PROTEOMIC AND METABOLOMIC CHARACTERISATION OF NOVEL WINE YEASTS: TOWARDS THE EVALUATION AND IMPROVEMENTS OF THEIR ABILITY TO PRODUCE AROMATIC SAUVIGNON BLANC WINES

By

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KEYWORDS

Hybrid wine yeasts

MALDI-TOF MS Biotyping

Metabolome

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Proteome

Sauvignon blanc

Tropical fruit aroma

Volatile acidity

Volatile thiols

ABSTRACT

Wine yeast *Saccharomyces cerevisiae* and wild yeasts *e.g. Torulaspora delbrueckii* forms an integral part of wine production by converting relatively 'neutral' flavoured Sauvignon blanc grape must into varietal aromatic wines. Yeast derived and mediated metabolites which contribute to Sauvignon blanc wine aroma and flavour, are regulated by yeast proteins (enzymes) that are differentially expressed during the course of fermentation. Inoculation with an appropriate yeast strain can, therefore, increase commercial wines sales as resultant wines will have sought-after aromas and flavours. Likewise, inoculation with the incorrect strain can have an undesirable effect on wine quality. Subsequently, the development of yeasts for the production of varietal aromatic Sauvignon blanc with lower volatile acidity (VA) was also identified as a South African industry priority. Although genetic modification can address this, the use of genetically modified organisms (GMO) is illegal. The South African wine industry is also too dependent on the highly sensitive European market for exports, which are largely against GM food products. Therefore, classical mating was deployed to breed *S. cerevisiae* intra-genus and *S. cerevisiae*/*T. delbrueckii* inter-genus hybrids with desired traits, whilst maintaining the green image of wine production. Subsequently, a trial was undertaken during 2013 to evaluate intra-genus hybrids for the production of laboratory-scale Sauvignon blanc wine. Recommended commercial 'thiol-releasing' wine yeast (TRWY) were included as references. Most intra-genus hybrids produced wines with lower VA levels than wines produced with TRWY. Some of these intra-genus hybrids produced wines with even less acetic acid, but more enhanced tropical fruit aroma compared to wines produced by all TRWY references. Contour clamped homogeneous electric field (CHEF) DNA karyotyping and matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF/MS) biotyping was successfully deployed to accurately differentiate wine yeast strains in this study. Small-scale winemaking trials during 2014 to evaluate protein expression and metabolite release of promising *S. cerevisiae* intra-genus hybrids identified during lab-scale vinification trials were initiated. Promising intra-genus hybrids were identified as they showed enhanced thiol-releasing, specifically 3-mercaptohexan-1-ol (3MH), and lower volatile acidity (VA) formation during the production of

Sauvignon blanc wines compared to commercial TRWY references. It is noteworthy that, intra-genus hybrid NH 56 produced wines with the second highest 3MH levels after intra-genus hybrid NH 84, and lowest acetic acid of all strains included in this study. This yeast was also the only strain to have down-regulated, amongst others, dehydrogenases previously reported to be involved in the production of acetic acid. Therefore, dehydrogenases are potential biomarkers for VA formation. Furthermore, differences in protein expression were reflected in the variation of metabolite release by different strains, thereby complementing notion that proteins are the final effectors for metabolite release. Sequential inoculation of grape must using *T. delbrueckii* and *S. cerevisiae* strains was previously reported to result in aromatic wines with lower VA levels, similar to results generated in this study. Additionally, a *S. cerevisiae*/*T. delbrueckii* inter-genus hybrid previously produced white wine with enhanced aroma. Therefore, intra-genus and inter-genus hybrids were trialled for the production of varietal aromatic small-scale Sauvignon blanc wines with lower VA during 2015. The inter-genus hybrid, NH 07/1 produced wine with a more positive association with the volatile thiol 3MHA than wines produced with commercial TRWY, Zymaflore X5 and Zymaflore VL3, and the wine also had a negative association with VA and acetic acid. Three intra-genus hybrids, NH 56, NH 57 and NH 88 produced wines with a negative association with VA and acetic acid, whilst having a positive association with tropical fruit aroma. These wines also had a stronger association with 3MH than all commercial TRWY included in this study. Hybrid NH 07/1 and Zymaflore VL3 also over-expressed the lactoylglutathione lyase protein responsible for the release of the volatile thiol, 4-mercapto-4-methyl-pentan-2-one (4MMP) by cleaving its carbon-sulfur bonds. Therefore, lactoylglutathione lyase is a potential biomarker for 3MH-release, as this thiol also contains a carbon-sulfur bond. Dehydrogenase proteins might also be useful biomarkers for VA formation by fermenting wine yeasts. Three intra- and one inter-genus hybrids with the abiliy to produce aromatic Sauvignon blanc wines with lower VA compared to commercial TRWY references were identified.

DECLARATION

I declare that “Proteomic and Metabolomic Characterisation of Novel Wine Yeasts: Towards the Evaluation and Improvements of their Ability to Produce Aromatic Sauvignon blanc Wines” is my own work and that it has not been submitted for any degree or examination at any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Signature : _____ Date : 15-03-2018

R. S. Hart

DEDICATED TO MY LOVING WIFE, MONIQUE

HART AND SONS, ZACHARY & MATEO HART

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LIST OF ABBREVIATIONS

2D-PAGE	Two-dimensional poly-acrylamide gel electrophoresis
3MH	3-mercaptohexan-1-ol
3MHA	3-mercaptohexyl acetate
4MMP	4-mercapto-4-methylpentan-2-one
ANOVA	Analysis of variance
CAF	Central Analytical Facility
CFAA	Cellular fatty acids analyses
CHEF	Contour clamped homogeneous electric field
DAP	Diammonium phosphate
FAN	Free amino nitrogen
FIGE	Field inversion gel electrophoresis
FLPH	Hybrids with ability to produce wines with floral aroma
FTIR	Fourier transform infra-red
GC-FID	Gas chromatography coupled to a flame ionisation detector
GC-MS	Gas chromatography coupled with mass spectrometry
GC-MS/MS	GC coupled to tandem mass spectrometry
GMO	Genetically modified organism
iBMP	2-isobutyl-3-methoxypyrazine
iTRAQ	Isobaric tags for relative and absolute quantitation
ITS	Internal transcribed spacers
LC-MS/MS)	Liquid chromatography-tandem mass spectrometry
LVPH	Low volatile acidity producing hybrids
LTQ	Linear trap quadrupole
MALDI-TOF/MS	Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry
MMTS	Methane methylthiosulphonate
NAPRSU	National Agricultural Proteomics Research & Services Unit
NIR	Near-infrared
OFAGE	Orthogonal-field-alternation gel electrophoresis
PACE	Programmable autonomously controlled gel electrophoresis
PANTHER	Protein ANalysis THrough Evolutionary Relationships
PCA	Principal component analysis
PFGE	Pulsed-field gel electrophoresis

PHOFE	Pulsed homogeneous orthogonal field gel electrophoresis
REG	Rotating gel electrophoresis
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SO_2	Sulphur dioxide
SPE	Solid-phase extraction
TA	Total acidity
TAFE	Transverse alternating field electrophoresis
TBE	Tris, boric acid and EDTA
TEAB	Triethylamonium bicarbonate
TFPH	Hybrids with ability to produce wines with tropical fruit aroma
TFPP	Parents with ability to produce wines with tropical fruit aroma
TRWY	Thiol-releasing wine yeasts
VA	Volatile acidity
YAN	Yeast assimilable nitrogen
YPD	Yeast extract, peptone and dextrose

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Chapter 1

General introduction and aims

Chapter 1: General introduction and aims

1.1. INTRODUCTION

South African enologists are continually striving to increase wine quality in an ever-increasing competitive market (Hart & Jolly, 2008; Moore *et al*., 2008; Alonso *et al*., 2013). The selection and development of new yeast is directly linked to improvement of wine quality as the wine yeast strain is responsible for fermenting 'neutral' grape must (juice) to wine with enhanced aroma and flavour (Swiegers *et al.,* 2006b; 2009; King, 2010; King *et al.,* 2011; Dennis *et al.,* 2012; Van Breda *et al.,* 2013; Jolly *et al.,* 2014). A criterion for yeast improvement identified by industry involves yeast specifically targeted for the production of varietal white wine. The full aroma potential of white wine, especially Sauvignon blanc, is often not achived due to the inability of yeasts to release bound aroma-inactive non-volatile thiols (metabolites) (Swiegers *et al.,* 2006b; 2007b; King, 2010; King *et al.,* 2011). As a result, a large source of aroma and flavour associated with volatile thiols *e.g.* 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA), primarily responsible for passion fruit, tropical fruit and citrus nuances originating from Sauvignon blanc grapes remains unexpressed. Subsequently, the use of commercial 'thiol-releasing' wine yeasts (TRWY) for the production of aromatic Sauvignon blanc increased in popularity. However, anecdotal evidence suggests that some TRWY might be associated with formation of undesirable metabolites *e.g.* volatile acidity (VA), which could have a negative commercial impact. Excessive VA in wine is perceived as vinegar-like off-flavours (Du Toit and Pretorius, 2000; Swiegers *et al.,* 2005; Ugliano *et al.,* 2007; Vilela-Moura *et al.,* 2011).

The South African wine industry is an important contributor to the economy as wine forms an integral part of exports from the agricultural sector (Ponte & Ewert, 2009). The wine industry is estimated to contribute more than R36 billion to the local Gross Domestic Product (GDP), whilst offering employment to more than 300 000 people *e.g.* farm workers, technical assistants, researcher etc.

(SAWIS, 2015). International wine production exceeds consumption thereof by approximately 15%, due to competition from other alcoholic beverages (Pretorius, 2016). However, international wine sales is estimated to be R1,3 trillion per year, which highlights the commercial importance of the wine industry. Therefore, the industry caused by *e.g.* inferior wine, can have a large snowball effect on income, job stability and the image of the South African wine industry.

1.2. RESEARCH AIMS AND OBJECTIVES

This study was undertaken to evaluate a selection of intra-genus *Saccharomyces cerevisiae* hybrid yeasts and commercial reference thiol-releasing wine yeasts (TRWY) for the improvement of Sauvignon blanc wine organoleptic quality with regard to tropical fruit aroma and lower VA formation. A second objective was to investigate wine yeast protein expression and whether regulated proteins correlate with metabolites released and/or produced by different yeast strains during fermentation. A third objective of this study were to breed *S. cerevisiae/T. delbrueckii* inter-genus hybrids using classical mating, characterise and evaluate these inter-genus hybrids for their fermentation potential, thiol-releasing abilities and low VA formation during the production of Sauvignon blanc wines.

Wine yeast regulated proteins and aroma compounds, especially volatile thiols *viz*. 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexylacetate (3MHA) as well as volatile acidity *viz*. acetic acid present at the end of fermentation and their association with final wine aroma and flavour was also investigated. It is envisioned that potential protein biomarkers associated with aroma-enhancing metabolites and VA will be identified. These biomarkers could be used in rapid screening of wine yeasts to identify promising yeast strains with sought after properties and, therefore, eliminate costly wine production experiments that have to be repeated over numerous vintages before a yeast will be considered for semi-commercial winemaking trials by yeast manufacturers.

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Chapter 2

Literature review

Chemical and organoleptic evaluation of Sauvignon blanc wine in conjunction with metabolomic and proteomic approaches towards enhancing varietal aroma

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Chapter 2: Chemical and organoleptic evaluation of Sauvignon blanc wine in conjunction with metabolomic and proteomic approaches towards enhancing varietal aroma

2.1. ABSTRACT

The wine yeast *Saccharomyces cerevisiae* forms an integral part of wine production by converting relatively 'neutral' flavoured grape juice (must) into varietal aromatic wines. Additionally, non-*Saccharomyces* yeast strains can also be used in co-inoculation strategies with *S. cerevisiae* for the production of wines with more complexity. Yeast strains, to varying extents produce and/or mediate the release of a range of key metabolites, which in turn contribute to enhanced wine aroma and flavour of final wine. These metabolites, amongst others, volatile thiols can impart desired aroma notes to Sauvignon blanc, but are dependent on yeast-expressed enzymes (proteins) during fermentation. Inoculation with an appropriate yeast will therefore lead to more commercial wines sales due to resultant wines with sought-after aroma and flavour. Likewise, inoculation with the incorrect yeast can lead to less desirable wines that will have a negative effect on sales. It is also important that the inoculated yeast strain dominates the fermentation. This should be monitored throughout the fermentation process by having quality control measures in place. DNA karyotyping by contour clamped homogeneous electric field gel electrophoreses (CHEF) and matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) biotyping was shown to be reliable in this regard, as both methods could accurately differentiate yeast strains. Standard chemical and descriptive sensory analyses of wine also served as evaluation and characterisation tools of yeast starter cultures. Metabolomic and proteomic approaches using gas chromatography (GC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) also proved to be instrumental yeast evaluation tools. The association between final wine chemical and sensory parameters as well as wine

yeast expressed proteins and released metabolites, and their effect on final wine organoleptic quality will be discussed.

2.2. INTRODUCTION

The production of varietal wines with enhanced aromatic characteristics from relatively 'neutral' flavoured grape juice (must) can be achieved by deploying the wine yeast *Saccharomyces cerevisiae* (Swiegers *et al.,* 2006a; 2009; King, 2010; King *et al.,* 2011; Dennis *et al.,* 2012; Erten *et al.,* 2016). Final wine aroma and flavour can, subsequently be attributed to the yeast starter culture's metabolism during alcoholic fermentation (Nedović *et al.,* 2015). Wine yeasts are, therefore, important for the production of aromatic white wines from grapes of non-aromatic grape cultivars such as Sauvignon blanc (Lambrechts & Pretorius, 2000; Cadière *et al.,* 2012). However, the onus rests with the winemaker to make knowledgeable decisions as to what wine yeast starter culture to use for wine production in order to harvest the maximum potential of wine grapes (Pretorius, 2016).

Sauvignon blanc grapes gained popularity in the Loire Valley and Bordeaux vineyards in France during the early 18th century (Bowers & Meredith, 1997). This cultivar continues to grow in popularity, especially in new world winemaking countries *e.g.* South Afica, Chile, New Zealand, Australia and the United States renowed for Sauvignon blanc production (Cozzolino *et al*., 2011; Benkwitz *et al*., 2012). Sauvignon blanc is estimated to be the second most planted white cultivar in the world after Chardonnay . Internationally, South Africa is only surpassed by France and New Zealand in terms of total Saugvignon blanc vines planted (Sweet, 2010). Although nationally, Chenin Blanc still remains the most planted cultivar, local viticulturalists are producing higher proportions of higher quality Sauvignon blanc grapes (Ponte & Ewert, 2009). Resultant Sauvignon blanc grapes are used to produce wines with diverse sensory profiles or styles *e.g.* vegetative/herbaceous aroma nuances (*e.g.* grassy, green bellpeppers), especially when grapes originate from cooler climatic regions (Marais, 1994). Other styles vary from floral (*e.g.* orange blossom, elderflower) to tropical aroma notes (*e.g.* passion

fruit, guava), especially when grapes orginates from warmer climatic regions (Swiegers *et al*., 2009; Van Wyngaard, 2013; Von Mollendorf, 2013). Another distinguished Sauvignon blanc style that is synonymous with Bordeaux, France and California, United States known as Pouilly-Fumé and Fumé Blanc due to pronounced "smoky" aroma nuances following barrel fermentation (Tominaga *et al*., 2003; Sweet, 2010). Aforementioned complexity of Sauvignon blanc is key to its worldwide appeal to viticulturists, enologist and consumers.

2.3. YEAST USED FOR SAUVIGNON BLANC WINE PRODUCTION

Wine yeasts are instrumental in the amplification of Sauvignon blanc grape aroma during fermentation (Swiegers *et al*., 2005; Murat *et al*., 2009). However, wine yeasts vary in their ability to develop the full aroma potential of Sauvignon blanc, due to differences in their ability to release volatile aromatic metabolites from their bound aroma-inactive precursors, an important contributor to the tropical fruit and floral aromas expected in these wines (Roland, *et al.,* 2011; Pinu *et al.,* 2012; Van Wyngaard, 2013; Von Mollendorf, 2013). However, the presence of *S. cerevisiae* on wine grapes is negligible compared to other ubiquitous non-*Saccharomyces* genera *e.g. Cryptococcus* spp., *Rhodotorula* spp. *Sporobolomyces* spp., *Candida* spp., *Hanseniaspora* spp., *Metschnikowia* spp., *Pichia* spp., *Zygosaccharomyces* spp., and *Torulaspora* spp. (Fleet, 2003; Jolly *et al.,* 2006; Barata *et al.,* 2012; Cray *et al.,* 2013; Jolly *et al.,* 2014). This observation, therefore, highlights the vigour required by *S. cerevisiae* to be the dominant yeast strain at the end of fermentation, and so be responsible for the organoleptic quality and profile of the final wine (Carrau *et al.,* 2008; Álvarez-Pérez *et al.,* 2014). As the organoleptic profile of wines produced by means of spontaneous fermentation varies between fermentations, commercial *S. cerevisiae* starter cultures are used to obtain a more consistent product. As mentioned above, spontaneous fermentations have shortcomings, as the chemical and sensory profiles of resultant wines are unpredictable, and in some instances less preferred than wines produced solely with *S. cerevisiae* strains (Sturm *et al.,* 2006; Navarrete-Bolaños, 2012; Velázquez *et*

al., 2015). Spontaneous fermentations also open the doors for spoilage organisms (*e.g. Brettanomyces*) to proliferate, resulting in wines with undesirable metabolites *e.g.* acetic acid (Romano *et al*., 2008) and 4-ethylphenol (Petrozziello *et al*., 2014), which impart 'vinegar-like' and 'horse sweat' odours, respectively.

Non-*Saccharomyces* spp. nonetheless are still being explored for production of wine with more complexity (Pretorius, 2000; Fleet, 2003; Ocón *et al.,* 2010; Jolly *et al.,* 2014). However, the use of non-*Saccharomyces* commercial starter cultures still requires co-fermentation by an *S. cerevisiae* strain to complete fermentation, as non-*Saccharomyces* are not able to completely ferment grape must due to lower alcohol tolerance. However, Jolly *et al.* (2003) and Van Breda *et al.* (2013) reported that some non-*Saccharomyces* spp. produced wine with similar chemical and sensory quality compared to wines produced with a *S. cerevisiae* strains. Non-*Saccharomyces* such as *Torulaspora delbrueckii* yeast starter cultures have also became commercially available to enhance wine complexity.

The use of a *T. delbrueckii* starter culture was shown to be advantageous as it resulted in wines with lower volatile acidity (VA) levels, and thereby enhanced final wine organoleptic quality (Bely *et al*., 2008; Renault *et al*., 2009). Another *T. delbrueckii* strain was reported to produce wine with enhanced varietal aromas as a single inoculum or in conjunction with *S. cerevisiae* (Van Breda *et al.,* 2013; Belda *et al.,* 2015; Renault *et al.,* 2016). Therefore, *T. delbrueckii* would also be a good candidate to be utilised in a hybrid breeding program to develop new yeast strains towards the production aromatic white wines, especially Sauvignon blanc with lower VA, a yeast development criterion identified by industry (M Fundira, Personal communication, 2015).

Wine yeasts produce a whole range of metabolites during fermentation, referred to as its metabolome, which include monosaccharide sugars, organic acids, fatty acids, amino acids, volatile thiols (imparts tropical fruitiness in Sauvignon blanc and Chenin Blanc), esters (imparts fruitiness), and alcohol which all contribute to the organoleptic characteristics of the wine (Carrau *et al.,* 2008;

Chambers *et al.,* 2009; Von Mollendorf, 2013; Rollero *et al.,* 2016; Varela, 2016). Wine is, therefore, the 'metabolic footprint' of the yeast strain that carried out the fermentation (Howell *et al.,* 2006; Mapelli *et al.,* 2008). Wine yeasts, however, can also produce undesirable metabolites *e.g.* acetic acid and/or VA, which impart unpleasant off-flavours as previously mentioned (Vilela-Moura *et al.,* 2011; Luo *et al.,* 2013). Subsequently, wine organoleptic quality will be negatively affected.

Yeast derived and mediated metabolites are dependent on enzymes (proteins) expressed by yeasts during fermentation (De Klerk, 2009; Holt *et al.,* 2011; Roncoroni *et al.,* 2011). Anecdotal evidence suggests that different yeast strains express proteins differentially despite being in the same growth medium. Therefore, different yeast strains will produce wines with different sensory properties, since expressed proteins will affect metabolite release responsible for final wine organoleptic quality (Roncoroni *et al.,* 2011; Juega *et al.,* 2012; Moreno-García *et al.,* 2015).

2.4. HYBRID YEAST AND THEIR ROLE IN WINE PRODUCTION

The importance of wine yeasts for the production of quality wines were neglected until the late 1980's, as improvement of wine quality focused exclusively on viticultural practises (Pretorius & van der Westhuizen 1991). As a result, the development of novel yeast strains was not a priority. Paradigm shifts were, however observed as more research showed the importance of yeast starter cultures to improve wine quality (Pretorius, 2000; Mateo *et al.,* 2001; Bartowsky, 2009; Meersman, 2015; Pretorius, 2016). Traditionally, wines were the product of spontaneous fermentations due to the natural micro-flora, but microbiological advantages during the late 1800's saw the use of pure *S. cerevisiae* wine yeast starter cultures to improve wine organoleptic quality and reproducibility thereof (Pretorius & van der Westhuizen 1991). These starter cultures were natural isolates originating from vineyard soil and vines (Martini, 1993). However, the use of single inoculums for wine production was reported to produce wines lacking complexity and mouthfell (Bellon *et al*., 2012). This dilemma was resolved by deploying non-genetic modification tools to improve underperformances of pure

starter cultures. Crossbreeding (classical mating) was shown to be a powerful tool that enabled biotechnologists to select desirable wine yeast characteristics, whilst eliminating undesirable characteristics of natural isolates in resultant intra-genus *S. cerevisiae* hybrid yeasts (Van der Westhuizen & Pretorius, 1992; Bellon *et al*., 2011; Cordente *et al*., 2012). A good example was the breeding of intra-genus *S. cerevisiae* hybrids with lower hydrogen suphide (imparts rotten egg odours) production (Bizaj *et al*., 2012). One of the first hybrid yeasts to be bred and commercialised was strain VIN 13 (Van der Westhuizen, 1990), and was soon followed by other yeasts as yeast producers realised the vigour and advantages of hybridisation.

Hybrids have since then developed into innovative tools for improved wine quality. Intra-genus hybridisation was followed by inter-genus hybridisation, as a *S. cerevisiae* and non-*Saccharomyces* hybrid was reported to positively effect wine aroma and flavour due to its metabolic activity (Santos *et al.,* 2008). Indications, therefore, are that *T. delbrueckii* in conjunction with *S. cerevisiae* have a commercial role to play in the production of wines. Overall, inter-genus hybrids potentially can produce aromatic wines with more complexity, whilst showing the robustness associated with the wine yeast *S. cerevisae* (Bellon, 2013).

WINE YEAST CHARACTERISATION

The use of commercial wine yeast starter cultures, *S. cerevisiae* in particular, have revolutionised earlier methods of winemaking by allowing winemakers to consistently produce wines with comparable organoleptic properties (Mateo *et al.,* 2001; Bartowsky, 2009; Meersman, 2015; Pretorius, 2016). The wine yeast *S. cerevisiae,* whether a natural isolate or a hybrid has an unsurpassed fermentation ability in comparison to yeasts from other genera due to, amongst others, higher alcohol tolerance (Pretorius, 2000). Yeast starter cultures commercially available undergo thorough evaluation that involves fermentation trials and classical and molecular characterisation prior to release (Garcia *et al.,* 2012; Bonciani, 2016). Currently enologists and wine technologists have a large variety of molecular tools at their disposal for the rapid characterisation of food related microbes, amongst others, wine yeast strains (Ivey & Phister, 2011). This enables them to follow their presence during fermentation and their contribution to the wine.

Wine organoleptic quality varies based on the yeast starter culture used to conduct the fermentation (Capece *et al.,* 2010; Sharma *et al.,* 2012; Usbeck *et al.,* 2014). Furthermore, some wine microorganisms are implicated in spoilage of wine, resulting in financial loss (Loureiro and Malfeito-Ferreira, 2003; Bartowsky, 2009). Differentiation of yeast strains during winemaking, therefore, is of utmost importance (Sharma *et al.,* 2012; Usbeck *et al.,* 2014). Inoculation with the incorrect starter culture can result in wine with undesirable chemical parameters and sensory flavours, which in turn will have undesirable financial implications. Similarly, inoculation with the "correct" starter culture resulting in wines with sought-after aroma and flavour is always lucrative, since more than a million litres of wines are annually produced and sold on a commercial-scale (South African Wine Industry Information and Systems [SAWIS], 2015). Yeast manufacturers also benefit from increased commercial sales of yeast starter cultures, especially those exhibiting characteristics of producing sought-after varietal aromatic wines (M. Fundira, Personal communication, 2015). Therefore, a reliable and fast differentiation method is a useful tool to ensure the correct identification of microorganisms during the production of varietal wines (Chovanová *et al.,* 2011; Panda *et al.,* 2015).

Chemical and sensory analyses of wine are routinely conducted, and fundamentally serve as a means of evaluating and characterising experimental yeast strains used for wine production (Hart & Jolly, 2008; Ezeama & Ebia, 2015). Coupled to this are metabolomic and proteomic characterisation, as some yeast expressed proteins during fermentation are responsible for the release and mediation of sought-after and undesirable metabolites, which in turn have an effect on wine organoleptic quality (De Klerk, 2009; Holt *et al.,* 2011; Roncoroni *et al.,* 2011; Juega *et al.,* 2012; Moreno-García *et al.,* 2015). Aforesaid yeast evaluation and characterisation tools will be discussed further.

2.3.1. Contour clamped homogeneous electric field (CHEF) DNA karyotyping

A popular molecular-based tool to characterise wine yeast, namely pulsed-field gel electrophoresis (PFGE) has been used over many decades for the evaluation of experimental yeast strains (Van der Westhuizen and Pretorius, 1992; Hoff, 2012; Van Breda *et al*., 2013). Contour clamped homogeneous electric field (CHEF) DNA karyotyping, a variation of PFGE, is a reliable technique to distinguish between yeast strains based on chromosomal DNA karyotypes (Hoff, 2012; Choi, Woo, 2013). The CHEF system comprises of electrodes that are hexagonally arranged (Carle, Olson, 1985; Basim and Basim, 2001; Parizad *et al.,* 2016). The DNA sample is placed in an agarose gel and separated by sequentially alternating the electric field between electrodes (Figure 2.1) based on pre-programmed pulse times (McEllistrem *et al.,* 2000). Subsequently, smaller and larger DNA chromosomes are efficiently separated as the latter requires more time to re-orientate and migrate every time the electric field is alternated, whilst the reverse applies to the former.

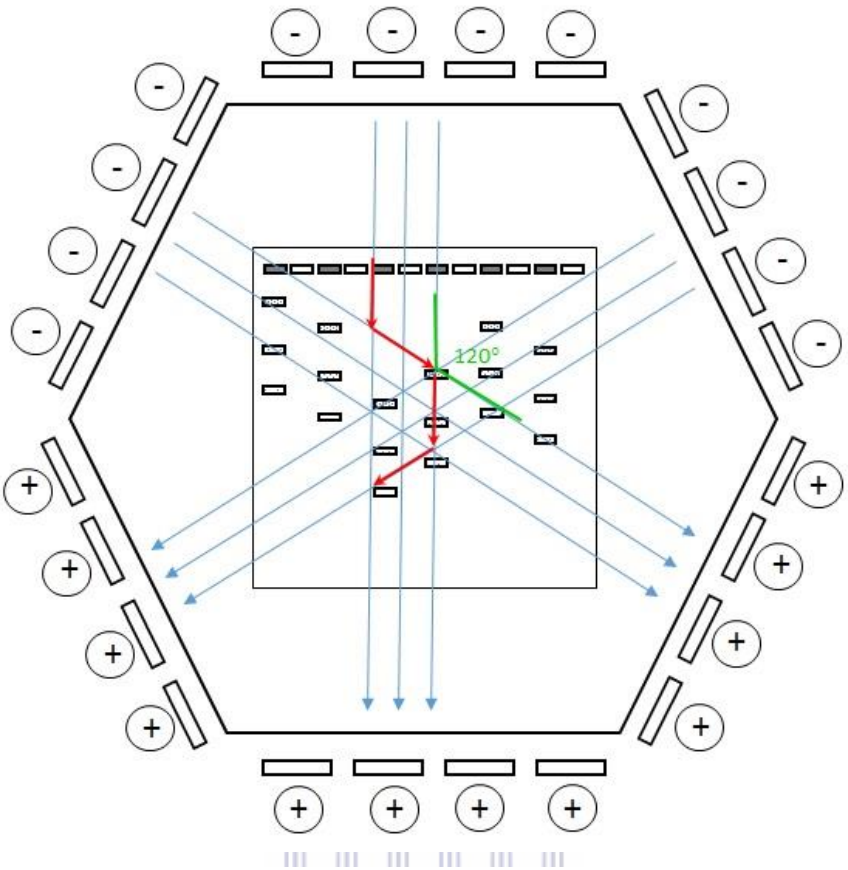


FIGURE 2.1: Schematic depiction of principle behind pulsed-field gel electrophoresis (PFGE)/contour clamped homogeneous electric field (CHEF) used to separate large chromosomal DNA bands by alternating the direction of the electric field at an angle of 120° that is regulated by programmed pulse times.

Other gel electrophoresis-based yeast differentiation tools that involve alteration of the electric field during separation of DNA include orthogonal-field-alternation gel electrophoresis (OFAGE), field inversion gel electrophoresis (FIGE) and transverse alternating field electrophoresis (TAFE), programmable autonomously controlled gel electrophoresis (PACE), pulsed homogeneous orthogonal field gel electrophoresis (PHOFE) and rotating gel electrophoresis (REG) (de Jonge *et al.,* 1986; Pretorius, Van der Westhuizen, 1991; Van der Westhuizen, Pretorius, 1992). Amongst these, CHEF DNA karyotyping has been widely and successfully deployed to differentiate wine yeasts to ensure that the correct yeast strain was used to inoculate and complete the fermentation (Maoura *et al.,* 2005; Hage and Houseley, 2013). Subsequently, a positive association between final wine organoleptic quality and yeast starter culture/s could be established. A drawback of CHEF DNA karyotyping is that

it is a lengthy process, requiring up to eight days to yield the identity of a pure culture (Van Breda *et al.,* 2013).

2.3.2. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR), which entails the amplification of internal transcribed spacers (ITS) is a faster molecular characterisation tool compared CHEF DNA karyotyping (Hoff, 2012; Colabella & Libkind, 2016). However, PCR was repeatedly shown to have a limitation in differentiating closely related yeasts strains as rDNA amplicons every so often have similar sizes that can't be resolved by gel electrophoresis (Pincus *et al.,* 2007; Hierro *et al.,* 2004). This predicament is circumvented by subjecting amplicons to expensive sequencing procedures (Chen *et al.,* 2004; Hulin and Wheals, 2014). Contour clamped homogeneous electric field DNA karyotyping does not have this limitation and it can be envisioned that the longer time required for PCR and sequencing will be similar to that required for CHEF DNA karyotyping.

2.3.3. Phenotypic and biochemical tests

Conventional microbiology *e.g.* sub-culturing, phenotypic characterisation, biochemical tests etc. was shown to be a relative cheaper alternative compared to CHEF DNA karyotyping (Van Breda *et al.,* 2013). However, conventional microbiology also have limitations, as it is time-consuming and labour intensive. Moreover, some of those techniques are also susceptible to misidentification (Ciardo *et al.,* 2006). A commonly used biochemical characterisation approach, namely ID 32 C AUX system (BioMérieux, South Africa) in conjunction with apiweb™ identification software was previously shown to differentiate yeasts on a genus level (Pincus *et al.,* 2007; Van Breda *et al.,* 2013). However, anecdotal evidence suggests that the ID 32 C approach can incorrectly identify some yeast strains used

for wine production due to a clinical yeast orientated database. It is, therefore, advised that the ID 32 C AUX technique be used in conjunction with other complementary yeast differentiation tools.

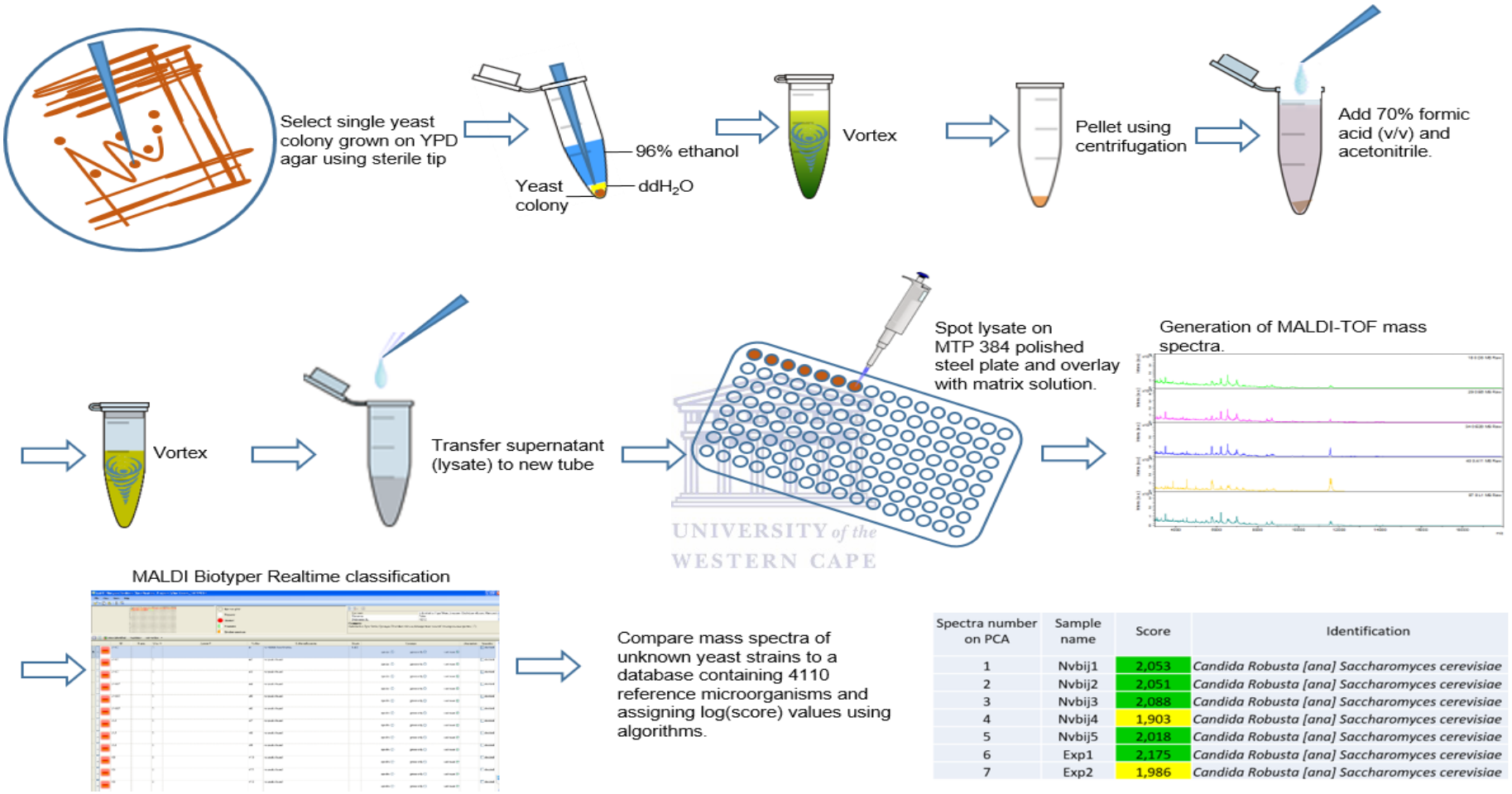
Cellular fatty acids analyses (CFAA) was shown to be a reliable tool to differentiate between closely related yeast strains (Augustyn, 1989; Augustyn & Kock, 1989). However, the physiological conditions are to be completely standardised for successful differentiation. If not, the cellular fatty acid profile of any given yeast strain will vary, which essentially means that a single strain will have different profiles in different fermentation matrices. Therefore, CHEF DNA karyotyping is a more practical and reliable characterisation method as this level of rigidity is not required (Van der Westhuizen and Pretorius, 1992; Van Breda *et al.,* 2013).

2.3.4. Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF/MS) biotyping

Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry biotyping was shown to be a relatively fast, accurate and dependable method to differentiate between closely related microorganisms (Chalupová *et al.,* 2013; Moothoo-Padayachie *et al.,* 2013; Pavlovic *et al.,* 2013; Deak *et al.,* 2015). Biotyping involves the extraction of ribosomal protein and subsequent generation of peptide mass spectral fingerprints using a tandem mass spectrometry (Figure 2.2). Ribsomal proteins are praticable for this application, as they are constitutively expressed even when the test organism are grown under different physiological conditions (Valentine *et al*., 2005; Clark *et al*., 2013; Gekenidis *et al*., 2014; Emami *et al*., 2015). These proteins are frequently expressed in abundance, and expression thereof is reproducible (Gekenidis *et al*., 2014). Peptide spectra are automatically processed with the appropriate software *e.g.* MALDI Biotyper Real Time Classification (Bruker Daltonics, Bremen, Germany). The software performs smoothing and baseline correction using Savitsky-Gloay, Multipolygon algorithms. Identification of unknown yeasts are achieved by comparing their mass spectra to a Bruker Daltonics BioTyper library database (Rizzato *et al.,* 2016). Furthermore,

yeast cells cultivated under different nutritional conditions differentially expressed proteins involved in biological processes (Querin *et al*., 2008; Zhao *et al*., 2014). As a result, proteins required for biological processes are impractical for MALDI-MS biotyping.

A further advantage of ribosomal proteins is that a single yeast strain isolated from different matrices, *e.g.* wine, beer or synthetic medium etc. can be accurately identified by the generated spectra. The different phenotypes when yeast is subjected to different physiological conditions, is a difficulty that is easily circumvented by deploying MALDI-TOF MS biotyping (Gekenidis *et al.,* 2014; Emami *et al.,* 2015). Another advantage is that the database includes both the anamorphic and teleomorphic species names (Turvey *et al.,* 2016; Du Plessis *et al*. 2017). However, despite the faster yeast identification obtainable using MALDI-TOF MS biotyping, this method still remains relatively expensive, especially if the unknown isolate is not in the database (Croxatto *et al.,* 2012; Panda *et al.,* 2015). Singhal *et al.* (2015) also highlighted that identification of unknown isolates is highly unlikely if the database does not contain a peptide mass spectral fingerprint of the relevant strain. Unfortunatelty, the preloaded database cannot be extended by inclusion of novel ribosomal peptide spectra (G Mohamed, Personal communication, 2016). A novel database can, however, be developed from scratch to accodomate spectra originating from excluded or novel microbes (Rizzato *et al*., 2016). This aspect warrants the establishment of a wine yeast database, as wine is an important contributor to the agricultural sector economy. Nevertheless, MALDI-TOF-MS biotyping provides fast and reliable differentiation of microorganisms, more so in circumstances where molecular differentiation of different isolates is impractical due to identical internal transcribed spacer (ITS) regions (Korabecná *et al.,* 2003; Emami *et al.,* 2015).



Spectra number on PCA	Sample name	Score	Identification
1	Nvbij1	2,053	*Candida Robusta [ana] Saccharomyces cerevisiae*
2	Nvbij2	2,051	*Candida Robusta [ana] Saccharomyces cerevisiae*
3	Nvbij3	2,088	*Candida Robusta [ana] Saccharomyces cerevisiae*
4	Nvbij4	1,903	*Candida Robusta [ana] Saccharomyces cerevisiae*
5	Nvbij5	2,018	*Candida Robusta [ana] Saccharomyces cerevisiae*
6	Exp1	2,175	*Candida Robusta [ana] Saccharomyces cerevisiae*
7	Exp2	1,986	*Candida Robusta [ana] Saccharomyces cerevisiae*

FIGURE 2.2: Schematic depiction of steps involved during matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF/MS) biotyping.

2.3.5. Fermentation potential

Establishment of the fermentation potential of a yeast strain involves various criteria such as determination of fermentation rate, measurement of wine chemical and sensory parameters, metabolite levels. As formerly mentioned, evaluating the fermentation potential of promising experimental yeast strains is critical before commercial trials and subsequent commercialisation of the yeast (Gonzalez *et al.,* 2007; Pérez-Torrado *et al.,* 2015). However, determining the fermentation potential of yeast strains through a series of laboratory-, small-, pilot- and semi-commercial (≥1000 L) scale vinification (winemaking) trials at *e.g.* a research cellar have drawbacks, and can easily take up to 10 years to identify an experimental yeast strain worthy for commercialisation purposes (Hart and Jolly, 2008). Firstly, a new criterion for yeast development will be identified by industry, whereafter fermentation trials are initiated using a large number of experimental and commercial reference yeast strains (Hart & Jolly, 2008; Heymann *et al.,* 2013). Promising experimental yeast strains will be re-evaluated during subsequent trials and/or vintages to establish that results obtained are repeatable, and to eliminate the effect of vintage. Subsequently, trials will be scaled-up to *e.g.* semi-commercial scale at commercial cellars as opposed to a research cellar, once observations during re-evaluations are satisfactory. This approach in all probability will ensure the development and identification of the most promising yeast strains with the ability to produce good quality wines irrespective of vintage (Von Mollendorf, 2013; Canonico *et al.,* 2015; Padilla *et al.,* 2016).

2.3.6. Fermentation rate

A classical method to investigate the fermentation rate following inoculation with a yeast strain, in laboratory-scale fermentations involves labour-intensive weighing of the fermentation vessels to monitor carbon dioxide (CO_2) -weight loss (Rosenfeld *et al.,* 2003; Fairbairn, 2012; Parcunev *et al.,* 2012). Other technological advances that also allow for monitoring of fermentation rate and wine parameters involves the use of state of the art bioreactors (fermenters) in conjunction with computer

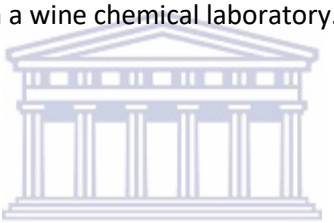
software (Nerantzis *et al.,* 2007; Sonego *et al.,* 2016). This approach is advantageous, as data are rapidly generated and physical weighing circumvented. However, installation and operation of bioreactors are expensive, making this approach difficult especially in developing countries where budget constraints applies (Svasti, 2001; Hashimura *et al.,* 2012; University World News [UWN], 2013). Therefore, traditional monitoring of CO_2-weight loss remains a viable and cheaper option.

2.3.7. Chemical characterisation

Monitoring of chemical parameters *e.g.* sugar utilisation, alcohol production, progression of pH, free amino nitrogen (FAN), yeast assimilable nitrogen (YAN), total sulphur dioxide (SO_2) etc. of ferments constitutes chemical characterisation of the wine yeast strain used to conduct the alcoholic fermentation (Heymann *et al.,* 2013; Cramer *et al.,* 2014). Traditionally, analyses of fermenting grape must and/or wine basic chemical parameters involves wet chemistry techniques that are still useful today to monitor sugar utilisation and alcohol accumulation etc. by deploying *e.g.* Rebelein titrations and a hydrometer (Jones & Ough, 1985; Hoon, 2015). Wet chemistry techniques are advantageous, because they do not require expensive computerised equipment that deploy, amongst others, near-infrared (NIR) based technologies (King & Heymann, 2014; Friedel *et al.,* 2016). Unfortunately, some wet chemical techniques *e.g.* the Rebelein method involves observing a critical colour change that is indicative of the reducing sugar level, which is in essence an anomaly as different technicians will perceive the critical colour-change differently.

The hydrometer is a sealed glass tube with a weight at the bottom and a potential alcohol (%) or specific gravity scale on the opposite end. Readings are taken at the start of fermentation and again towards the end of fermentation by observing the depth (meniscus) at which the tube is floating in the ferment, and calculating the actual alcohol from the difference in the depths (Figure 2.3). As with the Rebelein titrations, different technicians might interpret the floating level differently. This can potentially give contradictory results. Another drawback of wet chemistry is the reagents required for

the analysis, some of which are costly or the preparation and discarding of the resultant waste are time-consuming. Nonetheless, both methods are relatively cheap and can serve as guidelines whereafter results can be confirmed using more technologically advanced tools mentioned above. It is noteworthy that above-mentioned NIR-based instruments are calibrated using wet chemistry and/or hand analytical methods, which in itself is a predicament. A major advantage of NIR-based instruments is that they give quick and relative accurate results, whilst requiring little sample (analyte). These instruments also do not require chemical reagents. However a drawback is that they are digital instruments, and like any computer are prone to software malfunctioning. Lack of regular calibrations can result in less accurate measurements of relevant chemical parameters. Consequently, both wet chemical and more advanced technologies have advantages as well as disadvantages, and both approaches still have a role to play in a wine chemical laboratory.



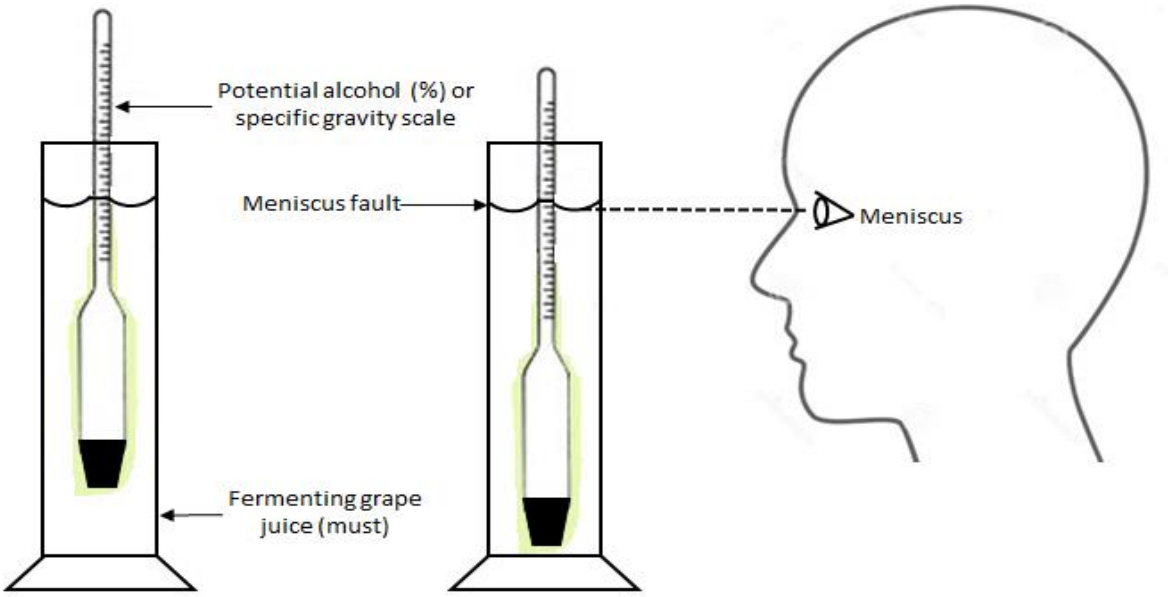


FIGURE 2.3: Schematic depiction of steps involved to determine the potential alcohol (%) in fermenting grape must by measuring the specific gravity using a hydrometer. The hydrometer floats higher in higher °Balling grape must (A), and with gradually floats lower as density of ferment decreases due to sugar depletion and alcohol (%) accumulation (B)

2.3.8. Sensory characterisation

Sensory evaluation of ferments and wine by a panel of trained judges also fundamentally constitutes sensory characterisation of the wine yeast strain used to conduct the alcoholic fermentation (Noble & Shannon, 2005; Molina *et al.,* 2009; Campo *et al.,* 2010). Arroyo *et al.* (2009), and Bhattacharyya and Bandhopadhyay (2010) reported that the human olfactory system, has a wider range for detecting aroma and flavour compounds compared to sophisticated analytical gas chromatographic equipment. Perception of wine aroma and flavour involves smelling and tasting of wines, which enables multiple nerve fibers (filaments) within the olfactory system, referred to as the olfactory bulb (receptor) cells, that detects aroma compounds *e.g.* esters, thiols (Araneda *et al.,* 2000; Rolls, 2005; Swiegers *et al.,* 2005; Shepherd, 2006; Tham *et al.,* 2009; Bushdid *et al.,* 2014). These receptor cells in conjunction with various regions within the brain makes an association with preconceived aromas and flavours *e.g.* banana, passionfruit etc. as schematically depicted in Figure 2.4. Various approaches are deployed to conduct experimental wine sensory analyses *e.g.* descriptive sensory analyses using an intensity

scale, unipolar numerical scale, multi-wine preference sorting, sensory profiling using "check-all-that-apply" (CATA) questions etc. Descriptive sensory evaluation involves visual (colour), aroma (nose) and flavour (taste) characterisation of wine, based on the perceived intensity on an unstructured line-scale of aroma and flavour descriptors. For Sauvignon blanc these include grassy, herbaceous, vegetative, green bellpeppers, floral (*e.g.* orange blossom, elderflower), passion fruit, grapefruit, peach, guava, banana etc. Aforementioned aroma notes may vary based on *e.g.* climatic conditions and the yeast strain used to conduct the alcoholic fermentation (Marais, 1994; Coetzee & Du Toit, 2015; Van Wyngaard, 2013; Von Mollendorf, 2013). Sensory evaluation using a unipolar numerical scale are conducted in a similar manner, except that judges on sensory panel are required to indicate aroma intensities on *e.g.* a unipolar six-point numerical scale (absent [0], very low [1], low [2], medium [3], high [4] and very high [5]). Additonally, judges must also specify the most prominent aromas and flavours perceived.

Multi-wine preference sorting instead, is a discriminative analysis which involves sorting of wines in at least two groups based on similarities and differences (Valentin *et al.,* 2016). Other variants of method allows for more than two groups. This is a useful approach to rapidly determine whether treatments (*e.g.* yeast inoculum) differ or are similar (Singh-Ackbarali & Maharaj, 2013; Weightman *et al.,* 2016). Favoured wines can subsequently be subjected to descriptive sensory evaluation in order to generate data that can be statistically analysed. Napping (Projective mapping), another advantageous wine sensory evaluation tool that is comparable to sorting, requires a sensory panel to evaluate wines based on similarities as well as their differences (Brand & Nieuwoudt, 2016a). Wines are also described separately, whilst sorting allows for wines that share similar aroma and flavour notes to be clustered together. Nonetheless, both approaches have a role to play as practicable wine sensory evaluation tools.

Wine sensory profiling using "check-all-that-apply" [CATA] questions has also increased in popularity (Brand & Nieuwoudt, 2016b). As the name says, CATA requires judges to highlight all perceived aroma

and flavour descriptors from a provided list (Ares *et al*., 2015). However, this approach differs from descriptive sensory evaluation as it is not quantitative. As with descriptive sensory analyses, wines are served individually to the judges in a randomised order. Check-all-that-apply is a useful tool to rapidly profile wines as a trained panel is used and their interpretation of the aroma and flavour descriptors are similar or assumed to vary marginally. Combining CATA and descriptive sensory analyses might lead to paradigms shifts with regard to wine sensory profiling, as the benefits of both techniques can be amalgamated into one protocol. Statistical analyses of data might be more complicated, but this prospect merits futher investigations.

FIGURE 2.4: Schematic depiction of the human olfactory system and the process of olfaction that allows for the perception of wine aroma and flavour. Olfactory bulb (receptor) consisting of multiple fibers transfer regular ortho-nasal (blue arrow) and retro-nasal (orange arrow) smell to brain for interpretation and/or identification.

2.3.9. Metabolomic characterisation

Wine contains various metabolites referred to as the yeast's exo-metabolome *e.g.* monosaccharide sugars, organic acids, fatty acids, amino acids, volatile thiols, esters, higher alcohols, some of which are yeast-derived (Howell *et al.,* 2006; Mapelli *et al.,* 2008; Chambers *et al.,* 2009; Varela, 2016). Various gas chromatographic (GC) based techniques *e.g.* gas chromatography coupled to a flame ionisation detector (GC-FID), gas chromatography-mass spectrometry (GC-MS), solid-phase extraction (SPE) coupled with GC-MS/MS analysis, and ultra-pressure liquid chromatography-tandem mass spectrometry (UPLC–MS/MS) are routinely used to measure these metabolite levels in wines following fermentation (Carrau *et al.,* 2008; Von Mollendorf, 2013; Schueuermann *et al.,* 2016). These analyses, in essence, constitute metabolomic characterisation of the yeast starter culture used to produce the wine (Dziadas & Jelen 2010; Jewison *et al.,* 2012; Kong *et al.,* 2014).

Tominaga *et al.* (1998), Aggio *et al.* (2014), and Savolainen *et al.* (2016) reported that GC-MS based methods are advantageous, since they allow for the simultaneous identification and quantification of various volatile thiols in Sauvignon blanc wines. Gas chromatography coupled with mass spectrometry also complements sensory descriptive analyses, as correlations between detection thresholds and sensory perception thresholds can easily be established (Schmidtke *et al.,* 2013). Vas & Vekey (2004) and Howard *et al.* (2014) reported that capillary GC-MS in conjunction with solid-phase microextraction (SPME) allows for fast and reliable characterisation of metabolites in wines. Gas chromatography-mass spectrometry based metabolite analyses can also distinguish between wines produced from different cultivars *e.g.* Chardonnay, Sauvignon blanc and Chenin Blanc, as well as wine produced from the same cultivar grown in different terroirs (Vas *et al.,* 1998; Bosch-Fusté *et al.,* 2007; Sagratini *et al.,* 2012).

2.3.9.1. Metabolites associated with tropical fruit aroma in Sauvignon blanc

A bottled wine with a desirable organoleptic profile can mostly be attributed to the yeast starter culture produced and mediated metabolites (Carrau *et al.,* 2008; Von Mollendorf, 2013; Rollero et al 2016). It has been reported that volatile thiols such as 4-mercapto-4-methylpentan-2-one [4MMP], 3-mercaptohexan-1-ol (3MH), 3-mercaptohexyl acetate [3MHA] are important contributors to the tropical fruit aroma associated with Sauvignon blanc wines (Swiegers *et al.*, 2006; King, 2010, Coetzee & Du Toit, 2012; Bovo *et al.,* 2015). Vela *et al*. (2017) echoed the association of these volatile thiols (mercaptans) with the varietal tropical fruit aromas of Sauvignon blanc. It is noteworthy that, that the new convention pertaining to 4MMP, 3MH, and 3MHA is 4-methyl-4-sulfanylpentan-2-one (4MSP), 3-sulfanylhexan-1-ol (3SH), and 3-sulfanylhexyl acetate (3SHA), respectively (Renault *et al*., 2016). However, Williams (2018) reported that the former conventions is still acceptable, hence they will be used hereafter. These aromatic compounds emanate directly from wine grapes where they are found as aroma-inactive, cysteine-bound precursors that are released through wine yeast mediated activity during fermentation. Other compounds are a direct result of yeast metabolic activity *e.g.* ethyl acetate, ethyl butyrate, 2-phenyl ethyl acetate, 2-phenyl ethanol, i-amyl alcohol and hexanol. Subsequently, Sauvignon blanc wine aroma and flavour can be enhanced by using a wine yeast starter culture with the ability to release bound non-volatile aroma compounds (Swiegers *et al.,* 2006; 2009). It is noteworthy that a non-*Saccharomyces* yeast strain was reported to increase the release of the volatile thiol *i.e.* 3MH during the production of Sauvignon blanc (Anfang *et al*., 2009). As mentioned previously, non-*Saccharomyces* yeast strains are important for producing aromatic wines, and to be used for genetic improvement with regard to *e.g.* volatile release. Additionally, yeast synthesized esters *e.g.* isoamyl acetate, ethyl octanoate and isobutyl acetate were reported to contribute to the tropical fruit aromas *e.g*. banana associated with warmer climate Sauvignon blanc wines (Benkwitz *et al*., 2012).

2.3.9.2. Metabolites associated with vegetative and/or herbaceous aroma in Sauvignon blanc

Marais (1994), Ryona *et al.* (2010), Sidhu *et al.* (2015), and Gregan and Jordan (2016) reported that besides tropical fruit aromas, vegetative and herbaceous aromas are also associated with Sauvignon blanc wine due to metabolites *e.g.* methoxypyrazines commonly found in Sauvignon blanc grapes. This is especially apparent when grown and harvested under cooler climatic conditions. Sauvignon blanc grapes originating from vineyards in the Constantia valley situated between the Table Mountain range and False Bay, Western Cape, South Africa comes to mind due to resultant wines' prominent vegetative and herbaceous aromas (Marais, 1994). Other cooler climate regions within the Cape Winelands include Overberg (Elgin valley), Cape Agulhas (Strandveld) and West Coast (Doringbaai). Vegetative Sauvignon blanc is not limited to South Africa as wines originating from the world renowed Malborough region, New Zealand, are known for their vegetative aromas and flavour namely, mint, peas and asparagus due to higher methoxypyrizaine levels (Benkwitz *et al*., 2012). Yeast synthesised metabolites namely, higher alcohols *e.g.* 3-hexenol (E), 3-hexenol (Z), 2-hexenol (E) and 2-hexenol was also reported to contribute to the vegetative aromas *e.g*. green cut grass usually associated with cooler climate Sauvignon blanc wines (Benkwitz *et al*., 2012; Harsch *et al*., 2013).

2.3.9.3. Metabolites associated with floral aroma in Sauvignon blanc

Besides the traditional vegetative and tropical fruit aromas, floral aromas are also associated with Sauvignon blanc wine due to grape derived metabolites *e.g.* monoterpenes (Marais 1994; Van Wyngaard, 2013; Von Mollendorf, 2013). Monoterpenes were also shown to be associated with citrus aromas in Sauvignon blanc wines (Marais, 1994; Carrau *et al.,* 2008; Coetzee & du Toit, 2015). Aromatic monoterpenes present in wine are metabolised by yeasts from their grape-derived aroma-inactive glucose-bound precursors (Carrau *et al.,* 2005; Swiegers *et al.,* 2005; Pardo *et al.,* (2015). These citrus-aroma enhancing metabolites are also de novo synthesised by wine yeast during fermentation (Gamero *et al.,* 2011; Pardo *et al.,* 2015). Various cellars within the Cape Winelands,

South Africa produce Sauvignon blanc wines with floral aroma notes, which can be attributed to aforementioned monoterpenes. Sauvignon blanc wines with floral, rose and geranium nuances and hints of citrus aromas are also commonly produced in the Malborough region due to various yeast-derived monoterpenes *e.g*. terpineol, nerol, citronellol, geraniol and linalool (Benkwitz *et al*., 2012).

2.3.9.4. Undesirable yeast produced and/or mediated metabolites in Sauvignon blanc

Du Toit and Pretorius (2000), Ugliano *et al.* (2007), Vilela-Moura *et al.* (2011) and Fairbairn *et al.* (2015) reported that wine yeast strains, some of which are commercially available, can produce wines with undesirable volatile acidity (VA) levels. Volatile acidity *e.g.* acetic acid, lactic acid, formic acid, butyric acid, propionic acid, octanoic acid, are produced as by-products of wine yeast metabolic processes during the fermentation process (Swiegers *et al.,* 2006; King, 2010; Bovo *et al.,* 2015; Moss, 2015). However, acetic acid is the main VA in wine with a sensory detection threshold of at least 0.7 g/L (Byarugaba-Bazirake, 2008; Vilela-Moura *et al.,* 2010). Acetic acid levels in faulty wine can be as high as 3 g/L, which is almost thrice the legal limit of 1,2 g/L permitted for VA in a white wine (OIV, 2012; Sirén *et al.,* 2015).) Excessive acetic acid levels can impart vinegar-like and nail polish odours in wines (Molina *et al.,* 2007). Subsequently, wine chemical and sensory quality are negatively affected (Du Toit, 2000; Ferreira, 2004; Muratore *et al.,* 2007), and are undesirable to consumers. Financial loss can, therefore, be incurred by winemakers due to lower wine sales and use of expensive techniques *e.g.* reverese osmosis to remove the excessive VA from wine (Zoecklein *et al.,* 1999; Vilela *et al.,* 2013).

Excessive acetic acid also has an inhibitory effect on wine yeasts during winemaking, causing sluggish or stuck fermentations (Mira *et al.,* 2010; Ding *et al.,* 2013; Shang *et al.,* 2016). In worst case scenarios the entire affected wine tank may need to be discarded, which will also result in financial loss. Furthermore, commercial yeast manufacturers will also incur financial losses if, and when a commercial yeast strain is implicated in excessive VA formation. Preventative measures involve the use of vigorous wine yeast strains that are known to be low VA producers. Vigorous yeast strains will

also inhibit the growth of acetic acid bacteria (AAB) that have the ability of producing acetic acid from glucose and ethanol.

Another undesirable compound namely, hydrogen suphide (H_2S) associated with all wines, including Sauvignon blanc, imparts rotten egg and sewage odours (Ugliano *et al*., 2011; Bizaj *et al*., 2012). Some wine yeasts inherently produce H_2S as a stress response when fermenting a nitrogen deficient grape must (Ogata, 2013). Therefore, addition of relatively costly yeast nutrients during rehydration or early during the fermentation are preventative measures to reduce the risk of H_2S formation during fermentation. Contrariwise, H_2S was reported to act as precursor for the sought-after volatile thiols mentioned above when available in excess in grape juice (Harsch *et al*., 2013). This is a proverbial double-edged sword, as H_2S are also known to mask wine fruity aromas, even when levels are below its sensory detection threshold. Nonetheless breeding of novel hybrids that are not predisposed to H_2S production when challenged with low nitrogen grape musts is an avenue of yeast development that should be investigated.

2.3.10. Proteomic characterisation

Wine yeast expressed proteins are instrumental in the production of varietal aromatic wines, as yeast derived and mediated aroma compounds (metabolites) derives from the activities of these proteins (enzymes), which play a key role in metabolic pathways of yeast cells (De Klerk, 2009; Holt *et al.,* 2011; Roncoroni *et al.,* 2011; Juega *et al.,* 2012; Moreno-García *et al.,* 2015). Yeast strains differentially express proteins even though a standard medium or grape juice is used for fermentation (Mostert, 2013). Additionally, proteins that are always associated with a certain physiological condition *e.g.* high aroma compounds, can be used as biomarkers for said conditions (Zhu *et al.,* 2000; Tao *et al.,* 2007). The advantage of using a protein biomarker above a gene biomarker is that an organism's genome is relatively constant, irrespective of the physiological condition, whilst protein expression varies based on physiological conditions (Herrero *et al.,* 2003; Bisson *et al.,* 2007; Gómez-Pastor, 2010).

Transcriptomics (study of the sum of all RNA transcripts of a organism referred to as its transcriptome) is also a viable characterisation tool, however, mRNA, which is directly transcribed from an expressed gene are less stable compared to proteins and are degraded more rapidly than proteins (Belinchón *et al.,* 2004; Parker, 2014). Furthermore, mRNA can be translated into more than one protein, each with a different function (Gygi *et al.,* 1999; Gingold & Pilpel, 2011). It is therefore, evident that proteomic analyses of wine yeasts can be used as a practical characterisation tool to differentiate between fermenting yeast strains (Kobi *et al.,* 2004; Hansen *et al.,* 2006; Mostert, 2013).

Proteomic characterisation involves qualitative and quantitative analyses of protein extracts (Rigaut *et al.,* 1999; Goodlett & Eugene, 2002; Gillet *et al.,* 2012). Protein quality can be determined using basic gel-based approaches in conjunction with innovative liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques (Silva *et al.,* 2006; Jiang *et al.,* 2008; Pressey *et al.,* 2011; Milac *et al.,* 2012; Nowakowski *et al.,* 2014). The most basic gel-based proteomic analyses involve one-dimensional (1D) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), whereby extracted proteins are separated based on their molecular weight (MW) (Kolkman *et al.,* 2005; 2006). However, the disadvantage of 1D-PAGE is that proteins with a similar molecular weight cannot be separated and are visualised as one protein band.

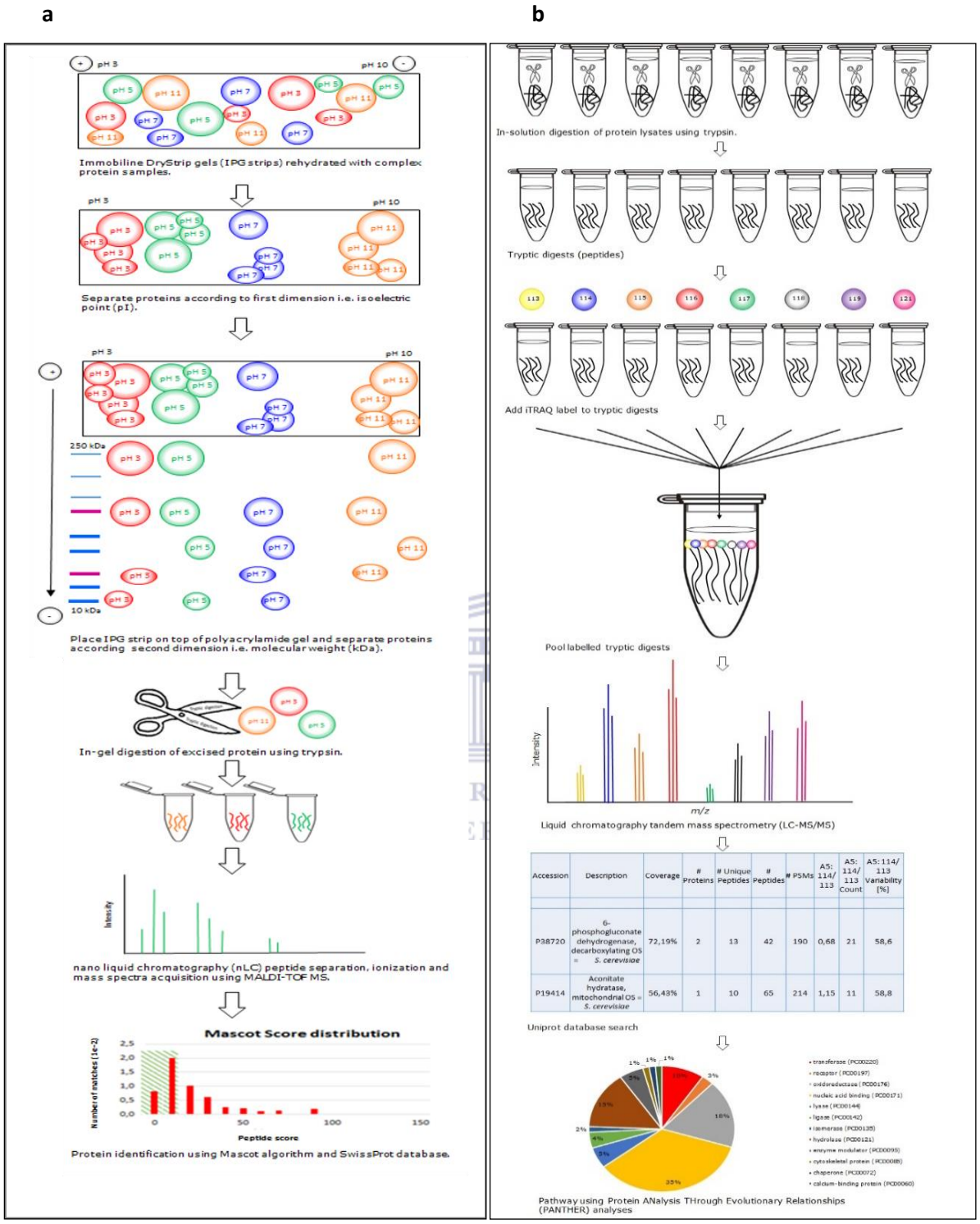
Abovementioned predicament is easily circumvented by means of two-dimensional (2D) PAGE, whereby proteins are separated in two steps based on two independent properties (Ndimba *et al.,* 2010; Pressey *et al.,* 2011). The first step entails separation of proteins according to their isoelectric points (pH where protein have a neutral net charge) followed by the second step which is a basic SDS-PAGE (Kolkman *et al.,* 2005; 2006; Pressey *et al.,* 2011). Individual proteins normally manifest as different spots on poly-acrylamide gels following a staining technique using, *e.g.* Coomassie blue, Silver staining etc. (Shevchenko *et al.,* 1996; Westermeier, 2006). It is improbable that two or more expressed proteins from the same organism will have identical molecular weights and isoelectric points (Shevchenko *et al.,* 1996). Differentially expressed proteins are then excised and characterised

by means of peptide mass fingerprinting (PMF) using MALDI-TOF/MS (Ngara *et al.,* 2012; Zhou *et al.,* 2012). The major drawback of gel-based proteomics is that protein identification is limited to a specific molecular weight- and pH-range (Aggarwal *et al.,* 2006). Nonetheless, 2D-PAGE is a useful tool to identify and characterise single proteins associated with certain wine chemical (lower VA) and sensory profiles (enhanced tropical fruit aroma), and these proteins can serve as biomarkers for experimental yeast starter cultures during winemaking trials.

Protein quantitation can be conducted by subjecting protein lysate to a rapid and simple spectrophotometric analyses *e.g.* Bradford Coomassie G-250 assay (Zor & Selinger, 1996; Ngara *et al.,* 2012). A major drawback of this type of protein quantitation is that complex protein lysates might have different adsorption properties compared to bovine serum albumin (BSA) protein used to construct the standard curve. As a result, various proteins will bind differently to the Coomassie G-250 compared to BSA, therefore giving incorrect measurements when extrapolated on a BSA-based standard curve (Zaia *et al.,* 2005). Cutting-edge liquid chromatography-mass spectrometry (LC-MS) based isobaric peptide tags for relative and absolute quantification (iTRAQ), where unknown protein peptides are tagged with reporter molecules that allows for quantification, is a practical alternative (Ross *et al.,* 2004; Ernst & Zor, 2010; Unwin *et al.,* 2010; Kim *et al.,* 2012; Nie *et al.,* 2013). A key advantage of iTRAQ is that complex protein samples originating from different yeast strains can be analysed simultaneously (Aggarwal *et al.,* 2006; Latosinska *et al.,* 2015). Therefore, protein expression of an experimental yeast strain can simultaneously be compared to various commercial yeast strains, respectively.

Unlike gel-based proteomics, iTRAQ protein identification is not limited to a given molecular weight (MW)- and pH-range, thereby allowing for quantification and identification of vast numbers of proteins during a single analysis. This approach does, however, require expensive state-of-the-art equipment *e.g.* Linear trap quadrupole (LTQ) Orbitrap Velos MS that is not always readily available, especially in developing countries. Label-free MS, contrariwise to iTRAQ-labelling, is also a practical

alternative for identifying complex protein samples originating from different sources simultaneously, but also requires above-mentioned LTQ Orbitrap Velos MS (Ross *et al.,* 2004; Li *et al.,* 2012; Latosinska *et al.,* 2015). Nevertheless, label-free approaches are significantly cheaper, as the use of expensive iTRAQ labels is not required (M Vlok, Personal communication, 2016). Quantitative and qualitative proteomic analysis procedures discussed are graphically illustrated in Figure 2.5.



Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	A5: 114/113	A5: 114/113 Count	A5: 114/113 Variability [%]
P38720	6-phosphogluconate dehydrogenase, decarboxylating OS = *S. cerevisiae*	72,19%	2	13	42	190	0,68	21	58,6
P19414	Aconitate hydratase, mitochondrial OS = *S. cerevisiae*	56,48%	1	10	65	214	1,15	11	58,8

FIGURE 2.5: Schematic depiction of qualitative and quantitative proteomic analysis tools. **a)** Two-dimensional polyacrylamide gel-electrophoresis (2D-PAGE), followed by peptide mass fingerprinting (PMF) using matrix-assisted laser desorption ionization-time of flight coupled mass spectrometry (MALDI-TOF/MS) to identify differentially expressed proteins; and **b)** quantification and identification of proteins using liquid chromatography-mass spectrometry (LC-MS) based isobaric peptide tags for relative and absolute quantification (iTRAQ).

2.4. CONCLUSION

The wine industry is an important contributor to the South African economy, as wine forms an integral part of exports from the agricultural sector. Furthermore, anecdotal evidence suggests that many of the yeasts commercially available are inadequate to fully meet the requirements of both South African winemakers and international winemakers. Therefore, South African oenologists are continually striving to increase wine quality in an ever increasing competitive market, especially in light of varietal aromatic new world wines lacking undesirable attributes becoming increasingly popular. Swiegers *et al.* (2007) and Holt *et al.* (2011) reported that yeasts strains with sought-after characteristics can be generated by deploying genetic modification (GM). However, the use of genetically modified organisms (GMO) for wine production is illegal. Furthermore, the Cape Winemakers Guild (CWG) and South African Wine Industry Council (SAWIC) is also largely against the use of GMO in wine production (CWG, 2015). Both organisations emphasised the dependence of the SA wine industry on the highly sensitive European market, which are largely against GM food products. Nonetheless, improvements in yeast starter cultures used for the wine production is of utmost importance, as it is directly linked to wines with improved organoleptic quality. Failure to improve yeast strains as industry criteria are changing will result in financial loss due to lower wine and yeast sales. In future, climate change with its impact on viticulture and possibly grape physiology, will create new fermentation challenges for the winemaker. As a result, continuous yeast development by means of classical mating is important and relevant in order for the SA industry to remain globally competitive.

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Chapter 3

Research results

Characterisation of *Saccharomyces cerevisiae* hybrid yeasts selected for low volatile acidity formation and the production of aromatic Sauvignon blanc wine

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Chapter 3: Characterisation of *Saccharomyces cerevisiae* hybrid yeasts selected for low volatile acidity formation and the production of aromatic Sauvignon blanc wine

3.1. ABSTRACT

Wine yeasts, namely *Saccharomyces cerevisiae* vary in their ability to develop the full aroma potential of Sauvignon blanc wine due to an inability to release volatile thiols. Therefore, the use of 'thiol-releasing' wine yeasts (TRWY) has increased in popularity. However, anecdotal evidence suggests that some commercially available TRWY intermittently exhibit undesirable characteristics *e.g.* volatile acidity (VA) formation. Therefore, a trial was undertaken to select and evaluate *S. cerevisiae* hybrid yeasts for the production of Sauvignon blanc wine with enhanced fruity and tropical aromas, but low VA. Hybrids were characterised by CHEF DNA karyotyping and MALDI-TOF MS biotyping, and subsequently trialled against top commercial TRWY *i.e.* Zymaflore VL3 and Zymaflore X5 (Laffort Oenologie, France), and Fermicru 4F9 (DSM Oenology, Netherlands) in laboratory-scale Sauvignon blanc vinifications during 2013. Most hybrids produced wines with VA levels significantly lower than those produced with Zymaflore VL3, Zymaflore X5 and Fermicru 4F9. Low VA forming hybrids also produced wines with tropical wine aroma notes. Wines produced by Fermicru 4F9 had the lowest acetic acid (the main volatile acid) of the commercial TRWY in this study. However, some hybrid yeasts produced wines with less acetic acid on average than wines produced by Fermicru 4F9. Overall, hybrids NH 6, NH 48, NH 56, NH 88 and NH 145 produced wines with enhanced tropical fruity aroma, but lower VA compared to wines produced by commercial TRWY.

3.2. INTRODUCTION

Wine aroma is comprised of compounds emanating directly from the grapes, compounds produced by the yeast such as esters and higher alcohols, and yeast mediated compounds *e.g.* volatile thiols (King, 2010; Bovo *et al.,* 2015). Wine yeasts (*Saccharomyces cerevisiae*) vary in their ability to develop the full aroma potential of Sauvignon blanc wine due to an inability to release volatile thiols (King *et al.,* 2011). Retention of these bound thiols implies that the full aroma potential of the wine is not realised, as the bound thiols can only be released by wine yeasts during fermentation (Swiegers *et al.,* 2006; Holt *et al.,* 2011). Therefore, the use of 'thiol-releasing' *S. cerevisiae* commercial wine yeasts (TRWY) for the production of aromatic Sauvignon blanc wine has increased in popularity (Swiegers *et al.,* 2009). These yeast strains can release 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) from the respective cysteine-bound precursors. Other yeast strains can convert the aromatic 3MH (passion fruit aroma) to 3MHA *(*tropical and citrus *aromas).* However, anecdotal evidence suggests that some commercial TRWY intermittently produce undesirable high levels of volatile acidity (VA), which imparts vinegar-like nuances to the wines (Du Toit & Pretorius, 2000; Ugliano *et al.,* 2007; Vilela-Moura *et al.,* 2011). Acetic acid is the main contributor to VA in wine with odour detection levels ranging between 0.7 to 1.1 g/L (Byarugaba-Bazirake, 2008; Vilela-Moura *et al.,* 2010). Even though, excessive levels of VA are mainly caused by lactic acid bacteria, acetic acid bacteria and wild yeasts, wine yeasts also contribute to VA, by producing acetic acid during alcoholic fermentation (Cordente *et al.,* 2013; Luo *et al.,* 2013). Other steam distillable acids *i.e.* lactic, formic, butyric, and propionic acids can also contribute to VA (Erasmus *et al.,* 2004; Moss, 2015). Currently, in South Africa the legal limit of VA permissible in wine is 1.2 g/L (OIV, 2012; Sirén *et al.,* 2015). However, the sensory threshold of VA is generally accepted to be 0.8 g/L (Du Toit, 2000).

Reduction of yeast derived VA formation can be done by using genetically modified (GM) yeasts (Swiegers *et al.,* 2007) or improved *S. cerevisiae* hybrid yeasts bred through classical mating (Pérez-

Torrado *et al.,* 2015). Although genetic modification can address VA formation by wine yeasts, the use of genetically modified organisms (GMO) is illegal (Berrie, 2011). The Cape Winemakers Guild (CWG) and South African Wine Industry Council (SAWIC) is also largely against the use of GMO in wine production (CWG, 2015). Both CGW and SAWIC emphasises that the SA wine industry is too dependent on the highly sensitive European market for exports, which are largely against GM food products. Sauvignon blanc was chosen for this study because this cultivar was previously shown to produce grapes containing aroma-inactive, non-volatile, bound thiols (metabolites) that can only be released by the wine yeast *Saccharomyces cerevisiae* during fermentation (von Mollendorf, 2013). Therefore, the aim of this study was to select and evaluate *Saccharomyces cerevisiae* hybrid yeasts for the production of wine with enhanced fruity and tropical fruit aromas, but low VA.

3.3. MATERIALS AND METHODS

3.3.1 Wine yeast strains

One hundred and thirty-six hybrid strains (NH 1 to 10, 12, 13, 15 to 18, 20, 22 to 25, 27 to 78, 80 to 95, 97 to 104, 106 to 119, and 121 to 145), four *S. cerevisiae* parental yeast strains (PS 1 to 4), three commercial TRWY references (Zymaflore VL3, Zymaflore X5 [Laffort Oenologie, France], and Fermicru 4F9 [DSM Oenology, Netherlands]) used in this study are conserved in the ARC Infruitec-Nietvoorbij micro-organism culture collection (ARC Inf-Nvbij CC). Hybrids were bred at the ARC Infruitec-Nietvoorbij microbiology laboratory through classical mating, as part of an ongoing hybrid breeding programme similar to that described by Steensels *et al.* (2014) and Snoek *et al.* (2015).

3.3.2 Pulsed-field gel electrophoresis (PFGE)/Contour clamped homogeneous electric field (CHEF) DNA karyotyping

DNA karyotyping of yeast strains was conducted according to the embedded agarose procedure described by Carle & Olson (1985), and Van der Westhuizen *et al.* (1992). The procedure was adapted by conducting chromosome separation in TBE (50 mM Tris, 41.3 mM boric acid, and 0.5 mM EDTA [Sigma-Aldrich, USA]) buffer at 14 °C with pulse-times of 30 and 215 sec for 34 hours using clamped homogenous electric field (CHEF) gel electrophoresis (CHEF-DR II, Bio-Rad Laboratories, Richmond, USA). Yeast strain PS1 was run parallel to CHEF DNA size marker #1703605 (Bio-Rad, Madrid, Spain) as an internal standard to determine respective chromosomal band sizes. Chromosomal banding patterns were visualised on a Bio-Rad image analyser following staining with 0.01% (v/v) ethidium bromide. Subsequently, the genetic relatedness of the various yeast strains was determined by subjecting CHEF DNA karyotypes to cluster analysis using FP Quest software FP 4.5 software (Bio-Rad, Madrid, Spain). Cluster analysis was based on the Dice coefficient and an un-weighted pair group method with arithmetic mean (UPGMA), with 1% tolerance and 0.5% optimisation.

3.3.3 Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF/MS) biotyping

Yeast strains were also identified by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF/MS) biotyping as an alternative to CHEF DNA karyotyping. Formic acid protein extraction for subsequent MALDI-TOF biotyping was conducted as described by Pavlovic *et al.* (2013). One microliter of wine yeast protein extract was spotted onto a MTP 384 polished steel target plate as described by Moothoo-Padayachie *et al.* (2013) and Deak *et al.* (2015). Thereafter, the spotted target plate was inserted into a Bruker UltrafleXtreme MALDI-TOF/MS (Bruker Daltonics, Bremen, Germany) apparatus. Generation of yeast protein mass spectra using MALDI-TOF/MS was conducted according to the standard National Agricultural Proteomics Research & Services Unit method

(obtainable from the National Agricultural Proteomics Research & Services Unit (NAPRSU), University of the Western Cape, South Africa). Mass spectra for all strains were acquired in triplicate.

3.3.4 Laboratory-scale fermentation trials

Wet culture wine yeasts were evaluated in laboratory-scale fermentation trials as described by Rossouw *et al.* (2010) and Maarman *et al.* (2014). Frozen Sauvignon blanc grape must (total sugar = 21.9 °B; total acidity = 9.3 g/L; pH = 3.28) was thawed and 250 mL aliquots were transferred into fermentation vessels (340 mL glass bottles). The yeast cultures were grown at 28 °C for 48 h in 10 mL YPD (1% [w/v] yeast extract, 2% [w/v] peptone, and 2% [w/v] dextrose [Biolab, Merck, South Africa]), and subsequently used to inoculate the Sauvignon blanc grape must at a concentration of 2% (v/v). Commercial TRWY Zymaflore VL3, Zymaflore X5 (Laffort Oenologie, France), and Fermicru 4F9 (DSM Oenology, Netherlands) were included in fermentation trials as references. Fermentation vessels were stoppered with a fermentation lock filled with water. Fermentations were conducted on an orbital shaker in an insulated temperature-controlled room, which were electronically regulated at 14.5 °C, and monitored by CO_2 weight loss for 30 days. All fermentations were conducted in triplicate in a completely randomised block design (Addelman, 1970).

3.3.5 Fourier transform infra-red (FTIR) spectroscopy

Wines were subjected to residual glucose/fructose, ethanol, VA, total acidity (TA) and pH analyses using an Oenofoss™ Fourier transform infrared (FTIR) spectrometer (FOSS Analytical A/S, Denmark) after fermentations stabilised.

3.3.6 Gas chromatography (GC) analysis

Wines with the most prominent fruity aromas as determined by the sensory panel were subjected to GC-MS analysis. Flavour compounds *viz*. esters, total fatty acids and higher alcohols were quantified by means of calibration mixtures of the applicable aroma compounds in conjunction with gas chromatography (GC) as described by Zhang *et al.* (2012) and Vilanova *et al.* (2013).

3.3.7 Sensory evaluation

Wines were subjected to descriptive sensory evaluation by a panel of 14 experienced wine judges. Judges were requested to indicate aroma intensities on a unipolar six-point numerical scale (absent [0], very low [1], low [2], medium [3], high [4] and very high [5]), and also to specify the most prominent aroma/s perceived *i.e.* 'tropical fruit' *e.g.* banana, guava, peach, passion fruit and citrus; 'vegetative' *e.g.* asparagus, herbaceous, green pepper, green beans, cut grass, green olive and gooseberry; or 'floral' *e.g.* rose, orange blossom etc. The wines were served as coded samples in international wine tasting glasses (approximately 50 mL) in a completely randomised order for each judge.

3.3.8 Statistical analyses

Chemical and sensory analyses data were subjected to principal component analysis (PCA) to determine the relationship between variables and treatments (yeasts) (Pearson, 1896; 1901; Zou *et al.,* 2006). The data matrix consisted of four chemical variables *i.e.* VA, ethanol, total acidity and pH; and three sensory aroma descriptors *i.e.* 'tropical fruit', 'vegetative' and 'floral'. Pearson's correlation was performed to study the linear relationship between the chemical and sensory variables. The Pearson's correlation matrix was used to standardise the data before performing the PCA. The PCA was performed using XLSTAT software (Addinsoft, 2013) with the principal components (PC's) as factors (*i.e.* F1 and F2).

3.4. RESULTS AND DISCUSSION

3.4.1 Pulsed-field gel electrophoresis (PFGE)/Contour clamped homogeneous electric field (CHEF) DNA karyotyping

Wine chemical and sensory quality are affected by the yeast strain used to carry out the alcoholic fermentation (Sharma *et al.,* 2012; Usbeck *et al.,* 2014). As a result, differentiation of yeast strains is essential to ensure that the correct yeast strain is used to inoculate grape must. Previous studies showed that Pulsed-field gel electrophoresis (PFGE)/Contour clamped homogeneous electric field (CHEF) DNA karyotyping DNA karyotyping allowed for the delineation of closely related yeast strains (Sheehan *et al.,* 1991; van Breda *et al.,* 2013). Similarly, CHEF DNA karyotyping was useful in this investigation to differentiate closely related *S. cerevisiae* hybrid strains descending from mutual parental yeast strains (Figure 3.1). Distinctive variations in the DNA karyotypes between hybrids can be seen, especially for the smaller chromosomes (bottom bands). Four pairs of hybrids that is, NH 33 and NH 34; NH 63 and NH 64; NH 75 and NH 76; and NH 86 and NH 89 had similar DNA karyotypes, whilst the remainder of yeast strains had distinguishable DNA karyotypes. Therefore, 139 CHEF DNA karyotyping profiles of the 143 strains were generated with genetic similarity ranging from 58 to 100%. The larger chromosomes (top bands) were common to most hybrids and parental yeast strains. It is evident that chromosomal DNA of the hybrids originated from more than one parental strain. It can be envisaged that some characteristics, including flavour compound (metabolite) release during fermentation, should be similar, different or enhanced compared to parental strains.

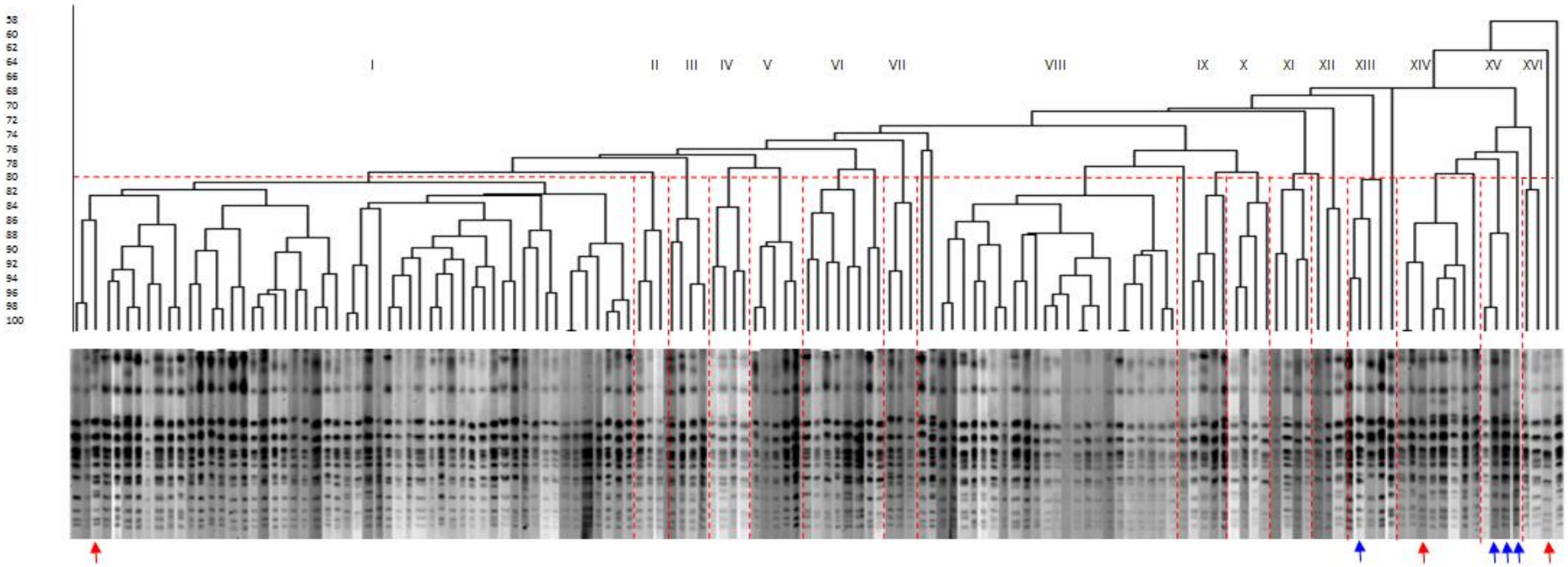


FIGURE 3.1: Dendogram showing the genetic similarity among three commercial 'thiol-releasing' wine yeasts (TRWY)(red arrows), four parental yeast (PS)(blue arrows) and 136 hybrid yeast (NH) strains. Cluster analyses was performed using a UPGMA algorithm. Yeast strains with 80% similarity (dotted line) were assigned to the same cluster indicated by Roman numerals. Dice (Opt:0.50%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%] **Cluster I:** NH 143, NH 132, ZYMAFLORE X5, NH12, NH 68, NH 66, NH 67, NH 125, NH 69, NH 24, NH 73, NH 10, NH 112, NH 107, NH 42, NH 113, NH 114, NH 133, NH 135, NH 134, NH 138, NH 35, NH 36, NH 117, NH 6, NH 9, NH 3, NH 47, NH 41, NH 15, NH 17, NH 54, NH 53, NH 94, NH 5, NH 7, NH 16, NH 52, NH 22, NH 25, NH 1, NH 106, NH 37, NH 13, NH 20, NH 91, NH 99, NH 33, NH 34, NH 32, NH 31, NH 4, NH 70, NH 30; **Cluster II:** NH 43, NH 55, NH 78; **Cluster III:** NH 130, NH 98, NH 48, NH 62; **Cluster IV:** NH 27, NH 81, NH 49, NH 97; **Cluster V:** NH 108, NH 127, NH 129, NH 136, NH 137; **Cluster VI:** NH 28, NH 50, NH 110, NH 29, NH 139, NH 142, NH 11, NH 18; **Cluster VII:** NH 39, NH 40, NH 38; **Cluster VIII:** NH 109, NH 2, NH 115, NH 116, NH 100, NH 45, NH 88, NH 23, NH 95, NH 44, NH 72, NH 77, NH 92, NH 93, NH 61, NH 75, NH 76, NH 74, NH 128, NH 86, NH 89, NH 83, NH 85, NH 87, NH 90; **Cluster IX:** NH 80, NH 101, NH 144, NH 71, NH 118; **Cluster X:** NH 123, NH 141, NH 103, NH 119; **Cluster XI:** NH 122, NH 8, NH 126, NH 82; **Cluster XII:** NH 121, NH 131, NH 84; **Cluster XIII:** NH 124, PS 2, NH 145, NH 140, NH 46; **Cluster XIV:** NH 63, NH 64, FERMICRU 4F9, NH 56, NH 59, NH 58, NH 60, NH 65; **Cluster XV:** NH 51, PS 1, PS 3, PS 4; **Cluster XVI:** NH 102, NH 104, ZYMAFLORE VL3, NH 57.

Cluster analysis of yeast CHEF DNA karyotypes allowed for the differentiation of yeast strains with common ancestry as described by Hoff (2012), Choi & Woo (2013) and Gallego *et al.* (2014). A dendogram comprising of sixteen clusters (I – XVI) was observed at a genetic similarity limit of 80% for all 143 strains (Figure 3.1). Four hybrids that is, NH 6, NH 67, NH 73 and NH 112 exhibiting the ability to produce wines with tropical fruit aroma (hereafter abbreviated as TFPH) clustered with the commercial TRWY reference Zymaflore X5, whilst another two TFPH and low VA producing hybrids (LVPH) that is, NH 56 and NH 57 cclustered with the commercial TRWY references Fermicru 4F9 and Zymaflore VL3, respectively. Both hybrids also clustered with tropical fruit wine producing PS 1 at a 74% genetic similarity cut-off. Moreover, both hybrids clustered with the lower VA producing PS 2, PS 3 and PS 4 (Figure 3.1). Therefore, these hybrids exhibiting the sought-after tropical fruit aroma enhancing and low VA forming qualities, inherited it from the respective parental strains.

3.4.2 Yeast profiling with MALDI-TOF/ MS Biotyping

Biotyping using MALDI-TOF/MS was successfully deployed to match ribosomal protein originating from commercial TRWY references, PS, and NH strains to that of a database described by Ghosh *et al.* (2015). All strains were identified as *Candida robusta,* the anamorph to *S. cerevisiae* (Diddens and Lodder, 1942; Kurtzman *et al.,* 2011) following biotyping (mass spectra can be requested from the National Agricultural Proteomics Research & Services Unit (NAPRSU), University of the Western Cape, South Africa). Overall 79.72 % of the strains were reliably identified as *Candida robusta* with scores of >2 as described by Moothoo-Padayachie *et al.* (2013). Nonetheless no cut-off score for reliable MALDI-TOF/MS biotyping was established, as all strains were shown by DNA karyotyping to be *S. cerevisiae*. Also noteworthy is that Cheng *et al.* (2013) showed that a lower cut-off score (1.7) sufficiently differentiate different *Candida* yeast strains. Therefore, the lowest cut-off score (>1.8) for some strains used during this trial is acceptable.

A dendogram consisting of nine clusters (I – IX) was generated following cluster analysis of the mass spectra at phylogenetic distance level of 0.80 indicated by dotted line (Figure 3.2). Hybrid strains were spread throughout the various mass spectral clusters. Some of the mass spectral clustering complemented DNA karyotype clustering, since TFPH, that is, NH 56 and NH 57, clustered with the commercial TRWY reference Zymaflore VL3. Moreover, LVPH, that is, NH 124; and NH 3, NH 88, NH 140, NH 13 and NH 81, were shown by MALDI-TOF/MS biotyping (Bruker Daltonics, Bremen, Germany) to have a close phylogenetic relationship with the low VA producing PS 3 and PS 4, respectively (Figure 3.2). Also noteworthy, is that TFPH and LVPH, that is, NH 6, NH 132 and NH 134 was shown by biotyping to have a close phylogenetic relationship with parental strains that is, PS 1 and PS 2, and PS 3, which was shown to produce wines with tropical fruit aroma (hereafter abbreviated to as TFPP). This provides more evidence supporting the notion that promising hybrids inherited desirable traits from the respective PS.

Identification of microorganisms according to ribosomal protein spectra was reported by Gekenidis *et al.* (2014) and Oumeraci *et al.* (2015). In this study, distinctive ribosomal protein mass spectra of hybrid yeasts compared to parental strains were observed (Figure 3.3) (all data can be obtained from the National Agricultural Proteomics Research & Services Unit (NAPRSU), University of the Western Cape, South Africa). This study complemented research done by Bărbulescu *et al.* (2015), and shows that MALDI-TOF/MS biotyping is a reliable yeast strain identification method that complemented CHEF DNA karyotyping. Biotyping proved to be a rapid identification method resulting in 143 mass spectra, whilst the laborious CHEF DNA karyotyping generated 139 karyotypes. However, CHEF DNA karyotyping still remains the cheaper option. Both techniques allowed for the delineation of genetically related hybrids.

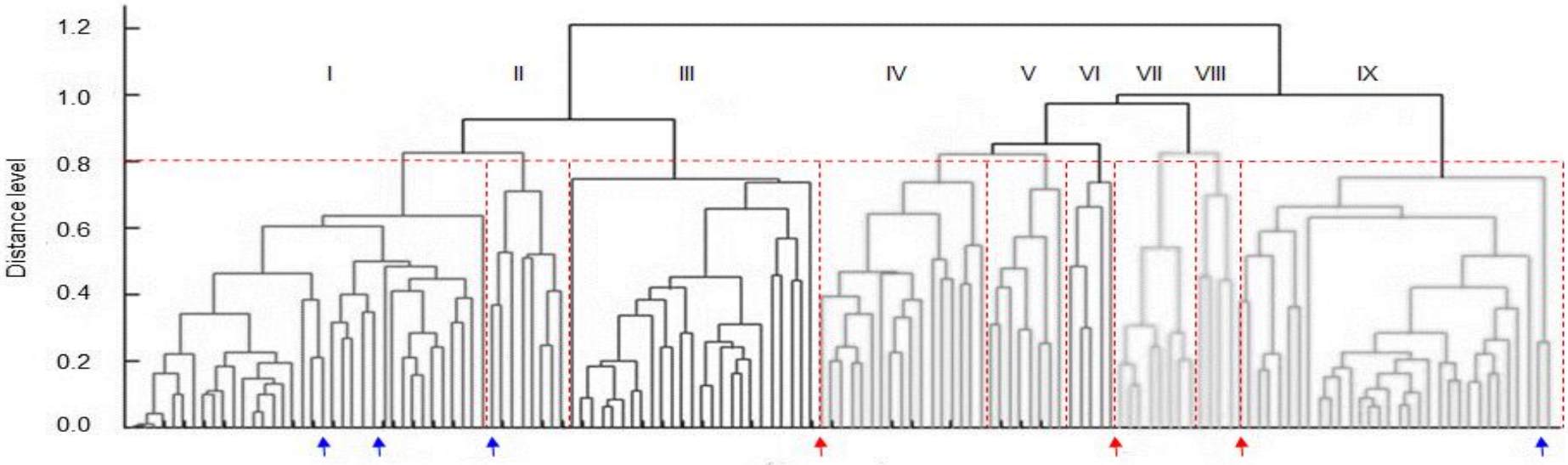


FIGURE 3.2: Principal component analysis (PCA) dendogram generated from matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-TOF MS) biotyping spectra of three commercial 'thiol-releasing' wine yeasts (TRWY)(red arrows), four parental yeast (PS)(blue arrows) and 136 hybrid yeast (NH) strains, generated by cluster analysis using BIOTYPER software (Bruker Daltonics). Dendrogram based on identification score values and distance level is indicative of phylogenetic distance amongst yeast strains. Blue and red arrows indicate parental and commercial reference strains, respectively. Yeast strains were assigned to the same cluster at a 0.80 distance level (dotted line) indicated by Roman numerals.

Cluster I: NH 104, NH 123, NH 24, NH 16, NH 47, NH 108, NH 90, NH 2, NH 9, NH 20, NH 23, NH 22, NH 44, NH 111, NH 75, NH 89, NH 145, NH 52, NH 114, NH 126, PS 1, NH 92, NH 144, NH 66, NH 107, PS 2, NH 6, NH 43, NH 82, NH 127, NH 72, NH 106, NH 29, NH 122, NH 115, NH 18 ; **Cluster II:** PS 3, NH 141, NH 103, NH 15, NH 124, NH 41, NH 93, NH 143; **Cluster III:** NH 37, NH 73, NH 86, NH 74, NH 31, NH 68, NH 132, NH 78, NH 125, NH 55, NH 87, NH 35, NH 38, NH 39, NH 134, NH 139, NH 137, NH 83, NH 36, NH 77, NH 131, NH 133, NH 46; **Cluster IV:** ZYMAFLORE VL3, NH 84, NH 91, NH 110, NH 33, NH 17, NH 56, NH 109, NH 57, NH 76, NH 61, NH 94, NH 135, NH 69, NH 112, NH 85; **Cluster V:** NH 7, NH 25, NH 48, NH 49, NH 102, NH 60, NH 65, NH 59; **Cluster VI:** NH 8, NH 50, NH 71, NH 42, NH 53; **Cluster VII:** FERMICRU 4F9, NH 40, NH 142, NH 54, NH 129, NH 51, NH 99, NH 100; **Cluster VIII:** NH 1, NH 10, NH 97, NH 101; **Cluster IX:** ZYMAFLORE X5, NH 30, NH 36, NH 140, NH 3, NH 13, NH 4, NH 5, NH 138, NH 80, NH 63, NH 32, NH 58, NH 67, NH 81, NH 98, NH 130, NH 116, NH 64, NH 45, NH 62, NH 88, NH 27, NH 28, NH 70, NH 95, NH 34, NH 118, NH 119, PS 4, NH 11.

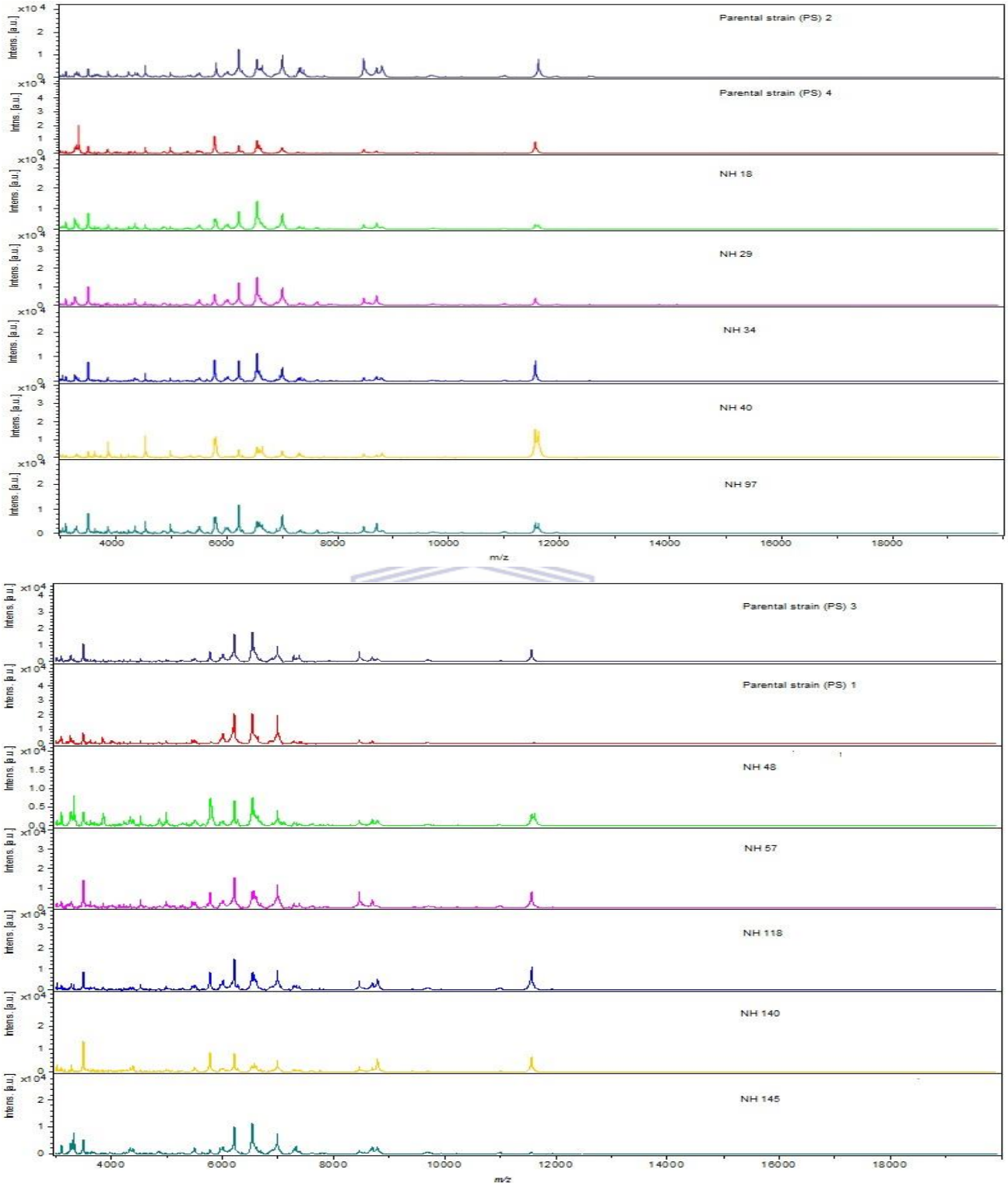


FIGURE 3.3: Matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) spectral fingerprints of four parental strains (PS) and ten hybrid strains (NH). The absolute intensities of the ions and mass-to-charge (m/z) ratios are represented on the y- and x-axis, respectively.

3.4.3 Laboratory-scale fermentation trials

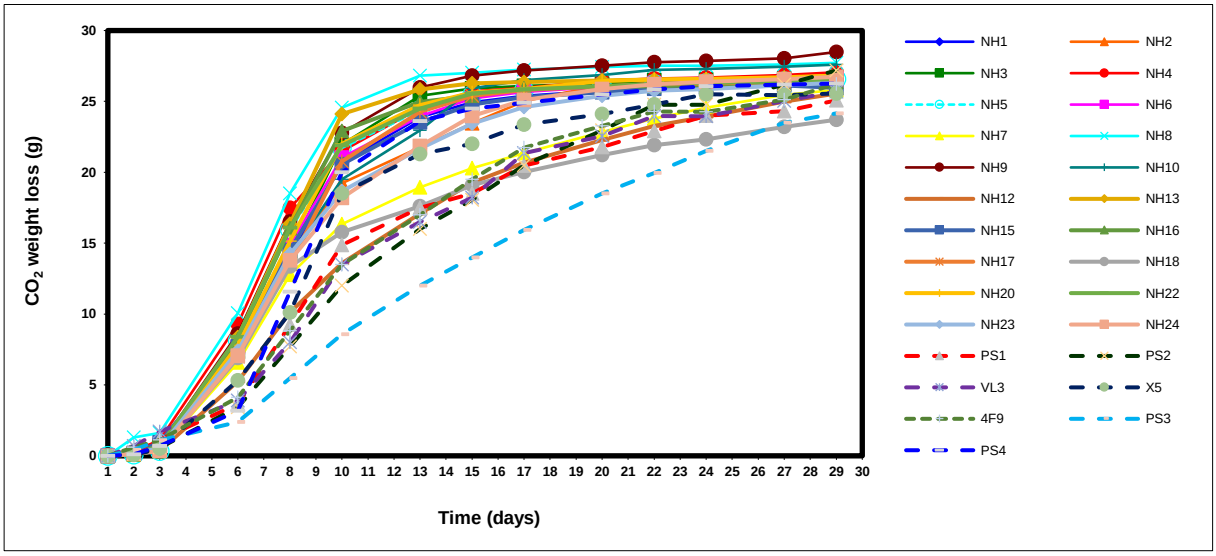
Most hybrids were able to ferment the grape must at a rate similar to commercial TRWY references and PS 1, PS 2, PS 3 and PS 4 (Figure 3.4). Most fermentations were shown to stabilise after 25 days following inoculation with the respective yeast strains. However, hybrids NH 36 and NH 34 fermented at rates noticeably different than the remaining strains included in this trial. Both hybrids produced wines with more vegetative aroma descriptors. Therefore, it can be tentatively surmised that fermentation rates nearby those of commercial TRWY references and TFPP are linked to production of wines with the sought-after fruity and tropical fruit aroma notes, since TFPH (for example, NH 56, NH 48, NH 88, NH 57, NH 3, NH 77, NH 124, NH 24, NH 29, NH 6) had similar rates to that of the commercial references and parental strains. This study complemented previous research which showed that faster fermentation rates improved the sensory quality of wines (Bell & Henschke, 2005). Also noteworthy is that, Shinohara *et al.* (1994) showed that hybrid yeast strains with similar fermentation rates as aromatic wine producing parental strains, was able to produce wines with aroma enhancing metabolites. Nonetheless, both NH 36 and NH 34 were shown to be LVPH and will be used in further breeding programs to improve progeny in this regard.

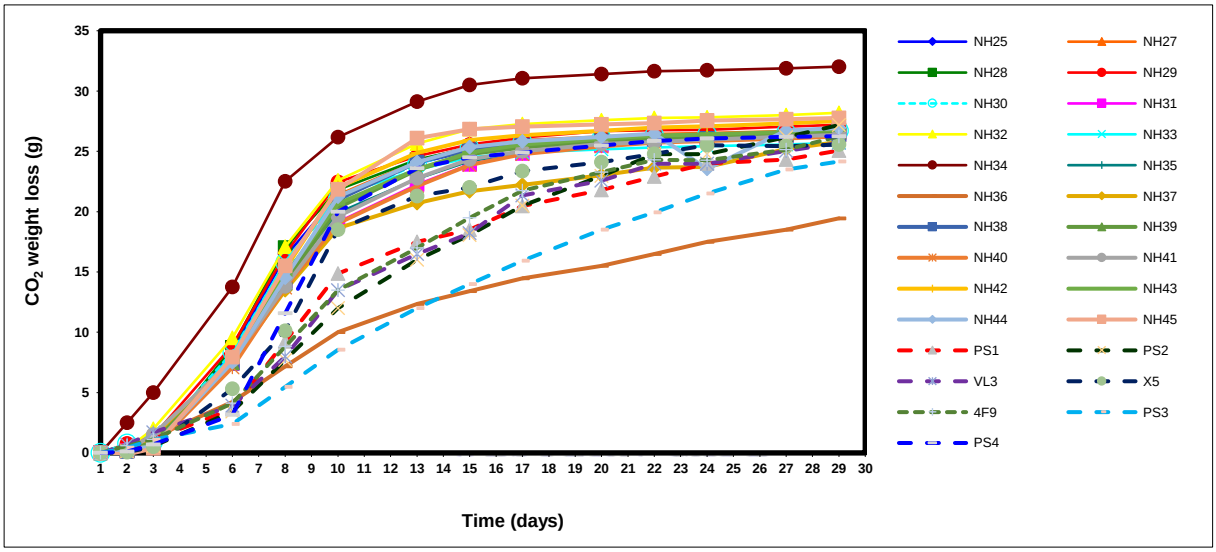
3.4.4 Fourier transform infra-red (FTIR) spectroscopy

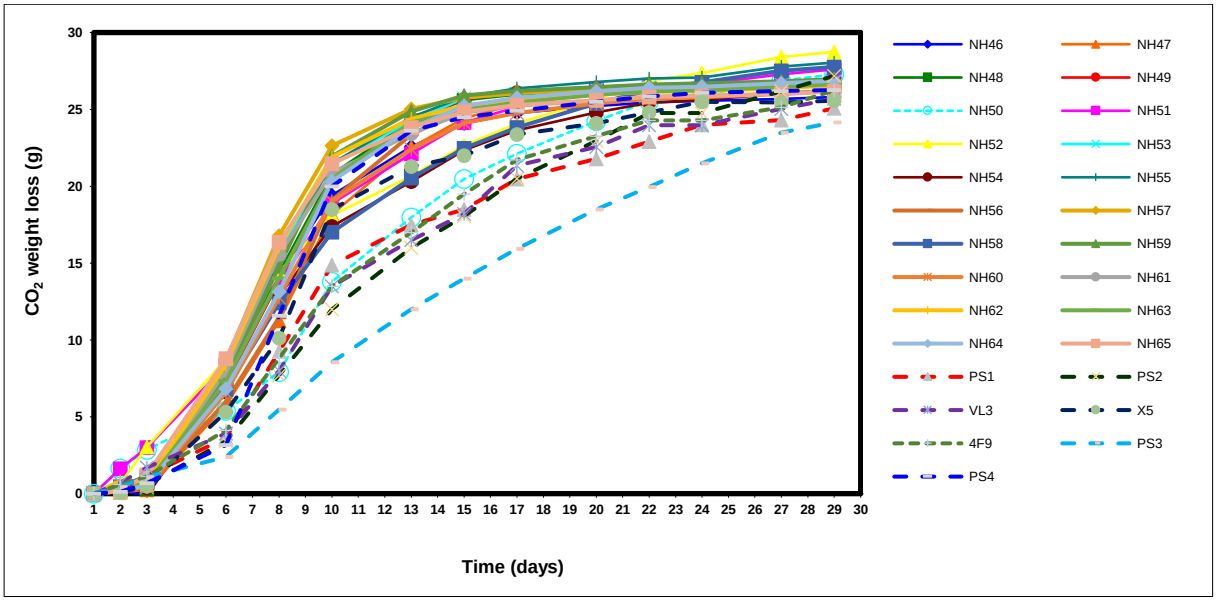
The PCA biplot of standard wine chemical data showed that promising hybrids, including NH 56, NH 48, NH 88, NH 57, NH 3, NH 77, NH 124, NH 24, NH 29, NH 6 situated in the left quadrants produced wines had a negative correlation with VA (Figure 3.5). The same observation was made with regard to PS 3, PS 2, PS 4, which was shown to be low VA producers (hereafter referred to as LVPP) and the commercial TRWY reference Fermicru 4F9. Overall, most hybrid strains produced wine with VA below 0.20 g/L (data not shown), whereas commercial TRWY references Zymaflore VL3 (0.31 ± 0.20 g/L) and Zymaflore X5 (0.50 ± 0.21 g/L) produced wines with significantly higher VA. These results support anecdotal evidence that some commercially available yeast strains can be implicated in VA formation.

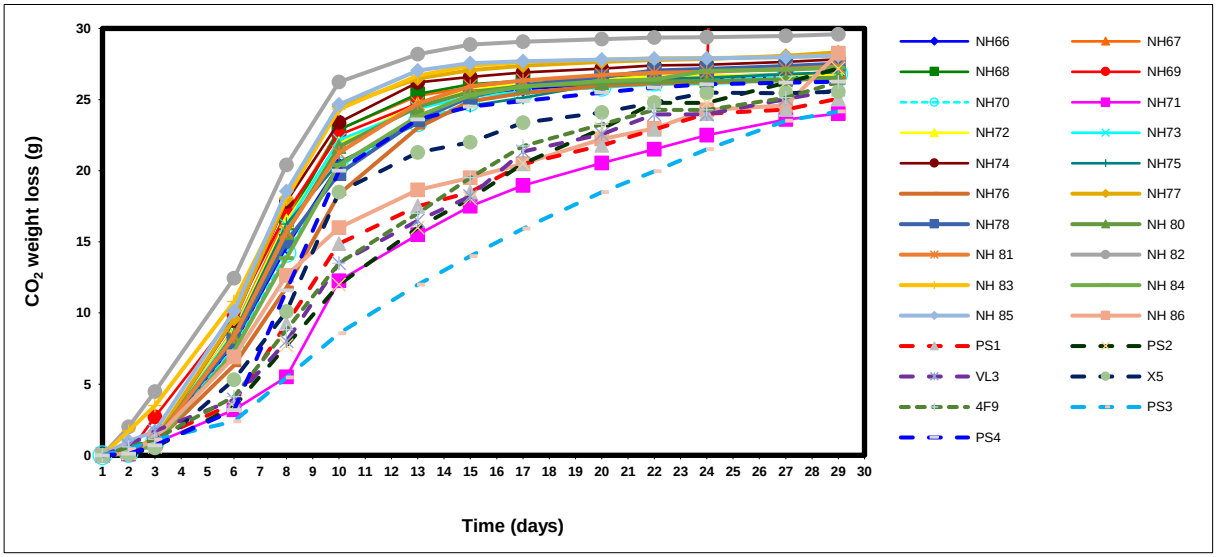
However, all commercial references produced wines with VA levels that comply with legislation. Strain PS 3 (0.02 ± 0.02 g/L) produced wines with the lowest VA of all the PS included in this study. Low VA forming hybrids must have inherited this trait from the respective PS that displayed this quality. Most TFPH produced wines with a more positive association with pH compared to wines produced with commercial TRWY references, Fermicru 4F9, Zymaflore VL3 and Zymaflore X5 (Figure 3.5). However, all yeast strains included in this study on average produced wines with desired pH values (pH 3.3 ± 0.01) as described (Gauntner, 1997; Pambianchi, 2001). It was also observed that plenty of hybrids, including the TFPH mentioned above, produced wines that was perceived to be "fruity", a wine aroma that can be accentuated at this pH.

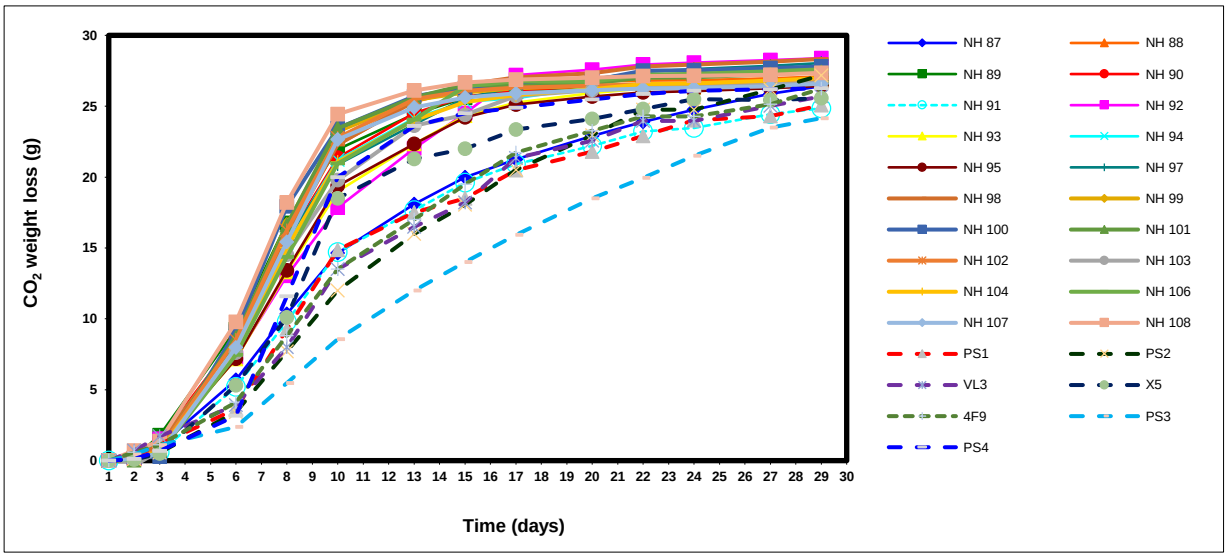
Also noteworthy is that, climate change together with a desire by wine producers to harvest grapes at optimal ripeness has led to grapes harvested with high sugar levels (Palliotti *et al.,* 2014). Consequently, these wines have undesirable high alcohol levels. Wine yeast strains suitable for the production of lower alcohol from grapes with higher sugar were identified as a global industry priority (Gardner *et al.,* 2007; Contreras *et al.,* 2014). Therefore, this study adds value to this priority, since promising LVPH (for example, NH 24, NH 73, NH 77, NH 124 and NH 145) also produced wines with lower alcohol levels (negative association ethanol). It is envisioned that this observation will be investigated further as part of another study.

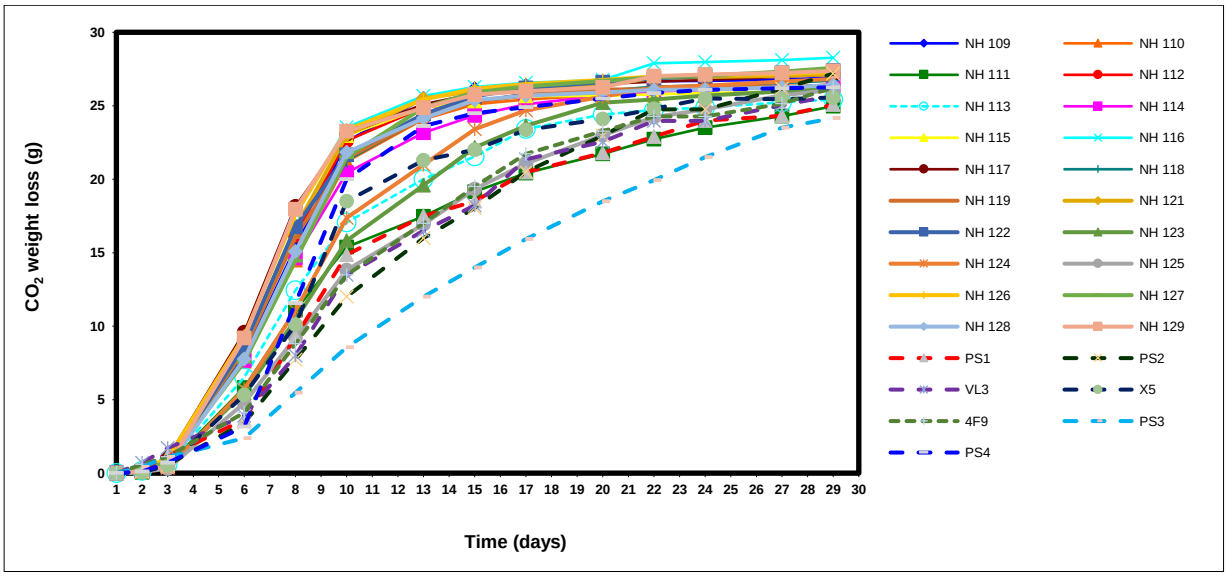












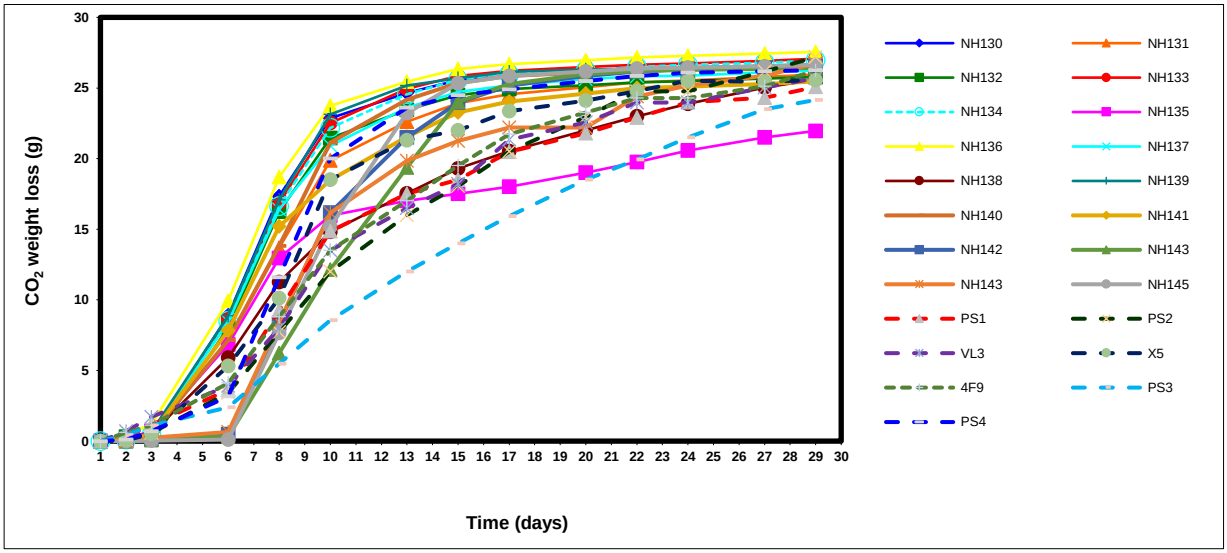


FIGURE 3.4: CO_2 weight loss of Sauvignon blanc grape must fermented at an ambient temperature of 14.5°C at the ARC Infruitec-Nietvoorbij microbiology laboratory using three commercial 'thiol-releasing' wine yeasts (TRWY), four parental yeast (PS) and 136 hybrid yeast (NH) strains.

3.4.5 Sensory evaluation

The biplot of wine sensory data showed no distinct clusters, but rather a spread over the entire sensory space (Figure 3.6). Both commercial TRWY references Zymaflore VL3 and Zymaflore X5 produced wines with a positive association with tropical fruit aromas (Figure 3.6). Moreover, both TRWY were previously recommended for the production of aromatic white wines due to the yeast's 'thiol-releasing' abilities (Anonymous, Personal communication, 2005). The TRWY Fermicru 4F9 produced wines with relative less tropical fruit aroma than afore-mentioned TRWY, however the wines had a greater association with tropical aroma compared to wine produced with for example, PS 3. It is noteworthy that the Zymaflore VL3 produced wines had hints of vegetative aromas, whilst Fermicru 4F9 produced wine with a slight hint of floral aroma. It can tentatively be said that marginal vegetative aromas perceived in the Zymaflore VL3 produced wine is the result of the positive association with VA (Figure 3.5), whilst the hints of floral aroma perceived in the Fermicru 4F9 produced wines were due to overpowering tropical aroma. Therefore, higher VA levels observed in wines produced by Zymaflore X5 and Zymaflore VL3 were somehow masked by the overall positive aromas perceived. Nevertheless, commercial references produced wines with desired aroma notes and VA levels that complies with legislation.

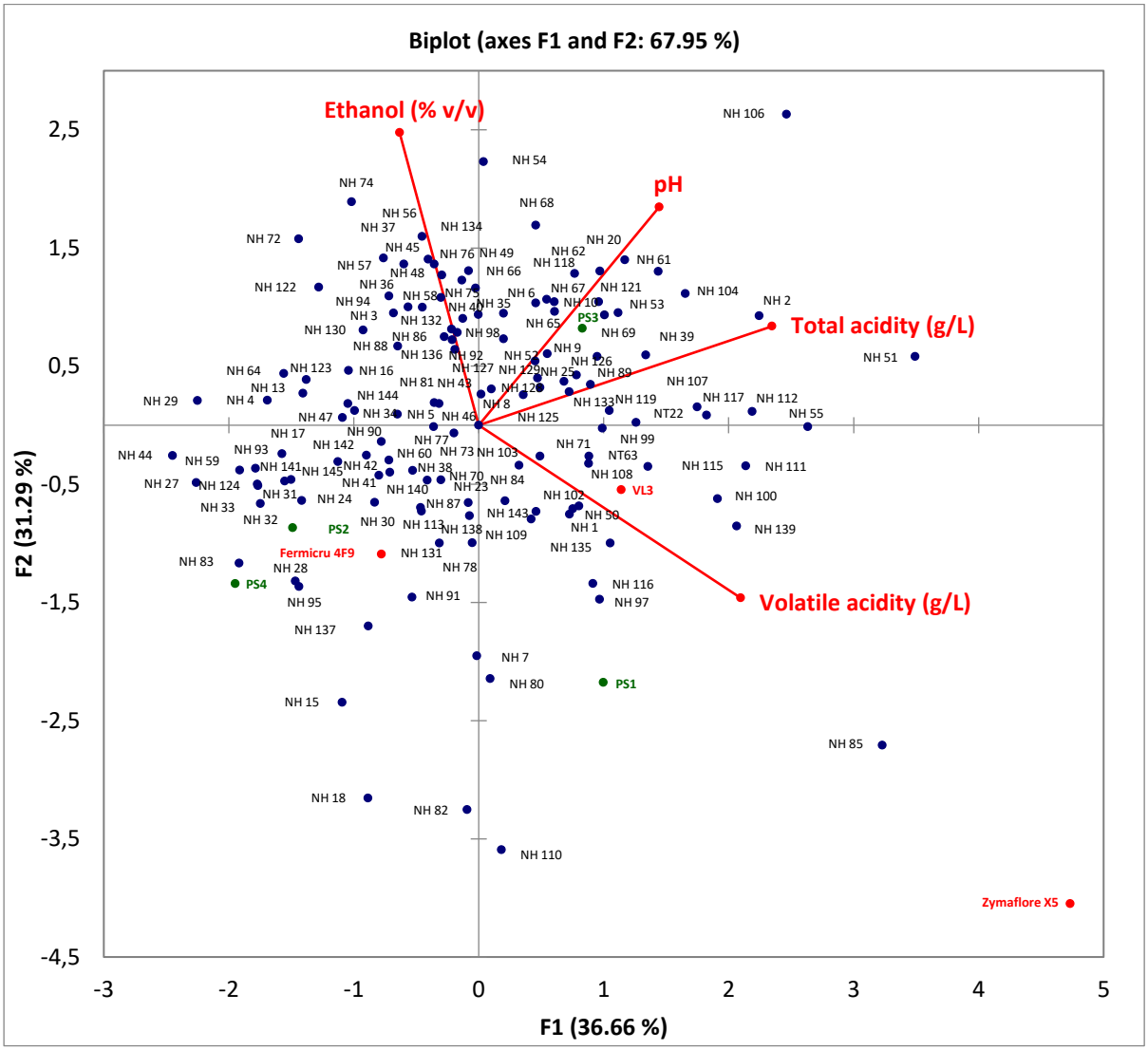


FIGURE 3.5: Biplot of basic chemical parameters of laboratory-scale Sauvignon blanc wine following fermentation by three commercial 'thiol-releasing' wine yeasts (TRWY), four parental yeast (PS) and 136 hybrid yeast (NH) strains. Average values of triplicate fermentations.

Numerous hybrids, amongst others, NH 112, NH 98, NH 88, NH 84, NH 73, NH 67, NH 57, NH 56, NH 48 and NH 6 are considered TFPH, since they produced wines with enhanced tropical fruit aromas compared to commercial TRWY and TFPP. Some of these TFPH were similarly identified as LVPH (Figure 5). These hybrids, therefore, comply with both criteria put forward in the overall objective of this study. Wines with tropical fruit aroma and low VA levels are an industry priority, and the production thereof was previously achieved using co-inoculations and/or GMO (Swiegers *et al.,* 2007). However, the use of GMO for wine production is currently illegal (Berrie, 2011). Therefore, it is

envisioned that afore-mentioned TFPH and LVPH have a commercial role to play, since the fermentation potential of the parental strains were improved through natural occurring classical mating. Moreover, other hybrid strains, NH 78, NH 46, NH 40, NH 34, NH 29, NH 28 and NH 18; and NH 136, NH 130, NH 124, NH 123, NH 92, NH 87, NH 82 and NH 77 that produced wines with pronounced vegetative and floral aromas, were also identified as LVPH. Two TFPP *i.e.* PS 1 and PS 2 produced wines with tropical fruit and floral aromas, whilst the two LVPP *i.e.* PS 3 and PS 4 produced wines with vegetative and floral aromas, respectively. In general, LVPH strains were evenly distributed on the sensory biplot, irrespective of wine sensory attributes.

3.4.6 Gas chromatography-mass spectrometry (GC-MS)

Gas chromatography-mass spectrometry analyses were performed on wines with the most prominent fruity aromas according to the descriptive sensory evaluation to determine aroma compounds that is, esters, total fatty acids and higher alcohols (Lambrechts and Pretorius, 2000). The PCA biplot of GC-MS data showed that the commercial TRWY reference strains Zymaflore VL3 and Fermicru 4F9 produced wines with high ester levels (Figure 3.7). In contrast, Zymaflore X5 and PS 1 produced wines with a positive association with total acids, amongst others, acetic acid. Three TFPH (for example, NH 56, NH 118, and NH 145) produced wines with a negative association with total fatty acids, and therefore comply with both criteria indicated in the aims. The commercial TRWY reference Fermicru 4F9 produced wines with the highest ester levels (5.58 $\pm$ 1.42 mg/L). However, NH 48 produced wines with ester levels (4.07 $\pm$ 0.17 mg/L) that were comparable to wines produced by Zymaflore VL3 (4.80 $\pm$ 0.94 mg/L) and Zymaflore X5 (4.02 $\pm$ 0.80 mg/L), respectively. It is noteworthy that aforesaid TFPH viz. NH 56 (48.74 $\pm$ 0.11 mg/L); NH 118 (63.75 $\pm$ 1.03 mg/L); and NH 145 (75.26 $\pm$ 2.43 mg/L) produced wines with less acetic acid, the main volatile acid than wines produced by Fermicru 4F9 (79.01 $\pm$ 1.23 mg/L). The latter produced wines with the lowest acetic acid of the all commercial references included

in this study. Therefore, GC-MS complemented FTIR spectroscopy, since LVPH also produced wines with lower acetic acid.

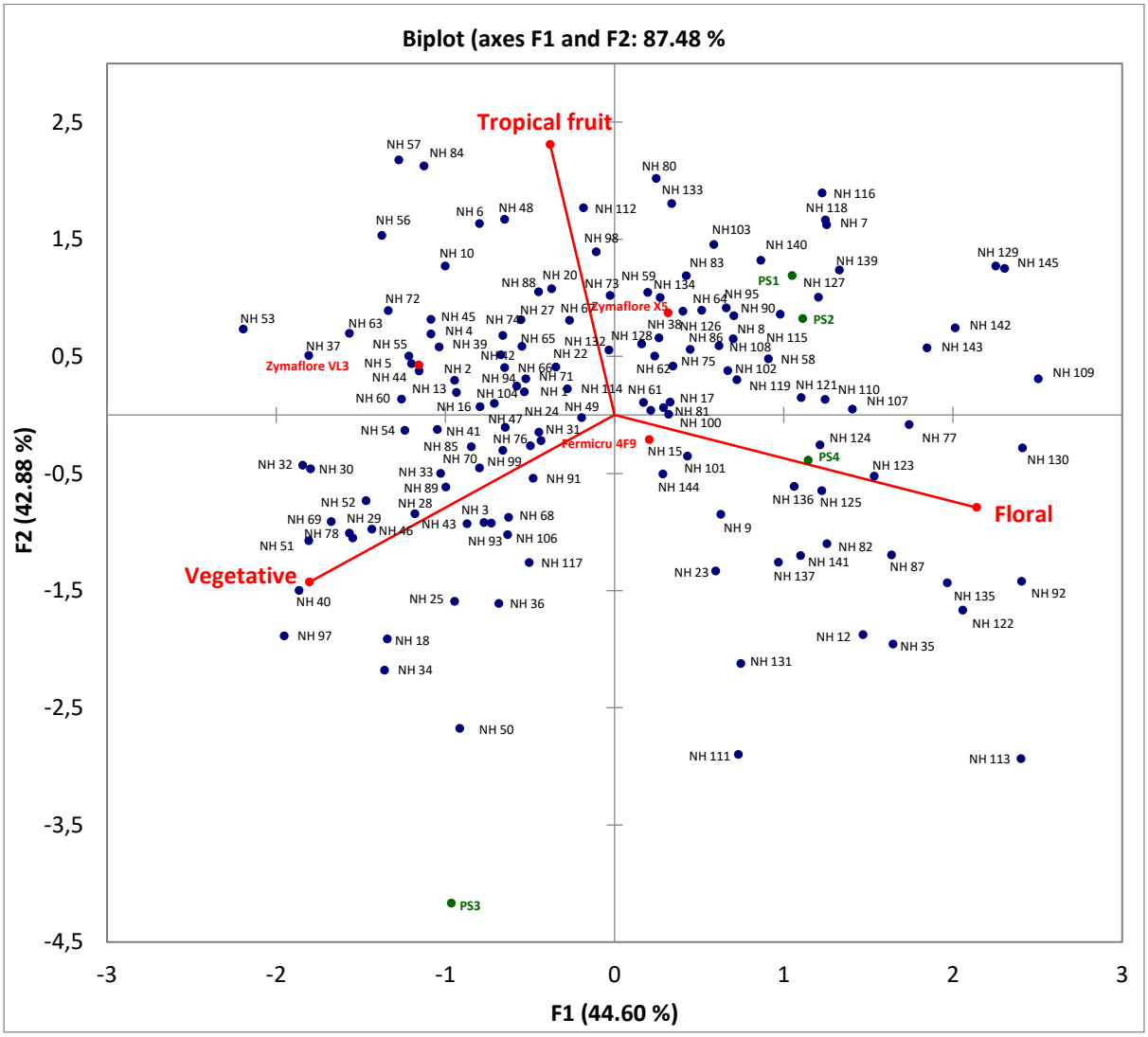


FIGURE 3.6: Biplot of descriptive sensory evaluation of laboratory-scale Sauvignon blanc wine following fermentation by three commercial 'thiol-releasing' wine yeasts (TRWY), four parental yeast (PS) and 136 hybrid yeast (NH) strains. Average values of triplicate fermentations.

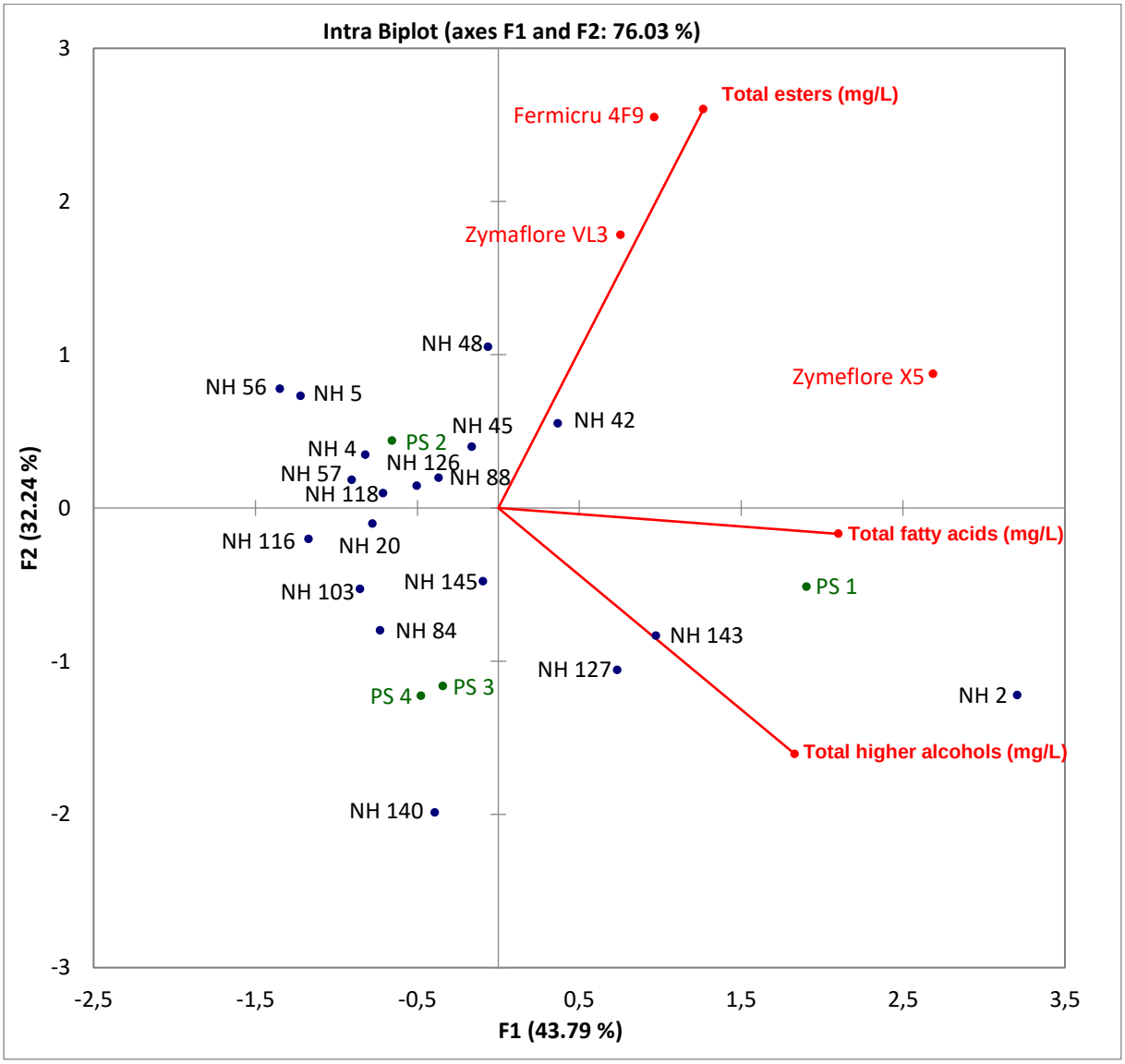


FIGURE 3.7: Biplot of aroma compounds in laboratory-scale Sauvignon blanc wine following fermentation by three commercial 'thiol-releasing' wine yeasts (TRWY), four parental yeast (PS) and selected hybrid yeast (NH) strains that produced wines with the fruitiest aroma. Average values of triplicate fermentations.

3.5. CONCLUSIONS

In conclusion, improved hybrid strains were identified compared to commercial TRWY references and TFPP (for example, PS 1 and PS 2) and LVPP (for example, PS 3 and PS 4) included in this study. These hybrids showed lower VA formation, whilst producing aromatic and/or typical Sauvignon blanc wines. Moreover, observations during this study indicate that some commercially available yeast strains can be associated with VA formation. However, VA formation is also dependent on vintage and generalisation should be avoided. This study showed that classical mating is still practical to produce

novel yeast strain with desired traits, whilst maintaining the green image of wine production. As it was also reported that yeast derived enzymes (proteins) are involved in the release of wine quality enhancing or reducing metabolites during fermentation (Holt *et al.,* 2011; Moreno-García *et al.,* 2015), it is envisioned that wine yeast protein expression will be investigated to establish an association with regulated proteins and metabolites released and/or produced during fermentation.

3.6. CONFLICT OF INTEREST

The authors have not declared any conflict of interest.

3.7. ACKNOWLEDGEMENTS

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Chapter 4

Research results

Characterisation of thiol-releasing and lower volatile acidity forming intra-genus hybrid yeast strains for Sauvignon blanc wine

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Chapter 4: Characterisation of thiol-releasing and lower volatile acidity forming intra-genus hybrid yeast strains for Sauvignon blanc wine

4.1. ABSTRACT

A single *Saccharomyces cerevisiae* wine yeast strain produces a range of aroma and flavour metabolites (*e.g.* volatile thiols), as well as unfavourable metabolites (*e.g.* volatile acidity [VA]) during the alcoholic fermentation of white wine, especially Sauvignon blanc. The former contributes to the organoleptic quality of the final wine. Previous research showed that yeast derived enzymes (proteins) are involved in the release of wine quality enhancing or reducing metabolites during fermentation. Small-scale winemaking trials were initiated to evaluate protein expression and metabolite release of tropical fruit aroma wine producing *S. cerevisiae* hybrid yeasts. Commercial 'thiol-releasing' wine yeasts (TRWY) were included in winemaking trials as references. Improved hybrids which showed enhanced thiol-releasing abilities, specifically 3-mercaptohexanol (3MH), and lower VA formation during the production of Sauvignon blanc wines, were identified and compared to some commercial TRWY references. It is noteworthy that the hybrid NH 56 produced wines with the second highest 3MH levels after hybrid NH 84, and lowest acetic acid of all strains included in this study. This yeast was also the only strain to have down-regulated proteins linked to amino acid biosynthesis, pentose phosphate pathway, glycolysis and fructose and galactose metabolism during the lag phase. Furthermore, differences in protein expression were reflected in the variation of metabolite release by different strains, thereby confirming that enzymes (proteins) are the final effectors for metabolite release.

4.2. INTRODUCTION

During alcoholic fermentation of grape must, the metabolic activity of *Saccharomyces cerevisiae* leads to a range of compounds (metabolites) in wine (Pinu *et al.,* 2015). These metabolites include, amongst others, monosaccharide sugars, organic acids, fatty acids, amino acids, esters, higher alcohols (Chambers *et al.,* 2009). In addition, wine yeasts also mediate the release of metabolites that enhance tropical fruit aroma (*e.g.* volatile thiols) from their grape-derived bound aroma-inactive precursors (King, 2010; Bovo *et al.,* 2015). Some wine yeast strains are implicated in the over-production of undesirable metabolites (*e.g.* acetic acid). Acetic acid accounts for 90% of volatile acidity [VA]), and results in vinegar-like off-flavours in wine (Du Toit & Pretorius, 2000; Swiegers *et al.,* 2005; Ugliano *et al.,* 2009). All these yeast derived and mediated metabolites contribute to the organoleptic character of the wine, and it has been suggested that wine is the 'metabolic footprint' of the wine yeast strain used to carry out the fermentation (Howell *et al.,* 2006; Mapelli *et al.,* 2008). Excessive VA levels in wines will have a negative commercial impact, as these wines will not be marketable. Financial loss can also be incurred by commercial yeast manufacturers, should a commercial wine yeast strain within their portfolio be implicated in excessive VA formation.

Strains of *S. cerevisiae* vary in their ability to produce and/or release volatile thiols, an important aroma compound in Sauvignon blanc, as well as the unfavourable metabolite, acetic acid (Swiegers *et al.,* 2006a; 2007a; Holt *et al.,* 2011). Therefore, the preferred wine yeast strain for Sauvignon blanc should produce varietal aromatic compounds, without production of undesirable off-flavours (Vilela-Moura *et al.,* 2011; Coetzee & du Toit, 2012). Wine yeast strains suitable for the production of aromatic wine with enhanced tropical fruit aroma were identified as a wine industry priority (Swiegers *et al.,* 2006b; 2007b; King, 2010; King *et al.,* 2011). Previous laboratory scale vinification trials with intra-genus hybrids, showed differences in wine chemical and sensory properties (Hart *et al.,* 2016). It was also reported that yeast derived enzymes (proteins) are involved in the release of wine quality enhancing

or reducing metabolites during fermentation (Holt *et al.,* 2011; Roncoroni *et al.,* 2011; Juega *et al.,* 2012; Moreno-García *et al.,* 2015). However, protein expression and metabolite production and/or release of above-mentioned intra-genus hybrids during fermentation of Sauvignon blanc must, was never investigated. This necessitated an in-depth study into wine yeast protein expression and metabolite release and the effect thereof on the organoleptic quality of wine, especially if the South African wine industry is to remain globally competitive. The Sauvignon blanc cultivar is ideally suited for this type of investigation as the grapes contain bound aroma-inactive metabolites that can only be released by wine yeast during fermentation (Swiegers *et al.,* 2006a; Holt *et al.,* 2011). Therefore, this study was undertaken to evaluate a selection of yeasts for the improvement of Sauvignon blanc wine organoleptic quality with regard to tropical fruit aroma and low VA formation. A second objective was to investigate wine yeast protein expression and whether regulated proteins correlate with metabolites released and/or produced during fermentation. Isobaric tags for relative and absolute quantitation (iTRAQ) in conjunction with liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to classify down-regulated and overexpressed proteins originating from three commercial 'thiol-releasing' wine yeasts (TRWY) and five hybrid yeast strains.

4.3. MATERIALS AND METHODS

4.3.1 Yeast strains

A hybrid that was provisionally characterised as having the ability to produce wines with tropical fruit aroma will henceforth be referred to as TFPH. Subsequently, nine TFPH *i.e.* NH 48, NH 56, NH 57, NH 84, NH 88, NH 118, NH 140, NH 143 and NH145. Likewise, a hybrid that was provisionally characterised as a low VA producer will henceforth be referred to as LVPH. Subsequently, five LVPH *i.e.* NH 18, NH 29, NH 34, NH 40 and NH 97 were used in this study. These hybrids are conserved in the ARC Infruitec-Nietvoorbij micro-organism culture collection (ARC Inf-Nvbij CC). Top TRWY strains, *i.e.* VIN 7 and VIN 13 (Anchor Yeast, South Africa), Zymaflore VL3, Zymaflore X5 (Laffort Oenologie, France), and Fermicru 4F9 (DSM Oenology, Netherlands) were included as references. The commercial strain N 96 (Anchor Yeast, South Africa) and an additional experimental hybrid P 35, were also included in this study as they have the ability to produce wine with tropical fruit aromas.

4.3.2 Matrix-assisted laser desorption/ionisation (MALDI) biotyping

All yeast strains were characterised by matrix-assisted laser desorption/ionisation (MALDI) biotyping using a Bruker UltrafleXtreme MALDI-TOF/MS (Bruker Daltonics, Bremen, Germany) as described (Hart *et al.,* 2016).

4.3.3 Small-scale winemaking trials

Sauvignon blanc grapes were routinely sampled and submitted to the cellar laboratory for sugar (°B) and total acidity (TA) analyses. The grapes were harvested from vines grown on the ARC Nietvoorbij Research farm once the °B/TA ratio was ± 2.5. Subsequently, small-scale wines were made in triplicate according to the standard cellar method included in the ARC Infruitec-Nietvoorbij harvest programme

2014 (ARC Infruitec-Nietvoorbij experimental wine evaluation committee). Briefly, the harvested grapes were chilled to 15 °C prior to mechanical destemming. Destemmed grape berries were crushed, followed directly by pressing grape must and skins at 1 Bar. Free-run grape must (juice) was transferred to a sedimentation tank, where after Novozymes (0.5 g/hL) and SO_2 (50 mg/L) were added to aid sedimentation and prevent oxidation, respectively. The skins were discarded. A must sample was subjected to pH, TA, sugar and SO_2 analyses. Thereafter the total SO_2 of the must was adjusted to 50 mg/L and allowed to clarify overnight at 14 °C. Subsequently, clarified must was racked off the sediments by siphoning into a new fermentation container. Nine litres Sauvignon blanc grape must (total sugar 21.9 °B; TA 9.3 g/L; pH 3.28) were dispensed into stainless steel canisters and inoculated with the respective wine yeast starter cultures as described by Hart *et al.* (2016). The method was adjusted by having the respective yeast inoculums cultured for 24 hours in 800 mL yeast extract, peptone, and dextrose (YPD) broth (Biolab, Merck) medium. Subsequently, 180 mL of the 24 hour cultures were used to inoculate clarified Sauvignon blanc grape must (2% inoculum). Thereafter, 50 g/hL diammonium phosphate (DAP) was added. Ninety mL bentonite solution (7.5 %) was added to fermenting must after 48 hours following inoculation. Fermenting must samples were taken every 48 hours using food-grade CO_2 to analyse residual glucose/fructose, ethanol, VA, total acidity and pH using an Oenofoss™ Fourier transform infrared (FTIR) spectrometer (FOSS Analytical A/S, Denmark) until fermentations went to dryness (R/S < 5 g/L). The Oenofoss™ FTIR was calibrated using wine chemical parameters, determined by wet chemistry and external accredited laboratories. The SO_2 was analysed upon completion of the respective fermentations. The wines were racked off the yeast lees and the free-SO_2 adjusted to 35 mg/L, followed by cold stabilisation at 0 °C for at least two weeks.

4.3.4 Gas chromatography (GC) analysis

Wine aroma compounds (*e.g.* esters, total acids and higher alcohols [fusel oils]), were quantified by means of calibration mixtures in conjunction with gas chromatography (GC) as described by Louw (2007). The GC system used in this study comprised of a HP 5890 Series II GC equipped with an HP 7673 Injector and HP 3396A Integrator (Hewlett Packard, Vienna, Austria). Aroma compounds were separated using 60 m x 0.32 mm x 0.5 µm Polyethylene Glycol column (Lab Alliance, State College, PA, USA).

4.3.5 Solid-phase extraction (SPE) and GC-MS/MS analysis

Solid-phase extraction was used to pre-concentrate key wine volatile thiols, *i.e.* 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexylacetate (3MHA) in wines as described by Mateo-Vivaracho *et al.* (2009). Subsequently, GC coupled to tandem mass spectrometry (GC-MS/MS) was used to quantify thiol levels as described by Mattivi *et al.* (2012). However, the GC-MS/MS system used in this study comprised of a GC Trace 1300/ TSQ8000 mass selective detector equipped with an AI 1310 auto sampler (Thermo Scientific™ Inc, USA). Aroma compounds were separated using 30 m x 0.25 mm x 0.25 µm Zebron WAX plus column (Phenomenex Inc., Torrance, CA, USA). Volatile thiol analyses was conducted an accredited laboratory.

4.3.6 Sensory evaluation

Descriptive sensory evaluation of bottled wines was conducted by an experienced panel consisting of 14 members who were requested to indicate the prominence of listed aroma descriptors on a unipolar six-point numerical scale (absent [0], very low [1], low [2], medium [3], high [4] and very high [5]). In addition, panel members had to specify listed aroma descriptors associated with Sauvignon blanc that

they perceived. All wines were coded and served in a randomised order using international wine tasting glasses (approximately 50 mL).

4.3.7 Quantitative LC-based iTRAQ proteomic analysis

4.3.7.1 Protein sample preparation

Eight yeast strains (VIN 7, Zymaflore X5, Zymaflore VL3, N 96, P 35, NH 56, NH 57 and NH 97) were subjected to protein extraction based on chemical (lower VA and total fatty acids) and sensory (tropical fruit aroma) analyses. Fermenting Sauvignon blanc grape must were sampled (50 mL) during the lag phase (48 hours following inoculation) and stationary phase (end of fermentation). Thereafter, samples were aliquoted into micro-centrifuge and pelleted at 14000 rpm for 30 seconds. The supernatant was discarded and the previous steps repeated until the yeast pellet weighed 50 mg, whereafter protein extraction was conducted according to the method described by Von den Haar (2007). Briefly, yeast pellet were dissolved in 400 µL lysis buffer (0.1 M NaOH, 0.05 M EDTA, 2 % (w/v) SDS and 2 % (v/v) 2-mercaptoethanol and sterile distilled water). Cell mixture was heated for 10 min at 90 °C to disrupt cells, whereafter 10 µL of acetic acid was added to the lysates and heated for an additional 10 min at 90 °C. Protein quantification and characterisation were conducted by deploying an iTRAQ 8-plex reagent kit (AB Sciex, USA), in accordance with the manufacturer's instructions.

4.3.7.2 Protein alkylation, digestion and isobaric labelling

Cysteine-residues of proteins were alkylated using 200 mM methane methylthiosulphonate (MMTS) as described by Boutureira & Bernardes (2015). Thereafter, proteins were digested overnight at 37 °C using 1 µg/µL trypsin solution (Promega, Madison, WI, USA). Subsequently, tryptic digests were air-dried and dissolved in water containing 2% acetonitrile/0.1% formic acid (Sigma-Aldrich, South Africa). The solution was again air-dried with the exception that it was re-suspended in triethylamonium

bicarbonat (TEAB). Tryptic digests extracted from the yeast strains were tagged with iTRAQ labels 113, 114, 115, 116, 117, 118, 119 and 121, respectively as described by Kim *et al.* (2012).

4.3.7.3 Liquid chromatography–tandem mass spectrometry (LC-MS/MS)

Mass spectrometry for protein characterisation was performed using a mass spectrometer equipped with a Nanospray flex ionisation source (Thermo Scientific™ Inc, USA). Samples were introduced through a stainless steel emitter as described by Vehus *et al.* (2016). Data was generated using synchronised pre-cursor selection with MS3 reporter ion generation. Subsequently, the raw files were imported into Proteome Discoverer v1.4 and processed using the Mascot algorithm (Matrix Science, London, UK), as well as the SequestHT algorithm included in Proteome Discoverer. Protein quantitation was performed using the iTRAQ quantitation algorithm. Only proteins with more than 2 peptides, but less than 20% variation, and iTRAQ ratios below 0.5 and above 2 were considered down-regulated and over-expressed, respectively.

4.3.7.4 Bioinformatics analysis

Proteins that were shown to be differentially expressed using quantitative iTRAQ were subjected to Protein ANalysis THrough Evolutionary Relationships (PANTHER, www.pantherdb.org/) to establish their involvement in biological processes, molecular function and protein classes (Sharma *et al.,* 2014).

4.3.7.5 Statistical analyses

Data generated following chemical, sensory and metabolomic analyses were subjected to analysis of variance (ANOVA) and principal component analysis (PCA) as described (Pearson,1896; 1901; Zou *et al.,* 2006). A Pearson's correlation was performed to study the linear relationship between the

chemical, sensory and metabolomic variables and standardise data prior to performing the PCA using XLSTAT software (Addinsoft, 2013) with the principal components (PC's) as factors (*i.e.* F1 and F2).

4.4. RESULTS AND DISCUSSION

4.4.1 Matrix-assisted laser desorption/ionisation (MALDI) biotyping

All 21 yeast strains were shown to have distinctive mass spectra (Figure 4.1), and belonged to the species *S. cerevisiae* (Table 4.1) as decribed (Hart *et al.,* 2016).

4.4.2 Fourier transform infra-red (FTIR) spectroscopy

The PCA biplot of standard wine chemical showed that the LVPH NH 18, NH 29, NH 34 and NH 97 and the TFPH NH 48, NH 56, NH 57, NH 88, NH 143, and NH 145, were positioned in the left and bottom quadrants, and the wines had a negative association with VA (Figure 4.2). Indications, therefore, are that these TFPH are also LVPH. The same observation was made with regard to commercial TRWY references, despite some producing wines with a more positive association with VA. However, Hart *et al.* (2016) reported that aforesaid yeast strains produced wines with VA levels that comply with legislation. None of the wines were perceived to be undesirable during descriptive sensory evaluation (Figure 4.3).

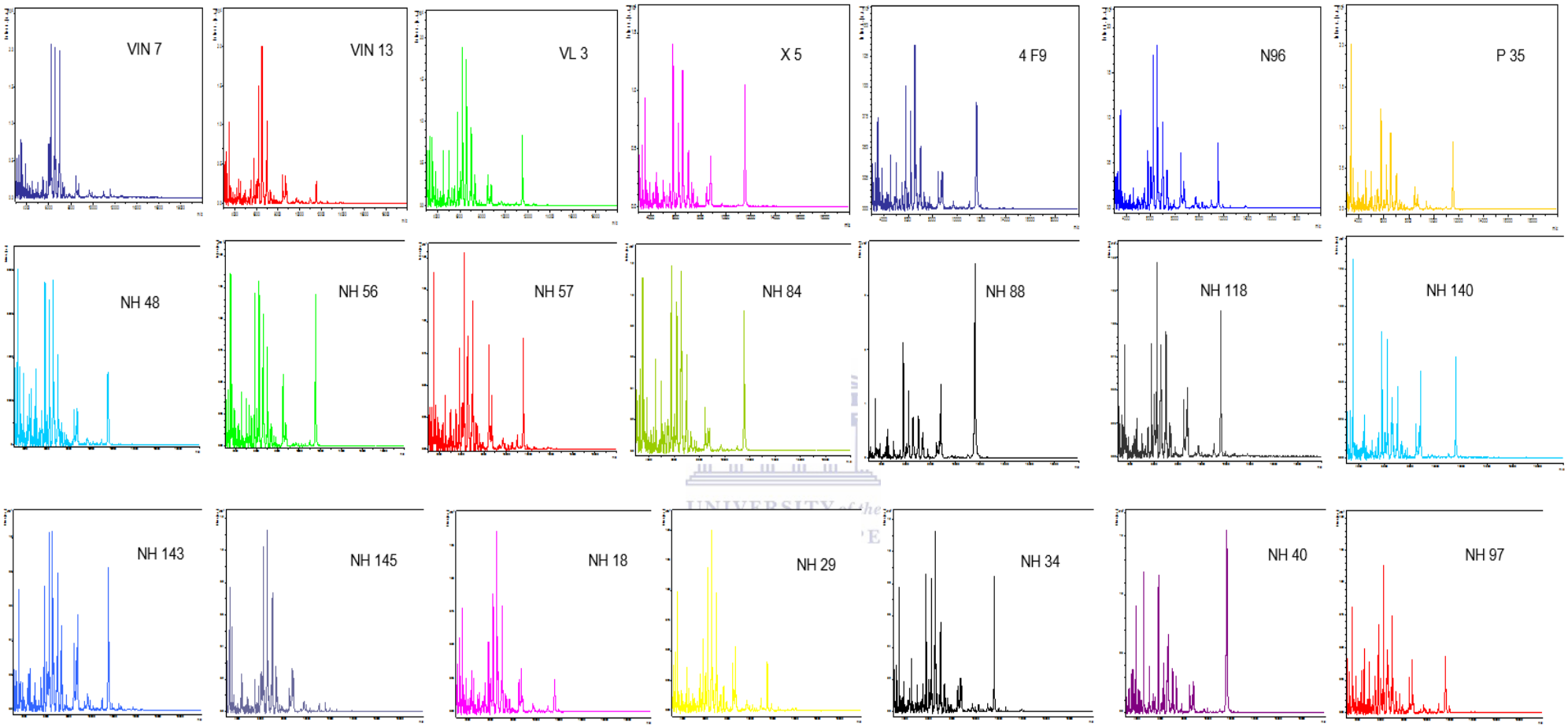

Figure 4.1: Matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF/MS) biotyping of commercial wine yeasts and experimental yeast strains conserved in the ARC Infruitec-Nietvoorbij microbial culture collection, selected for the production of aromatic white.

TABLE 4.1: Matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) real time classification of yeast strains used for the production of varietal Sauvignon blanc wines.

Mass spectra number	Yeast strain	MALDI-TOF MS log (score) value	Identification
1	**VIN 7[2] (Reference 1)**	2,1	*Candida Robusta*[1]
2	**VIN 13[2] (Reference 2)**	2,1	*Candida Robusta*[1]
3	**Zymaflore VL3[3] (Reference 3)**	2,1	*Candida Robusta*[1]
4	**Zymaflore X5[3] (Reference 4)**	1,9	*Candida Robusta*[1]
5	**Fermicru 4F9[4] (Reference 5)**	2,2	*Candida Robusta*[1]
6	**N 96[2] (Reference 6)**	2,0	*Candida Robusta*[1]
7	**P 35[5] (Reference 7)**	2,0	*Candida Robusta*[1]
8	**NH 18[5]**	2,0	*Candida Robusta*[1]
9	**NH 29[5]**	2,1	*Candida Robusta*[1]
10	**NH34[5]**	2,1	*Candida Robusta*[1]
11	**NH 40[5]**	1,9	*Candida Robusta*[1]
12	**NH 48[5]**	2,0	*Candida Robusta*[1]
13	**NH 56[5]**	2,0	*Candida Robusta*[1]
14	**NH 57[5]**	2,0	*Candida Robusta*[1]
15	**NH 84[5]**	1,9	*Candida Robusta*[1]
16	**NH 88[5]**	2,1	*Candida Robusta*[1]
17	**NH 97[5]**	2,1	*Candida Robusta*[1]
18	**NH 118[5]**	2,1	*Candida Robusta*[1]
19	**NH 140[5]**	2,0	*Candida Robusta*[1]
20	**NH 143[5]**	2,0	*Candida Robusta*[1]
21	**NH 145[5]**	2,1	*Candida Robusta*[1]

[1]*Candida robusta* (anamorph of *Saccharomyces cerevisiae*); [2]Commercial yeast (Anchor Wine Yeast, South Africa); [3]Commercial yeast (Laffort Oenologie, France); [4]Commercial yeast (DSM Oenology, Netherlands). [5]Experimental yeast (ARC Infruitec-Nietvoorbij).

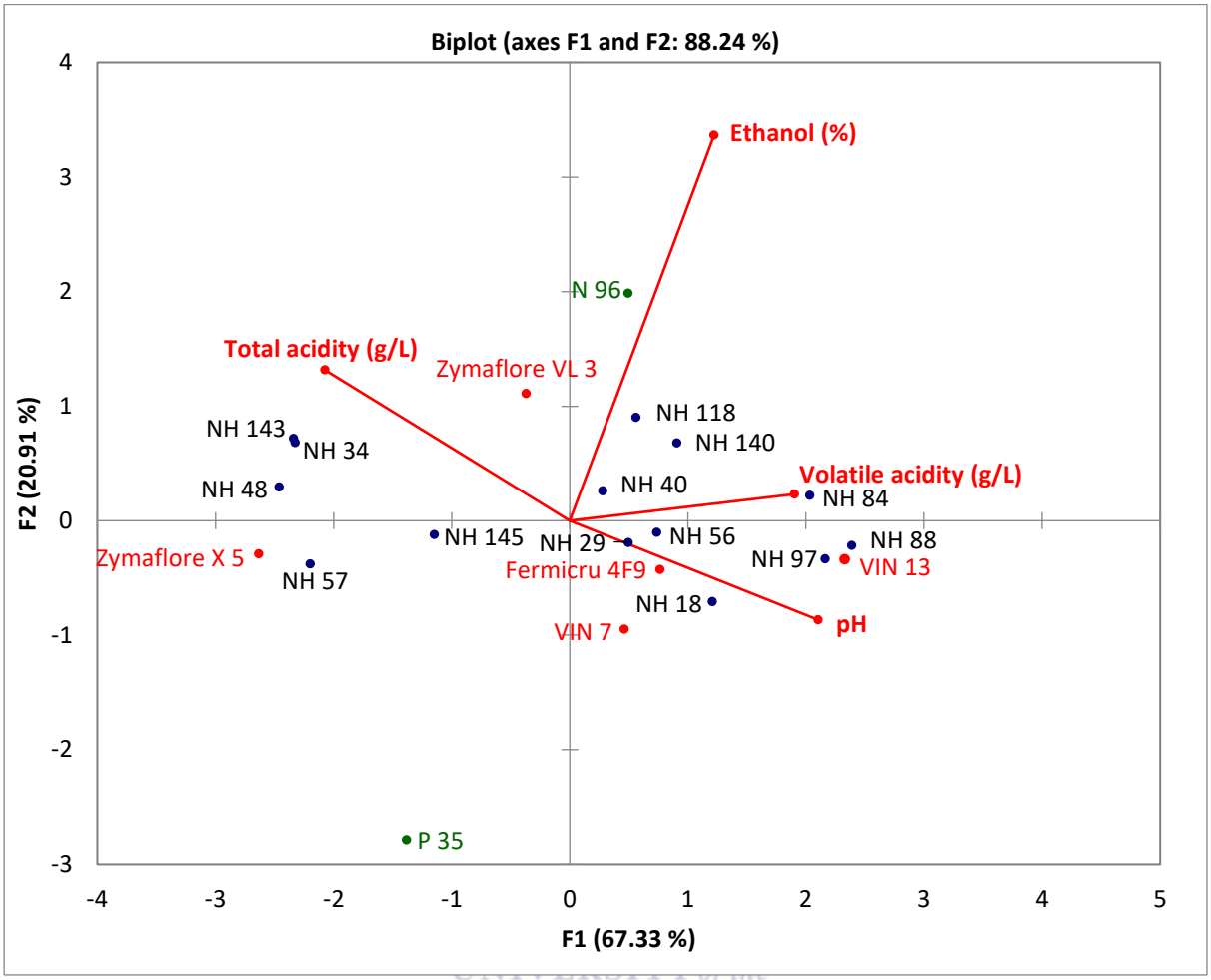

Figure 4.2: Biplot of basic chemical parameters of small-scale Sauvignon blanc wine following fermentation by five 'thiol-releasing' commercial wine yeasts (TRWY), two 'neutral' yeasts strains, nine hybrids shown to produce wines with tropical fruit aroma (TFPH), and five low VA producing hybrids (LVPH). Average values of triplicate fermentations.

4.4.3 Sensory evaluation

The PCA biplot of descriptive sensory evaluation showed that two commercial TRWY references Zymaflore X5 and VIN 7 produced wines with a positive association with tropical fruit aromas, whilst Zymaflore VL3 and Fermicru 4F9 produced wines positively associated with floral and vegetative aromas (Figure 4.3). The TRWY VIN 13 produced wines positively associated with both tropical fruit and vegetative aromas. Marais (1994) and Lapalus (2016) reported that green pepper and/or vegetative aromas can be attributed to Sauvignon blanc grape-derived aroma compounds (*e.g.* 2-isobutyl-3-methoxypyrazine [IBMP]). In this trial, these compounds could have masked the sought-

after tropical fruit aroma of the VIN 13 produced wine. Nonetheless, production of wines with tropical fruit aromas, is indicative of volatile thiol release and/or production which supports yeast manufacturers recommendations that above-mentioned TRWY strains are volatile "thiol-releasers" (Swiegers *et al.,* 2009). This was further confirmed by Swiegers *et al.* (2006a) and Srisamatthakarn (2011) who reported that commercial TRWY references included in this study do in fact have enhanced thiol releasing abilities.

Reference strains, namely N 96 and P 35 also produced wines with a positive association with tropical fruit and vegetative aromas, respectively as previously reported by Hart *et al.* (2016). Two TFPH namely, NH 57 and NH 140, produced wines that were perceived to have tropical fruit aromas, whilst NH 56 produced wines with almost equally intense tropical fruit and vegetative aromas. In contrast, three TFPH, namely NH 48, NH 118 and NH 145, produced wine with a positive association with floral aromas. The remainder of the TFPH, namely NH 84, NH 88 and NH 143, produced wines with prominent tropical aroma with hints of floral aromas. Floral aroma can also be a characteristic of new world Sauvignon blanc wines (Von Mollendorf, 2013). These hybrids produced wines that also had a negative association with vegetative aromas.

Sensorially, most TFPH included in this trial produced wine with equivalent and more pronounced tropical fruit and floral aromas compared to wines produced by TRWY references. The LVPH, namely NH 29 and NH 40, produced wines that had a positive association with tropical fruit aromas, whereas wines produced by NH 18 and NH 97 had a positive association with vegetative and tropical fruit aromas. The LVPH, namely NH 34 on the other hand produced wines with floral aroma and tropical fruit aromas. Indications, therefore, are that some LVPH are also TFPH. Overall, most TFPH produced wines with more tropical fruit than vegetative aromas.

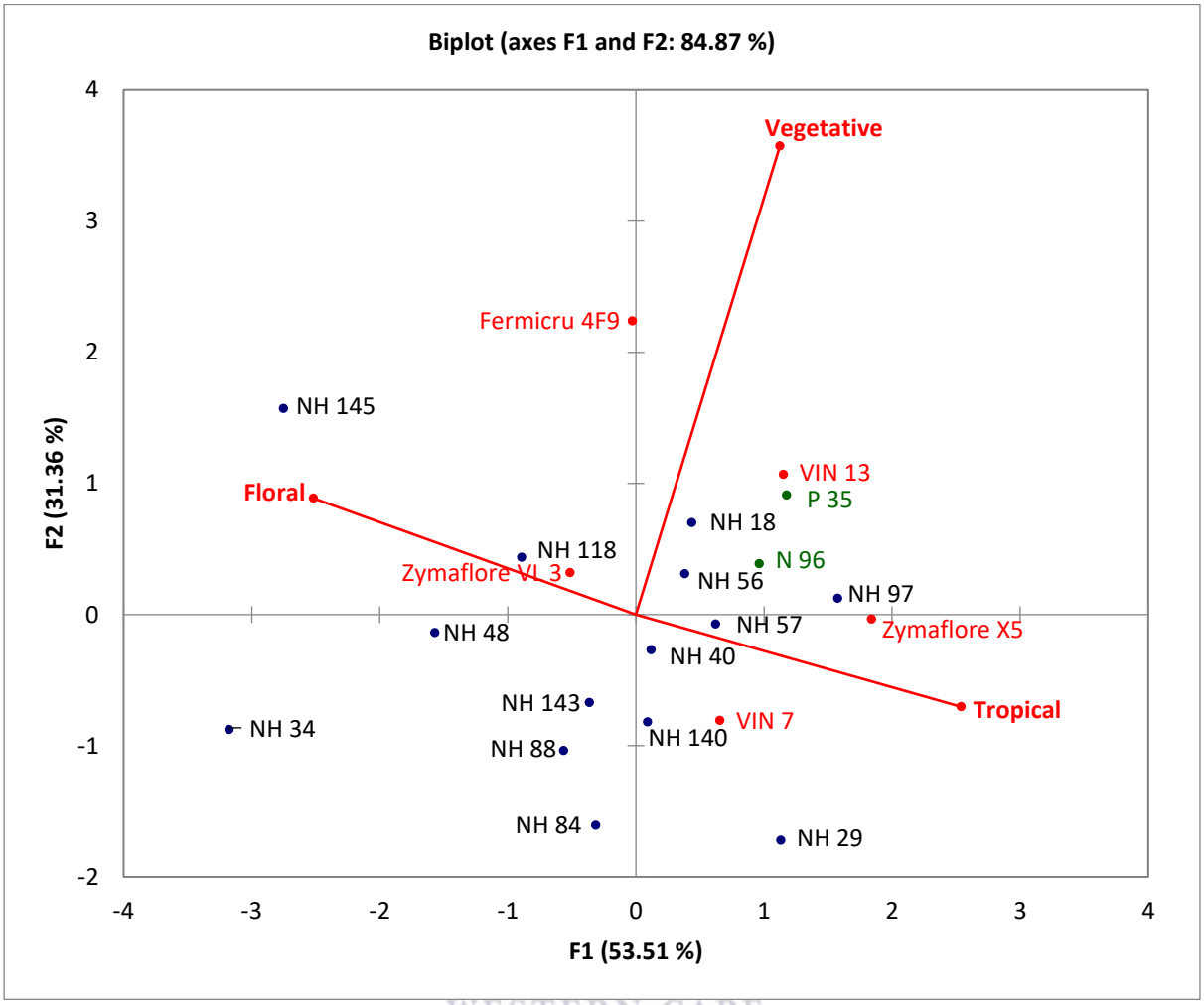

Figure 4.3: Biplot of descriptive sensory evaluation of small-scale Sauvignon blanc wine following fermentation by five 'thiol-releasing' commercial wine yeasts (TRWY), two 'neutral' yeasts strains, nine hybrids shown to produce wines with tropical fruit aroma (TFPH), and five low VA producing hybrids (LVPH). Average values of triplicate fermentations.

4.4.4 Gas chromatography (GC) analysis

Esters, total fatty acids and higher alcohol levels of all small-scale wines were analysed, as these aroma compounds (metabolites) contribute positively to 'fermentation bouquet' when present in moderate concentration (Lambrechts & Pretorius, 2000; Coetzee & du Toit, 2015). The PCA biplot showed that the TRWY reference, VIN 7, produced wines with the most positive association with esters compared to any other yeast strain included in this study (Figure 4.4). The TRWY references Zymaflore X5, Fermicru 4F9 and VIN 13 and the commercial reference strain N 96, positioned in the upper quadrants, also produced wines with a positive association with esters. This observation complements the sensory

evaluation results, where aforesaid yeast strains had a positive association with fruity aroma (Fig. 3). Wine esters are the main contributors to wine fruity aromas (Coetzee & du Toit, 2015). In contrast, TRWY reference, Zymaflore VL3, and the hybrid, P 35, produced wines with a negative association with esters, although P 35 produced wine that had a positive association with fruity aromas following sensory evaluation.

Hybrids NH 34 and NH 48 produced wines with a positive association with volatile fatty acids (*e.g.* octanoic acid, decanoic acid), which can be associated with faint fruity and citrus wine aroma (Lambrechts & Pretorius, 2000). However, wine fatty acids include acetic acid, the main acid responsible for vinegar-like off-flavours at higher concentrations (Vilela-Moura *et al.,* 2011). Furthermore, Zoecklein *et al.* (1995) reported that other steam distillable carboxylic acids (*e.g.* lactic acid, formic acid, butyric acid and propionic acid) also contribute to total acid content of wine. Volschenk *et al.* (2006) and Jackson (2014) reported that besides distillable acids, nonvolatile acids (*e.g.* malic and tartaric acid) also contribute to TA that was traditionally measured as an indicator of VA. Hybrids NH 34 and NH 48 are not necessarily high VA producers, since they were reported to be LVPH (Hart *et al.,* 2016). The TRWY references, namely Zymaflore VL3, Fermicru 4F9, VIN 13 and hybrid P 35, produced wines with a negative association with total fatty acids. The same observation was made with regard to five hybrids, namely NH 29, NH 57, NH 84, NH 88, and NH 140 that also produced wines with prominent tropical fruit aromas (Figure 4.3). Therefore, these strains comply with yeast selection criteria based on the ability to produce wine with tropical fruit aroma and low VA formation.

Overall, the level of higher alcohols in all wines were 242 ±37.18 mg/L. Five hybrids, namely NH 34, NH 40, NH 48, NH 143 and NH 145 were shown to produce wines with a positive association with higher alcohols. Higher alcohol concentrations below 300 mg/L add complexity to wines (Muñoz *et al.,* 2006; Styger, 2011; Von Mollendorf, 2013; Moss, 2015). Complexity of above-mentioned wines is evident as NH 48 and NH 145 produced wines with a positive association with floral aromas (Figure 4.3), whilst NH 34, NH 40 and NH 143 produced wines associated with tropical fruit and floral

aromas (Figure 4.3). Lambrechts & Pretorius (2000) reported that higher alcohols are key precursors involved in ester formation during alcoholic fermentation, which complements this study as these hybrids produced wines with a positive association with tropical fruit aroma (Figure 4.3) and esters (Figure 4.4).

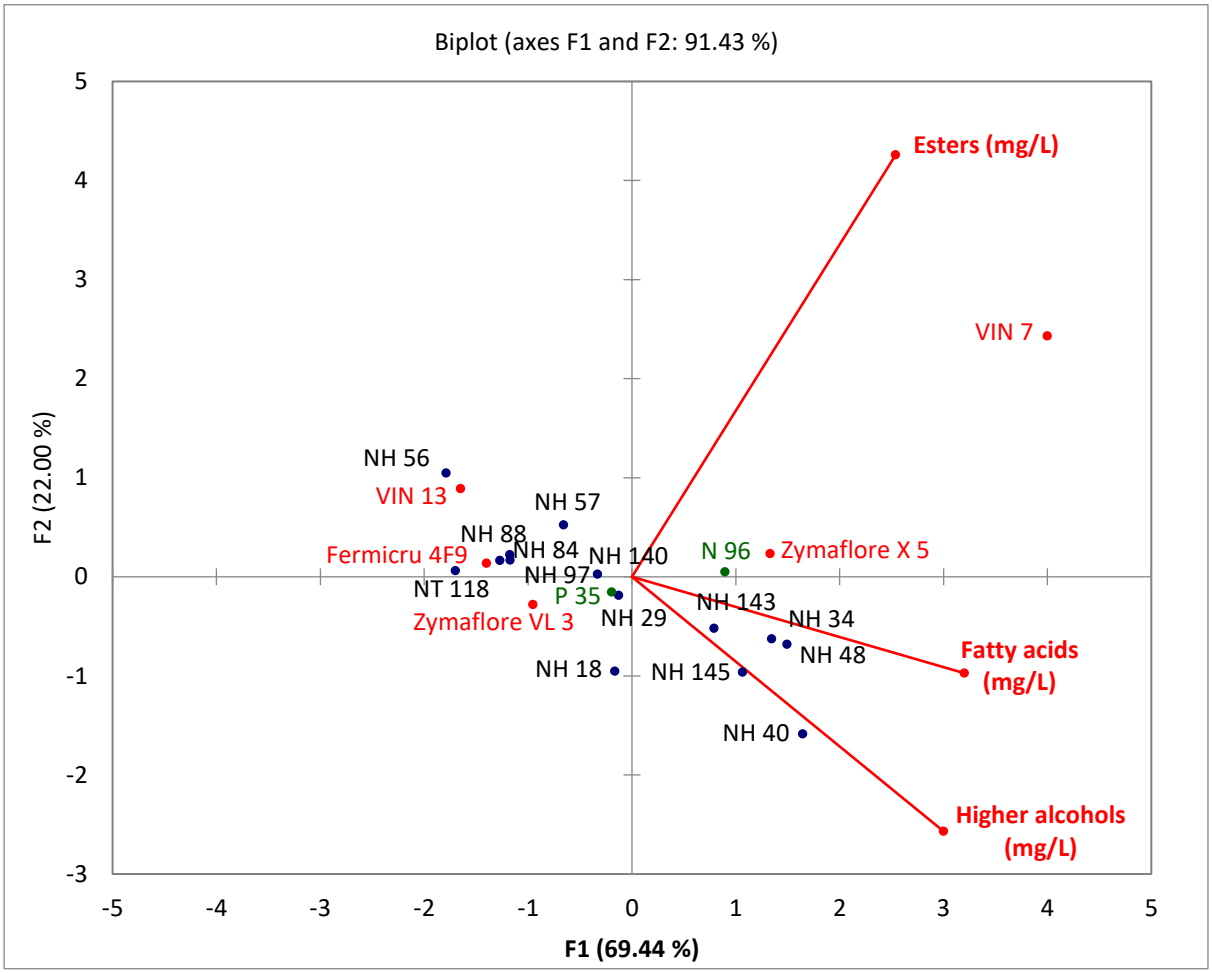

Figure 4.4: Biplot of aroma compounds, i.e. esters, higher alcohols and fatty acids in small-scale Sauvignon blanc wine following fermentation by five 'thiol-releasing' commercial wine yeasts (TRWY), two 'neutral' yeasts strains, nine hybrids shown to produce wines with tropical fruit aroma (TFPH), and five low VA producing hybrids (LVPH). Average values of triplicate fermentations.

4.4.5 Solid-phase extraction (SPE) and GC-MS/MS analysis

Volatile thiols contribute positively to 'varietal aroma' of Sauvignon blanc wines (Fedrizzi *et al.,* 2007; Pinu *et al.,* 2012). Two thiols in particular *viz.* 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) are the key aroma enhancing metabolites in white wines, especially Sauvignon blanc,

and are produced by wine yeasts during fermentation from odourles precursors present in grape juice (Swiegers *et al.,* 2007b; Roland *et al.,* 2011; Harsch *et al.,* 2013). The SPE-GC-MS/MS data of the analysed wines is presented as a PCA biplot in Figure 4.5. The TRWY references, VIN 7 and Fermicru 4F9, and hybrid P 35, produced wines with a positive association with both thiols. The TRWY namely, VIN 13 and Zymaflore VL3 produced wines positively associated with 3MH, whilst the TRWY Zymaflore X5 and the commercial reference strain N 96 produced wines positively associated with 3MHA (Figure 4.5). Overall, 3MH levels in these wines were, 270, 258, 280, 266, 155, 140 and 150 ng/L. It is noteworthy that 3MH levels in the VIN 7 produced wine was at least five times its sensory detection threshold (van Wyngaard, 2013), explaining the positive association with tropical fruit aroma (Figure 4.3). The TRWY references, Zymaflore X5 and Zymaflore VL3 produced wines with the lowest 3MH and 3MHA of all TRWY references. This is contrary to results from Dubourdieu (2006) and Bowyer *et al.* (2008) who reported both yeasts to be high thiol-releasers. Nonetheless, 3MH levels in both wines were twice their sensory detection thresholds and these wines had a positive association with tropical fruit and floral aromas (Figure 4.3).

Seven hybrids (NH 48, NH 56, NH 57, NH 84, NH88, NH 118, NH 140 and NH 143) produced wines with varying 3MH levels (264, 360, 196, 493, 315, 234, 217 and 206 ng/L). These hybrids also produced wines with the strongest association with 3MH and 3MHA compared to wines produced with Zymaflore X5, VIN 13 and Zymaflore VL3 (Figure 4.5). The 3MH levels in the TFPH NH 84 produced wine was at least eight times its sensory detection threshold (van Wyngaard, 2013), and twice that observed for the commercial TRWY reference VIN 7. Some of these hybrids were shown to produce wines with a more positive association with tropical fruit aromas (Figure 4.3) and lower total fatty acids (Figure 4.4) than wines produced with the commercial references namely Zymaflore VL3 and Fermicru 4F9. Hybrids NH 56, NH 84 and NH 88 that produced wines with a positive association with esters (Figure 4.4) also produced wines with the highest 3MH levels of all yeast strains included in this study. The remaining TFPH also produced wines with a stronger association with 3MH and 3MHA than some of the commercial references. The hybrid yeast P 35 that was also utilised as a parental yeast in the hybrid

breeding programme produced wines with higher 3MH levels than any of the commercial TRWY references. Therefore, TFPH that produced wines with a strong association with previously mentioned thiols, could have inherited this trait from P 35.

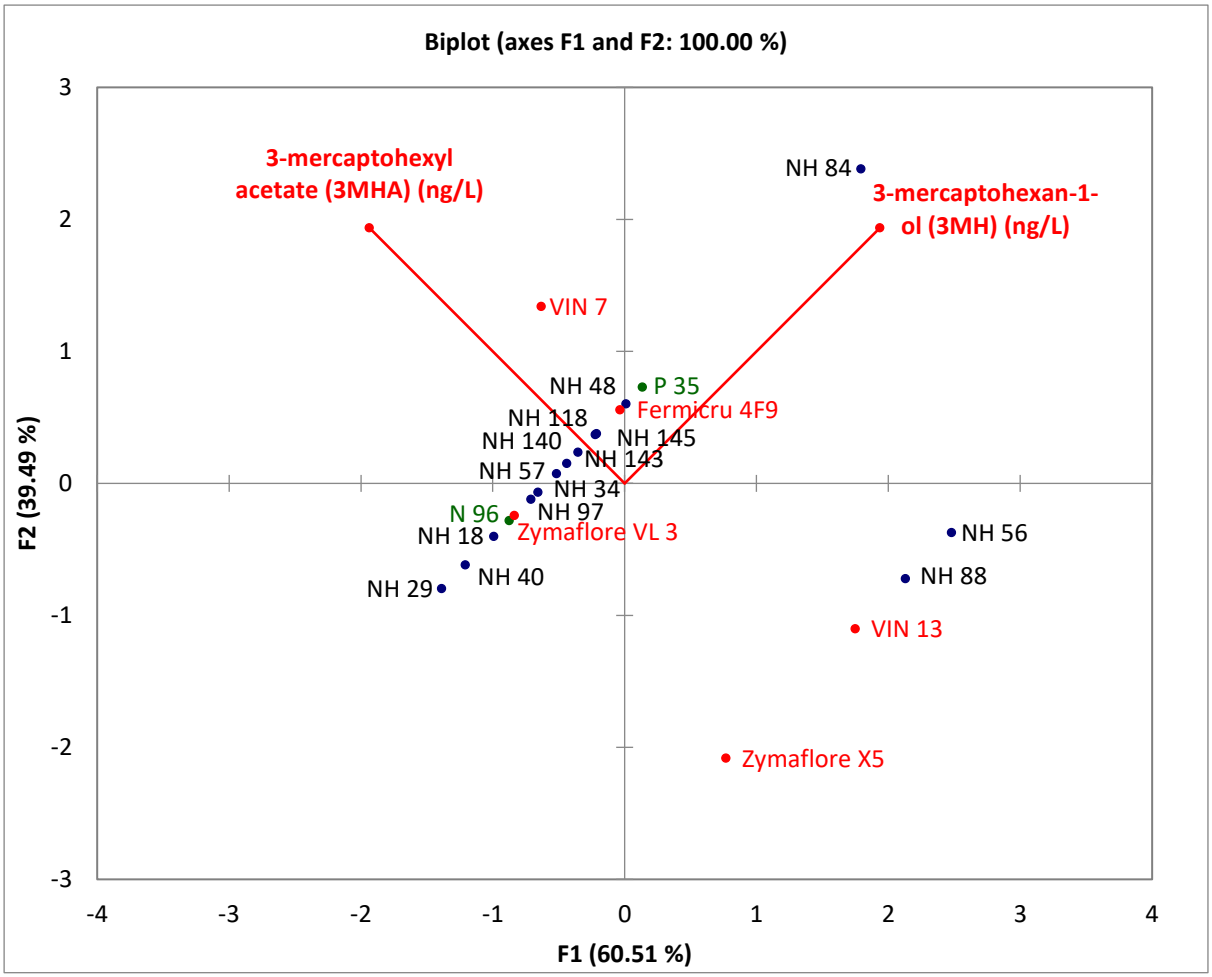

Figure 4.5: Biplot of volatile thiols, i.e. 3MH and 3MHA in small-scale Sauvignon blanc wine following fermentation by five thiol-releasing commercial wine yeasts (TRWY), two 'neutral' yeasts strains, nine hybrids shown to produce wines with tropical fruit aroma (TFPH), and five low VA producing hybrids (LVPH). Average values of triplicate fermentations.

4.4.6 Quantitative LC-based iTRAQ proteomic analysis

Yeast derived enzymes (proteins) are involved in the release of wine quality enhancing or reducing metabolites during fermentation (Juega *et al.,* 2012; Moreno-García *et al.,* 2015). In this study, selected TRWY references VIN 7, Zymaflore VL3 and Zymaflore X5, commercial yeast reference strain N 96, experimental hybrid yeast P 35, and promising TFPH NH 56, NH 57 and one LVPH NH 97, were selected

for quantitative LC-based iTRAQ analysis to investigate protein expression during the lag phase (48 hours following inoculation) and stationary phase (end of fermentation). The TRWY VIN 7 was chosen as reference for iTRAQ quantification, as it is a known 'thiol-releaser' and producer of wines with tropical aroma (Swiegers *et al.,* 2006b; Borneman *et al.,* 2012; Howe, 2016).

In order to minimise false positives during relative iTRAQ quantification and identification of proteins, cut-off ratios of 0.5 and 2.0 with less than 20% variation, were applied for down-regulated and overexpressed proteins, respectively. Analysis of the combined datasets in conjunction with Uniprot *S. cerevisiae* database (2015 edition) identified a total of 808 and 658 *S. cerevisiae* derived proteins during the lag and stationary phases of fermentation, respectively (Table 4.2). All yeast strains showed variation in the number of up- and down-regulated proteins in comparison to the VIN 7 reference expressed proteins. Overall 54 proteins (6.68%) were down-regulated and 201 proteins (24.88%) were overexpressed during the lag phase of fermentation, whilst 113 proteins (17.17%) were down-regulated and 28 proteins (4.25%) were overexpressed during the stationary phase of fermentation. Non-*Saccharomyces* expressed proteins originating from *Schizosaccharomyces pombe* and *Candida tropicalis* were observed during the lag phase, whilst none were detected at the end of fermentation. This observation complements previous research, since non-*Saccharomyces* genera are prominent in grape must, but are vanquished by *Saccharomyces* spp. as the fermentation progresses (Varela *et al.,* 2012; Jolly *et al.,* 2014; Wang *et al.,* 2016). Only three and 20 of the non-*Saccharomyces* derived-proteins were down-regulated and over-expressed, respectively. As the base must (juice) was homogenous and standard for all strains evaluated, the different reference and hybrid strain inoculums is the only variable (treatment).

TABLE 4.2: Number of proteins originating from fermenting commercial thiol-releasing wine yeasts (TRWY) and experimental hybrid yeast strains during the fermentation of 2013 Sauvignon blanc grape juice that was differentially expressed.

Yeast strains	Lag phase		Stationary phase	
	Down-regulated	Over-expressed	Down-regulated	Over-expressed
VIN 7 TRWY Reference	808 characterised		658 characterised	
VL3 TRWY Reference	0	22	2	4
X5 TRWY Reference	0	43	22	0
N 96 Reference	0	57	25	0
P 35 Reference	0	45	35	0
NH 56 TFPH	35	0	18	0
NH 57 TFPH	0	30	11	0
NH 97 LVPH	19	4	0	26

Proteomic analyses showed that all yeast strains, except NH 56, over-expressed between four and 57 proteins during the lag phase, whilst NH 56 and NH 97 were the only strains to have down-regulated 35 and 19 proteins, respectively. Over-expression of proteins linked to the rate and process of glycolysis, nitrogen and carbohydrate metabolism during the lag phase of fermentation is to be expected, because the yeast is actively generating energy whilst adapting to the new environment prior to proliferation (Zuzuarregui *et al.,* 2006; Salvado *et al.,* 2008). The yeast NH 56 down-regulated proteins were linked to the amino acid biosynthesis, pentose phosphate pathway, glycolysis and fructose and galactose metabolism pathways (*e.g.* dehydrogenases, reductases, synthetases, hydrolases, proteases, signalling molecules, dehydratases and transfer/carrier proteins). It can, therefore, be speculated that the yeast strain reduced its metabolic rate compared to the VIN 7 TRWY reference as well as to other strains included in this trial. Anecdotal evidence suggests that this delay might be instrumental in volatile thiol-release, since NH 56 produced wines had the highest positive association with 3MH (Figure 4.5). It is noteworthy that NH 56 did not regulate β-lyases (carbon-sulfur

lyases), enzymes previously reported to be responsible for the release of volatile thiols (Howell *et al.,* 2005; Swiegers *et al.,* 2007a).

Proteomic analyses showed that all strains down-regulated proteins during the stationary phase, except for Zymaflore VL3 and NH 97, that over-expressed four and 28 proteins, respectively. It was also observed that both strains produced wines with significantly lower 3MH compared to NH 56 produced wines. The remaining strains that did not over-express any proteins during the stationary phase produced wines with similar 3MH levels. Indications, therefore, are that down-regulation of proteins linked to, amongst others, amino acid biosynthesis, pentose phosphate pathway, glycolysis and fructose and galactose metabolism during the lag phase will enhance volatile thiol-release. Furthermore, Holt *et al.* (2011) and Ljungdahl & Daignan-Fornier (2012) reported that amino acids and their linked metabolic pathways are directly linked to the release and production of wine aroma compounds (*e.g.* thiols).

Hybrid yeast NH 56 produced wines with the least association with total acids compared to wines produced with the remaining yeast strains. Vilela-Moura *et al.* (2011) and Vilela *et al.* (2013) reported that acetic acid, the main VA responsible for vinegar-like off-flavours, is a major contributor to total fatty acids. Therefore, down-regulation of proteins associated with, amongst others, pentose phosphate pathway, glycolysis and fructose and galactose metabolism, and lack of overexpression of any other proteins at the start of fermentation, was instrumental in lower VA levels observed in wines produced with NH 56. Varela *et al.* (2012) and Walkey *et al.* (2012) reported that dehydrogenase enzymes are involved in excessive acetic acid production. Yeast strains that produced wines with a stronger association with acetic acid than NH 56, over-expressed dehydrogenases at the start of fermentation. Hybrid yeast NH 56 contrariwise down-regulated dehydrogenase at the start of fermentation. It is evident that different yeast strains expressed proteins differentially in response to the same grape must matrix. Subsequently, final wine organoleptic quality is affected by protein expression of the yeast starter culture.

Properties and relative expression of down-regulated and overexpressed proteins of promising TFPH (*e.g.* NH 56, NH 57) and LVPH (*e.g.* NH 97) during the lag and stationary phases of fermentations following iTRAQ quantification, are summarised in Tables 4.3, 4.4, 4.5, 4.6, 4.7 and 4.8. Isobaric tags for relative and absolute quantification proved to be a practical approach for identification of proteins extracted from eight different yeast strains simultaneously, as opposed to the laborious gel-based proteomics which only makes provision for visualisation of one sample at a time (May *et al.,* 2012). Consequently, the molecular weights (MW) and isoelectric points (pI) of regulated proteins during the lag (88) and stationary (55) phases originating from NH 56, NH 57 and NH 97, were determined using two iTRAQ runs as opposed to six two-dimensional poly-acrylamide gel electrophoresis (2D-PAGE) runs. Blasco *et al.* (2011) reported that yeast derived proteins present in must and/or wine are mainly glycoproteins. This study supports those observations, as regulated proteins include those associated with glycolysis. In general, regulated proteins during the different phases of fermentation were shown to be heterogeneous, as their molecular weight ranged from 7.10 to 351.40 kDa and pI ranged from pH 4.10 to 11.71.

Proteins differentially expressed by the yeast strains during the lag and stationary phases of Sauvignon blanc grape must fermentation were classified according to molecular function (Figure 4.6a & b), biological process (Figure 4.6c & d), and protein class (Figure 4.6e & f) using PANTHER (Sharma *et al.,* 2014). Classification of proteins showed that differentially expressed proteins during the lag phase of fermentation related to seven molecular functions *viz*. transporter, translation regulator, structural molecule, receptor, catalytic, binding and antioxidant activities (Figure 4.6a), whilst differentially expressed proteins during the stationary phase of fermentation are associated with the same molecular functions (Figure 4.6b). Therefore, regulated proteins during the start and end of fermentation are associated with the same molecular functions.

Classification of proteins showed that differentially expressed proteins during the lag phase of fermentation are associated with six biological processes viz. response to stimulus, metabolic,

localization, cellular, biogenesis and biological regulation (Figure 4.6c), whilst differentially expressed proteins during the stationary phase of fermentation are associated with five biological processes viz. metabolic, localisation, cellular, biogenesis and biological regulation (Figure 4.6d). Therefore, no proteins associated with 'stimulated response' were regulated towards the end of fermentation. It can tentatively be speculated that aroma-inactive bound thiol pre-cursors typically found in abundance in Sauvignon blanc grape must at the start of fermentation were depleted (Helwi *et al.,* 2016; O'Kennedy, 2016), hence the fermenting yeast strains were not stimulated to express proteins associated with the release volatile aromatic thiols. Classification of proteins showed that differentially expressed proteins during the lag phase clustered into sixteen protein classes (Figure 4.6e), whilst differentially expressed proteins during the stationary phase are associated with twelve different protein classes (Figure 4.6f).

It was observed that regulated proteins were mutual to eleven protein classes during the two phases of fermentation. However, five protein classes were exclusively observed during the lag phase, *i.e.* transporter, transfer/carrier, transcription factors, signalling molecule and defence. Luyten *et al.* (2002), Henricsson *et al.* (2005) and Young *et al.* (2011) reported that transporter proteins that play a pivotal role during the transport of molecules (*e.g.* hexose sugars) from the grape must into the cell are over-expressed during the lag phase, as cells are adapting to their environment prior to active proliferation. Perez *et al.* (2005) reported that carrier proteins responsible for the transport of glucose and fructose into cells were only expressed during the lag phase. Rossignol *et al.* (2006) reported that many genes associated with transcription and/or protein synthesis were regulated during the initiation of fermentation. It can be concluded that resultant protein expression will be higher. In accordance with Rossignol *et al.* (2006) transcription factors (proteins) in this study were also regulated during the lag phase. The wine yeast *S. cerevisiae* is known to secrete signalling molecules (proteins) to communicate to surrounding haploid *S. cerevisiae* with the intention to mate whilst growing in a rich medium (Lodish *et al.,* 2000; Merlini *et al.,* 2013). Proteomic analyses of fermenting must during this study showed that signalling molecules were also regulated during the lag phase (Figure 5e). Wine yeasts were also shown to undergo oxidative stress during fermentation, resulting in

the up-regulation of defence related genes during fermentation of a high-sugar-containing medium (Gómez-Pastor *et al.,* 2010; Navarro-Tapia *et al.,* 2016). The Sauvignon blanc base must used in this study had an initial sugar content of 221 g/L. It can, therefore, be concluded that this physiological condition also induced oxidative stress in yeast investigated as the data showed that defence proteins were only expressed during the lag phase.

TABLE 4.3: Differentially expressed proteins by the TFPH NH 56 relative to the commercial TRWY reference VIN 7 during the lag phase of fermentation in 2013 Sauvignon blanc grape must.

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	118/113 ratio
P04807	Hexokinase-2	111,96	41,15%	7	53,90	5,30	0,06
P51401	60S ribosomal protein L9-B	106,12	70,68%	2	21,60	9,66	0,07
P40581	Peroxiredoxin HYR1	25,70	47,24%	3	18,60	8,19	0,08
P05738	60S ribosomal protein L9-A	107,89	74,35%	2	21,60	9,73	0,08
P15891	Actin-binding protein	126,05	39,86%	9	65,50	4,68	0,08
P0CX33	P0CX33 40S ribosomal protein	28,70	46,03%	2	7,10	11,68	0,10
P35997	40S ribosomal protein S27-A	37,76	56,10%	3	8,90	9,14	0,10
P00815	Histidine biosynthesis trifunctional	183,54	41,80%	14	87,70	5,29	0,11
P0CX43	60S ribosomal protein L1-A	63,76	46,08%	5	24,50	9,72	0,11
P0C2H8	60S ribosomal protein L31-A	197,45	70,80%	12	12,90	9,99	0,12
P53912	Uncharacterised protein YNL134C	100,98	47,34%	6	41,10	6,21	0,13
P39522	Dihydroxy-acid dehydratase	121,14	44,10%	10	62,80	7,83	0,13
P0CX35	40S ribosomal protein S4-A	165,32	55,56%	11	29,40	10,08	0,13
P06105	Protein SCP160	125,71	23,16%	6	134,70	5,85	0,14
P14120	60S ribosomal protein L30	301,21	77,14%	13	11,40	9,80	0,15
P32445	Mitochondrial ssDNA-binding	95,40	72,59%	7	15,40	8,34	0,17

Table 4.3: (continued).

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	118/113 ratio
P41056	P41056 60S ribosomal	49,24	56,07%	1	12,20	11,08	0,17
P05744	P05744 60S ribosomal protein	73,64	55,14%	2	12,10	11,08	0,17
Q12118	SGT2 / YOR007C Protein	186,28	68,21%	9	37,20	4,79	0,17
P13663	Asp-semialdehyde_DH	128,70	44,38%	8	39,50	6,73	0,19
P31539	Heat shock protein 104	130,12	39,43%	7	102,00	5,45	0,20
P35691	TCTP	112,58	48,50%	7	18,70	4,56	0,20
Q12363	Transcriptional modulator WTM1	64,72	37,53%	7	48,40	5,36	0,21
P14772	Bile pigment transporter 1	130,37	33,10%	1	176,80	7,65	0,22
A6ZZH2	NADH-cytochrome b5 reductase 2	42,26	32,78%	6	34,10	8,65	0,24
P53163	Mitochondrial-nucleoid protein 1	55,51	41,75%	6	20,60	9,19	0,25
P49723	Ribonucleoside-diphosphate reductase	67,61	30,14%	4	40,00	5,21	0,26
P32463	Acyl carrier protein, mitochondrial	147,68	73,60%	3	13,90	4,97	0,30
P0C0T4	40S ribosomal protein S25-B	41,22	62,04%	4	12,00	10,32	0,31
P11633	Non-histone chromosomal protein 6B	51,80	41,41%	2	11,50	9,89	0,34
Q12513	ADC17	229,44	84,00%	9	16,80	4,73	0,34
Q12287	Cyt_c_oxidase_Cu-chaperone	27,45	14,49%	1	8,10	4,92	0,35
P39939	40S ribosomal protein S26-B	51,90	33,61%	1	13,40	10,90	0,35
P38999	Saccharopine reductase	134,12	50,00%	7	48,90	5,27	0,36
P26637	Leucine--tRNA ligase, cytoplasmic	82,03	21,65%	5	124,10	5,85	0,49

TABLE 4.4: Differentially expressed proteins by the TFPH NH 56 relative to the commercial TRWY reference VIN 7 during the stationary phase of fermentation in 2013 Sauvignon blanc grape must.

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	118/113 ratio
P32911	Protein translation factor SUI1	54,45	68,52%	3	12,30	7,97	0,23
P39994	Putative 2-hydroxyacyl-CoA lyase	78,84	49,82%	1	61,20	7,66	0,29
P05744	60S ribosomal protein L33-A	81,02	64,49%	1	12,10	11,08	0,30
P41277	(DL)-glycerol-3-phosphatase 1	232,6	80,80%	11	27,90	5,55	0,33
P40106	(DL)-glycerol-3-phosphatase 2	142,33	84,00%	5	27,80	6,16	0,33
P23301	Eukaryotic translation initiation factor 5A	42,43	50,32%	2	17,10	4,96	0,36
P19211	Hypusine-containing protein HP2	44,02	64,33%	2	17,10	4,96	0,36
P49089	ASNS1	58,73	36,89%	3	64,40	6,11	0,36
P07246	Alcohol dehydrogenase III	111,34	51,20%	1	40,30	8,43	0,37
Q756A9	Acyl-coenzyme A oxidase OS	103,61	58,25%	1	81,80	8,16	0,38
P41920	Ran-specific GTPase-activating protein	23,04	16,92%	2	22,90	6,10	0,39
P0CX48	40S ribosomal protein S11-B	109,24	57,69%	7	17,70	10,78	0,40
Q3E841	Uncharacterized protein YNR034W-A	85,68	76,53%	4	10,80	8,97	0,42
P14120	60S ribosomal protein L30 OS	145,86	83,81%	6	11,40	9,80	0,42
Q74ZK3	40S ribosomal protein S6 OS	102,94	46,19%	1	26,80	10,42	0,42
P15019	Transaldolase	75,2	53,43%	6	37,00	6,43	0,45
P0CX27	60S ribosomal protein L42-A	35,08	48,11%	2	12,20	10,59	0,47
P02400	60S acidic ribosomal protein P2	165,09	66,36%	5	11,00	4,15	0,49

TABLE 4.5: Differentially expressed proteins by the TFPH NH 57 relative to the commercial TRWY reference VIN 7 during the lag phase of fermentation in 2013 Sauvignon blanc grape must.

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	119/113 ratio
P43612	SIT4-associating protein	65,87	24,65%	1	114,90	4,72	2,03
P19262	Acyltransferase	71,56	41,90%	4	50,40	8,85	2,23
P33317	dUTP pyrophosphatase	50,73	57,14%	3	15,30	7,25	2,46
Q12306	Ubiquitin-like protein SMT3	109,69	46,53%	3	11,60	5,02	2,63
P25443	40S ribosomal protein S2	114,12	62,99%	9	27,40	10,43	2,70
P11633	Non-histone chromosomal protein 6B	51,80	41,41%	2	11,50	9,89	2,73
P37302	Aminopeptidase Y	65,43	22,16%	2	60,10	5,31	2,89
P00812	Arginase	36,95	30,93%	1	35,60	5,64	2,97
P0CX55	40S ribosomal protein S18-A	57,36	39,73%	3	17,00	10,27	3,13
C7GL62	Arginine biosynthesis protein ArgJ	66,33	47,62%	2	47,80	7,14	3,38
P47178	Cell wall protein DAN1	98,82	29,53%	1	29,60	4,56	3,53
P38137	Peroxisomal-coenzyme A synthetase	51,55	45,86%	5	60,50	9,20	3,74
P32472	Peptidyl-prolyl cis-trans isomerase FPR2	11,25	38,52%	1	14,50	5,50	4,30
P32179	3'(2'),5'-bisphosphate nucleotidase	17,26	19,33%	1	39,10	6,20	4,35
P38791	Deoxyhypusine synthase	64,22	45,99%	2	42,90	5,77	4,45
P26637	Leucine-tRNA ligase, cytoplasmic	82,03	21,65%	5	124,10	5,85	4,47

Table 4.5: (continued).

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	119/113 ratio
Q03973	High mobility group protein 1	37,20	32,52%	4	27,50	8,51	4,91
P00447	Superoxide dismutase mitochondrial	27,16	54,08%	1	25,80	8,48	4,97
P39111	V-type proton ATPase subunit F	54,17	47,46%	4	13,50	5,05	5,09
P32628	UV excision repair protein RAD23	63,73	46,48%	5	42,30	4,32	5,23
P53301	Probable glycosidase CRH1	52,61	40,24%	4	52,70	4,65	5,26
Q05016	L-allo-threonine dehydrogenase	45,18	57,30%	4	29,10	6,81	5,51
P17536	Tropomyosin-1	111,43	35,18%	8	23,50	4,61	6,45
P17695	Glutaredoxin-2, mitochondrial	58,04	76,92%	6	15,90	7,28	7,39
Q12305	Thiosulfate sulphurtransferase RDL1	55,15	58,27%	3	15,40	6,38	10,27
P01094	Protease A inhibitor 3	42,39	51,47%	4	7,70	7,40	11,96
P38077	ATP synthase subunit gamma	63,73	44,05%	3	34,30	9,31	12,49
P19158	Inhibitory regulator protein IRA2	351,71	29,78%	1	351,40	7,05	13,34
P34227	Mitochondrial peroxiredoxin PRX1	67,02	49,81%	6	29,50	8,87	15,30
P20081	FK506-binding protein 1	62,06	56,14%	3	12,20	6,04	22,61

TABLE 4.6: Differentially expressed proteins by the TFPH NH 57 relative to the commercial TRWY reference VIN 7 during the stationary phase of fermentation in 2013 Sauvignon blanc grape must.

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	119/113 ratio
P39994	Putative 2-hydroxyacyl-CoA lyase	78,84	49,82%	1	61,20	7,66	0,10
P0CX41	60S ribosomal protein L23-A	80,90	74,45%	5	14,50	10,33	0,18
P07260	mRNA cap-binding protein	11,46	24,88%	1	24,20	5,49	0,23
E7KS00	Protein HRI1	21,88	51,23%	1	27,50	5,21	0,28
P53334	Probable family 17 glucosidase SCW4	33,57	59,33%	3	40,10	4,83	0,29
Q756A9	Acyl-coenzyme A oxidase	103,61	58,25%	1	81,80	8,16	0,31
P04807	Hexokinase-2	78,46	67,28%	3	53,90	5,30	0,33
O43137	Uncharacterized protein YBR085C-A	11,62	27,06%	1	9,40	5,35	0,36
P49166	60S ribosomal protein L37-A	11,89	40,91%	2	9,80	11,63	0,38
P02293	Histone H2B.1	132,25	64,12%	6	14,20	10,10	0,45
P04911	Histone H2A.1	65,32	80,30%	3	14,00	10,67	0,47

TABLE 4.7: Differentially expressed proteins by the LVPH NH 97 relative to the commercial TRWY reference VIN 7 during the lag phase of fermentation in 2013 Sauvignon blanc grape must.

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	121/113 ratio
P40303	Proteasome subunit alpha type-4	11,36	15,35%	1	28,40	7,36	0,06
P36105	60S ribosomal protein L14-A	198,49	55,07%	12	15,20	10,93	0,07
P39938	40S ribosomal protein S26-A	53,94	33,61%	1	13,50	10,76	0,10
Q12447	Polyamine N-acetyltransferase 1	24,95	25,13%	2	21,90	5,82	0,13
P11633	Non-histone chromosomal protein 6B	51,80	41,41%	2	11,50	9,89	0,14
P21243	Proteasome subunit alpha type-1	73,67	36,90%	2	28,00	6,24	0,19
P06738	Glycogen phosphorylase	115,60	32,37%	6	103,20	5,62	0,20
P41921	Glutathione reductase	116,67	37,89%	4	53,40	7,83	0,21
P0CX55	40S ribosomal protein S18-A	57,36	39,73%	3	17,00	10,27	0,22
P32610	V-type proton ATPase subunit D	86,68	65,63%	8	29,20	5,92	0,24
P16521	Elongation factor 3A	136,38	34,39%	5	115,90	6,05	0,24
P27616	SAICAR synthetase	99,87	53,59%	9	34,60	5,95	0,25
Q12306	Ubiquitin-like protein SMT3	109,69	46,53%	3	11,60	5,02	0,27
C7GL62	Arginine biosynthesis protein ArgJ	66,33	47,62%	2	47,80	7,14	0,27
P0CX27	60S ribosomal protein L42-A	46,59	27,36%	2	12,20	10,59	0,29
P53163	54S ribosomal protein L12	55,51	41,75%	6	20,60	9,19	0,39

Table 4.7: (continued).

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	121/113 ratio
Q01976	ADP-ribose pyrophosphatase	29,57	42,42%	3	26,10	6,38	0,47
P32288	Glutamine synthetase	112,72	53,51%	5	41,70	6,34	0,48
P47178	Cell wall protein DAN1	98,82	29,53%	1	29,60	4,56	2,31
P46672	tRNA-aminoacylation cofactor ARC1	43,50	18,88%	3	42,10	7,88	2,92
P40513	Mitochondrial acidic protein MAM33	101,37	45,86%	7	30,10	4,58	5,29
P14772	Bile pigment transporter 1	130,37	33,10%	1	176,80	7,65	23,43

TABLE 4.8: Differentially expressed proteins by the LVPH NH 97 relative to the commercial TRWY reference VIN 7 during the stationary phase of fermentation in 2013 Sauvignon blanc grape must.

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	121/113 ratio
P0CX37	40S ribosomal protein S6-A	249,14	67,37%	6	27,00	10,45	2,05
O13516	40S ribosomal protein S9-A	186,60	79,19%	1	22,40	9,98	2,07
P07260	mRNA cap-binding protein	11,46	24,88%	1	24,20	5,49	2,09
P38788	DnaK-related protein SSZ1	88,79	51,49%	6	58,20	5,05	2,11
P00817	Inorganic pyrophosphatase	204,58	69,34%	9	32,30	5,58	2,12
P0CX23	60S ribosomal protein L20-A	168,44	60,47%	6	20,40	10,30	2,14
P40106	(DL)-glycerol-3-phosphatase 2	142,33	84,00%	5	27,80	6,16	2,15
P04911	Histone H2A.1	65,32	80,30%	3	14,00	10,67	2,15
A6ZYI0	Adenylate kinase	89,48	81,98%	5	24,20	6,70	2,25
P46784	40S ribosomal protein S10-B	60,57	71,43%	3	12,70	9,07	2,27
P48570	Homocitrate synthase cytosolic isozyme	115,15	64,72%	10	47,10	7,27	2,33
P04147	Poly(A)-binding protein	152,75	60,49%	11	64,30	5,97	2,40
Q12458	Putative reductase 1	64,36	62,82%	3	34,70	7,12	2,45
P39994	Putative 2-hydroxyacyl-CoA lyase	78,84	49,82%	1	61,20	7,66	2,47
P0CX49	60S ribosomal protein L18-A	43,43	52,15%	3	20,60	11,71	2,54
P32590	Heat shock protein homolog SSE2	72,03	44,16%	2	77,60	5,63	2,63

Table 4.8: (continued).

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	121/113 ratio
P27616	SAICAR synthetase	36,19	54,58%	3	34,60	5,95	2,63
P12695	Pyruvate dehydrogenase complex E2	91,44	51,45%	3	51,80	7,80	2,71
P21243	Proteasome subunit alpha type-1	42,58	45,63%	3	28,00	6,24	2,72
P38616	Protein YGP1	182,36	51,13%	7	37,30	5,44	2,84
P34227	Mitochondrial peroxiredoxin PRX1	114,37	43,30%	5	29,50	8,87	3,06
P40185	Protein MMF1, mitochondrial	64,68	69,66%	5	15,90	9,28	3,10
P53184	Nicotinamidase	43,10	45,37%	4	25,00	6,27	3,28
Q03496	tRNA methyltransferase	114,01	40,99%	2	163,50	6,60	3,33
P43616	Cys-Gly metallodipeptidase DUG1	43,56	49,06%	2	52,80	5,67	3,35
P0CX41	60S ribosomal protein L23-A	80,90	74,45%	5	14,50	10,33	3,41

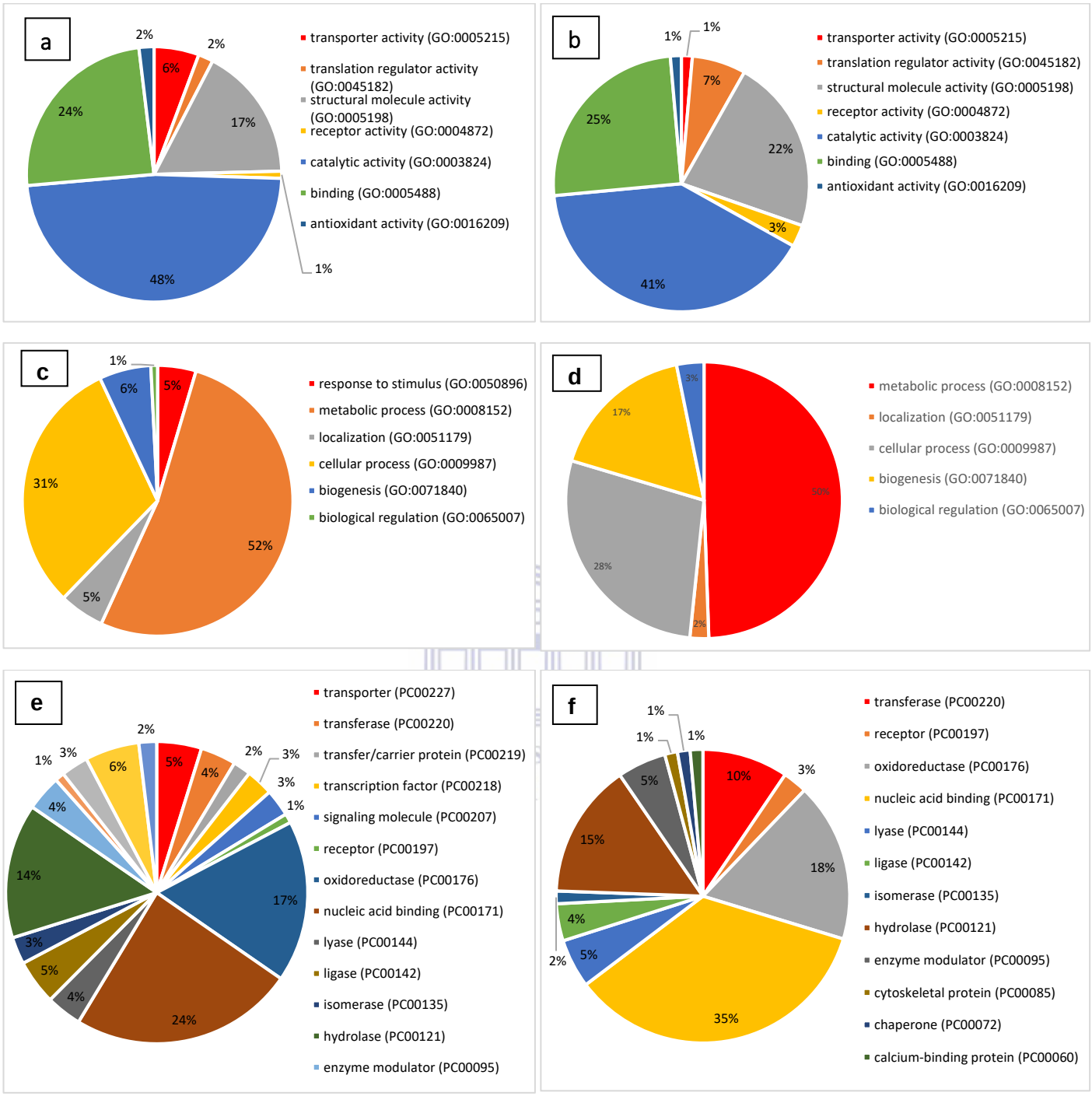

Figure 4.6: Classification of differentially expressed proteins by commercial TRWY, Zymaflore X5 and Zymaflore VL3, two 'neutral' wine yeast strains, N 96 and P 35; and two TFPH, NH 56 and NH 57 and one LVPH, NH 97 during the lag and stationary phases of Sauvignon blanc grape must fermentation according to a & b) Molecular function, c & d) Biological process, and e & f) Protein class using Protein ANalysis THrough Evolutionary Relationships (PANTHER, www.pantherdb.org/).

4.5. CONCLUSIONS

In conclusion, seven LVPH (NH 29, NH 56, NH 57, NH 84, NH 88, NH 118 and NH 140) were identified, of which all but strain NH 29 were also identified as TFPH. These strains, therefore, conform to the initial yeast selection criteria set forth in the aims of study viz. enhanced thiol-releasing or tropical fruit aroma wine producing abilities and lower VA formation. The TFPH were shown to produce wines with a more positive association with 3MH and 3MHA compared to wines produced with commercial TRWY references. The LVPH and TFPH, therefore have the potential to play a commercial role in the production of varietal aromatic white wine. The TFPH NH 56 produced wines with the second highest 3MH levels after NH 84, but with the lowest acetic acid of all strains included in this study. This yeast was also the only strain to have down-regulated proteins during the lag phase, which were linked to amino acid biosynthesis, pentose phosphate pathway, glycolysis and fructose and galactose metabolism. Differences in protein expression were reflected in the variation of metabolite release by the different strains, thereby confirming that proteins are the final effectors for metabolite release. This study highlights the importance of said protein classes, besides β-lyases, in the release of volatile thiols.

4.6. CONFLICT OF INTEREST

The authors have not declared any conflict of interest.

4.7. ACKNOWLEDGEMENTS

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Chapter 5

Research results

Characterisation of thiol-releasing and lower volatile acidity forming intra-genus and inter-genus hybrid yeast strains for Sauvignon blanc wine

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Chapter 5: Characterisation of thiol-releasing and lower volatile acidity forming intra-genus and inter-genus hybrid yeast strains for Sauvignon blanc wine

5.1. ABSTRACT

Wine yeast expressed proteins are influential during the production of varietal aromatic Sauvignon blanc wines as they release or mediate aroma compounds and undesirable volatile acidity (VA). As *Torulaspora delbrueckii* in conjunction with *Saccharomyces cerevisiae* as well as a *S. cerevisiae*/*T. delbrueckii* inter-genus hybrid were previously shown to produce white wine with enhanced aroma and/or lower VA, intra- and novel inter-genus hybrids were trialled for the production of aromatic Sauvignon blanc with lower VA. The inter-genus hybrid NH 07/1 produced wine with a more positive association with the aroma compound 3-mercaptohexylacetate (3MHA) than two commercial thiol-releasing wine yeast (TRWY) strains, Zymaflore X5 and Zymaflore VL3. The wine also had a negative association with VA, and a positive association with floral and tropical fruit aromas. Three intra-genus hybrids, NH 56, NH 57 and NH 88, produced wines with a negative association with VA, and a positive association with tropical fruit aroma. These wines also had a stronger association with the aroma compound, 3-mercaptohexan-1-ol (3MH) than wines produced with all commercial TRWY. The hybrid NH 07/1 and Zymaflore VL3 also over-expressed the lactoylglutathione lyase protein responsible for the release of the volatile thiol, 4-mercapto-4-methyl-pentan-2-one (4MMP) by cleaving its carbon-sulfur bonds. Therefore, lactoylglutathione lyase is a potential biomarker for 3MH-release, as this thiol also contains a carbon-sulfur bond. Dehydrogenase proteins might also be useful biomarkers for VA formation by fermenting wine yeasts. Three intra- and one inter-genus hybrids with the abiliy to produce aromatic Sauvignon blanc wines with lower VA compared to commercial TRWY references were identified.

5.2. INTRODUCTION

Sauvignon blanc wines are associated world-wide with either vegetative (herbaceous) or tropical fruit and/or floral aromas (Marais, 1994; Von Mollendorf, 2013; Hart *et al.,* 2016). Key to the production of high quality Sauvignon blanc wines with the desired properties are wine yeasts, namely *S. cerevisiae* that can convert relatively "neutral" grape must lacking varietal aromas into varietal-typical aromatic wines through their metabolic activity (Swiegers *et al.,* 2006a; 2007a). Sauvignon Blanc wine aroma and flavour are the result of grape derived compounds (metabolites), *e.g.* methoxipyrazines, *de novo* synthesised metabolites or compounds released from aroma-inactive, non-volatile grape-derived precursors by wine yeast during fermentation (Bovo *et al.,* 2015; Pinu *et al.,* 2015). However, yeast also produce undesirable metabolites, *e.g.* acetic acid the main contributor to volatile acidity (VA). These compounds are responsible for vinegar-like off-flavours that are detrimental to overall wine organoleptic quality (Du Toit & Pretorius, 2000; Swiegers *et al.,* 2005). Such wines will have negative financial implications as expensive reverse osmosis techniques have to be used to remove the excessive VA. Commercial yeast strains implicated in the production of wines with higher VA values will create negative perceptions for the yeast manufacturer and result in loss of revenue due to lower yeast sales (Margaret Fundira, Personal communication, 2016).

Wine yeast expressed enzymes (proteins) during winemaking were previously reported to be key effectors of wine aroma and flavour compounds present in wines (Holt *et al.,* 2011; Roncoroni *et al.,* 2011). Furthermore, Holt *et al.* (2012) and Pretorius (2016) reported that yeast expressed proteins with carbon-sulfur β-lyase activity are involved in the release of the aroma enhancing volatile thiol *i.e.* 4-mercapto-4-methyl-pentan-2-one (4MMP). Dehydrogenase enzymes were also reported to be involved in the production of acetic acid, the main contributor to total fatty acids (Varela *et al.,* 2012; Walkey *et al.,* 2012). Additionally, it was reported that over-expression of dehydrogenase enzymes by wine yeast during fermentation of Sauvignon blanc grape must resulted in wines with elevated total fatty acids (Hart *et al.,* 2016; 2017).

The use of the yeast *Torulaspora delbrueckii* was shown to produce wines with lower VA levels, and enhancing varietal aromas when inoculated singly or sequentially with *S. cerevisiae* (Albertin *et al.,* 2014; Renault *et al.,* 2016). *S. cerevisiae*/*T. delbrueckii* inter-genus hybrids also produced wine with enhanced aroma and flavour upon completion of fermentation (Santos *et al.,* 2008). Therefore, *T. delbrueckii* can be advantageous for the development of new hybrid strains with the ability to produce aromatic white wines with lower VA. For that reason, the aims of this study were to breed *S. cerevisiae/T. delbrueckii* inter-genus hybrids using classical mating which is naturally occuring phenomenon, characterise and evaluate these inter-genus hybrids for their fermentation potential, thiol-releasing abilities and low VA formation during the production of Sauvignon blanc wines. Promising *S. cerevisiae* intra-genus hybrids previously identified by Hart *et al.* (2016) for their ability to produce wines with enhanced tropical fruit aroma (henceforth referred to as TFPH) and lower VA (henceforth referred to as LVPH) compared to commercial 'thiol-releasing' wine yeasts (TRWY) were included in this study. Additionally, wine yeast regulated proteins and aroma compounds, especially volatile thiols *viz*. 3-mercaptohexanol (3MH) and 3-mercaptohexylacetate (3MHA) as well as volatile acidity *viz*. acetic acid present at the end of fermentation and their association with final wine aroma and flavour were investigated. It is envisioned that potential protein biomarkers associated with aroma-enhancing metabolites and VA will be identified.

5.3. MATERIALS AND METHODS

5.3.1. Origin of yeast strains

5.3.1.1. Reference yeast strains

The following commercial *S. cerevisiae* hybrid strains, namely NT 112 and NT 116 (Anchor Yeast, South Africa) served as references for the laboratory-scale fermentations, whilst the commercial thiol-releasing wine yeast (TRWY) strains, VIN 7 and VIN 13 (Anchor Yeast, South Africa), Zymaflore VL3, Zymaflore X5 (Laffort Oenologie, France), and Fermicru 4F9 (DSM Oenology, Netherlands), were

included as references for the small-scale fermentations. All TRWY were previously recommended for the production of aromatic white wines due to the yeast's 'thiol-releasing' abilities (Anonymous, Personal communication, 2005a; 2005b; 2017a; 2017b; 2017c). Another commercial strain, N 96 (Anchor Yeast, South Africa) and an experimental strain, P 35 (ARC Infruitec-Nietvoorbij, South Africa) used in hybrid breeding programmes, were also included in this study as references. The latter strains have the ability to produce wine with tropical fruit aromas (henceforth abbreviated as TFPP).

5.3.1.2. Intra-genus hybrids

Ten *S. cerevisiae* intra-genus hybrids, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145, previously characterised as TFPH and LVPH were included in this study (Hart *et al.,* 2016).

5.3.1.3. Inter-genus hybrids

Two inter-genus hybrids, NH 07/1 and NH 07/2, were generated through classical mating by fusing protoplasts originating from a *S. cerevisiae* strain MCB C6, isolated from Madeba cellar winery equipment, Robertson, South Africa and *T. delbrueckii* strain M2/1 (Van Breda *et al.,* 2013), resulting in inter-genus hybrids as shown in figure 5.1. Briefly, freeze cultures containing diploid *S. cerevisiae* strain MCB C6 and the haploid *T. delbrueckii* (Sasaki & Ohshima, 1987; Kurtzman *et al.,* 2011) strain M2/1 were thawed and streaked onto yeast extract peptone dextrose (YPD) agar (Biolab, Merck, South Africa). Agar plates were incubated at 28 °C for at least 48 h until single yeast colonies were visible. A single colony from the diploid (2n) *S. cerevisiae* yeast strain was aseptically transferred onto plates containing nitrogen-limiting growth media (0.25 % [w/v] yeast extract, 0.1 % [w/v] dextrose, 1 % [v/v] potassium acetate, and 1 % [w/v] agar) and incubated for 72 h at 28 °C until asci, each containing four haploid (n) spores could be observed. Thereafter, a single colony from the sporulated *S. cerevisiae*

MCB C6 culture and the *T. delbrueckii* M2/1 culture were transferred into separate tubes containing 10% (w/v) β-d-glucuronidase enzyme, and mixed until the suspension appeared milky. Protoplasts were generated by incubation of suspensions at 30 °C for 30 min. Thereafter, sterile water was added to each micro-centrifuge tube to rinse cell residue from protoplasts. Respective supernatants were gently removed and transferred into new tubes. Thereafter, 100 µL of each protoplast-containing supernatant were streaked onto different sections of a YPDA-plate, and placed on a Singer MSM system series 200 micro-manipulator (Singer Instruments, Watched, Somerset, UK) as described (Morin *et al.,* 2009). Protoplasts were physically disrupted using a micro-fine needle, whereafter haploid spores from the two parental strains were placed in close proximity on the YPDA. Thereafter, the plates were incubated at 28 °C for at least 48 h to allow haploids to fuse (karyogamy) to form diploid (2n) inter-genus hybrids.

5.3.2. Characterisation techniques

5.3.2.1. Contour clamped homogeneous electric field (CHEF) DNA karyotyping

The CHEF DNA karyotyping was conducted according to the embedded agarose procedure used for commercial TRWY and intra-genus hybrids described by Hart *et al.* (2016). A Bio-Rad image analyser (Bio-Rad, Madrid, Spain) was used to visualise chromosomal banding patterns on 0.01% (v/v) ethidium bromide-stained agarose gels.

5.3.2.2. Matrix-assisted laser desorption/ionisation (MALDI) biotyping

Yeast strains were characterised by MALDI biotyping using a Bruker UltrafleXtreme MALDI-TOF/MS (Bruker Daltonics, Bremen, Germany) used for commercial TRWY and intra-genus hybrids described by Hart *et al.* (2016).

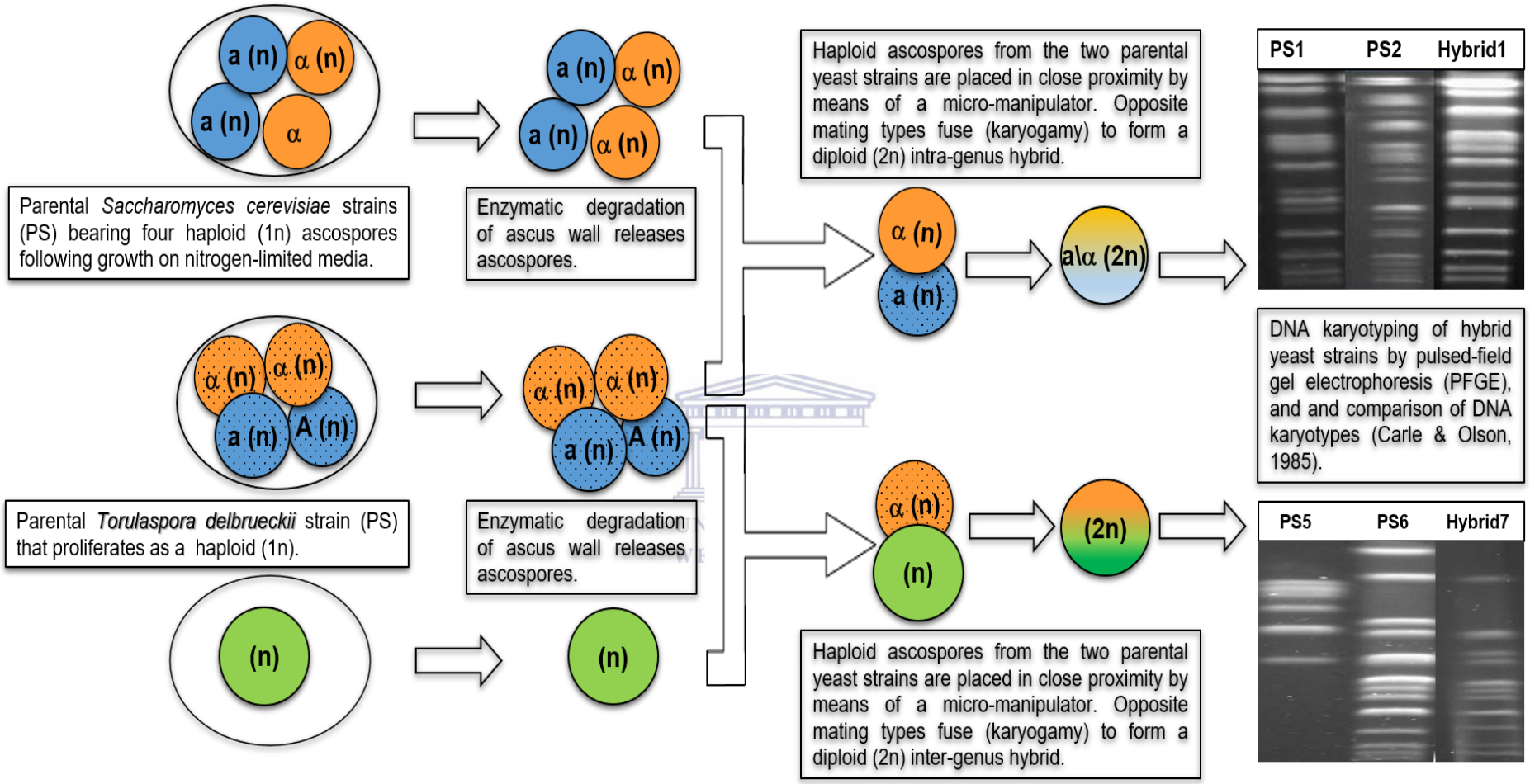

FIGURE 5.1: Schematic depiction of intra- and inter-genus hybrid yeast breeding by means of classical mating in conjunction with protoplast fusion, using *Saccharomyces cerevisiae* and *Torulospora delbrueckii* as parental yeast strains.

5.3.3. Evaluation techniques

5.3.3.1. Laboratory-scale fermentation trials

Fermentation potential of wet culture inter-genus hybrids was evaluated in laboratory-scale vinifications of Chardonnay clarified grape must (juice) (total sugar 21.3 °B; total acidity (TA) 8.1 g/L; pH 3.10), similar to vinifications with TRWY and intra-genus hybrids as described by Hart *et al.,* (2016). Commercial yeast strains, NT 112 and NT 116 (Anchor Yeast, South Africa) were included in the trials as references. All fermentations were conducted in triplicate in a completely randomised order (Addelman, 1970) at 15 °C, whilst gently shaking on an orbital shaker. Fermentations were monitored by CO_2 weight loss. Subsequently, both inter-genus hybrids were trialled in small-scale winemaking after it was established that they fermented the grape must (juice) to dryness (residual sugar <5 g/L) using a portable DMA 35 density meter (Anton Paar, Southern Africa).

5.3.3.2. Small-scale winemaking trials

Small-scale Sauvignon blanc wines were made in triplicate using commercial TRWY, intra- and inter-genus hybrids according to a standardised cellar method as described by Hart and Jolly (2008). For each treatment replicate, nine litres Sauvignon blanc grape must (total sugar 21.9 °B; TA 9.3 g/L; pH 3.28) were dispensed into 10 L stainless steel canisters with fermentation caps, and inoculated with the respective wine yeast starter cultures. The method was adjusted by having the respective yeast inoculums cultured for 24 hours in 600 mL YPD broth (Biolab, Merck, South Africa) medium. Subsequently, 180 mL of the 24 hour cultures (optical density at 600 nm = 0.92 ± 0.05; cfu/mL = 10^7 ± 10^6; viability = 97.93% ± 1.67) were used to inoculate clarified Sauvignon Blanc grape must (2% inoculum). Fermenting must was sampled every 48 hours to measure residual glucose/fructose (R/S), ethanol, VA, TA and pH, using an Oenofoss™ Fourier transform infrared (FTIR) spectrometer (FOSS Analytical A/S, Denmark) until fermentations went to dryness. This was repeated until the R/S

concentration were below 5 g/L, whereafter the free-SO_2 of the wines was adjusted to 35 mg/L, following racking. Wines were cold stabilised at 0 °C for at least two weeks prior to bottling.

5.3.3.3. Gas chromatography (GC) analysis

Wine aroma metabolites, namely esters, total fatty acids and higher alcohols (fusel oils), were analysed by gas chromatography (GC) on wine samples (50 mL) taken on day 15 of fermentation (stationary phase) as described by Hart *et al.* (2016; 2017).

5.3.3.4. Solid-phase extraction (SPE) and GC-MS/MS analysis

The main wine volatile thiols, 3MH and 3MHA, were pre-concentrated by deploying solid-phase extraction (SPE) as described by Hart *et al.* (2016; 2017). Subsequently, GC coupled to tandem mass spectrometry (GC-MS/MS) as described by Mattivi *et al.* (2012) was used to quantify volatile aromatic thiols. The GC-MS/MS system used in this study comprised of a GC Trace 1300/ TSQ8000 mass selective detector equipped with an AI 1310 auto sampler (Thermo Scientific™ Inc, USA). Aroma compounds were separated using a 30 m x 0.25 mm x 0.25 µm Zebron WAX plus column (Phenomenex Inc., Torrance, CA, USA).

5.3.3.5. Sensory evaluation

An experienced panel consisting of 14 members as described by Hart *et al.* (2016) conducted descriptive sensory evaluation of bottled wines. The panel was requested to indicate the intensity of aroma descriptors on a unipolar six-point numerical scale (absent [0], very low [1], low [2], medium [3], high [4] and very high [5]). Panel members also had to specify the most prominent aromas associated with Sauvignon blanc wines *viz.* 'tropical fruit' (*e.g.* banana, guava, peach, passion fruit and

citrus); 'vegetative' (*e.g.* asparagus, herbaceous, green pepper, green beans, cut grass, green olive and gooseberry); or 'floral' (*e.g.* rose, orange blossom), they perceived.

5.3.3.6. Quantitative LC-based iTRAQ proteomic analysis

Based on chemical (lower VA and total fatty acids) and sensory (tropical fruit aroma) analyses of final wines, yeast-containing ferments sampled (50 mL) in triplicate on day 15 of fermentation were selected for quantitative proteomic analysis using an iTRAQ 8-plex reagent kit (AB Sciex, USA) in conjunction with LC-MS/MS at the Mass spectroscopy unit, Proteomics laboratory, Central Analytical Facility (CAF), University of Stellenbosch (US). Briefly, proteins were extracted from the different strains, followed by alkylation in methylthiosulphonate (MMTS) and digestion at 37°C using 1 µg/µL trypsin solution (Promega, Madison, WI, USA) as described by Boutureira & Bernardes (2015). Tryptic digests originating from the eight yeasts (TRWY: VIN 7, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9; the intra-genus TFPH and LVPH: NH 84; two promising natural isolates; MCB C6 and M 2/1; and one inter-genus hybrid: NH 07/1), were tagged according to manufacturer's recommendations with iTRAQ labels 113, 114, 115, 116, 117, 118, 119 and 121, respectively, as described by Kim *et al.* (2012). The TRWY VIN 7 served as reference as the yeast was reported to be a high 'thiol-releaser' used for the production varietal aromatic Sauvignon blanc wines with enhanced tropical fruit aroma (Swiegers *et al.,* 2006b; Howe, 2016). Subsequently, proteins were characterised using a mass spectrometer equipped with a Nanospray flex ionisation source (Thermo Scientific™ Inc, USA) in conjunction with Mascot algorithm (Matrix DiffScience, London, UK), and SequestHT algorithm included in Proteome Discoverer v1.4. Isobaric tags for relative and absolute quantitation algorithm were used for protein quantitation. Only proteins with more than two (2) peptides, but less than 20% variation, and iTRAQ ratios below 0.5 and above two (2) were considered down-regulated and over-expressed, respectively. Differentially expressed proteins were also subjected to Protein ANalysis

THrough Evolutionary Relationships (PANTHER, www.pantherdb.org/) to establish their involvement in biological processes, molecular function and protein classes (Sharma *et al.,* 2014).

5.3.3.7. Statistical analyses

Analysis of variance (ANOVA) and principal component analysis (PCA) were conducted on data from chemical, sensory and metabolomic analyses data (Pearson, 1896; 1901; Zou *et al.,* 2006). The linear relationship between the chemical, sensory and metabolomic variables was analysed by means of a Pearson's correlation using XLSTAT software (Addinsoft, 2013) with the principal components (PC's) as factors (*i.e.* F1 and F2).

5.4. RESULTS AND DISCUSSION

5.4.1. Characterisation of yeast strains

5.4.1.1. Contour clamped homogeneous electric field DNA karyotyping

The CHEF DNA karyotyping technique was previously used to successfully differentiate between *S. cerevisiae* and *T. delbrueckii* yeast strains (Van Breda *et al.,* 2013). Additionally, CHEF could also differentiate between commercial TRWY strains, *S. cerevisiae* parental strains and *S. cerevisiae* intra-genus hybrids, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145 (Van der Westhuizen & Pretorius, 1992; Hoff, 2012; Hart *et al.,* 2016). Subsequently, CHEF successfully differentiated *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1 parental strains from inter-genus hybrids, NH 07/1 and NH 07/2 during this investigation. The inter-genus hybrid strains shared similar (yellow and blue text box) and different (red text box) chromosomes in terms of size with both parental strains (Figure 5.2). Both inter-genus hybrids had matching DNA karyotypes, so they may be the same strain, hence MALDI biotyping was deployed as a complementary characterisation tool.

5.4.1.2. Matrix-assisted laser desorption/ionisation (MALDI) biotyping

Biotyping successfully differentiated between commercial TRWY strains, *S. cerevisiae* parental strains and *S. cerevisiae* intra-genus hybrids, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145 (Hart *et al.,* 2016). Ribosomal proteins extracted from *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 were matched to that of a database described by Hart *et al.* (2016) and (2017). Strains MCB C6, NH 07/1 and NH 07/2 were identified as *Candida robusta,* the anamorph to *S. cerevisiae* (Diddens & Lodder, 1942; Kurtzman *et al.,* 2011), whilst strain M2/1 was identified as *C. collucilosa,* the anamorph to *T. delburueckii* (Table 5.1) (Van Breda *et al.,* 2013; Jolly *et al.,* 2014). It can tentatively be speculated that inter-genus hybdrids were classified as *C. robusta,* as the database does not have inter-genus reference accessions. It is envisioned that the database will be extended by including spectral data of both novel inter-genus hybrids. Parental strains MCB C6 and M2/1 and inter-genus hybrids *i.e.* NH 07/1 and NH 07/2 also had distinctive mass spectra (Figure 5.3). Therefore, MALDI-TOF/MS biotyping proved more reliable to distinguish closely related inter-genus hybrids compared to CHEF karyotyping. Nonetheless, the two methods were complementary, as inter-genus hybrids were distinguished from parental strains.

5.4.2. Evaluation of yeast strains

5.4.2.1. Laboratory-scale fermentation trials

Hart *et al.* (2016) and (2017) previously reported on the fermentation potential of intra-genus hybrids NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145 compared to commercial TRWY references used in this study. Laboratory-scale white wine fermentations showed that both inter-genus hybrids, NH 07/1 and NH 07/2 were also able to ferment grape must at a similar rate to commercial references, NT 112 and NT 116 as well as the *S. cerevisiae* strain MCB C6 parental yeast (Figure 5.3). The parental strain MCB C6 and inter-genus hybrids fermented at a similar rate, whilst the *T. delbrueckii* parental strain M2/1 fermented at a slower rate. Nonetheless, the latter was

chosen as parental strain for its lower VA formation as reported by Van Breda *et al.* (2013). Subsequently, both inter-genus strains were compared to intra-genus hybrids and commercial TRWY references for small-scale production of varietal aromatic Sauvignon blanc wines with lower VA.

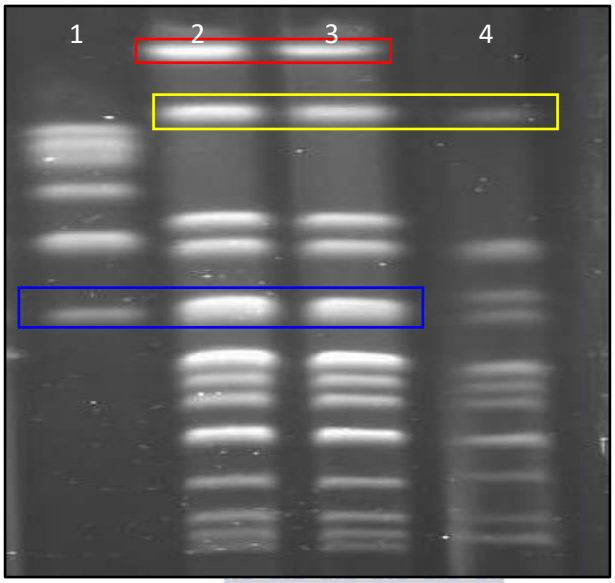

FIGURE 5.2: Contour clamped homogeneous electric field (CHEF) DNA karyotypes of parental strains, *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1, and inter-genus hybrids, NH 07/1 and NH 07/2 conserved in the ARC Infruitec-Nietvoorbij microbial culture collection (ARC Inf-Nvbij CC). Lane 1 - M2/1; Lanes 2 & 3 - NH 07/1 & NH 07/2; Lane 4 – MCB C6.

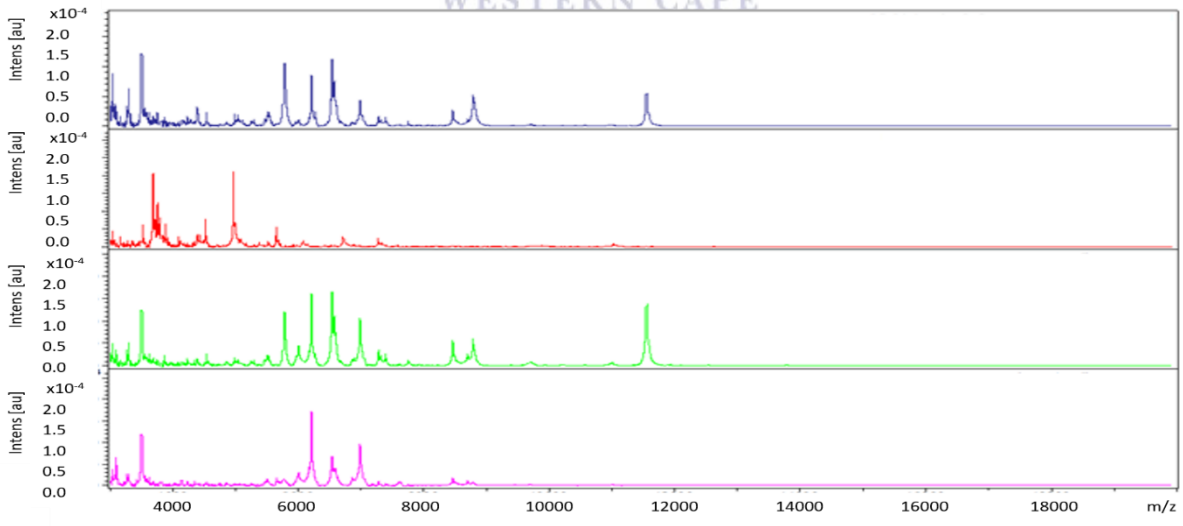

FIGURE 5.3: Matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) spectral fingerprints of parental strains, *S. cerevisiae* MCB C6 (blue-coloured spectrum) and *T. delbrueckii* M2/1 (red-coloured spectrum) and inter-genus hybrids, NH 07/1 (green-coloured spectrum) and NH 07/2 (purple-coloured spectrum) conserved in the ARC Infruitec-Nietvoorbij microbial culture collection. Inter-genus hybrids were selected for the production of aromatic white wine, especially Sauvignon blanc. The absolute intensities of the ions and mass-to-charge (m/z) ratios are represented on the y- and x-axis, respectively.

TABLE 5.1: Matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF/MS) real time classification of parental strains, *Saccharomyces cerevisiae* MCB C6 and *Torulaspora delbrueckii* M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 used for the production of aromatic Sauvignon blanc wine.

Mass spectra number	Yeast strain	MALDI-TOF MS log (score) value	Identification
1	**MCB C6[3]**	1.7	*Candida robusta*[1]
2	**M2/1[3]**	2.0	*Candida colliculosa*[2]
3	**NH 07/1[3]**	2.1	*Candida robusta*[1]
4	**NH 07/2[3]**	1.9	*Candida robusta*[1]

[1]*C. robusta* (anamorph of *S. cerevisiae*); [2]*C. colliculosa* (anamorph of *T. delbrueckii*); [3]Experimental yeast (ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa). *It can tentatively be speculated that inter-genus hybdrids were classified as C. robusta, as the database does not have inter-genus reference accessions.

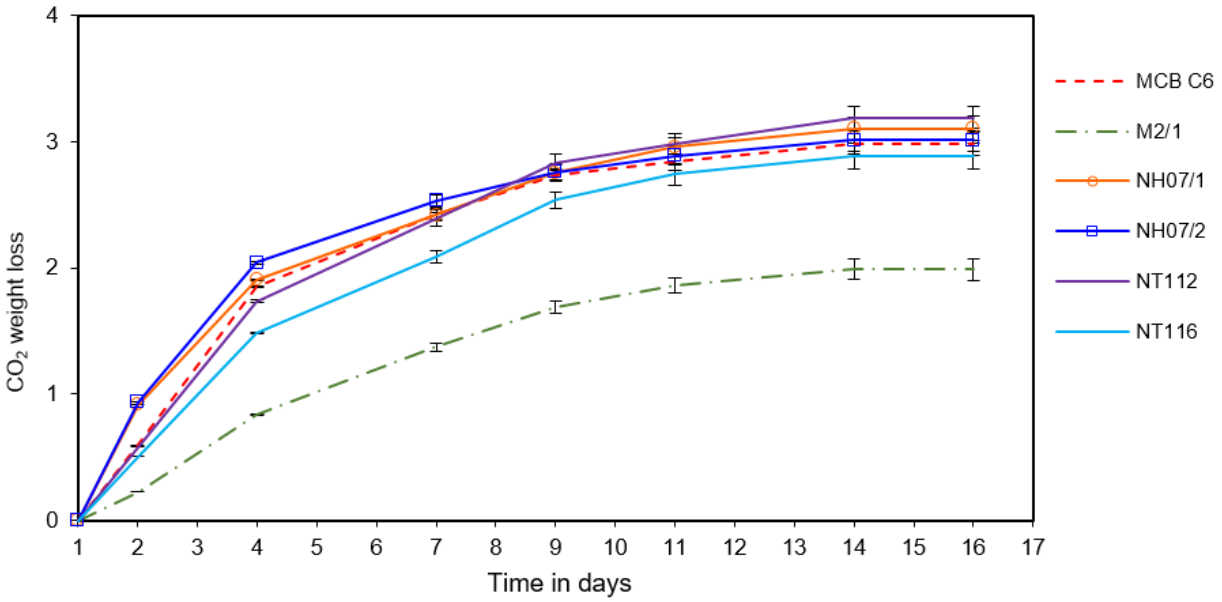

FIGURE 5.4: Carbon dioxide (CO_2) weight loss of Chardonnay grape must (juice) fermented at an ambient temperature of 15°C using *S. cerevisiae* strain MCB C6, *T. delbrueckii* strain M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 in laboratory-scale vinifications.

5.4.3. Small-scale winemaking

5.4.3.1. Fourier transform infra-red (FTIR) spectroscopy

Principle component analysis biplot of standard wine chemical parameters showed that both parental strains *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1 and inter-genus hybrids NH 07/1 and NH 07/2 produced final Sauvignon blanc with a negative association with VA (Figure 5.5). This observation with regard to *T. delbrueckii* M2/1 complements observations made by Jolly *et al.* (2003), Van Breda *et al.* (2013). The inter-genus hybrids can also provisionally be classified as LVPH a trait inherited from the non-*Saccharomyces* parental strain. Intra-genus hybrid strains provisionally characterised as LVPH, NH 48, NH 57, NH 143, and NH 145 were positioned in the left quadrants (Figure 5.5), and the wines also had a negative association with VA. The yeast Zymaflore VL3, positioned in the top-right quadrant, was the only commercial TRWY reference that produced wine with a positive association with VA (Figure 5.5).

5.4.3.2. Sensory evaluation

Overall, none of the wines were perceived to be undesirable during descriptive sensory evaluation, but differences were evident regarding expression of tropical fruit, floral and vegetative aroma notes (Figure 5.6). The PCA biplot of descriptive sensory evaluation data showed that two commercial TRWY references, VIN 7 and Zymaflore X5 positioned in the bottom left quadrant, produced Sauvignon blanc wines with a positive association with tropical fruit aromas, thereby supporting recommendations by yeast manufacturers for their use in the production of aromatic white wines, especially Sauvignon blanc (Figure 5.6). The commercial TRWY, Zymaflore VL3 and Fermicru 4F9 positioned in the top quadrants, on the other hand produced wines that positively associated with floral and vegetative aromas. The commercial TRWY VIN 13, two intra-genus TFPH and LVPH, NH 56 and NH 97 as well as the intra-genus hybrid parental strains, N 96 and P 35 positioned in the top left quadrant, produced wines that positively associated with both vegetative and tropical fruit aromas. Vegetative aromas

associated with Sauvignon blanc wines can be attributed to grape-derived aroma compounds *e.g.* 2-isobutyl-3-methoxypyrazine (IBMP), especially when grapes were harvested and processed under cooler conditions (Marais, 1994; Lapalus, 2016). It can, therefore, be concluded that these compounds masked the effect of the sought-after volatile thiols (Marais, 1994) associated with tropical fruit aroma, as VIN 13 is a known TRWY strain (Swiegers *et al.,* 2009; Von Mollendorf, 2013).

Both inter-genus hybrids, NH 07/1 and NH 07/2 as well as two intra-genus TFPH, NH 118 and NH 145 produced wines with a positive association with floral aromas (Figure 5.6). These hybrids can provisionally be characterised as having the ability to produce wines with floral aroma (henceforth referred to as FLPH). Both MCB C6 and M2/1, positioned in the bottom quadrants, produced wines that associated with floral and tropical fruit aromas. This supports previous observations that a *T. delbrueckii* strain (Belda *et al.,* 2015; Renault *et al.,* 2016) as well as an *S. cerevisiae*/*T. delbrueckii* inter-genus hybrid (Santos *et al.,* 2008) produced aromatic wines. Floral aromas, frequently associated with Sauvignon blanc wines, are the result of yeast-mediated metabolites, namely monoterpenes produced from pre-cursors present in grape must (Von Mollendorf, 2013; Hart *et al.,* 2017). It is apparent that the inter-genus hybrids inherited the ability to release monoterpenes from the parental strains. The intra-genus TFPH, NH 48, NH 84, NH 88 and NH 143 some of which were also shown to be LVPH (Figure 5.5), produced wines with a positive association with tropical fruit and floral aromas (Figure 5.5). These TFPH also produced wines with a negative association with vegetative aromas. Both inter-genus FLPH, NH 07/1 and NH 07/2, also produced Sauvignon blanc wines with a negative association with VA (Figure 5.5) and can provisionally be characterised as LVPH. Two intra-genus TFPH and LVPH, NH 57 and NH 145 produced wines with a negative association with VA (Figure 5.5), and a positive association with tropical fruit aroma (Figure 5.6). Therefore, these intra- and interspecfic TFPH, FLPH and LVPH yeasts showed promise for the production of typical varietal aromatic Sauvignon blanc wines with lower VA.

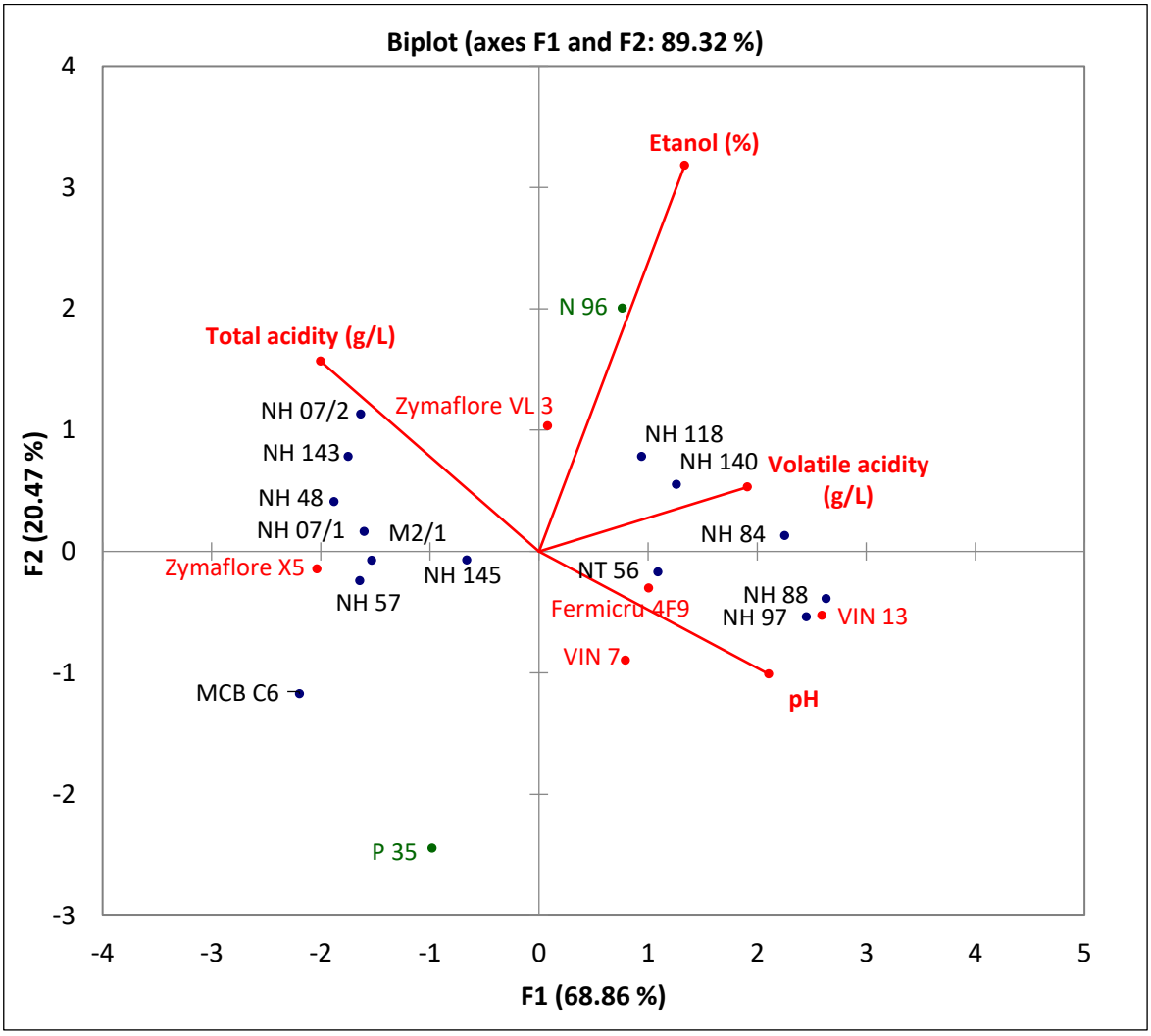

FIGURE 5.5: Biplot of basic chemical parameters of small-scale Sauvignon blanc wine following fermentation by five 'thiol-releasing' commercial wine yeasts (TRWY), VIN 7 and VIN 13, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9, two yeast strains with ability to produce wine with tropical fruit aromas, N 96 and P 35, ten intra-genus hybrids with the ability to produce wines with enhanced tropical fruit aroma and low VA, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145; and MCB C6 and M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 conserved in the ARC Infruitec-Nietvoorbij microbial culture collection. Average values of triplicate fermentations.

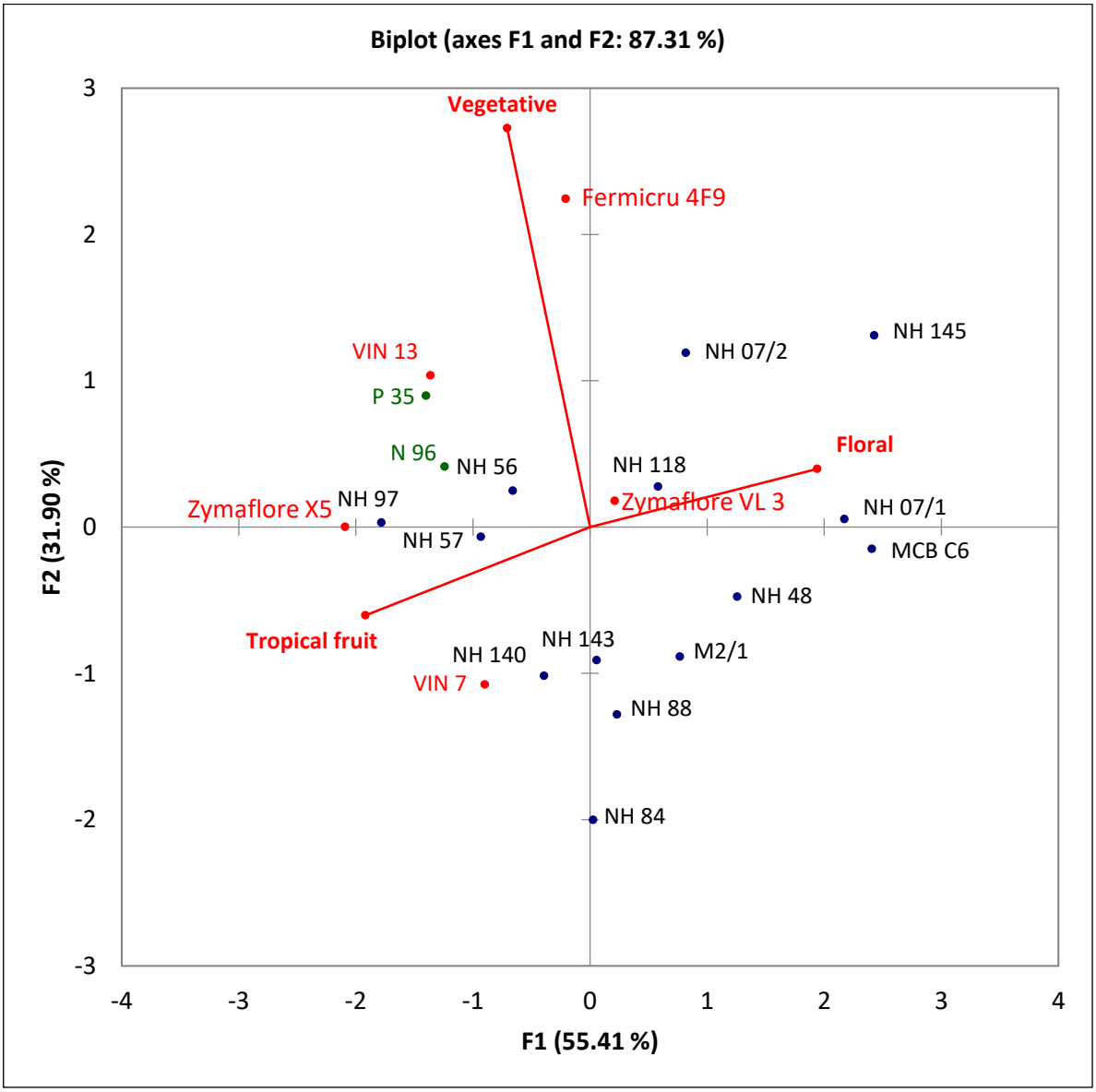

FIGURE 5.6: Biplot of descriptive sensory evaluation of small-scale Sauvignon blanc wine following fermentation by five 'thiol-releasing' commercial wine yeasts (TRWY), VIN 7 and VIN 13, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9, two yeast strains with ability to produce wine with tropical fruit aromas, N 96 and P 35, ten intra-genus hybrids with the ability to produce wines with enhanced tropical fruit aroma and low VA, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145; and MCB C6 and M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 conserved in the ARC Infruitec-Nietvoorbij microbial culture collection. Average values of triplicate fermentations.

5.4.3.3. Gas chromatography (GC) analysis

Gas chromatography was deployed to quantify wine aroma compounds, namely esters, total fatty acids and higher alcohols, most of which are associated with wine 'fermentation bouquet' and/or 'fruitiness' (Lambrechts & Pretorius, 2000; Coetzee & du Toit, 2015). The PCA biplot showed that strain *T. delbrueckii* M2/1 produced wines with a positive association with esters and higher alcohols compared to the remaining *S. cerevisiae* and hybrid yeast strains (Figure 5.7). Strains of *T. delbrueckii* were previously reported to produce wines with enhanced aroma (Van Breda *et al.,* 2013; Renault *et al.,* 2016). The TRWY reference VIN 7 and the intra-genus hybrids, NH 48 and NH 145, positioned in the right quadrant, also produced wines with a positive association with esters and higher alcohols, which imparts fruity aromas and complexity. These wines had positive associations with, amongst others, tropical fruit and floral aromas (Figure 5.6).

The TRWY reference, Zymaflore X5, the TFPP, N 96 (Figure 5.7) as well as the intra-genus hybrid NH 143, positioned in the top right quadrant, produced wines with a positive association with total fatty acids also referred to as volatile fatty acids. Some volatile fatty acids (*e.g.* octanoic acid, decanoic acid) were reported to be associated with faint fruity and citrus wine aromas (Lambrechts & Pretorius, 2000). However, acetic acid, responsible for vinegar-like off-flavours at higher concentrations still remains the main contributor to total fatty acids (Swiegers *et al.,* 2005; Ugliano *et al.,* 2009; Vilela-Moura *et al.,* 2011). Nevertheless, Zymaflore X5 still produced wines with a positive association with fruity aroma, specifically tropical fruit (Figure 5.6). The TRWY references VIN 13, Zymaflore VL3 and Fermicru 4F9, positioned in the left quadrants, produced wines with a negative association with total fatty acids (Figure 5.7). These wines also had a positive association with, amongst others, tropical fruit and floral aromas (Figure 5.6).

Both inter-genus FLPH, NH 07/1 and NH 07/2 also produced wines with a negative association with volatile fatty acids. This can also be seen in Figure 5.5 and 5.6 respectively, where there was a negative association with VA (Figure 5.5) and positive association with floral aroma (Figure 5.6). The lower

production of VA by the inter-genus hybrids can be attributed to inheritance from the *T. delbrueckii* parent strain.

Seven intra-genus hybrids provisionally characterised as TFPH and LVPH, namely NH 56, NH 57, NH 84, NH 88, NH 97, NH 118 and NH 140 also produced wines with a negative association with volatile fatty acids, including acetic acid (Figure 5.7). Two of these intra-genus hybrids were also shown to produce Sauvignon blanc wines with a negative association with VA (Figure 5.5) and positive association with floral aroma (Figure 5.6). These intra-genus hybrids also produced wines with a postive association with esters and higher alcohols (Figure 5.7). Yeast strains, namely VIN 7, M2/1, NH 48, NH 57, NH 84, NH 88, NH 140 and NH 143 produced wines with a positive association with 'fruitiness' (tropical fruit aroma) (Figure 5.6) and higher alcohols (Figure 5.7). Therefore, this observation compliments a previous study that showed higher alcohols to be the key precursors involved in ester formation (Patrianakou and Roussis, 2013). Based on this data set, intra- and inter-genus TFPH, FPH and LVPH have great potential for the production of varietal aromatic Sauvignon blanc wines with lower VA, as they comply with yeast selection criteria set forth in the objectives.

5.4.3.4. Solid-phase extraction (SPE) and GC-MS/MS analysis

Volatile aromatic thiols *e.g.* 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA), primarily responsible for passion fruit, tropical fruit and citrus aromas in Sauvignon blanc wines, are released by fermenting wine yeasts from aroma-inactive, bound precursors present in grape juice (Swiegers *et al.,* 2007b; Roland *et al.,* 2011; Harsch *et al.,* 2013). The PCA biplot of volatile thiol analyses showed that the inter-genus hybrid NH 07/1 produced wine with a more positive association with volatile thiols, 3MHA in particular, than either parental strain as well as two known commercial TRWY yeasts, Zymaflore X5 and Zymaflore VL3 (Figure 5.8). It is noteworthy that NH 07/1 also produced wines with a negative association with VA (Figure 5.5) and acetic acid (Figure 5.7), whilst having a positive association with floral aroma (Figure 5.6). These wines also had hints of tropical fruit aroma, and data

suggests that inter-genus hybrids can be applied for the production of varietal aromatic Sauvignon blanc wines with lower VA. The inter-genus hybrid NH 07/2, on the other hand, produced wines with a negative association with 3MH and 3MHA (Figure 5.8). This observation compliments the descriptive sensory evaluation, as NH 07/2 produced wines with a positive association with vegetative aroma (Figure 5.6). Nonetheless, NH 07/2 produced wines with chemically detectable 3MH and 3MHA levels, albeit it lowest of all yeast strains included in this study. It can, therefore, be speculated that the tropical fruit aroma and effect of volatile thiols were masked by methoxypyrazines, another aroma compound naturally associated with this cultivar (Marais, 1994; Lapalus, 2016).

Three intra-genus hybrids, NH 56, NH 84 and NH 88 produced wines with stronger association with the volatile thiol 3MH than the commercial TRWY VIN 7 reference. The latter was also reported to be the highest producer of another volatile thiol, 4MMP, associated with 'fruity' aroma in wine during previous studies (Swiegers *et al.,* 2009; Borneman *et al.,* 2012). Five more intra-genus yeasts, NH 48, NH 118, NH 140, NH 143 and NH 145 also produced wines with a stronger association with 3MH than wines produced with the commercial TRWY Zymaflore X5 and Zymaflore VL3 (Dubourdieu, 2006; Bowyer *et al.,* 2008). These hybrids also produced wines with a stronger association with 3MHA than wines produced with the commercial TRWY VIN 13. Three intra-genus TFPH and LVPH, NH 56, NH 57 and NH 88 also produced wines with a negative association with VA (Figure 5.5) and acetic acid (Figure 5.7), whilst having a positive association with tropical fruit aroma (Figure 5.6). Therefore, observations made during this study is indicative that these intra-genus hybrids can be used for the production of varietal aromatic Sauvignon blanc wines with low VA. It is noteworthy that the intra-genus TFPH, NH 84 produced wines with 3MH levels that were significantly higher than its sensory detection threshold (Van Wyngaard, 2013). The 3MH levels in these wines were also discernibly higher compared to wines produced by the best commercial TRWY reference VIN 7 in this study. The TFPP N 96, considered to be a 'neutral' yeast by the manufacturer (Anchor Yeast, South Africa - N 96 product data sheet), produced wines with a more positive association with 3MH (Figure 5.8) than the *T. delbrueckii* M2/1 previously shown to produce aromatic wines (Jolly *et al.,* 2003; Van Breda *et al.,*

2013). Indications, therefore, are that intra-genus TFPH inherited the 'thiol-releasing' abilities from both *S. cerevisiae* parental strains *i.e.* N 96 and P 35. The latter produced wines with a stronger association with 3MH than all commercial TRWY references included in this study (Figure 5.8).

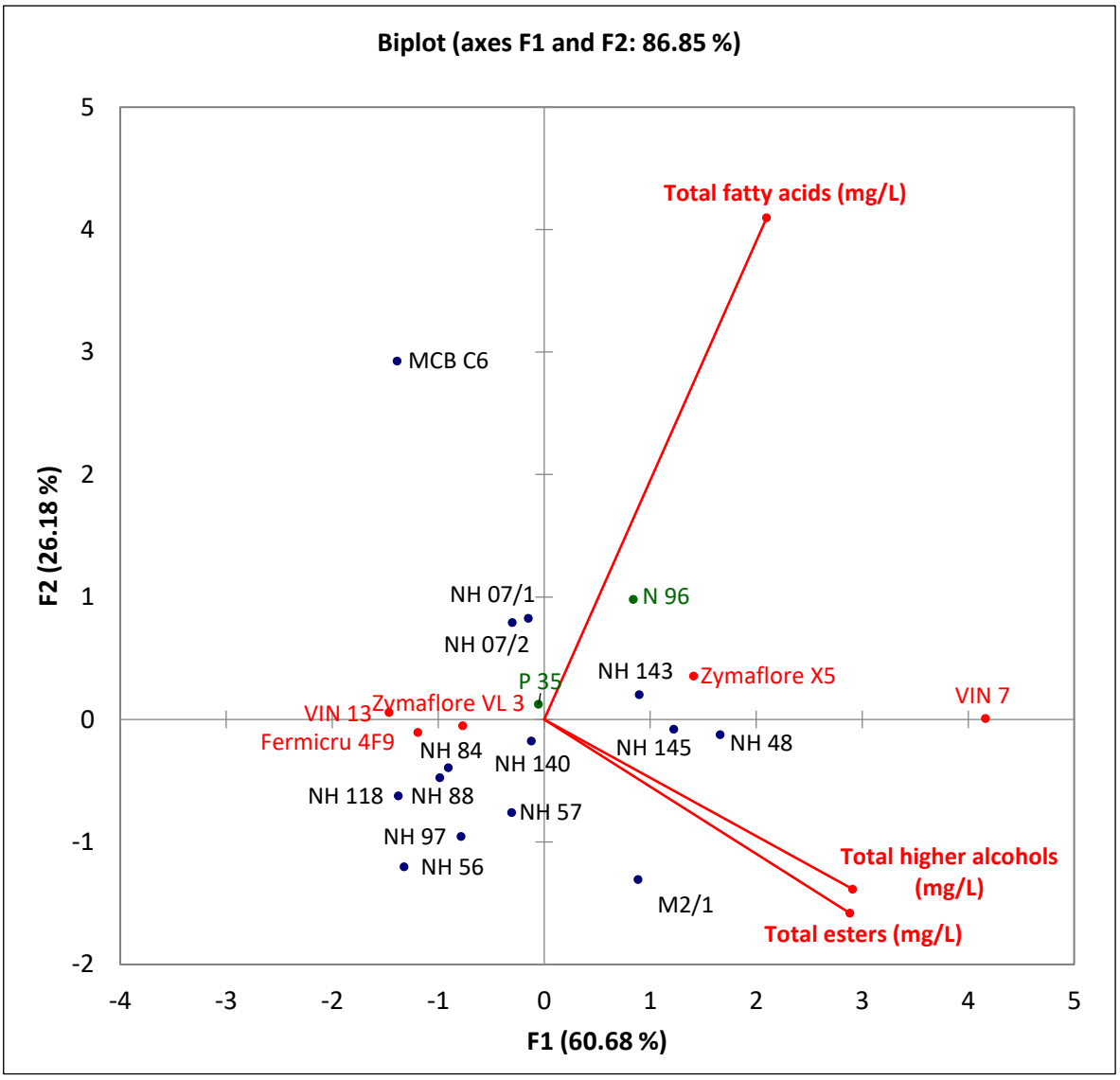

FIGURE 5.7: Biplot of aroma compounds, esters, higher alcohols and fatty acids in small-scale Sauvignon blanc wine following fermentation by five 'thiol-releasing' commercial wine yeasts (TRWY), VIN 7 and VIN 13, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9, two yeast strains with ability to produce wine with tropical fruit aromas, N 96 and P 35, ten intra-genus hybrids with the ability to produce wines with enhanced tropical fruit aroma and low VA, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145; and MCB C6 and M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 conserved in the ARC Infruitec-Nietvoorbij microbial culture collection. Average values of triplicate fermentations.fermentations.

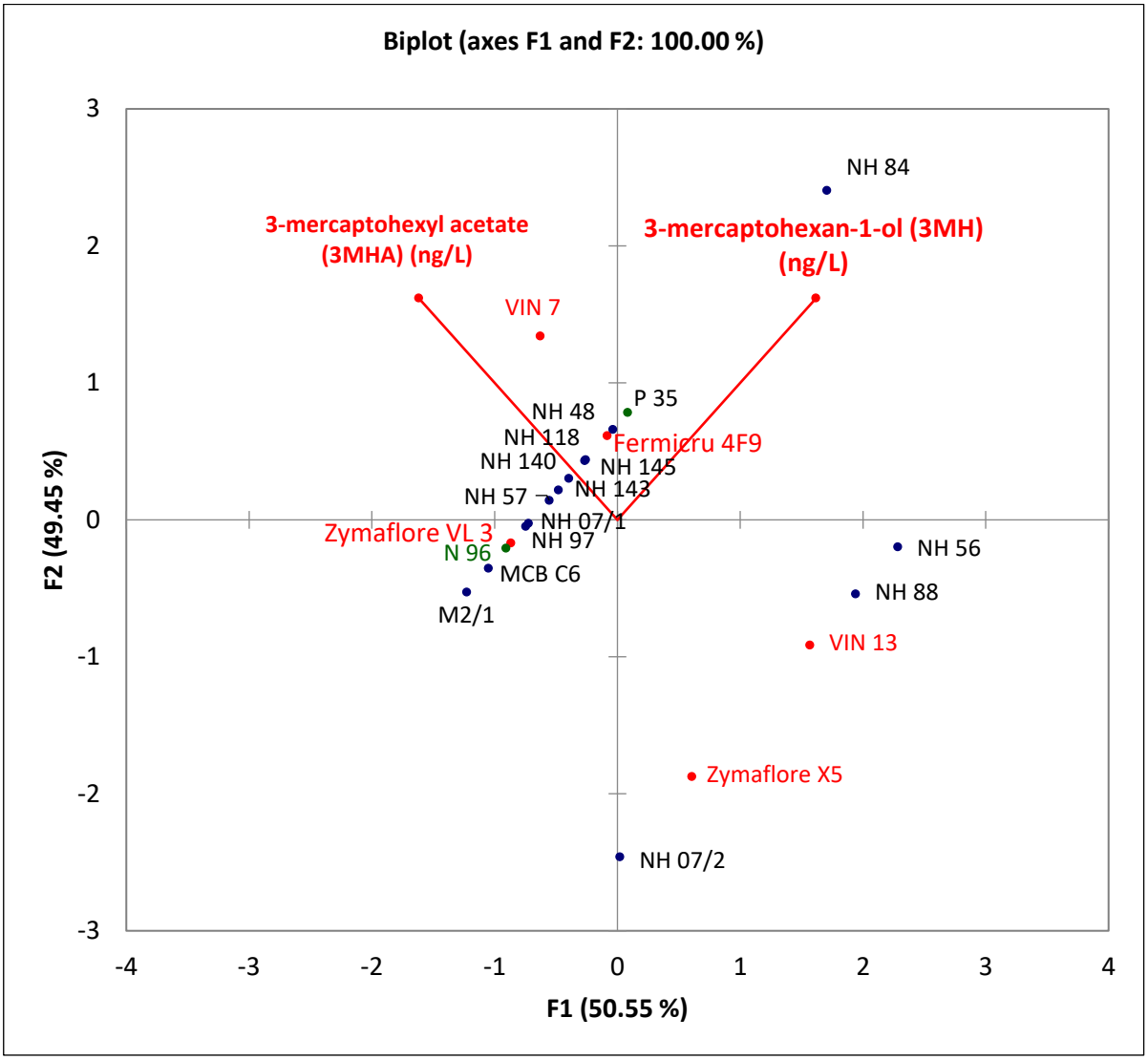

FIGURE 5.8: Biplot of volatile thiols, 3MH and 3MHA in small-scale Sauvignon blanc wine following fermentation by five 'thiol-releasing' commercial wine yeasts (TRWY), VIN 7 and VIN 13, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9, two yeast strains with ability to produce wine with tropical fruit aromas, N 96 and P 35, ten intra-genus hybrids with the ability to produce wines with enhanced tropical fruit aroma and low VA, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145; and MCB C6 and M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 conserved in the ARC Infruitec-Nietvoorbij microbial culture collection. Average values of triplicate fermentations.

*5.4.3.5. Quantitative LC-based iTRAQ p*roteomic analysis

Yeast-expressed enzymes (proteins) during fermentation regulate wine aroma compounds (metabolites) responsible for wine aroma and flavour (organoleptic quality) (Moreno-García *et al.,* 2015). Analysis of the combined datasets in conjunction with Uniprot *S. cerevisiae* database identified a total of 998 yeast derived proteins (Table 5.2) on day 15 of fermentation when fermentations stabilised and/or were dry. Commercial TRWY (VIN 7, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9), naturally isolated parental strains (MCB C6 and M2/1), as well as both intra- and inter-genus hybrids (NH 84, NH 07/1) were shown to vary in their up- and down-regulated proteins compared to the TRWY VIN 7 reference expressed proteins. Overall 25 proteins (2.51%) were down-regulated and 122 proteins (12.22%) were overexpressed.Properties and relative expression of down-regulated and overexpressed proteins of yeast strains, amongst others, the intra-genus TFPH and LVPH NH 84 and inter-genus FLPH and LVPH, NH 07/1 were established by using quantitative LC-based iTRAQ proteomic analysis (Tables 5.3, 5.4, 5.5, 5.6, 5.7, 5.8 & 5.9).

Proteomic analyses showed that the TRWY Zymaflore VL3 reference over-expressed the lactoylglutathione lyase protein (Table 5.3), an enzyme responsible for cleaving a carbon-sulfur bond to release the volatile thiol 4MMP from its bound aroma-inactive precursor (Howell *et al.,* 2005). Unfortunately, 4MMP was not quantified during this study. However, the TRWY produced wine with a moderate association with 3MH. It can, therefore, be speculated that above-mentioned carbon-sulfur lyase enzyme is also involved in the release of 3MH from its carbon-sulfur-containing precursor (Howell *et al.,* 2005; Swiegers *et al.,* 2007a). A gene encoding for β-lyase involved in the release of volatile thiols, 3MH and 4MMP was previously reported (Holt *et al.,* 2011). Therefore, lactoylglutathione lyase might be used as a protein biomarker for volatile thiol release during production of varietal Sauvignon blanc wines. Additionally, Zymaflore VL3 produced wines with a positive association with floral aroma that is influenced by yeast-mediated released monoterpenes, which essentially are hydrocarbons and/or glycoconjugates, from its bound aroma-inactive precursors in grape juice (Von Mollendorf,

2013). Monoterpenes was shown to be released in abundance by using genetically modified (GM) *S. cerevisiae* strains expressing a S-linalool synthase (Pardo *et al.,* 2015). Nevertheless, non-GM *S. cerevisiae* can release moderate geraniol and linalool levels (Lambrechts & Pretorius, 2000). The TRWY was also shown to produce wines with a moderate association with VA (Figure 5.5), which comprise acetic acid, an intermediate of long chain fatty acid production catalysed by fatty acid synthases. However, the yeast did not regulate any synthases. Nonetheless, the association between regulated proteins, especially synthases, and their effect on VA formation should be further investigated.

TABLE 5.2: Number of differentially expressed proteins originating from fermenting commercial 'thiol-releasing' wine yeasts (TRWY), with ability to produce wine with tropical fruit aromas, naturally isolated parental strains, intra- and inter-genus hybrid yeast strains during the fermentation of 2013 Sauvignon blanc grape juice.

Yeast strain	Stationary phase	
	Down-regulated	Over-expressed
VIN 7 TRWY Reference	998 proteins characterised	
VL3 TRWY	2	27
X5 TRWY	4	9
4F9 TRWY	1	60
NH 84 TFPH & LVPH	0	6
MCB C6	6	9
M2/1	11	3
NH 07/1 FLPH & LVPH	1	8

[1]TRWY: thiol-releasing wine yeast
[2]TFPH: hybrid with the ability to produce wines with tropical fruit aroma
[3]LVPH: hybrid with the ability to produce wines with lower volatile acidity
[4]FLPH: hybrid with the ability to produce wines with floral aroma

The TRWY Zymaflore X5 was shown not to regulate any lyases and synthases (Table 5.4), which complements FTIR analyses (Figure 5.5), descriptive sensory evaluation (Figure 5.6), GC-analyses (Figure 5.7) and SPE-GC/MS analyses (Figure 5.8), as the yeast produced wines with a negative association with VA and total fatty acids (comprised mainly of acetic acid), a positive association with tropical fruit aroma, and a negative association with volatile thiols, respectively. The TRWY Fermicru 4F9 on the other hand was shown to regulate dehydrogenases (Table 5.5). Proteins in the same class were previously implicated in excessive acetic acid production (Varela *et al.,* 2012; Walkey *et al.,* 2012). However, regulated dehydrogenases by Fermicru 4F9 do not seem to have stimulated VA formation as the yeast produced wines with a negative association with VA and total fatty acids (Figure 5.6 & 5.7). The yeast also did not regulate any carbon-sulfur lyases responsible for volatile 'thiol-release' and, therefore, complements descriptive sensory evaluation as the yeast produce wines with a negative association with tropical fruit aroma (Figure 5.6). The SPE-GC/MS analyses revealed that the TRWY produced wines with a positive association with volatile thiols 3MH and 3MHA (Figure 5.7). This therefore, implies that more proteins might be involved in volatile thiol-release.

Proteomic analyses further showed that the intra-genus TFPH and LVPH NH 84 was the only strain not to have down-regulated any proteins, whilst the remaining strains down-regulated from one to 11 proteins (Table 5.6). Furthermore, NH 84 only overexpressed six proteins classed as nucleic acid 'binders', hydrolases and transporters, some of which are associated with cell proliferation and protein synthesis. As NH 84 was the only strain to have regulated these proteins, they could also be associated with higher 3MH released by this strain. These proteins will in future be further investigated as potential biomarkers, as the yeast also produced wines with a positive association with tropical fruit aroma (Figure 5.6) and volatile thiols, especially 3MH (Figure 5.8).

Both inter-genus parental strains, *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1 were shown not to regulate the lactoylglutathione lyase protein (Tables 5.7 & 5.8). Nevertheless, the inter-genus hybrid, NH 07/1 provisionally characterised as a FLPH and LVPH, as observed with the TRWY Zymaflore VL3,

over-expressed the lactoylglutathione lyase protein responsible for the release of the volatile thiol 4MMP (Table 5.9) (Howell *et al.,* 2005). As previously mentioned, 4MMP was not quantified during this study. The inter-genus hybrid not only produced wine with a positive association with 3MH, it was more pronounced than wines produced with both parental strains MCB C6 and M2/1 and TRWY Zymaflore VL3 and Zymaflore X5. As 3MH release also involves enzymatic cleavage by a carbon-sulfur lyase, there is a possibility that this over-expressed protein could also be involved in the release of 3MH from its carbon-sulfur-containing precursor (Howell *et al.,* 2005; Swiegers *et al.,* 2007a). This observation further supports the notion that lactoylglutathione lyase might be a useful biomarker for volatile thiol release by NH 07/1 during production of varietal Sauvignon blanc wines.

The inter-genus FLPH and LVPH, NH 07/1 also produced wines with a positive association with floral aroma that is influenced by yeast-released monoterpenes, a metabolite that was released in abundance by a genetically modified (GM) *S. cerevisiae* strains expressing a S-linalool synthase as mentioned above (Von Mollendorf, 2013; Pardo *et al.,* 2015). However, the inter-genus FLPH did not regulate any synthases, which suggests that other proteins are involved in monoterpene release. Strains belonging to the same species of parental strains *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1 are known to produce monoterpenes (King & Dickinson, 2000). This warrants further investigation to identify protein biomarkers associated with floral wine aroma and monoterpene release.

Differentially expressed proteins by the intra-genus TFPH and LVPH, NH 84 and inter-genus FLPH and LVPH, NH 07/1 during the stationary phase of Sauvignon blanc grape must fermentation were classified according to molecular function, biological process and protein class using PANTHER (Sharma *et al.,* 2014). Classification of proteins according to molecular function showed that NH 84 regulated proteins were linked to translation regulator activity, catalytic activity and transporter activity, whilst that of NH 07/1 were linked to binding activity, structural molecule activity, catalytic activity and antioxidant activity (Figure 5.9a & b). Classification of proteins according to biological processes showed that NH 84 regulated proteins were linked to cellular processes, metabolic processes and

localisation, whilst those of NH 07/1 were linked to response to stimuli, cellular processes, metabolic processes and cellular biogenesis (Figure 5.9c & d). Furthermore, NH 84 regulated proteins clustered into three protein classes *viz.* nucleic acid binding, hydrolase and transporter, whilst those of NH 07/1 also clustered into different protein classes *i.a.* hydrolases, and oxidoreductases (Figure 5.9e & f). It is evident that regulated proteins differed between intra-genus and inter-genus strains, explaining the production of wines with different chemical (Figure 5.5) and sensory (Figure 5.6) properties, as well as differences in aroma and off-flavour compound levels (Figure 5.7 & 5.8).

FIGURE 5.9: Classification of differentially expressed proteins by intra-genus hybrids with the ability to produce wines with enhanced tropical fruit aroma (abbreviated as TFPH) and low VA (abbreviated as LVPH), NH 84 and the inter-genus hybrid, NH 07/1 during the end of Sauvignon blanc grape must fermentation according to a & b) Molecular function, c & d) Biological process, and e & f) Protein class using Protein ANalysis THrough Evolutionary Relationships (PANTHER, www.pantherdb.org/).

TABLE 5.3: Differentially expressed proteins by the TRWY Zymaflore VL3 relative to the commercial TRWY reference VIN 7 during the stationary phase of fermentation in 2013 Sauvignon blanc grape must.

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	114/113 ratio
A6ZQH2	Cell wall protein	157,45	68,29%	1	30,30	8,81	0,19
P36110	Protein PRY2 OS	41,81	48,94%	2	33,80	4,60	0,40
A5Z2X5	UPF0495 protein YPR	13,73	50,00%	1	7,90	8,73	2,13
P12695	Dihydrolipoyllysine-residue acetyltransferase	98,66	68,26%	5	51,80	7,80	2,16
P40961	Prohibitin-1 OS=Saccharomyces	42,49	67,94%	1	31,40	8,25	2,19
P38791	Deoxyhypusine synthase OS	39,81	59,43%	2	42,90	5,77	2,22
P07251	ATP synthase subunit	84,47	73,76%	3	58,60	9,04	2,23
P32474	Protein disuphide-isomerase	40,73	38,88%	1	58,90	4,91	2,25
P00445	Superoxide dismutase [Cu	406,09	73,38%	14	15,80	6,00	2,28
P32347	Uroporphyrinogen decarboxylase OS	33,97	67,68%	2	41,30	7,08	2,35
P38972	Phosphoribosylformylglycinamidine synthase	230,63	54,12%	2	148,80	5,27	2,41
P50107	Lactoylglutathione lyase OS	25	25,46%	2	37,20	6,84	2,42
P24000	60S ribosomal protein	21,73	56,13%	1	17,50	11,37	2,53
P25567	RNA-binding protein	79,49	71,20%	2	48,00	8,90	2,53
P25372	Thioredoxin-3, mitochondrial OS	4,76	65,35%	2	14,40	8,90	2,56

Table 5.3: continued.

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	114/113 ratio
P47068	Myosin tail region	148,86	62,58%	2	128,20	5,26	2,58
P38999	Saccharopine dehydrogenase [NADP	91,22	61,21%	8	48,90	5,27	2,72
P00812	Arginase OS=Saccharomyces	60,79	73,27%	1	35,60	5,64	2,87
P05626	ATP synthase subunit	42,37	72,13%	2	26,90	9,13	3,03
P38077	ATP synthase subunit	107,73	71,38%	2	34,30	9,31	3,26
P05744	60S ribosomal protein	24,5	58,88%	1	12,10	11,08	3,32
C8ZCR2	ABC transporter NFT	167,29	55,97%	1	176,20	8,60	3,95
P33317	Deoxyuridine 5'-triphosphate	94,91	88,44%	2	15,30	7,25	4,25
P05740	60S ribosomal protein	188,92	79,89%	1	20,50	10,92	4,26
P40202	Superoxide dismutase 1 copper	61,52	68,67%	3	27,30	6,67	4,48
P40581	Peroxiredoxin HYR1 OS	15,24	57,06%	4	18,60	8,19	4,93
P39016	Suppressor protein MPT	233,23	73,22%	1	95,40	8,03	5,13
P07560	Ras-related protein	47,27	64,65%	3	23,50	7,09	5,51
P38788	Ribosome-associated complex	122,09	68,03%	4	58,20	5,05	11,90

TABLE 5.4: Differentially expressed proteins by the TRWY Zymaflore X5 relative to the commercial TRWY reference VIN 7 during the stationary phase of fermentation in 2013 Sauvignon blanc grape must.

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	115/113 ratio
A6ZQH2	Cell wall protein	157,45	0,68	1,00	30,30	8,81	0,14
P53297	PAB1-binding protein	148,86	0,61	3,00	78,70	6,92	0,30
P36110	Protein PRY2 OS	41,81	0,49	2,00	33,80	4,60	0,38
A6ZP47	ATP-dependent RNA	204,95	0,80	2,00	65,50	7,83	0,42
Q08193	1,3-beta-glucanosyltransferase GAS	68,61	0,67	5,00	51,80	4,64	2,07
P38065	AP-2 complex subunit	172,82	0,66	1,00	114,90	8,63	2,07
C8ZEN7	Eisosome protein 1 OS	74,59	0,56	2,00	93,20	6,42	2,13
P43598	Inhibitor of glycogen	40,88	0,78	2,00	21,80	7,18	2,25
P38841	Uncharacterized protein YHR	33,56	0,89	7,00	12,70	5,06	2,30
P38431	Eukaryotic translation initiation	45,87	0,65	4,00	45,20	5,06	2,42
Q07897	Protein CMS1 OS	15,81	0,43	1,00	33,40	8,19	2,61
P30402	Orotate phosphoribosyltransferase 2 OS	77,50	0,78	3,00	24,80	8,51	4,67
P15303	Protein transport protein	220,61	0,69	9,00	85,30	5,66	5,07

TABLE 5.5: Differentially expressed proteins by the TRWY Fermicru 4F9 relative to the commercial TRWY reference VIN 7 during the stationary phase of fermentation in 2013 Sauvignon blanc grape must.

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	116/113 ratio
P43612	SIT4-associating protein	139,55	0,64	2,00	114,90	4,72	0,35
P07267	Saccharopepsin OS=Saccharomyces	36,79	0,70	9,00	44,50	4,84	2,04
P38219	Obg-like ATPase	32,52	0,46	1,00	44,10	7,43	2,05
Q08548	Lysophospholipid acyltransferase OS	34,90	0,46	1,00	72,20	9,48	2,07
P53044	Ubiquitin fusion degradation	52,38	0,57	1,00	39,80	6,21	2,09
P28737	Protein MSP1 OS	40,66	0,60	1,00	40,30	5,72	2,10
P40312	Cytochrome b5 OS	9,95	0,83	1,00	13,30	4,49	2,10
P07342	Acetolactate synthase catalytic	137,74	0,78	3,00	74,90	8,51	2,10
P32473	Pyruvate dehydrogenase E	36,51	0,64	2,00	40,00	5,30	2,15
P32347	Uroporphyrinogen decarboxylase OS	33,97	0,68	2,00	41,30	7,08	2,16
P26309	APC/C activator	83,93	0,69	2,00	67,30	8,82	2,21
P05744	60S ribosomal protein	24,50	0,59	1,00	12,10	11,08	2,22
P24000	60S ribosomal protein	21,73	0,56	1,00	17,50	11,37	2,24
P38075	Pyridoxamine 5'-phosphate	11,60	0,41	1,00	26,90	7,49	2,27
P26785	60S ribosomal protein	18,06	0,54	2,00	22,20	10,55	2,30
P0C0V8	40S ribosomal protein	40,64	0,64	2,00	9,70	6,06	2,40

TABLE 5.5: continued.

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	116/113 ratio
P38115	D-arabinose dehydrogenase	35,10	0,72	4,00	38,90	5,96	2,40
P45978	Protein SCD6 OS	53,11	0,64	1,00	39,20	7,42	2,41
P54838	Dihydroxyacetone kinase 1 OS	130,43	0,74	7,00	62,20	5,41	2,43
P43603	LAS seventeen-binding	100,28	0,85	3,00	49,30	7,96	2,44
A6ZPY2	Translation machinery-associated	19,03	0,45	2,00	22,50	8,60	2,54
P39676	Flavohemoprotein OS=Saccharomyces	60,50	0,62	3,00	44,60	6,28	2,63
P39929	Vacuolar-sorting protein	32,57	0,59	3,00	27,00	4,72	2,66
P36047	Protein phosphatase 1 regulatory	38,66	0,66	2,00	38,90	5,52	2,68
P11633	Non-histone chromosomal	26,18	0,58	1,00	11,50	9,89	2,71
P02294	Histone H2B	69,05	0,58	8,00	14,20	10,07	2,74
Q12017	Phosducin-like protein	52,54	0,66	1,00	32,80	4,72	2,78
Q02979	Glycerophosphodiester phosphodiesterase GDE	146,98	0,68	2,00	137,90	6,79	2,85
Q12218	Cell wall protein	33,24	0,26	2,00	47,80	4,15	2,88
Q05016	Uncharacterized oxidoreductase YMR	64,84	0,84	3,00	29,10	6,81	2,94
P40472	Probable secreted beta	83,48	0,52	4,00	48,20	4,60	2,94
P09064	Eukaryotic translation initiation	56,65	0,71	9,00	31,60	9,50	2,95
P32628	UV excision repair	154,20	0,70	7,00	42,30	4,32	2,96
Q12497	Protein FMP16, mitochondrial	12,41	0,53	1,00	10,80	9,64	2,96
P05740	60S ribosomal protein	188,92	0,80	1,00	20,50	10,92	3,03

TABLE 5.5: continued.

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	116/113 ratio
P40961	Prohibitin-1 OS=Saccharomyces	42,49	0,68	1,00	31,40	8,25	3,10
P34730	Protein BMH2 OS	509,64	0,88	2,00	31,00	4,88	3,17
P00812	Arginase OS=Saccharomyces	60,79	0,73	1,00	35,60	5,64	3,18
Q00055	Glycerol-3-phosphate dehydrogenase	121,94	0,69	2,00	42,80	5,47	3,22
P25372	Thioredoxin-3, mitochondrial OS	4,76	0,65	2,00	14,40	8,90	3,26
P33331	Nuclear transport factor	27,65	0,70	2,00	14,40	4,70	3,35
P38788	Ribosome-associated complex	122,09	0,68	4,00	58,20	5,05	3,42
P37302	Aminopeptidase Y OS	56,17	0,59	7,00	60,10	5,31	3,51
P11972	Protein SST2 OS	77,95	0,55	2,00	79,70	8,57	3,56
P43598	Inhibitor of glycogen	40,88	0,78	2,00	21,80	7,18	3,66
O74700	Mitochondrial import inner	44,95	0,59	2,00	10,20	8,18	3,66
P42222	Enolase-related protein	145,18	0,68	2,00	47,30	5,44	3,85
P09457	ATP synthase subunit	35,29	0,76	4,00	22,80	9,57	3,90
P20435	DNA-directed RNA	43,81	0,54	1,00	17,90	4,63	3,95
P47068	Myosin tail region	148,86	0,63	2,00	128,20	5,26	4,32
P23638	Proteasome subunit alpha	46,79	0,58	3,00	28,70	5,22	4,62
A5Z2X5	UPF0495 protein YPR	13,73	0,50	1,00	7,90	8,73	4,77
P40581	Peroxiredoxin HYR1 OS	15,24	0,57	4,00	18,60	8,19	4,91
Q06135	1,3-beta-glucanosyltransferase GAS	35,18	0,33	1,00	62,30	4,97	5,36

TABLE 5.5: continued.

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	116/113 ratio
P33317	Deoxyuridine 5'-triphosphate	94,91	0,88	2,00	15,30	7,25	6,29
Q07897	Protein CMS1 OS	15,81	0,43	1,00	33,40	8,19	6,84
Q04951	Probable family 17 glucosidase	60,95	0,46	1,00	40,40	4,65	7,21
Q04409	Putative glucokinase-2 OS	103,62	0,61	3,00	55,90	6,27	7,40
P32773	Transcription initiation factor	40,40	0,61	2,00	32,20	4,39	8,78
C8ZCR2	ABC transporter NFT	167,29	0,56	1,00	176,20	8,60	13,68
Q08969	Protein GRE1 OS	223,34	0,89	7,00	19,00	4,77	25,08

TABLE 5.6: Differentially expressed proteins by the intra-genus TFPH and LVPH NH 84 relative to the commercial TRWY reference VIN 7 during the stationary phase of fermentation in 2013 Sauvignon blanc grape must.

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	117/113 ratio
P16521	Elongation factor 3A	217,18	0,66	4,00	115,90	6,05	2,00
Q07653	Protein HBT1 OS	220,42	0,60	9,00	113,50	6,38	2,01
Q12271	Polyphosphatidylinositol phosphatase INP	171,30	0,66	1,00	124,50	7,20	2,01
P53094	Negative regulator of sporulation	244,84	0,56	1,00	166,90	7,43	2,06
C8ZCR2	ABC transporter NFT	167,29	0,56	1,00	176,20	8,60	2,15
P39676	Flavohemoprotein	60,50	0,62	3,00	44,60	6,28	4,47

TABLE 5.7: Differentially expressed proteins by the promising natural isolate MCB C6 relative to the commercial TRWY reference VIN 7 during the stationary phase of fermentation in 2013 Sauvignon blanc grape must.

Accession	Description	Score	Coverage	Unique peptides	MW [kDa]	calc, pI	118/113 ratio
P36110	Protein PRY2 OS	41,81	0,49	2,00	33,80	4,60	0,28
Q12287	Cytochrome c oxidase	51,50	0,45	2,00	8,10	4,92	0,39
P53221	60S ribosomal protein	75,36	0,76	2,00	14,20	10,46	0,39
Q03219	Uncharacterized protein YMR	27,86	0,64	1,00	31,10	6,05	0,42
P39743	Reduced viability upon	57,40	0,61	2,00	52,70	6,01	0,46
P07342	Acetolactate synthase catalytic	137,74	0,78	3,00	74,90	8,51	0,49
Q00055	Glycerol-3-phosphate dehydrogenase	121,94	0,69	2,00	42,80	5,47	2,12
P05740	60S ribosomal protein	188,92	0,80	1,00	20,50	10,92	2,25
P23638	Proteasome subunit alpha	46,79	0,58	3,00	28,70	5,22	2,46
P40581	Peroxiredoxin HYR1 OS	15,24	0,57	4,00	18,60	8,19	2,49
P39676	Flavohemoprotein OS=Saccharomyces	60,50	0,62	3,00	44,60	6,28	2,59
P16521	Elongation factor 3A	217,18	0,66	4,00	115,90	6,05	2,61
Q04409	Putative glucokinase-2 OS	103,62	0,61	3,00	55,90	6,27	6,90
Q12458	Putative reductase 1 OS	26,94	0,52	3,00	34,70	7,12	7,38
P39016	Suppressor protein MPT	233,23	0,73	1,00	95,40	8,03	24,61

TABLE 5.8: Differentially expressed proteins by the promising natural isolate M2/1 relative to the commercial TRWY reference VIN 7 during the stationary phase of fermentation in 2013 Sauvignon blanc grape must.

Accession	Protein	Score	Coverage	Unique Peptides)	MW [kDa]	calc. pI	A3:119/113
P36110	Protein PRY2 OS	41,81	0,49	2,00	33,80	4,60	0,33
P40159	Uncharacterized protein YNL	174,98	0,97	2,00	20,10	7,64	0,39
P38707	Asparagine--tRNA ligase	39,99	0,62	2,00	62,20	5,85	0,41
P53903	Processing of GAS	26,59	0,55	1,00	15,00	5,76	0,42
Q08972	[NU+] prion formation	176,50	0,49	1,00	134,20	5,86	0,43
Q12497	Protein FMP16, mitochondrial	12,41	0,53	1,00	10,80	9,64	0,43
Q04344	Hit family protein	14,31	0,52	3,00	17,70	6,95	0,44
P26785	60S ribosomal protein	18,06	0,54	2,00	22,20	10,55	0,44
P11632	Non-histone chromosomal	54,16	0,70	2,00	10,80	9,76	0,44
Q12213	60S ribosomal protein	17,35	0,66	3,00	27,70	10,15	0,47
P11633	Non-histone chromosomal	26,18	0,58	1,00	11,50	9,89	0,47
Q07897	Protein CMS1 OS	15,81	0,43	1,00	33,40	8,19	2,62
P15303	Protein transport protein	220,61	0,69	9,00	85,30	5,66	4,56
Q04409	Putative glucokinase-2 OS	103,62	0,61	3,00	55,90	6,27	4,85

TABLE 5.9: Differentially expressed proteins by the promising inter-genus FLPH and LVPH NH 07/1 relative to the commercial TRWY reference VIN 7 during the stationary phase of fermentation in 2013 Sauvignon blanc grape must.

Accession	Protein	Score	Coverage	Unique Peptides	MW [kDa]	calc. pI	A3:121/113
P0CE86	Seripauperin-21 OS=Saccharomyces	55,48	0,72	3,00	17,70	6,79	0,17
P32599	Fimbrin OS=Saccharomyces	82,48	0,76	4,00	71,70	5,48	2,02
Q08193	1,3-beta-glucanosyltransferase GAS	68,61	0,67	5,00	51,80	4,64	2,07
Q04894	NADP-dependent alcohol	55,82	0,56	2,00	39,60	6,74	2,09
P50107	Lactoylglutathione lyase OS	25,00	0,25	2,00	37,20	6,84	2,12
Q05584	Hydroxyacylglutathione hydrolase,	60,86	0,48	4,00	31,30	6,19	2,40
P23638	Proteasome subunit alpha	46,79	0,58	3,00	28,70	5,22	2,58
P40202	Superoxide dismutase 1 copper	61,52	0,69	3,00	27,30	6,67	2,91
P30402	Orotate phosphoribosyltransferase 2 OS	77,50	0,78	3,00	24,80	8,51	4,49

5.5. CONCLUSIONS

The inter-genus FLPH and LVPH, NH 07/1, produced wine with a more positive association with volatile thiols, 3MHA in particular, than both parental strains, *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1, as well as commercial TRWY, Zymaflore X5 and Zymaflore VL3. This hybrid also produced wines with a negative association with VA and acetic acid, but a positive association with floral aroma with hints of tropical fruit aroma. Three intra-genus TFPH and LVPH, NH 56, NH 57 and NH 88 produced wines with a negative association with VA and acetic acid, but with a positive association with tropical fruit aroma. These intra-genus hybrids also produced Sauvignon blanc wines with a stronger association with 3MH than the commercial reference. Five more intra-genus yeasts, NH 48, NH 118, NH 140, NH 143 and NH 145 also produced wines with a stronger association with 3MH than wines produced with the commercial TRWY Zymaflore X5 and Zymaflore VL3. These hybrids also produced wines with a stronger association with 3MHA than wines produced with the commercial TRWY VIN 13. Proteomic analyses showed that NH 07/1 and Zymaflore VL3 over-expressed the lactoylglutathione lyase protein responsible for the release of the volatile thiol 4MMP by cleaving its carbon-sulfur bonds. Since 3MH release also involves enzymatic cleavage by a carbon-sulfur lyase, there is a possibility that the above-mentioned over-expressed protein could also be involved in the release of 3MH from its carbon-sulfur-containing precursor. Lactoylglutathione lyase might be a useful protein biomarker for volatile thiol release by especially NH 07/1 during production of varietal Sauvignon blanc wines. As dehydrogenases were previously implicated in VA formation, these proteins might also be useful biomarkers for VA and/or acetic acid formation by fermenting wine yeasts.

5.6. CONFLICT OF INTEREST

The authors have not declared any conflict of interest.

5.7. ACKNOWLEDGEMENTS

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Chapter 6

General discussion

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6.1. CONCLUDING REMARKS

South African enologists are continually striving to increase wine quality in an ever-increasing competitive market (Moore *et al*., 2008; Alonso *et al*., 2013). The selection and development of new yeast is directly linked to improvement of wine quality as the wine yeast strain used to ferment 'neutral' grape must (juice) enhances wine aroma and flavour (Swiegers *et al.,* 2006b; Dennis *et al.,* 2012; Van Breda *et al.,* 2013; Jolly *et al.,* 2014). The production of varietal wines with enhanced aromatic characteristics from relatively 'neutral' flavoured grape juice (must) can be achieved by deploying the wine yeast *Saccharomyces cerevisiae* (Erten *et al.,* 2006; Swiegers *et al.,* 2009). Subsequently, the main objectives of this study was to evaluate a selection of intra-genus *Saccharomyces cerevisiae* hybrid yeasts and commercial thiol-releasing wine yeasts (TRWY) for the improvement of Sauvignon blanc wine organoleptic quality with regard to tropical fruit aroma and lower VA formation. A second objective was to investigate wine yeast protein expression and whether regulated proteins correlate with metabolites released and/or produced by different yeast strains during fermentation by on deploying metabolomic and proteomic approaches. A third objective of this study was to breed *S. cerevisiae/T. delbrueckii* inter-genus hybrids using classical mating, characterise and evaluate these inter-genus hybrids for their fermentation potential, thiol-releasing abilities and low VA formation during the production of Sauvignon blanc wines.

Intra-genus hybrid yeast strains, with similar fermentation rates as aromatic wine producing parental and commercial strains, produced laboratory-scale wines with aroma enhancing metabolites (Chapter 3). Additionally, MALDI-TOF/MS biotyping was proven to be a faster alternative than CHEF DNA karyotyping for the identification and characterisation of these hybrids, despite the existing database comprising primarily of clinical strains (Hart *et al.,* 2016). Furthermore, biotyping successfully differentiated inter-genus hybrids that could not be resolved using DNA karyotyping analyses. This study also pioneered the establishment of a MALDI-TOF/MS database pertaining to wine, wild and

hybrid yeasts that will receive further attention in another post-graduate study. It was also observed that relative faster fermentation rates resulted in wines with improved sensory quality (Hart *et al.,* 2016). Different commercial TRWY references and hybrid strains was shown to produce wines with different chemical and sensory profiles, due to varying levels of yeast derived and mediated metabolites. Improved intra-genus hybrids with the ability to produce laboratory-scale wines with positive correlation with tropical fruit aroma (abbreviated as TFPH) were identified during this study. These wines also had enhanced tropical fruit aromas compared to wines produced with commercial 'thiol-releasing' wine yeasts (TRWY), and parental strains which produced wines with tropical fruit aromas (abbreviated as TFPP). Some of these TFPH were also identified as lower volatile acidity producing hybrids (abbreviated as LVPH) as they also produced wines with a negative association to VA.

Overall intra-genus TFPH and LVPH, therefore, comply with both criteria put forward in the overall objective of this study in that they showed lower VA formation, whilst producing typical Sauvignon blanc wines with enhanced aromas. Moreover, observations during this study show that some commercially available yeast strains are associated with VA formation. This study further showed that classical mating is still a practical way to produce novel inter-genus hybrids with desired traits, thereby avoiding the controversial issue of the use of genetic modified organisms (GMOs) for wine production. Small-scale Sauvignon blanc wine production during the latter part of project confirmed the provisional characterisation of the hybrids as tropical fruit and lower VA producing yeast strains (Chapter 4). Commercial TRWY, references Zymaflore X5 and VIN 7 were shown to produce wines with a positive association with tropical fruit aromas, whilst Zymaflore VL3 and Fermicru 4F9 produced wines positively associated with floral and vegetative aromas. The TRWY, VIN 13 produced wines positively associated with both tropical fruit and vegetative aromas. Indications are that the lack of sought-after tropical fruit aroma in wines with vegetative nuances, could have been masked by Sauvignon blanc grape-derived aroma compounds, amongst others, 2-isobutyl-3-methoxypyrazine (IBMP) responsible for green pepper and vegetative aromas (Lapalus, 2016). Nonetheless, this study showed that the

production of wines with tropical fruit aromas positively associated volatile thiol release and/or production (Hart *et al*., 2017b), which supports yeast manufacturers assertions that above-mentioned TRWY strains are volatile thiol-releasers.

Wines produced with the TFPH, NH 56 also had a more positive association with key aroma enhancing metabolites *viz.* 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) in Sauvignon blanc in comparison to wines produced with top commercial TRWY references included in this study (Hart *et al*., 2017b). Strain NH 56 also produced wines with the second highest 3MH levels after NH 84, whilst producing wines with the lowest acetic acid of all strains included in this study. This yeast was also the only strain to have down-regulated proteins during the lag phase, which were linked to amino acid biosynthesis, pentose phosphate pathway, glycolysis and fructose and galactose metabolism. Differences in protein expression were reflected in the variation of metabolite release by the different strains, thereby confirming that proteins are the final effectors for metabolite release. This study is also the first to show a positive association between overexpression of other protein classes, besides β-lyases and higher volatile thiol release by hybrid strains. Similar to the findings of others (Varela *et al.,* 2012; Walkey *et al.* 2012) who reported that dehydrogenase enzymes are involved in excessive acetic acid production, yeast strains that produced wines with a stronger association with acetic acid than NH 56, over-expressed dehydrogenases at the start of fermentation. Hybrid yeast NH 56, contrariwise down-regulated dehydrogenase at the start of fermentation. This supports the sensory data that this hybrid is also a LVPH. This study, therefore, successfully developed an improved *S. cerevisiae* intra-genus hybrid due to its enhanced 3MH release and lower acetic acid formation compared to commercial thiol-releasing reference strains. The novelty of this aspect of study was underlining the over-expression of the dehydrogenase protein as a potential biomarker for excessive acetic acid and VA formation by hybrids during fermentation. This yeast, as both a LVPH and TFPH, therefore has the potential to play a commercial role in the production of varietal aromatic white wine.

The non-S*accharomyces* yeasts, namely *Torulaspora delbrueckii* was previously shown to produce white wine with enhanced varietal aromas following fermentation of a “neutral” base must (juice)

when inoculated singly or sequentially to *S. cerevisiae* (Jolly *et al.,* 2003; Van Breda *et al.,* 2013). The use of *T. delbrueckii* was also shown to produce wine with lower VA levels. Additionally, a *S. cerevisiae*/*T. delbrueckii* inter-genus hybrid was shown to enhance wine aroma and flavour upon completion of fermentation (Santos *et al.,* 2008). Subsequently, this study developed novel *S. cerevisiae*/*T. delbrueckii* inter-genus hybrids, NH 07/1 and NH 07/2 and investigated their role in the production of varietal aromatic Sauvignon blanc wines with lower VA and acetic acid levels (Chapter 5) alongside promising intra-genus TFPH and/or LVPH (Chapter 4). Chemical analyses showed that both inter-genus hybrids, NH 07/1 and NH 07/2 produced Sauvignon blanc with a negative association with VA, whilst intra-genus hybrid strains provisionally characterised as LVPH, NH 48, NH 57, NH 143, and NH 145 also produced wines with a negative association with VA (Hart *et al*., 2017a). This study showed that inter-genus hybrid strains are also LVPH, and it can be concluded that they inherited this trait from the non-*Saccharomyces* parental strain. Sensorially, both inter-genus, NH 07/1 and NH 07/2 produced Sauvignon blanc wines with a positive association with floral aroma, hence they were provisionally classified as FLPH. Nevertheless, these wines also had hints of tropical fruit aroma, and it can tentatively be said that inter-genus hybrids have application for the production of varietal Sauvignon blanc wines with low VA.

Two intra-genus hybrids characterised as TFPH and LVPH, NH 57 and NH 140 also produced Sauvignon blanc wines with a positive association with tropical fruit aroma. Furthermore, both inter-genus, NH 07/1 and NH 07/2 and seven intra-genus hybrids characterised as TFPH and/or LVPH, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118 and NH 140 produced wines with a negative association with volatile fatty acids *e.g.* acetic acid, which are responsible for undesirable vinegar-like off-flavours. Three intra-genus hybrids, namely NH 56, NH 84 and NH 88 produced wines with a stronger association with the volatile thiol 3MH than the top commercial reference TRWY VIN 7. This TRWY was also reported to be the highest producer of another volatile thiol, namely 4MMP associated with fruit aroma in wine (Swiegers *et al., et al.,* 2009; Borneman *et al.,* 2012). Five more intra-genus namely NH 48, NH 57, NH 118, NH 140, NH 143 and NH 145 also produced wines with a stronger association

with 3MH and 3MHA than wines produced with the commercial TRWY, namely VIN 13, Zymaflore X5, and Zymaflore VL3 known for their volatile thiol-releasing abilities (Dubourdieu, 2006; Bowyer *et al.,* 2008). The inter-genus hybrid NH 07/1 produced wine with a more positive association with 3MHA than *T. delbrueckii* M2/1 as well as top two commercial TRWY Zymaflore X5 and Zymaflore VL3 previously shown to produce aromatic wines and/or have 'thiol-releasing' abiities abilities. This observation supports the third objective of this study as an improved *S.cerevisiae*/*Torulaspora delbrueckii* inter-genus hybrid was developed, due to its enhanced 3MHA and lower acetic acid formation compared to commercial thiol-releasing reference strains mentioned above.

Proteomic analyses showed that the TRWY Zymaflore VL3 that produced wine with a moderate association with 3MH, over-expressed the lactoylglutathione lyase protein (Chapter 5), an enzyme responsible for cleaving a carbon-sulfur bond to release the volatile thiol 4MMP from its bound aroma-inactive precursor (Howell *et al.,* 2005). It is noteworthy that, the inter-genus hybrid, NH 07/1 characterised as a FPH and/or LVPH, as observed with the TRWY Zymaflore VL3, also over-expressed the lactoylglutathione lyase protein (Hart *et al*., 2017a). It can, therefore, tentatively be said that above-mentioned carbon-sulfur lyase enzyme is involved in the release of said volatile thiol from its carbon-sulfur-containing precursor. Furthermore, as a β-lyase encoding gene was reported to be involved in the release of volatile thiols *viz.* 3MH and 4MMP (Holt *et al.,* 2011), this study indicated that above-mentioned lactoylglutathione lyase protein can potentially be used as a biomarker for volatile thiol release during production of varietal Sauvignon blanc wines.

This study, therefore, is an architype (model) as it is the first to report on deploying metabolomic and proteomic approaches in conjunction with wine chemical and sensory evaluation to characterise hybrid wine yeasts, towards production of varietal Sauvignon blanc wine with higher volatile thiols and lower VA. Furthermore, differential protein expression by *S. cerevisiae* intra-genus wine yeast starter cultures (strains) at the beginning of fermentation compared to the stationary phase of fermentation was elucidated. Intra- and inter-genus hybrid strains was also observed to "respond" differently to the same grape matrix during the same phase of the fermentation, as differences in protein down-

regulation and over-expression was observed. The potential downside is that only 600 to 1000 regulated yeast proteins was characterised and quantified, which represents altogether ~20% of the total yeast proteome. Nonetheless, a significant innovation of this study was the development of a *S. cerevisiae*/*T. delbrueckii* inter-genus hybrid, and showing its advantage compared to parental reference strains by deploying metabolomic and proteomic approaches in conjunction with chemical and sensory evaluation.

In general, this study clearly demonstrated that intra- and inter-genus TFPH, LVPH and FPH have the potential to play a commercial role in the production of varietal aromatic white wines, especially Sauvignon blanc as they comply with yeast selection criteria set forth in the objectives. The commercialisation of an inter-genus hybrid for wine production will also result in paradigm shifts, as none are currently commercially available. Therefore, this study was successful in developing novel intra- and inter-genus hybrids that met yeast selection criteria using classical mating to avoid the contentious issue of genetic modified organisms (GMOs) usage, thereby maintaining the green image of South African wine industry.

6.2. LITERATURE CITED

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