Evaluating the influence of winemaking practices on biogenic amine production by wine microorganisms

by

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DECLARATION

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

_____ _____

Anita Yolandi Smit **Date**

SUMMARY

Biogenic amines are nitrogenous compounds of low molecular weight found in most fermented foods, including wine. These biologically produced amines are essential at low concentrations for normal metabolic and physiological functions in animals, plants and micro-organisms. However, biogenic amines can have adverse effects at high concentrations and pose a health risk for sensitive individuals. Symptoms include nausea, hot flushes, headaches, red rashes, respiratory distress and fluctuations in blood pressure. A number of countries have implemented upper limits for histamine in food and wine. This development has already started to threaten commercial export transactions and may become more serious in the near future, especially in the competitive wine industry of today. The most important biogenic amines in wine include histamine, tyramine, putrescine, cadaverine and phenylethylamine which are produced from the amino acids histidine, tyrosine, ornithine, lysine and phenylalanine respectively.

Biogenic amines are mainly produced in wine by microbial decarboxylation of the corresponding precursor amino acid. It may be produced by yeast during alcoholic fermentation, by lactic acid bacteria during malolactic fermentation, or potentially by spoilage microbes such as acetic acid bacteria and *Brettanomyces*. However, lactic acid bacteria are widely accepted as the main causative agents.

Inoculation with commercial malolactic fermentation starter cultures that do not possess the relevant decarboxylase genes may inhibit the growth and activity of decarboxylase positive indigenous bacteria and as such control the production of biogenic amines in wine. In this study it was shown that co-inoculation of malolactic starter cultures together with alcoholic fermentation could reduce the incidence of biogenic amines in wine compared to conventional inoculation protocols; presumably because undesirable activities were restrained at an earlier stage during co-inoculation. It was also indicated in this work that in some cases the effect of co-inoculation on biogenic amine reduction may only be visible after a period of ageing. The frequency of biogenic amine occurrence in wines aged for a short period was generally higher in the presence of fermentation lees than in its absence.

This work also included a preliminary investigation into the contribution of commercial wine yeast starter cultures to biogenic amine production. Diamines and polyamines (putrescine, spermidine and cadaverine) were produced to variable extents by all yeasts with very little or no production of physiologically important biogenic amines (histamine, tyramine and phenylethylamine).

Another objective of this study was to evaluate the influence of common winemaking practices on biogenic amine production under winemaking conditions. We have shown that biogenic amine production by lactic acid bacteria could be influenced, amongst others, by the presence of precursor amino acids in the grape must or wine, the time of contact between juice or wine and grape skins, the time of contact between wine and yeast lees, the presence of microbial nutrients, wine pH, sulphite and ethanol

levels, the phenolic composition of the wine and the number of decarboxylase positive lactic acid bacteria present in the wine.

Lately, the wine industry is under increasing pressure to increase measures to ensure food safety and security and to eliminate any compound, present even in trace amounts that could reduce the wholesomeness of the wine. The need arises for a rapid and inexpensive method for quality control. In this study we investigated the potential to use Fourier transform infrared spectroscopy to rapidly screen for the presence of elevated levels of biogenic amines. This presents a novel method for the detection and quantification of total biogenic amines in wines.

OPSOMMING

Biogeenamiene is stikstofbevattende verbindings met ‘n lae molekulêre gewig wat in meeste gefermenteerde voedselsoorte, insluitend wyn, voorkom. Hierdie biologies aktiewe amiene is noodsaaklik teen lae konsentrasies vir nomale metabolisme en fisiologiese funksies in diere, plante en mikroörganismes.

Biogeen amiene kan egter nadelige gevolge hê wanneer dit ingeneem word teen hoë konsentrasies en kan ‘n ernstige gesondheidsrisiko vir sensitiewe individue inhou. Algemene simptome van biogeenamiene sluit naarheid, warm gloede, hoofpyn, ‘n rooi uitslag, respiratoriese afwykings en fluktuasies in bloeddruk in. ‘n Aantal lande het reeds maksimum limiete vir die konsentrasie van histamien teenwoordig in voedsel en wyn geïmplimenteer. Hierdie ontwikkeling het alreeds begin om kommersiële uitvoertransaksies te bedreig en mag in erns toeneem in die nabye toekoms, veral weens die kompeterende aard van die hedendaagse wynindustrie. Die mees belangrike biogeenamiene in wyn sluit in histamien, tiramien, putresien, kadawerien and fenieletielamien, wat onderskeidelik geproduseer word vanaf die aminosuur voorlopers histidien, tirosien, ornitien, lisien en fenielalanien.

In wyn word biogeenamiene hoofsaaklik geproduseer deur die mikrobiese dekarboksilasie van die ooreenstemmende aminosuurvoorloper. Biogeenamiene kan deur gis geproduseer word tydens alkoholiese fermentasie, deur melksuurbakterieë gedurende appelmelksuurgisting of potensieël deur bederforganismes soos asynsuurbakterieë en *Brettanomyces*. Melksuurbakterieë word egter wyd aanvaar as die hoofbron van biogeen amien produksie.

Inokulasie van wyn vir appelmelksuurgisting met kommersiële aanvangskulture wat nie die relevante dekarboksilase gene besit nie kan die groei en aktiwiteit van dekarboksilase positiewe inheemse melksuurbakterieë inhibeer en sodoende die probleem van biogeenamiene in wyn beheer. In hierdie werk het ons gewys dat die ko-inokulasie van appelmelksuurgisting met alkoholiese fermentasie die voorkoms van biogeen amiene kan verlaag in vergelyking met konvensionele inokulasie-metodes; waarskynlik omdat ongewenste aktiwiteit reeds op ‘n vroeë stadium uitgeskakel word met ko-inokulasie. Dit is ook in hierdie werk gewys dat die effek van ko-inokulasie op biogeen amien vermindering soms eers na ‘n verouderingsperiode waarneembaar is. Die voorkoms van biogeen amiene in wyne wat vir ‘n kort tydperk veroudering ondergaan het was oor die algemeen hoër in die teenwoordigheid van gismoer as in die afwesigheid daarvan.

‘n Voorlopige ondersoek in die bydrae wat kommersiële wyngisaanvangskulture tot biogeen amien produksie kan lewer word ook in die werk vervat. Diamiene en poliamiene (putresien, spermidien en kadawerien) is in wissellende vlakke deur alle giste geproduseer, met min tot geen produksie van fisiologies belangrike biogeenamiene (histamien, tiramien en fenieletielamien).

Nog ‘n oogmerk van hierdie studie was om die invloed van algemeen toegepaste wynmaakpraktyke op biogeen amien produksie onder wynmaakkondisies te evalueer.

Ons het gewys dat biogeen amien produksie deur melksuurbakterieë onder meer beïnvloed kan word deur die teenwoordigheid van voorloper aminosure in die druiwemos of wyn, die kontaktyd tussen sap of wyn en die druiwedoppe, die kontaktyd tussen die wyn en die gismoer, die teenwoordigheid van mikrobiese voedingstowwe, wyn pH, sulfiet- en etanolvlakke, die fenoliese samestelling van die wyn en die aantal dekarboksilase positiewe melksuurbakterieë teenwoordig in die wyn.

Die wynindustrie is tans onder toenemende druk om maatstawwe in plek te stel om voedselveiligheid en -sekuriteit te verseker, en om die teenwoordigheid van enige komponent wat die heilsaamheid van wyn kan verminder te beperk, selfs in spoorhoeveelhede. Die behoefte ontstaan dus vir ‘n vinnige en goedkoop metode om kwaliteitskontrole toe te pas. In hierdie studie het ons ondersoek ingestel om Fourier-transformasie-infrarooi-spektroskopie te gebruik as vinnige siftingsmetode om hoë vlakke van biogeen amiene in wyn aan te dui. Hiermee rapporteer ons dan ook ‘n nuwe metode om totale biogeen amiene in wyn op te spoor en te kwantifiseer.

This thesis is dedicated to my family

Hierdie tesis is opgedra aan my familie

BIOGRAPHICAL SKETCH

Anita Smit was born on 25 March 1983 and lived for most of her childhood in George. She matriculated at Oakhill School, Knysna in 2001. Anita obtained her BScAgric degree (Oenology Specialised) at the Stellenbosch University in 2005. In 2006, she enrolled at the same university for a Masters degree in Oenology.

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PREFACE

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

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Chapter 2 **LITERATURE REVIEW**
Biogenic amines in wine: Understanding the headache

Chapter 3 **RESEARCH RESULTS**
Evaluating the influence of malolactic fermentation inoculation times and ageing on lees on the production of biogenic amines by wine lactic acid bacteria and preliminary quantification of biogenic amines by Fourier transform infrared spectroscopy

Chapter 4 **RESEARCH RESULTS**
Evaluating the influence of maceration practices on biogenic amine production by wine lactic acid bacteria

Chapter 5 **RESEARCH RESULTS**
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1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Amines are compounds that occur widely in nature, containing one or more organic substituent bonded to a nitrogen atom. Amines are endogenous to plants and may therefore occur in fresh fruits and vegetables. However, in foods they are mostly formed during fermentation, ageing and storage as a result of microbial decarboxylation of amino acids, and are as such designated biogenic (Shalaby, 1996). Fermented foods and beverages are produced primarily for its desirable properties such as preservation, hygienic safety and to obtain a high level of sensory and nutritive value (Straub, 1995). However, during fermentation various microorganisms can flourish in foodstuffs, increasing the risk of biogenic amine formation.

Malolactic fermentation in wine is by definition the enzymatic conversion of L-malic acid to L-lactic acid; a secondary process which usually follows after the primary (alcoholic) fermentation of wine but may also occur concurrently. This reduction of malic acid to lactic acid is not a true fermentation, but rather an enzymatic reaction performed by lactic acid bacteria after their exponential growth phase. Malolactic fermentation is normally mainly performed by *Oenococcus oeni*, a species that can withstand the low pH (<3.5), high ethanol (>10% v/v) and high SO_2 levels (50 mg/L) found in wine. Additionally, more resistant strains of *Lactobacillus*, *Leuconostoc* and *Pediococcus* can also grow in wine and contribute to the malolactic fermentation; especially if the wine pH exceeds 3.5 (Davis *et al.*, 1985; Wibowo *et al.*, 1985). The most important benefits of malolactic fermentation is the deacidification of high acid wines mainly produced in cool climates; the contribution of lactic acid bacteria to wine flavour and aroma complexity and to acquire improved microbiological stability (Lonvaud-Funel, 1999).

Unfortunately, uncontrolled malolactic fermentation also presents a risk of wine spoilage by compounds that can result in off-flavours (including acetic acid, volatile phenols and mousiness) or that may be hazardous to human health (such as ethyl carbamate and biogenic amines) (Liu & Pilone, 1998; Chattonet *et al.*, 1999; Costello *et al.*, 2001; Lonvaud-Funel, 2001). Biogenic amines will be the subject of investigation in this work.

It was already suggested in 1971 that histamine formation may be prevented by controlling the bacterial flora in wines to be used as inoculum for malolactic fermentation (Smith, 1980). Spontaneous malolactic fermentation is still widely applied in commercial wine cellars across the world, but can be more difficult to control than induced malolactic fermentations. Spontaneous malolactic fermentation can occur at any time during or after alcoholic fermentation, take a long time to reach completion and cause unpredictable changes in the organoleptic profile of wines due to the unknown metabolic properties of indigenous lactic acid bacteria. The inoculation of commercial malolactic starter cultures reduces the risk of spoilage by undesirable lactic acid bacterial strains, and is usually performed after alcoholic fermentation, but can also be

induced during alcoholic fermentation. When lactic acid bacteria are inoculated in the conventional manner after alcoholic fermentation their growth and/or metabolism could be inhibited by the high levels of ethanol present in the wine. This inhibition can be prevented by the concurrent inoculation of lactic acid bacteria and yeast at the start of alcoholic fermentation (Davis *et al.*, 1985). The control of malolactic fermentation was reviewed recently by Bauer & Dicks (2004). Apart from discussing the role of malolactic fermentation starter cultures, they also explore alternative technologies to promote and prevent malolactic fermentation. These include the use of bioreactors, recombinant yeast strains and bacteriocins.

The production of various biogenic amines by lactic acid bacteria during malolactic fermentation have been widely reported in the literature (Cilliers & Van Wyk, 1985; Lonvaud-Funel, 2001; Granchi *et al.,* 2005; Marcobal *et al.,* 2006), while reports on the contribution of yeasts during alcoholic fermentation are scant and contradictory (Goñi & Ancín Azpilicueta, 2001; Torrea & Ancín, 2002; Garde-Cerdán *et al.,* 2006). Biogenic amine formation has been associated with unsanitary winemaking conditions, implying the involvement of spoilage microorganisms, including spoilage lactic acid bacteria (Zee *et al.*, 1983; Delfini, 1989; Vidal-Carou *et al.*, 1991). Only one account has been given on the potential of acetic acid bacteria isolated from wine to form biogenic amines (Landete *et al.*, 2007), while the contribution by *Brettanomyces bruxellensis* have been investigated by Caruso *et al.* (2002) and Granchi *et al.* (2005). The final contribution to biogenic amines in wine by microorganisms other than lactic acid bacteria seems to be small. All four genera of wine lactic acid bacteria have been implicated in biogenic amine formation (Guerrini *et al.*, 2002; Moreno-Arribas *et al.*, 2003; Landete *et al.*, 2007).

Biogenic amines in alcoholic beverages have been paid special public attention due to the fact that alcohol may enhance the effect of amines by directly or indirectly inhibiting amine oxidases (Maynard & Schenker, 1996). Low concentrations of biogenic amines can easily be tolerated by the human body as they are efficiently detoxified by mono- and diamine oxidases in the intestinal tract. Although individual susceptibilities to amine intoxication vary, excessive amounts of ingested biogenic amines are able to initiate various pharmacological reactions. The most notorious intoxications are caused by histamine. More specifically, some symptoms caused by histamine are rash, oedema, headaches, low blood pressure, vomiting, diarrhoea and heart palpitations. Tyramine and phenylethylamine can cause high blood pressure and symptoms associated with vasoconstriction caused by the release of noradrenalin (such as migraine and brain haemorrhage) (Shalaby, 1996). Putrescine and cadaverine, although not toxic themselves, can enhance the toxicity of histamine, tyramine and phenylethylamine as they interfere with the catabolic enzymes (amine oxidases). Moreover, putrescine and cadaverine can cause a depreciation of wine aroma by imparting flavours of putrefaction and rotten meat respectively (Palacios, 2006).

Most wine-producing countries of the world have surveyed their commercial (grape) wines for the presence and concentrations of biogenic amines. These countries include

Greece (Soufleros *et al.*, 2007); Spain (Vidal-Carou *et al.*, 1990; Landete *et al.*, 2005; Bover-Cid *et al.*, 2006); Hungary (Sass-Kiss *et al.*, 2000; Hajós *et al.*, 2000); Oregon, USA (Glória *et al.*, 1998); South Africa (Cilliers & Van Wyk, 1985); Argentina (Díaz *et al.*, 2006); Portugal (Leitão *et al.*, 2005); Turkey (Anlı *et al.*, 2004); China (Zhijun *et al.*, 2007); Canada (Soleas *et al.*, 1999); Italy (mo Dugo *et al.*, 2006) and France (Bauza *et al.*, 1995; Soufleros *et al.*, 1998). Consumer demands for increased food safety, security and healthier products have promoted interest in trace compounds that can be harmful to human health. Still, no legal limits have been defined for biogenic amines although guidelines exist for some countries (Lehtonen, 1996). Therefore wine exports may be hampered to such countries in the future, making biogenic amines a potential economic threat. For the most part it is the responsibility of winemaking teams to control the production of biogenic amines in commercial wines by controlling the factors that could influence its formation.

A wide variety of viticultural and oenological factors may have an impact on the levels of biogenic amines produced in wine. While some factors can increase the precursor amino acid concentration in the grape and wine (grape variety, geographical region, vintage, extraction methods, grape skin maceration, ageing practices and nutrient additions), other factors influence diversity, growth, decarboxylase activity and gene expression of microorganisms that can potentially produce the biogenic amines (mostly wine physiochemical factors such as nutrient status, pH, temperature, SO_2 and a variety of substrates and products of fermentation).

In this work we will exploit some of the products and conditions used in commercial wineries on a smaller scale to better our understanding of the relationship between winemaking practices and biogenic amine production by lactic acid bacteria.

1.2 PROJECT AIMS

The principal objective of this work was to gain understanding of which winemaking parameters and practices influence the production of biogenic amines by microorganisms (lactic acid bacteria and/or yeasts) in wine. This knowledge will aid us to combat their formation under complex winemaking conditions.

The specific aims of this research project were to evaluate the risk of biogenic amine formation in wine due to:

a) spontaneous versus inoculated malolactic fermentation;

b) different times of inoculation with lactic acid bacteria for malolactic fermentation;

c) winemaking practices such as grape skin contact, additional maceration and ageing on fermentation lees (and the relationship with precursor amino acids);

d) the influence of complex bacterial and yeast nutrients; and

e) commercial yeast starter cultures.

This study also explored the development of new analytical methods to rapidly determine biogenic amines in wine, as a secondary aim. A preliminary evaluation of the influence of malolactic fermentation practices on volatile aroma compounds in wine was also conducted.

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2. LITERATURE REVIEW

Biogenic amines in wine: Understanding the headache

2.1 INTRODUCTION

A group of organic nitrogenous compounds formed and degraded by the metabolisms of living organisms (microorganisms, plants and animals) are called biogenic amines. The main biogenic amines associated with wine are putrescine, histamine, tyramine and cadaverine, followed by phenylethylamine, spermidine, spermine, agmatine and tryptamine. Biogenic amines in wine can be formed by various microorganisms associated with the different stages of wine production and storage.

The vinification of grapes consists of two main fermentation steps. Alcoholic fermentation of grape must, usually performed by the wine yeast *Saccharomyces cerevisiae*, involves the conversion of grape sugar to ethanol and CO_2. Malolactic fermentation is conducted in most red and some white wines by lactic acid bacteria of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Oenococcus*; principally to deacidify the medium by the conversion of L-malic acid to L-lactic acid. Malolactic fermentation also ensures a certain degree of microbial stability to the wine and modifies the sensory characteristics of the wine by the production of secondary bacterial metabolites (Lonvaud-Funel, 1999). Spoilage organisms such as acetic acid bacteria and the yeast *Brettanomyces bruxellensis* can grow during fermentations or storage and produce compounds that impart negative characteristics to the wine.

Apart from the primary metabolic products and many flavour compounds (both desirable and undesirable) released during the fermentations, some microorganisms can produce secondary metabolic products that can affect the wholesomeness of the wine. Biogenic amines are one such group of compounds. Other secondary metabolites associated with winemaking that could pose a health risk to humans include sulphur dioxide (SO_2), ethyl carbamate and the mycotoxin, Ochratoxin A.

The focus of this review will be biogenic amines. Their formation by various wine microorganisms will be discussed with specific reference to the enzymes and genes involved. The chemical, biochemical and toxicological properties of biogenic amines are considered. Factors influencing the formation of biogenic amines, covering the entire production chain, are critically reviewed. Analytical and molecular methods used in biogenic amine identification are also discussed.

2.2 MICROORGANISMS ASSOCIATED WITH BIOGENIC AMINE FORMATION IN GRAPES AND WINE

2.2.1 LACTIC ACID BACTERIA

Lactic acid bacteria are present on healthy grapes in low numbers and the population generally declines during alcoholic fermentation. Lactic acid bacteria are also transferred to winery equipment, where they can be present in significant numbers (Wibowo *et al*., 1985). *Oenococcus oeni* is normally the dominant species that survives to the end of alcoholic fermentation and is predominantly responsible for malolactic fermentation. However, if the pH of the wine is above pH 3.5 species of *Pediococcus* and *Lactobacillus*, generally associated with spoilage, may survive and grow to levels of 10^6 to 10^8 cells/mL and have antagonistic interactions with *Oenococcus oeni* (Wibowo *et al*., 1985; Lonvaud-Funel, 1999).

Extensive research has been done to correlate biogenic amine production in wine with species of lactic acid bacteria involved in the winemaking process. In the past, spoilage bacteria, mainly *Pediococcus* species such as *Pediococcus cerevisiae*, were held responsible for histamine production in wine (Delfini, 1989). Recent results by Landete *et al*. (2007b) show an agreement that, although the percentage of *Pediococcus* species capable of producing histamine seems to be low, some strains (for example *Pediococcus parvulus* in this study) are responsible for the highest concentrations of histamine. However, today it is widely known that *Lactobacillus*, *Leuconostoc* and *Oenococcus* species are also implicated in biogenic amine production in wine. Different strains of *Lactobacillus hilgardii, Lactobacillus buchneri, Lactobacillus brevis* and *Lactobacillus mali* have been found able to produce a variety of biogenic amines in wine (Moreno-Arribas & Lonvaud-Funel, 1999; Moreno-Arribas *et al*., 2000; Downing, 2003; Moreno-Arribas *et al*., 2003; Costantini *et al*., 2006; Landete *et al*., 2007b). Until 2003 there had been no reports on the role of *Leuconostoc* strains in the formation of biogenic amines in wine. Then, in a study by Moreno-Arribas *et al*. (2003), *Leuconostoc mesenteroides* was found to have a high potential to produce tyramine. Another strain of *Leuconostoc mesenteroides* isolated from wine, capable of producing relatively high levels of histamine, has also been identified by Landete *et al*. (2007b).

Commercial *O. oeni* strains are selected for their oenological parameters, including the absence of amino acid decarboxylases. According to *in vitro* studies done by Moreno-Arribas *et al*. (2003) none of four commercial malolactic starter cultures tested could produce histamine, tyramine or putrescine. Martín-Álvarez *et al*. (2006) also compared inoculated with spontaneous malolactic fermentation in 224 samples of Spanish red wine. They found that inoculation with a commercial starter culture of lactic acid bacteria could reduce the incidence of biogenic amines compared to spontaneous malolactic fermentation in wines. Starter cultures could eliminate indigenous bacteria, or might be able to degrade biogenic amines produced by the undesirable strains.

Other authors reported that no biogenic amine producers were present among naturally occurring *O. oeni* strains isolated from wine and must. Moreno-Arribas *et al*. (2003) tested 39 strains in a decarboxylase assay broth medium. HPLC was used to quantify biogenic amines. They report that similar results were also obtained by Straub *et al*. (1995), who tested for histamine producers among 88 strains of *O. oeni.* Costantini *et al*. (2006) found no *O. oeni* among 92 strains able to produce biogenic amines in a broth medium. PCR screening was used by these authors to confirm the absence of the respective decarboxylase genes.

In contrast, Guerrini *et al*. (2002) found that *O. oeni* is able to significantly contribute to the overall biogenic amine content of wines and that the ability of *O. oeni* to produce biogenic amines varies among strains. Twenty six of 44 strains tested were able to produce up to 33 mg/L histamine under optimal growth conditions in a synthetic medium. Putrescine and cadaverine were also produced by some strains. The high frequency of histamine-producing strains found in this study is in accordance with a study done by Coton *et al.* (1998a), who reported that most *O. oeni* strains isolated from wine have histamine producing capability. The latter research group found that almost half of the 118 wines tested from the Southwest of France contained histamine in varying amounts and possessed histidine decarboxylase positive bacteria belonging to *O. oeni*.

Thus, the ability of lactic acid bacteria to produce amines seems to be strain-dependent, and not a species specific characteristic. It may be that decarboxylase activities are randomly distributed within the different species of *Lactobacillus, Leuconostoc, Pediococcus and Oenococcus*. Some lactic acid bacteria strains have the ability to simultaneously produce different amines (Coton *et al.,* 1998a; Moreno-Arribas *et al*., 2000; Guerrini *et al.,* 2002) supporting the suggestion by Gale (1946) that some strains might possess more than one amino acid decarboxylase activity under specific culture conditions.

According to literature (Lonvaud-Funel, 2001) only lactic acid bacterial strains with histidine-, tyrosine- and ornithine decarboxylase activities have been isolated from wine. The properties of each of the decarboxylase enzymes will be discussed briefly.

2.2.1.1 Histidine decarboxylase

Two different kinds of decarboxylase enzymes exist. The majority of amino acid decarboxylase enzymes - those belonging to animals and Gram-negative bacteria - require pyridoxal 5'-phosphate as a cofactor. However, some bacterial histidine decarboxylase enzymes use a covalently-bound pyruvoyl group as a cofactor in the reaction and are pyridoxal 5'-phosphate independent (Coton *et al*., 1998b; Lonvaud-Funel, 2001). The latter group include well-studied Gram-positive bacteria: *Lactobacillus 30a* (Chang & Snell, 1968); *Lactobacillus buchneri, Clostridium perfringens* (Recsei *et al*., 1983); *Micrococcus* sp. (Prozorouski & Jörnvall, 1975) and *Oenococcus oeni* (Rollan *et al*., 1995). Yet, Landete *et al*. (2006) demonstrated

experimentally that pyridoxal 5'-phosphate does enhance amino acid decarboxylase activity in Gram-positive lactic acid bacteria and could therefore also be considered a decarboxylase cofactor for this group of bacteria.

The first histidine decarboxylase (HDC) enzyme of a wine lactic acid bacterium was isolated from a histamine producing strain (*O. oeni* 9204) by Lonvaud-Funel & Joyeux (1994) and has since been studied by various authors. Coton *et al*. (1998b) purified this histidine decarboxylase enzyme to homogeneity and provided valuable molecular data. This HDC enzyme is a single polypeptide of 315 amino acids, comprised of α and β subunits. The gene sequence aided researchers to develop rapid and specific detection systems based on polymerase chain reaction (PCR) to detect potential histamine-producing bacteria from wine (Le Jeune *et al*., 1995; Coton *et al*., 1998a).

The HDC enzyme has a high degree of cooperativity; at low histidine concentrations HDC has a low substrate affinity, but as histidine concentration increases, binding to the active site is favoured. The product, histamine, acts as a competitive inhibitor of the antiport histidine/histamine at the cell membrane and decreases the HDC activity (Rollan *et al*., 1995). The pH optimum of HDC is 4.8. These findings are the same as described previously for HDC of *Lactobacillus* 30a (Chang & Snell, 1968).

Lucas *et al*. (2005) related histidine decarboxylase enzyme activity of *Lactobacillus hilgardii* 0006 (isolated from wine), to the presence of an 80 kb plasmid on which the decarboxylase gene was located as part of a four-gene cluster. They proposed that this discovery is possibly the first described histamine-producing pathway. The four genes, *hdcP*, *hdcA*, *hisRS* and *hdcB* code for a histidine/histamine exchanger, a histidine decarboxylase, a histidyl-tRNA synthetase and an unknown product respectively. There is evidence to suggest that this same gene cluster may also be present in other histamine producers, such as *Lactobacillu*s 30a and *O. oeni* 9204. Also, *Lactobacillus* bacteria are able to transfer a conjugative plasmid to bacteria of the same or different genera (Gevers *et al*., 2003). Lucas *et al*. (2005) suggest that a plasmid-encoded HDC system could be transferred horizontally; and that the location of the gene on an unstable plasmid may explain the random distribution of histidine decarboxylase positive bacteria.

Some authors found that histamine decarboxylase activity is present in a high proportion of wine lactic acid bacterial strains, even if the levels of histamine produced in wine are generally low (Lonvaud-Funel & Joyeux, 1994; Le Jeune *et al*., 1995; Coton *et al*., 1998a; Guerrini *et al*., 2002; Landete *et al*., 2005a, 2007b). Other authors found the frequency of histamine producers in wine to be very low (Moreno-Arribas *et al*., 2003).

Landete et al. (2005a) screened 136 strains of wine lactic acid bacteria for the presence of the HDC gene and the ability to produce histamine in a synthetic medium. These included strains of *Lactobacillus* (54), *Oenococcus* (32), *Pediococcus* (34) and *Leuconostoc* (16). Their results showed that *Lactobacillus* (13%), *Oenococcus* (78%), *Pediococcus* (12%) and *Leuconostoc* (6%) were able to produce histamine. *O. oeni* had the highest frequency of histamine production, but produced the lowest concentrations.

Lactobacillus hilgardii and *Pediococcus parvulus* produced histamine at the highest concentrations (up to 200 mg/L) and can therefore be regarded as potential spoilage bacteria in wine. According to this study, other strains able to contribute to histamine in wine are *O. oeni*, *Lactobacillus mali* and *Leuconostoc mesenteroides*.

2.2.1.2 Tyrosine decarboxylase

Tyrosine decarboxylase (TDC) in bacteria had only been investigated in *Enterococcus faecalis* (Allenmark & Servenius, 1978) until 1999, where after a tyramine-producing strain was isolated from wine and identified as *Lactobacillus brevis* IOEB 9809 (Moreno-Arribas & Lonvaud-Funel, 1999).

In 2002 and 2003 the tyrosine decarboxylase gene of *Lactobacillus brevis* IOEB 9809 was purified and sequenced (Lucas & Lonvaud-Funel, 2002; Lucas *et al*., 2003). The sequence (7979 bp) contained four complete genes encoding a tyrosyl-tRNA synthetase, the tyrosine decarboxylase, a probable tyrosine permease and a Na+/H+ antiporter. The authors suggest that the tyrosine decarboxylase gene of *L. brevis* 9809 is the first well-characterised bacterial tyrosine decarboxylase gene. The TDC gene encodes for the 264 amino acids of the enzyme.

Tyrosine decarboxylase was found to be active in a pH range of 3.0 to 7.0 with an optimum at pH 5.0. Tyrosine decarboxylase enzyme activity was reported to be pyridoxal 5'-phosphate dependent (Moreno-Arribas & Lonvaud-Funel, 1999; Coton & Coton., 2005). Its activity is enhanced by the substrate (L-tyrosine) and the coenzyme (pyridoxal 5'-phosphate). These findings are similar to the properties described for TDC of *Enterococcus faecalis* (Allenmark & Servenius, 1978). As with histamine and HDC activity, tyramine acts as a competitive inhibitor of TDC and therefore the presence of the formed product will decrease the enzyme activity.

A rapid PCR assay was developed to detect *L. brevis* strains carrying the tyrosine decarboxylase gene (Lucas & Lonvaud-Funel, 2002). Downing (2003) determined that the described primers could be used to amplify a fragment of the TDC gene in tyrosine decarboxylating *L. brevis* as well as *L. hilgardii* strains from South African wines.

A number of wine lactic acid bacteria strains have since been identified as tyramine producers. Moreno-Arribas *et al*. (2000) isolated and identified a number of tyramine producing lactic acid bacteria in wine that had undergone malolactic fermentation. None of the strains in their study were identified as *O. oeni*, but all as *Lactobacillus brevis* and *Lactobacillus hilgardii*. Downing (2003) found tyramine to be the main amine formed in synthetic medium by *Lactobacillus brevis* and *Lactobacillus hilgardii* strains isolated from South African wines. As noted elsewhere in this review, *Leuconostoc mesenteroides* was also found to be a tyramine producer (Moreno-Arribas *et al.*, 2003). *O. oeni* seems to have a low distribution of the metabolic ability to produce tyramine (Moreno-Arribas *et al*., 2000; Geurrini *et al*., 2002). As far as literature suggested in 2003, no tyramine producing *O. oeni* strain had yet been reported (Moreno-Arribas *et*

al., 2003), with the exception of a strain (*O. oeni* DSM 2025) that was shown to be able to produce tyramine in a laboratory medium (Choudhury *et al.*, 1990).

2.2.1.3 Ornithine decarboxylase

Marcobal *et al.* (2004) reported that a putrescine producing strain of *O. oeni* (IOEB 8419) was isolated from ropy red wine and was suspected of having ornithine decarboxylase (ODC) activity. Later, seven strains of *O. oeni* (out of 44) were reported to be able to produce putrescine in culture media (Guerrini *et al.*, 2002). Then, a putrescine producing strain (*O. oeni* BIFI-83) was isolated from the fermentation lees of a Spanish wine which showed a high concentration of putrescine. This led to the first report of the ODC gene to be present in the genome *O. oeni* and detectable by PCR (Marcobal *et al*., 2004, 2005a). The ornithine decarboxylase gene encodes a 745 amino acid residue protein. Ornithine decarboxylase is also a pyridoxal 5'-phosphate dependent enzyme. The amino acid sequence of this ODC gene shares a 67% identity with that of *Lactobacillus* 30a (Marcobal *et al*., 2004).

Putrescine is reported to be the most abundant biogenic amine in wine, both qualitatively and quantitatively. Putrescine could be observed in high concentrations (up to 200 mg/L), especially after malolactic fermentation (Glória *et al*., 1998). Although putrescine has been found to be present in almost 100% of samples analysed by many authors (Glória *et al.,* 1998; Soufleros *et al*., 1998; Vasquez-Lasa *et al*., 1998; Soleas *et al*., 1999; Moreno & Azpilicueta, 2004; Landete *et al*., 2005b; Bover-Cid *et al*., 2006; González Marco & Ancín Azpilicueta, 2006; Alcaide-Hidalgo *et al*., 2007), only one strain of wine lactic acid bacteria has been reported in literature to posses the ornithine decarboxylase gene: *O. oeni* BIF-I83 (Marcobal *et al*., 2004). Marcobal *et al.* (2004) found that the ornithine decarboxylase gene is rarely present in the genome of *O. oeni* – they found none of 42 strains tested to possess the gene. Also, ornithine is usually only present at low levels in wine and it would be expected that the levels of putrescine would be correspondingly low.

2.2.1.4 The arginine deiminase pathway

A possible explanation for the production of high levels of putrescine in wine has been proposed by Mangani *et al*. (2005). The authors proved that putrescine, the most abundant biogenic amine in wine, could be produced by *O. oeni* from ornithine and also from arginine at wine pH (3.2). Some strains that can produce putrescine possess the complete enzyme system to convert arginine, a major amino acid in wine, to putrescine. In this case arginine is catabolised via the arginine deiminase (ADI) pathway. For this to occur, all three enzymes of this pathway must be present in the bacterial strain and active under wine conditions. These enzymes are arginine deiminase (ADI), ornithine transcarbomoylase (OTC), and carbamate kinase (CK). Some *O. oeni* strains can be deficient in one or two of these enzymes or arginine catabolism could be inhibited by low pH (Mangani *et al*., 2005). However, metabiosis can occur among *O. oeni* species.

This process is an association and exchange between strains able to metabolise arginine to ornithine but unable to produce putrescine and strains that cannot degrade arginine but can produce putrescine from ornithine. Putrescine production by metabiostic association can occur slowly after the completion of malolactic fermentation, whereas conversion of ornithine to putrescine by the ornithine decarboxylase enzyme of a single *O. oeni* strain was found to occur simultaneous with malic acid degradation and at a faster rate (Mangani *et al*., 2005). Thus, the authors concluded that despite commercial starter cultures being unable to use arginine or possess no ornithine decarboxylase, metabiosis can occur with indigenous strains and may play an important role in putrescine accumulation in wine.

Similarly, Arena & Manca de Nadra (2001) showed previously that a *Lactobacillus* species (*Lactobacillus hilgardii* X_1B), isolated from wine was able to degrade arginine via the arginine deiminase pathway and the arginine decarboxylase pathway. This enables the bacteria to produce putrescine from the intermediates ornithine and agmatine.

2.2.1.5 Other decarboxylases

De Las Rivas *et al.* (2006) described the first oligonucleotides for the PCR detection of lysine decarboxylase encoding genes in foodborne bacteria. Primers were designed to amplify the genes coding for lysine decarboxylase by alignment of lysine decarboxylase proteins in two groups: first for *Enterobacteriaceae* and second for bacteria from the genera *Bacillus*, *Clostridium*, *Listeria* and *Staphylococcus*. The possibility exists to extend this PCR method to detect cadaverine-producing bacteria in wine.

It is known that phenylethylamine production is often associated with tyramine production in lactic acid bacteria. This could be explained by the fact that phenylalanine is also a substrate for tyrosine decarboxylase; producing phenylethylamine in a secondary reaction (Landete *et al*., 2007c). The ability of wine lactic acid bacteria to form phenylethylamine seems to be rare, with *Lactobacillus brevis* and *Lactobacillus hilgardii* being the only strains to date associated with high levels of phenylethylamine in wines (Moreno-Arribas *et al.*, 2000; Landete *et al*. 2007c). According to Landete *et al*. (2007c) there have been no reports on studies regarding other phenylethylamine decarboxylase enzymes.

In general, lactic acid bacteria are the main microorganisms held responsible for biogenic amine production in wine. Soufleros *et al*. (1998) determined that the levels of biogenic amines (histamine, tyramine and putrescine) are low after alcoholic fermentations and increase in most wines during and after spontaneous malolactic fermentations with a corresponding decrease in the respective precursor amino acid concentration.

Granchi *et al*. (2005) examined biogenic amine production and the dynamics of microorganisms throughout the whole winemaking process of commercial vinifications.

They found a decrease of biogenic amines (especially putrescine) during both spontaneous and induced alcoholic fermentations while yeast dominated. Biogenic amine concentrations (putrescine and histamine and/or cadaverine and tyramine) increased significantly during both spontaneous and inoculated malolactic fermentations conducted by *O. oeni* strains. Other authors who observed the most important increase of biogenic amines during malolactic fermentation, when compared to the contributions by alcoholic fermentation and/or ageing, include Cilliers & Van Wyk (1985), Landete *et al*. (2005b), Marcobal *et al*. (2006b) and Alcaide-Hidalgo *et al*. (2007) **(Figure 2.1**). According to the results of Landete *et al*. (2007b), lactic acid bacteria can be considered the microorganisms responsible for the production of biogenic amines in wine, since in this study (involving 231 microorganisms), no yeasts or acetic acid bacteria were found capable of producing biogenic amines.

2.2.2 YEASTS

A large variety of indigenous yeast species can grow and perform the alcoholic fermentation in wine, along with commercial *Saccharomyces cerevisiae* strains. Few studies have been conducted on the formation of biogenic amines by yeasts, of which most only compared different yeasts species and quantified only histamine (Torrea & Ancín, 2002).

A number of authors reported that no remarkable increase in the concentration of biogenic amines could be observed during alcoholic fermentation, with the conclusion that yeasts do not appear to be responsible for the production of most amines found in industrial commercial red wines (Herbert *et al*., 2005; Marcobal *et al.,* 2006b). Granchi *et al.* (2005) even report a decrease of biogenic amines (especially putrescine) during alcoholic fermentation while yeast dominates, for both spontaneous and induced commercial vinifications. In another study, no potential to produce biogenic amines in synthetic medium, grape must or wine was found among 36 strains of yeast isolated from grape must and wine. The yeasts tested included strains belonging to the genera *Aureobasidium, Candida, Hanseniaspora, Hansenula, Kloeckera, Metschnikowia, Pichia, Rhodotorula* and strains of the species *Saccharomyces cerevisiae, Saccharomyces cerevisiae* var. *bayanus, Saccharomyces cerevisiae* var*. chevalieri* and *Saccharomyces cerevisiae* var*. steiner* (Landete *et al*., 2007b).

Any contribution of yeast to biogenic amine production could therefore be indirect: yeasts can alter the composition of grape musts by using some amino acids and secreting others during alcoholic fermentation and autolysis, thereby changing the concentration of precursor amino acids in the wine that can be used by other microorganisms in subsequent fermentation steps (Soufleros *et al*., 1998).

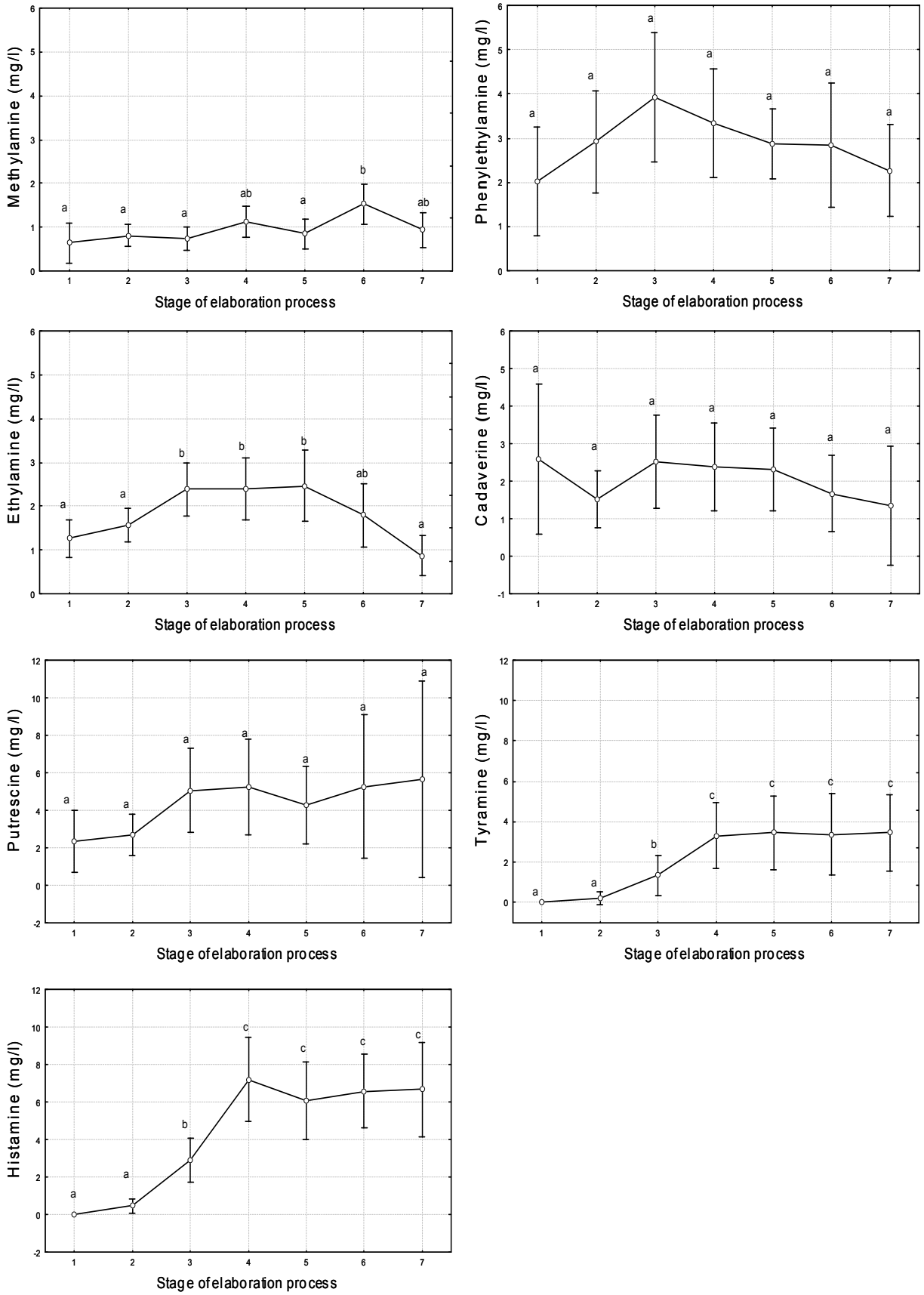

Figure 2.1 The average concentrations of oenologically important biogenic amines at different stages of industrial winemaking: 1, must; 2, after alcoholic fermentation; 3, during MLF; 4, just after MLF; 5, after MLF and addition of SO_2; 6, after 6 months of ageing; 7, after 12 months of ageing. Means with different letters are significantly different according to Fisher's test (Marcobal *et al*., 2006b).

In contrast, a number of studies show that an increase in biogenic amine concentration in wine by the direct action of yeast during both spontaneous and inoculated fermentations on experimental and commercial scale is possible. The results of a study performed by Buteau *et al*. (1984) disagree with the notion that biogenic amines (and particularly histamine) are formed by lactic acid bacteria during malolactic fermentation. These authors found that agmatine, cadaverine, ethanolamine, histamine, putrescine and tyramine were produced in the highest quantities during alcoholic fermentation. Moreover, some biogenic amines decreased during malolactic fermentation.

Goñi & Ancín Azpilicueta (2001) examined the concentration of biogenic amines produced by different *Saccharomyces cerevisiae* strains in rosé wines. They found a slight increase in biogenic amines (putrescine, spermine, spermidine, phenylethylamine and tyramine) depending on the strain. Wines fermented by killer strains showed the highest concentration of biogenic amines, when compared to those with a killer neutral phenotype. The variation in the concentrations of the biogenic amines in this study was ascribed to differences in the extent of production or use of the amines by the yeasts as a source of nitrogen.

Torrea & Ancín (2002) and Garde-Cerdán *et al.* (2006) proceeded with similar studies and found that inoculated musts produced wines with higher concentration of biogenic amines compared to spontaneous fermentations performed by indigenous yeasts. They attribute this to the greater consumption of precursor amino acids during fermentation by the commercial yeasts, and the lower yeast population in spontaneous fermentations. Garde-Cerdán *et al.* (2006) determined that biogenic amines are mainly synthesised in wine in small quantities after the consumption of the first 25% of sugars.

In a study conducted by Caruso *et al*. (2002), 50 yeast strains of five different genera isolated from grapes and wine were screened for biogenic amine production. *Brettanomyces bruxellensis* formed the highest concentration of total biogenic amines, followed by *Saccharomyces cerevisiae*. The ability of *Saccharomyces cerevisiae* to produce biogenic amines seems to be strain dependent and not a constant characteristic of the species. Some strains of *Saccharomyces cerevisiae*, particularly those present during spontaneous alcoholic fermentations, can therefore be considered as wine spoilage microorganisms due to their ability to produce biogenic amines. *Saccharomyces cerevisiae*, *Kloeckera apiculata*, *Candida stellata*, *Metschnikowia pulcherrima* and *Brettanomyces bruxellensis* were also tested by Granchi *et al.* (2005) in another study. **Table 2.1** gives an indication of the total biogenic amines produced by these yeasts, of which *Brettanomyces bruxellensis* produced the highest concentration in both studies, followed by *Saccharomyces cerevisiae*. Agmatine, followed by phenylethylamine and ethanolamine were produced in the highest amounts by most species.

Table 2.1 Production of biogenic amines by different wine yeast species in sterile grape must under laboratory conditions in two different studies.

Yeast species	**Average total biogenic amines (mg/L)**	
	Caruso *et al.,* 2002	Granchi *et al.,* 2005
Saccharomyces cerevisiae	12.14	13.7
Kloeckera apiculata	6.21	9.7
Candida stellata	7.73	7.8
Metschnikowia pulcherrima	9.6	13.3
Brettanomyces bruxellensis	15.01	20

It is clear that yeasts form different biogenic amines than lactic acid bacteria. Little is available in literature regarding the biochemistry, genetics and regulation of biogenic amine production of wine yeasts. The polyamines, spermine and spermidine, are formed from putrescine. Unlike putrescine itself, these polyamines have been found to be an absolute requirement for optimal growth in yeasts, in particular for meiotic sporulation and for maintenance of the double-stranded RNA killer plasmid (Cohn *et al*., 1978). Yeast mutants with decreased ornithine decarboxylase activity do not decarboxylate sufficient ornithine to putrescine and consequently grow slower because they synthesise fewer polyamines (Cohn *et al*., 1980). Cohn *et al.* (1980) also suggest that putrescine in yeast serves primarily as precursor for spermidine and spermine biosynthesis. Ornithine decarboxylase in yeast is the key regulatory enzyme of the polyamine biosynthetic pathway (Gupta *et al.,* 2001). Yeast ornithine decarboxylase activity requires a thiol and pyridoxal 5'-phosphate for activity and is rapidly decreased by the presence of spermine and spermidine (Tyagi *et al.*, 1981).

No mention could be found in literature of other decarboxylase enzymes studied in wine yeasts or yeasts from related fields.

2.2.3 FUNGI

Biotic stresses to the grapevine, such as caused by the fungus *Botrytis cinerea*, can lead to an increase of amine content of the grape berries (Hajós *et al*., 2000). *Botrytis cinerea* is responsible for the formation of aszu grapes, which are characteristic of the famous Tokaj wines from Hungary. The fungus penetrates the grape skin and significantly alters the composition and concentration of amino acids, carbohydrates and amines in the grape berries by increasing its concentrations while the water content of the grape decreases and the berry becomes raisin-like. Aszu wines are made by adding different quantities (butts) of these noble rot grapes to the ordinary wine grapes. Amine concentrations in aszu wines were found to be higher in three different cultivars studied when compared to corresponding normal wines from the same varieties. A positive

correlation could be made between the biogenic amine content and the number of butts used for the aszu wine (Sass-Kiss *et al*., 2000).

Marques *et al*. (2007) tested the potential of the fungicides carbendazyme, iprodione and procymidone (applied every three weeks to vines) to reduce the incidence of biogenic amines in grape musts and wine. It was found that control wine (that received no fungicide treatments) presented the highest mean concentrations of biogenic amines at the end of malolactic fermentation. They concluded that fungal metabolic activity could directly contribute to biogenic amine formation (especially for isoamylamine) or lead to the activity of bacteria other than those normally present in healthy grapes.

2.2.4 ACETIC ACID BACTERIA

Forty strains of acetic acid bacteria isolated from grape must and wine were screened in synthetic medium and wine by Landete *et al*. (2007b) for their ability to form histamine, tyramine, phenylethylamine, putrescine, cadaverine and tryptamine, but no positive results were obtained.

No further mention regarding the formation of biogenic amines by acetic acid bacteria in wine could be found in literature.

2.3 CHEMISTRY, BIOCHEMISTRY AND TOXICOLOGY OF BIOGENIC AMINES

Biogenic amines are compounds of low molecular weight, derived from aromatic or cationic amino acids and all have one or more positive charge and a hydrophobic skeleton (ten Brink *et al*., 1990; Shalaby, 1996). The chemical structure of biogenic amines can be aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenylethylamine) or heterocyclic (histamine, tryptamine) (ten Brink *et al*., 1990). **Figure 2.2** shows the chemical structures of some biogenic amines.

Putrescine, spermine and spermidine are present in plants, where they are important for physiological processes such as flowering and fruit development, cell division, stress responses and senescence (Halász *et al.,* 1994). A large number of volatile amines (for example ethylamine, methylamine, dimethylamine, pyrrolidine) are also present in grapes (Ouch *et al*., 1981).

Biogenic amines are likely to be found in food and beverages that contain proteins or free amino acids; if conditions persist that favour microbial or biochemical activity. Such foodstuffs include fish, fish products, meat products (sausages), eggs, cheeses, nuts, fermented and fresh fruits and vegetables such as sauerkraut and soy bean products, beers and wines (Halász *et al.,* 1994; Silla Santos, 1996).

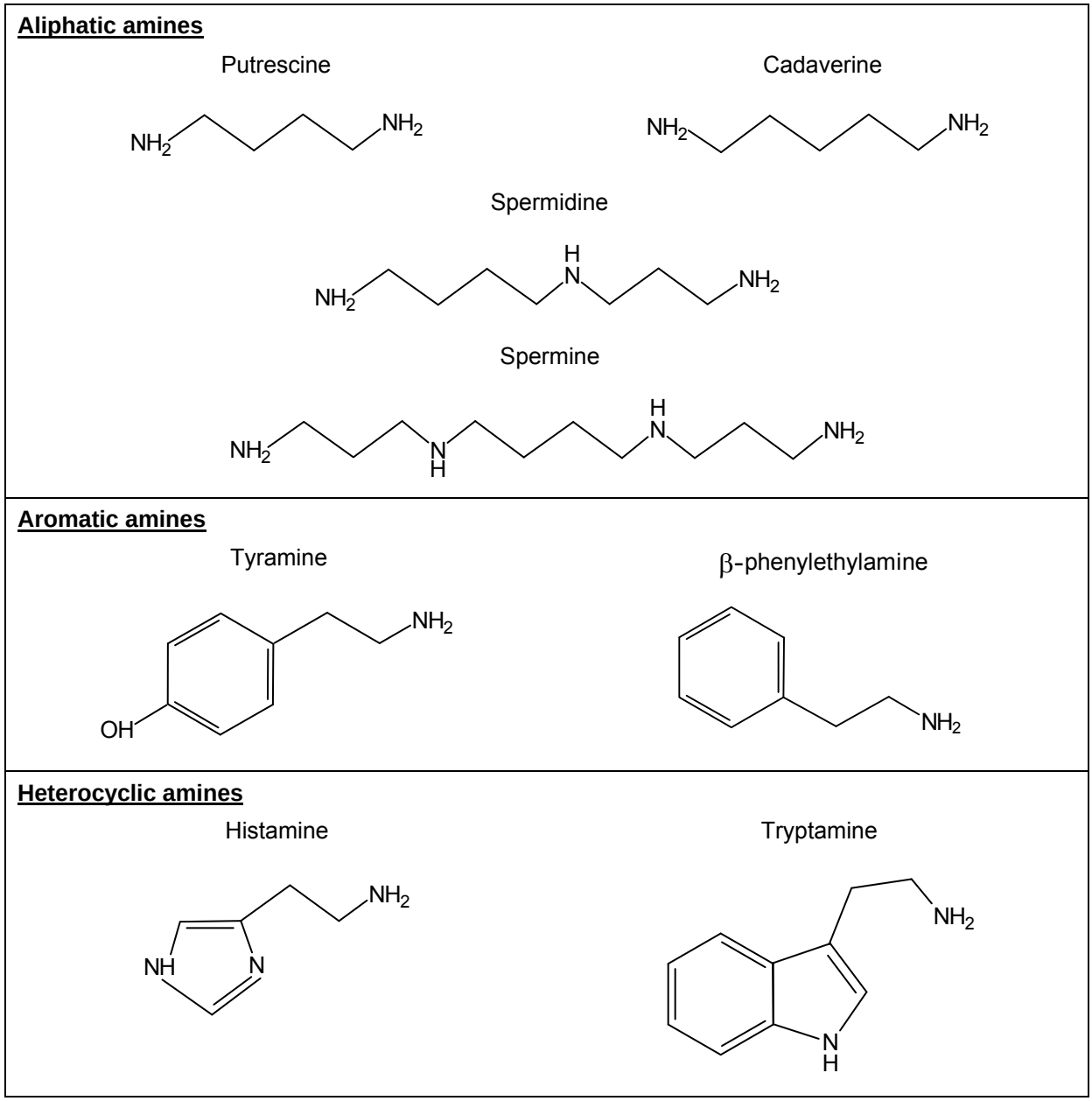

Figure 2.2 Chemical structures of some of the most oenologically important biogenic amines.

In fermented foods, the non-volatile or biogenic amines (histamine, putrescine, cadaverine, spermine, spermidine, agmatine, tyramine, tryptamine) and phenylethylamine (a volatile amine) are formed mainly by the microbial decarboxylation of the corresponding amino acids (Zee *et al*., 1983; ten Brink *et al*., 1990; Halász *et al*., 1994). Volatile amines are believed to be formed by the reductive amination or transamination of the corresponding aldehyde or ketone (Smith, 1980; Ouch *et al*., 1981).

Gram-positive bacteria are usually associated with spoilage of fermented foods (Silla Santos, 1996). In non-fermented foods, the presence of biogenic amines above a certain level can indicate microbial spoilage (ten Brink *et al*., 1990). In raw fish and

meat, Gram-negative bacteria are known to produce histamine when exposed to extreme temperatures.

Biogenic amines in wine can originate from the plant (grape berries) itself or be produced during the fermentation processes, ageing or storage when wine is exposed to undesirable activity of decarboxylase positive microorganisms. Contamination may occur due to poor sanitary conditions of both grapes and processing equipment (Zee *et al.,* 1983; ten Brink *et al*., 1990; Shalaby, 1996; Leitão *et al*., 2005). Most biogenic amine contamination of wine is believed to take place during malolactic fermentation.

Many genera of bacteria are able to decarboxylate free amino acids by the action of decarboxylase enzymes. Because the amino acids are obtained by the bacterium from the extracellular medium (wine), it is believed that decarboxylase enzymes operate together with a transporter protein (Lucas *et al.*, 2005). During amino acid uptake, a membrane potential is generated by the transporter because there is a net charge difference between the precursor (for example monovalent histidine) and the product (divalent histamine). A pH gradient is generated when a proton is consumed in the decarboxylation reaction. These two steps together generate a proton motive force. This reaction is thought to favour growth and survival in acidic media such as wine, since it produces metabolic energy by the described precursor/product antiport (Molenaar *et al*., 1993), and regulates (increases) the pH - thereby extending the growth period by rendering the extracellular medium less toxic to the cell (Gale, 1946).

Histamine is present at low levels in the human body and is involved in key functions such as the allergic response, neurotransmission and vascular permeability (Ohtsu & Watanabe, 2003). Other normal physiological functions of biogenic amines in humans include the regulation of body temperature, stomach volume, stomach pH and brain activity (ten Brink *et al.*, 1990).

Normally, if a low concentration of biogenic amines is ingested, they are quickly detoxified in the human body by amine oxidases or through conjugation. Amine oxidases catalyse the oxidative deamination of biogenic amines to produce an aldehyde, hydrogen peroxide and ammonia (Gardini *et al*., 2005). However, if an excessive amount of biogenic amines is ingested or if the normal catabolic routes are inhibited or genetically deficient, several physiological disorders can occur (ten Brink *et al.*, 1990). Histamine poisoning is sometimes referred to as “scombroid fish poisoning” due to illness resulting from consumption of fish such as tuna, mackerel and sardines; while high levels of tyramine in cheese causes a phenomenon known as the “cheese reaction” (Taylor, 1986; ten Brink *et al*., 1990). These false food allergies are of particular importance in wine, because the presence of ethanol, acetaldehyde and other biogenic amines may promote the harmful effects of histamine and tyramine by inhibiting their normal metabolism in humans (Landete *et al*., 2006). Histamine is often described as the most important biogenic amine since it is one of the most biologically active amines (Halász *et al*., 1994). Histamine causes dilation of peripheral blood vessels, capillaries and arteries, thus resulting in hypotension, flushing and headache

(Silla Santos, 1996). It also causes contraction of intestinal smooth muscle, resulting in abdominal cramps, diarrhoea and vomiting (Taylor, 1986).

Apart from allergic response, other serious human pathologies caused by biogenic amines include carcinogenesis and tumor invasion (ornithine-derived polyamines and histamine), immune response and neurological disorders (histamine), the formation of carcinogenic nitrosamines by reaction between nitrite and secondary amines (putrescine, cadaverine, agmatine), migraines and hypertension (tyramine and phenylethylamine) and Parkinson's disease, Schizophrenia and mood disorders (tyramine) (Smith, 1980; ten Brink *et al.*, 1990; Silla Santos, 1996; Medina *et al*., 1999).

The toxic level of biogenic amines depends on the tolerance of the individual for the compound, the concentration of total biogenic amines and the consumption of ethanol and/or drugs. The toxicity of histamine and tyramine depends on the effectivity of the catabolic path which employs monoamine oxidase (MAO) and diamine oxidase (DAO) enzymes, which again varies in individuals (ten Brink *et al*., 1990). Biogenic amines such as tyramine, putrescine and cadaverine that may also be present in the wine can inhibit the metabolism of histamine. These amines favour the passage of histamine from the intestines into the systemic circulation by competing for binding sites in the gastrointestinal tract or by interfering with the catabolism of histamine by saturating the activity of mono- or diamine oxidases (Kanny *et al*., 2001). The amine oxidase enzymes are not very specific and alcohol, acetaldehyde and anti-depressive drugs can also cause interference (ten Brink *et al*., 1990; Straub *et al*., 1995).

Generally the toxic dose in alcoholic beverages is considered to be between 8 and 20 mg/L for histamine, 25 and 40 mg/L for tyramine, while as little as 3 mg/L phenylethylamine can cause negative physiological effects (Soufleros *et al.,* 1998). Kanny *et al*. (2001) reports that a normal individual can tolerate 120 mg/L of histamine taken orally before symptoms occur, but only 7 µg administered intravenously.

However, there are studies that conclude that no relationship exists between the oral ingestion of biogenic amines and wine intolerance (Kanny *et al*., 1999, 2001; Jansen *et al*., 2003). Rather, these authors propose that wine may contain compounds (ethanol and acetaldehyde) that stimulate the release of histamine within the body.

Other than toxic effects, some biogenic amines also have other negative consequences, particularly regarding the sensory characteristics of the wine and economic implications.

A study by Rohn *et al.* (2005) suggests that histamine can be identified at high concentrations in commercial wines by well trained wine assessors. The study employed mouthfeel descriptors such as "irritation at the deep throat" and "crawling of the tongue". No specific taste could be attributed to histamine. Putrescine, the most abundant biogenic amine in wine can reduce sensorial quality at 15 to 20 mg/L and 20 to 30 mg/L in white and red wines respectively (Arena & Manca de Nadra, 2001). In wine at a low pH, volatile amines occur as odourless salts, but in the mouth they are partially liberated and their flavours become apparent (Lehtonen, 1996). Volatile amines could therefore also influence wine aroma.

Biogenic amines in wine could also cause commercial import and export difficulties. Certain countries will reject wines that contain more than a certain concentration of histamine. The upper limits for histamine in wine in some European countries are (mg/L histamine): Germany (2), Holland (3), Finland (5), Belgium (5 to 6), France (8), Switzerland and Austria (10) (Lehtonen, 1996).

2.4 FACTORS AFFECTING THE FORMATION OF BIOGENIC AMINES IN GRAPES AND WINE

The levels of biogenic amines produced in wine largely depend on the abundance of amino acid precursors in the medium, since on the whole, biogenic amines increase with an increase in amino acids. Amino acid content may be influenced by vinification methods, grape variety, geographical region and vintage (Lonvaud-Funel & Joyeux, 1994; Soufleros *et al*., 1998; Moreno Arribas *et al*., 2000). While some factors increase the precursor amino acid concentration, other factors influence the growth and enzyme activity of microorganisms that can form the biogenic amines.

2.4.1 VITICULTURAL INFLUENCES

Some amines, such as putrescine and other polyamines, may already be present in grape berries (Halász *et al.,* 1994; Bover-Cid *et al*., 2006). Cabernet Sauvignon, for example, was found to have high concentrations of putrescine, cadaverine and spermidine in the pericarp of the berries (Glória *et al*., 1998). Putrescine, cadaverine and spermidine have also been found present in high concentrations in the seeds of grape berries (Kiss *et al*., 2006). Therefore, putrescine concentration in wine may be influenced more by geographical region and grape variety than by winemaking practices (Landete *et al*., 2005b). Potassium deficiencies in the soil have been linked to an increase in putrescine concentration in plants (Adams, 1991); while water deficiencies do not seem to influence the content of biogenic amines in grape berries and wines (Bover-Cid *et al*., 2006). The degree of maturation of the grape and the soil type can also influence amine levels in the final product (Glória *et al*., 1998).

Biogenic amines are also dependent on grape variety and vine nutrition, which will determine the concentration and composition of precursor amino acids in grape must and finally, together with yeast metabolisms, in the wine (Soufleros *et al*., 1998). Nitrogen fertilization treatments can cause an increase in grape amino acids and amine concentrations (Spayd *et al*., 1994; Soufleros *et al*., 2007). Grape varieties with higher levels of assimilable amino acids have been found to yield the highest final concentrations of biogenic amines (Herbert *et al.*, 2005).

An experiment was conducted by Cecchini *et al.,* (2005) to determine whether red grape cultivars have an effect on the content of biogenic amines in wines. Merlot, Syrah, Sangiovese, Cesanese d'Afflile and Cabernet Franc were studied. Analysis of variance

showed a significant difference between individual and total amine contents in wines obtained from musts of the different cultivars. Cabernet Franc was found to have significantly higher total amine content than any of the other cultivars studied.

More than one study has reported that the grape variety Pinot noir contains higher levels of biogenic amines when compared to Cabernet Sauvignon. Ough (1971) observed that significantly higher histamine concentrations were present in Pinot noir in California compared to Cabernet Sauvignon, while Glória *et al.* (1998) found that Pinot noir (Oregon, USA) contained significantly more total biogenic amines compared to Cabernet Sauvignon. Soleas *et al*. (1999) also observed higher amine concentrations in Pinot noir from Ontario (Canada) compared to other red wines. Other authors have observed cultivar related differences in biogenic amine content in Hungarian (Hajós *et al*., 2000) and Greek (Soufleros *et al*., 2007) wines.

The vintage and the region of production can also affect the free amino acid and amine content in must and wine (Herbert *et al.*, 2005). The concentrations of precursor amino acids can vary significantly over years. The influence of vintage on biogenic amine levels could also be attributed to the diversity of wine microorganisms that are naturally selected - their growth could again be correlated to the pH of the grapes and wines that can differ accordingly with the vintage (Martín-Álvarez *et al*., 2006). These authors found Spanish Tempranillo wines from a specific region had significantly more biogenic amines in 2001 than in 2002. Amine levels of aszu wines were also found to be influenced by vintage and were significantly different for the 1993, 1997 and 1998 vintages (Sass-Kiss *et al*., 2000). On the contrary, Glória *et al.* (1998), found no difference in amine concentrations from two vintages (1991 and 1992).

2.4.2 GRAPE SKIN MACERATION PRACTICES

Grape skin maceration promotes extraction of grape components such as phenolic compounds, proteins, amino acids and polysaccharides. During cold maceration, grape must is left in contact with the grape skins at a cold temperature prior to alcoholic fermentation. Most red wines undergo alcoholic fermentation in contact with grape skins. Extended maceration after alcoholic fermentation can also be applied at cool temperature to extent the extraction period. Pectolytic enzymes are added to grape musts to increase the yield of juice, to clarify the must or wine, to extract more grape derived compounds such as phenols and to facilitate pressing and filtration.

Some authors could find no connection between grape skin maceration practices and the levels of biogenic amines in wines. Soleas *et al*. (1999) found no correlation between length of skin contact and concentration of biogenic amines, while Martín-Álvarez *et al*. (2006) concluded that the addition of pectolytic enzymes to the grapes at 2 g/100 kg did not promote biogenic amine accumulation in wine in their study.

However, others found that the duration of skin maceration to be a very important variable that affects biogenic amine content in wines, and that longer maceration time could favour increased production of biogenic amines (Bauza *et al*., 1995; Martín-

Álvarez *et al*. 2006). Results from the latter research group are represented in **Table 2.2**.

Table 2.2 Average biogenic amine concentrations (mg/L) for different levels of vintage, pectolytic enzymes, ageing on lees, maceration time and bacteria inoculation (Martín-Álvarez *et al*. 2006).

	Number of samples	Histamine	Methylamine	Ethylamine	Tyramine	Phenylethyl-amine	Putrescine	Cadaverine
Vintage		**	**	**	**	*	**	
2001	117	4.87±0.67	1.36±0.12	3.07±0.20	2.12±0.55	3.36±0.50	9.69±1.24	1.16±0.44
2002	107	1.44±0.69	0.39±0.12	0.44±0.20	0.62±0.57	2.29±0.52	4.70±1.29	1.60±0.46
Pectolytic enzymes						**		*
No	162	2.76±0.59	1.01±0.11	1.66±0.17	0.96±0.49	3.86±0.44	8.08±1.10	1.93±0.39
Yes	62	3.55±0.81	0.74±0.14	1.85±0.24	1.77±0.67	1.79±0.61	10.46±1.45	1.55±0.51
Ageing with lees			*		**		**	
No	159	3.82±0.63	0.71±0.11	1.92±0.19	2.30±0.52	2.68±0.47	3.93±1.1	1.21±0.42
Yes	65	2.49±0.78	1.05±0.14	1.58±0.23	0.43±0.64	2.97±0.58	10.46±1.45	1.55±0.51
Skin maceration (time)		**	*		**		**	
<10 days	123	1.83±0.58	0.71±0.10	1.83±0.17	0.51±0.47	2.70±0.43	3.32±1.07	1.02±0.38
>10 days	101	4.89±0.40	1.04±0.14	1.68±0.23	2.22±0.65	2.95±0.59	11.07±1.46	1.74±0.52
MLF inoculation		**			*			*
No	193	4.89±0.40	1.01±0.07	2.00±0.12	2.37±0.33	2.67±0.30	7.34±0.74	2.09±0.26
Yes	31	1.42±1.04	0.75±0.19	1.50±0.3	0.37±0.86	2.99±0.78	7.05±1.94	0.67±0.69
	224							

*The factor has a statistically significant effect on the variable, P<0.05.

**The factor has a statistically significant effect on the variable, P<0.01.

An investigation on musts and wines of elderberry fruit showed that histamine content was dependent on the method of pulp treatment prior to processing, namely hot maceration, hot maceration and depectinisation and fermentation on the pulp (**Table 2.3**). Their results show that histamine is formed by the action of native histidine decarboxylase of the berries, as well as by enzymes present in the Polish pectolytic preparation (Pectopol PT), and during fermentation by the action of decarboxylases produced by yeasts. The highest decarboxylase enzyme activity was found in Bordeaux yeast, followed by Burgundy, Malga, Tokay and Syrena yeasts. (These yeasts seem to be unspecified *Saccharomyces cerevisiae* wine yeast starter cultures.) None of the wines had undergone any malolactic fermentation, thus excluding the possibility of biogenic amine formation by the action of bacterial decarboxylases (Pogorzelski, 1992). The presence of and possible contribution by indigenous lactic acid bacteria on the elderberry fruit was not reported.

Table 2.3 Histamine in musts and wines from elderberry fruit depending on the pulp treatment prior to pressing. Wines were fermented with Bordeaux yeast (Pogorzelski, 1992).

	Must Range (mg/L)	Must Mean (mg/L)	Wine Range (mg/L)	Wine Mean (mg/L)
After depectinisation	2.20-4.70	3.20	7.60-12.36	10.40
After hot maceration	0.00-0.80	0.15	3.65-6.97	4.24
After fermentation in the pulp	1.20-3.34	2.12	5.77-8.30	7.40

2.4.3 PHENOLIC COMPOUNDS

Grape phenolics are also extracted from the grape skins (and seeds) during maceration and fermentation. These compounds are responsible for many chemical reactions in grape must and wine. They are involved in oxidation reactions (they are strong antioxidants), and influence the flavour and colour of wine. Even though it has been known for some time that phenolic compounds may have an effect (inhibiting or stimulatory) on the growth, metabolism and malolactic activity of lactic acid bacteria (Vivas *et al.*, 1997; Alberto *et al*. 2001, 2007), the first study to ascertain whether phenolic compounds can affect biogenic amine production by wine lactic acid bacteria was done in 2007 by Alberto *et al*., with specific focus on the influence of phenolic compounds on agmatine metabolism of *Lactobacillus hilgardii* X_1B. The formation of putrescine from agmatine does not involve amino acid decarboxylase. Alberto *et al*. (2007) observed the conversion of agmatine into putrescine was lower in the presence of all phenolic compounds, with the exception of gallic acid and quercetin. Gallic acid and quercetin also had no influence on bacterial growth or survival in the presence of agmatine and therefore had no interactions with agmatine metabolism.

Putrescine, the end product of this pathway, is able to protect the DNA damaged by reactive oxygen species and increase cell survival of *Escherichia coli* under conditions of oxidative stress (Tkachenko, 2004). Phenolics are powerful antioxidants and the reduction of putrescine formation in the presence of phenolics is attributed to the ability of the phenolics to protect the cells against oxidative stress themselves (Alberto *et al.*, 2007). Phenolic decarboxylation could also compete with the enzyme involved in the conversion of agmatine into putrescine, *N*-carbamoylputrescine decarboxylase, and thus inhibits the formation of putrescine. According to this study, the presence of phenolic compounds could pose a natural solution to reduce putrescine formation in red wine.

2.4.4 WINE PHYSIOCHEMICAL COMPOSITION

Wine physiochemical factors such as pH, temperature, SO_2 and a variety of substrates and products of fermentation can influence the concentration and diversity of microorganisms in the wine, but can also affect decarboxylase enzyme activity and gene expression.

2.4.4.1 Influence of wine composition on enzyme activity and decarboxylase gene expression

In wine, the presence of the HDC gene does not mean that the enzyme is functional and that histamine will necessarily be formed (Coton *et al*., 1998a). As discussed earlier in this review, histidine decarboxylation can be used by bacterial cells to generate additional energy under poor growth conditions (Konings *et al.,* 1997). It seems as if though the production of histamine by lactic acid bacteria is always enhanced under poor growth conditions, for example when the medium has a shortage of fermentable substrates such as L-malic acid and glucose (Lonvaud-Funel & Joyeux, 1994). However, the presence of glucose and L-malic acid can also have a stimulating effect on decarboxylation. Moreno-Arribas *et al*. (2000) reported that more tyramine was produced by a tyrosine decarboxylase positive *Lactobacillus* strain in the presence of glucose, possibly due to the energy provided to aid the enzyme activity. In contrast, glucose was found to have no effect on the histidine decarboxylase enzyme of *O. oeni* (Rollan *et al.,* 1995). Malic acid was found to play a key role in activating arginine catabolism by *O. oeni*, increasing the production of putrescine (Mangani *et al*., 2005).

The product of malolactic fermentation, lactic acid, was found to inhibit histidine decarboxylase activity (Rollan *et al.,* 1995; Lonvaud-Funel, 2001); while on the contrary, lactic acid does not appear to inhibit ornithine decarboxylase activity (Mangani *et al*., 2005). Citric acid may also inhibit HDC and TDC activity to a small extent at levels normally present in wines after malolactic fermentation (Rollan *et al.,* 1995; Moreno-Arribas & Lonvaud-Funel, 1999). Other compounds found to inhibit TDC activity to different extents include glycerol, β-mercaptoethanol, lactic acid and ethanol. However, Moreno-Arribas & Lonvaud-Funel (1999) conclude that even the highest concentrations of these compounds likely to be present in wine will not be sufficient to prevent the formation of tyramine.

pH and ethanol at levels found in wine could inhibit decarboxylase enzyme activity (Leitão *et al.,* 2000). HDC activity and consequent histamine production is enhanced at pH 3.5 and by ethanol concentrations up to 10%, where the conditions for histidine transport inside the cells are more favourable due to the fluidification of the cell membrane by ethanol (Lonvaud-Funel & Joyeux, 1994). A high ethanol concentration (12% or more), as most often found in wine, reduces the HDC activity by altering the physicochemical properties of the membrane and slowing down histidine transport (Rollan *et al.,* 1995).

Histamine production was further found to be regulated by the presence of histidine, histamine, pyridoxal 5'-phosphate and the bacterial growth phase (Landete *et al*., 2006). It was observed that HDC gene expression is induced by histidine (at 1 or 2 g/L) and decreased by histamine (at 1 or 2 g/L), while pyridoxal 5'-phosphate enhanced HDC activity (at 0.5 g/L).

During a study performed by Lucas *et al*. (2005) it was discovered that the histidine decarboxylase positive phenotype disappeared under certain culture conditions in *Lactobacillus hilgardii* 0006, isolated from wine. In a poor, acidic medium the number of

histidine decarboxylase positive cells increased, presumable due to the energetic and growth advantage of these cells. In a rich medium with a higher pH, the number of mutant cells lacking histidine decarboxylase activity increased. Loss of enzyme activity was found to correspond to the loss of a large plasmid (80 kb) on which the histidine decarboxylase gene was located.

In addition to decarboxylase activity, a small number of *O. oeni* strains (six of 220 strains tested) were found to also have proteolytic activity – the ability to release amino acids from peptides and proteins. The proteolytic activity is also dependent on nutritional and energetic composition of the medium, and generally increases when high-energy nutrients are exhausted in the late exponential growth phase (Leitão *et al.,* 2000). Decarboxylase activity is also expressed when the cells need the additional energy produced during amino acid transport. Northern blot analysis confirmed that HDC expression appeared during the early growth phase, reached a peak during the exponential growth phase (because the decarboxylation generates metabolic energy for this time of growth and cell division) and decreased significantly during the stationary phase when growth and cell division decrease (Molenaar *et al.,* 1993; Landete *et al*., 2006). Other researchers also found that decarboxylase activity increases or could be biosynthesised towards the end of the active growth phase and during the exponential phase of bacterial growth under experimental and industrial winemaking conditions (Gale, 1946; Moreno-Arribas *et al*., 2000; Marcobal *et al*., 2006b). *O. oeni* expresses proteolytic and decarboxylase activities only when there is no easier strategy for cell survival (Molenaar *et al*., 1993).

2.4.4.2 Influence of wine composition on bacterial growth and survival

A study was performed to analyse the effects of five physiochemical factors (incubation temperature, incubation time, environmental pH, added tyrosine concentration and pyridoxal 5'-phosphate supplementation) on cell growth and tyramine production of *Lactobacillus brevis* CECT 4669 and *Enterococcus faecium* BIFI-58 (isolated from grape must) under anaerobic and aerobic conditions. A multiple linear regression model was used to predict that the optimum conditions for growth and tyramine production was anaerobic incubation at acidic pH (4.4) in the presence of a high tyrosine concentration (Marcobal *et al*., 2006a).

According to Garde-Cerdán *et al.* (2006), SO_2 added to must does not affect the formation of biogenic amines during the alcoholic fermentation. Studies of the complete vinification of industrial wines indicated that SO_2 does prevent the formation of biogenic amines by reducing lactic acid bacteria in the wine (Marcobal *et al.* 2006b). Vidal-Carou *et al.* (1990) found that the highest levels of biogenic amines are found in red wines with low SO_2 levels and that increased levels of SO_2 was correlated with a decrease in the concentration of histamine and tyramine. The effect of SO_2 on tyramine accumulation was also found to be dependent on pH. At a higher pH, an increase of SO_2 was found to cause a decrease in tyramine concentration, while at a lower pH tyramine increased

with an increase of SO_2 (Gardini *et al*., 2005). This response to pH is contrary to what is usually encountered during biogenic amine production. Normally, at a higher wine pH, the bacterial microflora is more diverse and the growth and survival of decarboxylase positive bacteria becomes more likely. Hence, at higher pH, higher levels of biogenic amines are produced in most cases (Wibowo *et al*., 1985; Lonvaud-Funel & Joyeux, 1994; Gardini *et al*., 2005; Landete *et al*., 2005b; Martín-Álvarez *et al*., 2006). Landete *et al*. (2005b) observed that wines from La Rioja with a high histamine content had a pH of 3.6 or higher. Cilliers & Van Wyk (1985) also noted that the pH of all the red wines containing large amounts of histamine (>10 mg/L) in their study exceeded 3.7.

Lower levels of biogenic amines were produced in conditions of high ethanol concentrations and low pyridoxal 5'-phosphate concentrations (Gardini *et al*., 2005). The decrease in tyramine production by *O. oeni* T56 (a tyramine producer) under conditions of high ethanol and low pH was attributed to reduced metabolic activity and cellular viability and not to the specific decarboxylase activity in this study.

Acetic acid (volatile acidity) has been correlated with high levels of histamine in white and rosé wines in one study. However, the reason for this correlation was not determined (Vidal-Carou *et al.*, 1990).

2.4.5 CONDITIONS DURING AGEING AND STORAGE OF WINE

After malolactic fermentation Landete *et al*. (2005b) noticed a further increase in histamine during the first six months of storage in bottles. Gerbaux & Monamy (2000) also found that the concentration of histamine increases between four and eight months after malolactic fermentation in Pinot Noir and Chardonnay. A third study (Herbert *et al.* 2005) showed a consistent increase of histamine in red and white wines 18 months after the completion of malolactic fermentation, whilst tyramine and putrescine seemed to increase immediately following malolactic fermentation in red wines in this study.

The reason for the initial increase following the completion of malolactic fermentation could be that SO_2 added to the wine after malolactic fermentation does not completely stop all biochemical reactions and enzyme activity. Also, due to the high pH of many wines, SO_2 is less effective and hence biogenic amines can increase in sulphated wines during ageing (Gerbaux & Monamy, 2000).

Another reason for increase in biogenic amines following fermentations is derived from the winemaking practice of ageing wine in contact with yeast lees. Yeast autolysis favours the growth and activity of lactic acid bacteria due to the release of vitamins and nitrogen compounds. Lactic acid bacteria are able to hydrolyse and metabolise proteins and peptides and use the released amino acids as nutrients or energy sources. These amino acids may include the precursors of biogenic amines (Lonvaud-Funel & Joyeux, 1994). Yeast and bacterial lees can also be the source of decarboxylase positive lactic acid bacteria. Bauza *et al*. (1995) observed an increased production of tyramine and putrescine when wines are inoculated with bacteria through the addition of lees.

Incidentally, the first ornithine decarboxylase gene from a putrescine producing wine lactic acid bacterium was isolated from wine lees (Marcobal *et al.,* 2004).

Martín-Álvarez *et al*. (2006) left their wines in contact with lees for two months after alcoholic fermentation, before ageing in barrels. The mean concentrations of methylamine and putrescine were higher in wines aged on lees. In contrast, tyramine concentrations were significantly lower (Table 2). The authors postulate that tyramine could be consumed by residual microorganisms from lees for the production of carbon skeletons or amino groups. Coton *et al*. (1998a) also noted that even when no more culturable cells were detectable, histidine decarboxylase could still be active – thus biogenic amines can be produced during ageing. Moreover, most amines as well as decarboxylase enzymes are heat stable and will not be reduced during processing (such as pasteurisation), and can for this reason even increase during storage (ten Brink *et al.*, 1990).

After the initial increase of biogenic amines during storage, a general decrease or stabilisation in concentration could be observed by various research groups (Gerbaux & Monamy, 2000; Landete *et al*., 2005b; Marcobal *et al.*, 2006b). Biogenic amines can be degraded by oxidase enzymes present in some bacteria towards the end of the ageing period, even at wine pH (Vidal-Carou *et al*., 1991; Moreno & Azpilicueta, 2004). The general decrease of biogenic amines during ageing could explain why the highest histamine content (average 8.72 mg/L) was found by Vasquez-Lasa *et al*. (1998) in young red wines compared to red wines subjected to different traditional ageing prescriptions in Rioja, Spain. No statistically significant differences were found between histamine levels in crianza red wines (6.67 mg/L), reserva red wines (6.92 mg/L) or gran reserva red wines (5.12 mg/L).

Other factors present during ageing may or may not play a role in the accumulation of biogenic amines. Moreno & Azpilicueta (2004) compared the biogenic amine concentrations of filtered and unfiltered wines aged in barrels for 243 days. Diatomaceous earth can adsorb cationic amino acids and proteins on its surface; which can influence the evolution of biogenic amines during ageing. Unfiltered wine can contain skin residues which can be rich in amino acid precursors. However, it was found that the degree of turbidity did not influence the accumulation of biogenic amines during ageing. Also, the type of barrel (American, French Allier and French Nevers oak) did not influence the content of biogenic amines. In another study, the highest mean values for histamine were acquired in wines where malolactic fermentation was performed in tanks (not barrels), followed by ageing in the presence of lees stirred weekly or monthly. In this study, putrescine increased during ageing in wines aged in presence of yeast lees, but remained stable in wines without lees (Alcaide-Hidalgo *et al*., 2007).

Normally, the storage of wine at elevated or fluctuating temperatures can cause unwanted chemical, microbial or enzymatic reactions of wine components and seriously decrease product quality. However, it was found that wine storage temperature only has a small effect on amine concentration. Histamine concentration was found to increase slightly when wines were stored for 105 days, more so at 20°C than at the more

extreme temperatures of 4°C or 35°C. The formation or degradation of amines in wine mainly took place during the first 45 days of storage for all temperatures studied, due to the presence of residual decarboxylase activity after alcoholic and malolactic fermentations (González Marco & Ancín Azpilicueta, 2006). Vidal-Carou *et al*. (1991) found no formation or increase in biogenic amines (histamine or tyramine) at various temperatures ranging from 4°C to 35°C in wines stored for 93 to 125 days under spoilage conditions. The only changes observed in biogenic amine content were the decrease in histamine and tyramine, independent of temperature. The authors could not explain this phenomenon.

2.4.6 WINE STYLE AND TYPE

Red wines generally show a higher concentration of biogenic amines than white and rosé wines. The higher values are attributed to the presence of lactic acid bacteria and malolactic fermentation (Landete *et al.,* 2005b); since in the case where white and rosé wines did undergo malolactic fermentation in this study, the amine values were close to those observed in red wines after malolactic fermentation. White wines, in general, also contain fewer amino acid precursors and have lower pH due to the absence of skin contact during fermentation and the absence of malolactic fermentation, and may consequently have lower biogenic amine concentrations than red wines (Zee *et al*., 1983; Cilliers & Van Wyk, 1985; Vazquez-Lasa *et al*., 1998; Leitão *et al*., 2005). Buteau *et al*. (1984) attributed the higher levels found in red wines due to the lack of bentonite treatments (which adsorbs amines) and the release of cellular amines by autolysing yeast cells in lees during malolactic fermentation.

Rupasinghe & Clegg (2007) analysed and compared the biogenic amine content of ten different types of fruit wines (apple, black currant, blueberry, cherry, cranberry, elderberry, peach, pear, plum, raspberry) to wines made from grapes (Cabernet Sauvignon, Chardonnay, Riesling and icewine made from Vidal Blanc). The results indicate that Cabernet Sauvignon grapes contain significantly higher levels of biogenic amines (11 143 µg/L histamine) than all other fruit wines. Again, this was attributed to the fact that this red wine was the only wine that had undergone malolactic fermentation.

In Chinese rice wines, the average amount of total biogenic amines was found to be 107 mg/L, which is higher levels than reported in wine made from grapes. Interestingly, no putrescine was detected in any of the 42 samples of rice wines tested, while histamine was detected in all samples in the range of 5.02 to 78.50 mg/L, followed by spermine (present in 93% of samples), cadaverine (87%), tyramine (79%) and spermidine (79%). The highest levels of total biogenic amines (100 to 241 mg/L) were observed in Shaoxing rice wine. The vinification procedure followed in Shaoxing includes a soaking step where the rice is soaked in a fermentation substrate (soaking water) containing a large amount of free amino acids and active decarboxylase positive

lactic acid bacteria. No significant correlation was found between the pH of rice wines (4.04 to 4.33) and biogenic amines in this work (Yongmei *et al*., 2007).

More studies on the levels of biogenic amines in traditional alcoholic beverages (Lasekan & Lasekan, 2000), natural ciders (Garai *et al*, 2006) and beer have also been published. For the purpose of this review it is only important to note that the growth and activity of lactic acid bacteria are implicated in biogenic amine production in all these products.

2.5 ANALYTICAL AND MOLECULAR METHODS USED IN BIOGENIC AMINE IDENTIFICATION

Analysis of biogenic amines, individually or simultaneously, is important because of their toxicity potential and their potential to be applied as indicators of food spoilage or authenticity. Biogenic amines can be quantified by a variety of analytical methods that require sophisticated equipment. Qualitative measurements can also indicate the presence of amines in wine. For review papers on analytical methods used in the determination of biogenic amines, refer to Lehtonen (1996) and Önal (2007). The potential of biogenic amines to appear in wine can also be determined by using molecular tools which can detect the presence of decarboxylase positive microorganisms.

2.5.1 QUALITATIVE AND SEMI-QUANTITATIVE METHODS

2.5.1.1 Screening methods using selective media

Some of the first methods developed for qualitative biogenic amine detection (initially for histamine) are microbiological screening methods that involve the use of a differential agar medium with a pH indicator (bromocresol purple), where an increase in pH as a result of amine formation can be easily observed by a change in colour. Amino acid precursors are contained in the decarboxylase assay medium. Modifications of the screening plate medium have been made by various researchers to make it more suitable for the growth of lactic acid bacteria and activity of decarboxylase enzymes. Improvements have also led to greater sensitivity and reliability of the screening plate method, and it has shown good correlation with other chemical analytical methods (Choudhury, 1990; Maijala, 1993; Bover-Cid & Holzapfel, 1999). Yet, it is still advisable to confirm the results of simple screening methods by another analytical method (such as HPLC), because false positive results can occur due to the formation of other alkaline compounds (such as ammonia), and failure to detect amine production has also been reported (Moreno-Arribas *et al*., 2003).

2.5.1.2 Enzymatic methods

Enzymatic methods to quantify histamine were first reported for use in fish. When these enzymatic methods were applied to musts and wines, many false positive results were recorded. A direct enzymatic test for the use in wine was developed by Landete *et al*. (2004). This test is performed by the sequential activity of the two enzymes, diamine oxidase (to break down histamine) and peroxidase (to produce a colour change). A linear correlation between optical density and histamine concentration is used for quantification. A very good correlation (r^2=0.9984) could also be established between biogenic amine quantification by this enzymatic method versus HPLC analysis. The advantage of this method is its limited sample preparation and time-consumption, and it does not require expensive or sophisticated equipment or training.

Enzyme-linked immunosorbent assays (ELISA) are commonly used for the quantitative analysis of histamine in scombroid fish. Such a test was applied to wine samples for the first time by Marcobal *et al*. (2005b). No false negative results were obtained by the ELISA test, although there was a slight overestimation of histamine in a few samples when correlated to the results obtained by reverse phase-HPLC (r=0.91). This rapid, easy method could be used for screening in laboratories that are not equipped with an HPLC, in order to distinguish between wines with a histamine content of more or less than 10 mg/L.

2.5.1.3 Thin-layer chromatography

Thin-layer chromatography (TLC) was one of the first techniques used for the determination of biogenic amines in foods (Halász *et al*., 1994). Despite the advantage of not requiring special or expensive equipment, TLC is known to be time consuming and only semi-quantitative.

A simple and rapid qualitative TLC method for the determination of the ability of bacteria to produce biogenic amines in liquid culture media containing the amino acid precursor is described by Gárcia-Moruno *et al.* (2005). This method improves on previous TLC methods due to the omition of an extraction step from the bacterial supernatants. The fluorescent dansyl derivatives of histamine, tyramine, putrescine and phenylethylamine can be separated and identified using TLC. This method is not known to give false positive results. The only equipment involved is a photo camera and a UV transilluminator.

2.5.2 QUANTITATIVE METHODS

Wine is a complex matrix and biogenic amines are present at low concentrations, hindering the ease of determination. Biogenic amines are non-volatile, polar compounds, which makes their isolation from wine (an aqueous matrix) complicated. In addition, the aliphatic biogenic amines are difficult to detect as they exhibit no

characteristic ultraviolet (UV) absorption, fluorescent properties or electrochemical activity. These properties prohibit the direct detection of biogenic amines, for example by spectrophotometric or fluorimetric methods. To solve this problem, biogenic amines have to be derivatised to enable their detection by higher absorbance wavelengths. Samples usually also require a clean-up and pre-concentration step. Solid phase extraction (SPE) is widely used and has even been automated. Liquid-liquid extraction of the analyte with organic solvent can also be exploited. Both these method have their disadvantages such as losses of amines and low recoveries (García-Villar *et al*., 2006).

2.5.2.1 Liquid chromatography

Presently, high-performance liquid chromatography (HPLC) methods still seem to be the most widely used analytical approach to test for the presence of biogenic amines. These methods usually include pre- or post-column derivatisation and fluorimetric detection of the corresponding derivatives. Various HPLC methods have been described. Modifications and improvements are consistently being made to pre- and post column treatments and reagents in order to reduce preparation and analysis time and to improve resolution of biogenic amine peaks in the chromatogram (Soleas *et al*., 1999; Marcobal *et al*., 2005b).

O-phthalaldehyde (OPA) is one of the preferred derivatising agents, giving rise to highly fluorescent derivatives. However, OPA can react with primary amines only. Polyamines such as spermine and spermidine can therefore not be detected by using this reagent. Dansyl-chloride, the other popular derivatising reagent, can react with primary, secondary and even tertiary amino groups under selected conditions. These derivatives are stable and also fluorescent and therefore detectable in the UV region.

Fluorenylmethylchloroformate (FMOC) can be employed as derivatisation reagent to determine spermine and spermidine in addition to other biogenic amines, along with the simultaneous detection of their precursor amino acids in wine (Bauza *et al*., 1995).

Another derivatisation reagent that can react with primary, secondary and aromatic primary amino groups is 1,2-naphthoquinone-4-sulfonate (NQS). García-Villar *et al*. (2006) used liquid-liquid extraction with chloroform to preconcentrate biogenic amines and to remove polar compounds such as amino acid derivatives which interfere with subsequent separation steps.

8-phenyl-(4-oxy-acetic acid *N*-hydroxysuccinimide ester)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene (TMPAB-OSu) is another recently synthesised fluorescent reagent that can be used for rapid pre-column derivatisation for HPLC analysis of primary as well as secondary biogenic amines in wine (Li *et al*., 2006).

Another HPLC method proposes the direct injection of samples derivatised with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC). No other pre-treatment (extraction) is required. Histamine, putrescine, cadaverine and tyramine in musts and wines can be analysed with this method (Hernández-Orte *et al*., 2006).

Gómez-Alonso *et al*. (2007) use diethyl ethoxymethylenemalonate (DEEMM) as derivatising agent to form aminoenones which can be detected in the UV-visible region. By this method, nine biogenic amines, 24 amino acids and the ammonium ion can be analysed simultaneously in a single injection by reverse phase-HPLC. The authors claim that this method is an improvement to previous HPLC methods that can simultaneously quantify amines and amino acids.

Loukou & Zotou (2003) presented the first HPLC-fluorescence method (as opposed to HPLC-UV) to simultaneously assay 11 oenologically important biogenic amines. An HLPC-diode array detection-atmospheric pressure chemical ionisation mass spectrometry system (HPLC-DAD-APCI-MS) was used to characterise dansylamides for the first time in wine and other alcoholic beverages after pretreatment with polyvinylpyrrolidone (PVP). Pretreatment is used to remove substances from the matrix that can interfere in the derivatisation and quantification. A similar method (reverse phase HPLC-DAD) was described by mo Dugo *et al*. (2006) that does not require any sample pretreatment before derivatisation with dansyl chloride.

Alberto *et al*. (2002) described two reverse phase HPLC methods, one of which uses pre-column derivatisation with OPA to separate and quantify both biogenic amines and amino acids in a single run.

Micellar liquid chromatography (MLC) uses a surfactant solution instead of aqueous-organic solvents as is used by HPLC. The method described by Gil-Agustí *et al*. (2007) does not require pre-treatment of samples (other than filtration) or an extraction procedure prior to analysis, and can be performed with a single direct injection. With this method, tryptamine and tyramine and their precursors, tryptophane and tyrosine, can be determined simultaneously in wine samples. Micellar electrokinetic chromatography (MECC) separation with laser-induced fluorescence (LIF) detection is another method that can be applied for the quantification of a large number of biogenic amines and amino acids in wine (Nouadje *et al*., 1997).

A more liquid chromatography method to simultaneously analyse ten oenologically important biogenic amines has been proposed (Hernández-Borges *et al*., 2007). Nano-liquid chromatography with UV detection was used to quantify the biogenic amines with dansyl-chloride as derivatising agent and employing SPE for post-derivatisation clean-up and to extract the analytes from the wine. Other liquid chromatography techniques that are applicable to biogenic amine analysis in wine have been described by Ibe *et al*. (1991); Hlabangana *et al*. (2006) (ion-pair liquid chromatography) and Millán *et al*. (2007) (liquid chromatography-electronspray ionisation ion trap mass spectrometry).

2.5.2.2 Capillary electrophoresis

Capillary electrophoresis (CE) methods are attractive due to their short analysis time and high resolution. One problem is their lack of sensitivity, which can be overcome by coupling the CE to mass spectrometry (MS) detection, instead of UV detection. Other detection systems are also available. For example, a CE method using conductometric

detection and which requires no derivatisation or sample cleaning steps was developed recently by Kvasnicka & Voldrich (2006). This direct method is sensitive and can detect biogenic amines in foods and wine in less than 15 minutes. A high-performance capillary electrophoresis (HPCE) method exists to determine biogenic amines in wine, amongst other foodstuffs (Kovács *et al*., 1999).

An automated method for the determination of biogenic amines in wine has been described in order to exclude manual pretreatment of the sample (Acre *et al*., 1998). This method employs the use of a minicolumn for solid-phase extraction (SPE) to simultaneously clean-up and concentrate the sample prior to analysis by capillary electrophoresis (CE) with indirect UV detection, in a flow system. This method allows for the separation and determination of a wide range of biogenic amines present in wine in less than 15 minutes, compared to 25 minutes by HPLC. Another advantage over HPLC is the direct detection. As noted, HPLC determination requires derivatisation of the amine due to aliphatic biogenic amines containing no chromophores that absorb significantly in the UV-visual region.

Another automated method is described by Santos *et al*. (2004) for biogenic amine determination in white and red wines. They proposed the use of capillary electrophoresis-electronspray mass spectrometry (CE-ESI-MS) for the separation and quantification of nine biogenic amines.

2.5.2.3 Gas chromatography

Volatile amines have been measured in the past by gas chromatography. A very sensitive and accurate gas-chromatography/mass spectrometry method was developed by Fernandes & Ferreira (2000), with a run time of only 18 minutes. This method is the first GC-MS method that allows for the simultaneous determination of diamines, polyamines and aromatic amines in grape juice and various wine styles tested. The conversion of biogenic amines to their corresponding volatile (*o*-heptafluorobutyryl) derivatives allows for determination by gas chromatography.

2.5.3 POLYMERASE CHAIN REACTION

The use of methods that can rapidly detect the presence of biogenic amines and biogenic amine producing lactic acid bacteria at an early stage is important for preventing the accumulation of biogenic amines in wine and other food products. PCR techniques have been developed to detect bacterial amino acid decarboxylases in a rapid, sensitive and accurate manner. Thus, PCR cannot determine quantitative (or qualitative) amounts of biogenic amines; it can only be used to estimate the potential risk of amine formation (Coton et al., 1998a). Since molecular methods are independent of the culture conditions, it can be used to detect biogenic amine producers even under circumstances where the lactic acid bacteria had lost the ability to produce biogenic

amines. Such instances have been reported after prolonged storage or cultivation in synthetic media (Lonvaud-Funel & Joyeux, 1994; Leitão *et al.*, 2000).

In principle, some DNA sequences of some genes are highly conserved, so PCR can be applied to detect specific genes in various organisms. Specific oligonucleotides for the amplification of histidine-, tyrosine-, ornithine- and lysine decarboxylase genes have been designed to detect the presence of bacteria which can possibly produce the corresponding biogenic amine in wine.

Le Jeune *et al*. (1995) developed degenerate primers to detect histidine decarboxylase positive lactic acid bacteria. These primers were based on nucleotide and amino acid sequences of *Lactobacillus* 30a and *Clostridium perfringens* and the amino acid sequences of *Lactobacillus buchneri* and *Micrococcus* sp. The primers allowed for amplification of a HDC gene fragment using DNA of these bacterial strains. The HDC gene of *Oenococcus oeni* has also been described (Coton *et al*., 1998b). Another set of universal primers for the detection of HDC genes in Gram-positive bacteria was developed by Coton & Coton (2005). These primers are based on HDC gene nucleotide sequences from a variety of lactic acid bacteria such as *O. oeni* IOEB 9204, *Lactobacillus* 30a and *C. perfringens*.

By a similar approach, universal primers were designed by Coton *et al*. (2004) for the early detection of potential tyramine-producing bacteria in wine. Marcobal *et al*. (2005a) designed the first complete set of primers to amplify the gene coding for ornithine decarboxylase by aligning amino acid sequences of ornithine decarboxylases from Gram-positive and Gram-negative bacteria. De las Rivas *et al*. (2006) described the first PCR assay to determine the presence of lysine decarboxylase positive, cadaverine producing microorganisms in food.

Multiplex amplification methods were developed to reduce the quantity of reagent, labour costs and time, because more than one gene species (target amines) can be detected simultaneously using multiplex PCR, as opposed to uniplex PCR (Coton & Coton, 2005; De las Rivas *et al*., 2005; Marcobal *et al*., 2005a). **Figure 2.3** shows an example of a multiplex PCR assay.

Molecular methods for the detection of biogenic amine producing bacteria have been reviewed recently by Landete *et al.* (2007a).

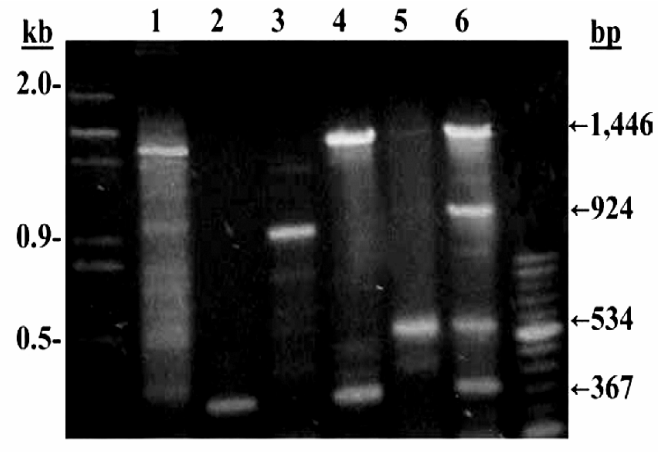

Figure 2.3 Multiplex PCR detection of histamine-, tyramine- and putrescine- producing bacteria. The assay is performed by using primer pairs for HDC (two different pairs showing the amplicon sizes of 367 bp and 534 bp respectively), TDC (924 bp) and ODC (1446 bp) simultaneously on chromosomal DNA from a number of bacterial species (lanes 1 to 6) (De las Rivas *et al.*, 2005).

2.5.4 USING BIOGENIC AMINES AS AN ANALYTICAL TOOL

Different chemometric procedures have been applied to establish criteria for differentiation in wines (Rius & Massart, 1991). Principal Component Analysis (PCA) has proved to be a useful tool for pattern recognition, classification, modelling and data evaluation. In a number of recent studies, wines have been successfully classified on the basis of biogenic amines.

Soufleros *et al.* (1998) used PCA to classify French wines of four regions according to wine style and origin by analyses of amino acids, biogenic amines, volatile compounds, and organic acids. Wines made from botrytised grapes formed a separate group.

Csomo´s *et al*. (2002) distinguished Hungarian red and white wines from the same geographic origin and vintage using PCA, according to their biogenic amine and polyphenol content. A further study concerning Hungarian wines concluded that, on the basis of the results of chemometric analyses (principal component analysis and linear discriminant analysis), free amino acid and biogenic amine contents seem to be useful to differentiate wines according to winemaking technology, geographic origin, grape variety, and year of vintage (Héberger *et al*., 2003).

Kiss *et al*. (2006) performed a study with the objective to compare the amine composition of Aszu grapes (noble rotten grapes) with that of gray rotten berries (infected mainly with *Botrytis cinerea*) and of berries infected with other local green molds (mainly *Penicillium*). Using multivariate statistical analyses (principal component analysis and linear discriminant analysis) intact grapes, Aszu grapes, and grape berries infected mainly with *Penicillium* species could be separated from each other in grape samples with the same origin (**Figure 2.4**). The composition and concentration of biogenic amines might also provide useful information on the authentication of the Tokaj

aszu wines (Hajós *et al*., 2000) and could be used in grapes, wines and aszu wines for quality control purposes (**Figure 2.5**) (Sass-Kiss *et al*., 2000; Kiss & Sass-Kiss, 2005).

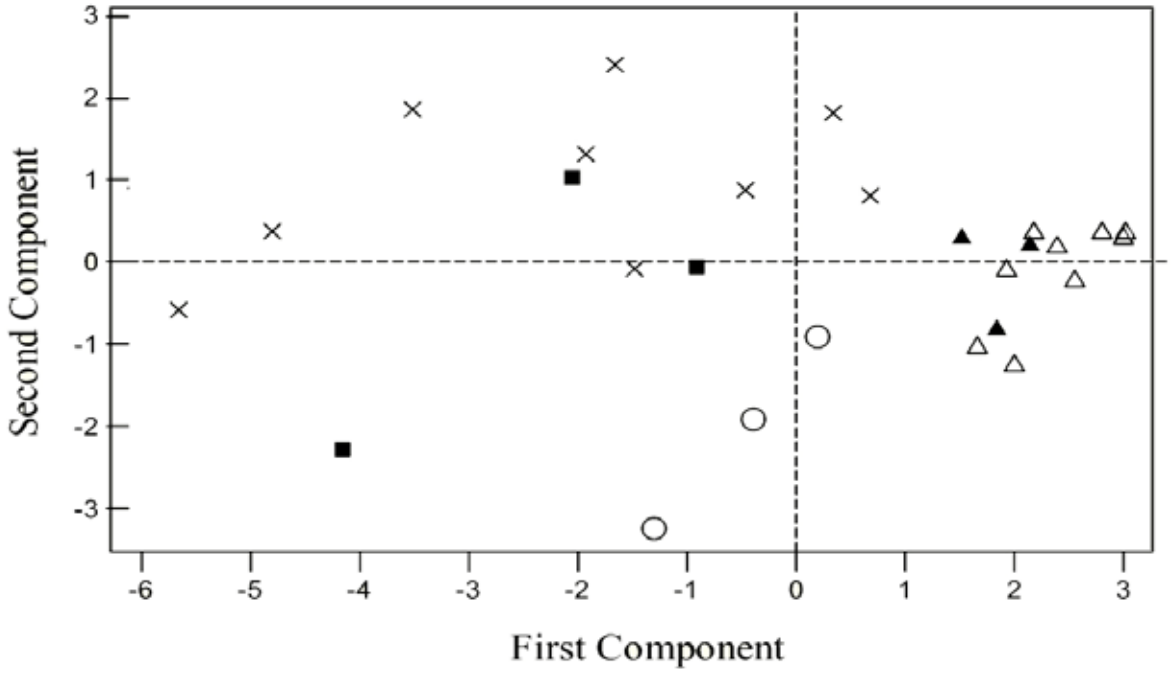

Figure 2.4 PCA of biogenic amines (putrescine, *i*-butyl amine, cadaverine, tyramine, histamine, agmatine, 2-methyl-butyl amine, 3-methyl-butyl amine, spermidine and phenylethylamine) of vintage 2004. Aszu grapes, □; intact grapes, ∆; dried grapes, ▲; *Penicillium* rotten grapes, ■; and gray rotten grapes, O (Kiss *et al*., 2006).

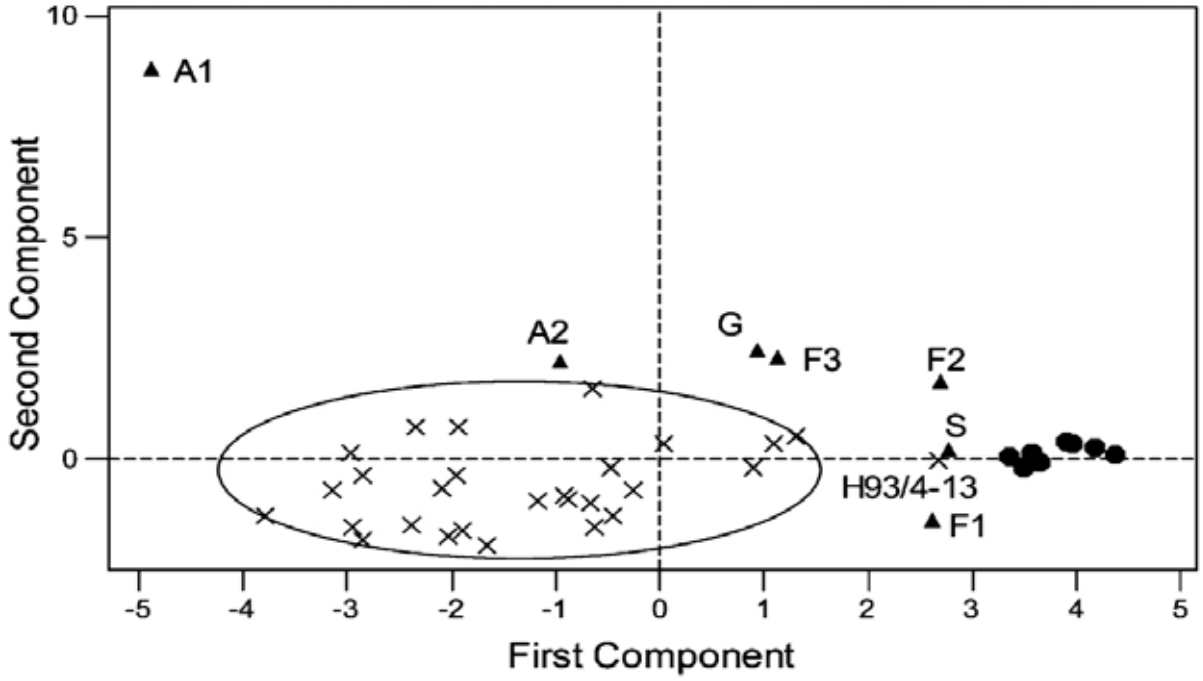

Figure 2.5 PCA of nine biogenic amines and two organic acids to distinguish between Hungarian botrytised wines, □; foreign botrytised wines, ▲; and Hungarian nonbotrytised wines, ● (Kiss & Sass-Kiss, 2005).

2.6 CONTROL OF BIOGENIC AMINE PRODUCTION

Due to indigenous lactic acid bacteria, including *O. oeni*, being able to produce biogenic amines during malolactic fermentation, the application of a genetically engineered malolactic wine yeast strain, capable of the complete degradation of L-malic acid in wine, has been proposed (Husnik *et al*., 2006). According to the authors, this yeast could prevent the formation of biogenic amines in wine by omitting the need for lactic acid bacteria to perform malolactic fermentation. Currently, consumer rejection of genetically modified organisms in wine producing countries still renders this option unavailable in most countries.

It is known that some microorganisms, among them lactic acid bacteria, are capable of degrading biogenic amines by amine oxidase enzyme activity. Consequently, Leuschner *et al*. (1998) studied a large number of food fermenting organisms to screen for the potential to degrade histamine and tyramine. Unfortunately, at this stage, amine degradation seems to be restricted to aerobic microorganisms which are of limited use in fermented foods such as wine which harbours an anaerobic environment.

Presently, the only realistic option to control the potential problem of biogenic amines is by inhibiting the growth of decarboxylase positive indigenous bacteria and other spoilage microorganisms. Sulphur dioxide is known to have antimicrobial properties. Molecular SO_2 can act to inhibit lactic acid bacteria in wine. The antibacterial activity of SO_2 is pH-dependent and will decrease with an increase in pH. Lysozyme is an enzyme that can cause lysis of the cell walls of Gram-positive bacteria, including wine lactic acid bacteria. Lysozyme retains its activity in higher pH wines. It can be successfully used to delay or inhibit the growth of lactic acid bacteria, especially when used in combination with SO_2 (Delfini *et al*., 2004; Ribérau-Gayon *et al*., 2006). Bacteriocins are antimicrobial peptides produced by some strains of lactic acid bacteria. Nisin is a commercially available bacteriocin that acts on the cytoplasmic membrane of Gram-positive bacteria. The possibility exists to exploit the synergistic effect of nisin and metabisulphite (SO_2) on growth inhibition of wine spoilage lactic acid bacteria for wine preservation (Rojo-Bezares *et al.*, 2007) but it has not been authorised for application in wine. The potential use of natural phenolic compounds as antimicrobial agents to control the growth of lactic acid bacteria in wine has also been proposed (García-Ruiz *et al.*, 2007).

Inoculation with *O. oeni* starter cultures that are unable to produce biogenic amines is a viable option for the control of these compounds in wine (Martín-Álvarez *et al*., 2006). It seems that co-inoculation of *O. oeni* starter cultures together with alcoholic fermentation has the potential to curb biogenic amine formation even more than conventional inoculation for malolactic fermentation after the completion of alcoholic fermentation (Van der Merwe, 2007).

While regulating the soil and vine nutritional status are the only viticultural manipulations that could be made to control the accumulation of biogenic amines in grapes, their occurrence in musts or wine could possibly also be controlled by reducing

practices that increase amino acid extraction such as grape skin maceration and lees contact.

2.7 CONCLUSION

The distribution of biogenic amine producers amongst wine microorganisms seems to be random and not a species specific quality. Lactic acid bacteria are the wine microorganisms that are, for the most part, associated with amino acid decarboxylation and biogenic amine formation. However, *O. oeni* seems to have a low distribution of tyrosine-, histidine-, and ornithine decarboxylase genes in its genome. The most important increase of biogenic amines takes place during malolactic fermentation, when compared to the contributions by alcoholic fermentation and ageing respectively. The contribution of yeast to biogenic amine production could be indirect (by amino acid secretion and autolysis) or direct, where killer positive strains produce the highest concentration of biogenic amines. Strains of *Brettanomyces bruxellensis*, followed by strains of *Saccharomyces cerevisiae* were found to produce significant concentrations of biogenic amines.

Biogenic amine concentrations in wine may be influenced by their presence in grape berries; dictated by factors such as soil potassium deficiencies, grape variety, geographical region and vintage. The concentrations of precursor amino acids are influenced by winemaking practices such as grape skin maceration. Biogenic amine formation is also determined by wine parameters and components of which pH, ethanol, SO_2 and pyridoxal 5'-phosphate have the most important effect on the diversity of microorganisms, decarboxylase enzyme activity and decarboxylase gene expression. The concentration of biogenic amines is dependent on wine type and style, but the presence of biogenic amines seems to be attributed to the presence of lactic acid bacteria in all cases.

PCR reactions can detect the presence of biogenic amine producing lactic acid bacteria and thereby estimate the potential risk of formation of histamine, tyrosine, cadaverine and putrescine in wine. Biogenic amines can be measured qualitatively (with screening and enzymatic methods), semi-quantitatively (by thin-layer chromatography) or quantitatively (using liquid chromatographies, capillary electrophoresis or gas chromatography).

Biogenic amine formation in wine is most likely to be prevented by inhibition of indigenous lactic acid bacteria and other spoilage microorganisms that could possess decarboxylase activity.

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3. RESEARCH RESULTS

Evaluating the influence of malolactic fermentation inoculation times and ageing on lees on the production of biogenic amines by wine lactic acid bacteria and preliminary quantification of biogenic amines by Fourier transform infrared spectroscopy

3.1 INTRODUCTION

Biogenic amines are a group of nitrogenous compounds likely to occur in fermented foods and beverages that contain the precursors of biogenic amines (free amino acids), can sustain the growth and metabolism of the causative microorganisms and favour the activity of the relevant decarboxylase enzymes. Wine is a medium in which these conditions are potentially satisfied (ten Brink *et al.*, 1990; Silla Santos, 1996). The main biogenic amines associated with wine are putrescine, histamine, tyramine and cadaverine. These are mainly the products of microbial decarboxylation of ornithine, histidine, tyrosine and lysine respectively (ten Brink *et al.*, 1990), although putrescine can also be formed via the arginine deiminase pathway from arginine (Arena & Manca de Nadra, 2001; Mangani *et al.*, 2005). Some biogenic amines are also formed by the metabolisms of plants and may be transferred from the grape berry to the must (and finally to the wine). These biogenic amines include putrescine, spermine and spermidine, which are associated with vine physiological processes such as flowering and fruit development, cell division, stress responses and senescence (Halász *et al.,* 1994). Many other biogenic amines such as phenylethylamine, agmatine, tryptamine, isoamylamine, methylamine and ethylamine have also been associated with wine (Glória *et al*., 1998; Marcobal *et al*., 2006; Soufleros *et al*., 2007).

Some biogenic amines are present at low levels in the human body and are involved in normal physiological functions. However, if an excessive amount of biogenic amines is ingested or if the normal detoxification routes via amine oxidases are deficient or inhibited by substances such as anti-depressant drugs or ethanol, several physiological disorders can occur in sensitive humans (ten Brink *et al.*, 1990; Maynard & Schenker, 1996; Shalaby, 1996). Thus, the presence of elevated levels of biogenic amines in wine should be avoided. Biogenic amines can be produced by various microorganisms associated with the different stages of wine production and storage.

In the literature it is generally agreed that the contribution to biogenic amines by yeast during alcoholic fermentation is less significant when compared to that by malolactic fermentation (Granchi *et al.,* 2005; Herbert *et al.*, 2005; Marcobal *et al.,* 2006). The highest concentrations of total biogenic amines contributed by yeasts during alcoholic fermentation (in sterilised grape musts) were associated with *Brettanomyces bruxellensis* and *Saccharomyces cerevisiae*, according to Caruso *et al.* (2002) and

Granchi *et al.* (2005). The ability of *S. cerevisiae* to produce biogenic amines seems to be strain dependent and not a constant characteristic of the species.

Most biogenic amine contamination of wine is believed to take place during malolactic fermentation. Lactic acid bacteria are present on healthy grapes in low numbers and can be transferred to winery equipment, where they can remain present in significant numbers (Wibowo *et al.*, 1985). These indigenous lactic acid bacteria are usually held responsible for spontaneous malolactic fermentations. However, the metabolic characteristics of such a flora are usually unknown and may contain decarboxylase activities which may lead to the production of biogenic amines from amino acids in the wine. *Oenococcus oeni* is predominantly responsible for malolactic fermentation, but species of *Pediococcus* and *Lactobacillus*, generally associated with spoilage, may survive and grow during malolactic fermentation, particularly if the pH of the wine is above pH 3.5 (Wibowo *et al.*, 1985; Lonvaud-Funel, 1999). Biogenic amine formation in wine has been associated with lactic acid bacterial species from all four genera, including *O. oeni* (Guerrini *et al.*, 2002; Coton *et al.,* 1998), *Pediococcus cerevisiae* and *Pediococcus parvulus* (Delfini, 1989; Landete *et al.* 2007), *Lactobacillus hilgardii, Lactobacillus buchneri, Lactobacillus brevis* and *Lactobacillus mali* (Moreno-Arribas & Lonvaud-Funel, 1999; Moreno-Arribas *et al.*, 2000; Downing, 2003; Moreno-Arribas *et al.*, 2003; Costantini *et al.*, 2006; Landete *et al.*, 2007) and *Leuconostoc mesenteroides* (Moreno-Arribas *et al.*, 2003; Landete *et al.*, 2007). As is the case with yeasts, the ability of lactic acid bacteria to produce biogenic amines seems to be randomly distributed among strains of different species.

Commercial preparations of *O. oeni* are said to be selected for the absence of amino acid decarboxylases and are therefore unable to produce biogenic amines (particularly histamine, tyramine and putrescine). This has been confirmed by a number of studies performed on commercial malolactic starter cultures (Moreno-Arribas *et al.*, 2003; Martín-Álvarez *et al.*, 2006). Inoculation of wine with a commercial malolactic fermentation starter culture could reduce the incidence of biogenic amines compared to spontaneous malolactic fermentation in wines, particularly by eliminating (by domination) indigenous bacteria which may contain decarboxylase activity.

Malolactic fermentation inoculation time could also impact on the sensory properties of the wine. One of the possible positive attributes associated with malolactic fermentation (other than microbial stability and natural wine deacidification by the enzymatic conversion of L-malic to L-lactic acid) is the modification of the sensory characteristics of the wine by the production of (volatile) secondary bacterial metabolites (Lonvaud-Funel, 1999). Interactions between lactic acid bacteria and wine yeasts during co-inoculated fermentations could potentially change the quantity or quality of volatile aroma compounds produced by these microorganisms.

After malolactic fermentation wine is usually submitted to clarifying and stabilising treatments and is aged (traditionally in oak barrels) from a few months to more than a year. White wines (such as from Burgundy) and sparkling wines produced by *méthode champenoise* are the only wine styles traditionally aged in contact with fermentation

lees. Today, it is becoming a common practice to age red wines on the lees to increase the organoleptic complexity and balance of the wine by release of compounds during yeast and bacterial autolysis (Hernández *et al.*, 2007; Alcaide-Hidalgo *et al.*, 2007b and references therein).

In order to accurately evaluate the evolution of biogenic amines during the course of wine fermentation and ageing, the need arises for a rapid and inexpensive method that does not require a large amount of sample preparation. Fourier transform infrared (FT-IR) spectroscopy is based on the measurement of the absorbance of radiation in the mid infrared region (4000 cm^{-1} to 400 cm^{-1}) by molecules that contain covalent bonds such as C−C, C−H, O−H, C═O and N−H (Smith, 1999). The final composite transmittance spectrum obtained by this technology can be used for calibration processes involving chemometric techniques such as partial least square regression (PLS) by which future spectra can be converted to quantitative data (Wehling, 1998). FT-IR technology can be employed for routine wine chemical analysis and a number of major fermentation products can be accurately and rapidly quantified with minimal sample preparation using FT-IR (Nieuwoudt *et al.*, 2004; Malherbe, 2007).

Chemometric procedures such as Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) have been applied to wines as tools to facilitate the extraction of relevant information, for pattern recognition and classification. Biogenic amines (in some cases in combination with other wine chemical compounds) have been employed to classify French, Hungarian and Spanish wines according to oenological parameters including wine style, winemaking technology, geographic origin, grape variety, year of vintage and ageing period (Soufleros *et al.,* 1998; Csomo´s *et al.*, 2002; Héberger *et al.*, 2003; García-Villar *et al*., 2007). Also, the composition and concentration of biogenic amines can be used to provide useful information on the authentication of wines (specifically the Tokaj aszu wines) and be used for quality control purposes (Hajós *et al.*, 2000; Sass-Kiss *et al.*, 2000; Kiss & Sass-Kiss, 2005; Kiss *et al.,* 2006).

The first objective of this study was to evaluate whether the timing of inoculation for malolactic fermentation could influence the final biogenic amine content in wines, and have an advantage over spontaneous malolactic fermentation. The direct or indirect influence of lactic acid bacteria, inoculated at different winemaking stages, on wine aroma was preliminarily evaluated in this study. The risk of biogenic amine formation during ageing on the lees was also determined. Finally, the quantification of biogenic amines by FT-IR, together with chemometric analyses was also explored in this work.

3.2 MATERIALS AND METHODS

3.2.1 VINIFICATION AND MICROBIOLOGY

3.2.1.1 Vinification procedures, malolactic fermentation treatments and sampling

The experimental procedure was repeated as a whole with Pinotage and Cabernet Sauvignon grapes purchased from commercial wine cellars in the Paarl region, South Africa over two vintages (2006 and 2007) and with two different commercial malolactic fermentation starter cultures. All treatments were repeated in duplicate in the 2006 season and in triplicate in 2007.

After grapes were destemmed and crushed, the skins and free-run juice were separated and homogenised. Equal volumes of the homogenised free run juice and equal weights of the homogenised skins were aliquoted to each treatment (approximately 6 L in total) in 10 L plastic buckets. A minimal amount of sulphur dioxide (10 to 20 mg/L) was added to the must of all treatments to inhibit the growth of spoilage organisms, unless otherwise stated. In the 2007 season, 50 mg/L of each of histidine, ornithine, tyrosine and lysine were added to the must to ensure the presence of precursors for potential biogenic amine synthesis.

Treatment 1 served as negative control, with a total of 50 mg/L of SO_2 and 25 to 30 mg/L of lysozyme (DSM Food Specialties Oenology, Servian, France) added to must to delay or inhibit the growth of indigenous lactic acid bacteria. An additional 50 mg/L of SO_2 was added to Treatment 1 after the completion of alcoholic fermentation. In treatment 2, the natural grape lactic acid bacteria flora was allowed to flourish and proceed with spontaneous malolactic fermentation. In the 2007 season, precultured amino acid decarboxylase positive lactic acid bacteria (section **3.2.1.2**) were inoculated into the grape must of treatments 3 to 7 at 10^6 cells/mL to simulate a natural flora with the potential to produce biogenic amines, in combination with the natural grape lactic acid bacterial flora. The addition of decarboxylase positive lactic acid bacteria, as well as treatment 3 was omitted from the 2006 experimental design. Spontaneous malolactic fermentation proceeded in treatment 3. Commercial malolactic fermentation starter cultures (Lalvin VP41, Lallemand, South Africa and Viniflora Oenos, Chr. Hansen, Denmark) were inoculated into the fermenting grape musts of treatments 4 and 5 respectively, 24 hours after inoculation with the yeast starter culture (co-inoculation). Treatments 6 and 7 were inoculated with Lalvin VP41 and Viniflora Oenos respectively after the completion of alcoholic fermentation (conventional inoculation). All commercial yeast and malolactic fermentation starter cultures added to wine during small scale vinifications were inoculated according to the instructions of the manufacturer at the maximum recommended dosage. Abbreviated descriptions of the treatments are given in **Tables 3.1** and **3.2** for each vintage separately.

Table 3.1 Description of the malolactic fermentation treatments performed in duplicate in 2006 with Pinotage and Cabernet Sauvignon. All treatments were inoculated with *Saccharomyces cerevisiae* NT202 for alcoholic fermentation.

Treatment number	Lysozyme & SO_2 addition	Natural LAB flora (grapes / cellar)	Must spiked with DC+ LAB & AA	Co-inoculation with AF (VP41)	Co-inoculation with AF (Oenos)	Normal inoculation after AF (VP41)	Normal inoculation after AF (Oenos)	Spontaneous MLF
1	X	X						X
2		X						X
~~3~~								
4		X		X				
5		X			X			
6		X				X		
7		X					X	

LAB: lactic acid bacteria; DC+: decarboxylase positive; AA: amino acid precursors; AF; alcoholic fermentation; MLF: malolactic fermentation

Table 3.2 Description of the malolactic fermentation treatments performed in triplicate duplicate in 2007 with Pinotage and Cabernet Sauvignon. All treatments were inoculated with *Saccharomyces cerevisiae* NT202 for alcoholic fermentation.

Treatment number	Lysozyme & SO_2 addition	Natural LAB flora (grapes / cellar)	Must spiked with DC+ LAB & AA	Co-inoculation with AF (VP41)	Co-inoculation with AF (Oenos)	Normal inoculation after AF (VP41)	Normal inoculation after AF (Oenos)	Spontaneous MLF
1	X	X						
2		X						X
3		X	X					X
4		X	X	X				
5		X	X		X			
6		X	X			X		
7		X	X				X	

LAB: lactic acid bacteria; DC+: decarboxylase positive; AA: amino acid precursors; AF; alcoholic fermentation; MLF: malolactic fermentation

Alcoholic fermentation was performed by *S. cerevisiae* strain NT202 (Anchor Yeast, South Africa) at room temperature (20 to 25°C). 0.2 to 0.5 g/L diammonium phosphate

was added to wines as nitrogen source for yeasts to avoid sluggish alcoholic fermentation. Sugar density was measured at least once daily with a Brix hydrometer to monitor the progression of alcoholic fermentation. Grape skins were punched through the wines daily throughout alcoholic fermentation. Wines were pressed with a hydrolic basket press to same pressure at the completion of alcoholic fermentation and transferred to 2 L or 4.5 L glass bottles (sealed with airlocks) to complete spontaneous or induced malolactic fermentation. Malolactic fermentation was monitored by obtaining the concentrations of malic acid and lactic acid on a regular basis (approximately every seven to 14 days) by FT-IR (WineScan FT120, FOSS Analytical, Denmark). At these intervals spectra were simultaneously acquired by FT-IR (section **3.2.5**). Fifty to 80 mg/L SO_2 was added to all wines at the completion of malolactic fermentation, prior to ageing. All wines (each replicate of each treatment) were aged in two 750 mL glass bottles with screw caps for 4 months in the presence or absence of fermentation lees respectively. Fermentation lees were removed from the treatments to be aged without lees by racking the wine after settling of the lees at a cool temperature (15°C). The settled lees were subsequently resuspended in the wine, ensuring a higher lees to wine ratio than was originally present in the wines to be aged with lees. Wines were aged at a cool temperature (≤15°C).

Representative samples for analyses of biogenic amines (section **3.2.4**) and microbial enumeration (section **3.2.2**) were drawn in sterile sample vials of the grape must, after alcoholic fermentation and after the completion of malolactic fermentation for both cultivars in 2006 and 2007. After the four month ageing period wines were sampled for the analyses of biogenic amines in both seasons and also for FT-IR spectroscopy (Section **3.2.5**) in 2006. Samples of all the grape musts, as well as of Pinotage 2007 treatments after alcoholic and malolactic fermentations were taken for amino acid analysis (section **3.2.4**). Pinotage and Cabernet Sauvignon treatments from 2007 were also analysed for aroma compounds at the end of malolactic fermentation (section **3.2.3**).

3.2.1.2 Lactic acid bacteria, preculture conditions and polymerase chain reaction

Amino acid decarboxylase positive lactic acid bacteria previously isolated from South African brandy base wines (Downing, 2003; Du Plessis *et al.*, 2004) (a combination of equal amounts of *Lactobacillus hilgardii* B74, *Lactobacillus hilgardii* M59 and *Lactobacillus brevis* M58) were inoculated into the grape must before SO_2 addition at 10^6 cells/mL to simulate a natural flora with the potential to produce biogenic amines. Prior to inoculation, the lactic acid bacteria were precultured separately for 36 hours in MRS broth followed by 48 hours in a MRS-based growth medium that facilitates adaptation to wine conditions (pH 4.6) supplemented with 0.1% amino acid precursor (of each of L-ornithine, L-tyrosine and L-histidine) and 0.005% pyridoxal-5'-phosphate (Sigma-Aldrich, Germany). The presence of decarboxylase genes were verified by the multiplex polymerase chain reaction (PCR) assay described by Marcobal *et al.* (2005a).

Genomic DNA of the *Lactobacillus* species was isolated according to the protocol of Dellaglio *et al.* (1973). *Lactobacillus* 30a was used as a positive control for histidine- and ornithine decarboxylase (Le Jeune *et al.*, 1995; Marcobal *et al.*, 2005a) and *Lactobacillus brevis* M58 for tyrosine decarboxylase (Downing, 2003). A 100 base pair (bp) ladder (MWM XIV, Roche) was used to identify amplification products in this reaction.

3.2.2 ENUMERATION OF MICOORGANISMS

The wine microbiological status was monitored in the must and at the completion of the alcoholic and malolactic fermentations by plate counts of colonies formed (cfu/mL) on selective agar media, followed by identification of cell morphology under a light microscope. One hundred µL of must or wine, diluted in water in a dilution series, was plated on selective media. All media were purchased from Biolab, Merck, Wadeville, Gauteng (South Africa) unless otherwise indicated. All plates were incubated at 30°C for 5 to 10 days depending on the growth rate of the microorganism.

3.2.2.1 Bacterial species

De Man, Rogosa and Sharpe (MRS) agar was used for the enumeration of *Lactobacillus, Pediococcus* and *Leuconostoc* species (De Man *et al.*, 1960). *Oenococcus oeni* was enumerated on MRS supplemented with 20% v/v apple juice at pH 5.2. Fifty mg/L pimaricin (Actistab, Gistbrocades, Anchor Bio-Technologies, South Africa) and 25 mg/L kanamycin sulphate (Roche, South Africa) dissolved in 1 mL of water were added to each of the media to inhibit the growth of yeast and acetic acid bacteria respectively. The MRS plates supplemented with apple juice were incubated anaerobically.

MRS agar supplemented with 2% v/v ethanol was used to isolate acetic acid bacteria in wine. GYC agar (5% w/v glucose, 1% w/v yeast extract, 3% v/v $CaCO_3$ and 2% w/v agar) was used to isolate acetic acid bacteria from grape must. Pimaricin (50 mg/L Actistab) and chloramphenicol (Sigma-Aldrich, Germany) (30 mg/L dissolved in 1 mL of 100% v/v ethanol) were added to both media to inhibit the growth of yeast and lactic acid bacteria respectively.

3.2.2.2 Yeast species

Lysine agar medium (lysine with 0.01% v/v potassium lactate) was used to establish non-*Saccharomyces* yeasts counts and yeast peptone dextrose (YPD) agar (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) to establish the total yeast population. Acetic acid bacterial growth was inhibited by the addition of kanamycin sulphate (25 mg/L) and lactic acid bacterial growth by chloramphenicol (30 mg/L).

WL nutrient agar for the enumeration of *Brettanomyces* species was purchased from Merck, Darmstadt, Germany. The pH of this medium was adjusted to 5.0 and 100 mg/L *p*-coumaric acid (a precursor for volatile phenol formation) was added to increase the selectivity of this medium. In addition to kanamycin sulphate and chloramphenicol, 50 mg/L cycloheximide (Sigma-Aldrich, Germany) (dissolved in 1 mL of 50% v/v ethanol) was added to inhibit the growth of yeast species other than *Brettanomyces*.

3.2.3 AROMA COMPOUND ANALYSIS

3.2.3.1 Chemicals and extraction of volatile compounds

Samples were drawn from the wines of the 2007 vintage straight after the completion of malolactic fermentation and were kept at -20°C until analysis. Volatile alcohols, esters and acids were quantified in wine samples by gas chromatography with flame ionisation detection (GC-FID). Prior to analysis the volatile compounds were extracted from the wine samples with 100 µL of internal standard followed by 1 mL of diethylether (Merck) added to 5 mL of wine sample. The internal standard constituted 0.5 mg/L 4-methyl-2-pentanol (Fluka) in a model wine solution (12% v/v ethanol, Merck; 2.5 g/L tartaric acid, Merck; de-ionised water from a MilliQ system, pH adjusted to 3.5 with 0.1 M NaOH, Merck). The wine/ether mixture was sonicated for 5 minutes followed by centrifugation at 3600 *g* for 3 minutes. The ether layer was removed and dried on $NaSO_4$ (Merck). Each extract was injected into the GC-FID in triplicate or duplicate. All chemicals used were analytical grade.

3.2.3.2 Gas Chromatography conditions

The gas chromatograph used for analysis (Hewlett Packard 6890 Plus GC) was equipped with a split/splitless injector and a FID detector (Agilent, Little Falls, Wilmington, USA). Compounds were separated on a AJ & W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with dimensions 60 m length x 0.32 mm internal diameter x 0.5 µm film thickness. The initial oven temperature was 33°C held for 17 minutes after which the temperature was increased to 240°C at 12°C/min and held for 5 minutes. The injection volume was 3 µL, at an injector temperature of 200°C. The split ratio was 15:1 and the split flow rate 49.5 mL/min. The column flow rate was 3.3 mL/min and the total run time was 50 minutes per sample. The detector temperature was 250°C. After each sample run, a post run of 5 minutes at oven temperature 240°C was performed with a gas flow of 6 mL/min to clean the column. After every 30 samples the column was thermally and chemically cleaned by injecting hexane at oven temperature 220°C and holding it for 10 minutes.

3.2.4 BIOGENIC AMINE AND AMINO ACID ANALYSES

Analyses of the histamine, tyrosine, putrescine and cadaverine content of wine fermentations from the 2006 season were performed by high performance liquid chromatography (HPLC) based on the method described by Alberto *et al.* (2000). Major changes included the use of different chemicals for the preparation of the derivatising solution, the amount of sample used for derivatisation and the derivatisation reaction time. Samples were diluted 10 times and filtered through a 0.22 µm syringe filter prior to derivatisation and column injection. The derivatising reagent used was 200 mg *o*-phtaldialdehyde (OPA) (Sigma, Germany) dissolved in 9 mL methanol, 1 mL 0.1M sodium tetra-borate (pH 10) and 160 µL 2-mercaptoethanol. 25 µl of the diluted sample reacted with 25 µl of derivatising reagent for exactly 45 seconds and 25 µl of this solution was injected immediately thereafter. The derivatisation process was automated by the use of an autosampler.

Biogenic amines (histamine, tyrosine, putrescine, cadaverine and spermidine) were quantified in 2007 by liquid chromatography mass spectrometry (LCMSMS). Analyses were performed either according to the method described by Millán *et al.* (2007) or according to the method described in **Chapter 4**, section **4.2.5**.

Free amino acids were analysed using the EZ: Faast liquid chromatography method with the EZ: Faast column as described in the EZ: Faast user's guide. Prior to analysis, wine samples were centrifuged at 13 000 *g* for 10 minutes. 100 µL of the supernatant was freeze dried after which it was subjected to the EZ: Faast protocol. Labelled homoarginine and methionine-D3, as part of the EZ: Faast kit, were included as internal standards. Amino acids were subsequently quantified by LCMSMS.

3.2.5 FT-IR SPECTROSCOPY AND MULTIVARIATE DATA ANALYSIS

3.2.5.1 Sample preparation and acquisition of FT-IR spectra

A WineScan FT120 spectrometer (FOSS Analytical, Denmark) was used for the generation of spectra in the wavenumber region 929–5011 cm^{-1}. The quality of mid-infrared spectra can be negatively influenced by high levels of carbon dioxide (CO_2) potentially present in wine samples that are still in active fermentation stages (alcoholic or malolactic fermentations). Must and wine samples were filtered using filter paper with a grading of 20 to 25 µm and a diameter of 185 mm (Schleicher & Schuell, catalogue number 10312714) and a filtration unit (type 79500, FOSS Electric, Denmark) connected to a vacuum pump. One to four successive filtrations were performed to effectively reduce CO_2 gas in samples to 300 mg/L or less, as indicated with FT-IR. At winemaking stages where the initial level of gas in the samples exceeded 2000 mg/L, samples were sonicated in an ultrasonic bath for 5 minutes in addition to repeated filtration.

All treatments (duplicates or triplicates) of the Pinotage and Cabernet Sauvignon of the two vintages (2006 and 2007) were scanned with FT-IR before and after alcoholic fermentation and on a regular basis (approximately once every week or every two weeks) during malolactic fermentation until its completion (taken as the point when the malic acid concentration was equal to or lower than 0.3 g/L). The primary aim of these scans was for routine monitoring of wine chemical compounds (particularly malic acid and lactic acid concentrations). Wines of the 2006 vintage were scanned again after an ageing period of four months. Duplicate scans were obtained for each sample.

The samples (7 mL) were pumped through the Ca_2F-lined cuvette (37 µm) at a constant temperature of 40°C. The samples were scanned at 4 cm^{-1} intervals in the wavenumber region 929–5011 cm^{-1}. The amount of infrared radiation transmitted by the sample at all the infrared wavenumbers were captured simultaneously at the detector in the form of an interferogram which is converted with the Fourier Transform algorithm to a single beam transmittance spectrum (WineScan FT120 Type 771100 and 77310 Reference Manual, FOSS Analytical, Denmark, 2001) which can be used for calibration, together with chemometric techniques (Wehling, 1998).

3.2.5.2 Selection of wavenumbers and sample sets

Although the whole spectral range (929–5011 cm^{-1}) is stored for each sample, the wavenumbers 964–1532 cm^{-1}, 1716–2731 cm^{-1} and 3300–3500 cm^{-1} were selected to exclude spectral noise largely caused by the absorption of water (Nieuwoudt *et al*., 2004) and to include the absorption area of primary and secondary amines (3300–3500 cm^{-1}) (McMurry, 2000) in the spectra used for multivariate data analysis.

The reference sample set was divided into a calibration set and a randomly selected validation set comprised of 87% and 13% respectively of the 489 samples that were scanned in total. This reference sample set comprised all samples on which biogenic amine analyses were performed by the analytical methods described in section **3.2.5**. These were the samples that were obtained at the end of alcoholic fermentation, at the end of malolactic fermentation (both seasons) and at the end of the four month ageing period (2006 samples only). The calibration was performed with The Unscrambler 9.2 software (Camo ASA, Trondheim, Norway) using PLS1 regression and a larger wavenumber selection than recommended by the WineScan software. A second model was composed using the full reference sample set with two-segmented cross validation. In order to make variable variances comparable, the data was first scaled (weighted) by the software by multiplication with the inverse of the standard deviation (Esbensen, 2002).

3.2.6 STATISTICAL ANALYSIS

Repeated measures analysis of variance (ANOVA) was performed on the data to evaluate main effects for fermentation treatment and phase, as well as their interactive effects. The Tukey HSD test was used to determine the significant differences between group means. A significance level of 5% was used in all cases. Statistical analyses were performed using STATISTICA (StatSoft, Inc. (2006)., version 7.1. www.statsoft.com).

3.3 RESULTS AND DISCUSSION

3.3.1 SELECTION OF BIOGENIC AMINE PRODUCING LACTIC ACID BACTERIA

Lactobacillus 30a is a histidine decarboxylase (HDC) and ornithine decarboxylase (ODC) positive lactic acid bacterium. This strain was used as a positive control for these two gene products during the PCR assay to identify lactic acid bacteria with the ability to produce these biogenic amines. Histidine decarboxylase was amplified at 367 bp and ornithine decarboxylase at 1446 bp in this PCR reaction.

Downing (2003) identified ten tyramine and four putrescine producing strains of *Lactobacillus* among 400 strains that were screened for the ability to produce histamine, tyramine, putrescine and cadaverine with a screening plate assay. *Lactobacillus brevis* M58, a tyramine producer, is one of the strains that showed a positive result with PCR amplification for the tyrosine decarboxylase gene. This strain was selected as a positive control for tyrosine decarboxylase (TDC). Tyrosine decarboxylase was amplified at 924 bp in this PCR assay.

Further work was done by Downing (unpublished data) on the identified biogenic amine producing strains, and HPLC analysis showed that some strains were able to produce tyramine, putrescine and histamine at high levels. For the present study *Lactobacillus hilgardii* M59 was selected as the highest producer of putrescine (1000 mg/L putrescine was produced from ornithine in a synthetic medium). *Lactobacillus brevis* M58 was selected since it was one of the highest producers of tyramine (720 mg/L tyramine was produced from tyrosine in a synthetic medium). *Lactobacillus hilgardii* B74 proved to be able to produce histamine from histidine (50 mg/L), despite showing a negative result with the screening plate method. Additionally, this strain was able to produce 200 mg/L tyramine from tyrosine and 637 mg/L putrescine from ornithine in a synthetic medium.

According to the result obtained in **Figure 3.1**, *Lactobacillus brevis* M58 is potentially able to produce tyramine as well as putrescine and *Lactobacillus hilgardii* M59 showed a positive result for the presence of histidine- and putrescine decarboxylase. The bands obtained for *Lactobacillus hilgardii* B74 are not clearly distinguishable, but can be observed for histidine- and putrescine decarboxylase. Presumable, due to its ability to produce tyramine, the tyrosine decarboxylase band at

924 bp is too faint to observe on this figure. The *Lactobacillus* strains in lanes 6 and 7 were identified as putrescine producers by Downing (2003) and were screened here for the presence of ornithine decarboxylase. However, these were not included for winemaking purposes in the present study. The abilities of the strains to produce cadaverine were not evaluated on genetic level in this study (due to the unavailability of the relevant primers at the time).

A combination of the three selected indigenous decarboxylase positive *Lactobacillus* strains was inoculated (spiked) into the wines of 2007 to simulate a natural flora with the genetic potential to produce biogenic amines.

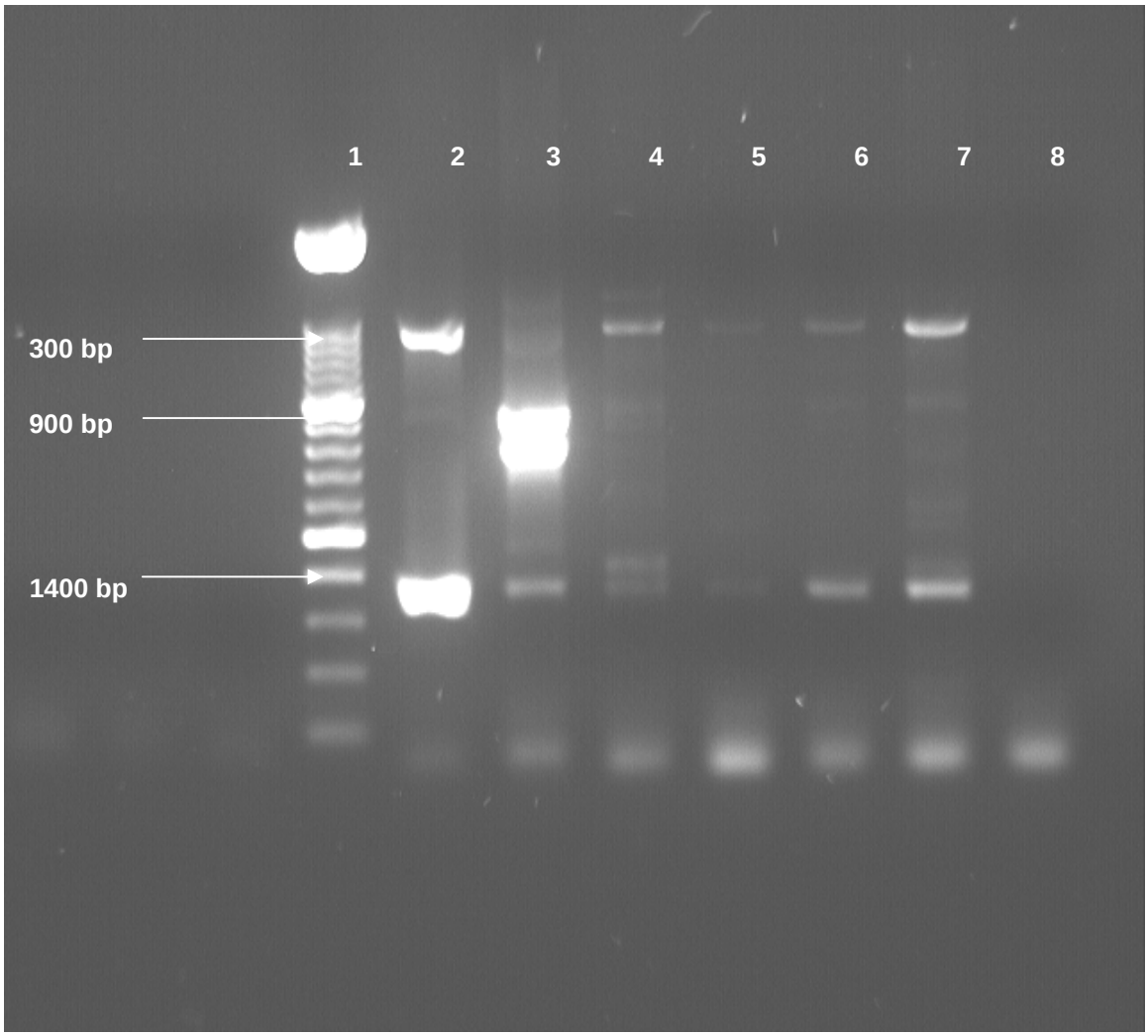

Figure 3.1 Multiplex PCR assay performed to identify biogenic amine producing lactic acid bacteria to be used in this study. Lane 1, 100 bp ladder; Lane 2, *Lactobacillus* 30a (positive control for HDC of 367 bp and ODC of 1446 bp); Lane 3, *Lactobacillus brevis* M58 (positive control for TDC of 924 bp); Lane 4, *Lactobacillus hilgardii* M59; Lane 5, *Lactobacillus hilgardii* B74; Lane 6, *Lactobacillus brevis* K24; Lane 7, *Lactobacillus brevis* G79; Lane 8, negative control.

3.3.2 ENUMERATION OF MICROORGANISMS

3.3.2.1 2006

In 2006 (**Table 3.3**) only the naturally occurring flora was present in the must (no decarboxylase positive lactic acid bacteria were spiked into the wine) along with the inoculated commercial *O. oeni* starter cultures (Lalvin VP41 and Viniflora Oenos). In Pinotage, no *Lactobacillus* (or other indigenous) species were detected once fermentations commenced. *Oenococcus* could also only be detected at the end of alcoholic fermentation in the two treatments (4 and 5) that were co-inoculated together with alcoholic fermentation. The natural lactic acid bacterial flora seemed to be present in low numbers. Spontaneous malolactic fermentation was completed in treatments 1 and 2 and cell densities were similar for most treatments in Pinotage at the time when they completed (spontaneous or inoculated) malolactic fermentation.

Table 3.3 Cell growth (cfu/mL) of wine lactic acid bacteria as recorded at three stages of the winemaking process in Pinotage and Cabernet Sauvignon in 2006. Each enumeration represents the average of duplicate treatments (standard deviations not shown).

	(a) Pinotage		(b) Cabernet Sauvignon	
	Oenococcus	***Lactobacillus***	***Oenococcus***	***Lactobacillus***
Grape must*				
All treatments	nd	4.60×10^4	2.40×10^2	8.00×10^1
End AF				
Treatment 1**	nd	nd	nd	nd
Treatment 2	nd	nd	4.55×10^3	1.44×10^3
Treatment 4	2.80×10^5	nd	4.70×10^6	1.22×10^3
Treatment 5	7.40×10^5	nd	1.97×10^7	1.12×10^3
Treatment 6	nd	nd	1.29×10^3	6.95×10^2
Treatment 7	nd	nd	1.95×10^3	1.42×10^3
End MLF				
Treatment 1	8.20×10^5	nd	2.23×10^7	nd
Treatment 2	6.00×10^6	nd	2.72×10^7	nd
Treatment 4	1.49×10^5	nd	1.22×10^6	nd
Treatment 5	1.95×10^5	nd	1.70×10^3	nd
Treatment 6	1.47×10^6	nd	1.91×10^7	nd
Treatment 7	2.40×10^5	nd	1.78×10^7	nd

*must was not spiked at any stage with decarboxylase positive Lactobacilli in 2006 treatments

Refer to **Table 3.1 for a description of the treatments

nd: not detected; AF: alcoholic fermentation; MLF: malolactic fermentation

Cabernet Sauvignon grapes contained some indigenous *Lactobacillus* species that increased during alcoholic fermentation and survived only until the end of this phase in treatments 2 to 7. *Oenococcus* cell density was higher in co-inoculated treatments and absent from the negative control containing lysozyme (treatment 1) at the end of alcoholic fermentation. Lower numbers of indigenous *Oenococcus* species were also present at the end of alcoholic fermentation in Cabernet Sauvignon treatments that

were uninoculated at this stage (as can be observed in treatments 2, 6 and 7). *Oenococcus* species that were inoculated at the end of alcoholic fermentation (treatments 6 and 7) and those that were allowed to grow from the indigenous flora (treatments 1 and 2) were present in similar numbers, at higher cell densities than *Oenococcus* remaining in co-inoculated treatments (4 and 5) at the end of malolactic fermentation.

Lysozyme successfully inhibited the growth of indigenous *Lactobacillus* species during alcoholic fermentation (and malolactic fermentation), and delayed the growth of indigenous *Oenococcus oeni* strains until after alcoholic fermentation in both cultivars.

No cell enumeration was performed for other wine microorganisms in 2006.

3.3.2.2 2007

In 2007 in both Pinotage (**Table 3.4**) and Cabernet Sauvignon (**Table 3.5**) the wine microflora was dominated by the inoculated *Saccharomyces cerevisiae* strain NT202 yeast at the end of alcoholic fermentation. *Saccharomyces* species were already present at a high cell density in the must of Pinotage. *Saccharomyces* cell numbers of Cabernet Sauvignon must could not be determined on YPD agar plates due to fungal contamination on the plates. A relatively high cell density (10^5 cfu/mL) of non-*Saccharomyces* yeast strains were also present in the musts of both cultivars and survived at 10^3 to 10^4 cfu/mL to the end of alcoholic fermentation. From two (Pinotage) and up to eight (Cabernet Sauvignon) different colony morphologies could be distinguished on lysine agar plates. No surviving acetic acid bacteria could be detected after wine fermentations had commenced and no culturable cells of *Brettanomyces* could be detected at any stage of cell enumeration.

Indigenous *Oenococcus oeni* strains were detected in the must of both cultivars. These strains presumably survived through alcoholic fermentation and completed the malolactic fermentation in treatment 2 and at least to some extent in treatment 3. No measurable malolactic fermentation occurred in treatment 1 (negative control) despite the presence of low numbers of *Oenococcus* in both cultivars at the end of malolactic fermentation. *Oenococcus* cell numbers were higher after alcoholic fermentation and lower at the end of malolactic fermentation for co-inoculated treatments than in other treatments. Malolactic fermentation was completed in co-inoculated treatments before conventional inoculated and spontaneous malolactic fermentation treatments, as could be expected.

Indigenous *Lactobacillus* species were present in the must of both cultivars. The three decarboxylase positive *Lactobacillus* strains were inoculated at a total of 10^6 cells/mL into the grape must shortly after the must sample was taken for cell enumeration. Lactobacilli could be detected at 10^6 cells/mL after three days (data not shown); indicating their viability in the wines. These *Lactobacillus* species were still present at relatively high cell numbers (10^4 cfu/mL) at the end of alcoholic fermentation when compared to treatments 1 and 2 which were not spiked with Lactobacilli. Even

though no more *Lactobacillus* cells could be detected at the end of malolactic fermentation in Cabernet Sauvignon wines, it is possible that these species had still contributed to compounds produced during fermentations in the wine (such as biogenic amines). Lactobacilli survived to the end of malolactic fermentation in Pinotage only in the co-inoculation treatments.

Lysozyme successfully inhibited the growth of indigenous *Lactobacillus* species during alcoholic fermentation (and malolactic fermentation), and reduced or delayed the growth of indigenous *Oenococcus oeni* strains in both cultivars.

Table 3.4 Cell growth (cfu/mL) of wine microorganisms as recorded at three stages of the winemaking process in Pinotage 2007. Each enumeration represents the average of triplicate treatments (standard deviations not shown).

	Saccharomyces	**non-*Saccharomyces***	***Brettanomyces***	**Acetic acid bacteria**	***Oenococcus***	***Lactobacillus***
Grape must*						
All treatments	3.24×10^5	6.39×10^5	nd	5.80×10^3	6.67×10^3	5.00×10^2
End AF						
Treatment 1**	1.77×10^8	1.49×10^4	nd	nd	nd	nd
Treatment 2	1.53×10^8	2.22×10^4	nd	nd	3.70×10^2	2.00×10^2
Treatment 3	1.74×10^8	1.54×10^4	nd	nd	nd	2.21×10^4
Treatment 4	1.63×10^8	3.53×10^3	nd	nd	1.66×10^6	1.37×10^4
Treatment 5	1.43×10^8	1.28×10^4	nd	nd	1.09×10^6	2.13×10^4
Treatment 6	1.74×10^8	1.54×10^4	nd	nd	nd	2.21×10^4
Treatment 7	1.74×10^8	1.54×10^4	nd	nd	nd	2.21×10^4
End MLF						
Treatment 1	nd	nd	nd	nd	4.67×10^2	nd
Treatment 2	nd	nd	nd	nd	1.11×10^7	nd
Treatment 3	nd	nd	nd	nd	1.57×10^7	nd
Treatment 4	nd	nd	nd	nd	4.54×10^5	3.88×10^2
Treatment 5	nd	nd	nd	nd	2.53×10^7	5.83×10^2
Treatment 6	nd	nd	nd	nd	2.18×10^7	nd
Treatment 7	nd	nd	nd	nd	4.26×10^6	nd

*before must was spiked with decarboxylase positive Lactobacilli

Refer to **Table 3.2 for a description of the treatments; no MLF occurred in treatment 1

nd: not detected; AF: alcoholic fermentation; MLF: malolactic fermentation

Table 3.5 Cell growth (cfu/mL) of wine microorganisms as recorded at three stages of the winemaking process in Cabernet Sauvignon 2007. Each enumeration represents the average of triplicate treatments (standard deviations not shown).

	Saccharomyces	non-*Saccharomyces*	*Brettanomyces*	Acetic acid bacteria	*Oenococcus*	*Lactobacillus*
Grape must*						
All treatments	nd	1.20×10^5	nd	8.00×10^2	1.00×10^4	3.50×10^3
End AF						
Treatment 1**	2.20×10^8	1.50×10^4	nd	nd	6.43×10^2	nd
Treatment 2	2.00×10^8	2.00×10^4	nd	nd	3.27×10^4	5.00×10^1
Treatment 3	2.00×10^8	8.70×10^3	nd	nd	2.60×10^4	1.57×10^4
Treatment 4	2.20×10^8	8.80×10^3	nd	nd	6.00×10^7	1.07×10^4
Treatment 5	2.00×10^8	5.50×10^3	nd	nd	7.93×10^7	1.53×10^4
Treatment 6	3.00×10^8	1.14×10^4	nd	nd	3.62×10^5	2.07×10^4
Treatment 7	2.00×10^8	9.80×10^3	nd	nd	1.68×10^5	3.05×10^4
End MLF						
Treatment 1	nd	nd	nd	nd	4.81×10^3	nd
Treatment 2	nd	nd	nd	nd	6.33×10^6	nd
Treatment 3	nd	nd	nd	nd	1.04×10^6	nd
Treatment 4	nd	nd	nd	nd	3.51×10^4	nd
Treatment 5	nd	nd	nd	nd	1.49×10^3	nd
Treatment 6	nd	nd	nd	nd	7.37×10^5	nd
Treatment 7	nd	nd	nd	nd	2.67×10^6	nd

*before must was spiked with decarboxylase positive Lactobacilli

Refer to **Table 3.2 for a description of the treatments; no MLF occurred in treatment 1

nd: not detected; AF: alcoholic fermentation; MLF: malolactic fermentation

3.3.3 PRODUCTION OF VOLATILE AROMA COMPOUNDS

The choice of microorganisms (yeast and bacteria) to conduct fermentation can influence the aroma and flavour profile of the wine. The contribution by a particular strain, species or genera of microorganisms can in turn be varied by increasing or restricting its time in contact with the wine. Wine yeast and lactic acid bacterial species and strains can be selected to optimise the synthesis of target flavour-active compounds. Lactic acid bacteria can degrade or produce odour-active compounds, modify grape- or yeast derived volatile flavour precursors and compounds, or produce compounds that may act by masking or enhancing other aroma compounds.

Apart from an increased smoothness and softened palate due to decreased wine acidity, the most frequently reported aroma modification connected with malolactic fermentation is the buttery character associated with diacetyl (reviewed by Bartowsky & Henschke, 2004). Additionally, malolactic fermentation can also involve changes in the nutty, fruity and flowery aromas as well as the reduction of vegetative or herbaceous aromas (Bartowsky & Henschke, 1995; Henick-Kling, 1995). Little research is available on the exact sensory contribution of lactic acid bacteria to wine during malolactic fermentation, although it seems that the type of bacterial strain or starter culture may play an important role. Presently, it is reported that malolactic fermentation can result in a significant increase or decrease in the concentration of esters in wine; while lactic acid

bacteria are known to also produce other volatile flavour-active compounds such as higher alcohols, fatty acids, lactones, sulphur compounds and nitrogen compounds (Edwards & Peterson, 1994; Macais *et al.*, 1999; Osborne *et al.*, 2000; Pripis-Nicolau *et al.*, 2004).

In the present study, the concentrations of most volatile aroma compounds were higher in one cultivar than in the other, or even absent in one of the cultivars and produced in the other. In general, the concentration of total esters was higher in Pinotage than in Cabernet Sauvignon while the concentration of total higher alcohols was higher in Cabernet Sauvignon. In some cases a difference in the concentration of volatile components could be observed as a result of the two commercial starter cultures, for example ethyl acetate, isoamyl acetate, methanol and decanoic acid production in Pinotage was higher in the treatments fermented with Lalvin VP41 (treatments 4 and 6) than those fermented with Viniflora Oenos (treatments 5 and 7). The opposite trend was observed in both cultivars for acetic acid production. However, these differences will not be discussed in detail as part of this work. Compounds that exhibit noticeable trends between different malolactic fermentation treatments will be discussed individually with reference to cultivar (**Table 3.6)**. Flavours associated with volatile compounds are indicated in parentheses in the text.

3.3.3.1 Esters

Esters in the wine may be grape derived and therefore depend on grape variety. Esters are mainly produced by yeasts and include ethyl esters of organic acids, ethyl esters of fatty acids and acetate esters from higher alcohols. Esters produced in wine can therefore largely depend on and vary with the commercial *Saccharomyces cerevisiae* strain used for fermentations as well as the presence of non-*Saccharomyces* yeasts.

Ethyl acetate is usually quantitatively the predominant ester in wine, like in the present study. It has a low sensory threshold and its aroma can be pleasant and fruity at low concentrations or resemble nail polish remover at higher concentration. In Pinotage, ethyl acetate in all inoculated treatments (co-inoculation and normal inoculation) was always lower in concentration than the uninoculated treatments 1 and 3, while ethyl acetate concentrations in the uninoculated treatment 2 was also higher than all inoculated treatments with the exception of one (treatment 4). Isoamyl acetate (banana, pear), hexyl acetate (sweet, fruity), ethyl hexanoate (green apple, fruity, violets), ethyl caprylate (apple) and 2-phenylethyl acetate (rose, fruity) were produced at higher levels in co-inoculated treatments in Pinotage. 2-phenylethyl acetate was only produced in detectable amounts in Cabernet Sauvignon in the co-inoculated treatments and treatments showed similar production of these esters. Diethyl succinate was the only ester consistently produced at the lowest levels in co-inoculated treatments for both cultivars when compared to spontaneous malolactic fermentation (treatments 2 and 3) and normal inoculated malolactic fermentation (treatments 6 and 7). Ethyl lactate (milky) production was inconsistent between treatments, but was produced at significantly

Table 3.6 Esters, higher alcohols and fatty acids (mg/L) measured in 2007 in Pinotage and Cabernet Sauvignon after malolactic fermentation. Concentrations represent the average of triplicate treatment repeats, each analysed in duplicate by GC-FID (six replicates in total). Standard deviations are not shown.

	Pinotage							Cabernet Sauvignon						
Treatment*	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Esters														
Ethyl Butyrate	0.885	0.875	0.943	0.918	0.901	0.886	0.825	0.729	0.754	0.740	0.604	0.720	0.736	0.740
Ethyl Hexanoate	0.787	0.897	0.972	1.410	1.520	1.038	0.959	0.630	0.698	0.685	0.755	0.679	0.629	0.650
Ethyl Lactate	1.707	45.816	48.353	27.356	44.532	34.833	43.404	2.264	33.386	22.227	22.336	58.274	12.933	8.685
Ethyl Caprylate	0.347	0.432	0.487	1.173	1.389	0.718	0.607	0.411	0.510	0.503	0.596	0.504	0.432	0.432
Ethyl Caprate	0.337	0.338	0.344	0.343	0.383	0.359	0.350	0.294	0.290	0.283	0.291	0.286	0.288	0.290
Diethyl Succinate	1.303	1.518	1.437	0.423	0.664	1.828	1.565	1.289	3.445	3.285	2.364	2.274	2.795	2.527
Ethyl Acetate	128.094	120.461	143.634	126.809	98.940	100.460	86.142	41.117	49.138	46.606	44.782	53.905	56.591	50.421
Isoamyl Acetate	5.131	4.738	6.046	8.204	8.229	5.279	4.526	1.334	1.168	1.043	1.007	0.898	0.998	1.087
Hexyl Acetate	0.305	0.285	0.305	0.494	0.506	0.300	0.287	0.127	nd	nd	0.030	0.019	nd	nd
2-Phenylethyl Acetate	0.041	0.010	0.022	0.431	0.465	0.049	0.002	nd	nd	nd	nd	nd	nd	nd
Total	**138.937**	**175.369**	**202.544**	**167.560**	**157.528**	**145.750**	**138.668**	**48.195**	**89.389**	**75.372**	**72.764**	**117.560**	**75.404**	**64.833**
Alcohols														
Methanol	68.993	61.023	65.637	36.677	29.427	44.162	39.927	52.726	64.300	56.838	55.072	72.653	81.651	73.945
Propanol	129.280	116.308	121.880	111.347	107.730	100.871	94.483	34.661	43.317	43.336	37.499	45.423	54.317	44.307
Isobutanol	21.117	18.777	17.929	18.306	18.185	16.059	17.701	26.018	25.902	27.423	22.931	27.426	30.277	26.672
Butanol	1.739	1.632	1.788	1.599	1.699	1.558	1.364	1.833	1.784	1.772	1.649	1.856	1.956	1.806
Isoamyl alcohol	152.543	148.461	150.010	148.505	159.058	144.194	140.910	301.424	316.506	308.282	295.898	302.330	321.423	321.293
Hexanol	1.282	1.501	1.440	1.419	1.656	1.601	1.528	2.515	2.740	2.526	3.313	2.843	2.075	2.334
2-Phenyl Ethanol	17.157	17.514	15.312	18.361	21.390	18.020	18.607	66.862	78.822	74.375	75.490	76.050	77.549	80.142
Total	**392.110**	**365.217**	**373.996**	**336.214**	**339.145**	**326.465**	**314.520**	**486.038**	**533.371**	**514.554**	**491.852**	**528.582**	**569.248**	**550.500**
Volatile acids														
Acetic Acid	205.029	349.185	343.341	225.334	377.538	323.895	330.465	197.404	281.872	262.402	382.614	454.398	291.596	303.527
Propionic Acid	6.644	6.641	7.524	6.058	7.363	7.459	8.112	6.079	5.667	5.693	5.997	6.562	6.424	6.030
Iso-Butyric Acid	0.500	0.496	0.395	0.534	0.531	0.479	0.690	1.468	1.233	1.400	1.325	1.331	1.413	1.313
Butyric Acid	0.259	0.292	0.373	2.073	2.144	0.625	0.373	0.676	0.798	0.816	1.014	0.951	0.763	0.712
Iso-Valeric Acid	0.507	0.548	0.437	0.581	0.714	0.552	0.686	3.805	3.571	3.636	3.595	3.410	3.608	3.617
Valeric Acid	0.019	0.012	0.003	0.024	nd	0.012	0.015	nd	nd	nd	nd	nd	nd	nd
Hexanoic Acid	2.398	2.635	2.726	2.642	3.109	3.138	2.860	1.651	1.734	1.652	1.822	1.563	1.467	1.581
Octanoic Acid	2.047	2.335	2.519	2.888	3.117	2.808	2.334	0.823	0.842	0.841	0.882	0.648	0.601	0.712
Decanoic Acid	nd	nd	nd	0.815	0.670	0.102	nd	nd	nd	nd	nd	nd	nd	nd
Total	**217.404**	**362.145**	**357.318**	**240.949**	**395.186**	**339.069**	**345.536**	**211.905**	**295.718**	**276.441**	**397.250**	**468.864**	**305.872**	**317.492**

Nd: not detected

*Refer to **Table 3.2** for a description of the treatments

lower levels in negative controls (treatment 1) than in all other treatments, indicating that malolactic fermentation contributed to its production regardless of inoculation practice. This is consistent with the result obtained by Ugliano & Moio (2005). Ethyl lactate formation can be associated with the production of lactic acid by lactic acid bacteria during malolactic fermentation in wine.

No specific references regarding the differences in flavour compounds caused by different malolactic fermentation times could be found in the present literature. Significant changes in the content of volatile esters as a result of (conventional) malolactic fermentation with different malolactic starter cultures were reported by Ugliano & Moio (2005). Ethyl fatty acid esters and acetate esters also increased in their study, especially isoamyl acetate and to a lesser extent, 2-phenylethyl acetate due to inoculated malolactic fermentation. Esterase activity has been investigated in wine lactic acid bacteria and the potential exists that the aroma profile of wine could be altered significantly during malolactic fermentation (Mtshali, 2007). Our results indicate that the time of inoculation could play a role in the expression or activity of some yeast or bacterial esterases.

3.3.3.2 Alcohols

Higher alcohols are alcohols that contain more than two carbons. They are secondary yeast metabolites produced mainly from amino acids by the Ehrlich pathway. High levels of higher alcohols generally impart strong pungent flavours and can have a negative impact on wine, while lower optimal levels can lend the wine a positive, fruity character. Methanol can have serious toxicity implications for humans if it is ingested at high concentrations. Methanol in wine is the product of the hydrolysis of grape pectins by the enzyme, methylesterase. The concentration of methanol was much lower in the co-inoculation treatments than in the uninoculated controls, and also lower than with normal inoculation after alcoholic fermentation in Pinotage. Co-inoculation also exhibited slightly lower concentrations of isobutanol (fusel, spirituous) and isoamyl alcohol (harsh, nail polish, herbaceous) than normal inoculation in Cabernet Sauvignon. It seems as if propanol (pungent, harsh) reached slightly higher concentrations in co-inoculated treatments than for normal inoculation in Pinotage, while the same trend was observed for hexanol (green, grass) in Cabernet Sauvignon.

However, most alcohols were not affected to a large extent by fermentation treatments. It also seems that the presence of lactic acid bacteria in co-inoculated treatments during alcoholic fermentation does not influence higher alcohol production significantly. Ugliano & Moio (2005) reported that in general, conventional malolactic fermentation did not have major effects on higher alcohol concentrations, although small increases were observed for a few higher alcohols. They found that important wine odorants such as isoamyl alcohol and 2-phenylethanol (floral) were sometimes increased significantly, depending on the commercial strain used for malolactic fermentation.

3.3.3.3 Fatty acids

Fatty acids are produced by fatty acid metabolism by yeast and bacteria. In the present study, butyric acid (pungent, sweaty) reached significantly higher concentrations in co-inoculated treatments, followed by normal inoculation, compared to the controls for both cultivars. Octanoic acid (oily, rancid, soapy, faint fruity) also shows a slightly higher concentration in co-inoculated treatments in Pinotage. Decanoic acid (phenolic, citrus) was absent from most treatments, with the highest levels present in co-inoculated treatments in Pinotage.

A high volatile acidity (acetic acid concentration above 0.7 g/L) can impart an undesirable vinegar-like and bitter character to wine (Ribéreau-Gayon *et al*., 2006b). Acetic acid was present in all treatments from both cultivars, but reached the lowest concentrations in treatment 1 (the negative control). In this treatment no malolactic fermentation took place. Only minimal growth of *Oenococcus* and no growth of *Lactobacillus* or *Pediococcus* was observed on selective media (see **Table 3.4** and **Table 3.5**). Thus, the concentration of acetic acid reached in treatment 1 (approximately 200 mg/L for both cultivars) could represent the contribution by yeast. Conflicting trends were observed in the two cultivars, with co-inoculation resulting in lower acetic acid concentrations than normal inoculation in Pinotage, while the opposite effect was observed in Cabernet Sauvignon wines. Strain Lalvin VP41 also produced lower levels of actic acid than Viniflora Oenos, although the contribution of the latter was still low in genenal winemaking terms.

Quantification of acetic acid produced in treatments of the two cultivars for both vintages were obtained by FT-IR (**Figure 3.2**). For the 2007 data, the trend is the same as obtained by GC-FID for acetic acid. It seems that, since three of the four experimental repeats show the lowest values of acetic acid present in the co-inoculated treatments when compared to both spontaneous and conventional inoculation methods, that co-inoculation is the most effective way to reduce lactic acid bacterial associated volatile acidity. This is contrary to what is generally feared in the wine industry due to the belief that *O. oeni*, as a heterolactic organism, would use the fermentable sugars present in the fermenting must with the production of high concentration of acetic acid as a consequence (Ribéreau-Gayon *et al*., 2006a). Beelman & Kunkee (1985) also showed that malolactic fermentation in the presence of fermentable sugars does not necessarily lead to excessive acid production.

The results obtained in this study for volatile compound production are based upon a very limited scope of study (two cultivars and one vintage) and are therefore not conclusive, but address the necessity for a larger study of this sort. Some interesting trends can be observed and it is clearly demonstrated that different times of inoculation with commercial starter cultures can result in different aroma profiles in the final wines. From this limited data it seems as if though co-inoculation may increase the fruity aromas in wine and lead to a decrease in some negative flavour-impact compounds such as acetic acid, methanol and fusel alcohols. Sensory thresholds of volatile compounds should also be addressed in future work, in order to determine the real

significance of the impact of malolactic fermentation inoculation times on aroma compounds that can actually be perceived in the wines. Unfortunately, sensory evaluation of wines using a trained panel could not be conducted during this study due to the anticipated high levels of biogenic amines that could be present in these experimental wines and have harmful effects on sensitive individuals. A study by Jussier *et al.* (2006) on the influence of co-inoculation on the sensory profiles and key wine parameters concluded that co-inoculation had no negative sensory impact on wines, which correlates with our results.

Finally, the impact of cultivar and commercial starter culture strain cannot be underestimated when evaluating the sensory contribution to aroma by lactic acid bacteria.

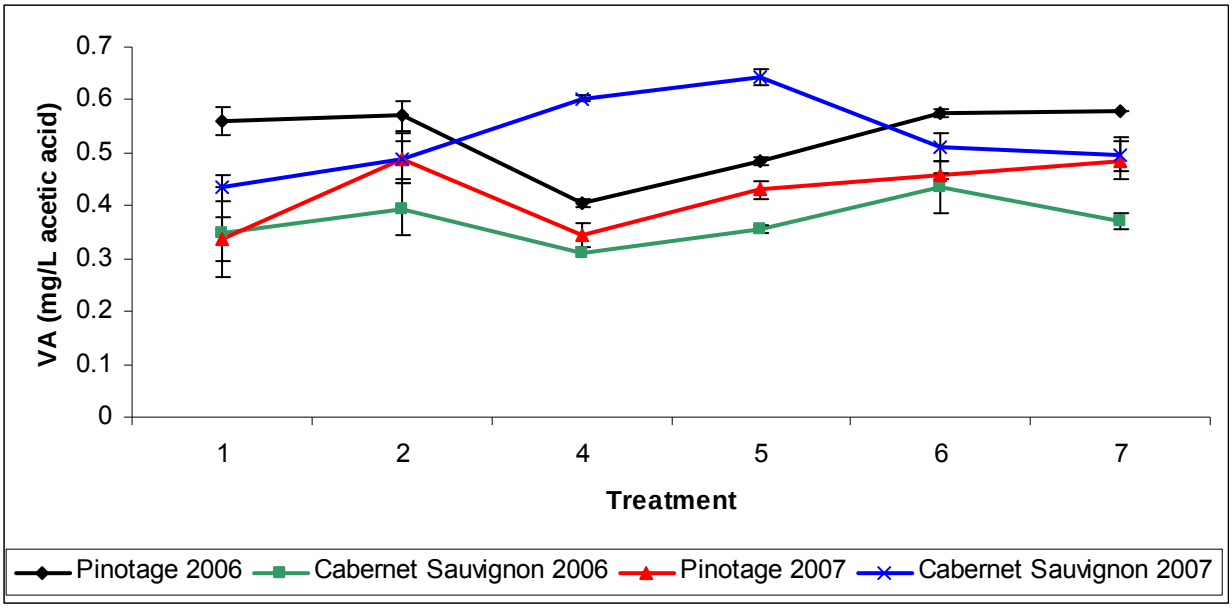

Figure 3.2 Volatile acidity (reported as mg/L acetic acid) of the different malolactic fermentation treatments (refer to the text for treatment descriptions) for the two cultivars and two vintages at the end of malolactic fermentation. Each value represents the average of duplicate (2006) or triplicate (2007) treatment repeats, each scanned in duplicate by FT-IR spectroscopy.

3.3.4 PRODUCTION OF BIOGENIC AMINES

Since histamine, tyramine, putrescine and cadaverine are the four main biogenic amines associated with wine, only these biogenic amines (and spermidine, a product of putrescine) will subsequently be included in discussions.

The amino acid concentrations of the must reported in **Table 3.7** represents the concentration before an additional 50 mg/L of each of histidine, tyrosine, ornithine and lysine (the precursors of the studied biogenic amines) was added to the must. Quantification of these four amino acids after addition resulted in concentrations above the respective detection limits, indicating a significant increase in concentrations.

For the Pinotage treatments of 2007, amino acids quantified at three stages of production are reported; in the must (**Table 3.7**), in the wine after alcoholic fermentation and in the wine at the completion of malolactic fermentation (**Table 3.8**). These analyses are to serve as a general example of the possible relation between amino acids and their associated biogenic amines.

The free amino acids that could serve as precursors of biogenic amines decreased in concentration from the must to the end of alcoholic fermentation. This was likely due to yeast metabolism of the amino acids as a source of nitrogen. It does not seem that amino acid changes were different in co-inoculated treatments (Treatments 4 and 5) than in other treatments at this stage.

Table 3.7 Amino acid and biogenic amine content of musts of the two cultivars in 2006 and 2007. Concentrations represent the averages of duplicate treatment analysis (standard deviations not shown).

Compound (mg/L)	2006		2007*	
	Pinotage	Cabernet Sauvignon	Pinotage	Cabernet Sauvignon
Alanine	172.54	87.46	140.70	55.20
Threonine	145.77	33.87	81.90	4.60
Serine	97.68	41.22	49.50	17.90
Glutamine	429.12	81.74	228.30	23.50
Arginine	848.78	27.01	395.40	10.90
Tryptophan	21.42	15.17	11.40	1.80
Glutamic acid	268.35	41.69	157.10	33.80
Valine	32.75	46.24	66.60	36.00
Histidine	91.47	53.57	46.60	12.50
Aspartic acid	149.50	27.54	180.00	17.90
Lysine	14.68	4.13	14.00	2.30
Proline	279.15	550.96	398.10	396.40
Methionine	10.91	2.63	0.40	0.30
Tyrosine	35.74	35.47	40.70	9.70
Cystine	0.26	0.22	4.80	1.60
Isoleucine	16.05	28.51	48.80	20.30
Phenylalanine	16.89	20.62	25.20	9.00
Leucine	38.43	41.36	52.90	21.20
Asparagine	10.70	1.76	7.70	2.70
Glycine	8.67	4.15	6.50	1.00
Ornithine	20.91	3.15	10.60	0.20
total	2709.77	1148.47	1967.20	678.80
Histamine	nd	nd	0.012	0.019
Putrescine	9.287	35.663	1.140	3.690
Spermidine	nd	nd	0.570	0.530
Tryptamine	nd	nd	nd	nd
Phenylethylamine	nd	nd	0.004	0.012
Tyramine	nd	nd	0.006	0.003
Cadaverine	nd	nd	nd	nd

* Amino acid concentrations of grape must before addition of precursors at a total of 200 mg/L

Table 3.8 Amino acid concentrations (mg/L) of the experimental repetition of Pinotage 2007 present in the malolactic fermentation treatments at the end of alcoholic (AF) and malolactic (MLF) fermentations.

Treatment*	1	2	3	4	5	6	7
End AF							
Alanine	12.7	13.2	10.3	12.0	7.5	10.3	10.3
Threonine	1.1	1.0	1.0	0.9	0.8	1.0	1.0
Serine	1.2	1.2	1.1	1.2	0.9	1.1	1.1
Glutamine	5.3	4.9	3.3	4.4	3.0	3.3	3.3
Arginine	5.8	5.8	5.6	6.9	3.3	5.6	5.6
Tryptophan	0.2	0.2	0.2	0.1	0.1	0.2	0.2
Glutamic acid	10.3	14.3	10.2	9.7	7.1	10.2	10.2
Valine	2.0	1.9	1.3	2.1	1.3	1.3	1.3
Histidine	2.9	3.4	2.6	3.6	2.1	2.6	2.6
Aspartic acid	2.6	3.0	2.4	2.9	1.9	2.4	2.4
Lysine	1.8	2.2	1.9	2.6	1.7	1.9	1.9
Proline	396.6	452.2	745.4	399.1	353.8	745.4	745.4
Methionine	0.2	0.3	0.4	0.2	0.2	0.4	0.4
Tyrosine	1.2	1.3	0.9	1.3	0.7	0.9	0.9
Cystein	4.2	3.8	3.2	3.2	3.0	3.2	3.2
Isoleucine	0.9	0.9	0.6	1.0	0.5	0.6	0.6
Phenylalanine	0.9	1.1	1.0	1.2	0.6	1.0	1.0
Leucine	1.2	1.8	1.3	1.8	1.0	1.3	1.3
Asparagine	7.1	8.6	7.7	8.3	5.8	7.7	7.7
Glycine	2.7	4.4	4.1	2.7	2.3	4.1	4.1
Ornithine	8.0	7.8	6.5	8.3	4.6	6.5	6.5
total	468.5	532.8	808.0	473.0	401.7	808.0	808.0
End MLF							
Alanine	49.7	73.8	82.3	83.0	89.8	87.1	94.4
Threonine	13.0	18.3	17.8	20.2	16.4	8.4	10.8
Serine	14.0	19.0	22.9	17.0	16.9	22.2	25.8
Glutamine	6.5	9.1	12.6	13.5	16.8	9.9	11.8
Arginine	41.0	3.9	3.1	36.4	9.6	19.4	2.8
Tryptophan	3.9	6.3	8.2	3.2	5.4	8.2	8.4
Glutamic acid	40.4	44.1	47.5	44.9	44.1	36.5	50.3
Valine	48.7	63.6	74.2	64.3	77.6	80.9	76.8
Histidine	12.4	15.3	19.2	10.8	13.5	14.8	13.2
Aspartic acid	36.6	50.9	59.9	47.8	56.3	60.6	66.2
Lysine	44.5	55.4	68.2	51.7	52.1	59.3	64.4
Proline	526.4	552.7	860.7	665.2	656.6	679.4	678.3
Methionine	2.7	2.4	4.4	0.4	0.4	7.3	5.4
Tyrosine	28.8	36.0	43.1	32.3	36.9	37.5	38.2
Cystein	19.4	10.8	8.2	5.8	9.6	5.8	9.0
Isoleucine	42.2	53.1	59.0	54.4	60.5	57.7	55.2
Phenylalanine	34.6	48.5	57.4	50.1	53.1	54.2	56.6
Leucine	71.8	105.9	120.7	102.1	114.7	122.2	120.1
Asparagine	31.5	38.5	48.7	42.2	48.1	49.6	50.4
Glycine	16.7	27.1	28.0	31.5	27.4	12.0	16.4
Ornithine	17.5	56.4	73.7	27.7	54.1	44.1	51.9
total	1101.7	1290.6	1712.9	1404.0	1459.3	1476.6	1506.0

*Refer to **Table 3.2** for a description of the treatments

All of the amino acid precursors increased again significantly during malolactic fermentation. Free amino acids can be released from peptides and proteins from grape skin derived components or autolysed yeast cells by peptidases and proteinases of lactic acid bacteria (Manca de Nadra et al., 1997; Remize et al., 2005). This increased pool of free amino acid precursors could potentially serve as substrates for biogenic amine production by residual decarboxylase activity during ageing. For this reason, an analysis of biogenic amines after a short ageing period (four months) was conducted to indicate whether the increase in biogenic amines occurred during ageing. Although not determined in the present work, Alcaide-Hidalgo *et al.* (2007a) indicated that free amino acids were increased during ageing of wines with lees in their study, and this could result in an additional risk of biogenic amine formation.

Figures 3.3 to 3.6 present the concentrations of histamine, cadaverine, putrescine and spermidine (2007 only) at each winemaking phase, namely at the end of alcoholic fermentation, after malolactic fermentation and after ageing for four months. Concentrations of biogenic amines at the end of the ageing period are reported on two different line graphs representing wines aged with lees and wines aged without lees (wines racked before ageing) respectively. No tyramine was detected in Pinotage or Cabernet Sauvignon in 2006 or 2007.

No histamine was detected in the grape must (**Table 3.7**) or produced in Pinotage in detectable amounts during fermentations in 2006 (**Figure 3.3**). Only during ageing in the presence of yeast lees was histamine detected at a low concentration in one of two replicates of the spontaneous malolactic fermentation treatment (treatment 2). This could indicate the potential variability and unreliability of spontaneous malolactic fermentation in which the metabolic properties of the flora is generally unknown and may differ from one wine to the next (for example between replicates).

Cadaverine was produced in all treatments only when aged on the fermentation lees. Analysis of variance indicated that there were no significant differences between the malolactic fermentation treatments ($p=0.3$) for cadaverine at any of the examined winemaking stages.

The lowest means for putrescine was obtained at the end of malolactic fermentation, and the highest at the end of ageing in the presence of lees. Repeated measures analysis of variance indicated that only these two phases differed significantly from each other ($p=0.02$), but that there were no statistical differences between putrescine production in the different fermentation treatments at any stage ($p=0.4$). The large difference in duplicate treatment analyses remained when sample analysis was repeated, but no reason could be found to explain this result.

Malolactic fermentation itself did not seem to have a large influence on the increase of biogenic amines in Pinotage in the 2006 vintage and no significant trends could be observed between malolactic fermentation inoculation treatments and biogenic amines. The grapes used in this experiment were of high quality and in a very healthy condition, with a low incidence of indigenous lactic acid bacterial flora that survived in the wine (**Table 3.1**). The health condition of the grapes could have played a big role in the

occurrence of indigenous decarboxylase positive microorganism and the physiological need to decarboxylate amino acids. For this reason it was decided to induce the relevant decarboxylase lactic acid bacteria, together with the precursors of biogenic amines, into the wine in the following season.

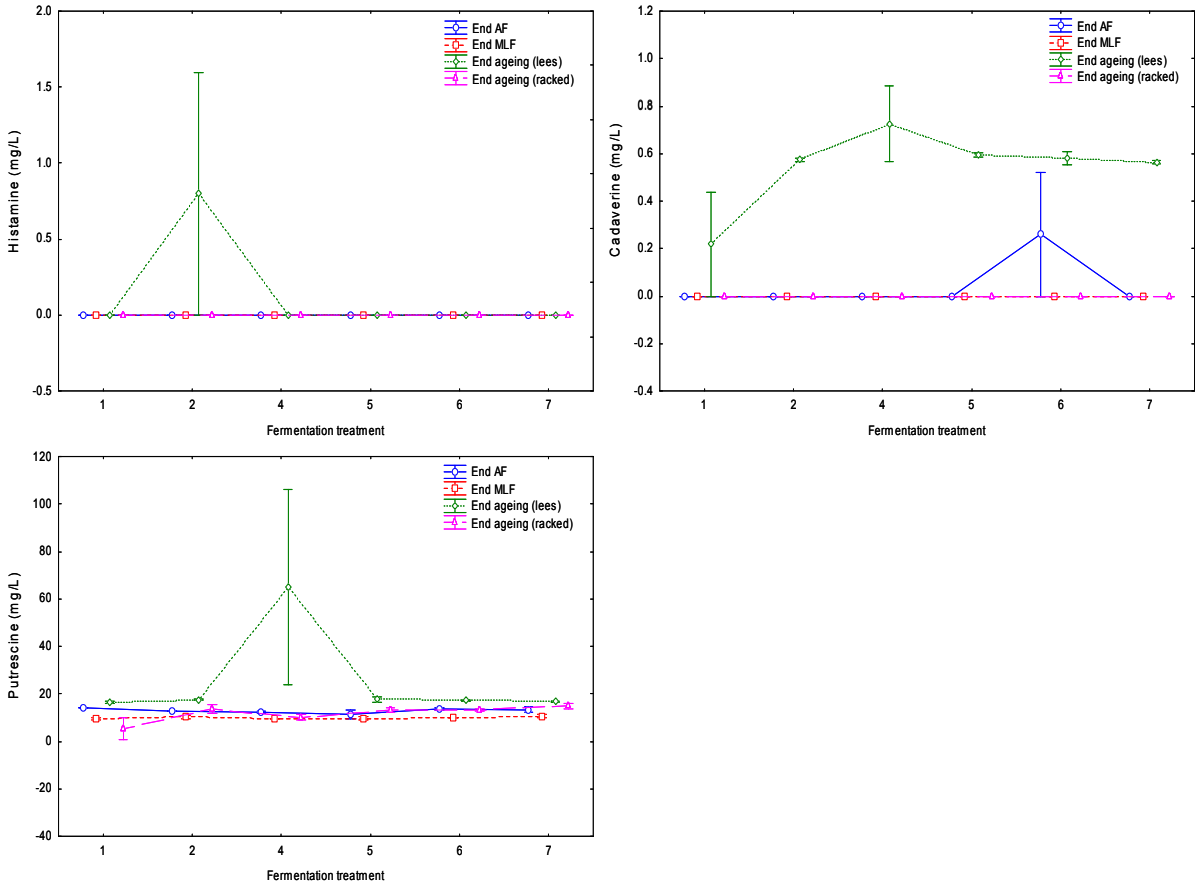

Figure 3.3 Biogenic amine production in malolactic fermentation and ageing treatments from Pinotage 2006. Error bars indicate the minimum, average and maximum values obtained for histamine, cadaverine and putrescine by analyses of duplicate treatments. Refer to the text for treatment descriptions.

In Cabernet Sauvignon in 2006 (**Figure 3.4**) histamine was increased significantly during malolactic fermentation ($p<0.01$) when compared to the other reported winemaking phases. Marcobal *et al.* (2006) and Alcaide-Hidalgo *et al.* (2007a) also indicated that histamine did not increase significantly during storage in their respective studies when compared to malolactic fermentation. Although differences were not statistically significant for malolactic fermentation treatments ($p=0.3$), the highest average concentration of histamine could be observed in treatment 2 (spontaneous malolactic fermentation), while co-inoculation resulted in higher histamine levels than normal inoculation after alcoholic fermentation or the negative control. The levels of histamine at the end of malolactic fermentation in this experimental repeat was higher than the accepted upper limits for histamine in wine in Germany (2 mg/L) and Holland (3 mg/L) and in the case of co-inoculated and spontaneous malolactic fermentation

treatments, it was also higher than the limits suggested by Finland (5 mg/L) and Belgium (5 mg/L to 6 mg/L). The upper limits are 8 mg/L for France and 10 mg/L for Switzerland and Austria (Lehtonen, 1996). Histamine decreased again during ageing in all treatments. In treatment 2 (spontaneous malolactic fermentation) aged in the presence of lees, some residual histamine was left after four months of ageing in one of the replicates. This again may indicate that spontaneous fermentation may be unpredictable and pose a larger risk of biogenic amine occurrence than in commercially inoculated malolactic fermentations.

Cadaverine also increased significantly during malolactic fermentation ($p<0.01$) and reached the highest concentration in co-inoculated treatments (trend only; $p=0.09$). Although this pattern was still observable at the end of ageing, the concentration of all treatments decreased in the wines regardless of the presence or absence of lees.

Putrescine was already present at significant levels in the grape must of Cabernet Sauvignon in 2006 (**Table 3.7**). Like histamine and cadaverine, putrescine also increased significantly during malolactic fermentation ($p<0.01$). Putrescine was the only biogenic amine that was present in the lowest concentrations in co-inoculated treatments, and the highest in conventional inoculated treatments at the end of malolactic fermentation. Statistically, co-inoculation and conventional inoculation with Lalvin VP41 differed significantly from other treatments ($p=0.02$) and led to the lowest and highest mean putrescine concentration respectively. Putrescine concentration decreased during ageing in all treatments inoculated with commercial starter cultures to varying degrees, both in the presence and absence of lees.

Lysozyme is an enzyme that can cause lysis of the cell walls of Gram-positive bacteria, including wine lactic acid bacteria. Lysozyme retains its activity in higher pH wines. It can be used successfully to delay or inhibit the growth of lactic acid bacteria, especially when used in combination with SO_2 (Delfini *et al.*, 2004; Ribéreau-Gayon *et al.*, 2006a) as it was observed in this study (**Table 3.3, 3.4** and **3.5**). It is important to note that in both cultivars in 2006 growth of indigenous *Oenococcus* strains and spontaneous malolactic fermentation occurred in the negative control with lysozyme (treatment 1), therefore biogenic amines produced in these treatments cannot be attributed to the metabolism of yeasts alone. The pattern of biogenic amine formation in negative control treatments (treatment 1) was mostly different from that observed in the spontaneous malolactic fermentation treatments (treatment 2), indicating that lysozyme inhibition or delay of particular lactic acid bacteria might have taken place and influenced subsequent biogenic amine formation.

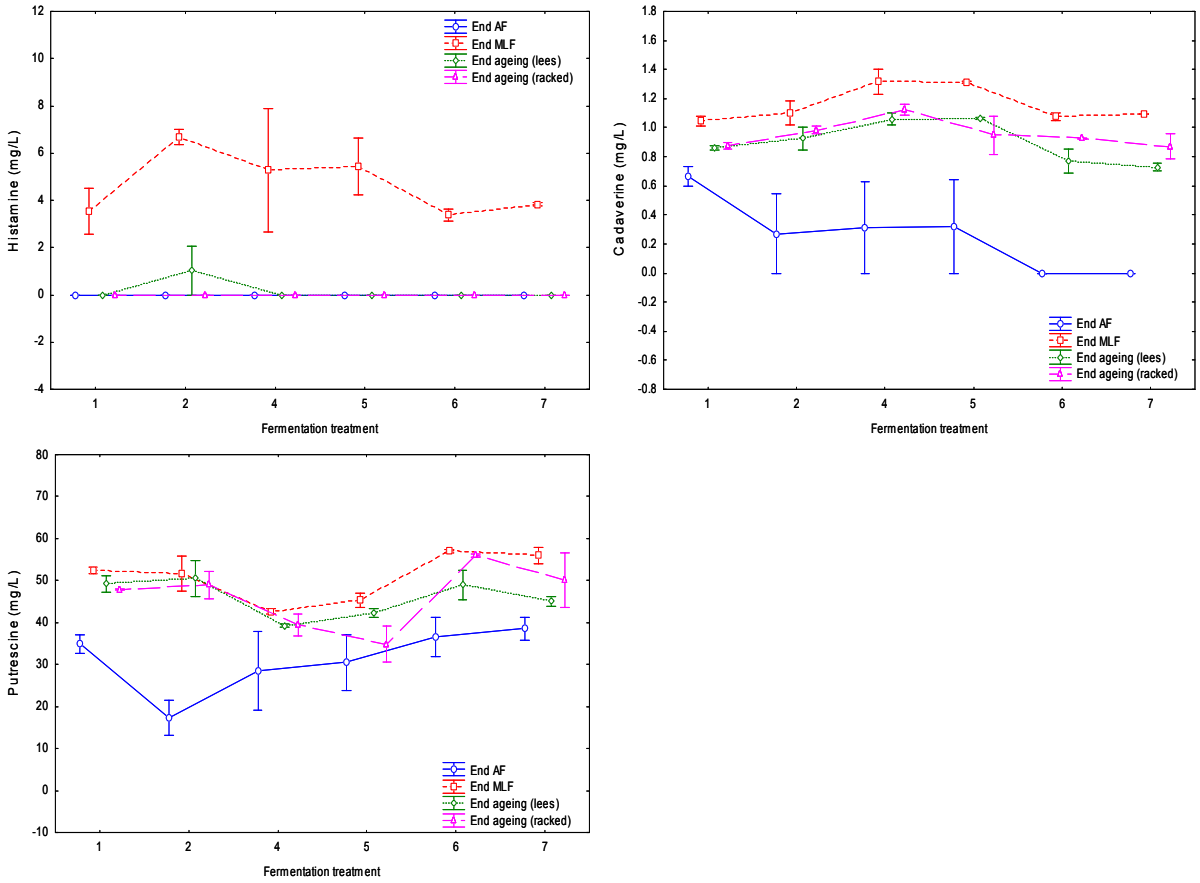

Figure 3.4 Biogenic amine production in malolactic fermentation and ageing treatments from Cabernet Sauvignon 2006. Error bars indicate the minimum, average and maximum values obtained for histamine, cadaverine and putrescine by analyses of duplicate treatments. Refer to the text for treatment descriptions.

Histamine was present only at very low concentrations in all Pinotage 2007 treatments (**Figure 3.5**) and far below any suggested upper limits (Lehtonen, 1996). At the end of alcoholic fermentation histamine seemed to have increased from the must (**Table 3.7**) to a small extent in co-inoculated treatments (treatments 4 and 5) and treatments containing no spiked Lactobacilli (treatments 1 and 2). Treatments 3, 6 and 7, which had the same composition at the end of alcoholic fermentation seemed not to support an increase in histamine. Histamine increased during malolactic fermentation and increased further during ageing. After ageing all fermentation treatments of both ageing practices had significantly higher concentrations of histamine ($p<0.01$) compared to treatments before ageing (with end alcoholic and end malolactic fermentation phases being statistically the same). Jiménez Moreno *et al.* (2003) and Jiménez Moreno & Ancín Azpilicueta (2004) both found that after histamine had reached a maximum concentration after the initial stages of ageing, it was progressively decomposed. There were no trends or statistical differences ($p=0.3$) between malolactic fermentation treatments for histamine.

Cadaverine only showed a significant increase during ageing and more so in treatments in the presence of lees than in its absence ($p<0.01$). Final concentrations of

cadaverine after ageing in the presence or absence of lees were statistically similar for most treatments with the exception of the three control treatments (1, 2 and 3) in the absence of lees ($p<0.01$), which yielded significantly lower mean values.

Putrescine showed an increase during malolactic fermentation and was decreased significantly during ageing; more so in the absence than in the presence of fermentation lees. While spermidine also increased significantly during malolactic fermentation, it continued to increase during ageing ($p<0.01$). All the changes in putrescine and spermidine concentrations (in all treatments) between phases and ageing treatments were significant ($p<0.01$). However, malolactic fermentation treatments were never different from each other with regards to putrescine ($p=0.8$) or spermidine ($p=0.9$) production. The decrease in putrescine during ageing can be correlated inversely with an increase in spermidine. Spermidine (and spermine which could not be quantified by the LCMSMS method used in this study) arises from putrescine as a secondary product by the donation of aminopropyl groups from decarboxylated *S*-adenosylmethionine. Putrescine, spermidine and spermine and are involved in nucleic acid and protein synthesis and the stabilisation of membranes (Smith, 1980). Spermidine was also formed only towards the end of ageing in a study by Jiménez Moreno & Ancín Azpilicueta (2004).

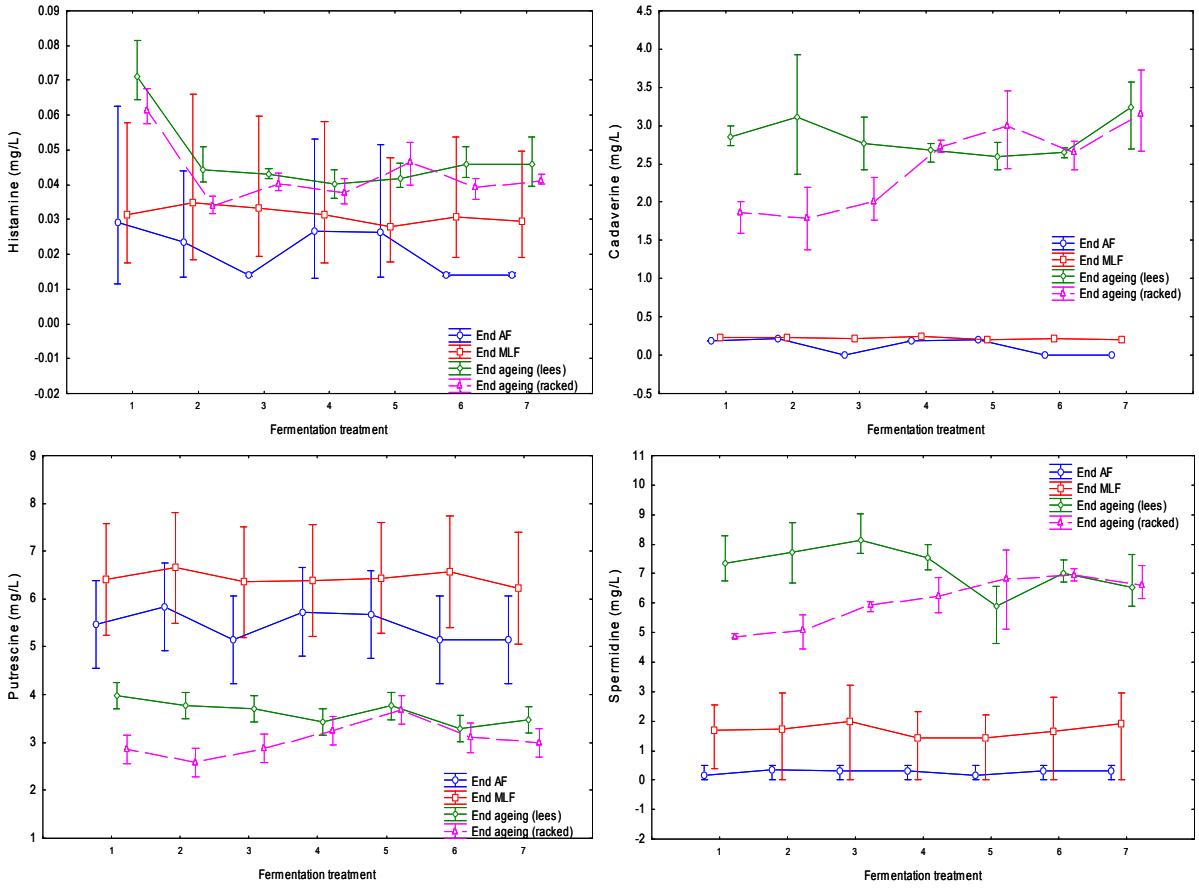

Figure 3.5 Biogenic amine production in malolactic fermentation and ageing treatments from Pinotage 2007. Error bars indicate the minimum, average and maximum values obtained by analyses of triplicate treatments for histamine, cadaverine, putrescine and spermidine. Refer to the text for treatment descriptions.

At the end of malolactic fermentation all four biogenic amines in all treatments in Cabernet Sauvignon had increased the most in uninoculated treatments (treatments 2 and 3) regardless of the presence or absence of spiked decarboxylase bacteria (**Figure 3.6**). It seems that the presence of commercial *O. oeni* starter cultures reduces the incidence of biogenic amine formation during malolactic fermentation regardless of time of inoculation.

Ageing of treatments in the absence of lees reduced the concentration of histamine significantly ($p<0.01$) when compared to wines at the end of malolactic fermentation and aged with lees but to a similar extent in all malolactic fermentation treatments aged without lees. Treatments 2, 3, 4, 5 aged with lees also showed a decrease in histamine concentration while treatments 6 and 7 (inoculated by conventional methods after alcoholic fermentation) showed an increase in histamine after ageing on lees. The treatments were significantly different from each other at this stage ($p<0.01$). The high concentration of histamine produced in treatment 1 cannot be explained at this stage. Means for fermentation treatments at other stages indicate that histamine was produced in lower levels on average in co-inoculated treatments than in the control treatments, with conventional inoculation yielding the highest means of histamine. However, these observations were not statistically significant ($p=0.06$), but exhibited a strong trend.

Cadaverine showed a significant increase during ageing ($p<0.01$). This increase was statistically identical for the two ageing treatments but different for fermentation treatments ($p<0.01$) with co-inoculation and the negative control having significantly lower mean values than spontaneous and conventional malolactic fermentation treatments.

Putrescine decreased during ageing; significantly more so in the absence of lees ($p<0.01$) than in the presence of lees. Analysis of the interaction between phase and malolactic fermentation treatment indicate that uninoculated treatments (treatments 1, 2 and 3) decreased significantly more than other treatments in the absence of lees during ageing ($p<0.01$). The decrease in putrescine concentrations could again be correlated with the increase in spermidine concentration. Contrary to the results obtained in all four of the experimental repeats, Jiménez Moreno *et al.* (2003) and Jiménez Moreno & Ancín Azpilicueta (2004) both reported that putrescine was accumulated and was not degraded during ageing.

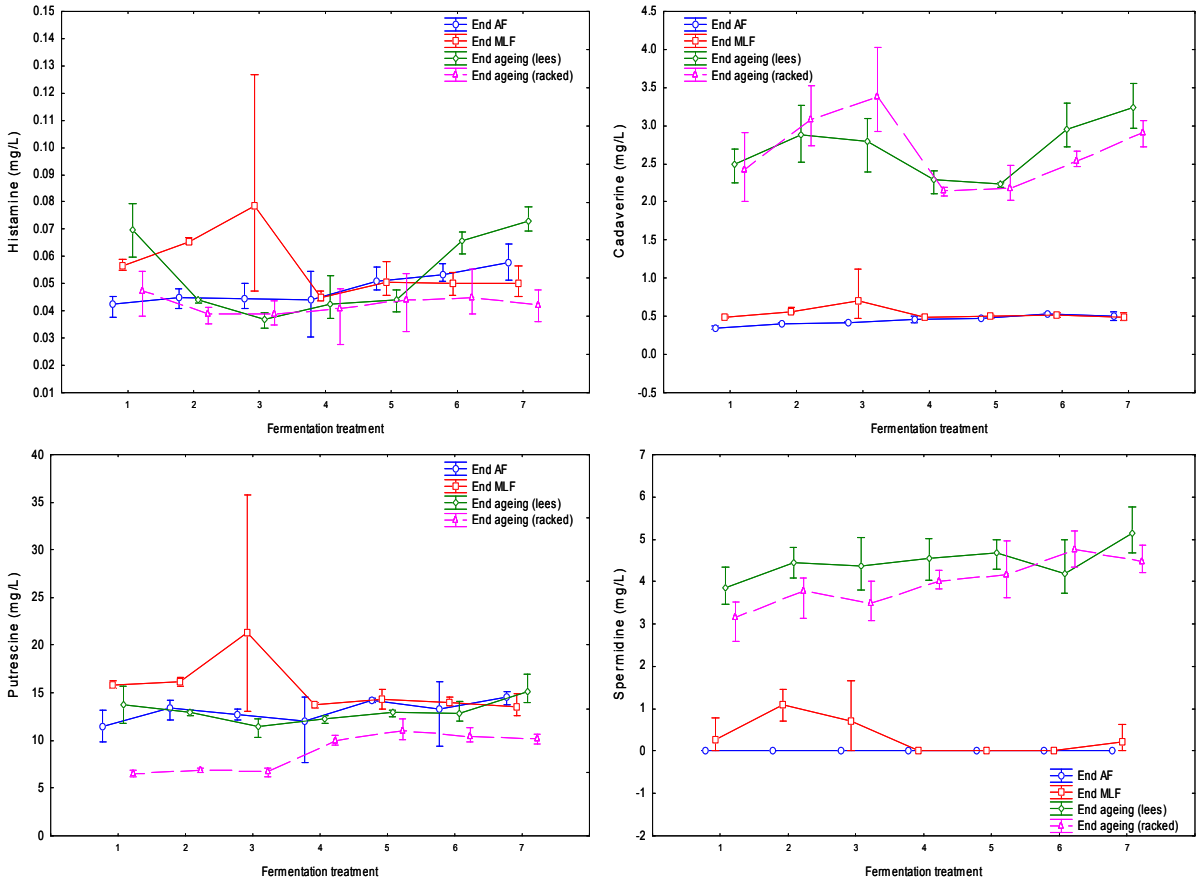

Figure 3.6 Biogenic amine production in malolactic fermentation and ageing treatments from Cabernet Sauvignon 2007. Error bars indicate the minimum, average and maximum values obtained by analyses of triplicate treatments. Refer to the text for treatment descriptions.

The reason for the initial increase following the completion of malolactic fermentation could be that SO_2 added to the wine after malolactic fermentation does not completely stop all biochemical reactions and enzyme activity. Lonvaud-Funel (2001) attributed the increase in biogenic amines (particularly histamine) at the beginning of a storage period to the microorganisms with decarboxylase activities that are still present in the wines after fermentations. Coton *et al.* (1998) also noted that even when no more culturable cells were detectable, histidine decarboxylase could still be active and thus biogenic amines can be produced during ageing. Another reason for increase in biogenic amines following malolactic fermentation is the release of vitamins and nitrogen compounds (that stimulate lactic acid bacterial activity) and free amino acids (that can act as biogenic amine precursors) due to yeast autolysis. Moreover, most biogenic amines as well as decarboxylase enzymes are heat stable and will not be reduced during processing (such as pasteurisation), and can for this reason even increase during storage (ten Brink *et al.*, 1990).

The decrease of some biogenic amines during ageing is presumable due to amine oxidases present in some bacterial strains (Leuschner *et al.*, 1998 and references therein) that act by breaking down biogenic amines to an aldehyde, hydrogen peroxide and ammonia.

In the 2007 vintage in both cultivars, the addition of lysozyme in combination with high levels of SO_2, decreased the incidence of lactic acid bacterial growth in the negative control treatments; and successfully inhibited malic acid degradation (results not shown) to any observable degree in these treatments (**Tables 3.4** and **3.5**). Therefore, these treatments may indicate the levels of biogenic amines formed by yeasts in this study or by indigenous lactic acid bacteria that were not fully inhibited by lysozyme and had residual enzyme activity in the wine even if it did not perform malolactic fermentation.

The decarboxylase positive *Lactobacillus* strains used in this study were adapted to wine conditions, but although the relevant amino acid precursors and cofactor for biogenic amine formation were included in the adaptation medium, the specific activity of the decarboxylase enzymes were not investigated before inoculation into the wines. Leitão *et al.* (2000) also reported that biogenic amines were not produced at significant concentrations, despite the presence of decarboxylase positive bacteria. Decarboxylase activity and/or cell viability of lactic acid bacteria can be decreased or lost due to prolonged storage or repeated subcultivation in synthetic media (Lonvaud-Funel & Joyeux, 1994; Leitão *et al.*, 2000) or inhibited by wine conditions such as low pH and ethanol concentrations exceeding 12% v/v (Rollan *et al.,* 1995; Leitão *et al.*, 2000; Gardini *et al.*, 2005). In particular, tyrosine decarboxylation never occurred in this study and histamine was produced at very low concentrations in Pinotage 2006, Pinotage 2007 and Cabernet Sauvignon 2007. It does not seem that the decarboxylase activities of the spiked *Lactobacillus* species contributed significantly to biogenic amine production in 2007 (treatment 3 versus treatment 2).

3.3.5 PREDICTION OF BIOGENIC AMINES USING FT-IR SPECTROSCOPY AND MULTIVARIATE DATA ANALYSIS

3.3.5.1 Preliminary calibration and results

Partial least squares (PLS) regression is a bilinear regression modeling method which projects the original x-variables (the spectra obtained by FT-IR spectroscopy) onto a smaller number of PLS components (Esbensen, 2002). PLS regression with one y-variable (the sum of all biogenic amines measured with analytical techniques) was performed on the data, using leverage corrected validation to identify outliers. The X-Y relation outliers plot (T versus U scores) was investigated to identify outliers. In **Figure 3.6** these outliers are shown in the X-Y relation outliers plot of the third PLS component (PC3), but one or both of the outliers could also be identified in PC1, PC2 and PC4. **Figure 3.6** indicates the scores plot of the first two PLS components which explain 43% and 35% of the variance in the data respectively; the T-U scores plot with the two outliers to be removed from the data set; the residual validation variance and the initial model obtained by PLS regression.

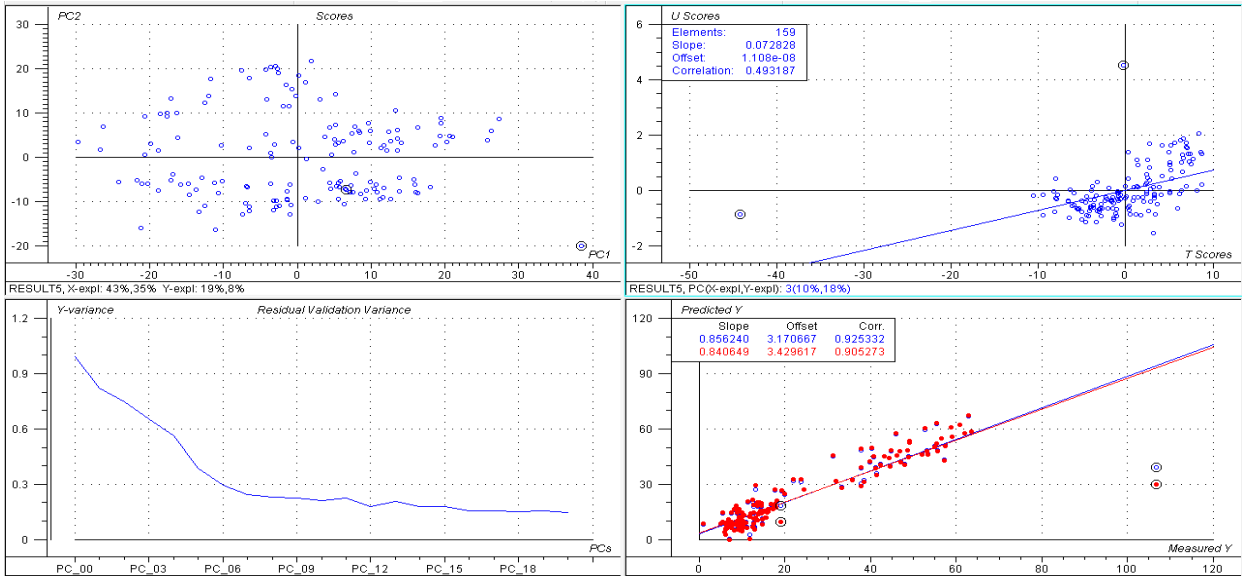

Figure 3.6 The PLS regression model obtained with leverage corrected validation for total biogenic amines (Y-variable) and selected spectra obtained by FT-IR spectroscopy (X-variables). The circled data points were identified as outliers.

After the removal of the two outliers, the validation of the calibration was attempted by an independent test set (validation set), of which samples were in the same concentration range as the calibration set. This independent test set was randomly selected from the calibration set (62 of 489 randomly selected samples) by the Unscrambler software and the improved model is shown in **Figure 3.7**. Cultivar (P for Pinotage and C for Cabernet Sauvignon) and vintage are indicated on the scores plot, where blue represents the 2006 data and red the 2007 data. A clear separation of these variables can be seen in the first two PLS components on the scores plot. On the residual validation variance plot, it is indicated that most of the Y-variance is explained by the first nine PLS components. The best model (Measured Y vs. Predicted Y) is therefore obtained using nine PLS components. The X-loading Weights of the first three PLS components, explaining most of the residual validation variance, are reported. The loading weights show the relative importance of each variable (wavenumber); how much each X-variable (wavenumbers in the spectra) contribute to the regression relationship between X and Y. The wavenumbers at which primary and secondary amines absorb in infrared spectra (3300–3500 cm^{-1}) are indicated on this plot. Even though these variables (wavenumbers) show high loadings on the loading weights plot, there are clearly other variables that contributed equally or more to the regression model and also show a strong correlation with total biogenic amines.

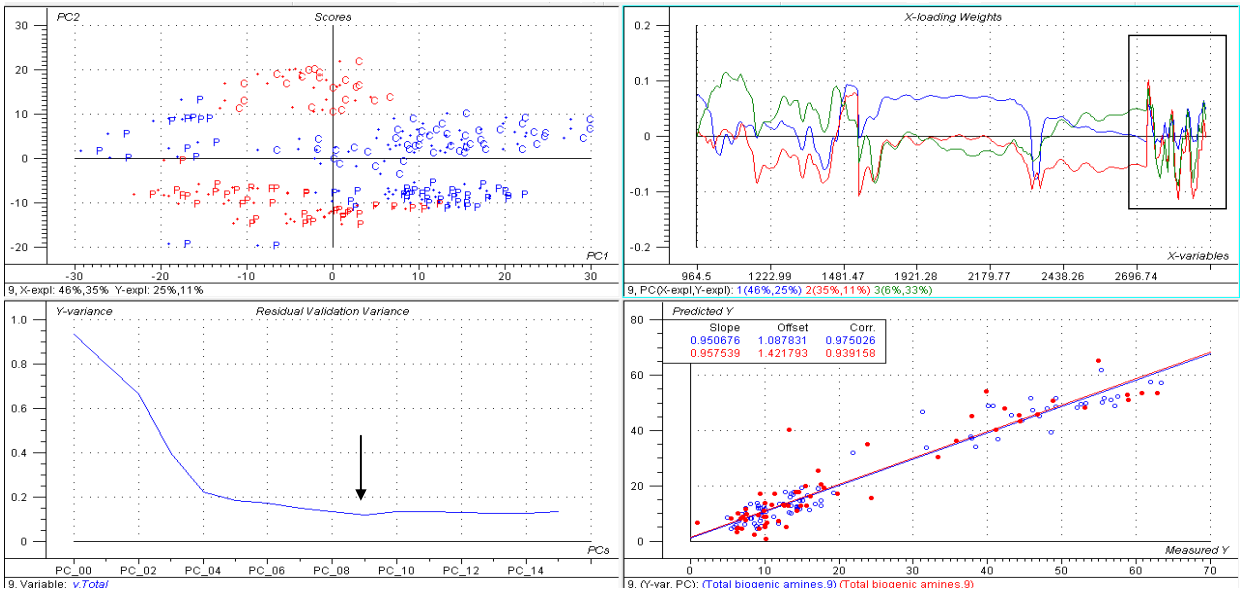

Figure 3.7 An improved model obtained by PLS regression after the removal of outliers and recalculation with independent test set validation. Cultivars and vintages are separated on the scores plot: P (Pinotage), C (Cabernet Sauvignon), blue (2006), red (2007).

The spectra obtained by FT-IR in this work was automatically generated during routine monitoring of wine chemical components and it was only decided at a later stage to evaluate the potential of FT-IR to predict biogenic amines. Therefore, the data was not originally selected or separated to form a calibration set and an independent validation set. In order not to subtract from the calibration set by taking out a test set, a second model was established (**Figure 3.8**) using two segmented cross-validation (a method performed by the software that keeps out either half of the samples from the calibration set successively and uses these subsets for prediction) which is the second best option for validation, following independent test set validation, according to Esbensen (2002). The best model was obtained using ten PLS components in this case.

The validation data of the two models (**Figure 3.9**) show that both are suitable to use for prediction purposes. The prediction accuracy of the calibration model, relative to the reference data is expressed as standard error of prediction (RMSEP). The bias is an indication of the systematic error in the predicted data and is used to evaluate the performance of the calibration. The residual predictive deviation (RPD) is the ratio of the standard deviation of the reference values to the standard error of the predicted values.

The coefficient of determination (R^2; correlation) indicated good precision for both models. According to Shenk & Westerhaus (1996) a coefficient of determination greater than 0.9 is excellent and the model is fit for quantification purposes. The RPD value for both models was between 3 and 5, judging the models suitable for screening purposes (Williams, 1995). The bias was close to zero in both cases indicating a small systematic error, but slightly more so with cross validation than with independent test set validation.

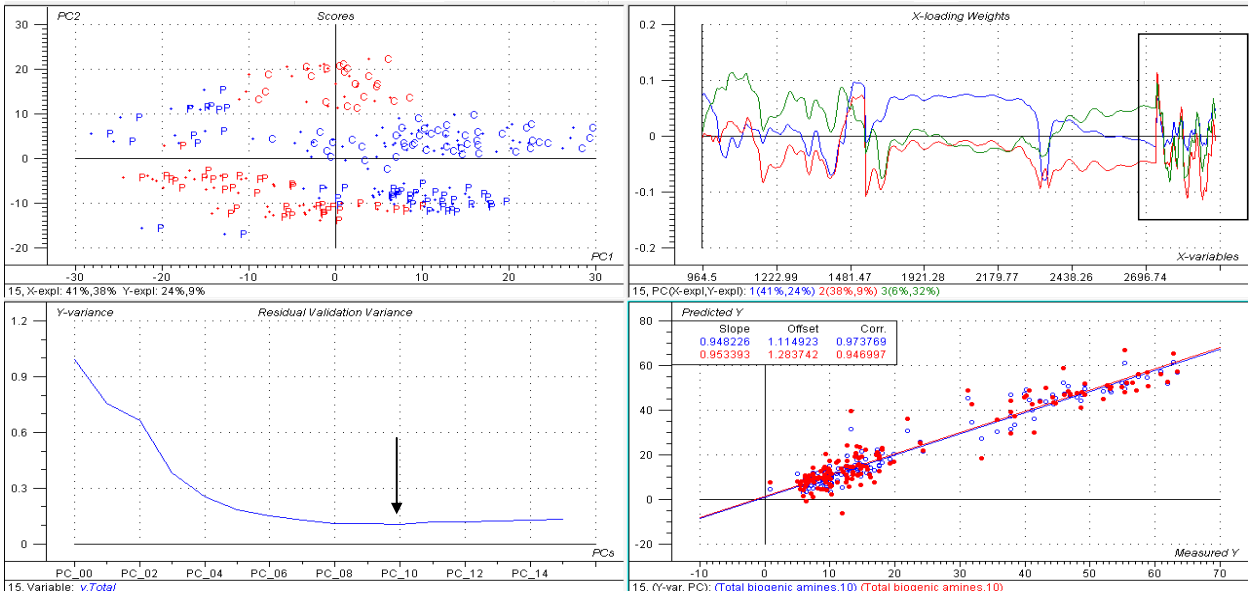

Figure 3.8 The model obtained by PLS regression after the removal of outliers and recalculation with two-segmented cross validation. Cultivars and vintages are separated on the scores plot: P (Pinotage), C (Cabernet Sauvignon), blue (2006), red (2007).

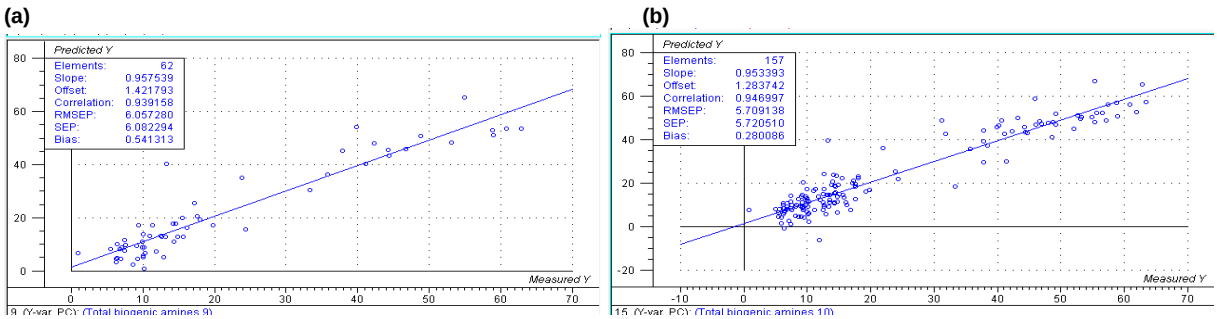

Figure 3.9 Validation data of the two models obtained by independent test set validation (a) and two-segmented cross validation (b). Refer to the text for a description of statistical indicators.

Even though the preliminary calibration attempt using test set validation performed well, further studies on the calibration of total biogenic amines will expand the sample set to permit test set validation with a larger validation set that also include more values in the middle of the represented range (0 to 70 mg/L). Calibrations were also attempted for individual biogenic amines (results not shown). Good results were obtained for putrescine, which in most cases represents the greater part of total biogenic amine concentrations. Results proved promising for cadaverine and histamine, but the current data set does not contain concentrations over a large enough range to represent all wines that will potentially be tested with the models in the future. Again, a larger calibration set should be set up during future work, perhaps using commercial and experimental wines that show a broader range (especially at the higher end) of histamine concentrations.

3.3.5.2 Prediction of biogenic amines produced during malolactic fermentation

Total biogenic amines were predicted over the course of malolactic fermentation, using the PLS model obtained by two-segmented cross validation (**Figures 3.8** and **3.9b**). **Figure 3.10** shows the predicted values for 2006; comparing total biogenic amines produced over time in spontaneous malolactic fermentation (treatment 2), co-inoculated malolactic fermentation with Lalvin VP41 (treatment 4) and conventional malolactic fermentation with Lalvin VP41 (treatment 6). The same trends were observed between treatments 2, 5 and 7 where malolactic fermentations were conducted by Viniflora Oenos.

According to the data obtained by the PLS prediction, the maximum biogenic amine content was reached after approximately 24 days (from the start of alcoholic fermentation) in Pinotage 2006 (**Figure 3.10a**). This implies that the maximum concentration of total biogenic amines reached during malolactic fermentation (approximately 20 mg/L) was almost double the concentrations observed at the two points representing the chemical analysis by HPLC (approximately 10 mg/L). Therefore, conventional chemical analyses performed at the end of key stages may be insufficient to monitor the development of biogenic amines over time in wines for certain study purposes. In this case chemical measurements were performed on day 7 (at the end of alcoholic fermentation) and on day 60 (at the completion of malolactic fermentation). No particular differences could be observed between malolactic fermentation treatments. It seems that the maximum concentration was reached earlier in the co-inoculated treatment (day 17 to 24) and later in the normally inoculated treatment (day 24 to 33), which might indicate a relationship between biogenic amine formation and bacterial growth phase. Various other research groups have indicated that the expression and/or activity of decarboxylase enzymes (mostly referring to histidine decarboxylase) appeared during the early growth phase, peaked during the exponential growth phase and decreased during the stationary phase (Moreno-Arribas *et al.*, 2000; Marcobal *et al.*, 2006; Landete *et al.*, 2006). Decarboxylase activity is expressed at stages when the bacterial cells need the additional energy produced during amino acid transport (Molenaar *et al.,* 1993).

This phenomenon was not observed for Cabernet Sauvignon in 2006, which showed a general increase in total biogenic amines, reaching a similar final concentration in the spontaneous and conventionally inoculated treatments and the lowest final concentration in the co-inoculated treatment (**Figure 3.10b**).

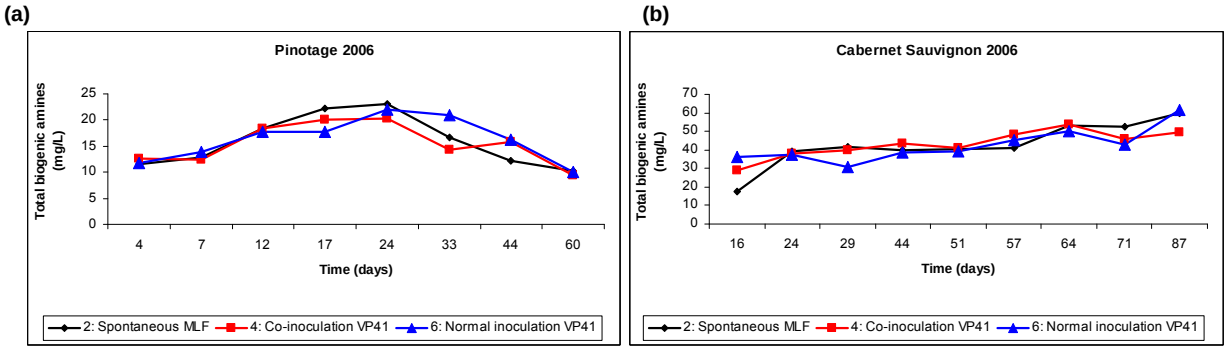

Figure 3.10 The evolution of total biogenic amines during alcoholic and malolactic fermentations with Lalvin VP41 for Pinotage (a) and Cabernet Sauvignon (b) in 2006. Concentrations of biogenic amines were predicted by PLS regression, except for values obtained by HPLC on days 7/60 and days 16/87 representing the end of alcoholic/malolactic fermentations in Pinotage and Cabernet Sauvignon respectively.

For 2007 the chemical measurements were performed by LCMSMS for Pinotage on day 13 (end alcoholic fermentation) and days 35/77 (end malolactic fermentation for treatment 4 and treatments 3 and 6 respectively); and for Cabernet Sauvignon on day 14 (end alcoholic fermentation) and day 66 (end malolactic fermentation). The results are reported for malolactic fermentations performed by Lalvin VP41. The same trends were observed between treatments 2, 5 and 7 where malolactic fermentations were conducted by Viniflora Oenos.

Cabernet Sauvignon (**Figure 3.11b**) followed a trend that was expected from the three control treatments. The treatment spiked with decarboxylase positive lactic acid bacteria (treatment 3) showing the highest values of biogenic amines, increasing over time, while the treatment containing only natural indigenous bacteria (treatment 2) showed lower concentrations, and the negative control (no malolactic fermentation; treatment 1) showed the lowest production of total biogenic amines. In the Pinotage 2007 (**Figure 3.11a**) this trend was not repeated. Treatments inoculated with commercial starter cultures (treatments 4 and 6) showed lower concentrations of total biogenic amines over time than the spiked spontaneous malolactic fermentation treatments (treatment 3) in both cultivars (**Figure 3.11c** and **d**). In Pinotage 2007 it seems as if though co-inoculation may yield the lowest final concentration of biogenic amines. Unfortunately no spectra were obtained of co-inoculation treatments after day 49, since malolactic fermentation had been completed in the previous two weeks and there was no further need to confirm the concentration of malic acid by FT-IR by which spectra could have been generated. As in the case of Pinotage in 2006, maximum values of total biogenic amines were obtained by FT-IR at certain points during malolactic fermentation, at which quantification by analytical methods was not possible due to financial and/or time constraints.

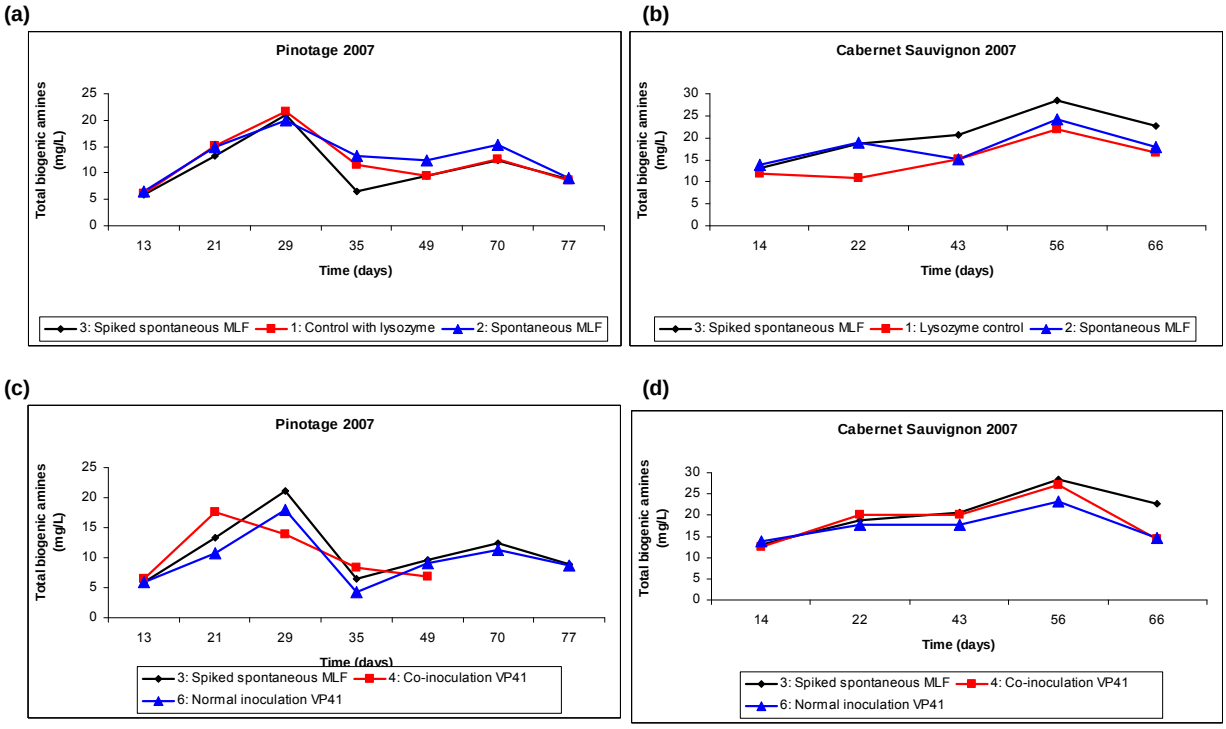

Figure 3.11 The evolution of total biogenic amines during alcoholic and malolactic fermentations with Lalvin VP41 for Pinotage (a and c) and Cabernet Sauvignon (b and d) in 2007. Concentrations of biogenic amines were predicted by PLS regression, except for values obtained by LCMSMS on day 13 (end alcoholic fermentation), day 35 for treatment 4 (end malolactic fermentation) and day 77 for treatments 3 and 6 (end malolactic fermentation) in Pinotage; and days 14/66 for the end of alcoholic/malolactic fermentations in Cabernet Sauvignon.

3.4 CONCLUSION

The concurrent inoculation of commercial strains of wine yeast and lactic acid bacteria in order to induce simultaneous alcoholic and malolactic fermentations can be a means to overcome potential inhibition of lactic acid bacteria in finished wines by toxic effects of high ethanol concentration and a shortage of nutrients. In our study, no antagonistic effects were observed during alcoholic fermentations that may have caused a delay in either alcoholic or malolactic fermentation. Moreover, we have shown that concurrent inoculations do not reduce the quality of volatile flavour compounds and may even increase certain esters associated with fruity character of young wines.

Inoculation with *O. oeni* starter cultures that are unable to produce biogenic amines and that may inhibit the growth and activity of decarboxylase positive indigenous bacteria is a realistic option for the control of biogenic amines in wine. Inoculation with malolactic fermentation starter cultures simultaneous with alcoholic fermentation may restrain these undesirable activities at an earlier stage than with conventional methods, decreasing the levels of biogenic amines in finished wine. In this study it was shown that co-inoculation could reduce the incidence of biogenic amines in wine compared to conventional inoculation protocols. In a recent study by Van der Merwe (2007) it was

shown that co-inoculation of *O. oeni* starter cultures together with alcoholic fermentation was able to significantly reduce the incidence of biogenic amine formation compared to conventional inoculation for malolactic fermentation after alcoholic fermentation. However, the opposite effect was also observed and higher levels of biogenic amines were produced during co-inoculation in some cases. It was also indicated in this work that in some cases the effect of co-inoculation on biogenic amine reduction may only be visible after a period of ageing. The inconsistent results obtained in the present study could be an indication of the variability that exists among indigenous strains of lactic acid bacteria to produce biogenic amines and the large influence of growth conditions, cultivar and vintage on decarboxylase enzyme expression and/or activity.

The frequency of biogenic amine occurrence in aged wines was generally higher in the presence of fermentation lees than in its absence. Biogenic amines were degraded in some cases during ageing (usually more so in the absence of lees). In other cases biogenic amines increased during ageing.

Today, the wine industry is under increasing pressure to eliminate or limit any substances that could reduce the wholesomeness of wine, to meet the demands of the consumers and traders alike of this market-orientated industry. The potential to use FT-IR spectroscopy as a tool to rapidly screen for the presence of elevated levels of biogenic amines as a quality control measure would therefore be a major advantage and was preliminarily developed in this study. Presently, quantification of biogenic amines is reliant on either time-consuming qualitative methods or requires expensive and sophisticated analytical equipment (Bover-Cid & Holzapfel, 1999; Marcobal *et al.*, 2005b; Önal, 2007). Using FT-IR the evolution of biogenic amines during the winemaking process could be followed in a cost-effective way at regular time intervals (weekly, daily or even more regularly), since one sample is scanned within 30 s and requires less than 2 min for sample preparation (filtration). Since no references to FT-IR used for the detection of biogenic amines could be found in the current literature, this work describes a novel method for the detection of biogenic amines in wines.

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4. RESEARCH RESULTS

Evaluating the influence of maceration practices on biogenic amine production by wine lactic acid bacteria

4.1 INTRODUCTION

The maceration of grape skins during process of vinification promotes extraction of grape components such as phenolic compounds, proteins, amino acids and polysaccharides. Grape skin maceration practices commonly applied are cold maceration (cold soaking before alcoholic fermentation), alcoholic fermentation in contact with grape skins (conventional maceration) and extended maceration after alcoholic fermentation. Pectolytic enzymes are added to grape musts to increase the yield of juice, to clarify the must or wine, to extract compounds from the grape skins and to facilitate pressing and filtration. Carbonic maceration and thermovinification are less conventional grape skin extraction methods that can also be applied during winemaking (Boulton *et al.*, 1996).

Biogenic amines are organic nitrogenous compounds of low molecular weight formed by the metabolisms of living organisms (microorganisms, plants and animals) from amino acid precursors. In fermented foodstuffs, such as wine, the non-volatile or biogenic amines (histamine, putrescine, cadaverine, spermine, spermidine, agmatine, tyramine, tryptamine) and phenylethylamine (a volatile amine) are formed mainly by the microbial decarboxylation of the corresponding amino acids (ten Brink *et al.*, 1990; Zee *et al.*, 1983, Halász *et al.*, 1994). In wine, biogenic amines can originate from the grape berries of the vine itself or be produced during the fermentation, ageing or storage processes if conditions persist that favour the growth of decarboxylase positive microorganisms and the activity of the relevant decarboxylase enzymes (Zee *et al.,* 1983; ten Brink *et al.*, 1990; Shalaby, 1996; Leitão *et al.*, 2005). Lactic acid bacteria are the main microorganisms held responsible for biogenic amine production in wine (Cilliers & Van Wyk, 1985; Soufleros *et al.*, 1998; Granchi *et al.*, 2005; Landete *et al.*, 2005a; Marcobal *et al.*, 2006; Alcaide-Hidalgo *et al.*, 2007; Landete *et al.,* 2007a). It is possible that yeasts may directly contribute to biogenic amine production during alcoholic fermentation (Goñi & Ancín Azpilicueta, 2001; Torrea & Ancín, 2002; Garde-Cerdán *et al.*, 2006). However, their contribution seems to be small compared to that made by lactic acid bacteria. Indirectly, yeasts could play an important role in the subsequent production of biogenic amines by decarboxylase positive lactic acid bacteria, by altering the amino acid composition of the fermenting must during alcoholic fermentation. Yeasts can also release amino acids that may serve as precursors of biogenic amines during yeast autolysis during malolactic fermentation and/or ageing periods (Soufleros *et al.*, 1998).

The levels of biogenic amines produced in wine greatly depend on the abundance of amino acid precursors in the medium; since on the whole, biogenic amine production will increase with an increased availability of free amino acids. Amino acid content in the grape must, and subsequently in the wine, may be influenced by vinification methods, grape variety, geographical region, vintage and vine nutrition (Lonvaud-Funel & Joyeux, 1994; Soufleros *et al.*, 1998; Moreno Arribas *et al.*, 2000; Herbert *et al.*, 2005). Grape varieties with higher levels of assimilable amino acids have been found to yield the highest final concentrations of biogenic amines (Herbert *et al.*, 2005). The particular distribution of amino acids between the skin, pulp and seeds within the grape berry is possibly also influenced by vineyard practices, terroir and grape variety. The amino acid content in the skins of Cabernet Sauvignon berries in France was found to represent 53% of the total amino acids in the grape berry, with 40% occurring in the pulp and 7.8% in the seeds (Miele *et al.*, 2000). In a similar study, Australian Cabernet Sauvignon berries were found to contain only 15 to 23% of the total grape amino acids in the grape skins, while approximately 77% was located in the pulp and 8.5% in the seeds (Stines *et al.*, 2000). Thus, it is possible that a large proportion of amino acids may be located in the grape skins and that prolonged contact between must or wine and the skins could increase their extraction into the wine.

Some biogenic amines, such as putrescine and other polyamines, may already be present in itself in grape berries (Halász *et al.,* 1994; Bover-Cid *et al.*, 2006) and therefore their concentration in wine may be influenced more by geographical region and grape variety than by winemaking practices (Landete *et al.*, 2005b).

Regarding the present literature, some authors could make no connection between grape skin maceration practices and the levels of biogenic amines in wines. Soleas *et al.* (1999) found no correlation between the length of skin contact and concentration of biogenic amines in their study. Also, Martín-Álvarez *et al.* (2006) concluded that pectolytic enzymes added to the grapes did not promote biogenic amine accumulation in their wine. However, other authors found the duration of skin maceration to be a very important variable to affect biogenic amine content in wines, and that longer maceration time could favour increased production of biogenic amines (Bauza *et al.*, 1995; Martín-Álvarez *et al.* 2006). An investigation of musts and wines of elderberry fruit also showed that histamine content was increased by pulp treatments such as hot maceration, depectinisation with a commercial pectolytic enzyme preparation, and fermentation on the pulp (Pogorzelski, 1992).

The analysis of biogenic amines is of importance to the wine industry because of the toxicity implications of these compounds to humans, as well as the potential to apply biogenic amines as indicators of spoilage or authenticity (ten Brink *et al.*, 1990; Hajós *et al.*, 2000). In this study we quantified seven oenologically important biogenic amines (histamine, tyramine, putrescine, spermidine, cadaverine, phenylethylamine and tryptamine) and their respective precursor amino acids (histidine, tyrosine, arginine, ornithine, lysine, phenylalanine and tryptophan) in wines spiked with a flora containing decarboxylase enzymes. This was done to ascertain the effect of five different grape

skin maceration treatments on the occurrence and/or production of these amino acids and biogenic amines.

4.2 MATERIALS AND METHODS

4.2.1 VINIFICATION AND MICROBIOLOGY

The experimental procedure was repeated in its entirety with Cabernet Sauvignon and Shiraz grapes; purchased from a commercial wine cellar in the Paarl region, South Africa. After grapes were destemmed and crushed, the skins and free-run juice were separated and homogenised.

Amino acid decarboxylase positive lactic acid bacteria previously isolated from South African brandy base wines (Downing, 2003; Du Plessis *et al.*, 2004) (a combination of equal amounts of *Lactobacillus hilgardii* B74, *Lactobacillus hilgardii* M59 and *Lactobacillus brevis* M58) were inoculated into the grape must before SO_2 addition at 10^6 cells/mL to simulate a natural flora with the potential to produce biogenic amines. The presence of decarboxylase genes in these lactic acid bacteria were verified by polymerase chain reaction (PCR) in **Chapter 3, section 3.3.1**, using the multiplex PCR assay described by Marcobal *et al.* (2005a). Prior to inoculation, the lactic acid bacteria were precultured separately for 36 hours in MRS broth followed by 48 hours in a MRS-based growth medium (pH 4.6) supplemented with 0.1% amino acid precursor (of each of L-ornithine, L-tyrosine and L-histidine) and 0.005% Pyridoxal-5'-Phosphate (Sigma-Aldrich, Germany) to facilitate adaptation of lactic acid bacteria to wine conditions.

Twenty mg/L sulphur dioxide was added to the must. All treatments were repeated in triplicate. Five litres of free-run juice was used for treatment 1 (control treatment), which did not receive any contact with grape skins. Equal amounts of free-run juice plus grape skins were assigned to each of treatments 2 to 5. Treatments 2 to 5 received full skin contact during alcoholic fermentation. Additionally, treatment 5 underwent extended grape skin maceration after alcoholic fermentation for two weeks at 15°C. Treatments 2 and 3 were subjected to cold maceration with and without the addition of a commercial maceration enzyme respectively (3 g/hL Rapidase® EX-Color, DSM Food Specialties, Delft, The Netherlands) for two days at 4°C before the commencement of alcoholic fermentation. Treatment 4 thus consisted of normal skin maceration during alcoholic fermentation without any enzyme addition or maceration prior to or after alcoholic fermentation. An abbreviated description of the treatments is given in **Table 4.1**. Daily punch-downs proceeded throughout alcoholic fermentation and during all grape skin maceration practices in all relevant treatments. Alcoholic fermentation was carried out in all treatments by *Saccharomyces cerevisiae* strain NT202 (Anchor Yeast, Cape Town, South Africa) at room temperature (20 to 25°C). 0.2 g/L of diammonium phosphate was added to all treatments as yeast nitrogen supplement on day three of alcoholic fermentation. Sugar density was measured at least once daily with a Brix hydrometer to

monitor the course of alcoholic fermentation. Following alcoholic fermentation (or extended maceration in the case of treatment 5), wines were pressed with a hydrolic basket press. Malolactic fermentation (MLF) was allowed to proceed spontaneously in 4.5 L glass bottles in all treatments, which were sealed with airlocks. Malic acid, lactic acid and selected other wine chemical compounds were monitored by Fourier Transform Infrared Spectroscopy (WineScan FT120, FOSS Analytical, Denmark). Samples for analyses of wine components (including biogenic amines and amino acids) and for microbial enumeration were drawn in sterile sample vials of the grape must (before inoculation of the three decarboxylase positive *Lactobacillus* strains), at the end of cold maceration, after alcoholic fermentation, after extended maceration and after the completion of malolactic fermentation (<0.3 g/L malic acid) from the relevant treatments. Nitrogen gas was blown into the 4.5 L glass bottles each time after samples were taken.

Table 4.1 Description of the five different maceration treatments performed in triplicate with Cabernet Sauvignon and Shiraz. The must of all treatments were spiked with decarboxylase positive lactobacilli and all treatments underwent spontaneous malolactic fermentation.

Treatment number	No skin contact	CM with enzyme (2 days, 4°C)	CM without enzyme (2 days, 4°C)	AF in contact with grape skins	EM after AF (2 weeks, 15°C)
1	X				
2		X		X	
3			X	X	
4				X	
5				X	X

CM: cold maceration; AF: alcoholic fermentation; EM: extended maceration

4.2.2 ENUMERATION OF MICOORGANISMS

The wine microbiological status was monitored in the must and at the completion of the alcoholic and malolactic fermentations by plate counts of colonies formed (cfu/mL) on selective agar media, followed by identification of cell morphology under a light microscope. One hundred µL of must or wine, diluted in water in a dilution series, was plated on selective media. All media were purchased from Biolab, Merck, Wadeville, Gauteng (South Africa) unless otherwise indicated. All plates were incubated at 30°C for 5 to 10 days depending on the growth rate of the microorganism.

4.2.2.1 Bacterial species

De Man, Rogosa and Sharpe (MRS) agar was used for the enumeration of *Lactobacillus, Pediococcus* and *Leuconostoc* species (De Man *et al.*, 1960). *Oenococcus oeni* was enumerated on MRS supplemented with 20% v/v apple juice at pH 5.2. Fifty mg/L pimaricin (Actistab, Gistbrocades, Anchor Bio-Technologies, South Africa) and 25 mg/L kanamycin sulphate (Roche, South Africa) dissolved in 1 mL of water were added to each of the media to inhibit the growth of yeast and acetic acid bacteria respectively. The MRS plates supplemented with apple juice were incubated anaerobically.

MRS agar supplemented with 2% v/v ethanol was used to isolate acetic acid bacteria in wine. GYC agar (5% w/v glucose, 1% w/v yeast extract, 3% v/v $CaCO_3$ and 2% w/v agar) was used to isolate acetic acid bacteria from grape must. Pimaricin (50 mg/L Actistab) and chloramphenicol (Sigma-Aldrich, Germany) (30 mg/L dissolved in 1 mL of 100% v/v ethanol) were added to both media to inhibit the growth of yeast and lactic acid bacteria respectively.

4.2.2.2 Yeast species

Lysine agar medium (lysine with 0.01% v/v potassium lactate) was used to establish non-*Saccharomyces* yeasts counts and yeast peptone dextrose (YPD) agar (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) to establish the total yeast population. Acetic acid bacterial growth was inhibited by the addition of kanamycin sulphate (25 mg/L) and lactic acid bacterial growth by chloramphenicol (30 mg/L).

WL nutrient agar for the enumeration of *Brettanomyces* species was purchased from Merck, Darmstadt, Germany. The pH of this medium was adjusted to 5.0 and 100 mg/L *p*-coumaric acid (a precursor for volatile phenol formation) was added to increase the selectivity of this medium. In addition to kanamycin sulphate and chloramphenicol, 50 mg/L cycloheximide (Sigma-Aldrich, Germany) (dissolved in 1 mL of 50% v/v ethanol) was added to inhibit the growth of yeast species other than *Brettanomyces*.

4.2.3 SPECTROPHOTOMETRIC ANALYSES OF PHENOLIC COMPOUNDS

Colour density (the sum of the absorbance units at 420, 520 and 620 nm), total red pigments (the absorbance units of all pigments measured at a low pH) and total phenolics (the absorbance units as measured at 280 nm at a low pH) were determined for all treatments of the grape must, at the end of alcoholic fermentation and at the completion of malolactic fermentation, as well as at the end of cold maceration and after extended maceration for the relevant treatments (Iland *et al*., 2000).

4.2.4 AMINO ACID ANALYSIS

Amino acids were analysed using the EZ: Faast liquid chromatography (LC) method with the EZ: Faast column as described in the EZ: Faast user's guide. Prior to analysis, wine samples were centrifuged at 13 000 *g* for 10 minutes. 100 µL of the supernatant was freeze dried after which it was subjected to the EZ: Faast protocol. Labelled homoarginine and methionine-D3, as part of the EZ: Faast kit, were included as internal standards. Amino acids were subsequently quantified by LCMSMS.

4.2.5 BIOGENIC AMINE ANALYSIS

4.2.5.1 Sample preparation

Samples were filtered through a 0.45 µm syringe filter and were diluted three times in an aqueous solution containing 1.33 µg/mL heptylamine (Fluka), which was used as the internal standard.

4.2.5.2 Liquid chromatography and mass spectrometry methods

A Waters Alliance High Performance Liquid chromatograph (HPLC) was used. Separation was achieved on a Waters Atlantis dC18, 2.1x150 mm, 3.5 µm column with the gradient indicated in **Table 4.2** using 0.4% trifluoro acetic acid as solvent A and methanol as solvent B at a flow rate of 0.2 mL/min at a column temperature of 30°C.

Table 4.2 HPLC gradient using 0.4% trifluoro acetic acid as solvent A and methanol as solvent B.

Time (min)	Solvent A	Solvent B	Curve
0	100	0	1
4	100	0	6
4.1	40	60	6
8	5	95	6
10	5	95	6
10.01	100	0	6
17.0	100	0	6

A Waters Quattro Micro triple quadrupole mass spectrometer (MS) was used with electrospray ionisation in the positive mode. The instrument settings were optimised for the best sensitivity. The desolvation temperature was 380°C, the source temperature was set at 120°C, the cone voltage at 18kV and the capillary voltage at 3.5kV. Collision energies and Multiple Reaction Monitoring (MRM) transitions for the different ions and the retention times are given in **Table 4.3**. The limit of quantitation was determined as

the minimum concentration (before dilution) that yields a percentage relative standard deviation of better than 20%.

Table 4.3 MRM functions, retention times and mass spectrometry (MS) parameters for each of the biogenic amines.

	Molecular weight	MRM	Cone voltage	Collision Energy	Retention time	Limit of quantitation
Biogenic amine						(ug/ml)
Tyramine	137	138>121	18	10	10.78	0.02
Agmatine	130	131>72	18	20	3.89	0.05
Phenylethylamine	121	122>105	18	10	11.62	0.001
Histamine	111	112>95	18	15	3.07	0.005
Cadaverine	102	103>86	18	10	2.96	0.05
Putrescine	88	89>72	18	15	2.74	0.1
Spermidine	145	146>72	18	15	3.02	0.5
Tryptamine	160	161>144	18	5	11.76	0.005
Spermine	202	203>129	18	15	3.53	1.5
Internal standard	115	116>58	18	15	12.46	N/A

MRM: Multiple Reaction Monitoring

4.2.6 STATISTICAL ANALYSIS

Repeated measures analysis of variance (ANOVA) was performed on the data to evaluate main effects for fermentation treatment and phase, as well as their interactive effects. The Tukey HSD test was used to determine the significant differences between group means Pearson and Spearman rank correlation coefficients were used to determine the relationship between biogenic amines and amino acids. A significance level of 5% was used in all cases. Statistical analyses were performed using STATISTICA (StatSoft, Inc. (2006)., version 7.1. www.statsoft.com).

4.3 RESULTS AND DISCUSSION

4.3.1 LCMSMS METHOD

This work gives a preliminary report on a quick LCMSMS method (17 minutes) that does not require any sample preparation apart from dilution and that does not use complex buffer systems that contaminate the ion source of the mass spectrometer.

4.3.2 ENUMERATION OF MICOORGANISMS

The microbial composition of Cabernet Sauvignon and Shiraz during the course of the experiments can be seen in **Tables 4.4** and **4.5** respectively. In both Cabernet Sauvignon and Shiraz the wine microflora was presumably dominated by the inoculated *Saccharomyces cerevisiae* strain NT202 yeast at the end of alcoholic fermentation. However, a large proportion of non-*Saccharomyces* yeast strains also survived at the end of alcoholic fermentation (up to eight different colony morphologies could be detected on lysine agar plates). Cold maceration (treatments 2 and 3) was carried out before inoculation with the commercial yeast starter culture. Even though low temperature can delay microbial growth, this period may have influenced the higher survival rate of some non-*Saccharomyces* species, present at higher cell densities at the end of alcoholic fermentation in treatments 2 and 3 of both cultivars. Certain *Kloeckera* and *Candida* species have been known to be able to grow at low temperatures (Heard & Fleet, 1988). No surviving acetic acid bacteria could be detected after wine fermentations and no culturable cells of *Brettanomyces* could be detected at any stage of cell enumeration. Indigenous *Oenococcus oeni* strains were already detectable in the must, survived through alcoholic fermentation and presumably completed the malolactic fermentation. Indigenous *Lactobacillus* species were present in the must. The three decarboxylase positive *Lactobacillus* strains were inoculated at 10^6 cells/mL into the grape must shortly after the must sample was taken for cell enumeration. Lactobacilli could be detected at 10^6 cells/mL after three days (data not shown); indicating their viability in the wines, and were still present at high cell numbers at the end of alcoholic fermentation. Even though no more *Lactobacillus* cells could be detected at the end of malolactic fermentation in Cabernet Sauvignon wines, it is possible that these species had still contributed to compounds produced during malolactic fermentation in the wine (such as biogenic amines). The lactobacilli survived to the end of malolactic fermentation in Shiraz wines, presumably due to unknown but more optimal culture conditions. The average concentration of ethanol present in the finished wines (at the end of malolactic fermentation) was high; at 14.98 % v/v for Cabernet Sauvignon and 15.36 % v/v for Shiraz. The average pH reached almost 4.6 in Shiraz wines and 4.0 in Cabernet Sauvignon. The apparent more unfavourable environmental conditions in Shiraz do not aid to explain why lactobacilli survived in Shiraz but not in Cabernet Sauvignon. Significant differences were found between South African Shiraz and Cabernet Sauvignon wines in terms of their phenolic composition, in a study done by Rossouw & Marais (2004), during which 76 samples of Shiraz and 84 samples of Cabernet Sauvignon were evaluated for 39 individual phenols and 2 polymeric phenolic groups. It is reported in the works of Vivas *et al*. (1997) and García-Ruiz *et al*. (2007) that to date gallic acid, catechin, caftaric acid, coutaric acid, ferulic acid, *p*-coumaric acid and free anthocyanins have been found able to stimulate the growth of lactic acid bacteria. According to the work of Rossouw & Marais (2004) coutaric acid, *p*-coumaric acid, some free anthocyanins and some unknown phenolic compounds were significantly higher in Shiraz wines than in Cabernet Sauvignon wines.

It could be that one or more of these phenolic compounds could have led to significant differences in the growth and/or decarboxylase expression and activity of lactic acid bacteria in the two cultivars used in this study.

Table 4.4 Cell growth (cfu/mL) of wine microorganisms as recorded at three stages of the winemaking process in Cabernet Sauvignon. Each enumeration represents the average of triplicate treatments.

	Saccharomyces	non-*Saccharomyces*	*Brettanomyces*	Acetic acid bacteria	*Oenococcus*	*Lactobacillus*
Grape must*						
All treatments	nd	1.20×10^5	nd	8.00×10^2	1.00×10^4	3.50×10^3
End AF						
Treatment 1**	1.80×10^8	1.20×10^4	nd	nd	7.53×10^4	4.73×10^4
Treatment 2	1.37×10^8	3.00×10^5	nd	nd	7.90×10^4	3.67×10^4
Treatment 3	1.38×10^8	1.38×10^5	nd	nd	2.38×10^5	2.00×10^5
Treatment 4	1.80×10^8	3.90×10^3	nd	nd	5.47×10^4	4.20×10^4
Treatment 5	1.30×10^8	2.12×10^4	nd	nd	6.53×10^4	4.50×10^4
End MLF						
Treatment 1	nd	nd	nd	nd	6.07×10^6	nd
Treatment 2	nd	nd	nd	nd	6.60×10^6	nd
Treatment 3	nd	nd	nd	nd	7.90×10^6	nd
Treatment 4	nd	nd	nd	nd	1.50×10^7	nd
Treatment 5	nd	nd	nd	nd	3.23×10^7	nd

*before must was spiked with decarboxylase positive lactobacilli

Refer to **Table 4.1 for a description of the treatments

nd: not detected; AF: alcoholic fermentation; MLF: malolactic fermentation

Table 4.5 Cell growth (cfu/mL) of wine microorganisms as recorded at three stages of the winemaking process in Shiraz. Each enumeration represents the average of triplicate treatments.

	Saccharomyces	non-*Saccharomyces*	*Brettanomyces*	Acetic acid bacteria	*Oenococcus*	*Lactobacillus*
Grape must*						
All treatments	nd	1.60×10^6	nd	1.20×10^4	6.00×10^3	1.10×10^4
End AF						
Treatment 1**	6.23×10^7	3.13×10^3	nd	nd	7.77×10^5	4.63×10^4
Treatment 2	1.77×10^8	1.91×10^4	nd	nd	7.33×10^5	4.10×10^4
Treatment 3	2.23×10^8	2.47×10^4	nd	nd	9.50×10^5	5.20×10^4
Treatment 4	1.57×10^8	5.90×10^2	nd	nd	4.63×10^5	6.73×10^3
Treatment 5	1.93×10^8	1.50×10^3	nd	nd	1.09×10^6	4.80×10^3
End MLF						
Treatment 1	nd	nd	nd	nd	7.40×10^6	4.48×10^3
Treatment 2	nd	nd	nd	nd	4.17×10^7	6.10×10^2
Treatment 3	nd	nd	nd	nd	3.80×10^7	7.00×10^2
Treatment 4	nd	nd	nd	nd	1.47×10^7	9.37×10^2
Treatment 5	nd	nd	nd	nd	1.03×10^7	1.10×10^3

*before must was spiked with decarboxylase positive lactobacilli

Refer to **Table 4.1 for a description of the treatments

nd: not detected; AF: alcoholic fermentation; MLF: malolactic fermentation

4.3.3 SPECTROPHOTOMETRIC ANALYSES OF PHENOLIC COMPOUNDS

Variation between treatments followed a similar trend for colour density, total phenolics and total red pigments in the Cabernet Sauvignon and Shiraz wines (**Table 4.6**). Treatment 1 had significantly lower means of colour and phenolic measurements than all other treatments at the end of both fermentation phases in both cultivars. This can be expected due to the absence of grape skins and seeds and related phenolic compounds (such as anthocyanins and tannins) in this treatment. The presence of low concentrations of total phenolics were due to cinnamic acid derivates, which are located in the juice (Boulton *et al.*, 1996). At the end of alcoholic fermentation (before the application of extended maceration in treatment 5) treatments 4 and 5 showed lower means for most phenolic measurements than treatments 2 and 3 to which an additional maceration practice (cold maceration) had already been applied. For Shiraz wines the phenolic measurements of treatment 2 (cold maceration with a maceration enzyme) also showed slightly higher means than treatments 3, 4 and 5 at the end of alcoholic fermentation. At the end of malolactic fermentation similar trends could be observed but with lower mean values. These results shows that the different treatments did have an effect on the extraction of different compounds, including phenolics, from the skins. A decrease in the colour density after malolactic fermnation is normally due to an increase in the pH of the wine, which leads to a larger proportion of anthocyanins being in the colourless carbinol pseudobase form (Ribéreau-Gayon *et al*., 2006). Similar decreases in total red pigments and total phenolics after malolactic fermentation were also observed by Keulder (2006).

Measurement of phenolic compounds provided an indication on the success of extraction from grape skins and seeds that may have taken place as a result of the different maceration treatments. For the most part in this study the observed differences were small (trends rather than statistically significant differences) and therefore it could possible be expected that only small differences in amino acids or biogenic amines may result from different maceration practices. However, even when the differences between treatments are small for individual compounds, a few mg/L of each biogenic amine can add up and contribute to a high final total biogenic amine content in the wine due to a particular maceration treatment. Treatment 1 is the exception and always displayed results that were significantly different from the other treatments.

Table 4.6 Means and standard errors of phenolic measurements of the five grape skin maceration treatments applied to Cabernet Sauvignon and Shiraz. Refer to the text for a description of treatments.

Phase / Treatment	(a) Cabernet Sauvignon			(b) Shiraz		
	Colour density ($A_{420}+A_{520}+A_{620}$)	Total phenolics (A_{280})	Total red pigments (A_{520})	Colour density ($A_{420}+A_{520}+A_{620}$)	Total phenolics (A_{280})	Total red pigments (A_{520})
End AF*						
Treatment 1**	0.56 ± 0.06^{e}	6.20 ± 0.54^{c}	1.21 ± 0.18^{d}	0.89 ± 0.07^{d}	8.69 ± 0.28^{b}	2.36 ± 0.11^{b}
Treatment 2	15.03 ± 0.46^{d}	51.64 ± 0.37^{ab}	32.94 ± 0.27^{bc}	9.38 ± 0.17^{c}	43.06 ± 2.62^{a}	29.53 ± 1.75^{a}
Treatment 3	15.19 ± 0.55^{d}	52.09 ± 1.12^{b}	33.90 ± 0.62^{c}	8.78 ± 0.10^{abc}	41.82 ± 1.37^{a}	28.86 ± 0.92^{a}
Treatment 4	14.09 ± 0.31^{cd}	49.18 ± 1.00^{ab}	32.53 ± 0.58^{bc}	8.77 ± 0.53^{abc}	38.21 ± 4.21^{a}	26.04 ± 2.82^{a}
Treatment 5	13.50 ± 0.11^{bcd}	47.65 ± 2.45^{ab}	28.17 ± 1.64^{abc}	9.29 ± 0.32^{bc}	43.02 ± 0.59^{a}	28.33 ± 0.42^{a}
End MLF**						
Treatment 1	0.38 ± 0.04^{e}	6.09 ± 0.23^{c}	0.65 ± 0.02^{d}	0.88 ± 0.10^{d}	10.98 ± 0.61^{b}	1.93 ± 0.22^{b}
Treatment 2	12.02 ± 0.55^{abc}	37.07 ± 8.21^{a}	20.25 ± 4.45^{a}	8.15 ± 0.35^{abc}	41.92 ± 3.06^{a}	25.98 ± 1.84^{a}
Treatment 3	12.32 ± 0.78^{abc}	49.42 ± 2.19^{ab}	27.36 ± 1.27^{abc}	7.94 ± 0.16^{a}	41.98 ± 2.67^{a}	26.91 ± 2.41^{a}
Treatment 4	11.38 ± 0.58^{ab}	44.79 ± 1.97^{ab}	25.29 ± 0.90^{ab}	8.22 ± 0.06^{abc}	41.32 ± 2.56^{a}	25.51 ± 1.47^{a}
Treatment 5	10.45 ± 0.09^{a}	45.62 ± 1.28^{ab}	20.49 ± 0.57^{a}	8.11 ± 0.08^{ab}	40.81 ± 2.34^{a}	23.90 ± 1.27^{a}

Different superscripts in each column indicate significant differences between the mean values of treatments and phases at $P< 0.05$.

* AF (alcoholic fermentation); ** MLF (malolactic fermentation)

Refer to **Table 4.1 for a description of the treatments

4.3.4 AMINO ACID AND BIOGENIC AMINE COMPOSITION OF TREATMENTS

Amino acids are utilised as a source of nitrogen for growth and metabolism to different extents during wine fermentations by yeasts and lactic acid bacteria. Therefore, the concentrations of amino acids in general, and potential precursors of biogenic amines in particular, may change considerably during wine fermentations. Changes in amino acid content may be due to the actions of yeasts, including the secretion of excess amino acids by yeasts and the release of amino acids by yeast autolysis. Amino acid composition and concentration can also be changed by lactic acid bacteria. As a result of maceration practices amino acids can be released from grape skins or extracted from grape skin derived components (or from yeast autolysates) by the action of proteolytic and peptolytic enzymes of lactic acid bacteria. Manca de Nadra *et al.* (1999) found that lactic acid bacteria exhibit exocellular protease activity in model solutions, while Leitão *et al.* (2000) showed that some *O. oeni* strains exhibit proteolytic activity wine media. Commercial pectolytic enzyme preparations may contain proteolytic activity which can lead to the release of amino acids from peptides and proteins (the exact composition of Rapidase® EX-Color used in this study could not be obtained).

Table 4.7 (Cabernet Sauvignon) and **Table 4.8** (Shiraz) present the concentrations of amino acids and biogenic amines in the musts and in the wines of the respective

treatments at different winemaking stages. Amino acids that serve as the precursors for the biogenic amines analysed in this study are highlighted in the Tables. These results represent the general trends and therefore the averages of two (amino acids) or three (biogenic amines) replicates of each treatment are reported (the standard deviations or standard errors are not shown). Selected results from **Tables 4.7** and **4.8** are also presented graphically with the relevant statistical analyses in **Figure 4.2** (Cabernet Sauvignon) and **Figure 4.2** (Shiraz).

Amino acid concentration differed significantly ($p<0.05$) between the fermentation phases (end alcoholic fermentation and end malolactic fermentation) for both cultivars. In both Cabernet Sauvignon and Shiraz the concentration of precursor amino acids for biogenic amine formation (tyrosine, ornithine, arginine, histidine, lysine, phenylalanine, tryptophan) decreased from the must to the end of alcoholic fermentation and increased again towards the end of malolactic fermentation in all treatments, but particularly in the control (treatment 1). This increase could be attributed to (early) autolysis of yeast cells and the release of free amino acids in the wine by the protease activity of lactic acid bacteria, as was also described by Alcaide-Hidalgo *et al.* (2007) who found that amino acids did not change significantly during malolactic fermentation in their study due to this reason. On the contrary, it has been found in at least one other study (Soufleros *et al.*, 1998) that amino acid concentrations decline significantly during malolactic fermentation.

All amino acid precursors in both cultivars showed an increase during cold maceration with the exceptions of phenylethylamine and lysine in Shiraz and lysine in Cabernet Sauvignon, while all amino acids in both cultivars increased when extended maceration was applied after fermentation.

Biogenic amine concentration also differed significantly ($p<0.05$) between the phases, showing an increase from the end of alcoholic fermentation to the end of malolactic fermentation in at least one treatment for both cultivars with the exceptions of tyramine in Cabernet Sauvignon and putrescine, cadaverine and tryptamine in Shiraz. The increase of biogenic amines during malolactic fermentation has been recorded by many other research groups, among them Soufleros *et al.* (1998) and Marcobal *et al.* (2006). The increase of biogenic amines during malolactic fermentation may indicate their formation by decarboxylase positive lactic acid bacteria (as were introduced into the wine in this study).

Cadaverine and tryptamine showed a decrease in some treatments in Shiraz from the end of alcoholic fermentation to the end of malolactic fermentation. The decrease of some biogenic amines could possibly be explained by two mechanisms. Firstly, yeasts are able to use biogenic amines as a source of nitrogen (Goñi & Ancín Azpilicueta, 2001). Secondly, biogenic amines can be broken down by oxidative deamination by amine oxidases to produce an aldehyde, hydrogen peroxide and ammonia (Gardini *et al.*, 2005). Amine oxidases have been described for higher organisms (such as humans) but also for bacteria (Leuschner *et al.*, 1998 and references therein). For example, tyramine oxidase has been found in wines in a previous study and it is suggested that

Table 4.7 Concentrations (mg/L) of amino acids and biogenic amines analysed in the must and wines of the five maceration treatments at the end of different winemaking stages in Cabernet Sauvignon. The concentrations represent the averages of duplicate treatment repeats for amino acids, and triplicate treatment repeats for biogenic amines.

Amino acid and biogenic amine concentrations (mg/L)														
Phase	**Grape must**	**End cold maceration**		**End alcoholic fermentation**					**End extended maceration**	**End malolactic fermentation**				
Treatment*	**All**	**2**	**3**	**1**	**2**	**3**	**4**	**5**	**5**	**1**	**2**	**3**	**4**	**5**
Alanine	55.20	115.70	93.65	6.56	1.92	2.03	8.14	4.51	21.95	22.66	10.81	10.51	18.04	16.55
Threonine	4.60	9.40	5.50	0.60	0.15	0.14	0.45	0.27	0.80	4.72	0.83	1.60	2.40	1.93
Serine	17.90	30.85	27.40	1.15	0.25	0.30	0.76	0.48	1.90	7.49	1.75	1.77	2.82	2.78
Glutamine	23.50	36.60	39.25	9.87	3.76	3.68	5.87	5.42	8.05	7.72	4.17	3.32	3.95	6.48
Arginine	10.90	30.30	25.70	2.42	1.50	1.42	2.17	1.87	4.30	9.33	4.79	4.57	0.74	0.61
Tryptophan	1.80	4.10	4.00	0.10	0.03	0.03	0.32	0.22	1.80	1.95	0.47	0.39	0.41	1.22
Glutamic acid	33.80	33.55	32.70	12.00	10.34	11.62	16.06	7.25	8.85	11.08	4.88	4.17	7.30	7.93
Valine	36.00	68.55	66.65	0.32	0.09	0.10	0.16	0.12	2.75	5.87	1.46	1.16	1.63	2.01
Histidine	12.50	23.55	22.65	1.56	0.74	0.76	1.51	1.56	7.40	8.17	4.15	3.90	4.98	10.00
Aspartic acid	17.90	24.70	24.90	1.98	1.03	1.08	0.78	0.42	2.25	14.86	3.87	4.08	5.03	4.66
Lysine	2.30	0.70	0.45	1.73	1.44	1.49	1.56	1.46	3.45	16.16	4.72	5.17	5.96	5.39
Proline	396.40	1068.90	753.80	182.68	171.33	189.23	213.95	209.64	590.25	186.20	190.79	185.91	218.37	195.06
Methionine	0.30	0.70	0.50	0.20	0.13	0.14	0.16	0.16	0.35	1.57	0.16	0.17	0.17	0.15
Tyrosine	9.70	19.20	19.00	0.79	0.34	0.35	0.69	0.50	2.10	8.96	2.72	2.38	3.01	2.93
Cystein	1.60	1.80	2.00	0.96	0.16	0.14	0.24	0.24	1.20	0.80	0.12	0.16	0.28	0.20
Isoleucine	20.30	30.30	33.05	0.39	0.27	0.27	0.32	0.28	1.00	4.84	1.07	1.11	1.42	1.47
Phenylalanine	9.00	17.70	15.55	0.30	0.06	0.07	0.20	0.10	1.35	8.56	1.81	1.79	2.30	2.35
Leucine	21.20	36.10	33.15	0.39	0.09	0.10	0.26	0.14	2.55	16.69	3.15	3.01	4.00	4.44
Asparagine	2.70	3.55	5.20	6.74	1.75	2.48	6.40	4.34	15.10	20.23	6.95	6.25	10.87	14.17
Glycine	1.00	2.70	1.50	1.79	0.08	0.18	1.79	0.61	2.10	9.13	2.96	5.42	7.39	4.56
Ornithine	0.20	1.00	0.90	0.54	0.14	0.15	0.49	0.24	1.55	12.61	6.10	6.08	10.26	13.39
Total	**678.80**	**1559.95**	**1207.50**	**233.03**	**195.55**	**215.73**	**262.23**	**239.78**	**681.05**	**379.55**	**257.68**	**252.88**	**311.27**	**298.24**
Histamine	0.0193	0.0443	0.0390	0.0841	0.0162	0.0336	0.1013	0.1028	0.0607	0.1251	0.0328	0.0468	0.1351	0.2182
Putrescine	3.6900	9.1207	8.2223	15.0170	2.5341	2.6250	18.9577	19.1852	15.4462	19.7185	2.9210	3.5102	24.3422	23.9069
Spermidine	0.5300	1.8286	1.5970	1.0183	0.6963	0.9924	2.1020	0.7707	1.6537	5.6633	5.9075	6.1024	7.4736	6.8033
Tryptamine	nd	0.0046	0.0036	0.0030	0.0145	0.0156	0.0143	0.0146	0.0171	0.0091	0.0212	0.0211	0.0153	0.0198
Phenylethylamine	0.0124	0.0146	0.0144	nd	0.0095	0.0089	0.0027	0.0024	0.0061	nd	0.0129	0.0128	0.0042	0.0042
Tyramine	0.0033	0.0053	0.0037	0.0002	0.0002	0.0025	0.0002	0.0002	0.0028	0.0013	0.0018	0.0051	0.0013	0.1023
Cadaverine	nd	0.0833	0.0836	0.1266	0.4255	0.4954	0.6251	0.9354	0.4613	0.1647	0.5972	0.5554	0.8124	1.4213
Total	**4.2550**	**11.1014**	**9.9636**	**16.2492**	**3.6962**	**4.1732**	**21.8033**	**21.0113**	**17.6479**	**25.6821**	**9.4945**	**10.2537**	**32.7840**	**32.4760**

nd: not detected *Refer to **Table** for a description of the treatments

Table 4.8 Concentrations (mg/L) of amino acids and biogenic amines analysed in the must and wines of the five maceration treatments at the end of different winemaking stages in Shiraz. The concentrations represent the averages of duplicate treatment repeats for amino acids, and triplicate treatment repeats for biogenic amines.

Amino acid and biogenic amine concentrations (mg/L)														
Phase	**Grape must**	**End cold maceration**		**End alcoholic fermentation**					**End extended maceration**	**End malolactic fermentation**				
Treatment*	**All**	**2**	**3**	**1**	**2**	**3**	**4**	**5**	**5**	**1**	**2**	**3**	**4**	**5**
Alanine	90.10	58.63	57.17	5.39	2.48	1.88	8.63	6.44	13.57	40.59	28.07	24.57	30.68	34.13
Threonine	31.80	78.89	50.92	1.19	0.44	0.52	1.49	0.92	2.42	18.44	8.69	7.94	10.75	11.30
Serine	53.50	70.50	71.87	1.91	0.60	0.45	1.87	1.76	3.46	25.71	10.95	9.99	13.86	14.63
Glutamine	276.00	902.28	1025.56	7.00	3.86	3.11	5.79	6.10	7.07	22.81	7.43	6.90	12.86	15.77
Arginine	363.50	573.50	592.01	2.64	2.29	2.21	5.87	4.36	9.72	42.14	23.25	22.55	28.02	32.03
Tryptophan	19.00	33.41	33.79	0.53	0.19	0.12	0.51	1.47	2.20	8.18	2.78	2.06	3.29	5.88
Glutamic acid	97.60	77.58	72.35	10.93	3.40	3.58	8.23	8.60	13.30	49.00	30.84	34.18	49.27	28.31
Valine	152.20	49.86	54.44	1.04	0.35	0.45	1.24	1.22	2.17	17.23	6.81	5.93	8.17	9.95
Histidine	46.30	74.02	81.34	2.20	1.06	0.66	3.57	3.64	4.90	4.51	0.91	0.58	0.44	1.38
Aspartic acid	80.70	62.39	64.31	6.87	1.21	1.08	5.76	3.29	8.50	62.27	29.49	25.82	40.76	44.41
Lysine	10.40	7.62	6.96	3.60	1.71	1.46	4.18	2.99	5.19	41.52	20.16	19.88	21.64	21.88
Proline	821.60	468.49	498.71	207.82	280.04	268.87	270.38	252.59	324.07	376.95	424.90	352.79	360.02	383.97
Methionine	1.00	0.96	0.83	0.30	0.25	0.20	0.26	0.22	0.26	4.58	0.77	0.33	0.74	1.02
Tyrosine	24.90	36.11	35.76	2.14	0.63	0.46	2.10	1.62	2.92	17.68	7.24	7.01	7.59	11.06
Cystein	3.20	2.88	2.58	2.22	1.62	1.76	2.20	2.14	1.76	2.80	1.30	1.54	3.04	2.34
Isoleucine	89.10	43.60	44.06	0.84	0.36	0.36	0.79	0.66	1.46	18.38	5.47	5.20	8.81	9.48
Phenylalanine	24.70	16.99	16.68	1.19	0.25	0.27	1.18	0.90	2.73	28.75	10.67	9.11	13.25	15.53
Leucine	93.20	59.16	52.58	2.30	0.43	0.39	2.08	1.71	4.49	48.34	18.97	16.73	23.23	26.83
Asparagine	5.60	6.44	2.85	3.19	4.66	1.65	5.85	4.95	7.92	1.57	3.51	2.83	3.36	4.10
Glycine	3.90	8.77	5.61	3.32	1.37	1.20	5.27	2.99	8.69	30.72	16.13	13.45	19.59	21.80
Ornithine	4.10	7.35	7.94	0.53	0.27	0.18	1.78	1.77	2.69	5.55	3.07	3.16	4.48	5.82
Total	**2292.40**	**2639.43**	**2778.28**	**267.09**	**307.42**	**290.82**	**339.00**	**310.28**	**429.45**	**867.69**	**661.34**	**572.52**	**663.80**	**701.56**
Histamine	nd	0.0693	0.0888	0.0801	0.0630	0.0778	0.0656	0.0668	0.0666	10.7220	4.7799	5.4219	5.1566	6.2016
Putrescine	1.9900	18.5825	14.9269	6.9063	8.2166	9.6078	9.5274	12.6415	14.9078	7.2726	6.2896	7.1485	9.9742	9.7171
Spermidine	nd	3.8989	3.6978	1.0379	0.6354	0.4887	1.6344	0.9211	2.1123	5.5259	4.4391	4.7413	3.4062	3.5901
Tryptamine	0.0010	0.0113	0.0100	0.0013	0.0041	0.0064	0.0029	0.0013	0.0035	nd	0.0006	0.0023	nd	nd
Phenylethylamine	0.0102	0.0554	0.0763	0.0088	0.0252	0.0298	0.0178	0.0224	0.0255	0.0229	0.0181	0.0203	0.0140	0.0190
Tyramine	nd	nd	0.0057	0.0014	0.0034	0.0067	0.0015	0.0033	0.0041	3.2875	0.7472	0.5003	1.3230	0.9412
Cadaverine	nd	0.6180	0.7312	0.1293	0.2144	0.2950	0.2426	0.2214	0.2712	0.1670	0.1904	0.2577	0.2159	0.2055
Total	**2.0012**	**23.2354**	**19.5365**	**8.1661**	**9.1620**	**10.5121**	**11.4923**	**13.8779**	**17.3910**	**26.9979**	**16.4648**	**18.0923**	**20.0899**	**20.6745**

nd: not detected

*Refer to **Table** for a description of the treatments

its presence could prevent the accumulation of tyramine in wine by breaking it down (Alcaide-Hidalgo *et al.*, 2007).

All biogenic amines increased to different extents during cold maceration in both cultivars. Most biogenic amines, with the exception of histamine, also showed an increasing tendency during extended maceration. However, total biogenic amine concentrations were the lowest where cold maceration were applied in both the Cabernet Sauvignon and the Shiraz. This was mainly due to lower putrescine levels in the Cabernet Sauvignon after alcoholic and subsequent malolactic fermentation. The total amino acid and total biogenic amine concentrations were the highest in treatment 1. This could be due to high conctrations of amino acids occurring in the pulp. The absence of skins and seeds in this treatment also led to much lower total phenolics being released into this wine. Proteins and amino acids, having a positive charge in wine, can also bind with negatively charged condensed tannins and other catechin moieties, thus being settled out from the wine (Du Toit *et al*., 2006).

Grape phenolic compounds have been found to affect the growth, metabolism and malolactic activity of lactic acid bacteria (Vivas *et al.*, 1997; Alberto *et al.* 2001, 2007). It has been suggested that phenolic compounds can either activate or inhibit the growth of bacteria by complex mechanisms (Vivas *et al.*, 1997). Vivas *et al.* (1997) found that anthocyanins had a stimulating effect on the growth of lactic acid bacteria, presumably due to usage of the glucose moiety of the anthocyanin as a source of energy. Similarly, bacterial growth is stimulated by gallic acid and catechin present at concentrations found in wine, which are also used as energy sources (Alberto *et al.*, 2001). Alberto *et al.* (2007) conducted a study to investigate whether phenolic compounds can affect biogenic amine production by wine lactic acid bacteria and observed the conversion of agmatine (an intermediate product of arginine and ornithine metabolism) into putrescine (which does not involve a decarboxylation step) was lower in the presence of all phenolic compounds, with the exception of gallic acid and quercetin. The specific effects of phenolic compounds on other biogenic amine production pathways have not been investigated to our knowledge, but could possibly have an inhibiting effect on the enzyme systems involved.

4.3.5 CORRELATIONS AND TRENDS BETWEEN BIOGENIC AMINES AND THEIR PRECURSOR AMINO ACIDS

Refer to **Figure 4.1 (a)** to **(h)** (Cabernet Sauvignon) and **Figure 4.2 (a)** to **(h)** (Shiraz) for graphical comparisons of amino acid precursors and biogenic amines in treatments and phases described in this section.

Certain general trends and/or statistical differences ($p<0.05$; indicated by different letters on **Figures 4.1** and **4.2**) could be observed for the amino acid precursor concentrations of the five treatments in the finished wines (at the end of malolactic fermentation). Differences in amino acid precursors between treatments were small and sometimes insignificant at the end of alcoholic fermentation. The control (treatment 1)

had a higher content of all amino acid precursors than the majority of the other treatments (for all amino acid precursors) for both cultivars in the finished wine.

In some cases the concentration of the amino acid precursors present at the end of malolactic fermentation were higher in treatment 5 than in treatments 2, 3 and 4 (and treatment 1 for some amino acids). These included ornithine, histidine, tryptophan and phenylalanine for Cabernet Sauvignon and Shiraz and also arginine and tyramine for Shiraz. It appears as if extended maceration leads to a greater increase in free amino acid precursors in the finished wine than any of the other maceration practices examined in this study.

In Cabernet Sauvignon no significant differences between treatments 2 and 3 (the two cold maceration treatments) could be observed. In Shiraz a slightly higher concentration in treatment 3 than in treatment 2 could be seen for histidine, tyrosine, tryptophan and phenylethylamine.

For tyrosine, ornithine, phenylalanine, histidine (Cabernet Sauvignon and Shiraz); lysine (Cabernet Sauvignon) and arginine and tryptophan (Shiraz) treatments 4 and 5 yielded higher final average concentrations of the amino acid than treatments 2 and 3, indicating that some factor during cold maceration reduces the concentration of amino acid precursors left over in the final wine. However, these observations were mostly trends and in some cases the amino acid concentration in treatments 2, 3, 4 and 5 were fairly similar (tyrosine, phenylalanine and lysine in both cultivars).

For arginine opposite trends to all of the other amino acid precursors could be observed, but only for Cabernet Sauvignon. The concentration of arginine in treatments 2 and 3 were similar to each other but higher than in treatments 4 and 5. Since arginine can be converted by *O. oeni* and by *Lactobacillus* to ornithine via the deiminase pathway (Arena & Manca de Nadra, 2001; Mangani *et al.*, 2005) the low levels of arginine observed at the end of malolactic fermentation in treatments 4 and 5 could possibly be correlated with the increased ornithine concentration in these treatments at the end of malolactic fermentation. **Table 4.9** indicates that the correlation between arginine and ornithine was not significant at the end of malolactic fermentation (even though it was weakly negative). Also, the decrease of arginine could be because it is an essential amino acid for *O. oeni*, and can be degraded to be used as a source of ATP (Fourcassie *et al.*, 1992).

The production of biogenic amines could also be grouped according to certain trends and/or statistical differences that were apparent between treatments in both cultivars.

Treatment 1 contained in some cases the highest final biogenic amine concentrations for Cabernet Sauvignon (putrescine, histamine, total biogenic amines) and Shiraz (tyramine, histamine, spermidine, phenylethylamine, total biogenic amines). In addition, tyramine was also produced at the second highest concentration in treatment 4 in Shiraz, (also a control without maceration additional to conventional maceration). It could be suggested that the absence of phenolic compounds improve conditions for biogenic amine formation, in particular for microbial or decarboxylase

activity. However, no reference to any study concerning the influence of phenolic compounds in wine on decarboxylase enzymes could be found. The only biogenic amine that consistently exhibited the lowest concentration in treatment 1 in both cultivars at the end of malolactic fermentation was cadaverine (despite the high concentration of its precursor, lysine, present at this stage). Lysine is formed as a result of yeast autolysis and therefore can be expected to increase in concentration during (and possibly after) malolactic fermentation performed on the yeast lees (Soufleros *et al.*, 1998). These authors, as well as Goñi & Ancín Azpilicueta (2001) found that despite the increase of lysine, cadaverine did not increase similarly during malolactic fermentation, which correlates with our results. The reason why this particular trend was only evident in treatment 1 in this study is not clear at this stage.

A number of biogenic amines in Cabernet Sauvignon (tyramine, cadaverine, histamine, total biogenic amines) exhibited a trend towards the highest concentration being present in the finished wine of treatment 5. In Shiraz, histamine as well as the total concentration of biogenic amines shows a similar trend, except that treatment 5 shows higher concentrations than all other treatments except treatment 1. The highest production of histamine in treatment 5 (Cabernet Sauvignon) correlated with the highest level of the precursor amino acid, histidine present in treatment 5 in the finished wine (after malolactic fermentation). This higher concentration of cadaverine in treatment 5 in Cabernet Sauvignon could already be observed following alcoholic fermentation, before the extended maceration treatment was applied. No significant differences could be observed between treatments 4 and 5 (which should be the same at this stage) with regards to cell counts (**Table 4.4**), phenolic and colour measurements (**Table 4.6**) or amino acid concentrations (**Table 4.7**).

No increase in tyramine could be observed during the winemaking process in Cabernet Sauvignon in any treatment other than in one replicate (of triplicates) of treatment 5 during extended maceration, despite the increase of its amino acid precursor in all treatments. Similarly in Shiraz, even though there was an increase in tyrosine concentration in treatments 3 and 5, there was no corresponding increase in tyramine. It is possible that the tyrosine decarboxylase enzyme present in the spiked *Lactobacillus* strains was not active or not expressed under the conditions that existed in this study. Also, tyrosine has been found to be an essential amino acid for *Oenococcus*, and therefore it may be preferentially used for purposes other than tyramine production and disappear during malolactic fermentation under growth conditions with sufficient energy-providing substrates and where *Oenococcus* is present (such as in this study) (Garvie, 1967; Remize *et al*., 2006). Several factors, such as the presence of the precursor amino acid, the growth medium composition, anaerobiosis and pH have been shown to affect bacterial growth and decarboxylase activity (Marcobal *et al.*, 2006).

For the biogenic amines histamine, cadaverine (Cabernet Sauvignon), tyramine (Shiraz) and putrescine and the sum of all biogenic amines (both cultivars), treatments 2 and 3 showed an increase in concentration during cold maceration, followed by no

further increase of these compounds during subsequent fermentation phases when compared to other maceration treatments. Ultimately, treatments 2 and 3 showed the lowest concentration of these biogenic amines in the finished wine when compared to other maceration treatments (treatments 4 and 5). It therefore appears as if cold maceration serves not only as a means to increase amino acid extraction, but it could simultaneously inhibit the formation of high levels of biogenic amines from these amino acid precursors during further fermentation stages, even in the presence of decarboxylase positive microorganisms. Since the control (treatment 1) also showed a similar high concentration of these biogenic amines (except for cadaverine) to treatments 4 and 5, it could be postulated that it was not the maceration treatments applied in treatments 4 and 5 that led to an increase of biogenic amines, but the absence of cold maceration. It could be that specific phenolic compounds and/or the cold temperature at which the cold maceration was applied decreased or inhibited the activity of the decarboxylase enzymes or delayed or inhibited the growth or activity of the indigenous lactic acid bacteria (including the spiked decarboxylase positive *Lactobacillus* species). Unfortunately no plate counts were done after cold maceration, before the onset of alcoholic fermentation to determine the lactic acid bacteria cell numbers.

In the case of putrescine (and to a lesser extent cadaverine and tyramine), which can already be present at high concentrations in the grape skin and grape seeds (Glória *et al.*, 1998; Kiss *et al.*, 2006), the concentration in the final wine in treatments 2 and 3 could resemble the putrescine extracted directly from the grape berry skins or seeds into the must and not produced by any microorganisms from the precursor amino acids. It is possible to positively correlate the higher levels of putrescine in the finished wines to a similar high concentration of the precursor ornithine (**Table 4.7**). The high production levels of putrescine in treatments 1, 4 and 5 in Cabernet Sauvignon and in all treatments in Shiraz at the end of alcoholic fermentation could indicate that putrescine was produced by yeast metabolism or by the indigenous or spiked lactic acid bacteria flora in these treatments.

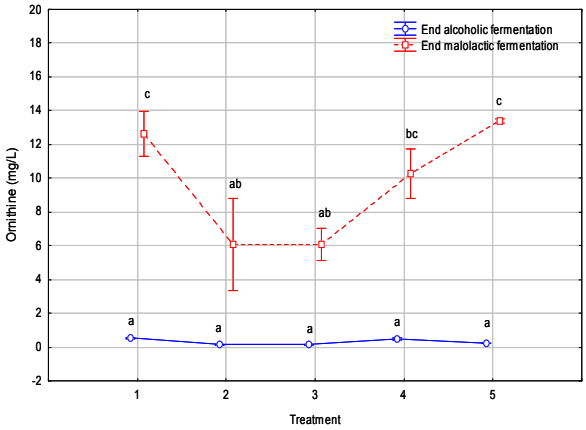
The polyamine spermidine may be produced in a secondary reaction from putrescine by yeasts or lactic acid bacteria. However, the production of spermidine and spermine (not quantified in this study) does not depend on a simple amino acid decarboxylation but it is the result of a complex biosynthetic pathway. Spermidine (and spermine) has been found to increase under restricted environmental conditions since its cellular function involves a protective and anti-oxidative role (Gardini *et al.*, 2005). In this study spermidine increased in concentration in both cultivars during malolactic fermentation, but this increase is similar for most of the treatments. Garde-Cerdán *et al.* (2006) have also observed that spermidine is formed during later fermentation stages than its precursor, putrescine.

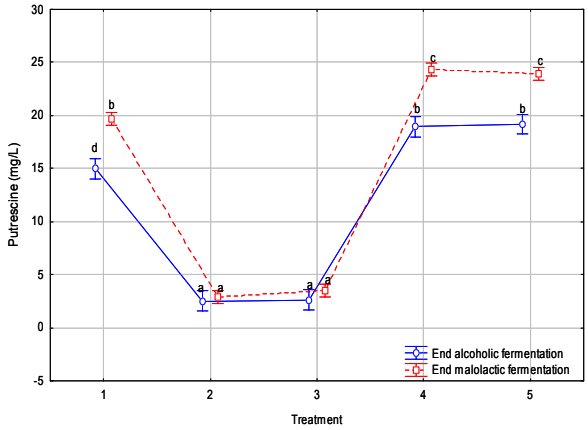
Phenylethylamine and tryptamine were present in the analysed samples at very low concentrations (less than 0.14 mg/L). However, since the described LCMSMS method is sensitive enough to quantify biogenic amines at low concentrations, these biogenic

amines are reported and were also submitted for statistical analysis, even if they are not present at levels than are of physiological importance. (The limits of detection for phenylethylamine and tryptamine with the LCMSMS method used in this study were 0.001 mg/L and 0.005 mg/L respectively.) Contrary to the pattern observed for many of the other biogenic amines, phenylethylamine as well as tryptamine were present at higher concentrations in the final wines of treatments 2 and 3 than for treatments 4 and 5. The low levels of phenylethylamine could possibly be attributed to the absence of relevant decarboxylase enzymes present in active form to convert the amino acid to the biogenic amine in this study. Phenylethylamine is also a substrate for tyrosine decarboxylase (Landete *et al.*, 2007b) and it seems, from the lack of tyramine production in treatments 1 to 4, that tyrosine decarboxylase was not active during this study.

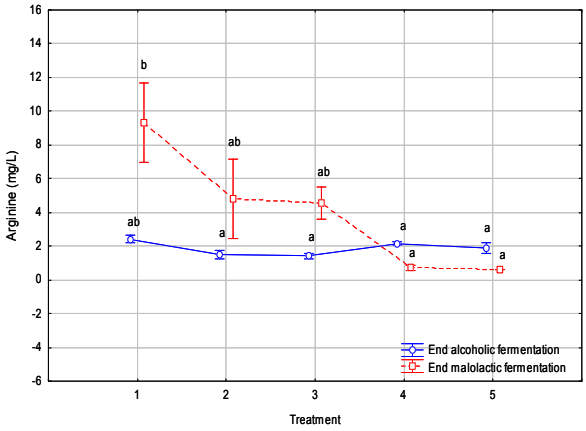
No significant differences in biogenic amine production could be observed between treatment 2 (with maceration enzyme) and treatment 3 (without maceration enzyme). Similar to our results, Martín-Álvarez *et al.* (2006) concluded that pectolytic enzymes added to the grapes did not increase biogenic amines in wine. On the contrary, Pogorzelski (1992) found that maceration (hot maceration) with depectinisation with a commercial pectolytic enzyme preparation led to the highest amount of histamine formed in wines from elderberry fruit (with a mean histamine concentration of 10.4 mg/L) when compared to fermentation in the pulp (7.4 mg/L) and hot maceration without depectinisation (4.3 mg/L).

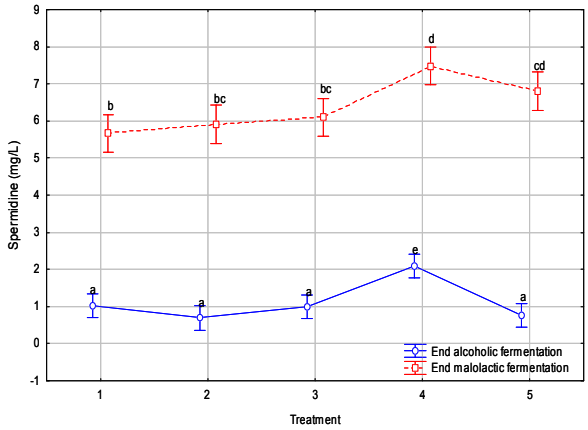
(a)



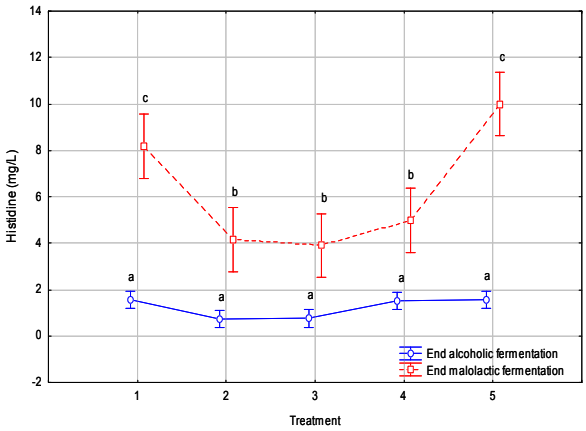


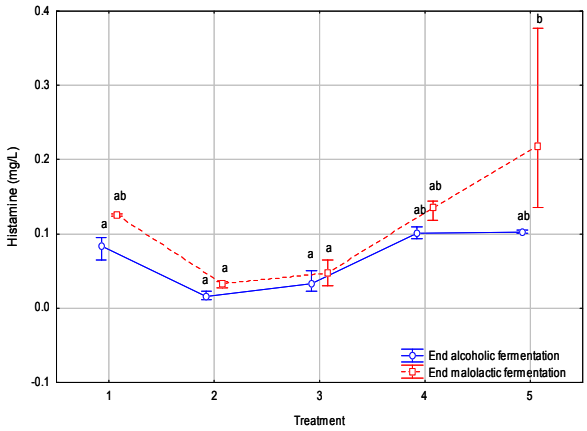
(b)



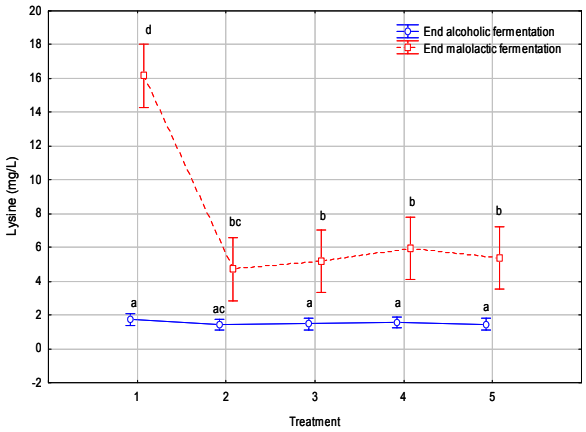


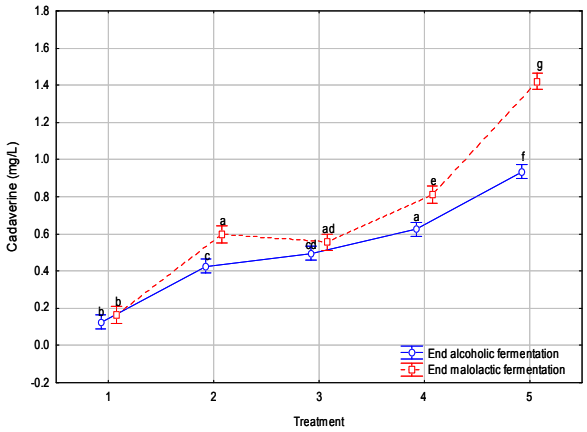
(c)


Histidine (mg/L)
Treatment
End alcoholic fermentation
End malolactic fermentation

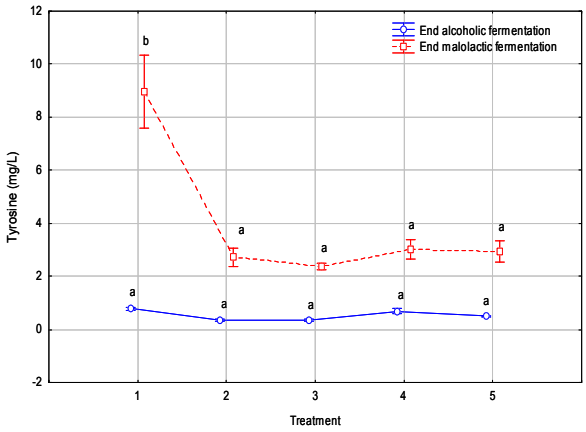

Histamine (mg/L)
Treatment
End alcoholic fermentation
End malolactic fermentation

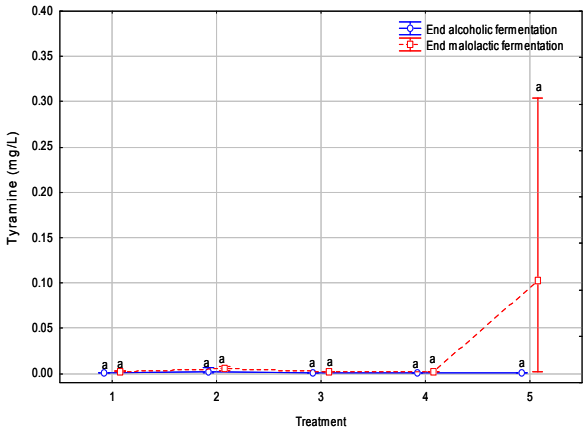
(d)


Lysine (mg/L)
Treatment
End alcoholic fermentation
End malolactic fermentation

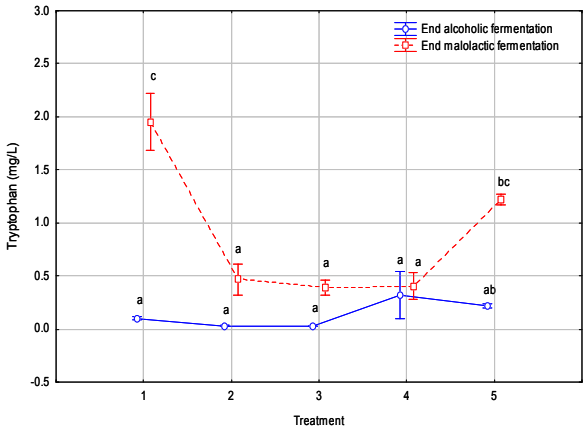

Cadaverine (mg/L)
Treatment
End alcoholic fermentation
End malolactic fermentation

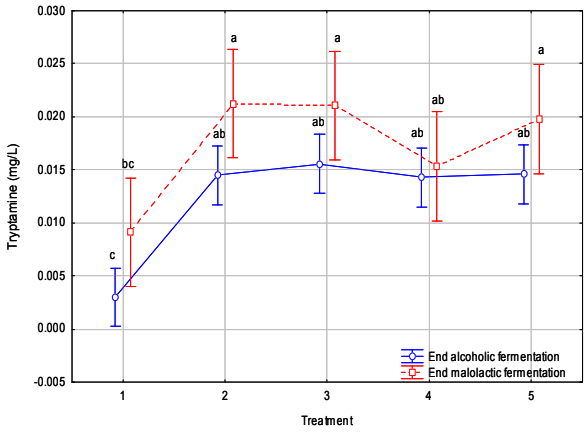
(e)


Tyrosine (mg/L)
Treatment
End alcoholic fermentation
End malolactic fermentation


Tyramine (mg/L)
Treatment
End alcoholic fermentation
End malolactic fermentation

(f)


Tryptophan (mg/L)
Treatment
End alcoholic fermentation
End malolactic fermentation


Tryptamine (mg/L)
Treatment
End alcoholic fermentation
End malolactic fermentation

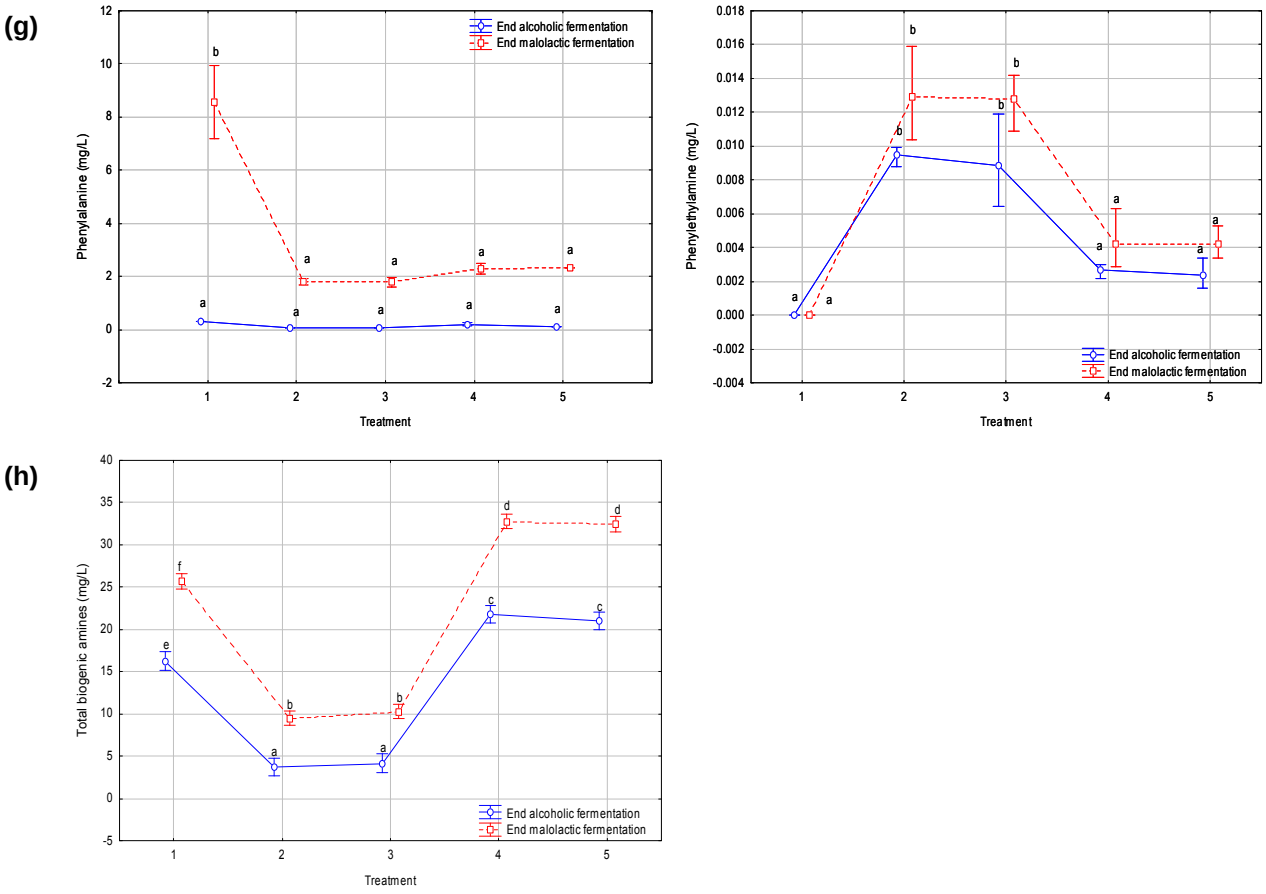
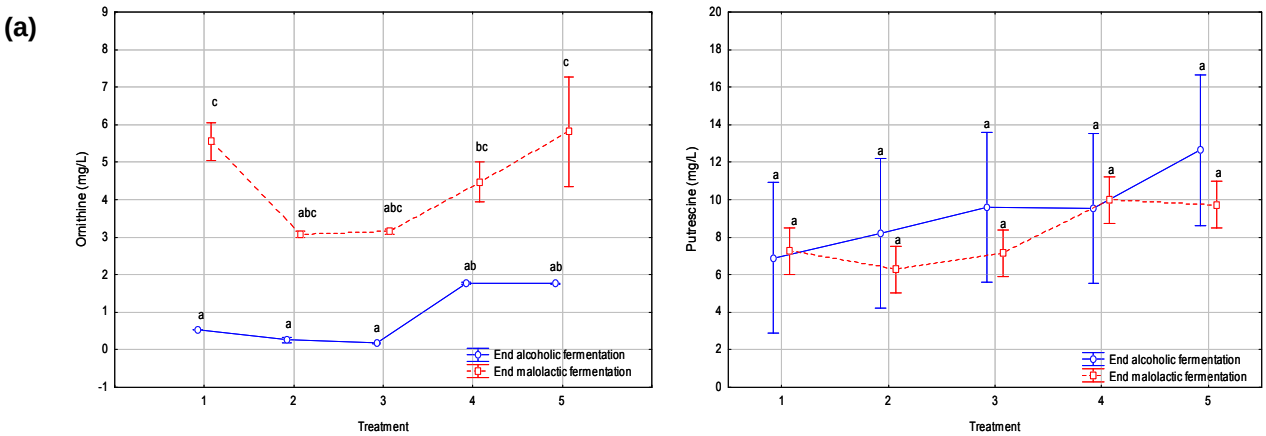
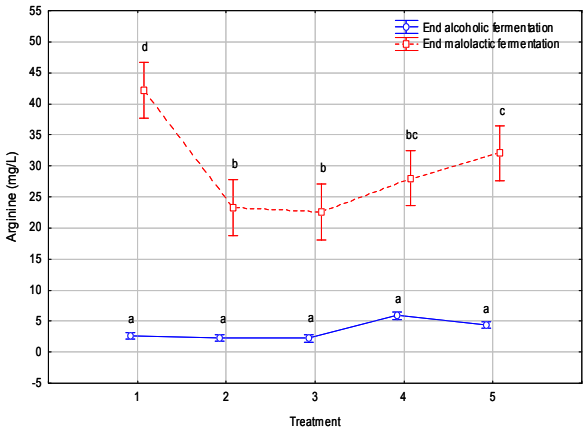
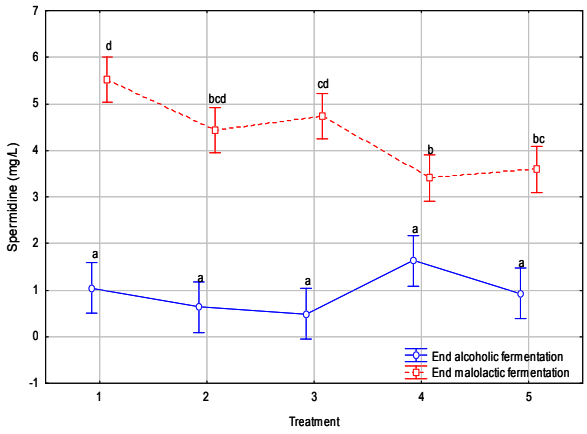


Figure 4.1 Minimum, average and maximum values of amino acids and related biogenic amines concentrations are reported as analysed at the end of alcoholic and malolactic fermentations in Cabernet Sauvignon treatments (refer to the text for treatment descriptions). Different letters on the line graphs indicate significant differences (P<0.05) between treatments and/or phases.

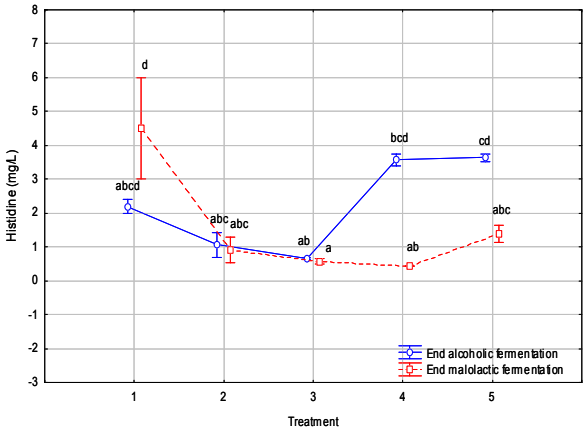


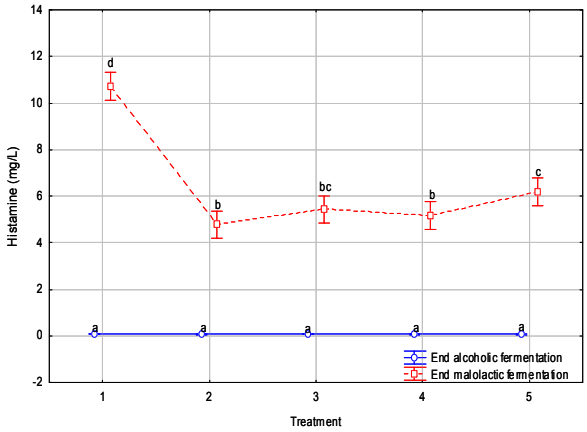
(b)


Arginine (mg/L)
Treatment
End alcoholic fermentation
End malolactic fermentation

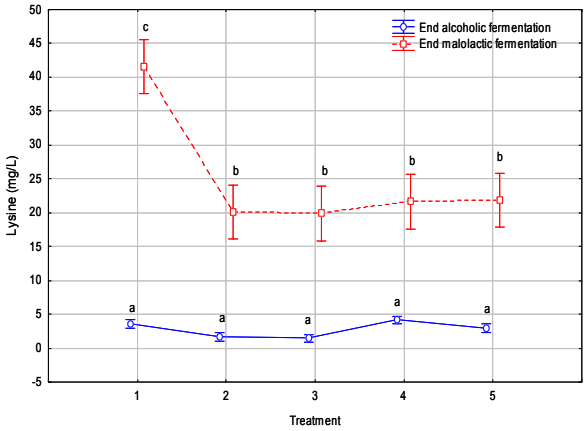

Spermidine (mg/L)
Treatment
End alcoholic fermentation
End malolactic fermentation

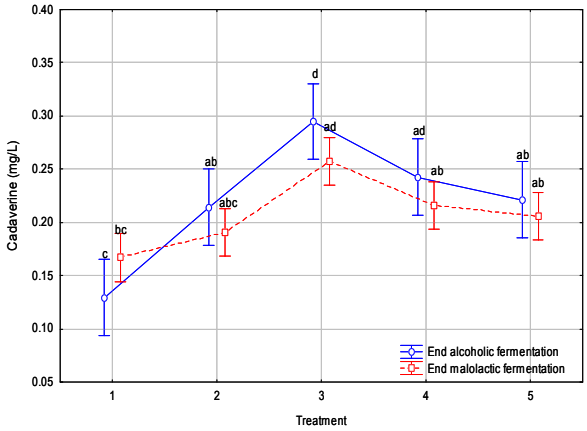
(c)


Histidine (mg/L)
Treatment
End alcoholic fermentation
End malolactic fermentation

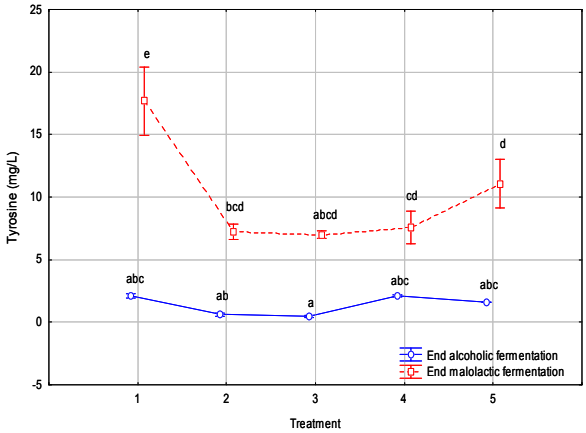

Histamine (mg/L)
Treatment
End alcoholic fermentation
End malolactic fermentation

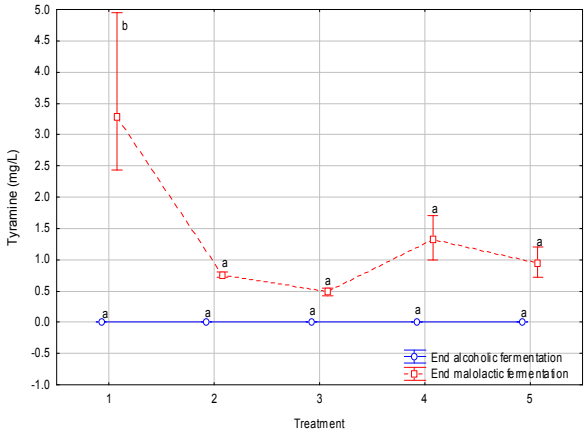
(d)


Lysine (mg/L)
Treatment
End alcoholic fermentation
End malolactic fermentation


Cadaverine (mg/L)
Treatment
End alcoholic fermentation
End malolactic fermentation

(e)


Tyrosine (mg/L)
Treatment
End alcoholic fermentation
End malolactic fermentation


Tyramine (mg/L)
Treatment
End alcoholic fermentation
End malolactic fermentation

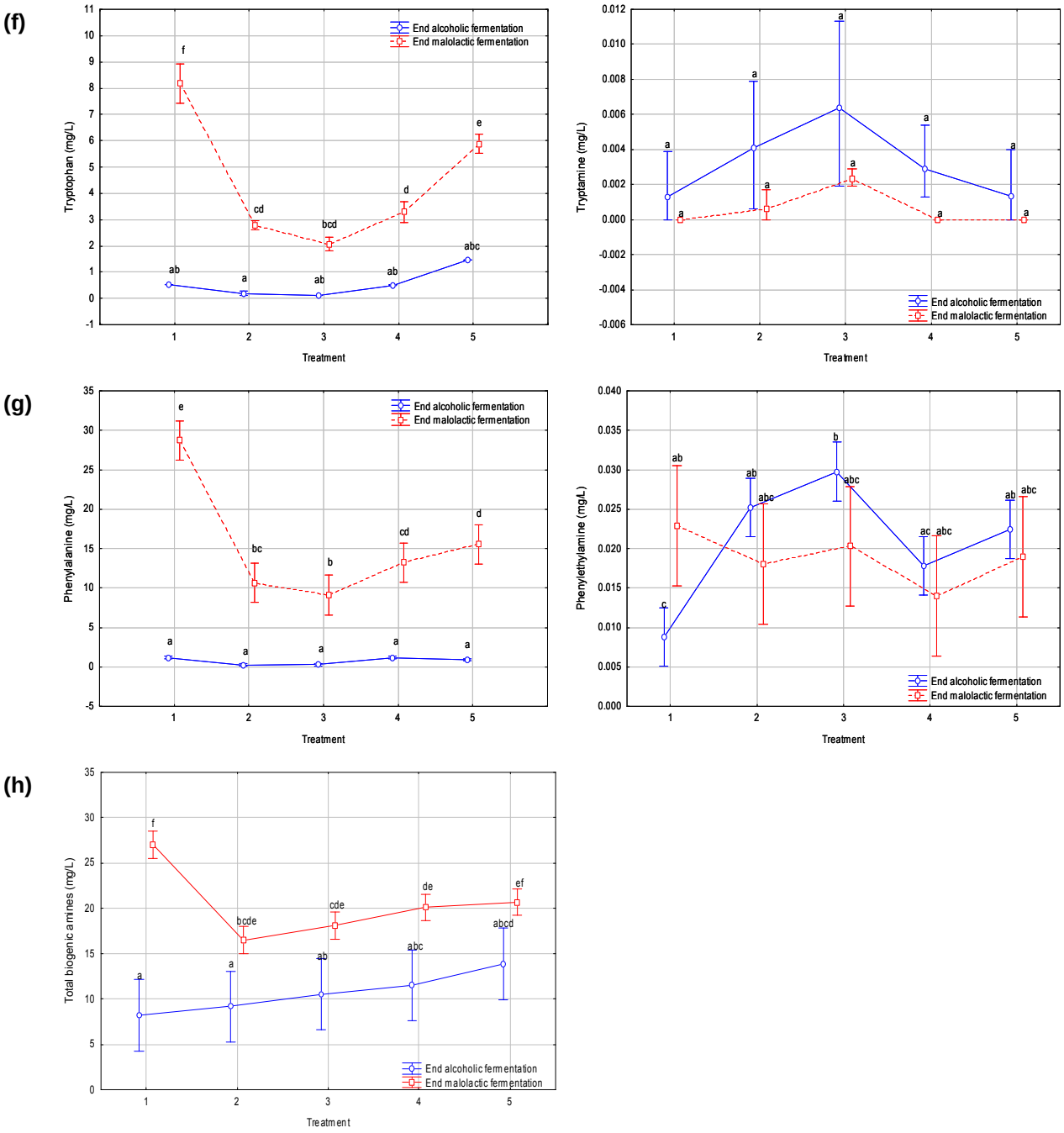


Figure 4.2 Minimum, average and maximum values of amino acids and related biogenic amines concentrations are reported as analysed at the end of alcoholic and malolactic fermentations in Shiraz treatments (refer to the text for treatment descriptions). Different letters on the line graphs indicate significant differences (P<0.05) between treatments and/or phases.

The relationship between biogenic amines and their amino acid precursors at a specific phase of winemaking (at the end of alcoholic fermentation and again end of malolactic fermentation) was determined by calculating correlation coefficients for each cultivar (**Tables 4.9** and **4.10**). All five treatments were included to determine these correlations. Spearman rank correlation coefficients were used between each pair of variables (amino acid and biogenic amine) since it is less sensitive to outliers (such as treatment 1) than Pearson coefficients. The Pearson coefficients are also reported in

parentheses. Only significant correlation coefficients (P<0.05) are reported in **Tables 4.9** and **4.10**. If P>0.05 for the Spearman and/or Pearson correlations coefficients it is indicated as such in the Tables.

In Cabernet Sauvignon, significant negative correlations were obtained for phenylalanine vs. phenylethylamine and for tryptophane vs. tryptamine at the end of alcoholic and malolactic fermentations. The strong positive correlation between arginine and ornithine at this stage could indicate a relationship between the two amino acids, as described by the arginine deiminase pathway. At the end of malolactic fermentation arginine also correlated negatively with both of its possible products, putrescine and spermidine. Tyrosine and tyramine also showed a negative correlation; this correlation was strongly influenced by the production of tyramine in treatment 5. These results could indicate that a correlation exists between the disappearance of amino acid precursor and the production of the corresponding biogenic amine.

Significant positive correlations were always obtained between histidine and histamine in Cabernet Sauvignon. A positive correlation could mean that the precursor is being used for biogenic amine formation, but is simultaneously being replenished by processes in the wine such as yeast autolysis.

Table 4.9 Significant (P<0.05) Spearman rank (and Pearson) correlation coefficients between amino acid precursors and biogenic amines for all treatments after alcoholic fermentation (AF) and malolactic fermentation (MLF) for Cabernet Sauvignon.

	Putrescine	Spermidine	Histamine	Phenylethyl-amine	Tryptamine	Cadaverine	Tyramine	Ornithine
END AF								
Arginine	ns*(0.70)	0.62(ns)						0.93(0.88)
Histidine			0.79(0.89)					
Ornithine	0.59(0.69)	ns(0.66)						
Phenylalanine				-0.84(-0.73)				
Tryptophan					-0.63(ns)			
Lysine						ns(ns)		
Tyrosine							ns(ns)	
Putrescine		ns(ns)						
END MLF								
Arginine	-0.64(ns)	-0.87(-0.72)						ns(ns)
Histidine			0.62(0.70)					
Ornithine	ns(0.81)	ns(ns)						
Phenylalanine				-0.87(-0.70)				
Tryptophan					-0.81(-0.86)			
Lysine						ns(ns)		
Tyrosine							-0.79(ns)	
Putrescine		0.64(0.55)						

ns: no significant correlation (P>0.05)

In Shiraz, significant negative correlations were obtained for phenylalanine vs. phenylethylamine and for tyrosine vs. tyramine at the end of alcoholic and malolactic

fermentations. At the end of malolactic fermentation putrescine was negatively correlated with its secondary product, spermidine. Tryptophan and tryptamine also showed a negative correlation at the end of malolactic fermentation. These results could indicate that a correlation exists between the disappearance of amino acid precursor and the production of the corresponding biogenic amine; and that spermidine was formed from putrescine in the later fermentation stages. Again, a strong positive relationship between arginine and ornithine could be observed at the end of both fermentation phases.

A significant positive correlation was again obtained between histidine and histamine at the end of malolactic fermentation. Histamine was always produced (increased) significantly only during malolactic fermentation in this study using Shiraz.

Table 4.10 Significant (P<0.05) Spearman rank (and Pearson) correlation coefficients between amino acid precursors and biogenic amines for all treatments after alcoholic fermentation (AF) and malolactic fermentation (MLF) for Shiraz.

	Putrescine	Spermidine	Histamine	Phenylethyl-amine	Tryptamine	Cadaverine	Tyramine	Ornithine
END AF								
Arginine	0.83(0.67)	ns(0.63)						0.88(0.93)
Histidine			ns(ns)					
Ornithine	0.78(0.67)	0.68(ns)						
Phenylalanine				-0.79(-0.77)				
Tryptophan					ns(ns)			
Lysine						ns(ns)		
Tyrosine							-0.78(ns)	
Putrescine		ns(ns)						
END MLF								
Arginine	ns(ns)	ns(ns)						0.81(0.70)
Histidine			0.65(0.87)					
Ornithine	ns(ns)	ns(ns)						
Phenylalanine				ns(ns)				
Tryptophan					-0.84(ns)			
Lysine						ns(ns)		
Tyrosine							ns(ns)	
Putrescine		-0.54(-0.56)						

ns: no significant correlation (P>0.05)

These results are in agreement with other studies. Marcobal *et al.* (2005) also found negative correlations between some biogenic amines and their amino acid precursors and positive correlations between others. Soufleros *et al.* (1998) found that on the whole, biogenic amines were positively correlated with amino acids, which indicates that the abundance of amino acids have an effect on the overall content of biogenic amines. However, contrary results were obtained by Goñi & Ancín Azpilicueta (2001), who found no relationship between utilisation of precursors and biogenic amine content.

4.4 CONCLUSION

The high levels of amino acids and consequently of biogenic amines observed in treatment 1 could imply the possibility that these compounds could reach high levels in white or rosé wines, in the absence of any grape skin contact or with limited skin contact. Also, since up to 77% of amino acids have been found in one study to be located in the pulp of Cabernet Sauvignon berries (Stines *et al.*, 2000), it could be that a significant proportion of amino acids were in fact not located in the grape skins in this study in one or both cultivars.

The presence of skin contact, in particular the grape phenols extracted by skin maceration, seems to lead to lower levels of biogenic amines produced in final red wines in some cases. In red wines the free amino acids, peptides and proteins released from yeast cells by autolysis, as well as from grape skins during maceration practices, could be bound in complex reactions by grape phenolics (polyphenols). It could be proposed that this could render potential biogenic amine precursors unavailable to the action of decarboxylase positive microorganisms (such as lactic acid bacteria).

Despite the actual concentrations of biogenic amines in this study being low (and physiologically insignificant to humans in most cases), differences between maceration treatments could be observed, albeit small. The most important observations include increased levels of some biogenic amines in the final wines of treatments that received extended maceration after alcoholic fermentation. The levels of some biogenic amines also seem to be reduced in the final wines in treatments that received cold maceration.

In this study the influence of the commercial maceration enzyme used on biogenic amine accumulation in wine seems to be small. However, no definite conclusion can be made in this regard without further investigation with a larger number of commercial enzyme preparations.

A number of possible reasons for the low levels of biogenic amines generally produced in this study can be proposed. Histamine is primarily produced by bacteria when grown in a poor growth media such as in the absence of glucose or malic acid, where histidine decarboxylation contributes to additional energy production for the lactic acid bacteria by generation of a proton motive force (Lonvaud-Funel & Joyeux, 1994; Molenaar *et al*., 1993). A similar energy function has been described for the decarboxylation of tyrosine to tyramine in lactic acid bacteria by Wolken *et al.* (2006). In our study, sufficient energy sources were presumably available to the lactic acid bacteria.

It is also known that lactic acid bacteria can loose the ability to produce biogenic amines, such as have been reported during prolonged storage or after repeated subcultivation in synthetic media (Lonvaud-Funel & Joyeux, 1994; Leitão *et al.*, 2000). The strains use in this study were adapted to grow in a wine medium and, although the precursors and cofactor for biogenic amine formation were included in the adaptation medium, the activity of the decarboxylase enzymes were not investigated before inoculation into the wines.

In other studies, despite using decarboxylase positive bacteria, no significant biogenic amine production could be detected in wines (Leitão *et al.*, 2000). These authors attribute the lack of biogenic amine production to a low pH (3.5 as opposed to the average optimum at 6) and/or ethanol inhibition of decarboxylase enzyme activities. According to Rollan *et al.* (1995) an ethanol concentration of 12% or more reduces histidine decarboxylase activity by altering the physiochemical properties of the cell membrane which slows down histidine transport. Gardini *et al.* (2005) also found that higher wine pH promoted biogenic amine (tyramine) formation, while it was inhibited by a higher ethanol concentration. However, they attributed this to lower metabolism and cell viability and not to decarboxylase activity. Moreno-Arribas & Lonvaud-Funel (1999) found that ethanol had no influence on tyrosine decarboxylase in cell-free extract. It could therefore be postulated that the high alcohol levels present in the final wines in this study could have had a certain influence on lactic acid bacteria cell viability and/or metabolism but possibly also on decarboxylase gene expression and/or enzyme activity. Thorough investigation in this area will be necessary for further investigations on biogenic amine production in high alcohol wines.

4.5 LITERATURE CITED

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5. RESEARCH RESULTS

Evaluating the influence of complex commercial yeast and bacterial nutrients on biogenic amine production by wine microorganisms

5.1 INTRODUCTION

Biogenic amines are nitrogenous organic compounds produced in wine from amino acid precursors by microbial decarboxylation (ten Brink *et al*., 1990). The concentration of biogenic amines that can potentially be produced in wine largely depends on the abundance of amino acid precursors in the medium, the presence of decarboxylase positive microorganisms (mainly lactic acid bacteria) and conditions that enable microbial or biochemical activity (Silla Santos, 1996).

Vintage, grape variety, geographical region and vinification methods such as grape skin maceration are some of the variables that can lead to an increase of precursor amino acids and subsequently the biogenic amine content in wine. Ageing of wine on yeast lees involves autolysing yeast cells that release vitamins and nitrogenous compounds into the wine. The latter may include amino acids that are the precursors of biogenic amines (Lonvaud-Funel & Joyeux, 1994; Bauza *et al*., 1995; Soufleros *et al*., 1998; Moreno Arribas *et al*., 2000; Martín-Álvarez *et al*. 2006).

The yeasts and lactic acid bacteria responsible for wine fermentations have certain basic nitrogen nutrient requirements. *Saccharomyces* species can utilise the ammonium ion (NH_4^+) and free alpha amino acids as nitrogen sources. Moreover, yeasts can synthesize all required nitrogen compounds, including amino acids, from ammonium. However, if amino acids are present yeasts will use it very efficiently after ammonium has been depleted. Yeasts strains display different preferences for the uptake of different amino acids, and can also secrete certain amino acids into the wine. In general, yeasts require at least a minimum of 140-150 mg/N/L to prevent stuck fermentations, but 200 mg/L is recommended to avoid the formation of off-flavours (H_2S) (Ribérau-Gayon *et al*., 2006). Because ammonium alone does not meet all the nutritional requirements of yeast, many wine yeast manufacturers recommend the use of complex yeast nutrients that include a nitrogen supplement. Another disadvantage of excessive ammonium nitrogen is the potential accumulation of undesirable substances in the wine such as ethyl carbamate and urea (Gonzalez Marco *et al*., 2006).

Complex yeast nutrients contain mainly ammonium salts (NH_4^+), but also include inactivated yeast cells, a complex nutrient source rich in vitamins, minerals, trace elements, sterols and long chain fatty acids (product catalogues of Lallemand, Canada; Anchor Yeast, South Africa; and Chr. Hansen, Denmark). Inactivated yeast cell walls can adsorb medium chain fatty acids that are toxic to the yeast cell. However, these inactivated yeast cells could contain proteins or free alpha amino acids that could be used by lactic acid bacteria in later fermentation stages for biogenic amine production.

Lactic acid bacteria require and are able to use only complex organic nitrogen sources, such as amino acids. They can also utilise peptides or proteins as nitrogen sources by the breakdown to amino acids by proteolytic enzyme activity (Leitão *et al*., 2000). Generally complex malolactic nutrients include inactivated yeast cells rich in alpha amino acids as well as casein, vitamins, minerals, polysaccharides and cellulose. As with commercial yeast preparations, commercial malolactic nutrients are recommended for use with fermentation starter cultures (selected *Oenococcus oeni* strains) unable to produce biogenic amines according to the manufacturers. However, in practice winemakers might add nutrients to a sluggish spontaneous malolactic fermentation or allow spontaneous malolactic fermentation to proceed after complex yeast nutrients had been added during alcoholic fermentation. The question arises whether residual precursor amino acids from complex nutrients could be present in the wine for the natural lactic acid bacteria flora, which could include decarboxylase positive strains, to use.

In this study, the influence of complex commercial yeast and bacterial nutrients on biogenic amine production by yeast and natural lactic acid bacteria (partially supplemented by decarboxylase positive *Lactobacillus* species) in the wine or added to a synthetic medium are evaluated.

5.2 MATERIALS AND METHODS

5.2.1 SMALL SCALE WINE FERMENTATIONS WITH COMPLEX NUTRIENTS

5.2.1.1 General vinification procedure and sampling for wine analyses

Cabernet Sauvignon and Shiraz grapes were obtained from a commercial wine cellar in the Paarl region and an experimental vineyard in the Stellenbosch region respectively. All treatments were repeated in duplicate for each of the two cultivars.

After grapes were destemmed and crushed, the skins and free-run juice were separated and homogenised by allocating equal amounts of free-run juice (per volume) and grape skins to each treatment vessel. A minimal amount of sulphur dioxide (SO_2) (20 mg/L) was added to the must of all treatments. Alcoholic fermentation was performed by *Saccharomyces cerevisiae* strain NT202 (Anchor Yeast, South Africa) in all treatments. NT202 was rehydrated and inoculated at 30 g/hL according to the instructions of the manufacturer. Sugar density was measured daily in all treatments with a Brix hydrometer to monitor the progression of alcoholic fermentation, which proceeded in 10 L buckets at room temperature. Grape skins were punched through the wines on a daily basis throughout alcoholic fermentation. All treatments were pressed with a hydrolic basket press at the completion of alcoholic fermentation and wines (treatments) were transferred to 4.5 L glass bottles to complete spontaneous malolactic

fermentation at room temperature (20 to 25°C). Eighty mg/L SO_2 was added to all wines at the completion of MLF, prior to bottling.

Representative samples for analyses of selected wine chemical components and the presence of microorganisms were drawn in sterile sample vials. The stages at which these samples were taken were of the grape must, after alcoholic fermentation, during the course of malolactic fermentation and after the completion of malolactic fermentation.

The presence and growth of lactic acid bacteria species in the wine was monitored by plate counts on agar media selective for the growth of *Lactobacillus*, *Pediococcus* and *Leuconostoc*, and *Oenococcus oeni* respectively (refer to **Chapter 3**, section **3.2.2**). Genera were distinguished microscopically based on identification of cell morphology.

Malolactic fermentation was also monitored at these stages using Fourier Transform Infrared Spectroscopy (FT-IR) (WineScan FT120, FOSS Analytical, Denmark) to determine malic acid and lactic acid concentrations in the wine. A sample of the must was submitted for analysis of free amino acids. Samples of the must, the wine after alcoholic fermentation and of the wine after malolactic fermentation were submitted for biogenic amine analysis (refer to section **5.2.3**).

5.2.1.2 Description of treatments

Treatments consisted of the addition of commercial preparations of complex yeast or bacterial nutrients to the fermenting must or wine. Yeast nutrients were added after the exponential growth phase (one to three days after inoculation with yeast for alcoholic fermentation). Bacterial (malolactic) nutrients were added after alcoholic fermentation, before the start of malolactic fermentation. All complex nutrients were added to the fermenting must or wine at the maximum dosage recommended by the manufacturers (**Table 5.1**). The complex yeast nutrients included in this study were Nutrivin and Nutrivin Super (Anchor Bio-Technologies, Cape Town, South Africa) and Fermaid K (Lallemand, South Africa). The complex bacterial nutrients used were Optimalo Plus (Lallemand, South Africa) and Bactiv-aid (Chr. Hansen, Denmark).

Treatment 1 was the negative control to which no nutrients were added, only alcoholic fermentation was allowed to proceed while the growth of lactic acid bacteria and/or malolactic fermentation was delayed or inhibited by the addition of 250 mg/L lysozyme (DSM Food Specialties Oenology, Servian, France). Lysozyme was added to the must of Cabernet Sauvignon and into the wine just before the end of alcoholic fermentation for Shiraz. Treatment 2 was a control treatment with no nutrient addition in which the natural lactic acid bacteria flora from the grapes and/or winery equipment were allowed to flourish and perform spontaneous malolactic fermentation. Decarboxylase positive lactic acid bacteria (a combination of equal cell concentrations of *Lactobacillus hilgardii* B74, *Lactobacillus hilgardii* M59 and *Lactobacillus brevis* M58) (refer to **Chapter 3**, section **3.2.1.2**) were precultured and all inoculated into treatments 3 to 8 at a total of 10^6 cells/mL after the completion of alcoholic fermentation and prior to

the start of spontaneous malolactic fermentation. No complex nutrients were added to treatment 3. Treatments 4, 5 and 6 were supplemented with complex yeast nutrients, and treatments 7 and 8 with complex bacterial nutrients (**Table 5.1**).

Table 5.1 Description of treatments 1 to 8 performed in grape must and wine, as referred to in the text. The maximum recommended dosages of complex nutrients are indicated in brackets. Shaded areas indicate the absence of the represented microorganism or nutrient in the treatment.

Treatments (in must/wine)	Yeast (NT202)	Spontaneous LAB flora	*Lactobacillus* species	Yeast nutrient	Bacterial nutrient
1	X				
2	X	X			
3	X	X	X		
4	X	X	X	Nutrivin (0.7 g/L)	
5	X	X	X	Nutrivin Super (0.5 g/L)	
6	X	X	X	Fermaid K (0.4 g/L)	
7	X	X	X		Optimalo Plus (0.2 g/L)
8	X	X	X		Bactiv Aid (0.15 g/L)

5.2.1.3 Progression of fermentation, malic acid degradation and the growth of lactic acid bacteria

a) Cabernet Sauvignon

With reference to **Figure 5.1** (section **5.3.1.2**), day 0 represents the natural flora present in the must from the grapes. Alcoholic fermentation was completed on day 16, after which wines were kept at a cool temperature (15°C) to inhibit an increase in lactic acid bacteria and the start of spontaneous malolactic fermentation before inoculating treatments 3 to 8 with *Lactobacillus* species. This step was not ideal, but preparation of the *Lactobacillus* cultures had been delayed. Plating and FT-IR analyses were performed on day 29 to monitor whether further increase in numbers of lactic acid bacteria and/or spontaneous malolactic fermentation had taken place, which did not occur. The three *Lactobacillus* species were spiked into the wines of treatments 3 to 8 on day 38. Malolactic fermentation was completed (below 0.3 g/L residual malic acid) between day 59 and day 71, depending on the treatment. No microbial counts were performed on the wines on day 71.

b) Shiraz

In **Figure 5.2** (section **5.3.1.2**), day 1 represents the natural flora present in the must of the grapes. Alcoholic fermentation was completed on day 13, after which wines were kept at a cool temperature (15°C) to inhibit an increase in lactic acid bacteria and the start of spontaneous malolactic fermentation before inoculating treatments 3 to 8 with *Lactobacillus* species. The three *Lactobacillus* species were spiked into the wines of treatments 3 to 8 on day 22. Malolactic fermentation was completed (below 0.3 g/L residual malic acid) on day 49 or 55, depending on the treatment. No microbial counts were performed on day 55.

5.2.2 FERMENTATIONS IN A SYNTHETIC MEDIUM WITH COMPLEX NUTRIENTS

5.2.2.1 Description of treatments and synthetic medium composition

A modified version of MS300 (Bely *et al.*, 1990) at pH 3.5 was used as a synthetic screening medium. In this medium, 120 mg/L ammonium chloride was included as the only nitrogen source (no amino acids were added). 0.005% of pyridoxal 5'-phosphate (Sigma-Aldrich, Germany) was also added to the medium. All chemicals used for the preparation of modified MS300 (mMS300) were purchased from Saarchem, Merck, South Africa except for malic acid, glucose and ammonium chloride (Sigma-Aldrich, Germany).

Control treatments comprised of the synthetic medium incubated without the addition of any complex nutrients, in the absence of yeast and lactic acid bacteria (1), in the presence of yeast and lactic acid bacteria (2), in the absence of yeast but presence of lactic acid bacteria (3), and in the absence of lactic acid bacteria but presence of yeast (4). The yeast and bacterial strains were the same as those mentioned in section **5.2.1.2** and were precultured in the same manner as described in **Chapter 6**, section **6.2.1** and **Chapter 3**, section **3.2.1.2** for the yeasts and bacteria respectively.

Treatments consisted of the addition of commercial preparations of complex yeast or bacterial nutrients to the synthetic medium at the maximum dosage prescribed by the manufacturers. Treatments are described in **Table 5.2** (control treatments included).

Table 5.2 Description of treatments 1 to 12 performed in synthetic must and wine (mMS300) as referred to in the text. The maximum recommended dosages of complex nutrients are indicated in brackets. Shaded areas indicate the absence of the represented microorganism or nutrient in the treatment.

Treatments (in mMS300)	Yeast (NT202)	*Lactobacillus* species	Yeast nutrient	Bacterial nutrient
1				
2	X	X		
3		X		
4	X			
5	X	X	Nutrivin (0.7 g/L)	Optimalo Plus (0.2 g/L)
6	X	X	Nutrivin (0.7 g/L)	Bactiv Aid (0.15 g/L)
7	X	X	Nutrivin Super (0.5 g/L)	Optimalo Plus (0.2 g/L)
8	X	X	Nutrivin Super (0.5 g/L)	Bactiv Aid (0.15 g/L)
9	X	X	Fermaid K (0.4 g/L)	Optimalo Plus (0.2 g/L)
10	X	X	Fermaid K (0.4 g/L)	Bactiv Aid (0.15 g/L)
11	X	X		Optimalo Plus (0.2 g/L)
12	X	X		Bactiv Aid (0.15 g/L)

Prior to the start of this experiment, samples were taken of each complex nutrient individually dissolved in the modified MS300 medium and submitted for biogenic amine and amino acid analyses (section **5.2.3**).

5.2.2.2 Alcoholic fermentation and growth of decarboxylase bacteria

Saccharomyces cerevisiae strain NT202 was again used as commercial yeast starter culture; rehydrated and inoculated according to the manufacturer's instructions. Complex yeast nutrients were added to the fermenting synthetic must after 48 hours. Alcoholic fermentation proceeded under semi-anaerobic static conditions in 100 mL Erlenmeyer flasks at 30°C. After 14 days gas formation seized and all yeast cells were removed by centrifugation (8000 rpm for 5 minutes) to prevent further fermentation or autolysis and release of nutrients from the yeast cells. The supernatant was collected, the complex bacterial nutrients were added and 1% of precultured decarboxylase positive lactic acid bacteria (**Chapter 3**, section **3.2.1.2**) was inoculated into the

medium. Bacteria were allowed to grow for 14 days at 30°C under static, semi-anaerobic conditions.

Samples were drawn from the fermentation medium after alcoholic fermentation and at the end of growth of lactic acid bacteria for biogenic amine analysis. Chemical analysis of selected wine components (pH, volatile acid, malic acid, lactic acid, glucose, fructose, ethanol and glycerol) was performed by FT-IR at the end of the growth period of the lactic acid bacteria (Nieuwoudt *et al.*, 2006).

5.2.3 AMINO ACID AND BIOGENIC AMINE ANALYSES

Amino acids were analysed using the EZ:Faast liquid chromatography method as described in the EZ:Faast user's guide. For sample preparation, derivatisation, solid phase extraction and standards, refer to **Chapter 3**, section **3.2.4**.

Biogenic amines (histamine, tyramine, putrescine and cadaverine) were quantified for the samples taken from the small scale wine fermentations by high performance liquid chromatography (HPLC), using the method described by Alberto *et al*. (2002) with modifications (**Chapter 3**, section **3.2.4**). Histamine, tyramine, putrescine and cadaverine present in the synthetic medium were determined using liquid chromatography mass spectrometry (LCMS) as described by Millán *et al.* (2007).

5.3 RESULTS AND DISCUSSION

5.3.1 SMALL SCALE WINE FERMENTATIONS WITH COMPLEX NUTRIENTS

5.3.1.1 Presence of amino acids and biogenic amines in grape must

Vine nutrition and grape variety are significant contributors to the concentration and composition of grape amino acids, which could determine the final biogenic amine concentration that is found or formed by microorganisms in wine (Soufleros *et al*., 1998; Herbert *et al*., 2005). Average concentrations and composition of amino acids in grape musts of 384 samples representing eight countries (Henschke & Jiranek, 1993) are reported in **Table 5.3** along with the concentration of amino acids measured in the musts of Cabernet Sauvignon and Shiraz grapes used in this study. Other than a very low concentration of arginine present in the must of Cabernet Sauvignon, the amino acids in this study seem to fall close to the average found by (Henschke & Jiranek, 1993).

Some biogenic amines, particularly polyamines such as putrescine, are often present in grape berries since they are produced by the metabolism of plants. Polyamines are important for plant physiological processes such as flowering and fruit development, cell division, stress responses and senescence (Halász *et al*., 1994; Bover-Cid *et al*., 2006). Cabernet Sauvignon was found to have high concentrations of

putrescine, cadaverine and spermidine in the pericarp of the berries (Glória *et al*., 1998). These three biogenic amines have also been found in the seeds of grape berries (Kiss *et al*., 2006).

Vine nutrition, such as potassium deficiencies in the soil, has been linked to elevated concentrations of putrescine (Adams, 1991). **Table 5.3** indicates that putrescine is the only biogenic amine (of the four biogenic amines tested in this study) present in the grapes at relatively high concentrations.

Table 5.3 Concentrations (mg/L) of biogenic amines and their precursor amino acids present in musts of Cabernet Sauvignon and Shiraz used in this study. Relevant compounds are highlighted. A single representative sample of the must of each cultivar was submitted for analyses. Average amino acid concentrations determined by Henschke & Jiranek (1993) are also reported.

Compound mg/L	Cabernet Sauvignon must	Shiraz must	Average in grape must
Alanine	87.46	89.34	153
Threonine	33.87	125.92	91
Serine	41.22	65.87	104
Glutamine	81.74	183.20	118
Arginine	27.01	440.98	394
Tryptophan	15.17	28.14	7
Glutamic acid	41.69	176.12	151
Valine	46.24	57.54	40
Histidine	53.57	87.55	28
Aspartic acid	27.54	46.51	56
Lysine	4.13	15.88	20
Proline	550.96	505.15	651
Methionine	2.63	2.98	7
Tyrosine	35.47	40.02	16
Cystine	0.22	nd	6
Isoleucine	28.51	29.38	30
Phenylalanine	20.62	17.13	32
Leucine	41.36	60.88	40
Asparagine	1.76	4.57	21
Glycine	4.15	7.16	18
Ornithine	3.15	5.77	6
Histamine	nd	nd	
Putrescine	35.66	30.38	
Tyramine	nd	nd	
Cadaverine	nd	nd	

nd: not detected

5.3.1.2 Malic acid degradation and the growth of lactic acid bacteria

a) Cabernet Sauvignon

When comparing the rate of decrease of malic acid concentration between treatments, it can be observed that malolactic fermentation initially proceeded faster in some treatments that received added complex nutrients than in others. When comparing the

three treatments that received additional complex yeast nutrients; not only did treatments 4 and 5 complete spontaneous malolactic fermentation at an earlier stage than treatment 6 (comparing day 51), the rate of alcoholic fermentation was also enhanced in these two treatments (data not shown). Malolactic fermentation in treatments 7 and 8 initially proceeded faster than in treatment 6 and the controls (treatments 1, 2 and 3), suggesting that malolactic nutrients can act to increase the initial rate of spontaneous malolactic fermentation.

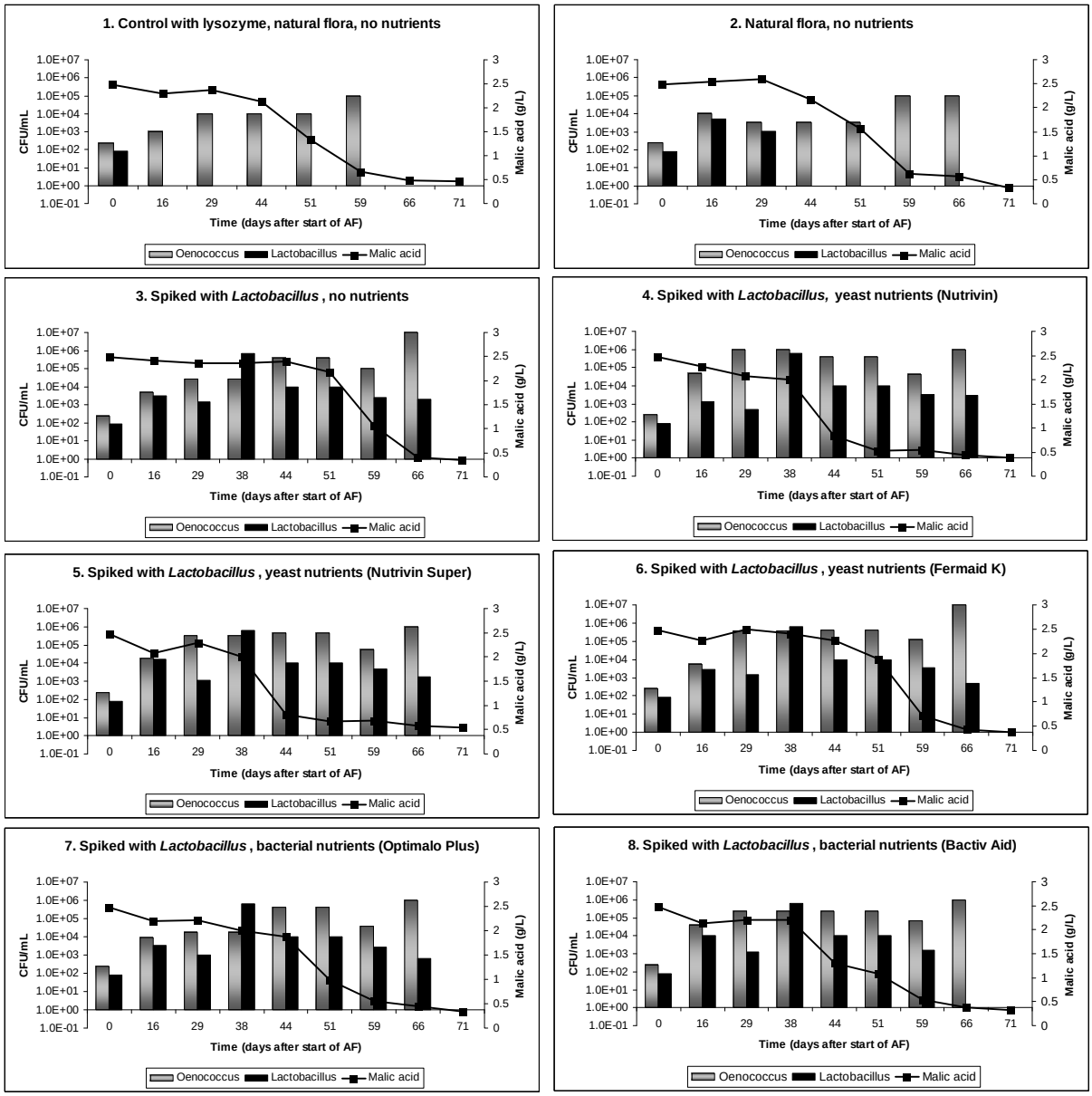

Figure 5.1 Cell counts (log cfu/mL) of lactic acid bacteria, and malic acid concentrations (g/L) in Cabernet Sauvignon wines. Stages represented on the time scale (days after start of AF) are explained in the text. The concentrations represent the average of duplicates for each treatment.

b) Shiraz

For Shiraz treatments the rate of malolactic fermentation was similar in all treatments with malic acid showing a sharp decrease between days 35 and 55. No malolactic fermentation occurred in treatment 1. This indicates that the addition of lysozyme (at 250 mg/L) at the end of alcoholic fermentation is more successful to inhibit the growth of lactic acid bacteria and the onset of malolactic fermentation than the addition of lysozyme at the same concentration to the grape must as was done for Cabernet Sauvignon treatment 1. Control treatment 2 showed a longer lag phase of malolactic fermentation and cell growth than other treatments (see day 43).

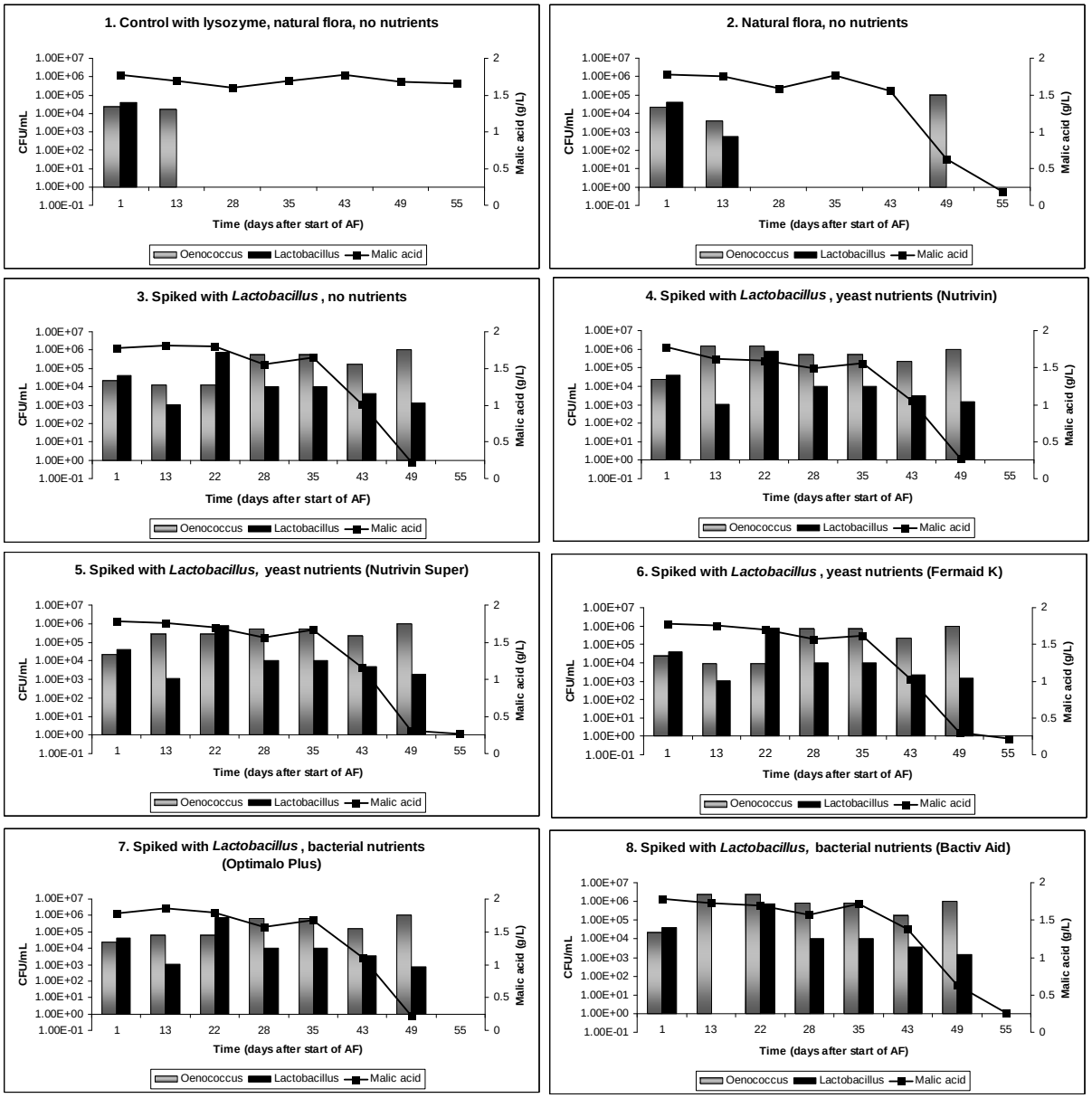

Figure 5.2 counts (log cfu/mL) of lactic acid bacteria, and malic acid concentrations (g/L) in Shiraz wines. Stages represented on the time scale (days after start of AF) are explained in the text. The concentrations represent the average of duplicates for each treatment.

5.3.1.3 Biogenic amine production in small scale experimental wines

Figures 5.3 to **5.7** represents the concentrations of each biogenic amine detected in samples of the grape must, at the end of alcoholic fermentation (AF) and at the end of malolactic fermentation (MLF) in (a) Cabernet Sauvignon and (b) Shiraz wines. The concentrations represent the average of duplicates for each treatment. In the case of histamine measured at the end of malolactic fermentation and cadaverine measured at the end of alcoholic fermentation, one of the two duplicate treatments showed no presence of the amine (even when resubmitted for a second test by HPLC), while the other repetition showed a much higher concentration. For this reason, the standard error bars show a very large variation in concentration on **Figures 5.3, 5.5** and **5.6.** No tyramine was detected in any of the treatments for either of the cultivars.

a) Cabernet Sauvignon

Despite the fact that only one of the duplicates showed positive values for histamine in some treatments, it seems that it may be possible that there are differences between treatments and that treatments 6, 7 and 8 (Cabernet Sauvignon and Shiraz) and treatment 4 (Shiraz) influenced the final histamine concentration in the wine (**Figures 5.3** and **5.6**). Due to the small volumes used in small scale winemaking, it is possible that the inherent heterogeneity in a product such as complex nutrients could have caused the discrepancy in treatment duplicates. Since the increase in histamine occurred during malolactic fermentation, the formation of this biogenic amine can most likely be attributed to the decarboxylation of histidine by lactic acid bacteria. It cannot be deduced from the available data which of the spiked *Lactobacillus* species or the natural *Oenococcus oeni* flora responsible for malolactic fermentation were also responsible for histamine formation. Of the four biogenic amines analysed in this study histamine is the most important to human physiology, since it is one of the most biologically active amines (Halász *et al*., 1994). Histamine can cause hypotension, flushing and headache (Silla Santos, 1996) as well as abdominal cramps, diarrhoea and vomiting (Taylor, 1986). The levels of histamine produced in some treatment replicates in both cultivars during this study are above the upper limits suggested for histamine in wine in Germany (2 mg/L) and Holland (3 mg/L) (Lehtonen, 1996).

Cadaverine concentrations increased more or less equally in all treatments in Cabernet Sauvignon wines (especially if taking into account the standard deviation of duplicate treatments) (**Figure 5.5**). There seems to be an increase in all treatments across the phases of fermentation. It appears that yeast produced a small amount of cadaverine and it is possible that indigenous yeasts could have produced cadaverine at the start of alcoholic fermentation. The increase in cadaverine concentration during malolactic fermentation is discussed below. No cadaverine was produced in Shiraz wines.

While alcoholic fermentation did not seem to produce a change in putrescine levels in Cabernet Sauvignon, all treatments showed a similar increase in putrescine

concentration from the end of alcoholic fermentation to the end of malolactic fermentation (**Figure 5.4**). When disregarding the standard error bars the only exceptions might be treatment 6 (Nutrivin Super addition) and treatment 8 (Bactiv-aid addition) that seems to be associated with a lower concentration of putrescine after alcoholic fermentation and malolactic fermentation respectively. Overall, differences in final putrescine concentrations in the Cabernet Sauvignon wines cannot be ascribed to treatments; only to the phase of winemaking.

Figure 5.3 Histamine concentrations in treatments 1 to 8 (refer to the text for the treatment descriptions) present in the grape must, at the end of alcoholic fermentation and at the completion of malolactic fermentation in Cabernet Sauvignon. The concentrations represent the average of duplicates for each treatment.

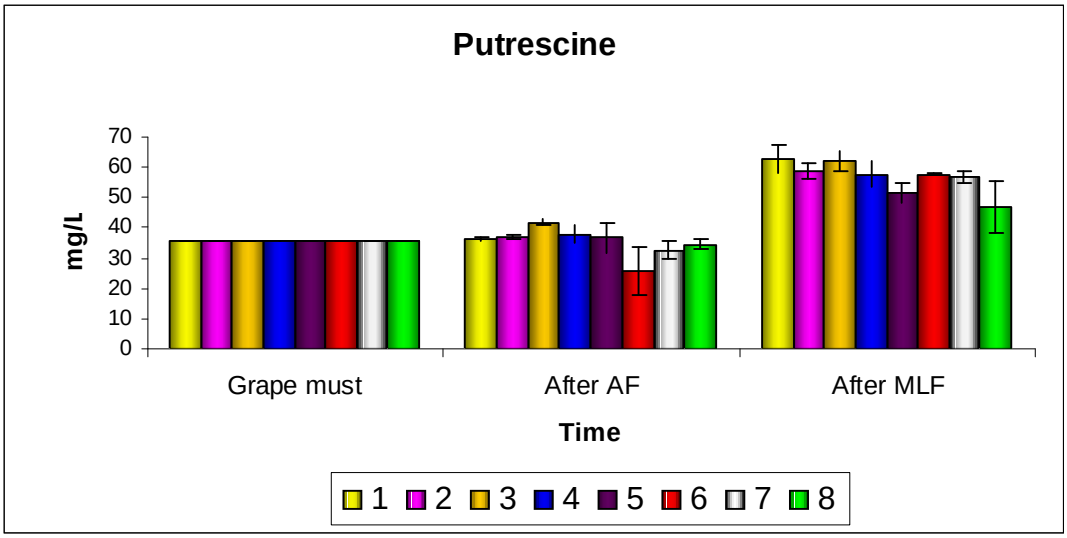

Figure 5.4 Putrescine concentrations in treatments 1 to 8 (refer to the text for the treatment descriptions) present in the grape must, at the end of alcoholic fermentation and at the completion of malolactic fermentation in Cabernet Sauvignon. The concentrations represent the average of duplicates for each treatment.

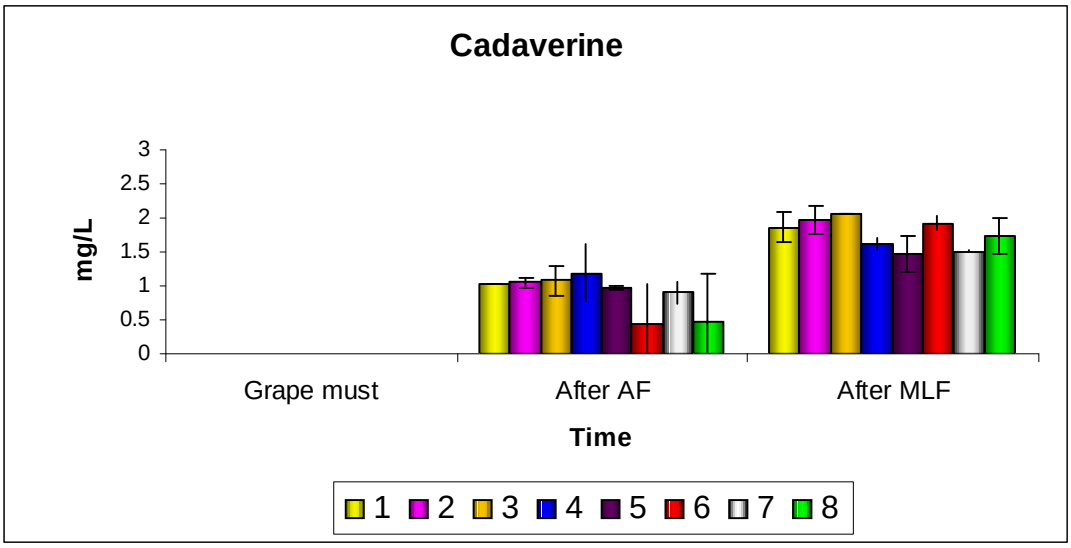

Figure 5.5 Cadaverine concentrations in treatments 1 to 8 (refer to the text for the treatment descriptions) present in the grape must, at the end of alcoholic fermentation and at the completion of malolactic fermentation in Cabernet Sauvignon. The concentrations represent the average of duplicates for each treatment.

b) Shiraz

For Shiraz wines the putrescine concentration decreased from the must to the end of alcoholic fermentation, after which the concentration remained more or less unchanged during malolactic fermentation (**Figure 5.7**). The decrease of putrescine during alcoholic fermentation is in accordance with results obtained by Granchi *et al*. (2005). The decrease of putrescine concentration can be explained by a conversion to the polyamines spermine and spermidine, which are in turn consumed by yeast as absolute requirements for optimal growth (Cohn *et al*., 1978). Only treatment 1 (negative control with lysozyme) seems to have a lower concentration of putrescine at the end of alcoholic fermentation. Indigenous *O. oeni* could have been responsible for biogenic amine production, since *O. oeni* strains were present in all treatments in Shiraz and Cabernet Sauvignon wines (**Figures 5.1** and **5.2**).

The increase of putrescine and cadaverine during malolactic fermentation is presumably due to decarboxylation by *O. oeni* or indigenous lactic acid bacteria of other genera of ornithine, arginine (**Chapter 2**, section **2.2.1.4**) and lysine present already in must or released from autolysing yeast cells and not from any of the added complex nutrients. Control treatments containing no added nutrients and not spiked with lactobacilli (treatments 1 and 2) showed a similar increase in putrescine and cadaverine when compared to the other treatments. Plate counts on selective media showed that no contamination with the three *Lactobacillus* species inoculated into treatments 3 to 8 took place in control treatments 1 and 2 (**Figures 5.1** and **5.2**).

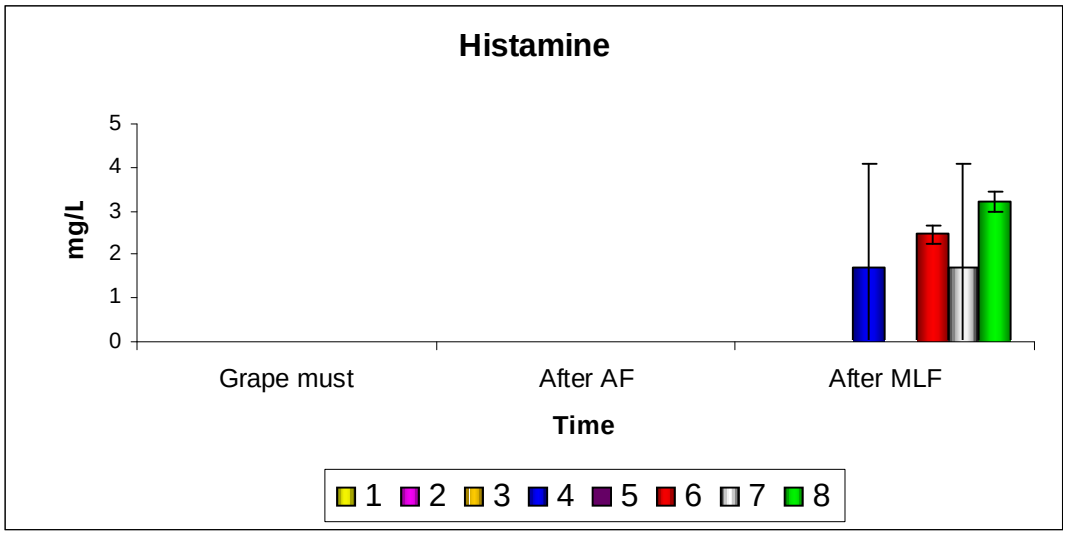

Figure 5.6 Histamine concentrations in treatments 1 to 8 (refer to the text for the treatment descriptions) present in the grape must, at the end of alcoholic fermentation and at the completion of malolactic fermentation in Shiraz. The concentrations represent the average of duplicates for each treatment.

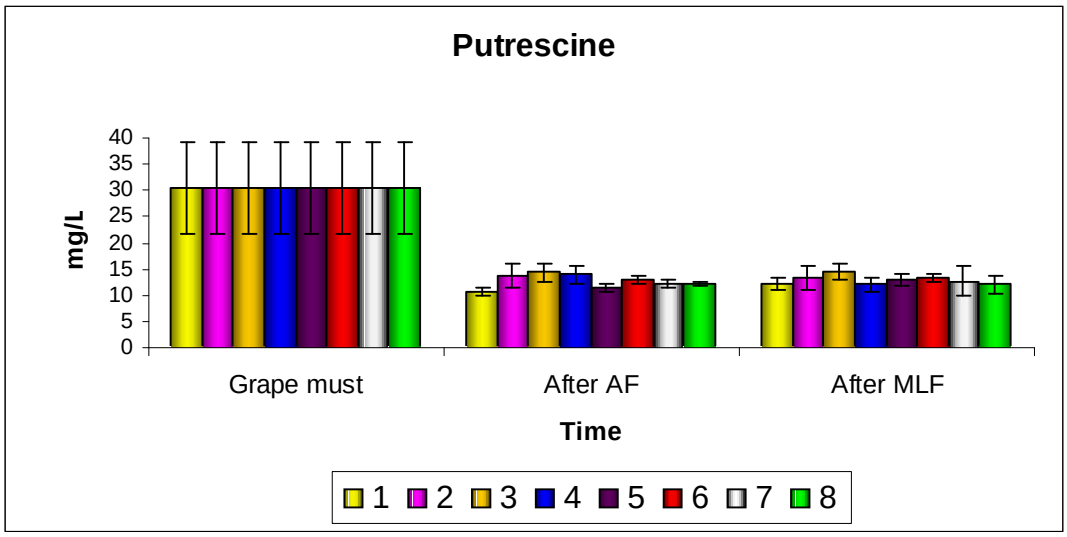

Figure 5.7 Putrescine concentrations in treatments 1 to 8 (refer to the text for the treatment descriptions) present in the grape must, at the end of alcoholic fermentation and at the completion of malolactic fermentation in Shiraz. The concentrations represent the average of duplicates for each treatment.

5.3.2 FERMENTATIONS IN A SYNTHETIC MEDIUM WITH COMPLEX NUTRIENTS

5.3.2.1 Presence of amino acids and biogenic amines in synthetic must

The measured biogenic amine and amino acid concentrations in the synthetic medium with or without dissolved complex nutrients are very low and should not (according to the results represented in **Table 5.4**) significantly influence biogenic amine formation. The absence of biogenic amines and amino acids were expected in the chemically

defined mMS300 medium without added nutrients, but at least some amino acids were expected to be present when complex nutrients were dissolved in this medium. It could be that most of the nitrogen from the inactivated yeast cells is still in protein form and had not yet been broken down to free alpha amino acids. Also, since a granular residue was noted in most of the treatments at the end of fermentations, it is possible that the complex nutrients simply did not dissolve well in the synthetic medium. The small quantities of complex nutrients used in synthetic wine fermentations (only 50 mL each) could have been heterogeneous and product variability could have been a factor influencing the final results, even more so than with small scale wine fermentations.

Table 5.4 Biogenic amines and their precursor amino acid concentrations (mg/L) present in the synthetic medium before inoculation. Compounds most relevant to this study are highlighted. Analyses are shown for the modified MS300 (mMS300) and for each complex nutrient dissolved in mMS300. Concentrations represent the average of analyses performed in duplicate.

Compound mg/L	mMS300	Nutrivin in mMS300	Nutrivin Super in mMS300	FermaidK in mMS300	Optimalo Plus in mMS300	Bactiv-aid in mMS300
Alanine	1.015	0.871	1.275	1.767	1.649	1.148
Threonine	0.728	0.237	0.172	0.336	0.403	0.258
Serine	1.197	0.381	0.214	0.407	0.507	0.290
Glutamine	nd	0.617	nd	nd	nd	0.714
Arginine	nd	0.747	0.699	0.764	0.349	0.430
Tryptophan	nd	nd	nd	nd	nd	nd
Glutamic acid	3.303	3.503	3.972	2.862	2.381	3.320
Valine	nd	0.524	0.148	0.536	0.775	0.572
Histidine	nd	0.044	nd	nd	nd	0.109
Aspartic acid	nd	0.884	1.306	1.025	0.489	0.005
Lysine	0.834	0.668	0.378	0.514	0.658	0.526
Proline	nd	0.154	1.050	0.393	0.387	0.444
Methionine	nd	0.019	nd	nd	0.044	0.125
Tyrosine	nd	0.204	nd	nd	0.242	0.362
Cysteine	nd	nd	nd	nd	nd	nd
Isoleucine	2.926	0.395	0.215	0.580	0.524	0.239
Phenylalanine	nd	0.167	0.039	nd	0.218	0.185
Ornithine	nd	0.051	0.018	0.456	0.432	0.482
Histamine	nd	nd	nd	0.002	nd	nd
Putrescine	nd	nd	0.003	0.019	0.012	nd
Spermidine	0.060	0.081	0.094	0.014	0.026	0.041
Tyramine	nd	0.000	0.000	0.013	0.005	nd
Cadaverine	nd	nd	nd	0.015	0.023	nd

nd: not detected

5.3.2.2 Chemical profile of finished synthetic wine

In order to evaluate to what extent growth and metabolism of the yeasts and lactic acid bacteria inoculated into the synthetic medium occurred, a routine analysis of chemical components was performed at the end of the experimental time. Chemical data of the treatments in the synthetic medium could only be extracted at the end of the

experimental time, since the volume used for this small scale screening was too little to allow for more than one measurement of standard chemical compounds by FT-IR. The end of alcoholic fermentation was therefore simply taken to be at the time when no more gas (CO_2) formation could be observed. Lactobacilli were allowed to grow for 14 days. It is highly unlikely that *Lactobacillus* species belonging to the natural lactic acid bacteria flora present in wine would grow for this long before natural *O. oeni* strains would dominate spontaneous malolactic fermentation in wine. Thus, 14 days was regarded as sufficient to observe the potential contribution of these *Lactobacillus* species to biogenic amine formation in a wine-like medium.

Chemical analysis indicated that the spiked *Lactobacillus* species were able to perform malolactic fermentation (convert L-malic acid to L-lactic acid) to some extent in some treatments (**Table 5.5**). Malic acid was initially added at 6 g/L to the synthetic medium. Residual malic acid was the lowest in treatment 3 (1.45 g/L) with a corresponding highest value of lactic acid (3.65 g/L). Comparing the residual malic acid of treatments 5 to 12 (5.13 to 5.96 g/L) to that of control treatments 1 and 4 where no lactic acid bacteria were present (7.19 and 9.37 g/L) it can be concluded that malolactic fermentation proceeded to a small extent in treatments (see also lactic acid concentration). It appears as if though an additional amount of malic acid was produced in treatments 1 and 4. Malic acid could be produced during sugar metabolism by yeasts by the reductive arm of the tricarboxylic acid cycle which is active under fermentative conditions (Ribéreau-Gayon *et al.*, 2006). However, the decrease of malic acid does not correspond to an equal increase in lactic acid as converted by lactic acid bacteria. It is possible that small quantities of malic acid could be degraded by *Saccharomyces* yeasts during alcoholic fermentation (Redzepovic *et al*., 2003).

Other observations worth noting in the chemical data table (**Table 5.5**) are the highest production of volatile acid (VA), higher pH and lower total acid in treatment 3 when only the *Lactobacillus* species were incubated in the mMS300 without any complex nutrients or yeast. Acetic acid (the main component of volatile acid) formation correlated with the high fructose utilisation by lactic acid bacteria in the absence of yeast. The residual sugar concentrations (100 g/L of each of glucose and fructose was initially included in the synthetic medium) could also be used as an indicator of the success of fermentation treatments. The highest residual sugar concentrations remained in the control treatment 1 where no microorganisms were included by inoculation; and where ethanol production was correspondingly low. However, contamination to a small extent with yeast or fungi was noted and could have caused the 3% and 9% v/v ethanol increase in control treatments 1 and 3.

Table 5.5 Chemical data obtained by FT-IR of treatments in synthetic medium at the end of the experiment. Concentrations represent the average of duplicate treatments and standard deviations are shown.

Treatment	pH	VolatileAcid (g/L)	MalicAcid (g/L)	LacticAcid (g/L)	Glucose (g/L)	Fructose (g/L)	Ethanol % v/v	Glycerol (g/L)
1	3.66±0.06	0.36±0.04	9.37±0.34	0.00±0.00	67.04±12.72	79.83±9.26	3.04±1.17	22.70±3.37
2	3.70±0.00	0.51±0.01	6.22±0.40	0.51±0.13	5.08±4.77	6.19±5.75	10.92±0.26	8.74±1.17
3	3.92±0.02	2.03±0.21	1.45±0.55	3.65±0.28	11.82±1.48	38.82±0.02	9.01±0.16	9.31±0.36
4	3.60±0.03	0.49±0.10	7.19±0.40	0.02±0.03	0.72±0.55	2.79±2.32	11.20±0.00	7.74±0.08
5	3.62±0.03	0.42±0.03	5.65±0.20	0.77±0.10	0.55±0.30	0.44±0.10	11.23±0.08	8.18±0.08
6	3.61±0.01	0.42±0.01	5.79±0.08	0.69±0.04	0.47±0.12	0.26±0.01	11.17±0.14	8.14±0.10
7	3.67±0.00	0.45±0.01	5.93±0.15	0.65±0.12	0.61±0.03	0.57±0.01	11.36±0.02	7.86±0.11
8	3.68±0.01	0.42±0.01	5.79±0.19	0.67±0.13	0.52±0.06	0.58±0.01	11.38±0.04	7.92±0.04
9	3.73±0.01	0.52±0.01	5.13±0.04	1.02±0.03	0.29±0.05	0.02±0.03	11.34±0.18	8.03±0.11
10	3.69±0.00	0.47±0.00	5.60±0.05	0.79±0.09	0.15±0.01	0.00±0.12	11.20±0.16	8.19±0.06
11	3.74±0.01	0.55±0.01	5.37±0.14	1.00±0.07	0.40±0.07	0.28±0.02	11.48±0.06	7.90±0.08
12	3.69±0.03	0.54±0.00	5.96±0.30	0.58±0.25	0.38±0.12	0.23±0.30	11.24±0.15	7.64±0.06

5.3.2.3 Biogenic amine production in synthetic wine

Figures 5.8 to **5.11** represents the concentrations of each biogenic amine detected in samples of the synthetic must, at the end of alcoholic fermentation (AF) and at the end of the 14 day growth period of lactic acid bacteria (LAB) in the synthetic wine. The concentrations represent the average of duplicates for each treatment. In order to simplify figures, treatments that were supplemented with the same complex yeast nutrient are presented successively in the same colour. Refer to **Table 5.2** for a description of each treatment number.

It seems as if though the *Lactobacillus* species produced much higher levels of histamine and tyramine when their growth was not preceded by the growth and fermentation of yeast (treatment 3; **Figures 5.8** and **5.9**). Also, the absence of any complex nutrients seemed to stimulate histamine and tyramine formation by *Lactobacillus* species (treatment 3). Whether complex nutrients were absent from the medium (treatment 2) or present in any combination or alone, similar concentrations (histamine and tyramine) were reached between treatments in the presence of both yeast and bacteria.

When no lactic acid bacteria were present (treatments 1 and 4), no significant production of any biogenic amines took place in this study. It can therefore be deduced that any major contribution to the increase of biogenic amines was contributed by the *Lactobacillus* species and that they likely produce more histamine and tyramine in an environment that lacks complex nutrients. Lactic acid bacteria produce biogenic amines in order to generate metabolic energy or regulate (increase) the pH of the growth medium (Molenaar *et al*., 1993). How they obtained the relevant precursors cannot be explained at this stage.

More putrescine was produced in treatments that were supplemented with complex nutrients than in control treatments 2 and 3 containing *Lactobacillus* species but no added nutrients (**Figure 5.10**). No significant amounts of putrescine were produced in the absence of the lactic acid bacteria (treatments 1 and 4). Therefore, all of the tested nutrient combinations had an influence on putrescine production, with bacterial nutrients added alone (treatments 11 and 12) leading to a slightly higher production when compared to any combination of yeast and bacterial nutrients. Unfortunately the impact of yeast nutrients alone was not evaluated in synthetic medium as was done in the experiment in wine.

Again, it appears that the yeast NT202 produced low levels of cadaverine during alcoholic fermentation (treatments 2 and 4), more so in the presence of complex yeast nutrients (treatments 5 to 10) (**Figure 5.11**). After the introduction of *Lactobacillus* species an increase of cadaverine for all treatments where *Lactobacillus* species were present could be observed. As with histamine and tyramine, it appears that the *Lactobacillus* species produced much higher levels of cadaverine when their growth was not preceded by the growth and fermentation of yeast. Comparison between treatment 2 (no complex nutrients in the presence of yeast) and treatments 11 and 12 show that the nutrients could have influenced cadaverine production by lactic acid bacteria when yeast was also present (**Figure 5.11**).

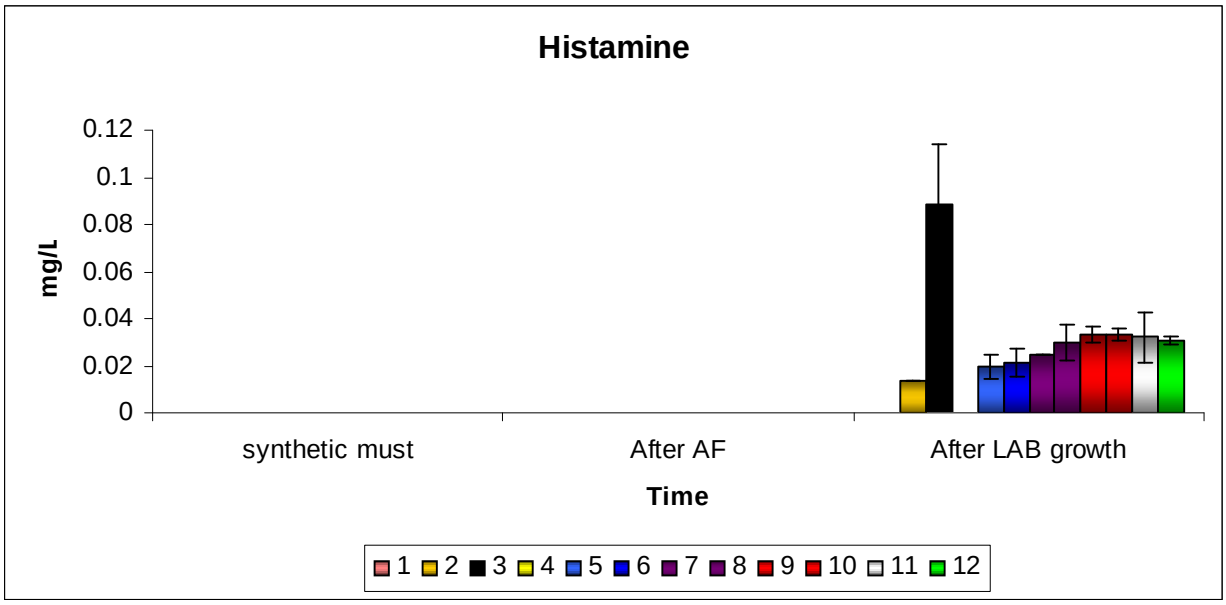

Figure 5.8 Histamine concentrations measured in treatments 1 to 12 (refer to the text for the treatment descriptions) in the synthetic must (modified MS300), at the end of alcoholic fermentation and after a 14 day growth period of decarboxylase positive *Lactobacillus* species. The concentrations represent the average of duplicates for each treatment.

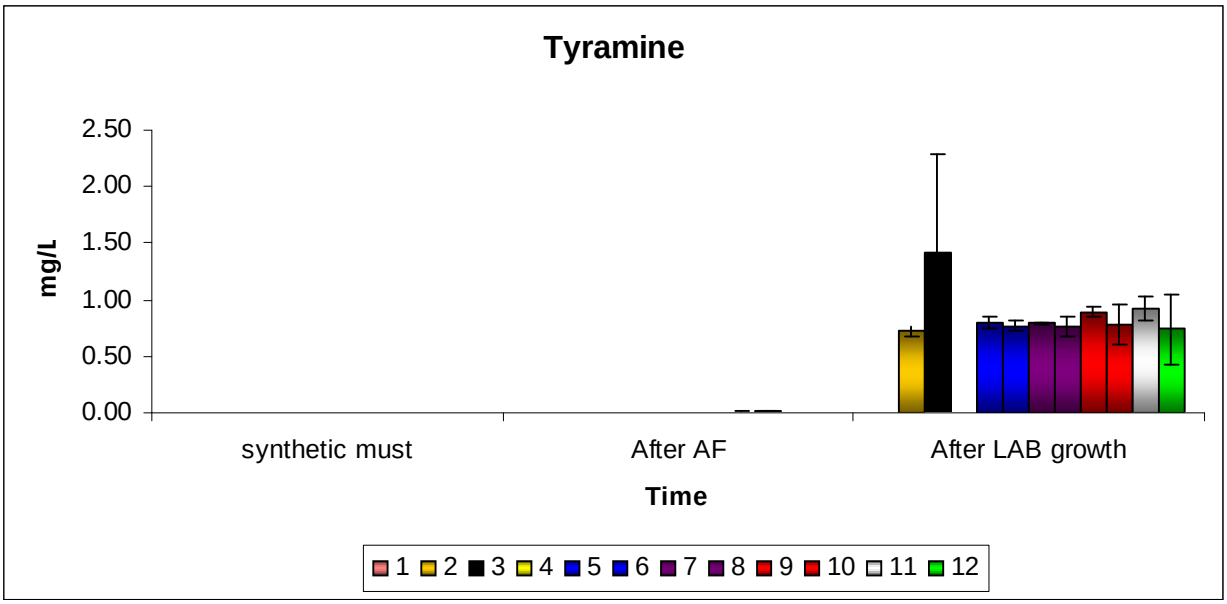

Figure 5.9 Tyramine concentrations measured in treatments 1 to 12 (refer to the text for the treatment descriptions) in the synthetic must (modified MS300), at the end of alcoholic fermentation and after a 14 day growth period of decarboxylase positive *Lactobacillus* species. The concentrations represent the average of duplicates for each treatment.

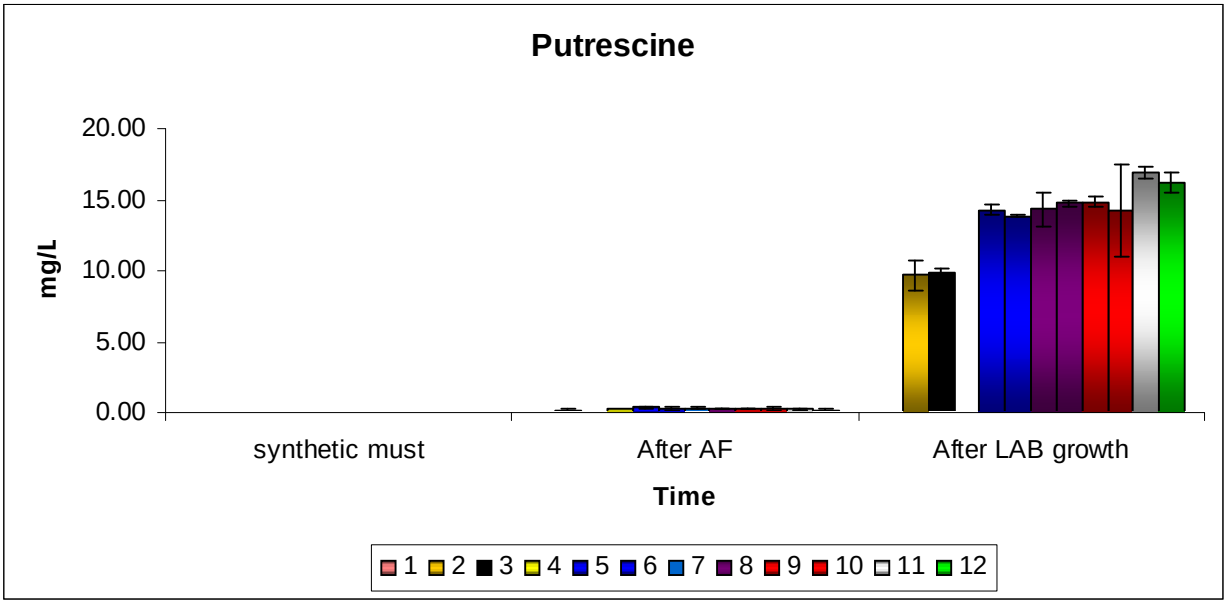

Figure 5.10 Putrescine concentrations measured in treatments 1 to 12 (refer to the text for the treatment descriptions) in the synthetic must (modified MS300), at the end of alcoholic fermentation and after a 14 day growth period of decarboxylase positive *Lactobacillus* species. The concentrations represent the average of duplicates for each treatment.

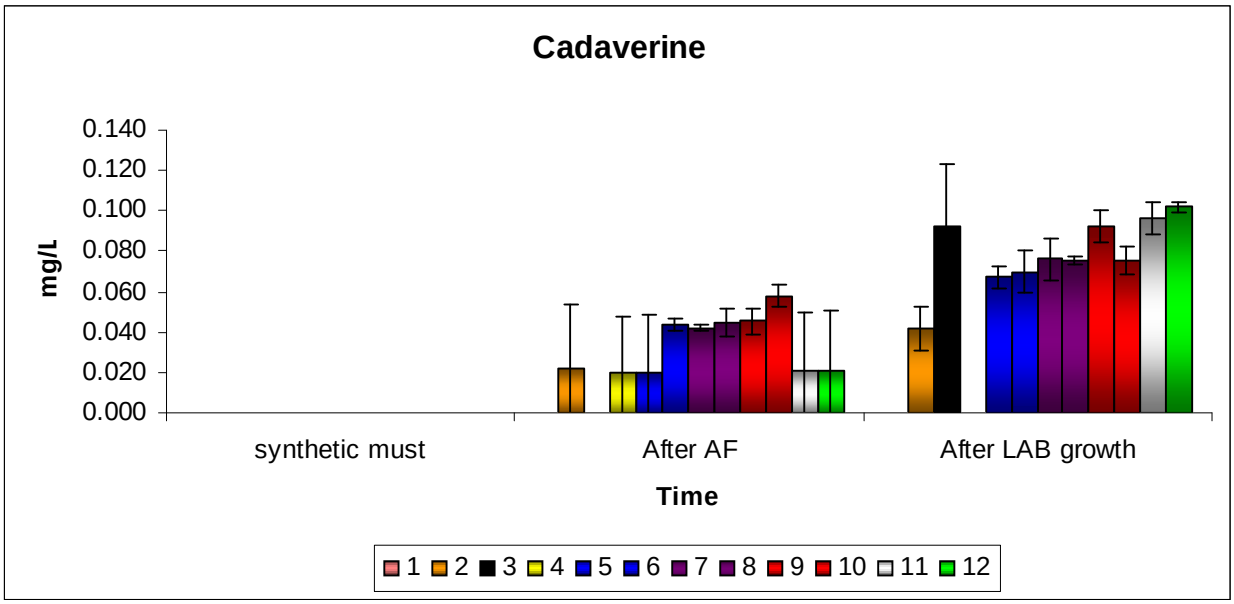

Figure 5.11 Cadaverine concentrations measured in treatments 1 to 12 (refer to the text for the treatment descriptions) in the synthetic must (modified MS300), at the end of alcoholic fermentation and after a 14 day growth period of decarboxylase positive *Lactobacillus* species. The concentrations represent the average of duplicates for each treatment.

5.4 CONCLUSION

During this study, a few trends regarding biogenic amine production in relation to complex yeast and bacterial nutrients could be observed. The production of relatively high levels of histamine in wine during or after malolactic fermentation (by decarboxylase positive lactic acid bacteria) as a result of complex nutrient addition could significantly impact the wholesomeness of the wine as well as have negative trade implications. However, the trend observed in the small scale wine fermentations was not confirmed in the fermentations in synthetic medium. More experiments with more control treatments will be necessary before any conclusive deductions should be made. Wine is a complex medium in which many compounds and microorganisms can inhibit the growth and metabolisms of one another or produce synergistic effects that are not observable in a simple chemically defined synthetic medium. It has been observed in a number of studies that biogenic amine production by wine microorganisms in various synthetic media can show a different set of results when evaluated in wine (Guerrini *et al*., 2002; Granchi *et al*., 2005; Landete *et al*., 2007).

The results obtained in this study in wine are in accordance with a similar study reported in the literature that examined the impact of yeast autolysate, a component of complex yeast nutrients, on biogenic amine production during winemaking. The enrichment of Chardonnay must by yeast autolysate did not lead to an increase of biogenic amines during alcoholic fermentation. However, the concentration of biogenic amines, particularly tyramine and cadaverine, were higher after malolactic fermentation. Tyrosine and lysine were the only amino acids that are also biogenic amine precursors

that were detected in free form after addition of the yeast autolysate to the must (Gonzalez Marco *et al*., 2006).

Another study that yielded similar results to those obtained in this study with regards to yeast nutrients was performed by Marques *et al.* (2007), who tested two commercial fermentation activators. A nutritive factor used for alcoholic fermentation was added to the must, and a nutritive factor used for malolactic fermentation was added at the end of alcoholic fermentation to the respective treatments. In this study, a slight increase in biogenic amines (particularly isoamylamine and tyramine) resulted from the addition of the alcoholic fermentation activator. This increase was especially noticeable at the end of malolactic fermentation. No significant differences could be observed between wines to which the malolactic fermentation activator were added and the control wines.

The validity of the experiment performed in this study in the synthetic medium should also be evaluated. When working on such a small scale, the heterogeneity of the chemically unspecified complex nutrients may largely influence the results. Also, the solubility of the products in the synthetic medium can be questioned. The nitrogen composition of the different complex nutrients should rather be submitted for analysis with the nutrients dissolved in water and/or wine and should include free amino acids as well as larger peptide or protein fractions. A relationship seems to exist between yeast and bacterial growth and biogenic amine concentrations produced in the synthetic medium. It appears from this experiment in synthetic medium that the absence of complex nutrients (the medium composition) has a more pronounced impact on biogenic amine production than the presence of any complex fermentation nutrients.

On the whole, it does not seem that complex nutrient preparations produced for the use with fermentation starter cultures pose a serious threat to biogenic amine production by indigenous microorganisms, in particular lactic acid bacteria. The contribution of the yeast strain NT202 to biogenic amine production in this experiment seemed to be minimal.

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6. RESEARCH RESULTS

Preliminary screening of commercial yeast starter cultures for biogenic amine production

6.1 INTRODUCTION

Commercial yeast starter culture preparations of pure *Saccharomyces cerevisiae* strains are selected for certain positive attributes over indigenous species (wild yeasts). These attributes include a steady and reliable alcoholic fermentation; a positive and predictable sensory profile; low production of SO_2, sulphur compounds and volatile acids; higher resistance to unfavourable fermentation conditions such as extreme temperatures, high sugar concentrations, high alcohol concentrations and low nutrient content. Spontaneous alcoholic fermentations or the use of mixed starter cultures that may contain a variety of yeast species (including non-*Saccharomyces*), may compromise some positive characteristics of pure strain inoculated fermentations, but greater flavour diversity can be obtained in the final product.

In general, lactic acid bacteria are the wine microorganisms mainly held responsible for biogenic amine production in wine, particularly during and after malolactic fermentation. Any contribution by yeast to biogenic amine formation in wine could be indirect, according to Soufleros *et al.* (1998). Yeasts could alter the composition of fermenting musts and wines by utilising some amino acids and secreting others during alcoholic fermentation and autolysis, thereby changing the concentration of precursor amino acids in the wine that can be used by other microorganisms in subsequent fermentation steps to form biogenic amines. The variation in the concentration of biogenic amines formed in wine could also be ascribed to differences in yeast amine metabolism since yeast strains are able to produce or use amines to different extents as a source of nitrogen (Goñi & Ancín Azpilicueta, 2001). However, it is possible that yeasts could directly contribute to biogenic amine formation in wine.

Little experimental data have been reported in the literature regarding the potential of yeasts in general, and *Saccharomyces cerevisiae* in particular, to produce biogenic amines. A number of authors have reported the potential of yeasts to produce biogenic amines based on an increase (Buteau *et al.*, 1984; Goñi & Ancín Azpilicueta, 2001; Torrea & Ancín, 2002; Garde-Cerdán *et al.*, 2006) or a decrease or absence (Herbert *et al.*, 2005; Granchi *et al.*, 2005; Marcobal *et al.*, 2006) of these compounds during commercial or industrial alcoholic fermentations.

A study with one of its aims to determine the potential of biogenic amine production of yeasts was performed by Landete *et al.* (2007), who screened 36 strains of yeast isolated from must and wine. The yeasts tested included strains belonging to the genera *Aureobasidium*, *Candida*, *Hanseniaspora*, *Hansenula*, *Kloeckera*, *Metschnikowia*, *Pichia*, *Rhodotorula* and strains of the species *Saccharomyces cerevisiae*,

Saccharomyces cerevisiae var. *bayanus*, *Saccharomyces cerevisiae* var. *chevalieri* and *Saccharomyces cerevisiae* var. *steiner*. No biogenic amines were produced by any of the yeast strains tested under the conditions present in this screening test; in synthetic medium (YPD supplemented with amino acid precursors and pyridoxal 5'-phosphate), grape must or wine.

Two particular studies raise interest in the ability of *Saccharomyces cerevisiae* to contribute to biogenic amine production in wine. Caruso *et al.* (2002) and Granchi *et al.* (2005) both screened 50 yeast strains isolated from grapes and/or wine, representing five different genera/species (*Saccharomyces cerevisiae, Kloeckera apiculata, Candida stellata, Metschnikowia pulcherrima* and *Brettanomyces bruxellensis*) for biogenic amine production in two independent studies. *Brettanomyces bruxellensis* formed the highest concentration of total biogenic amines in sterile grape must under laboratory condition in both studies (15 mg/L to 20 mg/L), followed by *Saccharomyces cerevisiae* (12 mg/L to 14 mg/L). It is proposed that the ability of *Saccharomyces cerevisiae* to produce biogenic amines is strain dependent and not a constant characteristic of the species.

Whether wine yeasts are able to produce biogenic amines such as histamine, tyramine, cadaverine and phenylethylamine is unclear and seems poorly researched or unreported in literature. The only available information regarding decarboxylase enzymes is for ornithine decarboxylase (ODC) belonging to *Saccharomyces cerevisiae* species in general. ODC is responsible for the conversion of ornithine to putrescine. Putrescine serves primarily as a precursor for spermidine and spermine biosynthesis in yeast (Cohn *et al.,* 1980). These polyamines are an absolute requirement for optimal growth in yeasts, in particular for meiotic sporulation and for maintenance of the double-stranded RNA killer plasmid (Cohn *et al.*, 1978). Yeast ornithine decarboxylase activity requires pyridoxal 5'-phosphate for activity and is rapidly decreased by the presence of spermine and spermidine (Tyagi *et al.*, 1981).

This study aimed at using a controlled experiment in a chemically defined medium to screen for the potential of commercial *Saccharomyces cerevisiae* starter cultures, as well as a few mixed yeast starter cultures, to produce biogenic amines. This simple screening test provides an indication of which strains to use in further evaluation steps in a more complex medium such as wine.

6.2 MATERIALS AND METHODS

6.2.1 YEAST STRAINS, SYNTHETIC MEDIA COMPOSITION AND CHEMICALS

Thirty one commercial preparations (mostly *Saccharomyces cerevisiae* strains) were screened for biogenic amine production in a synthetic medium. The strains tested are listed in **Table 6.1**.

Table 6.1 Commercial *Saccharomyces cerevisiae* strains screened, listed according to manufacturer. The strains from Chr. Hansen denoted *nsac* consist of *Saccharomyces cerevisiae* strains in combination with non-*Saccharomyces* strains.

Anchor Yeast	Lallemand	Maurivin Yeasts	Chr. Hansen	Springer Oenologie
VIN 13	L2056	BP 725	RUBY.ferm	Saint Georges S101
VIN 7	L2323	AWRI 796	MELODY.nsac	
NT 45	L2226	EP2	HARMONY.nsac	
NT 50	EC-1118	AWRI 350	RHYTHM.nsac	
NT 112	RHONE4600		MERIT.ferm	
NT 116	BM45		SYMPHONY.nsac	
NT 202	ICV-D80			
N 96	CrossEvolution™			
WE 372	DV10			
WE 228				
WE 14				

The synthetic medium used in this screening experiment was MS300 (Bely *et al.*, 1990) with the following modifications: 0.005% w/v of pyridoxal 5'-phosphate (Sigma-Aldrich, Germany) was added to the medium and the stock of amino acids was omitted. The pH of the medium was adjusted with sodium hydroxide to pH 3.3 and pH 3.5 for the relevant treatments. 200 mg/L of each amino acid (L-tryptophane, L-lysine-monohydrochloride, L-ornithine hydrochloride, L-arginine, L-tyrosine, L-histidine and L-phenylalanine) was added individually to the synthetic medium in separate aliquots; in order to evaluate the consumption of each amino acid by each yeast strain separately. Thus in total, each of the 31 yeast strains was evaluated in seven different synthetic media, each containing a single amino acid.

For the screening test, all yeast strains except those from Chr. Hansen were precultured on YPD agar plates (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose, 2% w/v agar) (Biolab, Merck, South Africa), after which a single colony was inoculated into YPD broth and precultured aerobically for 24 hours at 30°C on a shaker. A 1% v/v inoculum of each yeast culture was inoculated into each synthetic medium. Commercial preparations from Chr. Hansen consisting of mixed yeast cultures were inoculated directly into the synthetic medium at a 1% w/v inoculum. The screening test was conducted in test tubes in aliquots of 5 ml each. Only single treatments were performed (no duplicates) for this first screening test. Yeast cell growth and/or fermentation were allowed to proceed for 11 days under static semi-anaerobic conditions at 30°C, with occasional agitation.

All chemicals used for the preparation of MS300 were purchased from Saarchem, Merck, South Africa except for malic acid, glucose and ammonium chloride (Sigma-Aldrich, Germany). All amino acids were purchased from Sigma-Aldrich.

6.2.2 CONTROL TREATMENTS

Each of the seven synthetic media containing a single amino acid precursor was incubated without any inoculated yeast along with the treatments to serve as a control treatment. As an additional control, six yeast strains (NT202, WE372, EC1118, L2226, NT45 and NT50) were inoculated in separate aliquots into the modified MS300 medium containing no amino acids, at pH 3.3 and pH 3.5. Each of these six strains were precultured both in YPD broth and in a minimal synthetic defined (SD) medium (containing 6.7 g/L yeast nitrogen base (Difco) with only ammonium as nitrogen source, and 20 g/L glucose) in the same manner as previously described.

6.2.3 BIOGENIC AMINE ANALYSIS

Biogenic amines (tyramine, tryptamine, putrescine, cadaverine, spermidine, histamine and phenylethylamine) were quantified in the synthetic medium using liquid chromatography mass spectrometry (LCMS) as described by Millán *et al.* (2007).

6.3 RESULTS AND DISCUSSION

6.3.1 CONTROL TREATMENTS

Tables 6.2 and **6.3** show data useful to compare the influence of two different preculture media (SD medium and YPD broth) and two pH levels (pH 3.3 and pH 3.5) on biogenic amine production by six selected yeast strains (NT202, WE372, EC1118, L2226, NT45 and NT50). From **Table 6.2** it seems as if either the yeast strain used for this control test (NT50) is able to produce high amounts of biogenic amines (particularly putrescine) when cultured in YPD and/or under aerobic conditions, or the complex YPD medium itself has the potential to contain these biogenic amines. The yeast extract contained in YPD is a rich source of complex nitrogen obtained from yeast cells. Putrescine, which performs physiological functions within the yeast cell, could be released during yeast autolysis and therefore be contained at high levels within yeast extract preparations. More strains will need to be tested for biogenic amine production in YPD, along with an uninoculated control, before further conclusions can be drawn. Nevertheless, the potential exists that these amines could be carried over directly or indirectly from one culture medium to the next. The biogenic amine concentrations (mg/L) presented in **Tables 6.2** and **6.3** are for histamine (His), phenylethylamine (Phe), tryptamine (Try), tyramine (Tyr), putrescine (Put), cadaverine (Cad) and spermidine (Spe).

Table 6.2 Analysis of histamine (His), phenylethylamine (Phe), tryptamine (Try), tyramine (Tyr), putrescine (Put), cadaverine (Cad) and spermidine (Spe) for each of the two preculture media itself after the yeast strain NT50 was precultured therein for 24 hours on a shaker. Biogenic amine concentrations are given in mg/L.

Preculture medium	Tyr	Try	Put	Cad	Spe	His	Phe
SD	nd	nd	nd	0.01	nd	nd	nd
YPD	4.47	0.872	55.30	nd	0.21	1.625	0.739

nd: not detected

Table 6.3 Concentrations (mg/L) of histamine (His), phenylethylamine (Phe), tryptamine (Try), tyramine (Tyr), putrescine (Put), cadaverine (Cad) and spermidine (Spe) produced by the yeast strains NT202, WE372, EC1118, L2226, NT45 and NT50 in the modified MS300 medium containing no amino acid precursors. Each yeast strain was tested after being precultured in two media (SD broth and YPD broth) at two pH values (pH 3.3 and pH 3.5).

Preculture medium	pH	Yeast strain	Tyr	Try	Put	Cad	Spe	His	Phe
NONE	3.5	NONE	nd	nd	nd	nd	nd	nd	nd
NONE	3.3	NONE	nd	nd	nd	nd	nd	nd	nd
SD	3.5	EC1118	nd	nd	nd	nd	nd	nd	nd
SD	3.3	EC1118	nd	nd	nd	nd	nd	nd	nd
SD	3.5	NT50	nd	nd	0.40	nd	nd	nd	nd
SD	3.3	NT50	nd	nd	nd	nd	nd	nd	nd
SD	3.5	NT45	nd	nd	nd	nd	nd	nd	nd
SD	3.3	NT45	nd	nd	nd	nd	nd	nd	nd
SD	3.5	L2226	nd	nd	nd	0.08	nd	nd	nd
SD	3.3	L2226	nd	nd	nd	nd	nd	nd	nd
SD	3.5	NT202	nd	nd	nd	nd	nd	nd	nd
SD	3.3	NT202	nd	nd	nd	0.01	nd	nd	nd
SD	3.5	WE372	nd	nd	nd	nd	nd	nd	nd
SD	3.3	WE372	nd	nd	nd	nd	nd	nd	nd
YPD	3.5	EC1118	0.07	nd	0.92	0.14	nd	0.007	nd
YPD	3.3	EC1118	0.10	nd	0.99	0.29	nd	0.014	nd
YPD	3.5	NT50	0.08	nd	1.13	0.19	nd	0.006	0.001
YPD	3.3	NT50	0.09	nd	2.11	0.24	nd	0.017	0.005
YPD	3.5	NT45	0.10	nd	1.26	0.17	nd	0.013	nd
YPD	3.3	NT45	0.09	nd	1.07	0.21	nd	0.012	nd
YPD	3.5	L2226	0.08	nd	0.41	0.18	nd	0.017	nd
YPD	3.3	L2226	0.07	nd	0.23	0.15	nd	0.016	nd
YPD	3.5	NT202	0.08	nd	0.66	0.23	nd	0.017	0.007
YPD	3.3	NT202	0.10	nd	0.76	0.17	nd	0.019	nd
YPD	3.5	WE372	0.09	nd	0.33	0.21	nd	0.008	0.008
YPD	3.3	WE372	0.09	nd	0.50	0.22	nd	0.012	nd

nd: not detected

Comparisons can also be drawn between biogenic amine production from the modified MS300 medium containing no added amino acids (**Table 6.3**) and the media that contain the different precursor amino acids (data table not shown). Thus, the contribution to biogenic amine formation from the base medium and from each added amino acid can be calculated. It thus seems that certain yeast strains could produce certain biogenic amines in the absence of the precursor amino acid. Putrescine

production will be used as an example since it was produced most consistently and at the highest concentrations in this study. **Figure 6.1** is a representation of putrescine production from all the treatments by the six selected yeast strains.

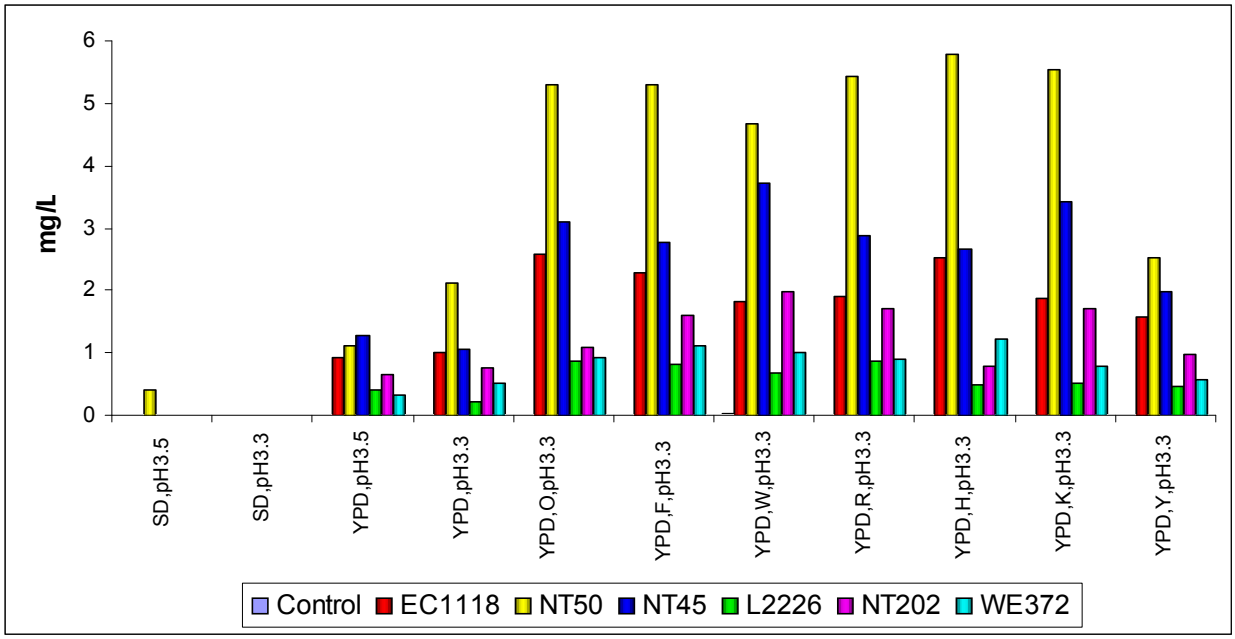


Figure 6.1 Putrescine production by the yeast strains NT202, WE372, EC1118, L2226, NT45 and NT50 in the modified MS300 media (mMS300). Each yeast strain was evaluated for putrescine production in mMS300 without any added amino acid precursors, after being precultured in both SD broth and YPD broth respectively at pH 3.3 and pH 3.5. In addition, the putrescine concentrations are shown as produced by each strain (precultured in YPD) in the synthetic media at pH 3.3 with individually added amino acid precursors: ornithine (O), phenylalanine (F), tryptophane (W), arginine (R), histidine (H), lysine (K) and tyrosine (Y).

No putrescine was produced in the medium containing no amino acids by any strains if they were precultured in a minimal medium (SD broth), with the exception of 0.4 mg/L putrescine produced by NT50. NT50 is the yeast strain that produced the highest concentrations of putrescine in all media in this study (**Figures 6.1** and **6.2**). When precultured in YPD, low levels of putrescine, tyramine, cadaverine and histamine but no spermidine were produced by the six strains in the medium containing no amino acid precursors (**Table 6.3**). Putrescine, tyramine, spermidine and cadaverine were produced in the media containing precursors (**Figure 6.2**, data table not shown).

The difference between biogenic amine production at pH 3.5 compared to pH 3.3 in the medium containing no amino acid precursors did not seem significant enough to repeat all treatments at both pH values.

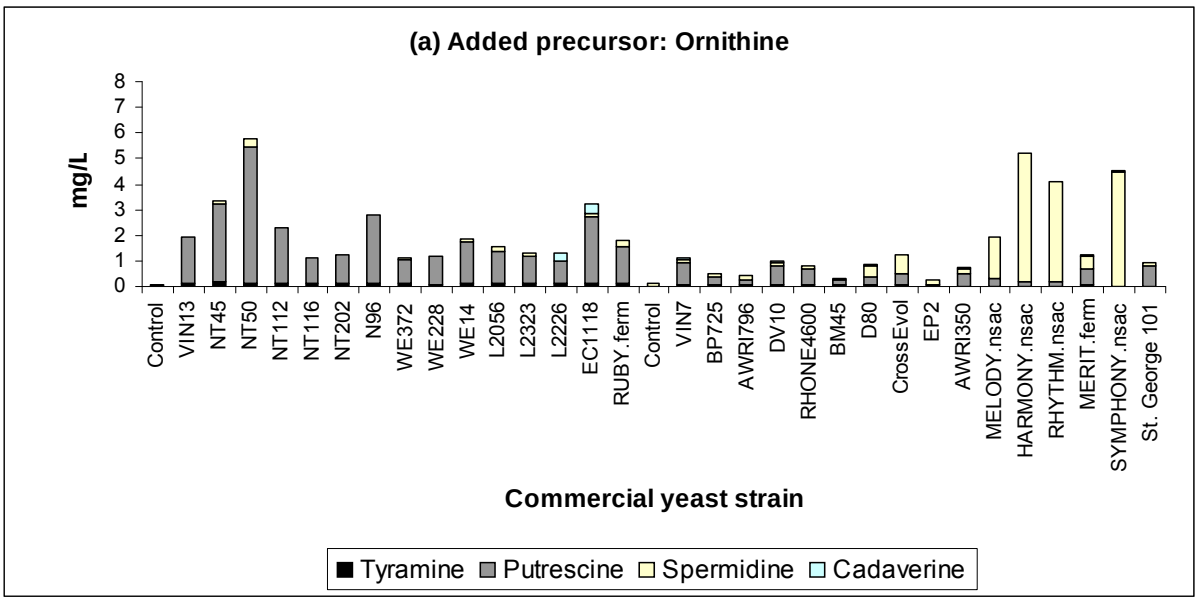
The average contribution to putrescine production from the medium containing no amino acid precursors was 48% and ranged from 38% (NT45) to 60% (NT202). From this it can be deduced that only 40% to 62% of putrescine production could potentially be contributed by the presence of the amino acids, regardless of whether or not the particular precursors of putrescine (arginine and/or ornithine) are present. This observation will be explained in section **6.3.2.**

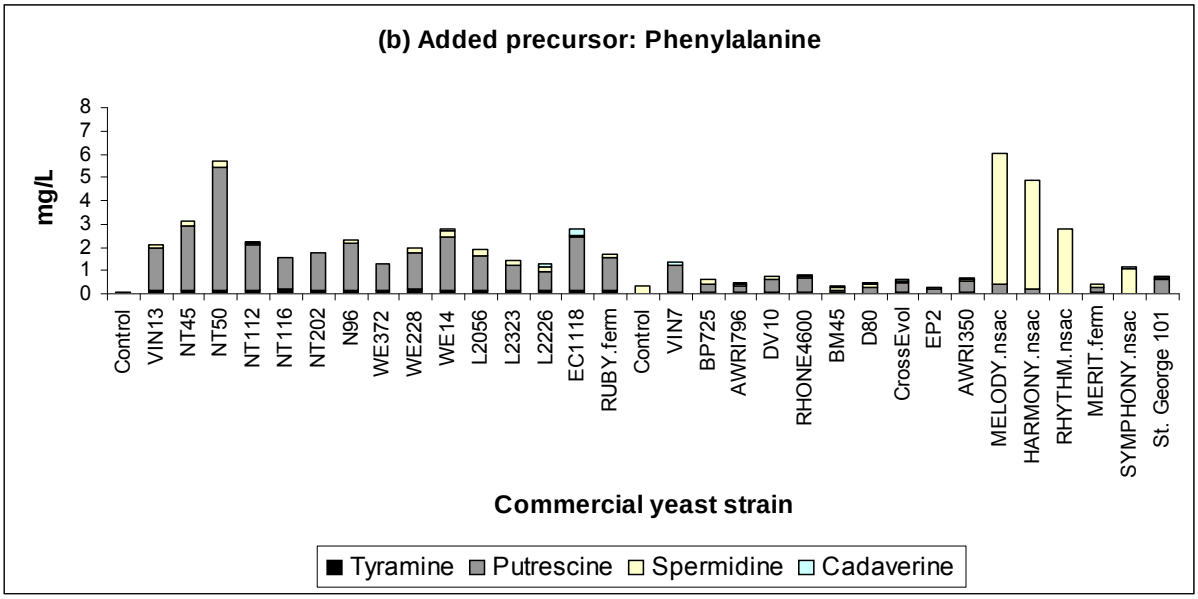
6.3.2 SCREENING OF COMMERCIAL STARTER CULTURES IN SYNTHETIC MEDIA CONTAINTING INDIVIDUAL AMINO ACID PRECURSORS

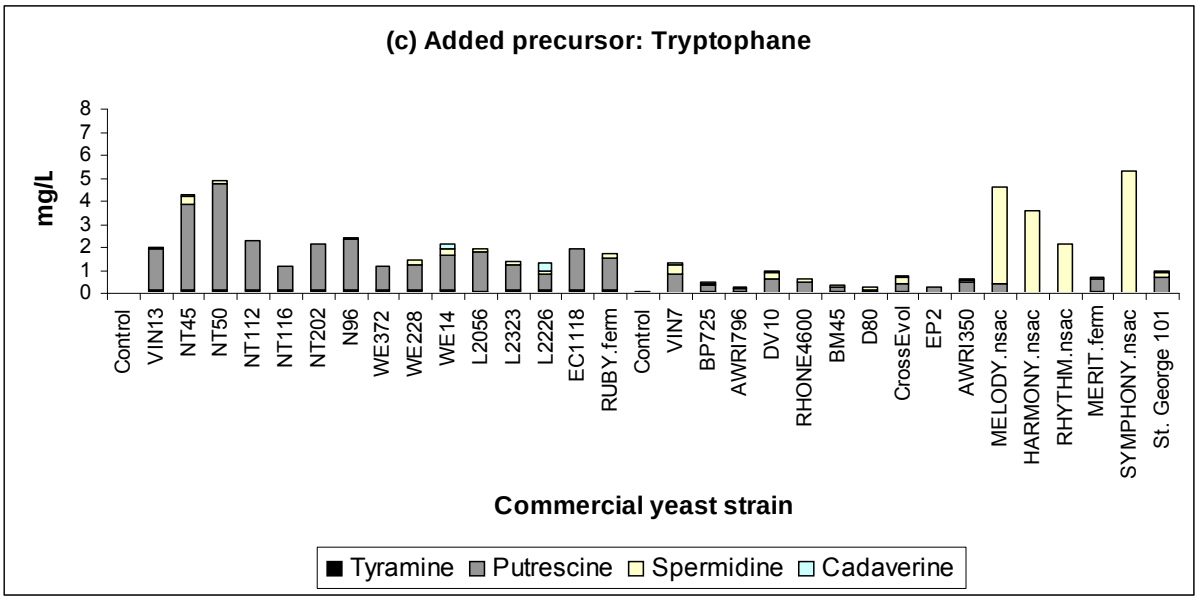
No histamine, phenylethylamine, tryptamine, agmatine or spermine were produced in detectable amounts in any of the synthetic media containing individual amino acid precursors by any of the yeast strains tested. Tyramine, putrescine, cadaverine and spermidine were produced in detectable amounts. **Figure 6.2** shows the relative amounts of biogenic amines produced by each yeast strain for each medium containing a single amino acid precursor. All strains were precultured in YPD broth at pH 3.3 as previously described.

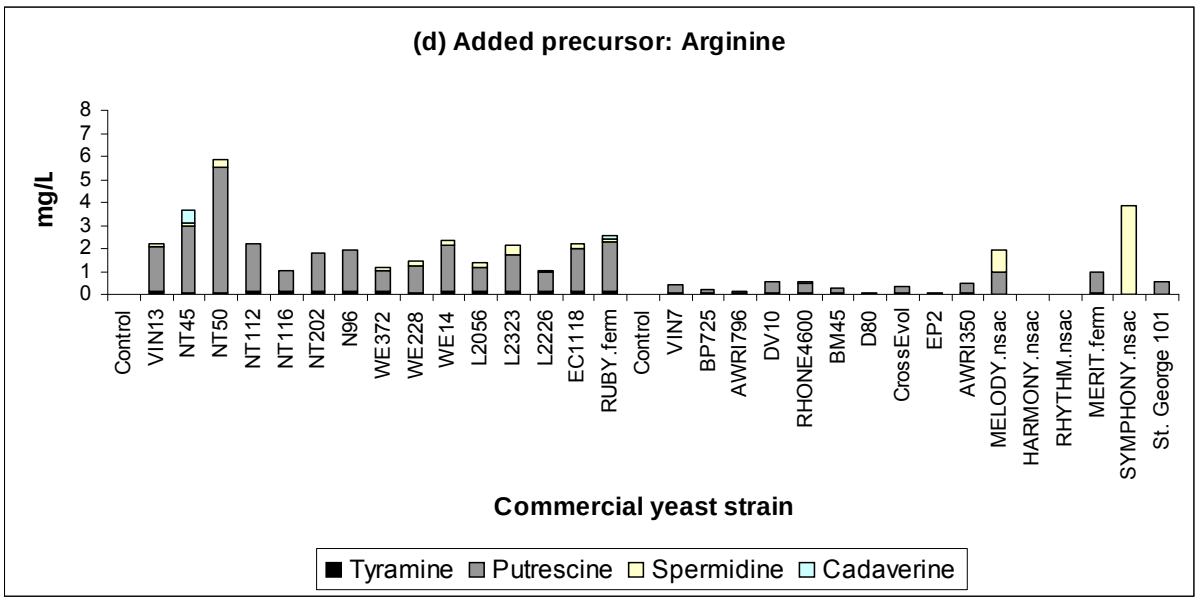
With reference to **Figure 6.2** it seems as if though all commercial yeast strains are able to produce putrescine as preferred product, followed by spermidine, cadaverine and tyramine, regardless of amino acid precursor added. Differences could be observed in biogenic amine concentrations when different amino acid precursors were added. For example when tyrosine was the added amino acid precursor [**Figure 6.2(g)]** the putrescine production by some strains (such as NT45 and NT50) was almost 2 mg/L lower than observed with other amino acid precursors. More spermidine was also produced by most strains. When arginine was added, many strains did not produce biogenic amines [**Figure 6.2(d)**].

Yeasts readily assimilate the ammonium ion and are able to utilise it to catabolise amino acids. Therefore, yeasts do not need to be supplied with amino acids for growth, but are stimulated by amino acids when it is present in the medium. Yeasts can fix an ammonium ion on a carbon skeleton which can be derived from sugar metabolism (glycolysis, the citric acid cycle or the pentose phosphate cycle). Glutamate is produced from an ammonium ion fixed onto α-ketoglutarate. In subsequent transamination reactions, glutamate serves as an amino group donor and pyridoxal 5'-phosphate serves as the cofactor to produce amino acids. The carbon skeletons of amino acids relevant to this study originate from α-ketoglutarate (arginine and ornithine), oxaloacetate (lysine), ribose 5-phosphate (histidine), phosphenolpyruvate and erythrose 4-phosphate (tyrosine, phenylalanine and tryptophane) (Ribérau-Gayon *et al.*, 2006). The ammonium ion is present as 120 mg/L ammonium chloride in the modified MS300 medium, but can also be obtained from available amino acids (such as the individually added precursors) through the transfer of an α-amino group onto α-ketoglutaric acid to form glutamate. Pyridoxine (vitamin B_6), the precursor of pyridoxal 5'-phosphate, as well as pyridoxal 5'-phosphate itself was also included in the modified MS300 medium. Therefore, all the components necessary to synthesize amino acids that could serve as precursors for biogenic amine formation were present in the synthetic medium used in this experiment. Usually, these components will also be present naturally in grape musts.


(a) Added precursor: Ornithine
mg/L
0
1
2
3
4
5
6
7
8
Control
VIN13
NT45
NT50
NT112
NT116
NT202
N96
WE372
WE228
WE14
L2056
L2323
L2226
EC1118
RUBY.ferm
Control
VIN7
BP725
AWRI796
DV10
RHONE4600
BM45
D80
CrossEvol
EP2
AWRI350
MELODY.nsac
HARMONY.nsac
RHYTHM.nsac
MERIT.ferm
SYMPHONY.nsac
St. George 101
Commercial yeast strain
Tyramine
Putrescine
Spermidine
Cadaverine


(b) Added precursor: Phenylalanine
mg/L
0
1
2
3
4
5
6
7
8
Control
VIN13
NT45
NT50
NT112
NT116
NT202
N96
WE372
WE228
WE14
L2056
L2323
L2226
EC1118
RUBY.ferm
Control
VIN7
BP725
AWRI796
DV10
RHONE4600
BM45
D80
CrossEvol
EP2
AWRI350
MELODY.nsac
HARMONY.nsac
RHYTHM.nsac
MERIT.ferm
SYMPHONY.nsac
St. George 101
Commercial yeast strain
Tyramine
Putrescine
Spermidine
Cadaverine


(c) Added precursor: Tryptophane
mg/L
0
1
2
3
4
5
6
7
8
Control
VIN13
NT45
NT50
NT112
NT116
NT202
N96
WE372
WE228
WE14
L2056
L2323
L2226
EC1118
RUBY.ferm
Control
VIN7
BP725
AWRI796
DV10
RHONE4600
BM45
D80
CrossEvol
EP2
AWRI350
MELODY.nsac
HARMONY.nsac
RHYTHM.nsac
MERIT.ferm
SYMPHONY.nsac
St. George 101
Commercial yeast strain
Tyramine
Putrescine
Spermidine
Cadaverine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mg/L
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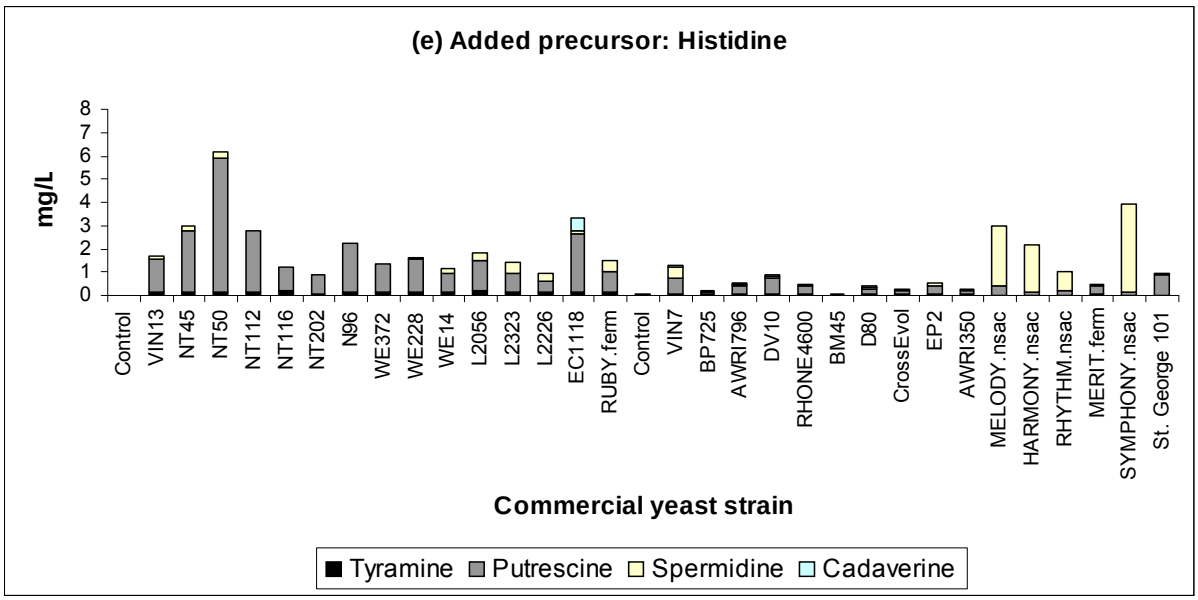
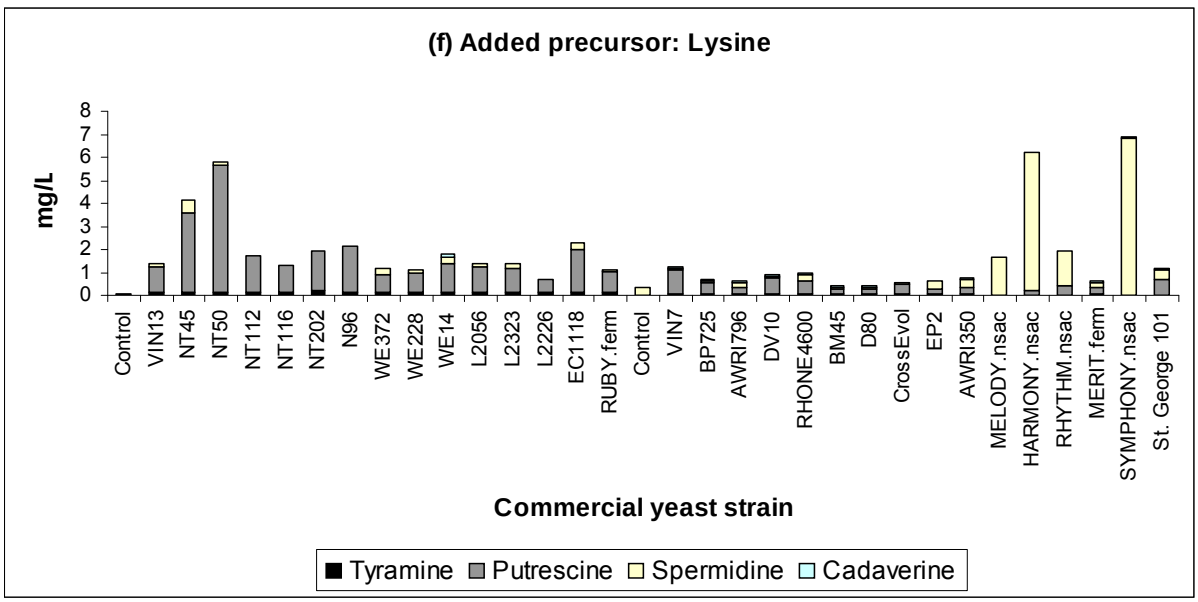
1
2
3
4
5
6
7
8
Control
VIN13
NT45
NT50
NT112
NT116
NT202
N96
WE372
WE228
WE14
L2056
L2323
L2226
EC1118
RUBY.ferm
Control
VIN7
BP725
AWRI796
DV10
RHONE4600
BM45
D80
CrossEvol
EP2
AWRI350
MELODY.nsac
HARMONY.nsac
RHYTHM.nsac
MERIT.ferm
SYMPHONY.nsac
St. George 101
Commercial yeast strain
Tyramine
Putrescine
Spermidine
Cadaverine


(e) Added precursor: Histidine
mg/L
0
1
2
3
4
5
6
7
8
Control
VIN13
NT45
NT50
NT112
NT116
NT202
N96
WE372
WE228
WE14
L2056
L2323
L2226
EC1118
RUBY.ferm
Control
VIN7
BP725
AWRI796
DV10
RHONE4600
BM45
D80
CrossEvol
EP2
AWRI350
MELODY.nsac
HARMONY.nsac
RHYTHM.nsac
MERIT.ferm
SYMPHONY.nsac
St. George 101
Commercial yeast strain
Tyramine
Putrescine
Spermidine
Cadaverine


(f) Added precursor: Lysine
mg/L
0
1
2
3
4
5
6
7
8
Control
VIN13
NT45
NT50
NT112
NT116
NT202
N96
WE372
WE228
WE14
L2056
L2323
L2226
EC1118
RUBY.ferm
Control
VIN7
BP725
AWRI796
DV10
RHONE4600
BM45
D80
CrossEvol
EP2
AWRI350
MELODY.nsac
HARMONY.nsac
RHYTHM.nsac
MERIT.ferm
SYMPHONY.nsac
St. George 101
Commercial yeast strain
Tyramine
Putrescine
Spermidine
Cadaverine

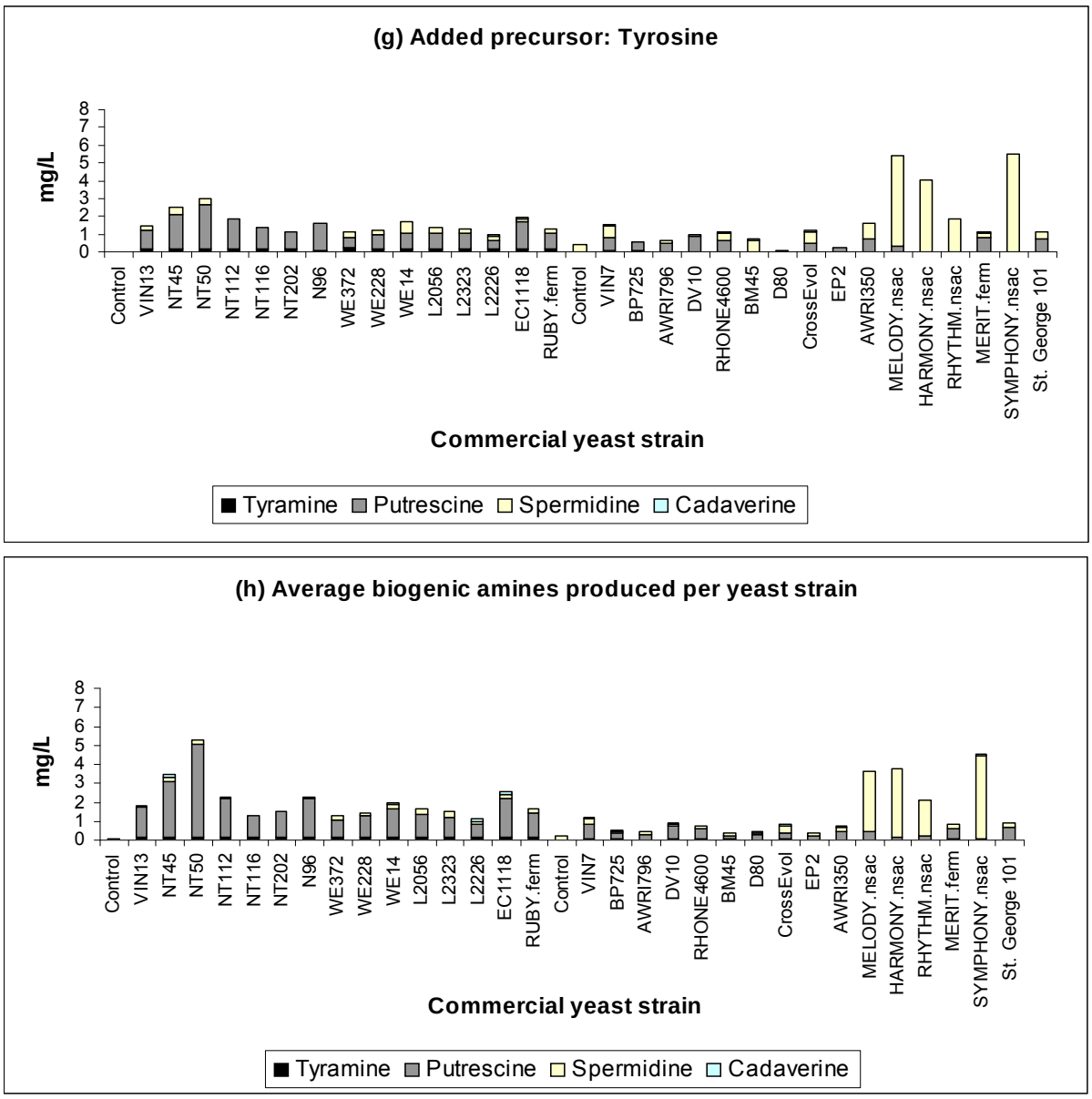


Figure 6.2 Biogenic amines (tyramine, putrescine, spermidine and cadaverine) produced by commercial *Saccharomyces cerevisiae* strains in synthetic media with individually added amino acid precursors: (a) ornithine, (b) phenylalanine, (c) tryptophane, (d) arginine, (e) histidine, (f) lysine and (g) tyrosine. Two uninoculated controls were included for each medium. (h) is a representation of the average concentration of biogenic amines produced in the seven synthetic media by each yeast strain.

A possible explanation for the preferential production of polyamines is that these compounds are required for optimal growth in yeasts. Putrescine, spermine and spermidine are necessary for meiotic sporulation and maintenance of the killer plasmid. Generally, meiotic sporulation (sexual reproduction) does not occur during fermentation of yeasts in a wine medium (Ribérau-Gayon *et al.*, 2006). Certain yeasts strains are able to secrete toxins into their environment that can kill sensitive strains. This is known as the killer factor. Neutral strains do not produce a toxin but are resistant to toxins secreted by killer positive strains. There does not seem to be a correlation between the presence of the killer factor in yeasts and the production of polyamines in this study.

The only strains used in this study that are killer sensitive are VIN 7, WE 228, BP 725, EP2 and AWRI 350. All other strains are either killer neutral or positive. The average concentration of total biogenic amines [**Figure 6.2(h)]** produced by killer positive strains range from the lowest (0.3 mg/L by BM45) to the highest (5.3 mg/L by NT50) levels obtained in this screening test. These results do not correspond to the findings by Goñi & Ancín Azpilicueta (2001), who found that wines fermented by killer strains showed the highest concentration of biogenic amines, when compared to those with a killer neutral phenotype. However, fermentations in grape must (as performed by Goñi & Ancín Azpilicueta) could provide a different result to fermentations in synthetic medium, as in our study.

The production of small amounts of tyramine, cadaverine and histamine during this experiment could possibly be attributed not to the commercial wine yeast strains, but to low numbers of bacterial contamination present in these commercial dried yeast preparations (contaminants of up to 10^2 cells/mL are allowed). It is possible that the culture conditions and growth phase may play an important role in the enzyme activity or gene expression that leads to the production of these biogenic amines, as is the case with lactic acid bacteria (Gale, 1946; Molenaar *et al.,* 1993). However, it is suggested that *Saccharomyces* do not use histidine or lysine as preferred nitrogen sources during anaerobic amino acid metabolism (Henschke & Jiranek, 1993).

It could also be postulated whether strains belonging to the subspecies *Saccharomyces cerevisiae* var. *bayanus* would produce different concentrations of biogenic amines when compared to *Saccharomyces cerevisiae* var. *cerevisiae*. From **Figure 6.2** it seems as if though EC1118 and N96 (*Saccharomyces cerevisiae* var. *bayanus*) could be biogenic amine producers on the higher end of the represented concentration range. However, the other two *Saccharomyces cerevisiae* var. *bayanus* strains present in this study, DV10 and AWRI350, showed the opposite tendency. No conclusion could therefore be made in this regard.

The only certain deductions that can be made from the available data, is that the commercial starter cultures from Chr. Hansen that are a combination of *Saccharomyces cerevisiae* with selected wild (non-*Saccharomyces*) yeasts (MELODY.nsac, HARMONY.nsac, RHYTHM.nsac and SYMPHONY.nsac) show a much higher production of spermidine than all other pure *Saccharomyces cerevisiae* preparations. It could be that the polyamine metabolisms of some of the non-Saccharomyces yeasts are different to that of *Saccharomyces cerevisiae*, or interactions between these strains could influence the polyamine profile of the media. Therefore, the ability of yeasts to produce biogenic amines seems to be very much a strain specific characteristic.

6.4 CONCLUSION

It is worthwhile to note that the varying levels of biogenic amines produced by the yeast strains in this study are interesting but preliminary data and by no means conclusive. It

seems possible that some commercial yeast preparations could contribute directly to the final levels of biogenic amines found in a wine-like medium or even wine. However, this ability could not be attributed to strains with a common characteristic (such as the killer factor or a certain subspecies) but rather seems to be randomly distributed among strains. The composite effect of all the precursor amino acids together in a synthetic medium was not evaluated. Results may prove completely different when yeasts are evaluated in a medium containing a combination of amino acids along with other complex nitrogen sources. Many other wine components (such as phenolic compounds) could have complex interactions with each other that could stimulate or suppress biogenic amine formation by yeasts. There is much room for further investigation into the ability of commercial yeast preparations to directly contribute to biogenic amine production. Screening in other chemically defined media with different compositions and after different preculture techniques are recommended, followed by evaluation in wine and if possibly, screening on a molecular level.

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7. GENERAL DISCUSSION AND CONCLUSIONS

7.1 CONCLUDING REMARKS AND FUTURE WORK

Presently, a great shortcoming exists in that no large scale or representative survey of the biogenic amine composition and concentration of commercial South African wines has been performed (to our knowledge) since 1985 (Cilliers & Van Wyk, 1985). Winemaking practices applied in commercial wine cellars, as well as the environmental conditions have seen some important changes in the past 22 years and the impact on the indigenous lactic acid bacterial flora and content of biogenic amines should be determined. Biogenic amines should be of concern to local wine producers and an awareness of the impact of practices applied during winemaking on the formation of these compounds should be encouraged in our wine industry.

In our research environment many strains of lactic acid bacteria indigenous to South African grapes and wines have been isolated and identified. However, this spontaneous lactic acid bacterial flora has not been fully characterised with regards to biogenic amine production using trustworthy molecular techniques. With the availability of new technologies and techniques it would be commendable to screen the available strains to determine the potential of South African wine isolates to produce biogenic amines.

During this study we made the first steps towards a calibration to use Fourier transform infrared (FT-IR) spectroscopy in the near future to qualitatively or quantitatively predict total biogenic amine concentration in wine in a rapid manner. Ideally, we should be able to separate the most oenologically important amines and quantify each one individually, of which the most crucial would be histamine due to its health and trade implications. It seems to be possible to set up the relevant calibration models if a large data set representative of South African wines could be obtained. The development of this proposed method could therefore be successfully performed using young and aged commercial South African wines which would simultaneous investigate the concentrations and composition of biogenic amines present in our wines. Finally, not only would a rapid, inexpensive method be of use to scan commercial and experimental wines for final levels of biogenic amines, but also for experimental studies which would aim to monitor the production of biogenic amines over time in small scale or laboratory fermentations in wine or synthetic medium.

During this study the largest accumulation of biogenic amines occurred in general during malolactic fermentation, indicating their formation by lactic acid bacteria. However, biogenic amine production could not be attributed in this study to specific strains of lactic acid bacteria, leading to inconclusive results in this regard. The causative agents could be any of the natural grape flora, the spiked *Lactobacillus* flora used in this work or even the commercial starter cultures. Developments in spectroscopy allow for the discrimination, classification and identification of microorganisms, using the FT-IR spectra of intact cells (Mariey *et al.*, 2001). This technology presents a valuable alternative to expensive molecular methods and time-

consuming plate counts or microscopy to identify and follow the contribution and growth of specific strains of microorganisms in wine and should be explored in future work concerning biogenic amine production.

Generally it was found in our investigation of malolactic fermentation practices that spontaneous malolactic fermentation presented the most variable results even under strictly controlled conditions, indicating its unpredictability and larger associated risk for biogenic amine occurrence than in commercially inoculated malolactic fermentations. The production of biogenic amines as a result of different malolactic fermentation and ageing treatments did not behave consistently. In Pinotage 2006 the only significant increase of any biogenic amines took place during ageing on the yeast lees, with only cadaverine increasing in all treatments. In Cabernet Sauvignon 2006, all biogenic amines increased significantly in all treatments during malolactic fermentation, and were degraded again during ageing in the presence or absence of lees. In Pinotage 2007 biogenic amine concentrations increased in general during malolactic fermentation and increased further during ageing in the presence or absence of lees (with the exception of putrescine), but more so in its presence. In Cabernet Sauvignon 2007 only treatments of spontaneous malolactic fermentation led to significant increases in biogenic amines during malolactic fermentation. Histamine and putrescine decreased during ageing (more so without lees) while cadaverine and spermidine increased during ageing (more so with lees).

In some cases co-inoculated treatments showed the production of significantly less biogenic amines than normal or spontaneous malolactic fermentation, but in a few cases treatments inoculated after alcoholic fermentation showed the lowest levels of biogenic amines. It was also indicated in this work that in some cases the effect of co-inoculation on biogenic amine reduction may only be visible after a period of ageing. During ageing, the presence of yeast lees may be a risk factor for subsequent biogenic amine formation. In general, it can be concluded that the presence of commercial *O. oeni* starter cultures reduced the incidence of biogenic amine formation during malolactic fermentation regardless of time of inoculation. However, the potential of co-inoculation to lower the risk of biogenic amine formation in comparison to conventional inoculation deserves further investigation.

On a chemical-analytical level, the preliminary results obtained in this study show that co-inoculation may lead to a different aroma profile than spontaneous malolactic fermentation or conventional malolactic inoculation. Moreover, it seems that concurrent inoculations do not reduce the quality of volatile flavour compounds and may even increase the pool of esters in the wine which could be advantageous for the production of fruity red wines. However, the impact of malolactic inoculation practices on wine aroma and flavour presents a relatively unexplored field of study, and much work is needed to better our understanding of this increasing popular winemaking application.

Regarding skin maceration practices, this work showed that in many cases the treatment in the absence of skins (control) had the highest levels of amino acids and biogenic amines in the final wines. Possibly, the presence of grape polyphenols largely

reduces the availability of amino acid precursors and also inhibits the mechanisms in lactic acid bacteria by which these are converted to biogenic amines. Other important observations included increased levels of some biogenic amines in the final wines of treatments that received extended maceration after alcoholic fermentation. The levels of some biogenic amines also seem to be reduced in the final wines in treatments that received cold maceration.

Histamine was the only biogenic amine produced that could be linked to the presence of complex nutrients during small scale fermentations. Unfortunately, this experiment was repeated on limited scale, spanning only one season. Judging from these restricted results, it does not seem that complex nutrient preparations produced for the use with fermentation starter cultures pose a serious threat to biogenic amine production by indigenous lactic acid bacteria. However, further investigations would be advisable.

Yeasts do not seem to contribute to any significant extent to the production of histamine or high levels of tyramine in wine. Also, yeasts do not seem able to use a particular amino acid precursor and convert it by decarboxylation to the corresponding amine. It seems possible that some commercial yeast preparations could contribute directly to the final levels of biogenic amines found in a wine-like medium or even wine. This could be due to release of the biogenic amines by secretion or when autolysis of the yeasts occurs and diamines and polyamines (cadaverine, putrescine, spermidine, spermine) which are essential to its physiological functioning are released into the wine medium. Indirect contribution by yeasts by the release of free amino acids, peptides or proteins during autolysis could be significant and it is unfortunate that biogenic amine increase could not be followed during ageing during the course of this work. This applies in particular to the maceration experiment where great increases in amino acid concentrations could be observed following malolactic fermentation.

While there is room for further investigation into the ability of commercial yeast preparations to directly contribute to biogenic amine production, the importance of this in comparison to the many unknown possibilities by lactic acid bacteria should be carefully evaluated.

The levels of biogenic amines were not very high for the most part in this experiment, despite the inclusion of decarboxylase positive lactobacilli in some fermentations. The levels of ethanol and pH presented in this study did not seem to be conducive to biogenic amine production in this study. Moreover, it seems that cultivar, vintage and phenolic compounds were some of the most important environmental variables that may have impacted the production of biogenic amines by lactic acid bacteria, and that these three factors may be linked. Complex reactions between grape phenolics and the nitrogen fraction of wine have been the subject of many investigations, yet the impact of phenolic compounds on biogenic amines, its precursors and its producers should make for very interesting future studies.

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