THE IMPACT OF NUTRIENTS ON AROMA AND FLAVOUR PRODUCTION DURING WINE FERMENTATION

by

Anita Yolandi Smit

Dissertation presented for the degree of

Doctor of Philosophy (Agricultural Sciences)

at

Stellenbosch University

Institute for Wine Biotechnology, Faculty of AgriSciences

Supervisor: Prof Florian F Bauer

March 2013

DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated) that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 18/02/2013

SUMMARY

During wine fermentation, numerous grape must constituents serve as nutrients to wine yeast (*Saccharomyces cerevisiae*), which enable their growth and successful completion of alcoholic fermentation. Many of these nutritional factors, in particular nitrogen, also act as precursors for yeast-derived flavour compounds such as higher alcohols, esters and volatile fatty acids. Yeast nitrogen metabolism thus plays a determining role in wine aroma and quality. Not only is the nitrogen source, concentration and supplementation timing important, but various environmental factors and the genetic constitution of the yeast strain used for fermentation will also contribute to fermentation outcomes.

The main goal of this work was to explore the complex interactions between a number of contributing factors; namely nitrogen source, timing of addition, yeast strain and fermentation matrix. Broadly, this study assessed the impact of seven different nitrogen combinations, added either to the initial grape must or after the onset of fermentation, on fermentation performance and aroma compound production by nine commercial wine yeast strains. Fermentations were done in synthetic grape must, and validated for a subset of parameters in real grape must. The nitrogen treatments were designed according to the generally established order of preference of *S. cerevisiae* for individual amino acids as source of nitrogen under fermentative conditions, and the potential of certain amino acids to participate in metabolic pathways that produce specific aroma compounds.

The results reveal that different nitrogen combinations can lead to unexpected aroma outcomes, depending strongly on the genetic background of individual yeast strains and the timing of nitrogen addition. Certain nitrogen treatments consistently resulted in significant increases or decreases in specific aroma compound concentrations in comparison to the treatment fermented on ammonium as only nitrogen source, for multiple yeast strains. These compounds were classified as nitrogen treatment dependent. Other aroma compounds were produced similarly for all nitrogen treatments and were designated as nitrogen treatment independent. The presence of specific amino acid groups (for example the branched-chain and aromatic amino acids) could be correlated to significantly altered production patterns of related (such as higher alcohols) or unrelated (diethyl succinate) aroma compounds relative to the other nitrogen treatments. Taken together, a number of interesting and novel hypotheses regarding the metabolic pathways involved could be derived from the data.

Ultimately, this initial assessment of interactive effects during fermentation will contribute to practical guidelines for winemakers to allow matching grape must constituents (such as nutrients) with the intrinsic aroma production capabilities of specific yeast strains in order to modulate wine aroma, style and quality.

OPSOMMING

Tydens wynfermentasie dien talle druiwemosbestanddele as voedingstowwe vir wyngis (*Saccharomyces cerevisiae*) wat hul groei bevorder en hul in staat stel om alkoholiese fermentasie suksesvol te voltooi. Baie van hierdie voedingstowwe, veral stikstof, dien ook as voorlopers vir geurkomponente afkomstig van gismetabolisme, soos hoër alkohole, esters en vlugtige vetsure. Die stikstofmetabolisme van gis speel dus ‘n bepalende rol in wynaroma en -kwaliteit. Nie net is die stikstofbron, konsentrasie en tydsberekening van stikstof toevoeging belangrik nie, maar verskeie omgewingsfaktore, asook die genetiese samestelling van die gisras aangewend vir fermentasie, sal bydra tot die fermentasie uitkomste.

Die hoofdoel van hierdie werk was om die komplekse interaksies tussen ‘n aantal bydraende faktore te ondersoek; naamlik die stikstofbron, tyd van stikstof toevoeging, gisras en fermentasiematriks. Breedweg het hierdie studie die impak van sewe verskillende stikstofkombinasies, toegedien tot die druiwemos voor of na die aanvang van fermentasie, op die suksesvolle verloop van fermentasie en die produksie van aromakomponente deur nege kommersiële wyngisrasse bepaal. Fermentasies is in sintetiese druiwemos uitgevoer, en ‘n deelversameling van die fermentasies in regte druiwesap te herhaal. Die stikstofbehandelings is ontwerp in ooreenstemming met die algemeen vasgestelde voorkeurvolgorde van *S. cerevisiae* vir individuele aminosure as stikstofbron onder fermentatiewe kondisies, en die potensiaal van sekere aminosure om mee te doen in metaboliese paaie wat spesifieke aromaverbindings produseer.

Die resultate toon dat verskillende stikstofkombinasies tot onverwagte aroma-uitkomste kan lei wat sterk afhanklik is van die genetiese agtergrond van individuele gisrasse en die tyd van stikstof byvoeging. Sekere stikstofbehandelings het konsekwent, vir veelvuldige gisrasse, tot beduidende toenames of afnames in die konsentrasies van spesifieke aromakomponente gelei in vergelyking met die behandeling wat ammonium as enigste stikstofbron bevat het. Hierdie verbindings is as stikstofbehandeling afhanklik geklassifiseer. Ander aromaverbindings is soortgelyk vir alle stikstofbehandelings geproduseer en is aangewys as stikstofbehandeling onafhanklik. Die teenwoordigheid van spesifieke aminosuurgroepe (byvoorbeeld die vertakte-ketting en aromatiese aminosure) kon gekorreleer word met beduidende veranderings in produksiepatrone van verwante (soos hoër alkohole) of onverwante (dietielsuksinaat) aromakomponente relatief tot die ander stikstofbehandelings. Alles inaggenome kon ‘n aantal interessante en nuwe hipoteses rakende die betrokke metabolise padweë van die data afgelei word.

Uiteindelik sal hierdie aanvaklike bepaling van interaktiewe effekte tydens fermentasie bydra tot praktiese riglyne vir wynmakers, wat hulle in staat sal stel om druiwesapbestanddele (soos nutriënte) te strook met die intrinsieke aromaproduksie kapasiteite van spesifieke gisrasse, en sodoende wynaroma, styl en kwaliteit te moduleer.

ACKNOWLEDGEMENTS

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

Florian Bauer, who acted as supervisor, for his guidance and valuable discussions during my studies, and for critical evaluation of this manuscript

The **Institute for Wine Biotechnology** and **Stellenbosch University** for affording me the opportunity to conduct this study whilst in their employ, and for financial support

Oenobrands and **THRIP** for funding this project

My colleagues **Dan Jacobson** for statistical analysis; **Samantha Fairbairn**, **Candice Stilwaney** and **Lynzey Isaacs** for technical support

The "**Sunbio girls"** (Anscha, Heidi, Elize, Charmaine and Lynn) for their daily support and encouragement

My **friends** and **family,** for believing in me and keeping hope alive

PREFACE

This dissertation is presented as a compilation of six chapters. Chapter 1 introduces the background and aims of this study. Chapter 2 provides an overview of the literature related to the topic of study. Chapters 3, 4 and 5 will be submitted for publication and are written according to a general style. Chapter 6 overviews the main findings of the study and concludes the work.

CHAPTER 1 **General introduction and project aims**

CHAPTER2 **Literature review**

Yeast aroma metabolism: From nutrients to flavour-active compounds

CHAPTER 3 **Research results**

Initial assessment of the combinatorial impacts of nitrogen source, addition time and yeast strain on fermentation performance and aroma production in synthetic grape must

CHAPTER 4 **Research results**

Linking grape must amino acid composition and aroma compound production pathways of wine yeast

CHAPTER 5 **Research results**

Comparative aroma production patterns of wine yeast strains in synthetic, white and red grape musts in the presence of different nitrogen treatments

CHAPTER 6 **General discussion and conclusions**

CONTENTS

CHAPTER 1. General introduction and project aims **1**

References 4

CHAPTER 2. Yeast aroma metabolism: From nutrients to flavour-active compounds **6**

2.1 Introduction 7

2.2 Pathways of formation of yeast-derived flavour compounds 8

2.3 The impact of nitrogen metabolism on yeast-derived flavour formation 9

2.4 The impact of essential nutrients and their interaction with nitrogen on yeast-derived flavour formation 12

2.4.1 Grape sugars *12*

2.4.2 Sulfur compounds *13*

2.4.3 Vitamins *15*

2.4.4 Minerals and metal ions *16*

2.4.5 Lipids and sterols *18*

2.5 Nutritional management strategies to optimise yeast-derived flavour in wine 19

2.5.1 Nutrient supplementation and yeast preconditioning *19*

2.5.2 Viticultural and winemaking practices *20*

2.5.3 Management of wine microflora and yeast strain selection *21*

2.6 Conclusions 22

References 22

CHAPTER 3. Initial assessment of the combinatorial impacts of nitrogen source, addition time and yeast strain on fermentation performance and aroma production in synthetic grape must **32**

Abstract 33

3.1 Introduction 33

3.2 Materials and methods 36

3.2.1 Fermentation medium *36*

3.2.2 Yeast strains and fermentation conditions *38*

3.2.3 Chemical analysis *38*

3.2.4 Statistical analysis *39*

3.3 Results and discussion 40

3.3.1 Fermentation of synthetic musts *40*

3.3.2 Multivariate data analysis *48*

3.3.3 Impact of nitrogen treatments on fermentation performance and aroma production *52*

3.3.3.1 Ammonium *52*

3.3.3.2 Preferred amino acids *52*

3.3.3.3 Branched-chain and aromatic amino acids *53*

3.3.3.4 Complete amino acids *54*

3.3.3.5 Non-preferred amino acids *54*

3.3.3.6 Non-utilised amino acids *55*

3.4 Conclusions 55

References 56

CHAPTER 4. Linking grape must amino acid composition and aroma compound production pathways of wine yeast **60**

Abstract 61

4.1 Introduction 61

4.2 Materials and methods 63

4.2.1 Fermentation medium, yeast strains and fermentation conditions *63*

4.2.2 Analysis of major volatile compounds *66*

4.2.3 Statistical analysis *66*

4.3 Results and discussion 67

4.3.1 Fermentation performance *67*

4.3.2 Detection of volatile aroma compounds by GC-FID *67*

4.3.3 Nitrogen treatment-independent aroma compound production *67*

4.3.4 Nitrogen treatment-dependent aroma compound production *70*

4.3.4.1 Higher alcohols and related compounds *71*

4.3.4.2 Ethyl esters *76*

4.4 Conclusions 78

References 79

CHAPTER 5. Comparative aroma production patterns of wine yeast strains in synthetic, white and red grape musts in the presence of different nitrogen treatments **82**

Abstract 83

5.1 Introduction 83

5.2 Materials and methods 85

5.2.1 Yeast strains and culture conditions *85*

5.2.2 Fermentation media and nitrogen treatments *86*

5.2.3 Fermentation conditions *86*

5.2.4 Amino acid composition of grape musts *87*

5.2.5 Chemical characterisation of grape musts and wines *87*

5.2.6 Analysis of volatile compounds at the end of alcoholic fermentation *89*

5.2.7 Statistical analysis *89*

5.3 Results and discussion 90

5.3.1 Comparison of synthetic and real grape must fermentation and aroma production *90*

5.3.1.1 Grape must chemical properties and amino acid composition *90*

5.3.1.2 Fermentation performance *91*

5.3.1.3 Multivariate analysis *94*

5.3.1.4 Conserved significant changes in aroma production due to treatment effects *99*

5.3.2 Production of volatile aroma compounds in real grape must *101*

5.4 Conclusions 103

References 106

CHAPTER 6. General discussion and conclusions **108**

References 112

Chapter 1

General introduction and project aims

CHAPTER 1

General introduction and project aims

The production of quality wine relies on the successful completion of alcoholic fermentation and the production of desirable flavour compounds by commercial wine yeast strains (*Saccharomyces cerevisiae*). Flavour compounds encompass all volatile and non-volatile compounds that contribute to the perception of aroma (smell), taste and touch in the mouth (Francis & Newton, 2005). The total flavour profile of any wine is the product of a multitude of compounds (more than 800 aroma-contributing compounds, according to Mendes-Pinto, 2009) some of which make significant individual contributions (impact compounds), while others often act synergistically or antagonistically. Flavour compounds can be derived directly from the grape berry, be transformed from non-volatile precursors in the berry to volatile products in the wine by chemical or enzymatic means, be produced by yeast and bacterial metabolisms or develop during wine ageing (Francis & Newton, 2005).

The most important flavour-active compounds produced by yeast during fermentation are primary (ethanol, glycerol, acetic acid and acetaldehyde) and secondary (higher alcohols, esters and fatty acids) fermentation products. The secondary metabolites are produced catabolically and anabolically via various interconnected metabolic pathways, which are regulated on genetic level and are therefore yeast strain dependent (Lambrechts & Pretorius, 2000; Lilly et al., 2006; Rossouw et al., 2008; Styger et al., 2011). Thus, the fermentation-derived flavour outcomes can be manipulated by the use of suitable commercial yeast strains. In the recent past, a multitude of "market orientated wine yeast strains" were isolated, engineered or improved by techniques such as hybridisation, mutagenesis and directed evolution to keep up with the growing wine market and changing consumer preferences (Pretorius & Bauer, 2002). However, new developments are now directed more towards the optimal exploitation of existing yeast strains in the market.

Although aroma compound production and fermentation performance of yeast are genetically determined, these characteristics are also greatly dependent on grape must composition (including nutritional factors) and environmental conditions. To date, many commercial wine yeast strains have been reasonably well characterised on a phenotypic, biochemical and even genotypic level, but the specific outcomes of individual wine fermentations remain largely unpredictable due to the unique chemical, physical and nutritional conditions in each grape must. However, the rapid development of new technologies and analytical tools should provide detailed chemical information regarding grape juice composition to winemakers in the near future which, together with strain characterisation, can be exploited to meet the dynamic and specific requirements of winemakers and wine consumers.

Currently, many winemaking practices and additives are directed at optimising the fermentation performance and aroma production by wine yeast. Incomplete or lagging alcoholic fermentations remain one of the great challenges in wine production. Numerous factors can be responsible for the decline of

fermentation rate and sugar consumption, including imbalances of macronutrients (nitrogen and phosphate) and micronutrients (such as vitamins and minerals) (Bisson, 1999). In addition, metabolites with undesirable organoleptic impacts such as acetic acid and hydrogen sulfide are often produced as a result of nutrient deficiencies, resulting in decreased wine quality (Wang et al., 2003; Bohlscheid et al., 2007). The addition of yeast nutrients, particularly nitrogen, is a common winemaking practice, aimed to alleviate or reduce the risk of fermentation problems. Historically, most problem fermentations were prevented or treated by supplementation of the total yeast assimilable nitrogen (YAN) with diammonium phosphate (DAP). Several past studies have focused on the impact of different YAN concentrations or sources (inorganic versus organic) on fermentation performance and, more recently, also on wine flavour or aroma profiles in synthetic and real grape musts (for example Radler & Shütz, 1982; Beltran et al., 2004; Hernández-Orte et al., 2005; Hernández-Orte et al., 2006a; Hernández-Orte et al., 2006b; Vilanova et al., 2007; Carrau et al., 2008). Of these studies, most evaluated the impact of individual or a limited number of parameters (such as single amino acids, complete organic nitrogen sources and/or a limited number of yeast strains) on aroma production in mostly mono-factorially designed experiments. What is evidently still lacking is the multi-factorial assessment of all factors involved; including the natural grape must chemical composition, the availability of nutrients, nutrient supplementation timing, environmental factors and yeast genetic ability. Without such comprehensive data, our ability to predict, control and direct fermentation outcomes remains limited. In this project we sought to address a number of parameters in order to overcome this limitation.

In particular, this study took an exploratory screening approach to provide an initial assessment of the impact of different nitrogen combinations on fermentation performance and aroma production of nine commercial wine yeast strains. In addition, the impacts of the timing of nitrogen addition and of different fermentation media (synthetic, white and red grape must) on the production of aroma compounds by some of these strains were investigated in more detail. The amino acid compositions of nitrogen treatments used in this study were based on the general order of preference of *S. cerevisiae* to utilise individual amino acids as source of nitrogen under fermentation conditions (Cooper, 1982; Beltran et al., 2004; Magasanik & Kaiser, 2002) and/or their potential impact on aroma production via specific metabolic pathways (for example sulfur-containing amino acids or branched-chain and aromatic amino acids).

Another important objective of this project was to integrate the results obtained in synthetic grape must into to the broader framework of real winemaking conditions. Furthermore, this project aims to aid the improvement of complex yeast nutrient formulations available as fermentation tools to the winemaker. In the long term, this study aims to contribute to practical guidelines for winemakers regarding nitrogen supplementation strategies for individual yeast strains to achieve desired flavour outcomes.

Ultimately, the goal of this study is to provide baseline data that will in future allow matching the chemical composition of grape musts (including nutritional factors) with the intrinsic fermentation and aroma production capabilities of specific yeast strains.

References

Beltran, G., Novo, M., Rozès, N., Mas, A., & Guillamón, J. M. (2004). Nitrogen catabolite repression in *Saccharomyces cerevisiae* during wine fermentations. *FEMS Yeast Research, 4*(6), 625–32.

Bisson, L. (1999). Stuck and sluggish fermentations. *American Journal of Enology and Viticulture, 50*(1), 107–119.

Bohlscheid, J. C., Fellman, J. K., Wang, X. D., Ansen, D., & Edwards, C. G. (2007). The influence of nitrogen and biotin interactions on the performance of *Saccharomyces* in alcoholic fermentations. *Journal of Applied Microbiology, 102*(2), 390–400.

Carrau, F. M., Medina, K., Farina, L., Boido, E., Henschke, P. A., & Dellacassa, E. (2008). Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: Effects of yeast assimilable nitrogen on two model strains. *FEMS Yeast Research, 8*(7), 1196–207.

Cooper, T.G. (1982). Nitrogen metabolism in *Saccharomyces cerevisiae*. In: Strathern, J.N., Jones, E.W., Broach, J.R. (Eds.). The molecular biology of the yeast *Saccharomyces*: Metabolism and gene expression, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 39– 99.

Francis, I. L., & Newton, J. L. (2005). Determining wine aroma from compositional data. *Australian Journal of Grape and Wine Research, 11*(2), 114–126.

Hernández-Orte, P., Bely, M., Cacho, J., & Ferreira, V. (2006). Impact of ammonium additions on volatile acidity, ethanol, and aromatic compound production by different *Saccharomyces cerevisiae* strains during fermentation in controlled synthetic media. *Australian Journal of Grape and Wine Research, 12*(2), 150–160.

Hernández-Orte, P., Ibarz, M., Cacho, J., & Ferreira, V. (2005). Effect of the addition of ammonium and amino acids to musts of Airen variety on aromatic composition and sensory properties of the obtained wine. *Food Chemistry, 89*(2), 163–174.

Hernández-Orte, P., Ibarz, M. J., Cacho, J., & Ferreira, V. (2006). Addition of amino acids to grape juice of the Merlot variety: Effect on amino acid uptake and aroma generation during alcoholic fermentation. *Food Chemistry, 98*(2), 300–310.

Lambrechts, M., & Pretorius, I. (2000). Yeast and its importance to wine aroma – A review. *South African Journal of Enology and Viticulture, 21*, 97–129.

Lilly, M., Bauer, F., Lambrechts, M., Swiegers, J., Cozzolino, D., & Pretorius, I. (2006). The effect of increased yeast alcohol acetyltransferase and esterase activity on the flavour profiles of wine and distillates. *Yeast, 23*, 641–659.

Magasanik, B., & Kaiser, C. A. (2002). Nitrogen regulation in *Saccharomyces cerevisiae*. *Gene, 290*(1-2), 1–18.

Mendes-Pinto, M. M. (2009). Carotenoid breakdown products the—norisoprenoids—in wine aroma. *Archives of Biochemistry and Biophysics*, *483*(2), 236–245.

Pretorius, I. S., & Bauer, F. F. (2002). Meeting the consumer challenge through genetically customized wine-yeast strains. *Trends in Biotechnology*, *20*(10), 426–32.

Radler, F., & Schütz, H. (1982). Glycerol production of various strains of *Saccharomyces*. *American Journal of Enology and Viticulture, 33*(1), 36–40.

Rossouw, D., Naes, T., & Bauer, F. F. (2008). Linking gene regulation and the exo-metabolome: A comparative transcriptomics approach to identify genes that impact on the production of volatile aroma compounds in yeast. *BMC Genomics, 9*, 530.

Styger, G., Jacobson, D., & Bauer, F. F. (2011). Identifying genes that impact on aroma profiles produced by *Saccharomyces cerevisiae* and the production of higher alcohols. *Applied Microbiology and* Biotechnology, 91(3), 713–30.

Vilanova, M., Ugliano, M., Varela, C., Siebert, T., Pretorius, I. S., & Henschke, P. A. (2007). Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by *Saccharomyces cerevisiae* wine yeasts. *Applied Microbiology and Biotechnology*, *77*(1), 145–57.

Wang, X. D., Bohlscheid, J. C., & Edwards, C. G. (2003). Fermentative activity and production of volatile compounds by *Saccharomyces* grown in synthetic grape juice media deficient in assimilable nitrogen and/or pantothenic acid. *Journal of Applied Microbiology*, *94*(3), 349–59.

Chapter 2

Yeast aroma metabolism:

From nutrients to flavour-active compounds

CHAPTER 2

Yeast aroma metabolism: From nutrients to flavour-active compounds

2.1 Introduction

The art and science of wine flavour has mystified winemakers, scientists, writers and consumers for centuries. Wine complexity and quality is largely characterised by its flavour, defined as the combined sensations of smell (orthonasal and retronasal aromas), taste and touch perceived due to the presence of volatile and non-volatile compounds in the wine (Francis & Newton, 2005). The terms flavour, aroma and bouquet are often used interchangeably to describe these individual or combined sensory experiences (Lambrechts & Pretorius, 2000).

Wine flavour is attributable to a myriad of combinations of volatile and non-volatile compounds. A large portion of these flavour compounds are metabolites from grapes, or derived from grapes through enzymatic release, biotransformation or *de novo* production by wine yeast and other wine microorganisms during alcoholic and malolactic fermentations. With more than 800 aroma-contributing compounds identified in wine (Mendes-Pinto, 2009), accurately capturing the unique character and total flavour complement of wine in a model fermentation system remains elusive (Keyzers & Boss, 2010), although approximate reconstruction of model wine has been achieved with a limited number of contributing aroma compounds (Ferreira et al., 2002). Recently, it was demonstrated that grape-derived compounds or activators, yet beyond our analytical grasp, modulate yeast-derived flavour compounds without acting as precursors themselves (Keyzers & Boss, 2010). Recent data also suggest that yeast-derived aroma compounds such as esters, higher alcohols and fatty acids play a role in varietal character (Pineau et al., 2009) and that the typical aroma profiles of certain grape cultivars can be correlated to their amino acid profiles (Hernández-Orte et al., 2002). Thus, the common classification of aroma-contributing compounds according to source (for example grape-derived or yeast-derived) becomes more indistinct the more we are able to grasp the complexity of wine.

Grape must constituents not only provide the raw materials for the production of flavour metabolites; they primarily serve as nutrients required by *Saccharomyces cerevisiae* for successful alcoholic fermentation, proliferation during the active growth phase and maintenance of metabolic activities during stationary phase (Bisson, 1999). These nutritional factors include sources of carbon, nitrogen and sulfur; essential vitamins, minerals and trace metals; and lipids or sterols.

When nutrients are present in insufficient amounts or in excess, major fermentation problems such as sluggish fermentation, fermentation arrest, or the production of metabolites perceived as off-flavour compounds can arise (Bisson, 1999; Fairbairn, 2012). Poor fermentation performance and off-flavour production are most often ascribed to shortages or excesses of yeast assimilable nitrogen (YAN). In the past it

was assumed that grape musts contain sufficient quantities of nutrients other than nitrogen for yeast growth and fermentation (Ough et al., 1989). However, all grape must nutritional factors, and not only nitrogen, can affect the growth and metabolism of yeast cells and can thus impact the composition of the final wine and its sensory properties.

When essential nutrients are limiting, cell growth slows down and an environmental stress response takes place, which is common to all nutrients (Gasch et al., 2000). Indeed, stress response pathways active during fermentation are complex and interlinked with other pathways such as metabolite production (Rossouw et al., 2008; Fairbairn, 2012). Changes in aroma compound production in a nutrient or nitrogen deficient medium could therefore be due to stress and not directly due to the nitrogen limitation; and many genes found to be related to nitrogen metabolism could in fact be related to other physiological stress responses and not directly to nitrogen metabolism (Contreras et al., 2012). Other than environmental stress (high sugar, osmotic stress, temperature etc.), many factors such as the availability of precursors, the redox and energy potential of the cell and nutrient availability in the growth medium could influence the metabolic pathways linked to the production of aroma compounds.

Current research is geared towards an understanding of all grape must nutritional factors, their interactions with each other and with environmental factors and their utilisation by individual yeast strains, in order to fully optimise the potential of the grape must to sustain fermentation while positively contributing to flavour production and wine quality (Wang et al., 2003; Bohlscheid et al., 2007; Fairbairn, 2012). This review will discuss the impact of the most relevant nutritional factors present in grape must and their interactions with nitrogen metabolism, on the production of major yeast-derived flavour compounds during wine fermentation.

2.2 Pathways of formation of yeast-derived flavour compounds

The production pathways of fermentation-derived volatile aroma compounds in wine have been reviewed extensively in the literature by authors such as Lambrechts and Pretorius (2000), Swiegers and Pretorius (2005) and Styger et al. (2011). In brief, the production of higher alcohols and their associated ester and volatile fatty acid derivatives can proceed via two routes; the Ehrlich (catabolic pathway) or *de novo* (anabolic) formation. In the Ehrlich pathway, branched-chain amino acids (valine, leucine and isoleucine) and aromatic amino acids (tryptophan, tyrosine, phenylalanine) undergo transamination to generate α-keto acids via transfer of the amino group of the amino acid to α-ketoglutarate (Dickinson et al., 1997; Dickinson et al., 1998; Dickinson et al., 2000; reviewed by Hazelwood et al., 2008). In the anabolic pathway, α-keto acids are provided by sugar metabolism via pyruvate (Dickinson et al., 1997; Dickinson et al., 1998). The α-keto acids are decarboxylated to yield an aldehyde intermediate which is subsequently reduced to yield the associated higher alcohols or oxidised to yield the associated fatty acid, depending on the requirements of the yeast cell for NAD/NADH regeneration (Lambrechts & Pretorius, 2000; Vuralhan et al., 2003). The

formation of esters and fatty acids are dependent on the availability of Coenzyme A (CoA). Volatile fatty acids can be formed via the fatty acid biosynthetic pathway by acetyl-CoA decarboxylation and condensation reactions (Nykanen, 1986; Lambrechts & Pretorius, 2000). Esters in wine are formed by the enzyme-catalysed condensation reactions between a fatty acid activated by CoA and an alcohol (either ethanol or higher alcohols) (Lynen 1967; Peddie, 1990). Carbonyl compounds form as intermediates of higher alcohol production from sugar or nitrogen (anabolic or catabolic), and include keto acids, aldehydes and related compounds such as diacetyl and acetoin (Nykanen et al., 1977).

The metabolic value of aroma compound production is still somewhat disputed, although it is well demonstrated that redox homeostasis is linked to the regulation of aroma producing networks (Lambrechts & Pretorius, 2000; Jain et al., 2011) and in particular to the production of higher alcohols and their fatty acids (Bisson & Karpel, 2010). Another explanation for higher alcohol production is the detoxification of aldehydes produced during amino acid catabolism which could negatively impact the cell (Boulton et al., 1995). To date, the physiological need for ester biosynthesis is still obscure and may not hold any advantage to the yeast cell. It could be that esters are formed from excess products available from sugar metabolism (Lambrechts & Pretorius, 2000). Ester formation could serve to remove toxic fatty acids from the yeast cell (Nordström, 1962; Nordström, 1964), to correct imbalances of CoA and acetyl-CoA (Lambrechts & Pretorius, 2000) or to maintain the redox balance when glycerol production is increased (Jain et al., 2011)

2.3 The impact of nitrogen metabolism on yeast-derived flavour formation

S. cerevisiae employs ammonium ions, free amino acids and occasionally low molecular weight peptides as nitrogen sources. Free amino acids can be directly incorporated into proteins or the amine functional group can be utilised as nitrogen source for various cellular functions. All essential amino acids can be synthesised by the yeast cell from ammonium nitrogen (Henschke & Jiranek, 1993).

The quality of amino acids as source for protein synthesis is unrelated to their quality as nitrogen source. For example glycine is required for the synthesis of sugar transporters, but is not a good nitrogen donor (Manginot et al., 1997). Yeast available nitrogen is taken up rapidly from the fermentation medium at the beginning of fermentation and stored in the cell cytoplasm and vacuole until required for cellular activities (Bisson, 1999). This uptake takes place before the accumulation of ethanol inhibits amino acid transport across the plasma membrane (Bisson, 1999). Sugar transporters are actively synthesised to maintain glycolysis throughout stationary phase, and therefore nitrogen availability remains crucial to the cell even after the active growth phase.

Good sources of nitrogen are accumulated more rapidly and are generally utilised earlier in fermentation than poor sources. This can be mainly attributed to the efficiency of the relevant transport systems (Jiranek et al., 1995). The pattern of nitrogen utilisation by yeast when a mixed source is supplied is controlled by nitrogen

catabolite repression (NCR), which dictates the preferred assimilation of good nitrogen sources, defined as those that support high growth rates and yield ammonium, glutamine or glutamate (Ter Schure et al., 2000; Magasanik & Kaiser, 2002; Marks et al., 2003; Beltran et al., 2004). Under the control of NCR the relevant transporters (permeases) of preferred (good) nitrogen sources are expressed while transporters of less preferred (poor) nitrogen sources are repressed and degraded in the presence of a more preferred source (Ter Schure et al., 2000; Magasanik & Kaiser, 2002). During wine fermentation, a nitrogen repressed condition is present at the beginning of fermentation which evolves into a derepressed state as preferred nitrogen sources are consumed by yeast. The conditions of nitrogen repression and derepression will determine the pattern of uptake of available nitrogen sources by their associated transporters (Beltran et al., 2004).

More specifically, under repressed conditions permeases transporting more preferred amino acids, branched-chain and aromatic amino acids as well as a number of constitutively expressed transporters are active. These include the basic amino acid permease (Can1p); the histidine permease (Hip1p); tryptophan (Tat1p), lysine (Lyp1p), branched-chain (Bap1p and Bap2p) and aromatic amino acid (Tat1p and Tat2p) transporters (Cooper, 1982; Tanaka & Fink, 1985; Hoffmann, 1985; Sychrova & Chevallier, 1993; Schmidt et al., 1994; Grauslund et al., 1995). Ammonium is generally considered a good nitrogen source and is assimilated under nitrogen repressed conditions, but yeast strains differ in amino acid transporter repression by ammonium (Rytka, 1975; Marks et al., 2003). Under derepressed conditions the general amino acid transporters (Gap1p and Agp1p) and proline permease (Put4p) allow uptake of less preferred nitrogen sources. It is suggested that ammonium permeases (Mep1p, Mep2p and Mep3p) are also expressed under derepressed nitrogen conditions in order to retrieve remaining low levels of ammonium (Beltran et al., 2004).

In grape must, preferred sources of YAN include ammonium, glutamate, glutamine, aspartate, asparagine and arginine (Cooper, 1982; Large, 1986; Henschke & Jiranek, 1993; Hofman-Bang, 1999; Ter Schure et al., 2000; Magasanik & Kaiser, 2002). Glutamate and glutamine contribute approximately 85% and 15% respectively to the cellular requirements of nitrogen and can be readily interconverted with each other and ammonium via various enzymes (Cooper, 1982; Ter Schure et al., 2000; Magasanik & Kaiser, 2002). Ultimately all amino acids contribute towards the formation of these two amino acids (Cooper, 1982). Asparagine is considered a good source of nitrogen as its hydrolysis yields ammonium and aspartate, which in turn easily yields glutamate (Sinclair et al., 1994). Arginine is a good source of nitrogen but is less preferred than glutamine, glutamate or ammonium. It is catabolised first to ornithine and urea, and finally to glutamate and ammonium (Large, 1986). Arginine transport has some NCR sensitivity, and although it is an average nitrogen source in supporting growth (Hofman-Bang, 1999), it is abundantly available in grape must and yeast is able to utilise it as a source of nitrogen under fermentative conditions (Henschke & Jiranek, 1993).

Together with arginine, proline constitutes the greatest part of total amino nitrogen in the grape (Ough & Bell, 1980; Stines et al., 2000). Proline is not used as nitrogen source during alcoholic fermentation, because the enzyme which catalyses the first step in proline catabolism (proline oxidase) requires oxygen. Similarly, tryptophan can only be degraded to a very limited extent by yeast strains under fermentative conditions, because its catabolism requires molecular oxygen. Histidine, glycine and lysine cannot be fully degraded by *S. cerevisiae* (Cooper, 1982; Large, 1986; Henschke & Jiranek, 1993; Beltran et al., 2004; Beltran et al., 2005).

Branched-chain and aromatic amino acids are accumulated during early fermentation and can be taken up throughout fermentation, even in a growth medium rich in preferred nitrogen sources, where repressed conditions are maintained (Forsberg & Ljungdahl 2001; Beltran et al., 2004; Beltran et al., 2005). Generally, branched-chain and aromatic amino acids are not considered the best nitrogen sources to support growth (Watson, 1976; Boer et al., 2007).

Evidently, the addition of different nitrogen sources can have a major impact on yeast growth and fermentation kinetics, can cause or alleviate fermentation problems and influence the formation of aroma compounds (Hernández-Orte et al., 2005, Hernández-Orte et al., 2006a; Hernández-Orte et al., 2006b; Garde-Cerdán & Ancín-Azpilicueta, 2008). Aroma compounds directly related to nitrogen metabolism (such as higher alcohols and their associated fatty acids and esters) are impacted by the total nitrogen concentration, source of nitrogen and the timing of nitrogen addition (Beltran et al., 2005; Hernández-Orte et al., 2005; Barbosa et al., 2009). Generally it is observed that in grape must with low nitrogen concentration there is a direct relationship between the nitrogen content and higher alcohol production, while an inverse relationship exists for moderate to high nitrogen levels (Äyräpää, 1971). Nitrogen limiting conditions cause increased production of higher alcohols via both catabolic and anabolic biosynthetic pathways. During nitrogen limitation, the majority of higher alcohols are produced from keto acids derived from sugars because few amino acids are available for transamination (Oshita et al., 1995). However, under conditions of sufficient nitrogen it is found amino acids are transaminated and the catabolic formation of higher alcohols is increased proportionally when additional branched-chain amino acids are supplied, while the anabolic formation is reduced. Therefore, the addition of nitrogen will decrease higher alcohol concentrations even when direct precursor amino acids are supplied (Äyräpää 1971; 2000; Schulthess & Ettlinger 1978). The proportion of branched-chain and aromatic amino acids are relatively low compared to other amino acids in natural grape musts (Giudici et al., 1993), and therefore fewer higher alcohols are produced form amino acids during nitrogen excess when higher alcohols are not really produced from sugars either. Thus, when nitrogen supplementations are made to the initial fermentation medium, higher alcohols are lower compared to when nitrogen additions are made during fermentation after a period of nitrogen limitation, during which yeast had the opportunity to produce higher levels of higher alcohols (Ough et al. 1980; Hernández-Orte et al. 2005).

Nitrogen metabolism also regulates other major pathways such as sugar and sulfur metabolism, as well as the utilisation of essential nutrients, and can thus impact on the production of many flavour-active intermediates and end-products.

2.4 The impact of essential nutrients and their interaction with nitrogen on yeast-derived flavour formation

2.4.1 Grape sugars

During alcoholic fermentation, carbon metabolism serves to generate energy and building blocks of cell constituents to sustain all cellular functions. Grape sugars (glucose and fructose) are the principle carbon sources used by *S. cerevisiae* during wine fermentation, but other fermentable hexose sugars and disaccharides can also be used (Walker, 2004; Zaman et al., 2008). Nitrogen impacts on all primary and secondary products of glycolysis as it regulates sugar accumulation, transport and metabolism (Boulton et al., 1995) and therefore a greater consumption of nitrogen is correlated with increased carbon catabolism (Jiranek et al., 1995). The primary products of alcoholic fermentation; ethanol, CO_2, glycerol and acetic acid make an important contribution to the aroma perception of wine (Albers et al., 1996; Styger et al., 2011).

The formation of glycerol during anaerobic fermentation by *S. cerevisiae* can be influenced by the source and quantity of nitrogen. Yeasts grown on amino acids generally produce lower glycerol concentrations than ammonium-grown cultures, because the need for *de novo* synthesis of amino acids and subsequently the need for the reoxidation of NADH by glycerol formation is reduced (Albers et al. 1996; Hohmann, 2007). However, amino acid composition can also impact glycerol formation. It was found that the addition of certain single amino acids (alanine, asparagine, serine and valine) as nitrogen source result in decreased levels of glycerol compared to a mixture of amino acids, while other amino acids (arginine, aspartic acid, glutamic acid, methionine and threonine) yield the same or higher glycerol concentrations than a mixture of amino acids (Radler & Schütz, 1982).

Acetic acid is produced via the oxidation of acetaldehyde and serves as precursor for acetyl-CoA (Bell & Henschke, 2005). An inverse relationship exists between initial nitrogen and acetic acid production, up to a maximum concentration of nitrogen (which depends in turn on the initial sugar concentration) from which point a direct relationship exists (Bely et al., 2003; Fairbairn, 2012). When nitrogen is freely available there is a reduced need to generate NADH through other redox reactions, such as the oxidation of acetaldehyde to acetic acid (or glycerol formation), resulting in lower levels of volatile acidity. Another possibility is that more acetyl-CoA is demanded for the synthesis of fatty acids (lipids) under stimulatory growth conditions, and hence less acetic acid is formed (Barbosa et al., 2009). Under conditions that are growth limiting such as nitrogen scarcity, acetic acid production will increase (Lambrechts & Pretorius, 2000).

Sugar metabolism generates the majority of carbon backbones required for the production of numerous yeast-derived volatile compounds, including esters, higher alcohols, aldehydes, polyols, organic acids keto acids and organic sulfur compounds (Rapp & Versini, 1995). In fact, many authors agree that the anabolic formation of higher alcohols and esters from sugars makes a more significant contribution to wine aroma than the catabolic formation from corresponding amino acids via the Ehrlich pathway (Lambrechts & Pretorius, 2000; Beltran et al., 2005; Miller et al., 2007). However, this observation appears to be dependent on strain and total nitrogen concentration. In a study by Hernández-Orte et al. (2005), similar concentrations of higher alcohols were produced by three yeast strains at very low (< 200 mg/l) or very high (>350 mg/l) total nitrogen concentrations in real grape must supplemented with amino acids. At intermediate nitrogen concentrations, yeast strains were differentiated in their higher alcohol producing capabilities, possibly attributable to a switch from anabolic to catabolic higher alcohols production at a nitrogen concentration which is strain and grape must dependent.

2.4.2 Sulfur compounds

Sulfur is used by yeast cells for the formation of various vital sulfur-containing compounds, such as co-factors (Swiegers & Pretorius, 2007), S-adenosylmethionine (a methyl-group donor) (Lambrechts & Pretorius, 2000) and the amino acids methionine and cysteine. For this purpose, wine yeasts take up various sulfurous compounds from the grape must, including inorganic sulfur sources such as elemental sulfur, sulfate and sulfite, and organic compounds such as glutathione and the amino acids cysteine and methionine (Henschke & Jiranek, 1993; Hallinan et al., 1999, Spiropoulos et al., 2000). Methionine and cysteine can be degraded to form sulfides, which are the precursors for various other volatile sulfur compounds (Swiegers & Pretorius, 2007). However, grape musts usually contain insufficient quantities of the two sulfur-containing amino acids to fulfil all the metabolic needs of the yeast cell, and therefore yeasts have to synthesise sulfur-containing cell constituents via the sulfate reduction sequence (SRS) pathway (Lambrechts & Pretorius, 2000). Various sulfur-containing aroma compounds are derived by yeast metabolism of sulfur-containing amino acids (Moreira et al., 2002) and other non-volatile sulfur-containing precursors (reviewed by Swiegers & Pretorius, 2007).

The interaction between sulfur and nitrogen metabolisms can lead to the accumulation of H_2S, a common and undesirable off-flavour in wine. When sufficient nitrogen is available during fermentation, the hydrogen sulfide ion (HS^-), an intermediate of the SRS pathway, will bind to nitrogen-derived receptor molecules (such as O-acetylhomoserine and O-acetyl serine) to form organic products (such as methionine and cysteine). In nitrogen deficient environments, HS^- can be reduced to free hydrogen sulfide (H_2S) (Rauhut, 1993; Spiropoulos et al., 2000). The total nitrogen concentration and source in the fermentation medium can influence H_2S production (Spiropoulos et al., 2000). However, various other nutritional factors can influence H_2S production and some studies even report poor correlation between nitrogen concentrations and H_2S

production (Sea et al., 1998; Spiropoulos et al., 2000). Thus, when H_2S is perceived under conditions of sufficient nitrogen, a different nutrient deficiency could be implicated (Wang et al., 2003; Bohlscheid et al., 2007).

Other sulfur-containing aroma compounds related to nitrogen nutrition are the volatile thiols. These are aroma impact compounds that play a prominent role in the aroma of Sauvignon blanc (Tominaga et al., 1998) and contribute to the aroma profiles of various other red and white cultivars (Thibon et al., 2008). The three thiols that mainly distinguish Sauvignon blanc character are 4-mercapto-4-methylpentan-2-one (4MMP) (cat's pee or broom), 3-mercaptohexanol (3MH) (grapefruit) and 3-mercaptohexyl acetate (3MHA) (passion fruit) (Darriet et al., 1995; Tominaga et al., 1998; Dubourdieu et al., 2006). The ability of a yeast strain to liberate the volatile thiol and amino acid acid moieties from the S-cysteine conjugate nonvolatile precursor, by carbon-sulfur β-lyase activity, is regarded the most important factor in the formation of thiols (Tominaga et al., 1998; Dubourdieu et al., 2006). However, the YAN content of the must can influence the presence of volatile thiols in wine, with high YAN levels possibly reducing thiol content of the wine. It has been proposed that NCR could repress the release of volatile thiols from S-cysteine conjugate precursors in synthetic medium, although the mechanism and plausibility are still disputed (Subileau et al., 2008b; Thibon et al., 2008; Deed et al., 2011).

In the work of Subileau et al. (2008), it is proposed that the uptake of the Cys-3MH precursor, which is structurally similar to cysteine, is induced by amino acid transporters and limited by the presence of preferred nitrogen sources such as ammonium. In their study, observations reminiscent of NCR were made. Production of the associated aromatic thiol (3MH) increased when yeast fermenting in synthetic must with a poor nitrogen source (urea) was replaced by a preferred source (diammonium phosphate; DAP). The authors also hypothesise that supplementation with DAP prolongs conditions of NCR and could delay the uptake of cysteinylated precursors of volatile thiols through GAP1p, thus resulting in a decrease of 3MH production in synthetic medium and Sauvignon blanc grape must. On the contrary, Thibon et al. (2008) found the release of volatile thiols from their cysteinylated precursors to be under general NCR control. Using a gene deletion approach, they demonstrated in synthetic grape must that NCR controlled the activity of the β-lyase enzyme and not the uptake of the precursors. However, Deed et al. (2011) showed by addition of DAP to real grape must or by deletion of NCR gene regulators in yeast, that NCR did not affect the concentration of volatile thiols in wine. Thus, at present the relationship between NCR and volatile thiol production is still unclear. S-glutathione conjugate precursors, rather than S-cysteine conjugate precursors may be quantitatively the major precursor of volatile thiols (Subileau et al., 2008a; Winter et al., 2011). It can be speculated that the presence of glutathionylated and other precursors could explain why volatile thiol release does not always seem subjected to NCR.

2.4.3 Vitamins

Vitamins are usually present in sufficient amounts in grape must for successful alcoholic fermentation, but their addition is beneficial to yeast cell growth and can play a role in the production of aroma compounds. In this regard, biotin and pantothenic acid are most often reported to influence the production of fermentation volatiles individually and in combination with nitrogen (Ough et al., 1989; Wang et al., Bohlscheid et al., 2007; Hagen et al., 2008).

Wine yeast is capable of synthesising all vitamins except biotin (Kunkee & Amerine, 1970; Oura & Suomalainen, 1978; Oura & Suomalainen, 1982; Monk, 1994). Biotin is required as cofactor in carboxylation reactions in sugar and amino acid metabolic pathways, for lipid synthesis, and the assimilation of sulfur compounds. Therefore, biotin could potentially impact on the production of all aroma compounds associated with various pathways such as higher alcohols, esters, medium-chain fatty acids (MCFA) and H_2S (Suomalainen & Keranen, 1963; Forch et al., 1975; Lynen, 1980). For example, biotin is an important cofactor for the enzyme pyruvate carboxylase which catalyses the transformation of pyruvate to oxaloacetate (Keech and Wallace 1985). Oxaloacetate serves as precursor for amino acid assimilation intermediates such as α-ketoglutarate and aspartic acid. A biotin deficiency can lead to insufficient α-ketoglutarate synthesis and subsequently reduced amino acid production, which influences the production of higher alcohols and related aroma compounds (Ahmad & Rose 1962; Cooper, 1982; Bohlscheid et al., 2007).

Biotin and pantothenic acid deficiencies can both result in reduced concentrations of MCFA and their associated esters in wine. Biotin is required for the activation of acetyl-CoA carboxylase during *de novo* fatty acid biosynthesis (Forch et al., 1975). Pantothenic acid is required as structural component of CoA. A pantothenic acid deficiency will result in decreased acetyl-CoA concentrations. In both cases this will lead to a reduction of fatty acid synthesis; thus lower concentrations of MCFA and their associated ethyl esters (Wang et al., 2003; Bohlscheid et al., 2007).

The combination of nitrogen and vitamin deficiencies could synergistically affect the accumulation of H_2S in wine (Bohlscheid et al. 2007). Biotin is required for aspartic acid production and pantothenic acid for CoA synthesis. In turn, both aspartic acid and CoA are required for the formation of receptor molecules of free sulfide ions in the SRS pathway, such as O-acetylhomoserine and O-acetylserine, to form sulfur-containing amino acids. When these receptor compounds are depleted due to a nitrogen or vitamin deficiency, H_2S will be produced (Wainwright 1970; Jiranek et al., 1995; Wang et al., 2003; Bohlscheid et al., 2007). The interactive effects of biotin or pantothenic acid with total nitrogen concentration illustrate that increasing the YAN will not “automatically” alleviate H_2S problems (Tamayo et al., 1999). In fact, Wang et al. (2003) suggests that an excess of YAN could stimulate the SRS pathway; which, when coupled with a shortage of pantothenic acid would result in a deficiency of O-acetylhomoserine and O-acetylserine, leading to excessive

H_2S production. Possibly, excess nitrogen increases the cellular demands for pantothenic acid (or rather, for acetyl-CoA) rather than increasing the sulfite/sulfate reductase activity. Thus, even when individual vitamins are available in sufficient concentrations to support maximum growth and fermentation rate, it may be insufficient to prevent sulfur off-flavours. On the other hand, it may be possible to reduce the production of H_2S under nitrogen deficient conditions with the addition of higher amounts of vitamins (Bohlscheid et al., 2007).

2.4.4 Minerals and metal ions

Minerals and metal ions are biologically essential micronutrients that play numerous important physiological roles during yeast growth and alcoholic fermentation (reviewed by Pereira, 1988 and Walker, 2004), yet these inorganic nutritional factors are often overlooked for their role in successful alcoholic fermentation and their contribution to the flavour of wine (Pohl et al., 2007; Ibanez et al., 2008). Major minerals are required by yeast in millimolar quantities (such as Na, Ca, K and Mg), while minor metals (including Al, Cu, Fe, Mn, Rb, Sr and Zn) and trace metals (Ba, Cd, Co, Cr, Li, Ni, Pb, V and others) are required in the micromolar range (Walker, 2004; Pohl et al., 2007).

During fermentation, metal ions act as catalysts or activators of glycolytic enzymes, thereby increasing biomass production and fermentative capacity. Furthermore, they participate in maintenance of cell integrity, cell-cell interactions (such as flocculation and foaming), osmoregulation, stress tolerance, gene expression, cell division, cell viability and growth (Pereira, 1988). The effects of minerals and metal ions on the aroma and flavour profile of the wine can be result of these cell activities. Minerals and metal ions can also cause direct alterations in the organoleptic properties of the wine including its flavour, aroma, taste, freshness and colour throughout the winemaking process. For example, metals can form complexes with polyphenols to stabilise colour in red wines acids (Cacho et al., 1995); participate in the browning of white wines with subsequent loss of freshness and aroma (Pohl et al., 2007); cause irreversible turbidity, haze or cloudiness (Russu et al., 1985; Green et al., 1997) and act as catalysts for oxidative spoilage during ageing (Pohl et al., 2007). Because metal ions have a tendency to chelate with other compounds such as proteins in the medium or cell cytoplasm, they are often unavailable to the yeast cell to use for cellular functions. Therefore, not only is the source and concentration of metal ions important, but their bioavailability is key to good fermentation performance (Walker, 2004).

Generally, wines with optimal concentrations and balanced ratios of micronutrients are described as balanced and full-bodied. Balanced levels of potassium are important for yeast growth and fermentation as a shortage or excess can lead to stuck fermentation and altered aroma profiles (Kudo et al., 1998; Perreira, 1988). Wines made with sufficient potassium reportedly have a pleasant acid taste. In contrast, a shortage of potassium can result in wines described as having a bland taste, while an excess can impart a bitter taste (Pereira, 1988). The

interactive effect of potassium and pH reportedly results in the production of metabolites such as acetic acid and glycerol in white wine, depending on the yeast strain (Schmidt et al., 2011). Elevated calcium to magnesium ratios can interfere with the uptake of magnesium by yeast cells and can result in the increase of undesirable metabolites such as acetic acid and acetaldehyde. Wines made from grape must optimally supplemented with magnesium reportedly contain reduced acetic acid and acetaldehyde, increased citric acid and glycerol and a desirable acid taste (Birch et al., 2003). Acetic acid, acetaldehyde and glycerol are also increased by increased sodium concentrations in the wine, and can make a negative flavour contribution when in excess (Pohl, 2007; Donkin et al., 2010).

Heavy metals, for example copper, can have positive or negative effects on yeast growth and aroma production. Heavy metals, although essential, may be toxic to yeast even in trace levels. Their optimal concentrations span a narrow concentration range above which they become inhibitory, mainly by causing a disruption of plasma membrane integrity (Azenha et al., 2000; Walker, 2004). For example, excessive copper will inhibit yeast activities, but minimum inhibitory concentrations differ between strains (Welch et al., 1983). Ferreira et al. (2006) determined that different yeast strains experience stress to different extents in the presence of copper ions, resulting in the production of significantly increased concentrations of acetic acid (volatile acidity) in wine.

When wines come into contact with heavy metals, for example fermentation containers and processing equipment made from heavy metals, reactions between the metal and sulfur dioxide or organic sulfur components in the wine can lead to the formation of H_2S, mercaptans and disulfides; all undesirable off-flavours (Eschenbruch & Kleynhans, 1974; Galani-Nikolakaki et al., 2002).

Various sulfur flavours can also be reduced by binding with metals. On the positive side, metal sulfates (typically CuSO4 or FeSO4) can be added to wine after fermentation to remove H_2S and other sulfur off-flavours through binding of these sulfur derivatives to form stable complexes, leading to an improvement in wine quality (Esparza et al., 2005). On the negative side, positive attributes can be diminished when heavy metals bind desirable sulfur compounds such as volatile thiols, which can dramatically decrease the varietal aroma of cultivars like Sauvignon blanc (Darriet et al., 2001).

Finally, zinc serves as cofactor for many fermentative enzymes and is able to modulate environmental stress (Walker, 2004). De Nicola et al. (2009) showed that zinc additions to a whisky distilling yeast strain of *S. cerevisiae* during malt fermentation increased the concentrations of esters and higher alcohols (especially those originating from branched-chain amino acids) and reduced formation of acetaldehyde. The authors propose that zinc could stimulate alcohol dehydrogenase, resulting in the conversion of acetaldehyde to ethanol. It is also proposed that acetaldehyde is reverted back to pyruvate and finally α-keto acids, which stimulates the production of higher alcohols.

2.4.5 Lipids and sterols

Lipids such as unsaturated long chain fatty acids and sterols such as ergosterol form part of the yeast plasma membrane and have various functions including the maintenance of membrane integrity and activities; particularly during stationary phase when fatty acids are required as "survival factors" to minimise ethanol disruption of plasma membrane activities (Lafon-Lafourcade et al., 1979). Palmitoleic and oleic acids constitute approximately 70% of the yeast cell membrane fatty acid content (Lambrechts &Pretorius, 2000).

During fatty acid biosynthesis, pyruvic acid is oxidatively decarboxylated to form acetyl-CoA, from which long chain saturated and unsaturated fatty acids are formed (Lynen, 1967). However, during wine fermentation, in the absence of oxygen, yeast depends on the uptake of exogenous unsaturated fatty acids (such as linoleic, oleic, linolenic, palmitic and palmitoleic acids) from grape must (Gallender & Peng, 1980; Ratledge & Evans, 1989). MCFA (such as hexanoic, octanoic and decanoic acids) are produced during the biosynthesis of long chain fatty acids, particularly under anaerobic fermentation conditions (Ravaglia & Delfini, 1993). These MCFA can inhibit yeast growth and alcoholic fermentation, depending on their solubility and the ethanol concentration in the medium (Walenga & Lands, 1975; Lafon-Lafourcade et al., 1984; Sa-Correia et al., 1989; Viegas et al., 1989; Ravaglia and Delfini, 1993), while long-chain fatty acids generally enhance growth (Soufleros and Bertrand, 1988). Furthermore, the composition of long chain unsaturated fatty acids in the grapes, grape must and yeast cell membranes affect the sensory attributes of wine as it is significantly correlated to the release of volatile compounds such as MCFA, esters and higher alcohols into the fermentation medium (Rosi & Bertuccioli, 1992; Torija et al., 2003; Yonuki et al., 2004; Yonuki et al. 2005).

A number of factors can influence the fatty acid composition of grapes, grape must and thus yeast cell membranes; such as the cryotolerance of grape varieties (Yonuki et al., 2005), pressing of the grapes, grape skin maceration, grape must clarification (Bertuccioli & Rosi, 1984), oxygen availability, yeast species or strain (Torija et al., 2003), fermentation temperature and nitrogen availability (Ratledge & Evans, 1989; Torija et al., 2003). Many of these factors impact on the degree of fatty acid unsaturation in the grapes and yeast cell membranes. When exogenous unsaturated fatty acids are available in abundance, yeast cells will incorporate it into cellular membrane lipids, with subsequent reduction of *de novo* fatty acids synthesis. Consequently, the production of related aroma compounds such as fatty acid ethyl esters and isoamyl acetate by yeast is reduced (Yonuki et al., 2005). In contrast, the *de novo* synthesis of fatty acids and ethyl esters is enhanced at low temperature fermentation (15°C) where exogenous unsaturated fatty acids are less readily incorporated by yeast cells than at higher fermentation temperature (25°C); potentially improving fruity aroma associated with esters (Yunoki et al., 2007). Thus, a higher degree of saturation in the yeast cell membrane is associated with increased elaboration of volatile compounds from the yeast (Rosi & Bertuccioli, 1992) particularly when fermented at cold temperature (Torija et al., 2003).

The fatty acid composition of the yeast cell membrane will also change in response to the nitrogen source (ammonium and/or amino acids) in the fermentation medium. Torija et al. (2003) showed that a high YAN fermentation medium consisting of a combination of amino acids and ammonium slowed growth, as also demonstrated by Fairbairn (2012). Torija et al. (2003) attributes this decrease in fermentation performance to a lower total fatty acid content but higher degree of unsaturation than when grown on ammonium or amino acids alone. The high degree of unsaturation also implies a reduction in aroma compound production.

2.5 Nutritional management strategies to optimise yeast-derived flavour in wine

2.5.1 Nutrient supplementation and yeast preconditioning

Nutritional management in the wine industry mainly constitutes routine supplementation of grape juice or fermenting grape must with inorganic nitrogen, and more recently also complex organic nitrogen sources, to reduce the risk of problem fermentations or correcting existing ones. It is generally indicated that many grape musts do not contain sufficient nitrogen for optimal fermentation performance, which results in problems such as sluggish fermentations and H_2S formation (Vos & Gray, 1979; Monk, 1982; Guidici & Kunkee, 1994; Jiranek et al., 1995; Hallinan et al. 1999; Spiropoulos et al. 2000; Wang 2003). Because ammonium is a preferred source of nitrogen to yeast cells, its presence will inhibit the uptake and utilisation of amino acids. This implies that when DAP additions are made during fermentation, it can cause major shifts in the pattern of amino acid accumulation, utilisation and transformation into aroma compounds. If a high concentration of DAP is added to the initial grape must, NCR may prevail throughout fermentation if the ammonium does not become fully depleted (Beltran et al., 2004).

However, the nitrogen requirements and preferences of individual yeast strains are inherently different and will also be differently affected by changing environmental conditions and the timing of nitrogen supplementation, factors which can be exploited as powerful flavour management tools. Thus empirical nitrogen supplementation without considering individual strain needs and natural grape must nitrogen composition is not best practice and has been rejected by several recent studies (Ugliano et al., 2007; Vilanova et al., 2007; Fairbairn, 2012).

Shortages of other nutrients, alone or in combination with nitrogen, could be the cause of fermentation problems and off-flavours. Various studies have determined that vitamin shortages have very little or no impacts on yeast growth and fermentation performance (Monk, 1982; Monk & Costello, 1984). However, shortages of individual vitamins could negatively impact on the production of aroma compounds, while combinations of vitamins and nitrogen shortages can also significantly alter the fermentation performance and organoleptic quality of the wine and should thus be considered in the diagnosis and correction of problem fermentations (Wang et al., 2003; Bohlscheid et al., 2007).

Similarly, it is generally assumed that mineral and metal concentrations are sufficient in grape must for the purposes of alcoholic fermentation. Therefore, grape must analysis of and supplementation with metals is still uncommon in the wine industry. In the beer industry, minerals such as zinc and magnesium are routinely analysed and optimised to improve yeast growth, fermentation performance and stress resistance (De Nicola et al., 2009). Yeast inorganic nutrition can be enhanced by mineral supplements in the form of inorganic salts. However, addition of external metal ions could result in a form that is not bioavailable to yeast, and may also be restricted by legislation (Walker, 2004). A viable alternative strategy is the use of preconditioned *S. cerevisiae* yeast cells enriched with metals. This intracellular enrichment strategy has been successfully applied for zinc and magnesium supplementation in brewer's and distilling yeast (De Nicola et al., 2009; Smith & Walker, 2000).

Inoculation of fermentation with commercial active dry wine yeast (ADWY) requires a rehydration step. The correct rehydration procedure, particularly when combined with rehydration nutrients, will ensure cell viability and vitality during fermentation. The concomitant use of inactive dry yeast products can aid cell membrane repair, suggesting the transfer of sterols between the inactive and the rehydrated yeast (Dulau et al., 2002; Soubeyrand et al., 2005). Another rehydration factor that was found to significantly enhance yeast vitality is magnesium; which had a greater impact than other rehydration factors studied by Rodriguez-Porrata et al. (2008), including carbon and nitrogen compounds, metallic ions, oxidant and antioxidant agents, and membrane fluidity agents.

For all reasons above, the use of complex commercial nutrients mainly comprised of inactivated yeast (a source of organic nitrogen) and other nutrients naturally present in the formulation or added (such as minerals, vitamins and lipids) is increasingly recommended by manufacturers to alleviate the characteristics of problem fermentations as well as for improving the general aroma profile of the wine (Munoz & Ingledew, 1990; Belviso et al., 2004).

2.5.2 Viticultural and winemaking practices

The quality and quantity of YAN in the fermentation medium, in particular the amino acid composition and concentration can already be manipulated in the vineyard through grape cultivar selection, grape maturity, the physical and chemical composition of the soil, and viticultural practices like canopy management (Spayd et al., 1994; Stines et al., 2000; Conradie, 2001). Apart from nitrogen, other nutritional factors required by yeast for successful alcoholic fermentation can be significantly influenced by the addition of grape vine nutrients (fertilizers), fungicides and insecticides in the vineyard; most often with detrimental implications for aroma compound production in the case of pesticides (Pohl et al., 2007). For example, copper can be transferred to wine as active ingredient of many pesticides; the bioavailability of zinc ions in grape musts could be negatively impacted by chemical additives such as fungicides (De Nicola et al., 2009) and residues of

elemental sulfur and other sulfur-containing fungicides and insecticides used in the vineyard can be reduced by wine yeast to form H_2S, mercaptans, disulfides and sulfur-containing esters (Shütz & Kunkee, 1977; Lambrecht & Pretorius, 2000).

During winemaking, any environmental parameter or winemaking practice that can affect the availability of nitrogen and carbon sources and subsequently the growth of the yeast strain could impact on the production of yeast-derived aroma compounds via catabolic and anabolic routes; including fermentation temperature (Torija et al., 2003; Beltran et al., 2008; Fairbairn, 2012), the exposure of grape must to oxygen prior to fermentation (Valero et al., 2002), the addition of lipids (Varela et al., 2012), and the amount of solids in the must (Ancín et al., 1996; Ayestarán et al., 1998). In addition, various winemaking parameters and practices can introduce metal contaminants into the wine. These include winemaking machinery and equipment made from metals (Eschenbruch & Kleynhans, 1974; Ibanez et al., 2008), processing water, clarifying and fining agents and other additives used in the cellar (Pohl et al., 2007; Ibanez et al., 2008).

All these viticultural and winemaking parameters should be carefully considered and managed for optimal wine flavour production by yeast.

2.5.3 Management of wine microflora and yeast strain selection

Many microorganisms are simultaneously and successively impacted by nutrient availability during and after alcoholic fermentation by *S. cerevisiae* (Fleet, 2003). At the beginning of fermentation, other yeast species will use a portion of the available nutrients, which could impact on the growth and fermentation performance of *S. cerevisiae*. For example, *Kloeckera apiculata* reportedly strips the grape must of micronutrients, particularly the vitamin thiamine, which can lead to sluggish or stuck fermentation, despite the fact that *S. cerevisiae* is able to synthesise this vitamin *de novo* (Bataillon et al.; 1996; Bisson, 1999; Mortimer, 2000). Indigenous non-*Saccharomyces* yeast could persist throughout alcoholic fermentation and thus compete with *S. cerevisiae* for nutrients and could significantly contribute to the production of volatile aroma compounds. Residual nutrients remaining after alcoholic fermentation can lead to the growth of spoilage yeast such as *Brettanomyces* and *Zygosaccharomyces* species. In addition to residual grape must nutrients, yeast autolysis releases organic nutrients which can support the growth and activities of spoilage yeast and lactic acid bacteria (Hernawan & Fleet, 1995), both of which could significantly affect the aroma profile of the wine.

The production of some compounds such as esters and higher alcohols has been potentially correlated with the nitrogen requirements of the yeast strains (Torrea et al., 2003). It is known that the nitrogen requirements, which regulate biomass production and fermentation rate of different wine yeast species and strains, are different. Generally, commercial yeasts have a relatively high nitrogen demand. A relationship exists between the nitrogen demand and the formation of volatile aroma compounds (Vilanova et al., 2007), where strains with a high demand have been found to produce more esters and fewer higher alcohols, volatile phenols,

monoterpenes and other (less volatile) alcohols. Indigenous yeasts have been shown to consume lower quantities of amino acids than commercial wine yeast strains and in particular ADY, indicating that industrial preparation of yeast strains increases their nutritional demands (Barrajón-Simancas et al., 2011).

Within the species *Saccharomyces cerevisiae*, production of many aroma compounds is evidently strain dependent (Lambrechts & Pretorius, 2000). Although most aroma compounds are produced by all wine yeast strains, the production of esters, fatty acids and higher alcohols (Mateo et al., 1992; Rossouw et al., 2008), H_2S formation (Jiranek et al., 1995, Spiropoulos et al., 2000, Mendes-Ferreira et al., 2002) and volatile thiol release and conversion (Howell et al., 2004; Dubourdieu et al., 2006) are all genetically and physiologically determined. Careful strain selection and management of indigenous yeast and bacterial populations should be employed to optimise flavour production and wine quality.

2.6 Conclusions

Modern winemaking necessitates the addition of yeast nutrients. Nutritional supplements to support fermentation to completion plays an increasingly important role as grape composition changes in response to vineyard stress and soil nutrients deficiencies emerging due to climate change (Mira de Orduña et al., 2010). In addition, it is recognised that the natural composition of grape musts may not be ideal for certain fermentation outcomes, particularly for wines produced from neutral grape varieties used to satisfy the preferences of diverse markets (Schmidt et al. 2011). For these reasons, proper chemical characterisation of grape must will assist winemakers to select the best nutrient supplementation strategy, favourably exploit its natural composition to optimise and individualise wine style and cultivar expression and/or to reduce fermentation problems. The appropriate choice of wine yeast strain, in order to match its inherent nutrient requirements to nutrient availability in the grape must should not be based solely on nitrogen efficiency and demand. In fact, in selecting both yeast strain and nutrient supplementation strategy, all individual and combinatorial nutritional and environmental factors encountered in grape must should be considered; an area of study which still requires much research.

References

Ahmad, F., & Rose, A.H. (1962). The role of biotin in the regulation of enzyme synthesis in yeast. *Archives of Biochemistry and Biophysics, 97*, 302–308.

Albers, E., Larsson, C., Lidén, G., Niklasson, C., & Gustafsson, L. (1996). Influence of the nitrogen source on *Saccharomyces cerevisiae* anaerobic growth and product formation. *Applied and Environmental Microbiology, 62*(9), 3187–95.

Ancin, C., Ayestarán, B., & Garrido, J. (1996). Clarification by vacuum filtration of Grenache must. Utilization of free amino acids during fermentation and bottle-aging of wine. *American Journal of Enology and Viticulture, 47*(3), 313–322.

Äyräpää, T. (1971). Biosynthetic formation of higher alcohols by yeast. Dependence on the nitrogen nutrient level of the medium. *Journal of the Institute of Brewing, 77*, 266–275.

Ayestarán, B., Garrido, J., & Ancín, C. (1998). Sedimentation clarification of viura musts. Utilization of amino acids during fermentation. *Food Chemistry, 63*(2), 191–197.

Azenha, M., Vasconcelos, M. T., & Moradas-Ferreira, P. (2000). The influence of Cu concentration on ethanolic fermentation by *Saccharomyces cerevisiae*. *Journal of Bioscience and Bioengineering, 90*(2), 163–7.

Bertuccioli, M.,& Rosi, I. (1984). Progress in flavour research. In: Adda, J. (Ed.), Developments in Food Science, Vol. 10. Elsevier, Amsterdam, p. 387.

Barbosa, C., Falco, V., Mendes-Faia, A., & Mendes-Ferreira, A. (2009). Nitrogen addition influences formation of aroma compounds, volatile acidity and ethanol in nitrogen deficient media fermented by *Saccharomyces cerevisiae* wine strains. *Journal of Bioscience and Bioengineering, 108*(2), 99–104.

Barrajón-Simancas, N., Giese, E., Arévalo-Villena, M., Úbeda, J., & Briones, A. (2011). Amino acid uptake by wild and commercial yeasts in single fermentations and co-fermentations. *Food Chemistry, 127*(2), 441–446.

Bataillon, M., Rico, A., Sablayrolles, J.-M., Salmon, J.-M., & Barre, P. (1996). Early thiamin assimilation by yeasts under enological conditions: Impact on alcoholic fermentation kinetics. *Journal of Fermentation and Bioengineering, 82*(2), 145–150.

Bell, S.-J., & Henschke, P. A. (2005). Implications of nitrogen nutrition for grapes, fermentation and wine. *Australian Journal of Grape and Wine Research, 11*(3), 242–295.

Beltran, G., Esteve-Zarzoso, B., Rozès, N., Mas, A., & Guillamón, J. M. (2005). Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and nitrogen consumption. *Journal of Agricultural and Food Chemistry, 53*(4), 996–1002.

Beltran, G., Novo, M., Guillamón, J. M., Mas, A., & Rozès, N. (2008). Effect of fermentation temperature and culture media on the yeast lipid composition and wine volatile compounds. *International Journal of Food Microbiology, 121*(2), 169–77.

Beltran, G., Novo, M., Rozès, N., Mas, A., & Guillamón, J. M. (2004). Nitrogen catabolite repression in *Saccharomyces cerevisiae* during wine fermentations. *FEMS Yeast Research, 4*(6), 625–32.

Belviso, S., Bardi, L., Bartolini, A. B., & Marzona, M. (2004). Lipid nutrition of *Saccharomyces cerevisiae* in winemaking. *Canadian Journal of Microbiology, 50*, 669–674.

Bely, M., Rinaldi, A., & Dubourdieu, D. (2003). Influence of assimilable nitrogen on volatile acidity production by *Saccharomyces cerevisiae* during high sugar fermentation. *Journal of Bioscience and Bioengineering, 96*(6), 507–12.

Birch, R. M., Ciani, M., & Walker, G. M. (2003). Magnesium, Calcium and Fermentative Metabolism in Wine Yeasts. *Journal of Wine Research, 14*(1), 3–15.

Bisson, L. (1999). Stuck and sluggish fermentations. *American Journal of Enology and Viticulture, 50*(1), 107–119.

Bisson, L. F., & Karpel, J. E. (2010). Genetics of yeast impacting wine quality. *Annual Review of Food Science and Technology, 1*, 139–62.

Boer, V. M., Tai, S. L., Vuralhan, Z., Arifin, Y., Walsh, M. C., Piper, M. D. W., De Winde, J. H., Pronk, J. T., & Daran, J-M. (2007). Transcriptional responses of *Saccharomyces cerevisiae* to preferred and nonpreferred nitrogen sources in glucose-limited chemostat cultures. *FEMS Yeast Research*, *7*(4), 604–20.

Bohlscheid, J. C., Fellman, J. K., Wang, X. D., Ansen, D., & Edwards, C. G. (2007). The influence of nitrogen and biotin interactions on the performance of *Saccharomyces* in alcoholic fermentations. *Journal of Applied Microbiology, 102*(2), 390–400.

Boulton, R.B., Singleton, V.L., Bisson, L.F., & Kunkee, R.E. (1995). Principles and Practices of Winemaking. Aspen Publishers Inc, USA.

Cacho, J., Castells, J., Esteban, A., Laguna, B., & Sagrista, N. (1995). Iron, copper, and manganese influence on wine oxidation. *American Journal of Enology and Viticulture, 46*(3), 380–384.

Conradie, W.J. (2001). Timing of nitrogen fertilisation and the effect of poultry manure on the performance of grapevines on sandy soil. II. Leaf analysis, juice analysis and wine quality. *South African Journal of Enology and Viticulture, 22*, 60−68.

Contreras, A., García, V., Salinas, F., Urzúa, U., Ganga, M. A., & Martínez, C. (2012). Identification of genes related to nitrogen uptake in wine strains of *Saccharomyces cerevisiae*. *World Journal of Microbiology & Biotechnology, 28*(3), 1107–13.

Cooper, T.G. (1982). Nitrogen metabolism in *Saccharomyces cerevisiae*. In: Strathern, J.N., Jones, E.W., Broach, J.R. (Eds.). The molecular biology of the yeast *Saccharomyces*: Metabolism and gene expression, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 39– 99.

Darriet, P., Bouchilloux, P., Poupot, C., Bugaret, Y., Clerjeau, M., Sauris, P., Medina, B., & Dubourdieu, D. (2001). Effects of copper fungicide spraying on volatile thiols of the varietal aroma of Sauvignon blanc, Cabernet Sauvignon and Merlot wines. *Vitis, 40*(2), 93–99.

Darriet, P., Tominaga, T., Lavigne, V., Boidron, J., & Dubourdieu, D. (1995). Identification of a powerful aromatic compound of *Vitis vinifera* L. var. Sauvignon wines: 4-Mercapto-4-methylpentan-2-one. *Flavour and Fragrance Journal, 10*, 385–392.

De Klerk, J. (2010). Succinic acid production by wine yeasts. MSc Thesis. Stellenbosch University. Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

De Nicola, R., Hall, N., Bollag, T., Thermogiannis, G., & Walker, G. (2009). Zinc accumulation and utilization by wine yeasts. *International Journal of Wine Research, 1*, 85–94.

De Nicola, R., Hall, N., Melville, S. G., Walker, G. M., & Brew, J. I. (2009). Influence of zinc on distiller's yeast: Cellular accumulation of zinc and impact on spirit congeners. *Journal of the Institute of Brewing, 115*(3), 265–271.

Deed, N. K., van Vuuren, H. J. J., & Gardner, R. C. (2011). Effects of nitrogen catabolite repression and di-ammonium phosphate addition during wine fermentation by a commercial strain of *S. cerevisiae*. *Applied Microbiology and Biotechnology, 89*(5), 1537–49.

Dickinson, J. R., Harrison, S. J., Dickinson, J. A., & Hewlins, M. J. (2000). An investigation of the metabolism of isoleucine to active Amyl alcohol in *Saccharomyces cerevisiae*. *The Journal of Biological Chemistry, 275*(15), 10937–42.

Dickinson, J. R., Harrison, S. J., & Hewlins, M. J. (1998). An investigation of the metabolism of valine to isobutyl alcohol in *Saccharomyces cerevisiae*. *The Journal of Biological Chemistry, 273*(40), 25751–6.

Dickinson, J. R., Lanterman, M. M., Danner, D. J., Pearson, B. M., Sanz, P., Harrison, S. J., & Hewlins, M. J. (1997). A 13C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*. *The Journal of Biological Chemistry, 272*(43), 26871–8.

Donkin, R., Robinson, S., Sumby, K., Harris, V., Mcbryde, C., & Jiranek, V. (2010). Sodium chloride in Australian grape juice and its effect on alcoholic and malolactic fermentation. *American Journal of Enology and Viticulture, 61*(3), 392–400.

Dubourdieu, D., Tominaga, T., Masneuf, I., Peyrot, C., & Murat, M. L. (2006). The Role of Yeasts in Grape Flavor Development during Fermentation: The Example of Sauvignon blanc. *American Journal of Enology and Viticulture, 57*(1), 81–88.

Dulau, L., Ortiz-Julien, A., & Trioli, G. (2002). Method for active dry yeast rehydration, and rehydration medium. International patent PCT/FR02/01949.

Eschenbruch, R., & Kleynhans, P. (1974). The influence of copper-containing fungicides on the copper content of grape juice and on hydrogen sulphide formation. *Vitis, 12*, 320–324.

Esparza, I., Salinas, Í., Santamaría, C., García-Mina, J. M., & Fernández, J. M. (2005). Electrochemical and theoretical complexation studies for Zn and Cu with individual polyphenols. *Analytica Chimica Acta, 543*(1-2), 267–274.

Fairbairn, S. C., (2012). Stress, fermentation performance and aroma production by yeast. MSc Thesis. Stellenbosch University. Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Ferreira, J., Du Toit, M., & Du Toit, W. J. (2006). The effects of copper and high sugar concentrations on growth, fermentation efficiency and volatile acidity production of different commercial wine yeast strains. *Australian Journal of Grape and Wine Research, 12*, 50–56.

Ferreira, V., Ortín, N., Escudero, A., López, R., & Cacho, J. (2002). Chemical characterization of the aroma of Grenache rosé wines: aroma extract dilution analysis, quantitative determination, and sensory reconstitution studies. *Journal of Agricultural and Food Chemistry, 50*(14), 4048–54.

Fleet, G. (2003). Yeast interactions and wine flavour. *International Journal of Food Microbiology, 86*(1-2), 11–22.

Forsberg, H., & Ljungdahl, P. O. (2001). Sensors of extracellular nutrients in *Saccharomyces cerevisiae*. *Current Genetics, 40*(2), 91–109.

Forch, M., Krauss, G., & Proksch, H. (1975). Wort composition and formation of lower free fatty acids. Part I. Effect of phosphorus, magnesium, calcium and biotin in the concentration of free fatty acids. *Journal of the Amercian Society of Brewing Chemists, 33*, 148–150.

Francis, I. L., & Newton, J. L. (2005). Determining wine aroma from compositional data. *Australian Journal of Grape and Wine Research, 11*(2), 114–126.

Galani-Nikolakaki, S., Kallithrakas-Kontos, N., & Katsanos, A. (2002). Trace element analysis of Cretan wines and wine products. *The Science of the Total Environment, 285*(1-3), 155–63.

Gallander, J. F., & Peng, A. C. (1980). Lipid and fatty acid compositions of different grape types. *American Journal of Enology and Viticulture, 31*(1), 24–27.

Garde-Cerdán, T., & Ancín-Azpilicueta, C. (2008). Effect of the addition of different quantities of amino acids to nitrogen-deficient must on the formation of esters, alcohols, and acids during wine alcoholic fermentation. *LWT - Food Science and Technology, 41*(3), 501–510.

Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D., et al. (2000). Genomic expression programs in the response of yeast cells to environmental changes. *Molecular Biology of the Cell, 11*(12), 4241–57.

Giudici, P., & Kunkee, R. E. (1994). The effect of nitrogen deficiency and sulpfur-containing amino acids on the reduction of sulfate to hydrogen sulfide by wine yeasts. *American Journal of Enology and Viticulture, 45*(1), 107–112.

Giudici, P., Zamponelli, C., & Kunkee, R. E. (1993). Increased production of n-propanol in wine by yeast strains having an impaired ability to form hydrogen sulfide. *American Journal of Enology and Viticulture, 44*(1), 17–21.

Graushmd, M., Didion, T., Kielland-brandt, M. C., & Andersen, H. A. (1995). BAP2, a gene encoding a permease for branched-chain amino acids in *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta, 1269*, 275–280.

Green, A. M., Clark, A. C., & Scollary, G. R. (1997). Determination of free and total copper and lead in wine by stripping potentiometry. *Fresenius' Journal of Analytical Chemistry, 358*(6), 711–717.

Hagen, K. M., Keller, M., & Edwards, C. G. (2008). Survey of Biotin, Pantothenic Acid, and Assimilable Nitrogen in Wine grapes from the Pacific Northwest. *American Journal of Enology and Viticulture, 59*(4), 432–436.

Hallinan, C. P., Saul, D. J., & Jiranek, V. (1999). Differential utilisation of sulfur compounds for H2S liberation by nitrogen-starved wine yeasts. *Australian Journal of Grape and Wine Research, 5*(3), 82–90.

Hazelwood, L. A., Daran, J.-M., van Maris, A. J. A., Pronk, J. T., & Dickinson, J. R. (2008). The Ehrlich pathway for fusel alcohol production: A century of research on *Saccharomyces cerevisiae* metabolism. *Applied and Environmental Microbiology, 74*(8), 2259–66.

Henschke, P.A & Jiranek, V. (1993). Yeasts – Metabolism of nitrogen compounds. In: Fleet, G. H. (Ed). Wine Microbiology and Biotechnology. Harwood Academic Publishers, Chur, Switzerland, pp. 77–164.

Hernawan, T., & Fleet, G. (1995). Chemical and cytological changes during the autolysis of yeasts. *Journal of Industrial Microbiology, 14*(6), 440–50.

Hernández-Orte, P, Ibarz, M. ., Cacho, J., & Ferreira, V. (2005). Effect of the addition of ammonium and amino acids to musts of Airen variety on aromatic composition and sensory properties of the obtained wine. *Food Chemistry, 89*(2), 163–174.

Hernández-Orte, P., Bely, M., Cacho, J., & Ferreira, V. (2006). Impact of ammonium additions on volatile acidity, ethanol, and aromatic compound production by different *Saccharomyces cerevisiae* strains during fermentation in controlled synthetic media. *Australian Journal of Grape and Wine Research, 12*(2), 150–160.

Hernández-Orte, P., Ibarz, M. J., Cacho, J., & Ferreira, V. (2006). Addition of amino acids to grape juice of the Merlot variety: Effect on amino acid uptake and aroma generation during alcoholic fermentation. *Food Chemistry, 98*(2), 300–310.

Hernández-Orte, P., Cacho, J. F., & Ferreira, V. (2002). Relationship between varietal amino acid profile of grapes and wine aromatic composition. Experiments with model solutions and chemometric study. *Journal of Agricultural and Food Chemistry, 50*(10), 2891–2899.

Hoffmann, W. (1985). Molecular characterization of the *CAN1* locus in *Saccharomyces cerevisiae*. A transmembrane protein without N-terminal hydrophobic signal sequence. *Journal of Biological Chemistry, 260*(21), 11831–11837.

Hofman-Bang, J. (1999). Nitrogen Catabolite Repression in *Saccharomyces cerevisiae*. *Molecular Biotechnology, 12*, 35–73.

Hohmann, S., Krantz, M., & Nordlander, B. (2007). Yeast osmoregulation. *Methods in Enzymology, 428*(7), 29–45.

Howell, K. S., Swiegers, J. H., Elsey, G. M., Siebert, T. E., Bartowsky, E. J., Fleet, G. H., Pretorius, I. S., De Barros Lopes, M.A. (2004). Variation in 4-mercapto-4-methyl-pentan-2-one release by *Saccharomyces cerevisiae* commercial wine strains. *FEMS Microbiology Letters, 240*(2), 125–9.

Ibanez, J. G., Carreon-Alvarez, A., Barcena-Soto, M., & Casillas, N. (2008). Metals in alcoholic beverages: A review of sources, effects, concentrations, removal, speciation, and analysis. *Journal of Food Composition and Analysis, 21*(8), 672–683.

Jain, V. K., Divol, B., Prior, B. A., & Bauer, F. F. (2012). Effect of alternative NAD^+-regenerating pathways on the formation of primary and secondary aroma compounds in a *Saccharomyces cerevisiae* glycerol-defective mutant. *Applied Microbiology and Biotechnology, 93*(1), 131–41.

Jiranek, V., Langridge, P., & Henschke, P. A. (1995a). Regulation of hydrogen sulfide liberation in wine-producing *Saccharomyces cerevisiae* strains by assimilable nitrogen. *Applied and Environmental Microbiology, 61*(2), 451–467.

Jiranek, V., Langridge, P., & Henschke, P. A. (1995b). Amino acid and ammonium utilization by *Saccharomyces cerevisiae* wine yeasts from a chemically defined medium. *American Journal of Enology and Viticulture, 46*(1), 75–83.

Keech, D.B., & Wallace, J.C. (1985). Pyruvate Carboxylase. Boca Raton, FL: CRC Press.

Keyzers, R. A., & Boss, P. K. (2010). Changes in the volatile compound production of fermentations made from musts with increasing grape content. *Journal of Agricultural and Food Chemistry, 58*(2), 1153–64.

Kudo, M., Vagnoli, P., & Bisson, L. (1998). Imbalance of pH and potassium concentration as a cause of stuck fermentations. *American Journal of Enology and Viticulture, 49*(3), 295–301.

Lafon-Lafourcade, S., Geneix, C., & Ribéreau-Gayon, P. (1984). Inhibition of alcoholic fermentation of grape must by fatty acids produced by yeasts and their elimination by yeast ghosts. *Applied and Environmental Microbiology, 47*(6), 1246–9.

Lafon-Lafourcade, S., Larue, F., & Ribereau-Gayon, P. (1979). Evidence for the existence of "survival factors" as an explanation for some peculiarities of yeast growth, especially in grape must of high sugar concentration. *Applied and Environmental Microbiology, 38*(6), 1069–1073.

Lambrechts, M., & Pretorius, I. (2000). Yeast and its importance to wine aroma – A review. *South African Journal of Enology and Viticulture, 21*, 97–129.

Large, P. J. (1986). Degradation of organic nitrogen compounds by yeasts. *Yeast, 2*, 1-34.

Lynen, F. (1967). The role of biotin-dependent carboxylations in biosynthetic reactions. *The Biochemical Journal, 102*(2), 381–400.

Lynen, F. (1980). On the structure of fatty acid synthetase of yeast. *European Journal of Biochemistry, 112*(3), 431–42.

Magasanik, B., & Kaiser, C. A. (2002). Nitrogen regulation in *Saccharomyces cerevisiae*. *Gene, 290*(1-2), 1–18.

Manginot, C., Sablayrolles, J. M., Roustan, J. L., & Barre, P. (1997). Use of constant rate alcoholic fermentations to compare the effectiveness of different nitrogen sources added during the stationary phase. *Enzyme and Microbial Technology, 20*(5), 373–380.

Marks, V., Van der Merwe, G., & Van Vuuren, H. (2003). Transcriptional profiling of wine yeast in fermenting grape juice: Regulatory effect of diammonium phosphate. *FEMS Yeast Research, 3*(3), 269–287.

Mateo, J., Jimenez, M., Huerta, T., & Pastor, A. (1992). Compasiron of volatiles produced by four *Saccharomyces cerevisiae* strains isolated from Monastrell musts. *American Journal of Enology and Viticulture, 43*(2), 206–209.

Mendes-Ferreira, A., Mendes-Faia, A., & Leao C. (2002). Survey of hydrogen sulphide production by wine yeasts. *Journal of Food Protection, 65*, 1033–1037

Mendes-Pinto, M. M. (2009). Carotenoid breakdown products the—norisoprenoids—in wine aroma. *Archives of Biochemistry and Biophysics, 483*(2), 236–245.

Miller, A., Wolff, S., Bisson, L., & Ebeler, S. (2007). Yeast strain and nitrogen supplementation: Dynamics of volatile ester production in Chardonnay juice fermentations. *American Journal of Enology and Viticulture, 58*(4), 470–483.

Mira de Orduña, R. (2010). Climate change associated effects on grape and wine quality and production. *Food Research International, 43*(7), 1844–1855.

Monk, P.R. (1982). Effect of nitrogen and vitamin supplements on yeast growth and rate of fermentation of Rhine Riesling grape juice. *Food Technology in Australia, 34*, 328–332.

Monk, P.R. (1994). Nutrient requirements of wine yeast. In: Proceedings of the New York Wine Industry Workshop. Henick-Kling, T. (Ed), Geneva, New York, pp. 57–64. Geneva, NY: Cornell University.

Monk, P.R., & Costello, P.J. (1984). Effect of ammonium phosphate and vitamin mixtures on yeast growth in preserved grape juice. *Food Technology in Australia, 36*, 25–28.

Moreira, N., Mendes, F., Pereira, O., Guedes de Pinho, P., Hogg, T., & Vasconcelos, I. (2002). Volatile sulphur compounds in wines related to yeast metabolism and nitrogen composition of grape musts. *Analytica Chimica Acta, 458*(1), 157–167.

Mortimer, R. K. (2000). *Kloeckera apiculata* controls the rates of natural fermentation. *Rivista di Viticolture e di Enologia, 53*, 61–68.

Munoz, E., & Ingledew, W. M. (1990). Yeast hulls in wine fermentations – A review. *Journal of Wine Research, 1*, 197–210.

Nordström, K. (1962). Formation of ethyl acetate in fermentation with brewer's yeast. III. Participation of coenzyme A. *Journal of the Institute of Brewing, 68*, 398–407.

Nordström, K. (1964). Formation of esters from acids by brewer's yeast. IV. Effect of higher fatty acids and toxicity of lower fatty acids. *Journal of the Institute of Brewing, 70*, 233–242.

Nykanen, L., & Nykanen, I. (1977). Production of esters by different yeast strains in sugar fermentations. *Journal of the Institute of Brewing, 83*, 30–31.

Nykanen, L. (1986). Formation and occurrence of flavor compounds in wine and distilled alcoholic beverages. *American Journal of Enology and Viticulture, 37*(1), 84–96.

Oshita, K., Kubota, M., Uchida, M. & Ono, M. (1995). Clarification of the relationship between fusel alcohol formation and amino acid assimilation by brewing yeast using 13C-labelled amino acid. Proceedings European Brewing Convention. Brussels (Oxford University Press: Oxford, UK) pp. 387–394.

Ough, C. ., Davenport, M., & Joseph, K. (1989). Effects of Certain Vitamins on Growth and Fermentation Rate of Several Commercial Active Dry Wine Yeasts. *American Journal of Enology and Viticulture, 40*(3), 208–213.

Ough, C., & Bell, A. (1980). Effects of nitrogen fertilization of grapevines on amino acid metabolism and higher-alcohol formation during grape juice fermentation. *American Journal of Enology and Viticulture, 31*(2), 122–123.

Ough, C. S., & Kunkee, R. E. (1968). Fermentation rates of grape juice V. Biotin content of juice and its effect on alcoholic fermentation rate. *Applied Microbiology, 16*(4), 572–6.

Oura, E., & Suomalainen, H. (1978). Biotin and the metabolism of baker's yeast. *Journal of the Institute of Brewing, 84*, 283–287.

Oura, E., & Suomalainen, H. (1982). Biotin active compounds, their existence in nature and the biotin requirements of yeast. *Journal of the Institute of Brewing, 88*, 299–308.

Peddie, H.A.B. (1990). Ester formation in brewery fermentations. *Journal of the Institute of Brewing, 96*, 327–331.

Pereira, C. (1988). The importance of metallic elements in wine. A literature survey. *Zeitschrift für Lebensmitteluntersuchung und- Forschung, 186*, 295–300.

Pineau, B., Barbe, J.-C., Van Leeuwen, C., & Dubourdieu, D. (2009). Examples of perceptive interactions involved in specific "red-" and "black-berry" aromas in red wines. *Journal of Agricultural and Food Chemistry, 57*(9), 3702–8.

Pohl, P. (2007). What do metals tell us about wine? *TrAC Trends in Analytical Chemistry, 26*(9), 941–949.

Ratledge, C., & Evans, C.T. (1989). Lipids and their metabolism. In: The yeasts, vol. III. Academic Press Ltd. pp 367-455.

Radler, F., & Schutz, H. (1982). Glycerol production of various strains of *Saccharomyces*. *American Journal of Enology and Viticulture, 33*(1), 36–40.

Rapp, A., & Versini, G. (1996). Influence of nitrogen compounds in grapes on aroma compounds of wines. *Developments in Food Science, 37*, 1659–1694.

Rauhut, D. (1993). Yeasts − Production of sulfur compounds. In: Fleet GH (Ed.). Wine microbiology and biotechnology. Harwood Academic, Chur, Switzerland, pp 183–223

Ravaglia, S., & Delfini, C. (1993). Production of MCFA and their ethyl esters by yeast strains isolated from musts and wines. *Italian Journal of Food Science, 1*, 21– 36.

Rosi, I., & Bertuccioli, M. (1992). Influences of lipid addition on fatty acid composition of *Saccharomyces cerevisiae* and aroma characteristics of experimental wines. *Journal of the Institute of Brewing, 98*, 305–314.

Rossouw, D., Naes, T., & Bauer, F. F. (2008). Linking gene regulation and the exo-metabolome: A comparative transcriptomics approach to identify genes that impact on the production of volatile aroma compounds in yeast. *BMC Genomics, 9*, 530.

Russu, G.I., Balanutse, A.P., Izmailova, D.N., Radchenko, Y.V., & Paraska, P.I. (1985). Content of heavy metal ions in cognacs. *Sadovodstvo, Vinogradarstvo i Vinodelie Moldavii, 11*, 36–38.

Rytka, J. (1975). Positive selection of general amino acid permease mutants in *Saccharomyces cerevisiae*. *Journal of Bacteriology, 121*(2), 562–70.

Sa-Correia, I., Salgueiro, S.P., Viegas, C.A. & Novais, J.M. (1989). Leakage induced by ethanol, octanoic and decanoic acids in *Saccharomyces cerevisiae*. *Yeast, 5*, 123-127.

Schmidt, A., Hall, M. N., & Koller, A. (1994). Two FK506 resistance-conferring genes in *Saccharomyces cerevisiae*, TAT1 and TAT2, encode amino acid permeases mediating tyrosine and tryptophan uptake. *Molecular and Cellular Biology, 14*(10), 6597–606.

Schmidt, S. A., Dillon, S., Kolouchova, R., Henschke, P. A., & Chambers, P. J. (2011). Impacts of variations in elemental nutrient concentration of Chardonnay musts on *Saccharomyces cerevisiae* fermentation kinetics and wine composition. *Applied Microbiology and Biotechnology, 91*(2), 365–75.

Schultess, D., & Ettlinger, L. (1978). Influence of the concentration of branched chain amino acids on the formation of fusel alcohols. *Journal of the Institute of Brewing, 84*, 240-243.

Schütz, M., & Kunkee, R. E. (1977). Formation of hydrogen sulfide from elemental sufur during fermentation by wine yeast. *American Journal of Enology and Viticulture, 28*(3), 137–144.

Sea, K., Butzke, C., & Boulton, R. (1998). Seasonal variation in the production of hydrogen sulfide during wine fermentations. In: Waterhouse, A.L., & Ebeler, S.E. (Eds.). Chemistry of Wine flavour pp. 81–95. Washington, D.C.: American Chemical Society.

Sinclair, K., Warner, J. P., & Bonthron, D. T. (1994). The ASP1 gene of *Saccharomyces cerevisiae*, encoding the intracellular isozyme of L-asparaginase. *Gene, 144*, 37–43.

Smith, G. D., & Walker, G. M. (2000). Fermentation performance of Mg-preconditioned yeast. In: Smart, K. A. (Ed.). Brewing Yeast Fermentation Performance. pp. 92–95. Blackwell Scientific Publications, Oxford.

Soubeyrand, V., Luparia, V., Williams, P., Doco, T., Vernhet, A., Ortiz-Julien, A., & Salmon, J.-M. (2005). Formation of micella containing solubilized sterols during rehydration of active dry yeasts improves their fermenting capacity. *Journal of Agricultural and Food Chemistry, 53*(20), 8025–32.

Soufleros, E., & Bertrand, A. (1988). Les acides gras libres du vin: observations sur leur origine. *Connaissance de la Vigne et du Vin, 22*, 251–260.

Spayd, S., Wample, R., Evans, R., Stevens, R., Seymour, B., & Nagel, C. (1994). Nitrogen fertilization of white Riesling grapes in Washington. Must and wine composition. *American Journal of Enology and Viticulture, 45*(1), 34–42.

Spiropoulos, A., Tanaka, J., Flerianos, I., & Bisson, L. F. (2000). Characterization of hydrogen sulfide formation in commercial and natural wine isolates of *Saccharomyces*. *American Journal of Enology and Viticulture, 51*(3), 223–248.

Stines, A. P., Grubb, J., Gockowiak, H., Henschke, P. A., Høj, P. B., & Heeswijck, R. (2000). Proline and arginine accumulation in developing berries of *Vitis vinifera* L. in Australian vineyards: Influence of vine cultivar, berry maturity and tissue type. *Australian Journal of Grape and Wine Research, 6*(2), 150–158.

Styger, G., Prior, B., & Bauer, F. F. (2011). Wine flavor and aroma. *Journal of Industrial Microbiology & Biotechnology, 38*(9), 1145–59.

Subileau, M., Schneider, R., Salmon, J.-M., & Degryse, E. (2008a). New insights on 3-mercaptohexanol (3MH) biogenesis in Sauvignon Blanc wines: Cys-3MH and (E)-hexen-2-al are not the major precursors. *Journal of Agricultural and Food Chemistry, 56*(19), 9230–5.

Subileau, M., Schneider, R., Salmon, J.-M., & Degryse, E. (2008b). Nitrogen catabolite repression modulates the production of aromatic thiols characteristic of Sauvignon Blanc at the level of precursor transport. *FEMS Yeast Research, 8*(5), 771–80.

Suomalainen, H., & Keraenen, A. (1963). The effect of biotin deficiency on the synthesis of fatty acids by yeast. *Biochimica et Biophysica Acta, 70*, 493–503.

Swiegers, J. H., & Pretorius, I. S. (2007). Modulation of volatile sulfur compounds by wine yeast. *Applied Microbiology and Biotechnology, 74*(5), 954–60.

Swiegers, J. H., & Pretorius, I. S. (2005). Yeast modulation of wine flavor. *Advances in Applied Microbiology, 57*(05), 131–75.

Sychrova, H., & Chevallier, M.R. (1993). Cloning and sequencing of the *Saccharomyces cerevisiae* gene LYP1 coding for the lysine permease. *Yeast, 9*, 771-782.

Tamayo, C., Ubeda, J., & Briones, A. (1999). Relationship between H_2S-producing strains of wine yeast and different fermentation conditions. *Canadian Journal of Microbiology, 45*(4), 343–346.

Tanaka, J., & Fink, G. (1985). The histidine permease gene (HIP1) of *Saccharomyces cerevisiae*. *Gene, 38*, 205–214.

Ter Schure, E. G., Van Riel, N. A. V, & Verrips, C. T. (2000). The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews, 24*(1), 67–83.

Thibon, C., Marullo, P., Claisse, O., Cullin, C., Dubourdieu, D., & Tominaga, T. (2008). Nitrogen catabolic repression controls the release of volatile thiols by *Saccharomyces cerevisiae* during wine fermentation. *FEMS Yeast Research, 8*(7), 1076–86.

Tominaga, T., Furrer, A., Henry, R., & Dubourdieu, D. (1998). Identification of new volatile thiols in the aroma of *Vitis vinifera* L. var. Sauvignon blanc wines. *Flavour and Fragrance Journal, 13*, 159–162.

Torija, M., Beltran, G., Novo, M., Poblet, M., Guillamón, J. M., Mas, A., & Rozès, N. (2003). Effects of fermentation temperature and *Saccharomyces* species on the cell fatty acid composition and presence of volatile compounds in wine. *International Journal of Food Microbiology, 85*, 127–136.

Torija, M., Beltran, G., Novo, M., Poblet, M., Rozès, N., Guillamón, J. M., & Mas, A. (2003). Effect of the nitrogen source on the fatty acid composition of *Saccharomyces cerevisiae*. *Food Microbiology, 20*, 255–258.

Torrea, D., Fraile, P., Garde, T., & Ancín, C. (2003). Production of volatile compounds in the fermentation of chardonnay musts inoculated with two strains of *Saccharomyces cerevisiae* with different nitrogen demands. *Food Control, 14*(8), 565–571.

Ugliano, M., Henschke, P. A., Herderich, M., & Pretorius, I. S. (2007). Nitrogen management is critical for wine flavour and style. *Wine Industry Journal, 22*(6), 24–30.

Valero, E., Moyano, L., Millan, M., Medine, M., & Ortega, J. (2002). Higher alcohols and esters production by *Saccharomyces cerevisiae*. Influence of the initial oxygenation of the grape must. *Food Chemistry, 78*, 57–61.

Varela, C., Torrea, D., Schmidt, S., Ancín-Azpilicueta, C., & Henschke, P. (2012). Effect of oxygen and lipid supplementation on the volatile composition of chemically defined medium and Chardonnay wine fermented with *Saccharomyces cerevisiae*. *Food Chemistry, 135*(4), 2863–2871.

Viegas, C. A., Rosa, M. F., Sá-correia, I., Novais, J. M., Correia, I. S. A., & Novais, J. M. (1989). Inhibition of yeast growth by octanoic and decanoic acids produced during ethanolic fermentation. *Applied and Environmental Microbiology, 55(*1), 21–28.

Vilanova, M., Ugliano, M., Varela, C., Siebert, T., Pretorius, I. S., & Henschke, P. A. (2007). Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by *Saccharomyces cerevisiae* wine yeasts. *Applied Microbiology and Biotechnology, 77*(1), 145–57.

Vos, P. J. A., & Gray, R. S. (1979). The origin and control of hydrogen sulfide during fermentation of grape must. *American Journal of Enology and Viticulture, 30*(3), 187–197.

Vuralhan, Z., Morais, M. A., Tai, S., Piper, M. D. W., & Pronk, J. T. (2003). Identification and characterization of phenylpyruvate decarboxylase genes in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology, 69*(8), 4534–4541.

Wainwright, T. (1970). Hydrogen sulphide production by yeast under conditions of methionine, pantothenate or vitamin B6 deficiency. *Journal of General Microbiology, 61*, 107–119.

Walenga, R. W., & Lands, W. E. (1975). Effectiveness of various unsaturated fatty acids in supporting growth and respiration in *Saccharomyces cerevisiae*. *The Journal of Biological Chemistry, 250*(23), 9121–9129.

Walker, G. M. (2004). Metals in yeast fermentation processes. *Advances in Applied Microbiology, 54*, 197–229.

Wang, X. D., Bohlscheid, J. C., & Edwards, C. G. (2003). Fermentative activity and production of volatile compounds by *Saccharomyces* grown in synthetic grape juice media deficient in assimilable nitrogen and/or pantothenic acid. *Journal of Applied Microbiology, 94(*3), 349–59.

Watson, T. G. (1976). Amino-acid pool composition of *Saccharomyces cerevisiae* as a function of growth rate and amino-acid nitrogen source. *Journal of General Microbiology, 96*(2), 263–8.

Welch, J. W., Fogel, S., Cathala, G. U. Y., & Karin, M. (1983). Industrial yeasts display tandem gene iteration at the Industrial Yeasts Display Tandem Gene Iteration at the CUP1 Region. *Molecular and Cellular Biology, 3*(8), 1353–1361.

Winter, G., Van Der Westhuizen, T., Higgins, V. J., Curtin, C., & Ugliano, M. (2011). Contribution of cysteine and glutathione conjugates to the formation of the volatile thiols 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) during fermentation by *Saccharomyces cerevisiae*. *Australian Journal of Grape and Wine Research, 17*(2), 285–290.

Yunoki, K., Hirose, S., & Ohnishi, M. (2007). Ethyl esterification of long-chain unsaturated fatty acids derived from grape must by yeast during alcoholic fermentation. *Bioscience, Biotechnology and Biochemistry, 71*(12), 3105–3109.

Yunoki, K., Tanji, M., Murakami, Y., Yasui, Y., Hirose, S., & Masao, O. (2004). Fatty acid compositions of commercial red wines. *Bioscience, Biotechnology and Biochemistry, 68*(12), 2623–2626.

Yunoki, K., Yasui, Y., Hirose, S., & Ohnishi, M. (2005). Fatty acids in must prepared from 11 grapes grown in Japan: comparison with wine and effect on fatty acid ethyl ester formation. *Lipids, 40*(4), 361–367.

Zaman, S., Lippman, S. I., Zhao, X., & Broach, J. R. (2008). How *Saccharomyces* responds to nutrients. *Annual Review of Genetics, 42*, 27–81.

Chapter 3

Initial assessment of the combinatorial impacts of nitrogen source, addition time and yeast strain on fermentation performance and aroma production in synthetic grape must

CHAPTER 3

Initial assessment of the combinatorial impacts of nitrogen source, addition time and yeast strain on fermentation performance and aroma production in synthetic grape must

Abstract

An initial framework is provided of the relative importance of yeast genetic background, nitrogen composition and nitrogen supplementation regimes in determining the fermentation performance and aroma outcomes in synthetic grape must. Firstly, the impact on fermentation of six nitrogen treatments (grouped according to their preference as nitrogen source or acting as aroma precursors) added either to the initial grape must or after the onset of fermentation was assessed for nine commercial wine yeast strains. Secondly, end-point aroma profiles were established and factors that significantly contribute to the aroma production patterns were identified. Unusual strain behavior was investigated. Our results indicate that preferred- and branched-chain and aromatic amino acid treatments most consistently supported fermentation completion when added to the initial fermentation medium. In general, highly variable fermentation performances were observed for different nitrogen treatment and yeast strain combinations, also depending on the timing of nitrogen addition. Multi-factorial analysis showed that the presence of amino acid precursors is the most distinguishing factor for higher alcohol, ester and fatty acid production. Although aroma compound production patterns per treatment were comparable for most strains, a few strains exhibited divergent behavior, particularly with regards to addition time points. Later addition of amino acid treatments generally resulted in a greater number and magnitude of significant changes in aroma compounds related to precursor amino acids. Our data indicate that different pathways of aroma compound formation (anabolic or catabolic) could be involved depending on the timing of nitrogen addition. Taken together, the fermentation performance and aroma data clearly indicate that wine quality can benefit from correct and carefully considered pairing between yeast strain and grape must nitrogen content; and that empirical addition at different fermentation stages can lead to adverse effects for particular yeast strains.

3.1 Introduction

Saccharomyces cerevisiae is the domesticated yeast of choice in the global wine industry, with more than 200 strains marketed internationally. Numerous strains of this species have been isolated from spontaneous fermentations or selected from breeding programmes and are used commercially for various specialised oenological applications. Wine yeast strain selection is usually aimed at the fermentation of different grape varieties and to obtain different styles of wine. More specifically, yeasts are chosen on the basis of characteristics such as optimised fermentation efficiency, aroma production, enzyme activities, cell wall properties and stress tolerance (Pretorius & Bauer, 2002). The highly divergent phenotypes

among wine yeast strains of the species *S. cerevisiae* are also evident in the significant genetic diversity of this species (Rossouw et al., 2008; Camarasa et al., 2011).

As of late, wine producers and product manufacturers are considering alternative strategies to produce individualised wines, rather than breeding more strains for an already saturated yeast market. The focus is shifting towards optimising the use of wine additives, such as enzymes and yeast nutrients, in combination with yeast strain selection and management of fermentation parameters to obtain particular styles of wine (Van Rensburg & Pretorius, 2000; Vilanova et al., 2007; Beltran et al., 2008; Carrau et al., 2008; Fairbairn, 2012).

Many nutrients required by industrial wine yeast strains during alcoholic fermentation may be present in grape must in insufficient supply to allow full sugar utilisation. Due to the highly diverse genetic backgrounds of wine yeast strains, the nutrient requirement for each strain will differ. For example, some reports suggest that many commercial yeast strains have a relatively high nitrogen demand compared to "indigenous" strains (Torrea et al., 2003; Hernández-Orte et al., 2005; Barrajón-Simancas et al., 2011), a characteristic associated with higher concentrations of esters and lower concentrations of higher alcohols and volatile phenols in wine (Vilanova et al., 2007). Nutrient research has indeed been focused mainly on nitrogen requirements because nitrogen in grape must is often growth-limiting, and certain nitrogen sources such as amino acids are direct precursors of important wine aroma compounds.

Ammonium, amino acids and other nitrogen compounds naturally occur in highly variable concentrations and combinations in grape juice, depending on viticultural factors such as grape cultivar (Hernández-Orte et al., 2002) and vineyard nutrition (Bell & Henschke, 2005), and oenological practices such as pressing, maceration of grape skins (Smit et al. 2013) and clarification of grape must (Ayestarán et al., 1998).

It has been established by several studies that nitrogen composition and concentration can influence yeast biomass formation, fermentation rate and sugar utilisation (reviewed by Henschke & Jiranek, 1993; Varela et al., 2004). For this reason, nitrogen addition to grape juice is one of the most common practices in commercial wine cellars. The first objective of adding nitrogen is usually to prevent or overcome fermentation problems such as stuck or sluggish fermentation and to avoid the production of unpleasant aromas such as hydrogen sulphide (H_2S). As a consequence, additions are often made empirically and without considering the impact of nitrogen addition on other oenological aspects such as the production of undesirable compounds affecting wine wholesomeness, such as urea and ethyl carbamate (Ough et al., 1988) and the overall aroma and flavour profile of the wine. The result of nitrogen supplementation in wine cellars will indeed alter the fermentation bouquet, since the changes induced in the nitrogen metabolism due to source; amount and timing of nitrogen addition could impact the production of aroma compound quality and quantity (Beltran et al., 2005; Hernández-Orte et al., 2005; Hernández-Orte et al., 2006a; Hernández-Orte et al., 2006b; Garde-Cerdán & Ancín-Azpilicueta, 2008; Barbosa et al., 2009).

Yeast will prefer a good source of nitrogen, and if present, will shut down transport and utilisation of poor sources, by a mechanism known as nitrogen catabolite repression (NCR). This mechanism enables the yeast to preferentially select nitrogen sources that best support growth (Ter Schure et al., 2000). According to Magasanik and Kaiser (2002) the growth rate supported by a nitrogen source and the extent of derepression (activation) of transport systems to enable the yeast cell to use alternative nitrogen sources determine how "good" a nitrogen source is.

Many studies have determined and described good (preferred) and poor (non-preferred) assimilable nitrogen sources for *S. cerevisiae*, and are generally in agreement. In laboratory studies reviewed by Hofman-Bang (1999), Ter Schure et al. (2000) and Magasanik and Kaiser (2002), ammonium, glutamine, glutamate and asparagine are generally considered good nitrogen sources, while proline, ornithine, γ-aminobutyrate, allantoin and urea are listed as poor sources. In grape must, arginine is also considered a reasonably good nitrogen source due to its abundance (Large, 1986; Hofman-Bang, 1999). A number of amino acids present in grape must are utilised poorly or not at all during wine fermentation, including proline, tryptophan, alanine, histidine, glycine and lysine (Cooper, 1982; Large, 1986; Henschke & Jiranek, 1993; Beltran et al., 2004; Beltran et al., 2005), although conflicting results are sometimes reported by different studies. Branched-chain and aromatic amino acids are not considered the best nitrogen sources as they do not support high growth rates (Watson, 1976; Boer et al., 2007), but are taken up from early fermentation stages throughout nitrogen repressed conditions during wine fermentation (Forsberg & Ljungdahl 2001; Beltran et al., 2004; Beltran et al., 2005).

NCR essentially affects the transport of amino acids. The permeases of amino acids subjected to NCR are repressed when yeast is grown on good nitrogen sources, and will be derepressed when the good sources (such as ammonium) are depleted and only poor nitrogen sources are available; for example the general amino acid permease Gap1p (transporting all naturally occurring amino acids) and Put4p (transporting proline) (Vandenbol et al., 1989; Jauniaux & Grenson, 1990). There are also permeases that are expressed constitutively and transport specific amino acids but also have variable degrees of specificity for particular groups of amino acids; for example Hip1p transports histidine, Can1p transports arginine and basic amino acids, Lyp1p transports lysine and Tat2p transports tryptophan (Cooper, 1982; Tanaka & Fink, 1985; Hoffmann, 1985; Sychrova & Chevallier, 1993; Schmidt et al., 1994).

Many amino acid assimilation studies for *S. cerevisiae* are focussed on laboratory strains or single industrial strains. Indications exist of strain dependent differences in preference for nitrogen sources, as demonstrated for ammonium preference and utilisation by laboratory strains (Rytka, 1975). The different patterns of nitrogen uptake and utilisation in wine reported by various research groups can possibly be attributed to variability in yeast strain and fermentation medium used in these different studies (Bell & Henschke, 2005). It is therefore necessary to keep in consideration the possible diversity of responses to nitrogen supplementation due to different yeast genetic backgrounds, as the yeast ecology and chemical composition of wine is by nature diverse.

It has been clearly shown that combined factors such as grape must chemical composition, nitrogen source and concentration, timing of nutrient addition, yeast genetic background and environmental stress have impacts that are significantly different from those that would be expected based on extrapolation of studies assessing single parameters. Most studies that have been reported thus far have only assessed the impact of individual or a limited number of parameters on fermentation performance and/or aroma production. However, the impacts of specific combinations of amino acids, deliberately grouped according to defined criteria, have not been reported. The aim of the present study was thus to explore the impact of six highly biased nitrogen supplementation treatments (grouped according to the preference of *S. cerevisiae* strains to utilise the amino acids as source of nitrogen and/or the potential impact the amino acids may have on aroma production) at two time points of nitrogen addition (initial fermentation media or after three days) on fermentation performance and the production of oenologically relevant aroma compounds by nine commercial yeast strains. The goal was to assess in these extreme conditions in model wine which parameters were most decisive in defining the aroma profile of the fermented product.

3.2 Materials and methods

3.2.1 Fermentation medium

The fermentation medium used in this study was a chemically defined synthetic grape must simulating a wine matrix, based on the medium described by Henschke & Jiranek (1993). The total sugar concentration was 200 g/l, consisting of equal amounts of glucose and fructose. The total nitrogen concentration of the medium was 200 mg N/l of yeast assimilable nitrogen. All treatments contained 50 mg N/l of ammonium nitrogen, supplied as ammonium chloride to the initial grape must. The remaining 150 mg N/l nitrogen varied for the six treatments and was added either to the initial grape must or at the beginning of the exponential phase (on day three of fermentation). Within a treatment, each amino acid contributed equally to the nitrogen content, to a total of 150 mg N/l. The composition of each treatment is described in **Table 1**. Ammonium chloride and amino acids were supplied by Sigma Aldrich and Fluka (Germany).

Table 1 The composition of nitrogen treatments applied in this study. Each treatment was initially supplied with 50 mg N/l of ammonium before the onset of fermentation. Additionally 150 mg N/l of different amino acid combinations was added to the synthetic grape must (initially or on day three of fermentation), with each amino acid contributing equally to the nitrogen content within a treatment.

Treatment	Compound	%N[a]	mg N/L	mg/L
Ammonium only				
Initial addition	**NH_4Cl**	**21.2**	**50.0**	189.3
Treatment addition	**NH_4Cl**	**21.2**	**150.0**	568.0

Table 1 (cont.)

Treatment	Compound	%N[a]	mg N/L	mg/L
Complete amino acids				
Initial addition	**NH_4Cl**	**21.2**	**50.0**	189.3
Treatment addition	**Amino acids**		**150.0**	
	ALA	15.7	7.5	47.8
	ARG	32.2	7.5	23.3
	ASN	21.2	7.5	35.4
	ASP	10.5	7.5	71.4
	CYS	11.6	7.5	64.9
	GLN	19.2	7.5	39.1
	GLU	9.5	7.5	78.9
	GLY	18.6	7.5	40.3
	HIS	27.1	7.5	27.7
	ILE	10.7	7.5	70.1
	LEU	10.7	7.5	70.1
	LYS	19.2	7.5	39.1
	MET	9.4	7.5	79.8
	PHE	8.5	7.5	88.2
	PRO	12.2	7.5	61.5
	SER	13.3	7.5	56.4
	THR	11.8	7.5	63.6
	TRP	13.7	7.5	54.7
	TYR	7.7	7.5	97.4
	VAL	12.0	7.5	62.5
Preferred amino acids				
Initial addition	**NH_4Cl**	**21.2**	**50.0**	189.3
Treatment addition	**Amino acids**		**150.0**	
	ARG	32.2	30.0	93.2
	ASN	21.2	30.0	141.5
	ASP	10.5	30.0	285.7
	GLN	19.2	30.0	156.3
	GLU	9.5	30.0	315.8
Branched-chain and aromatic amino acids				
Initial addition	**NH_4Cl**	**21.2**	**50.0**	189.3
Treatment addition	**Amino acids**		**150.0**	
	ILE	10.7	30.0	280.4
	LEU	10.7	30.0	280.4
	PHE	8.5	30.0	352.9
	TYR	7.7	30.0	389.6
	VAL	12.0	30.0	250.0
Non-utilised amino acids				
Initial addition	**NH_4Cl**	**21.2**	**50.0**	189.3
Treatment addition	**Amino acids**		**150.0**	
	HIS	27.1	50.0	184.5
	LYS	19.2	50.0	260.4
	PRO	12.2	50.0	409.8
Non-preferred amino acids				
Initial addition	**NH_4Cl**	**21.2**	**50.0**	189.3
Treatment addition	**Amino acids**		**150.0**	
	ALA	15.7	30.0	191.1
	GLY	18.6	30.0	161.3
	SER	13.3	30.0	225.6
	THR	11.8	30.0	254.2
	TRP	13.7	30.0	219.0

[a] Henschke & Jiranek (1993)

3.2.2 Yeast strains and fermentation conditions

The nine industrial wine yeast strains used in this study are listed in **Table 2**. Pure cultures of the strains were maintained on YPD agar. Two consecutive overnight pre-cultures were performed at 30°C ; first in YPD broth and thereafter in synthetic grape must containing only ammonium as nitrogen source. Fermentations were inoculated from pre-cultures into synthetic grape must treatments to a final OD_{600} of 0.1 (cell density of approximately 10^6 cfu/ml).

Fermentations were carried out in triplicate under static batch conditions at 20 to 22°C in a temperature controlled fermentation room. The fermentation vessels were 100 ml glass bottles containing 80 ml of fermentation medium, sealed with rubber stoppers and a CO_2 gas outlet. Fermentation activity was measured as CO_2 weight loss over a time course of 21 days, after which analyses of major volatile and non-volatile fermentation products were performed.

Table 2 Yeast strains used in this study.

Yeast strain	Commercial name and source	Species
VIN13	VIN13 Anchor Yeast, Cape Town, South Africa	*Saccharomyces cerevisiae* hybrid
NT50	NT50 Anchor Yeast, Cape Town, South Africa	*Saccharomyces cerevisiae* hybrid
NT202	NT202 Anchor Yeast, Cape Town, South Africa	*Saccharomyces cerevisiae* hybrid
PR7	Exotics Anchor Yeast, Cape Town, South Africa	*Saccharomyces cerevisiae x Saccharomyces paradoxus* hybrid
FXL	Fermicru XL DSM Food Specialities, Delft, The Netherlands	*Saccharomyces cerevisiae*
BM45	Lalvin BM45 Lallemand Inc., Montreal, Canada	*Saccharomyces cerevisiae*
285	Lalvin Cross Evolution Lallemand Inc., Montreal, Canada	*Saccharomyces cerevisiae* hybrid
DV10	Lalvin DV10 Lallemand Inc., Montreal, Canada	*Saccharomyces cerevisiae bayanus*
EC1118	Lalvin EC1118 Lallemand Inc., Montreal, Canada	*Saccharomyces cerevisiae bayanus*

3.2.3 Chemical analysis

Non-volatile fermentation products were analysed using a Winescan FT120 instrument (FOSS Analytical A/S, Hillerød, Denmark). Residual glucose and fructose concentrations were predicted from the mid-infrared spectra by in-house adjustments of commercial calibrations (FOSS Analytical A/S software version 2.2.1).

Volatile compounds were extracted from the synthetic wine samples by liquid-liquid extraction as described by Louw et al. (2009) with the following modification in order to optimise extraction in the synthetic wine matrix. The sample solvent mixture was centrifuged at 4000 rpm for 3 minutes, sodium sulfate was added to the mixture and the centrifugation repeated before removal and drying of the organic layer as previously described. Extracts were analysed in duplicate.

Major volatile compounds were quantified by gas chromatography with flame ionization detection (GC-FID). Calibrations for 39 volatile compounds were created with authentic standards (Merck, Cape Town, South Africa), using the internal standard method with 4-methyl-2-pentanol as internal standard. The volatile aroma compounds were analysed on a Hewlett Packard 6890 Plus gas chromatograph (Agilent, Little Falls, Wilmington, USA), equipped with a split/splitless injector. The split ratio was set to 15:1, the split flow rate 98.7 ml/min, and the temperature at 200°C. Compounds were separated on a J & W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with dimensions of 60 m × 0.32 mm with a 0.5 µm coating film thickness. Hydrogen was used as carrier gas with a flow rate of 6.6 ml/min. Three microliters of the extracted sample was injected into the gas chromatograph in duplicate. The oven temperature was held at 33°C for 8 minutes; increased by 21°C/min to 130°C and held for 1.3 min; increased by 21°C/min to 170°C and held for 1 min; and finally increased by 21°C/min to 240°C and held for 2.5 min. The FID was operated at 250°C with a hydrogen flow of 30 ml/min, oxygen 350 ml/min and make-up gas flow of nitrogen at 30 ml/min. A post run of 5 min at 240°C was performed between runs. The column was chemically and thermally cleaned by injection of hexane after approximately every 24 injections, with a holding time of 10 minutes per hexane injection. Manual data collection and peak integration was done using the HP ChemStations software (Rev. B01.03 [204]).

3.2.4 Statistical analysis

Statistical relationships between the variables (nitrogen treatments, addition times, yeast strains and aroma profiles) were explored using multivariate data analysis. The samples used in analysis are the individual fermentation replicates and include all six nitrogen treatments, two addition time points and nine yeast strains. The variables represented are the end point concentrations of aroma compounds and residual sugars. Yeast strain, nitrogen treatment and addition time were also included as categorical variables. Principal-component analysis (PCA) was performed on the data using The Unscrambler software (version 9.2, CAMO, Norway). Aroma data were pre-treated by autoscaling to account for variance in magnitude between the different compounds.

Significant differences between the ammonium treatment and each amino acid treatment were determined by pair-wise comparisons of aroma compounds produced by each strain. Cytoscape software (version 2.8.2, http://www.cytoscape.org) was used to visualise the data, presented from a strain-centric or compound-centric viewpoint. The figures (referred to as bubble graphs) contain blue nodes (ellipses) representing a statistically significant lower level in the amino acid treatment than in the ammonium treatment, or red nodes representing a significantly higher level. The colour intensity of the nodes

indicates the magnitude of the difference between treatments. A significance level of 5% ($p<0.05$) was used. Only significant differences are shown; non-significant data are omitted from the figures.

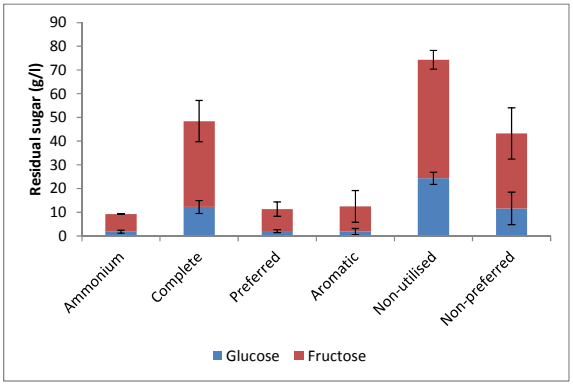
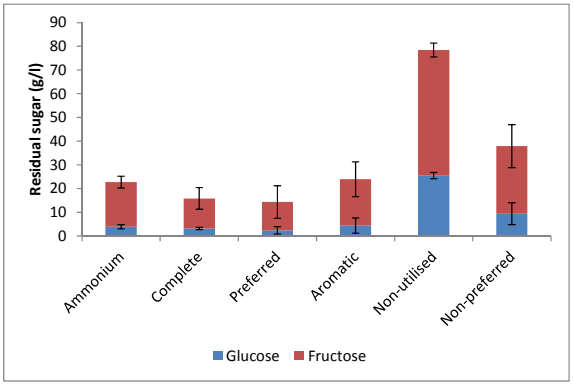
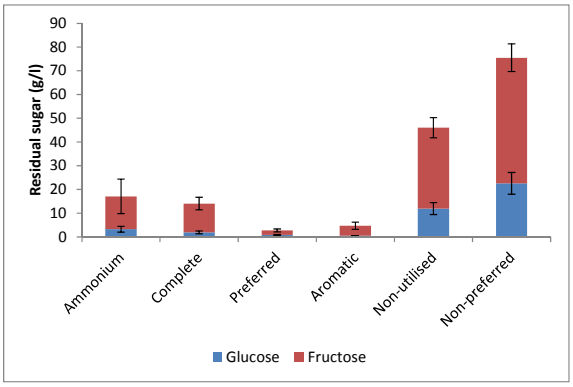
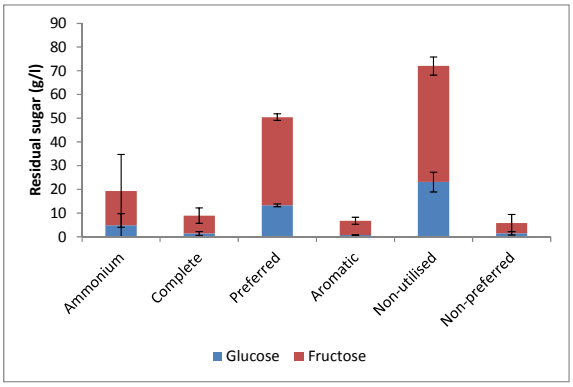
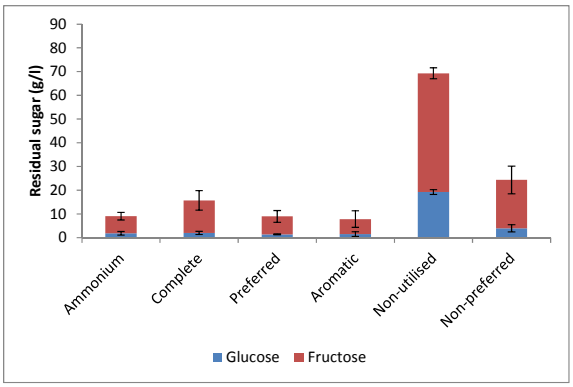
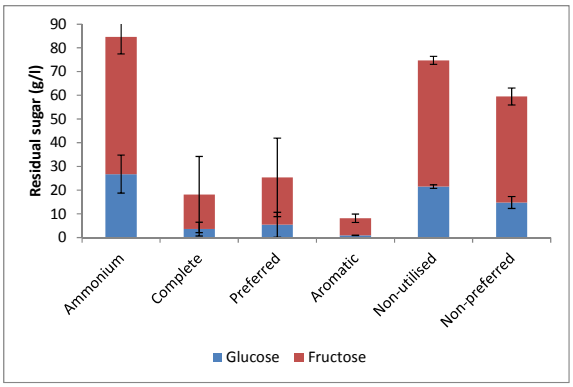
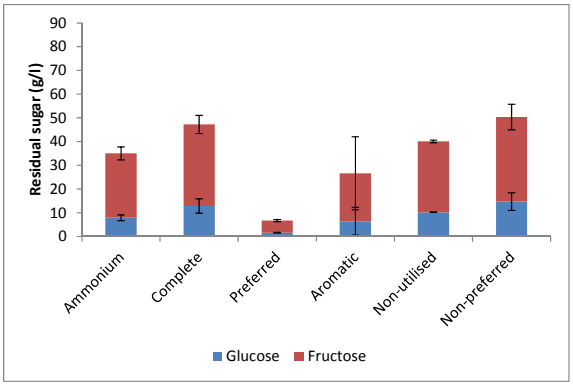
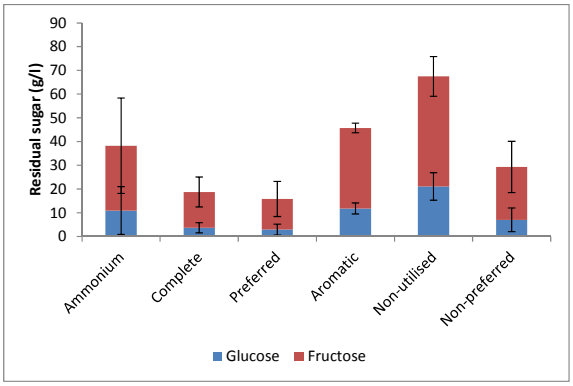
3.3 Results and discussion

3.3.1 Fermentation of synthetic musts

Six nitrogen treatments (**Table 1**) differing in their potential to support growth and aroma compound formation were studied in synthetic grape must under winemaking conditions. Two treatments contained ammonium as the sole assimilable nitrogen source, either at 200 mg N/l (ammonium only treatment), or at 50 mg N/l. In the latter case, the medium was also supplied with 150 mg N/l of amino acids that are reportedly not utilised as nitrogen source during fermentation (non-utilised amino acid treatment) (Cooper, 1982; Large, 1986; Henschke & Jiranek, 1993). Two treatments contained amino acids that differ in their efficiency to act as a nitrogen source in *S. cerevisiae* as described in the literature (preferred amino acid and non-preferred amino acid treatments) (Henschke & Jiranek, 1993; Hofman-Bang, 1999; Ter Schure et al., 2000; Magasanik & Kaiser, 2002; Beltran et al., 2004). A fifth treatment contained all branched-chain and aromatic amino acids (branched-chain and aromatic amino acid treatment); while a final treatment contained a combination of all 20 naturally occurring amino acids (complete amino acid treatment).

Fermentation performance was assessed through the measurement of weight loss during fermentation and residual sugar at the end of fermentation. Faster fermentation rates and greater total weight loss always equated to low residual sugars at the end of fermentation **(Figure 1**). Nitrogen treatments differed significantly with regards to their impact on fermentation performance (**Figure 1**) and aroma compound production (**Figures 2 and 3**), but impacts were significantly dependent on the timing of nitrogen addition and yeast strain. In general, when addition was made before inoculation, differences in fermentation kinetics between the treatments and strains were obvious from the start of fermentation. When nitrogen was administered on day three of fermentation, the fermentation rates of all strains were almost identical prior to addition. Differences between strains were more pronounced for the fermentations where nitrogen had been added on day three. For some combinations of strain and treatment, similar fermentation performances were observed but aroma profiles at the end of fermentation were different for the two addition points.

No single nitrogen treatment supported fermentation best for all strains. When nitrogen was supplied to the initial grape must the preferred and branched-chain and aromatic amino acids treatments most consistently led to fermentation completion (**Figure 1**).

Strain	Initial addition	Addition on day 3
VIN13		
NT50		
NT202		
DV10		

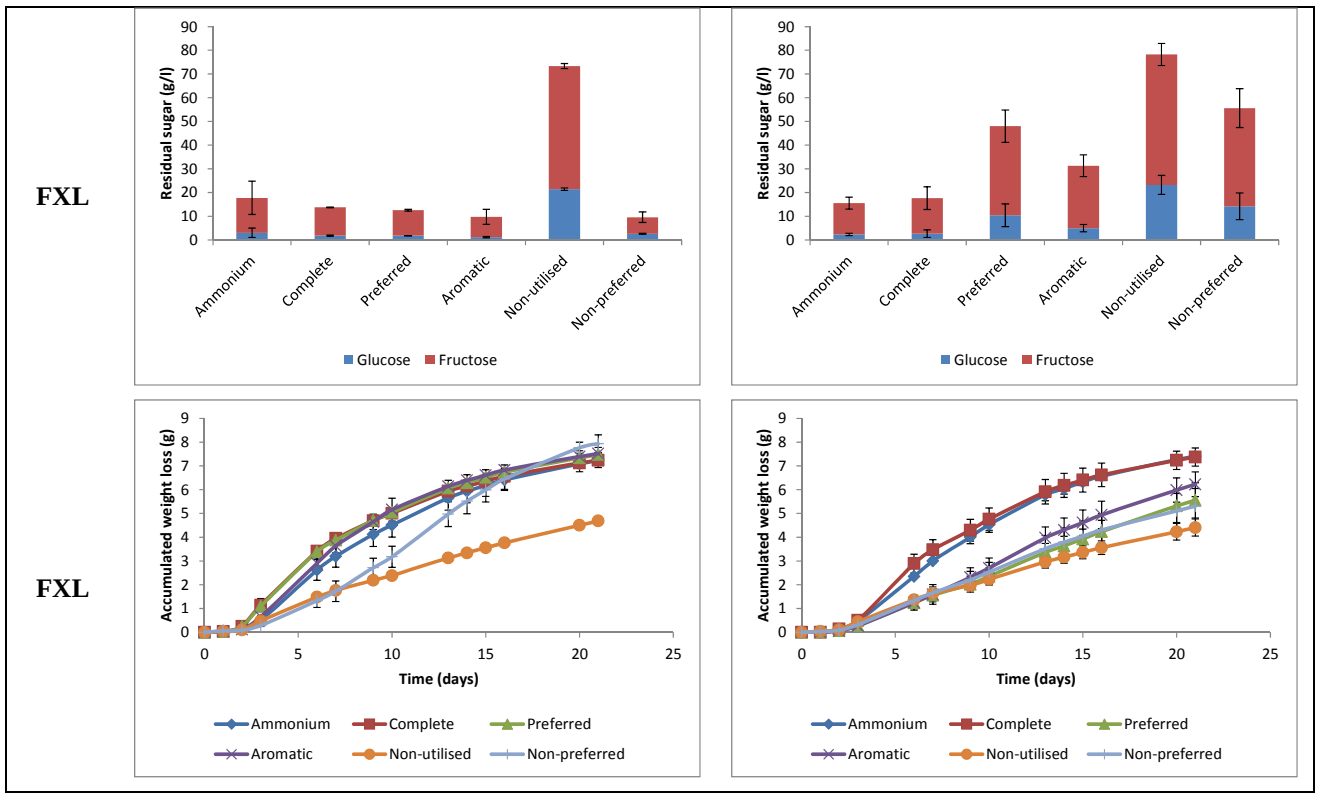


Figure 1 Fermentation performance of five representative strains included in the discussion; reported as residual sugar concentrations. The fermentation rate (weight loss) data correlated with the residual sugar concentrations measured at the end of fermentation, as illustrated for strain FXL. Error bars indicate the standard deviation between triplicate fermentation treatments.

Supplementation with the preferred amino acids generally led to the lowest residual sugars and fastest fermentation rates for most strains. Strains showed either a similar fermentation rate on preferred amino acids for both addition points (VIN13, EC1118, PR7) or a reduction in fermentation efficiency to different extents with later addition, with NT50 and FXL showing the most extreme reduction in fermentation performance of the strains tested (**Figure 1**).

Branched-chain and aromatic amino acids also supported fermentation to completion for the majority of strains (**Figure 1**). Most strains showed similar fermentation performances for the two nitrogen addition points when branched-chain and aromatic amino acids were provided, with the exception of strains FXL and DV10, which showed improvement in fermentation performance with initial addition.

Most strains displayed consistent fermentation behaviour when fermented with ammonium as only nitrogen source, irrespective of the time of addition (**Figure 1**) with the exception of strain NT202 which displayed a significantly lower fermentative ability when ammonium was added after the onset of fermentation (**Figure 1**). The fermentation rate on ammonium was very similar for the majority of strains tested, and only a few strains displayed relatively poor fermentation performance (DV10, BM45, and PR7).

Yeast strains showed varying responses in fermentation kinetics when supplied with all amino acids. In these conditions, fermentation performance was relatively poor for VIN13 when amino acids were present from the start, while later addition led to a significant improvement in performance (**Figure 1**). Other strains showing a similar behaviour were DV10, BM45 and PR7. The opposite behaviour (relative improved fermentation rate upon initial addition compared to later addition) was observed for EC1118 and 285.

The non-preferred amino acid treatment mostly resulted in incomplete fermentation and poor fermentation performance, with significant strain variation. When non-preferred amino acids were added to the initial fermentation medium, a few strains (NT50, DV10, PR7) showed even more sluggish fermentation than when non-utilised amino acids were added. Other strains exhibited efficient fermentation on non-preferred amino acids (FXL, 285). For a number of strains later addition of non-preferred amino acids significantly improved fermentation (NT50, DV10, PR7) while for others it caused greater sluggishness and lower sugar consumption (NT202, FXL, 285, EC1118). Only BM45 and VIN13 were not influenced by the timing of addition of this treatment.

The treatment containing non-utilised amino acids effectively had only 50 mg/l of available nitrogen in the form of initially supplied ammonium. This amount of nitrogen is too low to support a complete fermentation for most wine yeast strains, even under optimal sugar and temperature conditions (Fairbairn, 2012). Mendes-Ferreira et al. (2007) determined that 66 mg/l of initial ammonium was depleted within 24 h. For most strains, the fermentation rates for the two addition points were similarly low when non-utilised amino acids were supplied as nitrogen source. However, for a few strains (such as DV10, NT50, BM45) later addition led to even more sluggish fermentation and less complete sugar utilisation than initial addition.

The concentrations of selected aroma compounds produced by four representative strains are reported in **Figures 2 and 3**. These compounds are three medium-chain fatty acids and their ethyl esters (hexanoic acid, octanoic acid, decanoic acid, ethyl hexanoate, ethyl octanoate and ethyl decanoate); three higher alcohols (isobutanol, isoamyl alcohol and 2-phenylethanol), two related acetate esters (isoamyl acetate and 2-phenylethyl acetate) and compounds associated with volatile acidity (acetic acid and ethyl acetate).

Generally, the pattern of aroma production was similar for the preferred amino acid and ammonium treatments for the majority of strains (**Figures 2 to 4)** with fatty acid and ethyl esters produced in relatively high concentrations and higher alcohols and associated esters produced in lower concentrations than for other nitrogen treatments. This aroma production pattern was more pronounced for initial nitrogen addition, even when the fermentation kinetics observed for the two time point treatments was the same. **Figure 4** shows that not many significant changes are observed for preferred amino acids relative to ammonium.

Aroma compounds produced generally corresponded to the precursors supplied by the amino acid treatments. Dramatic increases in the concentrations of higher alcohols, and to a lesser extent acetate esters, were observed in response to supplementation with branched-chain and aromatic amino acids precursors for all strains at both addition points. This was seen in particular for the branched-chain and aromatic amino acid treatment, but also for the complete and non-preferred amino acid treatments, containing a lower percentage of branched-chain and aromatic amino acids. Later addition of the branched-chain and aromatic amino acid treatment generally led to higher concentrations of higher alcohols produced and a greater number of significant changes, as illustrated in **Figure 4**. Interestingly, the formation of medium-chain fatty acids and their associated ethyl esters were also influenced by branched-chain and aromatic amino acid addition. For most strains fatty acids and ethyl ester concentrations in the branched-chain and aromatic amino acid treatment were similar to or lower than for other nitrogen treatments, with the exception of strain FXL which displayed increased production of these compounds with early supplementation of branched-chain and aromatic amino acids (**Figure 2**).

The treatment containing non-utilised amino acids had a final aroma profile high in fatty acids and low in higher alcohols and acetate esters for most strains. However, a number of strains showed a significant increase for 2-phenylethanol for one or both time points of addition, and significant decrease for isobutanol when supplied with non-utilised amino acids supplied on day three of fermentation (**Figure 4**).

While most strains exhibited general aroma production behaviour, some strains displayed interesting deviations. An illustration is the highly divergent phenotypes displayed by strains DV10 and FXL when supplied with ammonium as only nitrogen source, depending on the timing of addition. Both strains showed identical fermentation performance with ammonium addition at the two time points of addition but very different aroma profiles (**Figure 1**). **Figure 5** shows all significant changes in aroma compound production relative to the treatment containing only ammonium for these two strains (DV10 and FXL) which displayed various interesting deviations from the rest of the strains and often exhibited opposite behaviour to one another in terms of aroma production.

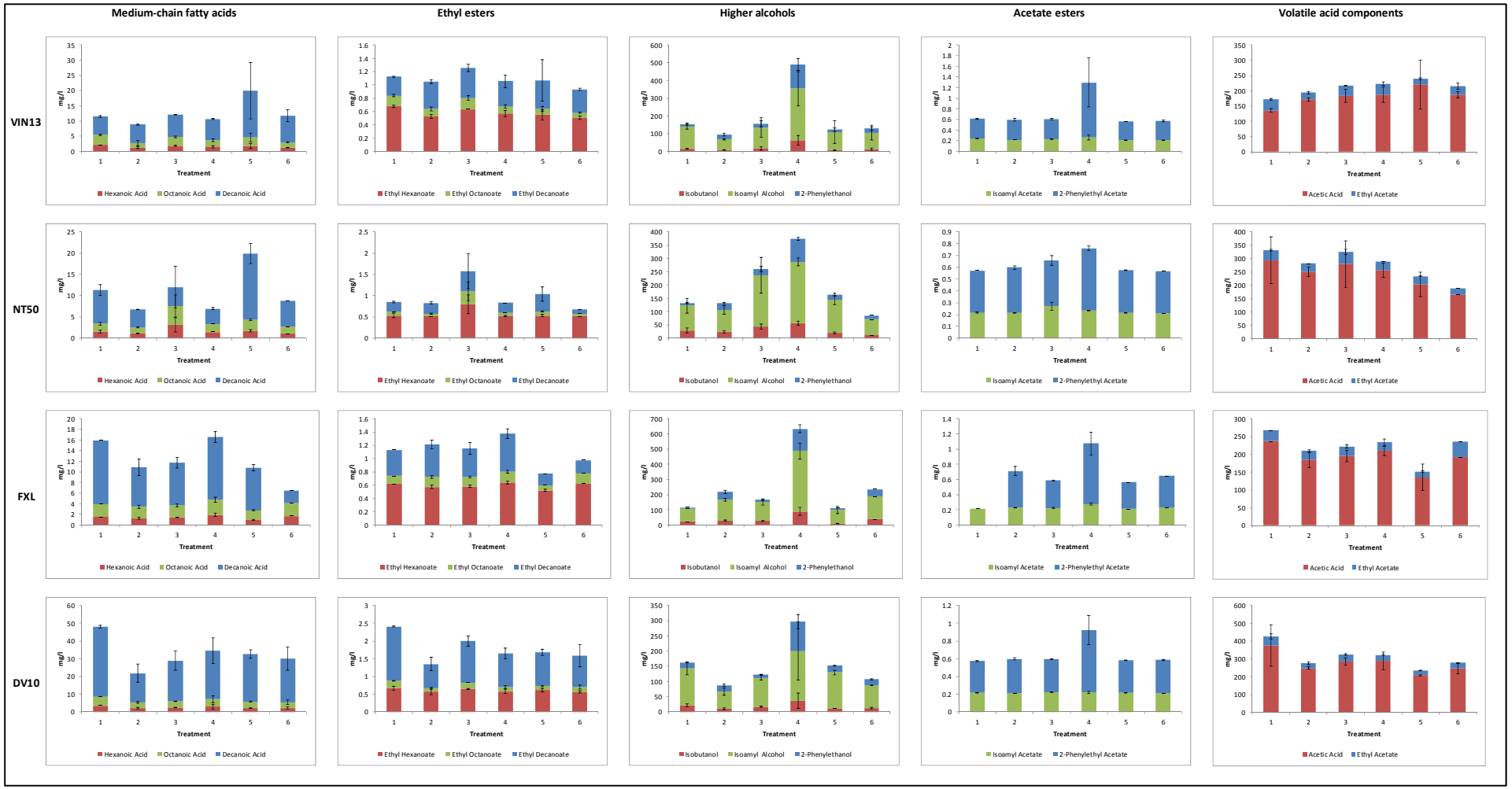


Figure 2 Aroma compounds produced by four different yeast strains, measured at the end of alcoholic fermentation in synthetic medium with nitrogen treatments applied to the initial grape must. For clarity of the graphs the scales of the y-axes are not standardised across yeast strains due to variation in magnitude of concentrations of compounds produced. Error bars indicate the standard deviation of three treatment replicates. Treatments are (1) ammonium only, (2) complete amino acids, (3) preferred amino acids, (4) branched-chain and aromatic amino acids, (5) non-utilised amino acids, (6) non-preferred amino acids.

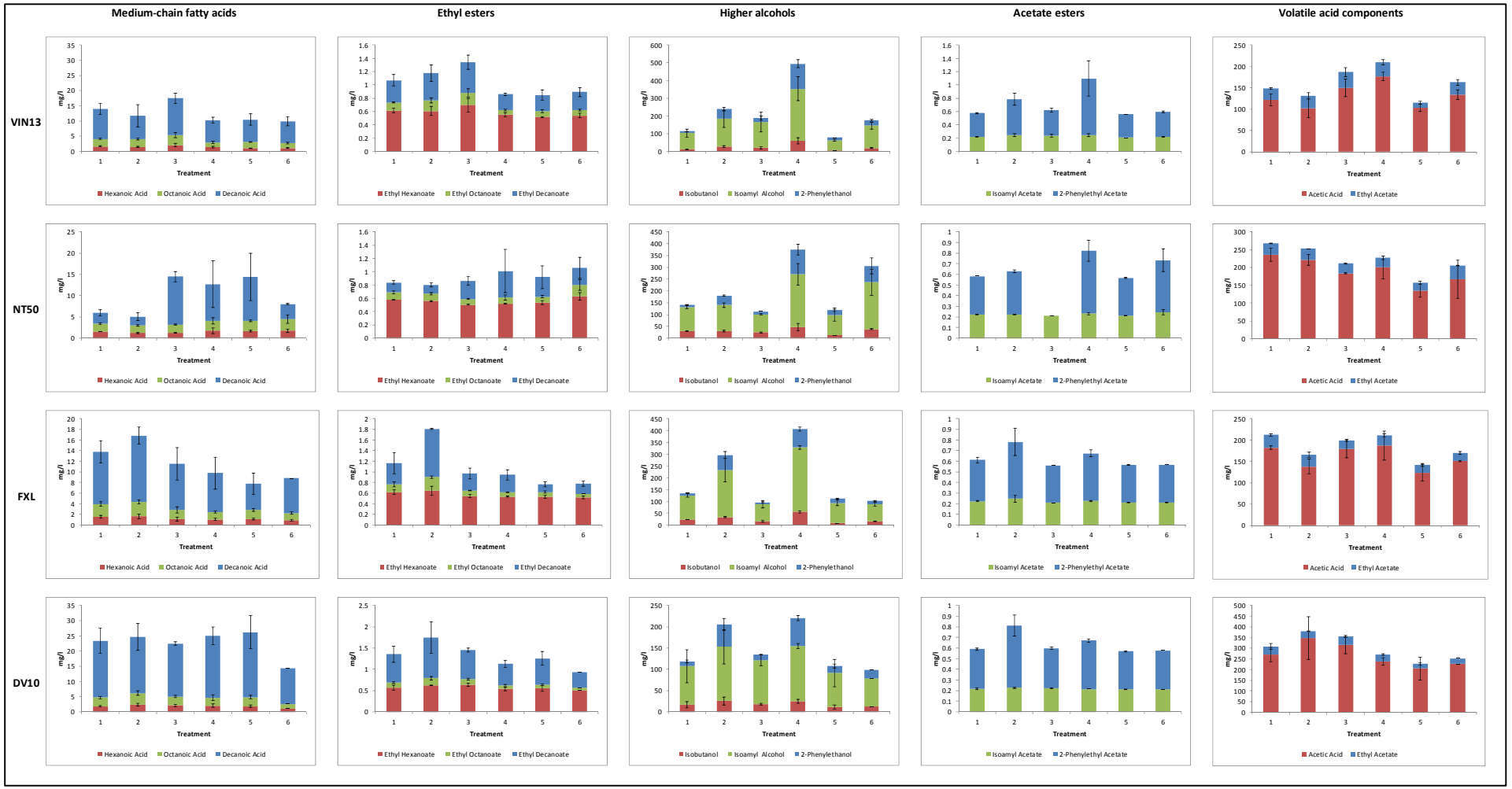


Figure 3 Aroma compounds produced by four different yeast strains, measured at the end of alcoholic fermentation in synthetic medium with nitrogen treatments applied on day three of fermentation. For clarity of the graphs the scales of the y-axes are not standardised across yeast strains due to variation in magnitude of concentrations of compounds produced. Error bars indicate the standard deviation of three treatment replicates. Treatments are (1) ammonium only, (2) complete amino acids, (3) preferred amino acids, (4) branched-chain and aromatic amino acids, (5) non-utilised amino acids, (6) non-preferred amino acids.

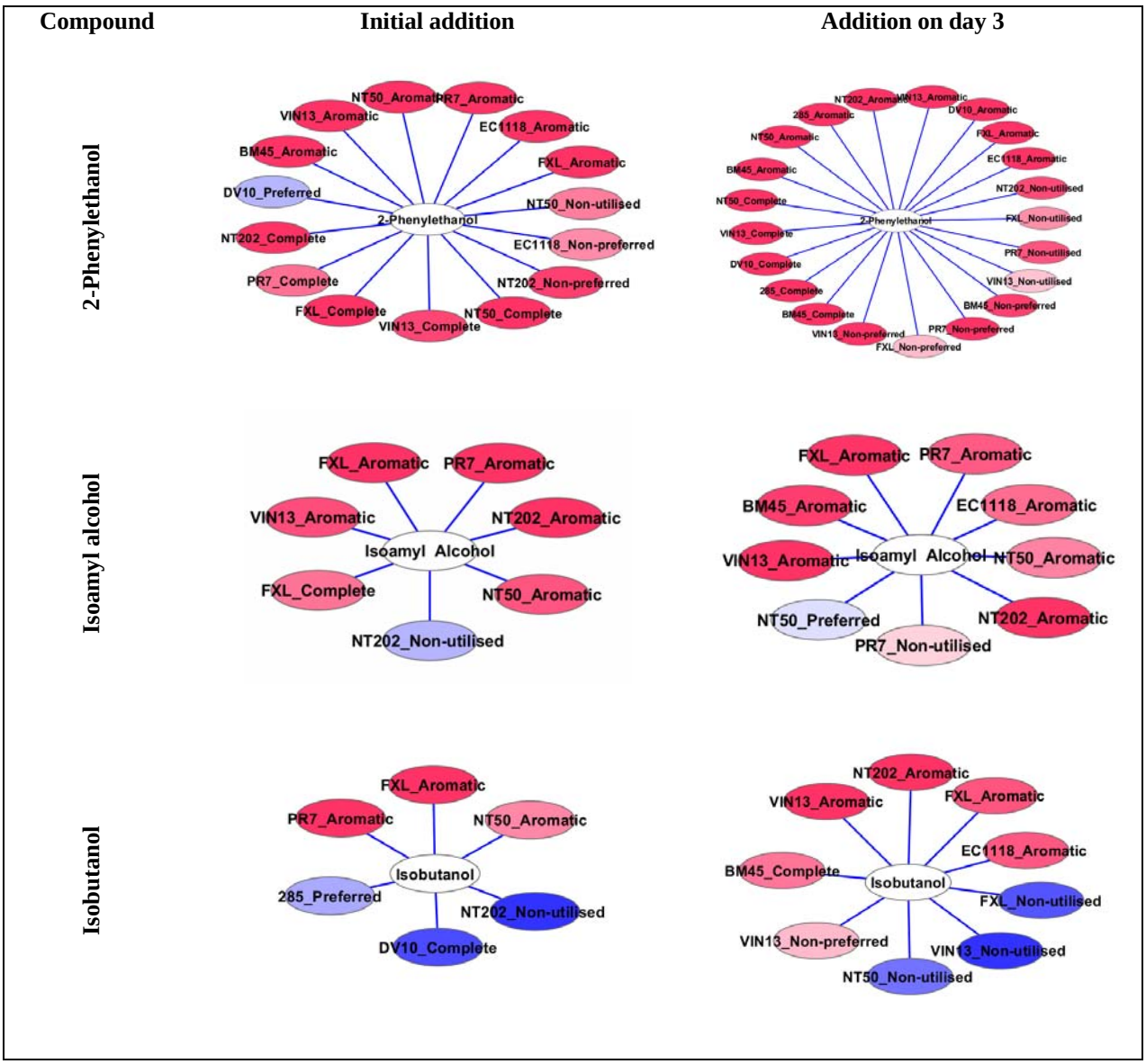


Figure 4 Significant differences in higher alcohol production in amino acid treatments relative to the ammonium treatment (designated reference treatment in pair-wise comparisons for statistical analysis). A significantly higher concentration of the compound in the amino acid treatment is indicated by a red node, while blue denotes a significantly lower concentration. The colour intensity indicates the magnitude of the relative change between treatments.

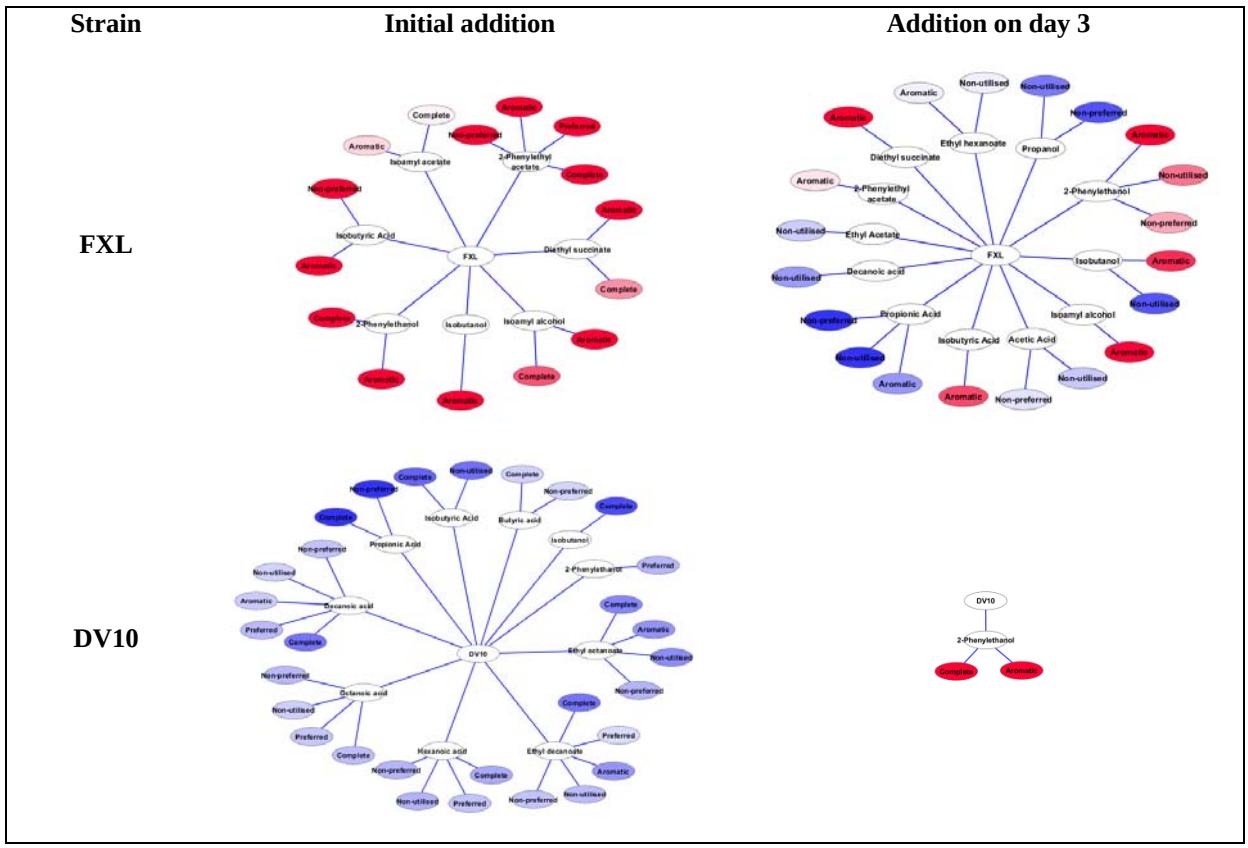


Figure 5 Comparison of all aroma compounds produced by two strains, DV10 and FXL, displaying divergent aroma production patterns. For each of the two strains, fermentation rates on ammonium were the same for the two addition points. Significant differences are indicated for each compound between the ammonium treatment and amino acid treatments. A red node indicates a significantly higher concentration of the compound in the amino acid treatment than in the ammonium treatment, and a blue node a significantly lower concentration. The colour intensity indicates the magnitude of the relative difference between treatments.

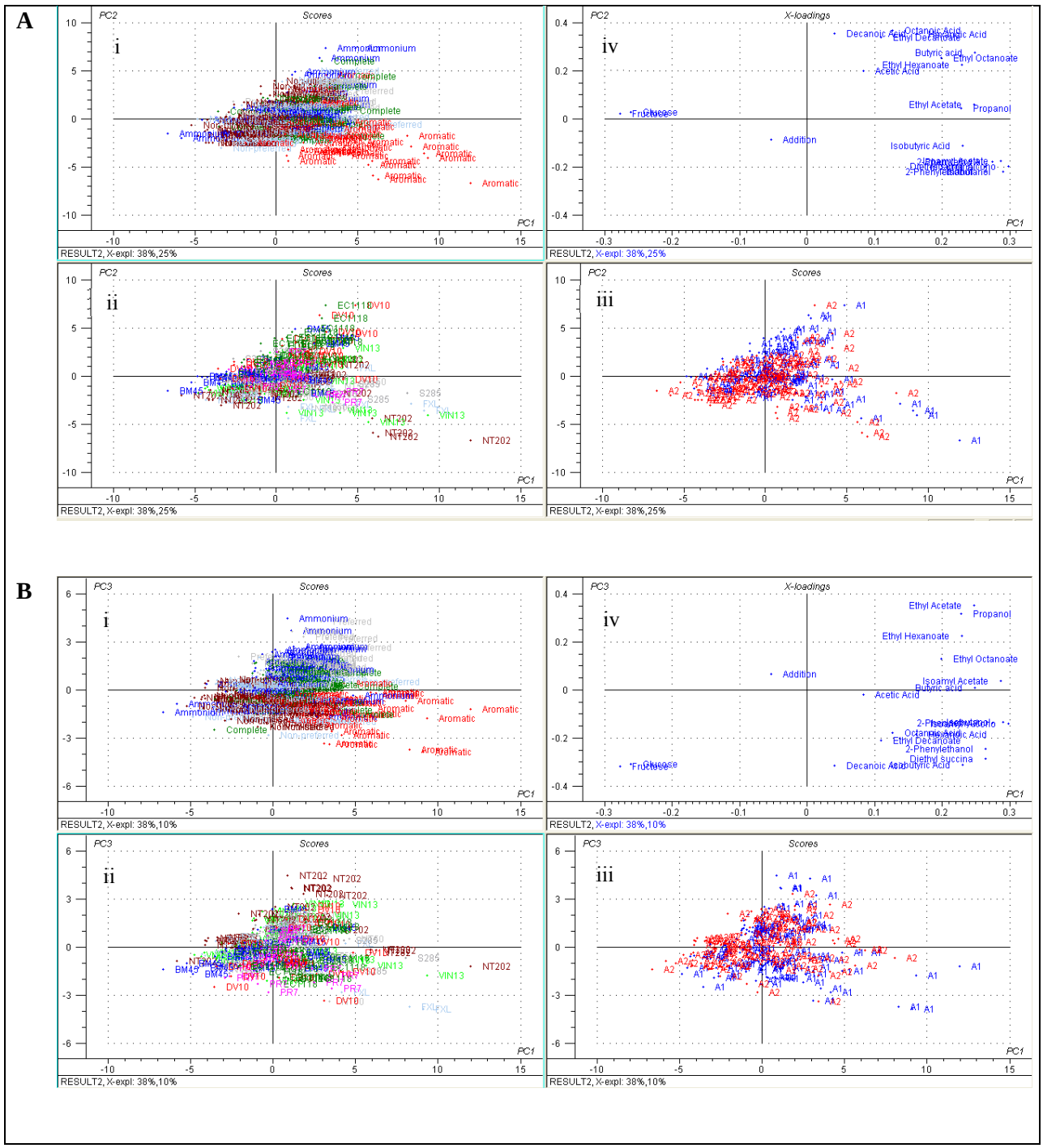
3.3.2 Multivariate data analysis

Multivariate analysis of the data was performed to reveal the relevant patterns in the data. A principal component analysis (PCA) plot of the quantified end-point metabolites of all samples was constructed to visualise the overall structure of the dataset.

The first two principal components (PCs) model 38% and 25% of the variance respectively. It is suggested in **Figure 6 A** that the presence of branched-chain and aromatic amino acids is the primary factor responsible for variation in the dataset, causing samples of this treatment to cluster together and separate from other samples along the first PC. It is evident from the loadings plot that there is a strong association between the branched-chain and aromatic amino acids and their associated aroma products (higher alcohols, acetate esters and volatile fatty acids). Importantly, this effect is more significant than the effect of yeast genetic background, although the strain impact is also partly reflected in the first PC.

Strains producing the highest overall concentrations of aroma compounds associated with branched-chain and aromatic amino acids (FXL, NT202 and VIN13) are grouped along the first PC. **Figure 4** shows the significant changes relative to other nitrogen treatments for the three higher alcohols which contributed greatly to the separation of the branched-chain and aromatic amino acid treatment from the rest of the dataset, according to the result in **Figure 1 A**. The magnitude of the increases in the concentrations of higher alcohols in the branched-chain and aromatic amino acid treatment relative to the ammonium treatment (indicated by the colour intensity of the red ellipses) for NT202, VIN13 and FXL also reiterates the yeast strain pattern observed in the PCA. Strains associated with higher concentrations of fatty acids and ethyl esters (EC1118 and DV10) form associations along PC2 on the scores and loadings plots of **Figure 1 A**.

In addition to samples supplied with branched-chain and aromatic amino acid precursors, a number of samples corresponding to specific combinations of nitrogen treatment, strain and addition time can be discriminated in the third and fourth PCs, together explaining an additional 16% of the variation in the dataset. For example, treatments containing ammonium and preferred amino acids can be associated with compounds such as ethyl acetate, propanol and ethyl esters, especially for strain NT202 (**Figure 6 B**). In the fourth PC (**Figure 6 C**) treatments with non-utilised amino acids, branched-chain and aromatic amino acids and complete amino acids form separate clusters, while the ammonium and non-preferred amino acid treatments are spread out over the multivariate space. Non-utilised amino acid treatments cluster together in the first three PCs but are centred on the intercept and are therefore not highly correlated with aroma compounds; but are correlated with residual sugars in the fourth PC. The fourth PC reveals an association between acetic acid production and certain treatments or yeast strains. The parameters that best explain the variance can be evaluated by assessing quantitative values. Strains DV10, BM45 and 285 produced significantly higher concentrations of acetic acid than other strains. These strains also displayed the poorest fermentation rate and completion among the strains tested (see **Figures 1 to 3** for DV10 as example). Only in the fifth PC (explaining only 5% of the variance) are samples from the two addition points separated (**Figure 6 D**).



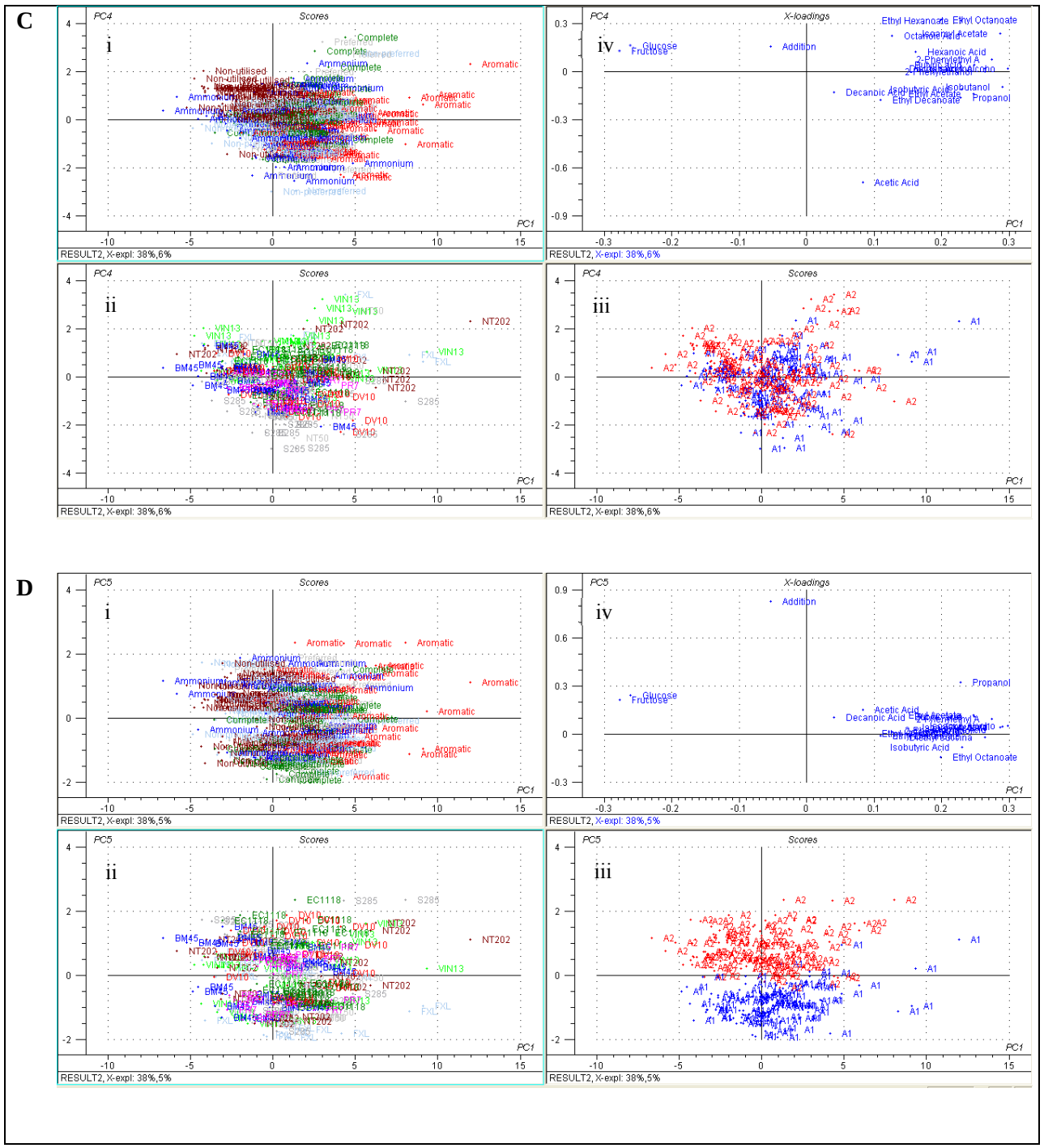


Figure 6 Principal component analysis of the end-point metabolites produced by nine yeast strains in synthetic grape must subjected to different nitrogen treatments. Samples are sorted in the scores plots (indicated in colour) according to (i) six nitrogen treatments, (ii) nine wine yeast strains and (iii) timing of addition (A1, initial addition; A2, addition on day three). The variables are shown on the loadings plot (iv). The principle components (PCs) represented are (A) PC1 and PC2, (B) PC1 and PC3, (C) PC1 and PC4, and (D) PC1 and PC5.

3.3.3 Impact of nitrogen treatments on fermentation performance and aroma production

3.3.3.1 Ammonium

The aroma production pattern observed for ammonium supplementation (increased fatty acids and ethyl esters and decreased higher alcohols and associated compounds) is in agreement with many studies showing that increased addition and consumption of ammonium by yeast, especially at the beginning of fermentation, result in increased levels of fatty acids and related esters and lower concentrations of higher alcohols and vice versa; often depending on yeast strain (Ough et al., 1980; Beltran et al., 2005; Hernández-Orte et al., 2005; Hernández-Orte et al., 2006b; Vilanova et al., 2007; Carrau et al., 2008; Barbosa et al., 2009).

In this study, the aroma producing capacity of some strains was influenced significantly by the timing of ammonium addition. This could have serious implications for industrial diammonium phosphate (DAP) additions which are often made empirically to the grape must or as fermentation problems arise. In the case of strain DV10, initial ammonium supplementation provided average fermentation support (**Figure 1**) but resulted in the production of high concentrations of aroma compound relative to other nitrogen treatments (**Figure 5**). Higher alcohols concentrations produced in the ammonium treatment were comparable to the branched-chain and aromatic amino acid treatment which supply direct aromatic precursors (**Figure 2**). This data suggests that, in this case, the anabolic formation of higher alcohols and associated aroma compounds from a sugar substrate takes precedence over their catabolic formation via the Ehrlich pathway, in accordance with similar observations made by authors Beltran et al. (2005) and Miller et al. (2007). However, when ammonium was supplied after a delay of three days, DV10 produced lower amounts of aroma compounds compared to initial supplementation (**Figures 3 and 5**), despite similar fermentation performances for the two time points. Beltran et al. (2005) also observed that when ammonium was added during the final fermentation stages, it was not consumed successfully by a strain in their study, even if it was the preferred nitrogen source to support growth (biomass production).

In contrast to DV10, FXL produced significantly lower concentrations of aroma compounds relative to other nitrogen treatments when supplemented with ammonium as initial nitrogen source (**Figures 3 and 5**). When ammonium was supplied later, FXL produced more aroma compounds despite near identical fermentation performances for the two addition points (as in the case of DV10).

3.3.3.2 Preferred amino acids

The treatment comprised of preferred amino acids is closely related to ammonium as it contains glutamate and glutamine, the main sources of cellular nitrogen to yeast (Cooper, 1982), which are directly synthesised from and easily interconverted with ammonium via α-ketoglutarate (Hofman-Bang, 1999, Ter Schure et al., 2000). Even though the ammonium and preferred amino acid treatments resulted in mostly similar aroma production patterns, there were a few exceptions.

An interesting phenomenon was that a number of strains (most notably NT50 but also FXL, BM45 and 285) showed a significantly reduced fermentation efficiency when preferred amino acids were supplied after the onset of fermentation rather than to the initial medium (**Figure 1**). For strain NT50, when addition of preferred amino acids was made on day three, this treatment was similar in fermentation properties to the treatment containing non-utilised amino acids; proposing the uptake of only the initial 50 mg N/l ammonium and a resulting sluggish fermentation. Correspondingly, ethyl esters of fatty acids, acetate esters and higher alcohols were produced in high concentrations by NT50 in the preferred amino acid treatment supplied to the initial medium, but were significantly reduced when added after the onset of fermentation (**Figures 2 to 4**).

3.3.3.3 Branched-chain and aromatic amino acids

Branched-chain and aromatic amino acids are consumed from the beginning of fermentation even under nitrogen repressed conditions, possibly due to the up-regulation of genes coding for branched-chain amino acid transporters in the presence of good nitrogen sources such as ammonium (Marks et al., 2003; Beltran et al., 2004). This stimulation of branched-chain and aromatic amino acid uptake by the presence of ammonium could explain why the branched-chain and aromatic amino acid treatment (which was supplied together with 50 mg N/l of initial ammonium) was more efficient at sustaining fast and complete fermentation than nitrogen sources subjected to NCR (for example the complete amino acid mixture for VIN13 and the preferred amino acid treatment for NT50) (**Figure 1**). Possibly, one or more component of the branched-chain and aromatic amino acid treatment also serves as a very good source of nitrogen to support growth, contrary to previous reports (Watson, 1976; Boer et al., 2007).

Branched-chain and aromatic amino acid supplementation resulted in significant increases in higher alcohols and associated acetate esters. In some instances the production of fatty acids and ethyl esters were correspondingly decreased in the presence of branched-chain and aromatic amino acids, such as for DV10 (initial addition) and VIN13, NT50 and FXL (addition on day three) (**Figure 3**). In a study by Trinh et al. (2010) it was found that the addition of branched-chain amino acids during Logan juice fermentation decreased the formation of acetate esters. These authors attribute this reduction to a diversion of acetyl-CoA to the formation of the acetate esters of the supplied amino acid products. Ethyl ester production, also requiring acetyl-CoA for its formation, could be decreased by a similar mechanism in the present study.

An interesting deviation from this trend was observed for strain FXL, the only strain showing an increase in fatty acids and ethyl esters when initially supplied with branched-chain and aromatic amino acids (**Figure 2**). This could suggest that FXL is able to utilise these amino acids efficiently as nitrogen source, for aroma compound production via the Ehrlich pathway, and for the production of fatty acids and ethyl esters when present in excess from the beginning of fermentation. The complex regulation between different groups of aroma compounds has been noted before, and it has been suggested that the regulation of ester formation by alcohol acetyl transferase (ATF) genes also affects branched-chain amino acid

metabolism, such as changes in higher alcohol formation (Lilly et al., 2006). FXL also performed the fastest fermentation and produced the greatest quantities of higher alcohols of the strains when supplied with branched-chain and aromatic amino acids to the initial medium. Its fermentation performance and aroma compound production abilities were significantly reduced when supplied with branched-chain and aromatic amino acids after the onset of fermentation (**Figures 1 and 3**).

3.3.3.4 Complete amino acids

Variable responses in fermentation performance and aroma production were observed for the complete amino acids treatment for different strains, as also revealed by the lack of clustering of this treatment in a multivariate space (**Figure 6**). This possibly illustrates the efficiency with which different strains are able to switch metabolism (especially transport systems) when a succession of nitrogen sources are available in the fermentation medium or introduced after the onset of fermentation. When a mixture of amino acids is present, *S. cerevisiae* will use the good, average and poor sources sequentially. Transport of certain amino acids in the complete amino acid treatment would be repressed under NCR and will not be utilised until all preferred amino acids and ammonium had been depleted. The derepression of transport of less preferred amino acids is clearly not equally responsive for all strains. For example, VIN13 showed similar fermentation performances when supplied initially with the complete amino acid treatment as with the non-preferred amino acid treatment (**Figure 1**). Evidently, VIN13 more readily uses ammonium as nitrogen source and would rather synthesise amino acids than transport directly supplied amino acids into the cell. Upon later addition, the complete amino acid treatment resulted in complete fermentation for VIN13 (and similarly for strains DV10, BM45 and PR7). These strains seem able to switch their metabolism efficiently after a short period of nitrogen limitation when initial ammonium is depleted. This could be explained by the induction of various metabolic pathways upon relief of nitrogen limitation, which would not be activated when a complete source of nitrogen is supplied from the beginning of fermentation. When nitrogen is added to a nitrogen depleted medium, genes from various pathways (nitrogen metabolism, glycolysis, stress response, thiamine and energy metabolism) will be expressed in order to restart fermentation and overcome the stress imposed by nitrogen depletion (Jiménez-Martí et al., 2007; Jiménez-Martí & Del Olmo, 2008).

The complete amino acid treatment contained 25% of the aromatic amino acid precursors. For most strains it is observed that second to the branched-chain and aromatic amino acid treatment, the complete amino acid treatment resulted in the highest levels of higher alcohols and their associated acetate esters produced, attributable to the presence of the direct precursors. This was already apparent in the data overview provided by PCA in **Figure 6** and can be seen for most strains in **Figures 2 and 3**.

3.3.3.5 Non-preferred amino acids

The amino acids present in the non-preferred amino acid treatment appeared to be utilised to different extents by different strains. A number of strains seemed able to overcome initial sluggish fermentation

encountered when non-preferred amino acids were provided as nitrogen source, indicating the efficient uptake and utilisation of these amino acids under nitrogen limiting conditions. When amino acids are scarce, yeast cells will increase transport to specifically target amino acids available in the grape must by increasing expression of genes encoding related transporters and inducing the enzymes required for the utilisation of the nitrogen sources (Rossouw & Bauer, 2009).

On the contrary, the non-preferred amino acid treatment caused more sluggish fermentation than the non-utilised amino acid treatment for some strain (such as NT50) when nitrogen was supplied to the initial fermentation medium. It is possible that the presence of one or more amino acids in the non-preferred amino acid treatment could decrease the ability of the strain to take up sufficient ammonium nitrogen to support fermentation to the same degree as for the non-utilised amino acid treatment.

In terms of aroma production, the non-preferred amino acid treatment resulted in the increased production of higher alcohols such as 2-phenylethanol (**Figure 4**), possibly due to an increased formation of this higher alcohol via the anabolic pathway under conditions of nitrogen limitation (Lambrechts & Pretorius, 2000) since its direct precursor (phenylalanine) was not supplied in this treatment.

3.3.3.6 Non-utilised amino acids

The non-utilised amino acid treatment contained only 50 mg N/l of assimilable nitrogen in the form of ammonium supplied to the initial medium. Therefore, nitrogen deficiency was experienced in this treatment, which could lead to stuck or sluggish fermentation and the formation of stress-related off-flavours (Fairbairn, 2012). The production of high concentrations of fatty acids and 2-phenylethanol in the non-utilised amino acid treatment (**Figure 4**) could possibly be related to the presence of specific amino acids such as threonine. According to Hernández-Orte et al. (2002), this amino acid is associated with fatty acid synthesis. Their results show that increased levels of threonine could result in increased formation of fatty acids and associated ethyl esters. Additionally, increased threonine concentrations in the initial grape must have been associated with increased 2-phenylethanol and reduced isobutanol production (Hernández-Orte et al., 2002), similar to what can be observed in the present study. Data showing this inverse relationship between 2-phenylethanol and isobutanol have also been reported but not explained by a number of studies (Hernández-Orte et al., 2002; Lilly et al., 2006; Jain et al., 2011; Styger et al., 2011).

3.4 Conclusions

This study assessed the combined impacts of yeast strain, nitrogen source and timing of nitrogen supplementation on fermentation performance and yeast-derived aroma compound production. The nitrogen treatments, grouped according to preference as source of nitrogen for *S. cerevisiae* and/or their potential impact on aroma production pathways, varied considerably in their ability to support fermentation to completion in synthetic medium; depending on yeast strain and timing of addition. The branched-chain and aromatic and preferred amino acid treatments most consistently resulted in complete

fermentation for the strains tested, while the complete and non-preferred amino acid treatments showed the most variable results, depending on strain and timing of addition. The treatment containing non-utilised amino acids resulted in incomplete fermentation due to nitrogen limitation for all strains.

Nitrogen treatment, in particular the presence of branched-chain and aromatic amino acids, had the greatest effect on the resultant aroma profiles of synthetic wines. The effect of this particular treatment on aroma production overruled the impact of yeast strain, but equal contributions and strong interactions between nitrogen treatments and yeast strains were evident for the remainder of the treatments. Timing of nitrogen addition had a lesser impact than nitrogen treatment or strain, but still had a significant impact on fermentation performance and aroma compound production. In particular, different pathways of aroma formation seem to be favoured depending on the timing of addition. With initial nitrogen addition, higher alcohols and related esters and fatty acids were produced at higher concentrations in the ammonium and preferred amino acid treatments than in the complete amino acid treatment (containing aromatic precursors), indicating that the anabolic formation of aroma compounds is probably favoured with initial nitrogen availability. With addition after the onset of fermentation, the complete amino acid treatment resulted in higher concentrations of aroma compounds than the ammonium and preferred amino acids, suggesting the greater formation of aroma compounds via the catabolic route. The later addition of the branched-chain and aromatic amino acid treatment also resulted in higher concentrations of associated aroma compounds and a greater number of significant changes relative to the ammonium treatment than with early addition.

Generally, the results of this study are in agreement with previous findings. However, interesting deviations from anticipated behaviour were also observed, such as the ability of branched-chain and/or aromatic amino acids to support fermentation; the opposite aroma producing behaviour of yeast strains when supplemented at different times with ammonium; the significant reduction in fermentation efficiency with later addition of preferred amino acids for particular yeast strains; the degree to which different strains are able to switch or activate amino acid uptake and catabolism in the presence of a complete or non-preferred nitrogen source, or after initial nitrogen limitation; and the possible interference of certain amino acids with the uptake of ammonium from the growth medium.

The data presented here provide an initial framework of the relative importance of yeast genetic background and of environmental (in this case nutritional) conditions in determining the aromatic profile of wines. In the long run, such data sets will contribute to improving our ability to predict fermentation outcomes and will pave the way to more judicious nutrient supplementation.

References

Ayestarán, B., Garrido, J., & Ancín, C. (1998). Sedimentation clarification of viura musts. Utilization of amino acids during fermentation. *Food Chemistry, 63*(2), 191–197.

Barbosa, C., Falco, V., Mendes-Faia, A., & Mendes-Ferreira, A. (2009). Nitrogen addition influences formation of aroma compounds, volatile acidity and ethanol in nitrogen deficient media fermented by *Saccharomyces cerevisiae* wine strains. *Journal of Bioscience and Bioengineering, 108*(2), 99–104.

Barrajón-Simancas, N., Giese, E., Arévalo-Villena, M., Úbeda, J., & Briones, A. (2011). Amino acid uptake by wild and commercial yeasts in single fermentations and co-fermentations. *Food Chemistry, 127*(2), 441–446.

Bell, S.-J., & Henschke, P. A. (2005). Implications of nitrogen nutrition for grapes, fermentation and wine. *Australian Journal of Grape and Wine Research*, *11*(3), 242–295.

Beltran, G., Esteve-Zarzoso, B., Rozès, N., Mas, A., & Guillamón, J. M. (2005). Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and nitrogen consumption. *Journal of Agricultural and Food Chemistry*, *53*(4), 996–1002.

Beltran, G., Novo, M., Rozès, N., Mas, A., & Guillamón, J. M. (2004). Nitrogen catabolite repression in *Saccharomyces cerevisiae* during wine fermentations. *FEMS yeast research*, *4*(6), 625–32.

Beltran, G., Novo, M., Guillamón, J. M., Mas, A., & Rozès, N. (2008). Effect of fermentation temperature and culture media on the yeast lipid composition and wine volatile compounds. *International Journal of Food Microbiology, 121*(2), 169–77.

Boer, V. M., Tai, S. L., Vuralhan, Z., Arifin, Y., Walsh, M. C., Piper, M. D. W., De Winde, J. H., Pronk, J. T., & Daran, J-M. (2007). Transcriptional responses of *Saccharomyces cerevisiae* to preferred and nonpreferred nitrogen sources in glucose-limited chemostat cultures. *FEMS Yeast Research*, *7*(4), 604–20.

Camarasa, C., Sanchez, I., Brial, P., Bigey, F., & Dequin, S. (2011). Phenotypic landscape of *Saccharomyces cerevisiae* during wine fermentation: Evidence for origin-dependent metabolic traits. *PloS One*, *6*(9), e25147.

Carrau, F. M., Medina, K., Farina, L., Boido, E., Henschke, P. A., & Dellacassa, E. (2008). Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: Effects of yeast assimilable nitrogen on two model strains. *FEMS Yeast Research, 8*(7), 1196–207.

Cooper, T.G. (1982). Nitrogen metabolism in *Saccharomyces cerevisiae*. In: Strathern, J.N., Jones, E.W., Broach, J.R. (Eds.). The molecular biology of the yeast *Saccharomyces*: Metabolism and gene expression, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 39– 99.

Fairbairn, S. C., (2012). Stress, fermentation performance and aroma production by yeast. MSc Thesis. Stellenbosch University. Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Forsberg, H., & Ljungdahl, P. O. (2001). Sensors of extracellular nutrients in *Saccharomyces cerevisiae*. *Current Genetics, 40*(2), 91–109.

Garde-Cerdán, T., & Ancín-Azpilicueta, C. (2008). Effect of the addition of different quantities of amino acids to nitrogen-deficient must on the formation of esters, alcohols, and acids during wine alcoholic fermentation. *LWT - Food Science and Technology*, *41*(3), 501–510.

Henschke, P.A and Jiranek, V., (1993). Yeasts – metabolism of nitrogen compounds. In: Wine Microbiology and Biotechnology. Fleet, G. H. (Ed). Harwood Academic Publishers, Chur, Switzerland, pp. 77–164.

Hernández-Orte, P, Ibarz, M., Cacho, J., & Ferreira, V. (2005). Effect of the addition of ammonium and amino acids to musts of Airen variety on aromatic composition and sensory properties of the obtained wine. *Food Chemistry*, *89*(2), 163–174.

Hernández-Orte, P., Bely, M., Cacho, J., & Ferreira, V. (2006a). Impact of ammonium additions on volatile acidity, ethanol, and aromatic compound production by different *Saccharomyces cerevisiae* strains during fermentation in controlled synthetic media. *Australian Journal of Grape and Wine Research*, *12*(2), 150–160.

Hernández-Orte, P., Ibarz, M. J., Cacho, J., & Ferreira, V. (2006b). Addition of amino acids to grape juice of the Merlot variety: Effect on amino acid uptake and aroma generation during alcoholic fermentation. *Food Chemistry*, *98*(2), 300–310.

Hernández-Orte, P., Cacho, J. F., & Ferreira, V. (2002). Relationship between varietal amino acid profile of grapes and wine aromatic composition. Experiments with model solutions and chemometric study. *Journal of Agricultural and Food Chemistry*, *50*(10), 2891–9.

Hoffmann, W. (1985). Molecular characterization of the *CAN1* locus in *Saccharomyces cerevisiae*. A transmembrane protein without N-terminal hydrophobic signal sequence. *Journal of Biological Chemistry*, *260*(21), 11831–11837.

Hofman-Bang, J. (1999). Nitrogen Catabolite Repression in *Saccharomyces cerevisiae*. *Molecular Biotechnology*, *12*, 35–73.

Jain, V. K., Divol, B., Prior, B. A., & Bauer, F. F. (2012). Effect of alternative NAD^+-regenerating pathways on the formation of primary and secondary aroma compounds in a *Saccharomyces cerevisiae* glycerol-defective mutant. *Applied Microbiology and Biotechnology, 93*(1), 131–41.

Jauniaux, J.-C., & Grenson, M. (1990). *GAP1*, the general amino acid permease gene of *Saccharomyces cerevisiae*. Nucleotide sequence, protein similarity with other bakers yeast amino acid permeases, and nitrogen catabolite repression. *European Journal of Biochemistry*, *190*, 39–44.

Jiménez-Martí, E., Aranda, A., Mendes-Ferreira, A., Mendes-Faia, A., & Del Olmo, M. L. (2007). The nature of the nitrogen source added to nitrogen depleted vinifications conducted by a *Saccharomyces cerevisiae* strain in synthetic must affects gene expression and the levels of several volatile compounds. *Antonie van Leeuwenhoek, 92*(1), 61–75.

Jiménez-Martí, E., & Del Olmo, M. L. (2008). Addition of ammonia or amino acids to a nitrogen-depleted medium affects gene expression patterns in yeast cells during alcoholic fermentation. *FEMS Yeast Research*, *8*(2), 245–56.

Lambrechts, M., & Pretorius, I. (2000). Yeast and its importance to wine aroma – A review. *South African Journal of Enology and Viticulture, 21*, 97–129.

Large, P. J. (1986). Degradation of organic nitrogen compounds by yeasts. *Yeast 2*, 1-34.

Lilly, M., Bauer, F., Lambrechts, M., Swiegers, J., Cozzolino, D., & Pretorius, I. (2006). The effect of increased yeast alcohol acetyltransferase and esterase activity on the flavour profiles of wine and distillates. *Yeast*, *23*, 641–659.

Louw, L., Roux, K., Tredoux, A., Tomic, O., Naes, T., Nieuwoudt, H. H., & van Rensburg, P. (2009). Characterization of selected South African young cultivar wines using FTMIR spectroscopy, gas chromatography, and multivariate data analysis. *Journal of Agricultural and Food Chemistry*, *57*(7), 2623–32.

Magasanik, B., & Kaiser, C. A. (2002). Nitrogen regulation in *Saccharomyces cerevisiae*. *Gene*, *290*(1-2), 1–18.

Marks, V., Van der Merwe, G., & Van Vuuren, H. (2003). Transcriptional profiling of wine yeast in fermenting grape juice: Regulatory effect of diammonium phosphate. *FEMS Yeast Research*, *3*(3), 269–287.

Mendes-Ferreira, A., Del Olmo, M., García-Martínez, J., Jiménez-Martí, E., Mendes-Faia, A., Pérez-Ortín, J. E., & Leão, C. (2007). Transcriptional response of *Saccharomyces cerevisiae* to different nitrogen concentrations during alcoholic fermentation. *Applied and Environmental Microbiology*, *73*(9), 3049–60.

Miller, A., Wolff, S., Bisson, L., & Ebeler, S. (2007). Yeast strain and nitrogen supplementation: Dynamics of volatile ester production in Chardonnay juice fermentations. *American Journal of Enology and Viticulture, 4*, 470–483.

Ough, C., & Bell, A. (1980). Effects of nitrogen fertilization of grapevines on amino acid metabolism and higher-alcohol formation during grape juice fermentation. *American Journal of Enology and Viticulture, 31*(2), 122–123.

Ough, C., Crowel, E., & Mooney, L. (1988). Formation of ethyl carbamate precursors during grape juice (Chardonnay) fermentation. I. Addition of amino acids, urea, and ammonia: Effects of fortification on intracellular and extracellular precursors. *American Journal of Enology and Viticulture*, *39*(3), 243–249.

Pretorius, I. S., & Bauer, F. F. (2002). Meeting the consumer challenge through genetically customized wine-yeast strains. *Trends in Biotechnology*, *20*(10), 426–32.

Rossouw, D., & Bauer, F. F. (2009). Comparing the transcriptomes of wine yeast strains: Toward understanding the interaction between environment and transcriptome during fermentation. *Applied Microbiology and Biotechnology*, *84*(5), 937–54.

Rossouw, D., Naes, T., & Bauer, F. F. (2008). Linking gene regulation and the exo-metabolome: A comparative transcriptomics approach to identify genes that impact on the production of volatile aroma compounds in yeast. *BMC Genomics*, *9*, 530.

Rytka, J. (1975). Positive selection of general amino acid permease mutants in *Saccharomyces cerevisiae*. *Journal of Bacteriology*, *121*(2), 562–70.

Schmidt, A., Hall, M. N., & Koller, A. (1994). Two FK506 resistance-conferring genes in *Saccharomyces cerevisiae*, *TAT1* and *TAT2*, encode amino acid permeases mediating tyrosine and tryptophan uptake. *Molecular and Cellular Biology*, *14*(10), 6597–606.

Smit, A. Y., Du Toit, W. J., Stander, M., & Du Toit, M. (2013). Evaluating the influence of maceration practices on biogenic amine production in wine. *LWT- Food Science and Technology,* (accepted for publication).

Styger, G., Jacobson, D., & Bauer, F. F. (2011). Identifying genes that impact on aroma profiles produced by *Saccharomyces cerevisiae* and the production of higher alcohols. *Applied Microbiology and Biotechnology*, *91*(3), 713–30.

Sychrova, H., & Chevallier, M.R., (1993). Cloning and sequencing of the *Saccharomyces cerevisiae* gene *LYP1* coding for the lysine permease. *Yeast 9,* 771-782.

Tanaka, J., & Fink, G. (1985). The histidine permease gene (*HIP1*) of *Saccharomyces cerevisiae*. *Gene*, *38*, 205–214.

Ter Schure, E. G., van Riel, N. A. V., & Verrips, C. T. (2000). The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews*, *24*(1), 67–83.

Torrea, D., Fraile, P., Garde, T., & Ancín, C. (2003). Production of volatile compounds in the fermentation of chardonnay musts inoculated with two strains of *Saccharomyces cerevisiae* with different nitrogen demands. *Food Control*, *14*(8), 565–571.

Trinh, T., Woon, W., Yu, B., Curran, P., & Liu, S.-Q. (2010). L-phenylalanine addition on aroma compound formation during Longan juice fermentation by a co-culture of *Saccharomyces cerevisiae* and *Williopsis saturnus*. *South African Journal of Enology and Viticulture, 31*(2), 116–124.

Vandenbol, M., Jauniaux, J. C., & Grenson, M. (1989). Nucleotide sequence of the *Saccharomyces cerevisiae PUT4* proline-permease-encoding gene: similarities between CAN1, HIP1 and PUT4 permeases. *Gene*, *83*(1), 153–159.

Van Rensburg, P., & Pretorius, I. (2000). Enzymes in winemaking: Harnessing natural catalysts for efficient biotransformations: A review. *South African Journal for Enology and Viticulture, 21*, 52–73.

Varela, C., Pizarro, F., & Agosin, E. (2004). Biomass content governs fermentation rate in nitrogen-deficient wine musts. *Applied and Environmental Microbiology, 70*(6), 3392–3400.

Vilanova, M., Ugliano, M., Varela, C., Siebert, T., Pretorius, I. S., & Henschke, P. A. (2007). Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by *Saccharomyces cerevisiae* wine yeasts. *Applied Microbiology and Biotechnology*, *77*(1), 145–57.

Watson, T. G. (1976). Amino-acid pool composition of *Saccharomyces cerevisiae* as a function of growth rate and amino-acid nitrogen source. *Journal of General Microbiology*, *96*(2), 263–8.

Chapter 4

Linking grape must amino acid composition and aroma compound production pathways of wine yeast

CHAPTER 4

Linking grape must amino acid composition and aroma compound production pathways of wine yeast

Abstract

The presence of amino acids in the fermentation medium largely influences the production of yeast-derived aroma compounds. While it is known that amino acids that act as direct precursors for the catabolic formation of higher alcohols, esters and fatty acids by yeast will impact wine aroma profiles, various other amino acids could potentially act as precursors or regulators of numerous metabolic pathways linked to aroma compound production. In this study we sought to explore these links between amino acid composition in model wine and aroma compound production by different strains of wine yeast. Synthetic grape musts containing seven different nitrogen combinations (chosen on the basis of preference as nitrogen source and/or potential to act as direct aroma precursors) were fermented by nine commercial yeast strains. Volatile aroma compounds (higher alcohols, esters and fatty acids) were analysed at the end of fermentation. Careful exploration of the data allowed sorting of aroma compounds that were produced in a conserved manner by the majority of yeast strains in this study into nitrogen treatment dependent and independent categories. The behaviour of individual aroma compounds that were dependent on amino acids present in nitrogen treatments, allowed the proposal of metabolic pathways involved in their formation and/or regulation. Interesting results include the exclusive production of a non-sulfurous ester and the strain-dependent inhibition of propanol and related compounds in the presence of sulfur-containing amino acids; the elucidation of prominent pathways of formation for higher alcohols associated with branched-chain amino acids (anabolic route) and aromatic amino acids (catabolic route) and the formation of diethyl succinate (possibly via the methyl citric acid cycle during later fermentation stages) in the presence of excess branched-chain and/or aromatic amino acids.

4.1 Introduction

Nitrogen is a vital macronutrient which sustains yeast cell biomass formation and alcoholic fermentation as it is a major constituent of macromolecules such as proteins and nucleic acids (Henschke & Jiranek, 1993). Yeast is able to manufacture all amino acids needed for protein synthesis from inorganic nitrogen (ammonium salts) when amino acids are not available or not successfully assimilated from the growth medium. Ammonium and amino acids are employed as nitrogen sources by yeast for general biosynthetic purposes through the transfer of the amine functional group (Cooper, 1982; Bisson, 1999).

Numerous laboratory studies performed in chemically defined grape must have indicated that balanced complex mixtures of amino acids result in improved yeast growth rates when compared to single preferred amino acids or ammonium, likely because the requirement for amino acid biosynthesis is reduced. However, different mixtures of ammonium and amino acids, individual amino acids or

ammonium alone yield different results, depending on the growth conditions and yeast strain (Torija et al., 2003; Beltran et al., 2004; Beltran et al., 2005; Hernández-Orte et al., 2006; Vilanova et al., 2007; Barbosa et al., 2009). Nevertheless, a general order of preference for amino acids as nitrogen source has been established in controlled laboratory studies, based on the ability of each amino acid to support growth and the extent to which transport of alternative nitrogen sources are derepressed in its presence (Cooper, 1982; Beltran et al., 2004; Magasanik & Kaiser, 2002). According to this definition ammonium, glutamate, glutamine, aspartate, asparagine and arginine are considered preferred nitrogen sources, while proline, ornithine, γ-aminobutyrate, allantoin and urea are considered poor nitrogen sources (Cooper, 1982; Henschke & Jiranek, 1993; Hofman-Bang, 1999; Ter Schure et al., 2000; Magasanik & Kaiser, 2002)

Under real winemaking conditions, the amino acid composition of each grape must is unique, and is dependent upon various factors, related mainly to grape cultivar and cultivation (Hernández-Orte et al., 2002; reviewed by Bell & Henschke, 2005). Free amino acids are the most important organic sources of nitrogen in grape must, constituting the majority of the yeast assimilable nitrogen, but the composition of free amino acids can vary considerably in individual grape musts (Henschke & Jiranek, 1993; Bell & Henschke, 2005). Also, every wine yeast strain, as well as indigenous microorganisms present during wine elaboration, has individual nutrient and nitrogen requirements. The grape must composition and yeast nitrogen needs, combined with external factors such as environmental stress and winemaking practices, result in numerous subtly different patterns of nitrogen uptake, which often contradict generally accepted results from laboratory studies (Bauer & Pretorius, 2000; Vilanova et al., 2007; Carrau et al., 2008).

Not only is the fermentation rate and completion influenced by the quality and quantity of amino acids in the grape must, but various volatile and non-volatile flavour compounds associated with varietal aroma and fermentation bouquet are also affected (Hernández-Orte et al., 2002; Hernández-Orte et al., 2005; Jiménez-Martí et al., 2007; Vilanova et al., 2007; Garde-Cerdán & Ancín-Azpilicueta, 2008). Of particular importance is the production of higher alcohols and their associated esters and fatty acids, derived directly from branched-chain and aromatic amino acid precursors via the Ehrlich pathway (Styger et al., 2011). Less obvious perhaps is the pivotal role amino acids and other yet unidentified grape constituents (possibly amino acid precursors or their degradation products) play in establishing the unique characters of individual wines (Hernández-Orte et al., 2002; Keyzers & Boss, 2010).

The success of routine and empirical application of nitrogen to prevent problem fermentations, while still commonly used in the global wine industry, is increasingly considered problematic since the use of inorganic nitrogen alone as well as nitrogen under- or over-supplementation have been suggested to have negative implications on fermentation performance and aroma compound production (Ugliano et al., 2007; Vilanova et al., 2007; Fairbairn, 2012). Instead, nitrogen addition should be a deliberately planned practice to favourably exploit varietal expression and/or wine style in neutral cultivars.

In this context, the outcome of the fermentation process largely determines final wine quality, particularly the concentration of flavour and aroma compounds. These outcomes depend on the availability of flavour and aroma precursor compounds in the must, the specific environmental conditions, and the intrinsic genetic constitution of the individual yeast strains responsible for fermentation. The chemical complexity of grape juice continues to challenge our ability to match specific grape juices with specific wine making conditions and specific yeast strains to predict, control and direct and the outcomes of fermentations.

The ultimate goal of this study is to provide baseline data that will in future allow matching the chemical composition of grape musts with the intrinsic fermentation and aroma production capability of specific yeast strains. In CHAPTER 3, different nitrogen treatments, added at two time points of fermentation (to the medium prior to fermentation or on day three), were assessed for their ability to support fermentation of nine commercial yeast strains in synthetic grape must. Most strains behaved in accordance with previously published findings regarding the preference of *Saccharomyces cerevisiae* for particular nitrogen sources. However, a number of interesting strain-specific deviations from the anticipated fermentation behaviour were observed for specific nitrogen treatments. In addition, the factors (nitrogen treatment, yeast strain and/or addition time) that most significantly impact on primary and secondary end-point metabolites were identified. Aroma compounds that were significantly affected by specific nitrogen treatments were described. General aroma production patterns could be established for each of the different nitrogen treatments, and significant deviations were highlighted for strains that showed the most diverse behaviour.

In the present chapter, the links between amino acid composition in model wine and aroma compound production by different strains of wine yeast will be further explored. The same six nitrogen treatments (ammonium, preferred amino acids, complete amino acids, branched-chain and aromatic amino acids, non-preferred amino acids, non-utilised amino acids) as well as an additional treatment (sulfur-containing amino acids) will be used, with supplementation to the initial grape must and fermentation with nine commercial yeast strains. Based on the outcomes of CHAPTER 3, which served to overview aroma profiles and identify strain exceptions, more careful analysis of aroma compounds will be performed, in order to elucidate the metabolic pathways involved for compounds that are produced in specific treatments in a conserved manner for multiple yeast strains. By grouping of aroma compounds in different ways, common and sometimes unexpected metabolic pathways or precursors could be identified.

4.2 Materials and methods

4.2.1 Fermentation medium, yeast strains and fermentation conditions

Fermentations were conducted in a chemically defined medium resembling grape juice. The composition of the synthetic grape must was based on the medium described by Henschke and Jiranek (1993) and differed only in nitrogen content. The total sugar concentration was 200 g/l, consisting of 100 g/l each of glucose and fructose. The total assimilable nitrogen concentration of the medium was 200 mg N/l. Seven

different nitrogen treatments were applied to the synthetic grape must prior to yeast inoculation, each containing a base level of 50 mg N/l ammonium nitrogen. The remaining 150 mg N/l was comprised of different amino acids, grouped according to two criteria: their reported order of preference to be utilised as nitrogen source by *S. cerevisiae* strains, and their specific oenological relevance as precursors of aromatic compounds. The detailed composition of each nitrogen treatments is provided in **Table 1**. Nine commercially available wine yeast strains were screened for aroma production at the end of alcoholic fermentation in the synthetic grape must. The yeast strains are Lalvin EC1118, Lalvin DV10, Lalvin BM45, Cross Evolution (285) (Lallemand Inc., Montreal, Canada); VIN13, NT202, NT50, Exotics (PR7) (Anchor Yeast, Cape Town, South Africa); and Fermicru XL (FXL) (DSM Food Specialities, Delft, The Netherlands). Pure cultures of the yeast strains were pre-cultured sequentially in YPD broth and synthetic grape must containing only ammonium as nitrogen source, at 30°C. Fermentations were inoculated from overnight cultures into synthetic grape must treatments at a final OD_{600} of 0.1 (a cell density of approximately 10^6 cfu/ml).

Fermentations were carried out in triplicate under static batch conditions in a temperature controlled fermentation room at 20 to 22°C. The fermentation vessels used were 100 ml glass bottles with a working volume of 80 ml of fermentation medium, sealed with rubber stoppers and a CO_2 gas outlet. Fermentation progress was monitored by CO_2 weight loss over a time course of 21 days, after which fermentation treatments were analysed for major volatile compounds.

Table 1 Nitrogen composition of treatments applied to synthetic grape must, each containing 50 mg N/l of ammonium and 150 mg N/l of various nitrogen sources.

Treatment	Compound	%N[a]	mg N/L	mg/L
Ammonium only				
Initial addition	**NH_4Cl**	**21.2**	**50.0**	189.3
Treatment addition	**NH_4Cl**	**21.2**	**150.0**	568.0
Complete amino acids				
Initial addition	**NH_4Cl**	**21.2**	**50.0**	189.3
Treatment addition	**Amino acids**		**150.0**	
	ALA	15.7	7.5	47.8
	ARG	32.2	7.5	23.3
	ASN	21.2	7.5	35.4
	ASP	10.5	7.5	71.4
	CYS	11.6	7.5	64.9
	GLN	19.2	7.5	39.1
	GLU	9.5	7.5	78.9
	GLY	18.6	7.5	40.3
	HIS	27.1	7.5	27.7
	ILE	10.7	7.5	70.1
	LEU	10.7	7.5	70.1
	LYS	19.2	7.5	39.1
	MET	9.4	7.5	79.8
	PHE	8.5	7.5	88.2
	PRO	12.2	7.5	61.5
	SER	13.3	7.5	56.4
	THR	11.8	7.5	63.6
	TRP	13.7	7.5	54.7
	TYR	7.7	7.5	97.4
	VAL	12.0	7.5	62.5
Preferred amino acids				
Initial addition	**NH_4Cl**	**21.2**	**50.0**	189.3
Treatment addition	**Amino acids**		**150.0**	
	ARG	32.2	30.0	93.2
	ASN	21.2	30.0	141.5
	ASP	10.5	30.0	285.7
	GLN	19.2	30.0	156.3
	GLU	9.5	30.0	315.8
Branched-chain and aromatic amino acids				
Initial addition	**NH_4Cl**	**21.2**	**50.0**	189.3
Treatment addition	**Amino acids**		**150.0**	
	ILE	10.7	30.0	280.4
	LEU	10.7	30.0	280.4
	PHE	8.5	30.0	352.9
	TYR	7.7	30.0	389.6
	VAL	12.0	30.0	250.0
Sulfur amino acids				
Initial addition	**NH_4Cl**	**21.2**	**50.0**	189.3
Treatment addition	**Amino acids**		**150.0**	
	CYS	11.6	75.0	649.4
	MET	9.4	75.0	797.9
Non-utilised amino acids				
Initial addition	**NH_4Cl**	**21.2**	**50.0**	189.3
Treatment addition	**Amino acids**		**150.0**	
	HIS	27.1	50.0	184.5
	LYS	19.2	50.0	260.4
	PRO	12.2	50.0	409.8
Non-preferred amino acids				
Initial addition	**NH_4Cl**	**21.2**	**50.0**	189.3
Treatment addition	**Amino acids**		**150.0**	
	ALA	15.7	30.0	191.1
	GLY	18.6	30.0	161.3
	SER	13.3	30.0	225.6
	THR	11.8	30.0	254.2
	TRP	13.7	30.0	219.0

[a] Henschke & Jiranek (1993)

4.2.2 Analysis of major volatile compounds

Liquid-liquid extraction was used to extract major volatile compounds from the synthetic wine matrix. The protocol described by Louw et al. (2009) was followed, with minor modifications as described in CHAPTER 3. Major volatile compounds (listed in **Table 2**) were quantified by gas chromatography with flame ionization detection (GC-FID) as described in CHAPTER 3.

Table 2 Aroma compounds analysed at the end of alcoholic fermentation in synthetic grape must (SGM).

Compounds quantified in SGM	Compounds below detection / quantification in SGM
Acetic acid	Acetoin
Butyric acid	Butanol
Decanoic Acid	Ethyl isovalerate
Diethyl succinate	Ethyl lactate
3-Ethoxy-1-propanol	Ethyl phenylacetate
Ethyl acetate	Ethyl propionate
Ethyl butyrate	Ethyl-2-methylpropanoate
Ethyl decanoate	Ethyl-3-hydroxybutanoate
Ethyl hexanoate	Hexanol
Ethyl octanoate	Hexyl acetate
Ethyl-2-methylbutyrate	Isovaleric acid
Hexanoic acid	Methanol
Isoamyl alcohol	3-Methyl-1-pentanol
Isoamyl acetate	4-Methyl-1-pentanol
Isobutanol	2-Methyl-propyl acetate
Isobutyric acid	1-Octen-3-ol
Octanoic acid	Pentanol
2-Phenylethanol	Butanol
2-Phenylethyl acetate	
Propanol	
Propionic acid	

4.2.3 Statistical analysis

Significant differences between treatments were determined using the ammonium-supplemented treatment as “control” (or reference treatment); at a significance level of 5% ($p<0.05$). Pair-wise comparisons were made between each amino acid treatment and the ammonium treatment for all aroma compounds produced by each strain. Significant differences were visualised on bubble graphs created with Cytoscape software (version 2.8.2, http://www.cytoscape.org). Data is presented from a compound-centric or a treatment-centric perspective. A colour scale was used to denote the significant changes, with blue nodes (ellipses) or edges (lines) representing a significant reduction relative to the ammonium

treatment, and red representing a significant increase. The magnitude of the fold difference between treatments is indicated by the intensity of the colour of the nodes or edges. Treatments, compounds and/or strains not represented on the graphs can be considered statistically similar (not significantly different from the ammonium treatment).

4.3 Results and discussion

4.3.1 Fermentation performance

The fermentation rate and completion (sugar utilisation) varied greatly between the different treatments and strains. For most strains, treatments supplied with ammonium, complete amino acids, preferred amino acid and branched-chain and aromatic amino acids showed comparable fermentation kinetics and resulted in the lowest residual sugars at the end of fermentation. The non-preferred amino acid treatment supported fermentation for some strains, but resulted in sluggish fermentation for others. Treatments supplemented with non-utilised and sulfur-containing amino acids led to slow and incomplete fermentations. Fermentation patterns of individual yeast strains under the different nitrogen treatment conditions are discussed in depth in CHAPTER 3.

4.3.2 Detection of volatile aroma compounds by GC-FID

The 39 compounds (comprised of higher alcohols, volatile fatty acids and esters) analysed in the synthetic grape must at the end of alcoholic fermentation are listed in **Table 2**. The detection and accurate quantification of 21 aroma compounds was possible in the synthetic wine matrix. The remaining 18 compounds were not reliably detected or were below the limit of quantification and were therefore not used in subsequent data analysis. The aroma compounds produced by yeast during alcoholic fermentation could be broadly grouped into two categories: compounds generally produced in a conserved manner regardless of nitrogen treatment, and compounds significantly influenced by nitrogen treatments. In both categories multiple yeast strains showed the same behaviour, although for some compounds yeast strain variation could be observed.

4.3.3 Nitrogen treatment-independent aroma compound production

Variation in the concentrations of a number of volatile aroma compounds could be attributed mainly to the influence of yeast strain and/or the impact of fermentation stress, including nitrogen limitation. This group of aroma compounds was mainly comprised of compounds associated with glycolysis (ethyl acetate and acetic acid), short- and medium chain fatty acids and related ethyl esters (**Figures 1 to 3**).

These compounds were produced by most yeast strains in comparable amounts independent of nitrogen treatment as long as sufficient assimilable nitrogen was available to sustain alcoholic fermentation. There were a few significant increases and decreases in the production of these compounds for the complete, aromatic, preferred or non-preferred amino acid treatments relative to the ammonium treatment, as shown

in **Figure 1**. (Non-utilised and sulfur amino acid treatment results are excluded from this figure.) The magnitude of variation was relatively low for these compounds, (as indicated by the colour intensities of the nodes), but variability due to yeast genetic background is demonstrated clearly for acetic acid, as also reported by other authors such as Vilanova et al. (2007). Mostly, the significant changes for this group of compounds are associated with strains DV10 and VIN13 and could be explained by the affinity of these two strains for ammonium as source of nitrogen (CHAPTER 3). **Figure 2** shows the concentrations of acetic acid (**A**) and ethyl hexanoate (**B**) to illustrate the similarity in final concentrations produced in the different fermentation treatments by different strains.

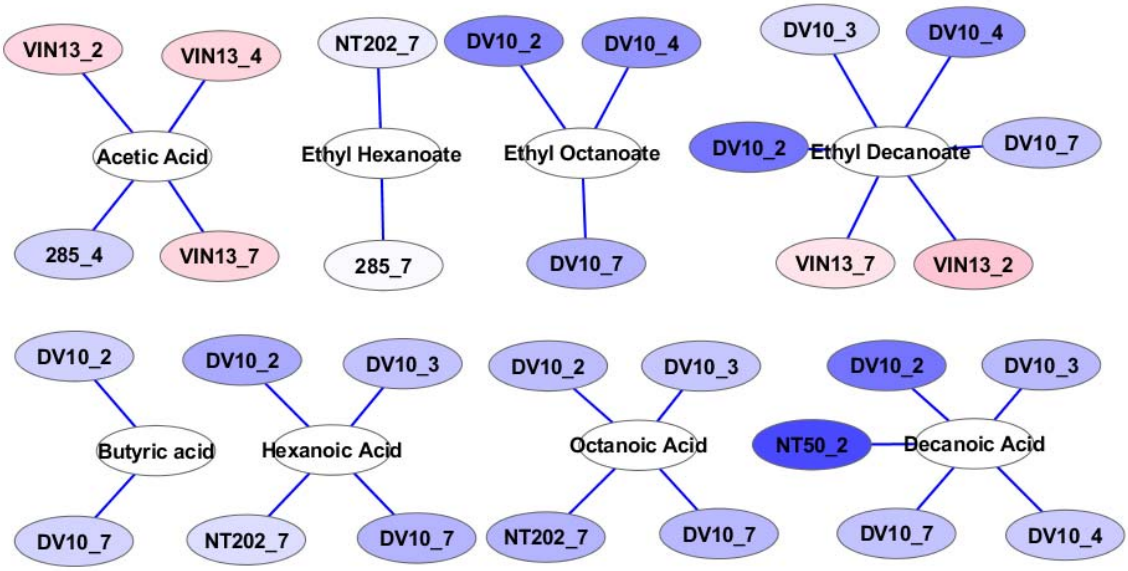


Figure 1 Significant differences in aroma compound production between amino acid treatments and the ammonium treatment (designated control) attributed mainly to yeast strain exceptions (such as DV10) but showing no conserved treatment effect. Significant increases of treatments relative to the ammonium treatment are indicated by red nodes, and decreases by blue nodes; with the colour intensity showing the magnitude of the difference. Treatments are (2) complete amino acids, (3) preferred amino acids, (4) branched-chain and aromatic amino acids, and (7) non-preferred amino acids. (Sulfur and non-utilised amino acid treatments are shown separately in **Figure 3**.)

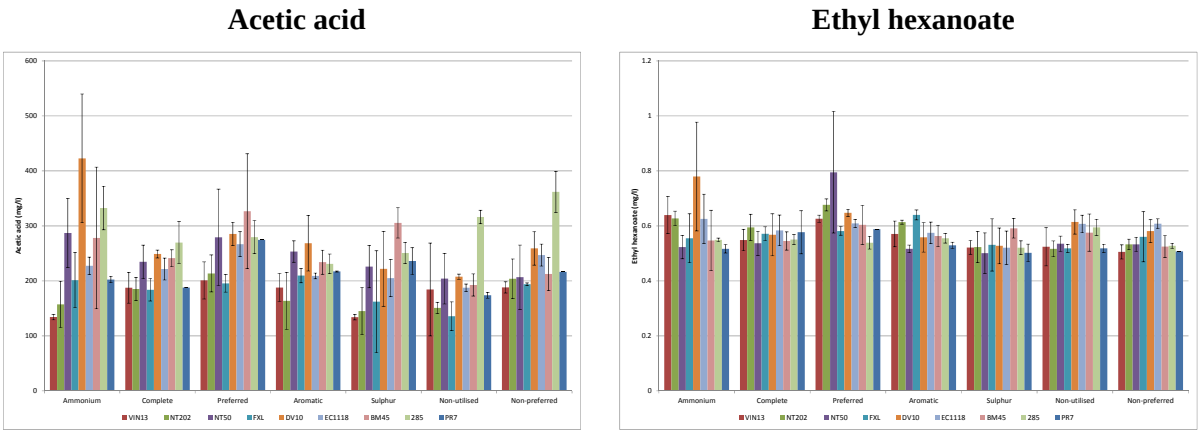


Figure 2 Concentrations of (**A**) acetic acid and (**B**) ethyl hexanoate measured at the end of alcoholic fermentation in the seven different nitrogen treatments, produced by nine different yeast strains. These two compounds illustrate the absence of major treatment effects observed for compounds shown in **Figure 1** while strain differences can be observed for acetic acid in particular. Error bars indicate the standard deviation between triplicate fermentation treatments.

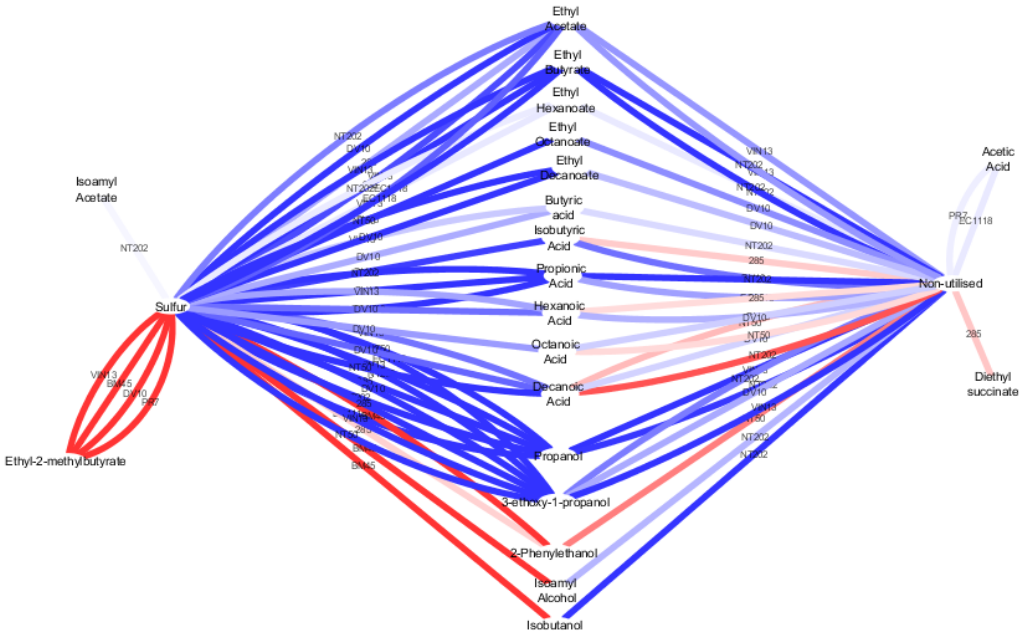


Figure 3 Significant differences in aroma compound production in the sulfur and non-utilised amino acid treatments relative to the ammonium treatment. Significant increases of aroma compounds in nitrogen treatments relative to the ammonium treatment are indicated per yeast strain by red edges (lines), and significant decreases by blue edges; with the colour intensity showing the magnitude of the relative change. Numerous aroma compounds were similarly affected by both treatments for multiple yeast strains, positioned in the centre of the figure and connected with edges to both treatment nodes.

Although the treatments containing non-utilised amino acids and sulfur amino acids no doubt impacted uniquely on the production of various secondary metabolites (for example sulfur compounds not measured in the present study), the two treatments had in common that they both induced fermentation stress, particularly nitrogen limitation. Thus, many of the compounds analysed in this study were affected similarly by the non-utilised and sulfur amino acid treatments (**Figure 3**). These "stressed treatments" showed significantly lower aroma compound concentrations in general, possibly owing to the lower overall metabolic activities. Only a few compounds, most notably medium-chain fatty acids and higher alcohols such as 2-phenylethanol, showed a relative increase in concentration for one or two strains. Interestingly, the compound ethyl-2-methyl butyrate was detected only in the sulfur amino acid containing treatment and will be further discussed.

4.3.4 Nitrogen treatment-dependent aroma compound production

When utilisable sources of nitrogen (ammonium; complete, aromatic, preferred or non-preferred amino acids) were supplied to the synthetic grape must, a number of aroma compounds were produced in a conserved manner for specific treatments, by multiple yeast genetic backgrounds. These compounds showed significant increases or decreases in concentration relative to the ammonium treatment (**Figure 4**); the magnitude of which was also more significant than for compounds showing mainly strain-related relative changes, as indicated by the colour intensity of the nodes on **Figure 1**.

Generally, the preferred amino acid treatment did not show many significant differences in aroma compound production compared to the ammonium treatment (**Figures 1 and 4**). The preferred amino acids in this treatment (specifically glutamine and glutamate) and ammonium are easily interconverted to provide cellular nitrogen to the cell, and would in most cases support similar growth and fermentation rates (CHAPTER 3). Although the preferred amino acid treatment could directly provide carbon skeletons (precursors) for aroma production, these are also easily derived anabolically from glycolytic precursors (Beltran et al., 2005; Miller et al., 2007). The remainder of the treatments displayed significantly different aroma profiles compared to the ammonium and preferred amino acid treatments.

The compounds that were the greatest role players could be further grouped into those that show mainly significant increases in concentration for amino acid treatments relative to ammonium (and preferred amino acids), and those that show mostly significant decreases in concentration. Three related compounds (propanol, 3-ethoxy-1-propanol and propionic acid) were significantly reduced in concentration in amino acid treatments relative to the ammonium treatment (and preferred amino acids). Compounds showing significant relative increases are the higher alcohols (isoamyl alcohol, isobutanol and 2-phenylethanol), related acetate esters and volatile fatty acids (isoamyl acetate, 2-phenylethyl acetate and isobutyric acid) and diethyl succinate. In addition, the presence of sulfur-containing amino acids resulted in the exclusive production of a non-sulfurous ester quantified in this study (ethyl-2-methylbutyrate).

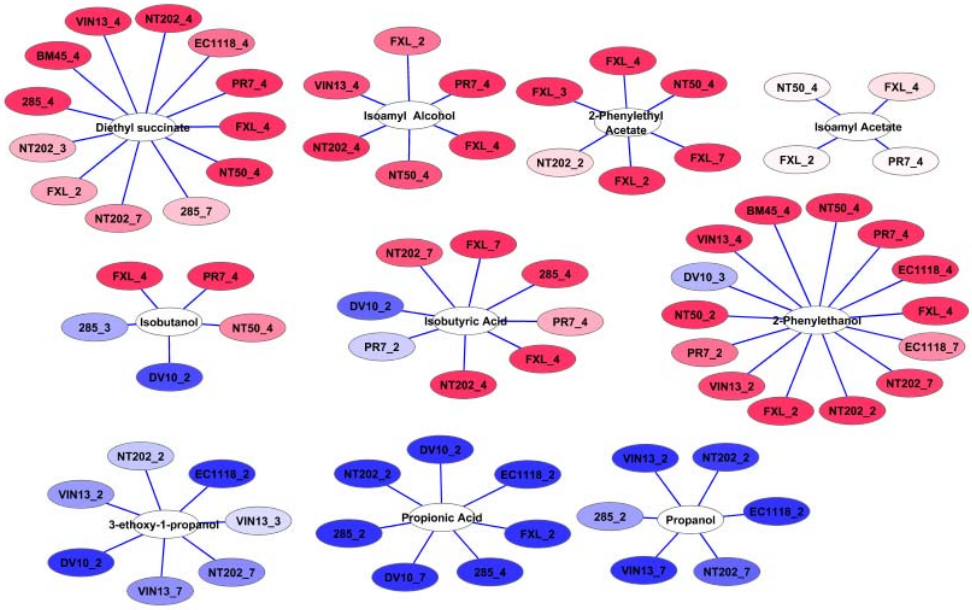


Figure 4 Significant differences in aroma compound production between amino acid treatments and the ammonium treatment (designated control) attributed mainly to nitrogen treatment effects, and conserved for multiple yeast strains. Significant increases of treatments relative to the ammonium treatment are indicated by red nodes, and decreases by blue nodes; with the colour intensity showing the magnitude of the difference. Treatments are (2) complete amino acids, (3) preferred amino acids, (4) branched-chain and aromatic amino acids, and (7) non-preferred amino acids. (Sulfur and non-utilised amino acid treatments are shown separately in **Figure 3**.)

4.3.4.1 Higher alcohols and related compounds

Propanol and related compounds

Generally, the formation of higher alcohols is known to increase upon nitrogen limitation (Lambrechts & Pretorius, 2000). Propanol production appears to be an exception, with higher concentrations formed with increasing available nitrogen and has been specifically correlated with large quantities of added ammonium (Giudici & Kunkee, 1994; Carrau et al., 2008). The associated amino acid precursor of propanol is threonine (**Figure 5**). Propanol production is related to aspartic amino acid metabolism, and is under metabolic control of methionine. The presence of methionine inhibits both the formation of the intermediate homoserine and its conversion to threonine. When methionine is deficient, threonine would be produced in high amounts, leading to an increased production of propanol (Giudici et al., 1993).

It has been proposed that 3-ethoxy-1-propanol is formed by *S. cerevisiae* via the Ehrlich pathway by the degradation of homoserine via O-acetylhomoserine, an intermediate of the biosynthetic pathway of methionine from aspartate. O-acetylhomoserine can react with sulfide to form homocysteine and finally methionine. Alternatively it can react with ethanol to form O-ethylhomoserine and 3-ethoxy-1-propanol

(**Figure 5**) (Irwin, 1992). The presence of the aspartic amino acids methionine and threonine inhibit the formation of 3-ethoxy-1-propanol, due to feedback inhibition of the relevant enzyme activities.

The data show that propanol was produced in high concentrations not only in the ammonium treatment, but also the preferred and branched-chain and aromatic amino acid treatments (**Figures 4 and 6**), and for some strains high concentrations were also produced in the non-preferred amino acid treatment (containing threonine). Large strain differences were observed. Some strains (VIN13 and NT202) appear to be relatively high producers of both propanol (**Figure 6 A**) and 3-ethoxy-1-propanol (**Figure 6 B**) in the presence of the ammonium, preferred amino acid and branched-chain and aromatic amino acid treatments. Other strains appear to produce relatively low amounts of both compounds, regardless of nitrogen treatment (FXL, 285). However, some strains are very low producers or even non-producers of 3-ethoxy-1-propanol (PR7 and BM45) even though they produce propanol at average concentrations. Interestingly, DV10 and EC1118 (both belonging to *S. cerevisiae* var. *bayanus*) are low producers of propanol but very high producers of 3-ethoxy-1-propanol, regardless of amino acid source.

In the presence of methionine (in the complete and sulfur amino acid treatments), production of these two compounds was inhibited similarly for all strains (**Figure 6**). In this study, methionine was provided at 75 mg N/l in the sulfur amino acids treatment. This amount substantially inhibited the formation of propanol, propionic acid and 3-ethoxy-1-propanol (**Figure 4**) relative to when ammonium was supplied as nitrogen source. No 3-ethoxy-1-propanol was detected in the sulfur amino acid treatment (for all yeast strains). The complete amino acid treatment contained 7.5 mg N/l of methionine, as well as the same amount of threonine, which was sufficient to cause significant inhibition of propanol produced by the majority of strains. The presence of threonine alone, at a concentration of 30 mg N/l in the non-preferred amino acid treatment caused significant reduction in propanol production, in particular by strains VIN13 and NT202.

As observed in the present study, it is also recorded in literature that the formation of high concentrations of propanol and 3-ethoxy-1-propanol in wine and other alcoholic beverages appear to be strain specific characteristics. Moreover, the production of propanol and 3-ethoxy-1-propanol in wine has been associated with non-hydrogen sulfide (H_2S) producing yeast strains (Antonelli et al., 1999). It is proposed that low sulfate reducing yeast strains produce methionine in insufficient amounts to cause feedback inhibition of the threonine pathway. As a result, high levels of propanol are produced by these strains (Giudici et al., 1993). The production of these compounds in wine has been associated with specific yeast strains of the species *S. cerevisiae* and *Saccharomyces bayanus* (Giudici et al., 1993; Antonelli et al., 1999; Carrau et al., 2008) and also with non-*Saccharomyces* yeast such as *Torulaspora delbreukii* (Herraiz et al., 1990).

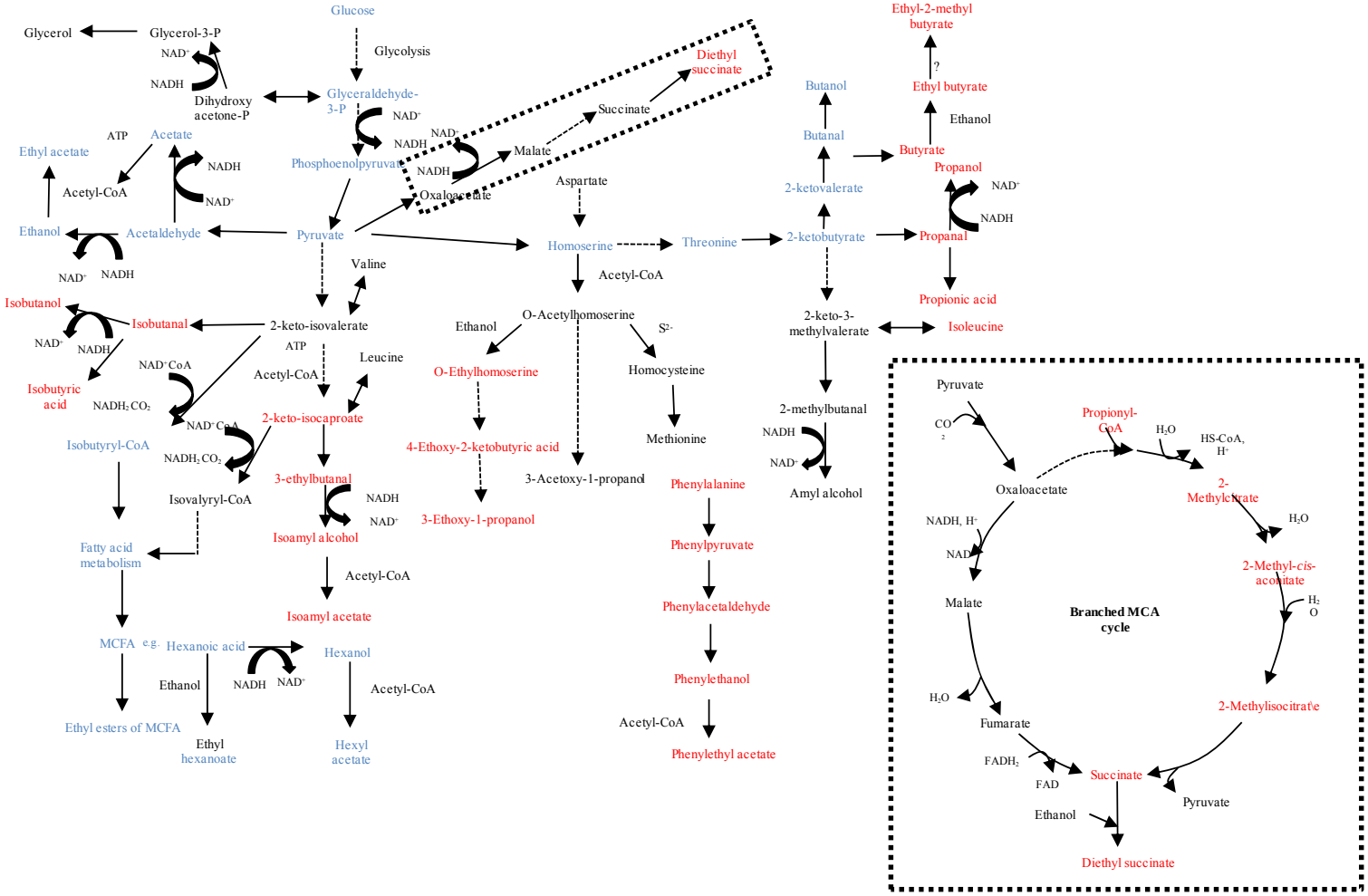


Figure 5 Diagram of metabolic pathways associated with the production of aroma compounds by *S. cerevisiae* described in this chapter. Compounds or pathways designated as nitrogen treatment independent are indicated in blue font, and nitrogen treatment dependent in red font. The biosynthetic pathway of 3-ethoxy-1-propanol in yeast was proposed by Irwin (1992). Succinic acid production via the methyl citric acid (MCA) cycle is described in De Klerk (2010). Dashed arrows indicate one or more omitted intermediates.

(A) Propanol **(B) 3-Ethoxy-1-propanol**

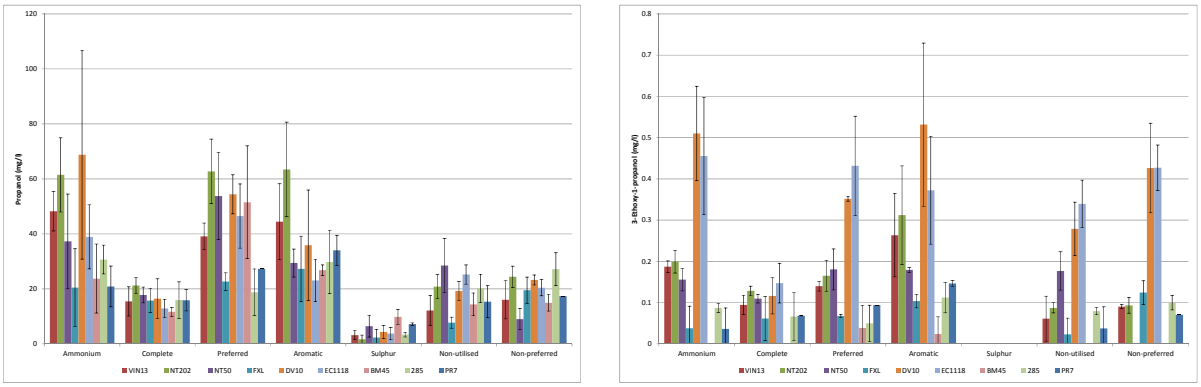


Figure 6 Concentrations of (**A**) propanol and (**B**) 3-ethoxy-1-propanol measured at the end of alcoholic fermentation in the seven different nitrogen treatments, produced by nine different yeast strains. Error bars indicate the standard deviation between triplicate fermentation.

2-Phenylethanol, isoamyl alcohol and isobutanol and related compounds

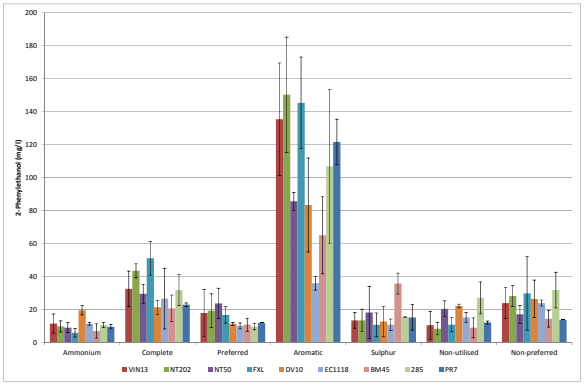
The production of elevated concentrations of higher alcohols due to the catabolism of branched-chain and aromatic amino acids via the Ehrlich pathway is overtly illustrated by the levels of 2-phenylethanol, isoamyl alcohol and isobutanol measured in the branched-chain and aromatic amino acid treatment at the end of alcoholic fermentation (**Figure 7**). **Figure 4** shows that for the majority of strains tested, concentrations of higher alcohols (2-phenylethanol, isoamyl alcohol and isobutanol), as well as their associated acetate esters (2-phenylethyl acetate and isoamyl acetate) and volatile fatty acids (isobutyric acid) were significantly increased in the branched-chain and aromatic amino acid treatment relative to the ammonium treatment. In addition, the complete amino acid treatment containing lower concentrations of branched-chain and aromatic amino acids (**Table 1**) also showed significant increases for most of these aroma compounds relative to the ammonium treatment (**Figure 4**). **Figure 7 A** shows the results for 2-phenylethanol, for which this result is conserved for the majority of strains. For isoamyl alcohol and isobutanol many strain-specific exceptions can be observed in comparison with 2-phenylethanol (**Figure 7 B and C**), with some other nitrogen treatments resulting in similar concentrations of these compounds as produced in the branched-chain and aromatic amino acid treatment. It appears that *de novo* synthesis from a sugar substrate plays an important role in the formation of higher alcohols associated with branched-chain amino acids (isoamyl alcohol and isobutanol) and is strongly influenced by the composition of nitrogen in the medium and the strain performing the fermentation. In contrast, the results of the present study show that higher alcohols directly associated with aromatic amino acids (in this case phenylalanine) are possibly produced more readily via the Ehrlich pathway.

The presence of amino acids in the non-preferred amino acid treatment also significantly increased the concentration of higher alcohols and their associated acetate esters for some strains. This could be attributed either to the increased production of higher alcohols under conditions of nitrogen limitation

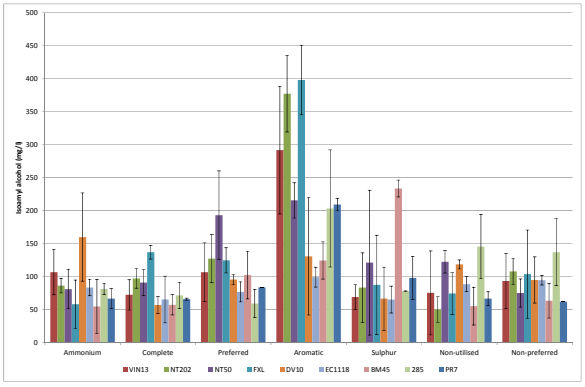
(Lambrechts & Pretorius, 2000) or could be related to the presence of specific amino acids in this treatment, for example tryptophan, an aromatic amino acid requiring molecular oxygen for its catabolism and thus not readily utilised under anaerobic conditions (Large, 1986).

A result that requires further study is the exceptionally high concentrations of higher alcohols produced by strain BM45 in the presence of sulfur amino acids relative to ammonium, but also in relation to the branched-chain and aromatic amino acids treatment (**Figure 7**). The low aroma compound production by strain EC1118 in the presence of branched-chain and aromatic amino acids (and most treatments) is also interesting, and may be a feature of *S. cerevisiae bayanus* strains since DV10 behaves similarly; as noted for propanol production in this study; as well as for overall aroma and gene expression profiles (Rossouw et al., 2008).

(A) 2-Phenylethanol



(B) Isoamyl alcohol



(C) Isobutanol

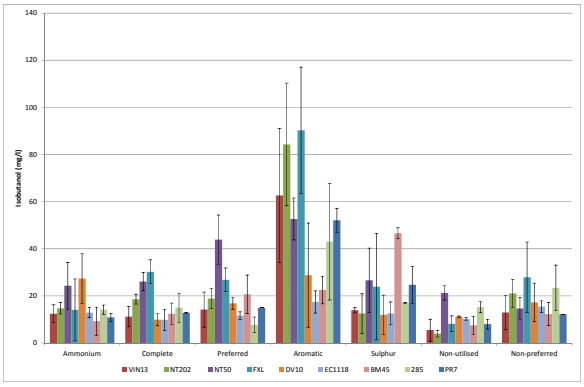


Figure 7 Concentrations of (**A**) 2-phenylethanol, (**B**) isoamyl alcohol and (**C**) isobutanol measured at the end of alcoholic fermentation in the seven different nitrogen treatments, produced by nine different yeast strains. Error bars indicate the standard deviation between triplicate fermentation treatments.

4.3.4.2 Ethyl esters

Ethyl-2-methylbutyrate

Like propanol, butanol is produced from the intermediate α-ketobutyrate, resulting from the metabolism of threonine, methionine and homocysteine (**Figure 5**). Butanol was below detection in synthetic grape must samples, but associated esters were detected, and although not always present in amounts that could be reliably or accurately quantified, certain trends could be established. While significantly lower concentrations of the ester ethyl butyrate was produced in the sulfur amino acid treatment relative to the ammonium treatment for all yeast strains, the related ester ethyl-2-methylbutyrate was detected only in the sulfur amino acid treatment (and not in the ammonium treatment) (**Figure 2**). The preferential production of the latter ester in the presence of sulfur amino acids should be further investigated, as it is does not seem to be documented in current literature.

Diethyl succinate

Diethyl succinate is one of the volatile esters of succinic acid, a major non-volatile by-product of alcoholic fermentation (De Klerk, 2010). According to Heerde & Radler (1978), high concentrations of succinic acid are formed when yeast cells are supplied with amino acids that are considered preferred sources of nitrogen, such as glutamate which is deaminated by NAD+-dependent glutamate dehydrogenase to release ammonium and α-ketoglutarate. In turn, the increase in the intracellular α-ketoglutarate concentration causes an increase of enzyme activities of the oxidative branch of the tricarboxylic acid (TCA) cycle, resulting in the production of succinate. It has been suggested that the carbon skeletons derived from deaminated "preferred" amino acids are often available to enter the TCA cycle and be transformed into succinate (De Klerk, 2010) since the carbon skeletons required by yeast for amino acid biosynthesis are largely obtained from α-keto acids derived from sugar catabolism (Jones et al., 1969; Henschke & Jiranek, 1993).

However, the oxidative branch of the TCA cycle is not the only metabolic pathway by which succinate is produced during alcoholic fermentation. A modified pathway, the methyl citric acid (MCA) cycle (**Figure 5**), is active under anaerobic conditions to metabolise propionyl-CoA, an intermediate in the catabolism of branched-chain amino acids (Shindo et al., 1993; Pronk et al., 1994). Shindo et al. (1993) specifically correlated the production of large concentrations of succinic acid by immobilised brewer's yeast (*S. cerevisiae*) to the consumption of isoleucine, rather than other more preferred amino acids such as glutamate.

The results of the present study clearly indicate that significantly elevated levels of diethyl succinate (and thus its precursor succinic acid) were obtained for all strains when supplied with branched-chain and aromatic amino acids as nitrogen source (**Figures 4 and 8**), with different strains producing different final concentrations. All other treatments, including the preferred amino acid treatment, contained statistically

similar concentrations of diethyl succinate, which was significantly lower than produced in the branched-chain and aromatic amino acid treatment.

The catabolism of "preferred" amino acids also provides amino groups for the biosynthesis of other amino acids (Jones et al., 1969; Henschke & Jiranek, 1993). Less succinic acid will be formed when preferred amino acids such as glutamate partake directly in transamination reactions to form other amino acids, instead of being converted to α-ketoglutarate. The results of the present study indicate the possibility that the branched-chain and/or aromatic amino acids directly enter the pathways of succinate production much more readily than other amino acids, or they stimulate *de novo* production of large quantities of α-ketoglutarate which enter the TCA cycle. It seems that the carbon skeletons of most other amino acids are equally directed towards or shared with other metabolic pathways for most strains.

Unlike for higher alcohol production, diethyl succinate was not produced in higher concentrations in the complete amino acid treatment (which also included branched-chain and aromatic amino acids) relative to other treatments containing amino acids (**Figure 8**). This further supports the argument that it is an excess of branched-chain and/or aromatic amino acids that will stimulate diethyl succinate production. It could be that compounds such as higher alcohols are produced first from available carbon skeletons (after requirements for amino acid biosynthesis have been met), likely to ensure redox balancing of the cell (Jain et al., 2011). Only thereafter will the remainder of α-ketoglutarate from branched-chain and aromatic amino acids be fed into the pathways of succinic acid production. In evidence, Rossouw et al. (2008) also noticed that diethyl succinate was only detectable towards the end of alcoholic fermentation in synthetic medium, and is not accumulated actively during the early stages of fermentation like most other aroma compounds, including higher alcohols.

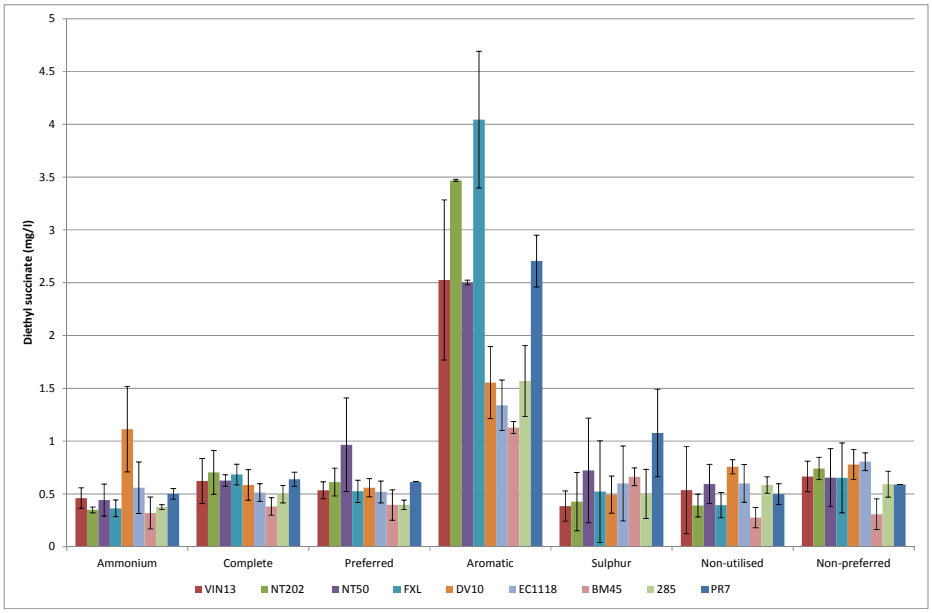


Figure 8 Concentrations of diethyl succinate measured at the end of alcoholic fermentation in the seven different nitrogen treatments, produced by nine different yeast strains. Error bars indicate the standard deviation between triplicate fermentation treatments.

4.4 Conclusions

This work was exploratory in nature and aimed to better describe the connection between amino acid composition in a wine-like matrix and aroma compound production by different strains of wine yeast.

In particular, CHAPTER 3 provided a general overview of aroma production profiles by different strains under different nitrogen conditions and major deviations from general behaviour could be identified. Here, a careful analysis of aroma compounds showing conserved behaviour for most yeast strains led to the sorting of compounds as either nitrogen treatment independent or dependent; and the attribution of potential metabolic pathways involved could be made. These findings (as summarised in **Figure 5**) can significantly contribute to future studies to understand interconnected metabolic pathway regulation.

The production of certain aroma compounds appeared to be mostly dependent on yeast strain differences. These compounds were produced in comparable amounts when sufficient nitrogen was available to support fermentation. When assimilable nitrogen was limited, such as in the sulfur and non-utilised amino acid treatments, yeast strains showed significantly lower aroma compound concentrations in general due to lower metabolic activity under stress conditions. The presence of excess sulfur amino acids also resulted in the production of a non-sulfurous ester (ethyl-2-methylbutyrate), which was not detected in any other treatment.

A number of aroma compounds were significantly affected by nitrogen treatments. Propanol and 3-ethoxy-1-propanol were produced in significantly higher amounts in the ammonium and preferred amino

acid treatments compared to other amino acid treatments, depending largely on yeast strain. However, the presence of methionine in the complete and sulfur amino acid treatments inhibited the production of these two compounds for all strains. Higher alcohols related to branched-chain and aromatic amino acid precursors were produced in elevated concentrations in the branched-chain and aromatic amino acid treatment; and to a lesser extent in the complete and non-preferred amino acid treatments. Our results indicate that the anabolic route of higher alcohol formation seems to be more prominent for higher alcohols associated with branched-chain amino acids (such as isoamyl alcohol and isobutanol), while the catabolic route is possibly favoured more for higher alcohols associated with aromatic amino acids (2-phenylethanol). The production of all higher alcohols analysed in this study appears to be highly strain dependent, with unique behaviour observed for EC1118 and DV10 (*S. cerevisiae* var. *bayanus*).

The production of large quantities of the ester diethyl succinate from excess concentrations of branched-chain and aromatic amino acids was observed in this study and could be related to the methyl citric acid (MCA) cycle. Possibly, the carbon skeletons of branched-chain and aromatic amino acids remaining after higher alcohol production enter this pathway directly whilst the carbon skeletons of most other amino acids are directed towards other metabolic pathways.

In conclusion, a number of aroma compounds showed interesting patterns of production that were not anticipated when the nitrogen treatments were designed. In addition, the conserved responses of different yeast genotypes to nitrogen treatments were demonstrated for these compounds. Of course, the proposed pathways of aroma compound formation require further investigation and confirmation, and may even prove insignificant in the complexity of real grape must.

References

Antonelli, A., Castellari, L., Zambonelli, C., & Carnacini, A. (1999). Yeast influence on volatile composition of wines. *Journal of Agricultural and Food Chemistry*, *47*(3), 1139–44.

Barbosa, C., Falco, V., Mendes-Faia, A., & Mendes-Ferreira, A. (2009). Nitrogen addition influences formation of aroma compounds, volatile acidity and ethanol in nitrogen deficient media fermented by *Saccharomyces cerevisiae* wine strains. *Journal of Bioscience and Bioengineering*, *108*(2), 99–104.

Bauer, F., & Pretorius, I. (2000). Yeast stress response and fermentation efficiency: How to survive the making of wine: A review. *South African Journal of Enology and Viticulture*, *21*, 27–51.

Bell, S.-J., & Henschke, P. A. (2005). Implications of nitrogen nutrition for grapes, fermentation and wine. *Australian Journal of Grape and Wine Research*, *11*(3), 242–295.

Beltran, G., Esteve-Zarzoso, B., Rozès, N., Mas, A., & Guillamón, J. M. (2005). Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and nitrogen consumption. *Journal of Agricultural and Food Chemistry*, *53*(4), 996–1002.

Beltran, G., Novo, M., Rozès, N., Mas, A., & Guillamón, J. M. (2004). Nitrogen catabolite repression in *Saccharomyces cerevisiae* during wine fermentations. *FEMS Yeast Research*, *4*(6), 625–32.

Bisson, L. (1999). Stuck and sluggish fermentations. *American Journal of Enology and Viticulture, 50*(1), 107–119.

Carrau, F. M., Medina, K., Farina, L., Boido, E., Henschke, P. A., & Dellacassa, E. (2008). Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: Effects of yeast assimilable nitrogen on two model strains. *FEMS Yeast Research*, 8(7), 1196–207.

Cooper, T.G. (1982). Nitrogen metabolism in *Saccharomyces cerevisiae*. In: Strathern, J.N., Jones, E.W., Broach, J.R. (Eds.). The molecular biology of the yeast *Saccharomyces*: Metabolism and gene expression, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 39– 99.

De Klerk, J. (2010). Succinic acid production by wine yeasts. MSc Thesis. Stellenbosch University. Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Fairbairn, S. C., (2012). Stress, fermentation performance and aroma production by yeast. MSc Thesis. Stellenbosch University. Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Garde-Cerdán, T., & Ancín-Azpilicueta, C. (2008). Effect of the addition of different quantities of amino acids to nitrogen-deficient must on the formation of esters, alcohols, and acids during wine alcoholic fermentation. *LWT - Food Science and Technology*, *41*(3), 501–510.

Giudici, P., & Kunkee, R. E. (1994). The effect of nitrogen deficiency and sulpfur-containing amino acids on the reduction of sulfate to hydrogen sulfide by wine yeasts. *American Journal of Enology and Viticulture*, *45*(1), 107–112.

Giudici, P., Zamponelli, C., & Kunkee, R. E. (1993). Increased production of n-propanol in wine by yeast strains having an impaired ability to form hydrogen sulfide. *American Journal of Enology and Viticulture*, *44*(1), 17–21.

Heerde, E., & Radler, F. (1978). Metabolism of the anaerobic formation of succinic acid by *Saccharomyces cerevisiae*. *Archives of Microbiology*, *117*, 269–276.

Henschke, P.A and Jiranek, V., (1993). Yeasts – Metabolism of nitrogen compounds. In: Wine Microbiology and Biotechnology. Fleet, G. H. (Ed). Harwood Academic Publishers, Chur, Switzerland, pp. 77–164.

Hernández-Orte, P, Ibarz, M., Cacho, J., & Ferreira, V. (2005). Effect of the addition of ammonium and amino acids to musts of Airen variety on aromatic composition and sensory properties of the obtained wine. *Food Chemistry*, *89*(2), 163–174.

Hernández-Orte, P., Bely, M., Cacho, J., & Ferreira, V. (2006). Impact of ammonium additions on volatile acidity, ethanol, and aromatic compound production by different *Saccharomyces cerevisiae* strains during fermentation in controlled synthetic media. *Australian Journal of Grape and Wine Research*, *12*(2), 150–160.

Hernández-Orte, P., Cacho, J. F., & Ferreira, V. (2002). Relationship between varietal amino acid profile of grapes and wine aromatic composition. Experiments with model solutions and chemometric study. *Journal of Agricultural and Food Chemistry*, *50*(10), 2891–9.

Herraiz, T., & Reglero, G. (1990). The influence of the yeast and type of culture on the volatile composition of wines fermented without sulfur dioxide. *American Journal of Enology and Viticulture*, *41*(4), 313–318.

Hofman-Bang, J. (1999). Nitrogen Catabolite Repression in *Saccharomyces cerevisiae*. *Molecular Biotechnology*, *12*, 35–73.

Irwin, A. (1992). 3-Ethoxy-1-propanol: A strain-and species-dependent yeast metabolite. *Journal of the Institute of Brewing*, *98*, 427–431.

Jain, V. K., Divol, B., Prior, B. A., & Bauer, F. F. (2012). Effect of alternative NAD^+-regenerating pathways on the formation of primary and secondary aroma compounds in a *Saccharomyces cerevisiae* glycerol-defective mutant. *Applied Microbiology and Biotechnology*, *93*(1), 131–41.

Jiménez-Martí, E., Aranda, A., Mendes-Ferreira, A., Mendes-Faia, A., & Del Olmo, M. L. (2007). The nature of the nitrogen source added to nitrogen depleted vinifications conducted by a *Saccharomyces cerevisiae* strain in synthetic must affects gene expression and the levels of several volatile compounds. *Antonie van Leeuwenhoek*, *92*(1), 61–75.

Jones, M., Pragnell, M. J., & Pierce, J. S. (1969). Absorption of amino acids by yeasts from a semi-defined medium stimulating wort. *Journal of the Institute of Brewing, 75*, 520-536.

Keyzers, R. A., & Boss, P. K. (2010). Changes in the volatile compound production of fermentations made from musts with increasing grape content. *Journal of Agricultural and Food Chemistry, 58*(2), 1153–64.

Large, P. J. (1986). Degradation of organic nitrogen compounds by yeasts. *Yeast 2*, 1-34.

Lambrechts, M., & Pretorius, I. (2000). Yeast and its importance to wine aroma – A review. *South African Journal of Enology and Viticulture, 21*, 97–129.

Louw, L., Roux, K., Tredoux, A., Tomic, O., Naes, T., Nieuwoudt, H. H., & Van Rensburg, P. (2009). Characterization of selected South African young cultivar wines using FTMIR spectroscopy, gas chromatography, and multivariate data analysis. *Journal of Agricultural and Food Chemistry*, *57*(7), 2623–32.

Magasanik, B., & Kaiser, C. A. (2002). Nitrogen regulation in *Saccharomyces cerevisiae*. *Gene*, *290*(1-2), 1–18.

Miller, A., Wolff, S., Bisson, L., & Ebeler, S. (2007). Yeast strain and nitrogen supplementation: Dynamics of volatile ester production in Chardonnay juice fermentations. *American Journal of Enology and Viticulture*, *58*(4), 470–483.

Pronk, J., Van der Linden-Beuman, A., Verduyn, C., Scheffers, W., & Van Dijken, J. (1994). Propionate metabolism in *Saccharomyces cerevisiae*: Implications for the metabolon hypothesis. *Microbiology*, *140*, 717–722.

Rossouw, D., Naes, T., & Bauer, F. F. (2008). Linking gene regulation and the exo-metabolome: A comparative transcriptomics approach to identify genes that impact on the production of volatile aroma compounds in yeast. *BMC Genomics*, *9*, 530.

Shindo, S., Sahara, H., & Koshino, S. (1993). Relationship of production of succinic acid and methyl citric acid pathway during alcohol fermentation with immobilized yeast. *Biotechnology Letters*, *15*(1), 51–56.

Styger, G., Jacobson, D., & Bauer, F. F. (2011). Identifying genes that impact on aroma profiles produced by *Saccharomyces cerevisiae* and the production of higher alcohols. *Applied Microbiology and Biotechnology*, *91*(3), 713–30.

Ter Schure, E. G., van Riel, N. A. V., & Verrips, C. T. (2000). The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews*, *24*(1), 67–83.

Torija, M., Beltran, G., Novo, M., Poblet, M., Rozès, N., Guillamón, J. M., & Mas, A. (2003). Effect of the nitrogen source on the fatty acid composition of *Saccharomyces cerevisiae*. *Food Microbiology*, *20*, 255–258.

Ugliano, M., Henschke, P. A., Herderich, M., & Pretorius, I. S. (2007). Nitrogen management is critical for wine flavour and style. *Wine Industry Journal*, *22*(6), 24–30.

Vilanova, M., Ugliano, M., Varela, C., Siebert, T., Pretorius, I. S., & Henschke, P. A. (2007). Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by *Saccharomyces cerevisiae* wine yeasts. *Applied Microbiology and Biotechnology*, *77*(1), 145–57.

Chapter 5

Comparative aroma production patterns of wine yeast strains in synthetic, white and red grape musts in the presence of different nitrogen treatments

CHAPTER 5

Comparative aroma production patterns of wine yeast strains in synthetic, white and red grape musts in the presence of different nitrogen treatments

Abstract

The impacts of seven nitrogen supplementation treatments on the fermentation performance and aroma compound production of three yeast strains were compared in three fermentation media: synthetic, white and red grape must. Real grape musts contain many known and unknown factors that increase its complexity relative to chemically defined fermentation media. Therefore, results from studies in synthetic grape must should be validated in real winemaking conditions. The results of this study indicate that not only are significant aroma impacts conserved for all fermentation media, but synthetic grape must could serve to eliminate unwanted "noise" and highlight only the most relevant contributing factors in multivariate studies. Interactive effects between nitrogen treatments and yeast strains, as well as proposed pathways of aroma compound formation and regulation previously described were confirmed in this study. The data presented here can be favourably employed to assist in the development of novel, unique and targeted commercial nitrogen supplements and/or practical fermentation management strategies to increase desirable fermentation attributes and decrease fermentation problems related to nitrogen nutrition in wine. Our results could, in the long term, enable wine producers to manipulate wine aroma profiles to match consumer preferences and improve wine quality.

5.1 Introduction

Grape juice serves as a reservoir of nutrients required for the proliferation of and successful alcoholic fermentation by wine yeast. Simultaneously, grape musts provide a pool of flavour and aroma precursors that can be produced or modified by the secondary metabolism of yeast. These secondary metabolic pathways form an intricate network of reactions with shared intermediates, where various fermentation parameters and environmental stress factors can influence the fate of each compound. Thus the availability of aromatic precursor compounds in the must, together with the specific environmental conditions and the intrinsic genetic constitution of the wine yeast strains performing fermentation will determine the flavour and aroma, and therefore the quality of the wine.

Unravelling the nature of these complex metabolic networks by systems biology-based approaches have led to improved understanding of the origins and regulation of compounds that contribute to the volatile aroma profile of wine on a genetic, enzymatic and metabolic level. Transcriptomic and multi-factorial fermentation studies have shown that significant differences exist in the way individual yeast strains respond to environmental stress (including nutrient availability) and alter their primary and secondary metabolisms (Rossouw & Bauer, 2009; Rossouw et al., 2009; Fairbairn, 2012). Optimal exploitation of such data leads to the development of predictive capacity regarding the interaction between yeast strain

genetic make-up, environmental factors and aroma production. Soon the need will arise for new technologies to provide us with detailed chemical information regarding grape juice composition, including nutritional factors available to yeast before the onset of fermentation. These new developments serve to enable wine producers to modify winemaking (and even vineyard) practices to produce wines of optimal quality and specific styles desired by target consumers.

Nitrogen composition and concentration in grape must is reasonably well studied and is known to have a major impact on fermentation rate and completion, but also on the aroma and flavour of the wine. Several studies have shown that the total available nitrogen, the specific sources of nitrogen, the timing of its addition and the pattern of selective nitrogen uptake by individual yeast strains are all factors that will impact on the accumulation of nitrogen-related volatile aroma compounds such as esters, higher alcohols and fatty acids (Torrea et al., 2003; Beltran et al., 2005; Hernández-Orte et al., 2005; Hernández-Orte et al., 2006b; Garde-Cerdán & Ancín-Azpilicueta, 2008; Barbosa et al., 2009). A number of non-volatile compounds contributing to wine flavour are also affected by yeast nitrogen metabolism and thus by nitrogen additions, including glycerol and organic acids (Albers et al., 1996; Vilanova et al., 2007; Torrea et al., 2011; Fairbairn, 2012).

Ammonium is widely used in wine production as a yeast nitrogen source. It is readily available in the form of diammonium phosphate (DAP), a comparatively inexpensive nitrogen supplement, which is often proactively added to grape musts without prior knowledge of its nitrogen status. In order to address the misconceptions around this standard fermentation management strategy, a number of recent studies have uncovered the effect of ammonium additions on yeast fermentation kinetics and aroma compound production and have addressed the nitrogen needs and demands of individual yeast strains (Albers et al., 1996; Hernández-Orte et al., 2002; Torrea et al., 2003; Hernández-Orte et al., 2005; Hernández-Orte et al., 2006a; Hernández-Orte et al., 2006b; Vilanova et al., 2007; Moreira et al., 2011b; Vilanova et al., 2012). In part as a result of such studies, the addition of complex yeast nutrients is becoming more common in commercial fermentation management. These more comprehensive nutrient formulations usually contain a combination of DAP and inactivated yeast cells or yeast cell walls, as well as other source of amino acids. Amino nitrogen also impacts on the fermentative and aroma producing capacities of yeast strains, as demonstrated by workers such as Hernández-Orte et al. (2005) and Garde-Cerdán and Ancín-Azpilicueta (2008). Most recent studies focus on the effects of adding different concentrations of ammonium, amino acids or a combination to grape must on the volatile and non-volatile composition of the product, with results generally showing strong interaction between yeast strain and fermentation conditions (Hernández-Orte et al., 2005; Hernández-Orte et al., 2006a; Hernández-Orte et al., 2006b; Garde-Cerdán & Ancín-Azpilicueta, 2008; Torrea et al., 2011; Fairbairn, 2012). Yet, even small fluctuations in the concentrations of numerous individual compounds due to combinatorial treatment effects can add up to significantly contribute to the final perceived fermentation bouquet (Pineau et al., 2009; Fairbairn, 2012). Considering that modern wine consumers are showing an increased preference towards fruitiness in both white and red wines (Pineau et al., 2009), modulation of yeast-derived aroma

compounds by making the correct choice of strain and nutritional strategy is becoming vital in order to remain competitive in the market. Major volatile compounds (esters, higher alcohols and fatty acids) derived from yeast amino acid metabolism can also play a pertinent role in varietal character, especially of young wines (Ferreira et al., 2000; Hernández-Orte et al., 2002).

Most studies regarding the effect of nitrogen have been conducted using synthetic must as a model. A real wine matrix is more complex than a chemically defined fermentation medium due to known and unknown factors including phenolic compounds, various organic acids and microorganisms. In CHAPTERS 3 and 4, the global fermentation performances and aroma production patterns as well as compounds and pathways uniquely influenced by specific nitrogen and strain combinations were established in synthetic grape must. However, it is imperative that results from fermentation studies in synthetic grape must can be reproduced successfully in real grape must.

Thus, the aim of this study is to validate the effects of supplementing synthetic grape must with different nitrogen treatments in real grape must for selected yeast strains. To do this, we compare the impact of the same seven nitrogen treatments added at the onset of fermentation on concentrations and significant changes of specific compounds in three fermentation matrices. Not only will this indicate the validity of synthetic grape must as representative fermentation environment, but also identify differences attributable to fermentation matrix effects (which could, in fact, be considered an additional factor). Three yeast strains were chosen based on the interesting fermentation properties they displayed for specific treatments in CHAPTER 3; yet these strains also conformed to general aroma production patterns as described in CHAPTER 4. We also investigate in this chapter the specific aroma production patterns in red and white grape must.

In the long term, this study aims to contribute to practical guidelines for winemakers regarding nitrogen supplementation strategies for individual yeast strains to achieve desired flavour outcomes.

5.2 Materials and methods

5.2.1 Yeast strains and culture conditions

Three commercial wine yeast strains were used in this study, namely *Saccharomyces cerevisiae* VIN13, NT50 (Anchor Yeast, Cape Town, South Africa) and Fermicru XL (FXL) (DSM Food Specialities, Delft, The Netherlands). Pure cultures of the yeast strains were maintained on YPD agar and were pre-cultured in YPD broth prior to inoculation of real grape must fermentations. Cultures used to inoculate synthetic grape must fermentations were pre-cultured additionally in synthetic grape must containing ammonium chloride as nitrogen source. All pre-cultures were performed at 30°C. Fermentations were inoculated from overnight cultures into fermentation media at a final optical density (OD_{600}) of 0.1 (a cell density of approximately 10^6 cfu/ml).

5.2.2 Fermentation media and nitrogen treatments

A chemically defined grape juice-like medium, previously described by Henschke and Jiranek (1993), was used for synthetic grape must fermentations, differing from the original composition only in its nitrogen content. The total assimilable nitrogen concentration of the synthetic medium was 200 mg N/l, of which 50 mg N/l was supplied to all treatments as ammonium nitrogen. The remaining 150 mg/l nitrogen was comprised of the different nitrogen treatments.

Real grape must fermentations were conducted in red and white grape musts. Red grapes of the cultivar Pinotage were crushed and destemmed, followed by the addition of 30 mg/l of sulfur dioxide (as potassium metabisulfite). The grape juice and skins were macerated at 4°C for three days to enhance extraction of red colour and other phenolic compounds from the grape skins. Grape skins were pressed in a manual basket press; the juice was collected and settled overnight at 4°C before storage. Hanepoot (Muscat d'Alexandrie) grape juice was obtained from a commercial wine producer. The white grape must was clarified by centrifugation at the commercial cellar and 30 mg/l of sulfur dioxide was added. The red and white grape musts were stored at -20°C until used (for approximately 2 months). Grape musts were thawed at 4°C, homogenised by magnetic stirring, dispensed into sterile fermentation vessels and warmed to ambient temperature (approximately 20°C) before inoculation with yeast.

A base level of nutrients other than nitrogen (vitamins and minerals) was supplied to the real grape must, in order to simulate the addition of a complex yeast nutrient. Ten percent of the vitamins and minerals described for the chemically defined grape juice media (Henschke & Jiranek, 1993) was added to the real grape must. This amount was calculated to provide vitamins and minerals to real grape must at a concentration intermediate between the full amount supplied to synthetic grape must and the dosage supplied by various complex commercial nutrients analysed previously in our laboratory (unpublished data). No dilutions and no other additions were made to real must.

The nitrogen treatments were comprised of seven different groups of amino acids added to the initial grape musts. The ammonium treatment contained ammonium as the only added nitrogen source, to a total addition of 200 mg N/l. The non-utilised amino acid treatment was supplied with only 50 mg N/l of ammonium and 150 mg N/l of amino acids that are not utilised during fermentation. Effectively, this treatment was supplied with only 50 mg N/l of available nitrogen. The remainder of the amino acid treatments were sorted according to the preference of yeast to utilise the amino acid as source of nitrogen (preferred amino acids, non-preferred amino acids or complete amino acids) or the potential impact the amino acids may have on aroma production (sulfur-containing amino acids or branched-chain and aromatic amino acids). **Table 1** shows the nitrogen composition of each treatment.

5.2.3 Fermentation conditions

Fermentations were carried out in triplicate, without agitation, in 100 ml glass bottles with a working volume of 80 ml of grape must. Fermentation vessels were fitted with rubber stoppers and a CO_2 gas

outlet. The temperature of the fermentation room was controlled at 20°C to 22°C. Fermentation progress was monitored by CO_2 release until completion (no further weight loss) for a maximum of 21 days, after which fermentation treatments were analysed for major non-volatile fermentation products. Samples of all fermentation treatments were stored at 4°C until analysed for volatile aroma compounds.

5.2.4 Amino acid composition of grape musts

Free amino acids present in grape must samples were analysed (after storage at -20°C) using a Waters AccQ Tag Kit (AccQ Tag™ Ultra derivatization kit, Waters, Milford, USA) with Norvaline as internal standard. Wines were diluted 10 x in water before analysis. Ten microliters of the diluted sample was added to the Waters AccQ Tag Kit constituents and heated in a heating block at 55°C for 10 minutes. Samples were analysed in duplicate on a Waters APE Quattro micro™ instrument (HPLC/MS/MS). One microliter of sample was injected onto a AccQ Tag C18 column with dimensions of 1.7 µm and 2.1 x 100 mm. MS conditions were as follows: capillary voltage, 3.5 kV; cone voltage, 15 V; source temperature, 120°C; desolvation temperature, 350°C; desolvation gas flow rate, 350 l/h and cone gas flow rate, 50 l/h.

5.2.5 Chemical characterisation of grape musts and wines

Basic chemical characterisation of the initial grape musts and the fermentation media at the end of alcoholic fermentation was performed with Fourier transform mid-infrared (FT-MIR) spectroscopy, using a Winescan FT120 instrument (FOSS Analytical A/S, Hillerød, Denmark). Compounds were quantified from mid-infrared spectra by in-house adjustments of commercial calibrations (FOSS Analytical A/S software version 2.2.1) and included major grape must components and fermentation products such as reducing sugars (grape must) or glucose and fructose (wine), total acid, pH, glycerol, and ethanol (Nieuwoudt et al., 2004; Louw et al., 2009). This analytical method is sufficient for screening purposes and accurate for non-volatile compounds, but does not provide absolute values for ethanol (Nieuwoudt et al., 2004). The titratable acid concentration was confirmed for real grape must using a Metrohm Titrino instrument.

Table 1 The amino acid composition of nitrogen treatments and natural grape musts used in this study, and the contribution of amino acids in treatments (T) and grape must (GM) to the total amino nitrogen (%); nd: not determined.

Amino acid treatment		White grape must (Hanepoot)			Red grape must (Pinotage)		
Amino acid	T (mg N/l)	GM (mg N/l)	T+GM (mg N/l)	%	GM (mg N/l)	T+GM (mg N/l)	%
Complete amino acids							
ALA	7.50	7.36	14.86	49.54	8.22	15.72	52.29
ARG	7.50	177.82	185.32	95.95	188.80	196.30	96.18
ASN	7.50	0.00	7.50	0.00	0.00	7.50	0.00
ASP	7.50	0.20	7.70	2.59	0.54	8.04	6.66
CYS	7.50	1.16	8.66	13.34	0.03	7.53	0.46
GLN	7.50	4.93	12.43	39.68	4.01	11.51	34.86
GLU	7.50	4.63	12.13	38.18	3.52	11.02	31.91
GLY	7.50	0.24	7.74	3.12	0.31	7.81	3.93
HIS	7.50	5.96	13.46	44.29	5.01	12.51	40.06
ILE	7.50	0.62	8.12	7.58	0.97	8.47	11.43
LEU	7.50	1.16	8.66	13.35	1.41	8.91	15.85
LYS	7.50	1.06	8.56	12.34	0.93	8.43	11.04
MET	7.50	1.29	8.79	14.70	0.62	8.12	7.64
PHE	7.50	0.90	8.40	10.72	0.86	8.36	10.32
PRO	7.50	37.29	44.79	83.25	41.66	49.16	84.74
SER	7.50	2.07	9.57	21.67	3.74	11.24	33.26
THR	7.50	2.35	9.85	23.89	4.47	11.97	37.32
TRP	7.50	2.98	10.48	28.43	1.60	9.10	17.55
TYR	7.50	0.72	8.22	8.72	0.75	8.25	9.06
VAL	7.50	2.74	10.24	26.73	2.37	9.87	24.01
Preferred amino acids							
ARG	30.00	177.82	207.82	85.56	188.80	218.80	86.29
ASN	30.00	Nd	nd	nd	nd	nd	nd
ASP	30.00	0.20	30.20	0.66	0.54	30.54	1.75
GLN	30.00	4.93	34.93	14.12	4.01	34.01	11.80
GLU	30.00	4.63	34.63	13.37	3.52	33.52	10.49
Branched-chain and aromatic amino acids							
ILE	30.00	0.62	30.62	2.01	0.97	30.97	3.13
LEU	30.00	1.16	31.16	3.71	1.41	31.41	4.50
PHE	30.00	0.90	30.90	2.92	0.86	30.86	2.80
TYR	30.00	0.72	30.72	2.33	0.75	30.75	2.43
VAL	30.00	2.74	32.74	8.36	2.37	32.37	7.32
Sulfur amino acids							
CYS	75.00	1.16	76.16	1.52	0.03	75.03	0.05
MET	75.00	1.29	76.29	1.69	0.62	75.62	0.82
Non-utilised amino acids							
HIS	50.00	5.96	55.96	10.65	5.01	55.01	9.11
LYS	50.00	1.06	51.06	2.07	0.93	50.93	1.83
PRO	50.00	37.29	87.29	42.72	41.66	91.66	45.45
Non-preferred amino acids							
ALA	30.00	7.36	37.36	19.71	8.22	38.22	21.50
GLY	30.00	0.24	30.24	0.80	0.31	30.31	1.01
SER	30.00	2.07	32.07	6.47	3.74	33.74	11.08
THR	30.00	2.35	32.35	7.28	4.47	34.47	12.96
TRP	30.00	2.98	32.98	9.04	1.60	31.60	5.05

5.2.6 Analysis of volatile compounds at the end of alcoholic fermentation

Volatile compounds were extracted from the wine samples by liquid-liquid extraction as described by Louw et al. (2009). To assist extraction from synthetic wines, modifications were made as described in CHAPTER 3. These modifications also improved extraction of the volatile fraction from real wine and were subsequently adopted for all samples.

Volatile compounds were analysed on a gas chromatograph (GC) with flame ionisation detection (FID). Each sample was extracted once and injected into the GC in duplicate. The instrument was calibrated for 39 compounds (**Table 2**) using standards (Merck, Cape Town, South Africa) and 4-methyl-2-pentanol as internal standard. For instrument specifications and chromatographic conditions, refer to CHAPTER 3. Data collection and peak integration was performed manually using the HP ChemStations software (Rev. B01.03 [204]).

5.2.7 Statistical analysis

In order to obtain a comprehensive statistical overview, explore patterns and extract the most influential information from the aroma datasets, multivariate data analysis was employed. Principal component analysis (PCA) was performed on the data using The Unscrambler software (version 9.2, CAMO, Norway). Data were normalised by autoscaling to account for differences in magnitude of individual aroma compounds. All samples from the three different fermentation media with all combinations of nitrogen treatments performed by the three yeast strains were included for PCA analysis. The variables were the end point concentrations of all volatile aroma compounds quantified by GC-FID that were detected in all three media. Fermentation medium, yeast strain and treatment were included as categorical data. The following compounds quantified in real grape must (**Table 2 A**) but below detection and/or quantification in synthetic grape must, were omitted from the multivariate models: ethyl phenylacetate, ethyl-2-methyl butyrate, hexyl acetate, butanol and hexanol.

Pair-wise comparisons of aroma compound concentrations were performed between each treatment and the ammonium treatment for all strains per fermentation medium. Graphs were visualised using Cytoscape software (version 2.8.2, http://www.cytoscape.org). A colour scale was assigned to the nodes of the bubble graphs, with blue representing a statistically significant lower compound concentration in the amino acid treatment than in the ammonium treatment, and red representing a significantly higher concentration. The graphs only display significant differences relative to the ammonium treatment, at a significance level of 5% ($p<0.05$). The colour intensity of the nodes indicates the magnitude of the fold difference between treatments.

Table 2 Aroma compounds analysed in real grape must; **(A)** quantified compounds grouped according to compound classes and **(B)** compounds below detection and/or quantification in real grape must.

A				
Ethyl esters	**Acetate esters**	**Fatty acids**	**Higher alcohols**	**VA components**
Ethyl butyrate	Isoamyl acetate	Propionic acid	Propanol	Ethyl acetate
Ethyl hexanoate	Hexyl acetate	Isobutyric acid	Isobutanol	Acetic acid
Ethyl octanoate	Ethyl phenylacetate	Butyric acid	Butanol	
Ethyl decanoate	2-Phenylethyl acetate	Hexanoic acid	Isoamyl alcohol	
Ethyl-2-methylbutyrate		Octanoic acid	Hexanol	
Diethyl succinate		Decanoic acid	3-Ethoxy-1-propanol	
			2-Phenylethanol	
B				
Ethyl esters	**Acetate esters**	**Fatty acids**	**Alcohols**	**Carbonyl compounds**
Ethyl propionate	2-Methyl-propyl acetate	Isovaleric acid	Methanol	Acetoin
Ethyl-2-methylpropanoate		Valeric acid	Pentanol	
Ethyl isovalerate			4-Methyl-1-pentanol	
Ethyl lactate			3-Methyl-1-pentanol	
Ethyl-3-hydroxybutanoate			1-Octen-3-ol	

5.3 Results and discussion

5.3.1 Comparison of synthetic and real grape must fermentation and aroma production

5.3.1.1 Grape must chemical properties and amino acid composition

The chemical characteristics of the three fermentation media used in this study were broadly similar. **Table 3** shows the initial sugar concentration, initial pH and total acidity of the grape musts, which were all within the same range. The contribution of each amino acid towards the total nitrogen concentration before and after amino acid addition is indicated in **Table 1**. White grape must contained a total of 255 mg N/l, and red grape must contained 270 mg N/l of amino nitrogen before treatment addition (calculated from the sum of all amino acids measured). Arginine and proline constituted the largest part of this amount, contributing 70% and 15% respectively to the amino nitrogen in both varieties (**Table 1**). Most of the amino acids, with the exception of arginine, proline and alanine, were present in low amounts in the natural grape must, and their contribution to the final concentrations of amino acids after addition was below 15%.

Table 3 Basic chemical characterisation of the three grape musts used in this study.

Grape must	pH	Initial sugar (g/l)	Total acid (g/l)
Synthetic	3.39	209.22	5.70
White (Hanepoot)	3.50	197.86	5.66
Red (Pinotage)	3.43	198.29	5.61

5.3.1.2 Fermentation performance

Chemical parameters related to fermentation performance (residual glucose, residual fructose, glycerol and ethanol concentrations) are reported in **Table 4**. Fermentations performance was comparable in the two real grape musts. All fermentations in white grape must (<4 g/l residual sugar) and red grape must (<5 g/l residual sugar) completed to dryness. In synthetic grape must, few treatments reached this level of completion in the 21 days of fermentation. Sulfur-containing and non-utilised amino acid treatments were least successful, while fermentation completion in treatments supplemented with complete and non-preferred amino acid treatments was strain-dependent (CHAPTER 3).

Ethanol yields were consistent across treatments in all three strains, in relation to the amount of sugar consumed and glycerol produced. The reported ethanol values serve as a general indication of relative fermentation completion (together with residual sugars) and are not intended to represent absolute concentrations.

Generally, the fermentation supplemented only with ammonium displayed slightly higher levels of glycerol than treatments with complete, preferred or branched-chain and aromatic amino acids. This is in agreement with other studies where increased ammonium consumption correlated with increased acetate and glycerol levels (Beltran et al., 2005). The absence of amino acids requires their *de novo* synthesis from a sugar substrate and ammonium, which requires reoxidation of NADH with the subsequent formation of glycerol (Albers et al,. 1996). In synthetic grape must, and to a lesser extent in red grape must, fermentation with high levels of sulfur amino acids showed elevated levels of glycerol at the end of fermentation, which can probably be attributed to stress experienced by yeast strains in a high sugar environment when poor nitrogen sources are supplied (Hohmann et al., 2007). The same might apply for some of the yeast strains when the fermentation medium was supplemented with non-utilised or non-preferred amino acids.

In all three media and for all three strains, lower glycerol levels were observed for the branched-chain and aromatic and preferred amino acid treatments, which were also the two treatments leading to the most successful fermentation completion in synthetic grape must for all three strains.

Table 4 Chemical analysis of major fermentation products at the end of alcoholic fermentation by three yeast strains in synthetic, white and red grape musts.

Medium	Strain	Treatment	Glucose (g/l)	Fructose (g/l)	Ethanol (%v/v)	Glycerol (g/l)
Synthetic grape must	VIN13	Ammonium	1.77 ± 0.61	7.45 ± 0.02	11.21 ± 0.21	5.78 ± 0.07
		Complete	12.20 ± 2.72	36.22 ± 8.71	9.04 ± 0.68	5.65 ± 0.24
		Preferred	2.03 ± 0.64	9.29 ± 3.00	11.27 ± 0.25	5.01 ± 0.15
		Aromatic	1.85 ± 1.31	10.61 ± 6.67	10.50 ± 0.52	4.71 ± 0.29
		Sulfur	27.35 ± 11.67	53.30 ± 12.97	6.93 ± 1.50	8.97 ± 1.46
		Non-utilised	24.33 ± 2.54	50.01 ± 3.95	7.41 ± 0.38	6.95 ± 0.18
		Non-preferred	11.60 ± 6.85	31.66 ± 10.82	9.24 ± 1.07	5.67 ± 0.61
	NT50	Ammonium	3.23 ± 1.22	13.83 ± 7.28	10.72 ± 0.52	8.41 ± 0.18
		Complete	1.92 ± 0.58	12.11 ± 2.67	10.87 ± 0.21	7.44 ± 0.13
		Preferred	0.79 ± 0.18	1.91 ± 0.63	11.57 ± 0.06	6.91 ± 0.22
		Aromatic	0.48 ± 0.01	4.19 ± 1.53	10.79 ± 0.08	7.20 ± 0.47
		Sulfur	31.14 ± 21.22	54.44 ± 19.56	6.28 ± 2.37	12.31 ± 1.30
		Non-utilised	11.92 ± 2.52	34.11 ± 4.25	8.97 ± 0.37	7.38 ± 0.32
		Non-preferred	22.55 ± 4.57	52.96 ± 5.85	7.08 ± 0.81	7.21 ± 0.69
	FXL	Ammonium	3.00 ± 1.97	14.72 ± 7.03	10.74 ± 0.45	6.03 ± 0.04
		Complete	1.80 ± 0.28	11.94 ± 0.13	11.02 ± 0.06	5.10 ± 0.13
		Preferred	1.77 ± 0.08	10.78 ± 0.41	11.05 ± 0.04	5.01 ± 0.14
		Aromatic	1.16 ± 0.30	8.59 ± 3.17	10.57 ± 0.17	5.06 ± 0.35
		Sulfur	7.66 ± 0.00	28.44 ± 0.00	9.77 ± 0.00	6.83 ± 0.00
		Non-utilised	21.41 ± 0.47	51.93 ± 1.07	7.50 ± 0.06	6.39 ± 0.10
		Non-preferred	2.58 ± 0.21	7.01 ± 2.21	11.23 ± 0.09	7.98 ± 0.07
Red grape must	VIN13	Ammonium	0.05 ± 0.09	1.20 ± 0.16	11.09 ± 1.67	5.57 ± 0.78
		Complete	0.05 ± 0.09	1.50 ± 0.33	11.97 ± 2.40	4.93 ± 0.88
		Preferred	0.09 ± 0.08	1.67 ± 0.06	13.65 ± 0.59	5.61 ± 0.12
		Aromatic	0.01 ± 0.01	1.98 ± 0.16	13.20 ± 0.29	4.88 ± 0.22
		Sulfur	0.19 ± 0.05	2.74 ± 0.28	10.83 ± 1.63	6.05 ± 1.38
		Non-utilised	0.28 ± 0.10	2.03 ± 0.25	12.90 ± 1.65	5.68 ± 0.53
		Non-preferred	0.68 ± 0.04	4.51 ± 0.02	9.18 ± 0.05	5.23 ± 1.42
	NT50	Ammonium	0.14 ± 0.20	2.09 ± 1.22	13.30 ± 1.05	10.22 ± 0.92
		Complete	0.01 ± 0.02	1.25 ± 0.24	13.05 ± 1.35	9.18 ± 0.47
		Preferred	0.00 ± 0.00	1.15 ± 0.34	11.61 ± 2.00	7.87 ± 1.19

		Aromatic	0.00	±	0.00	1.37	±	0.20	12.57	±	0.76	8.17	±	0.56
		Sulfur	0.00	±	0.00	1.15	±	0.29	11.86	±	2.46	8.04	±	1.66
		Non-utilised	0.00	±	0.00	1.30	±	0.17	13.26	±	0.99	9.42	±	0.50
		Non-preferred	0.05	±	0.07	1.53	±	0.06	13.83	±	0.15	9.50	±	0.13
		Ammonium	0.55	±	0.12	3.00	±	1.05	14.17	±	0.11	6.55	±	1.11
		Complete	0.47	±	0.01	3.98	±	1.25	13.85	±	0.16	6.59	±	2.24
		Preferred	0.20	±	0.26	3.21	±	1.39	13.10	±	1.39	5.10	±	0.36
	FXL	Aromatic	0.13	±	0.18	2.66	±	1.00	12.70	±	1.24	5.28	±	0.59
		Sulfur	0.31	±	0.19	2.77	±	1.16	13.73	±	0.01	6.40	±	0.75
		Non-utilised	0.68	±	0.21	4.47	±	2.44	14.26	±	0.15	5.85	±	0.22
		Non-preferred	0.53	±	0.19	4.06	±	0.69	14.16	±	0.17	5.69	±	0.16
White grape must		Ammonium	0.11	±	0.04	1.83	±	0.42	11.38	±	0.50	4.97	±	0.12
		Complete	0.02	±	0.03	1.36	±	0.67	11.62	±	0.01	4.92	±	0.10
		Preferred	0.00	±	0.00	1.37	±	0.12	11.67	±	0.02	4.48	±	0.06
	VIN13	Aromatic	0.01	±	0.02	1.03	±	0.09	11.03	±	0.17	4.15	±	0.26
		Sulfur	0.08	±	0.07	0.93	±	0.10	11.02	±	0.05	4.93	±	0.19
		Non-utilised	0.19	±	0.11	1.63	±	0.30	11.62	±	0.12	4.83	±	0.09
		Non-preferred	0.03	±	0.05	1.70	±	0.35	11.63	±	0.04	4.42	±	0.02
		Ammonium	0.00	±	0.00	1.45	±	0.15	11.38	±	0.04	8.63	±	0.21
		Complete	0.00	±	0.00	1.67	±	0.60	11.29	±	0.03	8.24	±	0.18
		Preferred	0.00	±	0.00	1.31	±	0.12	11.37	±	0.14	7.61	±	0.14
	NT50	Aromatic	0.00	±	0.00	1.26	±	0.13	10.80	±	0.04	7.03	±	0.33
		Sulfur	0.00	±	0.00	1.01	±	0.07	10.87	±	0.23	7.65	±	0.09
		Non-utilised	0.00	±	0.00	1.34	±	0.11	11.55	±	0.06	7.89	±	0.19
		Non-preferred	0.00	±	0.00	1.53	±	0.07	11.71	±	0.41	7.85	±	0.13
		Ammonium	0.17	±	0.11	1.47	±	0.16	11.59	±	0.04	5.18	±	0.09
		Complete	0.06	±	0.10	1.57	±	0.36	11.49	±	0.01	4.99	±	0.18
		Preferred	0.06	±	0.07	1.75	±	0.13	11.50	±	0.09	4.54	±	0.03
	FXL	Aromatic	0.00	±	0.00	1.49	±	0.07	10.92	±	0.13	4.09	±	0.07
		Sulfur	0.02	±	0.04	1.47	±	0.17	10.94	±	0.04	4.95	±	0.17
		Non-utilised	0.15	±	0.05	2.56	±	0.27	11.49	±	0.12	4.89	±	0.04
		Non-preferred	0.11	±	0.06	2.61	±	0.36	11.42	±	0.13	4.75	±	0.06

5.3.1.3 Multivariate analysis

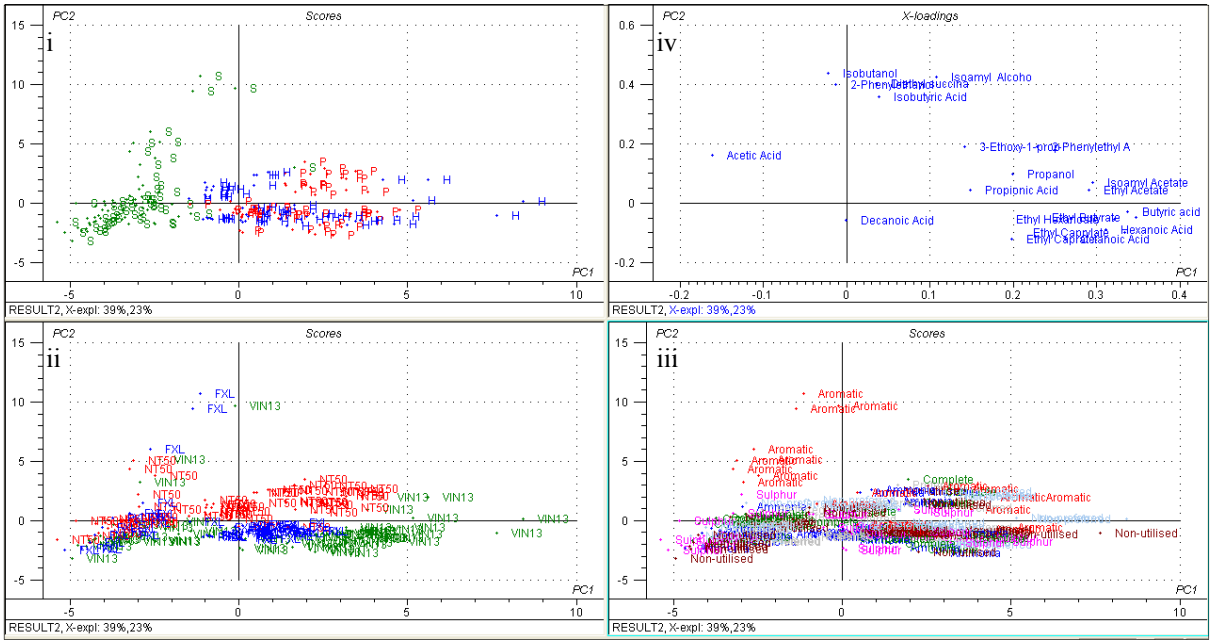
Principal component analysis (PCA) was performed on the dataset to evaluate which parameters most significantly impacted the overall structure of the data and to visualise volatile aroma profiles produced during fermentation.

The first principle component (PC), separates synthetic grape must from real grape must samples, with the majority of volatile aroma compounds positively correlated to real grape must samples (**Figure 1 A**). The first PC explains 39% of the variation in the data and shows that results obtained in synthetic and real grape musts are differently influenced by the variables in the dataset, but that white and red grape musts are similar in aroma compound composition. In the first PC there is prominent clustering of yeast strains in real grape must samples but not in synthetic grape must samples. Along the second PC (responsible for 23% of the variance), there is prominent discrimination of the branched-chain and aromatic amino acid treatment in synthetic medium. The positioning of variables in the loading plot suggests that this discrimination is strongly influenced by the volatile compounds associated with branched-chain and aromatic amino acids. The distinct clustering and separation of synthetic grape must samples, along with the grouping of yeast strains in real grape must samples obscure any further treatments effects in subsequent PCs. It therefore merits investigating the synthetic and real grape musts separately.

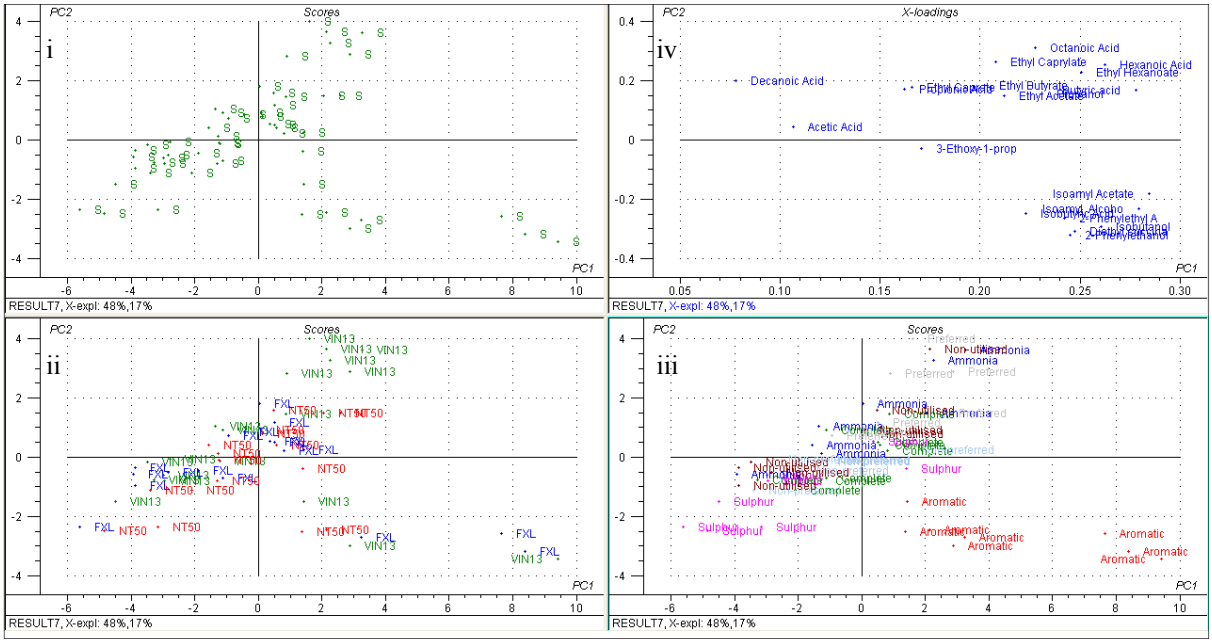
In synthetic grape must samples alone, 48% of the variance is explained by the first PC, which correlates the branched-chain and aromatic amino acid treatment samples with aroma compounds associated with branched-chain and aromatic amino acid metabolism (**Figure 1 B**) as also reported for a greater number of strains in CHAPTER 3. Other treatments (mainly ammonium and preferred amino acids) are positioned closely together in the scores plot and can be associated with high loadings for ethyl esters and fatty acids. Along subsequent PCs different treatments group together. Importantly, no discrete clustering of yeast strains is observed in synthetic medium.

In real grape must there is a very strong separation of the three yeast strains in first two PCs, together explaining 57% of the variation of the dataset (**Figure 1 C**). Treatments appear to cluster together for strains NT50 and VIN13 with a strong separation of aromatic and non-preferred amino acid treatments along the first PC in white grape must for VIN13. In the third PC, red and white grape musts are distinct (**Figure 1 D**). Certain amino acid treatments group together loosely in higher PCs, for example treatments containing aromatic and non-preferred amino acids in the third and fourth PCs (explaining 14 and 7% variance respectively) (**Figure 1 D**) and sulfur amino acid treatments in the sixth PC (explaining 4% variance) (**Figure 1 E**). Variables correlated with these treatments are: higher alcohols and diethyl succinate with aromatic and non-preferred amino acids (positive correlation) and propanol and 3-ethoxy-1-propanol with sulfur amino acids (negative correlation). However, prior knowledge facilitates assigning these correlations (CHAPTER 4).

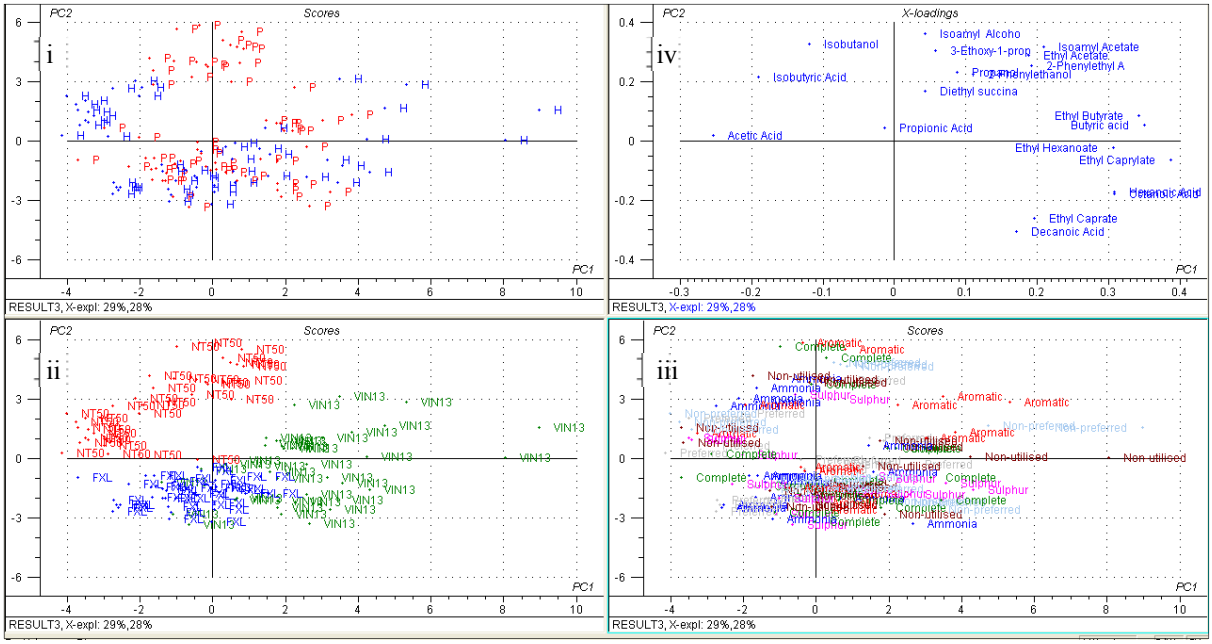
A



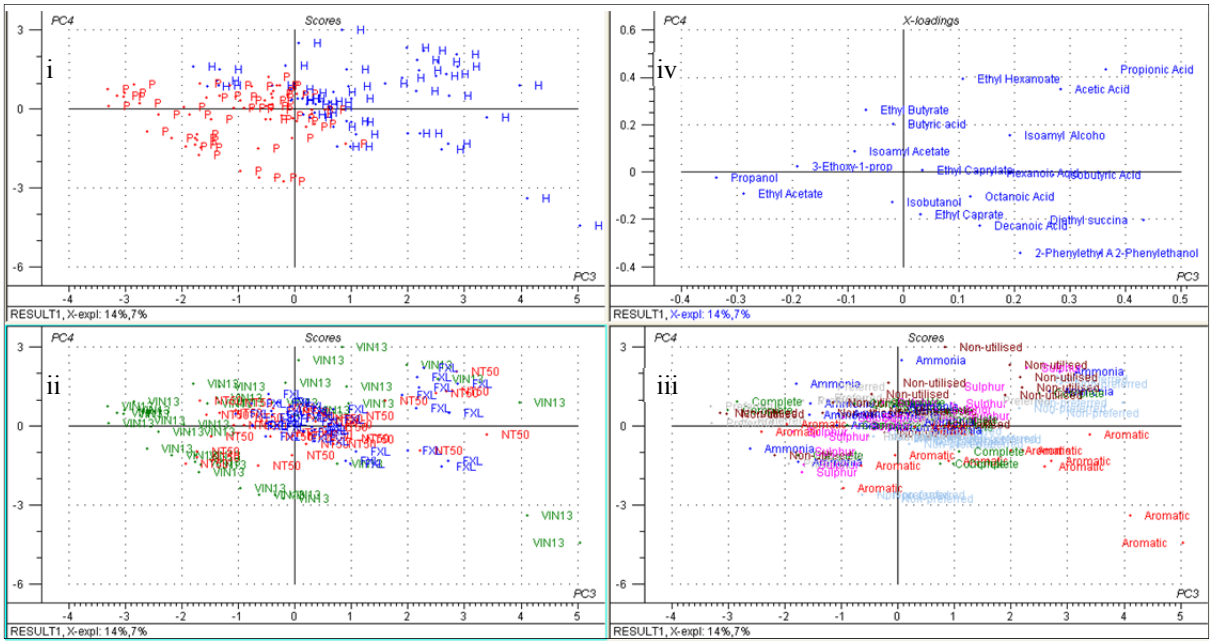
B



C



D



E

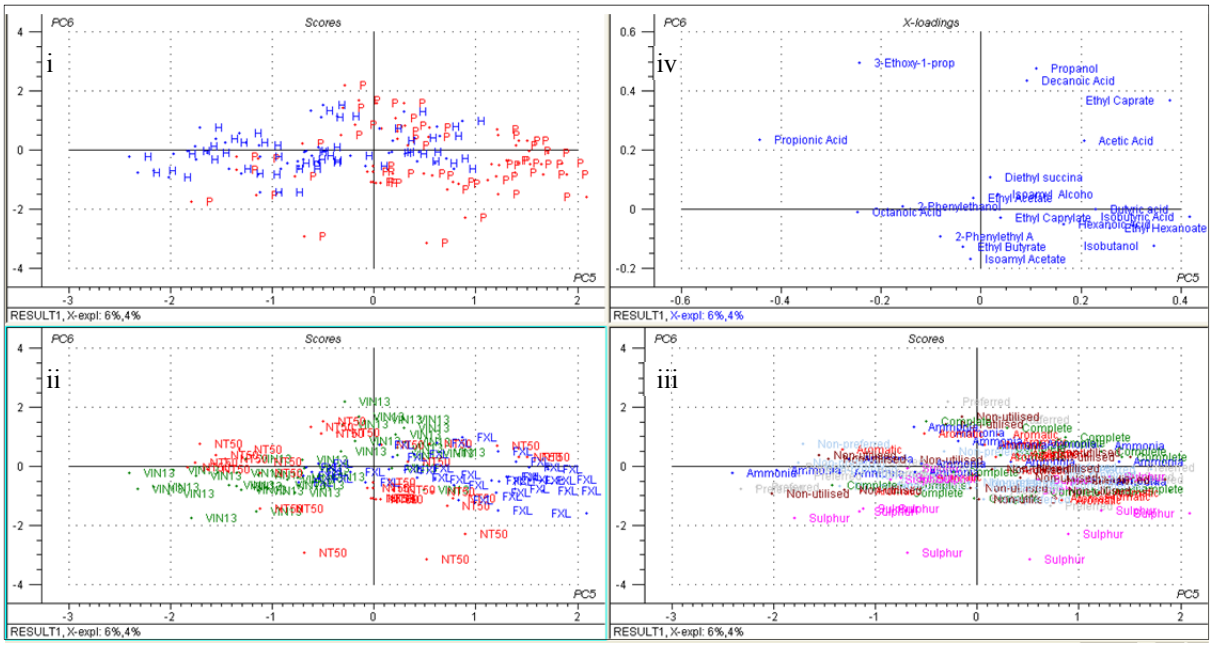


Figure 1 Principal component analysis of the end-point aroma compounds produced by three yeast strains in synthetic and real grape must subjected to different nitrogen treatments. Samples are sorted in the scores plots (indicated in colour) according to (i) fermentation medium, (ii) wine yeast strain and (iii) nitrogen treatment. The variables (aroma compounds) are shown on the loadings plot (iv). The principle components (PCs) investigated are PC 1 and PC 2 for all three fermentation media (A), PC1 and PC2 for synthetic grape must only (B), PC1 and PC 2 for real grape must (C), PC 3 and PC 4 for real grape must, and PC 5 and PC 6 for real grape must (E).

Many of the correlations in the dataset can be more specifically identified by looking at the average concentrations of metabolites produced by the three strains in the three fermentation media. In **Figure 2** aroma compounds are grouped into three compound classes (esters, higher alcohols and fatty acids) and the average across all treatments investigated (per strain and fermentation medium). Only compounds detected in all three media were included. The large standard deviations (error bars) account for the effect of the different nitrogen treatments. It can be seen that generally, synthetic grape must displayed the greatest treatment variation for all aroma compound classes, whereas the three strains produced similar average levels of volatile compounds in this matrix (**Figure 1 A**).

Strain discrimination in real grape must could be attributed to unique combinations of strain, treatment and grape must. For example, clustering of NT50 in the second PC (**Figure 1 C**) is most likely due to low levels of fatty acids produced by this strain, evident in the loadings plot and also in **Figure 2**. Fatty acids and their ethyl esters are strongly correlated in the first PC with VIN13 treatments in white grape must. The production of esters and fatty acids was also slightly lower in synthetic grape must than in real grape must. On average, higher alcohols were produced at the same concentrations in all three media. NT50,

showing the highest levels of higher alcohols in red grape must and slightly lower in white grape must, is positively correlated with these compounds along the second PC (**Figure 1 C and Figure 2**).

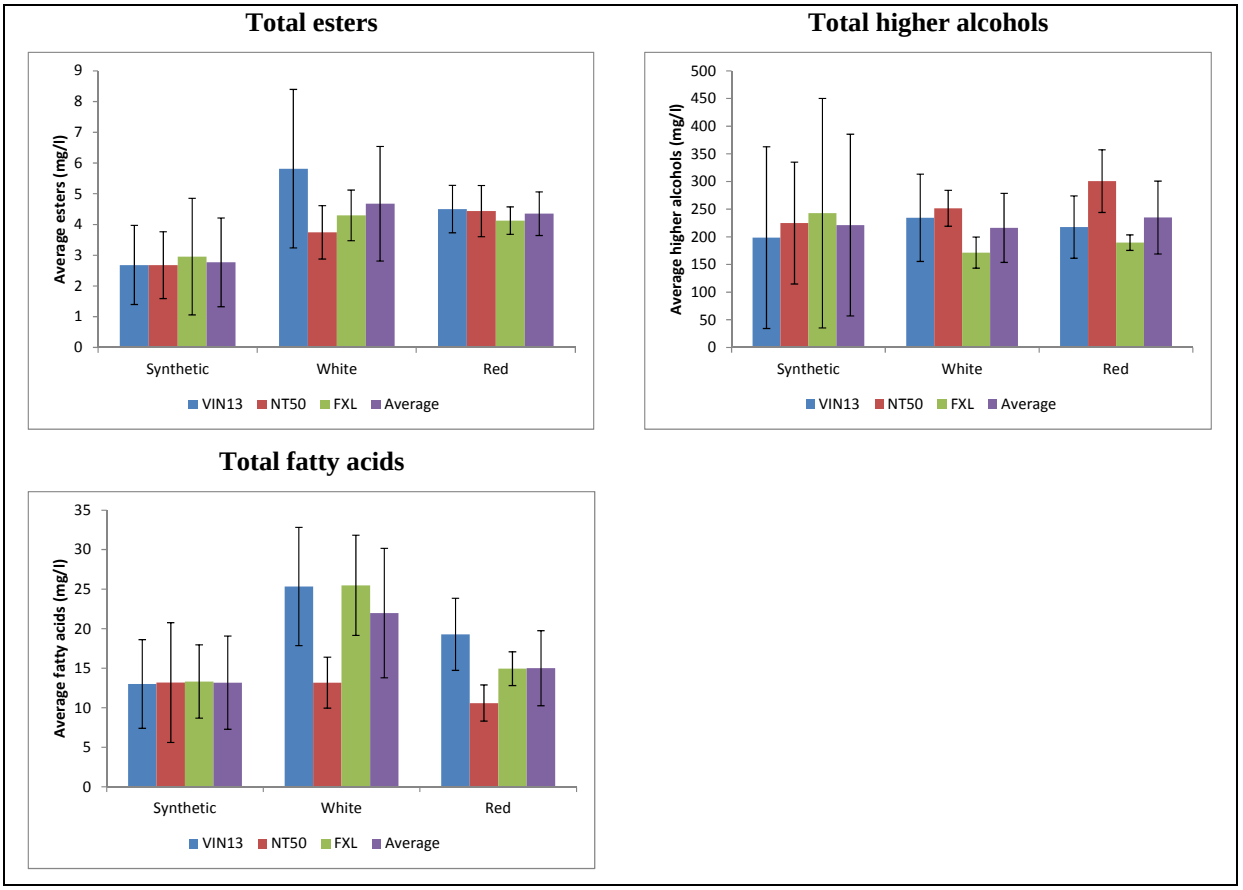


Figure 2 Aroma compound averages per strain and fermentation medium. Standard deviations account for the effect of the different nitrogen treatments.

While the amino acid composition of grape musts can account for a great portion of the variation observed in yeast-derived volatile aroma compound production, other studies that employed multivariate analysis of real grape must data have also shown that the genetic constitution of the particular strain can affect the metabolic profile of the wine more than nitrogen related variables in real grape must (Hernández-Orte et al., 2002; Hernández-Orte et al., 2005). Similar to our findings, Hernández-Orte et al. (2005) concluded that nitrogen supplementation (by amino acids or ammonium) can emphasise differential characteristics in aroma profiles for some but not all strains (different strains for different nitrogen sources). This strong interaction between yeast and nitrogen source is also evident in the present study and should be optimally exploited for optimal wine quality.

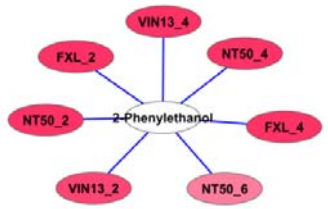
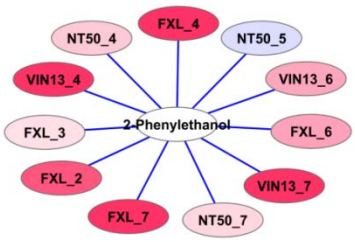
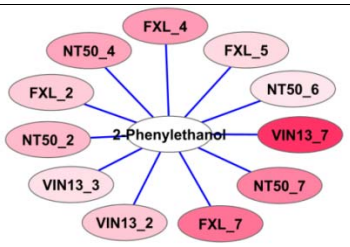
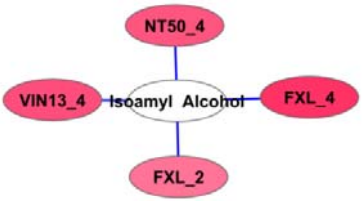
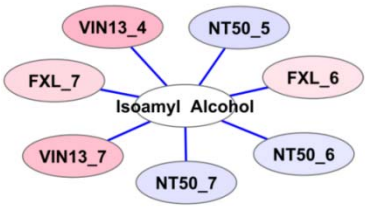
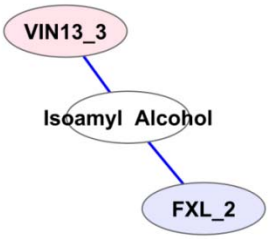
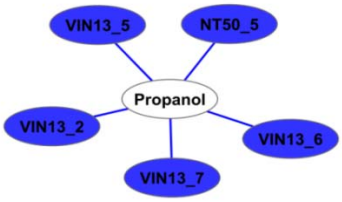
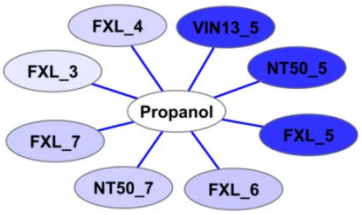
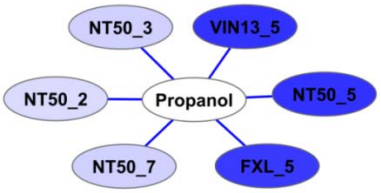
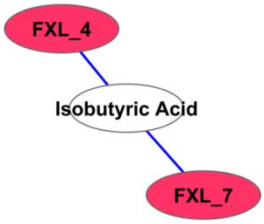
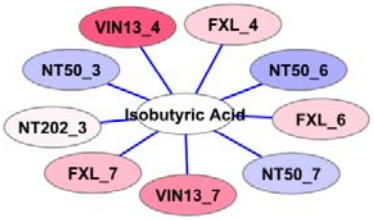
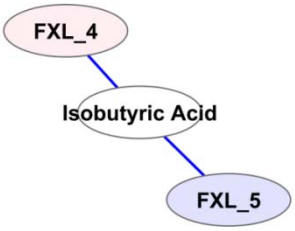
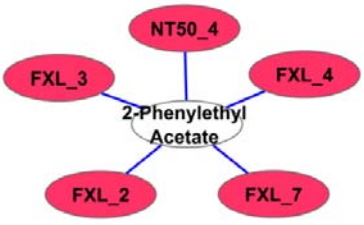
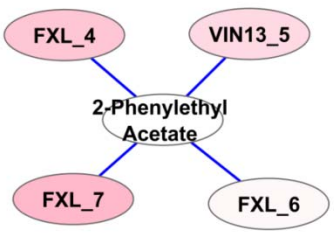
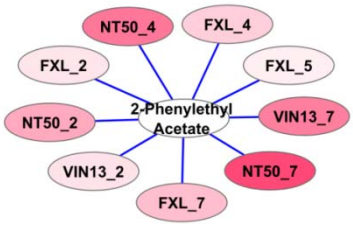
5.3.1.4 Conserved significant changes in aroma production due to treatment effects

The significant differences between amino acid treatments and the ammonium treatment for selected aroma compounds produced by the three yeast strains are shown in **Figure 3** for each of the three fermentation media. These selected compounds showed the most meaningful treatment effects in synthetic grape must (CHAPTER 4). The magnitude of the significant changes (shown by the colour intensity of the nodes on the figures) are generally less extreme in real grape must than in synthetic grape must, and a greater number of treatments show significant changes relative to ammonium. However, it is clear from the data that significant impacts caused by specific treatments on aroma compounds in synthetic grape must can be translated into a real wine matrix. The treatments that participated most are the branched-chain and aromatic amino acid treatment, and to a lesser extent the complete and non-preferred amino acid treatments (which contained lower concentrations of branched-chain and/or aromatic amino acids). The addition of sulfur-containing amino acids significantly impacted on the production of propanol (as well as related compounds propionic acid and 3-ethoxy-1-propanol). Metabolic pathways related to these significant changes are discussed in CHAPTER 4.

Amino nitrogen was added as nitrogen treatments at a concentration of 150 mg N/l, and together with the natural amino acid content of the grape must (255 mg N/l for white and 270 mg N/l for red grape must) the total amino nitrogen concentration would be approximately 400 mg N/l in real grape musts. However, not all of this nitrogen is equally accessible to the yeast, and approximately 24% of the amino nitrogen in the two real grape musts consisted of amino acids not utilised and/or not preferred during fermentation (according to our definition in **Table 1**). In addition, arginine is the most abundant “preferred" amino acid present in real grape must, but has an average ability to support growth and is subject to a degree of nitrogen catabolite repression (Hofman-Bang, 1999; Beltran et al., 2004) which means it may not be preferentially used by yeast when grape must is supplemented with more favourable sources of amino acids. The naturally occurring branched-chain and aromatic amino acids make only a small contribution in relation to those added in the form of nitrogen treatments. The amino acid treatment additions can therefore be expected to have a dominant and recognisable impact on the production of aromatic compounds and final aroma profiles of the fermentations.

Upon investigation of the results obtained by multivariate analysis of the data in isolation, it would appear that synthetic grape must does not suffice to represent a real winemaking environment, since different factors (treatment or strain) explain the main variation in the respective matrices. Importantly, in real grape must in our study the nitrogen was not limiting in treatments supplemented with less preferred amino acids, unlike in synthetic grape must. This could also justify the discrimination between real and synthetic grape must in a multivariate analysis. Even so, significant changes due to treatment are conserved for all yeast trains tested in all three fermentation media (**Figure 3**), proving synthetic grape must a valid system and representative environment to assess properties relevant to industrial fermentations. This is in line with previous studies that have shown that industrially relevant research can

be successfully translated from a synthetic wine medium to real grape must in systems biology studies (Rossouw & Bauer, 2009). Multi-factorial studies in synthetic grape must could serve to eliminate irregular variations in the data and prevent misinterpretation due to matrix complexity ("noise") of real grape must.

	Synthetic medium	White grape must	Red grape must
2-Phenylethanol			
Isoamyl alcohol			
Propanol			
Isobutyric acid			
2-Phenylethyl acetate			

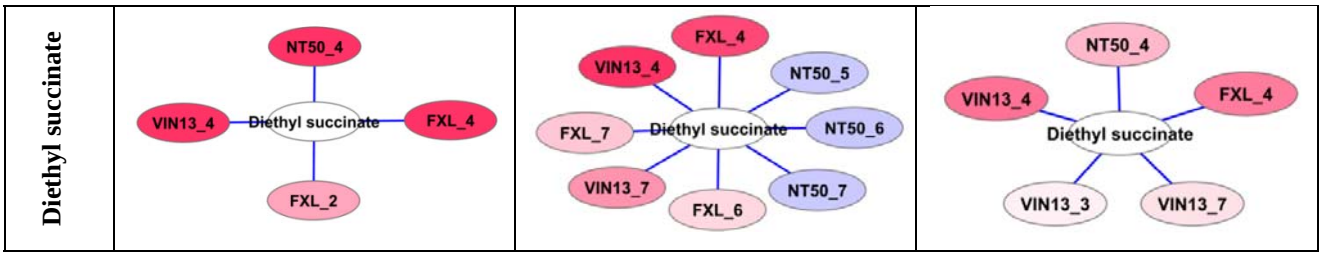


Figure 3 Significant differences in aroma compound production between nitrogen treatments and the ammonium treatment (designated control) for selected aroma compounds produced by the three yeast strains in the three fermentation media. Significant increases of amino acid treatments relative to the ammonium treatment are denoted by red nodes, and significant decreases by blue nodes. The colour intensity indicates the magnitude of the difference between treatment and control. Treatments are (2) complete amino acids, (3) preferred amino acids, (4) branched-chain and aromatic amino acids, (5) sulfur amino acids, (6) non-utilised amino acids, and (7) non-preferred amino acids.

5.3.2 Production of volatile aroma compounds in real grape must

Volatile compounds measured in white and red grape musts at the end of alcoholic fermentation were grouped together as listed in **Table 2 A**. Compounds that were below detection and/or quantification in grape must are listed in **Table 2 B**. **Figures 4 and 5** report the concentrations of total esters, total higher alcohols and total fatty acids for white and red grape musts respectively. Although individual yeast-derived aroma compounds can contribute significantly to perceived aroma and flavour of wine, the sum of related compounds provide a more realistic indication of the overall effect of nitrogen supplementation treatments on the flavour of the finished wine, due to the complex interactions and synergism between related compounds (Miller et al., 2007; Pineau et al., 2008).

Similar total concentrations of esters were produced by the three yeast strains in both grape musts, with the exception of a few treatment and strain combinations. For both grape cultivars and all three yeast strains, the branched-chain and aromatic amino acid treatment displayed higher levels of total esters produced than most other treatments. Exceptionally high levels of esters were produced by VIN13 in white grape must supplemented with aromatic and non-preferred amino acid treatments, which correlates with the PCA result observed in **Figure 1 C**. This finding is of importance since esters, having low perception thresholds are regarded as potent odorants that can significantly contribute to the sensory profile of wine even at low concentrations. Yeast strain, rather than nitrogen addition, has been reported as the more important variable determining the degree of ester formation, with individual esters correlating to specific yeast strains (Antonelli et al., 1999; Miller et al., 2007). Studies in real grape must have attributed ester formation mainly to mechanisms other than amino acid degradation, by observing that ammonium supplementation yields higher esters than amino acid addition (Miller et al., 2007). Our study clearly shows that the mechanism of ester formation is strain and grape must dependent, with NT50

favouring *de novo* production (elevated ester concentrations in the ammonium treatment) while the presence of the catabolic route is evident for VIN13 in white grape must.

In comparison to esters, higher alcohols have high perception thresholds and will impact on the fermentation bouquet only with significant alterations or variations in concentration (Ugliano et al., 2007; Pineau et al., 2009). For all three strains, slightly higher total concentrations were produced in red grape must relative to white grape must. Higher alcohols showed a number of treatment effects corresponding to ester production, for example significantly increased concentrations in aromatic and non-preferred treatments fermented by VIN13 and the ammonium treatment by NT50 in white grape must. Generally FXL showed little variation between treatments in its total higher alcohol production, as also shown in **Figure 3**.

The most consistent result for higher alcohol production in both grape musts for VIN13 and NT50 was the significant reduction in total higher alcohols observed in the sulfur amino acid treatment and complete amino acid treatment (also containing sulfur amino acids), due to the lower concentrations of propanol formed in these treatments. Propanol production is reduced in the presence of the sulfur-containing amino acid, methionine, which inhibits the formation of threonine, the direct precursor for propanol production (Giudici et al., 1993). The production of propanol is largely influenced by the genetic makeup of the strain performing fermentation (Giudici et al., 1993; Antonelli et al., 1999; Carrau et al., 2008 and CHAPTER 4). In synthetic grape must, VIN13 was found to be a high producer of propanol, while FXL produced relatively low amounts when supplemented with all but sulfur-containing amino acid combinations (CHAPTER 3).

No consistent pattern for nitrogen treatment could be observed for total fatty acid production in the two grape musts. Total concentrations were lower in red grape must than in white grape must, with strain NT50 generally producing lower amounts than the other two strains (also see **Figure 2**).

Total quantities of esters, higher alcohols and fatty acids in this study (**Figures 4 and 5**) are comparable to other totals reported in the literature (Torrea et al., 2003; Garde-Cerdán & Ancín-Azpilicueta 2008). Generally, studies exclude ethyl acetate and acetic acid from these reported totals as they impart distinct sensory qualities to the wines and are present in much greater quantities than other acetate esters or fatty acids respectively (Torrea et al., 2003). Together, acetic acid and ethyl acetate constitute the principal contributors to perceived volatile acidity (VA) (Hernández-Orte et al., 2006b), which could be detrimental to wine quality at elevated concentrations. In this study, strain differences in VA production were apparent, with VIN13 producing consistent low concentrations and NT50 producing the highest concentrations of VA of the three strains (particularly for ammonium and complete amino acid treatments). This result is also reflected in **Figure 1 C**, with NT50 positively correlated to acetic acid in the first PC and with ethyl acetate in the second PC. Interestingly, FXL is positively correlated with acetic acid, but negatively correlated with ethyl acetate, which is also evident in the quantitative values reported in **Figures 4 and 5**. It seems that the lowest overall concentrations of VA compounds were measured in

preferred (NT50) and aromatic (FXL) amino acid treatments, depending strongly on the yeast strain. The present study confirms previous work which showed that acetic acid production depends on yeast strain (Vilanova et al., 2007) and varies with nitrogen supplementation with regards to source and amount of nitrogen (Albers et al., 1996; Torrea et al., 2003; Beltran et al., 2005; Hernández-Orte et al., 2006a; Vilanova et al., 2007).

5.4 Conclusions

In this work, the impact of nitrogen supplementation on aroma production by different yeast strains in synthetic grape must was confirmed in real grape must. Multivariate analysis of the aroma datasets shows that different factors (treatment or strain) had the most significant impact on the aroma production in the respective matrices. Despite the complexity of real grape must potentially complicating the replication of results from a simple defined medium into a real wine, significant impacts on aroma production attributable to nitrogen treatments were conserved in all fermentation media (synthetic, white and red grape must). Moreover, aroma compounds were produced in comparable amounts in all three fermentation media. The results of this study confirm previous findings that have demonstrated the strong interaction between yeast strain and nitrogen source in determining aroma profile characteristics. The production pattern of individual and classes of aroma compounds also confirm that the proposed pathways of aroma compound production described in CHAPTER 4 are conserved in real grape must.

The integration of synthetic and real grape must datasets are the first steps towards providing predictive models that will allow the winemaker to match specific nitrogen supplementation regimes with specific yeast strains. Such “tailor-made” fermentation strategies will allow the manipulation of aroma profiles of individual wines towards desired flavour outcomes; and reduce the risk of incomplete fermentation and unwanted flavour production due to injudicious nitrogen supplementation practices and yeast strain incompatibilities.

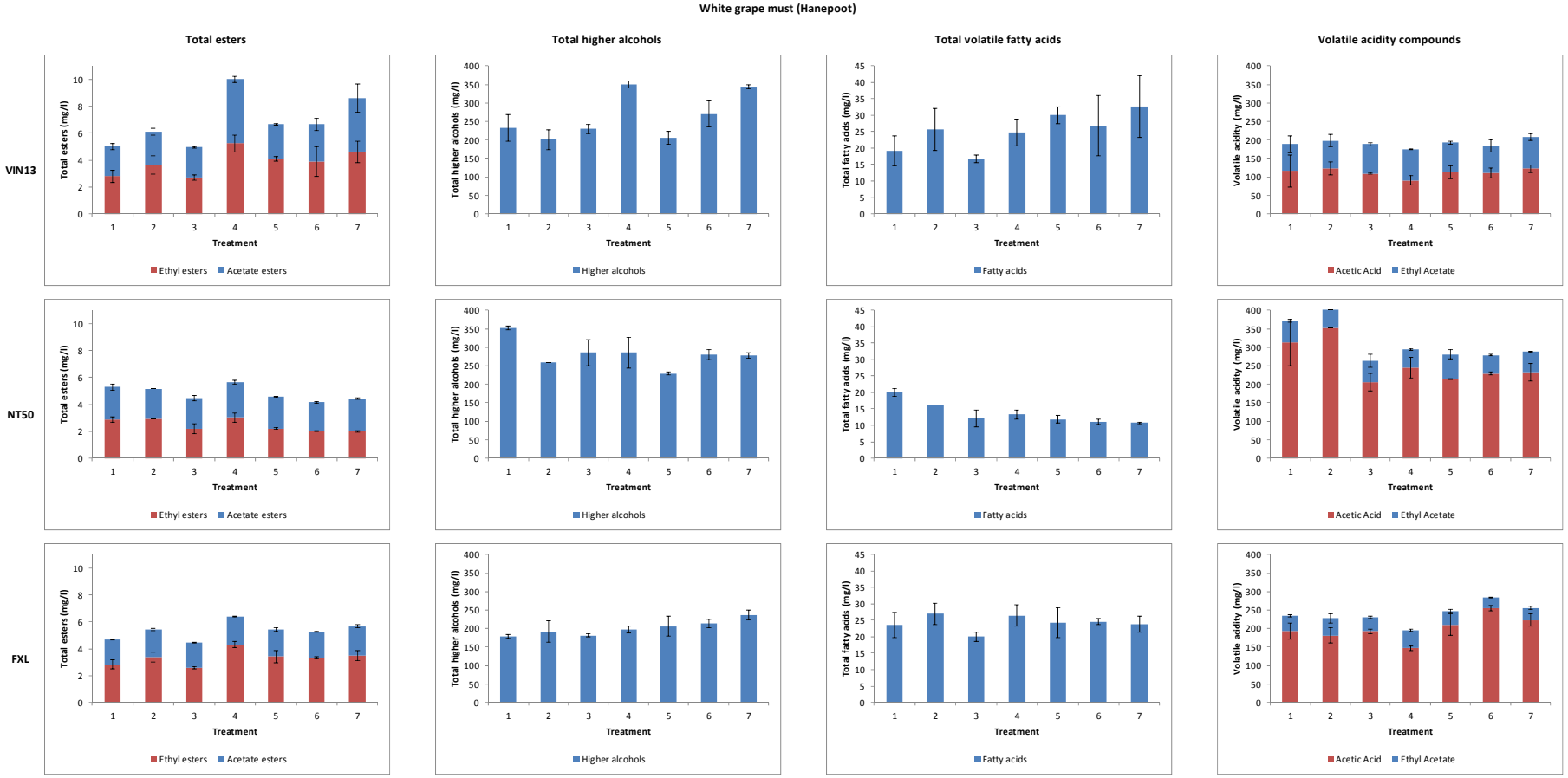


Figure 4 Total esters, total higher alcohols and total fatty acids produced by three yeast strains in white grape must. Treatments are (1) ammonium only, (2) complete amino acids, (3) preferred amino acids, (4) branched-chain and aromatic amino acids, (5) sulfur amino acids, (6) non-utilised amino acids, (7) non-preferred amino acids. Error bars indicate the standard deviation of three treatment replicates.

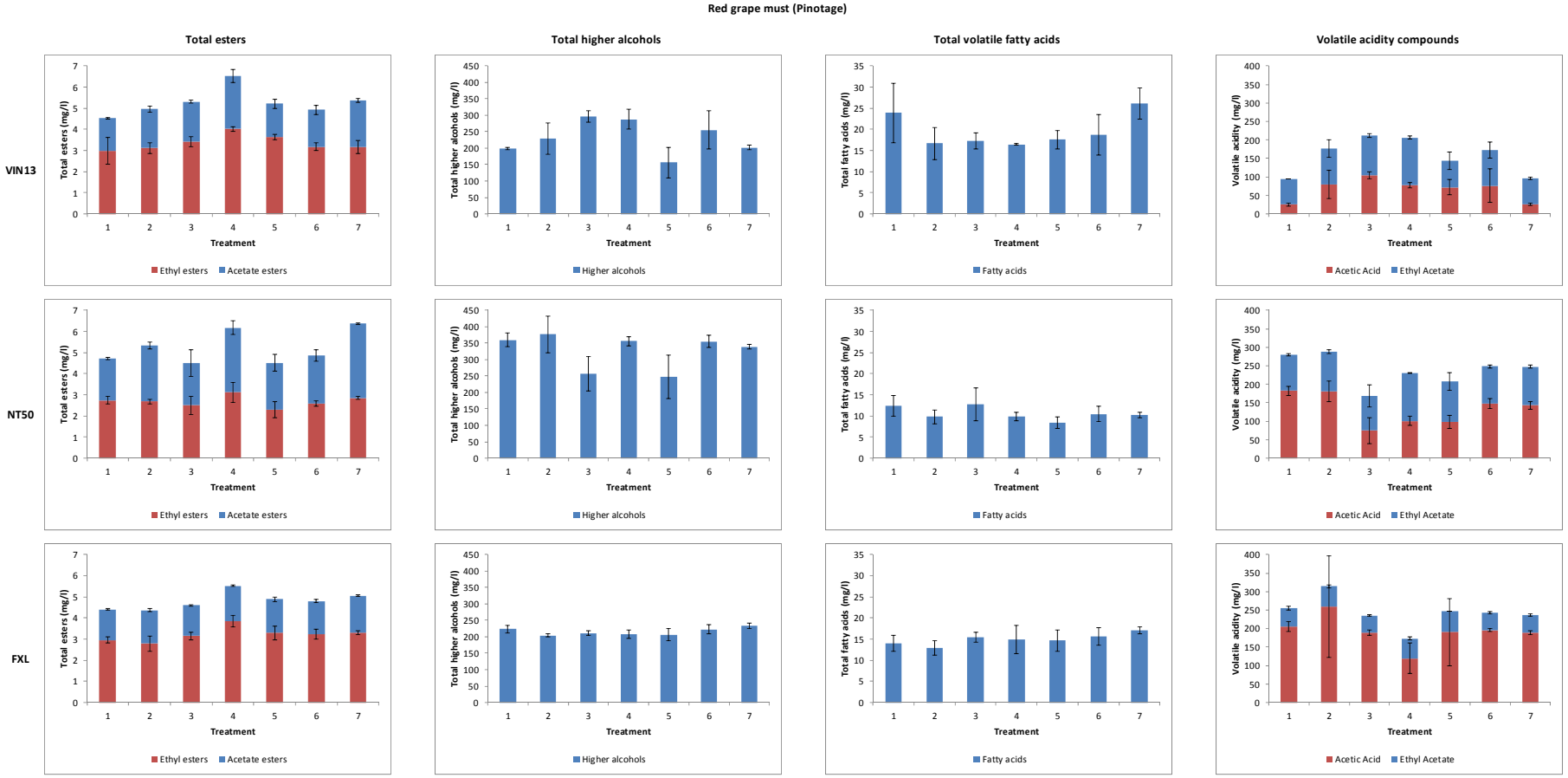


Figure 5 Total esters, total higher alcohols and total fatty acids produced by three yeast strains in red grape must. Treatments are (1) ammonium only, (2) complete amino acids, (3) preferred amino acids, (4) branched-chain and aromatic amino acids, (5) sulfur amino acids, (6) non-utilised amino acids, (7) non-preferred amino acids. Error bars indicate the standard deviation of three treatment replicates.

References

Albers, E., Larsson, C., Lidén, G., Niklasson, C., & Gustafsson, L. (1996). Influence of the nitrogen source on *Saccharomyces cerevisiae* anaerobic growth and product formation. *Applied and Environmental Microbiology*, *62*(9), 3187–95.

Antonelli, A., Castellari, L., Zambonelli, C., & Carnacini, A. (1999). Yeast influence on volatile composition of wines. *Journal of Agricultural and Food Chemistry*, *47*(3), 1139–44.

Barbosa, C., Falco, V., Mendes-Faia, A., & Mendes-Ferreira, A. (2009). Nitrogen addition influences formation of aroma compounds, volatile acidity and ethanol in nitrogen deficient media fermented by *Saccharomyces cerevisiae* wine strains. *Journal of Bioscience and Bioengineering*, *108*(2), 99–104.

Beltran, G., Esteve-Zarzoso, B., Rozès, N., Mas, A., & Guillamón, J. M. (2005). Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and nitrogen consumption. *Journal of Agricultural and Food Chemistry*, *53*(4), 996–1002.

Beltran, G., Novo, M., Rozès, N., Mas, A., & Guillamón, J. M. (2004). Nitrogen catabolite repression in *Saccharomyces cerevisiae* during wine fermentations. *FEMS Yeast Research*, *4*(6), 625–32.

Carrau, F. M., Medina, K., Farina, L., Boido, E., Henschke, P. A., & Dellacassa, E. (2008). Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: Effects of yeast assimilable nitrogen on two model strains. *FEMS Yeast Research*, *8*(7), 1196–207.

Fairbairn, S. C., (2012). Stress, fermentation performance and aroma production by yeast. MSc Thesis. Stellenbosch University. Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Ferreira, V., López, R., & Cacho, J. (2000). Quantitative determination of the odorants of young red wines from different grape varieties. *Journal of the Science of Food and Agriculture*, *80*, 1659–1667.

Garde-Cerdán, T., & Ancín-Azpilicueta, C. (2008). Effect of the addition of different quantities of amino acids to nitrogen-deficient must on the formation of esters, alcohols, and acids during wine alcoholic fermentation. *LWT - Food Science and Technology*, *41*(3), 501–510.

Giudici, P., Zamponelli, C., & Kunkee, R. E. (1993). Increased production of n-propanol in wine by yeast strains having an impaired ability to form hydrogen sulfide. *American Journal of Enology and Viticulture*, *44*(1), 17–21.

Henschke, P.A and Jiranek, V., (1993). Yeasts – Metabolism of nitrogen compounds. In: Wine Microbiology and Biotechnology. Fleet, G. H. (Ed). Harwood Academic Publishers, Chur, Switzerland, pp. 77–164.

Hernández-Orte, P, Ibarz, M., Cacho, J., & Ferreira, V. (2005). Effect of the addition of ammonium and amino acids to musts of Airen variety on aromatic composition and sensory properties of the obtained wine. *Food Chemistry*, *89*(2), 163–174.

Hernández-Orte, P., Bely, M., Cacho, J., & Ferreira, V. (2006a). Impact of ammonium additions on volatile acidity, ethanol, and aromatic compound production by different *Saccharomyces cerevisiae* strains during fermentation in controlled synthetic media. *Australian Journal of Grape and Wine Research*, *12*(2), 150–160.

Hernández-Orte, P., Ibarz, M. J., Cacho, J., & Ferreira, V. (2006b). Addition of amino acids to grape juice of the Merlot variety: Effect on amino acid uptake and aroma generation during alcoholic fermentation. *Food Chemistry*, *98*(2), 300–310.

Hernández-Orte, P., Cacho, J. F., & Ferreira, V. (2002). Relationship between varietal amino acid profile of grapes and wine aromatic composition. Experiments with model solutions and chemometric study. *Journal of Agricultural and Food Chemistry*, *50*(10), 2891–9.

Hofman-Bang, J. (1999). Nitrogen Catabolite Repression in *Saccharomyces cerevisiae*. *Molecular Biotechnology*, *12*, 35–73.

Hohmann, S., Krantz, M., & Nordlander, B. (2007). Yeast osmoregulation. *Methods in Enzymology*, *428*(07), 29–45.

Louw, L., Roux, K., Tredoux, A., Tomic, O., Naes, T., Nieuwoudt, H. H., & Van Rensburg, P. (2009). Characterization of selected South African young cultivar wines using FTMIR spectroscopy, gas chromatography, and multivariate data analysis. *Journal of Agricultural and Food Chemistry*, *57*(7), 2623–32.

Miller, A., Wolff, S., Bisson, L., & Ebeler, S. (2007). Yeast strain and nitrogen supplementation: Dynamics of volatile ester production in Chardonnay juice fermentations. *American Journal of Enology and Viticulture*, *58*(4), 470–483.

Moreira, N., De Pinho, P. G., Santos, C., & Vasconcelos, I. (2011). Relationship between nitrogen content in grapes and volatiles, namely heavy sulphur compounds, in wines. *Food Chemistry*, *126*(4), 1599–1607.

Nieuwoudt, H. H., Prior, B. A, Pretorius, I. S., Manley, M., & Bauer, F. F. (2004). Principal component analysis applied to Fourier transform infrared spectroscopy for the design of calibration sets for glycerol prediction models in wine and for the detection and classification of outlier samples. *Journal of Agricultural and Food Chemistry*, *52*(12), 3726–35.

Pineau, B., Barbe, J.-C., Van Leeuwen, C., & Dubourdieu, D. (2009). Examples of perceptive interactions involved in specific "red-" and "black-berry" aromas in red wines. *Journal of Agricultural and Food Chemistry*, *57*(9), 3702–8.

Rossouw, D., & Bauer, F. F. (2009). Comparing the transcriptomes of wine yeast strains: Toward understanding the interaction between environment and transcriptome during fermentation. *Applied Microbiology and Biotechnology*, *84*(5), 937–54.

Rossouw, D., Olivares-Hernandes, R., Nielsen, J., & Bauer, F. F. (2009). Comparative transcriptomic approach to investigate differences in wine yeast physiology and metabolism during fermentation. *Applied and Environmental Microbiology*, *75*(20), 6600–12.

Torrea, D, Fraile, P., Garde, T., & Ancín, C. (2003). Production of volatile compounds in the fermentation of chardonnay musts inoculated with two strains of *Saccharomyces cerevisiae* with different nitrogen demands. *Food Control*, *14*(8), 565–571.

Torrea, D, Varela, C., Ugliano, M., Ancin-Azpilicueta, C., Leigh Francis, I., & Henschke, P. A. (2011). Comparison of inorganic and organic nitrogen supplementation of grape juice – Effect on volatile composition and aroma profile of a Chardonnay wine fermented with *Saccharomyces cerevisiae* yeast. *Food Chemistry*, *127*(3), 1072–1083.

Ugliano, M., Henschke, P. A., Herderich, M., & Pretorius, I. S. (2007). Nitrogen management is critical for wine flavour and style. *Wine Industry Journal*, *22*(6), 24–30.

Vilanova, M, Ugliano, M., Varela, C., Siebert, T., Pretorius, I. S., & Henschke, P. A. (2007). Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by *Saccharomyces cerevisiae* wine yeasts. *Applied Microbiology and Biotechnology*, *77*(1), 145–57.

Vilanova, M., Siebert, T. E., Varela, C., Pretorius, I. S., & Henschke, P. A. (2012). Effect of ammonium nitrogen supplementation of grape juice on wine volatiles and non-volatiles composition of the aromatic grape variety Albariño. *Food Chemistry*, *133*(1), 124–131.

Chapter 6

General discussion and conclusions

CHAPTER 6

General discussion and conclusions

The principal goal of this project was to investigate the impacts of different nitrogen treatments added to synthetic and real grape must at two different time points on the fermentation performance and aroma profiles of multiple commercial wine yeast strains. Ultimately, the study aims to increase our knowledge and understanding of the complex interactions between grape must composition, yeast genetic background, nutritional (in particular nitrogen) requirements of wine yeast strains and environmental or winemaking factors.

In recent years, research efforts around the world have been aligned to examine the influence of nitrogen nutrition on fermentation performance and aroma production. Studies have focussed on the impacts of adding increasing concentrations of yeast assimilable nitrogen (YAN) to grape musts (Hernández-Orte et al., 2006; Vilanova et al., 2007; Carrau et al., 2008); the use of inorganic nitrogen, usually in the form of diammonium phosphate (DAP), compared to organic nitrogen (complex and complete sources of amino acids) (Beltran et al., 2005; Hernández-Orte et al., 2005; Jiménez-Martí et al., 2007; Garde-Cerdán & Ancín-Azpilicueta, 2008); and nitrogen addition at different times before and during fermentation (Barbosa et al., 2009). While all of these studies give invaluable insights into nitrogen regulation and its relationship with aroma production via catabolic and anabolic pathways, none provide a comprehensive screening of all contributing factors using multiple wine yeast strains. In this project we sought to begin to address this limitation by initiating a large exploratory experiment to provide an overview of the most important factors that influence fermentation performance and aroma production, covering a range of nitrogen conditions.

We hypothesised that particular combinations of nitrogen sources would cause different responses in primary fermentation performance as well as secondary metabolite production. Amino acids grouped together in this study are related in their capacity to support growth and be used as nitrogen source by *Saccharomyces cerevisiae* (ammonium only, a complete source of amino acids, preferred amino acids, non-preferred amino acids, non-utilised amino acids) (as reviewed by Hofman-Bang, 1999; Ter Schure et al., 2000; Magasanik & Kaiser, 2002), or would potentially participate in specific pathways of aroma compound production (sulfur-containing amino acids, branched-chain and aromatic amino acids) (reviewed by Lambrechts & Pretorius, 2000; Swiegers & Pretorius, 2005; Styger et al., 2011). Generating data from such nitrogen combinations could in future provide insights to design commercial nutrient formulations containing specific ratios of amino acids to modulate yeast-derived aroma production according to desired outcomes such as wine style.

Datasets were generated in three fermentation matrices. Fermentations ran to completion in red and white (real) grape must in the 21 day time frame, whereas in synthetic grape must the performances of individual yeast strains were differentially impacted by nitrogen treatments and timing of addition. Aroma

compounds were produced in comparable amounts in synthetic and real grape musts. Importantly, significant nitrogen treatment effects observed in synthetic grape must were repeatable in real grape must. In real grape must, a greater number of amino acid treatments showed significant differences relative to the ammonium treatment in aroma compound concentrations, but the intensity of the significant differences was less than in synthetic grape must. This could potentially be attributed to the presence of naturally occurring amino acids and other immeasurable irregularities or "noise". Therefore, in this study we could confirm previous work (Rossouw & Bauer, 2009) indicating that chemically defined grape must provides a representative wine-like environment to assess industrial fermentation properties in multi-factorial studies, in this case nitrogen utilisation and aroma compound production by wine yeast.

In this study, yeast strain differentiation in terms of aroma profiles was more prominent in real grape must than in synthetic grape must. However, in real and synthetic matrices, the employment of different yeast strains for fermentation significantly impacted on aroma compound production. The production of specific aroma compounds and composite aroma profiles were found to be highly dependent on (formerly classified) yeast subspecies (*S. cerevisiae bayanus* and *S. cerevisiae cerevisiae*) and individual strains tested in this study, even though many of the significant findings of this study were conserved for different yeasts.

In general, the preferred amino acid treatment resulted in the most complete fermentations for multiple strains and both time points of addition in synthetic grape must. Somewhat surprising was that the branched-chain and aromatic amino acid treatment generally supported fermentation completion to a similar degree; unlike previously reported for this class of amino acids (Watson, 1976; Boer et al., 2007). Besides consistently supporting good fermentation performance, these two treatments (for all three media and strains assessed) also led to lower formation of glycerol than other nitrogen treatments, possibly indicating lower metabolic stress and/or alternative pathways of redox balancing than via the glycerol production pathway, for example via the production of aroma compounds such as higher alcohols (Hohmann et al., 2007; Jain et al., 2011).

Aroma compounds could be broadly grouped into those that were influenced most by nitrogen treatments, and those that were influenced more by other factors, such as yeast strain or stress conditions (including nitrogen limitation). Stressed treatments, such as the sulfur amino acid and non-utilised amino acid treatments, displayed subdued aroma profiles; probably attributable to overall lower metabolic activity. The two treatments comprised of amino acids associated with specific aroma production pathways (sulfur amino acids and branched-chain and aromatic amino acids) resulted in the greatest production of "treatment dependent" aroma compounds. Moreover, a number of interesting associations between these nitrogen treatments and seemingly unrelated compounds came to the fore; the reasons for which should be investigated in more detail in future studies.

The most distinguishing impact on aroma production was caused by the presence of branched-chain and aromatic amino acids; especially in the branched-chain and aromatic amino acid treatment, but also

present in lower concentrations in the complete and non-preferred amino acid treatments. The branched-chain and aromatic amino acids are direct precursors in the catabolic formation of higher alcohols, fatty acids and esters via the Ehrlich pathway; but these aroma compounds can also be produced anabolically from a sugar substrate. Our results indicate the possibility that higher alcohols associated with branched-chain amino acids (such as isoamyl alcohol and isobutanol) are more likely produced via the anabolic route, while higher alcohols associated with aromatic amino acids (2-phenylethanol) are probably formed more readily via the catabolic route. Also, high concentrations of diethyl succinate were formed in this nitrogen treatment, and could potentially be linked to the presence of excessive amounts of branched-chain and aromatic amino acid catabolic intermediates fed into the methyl citric acid (MCA) cycle after other cellular needs have been met (including higher alcohol production).

The sulfur-containing amino acid treatment most notably impacted (decreased) specific compounds under the metabolic control of methionine, namely propanol, propionic acid and 3-ethoxy-1-propanol. In addition, sulfur amino acids could be exclusively linked with the production of a compound (ethyl-2-methylbutyrate), which was not detected in any other treatment.

In comparison to nitrogen treatment and yeast strain, the timing of nitrogen addition had a less prominent, yet significant impact. For example, the addition of the branched-chain and aromatic amino acid treatment after the onset of fermentation (thus following a period of nitrogen limitation) resulted in a greater number of significant changes relative to the ammonium treatment than with initial addition, and higher final concentrations of associated aroma compounds (in line with previous findings described in Lambrechts & Pretorius, 2000). Examining which treatments (excluding the branched-chain and aromatic treatment) resulted in the greatest production of higher alcohols and related compounds for each addition time point, we can postulate that the anabolic pathway of aroma compound formation was more important when nitrogen additions were made to the initial fermentation medium. In this regard, higher concentrations of aroma compounds were produced in the ammonium and preferred amino acid treatments (containing no direct precursors) than in the complete amino acid treatment (containing direct precursors). The opposite tendency was observed when additions were made after the onset of fermentation, suggesting the greater formation of aroma compounds via the catabolic route. Within a treatment, the effect of addition time on fermentation performance depended strongly on the yeast strain used. This interdependency was observed to different extents for all treatments.

The concentrations of individual amino acids could not be monitored throughout or at the end of fermentation in this study. Future work should include such quantitative measures to determine the extent of amino acid consumption by the yeast, as well as the role of specific amino acids within a treatment group.

While the scope of this study was more comprehensive than any of the published foregoing studies, it still falls far short of simultaneously addressing all intrinsic grape must factors (such as indigenous microorganisms, organic acids and phenolic compounds), other nutrients (such as vitamins and minerals),

and environmental or winemaking parameters (including pH, temperature and initial sugar) that could affect fermentation performance and aroma production interactively. The total nutrient composition, and not only nitrogen, should be considered in future experiments to optimise manipulation of aroma profiles, facilitate accurate diagnosis of problem fermentations (Bohlscheid et al. 2007) and to ensure the limitation of compounds harmful to human health such as ethyl carbamate, urea and heavy metals (Ough et al., 1988; Pohl et al. 2007).

Yeast-derived aroma compounds measured in this study (higher alcohols, esters and fatty acids) are known to influence wine aroma in combination and not only individually. In the present study, we did not consider the particular perception threshold or aroma contribution (associated flavour descriptors) of each individual compound, since the contributions of compounds to the overall aroma are easily overestimated due to complexity of wine (Pineau et al., 2009), and/or synergism and antagonism between related and unrelated compounds (Miller et al., 2007). For this reason, future studies should also link chemical analysis of aroma compounds to sensory data to determine the true organoleptic impact of multi-factorial interactions in a wine matrix.

To summarise, fermentation management directed at modulating aroma outcomes will require the generation of extensive datasets from experiments that consider all intrinsic and extrinsic fermentation factors. The results of this study provide a first step towards the characterisation of the complex interactions between a number of these factors (nitrogen source, timing of addition, yeast strain and fermentation matrix). The data show that combinations of nitrogen sources can lead to unexpected responses in metabolite production depending on the genetic background of individual strains and the timing of nutrient addition.

References

Barbosa, C., Falco, V., Mendes-Faia, A., & Mendes-Ferreira, A. (2009). Nitrogen addition influences formation of aroma compounds, volatile acidity and ethanol in nitrogen deficient media fermented by *Saccharomyces cerevisiae* wine strains. *Journal of Bioscience and Bioengineering, 108*(2), 99–104.

Beltran, G., Esteve-Zarzoso, B., Rozès, N., Mas, A., & Guillamón, J. M. (2005). Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and nitrogen consumption. *Journal of Agricultural and Food Chemistry, 53*(4), 996–1002.

Boer, V. M., Tai, S. L., Vuralhan, Z., Arifin, Y., Walsh, M. C., Piper, M. D. W., De Winde, J. H., Pronk, J. T., & Daran, J-M. (2007). Transcriptional responses of *Saccharomyces cerevisiae* to preferred and nonpreferred nitrogen sources in glucose-limited chemostat cultures. *FEMS Yeast Research, 7*(4), 604–20.

Bohlscheid, J. C., Fellman, J. K., Wang, X. D., Ansen, D., & Edwards, C. G. (2007). The influence of nitrogen and biotin interactions on the performance of *Saccharomyces* in alcoholic fermentations. *Journal of Applied Microbiology, 102*(2), 390–400.

Carrau, F. M., Medina, K., Farina, L., Boido, E., Henschke, P. A., & Dellacassa, E. (2008). Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: Effects of yeast assimilable nitrogen on two model strains. *FEMS Yeast Research, 8*(7), 1196–207.

Garde-Cerdán, T., & Ancín-Azpilicueta, C. (2008). Effect of the addition of different quantities of amino acids to nitrogen-deficient must on the formation of esters, alcohols, and acids during wine alcoholic fermentation. *LWT - Food Science and Technology, 41*(3), 501–510.

Hernández-Orte, P., Bely, M., Cacho, J., & Ferreira, V. (2006). Impact of ammonium additions on volatile acidity, ethanol, and aromatic compound production by different *Saccharomyces cerevisiae* strains during fermentation in controlled synthetic media. *Australian Journal of Grape and Wine Research, 12*(2), 150–160.

Hernández-Orte, P., Ibarz, M., Cacho, J., & Ferreira, V. (2005). Effect of the addition of ammonium and amino acids to musts of Airen variety on aromatic composition and sensory properties of the obtained wine. *Food Chemistry, 89*(2), 163–174.

Hofman-Bang, J. (1999). Nitrogen Catabolite Repression in *Saccharomyces cerevisiae*. *Molecular Biotechnology, 12*, 35–73.

Hohmann, S., Krantz, M., & Nordlander, B. (2007). Yeast osmoregulation. *Methods in Enzymology, 428*(07), 29–45.

Jain, V. K., Divol, B., Prior, B. A., & Bauer, F. F. (2012). Effect of alternative NAD^+-regenerating pathways on the formation of primary and secondary aroma compounds in a *Saccharomyces cerevisiae* glycerol-defective mutant. *Applied Microbiology and Biotechnology, 93*(1), 131–41.

Jiménez-Martí, E., Aranda, A., Mendes-Ferreira, A., Mendes-Faia, A., & Del Olmo, M. L. (2007). The nature of the nitrogen source added to nitrogen depleted vinifications conducted by a *Saccharomyces cerevisiae* strain in synthetic must affects gene expression and the levels of several volatile compounds. *Antonie van Leeuwenhoek, 92*(1), 61–75.

Lambrechts, M., & Pretorius, I. (2000). Yeast and its importance to wine aroma – A review. *South African Journal of Enology and Viticulture, 21*, 97–129.

Magasanik, B., & Kaiser, C. A. (2002). Nitrogen regulation in *Saccharomyces cerevisiae*. *Gene, 290*(1-2), 1–18.

Miller, A., Wolff, S., Bisson, L., & Ebeler, S. (2007). Yeast strain and nitrogen supplementation: Dynamics of volatile ester production in Chardonnay juice fermentations. *American Journal of Enology and Viticulture*, 58(4), 470–483.

Ough, C., Crowel, E., & Mooney, L. (1988). Formation of ethyl carbamate precursors during grape juice (Chardonnay) fermentation. I. Addition of amino acids, urea, and ammonia: Effects of fortification on intracellular and extracellular precursors. *American Journal of Enology and Viticulture, 39*(3), 243–249.

Pineau, B., Barbe, J.-C., Van Leeuwen, C., & Dubourdieu, D. (2009). Examples of perceptive interactions involved in specific “red-” and “black-berry” aromas in red wines. *Journal of Agricultural and Food Chemistry, 57*(9), 3702–8.

Pohl, P. (2007). What do metals tell us about wine? *TrAC Trends in Analytical Chemistry*, 26(9), 941–949.

Rossouw, D., & Bauer, F. F. (2009). Comparing the transcriptomes of wine yeast strains: Toward understanding the interaction between environment and transcriptome during fermentation. *Applied Microbiology and Biotechnology, 84*(5), 937–54.

Styger, G., Prior, B., & Bauer, F. F. (2011). Wine flavor and aroma. *Journal of Industrial Microbiology and Biotechnology, 38*(9), 1145–59.

Swiegers, J. H., & Pretorius, I. S. (2007). Modulation of volatile sulfur compounds by wine yeast. *Applied Microbiology and Biotechnology, 74*(5), 954–60.

Ter Schure, E. G., van Riel, N. A. V, & Verrips, C. T. (2000). The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews, 24*(1), 67–83.

Vilanova, M., Ugliano, M., Varela, C., Siebert, T., Pretorius, I. S., & Henschke, P. A. (2007). Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by *Saccharomyces cerevisiae* wine yeasts. *Applied Microbiology and Biotechnology, 77*(1), 145–57.

Watson, T. G. (1976). Amino-acid pool composition of *Saccharomyces cerevisiae* as a function of growth rate and amino-acid nitrogen source. *Journal of General Microbiology, 96*(2), 263–8.