Cape Peninsula University of Technology

# Fermentation rate, yeast protein and sensory profiles of wines from fungicide treated Chenin Blanc grapes

**by**

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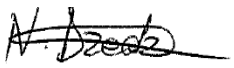
# DECLARATION

I, Ntombiyesicelo Dzedze, declare that the contents of this thesis represents my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.



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## ABSTRACT

Fungal diseases in vineyards are one of the main causes leading to economic losses within the viticultural sector and are continuously increasing over years. The most common of these fungal diseases are powdery mildew, downy mildew and grey mould. Commercial fungicides to treat the above-mentioned diseases are available and their usage is regulated under Act 36 of 1947 to comply with Good Agricultural Practises (GAP). However, the application of less-harmful, natural alternative fungicides to control vineyard diseases are currently an important research focus since the demand for organic products by consumers and retail companies are increasing. However, fungicide residues can alter the fermentation process and prevent some biochemical pathways of yeast metabolism involved in phenolic and/or aroma compound production that are critical for sensory quality. Therefore the aim of the study was to investigate the effect of fungicide treatments on the fermentation rate, yeast proteins expressed, aroma compounds released and sensory profile of wines produced.

In the study, Chenin Blanc grapes treated with chemical and natural fungicides (1x treatment and 2x treatment) under Good Agricultural Practises (GAP) were used to produce small-scale wines and laboratory-scale fermentations. Laboratory-scale fermentations were conducted in duplicate using the commercial *Saccharomyces cerevisiae (S. cerevisiae)* Active Dry Wine Yeast (ADWY) strains VIN 13 and VIN 7. The fermentations were monitored by frequently weighing until they stabilised ($CO_2$ weight loss).

Small-scale wines were produced using the commercial *S. cerevisiae* ADWY strain VIN 13 only. Wines were made according to the standard Nietvoorbij experimental winemaking procedure. At the end of fermentation, lees samples were plated onto Yeast Extract Peptone Dextrose (YPD) agar and colonies grown were subjected to CHEF gel electrophoresis to confirm that the *S. cerevisiae* yeast strain inoculated at the beginning of the fermentation completed it. Moreover, fermenting wine samples, collected at the start (lag phase) and at end of fermentation (stationary phase), were subjected to protein extraction, quantification and characterisation in order to investigate fermenting wine yeast proteins. Furthermore, the final wines were subjected to chemical analyses as well as measurement of aroma enhancing metabolites (esters, higher alcohols, fatty acids and thiol compounds) using GC – FID and MS. Additionally, duplicate samples of the wines were evaluated sensorially by a trained panel of 12 winemakers and researchers, using an unstructured line scale. Wines were compared to the control wine according to visual (colour), flavour (tree

fruit, tropical fruit, and wine foreign), taste (body mouthfeel, acidity) and overall quality. The data collected from the study was statistically analysed using a two-way analyses of variance (ANOVAs) and subject to a multiple factor analysis (MFA).

From the results obtained in the above study, it was concluded that yeast strains used for winemaking completed the fermentations at a similar rate to their respective controls. In addition, small-scale cellar fermentations showed that fungicide treatments (1x treatment and 2x treatment) compared to the controls had no notable negative effects on wine aroma and sensory profiles although differences were observed in the proteins expressed after the fermentation. Overall, the fungicide treatments did not negatively affect the yeast performance, yeast protein expressed, aroma compounds released and sensory profiles of the wines produced. Further studies are recommended on other white as well as red wine grape cultivars to fully assess the effects of the fungicides.

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And last, but not least, I acknowledge Almighty God for providing me with strength, guidance, faith and wisdom to fulfil my dreams and achieve my goals.

## DEDICATION

This thesis is dedicated to my parents for their faith and courage in me that everything is possible with God

I also dedicate this thesis to myself for being the first graduate from my whole family.

Ningekum aninakwenza nto
(Yohane 15:5)

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## GLOSSARY

| Terms/Acronyms/Abbreviations | Definition/Explanation |
|---|---|
| **ADWY** | Active Dried Wine Yeast |
| **YPD** | Yeast Peptone Dextrose |
| **EDTA** | Ethylene diamine tetra-acetic acid |
| **TBE** | Tris-borate-EDTA |
| **ANOVA** | Analysis of Variance |
| **PCA** | Principal Component Analysis |
| **MFA** | Multiple Factor Analysis |
| **Good Agricultural Practice (GAP)** | The registered (authorized) safe use of an agricultural remedy under actual conditions necessary for the effective and reliable pest/disease/weed/growth control. |
| **Maximum residue level (MRL)** | The maximum permitted concentration of a pesticide resulting from its use according to Good Agricultural Practice directly or indirectly for the production and protection of the commodity for which the limit is recommended. |
| **Defoliants** | Any chemical sprayed or dusted on plants to cause its leaves to fall off. |
| **Desiccants** | Chemicals that cause leaves to drop from plants. |
| **Vinification process** | The production of wine, starting with selection of the grapes and ending with bottling the finished wine. |
| **Chenin Blanc** | White grape cultivar most frequently planted in South Africa. |
| **CHEF** | Contour-clamped homogeneous electric field electrophoresis. |

# CHAPTER ONE: MOTIVATION AND DESIGN OF THE STUDY

## 1.1. Introduction

According to the U.S Federal Insecticides, Fungicides and Rodenticide Act of 1947, amended in 1988, a pesticide is defined as any chemical or mixture of chemicals proposed to prevent, destroy or alleviate any pest. Pesticides are also proposed as plant regulators, defoliants or desiccants and nitrogen stabilizers (Winter, 2000; Álvarez *et al.,* 2012). Also, according to South Africa legislation, Act 36 of 1947, amended in 2016, an agricultural remedy is defined as "any chemical substance or biological remedy, or any mixture or combination of any substance or remedy intended or offered to be used for the destruction, control, repelling, attraction or prevention of any undesired microbe, alga, nematode, fungus, insect, plant, vertebrate, invertebrate, or any product thereof, but excluding any chemical substance, biological remedy or other remedy in so far as it is controlled under the Medicines and Related Substances Control Act 1965 or the Hazardous Substances Act 1973 and also as plant growth regulator, defoliant, desiccant or legume inoculant" (DAFF Act No.36 of 1947).

Pesticides are classified according to various classes, depending on the type of pest to be controlled, thus herbicides are pesticides that control weeds, while insecticides control insects and fungicides control plant diseases (moulds) (Winter, 2000; Bostanian, 2004; Álvarez *et al.,* 2012; Zhang *et al*., 2015). In addition to these major classes of pesticides, there are many other classifications which include nematicides (for nematode control), acaracides (mite control), rodenticides (rodent control), molluscicides (snail and slug control), algacides (algal control), bacteriocides (bacterial control) and defoliants (leaf control) (Winter, 2000; Bostanian, 2004; Álvarez *et al.,* 2012; Zhang *et al*., 2015).

### 1.1.1. Fungicides

Fungicides are defined as a type of pesticide prepared from chemicals or biological agents with specific active ingredients to destroy or inhibit specific organisms on crops (Tadeo *et al*., 2004; Caboni & Cabras, 2010; Nollet *et al*., 2012; Paramasiyam, 2015). In the agricultural sector, these fungicides protect crops such as cereals, fruits and vegetables from fungal diseases. In vineyards, fungicides and pesticides play a major role in inhibiting the most common foliar diseases (i.e. grey mould, downy and powdery mildew) and insects (i.e. grape moths and citrus mealybugs) that negatively affect the

vineyard (Caboni & Cabras, 2010). In addition, the fungal diseases and insects mentioned above are the major known causes of economic losses in the viticultural sector. Fungicides used in vineyards include different chemical compounds such as acylalanine, anilinopyrimidine, azole, benzimidazole, dithio- and bis-dithio-carbamates, cyanopyrrole and more (Tadeo *et al*., 2004). However, when fungicides are applied in different agricultural sectors the residues can remain on the fruit. In the viticulture sector the possibility exists that they can be transferred to the must and wine during the vinification process (Tadeo *et al*., 2004; Caboni & Cabras, 2010; Nollet *et al*., 2012; Paramasiyam, 2015).

Consequently, the levels of fungicide residues found on grapes at harvesting depends on several factors, such as concentration of the fungicides used, the time-frame between the period of spraying to the time of harvesting, climatic conditions during that period, the vine growing region and the viticultural practices applied (i.e. grapes can be grown traditionally or organically) (Čuš *et al*., 2010; Ortiz *et al.*, 2010). Fungicide residues on grapes, must and wines differ with must having higher levels of fungicides than wine. This is due to the fact that some fungicides are water-soluble, for example benzimidazoles, and in such occurrences bentonite is used as a clarifying agent to reduce the residue level (Čuš *et al*., 2010; Ortiz *et al*., 2010). Moreover, during the vinification process, the fungicide residue decreases when the solids are separated from the liquid phase by adsorption. In addition, as the vinification process continues, other processes, such as the wine racking step also play a role in reducing the levels of fungicide residues. Moreover, later processes, such as filtration before bottling, also decreases the levels of residues although the effect is minimal (Álvarez *et al.,* 2012; Čuš *et al*., 2010; Ortiz *et al*., 2010; He *et al*., 2016).

Previous studies showed that fungicide residues can alter the fermentation process and prevent some biochemical pathways of yeast metabolism (Ortiz *et al*., 2010; González-Rodríguez *et al*., 2011; Noguerol-Pato *et al*., 2014). In addition, fungicide residues can also cause stuck and sluggish alcoholic fermentations and negatively affect malolactic fermentations. Yeast viability may gradually start diminishing and the fermentation process may completely stop in extreme cases and can also change phenolic and/or aroma compounds that are critical for sensory quality (Ortiz *et al*., 2010; González-Rodríguez *et al*., 2011). Therefore, it is necessary to investigate the effect they may have on the fermentation process. Inorganic fungicides, such as sulphur, has been used in several studies and showed that it does not have any negative effect on yeast. However, when used in high concentrations it may lead

to the development of off-flavours in wine (Halleen & Holz, 2001; Winter, 2005; Comitini & Ciani, 2008; Ortiz *et al*., 2010). The second type of inorganic fungicides namely copper-based fungicides had a negative impact by inhibiting the growth of *Saccharomyces cerevisiae* when used at concentrations of 10 $mg.kg^{-1}$ or more. Other studies using organic compounds obtained from Sulphoromides (dichlofunid) or Phthalimades (e.g. Folpet and Captofol) found it to be harmful to yeast strains such as *Hanseniaspora uvarum, S. bayanus* and *S. cerevisiae* (Ortiz *et al*., 2010; Dagostin *et al*., 2011). Comparing the aforementioned with other organic compounds, namely benzimidazole (carbendazim, benomyl and thiophanate methyl) found that they did not affect the yeast negatively when used in acceptable concentrations (Ortiz *et al*., 2010; Paramasiyam, 2015).

Several approaches have been followed as a solution to finding natural alternatives to the aforementioned fungicides (Romanazzi *et al*., 2012). The approaches (2006 – 2010) are grouped as follows: use of bio-control agents, natural antimicrobials (*Muscodor albus* and *Hanseniaspora uvarum*), generally regarded as safe (GRAS) decontaminating agents and combined treatments. In addition, these bio fungicides have been tested on citrus fruits, table grapes and wine grapes in Italy. In addition, plant essential oils have also been used by organic farmers on table grapes and vegetables. In SA, the biological control product being used so far is YieldPlus (*Cryptococcus albidus*) (Ippolito & Nigro, 2000; Mercier & Ben-Yehoshua, 2005; Romanazzi *et al*., 2012).

### 1.2. Statement of the research problem

Currently grape producers are using fungicide treatments to control fungi and various plant diseases during the growth of the vines. The use of these fungicides for the treatment of grapes are crucial, as the presence of fungi and plant disease can affect the grape harvest severely and result in economic losses. Furthermore, natural alternatives to control vineyard diseases are currently an important research focus since the demand for organic food by consumers and retail companies are increasing. Ozcan *et al.* (2016) stated that the food industry is focusing on the leading consumer trend which is a demand for healthy foods, especially foods that boost the immune system and that will further improve health. Natural alternatives include Kraalbos (*Galenia africana*), biocontrol agents (*Muscodor albus* and *Hanseniaspora uvarum)*, natural antimicrobials (salts and chitosan) and plant extracts (jojoba oil, rosemary oil, thyme oil, clarified hydrophobic extract of neem and cottonseed oil with garlic extract)

as fungicides. However, the effect on the fermentation rate of the yeast, yeast protein expression and sensory profiles of wines from fungicide-treated Chenin Blanc grapes have not been studied well, particularly in SA. Hence, this study will aim to monitor fermentation rate using laboratory-scale fermentations to investigate whether alternative fungicides affect the yeast *(S. cerevisiae*) performance. This will involve production of small-scale wines and monitoring of various indices of wine quality, including metabolites produced, proteins expressed during the fermentation process and their effect on the overall quality of the wine as well as sensorial acceptability.

### 1.3. Broad objective

The broad objective of the study was to monitor the fermentation rate of must from Chenin Blanc grapes subjected to different chemical and natural fungicide treatments, as well as inoculated with different yeast strains with a view to identify an effective alternative fungicidal treatment without negative effects on the vinification process and wine quality.

#### 1.3.1. Specific objectives

- The first specific objective was to monitor the *S. cerevisiae* (VIN 13 and VIN 7) activity during laboratory-scale fermentations measuring $CO_2$ weight loss, comparing the effect of fungicide treatments (control, chemical fungicide, and natural alternative fungicide, both at single and double dosages).
- The second specific objective was to compare the effect of fungicide treatments on standard chemical parameters of the wine (residual sugar, ethanol, volatile acidity, total sulphur and pH) before, during and after small-scale wine production.
- The third objective was to use the CHEF gel electrophoresis technique to confirm that the yeast *S. cerevisiae* (VIN 13) inoculated for small-scale winemaking at the start of the fermentation completed the fermentation process for all treatments.
- The fourth specific objective was to compare the effect of fungicide treatments on proteins released in small-scale wines produced by *S. cerevisiae* (VIN 13), during and after alcoholic fermentation.
- The fifth specific objective was to compare the effect of fungicide treatments on sensory profiles of small-scale wines.

- The sixth specific objective was to compare the effect of fungicide treatments on volatile metabolites released during the fermentation process of small-scale wines using GC – MS.
- The seventh specific objective was to collect, collate and analyse all the data sets statistically with a view to establish which alternative fungicide treatment is effective without negatively affecting the sensory and overall quality of the wine.

### 1.4. Hypothesis

It is hypothesized that the fermentation performance of the yeast strain *S. cerevisiae* (VIN 13) will not be negatively affected by the fungicide treatment. It is also hypothesised that the inoculated *S. cerevisiae* yeast strain will conduct the fermentation process. Hence, its presence will be verified using the CHEF DNA karyotyping technique in terms of the banding pattern of the inoculated yeast compared to the yeast isolated at the end of the fermentation. Moreover, it is hypothesized that the sensory profiles will not be negatively affected by either the chemical or natural fungicide in relation to volatile metabolites produced by *S. cerevisiae* (VIN 13) during the vinification process. Additionally, it is hypothesized that neither the chemical nor the natural fungicides will affect wine yeast protein expression negatively.

### 1.5. Delineations

To obtain reproducible results and to minimise experimental variation, the experiments were conducted over one vintage using Chenin Blanc grape must. The laboratory-scale fermentations were performed under standard laboratory conditions which may not reflect the actual conditions in the cellar, therefore small-scale wines were produced to address this deficiency.

### 1.6. Importance of the study

The study will determine the most effective alternative and/or natural fungicidal treatment that will not adversely affect the vinification process and the overall quality of the resultant wines produced.

### 1.7. Expected outcomes, results and contribution of the research

It is expected that fungicide treatments will not have a negative effect on the fermentation rate, wine yeast protein and metabolites produced across treatments

(control, 1x and 2x). It is also expected that the protein expressed and metabolites will correlate with the sensory profiles of the resultant wines. Moreover, it is expected that this investigation will lead to completing of a Master's degree in Food Science and Technology and will lead to the publication of a research article thus contributing to the research output of the Cape Peninsula University of Technology and the Agricultural Research Council. Additionally, it is expected that after the completion of the study, the results obtained will assist the wine industry with selecting alternative and/or natural fungicides that do not affect the vinification process and the overall quality of the wine negatively.

### 1.8. Thesis Overview

The research work presented in this thesis was conducted in the microbiology laboratory, of the Post-Harvest and Agro-processing Technologies Division, at the Agricultural Research Council; ARC Infruitec-Nietvoorbij (Fruit, Wine and Vine Institute), Western Cape, South Africa. The thesis is composed of 5 (five) chapters as highlighted below:

**Chapter 1:** Introduction: General introduction and background to the research project, objectives and the significance of the research.

**Chapter 2:** Literature review.

**Chapter 3:** Winemaking and sensory analysis**.**

**Chapter 4:** Proteins expressed and metabolites released during fermentation.

**Chapter 5:** General summary discussion and recommendations for future research.

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# CHAPTER 2: LITERATURE REVIEW

## 2.1. General wine grape cultivars in SA

The total acreage used for wine grape planting in South Africa was approximately 101 000 hectares in 2010, including both white and red cultivars. When compared to less developed, but rapidly growing countries such as Chile, Argentina, China and Portugal, the total acreage used in SA for wine grape production is less (Cusmano *et al*., 2010; Anon, 2012b; Anon, 2015). In addition, from 2001 the planting increased by 7% per annum but eventually dropped since new plantings could not keep up with the required replacement rate. Moreover, between the aforementioned cultivars, Chenin Blanc is the most planted compared to other white cultivars and Cabernet Sauvignon is the most common red cultivar (Figure 2.1). Additionally, the different white and red South African grape varieties are shown in Table 2.1.

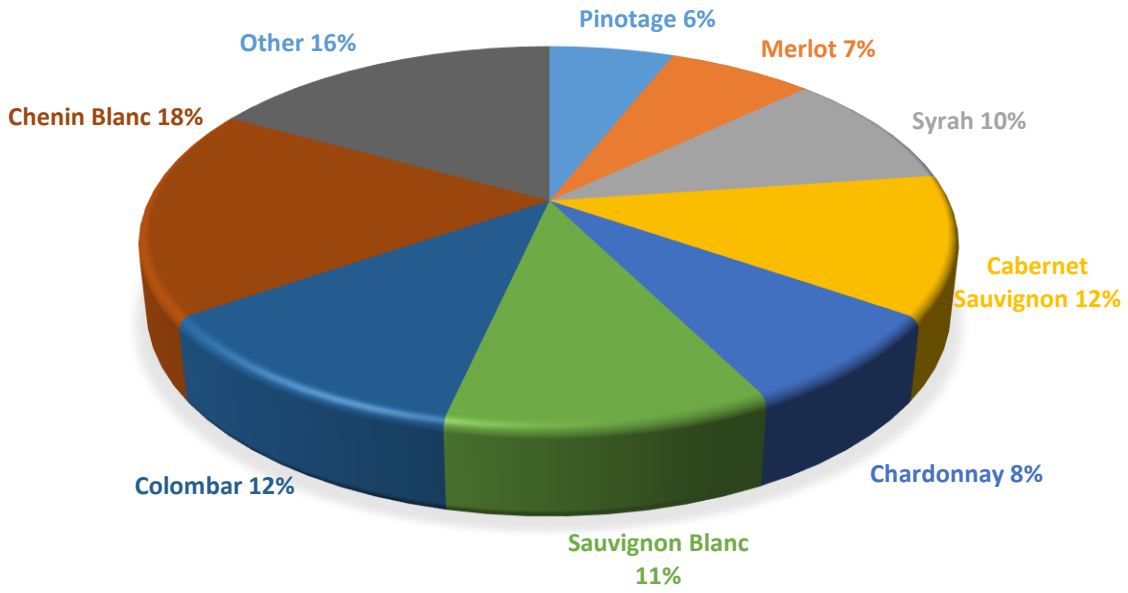


**Figure 2.1.** Wine grape cultivars commonly planted in SA (Anon, 2012b).

## 2.2. Chenin Blanc

Chenin Blanc is the most commonly or widely planted white grape cultivar in SA. It was first introduced by Jan Van Riebeek in 1655 along with other cultivars such as Semillon and Palomino. Chenin Blanc is used to produce many styles of wines including dry wines, sparkling wines and dessert wines (Warbrick-Smith & Edward, 2001; Loubser, 2008; Anon, 2012b; Hanekom, 2012; Aleixandre-Tudo *et al*., 2015). Initially, Chenin

Blanc was mainly used to produce grape juice and concentrate, brandy, spirits and inexpensive wine for drinking and distillation (Warbrick-Smith & Edward, 2001; Anon, 2012b; Hanekom, 2012). In the 1990's, wine producers started to discover the treasure of old bush vine Chenin Blanc's and was used to make high quality cultivar wines (Warbrick-Smith & Edward, 2001; Anon, 2012b; Aleixandre-Tudo *et al*., 2015). Moreover, this helped Chenin Blanc to be recognised in international markets. As SA's most widely planted cultivar with 46,500 acres, it is also the most planted cultivar in the world (Warbrick-Smith & Edward, 2001; Loubser, 2008; Anon, 2012b; Hanekom, 2012; Aleixandre-Tudo *et al*., 2015). Cultivar characteristics of Chenin Blanc grapes is the neutral taste found in the resultant wines, however, wines may also display fruity-estery aromas and guava-like aromas derived from volatiles formed during the fermentation process, especially in young Chenin Blanc wines (Augustyn & Rapp, 1982; Jolly *et al*., 2003; Anon, 2012b; Bester, 2011; Van Breda *et al*., 2018).

## 2.3. Fungal diseases affecting grapevines

Wine quality strongly depends on the quality of grapes used. The greater the quality of grapes, the higher the quality of wine produced (Caboni & Cabras, 2010). In order to produce quality wine, healthy grapes are harvested at a matured stage of ripeness. In addition, the farmers need to prevent plant diseases and pests that negatively affect the crops, e.g. downy and powdery mildew, grey mould, black rot and vine trunk diseases as well as dangerous insects such as grape moth and vine mealy-bugs (Saladin *et al*., 2003; Petit *et al*., 2008; González-Rodríguez *et al*., 2009 Caboni & Cabras, 2010; Gianessi & Williams, 2012; Noguerol-Pato *et al*., 2015).

### *2.3.1. Powdery mildew*

Powdery mildew is a disease of vineyards caused by the fungus *Uncinula nector* (Halleen & Holz, 2001; Ali *et al*., 2010). The outbreak of the disease was first seen in England in 1845 and was assumed to have come from North America (Caboni & Cabras, 2010). The disease spread to other countries including France in 1847, Belgium in 1848 and Italy in 1849 (Caboni & Cabras, 2010). The disease continued to affect vines in different countries for years. Jacob Cloete, who was the fourth son of Hendrick Cloete (the owner of Groot Constantia after Olof Berg's death), from Constantia in Cape Town first reported the disease in SA in 1880 (Halleen & Holz, 2001; Caboni & Cabras, 2010; Anon, 2012a).

**Table 2.1.** Wine grape cultivars in South Africa (Anon, 2012c; Anon, 2012d).

| White grape | Red grape |
|---|---|
| Chenin Blanc (Steen) | Cabernet Sauvignon |
| Cape Riesling (Crouchen Blanc) | Cabernet Franc |
| Chardonnay | Barbera |
| Chenel | Carignan |
| Bukettraube | Cinsaut (noir) |
| Clairette Blanche | Gamay noir ) |
| Colombar(d) | Grenache (noir) |
| Grenache (Blanc) | Malbec |
| Gewürztraminer | Merlot |
| Emerald Riesling | Muscadel |
| Weisser Riesling (Rhine Riesling) | Mourvèdre |
| Viognier | Pinot noir |
| Ugni Blanc (Trebbiano) | Pinotage |
| Sémillon (Green Grape) | Roobernet |
| Sauvignon Blanc | Ruby Cabernet |
| Roussanne | Sangiovese |
| Riesling (Rhine or Weisser Riesling) | Shiraz |
| Pinot gris (Grigio) | Souzão |
| Muscat d'Alexandrie (Hanepoot) | Tinta Barocca |
| Marsanne | Touriga Nacional |
| Muscadel | Zinfandel |
| Nouvelle | Nebbiolo |
| French Grape | Petit Verdot |
| Palomino (White) | Petit Sirah (Durif) |

Powdery mildew affects leaves, shoots and branches. The formation of an ash-grey white appearance on both upper and lower surfaces of the leaves indicate infection which results in crop loss (Halleen & Holz, 2001; Ellis & Nita, 2004; Caboni & Cabras, 2010). In addition, the disease negatively affects the grape yield, juice, wine quality, titratable acidity (TA), total phenolics, hydroxycinnamates and flavonoids. However, no off-flavours were detected in resultant wines. Darriet *et al*. (2012) reported volatile aroma compounds similar to mushroom and geranium-leaf in Cabernet Sauvignon and Sauvignon Blanc grapes affected by powdery mildew, but as previously reported these off-flavours were not detected in the resultant wines (Calonnec *et al.,* 2004; Stummer *et al.,* 2005; Barata *et al*., 2012; Gianessi & Williams, 2012).

*2.3.2. Downy mildew*

Downy mildew is the most common grape vine disease, caused by *Plasmopara viticola,* a fungus-like organism that affects all green tissues of the vines. The disease was first reported in France in 1878 and moved to Italy and other countries (Australia in 1919 and New Zealand in 1926) (Ellis & Nita, 2004; Ali *et al*., 2010; Caboni & Cabras, 2010; Francis & Keinath, 2010; Anon, 2012b). The disease spreads largely through seasonal rainfall which acts as a vector. The optimal conditions for primary infection are related to high humidity and low temperatures associated with unseasonal rainfall (Caboni & Cabras, 2010; Francis & Keinath, 2010; Anon, 2012b). Downy mildew affects leaves, shoots and berries resulting in defoliation of the vine and ultimate loss of the entire crop. The disease symptoms are shown by yellowish oilspots on top of the leaf seen within 12 days after infection (Ellis & Nita, 2004; Caboni & Cabras, 2010; Anon, 2012b). Infected berries changes to light brown and become soft, break easily and becomes covered by downy-like growth fungus in humid conditions. The infection normally starts during the early bloom until 4 weeks after the bloom. During this stage of infection, the fruit stems are the most susceptible to infection, and once infected it results in berries that do not mature normally (Madden *et al*., 2000; Ellis & Nita, 2004; Caboni & Cabras, 2010; Scott *et al*., 2010; Anon, 2012a; La Torre *et al*., 2014).

*2.3.3. Grey mould*

Grey mould also known as botrytis bunch rot in horticulture is a fungal disease caused by *Botrytis cinerea* that most commonly affect wine grapes (Couderchet, 2003; Cinquanta *et al*., 2015). The disease mostly infects the vineyards as conidia (short-lived propagules in the field) carried by air currents (Gabler *et al*., 2003; Holz *et al*., 2003; Van Schoor, 2004; Brink *et al*., 2006; Scott *et al*., 2010). The fungus firstly affects the leaves that show symptoms of infection at the end of the spring season by the appearance of irregular brown patches. Thereafter, the infection invades the grape berries where it causes bunch rot that covers the berries in a thick filamentous fungal layer (Coertze *et al*., 2001; Gabler *et al*., 2003; Šrobárová, & Kakalíková, 2007; Scott *et al*., 2010; Zhou *et al*., 2014). Moist weather with little windy conditions along with temperatures ranging from 15 – 25ºC favours filamentous fungal growth. Nevertheless, *B. cinerea* is also active at lower temperatures ranging from 0 – 10ºC, which highlights the versatility of the fungus to proliferate at various temperatures (Coertze *et al*., 2001; Gabler *et al*., 2003; Holz *et al*., 2003; Šrobárová & Kakalíková, 2007; Barata *et al*., 2012).

*2.3.4. Black rot*

Black rot is a fungal disease caused by *Guignardia bidwellii*, which affects the grapevine during spring, but mostly throughout the first month of vegetative growth (Harms *et al*., 2005; Molitor & Berkelmann-Loehnertz, 2011). The parts of the plants that are normally affected are immature leaves, clusters in bloom and the green berries. The infection is indicated by whitish dots that become surrounded by a reddish-brown ring and then the berries which become blue-black mummies (Šrobárová & Kakalíková, 2007). In addition, black rot is also one of the diseases that has an enormous negative impact on berry yield and wine quality (Molitor & Berkelmann-Loehnertz, 2011). However, it can be treated or prevented by using commercial chemical fungicides under GAP. A study by Molitor & Berkelmann-Loehnertz. (2011) was conducted by inoculating the grapes with the disease on a weekly interval until after bloom. Once the grapes showed disease severity, three fungicides i.e. dithianon, folpet and metrafenone that were not able to control black rot diseases in greenhouse trials at Geisenheim Research Center were applied. The results showed that the fungicide was able to control the black rot disease as well as other grape pathogens such as *Erysiphe necator* and *Plasmopara viticola* without the addition of other fungicides. Additionally, the diseases can be managed through proper agricultural practices such as cultural control by removing infected material from the trellis, canopy management and scouting (Ellis & Nita, 2004).

*2.3.5. Vine trunk disease*

Vine trunk disease is caused by various fungal pathogens originating from the fungal family, namely *Botryosphaerraceae* (Andolfi *et al*., 2011; Bertsch *et al*., 2013; Agustí-Brisach *et al*., 2015; Fontaine *et al*., 2016; Grozić, 2017). The most common being Eutypa dieback, Esca disease and Botryosphaeria dieback that firstly grows in mature wood. *Eutypa dieback* is caused by the fungus *Eutypa lata*, esca caused by *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* and botryosphoena caused by *Diplodia seriata, Diplodia mutila* and *Neofusicoccum parvum* (Andolfi *et al*., 2011; Bertsch *et al*., 2013; Agustí-Brisach *et al.,* 2015; Fontaine *et al*., 2016; Grozić, 2017). These can infect the vine in two forms: 1) through pruning, that causes wounds and that will lead to loss of production especially towards the maturation stage. 2) Through the material used for breeding, as a result, the new vine planted will be infected (Andolfi *et al*., 2011; Bertsch *et al*., 2013; Agustí-Brisach *et al*., 2015). Aforementioned diseases show similar symptoms in grapevines. These include:

wedge-shaped canker when cutting in the cross-section, external cankers, damage to the vascular system, dead arm, loss of spur position on the cordon, stunted shoots and bunch rot (Rolshausen *et al*., 2010; Bertsch *et al*., 2013; Grozić, 2017).

Furthermore, several parameters are available to prevent and/or treat these diseases. Once the vineyard is infected, the best intervention is to remove the infected and dead part of the vine and re-draft it. In addition, the use of biological and chemical protectors on the wounds caused by pruning is advised (Rolshausen *et al*., 2010; Amponsah *et al*., 2012; Bertsch *et al*., 2013; Grozić, 2017). These chemical products include fungicides such as tebuconazole, flusilazole, benomyl, prochloraz, prothioconazole and tebuconazole, fluazinam tyophanate methyl, mancozeb, fenarimol and procymidone. Moreover, in cases where the propagation material is infected a hot water treatment is recommended (Rolshausen *et al*., 2010; Amponsah *et al.,* 2012; Bertsch *et al*., 2013; Fontaine *et al*., 2016; Grozić, 2017).

### 2.4. Treatments for diseases

Vineyard diseases are treated using various forms of fungicides. They are categorised into two major groups, namely: chemical and biological-based fungicides. Chemical-based fungicides comprise organic and inorganic compounds and are classified according to their structure, topical activity and mode of action (Francis & Keinath, 2010; Paramasivam, 2015). Organic compounds found in chemical-based fungicides include acylalanine, anilinopyrimidine, azole, benzimidazole, dithio- and bisdithio-carbamates, cyanopyrrole and more, whilst inorganic compounds include sulphur and copper-based compounds (Francis & Keinath, 2010; Ortiz *et al*., 2010; Paramasivam, 2015). Biological-based fungicides include 1) natural antimicrobials i.e. *Muscodor albus* and *Hanseniaspora uvarum*, salts, chitosan, plant extracts and calcium chloride ($CaCl_2$), sodium carbonate/sodium bicarbonate ($NaHCO_3$), 2) generally regarded as safe (GRAS) type decontaminating agents i.e. acetic acid, electrolysed oxidising water and ethanol, 3) plant essential oils i.e. jojoba oil, rosemary oil, thyme oil, clarified extract of neem and cotton seed oil with garlic extract (Nigro *et al*., 2006; Jacometti *et al*., 2010; Romanazzi *et al*., 2012; Paramasivam, 2015). Thus far, the bio fungicides have been tested on table grapes and other fruits. In addition, plant essential oils have also been used by organic farmers on table grapes and vegetables (Schena *et al*., 2003; Liu *et al*., 2010; Romanazzi *et al*., 2012).

Biological controls currently used and registered in the United States include Aspire® (*Candida oleophila*) and BioSave® (*Pseudomonas syringae*), as well as

YieldPlus® (*Cryptococcus albidus*), also used in SA. However, there is still no biological control that have been used in Europe. As a result, the research for more biological control products is ongoing (Ippolito & Nigro, 2000; Mercier & Ben-Yehoshua, 2005).

Natural antimicrobials such as salts, chitosan and plant extracts are used to control grey mould on table grapes during the pre-harvest and post-harvest periods (Nigro *et al*., 2006). In addition, calcium chloride and sodium carbonate or sodium bicarbonate were also used to reduce grey mould from 64% to 29% when stored at 0°C for 30 days and were found to be more effective against crop diseases than fungicides containing cyprodinil and fluxodionil (Romanazzi *et al*., 2012). Moreover, boron was used in the form of potassium tetraborate at 0.1 – 1% and was found to effectively control grey mould in post-harvest table grapes stored under the same conditions. However, the best results were observed when 1% was used on berries inoculated with the grey mould, reducing the mould from 40% to 2% (Qin *et al*., 2010; Romanazzi *et al*., 2012).

Chitosan is known to be a natural biopolymer that can be used during the harvesting season to control decay. It can be dissolved in various acids to evaluate its effectiveness. Acetic acid was found to be the most effective acid for this application (Romanazzi *et al*., 2009; 2012). It was applied by immersing red globe grapes and storing them at 0 – 1°C for four weeks. The results showed that only 10 berries were infected per kg compared to 19 berries infected in the control (Lizardi-Mendoza *et al*., 2016). Additionally, the application of these salts are recommended to be applied pre-harvest since a visible salt residue on the surface of the grape berries appears as a white, waxy coating which is undesirable at the marketing stage. However, their use can cause darkening of the pedicels and dark brown spots on the berries (Ippolito & Nigro, 2000; Gabler & Smilanick, 2001; Nigro *et al*., 2006; Romanazzi *et al*., 2012).

Plant extracts currently being used include the application of an Aloe vera gel coating with a formulation under patent to control grey mould in table grapes pre-harvest and post-harvest (Serrano *et al*., 2006; Romanazzi *et al*., 2012). The Aloe vera gel was applied by spraying clusters of grapes a day before harvest and the grapes were stored at 2ºC for 35 days. The results showed that only 1% of berries treated with Aloe vera were infected compared to 15% in untreated berries (Castillo *et al*., 2010; Romanazzi *et al*., 2012).

Generally regarded as safe decontaminating agents include acetic acid, electrolysed oxidising water and ethanol. Acetic acid was used in a concentration of 0.25 M in 4 mL and 1 mL volumes respectively. The 1 mL of a 0.25 M solution

effectively controlled the grey mould in samples stored at 22ºC for 6 days. The US Food Drug Administration also listed ozone under the GRAS category since 2001 for table grapes. Ozone is termed fungistatic because of its effectiveness to control grape mould. However, at concentrations of 5000 mg $L^{-1}$ can also be phytotoxic (Sharpe *et al*., 2009; Gabler *et al.,* 2010; Romanazzi *et al*., 2012; Lizardi-Mendoza *et al*., 2016; Palou *et al*., 2016).

Generally, among these treatments there are cases where the treatment is not effective on its own, but when combined, following the multiple hurdle concept, they are able to control mould effectively (Romanazzi *et al*., 2012). Among the aforementioned treatments, the biopolymer chitosan was combined with ultraviolet C. The biopolymer was applied pre-harvest and post-harvest and their interaction produced a synergistic effect in grey mould control, reducing the mould in single berries treated with ozone from 22% in the control to 3% in treated samples. Additionally, for blue mould it was reduced from 13% in the control to 1% in single berries treated with ozone (Romanazzi *et al.,* 2006; 2012). This application is recommended in the pre-harvest cycle rather than the post-harvest cycle, as the table grapes are not normally washed post-harvest. The post-harvest application requires wetting which will need drying that could cause mechanical injuries in bunches, leading to economic losses (Romanazzi *et al*., 2012).

Plant essential oils are categorized as fungicides that are normally used by farmers that produce organic crops (Isman, 2000; Dayan *et al*., 2009; Vitoratos *et al*., 2013). These oils include jojoba oil, rosemary oil, thyme oil, clarified hydrophobic extract of neem and cotton seed oil with garlic extract. Although these oils are used to control fungal diseases, their actual active components and mode of action is unknown. Additionally, the following difficulties have been recognized when the product needs to be introduced into the market: the shortage of the relevant natural resources; quality control and chemical standards needed before these products are used commercially; and the complications when it comes to registration of the product. (Isman, 2000; Dayan *et al*., 2009; Vitoratos *et al*., 2013).

### 2.5. Proteins in grapes and wine

Proteins are known as a class of nitrogenous organic compounds that have large molecules consisting of one or more long chains of amino acids (Ferreira *et al*., 2001; Rusell, 2006; Wigand *et al*., 2009). In wine, proteins are present as minor constituents that originate from bacteria, fungi, grapes and yeasts. In addition, these proteins are

found in low amounts in wine compared to the must due to processes that occur during fermentation and by-products formed; as a result of proteolytic activity, precipitation by polyphenols and unfavourable conditions related to the low pH and the increasing ethanol content. Additionally, their concentration in white wine was reported to be between 10 – 500 mg $L^{-1}$ and their molecular weight as ranging from 9 – 99 kDa (Sauvage *et al*., 2010). Moreover, grapes contain pathogenesis-related protein (PR) specifically thaumatin-like protein and chitinases, while yeasts produce mannoproteins that are found in the yeast cell wall (Dupin *et al*., 2000; Caridi, 2006; Ndlovu, 2012; Rodrigues *et al*., 2012; Gazzola *et al*., 2017). However, the quantity of mannoproteins produced during the vinification process is relatively low and it ranges between 100 – 150 mg $L^{-1}$. Their presence in wine helps to stabilise wine from potassium bitartrate and protein haze as well as creating known mouthfeel characteristics. Additionally, their advantages include: 1) The ability to prevent the crystallisation of tartaric salts and protein haziness; 2) Interact with phenolic compounds and decrease red wine astringency; 3) Improve and interact with some wine aromas; 4) Improve the growth of malolactic bacteria; 5) Promote flocculation in sparkling wines and absorb ochratoxin (Howell, 2012; Ndlovu, 2012; Rodrigues *et al*., 2012).

In addition, a study conducted by Dupin *et al.* (2000) reported that mannoproteins from *S. cerevisiae* have the ability to decrease haze formation in white wines because of mannoprotein material (HPM). This will help to decrease the cost of vinifcation by replacing bentonite that is currently used as a fining agent in the cold tartaric acid stability process. However, the quantity produced during the vinification process is too low for industrial production. Hence, the suggestion was to extract the HPM from the cells rather than from the wine, because in a previous study, HPM was extracted from wine that did not produce high quantities. Moreover, further studies are needed to discover the variety of yeast mannoproteins and their effect on wine quality (Dupin *et al.,* 2000). Moreover, Ndluvu *et al*. (2018) reported that factors such as fermentation temperature, yeast strain or grape cultivar have an effect on total proteins levels reduction in Sauvignon Blanc and Chardonnay musts. However, yeast strain showed a significant change in concentration of the chitinase. Therefore, the results obtained from the study confirm the correlation between the levels of yeast cell wall chitin and changes in chitinase concentration. Also, recommend that the amount of lateral chitin is responsible for this activity not the chitin in bud scars.

This can be achieved by proteome analysis called proteomics (Jin *et al*., 2007; Sveinsdóttir *et al*., 2009; Ghen & Zhang, 2012). Proteomic studies are categorized into three parts as follows: large-scale protein identification including isoforms and post-translational modification (PTM's), global analysis of protein expression and the characterization of protein-protein relations (Sveinsdóttir *et al*., 2009; Ghen & Zhang, 2012). Proteomics can add great significance to food analysis studies by providing a valuable insight into aspects such as quality change within the product, before, during and after processing or storage. In addition, it can also add value to the understanding of the composition of the raw materials, the relations of proteins with one another or with other food constituents or any negative symptoms in the human system after ingesting (Jin *et al*., 2007; Sveinsdóttir *et al*., 2009; Ghen & Zhang, 2012). Proteomic studies in the wine industry was initially used to provide an improved explanation on the development of the grape berry under exceptional environmental conditions. Additionally, it was also used as a tool for relating the wine proteome from grapes and yeast with the sensory profiles of wine to advance the wine processing (Sarry *et al*., 2004; Giribaldi *et al*., 2007; Travis 2008; Ghen & Zhang, 2012; Hart *et al*., 2017).

### 2.6. Techniques used for protein quantification in wine

Extensive techniques for the study of protein quantification exist but there are limitations when it comes to wine and grapes because, some techniques are not suitable. These techniques include centrifugation, followed by filtration, followed by ultrafiltration with a cut-off of 10 kDa and finally obtaining the protein fraction by lyophilised ultra-concentration. The second method encompasses the use of ammonium sulfate to precipitate protein, followed by centrifugation to obtain the pellet (Marchal *et al.,* 1997; Le-Bourse *et al*., 2010). The unsuitability of these methods is due to low concentrations of protein present in grape juice and wine because they contain contaminants such as phenolic compounds and ethanol that will disturb the quantification analysis (Marchal *et al.,* 1997; Le-Bourse *et al*., 2010). However, direct quantification from the sample is not permitted due to the absence of standard grape or wine proteins. Therefore, this necessitates the use of techniques with very low detection limits such as the Bradford assay method. This method is mostly used for the analysis of grape protein, wine protein and polypeptides with molecular mass above 300 Da (Curioni *et al*., 2008; Le-Bourse *et al*., 2010).

The Bradford assay method is based on an absorbance shift in Coomassie Blue G-250. The technique is easy, rapid, reproducible, sensitive, low cost and widely used for wine protein analyses (Moreno-Arribas *et al*., 2002; Owusu-Apenten, 2002; Vincenzi *et al*., 2005; Le-Bourse *et al*., 2010). Alternatively, Lowry, Biuret or Smith (also called the bicinchoninic acid method assay) can also be used for wine protein analyses although they are likely to interfere with other compounds (Le-Bourse *et al*., 2010). Subsequently, proteins are identified and characterised using various chromatographic techniques (Le-Bourse *et al*., 2010; Weckwerth *et al*., 2004; Tantipaiboonwong *et al*., 2005; Powell *et al*., 2005). These include fast protein liquid chromatography (FPLC), ion exchange chromatography (IEC), affinity chromatography (AF), gel filtration chromatography (GFC) and chromatofocusing protocols, where proteins are separated according to their isoelectric points. Chromatography can be used as a tool to link full protein profiles of different samples and it is normally used as a procedure to prepare the entire purified protein fraction that will go through the characterisation step (Monteiro *et al*., 2007; Muhlack *et al*., 2007; Vanrell *et al*., 2007; Esteruelas *et al*., 2009; Le-Bourse *et al*., 2010).

Several studies have been conducted using the technique based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Recently, a study by Gazzola *et al*. (2017) on characterisation of chitinase isoforms from juice used SDS-PAGE to analyse the grape juice protein. The method showed that protein bands range from 20 – 30 kDa in juice, which were recognised as PR proteins that include thaumatin-like proteins and chitinase. Additionally, proteins with bands ranging from 45 – 80 kDa were also found. Of these proteins the ones with a molecular weight of 65 kDa are known to be grape vacuolar invertase and are known to be found in larger quantities in grapes. In Chardonnay, the vacuolar invertase makes up 14% of proteins. In addition, the SDS-PAGE also showed bands of 12 kDa that are identified as lipid transfer protein and are identified as one of the major allergens (Gazzola *et al*., 2017).

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a technique that combines mass spectrometry and assisted laser desorption ionization to analyse biomolecules and large organic molecules (Rossignol *et al*., 2008; Singhal *et al*., 2015; Gutiérreza *et al*., 2017). This technique makes use of laser energy to absorb a matrix that creates ions from large molecules with slight fragmentation. The technique is rapid and reliable when used to identify microorganisms, cost-effective, not labour intensive and does not require trained laboratory personnel. Additionally, the technique creates separately charged ions,

which helps to make data interpretation easy. Subsequently, the MALDI-TOF mass spectrometer has been applied to proteomics work on a large-scale, not only because of the aforementioned reasons but also because of the high throughput and speed associated with complete automation that the technique achieves (Ekström *et al*., 2000; Everley *et al*., 2008; Calderaro *et al*., 2014). Moreover, Hart *et al*. (2017) conducted a study that focused in thiol-releasing intra-genus hybrid yeast strains as well as proteins (yeast-derived enzymes) that also play a significant role in the release of the wine-enhancing metabolites during the fermentation of Sauvignon blanc wine using MALDI-TOF/MS to characterise the 9 yeasts used. The study reported that 1 yeast strain (TFPH NH 56) down-regulated the proteins during the lag phase that are associated to amino acid biosynthesis, the pentose phosphate pathway, glycolysis and fructose and galactose metabolism. Therefore, the differences reflected by protein expressed confirmed that proteins are the final effectors of metabolite release.

### 2.7. Gas Chromatography

Gas Chromatography (GC) is a chromatographic technique that separates the targeted compounds in the sample through a stationary and mobile phase (carrier gas). Moreover, the stationary phase consists of a packed column, which contains functional groups that enable separation of analytes based on interaction. Separation of analytes are influenced by temperature and retention time. When temperatures are very high poor separation of the target analytes are observed. Moreover, when the retention time is shorter, the analyte does not interact enough with the stationary phase, resulting in poor quality separation (Tadeo *et al*., 2004; Alañón *et al.*, 2015; Paramasivam, 2015; Thet & Woo, 2015). The GC is normally combined with a mass spectrometry detector (MS), Ultraviolet (UV), diode array (DAD) and fluorimetric detector to provide very sensitive tools for detecting and/or quantifying the analytes (Alañón *et al.*, 2015; Paramasivam, 2015; Thet & Woo, 2015).

The MS detector has advantages such as delivering information about the molecular structure of the specific compound at very low levels and it can be operated in two modes, i.e. total-ion scanning or selected-ion monitoring (SIM), both being suitable in food analysis. Using the GC in conjunction with MS operating in the SIM mode makes the MS highly sensitive and selective for the determination of residues. At present, MS with electron-impact ionization (EI) is used broadly to confirm fungicide residues in foods. Therefore, different groups of fungicides have been determined using GC-MS (Tadeo *et al*., 2004; Campillo *et al.*, 2012; Alañón *et al.*, 2015;

Paramasivam, 2015). Other detectors that can be used with GC for fungicides includes the flame photometric detector (FPD), pulsed flame photometric detector (PFPD), element-specific detector, such as microwave induced plasma atomic emission detector (MIP – AED) and inductively coupled plasma-mass spectrometry (ICP – MS) (Tadeo *et al*., 2004; Campillo *et al.*, 2012; Paramasivam, 2015; Anon, 2016).

Gas chromatography-mass spectrometry is characterised as a technique with high separation power and reproducibility, and as a result, it is called signature or spectral fingerprinting (Cubero-Leon *et al*., 2014). It is broadly used in wine genomic, transcriptomic, proteomic and metabolomic studies. Additionally, extraction processes to separate metabolites are required as it helps to improve the concentration of the metabolites in a sample. In addition, handling of the sample before analysis is essential because it could result in serious error if the extraction technique is not compatible with the GC technique (Cubero-Leon *et al*., 2014; Arbulu *et al*., 2015; Alañón *et al*., 2015). Various extraction techniques such as Liquid-liquid extraction (LLE) or Solid phase extraction (SPE) are used depending on the nature and properties of the analytes. Solid phase extraction is mostly used because it is more selective than LLE and can distinguish more specific molecular features of direct analytes (Cevallos-Cevallos *et al*., 2009; Alañón *et al*., 2015).

Previous studies effectively used GC with headspace solid-phase micro extraction (HS – SPME) using an atomic emission detector (AED) and MS detector for the detection of organotin compounds in honey and wine samples (Campillo *et al*., 2012). The results showed that the GC – MS tandem detected better signal ratios of the organotin compounds in the wine and honey samples, 2 – 5 times lower compared to the ratio produced by GC – AED. As a result, the MS detector was desired for the study (Campillo *et al*., 2012).

Historically, in the wine industry sensory evaluation is conducted to gather knowledge of the volatile composition of the wines by aroma evaluation. However, on some occasions the sensory evaluation would give poor results to profile the flavour of the wine because of variability, even amongst trained judges (Noble & Ebeler, 2002; González-Álvarez *et al*., 2013). This is caused by certain aroma compounds that interact and produce masking effects that will affect or supress the overall aroma profile of the wines (Francis & Newton, 2005; González-Álvarez *et al*., 2013). Therefore, in many cases GC – MS is used as an effective instrument in analysing the odour or aroma profile of wines (Noguerol-Pato *et al*., 2009; González-Álvarez *et al*., 2013). Additionally, the results obtained from both analytical instruments and sensory

evaluation assisted by multivariate statistical techniques that clarify the relationship between the sensory and instrumental data for wines (Aznar *et al*., 2003; Álvarez *et al.,* 2011; Pereira *et al.,* 2010; González-Álvarez *et al*., 2013).

### 2.8. Sensory analysis

Sensory evaluation was introduced as a scientific technique to evaluate different food products using all five senses (smell, touch, taste, sight and hearing) (Ebeler, 1999; Murray *et al*., 2001; Rousseau, 2004; Zoecklein, 2005; Hough, 2010). Additionally, sensory evaluation is categorised into affective and analytical methods. Affective methods require consumer panellists to answer for examples the following questions: 1) which product do you prefer? 2) which product do you like? 3) How well do you like this product? 4) How often would you buy/use this product? In addition, the panel must be large enough to ensure greater confidence about the validity of the results. Analytical methods, which are the most common sensory evaluation techniques used in the wine industry, are divided into descriptive and discrimination (or difference) test methods (Murray *et al*., 2001; Rousseau, 2004; Zoecklein, 2005; Hough, 2010).

Descriptive tests include the detection and measurement of different characteristics within the product (Murray *et al*., 2001; Rousseau, 2004; Zoecklein, 2005; Hough, 2010; Weightman, 2014). These tests can also be used to identify any product changes with regards to shelf-life and packaging effects. Examples of descriptive tests include Quantitative Descriptive Analysis (QDA®), Flavour Profile Analysis, Time-Intensity Descriptive Analysis, and Free-Choice Profiling. However, QDA® is most commonly used because it needs less training time than most of the other methods. Discrimination (or difference) tests include identifying the difference among the products and differentiating if one product differ from the other in terms of selected characteristics. The examples of discrimination tests include the triangle test, the paired comparison test and the duo-trio test. Furthermore, statistical analysis of variance and occasionally principle component analysis can be used to conclude if there is a statistically significant difference or similarity in specific characteristics among the wine samples or not (Murray *et al*., 2001; Rousseau, 2004; Zoecklein, 2005; Hough, 2010).

### 2.9. Flavour compounds in wine

Wine consists of extreme complex aroma compounds that can be identified at very low concentrations of $10^{-4}$ – $10^{-12}$ g $L^{-1}$ (Guadagni *et al*., 1963; Villamor & Ross, 2013). The

wine aroma complex consists of volatile compounds which include the groups of higher alcohols, esters, aldehydes, ketones, acids, terpenes, phenols and sulphur compounds that are found in various concentrations. The difference in concentration depends on various factors such as viticulture (climate, soil, water, cultivar, grape-growing practices) and oenology (condition of grapes, fermentation and post fermentation treatments) (Romano *et al*., 2003; Reynolds, 2010; Villamor & Ross, 2013). However, more than 800 volatile compounds are found in wine but only a few had concentrations above the perception threshold, hence only a few was found to be responsible for odour character (Perestrelo *et al*., 2006; Li *et al*., 2008; Villamor & Ross, 2013). The range of physicochemical and aroma compounds and their sensory characteristics found in young white, red and aged red wines were reported in Table 2.2.

## 2.10. Volatile compounds in wine

### *2.10.1. Classification*

Volatile compounds are classified into three categories: primary, secondary and tertiary aromas (Hartley, 2009; Ali *et al*., 2010; Villamor & Ross, 2013). Primary aromas are the aroma that is found mostly in grape skin tissue. Secondary aromas are by-products of fermentation that contributes to secondary aroma of wine (Lilly *et al*., 2000; Pisarnitskii, 2001; Hartley, 2009; Villamor & Ross, 2013). Moreover, during the fermentation process other metabolites such as ethanol, fusel oil substances (aliphatic alcohols, acids, aldehydes, esters, ketones, terpenes, phenols and sulphur compounds) are produced and are responsible for creating the background aroma of wines. Tertiary aroma is developed at the stage of wine aging since the aroma compounds in the grape skins and those produced during the fermentation process are lost (Pisarnitskii, 2001; Romano *et al*., 2003; Villamor & Ross, 2013).

Esters are defined as flavour compounds that are normally found in a range of food products. In fermented beverages, such as wines and beer, they are present in low concentrations with low aroma threshold concentrations (100 mg $L^{-1}$). In addition, the concentration of ester's, depends on several factors such as the yeast strain, fermentation temperature, insoluble material in the grape must, vinification methods, skin contact, pH, the amount of sulphur dioxide, amino acids present in the must and malolactic fermentation. Moreover, they are responsible for the tropical fruit and banana-like aromas in wines (Lilly *et al*., 2000; Pretorius & Lambrechts, 2000; Sumby *et al*., 2010; Vilanova *et al*., 2013).

**Table 2.2.** Physiochemical and sensory properties of selected aroma compounds in wine (Villamor & Ross, 2013).

| Compound | Molecular Weight (g $mol^{-1}$) | Boiling point (◦C) | Log P Value (d) | Concentration (µg $L^{-1}$) | Threshold (µg $L^{-1}$) | Aroma descriptor |
|---|---|---|---|---|---|---|
| ***Carbonyls*** | | | | | | |
| β-damascenone | 190 | 265 | 4.21 | 2, 29, 3.5 | 0.05 | apple, honey |
| β-ionone | 192 | 263 | 3.84 | 0.23 | 0.09 | seaweed, violet |
| 3-octanone | 128 | 168 | 2.22 | 17 | 21-50 | herb, butter |
| ***Esters*** | | | | | | |
| Ethyl 2-methylbutyrate | 130 | 135 | 2.26 | 32 | 18, 1 | apple |
| Ethyl 3-methylbutyrate | 130 | 135 | 2.26 | 20 | 3 | fruit |
| Ethyl butyrate | 116 | 122 | 1.85 | 680, 69 | 20 | apple |
| Ethyl hexanoate | 144 | 167 | 2.83 | 650, 140, 29 | 14, 5 | apple peel, fruit |
| Ethyl cinnamate | 176 | 271 | 2.99 | 1.22 | 1.1 | honey, cinnamon |
| Isoamyl acetate | 130 | 142 | 2.25 | 60, 142, 120 | 30 | banana |
| ***Alcohols*** | | | | | | |
| Isoamyl alcohol | 88 | 131 | 1.16 | 150,0, 1,41, 112,80 | 30,00 | whiskey, malt, burnt |
| 1-hexanol | 102 | 158 | 2.03 | 8,00, 617, 780 | 8,00 | resin, green |
| 2-phenylethanol | 122 | 218 | 1.36 | 34,00, 6,08, 60,30 | 14,00, 10,00 | honey, rose |
| Methionol | 106 | 178 | 0.44 | 3,75 | 1,00 | sweet, potato |
| 1-heptanol | 116 | 177 | 2.62 | 15 | 3 | chemical, green |
| ***Phenols*** | | | | | | |
| Guaiacol | 124 | 205 | 1.32 | 47.3 | 9.5, 10 | smoke, medicine |
| Eugenol | 164 | 253 | 2.27 | 60 | 6, 5 | clove, honey |
| 4-vinylguaiacol | 150 | 247 | 2.24 | 30 | 40,10[j] | clove, curry |
| ***Terpenes*** | | | | | | |
| β-citronellol | 156 | 224 | 3.91 | 21, 1.2 | 100 | rose |
| Linalool oxide | 170 | 233 | 2.08 | 3.0 | 4–10 | flower, wood |
| Geraniol | 154 | 230 | 3.56 | 19,3.2 | 20,30 | rose, geranium |
| ***Acids*** | | | | | | |
| 3-methylbutyric acid | 102 | 176 | 1.16 | 1,670 | 33 | sweat, acid |
| Hexanoic acid | 116 | 205 | 1.92 | 5,30, 120·2,730 | 420 | sweat |
| Octanoic acid | 144 | 239 | 3.05 | 26,00, 555, 910 | 500, | sweat, cheese |
| ***Lactone*** | | | | | | |
| Cis-whiskey lactone | 156 | 261 | 2.00 | 151 | 67 | coconut |

Alcohols are formed during ethanol production. Ethanol has a significant role in wine with concentrations ranging from 10 – 13 mL per 100 mL. It also has an important role in terms of wine stability, aging and sensory properties. Additionally, ethanol also has an effect on the type and amount of aromatic compounds by potentially disturbing yeast metabolic activity. Potentially significant higher alcohols in wine include n - propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, and 3-methyl-1-butanol. These are formed as by-products of yeast fermentation and influence the aroma and/or flavour of the wine. The factor affecting the aforementioned higher alcohol formation is the vinification process which includes temperature, presence of oxygen, suspended solids and the yeast strain (Pretorius & Lambrechts, 2000; Ali *et al*., 2010; Styger *et al*., 2011).

Organic acids are compounds that affect the organoleptic properties and microbiological stability of wine (Pretorius & Lambrechts, 2000; Ali *et al*., 2010; Styger *et al*., 2011). They may originate from the grapes, but processes such as alcoholic fermentation, malolactic fermentation and the oxidation of ethanol are involved in their production. In addition, wine contains volatile and fixed acids. The main volatile acid is acetic acid which can be eliminated by reverse osmosis. Fixed acids include tartaric, malic, citric acid (from grapes and fermentation process) along with lactic, succinic, oxalic and fumaric acid. The content of these fixed acids in wine influences the pH of the wine (Pretorius & Lambrechts, 2000; Ali *et al*., 2010; Styger *et al*., 2011).

Acetaldehyde is a by-product of yeast metabolism through alcoholic fermentation and is also formed as a result of oxidation during storage. It is also the most common aldehyde found in wine and constitutes 90% of the aldehyde content (Ebeler & Spaulding, 1998; Pretorius & Lambrechts, 2000; Styger *et al*., 2011; Nunes *et al*., 2017). In newly fermented wine, it is present in concentrations of 75 mg $L^{-1}$, while the sensory detection threshold is between 100 – 125 mg $L^{-1}$. Higher levels of acetaldehyde are detected in overripe, bruised fruit and result in sherry-like aromas (Ebeler & Spaulding, 1998; Pretorius & Lambrechts, 2000; Styger *et al*., 2011; Nunes *et al*., 2017). Moreover, factors such as temperature, pH, oxygen ($O_2$) level, sulphur dioxide ($SO_2$) level, and nutrient availability also have an effect on the formation of acetaldehyde. However, $SO_2$ has a significant role in the transformation of acetaldehydes into ethanol (Pretorius & Lambrechts, 2000; Frivik & Ebeler, 2003; Styger *et al*., 2011; Moss, 2015; Van Jaarsveld & October, 2015).

Volatile ketones are compounds found in grapes but not all of them endure the fermentation process, as a result, few of these are detected in the wine. These include

β - damascenone, α - ionone and diacetyl (Swipson & Miller, 1984; Rapp & Mandery, 1986; Pretorius & Lambrechts, 2000; Styger *et al*., 2011; Nunes *et al*., 2017). The β - damascenone is the ketone that plays an important role in various white wine aroma profiles. It was found that the threshold value of β - damascenone in white wine ranges between 4 – 7 μg $L^{-1}$; however, Li *et al*. (2008) reported a threshold of 4.5 μg $L^{-1}$ for white sweet wines (Pineau *et al.,* 2007; Li *et al*., 2008; Goins, 2015).

Sulphur compounds are known to be responsible for the various off-flavours (Fischer, 2007; Styger *et al.*, 2011). These compounds cause smells such as rotten egg, the odour of onions, green asparagus, burnt rubber or even garlic. They are formed as a result of the presence of methyl and ethyl sulphides, disulphides and thiols. However, these compounds can also have positive effects by creating fruity flavours in wine due to 3-mercaptohexanol formation. Additionally, the formation of hydrogen sulphites mostly depends on the yeast strain and less on the composition of the grape must (Fischer, 2007; Styger *et al.*, 2011).

### 2.11. Electrophoretic karyotyping (CHEF)

Electrophoretic karyotyping is a nucleic acid technique used to provide unique profiles of the DNA of a yeast strain or species for identification and characterisation purposes (Carle & Olson, 1985; Deák, 1995; Nair *et al*., 2014). The preparation of full-length chromosomal DNA includes growing yeasts in liquid media and subjecting the cells to direct DNA extraction until immobilised on gels. When the DNA is prepared, a powerful tool that enables separation of whole yeast chromosomes, such as pulsed field gel electrophoresis (PFGE), is used. When the technique was first introduced, it had limitations because of the poor quality apparatus used, extraction methods used, as well as parameters including field strength, pulse time, gel concentration and duration of electrophoresis (Johnston & Mortimer, 1986; Van Vuuren & Van Der Meer, 1987; Vaughan-Martini *et al*., 1993; Deák, 1993). However, apparatus by Beckman, together with improved extraction processes improved the overall technique. The apparatus developed included orthogonal field alternation gel electrophoresis (OFAGE), field inversion gel electrophoresis (FIGE), contour-clamped homogeneous electric field electrophoresis (CHEF), and transverse alternating field electrophoresis (TAFE) (Table 2.3). The improvements lead to the declaration that the technique could be used in industrial fermentations since it is a comparatively simple method to fingerprint strains of yeast (Vaughan-Martini *et al*., 1993; Deák, 1993).

**Table 2.3.** Apparatus used for pulsed field gel electrophoresis (Vaughan-Martini *et al*., 1993).

| Apparatus & electrodes | Lanes | Maximum kb separated | Observations |
|---|---|---|---|
| Orthogonal-non-uniform (OFAGE) | Curved | 1000 | Large chromosomes (> 1000 kb) are not separated. It is possible to compare only a few samples (5 – 6) per run |
| Uniform field inversion (FIGE) | Straight | 1000 | Ramping is required to avoid non-uniform movement of large molecules |
| Transverse alternating (TAFE) | Straight | 10000 | Gels are relatively small and few lanes are possible. Problems can be encountered with low agarose concentrations since gels are inserted vertically |
| Contour-clamped homogeneous (CHEF) | Straight | 12000 | Bubbles, which tend to distort lanes, can develop under the gel during longer runs |

## 2.12. Future trends

Fungal diseases in vineyards are continuously increasing with time and the notable disease that occur the most is powdery mildew (Halleen *et al*., 2000; Halleen & Holz, 2001; Ellis & Nita, 2004; Delaunois *et al*., 2014). Powdery mildew occurs annually when compared to other diseases and therefore the vineyards have to be treated on an ongoing basis (Halleen *et al*., 2000; Halleen & Holz, 2001; Ellis & Nita, 2004). Additionally, as mentioned, other grapevine diseases such as downy mildew and grey mould also affect the vineyards.The infection results in loss of crop that negatively affect the yield (production) leading to huge economic losses (Petit *et al*., 2008; Caboni & Cabras, 2010; González-Rodríguez *et al*., 2011; González-Álvarez *et al*., 2012; Delaunois *et al*., 2014). Therefore, the use of fungicides serves as a solution to the aforementioned negative effects along with good agricultural practices (Brink *et al*., 2006; Gianessi & Williams, 2012). The fungicides currently used are chemically based made from either organic or inorganic compounds. The organic compounds include acylalanine, anilinopyrimidine, azole, benzimidazole, dithio-and bisdithio-carbamates, cynapyrrole and more. The inorganic compounds include sulphur and copper-based compounds (Nigro *et al*., 2006; Jacometti *et al*., 2010; Romanazzi *et al*., 2012). Several studies have been conducted using chemically based fungicides (La Torre *et al*., 2002; Winter, 2005; Comitini & Ciani, 2008; Kretschmer & Hahn, 2008; Ortiz *et al*., 2010; Pitt *et al*., 2012). The challenge faced when these are continuously used is the evolvement of the target organisms causing their resistance to these fungicides. Various approaches have been researched where more than three fungicides were combined to treat the diseases (Kretschmer & Hahn, 2008; Čadež *et al*., 2010; Delaunois *et al*.,

2014). In addition, improving good agricultural practices such as pruning, scouting and then combining them with chemical fungicides was another approach used. These techniques were successful in certain applications and not in others (Ellis & Nita, 2004; Elmer & Reglinski, 2006; Ellis, 2008; Hartman & Beale, 2008).

Furthermore, natural alternative fungicides to control vineyard diseases are currently an important research focus since the demand for organic products by consumers and retail companies are increasing (Ellis & Nita, 2004; Mercier & Ben-Yehoshua, 2005; Delaunois *et al*., 2014). Natural alternatives include Kraalbos (*Galenia africana*), elicitors, biocontrol agents e.g. *Muscodor albus* and *Hanseniaspora uvarum*, natural antimicrobials (salts and chitosan) and plant extracts (jojoba oil, rosemary oil, thyme oil, clarified hydrophobic extract of neem and cottonseed oil with garlic extract) as fungicides to control vineyards diseases (Rabosto *et a*l., 2006; Castillo *et al*., 2010; Qin *et al*., 2010; Romanazzi *et al*., 2012; Delaunois *et al*., 2014; Lizardi-Mendoza *et al*., 2016). Moreover, alternative treatments currently used that meet standards are calcium chloride, chitosan and ozone but only in post-harvest storage in organic classification (Mercier & Ben-Yehoshua, 2005; Gabler *et al*., 2010; Romanazzi *et al*., 2012). However, little information is available on the use of natural alternative fungicide usage in wine grapes specifically on Chenin Blanc grapes in field trials and their effect on fermentation rate, wine sensory profile and yeast proteins expressed. Moreover, employing omics as a tools to evaluate the overall effects of fungicides on the release of aroma enhancing metabolites. The knowledge provided can be used to evaluate other white and red grape cultivars in future field trials.

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# CHAPTER 3: WINEMAKING, CHEMICAL AND SENSORY ANALYSIS OF THE WINES

## 3.1. Abstract

Commercial *Saccharomyces cerevisiae (S. cerevisiae)* yeast strain (VIN 13, Anchor Bio-Technologies) was used to inoculate small-scale wine fermentations. The small-scale fermentations were conducted at 15ºC for ±20 days. During the fermentations, samples were collected at the start, during and at the end of the fermentations. They were then analysed for glucose/fructose, total acidity (TA), alcohol and volatile acidity (VA) using the Oenofoss® (Denmark). In addition, the alcolyzer (Anton Paar) was used to verify the alcohol values obtained from the Oenofoss. The densitometer was also used to verify the total sugar content. Moreover, fermentation rate was monitored by conducting laboratory-scale fermentations with two yeast strains (VIN 13 and VIN 7) at 15ºC. All fermentations were conducted in duplicate. Monitoring entailed weighing the laboratory-scale bottles frequently ($CO_2$ weight loss) until the fermentations stabilised. Moreover, at the end of the small-scale fermentations, lees samples were plated onto Yeast Extract Peptone Dextrose (YPD) agar and selected colonies were subjected to contour-clamped homogeneous electric field (CHEF) gel electrophoresis to confirm that the *S. cerevisiae* yeast strain inoculated at the start of the fermentation was the same strain that completed the fermentations. The lees samples were subjected to CHEF running conditions. Resultant wines from the small-scale fermentations were bottled and stored for four months, after which they were chemically and sensorially assessed and the results statistically evaluated.

The results showed that the fungicide treatments compared to the controls had no notable negative effects on yeast fermentation rate and sensory quality of the wines.

## 3.2. Introduction

The winemaking process involves the conversion of grape juice into wine during which various biochemical reactions occur. These biochemical reactions start during the ripening phase of the grapes, proceeds during harvesting, the fermentation and lastly in the bottle (Romano *et al*., 2003). Wine quality strongly depends on the quality of grapes used, i.e. the greater the quality of the grapes, the higher the quality of the wine produced (Caboni & Cabras, 2010). However, fungal diseases in vineyards are one of the main factors leading to economic losses in the viticultural sector, therefore, it is important that these diseases are treated and controlled. Various fungicides are

therefore commercially available and are applied under Good Agricultural Practices (GAP). Moreover, in SA these fungicides have to be administered as stipulated in Act 36 of 1947 to meet safety regulations. For this reason, thorough testing is required before registration (DAFF Act No.36 of 1947; Nigro *et al*., 2006; Romanazzi *et al*., 2012).

Fungicides can be administered in three forms i.e. contact, translaminar or systemic (Dias, 2012; Petit *et al*., 2012). Contact fungicides protect the plant only where the spray is administered and is not taken up into the plant tissue. Translaminar fungicides can spread from upper surface of the sprayed leaf to the lower unsprayed surfaces. Systemic fungicides are taken up and spread through the xylem vessels and distributed from the roots to the rest of the plant. Moreover, it is important to note that some systemic fungicides are locally systemic and not taken upward through the vine (Dias, 2012; Petit *et al*., 2012).

Fungicides consist of two major groups: chemical-based and biological-based. Chemical-based fungicides are compounds that are synthesised from organic and/or inorganic chemicals for example acylalanine, benzimidazole, cynapyrrole, copper-based, sulphur etc. Biological-based fungicides include microorganisms and naturally occurring substances, such as *Muscodor albus* and *Hanseniaspora uvarum,* electrolysed oxidising water, acetic acid, jojoba oil, thyme oil and cottonseed oil with garlic extract (Nigro *et al*., 2006; Romanazzi *et al*., 2012). Crisp *et al*. (2006) conducted a study using milk (pasteurised full cream), mixed with whey powder and potassium bicarbonate, as well as the above mentioned ingredients mixed with canola-based oil. These preparations were used to control powdery and downy mildew diseases in *Vitis vinifera* 'Verdelho', Shiraz and Chardonnay grapes. Additionally, sulphur was used as an industry standard fungicide and untreated grapes as a control. The study found that the aforementioned milk-based treatments were able to control the diseases in all grape cultivars equivalent to those treated with sulphur. Nevertheless, further research on juice assessment and wine quality is essential. Moreover, according to Tripathi *et al*. (2008), essential oils extracted from *Ocimum sanctum, Prunus persica* and *Zingiber officinale* plants were able to control *Botrytis cinerea* when applied in a concentration of 200 and 100 ppm $mg^{-1}$, respectively. However, the concerns with their use as fungicides are that they will require accurate analysis in terms of their biological activity, development of a formula that will prevent the disease at a concentration that is non-toxic and how far the oils will spread in fruit tissues.

Although fungicides are applied under GAP, residues may be present in the grape must. Therefore, it should be highlighted that these can lead to stuck and sluggish alcoholic fermentations. Moreover, fungicide residues present on grapes and/or in must could affect wine sensory quality and stability, due to the fact that they can prevent some yeast metabolic pathways involved in phenolic and/or aroma compound production that are critical for sensory quality (Noguerol-Pato *et al*., 2014; 2015). Additionally, these fungicides (chemical and biological) are either water-soluble or water-insoluble. As a result, the water-soluble fungicides may be transferred from grapes to must and thus to the wine. The insoluble fungicides may be present in low quantities or not be present at all. Moreover, during the vinification process the fungicide residues may adsorb to the solids and therefore their levels decrease through the separation of solids from the liquid phase by racking, stabilisation and filtration (Cabras & Angioni, 2000; Álvarez *et al*., 2011; Čuš *et al*., 2010; Ortiz *et al*., 2010; He *et al*., 2016).

Tromp & Marais (2017) conducted a study using the chemical fungicide Triadimefon (triazole) to treat powdery mildew on grapes and investigated its effect on fermentation rate and overall wine quality. The results showed that the Triadimefon had no effect on the quality of wine and neither on the yeast fermentation rate. Calhelha *et al.* (2005) conducted a study using two fungicides individually (Dichlofluanid and Benomyl) to evaluate their effect on the following aspects: i) duration of fermentation, start and terminus; ii) physical and chemical parameters of the resultant wine and iii) organoleptic characteristics of the wine. The results showed that the two fungicides had no negative effects on the above parameters, however, the Benomyl caused a slight decrease in the fermentation rate during the start of fermentation.

Once the wines have been produced, they are subjected to various chemical analyses and evaluations to ensure that their quality is acceptable. Although these analyses are critical towards ensuring quality, it is also important to conduct sensory evaluations, which will be statistically analysed to identify significant sensorial differences among the wines or to describe the sensory profile. These evaluations require a trained panel of judges and typically involves three basic steps (Table 3.1) (Ugliano & Henschke, 2009; Romano *et al*., 2003). Regarding descriptive standards, Francis & Newton. (2005) and Coetzee & Du Toit. (2012) reported that certain compounds in wine could contribute positively or negatively to the overall sensory profile of the wine. In Chenin Blanc wine, aromas such as rotten egg, cooked vegetables, onion and cabbage are associated with off-flavours. Tropical fruit, passion

fruit and guava-like aromas are associated with positive flavours (Ugliano & Henschke, 2009; Álvarez *et al*., 2011).

Infection of vineyards by fungal diseases is a serious problem, therefore, effective fungicides, whether chemical or biological are important. However, fungi could develop resistance to existing fungicides over a prolonged period of time. Therefore, the application of less harmful, natural alternatives or to reduce the dosage of fungicides currently being administered should be strongly considered. Although extensive research is being conducted, the need for further research into the effects of specific fungicides used in control programs on the resultant quality of wines remains a priority.

With the above background the aim of the study was to monitor the *Saccharomyces cerevisiae* (VIN 13 and VIN 7) activity during laboratory-scale fermentations and small-scale winemaking with a view to compare the effect of fungicide treatments (control, 1x and 2x treatment of chemical and natural fungicides) on wine chemical parameters as well as on the sensory profile of wines produced.

**Table 3.1.** Basic steps involved in sensory evaluation (Anon, 2015).

| **Steps** | **Observations** |
|---|---|
| Sight | Observing colour and clarity |
| Swirl and smell | Aerate to free aroma (fruity notes and off odours) and smell the aromas. |
| Taste | Tasting to confirm the wine's bouquet and finish of the wine. |

## 3.3. Materials and methods

### *3.3.1. Grape and Must*

Chenin Blanc grapes were treated with chemical and biological fungicides at 1x treatment (T1) and 2x treatment (T2) under Good Agricultural Practises (GAP) by Agrochemical companies (Experiment A, B and C). Grapes for Experiment A were from the Groot Phesantekraal wine farm found in the Durbanville region. Grapes for Experiment B were from an Agter Paarl wine farm that is located in the Paarl region. In addition, grapes for Experiment C were sourced from the Nietvoorbij research farm situated in the Stellenbosch region. Control grapes treated with a previously approved chemical fungicide were also included to be compared to the new fungicides. The fungicide treatments have different active components, Experiment A contained Methyl-1H-pyrazole carboxylic acid phenylethyl amide, Experiment B, Boscalid and

Experiment C extract of *Gelania Africana* and penconazole. The Agrochemical companies applied the fungicides 14 – 28 days before harvesting, thereafter they were harvested and delivered to the ARC Nietvoorbij research cellar for laboratory-scale fermentations and small-scale wine production. The grapes were crushed and pressed to get the juice without skin contact. Pectolytic enzyme (0.5 g $hL^{-1}$) and $SO_2$ (0.5 g $hL^{-1}$) was added to the cloudy juice and it was left overnight at 14ºC to settle.

*3.3.2. Small-scale winemaking and laboratory-scale fermentations*

Small-scale wines were made following the standard vinification protocol of the ARC Infruitec-Nietvoorbij (Figure 3.1). The small-scale wine fermentations were conducted using a commercial *S. cerevisiae* ADWY strain (Active Dried Wine Yeast) VIN 13. The laboratory-scale fermentations were conducted in duplicate using the commercial *S. cerevisiae* ADWY strains (VIN 7 and VIN 13). Prior to inoculation, the yeast was re-hydrated by weighing 0.15 g into a sterile Erlenmeyer flask and adding 500 mL distilled water. The yeast suspension was incubated at 37°C for 20 minutes. The grape must was thoroughly mixed to ensure a homogeneous substrate and aliquots of 500 mL were transferred to 750 mL glass bottles and each inoculated with 10 mL of yeast suspension. Fermentation caps containing distilled water were placed on each bottle and were held in place with parafilm and the bottles placed at 15°C for fermentations. The bottles were weighed three times a week to monitor the $CO_2$ weight loss until the fermentations stabilised. Fermentation curves were constructed to monitor the yeast performance.

*3.3.3. Culturing*

Wine samples collected at the end of fermentation were streaked out onto Yeast Peptone Dextrose Agar plates (YPD agar) (Biolab, Merck), consisting of (g $L^{-1}$): Yeast extract (10); Peptone (20); Dextrose (20) and Agar (20), that was prepared by dissolving 70 g of the powder in distilled water and autoclaving at 121ºC for 15 minutes. After cooling down antibiotic chloramphenicol (Merck, Germany) was added to suppress other growth. A dilution series of $10^{-4}$ was plated to obtain single colonies. Plates were incubated at 28ºC for 48 hours. Then 10 colonies were randomly selected from each YPD and were subjected to electrophoretic karyotyping by CHEF gel electrophoresis to confirm that the inoculated *S. cerevisiae* yeast strain completed the fermentation.

*3.3.4. CHEF gel electrophoresis*

Samples were prepared according to the embedded agarose procedure of Carle & Olson (1985). Intact chromosomal DNA was separated using the CHEF method (CHEF-DR II, Bio-Rad Laboratories, Richmond, USA). The running conditions for *S. cerevisiae* yeasts according to the CHEF protocol (Oda & Tonomura, 1995; Hoff, 2012) (Block 1: 15 h, 60 s, 60 s, 6 V $cm^{-1}$ at 14ºC and Block 2: 11 h, 90 s, 90 s, 6 V $cm^{-1}$ at 14ºC) were applied to 1.2% agarose gels throughout this study.

*3.3.5. Buffers*

Various ethylene diamine tetra-acetic acid (EDTA) (Saarchem, Merck) buffers consisting of (g $L^{-1}$): 0.45 M pH 9 (83.75); 10 mM pH 7.5 (1.86); 50 mM pH 7.5 (9.3); 0.125 M pH 7.5 (23.25); and 0.5 M pH 9 (93.06) was prepared. A 10 mM Tris-HCl (hydroxymethyl aminomethane-hydrochloric acid) (Biolab, Merck) pH 8 (0.61) buffer; 1 N HCl solution containing 49.11 mL $L^{-1}$ concentrated HCl and a 10X Tris-borate-EDTA (TBE) buffer containing (g $L^{-1}$): Tris (121.1); EDTA (3.72); and boric acid (51.53) (Biolab, Merck) were also prepared using de-ionised water. The pH was adjusted to 8.4 with boric acid and the buffer diluted to 1 L. A 0.5X TBE buffer was prepared by mixing 100 mL of the 10X TBE buffer with 1.9 L of de-ionised water and mixing well. All EDTA buffers and the 1 N HCl solution was diluted to 500 mL in volumetric flasks with de-ionised water. The 10X TBE buffer was diluted to 1 L in a volumetric flask with de-ionised water. All buffers, except the 10X TBE buffer were sterilized by autoclaving.

*3.3.6. Agarose gels*

Agarose gels (1.2%) were prepared for CHEF pulsed field gel electrophoresis (PFGE) by dissolving 1.44 g Seakem® LE agarose (Lonza, USA) in 120 mL 0.5X TBE buffer and heating in the microwave until completely dissolved. A 1% low melting point gel was prepared for making the yeast plugs by dissolving 1 g of low melting point Seakem® agarose (Lonza, USA) in 100 mL of 0.125 M EDTA pH 7.5 buffer and heating in the microwave until completely dissolved. The gels were then stained with a 0.5X TBE buffer containing 15 µL (10 mg $mL^{-1}$) ethidium bromide solution (Promega Corporation, USA) for 30 minutes and de-stained with 0.5X TBE buffer for 45 minutes. Images were recorded of all CHEF gels using the Molecular Imager® Gel Doc™ XR system with FPQuest™ software (Bio-Rad Laboratories, USA).

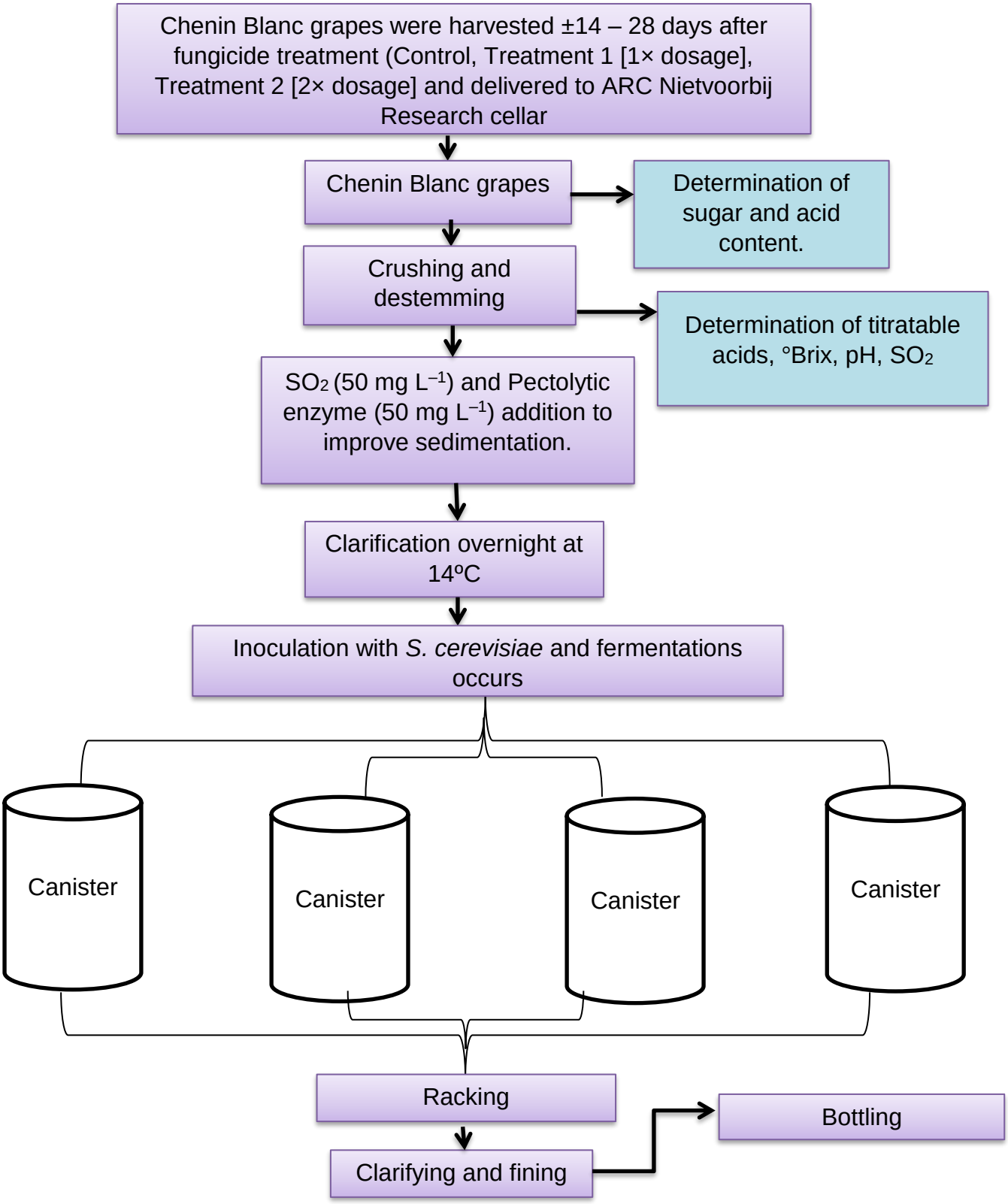


**Figure 3.1.** Flow chart of small-scale winemaking process according to the standard vinification protocol of the ARC Infruitec-Nietvoorbij.

*3.3.7. Chemical analyses and sensory evaluation*

Resultant small-scale wines were evaluated five months after bottling. Wines were analysed for glucose/fructose, total acidity (TA), alcohol, volatile acidity (VA) and malic acid using the Oenofoss (Rhine Ruhr), Alcolyzer (Anton Paar, Denmark) and Densitometer (Anton Paar, Denmark). In addition, wines were evaluated by a panel of 12 trained wine tasters sensorially using a descriptive sensory evaluation method. The panel were provided with tasting sheets that allowed them to evaluate the wine according to an unstructured line scale (Appendix A). Moreover, as part of the Cape Peninsula University of Technology's (CPUT) ethical standards policy, all wine tasters were required to sign a consent form before tasting (Appendix B).

*3.3.8. Statistical analyses*

The chemical and sensory data were analysed using a two-way Analysis of Variance (ANOVA) method. All sensory results obtained from the evaluation as well as the chemical data from the two-way Analysis of Variance (ANOVA) were subjected to a Principal Component Aanalysis (PCA) bi-plot and Multiple Factor Analysis (MFA) using the XLSTAT 2015 software.

## 3.4. Results and discussions

*3.4.1. Laboratory-scale fermentations*

In Experiment A (Figure 3.2), the fermentation curves of the VIN 13 and VIN 7 yeast strains for the 1x treatment (T1) and 2x treatment (T2) completed the fermentations at a similar rate to the respective controls. It is noteworthy that the VIN 13 and VIN 7 fermentations started on day 5 and both stabilised after 15 days. This trend was seen for both treatments as well as the control.

In Experiment B (Figure 3.3), the fermentation curves of the VIN 13 and VIN 7 yeast strains for the 1x treatment (T1) and 2x treatment (T2) also finished the fermentations at a similar rate to their respective controls. In this instance, VIN 13 and VIN 7 fermentations also started on day 5 and both stabilised after 10 days. This trend was also seen for both treatments as well as the control in this treatment.

In Experiment C (Figure 3.4), VIN 13 and VIN 7 fermentation curves showed that the fermentation was completed at a similar rate between 1x treatment (T1) and 2x treatment (T2) when compared to their controls. In this case, it took 5 days to initiate the fermentation for both yeast strains (5 days lag phase), but the fermentations also stabilised after 15 days.

*3.4.2. Small-scale wines*

Wines were bottled and chemical analyses was performed on the final wines. Average data is presented in Table 3.2. Glucose/fructose values for all the experiments were 0 g $L^{-1}$ which showed that the wines fermented to dryness (<5 g $L^{-1}$). Additionally, ethanol (alcohol) concentration of all samples ranged between 11 – 14% $^{v}/_{v}$, which falls within acceptable legal limits for white wines (≤15% $^{v}/_{v}$) (Anon, 1989). In addition, volatile acidity values for Experiment A (0.04 – 0.07 g $L^{-1}$), Experiment B (0.06 – 0.11 g $L^{-1}$) and Experiment C (0.017 – 0.19 g $L^{-1}$), fell within acceptable legal limits for SA white wines (≤1.2 g $L^{-1}$) (DAFF Act No.60 of 1989; Du Toit, 2001). Moreover, pH values obtained were as follows: Experiment A (3.10 – 3.20), Experiment B (3.11 – 3.47) and Experiment C (3.22 – 3.29). The overall pH values for all experiments ranged between 3.0 – 3.4 which is the typical range for SA white wines, although the 2x treatment (T2) of Experiment B, had a slightly higher pH. The total acidity for the various experiments was as follows: Experiment A (3.96 – 4.38 g $L^{-1}$), Experiment B (4.73 – 5.14 g $L^{-1}$) and Experiment C (5.82 – 6.15 g $L^{-1}$). Additionally, it can be observed that Treatment B and C have higher total acidity values when compared to Experiment A, however this was consistent within experiments. This could be attributed to the fact that grapes came from different wine regions and not necessarily an effect of the fungicides on the wine. It is further noteworthy that all the wine parameters fell within acceptable limits for SA wines.

*3.4.3. CHEF gel electrophoresis*

The DNA banding pattern of the control *S. cerevisiae* yeast inoculated at the start of the fermentation was similar to the banding pattern of the yeast isolated from the lees samples collected at the end of the fermentations (Figure 3.5). This confirmed that the inoculated yeast strain was not only present throughout but also conducted the alcoholic fermentations.

*3.4.4. Chemical and Sensory Analyses*

Sensory evaluation results were analysed using ANOVA and Multiple Factor Analysis (MFA). ANOVA results (Table 3.3) showed that there was no significant difference in all sensory attributes between the control and treatments (T1 and T2) for all experiments. Multiple factor analysis (MFA) for Experiment A (Figure 3.6) showed that the control and 1x treatment (T1) clustered in the top left quadrant produced wines with

a positive association with “Tropical fruit“ aroma. This is a good observation as tropical fruit is associated with Chenin Blanc wine flavour profiles due to the presence of volatile thiols. However, it is also seen that for T1 and the control “Wine Foreign” aromas were also perceived, which are aromas not usually associated with, or desirable in Chenin Blanc wines. This is not necessarily a reflection of the fungicides as the control was also associated with it. The 2x treatment (T2) clustered in the bottom left quadrant and showed a positive association with “Tree fruit”. The overall chemical analyses showed that all wines for Experiment A had a negative association with VA which was a desirable result, since VA is associated with an unpleasant vinegar-like aroma (Neeley, 2004; Torrea *et al*., 2011). This was the overall observation for all chemical parameters, indicating that the fungicide for Experiment A did not have any notable effect as the treatments grouped close to the control.

The MFA for Experiment B (Figure 3.7) shows that the control and 1x treatment (T1) clustered in the bottom right quadrant and produced wines with a positive association to “Overall Quality” and “Colour”. Nonetheless, although these wines had a negative association with “Tropical” and “Tree fruit” aromas, it also had a negative association with “Wine Foreign” aromas, some of which were highlighted as solvents (acetone like). In addition, these wines also showed a negative association with VA clustered in the top right quadrant and can be seen as a positive observation. The 2x treatment (T2) clustered in the top right quadrant and also showed a negative association with “Tropical”, “Tree fruit” and “Wine foreign” aromas. Moreover, the overall observation for Experiment B is that T1 grouped with the control and produced wines with high “Overall Quality” and “Colour”. It is noteworthy that this wine had a positive association with VA, so a 2x treatment of the fungicides would not be advisable.

In Experiment C, the MFA (Figure 3.8) showed that the control and 2x treatment (T2) clustered in the top right quadrant and produced wines with a negative association to “Tropical fruit” and “Wine Foreign”, which is desirable. It is also notable that these wines had a positive association with “Overall Quality” and “Colour” but also to VA which is less desirable. The 1x treatment (T1) clustered in the bottom right quadrant and had a negative association to VA and was perceived to have a higher quality and colour than the control and T2 wines. It can therefore, be concluded that because the control and T2 grouped together, the effects on the parameters cannot necessarily be attributed as a result of the fungicides, but possibly winemaking and vineyard effects.

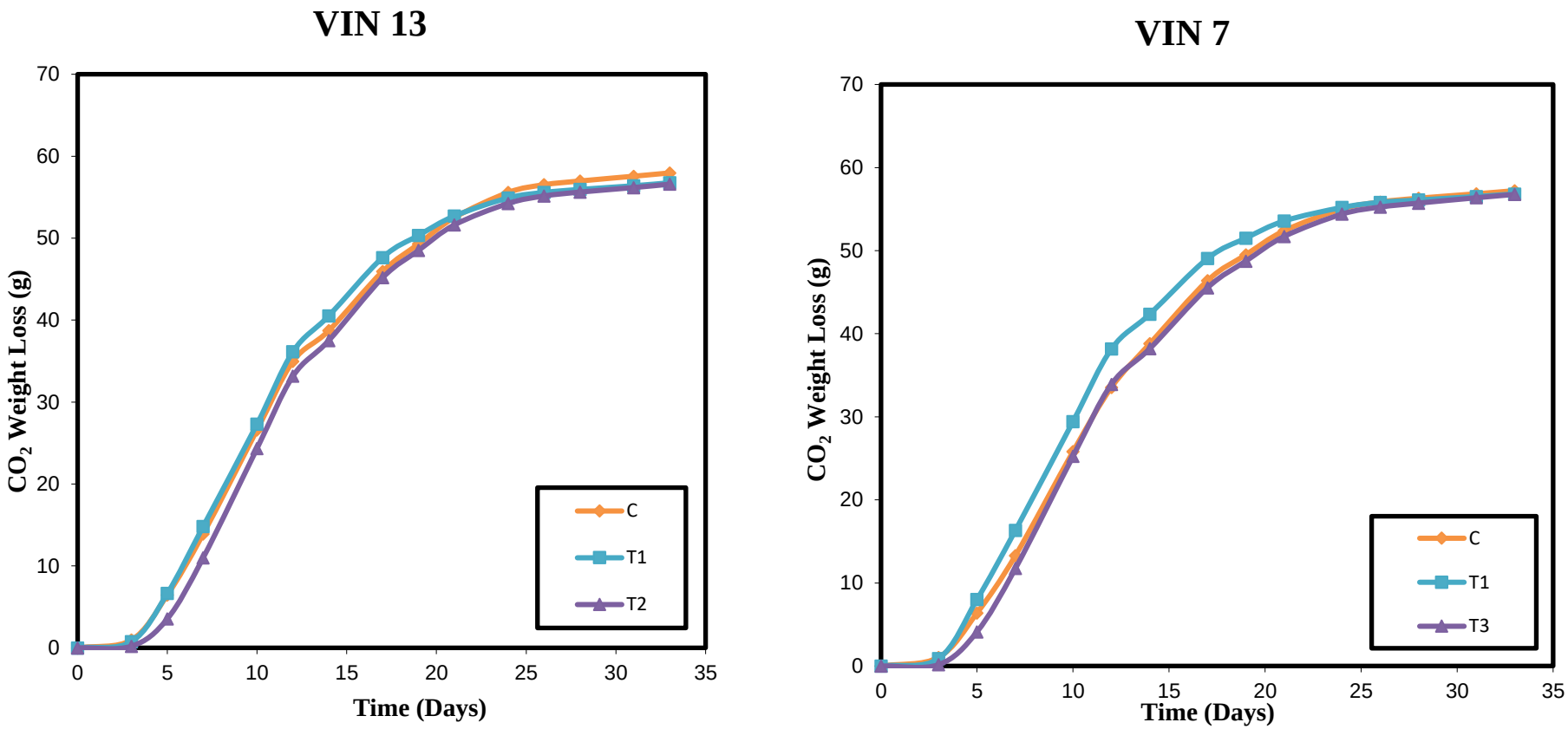


**Figure 3.2.** Experiment A: Fermentation curves of 1x treatment (T1) and 2x treatment (T2) in comparison to the control (untreated) at $15^0$C (Average values of duplicate fermentations).

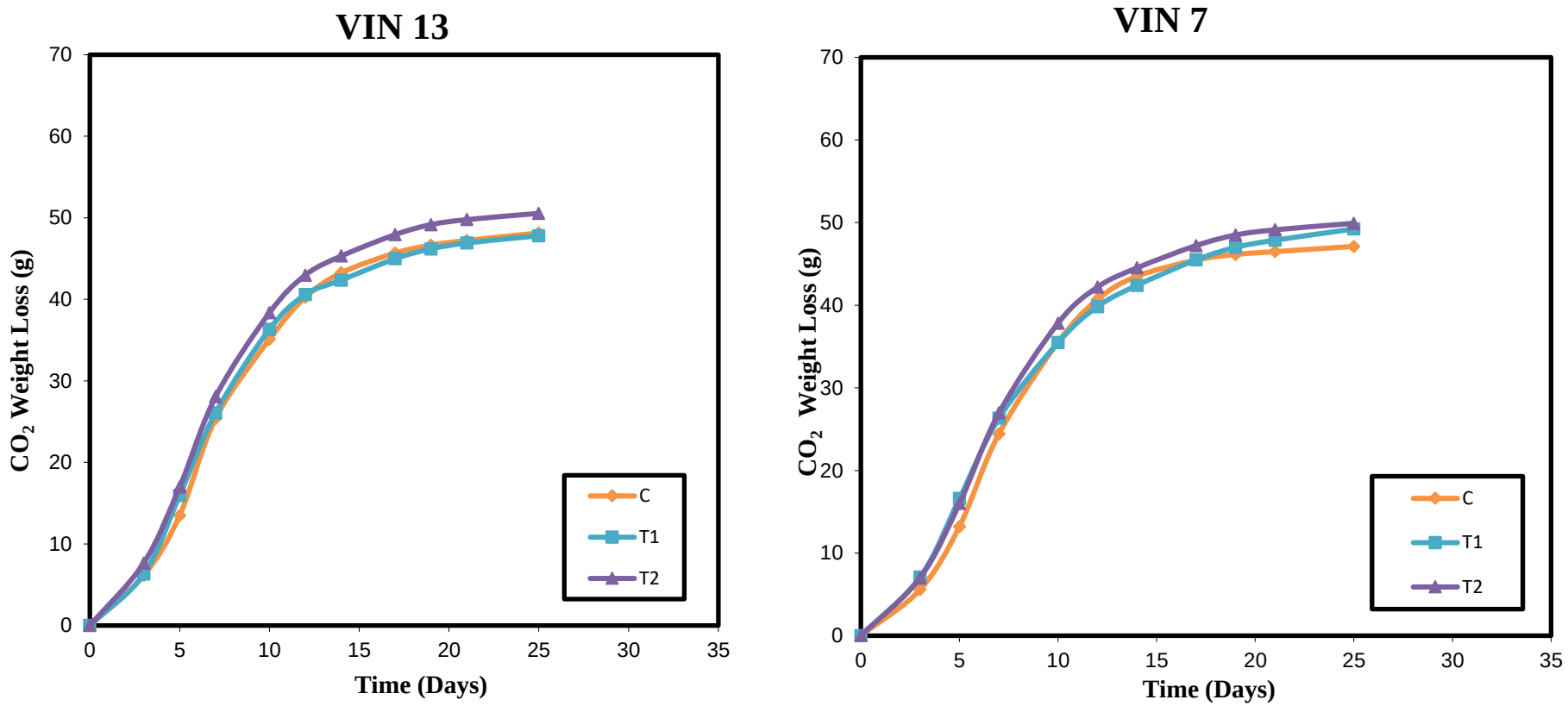


**Figure 3.3.** Experiment B: Fermentation curves of 1x treatment (T1) and 2x treatment (T2) in comparison to the control (untreated) at $15^0$C (Average values of duplicate fermentations).

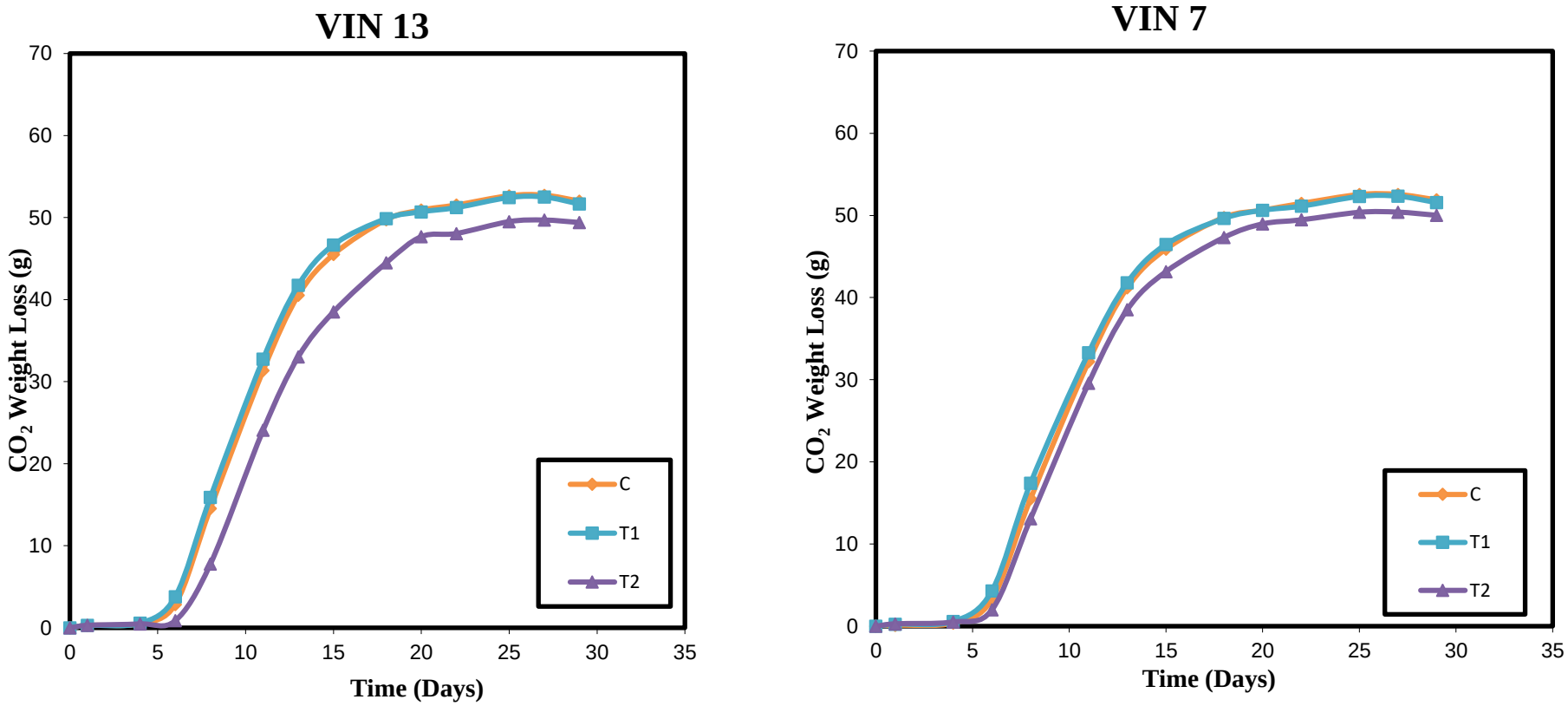


**Figure 3.4.** Experiment C: Fermentation curves of 1x treatment (T1) and 2x treatment (T2) in comparison to the control (untreated) at $15^0$C (Average values of duplicate fermentations).

**Table 3.2.** Chemical profile of Chenin Blanc wines produced in small-scale fermentations at 15ºC[1]

| Experiment | Treatments | Gluc/Fruc ($g\ L^{-1}$) | Total acidity ($g\ L^{-1}$) | pH | Ethanol ($\%^{v}/_{v}$) | Volatile acidity ($g\ L^{-1}$) |
|---|---|---|---|---|---|---|
| A | Control (C) | 0 ± 0 | 3.9 ± 0.2 | 3.2 ± 0.2 | 14.0 ± 0.2 | 0.0 ± 0.0 |
| | 1x Treatment (T1) | 0 ± 0 | 4.2 ± 0.6 | 3.1 ± 0.1 | 14.0 ± 0.2 | 0.1 ±0.0 |
| | 2x Treatment (T2) | 0 ± 0 | 4.4 ± 0.2 | 3.1 ± 0.0 | 13.0 ± 0.9 | 0.1 ±0.0 |
| B | C | 0 ± 0 | 5.1 ± 0.6 | 3.1 ± 0.0 | 11.0 ± 0.5 | 0.1 ± 0.2 |
| | T1 | 0 ± 0 | 5.0 ± 0.1 | 3.3 ± 0.1 | 12.0 ± 0.3 | 0.1 ± 0.1 |
| | T2 | 0 ± 0 | 4.7 ± 0.7 | 3.5 ± 0.2 | 13.0 ± 0.3 | 0.1 ± 0.1 |
| C | C | 0 ± 0 | 6.2 ± 0.5 | 3.3 ± 0.1 | 13.0 ± 0.6 | 0.2 ± 0.0 |
| | T1 | 0 ± 0 | 6.2 ± 0.4 | 3.2 ± 0.1 | 13.0 ± 1.3 | 0.2 ± 0.0 |
| | T2 | 0 ± 0 | 5.8 ± 0.2 | 3.3 ± 0.0 | 12.0 ± 1.1 | 0.2 ± 0.0 |

[1] Means ± standard deviation (n=3)

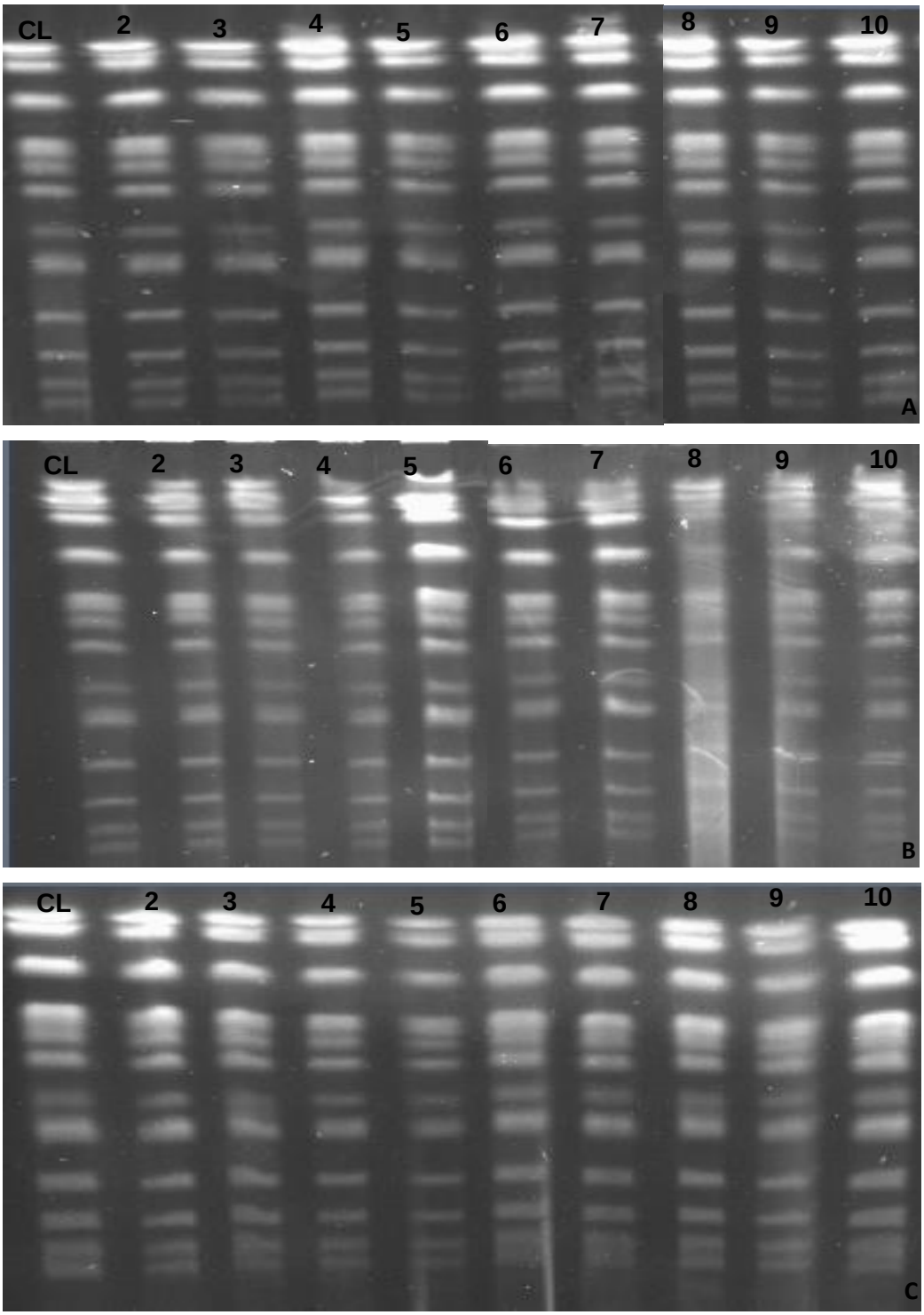


**Figure 3.5.** CHEF DNA profiles of the samples collected at the end of fermentation. Experiment A, Lane 1: *S. cerevisiae* (control), Lanes 2-10: End of fermentation samples. Experiment B, Lane 1: Control, Lanes 2-10: End of fermentation samples. Experiment C, Lane 1: Control, Lanes 2-10: End of fermentation samples.

### 3.5. Conclusion

It was concluded that fungicide treatments for Experiment A, B and C had no notable negative effects on the fermentation rate and overall quality of the wines produced. Additionally, wine chemical parameters also showed no significant difference between control and the different treatments. Furthermore, descriptive sensory evaluation of wines showed no significant difference between treatments and the respective controls except for the control of Experiment B, where the “Colour” and “Tree fruit” were significantly different to the treatments. It was also seen that for Experiment A, “Wine Foreign” aromas were perceived, but this was the same for the control as well. Therefore, it cannot be attributed to effects of the fungicides, but rather winemaking or viticultural factors. Wines for Experiment B and C did not associate with “Wine Foreign” aromas. However, wine foreign aromas are not necessarily undesirable, as it could be pleasant aromas not usually associated with Chenin Blanc wines. Overall, MFA results showed clear clusters between the different experiments. It will still, however, be necessary to conduct future studies to investigate whether fungicide treatments have an effect on the wine aroma enhancing metabolites, e.g. thiols, as well as yeast protein expression responsible for these metabolites, and ultimately their contribution to the overall quality of wines produced.

**Table 3.3.** ANOVA sensory analysis of Chenin Blanc wines produced in small-scale fermentations.

| Experiment | Treatment | Colour | Tree Fruit | Tropical | Wine Foreign | Overall Quality |
|---|---|---|---|---|---|---|
| A | Control (C) | 69.9a[1] | 54.3a | 57.4a | 20.7a | 52.1a |
| | 1X Treatment (T1) | 69.7a | 53.8a | 59.9a | 22.5a | 54.7a |
| | 2X Treatment (T2) | 71.4a | 55.2a | 56.7a | 21.1a | 56.0a |
| B | C | 76.4ab | 48.7ab | 51.7a | 15.5a | 57.3a |
| | T1 | 79.3a | 53.0a | 49.9a | 19.9a | 58.8a |
| | T2 | 76.3a | 45.5a | 47.0a | 16.0a | 58.0a |
| C | C | 76.8a | 47.6a | 52.4a | 15.9a | 58.59a |
| | T1 | 77.8a | 51.3a | 52.7a | 13.9a | 62.7a |
| | T2 | 74.1a | 46.9a | 53.3a | 15.9a | 57.2a |

[1] Values within columns followed by the same letter do not differ significantly ($p > 0.05$)

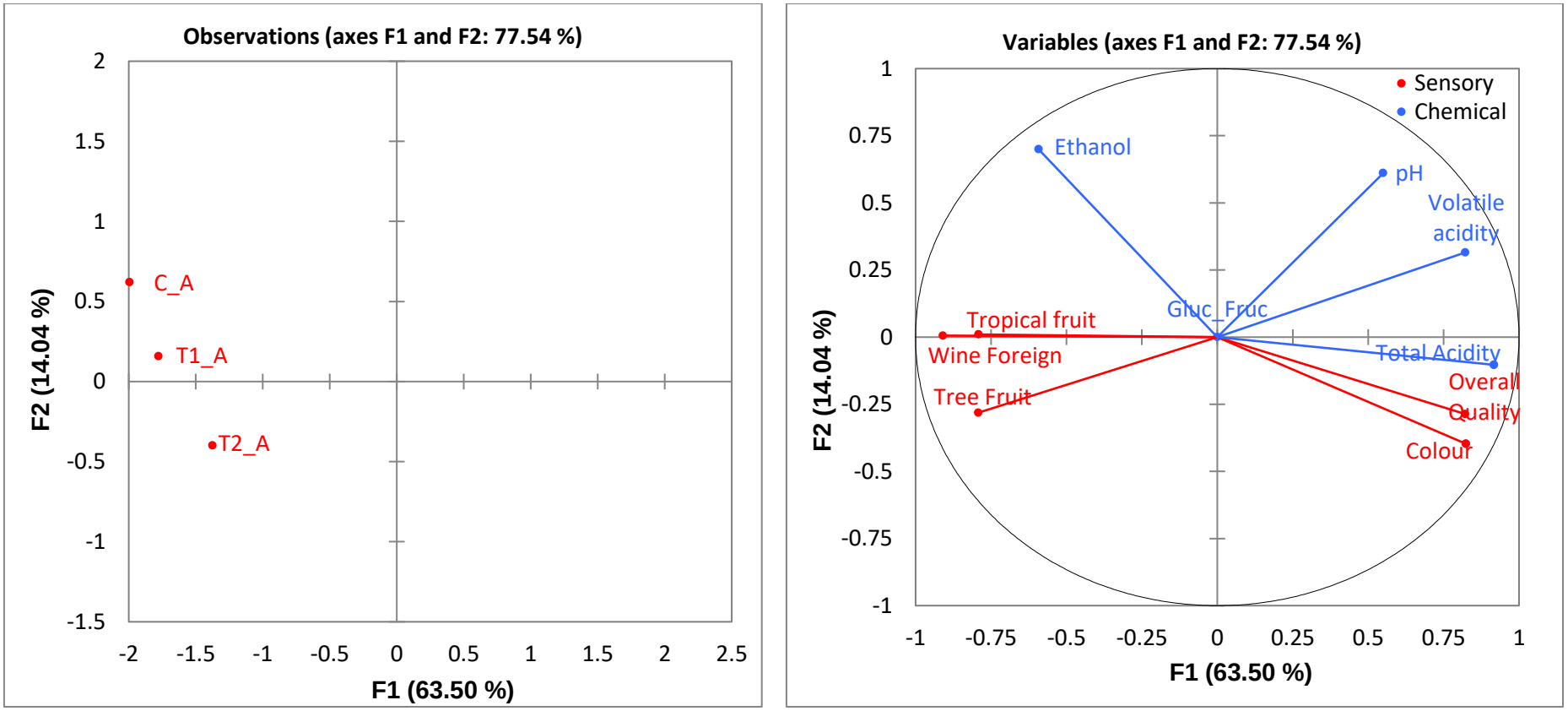


**Figure 3.6.** Experiment A: Multiple Factor Analysis (MFA) of chemical and sensory parameters of 2016 Chenin Blanc wines. This illustrates MFA bi-plots that were constructed to observe the correlation between FTIR spectroscopy (i.e. chemical parameters: total sugar, pH, ethanol, TA, MA and VA) and descriptive sensory analyses (i.e. aroma profiles) compared to chemical spray treatments (i.e. Control [C], 1x treatment [T1] and 2x treatment [T2]).

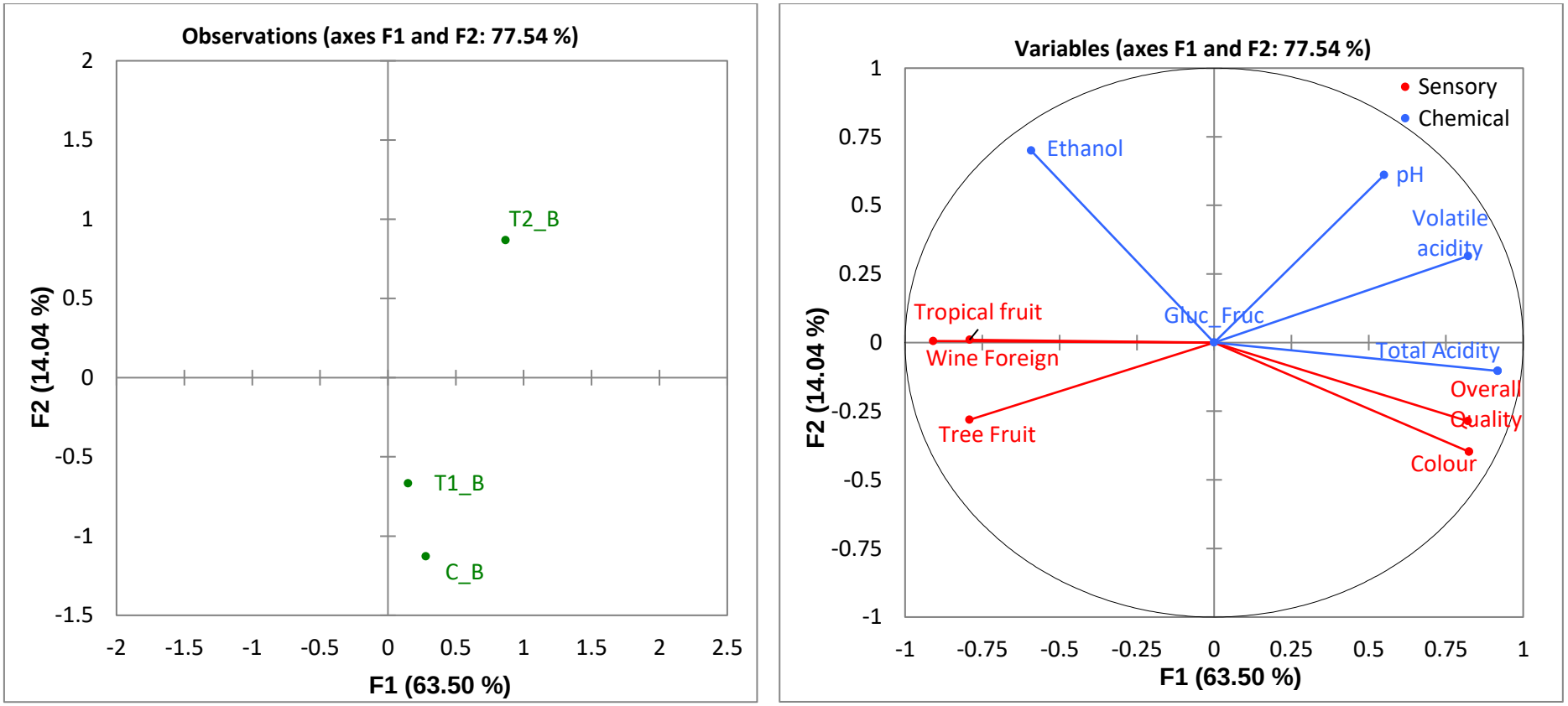


**Figure 3.7.** Experiment B: Multiple Factor Analysis (MFA) of chemical and sensory parameters of 2016 Chenin Blanc wines. This illustrates MFA bi-plots that were constructed to observe the correlation between FTIR spectroscopy (i.e. chemical parameters: total sugar, pH, ethanol, TA, MA and VA) and descriptive sensory analyses (i.e. aroma profiles) compared to chemical spray treatments (i.e. Control [C], 1x treatment [T1] and 2x treatment [T2]).

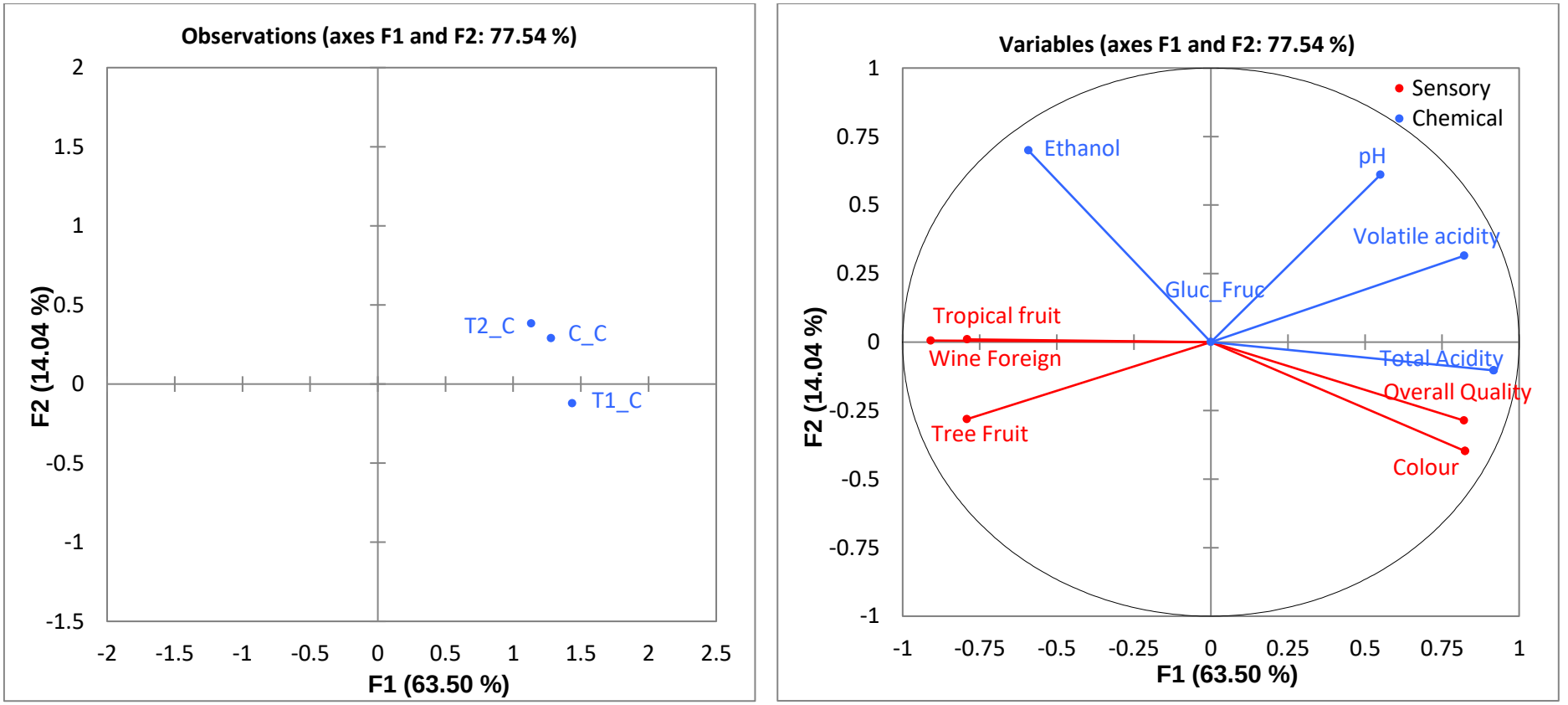


**Figure 3.8.** Experiment C: Multiple factor analysis (MFA) of chemical and sensory parameters of 2016 Chenin Blanc wines**.** This illustrates MFA bi-plots that were constructed to observe the correlation between FTIR spectroscopy (i.e. chemical parameters: total sugar, pH, ethanol, TA, MA and VA) and descriptive sensory analyses (i.e. aroma profiles) compared to chemical spray treatments (i.e. Control [C], 1x treatment [T1] and 2x treatment [T2]).

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CB4

## APPENDIX A

## WINE SCORING SHEET

Judge: _____ Date: _____

Cultivar: **Chenin Blanc** Wine number: 58

Judge the wine on the following line-scales:

| | Unacceptable | VISUAL | Excellent |
|---|---|---|---|
| **Colour** | _____ | | |

**FLAVOUR (NOSE/TASTE INTENSITY)**

| | Undetectable | Prominent |
|---|---|---|
| **Tree fruit** | _____ | |
| **Tropical fruit** | _____ | |
| **Wine foreign** | _____ | |

**TASTE (INTENSITY)**

| | Thin | Full |
|---|---|---|
| **Body (mouthfeel)** | _____ | |

| | Low | Balanced | High |
|---|---|---|---|
| **Acidity** | _____ | | |

**OVERALL QUALITY**

| | Unacceptable | Excellent |
|---|---|---|
| **Overall quality** | _____ | |

*Comments:* _____

_____

_____

**Describing term:**

| | | |
|---|---|---|
| Vegetative | - | Herbaceous, green grass, green pepper, *Eucalyptus*, mint, green beans, asparagus, green olive, black olive, artichoke, straw, tea, tobacco. |
| Tree fruit | - | Cherry, apricot, peach, apple. |
| Tropical fruit | - | Pineapple, musk-melon, banana, guava. |
| Dried fruit | - | Raisin, prune, peach, fig |
| Caramel | - | Honey, butter caramel, butter. |

# APPENDIX B

## Consent form for sensory evaluation


**Consent form** **Set no** [ ]

RESEARCH PARTICIPANT CONSENT FORM
Wine Evaluation
Ms N. Dzedze
(Department of Food Science and Technology/Cape Peninsula University of Technology)

Purpose of Research: To compare the wines to a standard.

Specific Procedures to be used: Sensory evaluation of wines

Duration of Participation: The wine evaluation will be conducted on one day. This will involve a tasting sessions of about 1 hour in the experimental cellar.

**Benefits to the Individual: Participants will have the satisfaction in knowing they have assisted with this research project.**

Risks to the Individual: The risk in tasting these wine samples will be no greater than tasting wine purchased in the retail market. The wine samples contain alcohol and sulphur.

Medical Liability: I understand that no financial compensation will be paid to me in connection with any physical injury or illness in the unlikely event of physical injury or illness as a direct or indirect result of my participation in this sensory project.

Confidentiality: Participants are not required to divulge any confidential data.

Voluntary Nature of Participation: You do not have to participate in this research project. If you do agree to participate you can withdraw your participation at any time without penalty.

Human Subject Statement:
If you have any questions about this research project, contact Ms N. Dzedze Tel: 021-8093144 or Ms V van Breda Tel: 021-8093039.

_____
Researcher's Signature

I HAVE HAD THE OPPORTUNITY TO READ THIS CONSENT FORM, ASK QUESTIONS ABOUT THE RESEARCH PROJECT AND AM PREPARED TO PARTICIPATE IN THIS PROJECT.

_____ 06 & 07/Sept/2016
Participant's Signature Date

_____
Participant's Name (print clearly)

# CHAPTER 4: PROTEINS EXPRESSED AND METABOLITES RELEASED DURING FERMENTATION

## 4.1. Abstract

It was previously reported that wine yeast expressed proteins play a significant role in the release of aroma enhancing compounds such as volatile thiols during fermentation. Hence fermenting small-scale wine samples collected at the start (lag phase) and at end of fermentation (stationery phase) were subjected to protein extraction, quantification and characterisation in order to investigate fermenting wine yeast protein. The SDS-PAGE showed no noticeable difference in protein band distribution and intensity between proteins extracted from control Chenin Blanc grapes and treated Chenin Blanc grapes for both the start and end of the fermentations (1x treatment (T1) and 2x treatment (T2)). However, at the end of fermentation, higher protein intensity was observed within the 50 to 75 kDa range for 2x treatment. Therefore, the 2x fungicide treatment used could have affected wine yeast protein expression, as this was a variable. Moreover, SDS-PAGE showed difference in protein band intensity between the control, 1x treatment (T1) and 2x treatment (T2) samples taken at the start of fermentation compared to their corresponding sample taken at the end of fermentation, respectively. This observation indicated that the yeast starter culture produced different proteins, due to changes that occurred in the grape must matrix as the fermentation progressed. Therefore, the fungicide treatment used could have affected wine yeast protein expression, as this was only variable. Overall, similar observations were seen for Experiment B and C.

The matrix-assisted laser desorption ionization with the time of flight mass spectrometry (MALDI-TOF/MS) showed noticeable difference in protein mass spectra between proteins extracted from control Chenin Blanc grapes and treated Chenin Blanc grapes for both the start and end of the fermentation (1x treatment (T1) and 2x treatment (T2)). Therefore, this indicates that the yeast starter behaved differently as a result of the treatment differing from the control, as well as difference in the treatments applied. Peptide mass fingerprinting (PMF) in conjunction with MALDI-TOF/MS characterised two over-expressed protein in the control at the start and end fermentation, namely Phosphoglycerate kinase (involved in glucose and fructose metabolism) and Polymerase suppressor protein 2 (a suppressor of wine yeast DNA

polymerases and/or DNA replication). Both observations resonate with the fact that the grape must used had relatively high glucose and fructose levels (23 ºBrix). Secondly, it can cautiously be said as the fermentation was in the stationary phase, yeast cell proliferation was inhibited by expressing this enzyme that suppresses DNA replication.

Wine aroma compounds, namely esters, higher alcohols and total fatty acids for Experiment A, B and C showed no significant difference for both treatments (1x treatment (T1) and 2x treatment (T2)) compared to the control. Overall, all treatments for all three Experiments (A, B & C) induced differential protein expression as well as releasing and producing different levels of aroma compounds (metabolites) relative to their controls. The sensory evaluation panel comprising trained and experienced wine judges did not perceive any negative or wine foreign aroma and flavours in these wines. Therefore, even though differences were observed in the protein expression, these results showed that the fungicide treatments did not produce wines that were significantly different to their respective controls.

### 4.2. Introduction

Wine proteins are usually a mixture derived from the grape proteins and yeast derived-proteins (Lamikanra & Inyang, 1988). Grapes contain pathogenesis-related protein (PR), specifically thaumatin-like protein and chitinases. Moreover, the grapes also contain numerous protein-based fruit aroma precursors that largely contribute to aroma during winemaking (Belancic *et al.,* 2003; Caridi, 2006; Ndlovu, 2012; Rodrigues *et al.,* 2012; Gazzola *et al.,* 2017). Protein from yeast is found in the cell wall which consists of the flexible network of β-1,3-glucan molecules with covalently attached β-1,6-glucan and chitin, and an external fibrillar layer of mannoproteins that have mannose glycoproteins and make 35 – 40% of the cell wall (Caridi, 2006). Amongst these proteins, mannoproteins have a significant effect on the wine organoleptic profile since it plays a major role in the overall wine quality. Moreover, the mannoproteins assist in surface assimilation of undesirable contaminants, yeast flocculation and autolysis (Caridi, 2006; Braconi *et al.,* 2011; Comuzzo *et al*., 2011; Juega *et al.*, 2012; Mostert & Divol, 2014). They also have a significant role in wine characteristics and processing such as membrane filtration and tartrate stabilization (Gonçalves *et al*., 2002; Howell, 2012; Ndlovu, 2012; Rodrigues *et al*., 2012). Protein concentrations in wine are low compared to the must because of the proteolytic activity

and pH changes which cause protein denaturation during fermentation. The proteins in must are found in heterogeneous shapes and ranges from 10 to 100 kDa, while grape proteins range from 14 to 60 kDa (Gonçalves *et al.,* 2002; Sauvage *et al.,* 2010). However, Gonçalves *et al*. (2002) conducted a study on characterization of mannoproteins in white wine and found mannoproteins with molecular masses of 53.4, 252 and 560 kDa respectively. These were found to have different chemical compositions since the highest molecular mass had 10% protein and 90% mannose, while the lowest contained 87.5% mannose and 2.5% protein.

The wine yeast *Saccharomyces cerevisiae* contributes to overall wine flavour and aroma by producing and releasing aroma enhancing metabolites during fermentation (Ugliano & Henschke, 2009; Hart *et al*., 2017 a & b). These synthesised metabolites includes esters, higher alcohols, volatile fatty acids, carbonyls and volatile sulphur compounds. However, their effects on the wine differs between yeast strains, due to the yeast starter culture's fermentation potential based on the grape must composition and fermentation conditions. Esters contribute mostly to fruitiness in the sensory profile of young wines. The specific compounds responsible for the fruitiness is acetate esters (i.e. ethyl acetate, active amyl and isoamyl acetate) and ethyl fatty acid esters (i.e. ethyl C3- ethyl C12) (Moio *et al*., 2004; Escudero *et al*., 2007; Ugliano & Henschke, 2009). Higher alcohols are the most important compounds produced by yeast during alcoholic fermentation and contribute to wine complexity in concentrations below 300 mg $L^{-1}$. However, at concentrations above 300 mg $L^{-1}$ they can have a negative effect on wine quality (Swiegers *et al*., 2005; Ugliano & Henschke, 2009). Volatile fatty acids consist of small, medium, long chain and branched-chain fatty acids. The most important fatty acid compound is acetic acid because it has a significant role in wine quality (Ugliano & Henschke, 2009). Additionally, aromatic metabolites, namely volatile thiols, also contribute to flavour and aroma of wine with odours such as "grapefruit," "passion fruit," and "boxwood". The aromatic volatile thiols of importance in white wine are 4-mercapto-4-methyl pentane-2-one (4MMP), 3-mercaptohexan- 1-ol (3MH), and 3-mercaptohexyl acetate (3MHA) (Holt *et al*., 2011; Herbst-Johnstone *et al*., 2013; Du Toit *et al.*, 2015; Piano *et al.,* 2015; Araujo *et al*., 2016).

Aroma and flavour are critical to the overall quality of the wine (Perestrelo *et al*., 2006; Li *et al.,* 2008; Villamor & Ross, 2013). The flavours and aromas originate from metabolites produced during the fermentation process and derive both from the

grapes and the yeast. During the formation and release of these aroma and flavour compounds, enzymes are involved (Perestrelo *et al.,* 2006; Li *et al*., 2008; Villamor & Ross, 2013). However, research done so far, focused predominantly on white cultivars such as Sauvignon Blanc and currently there was no study investigating metabolites in Chenin Blanc wines (Darriet *et al*., 2001; Masneuf-Pomarède *et al*., 2006; Tominaga *et al*., 2006; Parr *et al*., 2007; Swiegers *et al*., 2009; Allen *et al*., 2011). A study done by Wilson (2017) only included commercial Chenin Blanc wines comparing the levels of 3MHA and 3MH in response to different aspects (such as the use of barrels in vinification, wine age amongst others) of the wines.

Moreover, not much research in yeast proteome and how it may affect wine properties had been published with most research focusing on proteins responsible for haze formation and prevention, and foam stability in sparkling wines (Pocock *et al*., 2007; Rossignol *et al*., 2009; Falconer *et al*., 2010; Blasco *et al*., 2011; Marangon *et al.,* 2011; Juega *et al*., 2012).

Within the aforementioned background, Chenin Blanc is known for tropical aromas due to the presence of volatile thiols. However, these thiols can be released by yeast expressed proteins. Therefore, the aim of this study was to compare the profiles of proteins and metabolites released during fermentation, from grapes treated with both chemical and natural fungicides (control, 1x and 2x treatments) to identify their effect on the overall flavour and aroma of wines produced and ultimately the quality.

## 4.3. Material and methods

### *4.3.1. Protein analysis*

#### *4.3.1.1. Buffers and solutions*

##### *4.3.1.1.1. Lysis buffer*

The lysis buffer comprised 9.306 g of ethylene diamine tetra-acetic acid (EDTA) (Saarchem, South Africa) and 2 g NaOH (Merck, South Africa), dissolved in 1 L distilled water ($dH_2O$). The 2% sodium dodecyl sulfate (SDS) buffer comprised 10 g of SDS (Sigma-Aldrich, South Africa) and 10 mL β-mercaptoethanol (2% $^{v}/_{v}$) dissolved in 500 mL de-ionised water ($ddH_2O$) (Von Der Haar, 2007). The 4 M acetic acid buffer was prepared by adding 116.72 mL acetic acid (AnalaR (BHD) to de-ionised water to make up to a volume of 500 mL. All buffers were diluted to 500 mL in volumetric flasks with de-ionised water.

*4.3.1.1.2. SDS loading buffer*

The 1 M Tris hydrochloride (Tris-HCl) was prepared by weighing 12.114 g of Tris-HCl (Merck, SA) and dissolving it in 1 L distilled water ($dH_2O$). The 10% SDS solution was prepared by weighing 10 g SDS and dissolving it in 100 mL $dH_2O$. The 1% Bromophenol blue (BPB) solution was prepared by weighing 1 g BPB and dissolving it in 100 mL $dH_2O$. Thereafter, 1 mL of 1 M Tris-HCl, 4 mL of 10% SDS, 2 mL glycerol, 2.5 mL β-mercaptoethanol and 500 µl 1% BPB, was aliquoted into a 500 mL volumetric flask to make up 1 L SDS loading buffer.

*4.3.1.1.3. Staining solution*

Staining solution was prepared by weighing 1 g of Coomassie® Brilliant Blue (CBB) R – 250 (Merck, South Africa) into a 1 L autoclaved beaker stationed on a Thermolyne Cimarec 2 Magnetic Stirrer containing 500 mL de-ionised water ($ddH_2O$) and a stirrer bar. Thereafter, 400 mL methanol (Merck, South Africa) and 100 mL glacial acetic acid (Merck, South Africa) was added while continuously stirring to ensure a homogenous solution. The staining solution was then filtered through Whatman® filter paper into an autoclaved volumetric flask and stored at room temperature.

*4.3.1.1.4. Destaining solution*

Destaining solution was prepared by adding 700 mL $ddH_2O$ into a 1 L autoclaved beaker with a stirrer bar stationed on a Thermolyne Cimarec 2 Magnetic Stirrer. Thereafter, 200 mL methanol (KIMIX, South Africa), and 100 mL glacial acetic acid (Merck, South Africa) was gently added to $ddH_2O$ and solution was mixed by stirring for 5 minutes and transferring to an autoclaved volumetric flask.

*4.3.1.2. Grape juice sampling and protein extraction*

Fifty mL fermenting Chenin Blanc grape must (juice) were sampled aseptically at two day-intervals using food-grade $CO_2$ gas. Thereafter, 2 mL of the ferments were aliquoted into centrifuge tubes and centrifuged at 13000 rpm for 30 seconds to harvest yeast cells. The supernatant was discarded and the previous steps repeated until the yeast pellet weighed at least 50 mg. The pellet was re-suspended in 400 µl lysis buffer and the cell suspension was heated for 10 minutes at 90ºC to aid with the rupturing of

the yeast cells. Thereafter, 10 µl of acetic acid was added to the lysates, each sample was vortexed for 30 seconds and heated for a further 10 minutes at 90ºC.

*4.3.1.3. Protein precipitation*

Acetone was added to yeast cells at a ratio of 1:4 in an acetone-compatible tube. The mixture was vortexed and incubated overnight at -20ºC. The following day the mixture was centrifuged at 13000 rpm for 10 minutes. The supernatant was carefully discarded to avoid dislodging of the protein pellet and the tubes left open for 30 minutes at room temperature to allow the acetone to evaporate. Once the acetone was completely evaporated, the phosphate buffer was added to the pellet and it was roughly vortexed to dissolve the protein pellet.

*4.3.1.4. Protein quantification*

Protein concentration was determined using Bradford assays, as well as the NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Darmstadt, Germany) as a verification method. Bradford assay samples were prepared using Table 4.1. Thereafter, samples were incubated at room temperature for 5 minutes and the absorbance measured at 595 nm using a UV-visible spectrophotometer (CECIL, United Kingdom).

**Table 4.1.** Sample preparation for the Bradford protein assay analysis (adapted from Von Der Haar, 2007).

| Test sample | Sample (µl) | Water (µl) | Bradford reagents (µl) |
|---|---|---|---|
| Blank | 0 | 200 | 800 |
| BSA[1] standard (10 µg/mL) | 20 | 180 | 800 |
| BSA standard (20 µg/mL) | 40 | 160 | 800 |
| BSA standard (30 µg/mL) | 60 | 140 | 800 |
| BSA standard (40 µg/mL) | 80 | 120 | 800 |
| BSA standard (50 µg/mL) | 100 | 100 | 800 |
| Protein sample | 100 | 100 | 800 |

[1]Bovine Serum Albumin (BSA) (Sigma-Aldrich,Germany) used for standard curve calibration.

*4.3.1.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)*

Protein quality was determined using SDS-PAGE as previously described by Mostert (2013). Fifty ml polypropylene centrifuge tube were used to mix reagents required for

the polyacrylamide stacking and separation shown in Table 4.2. The mixture was carefully cast between two glass plates mounted in a mini-PROTEAN pre-casting chamber (Bio-Rad, South Africa). Thereafter, isopropanol was gently added to the top layer of the separation gel to ensure that it was smooth upon solidification and to prevent the formation of air bubbles. Once the separation gel solidified, the isopropanol was discarded and gel rinsed with $dH_2O$ prior to the addition of the stacking gel. Thereafter, the stacking gel was added between the glass plates and a ten-well comb was inserted before the stacking gel solidified.

The glass plates containing the solidified gels were transferred and mounted in the holding brackets of a mini-PROTEAN electrophoresis system (Bio-Rad, South Africa) that was in itself a mini-reservoir. The mini-resevoir was then mounted into the larger reservoir of the mini-PROTEAN electrophoresis system. The mini-resevoir was carefully filled with 1x SDS running buffer until it overflowed, and this was continued until the larger resevoir was filled to at least 50% of its capacity. Gentle filling of reservoirs minimises bubble formation which could interfere with protein separation on the SDS-PAGE. Subsequently, the ten-well comb was gently removed and protein samples were prepared by transferring ~10 µg of protein into a micro-centrifuge tube containing 5 µL 2x SDS loading buffer. The mixture was then briefly vortexed, centrifuged and boiled for 5 minutes at 94ºC prior to loading into the wells. The Precision Plus protein molecular weight marker (Bio-Rad, South Africa) was also heated prior to loading 3 µL into the appropriate well. The protein gels were initially run at 100 V for 20 minutes to stack proteins in stacking gel, whereafter the voltage was increased to 150 V to separate the proteins. The SDS-PAGE was stopped once the BPB dye migrated to the bottom of the gel. Gels were gently removed from glass plates with tap water, whereafter, they were transferred to a Tupperware container to be subjected to staining.

*4.3.1.6. Staining and destaining*

The SDS-PAGE gels were stained with Coomassie blue for 1 h. Thereafter, the Coomassie stain was poured off and the gel rinsed twice with de-ionised water to remove the excess staining solution. Gels were destained for 1 h using the destaining solution. To achieve even de-staining, pieces of laboratory paper towel were tied into knots and placed around the gel.

**Table 4.2.** Resolution (12%) and stacking (5%) gel preparation for the determination of protein bands (Von Der Haar, 2007).

| Content | Resolution gel (12%) | Stacking gel (5%) |
|---|---|---|
| Water | 4.3 mL | 3.0 mL |
| Acrylamide | 3.0 mL | 0.63 mL |
| Tris | 2.5 mL | 1.25 mL |
| 10% SDS (Sodium dodecyl sulfate) | 100 µl | 50 µl |
| 10% APS (ammonia persulphate) | 100 µl | 50 µl |
| Temed (Tetramethylethylenediamine) | 10µl | 5 µl |
| **Total** | **10 mL** | **5 mL** |

Thereafter, the stained laboratory paper towel pieces were discarded and replaced with fresh ones and the gel was de-stained overnight. After 24 hours, selected protein bands were cut using sterile blade from the SDS-PAGE gels for conducting Nanoscale Liquid Chromotography coupled to tandem mass spectrometry (Nano LC/MS) and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass spectrometry (MALDI-TOF/MS) analyses.

*4.3.1.7. In-gel digest and peptide extraction*

The 1D SDS-PAGE gels were placed on a clean glass plate and bands were cut out using a sterilized scalpel after which it was transferred to labelled 2 mL Eppendorf tubes. Following cutting, the gel pieces were de-stained by adding 200 µL of 50% acetonitrile (ACN)/25 mM ammonium bicarbonate (Sigma-Aldrich, Germany) and vortexed until they were clear. The destaining solution was removed and the gel pieces dried for 1 minute in a speed vacuum to remove the excess destaining solution. Thereafter, 100 µL of 2 mM Tris-carboxyethyl phosphine (TCEP) (Sigma-Aldrich, Germany)/25 mM ammonium bicarbonate and 100 µL of 50 mM iodoacetamide (Sigma-Aldrich, Germany) were added. The mixture was vortexed and placed in a dark room at temperature (20 – 25°C) for 15 minutes. The liquid was removed and the gel were speed-vacuumed for 2 minutes to remove the excess liquid, thus drying the gel strips. Thereafter, the gel pieces were rehydrated with 50 µL trypsin and stored on ice for 60 minutes. The trypsin (Promega, USA) was removed and the gel pieces were re-suspended in 70 µL of a 25 mM ammonium bicarbonate. The sample tubes were then wrapped with parafilm to avoid evaporation. Thereafter, the samples were incubated overnight at 37°C. The following day, 70 µL of trifluoroacetic acid (TFA) was added

and the suspension incubated for 45 minutes at room temperature. After incubation, the solution was removed from the gel pieces, placed in new sample tubes and speed-vacuumed until dry. The samples were rehydrated with 10 µL of 0.1% TFA and placed in a shaker for 5 minutes. Thereafter, the ZipTip clean-up process was performed as follows; a wetting step using 20 µL acetonitrile, an equilibrating step using 20 µL of 0.1% TFA, a binding step using 2 µL sample and 8 µl 0.1% TFA, a washing step using 20 µL 0.1% trifluoroacetic acid (TFA) and an eluting step using thioanisole (TA) 50 (50% ACN, 0,1% TFA). The sample tubes were speed-vacuumed until dry and re-suspended in 15 µL 0.1% TFA and stored at -80°C until further use.

*4.3.2. Metabolite analysis by GC – FID*

*4.3.2.1. Wines*

Twenty-seven Chenin Blanc wines were analysed by GC – FID for esters, fatty acids, higher alcohols and GC – MS for thiol compounds. The wines were produced from grapes treated with fungicides as mentioned in the previous chapter. Wines were stored at 4°C in the research cellar following the vinification process.

*4.3.2.2. Chemicals and standards*

The following internal standards were prepared by dispensing aliquots of 50 – 150 µL into 250 mL volumetric flasks and diluting to the mark using methanol:

Esters: Ethyl acetate, ethyl-3-hydroxybutanoate, ethyl phenylacetate, ethyl propionate, 2-methyl propyl acetate, ethyl decanoate (ethyl caprate), ethyl octanoate, ethyl 2-methylbutyrate, ethyl isovalerate, ethyl hexanoate, ethyl-2-methyl propanoate (ethyl isobutyrate) (Sigma-Aldrich, Germany), ethyl lactate, hexyl acetate, ethyl butyrate, butanol, 4-methyl-1-pentanol (internal standard) were purchased from Merck (Darmstadt, Germany).

Fatty acids: propionic acid, octanoic acid, valeric acid, acetic acid, hexanoic acid, butyric acid (Sigma-Aldrich, Germany). Diethyl succinate, Isovaleric acid, isobutyric acid and butyric acid were purchased from Fluka (Buchs, Switzerland).

Higher alcohols: pentanol, n-propanol, 3-methyl-1-pentanol, 1-octen-3-ol, isoamyl alcohol, acetaldehyde, methanol, acetoin, trans-2-hexenol and cis-3-hexen-1-ol (Sigma-Aldrich, Germany). 3-ethoxyl-1-propanol were purchased from Fluka (Buchs, Switzerland). The 2-phenylethanol and 1-hexanol were purchased from Merck (Darmstadt, Germany).

*4.3.2.3. Wine matrix simulant*

The wine matrix simulant is a solution used for the calibration and identification of the volatile compounds such as esters, alcohols and fatty acids. This was prepared by dissolving 2.5 g $L^{-1}$ tartaric acid in 12% $^{v}/_{v}$ ethanol in a 500 mL volumetric flask and making up to the mark with de-ionised water. The pH of the wine matrix simulant was adjusted to 3.5 using 0.1M NaOH (Ortega *et al*., 2001; Louw *et al*., 2010).

*4.3.2.4. Liquid-Liquid extraction*

In a 15 mL centrifuge tube, 10 mL wine, 2 mL diethyl ether and 200 µL 4-methyl-1-pentanol (100 µl of 0.50 mg $L^{-1}$ solution in wine stimulant) (internal standard) were added and vortexed. The mixture was extracted by sonicating for 30 minutes, and vortexing thereafter. The wine/ether mixture was centrifuged (Heraeus centrifuge, Kendro Laboratory Product, Germany) at 4 000 rpm for 5 minutes. After centrifugation, a clear layer was observed at the top of the centrifuge tube. This layer was then transferred to a small vial and sealed tightly with a manual vial cap crimper. The samples were then injected into the GC – FID. For calibration and identification purposes, 10 mL of wine matrix simulant solution was used instead of wine. The range of concentrations tested was between 50 – 5000 µg $L^{-1}$.

*4.3.2.5. Gas chromatographic conditions*

Gas chromatography was conducted using a Trude 1300 GC instrument (Thermo Scientific, Italy) fitted with a polar DB-FFAP capillary column (dimension 60 m length × 0.32 mm diameter × 0.5 µm film thickness) (Agilent Technologies, Wilmington, USA). The GC was also equipped with an auto sampler split or splitless injector (GC analytics, Switzerland) and a flame ionization detector (FID). The initial oven temperature was 45ºC and it was held for 5 minutes, after which it was increased by 3ºC $min^{-1}$ to 100ºC. Once the oven reached 100ºC, it was held for 5 minutes and finally increased to 250°C at 10°C $min^{-1}$, and held for 5 minutes. The sample was injected using the GC injection port at a temperature of 240°C operated in a 5:1 split mode. Helium gas was used as a carrier gas at a flow rate of 1.8 mL $min^{-1}$, and air (400 mL $min^{-1}$) and hydrogen (40 mL $min^{-1}$) were used as a hydrogen-air gas for the FID.

*4.3.3. Thiol analysis*

*4.3.3.1. Chemicals and reagents*

The internal standard 4-methoxy-2-methyl-2-mercaptobutane (4M2M2MB) was purchased from Acros Organics (Geel, Belgium). Hydrochloric acid (37%, reagent grade), sodium hydroxide (pellets, ≥99%, reagent grade) and sodium sulfate anhydrous (powder, extra pure, 98.5 – 100.5%) were obtained from Scharlau (Barcelona, Spain). Ethyl propiolate (ETP) and butylated hydroxyanisole (BHA) were purchased from Sigma-Aldrich. Methanol (HPLC grade, Scharlau, Barcelona, Spain) and dichloromethane (for gas chromatography, SupraSolv®, Merck, Darmstadt, Germany) were used as solvents. The SPE cartridges tested were Supelclean™ENVI-18 (6 mL cartridge volume; 1 g sorbent; Supelco, Castle Hill, NSW, Australia). Nitrogen (food grade) and helium (instrument grade) were sourced from BOC Gases New Zealand Ltd. (Auckland, NewZealand). Solutions were prepared using Grade 1 water (BARNSTEAD® NANOpure DIamond™ Water Purification System, Thermo Scientific, USA) with a resistivity of 18.2 MΩ/cm at 25ºC and absolute ethanol (≥99.5%, Univar, Ajax Finechem, Auckland, New Zealand) (Herbst-Johnstone *et al*., 2013; Araujo *et al*., 2017).

*4.3.3.2. Conditioning the cartridges*

The cartridges were prepared by placing into a filter and twisting a little to open them [LiChrolut EN (40 – 120 µm; 6 mL cartridge volume; 500 mg sorbent (Merck, Darmstadt, Germany)] to allow 10 mL of methanol to pass through at a pressure of 5 kPa. After methanol has passed through, 10 mL of de-ionised water was passed through at the same rate and the cartridge was closed to ensure that the filter did not dry.

*4.3.3.3. Sample preparation*

Chenin Blanc wines were poured into 50 mL volumetric flasks after which it was transferred to a 100 mL beaker containing a stirrer bar. The following chemicals were added respectively; 500 µl BHA 2mM, 50 µL internal standard 4-methoxy-2-methyl-2-mercaptobutane (4M2M2MB), 500 µl ETP 250 mM and the mixture stirred for 5 minutes at 500 rpm. The pH of the mixture was adjusted to 10 ± 0.05 using 10 N sodium hydroxide (NAOH). If over-adjusted, the pH was lowered using 0.1 M hydrochloric acid (HCL). The mixture was then stirred for 10 minutes at 500 rpm after

which it was transferred to 50 mL falcon tubes and centrifuged for 10 minutes at 6000 rpm. The centrifugation step helps to remove the precipitate that was formed during the pH adjustment. The supernatant was transferred to a beaker and the stirrer bar was removed. The entire mixture was then loaded into the conditioned cartridge. The samples were then filtered, and washed using 5 mL de-ionised water. Once the water passed through, the pressure was increased to 10 kPa, after which the filters were allowed to dry for 20 minutes. When the filters were dry, the analyte was recovered by passing 10 mL of dichloromethane through the filters. The eluate was dried on sodium sulphate anhydrous ($Na_2SO_4$) using long flat test tubes. The eluate was then filtered through glass wool using a Pasteur pipette into conical bottom vials. The organic phase was evaporated to 100 µL under Nitrogen gas and transferred into vials with inserts and closed tightly using a vial cap crimper.

*4.3.3.4. GC – MS analysis*

A Thermo Scientific TRACE 1300 gas chromatograph (Santa Clara, CA, USA) was used to perform the GC analyses. The GC was equipped with a Thermo Scientific TSQ 8000 triple quadrupole mass spectrometer (MS) detector. The sample was introduced on a polar Zebron ZB-FFAP capillary column (30 m x 0.25 mm x 0.25 µm, Phenomenex). The instrument was operated with parameters as proposed by Herbst-Johnstone *et al*. (2013), with the following changes: The initial oven temperature was 60ºC, held for 1 minute and it was increased by 25ºC $min^{-1}$ up to 100°C, held for 2 minutes and finally increased to 250°C at 12°C $min^{-1}$, and held for 5 minutes. The sample was injected using the GC injection port that was held at a temperature of 240°C operated in splitless mode with the split flow set at 50 mL $min^{-1}$ for 2 minutes. The gas saver was activated for 5 minutes at 20 mL $min^{-1}$. Helium was used as a carrier gas at a flow rate of 1.2 mL $min^{-1}$. The transfer line and ion source temperatures were both set at 250°C, respectively. The emission current was 75 µA and Argon was used as the collision gas.

## 4.4. Results and discussion

*4.4.1. Protein analyses*

*4.4.1.1. Protein quantification*

A Bovine Serum Albumin (BSA) standard curve with absorbance as a function of protein concentration using the Bradford assay in conjunction with spectrophotometry

(Absorbance (Abs) at 595 nm), showed a linear relationship with $R^2$ = 0.9817, where an increase in protein concentration will cause an increase in the absorbance (Figure 4.1.). The protein yield of the control, 1x treatment (T1) and 2x treatment (T2) for all the experiments was obtained by extrapolating spectrophotometry data (Abs 595 nm) on a BSA standard curve following the Bradford assay and ranged between 115 – 174 µg $\mu L^{-1}$ for Experiment A, 113 – 242 µg $\mu L^{-1}$ for Experiment B and 74 – 224 µg $\mu L^{-1}$ for Experiment C (Figure. 4.2). Fermenting Chenin Blanc grape must collected at the start of the fermentation for Experiment A showed that the 1x treatment (174.9 µg $\mu L^{-1}$) had higher protein expression when compared to 2x treatment (166.4 µg $\mu L^{-1}$) and control (156.9 µg $\mu L^{-1}$) (Figure 4.2). However, at the end of the fermentation, the protein expression was similar for 1x treatment (115.1 µg $\mu L^{-1}$) and 2x treatment (117.1 µg $\mu L^{-1}$), while the control (133.2 µg $\mu L^{-1}$) showed a higher value. Also, Experiment B showed similar results as Experiment A for the samples collected at the start of the fermentation, 237.5, 242.3 and 225.5 µg $\mu L^{-1}$ for the control, 1x treatment (T1), 2x treatment (T2), respectively (Figure 4.2). However, at the end of fermentation the 1x treatment (126.5 µg $\mu L^{-1}$) was slightly higher, while the control (113.6 µg $\mu L^{-1}$) and 2x treatment (113.5 µg $\mu L^{-1}$) were similar. Additionally, Experiment C showed higher protein expression for the control (224.1 µg $\mu L^{-1}$) compared to both treatments (216.4 and 164.8 µg $\mu L^{-1}$ for the 1x treatment (T1) and 2x treatment (T2), respectively) at the start of the fermentation (Figure 4.2). However, both treatments (80.7 and 80.8 µg $\mu L^{-1}$ for the 1x treatment (T1), 2x treatment (T2), respectively) at the end of the fermentation showed similar results while the control (74.3 µg $\mu L^{-1}$) was slightly lower. Moreover, threshold detection for the Coomassie Brilliant Blue G-250 is 30 ng (0.03 µg) (Kang *et al*., 2002), therefore all the samples had adequate proteins in order to proceed with SDS-PAGE assays. In addition to the Bradford test, the protein concentration was also determined by deploying another spectrophotometric approach, i.e. a NanoDrop™ UV-Vis spectrophotometric assay as a complementary approach. These results (not shown), also indicated a sufficiently high protein yield for all yeast strains by recording protein from 0.5 to 20 µg $\mu L^{-1}$ for all samples (Wiśniewski & Gaugaz, 2015).

*4.4.1.2. SDS-PAGE*

With one exception, the SDS-PAGE showed no noticeable difference in protein band distribution and intensity between proteins extracted from control Chenin Blanc grapes

and treated Chenin Blanc grapes for both the start and end of the fermentation (control, 1x treatment (T1) and 2x treatment (T2) of fungicide) (Figure 4.3). The exception was that, at the end of fermentation, higher protein intensity was observed within the 50 – 75 kDa range for the 2x treatment (T2).

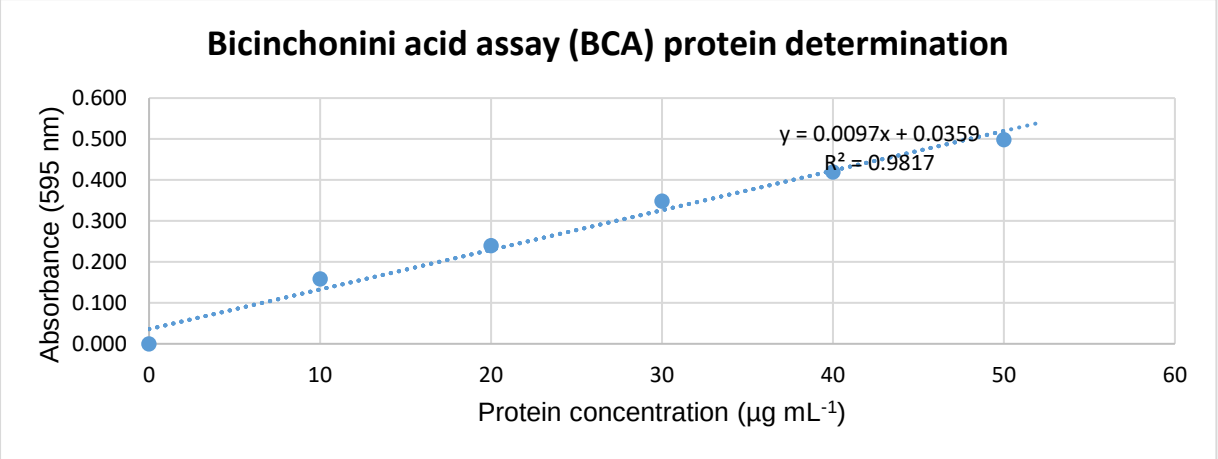
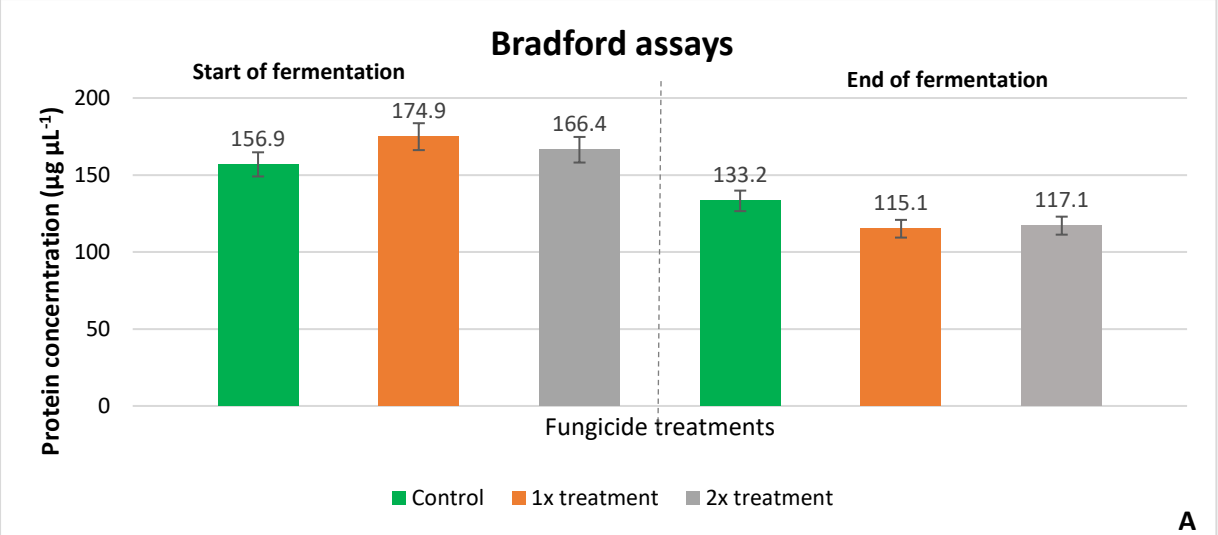
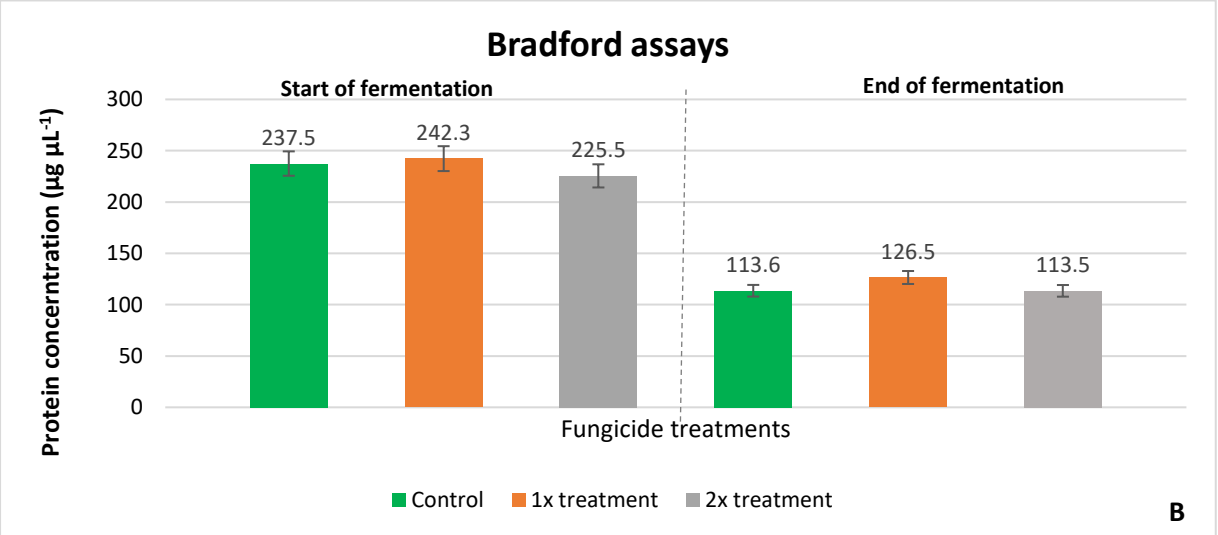



**Figure 4.1.** Bovine Serum Albumin (BSA) standard curve generated using Bradford assay spectrophotometric data measured at Abs 595 nm.

It is noteworthy that this occurred for Experiments A, B and C (Figure 4.3). This difference observed in protein band intensity between start and end of fermentation is suggestive of differential protein expression by the yeast starter culture possibly due to changes that occurred in the grape must matrix as the fermentation progressed. Therefore, the fungicide treatment used could have affected wine yeast protein expression, as this was the only variable compared to the control and 1x treatment (T1). This has potential significance, as previous research showed that certain yeast expressed proteins are involved in the release of aroma-enhancing metabolites, e.g. thiols (Caridi, 2006; Braconi *et al.,* 2011; Comuzzo *et al*., 2011; Juega *et al.*, 2012; Mostert & Divol, 2014; Hart *et al*., 2017 a & b). However, the overall sensory quality of the wines was not significantly different (refer to Figure 3.6, 3.7 and 3.8 in Chapter 3). Moreover, the proteins which are known to be involved in metabolite release was monitored during this study and the SDS-PAGE results also showed a difference in proteins between all samples, (i.e. the control, 1x treatment (T1) and 2x treatment (T2) taken at the start of fermentation, compared to the end of fermentation (Figure 4.3).



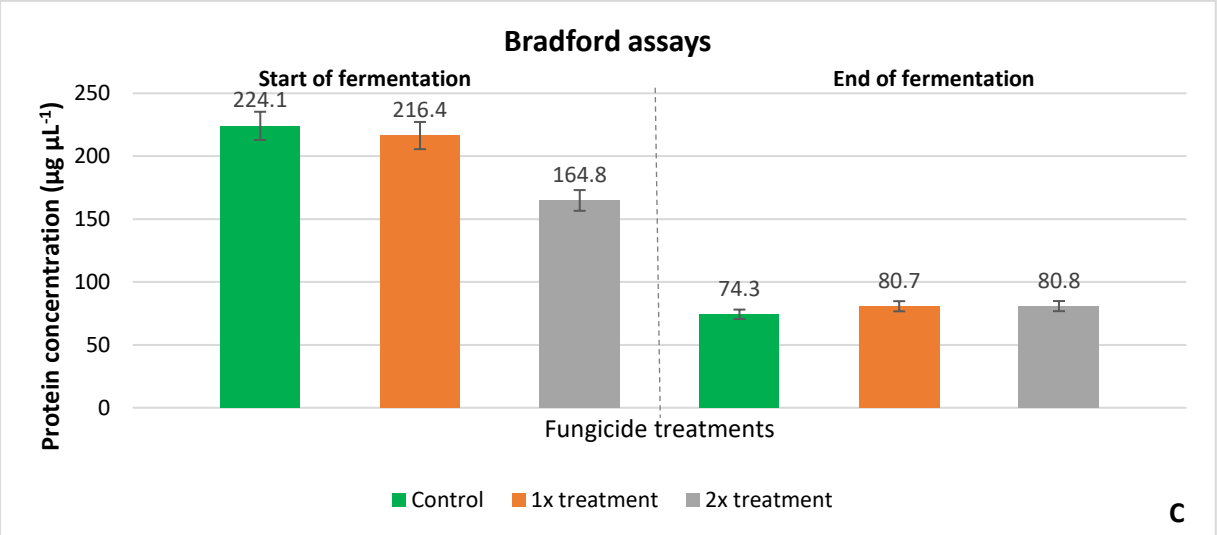




**Figure 4.2.** Experiment A, B & C: Protein concentration (µg µL$^{-1}$) of fermenting control, 1x treatment and 2x treatment. Protein extracts obtained were by extrapolating spectrophotometric data (Abs 595 nm) on a BSA standard curve following the Bradford assay.

Therefore, in all the cases the fermenting grape must matrix also influenced the yeast starter culture's metabolism, as the chemical parameters of grape must during the lag-phase of fermentation are known to differ from that of the same must during the lag-phase (exponential growth) (Vianna & Ebeler, 2001; Conde *et al*., 2007).

*4.4.1.3. Proteomic characterisation of yeast expressed proteins*

The MALDI-TOF/MS showed noticeable differences in protein mass spectra between proteins extracted from the control Chenin Blanc grapes and treated the Chenin Blanc grapes (1x treatment and 2x treatment) (Figure 4.4 a). Indications, therefore, are that the yeast starter behaved differently in the control as a result of the treatment and due to the different treatment applied. The same observation was made with regard to proteins extracted at the end of fermentation (Figure 4.4b). It is noteworthy that mass spectra of the control at the start of and end of fermentation also differed, which indicated that the yeast starter culture reacted to changes in the matrix of the fermenting grape must. The same observations were made for both treatments (T1 and T2). Experiment B (Figure 4.5a & b) and C (Figure 4.6a & b) showed similar results. Overall, all treatments for all three experiments seems to induce differential protein expression relative to the control. However, the sensory evaluation panel comprising trained and experienced wine judges did not perceive any negative or wine foreign aroma and flavours in the wines made from the treated grapes (refer Figure 3.6, 3.7 & 3.8 in Chapter 3).

Peptide mass fingerprinting (PMF) in conjunction with MALDI-TOF/MS characterised one protein for each treatment (control, 1x treatment (T1) and 2x treatment (T2)) at the start and end of the fermentation (Table 4.3). A general protein database search using UniProtKB (http://www.uniprot.org/uniprot/?query=HOSC&sort=score) identified three VIN 13 expressed proteins in all samples with the following assession numbers YD177, PGK1 and PSP2, respectively (Table 4.3). The protein, i.e. PGK identified as Phosphoglycerate kinase was expressed in the control at the start of fermentation (Table 4.3). The enzyme was reported to be involved in glycolysis through which a hexose sugar, e.g. glucose and fructose are metabolised into pyruvate and other metabolites, such as alcohol and glycerol (Ghaemmaghami *et al*., 2003).

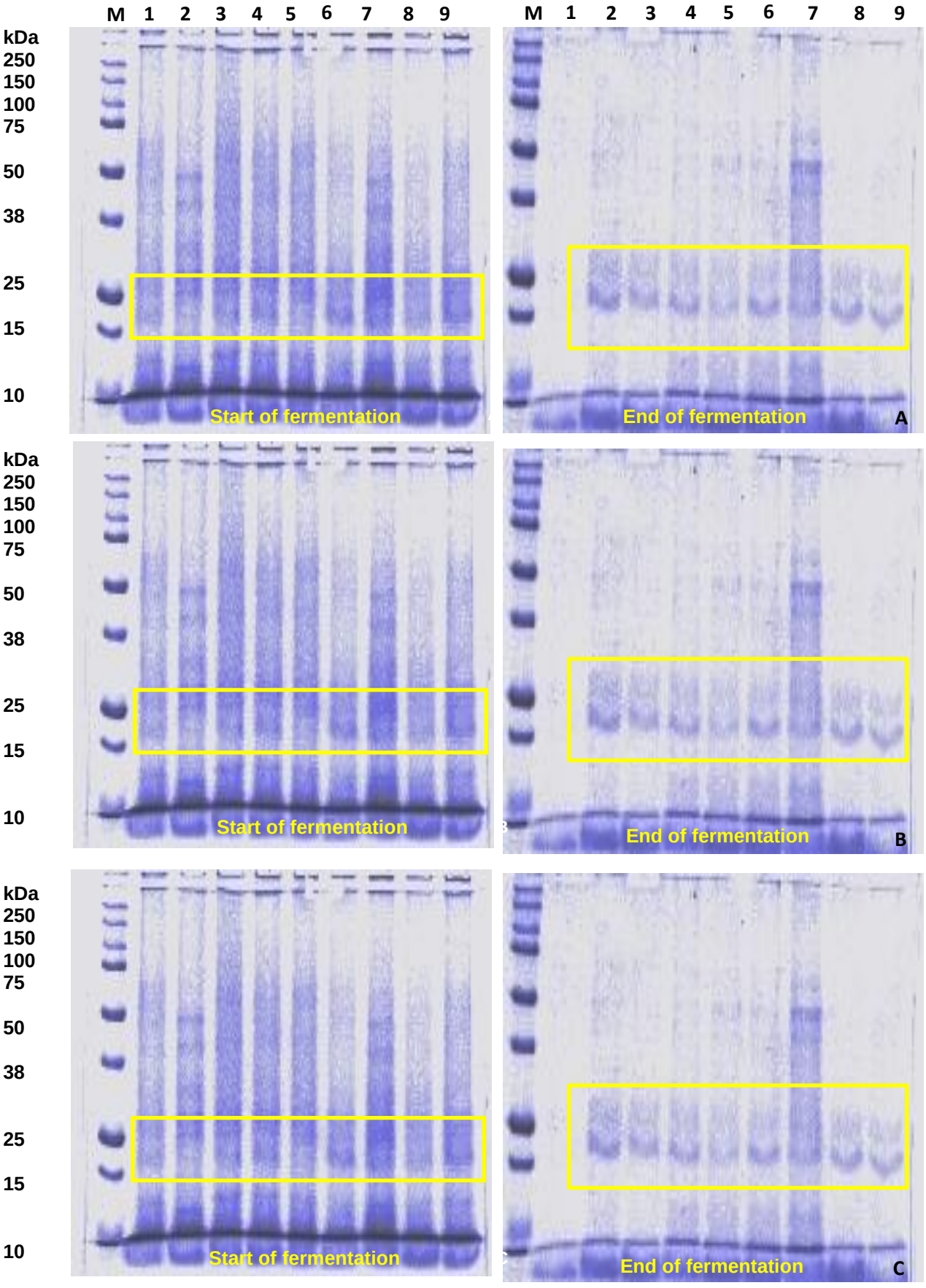



**Figure 4.3.** SDS-PAGE of protein extracts originating from samples collected at the start and end of fermentations for Experiment A, B and C: Lane M: Protein ladder (Precision Plus Protein™, Bio-Rad, Madrid, Spain); Lane 1-3: Control; Lane 4-6: 1x Treatment; Lane 7-9: 2x Treatment (start and end of fermentation as indicated).

This observation, therefore, complements the results of this study as the grape juice used for winemaking had a total sugar content of 23 g $L^{-1}$ (Chapter 3, Figure 3.1) which comprised mostly of glucose and fructose, which are metabolised into various metabolites as the fermentation progresses (Vianna & Ebeler, 2001; Conde *et al*., 2007). The Polymerase suppressor protein 2 (PSP2), on the other hand, was monitored in the control fermentation at the end of fermentation (Table 4.3). The PSP2 was reported as a suppressor of wine yeast DNA polymerases which are involved in DNA replication (Waldherr *et al*., 1993; Formosa & Nittis, 1998; Hałas *et al*., 1999). It can, therefore, be speculated that, as the fermentation was in the stationary phase, yeast cell proliferation was inhibited by expressing this enzyme that suppresses DNA replication. The last protein identified was IMPACT family member YDL177C in the initial stage and at the end of fermentation (Table 4.3). It was reported to be a miscellaneous enzyme that is found in the exponential growth phase where it is known to assist with breaking down grape pulp and skin cells (Ghaemmaghami *et al*., 2003). Therefore, it can be stated that the treatments did not have a negative effect since the fermentation was completed within the standard duration of a typical white wine fermentation (Figure 3.2, 3.3 and 3.4).

*4.4.2. Metabolite analysis*

Wines were bottled and allowed to mature and/or stabilise for four months, whereafter they were analysed for aroma compounds (metabolites) using GC-FID (Herbst-Johnstone *et al.,* 2013; Piano *et al.,* 2015; Araujo *et al*., 2016). Metabolites were categorised into; esters, higher alcohols and fatty acids, respectively.

The data was statistically analysed using Analysis of Variance (ANOVA) and Multiple Factor Analysis (MFA). The ANOVA results (Table 4.4) for Experiment A, showed that the metabolite compounds had no significant difference between the control and treatments ($p > 0.05$). The ester compounds present in the highest concentration were ethyl acetate (24.86, 20.89 and 19.89 mg $L^{-1}$ control, 1x treatment (T1), 2x treatment (T2), respectively), isoamyl acetate (2.39, 1.51 and 1.84 mg $L^{-1}$, respectively), ethyl hexanoate (6.94 mg $L^{-1}$ control and the rest were less than 1 mg $L^{-1}$), ethyl lactate (24.81, 24.68 and 23.93 mg $L^{-1}$, respectively) and diethyl succinate (2.21 mg $L^{-1}$ 1x treatment (T1), while control and 2x treatment (T2) were around 1 mg $L^{-1}$ or less). The remainder were present at concentrations around 1 mg $L^{-1}$ or less.

The odour threshold of ethyl acetate is 17.62 mg $L^{-1}$, hence in this study it was found in concentrations above the threshold value. It might be possible that the aromas contributed by this compound such as “solvent”, “varnish” and “fruity” were perceived in the wines (Lee & Noble, 2003; Escudero *et al*., 2007; Gomez-Miguez *et al*., 2007; King *et al*., 2008; Li *et al*., 2008). Plata *et al.* (2003) reported that ethyl acetate production is yeast-dependent and involves an enzymatic reaction. Therefore, it can be tentatively said that different treatments influenced the production of ethyl acetate, as differences in protein expression were observed between controls and treatment in this experiment following SDS-PAGE (Figure 4.3) and PMF (Figure 4.4a & b). Furthermore, the isoamyl acetate threshold is 0.03 mg $L^{-1}$, ethyl hexanoate 0.014 mg $L^{-1}$, ethyl lactate 0.58 mg $L^{-1}$ and diethyl succinate 200 mg $L^{-1}$ in white wine (Gomez-Miguez *et al*., 2007; Li *et al*., 2008). Therefore, the wines had aromas such as banana, pear, fruity and apple since the concentrations of the aforementioned compounds were above the threshold values. However, the diethyl succinate was detected in concentrations less than the threshold value of 200 mg $L^{-1}$ (Lee & Noble, 2003; Escudero *et al*., 2007; Gomez-Miguez *et al*., 2007; King *et al*., 2008; Li *et al*., 2008). A study by Pretorius & Lambrechts (2000) reported that acetate esters are responsible for the pleasantly fruity, flower and ester-like character in wines made from neutral cultivars such as Chenin Blanc and Colombar.

Higher alcohol compounds also showed no statistically significant difference ($p > 0.05$) between the control and treated wine in Experiment A (Table 4.4). However, the following compounds were present in the highest concentration, n-propanol (58.20, 110.50 and 143.80 mg $L^{-1}$ for the control, 1x treatment (T1), 2x treatment (T2), respectively) which can be associated with “ripe fruit” (Feng *et al*., 2015 ), isobutanol (4.55, 11.14 and 16.41 mg $L^{-1}$, respectively), isoamyl alcohol (49.09, 133.19 and 93.09 mg $L^{-1}$, respectively), 3-methyl-1-pentanol (4.97, 4.89 and 4.81 mg $L^{-1}$, respectively), hexanol (1.76, 1.41 and 1.29 mg $L^{-1}$, respectively), 3-ethoxyl-1-propanol (7.26, 9.24 and 10.93 mg $L^{-1}$, respectively) and 2-phenyl-ethanol (3.16, 8.76 and 4.05 mg $L^{-1}$, respectively). The rest were present at concentrations around 1 mg $L^{-1}$ or less. Additionally, the higher alcohols were still within the normal range with concentrations less than 400 mg $L^{-1}$, the threshold value beyond which higher alcohols cause the wine to have a harsh solvent-like odour.

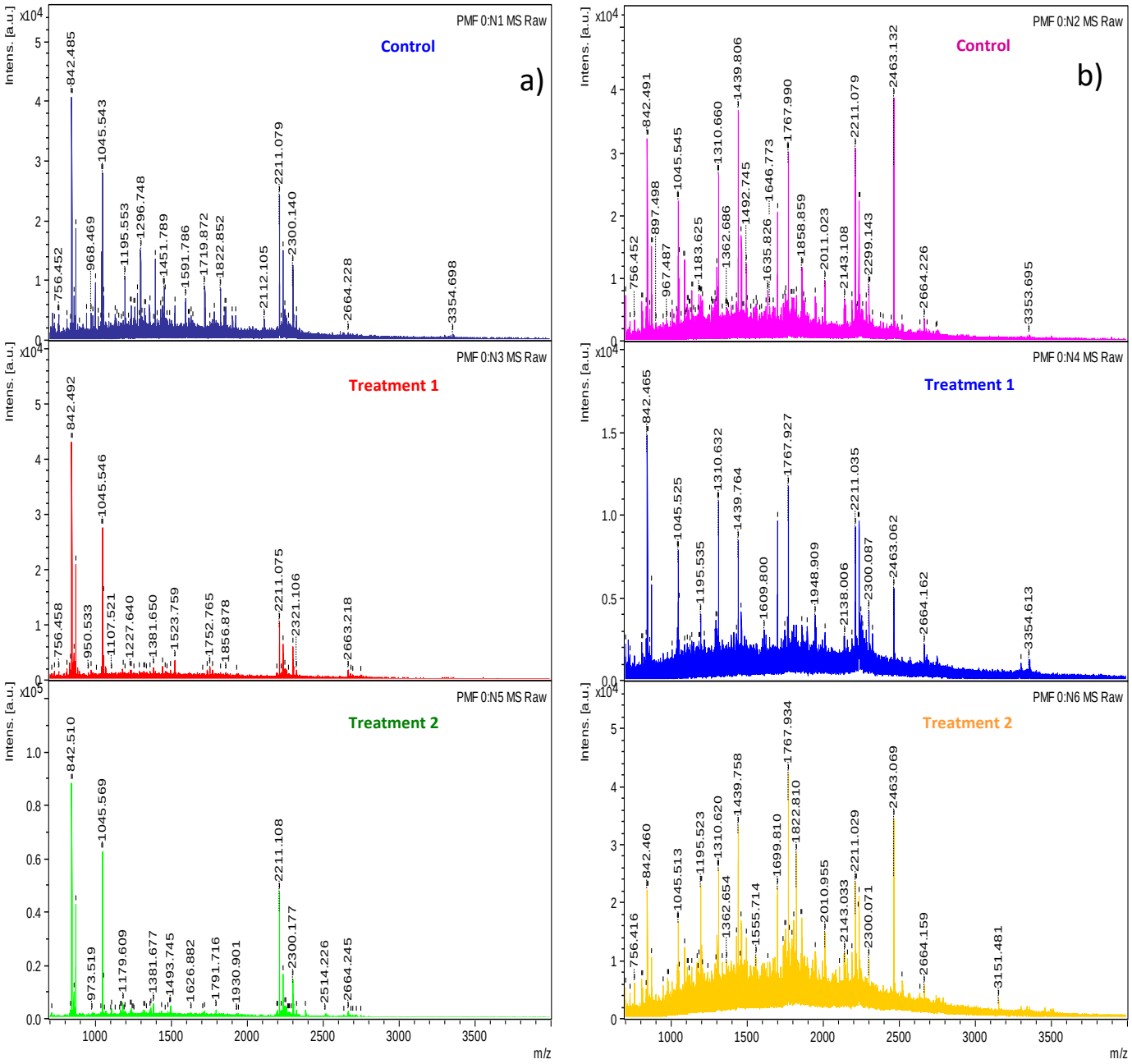



**Figure 4.4.** Experiment A: Mass spectra showing tripsinised wine yeast protein signals at the **a)** start of fermentation and **b)** end of fermentation following inoculation of Chenin Blanc grape juice (must) originating from grapes subjected to different treatments, i.e. control (grapes treated with a registered chemical spray), treatment 1 (1x treatment of experimental spray) and treatment 2 (2x treatment of experimental spray), respectively.

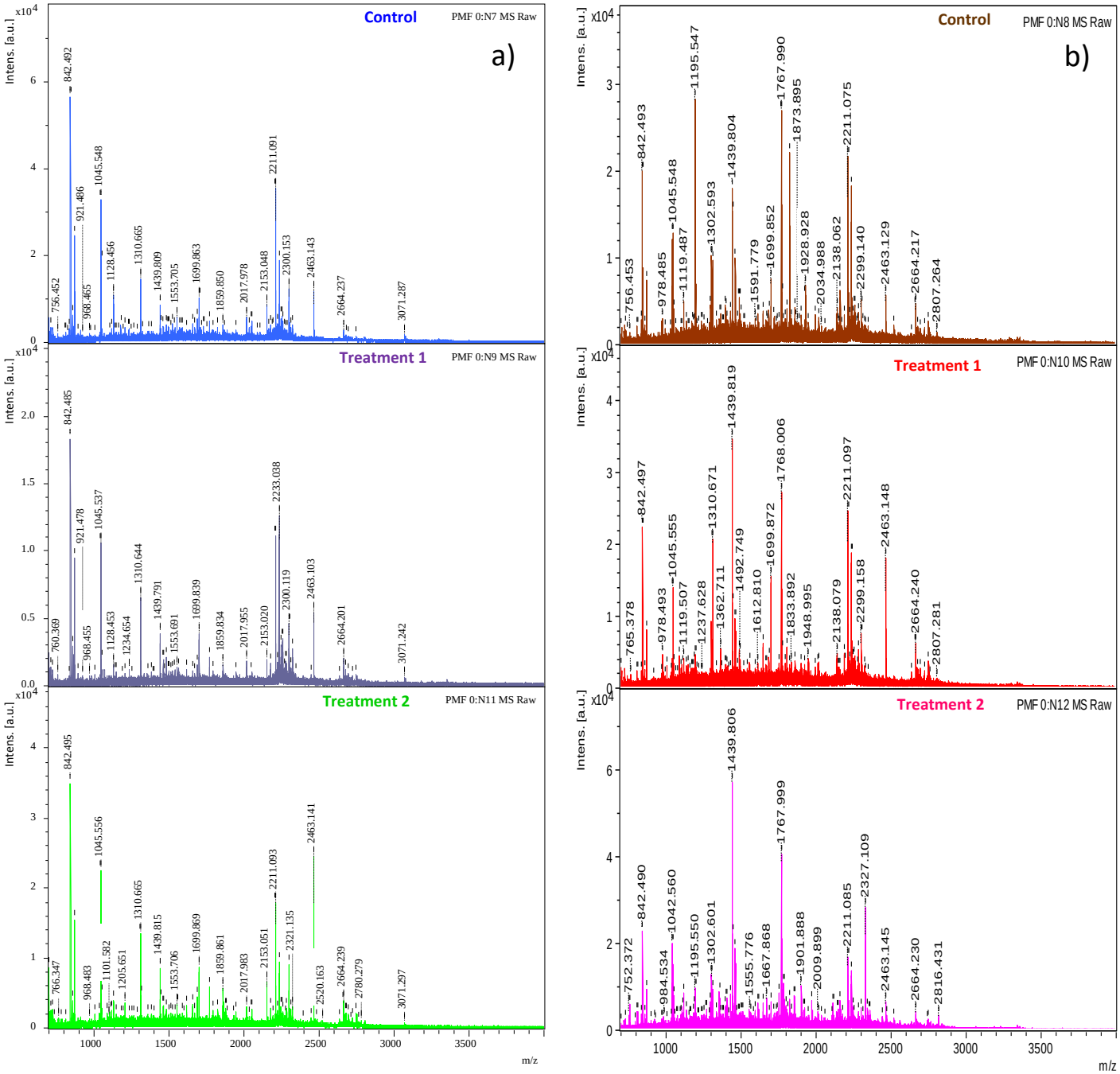



**Figure 4.5.** Experiment B: Mass spectra showing tripsinised wine yeast protein signals at the **a)** start of fermentation and **b)** end of fermentation following inoculation of Chenin Blanc grape juice (must) originating from grapes subjected to different treatments, i.e. control (grapes treated with a registered chemical spray B), treatment 1 (1x treatment of experimental spray) and treatment 2 (2x treatment of experimental spray), respectively.

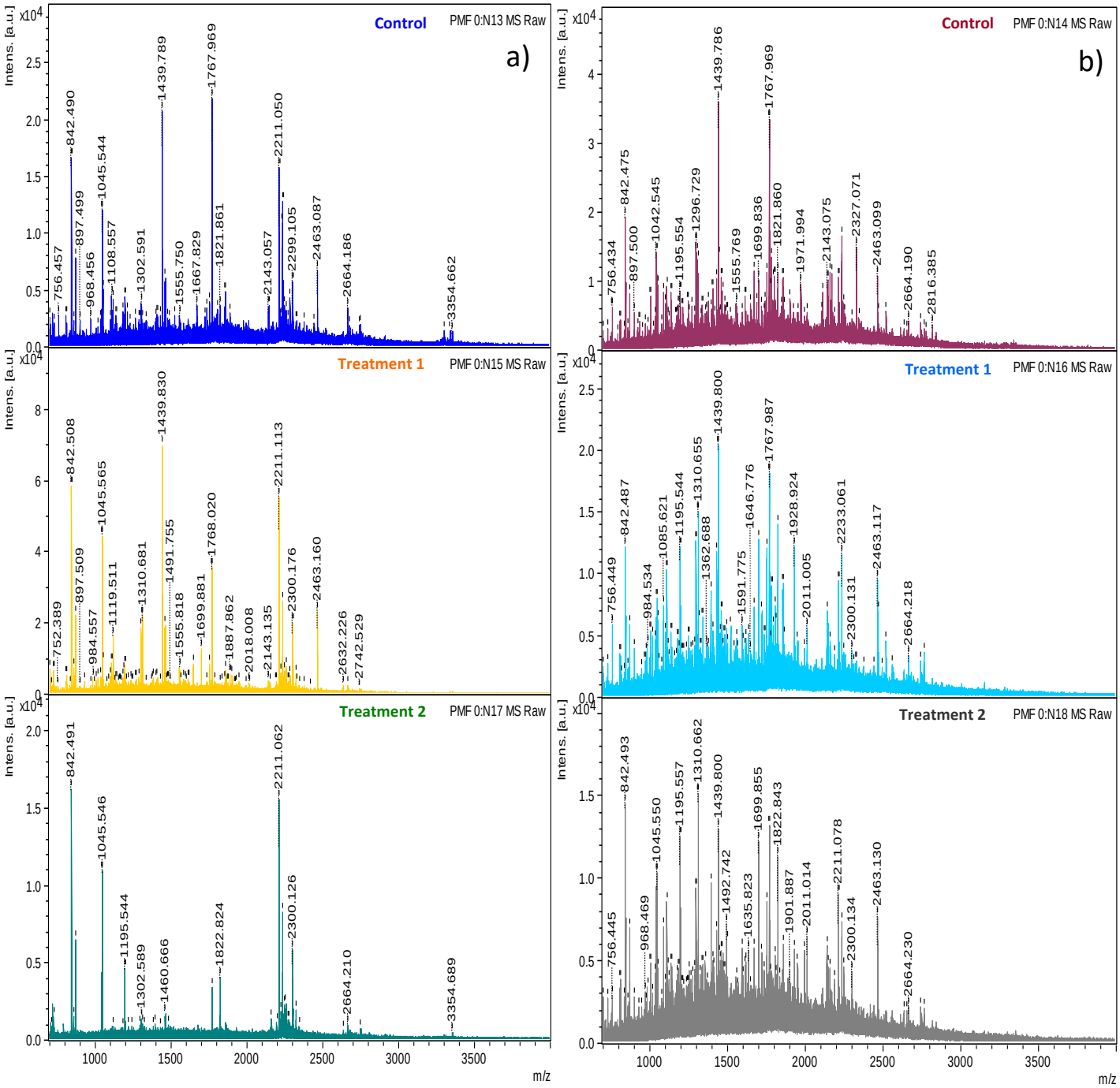



**Figure 4.6.** Experiment C: Mass spectra showing tripsinised wine yeast protein signals at the **a)** start of fermentation and **b)** end of fermentation following inoculation of Chenin Blanc grape juice (must) originating from grapes subjected to different treatments, i.e. control (grapes treated with a registered chemical spray), treatment 1 (1x treatment of experimental spray) and treatment 2 (2x treatment of experimental spray), respectively.

**Table 4.3.** Proteins expressed by the commercial yeast strain, namely VIN 13 during the fermentation of 2017 Chenin Blanc grape must that was identified by peptide mass fingerprinting (PMF) in conjunction with MALDI-TOF/MS.

| Sample | Spot position | Accession | Protein | Molecular weight (kDa) | Isoelectric point (pI) | Score | Peptides | Sequence coverage (%) |
|---|---|---|---|---|---|---|---|---|
| Control–start of fermentation (CC) | N13 | PGK_YEAST | Phosphoglycerate kinase OS=*Saccharomyces cerevisiae* (strain ATCC 204508 / S288c) GN=PGK1 PE=1 SV=2 | 44.7 | 7.1 | 32 | 2 | 7 |
| Control–start of fermentation (CCF) | N14 | PSP2_YEAST | Protein PSP2 OS=*Saccharomyces cerevisiae* (strain ATCC 204508 / S288c) GN=PSP2 PE=1 SV=2 | 65.5 | 8.7 | 28.4 | 1 | 2.2 |
| Treatment 1–start of fermentation (B1) | N9 | YD177_YEAST | IMPACT family member YDL177C OS=*S accharomyces cerevisiae* (strain ATCC 204508 / S288c) GN=YDL177C PE=1 SV=1 | 19 | 9 | 53.5 | 1 | 14.1 |
| Treatment 2–start of fermentation (B1F) | N10 | YD177_YEAST | IMPACT family member YDL177C OS=*S accharomyces cerevisiae* (strain ATCC 204508 / S288c) GN=YDL177C PE=1 SV=1 | 19 | 9.9 | 27 | 1 | 14 |
| Treatment 1–start of fermentation (B2) | N11 | YD177_YEAST | IMPACT family member YDL177C OS=*S accharomyces cerevisiae* (strain ATCC 204508 / S288c) GN=YDL177C PE=1 SV=1 | 19 | 9.9 | 35.5 | 1 | 14.1 |

However, in concentrations below 300 mg $L^{-1}$, higher alcohols contribute to aromas such as rose, grassy, honey and herb-like (Ferreira *et al*., 2002; Campo *et al.,* 2006; Gil *et al*., 2006).

Fatty acids present at highest concentrations were acetic acid (133.72, 142.20 and 107.94 mg $L^{-1}$ for the control, 1x treatment (T1), 2x treatment (T2), respectively), propionic acid (6.18, 1.26 and 1.62 mg $L^{-1}$, respectively), hexanoic acid (50.47, 21.95 and 31.72 mg $L^{-1}$, respectively) and octanoic acids (3.49, 4.48 and 4.10 mg $L^{-1}$, respectively), with the remainder present at concentrations around 1 mg $L^{-1}$ or less (Table 4.4). It is noteworthy that, acetic acid is the major contributor to total fatty acids and reported to impart undesired “vinegar-like” off-odours (Jiang, 2010; Hart *et al*., 2017 a & b). However, when detected at a threshold of 200 mg $L^{-1}$, it imparts the undesirable vinegar-like odour (Cheng *et al*., 2015), fortunately, in this experiment, it was detected in concentrations below the threshold value. Hexanoic acid and octanoic acid were within permissible concentration ranges for white wine (1 – 73 mg $L^{-1}$ and 2 – 717 mg $L^{-1}$, respectively). Hexanoic acid imparts “sweaty” nuances (Pretorius & Lambrechts, 2000; Francis & Newton, 2005; Lawrence, 2012), however, none of the wines were perceived to have these negative aromas by the sensory evaluation panel (Figure 3.6 in Chapter 3). Therefore, these results showed that the fungicide treatments did not produce wines that were sensorially significantly different to the control. Also, a study by Lawrence (2012) found that octanoic acids at concentrations between 3.5 – 12.0 mg $L^{-1}$ produced fresh and fruity Chenin Blanc wines. Furthermore, MFA data (Figure 4.7) showed that the control and treatments (T1 and T2), clustered in the top left quadrant and produced wines with a positive association to esters and higher alcohols which can contribute to positive aromas such as rose and honey. Also, the wines had a negative association with the fatty acids. Moreover, wines also had a positive association with “Tropical fruit” and “Tree fruit” aromas (Figure 4.7).

In Experiment B, generally, there was no statistical significant difference between the control and treatments ($p > 0.05$) (Table 4.5). The ester compounds present in the highest concentration were ethyl acetate (11.13, 17.07 and 5.69 mg $L^{-1}$ for the control, 1x treatment (T1), 2x treatment (T2), respectively) and ethyl lactate (17.23, 13.74 and 17.43 mg $L^{-1}$, respectively), with the rest once more present at concentrations around 1 mg $L^{-1}$ or less. Ethyl acetate contributes with varnish and solvent aromas at a threshold of 17.62 mg $L^{-1}$ (Pretorius & Lambrechts, 2000; Gomez-

Miguez *et al*., 2007; Li *et al*., 2008). In this study, ethyl acetate was found in concentrations lower than the threshold value (17.62 mg $L^{-1}$) when compared to Experiment A where it was above. Therefore, wine in this experiment did not have the aforementioned aromas as in Experiment A. Previous research showed that ethyl lactate at a threshold of 14 mg $L^{-1}$ contributes to fruity and flowery aromas (Li *et al*., 2008). Hence, there is no doubt that these wines will have the aromas mentioned above since the ethyl lactate was detected at concentrations above the threshold value 14 mg $L^{-1}$ (Pretorius & Lambrechts, 2000; Lee & Noble, 2003; Escudero *et al*., 2007; Gomez-Miguez *et al*., 2007; King *et al*., 2008; Li *et al*., 2008).

No statistical significant difference was observed between the higher alcohols of the control and treated wines in Experiment B (Table 4.5) ($p > 0.05$). The higher alcohols present at the highest concentration were n-propanol (50.45, 51.19 and 81.82 mg $L^{-1}$ for the control, 1x treatment (T1), 2x treatment (T2), respectively), isobutanol (7.71, 7.96 and 0.06 mg $L^{-1}$, respectively), isoamyl alcohol (59.12, 67.18 and 1.53 mg $L^{-1}$, respectively), 3-methyl-1-pentanol (3.47, 2.76 and 3.52 mg $L^{-1}$, respectively), 3-ethoxy-1-propanol (2.82, 2.89 and 5.09 mg $L^{-1}$, respectively), and 2-phenyl-ethanol (3.10, 3.44 and 0.79 mg $L^{-1}$, respectively). The aforementioned compounds could contribute to alcohol and ripe fruit aromas as also observed in Experiment A. Additionally, it was notable that isobutanol, isoamyl alcohol and 2-phenyl-ethanol had higher concentrations in the control and 1x treatment (T1), compared to the 2x treatment (T2), which as present at concentrations around 1 mg $L^{-1}$ or less (Tables 4.5). Moreover, 2-phenyl-ethanol, associated with floral and rose notes (Rocha *et al*., 2010; Knoll *et al*., 2011), were also detected in the wines at higher concentrations, but lower than the threshold value (400 mg $L^{-1}$). As seen in Experiment A, the higher alcohols were detected at concentrations lower than 300 mg $L^{-1}$ at which they contribute rose, grassy, honey and herb-like aromas, however when in concentrations above 400 mg $L^{-1}$ they give harsh solvent-like odours. Moreover, statistically, the data showed no significant difference ($p > 0.05$).

The overall fatty acids showed no significant difference between the control and treatments in Experiment B ($p < 0.05$) (Table 4.5), except for acetic acid. Acetic acid was significantly higher in the 2x treatment (149.92 mg $L^{-1}$) than in the control (91.81 mg $L^{-1}$) ($p > 0.05$). It is noteworthy that although the concentration was significantly higher than the control, it was still present below it's threshold value of 200 mg $L^{-1}$ (Jiang, 2010; Cheng *et al*., 2015; Hart *et al*., 2017 a & b). Therefore,

although statistically higher in the 2x treatment (T2), it can be concluded that the wines did not have the “vinegar-like” off-odours. Additionally, other fatty acids present at higher concentrations were hexanoic acid (62.42, 53.70 and 97.01 mg $L^{-1}$ for the control, 1x treatment (T1) and 2x treatment (T2), respectively) and octanoic acid (2.20, 1.44 and 0.29 mg $L^{-1}$, respectively). The rest were present at concentrations around 1 mg $L^{-1}$ or less (Table 4.5). As was observed in Experiment A, the aforementioned fatty acids are within the acceptable concentration ranges. Moreover, MFA data (Figure 4.8) showed that the control and 1x treatment (T1), clustered in the bottom left quadrant and produced wines with a positive association to higher alcohols which can contribute to rose and honey aromas. Additionally, wines also had a positive association with “Overall quality” and “Colour”. Furthermore, the 2x treatment (T2) clustered in the top right quadrant and although they showed an association with fatty acids, they also had a positive association with “Tropical Fruit” and “Tree fruit” aromas.

Esters produced in Experiment C (Table 4.6), showed no statistical significant difference between the control and the treatments ($p > 0.05$). The ester compounds present in the highest concentrations were ethyl acetate (4.44, 4.13 and 5.04 mg $L^{-1}$ for the control, 1x treatment (T1), 2x treatment (T2), respectively), ethyl lactate (14.12, 15.63 and 15.18 mg $L^{-1}$, respectively) and ethyl caprylate (1.05 mg $L^{-1}$, 2.01 mg $L^{-1}$ for the control, 1x treatment (T1), respectively and was not detected in 2x treatment (T2)), with the remainder present at concentrations around 1 mg $L^{-1}$ or less. It is noteworthy that, the ethyl acetate was detected at concentrations below its threshold value (17.62 mg $L^{-1}$) unlike in Experiments A (Table 4.4) and B (Table 4.5). Therefore, the undesirable aromas usually associated with it were not perceived in this experiment. Furthermore, ethyl caprylate was detected at concentrations higher than the threshold value (0.58 mg $L^{-1}$) and can thus contribute to the fruity and floral aromas perceived.

The higher alcohol compounds also showed no significant differences between the control and treatments ($p > 0.05$), except for acetoin (Table 4.6). Acetoin, was significantly lower in the control (0.24 mg $L^{-1}$) when compared to the treatments (0.31 mg $L^{-1}$, 0.32 mg $L^{-1}$ for the 1x treatment (T1) and 2x treatment (T2), respectively). However, it is important to highlight that acetoin contributes to the “buttery” character in wine at a threshold value of 150 mg $L^{-1}$ (Francis & Newton, 2005). This was substantiated buy the sensory results as “buttery” was not perceived by the sensory panel. The higher alcohols present at higher concentrations were n-propanol (13.72 mg $L^{-1}$ 1x treatment (T1), with the remainder less than 1 mg $L^{-1}$), isoamyl alcohol

(12.99, 20.33 and 8.78 mg $L^{-1}$ for the control, 1x treatment (T1), 2x treatment (T2), respectively), 3-methyl-1-pentanol (2.84, 3.19 and 3.23 mg $L^{-1}$, respectively), 3-ethoxyl-1-propanol (3.09, 2.23 and 2.22 mg $L^{-1}$, respectively) and 2-phenyl ethanol (1.22, 1.59 and 1.96 mg $L^{-1}$, respectively) (Table 4.6). The rest were present at concentrations around 1 mg $L^{-1}$ or less. However, these higher alcohols were also detected in lower concentrations than in Experiments A and B, and fell below the threshold values. Therefore, their aroma contribution to the wines was also below a detectable threshold.

Fatty acids showed no statistical significant difference between the control and the treatments ($p > 0.05$), except for iso-valeric acids (Table 4.6). Iso-valeric acid was significantly lower in the difference in control (0.25 mg $L^{-1}$) when compared to the treatments (0.33 mg $L^{-1}$, 0.35 mg $L^{-1}$ for the 1x treatment (T1) and 2x treatment (T2), respectively). Additionally, the threshold value of iso-valeric acid in wines is 0.033 mg $L^{-1}$ and it imparts sweet, acid, rancid, floral aromas to wine (Peťka *et al*., 2006; Sánchez-Palomo *et al*., 2010). Therefore, in this Experiment as with A and B it was detected in concentrations exceeding the threshold value. This however, was not perceived in the sensory results (refer to Figure 3.8 in Chapter 3). Fatty acids present at higher concentrations were acetic acid (195.64, 208.62 and 188.46 mg $L^{-1}$ for the control, 1x treatment (T1), 2x treatment (T2), respectively), propionic acid (1.09, 1.14 and 1.15 mg $L^{-1}$, respectively) and hexanoic acids (98.49, 99.09 and 94.34 mg $L^{-1}$, respectively), with the remainder present at concentrations around 1 mg $L^{-1}$ or less (Table 4.6). Furthermore, it was notable that acetic acid in 1x treatment was marginally higher than its threshold value (200 mg $L^{-1}$) while the control and 2x treatment (T2) were detected at lower concentrations (Table 4.6). As previously mentioned, acetic acid contributes to undesired “vinegar-like” off-odours when above the threshold value of 200 mg $L^{-1}$, hence the 1x treatment (T1) in this experiment would have been expected to have those off-odours**.** However, the overall sensory data showed positive associations to “Overall Quality” and “Colour” (Figure 3.8 in Chapter 3). Hexanoic acid was found in concentrations higher than the typical concentration range for white wine 1 – 73 mg $L^{-1}$ unlike in Experiment A, and is known to contribute to sweaty” nuances (Table 4.6) (Pretorius & Lambrechts, 2000; Francis & Newton, 2005; Lawrence, 2012). However, the MFA data (Figure 4.9) showed that the control and both treatments clustered in the top right quadrant and even though they are associated with fatty

acids, they also had a positive association with the desirable “Tropical Fruit” and “Tree fruit” aromas.

*4.4.3. Thiol analysis*

The thiol compounds 3-mercaptohexyl acetate (3MHA), 3-mercaptohexan-1-ol (3MH) and 4-mercapto-4-methylpentan-2-one (4MMP) were analysed using GC – MS. These compounds were selected because they are most commonly detected in white wines produced from Sauvignon Blanc and Chenin Blanc and are associated with positive aromas such as “box tree”, “tropical fruit”, “passion fruit” and “citrus” aromas (Du Toit *et al*., 2015; Herbst-Johnstone *et al*., 2013; Piano *et al*., 2015; Araujo *et al*., 2016). The presence of thiols in Chenin Blanc was first confirmed by Alexandre-Tudo *et al*. (2015). Recently, Wilson (2017) conducted a study where thiol compounds (3MHA and 3MH) were investigated in the following categories: vine age, trellis type, wine origin, lees contact, wood contact, wine age and price. The study found that there was no significant difference in 3MHA levels in all cases, except in response to trellis type and for 3MH wine origin significantly affected the levels.

The ANOVA results (Table 4.7) for Experiment A, showed that although there was no statistical significant difference between the control and the treatments ($p > 0.05$), 3MHA was present at high concentrations (527.30, 568.90 and 395.50 mg $L^{-1}$ for the control, 1x treatment (T1) and 2x treatment (T2), respectively) when compared to 4MMP (48.66, 28.91 and 98.72 mg $L^{-1}$, respectively) and 3MH (193.04, 181.12 and 185.38 mg $L^{-1}$, respectively) (Table 4.7). Moreover, in terms of the MFA data (Figure 4.7), the control and both treatments (T1 and T2) clustered in the top left quadrant. Hence they showed that the wines had a positive association with 3MHA, which is known to positively influence the wine aroma (associated with “box tree”, “tropical fruit”, “passion fruit” and “citrus” aromas) (Tominaga *et al*., 2000; King *et al*., 2008). Moreover, although the wines showed a negative association with 3MH and 4MMP (Figure 4.7), the wines had a positive association with “Tropical fruit” and “Tree fruit” aromas. Some judges detected an association with “Wine Foreign” aromas, which are usually not associated with Chenin Blanc. This could be the reason why the wines had a negative association to “Overall Quality” (Figure 4.7). However, when looking at the sensory analyses metabolites and thiols produced, indicates that the chemical sprays did not have a negative effect on the overall wine sensory quality as the treatments showed similar results to the control.

In Experiment B the ANOVA results (Table 4.7) showed no statistical significant difference in thiol compounds between the control and both treatments (T1 and T2) ($p > 0.05$), with the exception of the 3MH for 1x treatment (104.71 mg $L^{-1}$) which showed a significant difference when compared to the control (128.67 mg $L^{-1}$) and the 2x treatment (125.06 mg $L^{-1}$). Moreover, the MFA (Figure 4.8) showed that the control and 1x treatment (T1), clustered in the bottom left quadrant, and therefore had a negative association with 3MHA, 3MH and 4MMP. Additionally, it is important to note that the wines had a positive association with “Overall Quality” and “Colour”. Furthermore, the 2x treatment (T2) clustered in the top right quadrant, showing a positive association with 3MH and 4MMP that are associated with “box tree”, “tropical fruit”, “passion fruit” and “citrus” aromas (Tominaga *et al*., 2000; King *et al*., 2008; Piano *et al*., 2015). Therefore, the results indicated that the wines produced were not negatively affected by the chemical sprays in terms of wine sensory quality. It should however, be noted that although the 2x treatment (T2) had a lower association with quality but also had a negative association with “Wine Foreign” aromas therefore fungicide did not necessary negatively affect the wine.

The ANOVA results (Table 4.7) for Experiment C, also showed no statistical significant difference between the control and treatments (T1 and T2) ($p > 0.05$). However, the 3MHA compound in response to the 1x treatments and 2x treatment was not detected. The MFA data (Figure 4.9) showed that the control and both treatments (T1 and T2) clustered in the top left quadrant had a positive association with the 3MH and 4MMP compounds. As previously mentioned, these thiol compounds are known to contribute greatly to the aroma profile of the wine by producing aromas such as “box tree”, “tropical fruit”, “passion fruit” and “citrus” aromas, (Tominaga *et al*., 2000; King *et al*., 2008; Piano *et al*., 2015). Moreover, this is desirable as studies conducted by Du Plessis & Augustyn (1981) suggested 4MMP releases guava-like aromas. Therefore, the wines produced in this study had the aforementioned positive aromas. Furthermore, although the wines associated with fatty acids (Figure 4.9), they also had a positive association with “Tropical Fruit” and “Tree fruit” aromas. This observation complemented the sensory analyses and these results indicated that the chemical sprays did not have a negative effect on wine sensory quality as the treatments showed similar results to the control.

**Table 4.4.** Experiment A: Volatile metabolites of Chenin Blanc wines detected using Gas chromatography-flame ionisation detector (GC – FID).

| Aroma compounds (mg $L^{-1}$) | Odour description[2] | Control | 1x treatment | 2x treatment |
|---|---|---|---|---|
| ***Esters*** | | | | |
| Ethyl_Acetate | Varnish, fruity, solvent | 24.86a[1] | 20.89a | 19.89a |
| Isobutyl-Acetate | Banana | 0.02a | 0.02a | 0.02a |
| Ethyl_butyrate | Acidic, fruity, apple | 0.28a | 0.27a | 0.27a |
| Isoamyl_Acetate | Banana, pear | 2.39a | 1.51a | 1.84a |
| Ethyl_Hexanoate | Green apple | 6.94a | 0.27a | 0.34a |
| Hexyl_Acetate | Apple, cherry,pear,floral | 0.08a | 0.05a | 0.09a |
| Ethyl_Lactate | Lactic, buttery, fruity | 24.81a | 24.68a | 23.93a |
| Ethyl_Caprylate | Fruity, flower | 0.46a | 0.54a | 0.51a |
| Ethyl-3-hydroxybutanoate | Fruity, sweet | 1.11a | 0.85a | 0.77a |
| Ethyl_Caprate | Fruity, melon | 0.17a | 0.17a | 0.17a |
| Diethyl_Succinate | Wine, fruit | 0.49a | 2.21a | 1.14a |
| Ethyl_phenylacetate | Fruit, sweet | 0.26a | 0.24a | 0.22a |
| 2-Phenylethyl_Acetate | Rose, honey, tobacco, fruity, cooked apple | 0.98a | 1.13a | 1.19a |
| ***Higher alcohols*** | | | | |
| n-propanol | Alcohol, ripe fruit | 58.20a | 110.50a | 143.08a |
| Isobutanol | Fusel, alcohol | 4.55a | 11.14a | 16.41a |
| Butanol | Fusel odour, medicinal | 0.65a | 0.88a | 0.92a |
| Isoamyl_alcohol | Alcohol, harsh | 49.09a | 133.19a | 93.09a |
| Pentanol | Ripe banana | 0.00 | 0.00 | 0.00 |
| 4-methyl-1-pentanol | Tropical | 0.20a | 0.21a | 0.21a |
| Acetoin | Buttery | 0.20a | 0.20a | 0.18a |
| 3-methyl-1-pentanol | Fruity, Wine | 4.97a | 4.89a | 4.81a |
| Hexanol | Grassy | 1.76a | 1.41a | 1.29a |
| 3-ethoxy-1-propanol | Fruity | 7.26a | 9.24a | 10.93a |
| 2-Phenyl_Ethanol | Roses | 3.16a | 8.76a | 4.05a |
| ***Fatty acids*** | | | | |
| Acetic_Acid | Vinegar | 133.72a | 142.20a | 107.94a |
| Propionic_Acid | Rancid, pungent | 6.81a | 1.26a | 1.62a |
| Isobutyric_acid | Acidic | 0.44a | 0.68a | 0.49a |
| Butyric_Acid | Rancid, cheese, sweat | 0.68a | 0.62a | 0.64a |
| Iso-Valeric_Acid | Blue cheese | 0.67a | 0.99a | 0.65a |
| Valeric_Acid | Roast barley | 0.17a | 0.24a | 0.16a |
| Hexanoic_Acid | Sweat, cheesy | 50.47a | 2.95a | 31.72a |
| Octanoic_Acid | Rancid, harsh, sweaty | 3.49a | 4.48a | 4.10a |

[1] Values between columns followed by the same letter do not differ significantly ($p > 0.05$)
[2] Lee & Noble (2003); Escudero *et al*. (2007); King *et al*. (2008)

**Table 4.5.** Experiment B: Volatile metabolites of Chenin Blanc wines detected using Gas chromatography-flame ionisation detector (GC – FID).

| Aroma compounds (mg $L^{-1}$) | Odour description[2] | Control | 1x treatment | 2x treatment |
|---|---|---|---|---|
| ***Esters*** | | | | |
| Ethyl_Acetate | Varnish, fruity, solvent | 11.13a[1] | 17.07a | 5.69a |
| Isobutyl-Acetate | Banana | 0.03a | 0.03a | 0.00 |
| Ethyl_butyrate | Acidic, fruity, apple | 0.14a | 0.10a | 0.00 |
| Isoamyl_Acetate | Banana, pear | 0.32a | 0.20a | 0.08a |
| Ethyl_Hexanoate | Green apple | 0.12a | 0.00 | 0.00 |
| Hexyl_Acetate | Apple, cherry, pear, floral | 0.00 | 0.00 | 0.00 |
| Ethyl_Lactate | Lactic, buttery, fruity | 17.23a | 13.74a | 17.43a |
| Ethyl_Caprylate | Flower, fruity | 0.19a | 0.10a | 0.69a |
| Ethyl-3-hydroxybutanoate | Fruity, sweet | 0.52a | 0.59a | 0.77a |
| Ethyl_Caprate | Fruity, melon | 0.06a | 0.04a | 0.00 |
| Diethyl_Succinate | Wine, fruity | 0.64a | 0.67a | 0.14a |
| Ethyl_phenylacetate | Fruit, sweet | 0.21a | 0.23a | 0.23a |
| 2-Phenylethyl_Acetate | Rose, honey, tobacco | 0.63a | 0.45a | 0.07a |
| ***Higher alcohols*** | | | | |
| n-propanol | Alcohol, ripe fruit | 50.45a | 51.19a | 81.82a |
| Isobutanol | Fusel, alcohol | 7.71a | 7.96a | 0.06a |
| Butanol | Fusel odour, medicinal | 0.13a | 0.09a | 0.00 |
| Isoamyl_alcohol | Alcohol, harsh | 59.12a | 67.18a | 1.53a |
| Pentanol | Ripe banana | 0.00 | 0.00 | 0.00 |
| 4-methyl-1-pentanol | Tropical | 0.00 | 0.00 | 0.07a |
| Acetoin | Buttery | 0.07a | 0.07a | 0.00 |
| 3-methyl-1-pentanol | Fruity, Wine | 3.47a | 2.76a | 3.52a |
| Hexanol | Grassy | 1.82a | 1.49a | 1.22a |
| 3-ethoxy-1-propanol | Fruity | 2.82a | 2.89a | 5.09a |
| 2-Phenyl_Ethanol | Roses | 3.10a | 3.44a | 0.79a |
| ***Fatty acids*** | | | | |
| Acetic_Acid | Vinegar | 91.81b | 108.25ab | 149.92a |
| Propionic_Acid | Rancid, pungent | 0.07a | 0.04a | 0.18a |
| Isobutyric_acid | Acidic | 0.32a | 0.36a | 0.08a |
| Butyric_Acid | Rancid, cheese, sweat | 0.13a | 0.07a | 0.00 |
| Iso-Valeric_Acid | Blue cheese | 0.49a | 0.48a | 0.24a |
| Valeric_Acid | Roast barley | 0.17a | 0.17a | 0.13a |
| Hexanoic_Acid | Sweat, cheesy | 62.42a | 53.70a | 97,01a |
| Octanoic_Acid | Rancid, harsh, sweaty | 2.20a | 1.44a | 0,29a |

[1] Values between columns followed by the same letter do not differ significantly ($p > 0.05$)
[2] Lee & Noble (2003); Escudero *et al*. (2007); King *et al*. (2008)

**Table 4.6.** Experiment C: Volatile metabolites of Chenin Blanc wines detected using Gas chromatography-flame ionisation detector (GC – FID).

| Aroma compounds (mg $L^{-1}$) | Odour description[2] | Control | 1x treatment | 2x treatment |
|---|---|---|---|---|
| ***Esters*** | | | | |
| Ethyl_Acetate | Varnish, fruity, solvent | 4.44a[1] | 4.13a | 5.04a |
| Isobutyl-Acetate | Banana | 0.00 | 0.00 | 0.00 |
| Ethyl_butyrate | Acidic, fruity, apple | 0.01a | 0.01a | 0.03a |
| Isoamyl_Acetate | Banana, pear | 0.09a | 0.09a | 0.15a |
| Ethyl_Hexanoate | Green apple | 0.00 | 0.00 | 0.00 |
| Hexyl_Acetate | Apple, cherry, pear, floral | 0.00 | 0.00 | 0.00 |
| Ethyl_Lactate | Lactic, buttery, fruity | 14.12a | 15.63a | 15.18a |
| Ethyl_Caprylate | Fruity, flower | 1.05a | 2.01a | 0.00 |
| Ethyl-3-hydroxybutanoate | Fruity, sweet | 0.56a | 0.54a | 0.50a |
| Ethyl_Caprate | Fruity, melon | 0.01a | 0.01a | 0.01a |
| Diethyl_Succinate | Wine, fruit | 0.19a | 0.25a | 0.35a |
| Ethyl_phenylacetate | Fruit, sweet | 0.25a | 0.26a | 0.26a |
| 2-Phenylethyl_Acetate | Rose, honey, tobacco | 0.09a | 0.09a | 0.17a |
| ***Higher alcohols*** | | | | |
| n-propanol | Alcohol, ripe fruit | 0.05a | 13.72a | 0.05a |
| Isobutanol | Fusel, alcohol | 0.19a | 0.19a | 0.06a |
| Butanol | Fusel odour, medicinal | 0.00 | 0.00 | 0.00 |
| Isoamyl_alcohol | Alcohol, harsh | 12.99a | 20.33a | 8.78a |
| Pentanol | Ripe banana | 0.00 | 0.00 | 0.00 |
| 4-methyl-1-pentanol | Tropical | 0.22a | 0.22a | 0.27a |
| Acetoin | Buttery | 0.24b | 0.31a | 0.32a |
| 3-methyl-1-pentanol | Fruity, Wine | 2.84a | 3.19a | 3.23a |
| Hexanol | Grassy | 0.99a | 1.07a | 1.07a |
| 3-ethoxy-1-propanol | Fruity | 3.09a | 2.23a | 2.22a |
| 2-Phenyl_Ethanol | Roses | 1.22a | 1.59a | 1.96a |
| ***Fatty acids*** | | | | |
| Acetic_Acid | Vinegar | 195.64a | 208.62a | 188.46a |
| Propionic_Acid | Rancid, pungent | 1.09a | 1.14a | 1.15a |
| Isobutyric_acid | Acidic | 0.12a | 0.12a | 0.15a |
| Butyric_Acid | Rancid, cheese, sweat | 0.00 | 0.00 | 0.01a |
| Iso-Valeric_Acid | Blue cheese | 0.25b | 0.33a | 0.35a |
| Valeric_Acid | Roast barley | 0.13a | 0.15a | 0.14a |
| Hexanoic_Acid | Sweat, cheesy | 98.49a | 99.09a | 94.34a |
| Octanoic_Acid | Rancid, harsh, sweaty | 0.52a | 0.55a | 0.92a |

[1] Values between columns followed by the same letter do not differ significantly ($p > 0.05$)
[2] Lee & Noble (2003); Escudero *et al*. (2007); King *et al*. (2008)

**Table 4.7.** ANOVA of thiol compounds of 2016 small-scale Chenin Blanc wines using Gas chromatography-mass spectrometry (GC – MS).

| Experiment | Treatment | 4MMP[1] | 3MHA[2] | 3MH[3] |
|---|---|---|---|---|
| A | Control (C) | 48.66a[4] | 527.30a | 193.04a |
| | 1x Treatment (T1) | 28.91a | 568.90a | 181.12a |
| | 2x Treatment (T2) | 98.72a | 395.50a | 185.38a |
| B | C | 166.76a | 15.13a | 128.67a |
| | T1 | 156.67a | 15.99a | 104.71b |
| | T2 | 148.29a | 50.51a | 125.06a |
| C | C | 627.60a | 41.94a | 250.17a |
| | T1 | 752.20a | 0.00a | 222.58a |
| | T2 | 480.40a | 0.00a | 189.16a |

[1] 4MMP – 4-mercapto-4-methylpentan-2-one
[2] 3MHA – 3-mercaptohexyl acetate
[3] 3MH – 3-mercaptohexan-1-ol
[4] Values within columns followed by the same letter do not differ significantly ($p > 0.05$)

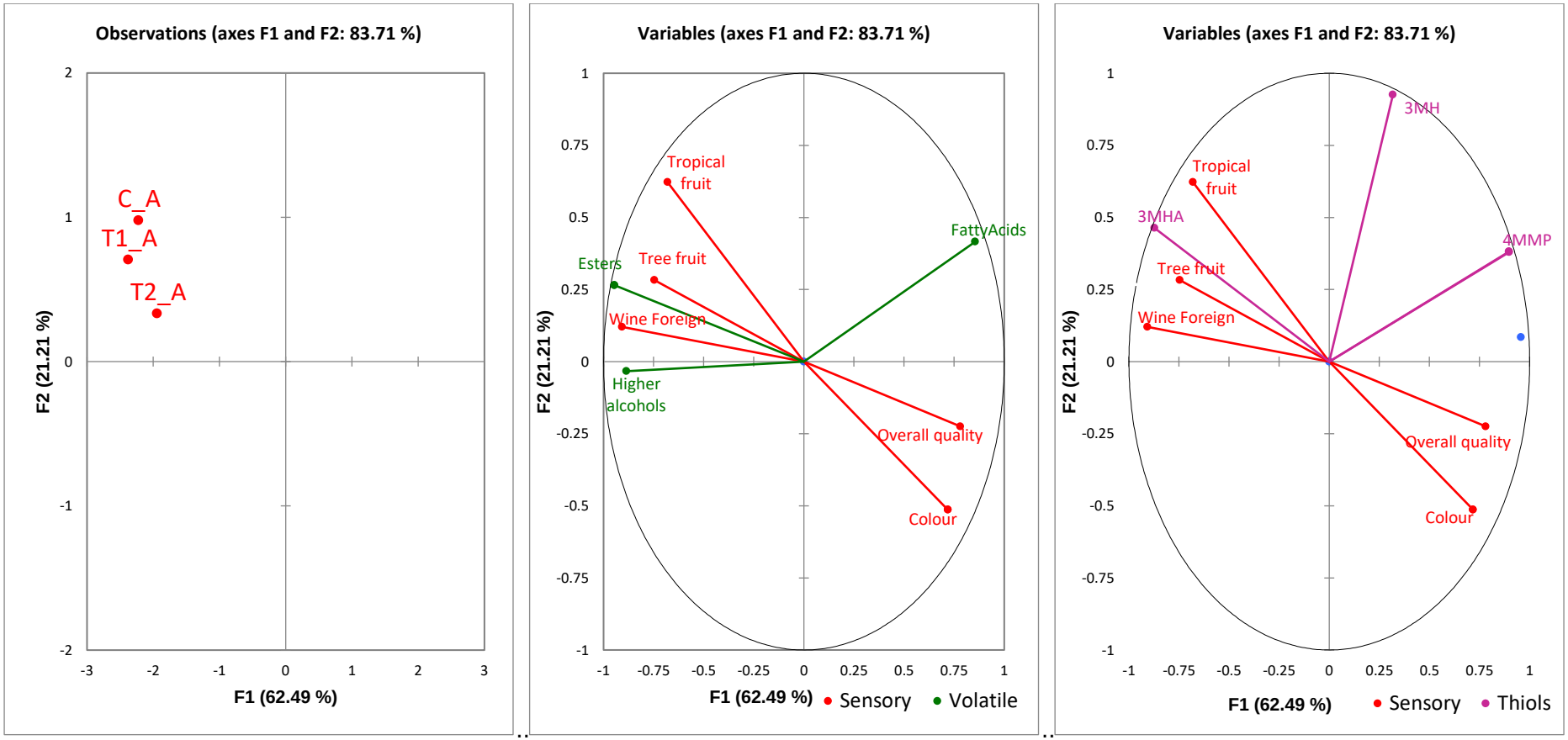



**Figure 4.7.** Experiment A: Multiple Factor Analysis (MFA) of wine sensory profiles and the association with esters, higher alcohols, fatty acids and thiol compounds i.e. 3-mercaptohexanol (3MH), 3-mercaptohexanol acetate (3MHA) and 4-mercapto-4-methylpentan-2-one (4MMP) when comparing chemical spray treatments (i.e. Control [C], 1x treatment [T1] and 2x treatment [T2].

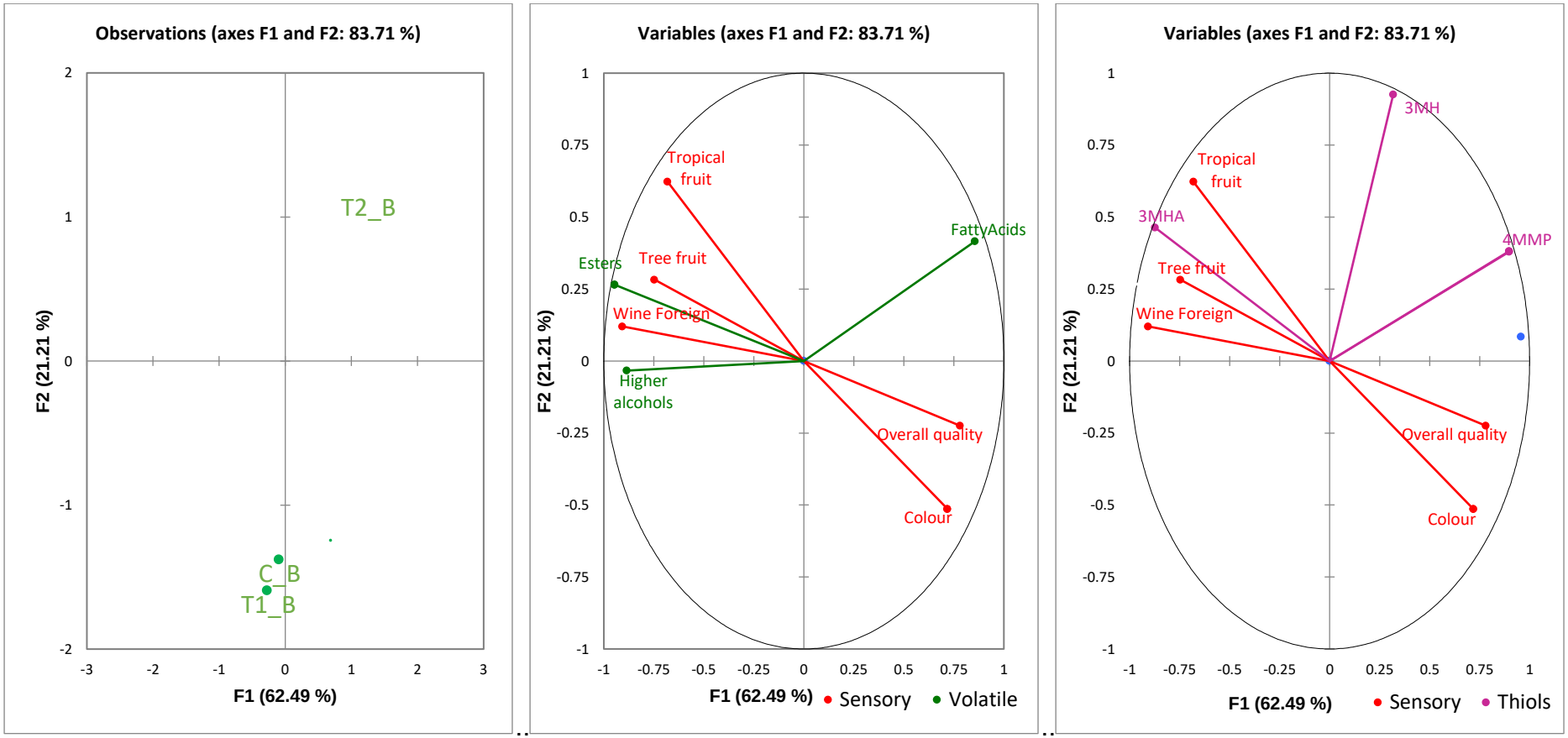



**Figure 4.8.** Experiment B: Multiple Factor Analysis (MFA) of wine sensory profiles and the association with esters, higher alcohols, fatty acids and thiol compounds i.e. 3-mercaptohexanol (3MH), 3-mercaptohexanol acetate (3MHA) and 4-mercapto-4-methylpentan-2-one (4MMP) when comparing chemical spray treatments (i.e. Control [C], 1x treatment [T1] and 2x treatment [T2].

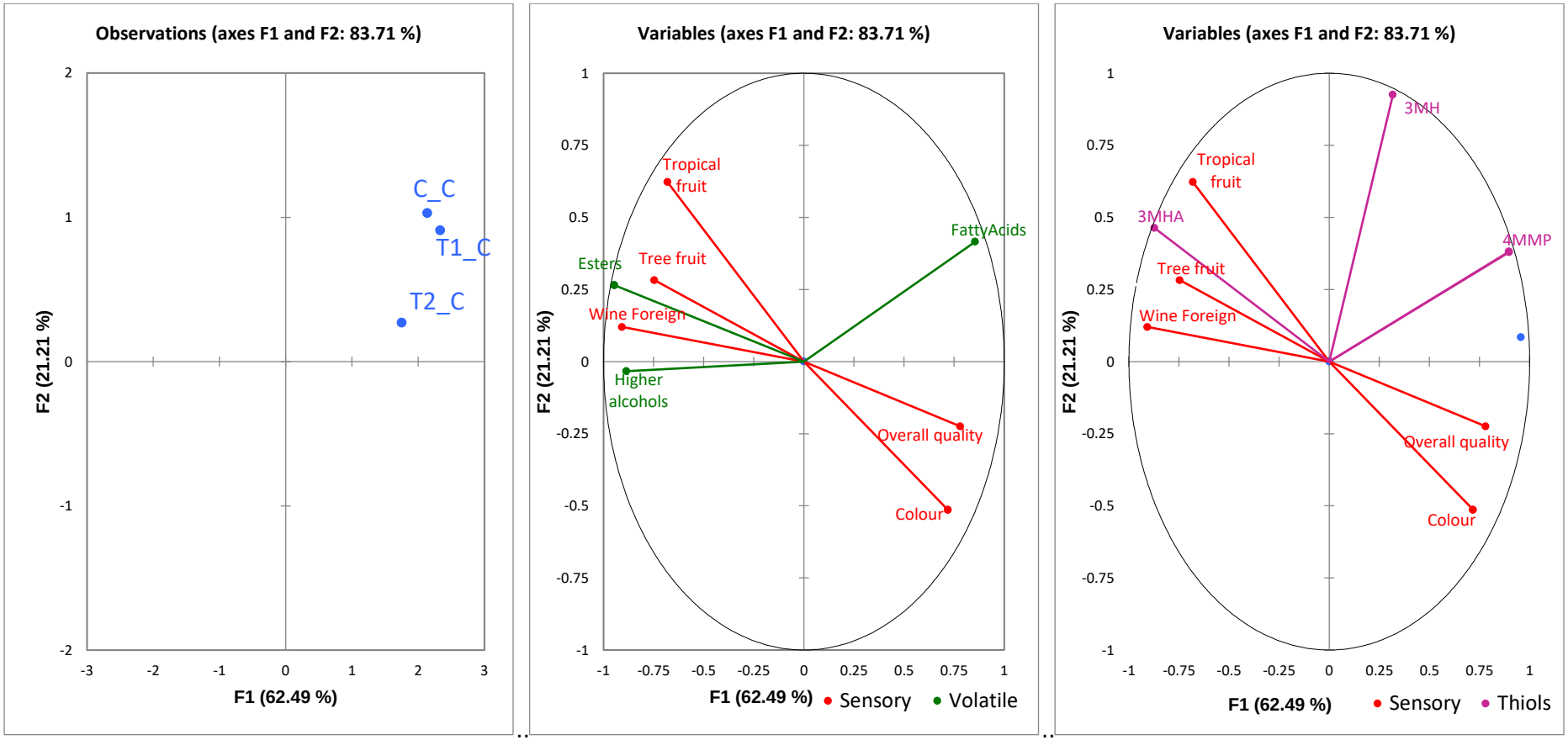



**Figure 4.9.** Experiment C: Multiple Factor Analysis (MFA) of wine sensory profiles and the association with esters, higher alcohols, fatty acids and thiol compounds i.e. 3-mercaptohexanol (3MH), 3-mercaptohexanol acetate (3MHA) and 4-mercapto-4-methylpentan-2-one (4MMP) when comparing chemical spray treatments (i.e. Control [C], 1x treatment [T1] and 2x treatment [T2].

## 4.5. Conclusion

In all the Experiments (A, B & C) fermenting wine samples collected at the start (lag phase) and at the end of fermentation (stationary phase) on both treatments when compared to the control showed no noticeable difference in terms of protein intensity and band distribution on SDS-PAGE. Therefore, the fungicide treatment used could have affected wine yeast protein expression, as this was only the variable. The peptide mass fingerprinting (PMF) in combination with MALDI-TOF/MS showed differences between the control and both treatments. It can be concluded that the treatments made the yeast behave differently, both at the start of fermentation and at the end of the fermentation. Furthermore, PMF with MALDI-TOF/MS also characterised two over-expressed proteins in the control at the start and end of fermentation, namely Phosphoglycerate kinase (involved in glucose and fructose metabolism) and Polymerase suppressor protein 2 (a suppressor of wine yeast DNA polymerases and/or DNA replication). These proteins also contributed to the release of aroma-enhancing metabolites.

Wine aroma compounds such as esters, higher alcohol and total fatty acids for all the Experiments showed no statistical significant difference between the control and the treatments. However, it is noteworthy that one ester compound detected in higher concentrations above its threshold value for Experiment A was ethyl acetate. This compound is known to contribute to “solvent” and “varnish” aromas above its threshold value. Furthermore, in Experiment A and B the fatty acid compound, acetic acid was also detected in higher concentrations above its threshold value. This compound imparts “vinegar-like” off-odours above its threshold value. Unfortunately, these aforementioned undesirable aromas were perceived by the judges during sensory evaluation (Figure 3.6 & 3.7 in Chapter 3). However, these aromas were also perceived in the control wines, therefore, it can be concluded that the fungicide treatments did not negatively affect the aroma of the wines but it could be attributed to production or viticultural practices. Hence, the overall conclusion for metabolites, the fungicide treatments had no prominent negative effect for Experiment A, B and C when comparing treatments (T1 and T2) to their respective controls. As a result, most wines showed positive associations with volatile thiol compounds (3MH, 3MHA and 4MMP) which are associated with tropical, fruity aromas, typical of Chenin Blanc wine.

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# CHAPTER 5: GENERAL DISCUSSIONS AND CONCLUSION

Wine has a significant role in South Africa's economy as it contributes 1.2% of the gross domestic product (GDP), which amounted to R36 billion in 2013. Additionally, wine exports increased from 50 million litres in 1994 to more than 400 million litres in 2014. For this reason, it is important to produce good quality wines. However, fungal infections of vines are known to decrease grape yield and quality. Therefore, fungal diseases in vineyards will lead to major economic losses (SAWIS, 2013). Furthermore, the most common diseases that adversely affect the vines include powdery mildew, downy mildew, grey mould and black rot (Caboni & Cabras, 2010; Ortiz *et al*., 2010; González-Rodríguez *et al*., 2011). While commercially available fungicides effectively inhibit fungal diseases (Tadeo *et al*., 2004; Caboni & Cabras, 2010; Nollet *et al*., 2012; Paramasiyam, 2015) and their usage is regulated under Act 36 of 1947 to comply with Good Agricultural Practises (GAP). However, they possess ingredients that may be harmful to the consumer or could negatively affect a fermentation process by affecting the yeast metabolic pathways that are involved in phenolic and/or aroma compounds production. Thus, the wine chemical parameters and the overall sensory profile could be affected (Čuš *et al*., 2010; Ortiz *et al*., 2010; González-Rodríguez *et al*., 2011; Álvarez *et al*., 2012; Noguerol-Pato *et al*., 2014; 2015).

Consequently, the application of less-harmful, natural alternatives should be strongly considered and investigated further. Furthermore, alternative treatments currently used that meet standards are calcium chloride, chitosan and ozone but only in post-harvest storage in organic classification (Mercier & Ben-Yehoshua, 2005; Gabler *et al*., 2010; Romanazzi *et al*., 2012). Considering the above mentioned facts, this study was initiated with the hypothesis that neither the fermentation performance of the yeast strain *S. cerevisiae* (VIN 13), nor the volatile metabolites, protein expressed and sensory profile of wines produced from Chenin Blanc treated grapes will be negatively affected by either the conventional, or the natural fungicide investigated in the study.

The study indeed showed that the fungicide treatments did not have a negative effect on fermentation performance of the yeast, chemical parameters and the sensory profiles of the resultant wine in both treatments when compared to the control. The performance of the yeast strains in both lab-scale and small scale fermentations showed that the yeast strain were able to complete the fermentations during the standard duration of a typical wine fermentation (± 15 days). Furthermore, the basic chemical analyses data, i.e. glucose/fructose, ethanol (alcohol) and volatile acidity

(VA) values showed that all three experiments produced wines with chemical values that were within acceptable limits. Therefore, the overall conclusion with regards to chemical analyses is that the fungicide treatments did not negatively affect the wines. Additionally, the treatments had no negative effect on the inoculated *S. cerevisiae* yeast strain as confirmed by the CHEF gel electrophoresis which showed that the yeast inoculated at the start of the fermentation had the same banding patterns as yeast inoculated from lees samples at the end of the fermentation.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for all the experiments showed that different yeast strain from fermenting wines was differentially expressed as protein banding distribution and intensity between proteins extracted was different. Moreover, this was confirmed by the protein mass spectra that showed a noticeable difference between proteins extracted from the control Chenin Blanc grapes and treated Chenin Blanc grapes for both the start and end of the fermentation (1x treatment and 2x treatment of fungicide). In addition, three over-expressed proteins were characterised by MALDI-TOF/MS and are known to contribute on the release of the aroma-enhancing metabolites. Therefore, it can be concluded that in both fermentations (at the start and at the end), yeast behaved differently because of the fungicide treatments.

Wine aroma compounds (esters, higher alcohols, total fatty acids and thiols) for control Chenin Blanc wines and treated Chenin Blanc (1x treatment and 2x treatment) contributed to aromas such as fruity, tropical and tree fruit aromas as perceived by the sensory panel during the sensory evaluations. Additionally, the wines also had the positive association with thiol compounds (3-mercaptohexyl acetate (3MHA), 3-mercaptohexan-1-ol (3MH) and 4-mercapto-4-methylpentan-2-one (4MMP)), which are known to contribute to tropical fruit, passion fruit and citrus aromas. Nonetheless, undesirable aromas (not associated with Chenin Blanc aromas) were also perceived in the wines including the control wines. Therefore, it can be concluded that the fungicide treatments showed no negative effect since the control also had similar aromas.

Considering the data obtained from the study it was seen that fungicides used to treat the Chenin Blanc grape under the GAP showed no negative effect on the yeast performance, chemical analyses, metabolites released, sensory profile and protein expressed. Moreover, one of the fungicides contained a natural extract and it showed no negative effects on the aforementioned parameters. Hence, the findings obtained from this study might help with the ongoing research focusing on natural alternative

fungicides (Elmer & Reglinski, 2006; Delaunois *et al*., 2014). In addition, omic's (proteomics and metabolomics) approaches proved to be a valuable tool to further assess the overall effect that fungicides had on proteins expressed during fermentations and ultimately their effect on the wine enhancing metabolites.

### 5.1. Future recommendations

It can be recommended that these fungicides be evaluated for other white grape cultivars as well as for red cultivars with the same treatments. It is also recommended that more natural fungicides be evaluated since there is ongoing research focused on natural alternatives to prevent fungal diseases of wine grapes. Moreover, the analysis of fungicide residue levels should be conducted prior to winemaking and also in the final product to establish actual remaining levels and to better assess their effects. Furthermore, omic's should be further utilised as a tool to investigate the effects of fungicides on other white as well as red cultivars.

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