Proteomic characterisation of wine yeast strains for the expression of arginases involved in urea formation during fermentation

By
Lucinda Jo-Anne Christians

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Supervisor: Prof Bongani Ndimba
Co-Supervisor: Mr. Rodney Hart (*Pri.Sci.Nat.*)
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Keywords

Wine yeast

Ethyl carbamate

Urea

Arginases

Protein expression

Descriptive sensory evaluation

ABSTRACT

Wine is a fermented beverage widely consumed all over the world as a recreational drink, but is known for its health benefits to humans. However, wine contain urea, a by-product of arginine hydrolysis by arginases expressed during fermentation by the wine yeast *Saccharomyces cerevisiae,* which reacts spontaneously with ethanol to form ethyl carbamate (EC). Ethyl carbamate was implicated in toxicity and carcinogenicity. Subsequently, small scale (18 L) Sauvignon Blanc and Cabernet Sauvignon winemaking trials using commercial wine yeasts were initialised during the 2014 and 2015 vintages to measure urea in final wines. The overall aim of this study was to investigate wine yeast protein expression during alcoholic fermentation and establish a possible correlation between urea formation by wine yeast and up/down regulated yeast proteins. Ion-exchange chromatography in conjunction with spectrophotometry was used to measure urea levels in bottled wines. The yeast strain, Prise de Mousse (PdM) was shown to be the lowest urea producer in both Sauvignon Blanc and Cabernet Sauvignon wines. The highest urea producing yeast strain i.e. UCD522 produced Cabernet Sauvignon and Sauvignon Blanc wines with urea exceeding 2 mg/L. Therefore, urea levels in these wines are above the Canadian legal limit, but comply with the USA voluntary limit (5 mg/L). Chemical analyses showed that all wines fermented to dryness and the sensory evaluations showed that none of the wines were negatively perceived by the judges. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed differential protein expression for the various yeast strains. Proteins of interest were, therefore, excised and characterised using matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF/MS). Characterisation of the proteins released from seven *Saccharomyces* yeasts during alcoholic fermentation were performed, by visualizing the protein profile on SDS-PAGE and identifying the differential expressed proteins in the fermentations with MALDI-TOF. It was observed that the yeast EC1118 that produced Sauvignon Blanc and Cabernet Sauvignon wines with a positive association with volatile acidity (VA), up-regulated the protein HSC82 associated with stress response. Therefore, this observation compliments previous research, since VA is known to be produced by wine yeast in response to stressful environments. It is also noteworthy, that the high urea producing reference UCD522 that produced Sauvignon Blanc wines with a negative association with VA also expressed the HSC82 stress response

protein. Indications, therefore, are that expression of the protein in different yeast strains has contrasting metabolic effects. It can be envisaged that Western blotting will be conducted in future to verify that the proteins and corresponding genes were in fact upregulated in the respective yeast strains.

DECLARATION

I declare that “Proteomic characterisation of wine yeast strains for the expression of arginases involved in urea formation during fermentation” is my own work and that it has not been submitted for any degree or examination at any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: ..... Signed at.....

Signature…… Date:.....

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: GENERAL INTRODUCTION

1.1. INTRODUCTION

Ethyl carbamate (EC) commonly found in fermented beverages has been verified to be a multisite carcinogen in experimental animals (Coulon *et al*., 2006). Ethyl carbamate was upgraded to Group 2A by the International Agency for Research on Cancer (IARC) in 2007, which indicates that EC is a probable carcinogen in humans. As EC is a proven carcinogen, discovery of its natural occurrence in products for human consumption caused quite a concern. Primary surveys indicated variable concentration levels in different products with some alcoholic beverages (mostly stone fruit distillates) containing extremely high levels (Zhao *et al*., 2013). Because of its threat to human safety, the presence of EC may be a big challenge in the alcoholic beverage industry. During the past few years, thorough and systematic research has been carried out in terms of the generation of EC in order to meet the allowed limitation levels in fermented beverages (Jiao *et al*., 2014). Previous studies also reported that EC primarily results from spontaneous reactions between precursors (e.g. ethanol and carbamyl group containing compounds) present in wine (Du Toit & Pretorius, 2000).

Said EC precursors are generally generated from arginine metabolism by *Saccharomyces cerevisiae* or lactic acid bacteria during alcoholic and malo-lactic fermentation, respectively (Jiao, 2014). Ethyl carbamate has, therefore, been banned in beverage alcohol production in some countries. Until recently, urea was not considered a major by-product of yeast metabolism that was residual in the finished wine. It is a chemical compound that can occur naturally or synthetically and is commonly used in fertilisers, animal feed and diuretics. Urea has been used for years as a nitrogen

supplement for yeast during fermentation (Zimmerli & Schlatter, 1991). However, urea was shown to react spontaneously with ethanol in wine to form ethyl carbamate (Ryu *et al*., 2015). Commercially, little attention was given to its potential formation in wine up until the Canadians found large amounts of EC in their sherries and heated dessert wines (Wu *et al*., 2012). Urea itself is not dangerous, but known to contribute to the production of EC. Reports by Jiao *et al* (2014) have shown that urea is the main source of ethyl carbamate in wines and other beverages. Other secondary sources are citrulline and possible other N-carbamyl amino acids.

Generally, red wines develop higher concentrations of EC than white wines. The bulk of EC present in wine is formed by the spontaneous reaction between urea and ethanol (Hart & Jolly, 2011). Urea is formed when the wine yeast metabolises arginine, a major alpha-amino acid in grape juice available to yeast. This reaction is therefore yeast strain dependent. Yeasts however differ in their ability to produce urea and to re-use urea secreted into the must / wine (Wu et al., 2012). The ability to use secreted urea is also affected by the overall nitrogen status of the must (Schehl *et al*., 2007). Lactic acid bacteria also metabolise arginine and liberate citrulline, an amino acid, which then reacts with ethanol to form EC. It is, therefore, evident that high arginine musts fermented by high urea producing yeasts will invariably contain high levels of urea, resulting in potentially elevated EC concentrations (Zhao *et al*., 2013). High urea levels can occur in wines produced from grapes of high (> 400 mg/L) arginine content. Such grapes tend to come from heavily fertilised vineyards. Fertilisers contain nitrogen in the form of urea, ammonium (NH_4) or nitrate (NO_3) (Ryu *et al*., 2015). It is thus important to understand the nitrogen cycle when applying nitrogenous fertilisers to the vines so that

the fate of the nitrogen can be predicted. Over-fertilised vineyards will, therefore, yield wines with higher urea and potentially higher EC concentrations. International alcoholic beverage producers have been motivated to lower EC levels as far as possible by, amongst others, Canadian legislation, which set maximum acceptable limits of 30 parts per billion (ppb) for natural wine, 100 ppb for fortified wine, 150 ppb for distilled spirits and 400 ppb for fruit brandies to be sold within their borders (Waldner & Augustyn, 2005). Subsequently, the United States of America (USA) Food and Drug Administration (FDA) established a voluntary target of 15 ppb for natural wine and less than 60 ppb for fortified wine produced in the USA (Zhao *et al*., 2013). Furthermore, the FDA informed all countries exporting wines to the United States that they need to develop programs/techniques to meet these proposed levels. However, Kodama *et al*. (1994); Hart & Jolly (2011) indicated that the urea content of wine should be below 5 mg/L and 2 mg/L in order to keep the EC levels below the Canadian legal and USA regulatory limits, respectively.

Subsequently, this research presents the metabolic mechanism of EC precursors and relevant metabolites, such as urea and arginine and the correlation between urea formation of yeast during fermentation and up/down regulation of protein expression. Therefore, the aim of this study is to investigate wine yeasts that express arginases during fermentation, and the effect on urea levels in final wines using a proteomic approach.

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CHAPTER 2: LITERATURE REVIEW

2.1. SOUTH AFRICAN WINE INDUSTRY

The wine industry in South African (SA) context is much wider than signified by the ordinary meaning of the word “wine” (SAWIS, 2013). Reason being, brandy and its building blocks (rabbat wine and/or distilling wine) for instance have always been a significant component of South African wine industry (Van Jaarsveld, 2009). Additionally, the South African wine industry from a wine grape perspective encompasses various wine styles (e.g. dry white and red, natural sweet, semi-sweet, fortified, sparkling, brandy and other spirits distilled from wine) as reported by SAWIS (2013). Furthermore, grape juice concentrate for use in non-alcoholic beverages have recently also come to the forefront.

Morokolo (2011) reported that the Western Cape wine industry (excluding tourism) provided close to 200 000 jobs during 2008, whilst contributing R14, 214 billion to the Western Cape provincial economy. This amounted to approximately 7.3% of the total provincial Gross Domestic Product (GDP) of the Western Cape. Nationally it amounted to more than R26 billion (equivalent of 1.95 %) of the GDP (Morokolo, 2011). Today, the South African wine industry has gone from strength to strength, with exports having more than doubled between 2005 and 2015. More than 99 463 hectares of agricultural land is currently being utilised to cultivate vines by more than 3300 farmers. According to a study, commissioned by the South African Wine Industry Information & Systems (SAWIS) published in January 2015, more than 300 000 people were directly and indirectly employed in the wine industry (e.g. farm labourers, labourers involved in

packaging, retailing and wine tourism). The annual 2013 harvest amounted to 1 498 240 tons of grapes (equivalent of 1 156.5 million litres of grape juice), of which 79 % was used for wine. The annual 2014 harvest saw a slight increase as 1 519 708 tons of grapes (equivalent of 1 181.1 million litres of grape juice) were harvested, of which 81% was used for wine (Wines of South Africa, 2016).

Contribution of wine industry to the GDP saw growth of at least 10 % per annum since 2003. The South African wine industry, therefore, not only contributes to the economy of the country, but also in job creation (Anonymous, 2016). As a result, continuous research to improve wine quality is important in order to maintain it's positive contribution to the SA economy. Furthermore, a key component of improving wine quality revolves around key wine microbes involved in alcoholic fermentation i.e. the wine yeast *Saccharomyces cerevisiae*.

2.2. ENOLOGY

2.2.1. Alcoholic fermentation

Enology, is the term used to describe the science of winemaking by deploying wine yeast and bacteria (Grainger & Tattersall, 2005). Therefore, the term "wine" can be defined as "the drink resulting from the alcoholic fermentation of wine yeasts" and in case of red wines, the malo-lactic fermentation (MLF) by lactic acid bacteria (LAB) (Peynaud, 1984). The grape vine *Vitis vinifera* is commonly cultivated with the aim of producing grapes for the production of wine (Grainger & Tattersall, 2005). *Vitis vinifera* spp. is used for the majority of the wines produced around the world (Swiegers *et al*., 2009). *Vitis vinifera* is a high climbing vine, growing to a height of 16 to 20 m if left

unpruned. It climbs by means of forked tendrils produced intermittently at two out of three vegetative nodes. Its leaves are palmately lobed, hairy/coarse on the underside as can be seen in FIGURE 1. Flowers form in dense panicles that develop into bunches of berries (‘grapes’), with or without seeds and are pollinated by wind, insects and self-pollination (Aradhya *et al.*, 2003).

FIGURE 1: Schematic illustration of the grape vine Vitis Vinifera. Grape Vine (Vitis vinifera) is a deciduous climber with ivy-like leaves which are coarsely toothed and clusters of tiny greenish flowers. The flowers are hermaphrodite (have both male and female organs) and are pollinated by insects. The fruits are succulent berries (grapes) green or red to black in colour. (Plants for A Future [PFAF], 2015).

Wine is a complex alcoholic beverage, which contains several compounds (metabolites) that influence the chemical and sensory quality of bottled wine (Pretorius, 2012). The metabolite compounds include water, alcohols, acids, sugars, phenolics, nitrogenous compounds, vitamins and various volatile compounds (FIGURE 2), where each

component contributing to unique aroma/s, taste and oral sensations of bottled wine (Ribéreau-Gayon *et al*., 2006).

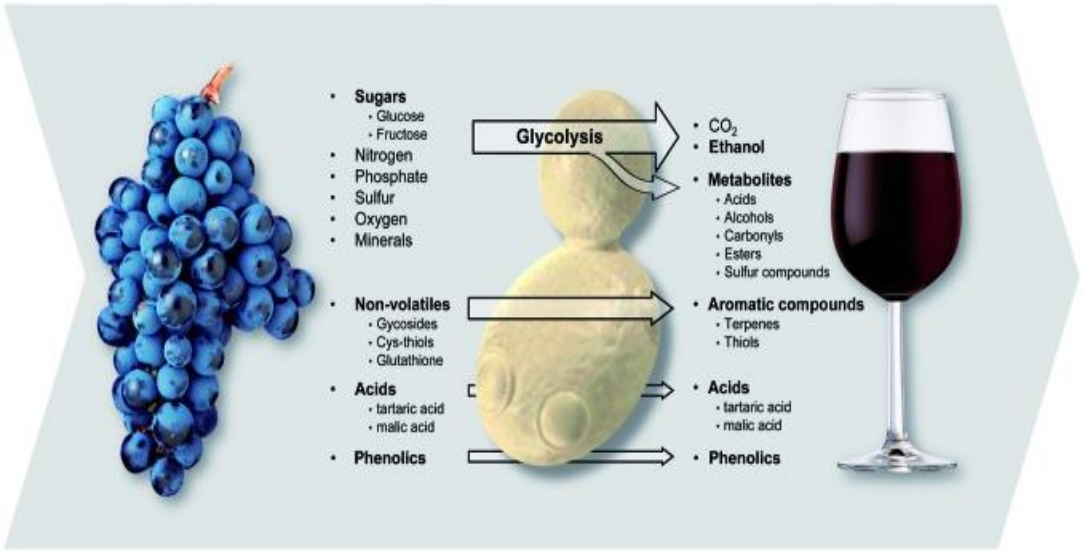

FIGURE 2: Schematic illustration of chemical compounds (precursors) present in grape must (juice). This figure shows the different compounds involved in grape must (before alcoholic fermentations) and resultant metabolites in final wines following alcoholic fermentation by the wine yeast Saccharomyces cerevisiae (Pretorius, 2012).

Once yeast is inoculated into glucose-containing solution, it will utilise the glucose as a carbon source and metabolise it into ethanol and carbon dioxide (Suarez-Lepe & Morata, 2012). The fermented product usually contains between 12-15 % ethanol, as yeast cells find it difficult to survive in higher ethanol concentrations (Pretorius, 2012). However, some strains can tolerate higher alcohol levels.

The process of fermentation is described by the reaction:

$$C_6H_{12}O_6 \xrightarrow{\text{yeast}} 2C_2H_5OH + 2CO_2$$

glucose (sugar) ➔ ethanol + carbon dioxide

FIGURE 3: The reaction process of alcoholic fermentation. Ethanol fermentation, also called alcoholic fermentation, is a biological process which converts sugars such as glucose, fructose, and sucrose into cellular energy, producing ethanol and carbon dioxide as a side-effect.

As the glucose is broken down, some of the energy stored in the chemical bonds of glucose is transferred to energy stored in the chemical bonds of ATP molecules (Pretorius, 2012). Sufficient sugar content is, therefore, essential for fermentation to proceed in a desirable manner.

2.2.2. Winemaking flow diagram (process)

Alcohol fermentation is a key component of the winemaking process (Suarez-Lepe & Morata, 2012). Furthermore, the choice of a yeast starter culture is just as important, as it has an impact on bottled wine chemical and sensorial properties (Rodney, 2016). Different wine style shares great commonalities (e.g. aroma and flavour), despite different winemaking conditions deployed e.g. Sauvignon Blanc and Cabernet Sauvignon can have similar herbaceous aroma and flavour (Curry, 2009). Furthermore,

red wines are exposed to oxygen during the primary fermentation, whilst white wines are “deprived” of oxygen (FIGURE 4) (Ribéreau-Gayon *et al*., 2006).

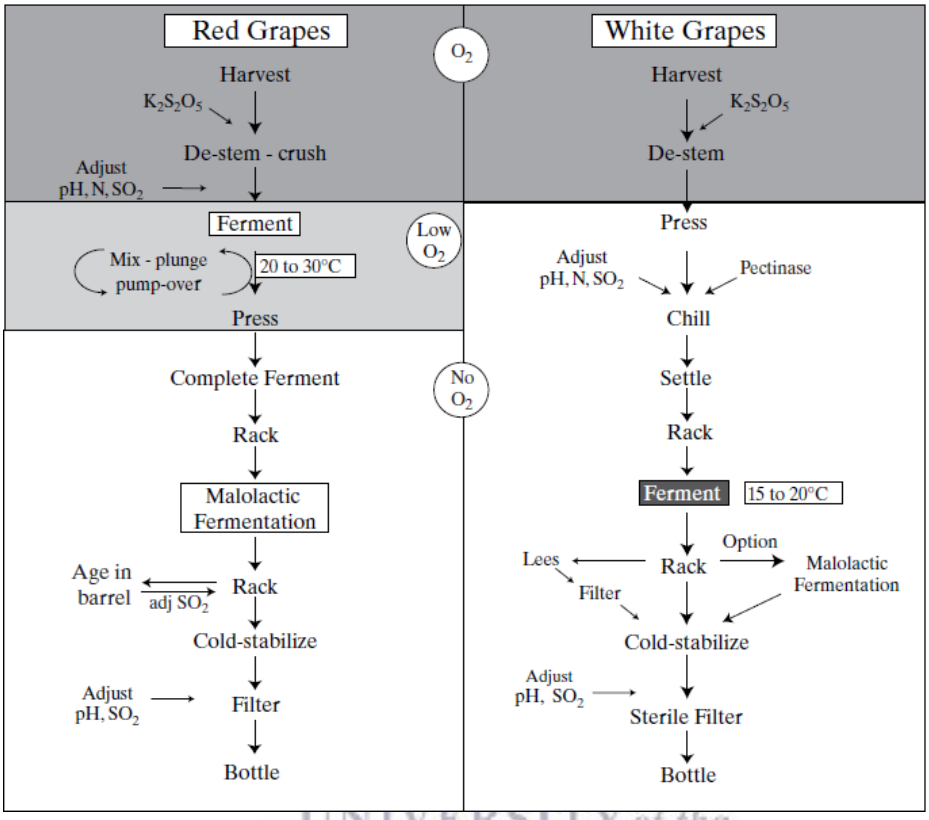

FIGURE 4: **Schematic presentation of the winemaking process**. The outline of the wine-making process for red and white wines illustrates the flow of the processes and their distinguishing features. (Ribéreau-Gayon *et al*., 2006).

2.3. WINE MICROBES

Yeasts are unicellular fungi that are commonly found in natural environments (Suarez-Lepe & Morata, 2012). There are approximately 1500 species currently known to science, and it is estimated that less than 1 % of all species have been described. Of this multitude of species, only a handful is known to be useful in the production of

alcoholic beverages (Curry, 2009; Suarez-Lepe & Morata, 2012). Yeast are commonly present on grape skins, and their population dynamics vary widely from place to place, which also contribute significantly to the aroma of the finished wine (van Breda, 2011). *Saccharomyces* strains are also common bio-flora of wine cellars and cellar equipment (Jolly, 2003; van Breda, 2011). The yeasts indigenous to a particular area are an important part of what gives its wine their character (Dubordieu *et al*., 2006). Yeasts are classified into two groups i.e. wine yeast *Saccharomyces* and the wild yeast non-*Saccharomyces* spp. As previously reported *Saccharomyces cerevisiae* is primarily responsible for alcoholic fermentation, and has been used for centuries to produce wine (Lleixà *et al*., 2016).

2.3.1. THE WINE YEAST *SACCHAROMYCES CEREVISIAE*

Saccharomyces cerevisiae, also known as baker's yeast is a single-celled eukaryote that is often used in scientific research (Lleixà *et al*., 2016). This species of yeast has been an invaluable model organism deployed in the understanding of fundamental cellular processes such as cell division and cell death, since its entire genome was sequenced and are routinely used for bio-engineering (Swiegers *et al*., 2009). Furthermore, as many yeast-expressed proteins are similar in sequence and function to those found in other organisms, studies performed in yeast can help us to determine how a particular gene or protein functions in higher eukaryotes (including humans). *Saccharomyces cerevisiae* metabolises glucose in one of two ways (e.g. aerobic respiration and anaerobic fermentation). Aerobic respiration requires the presence of oxygen, anaerobic fermentation occurs in the absence of oxygen. The net result of this

is two ATP, carbon dioxide and ethanol molecules, respectively (Ribéreau-Gayon *et al*., 2006). Both processes deploy haploid (a or ά) strains to prevent them from undergoing classical hybridisation or mating (Madigan, 2006; Gamero *et al*., 2015). In 1876 Louis Pasteur stated, “The taste and properties of the wine could depend on the special nature of yeasts which develop during the fermentation of the grapes” (Dubordieu *et al*, 2006).

2.4. YEAST METABOLITE PRODUCTION AND WINE QUALITY

Wine yeasts convert “neutral” grape juice into an aromatic wine consisting of various metabolites (Lambrechts & Pretorius, 2000; Hart & Jolly, 2008; Hart *et al*., 2016). Ethyl carbamate, a potential carcinogen for humans, is a natural constituent of fermented foods and beverages following a spontaneous chemical reaction between yeast derived metabolites i.e. urea and ethanol (Lachenmeier, 2010).

2.4.1. Significance of ethyl carbamate in wine

Recently, EC was reclassified by the International Agency for Research on Cancer (IARC) as "probably carcinogenic to humans" and occurs mainly in fermented beverages (Xue *et al*., 2015). Many countries have set limit values for EC in alcohol beverages. In this sense and taking into account the low concentrations found in alcoholic beverages, the scientific community has shown interest for the development of new analytical methods, whereby its simplification plays an important role in the EC control and prevention. Current advances in detection methods have led to the discovery of many potentially toxic substances present in fermented food and

beverages including wine, such as EC, which was shown to be harmful to humans (Jiao *et al*., 2014). It was proposed that EC was naturally formed in all fermented foods, possibly through the ethanolysis of carbamyl phosphate (Adams & van Vuuren, 2010). Subsequently, the formation of EC was investigated in model solutions and wines using radioactively labelled urea and monitoring the appearance of radioactivity in resultant ethyl carbamate (Monteiro *et al*., 1989). These studies proved that yeast metabolic activities are indirectly involved in EC formation, since one precursor urea are formed from the degradation of arginine by wine yeast expressed arginases (Lachenmeier, 2010; Leça *et al*., 2014).

It also was observed that EC is formed in wines following treatment with the yeast inhibitor i.e. diethyl pyro carbonate (DEPC), which was reported to serve as a precursor (Thoukis, 1962). During 1971 it was reported that DEPC reacts with ammonia at neutral or alkaline pH to produce EC. During this period, DEPC was widely used as an antimicrobial food additive for beverages (Stevens & Ough, 1993; Chen *et al*., 2015). Subsequently, DEPC was withdrawn as a food additive for all beverages and, was replaced with dimethyl pyro carbonate (DMDC) (de Orduña Heidinger, 2001). It was later demonstrated that urea, a natural by-product of fermentation, is the main precursor of EC in alcoholic products. Further investigations showed that actual EC levels in wines treated with DEPC were lower compared to that reported earlier (Stevens & Ough, 1993; Chen *et al*., 2015).

2.4.2. History of ethyl carbamate (EC)

Ethyl carbamate, also known as urethane, was used as a therapeutic agent for the treatment of leukaemia (Zhao & Kobashi, 1994). Furthermore, EC has also been used

as an intermediate in synthetic preparation and modification of resins and as a solvent for pesticides, fumigants and cosmetics (Leça *et al*., 2014). It was also known for its narcotic action, since it was used for many years as an anaesthetic (Zimmerli & Schlatter, 1991). However, this naturally occurring compound was later found to be carcinogenic and teratogenic to human beings (Adams & van Vuuren, 2010; Lachenmeier *et al*., 2012). Subsequently, its use was banned as a drug by many countries during 1970. Nonetheless, human beings still come into contact with urethane by consuming various fermented food and alcoholic fermented products such as wine, sherry, whisky and sake (TABLE 1) (Adams & van Vuuren, 2010; Pretorius, 2012). Therefore, consumption of fermented beverages can therefore significantly increase daily exposure to ethyl carbamate. It was also detected in cheese, tea, soy sauce and bread, with the levels of ethyl carbamate in toasted bread being six times higher than in the fresh counterpart.

TABLE 1: Ethyl carbamate concentration in various fermented beverages

(adapted from Leça *et al*., 2014).

Country	Product	Samples	EC content (µg/L)
Brazil	Sugar cane spirit	84	94
China	Rice wine	92	160
	White spirit	22	72
	Wine	30	16
Italy	Primitivo wine	160	6.81 to 15.62
Japan	Sake wine		100 to 250
North America and Europe	Brandy	137	78
	Cognac	256	30
	Gin	53	9 to 11
	Liqueur	356	21 to 22
	Whisky	1122	40
	Fortified wine	1000	39
	Wine	23278	5 to 7
Poland	Fruit spirits	3	130 to 390
South Africa	Wine(1 to 9 yr)	106	1.8 to 31
	Fortified wine(2 to 34y)	21	2.8 to 79
	Brandies (3 to 20y)	26	4.4 to 95
United Kingdom	Wine		11 to 24
	Sake wine	2	81 to 164
	Fortified wines		14 to 60

2.4.3. Urea the main ethyl carbamate precursor

Urea, a by-product of yeast metabolism, is the main precursor of EC, and can be measured in a young wine as an indicator for potential ethyl carbamate formation (Lachenmeier, 2010). Ethyl carbamate, the carcinogen, is formed when urea, a by-product of arginine metabolism by the wine yeast *S. cerevisiae* reacts spontaneously with ethanol during fermentation (Pretorius, 2012; Zhao *et al*., 2013; Chen *et al*., 2015). This reaction is affected by time and increased temperature, thereby, putting wines that

undergo long periods of maturation and storage at a higher risk of developing elevated levels of EC (De Orduna Heidinger, 2001). Several studies have shown that the formation of EC is significantly accelerated by high concentrations of ethanol, urea and citrulline. Stevens and Ough (1993) studied the relationship between urea, ethanol and the formation of EC at different temperatures, and reported that the urea concentration in wine should not exceed 2 mg/L. It is, therefore, crucial that a wine containing elevated levels of urea is not exposed to high temperatures during storage or shipment. Both urea and ethanol, the main precursors of EC in wine are released by yeast cells during alcoholic fermentation (Coulon *et al*., 2006; Dahabieh *et al*., 2010; Pretorius, 2012; Chen *et al*., 2015).

Alternative pathways have been identified for the formation of EC, particularly those that are present in fermented beverages as seen in FIGURE 5. Furthermore, major precursors contributing to EC formation was shown to contain a carbamyl group, which include urea, citrulline and carbamoyl phosphate (Dahabieh *et al*., 2010).

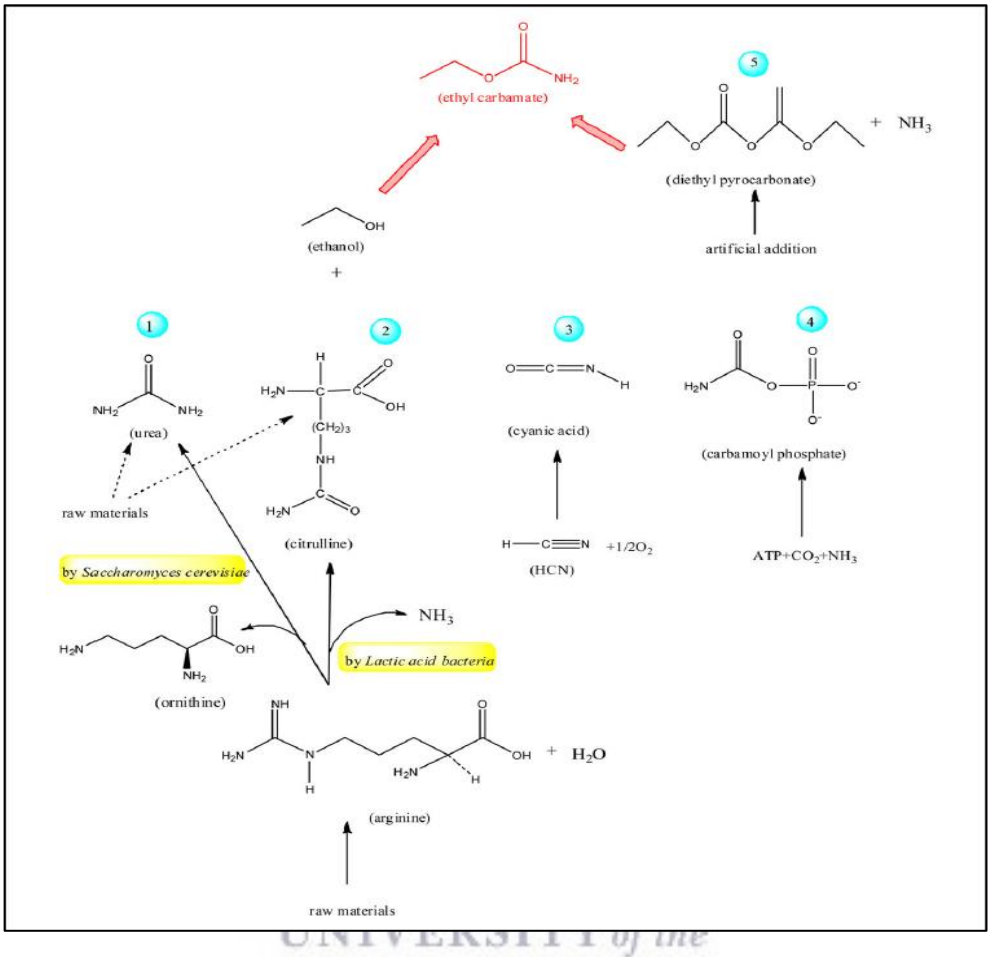

FIGURE 5: Pathways involved in ethyl carbamate (EC) formation in fermented beverages and/or spirits. The major precursors participating in the formation of EC have been identified to contain a carbamyl group, and these include urea, citrulline, and carbamoyl phosphate. Moreover, it has been demonstrated that cyanic acid and diethyl pyro carbonate are involved in EC formation (Jiao *et al*., 2014).

2.4.4. Effect of amino acids in wine on urea

The production of urea during fermentation is a direct consequence of arginine metabolism by the wine yeast *S. cerevisiae* (Monteiro & Bisson 1991; Lachenmeier, 2010; Zhao *et al*., 2013; Leça *et al*., 2014), which is one of the most abundant amino

acids in grape must and an important nitrogen source for yeast (Adams & van Vuuren, 2010).

In FIGURE 6 it shows how arginine is taken up by wine yeast as a nutrient and can be metabolised yielding urea if present in excess amounts (Dahabieh *et al*., 2010; Jiao *et al*., 2014). However, if the urea is not metabolised and accumulates above a critical concentration, yeast strains release it from their cells into the wine during or at the end of fermentation (Mohapatra & Bapuji, 1997; Pretorius, 2012). Subsequently, urea can spontaneously react with the alcohol present in wine to form EC. This chemical reaction between urea and ethanol is exponentially accelerated at elevated temperatures (Coulon *et al*., 2006). Citrulline, an amino acid which is formed during wine yeast arginine biosynthesis can also serve as an EC precursor (Pretorius, 2012). Lactic acid bacteria (LAB) were also shown to be a source of citrulline during winemaking. However, the main contributor of EC formation in wine results from the spontaneously reaction between urea and ethanol (Butzke & Bisson, 1997; Rossouw, 2010).

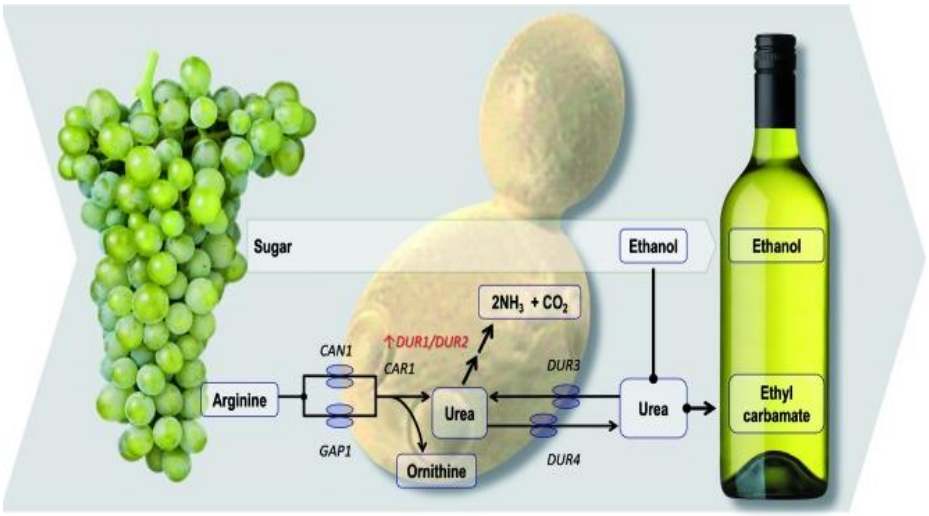

FIGURE 6: Schematic illustration of arginine metabolism into urea. Intracellular urea mainly results from the degradation of arginine through catalysis by arginase (CAR1). *S. cerevisiae* metabolises urea in 2 steps. First, urea is carboxylated to form allophanate by urea carboxylase. The resultant allophanate is degraded to CO2 and NH4 + by allophanate hydrolase urea carboxylase and allophanate hydrolase are performed by a bifunctional enzyme, urea amidolyase. Urea therefore serves as precursors for ethyl carbamate (EC) formation (Pretorius, 2012).

2.4.5. Metabolism of ethyl carbamate precursors in fermented beverages

Urea was shown to be the predominant precursor of EC in a variety of fermented beverages, and studies of wine, Chinese rice and sake wine due to metabolism of urea by *S. cerevisiae* (Marangon, 2010; Chen *et al*., 2015). Intracellular urea mainly results from the degradation of arginine. Arginine is cleaved by arginase (encoded by the *CAR1* gene) into ornithine and urea, which serves as source of nitrogen for *S. cerevisiae* (Wu

et al., 2012). As a toxic and poor nitrogen source for *S. cerevisiae*, the generated urea is usually collected and exported to the surrounding medium through a facilitated diffusion system (An & Ough, 1993; Pretorius, 2012). During wine fermentation, urea degradation does not always immediately follow arginine metabolism (Bisson, 1996). Therefore, urea is gradually excreted by yeast cells and can be re-absorbed at a later stage to be used as a nitrogen source (Jiao *et al*., 2014; Rabilloud *et al*., 2010).

Wine yeast strains differ in their ability to rapidly catabolise urea during fermentation. When excess urea accumulates in the cell's cytoplasm, it is released into its environment known as the must (Jiao *et al*., 2014; Giribaldi, 2010). Many factors, including grape juice composition, yeast strains, and vinification conditions, can affect urea formation, release, and reutilisation (Ough *et al*., 1990; Marangon, 2010). Subsequent studies highlighted the specific effect of each extraneous factor on urea excretion and reutilisation (An & Ough, 1993; Chen *et al*., 2015). It was also shown that yeast strains that take up arginine more rapidly tend to excrete more urea. Secondly, the presence of ethanol in the culture broth can inhibit metabolite transport over the cell wall. Therefore, at higher concentrations of initial ethanol, less arginine will be taken into the cells and less urea will be released (Wu *et al*., 2012). Furthermore, higher concentrations of ammonia and arginine were also shown to suppress urea reabsorption (Rossouw *et al*., 2010). High urea producing yeasts are those that have a high capacity to degrade arginine to urea and a low urea metabolising ability. Low urea metabolising ability may result from low activity of urea amidolyase, inhibition of amidolyase activity by the presence of high levels of ammonia, deficiencies of cofactors required by amidolyase, or apparently low activity due to hyperactive arginase (Adams

& Vuuren, 2010; Rossouw *et al*., 2010). Genetic as well as environmental factors influence the amount of urea released by the cells.

2.5. VINEYARD FERTILISATION

In addition to sugar, yeasts need access to certain nutrients in order to remain healthy (Ugliano *et al*., 2007). These nutrients include amino acids, fatty acids and nitrogen. Nitrogen has a major influence on vine development and grape composition. High nitrogen status increases vine vigour, yield and sensitivity to fungi, particularly *Botrytis cinere and* low vine nitrogen status increases berry sugar content and total phenolics (Lacroux, 2008).

Nitrogen (N) fertilisation in the vineyard has direct influence on the nitrogen contents of the grape berry and the resulting must (Weber & Sharypov, 2009). Excessive fertilisation with urea, ammonia and other N-fertilisers in the past is considered partially responsible for generally higher Ethyl Carbamate (EC) levels found in wines from traditional wine producing countries (Treeby *et al*., 2004). In turn, the concentration of arginine in grape musts depends on the production of this compound in the vineyard and nitrogenous compounds like arginine, increase proportionally with the fertilisation of vineyards (Butzke & Bisson, 1997; Ugliano *et al*., 2007).

Saccharomyces cerevisiae can grow in a wide variety of nitrogen containing media, as the rates of consumption and metabolism of nitrogen compounds in such media are dependent on the specific yeast strain, its physiological status and the physicochemical properties of the medium (Mauricio, 2001). Therefore *S. cerevisiae* can utilise amino acids to synthesize proteins and as a nitrogen source: the amino acids are degraded by yeast cells, and the nitrogen that they contain is released generally, but not always, as

ammonia is used to synthesise other nitrogen-containing cell constituents (Weber & Sharypov, 2009). The yeasts can therefore use the carbon in amino acids for synthetic purposes; these compounds act as carbon sources or are released into the medium (Mauricio, 2001).

Nitrogen compounds, particularly ammonium ion, amino acids, peptides, and small polypeptides found in grape must can be used as nitrogen sources by yeasts. These compounds are important to the vinification process, not only because they influence the growth of yeast but because they affect the formation of higher alcohols, which contribute to the aroma of wine and its quality (Ugliano *et al*., 2007). The concentration of nitrogenous components such as arginine in juice, and urea in wine increases proportionally with increased nitrogen fertilisation in the vineyard (Tesnière *et al.*, 2015). If arginine concentrations in juice exceed 1000 mg/L, the vineyard must be considered over-fertilised. Nitrogen status of grapes varies widely with vineyard site, soil, irrigation and fertilisation practices, vintage weather, scion and rootstock, and grape maturity (Ribéreau-Gayon *et al*., 2006). The two major sources for nitrogen in must are ammonia and amino acids with the exception of proline. Proline cannot be used as a yeast nitrogen source without molecular oxygen, which is not present in anaerobic grape juice fermentation (Tesnière, Brice & Blondin, 2015). Even though amino acids play a prominent role in the formation of EC, nitrogen-rich sources are important as nitrogen deficiencies in grape must and juices can lead to a series of difficulties during vinification, as it can limit yeast growth, and can result in sluggish or stuck fermentation (Lacroux *et al*., 2008).

2.6. PREVENTION OF EC PRECURSOR FORMATION

It has been specified that the formation of EC precursors, urea and citrulline by yeasts, depends on the presence of arginine in musts and wine (Chen *et al*., 2015). Therefore, the concentration of arginine in grape musts depends on the production of this compound in the vineyard and nitrogenous compounds like arginine, increase proportionally with the fertilisation of vineyards (Weber & Sharypov, 2009). A long term vineyard nitrogen fertilisation experiment done by Bisson (1991) showed that nitrogen fertilisation increased total nitrogen (+50 %) and arginine (+50 %) concentrations in musts compared with must from a non-fertilised control vineyard, concluding that as fertiliser increased so did arginine concentrations.

Because of its toxicity, carcinogenicity, and universality, EC is currently one of the biggest challenges in the alcoholic beverages industry (Chen *et al*., 2015). It is, therefore, clear that methods to decrease EC formation potential in wines have to start at the nitrogen status of the vineyards as suggested in the EC preventative action manual of Butzke and Bisson (1997). After alcoholic fermentation it still showed that arginine concentration was still high in wine from a nitrogen fertilised vineyard, compared with wine vinified from a controlled vineyard. Ough *et al.,* (1989) went further and showed that there is a direct relationship between vineyard fertilisation and formation of EC itself. Previous studies indicated that the amount of urea excreted and reabsorbed by yeast mainly depends on the remaining arginine level in the medium and was shown that yeast have different abilities to produce and re-utilise urea (An & Ough, 1993; Zhao *et al*., 2013; Wu *et al*., 2014; Li *et al*., 2015). Currently, there are still no

general methods for EC prevention in all foods and beverages but some different strategies have been developed and applied in industrial scale.

Based on recent research, the justification of EC in alcoholic beverages can be achieved by the modification of raw materials and by the optimisation of the fermentation parameters, like using commercial yeasts that excretes low concentrations of urea and by the addition of acid urease that degrades urea or even by the modification of the fermentation yeast cells itself (Lim & Lee, 2011; Zhao *et al*., 2013). Acid urease has an optimum pH compatible with wine, and its feasibility to remove urea has been demonstrated. However, the industrial application of acid urease in wineries is still limited because of longstanding and expensive procedures. There have been some new approaches to eliminating EC efficiently. Although these methods are not well developed and are even still at the assumption stage now, they provide some more effective and practicable ideas for EC elimination (Chen *et al*., 2015). Although acid urease can effectively degrade urea, once EC has formed it is very difficult to degrade it during storage. Nonetheless, another enzyme can degrade EC directly. Urethanase belongs to the category of amidases, which was named by Kobashi *et al*., (1990).

2.7. PROTEOMICS

The term proteomics describes the study and characterisation of complete set of proteins present in a cell, organ, or organism at a given time (Domon & Aebersold, 2006). In general, proteomic approaches can be used (a) for proteome profiling, (b) for comparative expression analysis of two or more protein samples, (c) for the localisation and identification of post-translational modifications, and (d) for the study of protein–protein interactions. Proteomics was previously shown to be a practical approach to study wine yeast protein expression in it natural habitats (Olineka, 2005; Feist & Hummon, 2015; Rahmad, 2014; Hart *et al*., 2016). Wine has a highly complex sample matrix and chromatographic techniques, which are suited for the separation of complex mixtures and quantitation of their components, are frequently used in wine analysis. Gas chromatography (GC) is primarily used in the analysis and research of the volatile fraction of wines. High performance liquid chromatography (HPLC) has found widespread application in wine analysis due to the versatility and scope of the technique, and it is primarily applied to the analysis of non-volatile wine components. Important research in this field increasingly requires analytical techniques that are capable of higher sensitivity and selectivity. As a consequence, conventional chromatographic detectors such as the flame ionisation detector (FID) in GC and the ultraviolet-visible spectroscopic detector in liquid chromatography (LC), increasingly fall short of experimental requirements.

A study done by Xiang-hong Shen (2013), made use of a proteomic technique to understand the content status of EC in yellow rice wine and the changes in storage period and shelf life in Zhejiang province. A total of 475 samples of yellow rice wine

purchased from supermarkets and food stores, and 49 samples collected from manufacturers were measured for EC content. The samples collected from manufacturers were placed for 400 d at 4 °C, room temperature and 37 °C, respectively. Subsequently shelf-like storage tests were conducted on the samples. The content was measured at every point in 2011. The EC of the samples was determined by gas chromatography-mass spectrometry (GS-MS) (Shen, 2013). The content of EC was correlated with storage time and temperature which resulted in an overall detection rate of EC was 99% (472/475) in yellow rice wine.

Even though the research done by Shen, did not require specific protein identification, a reliable and efficient proteomic technique was used to understand the content of EC in yellow rice wine and how storage and temperature plays a vital role. Due to the complex nature of the proteome, the continuous development of new methods and techniques for the chromatographic separation and detection (but also sample clean up, fractionation and pre-concentration) becomes a crucial prerequisite for the correct identification of peptides and proteins (Mishra, 2011). Real-time analysis of several thousands of different proteins from complex biological samples is often required in modern proteomics.

2.7.1. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Before the digestion, separation of proteins is performed using a gel. A basic overview of gel-based mass spectrometry protocol can be seen in FIGURE 7 (Feist & Hummon, 2015). In one of the most common proteomic sample preparation strategies, a denaturing sodium dodecyl sulfate in a polyacrylamide gel, (SDS-PAGE) is used for bottom–up proteomics, as the protein will be cleaved into peptides in later steps. Proteins are separated based in Molecular weight whereas proteins are stained and excised from the gel followed by de-staining. Thereafter proteins are subjected to digestion (Mishra, 2011).

One-dimensional gels are excellent for simple fractionation; as proteins often have specific molecular weights, allowing for a semi-targeted approach. Less interference is involved in this approach, as the sample is dramatically simplified (Chandramouli & Qian, 2009). The SDS-PAGE is possibly the most widely used proteomic technique today, having the ability to separate thousands of proteins in a single sample from a complex mixture. This technique is also a fairly simple procedure, and the gel provides a good vehicle for the safe storage of proteins for future analyses (Zhou et al., 2012; Rahmad, 2014).

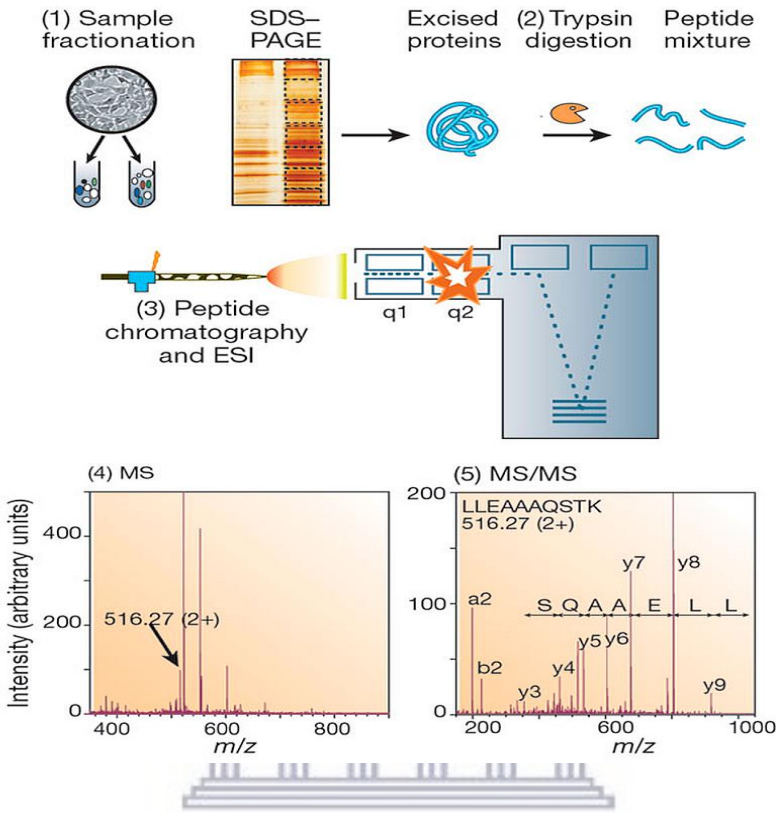

FIGURE 7: **Typical workflow for gel-based mass spectrometry analysis**. The gel is used to separate whole protein in one or two dimensions. After de-staining, the proteins are excised from the gel and subjected to enzymatic proteolysis. Peptides can then be analysed via mass spectrometry (Feist & Hummon, 2015).

2.7.2. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Conventional quantitative proteome analysis utilises two-dimensional (2D) gel electrophoresis (Chandramouli & Qian, 2009; Rahmad, 2014) to separate complex protein mixtures based on their Molecular Weight and pI followed by in-gel tryptic digestion and MS for the identification of protein. More than 1500 soluble proteins of yeast are detectable and well separated of 2D gels. This technique offers the

opportunity to detect alterations in protein synthesis, protein modifications, and protein degradation occurring in response to environmental or genetic changes (Zhou *et al*., 2012). However, 2D-PAGE has a limitation due to the low number of proteins that can be identified on the yeast protein map, as well as poor reproducibility between consecutive gels, the under representation of low-abundant and hydrophobic proteins and the poor dynamic range of detection (Rabilloud, 2002; Chandramouli & Qian, 2009; Feist & Hummon, 2015).

2.7.3. Protein spot excision

The ultimate goal of a 2D experiment is often the separation and identification of differentially expressed proteins. This is achieved by mass spectrometric analysis of individual protein spots identified on a 2D gel (Chandramouli & Qian, 2009; Feist & Hummon, 2015). The protein spots are then digested with trypsin and the resulting peptides are separated by high-performance liquid chromatography (HPLC).

2.7.4. Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

After gel electrophoresis, each eluted peptide is ionised by electrospray ionisation. It then enters the mass spectrometer through the first quadrupole mass filter (Q1) and is fragmented in a collision cell (Q2). The resulting spectrum is recorded (Q3). In the third step, the tandem MS spectrum of a selected ionised peptide contains sufficient specific sequencing information to identify the peptide and its associated protein. m/z ¼ mass to charge ratio.

2.7.5. Peptide Mass Fingerprint

Protein identification can be accomplished using a variety of approaches including peptide mass fingerprinting (PMF) using matrix-assisted laser desorption ionisation with time of flight mass spectrometry (MALDI-TOF MS), MALDI tandem MS using MALDI-TOF-TOF mass spectrometry, or by liquid chromatography tandem (LC) MS using reversed-phase chromatography coupled online to the mass spectrometer via an electrospray ionisation source (Figeys, 2004). In this approach, proteins are identified by comparing the peptide masses against a protein sequence database. In the latter two approaches, proteins are identified using peptide masses and their MS-MS fragmentation patterns to search the protein database (Feist & Hummon, 2015).

2.8. MOTIVATION FOR STUDY

To the winemaker, the yeast starter culture (inoculum) is essential to produce excellent, complex wines from simple, sugar-rich grape juice. Therefore, any improvements in wine quality, which involves developing yeast strains with enhanced fermentation and ability to produce wines with good chemical and sensory properties, will ultimately benefit the South African wine industry. As, EC is currently one of the biggest challenges in the alcoholic beverages industry due to its toxicity, carcinogenicity and universality, methodologies to decrease EC formation potential in bottled wines have to focus on the wine yeast strain used for alcoholic fermentation. Since the yeast derived metabolite i.e. urea is the main precursor of EC, refraining from researching this aspect of wine production, will have a negative impact on the South African wine industry GDP.

This research presents the metabolic mechanism of EC precursors and relevant metabolites, such as urea and arginine and the correlation between urea formation of yeast during fermentation and up/down regulation of protein expression. Therefore, the aim of this study is to investigate wine yeasts that express arginases during fermentation, and the effect on urea levels in final wines using a proteomic approach.

2.8.1 THE SPECIFIC AIMS OF THE STUDY WERE AS FOLLOWS

1.2.1. to determine urea formation of selected commercial yeast strains during fermentation;

1.2.2. to characterise wine yeast enzymes (proteins) that are expressed during the fermentation of grape must and synthetic must, rich in arginine;

1.2.3. to establish a correlation between urea levels at the end of fermentation and yeast proteins expressed; and

1.2.4. to develop a proteomic protocol to predict the potential of a yeast strain to form urea

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CHAPTER 3: SENSORY, PROTEOMIC AND METABOLOMIC CHARACTERISATION OF WINE YEAST FOLLOWING FERMENTATION OF SAUVIGNON BLANC AND CABERNET SAUVIGNON JUICE

3.1. INTRODUCTION

Ethyl carbamate, is considered a potential carcinogen for humans and shown to be a natural constituent of fermented foods and beverages (Coulon *et al*., 2006). Subsequently, Western countries like USA and Canada have set regulatory limits for the amounts of ethyl carbamate (EC) in alcoholic products. Urea, a by-product of yeast metabolism, is the main precursor of EC and can be measured in a young wine as an indicator for potential ethyl carbamate formation. Kodama *et al*., (1994) indicated that for wine the urea content should be below 2 mg/L to keep EC below the USA target. Identifying the causes of human cancer is the priority for its prevention and today the high incidence of this disease continues to increase. The EC raises some concerns in terms of public health as a “probably carcinogenic to humans” being a problem more associated with alcoholic beverages consumption (Jiao *et al*., 2014). The prevention and control of EC levels, used in the beverage industries, have obtained good results, however it seems to be important to keep implementing these effective preventive and control actions. There has been a clear reduction of this compound in commercial products over the past 20 years. These results are due to the efforts made in the identification of the main precursors, the understanding of its formation mechanisms, as well as the impact of external factors such as light, temperature and time of storage or ageing.

These main EC precursors are generally generated from arginine metabolism by *cerevisiae* or lactic acid bacteria (LAB) accompanied by the fermentation process (Jiao *et al*., 2014). Intracellular urea mainly results from the degradation of arginine. Arginine is cleaved by arginase (encoded by the CAR1 gene) into ornithine and urea, which serves as source of nitrogen for *cerevisiae* (Carrasco et al., 2003). During wine fermentation, urea degradation does not immediately follow arginine metabolism (Bisson, 1996). Therefore, urea is gradually excreted by yeast cells and can be re-absorbed at a later stage to be used as a nitrogen source (Jiao *et al*., 2014; Rabilloud *et al*., 2010).

In this sense and taking into account the low concentrations found in alcoholic beverages, the scientific community has shown interest for the development of new analytical methods, whereby its simplification in method and/or fermentation process plays an important role in the EC control and prevention. The choice of the EC for this investigation main purpose was based not only on the toxicological concerns but on the legislation limitations established for the EC occurrence in beverages, which already enforced some difficulties to wineries in exportation of wines. This in return leads to economic losses and the image of these wines in the international market can be compromised. The first objective of this work was the quantification of EC in wines using simple, fast and affordable analytical procedures, without needing sophisticated and expensive equipment that is available on the market.

Therefore, the aim of this study was to conduct comparative, chemical and descriptive sensory analyses of wines following fermentation with different yeast strains. Wine yeast proteome will also be investigated, since wine yeast expressed proteins e.g. arginases were reported to be involved in urea formation. Moreover, a comparative metabolome analyses of wines will also be conducted, since regulated/expressed proteins are also known to influence other yeast and derived metabolites.

3.2. MATERIALS AND METHODS

3.2.1. Yeast strains

The active dried wine yeast (ADWY) strains used in this study (TABLE 2) were selected and used for the production of white and red wine trialled in small-scale (20 L or 40 kg) wine production during the 2014 and 2015 harvest, and their effect on urea levels in final wines. Two commercial wine yeast strains i.e. a low urea producing yeast strain EC1118 (also known as Prise de Mousse [PdM]) and a high urea producing yeast strain UCD522 were included in this trial to serve as references (Ough *et al*., 1990).

TABLE 2: Commercial Saccharomyces cerevisiae yeast strains evaluated for urea formation during fermentation of, Sauvignon Blanc and Cabernet Sauvignon grape must.

Strain	Source
UCD522	High urea producer (Mauri Yeast, Australia)
EC1118	Low urea producer (Lallemand, France)
VIN7	Anchor Bio-Technologies, South Africa
VIN13	Anchor Bio-Technologies, South Africa
N96	Anchor Bio-Technologies, South Africa
NT50	Anchor Bio-Technologies, South Africa
NT112	Anchor Bio-Technologies, South Africa

3.2.2. Chemical analyses using Fourier transform infra-red (FTIR) spectroscopy

Wines should be analysed during production and storage to ensure that they comply with the requirements of regulatory bodies. In addition, the chemical analyses allow the winemaker to monitor the operations effectively to ensure a good quality wine (Boulton *et al*., 1995). Various chemical parameters i.e. alcohol, volatile acidity (VA), total acidity (TA, pH and urea were analysed on the final wines for both vintages.

3.2.3. Grape cultivars

Commercially ripe Sauvignon Blanc (SB) and Cabernet Sauvignon (CS) grapes originating from the ARC Infruitec-Nietvoorbij research farm in Stellenbosch were used for vinification trials during the 2014 and 2015 vintages.

3.2.4. Small-scale wine production

Wines were made in the ARC Nietvoorbij Research Cellar according to a standardised small-scale winemaking procedure (ARC Infruitec-Nietvoorbij harvest program, 2014; 2015) schematically depicted in FIGURE 8. The ADWY were rehydrated in water using the manufacturer's recommendations, 30 min at 37 ˚C, inoculated at 0.3 g/L, and fermented at 15 ˚C (Sauvignon Blanc) and 25 ˚C (Cabernet sauvignon) in stainless steel canisters sealed with a water-filled fermentation lock. Sugar concentrations were monitored daily during the fermentation process using a Oenofoss^{TM} Fourier Transform Infrared (FTIR) spectrometer. Once the fermentations were fermented to dryness (residual sugar <2 g/L), the wines were cold-stabilised, filtered and bottled according to a standard cellar methodical for white and red wine production, respectively. After bottling, the wines were stored at 14 ˚C to undergo bottle maturation until required for chemical analyses and sensory evaluation. Three repetitions of each treatment were conducted.

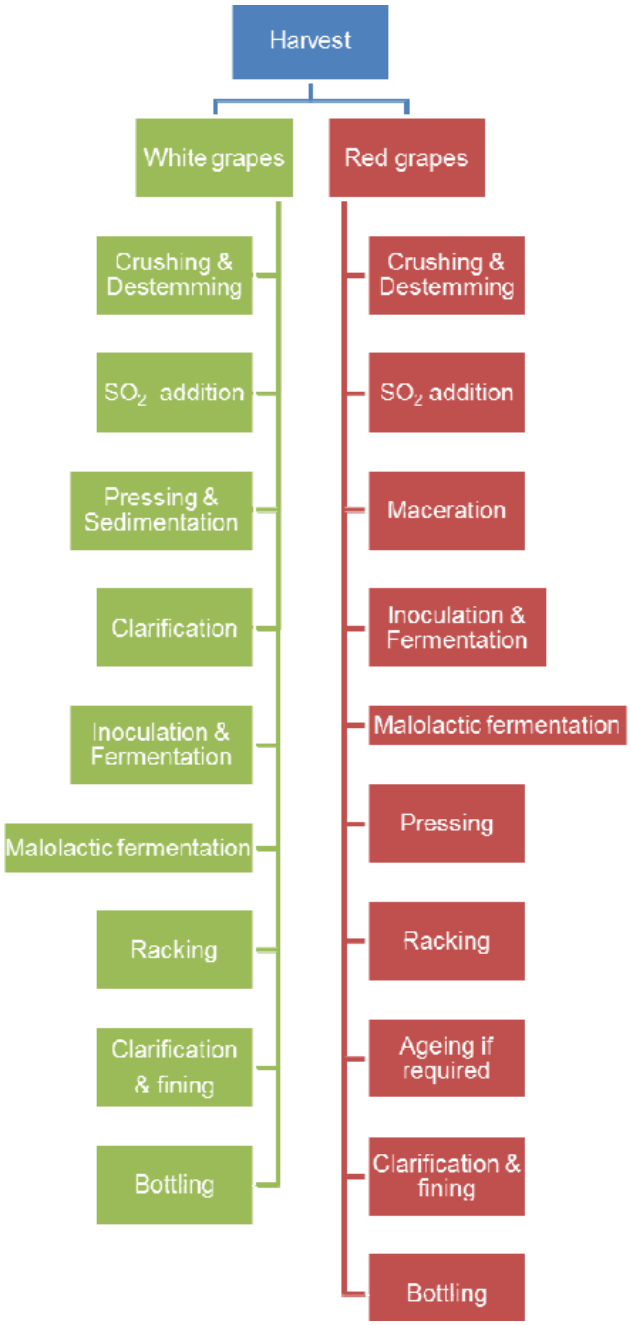

FIGURE 8: Production of white and red wine. This figure shows the process of white and red wines production according to the standard vinification protocol of the ARC Infruitec-Nietvoorbij (Van Breda, 2012).

3.2.5. Urea analysis using ion-exchange chromatography

An Ion-exchange apparatus (Supelco Visiprep ^{TM} 24) was used, and the columns (10 cm) were filled with 2 ml dH_2O to allow for easier sedimentation of ion-exchange resin. This was followed by adding ion-exchange resin (DOWEX, Merck) up to a height of 4 cm. Once the resin was settled, 21 ml 0.1 M HCl (Saarchem, Merck) was added to wash the resin. Thereafter 2 ml of standard calibration samples and wine were added to separate columns (activated charcoal was used to remove red wine colour before addition to columns). Three solutions were added to each tube containing wine and the standards as follows: 1) 320 µl 1 M HCl and drain, 2) 1 ml 0.1 M HCl and drain, and 3) 1.5 ml 0.1 M HCl/ 4 M NaCl (Saarchem, Merck). Glass tubes were placed within reservoir under the columns to ensure that column outlets flow directly into the corresponding glass tubes. A solution of 3.7 ml 0.1 M HCl/ 4 M NACI was added to respective columns and drained into the glass tubes. The glass tubes were removed from the reservoir and 1.75 ml of the analyte was aliquoted into a second brown glass tube respectively (both tubes thus contained 1.75 ml of the same analyte). 100 µl of 96 % by volume (Saarchem, Merck) was added to one of the brown tubes (blank) while 100 µl of 1-phenyl-1, 2-propandione 2-oxime (Merck) (0.2 g in 5 ml ethanol) was added to the remaining brown tube (experimental sample). Both the blank and experimental glass tubes were vortexed followed by adding 1.25 ml of a 1:3:1.25 solution of H_2SO_4 (18 M)-H_3PO_4 (14 M)-H_2O (Saarchem, Merck) to both glass tubes. Experimental and blank tubes were sealed and vortexed thoroughly and were placed in a water bath (Lasec, SA) to boil for 2 h at 90 °C. Thereafter the tubes were removed and allowed to cool down in a dark room (or cupboard). The optical density of the analytes were

determined at 540 nm using a spectrophotometer (Pharmacia Biotech Ultrospec 2000 UV-visible Spec) in which the glass tube containing the ethanol would always serve as a blank to zero the spectrophotometer for the second tube containing the 1-phenyl-1, 2-propandione 2-oxime. Yellow to pink colour indicates high absorbance values and high urea concentration expected. Absorbance readings were tabulated and the residual urea was determined from the calibration curve.

3.2.6. Sensory evaluation

In addition to chemical analyses, it is important to evaluate wines by sensory analyses followed by statistical evaluation of the data. All wines were evaluated sensorially after five months bottle-maturation by a panel consisting of seven trained wine judges (ARC Infruitec-Nietvoorbij staff). Judges were required to document wines' sensory parameters e.g. acidity, body, general quality, fruity, spicy, berry and tropical on a tasting sheet with a 10 cm line scales for the respective parameters, whilst 0 represented the absence of a specific aroma and/or flavour.

3.2.7. Statistical analyses

Statistical analysis of variance and principle component analysis can be used to determine whether a group of wines differ or are the same with regards to specific characteristics. When considering the chemical and sensory results, the differences can be graphically represented (Boulton *et al*., 1995). Chemical and sensory analyses data were subject to an appropriate factorial analysis of variance (ANOVA) and principle component analysis (PCA) using the XLSTAT (XLSTAT software version 2015.1.03,

Paris, France) (Addinsoft, 2015). All sensory evaluation data was analysed and subjected to Two-way Analysis of Variance (ANOVA) method (SAS Institute, Inc. (1999), SAS/STAT User's Guide, Version 9, 1st printing, Volume 2. SAS Institute Inc., SAS Campus Drive, Cary, North Carolina 27513). Data from the analysed wines was also subjected to multivariate analysis, i.e. PCA to establish clustering of the variables and their inter-relationships to the wine and treatments (yeast strains).

3.2.8. Proteome analyses

3.2.8.1 Protein extraction

Yeast cells were collected (2 ml) during the final stage of fermentation and harvested by centrifugation (Eppendorf centrifuge 5412) at 14000 x *g* for 1 min (4 °C) and then cells were re-suspended in 400 µl lysis buffer: 0.1 M NaOH, 0.05 M EDTA, 2 % (w/v) (Merck) SDS and 2 % (v/v) 2-mercaptoethanol (Sigma-Aldrich) made in distilled water (Von den Haar, 2007). The mixture was heated for 10 min at 90 °C, thereafter 10 µl of acetic acid was added to the lysate and heated for an additional 10 min at 90 °C.

3.2.8.2 Acetone precipitation

Cold (-20 °C) acetone (Merck, South Africa), at a volume four times that of the sample volume (1640 µl) were added to the samples, vortexed and incubated for 60 min at -20 °C followed by centrifugation for 10 min at 14000 × *g*. The supernatant was carefully, yet properly removed without dislodging the protein pellet, the tubes were left uncapped at room temperature for 30 min to allow the acetone to evaporate. For the downstream process to continue, urea buffer was added and vortexed thoroughly to dissolve protein

pellet. Bradford assays were conducted to determine the protein concentrations (data not shown), according to the results, different volumes of each yeast strain was loaded to achieve the standard concentration of 200 µg.

3.2.8.3 Sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins samples were separated on SDS-PAGE gels which consist of a stacking gel to concentrate the proteins into a thin line before they enter the separating gel, which then separates the proteins according to their molecular weight. Total protein extracts were analysed by electrophoresis on a 12 % polyacrylamide gel. The acrylamide gels were prepared as follows.

TABLE 3: Reagents used in preparation of SDS polyacrylamide gels

Reagents	12 % separating	4 % stacking
Deionised water	6.4 ml	3.6 ml
1.5 M Tris (Merck) pH 8.8	3.8 ml	-
0.5 M Tris (Merck) pH 6.8	-	630 µl
10 % SDS (Sigma-Aldrich)	150 µl	50 µl
10 % APS (Merck)	150 µl	50 µl
TEMED (Sigma-Aldrich)	8 µl	5 µl

The Minigel apparatus (Bio-Rad Laboratories, USA) was used to cast the gel. The separating gel was prepared in a 15 ml Greiner tube by combining the reagents in the order listed in TABLE 3. The solution was mixed by inverting the tube a few times and

immediately poured in between the gel plates. Isopropanol was added to ensure that the top of the gel was level when it solidified. The isopropanol was poured off and the gel was rinsed with distilled water. The stacking gel was then prepared in a 15 ml Greiner tube and immediately poured in between the gel plates. A ten well comb was placed in between the plates and the gel was allowed to solidify. The gel plates were removed from the casting trays and assembled on the electrophoresis system (Bio-Rad, Madrid, Spain). The samples were diluted with equal amount of 1 x Sample Treatment Buffer (2x Sample treatment buffer: 4 % SDS, 20 % Glycerol, 2 % 2-mercaptoethanol and 0.25 M Tris-HCl pH 6.8) followed by centrifugation on a bench top Eppendorf centrifuge 5412 at 14000 x g for another 5 minutes. Protein molecular weight marker was subjected to the same treatment before use.

Protein ladder and 200 µg of respective protein samples were loaded into wells following the removal of the combs. Electrophoresis was carried out in 1 x SDS Electrophoresis running buffer at a voltage of 100 V for 20 min using Bio-Rad power pack. The voltage was adjusted to 150 V when the Bromophenol blue dye reached the separating gel. Electrophoresis was stopped when the dye reached the bottom of the separating gel after which the gel was stained for 2 hours in Coomassie staining solution (0.02 % Coomassie blue, 40 % Methanol and 10 % Acetic acid made in distilled water) and de-stained for 1 hour in de-staining solution (10 % Methanol and 10 % Acetic acid made in distilled water) for visualisation. Selected differential expressed protein bands were excised from the SDS PAGE gels for conducting Nanoscale Liquid Chromatography coupled to tandem mass spectrometry (Nano LC/MS) and Matrix-

Assisted Laser Desorption/Ionisation Time-of-Flight Mass spectrometry (MALDI-TOF/MS) analyses.

Proteins extracts were evaluated according to (i) the amount of protein extracted (Von den Haar, 2007); (ii) the diversity of bands formation that are differentially expressed between the seven yeasts; (iii) the integrity of samples as well as the reproducibility of extraction.

3.2.8.4 In-gel digest and peptide extraction

One-dimensional Sodium Dodecyl Sulfate Polyacrylamide gels were placed on a clean glass plate and bands were removed with a sterilised scalpel and transferred to individual labelled and corresponding 2 ml eppendorf tubes.

All reagents were analytical grade or equivalent. Gel bands were destained with 200 µl of 50 % acetonitrile/25 mM Ammonium Bicarbonate until clear. Samples were dehydrated and desiccated with 100 µl acetonitrile (ACN) before reduction with 10 mM dithiothreitol (DTT) in 100 mM Ammonium hydrogen carbonate (NH_4HCO_3) for 1 hour at 60 °C. Cysteine residues were carbamidomethylated with 50 mM iodoacetamide (Sigma) in 100 mM NH_4HCO_3 for 30 minutes at room temperature (20 to 25 °C) in the dark. After carbamidomethylation the gel pieces were dehydrated and washed with 25 mM NH_4HCO_3. Proteins were digested by rehydrating the gel pieces in trypsin (Promega) solution (20 ng/uL) and incubated at 37 °C overnight. Peptides were extracted from the gel pieces once with 30 µl 30 % acetonitrile; 0.1 % trifluoroaceticacid (TFA) (Sigma) for 30 minutes at room temperature with occasional vortexing. The samples were dried down to remove residual NH_4HCO_3 and were re-dissolved in 0.1 %

TFA and were purified and concentrated using C_{18} ZipTip® according to manufacturer's instructions. The purified samples were eluted in 80 % acetonitrile/H_2O containing 0.1 % trifluoroacetic acid (TFA) and stored at -20 °C until further use.

3.2.8.5 Nanoscale liquid chromatography (nano-LC) procedure

Five purified samples (EC1118, VIN7, UCD 522, NT50 and N96) were dried in a speed vac and resuspended in 10 µl 0.1 % TFA. The peptides were then separated using nLC-MS. All experiments were performed on a Thermo Scientific EASY-nLC II connected to a Proteineer fc II protein spotter controlled through HyStar software. For liquid chromatography, separation was performed on an EASY column (2 cm, 75 µm ID, 5 µm, C18) pre-column followed by an analytical column (10 cm, 75 µm ID, 3 µm, C18) with a flow rate of 100 µl/hr using a 48 minute gradient run.

(For gradient run see TABLE 4).

3.2.8.6 Mass spectrometry (MS)

MALDI-TOF MS and LIFT MS/MS was performed using an UltrafleXtreme MALDI ToF/ToF system (Bruker Daltonics, Bremen, Germany) with instrument control through Flex control 3.4. Peptides were ionised with a 337 nm laser and spectra acquired in reflector positive mode at 28 kV using 100 laser shots per spectrum with a scan range of m/z =700-4000. Spectra were internally calibrated using peptide calibration standard II (Bruker Daltonics, Bremen, Germany). Peptide spectra of accumulated 4,000 shots were automatically processed using WARP LC 3.2 software (Bruker Daltonics, Bremen, Germany).

Data analysis

Database interrogation was performed with the Mascot algorithm using the SwissProt database on a ProteinScape 3.0 workstation. The search parameters were as follows: Taxonomy- *Saccharomyces cerevisiae*, Enzyme- trypsin, Missed cleavages- 1, Fixed modification- carbamidomethyl(C), Variable modification- oxidation (M), Precursor tolerance- 50 ppm and Fragment tolerance- 0.7 Da.

Candidate protein matches with molecular weight search (MOWSE) score greater than 22 were considered as identified proteins.

TABLE 4: **Gradient Run and process of MALDI-TOF MS analysis.** Consisting of time, function and value for each run

Time (min)	Function	Value
0	Flow rate	300nl/min
0	Solvent Mix	98 % A , 2 % B
44	Solvent Mix	65 % A , 35 % B
48	Solvent Mix	60 % A , 40 % B
48.10	Solvent Mix	0 % A , 100 % B
60	Solvent Mix	0 % A , 100 % B
60.10	Solvent Mix	98 % A , 2 % B
70	Solvent Mix	98 % A , 2 % B

A: 0.05 % TFA/H2O

B: 0.05 % TFA/ACN

3.3. RESULTS AND DISCUSSION

3.3.1 Yeast strains

Selected wine yeast strains were trialled in small-scale wine production during the 2014 and 2015 harvests using Sauvignon Blanc and Cabernet Sauvignon grape must respectively. Two commercial wine yeast strains i.e. a low urea producing yeast strain Prise de Mousse (PdM) and a high urea producing yeast strain UCD522 was included in this trial to serve as references (Ough *et al*., 1990). Fermenting yeasts sampled at the end of fermentation (final stage of wine production) were subjected to SDS-PAGE. Chemical and sensory analyses of 2014 and 2015 small-scale Sauvignon Blanc and Cabernet Sauvignon wines produced by various commercial yeasts following bottle maturation were conducted.

3.3.2 Sauvignon Blanc

3.3.2.1 Chemical analysis (FTIR and ion-exchange chromatography)

Chemical analyses using an Oenofoss™ Fourier Transform Infrared (FTIR) spectrophotometer were performed on the resultant wines to correlate and compare wines over two vintages. The following chemical parameters are tabulated in TABLE 5 with the main focus on Volatile Acidity (VA), ethanol and urea produced in Sauvignon Blanc wine. Oenofoss™ analyses of 2014 and 2015 final wines showed all yeasts fermented Sauvignon Blanc grape must to dryness (results not depicted).

During the 2014 harvest, all yeast strains, except VIN7 had a negative association with urea (FIGURE 9). However, VIN7 produced wines with urea levels (0.6 ± 0.39 g/L) that will comply with Canadian legal limit as well as the USA voluntary limit (TABLE 5).

Therefore, all wines do not have the potential to produce excessive ethyl carbamate levels. The low urea producing reference yeast strain EC1118 produced wines with the highest VA but lowest urea levels TABLE 5, whilst the high urea producing yeast UCD522 strain produced wines with both low VA and low urea (FIGURE 9). Statistical analysis using PCA bi-plot shows the correlation between yeast and the chemical parameters (FIGURE 9 & 10).

Oenofoss analyses of the 2015 harvest showed different yeast strains produced final wines with different chemical parameters (TABLE 5). NT50 produced wines with urea levels (0.37 ± 0.26 g/L), N96 (0.24 ± 0.13 g/L) and UCD522 (0.23 ± 0.07 g/L) and are compliant with the Canadian legal and USA voluntary limits (TABLE 5). It was also observed that NT112 produced wines with the least urea content (0.05 ± 0.04 g/L). The PCA bi-plot for the 2015 Sauvignon Blanc wines confirmed the results tabulated in table 4, as it can clearly be seen how different yeast group together according to association with the chemical parameters (FIGURE 10).

Ion-exchange chromatography (IEC) analyses showed that the low urea producing yeast strain i.e. Prise de Mousse (PdM) produced 2014 Sauvignon Blanc wines with the least urea (TABLE 5), while all yeast strains, including UCD522 except VIN7 had a negative association with urea (FIGURE 9). Overall the seven commercial yeast strains produced Sauvignon Blanc wines with lower urea levels during 2015 compared to wines produced in 2014.

Both spontaneous and inoculated wine fermentations are affected by the diversity of yeasts associated with the vineyard and winery. During primary alcoholic fermentation of sugar, the wine yeast, *S. cerevisiae*, together with other indigenous non-

Saccharomyces species, produce ethanol, carbon dioxide and a number of by-products. Of these yeast-derived metabolites, the alcohols, acetates and C4-C8 fatty acid ethyl esters are found in the highest concentration in wine. While the volatile metabolites contribute to the fermentation bouquet ubiquitous to all young wines, the production levels of these by-products are variable and yeast strain specific.

TABLE 5: Chemical profiles of Sauvignon Blanc wines produced in small-scale fermentations in 2014 and 2015.

	2014		Chemical Analyses			2015				
Yeast Strains	***Volatile acidity (g/L)***	***Total acidity (g/L)***	***pH***	***Alcohol (g/L)***	***Urea (g/L)***	***Volatile acidity (g/L)***	***Total acidity (g/L)***	***pH***	***Alcohol (g/L)***	***Urea (g/L)***
EC1118	0.6±0.03	5.8±0.08	3.3±0.00	11.8±0.05	0.1±0.06	0.3±0.03	6.15±0.01	3.2±0.01	13.9±0.22	0.19±0.17
UCD522	0.2±0.03	4.9±0.06	3.3±0.00	11.7±0.27	0.2±0.06	0.2±0.01	5.43±0.08	3.3±0.01	14.0±0.36	0.23±0.07
VIN7	0.2±0.03	6.3±0.05	3.3±0.00	11.5±0.08	0.6±0.39	0.4±0.02	6.01±0.21	3.3±0.00	14.1±0.11	0.13±0.05
VIN13	0.5±0.06	5.7±0.09	3.3±0.00	11.7±0.09	0.5±0.46	0.3±0.00	5.69±0.06	3.3±0.04	13.9±0.17	0.10±0.00
N96	0.4±0.06	5.6±0.24	3.3±0.01	11.7±0.30	0.2±0.15	0.3±0.01	6.04±0.07	3.3±0.03	13.9±0.12	0.24±0.13
NT50	0.5±0.08	5.6±0.05	3.3±0.00	11.5±0.1	0.2±0.21	0.3±0.05	6.40±0.66	3.3±0.09	13.5±0.43	0.37±0.26
NT112	0.5±0.02	5.8±0.09	3.3±0.00	11.7±0.12	0.1±0.13	0.4±0.01	6.18±0.01	3.3±0.01	13.7±0.04	0.05±0.04

Means ± standard deviation (n=2).

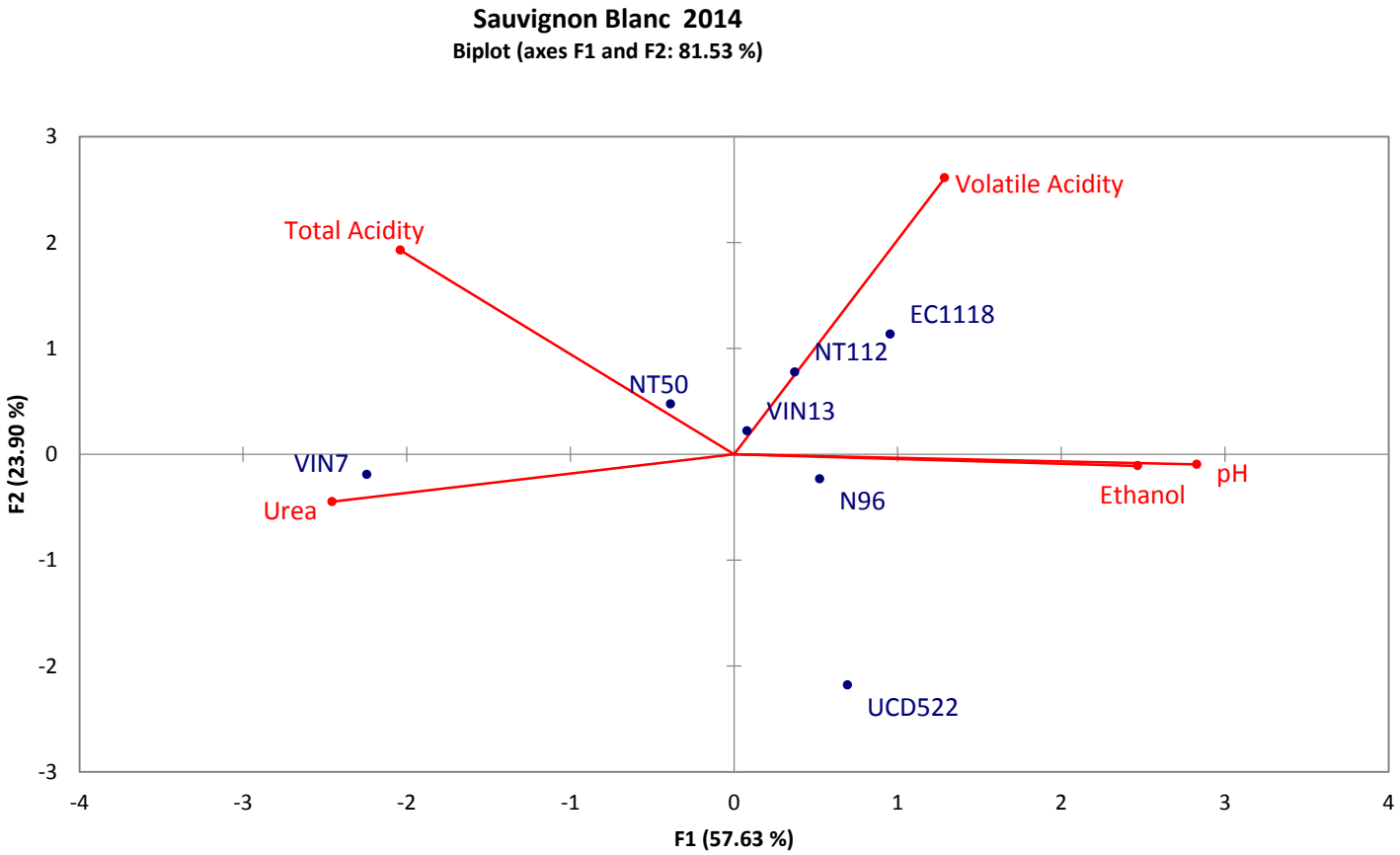

FIGURE 9: Principle Component Analysis (PCA) bi-plot of chemical analysis. The above bi-plot illustrates the grouping of seven yeasts according the specific chemical compounds that they produced within the wines during the 2014 alcoholic fermentation. F1 represents the First Principle Component and F2 represents the Second Principle Component.

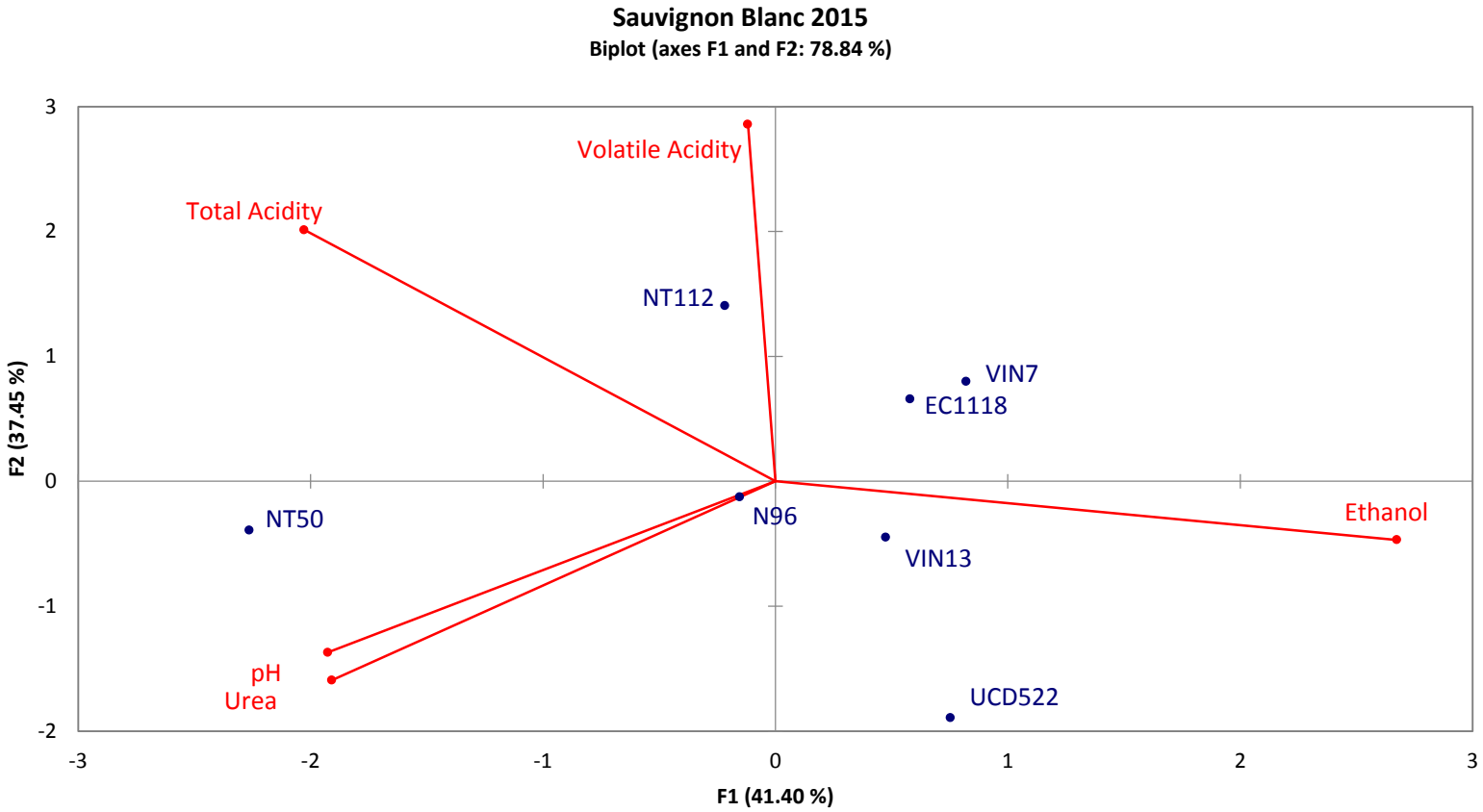

FIGURE 10: Principle Component Analysis (PCA) bi-plot of vintage 2015 chemical analysis. The above bi-plot illustrates the grouping of seven yeasts according the specific chemical compounds that they produced within the wines during the 2015 alcoholic fermentation. F1 represents the First Principle Component and F2 represents the Second Principle Component.

3.3.2.2 Sensory evaluation

Yeast and fermentation conditions are claimed to be the most important factors influencing the flavours in wine (Lambrechts & Pretorius, 2000). Wines were sensorially evaluated following bottle maturation for 5 months by a trained panel of seven judges consisting mostly of researchers. The sensory evaluation was carried out according to a randomised block design, using an unstructured line scale (Addinsoft, 2013). The wines were evaluated according to the descriptors applicable to Sauvignon Blanc e.g. “Tropical Fruit”, “Vegetative” and “Spices”. The results of the sensory evaluation following winemaking using seven ADWY are shown in the ANOVA (TABLE 6) and PCA bi-plot (FIGURE 11), respectively. The ANOVA highlights similarities and significant differences between the yeast as well as the wine aroma profiles. Sensory evaluation of the 2014 Sauvignon Blanc wines (TABLE 6 and FIGURE 11) showed that both UCD522 and NT50 produced wines with the most vegetative fresh aroma. The yeast NT50 then again produced wines that were perceived to be the most acidic even though it produced fruity wines, whilst wine produced with VIN13 had the highest vegetative cooked and dried aromas (TABLE 6). Therefore, the PCA showed that NT50 and VIN13 produced wines that had a positive association with vegetative fresh and vegetative cooked and dried aromas, respectively (FIGURE 11). The high urea producer UCD522 and NT112 produced wines that were similar to wines produced with NT50, whilst EC1118 produced wines that were similar to wines produced with VIN13. The commercial yeast strain N96 produced the fruitiest wines as can be seen in TABLE 6, even though they were acidic, these wines had a positive association with body and general quality similar to wines produced by yeast NT112. During the 2015 harvest ADWY produced wines with high aroma intensities (TABLE 7), of which EC1118 produced wines with the

highest vegetative, spices and general quality. The yeast NT112 produced wines with the fruitiest aroma and least spicy aroma. All seven yeast produced wines with great body and overall quality and were therefore, positively perceived. The PCA bi-plot (FIGURE 12) complements the data presented by ANOVA.

TABLE 6: Sensorial parameters for Sauvignon Blanc 2014 wines

Yeast strains	Vegetative Cooked	Vegetative Fresh	Vegetative Dried	Spices	White/Yellow Fruit	Tropical Fruit	Acid	Body	General Quality
EC1118	14a	23a	14a	6abc	12ab	33abc	54ab	54bc	51bc
UCD522	13a	33a	8a	4bc	13a	36abc	57a	57abc	56b
VIN7	12a	23a	9a	12a	7b	29c	50b	48c	42c
VIN13	16a	22a	17a	2c	14a	31bc	57a	61ab	56b
NT96	11a	26a	13a	5bc	17a	51ab	57a	63ab	65a
NT50	10a	34a	11a	7abc	15a	40abc	58a	55bc	53b
NT112	11a	28a	13a	9ab	13ab	54a	55ab	65a	66a

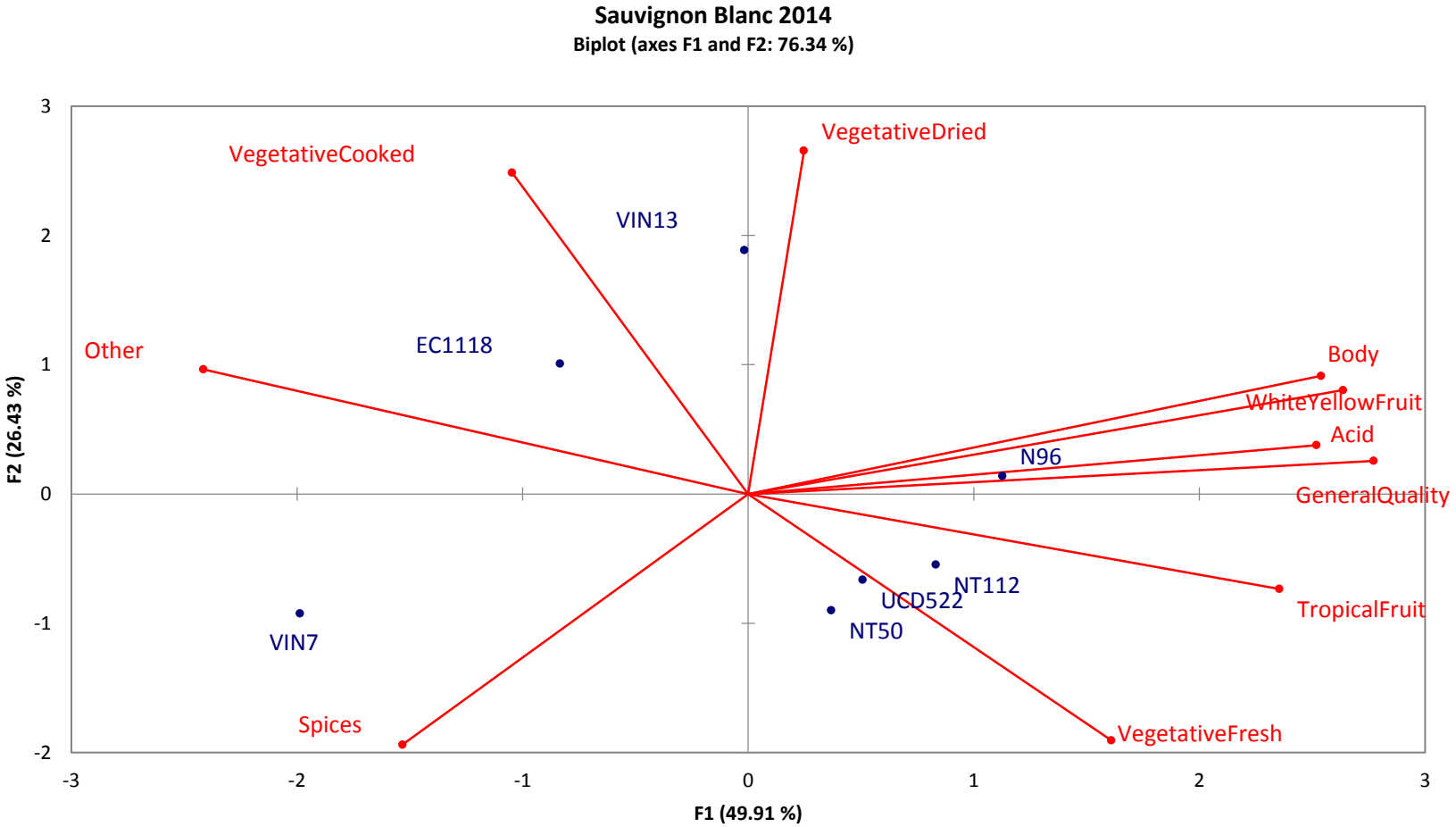

FIGURE 11: Principle Component Analysis (PCA) bi-plot of average values (n = 2) of sensory analysis descriptors for Sauvignon Blanc 2014. The above bi-plot illustrates the different aromas produced by the seven yeasts during the 2014 alcoholic fermentation. F1 represents the First Principle Component and F2 represents the Second Principle Component.

TABLE 7: Sensorial parameters for Sauvignon Blanc 2015 wines

Yeast strains	Vegetative Cooked	Vegetative Fresh	Vegetative Dried	Spices	White/Yellow Fruit	Tropical Fruit	Acid	Body	General Quality
EC1118	17a	42a	16a	15a	25a	36a	46ab	57a	60a
UCD522	16a	35a	13a	8abc	26a	39a	47ab	57a	59a
VIN7	21a	33a	11a	4c	23a	43a	46ab	51a	47a
VIN13	20a	33a	12a	4c	19a	31a	44b	55a	51a
NT96	10a	36a	13a	13ab	28a	32a	48a	57a	51a
NT50	16a	36a	21a	6bc	27a	28a	48a	55a	51a
NT112	24a	30a	19a	1c	36a	47a	46ab	51a	56a

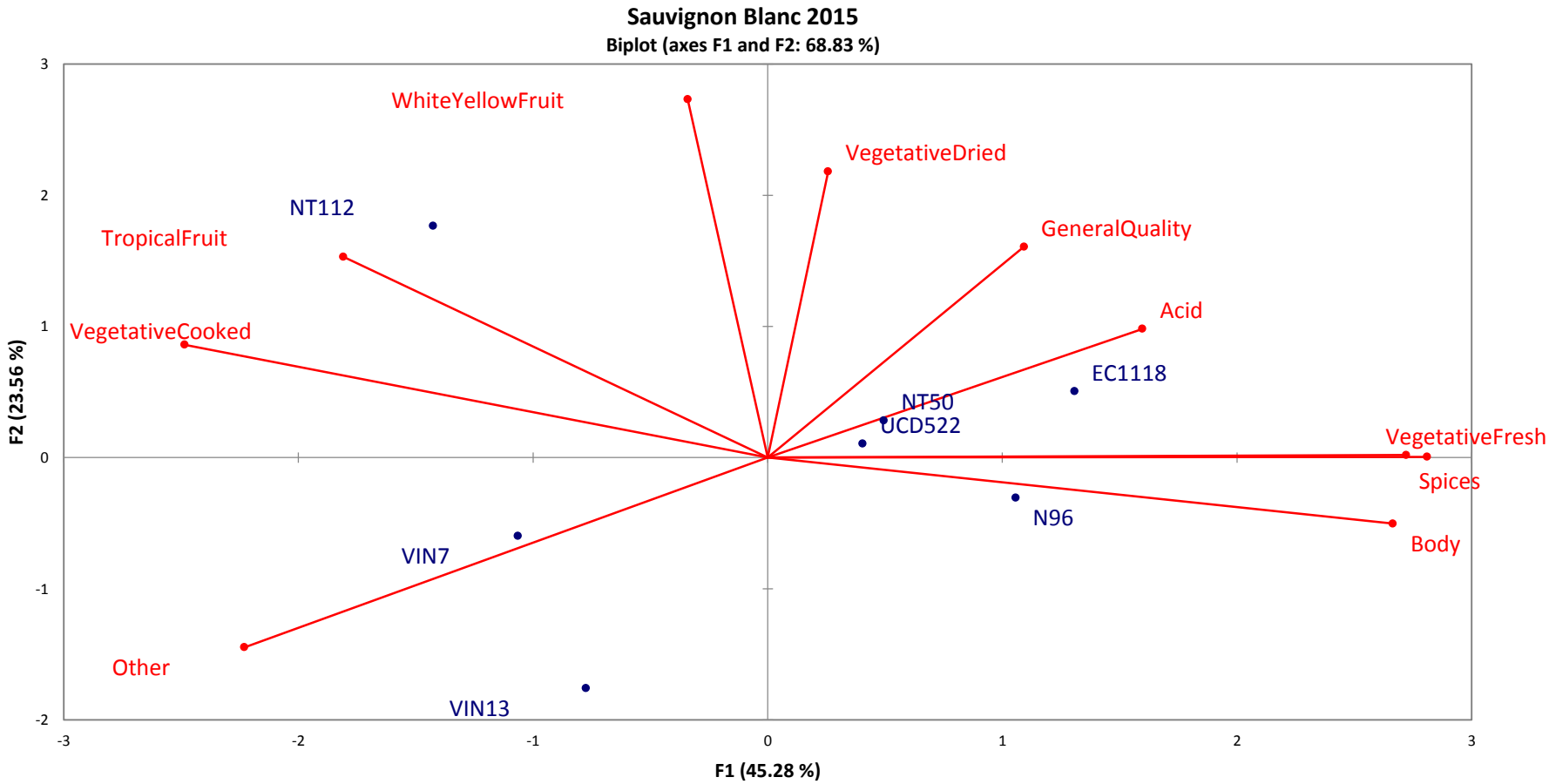

FIGURE 12: Principle Component Analysis (PCA) bi-plot of average values (n = 2) of sensory analysis descriptors for Sauvignon Blanc 2015. The above bi-plot illustrates the different aromas produced by the seven yeasts during the 2015 alcoholic fermentation. F1 represents the First Principle Component and F2 represents the Second Principle Component**.**

3.3.2.3 Proteomic characterisation

Nearly 200 µg of protein preparations were resolved by SDS-PAGE, yielding molecules ranging in size from 10 to 270 kDa with clear differences in protein profiles. Indications are that different yeast strains had differential protein expression as the SDS-PAGE showed various yeast strains had different protein banding patterns in terms of distribution and intensity (FIGURE 13). Subsequently, distinct protein bands were excised and subjected to in-gel trypsin digestion resulting peptides where after mass spectrometry (MS) analysis was conducted.

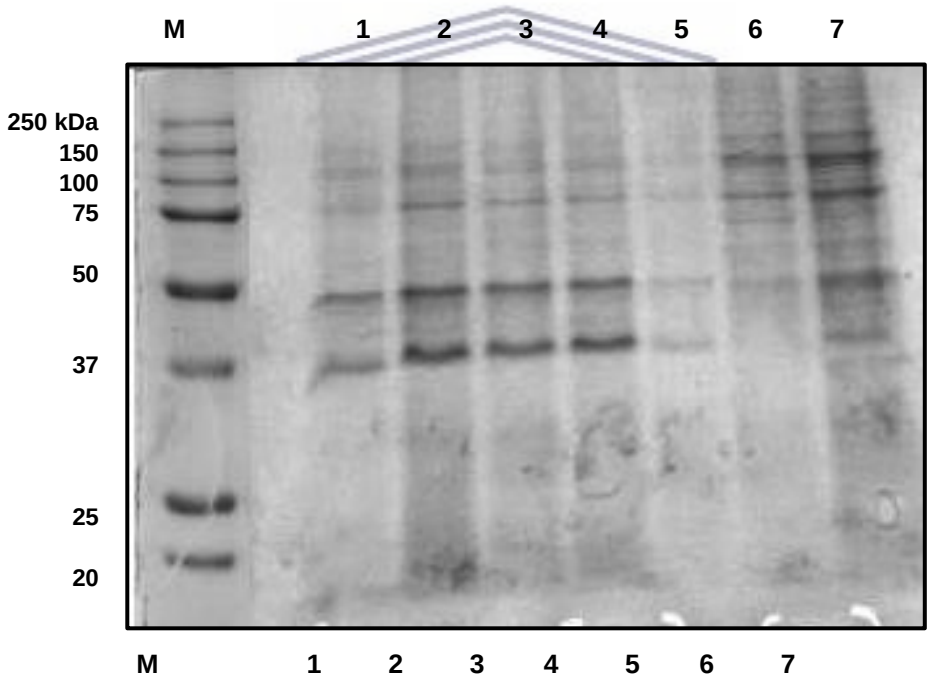

FIGURE 13: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Analyses of protein extracted from seven commercial yeasts i.e. Lanes 1 – 7: EC1118, UCD522, VIN7, VIN13, N96, NT50 and NT112 following the fermentation of Sauvignon Blanc grape must (juice).

A total of 3 proteins were characterised by deploying MALDI-TOF and nano LC/MS in final Sauvignon Blanc wine. Subsequently, various proteins were identified, after an intensive search on a universal protein database (UniProtKB database at http://www.uniprot.org/uniprot/?query=HOSC&sort=score). Expressed proteins during fermentation identified were shown to play a vital role during glycolysis, biosynthesis, glycogenesis and arginine synthesis (TABLE 12). The proteins tabulated below (Sauvignon Blanc) were identified in more than one yeast and proteins (TABLE 8). Distinct-protein bands (blue text box) originating from yeasts sampled at the end of fermentation were excised (VIN7 and N96) from the 1D SDS-PAGE gels (FIGURE 13). Various proteins that were differentially expressed were for the different yeast strains identified: VIN7, 2 proteins were identified, UCD522, 76 proteins and EC1118, 47 proteins NT50, 1 protein and N96, 1 protein. The Mascot score distribution (Figure 14) states that any score above 22 indicates identity/extensive homology. Therefore, each was extensively researched against a mascot data of yeast proteins. After the database search and validation of the obtained protein hits, main proteins that were observed in final wines produced by each yeast (mentioned above) were identified with high confidence.

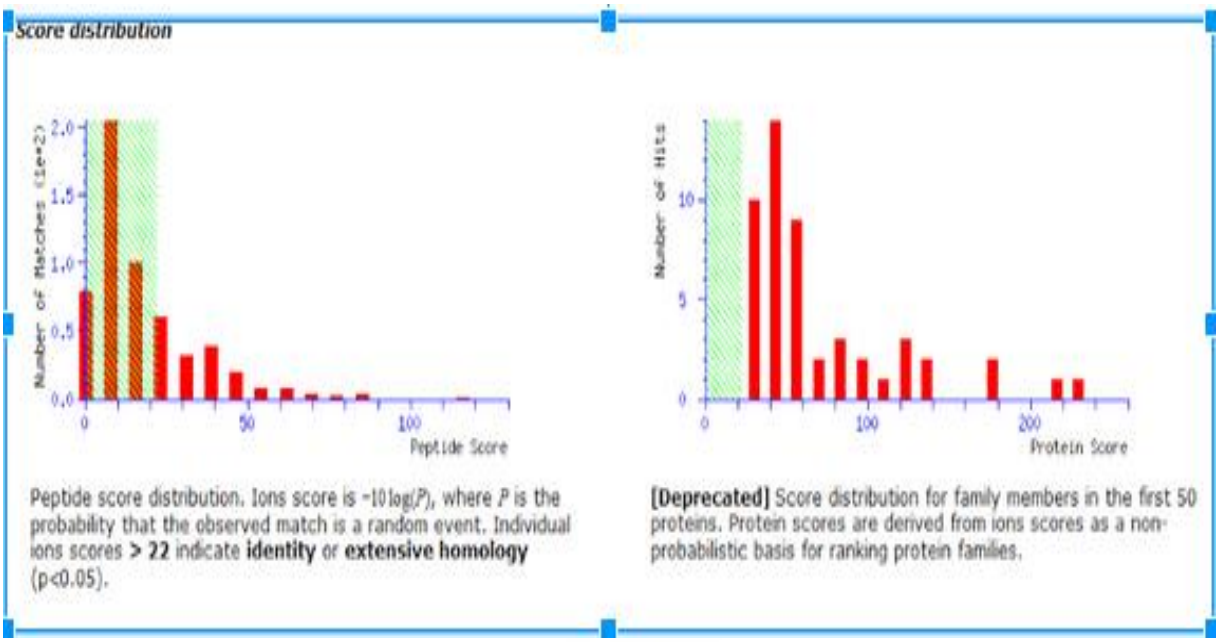

FIGURE 14: Nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC-MS/MS). The Mascot score distribution states that any score above 22 indicates identity/extensive homology.

Polymerase chain reaction (PCR) is genomic tool used to amplify the amount of DNA and increase the signal above the limit of detection (Kalle, *et al*., 2014). This approach however, is not applicable to proteomics. The difference between genomics and proteomics is simple as the names sounds. Genomics is the study of the genes in an organism while Proteomics is the study of the all the proteins in a cell. It is the large-scale experimental analysis of proteins (Feist & Hummon, 2015). It is more complex than genomics because genomes are more or less constant, whereas proteomes differ from cell to cell and from time to time. Cell expression is looked at via mRNA analysis (Fox, 2006). Proteomics, thus confirms the presence of protein content and provides a direct measure of the quantity present. Therefore, the intrinsic sensitivity of the analytical method applied to the analysis of biomolecules, generally liquid

chromatography–mass spectrometry (LC-MS), needs to be enhanced. Mass spectrometry has feasibly become the core technology in proteomics due to higher sensitivity obtained when used in conjunction with LC (Liebler, 2002). The application of techniques based on mass spectrometry for the qualitative and quantitative analysis of global proteome samples derived from complex mixtures. Mass spectrometers can be employed to identify unknown proteins by determining their molecular weight structure and chemical properties.

Their resulting score indicates high-quality MS/MS spectra and a high percentage of sequence coverage (TABLE 8 and 12). Despite the fact that only a few peptides were identified per protein, intra-cellular proteins identified were mostly related to glycolysis and cell wall proteins, which are involved in cell wall biogenesis/degradation. The proteins that scored above 100 has a key function, which is glycolysis. Glycolysis forms part of the cellular respiration whereby larger carbohydrates e.g. glucose, fructose etc. are enzymatically metabolised into smaller molecules to generate energy in the form of ATP and pyruvic acid (Dashty, 2013).

TABLE 8: Identified differentially expressed proteins found in Sauvignon Blanc (SB) final wines produced by VIN7 and N96 ADWY by MALDI-TOF/MS.

Accession	Yeast	Molecular weight (kDa)	Seq coverage %	Score	pI	Peptides	Function
PGK	VIN7	44.7	2.2	27.48	7.78	1	key enzyme in glycolysis and gluconeogenesis
ENO1	VIN7	46.8	4.3	40.41	6.16	2	Glycolysis
ENO1	N96	46.8	17.2	165.44	6.16	7	Glycolysis

3.3.3 Cabernet Sauvignon

3.3.3.1 Chemical analysis (FTIR and ion-exchange chromatography)

Chemical analyses using an OenofossTM Fourier Transform Infrared (FTIR) spectrophotometer were performed on the resultant wines to correlate and compare wines over two vintages. The following chemical parameters are tabulated in TABLE 9 with the main focus on Volatile Acidity (VA), ethanol and urea produced in Cabernet Sauvignon wine. Oenofoss analyses of 2014 and 2015 final wines showed all yeasts fermented Cabernet Sauvignon grape must to dryness (results not depicted).

During the 2014 harvest, all yeast strains, except the high urea producing yeast strain UCD522 produced Cabernet Sauvignon with highest levels of urea > 2 mg/L (3.3 ± 1.99 g/L) as well as the commercial yeast strain VIN13 (2.5 ± 0.05 g/L) (TABLE 9 and FIGURE 15). Subsequently these wines do not comply with Canadian legal limit (< 2 mg/L), The commercial yeast VIN7 produced wines with the lowest VA and urea concentrations compared to that of the low urea producing yeast EC1118.

The commercial yeast, VIN7 and NT112 produced Cabernet sauvignon wines during the 2015 harvest with high urea levels (VIN7: 2.66 ± 0.44 g/L) (NT112: 1.96 ± 1.34 g/L) > 2mg/L and the high urea producing yeast (UCD522) produced wines with low urea levels < 2mg/L (TABLE 9 and FIGURE 16). These wines therefore do not comply with the Canadian legal limit (< 2 mg/L). The PCA bi-plots for both vintages (FIGURE 15 and 16) is a representation of how the different yeasts are grouped together according to their chemical parameters. During the 2014 harvest the PCA plot clearly depicts the two yeast (VIN13 and UCD522) that produced wines with the highest urea content, but that

changed in the 2015 harvest with VIN7 producing wines with high urea content, (TABLE 9 and FIGURE 15) can justify the findings.

The highest urea producing yeast strain (UCD522) varied in both cases for Cabernet Sauvignon and Sauvignon Blanc. Generally, red wines develop higher concentrations of EC than white wines. The bulk of EC present in wine is formed by the spontaneous reaction between urea and ethanol (Hart & Jolly, 2011) and this can be evident in the graphs. Final wines for both vintages of Cabernet sauvignon were stored at 28 °C for 6 months to measure initial and final urea concentrations by Ion-exchange chromatography (FIGURE 19).

TABLE 9: Chemical profiles of Cabernet sauvignon wines produced in small-scale fermentations in 2014 and 2015

2014	Chemical Analyses					2015				
Yeast Strains	***Volatile acidity (g/L)***	***Total acidity (g/L)***	***pH***	***Alcohol (g/L)***	***Urea (g/L)***	***Volatile acidity (g/L)***	***Total acidity (g/L)***	***pH***	***Alcohol (g/L)***	***Urea (g/L)***
EC1118	0.3±0.05	3.6±0.03	3.2±0.01	12.9±0.76	1.7±1.93	0.3±0.01	5.74±0.02	4.4±0.02	13.0±0.13	1.03±0.35
UCD522	0.3±0.03	3.6±0.36	3.2±0.00	12.9±0.32	3.3±1.99	0.2±0.01	5.54±0.07	4.4±0.00	13.2±0.08	1.47±0.05
VIN7	0.1±0.03	4.2±0.12	3.2±0.01	12.8±0.43	0.5±0.61	0.2±0.04	5.55±0.18	4.4±0.01	13.7±0.21	2.66±0.44
VIN13	0.2±0.02	3.9±0.16	3.2±0.01	13.1±0.14	2.5±0.05	0.2±0.07	5.69±0.00	4.4±0.00	13.4±0.13	0.31±0.16
N96	0.3±0.04	4.2±0.15	3.2±0.00	12.8±0.09	1.7±0.05	0.2±0.01	5.73±0.06	4.4±0.01	13.2±0.06	0.67±0.12
NT50	0.3±0.09	4.3±0.21	3.2±0.01	12.9±0.41	1.4±0.02	0.2±0.04	5.91±0.06	4.4±0.01	12.9±0.13	0.23±0.05
NT112	0.4±0.01	4.1±0.05	3.2±0.00	12.8±0.08	1.7±0.04	0.3±0.01	5.85±0.16	4.4±0.04	13.5±0.71	1.96±1.34

Means ± standard deviation (n=2).

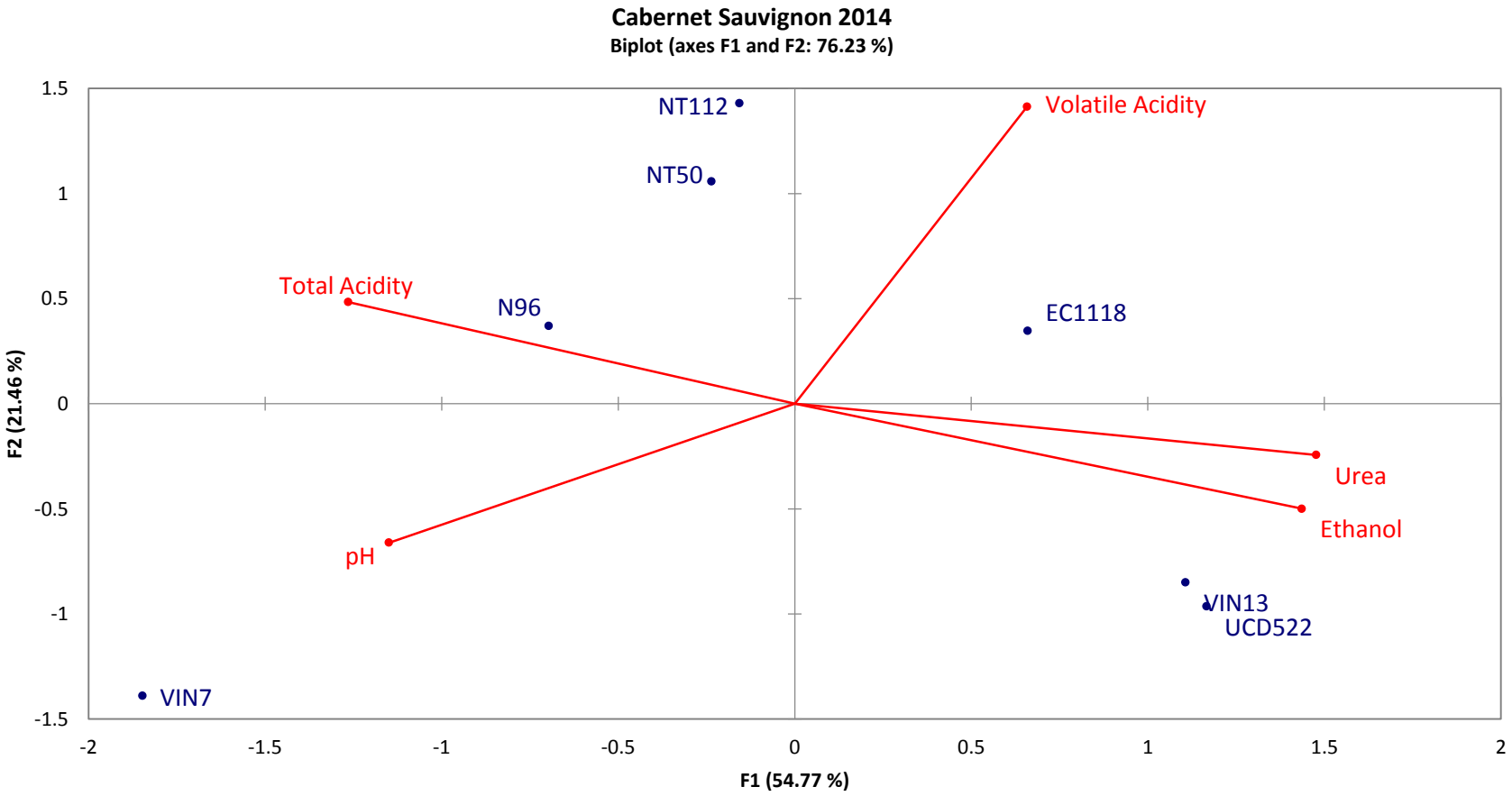

FIGURE 15: Principle Component Analysis (PCA) bi-plot of chemical analysis. The above bi-plot illustrates the grouping of seven yeasts according the specific chemical compounds that they produced within the wines during the 2014 alcoholic fermentation. F1 represents the First Principle Component and F2 represents the Second Principle Component**.**

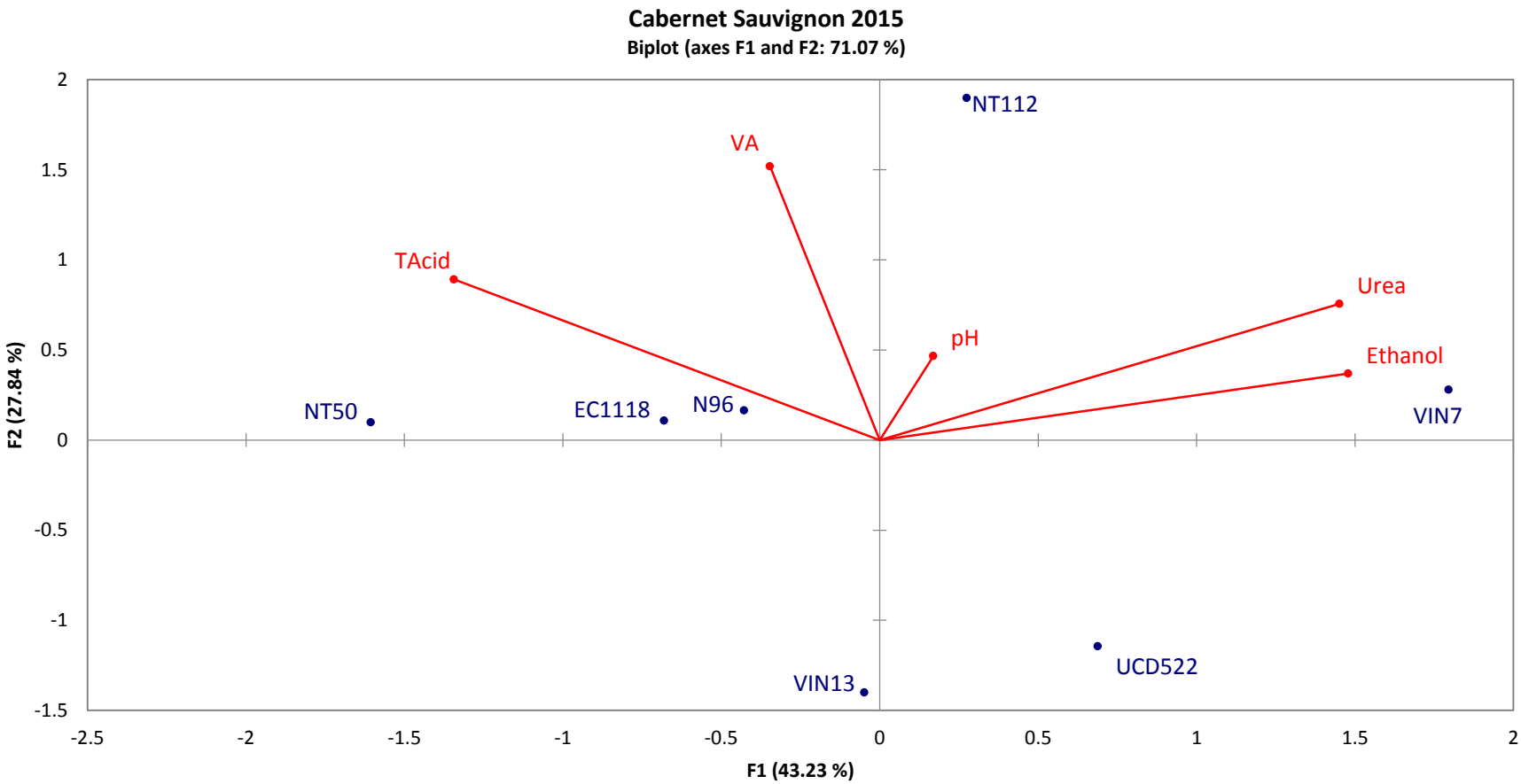

FIGURE 16 : Principle Component Analysis (PCA) bi-plot of chemical analysis. The above bi-plot illustrates the grouping of seven yeasts according the specific chemical compounds that they produced within the wines during the 2015 alcoholic fermentation. F1 represents the First Principle Component and F2 represents the Second Principle Component.

3.3.3.2 Sensory evaluations

Yeast and fermentation conditions were reported to be the most important factors influencing the flavours in wine (Lambrechts & Pretorius, 2000). Wines were sensorially evaluated following bottle maturation for 5 months by a trained panel of seven judges consisting mostly of researchers. The sensory evaluation was carried out according to a randomised block design, using an unstructured line scale (Addinsoft, 2013). The wines were evaluated according to the descriptors applicable Cabernet Sauvignon “Black and Red Fruit”, “Vegetative”, “Spices”, “Colour” and “General quality” wines, The results of the sensory evaluation, depicted by the ANOVA (TABLE 10, PCA bi-plot (FIGURE 17) highlights similarities and significant differences between the yeast together as well as the wine aroma profiles during the 2014 harvest. The low urea producing yeast strain EC1118, produced vegetative yet fruity aroma wines with deep colour and long lasting finish, UCD522 however produced wines with dry vegetative like aroma with a long finish as well. The latter of the yeasts, produced vegetative wines with spicy and fruity characteristics. During the 2015 harvest ADWY produced wines with high aroma intensities (TABLE 11), of which EC1118 produced vegetative and fruity wines, and UCD522 produced wines that were vegetative and spicy with the perfect colour intensity. All seven yeast produced wines with great body and long lasting finish. The PCA bi-plot (FIGURE 18) confirms the data presented by ANOVA.

TABLE 10: Sensorial parameters for Cabernet sauvignon 2014 wines

Yeast strains	Vegetative Cooked	Vegetative Fresh	Vegetative Dried	Spicy	Dried fruit	Colour	Black fruit	Red fruit	Finish
EC1118	20bc	28a	4c	22ab	11ab	86a	57a	16ab	61a
UCD522	23abc	20a	17a	24a	11ab	84ab	49ab	18ab	59ab
VIN7	27ab	22a	12ab	17ab	11ab	84ab	51ab	9b	61a
VIN13	23abc	24a	2c	14b	14a	83b	52a	17ab	58ab
NT96	25ab	21a	8bc	21ab	9ab	85ab	52a	16ab	59ab
NT50	31a	21a	1c	26a	6b	82b	38b	25a	52b
NT112	15c	18a	17a	22ab	9ab	85ab	50ab	16ab	57ab

P is (< 0.05)

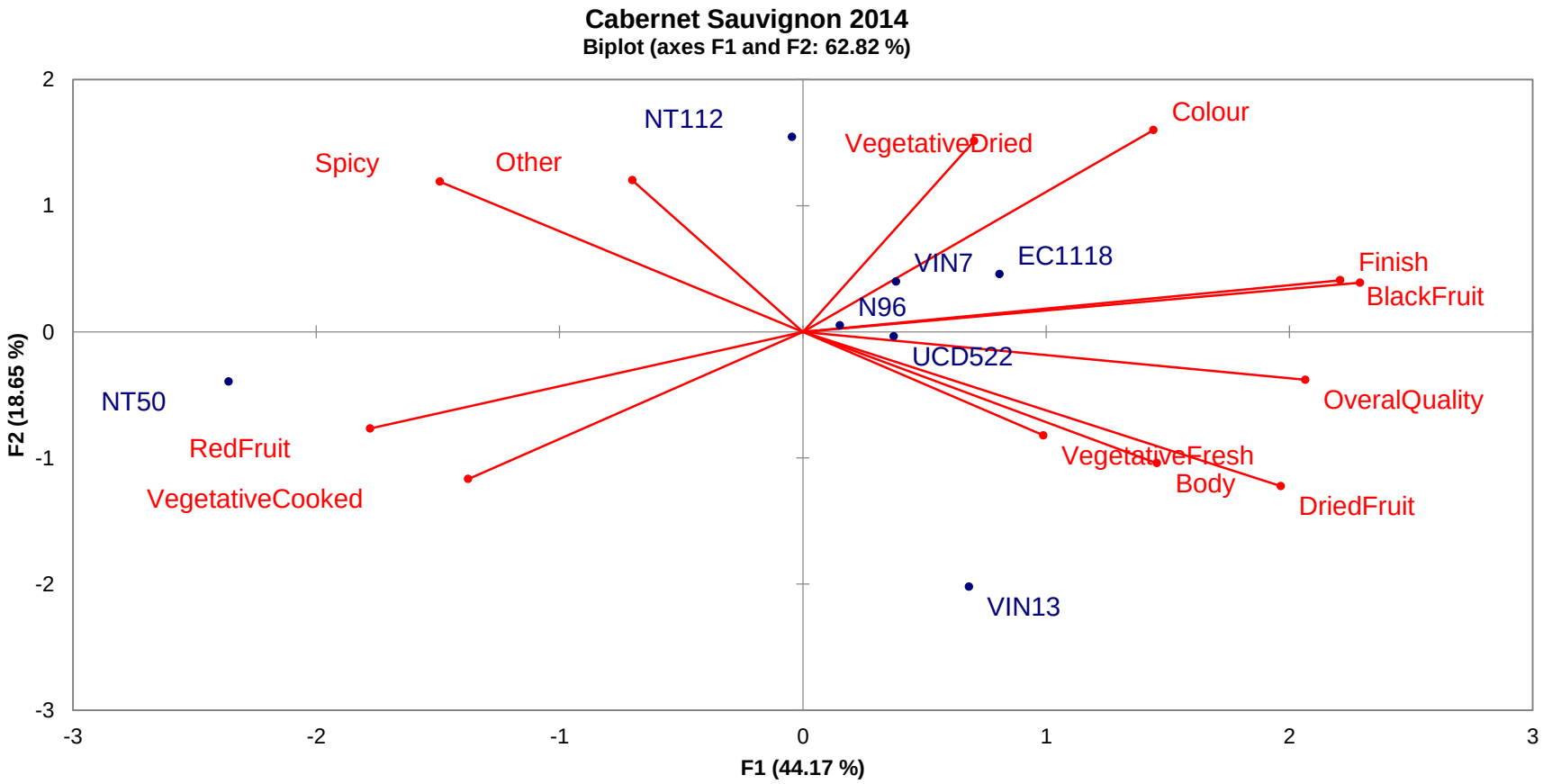

FIGURE 17: Principle Component Analysis (PCA) bi-plot of average values (n = 2) of sensory analysis descriptors for Cabernet sauvignon 2014. The above bi-plot illustrates the different aromas produced by the seven yeasts during the 2014 alcoholic fermentation. F1 represents the First Principle Component and F2 represents the Second Principle Component.

TABLE 11: Sensorial parameters for Cabernet sauvignon 2015 wines

Yeast strains	Vegetative Cooked	Vegetative Fresh	Vegetative Dried	Spicy	Dried fruit	Colour	Black fruit	Red fruit	Finish
EC1118	11b	32a	11a	28a	16a	60cd	40a	48a	51a
UCD522	16ab	34a	8a	32a	15a	68a	37ab	34ab	49a
VIN7	9b	29a	6a	29a	11a	58d	31ab	43a	47a
VIN13	17ab	28a	5a	26a	11a	58d	29ab	43a	52a
NT96	20ab	32a	5a	24a	14a	66ab	34ab	42ab	50a
NT50	33a	27a	8a	27a	11a	66ab	28b	28b	49a
NT112	20ab	34a	5a	27a	14a	63bc	30ab	38ab	52a

P is (< 0.05)

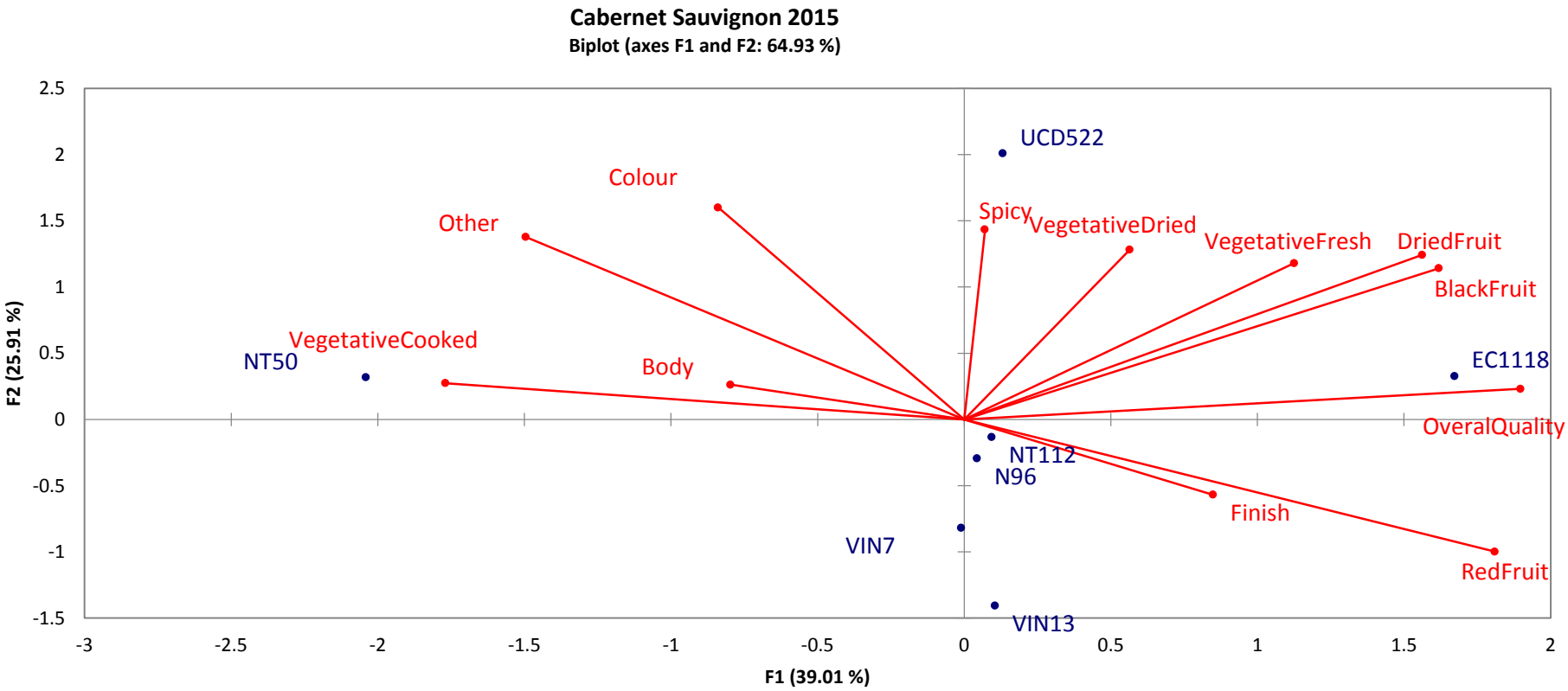

FIGURE 18: Principle Component Analysis (PCA) bi-plot of average values (n = 2) of sensory analysis descriptors for Cabernet sauvignon 2015. The above bi-plot illustrates the different aromas produced by the seven yeasts during the 2015 alcoholic fermentation. F1 represents the First Principle Component and F2 represents the Second Principle Component.

It is evident that the initial urea concentrations for both vintages were high in both cultivars produced for all ADWY. However, after storage for 6 months (FIGURE 19) at 28 °C, urea concentrations declined dramatically indicating that it reacted with ethanol to produce EC. Even though the yeast resulted in lower urea concentrations over time, for the final wines of 2014, yeast EC1118 and NT50 resulted in far higher urea concentrations than before. Kodama *et al*. (1994) clearly indicated that EC formation is closely related to urea content. These authors also determined that urea levels should be kept below 2 mg/L if EC levels in wine are to stay below the USA voluntary limit of 15 ppb. In wines the most part of EC is formed during or after fermentation, probably by the reaction of carbamoyl compounds with ethanol. This reaction is affected by time and increased temperature, thereby putting wines that undergo long periods of maturation and storage, good example are red wines, at a higher risk of developing elevated levels of EC. In fact, some of these compounds are formed during fermentation (Leça *et al*., 2014). One of the most common ways of EC occurring in acidic medium, such as wines, is the reaction of ethanol with urea. In turn, in distilled alcoholic beverages the major pathway for the formation of EC comes from cyanide volatile compounds, like cyanic acid, that are able to react in a gas phase or to pass into the distillate. External factors, such as temperature and pH influence the kinetics of these reactions. For wines to safely remain below the Canadian limit they indicated urea concentrations of < 5 mg/L. this standard can only be met if wines are stored at or below 20 °C. The data in TABLE 5 and 9 shows that generally red wines have higher concentrations of urea than that of white wines. Grape cultivar also seems to affect urea concentration levels as they are stored at different temperatures. In figure 19 it can be clearly seen how storage at high

temperatures and for a long period has an effect on the final urea concentration of the wine.

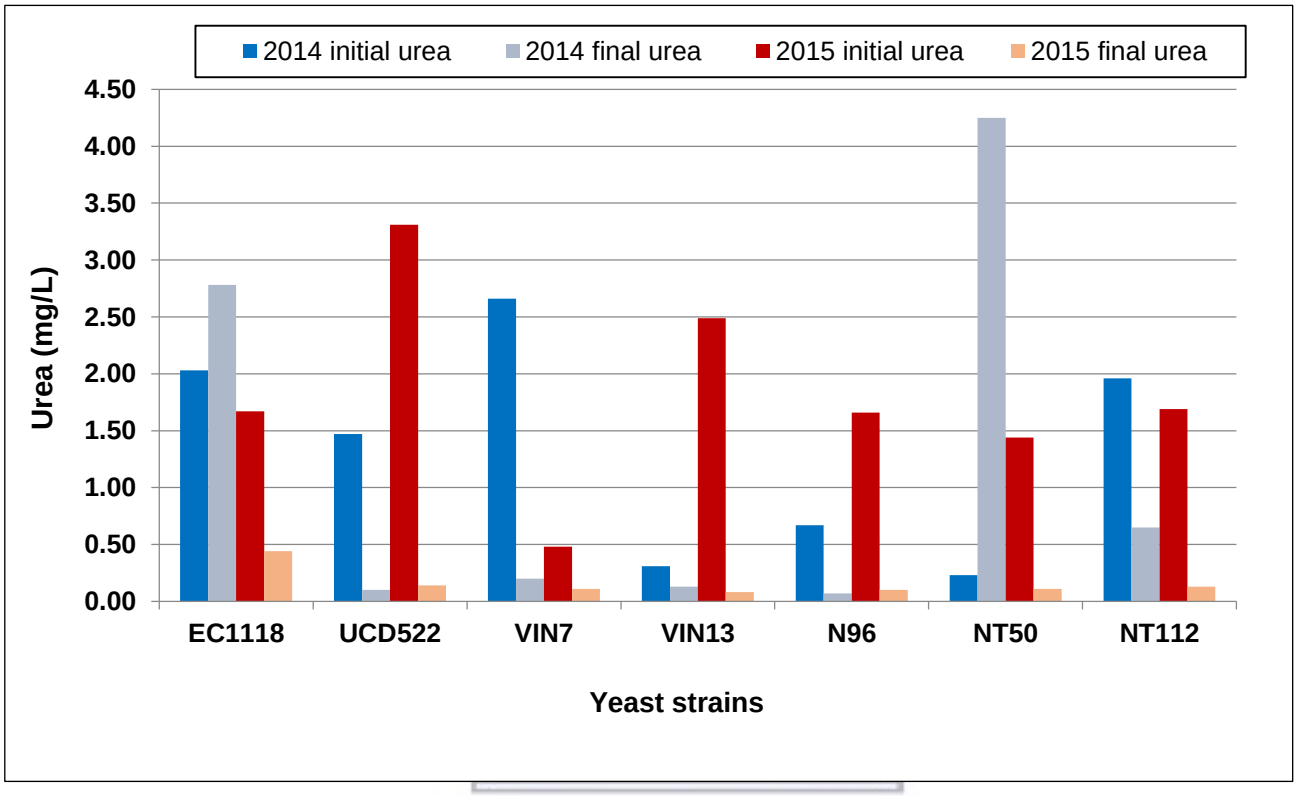

FIGURE 19: Residual urea in Cabernet sauvignon wines. The graph depicts Cabernet Sauvignon wines following fermentation/six months' storage at 28 °C using experimental and commercial active dried wine yeasts.

3.3.3.3 Proteomic characterisation

Approximately 200 µg of protein preparations were resolved by 1D SDS-PAGE, yielding molecules ranging in size from 10 to 270 kDa with clear differences in protein profiles (FIGURE 2). Indications are that different yeast strains had differential protein expression as the SDS-PAGE showed various yeast strains had different protein banding patterns in terms of distribution and intensity (FIGURE 20). Subsequently, these bands were excised and subjected to in-gel trypsin digestion resulting peptides where after mass spectrometry (MS) analysis was conducted. A total of 127 proteins were characterised by deploying MALDI-TOF and nano LC/MS in final Cabernet Sauvignon wine (Table 12). Proteins of interest produced by EC1118, UCD522 and NT50 yeasts were identified after an extensive search on a universal protein database (UniProtKB database at http://www.uniprot.org/uniprot/?query=HOSC&sort=score). Proteins identified with assession numbers CARB and ARLY found in EC1118 yeast, respectively are both involved in arginine synthetic processes as well as arginine biosynthesis (Interpro, 2016). Their score was less than 100% which compliments the low urea content observed within wines produced during and after alcoholic fermentation using the low urea producing reference EC1118. It was observed that the yeast EC1118 that produced Sauvignon Blanc and Cabernet Sauvignon wines with a positive association with VA, up-regulated the protein HSC82 associated with stress response. Therefore, this observation complements previous research, since VA are known to be produced by wine yeast in response to stressful environments. It is also noteworthy, that the high urea producing reference UCD522 that produced Sauvignon

Blanc wines with a negative association with VA also expressed the HSC82 stress response protein.

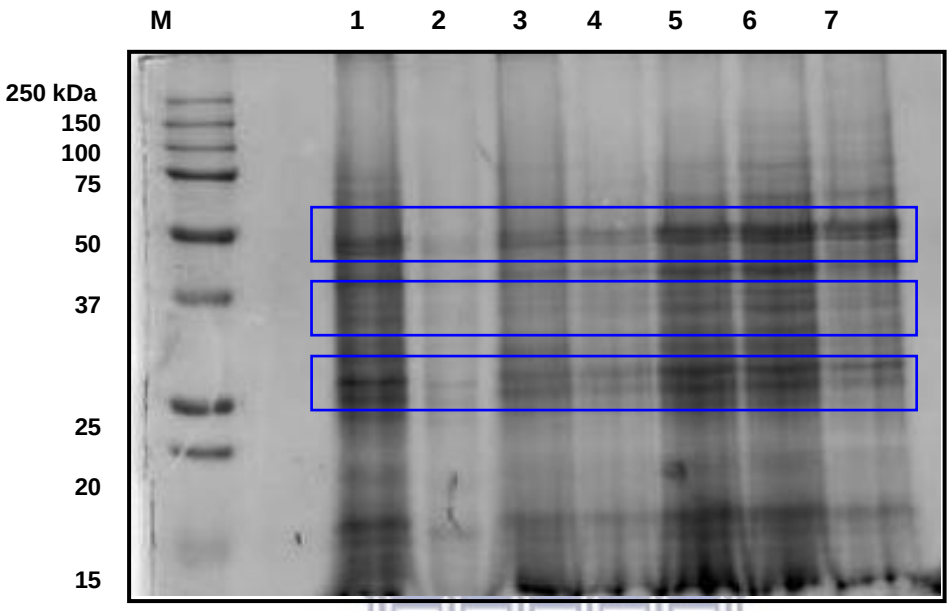

FIGURE 20: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The above figure is that of the analyses of protein extracted from seven commercial yeasts i.e. Lanes 1 – 7: EC1118, UCD522, VIN7, VIN13, N96, NT50 and NT112 following the fermentation of Cabernet Sauvignon grape must (juice).

In the above (TABLE 8) as described in 3.3.2.3 (proteomic characterisation) Identified differentially expressed proteins found in final wines produced by EC1118, UCD522 and NT50 yeast by MALDI-TOF/MS for both Sauvignon Blanc and Cabernet sauvignon final wines. Proteins that have been identified with high scores: PGK, ALF, ACT, AHP1, etc. are mostly associated with glycolysis, ATP-binding and protein folding. Indications therefore are that expression of the protein in different yeast strains has contrasting metabolic effects. It can be envisaged that Western blotting will be conducted in future

to verify that the proteins and corresponding genes were in fact upregulated in the respective yeast strains.

TABLE 12: Differentially expressed proteins found in Cabernet Sauvignon (CS) final wines produced by commercial active dried wine yeast (ADWY) i.e. EC1118, UCD522 and NT50 identified by deploying MALDI-TOF/MS.

Accession	Yeast	Molecular weight (kDa)	Sequence coverage %	Score	pI	Peptides	Function
PGK	EC1118	44.7	21.6	555.61	7.78	13	key enzyme in glycolysis and gluconeogenesis
	UCD522		7.9	156.98		3	
ALF	EC1118	39.6	12	258.23	5.44	5	Lyase, glycolysis, metal binding (Zn)
	UCD522		9.7	190.95		4	
AHP1	UCD522	19.1	15.9	261.92	4.87	5	thioredoxin peroxidase activity, cell redox homeostasis
BMH1	UCD522	30.1	11.2	150.3	4.67	3	DNA replication
CISY1	EC1118	53.3	6.9	93.47	8.81	3	Tranferase, tricarboxylic acid cycle, carbohydrate metabolism
G3P2	UCD522	35.8	22.6	362.4	6.52	8	Oxidoreductase, glycolysis
G3P3	EC1118	3.7	9.9	107.66	6.52	5	
ADH1	EC1118	36.8	9.5	168.34	6.23	4	required for the reduction of acetaldehyde to ethanol
ADH2	UCD522	36.7	2.3	28.34	6.29	1	catalyzes the conversion of ethanol to acetaldehyde
HSC82	EC1118	80.8	1.4	26.88	4.62	1	Chaperone, stress response, ATP binding
	UCD522		2.7	46.47		2	
TCTP	UCD522	18.7	5.4	46.36	4.28	1	Protein synthesis
CARB	EC1118	123.8	0.7	30.66	5.05	1	Arginine synthetic process, ligase
BCA1	EC1118	43.6	2	22.3	9.57	1	Amino transferase, amino-acid biosynthesis
ARLY	EC1118	52	2.8	26.89	5.38	1	Amino-acid biosynthesis, Arginine biosynthesis
HSP71	UCD522	69.6	8.4	249.26	4.84	4	Protein folding, stress response
	EC1118		4.8	129.55		2	
HSP72	EC1118	69.4	4.4	143.4	4.79	2	Stress response, ATP-binding, nucleotide-binding

	UCD522		10.3	299.4		6	

TABLE 12: Continued

Accession	Yeast	Molecular weight (kDa)	Sequence coverage %	Score	pI	Peptides	Function
ENO1	EC1118	46.8	24.7	762.17	6.16	16	Glycolysis
	UCD522						
	NT50		11.4	252.6		6	
ENO2	EC1118	46.9	22.2	718.21	5.61	14	Glycolysis, carbohydrate degradation
	UCD522		11.7	243.87		6	
ACT	EC1118	41.7	17.1	197.93	5.36	6	ATP-binding, Nucleotide-binding
	UCD522		2.7	29.74		1	
TSA1	UCD522	21.6	3.6	24.4	4.88	1	Antioxidant, Oxidoreductase, Peroxidase
ARPC3	UCD522	20.6	3.9	25.08	8.91	1	Actin-binding
VPS64	UCD522	67.2	1.2	22.73	7.16	1	Cell cycle, Protein transport, Transport
CYPC	UCD522	19.9	7.1	24.12	9.52	1	Isomerase, Rotamase
							Cyclosporin
HBN1	UCD522	21	6.2	25.79	6.53	1	Oxidoreductase
							Flavoprotein,
INP52	UCD522	133.2	0.7	25.88	9.4	1	Endocytosis, Lipid metabolism, Protein transport, Transport
COX2	UCD522	28.5	3.2	26.81	4.29	1	Oxidoreductase
							Electron transport, Respiratory chain, Transport
							Copper, Metal-binding
SEY1	UCD522	89.4	1	26.25	5.02	1	Stress response
							GTP-binding, Nucleotide-binding
CCC1	UCD522	34.2	2.2	28.29	4.82	1	Transporter

.

TABLE 12: Continued

Accession	Yeast	Molecular weight (kDa)	Sequence coverage %	Score	pI	Peptides	Function
TMA17	UCD522	16.8	8	28.08	4.43	1	Chaperone
SNX3	UCD522	18.8	5.6	27.93	9.9	1	Protein transport, Transport Lipid-binding
ACON	UCD522	85.3	2.1	30.45	8.87	1	Tricarboxylic acid cycle,Metal-binding
IPYR	UCD522	32.3	2.4	29.24	5.25	1	Magnesium, Metal-binding
IF5A1	UCD522	17.1	4.5	35.66	4.64	1	Elongation factor Protein biosynthesis RNA-binding
YL179	UCD522	22.1	5	35.17	4.62	1	Uncharacterized protein YLR179C
ATPB	UCD522	54.8	1.8	33.28	5.42	1	Hydrolase ATP synthesis, Hydrogen ion transport, Ion transport, Transport ATP-binding, Nucleotide-binding
HSP26	UCD522	23.9	4.7	45.46	5.19	1	major polypeptides produced on heat shock, Stress response
GPP2	UCD522	27.8	7.2	42.07	5.76	1	Hydrolase Stress response Magnesium, Metal-binding
NACA	UCD522	18.7	7.5	54.07	4.69	1	Protein transport, phosphatidic acid binding
PRTB	UCD522	69.6	1.4	46.77	5.93	1	Hydrolysis of proteins with broad specificity
RS7A	UCD522	21.6	7.9	62.23	10.29	2	Ribonucleoprotein, Ribosomal protein Ribosome biogenesis, rRNA processing
GIP4	UCD522	86.6	1.8	54.63	10.28	1	chromosome segregation regulation of phosphoprotein phosphatase activity
PMG1	UCD522	27.6	8.9	75.13	9.27	3	Isomerase, Glycolysis
ARF1	UCD522	20.5	10.5	70.18	7.65	2	GTP-binding, Nucleotide-binding

TABLE 12.: Continued

Accession	Yeast	Molecular weight (kDa)	Sequence coverage %	Score	pI	Peptides	Function
WTM1	UCD522	48.4	5.3	86.65	5.05	3	Meiosis, Transcription, Transcription regulation
TPIS	UCD522	26.8	8.9	84.37	5.67	2	Isomerase Gluconeogenesis, Glycolysis, Pentose shunt
HSP7E	UCD522	70	1.4	82.91	5.87	2	Chaperone ATP-binding, Nucleotide-binding
RSSA2	UCD522	27.9	10.7	113.72	4.54	3	Ribonucleoprotein, Ribosomal protein Ribosome biogenesis, rRNA processing
GPX3	UCD522	18.6	16	90.35	9.12	2	Oxidoreductase, Peroxidase
EF1A	UCD522	50	5.9	122.79	9.82	2	polypeptide chain elongation, Protein biosynthesis.
RS5	UCD522	25	12.9	114.1	9.35	4	Ribonucleoprotein, Ribosomal protein
RS15		16	9.2	39.27	11.14	1	RNA-binding, rRNA-binding
RS11A		17.7	4.5	37.8	11.49	1	
IF1A	UCD522	17.4	21.6	139.06	4.54	3	Initiation factor, Protein biosynthesis
BMH2	UCD522	31	14.3	137	4.67	4	DNA replication binding, Phosphoserine binding
KPYK1	UCD522	54.5	16.6	248.95	8.57	8	Glycolysis, ATP-binding, Kinase, metal-binding
ABF2	UCD522	21.5	27.3	242.1	10.06	7	DNA-binding
ECM5	EC1118	162.6	0.5	26.02	6.39	1	Cell wall biogenesis/degradation,Metal-binding, Zinc
METE	EC1118	85.8	1.3	23.67	6.04	1	Amino-acid biosynthesis, Methionine biosynthesis
YO387	EC1118	22.1	3.4	22.86	4.12	1	Ligand
H4	EC1118	11.4	12.6	28.97	11.85	1	DNA-binding
IDH1	EC1118	39.3	2.8	28.35	9.43	1	Tricarboxylic acid cycle, Oxidoreductase
IDH2		39.7	1.9	22.75	9.47		
EF2	EC1118	93.2	1.2	32.18	5.89	1	polypeptide chain elongation. Protein biosynthesis
	UCD522		1.9	58		2	
YJ00	EC118	84.2	0.9	31.98	6.82	1	hexose catabolic process

TABLE 12: Continued

Accession	Yeast	Molecular weight (kDa)	Sequence coverage %	Score	pI	Peptides	Function
CYS3	EC1118	42.5	2.8	29.4	6.06	1	Amino-acid biosynthesis, Cysteine biosynthesis
H2AZ	EC1118	14.3	6.7	32.93	11.11	1	Activator, Chromatin regulator, DNA-binding, transcription
UPF3	EC1118	44.9	1.8	32.28	10.42	1	Nucleotide binding, Protein ubiquitination, Nonsense-mediated mRNA decay
HXKA	EC1118	53.7	2.7	40.79	5.16	1	Enzyme regulation, catalytic activity, glycolysis
IF4A	EC1118	44.7	1.8	36.47	4.87	1	Protein biosynthesis, catalytic activity, ATP-binding
LSP1	EC1118	38	3.5	34.51	4.47	1	Lipid binding, Endocytosis, response to eat
QCR1	EC1118	50.2	2.8	42.77	6.93	1	metalloendopeptidase activity, aerobic respiration, electron transport
QCR2		4.5	2.4	25.35	8.6		
FPPS	EC1118	40.5	3.4	41.85	5.19	1	farnesyl diphosphate biosynthesis, geranyl diphosphate biosynthesis
GLYC	EC1118	52.2	3.6	49.69	7.18	2	tetrahydrofolate interconversion, One-carbon metabolism.
GLNA	EC1118	41.7	4.6	46.59	5.9	2	ATP-binding, Nucleotide-binding, Ligase
	UCD522		8.6	108.73		4	
CARP	EC1118	44.5	2.5	54.91	4.56	1	Aspartyl protease, Hydrolase, Protease
ILV5	EC1118	44.3	8.4	137.47	9.57	4	Oxidoreductase,amino-acid biosynthesis, metal-binding NADP
GPD1	EC1118	42.8	5.1	52.25	5.19	2	glycerol-3-phosphate dehydrogenase [NAD+] activity, stress response, oxidoreductase
HOSC	EC1118	47.1	5.1	68.85	7.02	3	Homocitrate synthase activity, transferase, amino-acid biosynthesis
RL4B	EC1118	39	6.9	56.66	11.17	2	RNA-binding, Cytoplasmic translation
EF1A	EC1118	50	8.7	120.85	9.82	4	polypeptide chain elongation, Protein biosynthesis.
PIL1	EC1118	38.3	11.2	153.47	4.39	4	Negative regulator of cell wall integrity, lipid binding
	UCD522		3.5	38.81		1	

TABLE 12.: Continued

Accession	Yeast	Molecular weight (kDa)	Sequence coverage %	Score	pI	Peptides	Function
OYE2	EC1118	45	9	86.98	6.13	3	Oxireductase, Flavoprotein, FMN,NADP. Apoptotic process
RIR4	EC1118	40	6.1	76.08	4.97	1	DNA replication, Oxidoreductase
RIR2	UCD522	46.1	7.3	70.77	5.01	3	Iron, metal binding
			4.5	53.31		2	
RL11A	UCD522	19.7	25.3	280.7	10.41	6	Ribonucleoprotein, Ribosomal protein
RL12A		17.8	24.8	219.6	9.99	5	RNA-binding,
RL21A		18.2	20	184.26	10.87	4	
RL13B		22.5	18.6	145.96	11.56	3	
RL5		33.7	8.4	81.55	6.39	2	
RL24B		17.5	12.3	77.77	11.87	1	
RL24A		17.6	12.3	62.07	11.78	1	
RL4B		39	3.9	57.3	11.17	1	
RL17A		20.5	3.8	41.71	11.37	1	
RL7A		27.6	4.9	30.27	10.56	1	
RL34A		13.6	6.6	29.5	11.93	1	
PDC5	UCD522	61.9	5.5	91.7	5.98	2	ethanol fermentation, Amino-acid degradation.Branched-chain amino acid catabolism
PDC1	EC1118	61.5	10.1	218.03	5.76	6	pyruvate decarboxylase activity, fermentation of glucose to ethanol

3.4. CONCLUSION

Final wines were chemically and sensorially analysed and compared to the previous vintage (harvest). The yeast strain, EC1118 was the lowest urea producer in both Sauvignon Blanc and Cabernet Sauvignon wines for both vintages. The yeast strains NT112 produced Sauvignon Blanc and Cabernet Sauvignon wines with the most 'tropical fruit" and "floral"; and "red fruit" aromas. However, wines produced with the remaining yeast strains were not negatively perceived.

Characterisation of the proteins released from seven *Saccharomyces* yeasts during alcoholic fermentation was performed and differential expressed proteins in the fermentations were identified with MALDI-TOF in conjunctions with nano-LC/MS. In this fermentation study, the most abundant proteins identified were associated with glycolysis and ethanol production. Indications, therefore, are that expression of the protein in different yeast strains has contrasting metabolic effects. Arginase (the product of the CAR1 gene), is the first enzyme required for the utilisation of arginine as nitrogen source. Optimal arginase production is only necessary when arginine is the sole nitrogen source available in the growth medium. When nitrogen sources such as ammonia, glutamine or asparagine supporting optimal growth are present, a poorer nitrogen source such as arginine is almost not utilized. Therefore, the synthesis of arginase is tightly modulated as a function of the presence of the inducer, the quality of the nitrogen source, and also the total amount of nitrogen present in the growth medium.

This information is crucial to our understanding of the influence these yeasts can have on the final product and how we can improve strains used for commercial wine

production (Marangon, 2010). Furthermore, Rossouw *et al*., (2010) showed that proteomic analyses can be a powerful means to interpret omics-related data and also to understand metabolic and physiological changes during the fermentation process. Clearly, analytical chemistry plays an important part in ongoing research aimed at improved understanding of wine production, with the ultimate goal of producing better products. In view of the chemical complexity of grapes and their derived products, these trends are expected to increase further in future, and in this manner analytical methods will continue to play an influential role in the understanding of the chemical composition of grapes, wine and their derived products on the African continent.

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CHAPTER 4

4.1 CONCLUDING REMARKS

Fermented beverages are widely consumed and highly appreciated all over the world. In fact, they provide considerable benefits to human nutrition (Chen *et al*., 2015). As a branch of fermented foods, they are the product of food substrates overgrown by edible microorganisms whose enzymes, mainly proteases, amylases, and lipases, hydrolyse nitrogen and carbon sources to absorbable low molecular nutrients. Several types of fermented beverages possess different nutrients, however they share similar fermentation processes, in which different microorganisms and complex conditions exist. These processes may produce toxic products as a result of metabolism and side reactions, including EC. These compounds are generated due to the incomplete metabolism of nitrogen-containing compounds during the fermentation process (Jiao *et al*., 2014).

Possible approaches to reduce the contents of ethyl carbamate in alcoholic beverages can be by adding acid urease, addition of diammonium phosphate (DAP), lower temperature or lower pH at initial stages of wine production. Apart from temperature and duration of storage, the main factors influencing urea in wines include the arginine content of the grape, yeast strain, method of yeasting, fortification and timing of fortification,

The majority of the urea formed comes from arginase-catalysed degradation of arginine during fermentation (as mentioned before). High urea levels occur in wines produced

from grapes of high (> 400 rng/L) arginine content. Such grapes tend to come from vineyards heavily fertilised or displaying high vigour (Ough, 1990). Yeast strains differ in their urea excretion and uptake during fermentation. Therefore, yeast selection plays a vital role in minimizing the potential for EC formation. The yeasts 71B (Lallenmand), SD 1120 (Red Star), and Prise de Mousse have been shown to release fairly low levels of urea during fermentation (An and Ough 1993). Yeasts that excrete little urea have slight but important differences in their arginine transport system and urea metabolizing enzymes. The main yeast genes that influence up or down regulation of proteins to produce urea are yeast CAR1 gene, which encodes arginase and DUR1,2 genes (encoding urea amidolyase, which can degrade urea into ammonia) or the DUR3 gene (encoding urea permease). By overexpressing these genes, urea-degrading strains produced 87% and 15% less EC than the original strain, respectively (Coulon *et al*., 2006). It can be envisaged that Western blotting will be conducted in future to verify that the proteins and corresponding genes that were identified were in fact upregulated in the respective yeast strains. Overall, the systems biology approach to the study of yeast metabolism during alcoholic fermentation opened up new avenues for further hypothesis-driven research and targeted engineering strategies for the genetic enhancement/ modification of wine yeast for commercial applications. It is envisioned that protein biomarkers associated with urea formation will identified in future.

In this sense, the study of the formation pathways has been playing a crucial role in the preventive and control actions. Multiple factors can affect the EC formation, depending on the fermented or distilled beverage. According to the Food and Drug Administration (FDA), urea, citrulline, and arginine are involved in the formation of EC in most

fermented beverages. The reaction between urea and ethanol seems to be the key reaction in its formation in wine. Vineyard fertilisation with urea, ammonia and other N-fertilisers has a direct influence in the nitrogen content of musts that potentiate the occurrence of EC (Ugliano, 2007). Nitrogen-fertilisers should only be used to provide sufficient nutrients for yeast cell growth. Although yeast is able to use many nitrogen sources for growth, the utilization rates of these components are different (Godard *et al*., 2007). In a study done by Zhao *et al* (2013), it was found that there were mainly two kinds of inhibitory effects on urea metabolism by preferred nitrogen sources. Asparagine, glutamine and ammonium can repress urea utilization, while aspartate, glutamate and serine can just slightly strongly repress urea utilisation. Global and in-depth investigations of the mechanism of nitrogen regulation that is involved in urea accumulation are essential to reveal the regulatory mechanisms controlling urea accumulation in *S. cerevisiae.* It is believed that the urea accumulation can be minimised by rational regulation of these negative or active regulators. The FDA also indicates the enzymatic hydrolysis of urea, with urease enzyme is an alternative to mitigate EC. Acid urease has an optimum pH compatible with wine, and its feasibility.

Techniques such as IEC and gel filtration are used to fractionate and analyse proteins (Giribaldi, 2010), and in recent years various methods have been tested and improved to collect, identify and quantify low abundance proteins present in wine (Rossouw *et al*., 2010). Techniques that were successfully used include combinatorial peptide ligand library (CPLL), ELISA, nano-LC/MS as well as combinations of these techniques (Marangon, 2010). For visual interpretation of yeast proteome, SDS-PAGE and 2D-PAGE analyses is still the most used method (Rabilloud, *et al*., 2010). Above-mentioned

techniques however have limitations as only proteins within a certain molecular weight and pH range can be visualised. Furthermore, the use of different stains will result in visualisation of different proteins. These challenges have not hampered researchers from investigating the wine proteome, with an increasing number of studies reporting on this subject in recent years. Previous studies regarding the EC control have focused their attention in the identification of the main precursors in alcoholic beverages, as well as understanding the impact of external factors in their formation such as light, reaction time and temperature (Hasnip, 2004). It is difficult to eliminate EC because of the lack of a comprehensive understanding of urea accumulation in *S. cerevisiae*. In fact, urea and most of the remaining nitrogen sources in fermented food can be degraded into non-harmful metabolites in *S. cerevisiae* under suitable conditions (Monteiro & Bisson, 1991). The key reason for urea accumulation is the inhibitory effect on urea utilisation of nitrogen regulation (Magasanik & Kaiser, 2002). Therefore, a promising approach for minimising urea accumulation is to control nitrogen regulation in *S. cerevisiae*

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