The Effects of Micro-oxygenation on the Evolution of Red Wine Composition and Sensory Properties

A Focus on Reductive Odours

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry

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Abstract

Micro-oxygenation (MOX) is a technique that continuously delivers a small metered amount of oxygen into a wine by means of micro-bubbling using a porous micro-diffuser. MOX is considered an effective oxygen management tool for winemakers to produce particular wines in a consistent style. While several beneficial effects of MOX on wine quality have been well examined in scientific research, information regarding the influence of MOX on the reductive sulfur containing off-odours, sulfur containing substances mainly produce unpleasant smells which impart organoleptic defects in wine, the well-known as rubbery or 'reduced' character, is very limited. Of note is that the term 'reductive' here refers to sulfur containing volatiles with detrimental effects on wine quality, to distinguish them with compounds responsible for characteristic varietal aromas.

An analytical method, using headspace-microextraction coupled with Gas Chromatography - Mass Spectrometry, was successfully developed and validated for the separation and quantification of up to fourteen reductive 'light' and 'heavy' sulfur containing volatiles. The effects of MOX were examined using different oxygen dosage rates before and after malolactic fermentation (MLF) on a Cabernet Sauvignon and a BORDEAUX blend, respectively, in replicated trials using 300 L stainless steel tanks and high density polyethylene tanks (Flextanks). A commercial scale MOX trial on an Australian Shiraz after MLF was also monitored. The analysis focused upon the evolution of the chemical composition, especially the reductive sulfur compounds. Effects on the wine post MLF at the end of the MOX treatments in terms of the perceived sensory characteristics have also been investigated.

Limited oxygen exposure stimulated wine colour development, with positive effects still observed when spontaneous MLF occurred during the MOX operation. A significant influence of oxygen on tannins was only seen in the Australian Shiraz with prolonged oxygenation. MOX had fairly limited effects on individual polyphenols, except for the highly oxidisable flavan-3-ol epicatechin and the monomeric anthocyanins. Oxygenation did not affect the desirable varietal thiol 3-mercaptohexanol (3-MH), but resulted in a lowering in

the concentrations of most reductive sulfur compounds. These observations were reflected in the sensory characteristics of the finished wines.

The research provides insights into the effects of MOX on the reductive sulfur compounds in terms of both quantitative and sensory perspectives, alongside the effect upon other wine constituents including the polyphenols. The results indicated that the chemical mechanism leading to a decrease in the off-odour methanethiol is more likely to be linked with oxidised wine polyphenols rather than from a direct oxidation to a disulfide. Increase in the content of dimethyl sulfide may come about from chemical transformations occuring during wine storage.

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Abbreviations

ºC	degree Celsius
3-MH	3-mercaptohexan-1-ol
A_{420}	Absorbance at 420 nm
A_{520}	Absorbance at 520 nm
A_{620}	Absorbance at 620 nm
AAS	Atomic Absorption Spectroscopy
AED	Atomic Emission Detector
AF	Alcoholic fermentation
AWRI	The Australian Wine Research Institute
BHA	Butylated hydroxyanisole
CaF_2	Calcium fluoride
Car-PDMS	Carboxen - Polydimethylsiloxane
CAS	Chemical Abstracts Services
cm	centimeter(s)
CMP	Chemical Measurement Process
CO_2	Carbon dioxide
CS2	Carbon disulfide
DAD	Diode Array Detector
DAP	Diammonium phosphate
DEDS	Diethyl disulfide
DES	Diethyl sulfide
DMDS	Dimethyl disulfide
DMS	Dimethyl sulfide
DMTS	Dimethyl trisulfide
DVB/Car-PDMS	Divinylbenzene/Carboxen-Polydimethylsiloxane
DVB-PDMS	Divinylbenzene-Polydimethylsiloxane
EDTA	Ethylenediaminetetraacetic acid
et al.	and others
ETA	S-Ethylthioacetate

EtSH	Ethanethiol
FDA	The U.S. Food and Drug Administration
FPD	Flame Photometric Detector
FTIR	Fourier Transform Infrared Spectrophotometer
GC-MS	Gas Chromatography - Mass Spectrometry
g	gram(s)
GM	Genetically Modified
GRP	Grape Reaction Product (2-S-glutathionyl caftaric acid)
HS-SPME	Head Space Solid Phase Micro-Extraction
ICP-MS	Inductively Coupled Plasma - Mass Spectrometry
IDL	Instrumental Detection Limit
INAO	Institut National d'Appelation d'Origine (*The French Regulatory body*)
IS	Internal standard
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
L	litre(s)
LAB	Lactic acid bacteria(um)
LLMV	Lowest Level of Method Validation
LOD	Limit of Detection
LOQ	Limit of Quantification
LSD	Least Significant Difference
M	Molarity
mAU	milli-Absorbance Unit
MCPT	Methyl Cellulose Precipitated Tannin
MDL	Method Detection Limit
mDP	mean degree of polymerisation
MeSH	Methanethiol
ME	2-Mercaptoethanol
MLF	Malolactic fermentation
MOX	Micro-oxygenation

MQL	Method Quantification Limit
MSD	Mass Spectrophotometric Detector
MTA	Methylthioacetate
MTB	4-Methylthio-1-butanol
MTE	2-Methylthio-1-ethanol
mth	month
MTP	3-Methylthio-1-propanol
m^2	square meter
mm	millimeter(s)
min	minute(s)
mL	milliliter(s)
mg	milligram(s)
mg/L	milligram per liter
mg/L/month	milligram per liter per month
N	Normality
NIST	National Institute of Standards and Technology (USA)
NSW	New South Wales
NZ	New Zealand
µL	microliter(s)
µg	microgram(s)
µg/L	microgram per liter
nm	nanometer(s)
PFPD	Pulsed Flame Photometric Detector
p-HMB	Na-4-(hydroxymercury)benzoate
PMS	Potassium metabisulfite
PTFE	Polytetrafluoroethylene
RP-HPLC	Reversed Phase - High Performance Liquid Chromatography
rpm	revolutions per minute
RSC	Reductive sulfur compounds
SCD	Sulfur Chemiluminescent Detector

sec	second
SMM	S-methyl methionine
SPME	Solid Phase Micro-Extraction
TIC	Total Ion Chromatogram
Tukey HSD	Tukey's Honestly Significant Difference
UAHPEC	University of Auckland Human Participants Ethics Committee
UV-Vis	Ultraviolet- Visible
% v/v	percentage volume per volume
% w/v	percentage weight per volume
% RSD	Relative standard deviation

CHAPTER 1. LITERATURE REVIEW

1.1. INTRODUCTION

More than a hundred years ago, Louis Pasteur stated that '*oxygen is the worst enemy of wine*' ('L'oxygene est le pire ennemi du vin'), but also pointed out that '*oxygen makes the wine, which ages under its influence*' ('C'est l'oxygene qui fait le vin, c'est par son qu'il vieillit'). Oxygen is introduced in an uncontrolled manner at various winemaking operations, such as pressing, racking, pumping over, filtering, bottling and also at a slower rate during barrel ageing and storage (Caillé *et al.*, 2010; Gonzalez-del Pozo *et al.*, 2010). Excessive oxygen is destructive to wine quality, but slow and continuous oxygen dissolution may contribute positively to wine ageing (Cheynier *et al.*, 2002).

Too much oxygen in any type of wine may cause unexpected oxidation. One example is an organoleptic defect known as 'flatness', in which ethanol is oxidised to acetaldehyde and its derivatives. Consequently, the wine develops a freshly cut apple note of acetaldehyde, and bitter and acid tastes (Ribéreau-Gayon *et al.*, 2006b, pp. 235,237). Excessive oxygen in red wines leads to the formation of large molecules that are unable to remain in

solution and precipitate, leaving the wine with a dry[1] and harsh[2] taste, and a diminished colour density (Paul, 2002; Cano-López *et al.*, 2006). Oxygen also promotes discolouration or browning due to condensation reactions between oxidised phenolic compounds, and initiates the growth of spoilage microorganisms, such as acetic acid bacteria and *Brettanomyces* (Gomez *et al.*, 1995; Cano-López *et al.*, 2006; Devatine *et al.*, 2007; Jackson, 2008, p. 435).

On the other hand, oxygen plays an indispensible part in various winemaking steps in the control of yeast activity, and in the management of sulfur off-odours and fermentation derived volatile composition (Jones *et al.*, 2004). Limited oxygen exposure is beneficial for the development of red wines, especially after alcoholic fermentation and during the early stages of ageing (Ribéreau-Gayon *et al.*, 2006b; Silva & Lambri, 2006; Perez-Magarino *et al.*, 2007; Devatine & Mietton-Peuchot, 2009).

Understanding the ways oxygen affects wine, and being able to control oxidation processes, is crucial for winemakers who want to produce high quality wines and who want to achieve consistency in producing wine of definable specifications and styles (Jones *et al.*, 2004). Modern winemaking technologies, including hyper-reduction, hyper-oxygenation and micro-oxygenation[3] are among those that provide winemakers with more powerful tools for oxygen management. While the first two techniques are often used for white wines, the later technique - micro-oxygenation (MOX) is commonly applied to red wines.

Since its invention, the MOX technique has been used for many years in wineries all over

[1] Astringency terms describe the feelings of lack of lubrication or desiccation in the mouth (Gawel *et al.*, 2000).

[2] A negative hedonic grouping suggesting aspects of unbalanced astringency, excessive roughness and/or bitterness (Gawel *et al.*, 2000).

[3] Another term is nano-oxygenation which refers to the oxygen occurring in wine after bottling (Caillé *et al.*, 2010).

the world, such as in France, Italy, Australia, New Zealand, the United States and Chile (Ortega-Heras *et al.*, 2008). Most of the published papers have focused on the effects of MOX on phenolic compounds in wine. However, studies on the impacts of MOX on wine sensory properties - tastes and aromas were much less frequent until quite recently, when more reports have become available in the literature (Ortega-Heras *et al.*, 2008; Hernandez-Orte *et al.*, 2009; Caillé *et al.*, 2010; Cejudo-Bastante *et al.*, 2011a, 2011b). Quantitative studies with regard to the effect of MOX on changes in 'reduced' or 'reductive'[4] sulfur compounds, which cause off-odours, or in other words, 'reduction' defects in wine, are also very limited, and only one study by McCord (2003) has been published. A recent investigation by Ortega-Heras *et al.* (2008) mentioned the application of a higher oxygen dose for a brief period of time in their trial setup to eliminate certain reductive compounds which sometimes appear just after alcoholic fermentation. However, no particular odouriferous volatiles were monitored in this study. Investigating the changes in the concentrations of reductive sulfur compounds in wines undergoing MOX treatments is the major focus of the research reported in this thesis.

1.2. ROLES OF OXYGEN

1.2.1. Oxygen dissolution in wine

Wine picks up oxygen when it is in contact with air. The longer and the more vigorously it is agitated, the more oxygen is dissolved (Ribéreau-Gayon *et al.*, 2006b, p.389). The

[4] These terms (reduced, reductive, reduction) are used interchangeably in wine literature to refer to the presence of disagreeable sulfur containing compounds, which tend to form under reductive conditions - oxygen is more or less excluded. It is also important to note that these compounds can also form in non-reductive conditions - in the presence of oxygen. For example, disulfides contribute to the 'reduced' aromas, as commonly understood by the odour impact they produce, but can be formed by the oxidation of thiols, as further discussed in Section 1.4 on page 22.

dissolved oxygen then may react with wine components through various oxidation processes that transform the wine (Ribéreau-Gayon *et al.*, 2006b; Jackson, 2008; Gonzalez-del Pozo *et al.*, 2010; Wirth *et al.*, 2010). In chemical processes, oxidation is the phenomena of losing one or more electron from an atom or groups of atoms. In other words, the substance is oxidised. Reduction reactions, on the other hand, are the reverse of oxidation reactions. Many chemical reactions in wine involve electron transfers, involving oxidation and reduction reactions (Ribéreau-Gayon *et al.*, 2006b, p.388; Waterhouse & Laurie, 2006).

Oxygen in its normal lowest energy, triplet state with two unpaired radical electrons in different orbitals, has a very limited reactivity towards organic substances[5], but is more reactive in its singlet form with no unpaired electron (Cheynier *et al.*, 2002; Waterhouse & Laurie, 2006). This reactivity is very unlikely under wine conditions in the absense of light, but oxygen is more reactive as a result of processes catalysed by iron and copper ions, leading to various free radicals being produced (Waterhouse & Laurie, 2006).

At room temperature and atmospheric pressure, the amount of oxygen that dissolves in air saturated wine is 6 mL/L, or 8.6 mg/L (Singleton, 1987; Kilmartin, 2009). Note that at 25 °C, an oxygen rate delivered at 10 mL/L/month is equivalent to 14 mg/L/month (Kilmartin, 2010). Red wines can tolerate larger amounts of oxygen before the phenolic content is exhausted, since they contain more phenolics, the primary substrates of wine oxidation, than white wines (Waterhouse & Laurie, 2006). White wines can typically take up around 10 air saturations (60 mL O_2/L) without being over-oxidised. Meanwhile, oxygen at levels up to 10 to 30 saturations (60 - 180 mL O_2/L) can improve red wine characteristics (Singleton, 1987). During alcoholic fermentation, the presence of oxygen helps to increase cell viability towards the end of the fermentation, thereby improving fermentation kinetics and decreasing the risks of fermentation failure (Devatine *et al.*,

[5] They are singlet compounds with no unpaired electron (Cheynier *et al.*, 2002).

2007). During wine maturation, oxygen may have beneficial effects on the wine, by favouring colour stabilisation, degradation of vegetal characteristics and the disappearance of a reductive note (Bosso *et al.*, 2000; Devatine *et al.*, 2007; Ortega-Heras *et al.*, 2008). While wine oxidation has traditionally been associated with sensory and/or microbiological degradation, a moderate level of wine oxidation can impart benefits to a broad range of wines (Waterhouse & Laurie, 2006).

1.2.2. Oxygen in fermentation

The process of fermentation by the wine yeast *Saccharomyces cerevisiae* does not require oxygen. However, trace amount of oxygen indirectly favour fermentation by allowing the biosynthesis of sterols and long-chain fatty acids which are required for the production and proper functioning of the yeast cell membrane (Jackson, 2008, p. 378). Oxygen also prevents stuck fermentation[6] (Silva & Lambri, 2006). Aeration, such as pumping over during the fermentation of red wine, can also increase the production of a yeast fermentation by-product, acetaldehyde, which is considered a key component of oxidative polymerisation. Aeration, therefore, favours colour stability by enhancing the formation of anthocyanin-tannin adducts and polymers (Salmon, 2006; Jackson, 2008, p.379). Oxygen addition has been found to be efficient only at the end of the cell growth phase, with typical low levels of oxygen ranging from 5 to 10 mg/L (Salmon, 2006). Short aeration at the beginning, or a few days after the commencement of fermentation, has also been recommended to offset the likelihood of hydrogen sulfide accumulation (Jackson, 2008, p.379). The influences of oxygen on the performance of wine yeast are not discussed in detail here as it is outside the scope of this study.

[6] Fermentation that has stopped before all the available sugars in the wine have been completely depleted (Ribéreau-Gayon *et al.*, 2006a, p.80).

1.2.3. Oxygen in maturation and ageing in oak barrels

Great red wines are traditionally aged in oak barrels from the end of fermentation until bottling in order to bring about positive effects on wine development, in terms of colour, clarity and flavour (Ribéreau-Gayon *et al.*, 2006b).

Barrel ageing accounts for 50 % of oxygen occurring in red wines. The remainder comes from handling operations, treatments and regular winemaking tasks (Ribéreau-Gayon *et al.*, 2006b). Kelly *et al.* (2003) calculated the barrels's own oxygen diffusion[7] potential to be 500 mL over a period of a month, which is equivalent to approximately 2.2 mL/L/month for a typical barrel that has a 225 L capacity, 2 m^2 surface area and 27 mm wall thickness. Oxygen diffusion from the headspace can only account for a very low rate of 0.4 mL/L/month (Kelly & Wollan, 2003).

The oxygen transferring into the wine during barrel maturation and ageing, although at a slow rate, nevertheless contributes to colour stabilisation and a lowering of astringency (Caillé *et al.*, 2010). Contact with oak wood also provides the wine with oak flavours and ellagic tannins, which appear to indirectly play a role in colour stability (Ribéreau-Gayon *et al.*, 2006b; Jackson, 2008, p.467). Oak maturation also lowers concentrations of some reductive sulfur compounds, including dimethyl sulfide and dimethyl disulfide, while methionyl acetate[8] also diminishes, but only in the presence of oxygen (Jackson, 2008, p.469).

[7] Oxygen diffusion is the process by which oxygen permeates through the oak wood of barrels into the wine, due to the pressure difference between the two sides. The partial pressure of oxygen in the atmosphere (18 kPa) is higher than the equilibrium vapour pressure of oxygen in the wine, assumed to be closed to zero due to oxygen being consumed by chemical reactions (Kelly & Wollan, 2003).

[8] Methionyl acetate or 3-(methylthio)propyl acetate is a heavy reductive sulfur volatile (boiling point 92 °C, perception threshold 50 µg/L) that can produce mushroom off-odours in wine (Ribéreau-Gayon *et al.*, 2006b, p. 265).

The oxygen permeability varies depending on the type of oak used, e.g. it was reported to be higher in French oak barrels and lower in American oak barrels (Nevares & del Alamo, 2008). The oxygen diffusion rate also decreases over time as the barrel ages, since most of the wood pores will be plugged with wine deposits, leading to inadequate levels of oxygenation for the optimum maturation of wines in barrels (Kelly & Wollan, 2003; Cano-López *et al.*, 2010). Barrel micro-oxygenation is believed to require a long ageing time during which the wine consumes practically all of the oxygen present (Nevares & del Alamo, 2008). It has been suggested that higher doses, e.g. 8 mL/L/month, than those naturally supplied by barrels might be necessary (Kelly & Wollan, 2003). The permament oxygen diffusion through the wood barrel walls and other technological inputs such as racking and topping up provides the wine with a slow and continuous dissolution of oxygen during barrel maturation. In fact, oxygen is consumed at a faster rate than it is provided to the wine in barrels. However, quantification and management of oxygen input during these conventional maturation process have never been controlled (Devatine *et al.*, 2007).

1.3. MICRO-OXYGENATION

1.3.1. Definition

Micro-oxygenation (MOX) is a technique developed in the Mediran region of southern France in the 1980s and commercially released in 1996 (Cano-López *et al.*, 2006). The principle of the MOX technique is that of continuously delivering a small and controlled amount of oxygen into the wine stored in tanks at different stages of the winemaking process, from fermentation to bottling (Pour-Nikfardjam & Dykes, 2003; Devatine *et al.*, 2007; Ortega-Heras *et al.*, 2008). The technique is contrasted to the widely used practice

of aerated racking[9] which involves the addition of large, discreted doses of oxygen into the wine (**Figure 1.1**) (Lesica & Kosmerl, 2009).

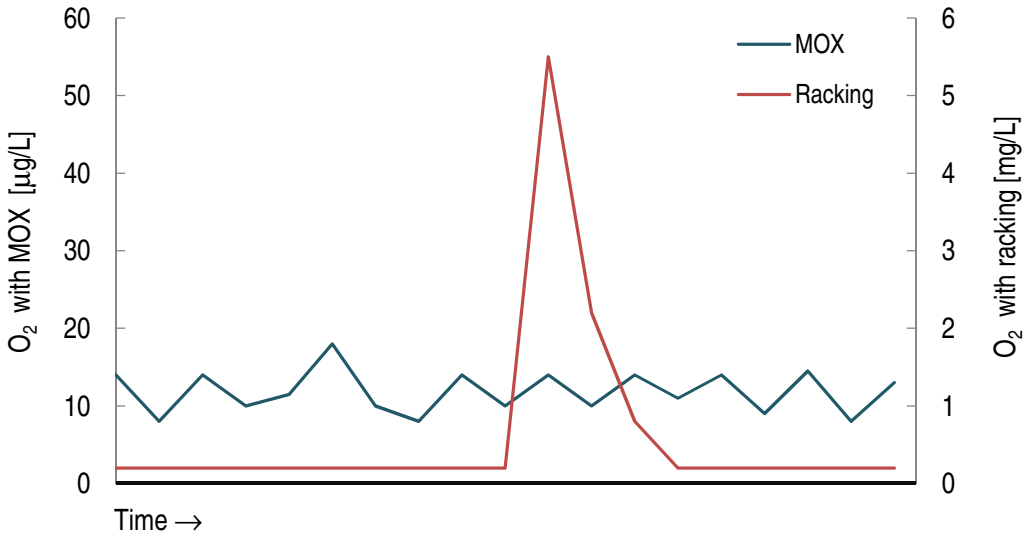


Figure 1.1. Dissolved oxygen during micro-oxygenation and racking of wine (Lesica & Kosmerl, 2009)

MOX is regarded as a modern technical innovation, as opposed to the use of oak barrels in the traditional art and craft of winemaking. The technique has gained considerable attention among both winemakers and researchers around the world, in their endeavour to understand how it affects various chemical processes that can positively transform the wine and improve its quality.

For winemakers, it is obvious that the ability to control the oxygen input would be of great assistance to master the vinification process (Devatine *et al.*, 2007). In fact, MOX has

[9] The operation of transferring the wine from one vat or barrel to another in order to separate clear wine from the sediments at the bottom of the container as well as the deposits on the sides, especially in wooden barrels (Ribéreau-Gayon *et al.*, 2006b).

existed as long as winemakers have put wine into barrels where oxygen slowly enters the wine via barrel staves and during topping operations (Cano-López *et al.*, 2006). However, oak barrel ageing may introduce the risks of microbiological contamination (e.g. *Acetobacter* and *Brettanomyces*) (Dykes, 2007; Rayne, 2007). In addition, it is inconvenient, laborious and hard to control, and for certain grades of wine is not cost effective. MOX, when used in conjunction with oak chips and staves, which are popular barrel flavour alternatives, can save millions of dollars when compared to the cost of purchasing and shipping of oak barrels (Goldfarb, 2007). Ageing in tanks is undoubtedly more adapted to large scale wine production and a consistent quality can be obtained (Devatine *et al.*, 2007). MOX, therefore, was proposed as an attempt to mirror and even accelerate the positive oxidative wine transformations occurring when oxygen diffuses through permeable barrel staves (Dykes, 2007).

In the increasingly competitive world wide market, it is without a doubt very important that winemakers can make particular wines in a consistent style. By carefully monitoring the oxygen dosage by means of MOX, an effective tool is therefore provided for their consideration. The most widely used technique, and now the accepted practice to micro-oxygenate wine, is micro-bubbling, using a porous gas distributor/micro-diffuser (Cano-Lopez *et al.*, 2007; Devatine *et al.*, 2007). The diffusers/spargers are designed to generate micro-bubbles in order to obtain maximum efficiency in the transfer for pure gaseous oxygen into the wine. They are made using porous ceramic diffusers, with pore diameters in the range of 2 - 4 μm, which generate micro-bubbles of around 400 μm in diameter (Devatine & Mietton-Peuchot, 2009).

MOX can be applied at any stage after alcoholic fermentation up until bottling, although it has been shown to be more effective after alcoholic fermentation and before malolactic fermentation (MLF) (Parish *et al.*, 2000; Jones *et al.*, 2004; Cano-López *et al.*, 2006; Lesica & Kosmerl, 2009). This could be explained by the fact that the indirect

condensation of anthocyanins and tannins requires the utilisation of the carbo-cationic form of acetaldehyde[10] (See **Figure 1.4**), therefore proceeds more rapidly at low pH before MLF (Parish *et al.*, 2000). In addition, sulfur dioxide added after MLF can bind with acetaldehyde to form acetaldehhyde hydroxysulfonates, thus making acetaldehyde less available for the condensation of anthocyanins and tannins.

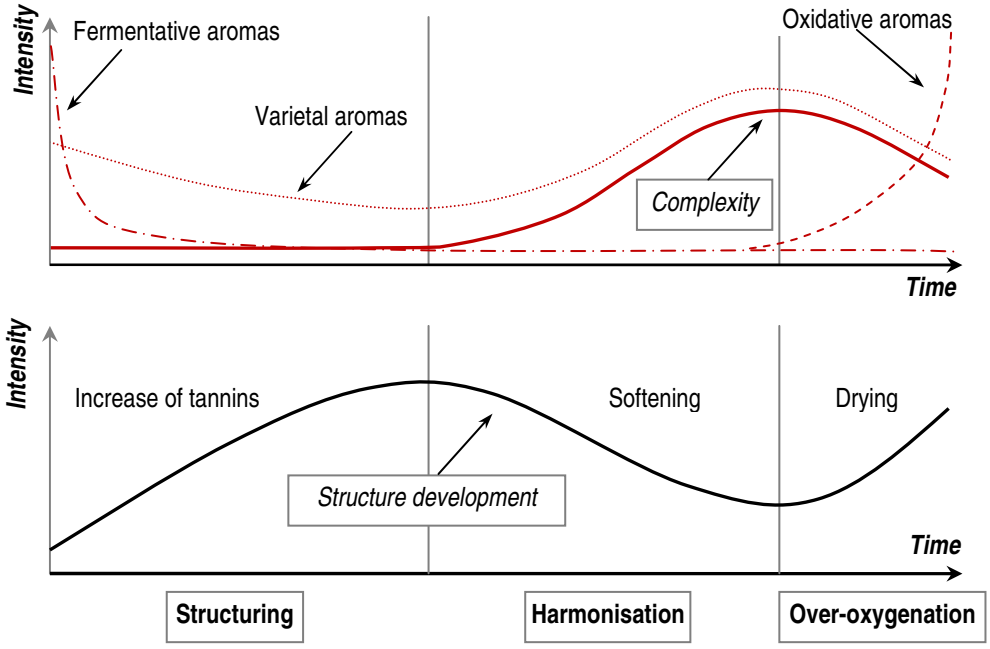


Figure 1.2. Development of the organoleptic profile of wine during the micro-oxygenation process

(Adapted from Parish *et al.*, 2000)

[10] The term acetaldehyde is preferentially used in this thesis as it is commonly used in wine literature, although ethanal is the correct chemical term for this aldehyde.

Table 1.1. Oxygen rates and duration of research micro-oxygenation trials

Wine	Oxygen rate and duration	References
	Before MLF	
3 reds	30 - 40 mL/L/month up to 4 weeks prior to completion of MLF	Loch (2002)
Monastrell	Low & high dose after AF and resume after MLF	Cano-López *et al.* (2006)
Monastrell	5 & 10 mL/L/month before AF; 3 & 5 mL/L/month after MLF for 2 months	Cano-López *et al.* (2007)
Span. reds	20 - 42 mL/L (total) for 18 - 23 days	Perez-Magarino *et al.* (2007)
Span. reds	50 - 60 mL/L/month for 10 days, then 20 - 30 mL/L/month for 8 - 10 days	Ortega-Heras *et al.* (2008)
CabSav.& Tempranillo	60 mL/L/month for 15 days	Hernandez-Orte *et al.* (2009)
CabSav.	90 mg/L/month for 3 days, then 10 mg/L/month for 17 days	Gonzalez-del Pozo *et al.* (2010)
Petit Verdot	45 mL/L/month for 20 days	Cejudo-Bastante *et al.* (2011a)
	After MLF	
Sangiovese	O_2 added till saturation levels at monthly & every 2 months for 6 months	Castellari *et al.* (2000)
Pinot noir	2 mL/L/month for 7 months	Castel *et al.* (2001)
CabSav.	10 mL/L/month for 1 month, then 5 mL/L/month for 4 months	McCord (2003)
CabSav.	5 mL/L (duration not specified)	Atanasova *et al.* (2002a)
CabSav.	5 mL/L/month	Pour-Nikfardjam & Dykes (2003)
CabSav.	3 mg/L/month for 3 months	Llaudy *et al.* (2006)
Merlot	4 mL/L/month; 3.1 mL/L/month (electrochemical MOX) for 12 weeks	Fell *et al.* (2007)
Merlot	10 mL/L/month for 112 days	Tao *et al.* (2007)
Sangiovese	3 and 9 mL/L/month for 90 days	Sartini *et al.* (2007)
Shiraz	2 mL/L/month till end of vintage time	Rudnitskaya *et al.* (2009)
CabSav.	2 mg/L/month for 3 months	Gonzalez-del Pozo *et al.* (2010)

Notes: *3 reds: 3 wines including Shiraz, Merlot & Cabernet Sauvignon; Span. reds: Spanish red grape varieties; CabSav.: Cabernet Sauvignon.*

Other studies seem to confirm that MOX applied after MLF gives better results for wine quality, especially with regard to colour and palate structure (Castellari *et al.*, 2000; Pour-Nikfardjam & Dykes, 2003). Nevertheless, Perez-Magarino *et al.* (2007) indicated that application of MOX after the alcoholic fermentation and before MLF was the best moment to commence, giving high colour stability of the colour and structure of wine, and doing that MOX wines were more coloured than the control after MLF.

Various oxygen doses have been applied in practice, but higher rates are normally used early pre MLF, while lower rates are employed post MLF (Table 1.1) (Jones *et al.*, 2004). Empirical results have shown that the application of MOX can improve the structure and body of wine, remove sulfides and reductive odours, stabilise colour, and lessen herbaceous characters (Parish *et al.*, 2000). The classic description of wine development during MOX is illustrated in Figure 1.2.

Changes in the organoleptic properties of the wine can be separated into two distinct stages, namely the structuring phase and the hamonisation phase (Parish *et al.*, 2000). An increase in the aggressiveness and intensity of tannins on the palate, together with a corresponding decline in the aromatic intensity and complexity of the wine, characterise the first phase of MOX. The harmonisation phase is characterised by an increase in tannin softness and general wine complexity. The disappearance of vegetal and reductive characters in the wine may also be observed during this second phase of MOX. Over-oxygenation leads to tannin dryness accompanied by the irreversible loss of wine freshness and the expression of oxidised aromas (Lemaire, 1995 in Parish *et al.*, 2000). Of the improved organoleptic characters, the capability of MOX to enhance palate structure, a parameter found to be highly correlated with the polyphenolic profile, is one of the principle reasons for employing the technique (Pour-Nikfardjam & Dykes, 2003).

1.3.2. The influence of MOX on wine colour and polyphenols

Since being initiated, most studies devoted to MOX have emphasized the influence of

different oxygenation treatments on wine colour and phenolics (Castellari *et al.*, 2000; Castel *et al.*, 2001; Atanasova *et al.*, 2002a; Pour-Nikfardjam & Dykes, 2003; Cano-López *et al.*, 2006; Llaudy *et al.*, 2006; Cano-Lopez *et al.*, 2007; Fell *et al.*, 2007; Perez-Magarino *et al.*, 2007; Sartini *et al.*, 2007; Tao *et al.*, 2007; Rudnitskaya *et al.*, 2009; Gonzalez-del Pozo *et al.*, 2010).

As wine matures and ages, the intinial red-violet colour of young red wines changes gradually to a brick red-orange colour characteristic of more aged wines (Alcalde-Eon *et al.*, 2006; Gonzalez-del Pozo *et al.*, 2010). Anthocyanins and flavan-3-ols are two groups of flavonoids which are particularly important to the quality of red wines. Anthocyanins (Greek: *anthos* - flower, *kyanos* - blue) are the principal red grape pigments (Sandler & Pinder, 2003; Cheynier *et al.*, 2006). Flavan-3-ols present in wine in the forms of catechin monomers, oligomers and polymers. In current grape and wine literature, the oligomers are generally called proanthocyanidins[11] and the polymers are also referred to as condensed tannins (Waterhouse, 2002; Cheynier *et al.*, 2006; Kennedy *et al.*, 2006). The colour of young red wines is ascribed to the monomeric anthocyanins, and co-pigmented[12] anthocyanins with various phenolic co-factors such as flavanols, flavonols and hydroxycinnamic acids (Boulton, 2001). During wine ageing, the amounts of these compounds diminish in favour of more stable polymeric pigments[13], such as pyranoanthocyanins and ethyl-linked compounds which are generated by different types of chemical reactions (Atanasova *et al.*, 2002a; Alcalde-Eon *et al.*, 2006; Gonzalez-del Pozo

[11] The name proanthocyanidins derives from the release of red anthocyanidins (cyanidin and/or delphinidin) when proanthocyanidins are heated in acidic solutions (Waterhouse *et al.*, 2000; Vidal *et al.*, 2004).

[12] Copigmentation is the phenomenon in which pigments and other non coloured organic molecules (co-factors) form molecular associations or complexes (Boulton, 2001). Copigments are considered as a storage form of anthocyanins, allowing more anthocyanins to be extracted from the grape skins and stabilised in solution until the polymeric pigments are formed (Sacchi *et al.*, 2005).

[13] A heterogeneous group of biomacro-molecules formed from the condensation reaction of anthocyanins with other grape polyphenols, including, in particular, flavan-3-ols (Remy *et al.*, 2000; Sacchi *et al.*, 2005).

et al., 2010). The increased stability of the resulting polymeric pigments compared with monomeric anthocyanins, are thus of great importance for the long term stability of wine colour (Sacchi *et al.*, 2005).

Research has confirmed the capability of MOX to stabilise wine colour, with increased colour density and a more rapid decrease in less stable monomeric anthocyanins (Atanasova *et al.*, 2002a; McCord, 2003; Perez-Magarino *et al.*, 2007). A recent study also found that these beneficial effects on wine colour stabilisation were not only observed during MOX treatment but were also maintained over time during many months of ageing (Gonzalez-del Pozo *et al.*, 2010). The colour enhancement impact of MOX has been attributed to various transformation reactions, including oxidation, condensation and polymerisation, of different compounds, mainly polyphenols, leading to the formation of pigments and polymeric compounds which can stabilise wine colour (Perez-Magarino *et al.*, 2007). Because the flavylium C4 of the anthocyanins is involved in bond formation, these polymeric forms are not reactive towards sulfur dioxide, nor are they responsive to changes in pH (Bakker & Timberlake, 1997).

Several mechanisms explaining the conversion of anthocyanins to more stable pigments have been proposed and confirmed (Atanasova *et al.*, 2002a; Cano-López *et al.*, 2006). The first mechanism is the *direct reaction between anthocyanins and flavanols*, leading to the formation of two types of products, denoted A^+-T (anthocyanin-flavanol) and T-A^+ (flavanol-anthocyanin), according to the position of the anthocyanin moiety (**Figure 1.3**) (Remy *et al.*, 2000; Atanasova *et al.*, 2002a; Sun *et al.*, 2008). The nucleophilic addition (1) of the flavanol on the flavylium cation anthocyanin produces either the colourless flavene (A-T adduct), which can be oxidised (a) to the red flavylium and proceed further (b) to a yellow xanthylium salt, or be converted (c) to a colourless bicyclic condensation product. The reaction (2) between the intermediate carbocation of proanthocyanidin (electrophile), released by acid-catalysed cleavage of their interflavanic bond, with the anthocyanin in its hemiketal form (nucleophile), produces the T-AOH adduct. This

colourless dimer in hemiketal form will further dehydrate (d) to the red flavylium form (Remy *et al.*, 2000; Sun *et al.*, 2008).

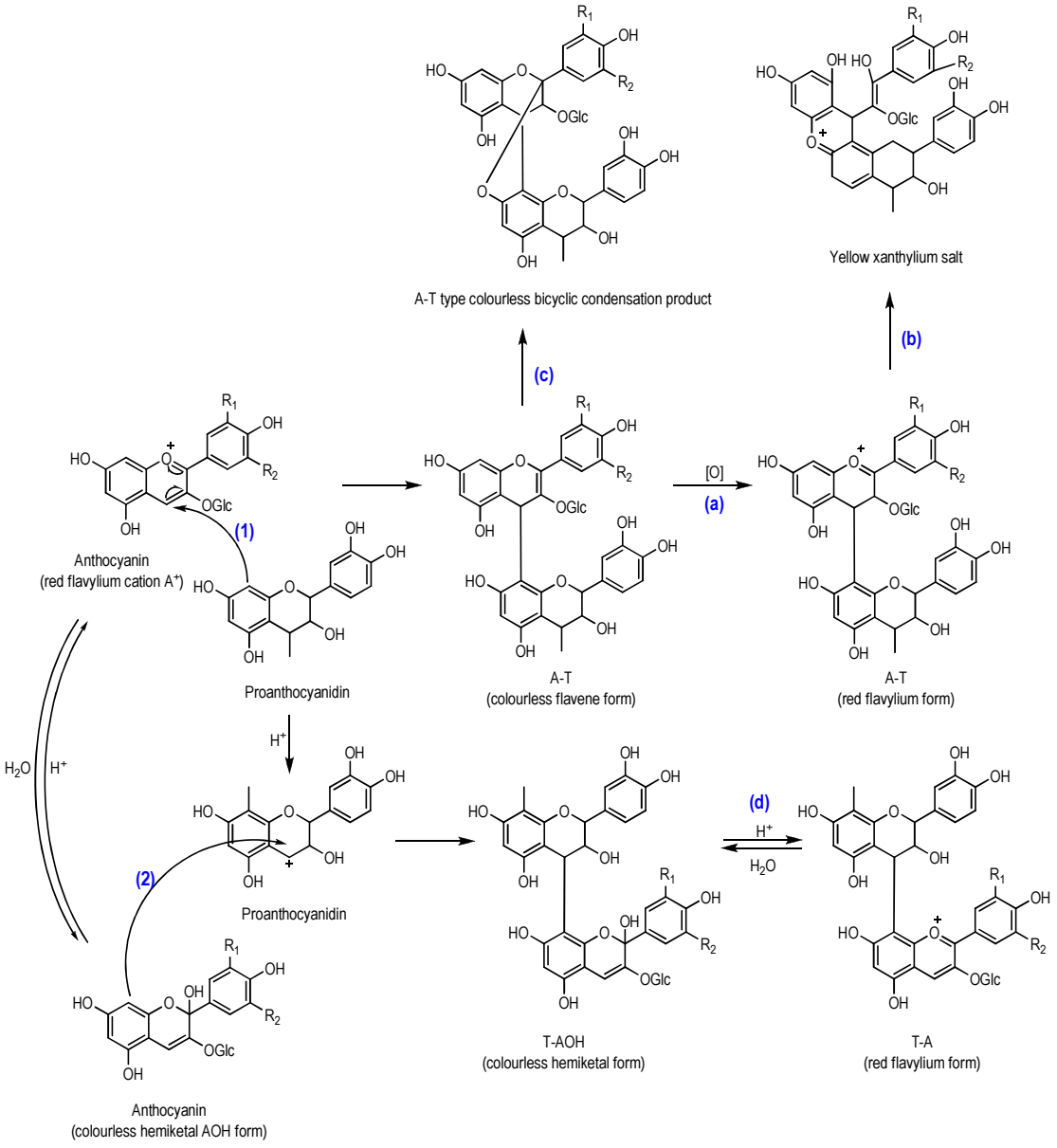


Figure 1.3. Mechanisms of direct reaction between anthocyanins and flavanols in wine

(Adapted from Cheynier, 2002, in Sun *et al.*, 2008)

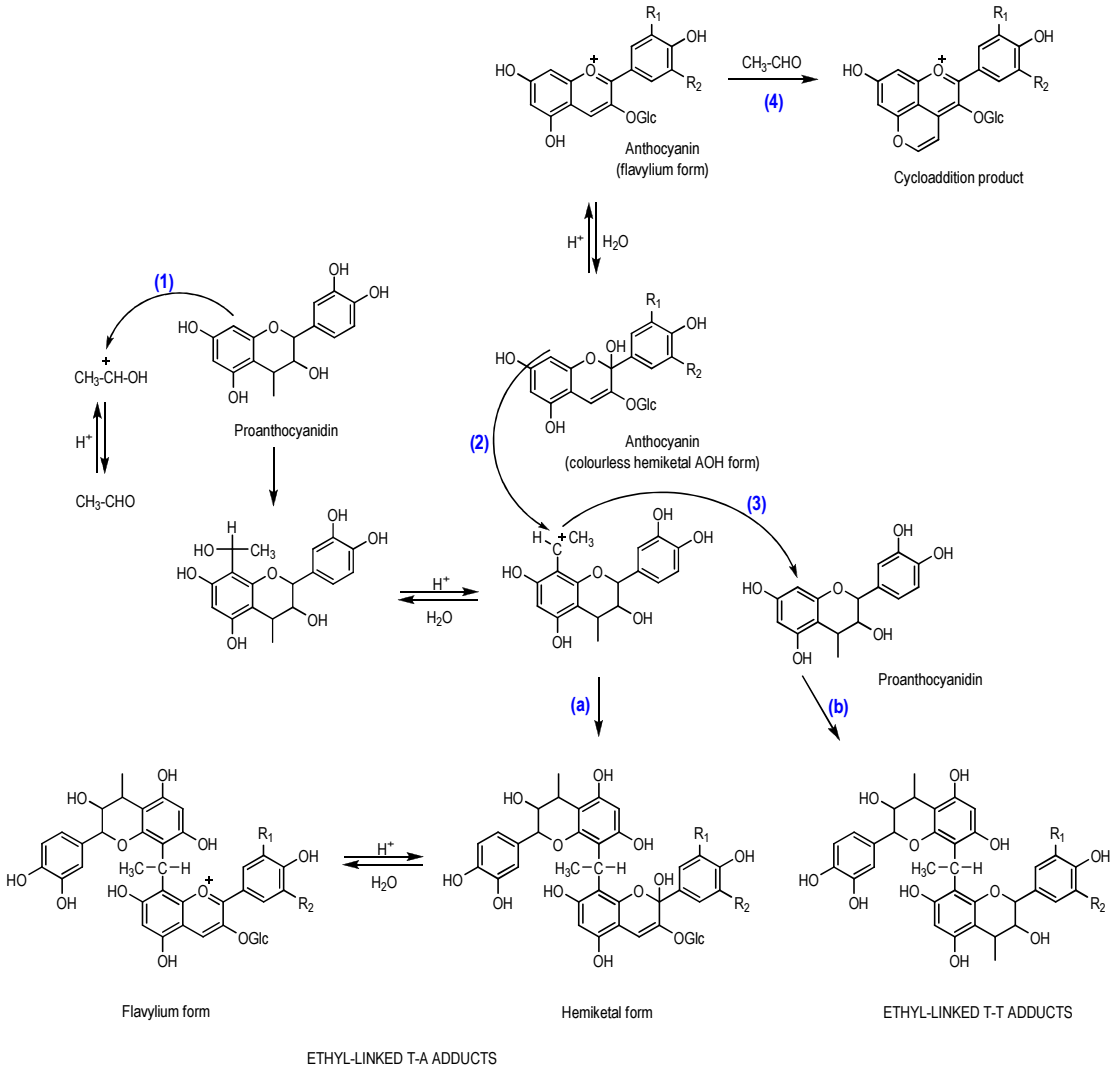


Figure 1.4. Mechanisms of acetaldehyde mediated indirect reaction between anthocyanins and flavanols in wine

(Adapted from Cheynier, 2002, in Sun *et al.*, 2008)

The second mechanism (**Figure 1.4**) is the *indirect condensation of anthocyanins and flavanols mediated by acetaldehyde*, starting with the nucleophilic addition of a flavanol to a protonated acetaldehyde (1). The product formed then loses a water molecule to yield a carbocation intermediate (electrophilic agent), which is involved in nucleophilic addition (2) by anthocyanins in the hemiketal form to produce ethyl linked T-A adducts (a). The

nucleophilic addition (3) may also proceed with other flavanols to form ethyl linked T-T adducts (b) (**Figure 1.4**) (Sun *et al.*, 2008).

The third mechanism (**Figure 1.4**) is the cycloaddition (4) of anthocyanins with acetaldehyde, leading to the formation of orange pigments structurally allied to the pyranoanthocyanins (Atanasova *et al.*, 2002a). These cycloaddition products are exceptionally resistant to sulfite bleaching, owing to substitution at the carbon 4 position (Bakker & Timberlake, 1997).

The condensation of anthocyanins with flavanols by the first two mechanisms, therefore, causes *bathochromical shifts* (to the violet direction) in the visible absorption maxima of the anthocyanins, providing a bluish-red hue to the wine. By contrast, cycloaddition reactions of anthocyanins with other compounds to produce pyranoanthocyanins cause a *hypsochromic shift* in the visible absorption maxima towards orange hue (Alcalde-Eon *et al.*, 2006).

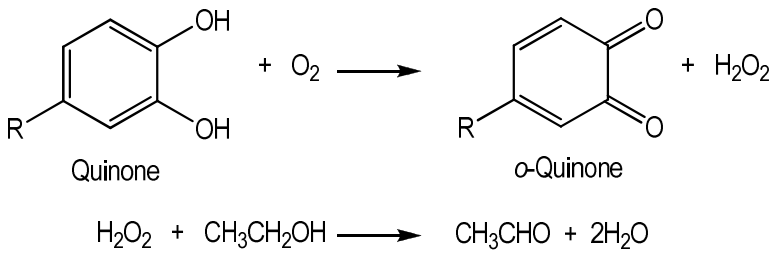


Figure 1.5. Autoxidation of vicinal dihydroxyphenols producing acetaldehyde (Adapted from Singleton, 1987)

Different factors, including the ratio of anthocyanins to flavanols, pH and temperature, oxygen availability and other cofactors such as metal ions have been shown to affect anthocyanin-flavanol interactions (Sun *et al.*, 2008). The indirect condensation reaction mediated by acetaldehyde is expected to be stimulated by oxygen addition. The reason is that in the presence of oxygen, vicinal dihydroxyphenols in grapes or wines are oxidised to

produce hydrogen peroxide as an intermediate oxidant. The hydrogen peroxide then reacts with ethanol, generating acetaldehyde (Figure 1.5) (Singleton, 1987). Even though acetaldehyde is consumed, and thus quantitative analyses are difficult to interpret, it is known to stimulate reactions of anthocyanins with other phenolic compounds, especially the flavanols (Jones *et al.*, 2004).

1.3.3. The influence of MOX on wine aroma and sensory characteristics

Research on the influence of MOX on wine aroma is very limited. Recently, some publications have reported the impact of MOX on wine aroma (Ortega-Heras *et al.*, 2008; Hernandez-Orte *et al.*, 2009; Cejudo-Bastante *et al.*, 2011a, 2011b). No significant changes in varietal aromas and fermentation derived volatile compounds in wine undergoing MOX were observed in the study of Ortega-Heras *et al.* (2008). However, some oak derived compounds were extracted in a way that facilitated a positive effect of MOX on the integration of woody notes with fruity and varietal aromas, similar to the findings more recently reported by Cejudo-Bastante *et al.* (2011a, 2011b), who studied the effects of MOX in addition to oak chips on Petit Verdot and Merlot wines.

Although MOX is credited with being able to oxidise and eliminate reductive sulfur containing volatiles, quantitative studies regarding the concentrations of sulfur compounds in micro-oxygenated wines is very limited in the literature, with a study by McCord (2003) being one example. The risks of objectionable smells caused by sulfur compounds in the final wine, along with their diverse possible origins, are reasons why the sulfur problem in must and wine is always a matter of concern for winemakers. Some research has been carried out on this topic but the current understanding of the phenomena involved is still unclear (Ribéreau-Gayon *et al.*, 2006b, p. 261). Regarding the effects of MOX on these undesirable sulfur compounds, only McCord (2003) has reported a significant decrease in the levels of methanethiol and ethanethiol in wine undergoing MOX. Levels of dimethyl sulfide were expected to increase in the absence of biological

processes in this study, yet its concentration instead decreased (McCord, 2003). Limited publications with regard to this important aspect could be due to a lack of a good analytical methodology in the past for the quantification of these highly sensitive trace components in a wine matrix. The influence of the MOX treatment on undesirable sulfur containing volatiles in wine, and first and foremost the task of establishing a good quantification method for these compounds, therefore, forms the major focus of the research presented in this thesis.

MOX is also proposed to enhance wine palate structure leading to less astringent characters through a softening of tannins. A wine may have a balanced impression or a certain aggressiveness that is either perceptible as bitterness (a perception of taste) on the end of the palate, or as astringency (a tactile sensation) on the aftertaste, depending on the type and concentration of tannins (Ribéreau-Gayon *et al.*, 2006b, p. 181). The mechanism of astringency perception is generally attributed to interactions of polyphenols with salivary proteins, which lessen mouth lubrication. The perception of bitterness is restricted to small molecules and depends on the interactions of these substances with taste receptors on the tongue (Vidal *et al.*, 2003; Cheynier *et al.*, 2006). The relative bitterness and astringency vary depending on the molecular size of polyphenolic compounds (Peleg *et al.*, 1999; Brossaud *et al.*, 2001). The monomeric catechins are bitter and astringent. On the other hand, in the polymers, the bitterness is minimal and the astringency remains (Peleg *et al.*, 1999; Brossaud *et al.*, 2001; Waterhouse, 2002). Figure 1.6 illustrates an example of a proanthocyanidin[14] with the sub-units linked through C4 and C8 positions (Waterhouse *et al.*, 2000). Studies have indicated that both galloylation and B-ring trihydroxylation contributed of the astringent sensation, providing that affinity of epigallocatechin gallate for immobilised salivary protein was higher than that of epicatechin gallate, which its affinity for salivary proteins was higher than those of

[14]Two sub-groups of proanthocyanidins, or once condensed, tannins, are procyanidins, consisting of (epi)catechin units (3',4' di-OH, R = H), and prodelphinidins, composing of (epi)gallocatechin (3',4',5' tri-OH, R = OH) (Waterhouse *et al.*, 2000).

epigallocatechin and of epicatehin (Brossaud *et al.*, 2001). The position of the galloy substituents also plays an important role in astringent sensation, because the major galloylated procyanidin in grape seeds, cyanidin dimer B3 3'-gallate bound less strongly to salivary proteins than the corresponding non galloylated species, B2 During wine maturation and ageing, the interactions of these highly reactive molecules with anthocyanins via polymerisation and condensation reactions may contribute to the loss of astringency (Brossaud *et al.*, 2001).

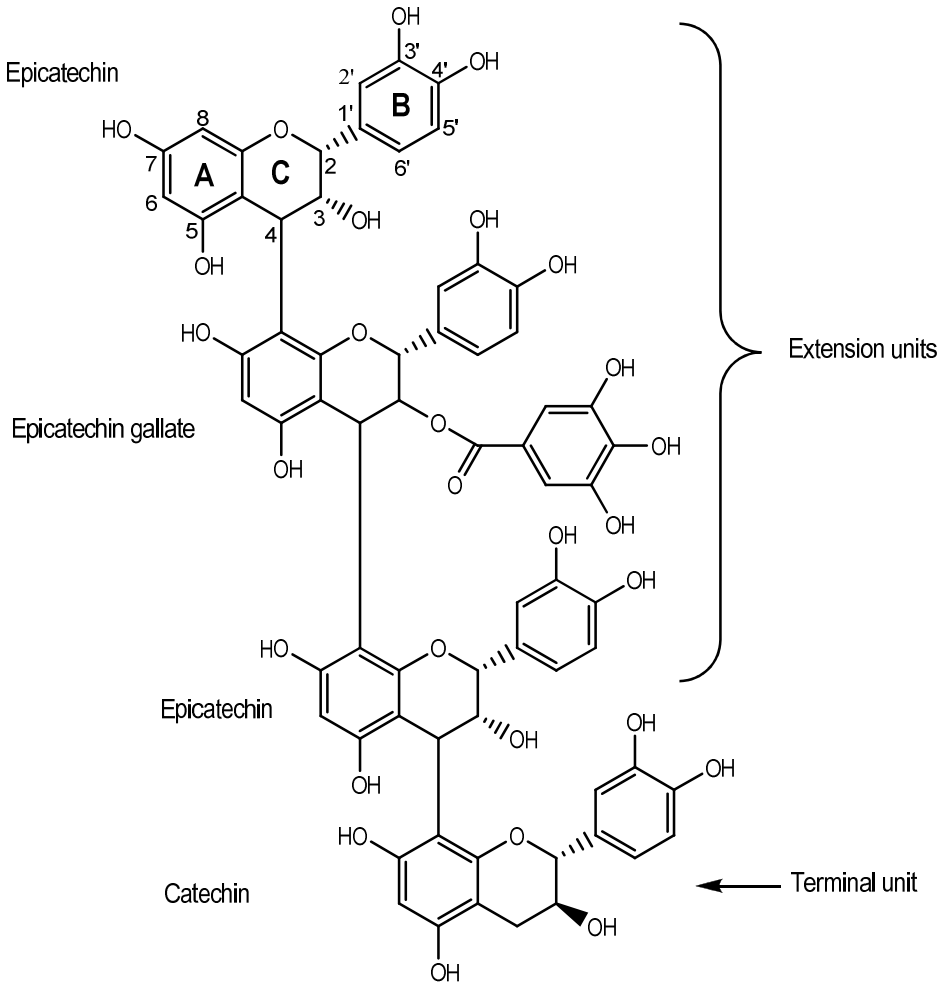


Figure 1.6. Structure of a proanthocyanidin consisting of possible catechol sub-units linked through C4-C8 bonds

Anthocyanins and most tannins are highly unstable. After being extracted by maceration in the fermenting must, they can undergo various enzymatic and chemical reactions as the wine is made and aged, thus bringing about changes in sensory properties and structural modifications to the wine (Cheynier *et al.*, 2006). The polymeric pigments or pigmented tannins, formed from the reactions of anthocyanins and tannins during ageing, as discussed in Section 1.3.2 and illustrated in Figure 1.3 and Figure 1.4 above, are thought to have different protein-binding properties, and may therefore contribute to the lowering of astringency (Villamor *et al.*, 2009).

The level of astringency was for a long time considered to increase to the decamer level but then decrease beyond this chain length as the polymers get too bulky, so are insoluble and unable to contribute to astringency (Ribéreau-Gayon *et al.*, 2006b). However, a study by Vidal *et al.* (2003) showed that larger tannins extracted from grape material were progressively more astringent and drying than smaller tannins. In addition, high molecular weight proanthocyanidins, with mean degree of polymerisation (mDP) greater than 20, were found to be removed from many wines because they are precipitated selectively from the wine by proteins added as fining agents. The longer tannin molecules interact strongly with proteins and are a major source of wine astringency (Cheynier *et al.*, 2006). Consequently, flavanol polymerisation reactions enhance rather than lower astringency, regardless of the polymers formed (proanthocyanidins, oxidation products, or ethyl-flavanols) (Cheynier *et al.*, 2006). However, Llaudy *et al*., (2006) reported a much lower astringency in a micro-oxygenated Cabernet Sauvignon wine, in which only a slight difference in the mDP of the proanthocyanidins was found. The relationship between bitterness, astringency and tannin length is extremely complex (Pour-Nikfardjam & Dykes, 2003). During wine ageing, cleavage reactions and incorporation of anthocyanins into small tannin structures, rather than polymerisation, may lead to a loss of astringency. Conversion of tannins to oligomeric tannin-anthocyanins adducts could well lower wine astringency. The astringency and mouthfeel of other polymers, such as ethyl-linked tannin-anthocyanin polymers and tannin-pyranoanthocyanins, have not yet been investigated. In a recent study, anthocyanins from red grape skin were isolated and added to different

white wine ferments in order to explain the complex mouthfeel difference of red and white wines (Oberholster *et al.*, 2009). It was found that the presence of anthocyanins during fermentation led to an increase in the intensity of astringency-related terms (Oberholster *et al.*, 2009). Nevertheless, other wine components such as polysaccharides and proteins can also form tannin complexes with salivary proteins, thus interfering with astringency perception (Cheynier *et al.*, 2006). Upon tasting, a combination of tannins and polysaccharides gives the impression of fullness and roundness which is highly desirable (Ribéreau-Gayon *et al.*, 2006b, p. 181). Positive evolution of sensory attributes, including mouthfeel and astringency, towards a more balanced and softer direction, has been reported in micro-oxygenated wine (Dykes, 2007).

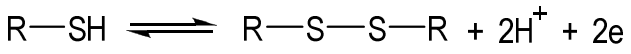
MOX has been also reported to affect the olfactory sensory properties of wine. Descriptive sensory analysis in a MOX study by Llaudy *et al.* (2006) revealed that MOX before oak ageing accentuates the oak aromatic notes, with significantly higher levels of toasting, spices and coffee aromas than the control wine. Another study (Hernandez-Orte *et al.*, 2009) also found the favourable effect of MOX on the toasty note of a Tempranillo wine after 8 months of maturation, but no increase was found for the herbaceous notes. On the other hand, the micro-oxygenated Cabernet Sauvignon wine in this study exhibited more herbaceous characters and less toasty, reduction, leather and animal odours (Hernandez-Orte *et al.*, 2009). These observations suggest that the extent to which MOX affects the aroma notes in wine may also vary depending on the grape varieties and the chemical composition of the wine.

1.4. 'REDUCTIVE' OR 'REDUCED' AROMAS

Aromatic substances are among the key determinants of food and beverage quality, owing to their interactions with the senses of smell and taste. These sensory impressions can then determine consumer acceptance or rejection of a product (Merken & Beecher, 2000; Mestres *et al.*, 2000). The aroma of foods is influenced by different compounds, among

which sulfur containing compounds are an important group due to their abundance and aromatic impact. Likewise, wine also contains various sulfur containing structures which have a great sensory impact and play a significant role in wine aroma and flavour (Mestres *et al.*, 2000; Swiegers & Pretorius, 2007; Moreira *et al.*, 2008). Some volatile sulfur compounds, notably with a thiol function, e.g. 3-mercaptohexan-1-ol (3-MH) with a passion fruit aroma, have been reported to contribute positively to the characteristic nuances of wine aromas (Darriet *et al.*, 1995; Bouchilloux *et al.*, 1998; Tominaga *et al.*, 1998a).

However, sulfur containing substances can produce unpleasant odours which impart organoleptic defects in wine, the well-known as rubbery or 'reduced' character, even at very low concentrations (Beloqui & Bertrand, 1995; Mestres *et al.*, 2000). The terms 'reduced' and 'reductive' used in this thesis refer to sulfur containing volatiles with detrimental effects on wine quality, to distinguish them with compounds responsible for characteristic varietal aromas. They are not used in the strict chemical sense to mean that the compounds in question are in their most 'reduced' state (and unable to be chemically reduced further), given, for example, that disulfides (R-S-S-R) can be formed from thiols (R-SH) by oxidation (loss of electrons), and can be reduced back again (**Equation 1.1**).



Equation 1.1. Oxidation of thiols to form disulfides

1.4.1. Classification of reductive sulfur compounds

Sulfur containing compounds found in wine are usually classified according to their volatility into two categories, the more volatile (light) and less volatile (heavy) sulfur compounds, with a boiling point below and above 90 °C, which is the boiling point of 3-methylthiopropanol (Beloqui & Bertrand, 1995; Karagiannis & Lanaridis, 1999; Mestres *et al.*, 2002). This classification is helpful for deciding an appropriate analytical technique to use because some techniques give better results than the others, depending on the

boiling points of the analytes (Mestres *et al.*, 2002). Based on their functional chemical structures, sulfur compounds can also be divided into different families, such as thiols, thioethers (e.g. sulfides and polysulfides), thioesters and heterocyclic compounds (described in detail in Section 1.4.2 below).

Table 1.2. Some important light and heavy off-odours sulfur compounds

(Spedding & Raut, 1982; de Mora *et al.*, 1987; Rauhut, 1993; Mestres *et al.*, 2000; Fang & Qian, 2005; Ribéreau-Gayon *et al.*, 2006b, pp. 263,265; Landaud *et al.*, 2008)

Sulfur compounds	Boiling point (ºC)	Perception threshold in wine (µg/L)	Odour description	Concentration range in wine (µg/L)
Light sulfur compounds				
Hydrogen sulfide	-61	0.001 - 150	Rotten eggs	nd - 370
Methanethiol	6	0.3**	Cooked cabbage	nd - 16
Ethanethiol	35	1.1	Onion, rubber, putrefaction	nd - 50
Dimethyl sulfide	35	10 - 160	Cabbage, asparagus, corn, molasses	nd - 910
Carbon disulfide	46	> 38	Cabbage, rubber	nd - 18
Dimethyl trisulfide	58	0.1*	Cabbage, onion, cooked vegetables	nd - 111
Heavy sulfur compounds				
Diethyl sulfide	90-92	0.93 - 18	Garlic	nd - 10
Dimethyl disulfide	109	20 - 45	Cooked cabbage, asparagus, onion	0 - 22
Diethyl disulfide	151-153	4.3 - 40	Garlic, onion, burnt rubber	nd - 85
2-Mercaptoethanol	157	130	Barnyard-like (*böxer*), poultry	nd - 400
Methylthioacetate	98	300*	Sulfury, rotten vegetables	nd - 115
S-Ethylthioacetate	116	40*	Sulfury	nd - 180
2-Methylthio-ethanol	170	250**	Cauliflower, French bean	88 - 139
3-Methylthio-propanol	90	1200	Cooked cabbage, cauliflower, meat-like	145 - 5655
4-Methylthio-butanol	96	100	Earthy, chive-garlic, onion	nd - 181
Benzothiazole	234	50 - 350	Rubber	0 - 30

** in beer; ** in hydroalcoholic solution; nd - not detected*

1.4.1.1. Light sulfur compounds

Light sulfur compounds are characterised by their high volatility and low perception thresholds (examples are given in Table 1.2). They can generally impart very unpleasant odours in wine. Nevertheless, most of these compounds can be easily eliminated by simple aeration (Beloqui & Bertrand, 1995; Mestres *et al.*, 2002). In 'reduced' wine, their concentrations are much higher than their perception thresholds (Ribéreau-Gayon *et al.*, 2006b, p. 262).

1.4.1.2. Heavy sulfur compounds

Many heavy sulfur compounds have been identified in wine, but only few have a significant impact on reduction defects (Ribéreau-Gayon *et al.*, 2006b, p. 265). Unlike the light compounds, heavy sulfur compounds are considered to be more detrimental to wine quality. This is due to their high boiling points making it impossible to eliminate these compounds from wine by simple aeration (Beloqui & Bertrand, 1995; Mestres *et al.*, 2002; Ribéreau-Gayon *et al.*, 2006b).

1.4.2. The odourant impacts of reductive sulfur compounds on wine aroma

Many sulfur compounds which cause off-odours have been found in wine. Each compound can produce different unpleasant odours when close to, or well above, their perception thresholds.

1.4.2.1. Thiols (mercaptans)

Mercaptans are the simplest organosulfur compounds, characterised by hydrocarbon chains attached to a sulfhydryl (-SH) group (Jackson, 2008, p. 307). The name 'mercaptan' for these compounds refers to their capacity to be precipitated by mercury

salts (Ribéreau-Gayon *et al.*, 2006b, p. 261). Of these compounds, hydrogen sulfide[15], methanethiol (methyl mercaptan) and ethanethiol (ethyl mercaptan) (**Figure 1.7**) are significant members, classified as light sulfur compounds, and play a decisive role in reduction defects (Silva Ferreira *et al.*, 2003; Ribéreau-Gayon *et al.*, 2006b, p. 262).

Hydrogen sulfide is the most common volatile sulfur compound in wine and can produce a very nauseating smell of rotten eggs (Mestres *et al.*, 2000; Jackson, 2008, p. 307). Although hydrogen sulfide (H_2S) can accumulate during fermentation to concentrations of a few hundred µg/L, well beyond its perception threshold (1.6 µg/L in white wine) (Park *et al.*, 2000; Siebert *et al.*, 2010), it is rapidly eliminated from the fermentation due to its high volatility and the purging action of CO_2 down to much lower concentrations of 1 - 20 µg/L at the end of fermentation (Ugliano *et al.*, 2010a). It was found that the formation of H_2S during fermentation was affected by yeast strains, YAN[16] and fermentation vigour[17]. Although the highest nitrogen fermentation always produced wines with the lowest H_2S, the residual concentration of H_2S in the finished wine did not depend on the total amount of H_2S produced during fermentation. For a given yeast, the one that produced the highest H_2S during fermentation did not lead to the wine with the most final H_2S (Ugliano *et al.*, 2010a). The results from this study implies that there is a need to establish the mechanisms that determine H_2S concentrations in wines at the end of fermentation and in the final wines, e.g. chemical reactions with wine constituents, such as phenolics and quinones (Ugliano *et al.*, 2010a).

Methanethiol is the simplest organic sulfur compound commonly found in wine. It has a very low perception threshold of only 0.3 µg/L in alcoholic solution and can generate an

[15] Hydrogen sulfide is not a thiol, but is a sulfide (having the sulfur anion in its lowest oxidation state of -2) in strict chemical classification. However, it can be considered as the simplest inorganic thiol (containing –SH group), whereas methanethiol is the simplest organic thiol.

[16] Yeast Assimilable Nitrogen

[17] The rate of CO_2 evolution and its purging effect

odour reminiscent of cooked cabbage (Mestres *et al.*, 2000). The concentration of methanethiol is usually lower than 1 µg/L after alcoholic fermentation but it can be greater than 10 µg/L in a wine which exhibits reductive characters (Landaud *et al.*, 2008).

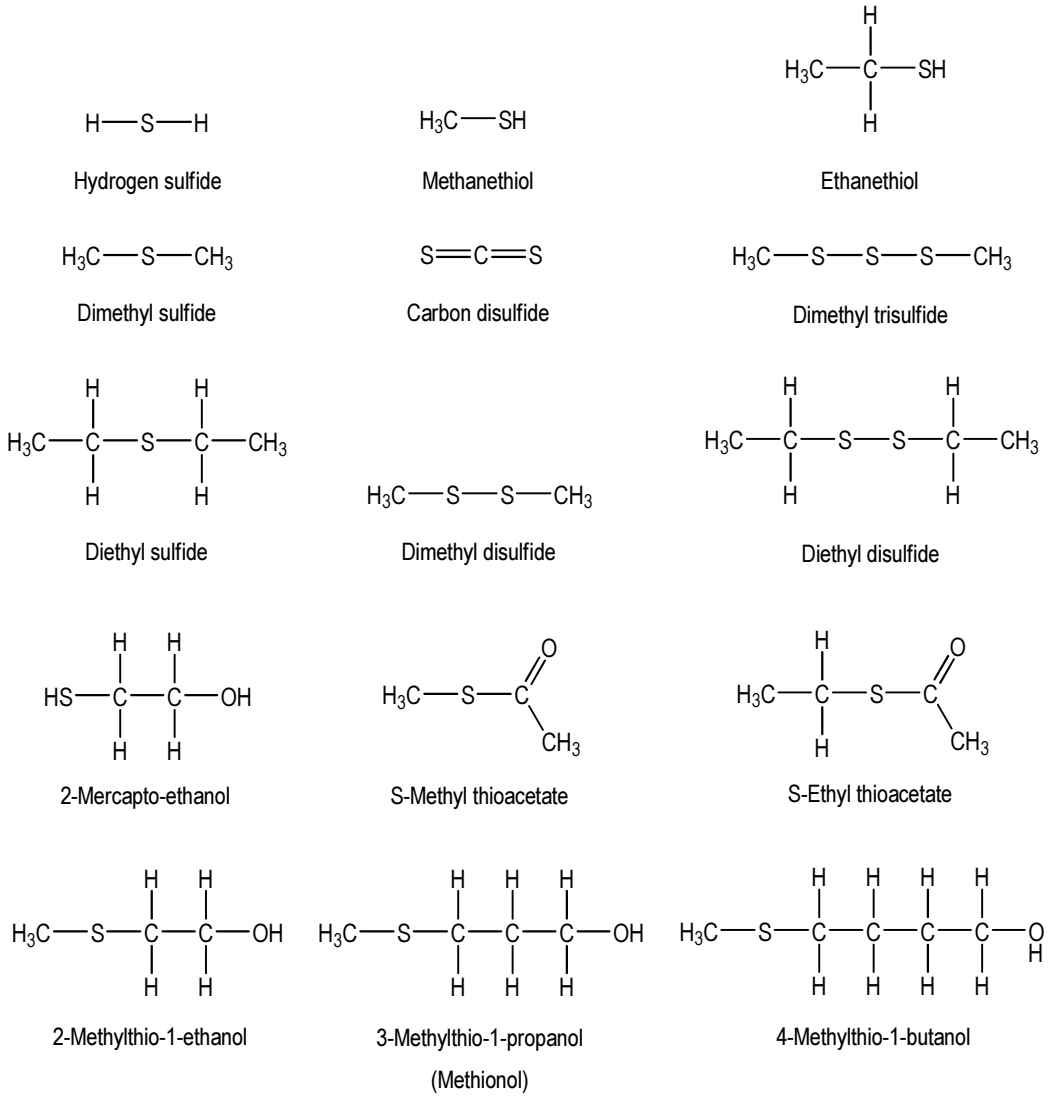


Figure 1.7. Chemical structures of some important reductive sulfur compounds

Ethanethiol produces rotten onion and burnt rubber odours at a concentration close to its threshold level, whereas at higher levels it has a skunky, fecal odour (Jackson, 2008, p. 307). These simple, low molecular weight thiols, namely methanethiol and ethanethiol,

can be easily oxidised to yield disulfides (Hofmann *et al.*, 1996) or react with other wine aroma compounds, giving rise to other off-odours (Mestres *et al.*, 2000). They can also form complexes and precipitates with many metal ions (Mateo-Vivaracho *et al.*, 2006).

2-Mercaptoethanol (Figure 1.7) is a heavy sulfur compound that is found in this group. It produces a powerful barnyard-like (*böxer*) odour (Rapp *et al.*, 1985; Jackson, 2008, p. 307). While keeping in mind that an aroma characteristic of wine is often brought about by a synergistic effect of a mixture of compounds, 2-mercaptoethanol is probably the principle component in the *böxer* off-odour aroma in wine (Rapp *et al.*, 1985).

Although most thiols have negative impacts on wine aroma, some of them, such as 3-mercaptohexan-1-ol, and 4-mercapto-4-methylpentan-2-ol are desirable wine aromas, producing typical varietal notes in some wines (Darriet *et al.*, 1995; Tominaga *et al.*, 1998a).

1.4.2.2. Thioethers (sulfides, disulfides, trisulfides and thioether alcohols)

Thioethers are organosulfur compounds characterised by the presence of one or more sulfur atoms bonded between two carbon atoms (Jackson, 2008, p. 308). These compounds include sulfides, polysulfides, and thioether alcohols. The incidence of sulfides and polysulfides, such as dimethyl sulfide, diethyl sulfide, dimethyl disulfide and diethyl disulfide (Figure 1.7) in wine has been reported (Spedding *et al.*, 1983; de Mora *et al.*, 1987; Goniak & Noble, 1987; Park *et al.*, 1994). Of these off-odours, dimethyl sulfide has been the cause of some differences in opinion regarding its influence on wine aroma (de Mora *et al.*, 1987). Above its perception threshold level, dimethyl sulfide contributes off-odours described as cooked cabbage and shrimp-like, whereas at low levels it produces odours reminiscent of asparagus, corn and molasses (Jackson, 2008, p. 308). On the other hand, low levels of dimethyl sulfide may contribute positively to the fruity bottle bouquet of some wines (Marais, 1979; Spedding & Raut, 1982; de Mora *et al.*, 1987).

Carbon disulfide (**Figure 1.7**) was found for the first time in wine in 1980 to be present at a level of 10 µg/L (Leppanen *et al.*, 1980). In the headspace of some New Zealand wines, carbon disulfide was found to reach 15 - 18.9 µg/L (Spedding *et al.*, 1983). Carbon disulfide can generate odours described as cabbage and rubbery smells (Mestres *et al.*, 2000). However, when considering the concentration of carbon disulfide usually found in wine, it should not actually be perceptible. Nonetheless, it may modify the perceived aroma of wine when present at a moderate concentration by raising the perception threshold of various pleasant aromas and accentuating unpleasant odours (Ribéreau-Gayon *et al.*, 2006b, p. 265). No other information regarding the perception threshold of carbon disulfide in aqueous matrices has been reported, except that in a study by Spedding *et al.* (1982), its perception threshold in distilled water was found to be higher than 38 µg/L. However, the concentration of carbon disulfide normally found in wine is typically well below this value, likely explaining why further examination of its perception threshold has not been undertaken.

Thioether alcohols including 2-methylthio-ethanol, 3-methylthio-propan-1-ol, 4-methylthio-butan-1-ol (**Figure 1.7**), classified as heavy sulfur compounds, have also been reported in wine. Of these compounds, **3-methylthio-propan-1-ol (methionol)** is considered the most important heavy sulfur compound playing a major role in reduction defects caused by yeast (Ribéreau-Gayon *et al.*, 2006b, p. 265). It was the first sulfur compound found in wine by Muller (1971) (Beloqui *et al.*, 1995). The methionol content of a wine is nearly always higher than its perception threshold (1200 µg/L) when a reduction defect attributed to heavy sulfur compounds during alcoholic fermentation develops. By contrast, the concentration of **2-methylthio-ethanol** in some wines with reductive odours may be very close to its perception threshold (Ribéreau-Gayon *et al.*, 2006b, p. 265). **4-Methylthio-butanol** was first identified in wine by Rapp *et al.* (1985). This thioether produces a sharp chive-garlic aroma similar to methionol, plus an apparent earthy aroma when present at levels near its perception threshold (0.1 to 1 mg/L in 15 % v/v ethanol water) (Rapp *et al.*, 1985).

1.4.2.3. Thioesters

Thioesters are simply the sulfur analogues of esters, characterised by the general formula R-S-CO-R', which result from the esterification between a thiol (R-SH), as opposed to an alcohol in a regular esters, and a carboxylic acid (R'-COOH) (Stanforth, 2006, p. 8). Among the thioesters, **methyl** and **ethyl thioacetates** (S-methyl and S-ethyl esters of ethanethioic acid) (**Figure 1.7**) were identified and determined for the first time in beers and wines by Leppanen *et al.* (1980). Despite their high sensory thresholds, these thioacetates are a potential source of off-odours because they can be hydrolysed to give free thiols (Leppanen *et al.*, 1980).

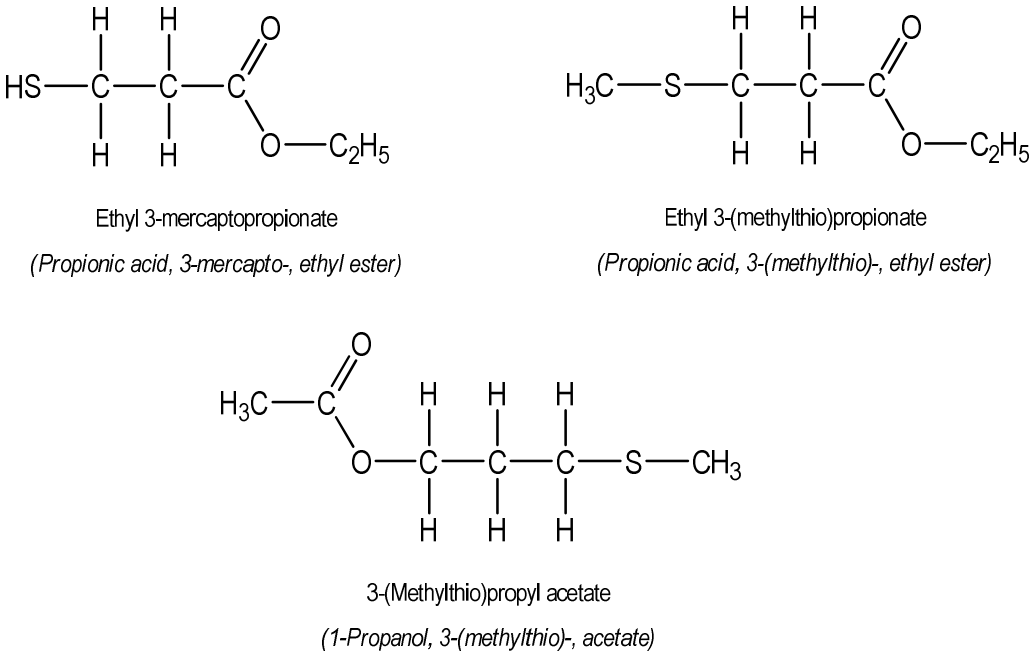


Figure 1.8. Sulfur containing ester derivatives of propionic and acetic acids in grape and wine

(Based on Kolor, 1983; Ribéreau-Gayon *et al.*, 2006b, p. 263)

Other related esters containing sulfur in their structures have also been reported in wine. An example is ethyl 3-mercaptopropionate, which was identified in *Vitis labrusca* grapes

(Kolor, 1983). At low levels, it has very pleasant fruity and fresh grape aromas, but a skunky or foxy, animal like odour at higher concentrations (Kolor, 1983). Other thioesters including ethyl 3-(methylthio)propionate and 3-(methylthio)propyl acetate may also be present in wine (Ribéreau-Gayon *et al.*, 2006b, p. 263). Structures of these compounds are shown in Figure 1.8.

1.4.2.4. Heterocyclic sulfur compounds

Heterocyclic sulfur compounds have also been detected in wine. Some examples are benzothiazole (with a rubber odour) (Herraiz *et al.*, 1991; Beloqui & Bertrand, 1995), 5-(2-hydroxyethyl)-4-methylthiazole (medicinal, peanut like smell) (Rapp *et al.*, 1985) and 2-methylthiolane-3-ol (faint onion-like smell) (Jackson, 2008, p. 308). Concentrations of benzothiazole commonly found in wine are in the range of 0.4 to 7 µg/L, while an addition of 24 µg/L is needed for a negative influence on the aroma of wine to be observed (Rauhut, 1993). 5-(2-Hydroxyethyl)-4-methylthiazole was also found in grape distillates (Strauss & Williams cited in Rapp *et al.*, 1985). Its odour threshold in 15 % v/v ethanol water was determined to be from 0.1 to 1 mg/L (Rapp *et al.*, 1985). However, whether it occurs at concentrations high enough to directly affect wine aroma is not yet known (Jackson, 2008, p. 308).

1.4.3. The origins of reductive sulfur compounds in wine

1.4.3.1. Wine yeast metabolism

Sulfur containing volatiles may have different origins, of which the metabolism of wine microorganisms is the principle source (Swiegers & Pretorius, 2007; Moreira *et al.*, 2008). This could be the metabolism of the wine yeast *Saccharomyces cerevisiae* during alcoholic fermentation (AF) and from lactic acid bacteria *Oenococcus oeni* during malolactic fermentation (MLF) (Lavigne *et al.*, 1992; Escudero *et al.*, 2000; Moreira *et al.*, 2002; Silva Ferreira *et al.*, 2003; Pripis-Nicolau *et al.*, 2004; Moreira *et al.*, 2005; Vallet *et al.*, 2008).

There are two pathways, the *sulfate assimilatory reduction* and the *sulfate dissimilatory reduction* pathway, involved in the metabolism of sulfur compounds by microorganisms. In the first pathway, sulfate is taken up and used for the biosynthesis of organic compounds such as cysteine and methionine. In the later one, a sulfate molecule is reduced as part of the respiratory pathway to sulfite or sulfide with little being metabolised and most being excreted, as occurs with sulfate reducing bacteria[18] in an anaerobic environment (Swiegers & Pretorius, 2007).

Volatile sulfur compounds in wine mainly come from the transformation of sulfur containing amino acids through the metabolism of wine yeast (Jackson, 2008, p. 307). Levels of light sulfur compounds may increase after the end of AF. By contrast, concentrations of heavy compounds remain stable during ageing in most cases (Ribéreau-Gayon *et al.*, 2006b, p. 265).

During fermentation, the *assimilatory reduction* of sulfate by wine yeast can lead to the excessive production of the hydrogen sulfide HS^- ion which causes the formation of the undesirable hydrogen sulfide (H_2S) in the wine (Spiropoulos *et al.*, 2000; Mendes-Ferreira *et al.*, 2002; Swiegers & Pretorius, 2007). The concentration of H_2S varies depending on the presence of various sulfur compounds, wine yeast strain, fermentation conditions and the nutritional status of the grape juice (Rauhut, 1993; Spiropoulos *et al.*, 2000; Swiegers & Pretorius, 2007). After being liberated, H_2S can also act as a precursor for other reductive sulfur compounds (Lambrechts & Pretorius, 2000). For example, it may combine with ethanol or with acetaldehyde to form ethanethiol (**Figure 1.9**), although this has not been proven (Rauhut, 1993; Landaud *et al.*, 2008).

[18] Bacteria that can use sulfate as an oxidising agent, reducing it to sulfide which is mostly released to the outside environment and substantially hydrolysed to free H_2S (Postgate, 1979).

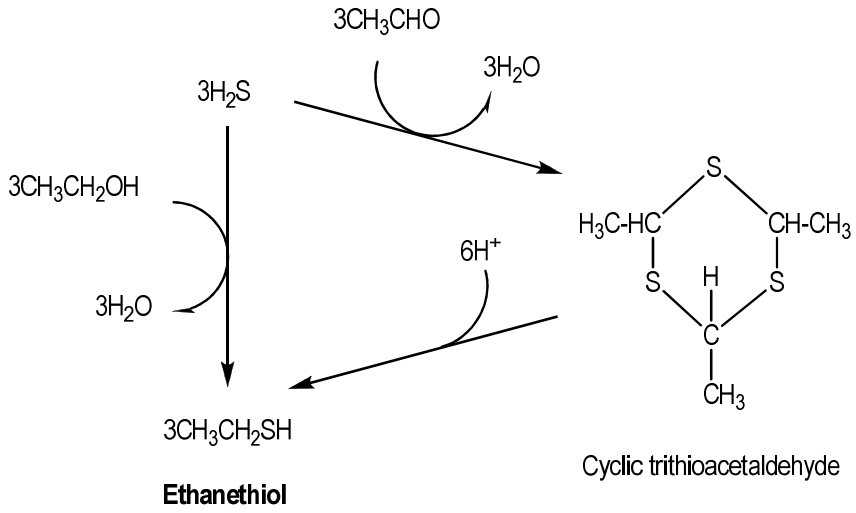


Figure 1.9. Proposed reactions of hydrogen sulfide with acetaldehyde and ethanol to form ethanethiol (Rauhut, 1993; Landaud *et al.*, 2008)

Ethanethiol, as well as other light sulfur compounds, including methanethiol, dimethyl sulfide, dimethyl disulfide and diethyl disulfide, can also originate from yeast metabolism acting on sulfur-containing amino acids (Boulton, 1996, pp. 173-174). The highly volatile compound, methanethiol, can be formed from yeast metabolism of methionine. It can either be oxidised to disulfides, such as dimethyl disulfide, or can lead to the production of thioesters, S-methylthioacetate and S-ethylthioacetate, via acetyl-CoA (Landaud *et al.*, 2008; Moreira *et al.*, 2008). The oxidation of methanethiol in wine can also lead to the formation of dimethyl trisulfide and dimethyl tetrasulfide (Rauhut, 1993).

The biosynthesis of dimethyl sulfide in wine has not yet been fully elucidated. It has been suggested that, under certain circumstances, yeast may produce S-methyl methionine, which subsequently can be enzymatically hydrolysed to dimethyl sulfide and homoserine (Figure 1.10) (Rauhut, 1993). On the other hand, it has been postulated that yeast cannot synthesize S-methyl methionine, but that cysteine, cystine and glutathione can be precursors of dimethyl sulfide (de Mora *et al.*, 1986; Rauhut, 1993; Landaud *et al.*, 2008). Dimethyl sulfoxide has also been demonstrated as a possible precursor of dimethyl sulfide during bottle storage and ageing (Figure 1.10) (Spedding *et al.*, 1980; de Mora *et al.*,

1986; de Mora *et al.*, 1993). A recent study (Loscos *et al.*, 2008), however, demonstrated that S-methyl methionine is a major dimethyl sulfide precursor in grapes.

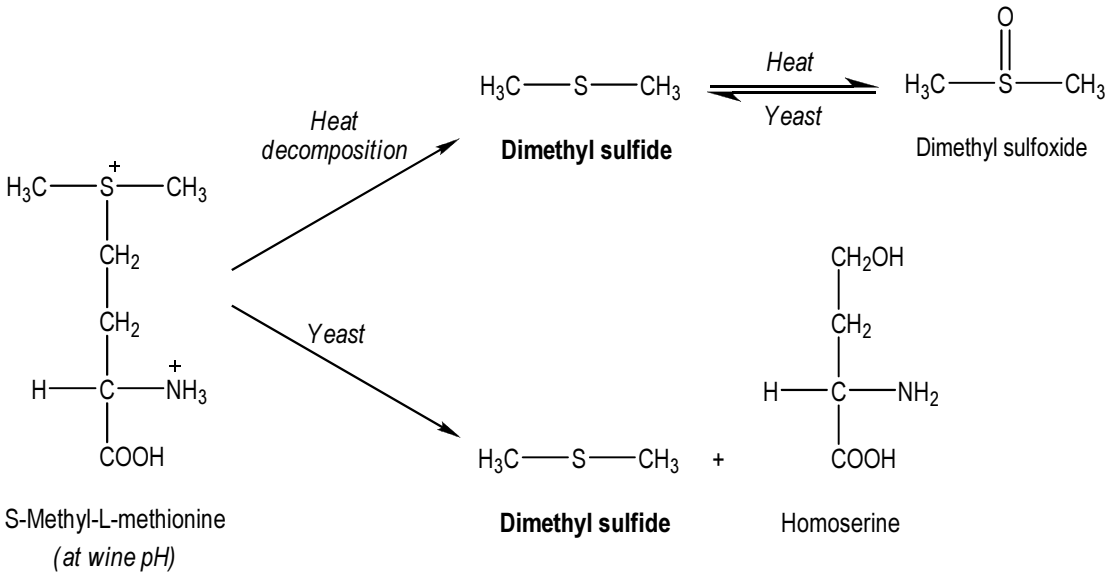


Figure 1.10. Suggested pathways for the formation of dimethyl sulfide from S-methyl methionine and from dimethyl sulfoxide by yeast and heating (Adapted from Rauhut, 1993)

The formation mechanisms of heavy sulfur compounds from yeast are still not yet well understood (Moreira *et al.*, 2008). A deficiency in the grape must of organic sulfur compounds, such as cysteine, glutathione and methionine, may cause yeast metabolism to produce these compounds, activating the *sulfate assimilation reduction* pathway (Figure 1.11) (Rauhut, 1993; Swiegers & Pretorius, 2007; Moreira *et al.*, 2008).

The sulfur containing amino acids and the amino acid precursors (*O*-acetyl serine and *O*-acetylhomoserine) then mediate the production of organic sulfur compounds in wine via the *Ehrlich* pathway (deamination, decarboxylation and reduction). One example of this is the well-known metabolic pathways of 3-methylthio-propanol (methionol), which first involves the deamination of methionine produced by yeast, followed by decarboxylation to form 3-methylthio-propanal (methional). This aldehyde is then reduced to methionol or

oxidised to the 3-methylthio-propionic acid. Methionol can react with acetic acid, producing 3-methylthio-propyl acetate (methionol acetate) (**Figure 1.11**). Ethyl (3-methylthio)propionate and 3-ethylthio-1-propanol also originate from the yeast metabolism of methionine. Homomethionine, methionine and cysteine are proposed to follow similar yeast metabolic pathways to methionol, to yield 4-methylthio-1-butanol, 3-mercapto-1-propanol, and 2-mercapto-1-ethanol (Mestres *et al.*, 2000; Moreira *et al.*, 2008).

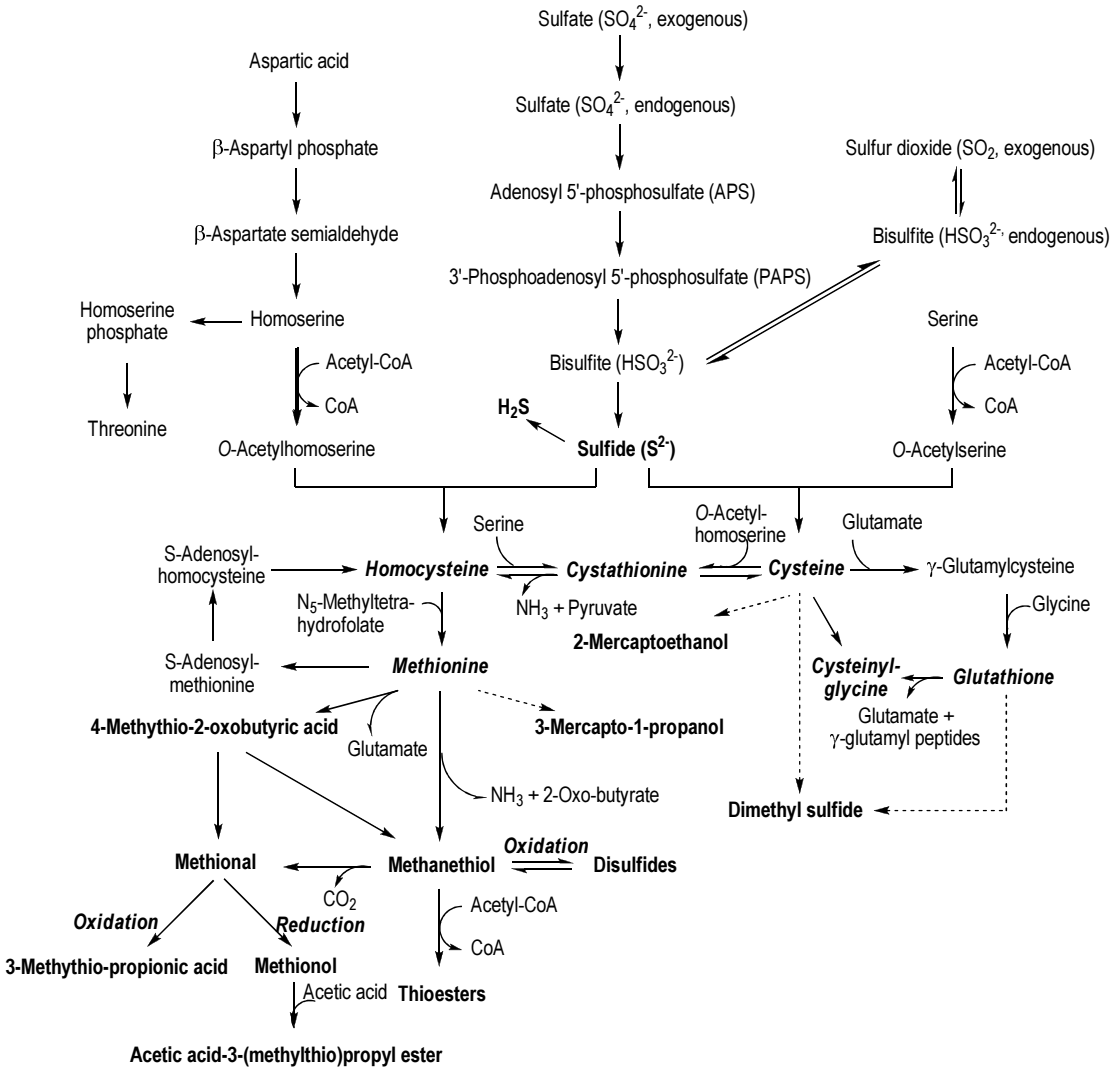


Figure 1.11. Sulfur metabolism by the wine yeast *Saccharomyces cerevisiae*

(Adapted from Moreira *et al.*, 2008)

The metabolism of lactic acid bacteria *Oenococcus oeni* during MLF also contributes to the formation of off-odour sulfur compounds (Vallet *et al.*, 2008). In addition to the key reaction that converts malic acid to lactic acid, *O. oeni* also transforms many sugars, organic acids and amino acids into products that can modify wine aromatic properties (Vallet *et al.*, 2008). However, the formation pathways of volatile sulfur compounds derived from methionine by *O. oeni* are still poorly understood. It was shown that strains of *O. oeni* convert methionine to different volatile sulfur compounds, such as 3-(methylthio)propionic acid, which contributes significantly to the lactic aromas of red wines (Pripis-Nicolau *et al.*, 2004). Recently, Vallet *et al.* (2008) demonstrated that cells of *O. oeni* grown in a medium supplemented with methionine produced methanethiol, dimethyl disulfide, methionol and 3-(methylthio)propionic acid. The intermediate produced during methionine metabolism of *O. oeni*, 2-oxo-4-(methylthio)butyric acid (KMBA), was shown to act as a precursor for methanethiol and dimethyl disulfide synthesis, through either chemical or enzymatic reactions (Vallet *et al.*, 2008). In cultures of *O. oeni*, preferentially with the presence of Mn^{2+}, KMBA can be oxidised chemically to methanethiol. It is also likely that a heat labile enzyme, possibly KMBA demethiolase, may contribute to the conversion of KMBA to methanethiol, yet no gene coding for this type of enzyme has been identified (Vallet *et al.*, 2008).

1.4.3.2. Vineyard sprays

Besides yeast metabolism as a major generator of reductive sulfur compounds, sulfur-containing pesticides are viewed as a source of these damaging off-odours in wine. The formation of sulfur compounds such as carbon disulfide, H_2S, sulfide and thiols through yeast metabolism of sulfur containing pesticides has been demonstrated (Mestres *et al.*, 2000). Some studies have reported that elemental sulfur present as a fungicide residue in the must can cause an increase in the production of H_2S during fermentation. However, the levels of elemental sulfur added during fermentation in these studies were several times higher than the residues normally observed with grapes harvested using normal

commercial vineyard practices. Furthermore, the analytical method used in some of the studies was not sensitive enough to detect H_2S (Thomas *et al.*, 1993).

1.4.4. Possible remedies for reductive aromas problems

1.4.4.1. Copper sulfate fining

Modern winemaking is trying to limit or eliminate the production of H_2S and reductive thiols while enhancing the production of favourable varietal thiols. Copper sulfate fining is being widely used to treat wine tainted with H_2S and reductive thiols (Swiegers & Pretorius, 2007). By reacting with copper sulfate and forming stable complexes, hydrogen sulfide, and likewise reductive thiols are eliminated from the wine through the formation of copper sulfide that precipitates out, and is therefore eliminated during filtration (**Equation 1.2**) (Rauhut, 1993).

$$H_2S + CuSO_4 \rightarrow CuS\downarrow + H_2SO_4$$

Equation 1.2. Formation and removal of precipitates in copper sulfate treatment of wine

However, treatment with copper also has the potential to lower the concentration of desirable thiols such as 3-mercaptohexan-1-ol (3-MH), but further research is needed to establish whether cupric ion (Cu^{2+}) discriminates between thiols in removing them from the wine. From this point of view, the use of copper sulfate in wine, therefore, is not always desirable (Swiegers & Pretorius, 2007). It was recently reported that copper addition at a rate of 0.1 mg/L at bottling, in fact, led to more H_2S after a few months for wine with limited oxygen present (e.g. under scewcaps), compared to the same wines without a pre-bottling copper treatment (Ugliano *et al.*, 2010b).

1.4.4.2. Selection and genetic modification of wine yeast

The use of copper sulfate is regarded as an interesting dilemma to winemakers due to the disadvantage mentioned above (Swiegers & Pretorius, 2007). The ultimate solution for reductive odours, therefore, would lie in the development of wine yeasts that produce desirable sulfur containing volatiles, imparting fruitiness to wine, without the formation of H_2S and reductive thiols (Swiegers & Pretorius, 2007). Significant progress in elucidating the metabolic pathways in yeast responsible for the formation of volatile sulfur compounds provides knowledge that can be used to develop wine yeast strains exhibiting lower H_2S production (Swiegers & Pretorius, 2007). Although consumers are still resistant to beverages produced by genetically modified (GM) micro-organisms, the first GM wine yeast generally regarded as safe by the FDA (U.S. Food and Drug Administration) has been commercialised by the North American wine industry (Husnik *et al.*, 2006; Swiegers & Pretorius, 2007). If GM yeasts are not widely used in the near future to produce commercial wines, they can still serve as useful models and prototypes by providing more information for scientists to apply in developing non-GM strains with altered sulfur metabolism using conventional techniques (Swiegers & Pretorius, 2007).

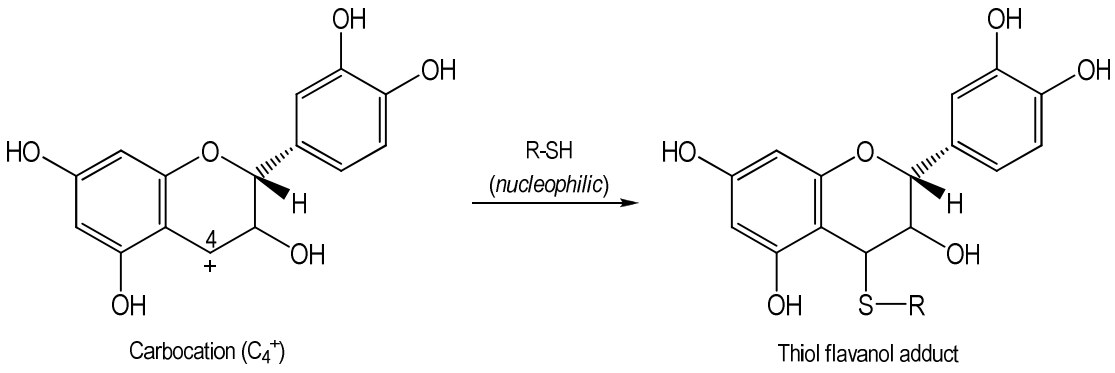
1.4.4.3. Micro-oxygenation

One of the beneficial claims made for MOX has been its capability to remove reductive aromas (Parish *et al.*, 2000; Cano-López *et al.*, 2006). The *o*-quinones formed from the oxidation of red wine polyphenols are extremely unstable. They are powerful oxidants and electrophilic species so they can react readily with reduced sulfur containing aroma compounds (Cheynier *et al.*, 2002). Oxygen, therefore, is expected to affect the concentrations of these aromas.

Majcenovic *et al.* (2002) has demonstrated that the levels of ethanethiol and diethyl disulfide in a young red wine, spiked with these sulfur compounds and oenological tannins, decreased during ageing, with oxygen having the major effect. In addition to the

reaction of oxidised polyphenols (*o*-quinones), it was shown that in a dilute acidic medium, comparable to wine (pH 3.2), the breakdown of a type B procyanidin (B_3 dimer) occurs, releasing catechin and a carbocation from the terminal and extension units of the procyanidin molecule. The carbocation form (C_4^+) is highly reactive, owing to its electrophilic centre, and can bind to various nucleophilic compounds, such as the thiols (R-SH). Off-odour sulfur compounds which are incorporated by the carbocation in this reaction (**Equation 1.3**), therefore, may be removed along with their unpleasant organoleptic characteristics (Ribéreau-Gayon *et al.*, 2006b, pp. 160-162). The reaction can occur in both red and white wines. The structure of 4-α-ethylthioflavanol (**Figure 1.12**), derived from (+)-catechin through its reaction with ethanethiol, has been elucidated (de Freitas, 1995 cited in Ribéreau-Gayon *et al.*, 2006b, p. 160).

The removal of ethanethiol and its disulfides may also involve reactions leading to the formation of other sulfur compounds, such as trisulfides in the presence of metals, and mixed disulfides of ethanethiol with other wine thiols (Majcenovic *et al.*, 2002). Therefore, limiting the formation of off-odours during winemaking would be the best way to get rid of these compounds (Majcenovic *et al.*, 2002).



Equation 1.3. Reaction of the carbocation (C_4^+) with nucleophilic compounds with a thiol function

(Adapted from de Freitas, 1995 cited in Ribéreau-Gayon *et al.*, 2006b, p. 161)

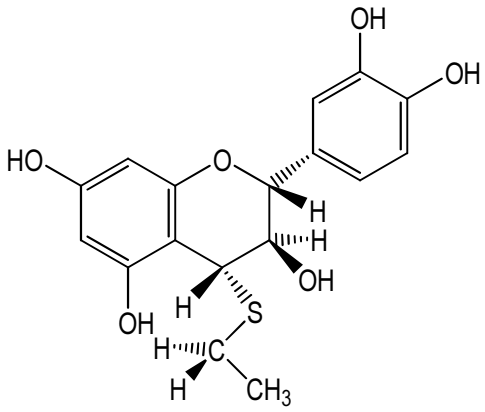


Figure 1.12. Structure of 4-α-ethylthioflavan-3-ol derived from (+)-catechin by reacting with ethanethiol

(Adapted from de Freitas, 1995 cited in Ribéreau-Gayon *et al.*, 2006b, p. 162)

1.5. RESEARCH OBJECTIVES

Since its commercial release in 1996, many scientific studies have devoted to the topic and proved the beneficial effects of MOX on wine colour and polyphenols. On the other hand, research reported on the impacts of MOX on the aromas in terms of both quantitative chemical analysis and sensory characteristics of wine is still very limited, although more publications have become available in recent years. Only one research has reported on the effects of MOX on the concentrations of a few reductive sulfur compounds found in the studied wine, whereas formal sensory evaluation with regard to reductive notes, however, to the best of my knowledge, has not been reported elsewhere. Therefore, the first objective of this study was to investigate the effect of oxygen at different dosage rates applied in traditional MOX on the chemical profiles and colour development of red wines, in replicated trials using 300 L stainless steel tanks. While Reverse Phase - High Performance Liquid Chromatography (RP-HPLC) measures of wine polyphenols, and spectrophotometric measures of wine colour provided information about the development of polymeric pigments which has been recognised as a key process brought about in MOX wines, the major focus in this thesis was placed on monitoring changes in the volatile compounds, especially the reductive sulfur containing volatiles. The initial task was to

develop a good analytical method for the quantification of the reductive sulfur compounds of interest. The impacts of MOX were examined for oxygenation treatments applied both before and after malolactic fermentation, in order to give an overall picture of how MOX affects these compounds during these particular steps of winemaking, when MOX can be applied. Effects of MOX on commercial red wines were also aimed to be investigated, when an access to commercial MOX was possible.

Nowadays, various storage and fermentation tanks made from polyethylene have been produced for use in the wine industry. Advantages of using polyethylene storage and maturation vessels over both traditional alternatives (oak and stainless steel) have been claimed (Flecknoe-Brown, 2005). The second objective of this research, therefore, was to investigate the effects of oxygen permeating through polyethylene tanks (300 L Flextanks) on the composition of red wine, in comparison with wine undergoing MOX treatments in stainless steel tanks, in order to quantitatively evaluate the capability and effectiveness of polyethylene tanks for wine maturation and ageing.

Besides the emphasis on monitoring the changes in the concentrations of reductive sulfur volatiles quantitatively, it was also necessary to correlate the chemical changes with the sensory characteristics of the final wine at the end of the MOX treatments. The third objective of this project was therefore to evaluate some relevant sensory attributes of the micro-oxygenated wines. This gives more insight into the effects of MOX on a wine's off-odours in terms of both chemical changes and sensory impacts.

CHAPTER 2. METHODOLOGY

2.1. DESIGN OF EXPERIMENTS

2.1.1. MOX trial after alcoholic fermentation

A red wine, commercially made from *Vitis vinifera* var. Cabernet Sauvignon grapes grown at Esk Valley, Hawkes Bay, New Zealand, was used for the 2008 micro-oxygenation (MOX) trial. The grapes were harvested at 22.0 °Brix on the 13th April, 2008, crushed, destemmed and inoculated with Bio Springer BCS103 yeast (Bio Springer, Maisons-Alfort, France) at Corbans Winery (Hawkes Bay, New Zealand). The must was left to ferment on skins for 17 days before being drained off, centrifuged and pasteurised. The wine was then pumped onto a combination of French oak staves and untoasted oak chips at a rate of 2 g/L. The wine was then kept at 12 °C for 2 days to stabilise before oxygen was delivered in a fully replicated MOX trial, conducted using twelve 300 L tanks. Malolactic fermentation (MLF) occurred spontaneously at day 42 after MOX had applied and was only completed towards the very end of the trial. Oxygen dosing was carried out using a PARSEC SAEn 4000 Micro-oxygenation Unit supplied by Kauri NZ Ltd. (Wellington, New Zealand) (Appendix 2-1).

The control (no oxygen) and the first two treatments, where oxygen was applied at a low rate of 5 mg/L/month and a higher rate of 20 mg/L/month, was conducted in triplicate using nine stainless steel tanks (Figure 2.1). The third treatment consisted of the same wine, but kept in three tanks (Flextank International Ltd., Abbotsford, Australia), made of a polyethylene blend, approximately 5 mm thick, allowing about 20 mg oxygen/L/year to pass through under atmospheric pressure at 17 °C (Smith, 2009).

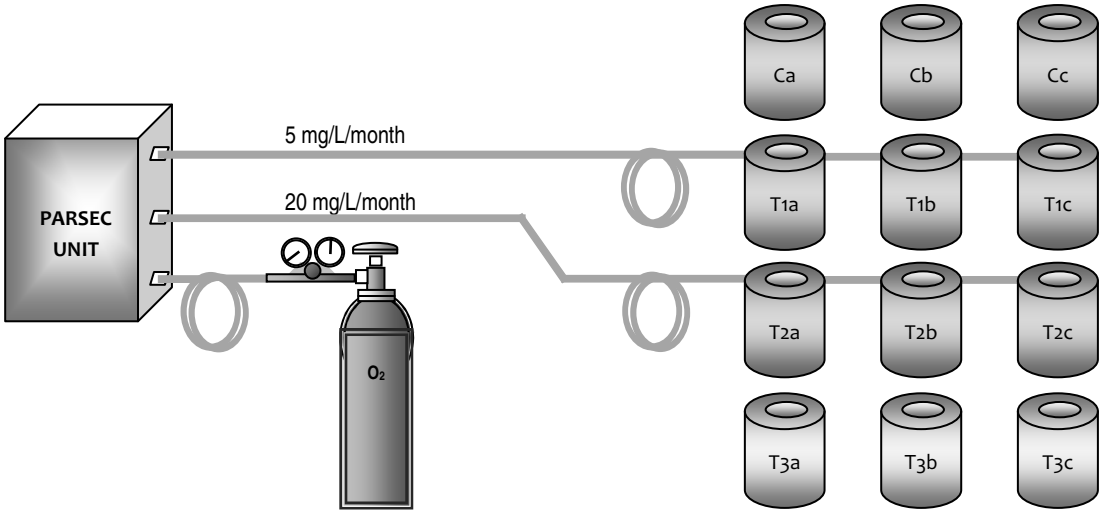


Figure 2.1. Design of the 2008 micro-oxygenation trial

Micro-oxygenation was conducted at 16 °C for 112 days at Corbans Winery's cellar in Hawkes Bay, NZ. Argon was purged briefly through the top of the tank on every two weeks to eliminate any oxygen that may present in the headspace above the wine, but the main purpose was to build up enough pressure to draw out the wine from the tank into collected containers when sampling. The total oxygen delivered to the wine received high oxygen rate of 20 mg/L/month was 56 mg/L or about 39 mL/L, which is lower than the tolerable oxygen limit of 50 mL/L (Perez-Magarino *et al.*, 2007). Dissolved oxygen levels measured in Cabernet Sauvignon after receiving oxygen doses of 5 mL/L/month for 6 months were found between 200 and 250 μg/L (Waterhouse & Laurie, 2006). The oxygen dosages, as delivered to the wine in this trial, therefore, is unlikely to cause any oxygen

accumulation to a level that can lead to undesirable oxidation of the wine, especially at the layer at the interface with the tank headspace, created after wine samples were collected. The actual set up at the winery is shown in Appendix 2-1.

50 mL wine samples were collected every two weeks and transported to the University of Auckland for polyphenol quantification and spectrophotometric analysis of colour within 24 hours of sampling. Another 150 mL of wine was kept frozen for later analyses of 3-mercapto-1-hexanol and the reductive sulfur containing compounds.

2.1.2. MOX trials after malolactic fermentation

2.1.2.1. 2009 trial on a BORDEAUX blend

The replicated 2009 MOX trial used a BORDEAUX blend wine commercially made from *Vitis vinifera* var. Cabernet Sauvignon, Merlot, Cabernet franc and Malbec grapes from a number of Hawkes Bay vineyards. Fermentation conditions were very similar as for the Cabernet Sauvignon used in 2008 MOX trial, with no sulfur dioxide addition before inoculation with Bio Springer BCS103 (Bio Springer, Maisons-Alfort, France) and a small addition of diammonium phosphate (DAP) at approximately 0.1 g/L. Ferments were conducted at a capacity from 15 to 60 tonnes at 25 - 28 °C for 15 - 20 days with air rummaging instead of cap plunging. The wines were then drained off skins, centrifuged, pasteurised and pumped into tanks containing French oak staves and untoasted oak chips (1 g/L). MLF was induced with Lalvin® B16 bacteria (Lallemand Inc., Ontario, Canada) and completed after 2 - 4 weeks. After treatment with sulfur dioxide post MLF to have the final concentration of free sulfur dioxide at 30 mg/L of, the wines were blended, fined with 0.1 g/L gelatine, racked, cold stabilised and filtered. The blend was then transported to the Pernod Ricard New Zealand Auckland Distribution Centre (Auckland, New Zealand) and portions transferred into 300 L tanks to have a fully replicated MOX trial. The design was similar to the 2008 trial, except that the oxygen dosage rate was selected as being suitable for a wine which had finished MLF. A low rate of 0.5

mg/L/month and a higher rate of 2.5 mg/L/month were applied in the first two treatments using 6 stainless steel tanks. Oxygen dosing was carried out using a PARSEC SAEn 4000 Micro-oxygenation Unit supplied by Kauri NZ Ltd. (Wellington, New Zealand) (Appendix 2-1). The Unit allows accurate delivery of oxygen by weight (mg/L) through a ceramic sparger (Appendix 1-1) placed 10 cm above the bottom of the tank. The other 3 stainless steel tanks were used for the control with no oxygen and treatment four was conducted using three 300 L polyethylene tanks (Flextank International Ltd., Abbotsford, Australia). Micro-oxygenation was conducted at Pernod Ricard Auckland winery cellar at 16 °C over 112 days. Argon was purged briefly through the top of the tank on a weekly basis to eliminate any oxygen present in the headspace above the wine.

50 mL samples were collected every two weeks for immediate spectrophotometric and polyphenols analyses. Another 150 mL of wine was kept frozen for later analyses of the volatile sulfur containing compounds.

2.1.2.2. Commercial trial on a SHIRAZ after MLF

Samples from a commercial MOX trial were also collected and supplied for analysis as part of the current research project. The commercial trial was conducted on a *Vitis vinifera* var. Shiraz, vintage 2007, grown at Geographe, Busselton, Western Australia. No sulfur dioxide was added in the vineyard or at the crusher before inoculation with a cross section of yeasts, mainly Lallemand products (Lallemand Inc., Ontario, Canada). Fermentation took place in fermentors with a capacity that ranged between 10,000 and 30,000 L. The ferments started at about 20 °C and were held at this temperature before allowing the majority of the fermentation to occur at 24 - 28 °C (maximum). Cap management and fermentation control was carried out through a combination of pumping over[19] and the

[19] Pumping-over is the operation which allows fermenting must flow from a faucet located at the lower part of the fermentor, and for the wine to be in contact with air, then pumps it back over the pomace cap at the upper part of the fermentor (Ribéreau-Gayon *et al.*, 2006a, pp. 341-342).

use of a powered irrigator. Primary fermentation took generally 6 days to complete. MLF was then induced using Lalvin 41 MBR® starter culture (Lallemand Inc., Ontario, Canada) with direct inoculation from the press tray, and this generally required four to six weeks in the winery to complete. The wine was treated with up to 30 mg/L of free sulfur dioxide at the beginning of MOX treatment. MOX was conducted in one 45,000 L tank at 0.5 mg/L/month using a PARSEC P2 Dual Output Micro-Oxygenation System supplied by Kauri NZ Ltd. (Wellington, New Zealand). Oxygen was stopped after 90 days but the wine was kept in the tank until further sample collection four months later (day 210). Micro-oxygenated samples were periodically taken from the sample cock and bottled at day 0, 15, 30, 50, 75, 90 and 210. Control samples were also collected at day 0, 90 and 210. Control wine was kept in a tank with the same capacity as the MOX tank. Shiraz samples were stored at 15 °C before being transported to the University of Auckland for analysis of colour, polyphenols, and reductive sulfur compounds.

2.2. CHEMICAL ANALYSES

Chemical analysis to monitor different MOX trials involved measurements of colour and tannin, quantification of individual polyphenols as well as the aroma compounds of interest. The analytical methods that were employed are described in the following sections.

2.2.1. Measurement of tannin by the Methyl Cellulose Precipitable (MCP) Tannin Assay

2.2.1.1. Principles of the method

By definition, tannins are compounds that have the ability to precipitate protein and to interact with a range of other biological molecules including polysaccharides and alkaloids. These properties have been exploited not only to remove excessive tannins

from wine but also to measure tannins in different applications including winemaking (Seddon & Downey, 2008).

Methyl cellulose (also methylcellulose), a commonly available polysaccharide, can react with wine tannins to form insoluble polymer tannin complexes which then precipitate. The Methyl Cellulose Precipitable (MCP) tannin assay allows the quantification of condensed tannins by precipitatation with methyl cellulose (Sarneckis *et al.*, 2006; Mercurio *et al.*, 2007).

The MCP tannin assay is based on the polymer (methyl cellulose) and tannin interactions, resulting in the formation of an insoluble polymer-tannin complex, which precipitates out and is separated by centrifugation (Mercurio *et al.*, 2007). It is a subtractive assay which involves preparing a control with no methyl cellulose added and a treated sample, and measuring the absorbance using a UV-Vis spectrophotometer. The absorbance at 280 nm (A_{280}) of the control provides a value for all of the phenolic compounds, while A_{280} of the treated sample gives a value for phenolic compounds remaining in solution after the MCP tannin has precipitated out. The MCP tannin is then determined by subtracting these two values and expressing this in terms of epicatechin equivalents .

2.2.1.2. Preparation of reagents

Reagents

Ultrapure reagent grade Type 1 water, with a resistivity of 18.3 MΩ.cm (conductivity < 0.055 μS/cm) at 25 ºC, was obtained daily when needed using a Thermo Scientific Barnstead® NANOpure DIamond™ Water Purification System (Barnstead Nanopure, Boston, MA, USA). Ammonium sulfate (Cas No. 7783-20-2) (≥ 99 %) was purchased from Univar (Ajax FineChem, Auckland, NZ) and methyl cellulose (Cas No. 9004-67-5) (M-0387, viscosity 1,500 centipoises, 2 % aqueous solution at 20 ºC) was from Sigma-Aldrich (St.

Louis, MO, USA). (-)-Epicatechin (Cas No. 490-46-0) (≥ 90 %) used to construct a working calibration curve was also obtained from Sigma-Aldrich.

Saturated ammonium sulfate solution

Excess ammonium sulfate crystals were added in a 500 mL Schott DURAN® glass bottle (Mainz, Germany) containing about 300 mL of ultrapure water with stirring until approximately 1.5 cm of crystals were no longer dissolved and rested on the bottom of the bottle. The solution was kept at room temperature for use for up to six months.

0.04 % methyl cellulose solution

0.4 g of methyl cellulose was weighed and small portions of the polymer were added into a 1 L volumetric flask, containing 300 mL of hot (80 ºC) ultrapure water, with continuous quick stirring until all the portions of the polymer had been added. The temperature of the solution was lowered using the remainder of the water (700 mL, 0 - 5 ºC). The flask was placed in ice water (0 - 5 ºC) and was stirred for 20 - 40 min until the solution became clear. The solution was allowed to reach room temperature before making up to a final volume of 1 L with ultrapure water, and was kept at room temperature for use within two weeks.

2.2.1.3. Preparation of samples and absorbance measurement

Control sample

Control sample was prepared by adding 2 mL of saturated ammonium sulfate $(NH_4)_2SO_4$ solution into 0.25 mL of wine sample, placed in a 10 mL volumetric flask. The total volume was made up to 10 mL with 7.75 mL ultrapure water. The solution was allowed to stand at room temperature for 10 min, before being centrifuged for 5 min at 4000 rpm. The solution was pipetted in to a UV-transparent disposable cuvette (Eppendorf UVette®)

(Eppendorf, Hamburg, Germany) for absorbance measurement at 280 nm. This value represents A_{280} (control) (Sarneckis *et al.*, 2006).

Treated sample

Treatment sample was prepared by adding 3 mL of 0.04 % methyl cellulose solution to 0.25 mL of red wine. The mixed solution was shaken several times and left to stand for 2 to 3 min. Saturated ammonium sulfate $(NH_4)_2SO_4$ solution (2 mL) was then added to this solution and the total volume was made up to 10 mL with 4.75 mL ultrapure water. The solution was allowed to stand for 10 min at room temperature before being centrifuged for 5 min at 4,000 rpm. After that, the solution was pipetted in to a UV-transparent disposable cuvette (Eppendorf UVette®) (Eppendorf, Hamburg, Germany) for absorbance measurement at 280 nm. This value represents A_{280} (supernatant) (Sarneckis *et al.*, 2006).

2.2.1.4. Calculation

The MCP tannin concentration is calculated following the formula below:

Tannin concentration (mg/L epicatechin eq.) = [tannin] * DF

where:

[tannin]: tannin concentration (mg/L epicatechin eq.); calculated from the epicatechin calibration curve, in which the A_{280} (tannin) = (A_{280} (control) – A_{280} (supernatant)) was placed into the calibration equation to calculate the tannin concentration;

DF: dilution factor, 40 in this case (0.25 mL of red wine in a total final volume of 10 mL solution).

2.2.1.5. Construction of a calibration curve for the MCP tannin

The calibration curve for the MCPT assay was constructed using 8 known concentrations of (-)-epicatechin ranging from 5 to 250 mg/L. These standard solutions were volumetrically made up in ultrapure water.

Table 2.1. Calibration curves used for the quantification of MCP tannin

Spectrophotometers	Epicatechin concentration range (mg/L)	Calibration curve equation	R^2
Varian Inc. Cary 50	5 to 250	86.407*x – 2.9562	0.9965
Genesis 10UV	5 to 250	85.874*x + 0.5907	0.9996

*Notes: *x is the spectral absorbance measured at 280 nm*

The absorbance values measured at 280 nm for these standard solutions were plotted against their corresponding concentrations to construct the calibration curve, which was used to report results of MCP tannin in epicatechin equivalents. The calibration curves were obtained using either a Varian Inc. Cary 50 Probe UV-Vis (Palo Alto, CA, USA) or a Genesis 10UV (Thermo Spectronic, Rochester, NY, USA) (Table 2.1). These calibration curves were used correspondingly to the specific spectrophotometer used for the samples.

2.2.2. Absorbance measurements of wine colour

Interest in the measurement of red wine colour started in the 1930s and its importance from the viewpoint of trade has been widely recognised since then (Heredia & Guzman-Chozas, 1993). Recently, absorbance measurement of wine colour has been used by a number of researchers (Atanasova *et al.*, 2002a; Otto, 2002; McCord, 2003; Rodríguez-Bencomo *et al.*, 2008; Pérez-Magariño *et al.*, 2009; Sanchez-Iglesias *et al.*, 2009; Ortega-

Heras *et al.*, 2010) as one of their major tools to study wine MOX. Spectral measurements were also employed in the current work to characterise the colour development of the research wines that underwent different MOX treatments. The method was adapted from Iland *et al.* (2000) and is briefly summarised below.

2.2.2.1. Spectral measures and wine properties

The development of wine colour was examined by monitoring wine colour density, wine colour hue and non-bleachable pigments or pigments resistant to SO_2 bleaching.

The absorbance measure at 420 nm (A_{420}) is related to the concentration of yellow brown pigments while the absorbance measure at 520 nm (A_{520}) gives an estimate of the concentration of red coloured pigments (anthocyanins and tannins) under natural wine conditions (actual pH and SO_2 level). For young red wines less than an year old, and for wines with a pH near 4, the absorbance measure at 620 nm (A_{620}), which is attributed to quinonic forms of free and combined anthocyanins, needs to be taken into account (Heredia & Guzman-Chozas, 1993). A_{620} relates to the concentration of blue coloured pigments under natural wine conditions. The sum of these three absorbances was proposed by Glories (1984) to give the value of ***wine colour density***.

Non-bleachable anthocyanins *(mainly polymeric pigments)* ($A_{520}^{SO_2}$) is provided by the absorbance at 520 nm after an excessive addition of SO_2, which bleaches out most of the monomeric anthocyanins.

2.2.2.2. Chemicals and reagents

Potassium metabisulfite (PMS) was purchased from Redox Pty Ltd. (Auckland, NZ), while hydrochloric acid (37 %, reagent grade) was provided by Scharlau (Global Science, North Shore, NZ).

PMS 25 % w/v solution was prepared by weighing approximately and with care 25 g of

potassium metabisulfite. The PMS powder was then placed in a 150 mL Schott bottle and mixed thoroughly until it was completely dissolved with 100 mL of ultrapure water. The bottle was then capped and stored at room temperature for further use.

Hydrochloric acid solution 1M was prepared by measuring 82 mL of concentrated hydrochloric acid (37 %, density 1.189 g/mL) and adding slowly with stirring to approximately 700 mL of ultrapure water placed in a 1 L volumetric flask. The solution was then brought up to volume with ultrapure water and mixed thoroughly before tranferring to a 1 L Schott bottle for storage until later use.

2.2.2.3. Colour measurements

The following procedures were adopted from Iland *et al.* (2000), to examine wine colour. For each wine sample, two glass test tubes were set up and labelled (i) and (ii), which contained wine sample and wine with sulfur dioxide added, respectively.

Sample preparation

To test tubes (i) and (ii) which contained 2 mL of the wine sample, add 30 µL of 25 % w/v potassium metabisulfite solution to the wine in test tube (ii). The test tubes were covered with parafilm and the solutions were mixed thoroughly. The solution were left to stand at room temperature for 45 min before taking the spectral measurements.

Absorbance readings

The absorbances of the solution in test tube (i) at 420, 520 and 620 nm were measured using a UV-transparent disposable cuvette (Eppendorf UVette®) (Eppendorf, Hamburg, Germany) with a 2 mm path-length (value to be multiplied by 5), to give the A_{420}, A_{520} and A_{620} of the wine under natural conditions. These values were used the calculate the colour density of the wine.

The absorbance of the solution in test tube (ii) at 520 nm using a cuvette with a 2 mm path-length (value to be multiplied by 5), to give the $A_{520}^{SO_2}$ value which represents the pigments resistant to sulfur dioxide bleaching.

The absorbance readings were made using the available spectrophotometers, either a Varian Inc. Cary 50 Probe UV-Vis (Palo Alto, CA, USA) or a Genesis 10UV (Thermo Spectronic, Rochester, NY, USA). The recorded values were converted to absorbance values with 10 mm path-length after correcting for the dilution. One specific spectrophotometer was used for all samples within a trial for the whole period for the sake of data consistency.

2.2.3. WineScan analysis to monitoring major wine parameters during MOX

2.2.3.1. Introduction

WineScan is a product of FOSS (Hillerod, Denmark), and has been widely used in many wineries all over the world for routine monitoring the concentrations of grape and wine compounds during the key stages of the wine making process. It allows robust measurements of several major parameters, including ethanol, total acidity, volatile acidity, malic acid, pH and reducing sugars (glucose/fructose) in a single analysis. Analysis based on Fourier Transform Infrared technique, the WineScan has also been employed in several published studies to characterise wines and alcoholic beverages (Palma & Barroso, 2002; Patz et al., 2004; Lachenmeier, 2007).

2.2.3.2. Principle of the method

In the current study, WineScan™ FT120 (FOSS, Hillerod, Denmark) instruments available at Pernod Ricard NZ laboratories were used to monitor the wines during the two fully replicated MOX trials.

The instrument is basically a Fourier Transform Infrared (FTIR) spectrophotometer fitted with a Michelson interferometer which generates the FTIR spectra and allows the scanning of the infrared spectrum wavenumbers from 5012 - 926 cm^{-1} (wavelengths 2 - 10.8 µm) (WineScan™ FT120, FOSS, Hillerod, Denmark).

Samples are pumped through a 37 µm path length CaF_2 - lined cuvette, scanned from 5012 - 926 cm^{-1} and the transmittance recorded is the detector at 4 cm^{-1} interval. An interferogram is generated from 20 scans before being processed by Fourier transformation and corrected for background absorbance to produce a single beam transmittance spectrum. The ratio of the single beam transmittance spectrum of the Zero liquid (Product No. S-6060, FOSS, Denmark) and that of the sample at each data point is used to generate the final transmittance spectrum for each sample. Two transmittance spectra are generated for each sample to monitor the repeatability of the spectral measurements, before being converted to linearised absorbance spectra through a series of mathematical procedures programmed in the Foss FT 120 software (FOSS, Hillerod, Denmark). The Zero liquid was set to be automatically scanned at regular intervals of samples to correct for the background absorbance.

2.2.3.3. Calibration

Calibrations of the WineScan™ FT120 were carried out on site where the trials were taking place (Corbans Winery, Hawkes Bay and the Pernod Ricard NZ Auckland Distribution Centre, Auckland). The company's laboratories used local materials, e.g grapes, juices and wines, as well as standard FOSS calibration standards, to predict the ranges/values of all of the parameters (e.g. ethanol, total acidity, volatile acidity, malic acid, pH and reducing sugars) for routine analysis of different grapes and wines at Pernod Ricard NZ wineries. The instruments therefore were ready for the analysis of the studied wines.

2.2.4. Reverse Phase - High Performance Liquid Chromatography analysis of polyphenols

2.2.4.1. Introduction

Reverse Phase - High Performance Liquid Chromatography (RP-HPLC) has been widely used for the analysis of individual phenolic compounds, first in 1976 by Fisher and Wheaton (Merken & Beecher, 2000; Ibern-Gomez *et al.*, 2002). Reversed phase silica-based columns have been commonly used by most researchers to analyse the phenolic composition of red wines (Lamuela-Raventos & Waterhouse, 1994). The elution system is usually composed of two solvents. One is an aqueous solvent acidified with acetic, perchloric, phosphoric, formic, or trifluoroacetic acid. The second is an organic solvent such as methanol or acetonitrile, possibly acidified with the same acid as used in the aqueous solvent system (Rivas-Gonzalo *et al.*, 1992; Gao & Mazza, 1994; Merken & Beecher, 2000).

Polyphenolic flavonoids are characterised by two absorption bands in the ultraviolet (UV) region. Band I with a maximum in the 300 - 550 nm range is believed to arise from the B-ring, while band II with a maximum in the 240 - 285 nm range arises from the A ring. The detection of these compounds, therefore, is generally carried out by UV-Vis with a Diode Array Detector (DAD). For example, anthocyanins are usually analysed and quantified at typical wavelengths such as 502 nm, 510 nm and 525 nm, while 210 nm, 278 nm and 280 are used to monitor catechins (Merken & Beecher, 2000). Wine non-flavonoids, such as hydroxycinnamic acids (e.g. caffeic acid, coumaric acid) and stilbenes (e.g. resveratrol) can be monitored at wavelength around 320 nm due to their characteristically high absorbance maxima in the ultraviolet (UV) region of 300 - 330 nm (Somers *et al.*, 1987). Benzoic acids (e.g. gallic acid) can be seen on the HPLC chromatogram at the wavelength of 280 nm (Zou *et al.*, 2002).

In the current research, analysis of individual wine polyphenols was conducted using RP-

HPLC, based on a method developed at the University of Auckland (Zou *et al.*, 2002). In the method, the use of a binary gradient elution system consisting of an acidified mobile phase (acetic acid) and an organic solvent (acetonitrile) gives an efficient separation of different wine polyphenolic groups. In addition, monitoring wine phenolics at four different wavelengths of 280 nm, 320 nm, 365 nm, and 520 nm also allows the simultaneous identification and quantification of various compounds in a single HPLC run.

Of note is that the current HPLC methodology did not use a particularly low pH buffer solution that enables better separation of anthocyanins, which are then all in their flavylium form[20]. The present method was designed to allow all groups of polyphenols to be analysed in a single run, and was not optimised for all of the anthocyanin classes present in smaller amounts, such as ethyl-linked compounds, but the present method was quite suitable for quantification of the main monomeric anthocyanins.

2.2.4.2. Chemicals and reagents

The solvents used in the HPLC mobile phase, including acetonitrile (CH_3CN) (gradient grade) and acetic acid glacial (HPLC grade, ≥ 99.8 %) were purchased from Scharlau (Global Science, NZ) and Merck (Merck Ltd, NZ), respectively. Ultrapure water was obtained daily using Barnstead® NANOpure DIamond™ Water Purification System (Thermo Scientific, USA). Argon gas was from BOC New Zealand (Auckland, NZ).

Absolute ethanol (≥ 99.5 %) and methanol (≥ 99.9 %) used to prepare standard solutions of polyphenols were of HPLC grade and provided by Univar (Ajax FineChem, NZ) and Scharlau (Global Science, NZ), respectively. Tartaric acid (≥ 99 %) came from Sigma-Aldrich.

[20] Red flavylium ions predominate only in very acidic solutions (pH <2) while the colourless, hydrated hemiketal form is the major form of anthocyanins occurring at wine pH (Cheynier *et al.*, 2006).

Commercial polyphenols, used as external standards, including gallic acid monohydrate (≥ 98 %), vanillic acid (≥ 97 %), syringic acid (≥ 95 %), caffeic acid (≥ 98 %), *p*-coumaric acid (≥ 98 %), *trans*-resveratrol (≥ 99 %), quercetin dihydrate (≥ 98 %), quercetin-3-β-D-glucoside (≥ 90 %), catechin hydrate (≥ 98 %), epicatechin (≥ 90 %), epigallocatechin (≥ 95 %) and epicatechin-*o*-gallate (≥ 98 %) were purchased from Sigma-Aldrich. Oenine chloride ($C_{23}H_{25}ClO_{12}$) (Cas No. 7228-78-6), used as an external standard for monomeric anthocyanins, was obtained from Extrasynthese (Genay Cedex, France).

2.2.4.3. The RP-HPLC system

Samples were injected into a Hewlett-Packard (Palo Alto, CA, USA) Agilent 1100 series instrument coupled to a Diode Array Detector (G1315B), a Column Heater (G1316A), an Auto-sampler (G1313A), a Quaternary Pump (G1311A) and a Degasser (G1379A). The chromatographic separation was performed on a Reversed-Phase Phenomex (Torrence, CA) Luna C18 column (5 µm particle size, 4.6 mm x 250 mm) maintained at 25 ºC.

The DAD was set at four different wavelengths, including 280 nm to monitor hydroxybenzoic acids and flavanols, 320 nm for hydroxycinnamic acids, grape reaction product (GRP) and stilbenes, 365 nm for flavonols, and 520 nm for the monomeric anthocyanins.

The ternary mobile phase used for the separation of phenolic compounds consisted of ultrapure water (solvent A), 5 % v/v acetic acid in ultrapure water (solvent B) and acetonitrile (solvent C) delivered at a flow rate of 1.0 mL/min. The solvent gradient was programmed as shown in Table 2.2.

2.2.4.4. Sample preparation

About 2 mL of wine was filtered through a Phenex RC membrane syringe filter (0.45 µm pore size, 15 mm diameter) (Part No. AFO-2103-12, Phenomenex, CA, USA) using

disposable Norm-Ject® 3 mL Luer syringes (Henke Sass Wolf, Tuttlingen, Germany) into a 2 mL screw top amber vial (Part No. 5182-0716, Agilent). After being purged with argon gas for a minute to displace the air in the headspace, the vial was closed with a polypropylene screwcap with integrated septum (blue/PTFE white silicone septa, Part No. 5182-0720) prior to direct injection of 20 µL into the HPLC system for immediate analysis.

Table 2.2. HPLC solvent gradient for red wine polyphenol elution

Time (min)	Solvent A (%) (ultrapure water)	Solvent B (%) (5 % acetic acid)	Solvent C (%) (acetonitrile)
0	45	55	0
10	25	75	0
20	0	100	0
30	0	100	0
50	0	90	10
70	0	85	15
80	0	80	20
95	0	60	40
105	0	55	45
115	45	55	0
125	45	55	0

2.2.4.5. Identification and quantification of polyphenols

Identification

The identification of polyphenols compounds in the research wines was determined by comparing the retention time, elution order and the absorbance spectra of the wine polyphenols with those of the external standards, as well as by their maximum

absorbance spectra as reported in the literature. Figure 2.2 depicts the HPLC separation of polyphenols obtained from a research Cabernet Sauvignon wine, monitored at four different wavelengths.

Calibration curves for polyphenol quantification

The quantification of polyphenols was carried out using external standards to construct calibration curves. Due to poor solubility of polyphenol compounds in water, all individual stock solutions of commercial standards, except quercetin, were prepared in absolute ethanol. The stock solution of quercetin was prepared in methanol due to its poor solubility in ethanol. Serial dilutions of stock solutions in model wine (5 g/L tartaric acid, 12 % v/v aqueous ethanol, pH adjusted to 3.6) were then performed to obtain different polyphenol concentrations, chosen to be in the range of these compounds usually found in red wines. Note that all stock solutions and model wine solution were filtered before proceeding to further dilutions to prepare standard solutions of individual polyphenols.

These standard solutions were then transferred into 2 mL amber vials and injected into the HPLC system for separation. The standard calibration curves consisted of eight points for each compound were constructed by plotting the peak areas of the external standards on the HPLC chromatograms against the corresponding external standard concentrations. Peak areas of the wine polyphenols were used to calculate/intrapolate the concentration of each component from their corresponding calibration curves.

Information about the polyphenol compounds monitored during the MOX trials, the wavelengths at which they were monitored, the external standards and calibration curves used for quantification can be seen in Table 2.3

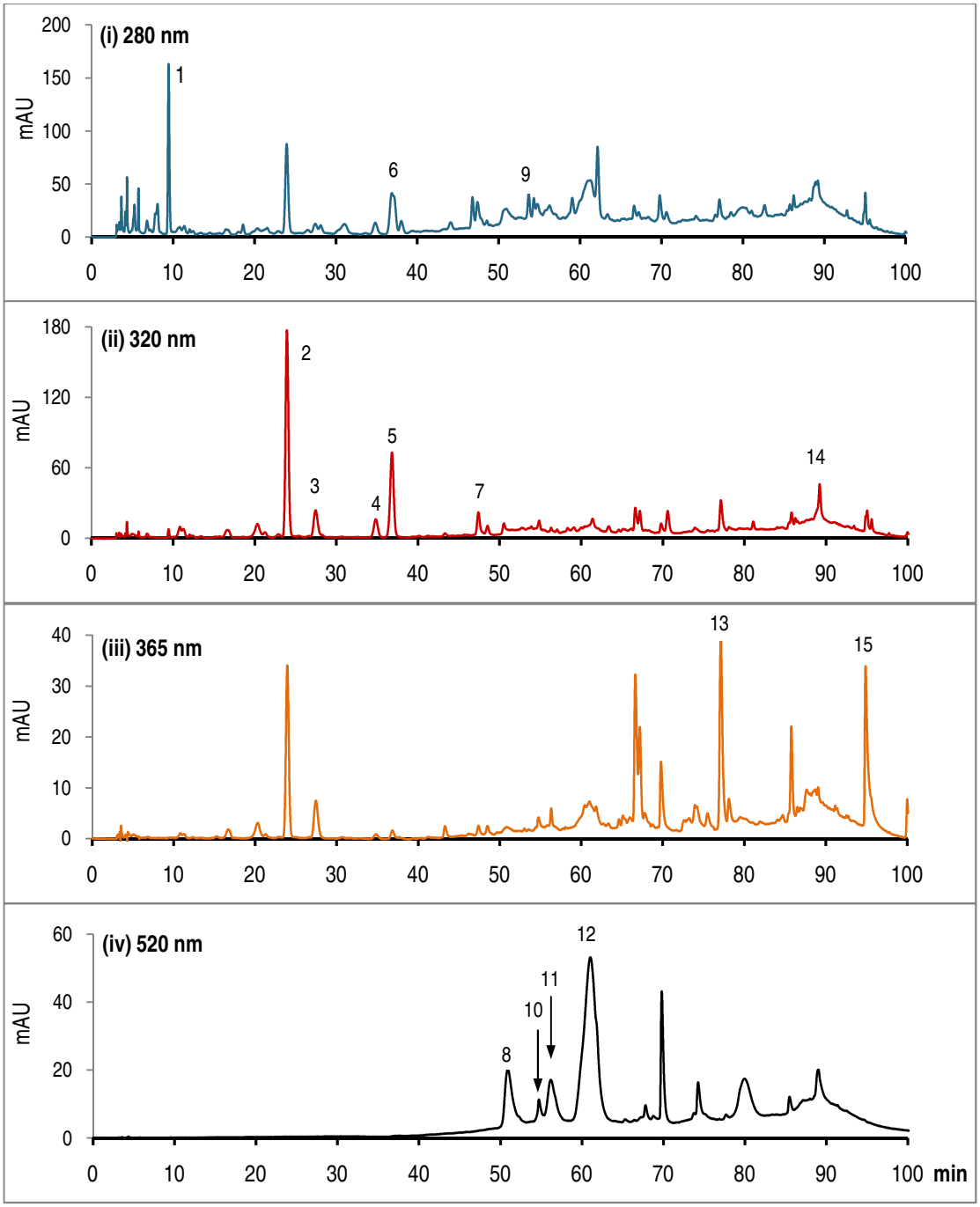


Figure 2.2. HPLC chromatogram of a Cabernet Sauvignon wine monitored at (i) 280 nm, (ii) 320 nm, (iii) 365 nm and (iv) 520 nm. Peaks are identified as: 1- gallic acid, 2- caftaric acid, 3- S-glutathionylcaftaric acid (GRP), 4- *cis*-coutaric acid, 5- *trans*-coutaric acid, 6- catechin, 7- caffeic acid, 8- delphinidin-3-glucoside, 9- epicatechin, 10- cyanidin-3-glucoside, 11- petunidin-3-glucoside, 12- malvidin-3-glucoside, 13- quercetin-3-glucoside, 14- *trans*-resveratrol, 15- quercetin.

Table 2.3. Polyphenol compounds monitored during the MOX trials using RP-HPLC

Polyphenol groups ⇨ Compound names	External standard	CAS No. of external standard	Wavelength (nm)	Retention time (min)	Concentration range (mg/L)	Linear regression	
						Equation	R^2
Benzoic acids							
⇨ *Gallic acid*	Gallic acid	149-91-7	280	9.4	1.5 to 200	0.0161*x – 1.2176	0.999
⇨ *Vanillic acid*	Vanillic acid	121-34-6	280	46.9	0.4 to 50	0.0242*x – 0.0967	0.9999
⇨ *Syringic acid*	Syringic acid	530-57-4	280	53.8	0.4 to 50	0.0134*x – 0.1022	0.9999
Hydroxycinnamic acids							
⇨ *S-Glutathionylcaftaric acid*	Caffeic acid	331-39-5	320	27.5	0.5 to 60	0.0075*x – 0.2433	0.9998
⇨ *Caffeic acid*	Caffeic acid		320	47.7			
⇨ *p-Coumaric acid*	*p*-Coumaric acid	501-98-4	320	61.7	0.5 to 60	0.006*x – 0.3371	0.9997
Hydroxycinnamic esters							
⇨ *t-Caftaric acid*	Caffeic acid		320	24.1			
⇨ *c-Coutaric acid*	*p*-Coumaric acid		320	35.0			
⇨ *t-Coutaric acid*	*p*-Coumaric acid		320	37.0			
Stilbenes							
⇨ *t-Resveratrol*	*t*-Resveratrol	501-36-0	320	89.7	0.3 to 40	0.0076*x – 0.0233	0.9998
Flavonols							
⇨ *Quercetin-3-glucoside*	Quercetin dihydrate	6151-25-3	365	77.2	0.6 to 85	0.0115*x + 2.1651	0.9989
⇨ *Quercetin*	Quercetin dihydrate		365	95.0			
Anthocyanins							
⇨ *Delphinidin-3-glucoside*	Malvidin-3-glucoside	7228-78-6	520	50.9	4.0 to 1000	0.0216*x + 2.8686	0.9989
⇨ *Cyanidin-3-glucoside*	Malvidin-3-glucoside		520	54.8			
⇨ *Petunidin-3-glucoside*	Malvidin-3-glucoside		520	56.1			
⇨ *Malvidin-3-glucoside*	Malvidin-3-glucoside		520	61.0			
Flavanols							
⇨ *(+)- Catechin*	(+)-Catechin hydrate	88191-48-4	280	38.4	3.0 to 350	0.0514*x – 1.0677	0.9998
⇨ *(-)-Epicatechin*	(-)-Epicatechin	490-46-0	280	54.5	2.0 to 300	0.0572*x – 1.1539	0.9998
⇨ *(-)-Epicatechin-o-gallate*	(-)-Epicatechin-*o*-gallate	1257-08-5	280	68.7	0.5 to 65	0.0243*x + 0.0661	0.9999

Note: *x is the peak area of the compound analysed*

2.2.5. Gas chromatography - Mass Spectrometry analysis of 3-mercaptohexan-1-ol

2.2.5.1. Introduction

The thiol 3-mercaptohexan-1-ol (3-MH) is thought to contribute to the fruity aromas of not only white wines such as Sauvignon blanc but also red wines such as Cabernet Sauvignon and Merlot (Bouchilloux *et al.*, 1998; Blanchard *et al.*, 2004). 3-MH possesses the thiol function (-SH) in its chemical structure so it might be affected by oxygen exposure. The current research, therefore, included monitoring of 3-MH during the first pilot MOX trial conducted in 2008.

Analysis of 3-mercaptohexan-1-ol (3-MH) was carried out using a Gas Chromatography-Mass Spectrometry (GC-MS) method similar to a published method (Tominaga *et al.*, 2006). The method is based on the properties of p-hydroxymercuribenzoate, in that it combines with compounds containing a thiol function in a reversible reaction (Jocelyn, 1987; Tominaga *et al.*, 1998b). The method, therefore, basically involves first fixing the thiols, extracted using an aqueous solution of *p*-(hydroxymercury)benzoate, on an anion exchange column, then releasing the bound complex thiol-*p*-(hydroxymercurybenzoate) by percolating a cysteine solution through the column (Tominaga *et al.*, 2006). The procedures were modified with the use of deuterated analogues of the varietal thiols as internal standards, i.e. 3-mercapto(1-$^{2}H_2$)hexan-1-ol as an internal standard for the quantification of 3-MH (Hebditch *et al.*, 2007). 3-Mercaptohexan-1-ol (Cas No. 51755-83-0) was purchased from Interchim (Montluçon Cedex, France) and the internal standard 3-mercapto(1-$^{2}H_2$)hexanol was synthesized at the University of Auckland, as reported in Hebditch *et al.* (2007).

2.2.5.2. Chemicals and reagents

Preparation of samples for thiol extraction involved the use of some chemicals and reagents. The information about their properties and suppliers is provided in Table 2.4.

Table 2.4. Chemicals and reagents used for the analysis of 3-mercaptohexanol

Chemicals and reagents	CAS No.	Product code	Supplier
Na-4-(hydroxymercury)benzoate (≥ 95 % Hg)	138-85-2	55540	Sigma-Aldrich
Butylated hydroxyanisole	25013-16-5	B1253-5G	Sigma-Aldrich
Sodium acetate trihydrate (99.5 - 100.5 %)	6131-90-4	SO0024	Scharlau.
(+)-L-Cysteine hydrochloride (99 %)	345909-32-2	C121800-100G	Sigma-Aldrich
TRIS (ultrapure, ≥ 99.9 %)	77-86-1	A0186,1000	AppliChem GmbH
Dowex® 1x2 Resin, Cl^-form, strongly basic, 50 -100 mesh	69011-19-4	44290-2.5KG	Sigma-Aldrich
Hydrochloric acid (reagent grade, 37 %)	4647-01-0	AC0741	Scharlau
Sodium hydroxide NaOH (pellets, reagent grade, ≥ 99 %)	1310-73-2	SO0420	Scharlau
Dichloromethane (for gas chromatography SupraSolv®)	75-09-2	106054	Merck
Ethyl acetate (LC-MS, ≥ 99.8 %)	141-78-6	AC0158	Scharlau
Sodium sulfate anhydrous (powder, 98.5 - 100.5 %)	7757-82-6	SO0670	Scharlau

A solution of Na-4-(hydroxymercury)benzoate (*p*-HMB) was prepared by first adding 6.057 g TRIS ultrapure in a 500 mL volumetric flask containing about 100 mL of ultrapure water (Barnstead® NANOpure DIamond™ Water Purification System) with stirring. After the TRIS was dissolved, 360 mg of *p*-HMB was carefully added and made up to volume with ultrapure water. The solution was stored at room temperature and kept away from light.

Butylated hydroxyanisole (BHA) 2mM solution, used to prevent the analyte from being oxidised, was made up by dissolving 0.036 g of BHA in 100 mL absolute ethanol. The solution was stored in a 4 ºC fridge.

The 0.1 M sodium acetate *wash buffer* was produced from 13.6 g sodium acetate trihydrate in 1 L of ultrapure water, with the pH adjusted to 6.00 using 10 N NaOH (400 g NaOH in 1 L of ultrapure water).

The 50 mM (+)-L-cysteine hydrochloride *elution buffer* was prepared daily and used fresh. A volume of 50 mL elution buffer, used for each sample, contained 400 mg (+)-L-cysteine hydrochloride in 50 mL of 0.1 M Na-acetate, with the pH adjusted to 6.00 using 10 N NaOH.

2.2.5.3. Extraction

3-MH was first separated from other wine constituents by column chromatography using Dowex ® 1x2 resin, and then extracted with dichloromethane.

To the prepare the column, the Dowex resin was activated with 1 M HCl (prepared as described in Section 2.2.2.2) and rinsed a few times with ultrapure water until the pH was around 5-6. The resin (12.5 mL) was then pipetted into a dry glass column already plugged loosely with a small amount of silanised glass wool (Sigma-Aldrich). A small filter paper was placed on top of the resin to keep it settled. The column was then washed with 100 mL of ultrapure water passed through at a flow rate of 1 drop every 3 seconds, adjusted via the stopcock.

The wine sample was first prepared by adding 5 mL of *p*-HMB and 0.5 mL of 2 mM BHA into 50 mL of wine. An aliquot of 50 µL mixture containing 22 µM of [1-$^{2}H_{2}$]3-MH as internal standard for 3-MH was then added. After that, the sample was mixed well at 400 rpm for 10 min, adjusted to pH 7.00 ± 0.05 and ran though the activated Dowex resin column at a rate of 1 drop every 5 seconds.

The column was then washed with 50 mL of 0.1 M Na-acetate wash buffer (pH 6.00) at 1 drop every 5 seconds. Bound thiols were eluted with 50 mL of (+)-L-cysteine hydrochloride

elution buffer at a flow rate of 1 drop every 7 seconds in a 100 mL volumetric flask. The collected eluate was extracted twice with 4 mL then 2 mL of dichloromethane for 5 minutes each and with stirring at 800 rpm. The effectiveness of the extraction was enhanced by adding 0.5 mL of ethyl acetate prior to the first extraction with dichloromethane. The lower organic phase was collected each time into a 15 mL clear glass vial and dried with anhydrous sodium sulfate. The dried organic phase was filtered through silanised glass wool tightly packed in a glass Pasteur pipette to remove sodium sulfate particles, which can clog the GC column. Finally, the collected organic phase was concentrated under nitrogen gas flow to about 200 µL before being transferred to a Qsert vial (12 x 32 mm, 300 µL) (Part No. AHO-7058, Phenomenex). The organic phase in the vial was further concentrated to ~25 µL ready for the injection of 3 µL onto the GC-MS system.

2.2.5.4. The GC-MS system

The GC-MS system consisted of an Agilent 6890 Gas Chromatography coupled with a 5973 *inert* Mass Spectrometry Detector (Santa Clara, CA, USA) and an Agilent G2614A Autosampler.

Separation of the analytes was performed on a HP-Innowax capillary column (60 m x 0.250 mm ID x 0.25 µm film thickness) (Part No. 19091N-136E, Agilent Technologies, Germany), using helium carrier gas at a flow rate of 1 mL/min. The sample obtained from an extraction of 50 mL of wine was desorbed at the injection port in pulsed splitless mode at 240 °C for 1 min, and the split vent was set up at 30 mL/min after 1.5 min.

The oven temperature program was initially set up at 50 °C and held for 5 min, then raised to 115 °C at 3 °C/min (held for 22 min), followed by an increase to 150 °C at 40 °C/min (held for 3 min) and raised to 173 °C at 3 °C/min, finally ramped up to 250 °C at 70 °C/min (held for 20 min) before dropping to 50 °C at 40 °C/min.

The Mass Spectrometer operated in electron impact (EI) mode at 70 eV. The temperature of the transfer line was 250 ºC. The temperatures of the MS source and quadrupole were set at 230 ºC and 150 ºC, respectively.

2.2.5.5. Identification and quantification of 3-MH

Identification was carried out using *m/z* 100 and 134 for 3-MH, while the respective deuterated ions *m/z* 102 and 136 were used for [1-$^{2}H_2$]3-MH. The calibration curve was obtained by adding increasing quantities of 3-MH into a Cabernet Sauvignon wine matrix to have the concentrations of 3-MH in the range of 150 to 4000 ng/L. Manual integration was carried out to obtain the peak areas of 3-MH and [1-$^{2}H_2$]3-MH. Because the presence of 3-MH in the base wine (Cabernet Sauvignon), the analyte/internal standard peak areas ratios (3-MH/[1-$^{2}H_2$]3-MH) of the standard solutions were corrected by subtracting the peak area ratio of the blank (no 3-MH addition), before they were plotted against the corresponding 3-MH concentrations to obtain the working calibration curve. The ten point calibration curve for 3-MH with the linear regression equation and the coefficient of determination (R^2) is displayed in Figure 2.3.

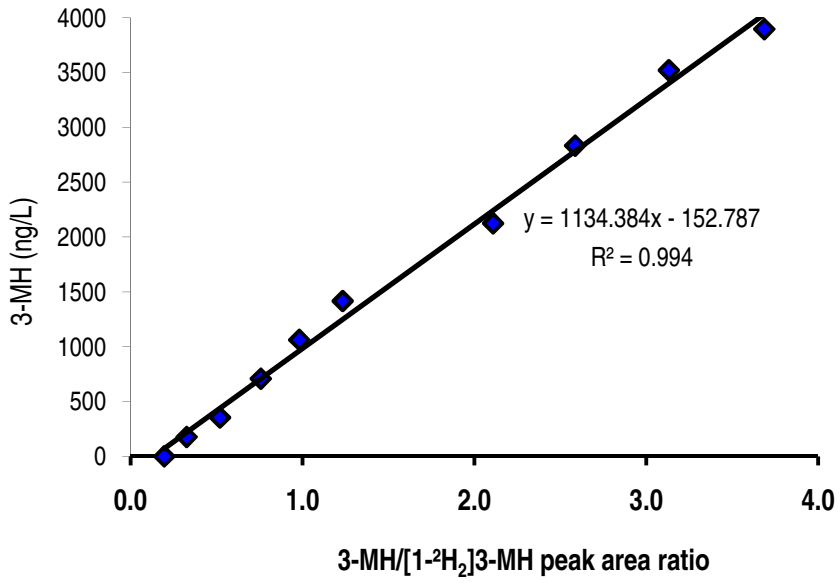


Figure 2.3. Calibration curve of 3-MH in a Cabernet Sauvignon wine

Quantification of 3-MH was performed by applying the ratio of the peak area of the quantifier ions of the analyte (134) and peak area of the internal standard (136), obtained from the GC-MS chromatogram of the analysed wine, in the calibration equation to calculate the concentration (ng/L).

2.2.6. Gas chromatography - Mass Spectrometry analysis of reductive sulfur compounds

An analytical method which is sensitive and reliable in order to analyse the reductive sulfur volatile compounds was needed for the current research. Development of a method was therefore conducted with reference to the available literature and making use of the laboratory facilities available at the Tamaki wine research laboratory at the University of Auckland. Details on the method finally adopted to quantify the reductive sulfur compounds of interest are provided in a separate chapter (CHAPTER 3).

2.3. SENSORY ANALYSIS

2.3.1. Ethics approval

Under the University of Auckland research policy, all projects which involve human participants must receive the approval of the University of Auckland Human Participants Ethics Committee (UAHPEC) before the research commences. A set of required documents was prepared and submitted to the UAHPEC. The ethics approval was granted (reference number 2009/444), allowing the sensory analysis to proceed. The documents prepared to enclosed with the UAHPEC form for the application can be found in Appendix 2.2.

2.3.2. Training and data collection

In order to correlate the quantitative chemical results with the perceived sensory characteristics, sensory evaluation of wines at the end of the MOX trial of a BORDEAUX blend after malolactic fermentation was conducted using the *triangle test* and the *multisample difference tests* with a *randomised (complete) block design*, as described in (Meilgaard *et al.*, 1999). The triangle test was employed to determine if there were any global sensory differences between any pair of samples. The later method was used to compare the control and the other three micro-oxygenated wine samples according to six single attributes selected, including four aromas (vegetal, fruity, reductive and oxidised notes) and two tastes (astringency and mouth-feel). The *multisample difference tests* with a *randomised (complete) block design* is considered appropriate for a small number of samples, generally less than six samples, thus the mental fatigue of the panellists is not a concern (Meilgaard *et al.*, 1999). In this method, panellists are the 'blocks' while samples are the 'treatments'. Because each panellist evaluates by ranking all of the samples which are presented to them in a balanced randomised order, so is the term *randomised block design* of the method derived (Meilgaard *et al.*, 1999).

2.3.2.1. Training the panel

A panel of 15 volunteers was assembled and trained, most of them being Wine Science Post Graduate students (University of Aukcland, NZ), who were familiar with the product or showed interest in tasting wine to improve their palate and skill. The goal of the training was to improve the ability of the panellists in recognising the wine sensory characteristics as well as building their ability and confidence in identifying, detecting and using the appropriate lexicon/description for the attributes.

Training was conducted initially by introducing six reference standards representing the attributes of interest to the panellists, followed by a triangle test in which the panellists were asked to identify and rank the intensity of each attribute in the different/odd sample

compared to that of the other identical/alike samples. Table 2.5 presents the commercial chemicals used to prepare the reference standards, while the lexicon describing the attributes, and information on how to prepare these standards can be found in Table 2.6. Score-sheets given to panellists during training sessions can be seen in Appendix 2.3.

Table 2.5. Chemicals used to prepare reference standards

Chemicals and reagents	CAS No.	Product code	Supplier
$Al_2(SO_4)_3.16H_2O$ (AnalaR®)	16828-11-8	10010	BDH Chemicals Ltd.
Tannin (tannic acid pure DAB, USP)	1401-55-4	A3619,0250	AppliChem
2-Isobutyl-3-methoxypyrazine (99 %)	24683-00-9	297666	Sigma-Aldrich
cis-3-Hexen-1-ol (98 %)	928-96-1	H12900	Sigma-Aldrich
Dimethyl sulfide (DMS) (≥ 99 %)	75-18-3	471577	Sigma-Aldrich
Sodium sulfide nonahydrate (98+ %, ACS reagent)	1313-84-4	208043-100G	Sigma-Aldrich

In the triangle test training session, panellists were presented with the base wines spiked with the sensory reference standard(s), which were chosen to well reflect the sensory attributes of interest. The base wines were either a model wine or were prepared from a Merlot diluted in a model wine (5 g/L tartaric acid, 13.5 % absolute ethanol in ultrapure water) at 1:1 ratio.

In order to lessen the work load on the panel and to avoid tiredness, this session was divided into two parts, where only two aromas and one taste samples were presented in each part. Panellists were instructed to only sniff the representative aromas, but taste then expectorate samples with standards for astringency and mouth-feel.

Table 2.6. Sensory attributes and the associated lexicon

	Attributes	Description/Lexicon	References
1	**Vegetal**	Herbaceous characters; smells of cut-grass	2-Isobutyl-3-methoxypyrazine (0.5 - 40 µg/L) and *cis*-3-hexen-1-ol (0.2 - 1 mg/L) in a diluted Merlot wine
2	**Fruitiness**	Berry characters	Watties (Hastings, New Zealand) blueberry, blackberry and plum currant juice in a diluted Merlot wine
3	**Oxidised**	Oxidised taint smell, rotting fruit, sweet-sickly fruit	Oxidised Merlot prepared by exposing a Merlot wine to air at room temperature until the oxidized note is noticeable
4	**Reductive**	Sulfury, onion, rotten eggs, canned vegetables	DMS (20 µg/L) and Na_2S (10 µg/L) in model wine
5	**Astringency**	Tactile sensation resulting from the precipitation of salivary protein, leading to a loss of mouth lubrication and a drying-puckering sensation in cheeks	$Al_2(SO_4)_3$ (2 g/L) in a diluted Merlot wine
6	**Mouthfeel**	A measure on how the wine fills the palate	A Cabernet Merlot 2009 (Mission Estate, Hawkes Bay, New Zealand) (62 % Cabernet Sauvignon, 34 % Merlot, 2 % Cabernet franc, 1 % Petit Verdot and 1 % Malbec)

2.3.2.2. Actual tasting for data collection

Data collection was conducted in the week following the training sessions. Samples containing aroma references were placed on a separate table where panellists could take a sniff to remind themselves of the perceived sensory characteristics of the aromas. Shortly before the data collection, 25 mL wine were poured from a 1 L Schott bottle used

to sample the wine into standard ISO/INAO (Institut National d'Appelation d'Origine – *the French Regulatory body*) wine tasting glasses, coded with three digit numbers correspondingly to the treatments and covered with plastic petri dishes. Panellists were provided with water to refresh their palate during the tasting. The tastings took place in the seminar room at the Ray Meyer Research Centre of the University of Auckland, Tamaki Campus. The room was sufficiently quiet, and the lighting, air and temperature were well regulated.

In the triangle test, each panellist received a suite of six rows of samples. Each row contained three glasses of wine from two different treatments. Samples were presented randomly to the panellists in a balanced way, so that each sample appeared in a given position an equal number of times. Panellists were asked to sniff and if needed taste the wine to evaluate the mouthfeel and astringency, then expectorate to identify a different sample in each row. In the score sheet provided (Appendix 2.4), the identified odd sample were indicated by circling the three digit code number corresponding to that sample. A short break was taken after the panellists finished their first three rows, before proceeding to the last rows of samples. For the triangle tests, a number of 20 to 40 subjects are usually required, although as few as 12 may also be employed (Meilgaard *et al.*, 1999). Taking into account the preparation load to be carried out to include six combinations from four different wines for tasting at the same day, as well as the availability of the panellists, the area of the tasting room and the number of glasses needed, a panel comprised of 15 panellists was considered appropriate for the purpose of this study.

In the ranking test using the *multisample difference test* with a *randomised (complete) block design*, all four samples including the control and three micro-oxygenated wines were introduced to each panellist at the same time in two consecutive ranking test sessions in the same day. Samples were coded with three digit numbers and served in a random, balanced order. In the first ranking test session, panellists were required to evaluate the wines according to the intensity of the vegetal and fruity notes as well as the

astringency of the samples presented to them in a random order. After completing the first tasting session, panellists were given a short break in order to minimise the mental fatigue of the panellists and to limit inaccuracy due to the evaporation of aromas in samples during tasting. The same samples (the control and three micro-oxygenated wines) were then freshly presented to the panellists in the second ranking test session for the evaluation of the oxidised and reductive notes, and the mouth-feel.

At the beginning of these ranking test sessions, panellists were given the scoresheets for use in each session (Appendix 2.4) and were instructed to arrange the samples in a provisional order according to the specific attribute. After that, they could verify and change the order based on further, more careful tasting. If the two samples were considered to be similar or the same, they were required either to make a 'best guess' as to their rank order or to indicate under 'comments' the identical samples. The average rank sum of the identical samples was assigned for later statistical analysis. After the order of the samples according to the intensity of each attribute was revealed, panellists were asked to note the three digit coded numbers in that order in the scoresheets provided (Appendix 2.4). There were 14 panellists who participated in this part of the study.

2.3.3. Analysis of sensory results

Analysis of the triangle test was evaluated by counting the number of correct responses and the number of total responses. The number of correct answers must be equal or larger than the minimum required number of correct responses for a specific number of total responses to generate a difference between samples (Appendix 2-15).

Analysis of the ranking test was performed using a Friedman's test. The current study involved the participation of 14 panellists, hence the requirement of a relatively large number of panellists, generally 12 or more, in order to provide reasonably accurate results, was met.

The rank sums for each sample (column sums) were calculated as well as was the value of the test statistic T, according to the following equation (Meilgaard *et al.*, 1999, p.292).

$$\mathrm{T} = \left([12/bt(t+1)] \sum_{j=1}^{t} x_{.j}^{2} \right) - 3b(t+1)$$

Where:

b - the number of the panellists;

t - the number of samples, and;

$x_{.j}$ - the rank sum of sample j (i.e., the column total for sample j)[21];

If the T valued calculated from this equation exceeds the $\chi^2_{\alpha,t-1}$ (the upper-α critical value of a χ^2 random variable with (t - 1) degrees of freedom), the null hypothesis of no sample differences is rejected or it can be concluded that significant difference does exist among samples. The $\chi^2_{\alpha,t-1}$ can be found in **Appendix 2-16**. If H_0 is rejected, a multiple comparison procedure is then performed to determine which of the samples are significantly different. The Fisher's LSD for rank sums from a randomised (complete) block design was calculated according to the following equation.

$$LSD_{rank} = t_{\alpha/2,\infty}\sqrt{bt(t+1)/6}$$

Here $t_{\alpha/2,\infty}$ is the two-sided critical value of a *t*-distribution with infinitive degrees of freedom. These values can be found in Field (2009, p.803), where $t_{0.05/2,\infty}$ = 1.96 and $t_{0.01/2,\infty}$ = 2.58.

[21] The 'dot' in $x_{.j}$ indicates that summing has been done over the index replaced by the dot, that is $x_{.j} = \sum_{i=1}^{b} x_{ij}$.

2.4. DATA ANALYSIS

Wine colour and chemical analyses were carried out once for each replicate of each treatment (in triplicate). The mean values and standard deviations of the mean for each parameter were calculated using Microsoft Excel 2007.

Linear regression analysis of all calibration standard curves was carried out on Microsoft Excel 2007. The One-way ANOVA Analysis, the Least Significant Difference (LSD) and the Tukey's Honestly Significant Difference (Tukey HSD) Post Hoc Test were performed to compare the mean values of the monitored compounds or parameters with a statistical significance level set at 0.05 using SPSS 17.0.

CHAPTER 3. AN AUTOMATED HS-SPME METHOD FOR THE ANALYSIS OF REDUCTIVE SULFUR COMPOUNDS

3.1. INTRODUCTION

3.1.1. The analysis of reductive sulfur compounds

Sulfur containing structures are among the most important class of food aromas, owing to their abundance and aroma impact (Mestres *et al.*, 2000). Wine aromas in particular consist of several hundreds of aroma compounds, occurring at concentrations ranging from several mg/L to a few ng/L (trace amount) (Ribéreau-Gayon *et al.*, 2006b, p.205). Among these, certain sulfur containing volatiles can contribute to the favourable sensory impacts (Bouchilloux *et al.*, 1998; Tominaga *et al.*, 1998a), while others can give unpleasant detrimental effects to the quality of wine (Landaud *et al.*, 2008). The later refers the reductive sulfur off-odours which have been a major concern for the wine industry, as discussed in CHAPTER 1.

Reductive sulfur containing volatiles belong to the group composed of heterogeneous compounds, which also include aldehydes, alkyl methoxypyrazines, furaneol and sotolon. The analysis of these compounds is very difficult due to their poor chemical stability and

extremely low concentrations, generally less than 0.1 µg/L (Genovese *et al.*, 2005). The analytical methods that can be used for the quantitative determination of these reductive sulfur compounds have been reviewed (Mestres *et al.*, 2000). The analysis of these compounds in wine is a challenging task, as a consequence of three main problems, namely the complexity of the wine matrix, the low concentrations of the compounds and their highly reactive nature (Mestres *et al.*, 2000). Therefore, working with these compounds is cumbersome and requires special care, especially when making standard solutions and samples containing thiols (Mestres *et al.*, 1997).

The methods that have been used to analyse these reductive sulfur compounds include amperometric, colourimetric, fluorimetric, potentiometric, titrimetric, and gas chromatographic techniques (Mestres *et al.*, 1997). In order to obtain good sensitivity, specificity and reliability, reductive sulfur compounds are usually analysed by gas chromatography (GC) coupled either with sulfur-specific detectors such as the flame photometric detector (FPD) (Mestres *et al.*, 1997; Pripis-Nicolau *et al.*, 2004), the pulsed flame photometric detector (PFPD) (Lopez *et al.*, 2007), the sulfur chemiluminescent detector (SCD) (Chang & Taylor, 1990), or with a non-sulfur specific detector such as a mass spectrophotometric detector (MSD) (Tominaga *et al.*, 1998b; Pripis-Nicolau *et al.*, 2004) or an atomic emission detector (AED) (Siebert & Pollnitz, 2007).

3.1.2. Headspace solid-phase microextraction

A pre-concentration step is required before chromatographic analysis of reductive sulfur compounds, due to their trace concentrations in wine. Traditional pre-concentration methods include solvent extraction and static headspace extraction[22] have been used widely to extract volatiles. However, these methods are often time consuming (solvent

[22] Static headspace extraction is the direct sampling and transferring of the vapour phase in the headspace above and in contact with a liquid or solid sample in a sealed container onto a GC system for separation, detection and quantification (Snow & Bullock, 2010).

extraction) and are low in sensitivity (static headspace extraction), and are thus not suitable for the analysis of reductive sulfur compounds in wine, as these compounds are extremely volatile and chemically reactive (Fang & Qian, 2005).

Solid phase micro-extraction (SPME), a technique introduced in 1980s, has been increasingly used as an alternative to traditional pre-concentration methods, for the extraction of volatile compounds (Mestres *et al.*, 2000; Fang & Qian, 2005). It is a 'solvent-less' technique that employs a polymer-coated fiber immersed into a liquid sample or the gas headspace (HS-SPME) to extract and concentrate analytes from the matrix onto the fiber (Mestres *et al.*, 2000). The fiber is then retracted and transferred into a GC system for desorption and analysis (**Figure 3.1**).

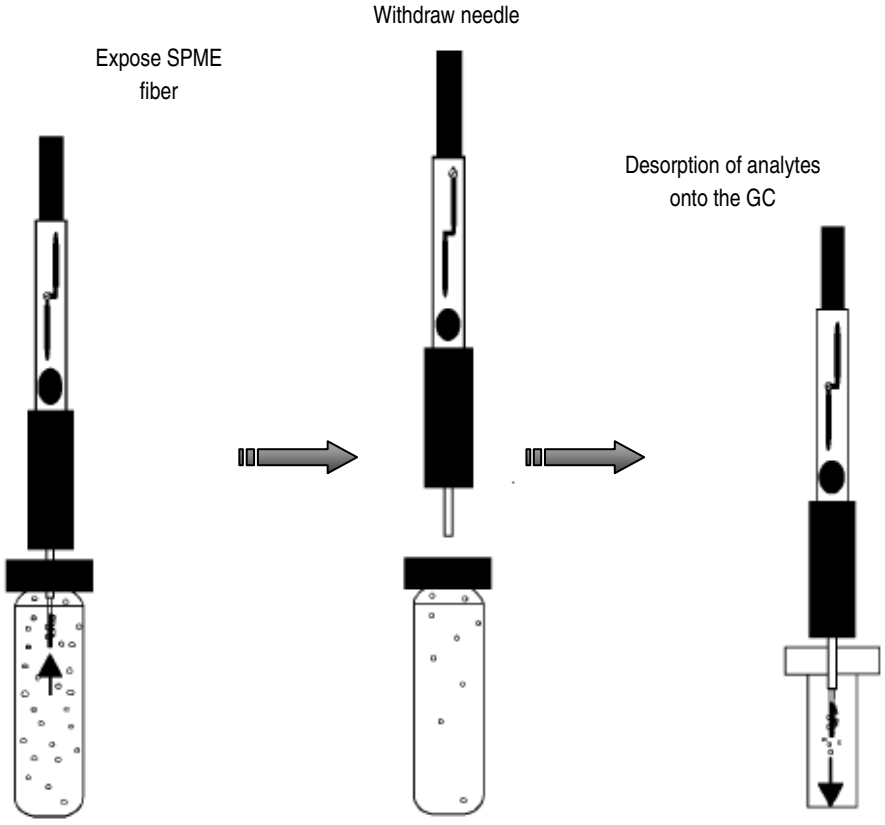


Figure 3.1. Basic steps in a SPME procedure

(Adapted from Supelco, 1998)

In SPME method development, four basic stages are involved, including the extraction technique, hardware, initial optimisation and finally calibration and validation (Pawliszyn, 1997). However, there has been increasing numbers of publication regarding SPME methods developed for the analysis of specific compounds, so not all listed steps (**Table 3.1**) will need to be repeated, particularly the initial steps of the extraction technique and hardware (Pawliszyn, 1997).

Table 3.1. Stages in SPME method development

(Adapted from Pawliszyn, 1997)

Stages	Steps involved
Extraction Strategies	Coating selection
	Derivatisation reagent selection
	Extraction mode selection
	Agitation method selection
	Selection of a manual vs. automated system
Hardware	Selection of separation and/or desorption conditions
	Optimisation of desorption conditions
Initial optimisation	Optimisation of sample volume
	Determination of extraction time profile in a pure matrix
	Determination of extraction time
	Calculation of the distribution constant
	Optimisation of extraction conditions (pH, salt, temperature)
Calibration and validation	Determination of the linear dynamic range of the method for a pure matrix at optimum extraction conditions
	Selection of calibration method
	Optimisation of extraction conditions for heterogeneous samples
	Verification of equilibration time, sensitivity, and linear dynamic range for complex sample matrices
	Method precision
	Method detection limits
	Validation
	Automation

3.1.3. Classification of fiber coating

Different fibers have been commercially available to use with SPME. Volatile analytes are retained better in a thicker fiber coating and can be transferred into the GC injection port without loss. On the other hand, a thin coating is used to ensure a rapid release of higher boiling point compounds during thermal desorption (Otles, 2009).

The commercially available SPME fibers can generally be classified into two groups. The first group involves a pure liquid polymer coating, such as polydimethylsiloxane (PDMS) and polyacrylate (PA). The second group are mixed films containing solid particles and *liquid polymers*, such as Carboxen-*polydimethylsiloxane* (CAR-PDMS) and divinylbenzene-*polydimethylsiloxane* (DVB-PDMS) (Otles, 2009). Carboxen acts as a carbon molecular sieve[23] and is often used with PDMS (CAR-PDMS) for low molecular weight polar analytes, while DVB-PDMS is more suited to semi-polar analytes (Otles, 2009).

3.2. GAS CHROMATOGRAPHY COUPLED WITH FLAME PHOTOMETRIC DETECTOR

3.2.1. Introduction

At the onset of this research, there was no method available which could be used to quantify different reductive sulfur compounds of interest, including light and heavy volatiles. In a previous Wine Science project at the University of Auckland (Ellett, 2006), modifications to a published method by Fang *et al*., (2005), using HS-SPME coupled with a GC-FPD system, had been carried out in order to improve repeatability and make it suitable for the analysis of reductive sulfur compounds in the Sauvignon blanc wines

[23] To trap very small molecular sized compounds (C_2-C_5)

studied in the project. The method still had very limited success due to the inteference of sulfur dioxide present in the wine with the detection of the reductive sulfur compounds, low sensitivity and repeatability, although it was an acceptable method used to compare peak area ratios of the analytes to internal standard in the same wine with different treatments (Ellett, 2006).

The first attempt of the current research was thus to optimise this previously developed method in order to fulfill the requirement of a good analytical tool for the quantification of the reductive sulfur compounds of interest in the MOX research wines.

3.2.2. Fiber coating and extraction of reductive sulfur compounds

In Ellett's method (2006), the same SPME fiber and similar extraction conditions were applied as in the method of Fang *et al.*, (2005). The fiber was a Carboxen/ polydimethylsiloxane (CAR-PDMS; d_f 85 µm x 1cm, StableFlex) fiber (Product No. 57334-U) (Supelco, Bellefonte, PA, USA), used with a manual fiber holder. The fiber was conditioned according to manufacturer's specifications at 300 °C for 1 hour before use. This fiber has been chosen for use in several studies on the quantitative determination of reductive sulfur compounds, because it has the strongest affinity for low molecular weight sulfur compounds (Mestres *et al.*, 1999; Fang & Qian, 2005; Lopez *et al.*, 2007).

Extraction was carried out in the headspace above 5 mL of sample placed in an amber screw top 20 mL vial (*Part No.* 5188-6537, Agilent Technologies) tightly capped with a certified ultraclean 18 mm screwcap with septum (*Part No.* 5188-2759, Agilent Technologies). Because the GC-FPD system available at the University of Auckland was not equiped with an automatic sampling system, extraction was carried out in a water bath kept at 30 °C. The sample was agitated with a magnetic stirrer bar placed in the vial at a speed of 480 rpm. The sample was initially incubated for 30 min before the fiber was introduced into the headspace to extract the sample for 15 min. The fiber was baked for 5

min at 300 ºC prior to the next exposure to the sample for extraction in order to clean off any heavier compounds remaining on the fiber from the sample matrix.

3.2.3. Chemicals and reagents

Most of chemicals and reagents were from the same suppliers, as detailed in Section 3.3.3 for those used in the method developed on a GC-MSD system, except for some chemicals that were only used in this part of the research. This included sodium chloride (Cas No. 7647-14-5, 99.0 - 100.5 %) and glyoxal (ethanedial) (40 wt.% solution in water (Cas No. 107-22-2) sourced from Sigma-Aldrich (Product code 128465-1KG). Thiophene (Cas No. 110-02-1, ≥ 99 %), used as an internal standard, was supplied by Sigma-Aldrich.

Stock solutions of individual commercial reductive sulfur compounds were prepared in absolute ethanol as described in Section 3.3.4.1. These individual stock solutions were then used to make mixtures of standards in groups, including, for example, methanethiol and ethanethiol (MeSH and EtSH) in the first group, dimethyl sulfide and diethyl sulfide (DMS and DES) in the second group, etc. Thiophene stock solution was prepared in absolute ethanol and stored at -80 °C. Serial dilutions were then made when necessary to have a thiophene concentration of 1 µg/L in the final sample (5 mL). All preparations of standard solutions of the reductive sulfur compounds, as well as sample handling, were carried out at 4 °C.

3.2.4. GC-FPD system and chromatographic conditions

The Gas Chromatography - Flame Photometric Detector (GC-FPD) system consisted of a Agilent 6890 GC system (G1540N) and a Flame Photometric Detector (No. CG4843, H9261). The separation of reductive sulfur compounds was performed using a DB-FFAP capillary column (30 m x 0.32 mm I.D., 1 µm film thickness) (Agilent, J&W Scientific, New Zealand).

After extraction, the fiber was manually retracted into its holder, transferred and inserted to the GC injection port for the desoprtion of the sulfur compounds at 240 ºC in splitless mode for 5 min. The oven temperature was initially set at 42 ºC for 5 min, then ramped at 7 ºC/min to 150 ºC and held at this temperature for 5 min. After that, the oven temperature was raised at a rate of 30 ºC/min to 220 ºC, and held for 1 min.

The carrier gas was helium at a constant flow rate of 3.1 mL/min, as selected in Ellett (2006), to provide optimum resolution of the reductive sulfur compounds analysed. The FPD detector (**Figure 3.2**) was kept at 220 ºC, and was supplied with 50 mL/min hydrogen, 50 mL/min nitrogen and 60 mL/min air. The carrier + make up flow was 40 mL/min, and nitrogen was the makeup gas.

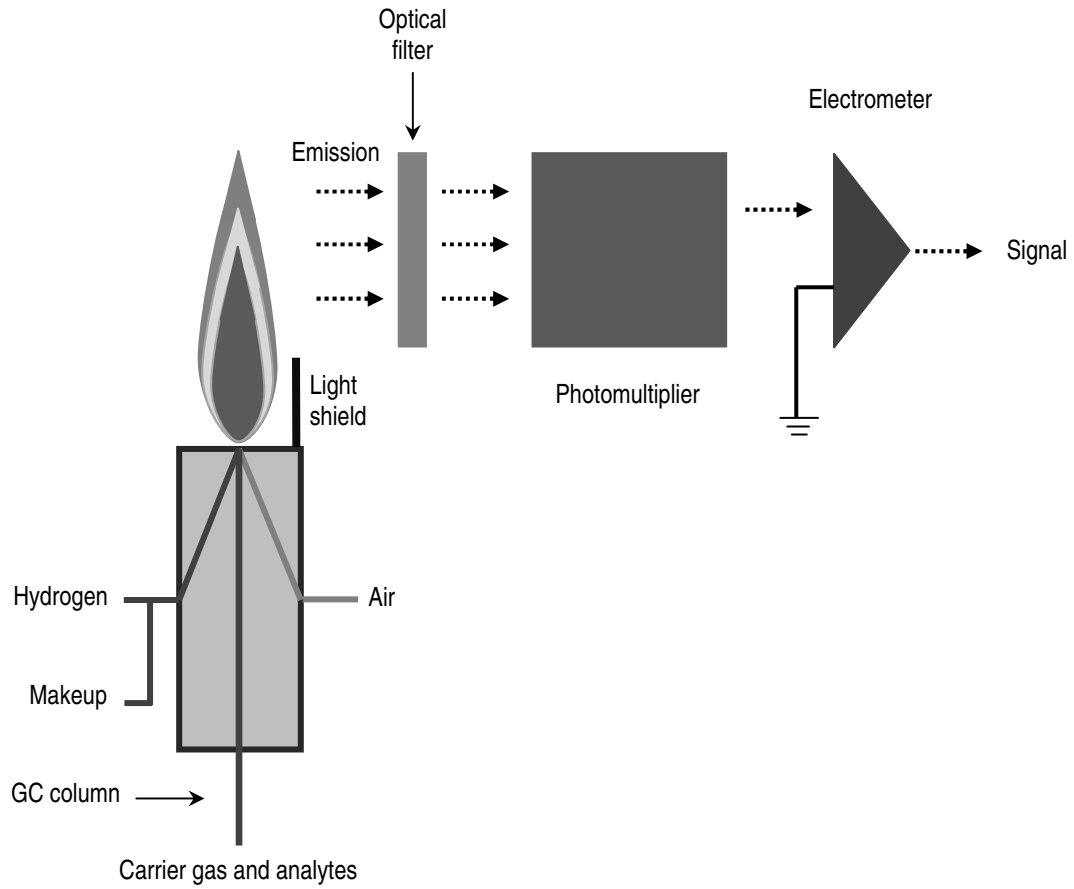


Figure 3.2. Schematic diagram showing a flame photometric detector

(Adapted from Nielsen, 2010)

3.2.5. Modification of sample preparation step

In both methods of Ellett (2006) and of Fang & Qian (2005), a wine sample of 5 mL was used for the extraction of reductive sulfur compounds onto the SPME fiber. It was reported in Ellett (2006) that the developed method still encountered some problems, such as the formation of artifacts due to the oxidation of thiols to form disulfides, and the interference of sulfur dioxide and the wine matrix on the extraction. In a later publication (Lopez *et al.*, 2007), it was suggested that a dilution factor of 50 (100 µL of wine in 4.9 mL of saturated NaCl brine) could minimise the matrix effect while the signal was kept sensitive enough. In order to limit the effects of oxidation, the author also emphasized the need for an immediate analysis on the GC system after the samples were prepared, and suggested the vial be flushed with nitrogen gas before the sample was introduced via a syringe (Lopez *et al.*, 2007). The presence of sulfur dioxide also affects the detection of sulfur compounds, as it produces a broad peak that obscures the signals of dimethyl sulfide and dimethyl disulfide (Lopez *et al.*, 2007). In Fang's method, acetaldehyde (ethanal) was used to overcome this problem, while in Lopez *et al.* (2007), ethanedial was selected for this purpose, given the argument that compounds like ethanal are highly volatile and could compete with the analytes for the adsoption sites of the fiber. Therefore, a modification to the extraction step in the method of Ellett (2006) was carried out, taking into consideration the suggestions in the report of Lopez *et al.* (2007).

Modified extraction

A 20 mL amber vial (*Part No.* 5188-6537, Agilent) containing 4.9 mL of saturated sodium chloride was purged with argon gas for 1 min, closed with a certified ultraclean 18 mm screwcap with septum (*Part No.* 5188-2759, Agilent). After that, a 100 µL of wine, 5 µL of ethanedial solution 8 % v/v and 50 µL of internal standard solution were injected into the vial through the septum with SGE gas tight syringes (Phenomenex NZ Ltd., New Zealand). The vial was then placed in a water bath kept at 35 °C to incubate for 5 min to

remove/scavenge sulfur dioxide by ethanedial[24]. The fiber was then introduced into the headspace via a manual holder that pierced through the septum to extract the analytes for 20 min. The sample was agitated during both incubation and extraction periods at 480 rpm with a magnetic stirrer bar placed in the vial.

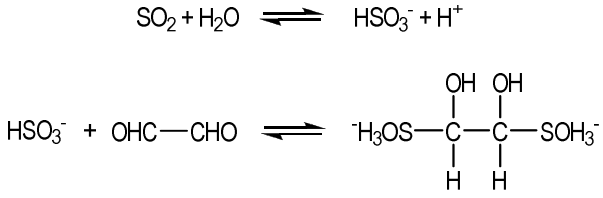
After extraction, the fiber was retracted from the vial for tranferring onto the GC-FPD system for desorption, detection and quantification at the same chromatographic conditions as in Ellett (2006), and as described in Section 3.2.4.

3.2.6. Calibration

A volume of mixed stock solution in model wine was introduced through a SGE gas tight syringe (Phenomenex NZ Ltd., New Zealand) into a 20 mL sealed vial containing saturated NaCl brine, already flushed with argon gas and closed with a septum. The total volume of the mixed stock solution and saturated NaCl brine was 5 mL. The vial also contained 25 µL of internal standard to have thiophene at 5 µg/L in the final sample, and 5 µL of glyoxal 8 % v/v. Both solutions were introduced through the septum via SGE syringes (Phenomenex NZ Ltd., New Zealand) just before the introduction of the mixed stock solution of the reductive sulfur compounds.

As the response of a FPD is quadratic, the calibration curve for specific sulfur compounds was constructed by plotting the concentrations of the analyte (sulfur compound) versus the square root of the ratio of the analyte peak area to internal standard peak area. As the analysis of MeSH and EtSH was the most challenging due to their highly volatile and

[24] The scavenging of sulfur dioxide is illustrated as below.



reactive nature towards oxidation, a preliminary attempt was made to construct the calibration curves in model wine for these two compounds.

Figure 3.3 showed the seven-point calibration plots obtained by the GC-FPD system for MeSH and EtSH. As can be seen from the graph that although the concentrations of the thiols were kept low in a fairly narrow concentration range, from 0 to 6 µg/L for both reductive thiols, the coefficient of determination R^2 of the calibration equations were not particularly good, at 0.961 and 0.956 for MeSH and EtSH, respectively. In addition, it is necessary that the concentrations of the standard solutions span a wider range, in order to cover the concentrations usually reported in the literature.

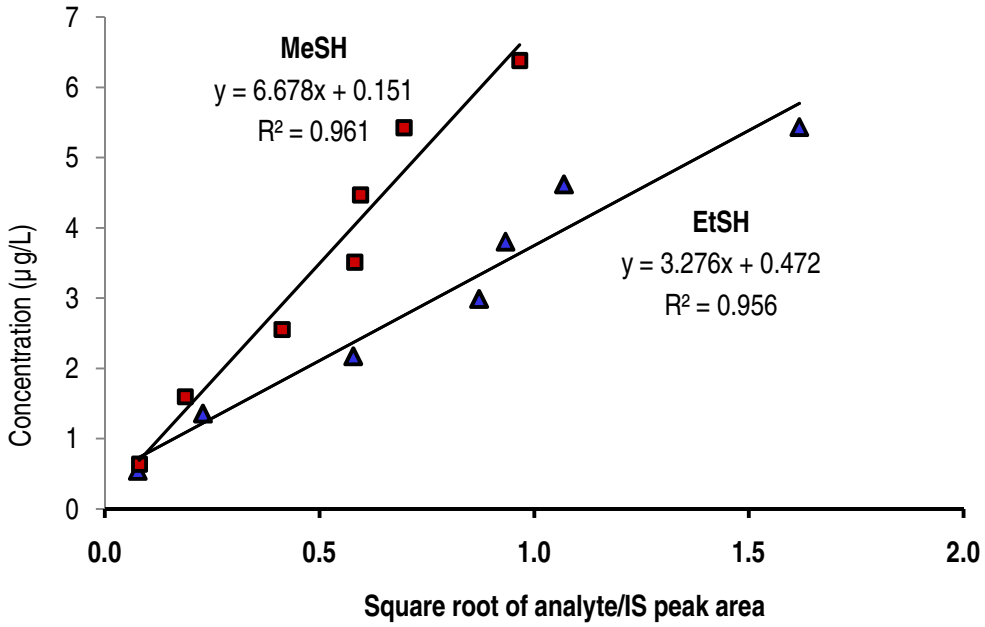


Figure 3.3. Calibration curves for MeSH and EtSH obtained by GC-FPD

3.2.7. Problems encountered with the GC-FPD method

During the attempt to optimise the available method at the University of Auckland for the analysis of reductive sulfur compounds, some problems with the use of the Flame Photometric Detector were observed. It was not a stable detector to work with, particularly

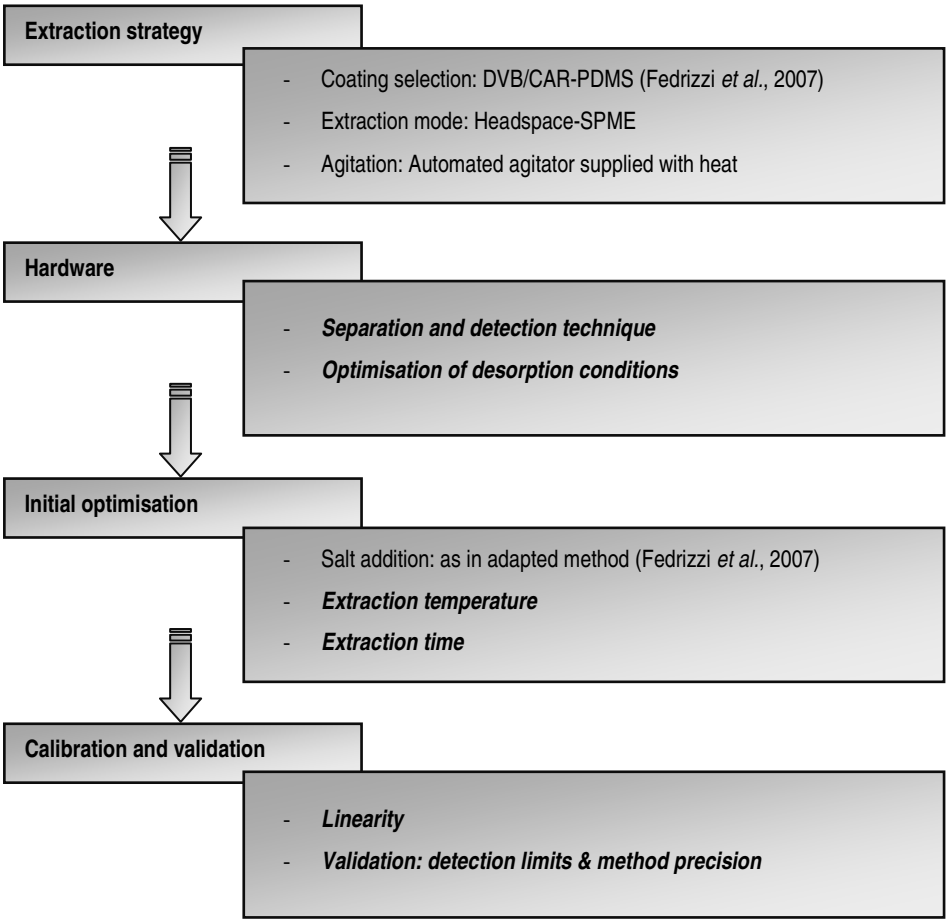
when the flame reignited during the run of a sample. Although an acceptable linear response could be obtained, such as in the case of MeSH and EtSH mentioned earlier, the preparation of calibration curves from mixture standard solutions containing only two compounds of a similar structure did not reflect the real adsorption competiveness of all reductive sulfur compounds taken onto the adsoption sites of the fiber, because these compounds may present simultaneously in a wine sample. In addition, the extraction and injection were carried out manually, which was hard to control and could consequently render more variations in the analysis. Manual extraction of samples also limits the number of samples that could be analysed in a certain period of time, and thus is time consuming. Taking into account these problems, and there was a Gas Chromatography - Mass Spectrometry system which allows automated SPME that became available, it was decided to develop an analytical method for the quantification of the reductive sulfur compounds of interest by employing this instrumentation. The method was fully established and is reported in detail in Section 3.3.

3.3. GAS CHROMATOGRAPHY COUPLED WITH MASS SPECTROMETRY DETECTOR

The Gas Chromatogaphy - Mass Spectrometry (GC-MS) method development for the analysis of reductive sulfur containing compounds was based on a publication of Fedrizzi *et al.* (2007). The steps employed in the development of a HS-SPME method needed for the research did not require the selection of a fiber used to extract the compounds of interest or the selection of salt addition at a specific concentration to enhance the extraction yield, as they have been reported in Fedrizzi *et al.* (2007).

Other steps were conducted to optimise the procedure for the quantification of all the reductive sulfur compounds of interest, with other light sulfur compounds being included. Scheme 3.1 summarises the strategies applied in the current GC-MS method

development, indicating steps that have been adapted from Fedrizzi *et al*., (2007) and those that were carried out in addition to the published method (in ***bold italics***).



Scheme 3.1. Strategies employed with bold italics indicate steps that were carried out in the current method development

3.3.1. Fiber coating

In the method Fedrizzi *et al.* (2007), fibers with six different coatings have been examined for their extraction efficiency with regard to the reductive sulfur containing compounds. Although other 'light' sulfur compounds were included in the current study, Divinylbenzene/Carboxen-Polydimethylsiloxane (DVB/CAR-PDMS; 50/30µm x 2 cm) fiber (Product No. 57298U, Supelco, Bellefonte, PA, USA), which proved to give the best results, was employed to extract the analytes of interest. The fiber is a mixed film coated with

Divinylbenzene/Carboxen on *polydimethylsiloxane*, bonded to a flexible fused silica which offers a less breakable fiber. The mixed film coating allows the extraction of the analytes by absorption with the liquid polymer and by adsorption with the porous solid particles (Otles, 2009).

3.3.2. Extraction and agitation

Incubation was selected in order to give similar headspace conditions before the fiber was exposed. Agitation using magnetic stirrer bars was employed during both incubation and extraction time in an available Gerstel Agitator/Stirrer, in which the agitation speed and temperature of samples were automatically controlled using MAESTRO Software (Version 1.2.0) (Gerstel, Mülheim an der Ruhr, Germany). Variations in agitation conditions, incubation and extraction, therefore, were eliminated.

The extraction temperature and time were optimised, as part of the current method development, to give the best extraction effectiveness for the reductive sulfur compounds of interest. The experimental protocols and results are presented in Section 3.3.7.

3.3.3. Chemicals and reagents

There were fourteen sulfur compounds of interest included in the current method development. The commercial standards of these compounds and the other three commercial standards, used as internal standards were purchased from either Sigma-Aldrich (Auckland, New Zealand) or Alfa Aesa (Ward Hill, MA, USA). A methanethiol gas cylinder was supplied by Matheson Coleman and Bell (East Rutherford, NJ, USA) (Table 3.2). These commerical standards were used to identify peaks on the chromatogram and to construct the standard calibration curves for quantification by GC-MS.

Absolute ethanol was of analytical grade and purchased from Ajax Finechem (Mt. Wellington, Auckland, New Zealand). Magnesium sulfate heptahydrate $MgSO_4.7H_2O$ (Cas

No. 10034-99-8) was obtained from Sigma (Sigma-Aldrich, Germany).

Table 3.2. Commercial standards used for analysis of reductive sulfur compounds

Chemicals and reagents	Purity (%)	CAS No.	Product code	Supplier
Analytes				
Methanethiol	99.5	74-93-1	-	Matheson Coleman & Bell
Ethanethiol	97	75-08-1	E3708-100ML	Sigma-Aldrich
Carbon disulfide	99	75-15-0	335266-100ML	Sigma-Aldrich
Dimethyl sulfide	99	75-18-3	471577-250ML	Sigma-Aldrich
Diethyl sulfide	98	352-93-2	107247-100ML	Sigma-Aldrich
Dimethyl disulfide	99	624-92-0	471569-250ML	Sigma-Aldrich
Diethyl disulfide	99	110-81-6	E26223-25G	Sigma-Aldrich
Dimethyl trisulfide	98	3658-80-8	W327506-100G-K	Sigma-Aldrich
S-Methyl thioacetate	98+	1534-08-3	A12400	Alfa Aesa
S-Ethyl thioacetate	98+	625-60-5	A10680	Alfa Aesa
2-Mercaptoethanol	99	60-24-2	M6250-250ML	Sigma-Aldrich
2-(Methylthio)-1-ethanol	99	5271-38-5	226424-10G	Sigma-Aldrich
3-(Methylthio)-1-propanol	98	505-10-2	318396-5G	Sigma-Aldrich
4-(Methylthio)-1-butanol	97	20582-89-3	319694-1G	Sigma-Aldrich
Internal standards				
$^{2}H_{6}$-Dimethyl sulfide	99	926-09-0	416452-1G	Sigma-Aldrich
Isopropyl disulfide	96	4523-89-8	W382701-100G	Sigma-Aldrich
3-(Methylthio)-1-hexanol	97	51755-66-9	W343803-25G-K	Sigma-Aldrich

3.3.4. Preparation of standard solutions

3.3.4.1. Stock solutions of reductive sulfur compounds

Stock solutions of all thirteen reductive sulfur compounds, as listed in Table 3.2, except

methanethiol, were separately prepared by introducing 50 µL of a commercial standard by a SGE 100 µL gas tight syringe (*Part No.* 005250 100R-GT, Phenomenex NZ Ltd., New Zealand) into an amber screw top 20 mL vial (*Part No.* 5188-6537, Agilent Technologies, Germany) containing 10 µL absolute ethanol and pre-flushed with an inert gas, closed with a certified ultraclean 18 mm screwcap with septum (*Part No.* 5188-2759, Agilent Technologies). Weight differences together with purities were then used to calculate the final concentrations. These stock solutions were stored at -80 °C.

Methanethiol was prepared by bubbling methanethiol gas through 10 mL ethanol in a collecting vial, which was placed in a dry ice container. The methanethiol stock solution was stored at -20 °C. All stock solutions had concentrations in the range between 1 and 15 g/L.

3.3.4.2. Internal standard solution

Individual stock solutions of internal standards were also prepared in similar way as for the reductive sulfur stock solutions, described in Section 3.3.4.1.

A mixture of internal standards was then prepared from the individual solutions to have the concentration of $^2H_6$-dimethyl sulfide at 5 mg/L, isopropyl disulfide 0.4 mg/L and 3-(methylthio)-1-hexanol 10 mg/L. Their concentrations in the wine samples were 25 µg/L, 2 µg/L and 50 µg/L, respectively. The internal standard mixture was prepared shortly before use and stored at -20 °C for daily usage.

$^2H_6$-Dimethyl sulfide was used to quantify methanethiol, ethanethiol, dimethyl sulfide, diethyl sulfide, methyl thioacetate, S-ethyl thioacetate. Isopropyl disulfide was used for dimethyl disulfide, diethyldisulfide, carbon disulfide, dimethyl trisulfide. Other compounds including 2-mercaptoethanol, 2-(methylthio)-1-ethanol, 3-(methythio)-1-propanol and 4-(methylthio)-1-butanol were quantified using 3-(methylthio)-1-hexanol as the internal standard.

3.3.5. GC-MS system and chromatographic conditions

Analysis of the reductive sulfur compounds was carried out on an Agilent Technologies 7890 GC system coupled with a 5975C inert XL MSD (Agilent, USA). Separation was performed on a tandem column composed of a 30 m x 0.320 mm x 0.25 µm HP-1MS[25] and a 30 m x 0.320 mm x 0.25 µm HP-Innowax[26] fused silica capillary column (Agilent, J&W Scientific, New Zealand). The transfer line temperature was set at 250 °C. Helium was used as the carrier gas at an initial flow rate of 1.8 mL/min, held for 5 min, then lowered at a rate of 1.6 mL/min to 1.5 mL/min for the rest of the run.

Different conditions, including injection temperature and oven temperature programs, have been trialled to obtain good peak separation. The final conditions chosen employed the temperature of the injection port at 250 °C. The oven temperature was initially set at 42 °C for 5 min, then ramped at 1.5 °C/min to 60 °C and at 4 °C/min to 150 °C, held for 5 min. After that, it was ramped at 40 °C/min to 230 °C and remained at this temperature for 10 min, until a last ramp at 70 °C/min back to 42 °C towards the end of the run.

3.3.6. Peak identification

The ions used for the identification and quantification of each compound were chosen according to the literature and NIST library. The resulting retention times of analysed compounds and of internal standards were revealed and are presented in Table 3.3. Figure 3.4 and Figure 3.5 display the peak separations of the internal standards and the investigated reductive sulfur compounds, obtained from an injection of a standard solution into the GC-MS system, as described in Section 3.3.5.

[25] Non-polar column with stationary phase made of 100 % dimethylpolysiloxane

[26] High polarity column with stationary phase made of polyethylene glycol (PEG)

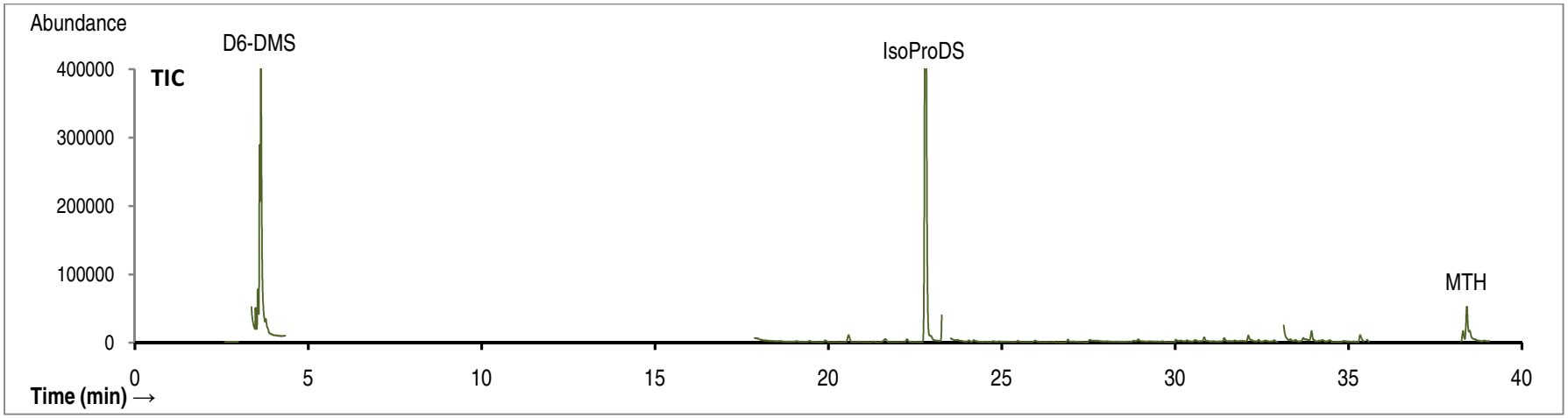


Figure 3.4. Total ion chromatogram (TIC) showing chromatographic peaks of the internal standards (D6-DMS, m/z 68; IsoProDS, m/z 150; MTH, m/z 148) obtained from an injection of a standard solution. The chromatographic conditions are described in Section 3.3.5.

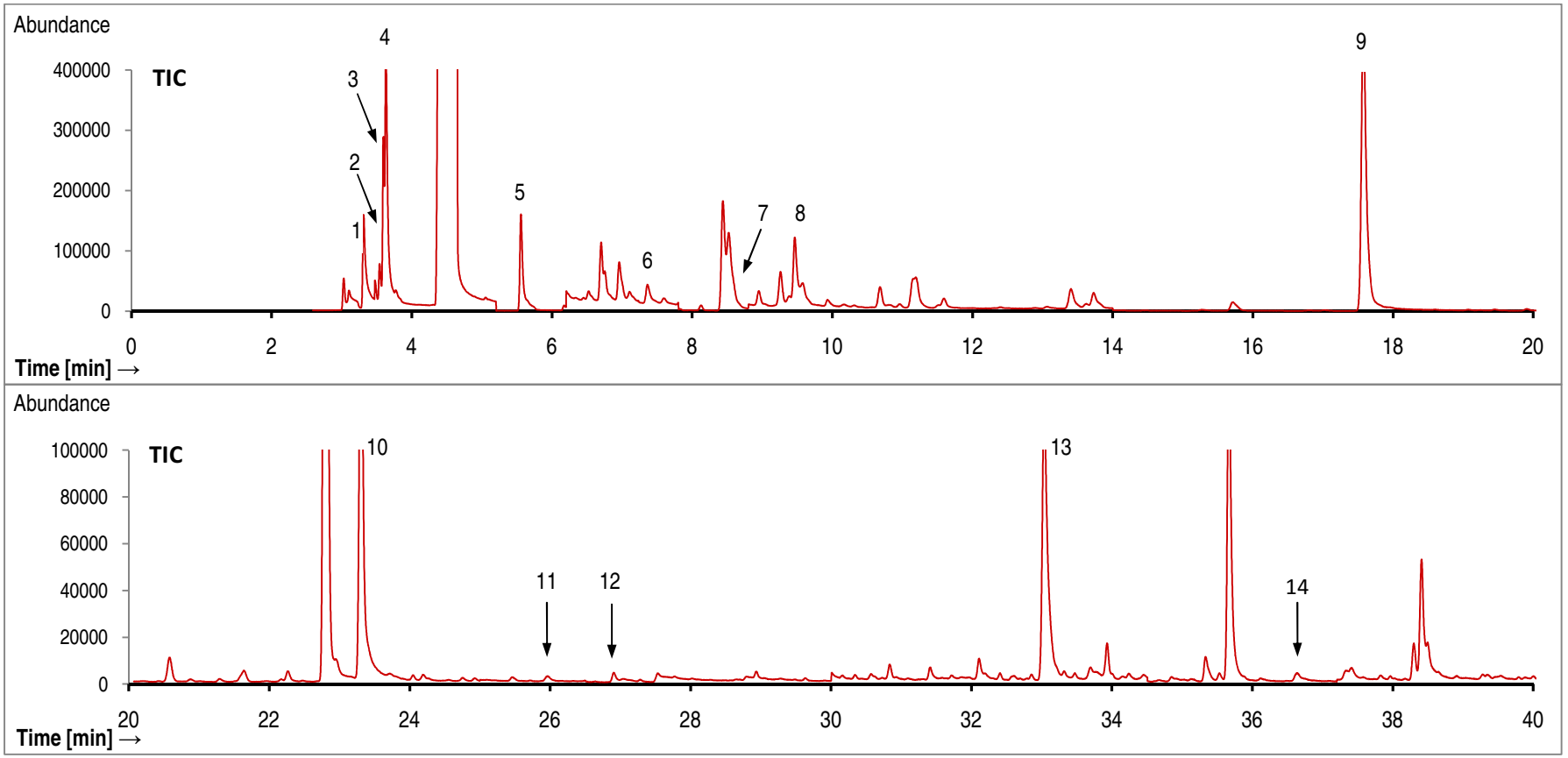


Figure 3.5. Total ion chromatogram (TIC) of a standard solution containing reductive sulfur compounds.

1- MeSH, m/z 47; 2- ETSH, m/z 62; 3- DMS, m/z62; 4- CS2, m/z 78; 5- DES, m/z 75; 6- MTA, m/z 90; 7- DMDS, m/z 79; 8- ETA, m/z 104; 9- DEDS, m/z 122; 10- DMTS, m/z 126; 11- ME, m/z 47; 12-MTE, m/z 92; 13- MTP, m/z 106; 14- MTB, m/z 120. The chromatographic conditions are described in Section 3.3.5.

Table 3.3. Retention times and ions used for the identification and quantification of reductive sulfur compounds

Chemicals and reagents	Abbreviations	Quantifier ion (Qualifier ions)	Retention time (min)
Analytes			
Methanethiol	MeSH	47 (45, 48)	3.3
Ethanethiol	EtSH	62 (34, 47)	3.53
Dimethyl sulfide	DMS	62 (47, 61)	3.61
Carbon disulfide	CS2	78 (44, 76)	3.62
Diethyl sulfide	DES	75 (61,62,90)	5.54
Methyl thioacetate	MTA	90 (43, 47, 75)	7.22
Dimethyl disulfide	DMDS	79 (61, 64, 94)	8.51
S-Ethyl thioacetate	ETA	104 (43, 60, 62)	9.45
Diethyl disulfide	DEDS	122 (66, 94)	17.56
Dimethyl trisulfide	DMTS	126 (64, 79)	23.29
2-Mercaptoethanol	ME	47 (60, 78)	25.96
2-(Methylthio)-1-ethanol	MTE	92 (47, 61)	26.82
3-(Methylthio)-1-propanol	MTP	106 (57, 58, 61)	33.02
4-(Methylthio)-1-butanol	MTB	120 (61, 87, 102)	36.62
Internal standards			
$^{2}H_{6}$-Dimethyl sulfide	D6-DMS	68 (66,50)	3.58
Isopropyl disulfide	IsoProDS	150 (108, 66)	22.78
3-(Methylthio)-1-hexanol	MTH	148 (61,75)	38.41

3.3.7. Optimisation of extraction conditions

3.3.7.1. Methods

Samples containing the same amounts of reductive sulfur compounds, prepared in a deodourised red wine, were extracted and injected onto the GC-MSD system. The chromatographic conditions are described in Section 3.3.5. A series of samples were analysed under the same extraction conditions, in which extraction time was kept unchanged while the extraction temperature was optimised, and vice versa.

3.3.7.2. Sample preparation

A 10 mL sample was put into a 20 mL amber headspace vial (*Part No.* 5188-6537, Agilent Technologies, Germany) that contained the required amount of magnesium sulfate salt (2.5 g $MgSO_4.7H_2O$) and a magnetic stirrer. The vial was then purged with argon gas and closed with a PTFE silicone cap (*Part No.* 5188-2759, Agilent, USA). After that, 50 µL of the internal standard solution was introduced by a SGE gas tight syringe (Phenomenex NZ Ltd., New Zealand). The vial was then placed on a Gerstel Multipurpose Sampler MPS tray before being transferred to a Gerstel Agitator/Stirrer for 5 min incubation before extraction.

3.3.7.3. Results and discussion

At room temperature, the concentration of semi-volatiles in the gaseous phase is small. The mass transfer rates are thus substantially lower, resulting in a longer extraction time using fiber coating, compared to direct extraction (Pawliszyn, 1997, p. 19). One of the options to shorten the extraction time is to increase the extraction temperature. An increased extraction temperature leads to greater diffusion coefficients and decreased distribution constants. An elevated temperature, therefore, can effectively dissociate analytes from the matrix and move them into the headspace. This leads to a faster

equilibration time for a more rapid extraction (Pawliszyn, 1997, p. 24). Temperature, therefore, is a very important parameter to optimise.

Figure 3.6 and Figure 3.7 present the chromatographic profile, based on peak areas, of the reductive sulfur compounds, extracted at five different temperatures from 30 °C to 55 °C. As can be seen from these graphs, increasing the temperature led to a decrease in the amounts of lighter sulfur compounds extracted onto the fiber. On the other hand, increasing the temperature up to 50 °C resulted in increased peak areas for the heavier compounds, including ME, MTE, MTB, MTP and MTH. In order to get larger amounts of heavier compounds onto the fiber, without too much decrease in the quantity of the lighter reductive sulfur compounds, 45 °C was chosen as a compromise temperature.

The optimisation of the extraction time was also based on a theory similar to the effect of increasing extraction temperature on the chromatographic profile of analytes. Similar results were found when samples were extracted for different periods of time, from 20 to 50 min (Figure 3.8 and Figure 3.9). The effect of extraction time on the chromatographic profile of the sulfur compounds was much less than the effect of temperature. As can be seen from the graphs, extraction time had very limited effects on the chromatographic profile of the reductive sulfur compounds. Prolonging the extraction time to 35 min gradually increased the peak areas of most compounds. Increasing the extraction time from 30 to 35 min did not increase the peak areas of the heavy sulfur compounds very much, but led to a decline in peak areas for some of the lighter compounds such as D6-DMS, CS2, and also DMDS and DEDS. Therefore, an extraction time of 30 min was selected, with a practical consideration being the desire to keep the extraction time reasonably short.

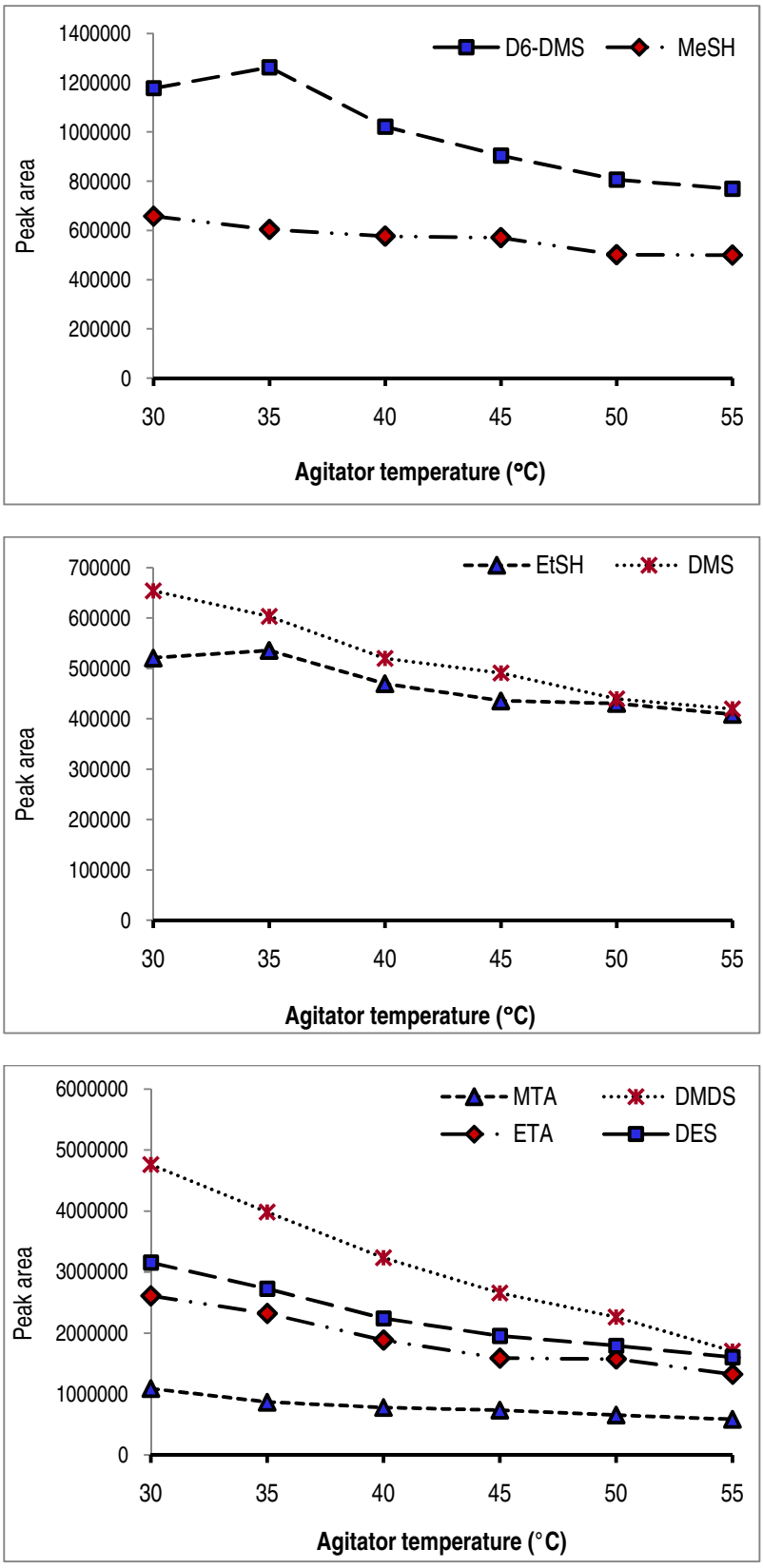


Figure 3.6. Effect of extraction temperature on the chromatographic profile of RSC

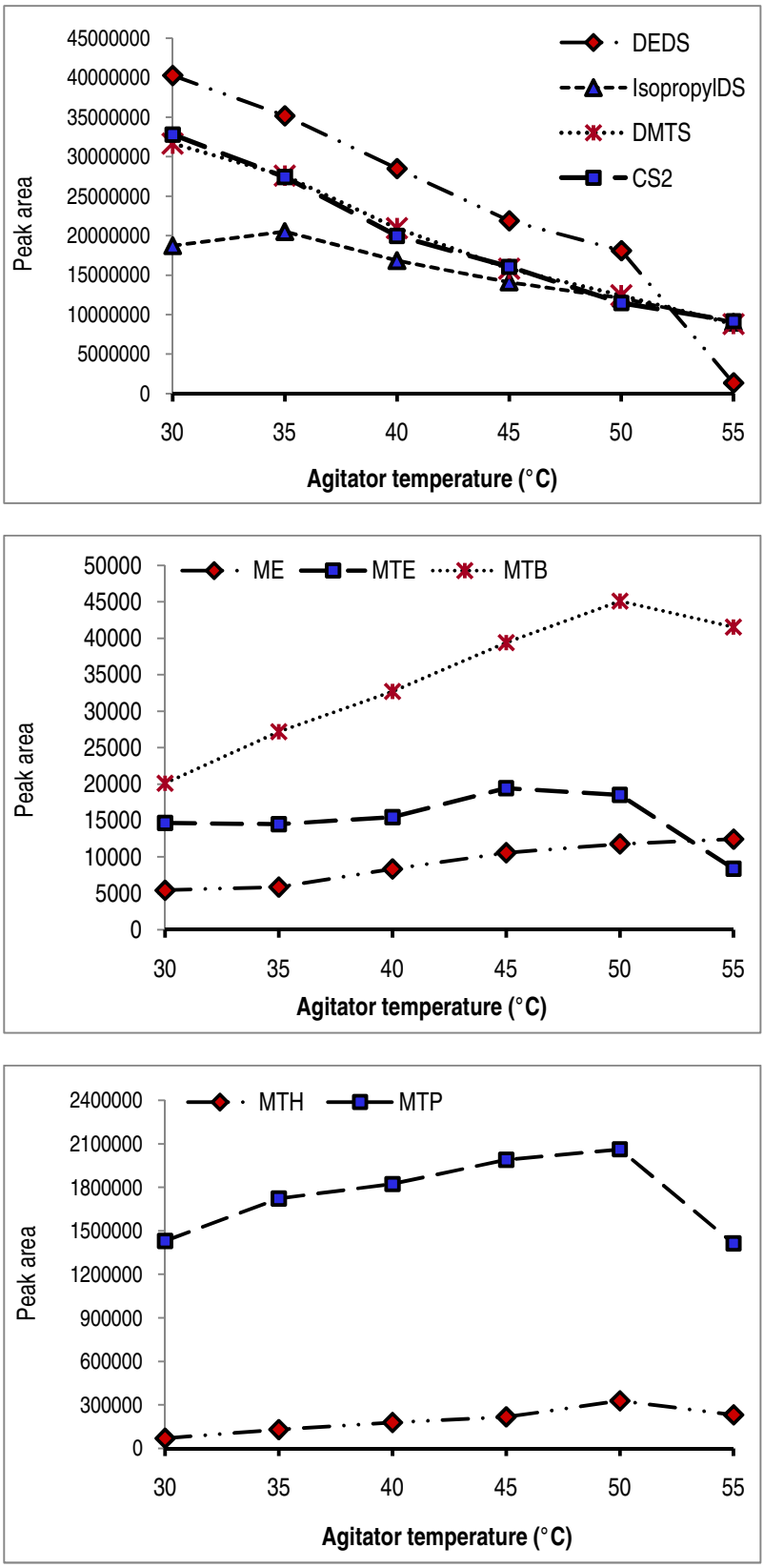


Figure 3.7. Effect of extraction temperature on the chromatographic profile of RSC (continued)

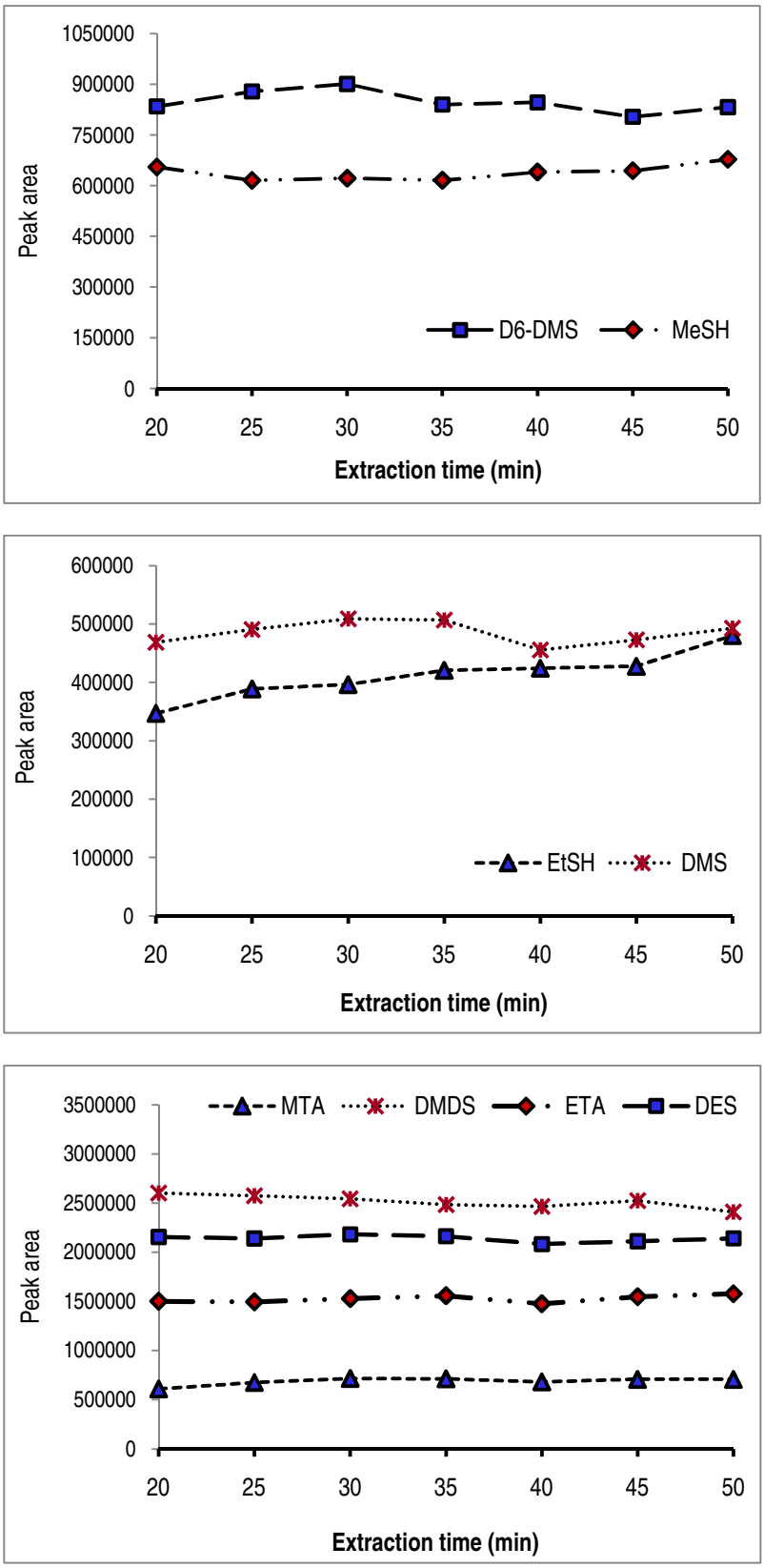


Figure 3.8. Effect of extraction time on the chromatographic profile of RSC

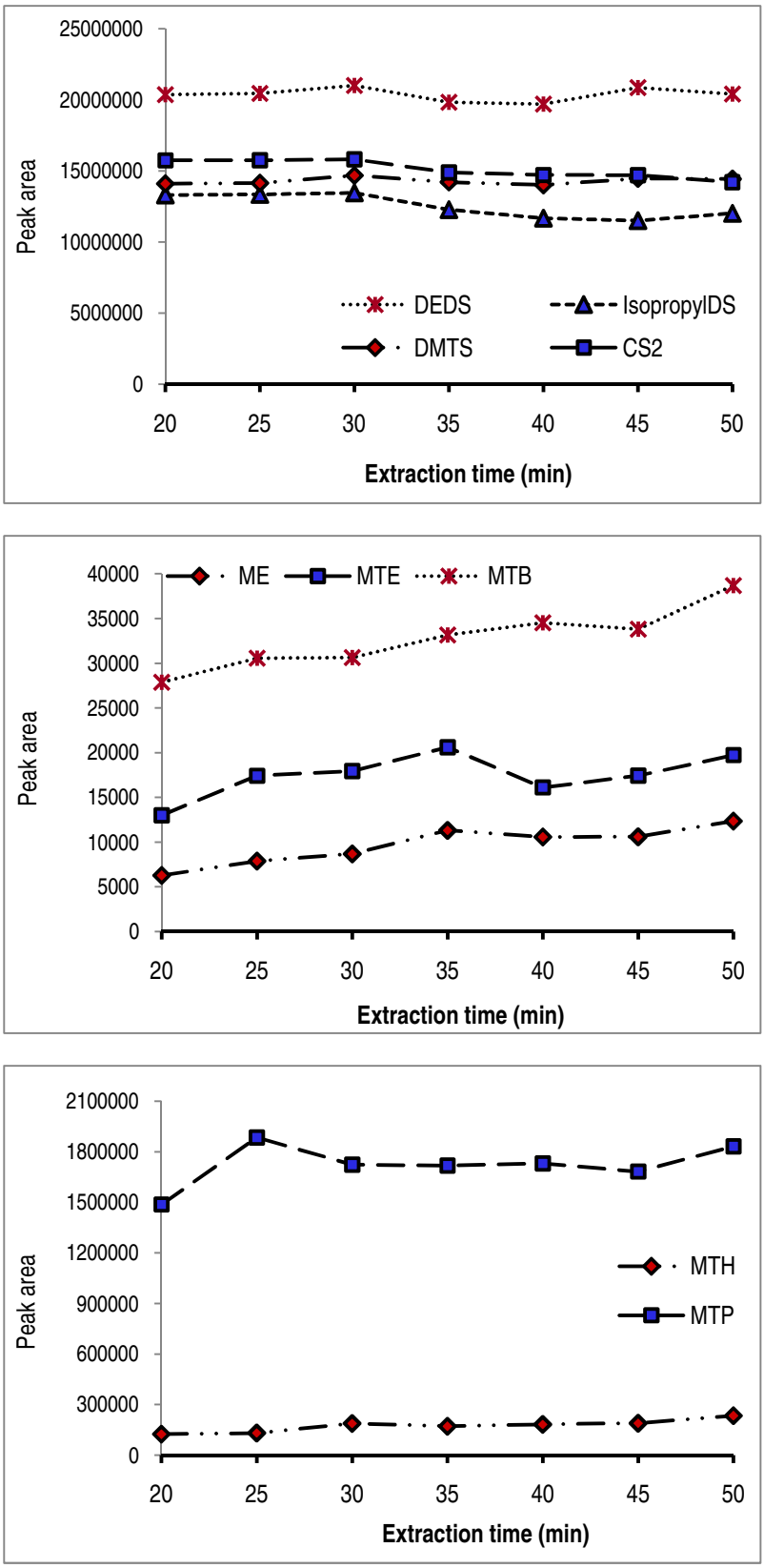


Figure 3.9. Effect of extraction time on the chromatographic profile of RSC (continued)

3.3.7.4. Selected sample preparation conditions

The optimised HS-SPME extraction conditions and other parameters finally selected for use in sample preparation prior to GC-MS analysis are given in Table 3.4.

Table 3.4. Selected sample preparation conditions for HS-SPME extraction of reductive sulfur compounds

Parameters	Conditions
Fiber coating	CAR-PDMS-DVB; 50/30 μm x 2 cm (Product No. 57928-U, Supelco)
Sample volume	10 mL
Salt addition	1M $MgSO_4.7H_2O$ (2.5 g in 10 mL sample)
Agitation speed	350 rpm (10 sec on, 3 sec off)
Incubation	
Incubation time	5 min
Incubation temperature	45 °C
Extraction	
Extraction time	30 min
Extraction temperature	45 °C

3.4. CALIBRATION AND VALIDATION OF THE GC-MS METHOD

3.4.1. Calibration

3.4.1.1. Preparation of deodourised wine

Deodourisation of wine was carried out in order to remove the volatile sulfur compounds detected by the method as much as possible along with the other main volatile

compounds, while keeping the other components of the wine to obtain a matrix effect similar to that of a wine sample.

A research red wine (Cabernet Sauvignon) was used to construct the calibration curves for the reductive sulfur compounds of interest. The wine was deodourised twice using a Büchi Rotavapor R (BÜCHI Labortechnik AG, Flawil, Switzerland) sourced from Watson Victor Ltd., Australia & NZ (**Figure 3.10**). An aliquot of the wine (200 mL) was put in an evaporation flask placed in a water bath and its temperature was kept at 30 °C. After the first evaporation, the wine was reconstituted with absolute ethanol and ultrapure water (Barnstead® NANOpure DIamond™ Water Purification System) and was evaporated for a second time. The wine was then reconstituted with absolute ethanol and ultrapure water to have 13.5 % v/v ethanol in the final reconstituted wine.

Figure 3.10. The setting up of a Büchi Rotavapor to deodourise wine

3.4.1.2. Preparation of reductive sulfur standard solutions for calibration curves

Stock mixture

In order to construct the calibration curves, a global stock mixture in absolute ethanol of all the reductive sulfur compounds of interest was prepared from the individual sulfur stock solutions already made up from commercial standards as detailed in Section 3.3.4.1. The volume of the individual stock solution needed was calculated so that the final concentrations of reductive sulfur compounds in the standard solutions fell in the concentration ranges for wines reported in the literature.

The global stock mixture of reductive sulfur compounds was prepared using a SGE gas tight syringe, by adding the required amounts of individual stock sulfur compounds into a 20 mL amber vial (*Part No.* 5188-6537) (Agilent Technologies, Santa Clara, CA, USA) containing the required volume of absolute ethanol. The vial had been previously purged with argon gas and closed with a certified ultraclean 18 mm screwcap with septum (*Part No.* 5188-2759, Agilent Technologies).

Standard solutions

After that, standard solutions used to build the calibration curves were prepared by adding a required volume of the global stock mixture into a 20 mL amber vial (*Part No.* 5188-6537, Agilent Technologies) containing reconstituted deodourised red wine (Section 3.4.1.1). The vial had been purged with argon gas and closed with a certified ultraclean 18 mm screwcap with septum (*Part No.* 5188-2759, Agilent Technologies) before introducing the global stock mixture of sulfur compounds through a SGE gas tight syringe to have a total sample volume of 10 mL. An aliquot of 50 µL internal standard solution (Section 3.3.4.2) was then placed in the vial before HS-SPME extraction and GC-MS analysis.

3.4.1.3. Data analysis

The regression analysis of calibration graphs for all analytes was performed using Microsoft Excel 2007 version to obtain the linear regression equations and the associated coefficients of determination (R^2).

3.4.2. Detection and quantification limits

3.4.2.1. Selection of a appropriate approach for the determination of detection and quantification limits

Detection and quantification capabilities are considered to be fundamental performance characteristics for a chemical measurement process (CMP). The importance of their objective measures have been well recognised (Currie, 1999). Many different models describing detection limits and quantification limits in the CMP are available in the literature (Kaus, 1998). The need for homogenous and international guidelines for the concepts of detection and quantification limits led to the publication in 1995 from the International Union of Pure and Applied Chemistry (IUPAC) entitled "Nomenclature in evaluation of analytical methods including detection and quantification capabilities" (Table 3.5) (Currie, 1995).

The detection and quantification capabilities of CMP derived from statistical theory of hypothesis testing and from the probability of two kinds of errors (type I and type II) were considered in the document of IUPAC. Type I error ("false positive") occurs when the signal for an analyte-free sample exceeds the critical value, leading to an incorrect conclusion that the sample contains a positive amount of the analyte (Currie, 1999). The error of the second type (Type II error or "false negative") occurs when a sample actually contains the analyte but the conclusion is made that it is not present. The probability of type I and II error is indicated as α and β, respectively. Their default values recommended by IUPAC are 0.05 each (Currie, 1999).

Table 3.5. International recommendations on analytical detection and quantification concepts and nomenclature

(Adapted from Currie, 1995)

Recommended terminology *(Alternates)*	Concepts	Definition
Critical Value (Lc) *(Critical Level)*	To distinguish a chemical signal against background noise	The probability of the estimated quantity ($\hat{L}$) which exceeds L_C is no greater than α if the analyte is absent (L = 0, null hypothesis)
Minimum Detectable (true) Value (L_D) *(Detection Limit)*	The measure of the inherent detection capability of a CMP	The true net signal for which the probability that the estimated value $\hat{L}$ does not exceed L_C is β
Minimum Quantifiable (true) Value (L_Q) *(Quantification Limit)*	The measure of the ability of a CMP to adequately quantify an analyte	The signal or analyte (true) value that will produce estimates having a specified relative standard deviation (RSD), commonly 10%

Table 3.6. Computational expression for L_C, L_D, L_Q

	L_C	L_D	L_Q
Paired observations	$2.33\sigma_B$	$4.65\sigma_B$	$14.1\sigma_B$
"Well known" blank	$1.645\sigma_0 = 1.645\sigma_B$	$3.29\sigma_0 = 3.29\sigma_B$	$10\sigma_0 = 10\sigma_B$

Note: *σ_0: standard deviation of the estimated quantity (net signal or concentration) under null hypothesis (true value = 0); σ_B: standard deviation of the blank.*

Taking into account the conventional assumption of a normal distribution of data, 5 % errors of the first and second kind ($\alpha = \beta = 0.05$), the uncertainty of the blank is

independent of the signal level ($\sigma_0 = \sigma_B$) and the relative standard deviation of the quantification at 10 % or better, recipes given by Currie (1999) for calculating critical, detection and quantification limits are summarised in **Table 3.6**.

The distribution and standard deviation of the blank (σ_B) are intrinsic to calculate the detection limit of any CMP (Currie, 1999). Precise determination of σ_B is a challenging task because the analyst has to distinguish between the noise that affects and does not affect the analyte peaks (Lee & Aizawa, 2003, p. 66). Under-calculation of σ_B may be due to the exclusion of matrix interferences (peaks eluting close to analyte) caused by integrating over a very narrow range (the width of the peak). Integrating over too wide a range may include non-interfering peaks in the value of σ_B. The determination of the value of σ_B, therefore is very subjective. It is the major drawback in using the IUPAC method for calculating detection limits in dynamic systems such as chromatography, even though it can be well applied to static systems (UV-Vis, AAS, ICP-MS, etc) (Lee & Aizawa, 2003, p. 66).

The Hubaux-Vos approach (Hubaux & Vos, 1970) is a method for determining detection limits, in which the probabilities for false positive and negative were also taken into account. This approach was employed by Fedrizzi *et al.* (2007) to determine detection and quantification capabilities in their method to analyse reductive sulfur compounds by HS-SPME coupled with GC-MS. The approach involves the generation of multiple calibration curves and factors in the variability in the slope and intercept of the calibration curve. Although these are important factors that must be considered in certain types of analyses, the method is complicated, very tedious and time consuming, and impractical for a complex matrix (Lee & Aizawa, 2003, p. 67&73).

Furthermore, both the IUPAC definition and the Hubaux-Vos approach do not take either variability in method efficiency or matrix effects into consideration (Lee & Aizawa, 2003, p. 73).

The IUPAC definition of detection limit (L_D) with additional recommendations, as also summarised in **Table 3.5**, can be read as '*The minimum detectable value of the net signal (or concentration) is that value for which the false negative error is β, given α. "α" is defined as the probability for a false positive ("analyte present" result when that is wrong) and "β" is defined as the probability of a false negative ("analyte absent" result when that is wrong)*' (Currie, 1995). Although this adds additional parameters to the IUPAC definition of detection limit, it does not solve the fundamental issue of variability in the calculated value, which depends on the analytical method used and the parameters included in calculating detection limit (Lee & Aizawa, 2003, pp. 59-75). The limit of detection (LOD), for most modern analytical methods, can be divided into two components, instrumental detection limit (IDL) and method detection limit (MDL). Each analytical instrument has a limitation in the amount of an analyte that they can detect. In addition, in complex matrices, interfering components cannot be completely eliminated so their effects must be taken into account when determining the LOD for an analyte-matrix combination. It is very important to have uniform definition for each of these terms because different detection limit definitions can result in value variation by up to an order of magnitude, which can lead to a meaningless value (Lee & Aizawa, 2003, pp. 59-75). For these reasons, proper definitions for some important terms were suggested by Lee *et al.* (2003) as follow:

1. IDL (Instrumental Detection Limit) is the smallest amount of an analyte than can be reliably detected or differentiated from the background of an instrument.
2. MDL (Method Detection Limit) is the smallest amount of an analyte that can be reliably detected or differentiated from the background for a particular matrix by a specific method. It should be applied to extraction and analysis methods developed for the analysis of specific analytes in a matrix.
3. MQL (Method Quantification Limit) can be defined as the smallest amount of an analyte that can be reliably quantified with a certain degree of reliability within a particular matrix by a specific method.

4. LLMV (Lowest Level of Method Validation) is the lowest concentration level expressed in terms of amount of analyte in the matrix, at which the method (extraction/analysis procedure) was validated or proven to be capable of reliably quantifying.

In the current method development, the two-step approach using $t_{99}\sigma_{LLMV}$ was employed to compute the limit of detection (LOD) and limit of quantification (LOQ) for the analytes of interest. This two-step approach using $t_{99}\sigma_{LLMV}$ is considered the best practice (Lee & Aizawa, 2003, p. 73) for calculating these values, taking into consideration several factors which affect the analyte signal, including instrumental noise, variability in instrumental sensitivity, variability in method efficiency, matrix effects and interference.

3.4.2.2. The two-step approach

The following part is directly adapted from Lee *et al.* (2003). The two step approach consists of two steps for the determination of the MDL and MQL. These include first the determination of the IDL and IQL and using these values to estimate the MDL and MQL, following by the calculation of the MDL and MQL for the extraction/analysis method.

Step 1- Determination of IDL and IQL following RMSE method

The Root Mean Square Error (RMSE) method, recommended by the US Environmental Protection Agency involves the generation of a calibration curve and calculating the RMSE. The steps involved are as follows:

1. Generate a 4-5 point calibration curve with standards of concentrations within an order of magnitude[27] of the estimated detection limit. The detection limit may be estimated as a concentration that would produce a signal three times the peak-to-

[27] The concentrations of these standards are within a factor of 10 of the estimated detection limits.

peak noise. The calibration curve should be generated by plotting the detector's response against concentration.

2. Perform a regression analysis on the calibration curve and calculate the values of the slope (m) and intercept (i) for a number of standards (n).

3. The calibration curve generated by plotting detector response (x) versus concentration (c) is:

$$x = m.c + i$$

4. Based on the values of slope (m) and intercept (i), calculate the predicted response (x_P) for each of the standards.

5. Calculate the error (E) associated with each measurement $|x_P-x|$.

6. Calculate the square of the errors for each standard and then calculate the sum of the square of the errors ($\sum E^2$) associated for the number of points (n).

7. After that, the RMSE is calculated as follows:

$$RMSE = [\sum E^2/n-2]^{½}$$

8. The predicted instrumental detection limit (IDL, c_L) is calculated as follows:

$$c_L=3.RMSE/m$$

9. The predicted instrumental quantification limit (IQL, c_Q) is calculated as below:

$$c_Q=10.RMSE/m$$

The detection and quantification limits determined here (c_L and c_Q) do not take the matrix interferences into account, because RMSE was determined from calibration standards. The value c_Q is used in the next step to spike the blank to compute the LOD and LOQ of the method, which incorporate instrumental variations. Consequently, both matrix/analytes and the extraction/analysis are taken into account for the determination of LOD and LOQ.

Step 2 – The $t_{99}\sigma_{LLMV}$ method to calculating the values of LOD and LOQ

⇨ Fortify the 'blank' with the analytes of interest (7 replicates) such that the concentration of the analytes in the matrix equals the estimated LOQ (eLOQ) as determined in the aforementioned step.

⇨ Extract and analyse these samples following the method used for sample analysis.

⇨ Determine the amount of each analyte in the fortified samples.

⇨ Calculate the standard deviation of these measurements (σ_{eLOQ}[28]).

⇨ Determine the 'one-tailed t-statistic' for n-1 observations at 99 % confidence level ($t_{99(n-1)}$). The $t_{99(n-1)}$ for 7 replicates (6 degrees of freedom) is 3.413.

⇨ The method detection limit or limit of detection (LOD) and method qualification limit or limit of quantification (LOQ) for the matrix/analytes combination and the extraction/analysis procedure is defined as:

$$LOD = t_{99(n-1)}.\ \sigma_{ELOQ} = 3.413\sigma_{eLOQ}$$

$$LOQ = 3.LOD$$

3.4.2.3. Detection and quantification limits

Table 3.7 summarises the parameters of the calibration graphs for all of the reductive sulfur analytes and the method detection and quantification limits for each compound. Linear regression analysis revealed that very good linearities (R^2 > 0.992) for the calibration graphs of all reductive sulfur compounds were obtained. The method also allowed good detection limits which were well below the sensory thresholds of the analysed sulfur compounds.

[28] According to the definition the lowest level of method validation (LLMV), the standard deviation of the concentration of the analyte in these fortified samples (σ_{eLOQ}) is the σ_{LLMV}, which explains the name of the method (the $t_{99}\sigma_{LLMV}$ method).

3.4.3. Recovery and repeatability

Two red wines and a white wine, before and after being deodorised and reconstituted with ethanol, were spiked with known amounts of sulfur compounds. The spiked and non-spiked wines were analysed in triplicate following the SPME extraction conditions, as summarised in Table 3.4 and GC-MSD separation and analysis conditions as in Section 3.3.5. Their concentrations were calculated by interpolation using the corresponding calibration curve. The recovery (R) (%) was estimated as:

$$R = [(C_s - C_0)*100]/C_a$$

where

C_s - Calculated concentration in spiked wine

C_o - Calculated concentration in the non-spiked wine

C_a - Concentration added

The repeatability of the method was also evaluated by calculating the relative standard deviation (% RSD) (n = 3) for each compound using the equation:

$$\%\ RSD = (STD*100)/Mean$$

where

% RSD - Relative standard deviation (%) of the mean

STD - Standard deviation of the mean

Mean - Mean value

Table 3.8 presents the calculated recoveries and the % RSD figures, which showed good precision of the method for all of the analysed sulfur compounds. The recovery values were close to 100 % and the % RSDs were less than 10 % for all compounds.

3.4.4. Reductive sulfur compounds in some New Zealand red and white wines

The HS-SPME coupled with GC-MS method was employed to analyse some New Zealand commercial wines, including five white wines and five red wines, produced from different vintages from 2004 to 2008. The old wines were chosen in order to see as many reductive sulfur compounds as possible using the method developed. High concentrations of these compounds in the older wines were also expected. The results obtained are dislayed in Table 3.9. As can be seen from this table, ten out of fourteen sulfur compounds that could be analysed using the established HS-SPME coupled with GC-MS method, in these wines. Some compounds were present at fairly high levels, compared to their perception thresholds. The concentration of methanethiol (MeSH) was found to be higher than its perception threshold (0.3 μg/L in alcoholic solution) in all of the analysed wines, while the concentrations of dimethyl sulfide were also relatively high compared to the reported threshold values which range from 10 - 160 μg/L. It was found that only small quantities of the other compounds, including disulfides, thioacetate and thioether alcohols, except 3-methylthio-1-propanol, were present in the examined wines. The levels of these compounds were below their respective sensory thresholds, as can be seen from Table 3.9. DMTS was only found in the red wines examined, and at concentrations well above its perception threshold (0.1 μg/L). Interestingly, the concentrations of 3-methylthio-1-propanol were generally higher in the red wines than that in the white wines, which followed the same trend as found in a report by Fang *et al.* (2005). Informal sensory evaluation by a group of Wine Science Post Graduate students (University of Aukcland, NZ) noticed that most of the white wines examined, except for the one Chardonnay (Chd1), exhibited quite strong reductive characters, especially those with higher concentrations of DMS. On the other hand, the reductive smells were only moderately noticeable in the Cabernet Sauvignon (CSav1) and the Merlot wines analysed. This was seen even though more reductive sulfur compounds were found in the red wines, and the concentrations of some compounds, such as 3-methylthio-1-propanol were

higher than those of the white wines, suggesting that the complex matrix of red wines could also play a role in the perception of the reductive notes.

Table 3.7. Parameters of calibration graphs and method detection and quantification limits

Compounds	Concentration range in calibration (µg/L)	Slope	Intercept	R^2	eLOQ (µg/L)	LOD (µg/L)	LOQ (µg/L)
MeSH	0.40-16.05	6.33	-0.799	0.993	0.37	0.16	0.49
EtSH	0.34-13.66	11.87	-1.015	0.994	0.28	0.12	0.36
DMS	0.80-32.13	26.09	0.228	0.993	0.14	0.14	0.42
CS2	0.44-17.67	110.7	0.321	0.994	0.14	0.08	0.23
DES	0.40-15.95	4.12	-0.139	0.995	0.25	0.03	0.10
MTA	0.85-34.01	32.94	0.079	0.998	0.39	0.15	0.44
DMDS	0.24-9.41	41.00	0.009	0.992	0.10	0.13	0.38
ETA	0.86-34.50	9.01	0.269	0.996	0.31	0.03	0.09
DEDS	0.39-15.54	4.61	0.210	0.996	0.12	0.06	0.19
DMTS	0.45-17.88	8.69	0.834	0.995	0.40	0.07	0.20
ME	11.86-237.11	2075	4.012	0.994	6.95	8.5	25.6
MTE	3.79-75.74	315.3	1.149	0.994	1.20	4.4	13.1
MTP	78.25-3130.12	172.4	-10.978	0.996	29.5	8.4	25.2
MTB	3.41-136.58	280.9	0.786	0.995	2.71	7.8	23.3

Table 3.8. Recovery (%) and repeatability (RSD) (%) (in brackets)

Compounds	Red wine 1	Red wine 2	White wine
MeSH	108.6 (5.0)	107.7 (2.6)	101.3 (0.7)
EtSH	94.7 (3.1)	96.6 (6.8)	104.8 (2.8)
DMS	97.2 (1.3)	95.5 (2.9)	90.9 (4.8)
CS2	98.9 (4.0)	104.8 (5.1)	105.9 (3.4)
DES	109.1 (3.5)	106.1 (0.39)	99.8 (2.1)
MTA	109.5 (4.8)	110.0 (2.5)	94.0 (0.10)
DMDS	96.3 (6.8)	97.4 (7.5)	94.6 (10.1)
ETA	99.6 (2.7)	109.7 (4.4)	91.7 (6.1)
DEDS	100.3 (5.0)	105.9 (5.3)	92.3 (7.1)
DMTS	105.5 (4.0)	94.5 (1.8)	90.4 (7.5)
ME	92.1 (8.8)	100.3 (13.8)	106.3 (0.10)
MTE	94.9 (4.4)	85.9 (6.2)	96.5 (1.3)
MTP	88.5 (6.7)	106.8 (6.0)	98.8 (1.9)
MTB	84.9 (4.3)	94.4 (9.3)	95.7 (0.85)

Table 3.9. Concentrations of reductive sulfur compounds (μg/L) ± standard deviations of the mean (n = 3) in some commercial New Zealand white and red wines

	MeSH	DMS	MTA	CS2	DMDS	DMTS	ME	MTE	MTP	MTB
Threshold (μg/L)	0.3	10-160	300	> 38	20 - 45	0.1	130	300	1200	100
SB1	3.17 ± 0.12	19.1 ± 0.9	12.76 ± 1.44	1.62 ± 0.06	1.19 ± 0.35	nd	nd	23.2 ± 0.7	282 ± 36	nd
SB2	4.40 ± 0.30	32.3 ± 0.6	12.34 ± 0.82	0.59 ± 0.07	1.06 ± 0.15	nd	nd	nd	237 ± 11	nd
Chd1	nd	9.1 ± 0.2	nd	0.94 ± 0.06	nd	nd	nd	26.0 ± 2.6	624 ± 50	nd
Chd2	2.20 ± 1.09	14.766 ± 0.01	5.42 ± 0.08	1.61 ± 0.01	nd	nd	nd	18.5 ± 1.4	230 ± 9	nd
Rslg	3.24 ± 0.02	39.3 ± 0.7	4.94 ± 1.06	0.71 ± 0.01	2.17 ± 0.14	nd	nd	nd	374 ± 33	nd
PN1	2.23 ± 0.19	36.3 ± 0.5	5.29 ± 0.29	1.48 ± 0.09	nd	nd	nd	26.7 ± 1.0	1064 ± 61	15.2 ± 4.0
PN2	4.13 ± 0.14	10.2 ± 0.05	8.16 ± 2.56	3.44 ± 0.14	5.71 ± 0.61	0.92 ± 0.06	nd	37.0 ± 3.2	625 ± 54	nd
CSav1	1.42 ± 0.01	54.4 ± 1.6	5.54 ± 0.61	7.18 ± 0.30	nd	0.87 ± 0.01	65.0 ± 9.5	27.8 ± 1.2	1119 ± 27	19.4 ± 4.0
CSav2	1.76 ± 0.15	11.5 ± 1.2	9.53 ± 0.36	2.67 ± 0.03	2.35 ± 0.30	0.90 ± 0.04	88.4 ± 5.2	32.4 ± 3.4	2350 ± 133	nd
Mer	4.85 ± 0.16	27.0 ± 0.4	5.97 ± 0.51	1.32 ± 0.03	2.28 ± 0.55	0.88 ± 0.03	43.6 ± 1.0	38.3 ± 2.5	1213 ± 8	nd

*** *Abbreviations:*** SB = Sauvignon blanc; Chd = Chardonnay; Rslg = Riesling; PN = Pinot noir; CSav = Cabernet Sauvignon; Mer = Merlot; nd = not detected

CHAPTER 4. MICRO-OXYGENATION AFTER ALCOHOLIC FERMENTATION[29]

4.1. INTRODUCTION

MOX is generally applied either pre-malolactic fermentation (MLF), or post-MLF and the addition of extra sulfur dioxide, but is generally not applied during MLF. However, the consequences of applying MOX during MLF have not been reported previously. One reason given for stopping the MOX application once MLF starts is because lactic acid bacteria can consume the acetaldehyde produced from the autoxidation of dihydroxyphenols (Cano-López *et al.*, 2006), making it less available for reactions among wine polyphenols and the formation of stable polymeric pigments.

[29] **Results from this chapter have been published as follows (Appendix 4-1):** Nguyen D-D., Nicolau L., Dykes S.I., Kilmartin P.A. (2010). Influence of Microoxygenation on Reductive Sulfur Off-Odors and Color Development in a Cabernet Sauvignon Wine. *American Journal of Enology and Viticulture, 61*(4), 457-464.

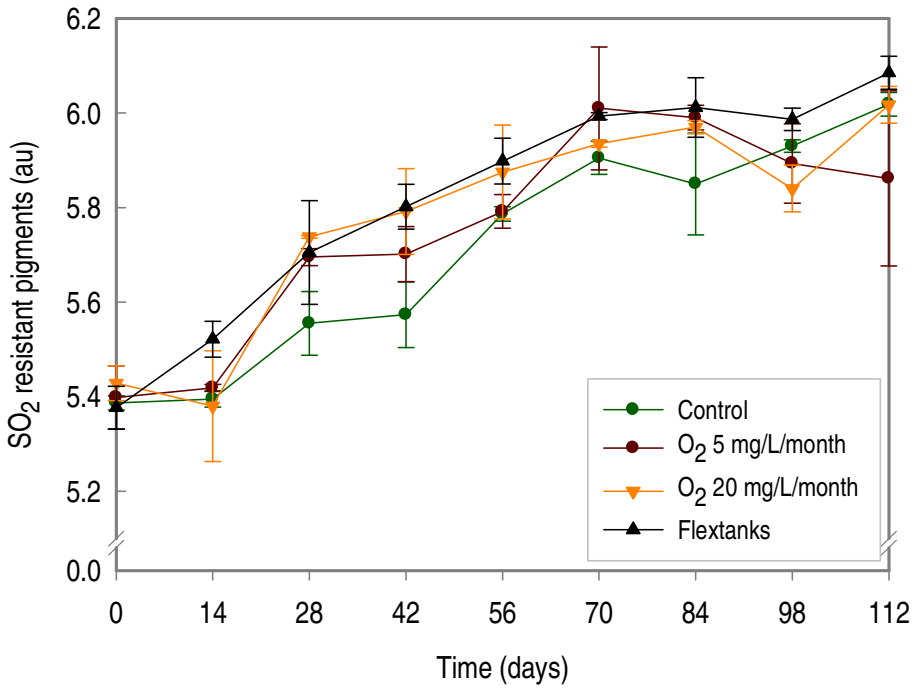
Apart from the volatile sulfur containing compounds responsible for characteristic varietal aromas, most volatile sulfur containing substances found in wine produce unpleasant odours which impart organoleptic defects in wine even at very low concentrations, including the well-known rubbery or reductive character (Beloqui & Bertrand, 1995; Mestres *et al.*, 2000). These compounds can be arbitrarily classified according to their volatility into two categories, the more volatile (light) and less volatile (heavy) sulfur compounds, with boiling points below and above 90 °C respectively, which is the boiling point of 3-methylthiopropanol (Beloqui & Bertrand, 1995; Karagiannis & Lanaridis, 1999; Mestres *et al.*, 2002). Although MOX is credited with being able to oxidise and eliminate reductive sulfur containing volatiles, quantitative studies regarding the concentrations of sulfur compounds in micro-oxygenated wines are very limited, with a study by McCord (2003) being one example. A recent investigation by Ortega-Heras *et al.* (2008) mentioned the application of a higher oxygen dose for a brief period of time to eliminate the reductive notes which sometimes appear just after alcoholic fermentation. However, no particular odouriferous volatiles were quantified in this report.

In this part of the project, an investigation was carried out on the effects of MOX applied after alcoholic fermentation in a fully replicated trial with an analysis of the reductive sulfur containing volatiles often found in wine, alongside changes in colour properties, polyphenols, and in the thiol 3-mercaptohexan-1-ol (3-MH), which is thought to contribute to the fruity aromas of white wines such as Sauvignon blanc and to red wines such as Cabernet Sauvignon and Merlot (Blanchard *et al.*, 2004). The application of MOX was also continued during MLF, which occurred spontaneously at 42 days after the MOX treatments commenced. The total oxygen dose applied to wines varies, ranging from 60 mL/L for lighter white wines up to 600 mL/L for tannic reds (Lesica & Kosmerl, 2009). Meanwhile, it is considered that a wine can adequately consume up to 50 mL/L without acquiring underisable oxidation (Perez-Magarino *et al.*, 2007). In our trial, the highest oxygen rate treated wine received an addition of 20 mg/L/month, which equals to a total oxygen of 56 mg/L (~39 mL/L), thus a lower level than the mentioned tolerable oxygen limit.

4.2. RESULTS AND DISCUSSION

4.2.1. Development of colour

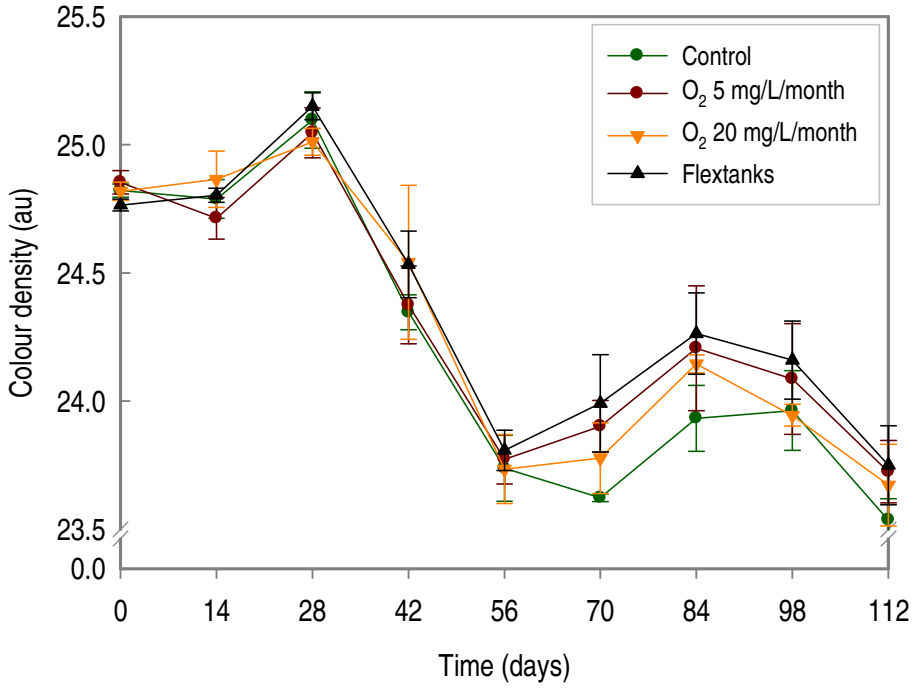
The development of colour properties in the control and treated wines (two micro-oxygenated and one in Flextank) over the trial period is shown in Figure 4.1 and Figure 4.2.



Time (days)	0	14	28	42	56	70	84	98	112
Control	5.39	5.40	5.56	5.57	5.79	5.91	5.85	5.93	6.02
O_2 5 mL/L/month	5.40	5.42	5.70	5.70	5.79	6.01	5.99	5.89	5.86
O_2 20 mL/L/month	5.43	5.38	5.74	5.79	5.86	5.82	5.87	5.73	5.90
Flexi tanks	5.38	5.52	5.71	5.80	5.90	5.99	6.01	5.99	6.09

Figure 4.1. Development of pigments resistant to sulfur dioxide bleaching in a Cabernet Sauvignon during MOX applied after AF.

Data points represent the mean values ± standard deviations (error bars) of triplicate treatments.



Time (days)	0	14	28	42	56	70	84	98	112
No oxygen	24.82	24.79	25.10	24.35	23.74	23.62	23.93	23.96	23.54
O_2 5 mL/L/month	24.85	24.71	25.05	24.38	23.77	23.90	24.21	24.09	23.73
O_2 20 mL/L/month	24.82	24.87	25.01	24.54	23.66	23.37	23.46	23.09	22.86
Flexi tanks	24.77	24.80	25.15	24.53	23.81	23.99	24.26	24.16	23.75

Figure 4.2. Development of colour density in a Cabernet Sauvignon during MOX applied after AF. Data points represent the mean values ± standard deviations (error bars) of triplicate treatments.

A significant increase up to day 42 in the pigments resistant to sulfur dioxide bleaching ($A_{520}^{SO_2}$) was observed in all treated wines, compared with the control (**Figure 4.1**). The differences in SO_2-resistant pigments did not continue after that point, and confounding effects from the occurrence of MLF could have affected the colour development. The scatter around the temporal trend seems larger than the differences between treatments, suggesting that the MLF fermentation of the wines may occur at different rates in different tanks. On the other hand, the positive effects of MOX on the wine colour density

(A_{420} + A_{520} + A_{620}) were more obvious in the second half of the trial period, in which the treated wines showed higher colour density values than the control wine (**Figure 4.2**). Interestingly, the colour density of wine stored in Flextanks was significantly higher ($P < 0.05$) than that of the control at every observation date after day 56. The increase in the SO_2-resistant pigments, and thus an expected improvement in colour stability, can be attributed to oxidative conditions favouring reactions of monomeric anthocyanins and other wine polyphenols to form coloured pigments. Of those compounds that could contribute to the improved colour characteristics are the pyranoanthocyanins (having higher A_{420} values than the anthocyanins), and ethyl-bridged anthocyanin-tannin adducts (with higher absorbance values at 420 nm and 620 nm relative to the 520 nm absorbance) (Bakker & Timberlake, 1997; Atanasova *et al.*, 2002a), as discussed in detail in Section **1.3.2**. The results found were in agreement with available literature reports which have demonstrated the favourable effects of oxygen on stabilising wine colour (Castellari *et al.*, 2000; Atanasova *et al.*, 2002a; Cano-López *et al.*, 2006; Cano-Lopez *et al.*, 2007). Although it is not recommended to apply MOX during MLF due to the consumption of acetaldehyde by the bacteria, our findings showed that MOX applied simultaneously with MLF could still provide positive impacts on wine colour, as observed in the colour density. In addition, acetaldehyde formation from the metabolism of some lactic acid bacteria has been demonstrated (Osborne *et al.*, 2000). While oxygen may still favour the release of acetaldehyde and its participation in wine colour stabilisation processes during MLF, the differences in colour properties between the treatment wines and the control wine were not as large as those seen in several recent MOX research reports (Cano-López *et al.*, 2006; Fell *et al.*, 2007).

4.2.2. Effects of MOX on 3-mercaptohexan-1-ol

The possible contribution of 3-mercaptohexan-1-ol (3-MH) to the aroma of red Bordeaux wines and to the fruity and citrus notes of rosé wines from Cabernet Sauvignon and Merlot has been reported (Ferreira *et al.*, 2002; Blanchard *et al.*, 2004). It was

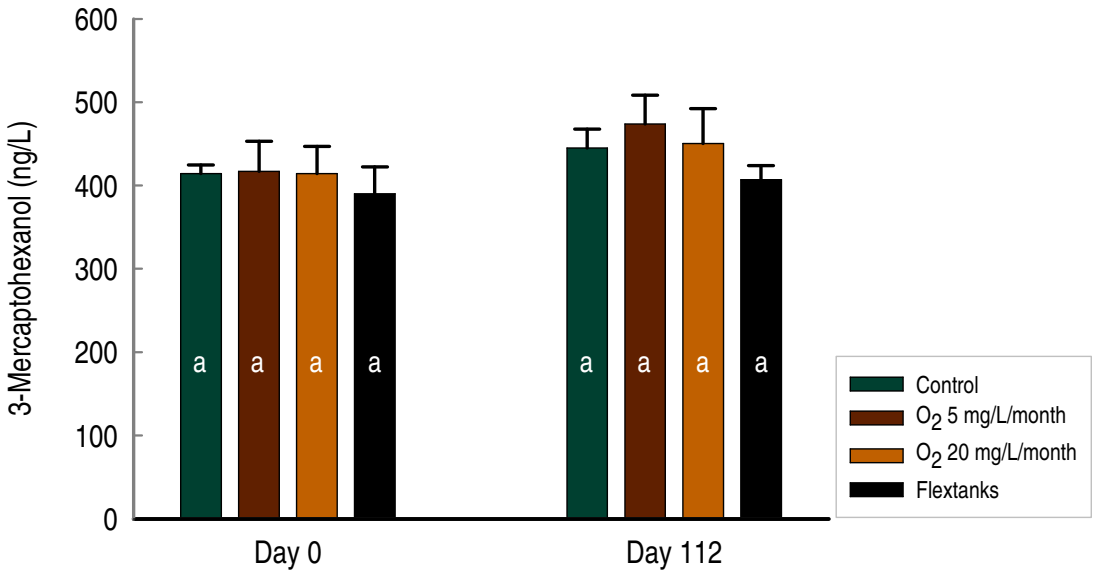


Figure 4.3. Effects of MOX applied after AF on 3-mercaptohexan-1-ol in a Cabernet Sauvignon.

Error bars represent the standard deviations of the mean (n = 3).

Columns sharing the same letters are not significantly different (P > 0.05).

demonstrated in the study of Blanchard *et al.* (2004) that oxygen present during handling operations such as racking, or ageing in oak barrels, could lower the concentration of 3-MH in red wine. During the present study, the MOX treatments and storage in Flextanks did not affect the concentration of 3-MH (**Figure 4.3**), but rather the concentration in the control and the three treated wines at day 0 and at the end of the trial period (day 112) were not significantly different (P > 0.05). In a model wine study, Blanchard *et al.* (2004) found that 3-MH concentration decreased in the presence of oxygen. Interestingly, the disappearance of 3-MH was more rapidly when catechin was also dissolved, yet 3-MH decrease was attenuated by adding sulfur dioxide (Blanchard *et al.*, 2004). The decrease of 3-MH concentration is expected to be associated with the breakdown on anthocyanins due to the antioxidant properties of anthocyanins and the highly oxidisable nature of thiols. However, it was demonstrated that the anthocyanin monoglucosides, e.g. malvidin-3-glucoside, produced a protective effect on 3-MH in a model wine medium (Murat *et al.*, 2003; Blanchard *et al.*, 2004). In the current study, although no sulfur dioxide was

present and the concentration of total monomeric anthocyanins were similar in all wines at the end of the MOX trial period (**Table 4.1**), the concentration of 3-MH in all wine were not significantly different ($P > 0.05$), while methanethiol (MeSH) were significantly lower ($P < 0.05$) in the oxygen treated wines than in the control (**Figure 4.4.i**), suggesting stoichiometric effect could be the reason explaining these observations.

4.2.3. Effects of MOX on reductive sulfur containing volatiles

Seven reductive sulfur compounds, out of fourteen that could be analysed using the GC-MS procedure, were found in the Cabernet Sauvignon wine, and their concentrations were monitored at the beginning and the end of the trial. The "highly volatile" sulfur compounds (boiling point less than 90 °C) that were found to be present were methanethiol (MeSH), and dimethyl sulfide (DMS). The wine also contained five "low volatile" compounds (boiling point above 90 °C), including dimethyl disulfide (DMDS), methyl thioacetate (MTA), S-ethyl thioacetate (ETA), 2-(methylthio)-1-ethanol (MTE) and 3-(methylthio)-1-propanol (MTP) (methionol).

4.2.3.1. Methanethiol, dimethyl sulfide, and dimethyl disulfide.

Methanethiol (MeSH) is one of the simplest sulfur compounds and plays a decisive role in reduction defects related to wine aromas (Ribéreau-Gayon *et al.*, 2006b). The perception threshold of methanethiol in a hydroalcoholic solution is 0.3 µg/L (ppb) and the compound can generate an odour reminiscent of cooked cabbage (Mestres *et al.*, 2000). The MeSH concentration after alcoholic fermentation is generally below 1 ppb but it can be greater than 10 ppb in a wine with reductive characters (Landaud *et al.*, 2008). In the current study, the concentration of MeSH in all of the wines was low and the wines were not overly reductive as evaluated by winemakers, although the concentrations were well above the perception threshold for MeSH. Our results indicated that at the end of the trial period, MOX did show some impact on the concentration of MeSH in the wine that received a higher oxygen dosage (20 mg/L/month) and in the wine stored in Flextanks, in

which the concentration of MeSH was significantly lower than those of the control and the wine treated with the lower oxygen rate (5 mg/L/month) (**Figure 4.4.i**). Our findings are in agreement with the study by McCord (2003) who reported a significant decrease in the concentration of MeSH and EtSH in a wine that underwent MOX. No biological activity was recorded in the wine used in the McCord study. However, spontaneous MLF did occur in the control and all treated wines during this MOX trial. Although the formation of methionine-derived reductive sulfur compounds from the metabolism of the lactic acid bacterium (LAB) *Oenococcus oeni* during MLF is still poorly understood, it was shown in a recent study that MLF may result in the formation of MeSH and DMDS (Vallet *et al.*, 2008). In addition, thiols such as MeSH are nucleophilic compounds very susceptible to oxidation, which can readily react with different electrophilic species such as *o*-quinones and the carbocation form of procyanidin molecules, formed from the oxidation of wine polyphenols (Majcenovic *et al.*, 2002; Ribéreau-Gayon *et al.*, 2006b). MeSH can also be oxidised to form DMDS and dimethyl trisulfide (DMTS) (Rauhut, 1993). The concentration of DMDS, which has a perception threshold of 20 - 45 μg/L in wine with cooked-cabbage and onion like odours (Mestres *et al.*, 2000), however, was significantly lower ($P < 0.05$) in the wine treated with 20 mg O_2/L/month and in the Flextank wines (**Figure 4.4.ii**), and DMTS was not detected in all of the wines.

MOX did not affect the concentration of DMS, but the Flextank wine had a lower DMS concentration than the control (**Figure 4.4.iii**). In McCord's study (2003) on the effect of toasted oak products with and without MOX on the ageing of a Cabernet Sauvignon wine, where no biological process occurred, the concentration of DMS was found to decrease in all treatments with added toasted oak, while no such decrease was found in the micro-oxygenated counterparts. DMS can produce off-odours described as cooked cabbage and shrimp-like at concentrations above its perception threshold (10 - 160 μg/L in wine), whereas at low levels it produces odours reminiscent of asparagus, corn and molasses (Mestres *et al.*, 2000; Jackson, 2008). On the contrary, it is thought to make a positive influence to the bouquet of some bottled wines when present at low levels (Spedding & Raut, 1982). The formation of DMS in wine has not yet been fully elucidated. Some

researchers consider that cysteine, cystine and glutathione can be precursors of DMS because yeasts cannot produce S-methyl methionine (SMM) (Landaud *et al.*, 2008). On the other hand, it has been suggested that yeast may produce SMM, which subsequently

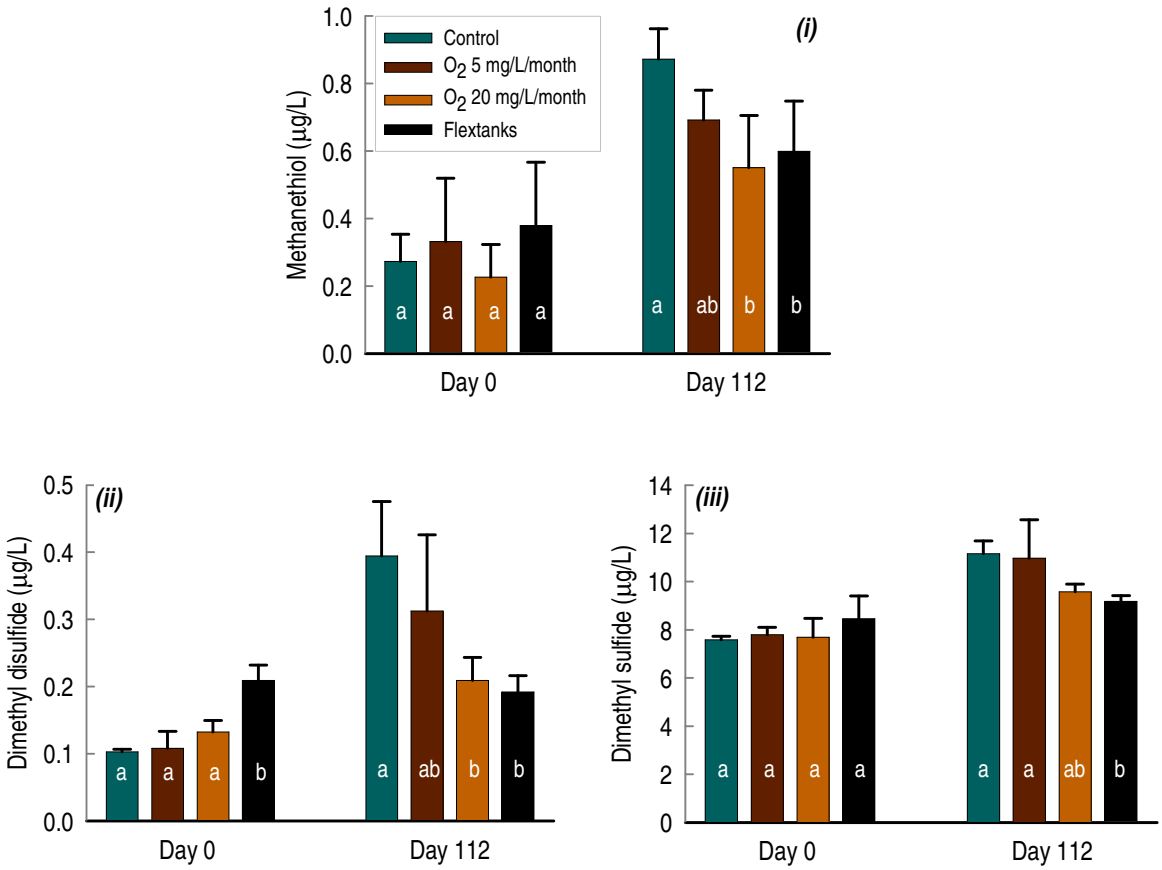


Figure 4.4. Effects of MOX applied after AF on (i) methanethiol, (ii) dimethyl disulfide and (iii) dimethyl sulfide in a Cabernet Sauvignon wine. Error bars represent the standard deviations of the mean (n = 3). Columns with different letters denote values which significantly differ ($P < 0.05$, LSD and Tukey Post Hoc Tests) at the same observation date.

can be enzymatically hydrolysed to release DMS and homoserine (Rauhut, 1993). Metabolism of LAB, e.g. *Oenococcus oeni*, during MLF, also produces diverse sulfur containing volatiles through methionine catabolism (Pripis-Nicolau *et al.*, 2004). Although

trace amounts of DMS was found in the basal medium inoculated with LAB strains, its formation however did not correlate with methionine metabolism (Pripis-Nicolau *et al.,* 2004). During wine ageing, the concentration of DMS can increase significantly, indicating that a chemical pathway could also be involved in its production (Landaud *et al.*, 2008). Reduction of dimethyl sulfoxide (DMSO) (odourless), present at a concentration up to 1230 µg/L in a survey of New Zealand wines, has been proposed as a DMS precursor during wine aging (de Mora *et al.*, 1993). However, it was found in a model ageing study of wine that DMSO is only a minor precursor of DMS, yet SMM appeared to be a good source of DMS during wine ageing (Segurel *et al.*, 2005). Loscos et al. (2008), for the first time, has isolated SMM from grapes and proved that it is a major DMS precursor in the grape. In the current MOX study, MLF started spontaneously after day 42, so either chemical or biochemical pathways could be sources of DMS, which could explain the increase in concentration values at day 112 compared to day 0 in all of the wines. This increase, however, was lower in the high oxygen treated wine and significantly lower in the Flextank wine, suggesting a limiting effect of oxygen on the enzymatic biosynthesis of DMS from the precursor SMM during the course of the trial. In this case, the LAB that predominated in the research wine during spontaneous MLF might have been a facultative anaerobiosis species, such as *Oenococcus oeni* and *Lactobacillus*, which are better adapted to the absence of oxygen (Ribéreau-Gayon *et al.*, 2006a), and thus enhanced the enzymatic hydrolysis of SMM in the control and the low oxygen treated wine.

4.2.3.2. S-thioesters

The S-methyl and S-ethyl esters of ethanethioic acid, methyl and ethyl thioacetates (MTA and ETA) were also found and monitored during the MOX trial. These compounds were identified and determined for the first time in beer and wine by Leppanen *et al.* (1980). Despite their high sensory thresholds, the thioacetates are a potential source of off-odours because they can be hydrolysed to give free thiols at low pH (Leppanen *et al.*, 1980), although they can produce rotten vegetable smells on their own (Landaud *et al.*,

2008). The formation of these esters could be due to the wine yeast metabolism of highly volatile MeSH via acetyl coenzyme A (acetyl-CoA) (Landaud *et al.*, 2008). In the present study, alcoholic fermentation had finished before oxygen was applied, so hydrolysis should have led to declines in MTA and ETA concentrations. In fact, the concentrations of both MTA and ETA in all wines did not show any difference among the treatments and the control (**Figure 4.5**). This indicates that oxygen did not influence this hydrolysis during the trial period, even though the ETA concentrations were different in the wines to start with and the spontaneous MLF may have contributed to an increase in ETA concentration in the control and the low oxygen treated wine, leading to similar levels of this compound in all wines at the end of the trial. During MLF, the metabolism of LAB can also transform sugars, organic acids or amino acids into aromatic compounds, including MeSH. The availability of MeSH, therefore, could lead to the formation of MTA through an enzymatic reaction between MeSH and acetyl-CoA. This mechanism has also been found to occur in beer and yeast metabolism (Landaud *et al.,* 2008). However, whether such an enzyme catalysed reaction occurs in the metabolism of wine LAB has not yet been established.

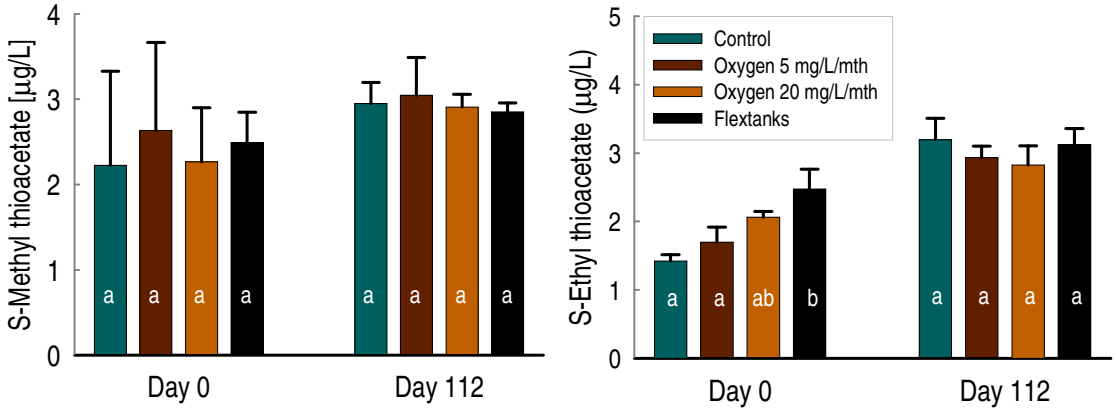


Figure 4.5. Effect of MOX applied after AF on the S-thioesters in a Cabernet Sauvignon wine. Error bars represent the standard deviations of the mean (n = 3). Columns with different letters denote values which significantly differ ($P < 0.05$, LSD and Tukey Post Hoc Tests) at the same observation date.

4.2.3.3. Thioether alcohols

Two other low volatile sulfur compounds included in this study were 2-methylthio-1-ethanol (MTE) and 3-methylthio-1-propanol or methionol (MTP). MTP is considered to be the most important heavy sulfur compound involved with reduction defects brought about by yeast activity (Ribéreau-Gayon *et al.*, 2006b). MTP can generate odours reminiscent of cauliflower and cabbage at a concentration above its perception threshold (1200 µg/L). MTE has a perception threshold of 250 µg/L in hydroalcoholic solution and has a French bean-like odour (Mestres *et al.*, 2000). The concentration of MTP in wines with reduction notes, due to the heavy sulfur compounds that develop during alcoholic fermentation, is typically found at levels above its perception threshold, while the concentration of MTE in wines with reductive characters may be very close to its perception threshold (Ribéreau-Gayon *et al.*, 2006b).

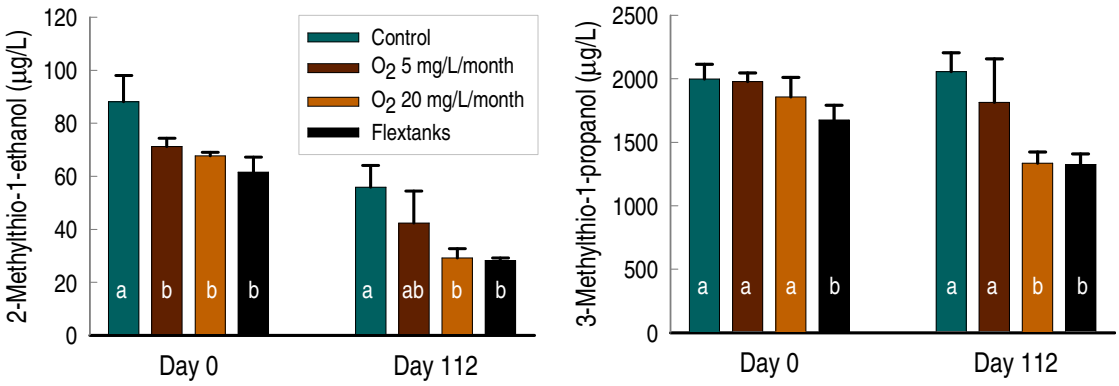


Figure 4.6. Effect of MOX applied after AF on 2-methylthio-1-ethanol and 3-methylthio-1-propanol.
Error bars represent the standard deviations of the mean (n = 3).
Columns with different letters denote values which significantly differ
($P < 0.05$, LSD and Tukey Post Hoc Tests) at the same observation date.

As can be seen from **Figure 4.6**, the concentration of MTE tended to decrease over time, with a greater drop in the high oxygen treated and Flextank wines shown at the end of the

trial. However, possibly because of some variations caused during pumping the wine from the bulk storage tanks to the 300 L tanks, the MTE concentration were different at day 0; so the question whether oxygen could have affected this compound during the trial is therefore undecided. Literature on the formation and evolution of MTE during winemaking is not well documented. On the other hand, the metabolic formation of MTP in wine, involving the deamination of methionine produced by yeast, decarboxylation, then reduction of methional to methionol (MTP) via the *Ehrlich* pathway, is well known. Because the alcoholic fermentation had finished, no additional MTP formation was expected during the course of the trial. Instead, the higher oxygen treated wine and the Flextank wine showed a significant drop in the concentration of MTP by the end of the trial (Figure 4.6). The loss of MTP could be due to direct oxidation of MTP to form methional. Some authors have demonstrated a decrease of methionol in the presence of oxygen along with methional formation (Escudero *et al.*, 2000). However, methional may also be formed from methionine via the Strecker mechanism in the presence of a dicarbonyl compound (e.g. methylglyoxal). This pathway was shown to be the main source of methional in white wines treated with oxygen (Silva Ferreira *et al.*, 2002). Silva Ferreira *et al.* (2003) in a further study did not find that methional was present in a Port wine treated with oxygen, and suggested that the decrease in MTP would lead to the formation of other compounds as yet unidentified.

4.2.4. Effects of MOX on tannins and polyphenols

Application of limited oxygen into wine can result in more coloured and improved wine palate structure. This involves many wine components, among which polyphenols are the main substrates/ key components for the oxidative reactions that transform the wine. Therefore, monitoring changes in tannin concentration, quantified using MCP Tannin Assay as detailed in Section 2.2.1 and individual polyphenols, analysed using HPLC described in Section 2.2.4, were included to provide more insight into the effects of MOX on the chemical profile of the wine.

Exposure of wine to a small amount of oxygen favours the interactions between anthocyanins and other polyphenols, especially flavan-3-ols, leading to the formation of pyranoanthocyanins and ethyl-linked compounds, by the cycloaddition of anthocyanins with acetaldehyde or indirect condensation of anthocyanins and flavanols mediated by acetaldehyde, as discussed in Section 1.3.2. In the indirect condensation mechanism, the carbocations formed from the nucleophilic addition of a flavanol to a protonated acetaldehyde may react with other flavanols to generate T-T adducts or new proanthocyanidin molecules that can increase the mean degree of polymerisation (mDP) depending on the level of monomeric flavanols available, e.g. it can be decreased if monomeric flavanols are present in an excess in the medium (Monagas *et al.*, 2005). These polymerisation reactions may be enhanced by the presence of some oxygen, leading to the formation of molecules large enough to effectively bind with methyl cellulose. Therefore, an increase in MCP tannin levels was expected as a result of MOX application to the Cabernet Sauvignon wine. However, it was found out that MOX applied after AF did not affect the MCP tannin concentration in the Carbernet Sauvignon wine, as can be seen in Table 4.1. Although the tannin concentrations in all wines increased throughout the trial period, the concentrations were not significantly different ($P < 0.05$, Tukey HSD) in different oxygen treated wines versus the control, at the same observation date. In another MOX trial at the University of Auckland (Fell *et al.*, 2007), no significant difference in tannin fraction, given by the mDP, was observed among the micro-oxygenated wines and the control with no oxygen. It is important to note that the ethyl-linked products formed from condensation reactions mediated by acetaldehyde are unstable in aqueous solution due to the breakage of the ethyl link (Monagas *et al.*, 2005). For example, the ethyl-linked flavanol oligomers can undergo a clevage of the ethyl bridge, leading to the formation of vinyl-flavanol adducts, which then participate in further steps in the formation of complex structures involved in the changes of colour in red wines, e.g reaction with pyranoanthocyanins to form portisins that provide blue colour to the wine (absorption maxima circa 575 nm) (Mateus *et al.*, 2003; Alcalde-Eon *et al.*, 2006). The formation of the larger sized tannins may exceed their disappearance due to the ethyl link

cleavage reaction, and thus may explain the increase in tannin levels measured in all wines at the end of the trial compared to these values at Day 0 (Table 4.1).

Regarding the individual polyphenols monitored by HPLC, differences ($P < 0.05$) at the end the the MOX trial among treatments were seen in the concentrations of only a few compounds, including gallic acid, (-)-epicatechin, *trans*-resveratrol and quercetin (Table 4.1). Concentrations of some other compounds, namely *trans*-caftaric acid, GRP, *cis* and *trans*-coutaric acid, and quercetin-3-glucoside differed among treatments in the middle of the trial (Day 56), but no significant difference ($P > 0.05$) was observed at the end the of the trial (Day 112). It can be seen from the data that epicatechin was the most oxygen affected monomeric polyphenol. Its concentrations in the treated wines, although not very much lower than that in the non-oxygen treated (control) wine, were statistically significant ($P < 0.05$), as a result of the high reactivity of this flavanol in an oxidative environment. A similar stabilisation effect of MOX on some wine flavanols ((+)-catechin and (-)-epicatechin) was also reported in a research by Sartini *et al.* (2007) (MOX after MLF) and more recently by Cejudo-Bastante *et al.* (2011a) (MOX applied after alcoholic fermentation), in which lower concentrations of these molecules were found in MOX wines compared to the control. A decrease in the concentrations of these easily oxidisable flavanols can also be observed during wine ageing (Sartini *et al.*, 2007). In our trial, a decrease in the concentration of (-)-epicatechin over time was observed in all of the wines. Only a small difference in *trans*-caftaric was seen among different treatments at day 56, although it is significant from a statistical point of view, but no difference was found at the end of the trial (Table 4.1).

It has been suggested that MOX is ideally applied either before or after MLF (Parish *et al.*, 2000; Cano-López *et al.*, 2006; Lesica & Kosmerl, 2009). The reason given was the instability of monomeric and oligomeric anthocyanins at the early phase of wine maturation, and the greater susceptibility of tannins to oxidation due to the lack of sulfur dioxide during this time. The addition of oxygen therefore is more efficient in stimulating polymerisation and increasing colour stability (Lesica & Kosmerl, 2009). Levels of total

monomeric anthocyanins were expected to decline as a result of oxygen exposure, yet no effects of MOX on this class of compounds was observed, as seen in Table 4.1. In a study on the effects of MOX applied before oak ageing, also to a Cabernet Sauvignon wine, Llaudy *et al.* (2006) reported significantly higher declines in monomeric anthocyanins in MOX wines than in the control, after 3 months MOX in stainless steel and 8 months in barrels. Although the formation of anthocyanin-ethyl-(epi)catechin are favoured by oxygen, these compounds have been demonstrated to be unstable in acidic aqueous solution and can be degraded as the ethyl link between anthocyanin and catechin is broken (Escribano-Bailon *et al.*, 2001). This reason, alongside the extremely complex processes of oxidation reactions involving phenolic compounds, as well as the confounding effects of the spontaneous MLF occuring during the course of the trial, may explain the limited effects of MOX on the monomeric anthocyanins, and on other phenolics, in this research Cabernet Sauvignon wine. Also, the large variation in the triplicate tanks due to MLF occuring at different time points, could be another reason why the difference in the monomeric anthocyanins was not apparent from a statistical viewpoint, while the quantitative data suggest a lowering in anthocyanins concentration for wines treated at a higher oxygenation rate (Table 4.1). This issue was also seen in the colour density and the SO_2-resistant pigment values, where the relative standard deviations of the mean values were quite high for the Cabernet Sauvignon wines treated with a higher oxygen dosage (Appendix 5-3).

Table 4.1. Changes in tannins and polyphenols in a Cabernet Sauvignon wine during MOX treatments applied after AF. Data present the mean values (mg/L) ± standard deviations of single measurements of triplicate treatments. Means sharing the same letter within a column representing each compound are not significantly different (P > 0.05) at the same observation date.

Times (days)	0	56	112
Tannins			
Control	1715^{a} ± 119	2119^{a} ± 122	2153^{a} ± 97
O_2 5 mg/L/mth	1913^{a} ± 81	2090^{a} ± 251	2102^{a} ± 17
O_2 20 mg/L/mth	1898^{a} ± 117	2417^{a} ± 159	2127^{a} ± 142
Flextanks	1841^{a} ± 174	2173^{a} ± 299	2208^{a} ± 174
Gallic acid			
Control	30.60^{a} ± 0.25	30.20^{a} ± 0.03	30.50^{a} ± 0.03
O_2 5 mg/L/mth	30.70^{a} ± 0.13	30.10^{a} ± 0.09	30.73^{ab} ± 0.07
O_2 20 mg/L/mth	30.71^{a} ± 0.36	30.21^{a} ± 0.14	31.03^{b} ± 0.23
Flextanks	30.65^{a} ± 0.30	30.28^{a} ± 0.05	31.00^{b} ± 0.06
Syringic acid			
Control	5.70^{a} ± 0.07	5.02^{a} ± 0.03	5.18^{a} ± 0.03
O_2 5 mg/L/mth	5.71^{a} ± 0.10	4.84^{a} ± 0.12	5.23^{a} ± 0.07
O_2 20 mg/L/mth	5.68^{a} ± 0.04	4.98^{a} ± 0.21	5.42^{a} ± 0.33
Flextanks	5.67^{a} ± 0.04	4.95^{a} ± 0.06	5.23^{a} ± 0.08
(+)-Catechin			
Control	14.26^{a} ± 0.09	13.24^{a} ± 0.33	15.28^{a} ± 0.42
O_2 5 mg/L/mth	14.87^{a} ± 0.55	13.36^{a} ± 0.31	15.30^{a} ± 0.23
O_2 20 mg/L/mth	15.15^{a} ± 0.14	13.04^{a} ± 0.34	15.40^{a} ± 0.41
Flextanks	14.61^{a} ± 0.74	12.67^{a} ± 0.09	15.89^{a} ± 0.05
(-)-Epicatechin			
Control	17.91^{a} ± 0.63	12.01^{a} ± 0.09	11.99^{a} ± 0.29
O_2 5 mg/L/mth	18.11^{a} ± 0.16	11.80^{ab} ± 0.18	10.78^{b} ± 0.34
O_2 20 mg/L/mth	17.94^{a} ± 0.61	11.39^{bc} ± 0.32	9.78^{bc} ± 0.67
Flextanks	17.96^{a} ± 0.21	11.26^{c} ± 0.06	9.40^{c} ± 0.29
trans-Caftaric acid			
Control	33.13^{a} ± 0.04	32.67^{a} ± 0.02	32.61^{a} ± 0.04
O_2 5 mg/L/mth	33.26^{a} ± 0.21	32.58^{ab} ± 0.05	32.59^{a} ± 0.14
O_2 20 mg/L/mth	33.33^{a} ± 0.13	32.48^{b} ± 0.10	32.69^{a} ± 0.13
Flextanks	33.26^{a} ± 0.18	32.44^{b} ± 0.02	32.73^{a} ± 0.03
GRP			
Control	6.13^{a} ± 0.03	5.85^{a} ± 0.01	5.04^{a} ± 0.01
O_2 5 mg/L/mth	6.13^{a} ± 0.01	5.81^{a} ± 0.02	5.05^{a} ± 0.05
O_2 20 mg/L/mth	6.13^{a} ± 0.03	5.82^{a} ± 0.01	5.01^{a} ± 0.09
Flextanks	6.14^{a} ± 0.04	5.75^{b} ± 0.02	5.03^{a} ± 0.03

Table 4.1. (Continued)

Times (days)	0	56	112
cis-Coutaric acid			
Control	$3.09^{a} \pm 0.02$	$2.89^{a} \pm 0.01$	$2.88^{a} \pm 0.05$
O_2 5 mg/L/mth	$3.11^{a} \pm 0.02$	$2.87^{b} \pm 0.00$	$2.91^{a} \pm 0.00$
O_2 20 mg/L/mth	$3.05^{a} \pm 0.07$	$2.87^{b} \pm 0.01$	$2.90^{a} \pm 0.01$
Flextanks	$3.07^{a} \pm 0.04$	$2.88^{ab} \pm 0.02$	$2.89^{a} \pm 0.02$
trans-Coutaric acid			
Control	$13.34^{a} \pm 0.05$	$13.19^{a} \pm 0.07$	$13.51^{a} \pm 0.03$
O_2 5 mg/L/mth	$13.43^{a} \pm 0.07$	$13.02^{b} \pm 0.02$	$13.51^{a} \pm 0.06$
O_2 20 mg/L/mth	$13.40^{a} \pm 0.09$	$12.99^{b} \pm 0.02$	$13.53^{a} \pm 0.02$
Flextanks	$13.47^{a} \pm 0.06$	$13.04^{ab} \pm 0.07$	$13.51^{a} \pm 0.02$
Caffeic acid			
Control	$2.99^{a} \pm 0.14$	$3.21^{a} \pm 0.04$	$3.18^{a} \pm 0.02$
O_2 5 mg/L/mth	$3.02^{a} \pm 0.06$	$3.17^{a} \pm 0.03$	$3.20^{a} \pm 0.00$
O_2 20 mg/L/mth	$3.02^{a} \pm 0.05$	$3.21^{a} \pm 0.01$	$3.20^{a} \pm 0.01$
Flextanks	$3.01^{a} \pm 0.07$	$3.20^{a} \pm 0.02$	$3.22^{a} \pm 0.02$
trans-Resveratrol			
Control	$3.01^{a} \pm 0.07$	$2.08^{a} \pm 0.15$	$3.32^{ab} \pm 0.10$
O_2 5 mg/L/mth	$3.08^{a} \pm 0.08$	$2.17^{a} \pm 0.12$	$3.35^{a} \pm 0.03$
O_2 20 mg/L/mth	$3.09^{a} \pm 0.29$	$2.25^{a} \pm 0.08$	$3.25^{ab} \pm 0.12$
Flextanks	$2.99^{a} \pm 0.21$	$2.22^{a} \pm 0.05$	$3.12^{b} \pm 0.06$
Quercetin-3-glucoside			
Control	$12.36^{a} \pm 0.12$	$11.48^{ab} \pm 0.10$	$11.89^{a} \pm 0.02$
O_2 5 mg/L/mth	$12.27^{a} \pm 0.11$	$11.30^{a} \pm 0.07$	$11.92^{a} \pm 0.08$
O_2 20 mg/L/mth	$12.32^{a} \pm 0.12$	$11.37^{ab} \pm 0.06$	$11.81^{a} \pm 0.18$
Flextanks	$12.27^{a} \pm 0.15$	$11.56^{b} \pm 0.04$	$11.92^{a} \pm 0.04$
Quercetin			
Control	$14.84^{a} \pm 0.53$	$11.01^{a} \pm 0.42$	$12.34^{a} \pm 0.27$
O_2 5 mg/L/mth	$15.28^{a} \pm 0.90$	$10.60^{a} \pm 0.31$	$13.40^{b} \pm 0.30$
O_2 20 mg/L/mth	$15.73^{a} \pm 1.49$	$10.92^{a} \pm 0.14$	$12.82^{ab} \pm 0.52$
Flextanks	$14.92^{a} \pm 0.59$	$10.84^{a} \pm 0.21$	$12.48^{ab} \pm 0.39$
Total monomeric anthocyanins			
Control	$202.48^{a} \pm 1.41$	$153.68^{a} \pm 0.93$	$138.45^{a} \pm 1.38$
O_2 5 mg/L/mth	$202.82^{a} \pm 0.71$	$151.10^{a} \pm 1.33$	$135.05^{a} \pm 5.35$
O_2 20 mg/L/mth	$207.86^{a} \pm 4.30$	$149.66^{a} \pm 3.92$	$128.92^{a} \pm 11.60$
Flextanks	$206.79^{a} \pm 1.32$	$151.79^{a} \pm 0.59$	$135.52^{a} \pm 1.38$

CHAPTER 5. MICRO-OXYGENATION POST-MALOLACTIC FERMENTATION

5.1. INTRODUCTION

The application of MOX can be carried out at any stage of the winemaking process, from primary fermentation to bottling. However, practical experience and experimental studies have suggested that MOX is more effective for red wine development, in terms of colour and palate structure, if the oxygen is added at the end of the alcoholic fermentation (Cano-López *et al.*, 2006; Perez-Magarino *et al.*, 2007). Other research has also been undertaken on the influence of MOX applied both before and after MLF (Cano-López *et al.*, 2008), after MLF (Pour-Nikfardjam & Dykes, 2003; Cano-López *et al.*, 2010) or in combination with oak chips (Gonzalez-del Pozo *et al.*, 2010). Whereas research on the effects of MOX on wine characteristics were initially more focused on the changes of

colour and polyphenols during and after MOX (Atanasova *et al.*, 2002a; Cano-López *et al.*, 2006; Cano-Lopez *et al.*, 2007; Fell *et al.*, 2007; Perez-Magarino *et al.*, 2007; Sartini *et al.*, 2007; Cano-López *et al.*, 2008), scientific studies on the impacts of oxygenation on the aroma and sensory properties of wine have only appeared more recently (McCord, 2003; Ortega-Heras *et al.*, 2008; Hernandez-Orte *et al.*, 2009; Cejudo-Bastante *et al.*, 2011a, 2011b). Except for the study by McCord (2003), no quantitative data regarding the effects of MOX on reductive sulfur compounds has appeared in the literature, although it has been mentioned elsewhere that oxygen was used to remove these reductive notes (Ortega-Heras *et al.*, 2008). The results from MOX reported in CHAPTER 4 indicated the influence of limited oxygenation applied before MLF on the sulfur volatile off-odours. Therefore, in this part of the research, changes in the concentrations of these undesirable compounds in red wines undergoing MOX treatments, but only after MLF, were investigated. The two sets of wines came from a commercial trial using larger scale tanks (an Australian Shiraz) and from a fully replicated pilot scale MOX trial using 300 L tanks (BORDEAUX blend wine). A sensory analysis of the BORDEAUX blend was undertaken after the MOX treaments to evaluate how MOX affected the perceived sensory characteristics of the wines, and in relation to the changes in the chemical profiles of the wines as a result of limited oxygen exposure after MLF.

5.2. RESULTS AND DISCUSSION

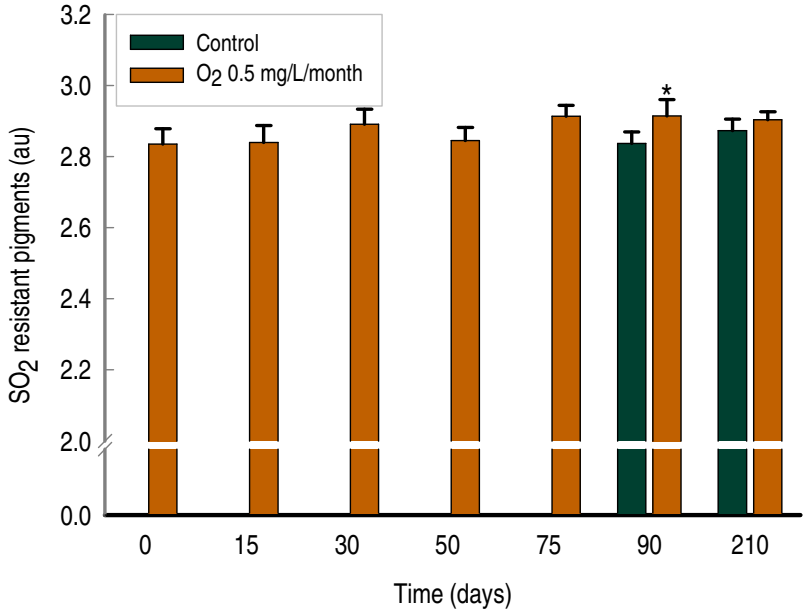
5.2.1. Development of colour

5.2.1.1. Developments of colour in a SHIRAZ during MOX after MLF

Figure 5.1 and Figure 5.2 show the colour development of a Shiraz wine, commercially micro-oxygenated at a rate of 0.5 mg/L/month for 90 days. It can be seen from these figures that the colour density increased gradually in the micro-oxygenated wine over the 90 day period. Samples for the control wine with no oxygen were not provided for the

intermediate treatment dates, and thus no colour measurements were made from day 15 to day 75 for the control. Although the difference in the SO_2-resistant pigments at the end of the MOX period was not remarkable, it was statistically significant higher ($P < 0.05$) in the oxygen treated wine than that of the no-oxygen counterpart. However, this difference was not seen at day 210, 4 months after stopping the oxygen supply (Figure 5.1). On the other hand, statistical differences ($P < 0.05$) in the colour density were found both at the end of the MOX period and also 4 months after stopping oxygenation (Figure 5.2), with the MOX wine showing a higher colour density than the control. The colour density of both wines showed a decrease at day 210, compared to those at the end of the MOX period (day 90), yet the colour density of the oxygen treated wine remained higher than that of the control. Interestingly, at day 210, while no significant difference in the SO_2-resistant pigments was observed, the colour density of the MOX Shiraz remained significantly higher ($P < 0.05$) than that of the control. This could be attributed to the likes of ethyl-linking cleavage reactions, leading to the release of monomeric anthocyanins, which are not resistant to sulfur dioxide bleaching, but still contribute to wine colour. In fact, the difference between the concentration of monomeric anthocyanins for the two treatments was smaller at day 210, compared to that at day 90 (Table 5.1). Gonzalez-del Pozo *et al.* (2010) found that the positive effects of MOX on colour density were only exhibited for a short period of time, i.e. only during the oxygen addition stage, and did not last during barrel storage and in bottles. On the other hand, it was demonstrated elsewhere that the improved colour characteristics were maintained, with MOX wines always showing higher colour density values than the control, irrespective of the ageing methods, either in barrels or bottles, for MOX treatment applied to Monastrell wines (Cano-Lopez *et al.*, 2007). Interestingly, colour density was found slightly less intense but significantly lower in the MOX wine, compared to the control, in a study by Llaudy *et al.* (2006), where MOX was applied before oak ageing. In this study, the treatment wine was initially kept in stainless steel tanks and was supplied with oxygen for 3 months before being transferred to oak barrels for 8 months. The control wine, however, was not kept in stainless tanks first before oak ageing to be comparable with the micro-oxygenated wine, but it was first kept in oak barrels for 8 months prior to 3 months in stainless steel tanks. The way the

experiment was designed in this study may explain the lower colour density of the treated wine compared to that of the control. It was found that non-flavonoid tannins extracted from oak appear to indirectly favour colour stabilisation by protecting anthocyanins and

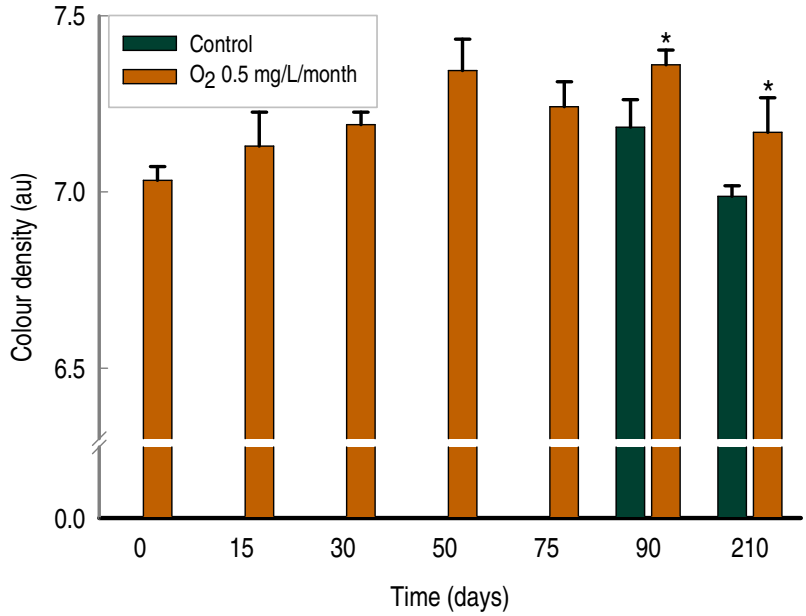


Times (days)	0	15	30	50	75	90	210
Control						2.84[a]	2.87[a]
O_2 0.5 mg/L/month	2.84[a]	2.84[ad]	2.89[abcd]	2.85[ad]	2.91[bcd]	2.92[cd]	2.90[d]

Figure 5.1. Development of pigments resistant to sulfur dioxide bleaching in a SHIRAZ during MOX at 0.5 mg/L/month applied after MLF. The asterisk indicates value which differs significantly from the control ($P < 0.05$, t-test). Values sharing the same letter are not significantly different ($P < 0.05$, Tukey HSD Post Hoc Test) across time within treatment.

flavanols from irreversible oxidative colour loss (Jackson, 2008, p. 290). From the intinital stage of this trial (the first 3 months), not only the oxygen ingressed through the oak

staves, but also the chemical components from the oak could both contribute to the stimulating effecs on colour stabilisation of the control wine. Meanwhile, the treated wine had the effects of only oxygen supplied by the micro-oxygenation process in stainless tanks. This suggests that early timing in the use of combined oak addition, such as in the forms of oak chips or staves, with MOX could play an important role in further enhancing the colour stabilisation.

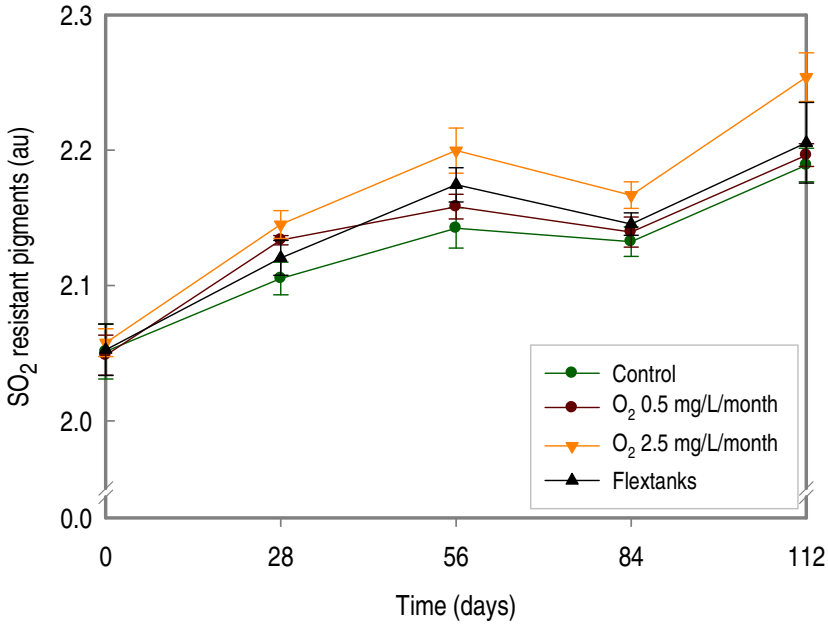


Time (days)	0	15	30	50	75	90	210
Control						7.18^{a}	6.99^{b}
O_2 0.5 mg/L/month	7.03^{a}	7.13^{abdf}	7.19^{bdf}	7.34^{cde}	7.24^{def}	7.36^{e}	7.17^{f}

Figure 5.2. Development of colour density in a SHIRAZ during MOX at 0.5 mg/L/month applied after MLF.

Data represent the mean value ± standard deviation of triplicate measurements. The asterisk indicates value which differs significantly from the control ($P < 0.05$, t-test). Values sharing the same letter are not significantly different ($P < 0.05$, Tukey HSD Post Hoc Test) across time within treatment.

5.2.1.2. Development of colour in a BORDEAUX blend during MOX after MLF



Time [days]	0	28	56	84	112
Control	2.05 a	2.11 a	2.14 a	2.13 a	2.19 a
O_2 0.5 mg/L/mth	2.05 a	2.13 bc	2.16 ac	2.14 a	2.20 a
O_2 2.5 mg/L/mth	2.06 a	2.15 c	2.20 b	2.17 b	2.25 b
Flextanks	2.05 a	2.12 ab	2.17 c	2.15 a	2.21 a

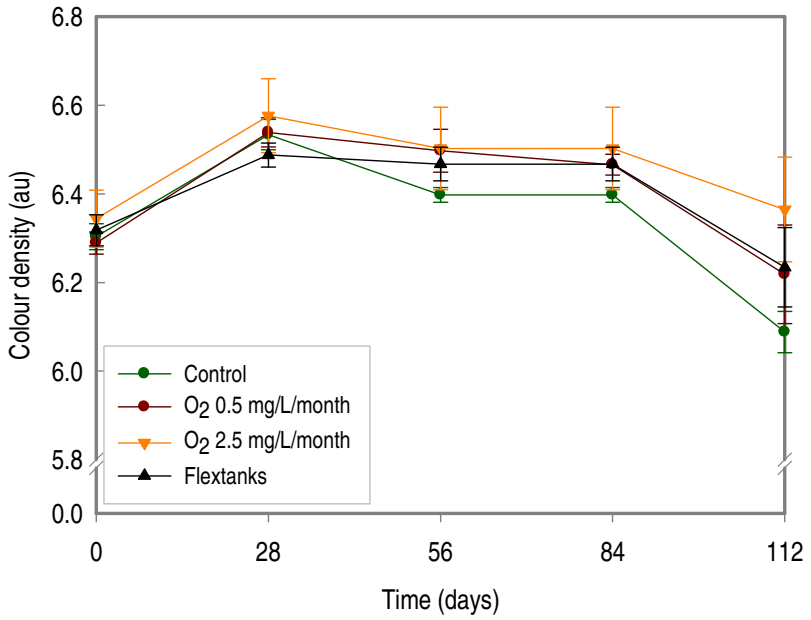
Figure 5.3. Development of pigments resistant to sulfur dioxide bleaching in a BORDEAUX BLEND during MOX applied after MLF.

Data points represent the mean values ± standard deviations (error bars) of triplicate treatments.

Same letters denote values which are not significantly different (P > 0.05, Tukey HSD Post Hoc Test) among treatments at a specific observation date

Spectrophotometric measures revealed differences in the chromatic development of the BORDEAUX blend treated with different oxygen rates (0.5 mg/L/month, and 2.5

mg/L/month), as well as of the wine stored in Flextanks, compared to the control with no oxygen addition. The effects of the MOX treatments on the evolution of wine colour, in terms of colour density and pigments resistant to sulfur dioxide bleaching, were more obvious in this trial, compared to those seen in the previous replicated MOX trial applied after alcoholic fermentation on a Cabernet Sauvignon wine, as reported in **CHAPTER 4.**



Time (days)	0	28	56	84	112
Control	6.30 a	6.53 a	6.40 a	6.40 a	6.09 a
O_2 0.5 mg/L/mth	6.29 a	6.54 a	6.50 a	6.47 a	6.22 ab
O_2 2.5 mg/L/mth	6.34 a	6.58 a	6.50 a	6.50 a	6.36 b
Flextanks	6.32 a	6.49 a	6.47 a	6.47 a	6.23 ab

Figure 5.4. Development of colour density in a BORDEAUX BLEND during MOX applied after MLF. Data points represent the mean values ± standard deviations (error bars) of triplicate treatments. Same letters denote values which are not significantly different (P > 0.05, Tukey HSD Post Hoc Test) among treatments at a specific observation date.

It can be seen from Figure 5.3 that there was a steady increase in the pigments resistant to sulfur dioxide bleaching in all of the BORDEAUX blend wines over time, although there were a small drop at day 84, possibly as a result of the ethyl link cleavage reactions. However, differences in the SO_2-resistant pigments among different oxygen treated wines were only seen at day 28 and day 56. After that, only the 2.5 mg/L/month oxygen added BORDEAUX blend wine showed a significantly higher level ($P < 0.05$) of SO_2-resistant pigments, as observed at day 84 and 112. This trend has been noted previously, including a very recent study of Gonzalez-del Pozo *et al.* (2010), in which spectrophotometric analyses indicated the positive impact of oxygen on the evolution of SO_2-resistant pigments, with a MOX wine showing a significantly higher value through all the stages of this study. In that study, oxygen was supplied after alcoholic fermentation, discontinued during spontaneous MLF, and resumed at a rate of 2 mg/L/month for 3 months thereafter (Gonzalez-del Pozo *et al.*, 2010). More SO_2-resistant pigments formed in MOX wines versus controls were also observed in other studies (Atanasova *et al.*, 2002a; Cano-López *et al.*, 2006).

The colour density of all BORDEAUX wines initially increased over the first 28 days, followed by a slowly decrease in this value towards the end of the trial (Figure 5.4). Similarly, a large fall in the colour density was clearly seen in both the control and MOX Cabernet Sauvignon wines during a subsequent MLF following MOX treatment after alcoholic fermentation in the study of Gonzalez-del Pozo *et al.* (2010). The decrease in colour density over time could be partially due to the changes in the colour contributed by the monomeric anthocyanins[30] as a result of an increase in the pH due to the consumption of malic acid (Gonzalez-del Pozo *et al.*, 2010). In our trials, the WineScan data showed that pH did increase in the research Cabernet Sauvignon wines from 3.84 at the beginning to 3.91 at the end of the trial, explaning a large drop as observed in the

[30] Anthocyanins occur in five major molecular states as an equilibrium, in which the red colour of wine is mostly attributed to a small proportion of flavylium cation (A^+) plus blue tones of quinoidal base (AO). The red flavylium cations predominates only in very acidic solution (pH < 2) (Cheynier *et al.*, 2006).

colour density at day 56 (Figure 4.2). Meanwhile, the pH remained at the same value of 3.84 for the BORDEAUX blend throughout the trial (Appendix 5-1). Of note is that the evolution of red wine colour density over time, e.g during maturation and ageing, along with the changes in tonality, from the intinial purple-red of young wines to a brick red-orange of more aged wines, are mainly attributed to the diverse reactions of monomeric anthocyanins[31] with other wine constituents (Salas *et al.*, 2004; Alcalde-Eon *et al.*, 2006). For example, the direct and indirect (acetaldehyde-mediated) condensations of anthocyanins with flavan-3-ols, leading to the formation of compounds such as ethyl-linked A-T and T-A pigments, cause *bathochromical shifts*[32] in the visible absorption maxima of the anthocyanins, providing a bluish-red hue to the wine (Rivas-Gonzalo *et al.*, 1995; Atanasova *et al.*, 2002a; Alcalde-Eon *et al.*, 2006). On the other hand, the cycloaddition of different wine components such as pyruvic acid, acetaldehyde and vinylphenol, onto the anthocyanin structure, form pyranoanthocyanins, which are more stable than ethyl-bridged compounds, and are more orange than malvidin-3-glucoside, owing to a new pyran ring in their structures, resulting in a *hypsochromical shift*[33] towards a more orange hue (Fulcrand *et al.*, 1996; Bakker & Timberlake, 1997; Fulcrand *et al.*, 1997).

However, although the colour density of all wines diminished over time, it is important to note that the decrease in colour density of the BORDEAUX wines was much smaller than that of the Cabernet Sauvignon wines that underwent MOX after alcoholic fermentation, as reported in CHAPTER 4. The stimulating effects of MOX on the evolution of wine colour

[31] The absorbance maxima (λmax) of monomeric anthocyanins range from 515 to 526 nm, while λmax for ethyl-linked compounds and pyranoanthocyanins are 528 to 540 nm, and 480 to 510 nm, respectively (Atanasova *et al.*, 2002b; Cheynier *et al.*, 2006).

[32] *Bathochromic shift* is a change of spectral band position in the absorption spectrum of a molecule to longer wavelength.

[33] *Hypsochromic shift* is a change of spectral band position in the absorption spectrum of a molecule to shorter wavelength.

intensity, leading to an increase of this chromatic value in MOX wines, has also been reported previously (Atanasova *et al.*, 2002a; Cano-López *et al.*, 2006; Gonzalez-del Pozo *et al.*, 2010). Atanasova *et al.* (2002a) studied the effects of oxygenation on a blended red wine after MLF and noted that despite the decrease in the colour density in both MOX and control wines, the decline was less in MOX wine than in the control (Atanasova *et al.*, 2002a). With regard to the effect of oxygen on this chromatic parameter, the drop in the colour density after day 28 of the control wine appeared to be the largest, yet it was not lower in a rigorous statistical sense than that of the two oxygen treated wines and of the Flextank wine until the end of the trial. In fact, statistical difference was only seen at day 112, when the highest oxygen added (2.5 mg/L/month) wine showed a significant higher ($P < 0.05$) colour density than the control wine. The colour density of the lower oxygen treated and the Flextank wines were similar and somewhat intermediate between the control and the highest oxygen treated wine (**Figure 5.4**). It is noteworthy that the consistency of the replicated MOX treatments post-MLF were better than that of the MOX trial applied after alcoholic fermentation as reported in **CHAPTER 4**, in which the spontaneous MLF occurred simultaneously during MOX. The relative standard deviations (% RSD) of the mean values of the colour parameters of the BORDEAUX blend trial were less than 2.0 %, while the % RSDs of the colour density and the pigments resistant to sulfur dioxide bleaching for the Cabernet Sauvignon wines were higher than 2.0 %, and as high as 6.41 %, especially at observation dates towards the end of the trial (See **Appendix 5-3** and **Appendix 5-4**). The greater consistency in the BORDEAUX replicates consequently led to a result that the colour density of the control and the high oxygen treated BORDEAUX blend were different at the end of the trial (day 112) from a statistical point of view, although they only differed moderately from a quantitative point of view, with values of 6.09 and 6.36, respectively (**Figure 5.4**). This reason could also be the explanation for the statistically significant difference in the colour density of the Cabernet Sauvignon wines as reported in **CHAPTER 4**, namely that the colour density of the wine stored in Flextanks was significantly higher ($P < 0.05$) than that of the control at every observation date after day 56, while the quantitative values were not remarkably different (**Figure 4.2** and **Appendix 5-3**).

5.2.2. Effects of MOX after MLF on reductive sulfur compounds

Both light and heavy reductive sulfur compounds were found in the commercial Shiraz wine micro-oxygenated at a rate of 0.5 mg/L/month and also in the control with no oxygen. Methanethiol and dimethyl sulfide were the "highly volatile" compounds, while other substances, including S-ethylthioacetate, 2-methylthio-1-ethanol and 3-methylthio-1-propanol were the four 'low volatile' compounds found to be present.

5.2.2.1. Effects of MOX after MLF on reductive sulfur volatiles in a Shiraz

Methanethiol and dimethyl sulfide

The highly volatile sulfur compound methanethiol (MeSH) was found in the commercial Shiraz at the beginning of the trial (Figure 5.5 i). MeSH concentration in the micro-oxygenated Shiraz fluctuated during the MOX period, and a large drop was observed after 15 and 30 days of MOX. It has been discussed previously in Section 4.2.3.1 that MeSH is a highly reactive compound (nucleophile), which can easily be oxidised to form other sulfur compounds such as disulfides, or can react with the oxidation products of wine polyphenols, and thus can be trapped by wine electrophiles, such as *o*-quinones and the carbocation form of procyanidin molecules (Equation 1.3) (Majcenovic *et al.*, 2002; Ribéreau-Gayon *et al.*, 2006b; Kilmartin, 2009). In this commercial MOX trial on an Australian Shiraz, no disulfide and trisulfide compounds were detected, and the wine had finished MLF before MOX, inferring that the chemical interaction of MeSH with oxidised wine polyphenols could lead to the remarkable fall in MeSH concentrations in the micro-oxygenated Shiraz, seen at day 15 and day 30. Comparison of the MeSH in the two wines at the end of the MOX period (day 90) indicated some impacts of MOX on this underisable sulfur compound. The concentration of MeSH in the MOX Shiraz was significantly lower ($P < 0.05$) than in the control at day 90 and the difference remained after 4 months (day 210), although the MeSH concentration in the control wine seemed to increase compared to its value at day 90 (but without statistical difference, $P > 0.05$).

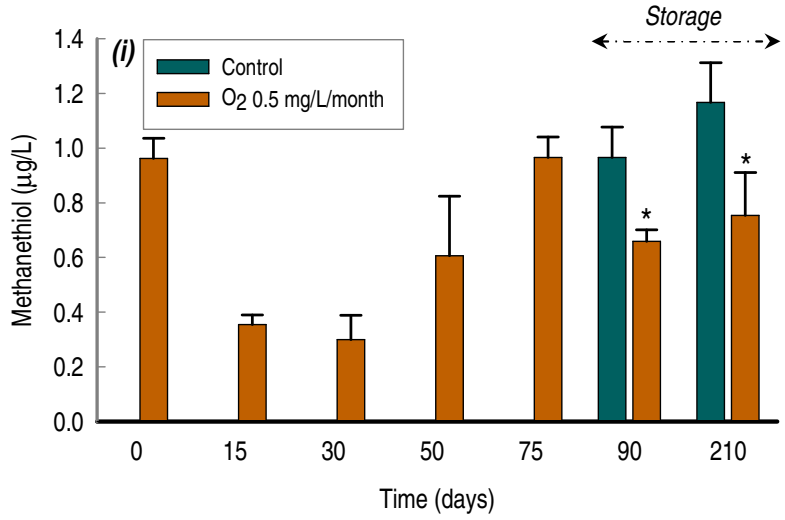
Another highly volatile compound present in the Shiraz wine was dimethyl sulfide (DMS). DMS is found in the wines of most grape varieties and is considered less significant in reduction defects. Concentrations of DMS are often low in freshly bottled wines but can increase remarkedly during ageing, suggesting the occurrence of other chemical pathways leading to DMS production during wine ageing (Marais, 1979; de Mora *et al.*, 1987). DMS can contribute positively or negatively to the 'ageing bouquet', depending on its concentration and the type of wine (Spedding & Raut, 1982; Moreira *et al.*, 2002; Segurel *et al.*, 2004; Landaud *et al.*, 2008). A concentration of DMS near 100 µg/L has been demonstrated to have an enhancing effect on the fruity notes of Syrah and Grenache noir wines from the Rhone Valley of France (Segurel *et al.*, 2004), while DMS levels lower than its perception threshold of 60 µg/L in Cabernet Sauvignon wine were found to improve the fruity character of this wine (de Mora *et al.*, 1987). In the commercial Shiraz wine, DMS was present at very low concentration, in the range of 6 - 8 µg/L. Its concentration seemed to increase with time in the MOX wine, but the increase was not statistically significant (**Figure 5.5 ii**). It was shown that DMS can increase during wine ageing, and a high storage temperature favours this formation (Marais, 1979). On the other hand, Silva Ferreira *et al.*(2003) demonstrated that higher temperatures tend to diminish DMS levels in Port wines, while high concentrations of DMS were strictly related to oxygen.

Statistical analysis indicated no significant difference in the concentration of DMS between the MOX Shiraz and the control at the end of the MOX treatment (day 90). However, quantitative data at day 210 showed a significantly lower concentration of DMS in the control compared to that at day 90. It has been demonstrated that DMS concentration can increase with storage time and temperature (Marais, 1979). On the other hand, Silva Ferreira *et al.*, (2003) showed that for the same oxygen treatment applied to a Port wine, there was a tendency for DMS to decrease with higher temperatures, suggesting that high temperatures, up to 60 °C in this study, were probably too high to maintain DMS in wine. High concentrations of DMS, however, were found to be strictly related to the presence of oxygen, and increase with time, when the wine was kept

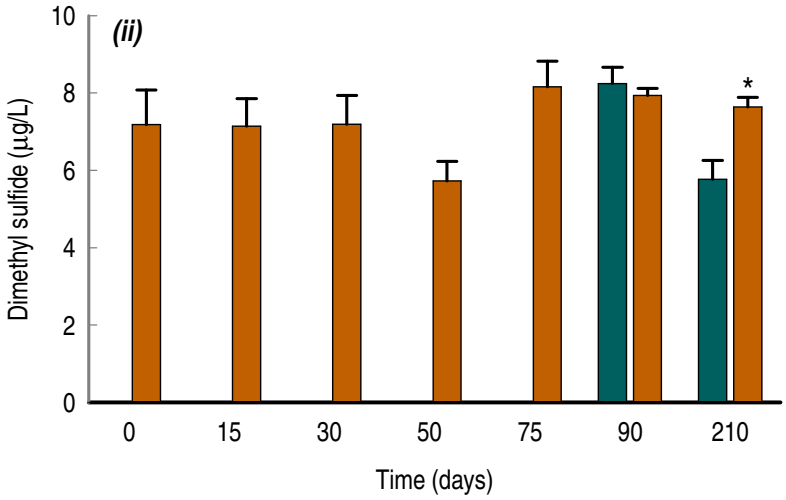
at 15 °C (Silva Ferreira *et al.*, 2003). In this part of the current study, it is unlikely that high temperature could be the reason leading to the decline in DMS concentration in the control Shiraz at day 210 compared to that at day 90, but rather the stroichiometric competition of substrates toward the completeness of many chemical reactions occurred in the wine matrix. There was also the posibility that the DMS concentration in both wines could have been lost at the same rate after the MOX period. However, the DMS concentration in the MOX wine remained higher than in the control, owing to the presence of oxygen that may favour the chemical transformations leading to the formation of this compound, as discussed in Section 4.2.3.1. Consequently, a significant higher DMS concentration in the MOX wine, than in the control, was observed at day 210, 4 months later after stopping oxygen supply, as presented in Figure 5.5.

S-thioesters

The low volatile S-thioester, S-ethyl thioacetate (ETA), was found to be present in the commerical Shiraz studied. ETA is the fermentation derived off-odour commonly found in bottled wines, as it does not respond readily to copper fining (Limmer, 2005). ETA can be hydrolysed under acidic condition during storage to liberate the corresponding free thiol, ethanethiol, that has lower perception threshold (1.1 μg/L compared to 40 μg/L). ETA, therefore, can be a source of off-odours in wine after fermentation (Leppanen *et al.*, 1980). Statistical analysis indicated that MOX did not affect the hydrolysis process of this S-thioester. At the end of the MOX period (day 90), and at 4 months of storage after stopping the MOX treatment (day 210), the was no significant difference ($P > 0.05$) in the concentration of ETA between the micro-oxygenated Shiraz and the control without oxygen addition (Figure 5.6). The concentration of ETA was less than 8 μg/L and diminished to concentrations close to approximately 4 μg/L at day 210 for both treatments. This decrease was statistically significant ($P < 0.05$) but there was no ethanethiol detected in any of the wines at any time.

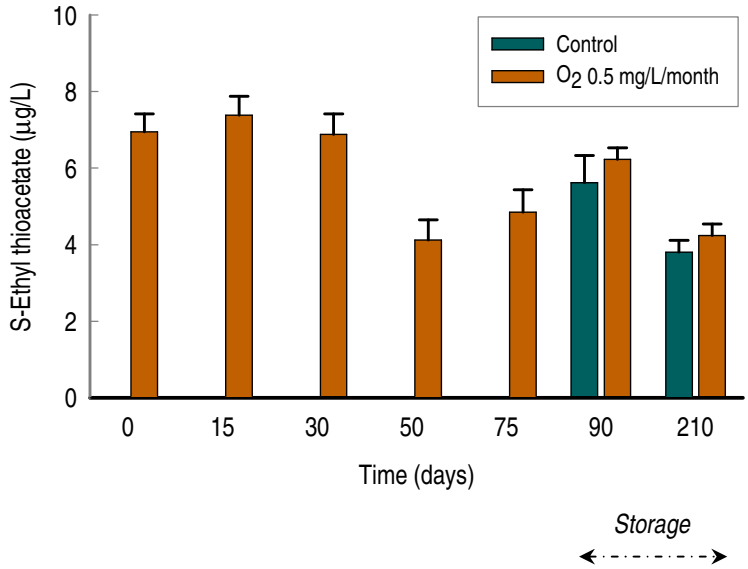


Time (days)	0	15	30	50	75	90	210
Control						0.97a	1.17a
O_2 0.5 mg/L/month	0.96a	0.36bc	0.30b	0.61bcd	0.97a	0.66acd	0.75ad



Time (days)	0	15	30	50	75	90	210
Control						8.24a	5.77b
O_2 0.5 mg/L/month	7.18ab	7.14ab	7.19ab	5.72a	8.16b	7.93b	7.64b

Figure 5.5. Effects of MOX on (i) methanethiol, (ii) dimethyl sulfide in a Shiraz after MLF. Error bars represent the standard deviations of the mean (n = 3). The asterisk indicates value which differs significantly from the control ($P < 0.05$, t-test). Values sharing the same letter are not significantly different ($P < 0.05$, Tukey HSD Post Hoc Test) across time within treatment.



Time (days)	0	15	30	50	75	90	210
Control						5.41a	3.81b
O_2 0.5 mg/L/month	6.95a	7.39a	6.88a	4.12b	4.85b	6.22a	4.24b

Figure 5.6. Effect of MOX on S-ethyl thioacetate in a Shiraz after MLF.

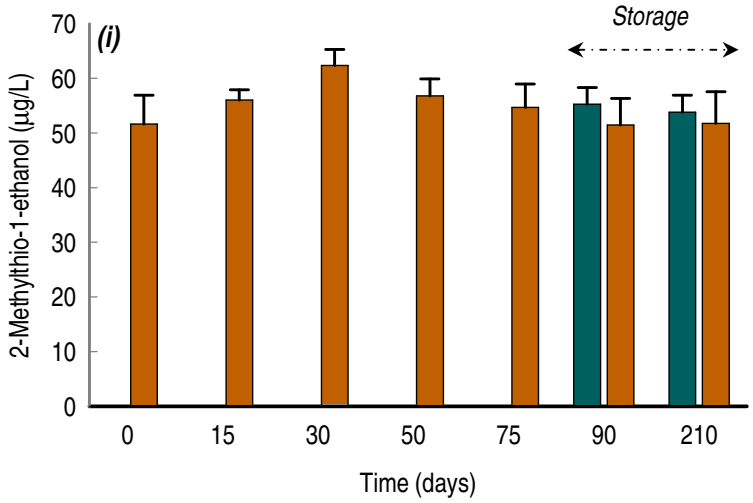
Error bars represent the standard deviations of the mean (n = 3). Values followed by different letters are significantly different ($P < 0.05$, Tukey HSD Post Hoc Test) across time within treatment.

Thioether alcohols

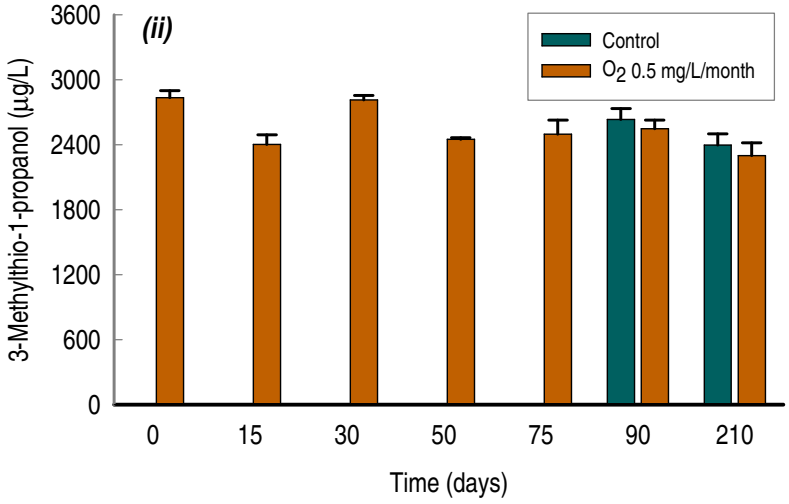
2-methylthio-1-ethanol (MTE) and 3-methylthio-1-propanol or methionol (MTP) were the low volatile sulfur compounds present in the Shiraz studied. The concentration of MTE did not change, while concentrations of MTP in both micro-oxygenated and control wines declined ($P < 0.05$) over time. MTE, with an odour reminiscent of the French bean, can present reduction defects in wines at concentrations very near its perception threshold (Ribéreau-Gayon *et al.*, 2006b). The origin and evolution of MTE during winemaking is not well-known. On the other hand, the biosynthesis of MTP by yeast through the *Ehrlich*

reaction has been established (See Section 4.2.3.3). The cauliflower odour-liked MTP can occur in wines at concentrations up to 5 mg/L (Moreira *et al.*, 2002; Landaud *et al.*, 2008), depending largely on the grape variety (Moreira *et al.*, 2002). In the commercial Shiraz, the concentration of MTP was well above its perception threshold of 1200 μg/L, yet the wine did not exhibit any obvious reduction notes in the initial sample, according to winemakers' comments, in the control and MOX wines at the end of the trial and after 4 months of storage, possibly due to its complexity. Concentrations of MTP were reported to increase following MLF (Ugliano & Moio, 2005). In the current trial, the Shiraz had finished MLF before applying MOX treatment, therefore, no formation of MTP was expected. Instead, its concentration gradually decreased, and the concentration at the end of the trial (day 90) was significantly lower ($P < 0.05$) than the initial levels (Figure 5.7).

In terms of MOX effects on these compounds, oxygen applied after MLF did not affect the evolution of the thioether alcohols in the studied Shiraz wine after MLF and during storage, as no significant difference ($P > 0.05$) was found when comparing the concentrations of MTE and MTP between the micro-oxygenated wine and the control at the end of the trial period (day 90) and after 4 months of storage (day 210) (Figure 5.7). It has been demonstrated that the presence of oxygen could lead to a loss of MTP, with or without methional formation (Escudero *et al.*, 2000; Silva Ferreira *et al.*, 2003). As the wine had completed MLF, it can be concluded that MOX applied at the low dosage rate (0.5 mg/L/month for 3 months) to the Shiraz after MLF did not affect the concentrations of MTE and MTP at the time the wine was examined.



Time (days)	0	15	30	50	75	90	210
Control						55.27a	53.82a
O_2 0.5 mg/L/month	51.63a	56.01a	62.40a	56.83a	54.72a	51.46a	51.78a



Time [days]	0	15	30	50	75	90	210
Control						2635a	2397b
O_2 0.5 mg/L/month	2833a	2401bc	2813a	2451bc	2497bc	2546c	2299b

Figure 5.7. Effect of MOX on (i) 2-methylthio-

-1-ethanol and (ii) 3-methylthio-1-propanol in a Shiraz after MLF.

Error bars represent the standard deviations of the mean (n = 3). Values following by different letters are significantly different ($P < 0.05$, LSD Post Hoc Test) across time within treatment.

5.2.2.2. Effects of MOX after MLF on reductive sulfur volatiles in a BORDEAUX BLEND

The impacts of MOX on the evolution of reductive compounds in the BORDEAUX blend red post-MLF were also investigated. A BORDEAUX blend made from Cabernet Sauvignon, Merlot, Cabernet franc and Malbec grapes was used in the fully replicated trial (See Section 2.1.2.1). Four common reductive sulfur compounds, namely methanethiol, dimethyl sulfide, 2-methylthio-1-ethanol and 3-methylthio-1-propanol were present in the wine and the changes in their concentrations during the course of the trial (112 days) were monitored.

Methanethiol and dimethyl sulfide

The simple reductive sulfur thiol methanethiol (MeSH) was again found in this BORDEAUX blend after MLF. The concentration of MeSH present in the BORDEAUX blend was low but still higher than its perception threshold of 0.3 μg/L in alcoholic solution (Mestres *et al.*, 2000). The concentration of MeSH in all of the BORDEAUX wines at the beginning of the trial were close to the level of this compound in the control Cabernet Sauvignon wine at the end of the MOX trial applied before MLF (See Section 4.2.3.1). The ANOVA statistic analysis indicates that the concentrations of MeSH in the two micro-oxygenated wines, where oxygen were supplied at a rate of 0.5 mg/L/month and 2.5 mg/L/month, were significantly lowered ($P < 0.05$), compared with the control and the Flextank storage wines in the middle of the trial period (day 56). These results indicated that oxygen did have some impacts on the evolution of this cooked-cabbage odour-liked thiol. Only chemical pathways, such as oxidation to disulfides or reaction with the products of polyphenol oxidation, could have been involved in lowering the level of MeSH in the studied wine, as MLF had been completed. The decrease in concentration of MeSH and ethanethiol were also reported in McCord's study (2003). Interestingly, at the end of the trial, only the low oxygen treatment wine showed a significantly lower ($P < 0.05$) concentration of MeSH compared with the other wines (Figure 5.8 i), which had higher MeSH concentrations at

day 112. It has been postulated that methional, formed during beer making from methionine via chemical means, the so called Strecker degradation[34], can be further oxidised to generate MeSH and propanal (Landaud *et al.*, 2008). Such a reaction, however, has not been documented in wine literature.

Dimethyl sulfide (DMS) was the other common highly volatile reductive sulfur compound found in the BORDEAUX blend wine. The concentration of DMS in the wine was low, compared to its odour threshold of 60 µg/L in Cabernet Sauvignon (de Mora *et al.*, 1987), and increased towards the end of the trial, as can be seen from **Figure 5.8 ii**. The concentration of DMS in the higher oxygen treated wine (2.5 mg/L/month) was significantly lower ($P < 0.05$) than that in the other wines at day 56. At the end of the trial (day 112), higher concentrations of DMS in the high oxygen treated and Flextanks stored wines were seen, relative to those in the low oxygen treated wine and the control. But the significant difference ($P < 0.05$) was only found for the low oxygen treated wine and not for the control. This finding is in agreement with Silva Ferreira *et al.* (2003) who demonstrated a decrease in DMS concentration over time in Port wines treated with oxygen at 15 °C, as previously mentioned in **Section 5.2.2.1**. The higher concentration of DMS in the high oxygen treated wine and in Flextanks observed at day 112 were also somewhat in accordance with the DMS results found in the MOX Shiraz wine presented above, indicating that MOX can increase the concentration of DMS over time. A lower concentration of DMS was found at day 112 in the low oxygen treated BORDEAUX wine, although treated at the same oxygen rate of 0.5 mg/L/mth as for the commercial Shiraz, suggesting that the extent to which MOX can affect this compound may depend on the grape variety and the wine matrix properties.

[34] Chemical reaction that converts an amino acid to an aldehyde, first discovered by A. Strecker in 1862 (Rizzi, 2008).

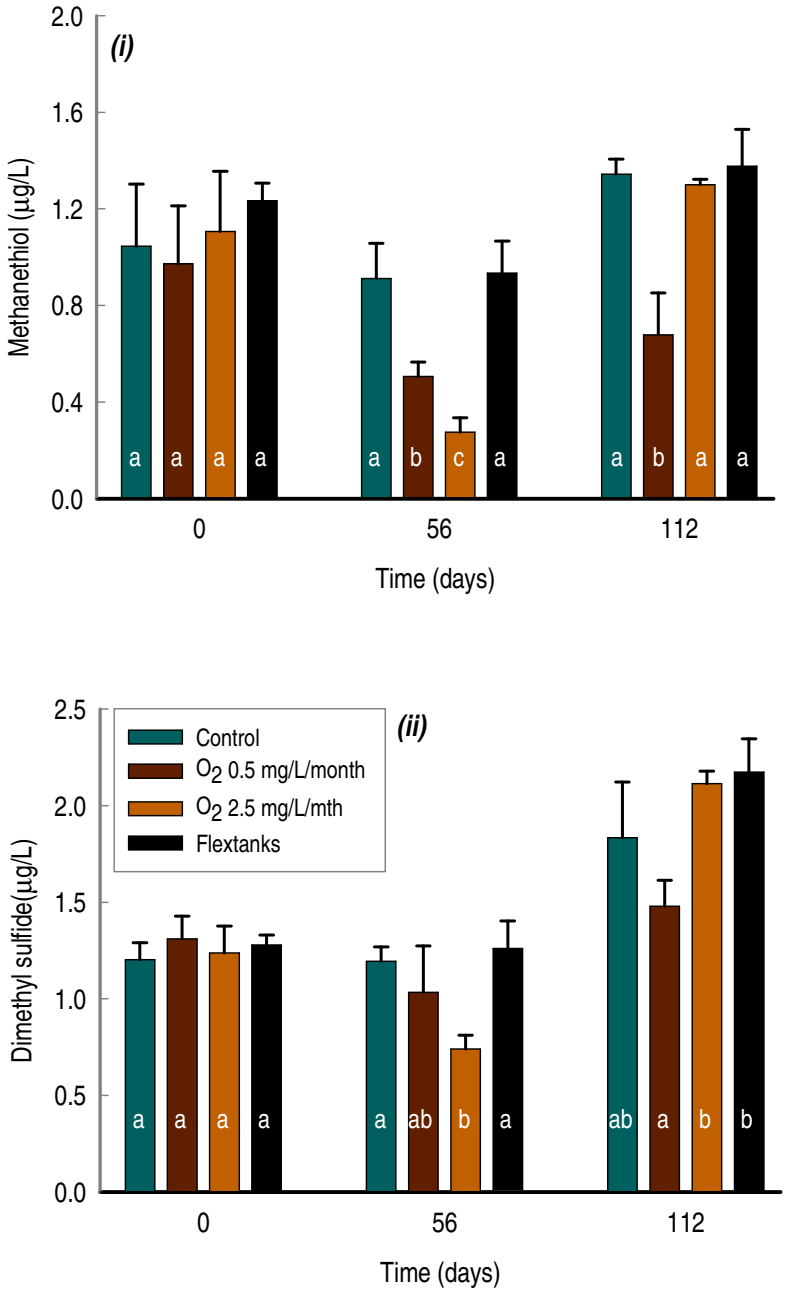


Figure 5.8. Effects of MOX on (i) MeSH and (ii) DMS in a BORDEAUX blend after MLF. Error bars represent the standard deviations of the mean (n = 3). Columns with different letters denote values which differ significantly ($P < 0.05$, LSD and Tukey HSD Post Hoc Tests) at the same observation date.

Thioether alcohols (2-methylthio-1-ethanol and 3-methylthio-1-propanol)

The evolution of two thioether alcohols, 2-methylthio-ethanol (MTE) and 3-methylthio-1-propanol (MTP), in the BORDEAUX blend wines are presented in Figure 5.9. Statistical analysis on the quantitative data for these two compounds indicates some impact of MOX treatments on these vegetable-liked odours in the middle of the trial (at day 56), when concentrations of MTE and MTP showed lower values ($P < 0.05$) in the both oxygen treated wines than that in the control and the Flextanks storage wines. At the end of the trial (day 112), the concentration of MTP was higher than in the middle of the trial and only the BORDEAUX wine that had a low oxygen dose (0.5 mg/L/month) exhibited a lower concentration ($P < 0.05$) of MTE and MTP, compared to the other wines.

It has been shown that methional at high levels was formed in white wine samples treated with oxygen via the Strecker mechanism involving the presence of methionine and methylglyoxal (Escudero *et al.*, 2000; Silva Ferreira *et al.*, 2002). In wine, methional can be either oxidised to form methylthio-propionic acid or reduced to form MTP. This explains that both the acid and the alcohol can be found concurrently in wine, while in beer methional is not oxidised to an acid (Landaud *et al.*, 2008). Whether methylthio-propionic acid can follow an esterification reaction to form the corresponding acetate ester 3-methylthio-propylacetate in wine has not yet been documented. However, it was demonstrated that this acetate ester formed from subsequent esterification of MTP, can also be a methionine-derived off-odour produced by yeast metabolism (Etschmann *et al.*, 2008). Being an ester, 3-methylthio-propylacetate can be hydrolysed to give free MTP, and this could explain the increase in the concentrations of MTP at day 112, compared to those at day 56 (Figure 5.9 ii).

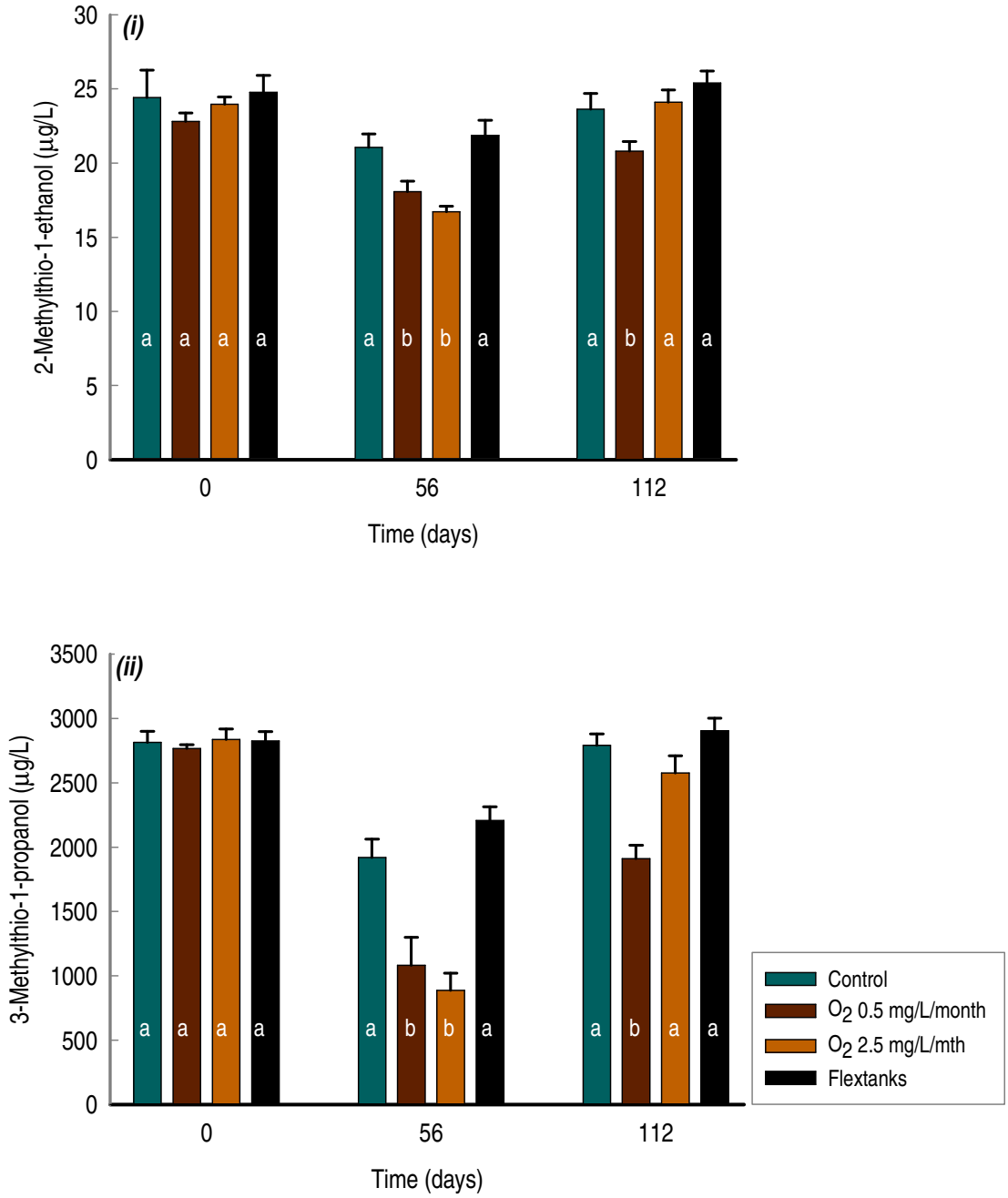


Figure 5.9. Effect of MOX on (i) MTE and (ii) MTP in a Bordeaux blend after MLF.

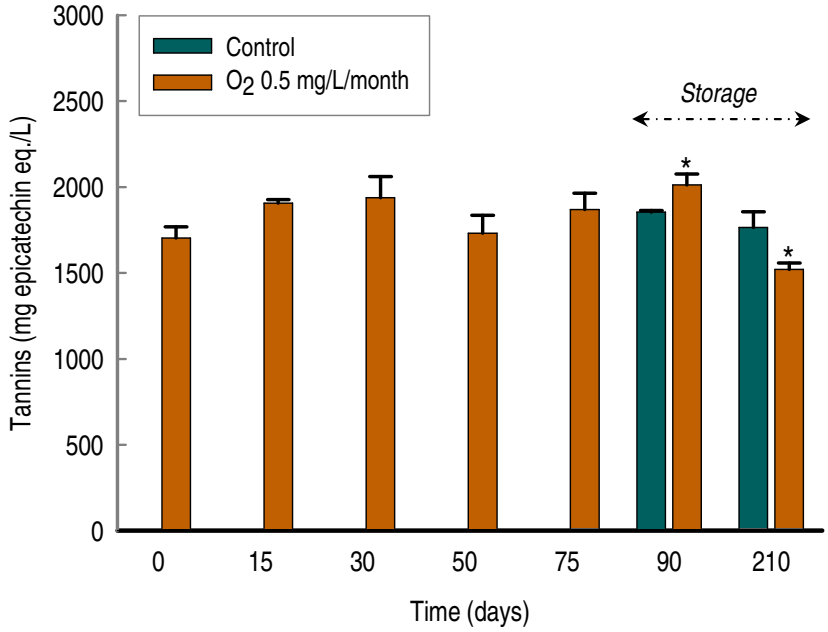
Error bars represent the standard deviations of the mean (n = 3). Columns with different letters denote values which differ significantly ($P<0.05$, Tukey HSD Post Hoc Test) at the same observation date.

It is very interesting to note that although MOX did show some clear impacts on lowering the detected reductive sulfur compounds found in the BORDEAUX blend wine after 56 days of oxygen application, its effects on these compounds only persisted in the low oxygen treat wines. This result suggests very complex chemical interactions and evolution of these volatiles in the complex red wine matrix after MLF in the presence of oxygen. The quantitative data here are also reflected in the sensory characteristics of the wines, and are discussed in the following part (See Section 5.2.5).

5.2.3. Effects of MOX after MLF on tannins

Changes in tannin concentration in the commercial Shiraz and BORDEAUX blend wines that underwent MOX were also monitored using the MCPT Assay, as detailed in Section 2.2.1. The results on the development of the MCP tannin in the Shiraz and BORDEAUX blend are presented in Figure 5.10 and Figure 5.11, respectively.

As can be seen from Figure 5.10, the concentration of MCP tannin in the commercial Shiraz fluctuated over time. The tannin concentration in the MOX Shiraz wine at day 90 showed a higher value than at day 0, while it decreased significantly ($P < 0.05$) after 4 months of storage. The tannin concentration in the control Shiraz also showed a decline from 1853 mg/L at day 90 to 1756 mg/L at day 210, but the decrease was not statistically significant ($P > 0.05$). An independent sample t-test was performed to compare the concentrations of tannin in the control and the micro-oxygenated wine at the end of the trial, and this indicated that MOX led to a significant increase in the concentration of tannin at the end of the MOX period. However, after 4 months of storage, the tannin concentration in the micro-oxygenated wine was significantly lower ($P < 0.05$) than that in the control. During wine maturation and ageing, monomeric anthocyanins and co-pigmented anthocyanins are transformed to more stable pigments via different types of chemical interactions, as previously discussed in Section 1.3.2 and 5.2.1.2. Some of these reactions are mediated by acetaldehyde, and are thus linked to the presence of oxygen (Atanasova *et al.*, 2002a; Gonzalez-del Pozo *et al.*, 2010)



Time (days)	0	15	30	50	75	90	210
Control						1853^{a}	1765^{a}
O_2 0.5 mg/L/month	1702^{ab}	1906^{bcd}	1938^{cd}	1731^{bc}	1869^{bcd}	2011^{d}	1519^{a}

Figure 5.10. Development of tannins in SHIRAZ during MOX applied after MLF.

Data present the mean values (mg/L) ± standard deviations of triplicate measurements. The asterisk indicates value which differs significantly from the control ($P < 0.05$, t-test). Values sharing the same letter are not significantly different ($P < 0.05$, Tukey HSD Post Hoc Tests) across time within treatment.

The monomeric anthocyanins are very unstable and can increase rapidly in size in the presence of available acetaldehyde, to form ethyl bridge-linked anthocyanin-flavanol compounds. These molecules can also be rapidly broken down, due to the relative high reactivity of the anthocyanin moiety, to release ethyl-flavanol units that may react again with anthocyanins or flavanyl dimers, leading to the formation of more condensed products or even polymers (Alcalde-Eon *et al.*, 2006). Hayasaka *et al.* (2003) have demonstrated the occurrence in wine of condensation products showing a degree of

polymerisation from dimers to octomers, which composed of an anthocyanin with a proanthocyanidin heptamer. This could explain the effects of oxygen on the increase in tannin concentration in the MOX Shiraz, observed at day 90, and the large drop in tannin concentration of the MOX Shiraz at day 210. Winemakers reported during a blind assessment of the wines, post trial, a preference for the MOX Shiraz at 45 and 60 days of treatments. The winemakers also considered that the treatment at 75 and 90 days had gone too far and that there had been a loss of aroma and the tannins were drying out.

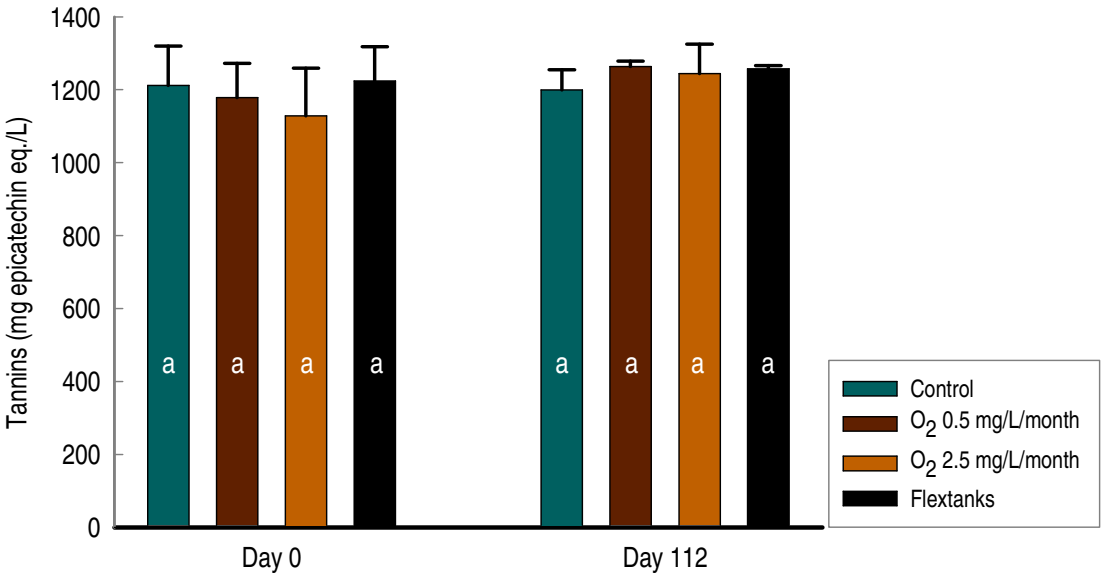


Figure 5.11. Tannins in a BORDEAUX blend micro-oxygenated after MLF.

Columns present the mean values (mg/L) ± standard deviations of triplicate measurements.

Columns sharing the same letter do not differ significantly (P > 0.05, Tukey HSD Post Hoc Tests) between treatments at the same time.

On the other hand, MOX showed no effect on the concentration of MCP tannin in the BORDEAUX blend wine during the course of the trial. The concentration of tannnin in the two micro-oxygenated wines and in the Flextank wine did not differ (P > 0.05) from each others' and from that in the control at day 112 (**Figure 5.11**). In our previous MOX trial

applied to a Cabernet Sauvignon wine after alcoholic fermentation, the concentration of MCP tannin were also not significantly different between treatments and the control, which may reflect the effects of both MOX and the confounding effect of spontaneous MLF. In the trial for both the commercial Shiraz and the BORDEAUX blend wines, MLF had been completed, and a higher concentration of tannins was thus expected as a result of the favourable effect of oxygen upon various chemical reactions, e.g. polymerisation and the incorporation of anthocyanin in tannin structure, assuming that the formations of these compounds may exceed the disappearance due to ethyl-linked cleavage reactions. However, the impact of MOX on tannin concentration was only significant for the Shiraz, while it did not show any effect on the tannin concentraton in the BORDEAUX blend. Results in a study by Cano-López *et al.* (2008) showed that higher percentage of anthocyanin derived pigments in the presence of oxygen is more favoured in a higher total phenol wine, indicating that different wines are differently affected by MOX, with the wine having lowest phenolic content being less influenced by MOX. In fact, the concentration of MCP tannin in the Shiraz was much higher than in the BORDEAUX blend to start with, at approximately 1700 mg/L compared to 1200 mg/L. The two wines used for these trials also had different concentrations of other monomeric polyphenols, with the Shiraz wine containing slightly higher concentrations of most of these compounds, except the monomeric anthocyanins. The effects of MOX on the development of the monomeric polyphenols are discussed in the following sections.

5.2.4. Effects of MOX after MLF on polyphenols

The development of monomeric polyphenols during the post-MLF MOX treatment, and after 4 months storage in the Australian Shiraz, and of the BORDEAUX blend during MOX, were also investigated. The results are present in Table 5.1 and Table 5.2.

It can be seen from Table 5.1 that the concentrations of most polyphenolic compounds in the Shiraz wines slightly decreased over time up to day 90, a process that usually occurs as a wine ages, although data are presented only for the micro-oxygenated wine. At day

210, concentrations of several polyphenolics, including gallic acid, (-)-epicatechin, *trans*-caftaric acid, *p*-coumaric acid, and the monomeric anthocyanins, showed slightly higher values than those at day 90, among which some were statistically different ($P < 0.05$) (Table 5.1), indicating some cleavage and hydrolysis reactions may have occured during storage. MOX did show some impact on the development of the polyphenols in the Shiraz wines. Lower concentrations ($P < 0.05$) of (-)-catechin, (+)-epicatechin, GRP, *p*-coumaric acid, quercetin-3-glucoside, quercetin, and monomeric anthocyanins were found in the oxygen treated, compared to the control (no oxygen) at the end of the MOX period (day 90).

Limited oxygen exposure promotes various chemical transformations which involve the participation of these compounds, such as flavanols and anthocyanins in the indirect acetaldehyde-mediated condensation reactions, giving pyranoanthocyanins, A-T and T-A adducts, ethyl-bridged (epi)catechin dimers and trimers (See Section 1.3.2). Lower concentrations of anthocyanins and flavanols has also been showed in other studies (Atanasova *et al.*, 2002a; Cano-López *et al.*, 2006; Tao *et al.*, 2007).

The concentration of the flavonol quercetin-3-glucoside was lowered slightly but significantly ($P < 0.05$) in the MOX Shiraz compared to the control at day 90, yet no significant difference in the concentration of the free quercetin was found. Regarding some of the hydroxycinnamic acids and their derivatives, the concentration of *trans*-caftaric acid (*trans*-caffeoyltartaric acid) was slightly lower in the MOX Shiraz, but it increased significantly as at day 210 to a level much higher than that in the control.The concentration of the related free caffeic acid in the MOX wine was lower, but did not differ from the control at day 210. In a study by Tao *et al.*, (2007), free caffeic acid was found to increase in accordance with a large drop in the level of caftaric acid, in MOX wines without added sulfur dioxide.

The difference in the levels of the individual polyphenolic compounds was diluted after 4 months of storage (day 210), when a significant difference was only observed in the

concentration of monomeric anthocyanins (Table 5.1). Gonzalez-del Pozo *et al.* (2010) also observed a small but significant increase in the degradation and transformation kinetics of anthocyanins. The effects, however, were only obvious in the short term, e.g. after MLF and during the MOX treatment after MLF (Gonzalez-del Pozo *et al.*, 2010).

Similarly, MOX treatments showed very limited effects on the development of monomeric polyphenols in the BORDEAUX wine (Table 5.2). Benzoic acids (gallic acid, benzoic acid) and hydroxycinnamic acids and esters (*trans*-caftaric acid, *cis*- and *trans*-coutaric acid, caffeic acid) were genarally unaffected by MOX, similar to the results found in Tao *et al.* (2007). No significant difference in the concentrations of flavanols, flavonols was found among the BORDEAUX wines that underwent different MOX treatments. MOX applied at the higher rate of 2.5 mg/L/mth show some impacts on the concentration of monomeric anthocyanins, and their concentration in the high oxygen treated wine was lower than in the other wines at the end of the trial (day 112). This may be due to their high reactivity, which results in their participation in different chemical reactions, leading to the transformation of wine colour and polyphenol structures, as well as the sensory characteristics of wine after MLF.

Table 5.1. Development of polyphenols in a SHIRAZ during MOX applied after MLF. Data present the mean values (mg/L) ± standard deviations of single measurements of triplicate treatments. Values followed by the same superscript letters do not differ significantly ($P > 0.05$, Tukey HSD Post Hoc Test) within treatment across time (lower case) and between treatments at the same time (upper case).

Time (days)	0	15	30	50	75	90	210
Gallic acid							
Control						$31.51^{aA} \pm 0.29$	$32.23^{bA} \pm 0.23$
O_2 0.5 mg/L/mth	$32.28^{ac} \pm 0.39$	$32.19^{ac} \pm 0.20$	$31.64a^{bc} \pm 0.30$	$31.52^{ab} \pm 0.35$	$31.17^{b} \pm 0.25$	$30.96^{bA} \pm 0.22$	$32.45^{cA} \pm 0.17$
Syringic acid							
Control						$4.72^{aA} \pm 0.06$	$4.80^{aA} \pm 0.03$
O_2 0.5 mg/L/mth	$4.95^{a} \pm 0.06$	$4.97^{a} \pm 0.17$	$4.65^{b} \pm 0.10$	$4.97^{a} \pm 0.01$	$5.30^{c} \pm 0.10$	$5.24^{cdB} \pm 0.01$	$5.02^{dB} \pm 0.04$
(+)-Catechin							
Control						$36.60^{aA} \pm 0.42$	$36.66^{aA} \pm 0.46$
O_2 0.5 mg/L/mth	$33.12^{a} \pm 0.54$	$34.96^{b} \pm 1.20$	$35.99^{bc} \pm 0.43$	$35.69^{b} \pm 0.43$	$35.88^{bc} \pm 0.41$	$35.51^{bB} \pm 0.18$	$37.37^{cA} \pm 0.19$
(-)-Epicatechin							
Control						$8.84^{aA} \pm 0.09$	$9.40^{bA} \pm 0.19$
O_2 0.5 mg/L/mth	$10.48^{a} \pm 0.48$	$10.69^{a} \pm 0.51$	$9.48^{b} \pm 0.32$	$8.03^{c} \pm 0.05$	$7.16^{d} \pm 0.19$	$7.22^{cdB} \pm 0.14$	$9.16^{bA} \pm 0.13$
trans-Caftaric acid							
Control						$0.98^{aA} \pm 0.00$	$1.10^{bA} \pm 0.00$
O_2 0.5 mg/L/mth	$0.90^{a} \pm 0.02$	$1.02^{bc} \pm 0.05$	$1.03^{c} \pm 0.01$	$1.02^{bc} \pm 0.01$	$0.96^{bd} \pm 0.01$	$0.95^{adB} \pm 0.01$	$2.16^{eB} \pm 0.00$
GRP							
Control						$6.97^{aA} \pm 0.01$	$6.52^{bA} \pm 0.01$
O_2 0.5 mg/L/mth	$7.14^{a} \pm 0.03$	$7.03^{b} \pm 0.05$	$6.94^{c} \pm 0.02$	$6.85^{d} \pm 0.01$	$6.85^{d} \pm 0.02$	$6.73^{eB} \pm 0.01$	$6.57^{fB} \pm 0.01$

Table 5.1. Continued

Time (days)	0	15	30	50	75	90	7 months
Caffeic acid							
Control						$27.03^{aA} \pm 0.31$	$26.90^{aA} \pm 0.95$
O_2 0.5 mg/L/mth	$28.12^{a} \pm 0.10$	$28.01^{ab} \pm 0.28$	$27.79^{abc} \pm 0.34$	$27.52^{abc} \pm 0.40$	$27.27^{bc} \pm 0.29$	$26.98^{cdA} \pm 0.21$	$26.31^{dA} \pm 0.33$
p-Coumaric acid							
Control						$12.90^{aA} \pm 0.11$	$13.47^{bA} \pm 0.10$
O_2 0.5 mg/L/mth	$13.22^{a} \pm 0.11$	$13.16^{a} \pm 0.03$	$13.01^{a} \pm 0.07$	$12.63^{b} \pm 0.11$	$12.32^{c} \pm 0.09$	$12.29^{cB} \pm 0.06$	$13.57^{dA} \pm 0.13$
Quercetin-3-glucoside							
Control						$10.14^{aA} \pm 0.16$	$10.05^{aA} \pm 0.12$
O_2 0.5 mg/L/mth	$10.37^{a} \pm 0.24$	$10.18^{ab} \pm 0.10$	$10.08^{abc} \pm 0.17$	$9.90^{bc} \pm 0.16$	$9.82^{bc} \pm 0.12$	$9.68^{cB} \pm 0.11$	$10.16^{abA} \pm 0.14$
Quercetin							
Control						$18.10^{aA} \pm 1.84$	$17.46^{aA} \pm 1.91$
O_2 0.5 mg/L/mth	$18.98^{a} \pm 2.86$	$18.87^{a} \pm 1.72$	$18.71^{a} \pm 2.31$	$17.48^{a} \pm 1.95$	$17.22^{a} \pm 1.68$	$16.14^{aA} \pm 1.36$	$18.14^{aA} \pm 2.06$
Total anthocyanins							
Control						$40.03^{aA} \pm 0.12$	$40.78^{bA} \pm 0.37$
O_2 0.5 mg/L/mth	$43.24^{a} \pm 0.22$	$38.85^{b} \pm 0.22$	$36.44^{c} \pm 0.52$	$30.67^{d} \pm 0.22$	$26.29^{e} \pm 0.07$	$26.15^{eB} \pm 0.12$	$39.37^{bB} \pm 0.22$

Table 5.2. Changes polyphenols during MOX applied to a BORDEAUX blend after MLF. Data present the mean values (mg/L) ± standard deviations of single measurements of triplicate treatments. The same superscript letters within a cell denote values that are not significantly different (P > 0.05, Tukey HSD Post Hoc Test) at the same observation date.

Time (days)	MOX treatments	Gallic acid	Syringic acid	Catechin	Epicatechin	*trans*-Caftaric acid	GRP
0	*Control*	$26.62^{a} \pm 0.34$	$5.22^{a} \pm 0.07$	$20.99^{a} \pm 0.13$	$20.50^{a} \pm 0.28$	$4.53^{a} \pm 0.05$	$4.12^{a} \pm 0.02$
	O_2 0.5 mg/L/month	$26.68^{a} \pm 0.08$	$5.24^{a} \pm 0.08$	$21.10^{a} \pm 0.66$	$20.50^{a} \pm 0.18$	$4.56^{a} \pm 0.06$	$4.13^{a} \pm 0.02$
	O_2 2.5 mg/L/month	$26.95^{a} \pm 0.10$	$5.28^{a} \pm 0.03$	$21.33^{a} \pm 0.38$	$20.66^{a} \pm 0.23$	$4.53^{a} \pm 0.01$	$4.13^{a} \pm 0.01$
	Flextanks	$26.87^{a} \pm 0.10$	$5.30^{a} \pm 0.08$	$21.57^{a} \pm 0.32$	$20.57^{a} \pm 0.08$	$4.53^{a} \pm 0.01$	$4.13^{a} \pm 0.01$
112	*Control*	$28.13^{a} \pm 0.78$	$4.92^{ab} \pm 0.04$	$20.08^{a} \pm 0.90$	$18.69^{a} \pm 0.60$	$4.77^{a} \pm 0.21$	$3.55^{a} \pm 0.08$
	O_2 0.5 mg/L/month	$28.53^{a} \pm 0.06$	$5.05^{a} \pm 0.06$	$19.76^{a} \pm 0.32$	$18.90^{a} \pm 0.33$	$4.68^{a} \pm 0.04$	$3.63^{bc} \pm 0.02$
	O_2 2.5 mg/L/month	$28.53^{a} \pm 0.10$	$4.84^{ab} \pm 0.17$	$20.07^{a} \pm 0.37$	$19.09^{a} \pm 1.93$	$4.67^{a} \pm 0.02$	$3.62^{b} \pm 0.02$
	Flextanks	$28.66^{a} \pm 0.11$	$4.70^{b} \pm 0.15$	$19.62^{a} \pm 0.10$	$17.54^{a} \pm 0.33$	$4.73^{a} \pm 0.02$	$3.71^{c} \pm 0.01$

Table 5.2. Continued

Time (days)	MOX treatments	*cis*-Coutaric acid	*trans*-Coutaric acid	Caffeic acid	Quercetin-3-glucoside	Quercetin	Total anthocyanins
0	*Control*	$0.78^{a} \pm 0.04$	$0.68^{a} \pm 0.05$	$5.61^{a} \pm 0.12$	$9.50^{a} \pm 0.05$	$6.29^{a} \pm 0.05$	$153.55^{a} \pm 1.60$
	O_2 0.5 mg/L/month	$0.80^{a} \pm 0.00$	$0.78^{a} \pm 0.08$	$5.64^{a} \pm 0.02$	$9.49^{a} \pm 0.02$	$6.37^{a} \pm 0.08$	$154.45^{a} \pm 0.67$
	O_2 2.5 mg/L/month	$0.80^{a} \pm 0.02$	$0.74^{a} \pm 0.03$	$5.70^{a} \pm 0.04$	$9.57^{a} \pm 0.04$	$6.56^{a} \pm 0.28$	$154.70^{a} \pm 0.27$
	Flextanks	$0.79^{a} \pm 0.02$	$0.76^{a} \pm 0.03$	$5.68^{a} \pm 0.02$	$9.54^{a} \pm 0.08$	$6.46^{a} \pm 0.10$	$152.43^{a} \pm 2.16$
112	*Control*	$0.74^{a} \pm 0.01$	$0.73^{a} \pm 0.86$	$5.70^{a} \pm 0.10$	$9.53^{a} \pm 0.13$	$5.92^{a} \pm 0.36$	$136.02^{a} \pm 6.55$
	O_2 0.5 mg/L/month	$0.75^{a} \pm 0.02$	$0.86^{ab} \pm 0.01$	$5.73^{a} \pm 0.01$	$9.55^{a} \pm 0.02$	$6.25^{a} \pm 0.22$	$135.20^{a} \pm 4.12$
	O_2 2.5 mg/L/month	$0.74^{a} \pm 0.01$	$0.84^{b} \pm 0.04$	$5.65^{a} \pm 0.08$	$9.58^{a} \pm 0.03$	$6.37^{a} \pm 0.20$	$125.92^{b} \pm 5.51$
	Flextanks	$0.75^{a} \pm 0.01$	$0.94^{c} \pm 0.02$	$5.59^{a} \pm 0.15$	$9.64^{a} \pm 0.03$	$6.21^{a} \pm 0.05$	$138.83^{a} \pm 0.56$

5.2.5. Effects of MOX on the sensory characteristics of the BORDEAUX blend

Red wines of high quality are traditionally stored in oak barrels for a long time to improve their sensory characteristics. The extraction of many oak substances then participate in wine transformation, enhancing colour stabilisation, lowering astringency and removing unwanted excess vegetative notes (Llaudy *et al.*, 2006; Ribéreau-Gayon *et al.*, 2006b; Caillé *et al.*, 2010). These changes are associated with small quantities of oxygen that ingress through the wood barrel during ageing, although the oxygen dissolution through barrel staves cannot be controlled. MOX, therefore, might be an alternative or complement to barrel maturation and ageing of wine, as a technique that can reproduce and stimulate the transformations of colour and polyphenolics development, as well as changes in aromas, that occur during oak ageing (Parish *et al.*, 2000; Llaudy *et al.*, 2006). Compared to the scientific research on the effects of MOX on wine colour and polyphenols, studies on the impacts of MOX on sensory characteristics of wine are still very limited, although more publications related to this topic have become available in recent years (Llaudy *et al.*, 2006; du Toit, 2007; Hernandez-Orte *et al.*, 2009; Cejudo-Bastante *et al.*, 2011a, 2011b). Formal sensory evaluation with regard to reductive notes, however, to the best of our knowledge, has not been reported elsewhere, except in a report by Ortega-Heras *et al.* (2008). It was mentioned in this research that informal sensory analysis carried out by a group of winemakers and technicians was employed to assess the wine's initial characteristics, including the reductive notes, which served as information to select the appropriate oxygen dosage rate (Ortega-Heras *et al.*, 2008). No chemical quantitative nor sensory data regarding the reductive notes was reported in this study.

Since the first MOX trial applied after alcoholic fermentation revealed some impacts of MOX on reductive sulfur containing compounds, it was decided to undertake sensory analysis of the Bordeaux wine that underwent different MOX treatments in the second

trial applied, but after MLF. This was undertaken in order to examine how MOX affected the sensory characteristics of the wine at the end of the MOX treatments, in correlation with the changes in the chemical profiles of the wine, especially those related with the flavours, reductive odours and mouth-feel. The addition of sensory analysis provides more insight into the effects of MOX on a wine's off-odours, in terms of both chemical changes and sensory impacts. The design of the sensory tests and statistical treatment of the results were described in Section 2.3.

The triangle test was initially employed to investigate the global sensory difference that may exist between any pair of samples, including the control BORDEAUX blend without oxygen addition, the two oxygen treated wines (at 0.5 mg/L/month and 2.5 mg/L/month), and the BORDEAUX blend wine kept in Flextanks. The results of this test are presented in Table 5.3. The triangle test results indicated that there was a significant overall sensory difference between the BORDEAUX blend treated with a low oxygen rate of 0.5 mg/L/month and the Flextanks stored wine. In the other words, there were perceived effects produced by the amounts and the different ways oxygen was supplied, namely through an oxygen diffuser placed at the bottom of the tank, and oxygen permeating through the dense polyethylene of the Flextanks. The sensory difference was significant at an α-risk[35] of 0.01 or 1 %[36] with 10 out of 15 panellists giving the correct answer (Table 5.3), providing strong evidence that there was an apparent difference between the two wines. A significant difference in the overall sensory characteristics was also found between the control and the Flextanks wine at α-risk of 0.10 or 10 %, indicating moderate evidence that the difference was apparent. In a MOX study by (Llaudy *et al.*, 2006), a

[35] α-risk is the probability of concluding that a perceptible difference exists when it does not (Type I error or false positive), while β-risk is the probability of concluding that no perceptible difference exists when it does (Type II error or false negative). In a basic triangle test for difference, the objective is purely to reveal whether the difference exists between two samples. The statistical analysis in this case is made under the assumption that only the α-risk matters and the β-risk is unimportant (Meilgaard *et al.*, 1999).

[36] The test will falsely conclude a difference only 1 % of the time.

triangular test was also employed to determine the difference between the MOX wine (micro-oxygenenated at 3 mg/L/month in 275 L stainless steel tanks for 3 month, followed by 8 months of oak ageing) and the control (8 months of oak ageing, then 3 months being kept in stainless steel tanks[37]). The test indicated that MOX applied before oak ageing in this study produced a diferent wine from the control, supported with 7 out of 10 tasters who could differentiate the two wines, corresponding to a statistically significant level of 0.05 (also see **Appendix 2-15**). Du Toit (2007) also found that tasters, composed mostly of enologists, were able to distinguish between the control and the 3 mg O_2/L/month Cabernet Sauvignon wine at 8 weeks after the treatments started, while after 12 weeks the panel could differentiate all wines (0, 1.5 and 3 mg O_2/L/month) from each other.

Table 5.3. The overall sensory differences of the BORDEAUX BLEND underwent different MOX treatments after MLF, by means of triangle test analysis.

Pair wines*	Number of panellists	Number correct	Existence of overall difference
Control vs. treatment 1	15	4	No
Control vs. treatment 2	15	4	No
Control vs. treatment 3	15	8	**Yes at α = 0.10**
Treatment 1 vs. treatment 2	15	6	No
Treatment 1 vs. treatment 3	15	10	**Yes at α = 0.01**
Treatment 2 vs. treatment 3	15	7	No

*The test were carried out in pair among four wines, including the control (no oxygen), treatment 1 (0.5 mg O_2 /L/month), treatment 2 (2.5 mg O_2 /L/month) and treatment 3 (Flextank stored wine).

[37] According to the authors' argument, this is to reproduce the typical conditions of commercial cellars, where oak ageing was often performed before storage in stainless steel tanks to enhance wine quality.

Table 5.4. Differences in the sensory characteristics of the BORDEAUX blend that underwent different MOX treatments after MLF, by means of a ranking test - multisample difference with a randomised (complete) block design. Numbers represent the rank sum given to each attribute by all panellists. Rank sums sharing the same letter are not different at $\alpha = 0.05$, Fisher's LSD ($t_{0.05/2,\infty}$) = 8.2. The asterisk denotes the rank sums that are different at $\alpha = 0.01$, Fisher's LSD ($t_{0.01/2,\infty}$) = 10.8.

	Vegetal	Fruity	Oxidised	Reductive	Astringency	Mouthfeel
Control	40 a	31.5 a	37 a	37.5 a	27.0 a	32.0 a
O_2 0.5 mg/L/month	**28 b***	32.5 a	29 a	**25.5 b***	**36.5 b**	37.0 a
O_2 2.5 mg/L/month	41 a	38.5 a	33 a	36.0 a	**41.0 b***	33.5 a
Flextanks	**31 b**	37.5 a	41 a	41.0 a	**35.5 b**	36.5 a

*The test were carried out in pair among four wines, including the control (no oxygen), treatment 1 (0.5 mg O_2 /L/month), treatment 2 (2.5 mg O_2 /L/month) and treatment 3 (Flextank stored wine).

Table 5.4 presents results from a *multisample difference tests* with a *randomised (complete) block design*, which was undertaken to test the sensory characteristics of the control and the other three micro-oxygenated BORDEAUX blend wine samples. There were six selected single attributes, including four aromas (vegetal, fruity, reductive and oxidised notes) and two tastes (astringency and mouth-feel).

It is one of beneficial claims that MOX could lower the vegetative note or the herbaceous/green character of wines (Parish *et al.*, 2000). Multisample difference testing indicated a significantly lower expression of perceived vegetal notes in the low oxygen added BORDEAUX blend (significant level $\alpha = 0.01$) and the Flextanks stored wine ($\alpha = 0.05$), compared to the control and the wine having high oxygen dosage rate (Table 5.4). C_6-alcohols, such as hexanol, *cis*- and *trans*-3-hexen-1-ol, are volatile compounds responsible for the vegetative characters of wines (Ortega-Heras *et al.*, 2008). Despite the lower concentration of the green flavoured *trans*-2-hexen-1-ol found in the oxygen-treated

Merlot wines, no significant lowering in the green attribute was observed in the study of Cejudo-Bastante *et al.* (Cejudo-Bastante *et al.*, 2011b). On the other hand, Ortega-Heras *et al.* (2008) found a similar or higher concentrations of the leafy C_6-alcohols, and suggested that the decrease of grassy notes in the MOX wines observed in other studies, such as reported in Pour-Nikfardjam & Dykes (2003), should be due to other compounds.

With regard to the impact of different MOX treaments on the perceived sensory properties of the BORDEAUX wine in terms of reductive and oxidised notes, the total rank sum of these attributes indicated that the reductive note was lowered in the low oxygen treated wine. The Flextanks wine received the highest rank in the reductive smell, followed by that of the control, and the high oxygen added wine, yet there was no significant difference in the reductive attribute of these wines. Changes in the reductive note in the different treatment wines at the end of the trial did not lead to the perception of any corresponding oxidised note, as the rank sum given by the panellists to the oxidised attribute was similar. It was suspected that the expression of the vegetal and reductive aromas may mask the perception of the fruity aromas. According to the results from the multisample ranking test, although the high oxygen added wine and Flextanks had the highest and the second highest in the fruity note rank sum, all the wines were ranked statistically similar in fruity character, despite a loss in the vegetal and reductive smells in some wines. The sensory effects of MOX on the reductive notes are somewhat correlated with the quantitative chemical results for these compounds, as discussed in Section 5.2.2.2. In a MOX trial applied at a rate of 60 mL/L/month for 15 days on a Cabernet Sauvignon wine, Hernandez-Orte *et al.* (2009) also reported a significant lowering in the intensity of the reduction attribute, given by panellists on a 7-point scale, in the micro-oxygenated wine compared to the control, 8 months after MLF. No quantitative data on the reductive sulfur compounds was presented in this study, except for 3-methylthio-propanol (methionol). The concentration of methionol in the MOX wine at 4 months after MLF was reported to be significantly higher than that of the control, yet no difference in the reductive note between the two wines was found. Concentrations of this compounds in the MOX and control wines at 8 months were not available in this study, although the perceived

reduction note was different (Hernandez-Orte *et al.*, 2009).

Since the beneficial effects of MOX are attributed to the interactions of polyphenols with other chemical components in the wine matrix, astringency and mouth-feel are expected to be influenced by the application of MOX, and thus were included in the sensory attributes examined.

Wine astringency is a tactile sensation resulting from the precipitation of salivary proteins leading to a loss of mouth lubrication and is mainly attributed to the presence of wine tannins (Vidal *et al.*, 2004). It is reported that astringency increases with an increase in the size of proanthocyanidins (Peleg *et al.*, 1999; Vidal *et al.*, 2003). Pour-Nikfardjam & Dykes (2003) found that when MOX is applied for too long, the wines become too astringent as a consequence of the increasing in mDP. Llaudy *et al.* (2006) demonstrated that MOX applied before oak ageing produced wines with a slightly but significantly higher mDP, and a considerably lower astringency, which was evaluated by both chemical analysis using ovalbumin as a precipitation and tannic acid solutions as standards (Llaudy *et al.*, 2004), and by sensory analysis. The astringency of a Petit Verdot wine was also found to be lowered by MOX treatment applied at 45 mL/L/month for 20 days before MLF in a study by Cejudo-Bastante *et al.* (2011a). On the other hand, du Toit (2007) found by means of an intensity test for astringency that tasters could not distinguish differences among different micro-oxygenated Pinotage wines supplied with 0, 1.5 and 3 mg O_2/L/month. All three Pinotage wines were in contact with American oak staves, plus the same Pinotage wine was kept in American oak barrels from the same cooperage in the fourth treatment (du Toit, 2007).

Mouth-feel is a general taste perception which measures how the wine fills the palate. A wine that is considered weak or thin mouth-feel is only perceived on the centre line of the tongue, while a big or full mouth-feel wine is felt strongly on the tongue and the inside of the cheeks (Dykes, 2007). Our results indicated that MOX did not result in any difference

in the mouth-feel property of the BORDEAUX blend wines, and a similar rank sum was given by the panel.

CHAPTER 6. CONCLUSION

6.1. CONCLUDING REMARKS

Among other beneficial effects that micro-oxygenation (MOX) can bring about to improve the quality of wine, the technique is believed to be capable of removing the undesirable reductive character of wine. The claim, however, has not been fully supported by scientific research, in terms of both quantitative and sensory perspectives. In order to examine this subject, fully replicated MOX treatments on red wines, employed after alcoholic fermentation, and also post malolactic fermentation, were conducted. Wines from a commercial MOX trial on a much larger scale were also monitored for the development of the wine's constituents through out the MOX period and after a few months of storage.

A demanding analytical procedure required to quantify the reductive sulfur containing volatiles of interest was developed. This was a prerequisite to the subsequent MOX trials

that investigated the impacts of limited oxygen exposure on the chemical profiles and sensory characteristics of red wines, with a focus on the unwanted off-odours. After an initial attempt to analyse these compounds by Gas Chromatography - Flame Photometric Detector, the analytical methodology employed in the research was an automated Head Space Solid Phase Micro-Extraction (HS-SPME) coupled with a Gas Chromatography - Mass Spectrometry Detector (GC-MSD), which was successfully established and validated to quantify the sulfur compounds. The method allows the quantification of both low and highly volatile sulfur compounds at levels below their perception thresholds.

6.1.1. Effects of MOX on wine colour development

MOX treatments were applied after alcoholic fermentation to a Cabernet Sauvignon wine, but spontaneous MLF occurred about half way through the oxygenation trial. The MOX and Flextank treatments showed a number of effects on the development of wine colour, and a higher value was seen with pigments resistant to SO_2 bleaching during the first half of the trial and in colour density during the second half of the trial. At the same time, the occurrence of MLF appeared to act as a moderating factor restricting the difference in colour properties that were able to develop in the oxygenation treatments.

MOX applied after malolactic fermentation to the BORDEAUX blend wine (0.5 and 2.5 mg/L/month), and oxygen diffusing through the polyethylene tanks, also resulted in improved wine colour characteristics, including increased colour density and pigment resistant to sulfur dioxide bleaching. The high oxygen rate treated wine had the most effect on the colour density and pigments resistant to sulfur dioxide bleaching. Commercial scale MOX trials, using oxygen at a rate of 0.5 mg/L/month with an Australian Shiraz, again indicated the beneficial influence of MOX on wine colour density, which persisted after oxygenation was completed, in this case after 4 months of storage.

Results from the two fully replicated trials and the commercial trial indicated that MOX induced the colour transformation in the wines towards a more intense colour and higher

sulfur dioxide resistant pigments. The extent of the effects also depends on the oxygen dosage and the time the MOX was applied, with increase in wine colour remained during storage after stopping oxygen supply.

6.1.2. Effects of MOX on 3-MH and the reductive sulfur containing compounds

Oxygen did not affect the concentration of the desirable sulfur containing varietal thiol 3-MH in the Cabernet Sauvignon, but did show some positive impacts on the sulfur compounds detrimental to wine aromas. The results indicated that oxygen could lower the concentration of MeSH, for example, through an interaction with oxidised polyphenol quinones, but without an associated increase in the concentration of the disulfide DMDS, expected through direct oxidation of MeSH. The concentrations of other reductive sulfur compounds, except the thioesters, were also affected by the presence of oxygen. These losses have the potential to impact in a favorable manner on the removal of unwanted reductive odours from red wines, an issue that needs to be examined in future studies combining sensory analysis with the chemical analysis of reductive sulfur compounds during MOX.

Similarly, MOX post malolactic fermentation showed some impacts of oxygen on the unwanted reductive sulfur compounds in both the commercial micro-oxygenated Shiraz and the BORDEAUX blend from the pilot scale replicated MOX trial. Lower concentrations of MeSH were found in both MOX wines, and a large decline in MeSH was clearly seen in the Shiraz during oxygenation, again without the concurrent formation of DMDS, indicating that the chemical reaction of this thiol with wine oxidised polyphenols is occuring. The low oxygen rate applied in the commercial trial on Shiraz did not show clear effects on other groups of sulfur compounds, but the concentration of dimethyl sulfide in the oxygen treated wine was surprisingly higher than that of the control after 4 months of storage. Although the Shiraz had a concentration of 3-methylthio-1-propanol higher than its perception threshold, the winemakers did not notice any reduction note throughout the

course of the trial. The MOX treatments in stainless steel tanks on the BORDEAUX blend led to a lowering in the concentrations of most of the reductive sulfur compounds throughout the trial, but the effects only persisted in the wine having a low oxygen rate of 0.5 mg/L/month, while the Flextanks behaved similar to the control in terms of off-odours evolution.

Our findings provide quantitative chemical data to support the claimed beneficial effects of MOX that it can eliminate the reductive odours in wine. Oxygen, whether being applied before for after MLF in this research could lower the concentrations of most reductive sulfur compounds, including thiols, thioesters and thioacohols. The decrease in the concentration of MeSH without concurrent formation of DMDS suggests the incorporation of the thiol into the structure of wine polyphenols.

6.1.3. Effects of MOX on tannins and monomeric polyphenols

Tannins and monomeric polyphenols, except the highly oxidisable flavanol epicatechin, were largely unaffected by MOX treatments applied to the Cabernet Sauvignon wine. Limited effects MOX on lowering the concentrations of monomeric anthocyanins were seen from a quantitative viewpoint, yet the difference was not statistical different, due to large variations in the triplicate tanks where malolactic fermentation occurred at different time points.

MOX treatment of a Shiraz at 0.5 mg/L/month for 90 days led to an increase in tannin concentration at the end of the trial. Howerver, a large drop in tannin concentration in the MOX Shiraz occurred after 4 months of storage. This can be related to the winemakers' note that the oxygenation at 75 and 90 days might have gone too far and that a loss of aroma and a drying out of tannins had occurred. On the other hand, tannin in the BORDEAUX blend was unaffected by MOX post malolactic fermentation. Limited effects of MOX were seen with regards to the concentration of individual polyphenols, in a way that significant lower levels were only found for highly reactive compounds like the monomeric

anthocyanins in the Shiraz wine and in the BORDEAUX blend treated with a higher oxygen rate of 2.5 mg/L/month.

6.1.4. Effects of MOX on the sensory characteristics

With regard to the influence of MOX on the perceived sensory characteristics, triangle tets revealed that panellists were able to distinguish wines from different treatments. It is especially interesting to note that the effects MOX on the reductive sulfur compounds from a quantitative perspective was somewhat reflected in the sensory analysis. This was undertaken with the BORDEAUX blend wine at the end of the trial, when the wine received low oxygen addition of 5 mg/L/month, and showed a significantly lower reductive note compared to all of the other wines. The effects of MOX on the other aroma notes, namely fruity flavour and oxidised smell, were not noticeable, and a lowered herbaceous note was only apparent in the Flextank wine. MOX did not result in any difference in the mouth-feel but a higher astringency taste was perceived in the oxygen added wines and the Flextank wine, compared to that of the control.

6.1.5. Impacts of oxygen permeating through the polyethylene Flextank

The Cabernet Sauvignon wine stored in the polyethylene Flextank had a consistently higher colour density than the control wine at every observation date, in the MOX trial applied after the alcoholic fermentation. However, when MOX was applied after MLF, the colour development of the BORDEAUX blend stored in Flextanks tracked between these of the two oxygen treated wines as expected because the oxygen rate permeated through the polyethylene of Flextank was reported to be equivalent to 2 mg/L/month, compared to the low and high oxygen rates of 0.5 and 2.5 mg/L/month in the two MOX wines.

The removal of reductive aromas was also as effective in the Flextank as in the higher MOX (20 mg/L/month) treated wines. The impact of the Flextank upon colour and aroma

could involve oxygen permeation through the tanks combined with a small interaction with the polyethylene surface. On the other hand, although it was expected that Flextank storage could have effects on the chemical development of the wine similar to the wine receiving high oxygen rate (2.5 mg/L/month), data from MOX trial after MLF indicated that the BORDEAUX blend stored in Flextanks behaved similar to that of the control with regard to the off-odours evolution, which was also somewhat reflected in the sensory characteristics of the wines evaluted at the end of the MOX period. An interpretation for these observation could lie in the fact the Flextank tanks were reused from the previous trial which may have wine deposits plugged in the polyethylene pores, thus diminishing the oxygen getting inside the tanks.

6.2. FUTURE PERSPECTIVES

The results found in this study imply the need for further investigation on the effects of MOX with regard to the development of reductive sulfur containing compounds, alongside the evolution of other wine components. This especially applies to the major oxidation substrate polyphenols, and would be best applied for an extented period of time after MOX treatment, such as during storage and bottling.

Given that the oxygenation applied after alcoholic fermentation, and even during spontaneous malolactic fermentation, did show some positive impacts on the detrimental sulfur off-odours, a study on the extent that MOX affects these compounds during the period just after alcoholic fermentation and before malolactic fermentation would be interesting to examine the effects of oxygen on these compounds, without the confounding effects of malolactic fermentation. Quantitative analysis of the reductive sulfur compounds, along with polyphenols in wine undergoing such a treatment, should also be accompanied with sensory evaluation in order to correlate the chemical composition with the perceived sensory characteristics of the wine.

The effects of MOX on wine did appear to vary depending on the grape variety, and the wine matrix may modify the perception threshold of the reductive notes. More study on the mechanism that occurs as reductive sulfur compounds are integrated with the wine matrix, and interact with wine polyphenols, e.g. during storage and bottling, therefore, is essential.

Since MOX has been used to simulate the behaviours of wine aged in oak barrels, it would be worthwhile to compare the observed changes in reductive sulfur containing compounds of wine micro-oxygenated in tanks to that of wine aged in oak barrels.

Keeping in mind that MOX could be used as an alternative or complement to oak ageing, the technique could also be used for wine with lighter tannin content but largely matured and aged in barrels. It should be noted that Pinot noir is becoming the most important red variety in New Zealand and is following the international success story of the unique New Zealand Sauvignon blanc. The effects of MOX on Pinot noir largely remained unexplored due to the smaller tannin content in this wine, thus is mainly aged in oak barrels. Considerable cost-savings, therefore, could be made, should a suitable MOX strategy be implemented with Pinot noir wines.

Appendices

Appendix 1-1. Ceramic oxygen spargers

(Photo courtersy of Kauri NZ Ltd, Wellington, NZ)

Appendix 2-1. Set-up of the micro-oxygenation trials

Appendix 2-2. Participant information sheet

THE UNIVERSITY OF AUCKLAND
NEW ZEALAND
Chemistry Department
Science Centre, Building 301
23 Symonds St, Auckland Central
The University of Auckland
Private Bag 92019
Auckland, New Zealand

PARTICIPANT INFORMATION SHEET

(To the participants of the sensory panel for examining the effects of micro-oxygenation - a winemaking technique on the sensory characteristics of red wines)

Project title: *Effects of micro-oxygenation on red wine – A focus on the reductive odours*
Name(s) of Researcher(s): Dang-Dung Nguyen (PhD Student)

My name is Dang-Dung Nguyen. I am a PhD student at the University of Auckland enrolled in the Department of Chemistry, Wine Science Program. I am carrying out research on the effects of micro-oxygenation on red wine, with a focus on its influence on the undesirable reductive sulfur containing off-odours in wines.

Micro-oxygenation is a winemaking technique that has been using in a number of wineries worldwide, including in New Zealand. As part of my research, which has been conducted in conjunction with Pernod Ricard New Zealand, there is a requirement to correlate chemical changes with sensory characteristics of the final wine at the end of different micro-oxygenation treatments. This will give more insights into the effects of micro-oxygenation on the off-odours in wines in terms of both chemical changes and sensory impacts.

You are invited to take part in a trained sensory panel that I am going to setup for my research since you might already have experience in wine tasting or be interested in gaining an enhanced understanding about the sensory attributes of wine, improving your own sense and awareness of the aromas and taste normally present in commercial wines, as well as the abilities in discriminating these attributes. As you will be tasting and sniffing wines, which usually contain sulfur dioxide as a preservative, you must inform the Principal Researcher before the sensory sessions if you are a chronic asthmatic or are allergic to sulfur dioxide.

You will be asked to participate in the training sessions before the actual tastings. In the training sessions, you will be given samples containing standards which represent the attributes, such as canned juices of different flavours to represent “fruitiness” and “cranberry juice” to represent “astringency”. The compounds given to you in a base red wine to sniff or taste occur naturally in wines. They are not hazardous at the concentration given and you are expected to expectorate the sample in any case as part of the tasting protocol. The training sessions will be 1-2 hours at the first two sessions and then 1 hour in following few weeks before the actual tasting sessions.

You will then participate in the actual tasting sessions for my data collection. In the first tasting session, you will be asked to taste four different research wines to identify if there is any overall difference among these samples. The next tasting session will involve tasting the research wines in order to rank the intensity of the attributes that you have been trained in the training sessions. This will be no more than 2 hours in each tasting sessions and you will be given a break of about 15 minutes before the next set of samples are presented to you.

Your participation is voluntary. You must be over 18 years of age in order to take part in this study.

The raw data as collected in the ballot sheets given to you in each sensory session will be stored in a locked draw at the University premises until 31.07.2010. After that, they will be destroyed by a shredder and disposed into a paper waste bin.

All participants have the right to withdraw from participation at any time without any reason. You also preserve your right to withdraw your information at any time before 31.07.2010.

Although the data collected will be used for my PhD thesis or publication, it will be done in a way that does not identify you as part of its source. Your identity and the data provided by you will be kept strictly confidential.

The company, Pernod Ricard New Zealand, does not provide any funding for this project. However, they have generously provided their wine and given access to their wineries to conduct the research. Although the knowledge gained from this study will be shared with the company, it will be done in a way that does not identify you as a source of its information.

Thank you very much for your precious time and help which makes this study possible. If you have any questions or wish to know more, please phone me at my number given below or write me at:

Wine Science

Department of Chemistry

The University of Auckland

Private Bag 92019, Auckland.

Tel: 373 7599 etxn. 84267 or 021269227

My supervisor is:

Assoc. Prof. Paul Kilmartin, Department of Chemistry, The University of Auckland, Private Bag 92019, Auckland. Tel. 373-7599 extn 88324

The Head of Department is:

Prof. James Metson, Department of Chemistry, University of Auckland, Private Bag 92019, Auckland. Tel. 3737599 extn 83877

APPROVED BY THE UNIVERSITY OF AUCKLAND HUMAN PARTICIPANTS ETHICS COMMITTEE ON ..... for (3) years, Reference Number 2009/444

Appendix 2-3. Consent form

THE UNIVERSITY OF AUCKLAND
NEW ZEALAND
Chemistry Department
Science Centre, Building 301
23 Symonds St, Auckland Central
The University of Auckland
Private Bag 92019
Auckland, New Zealand

CONSENT FORM

(To the participants of the sensory panel for examining the effects of micro-oxygenation - a winemaking technique on the sensory characteristics of red wines)

THIS FORM WILL BE HELD FOR A PERIOD OF 6 YEARS

Project title: *Effects of micro-oxygenation on red wine – A focus on the reductive odours*
Name(s) of Researcher(s): Dang-Dung Nguyen

I have read the Participant Information Sheet; have understood the nature of the research and why I have been selected. I have had the opportunity to ask questions and have them answered to my satisfaction.

- I agree to take part in this research.
- I am over the age of 18 years.
- I do not suffer from severe asthma. I am not allergic to sulfur dioxide.
- I understand that I am free to withdraw participation at any time, and to withdraw any data traceable to me up to 31/07/2010.

I have understood that the participation in this study is voluntary. Although the data collected will be used for the researcher's PhD thesis or publication, it will be done in a way that does not identify me as part of its source. My identity and the data provided will be kept strictly confidential.

Name _____
Signature _____ Date _____/_____/_____

APPROVED BY THE UNIVERSITY OF AUCKLAND HUMAN PARTICIPANTS ETHICS COMMITTEE ON ..... for (3) years, Reference Number 2009/444

Appendix 2-4. Advertisement for sensory panel

WINE SENSORY ANALYSIS

We are looking for volunteers to taste wine!

The aim of this study is to see if different maturation treatments (micro-oxygenation) can affect the sensory characteristics of wines.

Whether you have already got wine tasting experiences or are interested in tasting wines and wanting to improve your sense and ability in wine tasting, you are invited to participate in the sensory analysis of my research wines.

This involves several training sessions and three to four actual tasting sessions. Each will last no more than 2 hours. These sessions will be held in early December 2009 and some in late February 2010 at Wine Science Tasting Room, Building 740, University Tamaki campus, Glen Innes.

Any person who wish to know more information or is interested in participating in this wine tasting, please contact me at the address below:

Dang-Dung Nguyen (Zum)

Wine Science, Building 740 Room 229, Tamaki campus

Email: dngu031@aucklanduni.ac.nz

APPROVED BY THE UNIVERSITY OF AUCKLAND HUMAN PARTICIPANTS ETHICS COMMITTEE ON ..... for (3) years, Reference Number 2009/444

Appendix 2-5. Score sheet given to panellists during training session 1

Training session 1

Date: _____/_____/_____

Name:_____

In this session, samples with a single wine reference standard in model wine are prepared using the reference standards as listed in Table 1 below.

These samples are provided and you are asked to sniff ONLY samples containing the first four standard aromas. TASTE and then EXPECTORATE samples containing standards for astringency and mouthfeel.

Please read the description/lexicon provided in Table 1 to get familiar with the wine sensory characteristics (attributes). Try to remember how they smell or taste and how they are described by their corresponding lexicon.

Table 1. Sensory characteristics and lexicon

Attributes	Description/Lexicon	References
Vegetal	Herbaceous characters; smells of cut-grass	IBMP (0.5-40 µg/L in water); cis-3-hexen-1-ol (0.2-1 mg/L in water)
Fruitiness	Berry characters	Strawberry, blackcurrant juice in model wine
Oxidised	Oxidised taint smell, rotting fruit, sweet-sickly fruit	Oxidised wine
Reductive	Sulfury, onion, rotten eggs, canned vegetables	DMS (20 µg/L in model wine); MeSH (0.5 µg/L in model wine)
Astringency	Drying-puckering in cheeks	OceanSpray Cranberry juice or ***$Al_2(SO_4)_3$ (2 g/L in water)***
Mouthfeel	How the wine fills the palate; the tactile sensation caused by tannins	A Cab.Sav. Merlot

Appendix 2-6. Score sheet given to panellists during training session 2 - Part 1

Training session 2 – Part 1

Date: ____/____/____

Name:_____

This session involves a triangle test on the wine reference standards you are familiar with in the previous training session.

You are presented with three sets of three glasses; each set containing samples with a single wine reference standard in model wine. Two of the three samples in each set are at an identical concentration and the third is different (either a higher of lower attribute concentration than the other two).

Samples in the first two sets should be smelt ONLY and for the last set, TASTE and then EXPECTORATE.

All that is required is that the sample containing the different concentration be identified by writing the three digit code in the space provided below. Additionally, the panelist is required to indicate whether the different sample is of lower or higher concentration than the two matching samples in the particular suite. Finally, please try and name the attribute.

Example:

Set 1:
Samples codes: 851 422 698
Sample having different concentration_____*698*_____ Lower ☐ Higher ☒

Set 1:

Sample codes: 426 152 879

Sample having different concentration_____Lower ☐ Higher ☐

Name the attribute_____

Set 2:

Sample codes: 320 268 112

Sample having different concentration_____Lower ☐ Higher ☐

Name the attribute_____

Set 3:

Sample codes: 542 695 903

Sample having different concentration_____Lower ☐ Higher ☐

Name the attribute_____

Appendix 2-7. Score sheet given to panellists during training session 2 - Part 2

Training session 2 – Part 2

Date: ____/____/____

Name:_____

This session involves a triangle test on the wine reference standards you are familiar with in the previous training session.

You are presented with three sets of three glasses; each set containing samples with a single wine reference standard in model wine. Two of the three samples in each set are at an identical concentration and the third is different (either a higher of lower attribute concentration than the other two).

Samples in the first two sets should be smelt ONLY and for the last set, TASTE and then EXPECTORATE.

All that is required is that the sample containing the different concentration be identified by writing the three digit code in the space provided below. Additionally, the panelist is required to indicate whether the different sample is of lower or higher concentration than the two matching samples in the particular suite. Finally, please try and name the attribute.

Example:

Set 1:

Samples codes: 851 422 698

Sample having different concentration_____*698*_____ Lower ☐ Higher ☒

Set 4:

Sample codes: 245 458 396

Sample having different concentration_____Lower ☐ Higher ☐

Name the attribute_____

Set 5:

Sample codes: 183 266 522

Sample having different concentration_____Lower ☐ Higher ☐

Name the attribute_____

Set 6:

Sample codes: 614 941 797

Sample having different concentration_____Lower ☐ Higher ☐

Name the attribute_____

Appendix 2-8. An example of a score sheet given to panellists during data collection - triangle test

DIFFERENTIATION TEST

(Triangle test)

Name:_____ Date:___/___/____

Type of sample: ___*Wine*

Instructions:

You will be tasting wines presented to you in a suite of 6 rows. Each row contains a set of 3 glasses.

You are required to sniff and taste the samples in the order provided, from left to right. Expectorate the sample and wait at least 30 seconds between samples. Circle the three digit code of the different sample in each row.

Circle the code of the different sample in each row:

395	174 453
388	921 926
225	362 248
141	241 746
625	662 291
562	134 563

Appendix 2-9. Score sheet given to panellists during data collection - VEGETAL

RANKING TEST

Name:_____ **Date:** ___/___/____

Type of sample: ***Wine***

Characteristics studied: ***Vegetal***

Instructions

Smell the samples from left to right and note *the intensity of vegetal note*

Wait at least 30 seconds between samples. You may find it expedient to first arrange the samples in a provisional order, and then resolve the positions of adjacent samples by more careful sniffing.

Note the three digit codes of the samples according to the intensity of vegetal, starting *from the lowest to the highest*.

If two samples are the same, make a "best guess" as to their rank order and write your comments in the space provided below.

Sample codes _____ _____ _____ _____

Lowest Vegetal note *Highest*

Comments: _____

Appendix 2-10. Score sheet given to panellists during data collection - FRUITINESS

RANKING TEST

Name:_____ **Date:** ___/___/____

Type of sample: ***Wine***

Characteristics studied: ***Fruitiness***

Instructions

Smell the samples from left to right and note *the intensity of fruity note*

Wait at least 30 seconds between samples. You may find it expedient to first arrange the samples in a provisional order, and then resolve the positions of adjacent samples by more careful sniffing.

Note the three digit codes of the samples according to the intensity of fruitiness, starting *from the lowest to the highest*.

If two samples are the same, make a "best guess" as to their rank order and write your comments in the space provided below.

Sample codes _____ _____ _____ _____

Lowest Fruitiness *Highest*

Comments: _____

Appendix 2-11. Score sheet given to panellists during data collection - ASTRINGENCY

RANKING TEST

Name:_____ Date: ____/____/_____

Type of sample: *Wine*

Characteristics studied: *Astringency*

Instructions

Taste the samples from left to right and note *the intensity of astringency*

Wait at least 30 seconds between samples. You may find it expedient to first arrange the samples in a provisional order, and then resolve the positions of adjacent samples by more careful tasting.

Note the three digit codes of the samples according to the intensity of astringent taste, starting *from the lowest to the highest*.

If two samples are the same, make a "best guess" as to their rank order and write your comments in the space provided below.

Sample codes _____ _____ _____ _____

Lowest Astringency *Highest*

Comments: _____

Appendix 2-12. Score sheet given to panellists during data collection - OXIDISED

RANKING TEST

Name:_____ Date: ____/____/_____

Type of sample: ***Wine***

Characteristics studied: ***Oxidised note***

Instructions

Smell the samples from left to right and note *the intensity of oxidised note*

Wait at least 30 seconds between samples. You may find it expedient to first arrange the samples in a provisional order, and then resolve the positions of adjacent samples by more careful sniffing.

Note the three digit codes of the samples according to the intensity of oxidised note, starting *from the lowest to the highest*.

If two samples are the same, make a "best guess" as to their rank order and write your comments in the space provided below.

Sample codes _____ _____ _____ _____

Lowest Oxidised note *Highest*

Comments: _____

Appendix 2-13. Scoresheet given to panellists during data collection - REDUCTIVE

RANKING TEST

Name:_____ Date: ____/____/_____

Type of sample: ***Wine***

Characteristics studied: ***Reductive note***

Instructions

Smell the samples from left to right and note *the intensity of reductive note*

Wait at least 30 seconds between samples. You may find it expedient to first arrange the samples in a provisional order, and then resolve the positions of adjacent samples by more careful tasting.

Note the three digit codes of the samples according to the intensity of reductive note, starting *from the lowest to the highest*.

If two samples are the same, make a "best guess" as to their rank order and write your comments in the space provided below.

Sample codes _____ _____ _____ _____

Lowest Reductive note *Highest*

Comments: _____

Appendix 2-14. Scoresheet given to panellists during data collection - MOUTHFEEL

RANKING TEST

Name:_____ Date: ____/____/_____

Type of sample: ***Wine***

Characteristics studied: ***Mouthfeel***

Instructions

Taste then expectorate the samples from left to right and note *the intensity of mouthfeel.*

Mouthfeel is a measure of how the wine "fills" the palate. A wine with a weak mouthfeel will tend to only be perceived on the centre-line of the tongue. If the wine is felt strongly on the inside of the cheeks and the tongue, then it can be considered full or big.

Wait at least 30 seconds between samples. You may find it expedient to first arrange the samples in a provisional order, and then resolve the positions of adjacent samples by more careful tasting.

Note the three digit codes of the samples according to the perceived mouthfeel, starting *from the lowest (thin) to the highest (full)*.

If two samples are the same, make a "best guess" as to their rank order and write your comments in the space provided below.

Sample codes _____ _____ _____ _____

Thin Mouth-feel *Full*

Comments: _____

Appendix 2-15. Critical number of correct responses in a triangle test

(Adapted from Meilgaard *et al.*, 1999)

Instructions: Entries are the minimum number of correct responses required for significance at the stated α level (i.e., column) for the corresponding number of respondents, n (i.e., row). Reject the assumption of "no difference" if the number of correct responses is greater than or equal to the tabled value.

	α						
n	**0.40**	**0.30**	**0.20**	**0.10**	**0.05**	**0.01**	**0.001**
3	2	2	3	3	3	-	-
4	3	3	3	4	4	-	-
5	3	3	4	4	4	5	-
6	3	4	4	5	5	6	-
7	4	4	4	5	5	6	7
8	4	4	5	5	6	7	8
9	4	5	5	6	6	7	8
10	5	5	6	6	7	8	9
11	5	5	6	7	7	8	10
12	5	6	6	7	8	9	10
13	6	6	7	8	8	9	11
14	6	7	7	8	9	10	11
15	6	7	8	8	9	10	12
16	7	7	8	9	9	11	12
17	7	8	8	9	10	11	13
18	7	8	9	10	10	12	13

Appendix 2-16. Upper-α probability of the chi-square (χ^2) distribution

(Adapted from Meilgaard *et al.*, 1999)

Instruction:

(1) Enter the row of the table corresponding to the number of degrees of freedom (ν) for χ^2.

(2) Pick the values of χ^2 in that row from the column that corresponds to the predetermined α level.

				α			
ν	**0.500**	**0.250**	**0.100**	**0.050**	**0.025**	**0.010**	**0.005**
1	0.445	1.32	2.71	3.84	5.02	6.63	7.88
2	1.39	2.77	4.61	5.99	7.38	9.21	10.6
3	2.37	4.11	6.25	7.81	9.35	11.3	12.8
4	3.36	5.39	7.78	9.49	11.1	13.3	14.9
5	4.35	6.63	9.24	11.1	12.8	15.1	16.7
6	5.35	7.84	10.6	12.6	14.4	16.8	18.5
7	6.35	9.04	12.0	14.1	16.0	18.5	20.3
8	7.34	10.2	13.4	15.5	17.5	20.1	22.6
9	8.34	11.4	14.7	16.9	19.0	21.7	23.6
10	9.34	12.5	16.0	18.3	20.5	23.2	25.2
11	10.3	13.7	17.3	19.7	21.9	24.7	26.8
12	11.3	14.8	18.5	21.0	23.3	26.2	28.3
13	12.3	16.0	19.8	22.4	24.7	27.7	29.8
14	13.3	17.1	21.1	23.7	26.1	29.1	31.3
15	14.3	18.2	22.3	25.0	27.5	30.6	32.8
16	15.3	19.4	23.5	26.3	28.8	32.0	34.3
17	16.3	20.5	24.8	27.6	30.2	33.4	35.7
18	17.3	21.6	26.0	28.9	31.5	34.8	37.2
19	18.3	22.7	27.2	30.1	32.9	36.2	38.6
20	19.3	23.8	28.4	31.4	34.2	37.6	40.0

Appendix 4-1. Publication of chapter 4

Influence of Microoxygenation on Reductive Sulfur Off-Odors and Color Development in a Cabernet Sauvignon Wine

Dang-Dung Nguyen,[1] Laura Nicolau,[2] Stuart I. Dykes,[3] and Paul A. Kilmartin[4]*

Abstract: The effect of microoxygenation on the composition of a red wine was investigated, where oxygen was applied at both low and high rates (5 and 20 mg/L/month) through a diffuser or via permeation through polyethylene tanks. A control with no oxygen was also included. Each treatment was performed in triplicate, using a commercially made Cabernet Sauvignon wine, in nine 300-L stainless-steel and three 300-L polyethylene tanks at 16°C over 16 weeks. Spontaneous malolactic fermentation occurred simultaneously with the microoxygenation treatments. The results indicated that microoxygenation enhanced the wine color development, in which effects on pigments resistant to SO_2 were more marked in the first half of the trial and more obvious influences on color density were seen during the second half of the trial. Microoxygenation, as applied in this study, did not show any effect on the desirable varietal thiol 3-mercaptohexanol, yet led to some decreases in the concentrations of undesirable off-odors, including a lowering in the concentration of methanethiol without an increase in the concentration of dimethyl disulfide. Changes in the concentrations of other reductive sulfur compounds, except the thioesters, were also affected by oxygen and further by the influence of spontaneous malolactic fermentation, indicating the complicated interactions of these compounds in the wine matrix.

Key words: sulfur off-odors, microoxygenation, Cabernet Sauvignon, red wine

The addition of oxygen is an essential part of red winemaking. Oxygen has a role in regulating yeast activity and ensuring that sulfur off-odors and fermentation-derived volatiles are kept under control (Jones et al. 2004). Winemakers have long recognized the beneficial effects of limited oxygen exposure for red wines after the completion of alcoholic fermentation and early wine aging (Perez-Magarino et al. 2007). The microoxygenation (MOX) technique is an oxygen management tool that has been commercially available since the mid-1990s. It delivers a small and controlled amount of oxygen in a continuous manner to wine stored in stainless-steel tanks and can be applied at different stages in the winemaking process (Devatine et al. 2007, Heras et al. 2008, Pour-Nikfardjam and Dykes 2003). Microoxygenation has been used for many years worldwide (Heras et al. 2008), and recent research has mainly focused on the effect of MOX on the phenolic compounds in wine. Research has confirmed the capability of MOX to stabilize wine color and increase wine color density, together with a more rapid loss of less stable monomeric anthocyanins (Atanasova et al. 2002, McCord 2003, Perez-Magarino et al. 2007). Only a few studies have reported sensorial properties, tastes and aromas, of wines undergoing MOX (Heras et al. 2008). Microoxygenation is generally applied either premalolactic fermentation (MLF) or post-MLF with the addition of extra sulfur dioxide, but is generally not applied during MLF. However, the consequences of applying MOX during MLF have not been reported previously. One explanation for stopping the MOX application once MLF starts is that lactic acid bacteria can consume the acetaldehyde produced from the autoxidation of dihydroxyphenols (Cano-López et al. 2006), making it less available for reactions among wine polyphenols and the formation of stable polymeric pigments.

Apart from the volatile sulfur-containing compounds responsible for characteristic varietal aromas, most volatile sulfur-containing substances produce unpleasant odors which impart sensory defects in wines even at very low concentrations, including the well-known rubbery or reductive character (Beloqui and Bertrand 1995, Mestres et al. 2000). These compounds can be arbitrarily classified according to their volatility into two categories, the more volatile (light) and less volatile (heavy) sulfur compounds, with boiling points below and above 90°C, respectively, which is the boiling point of 3-methylthiopropanol (Beloqui and

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Appendix 5-1. Examples of WineScan data of the Cabernet Sauvignon and Bordeaux trials

(a) Cabernet Sauvignon

ID	Ethanol	Total Acidity	Volatile Acidity	Malic Acid	Reducing sugar	pH
Day 0						
C - a	12.86	6.1	0.26	1.86	1.99	3.82
C - a	12.96	6.2	0.25	1.86	2.06	3.8
C - a	12.9	6.22	0.25	1.9	2.12	3.8
STD	0.053	0.068	0.005	0.022	0.065	0.013
Mean	12.91	6.17	0.25	1.87	2.06	3.8
Day 112						
C-a	12.87	5.61	0.5	0.54	2.34	3.93
C-a	12.92	5.69	0.49	0.56	2.37	3.9
C-a	12.89	5.71	0.49	0.59	2.43	3.91
STD	0.025	0.054	0.006	0.03	0.046	0.013
Mean	12.89	5.67	0.49	0.56	2.38	3.91

(b) Bordeaux blend

ID	Ethanol	Total Acidity	Volatile Acidity	Malic Acid	Reducing sugar	pH
Day 0						
C - a	13.16	5.09	0.31	0.01	0.8	3.83
C - a	13.16	5.11	0.33	0.03	0.59	3.83
Mean	13.16	5.1	0.32	0.02	0.7	3.83
STD	0.001	0.009	0.012	0.012	0.15	0.006
Day 112						
C - a	13.13	5.22	0.39	0.07	0.72	3.85
C - a	13.11	5.24	0.38	0.15	0.72	3.84
Mean	13.12	5.23	0.38	0.11	0.72	3.84
STD	0.009	0.011	0.002	0.059	0.005	0.007

Appendix 5-2. Standard deviations (n = 3) of chromatic and chemical values of the Shiraz wines

Time [days]	0	15	30	50	75	90	210
SO_2 resistant pigments							
Control						0.03	0.03
MOX	0.04	0.05	0.04	0.04	0.03	0.05	0.02
Colour density							
Control						0.08	0.03
MOX	0.04	0.10	0.04	0.09	0.07	0.04	0.10
Tannins							
Control	66	21	122	104	94	9	89
MOX						64	38

Appendix 5-3. Colour changes of the Cabernet Sauvignon wines underwent MOX after alcoholic fermentation with relative standard deviations (% RSD) of the means for triplicate treatments

	DAYS	0	14	28	42	56	70	84	98	112
	SO_2-resistant pigments									
No oxygen	Mean	5.39	5.40	5.56	5.57	5.79	5.91	5.85	5.93	6.02
	STD	0.02	0.02	0.07	0.07	0.02	0.04	0.11	0.01	0.03
	% RSD	**0.28**	**0.32**	**1.22**	**1.24**	**0.26**	**0.59**	**1.84**	**0.22**	**0.42**
O_2 5 mg/L/month	Mean	5.40	5.42	5.70	5.70	5.79	6.01	5.99	5.89	5.86
	STD	0.07	0.01	0.02	0.06	0.04	0.13	0.03	0.08	0.19
	% RSD	**1.23**	**0.14**	**0.32**	**1.02**	**0.61**	**2.16**	**0.43**	**1.42**	**3.16**
O_2 20 mg/L/month	Mean	5.43	5.38	5.74	5.79	5.86	5.82	5.87	5.73	5.90
	STD	0.04	0.12	0.00	0.09	0.07	0.21	0.17	0.19	0.20
	% RSD	**0.68**	**2.17**	**0.05**	**1.57**	**1.27**	**3.58**	**2.86**	**3.38**	**3.38**
Flextanks	Mean	5.38	5.52	5.71	5.80	5.90	5.99	6.01	5.99	6.09
	STD	0.05	0.04	0.11	0.05	0.05	0.01	0.06	0.02	0.04
	% RSD	**0.84**	**0.69**	**1.92**	**0.82**	**0.82**	**0.13**	**1.05**	**0.39**	**0.58**
	Colour density									
Control	Mean	24.82	24.79	25.10	24.35	23.74	23.62	23.93	23.96	23.54
	STD	0.03	0.08	0.11	0.07	0.13	0.02	0.13	0.16	0.08
	% RSD	**0.10**	**0.30**	**0.44**	**0.28**	**0.54**	**0.06**	**0.54**	**0.65**	**0.35**
O_2 5 mg/L/month	Mean	24.85	24.71	25.05	24.38	23.77	23.90	24.21	24.09	23.73
	STD	0.05	0.08	0.10	0.15	0.10	0.10	0.24	0.22	0.12
	% RSD	**0.18**	**0.33**	**0.39**	**0.62**	**0.41**	**0.42**	**1.01**	**0.89**	**0.51**
O_2 20 mg/L/month	Mean	24.82	24.87	25.01	24.54	23.66	23.37	23.46	23.09	22.86
	STD	0.04	0.11	0.05	0.30	0.17	0.71	1.19	1.48	1.41
	% RSD	**0.14**	**0.44**	**0.21**	**1.23**	**0.70**	**3.04**	**5.07**	**6.41**	**6.15**
Flextanks	Mean	24.77	24.80	25.15	24.53	23.81	23.99	24.26	24.16	23.75
	STD	0.02	0.03	0.05	0.13	0.08	0.19	0.16	0.15	0.15
	% RSD	**0.09**	**0.11**	**0.21**	**0.53**	**0.33**	**0.79**	**0.65**	**0.63**	**0.65**

Appendix 5-4. Colour changes of the BORDEAUX blend wines underwent MOX after MLF with relative standard deviations (% RSD) of the means for triplicate treatments

	DAYS	0	28	56	84	112
	SO_2-resistant pigments					
Control	Mean	2.05	2.11	2.14	2.13	2.19
	STD	0.02	0.01	0.01	0.01	0.01
	% RSD	**0.99**	**0.57**	**0.69**	**0.52**	**0.57**
O_2 0.5 mg/L/month	Mean	2.05	2.13	2.16	2.14	2.20
	STD	0.01	0.00	0.01	0.01	0.01
	% RSD	**0.73**	**0.16**	**0.42**	**0.52**	**0.38**
O_2 2.5 mg/L/month	Mean	2.06	2.15	2.20	2.17	2.25
	STD	0.01	0.01	0.02	0.01	0.02
	% RSD	**0.50**	**0.48**	**0.76**	**0.45**	**0.80**
Flextanks	Mean	2.05	2.12	2.17	2.15	2.21
	STD	0.02	0.01	0.01	0.01	0.03
	% RSD	**0.93**	**0.61**	**0.58**	**0.39**	**1.35**
	Colour density					
Control	Mean	6.30	6.53	6.40	6.40	6.09
	STD	0.03	0.03	0.02	0.02	0.05
	% RSD	**0.47**	**0.53**	**0.26**	**0.26**	**0.77**
O_2 0.5 mg/L/month	Mean	6.29	6.54	6.50	6.47	6.22
	STD	0.03	0.03	0.05	0.02	0.11
	% RSD	**0.40**	**0.50**	**0.75**	**0.36**	**1.79**
O_2 2.5 mg/L/month	Mean	6.34	6.58	6.50	6.50	6.36
	STD	0.06	0.08	0.09	0.09	0.12
	% RSD	**1.01**	**1.27**	**1.44**	**1.44**	**1.86**
Flextanks	Mean	6.32	6.49	6.47	6.47	6.23
	STD	0.03	0.03	0.04	0.04	0.09
	% RSD	**0.55**	**0.42**	**0.59**	**0.59**	**1.44**

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