Defining the chemical features of wine perception

by

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Declaration

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Date: March 2018

Summary

All wines evoke a product recognition, regardless of quality and cultivar, but what is the origin of this feature? The prevalence of this wine concept suggests that its formation occurs independent of the varietal, and ageing-related aromas, and is therefore potentially a function of yeast metabolism. Yeast utilise the nutrients present in grape must to produce biomass, and metabolites which ultimately signify the conversion of grape juice to wine. Consequently, the nutrient composition is highly influential on the aromatic outcomes of alcoholic fermentation.

Synthetic grape must is widely used to evaluate all facets of the fermentation process but there remains much to learn. In this study, the impact of two nutrients, namely, amino acids and anaerobic factors, were evaluated with regard to their impact on yeast growth and aroma production under fermentative conditions. This work also examines the extent to which yeast *de novo* metabolism, both primary and secondary metabolism, contributes to the formation of the wine-like feature.

In a single amino acid context, a linear relationship was apparent between the amino acid concentration and the production of their associated volatile products. This relationship was evaluated in more complex amino acid mixtures and as expected, this linear relationship was lost. Nonetheless, a significant degree of responsiveness between the amino acid and its catabolites remained.

The impact of sterol (plant or yeast derived) or unsaturated fatty acid treatments, individually, as well as in combinations, were compared for their contributions to biomass formation and aroma production. Sterols had a greater impact on biomass development, as the fermentations treated with only unsaturated fatty acids displayed a poorer response. Moreover, they differently impacted aroma production. The unsaturated fatty acid lowered the production of acetate esters, medium chain fatty acids and their esters, whereas sterol supplementation generally bolstered the production of all compounds measured. This work highlights the importance of anaerobic factor management during winemaking.

Although these nutrients certainly impact wine aroma, this study also sought to examine the degree to which these nutrients contribute to wine (product) recognition. Using a novel fermentation-based approach, *Saccharomyces cerevisiae* converted a synthetic grape must into a wine-like product. These synthetic products underwent sensory evaluations to rate the product's resemblance to wine as well as to describe the aroma. This sensory data was used as a decision-making tool to decide upon treatments to be studied in subsequent fermentations. Ultimately, a wine-like character was created by altering the anaerobic factor composition of a synthetic grape must. The use of this synthetic grape must would allow for the more meaningful sensory characterisation of these synthetic products, in addition to providing a wine-like matrix used to evaluate the sensory implications of wine odorants.

Opsomming

Alle wyne kan as 'n wynproduk herken word, ongeag die kwaliteit en kultivar, maar wat is die oorsprong van hierdie kenmerk? Die voorkoms van hierdie wynkonsep dui daarop dat die vorming daarvan onafhanklik van die variëteit en verouderingsverwante aromas is, en daarom moontlik 'n funksie van gismetabolisme is. Gis gebruik die voedingstowwe wat in druiwe mos teenwoordig is om biomassa en metaboliete te produseer, wat uiteindelik die omskakeling van druiwesap na wyn aandui. Gevolglik het die nutrient-samestelling ‘n groot invloed op die aromatiese resultaat van alkoholiese fermentasie.

Sintetiese druiwe mos word algemeen gebruik om alle fasette van die fermentasieproses te evalueer, maar daar is nog baie om te leer. In hierdie studie is die impak van twee voedingstowwe, naamlik aminosure en anaërobiese faktore, op gisgroei en aroma produksie onder fermentatiewe toestande geëvalueer. Hierdie werk ondersoek ook die mate waartoe gis *de novo* metabolisme, beide primêre en sekondêre metabolisme, bydrae tot die vorming van die wynagtige kenmerk.

In 'n enkel aminosuur konteks, was daar 'n oënskynlike lineêre verhouding tussen die aminosuurkonsentrasie en die vorming van hul verwante vlugtige produkte. Hierdie verhouding is in meer komplekse aminosuurmengsels geëvalueer en soos verwag, is hierdie lineêre verhouding verloor. Nietemin het 'n beduidende mate van die verhouding tussen die aminosuur en die verwante kataboliete behoue gebly.

Die impak van sterole (plant of gis verwant) of onversadigde vetsuur, individueel, sowel as in kombinasie, is vergelyk vir hul bydraes met betrekking tot die vorming van biomassa en aroma produksie. Sterole het 'n groter impak op die vorming van biomassa gehad, aangesien die fermentasies wat slegs met die onversadigde vetsuur behandel was tot 'n swakker reaksie gelei het. Verder is die aroma produksie verskillend beïnvloed; die onversadigde vetsuur het die produksie van asetaat esters, mediumkettingvetsure en hul esters verlaag, terwyl die sterol aanvullings oor die algemeen die produksie van alle verbindings wat gemeet is bevorder het. Hierdie werk beklemtoon die belangrikheid van die bestuur van anaërobiese faktore tydens wynmaak.

Alhoewel hierdie voedingstowwe beslis wynaroma beïnvloed, het hierdie studie ook probeer om die mate waartoe hierdie voedingstowwe bydrae tot wyn (produk) herkenning te ondersoek. Met behulp van 'n nuwe fermentasie-gebaseerde benadering, het *Saccharomyces cerevisiae* 'n sintetiese druiwe mos in 'n wynagtige produk omskep. Hierdie sintetiese produkte is sensories geëvalueer om die produkte se ooreenkoms met wyn te bepaal, asook om die aroma te beskryf. Die sensoriese data het as 'n besluitnemende instrument gedien om te besluit watter behandelings in die daaropvolgende fermentasies bestudeer moet word. Uiteindelik is 'n wynagtige karakter geskep deur die samestelling van die anaërobiese faktore van 'n sintetiese druiwe mos te verander. Die gebruik van hierdie sintetiese druiwe mos sal ‘n meer betekenisvolle sintuiglike karakterisering van hierdie sintetiese produkte moontlik

maak, asook om 'n wynagtige matriks te bied wat gebruik kan word om die sensoriese implikasies van wyn aromas te evalueer.

This dissertation is dedicated to my family.

Biographical sketch

Samantha Fairbairn was born and raised in Cape Town and matriculated from Hottentots Holland High School in 1997. She enrolled at the University of Stellenbosch and obtained a BSc-degree in cellular and molecular biology, followed by a HonsBSc-degree in Microbiology in 2002. Samantha currently works as a technical officer at the Institute for Wine Biotechnology, at Stellenbosch University, where she completed her MSc in Wine Biotechnology.

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My **family** and **friends** for their love, empathy, and encouragement

My **Heavenly Father**, who renews my strength daily.

Preface

This dissertation is presented as a compilation of 6 chapters. Each chapter is introduced separately.

Chapters 2, 4 and 5 are written according to the style of the South African Journal of Enology and Viticulture, and chapter 3 to Frontiers in Microbiology to which it has been submitted for publication.

Chapter 1	General Introduction and project aims
Chapter 2	Literature review The contribution of the yeast volatome to wine aroma
Chapter 3	Research results The impact of single amino acids on growth and volatile aroma production by Saccharomyces cerevisiae strains
Chapter 4	Research results Evaluating of the impact of unsaturated fatty acids with yeast or plant sterols on aroma profiles
Chapter 5	Research results The importance of the yeast volatilome in creating a vinous character in synthetic grape must
Chapter 6	General discussion and conclusions

I hereby declare that I was a co-contributor to the multi-author manuscript presented in Chapter 3. Alexander McKinnon*, an MSc student at the Institute for Wine Biotechnology, performed all the single amino acid evaluations and I performed the fermentations with the more complex amino acid compositions. Additionally, I was responsible for the data presentation, analysis, and contributed to its interpretation. However, the writing of this chapter was a collaborative effort on the part of Alexander McKinnon, Dr Hannibal Musarurwa, my supervisors, and myself.

I was the primary contributor with respect to the experimental data presented in Research Chapters 4 and 5. I contributed to the conception of the experiment, its execution, data analysis, interpretation, and writing of these chapters. Prof Kidd performed the ANOVA and MFA analyses on the sensory data and Ana Rita Monforte performed the co-clustering analyses of the GC-MS data.

My supervisors, Prof F.F. Bauer and Prof A. Ferreira, were involved in the conceptual development of the study and continuous critical evaluation of the results, research in general, as well as the writing of this thesis.

*McKinnon, A. (2013). The impact of amino acids on growth performance and major volatile compound formation by industrial wine yeast. MSc thesis. Stellenbosch University, South Africa.

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Chapter 1

General introduction and aims

Chapter 1

General introduction and aims

1.1 Introduction

Wine aroma is of confounding complexity but is an essential contributor to wine quality (Charters & Pettigrew, 2007). Our current understanding of the grape must chemical matrix, its interaction with, and chemical transformation by, the wine microbiome during fermentation, and the complex interactions between many volatile compounds at the level of human perception does not allow us to predict the aromatic outcomes of a given grape must (Francis & Newton, 2005; Ferreira & Cacho, 2009). In simplified terms, wine aromas are a function of the grape variety (including winemaking practices) (Francis & Newton, 2005), alcoholic and malolactic fermentation (Swiegers *et al*., 2005; Styger *et al*., 2011; Hirst & Richter, 2016) and ageing-related modifications (Rapp & Versini, 1995).

All wines have a generic "wine-like" character, a concept which makes the product recognizable as "wine" to wine consumers. This wine-like character is prevalent in all wine, irrespective of wine quality and cultivar, suggesting that it stems from alcoholic fermentation, rather than the variable varietal or technical aspects associated with winemaking. "What makes wine, wine?", as grape juice does indeed not taste or smell like wine. Recently, wine-like aromas have been successfully produced via chemical reconstitution and have been used as the matrix to evaluate the influence of higher alcohols on aroma perception (de-la-Fuente-Blanco *et al*., 2016; de-la-Fuente-Blanco *et al*., 2017). Similarly, cultivar related aromas have also been successfully re-created to a certain degree (Guth 1997a; Guth 1997b; Ferreira *et al*., 2002; Mayr *et al*., 2016). In these instances, the use of chemical reconstitution to create a varietal aroma is limited by the comprehensiveness of the chemical characterisation of the prototype, as the omission of a crucial aroma compound could potentially alter the perceived aromas (Escudero *et al*., 2004; Ferreira & Cacho, 2009). Methods used to identify odorants that are influential on wine aroma invariably highlight the importance of a large number of volatile compounds derived from yeast metabolism (ethyl esters, acetate esters, fatty acids, and higher alcohols) in addition to ageing and varietal associated compounds (Guth 1997a; Guth 1997b; Ferreira *et al*., 2002; Benkwitz *et al*., 2012).

Yeast metabolism can be considered a cornerstone of wine character (Styger *et al*., 2011). Firstly, yeast enzymes transform non-volatile compounds into flavour and aroma active compounds, secondly, yeast utilise grape must components bio-transforming them into active odorants, and finally via *de novo* synthesis of aroma and flavour impacting compounds. This *de novo* synthesis occurs via

primary metabolism, which is the conversion of grape sugars into ethanol, glycerol, acetic acid, and acetaldehyde, in addition to the production of secondary metabolites such as higher alcohols, volatile fatty acids and esters (Lambrechts & Pretorius, 2000; Styger *et al*., 2011). The production of these secondary metabolites is influenced by the yeast strain (Lambrechts & Pretorius, 2000; Rossouw & Bauer, 2016), the fermentation conditions (Beltran et al., 2007; Fairbairn *et al*., 2014; Rollero *et al*., 2015), as well as the nutritional composition of the grape must (Ferreira *et al*., 2014; Rollero *et al*., 2015; Rollero *et al*., 2017). Consequently, by adjusting the fermentation parameters (nutrient composition), one should be able to alter this wine-like aroma.

Nitrogen is an essential nutrient for biomass production and is also the direct precursor for many of these secondary metabolites (Bell & Henschke, 2005), and as such has been widely studied (Hernández-Orte *et al*., 2006; Vilanova *et al*., 2007; Ferreira *et al*., 2014). However, due to the interconnectedness of nitrogen metabolic pathways (Crépin *et al*., 2017), it remains impossible to accurately predict the volatile aroma outcome of any given must nitrogen composition. Anaerobic factors have been shown to be important for biomass formation and consequently also fermentation kinetics (Taylor *et al*., 1979; Luparia *et al*., 2004; Ochando *et al*., 2017). Recently, it has also received more interest regarding its impact on aroma production (Mauricio *et al*.,1997; Varela *et al*.,2012; Duan *et al*., 2015; Rollero *et al*., 2015).

As a part of a broader wine aroma program, this study provides some new insights into the contribution of amino acids and anaerobic factors to wine aroma. Moreover, for the first time, the wine-like feature is examined, by evaluating the degree to which the *de novo* synthesis of aroma and flavour impacting compounds, produced by yeast, contributes to the formation of this vinous character.

1.2 Project aims

1.2.1 Wine-like feature

The primary aim of this project was to explore the sensory contribution of the yeast volitilome in creating a wine-like product (Figure 1). Using sensory approaches similar to ones used to evaluate wine typicality (Maitre *et al.,* 2010), this work evaluates whether *Saccharomyces cerevisiae* is able to convert a synthetic grape must into a product that evokes a wine-like recognition, in the absence of varietal and ageing-related aromas. To achieve this, various nitrogen and anaerobic factor compositions were evaluated, for their contribution to the wine-like feature.

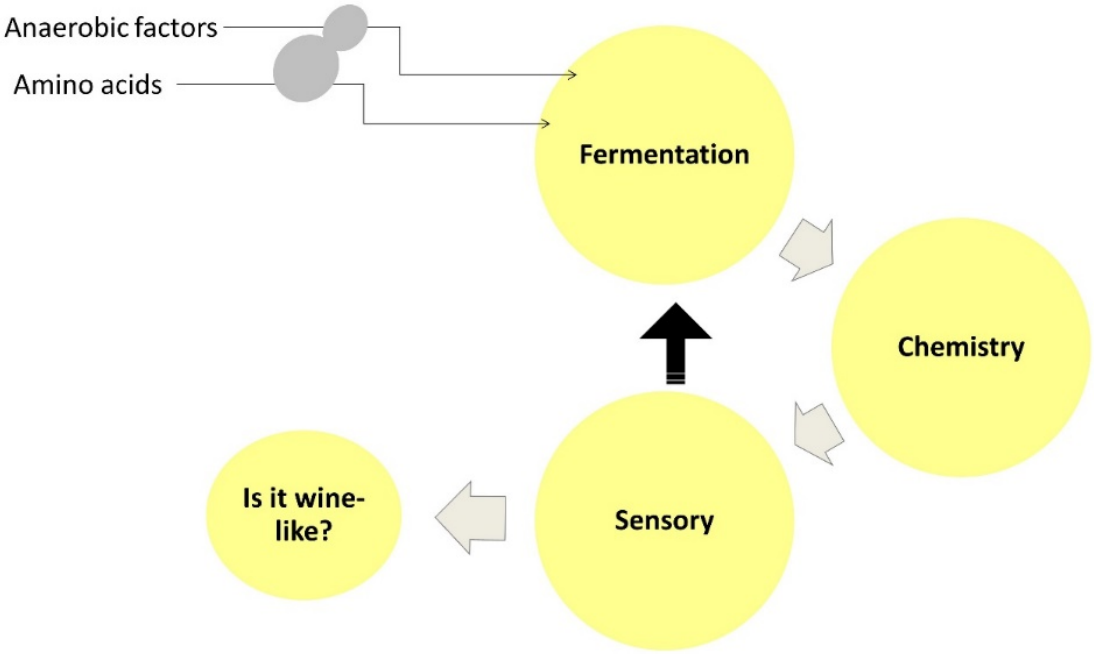


Figure 1.1. Schematic overview of the wine-like feature study. To explore the contribution of the yeast volatilome to the sensory recognition of wine-likeness, *Saccharomyces cerevisiae* was used to mediate the conversion of synthetic grape must into a fermented product. This product underwent chemical and sensory evaluation to determine whether its aromas were reminiscent of wine. This sensory data informed how the composition of the synthetic grape must would be altered to create a more wine-like aroma.

1.2.2 Additional research aims:

1.2.2.1 Amino acids and aroma

To evaluate baseline data describing the impact of single amino acids on growth kinetics under fermentative conditions, and their corresponding volatile profiles. In addition to evaluating the relevance of the trends observed in response to more complex amino acid mixtures.

1.2.2.2 Anaerobic factors and aroma

To explore the influence of single sterol or unsaturated fatty acid treatments, as well as in combinations, on biomass formation and aroma production.

1.3 References

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 Chem. 127, 2, 441–446.

Bell, S.J. & Henschke, P. a, 2005. Implications of nitrogen nutrition for grapes, fermentation and wine. Aust. J. Grape Wine Res. 11, 3, 242–295.

Beltran, G., Rozès, N., Mas, A. & Guillamón, J.M., 2007. Effect of low-temperature fermentation on yeast nitrogen metabolism. World J. Microbiol. Biotechnol. 23, 6, 809–815.

Benkwitz, F., Nicolau, L., Lund, C., Beresford, M., Wohlers, M. & Kilmartin, P.A., 2012. Evaluation of key odorants in sauvignon blanc wines using three different methodologies. J. Agric. Food Chem. 60, 25, 6293–6302.

Charters, S. & Pettigrew, S., 2007. The dimensions of wine quality. Food Qual. Prefer. 18, 7, 997–1007.

Crépin, L., Truong, N.M., Bloem, A., Sanchez, I., Dequin, S. & Camarasa, C., 2017. Management of multiple nitrogen sources during wine fermentation by *S. cerevisiae.* Appl. Environ. Microbiol. 83, 5, AEM.02617-16.

de-la-Fuente-Blanco, A., Sáenz-Navajas, M.P. & Ferreira, V., 2017. Levels of higher alcohols inducing aroma changes and modulating experts' preferences in wine model solutions. Aust. J. Grape Wine Res. 23, 2, 162–169.

de-la-Fuente-Blanco, A., Sáenz-Navajas, M.P. & Ferreira, V., 2016. On the effects of higher alcohols on red wine aroma. Food Chem. 210, 107–114.

Duan, L., Shi, Y., Jiang, R. & Yang, Q., 2015. Effects of adding unsaturated fatty acids on fatty acid composition of *Saccharomyces cerevisiae* and major volatile compounds in wine. South African J. Enol. Vitic. 36, 2, 285–295.

Escudero, A., Gogorza, B., Melús, M.A., Ortín, N., Cacho, J. & Ferreira, V., 2004. Characterization of the aroma of a wine from Maccabeo. Key role played by compounds with low odor activity values. J. Agric. Food Chem. 52, 11, 3516–3524.

Fairbairn, S.C., Smit, A.Y., Jacobson, D., Prior, B.A. & Bauer, F.F., 2014. Environmental stress and aroma production during wine fermentation. South African J. Enol. Vitic. 35, 2, 168–177.

Ferreira, A.C.S., Monforte, A.R. Teixeira, C.S., Martins, R., Fairbairn, S., & Bauer, F.F., 2014. Monitoring alcoholic fermentation: an untargeted approach. J. Agric. Food Chem. 62, 28, 6784–93.

Ferreira, V., Ortin, N., Escudero, A., Lopez, R. & Cacho, J., 2002. Chemical Characterization of the Aroma of Grenache Rose; Wines: Aroma Extract Dilution Analysis, Quantitative Determination, and Sensory Reconstitution Studies. J. Agric. Food Chem. 50, 14, 4048–4054.

Ferreira V., & Cacho J., 2009. Identification of Impact Odorants of Wines. In: Moreno-Arribas, M.V.& Polo M.C. (eds). Wine Chemistry and Biochemistry, vol I. Springer, New York, NY. pp. 293 -410

Francis, I.L., & Newton, J.L., 2005. Determining wine aroma from compositional data. Aust. J. Grape Wine Res. 11, 2, 114–126.

Guth, H., 1997a. Quantitation and Sensory Studies of Character Impact Odorants of Different White Wine Varieties. J. Agric. Food Chem. 45, 8, 3027–3032.

Guth, H., 1997b. Identification of character impact odorants of different white wine varieties. J. Agric. Food Chem. 45, 8, 3022–3026.

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 Chem. 98, 2, 300–310.

Hirst, M.B. & Richter, C.L., 2016. Review of aroma formation through metabolic pathways of *Saccharomyces cerevisiae* in beverage fermentations. Am. J. Enol. Vitic. 67, 4, 361–370.

Lambrechts, M.G. & Pretorius, I.S., 2000. Yeast and its Importance to Wine Aroma - A Review. South African J. Enol. Vitic. 21, Special Issue, 97–129.

Luparia, V., Soubeyrand, V., Berges, T., Julien, A. & Salmon, J.-M., 2004. Assimilation of grape phytosterols by *Saccharomyces cerevisiae* and their impact on enological fermentations. Appl. Microbiol. Biotechnol. 65, 1, 25–32.

Maitre, I., Symoneaux, R., Jourjon, F. & Mehinagic, E., 2010. Sensory typicality of wines: How scientists have recently dealt with this subject. Food Qual. Prefer. 21, 7, 726–731.

Mauricio, J.C., Moreno, J., Zea, L., Ortega, J.M. & Medina, M., 1997. The effects of grape must fermentation conditions on volatile alcohols and esters formed by *Saccharomyces cerevisiae*. J. Sci. Food Agric. 75, 2, 155–160.

Mayr, C.M., Geue, J.P., Holt, H.E., Pearson, W.P., Jeffery, D.W. & Francis, I.L., 2014. Characterization of the key aroma compounds in shiraz wine by quantitation, aroma reconstitution, and omission studies. J. Agric. Food Chem. 62, 20, 4528–4536.

Mendes-Pinto, M.M., 2009. Carotenoid breakdown products the-norisoprenoids-in wine aroma. Arch. Biochem. Biophys. 483, 2, 236–245.

Ochando, T., Mouret, J.R., Humbert-Goffard, A., Sablayrolles, J.M. & Farines, V., 2017. Impact of initial lipid content and oxygen supply on alcoholic fermentation in champagne-like musts. Food Res. Int. 98, 87–94.

Ough, C.., Davenport, M. & Joseph, K., 1989. Effects of Certain Vitamins on Growth and Fermentation Rate of Several Commercial Active Dry Wine Yeasts. Am. J. Enol. Vitic. 40, 3, 208–213.

Rapp, A. & Versini, G., 1995. Influence of nitrogen compounds in grapes on aroma compounds in wine. Dev. Food Sci. 37, 1659–1694.

Rollero, S., Bloem, A., Camarasa, C., Sanchez, I., Ortiz-Julien, A., Sablayrolles, J.M., Dequin, S. & Mouret, J.R., 2015. Combined effects of nutrients and temperature on the production of fermentative aromas by *Saccharomyces cerevisiae* during wine fermentation. Appl. Microbiol. Biotechnol. 99, 5, 2291–2304.

Rollero, S., Mouret, J.-R.R., Bloem, A., Sanchez, I., Ortiz-Julien, A., Sablayrolles, J.-M.M., Dequin, S. & Camarasa, C., 2017. Quantitative 13C-isotope labelling-based analysis to elucidate the influence of environmental parameters on the production of fermentative aromas during wine fermentation. Microb. Biotechnol. 10, 6, 1649–1662.

Rossouw, D. & Bauer, F.F., 2016. Exploring the phenotypic space of non-Saccharomyces wine yeast biodiversity. Food Microbiol. 55, 32–46.

Styger, G., Prior, B. & Bauer, F.F., 2011. Wine flavor and aroma. J. Ind. Microbiol. Biotechnol. 38, 9, 1145–1159.

Swiegers, J.H., Bartowsky, E.J., Henschke, P. a. & Pretorius, I.S., 2005. Yeast and bacterial modulation of wine aroma and flavour. Aust. J. Grape Wine Res. 11, 2, 139–173.

Taylor, G.T., Thurston, P. a. & Kirsop, B.H., 1979. The influence of lipids derived from malt spent grains on yeast metabolism and fermentation. J. Inst. Brew. 85, 4, 219–227.

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

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. Appl. Microbiol. Biotechnol. 77, 1, 145–157.

Chapter 2

The contribution of the yeast volatilome to wine aroma

Chapter 2

The contribution of the yeast volatilome to wine aroma

2.1 Introduction

Wine quality is of paramount importance to the wine consumer (Swiegers *et al.*, 2005), however, due to its subjective nature, the concept of wine quality is a contentious matter within the scientific community. Nonetheless, the importance of wine flavour (aroma, taste, and mouth-feel) to perceived wine quality is indisputable. More than 800 compounds have been reported to contribute to wine aroma (Mendes-Pinto, 2009). These volatile aromas have three origins, namely grape (varietal or grape processing), microbial i.e. formed during alcoholic and malolactic fermentation, and lastly ageing or storage associated aroma (Figure 2.1) (Rapp & Versini, 1995). Varietal compounds, distinctive to a specific cultivar, may be released or modified due to the action of yeast and bacteria during alcoholic and malolactic fermentation (Swiegers *et al.*, 2005; Styger *et al.*, 2011; Hirst & Richter, 2016). The extraction of these compounds is also influenced by oenological practices, such as the pressing method and maceration. During maturation and storage, the levels of varietal and fermentation aromas decrease, whereas wood contact results in the extraction of associated aromas (Styger *et al.*, 2011).

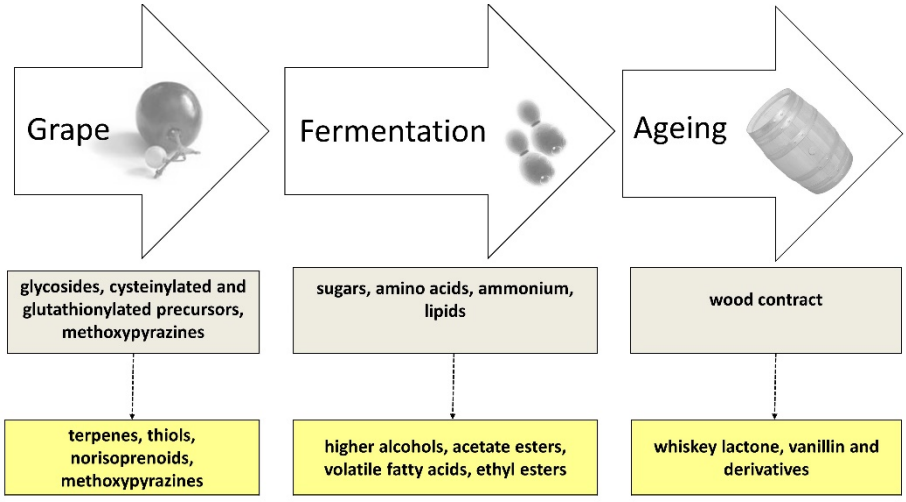
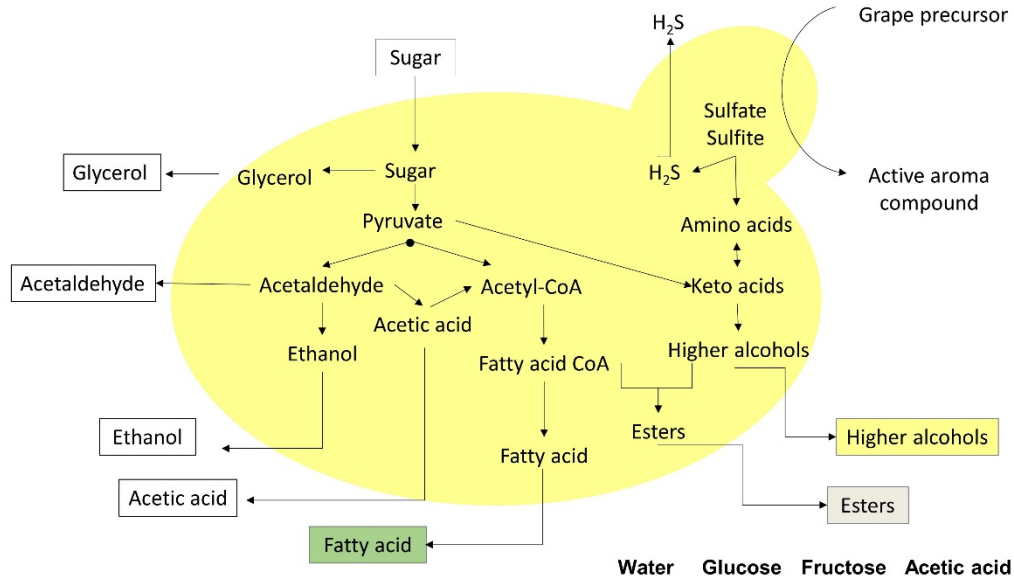


Figure 2.1. Wine aroma has three origins namely, the grape, fermentation, both alcoholic and malolactic fermentation, and the maturation processes (Adapted from Drawert, 1974; Ugliano and Henschke, 2009)

Saccharomyces cerevisiae is the yeast species primarily, though not exclusively, responsible for alcoholic fermentation and is also the major producer of non-volatile wine impact compounds (Figure 2.2 A). In addition to this, yeast also significantly contribute to wine's volatile aromas, producing the largest percentage of compounds, in comparison to grape and ageing-related volatiles (Figure 2.2 B). The compounds associated (Figure 2.2 A and B) with the yeast volatilome are synthesized from highly complex metabolic networks that also share intermediates (glycolysis, fatty acid synthesis, amino acid metabolism, and the TCA cycle) (Lambrechts & Pretorious, 2000). This review will focus on the fermentation-derived aroma compounds associated with yeast metabolism exclusively, as well as a few of the sensory methods employed to evaluate wine aroma.

(A)



	Water	Glucose	Fructose	Acetic acid	Ethanol	Glycerol
Juice (g/L)	700 – 850	80 – 130	70 – 120	Trace	Trace	Trace
Wine (g/L)	800 – 900	0.5 – 5	0.5 – 5	0.2 - 1.5	80 – 150	3 –14

(B)

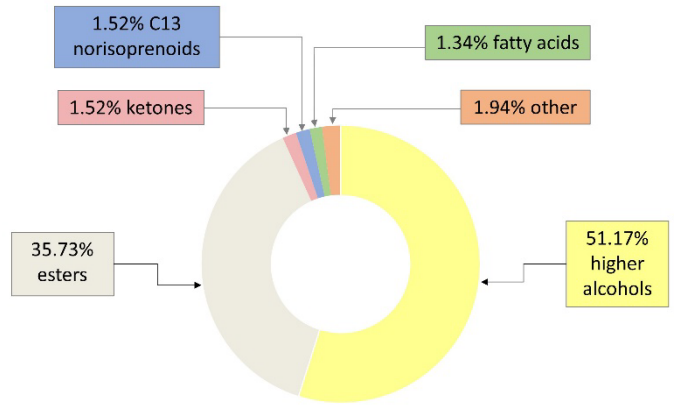


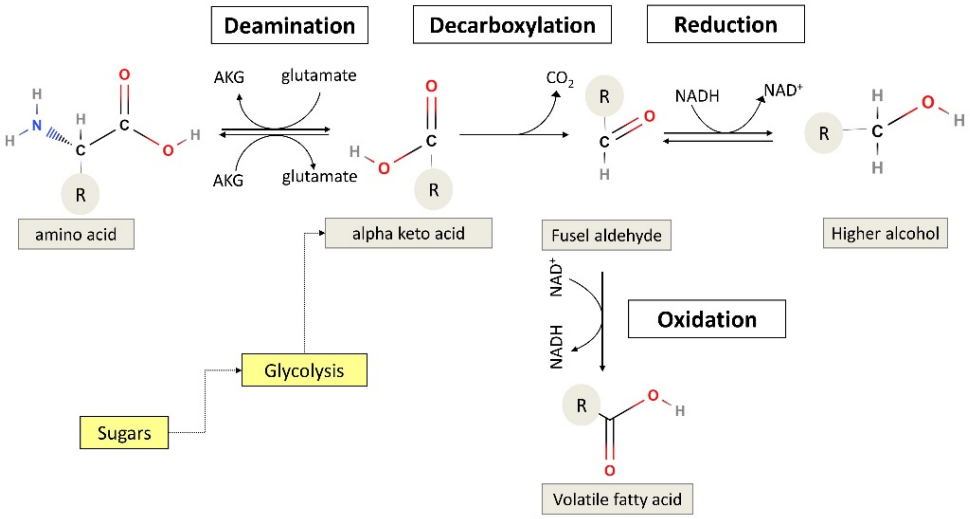
Figure 2.2. Overview of the metabolism of carbon and nitrogen by yeast **(A),** as well as the average composition of major wine volatile components **(B).** Adapted from (Lambrechts & Pretorius,2000; Sumby *et al.*, 2010; Coombe & Iland, 2004)

2.2 Yeast derived aroma compounds

2.2.1 Higher alcohols

Higher alcohols are the most abundant aromatic compounds present in wine and are synthesized via the Ehrlich pathway (Figure 2.3 A) (Hazelwood *et al*., 2008; Hirst & Richter, 2016). Briefly, branched-chain and aromatic amino acids undergo transamination, transferring the amino group to α-ketoglutarate (AKG) forming an α-keto acid and glutamate (Hazelwood *et al*., 2008). The α-keto acid undergoes decarboxylation forming a fusel aldehyde, which depending on the redox state of the cell, is either reduced to form a higher (fusel) alcohol, or oxidized forming a volatile fatty acid. The higher alcohol may also react with acetyl-CoA to produce an acetate ester. The α-keto acid used may be a product of amino acid catabolism, as described above, but may also be a by-product of central carbon metabolism.

(A)



AKG = α-ketoglutarate

(B)

Amino acid	Alpha-keto acid	Aldehydes	Higher alcohols	Volatile fatty acids
Leucine	α-ketoisocaproate	Isovaleraldehyde	Isoamyl alcohol	Isovaleric acid
Isoleucine	α-keto-methylvalerate	2-Methylbutyraldehyde	Amyl alcohol	2-Methylbutanoic acid
Valine	α-Ketoisovalerate	Isobutyraldehyde	Isobutanol	Isobutyric acid
Phenylalanine	Phenylpyruvate	Phenylacetaldehyde	Phenylethyl alcohol	Phenyl ethyl acetate
Tyrosine	ρ-OH-phenylpyruvate	ρ-OH-phenylacetaldehyde	ρ-OH-phenylethanol	ρ-OH-phenylethyl acetate
Tryptophan	Indole pyruvate	Indole-3-acetaldehyde	Tryptophol	Indol-3-acetic acid

Figure 2.3. An overview of the Ehrlich pathway **(A)**, which catabolises amino acids in to higher alcohols or volatile fatty acids, as well as a summary of the intermediated related to specific branched-chain and aromatic amino acids **(B)**.

Higher alcohol production is influenced by the yeast assimilable nitrogen content (YAN) (Bell & Henschke, 2005). Indeed, numerous studies have reported that at lower levels of nitrogen, a proportional relationship exists between nitrogen content and higher alcohol production, but that at high YAN levels an inverse relationship is observed (Äyräpää, 1971; Jiménez-Martí *et al*., 2007; Rollero *et al*., 2015; Rollero *et al*., 2017). A recent isotopic tracer study monitored the metabolism of ammonium and several amino acids, namely, glutamine, arginine, valine, threonine, leucine, and isoleucine (Figure 2.4), and reported some interesting findings (Crépin *et al.,* 2017). With few exceptions, they observed that the bulk of the amino acids assimilated are used for the *de novo* synthesis of other amino acids and that only a small fraction of the assimilated amino acids was converted into volatile compounds or incorporated into proteins. A follow-up study by the same research group, examined the flux of amino acid metabolism in response to various nitrogen (70, 250 or 425 mg N/L) and sterol (2 or 8 mg/L phytosterol) concentrations (Rollero *et al.*, 2017). In addition to affirming the abovementioned observations, it was also noted that irrespective of the sterol content, the increase in available nitrogen from 70 to 250 mg N/L resulted in an increase in the direct incorporation of leucine and valine at the latter stages of fermentation. However, the bulk of the proteogenic fraction was derived from *de novo* synthesis. Furthermore, the data show that the higher alcohols and volatile fatty acids produced were primarily products of central carbon metabolism. Interestingly, authors also reported an increase in higher alcohol production in response to increasing phytosterol concentrations, depending on the higher alcohol in question and the concentration of nitrogen provided. Taken together, these tracer studies raise concern that nitrogen supplementation may have little direct impact on wine aroma, and although Rollero *et al* (2017) explored the impact of proportionally increasing amino acid concentrations, nitrogen composition changes remain unexamined.

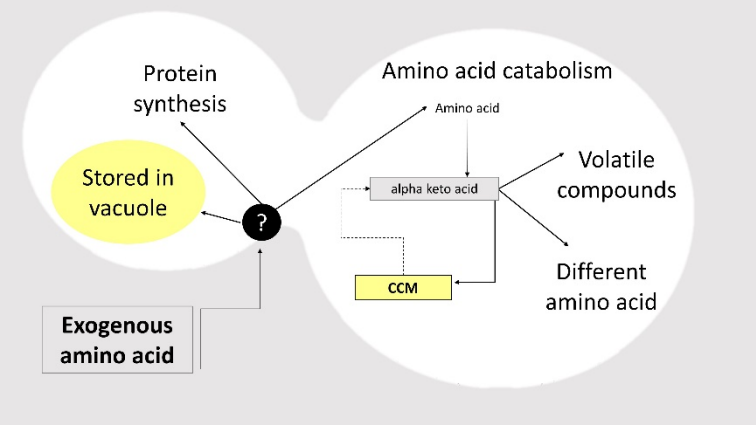


Figure 2.4. Following assimilation, amino acids may be metabolised in three possible pathways. Firstly, they may be stored in a vacuole for later use. Secondly, they can be utilised as is. Lastly, amino acids may be used as nitrogen sources, and be catalysed into an α-keto acid which in turn may be fed into central carbon metabolism, the Ehrlich pathway, or be used to synthesize a different amino acid (Adapted from Crépin *et al*., 2017).

2.2.2 Volatile fatty acids

Volatile fatty acids are by-products of fatty acid synthesis (Figure 2.5), or of aldehyde metabolism, as shown in figure 2.3 A and B. Acetic acid, the most abundant volatile fatty acid, is a product of glycolysis, whereby acetaldehyde is oxidised to generate acetic acid. It contributes positively to wine complexity at lower concentrations and has a negative impact on perceived wine quality at levels greater than 0.7 g/L (Lambrechts & Pretorius, 2000). Other short chain fatty acids include propionic acid and butanoic acids. In a similar manner, the aldehydes formed by the Ehrlich pathway also result in the formation of volatile fatty acids (Figure 2.3 A and B).

In contrast, medium chain (MCFA) and long chain fatty acids are by-products of the fatty acid biosynthetic pathways (Figure 2.5), during which, acetyl-CoA undergoes carboxylation to form malonyl-CoA, which requires one ATP molecule (Lambrechts & Pretorius, 2000). Malonyl-CoA and acetyl-CoA, bound to the fatty acid synthase (FAS) complex, undergo condensation reactions to elongate the acyl group with two carbons. In a reiterative process, the new acyl group replaces the acetyl group on the FAS complex and undergoes condensation with a new malonyl group. During alcoholic fermentation, the accumulation of long chain fatty acids inhibits acetyl-CoA carboxylase, resulting in the release of these acyl groups (medium and long chain fatty acids) from the FAS (Dufour *et al.*, 2003).

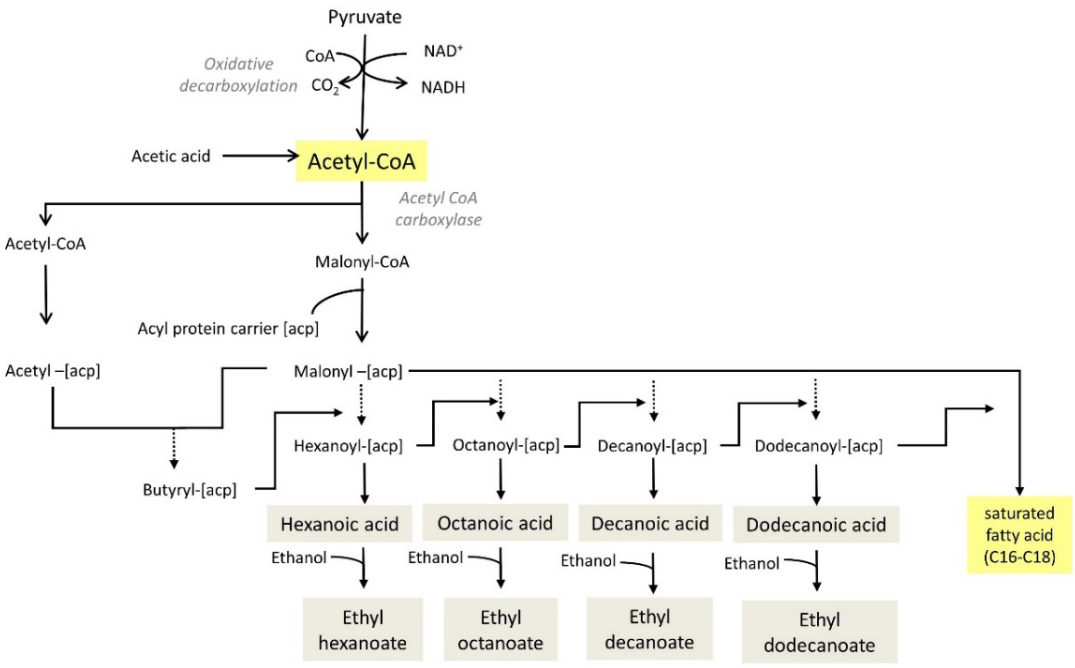


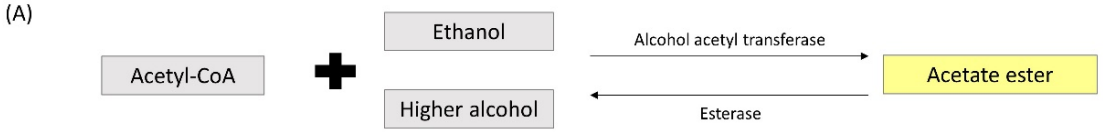
Figure 2.5. An abridged overview of fatty acid synthesis as well as their ethyl esters (Adapted from the KEGG database). The dashed lines indicate that intermediate steps have been omitted.

2.2.3 Acetate esters

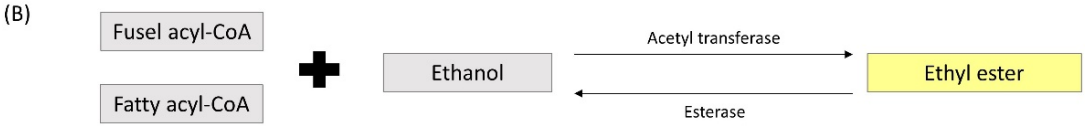
Acetate and ethyl esters are considered among the most important contributors to wine aroma, as they convey a fruity character (Ferreira *et al*., 2002).

Acetate esters are produced when acetyl-CoA undergoes a transferase reaction with ethanol or a higher alcohol (Bisson & Karpel, 2010) (Figure 2.6 A and C). The availability of these substrates, as well as the balance between esterase, IAH1, (Lilly *et al*., 2006) and the alcohol acetyl transferase, encoded by ATF1 and ATF2, activity all impact acetate ester concentrations (Yoshioka & Hashimoto, 1981; Saerens *et al*., 2010). Furthermore, the overexpression of ATF1 and ATF2 (Verstrepen *et al*., 2003), has been shown to bring about a concomitant increase in the production of acetate esters, with ATF2 being less influential than ATF1. Interestingly, the double deletion of these alcohol acetyl transferases resulted in the absence of only isoamyl acetate, indicating the presence of additional alcohol acetyl transferases (Verstrepen *et al*., 2003; Saerens *et al*., 2010).

(A)



(B)



(C)

Alcohol	Acetate ester	Acyl-CoA	Ethyl ester
Ethanol	Ethyl acetate	Isovaleric acid	Ethyl isovalerate
Isoamyl alcohol	Isoamyl acetate	Isobutyric acid	Ethyl isobutyrate
Isobutanol	Isobutyl acetate	2 methyl butanoic acid	Ethyl 2 methylbutanoate
Amyl alcohol	Active amyl acetate	Hexanoic acid	Ethyl hexanoate
2-Phenylethyl ethanol	2-Phenylethyl acetate	Octanoic acid	Ethyl octanoate
		Decanoic acid	Ethyl decanoate

Figure 2.6. An overview of acetate ester **(A)**, and ethyl ester production **(B)** which catabolises amino acids in to higher alcohols or volatile fatty acids, as well as a summary of the precursors related to their respective formation **(C** and **D)** adapted from Bisson & Karpel (2010) and Lambrechts & Pretorius (2000).

2.2.4 Ethyl esters

Ethyl esters are produced by a condensation reaction between ethanol and an acyl group formed via either fatty acids synthesis or nitrogen metabolism (Figure 2.6 B and C) (Bisson & Karpel, 2010). Their formation is dependent on the availability of substrates, namely, acyl-CoA groups and alcohol, as well as the balance between acyl transferase, EH1 (Mason & Dufour, 2000) and EEB1 (Saerens *et al.*, 2006), versus esterase activity (Saerens *et al.*, 2008). Deletion studies have shown that EH1 and EEB1 deletion resulted in a decrease in the production of ethyl esters (Saerens *et al.*, 2006). Interestingly, the over-expression of these genes, as well the increased availability of substrates (MCFA) had no discernible impact on ethyl ester levels. Moreover, the authors found that Eh1p and Eeb1p displayed both acyl transferase and esterase activity, and propose that this esterase activity prevented the accumulation of ethyl esters.

2.3 The sensory implications of chemical data

2.3.1 Sensory evaluation methods

The sensory profile of a given wine cannot be accurately predicted from its chemical composition alone (Francis & Newton, 2005), consequently, sensory evaluations are essential. Descriptive sensory analysis (DA) is one of the most trusted methods in sensory science, allowing both the qualitative and quantitative evaluation of products (Lawless & Heymann, 1998). In DA, the panel undergoes training, and is provided with discriminating descriptors, and has to agree on its perceived intensity. Descriptive analysis is time consuming and expensive, which has contributed to the introduction of alternative strategies to evaluate wine aroma. Rapid approaches, namely sorting, check-all-that-apply (CATA) and projective mapping (PM) among others, have been reviewed recently (Varela & Ares, 2012; Chollet *et al.*, 2011; Valentine *et al.*, 2012). As the name suggests, sorting involves making groups of wines based on their similarities and differences, and the assessors are also asked to "verbalise" the reasons for their groupings. In CATA, the panellist is provided with an appropriate list of descriptors and tasked with indicating which ones best describe the wines. In projective mapping, participants would spatially arrange wines on an A3 page, in a manner similar to sorting, they would group identical products close together, whereas wines that are the most different would be placed further apart. These approaches allow one to use novice wine consumers instead of a trained panel, which facilitates the rapid generation of data but the lack of training and consensus among assessors ensures that data analyses will be a lengthy process (Chollet *et al.*, 2011; Varela & Ares, 2012; Valentine *et al.*, 2012).

2.3.2 Aroma perception thresholds

The perception threshold is the lowest concentration at which at least one judge is able to perceive the odorant (Ferreira & Cacho, 2008). These perception levels (Table 2.1 and 2.2) can be used to infer the potential impact of volatile compounds on wine aroma, by calculating the odour activity values (Francis & Newton, 2005; Grosch, 2001).

$$\text{odour activity value (OAV)} = \frac{\text{concentration measured}}{\text{odour threshold}}$$

Compounds with OAVs greater than one are perceived. Although research has shown that compounds with OAV greater than 0.5 also contribute positively to wine aroma (Ferreira *et al*., 2002). The accuracy of the threshold values proposed is subject to the matrix used as well as the evaluation procedures followed (Robinson *et al*., 2014). For example, the thresholds reported for isobutyric acid vary greatly depending on the matrix used: 2.3 mg/L in model wine (Ferreira *et al*., 2000), 200 mg/L in 10% ethanol (Guth, 1997 a; Guth, 1997 b) and lastly 30 mg/L in beer (Etievant, 1991).

2.3.3 The non-volatile matrix

Most aroma research is focused on characterising the volatile fraction of wine, but this does not negate the importance of the non-volatile matrix (polyphenolic compounds, proteins, organic acids, and carbohydrates) (Villamor & Ross, 2013). Not only does the non-volatile matrix define taste parameters such as acidity, sweetness, bitterness, and astringency (Sáenz-Navajas *et al*., 2012), it has also been shown to alter white wine aromas so that they resemble that of red wine (Sáenz-Navajas *et al*., 2010). Using a reconstitution approach, the authors extracted the volatile and non-volatile constituents of 6 wines (red and white) and combined these fractions in all possible combinations. The authors showed that the white wine matrix, enhanced the perception of “typical” white wine descriptors (white and yellow fruit, citrus and exotic fruit) when paired with the volatiles of a red wine. Similarly, the use of a red wine matrix and white wine volatile fraction increased the frequency with which wines were described with red and black fruit descriptors. These differences in the perception may be due to several factors, including the greater capacity of a red wine matrix to retain esters and volatile fatty acids (Sáenz-Navajas *et al*., 2010).

Table 2.1. Odour perception values and descriptors of yeast derived volatile compounds

Aroma compound	Aroma descriptors	Odour threshold (mg/l)
Higher alcohols		
2-Phenyl Ethanol	floral, rose, honey, spice, lilac	14[A]
3-Ethoxy-1-propanol	chemical, solvent, fruity	0.1 [B]
Butanol	harsh, nail polish, whiskey	150[C]
Hexanol	herbaceous, green grass	8[D]
Isoamyl alcohol	fusel, alcoholic, solvent	30[D]
Isobutanol	fusel, alcoholic, medicinal	40[D]
Pentanol		64[C]
Propanol	fruity, alcohol, pungent	306[C]
Acetate esters		
2-Phenylethyl Acetate	floral, rose, fruity, cooked apple, marmalade, honey	0.25[D]
Ethyl Acetate	apple, glue, nail polish remover	12.27[C]
Hexyl Acetate	fruity, green, pear, apple, floral, herb	0.67[C]
Isoamyl Acetate	banana, pear	0.03[D]
Volatile fatty acids		
Acetic acid	sour, pungent, vinegar	200[D]
Butyric acid	spoiled cheese, sweaty, sour, pungent	0.173[A]
Isobutyric acid	rancid, cheese, sweaty, fruit, pungent	2.3[A]
Isovaleric acid	rancid, cheese, sweaty, putrid	0.033[A]
Propionic acid	pungent, vinegar, rancid	8.1[C]
Ethyl esters		
Diethyl Succinate	fruity	200[C]
Ethyl Butyrate	fruity, pineapple	0.02[D]
Ethyl Lactate	milky, buttery, sweet, fruity, strawberry	150[C]
Ethyl phenylacetate	rose, floral	0.25[D]
Medium chain fatty acids		
Octanoic acid	lactic, oily, sweaty, rancid, faint fruity	0.5[A]
Hexanoic acid	rancid, cheese, sweaty, metallic	0.42[A]
Decanoic acid	rancid, fatty, citrus	1[A]
Medium chain fatty acid ethyl esters		
Ethyl Caprate	fruity, floral, pleasant, soap	0.2[A]
Ethyl Caprylate	fruity, sweet, soap, pineapple, pear	0.005[A]
Ethyl Hexanoate	green apple, fruity, apple peel, strawberry, candy	0.014[A]

[A] Ferreira *et al* (2000) determined in 11% (vol/vol) ethanol, 7g/L glycerine and 5 g/L tartaric acid at pH 3.4
[B] Peindo *et al* (2004) determined in 10% (vol/vol) ethanol, pH 3.5 adjusted with tartaric acid
[C] Reviewed by Etievant (1991) determined in wine
[D] Guth *et al* (1997) determined in 10% ethanol (vol/vol)

Table 2.2. Odour perception values and descriptors of grape and ageing related volatile compounds

Aroma compound	Aroma descriptors	Odour threshold (mg/l)
Carbonyls		
β- damascenone	honey, apple	0.05[A]
β- ionone	seaweed, violet	0.09 [B]
Phenols		
guaiacol	smoke, medicine	10[A]
eugenol	clove, honey	5[A]
vanillin	vanilla	200[A]
4-vinylguaiacol	clove, curry	40[A]
Thiols		
4-mercapto-4-methylpentan-2-one	Box tree, passion fruit, broom, black current	0.0008E
3-Mercaptohexyl acetate	Passion fruit, grapefruit, box tree, gooseberry, guava	0.004E
3-Mercaptohexan-1-ol	Passion fruit, grapefruit, gooseberry, guava	0.06E
Terpenes		
β -citronellol	rose	100[C]
linalool	flower, wood	25[D]
cis-rose-oxide	lychee, rose, green	0.2[A]
geraniol	rose, geranium	7.4[B]
rotundone	pepper	0.008[F]
Lactone		
cis-oak lactone	coconut	67[C]
wine lactone		0.01[A]

[A]Guth *et al* (1997ab) determined in 10% ethanol (vol/vol)
[B]Ferreira *et al* (2000) determined in 11% (vol/vol) ethanol, 7g/L glycerine and 5 g/L tartaric acid at pH 3.4
[C] Reviewed by Etievant (1991) determined in wine
[D] Ferreira *et al* (2002) determined in 10% ethanol solution containing 7 g/L glycerine at pH 3.2
[E] Dubourdieu *et al* (2006) model wine
[F] Wood *et al* (2008) in an ethanol solution

2.3.4 Interactions

Not only do the volatile and non-volatile matrices interact with each other, the individual components found in the volatile fraction also interact, altering the resultant aroma perceptions (Francis & Newton, 2005) by enhancing or suppressing each other as illustrated when three different binary combinations of compounds with wood and fruity nuances were evaluated (Atanasova *et al*., 2005a). The authors report that despite having equal intensities as individual compounds, when mixed, the fruity aromas were invariably masked by those of the woody compounds. In a follow-up study, authors explored these interactions when woody associated compounds were at concentrations below or near the perception threshold levels, and the fruity related compounds above the perception thresholds (Atanasova *et al*., 2005b). Using a triangle sensory test, assessors were able to identify the samples with the additions of

woody character, from those without it, even at levels below its perception threshold. The assessors ascribed this difference to an increase in the intensity of the aromas present (Atanasova *et al*., 2005b).

In a synthetic matrix, norisoprenoids (β-ionone and β-damascenone), varietal compounds, have been shown to enhance fruity notes when present at low levels, whereas they alter aromas (plum-raisin character) at higher concentrations (Escudero *et al*., 2007). Interestingly, these trends were only observed in dearomatized wine, when DMS (dimethylsulfide), a compound formed during ageing, was also present. In contrast, Mayr *et al* (2016) reported that DMS masked the fruity aromas (Table S 2.2). Coetzee *et al* (2015) showed that an oxidation associated compound, methional, suppressed the fruity thiol notes in a model wine. Additionally, when paired with methoxypyrazines, it enhanced the perception of cooked beans and cooked potato aromas. San-Juan *et al* (2011) also examined the sensory impact of methional, which has a boiled potato character, and found that it altered the perceived fruity aromas to dried fruit. Furthermore, these authors also showed that volatile fatty acids enhance fruitiness, possibly by masking the deleterious animal aroma of 4-ethyl phenol (San-Juan *et al*., 2011).

2.3.5 Identification of odorants contributing to wine aroma

Most volatile compounds contribute to wine aroma, whereas key odorants are able to directly confer their aromas to the wine (Ferreira & Cacho, 2008). The most common means of identifying the compounds which most contribute to aroma is gas chromatography with olfactometric detection (GC-O) (Plutowska & Wardencki, 2008; Brattoli *et al*., 2013; Ferreira & Cacho, 2008). Gas chromatography separates the aromatic compounds, and a trained assessor is used as the detector to describe the aroma, and intensity perceived (Figure 2.7). As summarised in figure 2.7, GC-O has three applications: detection frequency methods, dilution to threshold methods, and direct intensity methods. Furthermore, mass spectrometry could be used in concert with olfactometry to allow the simultaneous identification of the odorant. As with any chemical analyses, sample preparation is crucial in obtaining as much relevant information regarding the sample as possible (Robinson *et al*., 2014; Ferreira & Cacho, 2008).

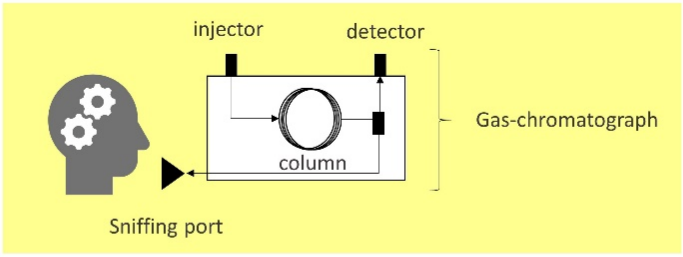
Aroma extract dilution analysis (AEDA), a dilution threshold method (Figure 2.8), is commonly used to identify the key odorants in wine (Ferreira & Cacho, 2008). In AEDA, the aroma compounds undergo serial dilutions, and assessors evaluate whether odorants are perceived at a given dilution or not. The flavour dilution (FD) is the highest dilution (R) at which an odorant is perceived (P) by at least one judge (Ferreira & Cacho, 2008).

$$\text{Flavour dilution} = R^{P+0.5}$$

Therefore, those compounds detected at the highest dilution rates are considered to be potentially influential on wine aroma. Following this, the OAVs are calculated for all quantified chemical compounds (Figure 2.8), providing a second list of candidate odorants (Ferreira *et al*., 2002). The AEDA and OVA analyses generally both result in a long list of compounds, few of which ultimately make a significant contribution to aroma individually. Furthermore, AEDA evaluates the volatile aromas in isolation, when odorants are known to interact with each other, as well as the non-volatile matrix, masking or enhancing the perception of certain descriptors (Ferreira & Cacho, 2008). Consequently, the reconstitution and omission analyses of aromas are of paramount importance. Briefly, wine aromas are re-created via chemical reconstitution whereby volatiles are added to a model or dearomatized wine (Grosch, 2001). Compounds identified by AEDA and OAV are strategically omitted from the reconstituted wines and the sensory profiles of these samples are compared to determine the importance of the omitted compounds. This approach has been applied to identify the influential odorants in Scheurebe (4-mercapto-4-methylpentan-2-one) and Gewürztraminer (cis-rose oxide, Table S2.1) (Guth, 1997 a; Guth, 1997 b), warmer (ethyl propanoate and dimethyl sulfide) and cooler (isovaleric acid and rotundone, Table S2.2) climate Shiraz (Mayr *et al*., 2016), Grenache Rosé (β-damascenone, and 3-mercapto-1-hexanol, Table S2.3) (Ferreira *et al*., 2002), Maccabeo (4-mercapto-4-methylpentan-2-one, Table S2.4) (Escudero, *et al.,* 2004) and Sauvignon blanc (β-damascenone, varietal thiols, esters, and higher alcohols, Table S2.5) (Benkwitz *et al*., 2012).

Generally, authors report that the reconstruction resulted in a reasonable approximation of the wines in question, but they also indicate that the reconstructed wine samples were lacking when compared to the wine reference (Escudero *et al*., 2004; Mayr *et al*., 2014; Benkwitz *et al*., 2012). Interestingly, in the case of Maccabeo (Table S2.4) the addition of a single compound 4-mercapto-4-methylpentan-2-one, a key odorant also identified in Scheurebe (Guth, 1997), masked the chemical nuances of the reconstituted wine and a fruity and fresh character, reminiscent of the reference wine, was more evident (Escudero *et al.,* 2004). This emphasizes the limitation of this workflow as, without extensive chemical characterisation of the sample, one would easily miss a compound present at ng/L, as the thiol in this instance, which is pivotal for the aroma reconstitution (Escudero *et al*., 2004).

Mayr *et al* (2016) sought to verify the importance of the identified compounds, by reconstituting these two Shiraz aroma profiles in a model wine matrix (Table S2.2) and evaluating them based on several descriptors. The absence of an essential constituent as observed by Escudero *et al* (2004), could explain why one of these wines was a poor approximation of its prototype. Nonetheless, the elimination of the rotundone or oak-related compounds brought about the expected decrease in the peppery or oaky character, respectively. Interestingly, for both "wines", the omission of oak-related compounds (guaiacol, cis-oak lactone, vanillin, eugenol, 4-methylguaiacol) also enhanced the perception of chocolate, as did the omission of the non-volatile fraction. This illustrates the complexity of wine aroma research, as the authors observed both expected and unexpected changes in aroma perception, in addition to a response to the non-volatile matrix.



Frequency detection methods	Dilution to threshold methods	Direct intensity methods
% Assessors perceiving an aroma at a specific retention time	Evaluate a dilution series of odour extracts	Determines intensity and duration of perception using different quantitative scales
Most influential aromas are perceived most frequently	Assessor identifies at which dilutions the compound can be sensed	
Indication of intensity only	Quantitative determination of perception threshold and a description	

Figure 2.7. A generic schematic, summarising gas chromatography with olfactometric detection (GC-O) instrumentation, and an overview of its applications (Plutowska &Wardencki, 2008; Brattoli *et al*., 2013).

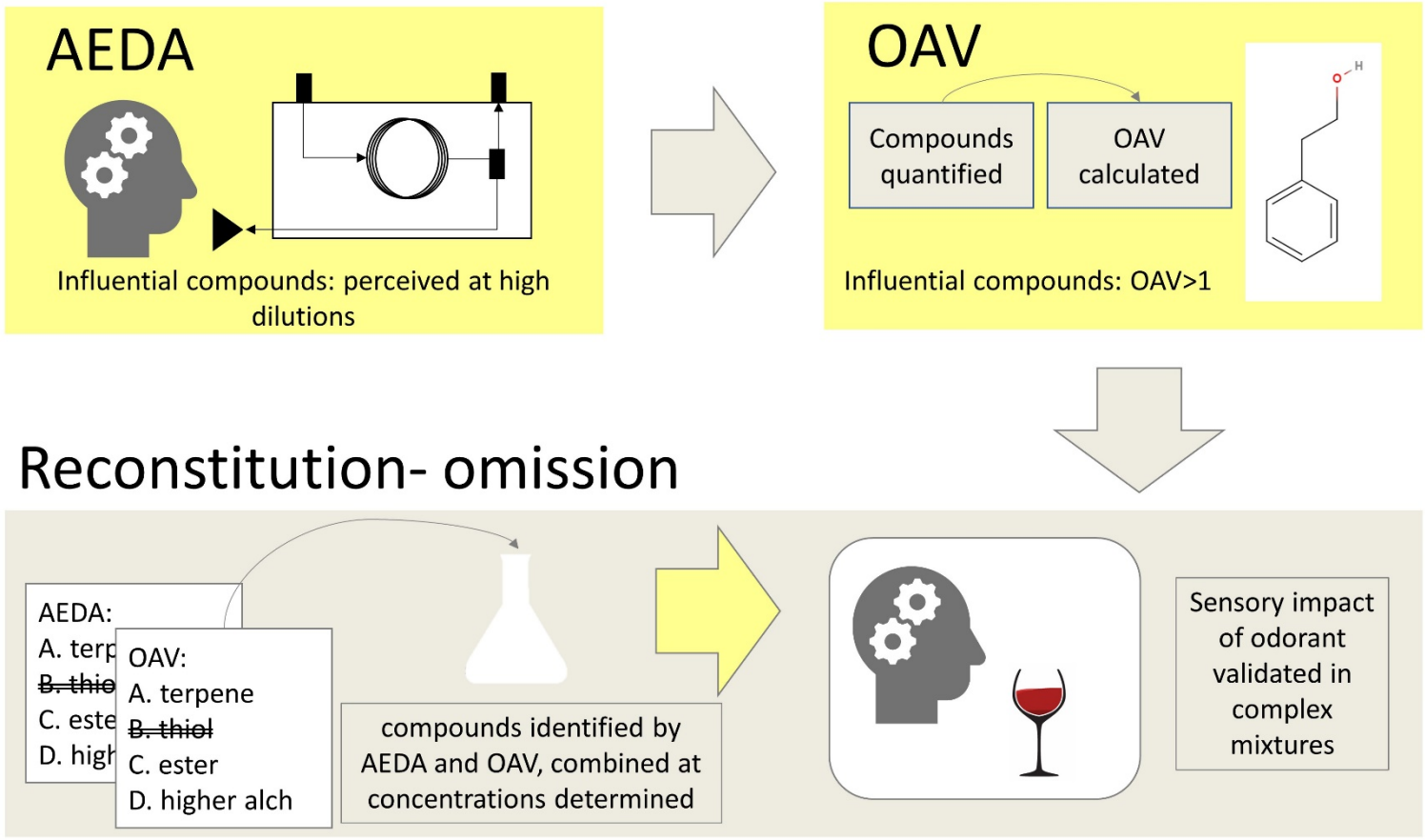


Figure 2.8. An overview of the procedures generally followed to identify key odorants in wine, however, in some instances authors only perform AEDA and OAV calculations.

2.4 Sensory impact of the yeast volatilome

Together with ethanol and acetaldehyde, esters, volatile fatty acids and higher alcohols have been described as having a vinous character, however the subtleties of the individual compounds are not identifiable in this diverse mixture (de-la-Fuente-Blanco *et al.,* 2016). Their individual contributions may be difficult to define, as they generally have related aroma properties and may be perceived as a unit (Escudero *et al.*, 2004). Nonetheless, as shown in tables S2.1 to 2.5, a large number of compounds derived from yeast metabolism (ethyl esters, acetate esters, fatty acids, higher alcohols) were identified as potentially being influential on aroma (AEDA and OIV data) and a few of which altered aroma (Table 2.3).

Table 2.3. Yeast derived metabolites identified as being influential on wine aroma in reconstitution studies.

Yeast metabolite		Reference
Higher alcohols		
	2-phenyl ethanol	Ferreira *et al.,* 2002
	isoamyl alcohol	Ferreira *et al.,* 2002
	higher alcohols collectively	Benkwitz *et al.,* 2012
Esters		
	isoamyl acetate	Guth 1997 a; Guth 1997 b; Ferreira *et al.*, 2002
	ethyl octanoate	Guth 1997 a; Guth 1997 b; Ferreira *et al.*, 2002
	ethyl hexanoate	Guth 1997 a; Guth 1997 b; Ferreira *et al.,* 2002; Benkwitz *et al.*, 2012
	esters collectively	Benkwitz et al., 2012
Volatile fatty acids		
	acetic acid	Guth 1997 a; Guth 1997 b
	butyric acid	Ferreira *et al.,* 2002
	octanoic acid	Ferreira *et al.*, 2002; Benkwitz *et al.,* 2012
	hexanoic acid	Ferreira *et al.,* 2002
	isovaleric acid	Ferreira *et al.*, 2002
	volatile fatty acids collectively	Benkwitz *et al.*, 2012

Higher alcohols are present in wine at comparatively high concentrations. Similarly, they also have high perception threshold concentrations (Table 2.1), which is beneficial due to their solvent or fusel character. It is widely reported that higher alcohols contribute positively to wine aroma at concentrations below 300 mg/L, but may have a deleterious influence at concentrations in excess of 400 mg/L (Lambrechts & Pretorius, 2000; Bell &and Henschke, 2005). A recent study sought to verify this assertion, by using a model wine-like solution (Table S2.6) with specific aroma characteristics, namely, fruity, woody, and animal, to which they added increasing concentrations of higher alcohols (de-la-Fuente-Blanco, *et al.*, 2017). In an earlier publication, the same research group reported, that

without a specific descriptor, the impact of higher alcohols was difficult to ascertain (de-la-Fuente-Blanco *et al.,* 2016). In response to increasing concentrations of higher alcohols, they observed that at levels greater than 284 mg/L (de-la-Fuente-Blanco, *et al*., 2017), higher alcohols contributed negatively to the perceived quality of samples, which suggests that higher alcohols may only contribute positively to wine complexity at levels below this. Panellists were also tasked with comparing these characteristic aromas to samples spiked with higher alcohols and note when the characteristic aromas changed. They concluded that in a woody or fruity aroma background, higher alcohols conferred a spirit-like character at levels above 281 mg/L. Moreover, higher alcohols suppressed red fruit (299 mg/L) nuances and woody aromas (288 mg/L) but enhanced the perception of animal (365 mg/L) and spirit-like (375 mg/L) aromas.

In contrast to higher alcohols, esters are described with positive fruit descriptors, and as such are highly coveted in wine (Pineau, *et al.,* 2009). In a reconstitution study, authors showed that even slight increases in the concentration of ethyl esters, red and dark berry notes were more pronounced (Pineau *et al.,* 2009). In a follow-up study, Lytra *et al* (2013), observed that in a reconstitution scenario, individually, all esters present above their perception thresholds were readily perceived. Moreover, they noted that when compared to the full complement of esters, the omission of related odorants and odorants present at subthreshold levels significantly altered the perceived aromas (Lytra *et al.,* 2013). Specifically, ethyl-3-hydroxybutanoate supplementation, at concentrations below its perception threshold, displayed a hyper-additive behaviour, significantly lowering the perception thresholds of fruity aromas.

2.5 Conclusion

Wine complexity limits the efficacy of the reductionist approaches applied to understanding the sensory impact of a wine's chemical composition. This is partly because wine aroma is not simply the sum of all its parts but rather a dynamic mixture of compounds evolving and interacting with each other, altering sensory perception and defying definition. These dynamic interactions could account for the often-conflicting assertions made regarding the impact of certain odorants.

An additional challenge is that the sensory evaluation of wine utilises a fallible instrument, that can be calibrated (trained) but is still vulnerable to both internal and external influences. There are several stages associated in wine tasting, the first being anticipatory of the experience, followed by a visual assessment, aroma, ingestion, and swallowing (Shepherd, 2015). All these elements act together, to inform the assessment of wine, and transform these perceptions into a descriptor. This entire

process has been described as being based on an illusion, as a few drops of food colouring has been shown to alter wine aroma perception from white wine to red (Morrot *et al.,* 2001).

Yeast metabolism is a powerful tool which converts grape juice into wine and produces many of the flavour impacting compounds via its primary and secondary metabolism, yet the question of what this contribution signifies within the broader wine sensory space remains somewhat unanswered.

2.6 References

Atanasova, B., Thomas-Danguin, T., Chabanet, C., Langlois, D., Nicklaus, S. & Etiévant, P., 2005. Perceptual interactions in odour mixtures: Odour quality in binary mixtures of woody and fruity wine odorants. Chem. Senses 30, 3, 209–217.

Atanasova, B., Thomas-Danguin, T., Langlois, D., Nicklaus, S., Chabanet, C. & Etiévant, P., 2005. Perception of wine fruity and woody notes: Influence of peri-threshold odorants. Food Qual. Prefer. 16, 6, 504–510.

Äyräpää, T., 1971. Biosynthetic formation of higher alcohols by yeast. Dependence on the nitrogenous level of the medium. J. Inst. Brew. 77, 3, 266–276.

Bell, S.J. & Henschke, P. a, 2005. Implications of nitrogen nutrition for grapes, fermentation and wine. Aust. J. Grape Wine Res. 11, 3, 242–295.

Benkwitz, F., Nicolau, L., Lund, C., Beresford, M., Wohlers, M. & Kilmartin, P.A., 2012. Evaluation of key odorants in Sauvignon blanc wines using three different methodologies. J. Agric. Food Chem. 60, 25, 6293–6302.

Bisson, L.F. & Karpel, J.E., 2010. Genetics of Yeast Impacting Wine. Quality Annu. Rev. Food Sci. Technol. 1, 1, 139–162.

Brattoli, M., Cisternino, E., Rosario Dambruoso, P., de Gennaro, G., Giungato, P., Mazzone, A., Palmisani, J. & Tutino, M., 2013. Gas chromatography analysis with olfactometric detection (GC-O) as a useful methodology for chemical characterization of odorous compounds. Sensors (Switzerland) 13, 12, 16759–16800.

Chollet, S., Lelièvre, M., Abdi, H. & Valentin, D., 2011. Sort and beer: Everything you wanted to know about the sorting task but did not dare to ask. Food Qual. Prefer. 22, 6, 507–520.

Coetzee, C., Brand, J., Emerton, G., Jacobson, D., Silva Ferreira, A.C. & du Toit, W.J., 2015. Sensory interaction between 3-mercaptohexan-1-ol, 3-isobutyl-2-methoxypyrazine and oxidation-related compounds. Aust. J. Grape Wine Res. 21, 2, 179–188.

Coombe, B.G. & Iland, P.G., 2005. Grape berry development and wine quality. In: Dry, P.R. & Coombe, B.G. (eds). Viticulture. Volume 1-resources, Second Edition, Winetitles. Pp. 210-248.

Crépin, L., Truong, N.M., Bloem, A., Sanchez, I., Dequin, S. & Camarasa, C., 2017. Management of multiple nitrogen sources during wine fermentation by *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 83, 5, e02617-16.

de-la-Fuente-Blanco, A., Sáenz-Navajas, M.P. & Ferreira, V., 2017. Levels of higher alcohols inducing aroma changes and modulating experts' preferences in wine model solutions. Aust. J. Grape Wine Res. 23, 2, 162–169.

de-la-Fuente-Blanco, A., Sáenz-Navajas, M.P. & Ferreira, V., 2016. On the effects of higher alcohols on red wine aroma. Food Chem. 210, 107–114.

Drawert, F., 1974. Winemaking as a Biotechnological Sequence. In: Webb, A.D. (eds). Chemistry of Winemaking, American Chemical Society: Washington. Pp. 1-10.

Dubourdieu, D., Tominaga, T., Masneuf, I., Des Gachons, C.P. & Murat, M.L., 2006. The role of yeasts in grape flavor development during fermentation: The example of Sauvignon blanc. Am. J. Enol. Vitic. 57, 1, 81–88.

Dufour, J.P., Malcorps, P.H. & Silcock, P., 2003. Control of Ester Synthesis During Brewery Fermentation. In: Smart, K. (ed). Brewing Yeast Fermentation Performance, Second Edition, Blackwell Science, Oxford. pp. 213–233

Etievant, P., 1991. Wine. In: Maarse, H (eds). Volatile compounds in food and beverages. Zeist, The Netherlands: TNO- CIVO Food Analysis Institute. pp. 486–546

Escudero, A., Gogorza, B., Melús, M.A., Ortín, N., Cacho, J. & Ferreira, V., 2004. Characterization of the aroma of a wine from Maccabeo. Key role played by compounds with low odor activity values. J. Agric. Food Chem. 52, 11, 3516–3524.

Escudero, A., Campo, E., Fariña, L., Cacho, J. & Ferreira, V., 2007. Analytical characterization of the aroma of five premium red wines. Insights into the role of odor families and the concept of fruitiness of wines. J. Agric. Food Chem. 55, 11, 4501–4510.

Ferreira V. & Cacho J., 2009. Identification of Impact Odorants of Wines. In: Moreno-Arribas, M.V.& Polo M.C. (eds). Wine Chemistry and Biochemistry, vol I. Springer, New York, NY. pp. 293 -410

Ferreira, V., López, R. & Cacho, J.F., 2000. Quantitative determination of the odorants of young red wines from different grape varieties J. Sci. Food Agric. 80, 11, 1659–1667.

Ferreira, V., Ortín, N., Escudero, A., López, R., Cacho, J., Ortin, N., Escudero, A., Lopez, R., Cacho, J., 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. J. Agric. Food Chem. 50, 14, 4048–4054.

Francis, I.L. & Newton, J.L., 2005. Determining wine aroma from compositional data. Aust. J. Grape Wine Res. 11, 2, 114–126.

Grosch, W., 2001. Evaluation of the key odorants of foods by dilution experiments, aroma models and omission Chem. Senses 26, 5, 533–545.

Guth, H., 1997a. Quantitation and Sensory Studies of Character Impact Odorants of Different White Wine Varieties. J. Agric. Food Chem. 45, 8, 3027–3032.

Guth, H., 1997b. Identification of character impact odorants of different white wine varieties. J. Agric. Food Chem. 45, 8, 3022–3026.

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. Appl. Environ. Microbiol. 74, 8, 2259–2266.

Hirst, M.B. & Richter, C.L., 2016. Review of aroma formation through metabolic pathways of *Saccharomyces cerevisiae* in beverage fermentations. Am. J. Enol. Vitic. 67, 4, 361–370.

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, Int. J. Gen. Mol. Microbiol. 92, 1, 61–75.

Lambrechts, M.G. & Pretorius, I.S., 2000. Yeast and its Importance to Wine Aroma - A Review South African J. Enol. Vitic. 21, Special Issue, 97–129.

Lawless, H. T., & Heymann, H., 2010. Sensory evaluation of food. Principles and practices. (2nd edition). New York: Springer

Lilly, M., Lambrechts, M.G. & Pretorius, I.S., 2000. Effect of increased yeast alcohol acetyltransferase activity on flavor profiles of wine and distillates. Appl. Environ. Microbiol. 66, 2, 744–753.

Lytra, G., Tempere, S., Le Floch, A., de Revel, G. & Barbe, J.-C.C., 2013. Study of sensory interactions among red wine fruity esters in a model solution. J. Agric. Food Chem. 61, 36, 8504–8513.

Mason, A.B. & Dufour, J.P., 2000. Alcohol acetyltransferases and the significance of ester synthesis in yeast. Yeast 16, 14, 1287–1298.

Mayr, C.M., Geue, J.P., Holt, H.E., Pearson, W.P., Jeffery, D.W. & Francis, I.L., 2014. Characterization of the key aroma compounds in shiraz wine by quantitation, aroma reconstitution, and omission studies. J. Agric. Food Chem. 62, 20, 4528–4536.

Mendes-Pinto, M.M., 2009. Carotenoid breakdown products the-norisoprenoids-in wine aroma. Arch. Biochem. Biophys. 483, 2, 236–245.

Morrot, G., Brochet, F. & Dubourdieu, D., 2001. The color of odors. Brain Lang. 79, 2, 309–320.

Pineau, B., Barbe, J.C., Leeuwen, C. Van & Dubourdieu, D., 2009. Examples of perceptive interactions involved in specific "Red-" and "Black-berry" aromas in red wines. J. Agric. Food Chem. 57, 9, 3702–3708.

Plutowska, B. & Wardencki, W., 2008. Application of gas chromatography-olfactometry (GC-O) in analysis and quality assessment of alcoholic beverages - A review. Food Chem. 107, 1, 449–463.

Rapp, A. & Versini, G., 1995. Influence of nitrogen compounds in grapes on aroma compounds in wine. Dev. Food Sci. 37, 1659–1694.

Robinson, A.L., Boss, P.K., Solomon, P.S., Trengove, R.D., Heymann, H. & Ebeler, S.E., 2014. Origins of Grape and Wine Aroma . Part 2 . Chemical and Sensory Analysis Am. J. Enol. Vitic. 1, 25–42.

Rollero, S., Bloem, A., Camarasa, C., Sanchez, I., Ortiz-Julien, A., Sablayrolles, J.M., Dequin, S. & Mouret, J.R., 2015. Combined effects of nutrients and temperature on the production of fermentative aromas by *Saccharomyces cerevisiae* during wine fermentation. Appl. Microbiol. Biotechnol. 99, 5, 2291–2304.

Rollero, S., Mouret, J.-R.R., Bloem, A., Sanchez, I., Ortiz-Julien, A., Sablayrolles, J.-M.M., Dequin, S. & Camarasa, C., 2017. Quantitative 13C-isotope labelling-based analysis to elucidate the influence of environmental parameters on the production of fermentative aromas during wine fermentation. Microb. Biotechnol. 10, 6, 1649–1662.

Sáenz-Navajas, M.P., Campo, E., Culleré, L., Fernández-Zurbano, P., Valentin, D. & Ferreira, V., 2010. Effects of the nonvolatile matrix on the aroma perception of wine. J. Agric. Food Chem. 58, 9, 5574–5585.

Sáenz-Navajas, M.P., Campo, E., Avizcuri, J.M., Valentin, D., Fernández-Zurbano, P. & Ferreira, V., 2012. Contribution of non-volatile and aroma fractions to in-mouth sensory properties of red wines: Wine reconstitution strategies and sensory sorting task. Anal. Chim. Acta 732, 64–72.

Saerens, S.M.G., Verstrepen, K.J., Laere, S.D.M. Van, Voet, A.R.D., Dijck, P. Van, Delvaux, F.R., Thevelein, J.M., Van Laere, S.D.M., Voet, A.R.D., Van Dijck, P., Delvaux, F.R. & Thevelein, J.M., 2006. The *Saccharomyces cerevisiae* EHT1 and EEB1 genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity. J. Biol. Chem. 281, 7, 4446–4456.

Saerens, S.M.G., Delvaux, F., Verstrepen, K.J., Van Dijck, P., Thevelein, J.M. & Delvaux, F.R., 2008. Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation. Appl. Environ. Microbiol. 74, 2, 454–461.

Saerens, S.M.G., Duong, C.T. & Nevoigt, E., 2010. Genetic improvement of brewer's yeast: current state, perspectives and limits. Appl. Microbiol. Biotechnol. 86, 5, 1195–1212.

San-Juan, F., Ferreira, V., Cacho, J. & Escudero, A., 2011. Quality and aromatic sensory descriptors (mainly fresh and dry fruit character) of spanish red wines can be predicted from their aroma-active chemical composition. J. Agric. Food Chem. 59, 14, 7916–7924.

Shepherd, G.M., 2015. Neuroenology: how the brain creates the taste of wine. Flavour 4, 1, 19.

Styger, G., Prior, B. & Bauer, F.F., 2011. Wine flavor and aroma. J. Ind. Microbiol. Biotechnol. 38, 9, 1145–1159.

Sumby, K.M., Grbin, P.R. & Jiranek, V., 2010. Microbial modulation of aromatic esters in wine: Current knowledge and future prospects. Food Chem. 121, 1, 1–16.

Swiegers, J.H. & Pretorius, I.S., 2005. Yeast modulation of wine flavor. Adv. Appl. Microbiol. 57, SUPPL. A, 131–175.

Ugliano, M.& Henschke, P.A., 2009. Yeasts and Wine Flavour. In: Moreno-Arribas, M.V. & Polo, M.C. (eds). Wine Chemistry and Biochemistry. Springer, New York, NY. pp. 313-392.

Valentin, D., Chollet, S., Lelièvre, M. & Abdi, H., 2012. Quick and dirty but still pretty good: A review of new descriptive methods in food science. Int. J. Food Sci. Technol. 47, 8, 1563–1578.

Varela, P. & Ares, G., 2012. Sensory profiling, the blurred line between sensory and consumer science. A review of novel methods for product characterization. Food Res. Int. 48, 2, 893–908.

Verstrepen, K.J., Van Laere, S.D.M., Vanderhaegen, B.M.P., Derdelinckx, G., Dufour, J.-P., Pretorius, I.S., Winderickx, J., Thevelein, J.M. & Delvaux, F.R., 2003. Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters. Appl. Environ. Microbiol. 69, 9, 5228–5237.

Villamor, R.R. & Ross, C.F., 2013. Wine matrix compounds affect perception of wine aromas. Annu. Rev. Food Sci. Technol. 4, 1–20.

Wood, C., Siebert, T.E., Parker, M., Capone, D.L., Elsey, G.M., Pollnitz, A.P., Eggers, M., Meier, M., Vössing, T., Widder, S., Krammer, G., Sefton, M. a. & Herderich, M.J., 2008. From wine to pepper: Rotundone, an obscure sesquiterpene, is a potent spicy aroma compound. J. Agric. Food Chem. 56, 10, 3738–3744.

Yoshioka, K. & Hashimoto, N., 1981. Ester formation by alcohol acetyltransferase from brewers' yeast. Yeast 45, 10, 2183–2190.

2.7 Supplementary material

Table S2.1. An overview of the aroma compounds that may have a potential impact on the aroma of Gewürztraminer and Scheurebe, as identified by AEDA and OAV evaluations, and the findings of the reconstitution-omission tests applied to verify their importance (Guth 1997 a and b).

White wines	Influential compounds identified by AEDA (FD >10)	Influential compounds with OAV above 1	Reconstitution/ omission (model wine)
Gewürztraminer	Compounds perceived at FD 1000: **wine lactone** Compounds perceived at FD 100: ethyl-2-methyl butyrate; 3-methylbutanol; 2-phenyl ethanol; 3-ethylphenol; sotolon Compounds perceived at FD 10: ethyl isobutyrate; ethyl butyrate; ethyl-3-methylbutyrate; 2-methylpropanol; **ethyl hexanoate; cis-rose oxide**; ethyl octanoate; *acetic acid*; linalool; butyric acid; 2/3-methyl butyric acid; 5-ethyl-4-hydroxy-2-methyl-3(2) furanone; ethyl cinnamate	1,1-diethoxyethane (8); ethyl isobutyrate (10); butane-2,3-dione (2); ethyl butyrate (11); ethyl 2-methylbutyrate (4); ethyl 3-methylbutyrate (1); 2-methylpropanol (1); **3-methylbutyl acetate** (97); 3-methylbutanol (4); **ethyl hexanoate** (98); **cis-rose oxide** (105); ethyl octanoate (315); **acetic acid** (1); linalool (12); 3-(methylthio)-1-propanol (3); β-damascenone (17); hexanoic acid (1); geraniol (7); 2-phenylethanol (2); ethyl cinnamate (2); eugenol (1); sotolon (10); (Z)-6-dodeceno-γ-lactone (3); acetaldehyde (4); dimethyl sulfide (1); ethyl acetate (8); dimethyl trisulfide (1)	* Reconstituted wines resembled their respective wine aromas *The Gewürztraminer reconstituted wines lost their similarity with the omission of **cis-rose oxide**, wine-lactone, **ethyl octanoate, acetic acid**; **3-methylbutyl acetate** (isoamyl acetate), and **ethyl hexanoate**. * For Scheurebe, the omission **4-mercapto-4-methylpentan-2-one** (4MMP) altered the aromas
Scheurebe	Compounds perceived at FD 1000: wine lactone Compounds perceived at FD 100: ethyl isobutyrate; ethyl-2-methyl butyrate; 3-methylbutanol; 2-phenyl ethanol; 3-ethylphenol; sotolon Compounds perceived at FD 10: ethyl butyrate; ethyl-3-methylbutyrate; 2-methylpropanol; ethyl hexanoate; **4-mercapto-4methylpentan-2-one**; ethyl octanoate; acetic acid; linalool; butyric acid; 2/3-methyl butyric acid; 5-ethyl-4-hydroxy-2-methyl-3(2) furanone; ethyl cinnamate	ethyl isobutyrate (32); butane-2,3-dione (2); ethyl butyrate (9); ethyl 2-methylbutyrate (5); ethyl 3-methylbutyrate (1); 2-methylpropanol (3); 3-methylbutyl acetate (48); 3-methylbutanol (4); ethyl hexanoate (56); cis-rose oxide (15)**; 4-mercapto-4-methylpentan-2-one** (667); ethyl octanoate (135); acetic acid (1); linalool (20); 3-(methylthio)-1-propanol (22); β-damascenone (20); 2-phenylethyl acetate (1); geraniol (1); 2-phenylethanol (2); ethyl cinnamate (2); wine lactone (10); (Z)-6-dodeceno-γ-lactone (1); acetaldehyde (4); ethyl acetate (3)	

Compounds listed were omitted in the reconstitution evaluations individually

Flavour dilution factor (FD) highest sample dilution at which the odour of the analysed compound is still detectable

Compounds in bold made a significant contribution to the appropriate cultivar's aroma

OAV are included in the parentheses

Table S2.2. An overview of the aroma compounds that may have a potential impact on the aroma of a cool and warm climate Shiraz, as identified by AEDA and OAV evaluations, and the findings of the reconstitution-omission tests applied to verify their importance (Mayr *et al.*, 2016).

Cultivar		Influential compounds identified by AEDA	Influential compounds with OAV above 1	Reconstitution/ omission observations in model wine
Shiraz	warm climate	Compounds perceived at the 1000 times dilution: ethyl 2-methyl propanoate; 2,3-butanedione ethyl butanoate; ethyl 2-methylbutanoate ethyl 3-methylbutanoate; isoamyl alcohol/active amyl alcohol; ethyl hexanoate furfuryl ethyl ether; furfural, butanoic acid; **β-damascenone; guaiacol;** 2-phenylethylethanol Compounds perceived at the 100 times dilution: 2/3-methylbutylacetate; ethyl pentanoate methyl hexanoate; **cis-oak lactone**; trans-oak lactone	acetic acid (3.4); ethyl lactate (2.4); ethyl acetate (13.4); isoamyl alcohol (5.4); 2-phenylethyl alcohol (1.9); methionol (2); **hexanoic acid** (4.4); **octanoic acid** (3); 2-methyl-1-butyl acetate (17); isovaleric acid (13); butanoic acid (2,5); ethyl octanoate (76); ethyl hexanoate (26); 2-methylbutanoic acid (10); **cis-oak lactone** (13); ethyl butanoate (15); vanillin* (1.5); ethyl-2-methylpropanoate (14); **dimethyl sulfide** (13); ethyl isovalerate (18); 4-ethylphenol* (3.5); **eugenol** (6.8); ethyl-2-methylbutanoate (36); **guaiacol (1.5); linalool*** (1); sotolon* (1.8); methanethiol* (1.4); **β-damascenone** (44)	*Warm climate sample was more wine-like than the cool climate reconstituted wine *It was also rated more highly for overall fruit, dark fruit, oak and chocolate *Cool climate samples had a nail polish trait not observed in the real wine, but was generally described with green and geranium descriptors *The omission of the non-volatile compounds (glucose, fructose, glycerol, lactic and succinic acid) increased the chocolate notes in both samples *Dimethyl sulphide or oak related compounds (guaiacol, cis-oak lactone, vanillin, eugenol, 4-methylguaiacol) lowered perceived fruitiness in the warm climate sample *The absence of linalool or rotundone increased the perception of dark fruit *In both samples, the omission of the oaky compounds reduced the oak character *The absence of rotundone displayed the expected decrease in the pepper aroma (Cool climate) *The omission of medium chain fatty acids (hexanoic, octanoic and decanoic acid) had little impact on wine aroma
	cool climate	Compounds perceived at the 1000 times dilution: ethyl butanoate; ethyl 2-methylbutanoate; ethyl 3-methylbutanoate; methyl hexanoate; isoamyl alcohol/active amyl alcohol; ethyl hexanoate; furfuryl ethyl ether; furfural; butanoic acid; **β-damascenone; guaiacol;** 2-phenylethylethanol Compounds perceived at the 100 times dilution: ethyl 2-methyl propanoate; ethyl pentanoate; ethyl 2-hydroxy-3-methylbutanoate; 5-methylfurfural; butanoic acid; **cis-oak lactone**	acetic acid (2.6); ethyl lactate (1.6); ethyl acetate (8.4); isoamyl alcohol (6.9); active amyl alcohol*(1.2); isobutanol*(1.4); 2-phenylethyl alcohol (4); methionol (3.1); **hexanoic acid (4.5); octanoic acid (3.4);** 2-methyl-1-butyl acetate (15.5); isovaleric acid (28.3); ethyl octanoate (87.8); ethyl hexanoate (27.9); 2-methylbutanoic acid (27.5); **cis-oak lactone (10);** ethyl butanoate (11.7); ethyl-2-methylpropanoate (23.5); **dimethyl sulfide (3.7);** ethyl isovalerate (33.3); **eugenol (4.7);** ethyl-2-methylbutanoate; (76); **guaiacol (1.2); β-damascenone (24);** hydrogen sulfide* (1.6); rotundone (4)	

The asterisk * highlights differences between warm and cool climate Shiraz

Compounds listed in bold were omitted in the reconstitution evaluations

OAV are included in the parentheses

Table S2.3. An overview of the aroma compounds that may have a potential impact on the aroma of Grenache Rosé, as identified by AEDA and OAV evaluations, and the findings of the reconstitution-omission tests applied to verify their importance (Ferreira *et al*., 2002).

Cultivar	Influential compounds identified by AEDA (FD >10)	Influential compounds with OAV above 1	Reconstitution/ omission observations in model wine
Grenache Rose	Compounds perceived at FD 50: **isoamyl alcohol**; 3-methyl-3-furanthiol; **ethyl octanoate**; linalool; isobutyric acid; **butyric acid**; **isovaleric acid**; **methionol**; **3-mercapto-1-hexanol**; **furananeol**; □-decalactone; sotolon; 4-vinylphenol; pheylacetic acid Compounds perceived at FD16: eyhyl isobutyrate; ethyl butyrate; **isoamyl acetate**; **ethyl hexanoate**; acetoin; acetic acid; **β-damascenone**; guaiacol; ethyl dihydrocinnamate; **2-phenylethyl alcohol**; ethyl cinnamate	**3-mercapto-1-hexanol** (67); **β-damascenone** (61); **isoamyl acetate** (42); **ethyl octanoate** (41); ethyl hexanoate (39); **isovaleric acid** (21); **butyric acid** (11); ethyl butyrate (9.8); **furaneol** (7.2); isobutyric acid (5.8); **isoamyl alcohol** (5.7); **octanoic acid** (5.1); **hexanoic acid** (4.9); **methionol** (1.8); **2-phenylethyl alcohol** (1.5); ethyl isobutyrate (1.2); ethyl isovalerate (1); ethyl acetate (3.2); γ-nonalactone (2.4); isobutanol (1.3); β-ionone (1.1)	*Three reconstitution models were created: The first (1) containing all the compounds identified, the second only compound with a OAV > 0.5 (2) and lastly using only compounds greater than 10 (3). *Models 1 and 2 were more similar to the reference wine than 3, suggesting that compounds with an OAV less than 0.5 contribute positively to wine aroma *The omission of the following compounds brought about a significant changed in the overall aroma: **2-phenyl ethanol, butyric acid, isoamyl alcohol; ethyl octanoate; methionol; octanoic acid; hexanoic acid; ethyl hexanoate; isovaleric acid; isoamyl acetate; □-damascenone**; **furaneol/ homofuraneol** and **3-mercapto-1-hexanol**.

The absence of the compounds listed in bold brought about a change in aroma profiles

Compounds listed were omitted in the reconstitution evaluations

Flavour dilution factor (FD) highest sample dilution at which the odour of the analysed compound is still detectable

Table S2.4. An overview of the aroma compounds that may have a potential impact on the aroma of Maccabeo, as identified by AEDA and OAV evaluations, and the findings of the reconstitution-omission tests applied to verify their importance (Escudero, et., 2004).

Cultivar	Influential compounds identified by AEDA	Influential compounds with OAV above 1	Reconstitution/ omission observations in dearomatized wine
Maccabeo	*Compounds perceived at FD 50:* **ethyl isobutyrate**; **ethyl butyrate**; **ethyl hexanoate**; **isovaleric acid**; **β-damascenone**; **hexanoic acid**; **guaiacol**; **2-phenylethylethanol**; **4,5-dimethyl-3-hydroxy-2(5H) furanone**; **2,6-dimethoxyphenol**; **phenylacetic acid** *Compounds perceived at 16 FD:* **isoamyl alcohol**; **isoamyl acetate**, **4-vinylguaiacol**; methyl anthranilate; 4-ethylphenol; **m-cresol**; **octanoic acid**; **2-phenylethyl acetate**; **ethyl octanoate**; 2-methyl-3-furanthiol	**ethyl octanoate** (139); **-damascenone** (110); **isoamyl acetate** (83); **ethyl hexanoate** (58); **4-vinylguaiacol** (31); **ethyl butyrate** (30); **isovaleric acid** (15); **octanoic acid** (9.9); **butyric acid** (7.7); **isoamyl alcohol** (6.9) ethyl acetate (6.9); **2,5-dimethyl-4-hydroxy-3(2H)-furanone** (6); **hexanoic acid** (5.9); **ethyl isobutyrate** (2.2); ethyl decanoate (2.1); ethyl isovalerate (1.7); (Z)-3-hexenol (1.5); isobutanol (1.4); ethyl cinnamate (1.4); **-**ionone (1.3); methionol (1.2); **2-phenethyl alcohol** (1); **4,5-dimethyl-3-hydroxy-2(5H)-furanone** (1)	*Authors reconstituted wines with all the compounds quantified (C), OAV > 1 (A), and OAV > 0.1 (B) *Reconstituted wines were compared to real (reference sample) and dearomatized wine (duo-trio test) *Overall the reconstituted wines were significantly different from the dearomatized wine, but not each other *Reconstituted wines were also significantly different from the wine reference samples due to an "alien" chemical character *Only the omission of **-**damascenone significantly altered the perceived aromas compared the reconstituted wine (C) *4-mercapto+-4-methylpenan-2-one, and 2-methyl-3-furanthiol, identified in the AEDA, but not included in the initial reconstitution was included in a forth reconstituted sample, and alleviated the chemical character referred to above

Compounds listed in bold were omitted in the reconstitution evaluations

Flavour dilution factor (FD) highest sample dilution at which the odour of the analysed compound is still detectable

Table S2.5. An overview of the aroma compounds that may have a potential impact on the aroma of Sauvignon blanc, as identified by AEDA and OAV evaluations, and the findings of the reconstitution-omission tests applied to verify their importance (Benkwitz *et al*., 2012).

Cultivar	Influential compounds identified by AEDA (FD >10)	Influential compounds with OAV above 1	Reconstitution/ omission observations in dearomatized wine
Sauvignon blanc	β-damascenone; phenylethanol; 3-mercaptohexyl acetate; isoamyl alcohol; 3-mercaptohexanol; 2-phenylethyl acetate; isoamyl acetate; ethyl hexanoate; 4-mercapto-4-methylpentan-2-one; ethyl butanoate	β-damascenone (32); phenylethanol (2); 3-mercaptohexyl acetate (338); isoamyl alcohol (2); 3-mercaptohexanol (154); 2-phenylethyl acetate (1.1); isoamyl acetate (111); ethyl hexanoate (17); 4-mercapto-4-methylpentan-2-one (12.5); ethyl butanoate (14.5); hexyl acetate (2); 3-hexenol (1); octanoic acid (4.2); 2-Methoxy-3-isobutylpyrazine (5); ethyl octanoate (2.1)	*Authors reconstituted wines with all the compounds quantified (complete), and omitted groups of related compounds (thiols, fatty acids, alcohols, C-6 alcohol, esters and terpenes) and individual compounds in a second experiment *The reconstituted wine was rated as more intense than the wine for several attributes (passionfruit skin stalk, sweet sweaty passionfruit, cat urine, capsicum, grassy, flinty, bourbon, and apple lolly) *The omission of ethyl hexanoate increased the apple and banana lolly character whilst decreasing the intensity of flinty, honey mead and apple aromas * The absence of octanoic acid lowered the intensity of flinty notes, but intensified the citrus character *Omission of esters decreased the intensity of cat urine, sweet-sweaty-passion fruit, passion fruit skin stalk, apple lolly, stone fruit, apple and tropical *The absence of terpenes the apple, stone fruit, apple lolly character was less intense, but the intensity of cat urine and sweet-sweaty-passion fruit notes were increased * The absence of higher alcohols or volatile fatty acids lowered the intensity of flinty and bourbon characters * β-damascenone omission alone had little sensory impact, but its absence paired with a thiol dramatically altered the perceived aromas

All compounds listed were omitted in the reconstitution evaluations

Flavour dilution factor (FD) highest sample dilution at which the odour of the analysed compound is still detectable

Table S2.6. The chemical composition of the model wines and the aromatic nuances used by de-la-Fuente-Blanco (2017) to evaluate the impact of increasing concentration of higher alcohols on aroma perception.

Model wine base aroma		mg/L
Volatile components		
	Acetic acid	150
	Ethyl acetate	50
	Hexanoic acid	2
	3-Methylbutanoic acid	0.3
	2,3-Butanedione	0.4
	Ethyl hexanoate	1
	Isoamyl acetate	1
	Ethyl -2-methylbutanoate	0.12
	Ethyl vanillate	0.25
	Vanilla	0.07
	γ-Nonalactone	0.02
	Guaiacol	0.01
	β -Damascenone	0.004
	β -Ionone	0.0003
Non-volatile		
	Tartaric acid	5000
	Glycerol	10000
	Tannic acid	50
	Quinine	7
	Arabic gum	75

Descriptive nuances		mg/L
Fruity nuance		
	2,3-Butanedione	14
	Isoamyl acetate	5.5
	Ethyl acetate	50
	Ethyl cinnamate	0.12
	β-Damascenone	0.003
Woody nuance		
	Whisky lactone	0.3
	Vanilla	0.1
	Eugenol	0.015
	Guaiacol	0.015
	4-Hydroxy-2,5-dimethyl-3(2H)-furanone	0.1
Animal nuance		
	4-Ethylphenol	100
	4-Ethylguaiacol	13

Chapter 3

The impact of single amino acids on growth and volatile aroma production by *Saccharomyces cerevisiae* strains

Chapter 3

The impact of single amino acids on growth and volatile aroma production by *Saccharomyces cerevisiae* strains

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Abstract

Nitrogen availability and utilization by *Saccharomyces cerevisiae* significantly influence fermentation kinetics and the production of volatile compounds important for wine aroma. Amino acids are the most important nitrogen source and have been classified based on how well they support growth. This study evaluated the effect of single amino acids on growth kinetics and major volatile production of two phenotypically different commercial wine yeast strains in synthetic grape must. Four growth parameters, lag phase, maximum growth rate, total biomass formation and time to complete fermentation were evaluated. In contrast with previous findings, in fermentative conditions, phenylalanine and valine supported growth well and asparagine supported it poorly. The four parameters showed good correlations for most amino acid treatments, with some notable exceptions. Single amino acid treatments resulted in the predictable production of aromatic compounds, with a linear correlation between amino acid concentration and the concentration of aromatic compounds that are directly derived from these amino acids. With the increased complexity of nitrogen sources, linear correlations were lost and aroma production became unpredictable. However, even in complex medium minor changes in amino acid concentration continued to directly impact the formation of aromatic

compounds, suggesting that the relative concentration of individual amino acids remains a predictor of aromatic outputs, independently of the complexity of metabolic interactions between carbon and nitrogen metabolism and between amino acid degradation and utilisation pathways.

3.1 Introduction

During fermentation, yeast take up and metabolize amino acids and other nutrients to support growth and produce biomass. In the process, a range of volatile aroma compounds, including esters, higher alcohols, volatile fatty acids, carbonyls and sulphur compounds are produced. The production of many of these aroma impact compounds is directly dependent on the nitrogen sources that are present during fermentation (Hernández-Orte et al., 2002; Gutiérrez et al., 2012; Torrea et al., 2011; Burin et al., 2015; Dickinson et al., 1997; Ferreira et al., 2015; Styger et al., 2012; Stribny et al., 2015; Miller et al., 2007). In part, for this reason, the metabolic fate and efficiency of amino acids have been studied extensively (Cooper 1982; Godard et al., 2007; Ljungdahl and Dignan-Fornier 2012; Monteiro and Bisson 1992; Crépin et al., 2012; Crépin et al., 2017). However, our understanding of the metabolic regulation of these pathways remains limited, as does our ability to predict the aromatic outcome of a fermentation based on the chemical composition of the grape must.

Grape must nitrogen concentration and composition are highly variable, affecting yeast metabolism and consequently wine aroma (Bell and Henschke, 2005). *Saccharomyces cerevisiae* differentially utilizes amino acids which have been classified according to their ability to support growth, measured as generation time, when present as the sole nitrogen source (Cooper, 1982; Ljungahl and Daignan-Fornier, 2012). Alanine, arginine, asparagine, aspartate, glutamate, glutamine, and serine were classified as preferred nitrogen sources and all other nitrogen sources as either intermediate or non-preferred (Ljundahl and Daignan-Fornier, 2012). These studies provide important foundational data sets for elucidating the impact of amino acid composition on yeast growth. However, such studies have mostly been carried out with laboratory strains and in conditions that are very different from those encountered during winemaking.

The impact of complex amino acid mixtures in real and synthetic grape must on the synthesis of aromatic metabolites has been the focus of extensive studies (Barbosa et al., 2009; Garde-

Cerdán and Ancín-Azpilicueta, 2007; Hernández-Orte et al., 2006; Hernández-Orte et al., 2002). Many of the findings link the formation of various aroma compounds such as fusel alcohols and fusel acids to the degradation of branched-chain and aromatic (BCAAs) amino acids, via the Ehrlich pathway (Hazelwood et al., 2008; Ehrlich, 1907). Nitrogen metabolism is highly complex, as its intermediates are also shared between other metabolic pathways, including carbon metabolism. Consequently, the different nitrogen treatments and fermentation media used by various studies have thus far yielded an incomplete understanding of the impact of amino acid composition on aroma compound formation under fermentative conditions. It remains impossible to predict the aromatic output of a yeast strain in a medium with a complex nitrogen mixture even when the fermentation medium is fully described. Therefore, it is important to establish a foundational data set to evaluate how individual amino acids affect yeast growth, and aroma formation under fermentative conditions, and to establish to what degree such data can be extrapolated to predict the production of aromatic compounds when more complex nitrogen sources are used. To our knowledge, this is the first study to provide a comprehensive analysis of the growth kinetics of commercial wine yeast strains in response to single amino acids, as well as assessing the relationships between amino acid concentration and the formation of volatile compounds in simple and more complex matrices.

3.2 Materials and Methods

3.2.1 Yeast strains

Two industrial *S. cerevisiae* strains VIN13 (Anchor Yeast, Cape Town, South Africa) and BM45 (Lallemand Inc., Montreal, Canada) were used, due to reported differences in fermentation kinetics and the resultant aroma profiles. The yeast pre-culture procedure was as follows: A single yeast colony was inoculated into YPD (100 mL) and incubated overnight at 30°C with agitation. Cultures were centrifuged and washed with distilled water and used to inoculate 100 mL YPD broth at an OD_{600nm} of 0.1 (approximately 10^6 CFU/mL) and again incubated overnight at 30°C. Cells were then centrifuged, washed, and resuspended in sterile distilled water. The fermentation media was then inoculated at an optical density of 0.1 at 600 nm.

3.2.2 Media

Two fermentation media were used for this study; Yeast Nitrogen Base (YNB) without amino acids and ammonium (Difco™ Laboratories) and synthetic grape must (SGM) (Henschke and Jiranek, 1993).

Fermentations (100 mL) were conducted in triplicate at 30°C. The medium contained 10% m/v glucose, to ensure that alcoholic fermentation would go to completion, and the initial pH was adjusted to 3.8 with KOH or HCl. The source of ammonium (NH_4^+) utilized was ammonium sulphate unless indicated otherwise.

The YNB media contained one of 19 amino acids or $NH4^+$ as the sole source of yeast assimilable nitrogen (YAN) at a concentration of 10.71 mmol N/L.

The SGM contained acids (3.0 g/L potassium hydrogen tartaric acid, 2.5 g/L L-malic acid, and 0.2 g/L citric acid), salts (1.14 g/L K_2HPO_4, 1.23 g/L $MgSO_4.7H_2O$, and 0.44 g/L $CaCl_2.2H_2O$), trace elements (200 µg/L $MnCl_2.4H_2O$, 135 µg/L $ZnCl_2$, 30 µg/L $FeCl_2$, 15 µg/L $CuCl_2$, 5 µg/L H_3BO_3, 30 µg/L $Co[NO_3]_2.6H_2O$, 25 µg/L $NaMoO_4.2H_2O$, and 10 µg/L KIO_3), vitamins (100 mg/L myo-inositol, 2 mg/L pridoxine.HCl, 2 mg/L nicotinic acid, 1 mg/L Ca-pantothenate, 0.5 mg/L thiamine.HCl, 0.2 mg/L para-aminobenzoic acid, 0.2 mg/L riboflavin, 0.125 mg/L biotin, and 0.2 mg/L folic acid), anaerobic factors (10 mg/L ergosterol and 0.5 mL/L Tween 80), and nitrogen sources as specified. The SGM contained a total YAN of 21.43 mmol N/L containing a single BCAA; phenylalanine, isoleucine, leucine, or valine at concentrations of 7.14, 14.28, and 21.43 mmol N/L (Table 3.1). The BCAA treatments were supplemented, when required, with NH_4^+ for a total nitrogen content of 21.43 mmol N/L. An additional set of valine treatments were supplemented with alanine, instead of ammonium to investigate the effect of varying ammonium additions. Additional control treatments contained only NH_4^+ or alanine as the sole nitrogen source at a concentration of 21.43 mmol N/L.

The third set of fermentations were conducted in SGM, containing 20% sugars and 200 mg N/L nitrogen. The nitrogen composition consisted of ammonium chloride (3.57 mg N/L), isoleucine (2.41 mg N/L), leucine (2.41 mg N/L), phenylalanine (2.41 mg N/L), tyrosine (2.41 mg N/L), and valine (2.41 mg N/L).

A final set of fermentations was conducted in SGM, as described above, however, in this instance, more complex mixtures of nitrogen (Table 3.2) were used. A YAN content of 14.3 mmol N/L was used, of which 3.57 mmol N/L was provided by ammonium chloride, the

remainder was made up of all the amino acids, where each amino acid provided equal amounts of fermentable nitrogen. Furthermore, a single BCAA (leucine, isoleucine, valine, phenylalanine, tyrosine, and tryptophan) was either present at the same concentration as the other amino acids, absent (0) or present at twice (2) the concentration of the other amino acids. Fermentations were conducted in triplicate at 20°C without agitation.

3.2.3 Growth parameters

3.2.3.1 Biomass

Optical density, measured at 600 nm (OD_{600nm}), was used as an indication of biomass accumulation during fermentation and samples were taken every 2 to 4 hours for the first 20 hours followed by every 6 to 24 hours until weight loss ceased. Two millilitres of the medium was aseptically collected from the fermentation vessel, centrifuged at 5000 rpm for 5 min, and the supernatant was removed. Pellets were resuspended in distilled water, centrifuged, and washed again. Samples were dried for 48 hours at 100°C and weighed. To obtain a calibration curve for the biomass, the dry weight was plotted against the OD reading at each of these sampling points.

3.2.3.2. Exponential growth rate and lag phase

The average exponential growth rate was determined by performing a semi-log transformation of the growth curve of each single amino acid treatment. From this transformed growth curve, the linear section was isolated, a trendline was added and its gradient represented the exponential growth rate. Since the lag phase ends where the exponential growth phase begins, the average lag phase was taken to be the period between the time of inoculation and the onset of exponential growth.

3.2.3.3 Time to complete fermentation

Fermentation vessels were weighed every 24 hours until no further weight loss was detected.

3.2.4 Gas chromatography with a flame ionization detector (GC-FID)

At the end of alcoholic fermentation, samples underwent a liquid-liquid extraction as described by Louw et al (2009) for analysis by gas chromatography. A 5 mL sample of media, 100 µl of internal standard (4-methyl-2-pentanol), and 1 mL of solvent (diethyl ether) were combined and then placed in an ultrasonic bath for 5 minutes to facilitate extraction. The mixture was then centrifuged for 3 min at 4000 rpm after which Na_2SO_4 was added to remove any water

from the non-polar layer and the sample was again centrifuged for another 3 min at 4000 rpm. A Hewlett Packard 6890 Plus GC-FID instrument (Agilent, Little Falls, Wilmington, USA) with a split/splitless injector was used for major volatiles quantification. The split flow rate was set at 49.4 ml/min and the split ratio was set to 15:1 at a temperature of 200°C. The separation of compounds was done using a J and B DBFFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with the dimensions of 60 m x 0.32 mm and a 0.5 µl coating film thickness with the flow rate of the hydrogen carrier gas set at 3.3 ml/min. Once the FID oven temperature reached the temperature of 240°C, 3 µl of extracted sample was injected into the gas chromatograph at an initial temperature of 33°C and held for 8 min; the temperature was then increased by 21°C/min to 130°C and then held for 17 min; increased by 12°C/min to 170°C and held for 5 min; increased by 21°C/min to 240°C and held for 2.5 min. A post run step at the end of each sample was carried out at 240°C for 5 min. Each sample was injected in duplicate. The column was cleaned with an injection of hexane after every 20 samples. Manual data collection and peak integration were done using the HP ChemStation software (Rev. B01.03 [204]).

3.2.5 Prediction of aroma compound production

To assess the functional relationship between the final BCAAs concentration and their associated volatile metabolites accumulation, a linear relationship was assumed, and regression analysis was carried out to determine the association.

The assumed relationship can be summarised by the following equation:

$$Y = a + bX$$

Where Y = metabolite concentration; b = gradient of the slope; X = concentration of amino acid; a= metabolite concentration when no amino acid is added.

Different concentrations of BCAAs were plotted against their corresponding aroma compounds and the strength of the regression model represents the predictability of aroma compound production as a function of initial concentration.

3.2.6 Statistical analysis

Heatmaps of autoscaled GC-FID data was constructed using the package Complex Heatmap in R studio, where applicable, rows and columns were clustered using Ward.D and the Euclidean distance metric. Similarly, cluster analysis was applied to the four yeast growth parameters measured.

Table 3.1. Amino acid treatments containing different concentrations of a branched-chain or aromatic amino acid and supplemented with either NH_4^+ or alanine to obtain a total YAN of 21.43 mmol N/L.

Nitrogen treatment	**BCAA concentration (mmol N/L)**	**NH_4^+ (mmol N/L)**	**Ala (mmol N/L)**
Leu 21.43	21.43	0	0
Leu 14.28	14.28	7.14	0
Leu 7.14	7.14	14.28	0
Ile 21.43	21.43	0	0
Ile 14.28	14.28	7.14	0
Ile 7.14	7.14	14.28	0
Val 21.43	21.43	0	0
Val 14.28	14.28	7.14	0
Val 7.14	7.14	14.28	0
Ala 14.28 + Val 7.14	7.14	0	14.28
Ala 21.43 + Val 0	0	0	21.43
Ala 7.14 + Val 14.28	14.28	0	7.14
Phe 21.43	21.43	0	0
Phe 14.28	14.28	7.14	0
Phe 7.14	7.14	14.28	0
NH_4^+	21.43	21.43	0

Table 3.2. Fermentations contained 14.3 mmol N/L of YAN, of which 3.57 mmol N/L^{-1} was provided by ammonium chloride, the remainder was made up of all the amino acids listed, where each one provided equal amounts of fermentable nitrogen except for leucine, isoleucine, valine, phenylalanine, tyrosine, and tryptophan which were also either absent (0) or present at twice (2) the concentration of the other amino acids.

Nitrogen sources (mmol N/L)	**All Amino acids**	**0 Ile**	**2 Ile**	**0 Leu**	**2 Leu**	**0 Val**	**2 Val**	**0 Trp**	**2 Trp**	**0 Tyr**	**2 Tyr**	**0 Phe**	**2 Phe**	**$NH4^+$**
Alanine	0.59	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.00
Arginine	0.59	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.00
Aspartic acid	0.59	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.00
Cysteine	0.59	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.00
Glutamic acid	0.59	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.00
Glutamine	0.59	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.00
Glycine	0.59	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.00
Histidine	0.59	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.00
Lysine	0.59	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.00
Methionine	0.59	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.00
Proline	0.59	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.00
Serine	0.59	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.00
Threonine	0.59	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.00
Isoleucine	0.59	0.00	1.13	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.00
Leucine	0.59	0.63	0.56	0.00	1.13	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.00
Valine	0.59	0.63	0.56	0.63	0.56	0.00	1.13	0.63	0.56	0.63	0.56	0.63	0.56	0.00
Tryptophan	0.59	0.63	0.56	0.63	0.56	0.63	0.56	0.00	1.13	0.63	0.56	0.63	0.56	0.00
Tyrosine	0.59	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.00	1.13	0.63	0.56	0.00
Phenylalanine	0.59	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.63	0.56	0.00	1.13	0.00
Ammonium	3.57	3.57	3.57	3.57	3.57	3.57	3.57	3.57	3.57	3.57	3.57	3.57	3.57	14.29
Total YAN	**14.3**	**14.3**	**14.3**	**14.3**	**14.3**	**14.3**	**14.3**	**14.3**	**14.3**	**14.3**	**14.3**	**14.3**	**14.3**	**14.3**

3.3 Results

3.3.1 Effect of single amino acids on growth kinetics

Individual amino acids were evaluated for their ability to support the growth of VIN13 and BM45 in YNB. Differences in growth kinetics were observed due to both the amino acid treatment and, to a lesser extent, the yeast strain. Four growth parameters were assessed: the duration of the lag phase, exponential growth rate, total biomass formation and time to complete fermentation (Figure 3.1 A to D). In most cases, and as expected, the four parameters showed a strong correlation, with short lag phase correlating with rapid exponential growth and high total biomass formation, as well as a shorter time of fermentation. In most cases; both strains also behaved similarly, but some significant exceptions were observed.

Glutamate, a preferred nitrogen source, resulted in the shortest lag phase (Figure 3.1 C) in both strains, as well as a rapid exponential growth rate (Figure 3.1 B), large biomass formation (Figure 3.1 A) and short fermentation duration (Figure 3.1 D). Glutamine, arginine, alanine, serine, aspartate, phenylalanine, and valine also generally supported similar patterns (Figure 3.1 E). However, some significant differences were observed between the two strains, as glutamate and asparagine led to very different exponential growth rates for BM45 while remaining very similar in the case of VIN13 (Figure 3.1 B).

For some amino acids, the general correlation between the four parameters was not maintained. This is illustrated by isoleucine, which yielded biomass comparable to the preferred amino acids such as arginine but resulted in a low exponential growth rate and long duration of fermentation (Figure 3.1 E).

Tryptophan, threonine, and asparagine treatments resulted in the lowest biomass production levels for both yeast strains (Figure 3.1 A). The yeast strain significantly impacted biomass production as seen in the arginine, isoleucine, leucine, threonine, tyrosine, methionine, and NH_4^+ treatments with VIN13 giving rise to lower final biomass (Figure 3.1 A).

As summarised in the heatmap, isoleucine, tyrosine, methionine, proline, leucine, and tryptophan displayed a comparatively weaker performance in all four growth parameters evaluated when compared to the preferred amino acids seen in the first cluster.

Asparagine, threonine, and ammonia are in the third cluster, as they displayed short lag phases, despite having low levels of biomass production. Ammonia is considered to be a preferred nitrogen source and therefore its poor performance was further investigated, and this deviant behaviour was ascribed to the buffering capacity of the YNB media used (data not shown).

Yeast strains also responded differently to certain nitrogen treatments, as shown by the longer lag phases observed for VIN13 when supplemented with only threonine, isoleucine, and tryptophan (Figure 3.1 C). The highest growth rate was observed with the BM45 strain in the glutamate treatment (Figure 3.1 B). Lower growth rates were observed in both strains for NH_4^+, threonine, proline, methionine, leucine, tyrosine, isoleucine, and tryptophan treatments.

3.3.2 Effect of single amino acids on aroma production

As anticipated, the volatile compound composition was strongly influenced by the amino acid (21.43 mmol N/L) provided (Figure 3.2). The branched-chain and aromatic amino acid treatments yielded high concentrations of fusel alcohols and fusel acids mostly in line with the known metabolic pathways. Valine treatment resulted in a high production of isobutanol, (4.4 mmol/L) and isobutyric acid, where VIN13 produced 1.57 mmol/L and BM45 1.99 mmol/L (Figure 3.2 I and J). The leucine and isoleucine treatments were linked to isoamyl alcohol, isovaleric acid and isoamyl acetate (Figure 3.2 E, F, and G) production. The products of isoleucine (amyl alcohol and 2-methylbutanoic acid) and leucine (isoamyl alcohol and isovaleric acid) catabolism have similar structures and consequently also similar retention times. The misidentification of metabolites is most likely the reason why the isoleucine profile so closely resembles that of leucine. Phenylalanine metabolism led to the production of more than 5 mmol/L of 2-phenylethanol, by both strains, and 0.17 mmol/L of 2-phenylethyl acetate by VIN13 and 0.23 mmol/L by BM45 (Figure 3.2 A and B). Threonine catabolism resulted in the production of propanol but also resulted in the highest levels of butanol and propionic acid, moderate levels of isoamyl alcohol, isovaleric acid, acetic acid, and ethyl acetate (Figure 3.2 C, D, E, and F).

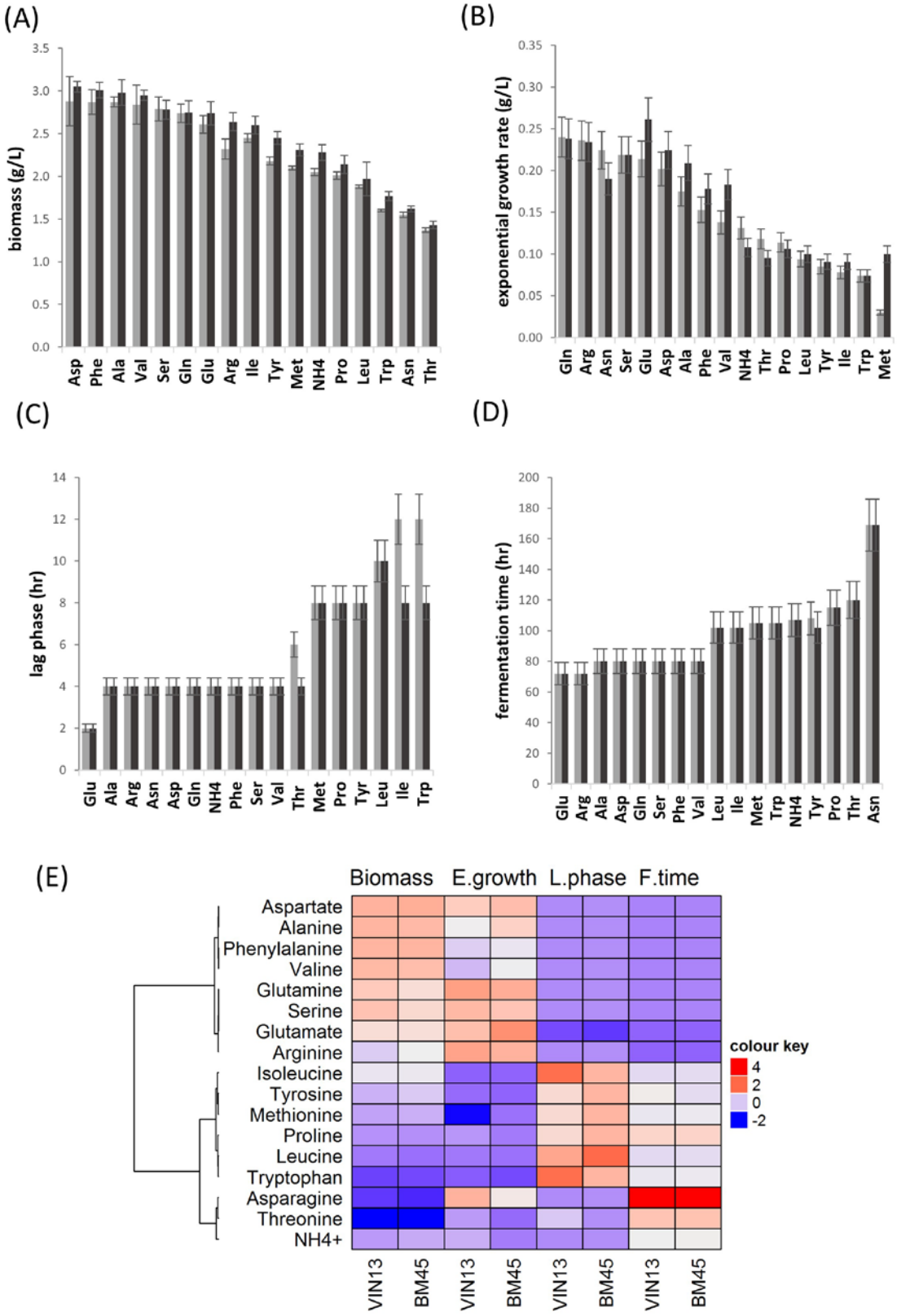

Figure 3.1. A comparison of biomass accumulation **(A)**, exponential growth rate **(B)** estimate of lag phase **(C)**, and fermentation duration **(D)** of VIN13 (light grey) and BM45 (dark grey) cultured in YNB containing a single amino acid or ammonium. Error bars denote the standard deviation of triplicate fermentations. The heatmap **(E)** summarizes all four growth parameters measured, where high values are coloured red and low blue and the colour intensity represents variation in the levels across the colour scale.

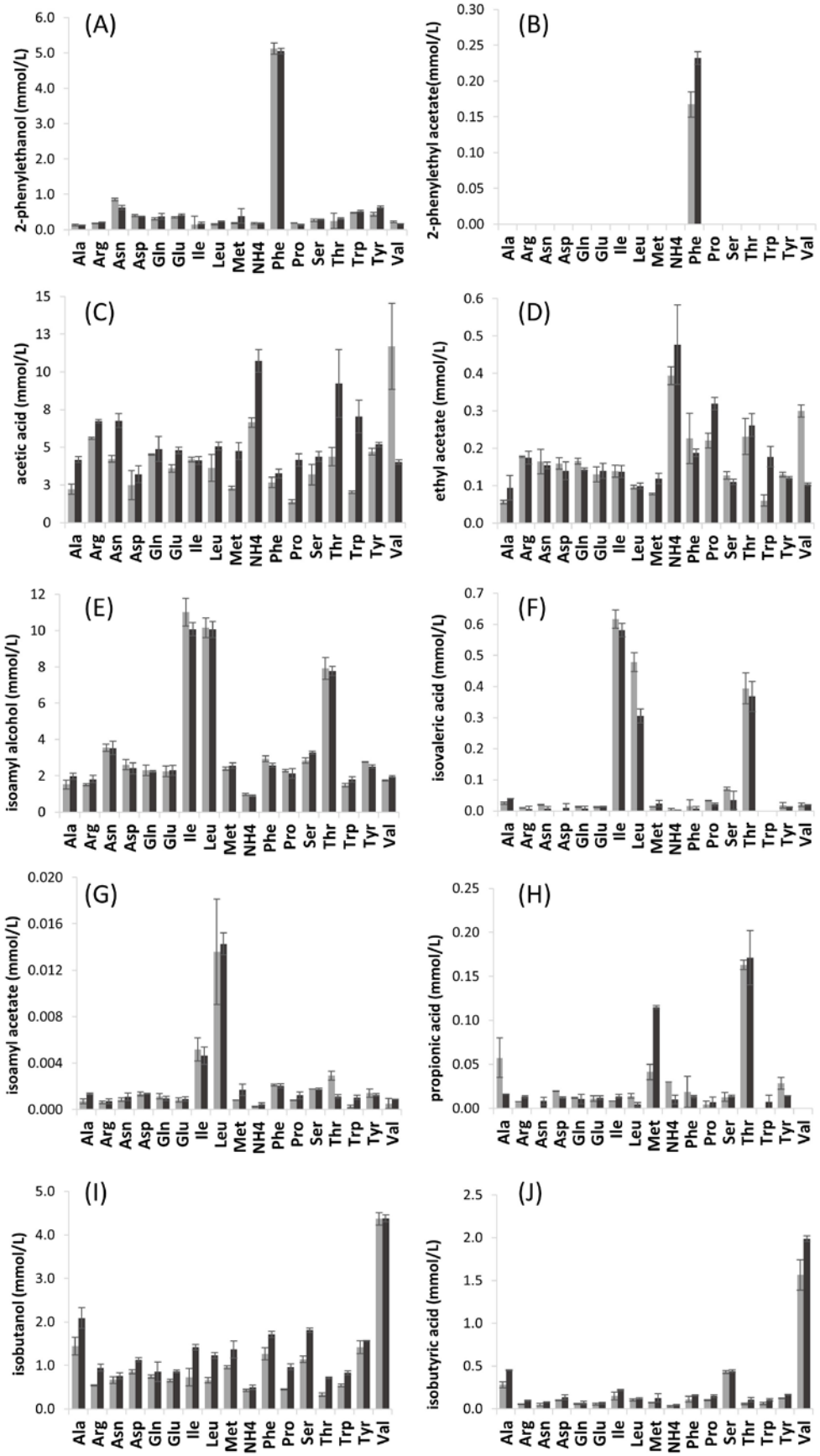

Figure 3.2. The impact of single amino acids in YNB on the production of volatile compounds by VIN13 (light grey) and BM45 (dark grey) on the production of volatile compounds (mmol/L) such as 2-phenylethanol **(A)**, 2-phenylethyl acetate **(B)**, acetic acid **(C)**, ethyl acetate **(D)**, isoamyl alcohol **(E)**,

isovaleric acid **(F)**, isoamyl acetate **(G)**, propionic acid **(H)**, isobutanol **(I)**, and isobutyric acid **(J)**. Error bars denote the standard deviation of triplicate fermentations.

3.3.3 Evaluating aroma production based on BCAAs concentration

The effect of single BCAAs on aroma biosynthesis was further investigated using VIN13 in synthetic grape must. Three amino acid concentrations were applied while maintaining total available nitrogen through ammonium addition. To evaluate whether changing ammonium concentrations might impact the result, the same data were also produced using alanine instead of ammonium. Volatile acidity (acetic acid and ethyl acetate) displayed a proportional response to ammonium supplementation (Supplementary table S 3.2), whereas propanol displayed an inverse relationship to supplementation (Figure 3.3 G).

The data suggested that a linear correlation existed, consequently, data were used to develop a simple regression model to evaluate aroma compound production based on the initial amino acid concentration (Table 3.3).

Table 3.3. Linear regression analysis of the relationship between BCAAs and related volatile metabolite produced by VIN13 during fermentation.

Amino acid	Aroma compound	R^2	regression equation	*P-value*
Val	isobutanol	0.9967	Y = -1.25 + 0.73X	0.052
	isobutyric acid	0.9991	Y = -0.40 + 0.12X	0.019*
	butyric acid	0.9999	$Y = 0.002 - (2.06 \times 10^{-5}) X$	0.008*
	propionic acid	0.9414	Y = 0.09 + 0.006X	0.156
Phe	2-phenylethanol	0.9998	Y = 0.87 + 0.95X	0.009*
	2-phenylethyl acetate	0.9980	Y = 0.0034X + 0.0128	0.028*
	decanoic acid	0.8878	Y = 0.01 – 0.0002X	0.217
	valeric acid	0.9989	Y = 0.007 + 0.0007X	0.022*
Leu	isoamyl alcohol	0.9999	Y = 0.69 + 0.95X	0.008*
	isovaleric acid	0.9596	Y = -0.04 + 0.02X	0.129
	isoamyl acetate	0.9927	Y = 0.02 + 0.002X	0.055
Ile	isoamyl alcohol	0.9767	Y = 1.2988X - 2.2464	0.098
	isovaleric acid	0.9869	Y = -0.03 + 0.02X	0.073
	isoamyl acetate	0.2181	Y = 0.01 + 0.0003X	0.691

* P-values with asterisk indicates a significant relationship between the amino acid concentration and the volatile compound produced (at 95% confidence level)

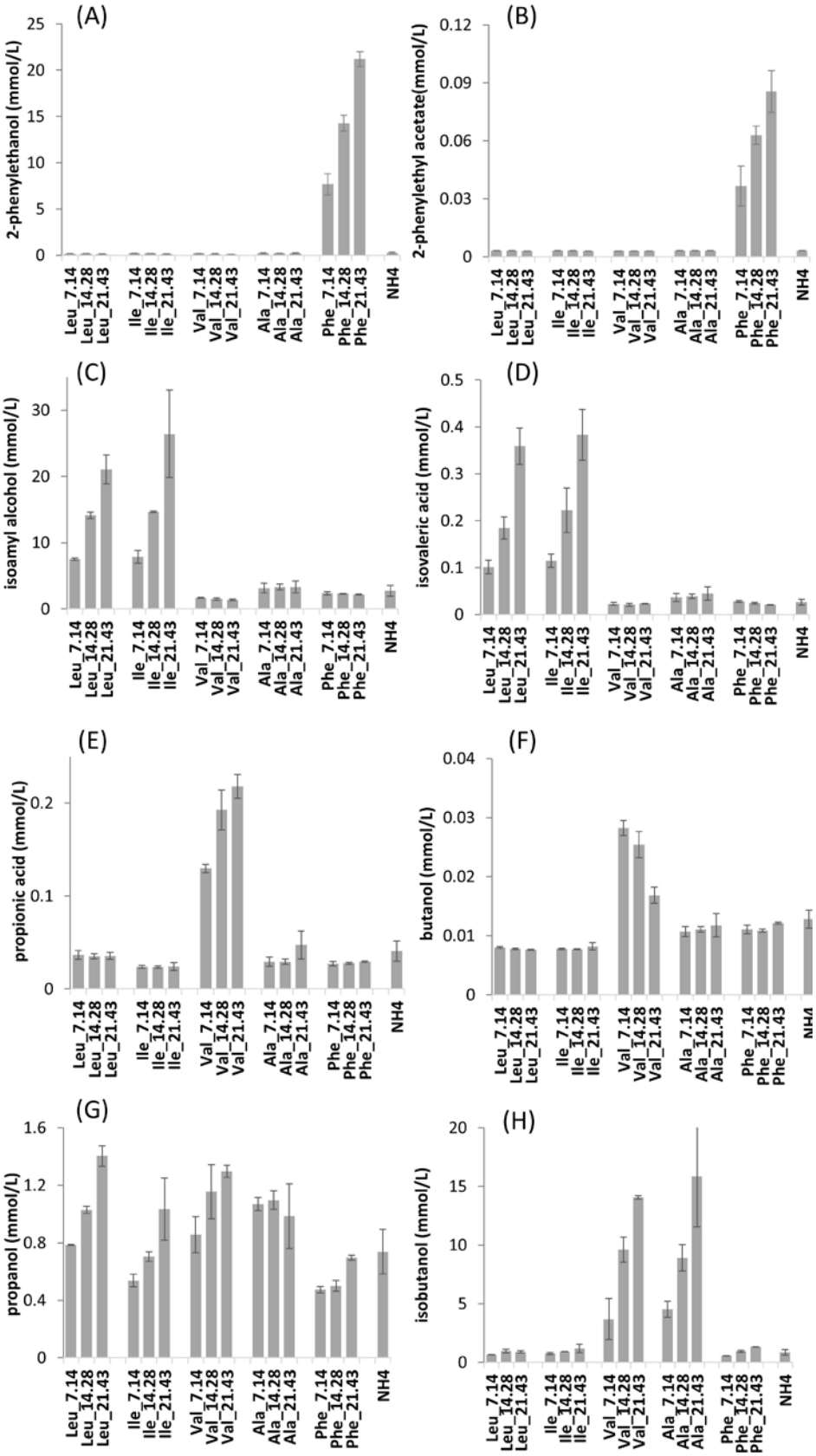

Figure 3.3. The impact of increasing concentrations of BCAAs in synthetic grape must on the production of volatile compounds by VIN13: 2-phenylethanol **(A)**, 2-phenylethyl acetate **(B)**, isoamyl alcohol **(C)**, isovaleric acid **(D)**, propionic acid **(E)**, butanol **(F)**, propanol **(G)** and isobutanol **(H)**. Error bars denote the standard deviation of triplicate fermentations.

Irrespective of the addition of ammonia or alanine, the valine fermentations responded in a similar manner with respect to isobutanol and isobutyric acid, whereas butanol and propionic acid levels were significantly impacted by ammonium levels (Figure 3.3 E, F, and H).

As shown in figure 3.2 (Supplementary Table 3.1), these SGM fermentations displayed a similar response to BCAA supplementation. A linear correlation between the concentration of an amino acid and the production of fusel alcohols and fusel acids was observed when the initial concentrations of BCAAs were varied individually (Figure 3.3, Supplementary Table 3.2). Some volatile compounds related to valine (isobutyric acid and butyric acid), phenylalanine (2-phenylethanol and valeric acid) and leucine (isoamyl alcohol) could be predicted using the correlation analyses. Although other aroma compounds had high regression coefficients (R^2), the slopes were insignificant (Table 3.3). Surprisingly, the total yield of aromatic compounds, primarily due to higher alcohol, was identical in concentration to the total amount of leucine and phenylalanine (Table 3.4). This may suggest that the contribution of other metabolic pathways to these aromatic compounds is negligible in these conditions, or could be a fortuitous coincidence. In the case of valine, the measured catabolic products account for between 58 and 76% of the initial concentration, but we did not measure some of the major breakdown products in this case.

Table 3.4. The volatile aroma yield (%) based on the BCAA concentration and related volatile metabolite produced by VIN13 during fermentation.

							Total yield (%)
	phenylethyl alcohol		**phenylethyl acetate**				
Phe_7.14	107.58	±16.14	0.51	±0.15			108.10
Phe_14.28	100.00	±5.91	0.44	±0.03			100.44
Phe_21.43	98.98	±3.77	0.4	±0.05			99.38
	isoamyl alcohol		**isovaleric acid**		**isoamyl acetate**		
Leu_7.14	105.08	±2.57	1.42	±0.2	0.42	±0.04	106.92
Leu_14.28	98.93	±3.32	1.29	±0.17	0.32	±0.04	100.54
Leu_21.43	98.20	±10.18	1.67	±0.18	0.27	±0.03	100.14
Ile_7.14	110.00	±13.42	1.61	±0.2	0.13	±0.01	111.74
Ile_14.28	102.57	±0.68	1.56	±0.33	0.13	±0.02	104.25
Ile_21.43	117.05	±7.9	1.79	±0.25	0.06	±0.02	118.90
	isobutanol		**isobutyric acid**				
Val_7.15	51.58	±2.09	7.03	±0.81			58.61
Val_14.28	67.23	±7.51	9.38	±1.02			76.61
Val_21.43	65.58	±8.1	10.59	±1.34			76.17

To evaluate the performance of these regression models, fermentations were conducted using more complex amino acid mixtures. These fermentations contained the BCAAs, isoleucine, leucine, phenylalanine, tyrosine, and valine, and the predicted concentrations of aromatic compounds were determined using the equations in table 3.3 (Table 3.5). In this comparatively simple matrix, the actual concentrations determined differed from the predicted values, indicating that the predictive ability of the linear correlational analyses has already been lost.

Table 3.5. Comparison between the actual and predicted concentrations of volatile metabolites in response to mixtures of BCAAs produced by VIN13 during fermentation.

Amino acid	Aroma compound	Measured concentration (mmol/L)	Predicted concentration (mmol/L)
Leu and Ile	isoamyl alcohol	4.70 ± 0.03	3.90
	isovaleric acid	0.04 ± 0.00	0.02
	isoamyl acetate	0.02 ± 0.00	0.03
Val	isobutanol	1.70 ± 0.01	0.5
	isobutyric acid	0.04 ± 0.00	-0.10
	propionic acid	0.02 ± 0.00	0.12
	butyric acid	0.01 ± 0.00	0.00
Phe	2-phenyl ethanol	2.30 ± 0.03	3.10
	2-phenylethyl acetate	0.02 ± 0.00	0.02
	valeric acid	0.01 ± 0.00	0.01
	decanoic acid	0.01 ± 0.00	0.01

3.3.4 Effect of changing the concentration of one amino acid in a complex mixture on aroma production

To evaluate the impact of relatively minor changes in amino acid composition, which more closely mimics grape must, fermentations were carried out using 19 amino acids, but varying only a single BCAA (leucine, isoleucine, valine, phenylalanine, tyrosine, and threonine) concentration. Three concentrations were used for each of these amino acids: Absent (0), same as all other amino acids and present at twice (2) the concentration of the other amino acids. The impact of this omission or twice the concentration of BCAAs on the production of aroma compounds was evaluated, in a more complex synthetic grape must (20% sugars). The amino acid treatments resulted in similar fermentation kinetics, with the ammonium treatment being comparatively slower, nonetheless, all fermentations proceeded to dryness (< 2 g/L residual sugar, data not shown).

The concentrations yielded differed from the predicted levels calculated, suggesting a loss of linear predictability. Nonetheless, the data indicates that these individual amino acid changes do indeed cause significant changes in the production of compounds associated with their catabolism (Figure 3.4, Supplementary table 3.3). This is clearly illustrated by a responsiveness to leucine (isoamyl acetate), phenylalanine (2-phenyl ethanol and 2-phenylethyl

acetate) and valine (isobutanol and isobutyric acid) supplementation, where the expected increases (2 treatments) or decreases (0 treatments) were observed. Relative to the all amino acid treatment (0.59 mmol N/L), the absence of phenylalanine resulted in a 56% decrease in the production of 2-phenyl ethanol, and a 46% reduction in 2-phenylethyl acetate. In contrast, twice the concentration of phenylalanine (1.13 mmol N/L) resulted in a 127% increase in the production of 2-phenyl ethanol and a 110% increase in 2-phenylethyl acetate. Similarly, the absence of valine resulted in a 24% decrease in isobutanol and 13% decrease in isobutyric acid, and twice the concentration of valine (1.13 mmol N/L) caused an 80% increase in isobutanol and isobutyric acid. The absence or twice the concentration of tryptophan and tyrosine only resulted in slight changes in the production of volatile fatty acids and ethyl esters. The ammonium treatment produced high levels of propanol, propionic acid, 3-ethoxy-1-propanol, and butanol, also seen in figure 3.3, and very low levels of metabolites derived from phenylalanine and valine metabolism.

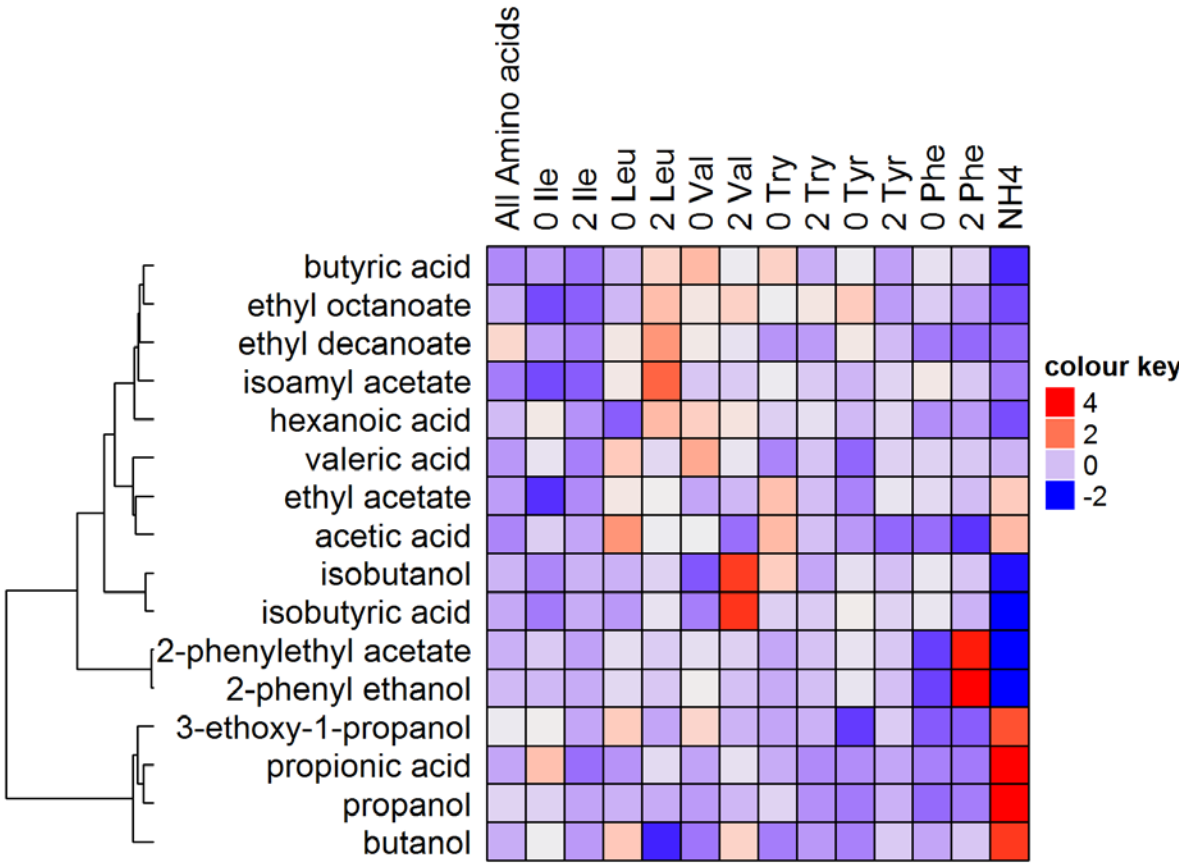

Figure 3.4. The impact of the absence (0) or twice the concentration of nitrogen (1.13 mmol N/L) (2) provided by BCCAs when all other amino acids provide the same amount of fermentable nitrogen on the production of volatile compounds (mmol/L). Fermentations, mediated by VIN13, were conducted in synthetic grape must.

3.4 Discussion

In a winemaking context, nitrogen has a dual role, promoting yeast growth to achieve completion of alcoholic fermentation, but also playing a central role in the production of volatile aromas. Previous studies used generation time (exponential growth rate) to evaluate the efficiency of nitrogen sources to support growth (Cooper, 1982; Godard 2007). This study included other growth (total biomass) and fermentation parameters (lag phase, fermentation time) providing additional information regarding the efficacy of single amino acids under winemaking conditions. The regulation of amino acid assimilation and utilization is eliminated by evaluating amino acids in isolation (Ljungdahl and Diagnan-Fornier, 2012; Godard et al., 2007). In general, the amino acid efficiency observed under fermentative conditions was in agreement with previous studies by Cooper (1982) and Godard et al (2007). The preferred nitrogen sources, which best supported fermentation kinetics (alanine, arginine, aspartate, glutamine, glutamate, and serine), are also the amino acids to be readily incorporated into the cell's metabolic pathways. In contrast with previous findings, the phenylalanine and valine treatments resembled the preferred amino acids whereas asparagine was a poor nitrogen source based on its capacity to support yeast growth. These differences in amino acid classification could be attributed to different fermentation conditions, wine yeast strains used, as well as the additional parameters assessed.

Overall, biomass formation was generally correlated with the duration of the lag phase and the exponential growth rate correlated with the fermentation time. Exceptions, due to strain differences, were found for both the biomass and lag phase (arginine, isoleucine, and threonine treatments) and exponential growth rate and fermentation time (valine and threonine treatments). Furthermore, asparagine was found to be an exception due to its irregular performance for all parameters evaluated. In a winemaking context, the inclusion of fermentation time and biomass provides a greater understanding of the effect of these individual amino acids on fermentation performance.

The type and quantity of volatile compounds produced are largely dependent on the amino acid, the yeast strain, and their interaction. Overall, similar responses to the nitrogen treatments were observed for most compounds, however, strains often differed in the magnitude of their

responses to some of the amino acid treatments employed. In agreement with literature, BCAAs are catabolised into their corresponding fusel alcohols and fusel acids (Hazelwood et al., 2008).

Consequently, when single BCAAs are used together with ammonium as the only other source of nitrogen, aroma compound production shows a strong linear correlation between the amino acid concentration and the corresponding volatile compound (Figure 3.3). Interestingly, valine was the only amino acid which resulted in less than a 100% conversion rate into its related volatile compounds. This suggests that the yeast redirected the alpha-keto acid (α-keto-isovalerate) toward a different metabolic pathway, possibly leucine biosynthesis. Whereas, irrespective of its concentration, leucine and phenylalanine resulted in the complete conversion of the amino acid to its volatile breakdown products, suggesting that all the amino acids were synthesized *de novo* (Crépin et al., 2017). Subsequent regression analyses highlighted the potential of using amino acid concentration to predict the concentration of the associated volatile compound (Table 3.4), however, only isoamyl alcohol, isobutyric acid, butyric acid, 2-phenylethanol and valeric acid could be reliably predicted using the regression models.

Considering the interconnectedness of nitrogen metabolism, it is not surprising that when all BCAAs are used together, the linear relationship was lost resulting in poor predictability (Table 3.5 and Figure 3.4). Generally, amino acids are catabolised yielding glutamate, which is central to amino acid biosynthesis, and a carbon skeleton which can enter the TCA cycle, the Ehrlich pathway, or be used for the biosynthesis of other amino acids, as is the case with leucine and valine (Magasanik and Kaiser, 2002). Consequently, in the absence of leucine, one would assume a decrease in the levels of volatile compounds associated with valine catabolism, as the cell would presumably use the carbon skeleton to produce leucine instead of a fusel alcohol or acid (Figure 3.4). This is not the case, as explained by Crépin et al (2017), in a recent study monitoring the fate of amino acids, which found that the α-keto acid precursors used for higher alcohol production were generally derived from sugar and not amino acid catabolism. Nonetheless, the data shown here (Figure 3.4) illustrates that even comparatively minor changes in BCAA concentrations do still result in significant impacts on the production of the associated volatile compounds, even when provided in complex mixtures and the presence of all amino acids. The work reported by Crépin et al (2017) raises a concern that the application of nutrients during winemaking may not make a direct contribution to the volatile profile. The data shown here clearly indicates that that is not the case, as a degree of responsiveness to even minor changes in individual amino acid supplementation led to a direct impact on the production of

associated aromatic compounds. Future work will explore prediction modelling of the impact of complex nitrogen composition on metabolic outputs.

This study provides a novel evaluation of the impact of single amino acids on several growth parameters, in addition to its effect on the production of volatile metabolites by two genetically different industrial *S. cerevisiae* strains, known to have differing fermentation kinetics and volatile aroma profiles (Rossouw et al., 2009). The various correlations between amino acid concentration and volatile compounds ranged from linear in the simplest of all cases (single amino acid) to unpredictable in more complex media. However, and importantly, relatively minor changes in the concentrations of single amino acids still led to changes in the variation of the derived volatile compounds.

3.5 Author contributions

SF and AM performed experiments, analysed data, and wrote the manuscript; HM and AF wrote the manuscript; FB designed the experiments, contributed to data interpretation, supervised the study, and co-wrote the manuscript.

3.6 Acknowledgements

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3.7 References

Barbosa, C., Falco, V., Mendes-Faia, A., and 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. J. Biosci. Bioeng. 108, 99–104. doi:10.1016/j.jbiosc.2009.02.017.

Bell, S. J., and Henschke, P. a (2005). Implications of nitrogen nutrition for grapes, fermentation and wine. Aust. J. Grape Wine Res. 11, 242–295. doi:10.1111/j.1755-0238.2005.tb00028.x.

Burin, V. M., Gomes, T. M., Caliari, V., Rosier, J. P., and Bordignon Luiz, M. T. (2015). Establishment of influence the nitrogen content in musts and volatile profile of white wines associated to chemometric tools. Microchem. J. 122, 20–28. doi:10.1016/j.microc.2015.03.011.

Cooper, Terrance G. "Nitrogen metabolism in *Saccharomyces cerevisiae."* Cold Spring Harbor Monograph Archive 11 (1982): 39-99

Crépin, L., Nidelet, T., Sanchez, I., Dequin, S., and Camarasa, C. (2012). Sequential use of nitrogen compounds by *Saccharomyces cerevisiae* during wine fermentation: a model based on kinetic and regulation characteristics of nitrogen permeases. Appl. Environ. Microbiol. 78, 8102–11. doi:10.1128/AEM.02294-12.

Crépin, L., Truong, N. M., Bloem, A., Sanchez, I., Dequin, S., and Camarasa, C. (2017). Management of multiple nitrogen sources during wine fermentation by *S. cerevisiae.* Appl. Environ. Microbiol. 83, AEM.02617-16. doi:10.1128/AEM.02617-16.

Dickinson, J. R., Lanterman, M. M., Danner, D. J., Pearson, B. M., Sanz, P., Harrison, S. J., et al. (1997). A 13C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae.* J. Biol. Chem. 272, 26871–26878. doi:10.1074/jbc.272.43.26871.

Ehrlich F (1907). Über die Bedingungen der Fuselölbildung und über ihren Zusammenhang mit dem Eiweissaufbau der Hefe. Ber Dtsch Chem Ges. 40, 1027-1047.

Ferreira Monteiro, F., and Bisson, L. F. (1992). Nitrogen Supplementation of Grape Juice. I. Effect on Amino Acid Utilization During Fermentation. Am. J. Enol. Vitic. 43, 1–10.

Garde-Cerdán, T., and 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 Sci. Technol. 41, 501–510. doi:10.1016/j.lwt.2007.03.018.

Godard, P., Urrestarazu, A., Vissers, S., Kontos, K., Bontempi, G., van Helden, J., et al. (2007). Effect of 21 different nitrogen sources on global gene expression in the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. 27, 3065–3086. doi:10.1128/MCB.01084-06.

Gutiérrez, A., Chiva, R., Sancho, M., Beltran, G., Arroyo-López, F. N., and Guillamon, J. M. (2012). Nitrogen requirements of commercial wine yeast strains during fermentation of a synthetic grape must. Food Microbiol. 31, 25–32. doi:10.1016/j.fm.2012.02.012.

Hazelwood, L. A, Daran, J. M., van Maris, J. A., Pronk, J. T., and Dickinson, J. R. (2008). The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism (vol 74, pg 2259, 2008). Appl. Environ. Microbiol. 74, 3920. doi:10.1128/AEM.00934-08.

Henschke, P. A., and V. Jiranek. "Yeasts-metabolism of nitrogen compounds." Wine microbiology and biotechnology (1993): 77-164.

Hernández-Orte, P., Cacho, J. F., and Ferreira, V. (2002). Relationship between varietal amino acid profile of grapes and wine aromatic composition. Experiments with model solutions and chemometric study. J. Agric. Food Chem. 50, 2891–2899. doi:10.1021/jf011395o.

Rossouw, D., and Bauer, F. F. (2009). Comparing the transcriptomes of wine yeast strains: toward understanding the interaction between environment and transcriptome during fermentation. Appl. Environ. Microbiol. 84, 937-954

Ljungdahl, P. O., and Daignan-Fornier, B. (2012). Regulation of amino acid, nucleotide, and phosphate metabolism in *Saccharomyces cerevisiae*. Genetics 190, 885–929. doi:10.1534/genetics.111.133306.

Louw, L., Roux, K., Tredoux, A., Tomic, O., Naes, T., Nieuwoudt, H. H., et al. (2009). Characterization of selected South African young cultivar wines using FTMIR Spectroscopy, Gas chromatography, and multivariate data analysis. J. Agric. Food Chem. 57, 2623–2632. doi:10.1021/jf8037456.

Magasanik, B., and Kaiser, C. (2002). Nitrogen regulation in *Saccharomyces cerevisiae.* Gene 290, 1–18. doi:10.1016/S0378-1119(02)00558-9.

Miller, A. C., Wolff, S. R., Bisson, L. F., and Ebeler, S. E. (2007). Yeast strain and nitrogen supplementation: Dynamics of volatile ester production in chardonnay juice fermentations. Am. J. Enol. Vitic. 58, 470–483.

Silva Ferreira, A. C., Monforte, A.R., Teixeira, C., Martins, R., Fairbairn, S., Bauer, F. Monitoring alcoholic fermentation: An untargeted approach. J. Agric. Food Chem., 2014, 62(28), 6784-6793. DOI: 10.1021/jf502082z

Stribny, J., Gamero, A., Pérez-Torrado, R., and Querol, A. (2015). *Saccharomyces kudriavzevii* and *Saccharomyces uvarum* differ from *Saccharomyces cerevisiae* during the production of aroma-active

higher alcohols and acetate esters using their amino acidic precursors. Int. J. Food Microbiol. 205, 41–46. doi:10.1016/j.ijfoodmicro.2015.04.003.

Styger, G., Prior, B., and Bauer, F. F. (2011). Wine flavor and aroma. J. Ind. Microbiol. Biotechnol. 38, 1145–1159. doi:10.1007/s10295-011-1018-4.

Torrea, D., Varela, C., Ugliano, M., Ancin-Azpilicueta, C., Leigh Francis, I., and 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 Chem. 127, 1072–1083. doi:10.1016/j.foodchem.2011.01.092

3.8 Supplementary material

Table S 3.1. The impact of single amino acids in YNB on the production of volatile compounds byVIN13 and BM45 (dark grey as determined by GC-FID (mmol/L). The data summarizes the average fermentations and standard deviation. Additionally, the letters denote significant differences (95%) between treatments using Fischer LSD.

	Ethyl acetate				Propanol				Isobutanol			
ALA_BM45	0.09	±	0.03	nop	0.22	±	0.03	efghijk	2.09	±	0.24	b
ALA_VIN13	0.06	±	0.01	p	0.30	±	0.03	def	1.44	±	0.21	ef
ARG_BM45	0.18	±	0.02	fghij	0.15	±	0.00	ijkl	0.94	±	0.08	jkl
ARG_VIN13	0.18	±	0.00	fghi	0.32	±	0.00	de	0.54	±	0.01	opq
ASN_BM45	0.15	±	0.01	ghijklm	0.11	±	0.02	l	0.76	±	0.07	lmn
ASN_VIN13	0.16	±	0.03	ghijk	0.18	±	0.06	ghijkl	0.66	±	0.08	nop
ASP_BM45	0.14	±	0.02	hijklmn	0.14	±	0.02	jkl	1.13	±	0.05	ij
ASP_VIN13	0.16	±	0.02	ghijkl	0.25	±	0.02	defgh	0.85	±	0.05	klm
GLN_BM45	0.14	±	0.00	hijklmn	0.13	±	0.00	fghijkl	0.85	±	0.22	klm
GLN_VIN13	0.17	±	0.01	ghijk	0.29	±	0.02	def	0.74	±	0.04	mn
ILE_BM45	0.14	±	0.02	hijklmn	0.25	±	0.01	defghi	1.42	±	0.06	efg
ILE_VIN13	0.14	±	0.02	ghijklmn	0.54	±	0.08	bc	0.73	±	0.21	mno
LEU_BM45	0.10	±	0.01	mnop	0.28	±	0.08	defg	1.23	±	0.07	hi
LEU_VIN13	0.10	±	0.01	nop	0.61	±	0.04	ab	0.66	±	0.06	nop
METH_BM45	0.12	±	0.01	klmno	0.27	±	0.02	defg	1.36	±	0.20	fgh
METH_VIN13	0.08	±	0.00	op	0.34	±	0.02	d	0.96	±	0.03	jk
NH4_BM45	0.48	±	0.11	a	0.12	±	0.02	kl	0.49	±	0.05	pqr
NH4_VIN13	0.39	±	0.02	b	0.29	±	0.04	defg	0.43	±	0.03	qr
PHE_BM45	0.19	±	0.01	fgh	0.23	±	0.01	defghij	1.71	±	0.07	cd
PHE_VIN13	0.23	±	0.07	def	0.34	±	0.06	d	1.27	±	0.14	ghi
PRO_BM45	0.32	±	0.02	c	0.51	±	0.04	bc	0.96	±	0.07	jk
PRO_VIN13	0.22	±	0.02	efg	0.66	±	0.02	a	0.45	±	0.01	qr
SER_BM45	0.11	±	0.01	klmnop	0.15	±	0.04	hijkl	1.81	±	0.05	c
SER_VIN13	0.13	±	0.01	ijklmno	0.30	±	0.04	def	1.14	±	0.07	i
THR_BM45	0.26	±	0.03	d	0.47	±	0.03	c	0.71	±	0.01	mno
THR_VIN13	0.23	±	0.05	def	0.46	±	0.08	c	0.33	±	0.04	r

TRP_BM45	0.18	±	0.03	fghi	0.12	±	0.01	kl	0.83	±	0.05	klmn
TRP_VIN13	0.06	±	0.01	p	0.15	±	0.01	hijkl	0.54	±	0.03	pq
TYR_BM45	0.12	±	0.00	jklmno	0.29	±	0.00	def	1.57	±	0.01	de
TYR_VIN13	0.13	±	0.01	ijklmno	0.47	±	0.03	c	1.42	±	0.15	efgh
VAL_BM45	0.10	±	0.00	lmnop	0.59	±	0.01	ab	4.38	±	0.08	a
VAL_VIN13	0.30	±	0.02	de	0.76	±	0.03	ab	4.37	±	0.14	a

Table S 3.1. continued

	Isoamyl acetate				Butanol				Isoamyl alcohol			
ALA_BM45	0.00	±	0.00	defg	0.00	±	0.00	ghij	1.98	±	0.17	klmno
ALA_VIN13	0.00	±	0.00	defg	0.00	±	0.00	fghij	1.53	±	0.24	op
ARG_BM45	0.00	±	0.00	defg	0.00	±	0.00	fghij	1.82	±	0.20	lmnop
ARG_VIN13	0.00	±	0.00	defg	0.00	±	0.00	fghij	1.50	±	0.05	op
ASN_BM45	0.00	±	0.00	defg	0.00	±	0.00	fghij	3.53	±	0.38	d
ASN_VIN13	0.00	±	0.00	defg	0.00	±	0.00	fghij	3.54	±	0.20	d
ASP_BM45	0.00	±	0.00	cdefg	0.00	±	0.00	hij	2.42	±	0.30	ghijk
ASP_VIN13	0.00	±	0.00	defg	0.00	±	0.00	ghij	2.61	±	0.29	fghi
GLN_BM45	0.00	±	0.00	defg	0.00	±	0.00	hij	2.23	±	0.05	ijklmn
GLN_VIN13	0.00	±	0.00	defg	0.00	±	0.00	fghij	2.30	±	0.29	hijkl
ILE_BM45	0.00	±	0.00	b	0.00	±	0.00	hij	10.07	±	0.37	b
ILE_VIN13	0.01	±	0.00	b	0.0007	±	0.0000	efgh	11.01	±	0.76	a
LEU_BM45	0.01	±	0.00	a	0.00	±	0.00	ij	10.06	±	0.45	b
LEU_VIN13	0.01	±	0.00	a	0.00	±	0.00	fghij	10.16	±	0.54	b
METH_BM45	0.00	±	0.00	cdefg	0.01	±	0.00	d	2.56	±	0.16	fghij
METH_VIN13	0.00	±	0.00	defg	0.01	±	0.00	c	2.40	±	0.10	ghijk
NH4_BM45	0.00	±	0.00	fg	0.00	±	0.00	fghij	0.90	±	0.06	q
NH4_VIN13	0.00	±	0.00	g	0.00	±	0.00	efg	0.98	±	0.06	q
PHE_BM45	0.00	±	0.00	cde	0.00	±	0.00	fghij	2.59	±	0.10	fghij
PHE_VIN13	0.00	±	0.00	cd	0.00	±	0.00	e	2.94	±	0.16	ef
PRO_BM45	0.00	±	0.00	defg	0.00	±	0.00	fghij	2.13	±	0.26	jklmn
PRO_VIN13	0.00	±	0.00	defg	0.00	±	0.00	ef	2.28	±	0.06	ijklm
SER_BM45	0.00	±	0.00	cdefg	0.00	±	0.00	j	3.28	±	0.08	de
SER_VIN13	0.00	±	0.000	cdef	0.00	±	0.00	hij	2.85	±	0.15	efg

THR_BM45	0.00	±	0.00	defg	0.01	±	0.00	b	7.78	±	0.25	c
THR_VIN13	0.00	±	0.00	c	0.04	±	0.00	a	7.92	±	0.59	c
TRP_BM45	0.00	±	0.00	defg	0.00	±	0.00	fghi	1.81	±	0.14	mnop
TRP_VIN13	0.00	±	0.00	g	0.00	±	0.00	efg	1.49	±	0.09	p
TYR_BM45	0.00	±	0.00	defg	0.00	±	0.00	fghij	2.52	±	0.08	fghij
TYR_VIN13	0.00	±	0.00	cdefg	0.00	±	0.00	ef	2.77	±	0.00	fgh
VAL_BM45	0.00	±	0.00	defg	0.01	±	0.00	d	1.96	±	0.05	klmnop
VAL_VIN13	0.00	±	0.00	efg	0.01	±	0.00	b	1.76	±	0.03	nop

Table S 3.1. continued

	Acetic Acid				Propionic Acid				Isobutyric Acid			
ALA_BM45	4.17	±	0.20	fghijk	0.02	±	0.00	ef	0.45	±	0.00	c
ALA_VIN13	2.22	±	0.32	mno	0.06	±	0.02	bcd	0.28	±	0.04	d
ARG_BM45	6.73	±	0.11	bc	0.01	±	0.00	ef	0.09	±	0.01	fghijk
ARG_VIN13	5.60	±	0.07	bcdef	0.01	±	0.00	ef	0.06	±	0.00	hijk
ASN_BM45	6.76	±	0.46	bcde	0.01	±	0.00	ef	0.08	±	0.00	ghijk
ASN_VIN13	4.22	±	0.22	efghij	0.00	±	0.00	f	0.05	±	0.01	ijk
ASP_BM45	3.20	±	0.57	ijklmno	0.01	±	0.00	ef	0.14	±	0.03	fg
ASP_VIN13	2.49	±	0.96	klmno	0.02	±	0.00	ef	0.10	±	0.01	fghij
GLN_BM45	4.88	±	0.83	defghi	0.01	±	0.01	ef	0.07	±	0.02	ghijk
GLN_VIN13	4.50	±	0.04	efghij	0.01	±	0.00	ef	0.06	±	0.00	hijk
ILE_BM45	4.11	±	0.25	fghijkl	0.01	±	0.00	ef	0.22	±	0.01	e
ILE_VIN13	4.20	±	0.16	efghij	0.09	±	0.14	ef	0.15	±	0.05	f
LEU_BM45	5.08	±	0.27	cdefgh	0.00	±	0.00	ef	0.12	±	0.01	fgh
LEU_VIN13	3.63	±	0.88	ghijklmn	0.01	±	0.00	ef	0.10	±	0.01	fghij
METH_BM45	4.74	±	0.56	efghi	0.04	±	0.01	cde	0.13	±	0.05	fg
METH_VIN13	2.29	±	0.14	lmno	0.11	±	0.00	b	0.07	±	0.00	ghijk
NH4_BM45	10.72	±	0.75	a	0.01	±	0.01	ef	0.04	±	0.00	jk
NH4_VIN13	6.65	±	0.31	bcd	0.01	±	0.02	def	0.03	±	0.01	k
PHE_BM45	3.26	±	0.29	hijklmn	0.01	±	0.00	ef	0.16	±	0.01	ef
PHE_VIN13	2.66	±	0.34	jklmno	0.02	±	0.02	ef	0.11	±	0.03	fghi
PRO_BM45	4.16	±	0.42	fghijk	0.01	±	0.01	ef	0.15	±	0.02	f
PRO_VIN13	1.40	±	0.14	o	0.00	±	0.00	ef	0.10	±	0.01	fghij
SER_BM45	4.39	±	0.33	efghij	0.01	±	0.00	ef	0.44	±	0.01	c

SER_VIN13	3.18	±	0.69	ijklmno	0.01	±	0.01	ef	0.43	±	0.02	c
THR_BM45	9.22	±	2.25	a	0.17	±	0.03	a	0.11	±	0.03	fghi
THR_VIN13	4.38	±	0.61	efghij	0.16	±	0.01	a	0.06	±	0.01	hijk
TRP_BM45	7.04	±	1.09	b	0.01	±	0.01	ef	0.10	±	0.01	fghij
TRP_VIN13	2.02	±	0.07	no	0.00	±	0.00	f	0.06	±	0.01	hijk
TYR_BM45	5.18	±	0.15	cdefg	0.01	±	0.01	ef	0.16	±	0.00	ef
TYR_VIN13	4.71	±	0.23	efghi	0.03	±	0.00	def	0.12	±	0.00	fgh
VAL_BM45	4.02	±	0.13	fghijklm	0.04	±	0.06	de	1.99	±	0.04	a
VAL_VIN13	11.70	±	2.87	a	0.07	±	0.07	bc	1.57	±	0.18	b

Table S 3.1. continued

	Isovaleric acid				**Valeric acid***				**2-Phenylethyl acetate**			
ALA_BM45	0.04	±	0.00	ef	0.00	±	0.00	b	0.00	±	0.00	c
ALA_VIN13	0.02	±	0.00	ef	0.00	±	0.00	b	0.00	±	0.00	c
ARG_BM45	0.01	±	0.01	f	0.00	±	0.00	b	0.00	±	0.00	c
ARG_VIN13	0.01	±	0.00	f	0.00	±	0.00	b	0.00	±	0.00	c
ASN_BM45	0.01	±	0.01	f	0.00	±	0.00	b	0.00	±	0.00	c
ASN_VIN13	0.02	±	0.00	ef	0.00	±	0.00	b	0.00	±	0.00	c
ASP_BM45	0.01	±	0.01	f	0.00	±	0.00	b	0.00	±	0.00	c
ASP_VIN13	0.00	±	0.00	f	0.00	±	0.00	b	0.00	±	0.00	c
GLN_BM45	0.01	±	0.01	f	0.00	±	0.00	b	0.00	±	0.00	c
GLN_VIN13	0.01	±	0.00	ef	0.00	±	0.00	b	0.00	±	0.00	c
ILE_BM45	0.58	±	0.02	a	0.00	±	0.00	b	0.00	±	0.00	c
ILE_VIN13	0.62	±	0.03	a	0.00	±	0.00	b	0.00	±	0.00	c
LEU_BM45	0.31	±	0.02	d	0.00	±	0.00	b	0.00	±	0.00	c
LEU_VIN13	0.48	±	0.03	b	0.00	±	0.00	b	0.00	±	0.00	c
METH_BM45	0.02	±	0.01	ef	0.00	±	0.00	b	0.00	±	0.00	c
METH_VIN13	0.01	±	0.00	ef	0.17	±	0.30	a	0.00	±	0.00	c
NH4_BM45	0.00	±	0.00	f	0.00	±	0.00	b	0.00	±	0.00	c
NH4_VIN13	0.00	±	0.00	f	0.00	±	0.00	b	0.00	±	0.00	c
PHE_BM45	0.01	±	0.01	f	0.00	±	0.00	b	0.23	±	0.01	a
PHE_VIN13	0.02	±	0.02	ef	0.01	±	0.00	b	0.23	±	0.11	b
PRO_BM45	0.02	±	0.00	ef	0.00	±	0.00	b	0.00	±	0.00	c

PRO_VIN13	0.03	±	0.00	ef	0.00	±	0.00	b	0.00	±	0.00	c
SER_BM45	0.03	±	0.03	ef	0.00	±	0.00	b	0.00	±	0.00	c
SER_VIN13	0.07	±	0.01	e	0.00	±	0.00	b	0.00	±	0.00	c
THR_BM45	0.37	±	0.05	c	0.00	±	0.00	b	0.00	±	0.00	c
THR_VIN13	0.39	±	0.05	d	0.00	±	0.00	b	0.00	±	0.00	c
TRP_BM45	0.00	±	0.00	f	0.00	±	0.00	b	0.00	±	0.00	c
TRP_VIN13	0.00	±	0.00	f	0.00	±	0.00	b	0.00	±	0.00	c
TYR_BM45	0.01	±	0.00	ef	0.00	±	0.00	b	0.00	±	0.00	c
TYR_VIN13	0.02	±	0.01	ef	0.02	±	0.01	b	0.00	±	0.00	c
VAL_BM45	0.02	±	0.00	ef	0.00	±	0.00	b	0.00	±	0.00	c
VAL_VIN13	0.02	±	0.01	ef	0.00	±	0.00	b	0.00	±	0.00	c

Table S 3.1. continued

	2-Phenylethanol			
ALA_BM45	0.13	±	0.01	c
ALA_VIN13	0.13	±	0.02	c
ARG_BM45	0.22	±	0.00	c
ARG_VIN13	0.19	±	0.00	c
ASN_BM45	0.48	±	0.26	c
ASN_VIN13	0.76	±	0.17	c
ASP_BM45	0.38	±	0.01	c
ASP_VIN13	0.40	±	0.03	c
GLN_BM45	0.38	±	0.08	c
GLN_VIN13	0.31	±	0.03	c
ILE_BM45	0.18	±	0.03	c
ILE_VIN13	0.28	±	0.24	c
LEU_BM45	0.24	±	0.01	c
LEU_VIN13	0.15	±	0.02	c
METH_BM45	0.39	±	0.21	c
METH_VIN13	0.19	±	0.01	c
NH4_BM45	0.18	±	0.01	c
NH4_VIN13	0.18	±	0.02	c
PHE_BM45	5.05	±	0.08	a
PHE_VIN13	4.31	±	1.43	b
PRO_BM45	0.14	±	0.02	c

PRO_VIN13	0.19	±	0.01	c
SER_BM45	0.29	±	0.01	c
SER_VIN13	0.27	±	0.03	c
THR_BM45	0.31	±	0.03	c
THR_VIN13	0.25	±	0.22	c
TRP_BM45	0.52	±	0.02	c
TRP_VIN13	0.47	±	0.01	c
TYR_BM45	0.64	±	0.03	c
TYR_VIN13	0.44	±	0.05	c
VAL_BM45	0.17	±	0.00	c
VAL_VIN13	0.22	±	0.02	c

Table S 3.2. The impact of increasing concentrations of leucine, isoleucine, valine, phenylalanine supplemented with either NH_4^+ or alanine in order to obtain a total YAN of 21.43 mM N L^{-1}. The data summarises the average fermentations (mmol/L) and standard deviation. Additionally, the letters denote significant differences (95%) between treatments using Fischer LSD.

	volatile acidity		higher alcohols				
	ethyl acetate	acetic acid	propanol	isobutanol	butanol	isoamyl alcohol	2-phenylethanol
Leu_7.14	0.31 ± 0.04 ^{a}	7.31 ± 0.38 ^{ab}	0.79 ± 0.00 ^{e}	0.67 ± 0.02 ^{d}	0.008 ± 0.000 ^{f}	7.50 ± 0.18 ^{d}	0.18 ± 0.01 ^{d}
Leu_14.28	0.23 ± 0.01 ^{bcd}	5.80 ± 0.61 ^{bcdef}	1.03 ± 0.02 ^{cd}	0.97 ± 0.16 ^{d}	0.008 ± 0.000 ^{f}	14.13 ± 0.47 ^{c}	0.17 ± 0.02 ^{d}
Leu_21.43	0.19 ± 0.01 ^{d}	4.39 ± 0.47 ^{fg}	1.40 ± 0.07 ^{a}	0.89 ± 0.10 ^{d}	0.008 ± 0.000 ^{f}	21.04 ± 2.18 ^{b}	0.12 ± 0.01 ^{d}
Ile_7.14	0.24 ± 0.01 ^{bcd}	7.28 ± 0.50 ^{abc}	0.54 ± 0.04 ^{fg}	0.75 ± 0.08 ^{d}	0.008 ± 0.000 ^{f}	7.85 ± 0.96 ^{d}	0.20 ± 0.03 ^{d}
Ile_14.28	0.24 ± 0.01 ^{bcd}	5.59 ± 0.26 ^{cdef}	0.70 ± 0.03 ^{ef}	0.92 ± 0.01 ^{d}	0.008 ± 0.000 ^{f}	14.65 ± 0.10 ^{c}	0.19 ± 0.01 ^{d}
Ile_21.43	0.20 ± 0.02 ^{d}	4.38 ± 1.12 ^{fg}	1.03 ± 0.22 ^{cd}	1.19 ± 0.36 ^{d}	0.008 ± 0.001 ^{f}	26.41 ± 6.59 ^{a}	0.12 ± 0.03 ^{d}
Val_7.14	0.28 ± 0.04 ^{ab}	5.87 ± 1.68 ^{bcdef}	0.86 ± 0.13 ^{de}	3.68 ± 1.74 ^{c}	0.028 ± 0.001 ^{a}	1.65 ± 0.06 ^{e}	0.19 ± 0.00 ^{d}
Val_14.28	0.24 ± 0.08 ^{bcd}	5.01 ± 0.77 ^{def}	1.16 ± 0.19 ^{bc}	9.60 ± 1.07 ^{b}	0.025 ± 0.002 ^{b}	1.53 ± 0.16 ^{e}	0.15 ± 0.03 ^{d}
Val_21.43	0.22 ± 0.09 ^{cd}	3.27 ± 0.65 ^{g}	1.30 ± 0.04 ^{ab}	14.05 ± 0.15 ^{a}	0.017 ± 0.001 ^{c}	1.37 ± 0.12 ^{e}	0.09 ± 0.02 ^{d}
Phe_7.14	0.31 ± 0.02 ^{a}	6.19 ± 0.46 ^{bcde}	0.47 ± 0.02 ^{g}	0.57 ± 0.03 ^{d}	0.011 ± 0.001 ^{e}	2.37 ± 0.23 ^{e}	7.68 ± 1.15 ^{c}
Phe_14.28	0.26 ± 0.03 ^{abc}	6.57 ± 0.66 ^{bcd}	0.50 ± 0.04 ^{g}	0.93 ± 0.08 ^{d}	0.011 ± 0.000 ^{e}	2.28 ± 0.04 ^{e}	14.28 ± 0.84 ^{b}
Phe_21.43	0.22 ± 0.00 ^{bcd}	5.58 ± 0.06 ^{cdef}	0.70 ± 0.02 ^{ef}	1.32 ± 0.02 ^{d}	0.012 ± 0.000 ^{de}	2.14 ± 0.05 ^{e}	21.21 ± 0.81 ^{a}
Ala_7.14	0.23 ± 0.00 ^{bcd}	4.88 ± 0.84 ^{defg}	1.07 ± 0.05 ^{c}	4.53 ± 0.69 ^{c}	0.011 ± 0.001 ^{e}	3.15 ± 0.72 ^{e}	0.21 ± 0.05 ^{d}
Ala_14.28	0.24 ± 0.01 ^{bcd}	4.59 ± 0.33 ^{efg}	1.10 ± 0.06 ^{c}	8.90 ± 1.11 ^{b}	0.011 ± 0.000 ^{e}	3.31 ± 0.44 ^{e}	0.21 ± 0.03 ^{d}
Ala_21.43	0.23 ± 0.02 ^{bcd}	5.46 ± 1.72 ^{def}	0.99 ± 0.22 ^{cd}	15.85 ± 4.30 ^{a}	0.012 ± 0.002 ^{de}	3.32 ± 0.86 ^{e}	0.23 ± 0.05 ^{d}
NH4	0.23 ± 0.02 ^{bcd}	8.57 ± 2.50 ^{a}	0.74 ± 0.15 ^{e}	0.85 ± 0.24 ^{d}	0.013 ± 0.002 ^{d}	2.72 ± 0.80 ^{e}	0.27 ± 0.08 ^{d}

Table S 3.2. continued

	acetate esters		volatile fatty acids				
	isoamyl acetate	2-phenylethyl acetate	propionic acid	isobutyric acid	isovaleric acid	valeric acid	decanoic acid
Leu_7.14	0.030 ± 0.003 ^{c}	0.003 ± 0.000 ^{d}	0.04 ± 0.00 ^{def}	0.05 ± 0.01 ^{d}	0.101 ± 0.014 ^{d}	0.006 ± 0.001 ^{i}	0.001 ± 0.001 ^{def}
Leu_14.28	0.046 ± 0.005 ^{b}	0.003 ± 0.000 ^{d}	0.04 ± 0.00 ^{def}	0.06 ± 0.00 ^{d}	0.184 ± 0.024 ^{c}	0.009 ± 0.000 ^{efg}	0.001 ± 0.000 ^{def}
Leu_21.43	0.057 ± 0.007 ^{a}	0.003 ± 0.000 ^{d}	0.04 ± 0.00 ^{def}	0.09 ± 0.01 ^{d}	0.359 ± 0.039 ^{a}	0.010 ± 0.001 ^{de}	0.002 ± 0.000 ^{cdef}
Ile_7.14	0.009 ± 0.001 ^{ef}	0.003 ± 0.000 ^{d}	0.02 ± 0.00 ^{f}	0.05 ± 0.01 ^{d}	0.115 ± 0.014 ^{d}	0.007 ± 0.000 ^{ghi}	0.001 ± 0.000 ^{def}
Ile_14.28	0.018 ± 0.003 ^{d}	0.003 ± 0.000 ^{d}	0.02 ± 0.00 ^{f}	0.06 ± 0.01 ^{d}	0.222 ± 0.047 ^{b}	0.008 ± 0.000 ^{fgh}	0.001 ± 0.000 ^{f}
Ile_21.43	0.013 ± 0.004 ^{de}	0.003 ± 0.000 ^{d}	0.02 ± 0.00 ^{f}	0.08 ± 0.01 ^{d}	0.383 ± 0.054 ^{a}	0.010 ± 0.002 ^{ef}	0.002 ± 0.002 ^{cdef}
Val_7.14	0.004 ± 0.000 ^{gh}	0.003 ± 0.000 ^{d}	0.13 ± 0.00 ^{c}	0.50 ± 0.29 ^{cd}	0.023 ± 0.003 ^{e}	0.006 ± 0.001 ^{hi}	0.001 ± 0.000 ^{ef}
Val_14.28	0.003 ± 0.001 ^{h}	0.003 ± 0.000 ^{d}	0.19 ± 0.02 ^{b}	1.34 ± 0.15 ^{b}	0.021 ± 0.003 ^{e}	0.007 ± 0.001 ^{ghi}	0.001 ± 0.000 ^{def}
Val_21.43	0.003 ± 0.001 ^{h}	0.003 ± 0.000 ^{d}	0.22 ± 0.01 ^{a}	2.27 ± 0.06 ^{a}	0.023 ± 0.001 ^{e}	0.007 ± 0.000 ^{ghi}	0.000 ± 0.000 ^{f}
Phe_7.14	0.009 ± 0.002 ^{fg}	0.037 ± 0.010 ^{c}	0.03 ± 0.00 ^{ef}	0.06 ± 0.00 ^{d}	0.028 ± 0.002 ^{e}	0.012 ± 0.001 ^{cd}	0.011 ± 0.002 ^{a}
Phe_14.28	0.008 ± 0.001 ^{fgh}	0.063 ± 0.005 ^{b}	0.03 ± 0.00 ^{ef}	0.08 ± 0.01 ^{d}	0.024 ± 0.002 ^{e}	0.017 ± 0.001 ^{b}	0.010 ± 0.001 ^{a}
Phe_21.43	0.006 ± 0.000 ^{fgh}	0.085 ± 0.011 ^{a}	0.03 ± 0.00 ^{ef}	0.10 ± 0.01 ^{d}	0.021 ± 0.001 ^{e}	0.022 ± 0.000 ^{a}	0.008 ± 0.003 ^{b}
Ala_7.14	0.006 ± 0.001 ^{fgh}	0.003 ± 0.000 ^{d}	0.03 ± 0.00 ^{ef}	0.41 ± 0.11 ^{cd}	0.036 ± 0.008 ^{e}	0.013 ± 0.002 ^{c}	0.002 ± 0.001 ^{cde}
Ala_14.28	0.006 ± 0.001 ^{fgh}	0.003 ± 0.000 ^{d}	0.03 ± 0.00 ^{ef}	0.65 ± 0.23 ^{c}	0.039 ± 0.005 ^{e}	0.013 ± 0.001 ^{c}	0.003 ± 0.001 ^{c}
Ala_21.43	0.008 ± 0.001 ^{fgh}	0.003 ± 0.000 ^{d}	0.05 ± 0.02 ^{d}	1.86 ± 1.08 ^{a}	0.045 ± 0.014 ^{e}	0.013 ± 0.002 ^{c}	0.002 ± 0.001 ^{cd}
NH4	0.006 ± 0.002 ^{fgh}	0.003 ± 0.000 ^{d}	0.04 ± 0.01 ^{de}	0.08 ± 0.02 ^{d}	0.026 ± 0.006 ^{e}	0.007 ± 0.001 ^{ghi}	0.002 ± 0.001 ^{cd}

Table S 3.3. The impact of changing the concentration of individual branched chain and aromatic amino acids in complex amino acid mixtures on the production of volatile compounds by VIN13, as determined by GC-FID (mmol/L). Fermentations contained 14.3 mmol N L^{-1} of YAN, of which 3.57 mmol N L^{-1} was provided by ammonium chloride, the remainder was made up of all the amino acids, where each one provided equal amounts of fermentable nitrogen except for leucine, isoleucine, valine, phenylalanine, tyrosine, and threonine which were also either absent (0) or present at twice (2) the concentration of the other amino acids. The data summarizes the average fermentations (mg/L) and standard deviation. Additionally, the letters denote significant differences (95%) between treatments using Fischer LSD.

	All Amino acids	**NH4**	**0 Isoleucine**	**2 Isoleucine**	**0 Leucine**	**2 Leucine**
2-phenyl ethanol	53.78 ± 3.78 cd	10.24 ± 0.69 a	53.64 ± 2.41 cd	50.40 ± 3.97 c	62.52 ± 0.51 f	57.50 ± 2.68 de
2-phenylethyl acetate	1.14 ± 0.06 bc	0.42 ± 0.00 a	1.25 ± 0.07 bcd	1.07 ± 0.12 b	1.36 ± 0.02 d	1.27 ± 0.08 cd
3-ethoxy-1-propanol	2.59 ± 0.22 de	3.43 ± 0.38 f	2.63 ± 0.16 de	2.29 ± 0.20 c	2.83 ± 0.03 e	2.18 ± 0.09 bc
acetic acid	575.27 ± 19.11 bc	656.16 ± 4.67 ef	609.67 ± 7.46 cd	590.48 ± 18.83 bcd	671.17 ± 8.13 f	623.85 ± 22.52 de
butanol	0.57 ± 0.02 bc	0.70 ± 0.04 f	0.60 ± 0.01 de	0.56 ± 0.01 bc	0.61 ± 0.02 e	0.50 ± 0.01 a
butyric acid	1.05 ± 0.05 ab	1.00 ± 0.01 a	1.06 ± 0.03 abc	1.04 ± 0.04 ab	1.04 ± 0.07 ab	1.13 ± 0.03 cd
ethyl acetate	45.74 ± 3.35 cde	48.26 ± 3.28 de	36.57 ± 0.43 a	32.80 ± 3.35 a	45.74 ± 1.49 cde	41.62 ± 2.93 bc
ethyl caprate	0.15 ± 0.07 d	0.07 ± 0.01 a	0.06 ± 0.00 a	0.08 ± 0.04 ab	0.13 ± 0.02 bcd	0.22 ± 0.02 e
ethyl caprylate	0.56 ± 0.29 bcd	0.32 ± 0.05 a	0.32 ± 0.04 a	0.37 ± 0.11 ab	0.60 ± 0.05 cde	1.03 ± 0.18 g
hexanoic acid	3.02 ± 0.16 bcde	2.71 ± 0.05 ab	3.18 ± 0.13 cde	2.90 ± 0.28 abcd	2.63 ± 0.27 a	3.34 ± 0.16 e
isoamyl acetate	1.23 ± 0.30 abc	1.23 ± 0.08 abc	1.02 ± 0.32 a	1.10 ± 0.29 a	1.73 ± 0.07 ef	2.21 ± 0.02 g
isobutanol	25.58 ± 2.73 d	13.21 ± 0.36 a	21.89 ± 2.80 bc	25.45 ± 3.52 d	25.47 ± 0.75 d	25.98 ± 0.72 d
isobutyric acid	1.78 ± 0.15 cd	0.87 ± 0.01 a	1.53 ± 0.20 b	1.80 ± 0.20 cde	1.64 ± 0.10 bc	1.94 ± 0.05 def
propanol	25.60 ± 2.48 f	48.17 ± 4.04 g	25.41 ± 0.51 ef	21.91 ± 1.44 cd	23.25 ± 0.60 cdef	22.44 ± 1.23 cde
propionic acid	1.29 ± 0.04 cd	1.97 ± 0.04 g	1.57 ± 0.03 f	1.17 ± 0.02 a	1.23 ± 0.04 abc	1.40 ± 0.06 e
valeric acid	0.53 ± 0.02 abc	0.51 ± 0.03 abc	0.56 ± 0.11 bcd	0.45 ± 0.10 ab	0.61 ± 0.02 cd	0.48 ± 0.06 ab

Table S3.3. continued

	0 Valine	2 Valine	0 Tryptophan	2 Tryptophan	0 Tyrosine	2 Tyrosine
2-phenyl ethanol	67.89 ± 5.28 g	55.68 ± 1.26 de	50.35 ± 1.37 c	55.35 ± 2.39 d	59.77 ± 1.42 ef	55.46 ± 2.24 de
2-phenylethyl acetate	1.35 ± 0.11 d	1.29 ± 0.09 cd	1.10 ± 0.03 bc	1.22 ± 0.08 bcd	1.37 ± 0.15 d	1.24 ± 0.08 bcd
3-ethoxy-1-propanol	2.76 ± 0.14 e	2.34 ± 0.10 cd	2.28 ± 0.06 c	2.34 ± 0.22 cd	1.80 ± 0.03 a	2.46 ± 0.22 cd
acetic acid	625.01 ± 26.08 de	564.03 ± 34.43 ab	656.47 ± 22.35 ef	603.27 ± 19.33 cd	584.12 ± 26.84 bc	560.63 ± 35.40 ab
butanol	0.54 ± 0.02 b	0.62 ± 0.02 e	0.54 ± 0.02 b	0.55 ± 0.01 bc	0.54 ± 0.02 b	0.58 ± 0.01 cd
butyric acid	1.15 ± 0.08 d	1.11 ± 0.05 bcd	1.13 ± 0.02 cd	1.07 ± 0.03 abcd	1.11 ± 0.08 bcd	1.07 ± 0.04 abc
ethyl acetate	44.10 ± 3.57 bcd	36.50 ± 0.26 a	49.22 ± 1.32 e	41.99 ± 3.36 bc	34.32 ± 2.51 a	41.22 ± 0.79 b
ethyl caprate	0.14 ± 0.03 cd	0.13 ± 0.02 bcd	0.09 ± 0.01 abc	0.09 ± 0.02 abcd	0.14 ± 0.06 cd	0.11 ± 0.06 abcd
ethyl caprylate	0.73 ± 0.12 de	0.79 ± 0.13 ef	0.70 ± 0.04 cde	0.73 ± 0.04 de	0.97 ± 0.06 fg	0.51 ± 0.16 abc
hexanoic acid	3.27 ± 0.35 de	3.20 ± 0.12 cde	3.07 ± 0.11 bcde	3.12 ± 0.15 cde	3.01 ± 0.27 bcde	3.09 ± 0.22 cde
isoamyl acetate	1.70 ± 0.04 ef	1.41 ± 0.11 bcd	1.69 ± 0.11 ef	1.56 ± 0.16 def	1.16 ± 0.05 ab	1.46 ± 0.08 cde
isobutanol	19.32 ± 1.62 b	46.04 ± 1.95 g	33.69 ± 1.90 f	24.50 ± 1.20 cd	26.92 ± 1.51 de	26.54 ± 2.78 de
isobutyric acid	1.55 ± 0.12 b	3.21 ± 0.18 g	1.98 ± 0.08 def	1.97 ± 0.14 def	1.90 ± 0.05 def	2.00 ± 0.07 ef
propanol	21.21 ± 1.24 bcd	23.41 ± 2.61 def	25.61 ± 1.00 f	20.33 ± 0.91 abc	18.67 ± 0.87 ab	22.92 ± 1.68 cdef
propionic acid	1.28 ± 0.05 cd	1.42 ± 0.08 e	1.30 ± 0.01 d	1.23 ± 0.03 abc	1.24 ± 0.02 bc	1.29 ± 0.02 cd
valeric acid	0.67 ± 0.11 d	0.56 ± 0.06 bcd	0.45 ± 0.04 ab	0.52 ± 0.12 abc	0.42 ± 0.12 a	0.54 ± 0.11 abcd

Table S 3.3. continued

	0 Phenylalanine			2 Phenylalanine		
2-phenyl ethanol	23.45 ± 0.77	b		122.33 ± 2.99	h	
2-phenylethyl acetate	0.61 ± 0.00	a		2.40 ± 0.34	e	
3-ethoxy-1-propanol	1.93 ± 0.13	ab		1.95 ± 0.13	ab	
acetic acid	563.43 ± 16.56	ab		537.57 ± 11.80	a	
butanol	0.56 ± 0.00	bc		0.58 ± 0.03	cd	
butyric acid	1.10 ± 0.02	bcd		1.09 ± 0.04	bcd	
ethyl acetate	43.87 ± 0.95	bc		41.92 ± 3.54	bc	
ethyl caprate	0.08 ± 0.00	ab		0.07 ± 0.01	a	
ethyl caprylate	0.62 ± 0.08	cde		0.51 ± 0.12	abc	
hexanoic acid	2.89 ± 0.12	abc		2.93 ± 0.43	abcd	
isoamyl acetate	1.74 ± 0.01	f		1.55 ± 0.17	def	
isobutanol	29.64 ± 0.88	e		26.92 ± 2.05	de	
isobutyric acid	2.10 ± 0.13	f		1.83 ± 0.14	cde	
propanol	17.45 ± 0.73	a		18.86 ± 1.98	ab	
propionic acid	1.21 ± 0.01	ab		1.20 ± 0.02	ab	
valeric acid	0.54 ± 0.02	abcd		0.53 ± 0.04	abc	

Chapter 4

Evaluating of the impact of unsaturated fatty acids with yeast or plant sterols on aroma profiles

Chapter 4

Evaluating of the impact of unsaturated fatty acids with yeast or plant sterols on aroma profiles

Abstract

The yeast volatilome makes a significant contribution to the organoleptic properties of wine. Lipids have been shown to significantly accelerate fermentation kinetics. Recently, the contribution of sterols and unsaturated fatty acids has received more interest as a means of modulating the formation of these volatile aromas. The objective of this study was to determine whether variations in lipid supplementation, yeast sterol, plant sterol or oleic acid, differently influence yeast growth as well as the production of fermentative aromas. In contrast with the sterols, oleic acid supplementation had little influence on yeast growth, but significantly altered the volatile profiles. Generally, sterol additions resulted in elevated levels of most of the volatile compounds measured, whereas oleic acid additions resulted in a reduction of acetate ester, medium chain fatty acids and their ethyl ester concentrations.

Keywords: volatile profiles, oleic acid, ergosterol, yeast growth, phytosterol

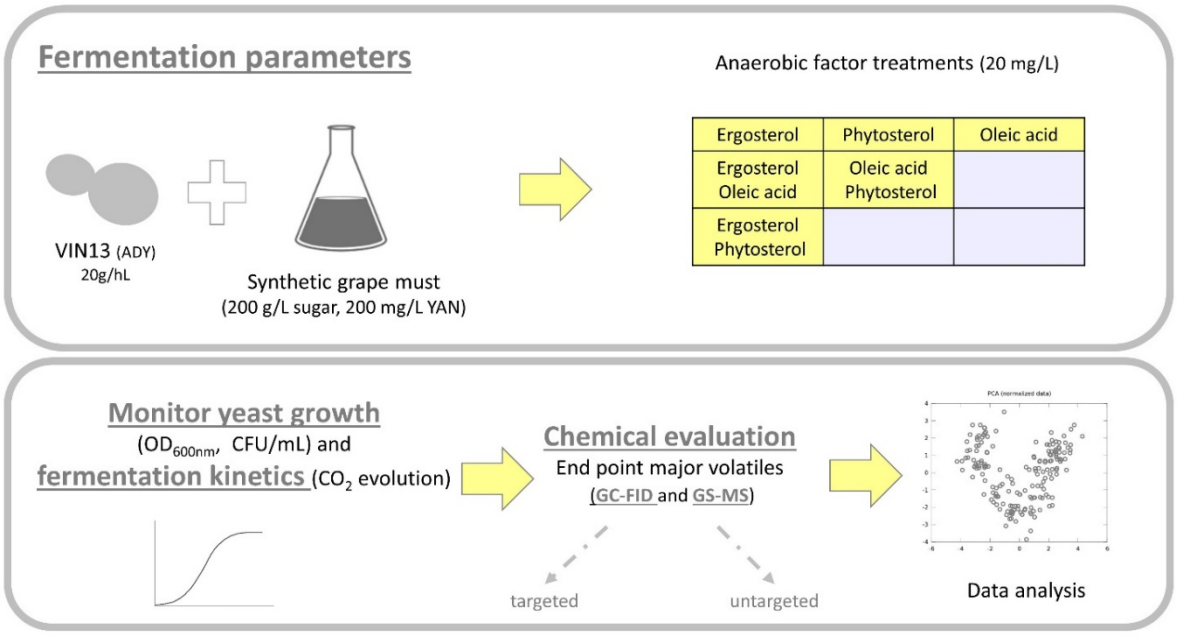

Figure 4A: *Schematic overview of experimental layout.*

4.1 Introduction

The plasma membrane composition plays an essential role in cell growth and survival (Daum 1998). The continuous adaptation of the plasma membrane to changing environmental conditions is particularly important during anaerobic batch fermentation, where it maintains membrane integrity and function in response to stresses such as high initial sugar concentrations, increasing levels of ethanol and a limited oxygen supply (Daum 1998). The cell achieves this by modulating the membrane fluidity via its composition of sterols as well as unsaturated fatty acids found in phospholipids (Mannazzu *et al*., 2008; You *et al*., 2003). There are conflicting reports regarding the importance of ergosterol content and ethanol tolerance, as high ergosterol content is not always correlated with improved tolerance (Redón *et al*., 2011; Mannazzu *et al*., 2008; You *et al*., 2003; Aguilera *et al*., 2006). In contrast, ethanol tolerant yeast cells generally contain higher levels of unsaturated fatty acids (You *et al*., 2003).

To produce one molecule of ergosterol, the yeast uses 12 molecules of molecular oxygen (Snoek & Steensma, 2007). Similarly, the desaturation of fatty acids also requires one molecule of molecular oxygen. *Saccharomyces cerevisiae* is only able to produce monounsaturated fatty acids, therefore, the most prevalent fatty acids found in yeast cells are palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), and oleic acid (C18:1) (Daum *et al.,* 1998). During alcoholic fermentation, oxygen is rapidly depleted, and the cell is no longer able to manufacture these protective compounds but is able to assimilate it from the environment. Consequently, research has moved from studying the impact of ergosterol, a yeast sterol, to the impact of phytosterol, plant sterols (β-sitosterol (70%), campesterol and stigmasterol (Delfini *et al.,* 1993)), found in grape must (Luparia *et al.,* 2004; Rollero *et al*., 2015; Ochando *et al*., 2017).

Phytosterol levels in grape must are greatly affected by winemaking practices, such as skin contact time and clarification, with reported levels of β-sitosterol ranging between 100 mg/L to 6 mg/L (Delfini *et al.,* 1993). A survey of Sauvignon blanc grape musts, over three vintages, reported total levels of free fatty acids (caproic acid (C6:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic (C18:1n-9), linoleic acid (C18:2n-6,9), and γ-Linolenic acid (C18:3n-6,9,12)) ranging from 0 to 28.45 mg/L (Tumanov *et al*., 2015).

Previous studies evaluating the impact of sterols and unsaturated fatty acids, have reported that supplementation, improves yeast growth and consequently also fermentation kinetics (Taylor *et al.*, 1979; Luparia *et al*., 2004; Ochando *et al*., 2017), changes in the plasma membrane composition

(Thurston *et al.,* 1981; Luparia *et al*., 2004, Mauricio *et al.,* 1998) as well as the production of volatile compounds (Rollero *et al.,* 2015; Duan *et al.,* 2015; Varela *et al*.,2012; Mauricio *et al*.,1997).

This study seeks to explore whether sterols, oleic acid, and their various combinations differently affect fermentation kinetics, yeast growth as well as the production of volatile aroma compounds, evaluated using targeted and untargeted chemical analyses. Fermentations were conducted in synthetic grape must supplemented with ergosterol, phytosterol (β-sitosterol), and oleic acid independently (20 mg/L) as well as in combination (10 mg/L of each component) with each other.

4.2 Methods and materials

4.2.1 Fermentation media, conditions, and treatments

Saccharomyces cerevisiae yeast strain, VIN13 (Anchor Yeast, Cape Town, South Africa), was rehydrated (20 g/hL) according to the supplier's instructions. Briefly, VIN13 was rehydrated for 20 min at 37 °C in warm water and subsequently cooled to within 10°C of the media's temperature before inoculation.

Fermentations were conducted using synthetic grape must (100 g/L glucose and 100 g/L fructose), as previously described (Henschke & Jiranek, 1993). The nitrogen sources (amino acids and ammonium) were added at a concentration of 200 mg/L of fermentable nitrogen (Bely *et al*., 1990). Fermentations were treated with anaerobic factors (20 mg/L): phytosterol (β-sitosterol), ergosterol, and oleic acid, and contained 10 mg/L of each constituent when used in combinations. Anaerobic factors were dissolved in ethanol and the untreated control received an addition of only ethanol.

Self-generated anaerobiosis was aided by replacing the oxygen found in the headspace with sterile nitrogen, in addition to conducting fermentations in modified Erlenmeyer flasks fitted with a sample port, sealed with a septum. The flasks were sealed with rubber stoppers and a CO_2 outlet was provided. Static fermentation took place at 20°C, and its progress was monitored twice a day for the first week and daily thereafter by weight loss, and growth by OD (600 nm) and plate counts (CFU/mL).

4.2.2 Targeted and untargeted chemical analyses

4.2.2.1 Gas chromatography flame ionization detector (GC-FID)

At the end of alcoholic fermentation, samples underwent a liquid-liquid extraction as described by Louw *et al* (2009) for analysis by gas chromatography. A 5mL sample of the fermented media, 100 µl of internal standard (4-methyl-2-pentanol) and 1 mL of solvent (diethyl ether) were combined and this mixture was placed in an ultrasonic bath for 5 minutes to facilitate extraction. The mixture was

centrifuged for 3 min at 4000 rpm after which Na_2SO_4 was added to remove any water from the non-polar layer and the sample was again centrifuged for another 3 min at 4000 rpm. A Hewlett Packard 6890 Plus GC-FID instrument (Agilent, Little Falls, Wilmington, USA) with a split/splitless injector was used for major volatiles quantification. The split flow rate was set at 49.4 ml/min and the split ratio was set to 15:1 at a temperature of 200°C. The separation of compounds was done using a J and B DBFFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with the dimensions of 60 m x 0.32 mm and a 0.5 µl coating film thickness with the flow rate of the hydrogen carrier gas set at 3.3 ml/min. Once the FID oven temperature reached the temperature of 240°C 3 µl of extracted sample was injected into the gas chromatograph at an initial temperature of 33°C and held for 8 min; the temperature was then increased by 21°C/min to 130°C and then held for 17 min; increased by 12°C/min to 170°C and held for 5 min; increased by 21°C/min to 240°C and held for 2.5 min. A post run step at the end of each sample was carried out at 240°C for 5 min. Each sample was injected in duplicate. The column was cleaned with an injection of hexane after every 20 samples. Authentic reference standards (Merck, Cape Town) were used to calibrate for each of the compounds using the internal standard compound 4-methyl-2-pentanol. Manual data collection and peak integration were done using the HP ChemStation software (Rev. B01.03 [204]).

4.2.2.2 Gas chromatography mass spectrometry (GC-MS)

The fermentative volatile compounds were extracted via headspace analysis (HS)-SPME using a DVB/CAR/PDMS fibre (Supelco, Bellefonte, PA). GC analysis was performed using a Trace GC Ultra gas chromatograph coupled with a TSQ Quantum Tandem mass spectrometer. GC separation was performed on a 30m Zebron capillary GC column (ZB-FFAP, Phenomenex) capillary column with an internal diameter of 0.25 mm and a film thickness of 0.25 µm.

A 5 mL sample, spiked with 50 µL of anisole D8 (50 mg/L) as the internal standard, and sodium chloride (1 g) were added to the 20mL vial. Samples were loaded onto the Triplus RSH autosampler (Thermo Electron Corporation, Waltham, MA), and in the agitator set at 50°C and 500 rpm for 5 min, after which the fibre was exposed to the sample headspace for 15 minutes. Desorption of volatiles took place over 10 minutes in the injector in split mode, with a ratio of 10. The initial oven temperature of 45°C, was held for 3 min, after which it was increased at a rate of 3 °C/min to 80 °C (held for 3 min), followed by an increase of 4°C/min to 110 °C (held for 5 min) and a final ramp of 15°C/min to 250 °C was reached and held for 3 min. The carrier gas, helium, was used at a constant flow of 0.8 mL/min, the split flow rate was 8 mL/ min. The inlet port was heated to 240°C. All full scan mass spectra were acquired in the electron impact (EI) mode (ionization energy, 70 eV; source temperature, 250°C). Data acquisition and analyses were performed using the Xcalibur Workstation software supplied by the

manufacturer. The identification of unknown compounds was achieved by comparing the retention times of standards, and mass spectra in the NIST library.

4.2.3 Data analyses

Analysis of variance (ANOVA) of chemical data was performed using XLSTAT 2017 (Addinsoft, Paris, France), using Fisher's LSD for the post-hoc testing ($p < 0.05$). Principal component analysis (PCA) was performed with the XLSTAT 2017 (Addinsoft, Paris, France).

The untargeted MS chromatographs underwent baseline correction and peak alignment. The resultant data were normalized with respect to the internal standard and autoscaled. The co-clustering of fermentation treatments and peak areas were used to identify odorants via their retention times. Unsupervised two-way agglomerative hierarchical cluster analysis (HCA) was applied to evaluate the similarities between individual profiles. This algorithm used a multivariate Euclidean distance metric and Ward's group linkage. The results are visualized as a heat map (green = low concentration, red = high concentration) with associated cluster dendrograms; the lower the linkage distances in the dendrogram the more similar the feature. All the statistical analyses were performed using the Matlab® scripting language, version R2014a (http://www.mathworks.com).

4.3 Results

4.3.1 Fermentation and growth kinetics

The application of sterols, either ergosterol or phytosterol, resulted in an increase in yeast growth, and consequently also fermentation kinetics relative to the untreated control. Interestingly, similar fermentation kinetics and population development profiles were observed for all sterol treatments (Figure 4.1 A and B), irrespective of dosage or combination of lipids applied. In contrast, for most of the exponential phase, the fermentations containing only unsaturated fatty acids were sluggish (Figure 4.1 C). Moreover, the addition of oleic acid resulted in only a slight, but significant, improvement in fermentation kinetics but not in yeast growth, suggesting a disconnection between these two parameters. The untreated control completed fermentation, probably due to the sterols and unsaturated fatty acids present in the inoculum, in addition to the dissolved oxygen present in the initial media. All the optical density observations were in agreement with the viable plate count data (data not shown).

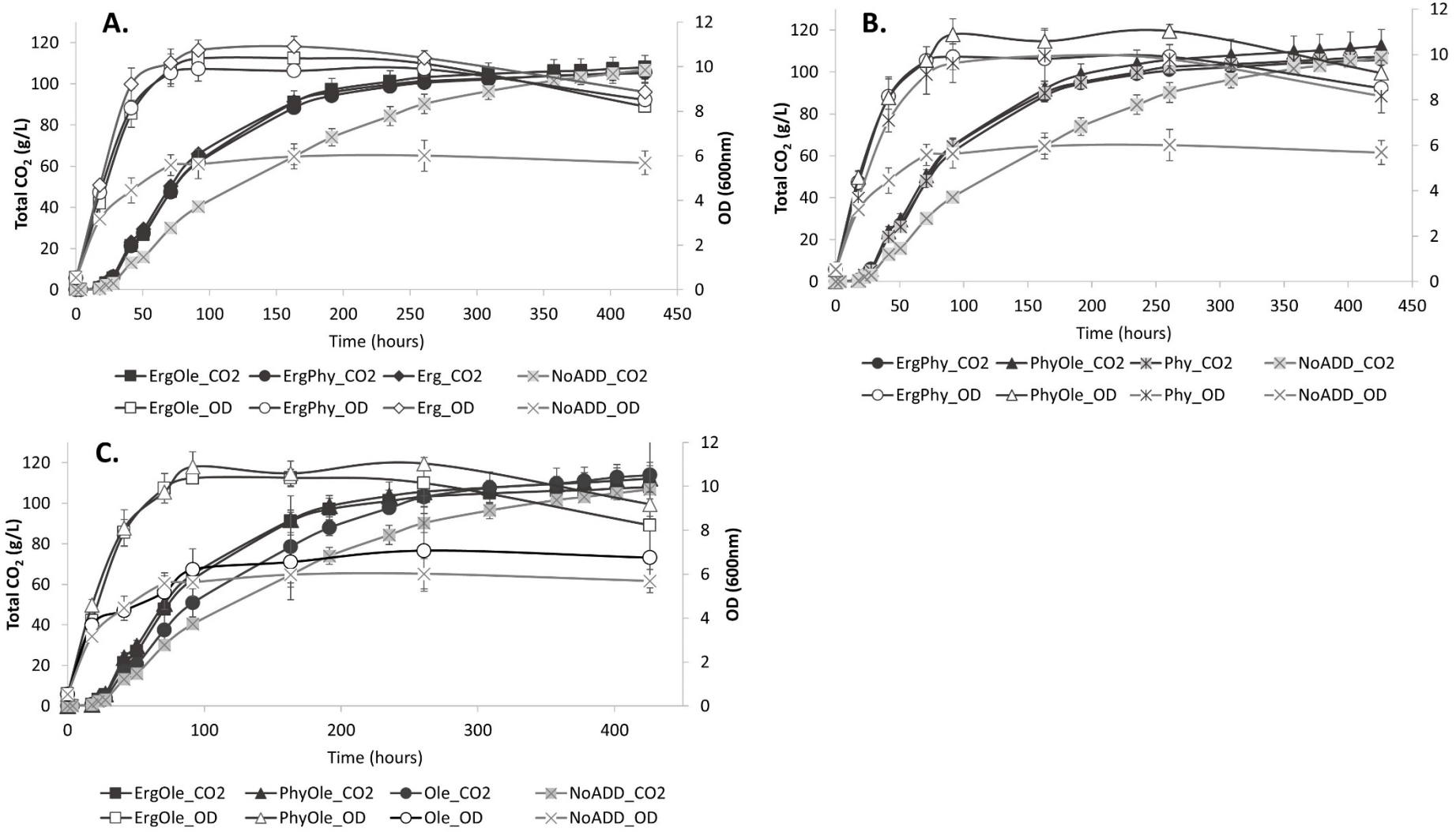

Figure 4.1. The impact of anaerobic factors on the fermentation kinetics (CO_2), as well as optical density (OD) of VIN13. The anaerobic factor treatments were as follows: **(A)** ergosterol (Erg), **(B)** phytosterol (Phy), and **(C)** oleic acid (Ole) used independently in addition to their use in combinations with each other. The error bars denote the standard deviation between triplicate treatments.

Table 4.1. The impact of anaerobic factor treatments on the production of volatile aroma compounds (mg/L), as determined by GC-FID. The anaerobic factors (20 mg/L) contain ergosterol (Erg), phytosterol (Phy), and oleic acid (Ole) used independently as well as in combinations with each other. The letters denote the significant differences between treatments as calculated by Fisher's LSD ($P < 0.05$).

	No addition	Ergosterol	Phytosterol	Oleic acid	Ergosterol + Oleic acid	Ergosterol + Phytosterol	Phytosterol + Oleic acid
Higher alcohols							
butanol	1.02 ± 0.05 ^{a}	0.91 ± 0.03 ^{bc}	1.06 ± 0.09 ^{a}	1.01 ± 0.08 ^{ab}	0.87 ± 0.07 ^{c}	0.90 ± 0.03 ^{c}	0.96 ± 0.04 ^{abc}
isobutanol	9.73 ± 0.72 ^{b}	12.18 ± 0.32 ^{a}	12.90 ± 1.20 ^{a}	13.02 ± 0.77 ^{a}	12.83 ± 1.06 ^{a}	12.10 ± 0.96 ^{a}	12.52 ± 0.39 ^{a}
propanol	33.45 ± 2.51 ^{d}	43.95 ± 1.69 ^{bc}	50.85 ± 2.36 ^{a}	41.12 ± 5.62 ^{c}	47.94 ± 4.47 ^{ab}	48.40 ± 4.53 ^{ab}	47.17 ± 1.39 ^{abc}
isoamyl alcohol	77.13 ± 4.75 ^{d}	97.17 ± 4.67 ^{abc}	105.19 ± 6.26 ^{a}	88.62 ± 6.57 ^{c}	101.90 ± 9.79 ^{ab}	92.71 ± 4.26 ^{bc}	106.38 ± 4.37 ^{a}
3-ethoxy-1-propanol	***2.13 ± 0.10*** ^{d}	***3.18 ± 0.10*** ^{c}	***4.29 ± 0.22*** ^{a}	***3.40 ± 0.72*** ^{bc}	***4.38 ± 0.43*** ^{a}	***3.96 ± 0.38*** ^{ab}	***4.59 ± 0.25*** ^{a}
2-phenyl ethanol	11.50 ± 1.18 ^{d}	26.39 ± 2.12 ^{ab}	25.46 ± 2.31 ^{b}	17.47 ± 5.46 ^{c}	28.35 ± 2.91 ^{ab}	24.36 ± 0.81 ^{b}	31.14 ± 4.08 ^{a}
Acetate esters							
isoamyl acetate	0.97 ± 0.03 ^{c}	1.65 ± 0.18 ^{b}	1.99 ± 0.27 ^{a}	0.65 ± 0.09 ^{d}	1.01 ± 0.13 ^{c}	1.45 ± 0.10 ^{b}	1.16 ± 0.15 ^{c}
2-phenylethyl acetate	***0.52 ± 0.01*** ^{e}	***0.93 ± 0.08*** ^{ab}	***1.01 ± 0.07*** ^{a}	***0.44 ± 0.03*** ^{e}	***0.64 ± 0.07*** ^{d}	***0.84 ± 0.02*** ^{b}	***0.74 ± 0.06*** ^{c}
Short chain fatty acids							
acetic acid	660.6 ± 29.74 ^{ab}	701.9 ± 24.90 ^{a}	594.2 ± 43.57 ^{bc}	664.9 ± 61.50 ^{ab}	689.6 ± 46.20 ^{a}	704.8 ± 18.99 ^{a}	538.3 ± 41.64 ^{c}
propionic acid	***1.03 ± 0.05*** ^{c}	***1.30 ± 0.09*** ^{a}	***1.35 ± 0.08*** ^{a}	***1.12 ± 0.12*** ^{bc}	***1.37 ± 0.10*** ^{a}	***1.30 ± 0.09*** ^{a}	***1.27 ± 0.06*** ^{ab}
isobutyric acid	***0.67 ± 0.03*** ^{b}	***0.82 ± 0.03*** ^{a}	***0.74 ± 0.04*** ^{b}	***0.67 ± 0.06*** ^{b}	***0.70 ± 0.05*** ^{b}	***0.73 ± 0.04*** ^{b}	***0.70 ± 0.03*** ^{b}
butyric acid	0.96 ± 0.02 ^{c}	1.18 ± 0.01 ^{a}	1.06 ± 0.05 ^{b}	0.60 ± 0.05 ^{e}	0.88 ± 0.06 ^{d}	1.14 ± 0.01 ^{a}	0.89 ± 0.06 ^{cd}
isovaleric acid	***0.88 ± 0.03*** ^{d}	***1.11 ± 0.03*** ^{a}	***1.00 ± 0.04*** ^{b}	***0.90 ± 0.09*** ^{cd}	***0.99 ± 0.06*** ^{bc}	***1.01 ± 0.06*** ^{b}	***0.96 ± 0.03*** ^{bcd}
valeric acid	***0.31 ± 0.01*** ^{d}	***0.41 ± 0.01*** ^{bc}	***0.44 ± 0.04*** ^{ab}	***0.38 ± 0.04*** ^{c}	***0.46 ± 0.01*** ^{ab}	***0.43 ± 0.02*** ^{b}	***0.48 ± 0.04*** ^{a}
Short chain ethyl esters							
ethyl acetate	44.20 ± 3.33 ^{c}	65.71 ± 3.53 ^{a}	69.37 ± 4.34 ^{a}	36.83 ± 4.03 ^{d}	51.93 ± 4.64 ^{b}	64.44 ± 2.52 ^{a}	47.98 ± 3.32 ^{bc}
ethyl butyrate	***0.53 ± 0.00*** ^{b}	***0.56 ± 0.02*** ^{a}	***0.56 ± 0.03*** ^{ab}	***0.48 ± 0.02*** ^{c}	***0.53 ± 0.02*** ^{b}	***0.55 ± 0.02*** ^{ab}	***0.54 ± 0.02*** ^{ab}
Medium chain fatty acids							
hexanoic acid	2.68 ± 0.19 ^{c}	3.57 ± 0.13 ^{a}	3.08 ± 0.16 ^{b}	1.46 ± 0.14 ^{e}	2.52 ± 0.31 ^{cd}	3.32 ± 0.17 ^{ab}	2.23 ± 0.21 ^{d}
octanoic acid	3.01 ± 0.61 ^{cde}	4.18 ± 0.27 ^{a}	3.88 ± 0.48 ^{ab}	2.29 ± 0.34 ^{e}	3.20 ± 0.61 ^{bcd}	3.69 ± 0.35 ^{abc}	2.81 ± 0.42 ^{de}
decanoic acid	***1.68 ± 0.20*** ^{bc}	***2.29 ± 0.11*** ^{a}	***1.83 ± 0.14*** ^{bc}	***1.19 ± 0.08*** ^{d}	***1.62 ± 0.26*** ^{c}	***1.92 ± 0.09*** ^{b}	***1.31 ± 0.16*** ^{d}
Medium chain ethyl esters							
ethyl hexanoate	***0.82 ± 0.04*** ^{a}	***0.81 ± 0.05*** ^{ab}	***0.82 ± 0.08*** ^{a}	***0.67 ± 0.02*** ^{c}	***0.74 ± 0.03*** ^{bc}	***0.81 ± 0.01*** ^{ab}	***0.72 ± 0.02*** ^{c}
ethyl caprate	***0.15 ± 0.01*** ^{a}	***0.13 ± 0.02*** ^{abc}	***0.14 ± 0.03*** ^{ab}	***0.10 ± 0.00*** ^{c}	***0.12 ± 0.01*** ^{bc}	***0.12 ± 0.00*** ^{abc}	***0.12 ± 0.01*** ^{bc}

Values in bold and italics were below limit of quantification

4.3.2 Volatile aroma profiles

At the end of alcoholic fermentation, the fermented media underwent chemical analysis (Table 4.1). Principal component analysis (PCA) was performed on the GC-FID data generated to evaluate whether the lipid treatments brought about reproducible overall changes to the production of aromatic compounds (Figure 4.2). The first PC describes the data (48.69%) with regard to the absence or presence of sterols. Whereas the second principal component (25.9%) separates the fermentations treated with oleic acid from the remaining treatments. The PCA suggests that relative to the untreated control, both the oleic acid and sterol treatments were influential in determining the volatile aromas produced.

Both PCA and co-clustering are unsupervised data analyses methods, however, PCA (Figure 4.2) maximises the variation between samples whereas co-clustering does not (Figure 4.2 and 4.3). Interestingly, both methods, PCA (GC-FID) and co-clustering (GC-MS), yield similar clustering patterns, namely, the (1) no addition control, (2) oleic acid, (3) sterols (individually and combined), and lastly the (4) sterols with oleic acid.

A lipid deficiency or oleic acid supplementation both displayed low levels of all volatile compounds measured, with some subtle differences between them (Table 1, Figure 4.2 and 4.3). The fermentations treated with oleic acid had comparatively lower levels of acetate esters, volatile fatty acids and their ethyl esters. Fermentation supplemented with a combination of sterols and unsaturated fatty acids, displayed a similar reduction in these compounds, whereas the sterol treatments, individual and combinations, both displayed elevated levels of most compounds.

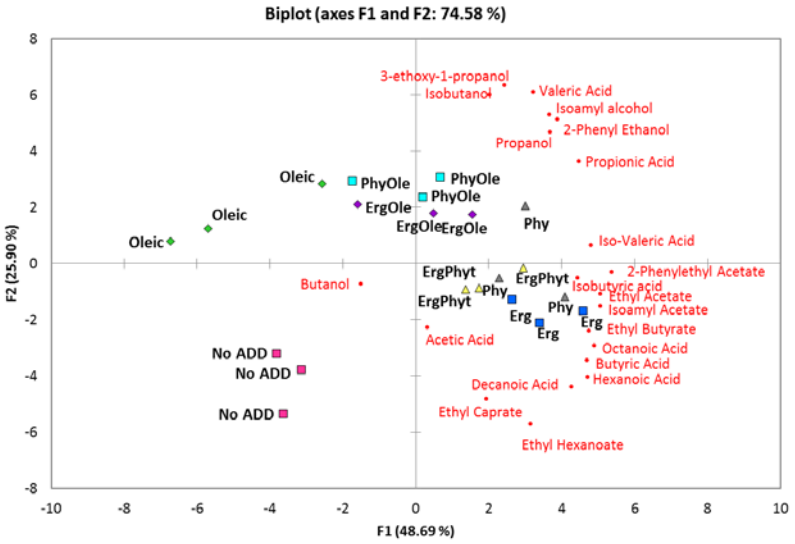

Figure 4.2. Principal component analyses of the GC-FID data of fermentations treated with lipids as follows: ergosterol (Erg), phytosterol (Phy), and oleic acid (Ole) added independently, in addition to their use in combinations with each other, as well as a no addition (No ADD) control.

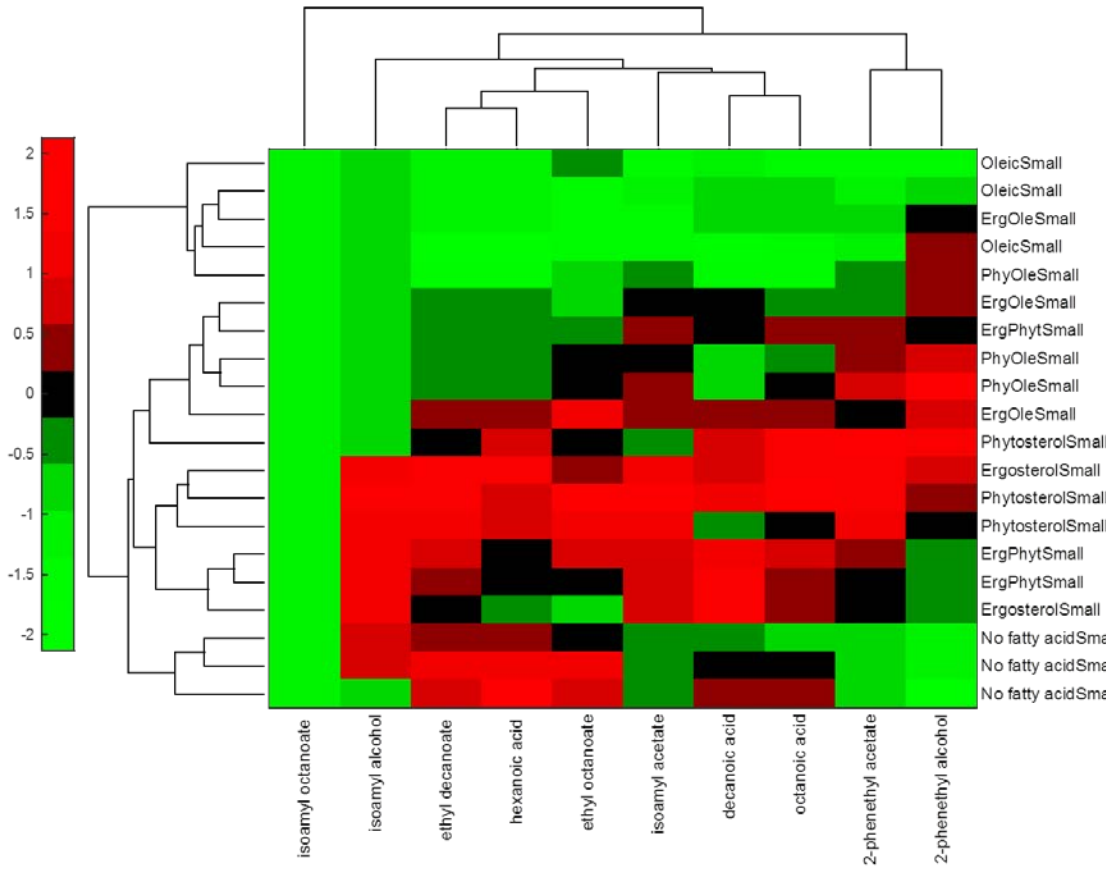

Figure 4.3. Unsupervised co-cluster analysis of untargeted GC-MS peak areas relating to ergosterol (Erg), phytosterol (Phy), and oleic acid (Ole) added independently, in addition to their use in combinations with each other, as well as a no addition (No ADD) control.

The ultimate aromatic outcomes in response to nutrient supplementation are relevant in a winemaking context (Figure 4.2 and 4.3). However, the cell's response to the supplementation is also of interest. Consequently, the GC-FID data obtained was normalised by the maximum optical density measurements. This normalisation is based on the assumption that the maximum OD is correlated with the total biomass produced. An overview of this normalised data is shown in figure 4.4, as seen in figure 4.2, the first PC describes the data (59.85%) with regard to the absence or presence of sterols. Whereas the second principal component (23.72%) separates the fermentations treated with oleic acid from the remaining treatments. Interestingly, compared to figure 4.2, the relative positioning of treatments in figure 4.4 have swapped in PC1. Following normalization, the negative control treatment displayed higher levels of volatile fatty acids and their ethyl esters. Additionally, the oleic acid and no addition treatments contained high levels of higher alcohols.

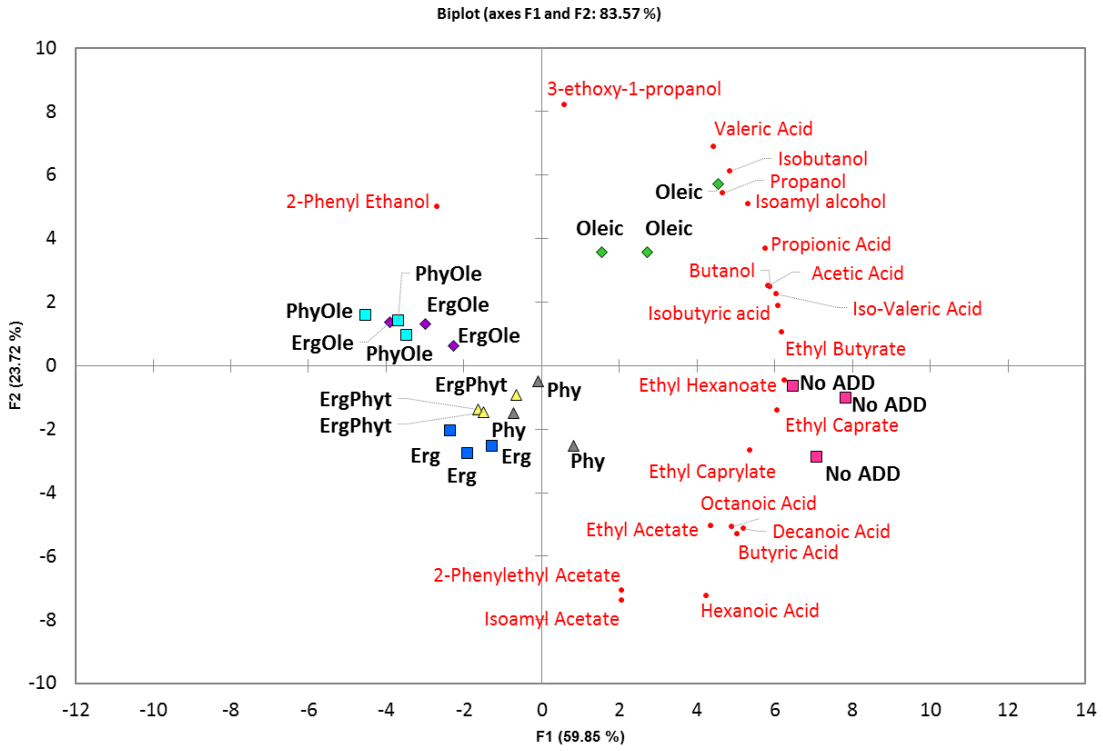

Figure 4.4. Principal component analyses of the GC-FID data normalised by the maximum optical density (OD) of fermentations treated with lipids as follows: ergosterol (Erg), phytosterol (Phy), and oleic acid (Ole) added independently, in addition to their use in combinations with each other, as well as a no addition (No ADD) control.

4.3.2.1 Higher alcohols

Generally, relative to the untreated control, the application of sterols, regardless of the combinations used, brought about an increase in the production of propanol, isoamyl alcohol, 3-ethoxy-1-propanol and 2-phenylethyl alcohol (Figure 4.5 A, Table 4.1). Despite having only, a slight impact on fermentation kinetics, oleic acid had a profound impact on the production of aromatic compounds. It is important to note that the concentrations of aromatic compounds produced were generally also different from the untreated control, indicating that the observations are not due to diminished growth alone. The supplementation of oleic acid alone also caused a significant increase in isobutanol and propanol.

Interestingly, following normalization, the data show that yeast cells treated with oleic acid as well as the no addition control actually produced greater concentrations of higher alcohols than the sterol treatments (Figure 4.6 A).

4.3.2.2 Acetate esters

The production of acetate esters was greatly influenced by the lipid treatments applied (Table 4.1). Ethyl acetate and isoamyl acetate levels were lower in the untreated control, and all oleic acid treatments (Figure 4.5 and 4.6, Table 4.1). Although 2-phenylethyl acetate levels were below the limit

of quantification, the data trends suggest a similar response. Furthermore, with increasing levels of oleic acid, decreasing levels of isoamyl acetate were observed when comparing oleic acid paired with either phytosterol or ergosterol to the oleic acid only treatment. This response is conserved to a large extent, following normalization (Figure 4.5 and 4.6 B). Additionally, the use of either or both sterols resulted in a significant increase in acetate esters.

4.3.2.3 Short chain fatty acids and their ethyl esters

The combination of lipid additions differently impacted the various short-chain fatty acids measured (Table 4.1). The presence of oleic acid, relative to its concentration, resulted in the production of lower levels of butyric acid, whereas sterol supplementation yielded increased levels of butyric acid (Figure 4.5 C).

4.3.2.4 Medium chain fatty acids and their ethyl esters

Interestingly, medium-chain fatty acid (MCFA), hexanoic acid, octanoic acid, and decanoic acid, production (Figure 4.5 C, Table 4.1) was affected in a similar manner. Whereby oleic acid decreased the levels of MCFA produced when used alone as well as when used in combination with a sterol. This pattern is mirrored in the ethyl ester data (ethyl hexanoate and ethyl caprate) as shown in figure 4.5 D (Table 4.1). Additionally, ergosterol treatments yielded greater concentrations of hexanoic and decanoic acid than those treated with phytosterol. Following normalization, the data indicated that the no addition control produced comparatively high levels of medium chain fatty acids and their ethyl esters (Figure 4.6 C and D).

Overall, ergosterol and phytosterol additions resulted in a similar response with regards to the production of volatile compounds, however, the use of phytosterol resulted in higher levels of propanol, butanol, isoamyl acetate and 3-ethoxy-1-propanol than that of ergosterol (Table 4.1). Interestingly, phytosterol treatments also produced lower levels of acetic acid, except when combined with ergosterol.

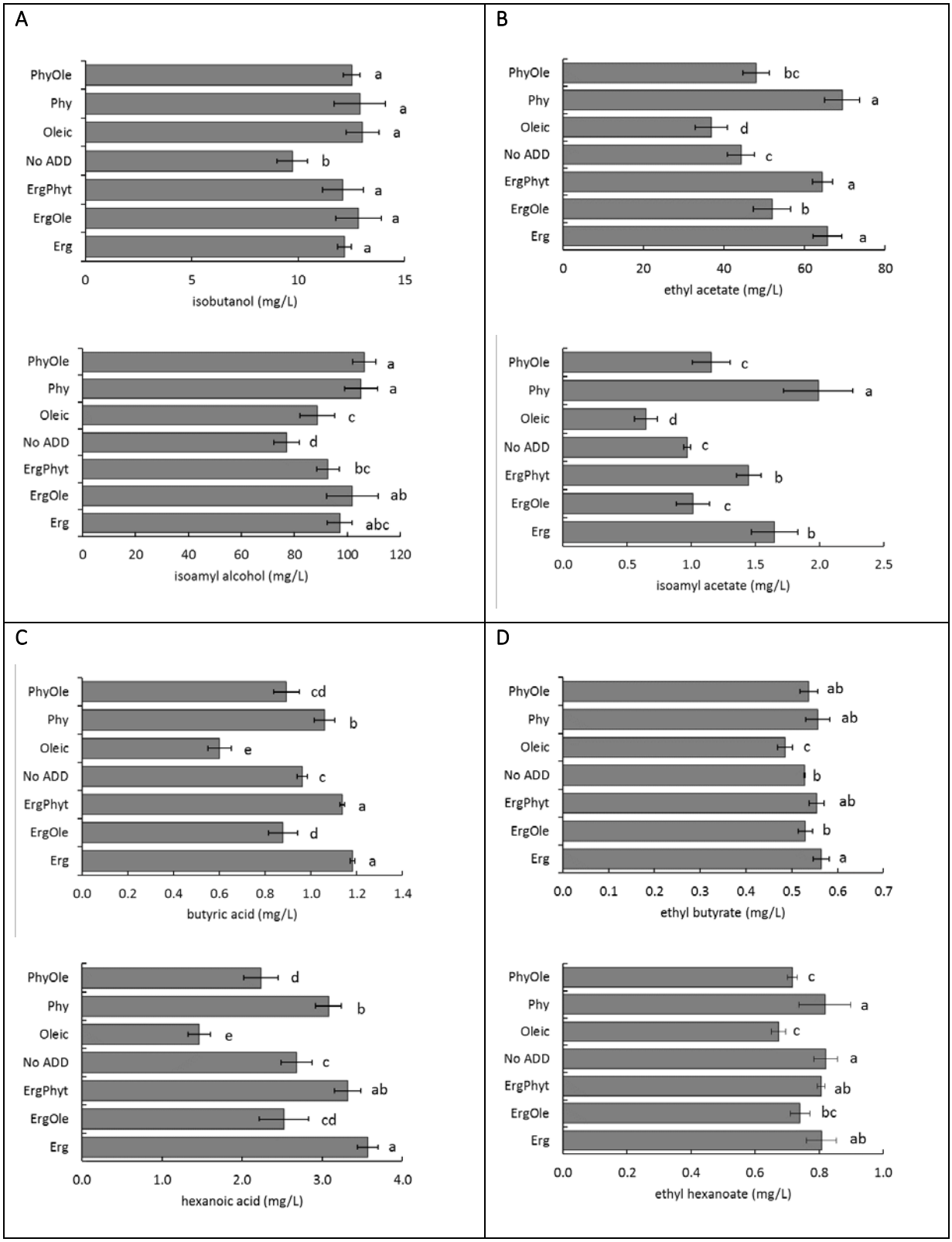

Figure 4.5. The impact of anaerobic factors on the production of **(A)** higher alcohols, isobutanol and isoamyl alcohol, **(B)** acetate esters: ethyl acetate and isoamyl acetate, **(C)** volatile fatty acids: butyric acid and hexanoic acid, **(D)** ethyl esters: ethyl butyrate and ethyl hexanoate, as determined by GC-FID. The anaerobic factors contain ergosterol (Erg), phytosterol (Phy), and oleic acid (Ole) added independently, in addition to their use in combinations with each other. The error bars denote the standard deviation between triplicate treatments, and the letters the significant differences as calculated by Fisher's LSD ($P < 0.05$).

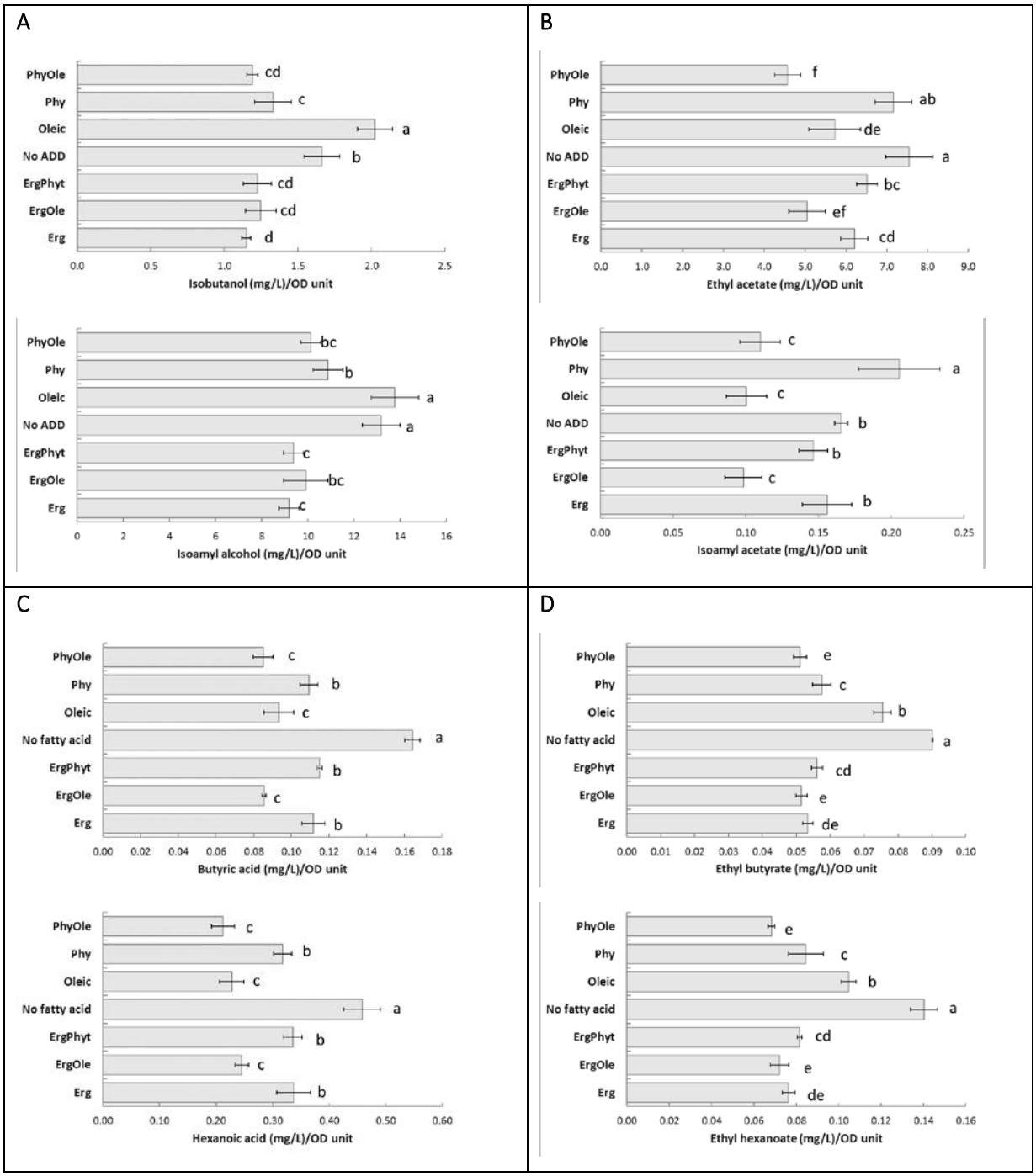

Figure 4.6. The impact of anaerobic factors on the production of **(A)** higher alcohols, isobutanol and isoamyl alcohol, **(B)** acetate esters: ethyl acetate and isoamyl acetate**, (C)** volatile fatty acids: butyric acid and hexanoic acid, (**D)** ethyl esters: ethyl butyrate and ethyl hexanoate, as determined by GC-FID and normalised by maximum OD. The anaerobic factors contain ergosterol (Erg), phytosterol (Phy), and oleic acid (Ole) added independently, in addition to their use in combinations with each other. The error bars denote the standard deviation between triplicate treatments, and the letters the significant differences as calculated by Fisher's LSD ($P < 0.05$).

4.4 Discussion

The impact of increasing concentrations of anaerobic factors has been the subject of several studies, and as such has highlighted their importance in yeast growth as well as their impact on the production of aromatic compounds (Taylor *et al*., 1979; Duan *et al*., 2015; Varela *et al*., 2012). This study explores whether combinations of yeast (ergosterol) and plant (phytosterol) derived sterols together with oleic acid differently affect yeast growth and volatile aroma production.

The formation of fermentation associated metabolites is inextricably linked to cell metabolism. Consequently, those factors which alter yeast growth could also influence the production of volatile compounds (Dufour *et al*., 2003). Indeed, phytosterol or ergosterol supplementation significantly enhanced both yeast growth, and consequently also fermentation kinetics relative to the untreated control. Luparia *et al* (2004) reported that only 5 mg/L of either sterol would result in similar maximum fermentation rates, and population sizes, but also found that phytosterol supplementation results in comparatively sluggish fermentations due to a decrease in yeast viability during the latter part of fermentation. In contrast, our study shows similar yeast growth and fermentation trends, irrespective of concentration (10 or 20 mg/L) of sterol applied. This may be as a result of differing fermentation conditions employed, particularly the high sugar concentrations used (240 g/L). In contrast with the sterol applications, oleic acid (C18:1, 20 mg/L) only slightly improved fermentation kinetics and resulted in no significant improvement in yeast growth compared to the untreated control. Furthermore, this divergent growth pattern occurred even before ethanol levels would have had an inhibitory effect, which suggests that the reduced growth was due to lower sterol levels. Similarly, the addition of linoleic acid (C18:2, 50 mg/L) reportedly brought about a slight increase in biomass formation and fermentation kinetics (Thurston *et al*., 1982). It is probable that the addition of unsaturated fatty acids, at the levels tested in this study (20 mg/L), only brought about a slight improvement in phospholipid production. In so doing, this allowed ATP to be diverted somewhere other than fatty acid synthesis, however, this energy saving and the potential improvement in ethanol tolerance was not sufficient to greatly impact cell growth.

Sterol supplementation enhanced yeast growth as well as increasing sugar metabolism, this may account for the elevated levels of higher alcohols observed (Dufour *et al*., 2003). Interestingly, after normalising the volatile data with respect to biomass produced, the untreated control and the oleic acid treatment produced comparatively higher concentrations of higher alcohols. The reason for this is unclear and warrants further exploration. However, under both conditions, VIN13 also produced

comparatively more acetic acid, therefore it is possible that higher alcohol production was favoured in order to restore the redox balance.

Amino acids or alpha-keto acids are metabolised via the Ehrlich pathway, resulting in the formation of either higher alcohols or fusel acids,-depending on the redox state of the cell (Hazelwood *et al*., 2008; Ehrlich, 1907). Subsequently, the higher alcohol may react with acetyl-CoA to produce an acetate ester. The availability of these substrates, acetyl-CoA, and an alcohol group, in addition to the activity of alcohol acetyl transferase (ATF1 and ATF2) enzymes, influence acetate ester formation (Yoshioka and Hashimoto, 1981; Saerens *et al*., 2010). Furthermore, the expression of ATF1 is repressed by the presence of unsaturated fatty acids and oxygen (Fujii *et al.,* 1997). The data shown here clearly indicates that the addition of oleic acid resulted in a decrease in the production of ethyl acetate, isoamyl acetate and 2-phenylethyl acetate in a concentration-dependent manner, which may be due to this inhibition of ATF1. As the formation of acetate esters is hindered by the presence of unsaturated fatty acids, an accumulation of higher alcohols would be expected. It is important to keep in mind that a significant change in acetate ester levels, such as isoamyl acetate present at 0.65 mg/L in the oleic acid treatment, would not necessarily translate to significant increases in its corresponding higher alcohol, isoamyl alcohol, which has a concentration of 88.62 mg/L, due to their relative abundance (Table 4.1). Therefore, relative to the untreated control, the fermentations treated with only oleic acid, contained a greater concentration of higher alcohols, although this increase was not necessarily significant (isoamyl alcohol and 2-phenyl ethanol).

Medium chain fatty acid synthesis is repressed by the presence of extracellular unsaturated fatty acids (Saerens *et al*., 2010), which corresponds with decreases in hexanoic acid, octanoic acid, and decanoic acid. In the absence of exogenous lipids, fatty acid biosynthesis is activated and once oxygen has been depleted, the accumulation of saturated fatty acids results in the release of what will become a medium chain fatty acid from the fatty acid synthase complex (Dufour *et al*., 2003). Ethyl ester production mirrors that of the medium chain fatty acids, as its production is limited by the precursors, medium chain fatty acid (acyl-CoA) and ethanol, concentration as well as enzyme activity (Saerens *et al.*, 2008). Following normalization, the untreated control displayed elevated levels of medium chain fatty acids, which is possibly indicative of an attempt to ultimately enhance the unsaturated fatty acid content of the cell to bolster its stress tolerance before oxygen is depleted.

Mouret *et al* (2014) reported that phytosterol and ergosterol differently affected the production of isoamyl alcohol. In this study, very few such differences were observed between sterol treatments due to vastly differing sterol additions (3.75 mg/L compared to our 10 or 20 mg/L). The most notable difference being the significant reduction in acetic acid levels observed due to phytosterol addition (Rollero *et al*., 2015; Ochanodo *et al*., 2017). It has also been shown that phytosterol additions promote the production of succinic acid; rather than acetic acid, potentially as a means to regenerate NADH. Additionally, when compared to phytosterol, ergosterol brought about a significant increase in the production of hexanoic acid and decanoic acid concentrations. This is possibly due to differences in acetyl-CoA metabolism, where phytosterol diverts it to the TCA cycle, and ergosterol directs it to fatty acid synthesis.

The untargeted analysis of GC-MS data yielded little new information but confirmed the trends already observed in the targeted GC-FID analysis. This suggests that the GC-FID could capture information relating to the most abundant metabolites and that the untargeted method should have been directed toward the identification of the trace metabolites instead.

To our knowledge, this is the first time that the impact of combinations of sterols and unsaturated fatty acids have been compared in this manner. Irrespective of application, oleic acid lowered the production of medium chain fatty acids, as well as ethyl and acetate esters. Whereas the specific sterol used altered the production of volatile fatty acids or organic acids. The data illustrates the profound impact the lipid composition has on fermentation kinetics and aroma production, and consequently, also the importance of managing the lipid composition of grape must during winemaking. This study provides a preliminary data-set to further explore the impact of these sterol and fatty acid interactions on cell compositional changes, and cell viability during alcoholic fermentation.

4.5 References

Aguilera, F., Peinado, R. a, Millán, C., Ortega, J.M. & Mauricio, J.C., 2006. Relationship between ethanol tolerance, H+ -ATPase activity and the lipid composition of the plasma membrane in different wine yeast strains. Int. J. Food Microbiol. 110, 1, 34–42.

Bely, M., Sablayrolles, J.M. & Barre, P., 1990. Description of alcoholic fermentation kinetics: its variability and significance. Am. J. Enol. Vitic. 41, 4, 319–324.

Daum, G., Lees, N.D., Bard, M. & Dickson, R., 1998. Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*. Yeast 14, 16, 1471–1510.

Delfini, C., Cocito, C., Ravaglia, S. & Conterno, L., 1993. Influence of clarification and suspended grape solid materials on sterol content of free run and pressed grape musts in the presence of growing yeast cells. Am. J. Enol. Vitic. 44, 4, 452–458.

Duan, L., Shi, Y., Jiang, R. & Yang, Q., 2015. Effects of Adding Unsaturated Fatty Acids on Fatty Acid Composition of *Saccharomyces cerevisiae* and Major Volatile Compounds in Wine. South African J. Enol. Vitic. 36, 2, 285–295.

Dufour, J.P., Malcorps, P., Silcock, P., 2003. Control of ester synthesis during brewery fermentation. In: Smart, K. (Ed.), 2003. Brewing Yeast Fermentation Performance, vol. 2. Blackwell Publishing, Oxford, pp. 213-233.

Ehrlich, F., 1907. Über die Bedingungen der Fuselölbildung und über ihren Zusammenhang mit dem Eiweissaufbau der Hefe. Ber Dtsch Chem Ges. 40, 1027-1047.

Fujii, T., Kobayashi, O., Yoshimoto, H., Furukawa, S. & Tamai, Y., 1997. Effect of aeration and unsaturated fatty acids on expression of the *Saccharomyces cerevisiae* alcohol acetyltransferase gene. Microbiology 63, 3, 910–915.

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. Appl. Environ. Microbiol. 74, 8, 2259–2266.

Henschke, P. A., and V. Jiranek. "Yeasts-metabolism of nitrogen compounds." Wine microbiology and biotechnology (1993): 77-164.

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. J. Agric. Food Chem. 57, 7, 2623–2632.

Luparia, V., Soubeyrand, V., Berges, T., Julien, A. & Salmon, J.-M., 2004. Assimilation of grape phytosterols by *Saccharomyces cerevisiae* and their impact on enological fermentations. Appl. Microbiol. Biotechnol. 65, 1, 25–32.

Mannazzu, I., Angelozzi, D., Belviso, S., Budroni, M., Farris, G.A., Goffrini, P., Lodi, T., Marzona, M. & Bardi, L., 2008. Behaviour of *Saccharomyces cerevisiae* wine strains during adaptation to unfavourable conditions of fermentation on synthetic medium: Cell lipid composition, membrane integrity, viability and fermentative activity. Int. J. Food Microbiol. 121, 1, 84–91.

Mauricio, J.C., Moreno, J., Zea, L., Ortega, J.M. & Medina, M., 1997. The effects of grape must fermentation conditions on volatile alcohols and esters formed by *Saccharomyces cerevisiae.* J. Sci. Food Agric. 75, 2, 155–160.

Mauricio, J.C., Millán, C. & Ortega, J.M., 1998. Influence of oxygen on the biosynthesis of cellular fatty acids, sterols and phospholipids during alcoholic fermentation by *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*. World J. Microbiol. Biotechnol. 14, 3, 405–410.

Mouret, J.R., Cadiere, A., Aguera, E., Rollero, S., Sablayrolles, J.M. & Dequin, S., 2014. Dynamics and quantitative analysis of the synthesis of fermentative aromas by an evolved wine strain of *Saccharomyces cerevisiae*. Yeast 32, 257–269.

Ochando, T., Mouret, J.R., Humbert-Goffard, A., Sablayrolles, J.M. & Farines, V., 2017. Impact of initial lipid content and oxygen supply on alcoholic fermentation in champagne-like musts. Food Res. Int. 98, 87–94.

Redón, M., Guillamón, J.M., Mas, A. & Rozès, N., 2011. Effect of growth temperature on yeast lipid composition and alcoholic fermentation at low temperature. Eur. Food Res. Technol. 232, 3, 517–527.

Rollero, S., Bloem, A., Camarasa, C., Sanchez, I., Ortiz-Julien, A., Sablayrolles, J.M., Dequin, S. & Mouret, J.R., 2015. Combined effects of nutrients and temperature on the production of fermentative aromas by *Saccharomyces cerevisiae* during wine fermentation. Appl. Microbiol. Biotechnol. 99, 5, 2291–2304.

Saerens, S.M.G., Delvaux, F.R., Verstrepen, K.J. & Thevelein, J.M., 2010. Production and biological function of volatile esters in *Saccharomyces cerevisiae*. Microb. Biotechnol. 3, 2, 165–177.

Snoek, I.S.I. & Steensma, H.Y., 2007. Factors involved in anaerobic growth of *Saccharomyces cerevisiae*. Yeast 24, 1-10.

Taylor, G.T., Thurston, P. A. & Kirsop, B.H., 1979. The influence of lipids derived from malt spent grains on yeast metabolism and fermentation. J. Inst. Brew. 85, 4, 219–227.

Thurston, P.A., Taylor, R. & Ahvenainen, J., 1981. Effects of linoleic acid supplements on the synthesis by yeast of lipids and acetate esters. J. Inst. Brew. 87, 2, 92–95.

Thurston, P.A., Quain, D.E. & Tubb, R.S., 1982. Lipid metabolism and the regulation of volatile ester synthesis in *Saccharomyces cerevisiae.* J. Inst. Brew. 88, 2, 90–94.

Tumanov, S., Zubenko, Y., Greven, M., Greenwood, D.R., Shmanai, V. & Villas-Boas, S.G., 2015. Comprehensive lipidome profiling of Sauvignon blanc grape juice. Food Chem. 180, 249–256.

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

Yoshioka, K. & Hashimoto, N., 1981. Ester formation by alcohol acetyltransferase from brewers' yeast. Yeast 45, 10, 2183–2190.

You, K.M., Rosenfield, C. & Knipple, D.C., 2003. Ethanol tolerance in the yeast *Saccharomyces cerevisiae* is dependent on cellular oleic acid content. Appl. Environ. Microbiol. 69, 3, 1499–1503.

Chapter 5

Evaluating the importance of the yeast volatilome in creating a vinous character in synthetic grape must

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Abstract

All wines possess an underlying wine-like aroma, which is easily recognised by wine consumers, yet this common sensory perception results from a chemical feature that remains largely unexamined. As yeast metabolism significantly contributes to wines' organoleptic properties, this study explores the possibility of creating a wine-like feature via alcoholic fermentation using a synthetic grape must. Various nitrogen and anaerobic factor conditions were evaluated, for their respective roles in modulating the formation of this sensory signature. Initial evaluations suggested that in terms of aroma, the synthetic products were far removed from wine-like aromas. Only after altering the anaerobic factor composition, was a more wine-like aroma evident. The results clearly show that the vinous character responsible for the recognition of wine is readily perceived in commercial wines, as well as to a certain degree in the synthetic products. Additionally, this work also highlights the importance of conducting the sensory evaluations of these synthetic products in the correct context, and the merit of using the sensory data as a decision-making tool, rather than the chemical data alone.

Keywords: amino acids, anaerobic factors, synthetic grape must, *Saccharomyces cerevisiae*, wine-like feature, wine aroma

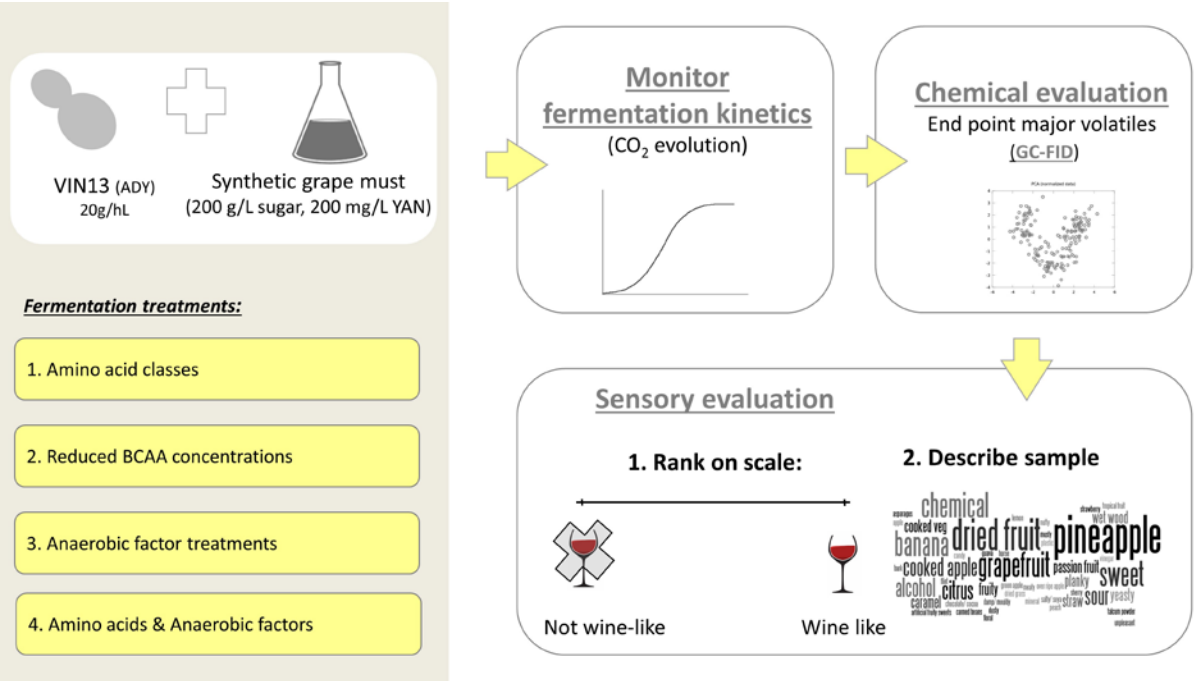


Figure 5A: *Schematic overview of experimental layout.*

5.1 Introduction

Wine aroma is the function of the interaction of several hundred compounds (Mendes-Pinto *et al*., 2009), which includes thiols, terpenes, higher alcohols, esters, and volatile fatty acids among others. These volatile compounds all interact with each other (Francis & Newton, 2005; Ferreira & Cacho, 2009), by masking (de-la-Fuente-Blanco, *et al*., 2017), enhancing (Atanasova *et al*., 2005) or altering each other's sensory perception (de-la-Fuente-Blanco, *et al*., 2017). This contributes to the difficulty associated with predicting the sensory outcome of chemical data alone (Francis & Newton, 2005).

The study of wine aroma impacting odorants is critical in identifying those compounds which influence aroma, but as alluded to, this has limited relevance without sensory evaluation. One means of contextualising the sensory implications of a given odorant is through chemical reconstitution (Escudero *et al*., 2004; Mayr *et al*., 2014; Benkwitz *et al*., 2012; Ferreira *et al*., 2002). Whereby the volatile profiles are recreated via the combination of volatile and non-volatile compounds at determined concentrations in order to simulate aroma profiles. A significant portion of the volatile compounds produced during alcoholic fermentation are products of yeast metabolism. The production of these compounds are greatly influenced by the yeast strain used (Barrajón-Simancas *et al*., 2011; Rollero *et al.,* 2016; Rossouw *et al.* 2008; Rossouw & Bauer, 2016; Vilanova *et al*., 2007) the concentration and composition of the fermentable nitrogen (Hernández-Orte *et al*., 2002; Gutiérrez *et al.,* 2012; Torrea *et al.,* 2011; Burin *et al.,* 2015; Dickinson *et al.,* 1997; Ferreira *et al.,* 2014; Styger *et al.,* 2011; Miller *et al.,* 2007), as well as the anaerobic factor composition (Rollero *et al.,* 2015; Duan *et al.*, 2015; Varela *et al.,*2012; Mauricio *et al.,*1997).

Sensory science has recently begun to explore wine typicality, due to its significant financial implications (Maitre *et al.,* 2010). Typicality is broadly characterised by either the cultivar, the geographical location of production, or combinations of both (Maitre *et al*., 2010). It finds expression in concepts such as Protected Designation of Origin (PDO), Protected Geographical Indication (Wine of Origin), or more simply, the degree to which a wine in question is perceived as a good representation of the cultivar and region. Various sensory methods have been employed to evaluate such wine characteristics including sorting (Ballester *et al.,* 2008, Parr *et al*., 2007), scaled ranking (Schüttler *et al*., 2015), and descriptive analyses (Cadot *et al.,* 2010) among others (Maitre *et al.*, 2010). In the evaluation of wine typicality, sensory assessors need to agree upon the criterion used and generally be experts on the wine region in question. Nonetheless, the heterogeneity of the sensory space may not allow a distinct classification with regard to typicality, as samples may resemble wine of different regions or cultivars (Perrin & Pagès, 2009).

Taking one step back from wine typicality, this study seeks to explore the volatile chemical feature required for the recognition of the product as wine. This recognition occurs in all wines, regardless of the cultivar or wine quality, and is, therefore, most likely a result of alcoholic fermentation. Importantly, it underlies and interacts with all other aromatic features discussed above. The aim of this study is to explore the extent to which the yeast volatilome contributes to this vinous character, referred to as the wine-like feature. To explore this, several novel approaches were employed: Firstly, rather than the traditional use of chemical reconstitution of wine aromas, a fermentation-based approach was used to convert the must into a wine-like product. Secondly, a synthetic grape must was used in order to reduce the complexity of the resultant product, to allow the easy manipulation of the matrix composition, as well as to examine the contribution of the yeast volatilome in isolation, without any ageing or varietal odorants. Finally, in a reiterative process, the study was driven by the sensory outcomes rather than the chemical data.

5.2 Methods and materials

5.2.1 Fermentation media, conditions, and treatments

Saccharomyces cerevisiae yeast strain, VIN13 (Anchor Yeast, Cape Town, South Africa), was rehydrated (20 g/hL) for 20 min at 37 °C in warm water and subsequently cooled to within 10°C of the media's temperature before inoculation, according to the supplier's instructions. Fermentations were conducted using synthetic grape must (100 g/L glucose and 100 g/L fructose), containing 10 mg/L ergosterol and 0.5 mL/L Tween 80 as the anaerobic factor (SGM), as previously described (Henschke & Jiranek, 1993), unless indicated otherwise. For the first two sets of fermentations, the nitrogen composition was varied, as summarised in tables 5.1 A and B. The concentration of fermentable nitrogen was maintained at 200 mg N/L and each amino acid provided equal amounts of fermentable nitrogen.

5.2.1.1 Amino acid classes

The first set of fermentations (1 L) contained amino acids classified on the basis of their ability to support yeast growth (Table 5.1 A), namely complete, preferred, branched chain and aromatic (BCAAs), not utilised, utilised but not preferred amino acids and ammonium (Smit, 2013). Static fermentations took place at 20°C, in triplicate, and were monitored daily (CO_2 evolution).

5.2.1.2 Reduced BCAAs concentrations

The second set of fermentations (1 L) contained all the amino acids but the concentration of BCAAs or sulphur containing amino acids were decreased (Table 5.1 B). Glutamine and alanine were used to compensate for this reduction in amino acid concentration to maintain the YAN content at 200 mg N/L. Static fermentations took place at 20°C, in triplicate, and were monitored daily (CO_2 evolution).

5.2.1.3 Anaerobic factors

The third set of fermentations (0.5 L) were conducted using 200 mg N/L of YAN (amino acids and ammonium, MS200) as described by Bely *et al* (1990). The anaerobic factors (20 mg/L) used in this fermentation consisted of phytosterol (β-sitosterol), ergosterol, Tween80 and oleic acid, added individually and in combinations with each other. Anaerobic factors were dissolved in ethanol and the untreated control received an addition of only ethanol. Static fermentations took place at 20°C, in triplicate, and were monitored daily (CO_2 evolution).

5.2.1.4 Combinations of amino acids and anaerobic factors

Based on the data obtained in the three previous fermentations, a final set of treatments were selected, as indicated in table 5.1 C, to be evaluated in combination with each other (0.5 L). Nitrogen treatments include 200 mg N/L of fermentable nitrogen in the form of ammonium, MS200 amino acids (Bely *et al.,* 1990) and the BCAAs (Table 5.1A). The SGM, ergosterol with oleic acid, phytosterol, and phytosterol with ergosterol treatments were used in combination with the nitrogen treatments as described in table 5.1 C. Static fermentations took place at 20°C, with four replicates, and were monitored daily (CO_2 evolution).

5.2.2 Chemical analyses

5.2.2.1 Gas chromatography flame ionization detector

At the end of alcoholic fermentation, samples underwent a liquid-liquid extraction as described by Louw *et al* (2009) for analysis by gas chromatography (detailed in Chapter 3).

Table 5.1 A. Fermentations were supplemented with different classes of amino acids, based on how well they support yeast growth, and ammonium.

Complete amino acids			Preferred amino acids			Branched chain & aromatic amino acids			Not utilised amino acids			Utilised but not preferred amino acids			Ammonium chloride		
	mg N/L	mg/L		mg N/L	mg/L		mg N/L	mg/L		mg N/L	mg/L		mg N/L	mg/L		mg N/L	mg/L
NH_4Cl	50.0	191.7	NH_4Cl	50.0	191.7	NH_4Cl	50.0	191.7	NH_4Cl	50.0	191.7	NH_4Cl	50.0	76.7	NH_4Cl	200	766.7
ALA	7.5	47.8	ARG	30.0	93.2	ILE	30.0	200.0	HIS	50.0	150.0	ALA	30.0	100.0			
ARG	7.5	23.3	ASN	30.0	141.5	LEU	30.0	300.0	LYS	50.0	250.0	GLY	30.0	50.0			
ASN	7.5	35.4	ASP	30.0	285.7	PHE	30.0	150.0	PRO	50.0	500.0	SER	30.0	400.0			
ASP	7.5	71.4	GLN	30.0	156.3	TYR	30.0	20.0				THR	30.0	350.0			
CYS	7.5	64.9	GLU	30.0	315.8	VAL	30.0	200.0				TRP	30.0	100.0			
GLN	7.5	39.1															
GLU	7.5	78.9															
GLY	7.5	40.3															
HIS	7.5	27.7															
ILE	7.5	70.1															
LEU	7.5	70.1															
LYS	7.5	39.1															
MET	7.5	79.8															
PHE	7.5	88.2															
PRO	7.5	61.5															
SER	7.5	56.4															
THR	7.5	63.6															
TRP	7.5	54.7															
TYR	7.5	97.4															
VAL	7.5	62.5															

Table 5.1 B. Fermentations were supplemented with ammonium, all amino acids, and decreasing concentrations of branched chain and aromatic amino acids, as well as a reduction in the concentration of sulphur containing amino acids.

Complete amino acids			50% S-containing amino acids			25% Branched chain & aromatic amino acids			50% Branched chain & aromatic amino acids			75% Branched chain & aromatic amino acids			Ammonium chloride		
	mg N/L	mg/L		mg N/L	mg/L		mg N/L	mg/L		mg N/L	mg/L		mg N/L	mg/L		mg N/L	mg/L
NH_4Cl	50	192	NH_4Cl	50	192	NH_4Cl	50	192	NH_4Cl	50	192	NH_4Cl	50	192	NH_4Cl	200	767
ALA	7.5	47.8	ALA	13.3	84.8	ALA	21.6	137.7	ALA	16.9	107.7	ALA	12.2	77.7			.
ARG	7.5	23.3	ARG	7.5	23.3	ARG	7.5	23.3	ARG	7.5	23.3	ARG	7.5	23.3			
ASN	7.5	35.4	ASN	7.5	35.4	ASN	7.5	35.4	ASN	7.5	35.4	ASN	7.5	35.4			
ASP	7.5	71.4	ASP	7.5	71.4	ASP	7.5	71.4	ASP	7.5	71.4	ASP	7.5	71.4			
CYS	7.5	64.9	CYS	3.8	32.5	CYS	7.5	64.9	CYS	7.5	64.9	CYS	7.5	64.9			
GLN	7.5	39.1	GLN	13.3	69.3	GLN	21.6	112.6	GLN	16.9	88.1	GLN	12.2	63.6			
GLU	7.5	78.9	GLU	7.5	78.9	GLU	7.5	78.9	GLU	7.5	78.9	GLU	7.5	78.9			
GLY	7.5	40.3	GLY	7.5	40.3	GLY	7.5	40.3	GLY	7.5	40.3	GLY	7.5	40.3			
HIS	7.5	27.7	HIS	7.5	27.7	HIS	7.5	27.7	HIS	7.5	27.7	HIS	7.5	27.7			
ILE	7.5	70.1	ILE	7.5	70.1	ILE	1.9	17.8	ILE	3.8	35.1	ILE	5.6	70.1			
LEU	7.5	70.1	LEU	7.5	70.1	LEU	1.9	17.8	LEU	3.8	35.1	LEU	5.6	70.1			
LYS	7.5	39.1	LYS	7.5	39.1	LYS	7.5	39.1	LYS	7.5	39.1	LYS	7.5	39.1			
MET	7.5	79.8	MET	3.8	39.9	MET	7.5	79.8	MET	7.5	79.8	MET	7.5	79.8			
PHE	7.5	88.2	PHE	7.5	88.2	PHE	1.9	22.3	PHE	3.8	44.1	PHE	5.6	66.2			
PRO	7.5	61.5	PRO	7.5	61.5	PRO	7.5	61.5	PRO	7.5	61.5	PRO	7.5	61.5			
SER	7.5	56.4	SER	7.5	56.4	SER	7.5	56.4	SER	7.5	56.4	SER	7.5	56.4			
THR	7.5	63.6	THR	7.5	63.6	THR	7.5	63.6	THR	7.5	63.6	THR	7.5	63.6			
TRP	7.5	54.7	TRP	7.5	54.7	TRP	7.5	54.7	TRP	7.5	54.7	TRP	7.5	54.7			
TYR	7.5	97.4	TYR	7.5	97.4	TYR	1.9	24.7	TYR	3.8	48.7	TYR	5.6	73.1			
VAL	7.5	62.5	VAL	7.5	62.5	VAL	1.9	15.8	VAL	3.8	31.3	VAL	5.6	46.9			

Table 5.1 C. Combinations of various nitrogen and anaerobic factor treatments used.

Nitrogen (200 mg/L)	Anaerobic factor
Ammonium	Standard anaerobic factors (SGM)[3]
Ammonium	Phytosterol
Ammonium	Ergosterol & phytosterol
Ammonium	Ergosterol & oleic acid
Branched chain & aromatic amino acids[1]	Standard anaerobic factors (SGM)[3]
Branched chain & aromatic amino acids[1]	Phytosterol
Branched chain & aromatic amino acids[1]	Ergosterol & phytosterol
Branched chain & aromatic amino acids[1]	Ergosterol & oleic acid
Standard amino acids (MS200)[2]	Standard anaerobic factors (SGM)[3]
Standard amino acids (MS200)[2]	Phytosterol
Standard amino acids (MS200)[2]	Ergosterol & phytosterol
Standard amino acids (MS200)[2]	Ergosterol & oleic acid

[1] Table 5.1 A
[2] Amino acids (Bely *et al*., 1990)
[3] Anaerobic factors (Henschke & Jiranek, 1993)

5.2.3 Sensory analyses

The sensory evaluations were conducted, in compliance with ASTM standards (8589) in an odourless, well-ventilated room with controlled lighting and temperature (20°C ±2). The room was secluded from excess noise. All samples and wines were served at room temperature and all assessments were conducted in off-white individual tasting booths. For each experiment, samples (25 mL) were presented simultaneously in black ISO glasses in a randomized order using a balanced complete block design. Each glass was labelled using a 3-digit code and covered with a Petri dish. The commercial wines used were unwooded white wines.

Samples were only evaluated via orthonasal olfaction. The sensory evaluation overview for each experiment are summarised in figure 5.1, and all data were collected on paper. Three different descriptive methods were used, namely, sorting (Cartier *et al*., 2006; Chollet *et al.,* 2011; Valentin *et al.,* 2012), free description and CATA (Valentin *et al.,* 2012). For the CATA evaluations, descriptors are listed in table 5.2, these lists were generated based on the initial sensory evaluations.

Finally, panellists ranked each sample with regards to the degree to which they resemble wine using an unstructured line scale. In the case of the combined evaluation of anaerobic factors and amino

acids, panellists first evaluated the synthetic product, and after a short break, they evaluated a second set of the same synthetic samples in addition to commercial wines (Figure 5.1).

For the sensory evaluation of amino acid classes, naive wine consumers were used between the ages of 18 and 65. In subsequent evaluations, the panel used was not trained on this matrix but had received regular and extensive training for other wine descriptive analyses projects. Consequently, we relied on their previous experience with line scale ranking to inform the intuitive ranking of the wine-like feature.

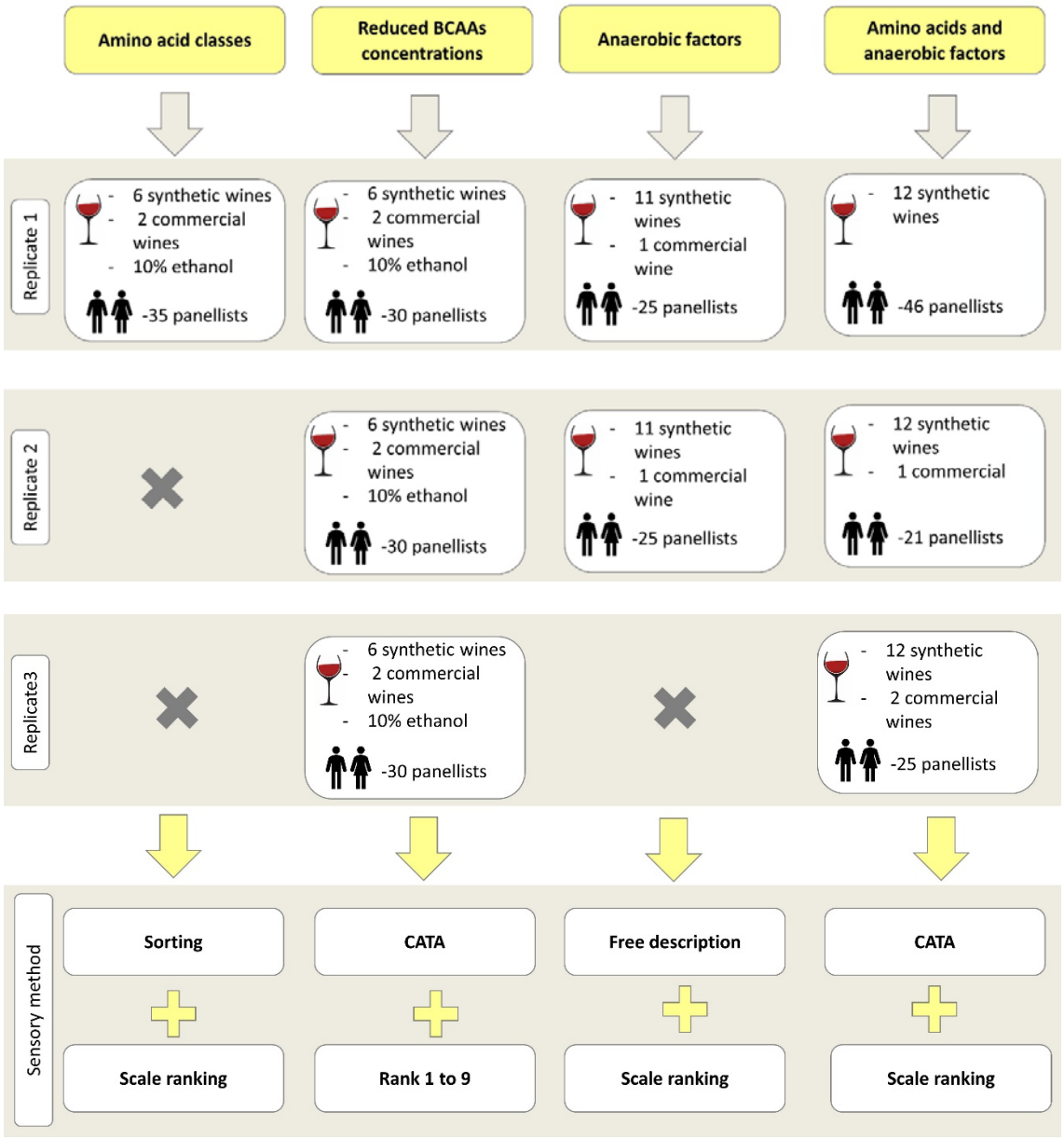


Figure 5.1. Schematic overview of the sensory evaluation procedures followed for each of the fermentation treatments.

Table 5.2. CATA descriptor lists for the reduced BCAA samples and the nitrogen and anaerobic factor treatments

Reduced BCAAs concentrations	Amino acids and anaerobic factors
Fruity	Chemical
Woody	Tropical fruit
Spicy	Petrol
Floral	Forest floor
Alcohol	Cooked vegetables
Sulphur	Fresh green
Toasted	Floral
Medicinal	Sweet
Yeast	Yeasty
Butter	Solvent
Nutty	Fruity
Forrest floor	Citrus
Solvent	
Chemical	
Fresh green	
Cooked vegetable	
Sweet	
Animal	
Oxidised	

5.2.4. Data analyses

5.2.4.1 Sensory data analyses

The sensory data was recorded for each participant, in a similarity matrix which was totalled for all participants. Where appropriate, similar descriptors were combined, and descriptors used by fewer than 15% of the panel were discarded (Lawrence *et al.,* 2013; Valentin *et al.*, 2012). This totalled similarity data matrix was evaluated using multidimensional scaling (MDS) in the case of the sorting data set, or correspondence analyses (CA) for CATA and free description data sets, using XLSTAT or Statistica. In multidimensional scaling (MDS), samples that are close to each other, are similar whereas those that are further apart, are dissimilar (Valentin *et al*., 2012). Additionally, the descriptors used to describe the products were projected on to the MDS plot using Pearson's correlation coefficient.

The ranking data were evaluated using analysis of variance (ANOVA) (type III) paired with the Fisher LSD post hoc test (P = 0.05) to determine which samples are significantly different with respect to the wine-like ranking (Statistica, version 13, Statsoft Inc., Tulsa, USA). A mixed model ANOVA was used with the judges treated as a random effect and treatments as fixed effects. Additionally, for the final set of fermentations, ANOVA was also used to investigate the interaction between each nutrient and the wine-like ranking.

5.2.4.2 Chemical data analyses

The chemical data analyses, namely, principal component analysis (PCA) and ANOVA, were conducted in XLSTAT.

5.3 Results and discussion

5.3.1 Amino acid classes

Panellists were asked to sort the synthetic samples, based on their similarities and differences, and to verbalize the reasons for their groupings (Figure 5.2 A). This data is visualised using a multidimensional scaling (MDS) plot, samples that are close to each other, are similar whereas those that are further apart, are dissimilar (Valentin *et al.,* 2012). The commercial wines were considered similar, and different from the 10% ethanol sample, with the synthetic products falling between these two poles.

Using only the descriptor data, a similar separation of samples is seen in the correspondence analyses (Figure 5.2 B). Due to the nature of the questions asked, wine was also used as a descriptor, for the commercial wine samples in addition to sweet and fruity. The not used and not preferred treatments were also described as fruity, in addition to chemical, and alcoholic. The remaining treatments were associated with chemical, unpleasant, and alcoholic aromas (Figure 5.2 A and B).

Furthermore, wine consumers readily identified the wine-like feature in the commercial wines provided, and its absence in the 10% ethanol samples (Figure 5.2 D), as illustrated by the wine-like ranking data. In contrast, the synthetic samples were ranked similarly, and closer to the ethanol than the wine sample. This first data set suggests that the ammonium treatment resulted in the most wine-like synthetic product, but not significantly more so than any of the other treatments evaluated (not-preferred, not-utilised and preferred).

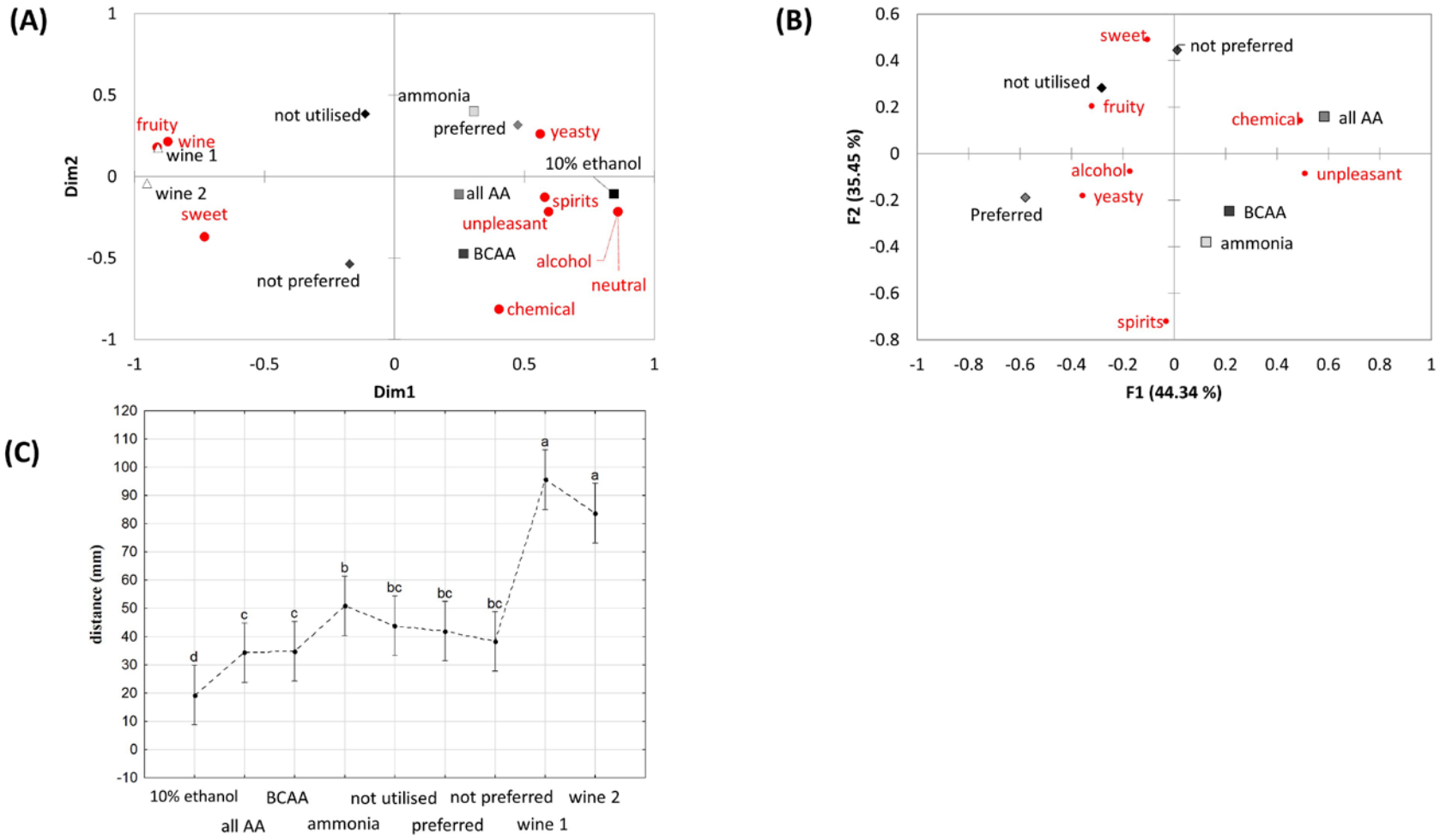


Figure 5.2. The multidimensional scaling (A) describing the sorting of treatments: Complete (all AA), preferred (preferred), branched chain and aromatic (BCAA), not utilised (not utilised), and the utilised but not preferred amino acids (not preferred), in addition to ammonium (ammonia), two commercial wines and 10% ethanol. The correspondence analyses including (B) and excluding the controls (C). The ranking data summarises how similar or dissimilar the products are to wine, the greater the distance ranking the more similar the product is to wine (D). The letters denote the significance level ($p < 0.05$)

Table 5.3. The impact of various amino acid treatments on the production of volatile compounds by VIN13 in synthetic grape must. The data summarizes the average of triplicate fermentations and their standard deviation. Additionally, the letters denote significant differences (95%) between treatments using Fischer LSD.

	Complete amino acids					Ammonium chloride					Branched chain & aromatic amino acids				
Aroma compound					OAV					OAV					OAV
2-phenyl ethanol	109.10	±	4.85	b	**7.79**	18.65	±	0.15	e	**1.33**	275.04	±	3.99	a	**19.65**
isoamyl alcohol	257.16	±	13.91	b	**8.57**	138.05	±	3.64	e	**4.60**	417.02	±	2.39	a	**13.90**
isobutanol	46.58	±	2.58	b	**1.16**	16.20	±	0.52	d	0.41	126.54	±	0.60	a	**3.16**
butanol	0.64	±	0.02	c	0.00	0.56	±	0.01	d	0.00	0.49	±	0.01	e	0.00
propanol	28.22	±	1.43	c	0.09	34.05	±	1.01	b	0.11	40.01	±	0.43	a	0.13
2-phenylethyl acetate	1.09	±	0.11	b	**4.38**	0.99	±	0.10	b	**3.95**	2.55	±	0.23	a	**10.19**
ethyl acetate	47.44	±	0.71	a	**3.87**	36.29	±	0.50	b	**2.96**	38.76	±	0.84	b	**3.16**
isoamyl acetate	1.26	±	0.04	b	**41.98**	0.80	±	0.00	c	**26.80**	2.66	±	0.11	a	**88.69**
acetic acid	379.76	±	21.77	c	**1.90**	397.45	±	10.32	c	**1.99**	402.70	±	1.63	c	**2.01**
butyric acid	1.02	±	0.03	b	**5.91**	0.99	±	0.00	b	**5.73**	1.11	±	0.01	a	**6.39**
isobutyric acid	1.67	±	0.07	b	0.73	0.79	±	0.00	e	0.34	3.45	±	0.02	a	**1.50**
isovaleric acid	2.17	±	0.08	b	**65.85**	1.37	±	0.02	d	**41.43**	4.25	±	0.09	a	**128.77**
propionic acid	1.25	±	0.03	c	0.15	1.55	±	0.01	b	0.19	1.69	±	0.01	a	0.21
valeric acid	0.62	±	0.01	c		0.55	±	0.00	d		0.74	±	0.00	a	
ethyl lactate	10.41	±	0.08	cd	0.07	11.53	±	0.24	a	0.08	10.32	±	0.07	d	0.07
ethyl phenylacetate	1.59	±	0.02	b	**6.36**	1.34	±	0.00	e	**5.37**	1.63	±	0.01	b	**6.53**
hexanoic acid	2.36	±	0.10	b	**5.62**	2.33	±	0.21	b	**5.55**	3.05	±	0.03	a	**7.27**
octanoic acid	3.52	±	0.05	c	**7.04**	3.80	±	0.04	bc	**7.60**	4.55	±	0.17	a	**9.10**
decanoic acid	1.84	±	0.23	b	**1.84**	2.31	±	0.00	a	**2.31**	2.34	±	0.30	a	**2.34**
ethyl caprylate	0.68	±	0.20	b	**136.84**	0.98	±	0.07	a	**195.96**	0.97	±	0.16	a	**194.96**

Table 5.3 continued. The impact of various amino acid treatments on the production of volatile compounds by VIN13 in synthetic grape must. The data summarizes the average of triplicate fermentations and their standard deviation. Additionally, the letters denote significant differences (95%) between treatments using Fischer LSD.

	Not utilised amino acids					Preferred amino acids					Utilised but not preferred amino acids				
Aroma compound					OAV					OAV					OAV
2-phenyl ethanol	35.18	±	1.10	d	**2.51**	22.23	±	0.50	e	**1.59**	79.60	±	4.82	c	**5.69**
isoamyl alcohol	157.75	±	1.85	d	**5.26**	140.57	±	4.03	de	**4.69**	213.73	±	11.46	c	**7.12**
isobutanol	16.37	±	0.42	d	0.41	14.54	±	0.58	d	0.36	28.86	±	1.01	c	0.72
butanol	0.77	±	0.01	b	0.01	0.73	±	0.02	b	0.00	1.00	±	0.03	a	0.01
propanol	40.36	±	1.02	a	0.13	36.06	±	0.99	b	0.12	39.13	±	1.37	a	0.13
2-phenylethyl acetate	0.65	±	0.00	c	**2.61**	0.84	±	0.15	bc	**3.38**	0.92	±	0.02	bc	**3.69**
ethyl acetate	31.88	±	1.80	c	**2.60**	45.64	±	1.30	a	**3.72**	45.64	±	1.08	a	**3.72**
isoamyl acetate	0.65	±	0.01	d	**21.59**	0.82	±	0.01	c	**27.24**	0.84	±	0.03	c	**28.14**
acetic acid	575.17	±	16.88	a	**2.88**	593.03	±	51.94	a	**2.97**	466.33	±	21.16	b	**2.33**
butyric acid	0.84	±	0.01	c	**4.86**	0.98	±	0.03	b	**5.69**	0.98	±	0.04	b	**5.67**
isobutyric acid	0.90	±	0.02	d	0.39	0.77	±	0.01	e	0.34	1.06	±	0.04	c	0.46
isovaleric acid	1.86	±	0.00	c	**56.40**	1.41	±	0.01	d	**42.79**	1.88	±	0.08	c	**56.96**
propionic acid	1.62	±	0.04	ab	0.20	1.62	±	0.08	ab	0.20	1.54	±	0.06	b	0.19
valeric acid	0.51	±	0.00	e		0.52	±	0.01	e		0.65	±	0.02	b	
ethyl lactate	11.02	±	0.08	b	0.07	10.69	±	0.09	c	0.07	10.44	±	0.01	cd	0.07
ethyl phenylacetate	1.50	±	0.02	c	**6.02**	1.43	±	0.03	d	**5.73**	1.71	±	0.03	a	**6.82**
hexanoic acid	1.66	±	0.02	d	**3.95**	2.38	±	0.06	b	**5.67**	2.05	±	0.11	c	**4.89**
octanoic acid	2.17	±	0.02	e	**4.34**	3.93	±	0.21	b	**7.86**	3.04	±	0.17	d	**6.08**
decanoic acid	1.36	±	0.01	c	**1.36**	2.31	±	0.18	a	**2.31**	1.99	±	0.07	ab	**1.99**
ethyl caprylate	0.34	±	0.02	c	**68.95**	0.65	±	0.06	b	**130.27**	0.68	±	0.04	b	**136.23**

Table 5.4. Odour perception values and descriptors of yeast derived volatile compounds

Aroma compound	Aroma descriptors	Odour threshold (mg/L)	Concentration range (mg/L) in South African white wines [E]	Concentration range (mg/L) in Spanish red wines [F]
Higher alcohols				
2-Phenyl ethanol	floral, rose, honey, spice, lilac	14[A]	5.26 – 53.01	40.093 – 153.269
3-Ethoxy-1-propanol	chemical, solvent, fruity	0.1 [B]	0.02 – 4.7	
Butanol	harsh, nail polish, whiskey	150[C]	0.20 – 2.54	
Hexanol	herbaceous, green grass	8[D]	0.05 – 4.11	
Isoamyl alcohol	fusel, alcoholic, solvent	30[D]	86.35 – 394.95	83.953 – 333.032
Isobutanol	fusel, alcoholic, medicinal	40[D]	2.26 – 66.32	25.7 – 86.9
Propanol	fruity, alcohol, pungent	306[C]	10.34 – 149.27	
Acetate esters				
2-Phenylethyl acetate	floral, rose, fruity, cooked apple, marmalade, honey	0.25[D]	0.04 – 1.47	0.00054 – 0.157
Ethyl acetate	apple, glue, nail polish remover	12.27[C]	6.24 – 233.58	
Hexyl acetate	fruity, green, pear, apple, floral, herb	0.67[C]	0.07 – 2.48	
Isoamyl acetate	banana, pear	0.03[D]	0.26 – 18.45	0.118 – 3.371
Volatile fatty acids				
Acetic acid	sour, pungent, vinegar	200[D]	80.91 – 1191.02	69.110 – 313.310
Butyric acid	spoiled cheese, sweaty, sour, pungent	0.173[A]	0.78 – 4.34	0.434 – 4.719
Isobutyric acid	rancid, cheese, sweaty, fruit, pungent	2.3[A]	0.20 – 2.74	
Isovaleric acid	rancid, cheese, sweaty, putrid	0.033[A]	0.13 – 2.52	
Valeric acid	-	-	0.10 – 0.37	
Propionic acid	pungent, vinegar, rancid	8.1[C]	0.73 – 50.06	
Ethyl esters				
Diethyl succinate	fruity	200[C]	0.09 – 4.89	
Ethyl butyrate	fruity, pineapple	0.02[D]	0.2 – 0.8	0.0692 – 0.371
Ethyl lactate	milky, buttery, sweet, fruity, strawberry	150[C]	0.92 – 80.24	
Ethyl phenylacetate	rose, floral	0.25[D]	0.2 – 0.6	
Medium chain fatty acids				
Octanoic acid	lactic, oily, sweaty, rancid, faint fruity	0.5[A]	1.15 – 12.24	0.562 – 4.667
Hexanoic acid	rancid, cheese, sweaty, metallic	0.42[A]	0.05 – 13.70	0.853 – 3.782
Decanoic acid	rancid, fatty, citrus	1[A]	0.40 – 5.79	0.0621 – 0.857
Medium chain fatty acid ethyl esters				
Ethyl caprate	fruity, floral, pleasant, soap	0.2[A]	0.23 – 2.58	0.0145 – 0.215
Ethyl caprylate	fruity, sweet, soap, pineapple, pear	0.005[A]	0.27 – 3.4	0.138 – 0.783
Ethyl hexanoate	green apple, fruity, apple peel, strawberry, candy	0.014[A]	0.07 – 2.32	0.153 – 0.622

[A] Ferreira *et al* (2000) determined in 11% (vol/vol) ethanol, 7g/L glycerine and 5 g/L tartaric acid at pH 3.4; [B] Peinado *et al* (2004) determined in 10% (vol/vol) ethanol, pH 3.5 adjusted with tartaric acid; [C] Reviewed by Etievant (1991) determined in wine; [D] Guth *et al* (1997a) determined in 10% ethanol (vol/vol); [E] Louw et al (2010, n =306); [E] Lawrence (2012, n= 120); [F] Ferreira et al (2002, n = 52)

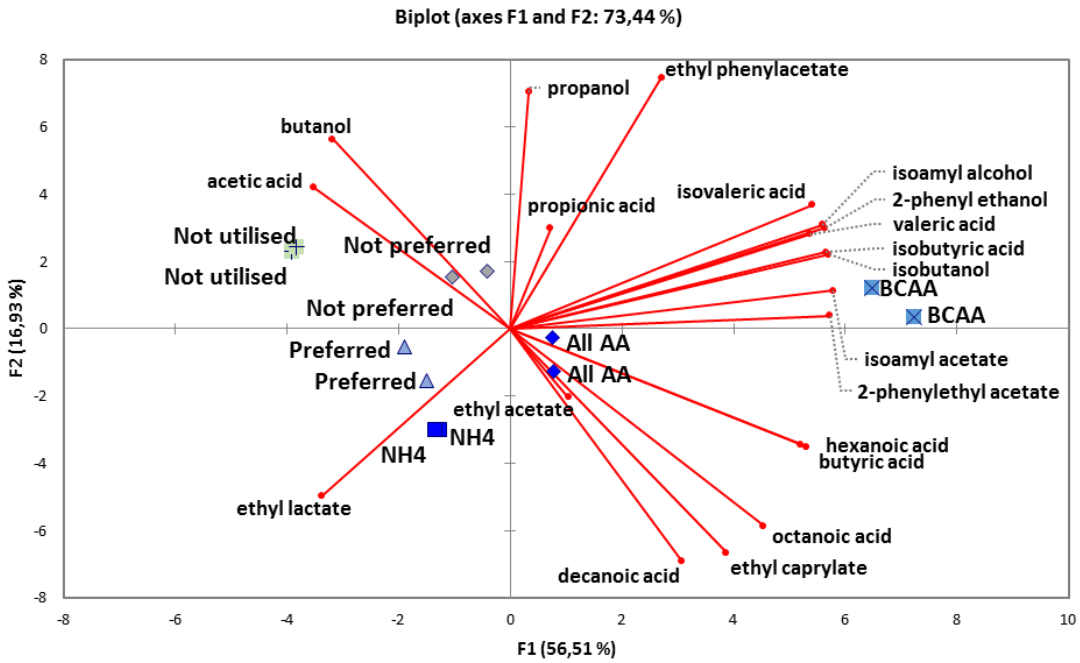


Figure 5.3. Principal component analyses of the GC-FID data of fermentations treated with various nitrogen treatments, namely, complete amino acids (All), preferred amino acids (Preferred), Branched chain & aromatic amino acids (BCAA); Not utilised amino acids (Not used), Utilised but not-preferred amino acids (Not preferred), Ammonium chloride (NH4).

Interestingly, all treatments resulted in distinct chemical profiles, as summarised in figure 5.3 (Table 5.3), despite their similar wine-association rating. Generally, the aromatic, all amino acid and used amino acid treatments resulted in elevated levels of most of the volatile compounds measured; consequently, these treatments also displayed higher odour activity values (Table 5.4). The odour activity values (OAV) (Table 5.3), are a means of identifying which compounds are above their perception thresholds and predicting whether they potentially contribute to wine aroma profiles (Guth, 1997b). This prediction is not definitive, as research has shown that compounds below their odour thresholds contribute positively to wine aroma (Ferreira *et al.*, 2002). Nonetheless, the BCAA treatments contained several compounds such as higher alcohols, volatile fatty acids and acetate esters at levels greater than those reported in wine (Table 5.4). These elevated levels of higher alcohols (in excess of 850 mg/L) in particular, may account for its association with chemical and spirit-like descriptors (Figure 5.2 A and B), as when present at concentrations greater than 280 mg/L higher alcohols have been shown to have a deleterious effect on aroma (de-la-Fuente-Blanco, *et al.*, 2017).

Overall, the sensory data revealed that wine consumers were readily able to recognise the wine-like feature in commercial wines, confirming that this approach could be satisfactory for the

identification of this wine-like feature in synthetic products. However, the synthetic products had an underlying chemical nature, which was tentatively ascribed to the higher alcohol concentration.

5.3.2 Reduced BCAA concentrations

To evaluate this hypothesis, the sensory space between the BCAA and the all amino acid treatments was evaluated (Figure 5.3), by systematically reducing the concentrations of BCAAs whilst providing all the other amino acids.

As seen in the previous data set, the correspondence analyses once again placed the synthetic samples between the commercial wine (positive control) and the 10% ethanol (negative control) (Figure 5.4 A). This is also reflected in the wine-like ranking, however, in this instance, the synthetic samples were significantly more wine-like than the 10% ethanol (Figure 5.4 B). The treatment containing 50% less of the sulphur containing amino acids was significantly more wine-like than all the synthetic products except for the ammonium fermentations (Figure 5.4 B).

The correspondence analysis of only the synthetic products suggests that all the synthetic samples were described similarly (overlapping confidence intervals), with the exception of ammonium (Figure 5.3 B). Increasing concentrations of aromatic and branched chain amino acids resulted in increasing concentrations of the corresponding higher alcohols, volatile fatty acids and their associated esters (Figure 5.5, Table S5.1). Interestingly, this progression was not seen in the correspondence analysis of the sensory data (Figure 5.4 C). Despite the use of more subtle nitrogen treatments, consumers were unable to perceive the wine-like feature in the synthetic samples, furthermore, the underlying chemical nature remained and was therefore not due to high levels of higher alcohols alone. In both previous experiments, treatments showed significant chemical differences which were potentially masked due to other components of the synthetic grape must formulation.

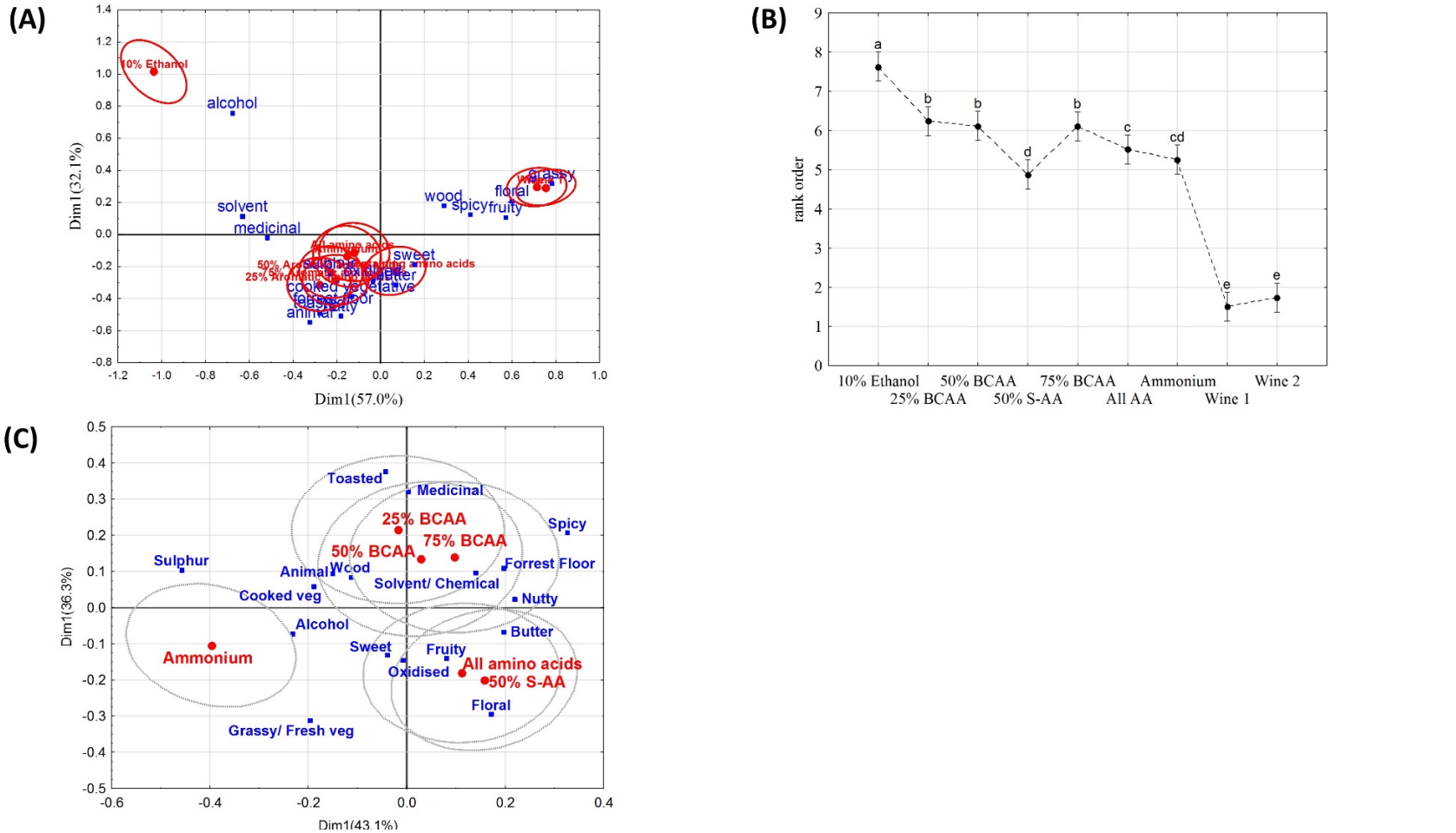


Figure 5.4. The correspondence analyses (A) describing the treatments: complete amino acids (ALL), 75% Branched chain & aromatic amino acids (75% BCAA); 50% Branched chain & aromatic amino acids (50% BCAA), 25% Branched chain & aromatic amino acids (25% BCAA), 50% S-containing amino acids (50% S), and Ammonium chloride (NH4), commercial wines and 10% ethanol, and the (B) ranking data of all samples. The letters denote the significance level ($p < 0.05$). The correspondence analyses (C) describing the abovementioned treatments excluding the controls

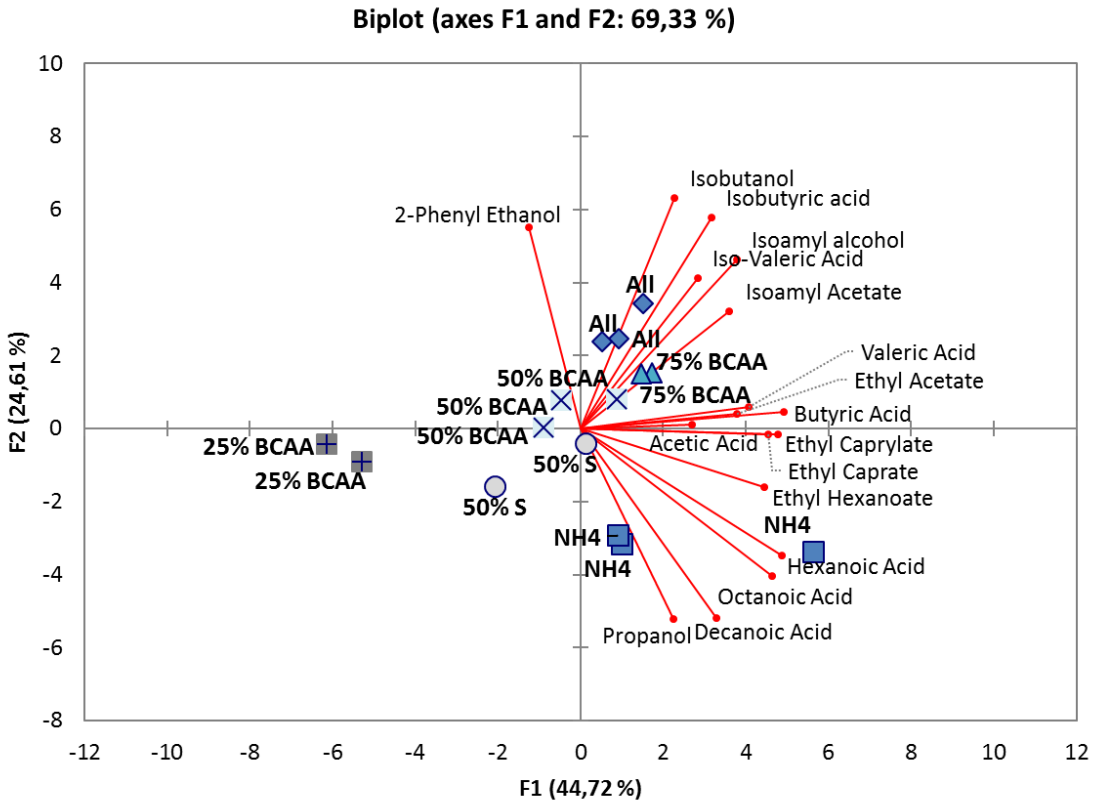


Figure 5.5. Principal component analyses of the GC-FID data of fermentations treated using various nitrogen treatments, namely, complete amino acids (ALL), 75% Branched chain & aromatic amino acids (75% BCAA); 50% Branched chain & aromatic amino acids (50% BCAA), 25% Branched chain & aromatic amino acids (25% BCAA), 50% S-containing amino acids (50% S), and Ammonium chloride (NH4).

5.3.3 Anaerobic factors

The alleviation of the chemical character observed previously was further explored by means of modulating the anaerobic factor composition. Consequently, fermentations were conducted to determine the chemical and sensory impact of supplementation with ergosterol (ERG), phytosterol (PHY), Tween80 (TWE) and oleic acid (OLE), independently and in combination with each other.

Sensory evaluations suggest that the synthetic samples were not entirely distinct from the adjacent treatments but that they formed a continuum, due to the overlapping of confidence intervals (data not shown). The products descriptions ranged from cleaning agent to chemical to green to fruity (Figure 5.6 A) depending on the anaerobic factor supplied. Overall, ERG, ERG+OLE, PHY+OLE and TWE were associated with chemical and forest floor descriptors (Figure 5.6 A). The TWE treatment also displayed a strong association with cleaning agents. The PHY and the ERG+PHY treatment were associated with sweetness, tropical and yellow fruit. Generally, multivariate analysis of sensory data shows a progression from fruity to green along the axis (Hein *et al.,* 2009), in previous iterations of our experiments, the descriptor progression was generally from fruity to chemical. This suggests that this

set of anaerobic fermentations may have alleviated some of the chemical traits, allowing the emergence of additional nuances.

Nonetheless, the commercial wines were as expected rated as the most wine-like and the synthetic products were rated as having a poor resemblance to wine (Figure 5.6 B). Generally, unsaturated fatty acid (oleic acid and Tween80) treatments resulted in the lowest wine-like ranking, with the PHY and ERG+PHY being most wine-like, but not significantly more so than the other products.

The chemical data does not show the same clear separation observed in chapter 4, this is possibly because of differences in fermentation volume, and the agitation used to allow sampling in the smaller fermentations. Nonetheless, similar trends are observed (Figure 5.7), whereby treatments are differentiated on the basis of their sterol and oleic acid content. Briefly, sterol additions promoted the production of most volatile compounds, and oleic acid lowered the production of medium chain fatty acids, as well as acetate and ethyl esters (Table S5.2).

Generally, the inclusion of the commercial wines was useful as a means of segmenting the panellists on the basis of whether they are able to recognise the wine-like concept, but it also serves as a reference for comparison. Due to their more chemical nature, these synthetic products may be more negatively judged, as hedonic judgements may take precedence or confound the wine-like typicality rankings (Charters & Pettigrew, 2007; Parr *et al.,* 2010). In subsequent fermentations, the synthetic samples were evaluated against a memorised abstraction of what a wine-like aroma is, and then with the inclusion of a commercial wine to determine whether this is the cause of the compression observed previously in the wine-like ranking.

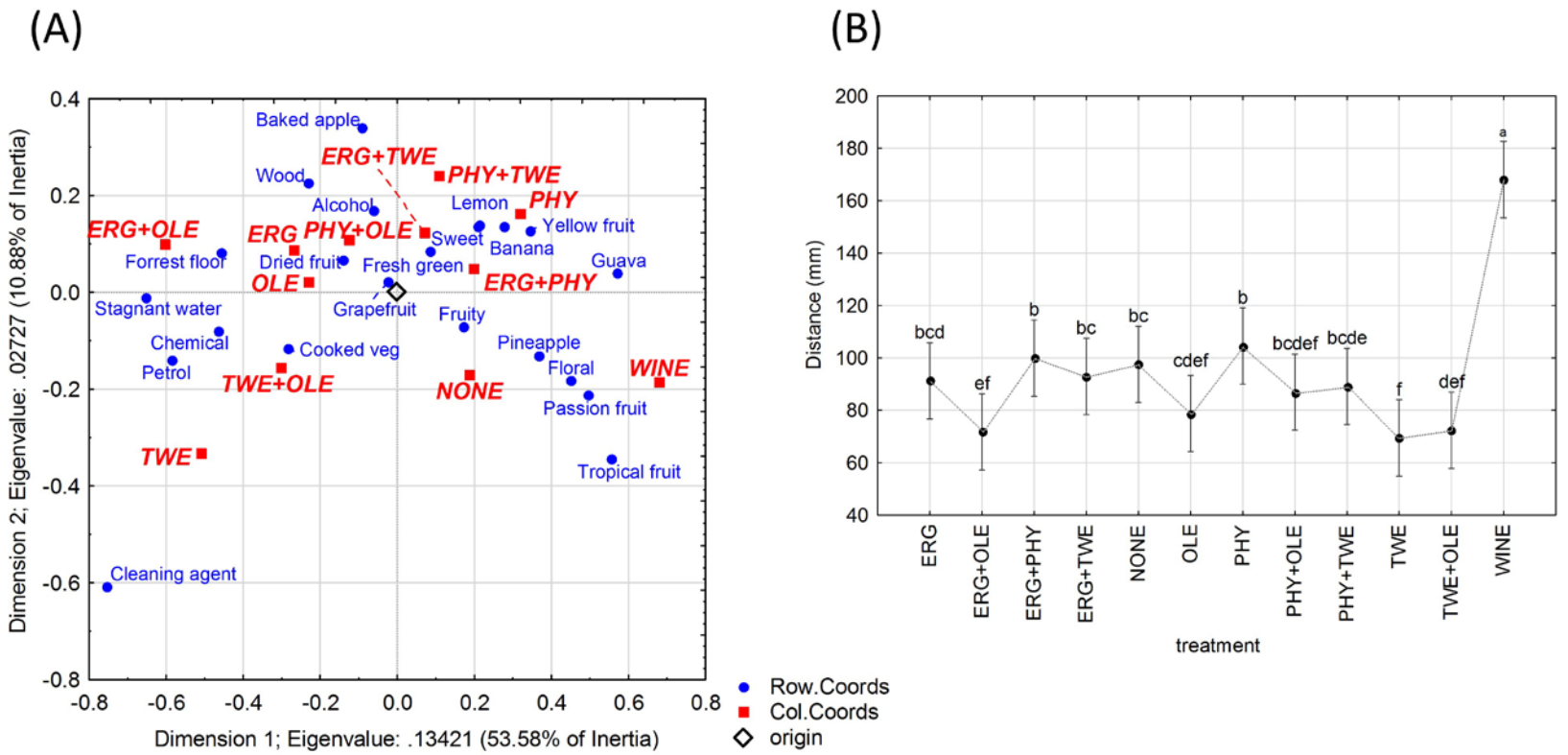
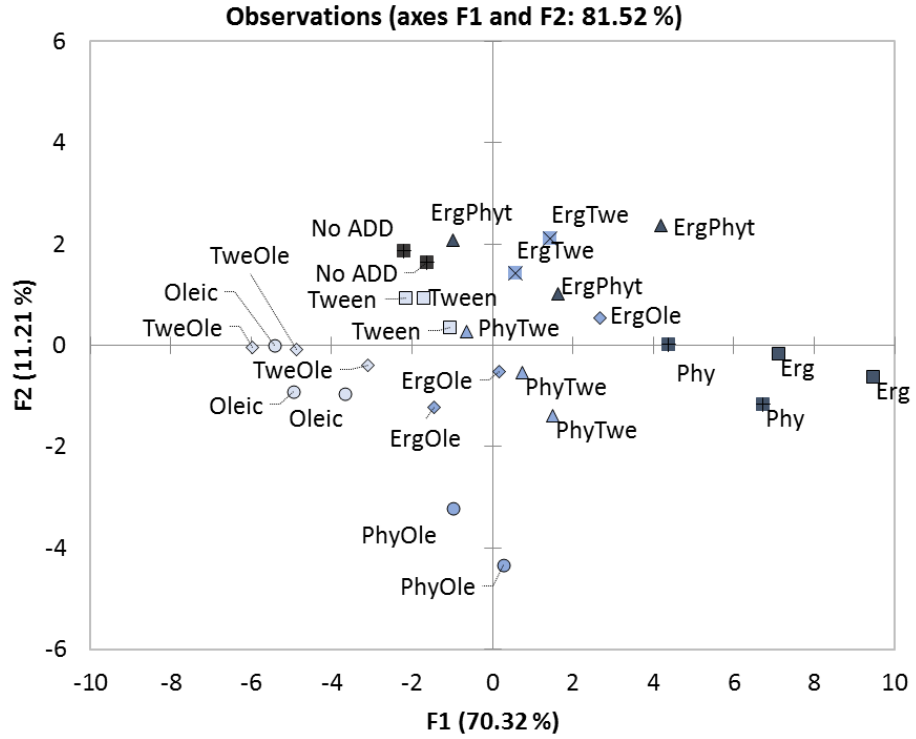


Figure 5.6. The correspondence analyses (A) describing the treatments: phytosterol (PHY), ergosterol (EGR), Tween80 (TWE) and oleic acid (OLE), used individually and in combination with each other, as well as without any supplementation (NONE). (B) The ranking data obtained using a line scale, the greater the distance ranking the more similar the product is to wine. The letters denote the significance level ($p < 0.05$).

(A)



(B)

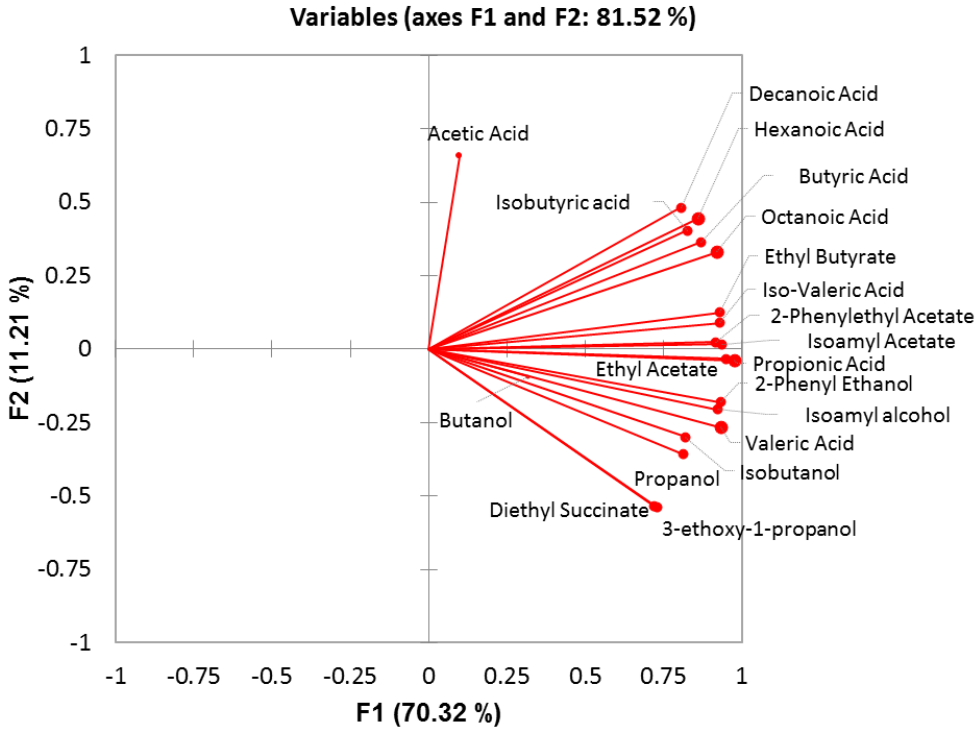


Figure 5.7. Principal component analyses of the GC-FID data ((A) scores and (B) loadings) of fermentations treated with lipids as follows: ergosterol (Erg), phytosterol (Phy), Tween80 (Twe), and oleic acid (Ole) added independently, in addition to their use in combinations with each other, as well as a no addition (No ADD)

5.3.4 Combinations of amino acids and anaerobic factors

Using the descriptor data obtained in the previous fermentations, various nitrogen and anaerobic factor treatments were selected, for combinatorial evaluations. The BCAA, ammonium and amino acid treatments (MS200) were selected for evaluation along with the SGM, phytosterol, phytosterol with ergosterol, and ergosterol with oleic acid anaerobic factors (Table 5.1 C). In previous fermentations, these treatments were described as chemical (BCAA), fruity (PHY and PHY+ERG), forest floor (ERG+OLE) and sweet/ chemical (ammonium). The aim was to evaluate whether these combinations would produce a predictable range of aromas from fruity to chemical and to determine the nature of the relationship, if any, between these nutrients. Particularly whether the fruitier anaerobic factor would ameliorate the impact of the more chemical character of amino acid treatments, or whether combinations would enhance positive or negative traits. Furthermore, the extent to which the commercial wines impacted the wine-like ranking of the synthetic samples was also evaluated. Whereby, sensory evaluations were first conducted on the synthetic samples alone and then with the inclusion of commercial wines.

In contrast with previous data sets, the data trends in figure 5.8 A shows that a sensory progression from least to most wine-like was obtained. The treatments containing the SGM anaerobic factor were rated as the least wine-like (Figure 5.8), followed by ERG+OLE, ERG+PHY and PHY. These trends are largely conserved irrespective of the nitrogen source, except for the BCAA treatment which brought about a significant increase in the wine-like rating when paired with the ERG+PHY treatment (Figure 5.8 A), however this was not apparent when the commercial wine samples were included (Figure 5.8 B and C). Interestingly, the inclusion of commercial wine had little impact on the relative distribution of the synthetic products (Figure 5.8 B and C). The commercial wine (Wine A) was rated as being more wine-like (Figure 5.8 B) than the SGM synthetic products, but there was a lack of agreement among judges. This was evaluated in a follow-up round of sensory evaluations, where a second commercial wine was included (Wine B) (Figures 5.8 C). In the case of Wine B, panellists displayed a greater consensus regarding the presence of the wine-like feature (Figure 5.8 C). However, it was not ranked as being more wine-like than several of the synthetic products.

Interestingly, the data trends suggest that the ERG+OLE treatments also resulted in a slight but not significant reduction in the wine-like ranking (Figure 5.8 B and C). Generally, this wine-like ranking data suggests that the nitrogen modality was less influential on the wine-like ranking than the anaerobic factors, with PHY being most consistently rated the most wine-like. Interestingly, the MS200_PHY, MS200_ERG+PHY and MS200_ERG+OLE, were rated favourably with regards to wine resemblance (Figure 5.8), whereas in the previous round of evaluations (Figure 5.6), these same treatments were described as not resembling wine (PHY, ERG+PHY, and ERG+OLE). This may be due to several factors,

such as the presence of the SGM anaerobic factors that were not considered to be wine-like, and the resultant contrast between treatments may have allowed for a more favourable ranking. Additionally, these differing results suggest that panellists may have been better prepared for evaluating the more neutral synthetic products when they first evaluated them in isolation and then with the commercial wine.

The descriptor data shows that irrespective of the nitrogen treatment, the SGM treatments were generally associated with petrochemical and forest floor descriptors (Figure 5.9). Furthermore, this was the same anaerobic factor composition used in the first two sets of experiments, which resulted in synthetic samples with an unpleasant chemical character (Figure 5.2 and 5.4). Wine A was described as green, which may account for the varied responses received for its wine-like ranking, as panellists may have struggled to separate the identification of the wine-like feature and their preference for fruity rather than green wine (Charters & Pettigrew, 2007; Parr *et al.,* 2010). Generally, the remaining samples were described as similar with respect to the descriptors provided, as seen by the relative positioning of the commercial wine and the remaining synthetic products (Figure 5.9).

The ANOVA data describing the interactions between the nitrogen or anaerobic factor treatment and the wine-like ranking suggests that only the anaerobic factor had a significant impact (ERG+PHY and PHY were statistically similar). However, it is difficult to identify the relationship between the anaerobic factor and amino acid treatment with regard to their combined impact on aroma. Interestingly, the descriptor data suggests that the BCAA_ERG+OLE treatment has a slightly higher association with chemical and lower association with fruity traits, which is also reflected in a slightly lower wine-like ranking relative to the other ERG+OLE treatments (Figure 5.8 and 5.9).

The chemical data trends (Figure 5.10) describe the amino acid treatment in the first principle component (44.92%), and the anaerobic factor addition in the second (18.68%). As seen previously, the higher concentration of BCAAs results in higher concentrations of their associated volatile products. It is interesting that the nitrogen treatments are the more influential treatments chemically, whereas the anaerobic treatments were of greater consequence in the sensory data set. Consequently, this sensory-driven approach offers new prospects for the study of wine aromas, as the sensory data displayed a poor correlation with the chemical data (Supplementary Figure S 5.1 to 5.3) in this study. A similar sensory-driven approach has recently been successfully applied, where authors first performed a sorting task to identify wines with differing aromas followed with the chemical characterisation of those wines to identify the compounds responsible (Alegre *et al*., 2017).

Nonetheless, the unsaturated fatty acids, found in both the SGM and ERG+OLE treatments, lowered the production of acetate esters (Yoshioka & Hashimoto, 1981; Saerens *et al*., 2010) (Figure

5.10), which are important contributors to fruitiness, these lower levels of esters may contribute to the dominance of the more chemical notes observed. The SGM treatment contains more oleic acid than ERG+OLE, therefore this inhibition of acetate ester formation is stronger also. However, this does not exclude the possibility that Tween80 itself also contributes negatively to the samples' aromas.

The actual concentrations of the volatile compounds measured (Table S 5.3) varied greatly depending on the nitrogen composition employed, nonetheless, this had little bearing on the wine-like ranking of these products in a PHY or ERG+PHY background. This suggests that the perception of the wine-like aroma is linked to the relative concentration of the acetate esters to other volatile aromas or be affected by volatile and non-volatile compounds that were not assessed.

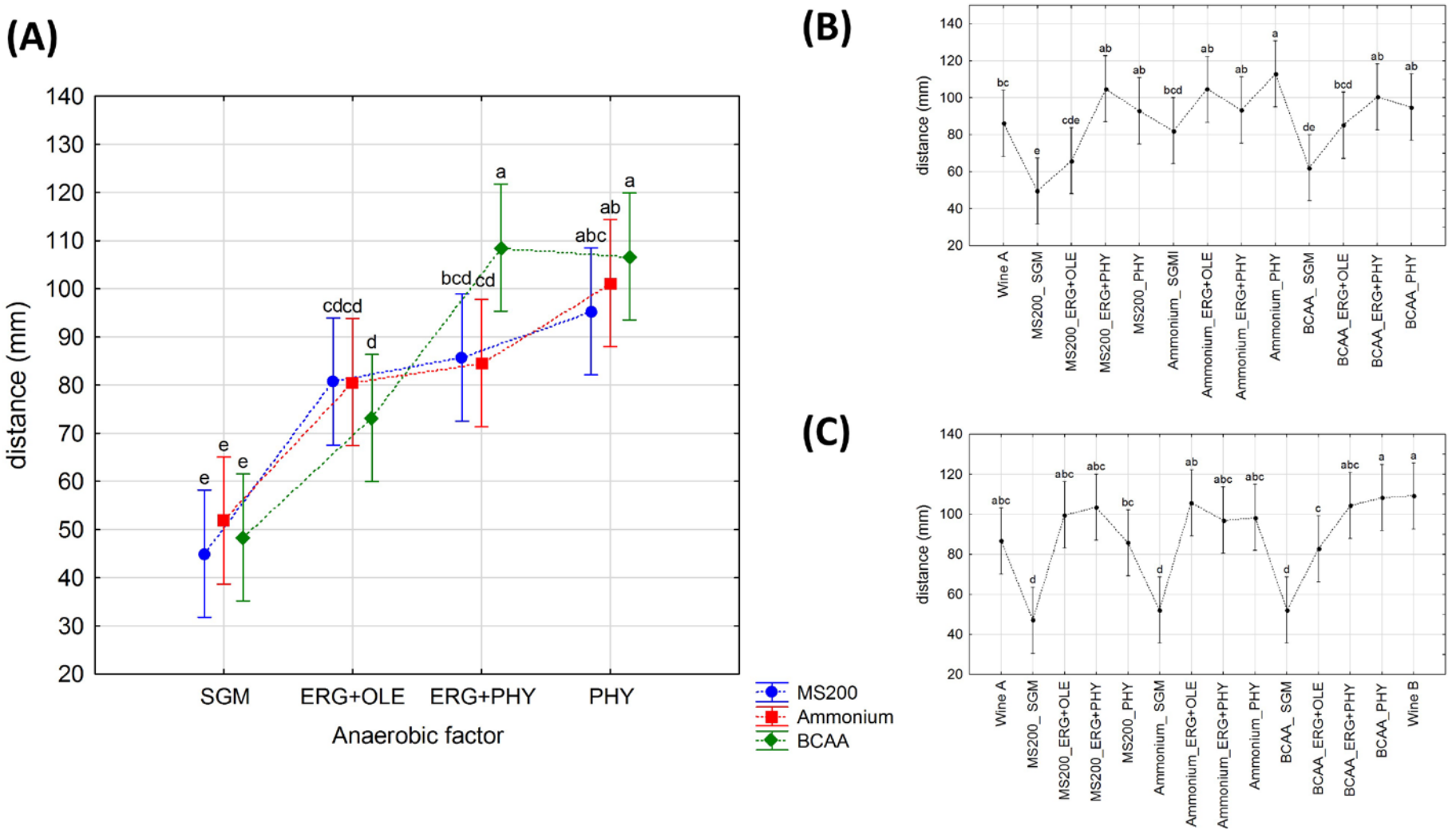


Figure 5.8. The wine-like distance was obtained using a line scale for only the synthetic samples treated with various combinations of anaerobic factors (A) (phytosterol (PHY), ergosterol and oleic acid (EGR+OLE), ergosterol and phytosterol (ERG+PHY), and SGM anaerobic factor mixture) and nitrogen compositions (BCAA, Ammonium, and MS200 amino acids), as well as ranking them relative one (B) or two commercial wines (C)

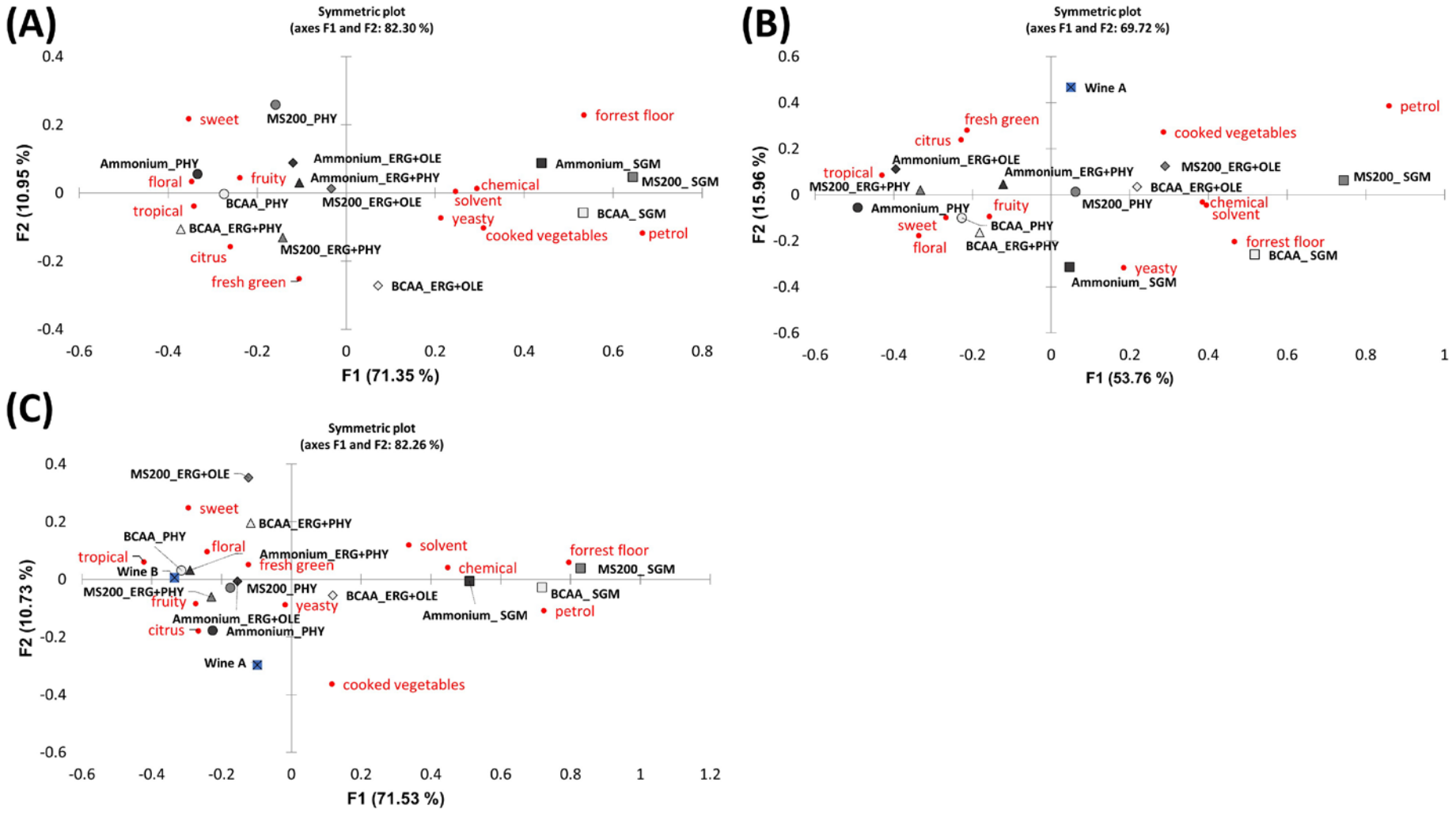


Figure 5.9. The correspondence analyses (A) describing only the synthetic samples treated with various combinations of anaerobic factors (phytosterol (PHY), ergosterol and oleic acid (EGR+OLE), ergosterol and phytosterol (ERG+PHY), and SGM anaerobic factor mixture) and nitrogen compositions (BCAA, Ammonium, and MS200 amino acids). Furthermore, these synthetic products were also described along with one (B) or two commercial wines (C).

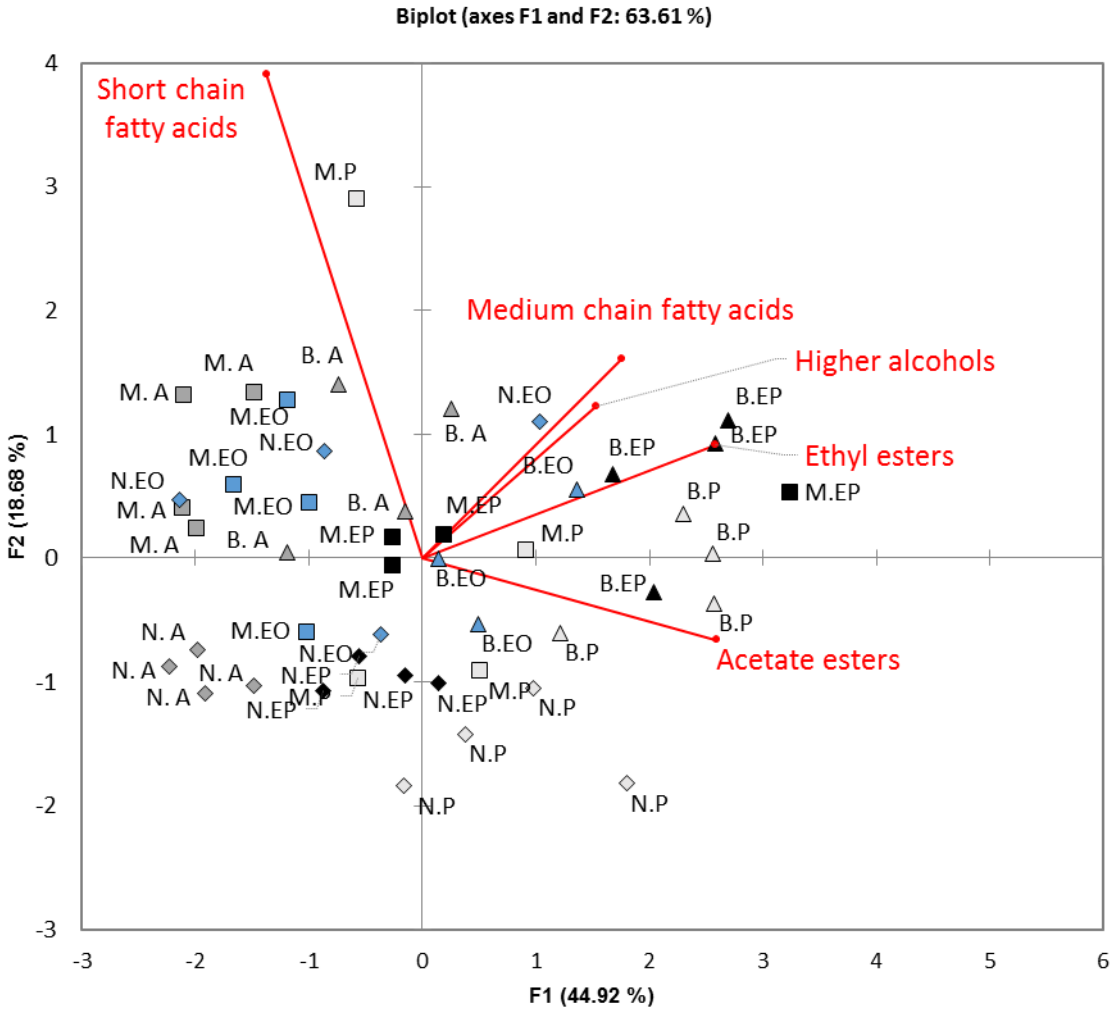


Figure 5.10. Principal component analysis of the GC-FID data of fermentations with various lipid and amino acid treatments, namely, branched-chain and aromatic amino acids (B), ammonium (N), and a complete mixture of amino acids (M), in addition to SGM anaerobic factors (A), phytosterol (P), ergosterol with phytosterol (EP) and ergosterol with oleic acid (EO)

5.4 Conclusion

This study sought to answer a deceptively simple question, "What makes wine, wine?", by exploring the wine-like concept. The fermentation products generated ultimately resembled wine. This suggests that the wine-like feature, or vinous character, is an entirely yeast "*de novo*" metabolism feature. Indeed, the simplified nature of synthetic must, which only contains non-volatile compounds, and no direct precursors or conjugated forms of aromatic products (even amino acids require multiple transformations) means that only *de novo* produced metabolites can impact the sensory perception of the wine-like feature. The data also provide for an optimised formulation of the synthetic must for future experiments which can expand on the current work to analyse and evaluate the sensory impact

of combinations of aroma compounds. The approach shown here can also be combined with chemical reconstitution approaches. Furthermore, the insights gained here, provide invaluable information needed to formulate better strategies to evaluate, verify and characterise the wine-like feature, one of the most significant being the context in which these synthetic products are evaluated, as when paired with a commercial wine the synthetic product was rated as a poor approximation of wine.

Anecdotal data described these synthetic samples as neutral, consequently, several panellists reported the commercial wines as being harsh in comparison. As a follow-up study, it would be interesting to determine whether varietal or ageing related compounds could enhance the perceived fruitiness of these synthetic products (Escudero *et al.*, 2007; Coetzee *et al.*, 2015), in addition to confirming the presence of the wine-like feature via chemical reconstitution.

This study certainly has several shortcomings, such as the inclusion of commercial wine in the initial experiments which magnified a bias against the synthetic wines resulting in a compression of the data. Additionally, it is likely that people have significantly variable precepts of what being wine-like means, and any such multidimensional quality is not easily translated into a simple linear scale. This could have been addressed with panel training for each experiment, however since follow-up treatments had to be designed based on the previous set of sensory data this would make a lengthy process even lengthier.

The results show that the vinous character responsible for wine recognition was perceived in the synthetic products. Consequently, this study has generated synthetic grape must formulations with a wine-like character by omitting unsaturated fatty acids and using either phytosterol or phytosterol with ergosterol as the anaerobic factor. This work has fallen short of being able to define the chemical signature responsible for wine recognition, however preliminary chemical data suggests that the relative rather than the actual concentrations of various odour families, particularly the acetate esters, may be important in establishing this feature. Additionally, this work highlights the value of using the sensory data as a decision-making tool, rather than the more commonly adopted chemistry-driven approach.

5.5 References

Alegre, Y., Sáenz-Navajas, M. P., Ferreira, V., García, D., Razquin, I. & Hernández-Orte, P., 2017. Rapid strategies for the determination of sensory and chemical differences between a wealth of similar wines. Eur. Food Res. Technol. 243, 8, 1295–1309.

Atanasova, B., Thomas-Danguin, T., Langlois, D., Nicklaus, S., Chabanet, C. & Etiévant, P., 2005. Perception of wine fruity and woody notes: Influence of peri-threshold odorants. Food Qual. Prefer. 16, 6, 504–510.

Ballester, J., Patris, B., Symoneaux, R. & Valentin, D., 2008. Conceptual vs. perceptual wine spaces: Does expertise matter? Food Qual. Prefer. 19, 3, 267–276.

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 Chem. 127, 2, 441–446.

Barrajón, N., Capece, A., Arévalo-Villena, M., Briones, A., & Romano, P., 2011. Co-inoculation of different *Saccharomyces cerevisiae* strains and influence on volatile composition of wines. Food Microbiol. 28, 5, 1080–1086.

Bely, M., Sablayrolles, J.M. & Barre, P., 1990. Description of alcoholic fermentation kinetics: its variability and significance. Am. J. Enol. Vitic. 41, 4, 319–324.

Benkwitz, F., Nicolau, L., Lund, C., Beresford, M., Wohlers, M. & Kilmartin, P.A., 2012. Evaluation of key odorants in Sauvignon blanc wines using three different methodologies. J. Agric. Food Chem. 60, 25, 6293–6302.

Burin, V.M., Gomes, T.M., Caliari, V., Rosier, J.P. & Bordignon Luiz, M.T., 2015. Establishment of influence the nitrogen content in musts and volatile profile of white wines associated to chemometric tools. Microchem. J. 122, 20–28.

Cadot, Y., Caillé, S., Samson, A., Barbeau, G. & Cheynier, V., 2010. Sensory dimension of wine typicality related to a terroir by Quantitative Descriptive Analysis, Just About Right analysis and typicality assessment. Anal. Chim. Acta 660, 1–2, 53–62.

Cartier, R., Rytz, A., Lecomte, A., Poblete, F., Krystlik, J., Belin, E. & Martin, N., 2006. Sorting procedure as an alternative to quantitative descriptive analysis to obtain a product sensory map. Food Qual. Prefer. 17, 7–8, 562–571.

Charters, S. & Pettigrew, S., 2007. The dimensions of wine quality. Food Qual. Prefer. 18, 7, 997–1007.

Chollet, S., Lelièvre, M., Abdi, H. & Valentin, D., 2011. Sort and beer: Everything you wanted to know about the sorting task but did not dare to ask. Food Qual. Prefer. 22, 6, 507–520.

Coetzee, C., Brand, J., Emerton, G., Jacobson, D., Silva Ferreira, A.C. & du Toit, W.J., 2015. Sensory interaction between 3-mercaptohexan-1-ol, 3-isobutyl-2-methoxypyrazine and oxidation-related compounds. Aust. J. Grape Wine Res. 21, 2, 179–188.

de-la-Fuente-Blanco, A., Sáenz-Navajas, M.P. & Ferreira, V., 2017. Levels of higher alcohols inducing aroma changes and modulating experts' preferences in wine model solutions. Aust. J. Grape Wine Res. 23, 2, 162–169.

Dickinson, J.R., Lanterman, M.M., Danner, D.J., Pearson, B.M., Sanz, P., Harrison, S.J. & Hewlins, M.J.E., 1997. A 13C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*. J. Biol. Chem. 272, 43, 26871–26878.

Duan, L., Shi, Y., Jiang, R. & Yang, Q., 2015. Effects of adding unsaturated fatty acids on fatty acid composition of *Saccharomyces cerevisiae* and major volatile compounds in wine. South African. J. Enol. Vitic. 36, 2, 285–295.

Escudero, A., Gogorza, B., Melús, M.A., Ortín, N., Cacho, J. & Ferreira, V., 2004. Characterization of the aroma of a wine from Maccabeo. Key role played by compounds with low odor activity values. J. Agric. Food Chem. 52, 11, 3516–3524.

Etievant, P., 1991. Wine. In H. Maarse (Ed.), Volatile compounds in food and beverages. Zeist, The Netherlands: TNO- CIVO Food Analysis Institute. pp. 486–546.

Ferreira, A.C.S., Monforte, A.R., Teixeira, C.S., Martins, R., Fairbairn, S., Bauer, F.F., 2014. Monitoring alcoholic fermentation: an untargeted approach. J. Agric. Food Chem. 62, 28, 6784–93.

Ferreira V. & Cacho J., 2009. Identification of Impact Odorants of Wines. In: Moreno-Arribas, M.V.& Polo M.C. (eds). Wine Chemistry and Biochemistry, vol I. Springer, New York, NY. pp. 293 -410

Ferreira, V., López, R. & Cacho, J.F., 2000. Quantitative determination of the odorants of young red wines from different grape varieties. J. Sci. Food Agric. 80, 11, 1659–1667.

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. J. Agric. Food Chem. 50, 14, 4048–4054.

Francis, I.L. & Newton, J.L., 2005. Determining wine aroma from compositional data. Aust. J. Grape Wine Res. 11, 2, 114–126.

Guth, H., 1997. Identification of character impact odorants of different white wine varieties. J. Agric. Food Chem. 45, 8, 3022–3026.

Gutiérrez, A., Chiva, R., Sancho, M., Beltran, G., Arroyo-López, F.N. & Guillamon, J.M., 2012. Nitrogen requirements of commercial wine yeast strains during fermentation of a synthetic grape must. Food Microbiol. 31, 1, 25–32.

Hein, K., Ebeler, S.E. & Heymann, H., 2009. Perception of fruity and vegetative aromas in red wine. J. Sens. Stud. 24, 3, 441–455.

Henschke, P. A. & Jiranek, V., 1993. Yeasts-metabolism of nitrogen compounds. In: Fleet, G.H. (ed) Wine microbiology and biotechnology. pp.77-164.

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. J. Agric. Food Chem. 50, 10, 2891–2899.

Lawrence, N. (2012). Volatile metabolic profiling of SA Chenin blanc fresh and fruity and rich and ripe wine styles: Development of analytical methods for flavour compounds (aroma and flavour) and application of chemometrics for resolution of complex analytical measurement. MSc Thesis. Stellenbosch University, South Africa.

Lawrence, G., Symoneaux, R., Maitre, I., Brossaud, F., Maestrojuan, M. & Mehinagic, E., 2013. Using the free comments method for sensory characterisation of Cabernet Franc wines : Comparison with classical profiling in a professional context. Food Qual. Prefer. 30, 2, 145–155.

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. J. Agric. Food Chem. 57, 7, 2623–2632.

Maitre, I., Symoneaux, R., Jourjon, F. & Mehinagic, E., 2010. Sensory typicality of wines: How scientists have recently dealt with this subject. Food Qual. Prefer. 21, 7, 726–731.

Mauricio, J.C., Moreno, J., Zea, L., Ortega, J.M. & Medina, M., 1997. The effects of grape must fermentation conditions on volatile alcohols and esters formed by *Saccharomyces cerevisiae*. J. Sci. Food Agric. 75, 2, 155–160.

Mayr, C.M., Geue, J.P., Holt, H.E., Pearson, W.P., Jeffery, D.W. & Francis, I.L., 2014. Characterization of the key aroma compounds in shiraz wine by quantitation, aroma reconstitution, and omission studies. J. Agric. Food Chem. 62, 20, 4528–4536.

Mendes-Pinto, M.M., 2009. Carotenoid breakdown products the-norisoprenoids-in wine aroma. Arch. Biochem. Biophys. 483, 2, 236–245.

Miller, A.C., Wolff, S.R., Bisson, L.F. & Ebeler, S.E., 2007. Yeast strain and nitrogen supplementation: Dynamics of volatile ester production in chardonnay juice fermentations. Am. J. Enol. Vitic. 58, 4, 470–483.

Parr, W. V, Green, J.A., White, K.G. & Sherlock, R.R., 2007. The distinctive flavour of New Zealand Sauvignon blanc: Sensory characterisation by wine professionals. Food Qual. Prefer. 18, 6, 849–861.

Parr, W. V., Valentin, D., Green, J.A. & Dacremont, C., 2010. Evaluation of French and New Zealand Sauvignon wines by experienced French wine assessors. Food Qual. Prefer. 21, 1, 56–64.

Peinado, R.A., Moreno, J., Medina, M. & Mauricio, J.C., 2004. Changes in volatile compounds and aromatic series in sherry wine with high gluconic acid levels subjected to aging by submerged flor yeast cultures. Biotechnol. Lett. 26, 9, 757–62.

Perrin, L. & Pagès, J., 2009. A methodology for the analysis of sensory typicality judgments. J. Sens. Stud. 24, 5, 749–773.

Rollero, S., Bloem, A., Camarasa, C., Sanchez, I., Ortiz-Julien, A., Sablayrolles, J.M., Dequin, S. & Mouret, J.R., 2015. Combined effects of nutrients and temperature on the production of fermentative aromas by *Saccharomyces cerevisiae* during wine fermentation. Appl. Microbiol. Biotechnol. 99, 5, 2291–2304.

Rollero, S., Mouret, J.-R., Sanchez, I., Camarasa, C., Ortiz-Julien, A., Sablayrolles, J.-M. & Dequin, S., 2016. Key role of lipid management in nitrogen and aroma metabolism in an evolved wine yeast strain. Microb. Cell Fact. 15, 1, 32.

Rossouw, D. & Bauer, F.F., 2016. Exploring the phenotypic space of non-*Saccharomyces* wine yeast biodiversity. Food Microbiol. 55, 32–46.

Rossouw, D., Næs, 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, 1, 530.

Saerens, S.M.G., Delvaux, F.R., Verstrepen, K.J. & Thevelein, J.M., 2010. Production and biological function of volatile esters in *Saccharomyces cerevisiae* Microb. Biotechnol. 3, 2, 165–177.

Schuttler, A., Friedel, M., Jung, R., Rauhut, D., Darriet, P., Schüttler, A., Friedel, M., Jung, R., Rauhut, D. & Darriet, P., 2015. Characterizing aromatic typicality of Riesling wines: Merging volatile compositional and sensory aspects. Food Res. Int. 69, 26–37.

Smit, A. (2013). The impact of nutrients on aroma and flavour production during wine fermentation. PhD Dissertation. Stellenbosch University, South Africa.

Styger, G., Prior, B. & Bauer, F.F., 2011. Wine flavor and aroma. J. Ind. Microbiol. Biotechnol. 38, 9, 1145–1159.

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 Chem. 127, 3, 1072–1083.

Valentin, D., Chollet, S., Lelièvre, M. & Abdi, H., 2012. Quick and dirty but still pretty good: A review of new descriptive methods in food science. Int. J. Food Sci. Technol. 47, 8, 1563–1578.

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

Varela, P. & Ares, G., 2012. Sensory profiling, the blurred line between sensory and consumer science. A review of novel methods for product characterization. Food Res. Int. 48, 2, 893–908.

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. Appl. Microbiol. Biotechnol. 77, 1, 145–157.

Yoshioka, K. & Hashimoto, N., 1981. Ester formation by alcohol acetyltransferase from brewers' yeast. Yeast 45, 10, 2183–2190.

5.6 Supplementary material

Table S 5.1. The impact of various amino acid treatments on the production of volatile compounds (mg/L) by VIN13 in synthetic grape must. The data summarizes the average of triplicate fermentations and their standard deviation. Additionally, the letters denote significant differences (95%) between treatments using Fischer LSD.

	Ammonium			OAV	All amino acids			OAV	75% Branched chain & aromatic amino acids			OAV
2-phenyl ethanol	7.52 ± 0.48	a		0.54	39.63 ± 2.00	e		**2.83**	20.76 ± 0.21	bc		**1.48**
isoamyl alcohol	81.55 ± 6.46	b		**2.72**	100.63 ± 7.75	cd		**3.35**	112.51 ± 0.35	d		**3.75**
isobutanol	9.90 ± 0.91	ab		0.25	17.74 ± 1.85	d		0.44	13.26 ± 0.85	c		0.33
propanol	33.71 ± 0.56	b		0.11	21.41 ± 1.79	a		0.07	21.72 ± 2.16	a		0.07
ethyl acetate	33.80 ± 3.75	b		**2.76**	32.93 ± 1.93	b		**2.69**	31.86 ± 4.51	b		**2.60**
isoamyl acetate	0.15 ± 0.03	bc		**5.03**	0.19 ± 0.06	c		**6.32**	0.33 ± 0.00	d		**10.92**
acetic acid	451.02 ± 17.34	b		**2.26**	454.78 ± 47.02	b		**2.27**	422.31 ± 0.98	ab		**2.11**
butyric acid	0.74 ± 0.07	b		**4.28**	0.70 ± 0.03	b		**4.06**	0.74 ± 0.00	b		**4.27**
isobutyric acid	0.56 ± 0.07	b		0.24	0.81 ± 0.06	d		0.35	0.68 ± 0.00	c		0.29
isovaleric acid	0.85 ± 0.05	ab		**25.66**	1.08 ± 0.05	b		**32.87**	1.08 ± 0.01	ab		**32.58**
valeric acid	0.32 ± 0.02	a			0.31 ± 0.02	a			0.31 ± 0.00	a		
hexanoic acid	2.09 ± 0.22	c		**4.98**	1.68 ± 0.05	b		**4.00**	1.74 ± 0.04	b		**4.15**
decanoic acid	2.01 ± 0.25	b		**2.01**	1.28 ± 0.11	a		**1.28**	1.25 ± 0.03	a		**1.25**
octanoic acid	3.18 ± 0.42	c		**6.37**	2.45 ± 0.05	b		**4.90**	2.50 ± 0.02	b		**4.99**
ethyl hexanoate	0.38 ± 0.02	cd		**26.90**	0.32 ± 0.01	b		**23.11**	0.35 ± 0.01	bcd		**24.73**
ethyl caprylate	0.34 ± 0.13	b		**68.65**	0.31 ± 0.03	ab		**61.62**	0.36 ± 0.02	b		**71.96**
ethyl caprate	0.17 ± 0.08	a		0.85	0.15 ± 0.02	a		0.77	0.15 ± 0.00	a		0.77

Table S 5.1 continued. The impact of various amino acid treatments on the production of volatile compounds (mg/L) by VIN13 in synthetic grape must. The data 1summarizes the average of triplicate fermentations and their standard deviation. Additionally, the letters denote significant differences (95%) between treatments using Fischer LSD.

	50% Branched chain & aromatic amino acids					25% Branched chain & aromatic amino acids					50% Sulphur containing amino acids				
					OAV					OAV					OAV
2-phenyl ethanol	20.69	±	1.71	b	**1.48**	24.12	±	2.23	c	**1.72**	35.40	±	1.33	d	**2.53**
isoamyl alcohol	92.02	±	4.17	bc	**3.07**	59.07	±	10.10	a	**1.97**	83.64	±	8.56	b	**2.79**
isobutanol	12.36	±	0.62	c	0.31	8.61	±	1.21	a	0.22	11.27	±	0.87	bc	0.28
propanol	18.78	±	1.01	a	0.06	18.41	±	4.25	a	0.06	31.23	±	1.81	b	0.10
ethyl acetate	28.46	±	2.02	b	**2.32**	18.94	±	1.49	a	**1.54**	33.81	±	5.06	b	**2.76**
isoamyl acetate	0.21	±	0.08	c	**6.98**	0.00	±	0.00	a	0.00	0.07	±	0.04	ab	**2.20**
acetic acid	437.04	±	18.85	ab	**2.19**	382.42	±	27.17	a	**1.91**	492.39	±	47.96	b	**2.46**
butyric acid	0.72	±	0.04	b	**4.18**	0.57	±	0.01	a	**3.28**	0.61	±	0.00	a	**3.54**
isobutyric acid	0.65	±	0.03	c	0.28	0.46	±	0.03	a	0.20	0.55	±	0.04	ab	0.24
isovaleric acid	0.77	±	0.29	ab	**23.20**	0.73	±	0.16	ab	**22.25**	0.71	±	0.28	a	**21.39**
valeric acid	0.30	±	0.01	a		0.28	±	0.00	a		0.27	±	0.01	a	
hexanoic acid	1.78	±	0.06	b	**4.23**	1.38	±	0.06	a	**3.29**	1.73	±	0.12	b	**4.12**
decanoic acid	1.09	±	0.07	a	**1.09**	1.12	±	0.27	a	**1.12**	1.30	±	0.07	a	**1.30**
octanoic acid	2.50	±	0.11	b	**5.00**	1.97	±	0.22	a	**3.94**	2.55	±	0.02	b	**5.10**
ethyl hexanoate	0.34	±	0.02	bc	**23.96**	0.22	±	0.00	a	**15.38**	0.39	±	0.06	d	**27.61**
ethyl caprylate	0.32	±	0.04	ab	**63.95**	0.19	±	0.05	a	**37.54**	0.31	±	0.04	ab	**62.65**
ethyl caprate	0.14	±	0.03	a	0.69	0.09	±	0.02	a	0.43	0.10	±	0.01	a	0.52

Table S 5.2. The impact of anaerobic factor treatments on the production of volatile compounds (mg/L) by VIN13 in synthetic grape must. The data summarizes the average of triplicate fermentations and their standard deviation. Additionally, the letters denote significant differences (95%) between treatments using Fischer LSD.

	Ergosterol					Ergosterol & Oleic acid					Ergosterol & Phytosterol					Ergosterol & Tween80				
					OAV					OAV					OAV					OAV
2-Phenyl ethanol	32.07	±	4.51	a	**2.29**	18.42	±	1.02	c	**1.32**	20.03	±	3.87	c	**1.43**	17.22	±	0.67	c	**1.23**
3-ethoxy-1-propanol	3.33	±	0.22	abc	**33.33**	2.77	±	0.14	cd	**27.65**	2.38	±	0.41	de	**23.84**	2.42	±	0.19	de	**24.18**
Isoamyl alcohol	113.08	±	3.46	a	**3.77**	83.24	±	11.49	b	**2.77**	70.82	±	11.79	bcd	**2.36**	71.63	±	1.71	bcd	**2.39**
Isobutanol	13.38	±	0.43	a	0.33	10.49	±	0.91	b	0.26	8.81	±	0.88	bcd	0.22	8.88	±	0.30	bcd	0.22
Butanol	0.96	±	0.03	a	0.01	0.59	±	0.08	c	0.00	0.59	±	0.06	c	0.00	0.57	±	0.00	c	0.00
Propanol	45.68	±	0.23	a	0.15	39.09	±	2.06	abc	0.13	32.70	±	3.70	cde	0.11	36.59	±	0.60	bcd	0.12
2-Phenylethyl acetate	1.20	±	0.32	a	**4.80**	0.48	±	0.05	cd	**1.90**	0.76	±	0.12	b	**3.05**	0.68	±	0.02	bc	**2.73**
Isoamyl acetate	3.27	±	0.71	a	**109.13**	0.91	±	0.24	cde	**30.48**	1.47	±	0.35	bc	**48.92**	1.52	±	0.06	b	**50.53**
Acetic acid	639.53	±	66.67	cd	**3.20**	759.52	±	33.43	a	**3.80**	706.24	±	63.01	ab	**3.53**	710.92	±	47.37	ab	**3.55**
Butyric acid	1.29	±	0.04	a	**7.44**	0.79	±	0.10	fg	**4.54**	1.11	±	0.12	bc	**6.44**	0.99	±	0.03	d	**5.72**
Isobutyric acid	0.76	±	0.03	a	0.33	0.69	±	0.08	abcd	0.30	0.75	±	0.10	a	0.33	0.70	±	0.02	abc	0.30
Isovaleric acid	1.07	±	0.02	a	**32.56**	0.96	±	0.05	bc	**28.99**	0.99	±	0.10	ab	**30.04**	0.93	±	0.01	bcd	**28.30**
Propionic acid	1.50	±	0.09	a	0.19	1.16	±	0.11	bc	0.14	1.15	±	0.14	bc	0.14	1.10	±	0.07	cd	0.14
Valeric acid	0.44	±	0.03	a		0.35	±	0.03	bc		0.35	±	0.03	bc		0.31	±	0.01	cde	
Diethyl succinate	0.36	±	0.00	ab	0.00	0.36	±	0.01	abc	0.00	0.34	±	0.02	cde	0.00	0.33	±	0.00	def	0.00
Ethyl acetate	78.98	±	2.16	a	**6.44**	47.99	±	1.54	de	**3.91**	51.76	±	6.35	cd	**4.22**	57.47	±	0.36	abc	**4.68**
Ethyl butyrate	0.64	±	0.01	a	**32.02**	0.54	±	0.02	de	**27.19**	0.55	±	0.04	cd	**27.63**	0.59	±	0.00	abc	**29.52**
Hexanoic acid	4.72	±	0.39	a	**11.24**	2.41	±	0.50	ef	**5.74**	4.22	±	0.58	ab	**10.04**	3.75	±	0.16	bc	**8.94**
Octanoic acid	6.40	±	0.86	a	**12.80**	3.91	±	1.45	cd	**7.82**	4.89	±	0.87	bc	**9.78**	4.72	±	0.66	bc	**9.43**
Decanoic acid	2.91	±	0.37	a	**2.91**	1.59	±	0.50	cd	**1.59**	2.21	±	0.15	b	**2.21**	2.38	±	0.28	b	**2.38**

Table S 5.2 continued. The impact of anaerobic factor treatments on the production of volatile compounds (mg/L) by VIN13 in synthetic grape must. The data summarizes the average of triplicate fermentations and their standard deviation. Additionally, the letters denote significant differences (95%) between treatments using Fischer LSD.

	Ergosterol				OAV	Ergosterol & Oleic acid				OAV	Ergosterol & Phytosterol				OAV	Ergosterol & Tween80				OAV
2-Phenyl ethanol	32.07	±	4.51	a	**2.29**	18.42	±	1.02	c	**1.32**	20.03	±	3.87	c	**1.43**	17.22	±	0.67	c	**1.23**
3-ethoxy-1-propanol	3.33	±	0.22	abc	**33.33**	2.77	±	0.14	cd	**27.65**	2.38	±	0.41	de	**23.84**	2.42	±	0.19	de	**24.18**
Isoamyl alcohol	113.08	±	3.46	a	**3.77**	83.24	±	11.49	b	**2.77**	70.82	±	11.79	bcd	**2.36**	71.63	±	1.71	bcd	**2.39**
Isobutanol	13.38	±	0.43	a	0.33	10.49	±	0.91	b	0.26	8.81	±	0.88	bcd	0.22	8.88	±	0.30	bcd	0.22
Butanol	0.96	±	0.03	a	0.01	0.59	±	0.08	c	0.00	0.59	±	0.06	c	0.00	0.57	±	0.00	c	0.00
Propanol	45.68	±	0.23	a	0.15	39.09	±	2.06	abc	0.13	32.70	±	3.70	cde	0.11	36.59	±	0.60	bcd	0.12
2-Phenylethyl acetate	1.20	±	0.32	a	**4.80**	0.48	±	0.05	cd	**1.90**	0.76	±	0.12	b	**3.05**	0.68	±	0.02	bc	**2.73**
Isoamyl acetate	3.27	±	0.71	a	**109.13**	0.91	±	0.24	cde	**30.48**	1.47	±	0.35	bc	**48.92**	1.52	±	0.06	b	**50.53**
Acetic acid	639.53	±	66.67	cd	**3.20**	759.52	±	33.43	a	**3.80**	706.24	±	63.01	ab	**3.53**	710.92	±	47.37	ab	**3.55**
Butyric acid	1.29	±	0.04	a	**7.44**	0.79	±	0.10	fg	**4.54**	1.11	±	0.12	bc	**6.44**	0.99	±	0.03	d	**5.72**
Isobutyric acid	0.76	±	0.03	a	0.33	0.69	±	0.08	abcd	0.30	0.75	±	0.10	a	0.33	0.70	±	0.02	abc	0.30
Isovaleric acid	1.07	±	0.02	a	**32.56**	0.96	±	0.05	bc	**28.99**	0.99	±	0.10	ab	**30.04**	0.93	±	0.01	bcd	**28.30**
Propionic acid	1.50	±	0.09	a	0.19	1.16	±	0.11	bc	0.14	1.15	±	0.14	bc	0.14	1.10	±	0.07	cd	0.14
Valeric acid	0.44	±	0.03	a		0.35	±	0.03	bc		0.35	±	0.03	bc		0.31	±	0.01	cde	
Diethyl succinate	0.36	±	0.00	ab	0.00	0.36	±	0.01	abc	0.00	0.34	±	0.02	cde	0.00	0.33	±	0.00	def	0.00
Ethyl acetate	78.98	±	2.16	a	**6.44**	47.99	±	1.54	de	**3.91**	51.76	±	6.35	cd	**4.22**	57.47	±	0.36	abc	**4.68**
Ethyl butyrate	0.64	±	0.01	a	**32.02**	0.54	±	0.02	de	**27.19**	0.55	±	0.04	cd	**27.63**	0.59	±	0.00	abc	**29.52**
Hexanoic acid	4.72	±	0.39	a	**11.24**	2.41	±	0.50	ef	**5.74**	4.22	±	0.58	ab	**10.04**	3.75	±	0.16	bc	**8.94**
Octanoic acid	6.40	±	0.86	a	**12.80**	3.91	±	1.45	cd	**7.82**	4.89	±	0.87	bc	**9.78**	4.72	±	0.66	bc	**9.43**
Decanoic acid	2.91	±	0.37	a	**2.91**	1.59	±	0.50	cd	**1.59**	2.21	±	0.15	b	**2.21**	2.38	±	0.28	b	**2.38**

Table S 5.2 continued. The impact of anaerobic factor treatments on the production of volatile compounds (mg/L) by VIN13 in synthetic grape must. The data summarizes the average of triplicate fermentations and their standard deviation. Additionally, the letters denote significant differences (95%) between treatments using Fischer LSD

	No Addition				OAV	Oleic acid				OAV	Tween80				OAV	Tween80 & Oleic acid				OAV
2-Phenyl ethanol	10.82	±	0.28	d	0.77	11.94	±	0.50	d	0.85	**11.58**	±	0.07	d	0.83	**11.63**	±	0.27	d	0.83
3-ethoxy-1-propanol	1.72	±	0.03	f	**17.19**	1.78	±	0.17	f	**17.84**	**2.09**	±	0.30	ef	**20.86**	**1.70**	±	0.09	f	**17.00**
Isoamyl alcohol	69.67	±	2.34	bcd	**2.32**	61.37	±	6.90	d	**2.05**	**68.66**	±	0.83	cd	**2.29**	**58.15**	±	**10.74**	d	**1.94**
Isobutanol	8.08	±	0.05	bcd	0.20	8.88	±	1.01	d	0.22	**9.13**	±	0.43	cd	0.23	**8.55**	±	**1.30**	d	0.21
Butanol	0.86	±	0.05	ab	0.01	0.73	±	0.15	bc	0.00	0.90	±	0.05	a	0.01	0.70	±	0.14	bc	0.00
Propanol	25.73	±	1.77	ef	0.08	20.89	±	3.82	f	0.07	**30.44**	±	**3.25**	de	0.10	**20.12**	±	**5.09**	f	0.07
2-Phenylethyl acetate	0.48	±	0.02	cd	**1.92**	0.39	±	0.00	d	**1.58**	0.47	±	0.02	d	**1.87**	0.40	±	0.01	d	**1.59**
Isoamyl acetate	0.99	±	0.11	bcde	**32.83**	0.53	±	0.06	e	**17.69**	0.84	±	0.02	de	**27.86**	0.54	±	0.10	e	**17.90**
Acetic acid	669.75	±	26.08	bcd	**3.35**	633.83	±	27.11	cd	**3.17**	**683.54**	±	**6.53**	bc	**3.42**	**658.74**	±	**7.23**	bcd	**3.29**
Butyric acid	1.01	±	0.03	cd	**5.84**	0.50	±	0.03	h	**2.91**	0.84	±	0.01	ef	**4.87**	0.51	±	0.06	h	**2.95**
Isobutyric acid	0.65	±	0.03	bcde	0.28	0.58	±	0.01	e	0.25	0.63	±	0.03	cde	0.27	0.58	±	0.04	e	0.25
Isovaleric acid	0.84	±	0.06	e	**25.38**	0.81	±	0.00	e	**24.54**	0.87	±	0.01	de	**26.40**	0.83	±	0.02	e	**25.02**
Propionic acid	0.91	±	0.06	ef	0.11	0.80	±	0.05	f	0.10	0.97	±	0.06	de	0.12	0.81	±	0.08	f	0.10
Valeric acid	0.28	±	0.00	e		0.28	±	0.02	e		0.30	±	0.02	de		0.28	±	0.01	e	
Diethyl succinate	0.32	±	0.01	f	0.00	0.32	±	0.01	ef	0.00	0.33	±	0.00	def	0.00	0.33	±	0.01	def	0.00
Ethyl acetate	43.99	±	4.27	f	**3.58**	33.51	±	2.42	f	**2.73**	**45.76**	±	**2.77**	ef	**3.73**	**32.63**	±	**3.99**	f	**2.66**
Ethyl butyrate	0.56	±	0.00	bcd	**27.91**	0.49	±	0.00	f	**24.42**	0.53	±	0.00	f	**26.34**	0.48	±	0.00	f	**23.87**
Hexanoic acid	3.24	±	0.06	cd	**7.72**	1.32	±	0.10	g	**3.13**	**2.67**	±	0.04	de	**6.35**	**1.36**	±	0.25	g	**3.25**
Octanoic acid	3.64	±	0.08	cde	**7.27**	2.06	±	0.23	f	**4.11**	**3.64**	±	0.07	cde	**7.29**	**2.33**	±	0.55	ef	**4.66**
Decanoic acid	1.95	±	0.07	bc	**1.95**	1.27	±	0.11	d	**1.27**	**1.97**	±	0.05	bc	**1.97**	**1.44**	±	0.25	d	**1.44**

Table S 5.3. The impact of anaerobic factor and nitrogen treatments on the production of volatile compounds (mg/L) by VIN13 in synthetic grape must. The data summarizes the average of four replicate fermentations and their standard deviation. Additionally, the letters denote significant differences (95%) between treatments using Fischer LSD

	propanol		isobutanol		butanol		isoamyl alcohol		3-ethoxy-1-propanol		2-phenyl ethanol	
MS300_ AWRI	26.77 ± 2.36	abc	8.26 ± 0.59	cd	0.43 ± 0.00	b	78.06 ± 9.13	b	2.83 ± 0.16	bcde	10.60 ± 0.34	c
MS300_ERG+OLE	26.95 ± 3.04	abc	7.45 ± 0.16	d	0.53 ± 0.03	b	76.13 ± 6.03	b	2.72 ± 0.13	bcdef	11.04 ± 0.61	c
MS300_ERG+PHY	26.56 ± 2.76	abc	8.15 ± 0.35	d	0.64 ± 0.06	b	77.08 ± 7.26	b	2.54 ± 0.08	ef	9.09 ± 0.67	c
MS300_PHY	27.00 ± 2.62	abc	7.33 ± 0.45	d	0.68 ± 0.06	b	76.72 ± 7.26	b	2.60 ± 0.28	def	10.92 ± 1.48	c
Ammonium_ AWRI	20.26 ± 2.29	e	7.50 ± 0.79	d	0.47 ± 0.02	b	74.80 ± 8.13	b	3.31 ± 0.35	a	5.94 ± 0.27	c
Ammonium_ERG+OLE	29.81 ± 2.08	a	7.78 ± 0.20	d	0.61 ± 0.03	b	88.47 ± 1.58	b	2.90 ± 0.24	abcd	4.39 ± 0.38	c
Ammonium_ERG+PHY	29.11 ± 3.50	ab	6.74 ± 0.55	d	0.69 ± 0.08	b	68.08 ± 5.78	b	2.93 ± 0.29	abc	4.38 ± 0.17	c
Ammonium_PHY	30.91 ± 4.03	a	7.43 ± 0.22	d	0.82 ± 0.02	b	81.80 ± 5.00	b	2.99 ± 0.14	ab	4.71 ± 0.23	c
BCAA_ AWRI	25.51 ± 3.28	bcd	44.19 ± 0.00	bc	2.05 ± 0.10	a	243.83 ± 21.44	a	2.41 ± 0.05	ef	154.46 ± 12.26	a
BCAA_ERG+OLE	23.25 ± 1.72	cde	48.87 ± 2.27	ab	1.99 ± 0.07	a	227.51 ± 14.07	a	2.58 ± 0.49	cdef	126.23 ± 22.15	b
BCAA_ERG+PHY	22.28 ± 1.45	de	42.11 ± 0.50	a	1.84 ± 0.04	a	197.72 ± 8.12	a	2.40 ± 0.15	f	93.10 ± 5.38	b
BCAA_PHY	25.12 ± 2.99	bcde	43.32 ± 0.69	ab	1.96 ± 0.08	a	216.07 ± 20.66	a	2.34 ± 0.03	f	128.85 ± 14.03	b

Table S 5.3 continued. The impact of anaerobic factor and nitrogen treatments on the production of volatile compounds (mg/L) by VIN13 in synthetic grape must. The data summarizes the average of four replicate fermentations and their standard deviation. Additionally, the letters denote significant differences (95%) between treatments using Fischer LSD

	ethyl acetate		ethyl butyrate		isoamyl acetate		ethyl phenylethyl acetate		2-phenylethyl acetate	
MS300_ AWRI	10.17 ± 0.63	ef	0.30 ± 0.01	fg	0.54 ± 0.01	def	0.49 ± 0.02	bcd	0.56 ± 0.05	b
MS300_ERG+OLE	9.66 ± 0.47	ef	0.38 ± 0.02	efg	1.25 ± 0.02	ef	0.58 ± 0.08	bc	1.18 ± 0.11	b
MS300_ERG+PHY	20.55 ± 1.31	cdef	0.44 ± 0.05	bcd	2.91 ± 0.40	cde	0.35 ± 0.02	cde	1.95 ± 0.17	b
MS300_PHY	22.97 ± 0.76	bcde	0.41 ± 0.03	cdef	2.78 ± 0.32	cde	0.53 ± 0.01	bcd	1.90 ± 0.14	b
Ammonium_ AWRI	6.20 ± 1.25	f	0.32 ± 0.03	g	0.46 ± 0.05	f	0.73 ± 0.05	a	0.34 ± 0.05	b
Ammonium_ERG+OLE	14.17 ± 0.75	def	0.42 ± 0.02	cde	1.51 ± 0.21	cdef	0.39 ± 0.04	cde	0.95 ± 0.04	b
Ammonium_ERG+PHY	30.36 ± 4.51	abc	0.38 ± 0.03	efg	2.36 ± 0.12	cdef	0.34 ± 0.04	e	1.18 ± 0.10	b
Ammonium_PHY	30.56 ± 4.69	a	0.47 ± 0.04	bc	3.40 ± 0.08	bcd	0.29 ± 0.07	e	1.48 ± 0.04	b
BCAA_ AWRI	10.98 ± 2.20	ef	0.39 ± 0.03	defg	2.09 ± 0.10	cdef	0.85 ± 0.06	a	1.40 ± 0.07	b
BCAA_ERG+OLE	18.50 ± 2.01	cdef	0.43 ± 0.04	cde	4.10 ± 0.09	abc	0.66 ± 0.24	ab	2.23 ± 0.40	b
BCAA_ERG+PHY	25.49 ± 1.48	abcd	0.50 ± 0.04	ab	7.30 ± 0.34	a	0.34 ± 0.02	de	4.22 ± 0.51	a
BCAA_PHY	29.94 ± 2.94	ab	0.52 ± 0.03	a	6.98 ± 0.28	ab	0.65 ± 0.06	ab	5.50 ± 1.31	a

Table S 5.3 continued. The impact of anaerobic factor and nitrogen treatments on the production of volatile compounds (mg/L) by VIN13 in synthetic grape must. The data summarizes the average of four replicate fermentations and their standard deviation. Additionally, the letters denote significant differences (95%) between treatments using Fischer LSD

	acetic acid		propionic acid		isobutyric acid		butyric acid		isovaleric acid		valeric acid	
MS300_ AWRI	379.57 ± 13.95	ab	1.96 ± 0.06	bcde	0.48 ± 0.06	c	0.58 ± 0.01	ef	0.46 ± 0.00	c	0.16 ± 0.02	bc
MS300_ERG+OLE	371.14 ± 29.36	a	2.37 ± 0.09	abc	0.36 ± 0.04	cd	0.74 ± 0.04	def	0.47 ± 0.04	c	0.12 ± 0.02	de
MS300_ERG+PHY	345.15 ± 19.33	abc	2.36 ± 0.05	abc	0.35 ± 0.02	cd	0.96 ± 0.10	abc	0.47 ± 0.03	c	0.12 ± 0.01	bcde
MS300_PHY	312.86 ± 19.56	cde	1.64 ± 0.22	e	0.33 ± 0.03	cd	1.01 ± 0.11	abc	0.43 ± 0.01	c	0.15 ± 0.01	bcde
Ammonium_ AWRI	310.47 ± 9.98	de	1.88 ± 0.08	cde	0.31 ± 0.05	cd	0.58 ± 0.06	f	0.43 ± 0.02	c	0.13 ± 0.02	cde
Ammonium_ERG+OLE	357.15 ± 35.29	a	2.39 ± 0.31	ab	0.28 ± 0.03	cd	0.74 ± 0.03	def	0.32 ± 0.02	c	0.10 ± 0.01	e
Ammonium_ERG+PHY	308.22 ± 5.50	de	2.41 ± 0.26	a	0.26 ± 0.02	d	0.80 ± 0.05	de	0.32 ± 0.03	c	0.12 ± 0.00	cde
Ammonium_PHY	269.86 ± 10.91	f	2.19 ± 0.15	abc	0.27 ± 0.02	d	0.88 ± 0.04	bcd	0.36 ± 0.03	a	0.11 ± 0.01	de
BCAA_ AWRI	352.41 ± 29.84	ab	2.12 ± 0.22	abcd	1.32 ± 0.32	a	0.80 ± 0.07	de	2.63 ± 0.27	b	0.24 ± 0.03	a
BCAA_ERG+OLE	302.15 ± 25.55	de	2.20 ± 0.54	abc	1.16 ± 0.10	b	0.82 ± 0.06	cde	2.08 ± 0.21	b	0.17 ± 0.03	b
BCAA_ERG+PHY	320.46 ± 25.57	bcd	1.97 ± 0.24	bcde	1.08 ± 0.06	b	1.04 ± 0.06	ab	1.92 ± 0.17	a	0.15 ± 0.01	bcd
BCAA_PHY	285.28 ± 24.85	ef	1.52 ± 0.11	de	1.34 ± 0.18	a	1.09 ± 0.17	a	2.50 ± 0.41		0.21 ± 0.02	a

Table S 5.3 continued. The impact of anaerobic factor and nitrogen treatments on the production of volatile compounds (mg/L) by VIN13 in synthetic grape must. The data summarizes the average of four replicate fermentations and their standard deviation. Additionally, the letters denote significant differences (95%) between treatments using Fischer LSD

	octanoic acid		**ethyl caproate**		**ethyl caprylate**		**ethyl caprate**	
MS300_ AWRI	2.91 ± 0.52	ef	0.46 ± 0.02	f	0.08 ± 0.02	bc	0.07 ± 0.01	bcd
MS300_ERG+OLE	5.62 ± 0.81	bcde	0.59 ± 0.05	ef	0.12 ± 0.02	bc	0.06 ± 0.01	cd
MS300_ERG+PHY	6.69 ± 0.11	abc	0.76 ± 0.08	bc	0.13 ± 0.01	bc	0.08 ± 0.02	bcd
MS300_PHY	7.19 ± 0.56	ab	0.72 ± 0.04	cd	0.14 ± 0.02	bc	0.09 ± 0.01	ab
Ammonium_ AWRI	3.04 ± 0.31	f	0.40 ± 0.03	g	0.07 ± 0.01	c	0.04 ± 0.01	d
Ammonium_ERG+OLE	3.75 ± 0.82	cde	0.57 ± 0.01	ef	0.12 ± 0.01	bc	0.07 ± 0.01	bcd
Ammonium_ERG+PHY	5.79 ± 0.58	bcde	0.62 ± 0.01	def	0.10 ± 0.01	bc	0.06 ± 0.03	cd
Ammonium_PHY	6.48 ± 0.33	abcd	0.67 ± 0.04	cde	0.14 ± 0.01	bc	0.07 ± 0.01	bcd
BCAA_ AWRI	4.07 ± 0.25	def	0.50 ± 0.02	f	0.12 ± 0.01	bc	0.06 ± 0.01	cd
BCAA_ERG+OLE	7.13 ± 1.29	ab	0.62 ± 0.03	def	0.17 ± 0.02	b	0.11 ± 0.04	ab
BCAA_ERG+PHY	9.28 ± 0.96	a	0.95 ± 0.12	a	0.29 ± 0.01	a	0.09 ± 0.00	abc
BCAA_PHY	8.65 ± 0.52	ab	0.81 ± 0.08	b	0.31 ± 0.05	a	0.10 ± 0.01	a

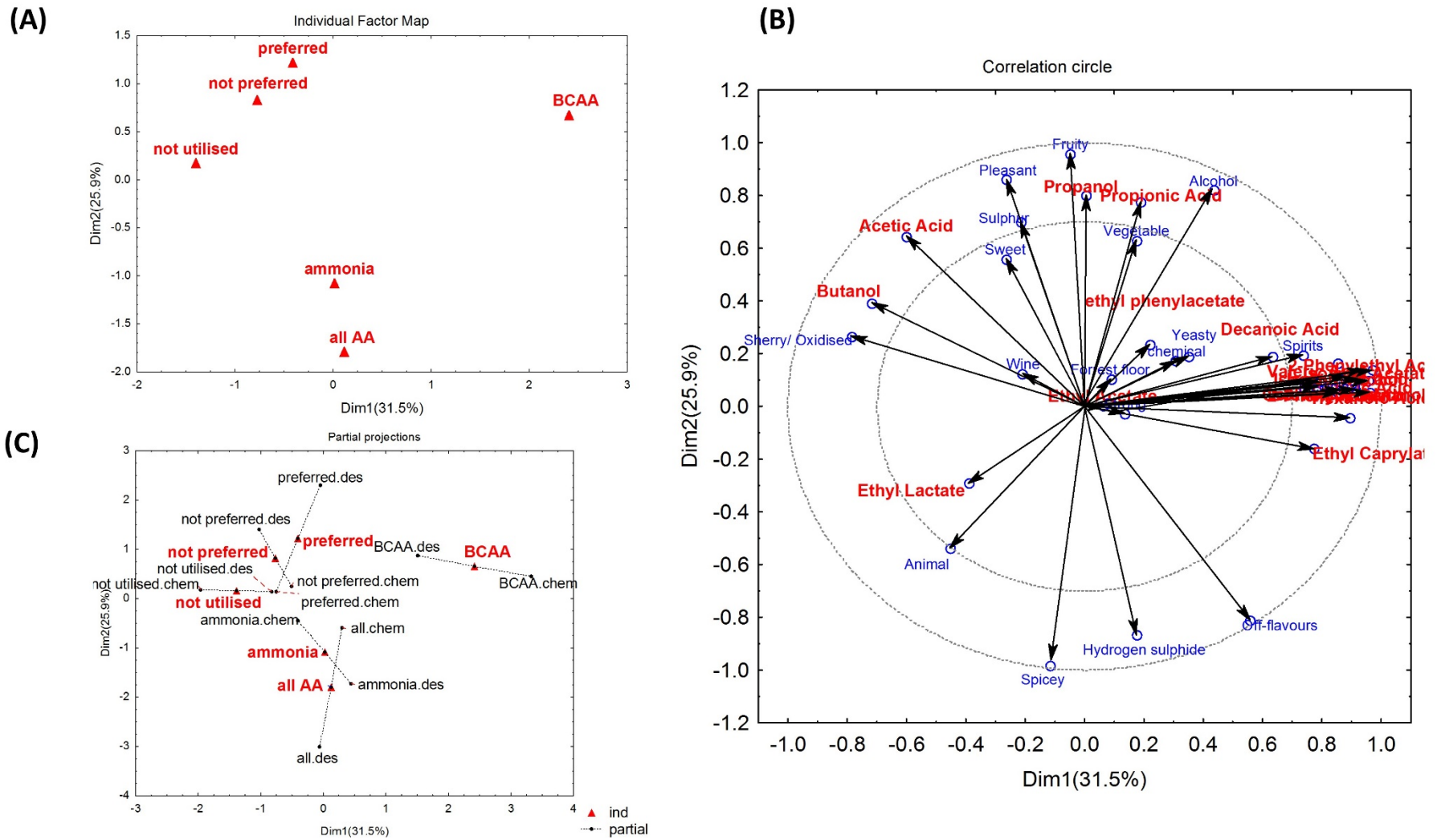


Figure S 5.1. Multiple factor analyses of chemical and sensory data: (A) scores, (B) the correlation circle, and the partial projections (C) of sensory and chemical data. Rv coefficient 0.5

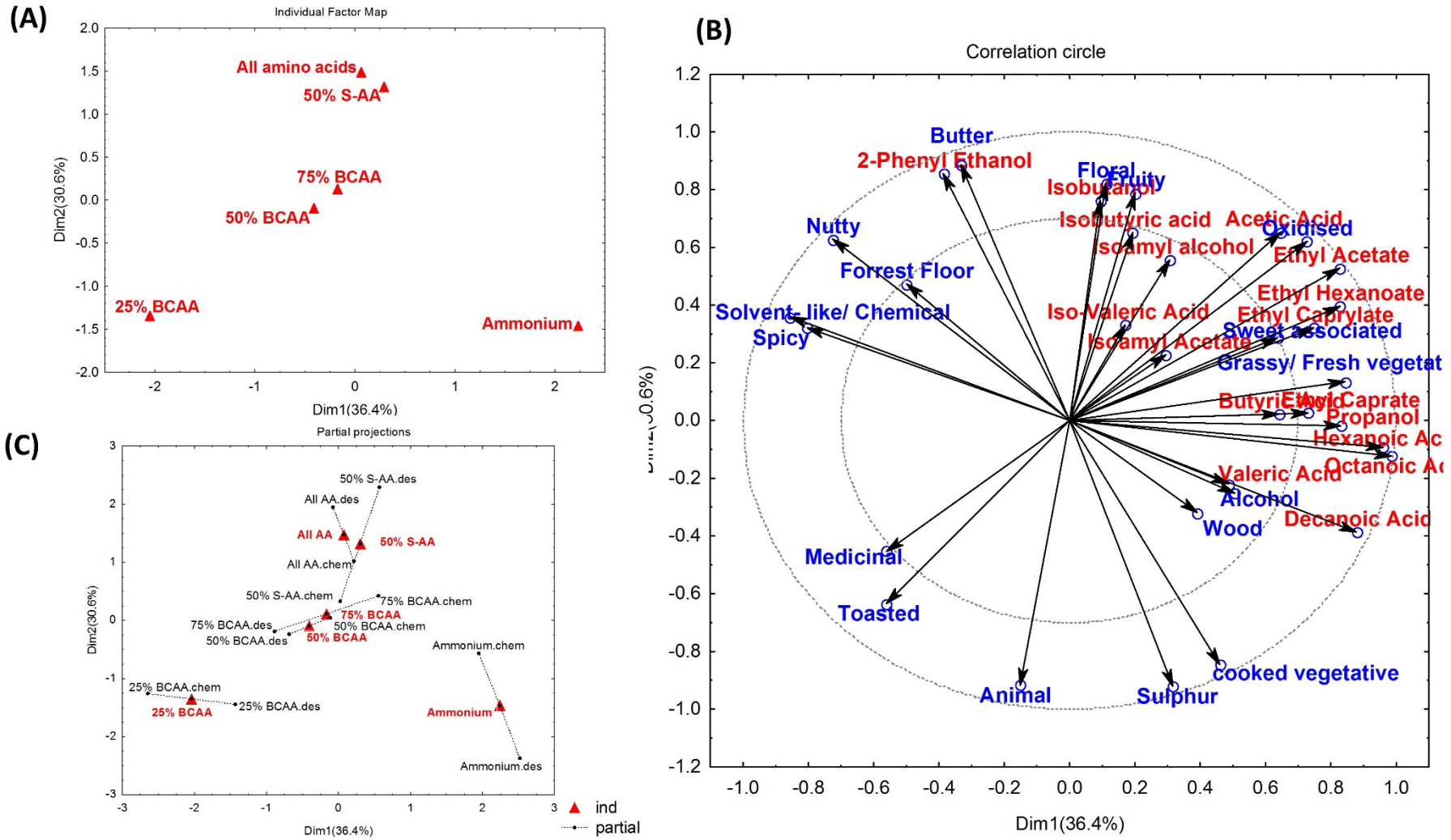


Figure S 5.2. Multiple factor analyses of chemical and sensory data: (A) scores, (B) the correlation circle, and the (C) partial projections of sensory and chemical data. Rv coefficient 0.61

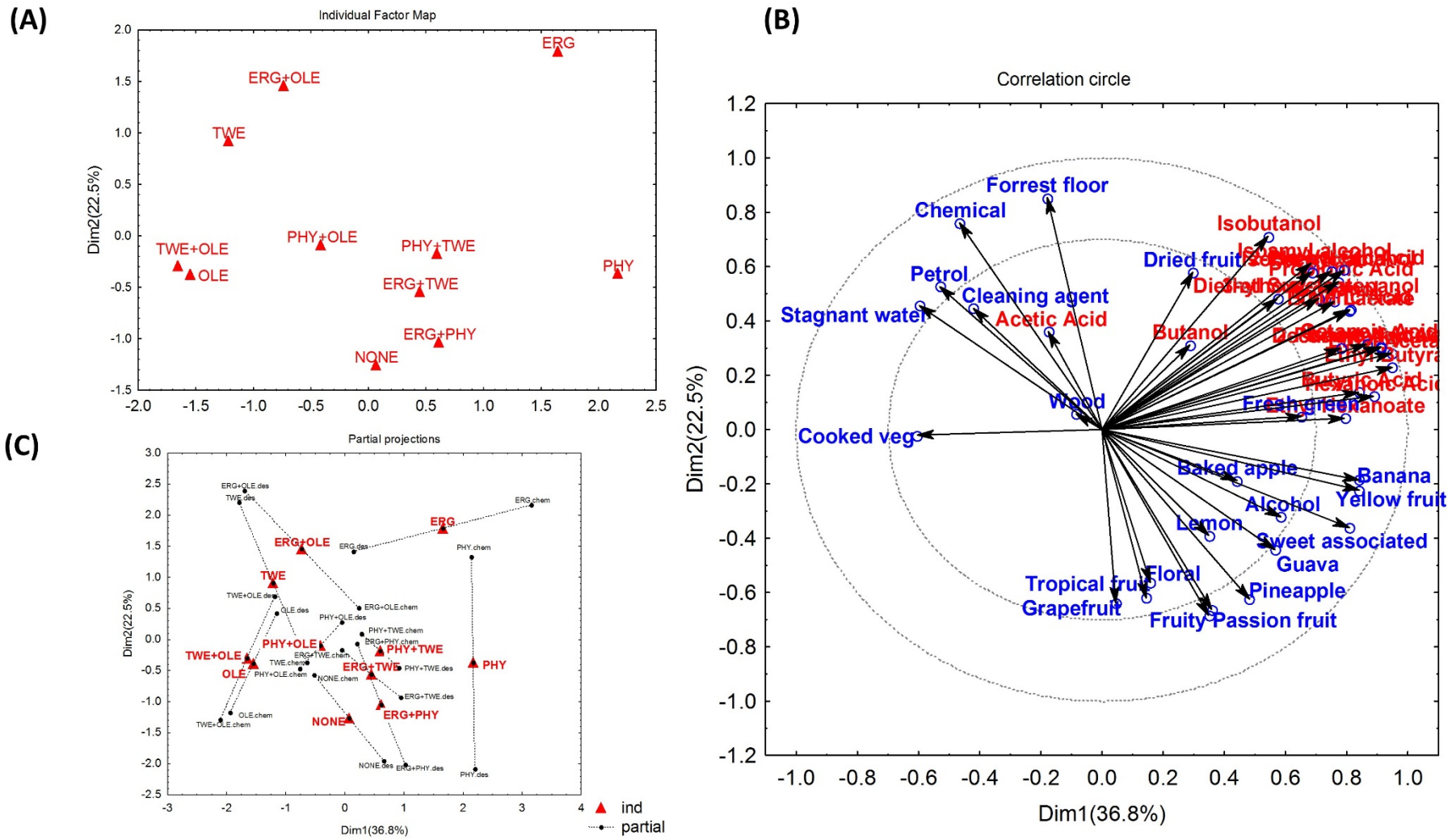


Figure S 5.3. Multiple factor analyses of chemical and sensory data: (A) scores, (B) the correlation circle, and the (C) partial projections of sensory and chemical data. Rv coefficient 0.33

Chapter 6

General discussion and conclusions

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6.1 General discussion and future work

What is wine? We intuitively know it when we see, smell, and taste it, but would give little thought to what has generated this perception. It is well established that much of the volatile vinous character of wine is comprised of important yeast derived metabolites, including ethanol, esters, volatile fatty acids, higher alcohols and acetaldehyde (de-la-Fuente-Blanco et al., 2016). Although these compounds may be present at concentrations below or above their respective perception thresholds, they are perceived as a single entity, and the omission of any one of them, rarely results in a perceptible change in aroma (Escudero *et al.,* 2004; Guth, 1997; Mayr *et al*., 2014; Ferreira *et al*., 2002). This vinous character has been created via chemical reconstitution and has been used to study how higher alcohols alter wine aromas (de-la-Fuente-Blanco *et al.,* 2016 and 2017).

The wine-like concept has however received relatively little attention, as most aroma research is focused on identifying the odorants which are influential on wine aroma (Escudero *et al*., 2004; Mayr *et al*., 2014; Benkwitz *et al*., 2012). This is somewhat surprising, since the fermentation-derived wine-like feature could be considered the foundation onto which varietal and ageing- related aromas are able to build a spectacular or mediocre wine. The principal goal of this project was to evaluate whether a fermentation-derived synthetic product, reflecting only the activity of yeast (synthetic must has no perceivable aromatic character) could result in a wine-like feature, or vinous character. In the final stages of the project, we were indeed able to create a fermented product which was rated as being wine-like. This would, however, need to be verified, by more fully characterising the volatile and non-volatile composition, and creating a reconstituted model of a few of these products, as well as compare it to other wine-like model solutions (de-la-Fuente-Blanco *et al.,* 2016 and 2017). It would also be beneficial to conduct omission tests, to verify the sensory importance of individual components in creating a wine-like aroma. Only once this is achieved, would one be able to propose a comprehensive description of the wine like chemical feature responsible for wine recognition, and the synthetic grape must formulation needed to produce it.

Synthetic grape must is widely used as an alternative to grape must, and has been shown to be an invaluable tool in evaluating the impact of fermentation conditions (Barrajón-Simancas et al., 2011; Rollero *et al*., 2015), yeast strain (Barrajón-Simancas *et al*., 2011; Rollero *et al*., 2015; Rossouw & Bauer, 2016) and yeast nutrition (Ough *et al.,* 1989, Rollero *et al.,* 2015; Rollero *et al.,* 2017; Ferreira *et al.,* 2014) on fermentation and population kinetics.

As nitrogen and anaerobic factors, are essential yeast nutrients for growth in addition to being influential on aroma production (Bell & Henschke, 2005; Ferreira *et al*., 2014; Hernández-Orte et al., 2006; Vilanova *et al.,* 2007; Taylor *et al.,* 1979; Luparia et al., 2004; Ochando *et al.,* 2017; Rollero *et al.,* 2015; Duan *et al.,* 2015; Varela *et al.,*2012; Mauricio *et al.,*1997), they were natural starting points as parameters that may be influential on the wine-like character.

Consequently, the study reports some baseline data regarding the impact of single amino acids on growth kinetics under fermentative conditions, and their corresponding volatile profiles. Additionally, it examines the aroma profiles resulting from increasing concentrations of branched-chain (leucine, isoleucine, valine) and aromatic amino acids (phenylalanine), whilst maintaining the concentration of fermentable nitrogen. The linear relationship between amino acid concentration and the production of their related aroma compounds were evaluated in more complex amino acid mixtures. It was not surprising that this linear relationship was lost, but interestingly, a strong degree of responsiveness to amino acid supplementation remained. This is an important finding considering recent works highlighting the minor contribution of amino acid catabolism to the production of volatile aromas (Rollero *et al.,* 2017; Crépin *et al.,* 2017), as our data clearly illustrates that this is not the case under our experimental conditions. Future work could certainly explore the possibility of creating models suitable for the prediction of aromatic outcomes of complex amino acid mixtures.

In this work, the influence of single sterol or unsaturated fatty acid treatments, as well as in combinations, were compared for their contributions to biomass formation and aroma production. This work illustrates that sterols are more influential on biomass development than unsaturated fatty acids, however both influence volatile aroma profiles. Overall, this highlights the importance of anaerobic factors in winemaking and their relative contribution to biomass formation and aroma production. In this and the amino acid study, GC-MS analyses were also performed, however, in both instances, these untargeted analyses yielded little new information. Consequently, these final studies would have benefited from adjustments made to the untargeted analyses to ensure a greater coverage of the volatile aromas.

To our knowledge, this is the first attempt at performing extensive sensory evaluations on the fermented products of synthetic grape must, in addition to using this sensory data for decision making. Thus, once this verification of our wine-like character has been finalised, with relatively minor alterations in the formulation, one would be able to gather meaningful sensory data from this wine-like matrix. Additionally, this would also be a useful vinous buffer to evaluate other odorants.

This project has provided some new insights into the contribution of anaerobic factors and amino acids into the development of wine aroma, in addition to formulating a more wine-like fermentation media.

6.2 References

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 Chem. 127, 2, 441–446.

Bell, S.J. & Henschke, P. a, 2005. Implications of nitrogen nutrition for grapes, fermentation and wine. Aust. J. Grape Wine Res. 11, 3, 242–295.

Benkwitz, F., Nicolau, L., Lund, C., Beresford, M., Wohlers, M. & Kilmartin, P.A., 2012. Evaluation of key odorants in sauvignon blanc wines using three different methodologies. J. Agric. Food Chem. 60, 25, 6293–6302.

Crépin, L., Truong, N.M., Bloem, A., Sanchez, I., Dequin, S. & Camarasa, C., 2017. Management of Multiple Nitrogen Sources during Wine Fermentation by *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 83, 5, e02617-16.

de-la-Fuente-Blanco, A., Sáenz-Navajas, M.P. & Ferreira, V., 2017. Levels of higher alcohols inducing aroma changes and modulating experts' preferences in wine model solutions. Aust. J. Grape Wine Res. 23, 2, 162–169.

de-la-Fuente-Blanco, A., Sáenz-Navajas, M.P. & Ferreira, V., 2016. On the effects of higher alcohols on red wine aroma. Food Chem. 210, 107–114.

Duan, L., Shi, Y., Jiang, R. & Yang, Q., 2015. Effects of adding unsaturated fatty acids on fatty acid composition of Saccharomyces cerevisiae and major volatile compounds in wine. South African J. Enol. Vitic. 36, 2, 285–295.

Escudero, A., Gogorza, B., Melús, M.A., Ortín, N., Cacho, J. & Ferreira, V., 2004. Characterization of the aroma of a wine from Maccabeo. Key role played by compounds with low odor activity values. J. Agric. Food Chem. 52, 11, 3516–3524.

Ferreira, A.C.S., Monforte, A.R., Teixeira, C.S., Martins, R., Fairbairn, S., Bauer, F.F., 2014. Monitoring alcoholic fermentation: an untargeted approach. J. Agric. Food Chem. 62, 28, 6784–93.

Ferreira, V., Ortin, N., Escudero, A., Lopez, R. & Cacho, J., 2002. Chemical characterization of the aroma of Grenache Rose; wines: Aroma Extract Dilution Analysis, quantitative determination, and sensory reconstitution Studies. J. Agric. Food Chem. 50, 14, 4048–4054.

Guth, H., 1997. Quantitation and Sensory Studies of Character Impact Odorants of Different White Wine Varieties. J. Agric. Food Chem. 45, 8, 3027–3032.

Hernandez-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. Aust. J. Grape Wine Res. 12, 2, 150–160.

Luparia, V., Soubeyrand, V., Berges, T., Julien, A. & Salmon, J.-M., 2004. Assimilation of grape phytosterols by *Saccharomyces cerevisiae* and their impact on enological fermentations. Appl. Microbiol. Biotechnol. 65, 1, 25–32.

Mauricio, J.C., Moreno, J., Zea, L., Ortega, J.M. & Medina, M., 1997. The effects of grape must fermentation conditions on volatile alcohols and esters formed by *Saccharomyces cerevisiae*. J. Sci. Food Agric. 75, 2, 155–160.

Mayr, C.M., Geue, J.P., Holt, H.E., Pearson, W.P., Jeffery, D.W. & Francis, I.L., 2014. Characterization of the key aroma compounds in Shiraz wine by quantitation, aroma reconstitution, and omission studies. J. Agric. Food Chem. 62, 20, 4528–4536.

Ochando, T., Mouret, J.R., Humbert-Goffard, A., Sablayrolles, J.M. & Farines, V., 2017. Impact of initial lipid content and oxygen supply on alcoholic fermentation in champagne-like musts. Food Res. Int. 98, 87–94.

Ough, C., Davenport, M. & Joseph, K., 1989. Effects of certain vitamins on growth and fermentation rate of several commercial active dry Wine yeasts. Am. J. Enol. Vitic. 40, 3, 208–213.

Rollero, S., Bloem, A., Camarasa, C., Sanchez, I., Ortiz-Julien, A., Sablayrolles, J.M., Dequin, S. & Mouret, J.R., 2015. Combined effects of nutrients and temperature on the production of fermentative aromas by *Saccharomyces cerevisiae* during wine fermentation. Appl. Microbiol. Biotechnol. 99, 5, 2291–2304.

Rollero, S., Mouret, J.-R.R., Bloem, A., Sanchez, I., Ortiz-Julien, A., Sablayrolles, J.-M.M., Dequin, S. & Camarasa, C., 2017. Quantitative 13C-isotope labelling-based analysis to elucidate the influence of environmental parameters on the production of fermentative aromas during wine fermentation. Microb. Biotechnol. 10, 6, 1649–1662.

Rossouw, D. & Bauer, F.F., 2016. Exploring the phenotypic space of non-*Saccharomyces* wine yeast biodiversity. Food Microbiol. 55, 32–46.

Taylor, G.T., Thurston, P. a. & Kirsop, B.H., 1979. The influence of lipids derived from malt spent grains on yeast metabolism and fermentation. J. Inst. Brew. 85, 4, 219–227.

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

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. Appl. Microbiol. Biotechnol. 77, 1, 145–157.