Effect of yeasts and oenological parameters on acetaldehyde production during alcoholic fermentation of South African grape musts

by

Francois Marc October

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science (Biochemistry) in the Faculty of Science at Stellenbosch University

Supervisor: Dr Francois van Jaarsveld

Co-supervisors: Prof. Johann Rohwer & Prof. Wessel du Toit

March 2020

Declaration

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

Date: *March 2020*

Summary

Acetaldehyde plays a role in the rate of fermentation and the quality of wine. High levels of acetaldehyde in fermenting juice may result in sluggish/stuck fermentations, and in wine, it may impart undesirable aromas usually associated with oxidative aromas. Depending on its levels, acetaldehyde has an effect on yeast metabolism and can therefore impact alcoholic fermentation.

The overall aim of this project was to investigate the effect of yeasts and oenological parameters on acetaldehyde production, to better understand the impact of acetaldehyde on alcoholic fermentation and wine sensorial composition. Ten commercial *Saccharomyces cerevisiae* strains and 10 non-commercial non-*Saccharomyces* yeasts were evaluated. These yeasts were screened in a laboratory trial for their acetaldehyde-producing ability during alcoholic fermentation, and resulted in the selection of a high-, medium- and low-acetaldehyde producing yeasts. The selected yeasts were the *S. cerevisiae* yeasts NT50 (high), NT116 (medium) and VIN13 (low); and the non-*Saccharomyces* yeasts *Torulaspora delbrueckii* (high), *Candida guilliermondii* (medium) and *Candida valida* (low).

The above-mentioned selection of *Saccharomyces* yeasts was used individually for vinification of grape must, as well as in all possible permutations with the non-*Saccharomyces* yeasts, and resultant wines analysed chemically and evaluated sensorially. The initial sensory results showed noticeable differences between treatments, in terms of aroma and sweetness. Statistical evaluation of the data from the screening and cellar trials showed that yeast strain and time of fermentation have an impact on levels of acetaldehyde. The ability of the yeast strains to produce acetaldehyde was affected differently by fermentation temperature during the screening trial. Wines co-inoculated with non-*Saccharomyces cerevisiae* yeasts have lower levels of acetaldehyde than wines only inoculated with *Saccharomyces cerevisiae* yeasts as observed in the cellar trial.

Sulphur dioxide (SO_2) has a very high affinity for acetaldehyde, therefore the impact of various concentrations of SO_2 on the levels of acetaldehyde in fermenting must was monitored in a second cellar trial. The resulting effects on fermentation and final wine quality were monitored. Although it is known that SO_2 impacts wine quality, it was also found that the varying levels of SO_2 have a direct effect on the acetaldehyde levels produced during fermentation.

During a separate fermentation trial (laboratory-scale), using three *Saccharomyces cerevisiae* yeast strains, the total enzyme activity of alcohol dehydrogenase (ADH) was monitored. The ADH activity showed a similar trend to acetaldehyde concentration, where high enzyme activity of the *Saccharomyces cerevisiae* yeasts correlated with high acetaldehyde levels.

In summary, there were significant differences in acetaldehyde levels between yeast strains tested in this study and the levels were within acceptable ranges normally found in wines. Higher acetaldehyde levels were found in wines inoculated with *S. cerevisiae*, exposed to high SO_2 levels, and fermented at higher temperatures. There was a direct correlation between total ADH activity and total acetaldehyde production of *Saccharomyces cerevisiae* yeasts.

To ensure lower levels of acetaldehyde in wine, winemakers should preferably co-inoculate with low ADH activity *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast strains, at low fermentation temperatures, while ensuring low levels of SO_2 before fermentation.

Opsomming

Asetaldehied speel ‘n rol by die fermentasietempo en wynkwaliteit. Hoë vlakke van asetaldehied in gistende druiwesap kan lei tot slepende/steekgistings, en in wyn kan dit lei tot wangeure wat gewoonlik met oksidatiewe aromas geassosieer kan word. Asetaldehiedvlakke het ‘n effek op gismetabolisme en kan dus fermentasiekinetika beïnvloed.

Die oorhoofse doel van hierdie studie was om spesifiek te kyk na die bydrae van verskillende gisrasse en wynkyndige parameters op asetaldehiedproduksie, om sodoende die impak van asetaldehied op alkoholiese fermentasie, asook op die sensoriese aspek van wyn, beter te kan verstaan. Tien kommersiële *Saccharomyces cerevisiae* rasse en 10 nie-*Saccharomyces* gisrasse was ondersoek. Hierdie giste was in laboratorium skaal proewe geëvalueer vir hul vermoë om asetaldehied te produseer tydens alkoholiese fermentasie en ‘n seleksie van hoog, -medium en lae asetaldehied produserende giste is gemaak. Die geselekteerde giste was *S. cerevisiae* yeasts NT50 (hoog), NT116 (medium) en VIN13 (laag), en die nie-*Saccharomyces* giste was *Torulaspora delbrueckii* (hoog), *Candida guilliermondii* (medium) en *Candida valida* (laag).

Die bogenoemde *Saccharomyces* giste was gebruik op hul eie en in alle moontlike kombinasies met die nie-*Saccharomyces* giste gedurende die eerste wynmaakproef in die kelder. Die wyne was ook chemies en sensories geëvalueer. Die aanvanklike sensoriese resultate het gewys dat die behandelings van mekaar verskil ten opsigte aroma en soetheid. Statistiese analise van die data het gewys dat gisras en fermentasie tyd ‘n impak op asetaldehiedvlakke gehad het. Die vermoë van die gisrasse om asetaldehied te produseer, was deur fermentasie temperatuur beïnvloed. Wyne wat met nie-*Saccharomyces* giste in kombinasie met *Saccharomyces* giste geproduseer was, het laer asetaldehiedvlakke gehad as wyne wat slegs met *Saccharomyces* giste geïnokuleer was.

Swaweldioksied (SO_2) het ‘n baie hoë affiniteit vir asetaldehied, daarom is die impak van verskeie SO_2 vlakke op asetaldehiedproduksie ondersoek in ‘n tweede kelderproef. Die

resultante effekte van voorgenoemde interaksies op fermentasie en wynkwaliteit was ook gemonitor. SO_2 het ‘n impak op wynkwaliteit en die variasie in SO_2 vlakke het ‘n direkte effek gehad op die asetaldehied vlakke gedurende fermentasie asook in die finale wyne.

Tydens ‘n onafhanklike fermentasieproef (laboratorium skaal) is die totale alkohol dehidrogenase (ADH) ensiemaktiwiteit van drie *Saccharomyces cerevisiae* gisrasse gemonitor. Die ADH aktiwiteit het ‘n soortgelyke tendens getoon as die asetaldehiedvlakke, waar hoë ensiemaktiwiteit van *Saccharomyces cerevisiae* giste gekorreleer het met hoë asetaldehiedvlakke.

Die asetaldehiedvlakke het betekenisvol verskil tussen die gisrasse wat getoets was en die vlakke was binne aanvaarbare perke wat normaalweg in wyne aangetref word. Hoër asetaldehiedvlakke was aangetref in wyne wat met *Saccharomyces cerevisiae* geïnokuleer was, aan hoë SO_2 vlakke blootgestel was en teen hoë temperature gegis was. Daar was ‘n direkte korrelasie tussen totale ADH aktiwiteit en totale asetaldehiedproduksie van *Saccharomyces cerevisiae* giste.

Om lae asetaldehiedvlakke in wyne te verseker, word wynmakers aangeraai om lae ADH aktiwiteit *Saccharomyces cerevisiae* en nie-*Saccharomyces* gisrasse te gebruik teen lae fermentasie temperature en ook om lae SO_2 vlakke voor gisting te handhaaf.

Dedications

This thesis is dedicated to:

My parents, ***Marty*** *&* ***Fanny****, and brother,* ***Michael-John****, who helped mould me into the person that I am today, and*

My loving wife, ***Feroza****, who solidly stood by my side through Life's highs & lows, together with our two beautiful children,* ***Samuel*** *(7 years old) &* ***Isabella*** *(2 years old),*

~ I love you all!

Acknowledgements

I would like to express my sincere thanks and gratitude to the following persons and institutions:

- Firstly, to my **Divine Creator** and **Saviour** for giving me Life in abundance.
- To my good friend and colleague, **Dr Heinrich du Plessis**, for patiently walking this path with me since *Day One*, and sacrificing his time and energy to get me over the last few hurdles towards completion.
- To my supervisors, **Dr Francois van Jaarsveld**, **Prof. Johann Rohwer** and **Prof. Wessel du Toit**, for their eternal patience with me, but more so for their valuable insight, knowledgeable suggestions and gentle guidance back on track when I occasionally drifted off with my thoughts and ideas.
- To my ARC colleagues, **Valmary van Breda**, **Justin Hoff**, **Dr Rodney Hart** and **Dr Neil Jolly** for their invaluable technical advice and support throughout the course of this study.
- To my colleagues at the *Nietvoorbij* Cellar, **Craig Paulsen** and **Jeremy Boonzaier**, for overseeing the vinification of this project's grapes, as well as for the additional support and encouragement throughout.
- To my ex-colleague and friend, **Dr Boredi Chidi**, for his valuable advice and practical assistance.
- To all other colleagues, students, interns and friends at **ARC Infruitec-Nietvoorbij** for their warm smiles, kind words of support, technical assistance and for sitting patiently through all my presentations.
- To the ARC's biometricians, **Dr Mardé Booyse** and **Dr Marieta van der Rijst** for their friendly advice, professional assistance and valuable contributions with all the statistical analyses of the data.
- To **Wernich Kühn** for all the acetaldehyde analyses on the *Arena Enzyme Robot* at the CAF (SU).
- To **Ludick Arnolds** (Distell) for the initial acetaldehyde analyses on the GC-FID.
- To **Valeria Panzeri** (IWBT/DVO, SU) for assistance and training with the relevant sensorial aspects.
- To the Systems Biology (SU)/*Triple-J* laboratory manager, **Arrie Arends**, for his friendliness, professionalism and patience, and for creating a temporary space for me during the enzyme assays.
- To **Susan Muiyser**, for teaching me the ropes in the *Triple-J* laboratory during the early days.
- To **Theo van Staden**, for sacrificing his time to consult with me and share valuable pointers.
- To **Tiaan Swanepoel**, for his laidback friendliness and eagerness to help, and who out of the blue, came to my rescue when things were not going as expected in the *Triple-J* laboratory.
- To all staff and post-graduate students of the **Department of Biochemistry (SU)** whom I have crossed paths with during the course of this study, and who have, also, patiently sat through my presentations.
- To my employer, the **Agricultural Research Council (ARC)**, for this opportunity and the availability of resources, infrastructure and financial support to complete this study.
- To **Winetech** and the **National Research Foundation (NRF)** for financial support.
- To my **parents, in-laws** and **extended family** for their constant love, support and encouragement.
- To my beautiful children, **Samuel** and **Isabella**, for giving me a reason to never give up.
- And, last but not least, to my dear wife, **Feroza**, for her unconditional love and support, and for remaining steadfast and believing in me, especially when the studies took its toll, at times.

THANK YOU!

Contents

List of Tables

List of Figures

List of Abbreviations and Acronyms

°B	Degrees Brix (sugar concentration)
°C	Degrees Celsius (temperature)
µL	Microlitre
µm	Micron (micrometer)
ADWY	Active dry wine yeast
ADH	Alcohol dehydrogenase
AF	Alcoholic fermentation
Al-DH	Aldehyde dehydrogenase
ANOVA	Analysis of variance
BSA	Bovine serum albumin
C_2H_4O	Acetaldehyde
Ca.	Circa (approximately)
Cells/mL	Cells per millilitre
C. guilliermondii (C.g.)	*Candida guilliermondii*
C. lambica (C.l.)	*Candida lambica*
C. pulcherrima (C.p.)	*Candida pulcherrima*
C. valida (C.v.)	*Candida valida*
CFU	Colony forming units
CFU/mL	Colony forming units per millilitre
CO_2	Carbon dioxide
cm	Centimeter
DA	Discriminant analysis
D.A.P.	Diammonium phosphate
dH_2O	Deionised water
FSO_2	Free sulphur dioxide
GC	Gas chromatograph
GC-FID	GC coupled with Flame Ionization Detector
g/L	Grams per litre
g/hL	Grams per hectolitre
GDP	Gross domestic product
H_2O_2	Hydrogen peroxide
hL	Hectolitre
HSP	Heat shock proteins

KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen orthophosphate
LAB	Lactic acid bacteria
L	Litre
LSD	Least significant difference
$MgSO_4$	Magnesium sulphate
mg/L	Milligram per litre
mg/kg	Milligram per kilogram
mL	Millilitre
mL/L	Millilitre per litre
mM	Millimolar
M	Molar
nm	Nanometers
NAD^+	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NAD(P)H	Nicotinamide adenine dinucleotide phosphate (reduced)
OD	Optical density
OIV	*International Organisation of Vine and Wine*
PIPES	Piperazine-1,4-bis(2-ethanesulphonic acid)
PMSF	Phenyl methanesulphonyl fluoride
P. kluyveri (P.k.)	*Pichia kluyveri*
rpm	Revolutions per minute
SAS	Statistical analysis system
SO_2	Sulphur dioxide
S. cerevisiae	*Saccharomyces cerevisae*
TA	Total acidity
T. delbrueckii (T.d.) -	*Torulaspora delbrueckii*
TSO_2	Total sulphur dioxide
VA	Volatile acidity
v/v %	volume/volume percentage
YPDA	Yeast peptone dextrose agar
YPD	Yeast extract peptone dextrose

Preface

This thesis is presented as a compilation of five (5) chapters. Chapter 2, or part thereof, has been published in Winelands (Van Jaarsveld & October, 2015).

This thesis consists of:

- CHAPTER 1 : General introduction and project aims
- CHAPTER 2 : Literature review
- CHAPTER 3 : Methodology
- CHAPTER 4 : Results and discussion
- CHAPTER 5 : Conclusion and references

All attempts to minimise inevitable repetitions were made.

CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 Introduction

The South African Wine Industry is a major role player in the South African economy and contributed approximately R36 billion to the country's gross domestic product (GDP) during 2018 and the total exports of wine were 420.2 million litres (SAWIS, 2018). Therefore, continuous research into improving wine quality has become vital in securing a sustainable industry, as well as stable livelihoods and employment security for many South Africans in the long term.

Acetaldehyde (ethanal) is a volatile chemical compound found to play a significant role in wine aroma, colour and stability (Osborne *et al.*, 2006). It is formed during the anaerobic fermentation of grape must to wine, and is the most important carbonyl compound produced by yeast metabolism during alcoholic fermentation (Nykänen *et al*., 1977; Romano *et al*., 1994). Post-fermentation activity, like the oxidation of ethanol in wine, can also lead to the production of acetaldehyde (Wildenradt & Singleton, 1974; Ribéreau-Gayon *et al*., 1983; Fleet & Heard, 1993; Elias, *et al*., 2009). At low levels (i.e. below the aroma threshold of 100 mg/L), acetaldehyde can contribute pleasant fruity aromas, and add flavour complexity in full bodied wines, especially red wines (Liu & Pilone, 2000; Swiegers *et al*., 2005; Aleixandre-Tudo *et al*., 2015). Excessive levels of acetaldehyde (i.e. above the aroma threshold of 100 mg/L) can leave the wine tasting flat with flor sherry characteristics, and cause a defective, pungent, irritating odour (Miyake & Shibamoto, 1993; Zea *et al.*, 2015; Coetzee *et al*., 2016a). It can also impart an undesirable green, grassy, apple-like aroma (Frivik & Ebeler, 2003; Coetzee *et al*., 2015), usually masked by the addition of sulphur dioxide (SO_2).

High levels of acetaldehyde in fermenting juice are unwanted as it may retard or inhibit ethanol formation by yeast, resulting in sluggish or stuck fermentations (Liu & Pilone, 2000). Acetaldehyde can affect fermentation kinetics through concentration-dependent inhibition or stimulation of the lag phase and growth rate of yeasts (Stanley *et al.*, 1993; Liu & Pilone, 2000; Vriesekoop, 2007; Hucker & Vriesekoop, 2008). It has been reported

that for ethanol-stressed *Saccharomyces cerevisiae* the lag phase was shortened and the growth rate stimulated at low acetaldehyde concentrations, also implying that acetaldehyde may play a role in preventing ethanol-induced stress/growth inhibition of yeast cells (Walker-Caprioglio & Parks, 1987; Stanley *et al.*, 1993; Stanley *et al*., 1997).

The accumulation of acetaldehyde during fermentation is dependent on the relative activity of the enzymes alcohol- and aldehyde dehydrogenase, each of which comprises of several isoenzymes (Cortes *et al.*, 1998; Ciani & Ferraro, 1998). The rate of acetaldehyde production is affected by the equilibrium between the oxidized and reduced coenzymes of ADH (Millán & Ortega, 1988).

1.2 Aims and objectives of this thesis

The overall aim of this project was to investigate the effect of yeasts and oenological parameters on acetaldehyde production, to better understand the impact of acetaldehyde on alcoholic fermentation and wine sensorial composition. The specific objectives of this study were:

i. To screen yeast strains for acetaldehyde production in South African context;
ii. To select and evaluate high-, medium- and low-acetaldehyde producing yeast strains;
iii. To evaluate the effect of winemaking practices on acetaldehyde levels;
iv. To evaluate the impact of acetaldehyde on sensory properties of wine, and;
v. To determine the ADH activity in yeasts during acetaldehyde production.

CHAPTER 2

LITERATURE REVIEW

2.1 Importance of acetaldehyde

Acetaldehyde (ethanal; C_2H_4O) is a low molecular weight, volatile compound found in a wide variety of aromatic foods and beverages that have, prior to their final stage of production, undergone a degree of fermentation (McCloskey & Mahaney, 1981; Liu & Pilone, 2000; Jackowetz *et al.*, 2011; Aguera, *et al.*, 2018). Acetaldehyde has been known to be a product of alcoholic fermentation by yeasts for more than a hundred years (Grey, 1913), but its presence in wine was confirmed by Dittrich and Barth (1984). It is formed during the first stages of alcoholic fermentation (Osborne *et al.*, 2000; Jackowetz *et al.*, 2011) and is one of the most important carbonyl compounds formed during alcoholic fermentation as it constitutes more than 90% of the total aldehyde content in wine (Nykänen, 1986). Wine aroma exists predominantly as a result of the presence of acetaldehyde, together with a large number of other volatile compounds, in wine (Liu & Pilone, 2000). Acetaldehyde levels in wines range from 4 mg/L to 493 mg/L (Table 2.1), with an average of 30 mg/L (red wine) and 80 mg/L (white wine) (McCloskey & Mahaney, 1981; Romano *et al.*, 1994; Aguera, *et al.*, 2018). The very high levels in sherry are due to the fact that this wine style is produced under oxidative conditions (Romano *et al.*, 1994, Coetzee *et al.*, 2016).

Table 2.1 Acetaldehyde levels normally found in wine[a]

Type of wine	Acetaldehyde range (mg/L)
Red wine	4 – 212
White wine	11 – 493
Sweet wine	188 – 248
Sherry (fortified wine)	90 – 500
Brandy (distilled wine)	63 – 308

[a]*Data summarised from: Liu & Pilone (2000).*

Acetaldehyde is the last precursor in yeast fermentation before ethanol is formed, and is produced when pyruvate, the end-product of glycolysis, is converted to acetaldehyde (Swiegers & Pretorius, 2005). Conversely, a secondary source of acetaldehyde production in red wine, which could occur during aging, is as a result of the oxidation

(exposure to air/oxygen) of ethanol. Oxidation of polyphenolics in wine yields hydrogen peroxide (H_2O_2) which oxidizes ethanol to acetaldehyde (Wildenradt & Singleton, 1974; Ribéreau-Gayon *et al*., 1983; Romano *et al.*, 1994; Jackowetz *et al.*, 2011). Acetaldehyde is also partially generated by decayed micro-organisms or low quality yeasts (Shin & Lee, 2019). Enzymatic oxidation, by alcohol dehydrogenase (ADH), of ethanol to acetaldehyde is also possible in wine (Millán & Ortega, 1988).

It must also be noted that the production levels of acetaldehyde during the early stages of fermentation differ widely from the final acetaldehyde concentration in wine (Cheraiti *et al.*, 2010). This is as a result of reutilisation by the yeast cells (Jackowetz *et al.*, 2011; Li & Mira de Orduña, 2010), as well as its degradation by bacteria (Osborne *et al.*, 2000; Jussier *et al.*, 2006) during the last stages of fermentation.

2.2 Effect of alcohol dehydrogenase (ADH) on acetaldehyde production

During alcoholic fermentation (AF), acetaldehyde production is linked to yeast fermentative metabolism of sugars via the action of pyruvate decarboxylase and alcohol dehydrogenase (Aguera, *et al.*, 2018). Alcohol dehydrogenases (ADHs) catalyse the interconversion of ethanol and NAD^+ to acetaldehyde and NADH, and they are commonly found in bacteria, yeasts, plants and animals (Pal, *et al.*, 2009). *Saccharomyces cerevisiae* possesses at least five genes (ADH1 to ADH5) that encode alcohol dehydrogenase isoenzymes involved in ethanol metabolism. The isoenzymes alcohol dehydrogenase I (ADH I), III, IV, and V reduce acetaldehyde to ethanol during alcoholic fermentation. In contrast, ADH II (EC 1.1.1.1) is glucose-repressed and catalyses the reverse reaction (i.e. the oxidation of ethanol to acetaldehyde). Therefore, when glucose in the fermentation medium is depleted, ADH II is the first enzyme to make use of ethanol. The accumulation of acetaldehyde during fermentation is dependent on the relative activity of the enzymes alcohol- and aldehyde dehydrogenase, each of which comprises of several isoenzymes (Maestre *et al*., 2008; Pal *et al*., 2008). The interconversion between ethanol and acetaldehyde is catalysed by the alcohol dehydrogenases (Aranda & del Olmo, 2003). In the glycolytic pathway, during alcoholic fermentation, sugars are converted to pyruvate. Pyruvate is decarboxylated to acetaldehyde, and subsequently reduced to ethanol by alcohol dehydrogenase (ADH), with NADH as reducing co-enzyme (Fig. 2.1).

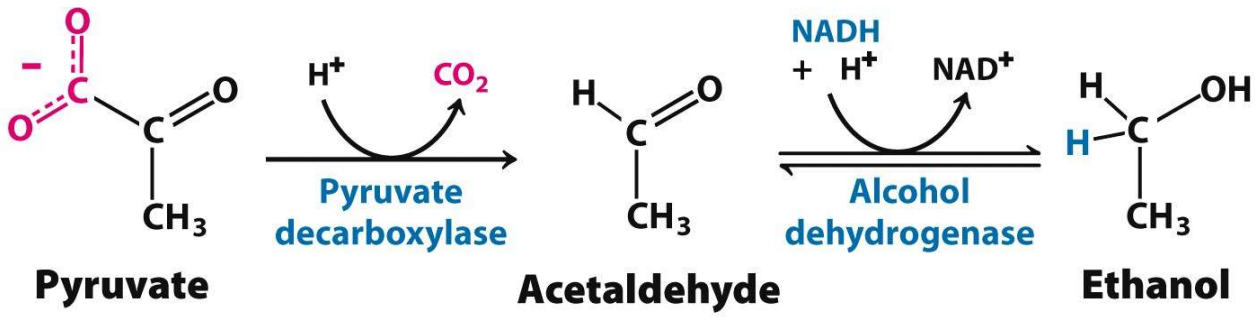


Figure 2.1 Schematic of the biochemical breakdown of pyruvate to acetaldehyde, and subsequently the conversion of acetaldehyde to ethanol.

During the latter conversion process NADH is oxidised to NAD^+. The use of SO_2 during winemaking can affect the activity of enzymes involved in acetaldehyde metabolism. The production of acetaldehyde is yeast dependent, but can also be directly related to the activity of ADH (Cortes *et al.*, 1998; Ciani & Ferraro, 1998). The rate of acetaldehyde production can also be affected by the equilibrium between the oxidised and reduced coenzymes of ADH, i.e. NAD^+ and NADH (Millán & Ortega, 1988).

2.3 Sensorial effects of acetaldehyde in wine

The oenological levels of acetaldehyde vary between different types of wine, e.g. white, red and sherry/port wines. Acetaldehyde has a low sensory threshold (Longo *et al.*, 1992). The threshold in wine ranges between 100-125 mg/L (Liu & Pilone, 2000; Osborne *et al.*, 2006; Aguera, *et al.*, 2018). In table wines, increased levels of acetaldehyde are undesirable, but at low wine levels acetaldehyde gives a pleasant, fruity aroma, whereas at higher levels it imparts typical oxidation-related nuances (Coetzee *et al.*, 2016a,b; 2018) and an irritating odour that has been described as a green, grassy, nutty or apple-like aroma (Liu & Pilone, 2000; Aguera, *et al.*, 2018). However, in sherry/port wines (fortified wines) the high acetaldehyde levels are considered to be a unique feature of that style (Liu & Pilone, 2000; Aguera, *et al.*, 2018).

High acetaldehyde levels in sherry/port wines also contribute to the increased colour observed in these wines, as compared to normal red wines (Liu & Pilone, 2000; Aguera, *et al.*, 2018). Rapid polymerisation of anthocyanins and other phenolics (e.g. catechins, tannins) occur in the presence of acetaldehyde, which assists in the formation of

pigmented condensation products that have higher colour intensity and stability (Osborne *et al.*, 2006; Sheridan & Elias, 2016). Furthermore, acetaldehyde also indirectly enhances and stabilises wine colour in that it strongly binds sulphur dioxide (SO_2) which is known to have a decolourising/bleaching effect on anthocyanins in wine (Liu & Pilone, 2000; Aguera, *et al.*, 2018).

In addition to changes brought about by acetaldehyde in the polymeric fraction of the phenolic substances, with corresponding effect on wine colour density and astringency, the volatiles fraction seem to be better protected in the presence of acetaldehyde during ageing, but needs to be confirmed sensorially. Acetaldehyde, therefore, leads to a clear difference in the chemical composition of the wines (Aleixandre-Tudo *et al.*, 2016).

2.4 Methods of acetaldehyde quantification

Total acetaldehyde concentration levels can either be determined chemically (iodimetry) or biochemically by utilizing an enzymatic assay kit (McCloskey & Mahaney, 1981; Longo *et al.*, 1992; Stanley *et al.*, 1993; Roustan & Sablayrolles, 2002; Osborne *et al.*, 2006; Jussier *et al.*, 2006; Li & Mira de Orduña, 2010; Jackowetz *et al.*, 2010; Cheraiti *et al.*, 2010), or quantitatively by traditional detection methods like gas or liquid chromatography (Ciani & Maccarelli, 1998; Romano *et al.*, 1994; Romano *et al.*, 2003; Peinado *et al.*, 2004; Paraggio & Fiore, 2004; Vriesekoop *et al.*, 2007; Hucker & Vriesekoop, 2008; Domizio *et al.*, 2011). The chemical method has proven to give results 1-20% higher than the enzymatic method, while the enzymatic method was considered more accurate and specific, as acetaldehyde is the predominant aldehyde in wine (Ough & Amerine, 1988; Liu & Pilone, 2000; Coetzee, 2014; Van Jaarsveld & October, 2015). Gas and liquid chromatography, however, usually require expensive instruments and complicated operations. Compared to traditional detection methods, fluorescence chemosensors offer a number of advantages including simplicity, quick response and real-time detection, however, reports of their usage in the literature are still rare (Yang *et al.*, 2019). It has also been found that acetaldehyde bound to SO_2 can affect the quantification of acetaldehyde, resulting in lower levels measured by titration and headspace gas chromatography (GC) than by enzymatic and OIV (International Organisation of Vine and Wine) methods. Selection of an appropriate analytical method is therefore important for the quantification of acetaldehyde in alcoholic beverages (Shin *et al.*, 2019).

2.5 Oenological parameters affecting acetaldehyde levels

2.5.1 Effect of sulphur dioxide (SO_2)

The total SO_2 content in wine may consist of varying levels of free and bound SO_2. Other than SO_2 being directly added to grape must/wine as a preservative during vinification, its presence in wine can also be attributed to yeasts, which also produce it to varying degrees (Osborne *et al.*, 2006). Furthermore, acetaldehyde is also chemically very active (Osborne *et al.*, 2006) and has a strong affinity for SO_2 (Liu & Pilone, 2000; Elias *et al.*, 2008). It therefore binds with free SO_2 (specifically the bisulphite ion, HSO_3^{-1}) to form a complex compound known as acetaldehyde hydroxy-sulphonate, which accounts for the largest percentage of the total SO_2 content (Liu & Pilone, 2000; Elias *et al.*, 2008). The reaction between acetaldehyde and bisulphite is rapid and, at pH 3.3, 98% of the acetaldehyde will bind with the sulphite within 90 minutes (Coetzee *et al.*, 2018), although it has been demonstrated that acetaldehyde remains reactive in the presence of bisulphite (Andorrà *et al.*, 2018). This bisulphite-acetaldehyde complex, consequently, reduces the potent sensory effects of acetaldehyde, but at the same time also reduces the antimicrobial, anti-enzymatic and antioxidant properties of SO_2 (Jackowetz *et al.*, 2011; Aguera, *et al.*, 2018). A lack of SO_2 could lead to spoilage of the wine. Therefore, due to this phenomenon more SO_2 is usually added to a wine containing high acetaldehyde levels, not only to bind it, but also to limit further formation of acetaldehyde, thereby making more free SO_2 available and subsequently protecting the wine's taste and aroma (Liu & Pilone, 2000; Osborne *et al.*, 2006; Coetzee *et al.*, 2018). However, as a result of escalating consumer awareness of the adverse health risks related to SO_2, efforts have been prioritised to reduce the SO_2 contents of wines (Osborne *et al.*, 2006).

Sulphur dioxide (SO_2) induces acetaldehyde formation by yeasts, and the final concentrations of acetaldehyde are higher in wines fermented with SO_2 than in wines fermented without SO_2 (Aguera, *et al.*, 2018). SO_2 either inhibits aldehyde dehydrogenase (so acetaldehyde is not converted to ethanol) or binds directly with acetaldehyde and thus reduces the amount of acetaldehyde that can be transformed to ethanol (Andorrà *et al.*, 2018).

2.5.2 Effect of fermentation temperatures

Some controversy exists regarding the effect of fermentation temperatures on the production levels of acetaldehyde. It was previously reported that acetaldehyde

concentration levels increased significantly at a fermentation temperature of 30°C, compared to 12°C, 18°C and 24°C (Romano *et al.*, 1994), which was in direct contrast to reports that fermentation temperature does not affect the final aldehyde content (Amerine & Ough, 1964). The increased level of acetaldehyde at 30°C could be due to an inhibitory effect of the temperature on the activity of alcohol dehydrogenase (ADH), the enzyme reducing acetaldehyde to ethanol (Romano *et al.*, 1994). However, it was also reported that cooler fermentation temperatures, in a strict oxygen-regulated environment, actually led to higher acetaldehyde levels, which could be due to a reduced reutilisation of acetaldehyde by the yeasts during the last stages of fermentation (Jackowetz *et al.*, 2011). Acetaldehyde is the major product of oxidation, and is also formed as an enzymatically-derived by-product of yeast metabolism during and after alcohol fermentation (Han *et al.*, 2017, 2019). Acetaldehyde is very reactive and takes part in a number of reactions with wine phenolics (i.e. anthocyanins/flavanols) during aging, which impact characteristics such as colour, flavour and astringency (Han *et al.*, 2019).

2.6 Role of yeasts

2.6.1 Effect of acetaldehyde on Saccharomyces cerevisiae

Saccharomyces cerevisiae is the most important wine yeast and is responsible for the metabolism of grape sugar to alcohol (ethanol) and carbon dioxide (CO_2) (Jolly *et al.*, 2006). It can grow in high sugar concentrations, as well as at low pH, and can survive in relatively high ethanol concentrations too, and as a result of these unique characteristics it is able to effectively ferment grape musts (with high sugar concentrations) to ethanol, giving it a competitive advantage over other yeasts (Swiegers & Pretorius, 2005; Ciani & Comitini, 2011).

The large variety of commercially available *S. cerevisiae* strains is partially responsible for the differences in acetaldehyde concentrations in wines, as this can be attributed to their varying rates of acetaldehyde production during alcoholic fermentation (Nykänen, 1986; Longo *et al.*, 1992; Romano *et al.*, 1994).

Although these wine yeasts are the primary producers of acetaldehyde during alcoholic fermentation, this metabolite, at high production levels, may have an inhibitory effect on the kinetics of *S. cerevisiae* by either lengthening its lag phase and/or slowing down its

growth rate. Conversely, it has been reported that for ethanol-stressed *S. cerevisiae* the lag phase was shortened and the growth rate stimulated at low acetaldehyde concentrations, also implying that acetaldehyde may play a role in preventing ethanol-induced stress/growth inhibition of yeast cells (Stanley *et al.*, 1993). However, this stimulatory and/or protective effect of acetaldehyde is not fully understood as yet, because the underlying mechanisms seem to be more complex than initially proposed by Stanley (1997) and Vriesekoop (2007), who both reported that the acetaldehyde effect was a redox-based mechanism (Hucker & Vriesekoop, 2008), and thus needs to be investigated more meticulously (Liu & Pilone, 2000).

From the above inhibitory effects of acetaldehyde and the stress induced on yeast cells by ethanol it is evident that, at certain concentrations, acetaldehyde and ethanol can be very toxic to yeast metabolism and growth. To resist these adverse growth conditions (acetaldehyde- and ethanol-stress), it has been found that acetaldehyde also triggers the transcription and expression of several HSP genes that are responsible for the synthesis of heat shock proteins (Hsp), of which one protective protein, Hsp104p, has been shown to resist *in vitro* stress factors (*e.g.* cold, glucose starvation, oxidative-, osmotic-, ethanol and/or acetaldehyde-stress) on certain yeast cells (Aranda *et al.*, 2002). High concentrations of acetaldehyde, intracellularly and extracellularly, may also retard/inhibit yeast ethanol formations, resulting in sluggish or stuck fermentations (Liu & Pilone, 2000).

2.6.2 Effect of acetaldehyde on non-Saccharomyces yeasts

Non-*Saccharomyces* yeast is a colloquial term, used mostly amongst wine microbiologists, and includes a wide variety of yeast species (Jolly *et al.*, 2014). This group of yeasts consists of many different genera frequently found on grapes, i.e. *Candida, Kloeckera, Hanseniaspora, Lachancea, Metschnikowia, Pichia* and *Torulaspora,* to name but a few (Jolly *et al.*, 2006; Domizio *et al.*, 2011; Beckner Whitener *et al.*, 2017; du Plessis *et al.*, 2017a, b). Non-*Saccharomyces* yeasts are naturally present in all wine fermentations and therefore their metabolites can impact wine quality, either negatively or positively (Jolly *et al.*, 2006, 2014). Historically, they are also known as wine spoilage yeasts, due to their ability to produce undesired compounds during the first stages of alcoholic fermentation (Ciani & Comitini, 2011). However, in recent years this perspective has started to change with more research being focused on its positive role

towards favourable volatile aroma profiles in mixed fermentations (Beckner Whitener *et al.*, 2017; du Plessis *et al.*, 2017b).

Acetaldehyde is one of many compounds produced by yeasts that positively or negatively contributes to wine aroma. The realisation that non-*Saccharomyces* yeasts can contribute significantly to the flavour and quality of wine has led to more detailed investigations into their properties (Romano *et al.*, 2003; du Plessis *et al.*, 2017b), as well as the studying of mixed fermentations, which involve the co-inoculation of *S. cerevisiae* with one or more different non-*Saccharomyces* strains (Beckner Whitener *et al.*, 2017; du Plessis *et al.*, 2017b). Metabolites normally produced by non-*Saccharomyces* yeasts at high concentrations, and considered detrimental to wine quality (i.e. negative aroma/flavour), does not reach detectable sensory levels in mixed fermentations (Domizio *et al.*, 2011).

Most of these non-*Saccharomyces* yeasts are susceptible to the adverse conditions of wine (e.g. pH, SO_2 & ethanol concentrations) and die off eventually during alcoholic fermentation (Jolly *et al.*, 2006). Other species, like *Saccharomycodes ludwigii* can produce large amounts of acetaldehyde that negatively affects wine aroma (Ciani & Maccarelli, 1998). Non-*Saccharomyces* strains of the species *H. uvarum*, and *M. pulcherrima* were found to lead to low acetaldehyde residues (less than 10 mg/L), while *C. stellata* and a *S. pombe* strain led to large residues (24 – 48 mg/L) (Romano *et al.*, 1997; Li & Orduña, 2017). However, the presence of *Starmerella bacillaris* reduced acetaldehyde and total SO_2 (Binati, *et al.*, 2020). Table 2.2 displays acetaldehyde levels for a few *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts.

Table 2.2 Acetaldehyde levels produced by some yeasts[a]

Yeast	Acetaldehyde (mg/L)
Saccharomyces cerevisiae	0.5–286
Saccharomyces uvarum	110–350
Saccharomyces bayanus	16–683
Saccharomyces oviformis	36–125
Saccharomyces fructuum	10–33
Saccharomyces ludwigii	30
Kloeckera apiculata	6–66
Torulaspora delbrueckii	0.5–5
Hanseniaspora guilliermondii	10.5–28
Metschnikowia pulcherrima	23–40

[a]*Data summarized from: Cortes et al. (1998), Di Stefano & Ciolfi (1982), Fleet & Heard (1993), Ibeas et al. (1997), Longo et al. (1992), Millán & Ortega (1988), Rankine & Pocock (1969), Romano et al. (1994, 1997), Stratford et al. (1987).*

2.7 Effect of lactic acid bacteria

Acetaldehyde levels in wine can be reduced by appropriate yeast strain selection, as well as the prevention of oxidation during vinification. In most cases, the reduction of acetaldehyde after alcoholic fermentation can be accomplished by wine lactic acid bacteria (LAB). Different strains of LAB (*Oenococcus*, *Lactobacillus*, and *Pediococcus*) have been found in wine during malolactic fermentation (MLF) (Wang *et al.*, 2018). Some LAB strains, such as *Lactobacilli*, *Leuconostocs*, *Pediococci* and *Streptococcus* spp., have ability to produce acetaldehyde (Liu & Pilone, 2000; Wang *et al.*, 2018). Homo- and heterofermentative wine LAB of the genera *Lactobacillus* and *Oenococcus* are capable of degrading free and SO_2-bound acetaldehyde (Osborne *et al.*, 2000). Acetaldehyde is normally consumed during MLF, since alcohol dehydrogenase (ADH) releases the LAB *Oenococcus oeni*, which has been reported to take charge of the acetaldehyde degradation (Wang *et al.*, 2018). Metabolism of the acetaldehyde moiety of SO_2-bound acetaldehyde by LAB result in the release of free SO_2 which in turn inhibit LAB growth.

2.8 Health related problems associated with high acetaldehyde levels in wine

It is crucial for winemakers to monitor and control acetaldehyde levels in wine since, in excess, it can pose several health-related problems. Besides its positive sensorial attributions in wines, numerous studies have shown that the administration of large concentrations of acetaldehyde can lead to a range of behavioural effects, notably those linked with symptoms of hangover such as vomiting, restlessness, nausea, confusion, sweating and headaches. Further, acetaldehyde has been shown to have several fundamental etiologic roles in the pathogenesis of liver fibrosis (Mello *et al.*, 2008) and fetal injury during pregnancy (Quertemont *et al.*, 2005). In addition, chronic alcohol consumption is often observed in patients who suffer oesophageal and gastric cancers as a result of the carcinogenic effect of high acetaldehyde levels in wines. Although no legal limits for concentration of acetaldehyde in wines are currently imposed, the importance of screening acetaldehyde levels in alcoholic beverages has been given special attention as a result of health concerns (Salaspuro, 2011).

2.9 Conclusion

Acetaldehyde plays an important role in the sensorial quality of wine. For table wines low levels of acetaldehyde contributes positively to the wine quality, while higher levels have

a negative impact. However, for fortified wines, high acetaldehyde levels contribute to the unique character of these wines. Recently, most studies focused on the biological and oxidative aging processes and the impact of acetaldehyde on the sensory properties of wine. Yeasts are the main contributors to acetaldehyde in wine, and the levels produced are species and strain dependent. Non-*Saccharomyces* yeasts have different attributes than *Saccharomyces* yeast and are currently utilised in the winemaking process to manipulate flavour and improve wine quality. The impact of mixed fermentations of *Saccharomyces* and non-*Saccharomyces* yeast interactions on acetaldehyde has received little attention. More research is also necessary regarding the correlation between intracellular ADH activity and the extracellular acetaldehyde levels. The role of fermentation temperature on acetaldehyde in wines have been studied, but there are some conflicting results. There is a strong relationship between acetaldehyde production and SO_2 levels in wine and with growing concerns about the health risks related to SO_2 further research is required. In the following chapters, the effect of yeast strain and oenological parameters on acetaldehyde production and sensory properties of wine will be discussed.

CHAPTER 3

METHODOLOGY

3.1 Screening of yeasts (laboratory-scale trials)

Pinotage grapes were harvested from ARC Infruitec-Nietvoorbij research farm (Stellenbosch, Western Cape, South Africa) and destemmed and crushed (macerated) as per the Nietvoorbij Cellar winemaking procedures (Section 3.4), but vinified according to a “blanc de noir” style. With no Chenin blanc (white cultivar) available at this point of the study, Pinotage (red cultivar) grapes was vinified according to the “blanc de noir” style in an attempt to minimise the polyphenol content in the must, and subsequently minimise its interactions with acetaldehyde. For this style the macerated grapes with minimal skin contact (less than 1 hour) was gently pressed at 1 Bar and later vinified according to the Nietvoorbij white wine preparation (Section 3.4.1).

A sample of the pressed must (before settling) was taken and the following parameters were analysed: residual sugar (°B), total acidity (TA), pH, free and total sulphur dioxide (FSO_2 and TSO_2). The SO_2 levels were determined using the Ripper method (Iland *et al*., 2000). Acetaldehyde concentrations (mg/L) were quantified using an Arena 20XT Enzyme Robot (Thermo Electron, Finland), which utilises an enzymatic kit (Acetaldehyde Assay Kit/K-ACHYD, Megazyme Ltd., Bray, Co. Wicklow, Ireland) for the analyses. A 20 L canister of must was used for this laboratory-based screening trial and the remainder of the must was frozen at -20°C for vinification until a later date (Section 3.2) after completion of the screening trial.

The laboratory-scale trials were performed to screen the yeasts for their ability to produce acetaldehyde and to select the highest, medium and lowest (relative to one another) acetaldehyde-producing yeast strains. Ten *Saccharomyces cerevisiae* and 10 non-*Saccharomyces* yeasts were screened for their acetaldehyde-producing abilities during the laboratory trials (Table 3.1).

Yeast strains were sourced from the ARC Infruitec-Nietvoorbij yeast genebank. Published literature (Romano *et al.*, 1994; Romano *et al.*, 2003; Swiegers *et al.*, 2005; Vriesekoop, 2007; Barrajon, 2011; Ciani & Comitini, 2011; Domizio *et al.,* 2011) was used as a guide

by ascertaining the levels of acetaldehyde produced by other yeasts in studies around the world. Before finalising the selection a consultation was held with the supervisor of the ARC Infruitec-Nietvoorbij yeast genebank, to confirm which of the yeasts of interest were available in the genebank. The final selection is tabulated in Table 3.1.

Standard laboratory protocol was followed for the culturing of the yeasts. All yeasts were cultured on yeast peptone dextrose agar (YPDA, Biolab, Merck, South Africa) at 30°C for 2-3 days, before a single colony or part of a single colony was transferred to 10 mL YPD broth (Biolab, Merck, South Africa) and grown for up to 2 days, with shaking, at 30°C. After sufficient growth was observed (i.e. when the growth medium became very turbid, and/or, a sizeable pellet of yeast cells was formed upon settling), the 10 mL yeast suspension was inoculated, into bottles containing 500 mL autoclaved grape juice, at the following cell densities: non-*Saccharomyces* yeasts at ~2 x 10^6 cells/mL; and, *Saccharomyces cerevisiae* strains at ~1 x 10^6 cells/mL.

Fermentations were conducted at two temperatures: 15°C and 25°C. The fermentation rates were monitored by weighing the bottles every second day and recording the mass-loss of fermenting must. Samples were taken to determine acetaldehyde levels (mg/L) and routine winemaking parameters, i.e. pH, ethanol (v/v %), total residual sugar (glucose and fructose) concentration, total acidity (TA) and malic acid. For the *S. cerevisiae* trial, samples were taken on day 0, day 2 (for 25°C fermenting musts only), day 3 (for 15°C fermenting musts only), and days 8, 15 and 22. Similarly, for the non-*Saccharomyces* trial, samples were taken on day 2 (for 25°C fermenting musts only), day 3 (for 15°C fermenting musts only), day 6 (for 25°C fermenting musts only), day 7 (for 15°C fermenting musts only), and days 8, 11, 15, 18 and 22. All fermentations were done in duplicate.

Yeast cell counts, unfortunately, were not done during the screening trial, as initially the focus was to only screen for acetaldehyde production by a variety of yeasts, and only thereafter select a smaller group of yeasts to continue with.

Table 3.1 Yeasts screened for their acetaldehyde-producing abilities

***Saccharomyces* yeast species**	**Strain name**	**Supplier/Source**
Saccharomyces cerevisiae	VIN13	Anchor Oenology, South Africa
Saccharomyces cerevisiae	EC1118	Lallemand, France
Saccharomyces cerevisiae	Zymaflore VL1	Laffort, France
Saccharomyces cerevisiae	Zymaflore VL3	Laffort, France
Saccharomyces cerevisiae	D47	Lallemand, France
Saccharomyces cerevisiae	QA23	Lallemand, France
Saccharomyces cerevisiae	NT202	Anchor Oenology, South Africa
Saccharomyces cerevisiae	NT116	Anchor Oenology, South Africa
Saccharomyces cerevisiae	NT50	Anchor Oenology, South Africa
Saccharomyces cerevisiae	NT112	Anchor Oenology, South Africa
Non-*Saccharomyces* yeast species	**Strain number**	**Supplier/Source**
*Hanseniaspora uvarum (Kloeckera apiculata)**	Y0858	ARC Infruitec-Nietvoorbij yeast genebank, Stellenbosch, South Africa
Metschnikowia pulcherrima (Candida pulcherrima)	Y0839	ARC Infruitec-Nietvoorbij yeast genebank, Stellenbosch, South Africa
Torulaspora delbrueckii (Candida colliculosa)	Y1031	ARC Infruitec-Nietvoorbij yeast genebank, Stellenbosch, South Africa
Pichia fermentans (Candida lambica)	Y0850	ARC Infruitec-Nietvoorbij yeast genebank, Stellenbosch, South Africa
Hanseniaspora valbyensis	Y0083	ARC Infruitec-Nietvoorbij yeast genebank, Stellenbosch, South Africa
Pichia guilliermondii (Candida guilliermondii)	Y0848	ARC Infruitec-Nietvoorbij yeast genebank, Stellenbosch, South Africa
Pichia membranifaciens (Candida valida)	Y0865	ARC Infruitec-Nietvoorbij yeast genebank, Stellenbosch, South Africa
Pichia kluyveri	Y0878	ARC Infruitec-Nietvoorbij yeast genebank, Stellenbosch, South Africa
Lachancea fermentati	Y0183	ARC Infruitec-Nietvoorbij yeast genebank, Stellenbosch, South Africa
Lachancea thermotolerans (Kluyveromyces thermotolerans)	Rhythm™	ARC Infruitec-Nietvoorbij yeast genebank, Stellenbosch, South Africa

**Yeast names in brackets are the anamorphic names of the preceding teleomorphic versions.*

3.2 Factors influencing acetaldehyde levels in must/wine (cellar trial 1)

3.2.1 Effect of winemaking treatments on acetaldehyde production

After 2 months, the vinification process commenced on the Pinotage must from the screening trial (Section 3.1). The acetaldehyde levels of the must were monitored from the defrosting step up until just before yeast inoculation, to ascertain whether there were any significant changes in the levels of acetaldehyde because of freezing. Must samples (50 mL) were taken immediately after it was thawed (after defrosting), after settling (by gravity, overnight at 4°C), as well as after racking and before yeast inoculation. All samples that were taken above were split into two equal volumes, and one portion was filtered through a 0.22 μm syringe filter, with the remaining portion left unfiltered. This was done to measure the effect of filtering on the acetaldehyde levels of the must/juice.

3.2.2 Effect of selected yeast strains/combinations on wine sensorial composition

The fermentation (cellar) trial commenced in which 20 L stainless steel canisters of must were inoculated with wet cultures of the three selected *S. cerevisiae* strains, individually, and in combination with the three selected non-*Saccharomyces* strains in all possible permutations (Table 3.2). The *in-house* reference yeast, VIN13 (ADWY, Anchor Oenology), normally used in the standard winemaking process at the Nietvoorbij Cellar, was used as a positive control for standard winemaking during this experimental trial. Standard laboratory protocol was followed for the culturing of the yeasts (as described in Section 3.1). Except for the ADWY control yeast which was rehydrated and inoculated at a dosage of 30 g/hL, as recommended by the supplier, all the other yeasts were wet cultures. All wet culture inoculations were done by hand for the non-*Saccharomyces* yeasts at ~2 x 10^6 cells/mL, and for the *S. cerevisiae* at ~1 x 10^6 cells/mL, and added to 20 L stainless steel canisters containing the must. All treatments were done in triplicate. The *S. cerevisiae* yeasts were NT50 (high acetaldehyde producer), NT116 (medium acetaldehyde producer) and VIN13 (low acetaldehyde producer); and, similarly, the non-*Saccharomyces* yeasts were *Torulaspora delbrueckii* (high), *Candida guilliermondii* (medium) and *Candida valida* (low). For the mixed culture fermentations, the non-*Saccharomyces* yeasts were inoculated 48 hours prior to *S. cerevisiae* inoculation. The standard Nietvoorbij winemaking protocol (Section 3.4) was followed, except for the yeast inoculations. Similar culturing conditions and procedures were followed as described in Section 3.1. Wine was fermented to dryness, i.e. when the residual sugar

(glucose/fructose) concentrations were below 5 g/L, whereafter it was cold stabilised at 0°C, bottled and stored until the sensorial evaluations.

Yeast cell count during fermentation was monitored by preparing a 5-step dilution series of each sample and plating out the two highest dilutions on YPDA and lysine agar, with both media containing 50 mg/L chloramphenicol (EMD Millipore Corp., Billerica, MA, USA) to inhibit bacterial growth. The plated yeast cells were allowed to grow aerobically for 2–3 days at 30°C until the colony forming units (CFUs) started forming on the agar plates. *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts grew on YPDA, while lysine was selective for non-*Saccharomyces* yeasts. The yeast cells were counted as soon as the CFUs became clearly visible on the plates, and was expressed as CFU/mL.

The first sensorial evaluation took the form of an informal tasting and discussion with 3 judges from the Department of Viticulture and Oenology (Stellenbosch University), and 7 judges from ARC Infruitec-Nietvoorbij in possession of the wine-evaluation certificate of the South African Wine & Spirits Board. During this evaluation, "free profiling" was done where each judge evaluated the wines and gave a list of various descriptors for each wine. This list of free-profiling descriptors also included some of the typical descriptors associated with the presence of free acetaldehyde, as obtained from literature, e.g. “green apple”, “bruised apple”, “grassy”, “pungent” (off-odour), “nutty” and “sherry”(-like) (Miyake & Shibamoto, 1993; Liu & Pilone, 2000; Frivik & Ebeler, 2003; Coetzee, 2014). Similar descriptors were grouped together under general headings (e.g. “rose petals” was grouped under Floral). For the formal sensory evaluation, the following descriptors were used on a 10 cm unstructured line scale: nutty, sherry-like, metallic, fruity flavours, off-flavour aroma, vegetative flavours, astringency, mouthfeel, sweet-associated, floral, alcohol, acidity and berry. Since the aforementioned “free profiling” exercise on the final wines did not yield clear differences amongst typical acetaldehyde descriptors (from literature), some of the acetaldehyde descriptors were thus grouped under “fruity flavours” and “vegetative flavours”. The intensity of the aroma descriptors were rated from “Undetectable” to “Prominent”, mouthfeel was rated from “Thin” to “Full”, and the other taste descriptors from “Low” to “High”, by the same panel of experienced wine judges from ARC Infruitec-Nietvoorbij. Thirty millilitres of each wine was presented in standard wine glasses, in a randomised order, to the judges.

Samples for acetaldehyde, and routine wine analyses, including ethanol analyses, were taken daily (from day 0 – 8) during the alcoholic fermentation to dryness. Acetaldehyde concentrations (mg/L) were quantified using an Arena 20XT Enzyme Robot. Free SO_2 and Total SO_2 were measured using the Ripper method (Iland *et al.*, 2000). Routine analyses, which included pH, ethanol (v/v %), residual sugar (g/L), malic acid (g/L), volatile- and total acidity (g/L), were performed on an OenoFoss™ (FOSS, Denmark).

Table 3.2 Yeast strains/combinations for cellar trial 1 in Pinotage must

Treatment	1st inoculation	2nd inoculation (48 h later)
1	Control*	*N.A.*
2	NT50	*N.A.*
3	NT116	*N.A.*
4	VIN13**	*N.A.*
5	*Torulaspora delbrueckii*	NT50
6	*Candida guilliermondii*	NT50
7	*Candida valida*	NT50
8	*Torulaspora delbrueckii*	NT116
9	*Candida guilliermondii*	NT116
10	*Candida valida*	NT116
11	*Torulaspora delbrueckii*	VIN13**
12	*Candida guilliermondii*	VIN13**
13	*Candida valida*	VIN13**

**Control refers to the commercial dry yeast (VIN13) used. **This VIN13 refers to the wet cultured strain.*

3.3 Effect of SO_2 on the acetaldehyde levels in must/wine (cellar trial 2)

In an independent cellar trial, Chenin blanc and Pinotage grapes were harvested from the ARC Infruitec-Nietvoorbij research farm and vinified as per the Nietvoorbij winemaking procedures (Section 3.4). Routine parameters of the must are listed in Table 3.3.

Table 3.3 Routine parameters of must for SO_2 trial

Pre-settling	Sugar (°B)	TA (g/L)	pH	Free SO_2 (mg/L)	Total SO_2 (mg/L)
Pinotage	21.8	8.2	3.19	6	21
Chenin blanc	21.4	6.3	3.30	11	23

Different concentrations of SO_2 (0, 50, 100 and 150 mg/L) were added to the Chenin blanc and Pinotage grape must prior to fermentation (day 0) to monitor its effect on acetaldehyde levels. All treatments were done in triplicate. In addition to the control (no SO_2 added), initial SO_2 concentrations were manipulated externally by addition of pre-calculated volumes from a stock SO_2 (potassium metabisulphite) solution to the must samples, to final concentrations of 50, 100 and 150 mg/L. The control (no SO_2 addition) had a baseline TSO_2 value of 23 mg/L for Chenin blanc, and 21 mg/L for Pinotage. The standard Nietvoorbij red and white wine-making protocols, with the exception of the SO_2 additions, were followed (Section 3.4). SO_2 additions were done before inoculation with the standard Nietvoorbij Cellar winemaking yeast, VIN13 (ADWY). Only the standard winemaking yeast was used during this trial, not only to, limit the number of samples (since 2 cultivars are also included), but also because the focus was mainly on the interaction between SO_2 and acetaldehyde during this trial.

Samples for SO_2, acetaldehyde and routine wine analyses were taken daily from days 1 – 7 until the acetaldehyde started levelling off, and then fermented to dryness (residual sugar < 5 g/L). FSO_2 and TSO_2 were measured using the Ripper method. Acetaldehyde concentrations (mg/L) were quantified using an Arena 20XT Enzyme Robot. Routine analyses were performed on an OenoFoss™. Wines did not undergo malolactic fermentation (MLF) (Supplementary Results, Table 1). Before bottling, SO_2 levels in the wines were adjusted as per the Nietvoorbij winemaking procedures, then bottled and stored at 15°C until the sensorial evaluations were performed. A formal sensory evaluation of the finished wines was performed as described in Section 3.2.

3.4 Nietvoorbij winemaking procedures

3.4.1 White wine preparation

White cultivar grapes were crushed, the juice and skins immediately pressed at up to 1 Bar. No skin contact was applied. For sedimentation 0.5 g/hL Ultrazym (Novozymes) was added to the turbid/cloudy juice. Fifty mg/L SO_2 was also added to the juice. A sample of the juice was taken for analyses (pH, total acid, sugar, SO_2) and the SO_2-level adjusted to a total of 50 mg/L, if necessary. Thereafter, the sediment was allowed to settle overnight at 14°C. The clear, settled juice was racked off the lees by siphoning into a fermentation container and the volumes of the juice and lees noted. The juice was

inoculated at 14°C with rehydrated pure yeast (VIN13) at a concentration of 30 g/hL, unless otherwise specified, as well as an addition of 50 g/hL diammonium phosphate (D.A.P.). After inoculation the fermentation continued at 14°C. Bentonite at 75 g/hL was added on the third day of fermentation (10 mL/L of 7.5% bentonite solution). Close to the end of fermentation, samples were taken (under CO_2 gas) and analysed for sugar content. After fermentation the wine was racked off the yeast lees. The FSO_2 and TSO_2 were adjusted to 45 mg/L FSO_2 whereafter the wine was cold stabilised at 0°C for at least two weeks. After cold stabilisation the wine was filtered by using filter mats (K900 and EK) and bottled into nitrogen-filled wine bottles at room temperature. The FSO_2 and TSO_2 were tested and the FSO_2 adjusted to 45 mg/L at bottling.

3.4.2 Red wine preparation

Red cultivar grapes were crushed, 50 mg/kg SO_2 was added, before the grape slurry was punched-down (Section 3.4.3). A sample of the must was taken for routine analyses (pH, total acidity and residual sugar). Skin contact was allowed for at least 1 hour before the grapes were inoculated with rehydrated pure yeast (VIN13) at a concentration of 30 g/hL, unless otherwise specified, as well as an addition of 50 g/hL diammonium phosphate (D.A.P.). Fermentation took place on the grape skins at a temperature of 25°C and the “cap” was punched down three times a day. Once the must had fermented to between 0°B and 5°B, the must/wine and skins were separated and pressed at 2 Bar. The pressed wine was added to the free-run wine, and fermented at 25°C until it was dry. Close to the end of fermentation, samples were taken (under CO_2 gas) and analysed for sugar content (°B). The fermentation was considered to be complete (dry) once the sugar concentration was less than 2 g/L. After fermentation the wine was racked off the yeast lees. The FSO_2 & TSO_2 was tested and adjusted to 45 mg/L FSO_2 (in accordance with the analysis). The wine should be analysed again to confirm the FSO_2. Bentonite was added to the wine before the wine was cold stabilised at 0°C for at least two weeks. After cold stabilisation (at least 2 weeks at 0°C) the wine was filtered using filter mats (K900 and EK), as well as a 0.45 μm membrane and bottled into nitrogen-filled wine bottles at room temperature. At bottling, the FSO_2 and TSO_2 were tested again, and the FSO_2 adjusted to 45 mg/L.

3.4.3 Punching-Down

To break up the cap that forms over fermenting red wine as the result of grape skins and solids rising to the surface, because of the carbon dioxide gas (CO_2) created by fermentation, the cap of skins and solids was “punched down” three times a day.

3.5 Acetaldehyde production and enzyme (ADH) activity

3.5.1 Alcoholic fermentation

The three *S. cerevisiae* yeast strains (i.e. NT50, NT116 & VIN13) were also used in a laboratory-scale fermentation trial to correlate the production of acetaldehyde with specific ADH activity. Standard laboratory protocol was followed for the culturing of the yeasts (see Section 3.1). After sufficient growth was observed, (i.e. when the growth medium became very turbid, and/or, a sizeable pellet of yeast cells was formed upon settling) the 10 mL yeast suspension was inoculated into bottles containing 500 mL autoclaved Chenin blanc juice and kept at room temperature (*ca.* 22°C) for 15 days. All fermentations were done in triplicate.

The fermentation rates were monitored by weighing the bottles every second day and recording the mass-loss of fermenting must. Samples for the enzyme assay, acetaldehyde concentration (mg/L) and routine winemaking parameters, i.e. pH, ethanol (v/v %), total residual sugar (glucose and fructose), total acidity and malic acid, were taken on days 2, 3, 4, 5, 9, 11 and 15 and stored at –20°C until needed.

3.5.2 Enzyme extraction

The stored yeast cells (from the fermentation trial) were thawed and centrifuged at 5000 rpm for 5 minutes at 4°C, according to Teusink *et al.* (2000). The supernatant was discarded and the pellets washed twice, with wash buffer (100 mM KH_2PO_4 at pH 7.0). Thereafter, the pellets (yeast cells) were resuspended in 0.5 mL extraction buffer. The extraction buffer consisted of 20 mM KH_2PO_4 at pH 7.0, 1 mM PMSF (1 M stock in DMSO) and approximately 0.5 g acid-washed glass beads were added. The 0.5 mm diameter glass beads were prepared by incubating them overnight in 1 M HCl, whereafter they were rinsed thoroughly with demineralised water and dried in an oven. The pellets and glass beads were vortexed for 30 seconds, with 30-second rest intervals on ice, over a

10-minute period, to lyse the cell walls so as to release the enzymes. Finally, the extracted enzymes were centrifuged at 10 000 rpm for 3 minutes at 4°C, and the supernatant (lysate) saved and stored at -20°C until the enzyme activity assay.

3.5.3 Enzyme plate assay

Each enzyme extract (lysate) was thawed and kept on ice while the assay stock solutions were being prepared. The assay buffer was made up of the following ingredients in 1 L dH_2O: 50 mM PIPES, 100 mM KCl, 5 mM $MgSO_4$, 50 mM KH_2PO_4, and adjusted to pH 7.0, according to Teusink *et al.* (2000). A 1:10 dilution (cocktail mix) of the following metabolite stock solutions was prepared using assay buffer: 525 mM (25 x K_m) ethanol; 40 mM NAD^+; 20 mM oxidised glutathione; 200 mM semicarbazide, and adjusted to pH 8.8. For the spectrophotometric reading, 10 µL of each lysate was placed in a microtitre plate well (Greiner 96 F-bottom) and immediately before the absorbance was taken, 90 µL of cocktail mix was added to the lysate to initiate the enzymatic reaction. Absorbance was measured at 340 nm on a SPECTROStar® Nano microplate reader (BMG LABTECH, Germany) at 30°C over a 10-minute period.

3.5.4 Protein assay

To calculate the specific enzyme activity (i.e. activity per mg protein) for each sample, the total protein concentration of the enzyme extract was determined using the protocol as described by Bradford (1976). One hundred microliters of the enzyme extract were transferred to a 2 mL cuvette, which contained 100 µL dH_2O and 800 µL bovine serum albumin (BSA) protein standard. Absorbance readings were done on a BioTek microplate reader (BioTek El*x*800, USA) at 595 nm, and protein concentration calculated using BSA standard calibration curve.

3.6 Statistical analyses

For the screening trial, with selected yeast strains, the experimental design was completely random with twenty yeast inoculations replicated two times at random. The treatment structure included 10 non-*Saccharomyces* yeasts and 10 *S. cerevisiae* single inoculations.

For the winemaking trial with selected yeast strains and combinations, the experimental design was completely random with thirteen yeast inoculations replicated three times at random. The treatment structure was a 3 x 3 factorial with three non-*Saccharomyces* yeasts and three *S. cerevisiae* co-inoculations, plus four (4) single inoculations (*S. cerevisiae* only, as well as the control (commercial active dry wine yeast).

For the trial assessing the effect of SO_2 on the levels of acetaldehyde, the experimental design was completely random with four SO_2 levels (0, 50, 100 and 150 mg/L SO_2 added to grape must before inoculation with VIN13), replicated three times at random.

Sensory data were pre-processed to test for panel reliability using a model that includes panellist, replicate and sample effects and interactions (Næs *et al.*, 2010). The Shapiro-Wilk test for normality was performed on the standardised residuals from the model, and outliers were removed when the standardised residual for an observation deviated more than three standard deviations from the model value. Following confirmation of panel reliability and normality, statistical analyses on the sensory data were conducted on means over judges.

Sensory and instrumental data were subjected to analysis of variance (ANOVA) according to the experimental design to test for treatment effects. Fisher's LSD was calculated at the 5% level to compare treatment means. A probability level of 5% was considered significant. Univariate analyses were performed using SAS software (Version 9.4, SAS Institute Inc., Cary, USA).

Multivariate statistical techniques were applied to elucidate patterns in data using XLStat (Version 2016, Addinsoft; New York, USA). Discriminant analysis (DA) was performed to clarify the association amongst treatments, sensory profile and instrumental data. Similarly, discriminant analysis (DA) was carried out to determine whether wines with different levels of SO_2 addition could be mathematically distinguished on the basis of the sensory and instrumental data.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Screening of yeasts (laboratory-scale trials)

The routine parameters for the Pinotage juice were: 24.6 °B; TA 5.1 g/L; pH 3.56; FSO_2 4 mg/L; TSO_2 12 mg/L, and the acetaldehyde concentration was 11.08 mg/L. The concentrations of acetaldehyde produced by 10 commercial *Saccharomyces* and 10 non-commercial non-*Saccharomyces* yeasts during fermentation of Pinotage at two temperatures (15°C or 25°C) are listed in Table 4.1.

For the *S. cerevisiae* yeast group, only strain VL3 showed significant differences ($p \leqq 0.05$) between acetaldehyde production at 15°C and 25°C. Almost all of *S. cerevisiae* yeasts produced lower acetaldehyde levels at 25°C than at 15°C. The exception to this trend was NT50, which produced higher acetaldehyde levels at 25°C than at 15°C. Significant differences in acetaldehyde levels were observed among some of the non-*Saccharomyces* yeast species at the two temperatures. *Candida pulcherrima* and *Torulaspora delbrueckii* produced significantly higher acetaldehyde concentrations at 15°C than at 25°C. In contrast, *Candida lambica*, *Pichia kluyveri* and *Candidia valida* produced significantly lower acetaldehyde concentrations at 15°C than at 25°C. Increases and decreases in acetaldehyde concentration at specific temperatures for different yeast species have been reported (Romano *et al*., 1994; Jackowetz *et al*., 2010). The fluctuation in acetaldehyde levels during fermentation and at the different fermentation temperatures could be ascribed to the yeast cells metabolising acetaldehyde at different rates, as well as being degraded or absorbed by various substances in the fermentation medium (Osborne *et al*., 2000; Li & Mira de Orduña, 2010).

Table 4.1 Mean acetaldehyde values of Pinotage wines after fermentation with different yeasts at 15°C and 25°C

***Saccharomyces* yeast species**	**Strain name**	**Temperature (°C)**	**Acetaldehyde (mg/L)**
Saccharomyces cerevisiae	D47	15	16.292^{cdef} * (15.857) **
Saccharomyces cerevisiae	D47	25	12.606^{ef} (6.064)
Saccharomyces cerevisiae	EC1118	15	17.363^{cde} (16.560)
Saccharomyces cerevisiae	EC1118	25	16.166^{cdef} (7.854)
Saccharomyces cerevisiae	NT112	15	17.884^{cd} (18.408)
Saccharomyces cerevisiae	NT112	25	13.450^{def} (6.534)
Saccharomyces cerevisiae	NT116	15	14.756^{cdef} (13.874)
Saccharomyces cerevisiae	NT116	25	14.687^{cdef} (7.899)
Saccharomyces cerevisiae	NT202	15	15.957^{cdef} (16.027)
Saccharomyces cerevisiae	NT202	25	15.508^{cdef} (8.176)
Saccharomyces cerevisiae	NT50	15	23.341^{ab} (22.568)
Saccharomyces cerevisiae	NT50	25	26.508^{a} (21.349)
Saccharomyces cerevisiae	QA23	15	16.410^{cdef} (12.275)
Saccharomyces cerevisiae	QA23	25	13.203^{def} (5.866)
Saccharomyces cerevisiae	VIN13	15	15.423^{cdef} (15.332)
Saccharomyces cerevisiae	VIN13	25	12.848^{def} (6.605)
Saccharomyces cerevisiae	VL1	15	14.723^{cdef} (10.163)
Saccharomyces cerevisiae	VL1	25	12.115^{f} (5.799)
Saccharomyces cerevisiae	VL3	15	18.922^{bc} (14.497)
Saccharomyces cerevisiae	VL3	25	13.531^{def} (7.265)
Non-*Saccharomyces* yeast species	**Strain name**	**Temperature (°C)**	**Acetaldehyde (mg/L)**
Kloeckera apiculata	Y0858	15	16.445^{ghij} (8.215)
Kloeckera apiculate	Y0858	25	14.809^{hijk} (8.999)
Hanseniaspora valbyensis	Y0083	15	17.374^{fghi} (4.068)
Hanseniaspora valbyensis	Y0083	25	14.401^{hijk} (3.383)
Lachancea fermentati	Y0183	15	13.139^{jk} (5.106)
Lachancea fermentati	Y0183	25	14.004^{ijk} (4.803)
Lachancea thermotolerans	Rhythm™	15	21.457^{ef} (4.317)
Lachancea thermotolerans	Rhythm™	25	23.931^{e} (2.508)
Candida pulcherrima	Y0839	15	20.610^{ef} (7.195)
Candida pulcherrima	Y0839	25	16.052^{ghij} (6.165)
Candida lambica	Y0850	15	11.672^{k} (6.093)
Candida lambica	Y0850	25	18.340^{fgh} (9.772)
Candida guilliermondii	Y0848	15	14.772^{hijk} (7.793)
Candida guilliermondii	Y0848	25	16.178^{ghij} (5.461)
Pichia kluyveri	Y0878	15	11.654^{k} (3.351)
Pichia kluyveri	Y0878	25	19.240^{fg} (10.052)
Candida valida	Y0865	15	5.424^{l} (1.333)
Candida valida	Y0865	25	16.193^{ghij} (6.775)
Torulaspora delbrueckii	Y1031	15	56.875^{c} (10.177)
Torulaspora delbrueckii	Y1031	25	40.248^{d} (5.710)

**Values in same column followed by a letter or group of letters (superscript), different from another, were significantly different from each other ($p \leq 0.05$, Student t-test); and, values in the same column followed by the same letter or group of letters (superscript), did not differ significantly ($p \leq 0.5$). **Standard deviation of the mean. For S. cerevisiae yeasts, the acetaldehyde values are the mean values of duplicate fermentations and five sampling times (n = 10); For non-Saccharomyces yeasts, the acetaldehyde values are the mean values of duplicate fermentations and seven sampling times (n = 14).*

Differences in acetaldehyde levels were observed for the yeast strain used, time of fermentation, and fermentation temperature (Figs 4.1 and 4.2). The highest acetaldehyde levels were produced on day 2 or day 3, after which it decreased and only increased at the end of fermentation. At 15°C, most of the *S. cerevisiae* yeast produced the highest acetaldehyde levels (>30 mg/L) on day 3. However, the same trend was not observed for the non-*Saccharomyces* yeasts. In general, the non-*Saccharomyces* yeasts produced lower acetaldehyde levels during fermentation than the *Saccharomyces* yeasts, which is in agreement with other reports (see Table 2.2).

From the screening trial data, the following yeasts were selected, based on statistical differences in acetaldehyde production, *S. cerevisiae* yeasts: NT50 (high), NT116 (medium), VIN13 (low), and non-*Saccharomyces* yeasts: *T. delbrueckii* (high), *C. guilliermondii* (medium), *C. valida* (low) (Table 4.2). Technically, VL1 was the lowest producer, but VIN13 being the second lowest producer, was selected instead, based on additional interest in it as the *in-house* reference yeast, since it was also used in the Nietvoorbij Cellar as the standard winemaking yeast.

4.2 Factors influencing acetaldehyde levels in must/wine (cellar trial 1)

4.2.1 Effect of winemaking treatments on acetaldehyde levels in juice

Winemaking treatments such as freezing, settling and racking of grape must before analysis, did not have a mentionable effect on acetaldehyde levels (Fig. 4.3). However, filtration reduced the acetaldehyde in the must/juice samples. The acetaldehyde levels in the unfiltered must/juice samples ranged between 12.1 and 13.6 mg/L, and between 8.5 and 11.1 mg/L for the filtered samples. These acetaldehyde levels in this must/juice are not necessarily high, since not much has been reported with regard to acetaldehyde levels in grape juice. However, McCloskey & Mahaney (1981) reported acetaldehyde levels of 61 mg/L in Riesling juice.

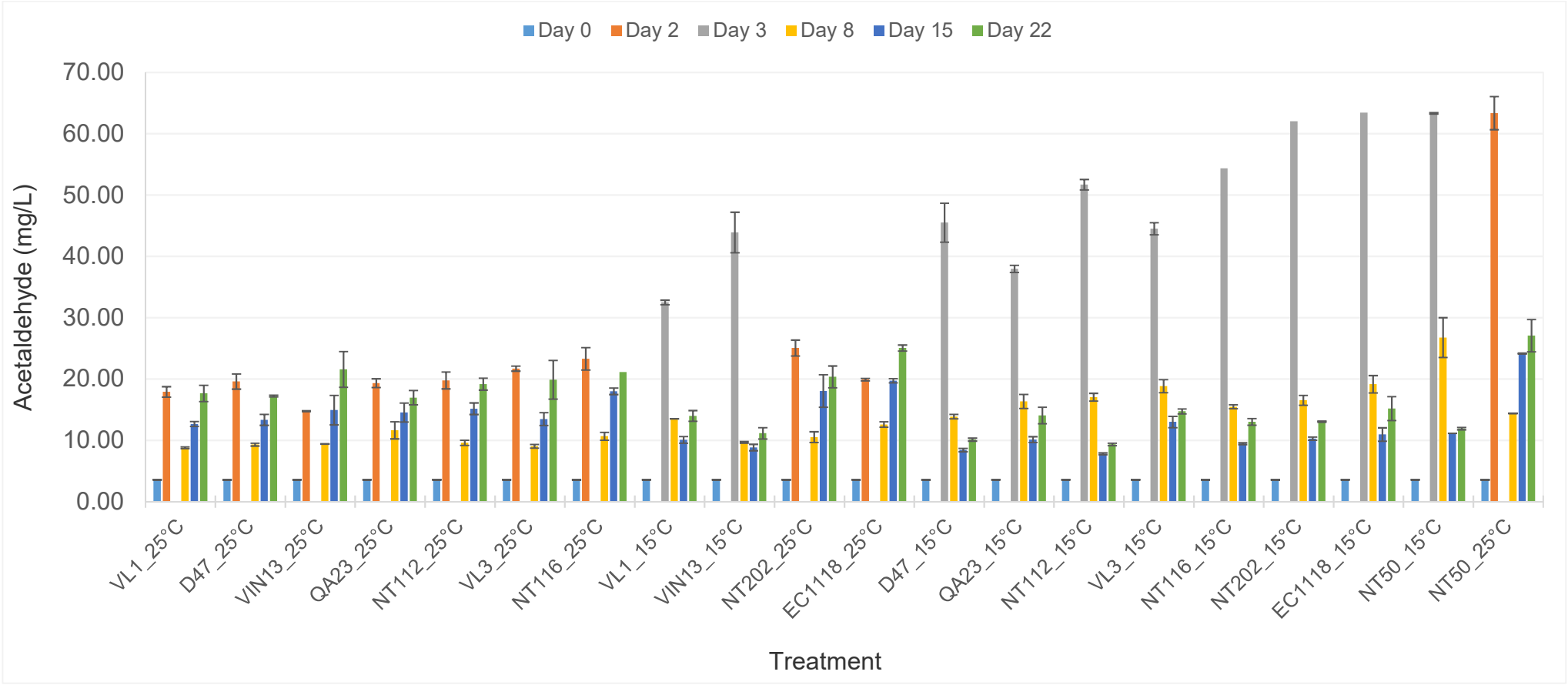


Figure 4.1 Impact of *Saccharomyces cerevisiae* yeasts, fermentation time and temperature on acetaldehyde levels. Each yeast was screened for acetaldehyde production during fermentation, in duplicate, over 5 sampling days, at 15°C and at 25°C (n = 10). Error bars represent the standard error of the mean (SEM).

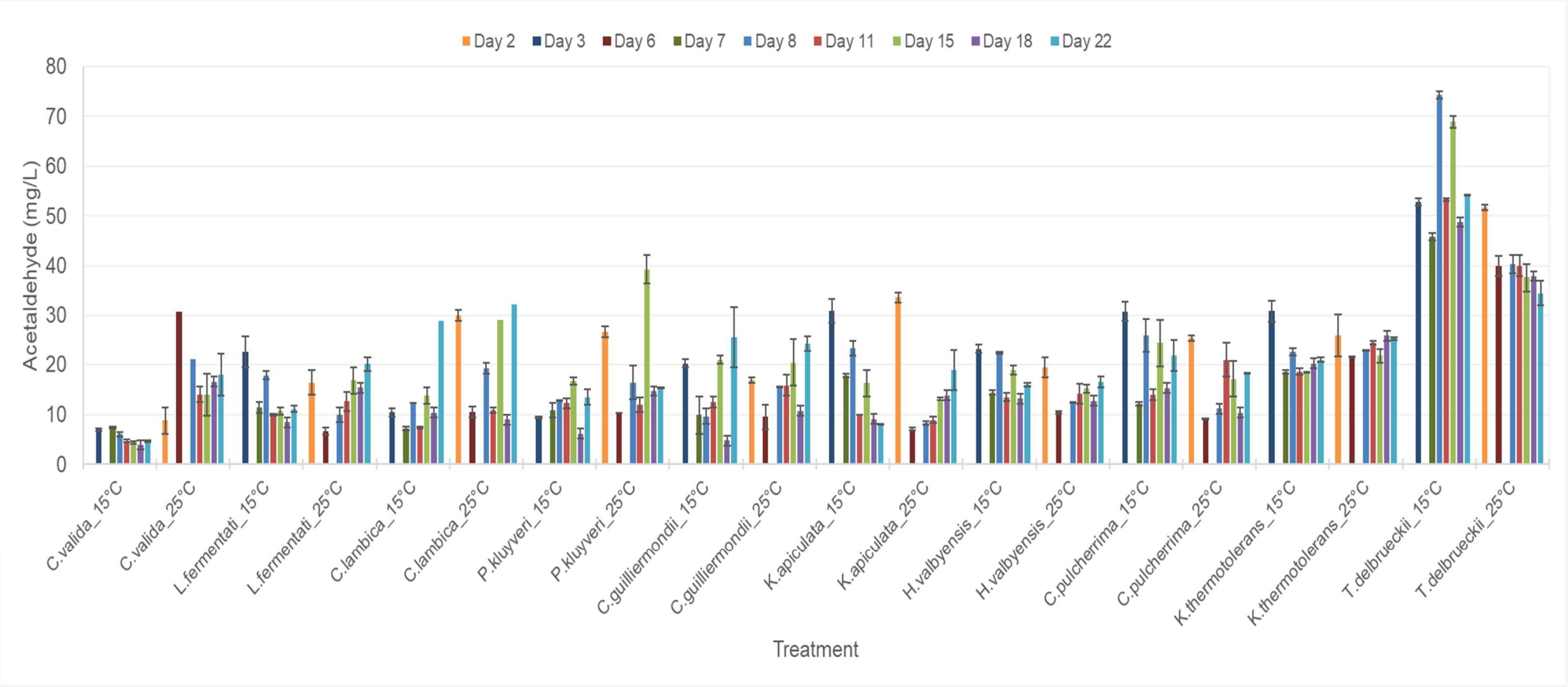


Figure 4.2 Impact of non-*Saccharomyces cerevisiae* yeasts, fermentation time and temperature on acetaldehyde levels. Each yeast was screened for acetaldehyde production during fermentation, in duplicate, over 7 sampling days, and at 15°C and at 25°C (n = 14). Error bars represent the standard error of the mean (SEM).

Table 4.2 Overall mean acetaldehyde values in Pinotage wine produced by different yeasts over the course of the fermentation trial

***Saccharomyces* yeast species**	**Strain name**	**Acetaldehyde (mg/L)**
Saccharomyces cerevisiae	D47	14.449[b] * (11.836) **
Saccharomyces cerevisiae	EC1118	16.733[b] (12.374)
Saccharomyces cerevisiae	NT112	15.667[b] (13.635)
Saccharomyces cerevisiae	NT116	14.722[b] (10.952) - *medium level*
Saccharomyces cerevisiae	NT202	15.721[b] (12.150)
Saccharomyces cerevisiae	NT50	24.925[a] (21.442) - *higher level*
Saccharomyces cerevisiae	QA23	14.806[b] (9.507)
Saccharomyces cerevisiae	VIN13	14.136[b] (11.566) - *lower level*
Saccharomyces cerevisiae	VL1	13.419[b] (8.163)
Saccharomyces cerevisiae	VL3	16.227[b] (11.498)
Non-*Saccharomyces* yeast species	**Strain name**	**Acetaldehyde (mg/L)**
Kloeckera apiculata	Y0858	15.627[de] (8.496)
Hanseniaspora valbyensis	Y0083	15.887[de] (3.971)
Lachancea fermentati	Y0183	13.571[e] (4.884)
Lachancea thermotolerans	Rhythm™	22.694[c] (3.686)
Candida pulcherrima	Y0839	18.331[d] (6.972)
Candida lambica	Y0850	14.873[e] (8.596)
Candida guilliermondii	Y0848	15.475[de] (6.642) - *medium level*
Pichia kluyveri	Y0878	15.447[de] (8.305)
Candida valida	Y0865	10.394[f] (7.148) - *lower level*
Torulaspora delbrueckii	Y1031	48.561[b] (11.715) - *higher level*

**Values in same column followed by a letter or group of letters (superscript), different from another, were significantly different from each other (p ≤ 0.05, Student t-test); and, values in the same column followed by the same letter or group of letters (superscript), did not differ significantly (p ≤ 0.5). **Standard deviation of the mean. For S. cerevisiae yeasts, the acetaldehyde values are the mean values of duplicate fermentations, five sampling times and two temperatures (n = 20); For non-Saccharomyces yeasts, the acetaldehyde values are the mean values of duplicate fermentations, seven sampling times and two temperatures (n = 28).*

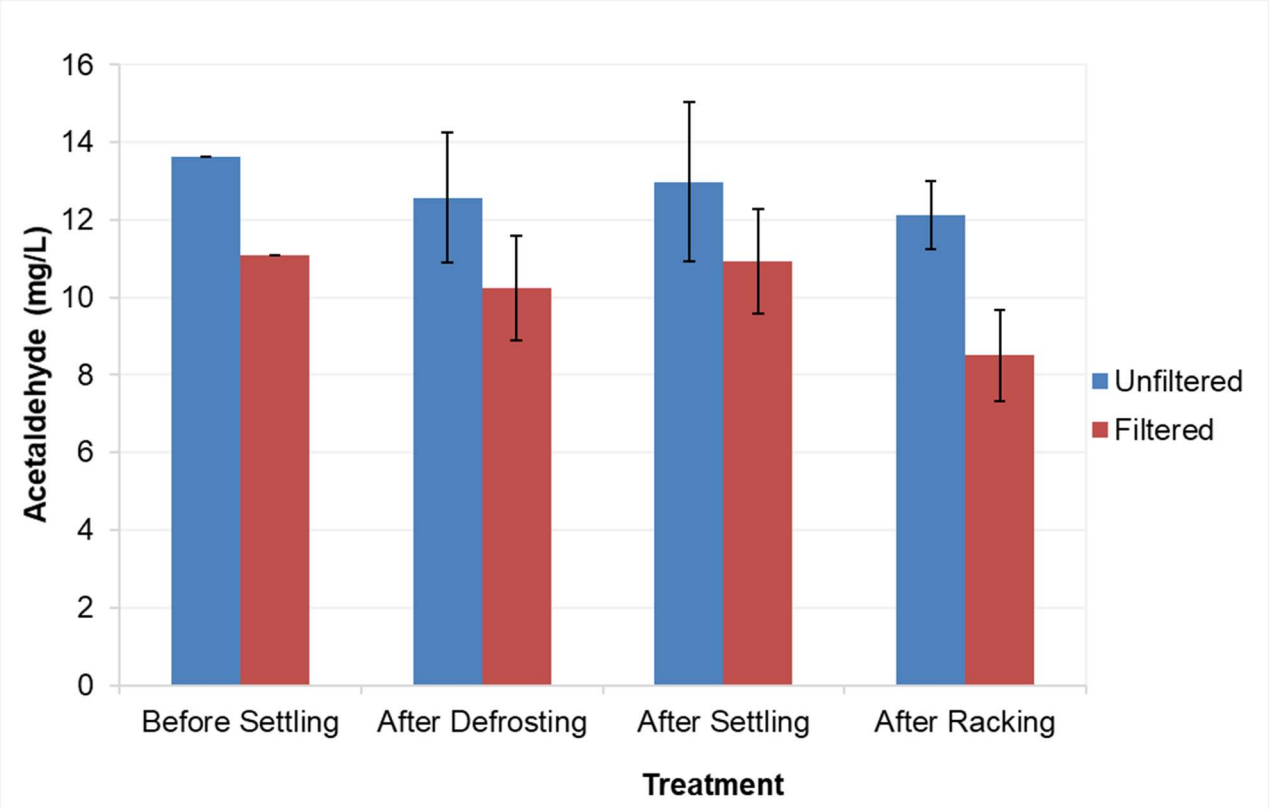


Figure 4.3 Effect of different Pinotage must treatments before fermentation on acetaldehyde concentrations. Samples per treatment (n) = 3. Error bars represent the standard error of the mean (SEM).

4.2 2 Effect of selected yeast strains/combinations on wine sensorial composition

The selection of high-, medium- and low-acetaldehyde producing yeast strains and combinations of these strains used in the cellar trial are listed in Table 4.3. For the treatments where non-*Saccharomyces* yeasts were used in combination with *S. cerevisiae* yeasts, the *S. cerevisiae* yeasts were inoculated 48 hours after the non-*Saccharomyces* yeasts.

Table 4.3 Mean acetaldehyde values (n = 3) for Pinotage finished wines (after bottling) produced by different yeast/combinations (cellar trial 1)

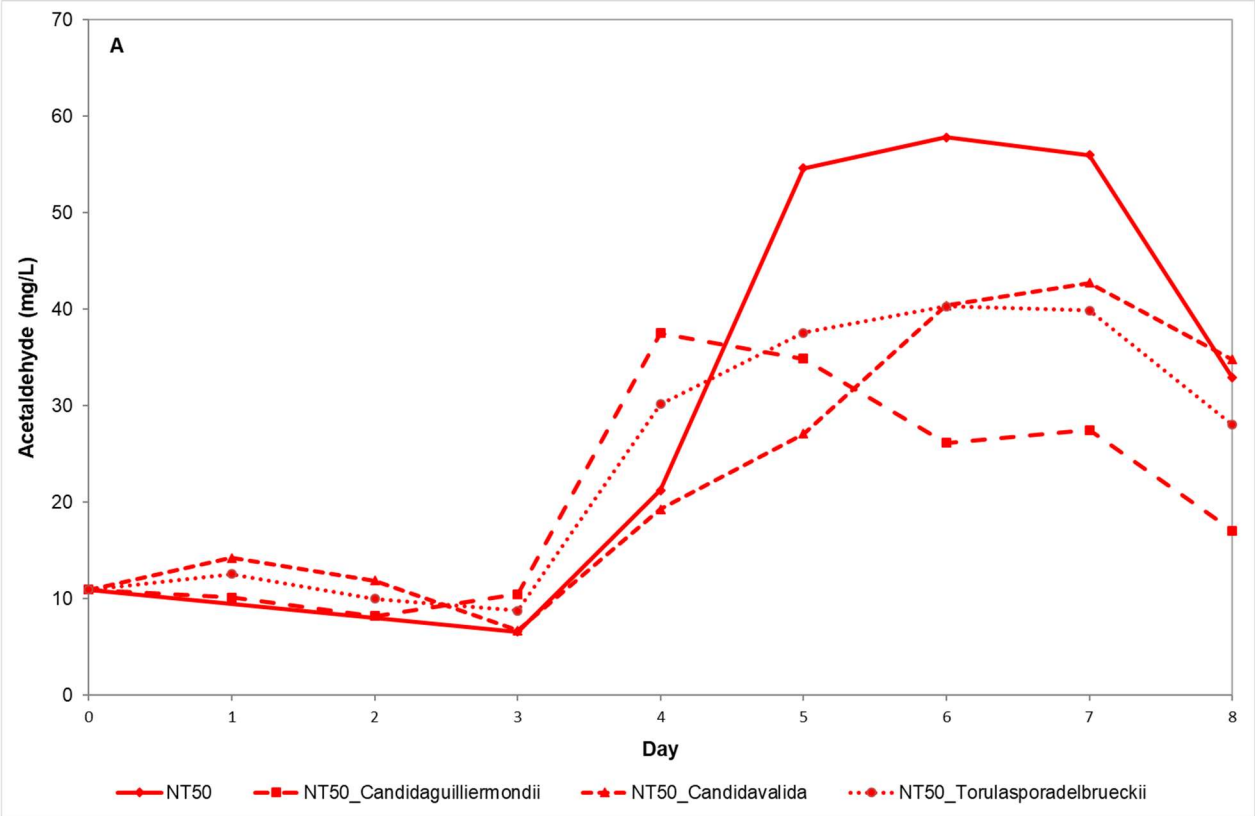
Yeast(s) and combinations	Acetaldehyde (mg/L)
Control*	28.35^{ab} (8.51)
NT50	38.18^{a} (15.67)
NT50-*Torulaspora delbrueckii*	25.90^{abc} (7.80)
NT50-*Candida guilliermondii*	21.47^{cde} (3.61)
NT50-*Candida valida*	24.63^{bcd} (8.28)
NT116	32.83^{ab} (7.38)
NT116-*Torulaspora delbrueckii*	24.84^{abc} (7.30)
NT116-*Candida guilliermondii*	26.93^{abc} (6.29)
NT116-*Candida valida*	19.56^{cde} (2.83)
VIN13	18.40^{de} (4.11)
VIN13-*Torulaspora delbrueckii*	22.43^{abc} (5.56)
VIN13-*Candida guilliermondii*	19.23^{bcde} (3.04)
VIN13-*Candida valida*	17.16^{e} * (3.19) **

**Values in same column followed by a letter or group of letters (superscript), different from another, were significantly different from each other ($p \leq 0.05$, Student t-test); and, values in the same column followed by the same letter or group of letters (superscript), did not differ significantly ($p \leq 0.5$). **Standard deviation of the mean (n = 3). Control indicates the commercial dry yeast VIN13 used by Nietvoorbij Cellar.*

The levels of acetaldehyde during alcoholic fermentation (AF) varied from 4.8 mg/L on day 3 (i.e. for one of the three NT50_*Candida valida* co-inoculations) up to 86.1 mg/L on day 7 (i.e. for one of the three single NT50 inoculations). This large variation confirmed that certain yeasts or yeast combinations play a role in the levels of acetaldehyde in fermenting musts and wines. Day 8 signified the last day of fermentation and was also the last day of sampling, whereafter the SO_2 levels were adjusted according to the Nietvoorbij winemaking protocol (Section 3.4). The acetaldehyde levels in the finished wines were determined before the wines were sensorially evaluated and these results are listed in Table 4.3.

During fermentation, acetaldehyde levels increased, levelled off, and decreased (Fig. 4.4). Yeast strains had an impact on the levels of acetaldehyde during fermentation and in the finished wine (Fig. 4.4 and Table 4.3). In most cases, co-inoculations with a non-

Saccharomyces yeast generally resulted in lower levels of acetaldehyde than the single inoculations with only *Saccharomyces cerevisiae* (except for VIN13 from culture collection) (Fig. 4.4 and Table 4.3). It has also been reported that mixed fermented wines also showed a reduction in acetaldehyde and were evaluated with the highest scores for 'colour', 'flavour' and 'taste' (Kim *et al.*, 2008; Jolly *et al.*, 2014). The decreasing levels of acetaldehyde could possibly be as a result of the acetaldehyde being re-utilised by the yeast cells, degraded or absorbed by various substances in the fermentation medium (Liu & Pilone, 2000; Li & Mira de Orduña, 2010; Jackowetz *et al.*, 2011; Shin *et al.*, 2019). Varying acetaldehyde levels as a result of either fermentation conditions and/or yeast strains, have been reported in literature, and in certain cases the mutant strains of *S. cerevisiae* accumulated more acetaldehyde in the medium than the parental strain (Casalone, *et al.*, 1992; Romano *et al.*, 1994).



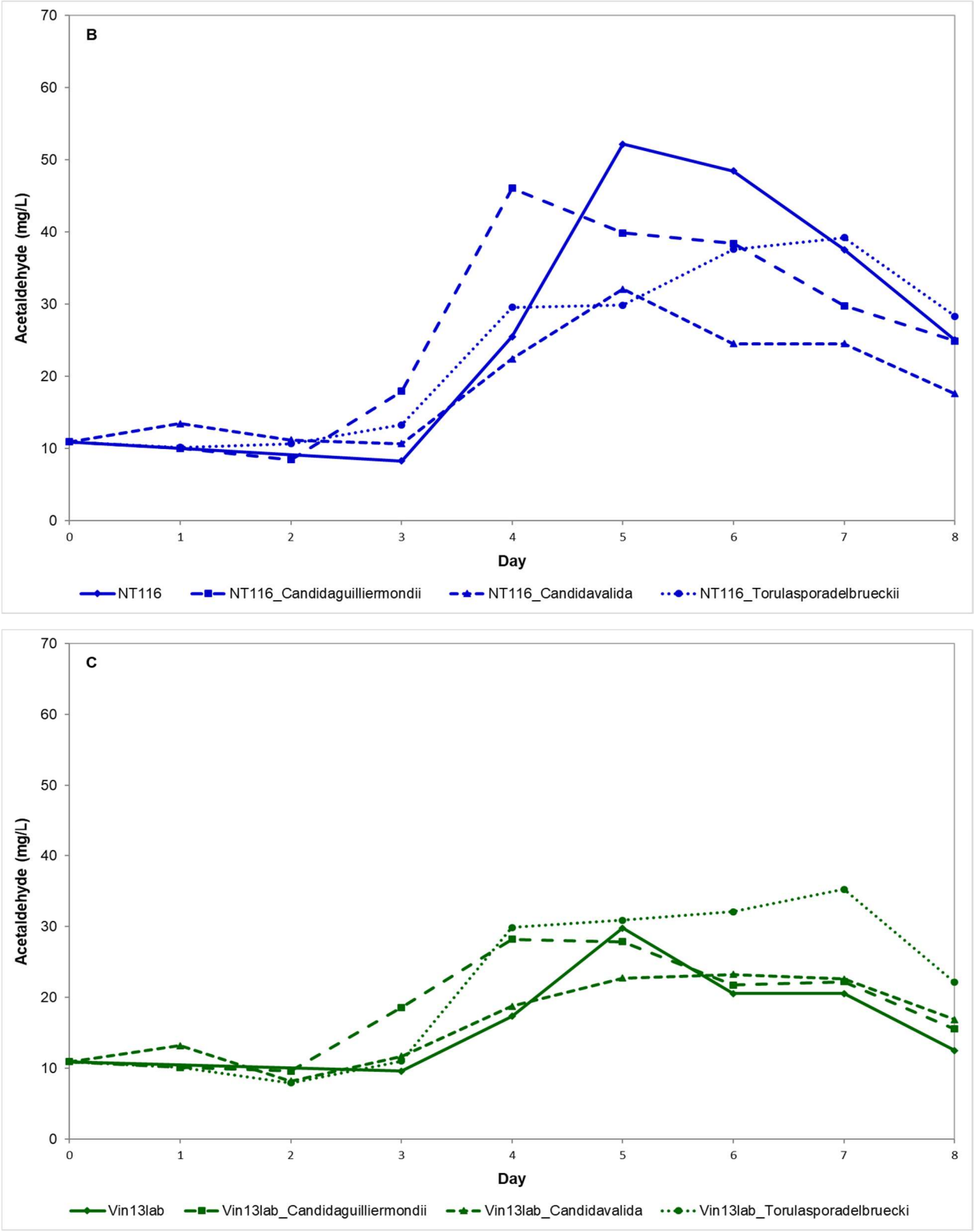


Figure 4.4 Acetaldehyde production by various *Saccharomyces cerevisiae* strains, and by the same strains co-inoculated with non-*Saccharomyces* yeasts, during the fermentation of Nietvoorbij Pinotage must. For improved visualisation, related yeasts and their combinations have been grouped together into figures *A* (NT50 and its combinations), *B* (NT116 and its combinations) and *C* (Vin13lab and its combinations).

A similar trend in acetaldehyde levels was also observed for most of the yeasts from day 3 onwards, which seemed to directly correlate with yeast cell counts from the YPDA plates for the same time interval (Supplementary Results, Figure 1). Yeast cell count data for the earlier time intervals were incomplete, and therefore not shown in these results.

The reason why two types of the VIN13 yeasts were used in this study was because the isolate from ARC Infruitec-Nietvoorbij yeast genebank ("Vin13lab") was associated with lower levels of acetaldehyde production during the screening trial. It was therefore selected to be used in the cellar trial. The commercial ADWY strain of VIN13 was used as a standard wine control, since it was the standard wine yeast used in the Nietvoorbij Cellar. However, a significant difference was observed between the acetaldehyde levels associated with these two types of VIN13, with the isolate from yeast genebank showing an average acetaldehyde concentration (n = 3) of 18.4 mg/L, while the commercial (ADWY) strain averaged significantly higher at 28.35 mg/L. The yeast preparation (ADWY or cultured) thus seems to have a significant impact on acetaldehyde levels, but one can also not rule out the possibility that one of these strains might have undergone a mutation, and therefore further research into these differences is needed.

Figure 4.5 shows the results of a discriminant analysis (DA) plot for the different yeast combinations quantifying the factors contributing the most to separating the results for the various sensory and chemical analyses. The three *Saccharomyces cerevisiae* yeasts i.e. NT50, NT116 and the ADWY, VIN13, associated with the higher acetaldehyde levels (Table 4.3), formed clusters with strong fruity and floral aromas, with berry and vegetative nuances present at lower intensities. In the same cluster the chemical attributes, acetaldehyde and TSO_2, are prominent at similar intensities, which is to be expected because of their high affinity for each other (Liu & Pilone, 2000; Elias *et al.*, 2008). Acetaldehyde at low wine levels, i.e. 30 – 60 mg/L (Miyake & Shibamoto, 1993; Zea *et al.*, 2010; Coetzee *et al*. 2016a) can impart pleasant, fruity aromas (Liu & Pilone, 2000; Coetzee *et al.*, 2016a,b; 2018), which could explain the cluster of the fruity descriptors with acetaldehyde levels in this study.

The negative aroma descriptors (metallic, sherry-like, cooked vegetative and off-flavour aroma) cluster around the co-inoculated treatments where the medium and lower acetaldehyde producing *S. cerevisiae* strains are in combination with the two *Candida* species. These unpleasant aromas are not necessarily an effect of acetaldehyde levels, but could rather be due to the wild yeasts. In the past, non-*Saccharomyces* yeasts were generally known as wine spoilage yeasts, due to their ability to produce undesired compounds during the first stages of alcoholic fermentation (Ciani & Comitini, 2011), and they were initially given 48 hours to interact with the grape must prior to the co-inoculation with the *S. cerevisiae* strains, which could explain the negative nuances.

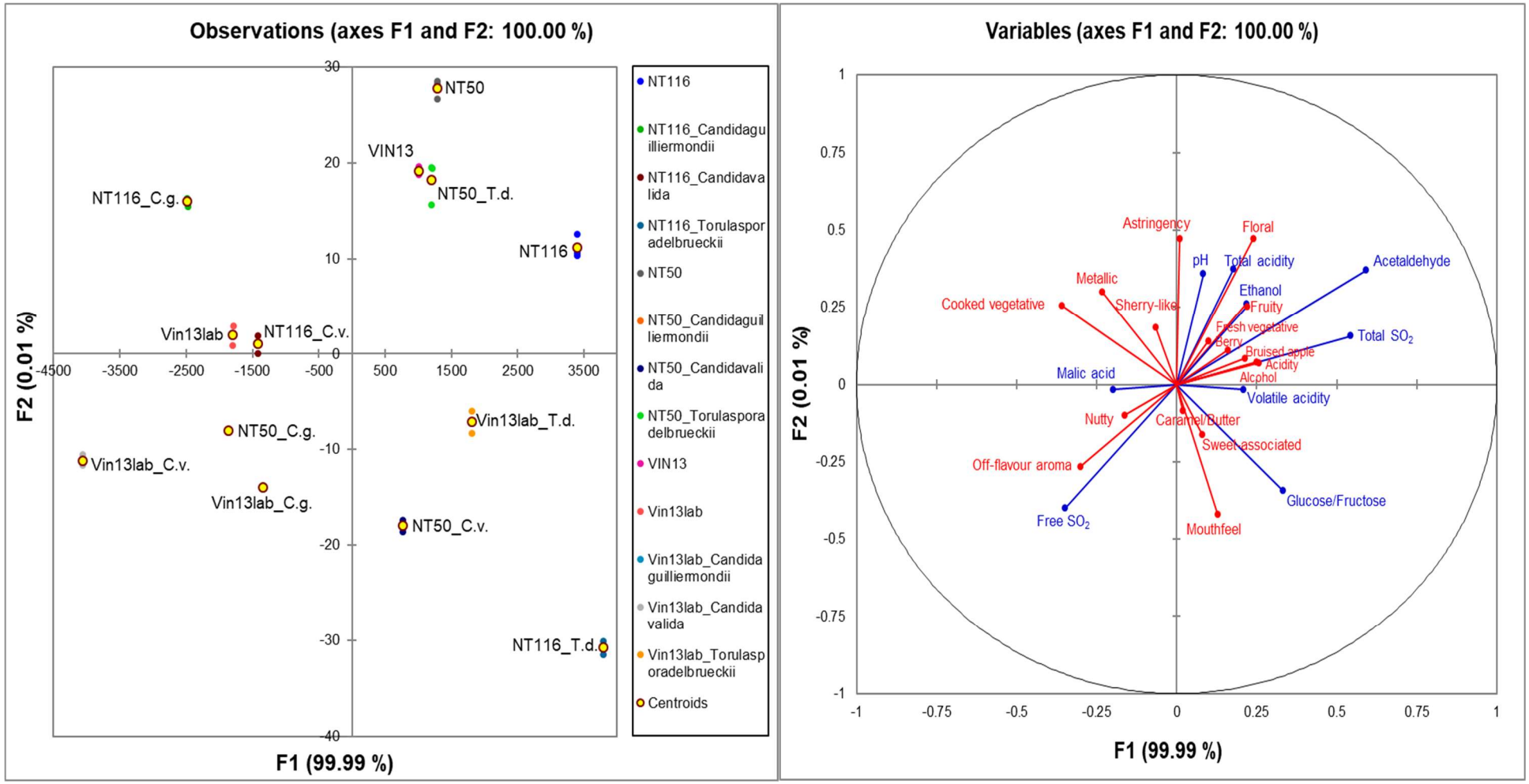


Figure 4.5 Discriminant analysis plot of Pinotage based on sensory and chemical attributes of finished wines fermented with different yeast inoculations, i.e. NT116, NT116 and *Candida guilliermondii (C.g.)*, NT116 and *Candida valida (C.v.)*, NT116 and *Torulaspora delbrueckii (T.d.)*, NT50, NT50 and *Candida guilliermondii (C.g.)*, NT50 and *Candida valida (C.v.)*, NT50 and *Torulaspora delbrueckii (T.d.)*, VIN13 (Control), VIN13lab, VIN13lab and *Candida guilliermondii (C.g.)*, VIN13lab and *Candida valida (C.v.)*, VIN13lab and *Torulaspora delbrueckii*

(T.d.). Chemical variables analysed include the routine wine parameters pH, TA, VA, ethanol, malic acid, total sugar (glucose + fructose), free and total SO_2 and acetaldehyde. Sensory descriptors include nutty, sherry-like, metallic, fruity flavours, off flavour aroma, vegetative flavours, astringency, mouthfeel, sweet-associated, floral, alcohol, acidity and berry. “Vin13lab” indicates the yeast sourced from the ARC Infruitec-Nietvoorbij yeast genebank at Nietvoorbij, Stellenbosch. “Vin13” indicates the commercial dry yeast (control).

4.3 Effect of SO_2 on the acetaldehyde levels in must/wine (cellar trial 2)

The varying levels of SO_2 had a direct effect on the acetaldehyde levels during fermentation of Chenin blanc grape must. The increased production of acetaldehyde in response to increased levels of SO_2 is evident from figure 4.6. The low levels of acetaldehyde in Chenin blanc and high levels in Pinotage at day 1 are possibly due to the fact that the fermentation rates of these two grape cultivars were affected by their respective fermentation temperatures (i.e. Chenin blanc at 15°C and Pinotage at 25°C). During the screening trial (Section 4.1) it was observed that for most *S. cerevisiae* yeast strains (Fig. 4.1), acetaldehyde levels peaked earlier at 25°C (day 2 of fermentation) than at 15°C (day 3 of fermentation). This could explain why Pinotage must, fermented at 25°C according to the red winemaking preparation (Section 3.4.1), displays higher levels of acetaldehyde (Fig. 4.6) than Chenin blanc must, fermented at 15°C according to the white winemaking preparation (Section 3.4.1). Reasons for the levelling off and decreasing trends, during the latter stages of fermentation, could be due to yeast cells dying in the presence of higher ethanol concentrations, exposure to oxygen (aldehydes degrade in air via the process of autoxidation), and because of the tendency of acetaldehyde to oligomerise, polymerise or hydrate. Fermentations under severely repressive conditions, such as juices treated with high SO_2 (metabisulfite) prior to the onset of fermentation, cause a correspondingly higher production of acetaldehyde by the yeast cells to bind the SO_2. A possible explanation for this observed trend could be that high SO_2 levels inhibits the enzyme responsible for metabolising acetaldehyde into ethanol (i.e. aldehyde dehydrogenase), by binding directly with acetaldehyde, thereby preventing its transformation to ethanol, and resulting in high levels of acetaldehyde remaining in the fermenting must (Frivik & Ebeler 2003; Andorrà *et al*., 2018).

Acetaldehyde is released by the yeast as the detoxification mechanism for sulphites (Jackowetz *et al*., 2011). Acetaldehyde has a strong affinity for SO_2 (Liu & Pilone, 2000; Elias *et al*., 2008), therefore, varying concentrations of the one, will affect the free or available levels of the other, with possible subsequent effects on wine quality (Coetzee *et al*., 2016a; Coetzee *et al*., 2018).

The fermentation of Pinotage took place on the grape skins and the “punching- down”” activity was performed to extract colour (anthocyanins) and aroma compounds. An additional reason for the high acetaldehyde levels on day 1 for the Pinotage fermenting

must (Fig. 4.6) could be as a result of there not being free SO_2 available to bind the acetaldehyde, since most SO_2 bound to anthocyanins, thereby reducing its availability to bind the acetaldehyde (Coetzee, 2014; Andorrà *et al*., 2018; Giacosa *et al*., 2019). As fermentation on the grape skins progressed, the acetaldehyde concentration decreased during the initial stages, while the latter part of the fermentation showed an increased and decreased trend up to the end of fermentation (Fig. 4.6). These decreases could be explained by the yeast cells producing more sulphites, which in turn would bind the acetaldehyde, or even as a result of acetaldehyde binding to anthocyanins, amongst other grape phenolics, during fermentation (Frivik & Ebeler, 2003; Aleixandre-Tudo, 2016; Andorrà *et al*., 2018; Giacosa *et al*., 2019). Increases in observed SO_2 and acetaldehyde levels (particularly noticeable for low or no added SO_2 treatments) after a few days of fermentation, could be reflective of SO_2 produced by the yeast, and auto-oxidation of alcohol to acetaldehyde (Wildenradt & Singleton, 1974; Ribéreau-Gayon *et al*., 1983; Andorrà *et al*., 2018). The concentrations of SO_2 used in the experiment were within the legal upper limit of 150 mg/L, imposed by South African regulations (SAWIS, 2018).

Groupings based on chemical and sensorial attributes of Chenin blanc and Pinotage finished wines could clearly be observed using Discriminant Analysis (DA) (Figs 4.7 and 4.8, respectively). It was observed that the varying SO_2 treatments did have an impact on the groupings observed. Higher acetaldehyde levels were observed to fall within the same quadrant on the DA plot (i.e. group together) as the higher SO_2 treatments.

The dynamics of acetaldehyde and SO_2 changed throughout fermentation and culminated in the finished wine, with red and white winemaking procedures impacting on these dynamics (Figs 4.6 – 4.8). Not only did the patterns of acetaldehyde levels differ during fermentation between red and white musts, but also the levels of acetaldehyde in the finished white wine (Chenin blanc) was more varied than in the case of red wine (Pinotage) (Table 4.4).

The levels of acetaldehyde in this study fell within reported levels for acetaldehyde in white (11 – 493, average 80 mg/L) and red (4 – 212, average 30 mg/L) wines (Liu & Pilone, 2000). All acetaldehyde levels in this study are the total acetaldehyde levels, and not the free acetaldehyde portion, since some challenges still exist in accurately

determining free acetaldehyde levels, which need to be investigated further (Coetzee, 2014).

Table 4.4 Acetaldehyde and SO_2 averages (n = 3) for bottled wines from the SO_2 cellar trial before sensorial evaluation of the wines

Finished wines	SO_2 Treatment (mg/L)	Acetaldehyde (mg/L)	Total SO_2 (mg/L)	Free SO_2 (mg/L)
Chenin blanc	**0**	21.128^{d} * (0.701) **	39.000^{d} * (0.000) **	15.000^{b} * (1.414) **
Chenin blanc	**50**	44.517^{c} (2.099)	63.333^{c} (2.082)	15.333^{ab} (1.528)
Chenin blanc	**100**	101.871^{a} (4.716)	85.000^{b} (2.000)	15.000^{b} (0.000)
Chenin blanc	**150**	68.196^{b} (1.288)	124.500^{a} (3.536)	18.000^{a} (1.414)
Pinotage	**0**	25.780^{a} (3.832)	45.667^{a} (6.110)	34.000^{a} (4.243)
Pinotage	**50**	24.761^{a} (3.036)	40.333^{a} (3.215)	30.667^{ab} (4.041)
Pinotage	**100**	28.783^{a} (9.451)	44.667^{a} (4.163)	24.667^{b} (5.131)
Pinotage	**150**	31.091^{a} (0.966)	41.667^{a} (2.081)	27.000^{ab} (0.000)

**Values in same column followed by a letter or group of letters (superscript), different from another, were significantly different from each other ($p \leq 0.05$, Student t-test); and, values in the same column followed by the same letter or group of letters (superscript), did not differ significantly ($p \leq 0.5$). **Standard deviation of the mean (n = 3).*

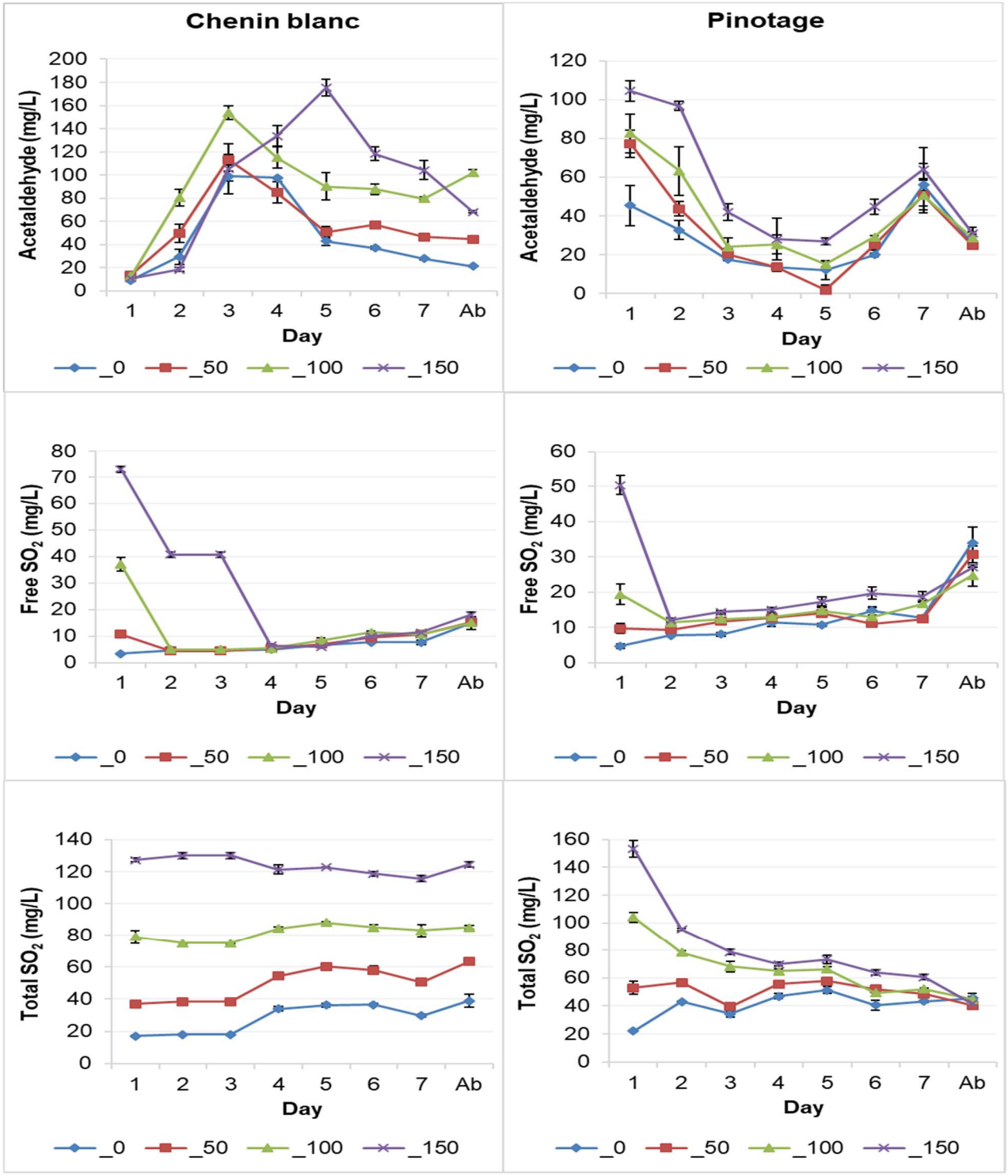


Figure 4.6 Impact of added SO_2 (0, 50, 100 and 150 mg/L) on acetaldehyde production by VIN13 during fermentation, and changes in the concentration of SO_2 during fermentation. Acetaldehyde, free and total SO_2 concentrations were measured during fermentation. SO_2 was added on day 0, and the first sample taken on day 1. The control (no SO_2 added) had a baseline SO_2 value (i.e. Chenin blanc: TSO_2 of 23 mg/L; Pinotage: TSO_2 of 21 mg/L) and was not 0 mg/L. Abbreviations: Ab, after bottling.

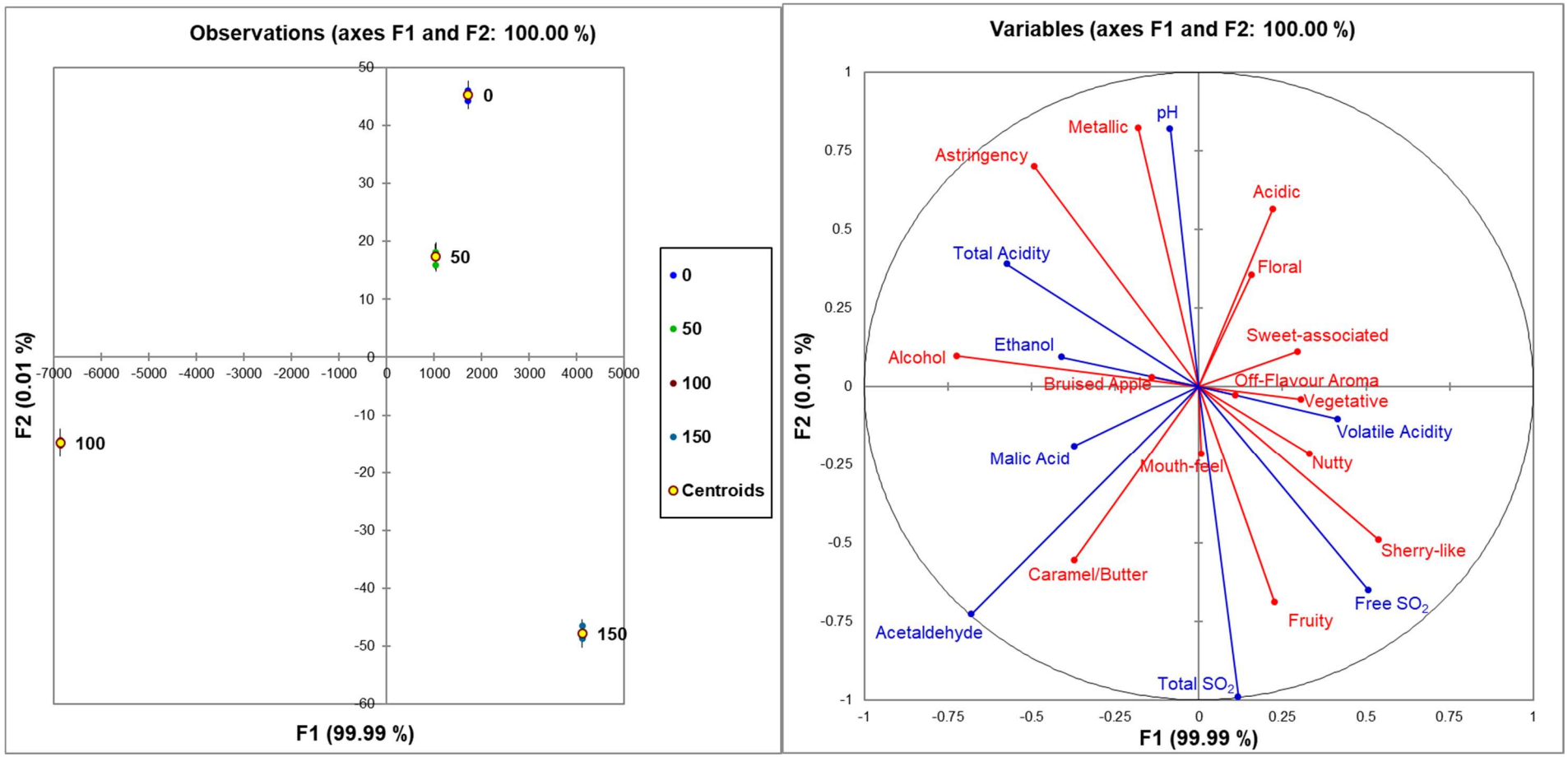


Figure 4.7 Discriminant analysis plot of Chenin blanc finished wines based on sensory and chemical variables for discrimination between classes, i.e. 0, 50, 100 and 150 mg/L SO_2 added before inoculation of grape must with VIN13. Chemical variables analysed for include the routine wine parameters pH, total acidity (TA), volatile acidity (VA), ethanol, malic acid, free and total SO_2, and acetaldehyde. Sensory descriptors include vegetative, sherry-like, acidic, metallic, astringency, nutty, caramel-butter, bruised apple, fruity, mouthfeel, off-flavour aroma, alcohol, sweet-associated aroma and floral.

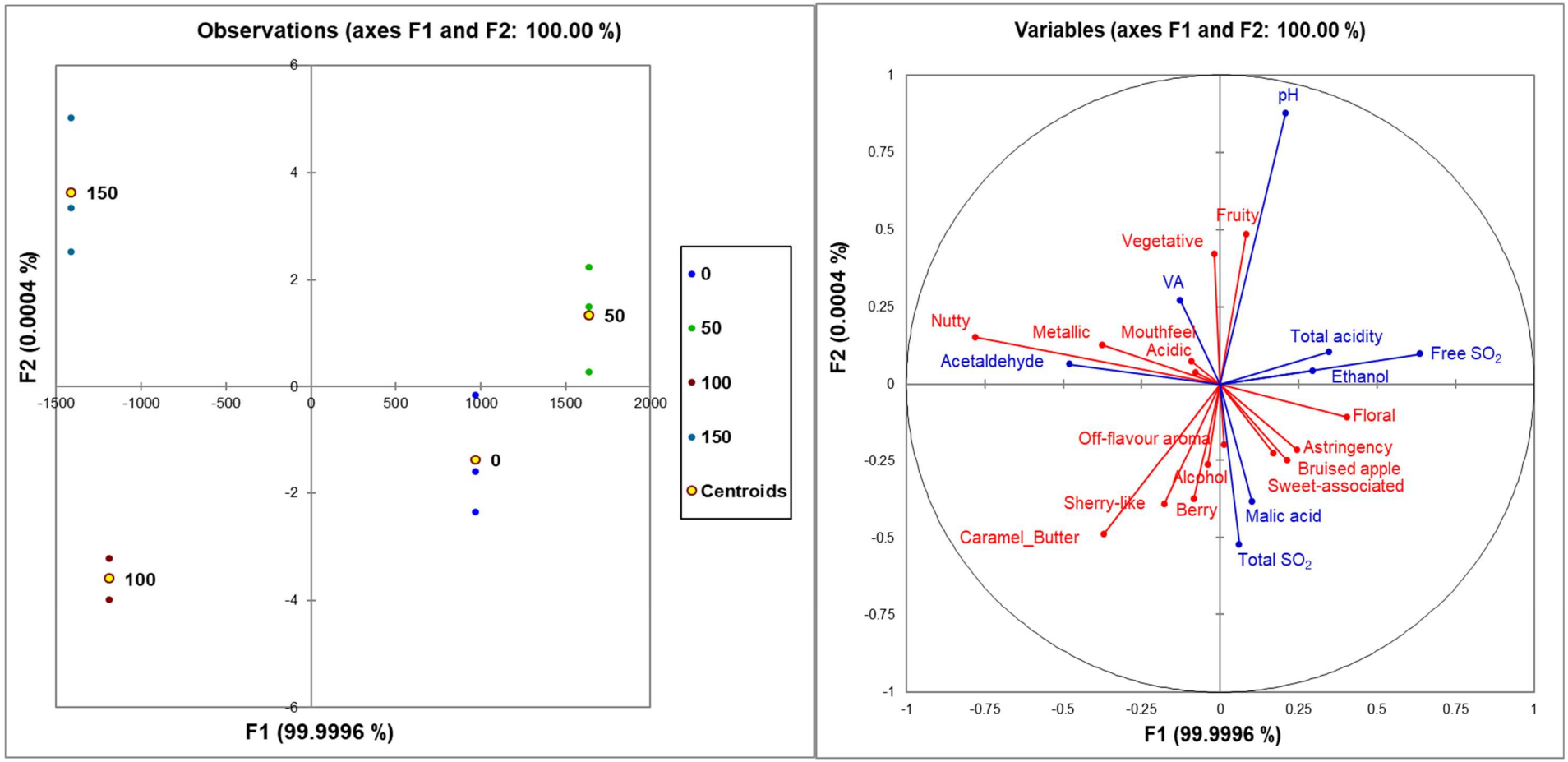
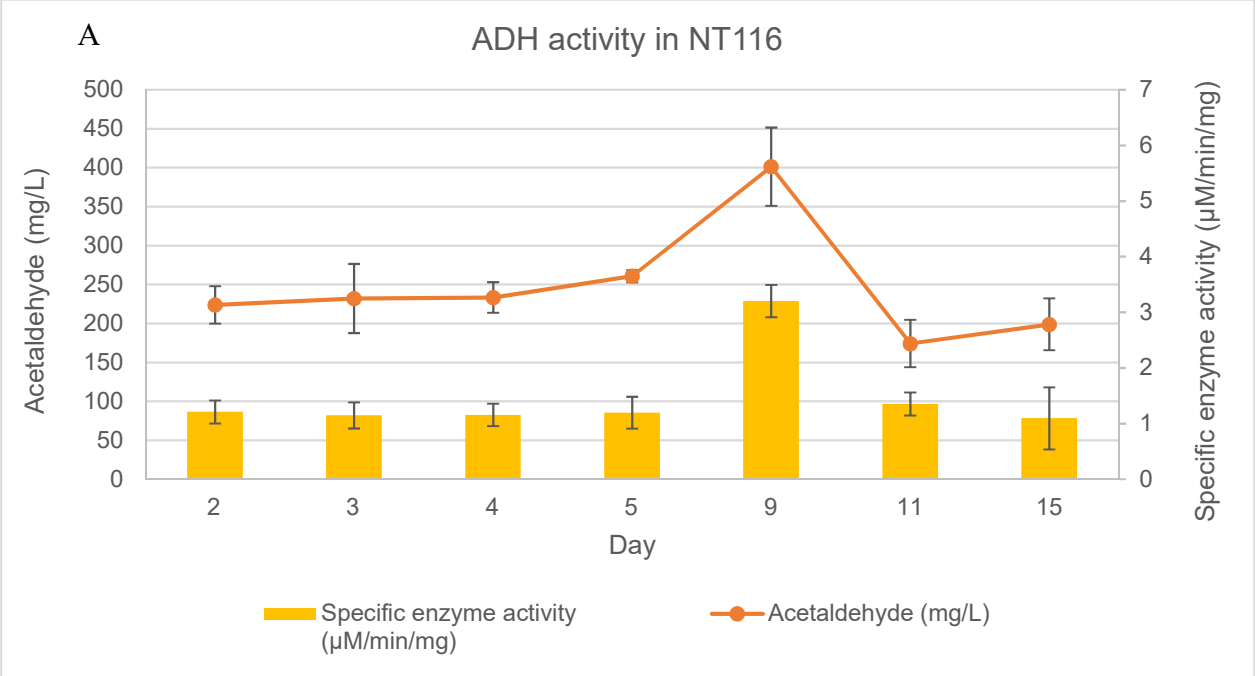


Figure 4.8 Discriminant analysis plot of Pinotage finished wines based on sensory and chemical variables for discrimination between classes, i.e. 0, 50, 100 and 150 mg/L SO_2 added before inoculation of grape must with VIN13. Chemical variables analysed for include the routine wine parameters pH, total acidity, VA, ethanol, malic acid, free and total SO_2, and acetaldehyde. Sensory descriptors include vegetative, sherry-like, acidic, metallic, astringency, nutty, caramel-butter, bruised apple, fruity, mouthfeel, off-flavour aroma, alcohol, sweet-associated aroma and floral. Most of the variation can be explained by Factor 1 (F1) which is mainly as a result of the total SO_2 concentration.

4.4 Acetaldehyde production and enzyme (ADH) activity

During a separate fermentation trial of Chenin blanc (laboratory-scale) evaluating yeast strains NT50, NT116 and VIN13, the specific enzyme activity of ADH showed a similar increasing/decreasing trend as that of acetaldehyde concentration. ADH activity and acetaldehyde concentration peaked at day 9 for NT116 and VIN13, while NT50 showed peaked ADH activity at day 11 (Fig. 4.9). Non-*Saccharomyces* yeasts were not included in this trial, since the idea was to initially monitor ADH activity in a more well-known yeast such as *Saccharomyces cerevisiae*.



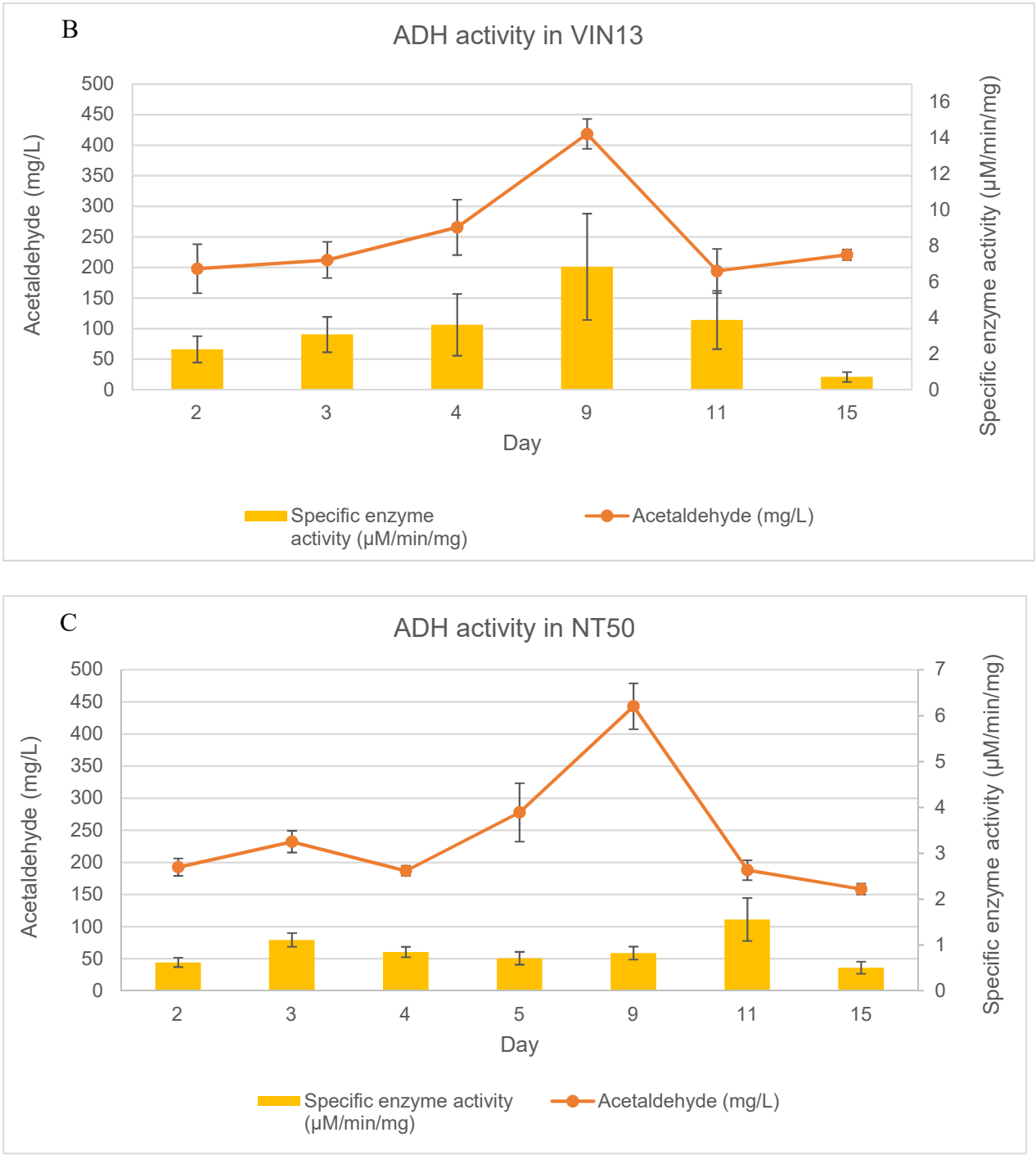


Figure 4.9 ADH activity and total acetaldehyde concentration during fermentation of Chenin blanc juice/must by (A) NT116, (B) VIN13 and (C) NT50. Each yeast treatment was done in triplicate (n = 3) and sampled at specific days during fermentation. Error bars represent the standard error of the mean (SEM).

It was observed that the *S. cerevisiae* strains, NT116 and VIN13, behaved similarly by both having peak acetaldehyde levels at day 9 (Fig. 4.9 A and B) of the fermentation trial, and this was on the same day that the total ADH enzymes had a peak in its specific activity too. This possibly was the point where most of the grape sugars (glucose/fructose)

had been depleted, and could be explained by induction of the ADH2 to start producing more acetaldehyde from the ethanol present in the fermentation. (Maestre *et al*., 2008; Pal *et al*., 2008).

However, the NT50 yeast strain did not follow the same pattern (Fig. 4.9 C) as that of its counterparts (NT116 and VIN13) for enzyme activity. The specific activity of ADH in the NT50 yeast was very low throughout the trial, especially at day 9, even though the acetaldehyde concentration peaked on day 9. This strange observation, especially since NT50 was the higher acetaldehyde producing strain in a prior trial (Section 4.2), could be as a result of there not being sufficient yeast cells present during the fermentation process, since the Bradford assay showed that the protein extract for this yeast yielded a lower concentration, as fermentation progressed, as compared to the other two.

However, the acetaldehyde levels during this trial were relatively higher than the previous trials (Sections 4.2 and 4.3). This could be as a result of an alternative method of quantification for acetaldehyde being attempted, since the Arena Enzyme Robot was rendered unavailable during this trial. For this trial the acetaldehyde quantification was done manually, using a slightly different enzymatic assay kit which was later found to be for total aldehyde quantification (Aldehyde Quantification Assay Kit (Colorimetric) #ab112113 by abcam®). It was assumed that the actual acetaldehyde levels would not be too far off, since acetaldehyde does make up 90% of the total aldehyde content in wine (Nykänen, 1986).

4.5 General discussion

The overall aim of this project was to investigate the effect of yeasts and oenological parameters on acetaldehyde production, and to better understand the impact of acetaldehyde on fermentation and wine sensorial composition. The specific objectives of this study were:

i. To screen yeast strains for acetaldehyde production;
ii. To select and evaluate high-, medium- and low-acetaldehyde producing yeast strains;
iii. To evaluate the effect of winemaking practices on acetaldehyde levels;
iv. To investigate the impact of acetaldehyde on sensory properties of wine, and;
v. To determine the total ADH activity in yeasts during acetaldehyde production.

Successful screening of various *S. cerevisiae* and non-*Saccharomyces* yeasts resulted in a statistically-based selection of high-, medium- & low acetaldehyde-producing yeast strains from each genus. The high-, medium- & low acetaldehyde-producing yeasts were combined in various permutations in a cellar trial. The impact of yeast strain and time of fermentation on levels of acetaldehyde is evident.

Yeast metabolism, specifically the production of acetaldehyde by different yeast strains, is also influenced by the fermentation temperature (screening/laboratory trial), but this needs to be investigated on a larger or cellar scale to obtain a more conclusive result.

In the cellar trial that monitored the effect of varying SO_2 levels on acetaldehyde levels in fermenting must, the varying levels of SO_2 had a direct effect on the acetaldehyde levels during fermentation and in the finished wine. Higher levels of SO_2 can trigger increased production of acetaldehyde by yeasts in fermenting musts. However, in the finished wines the acetaldehyde levels were below the flavour threshold. No significant organoleptic differences were found between the different SO_2 treatments.

Although there were differences in acetaldehyde levels between yeast strains used in this study, the levels were still within acceptable ranges or limits found in wines. Acetaldehyde levels in samples inoculated with were generally higher than in samples co-inoculated with non-*Saccharomyces*.

During the fermentation of Chenin blanc (laboratory-scale) by the *S. cerevisiae* yeasts, the increased/decreased acetaldehyde concentration is the result of up-/down-regulated ADH activity during alcoholic fermentation.

CHAPTER 5

CONCLUSION

This study investigated the effects of *Saccharomyces* and non-*Saccharomyces* yeasts, as well as oenological parameters, on acetaldehyde production and the impact they had on wine flavour. Yeast strain, fermentation temperature and SO_2 levels can affect acetaldehyde levels in wine. Selecting a commercial yeast strain for white or red wine production to reduce acetaldehyde levels, while enhancing flavour, is important. Co-inoculation of wine with a selected *S. cerevisiae* and non-*Saccharomyces* yeast, as well as maintaining low levels of SO_2 during fermentation, can result in wines with lower levels of acetaldehyde. For two of the *S. cerevisiae* strains studied, a positive trend was observed between total alcohol dehydrogenase activity and total acetaldehyde production. Generally, the acetaldehyde levels in our wines were below the sensory threshold values and within acceptable ranges for table wines. The above knowledge is valuable in optimising the alcoholic fermentation process and enhancing the safety and quality of wine. To ensure lower levels of acetaldehyde in wine, winemakers should preferably co-inoculate with low ADH activity *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast strains, at low fermentation temperatures, while ensuring low levels of SO_2 before fermentation. This research also provides information, which is both of fundamental and industrial importance, and confirms how complex wine production is. One of the limitations of this study was that other volatile compounds, in addition to acetaldehyde, were not analysed. Knowing the volatile chemical composition of the wines would have enabled us to study possible correlations with the sensory data.

RECOMMENDATIONS

This study showed that there is great variability among the yeast strains investigated. One of the limitations of this study was the number of *Saccharomyces* and non-*Saccharomyces* strains that were screened. Screening different non-*Saccharomyces* yeast species, and a larger number of *Saccharomyces* and non-*Saccharomyces* strains, could be useful in finding yeast strains that produce low acetaldehyde levels, while improving wine flavour. Future research should focus more on commercial (ADWY) *S. cerevisiae* and non-*Saccharomyces* yeast strains and their interactions.

This study did not investigate the interactions between *S. cerevisiae,* non-*Saccharomyces* yeasts and lactic acid bacteria (LAB) strains. Malolactic fermentation plays an important role in red wine production, and understanding how these interactions between different yeast and LAB strains affect acetaldehyde levels, could produce some interesting results.

This study mainly focused on acetaldehyde and future studies should include other volatile compounds to try and correlate chemical data to the sensory profiles that were obtained.

Only two wine grape cultivars/varieties were investigated in this study, but the results and trends observed might not be the same for other cultivars. Therefore, further research is needed to establish if the same trends, with regard to *Saccharomyces* and non-*Saccharomyces* yeast interactions, would be observed in different grape varieties.

Total alcohol dehydrogenase (ADH) activity showed a general correlation to total acetaldehyde production. Further work into gene expression (transcriptomics) is necessary to confirm which specific genes and/or isoenzymes are responsible for such correlations.

REFERENCES

Aguera, E., Sire, Y., Mouret, J.-R., Sablayrolles, J.-M. & Farines, V. (2018) Comprehensive study of the evolution of the gas-liquid partitioning of acetaldehyde during wine alcoholic fermentation. J. Agric. Food Chem. **66**, 6170-6178.

Aleixandre-Tudo, J.L., Lizama, V., Álvarez, I., Nieuwoudt, H., García, M.J., Aleixandre, J.L. & du Toit, W.J. (2016) Effect of acetaldehyde addition on the phenolic substances and volatile compounds of red Tempranillo wines. Austr. J. Grape & Wine Res. **22**, 205-214.

Amerine, M.A. & Ough C.S. (1964) Studies with controlled fermentation. viii. Factors affecting aldehyde accumulation. Am. J. Enol. Vitic. **15**, 23-33.

Andorrà, I., Martín, L., Nart, E., Puxeu, M., Hidalgo, C. & Ferrer-Gallego, R. (2018) Effect of grape juice composition and nutrient supplementation on the production of sulfur dioxide and carboxylic compounds by *Saccharomyces cerevisiae*. Austr. J. Grape & Wine Res. **24**, 260–266.

Aranda, A., Querol, A. & del Olmo, M. (2002) Correlation between acetaldehyde and ethanol resistance and expression of *HSP* genes in yeasts strains isolated during the biological aging of sherry wines. Arch. Microbiol. **177**, 304-312.

Aranda, A., & del Olmo, M. (2003) Response to acetaldehyde stress in the yeast *Saccharomyces cerevisiae* involves a strain-dependent regulation of several *ALD* genes and is mediated by the general stress response pathway. Yeast **20 (8)**, 747-759.

Barrajon, A., Capece, M., Arévalo-Villena, M., Briones, A. & Romano, P. (2011) Co-incolulation of different *Saccharomyces cerevisiae* strains and influence on volatile composition of wines. Food microbiology **28 (5)**, 1080 – 1086.

Beckner Whitener, M.E., Stanstrup, J., Carlin, S., Divol, B., du Toit, M. & Vrhovsek, U. (2017) Effect of non-*Saccharomyces* yeasts on the volatile chemical profile of Shiraz wine. Austr. J. Grape & Wine Res. **23**, 179-192.

Binati, R.L., Lemos Junior, W.J.F., Luzzini, G., Slaghenaufi, D., Ugliano, M. & Torriani, S. (2020) Contribution of non-*Saccharomyces* yeasts to wine volatile and sensory diversity: A study on *Lachancea thermotolerans*, *Metshchnikowia* spp. & *Starmerella bacillaris* strains isolated in Italy. Int. J. Food Microbiol. **318**, 1-12.

Blouin, J. (1966) Contribution to study of binding of sulphur dioxide in musts and wines. I. *Annales de Technologie Agricole*. **15**, 223-287.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248-254.

Casalone, E., Colella, C.M., Daly, S., Gallori, E., Moriani. L. & Polsinelli, M. (1992) Mechanism of resistance to sulphite in *Saccharomyces cerevisiae*. Current Genetics. **22**, 435 – 440.

Cheraiti, N., Guezenec, S. & Salmon, J.-M. (2010) Very early acetaldehyde production by industrial *Saccharomyces cerevisiae* strains: a new intrinsic character. Appl. Microbiol. Biotechnol. **86**, 693-700.

Ciani, M. & Comitini, F. (2011) Non-*Saccharomyces* wine yeasts have a promising role in biotechnological approaches to winemaking. Ann. Microbiol. **61**, 25-32.

Ciani, M. & Ferraro, L. (1998) Combined use of immobilized *Candida stellata* cells and *Saccharomyces cerevisiae* to improve the quality of wines. J. Appl. Microbiol. **85,** 247–254.

Ciani, M. & Maccarelli, F. (1998) Oenological properties of non-*Saccharomyces* yeasts associated with winemaking. World J. Microbiol. & Biotechnol. **14**, 199-203.

Coetzee, C., Brand, J., Jacobson, D. & du Toit, W.J. (2016)[a] Sensory effect of acetaldehyde on the perception of 3-mercaptohexan-1-ol and 3-isobutyl-2-methoxypyrazine. Austr. J. of Grape & Wine Res. **22**, 197–204.

Coetzee, C., Van Wyngaard, E., Šuklje, K., Ferreira, A.C.S. & du Toit, W.J. (2016)[b] Chemical and Sensory Study on the Evolution of Aromatic and Nonaromatic Compounds during the Progressive Oxidative Storage of a Sauvignon blanc Wine. J. Agricultural & Food Chem. **64**, 7979−7993.

Coetzee, C. (2014) Oxidation treatments affecting Sauvignon blanc wine sensory and chemical composition (Ph.D. dissertation). Department of Viticulture and Oenology, Faculty of AgriSciences, Stellenbosch University.

Coetzee, C., Buica, A. & du Toit, W. (2018) The Use of SO_2 to Bind Acetaldehyde in Wine: Sensory Implications. S. Afr. J. Enol. Vitic. **39 (2),** 157-162.

Cortes, M. B., Moreno, J., Zea, L., Moyano, L. & Medina, M. (1998) Changes in Aroma Compounds of Sherry Wines during Their Biological Aging Carried out by *Saccharomyces cerevisiae* Races *bayanus* and *capensis.* J. Agric. Food Chem. **46**, 2389-2394.

Di Stefano, R. & Ciolfi, G. (1982) *Trasformazione delle aldeidi e degli al-cooli C_6 ad opera dei lieviti nel corso del processo fermentativo.* Riv. Vitic. Enol. **35**, 431-435.

Dittrich, H.H. & Barth, A. (1984) The SO_2 – content, SO_2 – binding substances and malo-lactic fermentation in German wines. An investigation on 544 German wines. Vitic. Enol. Sci. **39,** 184 – 200.

Domizio, P., Romani, C., Lencioni, L., Comitini, F., Gobbi, M., Mannazzu, I. & Ciani, M. (2011) Outlining a future for non-*Saccharomyces* yeasts: Selection on putative spoilage wine strains to be used in association with *Saccharomyces cerevisiae* for grape juice fermentation. Int. J. Food Microbiol. **147,** 170-180.

Du Plessis, H.W., du Toit, M., Hoff, J.W., Hart, R.S., Ndimba, B.K. & Jolly, N.P. (2017)[a] Characterisation of non-*Saccharomyces* yeasts using different methodologies and evaluation of their compatibility with malolactic fermentation. S. Afr. J. Enol. Vitic. **38**, 46-63.

Du Plessis, H.W., du Toit, M., Nieuwoudt, H., van der Rijst, M., Kidd, M. & Jolly, N. (2017)[b] Effect of *Saccharomyces*, non-*Saccharomyces* yeasts and malolactic fermentation strategies on fermentation kinetics and flavor of Shiraz wines. Fermentation **3 (64)**, 1-24.

Elias, R.J., Laurie, V.F., Ebeler, S.E., Wong, J.W. & Waterhouse, A.L. (2008) Analysis of selected carbonyl oxidation products in wine by liquid chromatography with diode array detection. Analytica Chimica Acta **626,** 104-110.

Fleet, G.H. & Heard, G.M. (1993). Yeasts: growth during fermentation. In: Fleet, G.H. (ed). Wine microbiology and biotechnology. Harwood Academic Publishers, Chur, Switzerland, pp. 27-54.

Frivik, S.K. & Ebeler, S.E. (2003). Influence of sulfur dioxide on the formation of aldehydes in white wine. Am. J. Enol. Vitic. **54 (1)**, 31−38.

Giacosa, S., Rıo Segade, S., Cagnasso, E., Caudana, A., Rolle, L. & Gerbi, V. (2019) SO_2 in Wines: Rational Use and Possible Alternatives. Red Wine Technology, 309-321 (book - edited by Morata, A.).

Grey, E.C. (1913) The production of acetaldehyde during the anaerobic fermentation of glucose by *Bacillus coli communis (Escherich)*. Biochemical Lab. Lister Institute.

Han, G., Webb, M.R. & Waterhouse, A.L. (2019) Acetaldehyde reactions during wine bottle storage. Food Chem. **290**, 208-215.

Han, G., Webb, M.R., Richter, C., Parsons, J. & Waterhouse, A.L. (2017) Yeast alter micro-oxygenation of wine: Oxygen consumption and aldehyde production. J. Sci. Food Agric. **n/a**-n/a.

Hucker, B. & Vriesekoop, F. (2008) Acetaldehyde stimulates ethanol-stressed *Saccharomyces cerevisiae*, grown on various carbon sources. Folia Microbiol. **53 (6)**, 505-508.

Ibeas, J.I., Jimenez, J. (1997) Mitochondrial DNA loss caused by ethanol in *Saccharomyces* flor yeasts. Am. Soc. Microbiol. **63**, 7-12.

Iland, P., Ewart, A., Sitters, J., Markides, A. & Bruer, N. (2000) Techniques for chemical analysis and quality monitoring during winemaking. Patrick Iland Promotions, Campbelltown, Australia.

Jackowetz, J.N., Dierschke, S. & Mira de Orduña, R. (2011) Multifactorial analysis of acetaldehyde kinetics during alcoholic fermentation by *Saccharomyces cerevisiae*. Food Res. Int. **44**, 310-316.

Jolly, N.P., Augustyn, O.P.H. & Pretorius, I.S. (2006) The role and use of non-*Saccharomyces* yeasts in wine production. S. Afr. J. Enol. Vitic. **27 (1)**, 15-39.

Jolly, N.P., Varela, C. & Pretorius, I.S. (2014) Not your ordinary yeast: Non-*Saccharomyces* yeasts in wine production. FEMS Yeast Res. **14**, 215-237.

Jussier, D., Dubé Morneau, A. & Mira de Orduña, R. (2006) Effect of simultaneous inoculation with yeast and bacteria on fermentation kinetics and key wine parameters of cool-climate Chardonnay. Appl. Environ. Microbiol. **72 (1)**, 221-227.

Kim, D.-H., Hong, Y.-A. & Park, H.-D. (2008) Co-fermentation of grape must by *Issatchenkia orientalis* and *Saccharomyces cerevisiae* reduces the malic acid content in wine. Biotechnol. Lett. **30**, 1633-1638.

Lambrechts, M.G. & Pretorius, I.S. (2000) Yeast and its importance to wine aroma. S. Afr. J. Enol. Vitic. **21**, 97–129.

Li, E. & Mira de Orduña, R. (2017) Acetaldehyde kinetics of enological yeast during alcoholic fermentation in grape must. J. Ind. Microbiol. Biotechnol. **44**, 229-236.

Li, E. & Mira de Orduña, R. (2010) Evaluation of the acetaldehyde production and degradation potential of 26 enological *Saccharomyces* and non-*Saccharomyces* yeast strains in arresting cell model system. J. Ind. Microbiol. Biotechnol. (Springer Online)

Liu, S.-Q. & Pilone, G.J. (2000) An overview of formation and roles of acetaldehyde in winemaking with emphasis on microbiological implications. Int. J. Food Sci. Technol. **35,** 49-61.

Longo, E., Velázquez, J.B., Sieiro, C., Cansado, J., Calo, P. & Villa, T.G. (1992) Production of higher alcohols, ethyl acetate, acetaldehyde and other compounds by 14 *Saccharomyces cerevisiae* wine strains isolated from the same region (Salnés, N.W. Spain). World J. Microbiol. Biotechnol. **8**, 539-541.

Maestre, O., García-Martínez, T., Peinado, R.A. & Mauricio, J.C. (2008) Effects of ADH2 overexpression in *Saccharomyces bayanus* during alcoholic fermentation. Appl. Environ. Microbiol. **74**, 702 – 707.

McCloskey, L.P. & Mahaney, P. (1981) An enzymatic assay for acetaldehyde in grape juice and wine. Am. J. Enol. Vitic. **32 (2)**, 159-162.

Mello, T., Ceni, E., Surrenti, C. & Galli, A. (2008) Alcohol induced hepatic fibrosis: Role of acetaldehyde. Mol. Aspects Med. **29 (1-2),** 17-21.

Millán, C. & Ortega J.M. (1988) Production of Ethanol, Acetaldehyde, and Acetic Acid in Wine by Various Yeast Races: Role of Alcohol and Aldehyde Dehydrogenase. Am. J. Enol. Vitic. **39**, 107-112.

Miyake, T. & Shibamoto, T. (1993) Quantitative analysis of acetaldehyde in foods and beverages. J. Agric. Food Chem. **41 (11)**, 1968-1970.

Nykänen, L. (1986) Formation and occurrence of flavor compounds in wine and distilled alcoholic beverages. Am. J. Enol. Vitic. **37 (1)**, 85-96.

Næs, T., Brockhoff, P. & Tomic, O. (2010) Statistics for Sensory and Consumer Science. Wiley, New York, USA.

Osborne, J.P., Dubé Morneau, A. & Mira de Orduña, R. (2006) Degradation of free and sulfur-dioxide-bound acetaldehyde by malolactic lactic bacteria in white wine. J. Appl. Microbiol. **101**, 474-479.

Osborne, J.P., Mira de Orduña, R., Pilone, G.J. & Liu, S.-Q. (2000) Acetaldehyde metabolism by wine lactic acid bacteria. FEMS Microbiol. Lett. **191**, 51-55.

Ough, C.S. & Amerine, M.A. (1988) Methods for analysis of musts and wines. 2nd edition (book).

Pal, S., Park, D.H. & Plapp, B.V. (2008) Activity of yeast alcohol dehydrogenases on benzyl alcohols and benzaldehydes: Characterization of ADH1 from *Saccharomyces carlsbergensis* and transition state analysis. Chemico-Biological Interactions. **178 (1-3)**, 16-23.

Paraggio, M. & Fiore, C. (2004) Screening of *Saccharomyces cerevisiae* wine strains for the production of acetic acid. World J. Microbiol. Biotechnol. **20**, 743-747.

Peinado, R.A., Moreno, J.A., Muñoz, D., Medina, M. & Moreno, J. (2004) Gas chromatographic quantification of major volatile compounds and polyols in wine by direct injection. J. Agric. Food Chem. **52,** 6389-6393.

Quertemont, E., Tambour, S. & Tirelli, E. (2005) The role of acetaldehyde in the neurobehavioral effects of ethanol: A comprehensive review of animal studies. Prog. Neurobiol. **75 (4),** 247-274.

Rankine, B.C. & Pocock, K.F. (1969) Influence of yeast strain on binding of sulphur dioxide in wine, and on its formation during fermentation. J. Sci. Food Agric. **20 (2)**, 104-109.

Ribéreau-Gayon, P., Peynaud, E., Lafon, M. (1956a, 1956b) Investigations on the origin of secondary products of alcoholic fermentation. Am. J. Enol. Vitic. **7 (3**), 112-118.

Ribéreau-Gayon, P., Pontallier, P., Glories, Y. (1983) Some interpretations of colour changes in young red wines during their conservation. J. Sci. Food Agric. **34 (5)**, 505-516.

Romano, P., Suzzi, G., Turbanti, L. & Polsinelli, M. (1994) Acetaldehyde production in *Saccharomyces cerevisiae* wine yeasts. FEMS Microbiol. Lett. **118,** 213-218.

Romano, P., Suzzi, G., Domizio, P. & Fatichenti, F. (1997) Secondary products formation as a tool for discriminating non-*Saccharomyces* wine strains. Antonie van Leeuwenhoek **71,** 239-242.

Romano, P., Fiore, C., Paraggio, M., Caruso, M. & Capece, A. (2003) Function of yeast species and strains in wine flavour. Int. J. Food Microbiol. **86,** 169-180.

Roustan, J.L. & Sablayrolles, J.-M. (2002) Modification of the acetaldehyde concentration during alcoholic fermentation and effects on fermentation kinetics. J. Biosci. Bioeng. **93 (4)**, 367-375.

SAWIS (2018) South African Wine Industry & Information Systems (SAWIS), European Union Wine Labelling Guide (updated 9th Sept. 2017), *http://www.sawis.co.za/winelaw/download/EU_labelling_guide_July_2012.pdf*

Salaspuro, M. (2011) Acetaldehyde and gastric cancer. J. Digest. Disea. **12 (2)**, 51−59.

Sheridan, M.K. & Elias, R.J. (2016) Reaction of Acetaldehyde with Wine Flavonoids in the Presence of Sulfur Dioxide. J. Agric. Food Chem. **64**, 8615−8624.

Shin, K.-S. & Lee, J.-H. (2019) Acetaldehyde contents and quality characteristics of commercial alcoholic beverages. Food Sci. Biotechnol. **28 (4),** 1027-1036.

Stanley, G.A., Douglas, N.G., Every, E.J., Tzanatos, T. & Pamment, N.B. (1993) Inhibition and stimulation of yeast growth by acetaldehyde. Biotechnol. Lett. **15 (12),** 1199-1204.

Stanley, G. A., Hobley, T.J. & Pamment, N.B. (1997) Effect of acetaldehyde on *Saccharomyces cerevisiae* and *Zymomonas mobilis* subjected to environmental shocks. Biotechn. Bioeng. **53**, 71-78.

Stratford, M., Morgan, P. & Rose, A.H. (1987). Sulphur dioxide resistance in *Saccharomyces cerevisae* and *Saccaromycodes ludwigii*. J. Gen. Microbiol. **133**, 2173 – 2179.

Swiegers, J.H. & Pretorius, I.S. (2005) Yeast modulation of wine flavor. Advances in Appl. Microbiol. **57,** 131-175.

Teusink, B., Passarge, J., Reijenga, C.A., Esgalhado, E., van der Weijden, C.C., Schepper, M., Walsh, M.C., Bakker, B.M., van Dam, K., Westerhoff, H.V. & Snoep, J.L. (2000) Can yeast glycolysis be understood in terms of *in vitro* kinetics of the constituent enzymes? Testing biochemistry. Eur. J. Biochem. **267**, 5313-5329.

Van Jaarsveld, F. & October, F. (2015) Acetaldehyde in wine. Wineland, 1 June 2015.

Vriesekoop, F., Barber, A.R. & Pamment, N.B. (2007) Acetaldehyde mediates growth stimulation of ethanol-stressed *Saccharomyces cerevisiae*: evidence of a redox-driven mechanism. Biotechnol. Lett. **29,** 1099-1103.

Walker-Caprioglio, H.M. & Parks, L.W. (1987) Autoconditioning factor relieves ethanol-induced growth inhibition of *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **53**, 33–35.

Wang, S., Li, S., Zhao, H., Gu, P., Chen, Y., Zhang, B. & Zhu, B. (2018) Acetaldehyde released by *Lactobacillus plantarum* enhances accumulation of pyranoanthocyanins in wine during MLF. Food Res. Int. **108**, 254–263.

Wildenradt, H.L. & Singleton, V.L. (1974) The production of aldehydes as a result of oxidation of polyphenolic compounds and its relation to wine aging. Am. J. Enol. Vitic. **25 (2),** 119-126.

Yang, C., Li, Y., Wang, J., He, J., Hou, H. & Li, K. (2019) Fast and highly selective detection of acetaldehyde in liquor and spirits by forming aggregation-induced emission luminogen. Sensors & Actuators: B. Chemical **285**, 617–624.

Zea, L., Moyano, L., Ruiz, M.J. & Medina, M. (2010) Chromatography – Olfactorometry study of the aroma of fino sherry wines. Int. J. Anal. Chem. 1-5.

Zea, L., Serratosa, M.P., Mérida, J. & Moyano, L. (2015) Acetaldehyde as key compound for the authenticity of sherry wines: A study covering 5 decades. Compr. Rev. Food Sci. Food Saf. **14 (6),** 681 – 693.