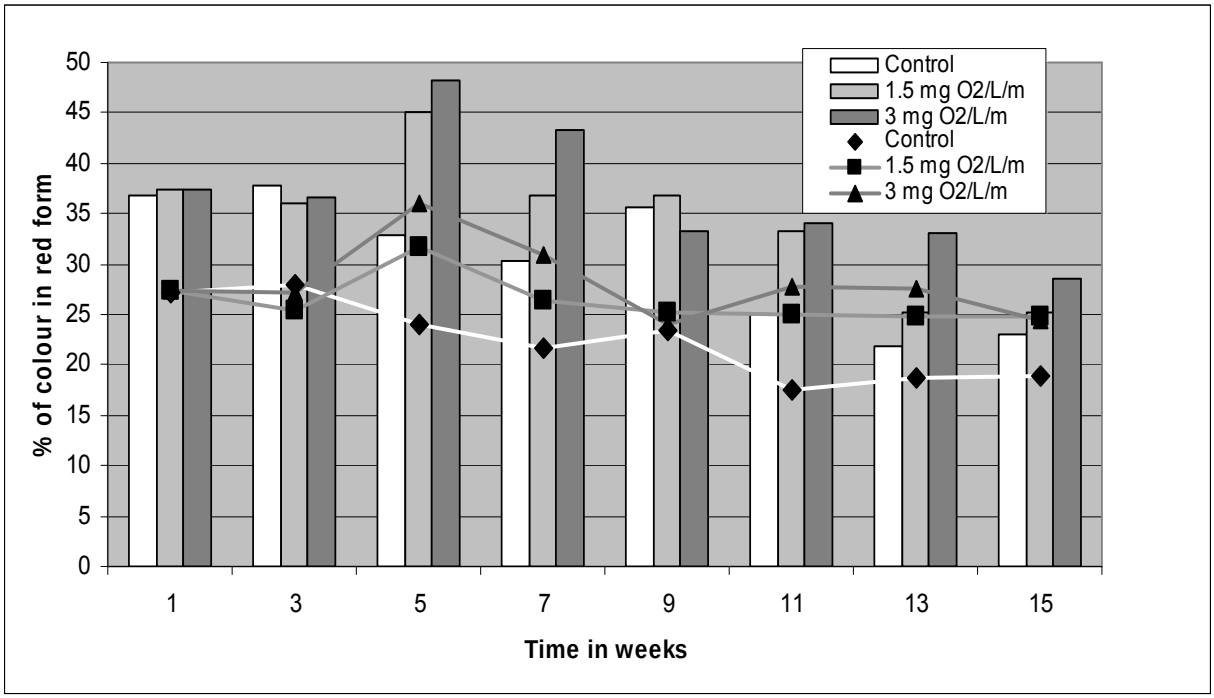


FIGURE 4.2

The percentage (lines) and modified percentage (bars) of colour in the red form of wine A (see Table 4.1) during micro-oxygenation treatment.

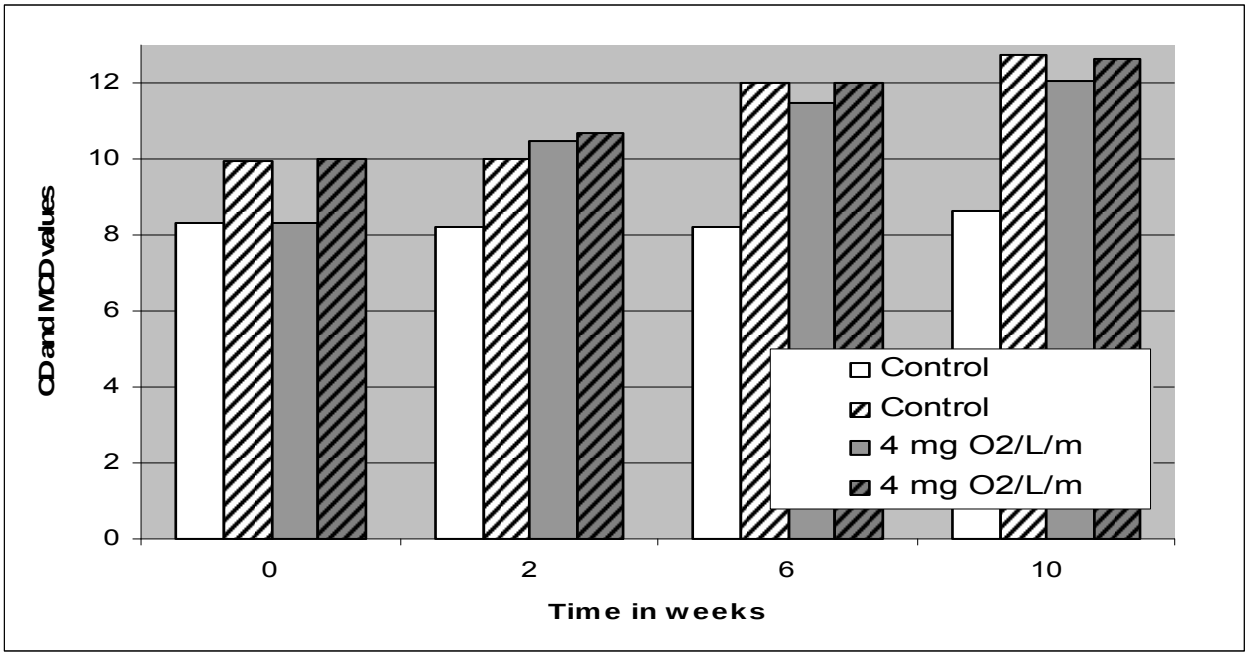


FIGURE 4.3

Colour (CD) and modified colour density (MCD) of wine B (see Table 1) during micro-oxygenation treatment.

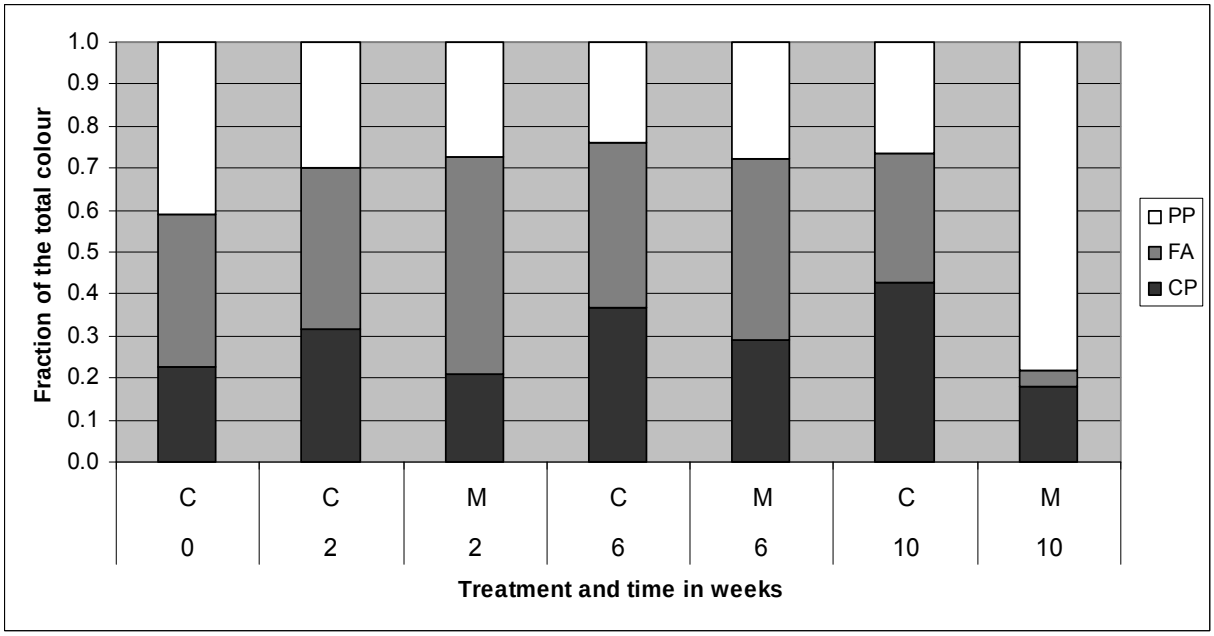


FIGURE 4.4

Development of the fraction of colour of wine B (see Table 1) during micro-oxygenation treatment. C: control tank, M: micro-oxygenation tank receiving 3 mg O_2/L/month (see Table 1). PP: Fraction of colour due to polymeric fraction, FA: Fraction of colour due to free anthocyanins, CP: Fraction of colour due to co-pigmentation.

The addition of O_2 with micro-oxygenation does not always lead to an increase in colour density, as other wines that we monitored showed. This was true for wine D,

which received 3 mg O_2/L/month (Fig. 4.5), as well as wine C, which received 1.5 and 3 mg O_2/L/month for five months (Fig. 4.6).

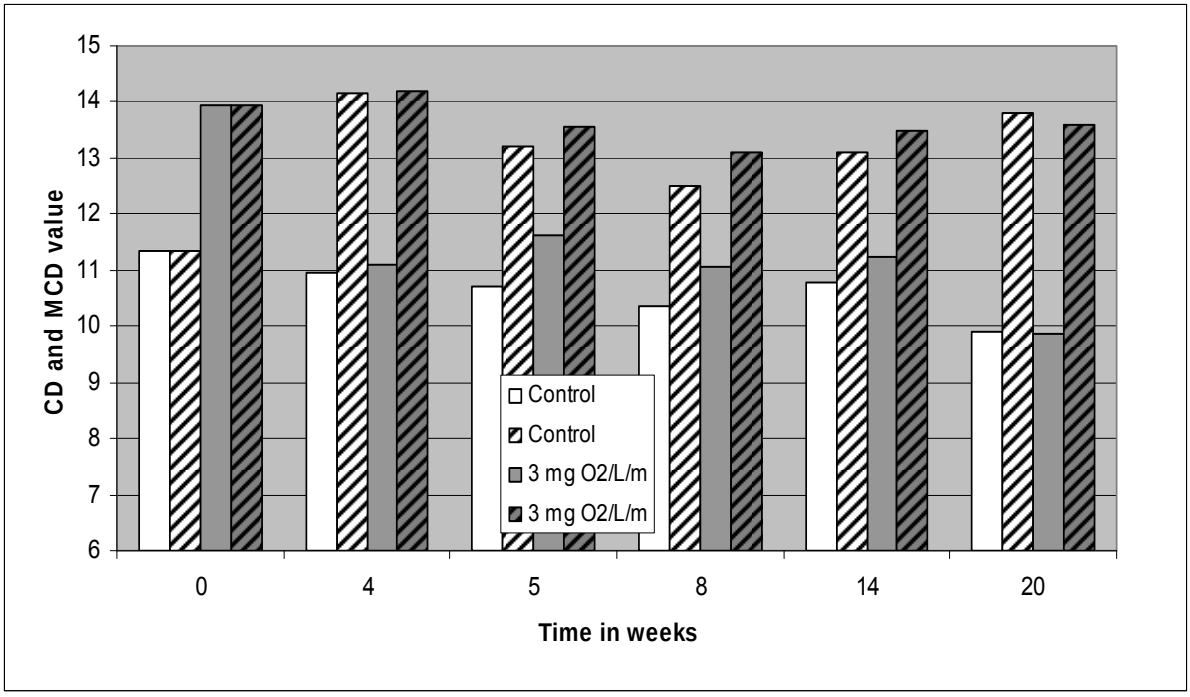


FIGURE 4.5

Colour (open bars) and modified (striped bars) colour density of wine D during micro-oxygenation treatment.

In wine C where 1.5 and 3 mg O_2/L/month were added to the wine, the colour and modified colour densities did not differ dramatically over the 18 weeks' treatment time from that of the wine matured in the control tank and the barrel (results not shown). The modified colour density actually decreased after 10 weeks in all the treatments, but was about 12 in the 3 mg O_2/L/month treatment and barrel wines compared to the control tank and 1.5 mg O_2/L/month treatment which were 11.5 after 18 weeks. Colour density of the 3 mg O_2/L/month tank was also 1 unit higher than that in the control tank at this stage (results not shown). It thus seems that micro-oxygenation does not always increase the colour intensities of red wine. It is more effective in younger red wines just after the completion of malolactic fermentation, when a large part of the anthocyanins are still in the colourless pseudobase form than in an older wine (Ribéreau-Gayon *et al.*, 2000). Total red pigments decreased as expected in all the treatments, but were slightly higher in the treated wines after 15-18 weeks than in the control (Fig. 4.6).

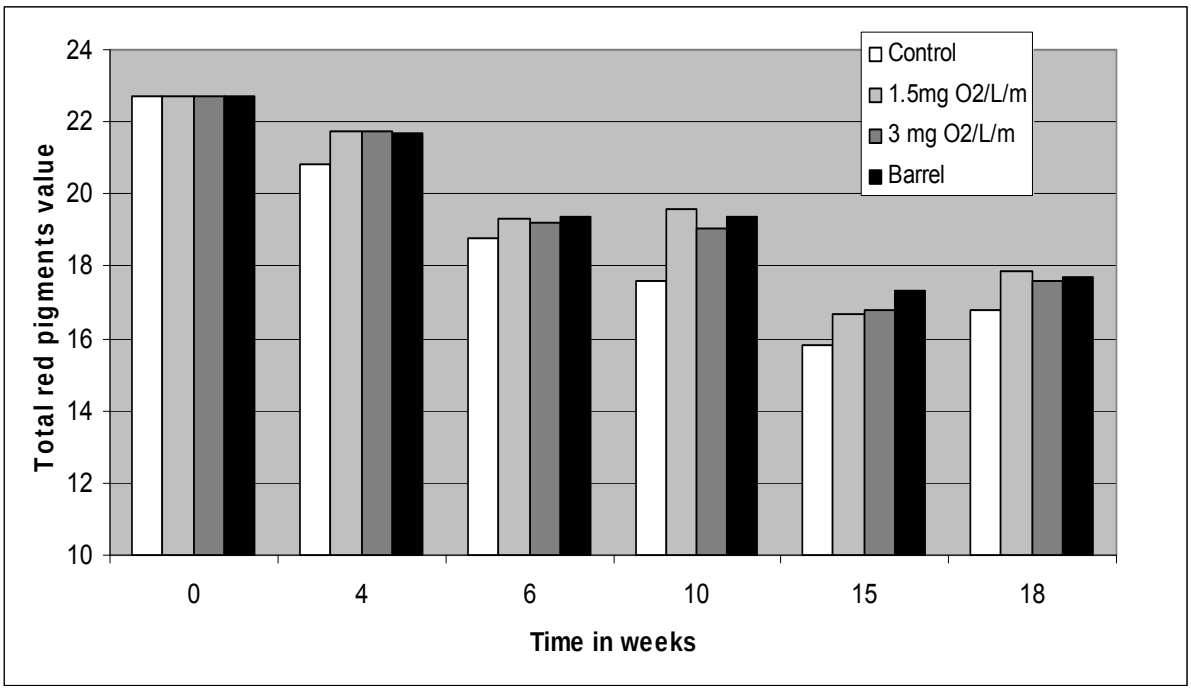


FIGURE 4.6

Total red pigment development of wine C (see Table 4.1) during micro-oxygenation treatment

The HPLC results for wine C can be seen in Table 4.3. Vanillic acid was much higher in the barrel treated wine than in the other treatments, probably due to the higher oak contact. Catechin and procyanidin B1 concentrations decreased with the increasing O_2 addition over time, with the malvidin-3-glucoside concentration being lower in the control wine. This correlates with the spectrophotometric results. The procyanidin B1 concentration can also increase over time due to catechin associations, explaining the higher concentrations in the control wine after 24 weeks. The polymeric pigment increased in the 3 mg O_2/L/month and barrel treatments and was also higher in regard to polymeric phenols after 24 weeks of treatment. This is probably due to more acetaldehyde being produced in these treatments because of the higher O_2 additions, which led to enhanced acetaldehyde polymerisation (Ribéreau-Gayon *et al*., 2000). The polymerisation of procyanidins in wine A was also reflected in an increase in the HCl index of the treated wines (Fig. 4.7).

TABLE 4.3

Concentrations of different phenolic compounds in wine C (see Table 1) initially and after 24 weeks treatment.

		Concentration (mg/L) for each treatment			
Compound	**Initially**	**Control**	**1.5 mg O_2/L/month**	**3 mg O_2/L/month**	**Barrel**
Gallic acid	46.4	56.1	50.3	57.2	47.3
Gentisic acid	1.5	2.6	nd	1.5	nd
Caftaric acid	17.3	16.3	17.5	16.8	17.3
Vanillic acid	2.6	4.1	5.1	3.3	43.7
Catechin	790.2	784.0	704.4	698.6	659.5
Caffeic acid	64.2	59.0	56.8	57.0	52.6
Procyanidin B1	60.0	91.0	93.5	55.6	78.5
p-Coumaric acid	3.6	3.8	4.2	6.4	12.2
Procyanidin B2	49.1	40.8	40.4	40.0	39.8
Epicatechin	90.1	73.6	76.2	68.8	80.5
Delphinidin-3-glucoside	11.2	7.7	7.4	9.6	7.2
Petunidin-3-glucoside	13.8	7.6	9.3	8.5	8.7
Peonidin-3-glucoside	5.5	2.9	4.1	3.4	4.1
Malvidin-3-glucoside	117.9	63.4	83.8	72.0	79.6
Ellagic acid	3.7	4.8	4.5	5.4	3.3
Quercetin-3-glucoside	15.8	12.3	13.6	11.1	14.7
Myricetin	4.3	3.2	5.1	5.1	4.3
Quercetin-3-rhamnoside	5.0	4.2	3.4	4.7	4.0
Malvidin-3-Acetate	40.1	18.4	23.8	24.2	22.4
Quercitin	7.8	4.8	6.4	5.8	5.2
Malvidin-3-p-coumaric acid	19.6	6.8	11.1	9.6	10.0
Polymeric pigment	28.8	29.0	33.9	34.8	35.3
Polymeric phenols	791.6	782.9	947.1	1021.5	1132.0

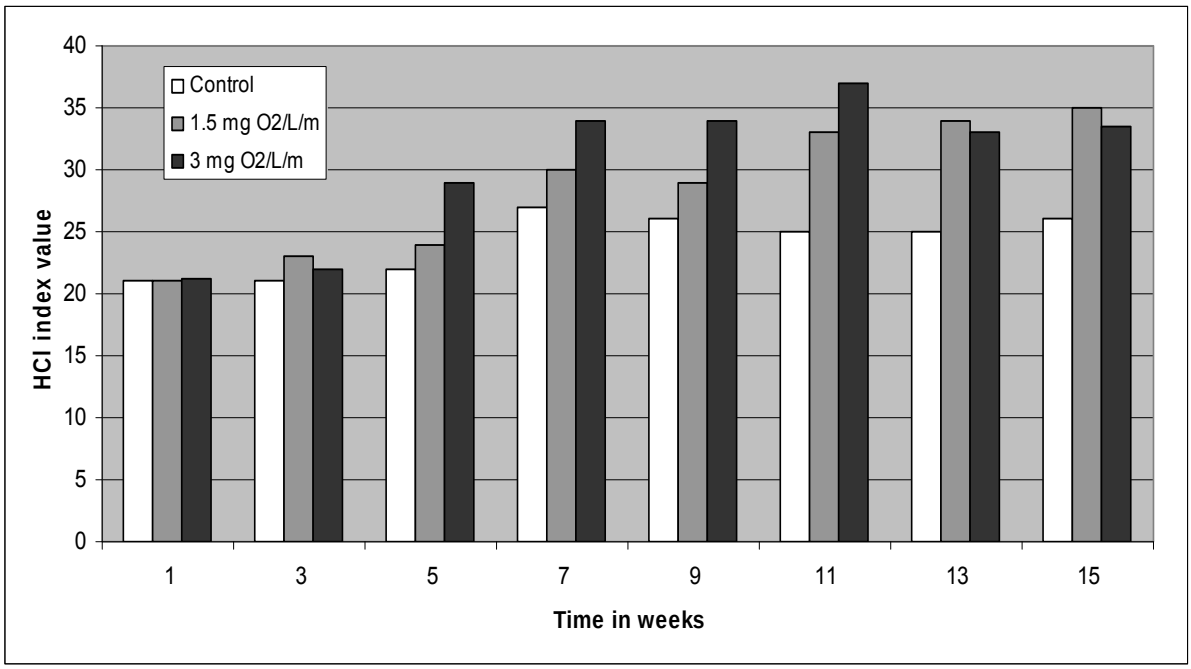


FIGURE 4.7

Degree of polymerisation of procyanidins (HCl index value) of wine A (see Table 1) during micro-oxygenation treatment.

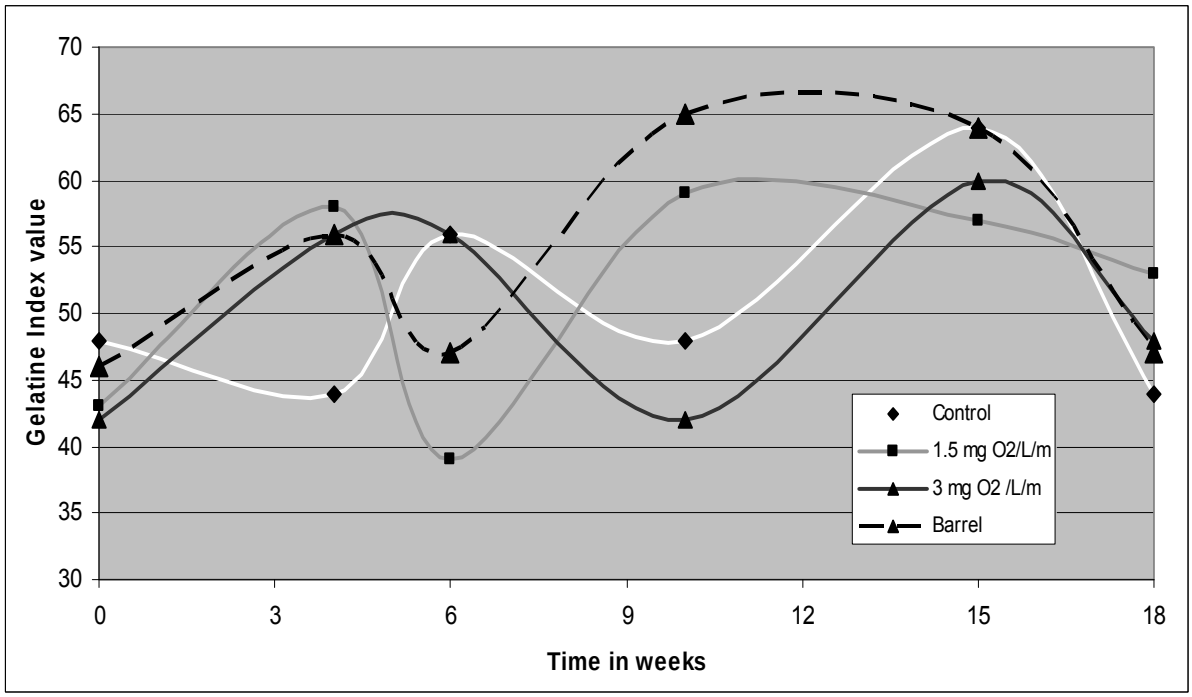


FIGURE 4.8

Reactivity of wine C towards proteins (gelatine index value, see Table 1) during micro-oxygenation treatment.

The formation of acetaldehyde from ethanol and glyoxylic acid from tartaric acid due to oxidation can lead to the polymerisation of catechin molecules (Drinkine *et al*., 2005). A small decrease in the total tannin concentration and a small increase in colour hue in the treated wines were observed (results not shown). In wine C the gelatine index varied over time and between the treatments (Fig. 4.8). This shows that the wine goes though different stages of reactivity towards proteins. The gelatine index is, however, only an indication of astringency and does not always correlates directly with the astringency of a red wine (Ribéreau-Gayon, *et al*. 2000). According to Parish *et al*. (2000), red wine first goes through a structuring stage after micro-oxygenation has started. During this stage the astringency increases. This is followed by the harmonisation phase, which is characterised by a decrease in astringency, but can be followed by a stage where the wine becomes too hard or “dried out” if the micro-oxygenation is applied for too long. This correlates with results of Nikfardjam & Dykes (2003). Our results also showed differences between the control and treated wines, but these were not correlated with astringency assessment through tastings. Clearly more research is needed regarding the effect of micro-oxygenation and the effect of O_2 addition to red wine on the sensory characteristics. In this investigation tendencies were observed, but trials investigating the effect of micro-oxygenation on the evolution of the taste of red wine should be carried out in future with adequate repeats.

4.2.2 MICROBIOLOGICAL STATUS

Acetic acid bacteria and *Brettanomyces* are both well-known spoilage microorganims of wine. Acetic acid bacteria can form unwanted elevated levels of acetic acid through the oxidative metabolism of ethanol. *Brettanomyces* can cause medicinal, barnyard characters in wine due to the production of volatile phenols. Both these organisms have been proven to grow in wine when levels increased (Du Toit & Pretorius, 2002; Du Toit *et al*., 2005). There has been no other report, according to our knowledge on the effect of micro-oxygenation on acetic acid bacteria and *Brettanomyces* cell numbers in wine. In Figs. 4.9 & 4.10, the acetic acid bacteria and *Brettanomyces* numbers in wine C can be seen during micro-oxygenation. Acetic acid bacterial numbers decreased in the control tank after four weeks, but were generally higher in the tanks receiving O_2 and in the barrel (Fig. 4.9). Acetic acid bacteria can go into a viable but non-culturable state in wine that can be negated by the addition of O_2 (Du Toit *et al*., 2005).

Brettanomyces counts increased in the 1.5 mg O_2/L/month treatment from 10^1 - 10^3 cfu/mL after 14 weeks (Fig. 4.10). At this time the free SO_2 concentration decreased in this tank to 18 mg/L and it was increased to 35 mg/L. This led to a decrease in *Brettanomyces* cell counts in this tank. The counts in the 3 mg O_2/L/month tank increased, with a correlating decrease in the free SO_2 (17 mg/L) at week 20. At this stage, the SO_2 was then adjusted to 35 mg/L in all the treatments. Therefore, micro-oxygenation does not seem to support the growth of acetic acid bacteria but possibly supports their survival. With the permeation of O_2 through oak staves (Vivas *et al*., 2003) small amounts of O_2 coming into contact with wine could hence support the survival of acetic acid bacteria and *Brettanomyces*. The judicial use and monitoring of SO_2 in combination with micro-oxygenation is important to prevent this, especially in the case of *Brettanomyces*. Overly high SO_2 concentrations could, however, lead to inhibition of favourable phenolic polymerisation reactions (Ribéreau-Gayon *et al*., 2000).

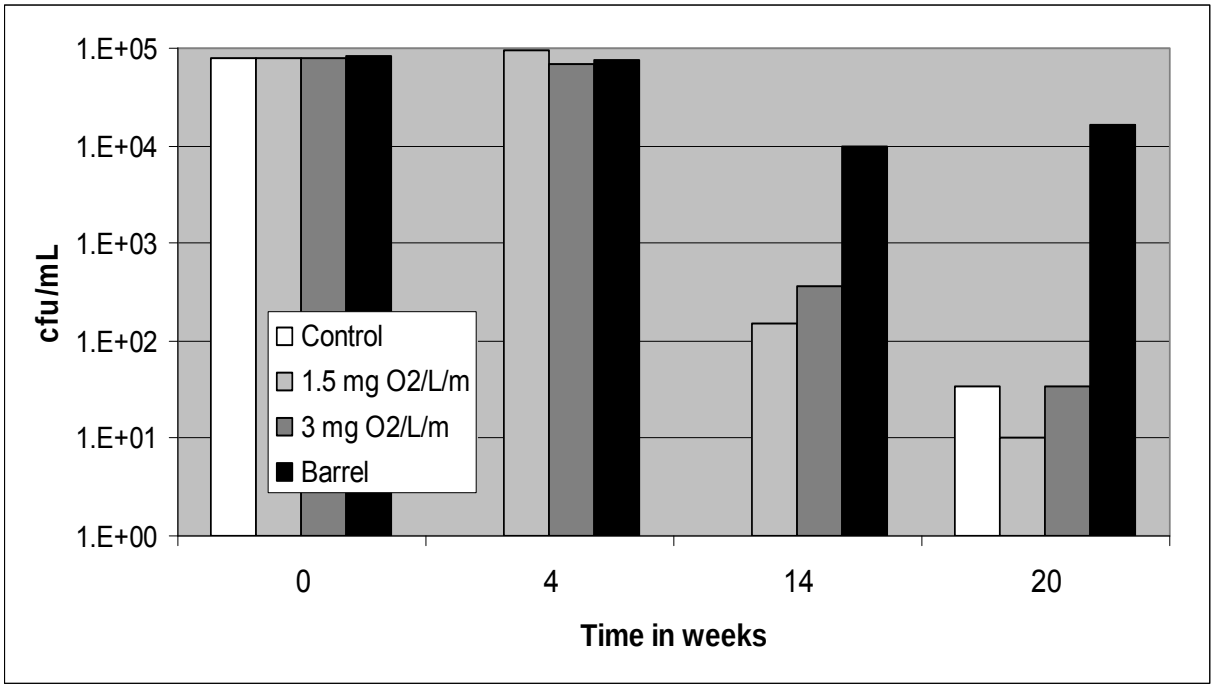


FIGURE 4.9

Acetic acid bacterial numbers in wine C during micro-oxygenation treatment

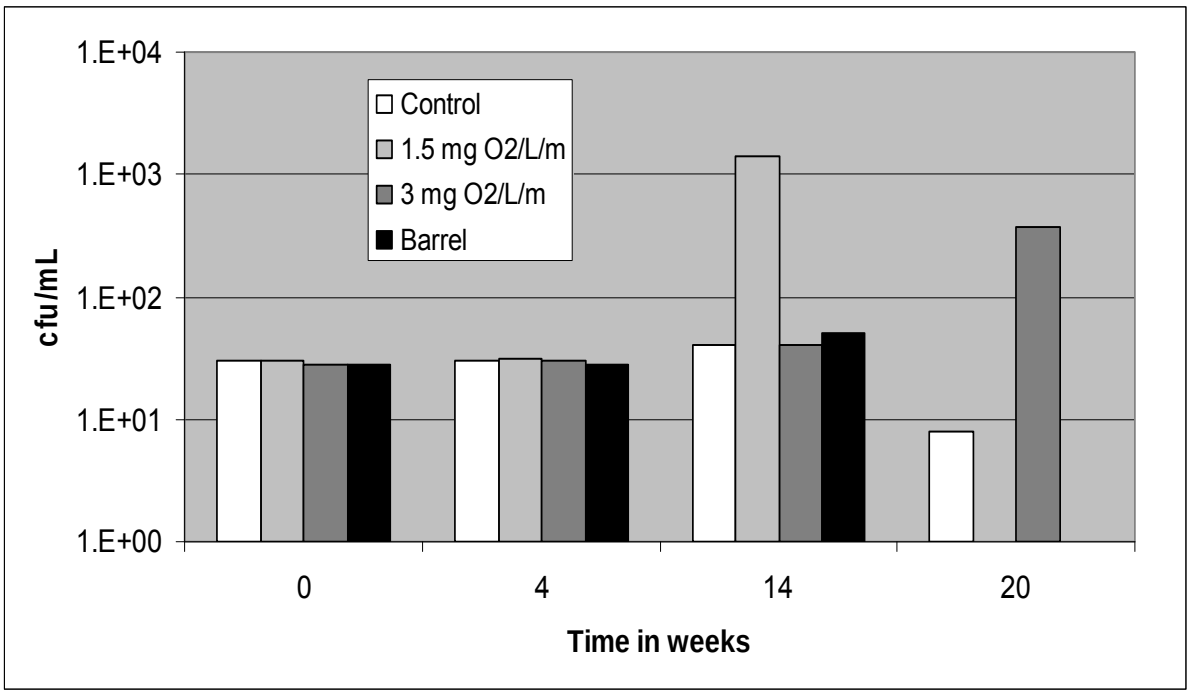


FIGURE 4.10

Brettanomyces numbers in wine C during micro-oxygenation treatment

4.2.3 SENSORY RESULTS

The triangle tasting of wine A after eight weeks of treatment revealed that there was a significant difference between the control and the 3 mg O_2/L/month treatment ($p \leq 0.05$, results not shown). The same level of significance was found when the panel had to distinguish between the 1.5 mg O_2/L/month and 3 mg O_2/L/month treatments. The panel was unable to distinguish between the control and the 1.5 mg O_2/L/month treated wines, but was able to distinguish between the control and the 3 g/L/month O_2 treated wine. At both significance levels ($p \leq 0.05$ and $p \leq 0.01$), the 3 mg O_2/L/month treated wine was preferred to the control and from the 1.5 mg/L/month O_2 treatment ($p \leq 0.05$). There was no significant difference regarding preference between the control and the 1.5 mg O_2/L/month treated wine at this stage.

After 12 weeks, the triangle tests showed that there was a significant difference ($p \leq 0.01$) level where the panel could distinguish between the control and the 1.5 mg/L/month O_2 treatment. The same level of significance was found when the panel had to distinguish between the control and the 3 mg O_2/L/month treatments and between the 1.5 mg O_2/L/month and 3 mg O_2/L/month treatments Wessel, hierdie sin is verwarrend en onduidelik. Maak seker date dit reg vertolk het. At a significance level of $p \leq 0.05$, the 3 mg O_2/L/month treatment was preferred to the 1.5 mg

O_2/L/month treatment. The 1.5 mg O_2/L/month treatment was also preferred to the control at this level of statistical significance ($p \leq 0.05$). At a significance level of $p \leq 0.01$ the 3 mg O_2/L/month treatment was preferred to the control after 12 weeks of micro-oxygenation. In this younger red wine micro-oxygenation thus led to these wines being preferred over the control wines and this phenomenon occurred faster at the higher O_2 dosage.

Wine C was tasted twice. In the initial tasting (after three months) the panel could not statistically distinguish between the control, the 1.5 and 3 mg O_2/L/month treatments, and the barrel wines on an intensity tasting for bitterness and astringency (results not shown). The panel rated the barrel matured wine much higher in terms of oak wood character and lower in terms of fruitiness. This was probably due to the masking effect that the oak flavour compounds had on the fruity character. This highlighted the need for further research regarding the use of oak staves in conjunction with micro-oxygenation to simulate storage in barrels, because the oak stave dosage was conducted according to the supplier's recommendations. The panel also did not have a significant preference for a certain treatment.

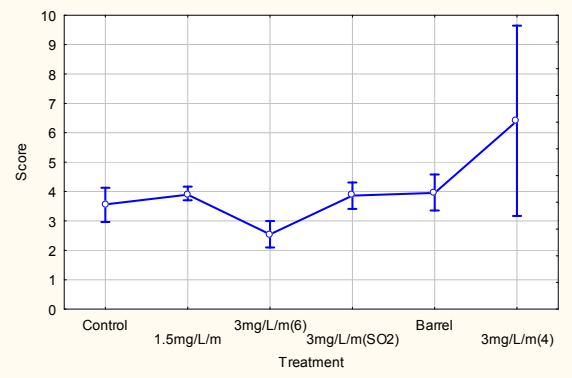
It is clear that the bootstrap means for the fruity and spicy attributes did not differ significantly between the treatments, although the former was slightly lower in the barrel treatment (second tasting after six months, Fig. 4.11). Astringency and bitterness also did not differ significantly, but the oak wood/coconut and vanilla/butterscotch attributes were significantly higher in the barrel treatment than in the other treatments. This also explains the previously mentioned lower perceived levels of fruitiness due to the masking effect of the oak wood aromas. Oxidised/aged and barnyard/medicinal flavours were higher in the 3 mg O_2/L/month treatment at this stage. The addition of SO_2 to these samples lowered the perception of the oxidised/aged character slightly. SO_2 binds acetaldehyde lowering its perception in wine, but it seems that the oxidised aroma of wine is not correlated with the acetaldehyde concentration (Silva Ferreira *et al*., 2002). The barnyard/medicinal character also increased in the 3 mg O_2/L/month treatment between four and six months of treatment.

At a significance level of $p \leq 0.05$, the panel preferred the control, 1.5 mg O_2/L/month tank, barrel treatments and the 3 mg O_2/L/month sample taken after four months rather than the 3 mg O_2/L/month treatment at this stage (six months, results not shown). The panel did not, however, have statistically significant preferences between the 3 mg O_2/L/month treatment taken after four months of micro-

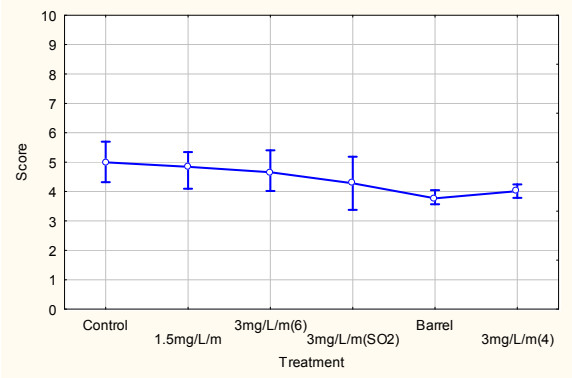
oxygenation, the control, the 1.5 mg O_2/L/month and barrel treatments. This highlights the fact that when micro-oxygenation is applied for too long the wine can become over-aged/developed, with a decrease in quality. The barnyard/medicinal aromas normally associated with *Brettanomyces* spoilage, also correlated with the increase in *Brettanomyces* counts after 14 and 20 weeks in the 1.5 mg O_2/L/month and 3 mg O_2/L/month treatments (Fig. 4.10). It is thus also doubtful whether micro-oxygenation is effective in an older red wine because the panel did not have a clear preference for the O_2 treated wines over the control. Nikfardjam & Dykes (2003) found that when micro-oxygenation is applied for too long that the wine becomes too astringent. This correlates with a mean degree of polymerisation of procyanidins that is too high.

4.2.4 STANDARD WINE ANALYSIS

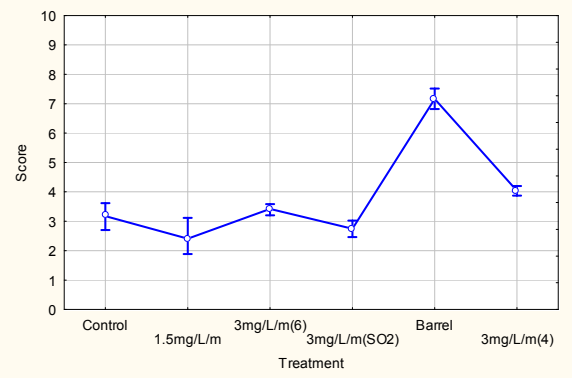
Minor changes took place during the different treatments regarding standard analysis (results not shown). The largest change took place in the free SO_2 concentration, where decreases from 25-30mg/L to 17-20 mg/L free SO_2 were observed after six to 12 weeks of micro-oxygenation in the different wines. Alcohol, volatile acidity, total acidity, residual sugar and pH values stayed the same during the treatments.



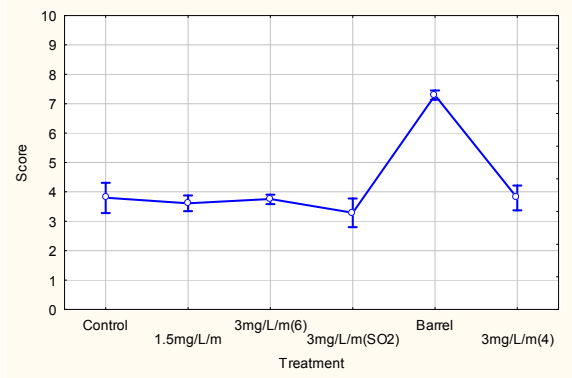
Spiciness (a)



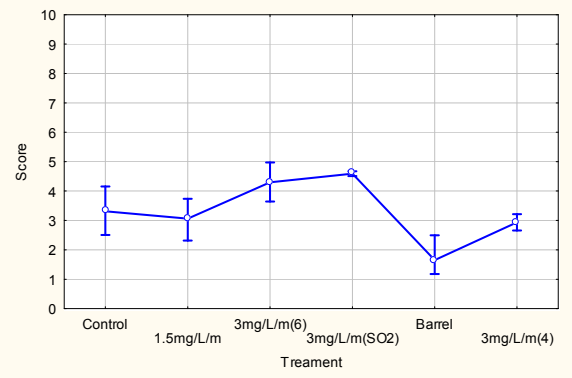
Fruitiness (b)



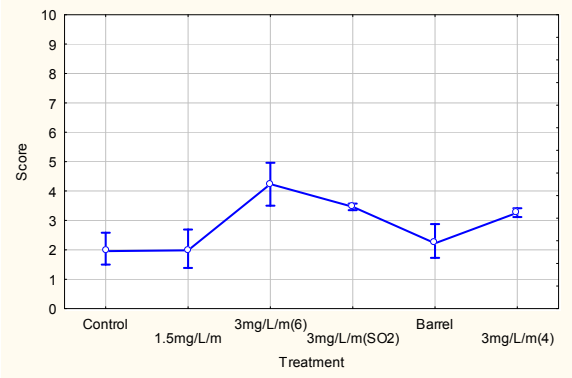
Vanilla/butterscotch (c)



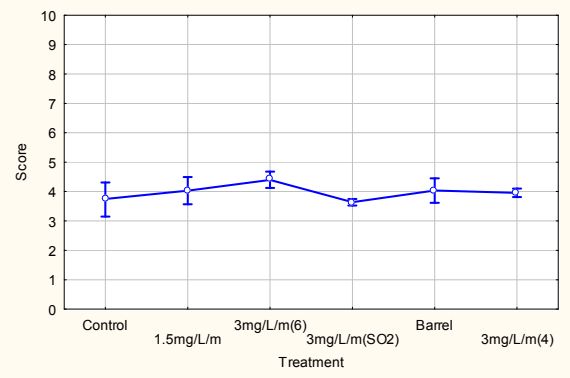
Oak/coconut (d)



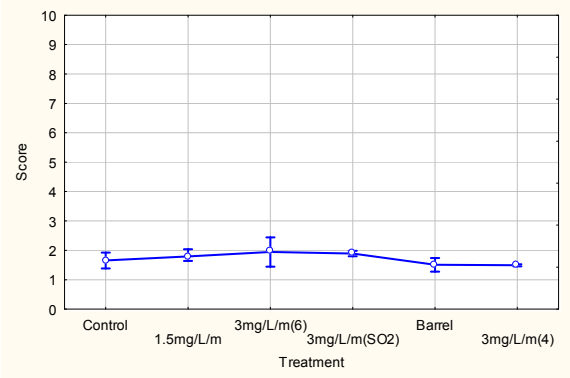
Barnyard/medicinal (e)



Oxidized/aged (f)



Astringency (g)



Bitterness (h)

FIGURE 4.11

Score plots of bootstrap confidence intervals of different sensory attributes of wine C after 6 months of micro-oxygenation (6). In the 3 mg O_2/L/month treatment wines were also evaluated (see Table 1) after 4 months (4), and included samples to which SO_2 was added prior to the tasting (SO_2). Vertical bars denote 0.95 bootstrap confidence interval.

4.3 CONCLUSIONS

Micro-oxygenation has an influence on the phenolic composition and quality of red wine. This technique that has not been investigated in detail under South African conditions. This study has shown that under certain circumstances micro-oxygenation can lead to enhanced colour densities and the colour becoming less prone to SO_2 bleaching, but this does not seem to be the case with older red wines. Micro-oxygenation can be used to enhance the quality of a younger red wine, but should be used with care in an older red wine because over-oxidation can lead to an faulty/spoiled character. The SO_2 levels of the wine should be checked regularly because *Brettanomyces* growth during micro-oxygenation can lead to unwanted medicinal flavours. Micro-oxygenation can also lead to higher acetic acid bacteria numbers. Phenolic development in a wine that received 3 mg O_2/L/month was on par with the same wine matured in an oak barrel. More research into this is required, especially to investigate the validity of the recommendations of suppliers of oak staves, where they are used in combination with micro-oxygenation, to simulate oak barrel storage. However, if used correctly, micro-oxygenation can be applied with success to bring about favourable changes in a red wine, if used correctly. Clearly, more research on micro-oxygenation as well as on the effect of oxygen on red wine composition in general is needed. The difficulties in relation to having sufficient repeats in a micro-oxygenation experiment can be overcome through the

manufacture of a system that can dose O_2 in small amounts accurately in small tanks, lowering the volumes of wine required.

4.4 ACKNOWLEDGEMENTS

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5. EVALUATING FOURIER TRANSFORM INFRARED SPECTROSCOPY AS A MEANS TO FOLLOW OXIDATION IN PINOTAGE WINES

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Oxidation status of red wine should be assessed to ensure consistent quality. The use of visible light to assess the progression of oxidation does not always yield satisfactory results. The aim of this study was to investigate whether Fourier transform infrared spectroscopy (FTIR) could be used to assess the progression of oxidation in Pinotage wine. Sensory development, such as a decrease in coffee aroma and fruitiness and an increase in potato skin and acetaldehyde aromas during oxidation, has been shown as well as the ability of FTIR, to distinguish between a control and wines receiving oxygen. Principal component analysis (PCA) revealed that FTIR could potentially be a more efficient means of assessing the progression of oxidation in certain red wines than measurements at the visible light spectrum. To our knowledge this is the first report on the use of FTIR to follow the relative degree of oxidation in red wine.

KEY WORDS: Oxidation, FTIR spectroscopy, Wine colour, Multivariate data analysis

5.1 INTRODUCTION

The production of red wine of consistent high quality is an important goal for all red wine producers. This is equally true in the case of Pinotage, a South African bred cultivar that is nowadays widely cultivated in South Africa. This cultivar is a cross between Hermitage (Cinsaut) and Pinot noir and contributes significantly to income generation for certain wine producers. Any process or factor leading to the lowering

of Pinotage quality should be identified and rectified in advance by the wine producer. Oxygen (O_2) comes into contact with wine throughout the production process. This can be due to pumping, racking, fining, filtration, barrel-ageing and bottling of the wine (*1*). At low concentrations, O_2 can contribute positively to red wine quality by inducing acetaldehyde-mediated polymerisation of tannin and anthocyanin molecules during aging in oak barrels (*2*). This leads to an increase in colour density as anthocyanins are being incorporated into the polymerised colour fraction, and results in a softening of tannins. For these positive changes to take effect, the addition of O_2 should take place in a controlled manner over a long period of time. This can happen during barrel-ageing when small amounts of O_2 permeate wine. Unwanted over-oxidation can occur when the O_2 is added at excessive concentrations in a short time. This can occur when faulty equipment, such as pumps and filters ,is used and the wine is exposed wine to O_2 concentrations that are too high (*1, 3*).

Oxidative degradation refers to the negative effect of O_2 on the colour and aroma of wine and more literature on this topic is available for white wine than red wine. Browning of white wine, which is due to the oxidation of phenols, is usually one of the first signs of this type of spoilage (*4, 5*). However, an oxidative aroma, reminiscent of potato skins or acetaldehyde can often be perceived prior to browning. This is particularly true for red wine where the development of a brown hue is not always visually detectable. The colour of red wine is often analysed spectrophotometrically by measuring brown, red and violet hues at 420, 520 and 620 nm, respectively (*6, 7*). This raises the question whether the measurement of brown colour alone provides a sensitive enough indication of over-oxidation, especially in the early stages of this spoilage. Due to the serious economic implications for the red wine producer if oxidative spoilage is not detected at the earliest possible stage, it is clear there is a need to develop additional, more sensitive analytical methods of tracing the degree of oxidation in red wine.

Near-infrared spectroscopy has been used for many years to measure sensory properties in foodstuffs and beverages and many quality control applications have been reported (*8, 9, 10, 11*). Fourier transform mid-infrared (FTIR) spectroscopy is increasingly being used in the grape and wine industry for the chemical analysis of wine, fermenting must and grape juice (*12, 13*). The technology is based on the measurement of the molecular vibrations of chemical bonds such as C-C, C-H, O-H,

C=O and N-H upon absorbance of light in the mid-infrared region (5000-929 cm^{-1} or 2500-50,000 nm) of the electromagnetic spectrum (*14*). The measured intensities of the vibrations recorded at the respective wavelengths are processed though a series of mathematical procedures (including Fourier transformation) to generate an absorbance spectrum (*15*). Large amounts of data related to the chemical composition of samples are captured in FTIR spectra and the information in the data can be extracted through the use of multivariate data analysis techniques, such as principal component analysis, discriminant analysis and partial least squares regression (*16, 17*).

The purpose of this study was to evaluate FTIR spectroscopy as a means of tracing the progression of oxidation in Pinotage wine and to compare this method with spectrophotometric analysis and sensory evaluation.

5.2 MATERIALS AND METHODS

5.2.1 Wine samples and accelerated oxidation of wines

Three different Pinotage red wines from different geographic origins were used; two from Stellenbosch, South Africa (wines A and B, 2004 vintage) and one from Paarl, South Africa (wine C, 2005 vintage). All the wines completed malolactic fermentation and were collected from the producing cellars by allocating them to 20 L steel tanks sparged with nitrogen (Afrox South Africa) prior to filling. The wines were filtered through a K300 mat filter (Columbit South Africa Pty, Ltd) under N_2 pressure with the aid of diatomaceous earth to remove remaining solids such as grape particles, yeast and Potassium bitartrate crystals (Columbit South Africa Pty, Ltd). The wine was tasted upon receival to confirm that no oxidative character could be detected.

5.2.2 Accelerated oxidation of wines

Wines A and B were divided into two batches of 18 L each and each batch was placed in a 20 L steel tank with an airlock top. Wine C was also divided into two equal batches of 45 L and placed in 50 L tanks. Batch one of each wine was was sparged with N_2 for 30 min to remove most of the dissolved O_2 from the wine. Batch two was sparged with O_2 (Afrox South Africa) for 30 min to ensure a high residual O_2 concentration in the wine. The O_2 concentration in the wines was not measured, but

the wines were tasted regularly during the course of the experiment to assess the progress of oxidation. All the oxidations of wines were conducted at 20°C.

5.2.3 Wines A and B

The N_2-sparged batches of respectively wines A and B were used to fill 120 mL screw-cap plastic bottles leaving no headspace and the caps were sealed with parafilm to prevent further air coming into contact with the wine. These samples are referred to as control samples in the text. The air-sparged batches of wines A and B were dispensed in 125 mL aliquots into sterile 750 mL glass bottles that were closed on the top with cotton wool. This ensured a large air headspace on top of the wine. Twenty-five repeats of the control and treated samples were prepared in this manner for wines A and B.

5.2.4 Wine C

The O_2-sparged batch (45 L) was used to fill five 10 L steel tanks with airtight locks (tanks a, b, c, d and e). The N_2-sparged batch was used to fill 750 mL glass bottles (in which the air was previously displaced with N_2). The glass bottles were then sealed with airtight screw caps and served as the control samples (K) in this experiment. The wines in the 10 L steel tanks were exposed to high O_2 additions every day by racking the wines four times from the tanks into 20 L plastic buckets,and then returning the wine to the steel tanks. After one (stage 2), two (stage 3) and three (stage 4) weeks of treatment, two 750 mL bottles were filled from each of tanks b, c, and d and sealed with screw tops. One of these samples, together with the control samples (designated K) collected at the beginning of the experiment and also at exactly the same stage (designated K2, K3 and K4) as the treated samples, were then analysed with FTIR and the visible light spectra (section 5.2.5) on the same day. The remaining samples from the respective treatments were used for sensory evaluation. The wines in the two remaining 10 L steel tanks (tanks a and e) were used to fill up the headspace generated by the sampling procedures. Tanks a and e received the same oxidative treatments as tanks b, c and d. All samples were kept at 20°C for the duration of each experiment.

5.2.5 FTIR and visible light spectral measurements

Wine samples were scanned using a WineScan FT 120 spectrometer (*18*), a purpose-built instrument designed specifically for chemical analysis of wine,

fermenting must and grape juice. The conditions of scanning, the number of scans generated per sample (n=20) and the processing of the spectra are predetermined by the manufacturer and cannot be changed by the user. For analysis, samples were pumped through a CaF_2-lined cuvette (37 µm path length) that is located in the heater unit of the instrument. The temperature of the samples was brought to 40ºC before analysis and samples were scanned from 5011–929 cm^{-1} at 4 cm^{-1} intervals, thereby collecting 1056 data points per spectrum. Zero liquid *(19)* was scanned before to the samples to correct for background absorbance. Prior to scanning samples were degassed to remove excessive CO_2 by vigorous shaking and sonication (Model UMC2, Ultrasonic Manufacturing Company, Krugersdorp, South Africa). Wines were scanned in duplicate.

In the visible light region, 50 µL sample volumes of the wines were scanned (400-640 nm at 2 nm intervals) using a Universal Microplate Spectrophotometer (µQuant model, Bio-Tek Instruments, USA). This volume ensured that spectral readings were lower than one.

5.2.6 Sensory evaluation and descriptive profiling of oxidised wines

A wine tasting panel consisting of nine staff members and postgraduate students of the Department of Viticulture and Oenology, Stellenbosch University was used for the sensory evaluation of the wines. All members had previously passed the Department's sensory exam, which is required to judge wines at the annual South African National Wine Show. The panel had five further training sessions using wine C before the official tasting.

This training consisted of descriptive profiling of wine C by initially supplying each panel member with control wine and wine that had undergone different degrees of oxidation. Panel members were encouraged to name the flavour and taste characteristics perceived in the wines. The descriptors were selected based on consensus reached by the entire panel. Unstructured line scales (10 cm) were then drawn up with the lower end of the scale marked as "none" and the higher end as "intense". Panel members had to indicate the intensity of each characteristic for each wine on the scale. By measuring the length of the indication on the line scale, a numerical value was obtained and these measurements were statistically analysed. Results obtained at the last two training sessions were discussed among panel members and used to reach consensus regarding the intensity of the different descriptors.

At the official tasting of wine C, each judge received the same amount of wine per glass and was asked to evaluate the colour characteristics of the wines before judging the aroma and taste profile. Tasting was conducted at 18 °C in standard ISO glasses. Tasting was done in separate booths to prevented contact between fellow judges during the official tasting. Randomly selected two-digit numbers between 20 and 99 were used to identify the wines. Samples for the tasting were drawn from the control, tank b after one week (stage 2) and tanks b and c after two weeks (stage 3) and three weeks (stage 4) of oxidative treatment, respectively.

5.2.7 Gaschromatographic analysis

Chromatographic analysis was conducted on an Agilent 6890N gas chromatograph fitted with a split/splitless inlet operated in splitmode, ratio 15:1, temperature 200°C**.** **Column:** DB-FFAP, 60 m × 0.32 mm × 0.5 μm f.t. **Detection:** Flame ionization detector (FID), temperature 250°C. **Oven Program**: 33°C (17 min), ramp at 12°C/min to 240°C, hold for 5 min., initial pressure 84.5 kPa, carrier H_2 (constant flow) at 3.3 mL/min.

Five mL of wine was extracted with one mL of diethyl ether by placing the ether/wine mixture in an ultrasonic bath for 5 min. The internal standard (100μl) was added with the diethyl ether to the wine. This consisted of 0.5 mg/L 4-methyl-2-pentanol, prepared in distilled H_2O with 12% ethanol, 2.5 g/L tartaric acid, pH adjusted to 3.5 with KOH. After extraction the wine/ether mixture was centrifuged at 4000 rpm for 3 min. The diether layer was removed and dried on $NaSO_4$. This extract was then injected into the GC (injection volume, 3 μl). Samples taken at stages 2, 3 and 4 were analysed.

5.2.8 Multivariate data analysis

Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) of the FTIR, visible light and sensory data was done with the Unscrambler Software (version 9.2, Camo AS, Norway, reference (*17*). For this purpose data were organised in one two-dimensional table where the columns were defined by **X-**variables (in this study the various wave numbers and wave lenghths at which absorbance was measured and the sensory descriptors used for wine evaluation) and a **Y-**variable (class membership of the wines, control or oxidised), while the rows were defined by the samples. PCA models the interrelationships between the different variables and facilitates the detection and interpretation of sample patterns,

similarities and differences (*15*,*16*). Each principal component (PC) is a linear function of the original **X-**variables and collectively describes in descending order the main structured information in the data. Every PC can be interpreted individually because they are calculated orthogonal to one another.

PLS-DA is a multivariate classification method based on modelling the differences between classes of samples with the PLS1 algorithm (*15*,*16*). Class membership of control wines was coded by –1 and oxidised wines by +1.

5.3 RESULTS AND DISCUSSION

Oxidative spoilage of white wine is frequently observed as a visible browning of the wine (*4,5*). The colour of red wine is also affected by this spoilage and the effect on the colour can be detected spectrophotometrically by measuring brown, red and violet hues at 420, 520 and 620 nm, respectively (*6, 7*). However, slight degrees of oxidation in red wine are usually easily perceived by the experienced wine taster, without any significant changes in the absorbance at the aforementioned visible light wave lengths. The aim of this work was to evaluate FTIR spectroscopy as an alternative analytical strategy to track oxidation in red wine with the long-term aim of developing a model that can be used to predict the the degree of oxidation in unknown wines.

5.3.1 Discrimination between oxidised and non-oxidised wines A and B using FTIR- and visible light spectral data

Principal component analysis of wines A and B showed two separate clusters for each wine that related to respectively, the control and oxidized wines (Figure 5.1A). PC1 (explaining 43% of the variance) captured the major differences in the spectral properties of the two wines from the Stellenbosch area. The control samples of wines A and B appeared as tight clusters while the treated samples were more dispersed and distributed diagonally along PC1 and PC2 (explaining 33% of the variance in the spectral data). Six PCs explained 95% of the variance in the X-variables (Figure 5.1B) indicating that the most significant variance in the data set was modelled in the analysis. Contiguous wave-number and wave-length intervals contributed with highest loadings to the separation of the samples (Figure 5.1 C), but a prominent seemingly “noisy” area was also observed from *ca* 2994 cm^{-1} to 5011

cm^{-1}. Water absorbs strongly in the wavenumber region 2970–3626 cm^{-1}, while considerable background noise is often associated with the region 3626–5011 cm^{-1} (*14*). PCA with wavenumbers 2970-5011 cm^{-1} deleted (Figure 5.2A) yielded a similar grouping of the control and oxidised wines as before, although only 2 PCs were now necessary to explain 95% of the variance in the sample set (Figure 5.2B). It was therefore considered justified to exclude these variables from all subsequent multivariate data analysis.

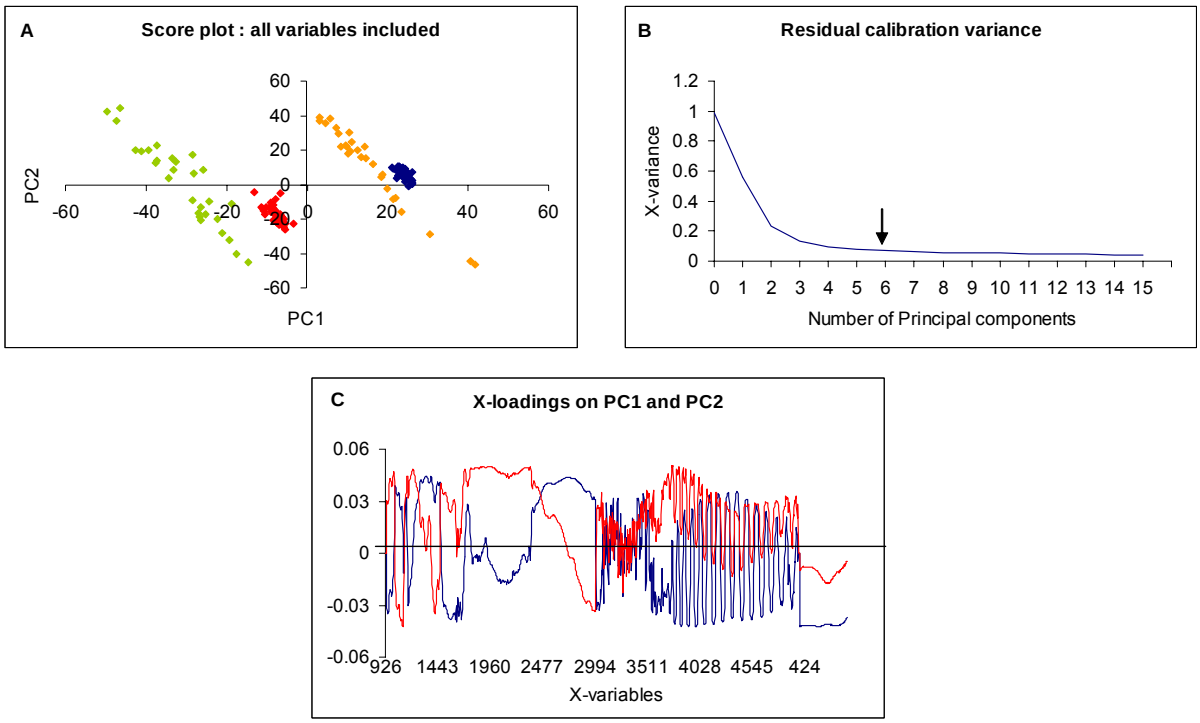

Figure 5.1 Principal component analysis (PCA) of wines A and B. A) Score plot showing that control wine A (red markers), oxidised wine A (green markers), control wine B (blue markers) and oxidized wine B (orange markers) separate along PC1. PC1: 43% explained variance, PC2 33% explained variance. B) Residual calibration plot showing that more than 95% of the X-variance in the data was described by 5 PC's. C) X-variables delineate the infrared region (925.92 - 5011 cm-1) and the visible region (420 –620 nm). Loadings on PC1 (blue line) and PC2 (red line) are shown.

The PCA x-loading plot for this data revealed that the FTIR data contributed to high x-loadings on PC1 and especially PC 2, with the visible spectrum contributing to PC1 (Figures 5.2C and D). Wave numbers in the so-called 'fingerprint area' (929-1800 cm^{-1}) contributed to the differences observed between the treatments. This area includes the absorbance due to infrared active covalent bonds of the C-O, C-C, C-H and C-N groups (*20)*. Phenolic compounds of wine consist basically of benzene derivatives,

which have a number of these covalent bonds (*7*). PCA using only the FTIR data (Figure 5.3A) resulted in a clear separation between the control and oxidized samples, without the diagonal distribution that was observed in Figure 5.3A. PCA using only visible light wave lenghts (Figure 5.3B) showed separation between the control and oxidised samples of wine B. The separation between wine A's control and the oxidised samples was not as clearly defined as with the FTIR data. Wine A underwent a shorter period of oxidation than wine B, which could have contributed to this. The diagonal distribution observed in Figure 5.1A is thus due to influence of the visible light spectrum. Differences between the amounts of O_2 that the oxidised samples received could possibly explain this.

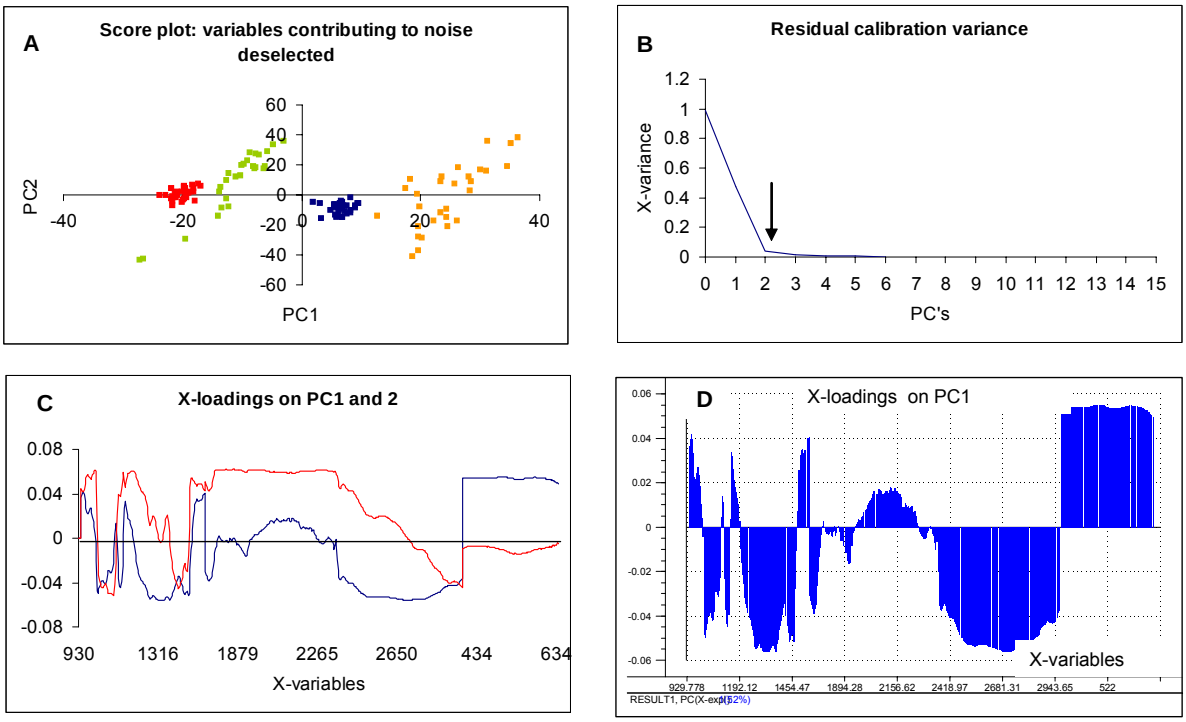

Figure 5.2 Principal component analysis (PCA) of wines A and B. The wavenumbers where water absorbs (2970 – 3626 cm-1) and the infrared noise peak (3626 – 5011 cm-1) were deleted before modelling. A) Score plot showing that control wine A (red markers), oxidised wine A (green markers), control wine B (blue markers) and oxidised wine B (orange markers) separate along PC1. B) Residual calibration plot showing that more than 95% of the X-variance in the data was described by 2 PCs. C) Loadings on PC1 (blue line) and PC2 (red line) are shown. X-variables delineate the infrared region (925.92 - 5011 cm-1) and the visible region (420 –620 nm). D) Contiguous variable intervals indicate that almost the entire infrared region and visible region contain information for the separation between different groups.

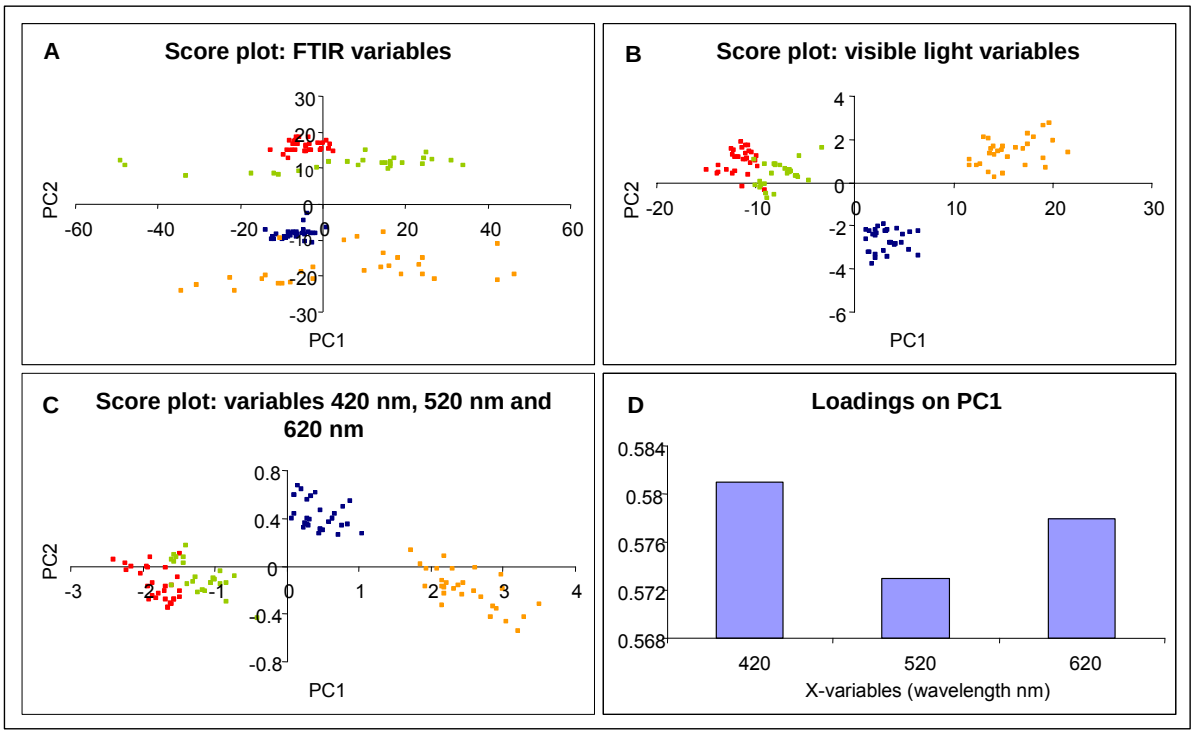

Figure 5.3 Principal component analysis (PCA) of wines A and B. Control wine A (red markers), oxidised wine A (green markers), control wine B (blue markers) and oxidised wine B (orange markers). Score plot based on FTIR variables only. Wave numbers 2970 – 3626 cm^{-1} and 3626 – 5011 cm^{-1} were deleted before modelling. B) Score plot based on visible light variables only. C) Score plot based on wave lenghts 420, 520 and 620 nm only. D) Absorbance at 420 nm and 620 nm contributed with highest loadings to the separation between groups of samples on PC1.

A PCA (Figure 5.3C) conducted with only the wavelengths commonly used to measure brown (420 nm), red (520 nm) and purple (620 nm) colour intensities in red wines showed that the FTIR distinguished more efficiently between the control and oxidised samples of wine A. The x-loading plot for this data revealed that 420 nm contributed the most to high x-loadings on PC1, which is due to phenolic compounds being oxidised to brown quinones (*21*). PLS- discriminant analysis of wines A and B (Figure 5.4) using FTIR and visible light variables found an excellent correlation of 0.979, with a slope of 0.969 between the oxidised and control samples.

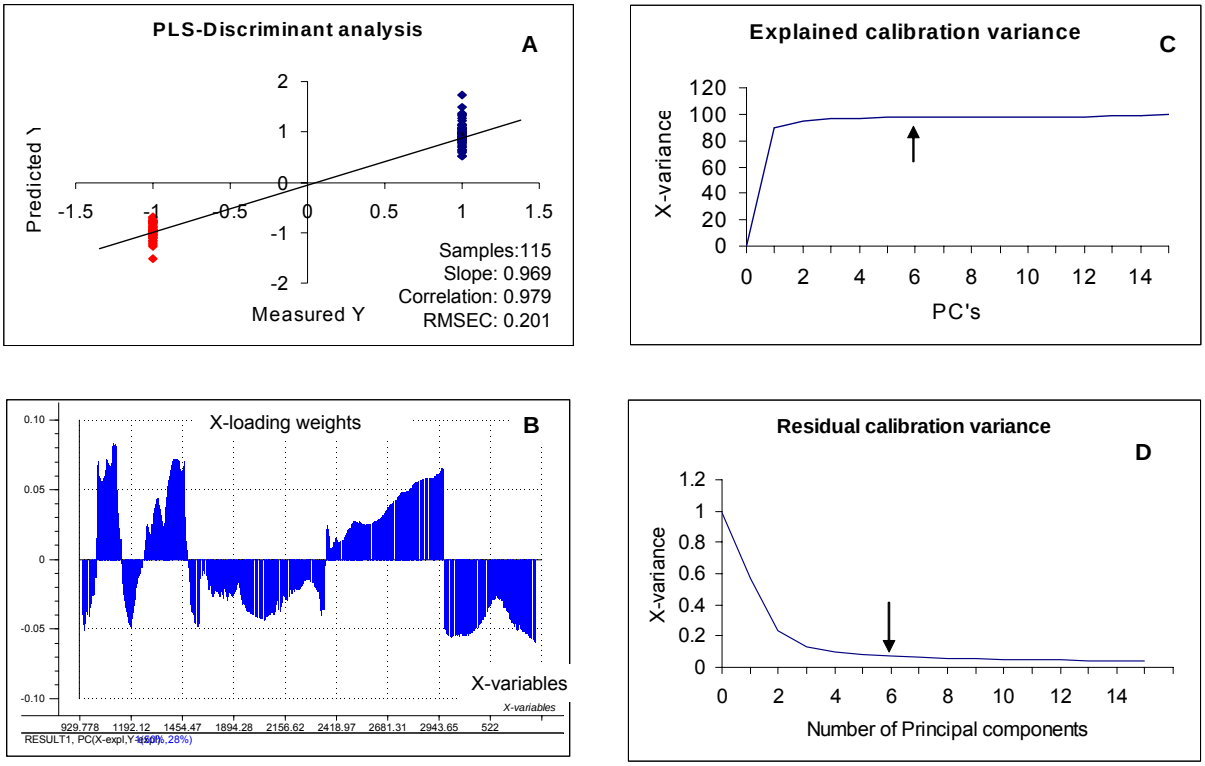

Figure 5.4 A) PLS-discriminant analysis (Y: -1 =oxidized samples, red markers, ; Y: +1 = control samples, blue markers) of wines A and B using FTIR (wave numbers 2970 – 3626 cm^{-1} and 3626 – 5011 cm^{-1} were deleted before PLS modelling) and visible light variables. B) Contiguous variable-intervals contributed with highest loading weights to the discrimination, signifying that essentially the entire range contains information pertaining to successful discrimination. C) and D) Full cross-validation indicates 6 significant principal components and 95% of the Y-variable were modelled, using 90% X-variance.

5.3.2 Sensory evaluation and descriptive profiling of oxidised wines

The set of descriptors the panel decided on for wine C were brown colour, red colour, berry fruit, coffee, banana, spiciness, potato skin, acetaldehyde, astringency, fullness and bitterness. The tasting data with these variables revealed a clear tendency for the treatments to separate along PC1 from the control samples (K) to stage two three and four as the oxidation progressed (Figure 5.5). This is due to the oxidation characteristics (initially potato skin and later acetaldehyde-like) characters becoming more prominent, with a concurrent decrease in the berry fruit, banana and coffee attributes (Figures 5.5 and 5.6). The coffee character of certain South African Pinotage wines is unknown, but is believed to be due to furfuralthiol because fermentation on oak staves increases this flavour (*22*). Berry fruit and coffee were positively correlated, with these characteristics being negatively correlated with potato skin/acetaldehyde (Figures 5.6 and 5.7). The honey like notes of oxidised

wines are caused by phenylacetaldehyde while methional causes the boiled potatoes aroma. Other unidentified compounds probably influence the aroma as well (*23*), although these results were obtained in white wine. The panel could, however, not distinguish between the red colour of the treatments, although brownness showed some correlation with the acetaldehyde/ potato skin characteristics. The contribution of the brown variable to the x-loadings was small (Figure 5.6 and 5.7**)**. Astringency and bitterness, both being caused by mainly phenolic compounds (*7*), were also positively correlated.

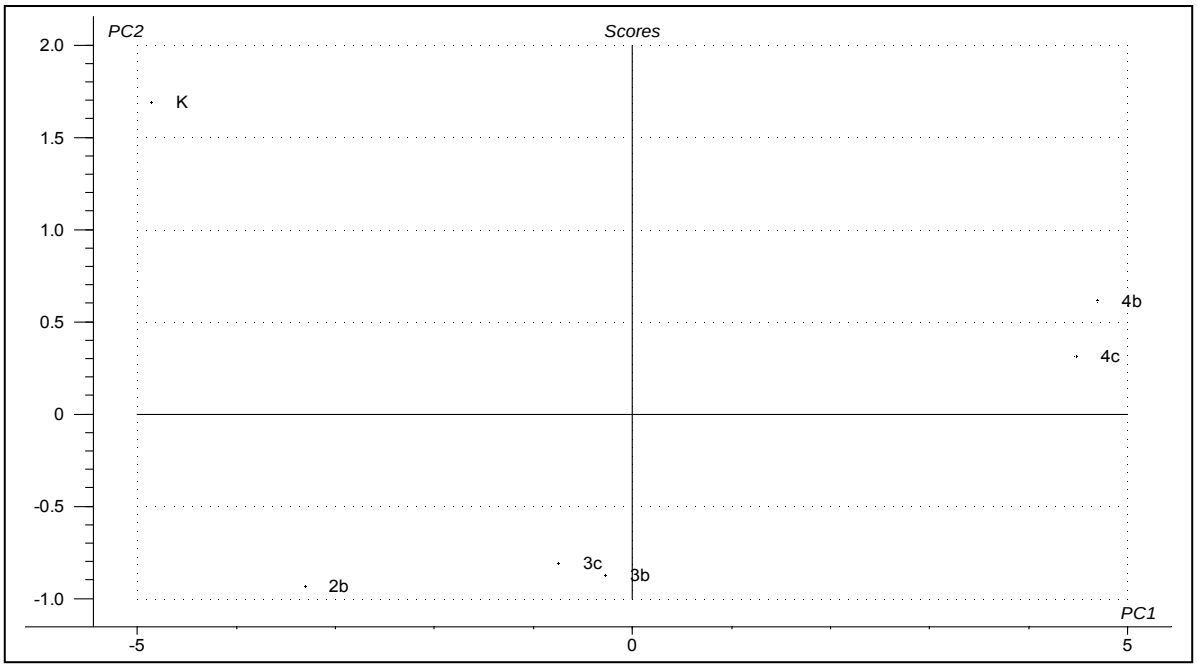

Figure 5.5 Principal component analyses (PCA) of wine C's sensory results. Score plot showing that control wine C (K), oxidised wine C stage 2, tank b (2b), oxidised wine C stage 3, tanks b and c (3b and 3c), oxidised wine C stage 4, tanks b and c (4b and 4c) separate along PC1.

The panel as a whole could not distinguish between individual tanks at a given time (for example between tanks b and c at stage 3), with both tanks b and c clustering together at both stages 3 and 4 (Figure 5A). During aeration colourless flavenes can be oxidized to the red form in wine, thereby increasing the colour density of the wine. Further oxidation can lead to the formation of brown quinones (*2, 21*). This process can, however, take a number of months in a wine barrel (*24*) and the panel could not distinguish the red colour in the oxidised wines from the control. This raises the need for developing a method of distinguishing between wines undergoing different levels of oxidation without taking into account sensory colour change because was shown

to not be an accurate assessment for wines that did not undergo extreme oxidation (as was the case with wine C).

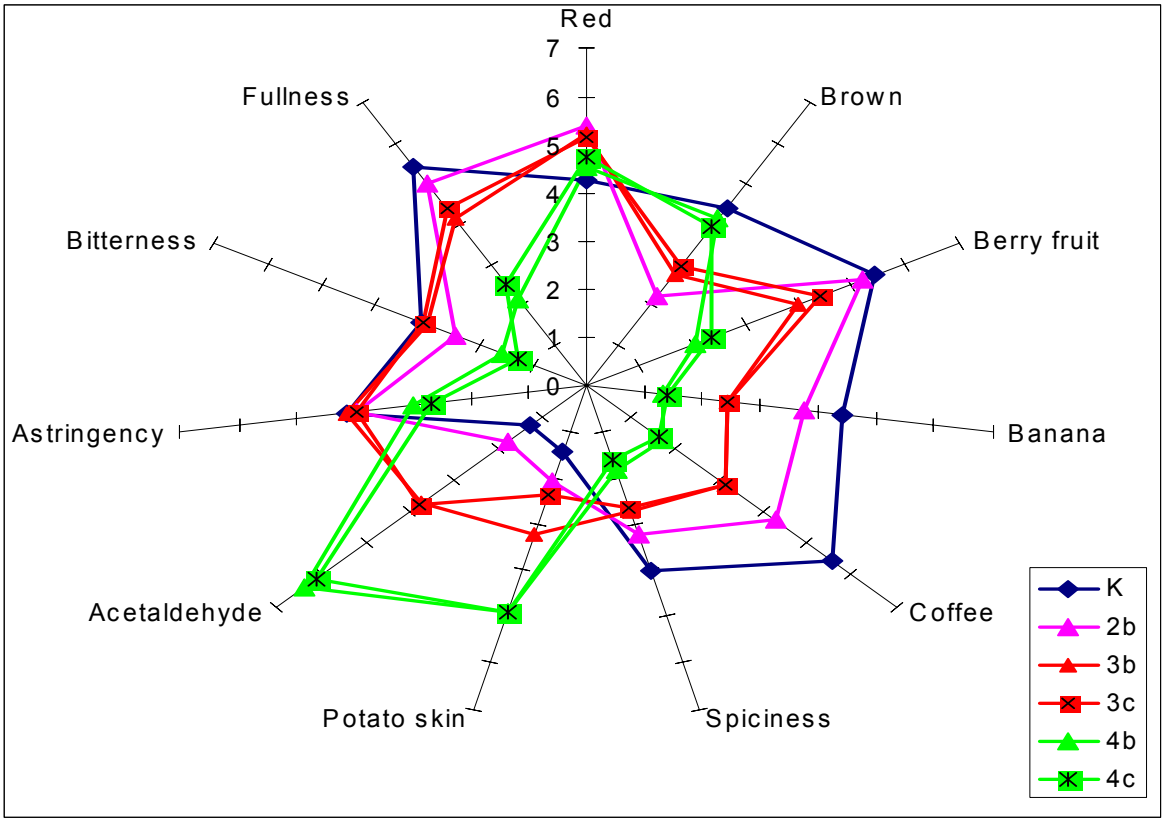

Figure 5.6 Spider diagram of the sensory results of wine C during oxidation. Wine C Control: (K), oxidised wine C stage 2, tank b (2b), oxidised wine C stage 3, tanks b and c (3b and 3c), oxidised wine C stage 4, tanks b and c (4b and 4c).

5.3.3 Discrimination between oxidised and non- oxidised wine C over time using FTIR and visible light spectral data.

As significant amount of noise is found in the FTIR spectrum, as mentioned earlier and in Figure the FTIR and visible light data yielded separation on PC1 between the different treatments over time with this noise removed (Figure 5.8). The PCA1 loading plot for this data revealed that the FTIR data contributed at different wavenumbers to this difference. The control clustered differently from the oxidised wines in the visible spectra alone (results not shown), but no separation could be observed for stages two, three and four. This could probably be due to colourless flavenes being transformed initially into the red form due to oxidation, as mentioned earlier, but this did not take place sufficiently for this technique to distinguish between the later stages. This separation was also not significant enough for the panel to be able to clearly observe it visually, as also mentioned before.

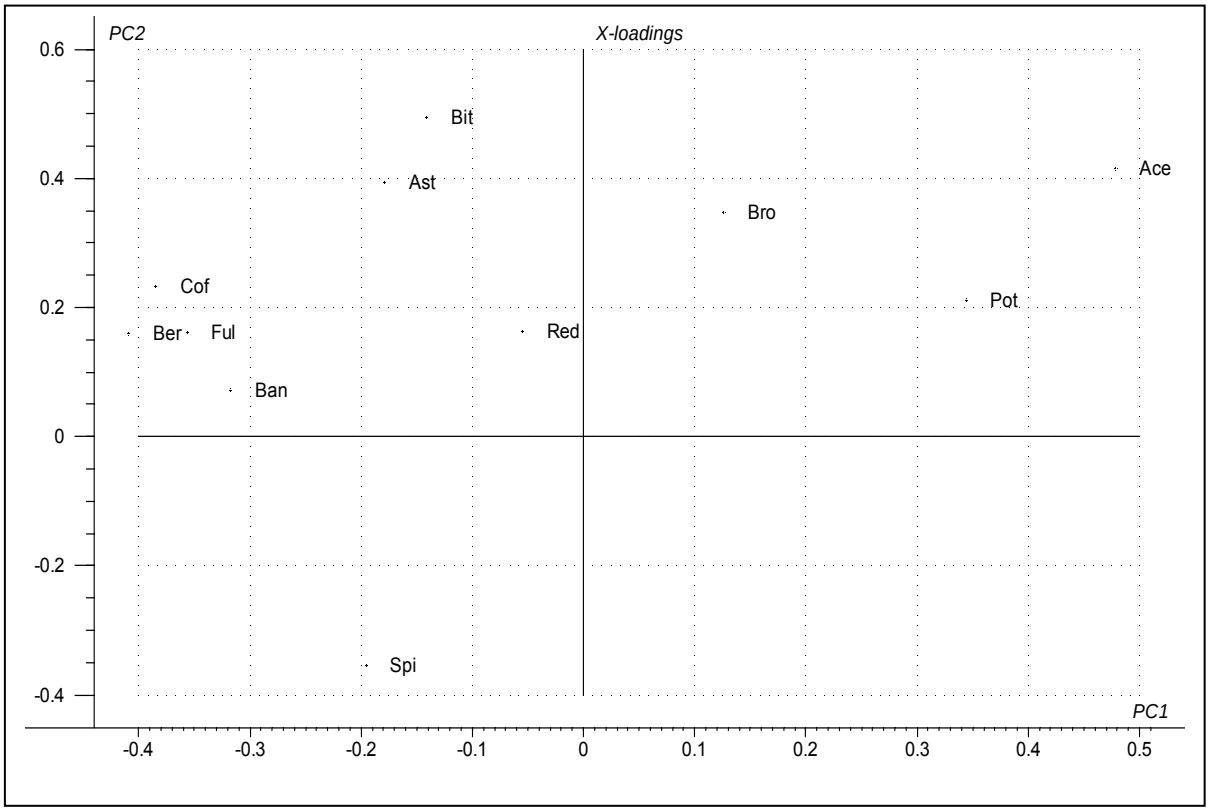

Figure 5.7 Loadings of the sensory results of wine C on PC1 and PC2. X-variables delineate the characteristics of Coffee (Cof), Berry fruit (Ber), Fullness (Full), Banana (Ban), Astringency (Ast), Bitterness (Bit), Spiciness (Spi), Red colour (Red), Brown colour (Bro), Potato skin (Pot) and Acetaldehyde (Ace).

5.3.4 Effect of oxidation on certain flavour compounds

GC analysis revealed that certain aroma compounds decreased during oxidation (Table 5.1). Iso-amylacetate is known to impart a banana, fruity character (*25*) to certain Pinotage wines and was lower in the oxidised wines. Concentrations of certain other compounds, including n-propanol, isobutanol, n-butanol, ethyl lactate, hexanol, iso-valeric acid, diethyl succinate, n- valeric acid, 2-phenyl acetate, hexanoic acid, 2-phenyl-ethanol, octanoic and decanoic acids did not differ significantly between the control and oxidised wines (data not shown). The sensory data revealed that the coffee character decreased also very quickly. This could be due to the decrease in furfuralthiol (*20*) as mentioned earlier, although the concentration

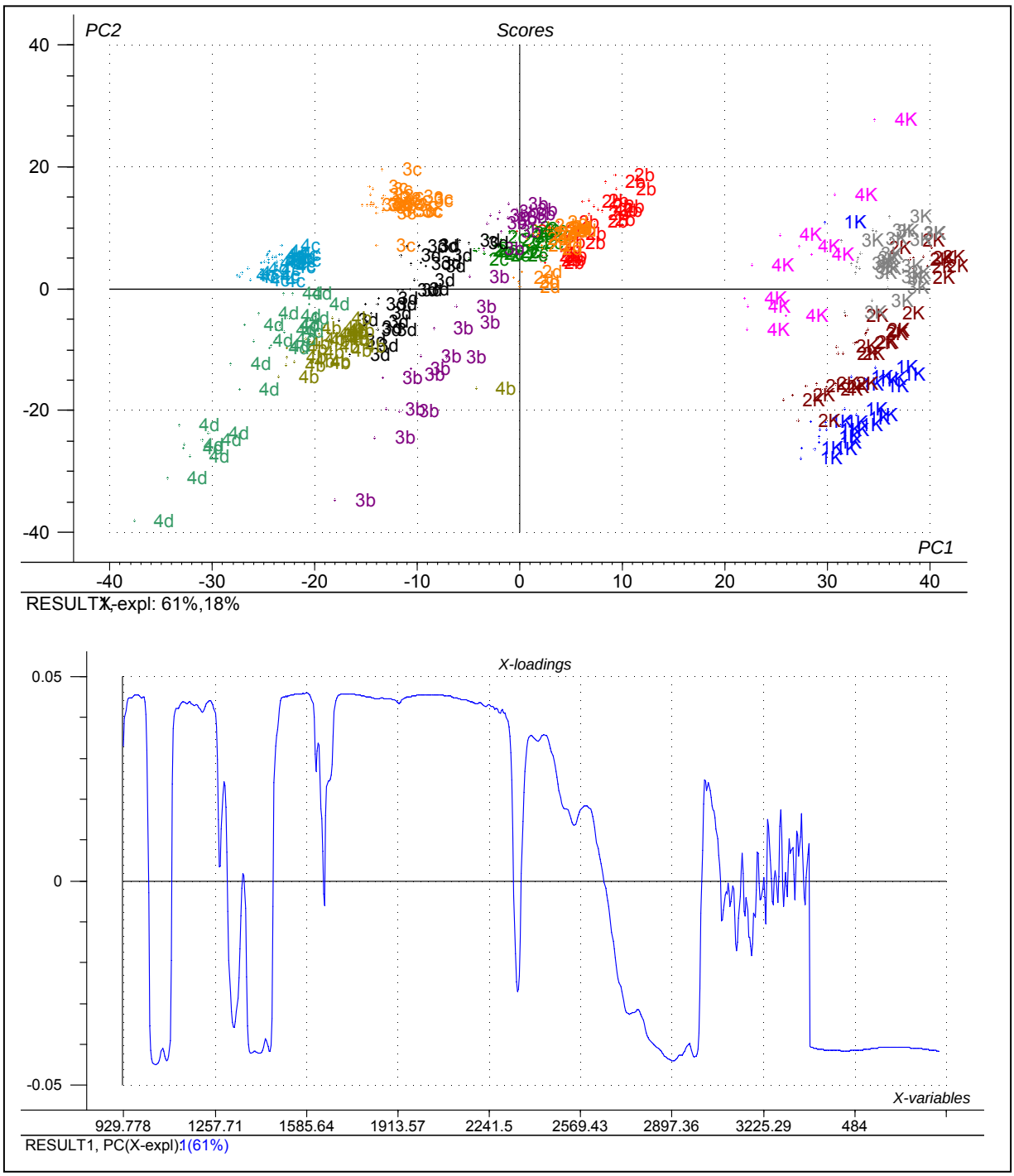

Figure 5.8 Principal component analysis (PCA) of wine C. The wave numbers where water absorbs (2970 – 3626 cm-1) and the infrared noise peak (3626 – 5011 cm-1) were deleted before modelling. A) Score plot showing that control wines K1 (dark blue markers), K2 (brown), K3 (grey), K4 (pink) and oxidised wines, 2b (red), 2c (dark green), 2d (yellow), 3b (purple), 3c (orange), 3d (black), 4b (khaki), 4c (light blue) and 4d (light green) separate along PC1. Loadings on PC1 (blue line) are also shown. X-variables delineate the infrared region (925.92 - 5011 cm-1) and the visible region (420 –620 nm).

of this compound was not measured in this study. Clearly more research is necessary on the effect of oxidation on the aroma compounds of especially red wine.

This work showed that the oxidation of red wine can be measured through different means, although the visible light measurements (420, 520 and 620 nm) now used by wine producers do not seem always to indicate oxidation efficiently, especially in the initial stages of oxidation. Red wines can differ drastically in their phenolic compositions and a brown colour does not always indicate an oxidative degradation. This is especially true for older red wines that underwent bottle ageing, where electrophilic carbocations, formed from procyanidins in a low pH medium like wine, could react with the nucleophilic C6 or C8 carbons of anthocyanin. This could eventually lead to a reddish-orange hue in the wine and oxygen is not required for this reaction (*7,26,27*). The use of visible and near infrared spectroscopy to measure the relative degree of oxidation in white wine has recently been demonstrated *(28)* but according to our knowledge this is the first report on the use of FTIR to follow the relative degree of oxidation in red wine. The development of alternative ways of measuring the progression of oxidation in red wine is thus important to complement sensory evaluations. FTIR seems be a possible method for doing this. More research is needed because the data presented here is preliminary work. Follow-up research could include testing this model on different wines, including red wines made from cultivars other than Pinotage.

Table 5.2 Concentration (mg/L) of different volatile compounds in wine C during oxidation. K, ox, 2, 3, 4 and 5: control, oxidised samples at stadiums 2, 3, 4 and 5 respectively. nd: not detected.

Compound	2K	2ox	3K	3ox	4K	4ox	5K	5ox
Ethyl acetate	76.50	57.48	69.01	54.09	69.01	44.54	69.55	19.34
Methanol	268.72	221.13	230.85	251.97	230.85	285.74	282.63	173.99
Ethyl butyrate	0.36	0.29	0.35	0.27	0.35	nd	0.27	nd
Iso-amylacetate	2.23	1.71	2.20	1.32	2.20	0.92	1.42	0.35
Iso-amylalcohol	231.04	222.81	228.60	226.56	228.60	240.89	205.18	180.48
Ethyl hexanoate	0.69	0.64	0.69	0.59	0.69	0.52	0.57	nd
Ethyl caprylate	0.28	0.27	0.28	0.25	0.28	0.20	0.17	0.12
Isobutyric acid	6.73	6.30	6.35	6.13	6.35	7.45	6.58	5.20
Butyric acid	0.83	0.85	0.79	0.80	0.79	0.88	0.74	0.64

5.4 ACKNOWLEDGEMENT

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6. GENERAL CONCLUSIONS AND FUTURE PROSPECTS

6.1. CONCLUSIONS AND FUTURE PROSPECTS

Oxygen can drastically influence the quality and consistency of wine. A decrease in white wine quality is often observed with O_2 addition, but it leads to an increase in colour and polymerisation of phenolic molecules in red wine if applied correctly.

In Chapter 3, it was shown that the addition of O_2 to wine can lead to the rapid growth of acetic acid bacteria and *Brettanomyces*. Free molecular SO_2 inhibits these two types of microorganisms, but it should be used with care because a world-wide trend to lower SO_2 concentrations is being experienced in the wine industry. This could explain the interest today in research into acetic acid bacteria and *Brettanomyces*. Lower SO_2 levels would require the development of other new preservatives to prevent the growth of these types of microorganisms in wine. Cellar practices should also be reviewed, including hygiene, barrel and O_2 management. This study investigated only one strain each of acetic acid bacteria and *Brettanomyces.* Due to strain differences, future research should include more strains tested in must and wine. This would give the winemaker a better idea of the minimum amount of molecular SO_2 required to inhibit these two spoilage microorganisms because bound SO_2 does not inhibit them. *Brettanomyces* seems to take up the molecular form of SO_2 relatively quickly and future work should expand on this to elucidate the exact mechanism of inhibition. Recently, relatively few wine-related research articles have been published on acetic acid bacteria. The effect that O_2 has on acetic acid bacterial numbers during fermentation and their interactions with yeast needs further study.

Micro-oxygenation was also investigated in this studyl (Chapter 4). Results showed that micro-oxygenation leads to increased colour density of certain wines, with certain other phenols being decreased. Micro-oxygenation also seems to prolong the survival of *Brettanomyces* and acetic acid bacteria in wine and should be conducted with care. The introduction of high amounts of O_2 into wine, as could happen during a subsequent racking, may lead to the growth of acetic acid bacteria and *Brettanomyces*. In younger red wines, micro-oxygenation increased the quality of the wine, but in older red wines it could lead to an over-aged character. Future research should focus on the effect of micro-oxygenation compared to use of oak

barrels because it is not known whether the recommendations of suppliers on the use of oak staves in conjunction with micro-oxygenation instead of the use of more expensive oak barrels are correct. In such a study, oak chips and oak dust, nowadays available on the South African wine market, could also be included. The effect that micro-oxygenation has on the sensory characteristics of red wine should also be further investigated because the 'structuring' and 'harmonisation' phases that the wine goes through during micro-oxygenation (as claimed by the suppliers) have also not been scientifically proved. Micro-oxygenation, however, when used correctly does seem to be a useful technique to increase the quality of certain wines.

The assessment of oxidation in wine is another field that needs more study. The oxidation of white wine has received more attention than that of red, probably due to the more complex phenolic nature of the latter. Light measurements in the visible spectrum of light are currently still used in wineries to assess colour changes in red wine. Oxidation of red wine may, however, occur before an excessive brown colour is observed. Fourier transform infrared spectroscopy (FTIR) may assist in future to better assess oxidation in red wine. This technique requires very little preparation and reaction time and has been modified and adjusted for the wine industry. This work (Chapter 5) showed that clear separation of oxidised Pinotage wines could be observed from the control even before drastic colour changes occur. Future work should include other red cultivars as well to compile a concise database of red wines undergoing oxidation. The phenolic complexity and diversity of different red wines might complicate such a study. Such a database could possibly be used as a tool to assess the sensitivity of a given red wine to oxidation.

It was clear from this study that O_2 plays a major role in the wine production process. O_2 can be applied by the winemaker in a positive manner, but careless practices regarding O_2 management can lead to the lowering of wine quality or even spoilage. More research is clearly needed on the complex interaction of wine with O_2.