Investigation of the impact of commercial malolactic fermentation starter cultures on red wine aroma compounds, sensory properties and consumer preference

by

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DECLARATION

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SUMMARY

Wine is the result of a variety of biochemical reactions and microbial interactions which contribute to the organoleptic properties of wine. Wine aroma and flavour encapsulate the sensory experience of wine and could ultimately determine wine quality and consequently influence consumer acceptance and preference of a product. A thorough understanding of potential factors influencing wine aroma and flavour is therefore needed in order to exploit such factors. The aim of this study was to evaluate the influence of commercial malolactic fermentation (MLF) starter cultures on wine composition, aroma and flavour and the potential impact on consumer preference of experimentally produced red wines.

An analytical platform was established to capture the compositional changes induced by different MLF bacteria in experimentally produced red wines. A fast gas chromatography flame ionisation detection (GC-FID) method was developed to determine 39 wine volatile compounds in less than 15 minutes per sample. A 3-fold reduction in analysis time was achieved in comparison to a conventional GC-FID method (40 minutes). Analytes quantified comprise a large boiling point and polarity range which illustrates the robustness of the method. A method was also developed for the direct quantification of carbonyl compounds including diacetyl, acetoin, 2,3-pentanedione and certain aldehydes using headspace solid phase microextraction coupled to gas chromatography mass spectrometry (HS-SPME GC-MS). Both analytical methods showed satisfactory linearity, repeatability and limits of quantification.

The contribution of four commercial *Oenococcus oeni* malolactic fermentation (MLF) starter cultures to the volatile composition, organic acid content and infrared spectral properties of experimentally produced South African red wines, showed significant strain-specific variations in the organic acid profiles, especially for the production of citric acid and lactic acid during MLF. Subsequently, concentrations of compounds related to citric acid metabolism, namely ethyl lactate, acetic acid, diacetyl and acetoin, were influenced accordingly. Bacterial metabolic activity increased the concentration of higher alcohols, fatty acids and esters, with a larger increase observed in ethyl esters compared to acetate esters. A strain-specific tendency to reduce total aldehyde concentrations was found at the completion of MLF, however, further investigation is needed to clarify this observation. Infrared spectral fingerprints were used to characterise the different bacteria and in addition, the prediction of MLF related compounds, diacetyl, acetoin and 2,3-pentanedione, from mid-infrared spectra was explored by partial least squares (PLS) models.

Quantitative descriptive analysis (QDA) results depicted significant differences between wines fermented with different starter cultures, in terms of sensory attributes including buttery, fruity, nutty and yoghurt/buttermilk aroma as well as smoothness and mouth-feel attributes. Consumer preference testing results indicate that sensory differences imparted by different MLF bacteria could influence consumer-liking. Preference mapping revealed interesting relationships between sensory attributes and consumer-liking, that can be used for preliminary interpretative purposes.

In conclusion, this study illustrated the potential impact of bacterial strains on wine aroma and flavour, resulting sensory properties and consumer preference through an integrative approach combining compositional, spectral, sensory and consumer data. The results presented in this study are of significance to the wine industry since they illustrate and reiterate the potential of different MLF starter cultures as an additional tool to contribute to wine aroma and flavour, and potentially influencing consumer preference and product liking.

OPSOMMING

Wyn is die resultaat van ‘n verskeidenheid biochemiese reaksies en mikrobiologiese interaksies wat tot die organoleptiese eienskappe van die finale produk bydra. Wynaroma en geur vang die sensoriese ervaring van wyn vas en dit kan dus wynkwaliteit bepaal en gevolglik verbruikersaanvaarding asook voorkeur van ‘n produk beïnvloed. Die potensiële faktore wat wynaroma en geur kan beïnvloed moet dus vir hierdie rede deeglik bestudeer word ten einde sulke faktore ten volle te benut. Die doel van hierdie studie was om die invloed van kommersiële applemelksuurgisting (AMG) aanvangskulture op wynsamestelling, die gevolglike aroma en geur eienskappe en die potensiële impak op verbruikersvoorkeure te ondersoek.

‘n Analitiese platform is gevestig om die veranderings in samestelling veroorsaak deur verskillende AMG bakterieë in eksperimenteel bereide rooi wyne vas te vang. ‘n Vinnige gas chromatografiese vlam geïoniseerde deteksie (GC-FID) metode is ontwikkel vir die meting van 39 vlugtige komponente in minder as 15 minute per wynmonster. In vergelyking met ‘n konvensionele GC-FID metode (40 minute) is ’n 3-voudige vermindering in analise tyd behaal. Gekwantifiseerde komponente bestaan uit ‘n wye kookpunt- en polariteitsreeks wat die robustheid van die metode illustreer. ‘n Metode vir die direkte kwantifisering van karboniel komponente, insluitende diasetiel, asetoïen, 2,3-pentanedioon en verskeie aldehiede is ontwikkel met die gebruik van dampfase soliede fase mikroekstraksie gekoppel aan gas chromatografie massa spektrometrie (HS-SPME GC-MS). Albei analitiese metodes besit voldoende lineariteit, herhaalbaarheid en lae deteksie limiete.

Die bydrae van vier kommersiële *Oenococcus oeni* AMG aanvangskulture tot die vlugtige samestelling, organiese suurinhoud en infrarooi spektrale eienskappe van Suid-Afrikaanse rooiwyn het beduidende ras spesifieke variasies in die organiese suur profiele, spesifiek vir die produksie van sitroensuur en melksuur gedurende AMG, vertoon. Gevolglik is die konsentrasies van komponente verwant aan sitroensuur metabolisme, naamlik etiellaktaat, asynsuur, diasetiel en asetoïen, dien ooreenkomstig beïnvloed. Bakteriese metaboliese aktiwiteit het ‘n toename tot gevolg gehad in die hoër alkohole, vetsure en algemene ester konsentrasies met ‘n groter toename in etiel-esters in vergelyking met asetaat-esters. ‘n Ras-spesifieke tendens om die totale aldehiedkonsentrasie te verminder na afloop van AMG, is waargeneem alhoewel verdere ondersoek in hierdie area nodig is. Infrarooi spektrale patrone is gebruik om verskillende bakterieë te karakteriseer asook om die voorspelling van spesifieke AMG verwante komponente soos diasetiel, asetoïen en 2,3-pentanedioon met die gebruik van mid-infrarooi spektrala parsiële kleinste kwadraat verskille (PLS) modelle te ondersoek.

Kwantitiewe beskrywende sensoriese analise illustreer beduidende verskille tussen wyne wat gefermenteer is met verskillende aanvangskulture in terme van geure soos botteragtigheid, vrugtigheid, neutagtigheid, joghurt/karringmelkgeur, asook gladheid en mondgevoel eienskappe. Verbruikersvoorkeur resultate illustreer die groot invloed wat sensoriese verskille veroorsaak deur verskillende AMG bakterieë op verbruikersvoorkeure kan hê. Voorkeur kartering het interessante verhoudings tussen sensoriese eienskappe en verbruikersvoorkeure uitgelig.

Hierdie studie illustreer die impak van bakteriese rasse op wynaroma en geur en verbruikersvoorkeure deur ‘n geïntegreerde benadering waarin samestellende, spektrale, sensoriese en verbruikersdata gekombineer is. Die resultate van hierdie studie is van belang vir die wynindustrie deurdat dit die potensiële bydrae van verskillende AMG kulture tot wynaroma en geur asook die potensiaal om verbruikersvoorkeure te beïnvloed, illustreer en beklemtoon.

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BIOGRAPHICAL SKETCH

Sulette Malherbe was born on 20 July 1980 and matriculated at Paarl Gymnasium High School in 1998. She obtained her BSc degree at Stellenbosch University in 2003, majoring in Chemistry. In 2004, Sulette enrolled at the Institute for Wine Biotechnology and obtained her BSc Honours degree in Wine Biotechnology in December of that year. In 2005 she enrolled for a Masters degree in Wine Biotechnology at the same university and obtained her MSc *cum laude* in March 2007. She enrolled for her PhD in Wine Biotechnology in the same year.

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PREFACE

This thesis is presented as a compilation of six chapters. Each chapter is introduced separately and is written according to the style of the *South African Journal of Enology and Viticulture.*

Chapter 1 **GENERAL INTRODUCTION AND PROJECT AIMS**

Chapter 2 **LITERATURE REVIEW**
Malolactic fermentation and wine aroma: a review

Chapter 3 **RESEARCH RESULTS**
High-throughput quantification of major volatile compounds in wine: fast GC method development, validation and application

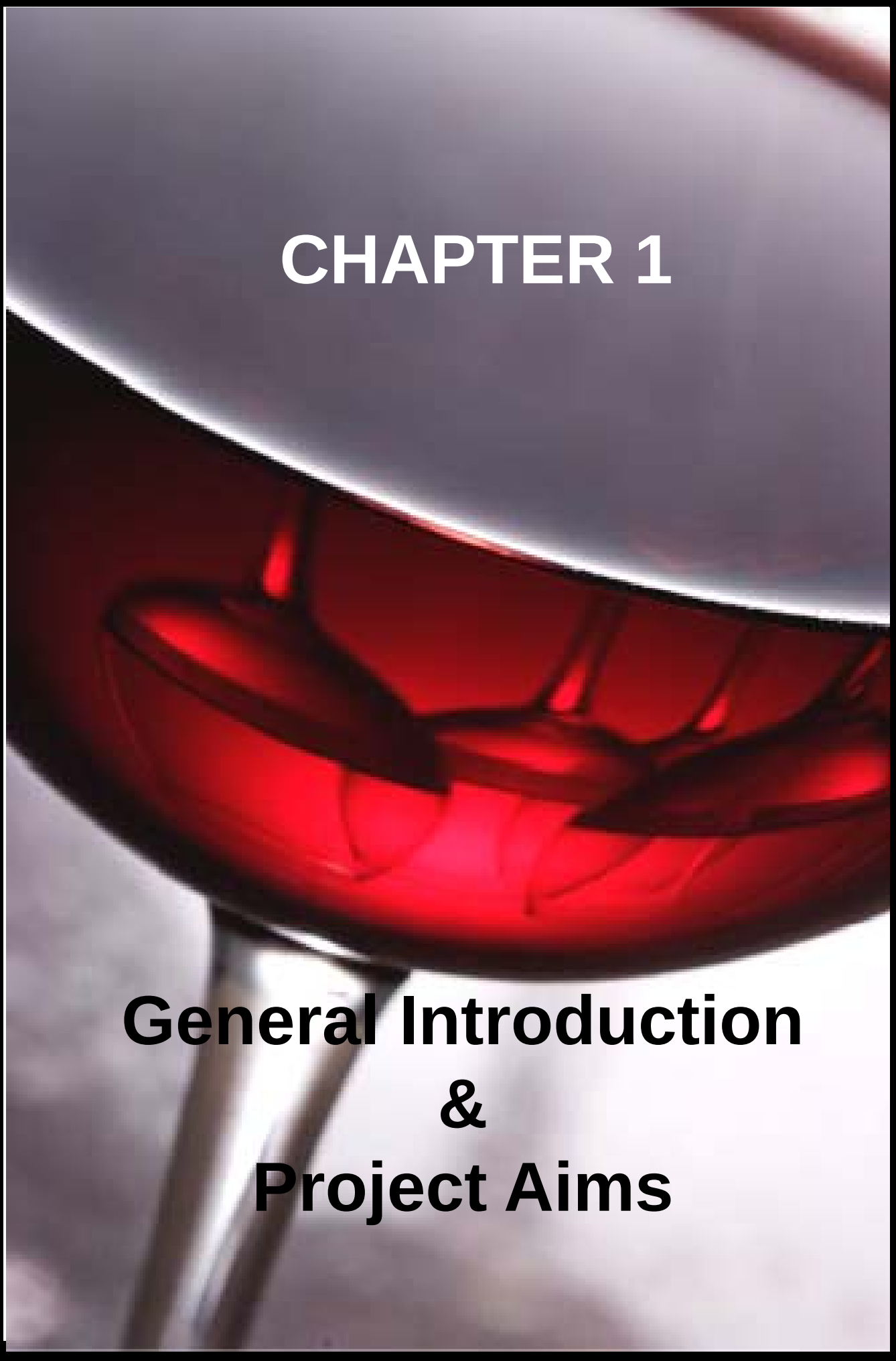
Chapter 4 **RESEARCH RESULTS**
Comparative metabolic profiling approach to investigate the contribution of malolactic fermentation starter cultures to red wine chemical composition

Chapter 5 **RESEARCH RESULTS**
Investigating the impact of malolactic fermentation starter cultures on sensory properties and consumer-liking of red wines

Chapter 6 **GENERAL DISCUSSION AND CONCLUSIONS**

These chapters were written as independent papers with the consequence that overlapping, especially in the introductory parts and in the materials and methods sections, was unavoidable.

CONTENTS



CHAPTER 1

General Introduction & Project Aims

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Wine is the result of biochemical processes involving microbiological and chemical interactions which ultimately determine the sensory properties of wine. These intrinsic properties are constituted by a multitude of aroma and flavour compounds that deliver the sensory experience to the wine consumer. The sensory perception of wine aroma and flavour is the result of a complex interaction with the human olfactory system (Swiegers *et al*., 2005a). In order to better understand consumer preferences in relation to the organoleptic quality of wine, a thorough understanding of potential factors influencing aroma and flavour is needed. Wine aroma compounds could originate from a number of potential sources and are distinguished accordingly as grape-derived flavour, pre-fermentative flavour, fermentative flavour and maturation or post-fermentative flavour (Rapp, 1998). Fermentation-derived aroma compounds constitute a major part of the volatile fraction of wine, since these compounds are present in the highest concentration (Lambrechts & Pretorius, 2000). Fermentation-derived aroma compounds are produced by yeast during alcoholic fermentation (Lambrechts & Pretorius, 2000) and to a certain extent by lactic acid bacteria (LAB) during the secondary fermentation process of malolactic fermentation (MLF) (Bartowsky & Henschke, 1995; Liu, 2002). Numerous studies (reviewed by Lambrechts & Pretorius, 2000; Swiegers *et al*., 2005b) have focused on the formation of yeast-derived aroma compounds and the contribution of yeast metabolites to the sensory properties of wine. However, insight regarding the contribution of LAB to wine aroma and flavour, as well as the potential influencing factors, is limited and merits further investigation.

MLF involves the conversion of dicarboxylic L-malic acid to monocarboxylic L-lactic acid and carbon dioxide, resulting in a limited increase in pH and a decrease in perceived acidity (Davis *et al*., 1985; Lonvaud-Funel, 1999). This reaction, catalysed by the malolactic enzyme (Lonvaud-Funel, 1999), could spontaneously occur by bacterial species of the genera *Leuconostoc*, *Lactobacillus*, *Pediococcus* as well as *Oenococcus oeni* (Dicks *et al*., 1995) present in the wine (Lerm *et al*., 2010). However, the introduction of freeze-dried starter culture preparations (Nielsen *et al*., 1996) for direct inoculation has improved the management of MLF, allowing for better control of the flavour contribution of MLF, through the use of selected strains and reduces the risk of potential biogenic amine production which has health implications (Lonvaud-Funel, 2001). In general, commercially available MLF strains are isolated from spontaneous fermentations and carefully evaluated for their fermentation ability, gene expression patterns, ability to produce biogenic amines and contribution to flavour and mouthfeel properties, to name but a few of the selection criteria (Ruiz *et al*., 2010; Solieri *et al*., 2010). *O. oeni* is recognised as the most suitable species as it is the most tolerant to the harsh wine conditions of low pH, high sulphur dioxide (SO_2) and high alcohol content (Versari *et al*., 1999). For this reason, *O. oeni* is mostly selected as starter culture as well as for its favourable flavour profile (Lerm *et al*., 2010).

Wine aroma and flavour could be influenced by bacteria via several mechanisms including (i) the removal of flavour compounds by metabolism and adsorption to the cell wall; (ii) the production of new volatiles from the metabolism of grape sugars, amino acids, organic acids and other nutrient

compounds; and (iii) the metabolism or extracellular modification of grape and yeast secondary metabolites, to either more or less flavoured metabolites (Bartowsky & Henschke, 1995). In support of these possible mechanisms, wine LAB have diverse genetic properties and possess a variety of enzymes that could potentially be involved in converting grape-derived (Hernandez-Orte *et al*., 2009), yeast-derived (Ugliano & Moio, 2005) or wood-derived (de Revel *et al*., 2005) precursor compounds into aroma compounds (Liu, 2002; Matthews *et al*., 2004; Mtshali *et al*., 2010). Many acids, alcohols, esters and carbonyl compounds have been associated with MLF and their production is greatly dependant on strain characteristics, cultivar selection and fermentation conditions (Bartowsky & Henschke, 1995; Lerm, 2010).

According to Henick-Kling *et al*. (1994), MLF contributes to the fruity and buttery aroma notes but reduces the vegetative, green, grassy herbaceous aroma. In relation to these sensory changes, the increased buttery note has been ascribed to the formation of diacetyl via citric acid metabolism of wine LAB during MLF (Bartowsky & Henschke, 2004). This aspect has been well studied and reviewed (Davis *et al*., 1985; Bartowsky & Henschke, 1995; Laurent *et al*., 1994; Martineau *et al*., 1995; de Revel *et al*., 1999; Lonvaud-Funel, 1999; Bartowsky *et al*., 2002; Bartowsky & Henschke, 2004; Bauer & Dicks, 2004; Versari *et al*., 1999). The increased fruity note is ascribed to the formation of esters by wine LAB however reports with regard to specific esters are contradictory (Maicas *et al*., 1999; Delaquis *et al*., 2000; Gámbaro *et al*., 2001). Furthermore, the reduction in vegetative aroma is attributed to the catabolism of aldehydes by wine LAB (Liu, 2002). Information related to this aspect is limited to the catabolism of acetaldehyde (Osborne *et al*., 2000). Additional descriptors associated with MLF include floral, nutty, yeasty, oaky, sweaty, spicy, roasted, toasty, vanilla, smoky, earthy and honey (Henick-Kling *et al*., 1994; Laurent *et al*., 1994; Sauvageot & Vivier, 1997). However, further research is required to relate these sensory attributes to the production or degradation of specific chemical compounds (Versari *et al*., 1999; Liu, 2002).

In terms of its contribution to the sensory properties of wine, the impact of MLF on the taste of wine as a result of deacidification is well recognised. As previously mentioned, evidence to support the observed aroma modifications in terms of chemical composition is often contradictory or inconclusive and the mechanisms responsible for these modifications are not completely understood. Typically, available reports on the effect of MLF are often very specific to countries and regions with respect to the cultivars and strains evaluated, for example Tannat (Uruguayan red cultivar; Boido *et al.*, 2009; Gámbaro *et al*., 2001), Aglianico (Southern Italy; Ugliano & Moio, 2005) and Tempranillo (Spanish cultivar; Hernandez-Orte *et al*., 2009). Due to limited reports on the sensory impact of MLF starter cultures used in two of the major red cultivars produced in South Africa (Lerm, 2010), Shiraz and Pinotage were selected for this study.

As a starting point, to a broad-range chemical profiling approach, an analytical platform had to be developed for the quantification of a number of relevant volatile compounds, presumably originating from MLF. In order to increase the sample throughput, simplify tedious analytical measurements and analyse carbonyl compounds, the development of fast gas chromatography (GC) and headspace solid phase microextraction gas chromatography mass spectrometry (HS SPME GC-MS) methods was required. The measured analytes could serve as a platform to link differences between chemical compounds and sensory perception. For this reason, sensory profiling by a trained panel was necessary to firstly determine whether differences amongst different MLF starter cultures could be

perceived and secondly, to establish the possible effect on consumer preference. The combination of chemical analysis, sensory profiling and consumer data will enable a more comprehensive evaluation of bacterial strains. This will ultimately assist winemakers in selecting optimal starter cultures for achieving the style attributes of cultivars targeted at specific consumer groups.

1.2 PROJECT AIMS

This project forms part of an extensive research program at the Institute for Wine Biotechnology, Stellenbosch University, regarding the metabolic profiling of LAB in the winemaking environment. The outcomes of this project will be used to establish future goals for projects and to evaluate the direction of the current research.

The principal aim of this work was to comparatively evaluate the influence of Enoferm alpha®, Lalvin VP41®, Viniflora oenos® and Viniflora CH16® MLF starter cultures on wine composition, sensory properties and consumer preference. An integrated approach was followed in order to obtain a comprehensive profile of chemical, spectral, sensory and consumer data which were subjected to multivariate data analysis and other statistical procedures for interpretation and prediction purposes. The nature of this approach along with the use of these powerful technologies could contribute to a better understanding of the influence of MLF, and specifically the use of starter cultures, on wine aroma. This study and its outcome would have both fundamental and industrial applications regarding bacterial strain development, characterisation, marketing and future research endeavours. The specific research objectives of this study were as follows:

a) to develop a simple and effective method for the high-throughput measurement of major volatile compounds in wine utilizing fast gas chromatography flame ionisation detection (fast GC-FID);
b) to develop an analytical method for the simultaneous determination of a selection of MLF related carbonyl compounds based on headspace solid phase micro-extraction (SPME) coupled to gas chromatography mass spectrometry (GC-MS);
c) to evaluate the contribution of four selected commercial MLF starter cultures to wine composition by the application of these newly established, as well as existing methods;
d) to evaluate the impact of different MLF bacterial starter cultures on wine sensory properties in two cultivars (Shiraz and Pinotage) over two vintages by means of a trained panel;
e) to determine the consequent effect on consumer perception and preference; and
f) to investigate whether drivers of liking could be identified by relating sensory data to consumer data, through preference mapping.

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CHAPTER 2

Literature Review

Malolactic fermentation and wine aroma: a review

2. LITERATURE REVIEW

Malolactic fermentation and wine aroma: a review

ABSTRACT

Wine aroma and flavour contribute to the intrinsic sensory properties which determine wine quality and consequently influence consumer acceptability and preference of a product. Understanding wine aroma and flavour requires having insight into an extremely complex system of interactions among many hundreds of compounds that are influenced by a variety of physical and biological factors. The chemical compounds involved in the final aroma and flavour of a wine could originate from the grape to the bottle at any stage and often involves microbial activity of some kind. The influence of malolactic fermentation (MLF) on wine aroma and flavour has received considerable attention in the last few years, and is of particular interest as it could provide an additional tool to winemakers to produce quality wines. However, the potential contribution of lactic acid bacteria to wine aroma and flavour is not yet fully understood. The main focus of this review includes; (i) a summary of MLF related compositional changes and their potential impact on wine aroma and flavour; (ii) an outline of the analytical techniques used to quantify specific compounds in wine; (iii) an overview of MLF related sensory research findings; and finally, (iv) some innovative applications for studying MLF and its influence on wine aroma and flavour. This review therefore also highlights the importance of exploiting the hidden wealth of possibilities for bacteria to improve the aroma and flavour profile of wine.

Keywords: malolactic fermentation, wine aroma, analytical techniques, sensory, wine composition

2.1 INTRODUCTION

Wine is a complex mixture consisting of hundreds of compounds formed as a result of successive biological processes and interactions by both yeast and bacteria (**Figure 2.1**). Wine aroma and flavour are of critical importance since it encapsulates the sensory experience of wine and influences consumer perceptions. Volatile compounds influence wine aroma which is perceived by the sense of smell, while wine flavour refers to the combination of both aroma and non-volatile compounds experienced by taste (Francis & Newton, 2005). Compounds contributing to wine aroma and flavour are classified according to the different sources from which they originate. These include varietal flavour (flavour compounds originating from the grapes), pre-fermentative flavour (compounds formed during operations of extraction and conditioning of must), fermentative flavour (produced by yeast and bacteria during alcoholic and malolactic fermentation) and post-fermentative flavour (compounds that appear during the ageing process through enzymatic or physicochemical actions in wood or in the bottle) (Schreier, 1979; Boulton *et al*., 1995; Rapp, 1998). Fermentation products usually dominate the volatile composition of wine as they constitute the largest concentration (Lambrechts & Pretorius, 2000) and therefore represent a critical aspect which influences wine aroma and flavour.

During the primary fermentation process, grape sugars are converted to alcohol and carbon dioxide by yeast, predominantly of the species *Saccharomyces cerevisiae*. Apart from the formation of alcohol, yeast also contributes to wine aroma by the formation of secondary metabolites such as esters, higher alcohols and other carbonyl compounds (**Figure 2.1**). Comprehensive reviews (Lambrechts & Pretorius, 2000; Fleet, 2003; Swiegers *et al*., 2005a) summarise the large amount of research directed to the importance of yeast strain selection, fermentation conditions and other factors affecting the contribution of yeast to wine aroma.

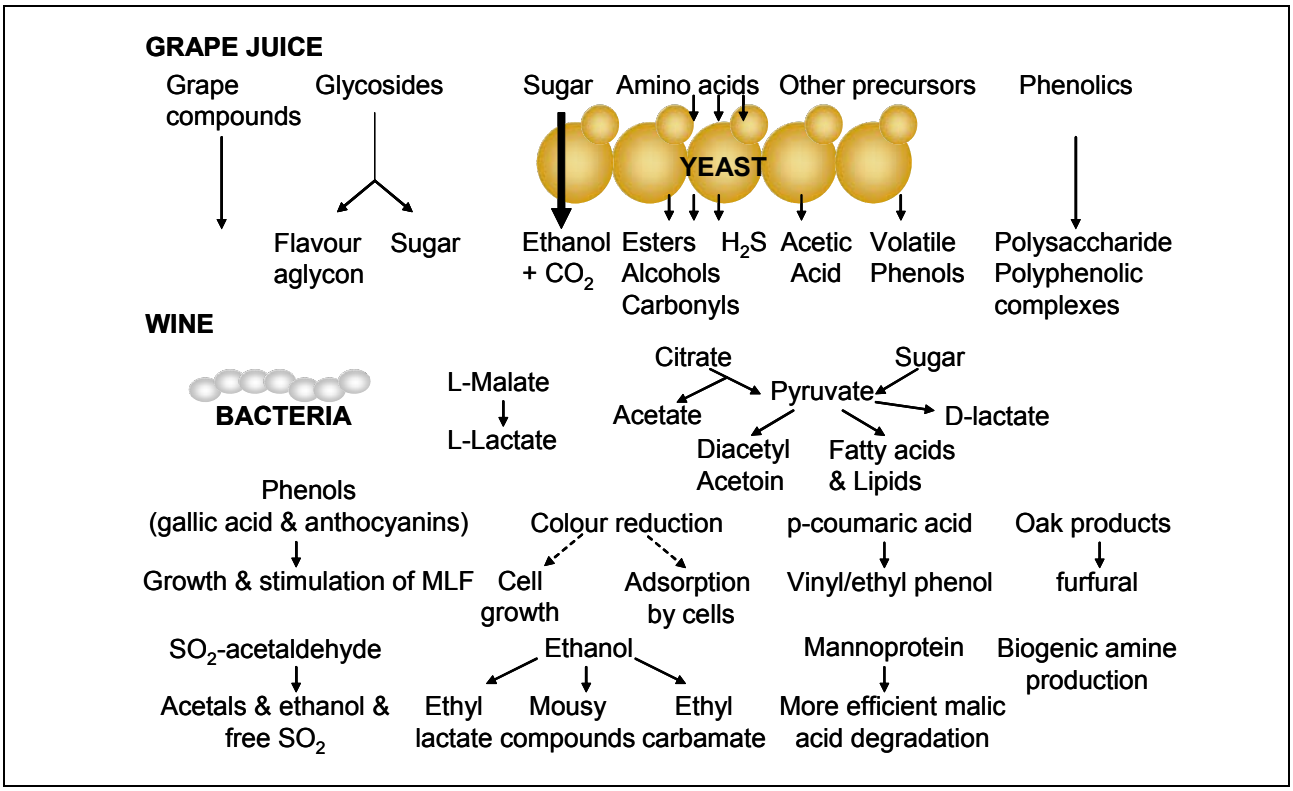

Figure 2.1 Summary of the major metabolism products of grape-derived compounds by yeast and bacteria during the vinification process (Bartowsky *et al*., 2002a).

The secondary fermentation process involved in winemaking, namely malolactic fermentation (MLF), involves the conversion of L-malic acid to L-lactic acid by lactic acid bacteria (LAB) (Davis *et al*., 1985). In addition to the biodeacidification, a large variety of other compounds are either increased or reduced by bacterial metabolism (**Figure 2.2**). The MLF process could spontaneously occur in wine by indigenous *Oenococcus oeni*, *Pediococcus* and *Lactobacillus* species present on the grapes and in the wine environment (Wibowo *et al*., 1985; Du Toit *et al*., 2010). However, spontaneous MLF does not ensure consistent outcomes in terms of MLF completion, organoleptic profile or resulting wine quality. The introduction of commercial freeze-dried bacterial cultures for direct inoculation into wine has improved the management of MLF (Nielsen *et al*., 1996). This ensures better control over the time of onset and rate of MLF, reduces the potential for spoilage by other bacteria, reduces the potential interference by bacteriophages, gives better control over the flavour contribution of MLF and reduces the risk of potential biogenic amine production which has health implications (Lonvaud-Funel, 2001).

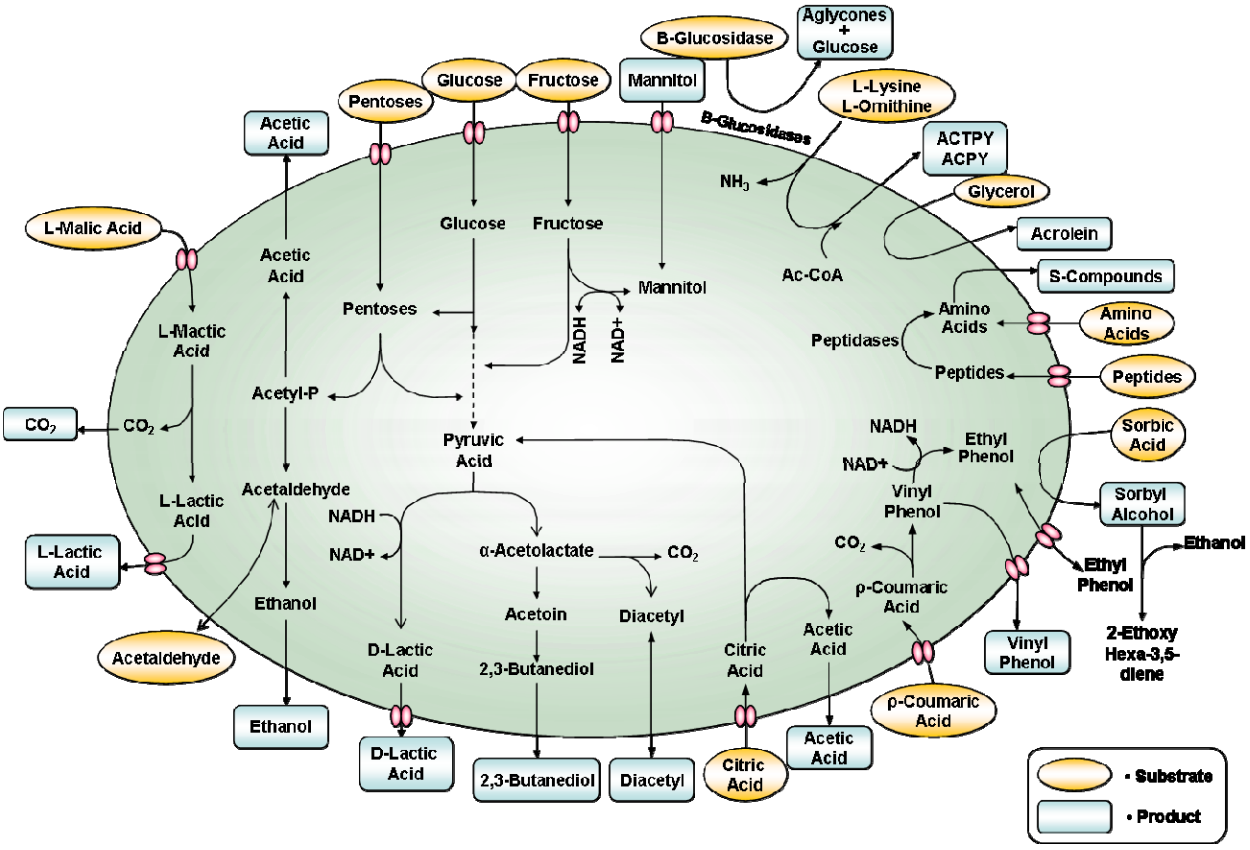

Figure 2.2 A simplified schematic representation of the potential biosynthesis and modulation of aroma compounds by lactic acid bacteria (schematic from Swiegers *et al*., 2005a).

Commercially available strains are usually isolated from spontaneous wine fermentations and consequently evaluated for their fermentation ability, flavour and mouthfeel contribution amongst other criteria (Solieri *et al*., 2010). The species *O. oeni* (previously *Leuconostoc oenos*, Dicks *et al*., 1995) is the preferred starter culture as it is especially well adapted to the harsh wine environment of low nutrient status, low pH, high alcohol and high SO_2 content (Wibowo *et al*., 1985; Versari *et al*., 1999). Research towards investigating the enzymatic capacity amongst LAB has shown the presence of a variety of enzymes, such as esterases, lipases and glucosidases, all of which could contribute to the formation of wine aroma compounds (Liu, 2002; Matthews *et al*., 2004). These findings directed interest towards isolation and genetic screening of other LAB genera for their potential use as commercial starter cultures (Lerm, 2010; Mtshali *et al*., 2010). In addition to the influence of bacterial strain selection on the outcome of MLF, the inoculation regime used for MLF induction could also influence the metabolism of the bacteria and hence impact on the organoleptic profile of the wine. The induction of MLF can typically occur at three main stages during winemaking, namely pre-alcoholic fermentation, during alcoholic fermentation and post-alcoholic fermentation. The availability of nutrients and grape secondary metabolites can vary greatly at these different stages and consequently, the resulting influence of bacterial strains also varies depending on the time of inoculation.

In addition to the biological deacidification reaction that characterises MLF, a diverse range of other metabolic activities are associated with the growth and development of LAB in wine, which significantly influence wine composition and possibly sensory properties of wine (Bartowsky & Henschke, 1995, 2004; Lonvaud-Funel, 1999; Liu, 2002; Bartowsky *et al*., 2002a). An overview of the current knowledge on MLF and wine aroma with specific focus on the biochemical compounds affected by MLF, the analytical techniques generally used for the quantification of these chemical compounds and the sensory research findings related to MLF, will be provided in the following sections.

2.2 BIOCHEMICAL COMPOUNDS INFLUENCED BY LAB METABOLISM

Malolactic fermentation is performed by LAB and as a result, the contribution to wine aroma depends largely on the bacterial strain used and other influencing factors, such as microbial interactions and fermentation conditions including temperature, pH, ethanol and sulphur dioxide levels (Bartowsky & Henschke, 1995). The formation of aroma compounds, such as esters, fatty acids, fatty acid esters and higher alcohols, by bacteria is intrinsically linked to their metabolism. Some of these aroma compounds have specific functions in the bacterial cell, however, the function and mechanism related to the formation of others are still speculative (Liu, 2002). This section will focus on biochemical changes imparted by MLF with a specific focus on their sensory significance pertaining to the knowledge currently available.

2.2.1 ORGANIC ACIDS

Malic acid and tartaric acid are the major organic acids present in grapes (Swiegers *et al*., 2005a). Concentrations of malic acid usually vary between 2-5 g/L, depending on geographic location and climatic conditions (Swiegers *et al*., 2005a). Malic acid metabolism, catalysed by the malolactic enzyme, forms the basis of MLF and involves the conversion of L-malic acid to L-lactic acid (Davis *et al*., 1985, 1988; Lonvaud-Funel, 1999).

Citric acid metabolism in LAB is initiated after the depletion of malic acid and results in the formation of one of the most important compounds associated with MLF, namely diacetyl (2,3-butandione) which confers a ‘buttery’ character to wine (Bartowsky & Henschke, 2004). Other consequences of citrate utilization by *O. oeni* are the formation of acetoin, 2,3-butanediol and acetic acid (**Figure 2.3**).

Acetic acid is described by a sour, pungent, vinegar-like aroma when present at concentrations above its odour threshold of 0.7 g/L (Francis & Newton, 2005). At concentrations between 0.2 and 0.6 g/L, this compound could contribute to the complexity of wine aroma depending on the type and style of wine (Bartowsky & Henschke, 1995; Lonvaud-Funel, 1999). Acetic acid production by heterofermentative LAB during MLF could occur via two potential mechanisms; (i) the conversion of hexoses to produce ethanol, CO_2, acetic acid and D-lactic acid via the phosphoketolase pathway (Lonvaud-Funel, 1999; Swiegers *et al.*, 2005a), and (ii) the formation during the first reaction of citric acid metabolism catalyzed by the citrate lyase enzyme (Bartowsky & Henschke, 2004). Generally, an increase in acetic acid concentration of 0.1 to 0.2 g/L is associated with MLF (Bartowsky & Henschke, 1995).

The metabolism of organic acids during malolactic fermentation can have a significant impact on the flavour of wine (Bartowsky *et al*., 2002a). The reduction of malic acid to lactic acid generally results in a softer, more palatable wine as a result of the reduction in acidity, while the formation of acetic acid and diacetyl, contributes to the volatile acidity and buttery character of wine, respectively (Bartowsky *et al*., 2002a).

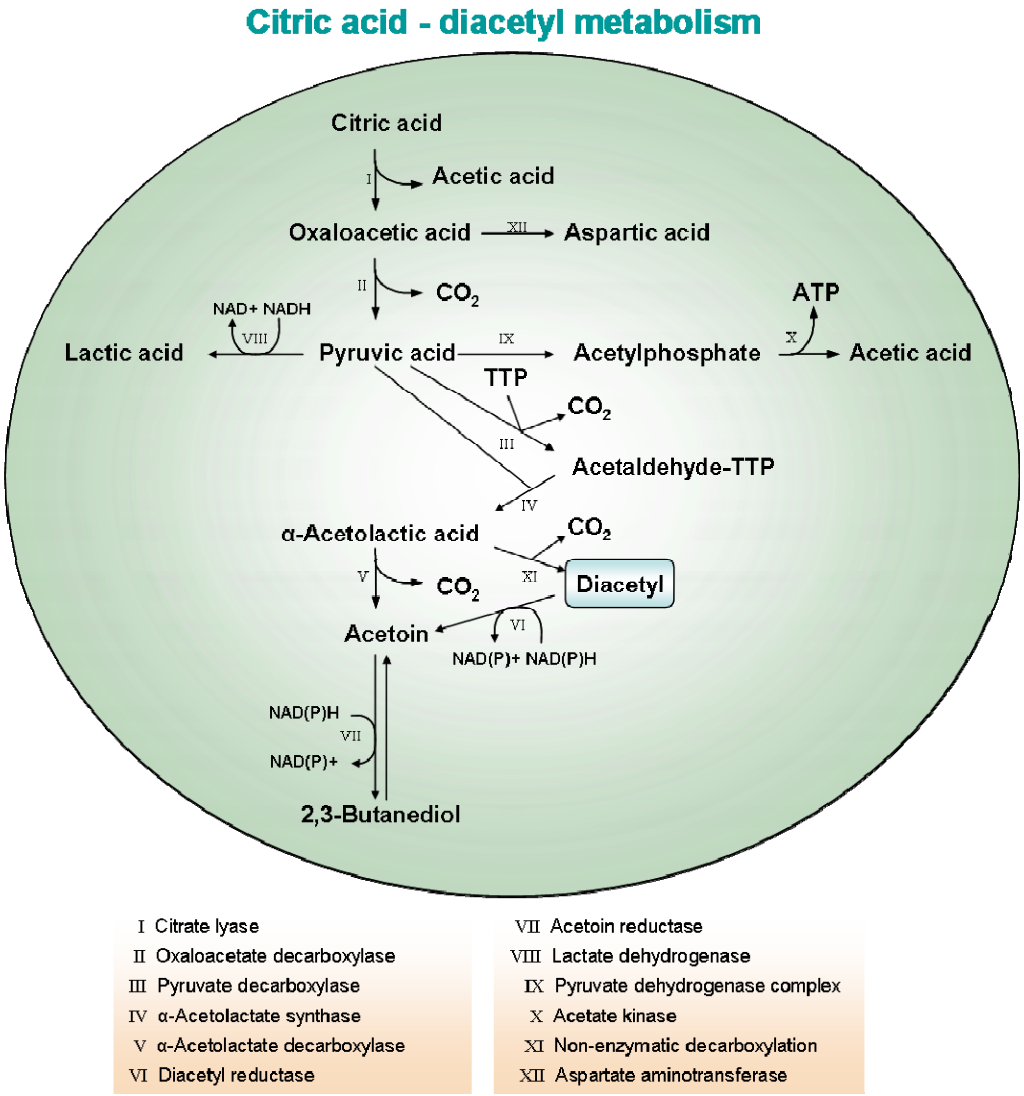

Figure 2.3 Schematic representation of citric acid metabolism and the synthesis of diacetyl by lactic acid bacteria (Swiegers *et al*., 2005a).

2.2.2 VOLATILE FATTY ACIDS

Volatile fatty acids, both straight chain and branched chain fatty acids, are produced by the action of lipases on lipids present in wine (Liu, 2002; Matthews *et al*., 2004) (**Table 2.1**). These compounds are of interest due to their low perception thresholds. As a result, they have the ability to add complexity when present in lower quantities and be detrimental to wine quality when present at higher concentrations, as they impart unpleasant odours of rancid, pungent, cheese, sweaty and

fat-like aromas (Francis & Newton, 2005). A positive contribution to the wine aroma profile can develop when volatile compounds such as esters, ketones and aldehydes are derived from these fatty acids (Matthews *et al*., 2004). In a study of LAB isolated from wine, Davis *et al*. (1988) observed lipase activity in several *O. oeni* strains and one *Lactobacillus* strain. In more recent surveys (Matthews *et al.,* 2006; Mtshali, 2007; Mtshali *et al*., 2010), lipase gene activity was absent in the *Lactobacillus* strains tested (Mtshali, 2007; Mtshali *et al*., 2010), or lipase enzymatic activity was restricted to three *Lactobacillus* isolates and absent in the 23 *O. oeni* strains tested (Matthews *et al*., 2006). Despite the evidence supporting the limited lipase activity of wine LAB, a number of studies have reported changes in the volatile fatty acid composition as a result of MLF and consequently LAB activity. A significant increase in the concentrations of octanoic, hexanoic and decanoic acids after completion of MLF was previously reported by Herjavec *et al.* (2001) and Maicas *et al*. (1999). In another study, Pozo-Bayón *et al*. (2005) reported significant differences for octanoic and decanoic acids depending on the MLF culture used. In a recent metabolic profiling study, differentiation between wines according to LAB strain was ascribed, amongst other factors, to differences in the concentrations of isobutyric and octanoic acids (Lee *et al*., 2009). In contrast, Maicas *et al.* (1999) found no significant increases in isovaleric, isobutyric and hexanoic acids after the completion of MLF. The lipolytic systems in wine LAB are not well known and further research is needed in this area (Liu, 2002; Matthews *et al.*, 2004).

Table 2.1 Volatile fatty acids present in wine. Concentrations, odour quality and thresholds are indicated (Francis & Newton, 2005).

Acid	Odour quality	Concentration (µg/L) in		Odour threshold[a] (µg/L)
		Young red wine	Aged red wine	
Isobutyric acid	Rancid, butter, cheese	434 - 2345	3510 - 7682	2300
Isovaleric acid	Sweat, acid, rancid	305 - 1151	1062 - 3507	33.4
Butyric acid	Rancid, cheese, sweat	434 - 4719	2020 - 4481	173
Propionic acid	Pungent, rancid, sweat	nr[b]	4160 - 11907	8100
Hexanoic acid	Sweat	853 - 3782	1441 - 5838	420
Octanoic acid	Sweat, cheese	562 - 4667	1095 - 4970	500
Decanoic acid	Rancid, fat	62.1 - 857	290 - 2000	1000

[b]nr = not reported above threshold in any study; [a]in 11% ethanol at pH 3.2 (Francis & Newton, 2005).

2.2.3 CARBONYL COMPOUNDS

2.2.3.1 Diacetyl and related compounds

During MLF, changes associated with carbonyl compounds are often reported (Sauvageot & Vivier, 1997). One of the most frequently reported aroma modifications and the most important flavour compound synthesized during MLF, is diacetyl (2,3-butanedione). At concentrations above its sensory threshold, diacetyl confers a buttery, butterscotch, nutty and/or toasty aroma to wine (Etiévant, 1991; Bartowsky & Henschke, 1995; 2004). The aroma detection threshold for diacetyl in a 10% ethanol solution was reported as 0.1 mg/L (Guth, 1997). In wine, the detection threshold for diacetyl is dependent on the wine style and has been reported to vary from 0.2 mg/L for Chardonnay, 0.9 mg/L for Pinot Noir and 2.8 mg/L for Cabernet Sauvignon (Martineau *et al*., 1995a). The perception of the buttery attribute is thus highly dependent on the presence of other compounds in the wine matrix (Martineau & Henick-Kling, 1995b; Bartowsky *et al*., 2002a). A

survey of Australian wines illustrates this aspect very clearly (Bartowsky *et al*., 1997, 2002b) where wines with similar concentrations of diacetyl received different intensity scores for the 'buttery' attribute (**Figure 2.4**).

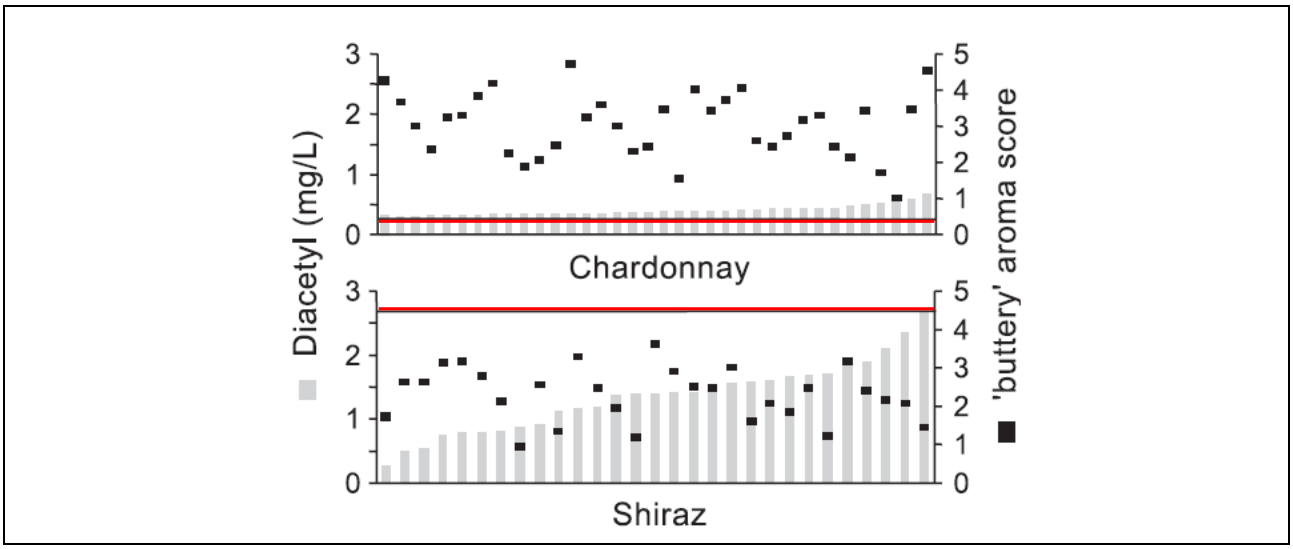

Figure 2.4 The diacetyl content (mg/L) and 'buttery sensory' perception of Australian Chardonnay (36) and Shiraz (29) wines (Bartowsky *et al*., 1997; 2002b). The 'buttery' aroma score for the wines were rated on a scale of 0 to 9 (0 indicated that the buttery attribute could not be perceived, while 9 was defined as high intensity). The red line at 0.2 mg/L (Chardonnay) and 2.8 mg/L (Shiraz) indicate the reported sensory thresholds for diacetyl in these wines.

Diacetyl is an intermediate product of citric acid metabolism and can be further metabolised to acetoin and 2,3-butanediol (**Figure 2.3**). Acetoin and 2,3-butanediol are considered to be flavourless in wine due to their high aroma thresholds (approximately 150 and 600 mg/l, respectively; Etiévant, 1991). Yeast also have the ability to produce diacetyl during alcoholic fermentation, however, the majority of this diacetyl is further metabolised to acetoin and 2,3-butanediol (Martineau & Henick-Kling, 1995a).

A considerable amount of research has focused on the manipulation of diacetyl concentrations during winemaking and comprehensive reviews regarding the influencing factors are available elsewhere (Martineau *et al*., 1995b; Bartowsky *et al*., 2002a; Bartowsky & Henschke, 2004). In brief, the bacterial strain and inoculation rate (i.e. cfu/mL), as well as the wine pH, citrate concentration and fermentation temperature, could influence diacetyl concentrations. The extent of lees contact after MLF, the sulphur dioxide concentration and the degree of aeration during winemaking, could all influence the diacetyl content of wine. Consequently, different wine styles with regards to the buttery attribute resulting from diacetyl, could be obtained by manipulating the mentioned factors (Martineau *et al*., 1995b; Bartowsky *et al*., 2002a; Bartowsky & Henschke, 2004).

Other dicarbonyl compounds such as glyoxal, methylglyoxal, hydroxypropandial and 2,3-pentanedione are involved in cellular redox systems and could be produced by microorganisms responsible for MLF (de Revel & Bertrand, 1993; Guillon *et al*., 1997; de Revel *et al*., 2000; Flamini & Dalla Vedova, 2003). Yeast could also synthesize dicarbonyl compounds, with the exception of 2,3-pentanedione, during alcoholic fermentation (Lambrechts & Pretorius, 2000). The reduction of these dicarbonyl compounds are advantageous for yeasts and bacteria since it renders them less

toxic and increases NAD^+ and $NADP^+$ levels (Okado-Matsumoto & Fridovich, 2000; Flamini & Dalla Vedova, 2003). In terms of their contribution to wine aroma, 2,3-pentanedione has some importance, while glyoxal and methylglyoxal have little sensory significance. However, their aroma properties are similar to that of diacetyl, namely buttery or lactic-like (de Revel *et al*., 2000).

2.2.3.2 Aldehydes

Volatile aldehydes constitute a group of compounds with detection thresholds in the low µg/L range and therefore possibly contribute to perceived wine aroma (de Revel & Bertrand, 1993). Acetaldehyde is quantitatively the most important carbonyl compound in wine and constitutes 90% of the total aldehyde concentration, with levels typically ranging between 10-200 mg/L (Romano *et al*., 1994). It contributes a pleasant fruity, nutty aroma to wine when present near its sensory threshold of 500 µg/L (Ferreira *et al*., 2000), but imparts a sharper, green, grassy, oxidative or apple-like aroma when present at higher concentrations (Miyake & Shibamoto, 1993). The metabolism of acetaldehyde by wine LAB is not well understood and it is still not clear whether wine LAB can produce acetaldehyde (Liu & Pilone, 2000), although dairy lactococci and lactobacilli can produce acetaldehyde (Liu & Pilone, 2000). However, Osborne and co-workers (2000) showed that all oenococci tested in a synthetic wine medium were able to degrade acetaldehyde, converting it to ethanol and acetate. In addition, a follow-up study by the same author, illustrated the ability of two commercial *O. oeni* starter cultures to degrade SO_2-bound acetaldehyde in white wine (Osborne *et al*., 2006). The degradation of acetaldehyde by LAB has important consequences in terms of the use of the wine preservative sulphur dioxide (SO_2) and impact on red wine colour development (reviewed by Bartowsky & Henschke, 1995; Liu & Pilone, 2000; Bauer & Dicks, 2004).

Apart from acetaldehyde, a large number of other aldehydes, mostly present at trace levels, have been reported in wine (**Table 2.2**). Aliphatic aldehydes containing 3-5 carbon atoms have been reported to be present in wine at concentrations of up to 5 mg/L while the expected levels of (*E*)-2-nonenal and other higher aldehydes are between 0.1 and 5 µg/L (Ferreira *et al*., 2004).

Table 2.2 Concentrations, odour quality and thresholds of some aldehydes found in wine (Culleré *et al*., 2007).

Compounds	Odour quality	Concentration (µg/L)	Odour threshold[a] (µg/L)
2-Methylpropanal	chocolate-like, malty	0.9 - 132	6.0
2-Methylbutanal	chocolate-like, malty	3.3 - 105	16
3-Methylbutanal	chocolate-like, malty	1.0 - 49	4.6
E-2-Hexenal	herbaceous, green[1]	0.02 - 1.6	4
E-2-Heptenal	herbaceous[1]	<0.16	4.6
E-2-Octenal	herbaceous[1], lemon[2]	0.04 - 4.1	3
E-2-Nonenal	sawdust, plank[3]	0.1 - 3.7	0.6
Phenylacetaldehyde	hawthorne (floral), honey, sweet	2.4 - 130	1

[1]de Revel & Bertrand, 1994; [2]Escudero *et al*., 2007; [3]Chatonnet & Dubourdieu, 1998 ; [a]in 11% ethanol at pH 3.2 (Francis & Newton, 2005).

A study related to the sensory properties of aldehydes revealed that aldehydes with 8-10 carbon atoms, such as (*E)*-2-nonenal, octanal, nonanal, decanal or (*E,Z)*-2,6-nonadienal, are strong odourants (Laska & Teubner, 1999). In wine, the odour properties of (*E*)-2-nonenal are particularly

important since it can be responsible for a "sawdust" or "plank" off-flavour (Chatonnet & Dubourdieu, 1998), while the herbaceous odour in wine is often associated with aliphatic aldehydes such as hexanal, (*E*)-2-hexenal, (*E*)-2-heptenal, octanal and (*E*)-2-octenal (de Revel & Bertrand, 1994). In previous reports, the aldehydes studied, namely octanal, nonanal, decanal, (*E*)-2-nonenal, (*E,Z*)-2,6-nonadienal (Ferreira *et al*., 2004) and phenylacetaldehyde, 3-methylbutanal, (*E*)-2-octenal, (*E*)-2-hexenal and (*E*)-2-heptenal (Culleré *et al*., 2004), were present in wine at concentrations above their respective odour thresholds, with the exception of (*E,Z*)-2,6-nonadienal, (*E*)-2-hexenal and (*E*)-2-heptenal. Subsequent assessments of oxidation-related aldehydes in wine (Ferreira *et al*., 2006; Culleré *et al*., 2007) confirm the active sensory role and revealed the existence of interactions, either additive or synergistic, between aldehydes and other volatile components. The importance of branched chain aldehydes, such as 3-methylbutanal, their relevance in the flavour of food products and the possible pathways involved, were recently reviewed by Smit *et al*. (2009). It is clear from the literature available that the exact role of aldehydes in wine aroma is not fully understood due to the lack of analytical data. The ability of wine LAB to degrade acetaldehyde (Osborne *et al*., 2000; 2006) demonstrate the potential of wine LAB to catabolise other aldehydes. This could possibly be related to the reduction in green or vegetative aroma attribute (Henick-Kling *et al*. 1994) often ascribed to MLF. No reports are currently available regarding the effect of MLF and different LAB strains on aldehyde concentrations and this merits further investigation.

2.2.4 ESTERS

Esters are formed by the esterification of an alcohol and carboxylic acid and the elimination of a water molecule, either enzymatically, or as a result of chemical esterification during wine ageing (Etiévant, 1991). This group of compounds is qualitatively one of the most important groups of volatile compounds in determining wine flavour (Ferreira *et al*. 1998; Lilly *et al*. 2006) and represents the primary source of fruity aroma characteristics in wine (Ebeler, 2001) (**Table 2.3**). Enzymatic ester synthesis is catalysed by esterases, lipases and alcohol acetyltransferases (Lilly *et al*., 2006), which are produced by microbial metabolism during winemaking (reviewed by Sumby *et al*., 2010). Esters are mainly produced as secondary products of yeast sugar metabolism during alcoholic fermentation (Lambrechts & Pretorius, 2000) and are generally categorised as either ethyl esters of fatty acids, acetate esters of higher alcohols or esters of organic acids. The latter being the predominant group in wine, followed by acetate esters and ethyl esters of fatty acids (Etiévant, 1991).

Table 2.3 A selection of esters found in wine. Concentrations found in wine, odour quality and thresholds are presented (Francis & Newton, 2005).

Ester	Odour quality	Concentration (µg/L) in		Odour threshold[a]
		Young red wine	Aged red wine	(µg/L)
Ethyl hexanoate	Apple peel, fruit	153 - 622	255 - 2556	5 - 14
Ethyl octanoate	Fruit, fat	138 - 783	162 - 519	2 - 5
Ethyl butyrate	Apple	69.2 - 371	20 - 1118	20
Isoamyl acetate	Banana	118 - 4300	249 - 3300	30
2-Phenylethyl acetate	Rose, honey, tobacco	0.54 - 800	nr[b]	250

[b]nr = not reported above threshold in any study; [a]in 11% ethanol at pH 3.2 (Francis & Newton, 2005).

The most important esters typically associated with MLF are ethyl lactate (ethyl-2-hydroxypropanoate) and diethyl succinate (Maicas *et al*., 1999; Herjavec *et al*., 2001; Ugliano & Moio, 2005). The contribution of MLF to the ester profile of wine has been shown by a number of wine volatile profiling studies (Laurent *et al.*, 1994; Maicas *et al*., 1999; Delaquis *et al.*, 2000; D'Incecco *et al*., 2004; Ugliano & Moio, 2005). Observations from these reports suggested that wine LAB possess enzymatic activity which could either synthesize or hydrolyze esters, depending on the bacterial strains, grape cultivar and fermentation conditions (Pozo-Bayón *et al*., 2005). Strain specific changes observed in ester concentration during MLF are summarised by Sumby *et al*. (2010). Generally, increases were observed in ethyl-2-methylpropanoate (fruity, strawberry, lemon), ethyl 2-methylbutanoate (apple, berry, sweet, cider, anise), ethyl 3-methylbutanoate (sweet fruit, pineapple, lemon, anise, floral), ethyl 2-hydroxypropanoate (milk, soapy, buttery, fruity), ethyl 3-hydroxypropanoate (fruity, green, marshmallow), ethyl hexanoate (fruity, strawberry, green apple, anise), 3-methylbutyl acetate (banana, fruity), ethyl 2-phenylacetate (rose, floral), 2-phenylethyl acetate (flowery, rose) and hexyl acetate (green, herbaceous, fruit, grape).

In a survey by Matthews *et al*. (2006), all 50 LAB isolates investigated and comprising of *Lactobacillus*, *Oenococcus* and *Pediococcus* spp., were found to hydrolyze esters. Increased esterase activity was found amongst oenococci, followed by lactobacilli and pediococci. A follow-up study showed that *O. oeni* esterase activity increased progressively with increasing ethanol up to 14% and was the least influenced by pH (Matthews *et al*., 2007). Recently, genetic studies identified and characterised genes involved in the esterase activity of *O. oeni* (Sumby *et al*., 2009) and wine-associated *Lactobacillus* spp. (Mtshali *et al*., 2010). It is clear from the mentioned findings that LAB possess an extensive collection of ester synthesizing and hydrolyzing activities (Matthews *et al*., 2004; Liu, 2002) which highlights the tremendous potential of this group of organisms to contribute to wine aroma.

2.2.5 HIGHER ALCOHOLS

Higher alcohols (referring to alcohol compounds with more than two carbon atoms) are synthesized as a consequence of amino acid metabolism and considered to contribute to the complexity and fruity aroma of wine when present at concentrations lower than 300 mg/L (Swiegers *et al*., 2005a). However, at concentrations above 400 mg/L, these compounds could impart harsh, solvent, chemical-like aromas detrimental to wine aroma (Swiegers *et al*., 2005a) (**Table 2.4**). The influence of MLF on concentrations of higher alcohols appears to be inconclusive. A number of studies reported no change (Laurent *et al*., 1994; Herjavec *et al*., 2001), or an insignificant increase (Pozo-Bayón *et al*., 2005) in the concentrations of 1-propanol, isobutanol, isoamyl alcohol and 2-phenylethanol. Maicas *et al.* (1999) observed the production of isobutanol, 1-propanol, 1-butanol and isoamyl alcohol to be dependent on the strain used to perform MLF. Other studies (de Revel *et al*., 1999; Jeromel *et al*., 2008) found an insignificant effect on the higher alcohol content of wine, with the exception of significant increases in the concentrations of isoamyl alcohol (de Revel *et al*., 1999), isobutanol and 2-phenylethanol (Jeromel *et al*., 2008).

Table 2.4 Higher alcohols produced by lactic acid bacteria. Odour quality, concentration in wine and odour threshold is provided (Francis & Newton, 2005).

Higher alcohol	Odour quality	Concentration (µg/L) in		Odour threshold[a]
		Young red wine	Aged red wine	(µg/L)
Isobutanol	Wine, solvent, bitter	25.7 - 86.9	57.2 - 230	40
Isoamyl alcohol	Whiskey, malt, burnt	83.95 - 333	165 - 472	30
2-Phenylethanol	Honey, spice, rose, lilac	9 - 153	24 - 166.6	10 - 14

[a]in 11% ethanol at pH 3.2 (Francis & Newton, 2005).

2.2.6 GLYCOSYLATED COMPOUNDS

Aroma compounds such as monoterpenes, C_{13}-norisoprenoids, benzene derivatives and aliphatic compounds could occur in grape and wine as odourless monoglycosides, linked to D-glucose, or as disaccharide glycosides. In the latter, the D-glucose is further conjugated with a second sugar unit of α-L-arabinofuranose, α-L-rhamnopyranose, β-D-xylopyranose or β-D-apiofuranose (Sefton *et al*., 1993b). The general structure of glycosides is shown in **Figure 2.5**. Wood-derived glycoconjugates will be discussed in section 2.2.10 which focuses on wood-related compounds.

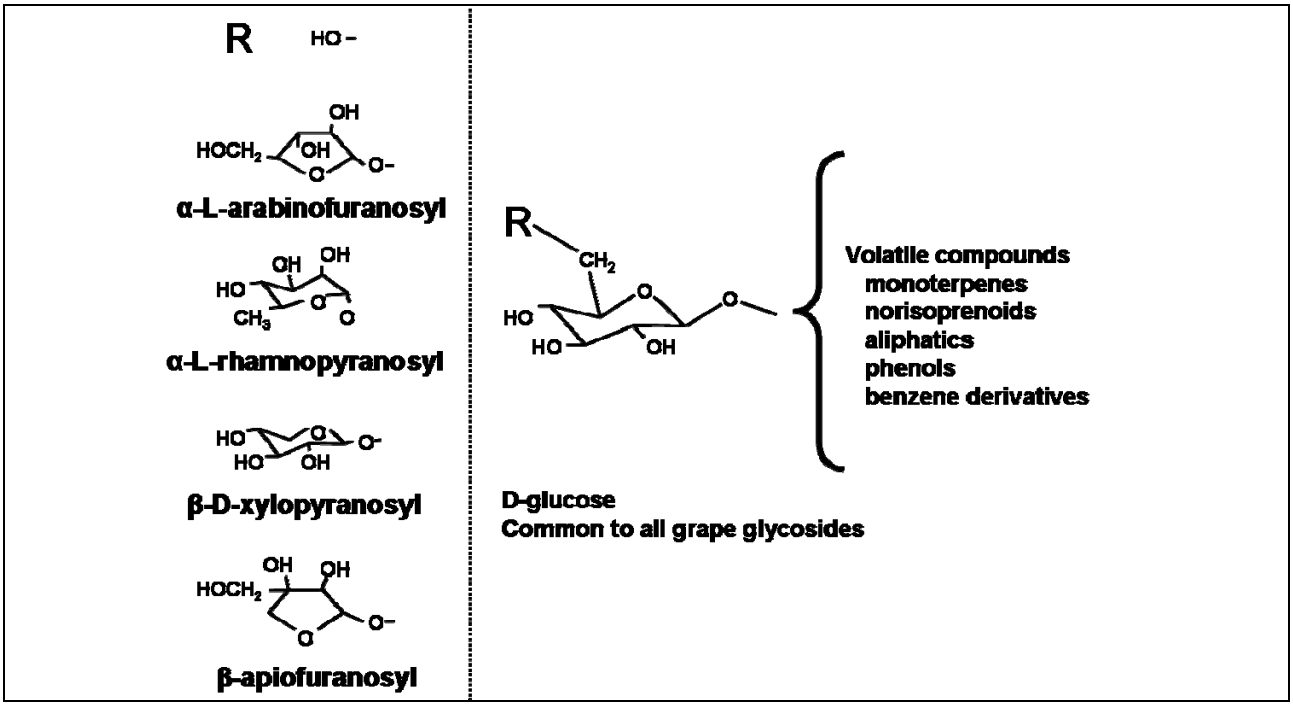

Figure 2.5 Mono- and disaccharide sugar moieties that have been identified as flavour precursors in grapes (D'Incecco *et al*., 2004).

Liberation of glycosidically bound aroma precursors may occur enzymatically by the action of glycosidases or *via* acid hydrolysis (Sefton *et al*., 1993b) to release odour-active aglycons, which could contribute to the sensory characteristics of wine. The release of glucose-bound volatiles requires the action of a β-glucosidase, while the release of volatile compounds from a disaccharide glycoside involves the sequential action of an appropriate *exo*-glycosidase (e.g. arabinosidase) followed by β-glucosidase to release the aglycon (Günata *et al*., 1988). Some of the sensory modifications associated with MLF, such as changes in the intensities of floral, fruity, spicy, and

honey attributes (Bartowsky & Henschke, 1995), might be related to the release of glycosidically bound volatile compounds.

A number of investigations have therefore focused on the glycosidase activities of wine LAB to release compounds with potential sensory significance (McMahon *et al*., 1999; Boido *et al*., 2002; Mansfield *et al*., 2002; Ugliano *et al*., 2003; Barbagallo *et al*., 2004; D'Incecco *et al*., 2004; Ugliano & Moio, 2006). Comprehensive research by Grimaldi and co-workers (2000; 2005a; 2005b) illustrated the *β*-glucosidase activity of a large selection of *O. oeni* strains, *Lactobacillus* spp. and *Pediococcus* spp. towards synthetic glycoside substrates. Observations found this activity to be substrate-specific and influenced by wine parameters such as pH, temperature, sugars and ethanol. Most *O. oeni* strains was found to have relatively high glycosidase activity at wine pH (3.0-4.0) (Grimaldi *et al*., 2005a), while *Lactobacillus* spp. and *Pediococcus* spp. were shown to possess different degrees of *β* - and α-D-glucopyranosidase activities, that were dependant on the wine parameters (Grimaldi *et al*., 2005b). Activity towards glycosides extracted from Muscat wines (Ugliano *et al*., 2003), non-floral Verdejo, Chardonnay, Garnacha and Tempranillo grapes (Hernandez-Orte *et al*., 2009) and Chardonnay (D'Incecco *et al*., 2004) all confirm the glycosidase activity of *O. oeni*. The absence of *β*-glucosidase activity on Viognier grape glycosides, imply that this cultivar has somehow an influence on enzymatic activity (Mansfield *et al*., 2002). This is supported by the limited release of glycosylated aroma compounds by MLF in Tannat wine (Boido *et al*., 2002). In contrast, Ugliano & Moio (2006) presented findings on the ability of four commercially available bacterial strains to modify the composition of the grape-derived volatile fraction of red wine, through the hydrolysis of glycosides and the release of the corresponding aglycons during winemaking. The effect of different bacterial strains on the liberation of aroma compounds from glycosidically bound compounds is clear from **Figure 2.6.**

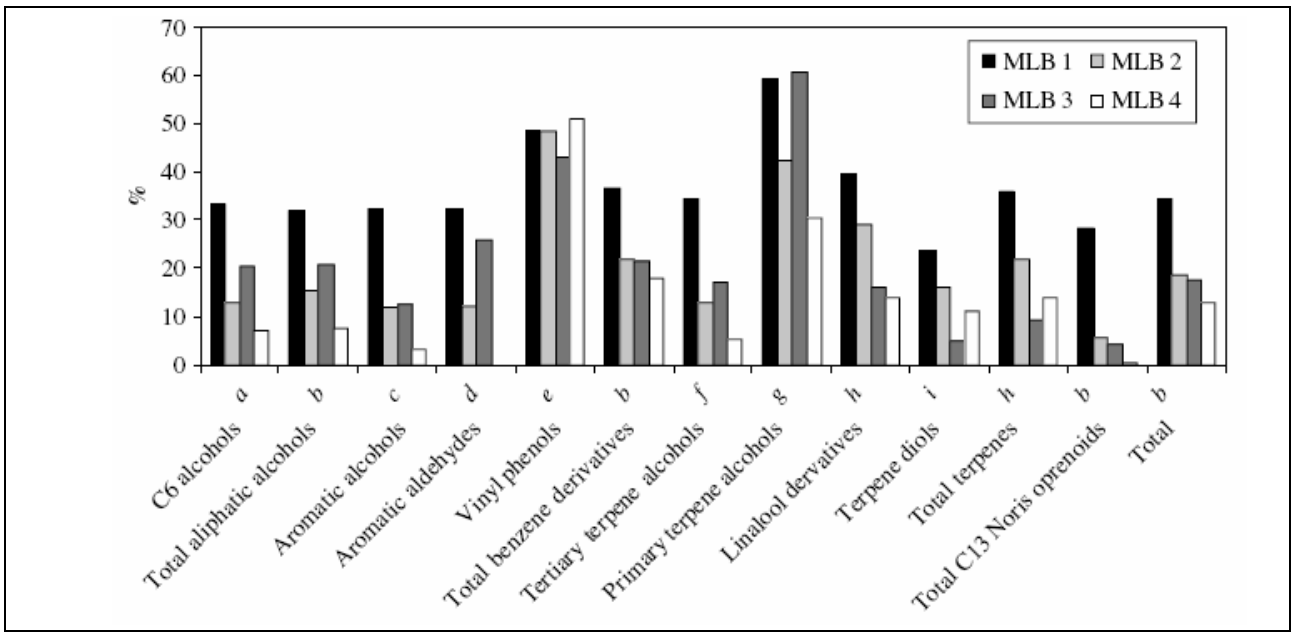

Figure 2.6 Extent of glycoside hydrolysis during MLF in red wine, calculated as a percentage ratio between the concentration of glycosides in MLF samples and in the control (no MLF) (Ugliano & Moio, 2006). Four commercial MLF bacteria were used, MLB 1, MLB 2, MLB 3 and MLB 4.

2.2.7 VOLATILE PHENOLS

Phenolic acids, mainly ferulic and *p*-coumaric acids, are natural constituents of wine which could be microbially metabolised to produce the volatile phenols 4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol (Liu, 2002). Due to their low detection thresholds and distinct flavours, volatile phenols can contribute positively or negatively to wine aroma, depending on their concentrations. These compounds contribute smokey, woody, clove-like, spicy, medicinal, animal and sweaty notes to wine (Lonvaud-Funel, 1999) which are characteristics typically associated with the presence of *Brettanomyces* in wines (Chatonnet *et al*., 1997). However, the increased concentrations of volatile phenols observed during MLF suggest the ability of wine LAB to produce some of these compounds (Liu, 2002).

The synthesis of these compounds involves the corresponding precursor to be transported into bacterial cells by active transport, decarboxylated to the vinyl derivatives by hydroxycinnamic acid decarboxylases and enzymatically reduced to the ethyl derivatives (Cavin *et al*., 1993) (**Figure 2.2**). Studies related to phenolic acid metabolism by wine LAB (reviewed by Liu, 2002; Swiegers *et al*., 2005a) suggest the ability of several wine lactobacilli and pediococci to metabolise phenolic acids, however, further work in this area is necessary with regards to *O. oeni* metabolism. It has been shown that *p*-coumaric and ferulic acids could stimulate *O. oeni* growth (Campos *et al*., 2003; Nelson, 2007). However, this microorganism lacks the gene necessary to decarboxylate *p*-coumaric acid to produce volatile phenols (Gámbaro *et al*., 2001; Swiegers *et al*., 2005a).

2.2.8 SULPHUR CONTAINING COMPOUNDS

Volatile sulphur compounds make an important contribution to the sensory properties of wine as a result of their low odour thresholds and characteristic aroma notes (**Table 2.5**). Some sulphur compounds contribute negatively to wine quality (e.g. hydrogen sulphide (H_2S), methanethiol (MTL) and ethanethiol) while other compounds have a positive effect on wine aroma (e.g. dimethyl sulphide (DMS), methional) or contribute to wine varietal aroma (e.g some volatile thiols, recently reviewed by Landaud *et al*., 2008).

Table 2.5 Concentrations (µg/L), odour quality and thresholds of some volatile sulphur compounds found in wine (Landaud *et al*., 2008).

Compounds	Odour quality	Probable precursor	Concentration (µg/L)	Odour threshold[a] (µg/L)
Methanethiol	Cooked cabbage, onion	Methionine	2.1-5.1	0.3
Dimethyl disulfide	Cooked cabbage, intense onion	Methanethiol	2	15-29
3-(methylsulphanyl)propan-1-ol	Cauliflower, cabbage	Methionine	140-5000	500
3-(methylsulphanyl)propionic acid	Chocolate, roasted	Methionine	0-1811	244

[a]determined in wine.

Pripis-Nicolau *et al.* (2004) provided the first evidence regarding the ability of wine LAB to metabolise methionine to produce volatile sulphur compounds during MLF, while the possible pathways involved in methionine metabolism were described by Vallet *et al*. (2008). Results indicated that all *Oenococcus oeni* strains and wine associated *Lactobacillus* species tested in

synthetic media, were able to metabolise methionine to MTL, DMS, 3-(methylsulphanyl)propan-1-ol (also known as methionol) and 3-(methylsulphanyl)propanoic acid (**Figure 2.7**) (Pripis-Nicolau *et al.*, 2004). However, 3-(methylsulphanyl)propan-1-ol and 3-(methylsulphanyl)propanoic acid were formed in more significant quantities by *O. oeni* than *Lactobacillus* and significant differences between strains within a species were also observed (**Figure 2.7**).

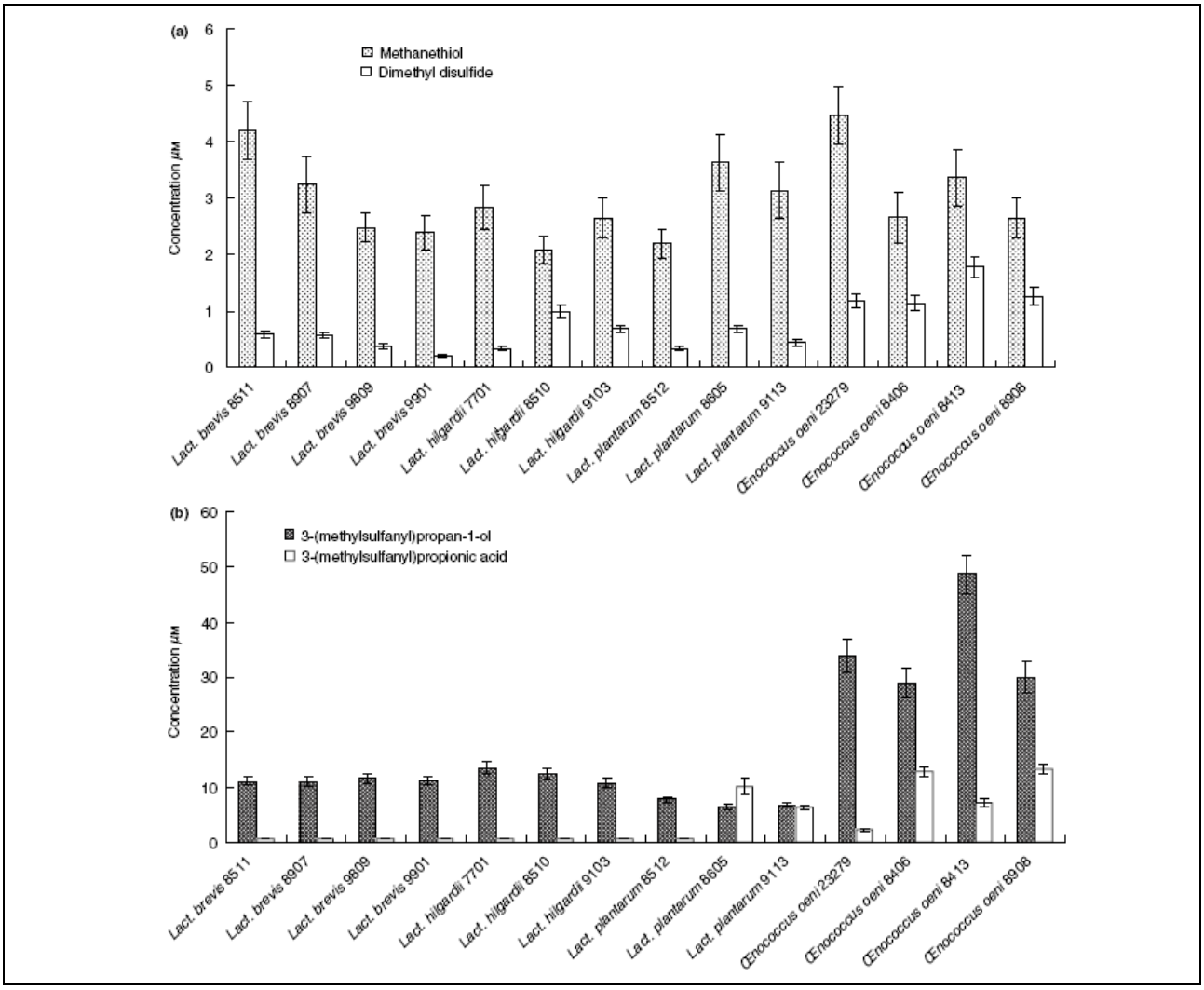

Figure 2.7 Production of volatile sulphur compounds by various species and strains of *Lactobacillus* and *Oenococcus*. The averages (histograms) ± standard deviation (bars) of three determinations are presented for (a) methanethiol and dimethyl disulphide, (b) 3-(methylsulphanyl)propan-1-ol and 3-(methylsulphanyl)propionic acid (Pripis-Nicolau *et al*., 2004).

In a trial evaluating four commercial LAB starter cultures in Merlot wine, increased concentrations of 3-(methylsulphanyl)propanoic acid were observed, suggesting the ability of *O. oeni* to metabolise methionine and produce volatile sulphur compounds. The odour detection threshold of 3-(methylsulphanyl)propanoic acid was determined to be 244 µg/L in red wine (Pripis-Nicolau *et al*., 2004) and increased concentrations were shown to affect the earthy and red fruit sensory descriptors of the wine (**Figure 2.8**).

In a recent study (Knoll *et al*., 2010), a cystathionine β/γ-lyase enzyme capable of degrading sulphur containing amino acids such as cystathionine, cysteine, homocysteine and methionine was cloned and characterised from two *O. oeni* strains of oenological origin. These results suggest that *O. oeni* may have the ability to contribute to the formation of volatile sulphur compounds. However, further work is needed to evaluate the enzymatic activity under harsh winemaking conditions in order to confirm its influence on the production of volatile sulphur compounds in wine.

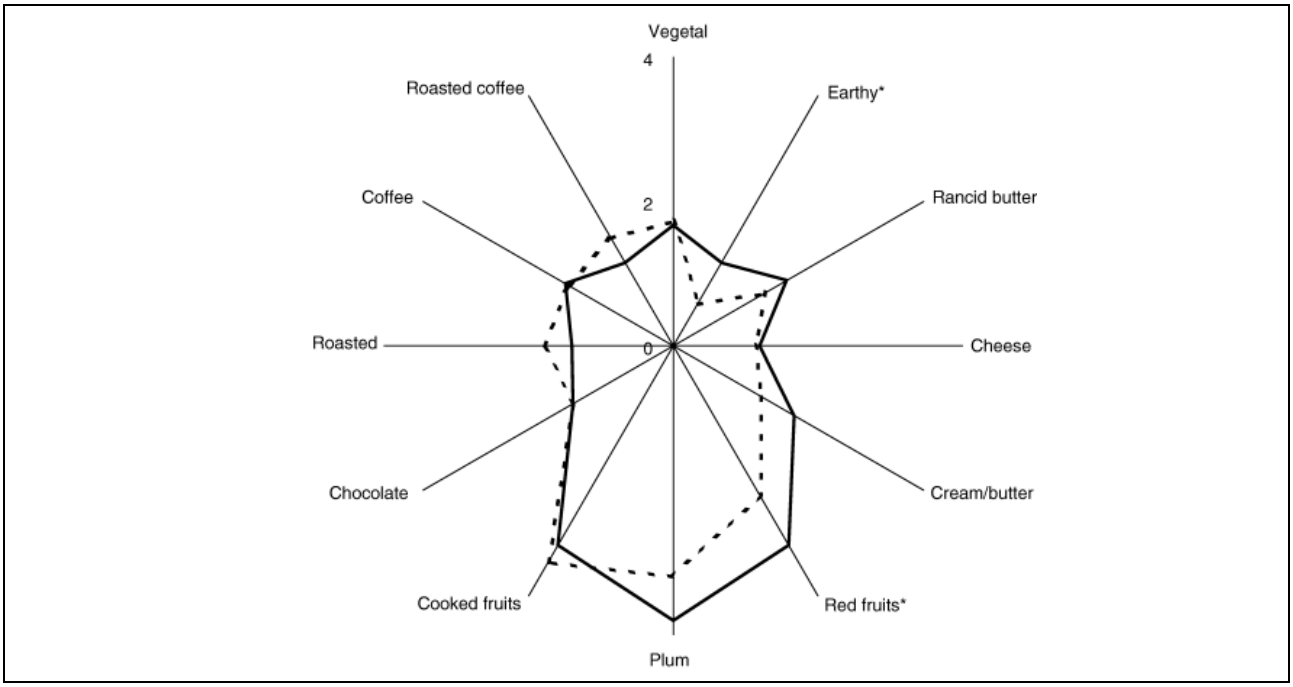

Figure 2.8 Graphic presentation of 12 descriptors (average of 15 tasters) selected for the quantitative descriptive sensory profiling analysis of a Merlot red wine, with (continuous line) or without (stopped line) addition of the 3-(methylsulphanyl)propionic acid. *Descriptors which differentiate the two wines (Pripis-Nicolau *et al*., 2004).

In wine, non-enzymatic reactions involving cysteine and α-dicarbonyl compounds could also occur after MLF. Diacetyl, an α-dicarbonyl compound related to the growth and metabolism of LAB, could react with cysteine and form methanethiol (MTL) and heterocyclic compounds such as tetramethylpyrazine and trimethyloxazole responsible for “toasted”, “sulphur” and “cabbage” aroma notes in wine (Pripis-Nicolau *et al*., 2000).

2.2.9 NITROGENOUS COMPOUNDS

The nitrogenous fraction of wine contains proteins which may be broken down by bacterial proteases and peptidases to generate peptides and amino acids which could potentially impact on wine flavour and stability (Liu, 2002). LAB are fastidious in their amino acid requirements and it has been reported that some LAB produce the enzymes needed to acquire peptides and amino acids to meet these requirements (Farias *et al*., 1996; Matthews *et al*., 2004; Mtshali *et al*., 2010). An early report (Davis *et al*., 1988) found no protease activity in several wine LAB, including several strains of oenococci, pediococci and lactobacilli. However, the production of extracellular proteases by strains of *O. oeni* has been subsequently demonstrated (Rollan *et al.*, 1993) and also partially

characterised (Rollan *et al*., 1995; Farias *et al*., 1996; 2000). Thus, wine LAB appears to have proteolytic activity, although not widespread among oenococci (Leitao *et al*., 2000). The production of peptides and amino acids during MLF in both red and white wines by an *O. oeni* strain (X_2L) was demonstrated by Manca de Nadra and colleagues (1997; 1999; 2005). Although peptides are an important source of amino acids, they can also contribute to bitterness (Habibi-Najafi & Lee 1996; Desportes *et al*., 2001).

Several nitrogen heterocyclic compounds can be produced by wine LAB through metabolism of certain amino acids, notably lysine and ornithine (Heresztyn, 1986; Costello *et al*., 2001) (**Figure 2.9**). The three known sensorially important compounds are 2-acetyltetrahydropyridine (ACTPY), 2-acetyl-1-pyrroline (ACPY) and 2-ethyltetrahydropyridine (ETPY) (Lonvaud-Funel, 1999; Costello *et al*., 2001) which could impart an unpleasant 'mousy' off-flavour to wine (Costello & Henschke, 2002).

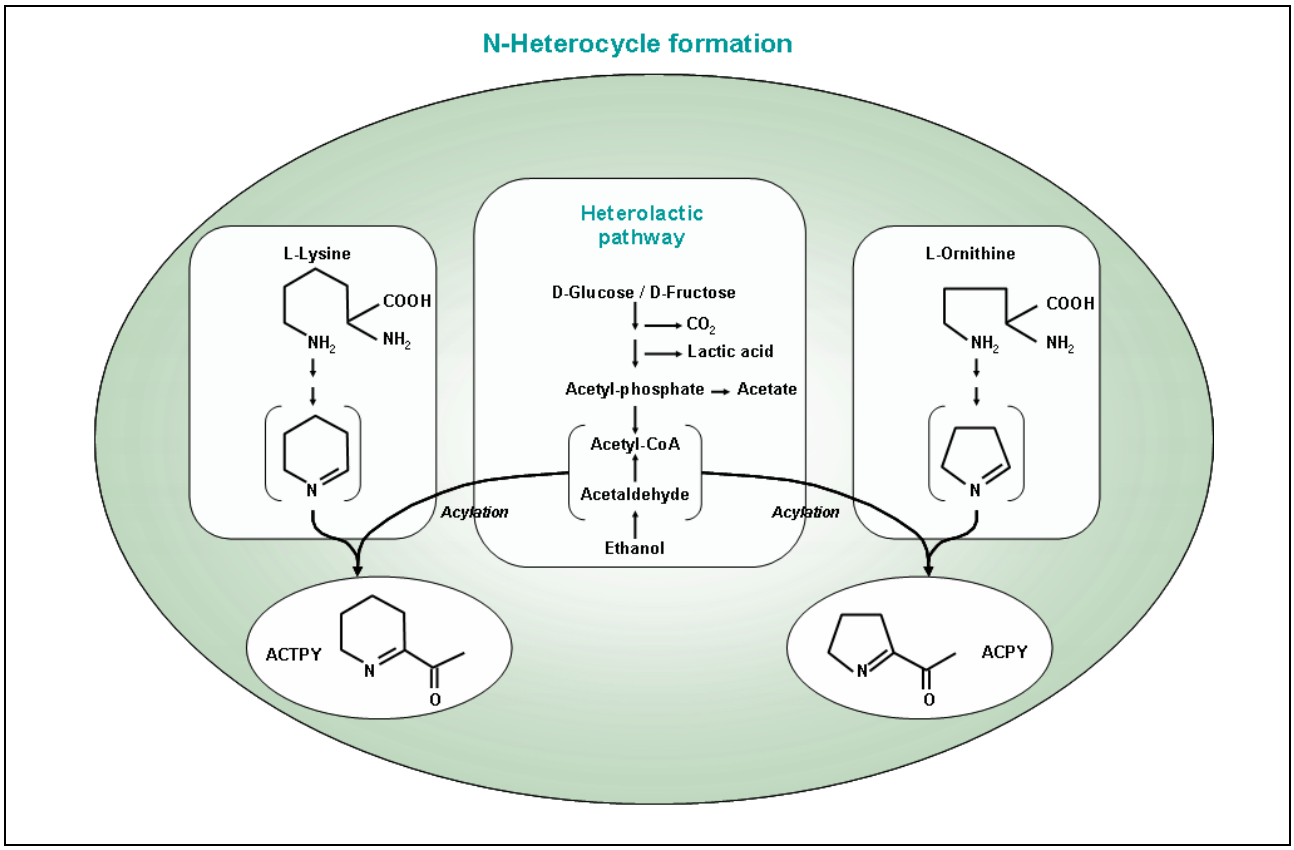

Figure 2.9 Schematic representation of the formation of potent and unpleasant nitrogen-heterocycle 'mousy' off-flavour compounds (2-acetyltetrahydropiridine (ACTPY) and 2-acetyl-1-pyrroline (ACPY)) by some lactic acid bacteria (Swiegers *et al*., 2005a).

The odour detection thresholds of these extremely potent compounds are 1.6 µg/L (determined in water) for ACTPY and 0.1 µg/L (determined in wine) for ACPY, while ETPY was reported to have a taste threshold of 150 µg/L in wine (Herderich *et al*., 1995; Costello & Henschke, 2002). Wines affected by mousy off-flavour have been found to contain individual amounts or combinations of ETPY (2.7 - 18.7 µg/L), ACPY (up to 7.8 µg/L) and ACTPY (4.8 - 106 µg/L) (Costello & Henschke, 2002).

The heterofermentative lactic acid bacteria, *Oenococcus oeni*, *Leuconostoc mesenteroides* and some *Lactobacillus* species were found capable of synthesising ACTPY, ETPY and ACPY (Costello *et al*., 2001). Differences in the formation of the different nitrogen heterocyclic compounds were observed. The heterofermentative lactobacilli favoured the formation of ACTPY, *O. oeni* produced increased amounts of the least flavour active compound, ETPY and the homofermentative pediococci favoured the production of the most flavour active compound, ACPY. In general, the heterofermentative LAB possessed the highest ability to produce a mousy off-flavour (Swiegers *et al*., 2005a). Costello and Henschke (2002) illustrated the significant impact of the precursors lysine and ornithine, as well as the stimulating effect of ethanol and acetaldehyde on the ability of LAB to produce these nitrogen heterocyclic compounds.

Amines are produced by the decarboxylation of their respective amino acids through specific amino acid decarboxylases which are not present in all bacteria (Bartowsky & Henschke, 1995; Lonvaud-Funel, 2001). Amine production varies with each strain of *Leuconostoc, Lactobacillus* and *Pediococcus* and it is generally believed that amines are insignificant in contributing to wine flavour since these compounds are not volatile at wine pH (Radler & Fäth, 1991). However, amines could contribute to the after-taste of wine in a similar manner to that shown for heterocyclic bases implicated in mousy taint (Heresztyn, 1986) where the increase in pH due to contact with saliva may render them sufficiently volatile to elicit a detectable flavour. In addition to amines, the catabolism of amino acids by wine LAB could result in the production of a range of compounds such as aldehydes, alcohols and acids that could have a significant impact on wine quality (Liu, 2002).

Despite various research efforts regarding MLF and nitrogenous compounds (included in reviews by Bartowsky & Henschke, 1995; Liu, 2002; Bauer & Dicks, 2004; Matthews *et al*., 2004; Swiegers *et al*., 2005a) the proteolytic and peptidolytic systems of wine LAB remains poorly defined and the consequent effect on wine sensory properties are unclear.

2.2.10 WOOD-RELATED COMPOUNDS

The fermentation and/or ageing of wines in oak barrels form integral parts of winemaking and could impart important oak-derived aroma attributes, such as woody (oak lactones), spicy (eugenol), smoky (isoeugenol) and vanilla (vanillin), to name a few (de Revel *et al*., 2005). The oak toasting process results in the thermal degradation of lignin/hemicellulose and the production of guaiacol, syringol, furfural, eugenol and increased vanillin levels (Wilkinson *et al*., 2004). These compounds represent a pool of wood-related compounds which could be inevitably affected by LAB metabolism as a result of wood and bacterial interactions during MLF and ageing in barrels.

Previous studies have shown that the metabolic interactions between LAB and oak-derived components could affect bacterial growth (Vivas *et al*., 2000; Bloem *et al*., 2007, 2008), as well as wine aroma (Sefton *et al*., 1993c; Spillman, 1995; de Revel *et al*., 1999, 2005; Bloem *et al*., 2007, 2008). Various authors (Sefton *et al*., 1993a; Spillman, 1995) noted a reduction in the impact of the oak-derived compound, furfural, as a result of MLF performed in barrels. The reduction of furfural to furfuryl alcohol was associated with the contribution of LAB to a large extent.

Extensive research by de Revel and co-workers found increased concentrations of oak lactones, isoeugenol, eugenol and vanillin in Sauvignon Blanc wines (de Revel *et al*., 1999), where MLF was performed in barrels compared to control wines without the intervention of bacterial

starter cultures. This effect was magnified in new barrels with 15% more *trans*-3-methyloctano-4-lactone, 150% more eugenol and 90% more vanillin reported in comparison to the control wine (de Revel *et al*., 1999). In addition, MLF in one-year-old barrels extracted comparable concentrations of these compounds compared to the control wine which was kept in new barrels without MLF (de Revel *et al*., 1999), which could be of practical importance to winemakers.

Similarly, the capacity of *O. oeni* to increase wood related compounds, was demonstrated in synthetic medium supplemented with wood shavings or wood extract, as well as MLF in Merlot wine performed in different containers (stainless steel tank, old barrel, new barrel) (de Revel *et al*., 2005). A higher content of oak lactone, eugenol, isoeugenol and vanillin (**Table 2.6**) was found when MLF occurred in barrels compared to tanks, particularly when new barrels were used for the wine (de Revel *et al*., 2005).

Table 2.6 Wood related compounds (µg/L) found in red wines after 2 months of barrel ageing (de Revel *et al*., 2005).

Compound	Ageing in old barrels		Ageing in new barrels	
	MLF in tank	MLF in old barrels	MLF in tank	MLF in new barrels
cis-Oak lactone	9	15	43	67
trans-Oak lactone	11	17	55	86
Eugenol	2.8	4.1	10	16
Isoeugenol	0.4	0.5	5.4	9.2
Vanillin	57	95	123	258

The effect of wood toasting on bacterial growth was also demonstrated by de Revel and co-workers (2005). Without toasting, the wood shavings inhibited bacterial growth, while in the presence of toasted shavings, bacterial growth was promoted. This suggests the release of possible precursors for bacterial metabolism from wood during the toasting process.

The potential mechanism related to the observed increase in vanillin concentrations by MLF was further investigated by Bloem and colleagues (2007; 2008). Although the conversion of simple phenolic compounds (ferulic acid, eugenol, isoeugenol and vanillic acid) to vanillin has been widely ascribed to microorganisms (Priefert *et al*., 2001), this conversion by *O. oeni* during MLF, was found insufficient to explain the increased concentrations of vanillin observed after fermentation (Bloem *et al*., 2007). A subsequent study (Bloem *et al*., 2008) illustrated the ability of *O. oeni* to hydrolyse glycosylated compounds in oak wood, which explains the dramatic increase of aroma, especially vanillin, when MLF is conducted in oak wood barrels.

Investigations regarding the influence of MLF on wood-derived compounds illustrate the potential effect of bacterial strain selection on the composition and resulting aroma characteristics of the wine (de Revel *et al*., 1999). During a study in Sauvignon blanc wine, the one bacteria (SBY) extracted increased amounts of volatile wood compounds compared to another bacteria (SBX) used (de Revel *et al*., 1999). This was supported by the sensory profiles (**Figure 2.10**) that illustrated more intense roasted, vanilla, buttered and smoked attributes for the SBY bacteria.

The sensory detection thresholds of oak lactone (67 µg/L), eugenol (5-6 µg/L) and vanillin (200 µg/L) (as reported by Francis & Newton, 2005) were exceeded by the respective concentrations found in studies pertaining to MLF and wood interactions (de Revel *et al*., 1999; 2005) and therefore could contribute to the increased perceptions of roasted and smoked attributes caused by eugenol or isoeugenol.

The potential liberation of aroma active compounds from wood through glycosidic hydrolysis by *O. oeni* was also suggested to be strain dependant (Bloem *et al*., 2008). These findings reiterate the importance of strain selection when considering the influence of *O. oeni* on the expression of the woody profile of wines.

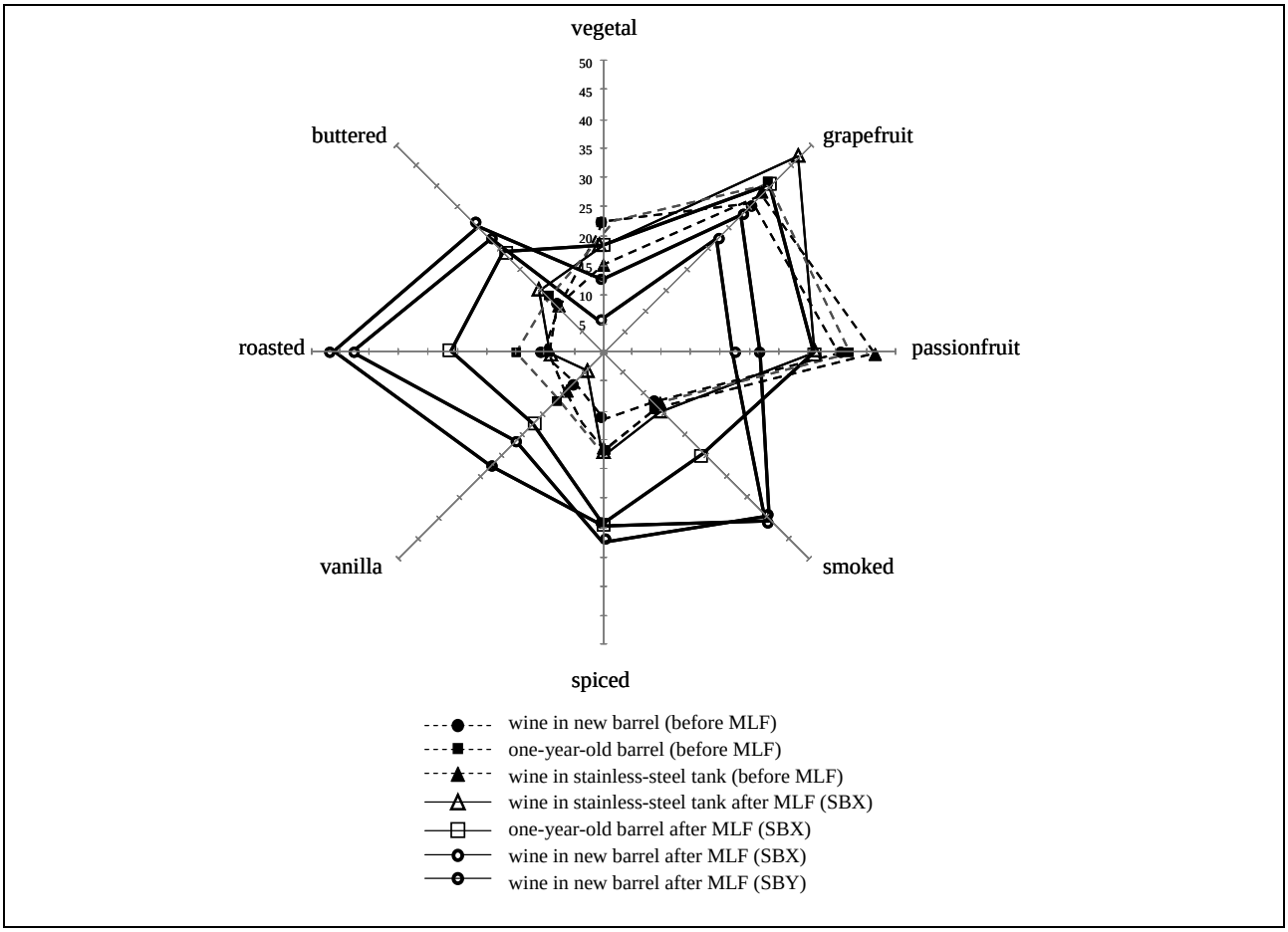

Figure 2.10 Descriptors used to differentiate between wines before and after MLF in stainless steel tanks or barrels with two starter culture preparations, SBX and SBY (de Revel *et al*., 1999).

2.3 WINE AROMA MEASUREMENT - APPLICATION TO MLF

Wine aroma and flavour is complex and many different sensory modalities and chemical compounds influence the sensory perception. Wine aroma is firstly influenced by the volatile composition of wine and secondly by the perception of the sensory attributes by the human olfactory system (Swiegers *et al*., 2005b). The complexity of wine aroma is derived from many sources and influenced by specific biological processes. Knowledge regarding the identity and quantity of compounds comprising the volatile fraction of wine, as well as information regarding the sensory properties of a wine, is therefore crucial to study and understand the effects of influencing factors, such as MLF, on wine aroma. Analytical approaches are continuously evolving as a result of improved technological advances and data processing technologies. This section provides a

synopsis of analytical techniques and sensory research findings related to the study of MLF, as well as some emerging technologies.

2.3.1 ANALYTICAL APPROACHES FOR VOLATILE COMPOUND QUANTIFICATION

A number of techniques are available for the quantification of volatile compounds. Several excellent reviews provide detailed information regarding the different sample preparation techniques, separation, identification and quantification of volatile flavours in wine (Ebeler, 2001; Polášková *et al*., 2008). Gas chromatography (GC) in combination with mass spectrometry (GC-MS) and/or additional sorptive extraction techniques such as solid phase micro extraction (SPME), solid phase dynamic extraction (SPDE) and solid phase extraction (SPE) have been used for the quantification of volatile compounds (Ebeler, 2001). A number of studies pertaining to the influence of different MLF starter cultures used SPE combined by GC-FID and GC-MS for the quantification and identification of yeast-derived or major volatile compounds (Ugliano & Moio, 2005; Boido *et al.*, 2009). Herjavec *et al*. (2001) investigated the effect of different MLF treatments by the quantification of higher alcohols and ethyl acetate (from a wine distillate) and other volatile compounds, including volatile fatty acids, ethyl esters of fatty acids and higher alcohol acetates (dichloromethane extract), using a GC-FID method. Another study quantified volatile compounds by direct injection (for major volatiles) and HS-SPME (for minor volatiles) in combination with GC-FID to compare the effect of different MLF starter cultures (Pozo-Bayón *et al*., 2005).

2.3.2 PROMISING TECHNOLOGIES

An improved understanding of grape and wine flavour chemistry necessitates continuous development of analytical methods. Currently, there is much interest in rapid, high-throughput methods for quantifying volatile components, relating chemical observations to sensory perceptions and monitoring qualitative changes in volatile composition as a result of viticultural practices, winemaking techniques or storage processes. Some of the more recent advances and approaches will be discussed in brief.

2.3.2.1 Gas chromatography olfactometry

Attempts to link the identification and quantification of odourants to their sensory impact have resulted in the development of quantitative gas chromatography-olfactometry (GC-O or GC-'sniff') (Delahunty *et al*., 2006). GC-O involves employing the human nose as a GC detector by sniffing of the individual separated volatiles as they elute from the GC column and recording the timing and qualitative perception of odourants (Ebeler, 2001). This technique represents a more comprehensive approach to investigate wine aroma and has received much attention in recent years (reviewed by Acree, 1997; Delahunty *et al*., 2006; Polášková *et al*., 2008). In the two widely used GC-O techniques, CHARM analysis (Acree *et al*., 1984) and Aroma Extract Dilution Analysis (AEDA), aroma extracts are diluted until no odour is perceived at the sniff port (Ebeler, 2001). Using these techniques, compounds with the highest odour activity require greater dilution before their odour can no longer be observed. Practical considerations and limitations of GC-O analysis have been reviewed (Delahunty *et al*., 2006; Polášková *et al*., 2008). Applications of this technique to the study of MLF could provide insight into compounds contributing to wine aroma or resulting

from the use of different MLF starter cultures. No literature in this regard is currently available, according to the author's knowledge.

2.3.2.2 Comprehensive GCxGC

Increased focus toward maximizing analytical outputs, in terms of selectivity and capacity, has directed the development of methods towards comprehensive or two dimensional (2D) GC methods (GCxGC; GCxGC-TOFMS) (Bushey & Jorgenson, 1990a, 1990b; Pierce *et al*., 2008). These approaches use two columns of different polarity to create an orthogonal 2D plane of separation based on two different compound properties, such as volatility and polarity (de Geus *et al*., 1996). Application to wine analysis has great potential as the problem of co-elution in complex mixtures is addressed by significantly increased peak capacity and hence a large quantity of data is obtained (Ebeler, 2001; Polášková *et al*., 2008; Pierce *et al*., 2008). However, data analysis is labour intensive, requires sophisticated software and a large degree of expertise. Advances in software development and chemometric applications could foresee that this problem is solved in the near future, as is evident from the increased number of publications related to optimizing 2D GC systems (Pierce *et al*., 2008; Vial *et al*., 2010).

2.3.2.3 Infrared spectroscopy

Another field of analytical methodology which shows potential with regard to relating chemical information with sensory responses is infrared (IR) spectroscopy. The use of this technology in the wine industry is currently restricted to the quantification of multiple wine parameters, such as pH, and residual sugar, from a single sample with minimal treatment. Both qualitative and quantitative information of the sample material is contained in the infrared spectrum since all molecules absorb infrared radiation at different wavelengths. Findings from Cozzolino *et al.* (2005) demonstrate the relationship between sensory analysis and visible (VIS) and near infrared (NIR) spectroscopy in two Australian white wine varieties. In another study (Cozzolino *et al*., 2008), the relationship between the sensory quality scores from a variety of red wines and the VIS and NIR spectroscopic data is shown with the use of multivariate regression methods. These studies illustrate the possibility of predicting sensory properties and wine quality scores from VIS and NIR spectra. Further studies involving other spectroscopic techniques (e.g. mid-infrared) or the combination of spectroscopy with other analytical techniques such as GC-MS, are needed to identify the chemical compounds that might explain the relationship between sensory and IR properties in wine.

2.3.2.4 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) (^{13}C and ^{1}H) spectroscopy have been used to determine amino acids, organic acids, sugars, alcohols, glycerol, polyphenols, catechin, epicatechin and gallic acid in grape juice or must, wine and phenolic extracts (reviewed by Clark *et al.*, 2006).

Recently, Son *et al.* (2009) illustrated the use of ^{1}H NMR spectroscopy coupled with multivariate statistics to investigate changes in Meoru wine metabolites, in order to characterise the contribution by MLF. In another study, the application of ^{1}H NMR for profiling the contribution of bacterial starter cultures to Meoru wine composition was reported (Lee *et al*., 2009). The authors illustrated the discrimination amongst different bacterial strains used for MLF by comparison of

NMR spectra. This technique shows potential to study the effects of MLF and characterise the contribution of different bacterial strains to wine composition.

2.3.2.5 Electrochemical sensors

One of the most promising directions for the development of innovative analytical methods is the use of electrochemical techniques. The electronic nose (e-nose) consists of an array of gas sensors with different selectivity, while the electronic tongue (e-tongue) comprises an array of sensors which are specific for liquids and based on conductometric, potentiometric and voltammetric techniques (Buratti *et al*., 2007). A number of investigations combining the application of these techniques with sensory aspects have been reported. Buratti *et al*. (2007) evaluated the prediction of red wine sensory descriptors from e-nose, e-tongue and spectrophotometric analysis. Another study (Cozzolino *et al*., 2006) combined the use of e-nose, VIS-NIR spectroscopy and chemometrics to assess the sensory properties of Riesling wines. Arroyo *et al*. (2009) showed that the perception threshold of the human nose is superior to the e-nose, whereas the e-nose provided better results in terms of the recognition threshold of some aromas.

2.4 SENSORY EVALUATION: IMPACT OF MLF

2.4.1 WINE AROMA AND FLAVOUR

Wine flavour refers to a combination of responses perceived as a result of both the basic tastes perceived on the tongue and the interaction of volatile compounds with the olfactory bulb (Francis & Newton, 2005; Swiegers *et al*., 2005b). Non-volatile compounds convey taste sensations such as sweet, sour, salty and bitter and affect mouthfeel. Volatile compounds of wine are responsible for the aroma, and are detected both orthonasally (through inhalation) and retronasally (through tasting a wine). Wine aroma and flavour consist of a large quantity of aroma-active compounds, interacting with each other and resulting in masking or suppressing effects as well as additive interactions for compounds (Buttery, 1999; Hein *et al*., 2008).

2.4.2 AROMA THRESHOLD AND ODOUR ACTIVITY VALUES

Aroma threshold values and odour activity values (OAVs) are often used to indicate the relative importance of chemical compounds measured, and suggest which compounds could have the greatest consequence to the wine aroma (Francis & Newton, 2005). Aroma thresholds, also called odour detection or perception thresholds, refer to the lowest concentration of odour components perceivable by the human sense of smell. Recognition thresholds refer to the lowest concentration at which an odour is identified and is generally higher than detection thresholds (Buttery, 1999). The concept of OAVs, also called aroma or odour units, or aroma values, is obtained by dividing the concentration of the odourant in the sample by the detection threshold concentration for that compound (Francis & Newton, 2005). This provides a useful measure to assess the relative importance of individual chemical components present in a sample. OAVs greater than 1 indicate a potential active aroma contribution of a specific compound in the sample under investigation. Although chemical compounds could be present at sub-threshold concentrations (i.e. OAVs < 1),

their potential contribution to wine aroma should not be excluded due to additive effects as has been previously discussed (Buttery, 1999).

2.4.3 SENSORY IMPACT OF MLF

A number of reviews on the general subject of MLF have been published, all with specific reference to the sensory effects of MLF (Davis *et al*., 1985; Bartowsky & Henschke, 1995; Martineau *et al*., 1995a; Lonvaud-Funel, 1999; Liu, 2002). The impact of MLF on the organoleptic qualities of wine is reflected in taste, mouthfeel, aroma and flavour as a result of the multitude of metabolites affected by bacterial metabolism.

The influence of MLF on the taste of wine due to deacidification is well recognised (Bartowsky & Henschke, 1995). Available evidence indicates that MLF can enhance mouthfeel or body and concurrently increase the smoothness or roundness of the wine (Bartowsky & Henschke, 1995; Bartowsky *et al*., 2002a). Trained panel assessments regarding the mouthfeel effects of MLF in Chardonnay wine (Krieger *et al.,* 2004), found that MLF not only affected perceived acidity and acid-mediated tactile sensations, but also increased the perception of attributes such as 'velvet' and 'small marshmallow' in a strain-dependant manner. MLF can also impact on the astringency sensation of red wines. Specifically, MLF can increase anthocyanin and tannin condensation, which in turn has been shown to reduce the astringency and also affect the colour of red wine (Vivas *et al*., 1997).

In general, the contribution of MLF to wine aroma has been noted to affect the fruity character, decrease the vegetative characters and increase the buttery aroma (Henick-Kling *et al*., 1994). A wide range of other descriptors has been used to describe wines that have undergone MLF. The extent of the contribution to aroma is related to the cultivar, fermentation conditions and bacterial strains employed. Investigations with the objective of evaluating the sensory contribution of MLF, excluding those studies only focussed on chemical analysis, have been reported in Chardonnay (Rodríguez *et al*., 1990; Avedovech *et al*., 1992; Laurent *et al*., 1994; Sauvageot & Vivier, 1997), Riesling (Herjavec *et al*., 2001), Chancellor (Delaquis *et al*., 2000), Sauvignon blanc (de Revel *et al*., 1999), Pinot Noir (McDaniel *et al*., 1985; Sauvageot & Vivier, 1997) and Tannat wines (Gámbaro *et al*., 2001; Boido *et al*., 2009).

A variety of sensory methodologies have been used during these investigations including informal sensory evaluation (Bartowsky *et al*., 2002b), difference from control method (Larmond, 1977), triangle tests, preference ranking and sensory profiling using Quantitative Descriptive Analysis (QDA) (Gámbaro *et al*., 2001). Studies related to the influence of MLF on wine aroma are often conducted in country specific cultivars, using different bacterial strains under variable fermentation conditions. Apart from sensory evaluation with trained panels, the effect of MLF on consumer preference has not received much attention.

2.5 CONCLUSIONS

The sensory characteristics perceived during wine tasting are influenced by chemical compounds that can be grape-derived or produced through a succession of biological, biochemical and technological factors, including MLF. Improved process control by implementing bacterial starter cultures offer winemakers another tool to obtain high quality wines.

In order to truly evaluate the impact of a bacterial starter culture selection, an integrated approach involving the chemical characterisation, sensory evaluation as well as consumer preference is of value and should be considered. Advances in technology allow for increased sample throughput as well as increased quality of data obtained. Implementation of multivariate data analysis to MLF-related sensory data (as reported by Gámbaro *et al*., 2001) provides a visualisation of the relationship among sensory terms as well as additional available data such as volatile compounds. Such methods are needed to investigate correlations amongst sensory attributes and chemical compounds.

There is a lack of published data related to the comparison of the wine aroma contribution, in terms of sensory and chemical data, made with LAB in different cultivars. Such data could provide insight with regard to the suitability of selected strains in specific cultivars to express more desired sensory attributes associated with different cultivars. Studies investigating the effect of MLF strain selection on consumer preference and acceptance are lacking. Additionally, information regarding the effect of different strains across different cultivars, with respect to consumer preference is needed.

Future studies involving a broad-range profiling approach and integrating the chemical information with sensory information would provide insight into the importance of the contribution of MLF to wine aroma and possibly consumer preference. Such information would be of significance to the wine industry and may contribute to a better understanding, management and exploitation of the MLF process to enhance wine quality.

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CHAPTER 3

Research Results

High-throughput quantification of major volatile compounds in wine: fast GC-FID method development, validation and application

3. RESEARCH RESULTS

High throughput quantification of major volatile compounds in wine: fast GC method development, validation and application

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ABSTRACT

Metabolic profiling and routine quality assessment of wine demand on reliable, rapid and cost-effective analysis techniques to evaluate the volatile composition of wine. This study proposes a fast gas chromatography flame ionisation detection (GC-FID) method for the quantification of volatile compounds such as higher alcohols, esters and fatty acids produced during alcoholic and malolactic fermentation. A simple liquid-liquid diethyl ether extraction procedure is followed for GC-FID analysis on a narrow-bore wax type GC column. Separation of 39 volatile compounds is achieved in less than 15 minutes per analysis in comparison to conventional methods (typically 40 minutes or longer). This constitutes a 3-fold reduction in analysis time. Good linearity for the quantification of the compounds was obtained with correlation coefficients (R^2) ranging between 0.998 and 0.999. The method exhibits a high level of accuracy and repeatability (RSD<10%) with limits of detection suitable for the expected concentrations in wine. The selection of analytes quantified comprises a large boiling point range (from ethyl acetate to decanoic acid) as well as a large polarity range (from polar acids/alcohols to less polar esters) which illustrates the applicability for wine analysis using this method. The method was evaluated and successfully applied for the analysis of several red and white cultivars as well as a selection of wines fermented with different malolactic fermentation starter cultures. The results suggest that the application of fast GC-FID based analysis to wine offers a valuable high-throughput tool for routine investigations of the volatile composition of wine and could be used as a research screening method ideally suited for metabolic fingerprinting in combination with multivariate data analysis techniques.

Keywords: Fast gas chromatography, wine, volatile aroma compounds

3.1 INTRODUCTION

The concept of increased separation speed of gas chromatography (GC) has been of ongoing interest since the introduction of GC in 1952 (Korytár, 2002). Faster GC offers the advantage of higher throughput, lower operational cost per sample, shorter “time-to-result” and in addition improved precision and sensitivity as a result of several replicate analyses in the same time as a single conventional capillary GC analysis (Korytár, 2002; Matisová & Dömötörová, 2003). The basic theory and principles of fast GC, based on the fundamental theory of chromatography, were already

established in the 1960's (Purnell & Quinn, 1960; Desty *et al*., 1962; Desty, 1965) but were mostly hampered by instrumental limitations. Continuous instrumental advances were followed by increasing use of the technique in a vast application area. This includes rapid profiling of complex mixtures such as polychlorinated biphenyls (PCBs), fats and essential oils or pesticide extracts from water samples, nutmeg, bacterial fatty acid methyl esters (David *et al*. 1999) and solvent mixtures (Chen *et al.*, 1998), to name a few. There is a revived interest in the development and implementation of methods for faster GC as a result of specific application requirements such as metabolic fingerprinting (Jumtee *et al*., 2009). Several emerging technologies, improved instrumentation and availability of commercial columns for high speed GC applications have been developed in the last decade (Sacks *et al.*, 1998).

Successful exploitation of any method for faster GC requires careful adjustment of the specific settings of the GC instrument and is highly dependant on the physical properties of the analytes. By utilising modern, but nowadays standard features available in gas chromatography such as accurately controlled high carrier gas pressures and shorter, highly sophisticated narrow-bore columns due to modern column production technologies, the possibility to reduce total analysis time by a factor of at least 2 - 3 exists. Furthermore, as a result of shorter analysis time, less band-broadening occurs and sharper peaks are obtained, leading to higher sensitivity and lower detection limits (David *et al.*, 1999; Maštovská & Lehotay, 2003). In depth reviews of the possibilities and limitations for fast GC applications in terms of advantages of faster GC analysis, general approaches to faster GC method development and practical aspects of fast GC have been published (Leclercq & Cramers, 1998; David *et al.*, 1999; Korytár, 2002; Matisová & Dömötörová, 2003; Maštovská & Lehotay, 2003; Dömötörová *et al*., 2006). There are generally three routes towards faster GC separation: (a) minimisation of the resolution to a value just sufficient, (b) maximisation of the selectivity of the chromatographic system, (c) implementation of a method that reduces analysis time at constant resolution (Korytár *et al.*, 2002; Matisová & Dömötörová, 2003). However, there is no universal method and the question of which approach to select is by no means trivial and very often dependent on the complexity of the analyte mixture. Trends in GC applications include the need for more flexible systems that allow the analysis of a wide variety of samples in one system (Dömötörová *et al*., 2006). In fast GC, mass spectrometric detection (MSD) (Van Deursen *et al.*, 2008) and time-of-flight (TOF) mass spectrometers (Cochran, 2002; Maštovská & Lehotay, 2003) are popular and often preferred detection methods because of its sensitivity, reproducibility, robustness and fast data acquisition rates. However, the instrumentation and maintenance cost is relatively expensive. Conversely, gas chromatography with flame ionization detection (GC-FID) is a comparatively less expensive instrument which is extensively available in most industrial laboratories and research institutions. FID is a universal detector, which provides wide coverage of carbon-containing compounds with high sensitivity and has been used most frequently for faster GC (Matisová & Dömötörová, 2003). The majority of faster GC applications for complex mixtures fall into the category of fast GC - defined as separation in the range of minutes with peak width 1-3s (Korytár *et al.*, 2002).

The major volatile composition of wine is monitored extensively in both research and routine industrial applications. Research projects are continuously more directed towards the metabolic fingerprinting of wine volatile composition which relies on the accurate quantification of large numbers of samples in a short period of time (Ortega *et al.*, 2001; Escudero *et al.*, 2004; Jonsson *et al.*, 2005). During strain development programs the assessment of potential aroma contribution by new yeast and

bacterial starter cultures for both alcoholic and malolactic fermentation also remains of critical importance. Industrial applications also require the measurement of a variety of volatile compounds for large quantities of samples during quality control (Mac Namara *et al.*, 2005; Louw *et al.*, 2009). The majority of available methods for the above-mentioned applications are relatively easy and uncomplicated to perform, however the analysis is often time-consuming. This demand for increased amounts of analysis in a shorter time period necessitates the development and optimisation of high-throughput analytical tools such as fast gas chromatography (GC). The advantages of this approach are reflected in considerably higher sample throughput and lower analysis cost.

This study proposes an analytical method for the high throughput quantification of potentially 39 major volatile compounds in wine with the use of a simple and rapid gas chromatography flame ionization detection (GC-FID) method. The first objective includes the translation of a conventional or standard GC-FID method to the proposed fast GC-FID method followed by method optimisation and validation. The method entails a fast and straightforward liquid-liquid extraction (Louw *et al*., 2009) prior to chromatographic analysis, adding to the speed and ease of the complete analytical protocol. Method optimisation is restricted to injection and GC conditions during this study. Subsequently, the method is applied for wine analysis of a variety of commercial red and white cultivars including Pinotage, Merlot, Cabernet Sauvignon, Sauvignon blanc and Chardonnay. Application of this method to investigate metabolic differences amongst different bacterial strains was also evaluated in the final section of this work. The proposed method could be used routinely for the high-throughput quantification of major volatile aroma compounds and be a useful tool for screening metabolic differences amongst bacterial strains.

3.2 MATERIALS AND METHODS

3.2.1 REAGENTS, STANDARDS AND SAMPLES

Diethyl ether (99.5%), absolute ethanol and sodium sulphate for liquid-liquid extraction purposes and GC analyses were purchased from Merck (Darmstadt, Germany). All working reference standards were of analytical grade (listed in **Table 3.1**) and purchased from Sigma-Aldrich (St. Louis, MO, USA), Fluka (Buchs, Switzerland), Riedel de Haën (Seelze, Germany) and Aldrich (Steinheim, Germany). These standards were dissolved in a wine simulant (also termed synthetic wine) (as described by Louw *et al*., 2009) consisting of 12.5% v/v ethanol (Merck) and 5 g/L tartaric acid (Merck) in deionised water from a Milli-Q purification system (Millipore, Billerica, MA, USA). The pH was adjusted to 3.2 using NaOH (Merck, Darmstadt, Germany). The internal standard solution contained 4-methyl-2-pentanol (≥97%, Fluka, Buchs, Switzerland) at 0.5 mg/L dissolved in the mentioned wine simulant.

All wine samples were from South Africa. During method development and validation two wines were used: Wine 1 (dry young red wine; 13.5% v/v ethanol) and Wine 2 (dry young white wine; 13.2% v/v ethanol).

Table 3.1 Chemical standards used for quantitative analysis.

Peak nr.	Analyte	Supplier	Purity (%)	CAS nr.
1	Ethyl acetate	Sigma-Aldrich	>99.7%, HPLC Grade	141-78-6
2	Methanol	Sigma-Aldrich	>99.9%	67-56-1
3	Ethyl propionate	Sigma-Aldrich	puriss.p.a., std for GC, >99.7%	105-37-3
4	Ethyl 2-methyl propanoate	Fluka	purum, ≥98.0% (GC)	97-62-1
5	2-Methyl propyl acetate	Fluka	puriss. p.a., std for GC, >99.8% (GC)	110-19-0
6	Ethyl butyrate	Fluka	purum.>98% (GC)	105-54-4
7	Propanol	Fluka	purum.>99% (GC)	71-23-8
8	Ethyl 2-methylbutyrate	Sigma-Aldrich	>98%, FCC, Kosher, FG	7452-79-1
9	Ethyl isovalerate	Sigma-Aldrich	puriss.p.a., std for GC, >99.7%	108-64-5
10	Isobutanol	Fluka	puriss.p.a.>99.5%	78-83-1
11	Isoamylacetate	Riedel de Haën	min. 98%	123-92-2
12	Butanol	Fluka	puriss.p.a.>99.5% (GC)	71-36-3
13	Isoamylalcohol	Aldrich	>99%	123-51-3
14	Ethyl hexanoate	Fluka	purum. 99% (GC)	123-66-0
15	Pentanol	Fluka	puriss. p.a., std for GC, ≥99.8% (GC)	71-41-0
16	Hexyl acetate	Fluka	puriss. 99% (GC)	142-92-7
17	Acetoin	Fluka	purum, ≥97.0% (GC)	513-86-0
18	4-Methyl-1-pentanol	Sigma-Aldrich	purum, >95% (GC)	626-89-1
19	3-Methyl-1-pentanol	Sigma-Aldrich	purum, >97% (GC)	589-35-5
20	Ethyl lactate	Fluka	purum. 99% (GC)	687-47-8
21	Hexanol	Merck	>98%	111-27-3
22	3-Ethoxy-1-propanol	Sigma-Aldrich	>97%	111-35-3
23	Ethyl octanoate	Fluka	purum.>98% (GC)	106-32-1
24	1-Octen-3-ol	Fluka	purum, ≥98.0% (GC)	3391-86-4
25	Acetic acid	Saarchem	min.98%	64-19-7
26	Ethyl 3-hydroxybutanoate	Fluka	purum, ≥97.0% (GC)	5405-41-4
27	Propionic acid	Fluka	puriss.p.a.>99.5% (GC)	79-09-4
28	Isobutyric acid	Fluka	puriss.p.a.>99.5% (GC)	79-31-2
29	Butyric acid	Fluka	puriss.p.a.>99.5% (GC)	107-92-6
30	Ethyl decanoate	Aldrich	>99%	110-38-3
31	Isovaleric acid	Fluka	purum.>99% (GC)	123-25-1
32	Diethyl succinate	Fluka	purum.>98% (GC)	503-74-2
33	Valeric acid	Fluka	puriss.>99% (GC)	109-52-4
34	Ethyl phenylacetate	Fluka	puriss., ≥99.0% (GC)	101-97-3
35	2-Phenylethyl acetate	Fluka	purum, ≥99.0% (GC)	103-45-7
36	Hexanoic acid	Aldrich	>99.5%	142-62-1
37	2-Phenylethanol	Merck	>99%	60-12-8
38	Octanoic acid	Aldrich	>99.5%	124-07-2
39	Decanoic acid	Sigma	>98%	334-48-5

Fifty commercial wines comprising of 26 white and 24 red cultivars from various vintages and viticultural areas in South Africa were analysed following the proposed procedure. The commercial white cultivars included Sauvignon blanc (6), Chenin blanc (4), Chardonnay (6), Sauvignon blanc/Semillon (2), Gewürztraminer (2), Rhine Riesling (2), Viognier (1) and three semi-sweet style wines. The commercial red cultivar distribution was as follows: Pinotage (4), Merlot (3), Cabernet Sauvignon (6), Shiraz (5), Petit Verdot (1), Ruby Cabernet (1), Claret (1), Malbec (1), Tinta Barocca

(1) and Touriga Nacional (1). These wines represented 5 different viticultural areas, 5 vintages (2004-2008) and different wine styles of which the majority was dry wines.

Secondly, wines fermented with four different malolactic fermentation starter cultures were included in the analysis to evaluate the detection of subtle differences between samples using the proposed method. Two cultivars were included in this experiment namely Pinotage and Shiraz.

3.2.2 SAMPLE PREPARATION

All samples were prepared according to the validated method described by Louw (2007). In brief, 5 mL of wine was extracted with 1 mL of diethyl ether after the addition of 100 µL (10 mg/L) internal standard, 4-methyl-2-pentanol. The same sample preparation extraction procedure was followed for both the conventional GC-FID and fast GC-FID methods.

3.2.3 GC-FID CONDITIONS: THE CONVENTIONAL AND THE FAST METHOD

Analysis of volatile aroma compounds was performed in triplicate using a Hewlett Packard 6890 Plus gas chromatograph (Little Falls, USA) equipped with a split\splitless injector and a FID detector. A J & W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with dimensions 60 m length $\times$ 0.32 mm inside diameter $\times$ 0.5 μm film thickness was used for the conventional method (described by Louw *et al*. 2009) and a similar column with dimensions 20 m length x 0.1 mm inside diameter x 0.2 μm film thickness was used for separation during the fast GC-FID method. The instrument parameters and chromatographic conditions are described in **Table 3.2**. Subsequently, the volatile compounds (listed in **Table 3.1**) were quantified by comparing their retention times and areas with those from calibration standard curves on a data handling system (HP GC Chemstation, Revision A.07.01 [682]). Method validation for the conventional GC-FID method in terms of selectivity, linearity, limits of detection, limits of quantification, recovery, robustness and repeatability has been described elsewhere (Louw, 2007).

3.2.4 CALIBRATION CURVES

Calibration curves were prepared by GC-FID analysis of diethyl ether extracts containing known amounts of the standards and of the internal standard in the synthetic wine. Six calibration points were used to construct the calibration graph for each individual compound. Each calibration point comprised the average of 3 different extractions injected in duplicate, resulting in 6 data points per concentration level for each analyte. The calibration range of concentrations can be seen in **Table 3.3**. The relative response areas for each of the individual wine volatile compounds to the internal standard were calculated and interpolated in the corresponding calibration graphs for quantification purposes.

3.2.5 FAST GC METHOD DEVELOPMENT AND VALIDATION

3.2.5.1 Sample preparation

A variety of solvents and solvent combinations were tested for optimum extraction of the volatile compounds from wine (data not shown). Diethyl ether and dichloromethane (DCM) had similar extraction efficiencies for the compounds of interest; however diethyl ether was selected for its uncomplicated extraction procedure. As mentioned previously, the validation of the diethyl ether sample preparation method was performed and reported in another study (Louw, 2007).

Table 3.2 Experimental conditions for major wine volatile analysis on a conventional GC system and on a narrow bore column (Fast GC method). The translated method, obtained from the translation software, prior to optimisation is also shown, together with detection parameters.

Parameters	Std GC method	Translated GC method	Fast GC method
Column			
Type	FFAP*	FFAP	FFAP
Length (m)	60	20	20
Internal diameter (mm)	0.32	0.1	0.1
Film thickness (µm)	0.5	0.2	0.2
Injection	Split	Pulsed split 420kPa; 1.5 min	Pulsed split 420kPa; 1.5 min
Injection volume	3 µL	1µL	1µL
Split ratio	15:1	15:1	10:1
Injection temperature (˚C)	250	250	250
Inlet pressure (kPa)	90	480	278
Carrier gas	Hydrogen	Hydrogen	Hydrogen
Average velocity (cm/s)	47	63.5	36.7
Column flow rate (mL/min)	3.3	1.12	0.5
Oven program			
Initial temperature (˚C)	33	40	40
Initial time (min)	17	3.4	3.5
Ramp	12	30.7	18
Final temperature (˚C)	240	240	240
Final time (min)	5	2.1	1
Detection	FID, 230˚C	FID, 230˚C	FID, 230˚C
Hydrogen flow (mL/min)	30	30	30
Air flow (mL/min)	350	350	350
Make-up (N_2) (mL/min)	30	30	30
Analysis time (min)	39	12.01	14.3

*Free fatty acid phase

3.2.5.2 Method translation and optimisation

The standard GC-FID method described in Section 2.3 was translated to a fast GC-FID method using Agilent GC method translation software (version 2.0.a.c) (Agilent Technologies, Little falls, Wilmington, USA) which is available free of charge from the Agilent web site. The translate only option was used for the method translation. The application of method translation software allows the translation from conventional to narrow bore columns with almost no changes in the resolution, selectivity and thus

overall separation (David *et al.,* 1999). Additional method optimisation to separate specific critical pairs in the analyte mixture was restricted to injection and GC conditions for this study. Optimisation included the evaluation of different injection conditions such as injection mode (split versus splitless, combinations with pulsed pressure injection, split ratio), injection temperature and type of liner (data not shown). GC oven temperature and temperature ramp rate was also evaluated during optimisation of the method to obtain completely resolved analytes. The final fast GC-FID method conditions are shown and compared to the standard GC method conditions in **Table 3.2**. After selection of the best conditions for faster GC-FID analysis the method was validated by studying the range of linearity, limits of detection and quantification, accuracy and repeatability.

3.2.5.3 Linearity and accuracy

Method linearity was studied as described in Section 3.2.4. Accuracy of the calibration graph was tested by injection of known amounts of analyte in a synthetic wine and evaluating the accuracy as a percentage recovery from the calibration curve. The recovery was estimated as:
Recovery (%) = amount calculated from graph/known amount of analyte x 100%.

3.2.5.4 Repeatability and intermediate repeatability

The repeatability and intermediate repeatability of the method was determined by duplicate extraction of wine 1, 2 and a synthetic wine containing known amounts of analyte on 3 different days. The precision of the method was evaluated by testing the within-day repeatability of measurements and the between-day repeatability (intermediate repeatability) of measurements. The repeatability was evaluated by injecting the same extract (both for red and white wine, respectively) for 5 consecutive analysis and expressing the results as the percentage relative standard deviation (%RSD) over the 5 analysis. The intermediate repeatability (between-day repeatability) was tested by analysing the same samples (both a red and white wine) on three different days. The results were calculated as the %RSD which indicated the repeatability for each sample.

3.2.5.5 Limit of detection and limit of quantification

The limit of detection (LOD) is reported as the concentration of analyte with a peak height three times as high as the baseline noise level (signal-to-noise (S/N) ratio = 3). Similarly, the limit of quantification (LOQ) is reported as the concentration at which the analyte had an S/N ratio of 10. These definitions are in accordance with the OIV recommendations for chromatographic measurements. These parameters ensured quantitation with no more than 10% error because of noise and the ability to differentiate a peak from random noise.

3.2.5.6 Evaluation of matrix effects

Existence of matrix effects was tested as follows: standard addition to white (wine 1) and red wine (wine 2) and the analysis of the spiked and unspiked solutions using the proposed extraction and chromatographic method. The recovery is expressed as a percentage (calculated as the spiked amount minus the unspiked amount divided by the amount of analyte spiked with). The existence of

matrix effects was checked by the replicate analysis of wines 1 and 2 and of those wines spiked with known amounts of analytes.

3.2.6 APPLICATION OF METHOD ON WINES

Fifty different commercial red (24) and white (26) wines from different regions, different vintages, different wine styles (dry, semi-sweet) and different cultivars from South Africa were analysed in this study following the methodology proposed. Each wine was extracted once as described in section 3.2.2 and analysed in triplicate. Principal component analysis (PCA) was performed on the fast GC-FID data to evaluate and illustrate whether sufficient differentiation amongst white and red cultivars could be observed using the fast GC-FID data.

In addition, 45 Pinotage and Shiraz wines produced with grapes from the Western Cape, South Africa in an experimental cellar and were inoculated with four different commercial MLF starter cultures and were analysed to test the ability of the fast GC method to differentiate subtle differences between wines, such as differences resulting from the use of various MLF starter cultures as compared to the more pronounced cultivar differences tested with the commercial wines. These experimental wines were analysed following the proposed fast GC-FID method as well as with the original standard GC-FID system and the data was then compared.

3.2.7 DATA PROCESSING AND MULTIVARIATE ANALYSIS

Descriptive statistical parameters including mean, standard deviation (SD), and relative standard deviation (%RSD) were calculated using Microsoft Excel 2003. The quantitative data obtained from the fast GC-FID method was exported to the *The Unscrambler* software (version 9.2, CAMO ASA, Norway) for the purpose of multivariate data analysis. A matrix was constructed with rows representing wine samples (objects) and variables in the columns corresponding to fast GC-FID data. Principal component analysis (PCA) was used for data exploration purposes and to extract information from the multivariate GC-FID data (Kettanah *et al.*, 2005). Partial least squares discriminant analysis (PLS-DA) models (Naes *et al*., 2002) were constructed to evaluate whether the generated fast GC data could be applied to differentiate between wines fermented with four different malolactic fermentation starter cultures. The models use a no metric dummy Y-variable as a reference value. The dummy variable is an arbitrary number assigned to a sample belonging to a particular group or category. Each sample is assigned a dummy variable (signified by -1 for not belonging to a specific bacterial group and +1 for belonging to a specific group) to test the ability of the fast GC data to discriminate between wine samples fermented with different bacteria. The PLS-DA model is developed by regression of the fast GC data (X-variables/matrix) against the assigned reference value (dummy variable).

3.3 RESULTS AND DISCUSSION

3.3.1 METHOD DEVELOPMENT FOR HIGH-THROUGHPUT ANALYSIS OF MAJOR WINE VOLATILE COMPOUNDS

Method translation of the standard GC-FID method was performed using Agilent method translation software (version 2.0.a.c) (Agilent Technologies, Little falls, Wilmington, USA) and the resulting translated method is shown in **Table 3.2**. As can be seen, the projected reduction in time is 3.25 fold, theoretically when using the 'translate only' option. However, in practise, these conditions did not provide complete resolution of some critical pairs occurring in this complex analyte mixture consisting of a diverse range of chemical classes of compounds. These included alcohols, acids, fatty acids and esters, differing significantly in physico-chemical properties such as polarity and boiling point. Consequently, as mentioned, the translated method resulted in insufficient separation of some analytes (data not shown). Improved separation of these analytes was obtained by re-evaluating a selection of different injection conditions and GC analysis conditions. Compared to conventional columns, the sample capacity and therefore injection volume possible for narrow bore columns is much smaller (David *et al.*, 1999). Sample introduction in high speed capillary GC using narrow-bore columns is therefore of critical importance and can severely affect the sensitivity and overall performance of the method. For the proposed method, a low volume, narrow bore GC injection port liner was used in combination with a high injection pressure pulse to facilitate fast transfer of the analytes onto the GC column and to prevent flashback into the septum purge line by compressing the evaporated solvent and thus reducing its vapour volume to not exceed the volume of the liner. This pulsed split automated injection allowed for fast sample introduction which was crucial to ensure narrow injection plugs (bands) for chromatographic separation, consequently leading to narrow peak widths associated with increased sensitivity. Split injection mode and injection volume was also evaluated. Generally, split injection results in better peak shapes compared to splitless injection, but with a loss in sensitivity. The split ratio was decreased to 10:1 compared to the split ratio of 15:1 suggested by the method translation software (shown in **Table 3.2**). This provided adequate sensitivity and chromatographic performance while still preventing column overloading and peak fronting (David *et al*., 1999). In this application, pulsed split injection using a pulsed pressure of 420 kPa resulted in the best sensitivity and effective separation of the analytes.

Temperature programming is essential to achieve high-speed chromatographic separation of analyte mixtures covering a wide boiling point range (Leonard *et al.*, 1999). Therefore, GC oven temperature and ramp was also evaluated in order to optimise resolution. Initial oven temperature of 40˚C was selected from the range tested (30˚C, 33˚C, 40˚C) since a decreased initial oven temperature resulted in larger peaks but worse peak shape. Ramp rates of 40˚C/min lead to sharper peaks and therefore theoretically better resolution, however this also lead to co-elution of certain analytes. After evaluation of a selection of oven temperature ramp rates, 18˚C/min produced optimum separation of all the analytes without losing too much time gain.

A conventional GC-FID chromatogram is shown in comparison to a chromatogram of the proposed faster GC-FID method for the quantification of major wine volatile compounds (see **Figure 3.1**). It is clear that there is comparable resolution with the use of a shorter column with smaller

internal diameter and thinner film thickness (David *et al*., 1999) even for a mixture consisting of various classes of chemical compounds with different physico-chemical properties.

Considering detection, the make-up gas flow rate was evaluated and 30mL/min was found to be sufficient for fast transfer of the column eluent to the detector to avoid extra-column band-broadening.

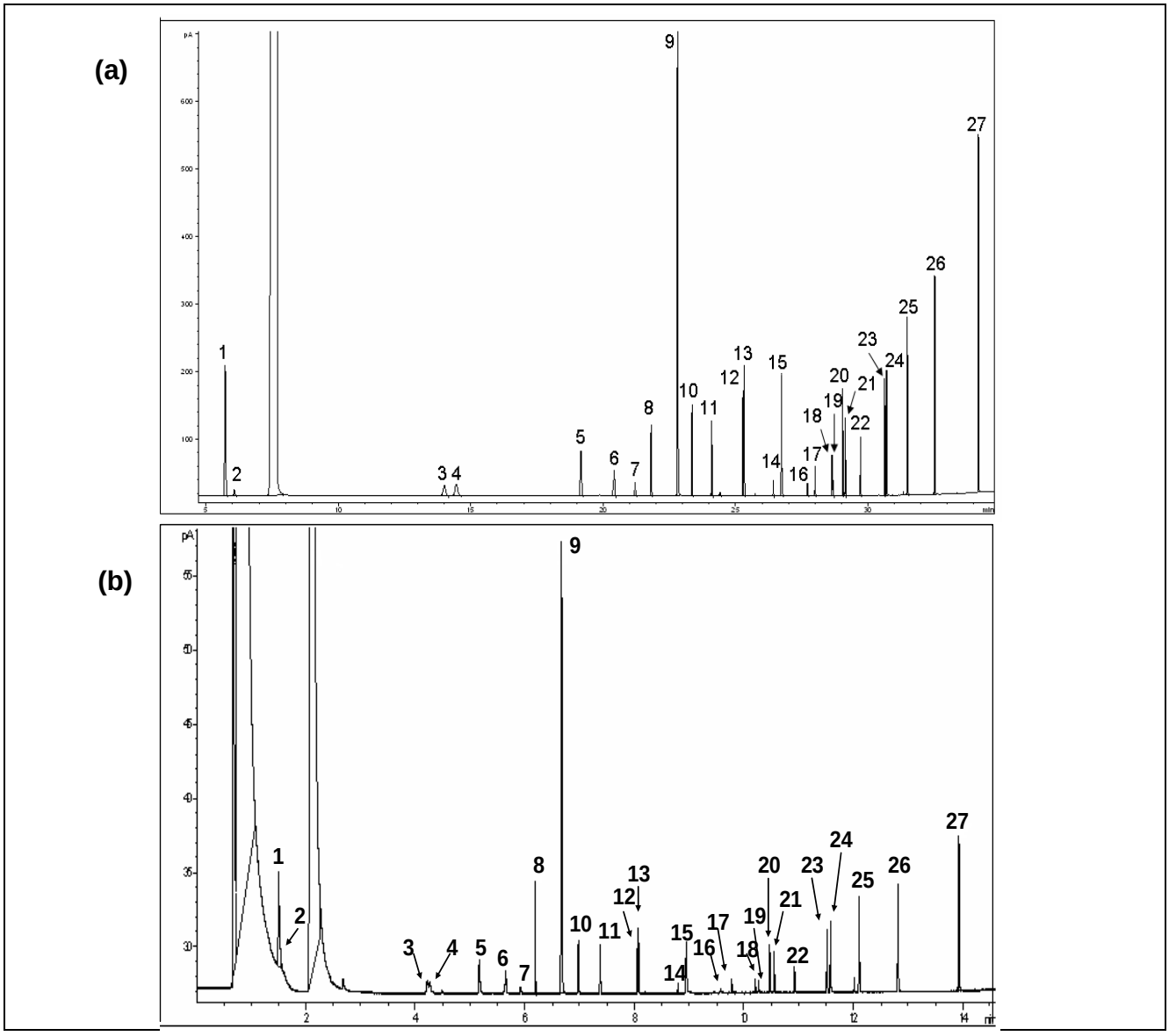

Figure 3.1 Chromatogram of (a) a conventional GC-FID method compared to the (b) fast GC-FID method for the quantification of 27 volatile compounds. The major volatiles include (1) ethyl acetate, (2) methanol, (3) ethyl butyrate, (4) propanol, (5) isobutanol, (6) isoamyl acetate, (7) butanol, (8) 4-methyl-2-pentanol (IS), (9) isoamyl alcohol, (10) ethyl hexanoate, (11) hexyl acetate, (12) ethyl lactate, (13) hexanol, (14) ethyl caprylate, (15) acetic acid, (16) propionic acid, (17) isobutyric acid, (18) butyric acid, (19) ethyl caprate, (20) isovaleric acid, (21) diethyl succinate, (22) valeric acid, (23) 2-phenylethyl acetate, (24) hexanoic acid, (25) 2-phenyl ethanol, (26) octanoic acid and (27) decanoic acid. Expansion of the fast GC-FID method to quantify potentially 39 volatile compounds in 15 minutes is shown in Figure 3.2.

Analysis time has been decreased from 40 minutes to 14.3 minutes. This speed gain of 2.73 will significantly increase sample throughput and be of great use in metabolic profiling of wines fermented with different yeasts and bacteria in order to compare their volatile profiles. After optimisation of the method using 27 analytes, a further 12 important fermentation compounds were selected from literature and were added to the analysis in order to exploit the full peak capacity. Separation of the total of 39 compounds was successfully achieved with the faster GC-FID analysis in less than 15 minutes (chromatogram shown in **Figure 3.2**).

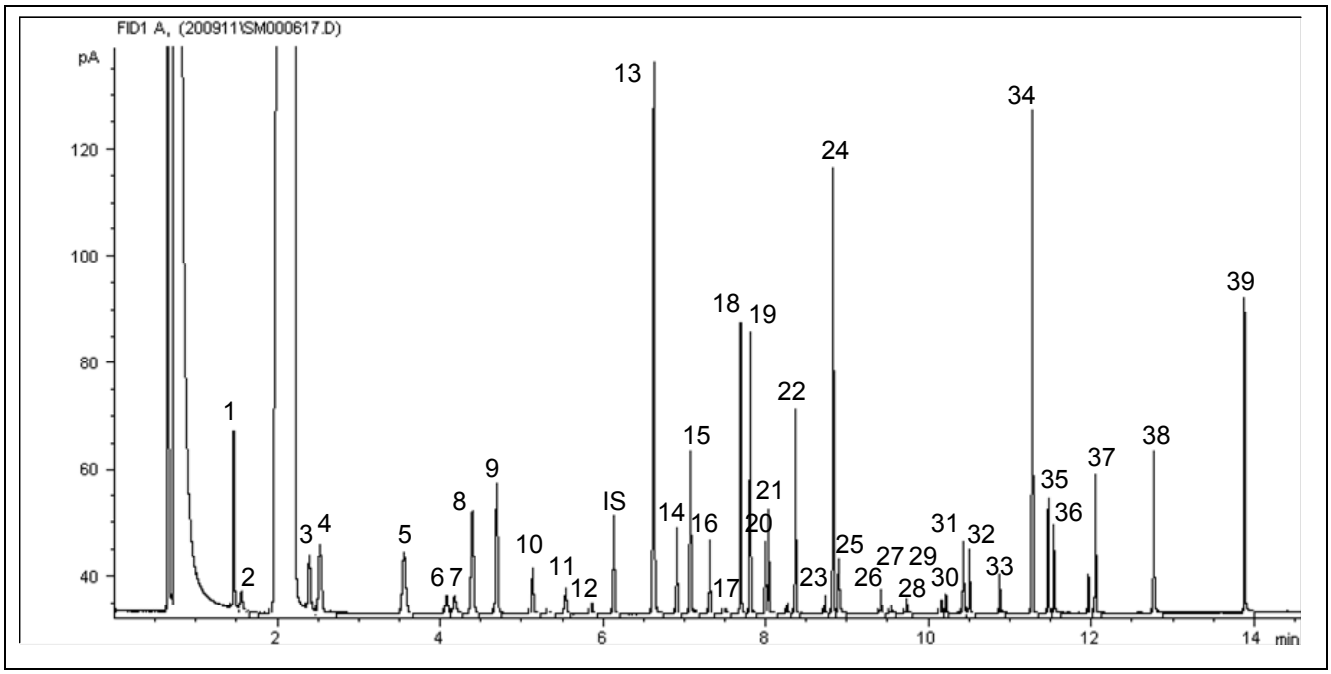

Figure 3.2 Chromatogram of the proposed fast GC-FID method showing separation for potentially 39 wine related volatile compounds in a synthetic wine medium. Peak identification is shown in Table 3.1. IS=Internal standard, 4-methyl-1-pentanol.

3.3.2 VALIDATION OF THE ANALYTICAL METHOD

After optimisation of the method in terms of injection parameters and GC conditions, calibration graphs using the internal standard method were constructed and the limits of detection and quantification of the method were calculated as described in section 3.2.5.5. All the experiments were carried out using the extraction method described in section 3.2.2 and the proposed analytical method described in **Table 3.2**. In order to validate the proposed method developed in this study, discussion in terms of method linearity, repeatability and reproducibility will be made.

3.3.2.1 Linearity and detection limits

Three replicates of six standard solutions in the range shown in **Table 3.3**, all of them with internal standard solution of 10 mg/L, were analysed. The mean values were used to construct the calibration graphs by plotting the peak area ratio (analyte peak area/internal standard peak area) against the standard concentration.

Table 3.3 Method linearity data, retention time (t_R), calibration range, limits of quantification (LOQ) and limits of detection (LOD) for the proposed fast GC-FID method.

Peak nr.	Compound	t_R (min)	R^2	Range (mg/L)	n	LOQ[a]	LOD[b]
1	Ethyl acetate	1.466	0.999	3.61 - 360.80	30	3.6	1.1
2	Methanol	1.560	0.997	9.02 - 901.74	30	9.0	2.7
3	Ethyl propionate	2.396	0.999	1.00 - 100.00	30	1.0	0.3
4	Ethyl 2-methyl propanoate	2.526	0.999	1.00 - 100.00	30	1.0	0.3
5	2-Methyl propyl acetate	3.559	0.999	1.00 - 100.00	30	1.0	0.3
6	Ethyl butyrate	4.082	0.999	0.22 - 21.95	30	0.2	0.08
7	n-Propanol	4.180	0.999	2.01 - 201.00	30	2.0	0.6
8	Ethyl 2-methylbutyrate	4.395	0.999	1.00 - 100.00	30	1.0	0.3
9	Ethyl isovalerate	4.695	0.999	1.00 - 100.00	30	1.0	0.3
10	Isobutanol	5.136	0.999	1.00 - 100.38	30	1.0	0.3
11	Isoamylacetate	5.543	0.999	0.19 - 19.27	30	0.2	0.05
12	n-Butanol	5.867	0.999	0.20 - 20.28	30	0.2	0.08
13	Isoamylalcohol	6.624	0.999	4.77 - 477.31	30	4.8	2.1
14	Ethyl hexanoate	6.909	0.999	0.31 - 30.56	30	0.3	0.1
15	Pentanol	7.075	0.999	1.00 - 100.00	30	1.0	0.5
16	Hexyl acetate	7.313	0.999	0.22 - 21.90	30	0.2	0.08
17	Acetoin	7.511	0.999	1.00 - 100.00	30	1.0	0.6
18	4-Methyl-1-pentanol	7.691	0.999	1.00 - 100.00	30	1.0	0.3
19	3-Methyl-1-pentanol	7.809	0.999	1.00 - 100.00	30	1.0	0.3
20	Ethyl lactate	7.996	1.000	5.00 - 500.16	30	5.0	1.5
21	Hexanol	8.034	0.999	0.31 - 30.93	30	0.3	0.1
22	3-Ethoxy-1-propanol	8.267	0.999	1.00 - 100.00	30	1.0	0.5
23	Ethyl octanoate	8.736	0.999	0.04 - 3.51	30	0.04	0.02
24	1-Octen-3-ol	8.827	0.999	1.00 - 100.00	30	1.0	0.5
25	Acetic acid	8.899	0.999	18.04 - 902.14	30	18.0	5.4
26	Ethyl 3-hydroxybutanoate	9.415	0.999	1.00 - 100.00	30	1.0	0.5
27	Propionic acid	9.545	0.999	0.30 - 29.79	30	0.3	0.1
28	Isobutyric acid	9.733	0.999	0.21 - 20.90	30	0.2	0.07
29	Butyric acid	10.162	0.999	0.21 - 21.21	30	0.2	0.06
30	Ethyl decanoate	10.214	0.999	0.03 - 3.45	30	0.03	0.01
31	Isovaleric acid	10.429	0.999	0.39 - 39.35	30	0.4	0.1
32	Diethyl succinate	10.505	0.999	0.31 - 31.41	30	0.3	0.1
33	n-Valeric acid	10.879	0.999	0.21 - 20.66	30	0.2	0.07
34	Ethyl phenylacetate	11.279	0.999	1.00 - 100.00	30	1.0	0.3
35	2-Phenylethyl acetate	11.476	0.999	0.21 - 20.60	30	0.2	0.07
36	Hexanoic acid	11.543	0.999	0.30 - 29.66	30	0.3	0.1
37	2-Phenylethanol	12.057	0.999	0.51 - 50.95	30	0.5	0.2
38	Octanoic acid	12.771	0.999	0.40 - 40.04	30	0.4	0.1
39	Decanoic acid	13.887	0.999	0.50 - 50.01	30	0.5	0.1
IS	4-methyl-2-pentanol	6.136					

[a]Limit of quantification (mg/L); [b]Limit of detection (mg/L).

Regression, slope and origin intercept (**Table 3.3**) were calculated by linear least-squares regression. The resulting six point calibration graphs were found to have good linearity in the tested concentration range with correlation coefficient (R^2) values greater than 0.997 for all compounds. Calibration ranges

are shown in **Table 3.3**. Concentrations of the calibration curves covered the concentration ranges expected for the mentioned volatile compounds in wine as previously reported in literature (Louw *et al*., 2009; Ortega *et al*., 2001). Detection and quantification limits were determined by analysing synthetic wine (described in section 3.2.1) containing major volatile compounds and calculating the concentration required to give a signal-to-noise ratio of 3 or 10, respectively (data shown in **Table 3.3**). Quantification limits ranged from 0.03 to 18 mg/L for ethyl decanoate and acetic acid respectively while detection limits ranged between 0.01 and 5.4 mg/L for the same compounds. These limits of quantification were acceptable for the purpose of wine analysis.

3.3.2.2 Repeatability and intermediate repeatability

Under the analytical conditions described above, the intermediate repeatability, expressed by the relative standard deviation (%RSD) obtained on six independent analyses of the volatile compound standard solution, standard addition to red and white wine over three different days were calculated. The intermediate repeatability values (**Table 3.4**) range between 0.3% (isoamyl alcohol) and 12.7% (valeric acid) in the white wine, between 0.3% (isoamyl alcohol) and 11.1% (ethyl 2-methylbutyrate) in the red wine and between 1.1% (ethyl isovalerate) and 13.1 % (methanol) in the standard solution. These values are satisfactory for the between-day repeatability of the method and no carryover or accumulation of high boiling point compounds was observed between the different injections.

The within-day repeatability (shown in **Table 3.4**) for the standard solution was 4.7% on average calculated from six replicates. The maximum values were near 12% for methanol and the minimum was 0.7% for isoamyl acetate. For wine samples the repeatability was 3.4% on average for the red wine and 2.1% on average for the white wine. The maximum values were near 11% for ethyl octanoate in the red wine and 10% for methanol in the white wine. The minimum values were 0.1% (ethyl decanoate) and 0.3% (isoamyl alcohol, acetic acid) in the red and white wine respectively.

3.3.2.3 Evaluation of matrix effects and recovery

The recovery of the overall analytical method was tested with a synthetic wine (standard solution containing known amounts of analyte) and wine samples (wine 1 and wine 2) spiked with the analytes at two different levels (lower and higher concentration within the calibration graph concentration range). Two samples of each were prepared and extracted according to the method described. The recoveries of the 39 compounds were calculated and are shown in **Table 3.5**.

For synthetic wine, the results show a very good recovery (72.9% - 132.2%) with a standard deviation of less than 20% with the exception of acetoin (24.7%).

The range of recoveries in wine samples is very wide, as could be expected for such a diverse mixture of chemical compounds, with good recovery in the range of 95% (4-methyl-1-pentanol) to 119% (methanol, ethyl acetate) as well as some poorly recovered compounds in the range of 2% (ethyl decanoate and ethyl octanoate) to 40% (decanoic acid). In general, the recoveries calculated in the synthetic wine were better than the extraction obtained in wine samples. However, standard addition experiments proved that this did not affect the overall performance of the method.

Table 3.4 Repeatability and intermediate repeatability data for two wines and a synthetic wine. Relative standard deviation (%RSD) as found for each analyte is an estimation of the repeatability of the method.

Compound	Repeatability (%RSD)			Intermediate repeatability (%RSD)		
	Wine 1[a]	Wine 2[b]	Synthetic wine	Wine 1[a]	Wine 2[b]	Synthetic wine
Ethyl acetate	5.0	4.5	8.0	4.3	5.2	5.7
Methanol	10.8	8.8	12.3	11.3	9.0	13.1
Ethyl propionate	4.5	4.9	8.7	6.4	8.3	8.2
Ethyl 2-methyl propanoate	2.4	4.1	8.2	5.2	8.0	4.9
2-Methyl propyl acetate	1.0	3.6	3.0	2.3	5.6	1.6
Ethyl butyrate	0.9	2.5	1.9	4.7	9.8	1.3
n-Propanol	2.4	4.5	1.6	2.8	5.7	2.8
Ethyl 2-methylbutyrate	0.8	7.0	1.5	1.0	11.1	1.2
Ethyl isovalerate	0.7	1.3	1.5	7.9	6.4	1.1
Isobutanol	1.4	3.4	3.2	3.8	3.4	2.6
Isoamylacetate	0.8	0.7	0.7	1.7	2.0	0.6
n-Butanol	0.8	6.3	3.0	3.4	4.3	1.8
Isoamylalcohol	0.3	0.3	2.1	0.3	0.3	1.3
Ethyl hexanoate	1.1	3.9	2.7	3.5	4.6	2.1
Pentanol	0.6	1.0	1.9	0.7	6.6	1.2
Hexyl acetate	1.1	3.8	2.7	3.5	9.3	2.1
Acetoin	3.3	3.5	8.6	7.8	2.5	10.3
4-Methyl-1-pentanol	1.2	3.7	2.9	2.8	2.0	2.0
3-Methyl-1-pentanol	1.3	3.5	3.0	2.9	8.8	2.1
Ethyl lactate	0.6	1.6	4.6	3.3	1.6	3.6
Hexanol	1.4	2.8	4.9	2.8	2.3	2.8
3-Ethoxy-1-propanol	1.6	1.0	5.6	4.5	6.2	5.3
Ethyl octanoate	1.4	11.6	1.5	5.5	8.4	3.7
1-Octen-3-ol	1.6	6.7	4.0	5.3	4.0	3.1
Acetic acid	0.3	0.8	3.7	2.1	1.0	3.9
Ethyl 3-hydroxybutanoate	2.6	3.9	6.5	4.3	8.0	7.8
Propionic acid	5.2	3.7	10.1	7.3	10.9	11.5
Isobutyric acid	2.8	2.6	2.3	6.1	5.6	4.6
Butyric acid	2.2	1.6	6.9	3.6	6.6	7.5
Ethyl decanoate	1.9	0.1	8.1	7.0	9.9	9.7
Isovaleric acid	1.8	1.6	2.6	5.6	4.7	3.6
Diethyl succinate	2.5	2.5	5.2	7.4	6.9	4.9
n-Valeric acid	1.7	2.1	5.1	12.7	4.2	5.6
Ethyl phenylacetate	1.8	2.9	5.3	6.9	4.3	5.7
2-Phenylethyl acetate	1.9	2.9	5.1	8.4	9.6	6.1
Hexanoic acid	2.4	2.1	6.2	6.7	5.5	6.9
2-Phenylethanol	2.5	2.6	5.8	5.6	3.8	5.9
Octanoic acid	2.4	3.2	4.4	8.5	3.6	8.2
Decanoic acid	2.8	3.5	6.7	9.8	4.0	11.5

[a]Wine 1: White wine used during method validation; [b]Wine 2: Red wine used during method validation.

Table 3.5 Average (n=4) recoveries (%) of major volatile compounds in white wine (Wine 1), red wine (Wine 2) and a standard solution (synthetic wine) at two different levels.

Compound	Level 1						Level 2					
	Wine 1		Wine 2		Synthetic wine		Wine 1		Wine 2		Synthetic wine	
	Added (mg/L)	Recovery (%)±RSD (%)	Added (mg/L)	Recovery (%)±RSD (%)	Added (mg/L)	Recovery (%)±RSD (%)	Added (mg/L)	Recovery (%)±RSD (%)	Added (mg/L)	Recovery (%)±RSD (%)	Added (mg/L)	Recovery (%)±RSD (%)
Ethyl Acetate	5.0	115.6 ± 2.4	5.0	28.4 ± 4.7	24.1	116.2 ± 8.0	20.0	112.3 ± 4.5	20.0	105.2 ± 5.0	90.2	107.1 ± 3.4
Methanol	5.0	118.9 ± 10.5	5.0	109.1 ± 12.5	60.1	132.2 ± 18.5	20.0	117.5 ± 8.8	20.0	119.3 ± 10.8	225.4	94.7 ± 16.5
Ethyl propionate	5.0	116.2 ± 12.5	5.0	101.6 ± 6.4	6.7	126.6 ± 13.2	20.0	111.1 ± 15.9	20.0	109.0 ± 4.5	25.0	102.7 ± 3.2
Ethyl 2-methyl propanoate	5.0	101.3 ± 14.0	5.0	99.6 ± 15.9	6.7	105.7 ± 8.2	20.0	100.5 ± 14.0	20.0	104.5 ± 2.4	25.0	98.2 ± 1.5
2-Methyl propyl acetate	5.0	89.1 ± 2.7	5.0	92.5 ± 3.2	6.7	100.5 ± 3.0	20.0	95.2 ± 3.6	20.0	102.0 ± 1.0	25.0	97.4 ± 0.1
Ethyl Butyrate	5.0	15.8 ± 5.5	5.0	20.1 ± 5.1	1.5	99.4 ± 1.9	20.0	19.5 ± 2.5	20.0	22.3 ± 0.9	5.5	97.6 ± 0.7
Propanol	5.0	108.3 ± 4.4	5.0	114.8 ± 2.1	13.4	111.0 ± 1.6	20.0	110.7 ± 4.5	20.0	103.6 ± 2.4	50.3	104.8 ± 4.0
Ethyl 2-methylbutyrate	5.0	92.4 ± 5.1	5.0	91.0 ± 1.2	6.7	93.1 ± 1.5	20.0	100.1 ± 7.0	20.0	102.1 ± 0.8	25.0	96.1 ± 0.8
Ethyl isovalerate	5.0	86.1 ± 1.5	5.0	89.4 ± 1.5	6.7	96.7 ± 1.5	20.0	94.1 ± 1.3	20.0	100.3 ± 0.7	25.0	96.3 ± 0.7
Isobutanol	5.0	109.3 ± 3.9	5.0	71.7 ± 7.5	6.7	109.1 ± 3.2	20.0	70.5 ± 3.4	20.0	86.5 ± 1.4	25.1	104.1 ± 2.0
Isoamylacetate	5.0	17.6 ± 2.2	5.0	14.3 ± 2.1	1.3	95.3 ± 0.7	20.0	17.9 ± 0.7	20.0	16.8 ± 0.8	4.8	95.0 ± 0.4
Butanol	5.0	26.7 ± 4.6	5.0	24.0 ± 4.4	1.4	104.6 ± 3.0	20.0	23.7 ± 6.3	20.0	22.9 ± 0.8	5.1	103.4 ± 0.6
Isoamylalcohol	5.0	102.0 ± 0.5	5.0	106.5 ± 1.1	31.8	103.2 ± 2.1	20.0	117.0 ± 0.3	20.0	101.7 ± 0.3	119.3	100.9 ± 0.4
Ethyl hexanoate	5.0	25.7 ± 3.0	5.0	26.6 ± 5.6	2.0	92.5 ± 2.7	20.0	27.8 ± 3.9	20.0	28.7 ± 1.1	7.6	92.8 ± 1.5
Pentanol	5.0	108.8 ± 0.9	5.0	106.6 ± 0.8	6.7	101.9 ± 1.9	20.0	110.3 ± 1.0	20.0	107.5 ± 0.6	25.0	100.3 ± 0.4
Hexyl Acetate	5.0	17.9 ± 7.3	5.0	19.2 ± 5.9	1.5	92.2 ± 2.7	20.0	19.5 ± 3.8	20.0	20.9 ± 1.1	5.5	92.1 ± 1.6
Acetoin	5.0	59.0 ± 2.0	5.0	105.8 ± 12.4	6.7	111.3 ± 24.7	20.0	39.0 ± 3.5	20.0	59.4 ± 3.3	25.0	72.9 ± 12.8
4-Methyl-1-pentanol	5.0	95.5 ± 1.2	5.0	97.4 ± 4.5	6.7	98.3 ± 2.9	20.0	100.3 ± 3.7	20.0	101.7 ± 1.2	25.0	96.5 ± 1.0
3-Methyl-1-pentanol	5.0	97.9 ± 1.0	5.0	98.3 ± 4.6	6.7	98.4 ± 3.0	20.0	101.3 ± 3.5	20.0	102.3 ± 1.3	25.0	96.7 ± 1.1
Ethyl Lactate	5.0	112.0 ± 0.6	5.0	99.8 ± 6.1	33.3	105.3 ± 4.6	20.0	111.5 ± 1.6	20.0	100.8 ± 0.6	125.0	99.8 ± 2.6
Hexanol	5.0	29.9 ± 1.5	5.0	31.4 ± 4.9	2.1	98.0 ± 4.9	20.0	30.5 ± 2.8	20.0	30.8 ± 1.4	7.7	95.9 ± 0.7
3-Ethoxy-1-propanol	5.0	76.3 ± 11.3	5.0	68.9 ± 7.5	6.7	99.7 ± 5.6	20.0	114.5 ± 1.0	20.0	95.3 ± 1.6	25.0	94.4 ± 5.1
Ethyl octanoate	5.0	2.2 ± 3.9	5.0	2.8 ± 9.6	0.2	88.5 ± 1.5	20.0	2.7 ± 11.6	20.0	2.7 ± 1.4	0.9	87.1 ± 6.0
1-Octen-3-ol	5.0	88.2 ± 1.4	5.0	90.6 ± 9.0	6.7	94.9 ± 4.0	20.0	95.2 ± 6.7	20.0	99.0 ± 1.6	25.0	93.8 ± 2.2
Acetic acid	5.0	100.5 ± 0.8	5.0	102.4 ± 2.6	120.3	106.7 ± 3.7	20.0	104.0 ± 0.8	20.0	107.8 ± 0.3	451.1	94.4 ± 4.0
Ethyl 3-hydroxybutanoate	5.0	85.6 ± 4.5	5.0	99.2 ± 6.0	6.7	118.7 ± 19.9	20.0	120.3 ± 11.2	20.0	108.1 ± 2.6	25.0	102.7 ± 5.4
Propionic acid	5.0	65.0 ± 3.2	5.0	47.1 ± 9.4	2.0	99.8 ± 12.1	20.0	22.3 ± 11.7	20.0	38.1 ± 5.2	7.4	102.0 ± 10.9
Isobutyric acid	5.0	16.6 ± 2.5	5.0	27.2 ± 9.4	1.4	100.9 ± 2.3	20.0	21.9 ± 2.6	20.0	22.8 ± 2.8	5.2	97.7 ± 6.8
Butyric acid	5.0	23.5 ± 2.5	5.0	24.7 ± 13.2	1.4	102.9 ± 6.9	20.0	23.6 ± 11.1	20.0	23.8 ± 2.2	5.3	97.5 ± 8.1
Ethyl decanoate	5.0	2.9 ± 6.4	5.0	2.5 ± 12.2	0.2	97.5 ± 18.3	20.0	3.0 ± 9.3	20.0	3.2 ± 1.9	0.9	83.8 ± 8.4
Isovaleric acid	5.0	42.6 ± 2.0	5.0	36.8 ± 11.8	2.6	99.6 ± 2.6	20.0	40.1 ± 8.4	20.0	39.9 ± 1.8	9.8	95.1 ± 4.6
Diethyl Succinate	5.0	16.6 ± 1.4	5.0	31.7 ± 11.1	2.1	96.4 ± 5.2	20.0	19.5 ± 12.2	20.0	32.2 ± 2.5	7.9	93.4 ± 4.7
Valeric acid	5.0	16.2 ± 3.0	5.0	12.7 ± 13.4	1.4	98.5 ± 5.1	20.0	21.1 ± 2.1	20.0	20.0 ± 1.7	5.2	94.2 ± 6.0
Ethyl phenylacetate	5.0	86.9 ± 1.4	5.0	89.4 ± 12.0	6.7	93.1 ± 5.3	20.0	96.6 ± 2.9	20.0	97.2 ± 1.8	25.0	89.9 ± 6.2
2-Phenylethyl acetate	5.0	17.5 ± 2.2	5.0	18.8 ± 12.9	1.4	93.1 ± 5.1	20.0	18.9 ± 10.0	20.0	20.1 ± 1.9	5.2	89.5 ± 7.0
Hexanoic acid	5.0	26.7 ± 1.4	5.0	32.0 ± 13.0	2.0	98.6 ± 6.2	20.0	27.7 ± 11.4	20.0	28.5 ± 2.4	7.4	91.3 ± 7.6
2-Phenylethanol	5.0	5.6 ± 2.2	5.0	52.4 ± 9.9	3.4	98.2 ± 5.8	20.0	26.0 ± 2.6	20.0	46.5 ± 2.5	12.7	94.2 ± 6.0
Octanoic acid	5.0	34.9 ± 1.7	5.0	38.1 ± 15.8	2.7	94.7 ± 4.4	20.0	38.2 ± 3.2	20.0	38.0 ± 2.4	10.0	87.7 ± 12.0
Decanoic acid	5.0	41.3 ± 3.2	5.0	42.2 ± 18.6	3.3	91.6 ± 6.7	20.0	44.4 ± 3.5	20.0	45.7 ± 2.8	12.5	82.7 ± 16.2

3.3.2.4 Application to wine

After validation, the analytical method was applied to 50 samples of commercially available wines to determine the volatile compound profile. Each sample was analysed in triplicate. Identification by pure standards was used to identify major volatile compounds in the wine samples. The method was successfully applied to both white and red wines. **Table 3.6** and **Table 3.7** show ranges and cultivars measured with this method for white and red wines respectively. Repeatability was less than 10% RSD between replicate analyses. Note that the large %RSD shown for each analyte is related to the variation observed between the different wine samples analysed for a specific cultivar. Since the commercial wines were representative of different viticultural areas, vintages and most probably winemaking practices, this explains the observed variation. Of the 39 compounds, 35 compounds were detected in all the white wines and 37 compounds were detected in all the red wines. The concentrations of the quantified compounds were in the expected ranges previously reported for white and young red wines (Francis & Newton, 2005). Differentiation between white and red cultivars was observed by performing PCA on the fast GC data (**Figure 3.3a**). Variables associated with white cultivars were situated to the negative side of the loadings plot (**Figure 3.3b**) and included hexanoic, decanoic and octanoic acids. Alcohols, ethyl lactate and acetic acid were amongst the compounds correlated with the red cultivars and situated to the positive side of the loadings plot (**Figure 3.3b**). Ethyl lactate and acetic acid concentrations have been shown to increase with malolactic fermentation (MLF) performed by lactic acid bacteria (LAB) (Bartowsky & Henschke, 1995). MLF is performed in the majority of red wines and in some white wines, mostly Chardonnay. The ethyl lactate concentrations in the red wine data set are significantly higher compared to that of the white wines. In the white wine data set, the Chardonnay wines exhibit a higher concentration of ethyl lactate, most probably indicating some MLF activity.

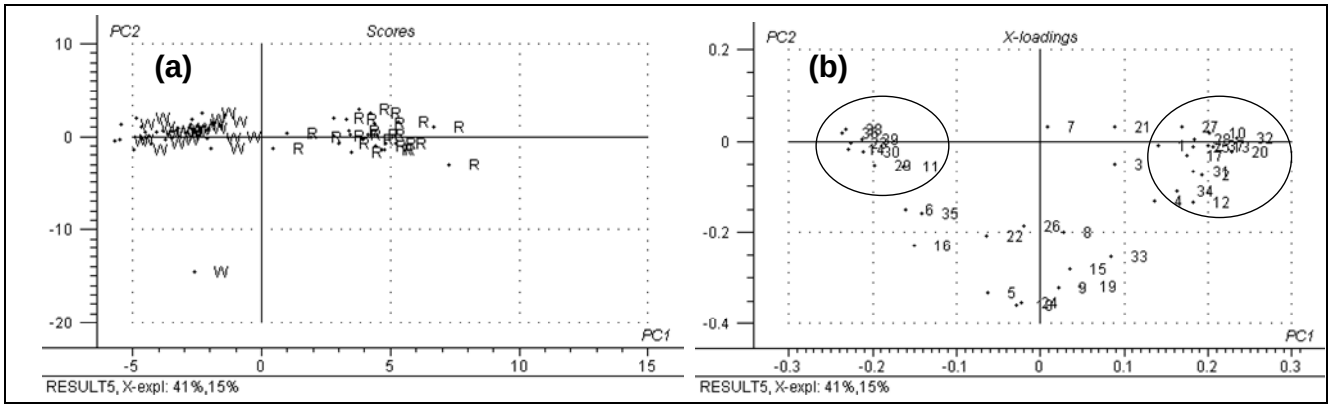

Figure 3.3 (a) PCA scores plot shows clear differentiation between red (indicated by R) and white (indicated by W) cultivars using the fast GC data. (b) Corresponding loadings for the PCA indicates which variables contribute significantly to the differentiation between red (variable in the circle on the right-hand side of the plot) and white cultivars (variables situated towards the left of the plot).

Table 3.6 Quantitative data of the commercial white wines analysed with the proposed faster GC-FID method. Means (mg/L), relative standard deviation (%RSD) and number of different wines analysed per cultivar are given.

Compound	Sauvignon blanc (n=6)	Chardonnay (n=6)	Dry white blend (n=2)	Gewürztraminer (n=2)	Semi-sweet white (n=3)	Riesling (n=2)	Chenin blanc (n=4)	Viognier (n=1)
	Mean (mg/L)±%RSD	Mean (mg/L)±%RSD	Mean (mg/L)±%RSD	Mean (mg/L)±%RSD	Mean (mg/L)±%RSD	Mean (mg/L)±%RSD	Mean (mg/L)±%RSD	Mean (mg/L)±%RSD
Ethyl acetate	96.9 ± 48.8	83.6 ± 39.6	108.5 ± 10.3	86.4 ± 7.0	84.9 ± 30.8	52.1 ± 21.0	112.1 ± 38.5	93.4 ± na
Methanol	57.4 ± 49.2	81.0 ± 19.0	56.9 ± 1.1	66.3 ± 3.5	111.6 ± 37.1	66.7 ± 8.8	72.0 ± 19.1	62.1 ± na
Ethyl propionate	0.5 ± 108.1	1.3 ± 71.4	0.9 ± 137.1	0.1 ± 75.4	0.6 ± 33.6	nd	0.5 ± 117.3	0.2 ± na
Ethyl 2-methyl propanoate	0.4 ± 69.1	0.7 ± 43.8	0.5 ± 2.7	0.3 ± 45.5	0.8 ± 68.0	0.3 ± 93.7	0.4 ± 74.9	1.3 ± na
2-Methyl propyl acetate	0.6 ± 9.7	0.6 ± 6.9	0.7 ± 3.3	0.6 ± 4.0	0.7 ± 29.8	0.6 ± 8.0	0.6 ± 7.2	0.6 ± na
Ethyl butyrate	0.5 ± 18.5	0.6 ± 19.3	0.4 ± 31.1	0.5 ± 11.3	0.6 ± 17.3	0.4 ± 21.8	0.6 ± 14.5	0.5 ± na
Propanol	44.1 ± 52.5	67.6 ± 42.2	53.8 ± 41.0	79.5 ± 11.6	35.6 ± 9.2	40.9 ± 22.0	45.8 ± 52.6	56.2 ± na
Ethyl 2-methylbutyrate	0.1 ± 57.3	0.1 ± 92.2	0.2 ± 11.7	0.1 ± 55.3	0.3 ± 69.5	0.4 ± 116.5	0.2 ± 32.4	0.2 ± na
Ethyl isovalerate	0.4 ± 11.2	0.4 ± 6.0	0.4 ± 16.7	0.4 ± 1.2	0.5 ± 34.9	0.4 ± 11.0	0.4 ± 12.0	0.5 ± na
Isobutanol	27.4 ± 23.0	20.0 ± 18.2	22.9 ± 4.7	27.5 ± 34.6	31.7 ± 5.7	31.3 ± 22.7	26.6 ± 9.4	19.7 ± na
Isoamylacetate	2.1 ± 44.2	2.5 ± 63.2	1.4 ± 31.0	2.3 ± 7.1	1.7 ± 50.7	1.1 ± 109.6	0.9 ± 94.3	0.9 ± na
Butanol	1.0 ± 38.8	1.0 ± 28.0	0.8 ± 61.1	1.3 ± 55.2	1.3 ± 59.1	0.8 ± 4.7	1.1 ± 18.9	1.6 ± na
Isoamylalcohol	201.1 ± 13.8	162.7 ± 15.4	185.1 ± 0.1	185.4 ± 10.5	187.0 ± 6.6	180.5 ± 20.8	190.1 ± 6.2	172.8 ± na
Ethyl hexanoate	1.2 ± 21.1	1.2 ± 22.8	1.1 ± 41.4	1.0 ± 23.3	1.1 ± 11.8	1.0 ± 18.5	0.9 ± 18.8	0.9 ± na
Pentanol	0.1 ± 23.6	0.1 ± 39.1	0.2 ± 63.8	0.1 ± 6.7	0.2 ± 66.5	0.1 ± 47.7	0.1 ± 34.8	0.2 ± na
Hexyl acetate	0.2 ± 17.4	0.2 ± 26.9	0.1 ± 9.8	0.2 ± 27.3	0.2 ± 31.1	0.2 ± 21.2	0.2 ± 34.6	0.1 ± na
Acetoin	5.6 ± 26.0	4.8 ± 47.1	8.2 ± 12.4	6.7 ± 15.4	9.5 ± 72.2	3.0 ± 115.1	6.9 ± 26.7	11.0 ± na
4-Methyl-1-pentanol	nd	0.01 ± 132.0	0.04 ± 141.4	0.02 ± 141.4	0.1 ± 173.2	nd	0.03 ± 88.5	0.1 ± na
3-Methyl-1-pentanol	0.02 ± 35.0	0.04 ± 14.3	0.1 ± 63.2	0.01 ± 141.4	0.1 ± 128.0	0.02 ± 126.5	0.03 ± 78.3	0.1 ± na
Ethyl lactate	17.3 ± 37.5	39.7 ± 149.1	17.1 ± 71.8	19.0 ± 5.8	25.6 ± 67.8	18.1 ± 2.2	20.3 ± 51.2	35.5 ± na
Hexanol	1.4 ± 27.5	1.3 ± 21.4	1.3 ± 54.7	1.3 ± 21.4	1.4 ± 13.0	1.6 ± 62.9	1.1 ± 25.5	0.6 ± na
3-Ethoxy-1-propanol	4.0 ± 37.5	4.6 ± 55.9	2.9 ± 31.0	5.7 ± 23.6	5.9 ± 46.7	2.2 ± 72.1	4.1 ± 36.0	2.1 ± na
Ethyl octanoate	1.2 ± 21.7	1.3 ± 27.5	1.1 ± 53.2	1.1 ± 32.9	1.1 ± 19.9	1.1 ± 31.5	0.7 ± 42.9	0.9 ± na
1-Octen-3-ol	nd	nd	nd	nd	0.1 ± 173.2	nd	nd	nd
Acetic acid	467.4 ± 48.8	401.4 ± 55.0	592.6 ± 13.7	416.4 ± 23.7	463.9 ± 31.3	290.3 ± 41.1	521.1 ± 32.4	418.1 ± na
Ethyl 3-hydroxybutanoate	1.7 ± 29.8	1.6 ± 34.1	1.4 ± 37.7	0.8 ± 38.3	3.0 ± 12.7	1.9 ± 90.6	2.1 ± 51.0	0.8 ± na
Propionic acid	16.3 ± 48.1	24.5 ± 18.2	18.2 ± 57.1	21.4 ± 32.8	18.1 ± 12.0	13.9 ± 3.6	15.7 ± 22.5	32.0 ± na
Isobutyric acid	1.5 ± 20.7	1.6 ± 34.5	1.2 ± 13.0	1.5 ± 2.8	1.6 ± 16.2	1.6 ± 2.7	1.3 ± 25.5	1.5 ± na
Butyric acid	2.2 ± 15.4	2.7 ± 15.6	2.2 ± 30.0	2.8 ± 10.5	2.6 ± 13.8	2.3 ± 6.3	2.4 ± 10.2	2.1 ± na
Ethyl decanoate	0.4 ± 37.9	0.4 ± 40.6	0.4 ± 90.9	0.3 ± 51.6	0.4 ± 15.6	0.3 ± 68.4	0.2 ± 112.3	0.2 ± na
Isovaleric acid	1.2 ± 8.2	1.3 ± 19.7	1.2 ± 30.8	1.1 ± 3.8	1.4 ± 23.3	1.1 ± 16.8	1.2 ± 7.9	1.4 ± na
Diethyl succinate	1.8 ± 54.3	2.4 ± 44.5	2.4 ± 71.9	1.6 ± 4.5	2.0 ± 34.9	3.3 ± 78.0	3.5 ± 53.1	6.9 ± na
Valeric acid	0.4 ± 15.4	0.4 ± 9.7	0.4 ± 34.1	0.4 ± 2.5	0.6 ± 44.1	0.4 ± 0.4	0.4 ± 14.2	0.4 ± na
Ethyl phenylacetate	0.02 ± 110.0	0.02 ± 103.8	0.02 ± 12.5	0.01 ± 141.4	0.1 ± 77.7	nd	0.02 ± 67.1	0.02 ± na
2-Phenylethyl acetate	0.1 ± 62.0	0.1 ± 55.6	0.1 ± 28.9	0.1 ± 3.8	0.2 ± 48.4	0.05 ± 59.0	0.1 ± 85.0	0.02 ± na
Hexanoic acid	5.7 ± 19.5	6.0 ± 16.4	5.2 ± 19.9	5.4 ± 20.4	5.4 ± 26.0	5.5 ± 9.8	4.9 ± 9.2	3.9 ± na
2-Phenylethanol	17.1 ± 16.4	13.7 ± 20.6	16.7 ± 10.2	12.5 ± 0.5	13.1 ± 27.8	11.0 ± 15.7	14.8 ± 15.0	10.1 ± na
Octanoic acid	9.5 ± 21.0	8.7 ± 21.3	7.1 ± 33.4	7.6 ± 30.8	7.5 ± 31.9	8.0 ± 23.1	6.2 ± 33.6	5.4 ± na
Decanoic acid	2.2 ± 33.8	2.3 ± 28.3	1.9 ± 60.2	2.0 ± 42.4	2.1 ± 29.1	1.8 ± 48.5	1.3 ± 69.1	1.3 ± na

nd=not detected; na=not applicable, only one wine analysed.

Table 3.7 Quantitative data of the commercial red wines analysed with the proposed faster GC-FID method. Means (mg/L), relative standard deviation (%RSD) and number of different wines analysed per cultivar are given.

Compound	Petit Verdot (n=1)	Pinotage (n=4)	Tinta Barocca (n=1)	Touriga Nacional (n=1)	Ruby Cabernet (n=1)	Cabernet Sauvignon (n=6)	Shiraz (n=5)	Malbec (n=1)	Merlot (n=3)	Claret (n=1)
	Mean (mg/L)±%RSD	Mean (mg/L)±%RSD	Mean (mg/L)±%RSD	Mean (mg/L)±%RSD	Mean (mg/L)±%RSD	Mean (mg/L)±%RSD	Mean (mg/L)±%RSD	Mean (mg/L)±%RSD	Mean (mg/L)±%RSD	Mean (mg/L)±%RSD
Ethyl acetate	141.9 ± na	169.4 ± 16.9	132.5 ± na	124.6 ± na	75.0 ± na	138.1 ± 37.1	151.5 ± 22.0	147.6 ± na	159.9 ± 22.8	85.9 ± na
Methanol	125.9 ± na	153.9 ± 21.2	180.7 ± na	155.8 ± na	166.7 ± na	146.8 ± 29.9	177.9 ± 16.5	126.8 ± na	233.6 ± 31.4	127.2 ± na
Ethyl propionate	2.1 ± na	1.3 ± 18.7	2.3 ± na	1.7 ± na	0.4 ± na	1.1 43.6	0.9 ± 126.1	2.5 ± na	0.9 ± 86.9	3.9 ± na
Ethyl 2-methyl propanoate	1.6 ± na	0.8 ± 40.3	1.3 ± na	0.7 ± na	0.4 ± na	0.9 ± 46.2	1.3 ± 63.6	1.0 ± na	1.3 ± 50.8	1.2 ± na
2-Methyl propyl acetate	0.6 ± na	0.6 ± 4.9	0.6 ± na	0.7 ± na	0.6 ± na	0.6 ± 9.0	0.6 ± 6.3	0.6 ± na	0.6 ± 10.6	0.5 ± na
Ethyl butyrate	0.4 ± na	0.5 ± 6.7	0.4 ± na	0.4 ± na	0.4 ± na	0.3 ± 19.9	0.4 ± 21.5	0.3 ± na	0.4 ± 12.9	0.4 ± na
n-Propanol	79.3 ± na	57.6 ± 7.9	97.1 ± na	115.4 ± na	32.1 ± na	42.7 ± 35.6	50.0 ± 15.5	72.1 ± na	55.7 ± 58.9	35.3 ± na
Ethyl 2-methylbutyrate	0.1 ± na	0.2 ± 69.6	0.4 ± na	0.2 ± na	0.3 ± na	0.2 ± 59.4	0.3 ± 78.2	0.2 ± na	0.2 ± 94.2	0.3 ± na
Ethyl isovalerate	0.6 ± na	0.4 ± 12.1	0.4 ± na	0.4 ± na	0.4 ± na	0.4 ± 22.6	0.4 ± 8.1	0.4 ± na	0.4 ± 6.5	0.4 ± na
Isobutanol	48.4 ± na	48.2 ± 12.6	41.1 ± na	37.2 ± na	60.6 ± na	80.1 ± 34.2	55.7 ± 20.8	55.3 ± na	70.7 ± 26.9	49.3 ± na
Isoamylacetate	0.6 ± na	1.1 ± 60.0	1.0 ± na	0.9 ± na	1.5 ± na	0.6 ± 17.5	0.5 ± 35.9	0.6 ± na	0.5 ± 16.3	0.6 ± na
n-Butanol	2.1 ± na	1.7 ± 14.1	2.0 ± na	2.2 ± na	2.6 ± na	1.8 ± 11.6	2.0 ± 21.7	2.1 ± na	1.7 ± 1.4	1.6 ± na
Isoamylalcohol	486.7 ± na	236.1 ± 6.5	322.7 ± na	180.0 ± na	510.1 ± na	452.4 ± 18.6	294.9 ± 11.4	324.7 ± na	325.7 ± 0.7	347.3 ± na
Ethyl hexanoate	0.4 ± na	0.5 ± 19.6	0.5 ± na	0.3 ± na	0.5 ± na	0.4 ± 14.8	0.4 ± 18.4	0.4 ± na	0.4 ± 25.3	0.5 ± na
Pentanol	0.1 ± na	0.2 ± 31.1	0.2 ± na	0.2 ± na	0.1 ± na	0.1 ± 59.1	0.1 ± 24.7	0.2 ± na	0.1 ± 46.4	0.2 ± na
Hexyl acetate	0.2 ± na	0.1 ± 18.2	0.1 ± na	0.2 ± na	0.1 ± na	0.1 ± 52.4	0.1 ± 27.2	0.1 ± na	0.1 ± 18.6	0.1 ± na
Acetoin	23.9 ± na	13.5 ± 31.2	22.2 ± na	7.7 ± na	8.3 ± na	26.6 ± 43.7	24.7 ± 43.0	24.5 ± na	15.1 ± 16.6	10.8 ± na
4-Methyl-1-pentanol	nd	nd	0.1 ± na	0.01 ± na	0.0 ± na	0.01 ± 191.3	0.01 ± 148.1	nd	nd	nd
3-Methyl-1-pentanol	0.1 ± na	0.1 ± 71.7	0.2 ± na	0.04 ± na	0.2 ± na	0.1 ± 77.4	0.05 ± 93.4	0.01 ± na	0.03 ± 31.9	0.04 ± na
Ethyl lactate	393.0 ± na	260.7 ± 28.5	257.8 ± na	186.1 ± na	282.3 ± na	222.7 ± 18.5	207.2 ± 23.8	271.6 ± na	195.9 ± 22.5	230.7 ± na
Hexanol	2.1 ± na	1.3 ± 46.5	1.3 ± na	1.4 ± na	1.6 ± na	1.8 ± 32.7	1.6 ± 35.7	3.4 ± na	1.2 ± 21.2	1.2 ± na
3-Ethoxy-1-propanol	6.5 ± na	3.7 ± 18.5	3.7 ± na	5.5 ± na	2.0 ± na	2.9 ± 42.3	2.6 ± 38.9	3.4 ± na	3.3 ± 28.5	3.5 ± na
Ethyl octanoate	0.2 ± na	0.4 ± 36.0	0.5 ± na	0.2 ± na	0.3 ± na	0.3 ± 36.9	0.3 ± 27.9	0.2 ± na	0.3 ± 21.9	0.4 ± na
1-Octen-3-ol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Acetic acid	888.4 ± na	855.2 ± 24.5	738.3 ± na	973.8 ± na	555.7 ± na	840.2 ± 25.1	811.3 ± 20.7	954.4 ± na	913.1 ± 23.0	532.5 ± na
Ethyl 3-hydroxybutanoate	1.2 ± na	1.6 ± 65.3	2.6 ± na	2.0 ± na	2.9 ± na	1.3 ± 42.8	2.2 ± 36.5	1.3 ± na	1.4 ± 41.5	2.0 ± na
Propionic acid	23.2 ± na	32.8 ± 21.0	41.3 ± na	56.5 ± na	32.5 ± na	31.8 ± 14.6	33.5 ± 16.6	53.3 ± na	34.8 ± 1.9	30.0 ± na
Isobutyric acid	3.9 ± na	2.1 ± 26.8	2.3 ± na	1.8 ± na	2.6 ± na	2.7 ± 20.9	1.9 ± 21.6	2.4 ± na	2.2 ± 12.2	2.2 ± na
Butyric acid	1.3 ± na	1.7 ± 13.6	1.9 ± na	1.2 ± na	1.4 ± na	1.5 ± 10.3	1.7 ± 11.5	1.6 ± na	1.8 ± 12.1	1.5 ± na
Ethyl decanoate	0.0 ± na	0.1 ± 88.5	0.1 ± na	0.1 ± na	0.1 ± na	0.04 ± 75.8	0.1 ± 53.1	0.02 ± na	0.1 ± 39.8	0.03 ± na
Isovaleric acid	8.9 ± na	2.0 ± 26.4	2.2 ± na	1.2 ± na	4.3 ± na	3.2 ± 6.8	2.1 ± 10.4	2.4 ± na	2.7 ± 3.0	3.2 ± na
Diethyl succinate	40.3 ± na	15.9 ± 46.4	35.1 ± na	14.0 ± na	30.0 ± na	28.8 ± 13.3	22.2 ± 30.7	40.4 ± na	24.6 ± 17.7	32.3 ± na
n-Valeric acid	1.0 ± na	0.5 ± 5.1	0.5 ± na	0.4 ± na	0.8 ± na	0.6 ± 32.9	0.4 ± 11.5	0.4 ± na	0.4 ± 35.5	0.4 ± na
Ethyl phenylacetate	0.6 ± na	0.05 ± 110.0	0.1 ± na	0.4 ± na	0.3 ± na	0.1 ± 74.0	0.1 ± 119.6	0.2 ± na	0.2 ± 36.7	0.1 ± na
2-Phenylethyl acetate	0.1 ± na	0.1 ± 28.6	0.1 ± na	0.05 ± na	0.1 ± na	0.04 ± 51.1	0.03 ± 66.5	0.03 ± na	0.02 ± 15.3	0.03 ± na
Hexanoic acid	2.0 ± na	2.3 ± 15.3	2.8 ± na	1.4 ± na	2.2 ± na	1.9 ± 19.9	1.9 ± 17.9	1.9 ± na	1.8 ± 19.5	2.4 ± na
2-Phenylethanol	101.3 ± na	23.2 ± 47.8	50.8 ± na	19.9 ± na	103.3 ± na	75.5 ± 25.8	37.4 ± 29.6	43.5 ± na	47.0 ± 27.6	68.5 ± na
Octanoic acid	1.6 ± na	2.9 ± 31.6	3.1 ± na	1.5 ± na	2.4 ± na	1.9 ± 29.7	2.1 ± 23.1	1.5 ± na	2.1 ± 17.5	2.8 ± na
Decanoic acid	0.5 ± na	0.9 ± 34.0	0.9 ± na	0.7 ± na	0.8 ± na	0.6 ± 24.4	0.7 ± 23.1	0.5 ± na	0.7 ± 12.6	0.6 ± na

nd=not detected; na=not applicable, only one wine analysed.

In order to further evaluate the potential application of the fast GC for metabolic profiling purposes, wines fermented with four different malolactic fermentation bacterial starter cultures were analysed with the proposed fast GC method. PLS-DA models (**Figure 3.4**) of the data obtained with fast GC (**Figure 3.4a**) and conventional GC-FID (**Figure 3.4b**) show similar separation patterns between the different bacterial starter cultures in Shiraz wine. Similar observations were made for the Pinotage wines tested (data not shown). The proposed faster GC-FID method could therefore be successfully used as a screening tool in metabolic studies.

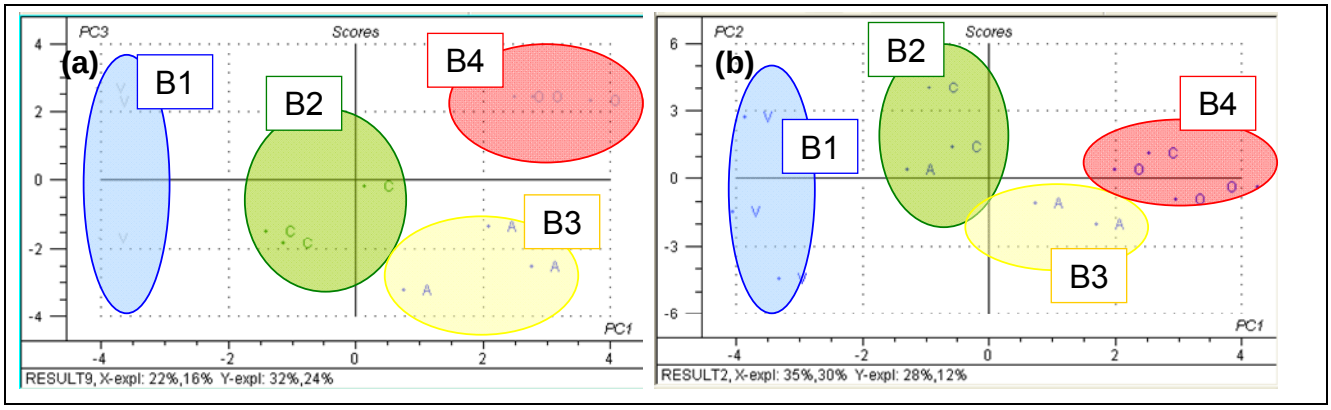

Figure 3.4 (a) PLS-DA scores plot shows differentiation of Shiraz wines fermented with different MLF starter cultures (indicated by B1, B2, B3, B4) based on (a) fast GC-FID data and (b) conventional GC-FID data. Similar differentiation patterns were also observed with both analytical methods.

3.4 CONCLUSIONS

In this contribution an in-house developed fast GC-FID method with the potential for the high throughput quantification of 39 volatiles in wine in less than 15 minutes is developed, validated and successfully applied for the analysis of several red and white cultivars popular in South Africa, including Pinotage, Shiraz, Cabernet Sauvignon, Sauvignon blanc, Chardonnay and Chenin blanc. Combining these instrumental advances with a fast and straightforward liquid-liquid extraction sample preparation method prior to chromatographic analysis, the speed of the complete analytical protocol is considerably enhanced and therefore sample throughput is significantly enhanced.

The results obtained indicate that the method is suitable for significantly faster routine analysis of wine volatiles, while high accuracy and repeatability comparable to conventional methods are maintained. Validation of this analytical method illustrated selectivity, linearity, repeatability and recovery of analytes which are acceptable for quantification of a wide range of chemical compounds involved in wine aroma. The importance of GC injection port liner selection, a clean injection port and critical injection parameters should be emphasized as this largely affects the sensitivity of the method.

The compounds determined in the selection of wines were used to successfully discriminate between white and red cultivars obtained from different viticultural areas and vintages. Applying multivariate data analysis techniques to the fast GC-FID data demonstrate the differentiation between wines fermented with different MLF bacterial starter cultures. The application of the proposed fast GC-FID analytical technique will enable researchers to more efficiently monitor the chemical and

biological processes that are ongoing during the fermentation of wine, as more samples can be analysed in a much shorter time-period targeting these important compounds. Yeast and bacterial starter culture development programs would definitely benefit from this technique as large numbers of isolates are often screened for their contribution to volatile composition of wines. Finally, this technique could be used during metabolic profiling research as a screening tool to investigate potentially interesting tendencies in wine since these compounds are some of the major contributors to wine aroma.

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CHAPTER 4

Research Results

Comparative metabolic profiling approach to investigate the contribution of malolactic fermentation starter cultures to red wine chemical composition

4. RESEARCH RESULTS

Comparative metabolic profiling approach to investigate the contribution of malolactic fermentation starter cultures to red wine chemical composition

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ABSTRACT

This research work investigates the changes associated with four commercial *Oenococcus oeni* malolactic fermentation (MLF) starter cultures in terms of the volatile composition, organic acid content and infrared spectral fingerprints of South African Shiraz and Pinotage red wines. Wines were produced under small scale standardised experimental conditions with four starter cultures and compared to a control wine where MLF was suppressed. The MLF progress was monitored with both mid and near infrared spectroscopy and also used to acquire a metabolic fingerprint of the wine. Gas chromatographic analysis and capillary electrophoresis were used to evaluate the volatile composition and organic acid profiles, respectively. Significant strain-specific variations were induced in the organic acid profiles, especially for the degradation of citric acid and the production of lactic acid during MLF. Subsequently, compounds directly and indirectly resulting from citric acid metabolism, namely diacetyl, acetic acid, acetoin and ethyl lactate, were also influenced depending on the bacterial strain used for MLF. Bacterial metabolic activity increased the higher alcohols, fatty acids and overall ester concentrations with a larger increase observed in ethyl esters compared to acetate esters. Ethyl lactate, diethyl succinate, ethyl octanoate, ethyl-2-methylpropanoate and ethyl propionate were increased by MLF. Hexyl acetate, isoamyl acetate, 2-phenylethyl acetate and ethyl acetate were decreased or unchanged, depending on the strain and cultivar evaluated. The formation of ethyl butyrate, ethyl propionate, ethyl-2-methylbutryate and ethyl isovalerate was related to specific bacterial strains used, illustrating a possible difference in terms of esterase activity. A strain-specific tendency to reduce total aldehyde concentrations was found with the completion of MLF, however further investigation is needed in this regard. Infrared spectral fingerprints were used to characterise the different bacteria and in addition, the prediction of MLF related compounds, diacetyl, acetoin and 2,3-pentanedione, from mid-infrared spectra was explored by PLS models. The current study provides insight regarding the metabolism of *O. oeni* starter cultures during MLF in red wine under warm climate conditions. The results presented in this study is of significance to the wine industry since it illustrate and reiterate the potential of different MLF starter cultures to contribute to wine aroma and flavour.

Keywords: malolactic fermentation, volatile composition, carbonyl compounds, bacterial starter cultures, infrared spectroscopy, organic acids

4.1 INTRODUCTION

Wine production involves a succession of biological processes including alcoholic fermentation performed by yeast and malolactic fermentation (MLF) conducted by lactic acid bacteria (LAB). MLF in wine is conducted, preferably by *Oenococcus oeni* [formerly known as *Leuconostoc oenos* (Dicks *et al*., 1995)], in order to decrease wine acidity through the biotransformation of the dicarboxylic L-malic acid to the monocarboxylic L-lactic acid by the malolactic enzyme (Davis *et al*., 1985; Lonvaud-Funel, 1995). MLF improves biological stability and influences organoleptic properties such as aroma, flavour and mouthfeel (Davis *et al*., 1985; Henick-Kling *et al*., 1993; Bartowsky *et al*., 2002).

The complexity and diversity of the metabolic activities associated with the growth of LAB suggest that MLF may affect wine quality both positively and negatively (Liu, 2002). Wine aroma and flavour could be influenced by bacteria via several mechanisms including (i) the reduction of flavour compounds by metabolism and adsorption to the cell wall; (ii) the production of additional volatiles from the metabolism of grape sugars, amino acids and other nutrient compounds; and (iii) the metabolism or extracellular modification of grape and yeast secondary metabolites to either more or less flavour and aroma active metabolites (Bartowsky & Henschke, 1995). Wine associated LAB have been shown to produce a variety of enzymatic activities which has the potential to affect or produce a range of volatile compounds (Liu, 2002; Matthews *et al*., 2004; 2006; Mtshali *et al*., 2010). Differences in enzymatic activity have been observed amongst LAB genera and strains evaluated (Matthews *et al*., 2006). The use of different bacterial starter culture strains during winemaking could therefore influence the volatile composition and possibly the resulting sensory properties in a strain-dependant manner. This provides winemakers with a state of the art tool to produce wines comprising of different styles and desired sensory properties. However, the impact of MLF on wine composition and sensory properties is not yet fully understood and depends largely on bacterial strain characteristics, varietal aroma of the wine and vinification techniques employed (Henick-Kling, 1995). In order to understand the wine aroma changes associated with bacterial growth a broad-range profiling approach is required to monitor a variety of chemical changes occurring during MLF.

One of the most important aroma compounds synthesized during MLF and the most frequently reported aroma modification associated with LAB is the production of diacetyl (2,3-butanedione) which, when present at concentrations above its sensory threshold, contributes a buttery, nutty and/or toasty aroma to wine (Davis *et al*., 1985; Etiévant, 1991; Laurent *et al*., 1994; Bartowsky & Henschke, 1995; Martineau *et al*., 1995b). The aroma threshold of diacetyl in wine is low, 0.2-2.8 mg/L, depending on the wine type (Martineau *et al*., 1995a). It is well-known that diacetyl, acetic acid, acetoin and 2,3-butanediol are formed by LAB through citric acid catabolism (Lonvaud-Funel, 1999; Bartowsky *et al*., 2002; Bartowsky & Henschke, 2004). Information regarding the factors influencing the production of diacetyl, managing concentrations in wine and the sensory effect on wine has been thoroughly studied for many years (Rankine *et al*., 1969; Davis *et al*., 1985; Martineau *et al*., 1995b; de Revel *et al*., 1999) and several comprehensive reviews are available (Lonvaud-Funel, 1999; Bartowsky *et al*., 2002; Bartowsky & Henschke, 2004; Bauer & Dicks, 2004; Versari *et al*., 1999). However, except for diacetyl, the influence of LAB on wine chemical and organoleptic properties has not been characterised.

According to previous reports, the modification of wine aroma induced by MLF is far more complex and often involves changes in fruity, flowery and nutty attributes, as well as the reduction of vegetative, green, grassy, herbaceous aromas (Bartowsky & Henschke, 1995; Henick-Kling, 1995). Evidence to support the observed aroma modifications in terms of chemical composition is often contradictory or inconclusive and the mechanisms responsible for these modifications are not completely understood. A significant increase in the concentration of several esters produced by bacteria metabolism has been reported (Maicas *et al*., 1999; Delaquis *et al*., 2000), while other studies report decreased ester concentrations (Gámbaro *et al*., 2001). The catabolism of acetaldehyde by wine LAB was reported by Osborne *et al*. (2000). This illustrates the potential of LAB to metabolise aldehydes (Liu, 2002) and consequently reduce the associated herbaceous aroma, however reports on these changes during MLF are few. Additional compounds such as higher alcohols, fatty acids, lactones, sulphur and nitrogen containing compounds may also be produced and could potentially contribute or alter wine aroma through either enhancing or masking effects (Ugliano & Moio, 2005).

Different analytical procedures have been described for the quantification of volatile compounds in wine and comprehensive reviews are available (Ebeler, 2001; Ortega-Heras *et al*., 2002). Gas chromatography (GC) methods in combination with a variety of extraction and detection techniques have been most extensively used for the quantification of wine volatile compounds. Headspace SPME (HS-SPME) (Arthur & Pawliszyn, 1990; Zhang & Pawliszyn, 1993) represents an effective and solventless sampling technique especially suitable for the quantification of highly volatile analytes since it reduces interferences from other, non-volatile, wine constituents. Hayasaka *et al*. (1999) described a simple and effective method for the quantification of diacetyl by using HS-SPME coupled to GC-MS. A number of other studies have described methods for the quantification of diacetyl as well as other dicarbonyl compounds (de Revel *et al*., 2000) and several aldehydes (Ferreira *et al*., 2004, 2006; Wang *et al*., 2005) by using derivatisation procedures. Since the influence of MLF on aldehydes are not yet characterised mainly due to a lack of analytical data, a robust method for the simultaneous determination of diacetyl and aldehydes in wine would provide insight into changes associated with LAB during MLF.

In order to exploit the potential contribution of LAB to wine, effective management of MLF is of critical importance to winemakers. MLF could occur spontaneously in wine as a result of *O. oeni*, *Pediococcus* spp. and *Lactobacillus* spp. present in the wine (Wibowo *et al*., 1985; Du Toit *et al*., 2010). However, spontaneous MLF provides no consistent outcome in terms of MLF completion, organoleptic profile or the resulting wine quality. The introduction of commercial freeze-dried bacterial cultures of *O. oeni* for direct inoculation into wine has improved the management of MLF (Nielsen *et al*., 1996) as this practice ensures better control over the time of onset and rate of MLF, reduces the potential for spoilage by other bacteria, reduces the potential interference by bacteriophages, gives better control over the flavour contribution of MLF and reduces the risk of potential biogenic amine production which has health implications (Lonvaud-Funel, 2001). Commercially available strains are usually isolated from wines undergoing spontaneous fermentation and then carefully evaluated for their fermentation ability, flavour and mouthfeel contribution properties (Ruiz *et al*., 2010; Solieri *et al*., 2010). *O. oeni* is recognised as the species most tolerant to the harsh wine conditions of low pH, high sulphur dioxide (SO_2) and high alcohol content (Versari *et al*., 1999) and for this reason *O. oeni* is mostly selected as starter culture in addition to its favourable flavour profile (Lerm *et al*., 2010).

Comparative studies regarding the influence of different commercial MLF bacteria on the concentration of wine volatiles often focus on selected groups of compounds whereas the cultivars and strains tested are often very specific to countries and regions. The effect of MLF activity in Tannat, the most important red wine in Uruguayan viticulture, was previously investigated (Boido *et al*., 2009) and focused on the comparison of different major volatile compounds. In other studies, the potential of four commercial MLF starter cultures to hydrolyse glycosides and release volatile compounds and the influence on yeast-derived volatile compounds during MLF in Aglianico grapes from Southern Italy were evaluated (Ugliano & Moio, 2005, 2006). Pozo-Bayón *et al*. (2005) found significant differences in the wine volatile and amino acid composition of Tempranillo wine, one of the most important Spanish red grape cultivars, after MLF with *O. oeni* and *Lactobacillus plantarum* starter cultures. Recently, metabolic profiling studies showed significant differences in the major volatile compounds after MLF by different starter cultures in Meoru wine, made from a wild Korean grape (Lee *et al*., 2009; Son *et al*., 2009). Some studies have reported on the sensory effects of MLF in Chardonnay (Rodriguez *et al*., 1990) and Pinot noir (Sauvageot & Vivier, 1997) by comparing the effect of different bacterial inoculations. However, these studies lack the complementation of supporting volatile composition data. Volatile aroma constituents including esters, aldehydes, alcohols, ketones, acids, and sulphur-containing compounds were investigated in Chancellor wine after MLF by two commercial starter cultures (Delaquis *et al*., 2000). De Revel *et al.* (1999) found increased concentrations of wood-derived volatile compounds after MLF performed in barrels with Sauvignon blanc must. Studies specifically focusing on the effect of different MLF scenarios on the diacetyl concentrations have also been reported in Chardonnay (Laurent *et al*., 1994).

Reports which evaluate the effect of MLF in Shiraz and Pinotage grapes from warm climatic regions are limited (Lerm, 2010). Pinotage [Pinot noir x Cinsaut (known as Hermitage in South Africa) hybrid variety) is a South African grape cultivar which has been previously found to differ from other red cultivars with regard to its volatile composition (Louw *et al*., 2009). Louw *et al*. (2009) reported observations suggesting that this cultivar is more comparable to white cultivars than other red cultivars in terms of specific volatile compounds. This is in support of another study (De Beer *et al*., 2005) that found Pinotage to compare better to white wines than red wines in terms of its antioxidant potential.

The purpose of this study was to investigate the influence of MLF by using four commercial *O. oeni* starter cultures on the chemical composition in terms of volatile compounds, organic acids and infrared spectral properties of Pinotage and Shiraz wine from South Africa, to provide a better understanding of the contribution of MLF to the composition of wine and potential contribution to wine aroma.

4.2 MATERIALS AND METHODS

4.2.1 CHEMICAL STANDARDS AND REAGENTS

All standards (**Table 4.1**) were of analytical grade (purity 95% - 99.9%) and purchased from Fluka (Buchs, Switzerland), Sigma-Aldrich (Steinheim, Germany), Riedel-de-Häen (Seelze, Germany) and Merck (Darmstadt, Germany) respectively. Sodium chloride (HPLC quality) and diethyl ether (99.5%) were purchased from Merck (Darmstadt, Germany) and pure water was obtained from a Milli-Q

purification system (Millipore, Bedford, MA, USA). The internal standards (2-pentanone and 4-methyl-2-pentanol, Fluka) and volatile standards were dissolved in a wine simulant (12% v/v ethanol) prepared according to Louw *et al*. (2009) and used for the respective calibration curves.

Table 4.1 Odour threshold (OTH) values (mg/L) and descriptions as reported in literature (source is given in parentheses).The source and analytical grade for the analytes are listed.

Analyte	OTH (mg/L)	Odour description	Source
Esters			
ethyl decanoate	0.2 *(1)*	grape, floral, soap *(16)*	Aldrich, >99%
ethyl hexanoate	0.014 *(1)*	fruity, anise *(17)*	Fluka, 99%
ethyl butyrate	0.02 *(2)*	fruity *(17)*, apple *(16)*	Fluka, >98%
ethyl octanoate	0.005 *(1)*	fruit, fat *(16)*	Fluka, >98%
ethyl lactate	154.6 *(3)*	butter, cream, fruit *(16)*	Fluka, 99%
ethyl propionate	1.8 *(4)*	fruity *(17)*	Fluka, >99.7%
ethyl-2-methylpropanoate	0.015 *(1)*	fruity *(17)*	Fluka, >98%
ethyl-2-methylbutyrate	0.018 *(1)*	fruity *(17)*, apple *(16)*	Aldrich, >98%
ethyl isovalerate	0.003 *(1)*	fruity, anise *(17)*	Fluka, >99.7%
ethyl-3-hydroxybutanoate	20 *(5)*	strawberry, burnt marshmallow *(18)*	Fluka, >97%
ethyl phenylacetate	0.65 *(6)*	rose, floral *(19)*	Fluka, >99%
ethyl acetate	12.26 *(3)*	fruit, nail polish *(16)*	Sigma-Aldrich, >99.7%
isoamyl acetate	0.03 *(2)*	banana, pear *(17)*	Riedel de Haën, >98%
hexyl acetate	1.5 *(4)*	sweet, perfume *(20)*	Fluka, 99%
2-phenylethyl acetate	0.25 *(2)*	roses *(17)*	Fluka, >99%
diethyl succinate	200 *(4)*	berry *(16)*	Fluka, >98%
2-methyl-propyl acetate	1.6 *(5)*	solvent *(17)*	Fluka, >99.8%
Alcohols			
hexanol	8 *(2)*	green, grass, resin *(17)*	Merck, >98%
butanol	150 *(4)*	fusel, spirituous *(16)*	Fluka, >99.5%
methanol	500 *(7)*	alcohol *(7)*	Sigma-Aldrich, >99.9%
2-phenylethanol	14 *(1)*	honey, spice, rose, lilac *(16)*	Merck, >99%
propanol	306 *(8)*	pungent, harsh *(16)*	Fluka, >99%
isobutanol	40 *(2)*	wine, solvent, bitter *(16)*	Fluka, >99.5%
isoamyl alcohol	30 *(2)*	fusel *(17)*, whiskey, malt, burnt *(16)*	Aldrich, >99%
pentanol	64 *(4)*		Fluka, >99.8%
4-methyl-1-pentanol	1 *(9)*		Sigma-Aldrich, >95%
3-methyl-1-pentanol	1 *(9)*		Sigma-Aldrich, >97%
3-ethoxy-1-propanol	0.1 *(7)*	fruity *(7)*	Sigma-Aldrich, >97%

[1]Ferreira *et al*., 2000; [2]Guth, 1997a; [3]Ferreira *et al*., 2001; [4]Etievant, 1991; [5]Aznar *et al*., 2003; [6]Fazzalari, 1978; [7]Peinado *et al*., 2004b; [8]Peinado *et al*., 2004a; [9]Zea *et al*., 2001; [10]Lambrechts & Pretorius, 2000; [11]Guth, 1997b; [12]Culleré *et al*., 2004; [13]Ferreira *et al*., 2004; [14]Chatonnet&Dubourdieu, 1998; [15]Boehlens & van Gemert, 1987; [16]Francis & Newton, 2005; [17]Escudero *et al*., 2007; [18]Ugliano & Moio, 2005; [19]Sumby *et al*., 2010; [20]Swiegers *et al*., 2005; [21]de Revel & Bertrand, 1994; [22]Hashizume & Samuta, 1997.
*Shown to range from 0.2 mg/L to 2.8 mg/L depending on wine style (Martineau *et al*., 1995a).

Table 4.1 (*continued)* Odour threshold (OTH) values (mg/L) and descriptions as reported in literature (source is given in parentheses).The source and analytical grade for the analytes are listed.

Analyte	OTH (mg/L)	Odour description	Source
Acids and fatty acids			
acetic acid	200 *(2)*	vinegar *(17)*	Saarchem, > 98%
propionic acid	20 *(10)*	pungent, rancid, sweat *(16)*	Fluka, >99.5%
isobutyric acid	2.3 *(1)*	rancid, butter, cheese *(16)*	Fluka, >99.5%
butyric acid	0.173 *(1)*	cheese *(17)*	Fluka, >99.5%
isovaleric acid	0.033 *(1)*	cheese *(17)*	Fluka, >99%
valeric acid			Fluka, >99%
hexanoic acid	0.42 *(1)*	sweat *(16)*	Aldrich, >99.5%
octanoic acid	0.50 *(1)*	sweat, cheese *(16)*	Aldrich, >99.5%
decanoic acid	1 *(1)*	rancid, fat *(16)*	Sigma, > 98%
Carbonyl compounds			
diacetyl (2,3-butanedione)	0.1* *(11)*	butter, cream *(17)*	Fluka, >99.5%
acetoin (3-hydroxy-2-butanone)	150 *(4)*	butter, cream *(16)*	Fluka, >97%
2,3-pentanedione	0.9 *(7)*	butter, cream *(17)*	Fluka, >95%
E-2-hexenal	0.01 *(12)*	herbaceous, green *(21)*	Fluka, >97%
E-2-heptenal	0.013 *(12)*	herbaceous *(21)*	Fluka, >96%
octanal	0.05 *(9)*	herbaceous *(21)*, fatty, citrus *(9)*	Fluka, >98%
E-2-octenal	0.0001 *(12)*	lemon *(17)*; herbaceous *(21)*	Aldrich, >94%
nonanal	0.001 *(13)*	herbal, floral	Fluka, >95%
E-2-nonenal	0.000068 *(14)*	sawdust, plank *(14)*	Aldrich, 97%
decanal	0.0001 *(13)*	citrus, fruity	Sigma, >98%
trans-2, *cis*-6-nonadienal	0.00001 *(15)*	cucumber, green *(22)*	Aldrich, 95%

4.2.2 BACTERIAL STRAINS

The four different commercial preparations used for this study were Lalvin VP41® (Lallemand, Stellenbosch, South Africa), Enoferm alpha® (Lallemand, Stellenbosch, South Africa), Viniflora® oenos (Chr Hansen, Hørsholm, Denmark) and Viniflora® CH16 (Chr Hansen, Hørsholm, Denmark). Bacterial preparations were kindly donated by Lallemand and Chr Hansen. These four commercial starter cultures were selected as they are frequently used in the South African wine industry. A summary of the respective sensory attributes according to the manufacturers' literature is given in **Table 4.2.**

Table 4.2 Sensory attributes of the commercial starter cultures according to the respective manufacturer. Abbreviations for the starter cultures used during this study are listed.

Starter culture	Abbreviation	Company	Sensory contribution description in brief[a]
Enoferm alpha®	A	Lallemand	Mouthfeel, lower perception of green and vegetative flavours, positive impact on wine complexity
Lalvin VP41®	V	Lallemand	Enhances complexity and mouthfeel, contribute to aroma and wine structure
Viniflora® oenos	O	Chr Hansen	Clean and classic flavour profile, Low production of volatile acidity
Viniflora® CH16	C	Chr Hansen	Low production of volatile acidity

[a]Information obtained from the respective technical data sheets: www.chr-hansen.com; www.lallemandwine.com

4.2.3 EXPERIMENTAL DESIGN OF WINEMAKING

The experimental design for the winemaking experiments was the same for both years (2008, 2009) and cultivars (Pinotage, Shiraz) (**Figure 4.1**). Alcoholic fermentation (1000 L) was followed by MLF performed in triplicate (4.5 L x 3) for each of the different treatments, namely control (no MLF), spontaneous (spon), Enoferm alpha® (A), Lalvin VP41® (V), Viniflora oenos® (O) and Viniflora CH16® (C). The control wine treatment (no MLF; lysozyme added to inhibit LAB growth) and spontaneous treatment (no MLF inoculation) were subjected to the same experimental design and included for comparative purposes. Subsequent chemical analysis of each of the biological replicates was performed in duplicate or triplicate, depending on the chemical analysis.

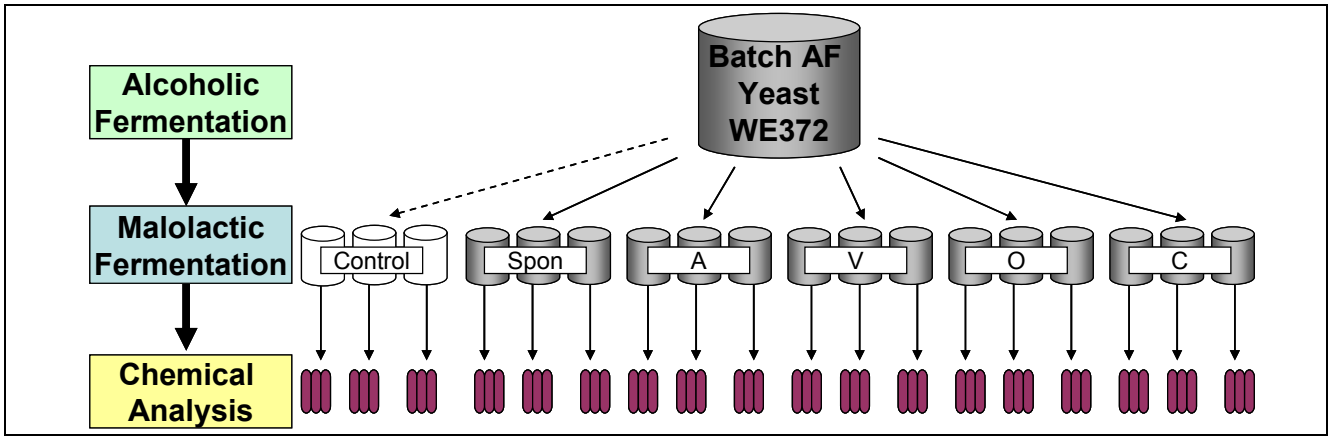


Figure 4.1 Summary of the experimental design followed during all winemaking experiments. Batch alcoholic fermentation (1000 L) was followed by MLF in triplicate (4.5 L x 3) for each of the different treatments namely control (no MLF), spontaneous (spon), Enoferm alpha (A), Lalvin VP41 (V), Viniflora oenos (O) and Viniflora CH16 (C). Subsequent chemical analysis of each of the biological replicates was performed in triplicate.

One hundred and seventy kilograms of Shiraz grapes were harvested at 25.0˚B during the 2008 season from the Wellington region, Western Cape, South Africa. Pinotage grapes were harvested from the same region in South Africa at 28.4˚B (170 kg) and 22.0˚B (175 kg) during the 2008 and 2009 vintages respectively. After crushing and destemming, 30 mg/L sulphur dioxide (SO_2) was added to the must in order to reduce possible growth from natural flora present on the grapes and avoid oxidation. Alcoholic fermentation was performed in a 1000 L stainless steel tank at 25˚C. A commercial strain of actively dried *Saccharomyces cerevisiae*, WE372 (Anchor biotechnologies, South Africa) was inoculated for alcoholic fermentation at 0.3 g/L after rehydration according to the manufacturer's specifications. Lysozyme (DSM Food Specialties, Oenology, France) was added at 0.25 g/L to inhibit indigenous LAB microflora. During alcoholic fermentation the skins were punched down manually twice a day. At 3˚B the wine was removed from the skins by light pressing using only the free-flow wine to reduce hard tannins being present in the wine. After completion of alcoholic fermentation (residual sugar less than 5 g/L) the wine was divided into 4.5 L glass bottles for malolactic fermentation. MLF was performed at 20˚C in triplicate for each of the respective treatments, namely spontaneous MLF, four commercial starter cultures and a control treatment. The spontaneous MLF treatment was not inoculated with a starter culture and no SO_2 was added. This treatment was

included to evaluate whether any natural flora could have potentially contributed to the MLF process and whether the lysozyme treatment was effective. Commercial starter cultures were rehydrated and inoculated according to the manufacturers' specifications at 0.01 g/L. For the control treatment, three of the 4.5 L glass bottles were racked and 50 mg/L SO_2 added directly after alcoholic fermentation to inhibit microbial growth and capture the chemical composition of the wines before MLF. Bacterial complex nutrients were added according to each manufacturer's instructions: 0.2 g/L Optimalo (Lallemand, Stellenbosch, South Africa) for the Lalvin VP41 and Enoferm alpha cultures and 0.1 g/L Bactiv-aid (Chr Hansen, Hørsholm, Denmark) for the Viniflora oenos and Viniflora CH16 cultures. MLF was regarded as complete at malic acid concentrations less than 0.3 g/L. After MLF, all wines were racked, SO_2 levels adjusted to 50 mg/L and bottled. Wines were stored at 15˚C prior to all chemical analysis.

4.2.4 MICROBIAL ENUMERATION

Microbial populations for LAB were monitored to evaluate the effectiveness of the inoculated commercial cultures and to establish if other LAB species survived and could potentially contribute to MLF. For this purpose, LAB were determined by plating 100 µL of a dilution series made in sterile water of wine on selective media. MRST plates contained 50 g/L De Man, Rogosa and Sharpe (MRS; Biolab, Merck, Wadeville, South Africa), 20 g/L Bacteriological agar (Biolab, Merck) supplemented with 10% preservative free tomato juice (All Gold, South Africa) and pH adjusted to 5.0 with hydrochloric acid (HCl). MRS plates contained 50 g/L MRS broth (Biolab, Merck) and 15 g/L Bacteriological agar (Biolab, Merck). All plates contained 50 mg/L Delvocid Instant (DSM Food Specialties, The Netherlands) to prevent yeast growth and 25 mg/L kanamycin sulphate (Roche Diagnostics GmbH, Mannheim, Germany) to suppress the growth of acetic acid bacteria. MRST, which favours the growth of *O. oeni,* was used for the enumeration of *O. oeni* while MRS agar was used for the enumeration of other wine LAB. Agar plates were incubated at 30˚C for 5 to 7 days after which colony forming unit per mL (cfu/mL) were determined. All LAB were anaerobically cultivated by using Microbiology Anaerocult pads in anaerobic jars (Merck, Darmstadt, Germany).

4.2.5 FOURIER TRANSFORM MID-INFRARED SPECTROSCOPY

FT-IR spectra were generated by using a Winescan FT120 instrument (FOSS Analytical A/S software version 2.2.1) equipped with a purpose-built Michelson interferometer (FOSS Analytical A/S, Hillerød, Denmark). Samples were filtered prior to analysis to remove particles and excess amounts of carbon dioxide which could potentially affect the accuracy of the results (Winescan Reference manual, FOSS Analytical, Denmark). A filtration unit (type 79500, FOSS Electric, Denmark) connected to a vacuum pump and filter paper circles graded at 20 - 25 µm with a diameter of 185 mm (Schleicher & Schnell, reference number 10312714) was used for this purpose. Duplicate spectra were acquired in the spectral range 4992 - 929 cm^{-1} for each sample and the spectra averaged for data processing. Samples were scanned at 4 cm^{-1} intervals at 40˚C using a CaF_2-lined cuvette with a fixed cuvette path length of 37 µm (Winescan FT120 Type 77110 and 77310 Reference manual, FOSS Analytical, Denmark). Each spectrum is based on an average of 20 repeat scans, recorded in transmittance

mode and converted to a linearised absorbance spectrum (Winescan FT120 Type 77110 and 77310 Reference manual, FOSS Analytical, Denmark).
Quantified chemical data including pH, ethanol, total acidity (TA), acetic acid, volatile acidity (VA), glycerol, malic acid and residual sugar (RS) were predicted from infrared spectra by commercial calibrations or in-house adjustments as described before (Louw *et al*., 2009) using the Winescan FT120 2001 version 2.2.1 software.

4.2.6 FOURIER TRANSFORM NEAR INFRARED SPECTROSCOPY

Near infrared (NIR) spectral data in the 12 498.9 - 3999.8 cm^{-1} region of the electromagnetic spectrum were acquired in transmission mode using a quartz cuvette of 1 mm path length with a Fourier transform (FT)-NIR spectrometer (MPA Multi Purpose FT-NIR analyzer instrument, Bruker Optics, Bryanston/Cramerview, South Africa) equipped with OPUS software version 6.5 (Bruker Optics). Prior to data acquisition, the cuvette was cleaned with absolute ethanol (Merck) and distilled water. Afterwards, the cuvette was first rinsed with the wine sample in question, then filled with the wine sample and data acquired (8 cm^{-1} resolution; air background, 16 sample scans) at controlled temperature (21˚C±2˚C) conditions.

4.2.7 ORGANIC ACID ANALYSIS

Malic acid, lactic acid, pyruvic acid, gluconic acid, acetic acid, succinic acid and citric acid were quantified before and after MLF using a modified version of the certified OIV reference method (Oeno-05, OIV, 2006). The original OIV method was modified (running buffer contains 5% acetonitrile as compared to 10% in the original) in order to include more analytes for quantification. Samples were diluted 1/25 in the running buffer before injection. A 3D CE instrument (Agilent Technologies, Waldbron, Germany) equipped with Agilent Chemstation software version B.01.03 [204] was used for the analysis and data processing according to the certified OIV method (Oeno-05, www.oiv.org, 2006). Calibration ranges were between 0.04 g/L and 2 g/L for all compounds except for pyruvic acid which had an upper limit of 1 g/L.

4.2.8 VOLATILE COMPOUND ANALYSIS

4.2.8.1 Major volatile compounds

Volatile higher alcohols, esters, fatty acids and carbonyl compounds was analysed in triplicate with a Hewlett Packard 6890 Plus gas chromatograph (Little Falls, USA) equipped with a split/splitless injector and an FID detector following the newly developed fast GC procedure described in Chapter 3. In brief, volatile compounds were extracted from 5 mL of wine after the addition of 10 mg/L internal standard, 4-methyl-2-pentanol (≥97%, Fluka, Buchs, Switzerland) using a liquid-liquid diethyl ether (99.5%, Merck, Darmstadt, Germany) extraction procedure. Analysis of the different compounds was achieved using a J&W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with dimensions 20 m length × 0.1 mm inside diameter × 0.2 μm film thickness. Analyte concentrations

were calculated by comparing their retention times and areas with those from calibration standard curves on a data handling system (HP GC Chemstation, Revision A.07.01 [682]).

4.2.8.2 Carbonyl compounds

A headspace solid phase microextraction gas chromatography mass spectrometry (HS-SPME GC-MS) was developed to quantify the content of the carbonyl compounds diacetyl, acetoin, 2,3-pentanedione and a selection of aldehydes including hexanal, *E*-2-hexenal, decanal, octanal, *E*-2-octenal, *E*-2-nonenal and *cis*-2, *trans*-6-nonadienal.

Headspace solid-phase micro-extraction was performed by using a 60 µm polyethylene glycol (PEG) SPME fiber (Supelco, Bellefonte, PA), specific for the extraction of polar compounds from the headspace. Glass screw-cap vials with polytetrafluoroethylene (PTFE)/silicone septa (20 mm) (Agilent Technologies, Little Falls, Wilmington, USA) were used. After optimisation, the SPME analyses were performed using 1 mL of wine, 9 mL distilled milli-Q water (Millipore) and 2 g of sodium chloride (NaCl; Sigma) in a 20 mL vial. The internal standard, 2-pentanone, was added at 10 mg/L to each vial. The wine was agitated to ensure that NaCl dissolved completely. Extraction of volatiles from the headspace was performed at 50˚C for 10 min. Subsequently, the fiber was desorbed in the hot injection port of the GC-MS at 220˚C for 2 min. The injector was operated in pulsed split mode (300 kPa, split ratio 10:1) at 220˚C for 2 min and 171 kPa afterwards. Each wine was analysed in duplicate.

Separation was performed on a 60 m L x 0.25 mm i.d. x 0.25 µm f.t. FFAP column (Agilent Technologies, Little Falls, Wilmington, USA) using a 6890 gas chromatograph coupled to a mass spectrometer 5975C (Agilent Technologies, Little Falls, Wilmington, USA) and equipped with Enhanced Chemstation version D.01.02.16 software (Agilent technologies, Little Falls, Wilmington, USA). For sample preparation and introduction, the instrument was equipped with a CTC CombiPal autosampler (CTC Analytics, Switzerland) and used with the SPME option. The carrier gas (helium) flow through the GC column was 1.7 mL/min and the oven programmed from 35˚C (2 min), ramped at 5˚C/min to 150˚C (2 min) and ramped at 15˚C/min to 240˚C (1 min). The mass spectrometer (MS) was operated in electron impact (EI) mode (70 eV). Data acquisition was performed in SIM mode by monitoring the mass-to-charge (m/z) ratio's representing unique ion fragments for the respective compounds: 2-pentanone (IS) [43, 86]; diacetyl [43, 86]; 2,3-pentanedione [57, 100]; *E*-2-hexenal [69, 83, 98]; octanal [69, 84, 110]; acetoin [45, 88]; nonanal [82, 98, 114]; *E*-2-octenal [70, 83, 97]; nonanal [82, 95, 112]; decanal [82, 95, 112]; *E*-2-nonenal [83, 70, 96]; 2-*trans*, 6-*cis*-nonadienal [69, 70, 81]. Peak identification of the volatile components was achieved by comparison of retention times when injecting pure, authentic standards.

4.2.9 DATA ANALYSIS

Data was subjected to one-way analysis of variance (ANOVA) followed by Fisher's Least significant difference (LSD) test to determine whether significant differences between samples existed, using the XLStat software version 2009.1.02 (Addinsoft, www.xlstat.com). Differences between samples with a significance level of 5 % ($p \leq 0.05$) were considered as significant (Otto, 1998; SAS, 2002). In order to obtain a more comprehensible overview of the volatile aroma compounds and to investigate possible correlations amongst the analytes, multivariate data analysis techniques (Naes *et al*., 2002), including

principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA), were performed with the use of *The Unscrambler* software (version 9.2.1, Camo ASA, Norway). Data were pretreated by autoscaling in order to avoid the differences in measurement units.

4.3 RESULTS AND DISCUSSION

4.3.1 MONITORING MLF

Malolactic fermentation was induced with four different LAB strains in the wines, following completion of alcoholic fermentation with *S. cerevisiae* WE372. **Table 4.3** shows the average chemical composition after alcoholic fermentation and the resulting averages changes observed after the completion of MLF for each of the different batches of wine, namely Pinotage 2008, Pinotage 2009 and Shiraz 2008. During MLF in the Pinotage 2008 wines the average titratable acidity (TA) decreased from 5.27 to 4.47 g/L and pH was increased from 3.95 to 4.24. Similarly, the TA was reduced by 0.8 g/L and the pH increased from 3.58 to 3.67 during MLF in the Pinotage wines during the 2009 vintage. In the Shiraz wine, pH increased from 4.14 to 4.19 and total acidity decreased from 5.52 to 4.97 g/L after the completion of MLF (**Table 4.3**). These results are in agreement with typical changes observed during MLF which include decreases in total acidity ranging from 1 to 3 g/L and pH increases by 0.1 to 0.3 units (Davis *et al*., 1985). Volatile acidity was increased from 0.31 to 0.55 g/L and from 0.32 to 0.45 g/L in the Pinotage wines of 2008 and 2009 respectively. In the Shiraz wines an increase from 0.36 to 0.42 g/L volatile acidity was observed. The increase of 0.25 g/L, 0.13 g/L and 0.06 g/L volatile acidity for the respective wines are in agreement with previous reports that MLF performed by *O. oeni* results in a 0.1 to 0.2 g/L increase in acetic acid concentration (Henschke, 1993) resulting in an increase in volatile acidity.

Table 4.3 Average values of the standard wine parameters[a] for the respective wines after alcoholic fermentation (indicated as before MLF) and changes observed in the composition after the completion of MLF development (indicated by after MLF column).

Parameter	Pinotage 2008		Pinotage 2009		Shiraz 2008	
	Before MLF	After MLF	Before MLF	After MLF	Before MLF	After MLF
alcohol (% v/v)	13.20	13.56	11.81	11.93	14.59	14.34
pH	3.95	4.24	3.58	3.67	4.14	4.19
titratable acidity (g/L)	5.27	4.47	6.95	6.15	5.52	4.97
volatile acidity (g/L)	0.31	0.55	0.32	0.45	0.36	0.42
residual sugars (g/L)	2.93	1.28	0.66	0.80	1.19	1.15
malic acid (g/L)	2.50	0.09	2.31	0.08	3.01	0.17
lactic acid (g/L)	0.12	1.29	0.63	1.36	0.12	0.95

[a]Determined by using the FOSS winescan; acid concentrations confirmed with CE as described in section 4.2.7.

Malolactic fermentation progress in terms of malic acid degradation and the evolution of lactic acid after inoculation with four different commercial strains compared to spontaneous MLF and the control sample is shown in **Figure 4.2** (Pinotage) and **Figure 4.3** (Shiraz). On the basis of changes in malic acid and lactic acid concentrations, spontaneous MLF did not occur in the Pinotage 2008 wine

(**Figure 4.2a)** and the Shiraz 2008 wine (**Figure 4.3**). For this reason, the inclusion of a spontaneous fermentation was omitted during the 2009 vintage (**Figure 4.2b**).

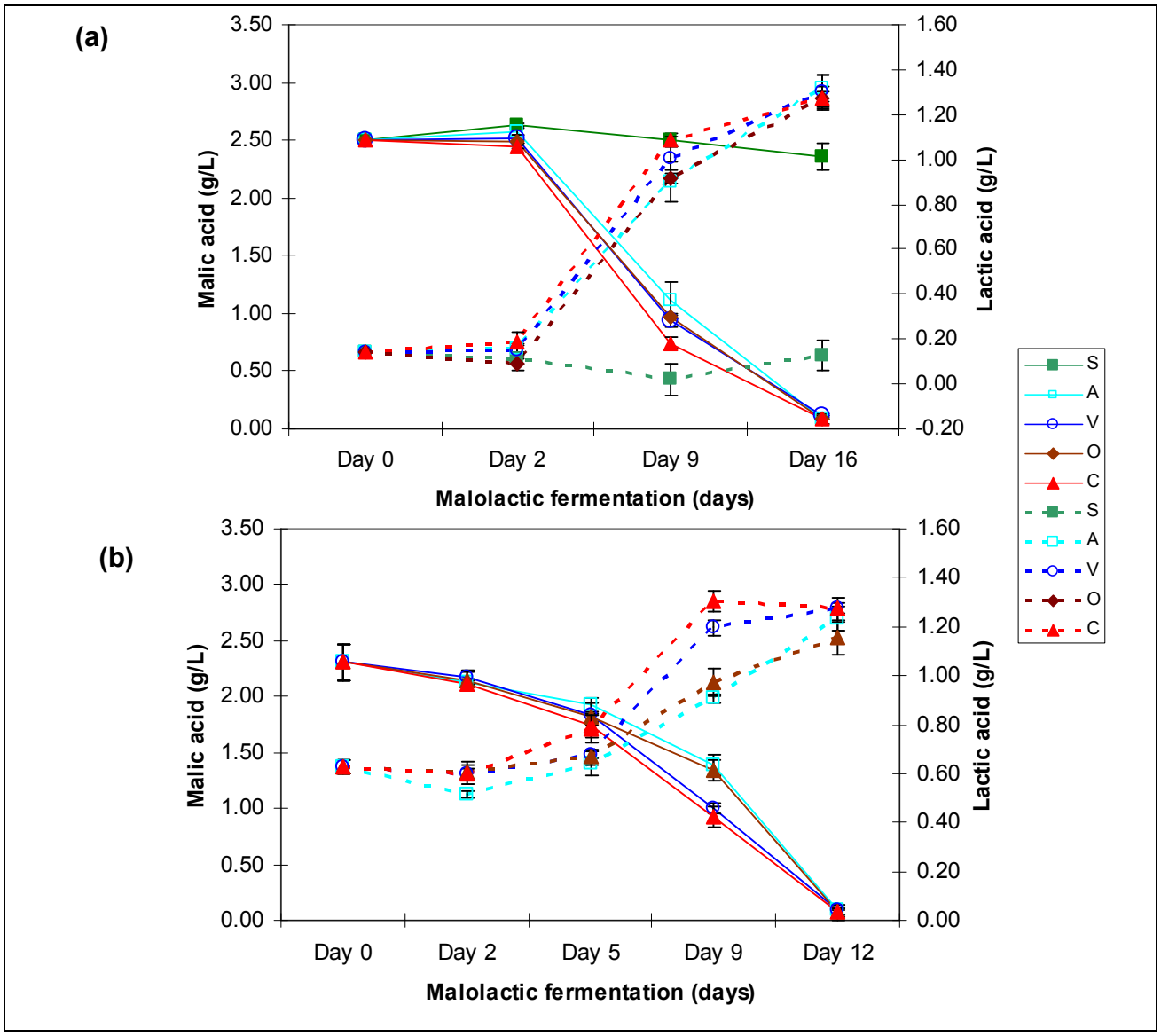


Figure 4.2 Changes in malic acid and lactic acid content during MLF in (a) 2008 Pinotage and (b) 2009 Pinotage with four *O. oeni* strains, namely Enoferm alpha (A); Lalvin VP41 (V); Viniflora oenos (O) and Viniflora CH16 (C) as well as including spontaneous MLF (S). Malic acid is indicated by a solid line and lactic acid by a dashed line.

All of the inoculated treatments completed MLF (<0.3 g/L malic acid) within 9-16 days in the respective wines tested. The MLF progression is shown in **Figure 4.2** for the Pinotage 2008 (**Figure 4.2a**), Pinotage 2009 (**Figure 4.2b**) and Shiraz 2008 (**Figure 4.3**) wines. In general, Viniflora oenos and Viniflora CH16 fermented slightly faster than the other two treatments. Although the Enoferm alpha

and VP41 strains initially fermented at a slower rate, both completed MLF successfully after 12-16 days. The average analytical parameters of the wines at the end of MLF are reported in **Table 4.3.** Microbial enumeration on the wines prior to MLF inoculation revealed no or very low lactic acid bacteria cell counts (~10^2 cfu/mL) present in the wine after alcoholic fermentation. After MLF inoculation, viable *O. oeni* cell counts were determined to be approximately 10^6 cfu/mL for the Viniflora oenos and Viniflora CH16 strains on day 2 while cell counts 10^5 cfu/mL were determined for Enoferm alpha and VP41 on day 2 of fermentation. In general, the fermentation rate was fast which could be a result of the higher pH of these wines.

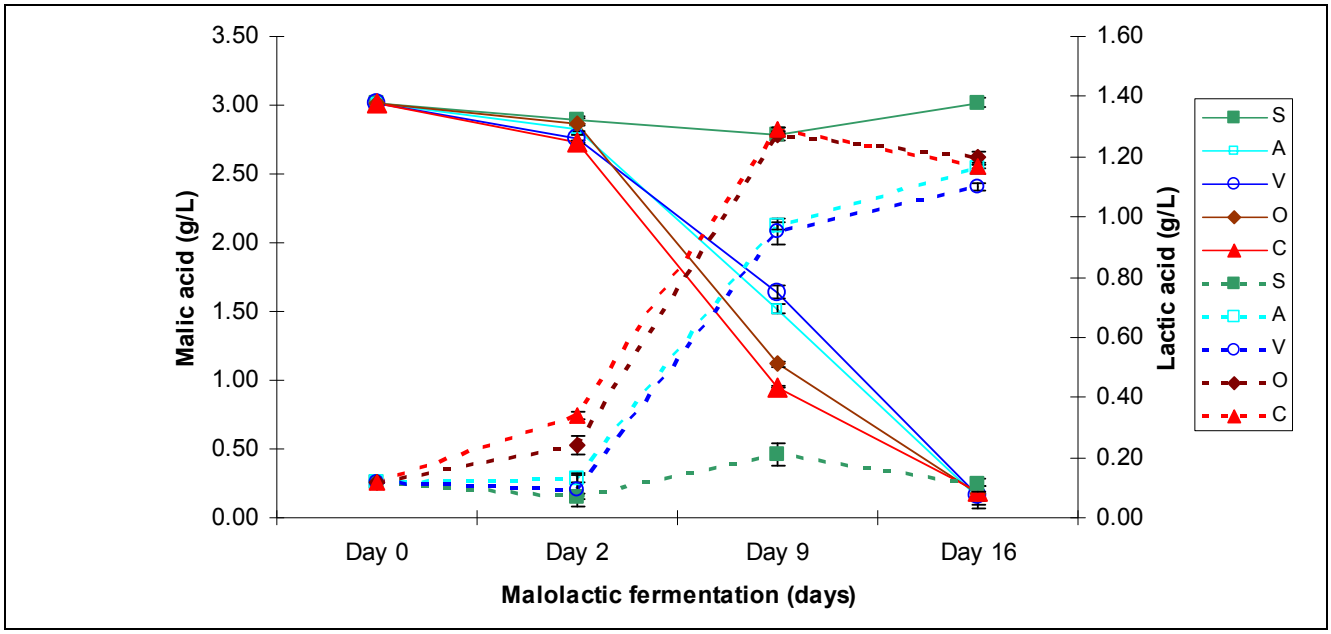


Figure 4.3 Changes in malic acid and lactic acid content during MLF in 2008 Shiraz wine wines with four *O. oeni* strains, namely Enoferm alpha (A); Lalvin VP41 (V); Viniflora oenos (O) and Viniflora CH16 (C) as well as including spontaneous MLF (S). Malic acid is indicated by a solid line and lactic acid by a dashed line.

4.3.2 ORGANIC ACID PROFILES

After completion of MLF in the Pinotage 2008 wine, no statistically significant (at 95% confidence level) differences were observed amongst the bacterial starter cultures for the malic acid (data not shown), lactic acid (data not shown) and citric acid (**Figure 4.4a**) profiles. Citric acid consumption is directly involved in the production of diacetyl via the citric acid pathway (Bartowsky & Henschke, 2004). No significant citric acid concentration differences were observed amongst the four commercial starter cultures, possibly pointing to the production of similar diacetyl concentrations, depending on the physiology of the bacteria (Bartowsky & Henschke, 2004). No changes in the tartaric acid concentrations were observed during MLF (data not shown). In terms of acetic acid formation (**Figure 4.4b**), the Enoferm alpha strain produced significantly more acetic acid compared to the control (in Pinotage 2008), while compared to the other bacterial strains showed no significant difference.

In the Pinotage 2009 wine and in the Shiraz 2008 wine, the Lalvin VP41 strain consumed significantly less citric acid during MLF in comparison to the other three strains (**Figure 4.4a**). This

could possibly suggest less metabolic activity towards citric acid and consequently lower diacetyl concentrations could be expected for this specific strain (Bartowsky & Henschke, 2004). No significant changes in the acetic acid (**Figure 4.4b**) and tartaric acid (data not shown) concentrations were observed in the Pinotage 2009 wine. After MLF in the Shiraz 2008 wine, significant differences were observed amongst the different bacterial starter cultures in terms of citric acid (**Figure 4.4a**) and acetic acid (**Figure 4.4b**). Viniflora CH16 produced the highest acetic acid concentration and the Enoferm alpha strain produced the lowest acetic acid concentration. The other bacteria, control wine and spontaneous ferment did not differ significantly from each other in terms of acetic acid concentration.

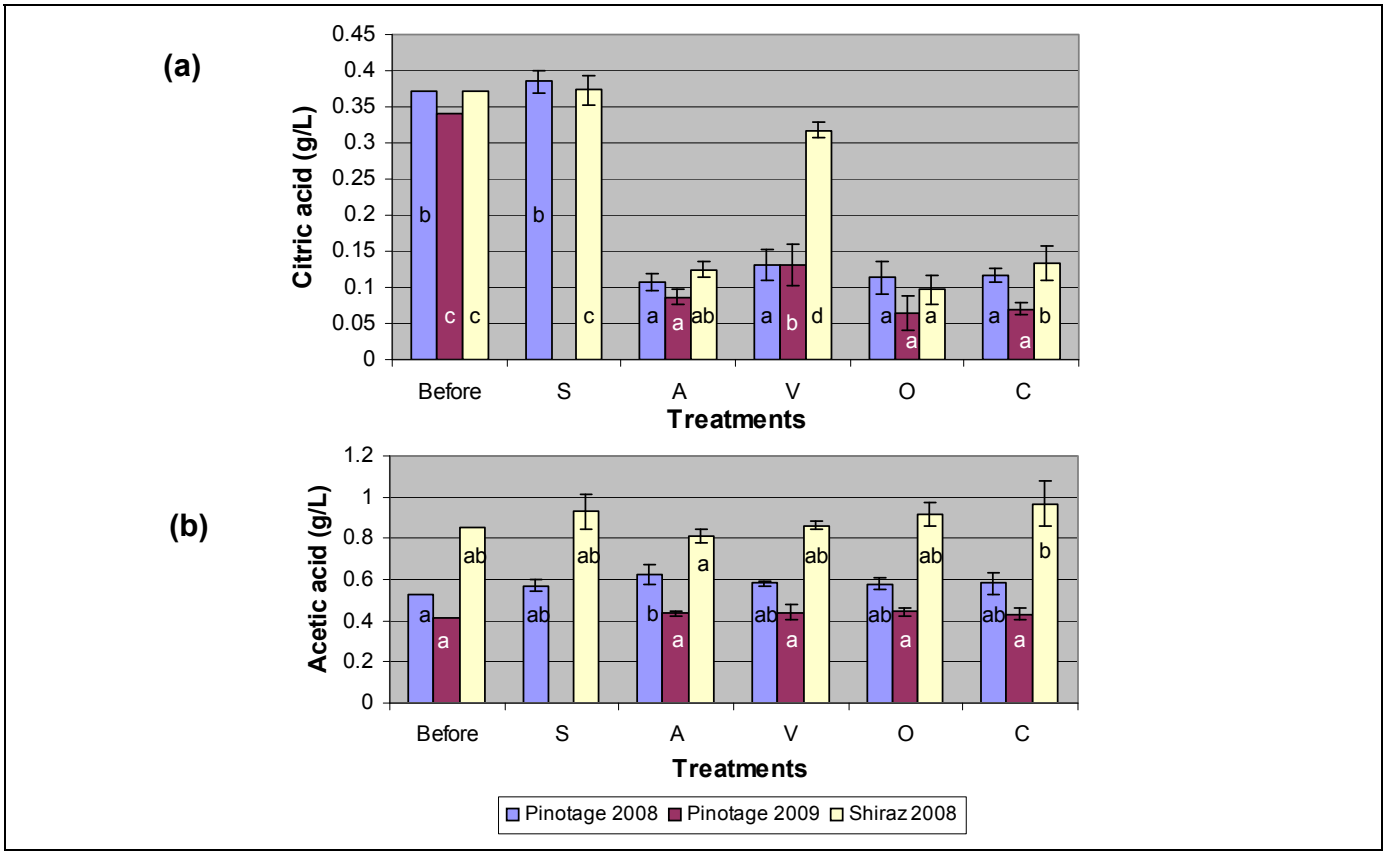


Figure 4.4 Changes observed in the (a) citric acid and (b) acetic acid concentrations as a result of MLF with different starter cultures (A, V, O, C) compared to a spontaneous (S) and control wine (no MLF: depicted by "before" text). Enoferm alpha: A; Lalvin VP41: V; Viniflora oenos: O; Viniflora CH16: C. Different alphabetical letters indicate significant differences.

4.3.3 HS-SPME METHOD OPTIMISATION AND VALIDATION

A method was developed for the quantification of diacetyl, acetoin, 2,3-pentanedione and a selection of aldehydes using a HS-SPME GC-MS method. The sample preparation procedure was optimized (using a PEG fiber) by evaluating different sample volumes (5 mL, 10 mL, 20 mL) (data not shown), sample dilutions (undiluted, 5x, 10x) (**Figure 4.5**) and sodium chloride (NaCl) concentrations (1 g, 2 g, 3 g) (**Figure 4.6**) to obtain optimal peak shape and sensitivity for all the analytes of interest. It was

found that although different combinations of dilution and salt addition provided variable sensitivity for different compounds, the combination of diluting the sample 10x together with the addition of 2g NaCl provided the best overall sensitivity and chromatographic performance by minimizing interfering matrix effects.

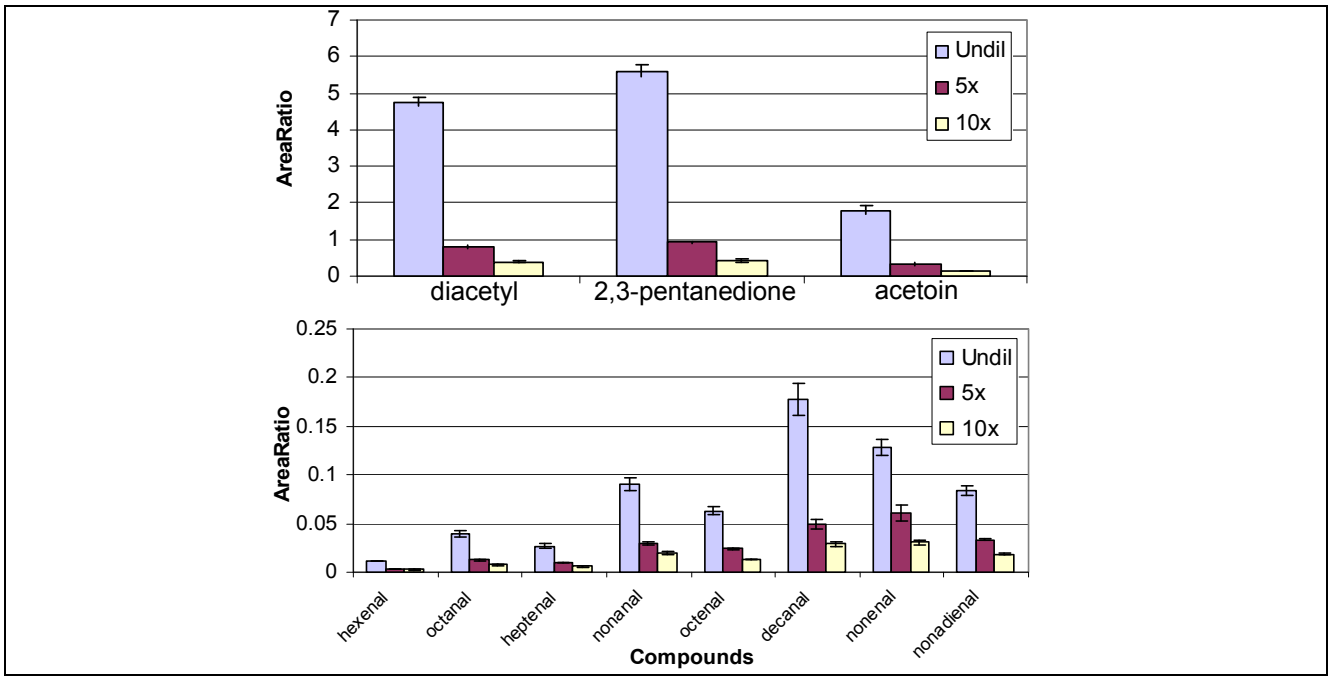


Figure 4.5 Different sample dilutions (undiluted, 5x, 10x) evaluated during sample preparation optimisation for the analysis of carbonyl compounds in wine.

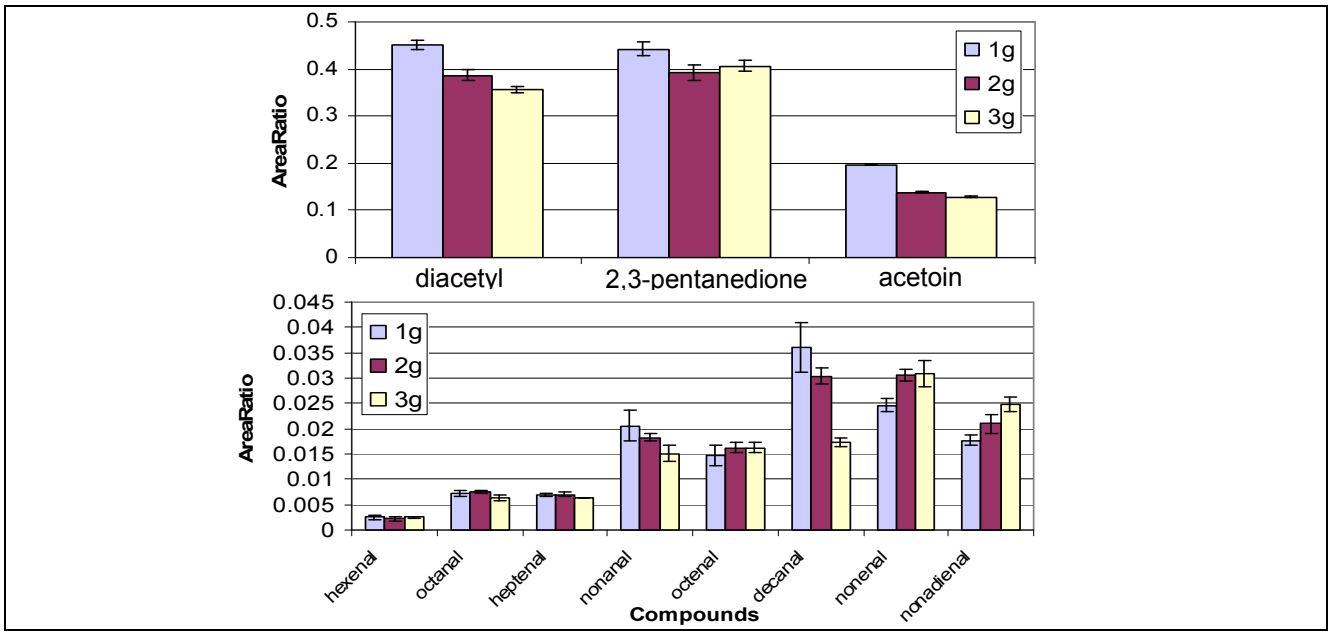


Figure 4.6 Different sodium chloride (NaCl) concentrations (1g, 2g, 3g) evaluated during sample preparation optimisation for the analysis of carbonyl compounds in wine.

In addition to the optimization of the sample preparation technique, a number of different fibers [carboxen/divinylbenzene/polydimethylsyloxane (CAR/DVB/PDMS), polyacrylate (PA), polydimethylsyloxane/divinylbenzene (PDMS/DVB) and carbowax/divinylbenzene (CW/DVB)], different extraction times (10 min, 30 min and 60 min), extraction temperatures (30˚C, 50˚C and 80˚C), injection modes (split, splitless and pulsed split), split ratios (1:2, 1:5 and 1:10), injection temperatures, desorption times and temperatures were evaluated during the method development stages (data not shown). The optimum conditions were selected according to selectivity in the case of the fiber selection and chromatographic peak shape and intensity obtained with regard to the other parameters tested. A chromatogram of the optimised method is shown in **Figure 4.7**. Method calibration and a selection of the validation data is shown in **Table 4.4**.

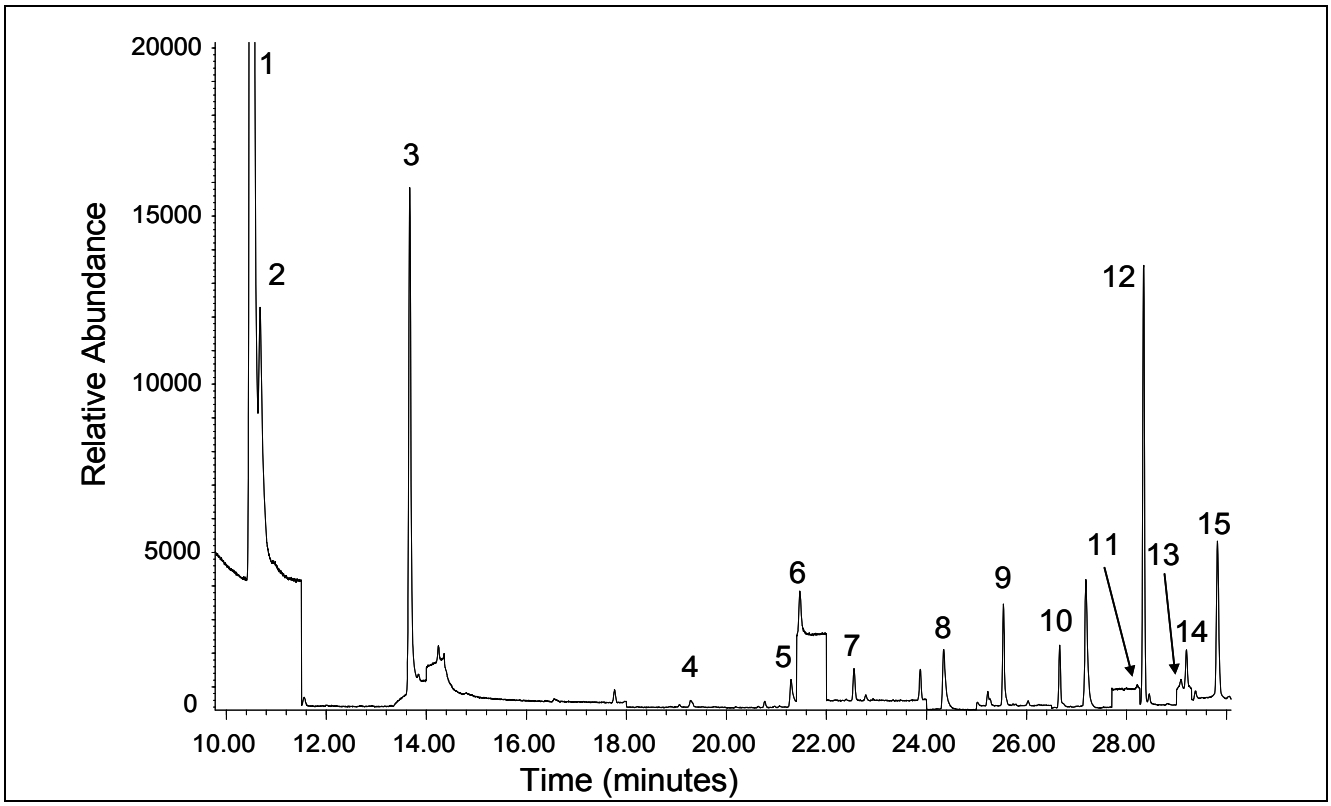


Figure 4.7 GC-MS chromatogram showing carbonyl compounds associated with metabolism of LAB in a synthetic wine that could be determined with a quantification limit of 5 µg/L for the compounds listed in Table 4.1. The chromatogram corresponds to a wine spiked with 15 µg/L analytes.

Table 4.4 Peak identification, slope of calibration graphs, linear dynamic ranges, coefficients (R^2), limits of detection (LODs) and reproducibility, R.S.D. (%)

Peak	Compounds	t_R (min)	m/z	Linearity[a]		Range	LOD	R.S.D.
				Slope	R^2	(µg/L)	(µg/L)	(%)[b]
1	2-pentanone*	10.5	43, 86					
2	diacetyl (2,3-butanedione)[c]	10.7	43, 86	0.0136	0.985	1-50	1	4.82
3	2,3-pentanedione [c]	13.6	57, 100	0.0204	0.998	1-50	0.1	6.03
4	*E*-2-hexenal	19.2	69, 83, 98	0.09	0.999	1-50	5	10.6
5	octanal	21.3	69, 84, 110	0.2684	0.994	1-50	0.1	3.36
6	acetoin (3-hydroxy-2-butanone) [c]	21.5	45, 88	0.0059	0.994	1-50	1	13.2
7	*E*-2-heptenal	22.5	55, 83, 112	0.2857	0.990	1-50	1.0	8.38
8	nonanal	24.3	82, 98, 114	0.5542	0.997	1-50	0.1	5.48
9	*E*-2-octenal	25.5	70, 83, 97	0.6607	0.995	1-50	1.0	10.8
10	decanal	27.2	82, 95, 112	0.8031	0.999	1-50	0.1	6.65
12	*E*-2-nonenal	28.3	83, 70, 96	1.5309	0.974	1-50	1.0	9.52
15	*trans-2, cis-6*-nonadienal	29.8	69, 70, 81	0.6419	0.992	1-50	0.1	8.35

[a]n = 5 aqueous/ethanol calibration solutions; [b]Mean of 6 determinations on 3 different days; [c]mg/L.

4.3.4 INFLUENCE OF MLF ON VOLATILE COMPOSITION

The concentrations of the 48 volatile compounds determined in the control wine (before MLF) and after MLF are listed in **Table 4.5** for the Pinotage 2008 and 2009 wines and in the **Table 4.6** for the Shiraz wine. Analysis of variance (ANOVA) showed significant differences ($p<0.05$) in the concentrations of 30 compounds in the Pinotage 2008 wine (**Table 4.5**), 28 compounds in the Pinotage 2009 wine (**Table 4.5**) and 34 compounds in the Shiraz wine (**Table 4.6**) as a consequence of MLF. This outcome is in agreement with previous studies on other red grape varieties (Pozo-Bayón *et al*., 2005; Maicas *et al*., 1999). Different alphabetical letters indicate significant differences ($p<0.05$) among the average values obtained for each of the LAB strains that performed MLF.

The odour thresholds (OTH) as reported in literature, aroma descriptor and supplier information of each compound are listed in **Table 4.1.** Odour activity values (OAVs), listed in **Tables 4.5** and **4.6**, were calculated by dividing the mean concentration value of a compound by its odour threshold value as reported in the literature (Guth, 1997). This indicates that these volatile compounds (with OAV>1) could potentially make an active contribution to the odour of the wine (Guth, 1997). Of the 48 volatile compounds quantified, 18 analytes had OAVs > 1 in the Pinotage 2008 wine (**Table 4.5**), 20 analytes had OAVs > 1 in the Pinotage 2009 wine (**Table 4.5**) and 23 analytes had OAVs > 1 in the Shiraz 2008 wine (**Table 4.6**). These compounds include ethyl hexanoate, ethyl butyrate, ethyl octanoate, ethyl-2-methylpropanoate, ethyl-2-methylbutyrate, ethyl isovalerate, ethyl acetate, isoamyl acetate, 2-phenylethanol, isoamyl alcohol, 3-ethoxy-1-propanol, acetic acid, butyric acid, isovaleric acid, hexanoic acid, octanoic acid, diacetyl (2,3-butanedione), 2,3-pentanedione, *E*-2-hexenal, *E*-2-octenal, *E*-2-nonenal and *trans*-2, *cis*-6-nonadienal. However, volatile compounds with high OAVs do not always have an effect on the aroma of wine and this information only shows the potential aroma contribution (Escudero *et al*., 2007) of individual analytes.

Table 4.5 Concentrations determined in this study for different bacterial starter cultures as well as odour activity values calculated for the Pinotage 2008 and 2009 wines. Averages are expressed as milligrams per liter (mg/L). Different alphabetic letters row-wise indicate significant differences ($p<0.05$).

	Pinotage 2008												Pinotage 2009									
	Control		Bacteria A		Bacteria V		Bacteria O		Bacteria C		Control		Bacteria A		Bacteria V		Bacteria O		Bacteria C	
Analyte	Average	OAV[a]	Average	OAV	Average	OAV	Average	OAV	Average	OAV	Average	OAV	Average	OAV	Average	OAV	Average	OAV	Average	OAV
Esters																				
ethyl decanoate	0.00 c	0.0	0.08 a	0.42	0.08 a	0.4	0.08 b	0.4	0.08 b	0.4	0.18 a	0.9	0.18 a	0.9	0.18 a	0.9	0.18 a	0.9	0.18 a	0.9
ethyl hexanoate	0.48 b	33.9	0.56 a	40.12	0.57 a	40.6	0.51 b	36.4	0.51 b	36.2	0.47 b	33.5	0.44 a	31.1	0.44 a	31.6	0.44 a	31.3	0.47 b	33.5
ethyl butyrate	0.48 a	24.0	0.48 a	24.18	0.49 a	24.4	0.49 a	24.5	0.49 a	24.3	0.11 a	5.6	0.11 a	5.5	0.11 a	5.6	0.12 ab	5.8	0.13 b	6.3
ethyl octanoate	0.18 d	36.7	0.29 a	58.69	0.30 a	59.5	0.22 c	43.2	0.21 c	41.3	0.25 a	49.7	0.25 a	49.7	0.25 a	49.7	0.25 a	49.2	0.28 a	55.2
ethyl lactate	0.00 e	0.0	33.05 a	0.21	29.55 b	0.2	22.35 c	0.1	19.93 d	0.1	13.73 b	0.1	36.70 a	0.2	40.27 c	0.3	37.37 a	0.2	36.54 a	0.2
ethyl propionate	0.000 b	0.0	0.355 ab	0.20	0.706 a	0.4	0.178 ab	0.1	0.819 a	0.5	0.00 b	0.0	0.28 ab	0.2	0.61 a	0.3	0.03 b	0.0	0.13 b	0.1
ethyl-2-methylpropanoate	0.000 b	0.0	0.191 b	12.71	0.099 b	6.6	0.121 b	8.0	0.697 a	46.4	0.19 a	12.8	0.36 a	23.7	0.47 a	31.4	0.30 a	19.8	0.30 a	19.8
ethyl-2-methylbutyrate	0.000 b	0.0	0.267 a	14.83	0.081 ab	4.5	0.041 ab	2.3	0.084 ab	4.7	1.09 a	60.5	0.13 b	7.2	0.05 b	2.6	0.04 b	2.5	0.06 b	3.3
ethyl isovalerate	0.407 a	135.6	0.392 a	130.55	0.406 a	135.5	0.420 a	139.9	0.437 a	145.7	0.38 a	125.2	0.29 a	97.4	0.37 a	124.4	0.40 a	131.9	0.43 a	141.7
ethyl-3-hydroxybutanoate	1.074 b	0.1	2.871 a	0.14	1.577 ab	0.1	1.758 ab	0.1	1.270 ab	0.1	3.72 a	0.2	1.19 b	0.1	1.01 b	0.1	1.22 b	0.1	1.94 b	0.1
ethyl phenylacetate	1.249 b	1.9	1.226 b	1.89	1.308 a	2.0	1.346 a	2.1	1.234 b	1.9	1.07 a	1.7	1.06 a	1.6	1.23 a	1.9	1.00 a	1.5	0.99 a	1.5
ethyl acetate	59.56 a	4.9	53.29 a	4.35	52.73 a	4.3	47.74 a	3.9	47.37 a	3.9	24.36 a	2.0	25.01 a	2.0	25.84 ab	2.1	27.72 b	2.3	27.93 b	2.3
isoamyl acetate	2.01 a	66.9	1.93 a	64.42	2.03 a	67.6	1.99 a	66.5	1.97 a	65.6	1.29 b	43.1	1.22 a	40.7	1.23 a	40.9	1.25 ab	41.6	1.29 b	43.0
hexyl acetate	0.166 a	0.1	0.120 b	0.08	0.126 ab	0.1	0.122 b	0.1	0.110 b	0.1	0.45 a	0.3	0.32 a	0.2	0.20 a	0.1	0.24 a	0.2	0.20 a	0.1
2-phenylethyl acetate	0.07 a	0.3	0.07 a	0.28	0.07 a	0.3	0.07 a	0.3	0.06 a	0.2	0.59 b	2.4	0.58 a	2.3	0.58 a	2.3	0.58 a	2.3	0.58 a	2.3
diethyl succinate	0.54 c	0.0	0.85 a	0.00	0.70 b	0.0	0.60 c	0.0	0.54 c	0.0	0.35 c	0.0	0.38 ab	0.0	0.38 ab	0.0	0.38 a	0.0	0.41 b	0.0
2-methyl-propyl acetate	0.622 a	0.4	0.614 a	0.38	0.661 a	0.4	0.677 a	0.4	0.659 a	0.4	0.59 a	0.4	0.52 a	0.3	0.53 a	0.3	0.60 a	0.4	0.64 a	0.4
Alcohols																				
hexanol	0.59 c	0.1	0.66 ab	0.08	0.67 a	0.1	0.59 c	0.1	0.56 c	0.1	1.50 a	0.2	1.52 ab	0.2	1.50 a	0.2	1.51 ab	0.2	1.54 b	0.2
butanol	1.81 ab	0.0	1.90 a	0.01	1.87 a	0.0	1.83 ab	0.0	1.68 b	0.0	0.96 a	0.0	0.97 a	0.0	0.97 a	0.0	1.00 a	0.0	0.99 a	0.0
methanol	29.02 ab	0.1	30.08 a	0.06	25.65 abc	0.1	22.36 bc	0.0	21.28 c	0.0	43.59 c	0.1	40.36 bc	0.1	36.89 abc	0.1	33.60 ab	0.1	31.35 a	0.1
2-phenylethanol	24.06 ab	1.7	25.14 a	1.80	24.93 a	1.8	23.07 b	1.6	21.38 c	1.5	32.20 a	2.3	32.29 a	2.3	31.72 a	2.3	31.57 a	2.3	32.02 a	2.3
propanol	79.80 a	0.3	80.12 a	0.26	76.32 ab	0.2	76.35 ab	0.2	69.37 b	0.2	43.93 a	0.1	42.86 a	0.1	44.27 ab	0.1	46.29 b	0.2	44.75 ab	0.1
isobutanol	24.34 ab	0.6	24.42 ab	0.61	23.72 ab	0.6	22.43 bc	0.6	20.90 c	0.5	24.92 ab	0.6	24.46 a	0.6	24.77 ab	0.6	25.69 b	0.6	25.02 ab	0.6
isoamyl alcohol	171.35 a	5.7	171.91 a	5.73	171.28 a	5.7	155.85 b	5.2	151.61 b	5.1	175.29 a	5.8	174.34 a	5.8	173.77 a	5.8	176.81 a	5.9	176.11 a	5.9
pentanol	0.21 a	0.0	0.17 ab	0.00	0.19 a	0.0	0.18 ab	0.0	0.13 ab	0.0	0.11 a	0.0	0.04 a	0.0	0.12 a	0.0	0.13 a	0.0	0.18 a	0.0
4-methyl-1-pentanol	0.02 a	0.0	0.02 a	0.02	0.01 a	0.0	0.01 a	0.0	0.02 a	0.0	0.15 a	0.1	0.01 b	0.0	0.05 b	0.0	0.04 b	0.0	0.05 b	0.0
3-methyl-1-pentanol	0.04 b	0.0	0.03 b	0.03	0.05 ab	0.0	0.08 a	0.1	0.06 ab	0.1	0.11 ab	0.1	0.06 b	0.1	0.09 ab	0.1	0.07 b	0.1	0.16 a	0.2
3-ethoxy-1-propanol	3.45 b	34.5	4.34 a	43.44	3.69 ab	36.9	3.84 ab	38.4	3.33 b	33.3	3.91 a	39.1	3.48 a	34.8	3.88 a	38.8	3.59 a	35.9	5.01 a	50.1
Acids and fatty acids																				
acetic acid	214.96 f	1.1	421.52 a	2.11	395.97 b	2.0	369.46 c	1.8	344.92 d	1.7	160.15 b	0.8	256.72 a	1.3	257.21 a	1.3	269.29 a	1.3	267.93 a	1.3
propionic acid	11.46 b	0.6	10.83 bc	0.54	10.18 bcd	0.5	9.83 cd	0.5	9.19 d	0.5	3.55 a	0.2	3.65 ab	0.2	3.89 ab	0.2	3.98 b	0.2	3.85 ab	0.2
isobutyric acid	0.82 bc	0.4	0.87 ab	0.38	0.89 a	0.4	0.79 c	0.3	0.76 c	0.3	1.59 a	0.7	1.77 a	0.8	1.77 ab	0.8	2.17 c	0.9	1.95 b	0.8
butyric acid	1.09 ab	6.3	1.16 a	6.71	1.15 ab	6.6	1.08 bc	6.2	1.00 c	5.8	0.68 a	3.9	0.71 b	4.1	0.70 ab	4.0	0.68 a	3.9	0.70 ab	4.0
isovaleric acid	0.71 bc	21.5	0.77 ab	23.35	0.78 ab	23.7	0.71 bc	21.6	0.65 c	19.6	1.83 a	55.6	2.00 b	60.7	1.77 a	53.7	1.79 a	54.2	1.79 a	54.1
valeric acid	0.42 ab		0.43 ab		0.45 a		0.43 ab		0.43 ab		0.72 a		0.72 a		0.72 a		0.72 a		0.72 a	
hexanoic acid	1.52 bc	3.6	1.68 a	3.99	1.68 a	4.0	1.60 ab	3.8	1.47 c	3.5	2.44 a	5.8	2.51 ab	6.0	2.45 ab	5.8	2.47 ab	5.9	2.54 b	6.0
octanoic acid	0.95 d	1.9	1.57 a	3.14	1.54 a	3.1	1.44 b	2.9	1.37 b	2.7	3.81 c	7.6	4.05 ab	8.1	3.95 a	7.9	3.99 ab	8.0	4.11 b	8.2
decanoic acid	0.23 d	0.2	0.41 a	0.41	0.40 ab	0.4	0.40 a	0.4	0.37 b	0.4	0.97 a	1.0	0.95 a	1.0	0.94 a	0.9	0.94 a	0.9	0.97 a	1.0
Carbonyl compounds																				
diacetyl (2,3-butanedione)	7.45 a	74.5	7.08 a	70.83	6.55 a	65.5	7.97 a	79.7	6.71 a	67.1	5.19 b	51.9	14.45 a	144.5	7.19 b	71.9	14.99 a	149.9	13.35 a	133.5
acetoin (3-hydroxy-2-butanone)	4.51 ab	0.0	4.35 ab	0.03	3.69 a	0.0	4.88 b	0.0	4.20 ab	0.0	3.08 a	0.0	12.01 b	0.1	2.94 a	0.0	5.81 a	0.0	4.82 a	0.0
2,3-pentanedione	2.51 e	2.8	1.29 c	1.43	1.23 bc	1.4	1.14 a	1.3	1.15 ab	1.3	1.40 b	1.6	1.29 a	1.4	1.33 ab	1.5	1.28 a	1.4	1.31 a	1.5
E-2-hexenal	0.002 a	0.2	0.0081 a	0.81	0.0 a	0.0	0.002 a	0.2	0.001 a	0.1	0.0015 a	0.1	0.0005 a	0.1	0.0009 a	0.1	0.0024 a	0.2	nd	
E-2-heptenal	nd		nd		nd		nd		nd		nd		nd		nd		nd		nd	
octanal	nd		nd		nd		nd		nd		nd		nd		nd		nd		nd	
E-2-octenal	nd		0.0003 a	3.33	nd		nd		nd		0.0009 ab	8.6	nd		0.0014 b	14.2	nd		nd	
nonanal	nd		0.0009 b	0.88	nd		nd		nd		nd		nd		nd		nd		nd	
E-2-nonenal	nd		nd		nd		nd		nd		nd		nd		nd		nd		nd	
decanal	nd		0.0002 a	2.27	nd		nd		nd		nd		0.0003 a	3.5	0.0001 a	1.4	nd		nd	
trans-2, *cis*-6-nonadienal	0.002 a	237.8	0.0018 a	181.98	0.0013 a	132.9	0.002 a	243.2	0.002 a	199.8	0.0017 a	172.8	0.0006 a	57.6	0.0012 a	121.9	0.0007 a	73.5	0.0011 a	111.8

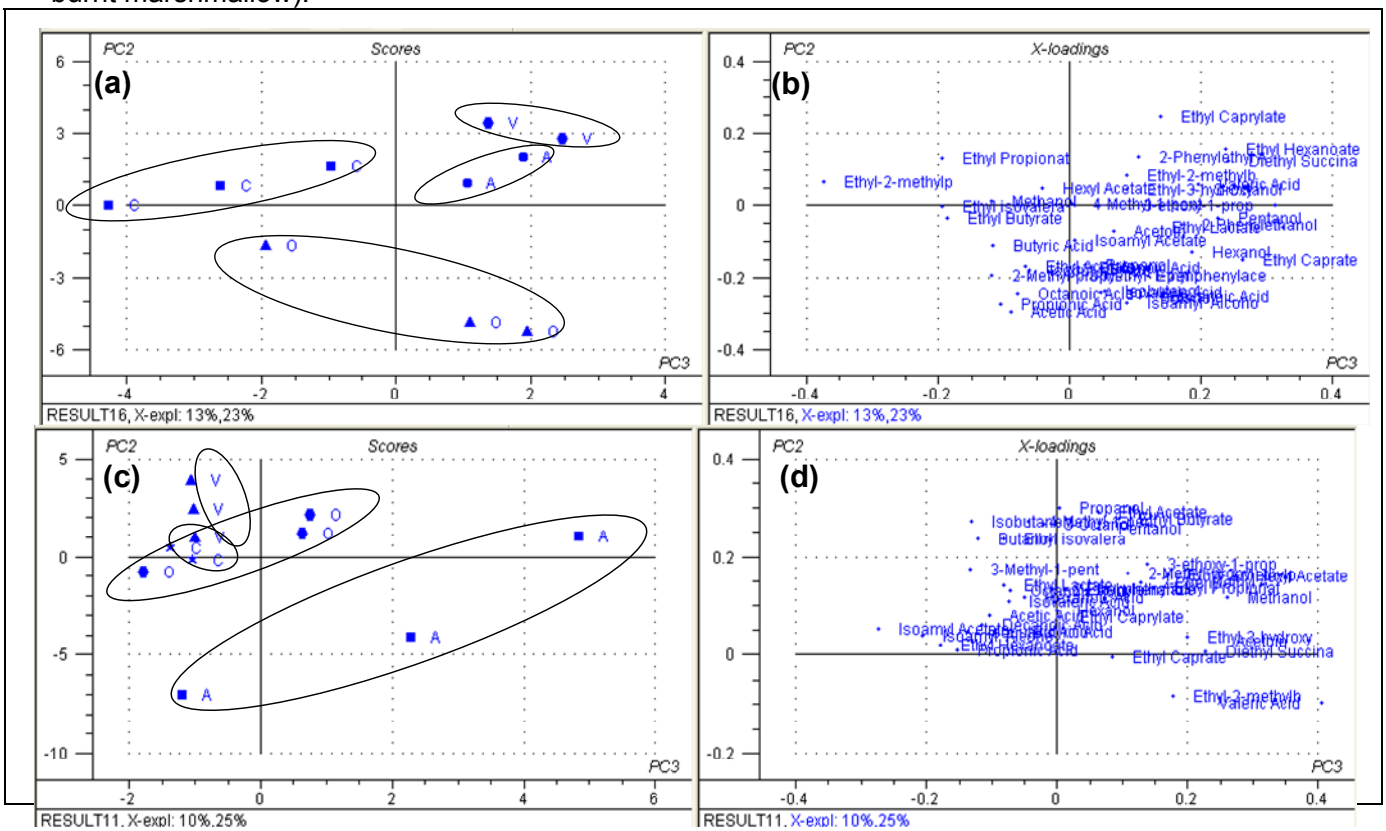
[a]OAV, odour activity value

Table 4.6 Volatile compounds determined in this study as well as odour activity values for calculated for the respective Shiraz wines. Averages are expressed as milligrams per litre (mg/L). Different alphabetic letters row-wise indicate significant differences ($p<0.05$).

	Control			Bacteria A			Bacteria V			Bacteria O			Bacteria C		
Analyte	**Average**		**OAV[a]**	**Average**		**OAV**	**Average**		**OAV**	**Average**		**OAV**	**Average**		**OAV**
Esters															
ethyl decanoate	0.038	a	0.2	0.028	a	0.1	0.032	a	0.2	0.025	a	0.1	0.040	a	0.2
ethyl hexanoate	0.402	c	28.7	0.454	ab	32.4	0.444	b	31.7	0.461	a	32.9	0.447	b	32.0
ethyl butyrate	0.443	b	22.1	0.469	a	23.4	0.464	ab	23.2	0.478	a	23.9	0.464	ab	23.2
ethyl octanoate	0.127	d	25.3	0.171	ab	34.1	0.163	bc	32.5	0.177	a	35.3	0.166	b	33.1
ethyl lactate	0.000	e	0.0	25.893	b	0.2	16.372	d	0.1	31.529	a	0.2	24.490	c	0.2
ethyl propionate	0.202	a	0.1	1.184	a	0.7	1.155	a	0.6	0.384	a	0.2	1.098	a	0.6
ethyl-2-methylpropanoate	0.305	b	20.3	0.811	a	54.1	0.413	ab	27.5	0.430	ab	28.7	0.261	b	17.4
ethyl-2-methylbutyrate	0.013	a	0.7	0.158	a	8.8	0.040	a	2.2	0.095	a	5.3	0.036	a	2.0
ethyl isovalerate	0.375	b	125.2	0.328	b	109.4	0.384	b	127.9	0.345	b	115.1	0.337	b	112.4
ethyl-3-hydroxybutanoate	1.971	ab	0.1	1.738	b	0.1	1.478	b	0.1	1.582	b	0.1	1.447	b	0.1
ethyl phenylacetate	2.271	bc	3.5	2.235	c	3.4	2.444	a	3.8	2.410	ab	3.7	2.420	ab	3.7
ethyl acetate	43.957	a	3.6	46.475	a	3.8	43.417	a	3.5	46.859	a	3.8	46.667	a	3.8
isoamyl acetate	1.486	a	49.5	1.476	ab	49.2	1.436	b	47.9	1.507	a	50.2	1.506	a	50.2
hexyl acetate	0.108	ab	0.1	0.109	ab	0.1	0.124	a	0.1	0.094	b	0.1	0.099	ab	0.1
2-phenylethyl acetate	0.028	a	0.1	0.030	a	0.1	0.033	a	0.1	0.030	a	0.1	0.027	a	0.1
diethyl succinate	0.334	b	0.0	0.519	a	0.0	0.490	a	0.0	0.493	a	0.0	0.463	a	0.0
2-methyl-propyl acetate	0.654	b	0.4	0.515	d	0.3	0.584	c	0.4	0.548	cd	0.3	0.561	cd	0.4
Alcohols															
hexanol	1.215	c	0.2	1.406	a	0.2	1.365	b	0.2	1.441	a	0.2	1.412	a	0.2
butanol	3.532	c	0.0	3.868	ab	0.0	3.824	ab	0.0	3.926	a	0.0	3.873	ab	0.0
methanol	114.136	b	0.2	128.928	a	0.3	123.490	ab	0.2	128.292	a	0.3	130.038	a	0.3
2-phenylethanol	36.787	b	2.6	40.939	a	2.9	39.661	ab	2.8	40.906	a	2.9	38.761	ab	2.8
propanol	68.792	b	0.2	76.253	a	0.2	74.049	a	0.2	74.242	a	0.2	75.362	a	0.2
isobutanol	25.897	b	0.6	28.209	a	0.7	27.890	a	0.7	28.104	a	0.7	28.291	a	0.7
isoamyl alcohol	225.917	b	7.5	243.408	a	8.1	243.053	a	8.1	246.364	a	8.2	245.353	a	8.2
pentanol	0.166	a	0.0	0.139	a	0.0	0.172	a	0.0	0.173	a	0.0	0.167	a	0.0
4-methyl-1-pentanol	0.024	ab	0.0	0.000	c	0.0	0.034	a	0.0	0.006	c	0.0	0.000	c	0.0
3-methyl-1-pentanol	0.113	b	0.1	0.112	b	0.1	0.134	a	0.1	0.129	ab	0.1	0.137	a	0.1
3-ethoxy-1-propanol	3.004	b	30.0	3.296	ab	33.0	3.936	a	39.4	3.808	ab	38.1	3.208	ab	32.1
Acids and fatty acids															
acetic acid	186.742	e	0.9	309.976	c	1.5	238.173	d	1.2	389.174	a	1.9	348.797	b	1.7
propionic acid	6.102	c	0.3	11.983	a	0.6	11.244	a	0.6	11.982	a	0.6	11.292	a	0.6
isobutyric acid	0.772	c	0.3	0.879	ab	0.4	0.866	ab	0.4	0.878	ab	0.4	0.851	b	0.4
butyric acid	0.683	b	3.9	0.741	a	4.3	0.760	a	4.4	0.757	a	4.4	0.735	a	4.2
isovaleric acid	1.022	c	31.0	1.220	ab	37.0	1.195	b	36.2	1.291	a	39.1	1.212	ab	36.7
valeric acid	0.607	a		0.436	cd		0.480	bc		0.475	bc		0.398	d	
hexanoic acid	0.987	a	2.3	0.997	a	2.4	0.974	a	2.3	1.015	a	2.4	0.962	a	2.3
octanoic acid	1.721	a	3.4	1.043	a	2.1	1.034	a	2.1	1.032	a	2.1	1.013	a	2.0
decanoic acid	8.189	a	8.2	0.492	b	0.5	0.491	b	0.5	0.423	b	0.4	0.422	b	0.4
Carbonyl compounds															
diacetyl (2,3-butanedione)	7.62	a	76.2	21.34	d	213.4	10.72	b	107.2	8.10	a	81.0	12.42	c	124.2
acetoin (3-hydroxy-2-butanone)	3.32	a	0.0	10.80	cd	0.1	4.53	b	0.0	10.08	c	0.1	11.21	d	0.1
2,3-pentanedione	2.32	b	2.6	1.62	c	1.8	1.88	d	2.1	1.28	a	1.4	1.34	a	1.5
E-2-hexenal	0.343	b	34.3	0.0005	a	0.0	0.0000	a	0.0	0.083	a	8.3	0.107	a	10.7
E-2-heptenal	nd		nd	nd			nd		nd	nd			nd		
octanal	nd		nd	nd			nd		nd	nd			nd		
E-2-octenal	nd	a	nd	0.0007	a	6.7	0.0003	a	2.5	0.0007	a	7.0	0.00002	a	
nonanal	nd		nd	nd			nd			nd			nd		
E-2-nonenal	nd	a	nd	nd	a		0.0004	a	5.9	nd			nd	a	
decanal	nd	a	nd	nd	a		nd	a		0.0001	a	0.6	0.00008	a	
trans-2, *cis*-6-nonadienal	0.003	a	327.2	0.0041	a	410.0	0.0029	a	293.1	0.003	a	343.4	0.003	a	288.9

[a]OAV, odour activity value.

The PCA scores plot and corresponding loadings plot in **Figure 4.8a** and **Figure 4.8b** provide an overview of the volatile profiles associated with the metabolic activity of the four starter cultures obtained during MLF in the Pinotage 2008 wine in terms of esters, alcohols and acids. Separation along the first principal component (PC1) appears to be driven by the association of the Viniflora CH16 strain (positioned to the left of the scores plot in **Figure 4.8a**) with a selection of ethyl esters, namely ethyl-2-methylpropanoate (fruity), ethyl propionate (fruity), ethyl isovalerate (fruity, anise) and ethyl butyrate (fruity, apple) (**Figure 4.8b**). The Enoferm alpha and Lalvin VP41 strains, as well as the Viniflora oenos strain to a certain extend, are positioned towards the right-hand side of the scores plot (**Figure 4.8a**) along PC1 and associated with ethyl hexanoate (fruity, anise), ethyl lactate (butter, cream, fruit), 2-phenylethanol (honey, rose), 3-ethoxy-1-propanol (fruity) and diethyl succinate (berry). Along the second PC, Viniflora oenos, positioned to the bottom of the scores plot (**Figure 4.8a**), is separated from the other bacteria in terms of its association with acetic acid (vinegar), propionic acid (pungent, rancid, sweat), octanoic acid (sweat, cheese), isovaleric acid (cheese), hexanoic acid (sweat), decanoic acid (rancid, fat), butyric acid (cheese), isobutyric acid (rancid, butter, cheese), isobutanol (wine, solvent), propanol (pungent, harsh), butanol (fusel, spirituous), hexanol (green, grass, resin), 3-methyl-1-pentanol, isoamyl alcohol (fusel, whisky, malt), ethyl acetate (fruit, nail polish), ethyl caprate/decanoate (grape, floral, soap), isoamyl acetate (banana, pear), ethylphenyl acetate (rose, floral) and 2-methyl-propyl acetate (solvent). The VP41, CH16 and Enoferm alpha strains are positioned to the top part of the scores plot (**Figure 4.8a**) along the second PC and associated with ethyl caprylate/octanoate (fruit, fat), 2-phenylethyl acetate (roses), ethyl-2-methylbutyrate (fruity, apple), diethyl succinate (berry) and ethyl-3-hydroxybutanoate (strawberry, burnt marshmallow).



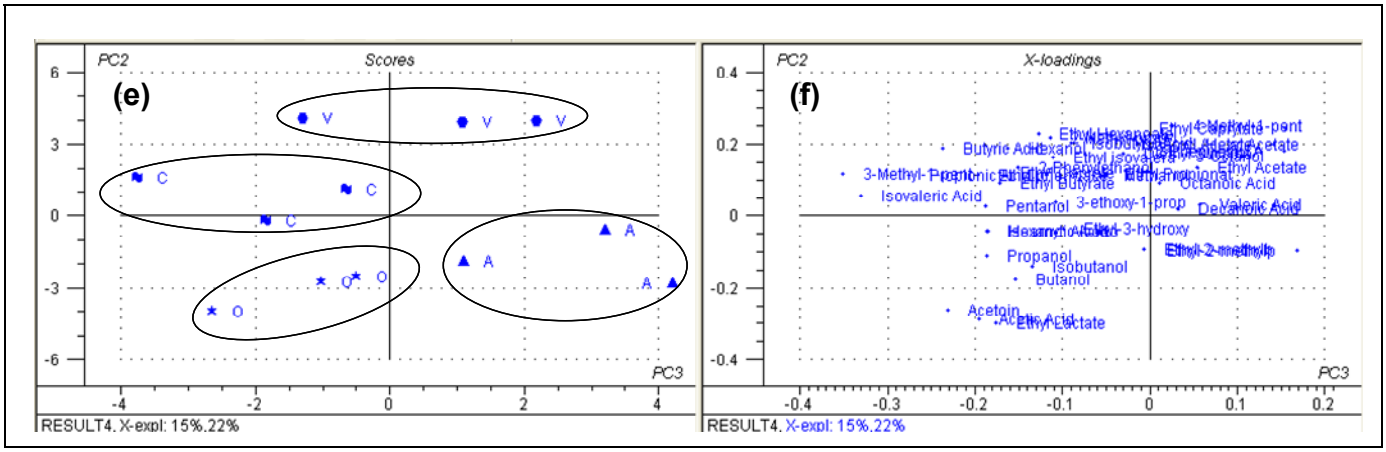


Figure 4.8 PCA provides a visual overview of changes in the esters, higher alcohol and acid composition imparted by bacterial metabolism during MLF. (a) Pinotage 2008 wine scores plot and (b) the corresponding loadings plot; (c) Pinotage 2009 wine scores plot and (d) the corresponding loadings plot; (e) Shiraz 2008 scores plot and (f) corresponding loadings plot. The different starter cultures including Enoferm alpha (indicated by A); Lalvin VP41 (indicated by V); Viniflora oenos (indicated by O) and Viniflora CH16 (indicated by C) are compared.

PCA results (**Figure 4.8c and 4.8d**) for the volatile profiles obtained during the Pinotage 2009 wine illustrate less prominent strain discrimination in terms of the esters, higher alcohols and acid profiles associated with the four different bacteria. The Enoferm alpha strain is positioned more to the right-hand side of the scores plot (**Figure 4.8c**) along PC3 as well as to the bottom of the plot along PC2. This position appears to be driven by association with acetoin (butter, cream), diethyl succinate (berry), ethyl propionate (fruity), ethyl caprate/decanoate (grape, floral, soap), valeric acid and ethyl-2-methylbutyrate (fruity, apple) (**Figure 4.8d**). The remaining three strains are positioned more to the left of the scores plot (**Figure 4.8c**) along PC3 and slightly to the top part of the plot along the second PC. These strains are associated with ethyl butyrate (fruity, apple), 4-methyl-1-pentanol, ethyl hexanoate (fruity, anise), ethyl caprylate/octanoate (fruit, fat), ethyl-3-hydroxybutyrate (strawberry, burnt marshmallow), hexanol (green, grass), acetic acid (vinegar), isoamyl alcohol (fusel, whisky), isoamyl acetate (banana, pear), octanoic acid (sweat, cheese), hexanoic acid (sweat), decanoic acid (rancid, fat), hexyl acetate (sweet, perfume) and 2-methyl-propyl acetate (solvent) with concentrations dependent on the strain used.

PCA results for the Shiraz 2008 wine shows clear differentiation amongst the four different bacterial starter cultures (**Figure 4.8e**). Separation along the second PC appears to discriminate VP41 and Viniflora CH16 from Viniflora oenos and Enoferm alpha. Along PC3, the Viniflora CH16 and oenos strains are positioned towards the left of the scores plot, while the VP41 and Enoferm alpha strains are positioned more to the right-hand side of the scores plot (**Figure 4.8e**). The corresponding loadings plot (**Figure 4.8f**) represents the volatile profiles associated with the respective bacteria. The Viniflora oenos strain is positioned to the bottom of the scores plot (**Figure 4.8e**) and associated with higher concentrations of acetoin, acetic acid, ethyl lactate, butanol, isobutanol, propanol, ethyl-3-hydroxybutanoate, ethyl-2-methylbutyrate, ethyl-2-methylpropanoate, hexanoic aicd and isoamyl alcohol. The VP41 strain is separated from Viniflora oenos along PC2 as a result of its association with a selection of esters, higher alcohols and fatty acids including ethyl caprylate/, ethyl hexanoate, ethyl isovalerate, 2-methyl-propyl acetate, diethyl succinate hexyl acetate isoamyl acetate,

2-phenylethyl acetate 2-phenylethanol, 4-methyl-1-pentanol, hexanol, 3-ethoxy-1-propanol, isobutyric acid, decanoic acid and octanoic acid. Enoferm alpha is positioned towards the right-hand side of the scores plot along PC3 (**Figure 4.8e**) and associated with decanoic acid, 2-phenylethyl acetate, hexyl acetate, isoamyl acetate and ethyl acetate. This strain is positioned to the bottom half of the score plot as a result of increased concentrations of ethyl-2-methylbutyrate, ethyl-2-methylpropanoate, ethyl-3-hydroxybutanoate and ethyl lactate produced during MLF. The CH16 strain was positioned to the left of the scores plot (**Figure 4.8e**) as a result of increased amounts of isovaleric acid, propionic acid, butyric acid, hexanoic acid, octanoic acid, acetic acid, 3-methyl-1-pentanol, hexanol, acetoin, ethyl lactate, ethyl hexanoate, ethyl caprate/, diethyl succinate and 2-methyl-propylacetate.

General observations in terms of the changes within the different chemical groups including esters, higher alcohols, volatile fatty acids and carbonyl compounds will be discussed in the following sections.

4.3.4.1 Esters

Changes observed in ester concentrations after the completion of MLF are illustrated in **Figure 4.9**. Synthesis and hydrolysis of esters during MLF were evident as the results indicate. Ethyl lactate, diethyl succinate, ethyl octanoate, ethyl-2-methylpropanoate and ethyl propionate concentrations were increased during MLF in comparison to the control wine, independent of the cultivar or bacterial strain evaluated (**Figure 4.9a and 4.9b**). For interpretation of the graphs, ethyl lactate is excluded due to its much higher concentration in comparison to the other esters.

In the Pinotage wines, the Viniflora CH16 strain produced consistently lower concentrations of ethyl lactate (**Table 4.5**) while the Enoferm alpha strain appears to produce consistently higher concentrations of diethyl succinate, independently of the cultivar tested (**Table 4.5 and 4.6**). Ethyl lactate and diethyl succinate are the most important esters typically associated with MLF (Maicas *et al*., 1999; Herjavec *et al*., 2001; Ugliano & Moio, 2005). The increased concentrations are the result of succinic acid and lactic acid produced through *O. oeni* metabolism followed by the consequent esterification of succinic acid and lactic acid respectively with ethanol present in the wine (Maicas *et al*., 1999). Although the increase in ethyl lactate concentration was quantitatively the largest, this compound was far below its aroma threshold and is therefore not necessarily contributing to wine aroma. Ethyl propionate was consistently produced at higher concentrations by the Lalvin VP41 strain and at lower concentrations by the Viniflora oenos in the Pinotage and Shiraz wines.

Changes in terms of ethyl hexanoate, ethyl decanoate and ethyl butyrate concentrations were dependant on both cultivar and bacterial strain used during MLF, however the Viniflora oenos strain showed a tendency to produce higher concentrations ethyl butyrate in general. Similarly, ethyl-2-methylbutyrate and ethyl-3-hydroxybutyrate were either increased or decreased with Enoferm alpha producing higher levels of ethyl-2-methylbutryate (fruity, apple) across the wines tested and increased levels of ethyl-3-hydroxybutyrate (strawberry or burnt marshmallow) in the Pinotage 2008 wine. After MLF, ethyl-2-methylbutyrate had an OAV>1 in all wines, illustrating the potential contribution to the fruity wine aroma. Ethyl isovalerate (ethyl-3-methylbutyrate) concentrations were decreased in the Shiraz wine while a slight increase was observed for VP41. However, in the Pinotage wines a characteristic behaviour was observed with the Viniflora oenos and Viniflora CH16 strains increasing

the ethyl isovalerate concentrations while Enoferm alpha showed a decrease and VP41 did not affect the concentration of this compound during both vintages.

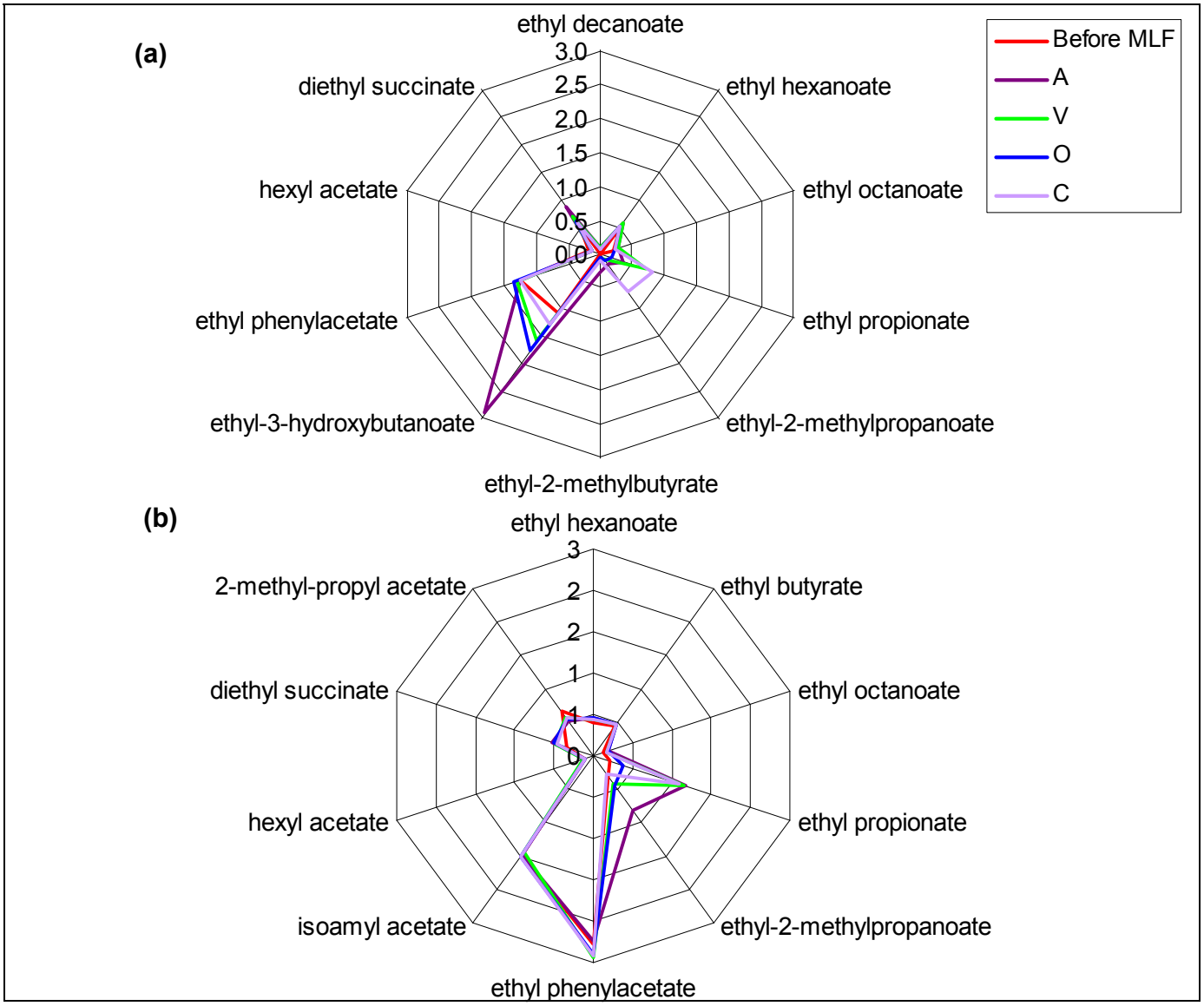


Figure 4.9 Changes in the esters profiles associated with MLF by different bacterial starter cultures in (a) Pinotage 2008 and (b) Shiraz 2008 wines.

The concentrations of hexyl acetate, isoamyl acetate, 2-phenylethyl acetate and ethyl acetate were generally decreased or remained unchanged, depending on the strain used or the cultivar tested. Ethyl-2-phenyl acetate and 2-methylphenyl acetate concentrations were increased or decreased depending on the cultivar tested, however a general trend for the Enoferm alpha strain to produce lower concentrations was observed. Contradictory to previous results (Ugliano & Moio, 2005; Maicas *et al*., 1999; Laurent *et al.*, 1994), the concentration of the powerful odourant isoamyl acetate, characterised by banana notes, was generally decreased following MLF in all three data sets, with the

exception of a strain specific increase observed in the Pinotage 2009 wine. The isoamyl acetate concentrations found in this study was far above its aroma threshold (0.03 mg/L; Guth, 1997) and could therefore potentially contribute to wine aroma. 2-Phenylethyl acetate was also decreased or not affected throughout, however the final concentration of this compound was well below its odour threshold (0.25 mg/L; Guth, 1997). Pozo-Bayón *et al*. (2005) also reported no differences in hexyl acetate and 2-phenylethyl acetate concentrations as a result of MLF.

Ethyl hexanoate, ethyl butyrate, ethyl octanoate, ethyl-2-methylpropanoate, ethyl-2-methylbutyrate, ethyl isovalerate, ethyl acetate and isoamyl acetate were all present at OAVs > 1 and could therefore contribute to the fruity aroma of the wines. The total ester production was increased by the bacterial starter cultures and it appears that two of the cultures (bacteria A and V) produced higher ester concentrations than the other two bacteria (bacteria O and C). The total amounts of esters found in these wines after MLF suggest their beneficial contribution to the wines' final aroma.

4.3.4.2 Higher alcohols

Increments in the concentrations of the majority of higher alcohols were observed in comparison with control wines where MLF was suppressed (**Table 4.5** and **4.6**). Higher alcohols are synthesized by yeasts through the degradation of amino acids and considered to impact on the aroma and flavour of wine (Swiegers *et al*., 2005). Isoamyl alcohol, isobutanol, 2-phenylethanol, propanol, butanol, hexanol, 3-methyl-1-pentanol and 3-ethoxy-1-propanol concentrations were significantly increased by MLF and showed a characteristic result depending on the strain used to perform MLF. For isoamyl alcohol and isobutanol, the contribution of bacterial strain selection appears to be more profound in the Pinotage wines compared to the Shiraz wine. Maicas *et al*. (1999) also previously found the production of isobutanol, propanol, butanol and isoamyl alcohol to be strain dependant. In contrast, other studies found no change in the isoamyl alcohol, 2-phenylethanol, isobutanol and propanol concentration after MLF (Herjavec *et al*., 2001; Laurent *et al*., 1994). Other authors (Jeromel *et al*., 2008; de Revel *et al*., 1999) found that MLF had no significant effect on the higher alcohol concentration of the wine, except for significant increases in isoamyl alcohol (de Revel *et al*., 1999) and isobutanol and 2-phenylethanol respectively (Jeromel *et al*., 2008). The observed increase in hexanol and 3-methyl-1-pentanol concentrations as a result of MLF is in agreement with a previous report by Ugliano & Moio (2005). Pozo-Bayón *et al*. (2005) also found MLF to increase the levels of higher alcohols, however none of these increases were significant.

In general, the concentrations of these alcohols, depending on the specific alcohol, were in agreement with the levels found in young red wines (Francis & Newton, 2005). Interesting to note that only 2-phenylethanol, isoamyl alcohol and 3-ethoxy-1-propanol, characterised by honey, spice, rose, lilac, fusel, whisky, malt and fruity aroma notes, had OAVs > 1 after the completion of MLF. This indicates the potential of higher alcohols to contribute to the complexity and fruity aromas in wine, however at higher concentrations (above 400 mg/L) these compounds are detrimental to wine aroma due to the harsh chemical-like aromas (Swiegers *et al.*, 2005).

4.3.4.3 Volatile fatty acids

All four commercial strains of *O. oeni* tested in the present study caused significant increases in the concentration of short-chain fatty acids (**Table 4.5 and 4.6**). Volatile short-chain fatty acids are

produced by yeast and bacteria as a result of fatty acid metabolism and despite their low concentrations in wine, these compounds can negatively affect the aroma quality of wine due to their low perception threshold value and odours reminiscent of cheese and rancid cheese (Rodriguez *et al*., 1990). However, in the present study, the degree to which these compounds were affected differed significantly from each other and was strain dependant for certain compounds. Hexanoic, decanoic and octanoic acids were increased by MLF (**Figure 4.10**), although the magnitude of change in the concentration was more strain dependant in the Pinotage wine (**Figure 4.10a**) than in the Shiraz wine (**Figure 4.10b**). In agreement with these results, Maicas *et al*. (1999) found increased levels of decanoic and octanoic acid after MLF. Herjavec *et al*. (2001) found significant increases in octanoic, hexanoic and decanoic acid concentrations and Pozo-Bayón *et al*. (2005) found significant differences for octanoic and decanoic acids depending on the MLF culture used.

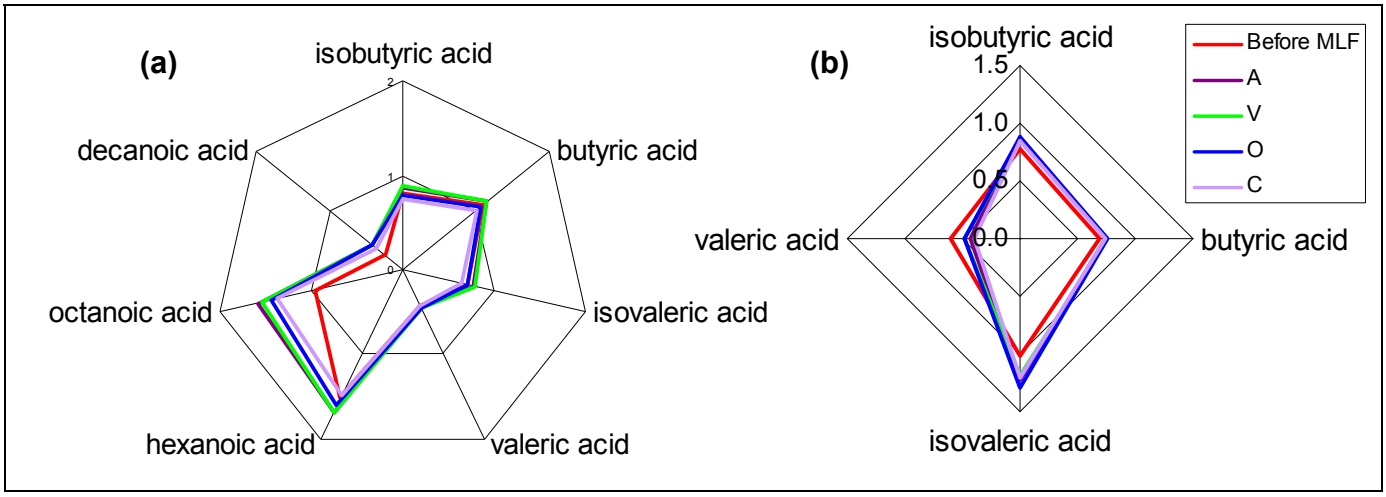


Figure 4.10 Changes in the volatile fatty acid profiles associated with MLF by different bacterial starter cultures in (a) Pinotage 2008 and (b) Shiraz 2008 wines.

The remainder of the measured fatty acids was either increased or unchanged by MLF (**Figure 4.10**). In a recent metabolic profiling study, Lee *et al*. (2009) reported differentiation between wines according to LAB strain used with regard to, amongst other compounds, differences observed in isobutyric and octanoic acids. In the present study, butyric acid, isovaleric acid, hexanoic acid and octanoic acid were the only fatty acids present at concentrations above their reported threshold values. This observation is in support by previous reports regarding the unlikely contribution of volatile fatty acids to cause flavour changes or cheesy off-flavours during MLF with *O. oeni* (Rodriguez *et al.*, 1990; Ugliano & Moio, 2005). It has been proposed that wine LAB have the metabolic capacity to produce volatile fatty acids through lipase activity (Davis *et al*., 1988) but lipolytic systems in wine LAB are not well known and further work is needed in this area (Liu, 2002; Matthews *et al.*, 2004).

4.3.4.4 Carbonyl compounds - Diacetyl and aldehydes

Changes related to aldehyde metabolism and citric acid metabolism in terms of the formation of carbonyl compounds in the Pinotage 2008 wine are listed in **Table 4.5** and graphically displayed in **Figure 4.11a**. No significant difference was found between the control and the wines fermented with

commercial starter cultures in terms of diacetyl concentration, although the Viniflora CH16 strain produced increased concentrations compared to the other bacteria (**Table 4.5**). Similarly, no significant difference was observed in acetoin concentrations, although the Viniflora CH16 strain produced the highest concentration of the bacteria tested. Principal component analysis (PCA) provides a summary of the changes observed (**Figure 4.11a**). Diacetyl and acetoin were correlated with each other and strongly associated with the Viniflora oenos bacteria as well as to a lesser extend with the Viniflora CH16 and VP41 strains. Enoferm alpha was positioned towards the bottom of the plot and associated with increased concentrations of *E*-2-octenal (herbaceous, lemon), decanal (citrus, fruity), nonanal (herbal, floral) and *E*-2-hexenal (herbaceous, green), all of which are associated with green or herbaceous aromas in wine (de Revel & Bertrand, 1994). However, neither of these compounds was present at concentrations above their individual aroma thresholds, but possible contribution to wine aroma should not be excluded since their cumulative effect might contribute to perceived wine aroma. The presence of *trans*-2, *cis*-6-nonadienal and 2,3-pentanedione was associated more with the control and spontaneous treatments during this experiment. Interesting to note the negative correlation between diacetyl and 2,3-pentanedione. The oxidation of diacetyl results in the formation of 2,3-pentanedione while the reduction of diacetyl by LAB results in the formation of acetoin. Diacetyl was present as concentrations above its range of reported aroma threshold values (0.2-2.3 mg/L; Martineau *et al.*, 1995b), 2,3-pentanedione was present at concentrations above its reported threshold while acetoin was present at levels below its reported threshold and would be less likely to contribute to wine aroma. All three compounds contribute to very similar aroma attributes in wine, therefore the cumulative effect on wine aroma should not be excluded.

During the Pinotage 2009 vintage, separation along the first PC was driven by the strong association of the Enoferm alpha (indicated by A in **Figure 4.11c** and **Table 4.5**) strain with diacetyl and acetoin positioned towards the far left of the plot. Viniflora oenos and Viniflora CH16 also produced significant levels of diacetyl and acetoin while the VP41 strain (indicated by V in the graph) produced slightly lower levels of these compounds. The control treatment was strongly associated with higher concentrations of the aldehyde compounds and positioned towards the far right of the plot, however the VP41 strain also showed association with these compounds.

Prominent discrimination between the different MLF treatments in terms of carbonyl compounds for the Shiraz 2008 experiment is shown in **Figure 4.11e** using PCA. The control, spontaneous and VP41 treatments were positioned to the left of the PCA scores plot and correlated with each other (**Figure 4.11e**) as a result of their association with higher concentrations of 2,3-pentanedione, *E*-2-hexenal and *E*-2-nonenal (**Figure 4.11f**) as well as lower concentrations of diacetyl and acetoin. Conversely, the Enoferm alpha, Viniflora oenos and Viniflora CH16 strains were positioned to the right-hand side of the scores plot and showed strong association with diacetyl and acetoin (**Figure 4.11f**). The Enoferm alpha strain is slightly separated to the top right-hand part of the scores plot (**Figure 4.11e**) as a result of its association with *trans*-2, *cis*-6-nonadienal and *E*-2-octenal. The Viniflora oenos and CH16 strains are more correlated and positioned slightly towards the bottom of the scores plot due to the presence of decanal. The Enoferm alpha strain produced significantly more diacetyl compared to the other three bacteria (**Table 4.5**) while VP41 imparted the smallest increase in diacetyl concentrations. All four strains produced diacetyl concentrations with OAVs>1, potentially contributing to the buttery aroma of the wine (Bartowsky & Henschke, 2004). Acetoin was produced at

higher concentrations by Enoferm alpha and Viniflora CH16 strains compared to VP41, however these concentrations did not exceed the reported threshold level of 150 mg/L (Etiévant, 1991).

Overall, MLF increased diacetyl concentrations significantly with the exception of the Pinotage 2008 wine. Strain dependant differences were observed with the VP41 strain generally producing lower concentrations of diacetyl independent of the cultivar tested. Acetoin concentrations were increased while 2,3-pentanedione level were decreased by MLF. Strain dependant differences were observed in the degree of 2,3-pentanedione reduction. In general, decreased concentrations were observed for the specific aldehydes depending on the bacterial strain and cultivar evaluated.

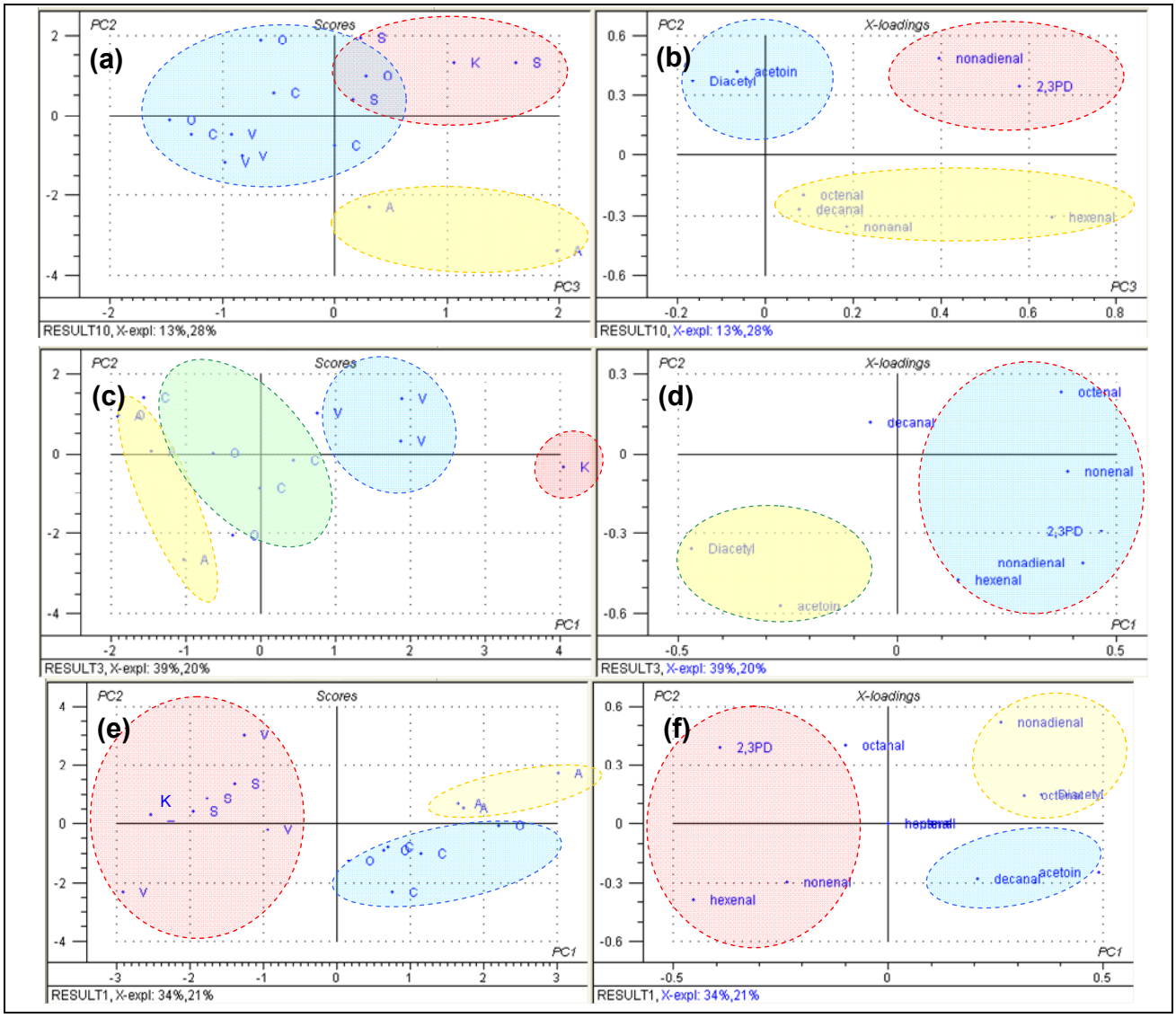


Figure 4.11 PCA illustrate the differences amongst the four strains (indicated by A, V, O, C) in terms of their effect on a selection of carbonyl compounds in the Pinotage 2008 wine (plot a and b), Pinotage 2009 wine (plot c and d) and Shiraz 2008 wine (plot e and f). Bacteria are depicted by A (Enoferm alpha), V (Lalvin VP41), O (Viniflora oenos), C (Viniflora CH16) and the control wine is indicated by the letter K. The letter S represents a spontaneous MLF treatment included in the study as explained in section 4.2.3.

4.3.5 CHARACTERISATION OF MLF STARTER CULTURES BY INFRARED SPECTROSCOPY

Differences observed in the infrared spectra of wines fermented with four different commercial MLF starter cultures are shown in **Figure 4.12** for the Shiraz wine. PCA models discriminate between the bacteria based on their spectral "fingerprint" obtained with both MIR (**Figure 4.12a**) and NIR spectroscopy at the end of MLF (**Figure 4.12b**). As a result of different metabolites produced or consumed by each bacterial strain, characteristic metabolic profiles associated with bacterial growth are captured by IR spectra. **Figure 4.12a** illustrate bacterial groupings based on their MIR spectral fingerprint with the Viniflora oenos and Viniflora CH16 strains (indicated by the dashed ellipse) more separated from the Enoferm alpha and VP41 strains (indicated by the solid ellipse). Similarly, PCA with NIR spectra also showed a tendency for Viniflora oenos and Viniflora CH16 (indicated by the dashed ellipse) to be more associated with each other while Enoferm alpha and VP41 (indicated by the solid ellipse) were more associated with each other.

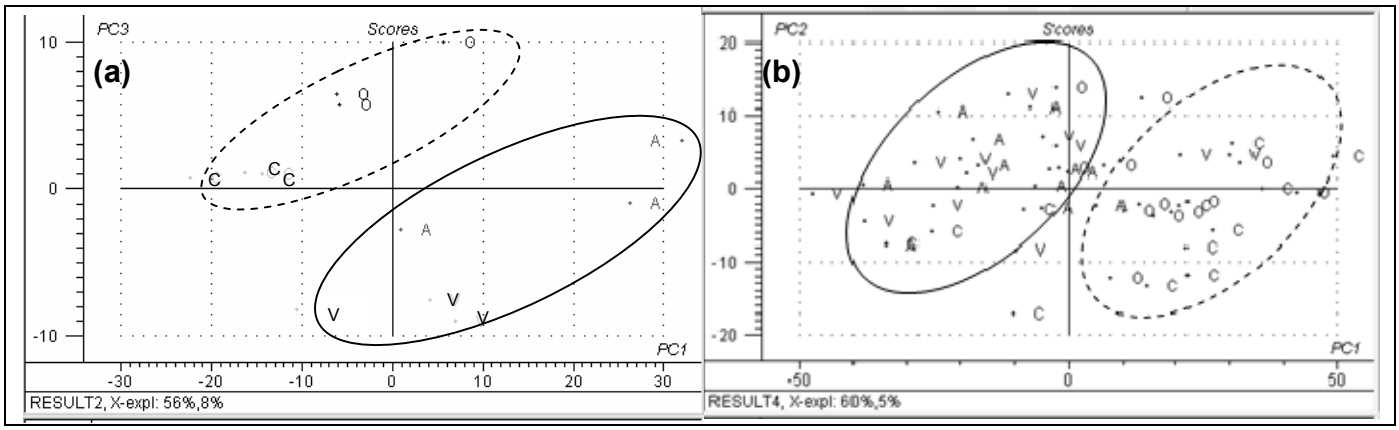


Figure 4.12 PCA scores plot illustrate the possibility of differentiating four commercial starter cultures with the use of (A) MIR and (B) NIR spectra. Enoferm alpha (denoted by A) and VP41 (denoted by V) tend to group together (indicated by the solid ellipse) while Viniflora oenos (denoted by O) and Viniflora CH16 (denoted by C) are grouped together (indicated by the dashed ellipse).

The typical changes imparted by MLF have been used to monitor MLF fermentation progress through the quantification of malic acid and lactic acid by MIR. The possibility to quantify other MLF relevant volatile metabolites using MIR spectra was explored. Preliminary PLS models were constructed to relate MIR spectra to diacetyl (**Figure 4.13d**), acetoin (**Figure 4.13c**), 2,3-pentanedione (**Figure 4.13a**) and nonadienal (**Figure 4.13b**) concentrations. The preliminary models show promise with R^2 values ranging between 0.985 and 0.999. This observation merits further investigation and could possibly be used in the future to monitor the evolution of MLF related aroma compounds more effectively.

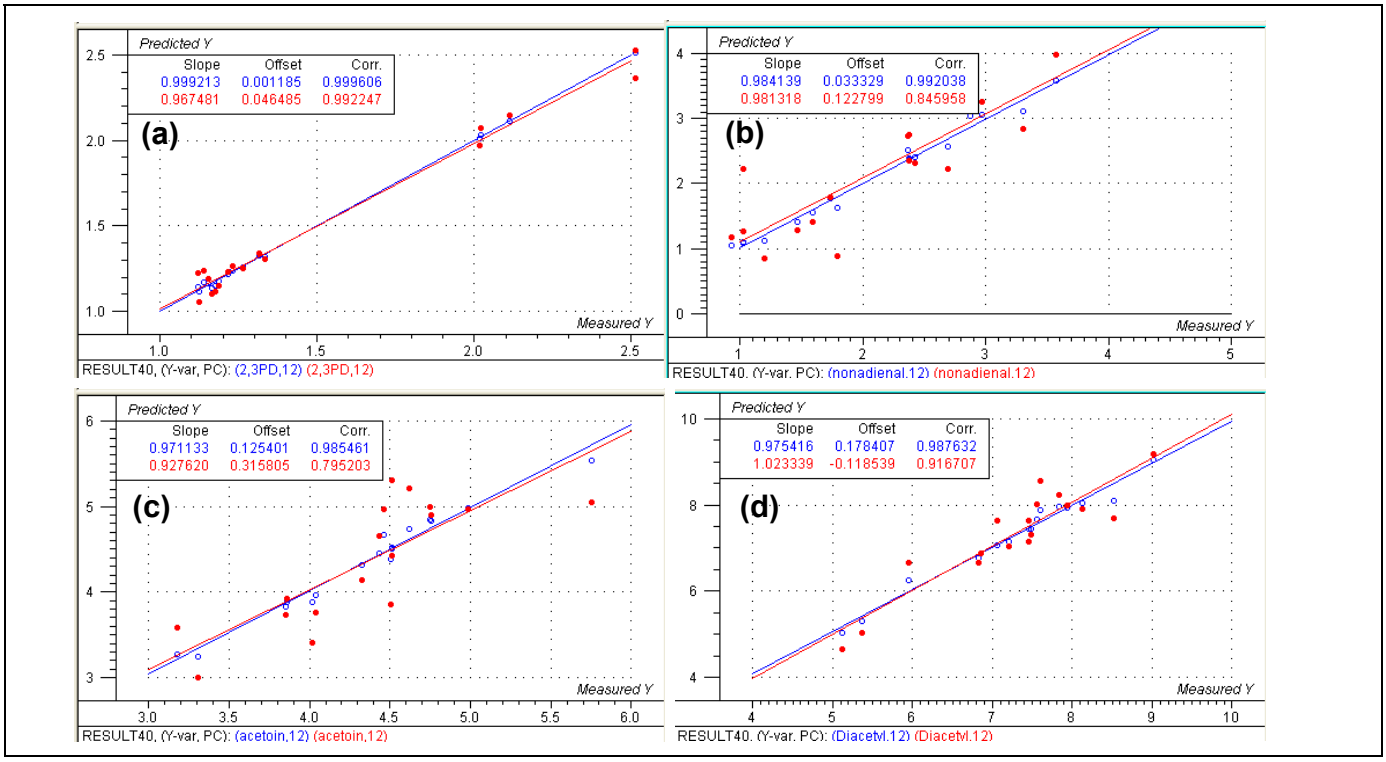


Figure 4.13 Preliminary prediction PLS models for (a) 2,3-pentanedione, (b) *trans-2*, *cis-6*-nonadienal, (c) acetoin and (d) diacetyl illustrate the potential for quantification or screening of MLF related volatile compounds with the use of IR.

4.4 CONCLUSIONS

In summary, this work analysed the compositional changes in terms of organic acids, volatile metabolites and infrared spectral properties of Pinotage and Shiraz wines produced by four different commercial MLF starter cultures under South African winemaking conditions.

The application of a variety of statistical techniques to the volatile compounds, organic acids and spectral data has revealed significant metabolic differences between the *O. oeni* strains. Results also suggest a degree of diversity in the *O. oeni* group, since wines also showed specific characteristics depending on the strain.

Significant differences in organic acid profiles corresponding to different bacterial starter cultures were shown to be a useful means to project possible differences in terms of specific metabolites such as ethyl lactate and diacetyl. As previously reported (Liu, 2002), differences in lactic acid production and citric acid metabolism could indicate different metabolic requirements and resulting volatile metabolites.

In terms of volatile composition, a general increase in the ester, higher alcohol and volatile fatty acid concentrations of all the wines were observed after the completion of MLF. This observation was independent of the bacterial strain used and independent of grape cultivar. However, specific strain dependent differences were observed for certain compounds. A large portion of the esters, which are important for the fruity aroma notes of wine, were found to have OAVs > 1 indicating their potential

aroma contribution. It could therefore be concluded that MLF using any of these four strains may contribute to wine quality by modifying the concentration of some of the aroma compounds. However, the influence of chemical changes on perceived aroma should further be investigated with the use of sensory evaluation techniques.

In addition to monitoring of MLF progress with infrared spectra, valuable spectral fingerprints captured differences amongst the wines prepared with different strains of *O. oeni* bacteria by using PCA models. Preliminary PLS models show promise for predicting specific MLF related aroma compounds with the use of MIR spectra. However, these possibilities need further exploration. Previous reports on relationships and prediction possibilities between infrared and sensory data also merit additional investigation (Cozzolino *et al*., 2005.).

The present research contributes to our current knowledge of malolactic fermentation and more specifically the contribution of different bacterial strains to wine composition and potentially wine aroma. The results therefore illustrate and reiterate the potential of MLF and specifically the use of different starter cultures to contribute to wine aroma and flavour. Future studies should include recently developed starter cultures, combination starter cultures and additional inoculation strategies in order to expand our current knowledge on MLF and wine aroma in different scenarios. Additional analytical applications, such as gas chromatography-olfactometry (GC-O) could be employed to investigate and better understand the formation of volatile compounds contributed by MLF.

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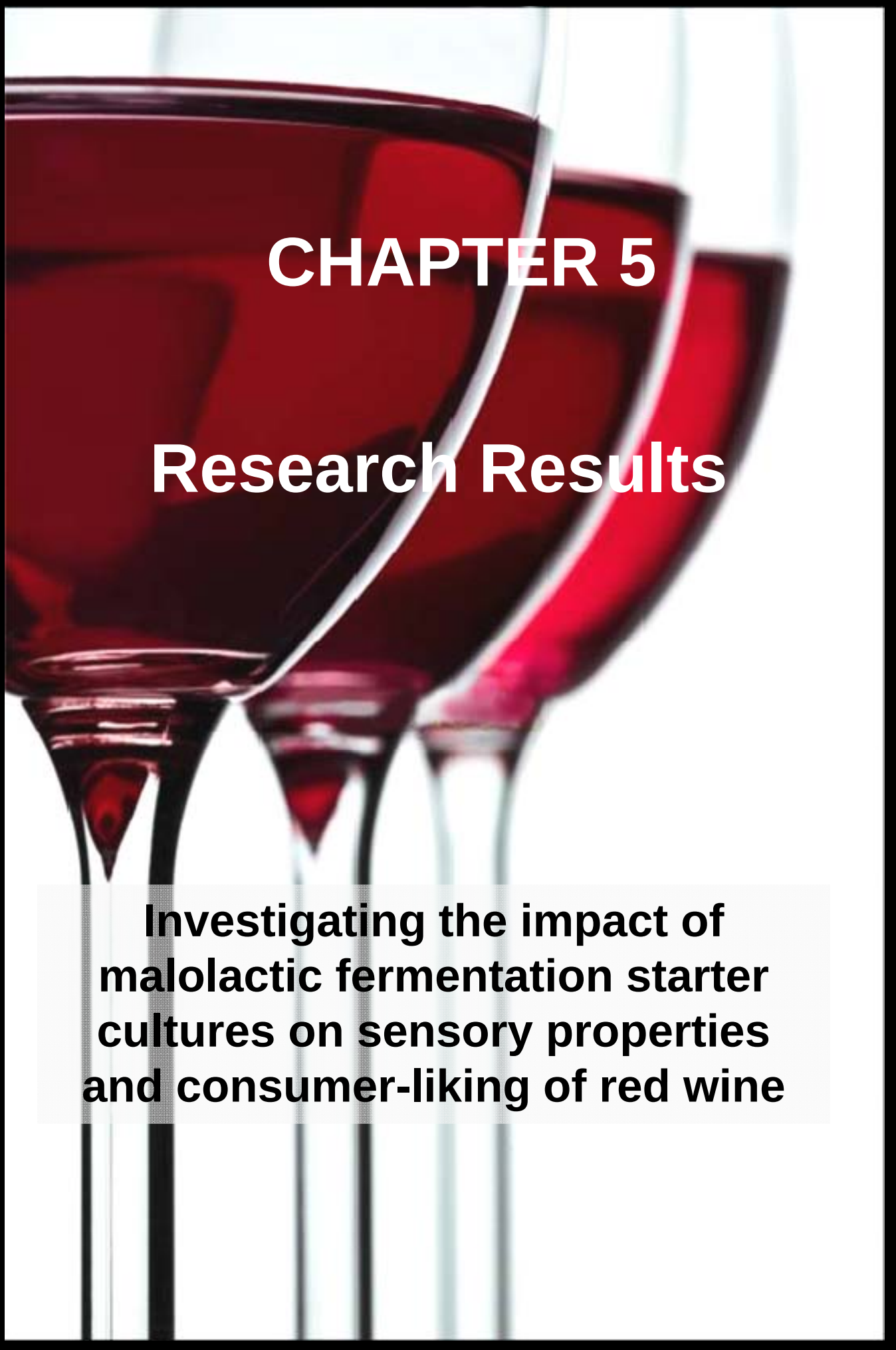

CHAPTER 5

Research Results

Investigating the impact of malolactic fermentation starter cultures on sensory properties and consumer-liking of red wine

5. RESEARCH RESULTS

Investigating the impact of malolactic fermentation starter cultures on sensory properties and consumer-liking of red wine

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ABSTRACT

Malolactic fermentation (MLF) has been shown to modify wine aroma and flavour. Limited studies have previously explored the relationship between bacterial strain selection, the sensory properties and hedonic responses. This study describes the influence of MLF bacterial starter culture selection on the sensory characteristics of experimentally produced South African Pinotage and Shiraz wine through both descriptive analysis and consumer testing. A trained panel (n=12) was used for the profiling of wines fermented with Enoferm alpha®, Lalvin VP41®, Viniflora oenos® and Viniflora CH16® starter cultures compared to a control (no MLF performed). The respective starter cultures are frequently used in the wine industry and were therefore selected for this study. Quantitative descriptive analysis was used to measure the intensity of specific sensory characteristics. Consumer-liking was tested with the use of the 9-point hedonic scale for overall-liking, aroma-liking and taste-liking. Consumer information included demographic details and wine consumption habits. Significant differences between wines fermented with different starter cultures were observed for sensory descriptors including buttery, fruity, nutty and yoghurt/buttermilk as well as smoothness and mouth-feel attributes. Sensory properties observed in relation to specific bacteria used were found to differ between the Pinotage and Shiraz wines. It was shown that the differences imparted by MLF bacteria could potentially influence consumer liking. Preference mapping highlighted interesting relationships between sensory attributes and consumer-liking. Observations suggest that wines produced by one of the starter cultures was more preferred in both the Pinotage and Shiraz, however the sensory drivers of liking were different in the respective wines. The importance of integrating sensory and consumer analysis in the assessment of MLF starter culture contributions to wine sensory properties was highlighted. This study illustrates the positive impact of MLF starter cultures on wine aroma and reiterates the importance of strain selection.

Keywords: Malolactic fermentation, sensory evaluation, consumer-liking, wine aroma, preference mapping

5.1 INTRODUCTION

Wine is the result of a multi-faceted process involving various biochemical reactions and chemical interactions. Apart from yeast, the other key microbes in this process are lactic acid bacteria involved in the bioconversion of malic acid to lactic acid during the secondary fermentation process, malolactic fermentation (MLF) (Bartowsky & Henschke, 1995). In addition to this deacidification, MLF is a desirable process to increase biological stability and to contribute to aroma and flavour complexity (Kunkee, 1974; Amerine *et al*., 1982; Davis *et al*., 1985). The contribution of MLF to wine aroma is often a topic of controversy with results and opinions differing between authors (Bartowsky & Henschke, 1995; Sauvageot & Vivier, 1997; de Revel *et al*., 1999; Maicas *et al*., 1999; Gámbaro *et al*., 2001; Ugliano & Moio, 2005; Boido *et al*., 2009). The use of commercial bacterial starter cultures is beneficial since it improves MLF control by preventing problems associated with spontaneous MLF such as potential product inconsistency. *Oenococcus oeni* strains are commonly used as starter cultures due to their tolerance and adaptation to low pH and high alcohol wine conditions (Davis *et al*., 1985; Henick-Kling, 1993; Henschke, 1993). However, these commercial starter cultures are primarily used by winemakers to ensure successful malic acid fermentation rather than for their potential to contribute positively to the wine quality in terms of aroma and flavour.

MLF starter cultures have the ability to influence wine aroma and flavour by producing secondary metabolites and by modifying grape and yeast-derived metabolites (Henick-Kling, 1993; Bartowsky & Henschke, 1995). However, the aroma and flavour impact of specific starter culture bacteria can be variable depending on the wine cultivar (Laurent *et al*., 1994; Sauvageot & Vivier, 1997; Gámbaro *et al*., 2001) and the particular strain of MLF bacteria used (McDaniel *et al*., 1987; Bartowsky & Henschke, 1995; Delaquis *et al*., 2000). Correlations between specific starter cultures and their exact aroma impact have not been studied in depth. Studies pertaining to the influence of MLF on wine aroma mostly focus on the production of diacetyl and other carbonyl compounds which contribute to a lactic and buttery character in wine (Martineau & Henick-Kling, 1995; Bartowsky & Henschke, 2004). However, in recent years research on the contribution of MLF to wine aroma has shifted to comprise a full spectrum of chemical compounds which include amongst other, compounds such as esters which are more related to the fruity attributes of wine (Boido *et al.*, 2009; Sumby *et al.*, 2010). A number of studies related to MLF and wine aroma have investigated changes in the volatile chemical composition (De Revel, *et al*., 1999; Lloret *et al.*, 2002; Fernandes *et al*., 2003; Ugliano & Moio, 2005) while the sensory impact of these compounds on the total aroma contribution of MLF is less well-known (McDaniel *et al*., 1987).

Sensory evaluation is a scientific discipline used to evoke, measure, analyse, and interpret reactions to stimuli perceived through the senses (ASTM 2005). Sensory panels are considered as equivalent to analytical instruments used to produce sensory data which are accurate, sensitive, repeatable and reproducible (Lesschaeve, 2007). The collection of high quality data from sensory panels involves a complex and time-consuming process where panel members are selected and trained to perform sensory tasks objectively and consistently (Issanchou *et al*., 1997). The application of sensory evaluation in wine research efforts is wide-spread with a major focus on characterising the sensory impacts of viticultural or oenological treatments on finished wine (Heymann & Noble, 1987; Francis *et al*., 1992; Cliff & Dever, 1996; Reynolds *et al*., 1996). Sensory attributes however, do not

provide any indication of consumers' product preferences. Preference mapping techniques (Greenhoff & MacFie, 1994; Schlich, 1995) are therefore often applied to relate consumer-liking data to sensory attribute data. Apart from the intrinsic sensory properties of a product, an array of extrinsic factors has been shown to influence preference such as appearance, packaging and price (Zajonc & Markus, 1982; Yegge, 2001; Simons & Noble, 2003). Contextual factors were also shown to influence consumer acceptance of wine (Hersleth *et al*., 2003) while another study highlighted the influence of the degree of wine knowledge on wine preference (Frøst & Noble, 2002). It is clear that consumer preference is a function of social, intrinsic and extrinsic requirements and integrative approaches which address these aspects are therefore critical to understand consumers' preferences. The influence of production parameters, such as MLF starter culture selection, therefore needs to be assessed since it could provide winemakers with another tool to manipulate the intrinsic sensory characteristics and produce products that meet consumer requirements.

The present research investigates the influence of Enoferm alpha®, Lalvin VP41®, Viniflora oenos® and Viniflora CH16® commercial MLF starter cultures on the sensory characteristics and consumer-liking of Pinotage and Shiraz wine. A trained panel was used to obtain a sensory profile of the respective wines followed by consumer testing to evaluate degree of liking. Statistical analysis was performed to test for significant differences in the sensory profiles and correlate the sensory data with the consumer data through preference mapping in order to identify possible drivers of liking. Consumer segmentation according to different demographic and behavioral information was also investigated. This study describes an integrative approach to evaluate the changes associated with Enoferm alpha®, Lalvin VP41®, Viniflora oenos® and Viniflora CH16® MLF starter cultures and the effect on sensory characteristics and consumer-liking in Shiraz and Pinotage wine.

5.2 MATERIALS AND METHODS

5.2.1 PREPARATION OF WINES

Experimental Pinotage and Shiraz wines prepared during the 2008 and 2009 vintages as described in **Chapter 4** were used for evaluation. Grapes from the Wellington region (Western Cape, South Africa) were crushed, destemmed and 30 mg/L of sulphur dioxide was added. The must was inoculated with the commercial yeast WE372 (Anchor Technologies, South Africa) according to the manufacture's recommendation. Alcoholic fermentation was performed at 25˚C with frequent punching down of the cap. The wine was pressed from the skins at 2˚Brix and divided into triplicate lots of 4.5 L glass carboys (during the 2008 vintage) and 20L stainless steel canisters (during the 2009 vintage) for each MLF treatment. The winemaking experiment consisted of four different MLF starter cultures (listed in **Table 5.1**), a control wine (no MLF) and a spontaneous MLF to monitor the indigenous microflora.

The sensory attributes of the MLF starter cultures, as described by the specific manufacturers, are listed in **Table 5.1**. Bacteria were inoculated to 1 x 10^6 cfu/mL after alcoholic fermentation, following manufacturers' instructions for rehydration of actively dried cultures. For the control treatment, three of the 4.5 L glass bottles were racked and 50 mg/L SO_2 added directly after alcoholic fermentation to inhibit microbial growth and capture the chemical composition of the wines before MLF. MLF was conducted at 20˚C and considered complete when the concentration of malic acid was below 0.3 g/L.

Upon completion of MLF, the wines were racked from the lees, stored at 0˚C for two weeks for cold stabilisation and then bottled. The experiments were repeated in Shiraz (2008) and Pinotage (2008, 2009). The bottled wines were stored at 15˚C for 1 year (Pinotage and Shiraz 2008 wines) and 5 months (Pinotage 2009 wines) respectively, prior to sensory evaluation by the trained panel. Information related to the samples tested as well as the chemical composition for the respective samples is listed in **Table 5.2.**

Table 5.1 Sensory attributes of the commercial starter cultures according to the respective manufacturer. Abbreviations for the starter cultures used during this study are listed.

Starter culture	Abbreviation	Company	Sensory contribution description in brief *
Enoferm alpha®	A	Lallemand[a]	Mouthfeel, lower perception of green and vegetative flavours, positive impact on wine complexity
Lalvin VP41®	V	Lallemand	Enhances complexity and mouthfeel, contribute to aroma and wine structure
Viniflora® oenos	O	CHR Hansen[b]	Clean and classic flavour profile, Low production of volatile acidity
Viniflora® CH16	C	CHR Hansen	Low production of volatile acidity

*Information obtained from the respective technical data sheets: www.chr-hansen.com; www.lallemandwine.com
[a]Lallemand, Stellenbosch, South Africa; [b]CHR Hansen, Hørsholm, Denmark

Table 5.2 Samples analysed by sensory evaluation and the standard wine parameters of the samples at the end of MLF. Chemical parameters are given in g/L, except alcohol (%v/v).

Samples tested[a]	Cultivar	Descriptive testing	Degree of liking	pH	Alcohol	Malic acid	Lactic acid	Volatile acidity
Control	Shiraz 2008	Trained panel of judges analysed all wines for sensory attributes	Consumer panel nr 1 (n=48)	4.14	14.59	3.01	0.12	0.36
Wine A				4.22	14.33	0.15	1.20	0.47
Wine V				4.24	14.33	0.16	1.23	0.41
Wine O				4.28	14.31	0.17	1.31	0.52
Wine C				4.27	14.27	0.19	1.26	0.49
Control	Pinotage 2008		Consumer panel nr 2 (n=52)	4.05	13.30	2.52	0.14	0.40
Wine A				4.23	13.25	0.08	1.32	0.54
Wine V				4.23	13.34	0.11	1.30	0.53
Wine O				4.25	13.83	0.09	1.27	0.57
Wine C				4.26	13.84	0.08	1.27	0.57
Control	Pinotage 2009		Consumer panel nr 3 (n=46)	3.58	11.81	2.31	0.63	0.32
Wine A				3.66	11.97	0.09	1.33	0.44
Wine V				3.65	11.93	0.10	1.38	0.43
Wine O				3.68	11.91	0.07	1.37	0.47
Wine C				3.68	11.92	0.07	1.37	0.47

[a]Details of the MLF starter cultures are listed in **Table 5.1.**

5.2.2 EXPERIMENTAL DESIGN

The design of the experiment was the same for both years. All 4 bacteria and the control [control, Enoferm alpha (wine A), Lalvin VP41 (wine V), Viniflora oenos (wine O), Viniflora CH16 (wine C)] were

used and three bottles from each were used for testing. In total the number of bottles was 3*5*3= 45, 15 for each of the 3 wines namely Pinotage 2008, 2009 and Shiraz 2008. For the chemical analysis, one sample was taken from each bottle and tested in triplicate. For the sensory analysis, one sample for each bottle was taken and served to all the assessors. The three replicates for the sensory panel thus correspond to three different bottles. The three replicates were taken after each other in a systematic way indicating the need for a systematic replicate effect in the ANOVA model. For the consumer test, all 5 samples (control, wine A, wine V, wine O, wine C) were given to each consumer.

5.2.3 SENSORY EVALUATION PROCEDURE

All samples were evaluated with the use of discriminative and descriptive testing procedures. A preliminary discriminative test was followed to establish whether differences could be perceived amongst the different treatments. Consequently, the description of the wine sensory properties was achieved by using a trained panel. The sensory evaluation procedure followed is explained in terms of discriminative testing, sensory panel selection and training and finally, descriptive testing procedures used.

5.2.3.1 Preliminary discriminative testing

Initial sensory testing involved the use of a discriminative technique to establish if there were perceived differences between the control wine (no MLF) and the MLF wines. The paired comparison test (Lawless & Heymann, 1998) was used for this purpose in which each taster is provided with two wine samples: one control and one MLF sample. A panel of 12 individuals who regularly taste wines were asked if a difference is perceived between the two samples. The Roussler table was used to assess the statistical significance of the answers (Lawless & Heymann, 1998). In addition, tasters were also asked if the differences observed were more prominent in terms of aroma (perceived smell), taste/mouth-feel (palate attributes) or both. Tasters were also prompted to provide a few descriptive terms that would capture the differences observed. These descriptive terms were used as an indication for possible reference standards to be prepared during formal training of the panel.

5.2.3.2 Sensory panel selection and training

A panel consisting of 10 women (ages 24-50) were used for the wine evaluation. Panellists were selected on the basis of availability, having an interest in wine sensory evaluation and previous experience (6 months) in wine evaluation. Panel members were trained according to the consensus method (Lawless & Heymann, 1998). Panel training consisted of 6-8 sessions of at least 1½ hours. During each session panellists were trained by round table discussion for the following: a) lexicon development, b) intensity training and c) reference association for aroma attributes and mouth-feel attributes. Three different sets of wine, namely Shiraz 2008, Pinotage 2008 and Pinotage 2009, were evaluated. Each of the sets consisting of 5 wines (control and four bacteria), were treated individually. That is, training and data capturing was completed for Shiraz 2008, followed by Pinotage 2008 and finally the same procedure was followed for the Pinotage 2009 wine.

Panelists were provided with the specific product set of 5 wines accompanied by an adapted version of the wine aroma wheel (Noble *et al*., 1987) (**Figure 5.1**), training reference standards in a

neutral red wine (**Table 5.3**) for specific aroma descriptors and pieces of cloth for describing mouth-feel attributes. This was used to encourage description of the specific wine and the reference standards could be used for aroma recognition or confirmation purposes. Although the focus of the sensory evaluation was on aroma attributes, the process of MLF is often associated with a contribution to mouth-feel properties of wine (Henick-Kling *et al*., 1994). It was therefore decided to include two additional mouth-feel properties for sensory evaluation. For the purpose of this study, the mouth-feel descriptors (Gawel *et al*., 2000) were kept very basic. The two mouth-feel descriptors included were body/mouth-feel and smoothness (definitions for this specific study in **Table 5.4**). Training involved the use of different pieces of cloth/material (Gawel *et al*., 2000), touching the material and relating that feeling back to the feeling in the mouth in the case of the smoothness descriptor. Body or mouth-feel was described as the weight of the wine in the mouth and anchored on the line scale by *thin* and *full* descriptors.

Once the recognition and description of possible aroma nuances and mouth-feel properties were finalised and definitions confirmed (**Table 5.4**) with the panel, the use of the 100 mm unstructured line scale was exercised. Intensity ranking was practiced and discussed for a number of sessions in order to calibrate the panel in the use of the line scale until consensus was reached amongst panelists.

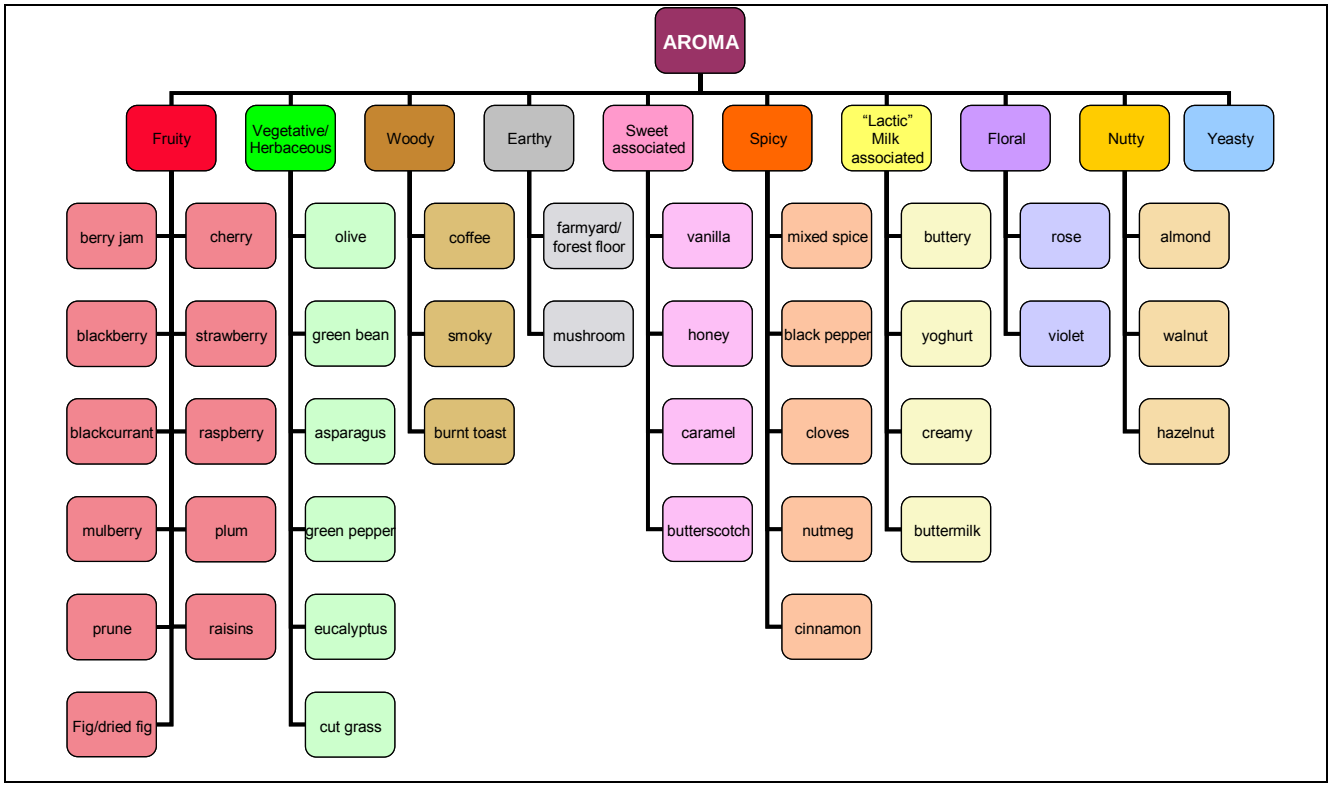


Figure 5.1 Adaptation of the wine aroma wheel used during training sessions for the description of aroma related to malolactic fermentation (Noble *et al*., 1987).

Table 5.3 Standards developed for malolactic fermentation related aroma description training.

Attribute		Reference[a]
Fruity	berry jam	3 tsp[b] mixed berry jam
	cherry	5 tsp cherry juice
	strawberry	2 medium sized fresh strawberries
	raspberry	3 tsp raspberry jam
	mulberry	5 fresh mulberries
	prune	2 prunes
	raisins	1 1/2 tablespoon[c] raisins
	fig	2 1/2 tsp fig jam
Vegetative	black olive	4 olives cut in pieces
	green bean	3 1/2 tsp of brine of green bean
	asparagus	1 tsp of brine of cooked asparagus
	green pepper	1 tsp of chopped pieces of bell pepper
	cut grass	fresh grass
Woody	coffee	1 tsp coffee
	chocolate	1 tsp cocoa
	earthy	1 tablespoon soil
	mushroom	1 large mushroom in pieces
Sweet associated	vanilla	1/4 tsp vanilla essence
	honey	1 tsp honey
	caramel	1/4 tsp caramel essence
	butterscotch	1 butterscotch candy
Spicy	mixed spice	1/4 tsp mixed spice
	black pepper	1/4 tsp grind black pepper
	cloves	1 clove in wine for 5 minutes
	nutmeg	1/4 tsp nutmeg
	cinnamon	1/4 tsp cinnamon
Lactic	butter	40 µL diacetyl (2,3-butanedione)
	yoghurt	2 tsp plain yoghurt
	creamy	4 tsp fresh cream
	buttermilk	1 1/2 tablespoon cultured buttermilk
Floral	rose	5 µL 2-phenylethyl acetate
	violets	0.2 µL β-ionone
Nutty	almond	1/4 tsp almond essence
	walnut	1 walnut cut in pieces
Yeasty		1/2 tsp bakers yeast in water; added

[a]References prepared in 200 mL neutral red wine; [b]teaspoon (tsp) = 5 mL; [c]tablespoon = 25 mL.

5.2.3.3 Descriptive testing

Quantitative descriptive analysis (QDA®, Stone *et al*., 1974) is used internationally as a profiling method for a variety of food products. This profiling technique was used to describe the sensory attributes perceived in the wine samples as identified by the trained panel. Wine evaluation was performed in tasting booths equipped with the electronic data capturing Compusense *five* system (release 4.8, Compusense Inc., Guelph, Canada). All evaluations were conducted in individual tasting

booths under white light and in temperature controlled conditions (21˚C±2˚C). Samples (30 mL) were presented in tulip shaped standard clear ISO wine tasting glasses covered with a Petri dish lid (Kimix, South Africa). Panellists received 5 samples with 3-digit codes in complete randomised order according to a balanced complete block design. The panel used a 100 mm unstructured line scale to analyse the five products for the 13 respective sensory attributes (**Table 5.4**). Panellists were asked to refresh their palate in between samples with distilled water and unsalted crackers (Water Biscuits, Woolworths, Stellenbosch, South Africa). The analysis was replicated during three identical consecutive sessions for each assessor on the same day. A time delay of 5 minutes was included in the questionnaire to ensure a resting period between each of the 3 tasting sessions.

Table 5.4 Aroma and taste descriptors used for the respective sensory attributes during trained panel sessions.

Descriptors	Definition	S08[a]	P08[b]	P09[c]
Fruity general	Aroma/flavour note associated with non-specific fresh fruit	√	√	√
Berry/berry jam	Slightly sweet aroma note associated with non-specific berries or berry jam	√	√	√
Prune	Dried fruit aroma nuance associated with prunes	nd	√	√
Vegetative	Aroma/flavour characteristic of cooked vegetables	nd	√	nd
Caramel/butterscotch	Sweet associated aroma/flavour note associated with caramel or butterscotch	√	nd	√
Buttery	Slightly rancid aroma/flavour associated with melted butter or butter buttons	√	√	√
Yoghurt/buttermilk	Sour milk associated aroma/flavour typical of yoghurt and buttermilk	√	√	√
Floral/rose	Floral aroma note associated with roses	nd	√	√
Nutty	Aroma associated with non-specific nuts	nd	√	nd
Sour	Sour taste associated with the taste of acids on the tongue	√	√	√
Butter taste	Oily taste in the back or the throat, reminiscent of butter	√	√	√
Smoothness	Feeling associated with the surface smoothness in the mouth cavity	√	√	√
Body/mouthfeel	Weight of the wine in the mouth - thin or full	√	√	√

[a]Shiraz 2008; [b]Pinotage 2008; [c]Pinotage 2009; nd = not detected.

5.2.3.4 Consumer testing

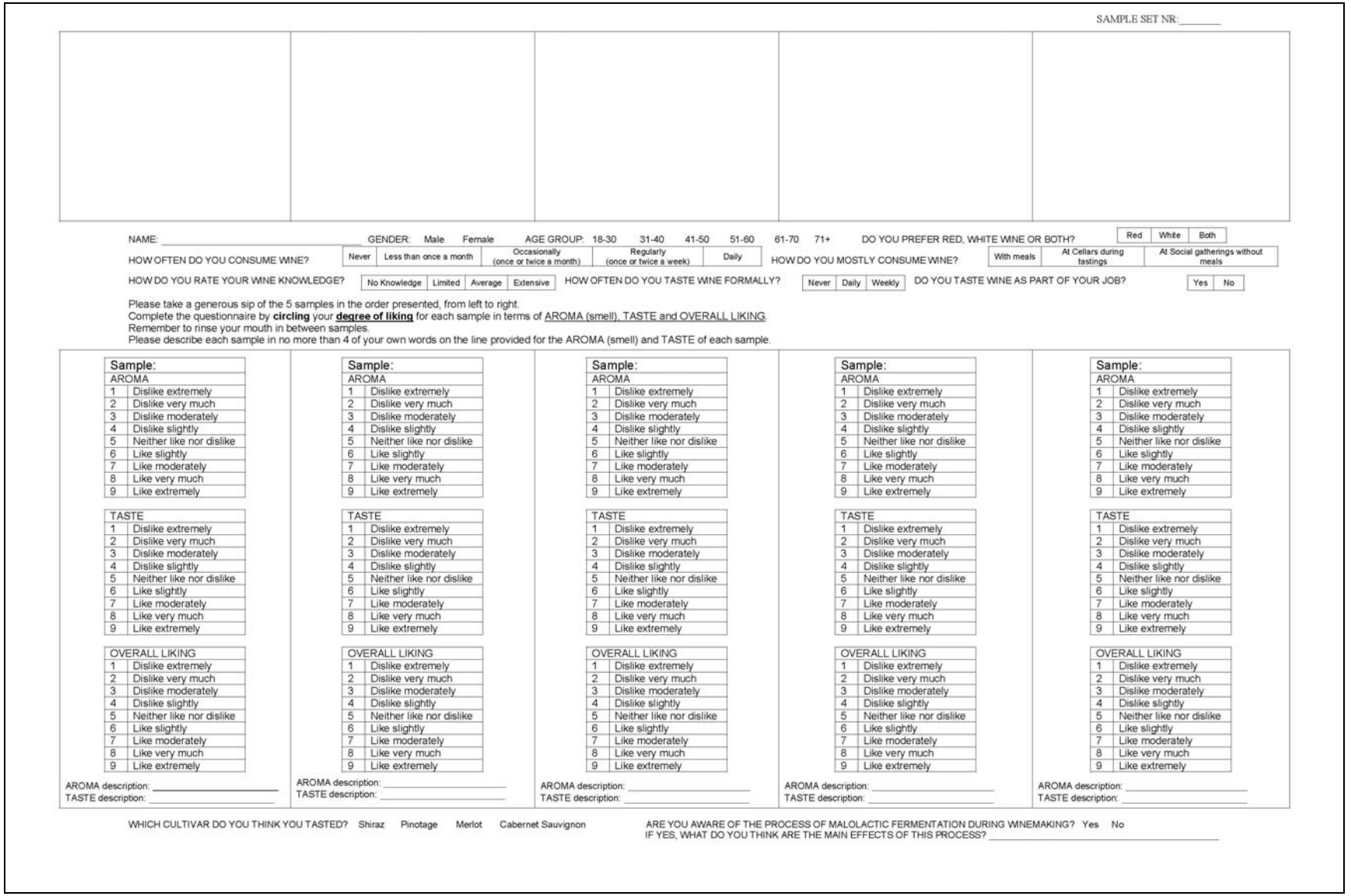
5.2.3.4.1 Consumer recruitment

Consumer testing of the respective wines (Shiraz 2008, Pinotage 2008 and Pinotage 2009) was also performed, complementary to the sensory evaluation, to investigate possible drivers of liking for a specific product and compare the results with QDA results. Consumers were recruited via email as well as by word of mouth. The target consumers had to (1) consume wine regularly (at least twice a month) and (2) prefer red wine to white wine or prefer both equally. A large percentage of the consumers contacted were from the Department of Viticulture and Oenology and from the Institute for Wine Biotechnology. Therefore, some questions regarding level of wine knowledge were included in the questionnaire in order to evaluate if the level of wine knowledge had an influence on the degree of liking for specific wine treatments.

5.2.3.4.2 Questionnaire design

Ideas for the questionnaire design were gathered from sensory evaluation text books (Lawless & Heymann, 1998) and research papers (Frøst & Noble, 2002). The questionnaire used in this study is shown in **Figure 5.2**. Demographic questions regarding gender, age and occupation were asked as well as questions related to wine consumption habits and wine knowledge. Consumers were presented with five samples (4 MLF treatments and one control with no MLF) in a complete randomised order using 3-digit codes.

The 9-point hedonic scale for degree of liking is used internationally for the evaluation of degree of liking for food products (Lawless & Heymann, 1998). Consumers were asked to indicate their degree of liking for the product tasted by using a 9-point category scale ranging from *Dislike extremely = 1* to *Like extremely = 9*. Overall liking, aroma liking and taste liking for each product was assessed by consumers. In addition, consumers were asked to describe each product in 4 words of their own. These words were used in a flash profiling (Siefferman, 2002) exercise and compared to the QDA results. Consumer testing was conducted over 3 consecutive days with evaluation of Shiraz 2008, Pinotage 2008 and Pinotage 2009 wine on separate days (**Table 5.2**). After completion of the tasting, information related to wine knowledge was obtained in the form of a mini quiz.



SAMPLE SET NR:_____

NAME: _____ GENDER: Male Female AGE GROUP: 18-30 31-40 41-50 51-60 61-70 71+ DO YOU PREFER RED, WHITE WINE OR BOTH? Red White Both

HOW OFTEN DO YOU CONSUME WINE? Never | Less than once a month | Occasionally (once or twice a month) | Regularly (once or twice a week) | Daily

HOW DO YOU MOSTLY CONSUME WINE? With meals | At Cellars during tastings | At Social gatherings without meals

HOW DO YOU RATE YOUR WINE KNOWLEDGE? No Knowledge | Limited | Average | Extensive

HOW OFTEN DO YOU TASTE WINE FORMALLY? Never | Daily | Weekly

DO YOU TASTE WINE AS PART OF YOUR JOB? Yes | No

Please take a generous sip of the 5 samples in the order presented, from left to right.
Complete the questionnaire by **circling** your **degree of liking** for each sample in terms of AROMA (smell), TASTE and OVERALL LIKING.
Remember to rinse your mouth in between samples.
Please describe each sample in no more than 4 of your own words on the line provided for the AROMA (smell) and TASTE of each sample.

Sample:	
AROMA	
1	Dislike extremely
2	Dislike very much
3	Dislike moderately
4	Dislike slightly
5	Neither like nor dislike
6	Like slightly
7	Like moderately
8	Like very much
9	Like extremely

TASTE	
1	Dislike extremely
2	Dislike very much
3	Dislike moderately
4	Dislike slightly
5	Neither like nor dislike
6	Like slightly
7	Like moderately
8	Like very much
9	Like extremely

OVERALL LIKING	
1	Dislike extremely
2	Dislike very much
3	Dislike moderately
4	Dislike slightly
5	Neither like nor dislike
6	Like slightly
7	Like moderately
8	Like very much
9	Like extremely

AROMA description: _____
TASTE description: _____

(Sample: / AROMA / TASTE / OVERALL LIKING tables identical to the first sample)

AROMA description: _____
TASTE description: _____

(Sample: / AROMA / TASTE / OVERALL LIKING tables identical to the first sample)

AROMA description: _____
TASTE description: _____

(Sample: / AROMA / TASTE / OVERALL LIKING tables identical to the first sample)

AROMA description: _____
TASTE description: _____

(Sample: / AROMA / TASTE / OVERALL LIKING tables identical to the first sample)

AROMA description: _____
TASTE description: _____

WHICH CULTIVAR DO YOU THINK YOU TASTED? Shiraz Pinotage Merlot Cabernet Sauvignon

ARE YOU AWARE OF THE PROCESS OF MALOLACTIC FERMENTATION DURING WINEMAKING? Yes No
IF YES, WHAT DO YOU THINK ARE THE MAIN EFFECTS OF THIS PROCESS? _____

Figure 5.2 Questionnaire presented to consumers during the tasting. Demographic information and information related to wine consumption habits and occupation were collected. Information related to wine knowledge was obtained in the form of a mini quiz after completion of the tasting.

5.2.4 STATISTICAL ANALYSIS OF DATA

5.2.4.1 Descriptive sensory analysis

A randomized complete block design was used with five treatments and three replications for the descriptive sensory analysis. Quantitative descriptive analysis data were exported from CompuSense *five* software (release 4.8, Compusense Inc., Guelph, Canada) to PanelCheck software (version 1.3.2, Nofima, Norway) for the purpose of initial data analysis and panel performance evaluation (as described by Tomic *et al*. (2010) using Tucker1 plots, profile plots and 3-way analysis of variance). Data were subjected to test-retest analysis of variance (ANOVA) using SAS® software (Version 9; SAS® Institute Inc, Cary, USA) to test for reliability, i.e. temporal stability (Judge*Replication interaction) and internal consistency (Judge*Level interaction) (SAS®, 2002). The Shapiro-Wilk test was used to test for non-normality (Shapiro & Wilk, 1965). If non-normality was significant ($P \leq 0.05$) and caused by skewness, the outliers were identified and removed until the data were normal distributed (Glass *et al.,* 1972). The ANOVA was retested after the above-mentioned procedures were done. Student's t-least significant difference (LSD) was calculated at the 5% significance level to compare treatment means for each sensory attribute. The analysis was performed for all three sets of wine tested, namely Shiraz 2008, Pinotage 2008 and Pinotage 2009.

5.2.4.2 Consumer sensory analysis

The consumer data were analysed using ANOVA (SAS®, Version 9; SAS® Institute Inc, Cary, USA.). The Shapiro-Wilk test was used to test for non-normality in the data (Shapiro & Wilk, 1965). If skewness appeared to be the result of outliers, the outliers were identified and removed until the data were considered normal (Glass *et al.,* 1972).
Overall differences in preference for the wines were initially tested by ANOVA of the wine liking scores for all consumers. Preferences among consumers were further investigated by segmenting based on wine knowledge and demographic information.

5.2.4.3 Multivariate statistical techniques

Multivariate statistical analysis was performed using *The Unscrambler* software (version 9.2, CAMO ASA, Norway) and *PanelCheck* (version 1.3.2, Nofima, Norway) was used to evaluate panel performance. Principal component analysis (PCA) was used to investigate the distribution of the wine samples relative to each other based on their sensory attributes. Observations were also compared between different cultivars as well as between different vintages (within the same cultivar). Preference mapping (Greenhoff & MacFie, 1994; Schlich, 1995) was performed by regressing consumer preference scores (y-space) onto the trained panel descriptive data (x-space) to investigate relationships between sensory attributes and consumer liking using principal component regression (PCR).

5.3 RESULTS AND DISCUSSION

5.3.1 PRELIMINARY DISCRIMINATIVE TESTING

Initial discriminative testing of the control wine that did not undergo MLF and a wine that did undergo MLF was conducted with a panel of regular wine tasters. This was done in order to evaluate whether sensory differences between the control wine and a MLF wine could be perceived. According to the Roussler table (Lawless & Heymann, 1998) a significant difference existed between the two presented samples since 9 out of the 12 tasters indicated a perceivable difference between the samples. Tasters also indicated that the difference was perceived in terms of both aroma and taste/mouth-feel which has been previously associated with MLF (Bartowsky & Henschke, 1995).

5.3.2 DESCRIPTIVE TESTING

In order to describe the specific sensory differences perceived between the samples, a quantitative descriptive analysis was performed on the wines.

5.3.2.1 Panel performance

Assessor and panel performance was assessed with the use of the Tucker-1 multivariate analysis method for the multiple sensory attributes evaluated in this study (data not shown). This method allows for the visual identification of assessors performing differently from the rest of the panel as well as identifying sensory attributes in need of additional training (Tomic *et al*., 2010). Complementary to this, profile and line plots as well as plots related to ANOVA results where used to investigate panel discrimination ability and repeatability (Tomic *et al*., 2010). Panel performance was found to be acceptable for wine evaluation during this study.

5.3.2.2 Sensory differences imparted by malolactic fermentation in Shiraz wine

Results from the PCA clearly show differentiation between the different bacteria and the control wine (**Figure 5.3**) for the measured sensory attributes. Three groups of samples were identified. The control sample, Lalvin VP41 (depicted as wine V) and Viniflora CH16 (depicted as wine C) grouped towards the top part of PC2, correlated with the body/mouth-feel, fruity and berry aroma and flavour attributes. Enoferm alpha (depicted as wine A) and Viniflora oenos (depicted as wine O) however, were positioned towards the bottom half of PC2 and were negatively correlated with the fruity attributes. Separation between Enoferm alpha (wine A) and Viniflora oenos (wine O) along PC1 seems to be driven by positive correlation of these wines with the buttery and yoghurt/buttermilk attributes, respectively. Enoferm alpha (wine A) is correlated with the buttery and smoothness attributes towards the right-hand side of PC1 while Vinifloral oenos (wine O) is correlated with the yoghurt/buttermilk and sour taste attributes towards the left-hand side of PC1. Enoferm alpha (wine A) and Viniflora oenos (wine O) displays more typical MLF aroma characteristics (Bartowsky & Henschke, 1995) in this particular Shiraz while the control, Lalvin VP41 (wine V) and Viniflora CH16 (wine C) display fruitier aroma characteristics.

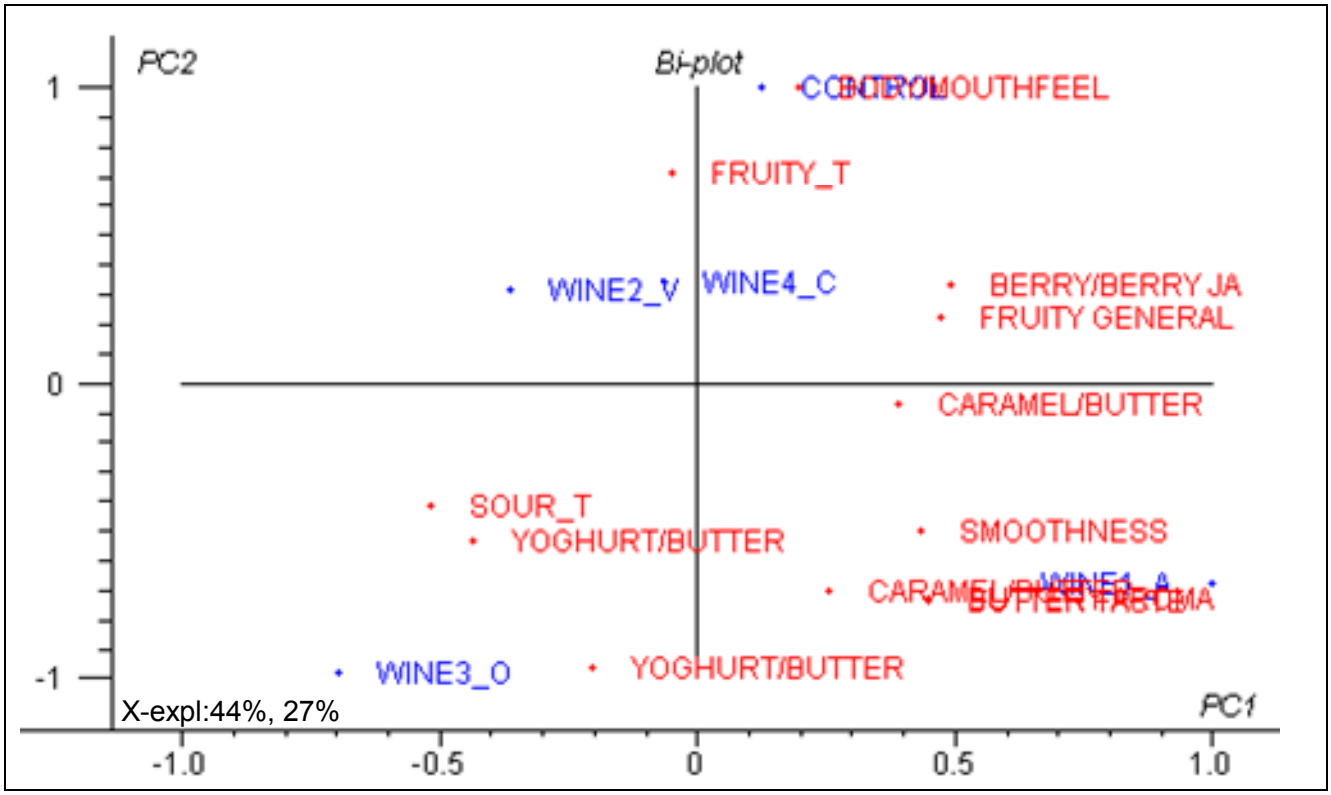


Figure 5.3 The PCA biplot show correlations between the Shiraz 2008 samples (in blue text) and the perceived sensory attributes (indicated in red text). PC1 and PC2 explain 44% and 27% of the variance, respectively.

Each sensory attribute was also tested for significant differences across the different wine treatments. Results for the ANOVA substantiate the PCA observations and are summarized in **Table 5.5** and discussed in brief. No significant differences were observed for fruity aroma, caramel/butterscotch aroma and flavour, sour taste, smoothness and body/mouth-feel sensory attributes. The control wine, and Enoferm alpha (wine A) had significantly higher levels of perceivable berry/berry jam aroma attribute than wine fermented with Viniflora oenos (wine O) and Lalvin VP41 (wine V). Viniflora CH16 (wine C) exhibited no significant difference in terms of berry/berry jam aroma from the other wines. The control wine and wine fermented with Viniflora oenos (wine O) had the higher perceived fruity flavour. Enoferm alpha (wine A), Lalvin VP41 (wine V) and Viniflora CH16 (wine C) did not have significantly less fruity flavour than Viniflora oenos (wine O), however these wines did differ significantly in this attribute from the control wine. Enoferm alpha (wine A) exhibited significantly higher buttery aroma compared to the rest of the wines. The control wine and Lalvin VP41 (wine V) were found to have the least perceived buttery aroma, while Viniflora oenos (wine O) and Viniflora CH16 (wine C) was perceived as significantly more buttery. Enoferm alpha (wine A) had a significantly higher butter taste compared to the other wines. Viniflora oenos (wine O) was perceived as the most intense in yoghurt/buttermilk aroma and flavour attributes. The other wines had significantly less perceived yoghurt/buttermilk attributes.

Table 5.5 Sensory attribute intensities perceived by the trained panel and ANOVA results for the different wine treatments evaluated in the Shiraz 2008 wine.

	Shiraz 2008 - Wine treatments				
AROMA	**Control**	**A**	**V**	**O**	**C**
Fruity general	18.13a*	18.92a	18.10a	17.47a	18.80a
Berry/berry jam	10.03a	9.97a	7.90b	7.60b	8.28ab
Prune	nd	nd	nd	nd	nd
Vegetative	nd	nd	nd	nd	nd
Caramel/butterscotch	0.68a	1.32a	1.13a	0.95a	0.70a
Buttery	7.28c	21.97a	8.03c	10.11bc	11.64b
Yoghurt/buttermilk	10.25b	10.27b	11.32b	15.21a	12.74ab
Floral/rose	nd	nd	nd	nd	nd
Nutty	nd	nd	nd	nd	nd
TASTE AND MOUTHFEEL					
Sour	23.02a	22.58a	24.73a	25.25a	23.30a
Fruity	15.73a	13.70b	13.92b	14.22ab	13.72b
Vegetative	nd	nd	nd	nd	nd
Yoghurt/buttermilk	2.88b	3.67b	2.38b	6.23a	2.40b
Caramel/butterscotch	0.47a	0.48a	0.25a	0.32a	0.21a
Butter taste	3.48b	8.38a	4.20b	4.37b	3.77b
Smoothness	51.07a	52.00a	50.22a	51.10a	51.30a
Body/mouthfeel	48.00a	47.23a	47.90a	45.17a	48.20a

*Means with different letters (a, b, c) row wise are significantly different ($p<0.05$); nd = not detected.

5.3.2.3 Sensory impact of MLF starter cultures over two vintages in Pinotage wine

Differentiation in the perceived sensory properties of different MLF starter cultures in Pinotage is evident from the PCA results shown in **Figure 5.4**. No caramel/butterscotch aroma or flavour was perceived in this set of wines. In comparison to the Shiraz wine experiment, additional sensory attributes were perceived in the Pinotage 2008 wine. During the 2008 vintage (**Figure 5.4a**), the control sample (no MLF) and Lalvin VP41 (wine V) separated towards the negative side of PC1 and showed strong correlation with the nutty aroma, vegetative aroma and flavour attributes while these attributes were negatively correlated with Viniflora oenos (wine O) and Viniflora CH16 (wine C). Viniflora oenos (wine O) separates towards the positive side of PC1 and is strongly correlated with the buttery aroma attribute. A combination between fruity and buttery-related attributes appears to drive the separation of Viniflora oenos (wine O), Viniflora CH16 (wine C) and Enoferm alpha (wine A) away from the more vegetative Lalvin VP41 (wine V) and the control wine along PC1. These observations are supported by the ANOVA results which are shown in **Table 5.6**.

One-way ANOVA (**Table 5.6**) for the Pinotage 2008 sensory attributes indicates that Viniflora oenos (wine O) and Viniflora CH16 (wine C) was perceived as the most intense in fruity aroma, however no significant difference was observed compared to Enoferm alpha (wine A) and the control wine. Lalvin VP41 (wine V) was found to have significantly less fruity aroma. No significant differences was observed for the fruity flavour, berry/berry jam aroma and flavour, prune aroma, yoghurt/buttermilk aroma and flavour and floral/rose aroma. The control wine and Lalvin VP41 (wine V) were perceived to have significantly more intense vegetative aroma notes, followed by Enoferm

alpha (wine A). Viniflora oenos (wine O) and Viniflora CH16 (wine C) were found to have the least intense vegetative aroma. The control wine had the most intense vegetative flavour while Viniflora CH16 (wine C) was perceived as the least vegetatively flavour wine. Viniflora oenos (wine O) was perceived as significantly more intense in buttery aroma compared to the other wines. No significant differences were observed amongst the wine in terms of the butter taste. The most intense nutty aroma characteristic was perceived in the control wine, followed by Enoferm alpha (wine A). Viniflora oenos (wine O) and Viniflora CH16 (wine C) had the lowest rating for nutty aroma. No significant differences were observed for the sour taste, smoothness and body/mouthfeel of the tested wines.

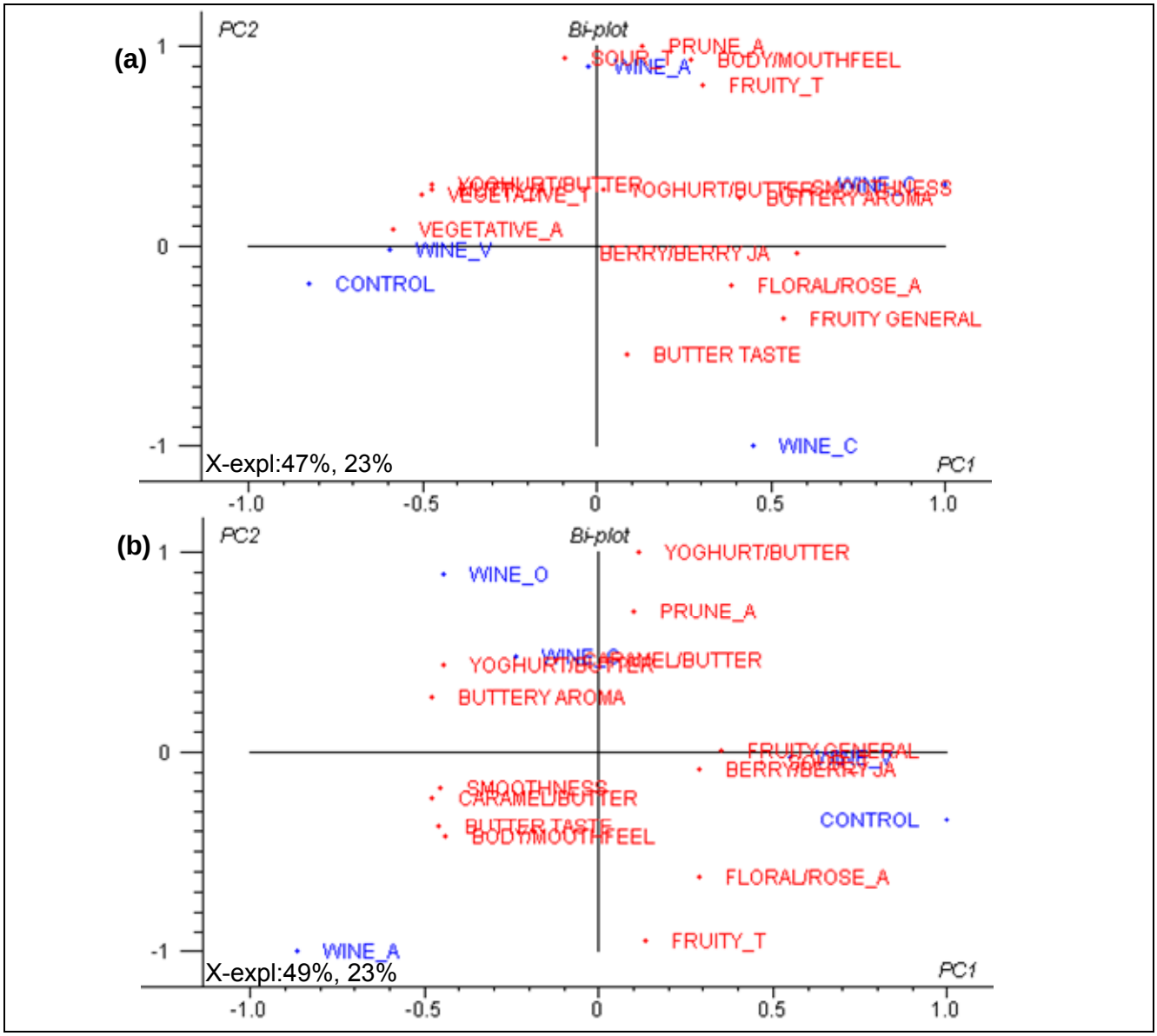


Figure 5.4 The PCA biplot for (a) Pinotage 2008 and (b) Pinotage 2009 samples based on the perceived sensory attributes. PC1 and PC2 cumulatively explained 70% and 72% of the total variance for Pinotage 2008 and Pinotage 2009, respectively.

Table 5.6 Sensory attribute intensities as perceived by the trained panel and ANOVA results for the different wine treatments evaluated in the Pinotage 2008 wine.

	Pinotage 2008 - Wine treatments				
AROMA	**Control**	**A**	**V**	**O**	**C**
Fruity general	16.81ab*	16.85ab	15.90b	19.67a	19.25a
Berry/berry jam	10.44a	10.48a	10.13a	12.46a	11.59a
Prune	4.73a	5.67a	4.17a	5.23a	3.96a
Vegetative	29.58a	19.79b	27.77a	8.73c	12.54c
Caramel/butterscotch	nd	nd	nd	nd	nd
Buttery	4.00b	3.73b	4.56b	9.15a	3.92b
Yoghurt/buttermilk	1.90a	2.18a	0.94a	1.40a	1.92a
Floral/rose	1.09a	1.04a	1.72a	2.00a	1.52a
Nutty	5.70a	3.83ab	2.10bc	0.44c	0.96c
TASTE AND MOUTHFEEL					
Sour	13.29a	13.75a	13.88a	13.75a	12.48a
Fruity	12.72a	14.46a	13.98a	14.40a	13.35a
Vegetative	13.69a	7.27bc	9.38b	5.15cd	2.65d
Yoghurt/buttermilk	0.94a	0.56a	1.02a	0.50a	0.21a
Caramel/butterscotch	nd	nd	nd	nd	nd
Butter taste	5.30a	3.06a	3.19a	5.00a	4.71a
Smoothness	38.52a	39.29a	38.40a	39.52a	39.17a
Body/mouthfeel	38.71a	39.27a	38.50a	39.23a	38.48a

*Means with different letters (a, b, c) row wise are significantly different ($p<0.05$); nd = not detected.

PCA results for the 2009 vintage (**Figure 5.4b**) show differentiation amongst the different MLF bacteria tested and the control wine in terms of sensory attributes. Similar to the 2008 vintage, the control and the wine fermented with Lalvin VP41 (wine V) exhibit similar sensory attributes and group together along PC1. The sensory characteristics responsible for this grouping differ from the previous season (**Figure 5.4a**) where vegetative aroma and flavour separated these wines from the rest. However, no vegetative sensory attributes were detected in the 2009 wines. The fruity and berry/berry jam sensory attributes are correlated with the control wine and Lalvin VP41 (wine V) as well as the sour taste attribute, all separating towards the positive side of PC1. Wine fermented with Viniflora oenos (wine O), Viniflora CH16 (wine C) and Enoferm alpha (wine A) separate towards the negative side of PC1 and has correlation with buttery aroma, yoghurt/buttermilk aroma and flavour, caramel/butterscotch aroma and flavour as well as with smoothness and body/mouth-feel. Viniflora oenos (wine O) and Viniflora CH16 (wine C) are more similar in sensory characteristics and separate to the top part of PC2 while Enoferm alpha (wine A) is positioned more to the bottom of PC2. This could be the result of Enoferm alpha (wine A) having increased smoothness, body/mouth-feel and buttery taste compared to Viniflora oenos (wine O) and Viniflora CH16 (wine C).

Substantiating ANOVA results are summarised in **Table 5.7** for the Pinotage 2009 wines. Fruity aroma and flavour attributes showed no significant differences perceived amongst the different wine treatments. Similarly, no significant differences were observed for prune and floral/rose aroma attributes. The control wine was perceived as having significantly more berry/berry jam aroma characteristics compared to the other wines, followed closely by Viniflora oenos (wine O). Enoferm

alpha (wine A), Viniflora oenos (wine O) and Viniflora CH16 (wine C) were perceived as having more caramel/butterscotch aroma than Lalvin VP41 (wine V) and the control wine, although these ratings were very low. Viniflora CH16 (wine C) had a significantly higher rating for caramel/butterscotch flavour than the rest of the wines.

Table 5.7 Sensory attribute intensities as perceived by the trained panel and ANOVA results for the different wine treatments evaluated in the Pinotage 2009 wine.

	Pinotage 2009 - Wine treatments				
AROMA	**Control**	**A**	**V**	**O**	**C**
Fruity general	32.18a*	27.77a	27.90a	29.03a	28.16a
Berry/berry jam	16.78a	13.63b	13.28b	14.73ab	13.03b
Prune	4.22a	3.26a	3.48a	4.43a	5.19a
Vegetative	nd	nd	nd	nd	nd
Caramel/butterscotch	0.02c	1.28a	0.42bc	0.73ab	0.88ab
Buttery	3.87c	12.15a	6.95bc	11.67ab	13.17a
Yoghurt/buttermilk	3.43b	5.55ab	3.42b	6.69a	5.50ab
Floral/rose	2.48a	2.81a	3.00a	1.82a	2.87a
Nutty	nd	nd	nd	nd	nd
TASTE AND MOUTHFEEL					
Sour	38.83a	13.75a	35.77a	35.73a	37.73a
Fruity	9.73a	9.60a	8.85a	8.32a	8.90a
Vegetative	nd	nd	nd	nd	nd
Yoghurt/buttermilk	2.32ab	1.43b	2.35ab	3.15a	2.37ab
Caramel/butterscotch	0.07b	0.08b	0.08b	0.12b	0.50a
Butter taste	3.87c	10.43a	3.17c	7.29b	5.57bc
Smoothness	27.22b	30.50a	25.90b	28.90ab	29.05ab
Body/mouthfeel	25.50b	29.15a	24.50b	26.85ab	26.35ab

*Means with different letters (a, b, c) row wise are significantly different ($p<0.05$); nd = not detected.

5.3.3 CONSUMER TESTING

5.3.3.1 Overall-liking, aroma-liking and taste-liking

In order to evaluate whether the sensory differences obtained with a trained panel are perceivable by consumers or influence their degree of liking, a 9-point hedonic test was performed. Consumers rated the overall-liking, aroma-liking and taste-liking for each of the five wines presented in each set of the cultivars tested over 3 consecutive days. Results are presented in **Figure 5.5** for the Pinotage 2008 wine (**Figure 5.5a**), Pinotage 2009 (**Figure 5.5b**) and Shiraz 2008 wine (**Figure 5.5c**).

In the Pinotage 2008 set of wines (**Figure 5.5a**), the control sample was least preferred for aroma and taste-liking while for overall-liking, Lalvin VP41 (wine V) and the control was least preferred. Viniflora CH16 (wine C) was significantly more preferred in terms of overall-liking, followed by Viniflora oenos (wine O). However, in terms of taste-liking, Viniflora oenos (wine O) was preferred significantly more compared to the rest of the wines. Differences between the aroma-liking of the wines were not

that observable with a tendency for wines fermented with Viniflora CH16 (wine C) and Viniflora oenos (wine O) to be more preferred in this data set.

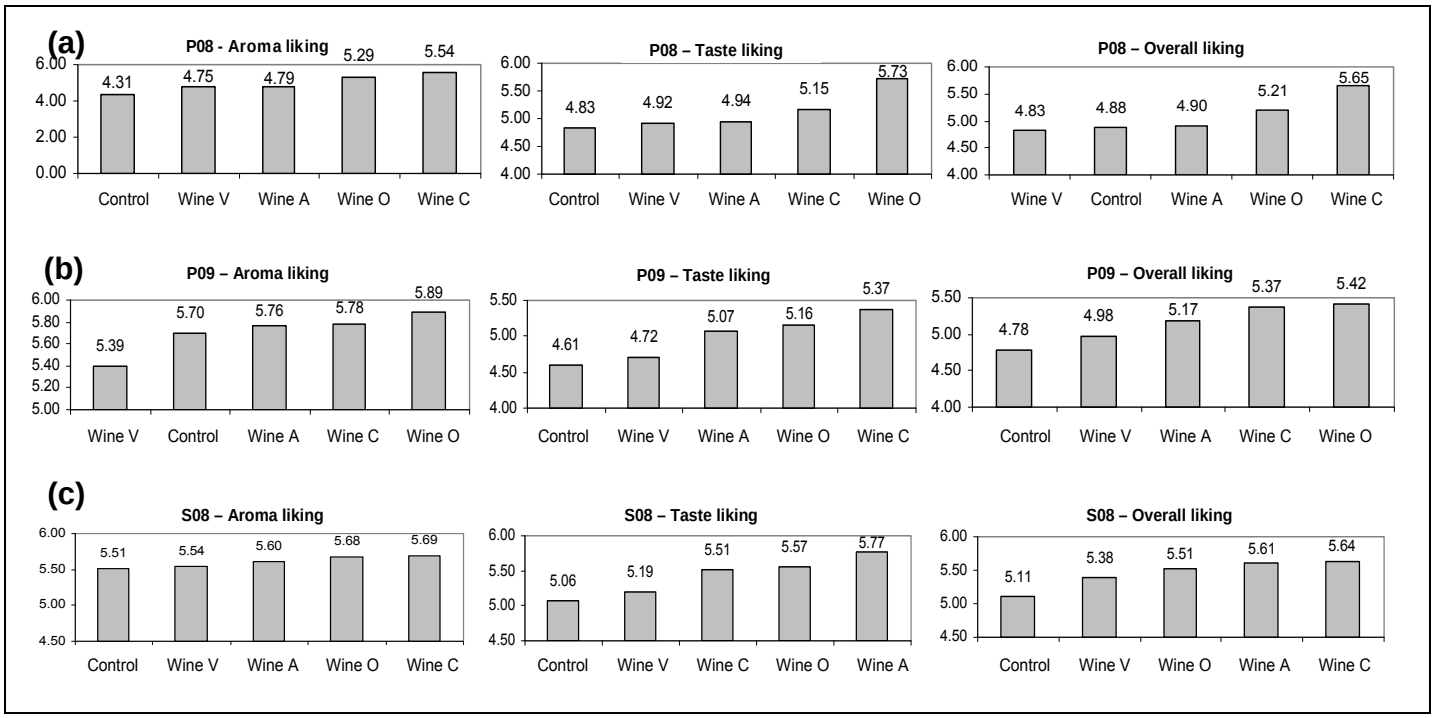


Figure 5.5 Mean aroma liking, taste liking and overall liking for the respective wines: (a) Pinotage 2008 wines, (b) Pinotage 2009 wine, (c) Shiraz 2008 wine. The control and wine V was less preferred, while wine C was most preferred over the different wines tested.

Aroma-liking in the Pinotage 2009 wines (**Figure 5.5b**) showed similar trends compared to the Pinotage 2008 wines with Viniflora oenos (wine O) and Viniflora CH16 (wine C) being most preferred. The aroma of Lalvin VP41 (wine V) was significantly less preferred than the other wines. Similar trends were observed in the taste and overall-liking. The control wines had the lowest rating, followed by Lalvin VP41 (wine V). Viniflora CH16 (wine C) received the highest rating for taste-liking. Overall-liking indicated that Viniflora oenos (wine O) followed closely by Viniflora CH16 (wine C) was most preferred.

The overall, aroma and taste that was least preferred in the Shiraz 2008 wine (**Figure 5.5c**), was that of the control wine, followed by Lalvin VP41 (wine V). Viniflora CH16 (wine C) and Enoferm alpha (wine A) received the higher overall-liking scores. Wine fermented with Enoferm alpha (wine A) scored the highest for taste-liking, while Viniflora CH16 (wine C) was most preferred in terms of aroma.

5.3.3.2 Results from segmentation of consumers according to wine knowledge

In view of the fact that consumers recruited for this study included personnel from a viticulture and oenology department as well as from a wine biotechnology institute, this factor was taken into account. After completion of the tasting, consumers were asked to complete a mini wine quiz. Scores from the wine quiz were used to segment consumers into 2 groups depending on their quiz score. Consumers

with scores between one and five were assigned to the low-medium group, while consumers with quiz scores ranging from six to ten comprised the medium-high segment. Additional segmentation techniques were also implemented in order to take demographic information into account, these are discussed elsewhere. For the purpose of illustration, the consumer segmentation results based on wine knowledge for the Shiraz 2008 wine is shown for overall-liking of the wines (**Figure 5.6**). Consumers with med-high score (**Figure 5.6a**) in the wine quiz preferred Enoferm alpha (wine A) for overall-liking, while the other group of consumers (**Figure 5.6b**) preferred Viniflora CH16 (wine C). The control wine was least liked by consumers in the low-medium group while consumers in the med-high segment least preferred Viniflora oenos (wine O). Similar differences in aroma and taste-liking was observed for these two different segments of consumers for the Pinotage wines (data not shown). The effect of wine knowledge on degree of liking of red wine was previously illustrated (Frøst & Noble, 2002) is supported by the results from this study.

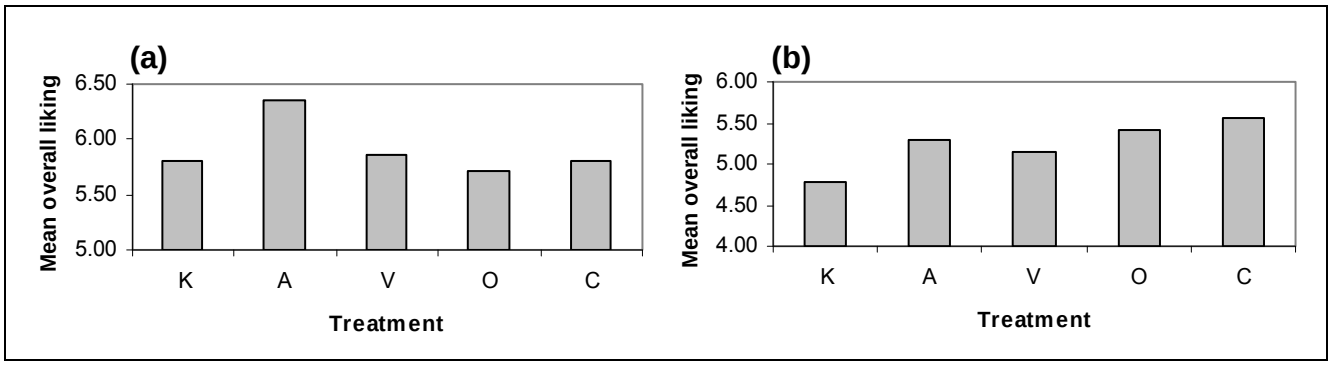


Figure 5.6 Consumer segmentation according to wine knowledge illustrates different preferences for Shiraz wine fermented with four commercial starter cultures. Overall liking for consumers with (a) medium-high quiz scores and (b) low-medium quiz scores are shown.

5.3.3.3 Preference mapping

In order to relate the overall-liking of the wines to the sensory attributes obtained with the trained panel, PCR models were constructed for the purpose of preference mapping. In the Shiraz wines the majority of the consumers are positioned on the left of the plot (**Figure 5.7**). This correlates with Viniflora oenos (wine O), Lalvin VP41 (wine V), Viniflora CH16 (wine C) and the control wine. However, within this group it is observed that some consumers preferred wine fermented with Viniflora oenos (wine O) while another group preferred wines fermented with Lalvin VP41 (wine V), Viniflora CH16 (wine C) and the control. According to the sensory attributes obtained for these wines using a trained panel, Viniflora oenos (wine O) is more associated with a sour taste and yoghurt/buttermilk aroma and flavour compared to wines fermented with Lalvin VP41 (wines V), Viniflora CH16 (wine C) and the control which had a more fruity flavour. A small number of consumers showed a preference for Enoferm alpha (wine A), positioned towards the positive side of PC1. Enoferm alpha (wine A) is characterised by a more buttery aroma, smoothness as well as some fruity and berry aroma attributes. It might be possible that the intense buttery aroma reduced the preference for Enoferm alpha (wine A) in this specific Shiraz.

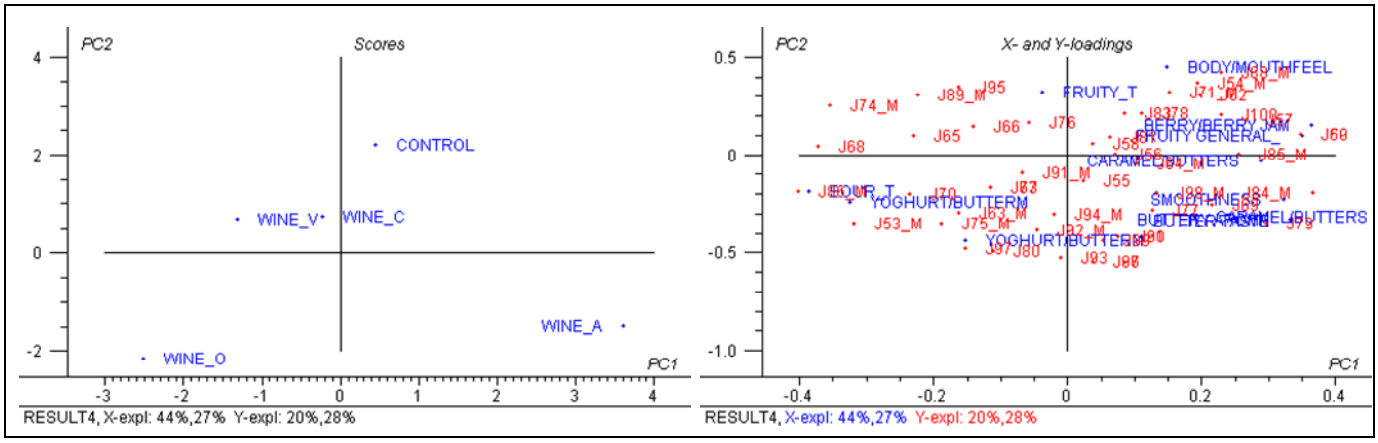


Figure 5.7 Preference mapping with the use of PCR relates the overall consumer liking of each judge to the observed sensory attributes of the different samples in the Shiraz 2008 wine. Judges indicated as numbers (in red text) and sensory attributes (indicated in blue text) are shown on the loadings plot.

The PCR of consumer liking scores (**Figure 5.8**) for the Pinotage 2008 wines showed the same trends as the mean liking calculated, but provide a relation to the sensory attributes of the samples. In this analysis, the first dimension discriminated between the most liked Viniflora CH16 (wine C) and Viniflora oenos (wine O) and the least liked wines, Lalvin VP41 (wine V) and the control. Very little consumers fell close to the two least liked wines (control and Lalvin VP41 (wine V)), consistent with their lower liking scores. These wines were correlated with sour taste, yoghurt/buttermilk aroma, vegetative aroma and flavour attributes. In contrast, there is a fairly high density of subjects around the most liked wines: wines fermented with Viniflora oenos (wine O) and Viiflora CH16 (wine C). This preference is driven by fruity, berry attributes as well as buttery aroma and taste. Interestingly, this is in contrast with the Shiraz 2008 wine where preference was reduced by the buttery attributes.

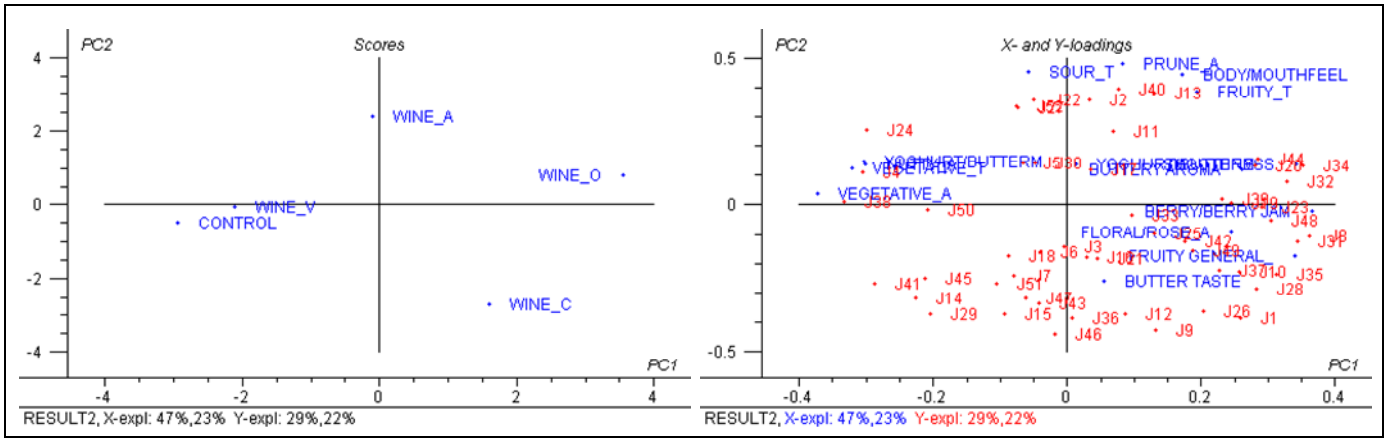


Figure 5.8 The overall liking scores of the Pinotage 2008 wines are related to the sensory attributes by means of PCR preference mapping of the different samples. Judges indicated as numbers (in red text) and sensory attributes (indicated in blue text) are shown on the loadings plot.

Preference mapping (**Figure 5.9**) with the use of PCR related sensory attributes to the consumer-liking scores of the Pinotage 2009 wine fermented with four different MLF starter cultures. Separation

along PC1 discriminates between least liked (wine fermented with Lalvin VP41 (wine V) and control) and most liked samples, Viniflora oenos (wine O) and Viniflora CH16 (wine C). This is in agreement with observations made in the Pinotage 2008 wine. Lalvin VP41 (wine V) and the control wine are correlated with sour taste, fruity and floral sensory attributes. There is a marked higher density of consumers located to the left of the plot, showing preference for aroma attributes related to yoghurt/buttermilk, buttery, caramel/butterscotch as well as smoothness and body/mouth-feel attributes. Buttery aroma and taste seems to impart a perception of smoothness or increased body/mouth-feel resulting in possible increased preference. However, this observation seems to be cultivar dependent as well as dependent on the intensity of the specific character. The same increased preference was not observed for wine with an overly buttery character in the Shiraz 2008 wines. In general, there is a rather low degree of explained variance for consumer liking ratings (~45-50%), indicating substantial individual differences in preferences for these wines. However, it is not uncommon that the explained variance is low in consumer testing (Schlich, 1995).

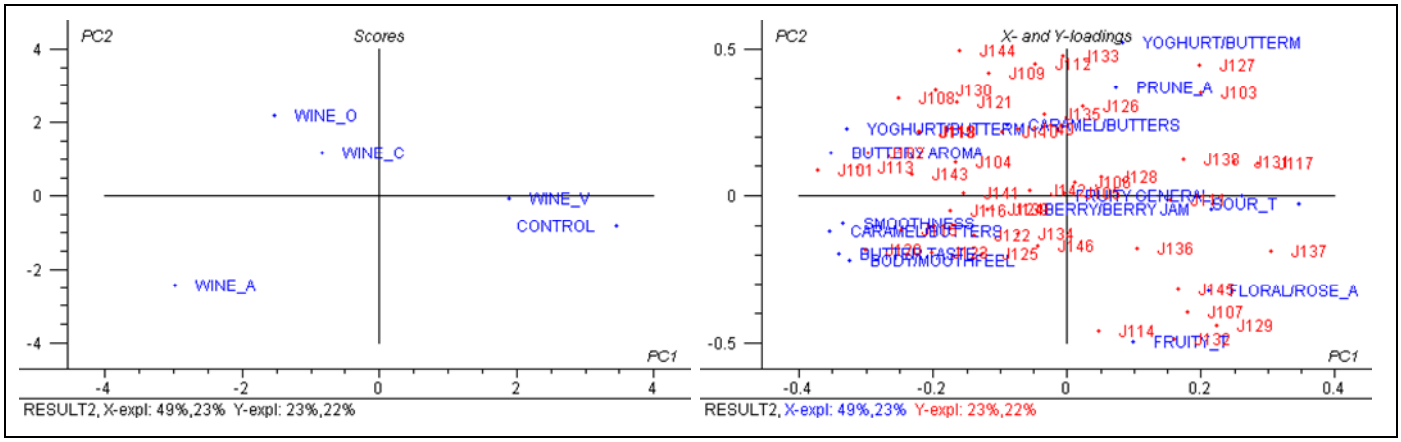


Figure 5.9 The overall liking for the Pinotage 2009 wines are related to the sensory attributes by means of preference mapping of the different samples. Judges indicated as numbers (in red text) and sensory attributes (indicated in blue text) are shown on the loadings plot.

5.4 CONCLUSIONS

Descriptive analysis of the wines revealed sensory differences amongst wine fermented with Enoferm alpha®, Lalvin VP41®, Viniflora oenos® and Viniflora CH16® MLF starter cultures. Pinotage 2008 wines differed significantly across the wine treatments in the buttery, nutty, fruity and vegetative aroma attributes. Pinotage 2009 wine sensory data indicated significant differences amongst the different bacteria and the control in terms of the berry/berry jam, caramel/butterscotch, buttery, smoothness and body/mouth-feel sensory attributes. Significant differences for berry/berry jam, buttery, and yoghurt/buttermilk aroma and flavour were observed in the Shiraz 2008 wine samples. Shiraz showed more pronounced sensory differences between the four bacteria compared to the Pinotage wines.

Differences in degree of liking were found for the wines using consumer panels. Apart form the small size of the consumer panel, interesting observations could be made regarding relationship between sensory attributes and consumer-liking. For each wine, substantial standard deviation in the

liking scores were observed, reflecting large individual differences in preference. Preference mapping techniques highlighted that overall, consumers preferred wines that have gone through MLF more than the control wines. Interestingly, overall-liking of the wines is not necessarily representative of the trends observed for aroma-liking or taste-liking individually, but rather a combination.

In this specific study, a number of questions related to wine knowledge were asked in order to investigate the influence of wine knowledge on consumer-liking. Segmentation based on wine knowledge showed differences in overall-liking scores, suggesting that consumers more exposed to wine tasting on a regular basis exhibit different preferences for the wine treatments than consumers with less wine knowledge. This is in agreement with findings from a previous study (Frøst & Noble, 2002).

In summary, this study provided considerable insight into the positive contribution of MLF starter cultures to wine aroma and flavour. Specific starter culture aroma contributions were observed in the 3 different wines studied. Between vintage variations also seem to be comparable between the different bacteria. Preference mapping of the sensory data in combination with the liking-scores provided extremely valuable information regarding the possible influence of bacterial strains on consumer-liking, something that has not been shown previously across different cultivars. Correlations between the changes observed in the sensory profiles and the changes observed in the chemical composition related to the use of specific starter cultures should be further investigated. Future research could also focus on sensory evaluation of wines fermented by different bacteria after a period of barrel ageing, which is a common practice in the wine industry. This would be of value to investigate whether ageing in combination with wood contact diminishes the differences observed amongst bacteria or enhances the differences.

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CHAPTER 6

General Discussion & Conclusions

6. GENERAL DISCUSSION AND CONCLUSIONS

This research work investigated the contribution of four commercial malolactic fermentation (MLF) bacterial starter cultures (*Oenococcus oeni*) to the volatile composition, organic acid content and infrared spectral properties of Shiraz and Pinotage red wines that were produced under small scale standardised experimental conditions. The different MLF wines, Enoferm alpha®, Lalvin VP41®, Viniflora oenos® and Viniflora CH16®, were compared to a control wine where MLF was suppressed. MLF progress was monitored with both mid and near infrared spectroscopy and the acquired data was used to establish metabolic fingerprints of the wine. In addition, gas chromatographic (GC) analysis, with flame ionisation detection (FID) or mass spectrometry (MS) detection, and capillary electrophoresis (CE) were used to evaluate the volatile composition and organic acid profiles respectively, of these wines. In an attempt to better understand the contribution of different MLF starter cultures to wine aroma in the context of observed chemical changes, the sensory properties of the resulting wines were assessed through quantitative descriptive profiling by a trained sensory panel. Furthermore, preliminary consumer testing was performed to investigate whether the selection of different bacterial starter cultures could possibly have an influence on consumer preference.

6.1 GENERAL DISCUSSION

Malolactic fermentation (MLF), performed by lactic acid bacteria (LAB), is one of the biological processes during winemaking which could contribute to the hundreds of chemical compounds influencing wine aroma and flavour (recently reviewed by Lerm *et al*., 2010). The primary reaction of MLF converts L-malic acid to L-lactic acid and carbon dioxide, resulting in a less acidic wine with sensory consequences such as the perception of a softer, rounder pallet (Bartowsky & Henschke, 1995). It is for this reason that winemakers use the process of MLF primarily for the deacidification of virtually all red wines, to a lesser extent sparkling base wines and several varieties of white wine, in particular Chardonnay. In addition to the mentioned primary MLF reaction, LAB could also alter the chemical composition of wine and potentially influence wine aroma and flavour through the formation of various other metabolites (Liu, 2002), such as esters, higher alcohols, acids and carbonyl compounds. In contrast to the information available on yeast and wine aroma in terms of genetics, metabolic mechanisms and considerations with regard to winemaking practices (Lambrechts & Pretorius, 2000; Swiegers *et al*., 2005), the influence of LAB on volatile composition and the resulting sensory impact are poorly understood.

This study was therefore initiated to investigate the changes associated with MLF and to establish if commercial LAB cultures contribute significantly to the organoleptic properties of red wine. In order to achieve this, multiple analytical approaches were implemented for the broad-range profiling of chemical compounds produced by the different commercial bacterial starter cultures Enoferm alpha®, Lalvin VP41®, Viniflora oenos® and Viniflora CH16® under controlled winemaking conditions. Due to limited reports on the sensory impact of MLF starter cultures used in red wines, two of the major red cultivars produced in South Africa (Lerm, 2010) namely, Shiraz and Pinotage, were selected for this study.

In an attempt to better understand the chemical changes imparted by the different MLF starter cultures studied in this dissertation, it was necessary that two analytical methodologies were developed, optimised and implemented for the quantification of volatile compounds.

The first methodology, a fast gas chromatography flame ionisation detection (GC-FID) method was developed during this study for the quantification of 39 major wine volatile compounds within a 15 minute GC run. A 3-fold reduction in analysis time compared to an in-house conventional GC method (45 minutes; Louw *et al*., 2009) was thus achieved. The analytes quantified within a single fast GC run include a diverse group of compounds including esters, higher alcohols and fatty acids produced during alcoholic and malolactic fermentation. This selection of analytes comprises a large boiling point range (from ethyl acetate to decanoic acid) as well as a large polarity range (from polar acids/alcohols to less polar esters) which illustrates the robustness of the method. Another advantage of this method is that it operates at pressure ranges which are acceptable for use on conventional instruments without the need for special high pressure systems. The fast GC-FID method developed is therefore suitable for wine analysis in laboratories with routine instrumentation since it quantifies such a diverse group of chemical compounds found in wine. This offers a valuable high-throughput tool ideally suited as a research screening method for metabolic profiling in combination with multivariate data analysis techniques. Other applications of fast GC have been reported for the rapid profiling of complex mixtures such as polychlorinated biphenyls (PCBs), fats and essential oils or pesticide extracts from water samples, nutmeg, bacterial fatty acid methyl esters (David *et al*. 1999), solvent mixtures (Chen *et al.*, 1998) and distilled alcoholic beverages (Mac Namara *et al*., 2005), to name a few. However, some of the reported methods only quantify a limited number of compounds of specific chemical classes. The method developed during this study is therefore more suitable for the variety of chemical compounds present in wine.

The second methodology in this dissertation presents a method for the direct quantification of diacetyl, acetoin, 2,3-pentanedione and a selection of aldehydes using headspace solid phase microextraction followed by gas chromatography mass spectrometry (HS SPME GC-MS). Although the quantification of diacetyl with SPME GC-MS has been previously reported (Hayasaka *et al*., 1999), the development of a suitable method for this study was a necessity at the time. After numerous failed attempts to derivatise the analytes with procedures previously described (Ferreira *et al.*, 2004, 2006; Wang *et al*., 2005), it was decided to evaluate the quantification of these compounds without derivatisation. Although the method validation results showed good linearity, repeatability and sufficient sensitivity for the expected concentrations of these compounds in wine, some of the aldehydes were not consistently detected in the wines tested. The possibility exist that these aldehydes are either present below the LODs (limits of detection) established with this method, or it could be speculated that they were completely degraded by bacterial metabolism. Nevertheless, this method provides a useful tool for investigating and monitoring the effect of MLF on the concentrations of diacetyl, acetoin and 2,3-pentanedione. Although the reduction in vegetative aromas have often been related to the possible degradation of aldehydes through bacterial metabolism (Henschke & Bartowsky, 1995), the effect of MLF on methoxypyrazines, also associated with green notes, merits further investigation.

In addition to the volatile chemical compounds, the fermentative behaviour of Enoferm alpha®, Lalvin VP41®, Viniflora oenos® and Viniflora CH16® MLF starter cultures revealed significant

differences in the fermentation rate and resulting organic acid profiles associated with each bacterial culture. The Lalvin VP41® starter culture consistently consumed the least amount of citric acid compared to the other three starter cultures. Since citric acid degradation is directly related to the formation of diacetyl (Henschke & Bartowsky, 1995), it was expected that this strain would exhibit the lowest production of diacetyl in comparison to the other strains. The production of lactic acid differed amongst the various strains, however no general trend in terms of strain characteristics was observed in both the cultivars tested over the seasons investigated. Subsequently, concentrations of compounds related to the production of citric acid and lactic acid, namely ethyl lactate, acetic acid, diacetyl and acetoin, were influenced accordingly. The Lalvin VP41® starter culture consistently produced the lowest concentration of diacetyl while Viniflora oenos® and Enoferm alpha® produced higher concentrations of this compound.

In terms of volatile composition, bacterial metabolic activity generally increased the higher alcohols, fatty acids and overall ester concentrations in comparison with the control wine in which MLF was suppressed. A larger increase was observed in ethyl esters compared to acetate esters which are in agreement with previous observations (Ugliano & Moio, 2005). This observation was independent of the bacterial strain and the grape cultivar tested. Overall, ethyl lactate, diethyl succinate, ethyl octanoate, ethyl-2-methylpropanoate and ethyl propionate were increased by MLF. Hexyl acetate, isoamyl acetate, 2-phenylethyl acetate and ethyl acetate were decreased or remained unchanged, depending on the bacterial strain and cultivar evaluated in this specific study. The differences observed in the concentrations of ethyl butyrate, ethyl propionate, ethyl-2-methylbutryate and ethyl isovalerate in the wines fermented with the respective bacterial strains could possibly suggest distinct differences in terms of esterase activity. A large portion of the esters, which are important contributors to the fruity aroma of wine, were found to have OAVs > 1 indicating their potential aroma contribution (recently reviewed by Sumby *et al*., 2010). The potential reduction of total aldehyde concentrations was observed with the completion of MLF, however further investigation is needed in this regard.

As an additional profiling approach, infrared spectral fingerprints were obtained which characterise wines produced by the different bacteria. In addition, the prediction of MLF related compounds, diacetyl, acetoin and 2,3-pentanedione, from mid-infrared spectra was explored by PLS models. The possibility of quantification of these compounds using infrared spectra merits further investigation with a larger data set spanning wider concentration ranges. Although some of the metabolites quantified could also be formed by yeast, the changes observed after the completion of MLF with different starter cultures in comparison with wines where MLF was suppressed, implicate the potential action of bacteria in the formation of these compounds.

In addition to investigating the differences observed in the chemical composition of wine fermented with different MLF starter cultures, this dissertation also observed the potential impact and influence of malolactic fermentation on the sensory characteristics of South African Pinotage and Shiraz wine. Quantitative descriptive analysis by a trained sensory panel was used to describe the sensory profiles of the respective wines. Significant differences between wines fermented with different starter cultures were observed for buttery, fruity, nutty and yoghurt/buttermilk sensory attributes as well as smoothness and mouth-feel descriptors and these were more preferred than the control wines that did not undergo MLF.

More importantly, this research contribution also observed that the sensory difference imparted by MLF bacteria could have the ability to potentially influence consumer preference of the resulting wines. The 9-point hedonic scale was used to determine consumers' degree of liking for the respective wines in terms of aroma, taste and overall-liking. The degree of liking for aroma appeared to play a major role in the overall-liking, since the wine most preferred on aroma was often also the wine most liked overall. The use of preference mapping to model the relationship between the sensory data and the consumer-liking scores highlighted potential drivers of liking which could potentially be linked to the bacterial strain used. Overall, the combination of buttery and berry or floral sensory attributes appears to drive the consumer-liking. It should be noted that apart from the aroma attributes, the smoothness attribute was also correlated with the most preferred samples. The consumer testing results could only be used as a preliminary assessment of the potential aroma contribution and impact of MLF starter cultures due to the possible interactions of bacteria with the wine environment. Apart from the different sensory attributes which was driving liking, an interesting trend was observed with the fact that the Viniflora CH16 starter culture was the culture consistently being more preferred in both the Shiraz and Pinotage and in both vintages evaluated.

6.2 SUMMARY OF RESEARCH FINDINGS

The main outcomes, results and research findings of this dissertation are:

- A simple and effective fast GC-FID method with a total run time of 15 minutes was developed for the high throughput quantification of 39 major volatile compounds;
- A headspace SPME GC-MS method for the direct quantification of diacetyl, acetoin, 2,3-pentanedione and a selection of aldehydes in a single run was established;
- Insight was gained regarding respective changes in the volatile composition of wines fermented with four different commercial starter cultures compared to a control;
- The potential impact of four LAB starter cultures on the wine sensory properties of Shiraz and Pinotage wine was shown;
- The possible effect of different MLF starter cultures on consumer perception and preference was observed;
- Trends in terms of consumer preference for a specific starter culture were observed irrespective of vintage or cultivar tested;
- Drivers of liking were identified by relating sensory data to consumer data through preference mapping and shown to differ between cultivars;
- Consumer segmentation highlighted the possible influence of the degree of wine knowledge on consumer preference; and
- Relationships between chemical compounds, spectral data, sensory and consumer data were observed through multi-way analysis.

6.3 FUTURE PERSPECTIVES

The focus of this investigation was to compare the contribution of four *O. oeni* strains to the volatile composition and sensory properties of the resulting wine by using the traditional method of sequential inoculation performed after the completion of alcoholic fermentation. However, the influence of different inoculation strategies on wine aroma merits further investigation as preliminary studies illustrate perceived differences. Early inoculation for MLF in German Pinot Blanc resulted in wines described as having more fruit, better 'structure', greater 'complexity' and less MLF character compared to wines inoculated post-alcoholic fermentation (Bartowsky *et al*., 2002). Another recent report (Lerm, 2010) also illustrated the potential use of co-inoculation during winemaking and further investigations with respect to the potential effect on wine aroma are therefore needed.

In addition to the use of single culture *O. oeni* starter cultures, a number of *O. oeni* combination starter cultures such as Biolact Acclimatée (AEB Group, Italy) and Biolact Acclimatée 4R (AEB Group, Italy), using 3 and 4 different *O. oeni*, have been introduced to the market. The effect of starter cultures other than *O. oeni*, for example *Lactobacillus plantarum* V22 (Lallemand), also needs thorough exploration. Recently, the use of a mixed starter culture, referring to a combination of different LAB genera, was introduced (Lerm, 2010) and this area of research also requires further characterisation in terms of impact on the chemical composition as well as the effect on sensory attributes through formal evaluation. Developments in terms of LAB starter cultures worldwide clearly indicate the realized potential of MLF to contribute to wine sensory attributes other than reducing acidity.

Apart from monitoring changes in the chemical and sensory properties, further genetic studies are needed to provide insight into the *O. oeni* metabolism in relation to its contribution to wine aroma. The *O. oeni* genome has been sequenced and analysed (Mills *et al*., 2005) and the genes that have been cloned and characterised include those involved in diacetyl synthesis (*alsD* and *alsS*; Garmyn *et al*., 1996), an esterase from *O. oeni* (*EstB*28; Sumby *et al*., 2009) and recently a cystathionine lyase involved in the formation of volatile sulphur compounds (Knoll *et al*., 2010). In a recent study which reported on the genetic diversity of 258 *O. oeni* strains from all over the world (Bridier *et al*., 2010), 3 groups of isolates showed distinct phylogenetic differentiation from the rest of the strains. These were isolates from South Africa, Chilli and Eastern France and this highlights the diversity amongst *O. oeni* and the potential of developing starter cultures for use in specific areas of origin. The development of strains with increased ability to express cultivar specific characteristics in a country is a concept that merits future investigation.

Following this study, it is clear that the characterisation of the changes in volatile aroma compounds related to MLF as a result of bacterial metabolism could be further explored with a number of analytical technologies. The use of comprehensive two-dimensional GC systems should be considered to gain insight into MLF and wine aroma. Preliminary results from a collaborative study to this project illustrate the potential to discriminate between a control wine and amongst wines fermented with different bacterial starter cultures using two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GCxGC-TOF-MS) (Vestner *et al*., 2010). Gas chromatography olfactometry could also be employed to investigate and gain a better understanding of the formation of aroma-active volatile compounds contributed by different bacteria during MLF. Another alternative

approach to investigate the effect of MLF on wine aroma includes modelling relationships between sensory and chemical data, as well as modelling the relationship between sensory attributes and infrared spectroscopy.

Finally, the long term effects of the influence of bacterial strains on volatile composition and resulting sensory properties of red wine should in future be determined in combination with winemaking practices that follow after MLF such as, barrel ageing regimes, lees contact and wood contact (Boido *et al*., 2009; de Revel *et al*., 1999; 2005). These factors also play a significant role on the organoleptic properties and overall quality of wine and the success thereof may unknowingly be linked with a "consumer-selected" MLF culture in future.

6.4 CONCLUDING REMARKS

The contribution of MLF to wine aroma has long been underestimated. However, concerted research efforts around the world have provided greater insight into the changes associated with the growth and metabolic activity of LAB during MLF in wine and reiterated its potential contribution to wine aroma.

This dissertation contributes to the knowledge currently available on MLF and more specifically the contribution of different bacterial strains to wine composition and potentially wine sensory attributes. A first approach to the organoleptic consequences of the use of commercial LAB starter cultures in warmer climate conditions has therefore been made for Pinotage and Shiraz wine. The specific contribution of MLF to the wine aroma attributes was supported by the changes observed in a large variety of chemical compounds. Moreover, the application of a variety of statistical techniques to the data generated revealed significant metabolic differences between *O. oeni* strains. Results also suggest a degree of diversity in the *O. oeni* group, since wines also showed specific characteristics depending on the LAB strain. These conclusions justify inducing MLF with selected strains that produce beneficial sensory attributes and no defects in wine. The supplementary consumer insight data provide additional evidence that MLF strain selection is an important contributor to the final sensory profile of wine and could influence consumer perception. This aspect could be exploited in order to produce wines suitable for specific consumer preferences in conjunction with the management of other important wine parameters.

In conclusion, the integrated analytical approach followed during this dissertation resulted in a comprehensive profile of chemical, spectral, sensory and consumer data and led to several interesting results. This research provides information which is both of fundamental and industrial importance and reiterates that MLF is more than deacidification.

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