Metabolomic profiling of non-*Saccharomyces* yeasts in wine

by

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Declaration

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Summary

Recent trends in wine making have led to the commercial production and use of non-*Saccharomyces* yeasts in wine making. Very little is understood however about how the use of these yeasts affects the final product. The purpose of this study was to evaluate the chemical and sensory characteristics of wine fermented with non-*Saccharomyces* yeasts using a sequential inoculation strategy. Targeted and untargeted analysis techniques were developed to help identify and quantify the volatile fraction of the wines produced. By combining this and sensory data we were able to build the most comprehensive picture to date of the volatile wine metabolome as it is influenced by various yeast species.

The first step was a literature review dedicated to summarizing the current knowledge surrounding the metabolomics of the yeasts used in the subsequent chapters. Specifically, we sought to understand what is currently known about the use of non-*Saccharomyces* yeasts in wine. Also investigated were the technologies currently being used in the fields of food, wine, and yeast metabolomics. The goal was to provide the background necessary to understand the research in the subsequent chapters, as well as aid in the development and planning of the experiments discussed here within.

Two stages of research were conducted. Not only did we want to understand the effects of non-*Saccharomyces* yeasts on wine aroma but we were interested in whether or not these effects were the same in both red and white wines. As such the first research stage, was a preliminary investigation of the yeast response to two different grape musts. Five different species of non-*Saccharomyces* yeasts, were chosen and grown in both Shiraz and Sauvignon blanc must and samples were collected for analysis just prior to the point at which *Saccharomyces cerevisiae* would usually be added to complete the fermentation. The fermentation rates were monitored and the chemical profile of the musts was evaluated. A solid-phase microextraction-Gas Chromatography-Mass spectrometry method that targeted 90 different compounds known to be found in wine was used to evaluate the headspace of the fermented musts.

The results obtained helped shape the experimental design for the next phase of the project. The scale was increased to full wine production to evaluate how the yeasts could influence a completed wine product. Again, Sauvignon blanc and Shiraz were chosen and an untargeted chemical analysis method was developed to ensure that the widest possible range of analytes could be evaluated. The finished Sauvignon blanc wine was also subjected to sensory analysis which provided even greater insight into how these inoculation strategies can change the sensory profile of the wine.

This research was undertaken in an attempt to answer the questions of ‘What will the wine smell and taste like if I use non-*Saccharomyces* yeasts during fermentation?’ and ‘Could it be superior to standard wines only inoculated with *S. cerevisiae*?’ The experiments conducted provided a great deal of insight that can help to begin answering these questions but there is much that remains unknown. In general, we were able to build a detailed volatiles chemical profile for each of the yeast treatments used in both Shiraz and Sauvignon blanc. While some treatments proved to be somewhat detrimental to the aroma and flavor of the wine, others showed promise in possibly enhancing its complexity. We were also able to demonstrate that the yeasts behave very differently in the two different musts. As comprehensive as these studies were, future work should be undertaken to improve the understanding of why and how these yeasts can make an impact on wine production. For example, our work did not include any genetic expression analysis of the yeasts used. Correlating genetic expression to quantitative chemical analysis would provide a much more complete picture of the wine yeast metabolome.

Opsomming

Onlangse tendense in wynbereiding het gelei tot die kommersiële vervaardiging en gebruik van nie-*Saccharomyces* giste in wynbereiding. Baie min word egter verstaan van hoe die gebruik van hierdie giste die finale produk affekteer. Die doel van hierdie studie was om die chemiese en sensoriese kenmerke te evalueer van wyn wat met nie-*Saccharomyces* giste gegis is deur gebruik te maak van ‘n opeenvolgende inentingstrategie. Geteikende en ongeteikende analise-tegnieke is ontwikkel om die vlugtige fraksie van die vervaardigde wyne te help identifiseer en kwantifiseer. Deur hierdie en die sensoriese data te kombineer, was ons in staat om die mees omvattende beeld tot op datum te bou van die vlugtige wynmetaboloom soos dit deur verskeie gisspesies beïnvloed word.

Die eerste stap was ‘n literatuuroorsig gemik op die opsomming van huidige kennis oor die metabolomika van die giste wat in die opeenvolgende hoofstukke gebruik is. Ons het spesifiek gepoog om te begryp wat tans bekend is oor die gebruik van nie-*Saccharomyces* giste in wyn. Ons het ook die tegnologieë ondersoek wat tans in die gebied van voedsel-, wyn en gismetabolomika gebruik word. Die doelwit was om die nodige agtergrond te verskaf om die navorsing in die daaropvolgende hoofstukke te kan verstaan, sowel as om te help in die ontwikkeling en beplanning van die eksperimente wat hierbinne bespreek word.

Twee stadiums van navorsing is onderneem. Nie net wou ons die effekte van nie-*Saccharomyces* giste op wynaroma verstaan nie, maar ons het ook daarin belanggestel om uit te vind of hierdie effekte dieselfde was in beide rooi- en wit wyne. As sulks was die eerste navorsingstadium ‘n voorlopige ondersoek na die gisrespons op twee verskillende druiwemoste. Vyf verskillende spesies van nie-*Saccharomyces* giste is gekies en in beide Shiraz- en Sauvignon blanc-mos gegroei en monsters vir analise is geneem net voor die punt waarop *Saccharomyces cerevisiae* gewoonlik bygevoeg sou word om die gisting te voltooi. Die gistingstempo’s is gemonitor en die chemiese profiel van die moste is geëvalueer. ‘n Soliede fase-mikroekstraksie–gaschromatografie massaspektrometrie metode wat 90 verskillende verbindings teiken wat daarvoor bekend is om in wyn voor te kom, is gebruik om die lugspasie van die gegiste moste te evalueer. Die resultate wat behaal is, het bygedra tot die opstel van die eksperimentele ontwerp vir die volgende fase van die projek. Die skaal is verhoog tot volledige wynproduksie om te evalueer hoe die giste ‘n voltooide wynproduk sou beïnvloed. Sauvignon blanc en Shiraz is weer gekies en ‘n ongeteikende metode van chemiese analise is ontwikkel om te verseker dat die breedste moontlike reeks analiete geëvalueer kon word. Die voltooide Sauvignon blanc wyn is ook aan sensoriese analise onderwerp wat nog groter insig verskaf het in hoe hierdie inentingstrategieë die sensoriese profiel van die wyn kan verander.

Hierdie navorsing is onderneem in ‘n poging om vrae te beantwoord soos: ‘Hoe sal die wyn ruik en proe as ek nie-*Saccharomyces* giste tydens gisting gebruik?’ en ‘Sou dit beter as standaard wyne wees wat net met *S. cerevisiae* ingeënt is?’ Die eksperimente wat uitgevoer is, het ‘n groot mate van insig verskaf wat ons kan help om te begin om hierdie vrae te beantwoord, maar daar is baie wat nog onbekend is. Oor die algemeen kon ons ‘n gedetailleerde chemiese profiel van vlugtige stowwe vir elk van die gisbehandelings wat in beide die Shiraz en Sauvignon blanc gebruik is, bou. Hoewel sommige van die behandelings ietwat nadelig was vir die aroma en geur van die wyn, het ander belofte getoon om moontlik die kompleksiteit te verhoog. Ons kon ook demonstreer dat die giste baie verskillend in die twee verskillende moste opgetree het. Hoewel hierdie studies omvattend was, moet verdere werk in die toekoms gedoen word om ons begrip van hoekom en hoe hierdie giste ‘n impak op wynproduksie kan maak, te verbeter. Byvoorbeeld, ons werk het nie enige analise van die genetiese uitdrukking van die giste wat gebruik is, ingesluit nie. ‘n Korrelasie van die genetiese uitdrukking met kwantitatiewe chemiese analises sou ‘n baie meer volledige beeld van die wyngismetaboloom kon verskaf.

This dissertation is dedicated to my family,
without whose support it would not have been possible.

Biographical sketch

Margaret Elizabeth Beckner Whitener was born on December 11th, 1986 in Frankfort, Kentucky, USA. The daughter of a United States Army officer and elementary school teacher, she grew up moving frequently. At the age of 16 she was accepted into a prestigious residential high school, North Carolina School of Science and Mathematics. After completing her studies there she attended the University of North Carolina at Chapel Hill and graduated with a Bachelor of Science degree in Biology in 2009. Margaret was then accepted to the North Carolina State University's Department of Food, Bioprocessing and Nutrition Sciences where she completed a Master's Degree with a thesis entitled: *Understanding Volatile Compound Production by S. cerevisiae and L. plantarum and their Role in Interactions between the Species.* In 2012, she was awarded a scholarship in order to pursue a PhD from Fondazione Edmund Mach's International Doctoral Programme, located in Trentino, Italy. The scholarship was funded through the Genomics and Molecular Physiology of Fruit Plants program. In 2013, she enrolled in the Institute for Wine Biotechnology at Stellenbosch University, South Africa and began an international collaborative PhD project studying the metabolomics of non-*Saccharomyces* yeasts in wine. Her research has taken her to Sweden, South Africa and Italy which have all helped to enhance her work and academic experience.

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Preface

This dissertation is presented as a compilation of six chapters. Each chapter is introduced separately, chapters 1, 2 and 6 are written according to the style of the *South African Journal of Enology and Viticulture.* Chapter 3 is written according to the style of *LWT- Food Science and Technology* and has been accepted for publication. Chapter 4 is written according to the style of the journal of *Metabolomics* and has been accepted for publication. Chapter 5 is written according to the style of the *Australian Journal of Grape and Wine Research.*

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Chapter 2 **Literature review**
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Chapter 3 **Research results**
Early fermentation volatile metabolite profile of non-*Saccharomyces* yeasts in red and white grape must: a targeted approach

Chapter 4 **Research results**
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Chapter 1

General introduction and project aims

1.1 Introduction

With a more than 6000 year history, wine or at least fermented grape juice is arguably the world's oldest fermented beverage. For the majority of that history spontaneous fermentation by native organisms has been the means by which this beverage has been produced. It is only relatively recently however, thanks to the efforts of Louis Pasteur which led to the advent of modern microbiology, that man has been able to gain more in-depth knowledge on the subject of fermentation. This understanding has allowed for meticulous and unprecedented control over all steps of the winemaking process. Where once musts were left to ferment with the natural yeasts present on the grape berries and on the cellar equipment, it is now possible, and in fact, common for vintners to add copious amounts of selected and mass produced dried strains of *Saccharomyces cerevisiae* to their crushed berries to ensure a successful and even fermentation (Boulton *et al.*, 1999). This is in part due to the fact that *S. cerevisiae* is the yeast primarily responsible for alcoholic fermentation but it is not the only yeast species found on grapes. In fact, at the time of harvest, *S. cerevisiae* typically accounts for 1% or less of the total yeast population found on healthy, undamaged grape berries (Barata *et al.*, 2012). Originally, the industrial strains of *S. cerevisiae* were selected solely on their ability to reliably perform alcohol fermentation without becoming stuck. Later, research was able to provide strains that could produce specific aromas or were adapted to certain cultivars or winemaking styles. This new-found ability to provide reliable and consistent product at a seemingly low cost has, in some ways, given birth to a whole new wine market. Though the benefits of this cannot be denied there is fear that the industry may become too homogenized. Research, however, remains largely focused on strains of different *Saccharomyces* species and has shown that when *S. cerevisiae* is inoculated at high levels at the beginning of fermentation it rapidly out-competes the native yeasts (Jackson, 2014). While this might mitigate the potential for contamination and stuck fermentation, studies have shown that the complexity of wine aroma and flavor profile and even the mouthfeel can suffer significantly (Lambrechts & Pretorius, 2000). Until only a few decades ago, non-*Saccharomyces* yeasts were considered detrimental (or at the very least inconsequential) to the winemaking process (Dubourdieu *et al.*, 2006; Snowdon *et al.*, 2006; Bartowsky & Pretorius, 2009). There are currently over twenty different yeast genera thought to be associated with grapes and wine (Kurtzman & Fell, 2011). With this abundance of biodiversity that has clearly always been present in this process, it stands to reason that there is significant oenological potential where the use of these yeasts is concerned. These yeasts, or a lack there of, could indeed account for the loss of sensorial complexity observed in wines where *S. cerevisiae* completely dominates the fermentation.

Studies have shown that the presence of certain non-*Saccharomyces* yeasts during fermentation can, under the right circumstances, contribute significantly to positive wine aroma (Herraiz *et al.*, 1990; Lema *et al.*, 1996; Pastor *et al.*, 1996; Rementeria *et al.*, 2003; Romano *et*

al., 2003; Comitini *et al.*, 2011). It has since been theorized that the presence and activity of native yeasts during fermentation may in fact be beneficial to wine production. Research has been undertaken to establish the behaviors and profiles of these yeasts in an effort to understand their role in contributing to flavor and aroma production.

To date this research has largely centered around the origin of the yeasts on the grape berry, their ethanol, acetate, and glycerol production as well as their ability to produce extracellular enzymes early in fermentation. This is of particular interest because extracellular enzymes have the capacity to liberate otherwise bound compounds that can contribute to the distinct varietal characteristics of wines. Some work has also investigated the production of major secondary metabolites, such as esters and higher alcohols, and their role in conferring organoleptic attributes in the wine (Charoenchai *et al.*, 1997; Ciani & Maccarelli, 1998; Manzanares Rojas, Genoves & Valles, 2000; Andorrà *et al.*, 2012; Azzolini *et al.*, 2015). Several reviews have been written on each of these subjects (Esteve-Zarzoso *et al.*, 1998; Jolly *et al.*, 2006, 2014; Cordero-Bueso *et al.*, 2012). While these are all highly useful in helping to piece together the larger picture of how these yeasts affect wine, there is still much that needs to be understood. Indeed, the majority of the studies that have been published thus far have taken a very targeted approach, focusing on a very narrow aspect of the subject and relatively few yeast species. A more holistic approach, looking at the metabolic interactions taking place between the yeasts and the grapes, the yeasts and the must, and between the yeasts themselves has yet to be fully realized. Likewise, there are entire genera of yeast whose potential role in winemaking has not yet been investigated.

In recent years, the advances in analytical chemistry techniques have allowed for a much higher resolution analysis of wine. Analytical chemists can employ a wide range of techniques in order to better understand its complex nature. To date, liquid and gas chromatography have been the primary means of separating individual compounds in mixed matrices. The identification and quantification of these compounds is typically achieved by coupling these systems to different detectors like mass spectrometers, photodiode array detectors, or ultraviolet–visible spectrometers. With regards to the analysis of wine and alcoholic beverage aroma, the most commonly employed sampling technique is Solid-Phase Microextraction (SPME) (Ebeler, 2001). Typically, this has been coupled to Gas Chromatography (GC) for separation of the compounds prior to detection. Mass spectrometry (MS) is the most common detection method used and can give a broad picture of the chemical make-up of a substance. Recent advances in MS have allowed for highly accurate targeting of analytes which can aid in quantitative work. In the last ten years, another separation technique known as GCxGC-Time of Flight Mass Spectrometry (GCxGC-TOF-MS) has proven to greatly increase the separation power and thus the detection of analytes. While still a fairly new technology, it is gaining popularity, particularly in the field of

metabolomics (Dunn & Ellis, 2005; Almstetter *et al.*, 2012). It has also shown promise in wine research, for example Vestner *et al.* (2011) successfully used it to profile Pinotage wines while Weldegergis *et al.* (2011) were able to tentatively identify over 200 compounds in different South African red wines. This would not have been possible with one dimensional GC due to the fact that many of the compounds co-elute on the primary, non-polar column, and are only able to be separated on the more polar secondary. The separation and identification potential of GCxGC-TOF-MS could be highly useful in helping to provide a better, more comprehensive, understanding of non-*Saccharomyces* yeast metabolism in wine.

1.2 Project Aims

This project is part of the Fruit Plants Genomics and Molecular Physiology (GMPF) international PhD Programme based in San Michele all'Adige, Trentino, Italy. Selected GMPF candidates carry out research projects involving at least two collaborative institutions. The project entitled "Mass spectrometry based metabolomics of non-conventional yeasts and its application in oenology" was written in collaboration with the University of Stellenbosch's Institute for Wine Biotechnology as well as the ITN EU project "Cornucopia" whose mission is to explore yeast biodiversity.

The primary objective of this project was to use metabolomics based methodologies to study non-*Saccharomyces* yeasts in the context of their potential contribution to wine aroma. The majority of yeasts associated with wine have so far remained largely unexplored both in fundamental studies and for possible commercialization with a few exceptions. As such, this project focused on two yeast groups: those that were already commercially available, and 'novel', relatively unknown species. *Torulaspora delbrueckii* (Biodiva®, Lallemand Inc., Quebec, Canada), *Metschnikowia pulcherrima* (Flavia®, Lallemand), *Pichia kluyveri* (Viniflora® FROOTZEN™, Chr. Hansen, Horsholm, Denmark), and *Lachancea thermotolerans* (Viniflora® CONCERTO™, Chr. Hansen), are all available commercially for use in wine production but have not been thoroughly metabolically characterized. *Candida zemplinina* (*Starmerella bacillaris*), *Zygosaccharomyces kombuchaensis, Kazachstania gamospora,* and *Kazachstania aerobia* were also investigated after preliminary, unpublished, data from the Cornucopia project indicated they may be promising in wine production. The study used a comprehensive, integrated, top-down approach that combined targeted and untargeted analytical chemistry methods with sensory analysis. The approach allowed for the metabolic and sensorial aspects of both red and white wine fermented with the above-mentioned yeast to be assessed. The specific research goals are outlined below:

1. Understand the current state of research with regards to:
    a. The role of non-*Saccharomyces* yeasts in wine,
    b. The field of metabolomics, specifically as it applies to the profiling of yeast metabolomes in complex matrices such as wine;
2. Characterize the behavior of select non-*Saccharomyces* yeasts in both red and white grape must using a targeted chemical analysis method;
3. Develop and apply an untargeted analytical method to consistently and accurately characterize the whole volatile profile of finished wine;
4. Perform untargeted GCxGC-TOF-MS and sensory analysis on wine to establish a more complete volatile metabolic profile of the selected yeast in wine;
5. Compare chemical and sensory analysis to evaluate whether differences in the chemical profile translate, or not, to differences in a sensory profile; and
6. Compare chemical analysis of both a white and red wine fermented with the same yeasts to establish how matrix differences can affect yeast metabolic output.

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Chapter 2

Literature review

Metabolomics of non-*Saccharomyces* yeasts in wine: A review

2.1 Introduction

Grapes were first fermented into wine, probably by accident, more than 7000 years ago in Mesopotamia (Chambers & Pretorius, 2010). Upon realizing that the juice of harvested grapes would transform into a pleasurable drink that did not spoil easily the practice of winemaking was born. Over the centuries the process of growing and harvesting grapes for wine production was refined. Advancements in technology made the process easier but the forces behind the transformation of juice into wine remained cloaked in mystery. This was the case until advancements in microbiology in the 19th century made it clear that yeasts were responsible for the conversion of sugars into ethanol. Further study quickly revealed that *Saccharomyces cerevisiae* was the yeast primarily responsible for alcoholic fermentation but it was not the only yeast species found on grapes and in the winery environment. In fact, at the time of harvest, *S. cerevisiae* typically accounts for 1% or less of the total yeast population found on healthy, undamaged grape berries (Martini, 1993). According to *The Yeasts, A Taxonomic Study* the following 15 genera of yeasts are known to be associated with wine: *Brettanomyces, Dekkera*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Torulaspora, Hanseniaspora, Kloeckera*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Saccharomycodes*, *Schizosaccharomyces*, and *Zygosaccharomyces* (Kurtzman & Fell, 2011). Since its publication further research has added the following genera to the list of 'grape/wine yeast': *Issatchenkia, Aureobasidium, Saccharomycopsis, Belleromyces, Sporidiobolus, Sporobolomyces,* and *Trichosporon* (Barata, Malfeito-Ferreira & Loureiro, 2012; Bezerra-Bussoli, Baffi, Gomes & Da-Silva, 2013; Duarte, Pimentel, Teixeira & Fonseca, 2012; Jolly, Varela & Pretorius, 2014; Ženišová *et al.*, 2014). In the past, species from many of these genera were considered spoilage organisms or simply inconsequential in the winemaking process due to their lack of fermentative capability (Chatonnet, Dubourdieu, Boidron & Pons, 1992; Dias *et al.*, 2003; Heresztyn, 1986; Loureiro, 2003; Moreira *et al.*, 2002). For these reasons many winemakers choose to overcome their presence by treating grape must with SO_2 and inoculating copious amounts of selected *S. cerevisiae* strains which very quickly outcompete native yeasts (Jackson, 2014). This practice is nearly ubiquitous in the wine industry because it can help ensure consistent product year to year which has a marked impact on both productivity and profitability (Boulton, Singleton, Bisson & Kunkee, 1999). Some winemakers however, will allow grapes to ferment with the naturally present microbes in pursuit of a more complex and unique product. Recent studies have confirmed this idea showing that the complexity of wine aroma, flavor and mouth feel can be altered significantly, often positively, when non-*Saccharomyces* yeasts are allowed to grow in grape must (Soden *et al.*, 2000; Varela *et al.,* 2009). This isn't wholly surprising given the amount of natural diversity on the grapes and in the wine environment. It's not a huge leap to reason that there could be significant oenological potential within these species. After all, historically speaking, they have been responsible for the majority of wine both produced and consumed throughout human history.

Though some research has been done on the role of non-*Saccharomyces* yeast in wine, it has largely centered around the origin of the yeast on the grape berry, the enzymatic production of these yeasts early in fermentation and some work investigating major secondary metabolites and their role in conferring organoleptic attributes. Reviews have been written summarizing each of these subjects (Jolly, Augustyn & Pretorius, 2006; Jolly *et al*., 2014; Lambrechts & Pretorius, 2000; Steensels & Verstrepen, 2014). While this research has been critical in helping to piece together the larger picture of how these yeasts affect wine, the picture is still incomplete. Most notably little is still understood about the metabolic interactions taking place between these yeasts and the grape must, the yeasts and the bacteria present, and between the yeasts themselves.

To date over 1300 chemical compounds have been identified in wine, most of which are present as a result of the yeast metabolism during fermentation (Ebeler, 2001; Hagman, Säll, Compagno & Piskur, 2013; Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008; Rapp, 1998; Zelle *et al*., 2008). In recent years, advances in both separation and detection technology have allowed for much more in-depth analyses of both wine and species metabolisms. The study of species metabolism or the metabolites within a cell, an organism or its tissues is collectively known as metabolomics. It is a field that is uniquely suited to aid in the search for compounds produced by different yeasts that can play in wine flavor and aroma. The following review summarizes the field of metabolomics as it currently applies to wine and yeast research. It also examines the current knowledge surrounding the yeast species used in the subsequent chapters. Specifically, the impact of recent advances in analytical chemistry on research related to aroma and flavor compound production of non-*Saccharomyces* yeasts during wine fermentation are discussed.

2.2 Wine Metabolomics

2.2.1 Introduction

Metabolomics is commonly defined as the “Non-biased identification and quantification of all metabolites in a biological system” (Dunn & Ellis, 2005). Ultimately, however, the main goal of any metabolic analysis is to attempt to characterize and understand the implications of the unique chemical profile or fingerprint of a given system. As such, metabolomic studies are typically as complex as the systems they study and often bring together multi-disciplinary teams comprised of individuals knowledgeable in microbiology, analytical chemistry, statistics and chemometrics, just to name a few. It is a structure that is being used to study a wide range of biological systems with various sample types, from human cancers, to plants and food systems (Cevallos-Cevallos, Etxeberria, Danyluk & Rodrick, 2009; Lyan, Migne, Bouveresse, Paris & Rutledge, 2015; Patel & Ahmed, 2015). Table 2.1 details some of the most common food systems studied using metabolomic techniques, including wine.

The mysteries surrounding the seemingly boundless organoleptic variety found in wine arise primarily from a set of complex interactions constantly taking place between thousands of chemical compounds (Gamero, Ferreira, Pretorius & Querol, 2014; Styger, Prior & Bauer, 2011). The presence or generation of these compounds can be affected by genetic or environmental factors affecting both the grapes and the microorganisms involved in the winemaking process (Dubourdieu, Tominaga, Masneuf, Peyrot des Gachons & Murat, 2006; Rossouw, Naes & Bauer, 2008; Tominaga, Furrer, Henry & Dubourdieu, 1998a). The system as a whole is exceedingly complex and thus requires a variety of research approaches. It shouldn't come as a surprise then that in the field of wine research, metabolomics can and has played a critical role in helping to identify the metabolic profile of both grape varieties and yeast species or strains (Antalick, Perello & de Revel, 2014; Ferreira *et al.*, 2014; Jose *et al.*, 2014; Mateo & Jiménez, 2000; Richter, Kennedy, Guo & Dokoozlian, 2015; Saerens *et al*., 2008; Spraul, 2013).

Metabolomic studies are generally either untargeted, describing as many analytes as possible, or targeted, identifying and quantitating a specific group of analytes. An untargeted analysis is generally attempting to create a fingerprint or identify a unique pattern of a given sample or treatment. In doing so, thousands of chemicals can be identified and thus are not typically quantified. In contrast, targeted analyses rely more heavily on specialized extraction techniques to aid in the quantitation and identification of certain groups of analytes. In doing so researchers are better able to establish a profile of the organism or treatment. In either case, targeted or untargeted, huge data sets are typically generated from metabolomic studies that require a considerable amount of multivariate statistical analysis, for which many reviews have been written (Gromski *et al*., 2015; Kemsley *et al*., 2007; van der Werf, Jellema & Hankemeier, 2005).

In addition to targeted or untargeted, metabolomic studies can be either informative and/or predictive. The former are generally targeted, quantitative, and discriminative works aimed at differentiating between populations or treatments; while in the latter, either targeted or untargeted data is used to create statistical models based on metabolic profiles or fingerprints. All of these study types are regularly employed in wine metabolomics (Ghanem *et al*., 2015; Mendes Ferreira, Climaco & Mendes Faia, 2001; Vestner *et al*., 2011). A detailed review of "Wine science in the metabolomics era" has recently been published which details many of these techniques (Alañón, Pérez-Coello, & Marina, 2015). A review has also been written which covers metabolomic analysis in food science including a broad discussion on many techniques and applications. Many different food systems are discussed including wine but it is not the main focus of the review. For full details see Cevallos-Cevallos *et al.*, (2009). The following section describes, in detail, the analytical methods that can be used in wine and yeast metabolomics.

Table 2.1 The most common metabolomics processes in food analysis (Cevallos-Cevallos *et al.*, 2009).

Table 1. Most common metabolomics processes in food analysis.					
Sample: Purpose of analysis	Type	Extraction and preparation	Separation–detection	Data treatment	Reference
Apples: light induced changes in peel	Untargeted/discriminative	MeOH Derivatization for GC–MS	GC–MS, LC–MS	PCA	Rudell et al., 2008
Berries: polyphenol composition	Targeted/informative	Acetic acid + water C18 and Sephadex LH 20 columns	LC–MS, DIMS	Compound identification	McDougall et al., 2008
Broccoli, mustard, and brassica: glucosinolates composition	Targeted/informative	Hot water (90 °C) + sonication	LC–MS^n	Compound identification	Rochfort et al., 2008
Broccoli: variety differentiation	Untargeted/discriminative	Freeze dried MeOH + H_2O	LC–UV–MS, DIMS	PCA, ANOVA	Luthria et al., 2008
Cheese: Production control	Untargeted/informative	–	IMS	Compound identification	Vautz et al., 2006
E. coli: glycolisis metabolites	Targeted/informative	Indirect thermal treatment	LC–MS	Compound identification	Schaub & Reuss, 2008
Ginseng: variety differentiation	Untargeted/discriminative	Deuterated MeOH + buffered water	NMR	PCA	Kang et al., 2008
Green: tea quality	Untargeted/predictive	Freeze dried MeOH + H_2O + $CHCl_3$	UPLC–TOF–MS	PCA, PLS	Pongsuwan et al., 2008
Honey: origin verification	Untargeted/discriminative/ predictive	Buffered water	NMR	PLS–GP	Donarski et al., 2008
Maize: GMO identification	Untargeted/discriminative	MeOH + water + ultrasonication	CE–TOF–MS	Student's t, PCA	Levandi et al., 2008
Meat: quality/safety	Untargeted/discriminative	Neutral desorption	EESI–MS	PCA	Chen et al., 2007
Olive oil: origin differentiation	Targeted/discriminative	SPME	GC–CI–MS	LDA Kruskal–Wallis, Wald–Wolfowitz tests	Cavaliere et al., 2007
Pine mushrooms: quality differentiation	Untargeted/discriminative	MeOH + H_2O + $CHCl_3$	NMR	PCA	Cho et al., 2007
Potato: GM differentiation	Untargeted/discriminative	MeOH + H_2O + $CHCl_3$, Derivatization for GC–MS, DIMS	GC–MS	PCA	Catchpole et al., 2005
Potato: identification of cultivars	Untargeted/discriminative/ informative	Freeze dried + MeOH + water + chloroform + derivatization	GC–TOF–MS	ANOVA, PCA	Dobson et al., 2008
Potato: variety differentiation	Untargeted/discriminative/ informative	MeOH + H_2O + $CHCl_3$ Derivatization for GC–MS, DIMS	GC–MS	RF	Beckmann et al., 2007
Soybean: GMO differentiation	Untargeted/informative	MeOH–EtOH–H_2O	CE–TOF–MS	Compound identification	Garcia-Villalba et al., 2008
Spinach: E. coli contamination	Untargeted/discriminative	Neutral desorption	EESI–MS	PCA	Chen et al., 2007
Tomato paste: changes during production	Targeted to antioxidants/informative	Targeted: H_2O–MeOH and MeOH–$CHCl_3$	LC–antioxidant detector	ANOVA, PCA	Capanoglu et al., 2008
	Untargeted/informative	Untargeted: Formic acid–MeOH–H_2O	LC–TOF–MS		
Tomato: metabolite correlations	Untargeted/predictive	Volatiles: EDTA–NaOH–H_2O + SPME	GC–MS	PCA, LDA, CN	Ursem et al., 2008
		Sugars and organic acids: MeOH + derivatization			
Tomato: variety differentiation	Untargeted/discriminative	Lyophilization + MeOH + sonication	LC–TOF–MS, NMR	PCA	Moco et al., 2008
Tomato: volatiles analysis	Targeted/discriminative	EDTA–NaOH–H_2O + SPME	GC–MS	PCA, HCA	Tikunov et al., 2005
Watermelon: quality evaluation	Untargeted/predictive	Buffered D_2O	NMR	PLS–LDA	Tarachiwin et al., 2008
Wine: metabolite characterization	Untargeted/discriminative	Lyophilized + buffered D_2O	NMR	PCA, PLS	Son et al., 2008
Yeast: aroma compounds production	Targeted/discriminative	Diethyl ether	GC–FID	PCA, PLS	Rossouw et al., 2008
Yeast: strain differentiation	Untargeted/discriminative	Lyophilization + derivatization	GC–TOF–MS	PCA, HCA	MacKenzie et al., 2008
Yeast: strain differentiation	Untargeted/discriminative	–	NIR	PCA, LDA	Cozzolino et al., 2006

2.2.2 Sample preparation/extraction

Before any type of analysis can take place, samples must be prepared in accordance with the type of analysis being conducted. Wine is a complex matrix created by a thriving ecosystem. Each part of this system can be analyzed to reveal how the system as a whole functions. Typically the yeast and bacterial species present in the wine are isolated and treated much the same way that other biological samples are. Namely, the most important consideration for the purpose of metabolomics is quenching. Cessation of all enzyme and metabolic activity within living tissue is of paramount importance. For yeast and bacterial samples, this can be done in one of two ways. The first is a methanol buffer solution. Dry ice and ethanol are used to maintain a constant temperature of either -40°C or -50°C while the cells are washed in a methanol buffer solution. Metabolites are then extracted in a solution of boiling absolute ethanol containing buffer at pH 7.5 (Castrillo, Hayes, Mohammed, Gaskell & Oliver, 2003; Gonzalez & Franc, 1997). The second method uses liquid nitrogen as a cooling agent which has the advantage of staunching cellular processes more quickly than dry ice/ethanol. Both methods ensure that metabolism is halted more or less immediately and metabolites are maintained at a concentration that is detectable after analyte separation. Fully fermented wine on the other hand, by comparison, typically needs little preparation prior to analysis and the details of various wine extraction methods are discussed below.

Solid phase microextraction (SPME) is an extraction method widely used in the analysis of volatile aroma compounds (Ebeler, 2001). Until its invention wine aroma was typically extracted by liquid-liquid, solid-liquid or solid phase extraction. Each of these techniques has their drawbacks, namely they are time consuming, require large amounts of various solvents as well as sample material, and can selectively extract certain compounds over others depending on the selectivity of the solvents used. Where wine is concerned they often also have trouble capturing low boiling point compounds and are thus combined with other extraction techniques such as dynamic headspace extraction (Mamede & Pastore, 2006). In spite of this, these techniques are still very useful for certain applications. They are uniquely suited to extract higher boiling point or molecular weight compounds such as volatile phenols and pesticides (Ghiselli, Nardini, Baldi & Scaccini, 1998). The *Quick, Easy, Cheap, Effective, Rugged and Safe* method developed by Anastassiades *et al.* (2003) combines liquid-liquid and solid extraction specifically for pesticide analysis.

As extraction technology has progressed SPME has become the common method by which wine aromas are assessed. Compared to its predecessors it is a low waste, highly sensitive, reproducible process that is easy to automate. This last point is critical for its ability to limit introduced human error. The process works by allowing a small fiber coated with a polymer, a sorbent or a combination of both, to come into contact with either the gas or liquid phase of a sample. The fiber is put in place of a needle in an autosampler which can insert the fiber into a prepared vial of wine. Vials usually contain NaCl to increase the concentration of volatiles in the headspace (Prosen & Zupančič-Kralj, 1999). The fiber can either remain in contact with the sample headspace or reach to the liquid phase for a given amount of time before being moved to a GC inlet where it is rapidly heated to release the collected volatiles onto a GC column for separation. An example of this is illustrated in Fig. 2.1. Various types of fibers are made with coatings that have a wide range of polarity allowing for selection of different analytes from a sample. This combined with the fact that samples can remain largely unadulterated by heat or solvents make it an ideal candidate for wine volatile analysis. A much more extensive review on SPME extraction techniques has been written by Jeleń *et al*. (2012) and Souza-Silva *et al.* (2015). As mentioned previously the SPME process is easily automated because it is tied directly to an autosampler. SPME does have its drawbacks however. For example, extractions of a sample can only be analyzed once. This means that more sample and different types of extraction techniques are required if a wine is to be analyzed with several different methods as is common in larger metabolomics profiling studies. This has the potential to introduce bias and make data difficult to correlate. However, once it is generated this big picture data can be used to target specific compounds and their possible influences on both sensory and quality characteristics of the wine can be assessed.

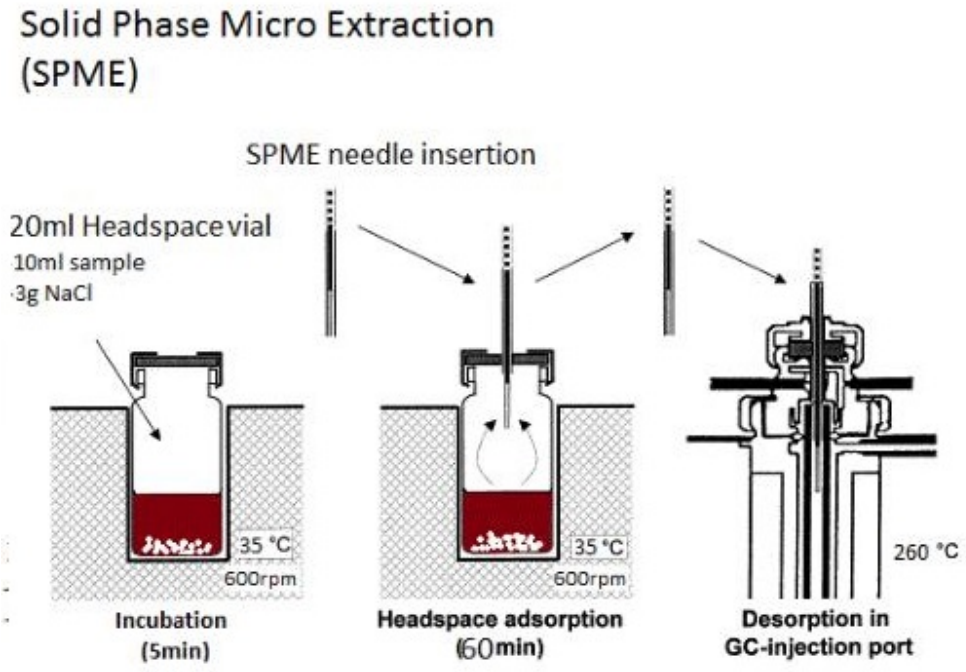

Figure 2.1 Example of solid phase microextraction method. Image was taken from Sporkert & Pragst, 2000 and adapted to fit the needs of this review.

2.2.3 Analyte separation and detection

Once samples have been prepared, they can be subjected to a number of different techniques depending on the desired outcome. These include: Nuclear Magnetic Resonance (NMR) spectroscopy, Gas Chromatography (GC), High-Performance Liquid Chromatography (HPLC), Ultra Performance Liquid Chromatography (UPLC), or Capillary Electrophoresis (CE). The chromatography separation techniques are often coupled to one of the following detectors: Mass Spectrometry (MS), Flame Ionization Detector (FID), Refractive Index Detector (RI), or Diode Array Detector (DAD). For wine metabolomics, NMR spectroscopy is the most commonly used technique followed closely by GC-MS and LC-MS (Alañón *et al*., 2015; Son *et al*., 2008, 2009). Two-Dimensional Gas Chromatography-Time of Flight Mass Spectrometry (GCxGC-TOF-MS) has also recently come into normal use for metabolomics in general, including wine research.

Gas Chromatography (GC) and multidimensional GC (GCxGC) have been reviewed extensively in terms of their applications in food science (Lehotay & Hajšlová, 2002; Pažitná, Jánošková & Špánik, 2013). The use of this, NMR and LC-MS specifically in wine analysis is also discussed at length by Alañón *et al*. (2015). To briefly summarize, however, NMR is highly prized for its

rapid and non-destructive capabilities in analysis. It also requires minimal sample prep; typically not requiring extractions or other pretreatments. NMR is generally non-selective and is used when a broad picture of the sample is desired. Compared to other techniques, however, it has relatively low sensitivity.

One way sensitivity is increased in analytical methods, is to separate compounds prior to detection. Different chromatographic techniques are used depending on the sample type and the type of data sought. LC comes in many forms and can couple to many different types of detectors. As the name suggests analytes are separated using a liquid mobile phase to carry the sample through a chromatographic column. For this to work the sample has to be soluble in the chosen mobile phase which is typically a water-solvent combination. LC is often chosen to separate samples that would otherwise degrade or be inextricably altered when heated as is the case for GC. For a more thorough review and description of LC-MS based metabolomics see Zhou *et al.* (2012)

Since its commercial introduction over fifty years ago, GC has become one of the most commonly used separation methods of wine and food volatiles. Advancements in capillary technology have led to much better separation capacity which in turn has aided in the identification of new compounds in wine. 1-Dimensional GC is limited however in its capacity to completely separate compounds in complex matrices due largely to overlap of similar compounds. This could be one reason why so few compounds were thought to be associated with wine aroma. Hardy & Ramshaw (1970) for example were only able to identify 45 compounds in a Riesling wine. Even as chromatographic techniques improved over the years the number of compounds thought to be associated with wine aroma grew slowly. Rapp & Mandery's review in 1986 and Stashenko *et al.* (1992) only show-cased a few more identified compounds, bringing the total to less than 100.

The continued advancements in separation technology and the invention of GCxGC separation helped to change this. As can be seen in Robinson *et al*. (2011) using a GCxGC-TOF-MS enabled them to identify over 360 compounds from the volatile headspace of Cabernet Sauvignon wines from Western Australia (Table 2.2 at the end of the chapter). This was possible as the second GC column allows for a secondary phase of separation to take place prior to analyte detection. An example of this is seen in Figure 2.2 taken from Welke *et al.* (2012). The figure illustrates how different column combinations can change how compounds

are separated depending on the polarity of the first and second column. Regardless of the columns used, before entering the second column compounds are modulated by hot and cold jets that trap and release the compounds as they leave the primary column. This is necessary to help maintain separation as secondary columns are much shorter than the primary. Knowing the timing of the pulses also allows the computer running the system to back calculate the retention time of the first column since analytes are only detected after they have passed through both. It is easy to see how, in general, GCxGC offers higher resolution, sensitivity and peak capacity compared to 1D-GC. This technique has proven most helpful in determining broad profiles of wine. It has been used to identify more than 300 different previously uncharacterized compounds in Brazilian Merlot, South African Pinotage, as well as Australian Cabernet Sauvignon as already mentioned (Vestner *et al.*, 2011; Weldegergis *et al.*, 2011a, b; Welke & Alcaraz Zini, 2011; Welke *et al.*, 2012; Naudé & Rohwer, 2013). It has also been used to identify twelve volatile compounds that can be used to differentiate between and thus classify Cabernet Sauvignon, Merlot, Chardonnay, Sauvignon blanc and Pinot Noir varieties (Welke, Manfroi, Zanus, Lazzarotto & Alcaraz Zini, 2013). Each of these studies used Solid-Phase-Micro-Extraction (SPME) to analyze the volatile fraction of the wine before analysis. Regardless of the separation method used, the next step in the analytical process is analyte detection.

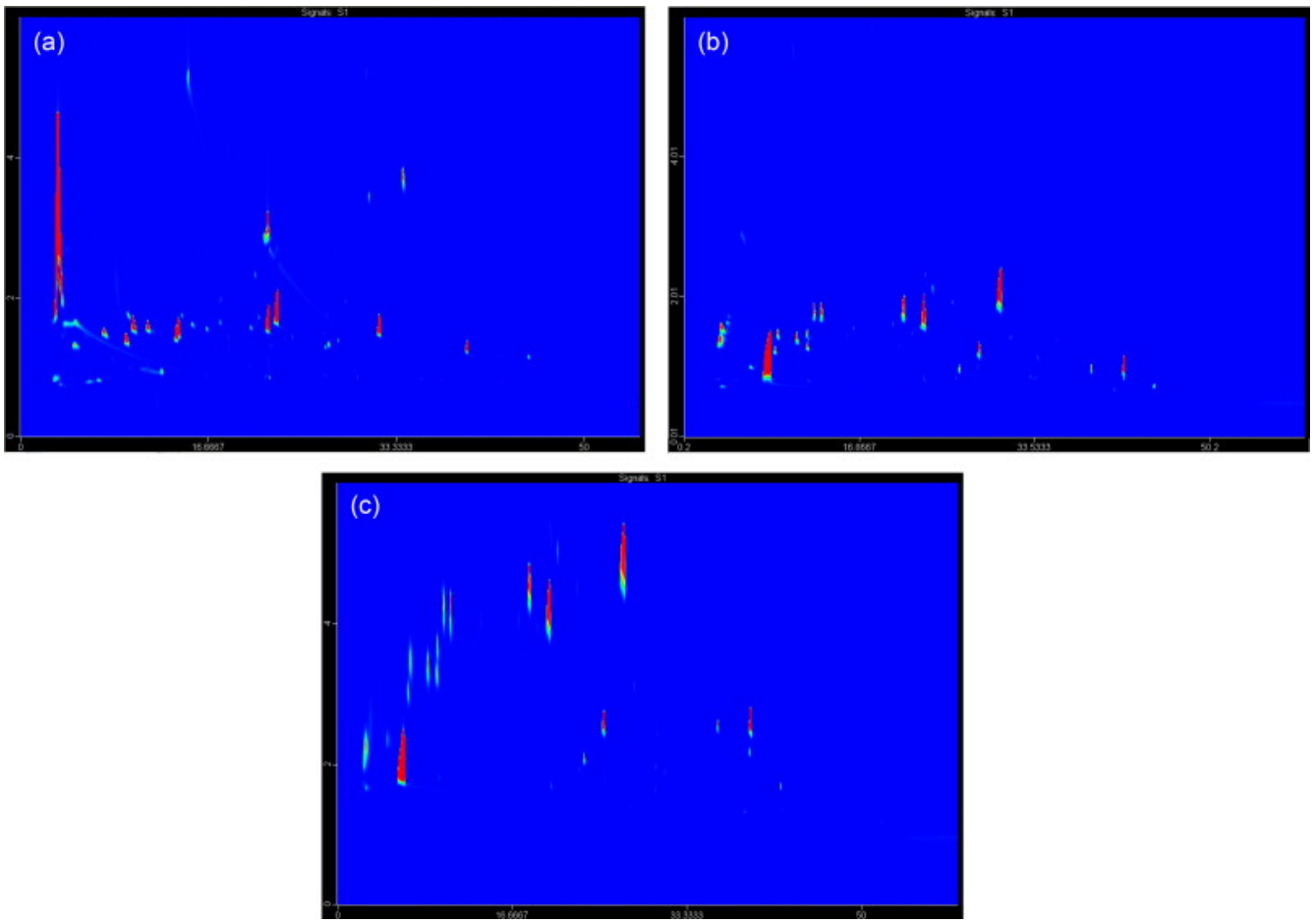

Figure 2.2: Separation of 22 volatile compounds from wine in different GC ×GC capillary column sets: (a) DB-5 × DB-WAX, (b) DB-WAX × DB1ms and (c) DB-WAX ×DB17ms (Welke et al., 2012)**.**

Of all the detection technologies to be employed mass spectrometry (MS) was the first to be developed and is still widely in use today. Advancements through the years have provided a significant leap forward in our ability to detect and identify compounds previously unknown in wine. This is largely due to increased sensitivity and speed of the instruments as well as systematic construction of libraries that aid in identification. The general pipeline of a mass spectrometer takes the separated analytes, ionizes and fractures them and then record the facture pattern. That pattern can then be compared to spectral libraries such as Wiley (~700,000 spectra) and NIST (~300,000 spectra) which allow for identification of the compounds present in a sample. The field of wine metabolomics has been an active area of research for decades and, as already stated, much has been written on the subject. This includes three thorough reviews on the use of MS and modern analytical techniques in metabolomics and wine research (Alañón *et al*., 2015; Dettmer, Aronov & Hammock, 2007; Zhang, Sun, Wang, Han & Wang, 2012). After analytes are separated and detected the final step in the experiment is the data analysis.

2.2.4 Data analysis

Once the raw data has been collected it must be converted into a standard format. Nowadays each instrument is equipped with its own software that can take the raw instrument data, filter out the noise and background, pick out individual peaks and then align the analytes across all samples (Almstetter, Appel, Dettmer, Gruber & Oefner, 2011; Castillo, Mattila, Miettinen, Orešič & Hyötyläinen, 2011). Several open source projects also exist to aid in peak picking alignment in cases where proprietary software may be unavailable (Koek *et al*., 2011; Lommen *et al.*, 2012). This is true for all separation and detection types the subject of which multiple reviews have been written (Fiehn, 2002; Katajamaa & Orešič, 2007). Once the data has been processed and aligned the peak tables and spectral chromatograms can be exported for further statistical analysis. In untargeted or discriminate work multivariate data analysis techniques such as principal component analysis or clustering are commonly used to differentiate between populations which in turn can aid in the evaluation of possible metabolic pathways. When data sets are extremely large however, these techniques can be difficult to interpret as the graphs generated are large and messy. Often other forms of statistical analysis are required to answer specific research questions. Several reviews have been written on the subject of metabolomics and ‘big’ data analysis covering the most common techniques used for doing multiple comparison work and data plotting (Franceschi, Giordan, & Wehrens, 2013; Liland, 2011). One of the major concerns, especially in untargeted work is controlling the level of false discovery

rate. Several methods have been written that can limit within a certain percentage the level of false positive results from a given data set (Strimmer, 2008a, b). The use and cutoff limit are at the discretion of the researcher and are often chosen based of they type of questions one hopes to answer from a study.

In targeted or informative work, quantitation and true positive identification is the goal and thus requires validated concentration curves of targeted analytes. In most cases the statistical analysis used for this type of data can follow a much more classic approach since the research questions usually revolve around known concetrations. Calibration curves are typically obtained by injecting a pure sample of the identified compound to confirm its mass spectral pattern and quantify the peaks based on their calculated area. A method of semi-quantitation is also sometimes used when pure standards cannot be obtained. An internal standard is added to the sample matrix in a known quantity and the relative quantity of a compound is expressed as the ratio between its peak areas and that of the internal standard. A review has been written by Smilde *et al.* (2010) that covers some of the more nuanced methodologies behind large data analysis.

2.3 Role of yeasts in wine aroma and flavor

2.3.1 Major flavors produced by wine yeasts: a brief overview

The chemical compound classes that contribute the most to the aroma of a wine are higher alcohols, acetate and ethyl esters, organic acids, volatile phenols, terpenes and sulfurous compounds (hydrogen sulfide, mercaptans, and volatile thiols). The synthesis of these compounds and the genes involved have been most studied in *S. cerevisiae* and while some studies have begun to investigate the presence of these compounds in fermentations partially driven by non-*Saccharomyces* yeasts they have not been nearly as well studied (Andorrà, Berradre, Mas, Esteve-Zarzoso, & Guillamón, 2012; Benito, Calderón, Palomero & Benito, 2015; Benito, Morata, Palomero, González & Suárez-Lepe, 2011; Ciani, Beco, & Comitini, 2006; Comitini *et al*., 2011; Dashko *et al*., 2015; Sadoudi *et al.*, 2012; Zott *et al*., 2011; Zott, Miot-Sertier, Claisse, Lonvaud-Funel & Masneuf-Pomarede, 2008).

Higher alcohols are produced in yeast by the very well characterized Ehrlich pathway which is responsible for amino acid catabolism (Hazelwood, Daran, van Maris, Pronk & Dickinson, 2008). Amino acids are the major source of nitrogen in wine fermentations and are taken up by the yeast sequentially throughout the fermentation (Crépin, Nidelet, Sanchez, Dequin &

Camarasa, 2012). After transamination, the resulting α-keto acid cannot be used in central carbon metabolism and is decarboxylated to an aldehyde which is then either reduced or oxidized to a fusel acid or alcohol depending on gene regulation governed by cultivation conditions and the redox balance of the cell.

Acetate esters are synthesized by a condensation reaction between higher alcohols and acetyl-CoA and production of these compounds has been shown to be highly species dependent (Gamero *et al*., 2014; Rojas, Gil, Piaga & Manzanares, 2001). The genes for the synthesis of these compounds are well documented in *Saccharomyces* species but not in non-*Saccharomyces* yeasts. These reactions are important especially considering that when higher alcohols are too highly concentrated in a wine they become detrimental to wine quality by imparting strong, fusel odors.

Similarly, ethyl ester production is mediated by acyltransferases that facilitate the condensation of an alcohol and acyl-CoA. And while studies have shown that in general *Saccharomyces* species tend to produce more esters overall in comparison to other wine yeasts, levels of specific esters will vary depending on the strain (Rossouw *et al*., 2008).

Acetic acid and many of the other organic acids contribute to volatile acidity in wine. In high concentrations (0.7-1.1 g/l), it imparts a vinegar odor and flavor to wine. It is produced via acetaldehyde oxidation in the pathway responsible for converting pyruvate into acetyl-CoA. As the only source of acetyl-CoA in the cytosol it is a highly important pathway in yeast metabolism. In *S. cerevisiae,* the Ald6p, Ald5p and Ald4p enzymes are dehydrogenases primarily responsible for converting acetaldehyde to acetate (Saint-Prix, Bönquist & Dequin, 2004). Remize *et al.* in 2000 showed that when the ALD6 gene is deleted there is a reduction in the amount of acetic acid produced but the resulting redox imbalance caused an increase in glycerol, succinate and 2,3-butanediol. It has also been shown that certain strains of *Torulaspora delbrueckii* can help produce wines that are lower in acetic acid and volatile acidity but also have higher amounts of glycerol than wines fermented with standard industrial *S. cerevisiae* strains (Van Breda, Jolly & Van Wyk, 2013). This may indicate that *T. delbrueckii* is naturally deficient in one or more of the dehydrogenase genes though this has yet to be studied.

Volatile phenols, like acetic acid, have a very low sensory threshold in wine, typically described as ‘barnyard’, ‘sweat’, or ‘medicinal’ odors. However, in concentrations just below the sensory

threshold they can contribute to the overall complexity of a wine. They are the result of non-oxidative decarboxylation of hydroxycinnamic acids carried out by two different enzymes. Hydroxycinnamic acids (e.g. *trans* ferulic, *trans-p*-coumaric, and caffeic acid) are first decarbozylated by hydroxycinnamate decarboxylase to form vinylphenols such as 4-vinylguaiacol and 4-vinylphenol. Then these can be reduced by vinylphenol reductase to form ethylphenols, 4-ethylguaiacol and 4-ethylphenol to be specific (Chatonnet *et al.*, 1992). The odors are most commonly associated with wines that have been contaminated by *Brettanomyces* and/or *Dekkera* yeast species. As such these were some of the first non-*Saccharomyces* yeasts to be investigated in a wine context and generally lead to the perception that non-*Saccharomyces* yeasts were undesirable in wine (Heresztyn, 1986; Jolly *et al.*, 2014; Loureiro, 2003; Rapp, 1998). Further research identified several other non-*Saccharomyces* species that were capable of at least the first conversion step, the formation of ethylphenols. These species include: *Pichia guilliermondii*, and various species of *Hanseniaspora* and *Zygosaccharomyces* (Chatonnet *et al.*, 1992). A review of ethylphenol formation was conducted by Suárez *et al.* (2007).

Terpenes often contribute most significantly to varietal floral aromas of wine. This is due to the fact that grape must has glycosylated precursors which can be cleaved by glycosidases. The production of these has been well studied in both *S. cerevisiae* and non-*Saccharomyces* yeast species (Arévalo Villena, Úbeda Iranzo, Cordero Otero & Briones Pérez, 2005; González-Pombo *et al*., 2008). Manzanares *et al.* (2011) gives an extensive review of the species that are capable of producing the necessary enzymes to liberate bound precursors.

Sulfur compounds are present in wine through a number of pathways and relay greatly on the fermentation conditions, the yeasts that are used, the presence of sulfur in the must, and the must quality in terms of available nutrition (Landaud, Helinck & Bonnarme, 2008; Linderholm, Findleton, Kumar, Hong & Bisson, 2008; Mestres, Busto & Guasch, 2000; Moreira *et a*l., 2002; Rauhut, 2009; Spiropoulos & Bisson, 2000). In general, sulfur containing compounds have a very low odor threshold and are very commonly a source of wine fault. Hydrogen sulfide for example has a threshold of 10-80 µg/l and is usually present in wine thanks to sulfur containing amino acid catabolism. Not all sulfur compounds are undesirable however. 3-Mercaptohexan-1-ol (3MH), 3-mercaptohexyl acetate (3MHA), and methyl-4-sulfanylpentan-2-one (4MSP) are the compounds responsible for the tropical aromas in Sauvignon blanc wine. They exist as non-volatile S-cysteine conjugate precursors in the must and like terpenes, yeast are able to release

these bound compounds during fermentation (Anfang, Brajkovich & Goddard, 2009; Murat *et al.*, 2001; Swiegers *et al.*, 2009; Tominaga, Furrer, Henry & Dubourdieu, 1998b; Tominaga, Peyrot des Gachons & Dubourdieu, 1998; Zott *et al*., 2011).

2.3.2 Specific contributions of intentionally inoculated yeasts

Of the over 20 genera of non-*Saccharomyces* yeasts associated with wine, historically many of them have been considered either inconsequential or potentially detrimental to winemaking (Loureiro, 2003; Steels, James, Bond, Roberts & Stratford, 2002).

However, numerous studies have been done and reviews written on the subject of yeast biodiversity and yeast potential in vineyards and the winery environment (Barata *et al*., 2012; Barnett, Delaney, Jones & Magson, 1972; Bezerra-Bussoli *et al.*, 2013; Bisson & Joseph, 2009; Cocolin, Bisson & Mills, 2000; Combina *et al.*, 2005; Martini, 1993; Parish & Carroll, 1985; Raspor, Mikli, Polanc & Smole, 2006; Rosini, Federici & Martini, 1982; Sabate, Cano, Esteve-zarzoso & Guillamón, 2002; Zagorc *et al.*, 2001). From them we have learned, for example, that apiculate yeasts such as *Kloeckera apiculata* and *Hanseniaspora uvarum* account for between 50 and 75% of culturable yeasts found on grape berries at the time of harvest and *Kloeckera*, *Hanseniaspora* and *Candida* species predominate the early stages of fermentation. Some species however, can have a detrimental effect on the *Saccharomyces* population (Oro, Ciani & Comitini, 2014; Torija, Rozès, Poblet, Guillamón & Mas, 2001; Wang, Mas & Esteve-Zarzoso, 2015; Zagorc *et al*., 2001). In the middle stages of the fermentation, if they were present on the berries, species of *Metschnikowia* and *Pichia* begin to dominate due to their relative ethanol tolerance. They are typically found in higher levels when the must reaches 3-4% ethanol (Fleet, 1993; González-Pombo *et al.*, 2008). These yeasts are also known to be high producers of esters (Rodriguez, Lopes, Barbagelata, Barda & Caballero, 2010). *Saccharomyces* species are the only yeasts known to consistently be extremely ethanol tolerant and therefore able to ultimately dominate alcoholic fermentations. *Brettanomyces, Kluyveromyces, Schizosaccharomyces, Torulaspora,* and *Zygosaccharomyces* are associated with wine but in far fewer reported numbers (Kurtzman & Fell, 2011). With the abundance of biodiversity that has clearly always been present in the winemaking process and indications from recent research, it is clear that there could be significant oenological potential within all of the species encapsulated by the mentioned genera.

Obviously, some research has been conducted on the contributions of non-*Saccharomyces* yeasts in wine but it has largely centered around the origin of the yeast on the grape berry, the enzymatic production of these yeasts early in fermentation and some work investigating major secondary metabolites and their role in conferring organoleptic attributes. Reviews have been written on each of these subjects (Cordero-Bueso, Esteve-Zarzoso, Cabellos, Gil-Díaz & Arroyo, 2012; Esteve-Zarzoso, Manzanares, Ramón & Querol, 1998; Jolly *et al.*, 2006, 2014). These are all highly useful in helping to understand how these yeasts affect wine and wine production. With so much species diversity however there is still large gaps in knowledge and understanding that warrants further research. Specifically very little is known about what metabolic interactions take place between different yeast species, between the yeasts and the grapes, and ultimately how these interactions can affect the final wine product. As previously mentioned, recent advances in analytical chemistry have allowed for a much higher resolution analysis of wine and other food and beverage products. It is now possible to characterize, in unprecedented detail, the broad chemical profiles of entire systems. These technologies are now being used to identify previously undetected compounds produced by different yeasts; specifically ones that may play a role in wine flavor and aroma. With regards to wine this includes aroma and flavor compound production. Subsequent chapters demonstrate the use of both targeted and untargeted metabolomics analysis methods to better understand the contribution that specific yeast species can make in wine. The next section of this review covers what is known to date about the species used in those studies with regard to wine.

2.3.2.1 The *Saccharomyces* genus

Where once musts were left to ferment with the natural yeast present on the grape berries it is now possible, and in fact common for vintners to add high levels of *S. cerevisiae* to their crushed berries to ensure successful and even fermentations. While *S. cerevisiae* is the best alcohol producer of its genus there are other *Saccharomyces* species that are commonly found in fermented beverages. *S. bayanus, S. bayanus* var. *uvarum, S. pastorianus, S. paradoxus, S. uvarum*, *S. kudriazevii* and some hybrids of these species have all been associated with the winemaking process (Kurtzman & Fell, 2011; López-Malo, Querol & Guillamon, 2013). Each species is known to contribute to the overall aroma and complexity of wine differently. It is well known that wines fermented at lower temperatures retain their flavor volatiles better. *S. bayanus* var. *uvarum* and *S. kudriavzevii* hybrids are known to be the most psychotropic species of the *Saccharomyces* genus and have slightly different metabolic activity than *S.*

cerevisiae that allow them to grow much better at much lower temperatures (Arroyo-López, Orlić, Querol & Barrio, 2009). Studies have shown that these species have different carbohydrate and lipid metabolism which can directly impact the organoleptic properties of wines (López-Malo *et al.,* 2013). This is just one example of the ways in which metabolomics has enhanced our understanding of not only yeast biology but how that biology can directly impact wine flavor formation.

In an industry that relies on agricultural products whose quality is subject to the variable forces of nature, the benefits of being able to produce a reliable and consistent product cannot be denied. It is not surprising then that the wine industry has slowly adopted the practice of inoculating *S. cerevisiae* in high numbers to quickly initiate fermentation. In many ways this practice has contributed to a whole new wine market. One in which consumers can now expect to find relatively good, relatively cheap wines that taste the same, year after year. Though financially this model may make a lot of sense, there is fear of a loss of diversity due to homogenization of the world wine market. Indeed, research has shown that when *S. cerevisiae* is inoculated at high levels at the beginning of fermentation, it rapidly outcompetes the native yeasts whose presence has been shown to significantly increase the complexity of both flavor and aroma of wine (Anfang *et al.*, 2009; Medina, Boido, Dellacassa & Carrau, 2012; Molina, Swiegers, Varela, Pretorius & Agosin, 2007; Styger *et al*., 2011).

2.3.2.2 *Torulaspora delbrueckii*

T. delbrueckii has been used in winemaking for several years and is one of few non-*Saccharomyces* species commercially available for use in wine and beer production. Since 2003 wine makers have been able to purchase mixes containing *T. delbrueckii* and *S. cerevisiae* (Vinoflora® Melody and Vinoflora® Harmony from CHR HANSEN). In 2009 a mono-culture of the *T. delbrueckii* was made available by CHR HANSEN and several other yeast suppliers quickly followed suit (Hansen, 2009; Laffort, 2013; Lallemand, 2012). While it may be the most well studied and most available species of the genus, like all wine-related non-*Saccharomyces* species, much remains unknown. Of the studies that have been conducted, it has been reported that wine fermented with *T. delbrueckii* in co-culture with *S. cerevisiae* were typically characterized by low volatile acidity, higher terpenols, 2-phenylethanol and C6 compound production (Ciani & Maccarelli, 1998; Sadoudi *et al*., 2012; Van Breda *et al.*, 2013). This combined with a low production of fault causing compounds like acetoin, acetic acid, acetaldehyde and ethyl acetate has made it a good candidate for the food and beverage

industry. Azzolini *et al.* (2012) produced Amarone wines via sequential inoculation of *T. delbrueckii* and *S. cerevisiae* and a sensory panel indicated the resulting wines had more intense aromas of 'ripe red fruit' than the *S. cerevisiae* only control. A study conducted by Renault *et al.* (2009) looked specifically at strain variability within this species and while differences were apparent between strains they found a few esters that might be indicative of *T. delbrueckii's* metabolic activity. These compounds were: ethyl propanoate, ethyl isobutanoate, ethyl dihydroxycinnamate and ethyl isobutyrate. Ethyl esters are the product of fatty acids reacting with ethanol which is mediated by acyltransferases. High concentrations of fatty acids are typically not desirable in wine as many of them have strong and unpleasant odors. Esters on the other hand are known for their fruity and floral characteristics and typically make up the bulk of identifiable aromas in wine. In *S. cerevisiae* acyltransferases are encoded by the genes *EHT*1 and *EEB*1 (Rossouw *et al.*, 2008; Saerens *et al*., 2006). *T. delbrueckii* has recently been sequenced and according the Kyoto Encyclopedia of Genes and Genomes (KEGG) database the same genes for these enzymes are present in the species (Gomez-Angulo *et a*l., 2015; Kanehisa & Goto, 2000). In time future research may be able to pinpoint the metabolic pathways in *T. delbrueckii* that contribute to the production of these and other desirable compounds in wine.

2.3.2.3 *Lachancea thermotolerans* (previously *Kluyveromyces thermotolerans*)

Similar to *T. delbrueckii*, *L. thermotolerans* has a commercially available strain used in wine production. Currently only CHR Hansen is producing a pure mono-culture (Viniflora® CONCERTO™) for use in winemaking (Hansen, 2011). Various studies have investigated its potential use in winemaking with regards to acetaldehyde, lactic acid, glycerol, 2-phenylethanol, and polysaccharide production as well as β-glucosidase activity. It is well established that this strain is capable of producing lactic acid and increasing the pH of wine while reducing its volatile acidity. It has also been shown to increase glycerol and 2-phenylethanol concentrations while being a low acetaldehyde producer (Ciani *et al*., 2006; Ciani & Comitini, 2010; Comitini *et al*., 2011; Cordero-Bueso *et al*., 2012; Kapsopoulou, Mourtzini, Anthoulas & Nerantzis, 2007). Gobbi *et al.* (2013) is the most extensive study of this species in wine to date. Fermentations were carried out in Sangiovese grape must in industrial fermentation trials. They report that even in sequential inoculation, *L. thermotolerans* was the dominant species during fermentation and that these fermentations showed reduced 2-methyl-1-propanol and 3-methyl-1-butanol, higher 2-phenylethanol, reduced acetate esters but higher ethyl acetate. The ethyl acetate was below the sensory threshold, however. The wines were also noted for their higher 'spicy' and

acidic notes compared to the *S. cerevisiae* solo fermentation it was compared to. A critical review of the Gobbi and Kapsopoulou study indicates that the amount of influence that *L. thermotolerans* can exert on a given fermentation is relative to the amount of time it spends in contact with the grape must alone. The sooner *S. cerevisiae* is added the less lactic acid and glycerol will be in the final wines. Even with all this there is still much that remains unknown about this species and its potential role in enology. *L. thermotolerans* has been sequenced and so like *T. delbrueckii* continued research will be able to indicate how and why this yeast produces wine critical compounds (Souciet *et al*., 2009).

2.3.2.4 *Metschnikowia pulcherrima*

A common isolate in vineyards and from grape must, *M. pulcherrima* has long been associated with grapes and wine and early research into the potential of this strain showed that certain isolates displayed a high β-glucosidase activity (Fernández, Úbeda & Briones, 2000; González-Pombo *et al*., 2008). Clemente-Jimenez *et al.* (2004) reported that *M. pulcherrima* produced high amounts of both ethyl caprilate and 2-phenyl ethanol. Romano *et al*. in 2003 characterized 2, 3-butanediol and acetoin isomer production ratios in several non-*Saccharomyces* species including *M. pulcherrima*. Sadoudi *et al*. (2012) is, to date, the most comprehensive study of *M. pulcherrima* in co-culture with *S. cerevisiae*. They observed that fructose was consumed more slowly over the course of co-culture fermentation and that less acetic acid was produced compared to the *S. cerevisiae* mono-culture fermentations. They also noted that *M. pulcherrima* in mono-culture was a low producer of volatile acidity and this confirmed previous findings (Comitini *et al*., 2011). Unlike the other yeasts mentioned previously *M. pulcherrima* has not been fully sequenced however, with so many potential positive attributes associated with this species it is not surprising to find that a pure strain has been produced for use in wine fermentation by Lallemand (Lallemand, 2013). It is called Flavia® and is described as a species that can help increase varietal characteristics and volatile thiol content especially in white wine.

2.3.2.5 *Pichia kluyveri* and other *Pichia* species

There is a very high amount of biodiversity in the *Pichia* genus some species of which have shown promise in winemaking (Domizio *et al*., 2011). In fact *P. kluyveri* is commercially available under the name of FrootZen™. Despite this however, comparatively little research has been published on this specific species' contributions to the winemaking process. Anfang *et al*. (2009) co-fermented Sauvignon blanc with a specific *P. kluyveri* isolate from New Zealand and

showed that the resulting wines had elevated levels of 3MHA, indicating that that specific isolate was capable of releasing more favorable volatile thiols from the Sauvignon blanc must. Beyond that the majority of *Pichia* work concerning wine has been conducted with *P. membranifaciens* and *P. guilliermondii. P. membranifaciens* was characterized as a good acetate ester producer by Viana *et al.* (2008). *P. guilliermondii* has shown high hydroxycinnamate decarboxylase activity even in the presence of *S. cerevisiae* which can lead to the formation of vinylphenolic pyranoanthocyanins which are desirable, highly stable anthocyanin pigments in wine *(Benito et al*., 2011). Clearly more research on these and perhaps other species of the genus is warranted.

2.3.2.6 *Candida* species

The *Candida* genus is large and extremely diverse with over 50 different identified species several of which have been associated with winemaking (Kurtzman & Fell, 2011). The most notable of these are *C. lambica, C. cantarellii, C. pulcherrima,* and *C. zemplinina* (Comitini *et al*., 2011; Magyar & Tóth, 2011; Sipiczki, 2003; Toro & Vazquez, 2002). With a genus as large as *Candida* it is unsurprising how many of the species have been reclassified as advancements in molecular identification techniques have improved (Csoma & Sipiczki, 2008). In fact the *Candida* species most commonly associated with wine, *C. zemplinina* used to be known as *C. stellata* and has just recently been reclassified to *Starmerella bacillaris* (Duarte *et al*., 2012). Regardless of its name research on *S. bacillaris*/*C. zemplinina* in wine has indicated that it has the capacity to reduce the amount of acetic acid in a wine fermentation especially when used in conjunction with *S. cerevisiae* (Englezos *et al*., 2015; Rantsiou *et al*., 2012; Sadoudi *et al*., 2012). The Englezos *et al.* (2015) and Sadoudi *et al.* (2012) studies also investigated the terpene content in single and mixed culture fermentations of *C. zemplinina* and *S. cerevisiae*. Englezos *et al*. (2015) tested 63 different strains and found that only 5% of the isolates showed β-glucosidase activity indicating a large amount of metabolic diversity within the species. Sadoudi *et al*. (2012) found that, in monoculture, *C. zemplinina* produced more norisoprenoids and terpenols but this trend did not hold in mixed fermentation with *S. cerevisiae*. *C. zemplinina* is a fructophilic yeast and has been noted for its ability to produce lower alcohol wines when used in conjunction with *S. cerevisiae* (Englezos *et al*., 2015; Maio *et al*., 2012; Zara *et al*., 2014). It has been theorized that the alterations in the sugar consumption/ethanol production capacity of this yeast is also what leads to higher glycerol content typically seen in wines fermented with *C. zemplinina*. Clearly this strain and others of the species show promise in wine

aroma and flavor modification but even when compared to other non-*Saccharomyces* yeasts there is still much to be learned.

2.3.2.7 *Kazachstania* species

The *Kazachstania* genus as a whole is fairly new; it was first mentioned in literature in 2003 when, based on analysis of the 18S rRNA gene sequence, it was determined to be a distinct genus of the *Saccharomycetaceae* family (Kurtzman & Robnett, 2003). From this study it was determined that the genus includes some new species as well as some species formally a part of *Kluyveromyces, Arxiozyma* and *Pachytichospora*. It has since been determined that the genus as a whole is the most closely related genus to *S. cerevisiae* evolutionarily speaking (Hagman *et al*., 2013). This makes this genus of particular interest to the food and beverage industry. *K. aerobia* was first identified in 2004 from corn silage while *K. gamospora* was discovered as a species in 2007 (Imanishi, Ueda-Nishimura & Mikata, 2007; Lu, Cai, Wu, Jia & Bai, 2004). *K. gamospora* demonstrated an ability to ferment both sucrose and raffinose but not galactose. It also proved to be able to assimilate ethanol and glycerol as carbon sources as well (Imanishi *et al*., 2007). Since then only a few other articles have been published in which these species are mentioned. Nisiotou & Nychas (2008) isolated *K. hellenica* in *Botrytis*-affected fermenting grape juice from Greece. Setati *et al.* (2012) isolated *K. aerobia* from healthy undamaged grape berries in South Africa. Dashko *et al.* (2015) used *K. gamospora* in wine fermentation after lab scale experiments showed it performed well and produced a unique aroma profile. Being relatively new yeasts and so closely related to *S. cerevisiae*, the genus is worth investigating for use in fermentation applications.

2.3.2.8 *Zygosaccharomyces* species

The genus *Zygosaccharomyces* is known for its ability to spoil wine, specifically sweet and sparkling wines (Loureiro, 2003). *Z. bailii* and *Z*. *rouxii* are often the source of spoilage in acidic and shelf-stable foods as well as sweet wines due to their ability to tolerate high acid, salt and sugar conditions. *Z. kombuchaensis* is a 'newer' species that was only isolated in kombucha tea in 2001 and characterized in terms of its relationship to other members of the genus in 2002 (Kurtzman, Robnett & Basehoar-Powers, 2001; Steels *et al.*, 2002). The species displayed properties significantly different from other species of the genus and may be worth investigating in fermentation conditions. Specifically, it has been shown that *Z. kombuchaensis,* much like *Z. lentus* its close genetic neighbor, is able to grow at much lower temperatures than *Z. bailii* and

Z. rouxii. These species were also unable to grow aerobically at temperatures much above 25°C and relative sensitivity to common preservatives such as scorbic acid, benzoic acid, high glucose, and high salt concentrations. *Z. bailii* and *Z. rouxii* are not overly sensitive to these conditions and thus are often found spoiling acidic and sweet products. Furthermore, since *Z. kombuchaensis* was isolated from Kombucha tea, a slightly alcoholic beverage made by fermenting sweetened brewed tea with a mixture of bacteria and fungi, it stands to reason it may be beneficial, organoleptically, to fermented beverages.

2.4 Conclusions

Metabolomics is a multifaceted field that has emerged to study the vast complexity of biological systems. With a nearly incalculable set of factors influencing the chemical matrix that is wine, it is not surprising that the field of wine research has already benefited greatly from metabolomic study techniques. Likewise the analysis of the yeast metabolism, both in general and with specific applications in wine, has begun to take off. Once, only 100 or fewer compounds were identified in wine and the fermentation mechanisms of *S. cerevisiae* were scarcely understood. We have now catalogued more than 1300 individual chemicals and know that many of them are the direct result of the yeast metabolome. Recent exponential growth in technology has led to increased capture, separation, detection and identification of wine associated yeast species and the analytes they impart. More than twenty genera of yeast are thought to be associated with grape berries, wine, and the winery environment. Though once thought to be at best inconsequential and at worst spoilage organisms initial research has begun to shed light on their potential for enhancing wine aroma and complexity. This research however has only scratched the surface and left many more questions than there are answers:

- What factors contribute to the presence of different yeast on the grape or in the winery environment?
- For each associated yeast what organoleptic properties can they impart on wine?
    - How exactly is this accomplished both chemically and genetically?
- How, if at all, do different wine matrixes affect yeast metabolic behavior?
- How, if at all, does the presence of these yeasts in the same system affect each other?

The technologies that have been developed thus far and their general rate of advancement give us both the necessary knowledge to ask these questions and the means by which to answer them. Doing this will not only increase our understanding of how wine is made, and why it

smells and tastes the way it does, but also potentially give winemakers more adept tools to tailor those properties to meet consumer expectations and the demands of an ever changing market. The current and future analytical techniques alone are not enough however. Systems as intricate and nuanced as wine, yeast biology and in general, metabolomics require teams from a variety of fields to fully realize the potential of the vast amounts of data collected. Not only do we need to continue refining and innovating the analytical techniques but we will also need biologists, chemists, statisticians, chemometricians and bioinformaticians who are able to communicate and work together to form a more cohesive picture.

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2.6 Tables and Figures

Table 2.2 Compound names, CAS numbers, unique masses, mean mass spectral match quality, retention times, and retention indices for compounds analyzed by GC × GC-TOFMS based on MS and RI matches for five commercial Cabernet Sauvignon wines from Western Australia. Table 3 from Robinson *et al.*, 2011**.**

Peak#	Compound	CAS	Unique mass[a]	MS match	1 RT(s)	2 RT(s)	RI[b] (calc)	RI[c] (lit)
1	Isobutyl alcohol	78-83-1	74	845	348	1.703	695	650
2	1-Butanol	71-36-3	56	823	396	1.819	711	662
3	1-Penten-3-ol	616-25-1	57	846	420	1.838	720	684
4	2-Ethylfuran	3208-16-0	81	767	432	1.838	724	720
5	1-Propene, 1-(methylthio)-, (E)-	42848-06-6	73	801	432	1.939	724	726
6	2,3-Pentanedione	600-14-6	57	800	432	2.088	724	697
7	2,5-Dimethylfuran	625-86-5	96	788	444	1.881	729	728
8	Ethyl propanoate	105-37-3	102	918	456	2.034	733	726
9	Propyl acetate	109-60-4	43	917	462	2.031	735	728
10	Acetal	105-57-7	47	812	486	1.786	744	726
11	2,4,5-Trimethyl-1,3-dioxolane	3299-32-9	101	838	486	1.938	744	735
12	Acetoin	513-86-0	88	819	486	2.662	745	743
13	Ethyl isobutyrate	97-62-1	116	841	552	2.147	768	756
14	Isobutyric acid	79-31-2	73	852	567	2.815	773	775
15	Toluene	108-88-3	91	919	570	2.404	774	771
16	2-Methylthiophene	554-14-3	97	831	582	2.676	778	775
17	Isobutyl acetate	110-19-0	56	881	588	2.223	781	780
18	3-Methylthiophene	616-44-4	98	778	600	2.744	785	786
19	Diethyl carbonate	105-58-8	91	854	618	2.762	792	765
20	2,3-Butanediol	513-85-9	47	899	636	3.304	798	789
21	Butanoic acid	107-92-6	60	726	636	3.365	798	789
22	Octane[d]	111-65-9	85	735	642	1.545	800	800
23	2-Ethyl-5-methylfuran	1703-52-2	95	775	642	2.36	800	802
24	Ethyl butanoate	105-54-4	89	913	648	2.47	803	803
25	Hexanal	66-25-1	82	682	654	2.662	805	804
26	Dibromochloromethane	124-48-1	129	849	654	3.402	806	800

27	Tetrachloroethylene	127-18-4	166	888	660	2.439	807	815
28	Butyl acetate	123-86-4	61	882	684	2.491	816	813
29	Ethyl lactate	97-64-3	75	795	690	3.068	818	815
30	1,3-Octadiene	1002-33-1	54	902	708	1.979	824	827
31	Methyl ethyl disulfide	20333-39-5	108	711	744	3.147	837	846
32	Furfural	98-01-1	96	930	744	4.513	838	835
33	Ethyl crotonate	10544-63-5	69	898	768	3	847	834
34	Chlorobenzene	108-90-7	112	836	774	3.19	848	852
35	Ethyl 2-methylbutyrate	7452-79-1	102	927	780	2.493	850	848
36	Isohexanol	626-89-1	56	812	780	2.684	851	838
37	S-Methylmercaptoethanol	5271-38-5	61	834	780	4.121	851	838
38	Isovaleric acid	503-74-2	60	843	786	3.126	853	839
39	Ethyl isovalerate	108-64-5	88	890	792	2.529	855	852
40	3-Hexen-1-ol, (E)-	928-97-2	67	851	792	2.936	855	853
41	3-Hexen-1-ol, (Z)-	928-96-1	67	939	804	2.932	860	860
42	Ethylbenzene	100-41-4	91	931	810	2.859	861	866
43	2-Furanmethanol	98-00-0	98	878	810	4.047	862	866
44	2-Methylbutanoic acid	116-53-0	74	903	816	3.196	864	850
45	2-Ethylthiophene	872-55-9	97	779	822	3.129	866	871
46	m-Xylene	108-38-3	91	907	834	2.842	870	874
47	1-Hexanol	111-27-3	56	893	840	2.821	873	863
48	Isoamyl acetate	123-92-2	70	797	858	2.707	879	876
49	3,4-Dimethylthiophene	632-15-5	111	804	858	3.291	879	887
50	2-Methylbutyl acetate	624-41-9	70	810	864	2.658	880	875
51	2-Butylfuran	4466-24-4	81	710	894	2.593	892	894
52	2-Heptanone	110-43-0	58	894	894	2.96	892	889
53	o-Xylene	95-47-6	91	901	900	3.109	894	894
54	Styrene	100-42-5	104	895	900	3.38	894	897
55	Nonane[d]	111-84-2	57	897	918	1.737	900	900
56	Propyl butanoate	105-66-8	71	801	918	2.715	900	896
57	Ethyl pentanoate	539-82-2	88	906	924	2.746	903	898
58	2-Heptanol	543-49-7	45	876	936	2.601	906	901
59	Heptanal	111-71-7	86	857	936	2.911	906	900
60	2-Acetylfuran	1192-62-7	95	917	960	4.74	915	914

61	Isobutyl isobutyrate	97-85-8	71	823	966	2.442	916	906
62	Pentyl acetate	628-63-7	70	828	966	2.769	916	916
63	γ-Butyrolactone	96-48-0	86	945	978	1.42	920	915
64	Anisole	100-66-3	108	813	978	3.921	921	920
65	Methyl hexanoate	106-70-7	74	893	996	2.84	926	923
66	Cumene	98-82-8	105	798	996	2.953	925	924
67	Ethyl tiglate	5837-78-5	113	820	1038	3.207	940	939
68	Ethyl 3-hydroxybutanoate	5405-41-4	71	875	1038	3.644	940	945
69	Camphene	79-92-5	93	746	1074	2.458	951	961
70	Propyl isovalerate	557-00-6	85	835	1074	2.634	951	949
71	Propylbenzene	103-65-1	91	884	1086	3.031	955	957
72	Isobutyl butanoate	539-90-2	71	850	1092	2.632	957	955
73	Ethyl 3-methylpentanoate	5870-68-8	88	794	1098	2.717	960	960
74	m-Ethyl toluene	620-14-4	120	883	1110	3.073	964	969
75	Ethyl isohexanoate	25415-67-2	88	883	1122	2.745	967	969
76	Ethyl 2-hydroxyisovalerate	6/7/2441	104	822	1122	3.112	967	987
77	Benzaldehyde	100-52-7	106	903	1122	4.959	968	969
78	5-Methylfurfural	620-02-0	110	893	1122	5.159	968	964
79	Dehydroxylinalool oxide A	7392-19-0	139	840	1134	2.506	971	971
80	Isoamyl propanoate	105-68-0	57	880	1134	2.744	971	969
81	1-Heptanol	111-70-6	56	891	1140	2.949	973	970
82	Dimethyl trisulfide	3658-80-8	126	871	1140	4.615	973	982
83	Methyl furoate	611-13-2	95	915	1158	4.97	979	985
84	o-Ethyltoluene	611-14-3	105	877	1164	3.278	980	988
85	Octen-3-ol	3391-86-4	57	843	1170	2.845	983	986
86	α-Methylstyrene	98-83-9	118	836	1176	3.517	985	988
87	Ethyl (methylthio)acetate	4455-13-4	134	739	1182	4.313	987	990
88	Methionol	505-10-2	106	918	1182	4.733	987	982
89	3-Octanone	106-68-3	99	842	1188	3.019	988	989
90	Methyl heptenone	409-02-9	108	740	1188	3.417	988	987
91	β-Myrcene	123-35-3	93	874	1194	2.461	990	991
92	2-Amylfuran	3777-69-3	81	800	1194	2.773	991	993
93	2-Octanone	111-13-7	58	781	1200	3.099	993	990
94	2-Carene	554-61-0	121	737	1212	2.685	997	1001

95	6-Methyl-5-hepten-2-ol	1569-60-4	95	842	1212	3.022	997	993
96	Pseudocumene	95-63-6	105	933	1212	3.217	997	1000
97	Phenol	108-95-2	94	803	1212	4.474	996	979
98	2-Methylthiolan-3-one	13679-85-1	116	849	1212	5.323	997	994
99	Decane[d]	124-18-5	43	896	1224	1.899	1000	1000
100	Benzofuran	271-89-6	118	848	1224	4.486	1001	1007
101	(Z)-3-Hexenyl acetate	3681-71-8	67	814	1236	3.12	1004	1006
102	Octanal	124-13-0	84	818	1242	3.08	1006	1003
103	α-Phellandrene	99-83-2	136	682	1248	2.624	1009	1005
104	Ethyl-3-hexanoate	2396-83-0	142	879	1248	3.213	1008	1007
105	α-Thiophenecarboxaldehyde	98-03-3	111	912	1254	0.076	1009	1010
106	m-Dichlorobenzene	541-73-1	146	796	1254	3.84	1010	1022
107	Ethylfurylketone	3194-15-8	95	851	1254	4.794	1011	1008
108	1-Methyl-2-formylpyrrole	1192-58-1	109	814	1254	5.53	1011	1010
109	Isoamyl isobutyrate	1/3/2050	89	844	1266	2.655	1014	1018
110	Hexyl acetate	142-92-7	84	894	1266	2.923	1014	1007
111	Hexanoic acid	142-62-1	60	910	1266	3.442	1015	978
112	α-Terpinene	99-86-5	93	854	1278	2.671	1019	1018
113	Isocineole	470-67-7	111	828	1278	2.794	1018	1016
114	Benzyl chloride	100-44-7	91	801	1278	4.542	1019	1023
115	p-Dichlorobenzene	106-46-7	146	892	1284	3.957	1020	1015
116	(S)-3-Ethyl-4-methylpentanol	0-00-0	84	883	1296	3.017	1024	1020
117	Hemimellitene	526-73-8	105	932	1296	3.527	1024	1033
118	p-Cymene	99-87-6	134	859	1308	3.1	1027	1026
119	Limonene	5989-27-5	68	884	1320	2.67	1032	1031
120	2-Ethyl hexanol	104-76-7	57	890	1320	2.883	1032	1030
121	Eucalyptol	470-82-6	108	869	1332	2.957	1036	1033
122	(Z)-Ocimene	3338-55-4	92	847	1338	2.661	1038	1040
123	Indane	496-11-7	117	862	1338	3.929	1038	1048
124	2-Acetyl-5-methylfuran	1193-79-9	109	849	1338	5.1	1039	1042
125	2,2,6-Trimethylcyclohexanone	2408-37-9	82	883	1344	3.464	1039	1035
126	Benzyl alcohol	100-51-6	108	916	1356	5.069	1044	1041
127	Lavander lactone	1073-11-6	111	755	1356	5.691	1045	1041
128	Ocimene quintoxide	7416-35-5	139	712	1362	2.828	1046	1049

129	Ethyl 2-hexenoate	27829-72-7	99	922	1362	3.371	1046	1036
130	(E)-Ocimene	3779-61-1	93	847	1368	2.68	1047	1051
131	3-Nonen-5-one	82456-34-6	83	801	1374	3.095	1050	1051
132	Salicylaldehyde	90-02-8	122	812	1374	5.092	1051	1057
133	Phenylacetaldehyde	122-78-1	120	900	1374	5.231	1051	1050
134	m-Propyltoluene	1074-43-7	105	850	1386	3.122	1053	1052
135	Ethyl furoate	614-99-3	95	908	1392	4.819	1056	1056
136	Isoamyl butyrate	106-27-4	71	892	1398	2.806	1057	1054
137	Butylbenzene	104-51-8	91	835	1398	3.185	1058	1058
138	Ethyl 2-hydroxy-4-methylpentanoate	10348-47-7	69	914	1404	3.224	1059	1060
139	γ-Hexalactone	695-06-7	85	876	1410	0.202	1060	1063
140	γ-Terpinene	99-85-4	93	817	1410	2.855	1061	1062
141	o-Cresol	95-48-7	108	851	1434	4.491	1069	1077
142	Diethyl malonate	105-53-3	115	862	1434	4.382	1070	1069
143	Ethyl 5-methylhexanoate	10236-10-9	88	722	1440	2.899	1071	1072
144	Acetophenone	98-86-2	105	926	1440	5.269	1072	1076
145	1-Octanol	111-87-5	56	904	1452	3.032	1075	1080
146	p-Tolualdehyde	104-87-0	119	835	1452	4.992	1075	1079
147	2-Ethyl-p-xylene	1758-88-9	119	673	1458	3.32	1078	1077
148	Terpinolene	586-62-9	93	915	1488	2.982	1087	1087
149	4-Ethyl-o-xylene	934-80-5	119	856	1488	3.348	1087	1093
150	p-Cresol	106-44-5	107	869	1500	4.501	1091	1077
151	Guaiacol	90-05-1	109	896	1500	5.055	1092	1102
152	2-Nonanone	821-55-6	58	793	1506	3.153	1093	1092
153	Dehydro-p-cymene	1195-32-0	117	927	1506	3.585	1093	1091
154	Propyl hexanoate	626-77-7	99	899	1512	2.909	1095	1079
155	Ethyl heptanoate	106-30-9	88	914	1524	2.932	1098	1093
156	Methyl benzoate	93-58-3	105	901	1524	4.768	1099	1100
157	Undecane[d]	1120-21-4	57	889	1530	1.947	1099	1100
158	Isopentyl 2-methylbutanoate	27625-35-0	85	872	1530	2.703	1100	1100
159	Ethyl sorbate	2396-84-1	140	854	1530	3.825	1101	1103
160	Linalool	78-70-6	93	893	1536	3.031	1103	1106
161	Ethyl methylthiopropanoate	13327-56-5	74	913	1536	4.373	1103	1098

162	2-Nonanol	628-99-9	45	906	1542	2.803	1105	1098
163	Isopentyl isovalerate	659-70-1	85	877	1548	2.707	1107	1105
164	Nonanal	124-19-6	95	893	1548	3.12	1107	1106
165	Heptyl acetate	112-06-1	43	862	1566	2.931	1113	1115
166	(Z)-Rose oxide	16409-43-1	139	830	1566	3.074	1113	1112
167	2-Methylcumarone	4265-25-2	131	887	1566	4.449	1113	1109
168	1,3,8-p-Menthatriene	21195-59-5	134	793	1572	3.406	1115	1111
169	α-Cyclocitral	432-24-6	81	772	1596	3.605	1124	1116
170	Methyl octanoate	111-11-5	127	879	1602	3.002	1126	1129
171	2-Ethylhexanoic acid	149-57-5	88	721	1620	3.3	1132	1128
172	α-Isophoron	78-59-1	82	737	1620	4.553	1132	1118
173	(E)-Rose oxide	876-18-6	139	680	1626	3.149	1133	1127
174	Ethyl 3-hydroxyhexanoate	2305-25-1	71	786	1626	3.617	1134	1133
175	p-Menth-3-en-1-ol	586-82-3	81	691	1650	3.349	1143	1138
176	N-Isopentylacetamide	13434-12-3	72	882	1668	4.786	1149	1150
177	o-Dimethoxybenzene	91-16-7	138	818	1674	5.389	1151	1154
178	Isobutyl hexanoate	105-79-3	99	907	1680	2.798	1152	1144
179	4-Oxoisophorone	1125-21-9	68	839	1680	4.994	1153	1142
180	Prehnitene	488-23-3	119	905	1686	3.753	1155	1120
181	Camphor	464-49-3	95	762	1686	4.207	1155	1151
182	Nerol oxide	1786-08-9	83	820	1692	3.462	1156	1151
183	Pentylbenzene	538-68-1	91	783	1704	3.214	1161	1154
184	(Z)-3-Nonenol	10340-23-5	81	812	1704	3.237	1161	1160
185	γ-Heptalactone	105-21-5	85	802	1704	5.818	1162	1144
186	Menthone	89-80-5	112	756	1710	3.577	1162	1154
187	2-Methylundecane	7045-71-8	85	847	1716	1.936	1165	1165
188	3-Cyclohexene-1-carboxaldehyde, 1,3,4-trimethyl-	40702-26-9	137	752	1722	3.571	1167	1171
189	3-Ethylphenol	620-17-7	107	710	1722	4.408	1168	1184
190	Benzyl acetate	140-11-4	150	880	1728	4.877	1170	1165
191	3-Methylundecane	1002-43-3	57	849	1734	1.968	1171	1169
192	(Z)-6-Nonenol	35854-86-5	67	872	1734	3.206	1171	1172
193	Isomenthone	491-07-6	112	814	1734	3.787	1171	1165
194	m-Dimethoxybenzene	151-10-0	138	864	1740	5.095	1174	1182

195	Ocimenol	5986-38-9	93	738	1746	3.309	1175	1179
196	Ethyl benzoate	93-89-0	105	906	1746	4.527	1177	1180
197	Isobutyl methoxypyrazine	24683-00-9	124	618	1758	3.703	1180	1179
198	m-Methylacetophenone	585-74-0	119	760	1758	5.071	1180	1183
199	1-Nonanol	143-08-8	70	907	1764	2.995	1182	1173
200	(E)-Linalool oxide	14049-11-7	59	797	1764	3.755	1181	1184
201	Phenethyl formate	104-62-1	104	890	1764	4.901	1183	1178
202	Methyl benzeneacetate	101-41-7	150	838	1764	5.175	1183	1194
203	Diethyl succinate	123-25-1	74	890	1770	4.325	1184	1191
204	4-Ethyl phenol	123-07-9	107	930	1776	4.682	1186	1178
205	Terpinen-4-ol	562-74-3	71	859	1782	3.532	1189	1177
206	1-Dodecene	112-41-4	69	903	1794	2.165	1192	1193
207	Octanoic acid	124-07-2	144	844	1800	3.435	1194	1202
208	Dill ether	74410-10-9	137	751	1800	3.861	1193	1184
209	Naphthalene	91-20-3	128	855	1800	5.179	1194	1191
210	p-Methylacetophenone	122-00-9	119	793	1806	5.064	1196	1179
211	Dodecane[d]	112-40-3	57	852	1818	2.227	1201	1200
212	Methyl salicylate	119-36-8	120	913	1824	4.894	1202	1201
213	p-Creosol	93-51-6	123	862	1836	4.863	1206	1188
214	α-Terpineol	98-55-5	136	850	1842	3.603	1210	1186
215	Safranal	116-26-7	150	799	1848	4.385	1211	1196
216	Decanal	112-31-2	82	869	1854	3.083	1213	1206
217	Benzofuran, 4,7-dimethyl-	28715-26-6	145	828	1860	4.364	1217	1220
218	4,7-Dimethylbenzofuran	28715-26-6	145	829	1878	4.378	1223	1220
219	Methyl nonanoate[e]	1731-84-6	141	892	1890	3.003	1226	1229
220	Ethyl nicotinate	614-18-6	106	812	1890	5.045	1226	1218
221	p-Menth-1-en-9-al	29548-14-9	94	764	1896	3.993	1228	1217
222	β-Cyclocitral	432-25-7	137	874	1896	4.196	1229	1220
223	Citronellol	106-22-9	156	899	1908	3.288	1233	1233
224	2-Hydroxycineol	18679-48-6	108	756	1914	4.201	1236	1227
225	Benzothiazole	95-16-9	135	911	1926	0.497	1239	1244
226	6-Ethyl-o-cresol	1687-64-5	121	859	1926	4.499	1239	1236
227	Benzenepropanol	122-97-4	117	851	1926	5.121	1241	1231
228	Isothiocyanatocyclohexane	1122-82-3	141	860	1932	4.925	1243	1260

229	Ethyl phenylacetate	101-97-3	164	908	1950	4.857	1249	1247
230	Ethyl 2-octenoate	2351-90-8	125	862	1956	3.309	1250	1243
231	2-Methylbutyl hexanoate	2601-13-0	99	874	1962	2.875	1252	1247
232	Isopentyl hexanoate	2198-61-0	99	898	1962	2.875	1252	1250
233	D-Carvone	2244-16-8	82	767	1962	4.509	1253	1254
234	2-Nitro-p-cresol	119-33-5	153	781	1968	5.031	1255	1250
235	Geraniol	106-24-1	69	818	1974	3.596	1257	1255
236	Carvotanacetone	499-71-8	82	764	1974	4.286	1258	1246
237	α-Ionene	475-03-6	159	629	1986	3.32	1261	1256
238	2-Phenylethyl acetate	103-45-7	91	906	1986	4.877	1262	1256
239	γ-Octalactone	104-50-7	85	850	1992	5.575	1264	1262
240	9-Decenol	13019-22-2	68	802	2010	3.258	1270	1267
241	3,5-Dimethoxytoluene	4179-19-5	152	842	2016	4.895	1273	1276
242	Nonanoic acid	112-05-0	60	696	2028	2.336	1277	1280
243	1-Decanol	112-30-1	70	921	2028	3.067	1277	1283
244	Ethyl salicylate	118-61-6	120	858	2028	4.511	1277	1267
245	4-Ethylguaiacol	2785-89-9	137	926	2040	4.755	1281	1282
246	Diethyl glutarate	818-38-2	143	915	2046	4.164	1283	1284
247	Vitispirane	65416-59-3	192	904	2058	3.493	1287	1272
248	Phellandral	21391-98-0	109	814	2058	4.303	1287	1273
249	δ-Octalactone	698-76-0	99	866	2070	0.069	1291	1287
250	p-Ethylacetophenone	937-30-4	133	689	2070	4.963	1292	1281
251	Propyl octanoate	624-13-5	145	895	2076	2.919	1294	1290
252	2-Undecanone	112-12-9	58	885	2082	3.143	1296	1295
253	(E)-Oak lactone	39638-67-0	99	827	2082	5.011	1297	1304
254	Ethyl nonanoate	123-29-5	88	895	2088	2.931	1298	1295
255	Perilla alcohol	536-59-4	68	760	2088	4.222	1299	1295
256	Thymol	89-83-8	135	831	2088	4.332	1298	1290
257	Tridecane[d]	629-50-5	57	849	2094	2.083	1300	1300
258	p-Cymen-7-ol	536-60-7	135	850	2094	4.722	1301	1295
259	Theaspirane A	0-00-0	138	844	2106	3.283	1305	1301
260	2-Undecanol	1653-30-1	45	886	2112	2.831	1306	1303
261	p-Menth-1-en-9-ol	18479-68-0	94	797	2112	4.021	1308	1295
262	Carvacrol	499-75-2	135	855	2112	4.433	1307	1304

263	Edulan I	41678-29-9	177	768	2136	3.705	1317	1309
264	4-Hydroxy-3-methylacetophenone	876-02-8	135	839	2136	5.715	1317	1323
265	4-Vinylguaiacol	7786-61-0	150	825	2142	5.287	1319	1317
266	Theaspirane B	0-00-0	138	822	2148	3.395	1322	1319
267	Methyl decanoate	110-42-9	74	873	2160	3.004	1325	1323
268	Methyl geranate	2349-14-6	114	868	2160	3.596	1325	1326
269	(Z)-Oak lactone	55013-32-6	71	920	2166	5.35	1329	1340
270	Isobutyl octanoate	6/3/5461	127	856	2220	2.811	1348	1348
271	Citronellol acetate	150-84-5	81	752	2226	3.191	1350	1352
272	Ethyl dihydrocinnamate	2021-28-5	104	858	2232	4.632	1354	1350
273	Syringol	91-10-1	154	859	2244	0.36	1356	1362
274	Eugenol	97-53-0	164	915	2250	4.933	1360	1359
275	TDN	30364-38-6	157	807	2256	4.137	1361	1364
276	(Z)-β-Damascenone	23696-85-7	121	786	2262	4.101	1364	1367
277	γ-Nonalactone	104-61-0	85	883	2268	5.315	1368	1361
278	Dihydroeugenol	2785-87-7	137	924	2274	4.6	1369	1365
279	Hydroxy citronellol	107-74-4	59	793	2286	2.817	1373	1359
280	1-Undecanol	112-42-5	126	855	2298	3.032	1378	1367
281	(E)-α-Ionol	25312-34-9	138	770	2304	3.464	1381	1376
282	(E)-β-Damascenone	23726-93-4	121	886	2316	4.263	1385	1387
283	Biphenyl	92-52-4	154	894	2322	5.345	1388	1385
284	Ethyl decanoate	110-38-3	101	620	2325	3.225	1388	1393
285	Methyl cinnamate	103-26-4	131	796	2334	5.381	1393	1397
286	2-Phenylethyl isobutyrate	103-48-0	104	771	2346	4.419	1397	1396
287	Tetradecane[d]	629-59-4	57	869	2358	2.129	1401	1400
288	α-Cedrene	469-61-4	119	685	2391	3.762	1414	1410
289	β-Damascone	85949-43-5	177	760	2394	4.098	1415	1419
290	Dihydro-α-ionone	31499-72-6	136	699	2406	3.819	1420	1406
291	α-Ionone	127-41-3	136	687	2424	3.931	1428	1426
292	1,7-Dimethylnaphthalene	575-37-1	156	896	2436	5.087	1433	1419
293	Aromadendrene	109119-91-7	161	809	2454	3.077	1439	1443
294	2-Phenylethyl butyrate	103-52-6	104	858	2466	4.506	1445	1439
295	Isoamyl octanoate	2035-99-6	127	859	2472	2.88	1447	1450

296	Dihydropseudoionone	689-67-8	69	838	2481	3.658	1451	1457
297	β-Farnesene	18794-84-8	93	854	2490	2.906	1454	1455
298	DBQ	719-22-2	220	833	2520	3.741	1467	1472
299	γ-Decalactone	706-14-9	85	792	2532	5.134	1472	1470
300	1-Dodecanol	112-53-8	97	874	2544	3.055	1477	1483
301	Cabreuva oxide D	107602-52-8	94	868	2556	3.403	1481	1479
302	dehydro-β-Ionone	1203-08-3	175	914	2556	4.447	1483	1485
303	δ-Decenolactone	54814-64-1	97	841	2556	5.71	1482	1483
304	α-Curcumene	644-30-4	132	795	2562	3.415	1484	1485
305	β-Ionone	79-77-6	177	828	2562	4.174	1485	1486
306	Propyl decanoate	30673-60-0	61	852	2580	2.911	1491	1489
307	Ethyl undecanoate	627-90-7	88	879	2586	2.922	1494	1491
308	(Z)-β-Guaiene	88-84-6	161	737	2586	3.393	1493	1492
309	1,10-Oxidocalamenene	143785-42-6	173	925	2586	4.228	1494	1491
310	Isoamyl phenylacetate	102-19-2	70	844	2586	4.4	1494	1490
311	Phenethyl isovalerate	140-26-1	104	831	2592	4.269	1496	1490
312	δ-Decalactone	705-86-2	99	831	2598	5.55	1500	1505
313	Pentadecane[d]	629-62-9	57	884	2604	2.159	1499	1500
314	α-Amorphene	483-75-0	105	882	2610	3.335	1504	1505
315	α-Farnesene	502-61-4	189	607	2616	3.755	1506	1511
316	Butylated hydroxytoluene	128-37-0	205	873	2616	3.806	1506	1533
317	2,4-Di-tert-butylphenol	96-76-4	191	863	2622	3.938	1510	1513
318	β-Bisabolene	495-61-4	204	783	2628	3.087	1512	1509
319	α-Alaskene	28400-12-6	136	632	2628	3.886	1511	1512
320	Methyl dodecanoate	111-82-0	74	846	2658	2.997	1524	1525
321	δ-Cadinene	483-76-1	134	737	2658	3.444	1524	1528
322	α-Panasinsen	56633-28-4	161	610	2658	3.45	1524	1518
323	(E)-Calamene	483-77-2	159	781	2670	3.787	1529	1530
324	Ethyl 4-ethoxybenzoate	23676-09-7	121	827	2670	4.969	1530	1522
325	β-Sesquiphellandrene	20307-83-9	93	668	2676	3.259	1532	1526
326	Isolongifolene, 4,5,9,10-dehydro-	156747-45-4	200	780	2682	4.192	1535	1544
327	Ethyl 3-hydroxytridecanoate	107141-15-1	117	824	2688	3.492	1537	1539
328	Dihydroactinidiolide	17092-92-1	111	860	2706	0.41	1543	1548
329	Isobutyl decanoate	30673-38-2	155	881	2706	2.814	1546	1545

	330	α-Calacorene	21391-99-1	157	926	2718	4.085	1550	1549
	331	Nerolidol	7212-44-4	93	814	2748	3.343	1563	1566
	332	β-Calacorene	50277-34-4	157	862	2766	4.189	1572	1564
	333	β-Vetivenene	27840-40-0	187	882	2772	4.728	1575	1554
	334	γ-Undecalactone	104-67-6	85	702	2784	4.977	1580	1573
	335	Hexyl octanoate	1117-55-1	127	816	2790	2.92	1583	1584
	336	Ethyl dodecanoate	106-33-2	101	865	2820	2.965	1595	1593
	337	Hexadecane[d]	544-76-3	57	887	2832	2.194	1600	1600
	338	Isopropyl laurate	10233-13-3	60	851	2892	2.759	1627	1618
	339	Cubenol	21284-22-0	161	762	2928	4.001	1643	1642
	340	Isopentyl decanoate	2306-91-4	70	885	2934	2.863	1646	1647
	341	Phenethyl hexanoate	6290-37-5	104	846	2934	4.363	1648	1650
	342	Cadalene	483-78-3	183	886	3018	4.763	1684	1684
	343	α-Bisabolo	515-69-5	119	893	3036	3.767	1694	1688
	344	Ethyl tridecanoate	28267-29-0	88	845	3042	2.915	1695	1687
	345	Heptadecane[d]	629-78-7	57	869	3054	2.222	1700	1700
	346	Methyl tetradecanoate	124-10-7	74	720	3108	2.992	1726	1722
	347	2,6-Diisopropylnaphthalene	24157-81-1	197	865	3120	4.307	1732	1728
	348	(Z)-Farnesol	3790-71-4	69	776	3132	3.173	1737	1718
	349	Ethyl 3-hydroxydodecanoate	126679-28-5	117	736	3144	3.412	1743	1743
	350	Ethyl tetradecanoate	124-06-1	88	866	3252	2.923	1795	1796
	351	Octadecaned	593-45-3	57	864	3264	2.249	1800	1800
	352	Isopropyl myristate	110-27-0	102	791	3312	2.777	1825	1823
	353	Isoamyl laurate	6309-51-9	70	826	3354	2.857	1846	1847
	354	Phenethyl octanoate	5457-70-5	104	860	3372	4.198	1856	1846
	355	Ethyl pentadecanoate	41114-00-5	88	884	3450	2.92	1897	1897
	356	Dibutyl phthalate	84-74-2	149	908	3582	5.233	1965	1967
	357	Ethyl 9-hexadecenoate	54546-22-4	79	808	3606	3.135	1976	1977
	358	Ethyl hexadecanoate	628-97-7	88	889	3642	2.932	1995	1994
	359	Eicosane[d]	112-95-8	57	867	3654	2.3	2000	2000
	360	Isopropyl palmitate	142-91-6	102	710	3696	2.778	2022	2027
	361	Ethyl octadecanoate	111-61-5	88	741	4008	2.912	2182	2194
T1		Mercaptoacetone	24653-75-6	90	898	438	2.342	726	
T2		2-(Methoxymethyl)furan	13679-46-4	81	861	720	3.204	829	

T3	Ethyl 3-furoate	614-98-2	95	864	1224	3.957	1000
T4	Pantolactone	599-04-2	71	874	1404	5.508	1060
T5	2-Thiopheneacetic acid	1918-77-0	97	758	1410	4.3	1061
T6	Ethyl levulate	539-88-8	99	777	1422	4.829	1066
T7	γ-Ethoxybutyrolactone	932-85-4	85	914	1428	5.955	1069
T8	Isoamyl lactate	19329-89-6	45	843	1440	3.21	1071
T9	Ethyl methyl succinate	627-73-6	115	903	1554	4.477	1109
T10	(E)-2-Ethyl heptenoate	54340-72-6	111	758	1680	3.305	1152
T11	(E)-6-Nonenol	31502-19-9	67	804	1764	3.296	1181
T12	Ethyl 2-pyrrolecarboxylate	2199-43-1	139	801	1836	5.51	1207
T13	Diethyl methylsuccinate	4676-51-1	143	799	1842	3.913	1209
T14	p-tert-Butylcyclohexanone	98-53-3	98	809	1920	4.216	1237
T15	3,9-Epoxy-p-menth-1-ene	70786-44-6	137	774	1932	4.115	1241
T16	Diethyl malate	626-11-9	117	880	2010	4.667	1270
T17	Ethyl 5-oxotetrahydro-2-furancarboxylate	1126-51-8	85	930	2112	1.342	1307
T18	2-Hexanoylfuran	14360-50-0	110	820	2112	4.47	1309
T19	Isoamyl 2-furoate	615-12-3	95	871	2136	4.389	1317
T20	3,4-Dihydro-3-oxoedulan	20194-67-6	193	849	2568	4.549	1487
T21	Megastigmatrienone	38818-55-2	148	782	2796	4.829	1587
T22	Heptyl ketone	818-23-5	57	870	2994	2.976	1674

Note: RI (calc) values for compounds 1-21 are extrapolated using ChromaTOF Software and RI (lit) values could not be found for compounds T1-T22 therefore identification is based on MS match only.
a Unique ion (m/z): used for peak area determination, identifiedas the unique ion by ChromaTOF data analysis.
b Retention indices calculated from C8 to C20 n-alkanes.
c Retention indices reported in the literature for 5% phenyl polysilphenylene-siloxane capillary GC columns or equivalent.
d Straight chain n-alkanes not present in the wine samples.
e Methyl nonanoate internal standard not present in wine samples.

Chapter 3

Research results

Early fermentation volatile metabolite profile of non-*Saccharomyces* yeasts in red and white grape must: a targeted approach

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Early fermentation volatile metabolite profile of non-Saccharomyces yeasts in red and white grape must: a targeted approach

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Abstract

Saccharomyces cerevisiae is the main driver of alcoholic fermentation. It is typically inoculated at high levels to ensure successful implantation as well as reduce the risks of stuck fermentations and off-flavor production. However, winemakers have found that wines produced with only *S. cerevisiae* can be lacking in complexity compared to fermentations where non-*Saccharomyces* yeasts are more active. This study sought to understand the early fermentation characteristics of *Kazachstania gamospora, Lachancea thermotolerans, Metschnikowia pulcherrima, Torulaspora delbrueckii* and *Zygosaccharomyces kombuchaensis* in both Sauvignon blanc and Syrah musts. *S. cerevisiae* was used as a control. Solid-phase microextraction coupled to GC-MS was used to evaluate the musts once they reached 2% ethanol concentration. The method targeted 90 different compounds known to occur in wine and/or be produced by yeast during fermentation. For the first time, *K. gamospora* and *Z. kombuchaensis* have been studied in the context of wine. While the other yeasts are commercially available starter cultures, they have never been profiled this extensively. Analysis showed that each yeast profile was unique and different based on the must. The non-S*accharomyces* yeasts produced lower concentrations of esters, alcohols and terpenes with the exception of *K. gamospora* which produced more total esters than the control.

Keywords

Non-*Saccharomyces* yeasts, *Kazachstania gamospora*, *Zygosaccharomyces kombuchaensis*, SPME-GC-MS, Wine

3.1 Introduction

Traditional winemaking practices rely on the microbiota naturally present on the grapes and in the winery environment to convert grape juice into wine, one of the most widely consumed alcoholic beverages in the world. Wine is the result of the biochemical process that takes place between grapes, microorganisms (yeasts, bacteria and fungi) and the wine cellar (Fleet, 2003). In order to mitigate product loss from stuck fermentation or the production of off-flavors, modern day winemaking commonly employs the use of commercially produced starter cultures, the vast majority of which are *Saccharomyces cerevisiae*. However, while this practice may reduce sources of microbial spoilage, some winemakers feel that the exclusive use of S. *cerevisiae* has resulted in a lack of organoleptic complexity when compared with successful spontaneous fermentations (Jolly, Augustyn, & Pretorius, 2006). Between 9 and 15 different yeast genera are typically reported to be associated with the winemaking process and compared to *S. cerevisiae*, their influence on wine in the wine production system is largely unknown (Johnson & Echavarri-Erasun, 2011). Recent research has nevertheless begun to show that certain non-*Saccharomyces* yeasts can have a positive impact on wine quality (Andorrà, Berradre, Mas, Esteve-Zarzoso, & Guillamón, 2012; Ciani & Comitini, 2011; Comitini et al., 2011; Gobbi et al., 2013; Sadoudi et al., 2012; Sun, Gong, Jiang, & Zhao, 2014; Zott et al., 2011). This has served to increase interest in the strategic use of non-*Saccharomyces* yeasts in winemaking and has even prompted commercial production of species belonging to the *Lachancea, Metschnikowia, Torulaspora,* and *Pichia* genera. There are two general practices when using these yeasts. The first is known as co-inoculation and some studies have been able to demonstrate that inoculating selected non-*Saccharomyces* yeasts at high cell concentration together with *S. cerevisiae* may produce wines with distinct characteristics while avoiding stuck fermentations (Comitini et al., 2011; Jolly et al., 2006; Soden, Francis, Oakey, & Henschke, 2000). Others have investigated the use of non-*Saccharomyces* yeasts in sequential inoculation though to a lesser extent (Contreras, Curtin, & Varela, 2015; Gobbi et al., 2013). This is where selected non-*Saccharomyces* yeasts are first inoculated at high levels and allowed to ferment on their own for a given amount of time before *S. cerevisiae* is added to take over the fermentation. This practice gives the non-*Saccharomyces* yeast more time to express their unique metabolic footprint uninhibited by the stress of *Saccharomyces* competition. This study sought to understand the early fermentation volatile metabolite profile or footprint of *Kazachstania gamospora, Lachancea thermotolerans, Metschnikowia pulcherrima, Torulaspora delbrueckii* and *Zygosaccharomyces kombuchaensis* in both Sauvignon blanc and Syrah musts prior to *S. cerevisiae* addition. *S. cerevisiae* meanwhile was used as a control. We present for the first time an in-depth extracellular metabolic characterization of five non-*Saccharomyces* yeasts by targeting and identifying 90 different compounds known to be present in wine due to yeast fermentation. To our knowledge this is the most comprehensive chemical profiling of volatile compounds for the yeast studied. Furthermore, *Kazachstania gamospora* and *Zygosaccharomyces kombuchaensis*

are relatively newly identified species capable of alcoholic fermentation and may therefore be of use to the wine making industry. They have however to date not been studied extensively in a winemaking capacity (Dashko et al., 2015; Imanishi, Ueda-Nishimura, & Mikata, 2007; Kurtzman, Robnett, & Basehoar-Powers, 2001; Nisiotou & Nychas, 2008; Steels, James, Bond, Roberts, & Stratford, 2002). The other three strains used in this study were commercial strains of *Lachancea thermotolerans, Metschnikowia pulcherrima,* and *Torulaspora delbrueckii*. Though these yeasts are commercial starter strains, they have not been profiled extensively in early fermentation with sequential inoculation practices in mind.

3.2 Materials and Methods

3.2.1 Samples, yeasts, chemicals and materials

Syrah and Sauvignon blanc grapes (vintage 2013) were obtained from the vineyards at Fondazione Edmund Mach in San Michele all 'Adige, Trentino, Italy. *S. cerevisiae* (Enoferm M2®, Lallemand), *T. delbrueckii* (Biodiva®, Lallemand), *M. pulcherrima* (Flavia®, Lallemand), *L. thermotolerans* (Viniflora® CONCERTO™, Chr. Hansen), *K. gamospora* (CBS-KNAW 10400) and *Z. kombuchaensis* (CBS 8849) were used. Twenty-milliliter glass screw cap vials, 60-mL screw cap vials, YPD, NaCl (ACS grade), sodium azide, internal standard 2-octanol, a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coating 50/30 µm, 2-cm length SPME fiber were purchased from (Supelco) Sigma-Aldrich S.r.l., Milan, Italy.

3.2.2 Fermentations, sample collection and preparation

The single culture fermentations were carried out in autoclave sterilized 60-mL vials equipped with screw caps fitted with 1.5-mm thickness PTFE/silicone septa. The grapes were crushed and the obtained must was frozen at -20°C until use. Before freezing the Syrah must was heated to 60°C for 6h to facilitate skin compound extraction and then centrifuged to remove particulates. Initial sugar, acidity and yeast assimilable nitrogen content, as well as pH were measured in each must (Table 1). Fifty-five milliliters of must were placed into each vial. The vials were inoculated at a density of approximately 10^6 cells/mL from pure yeast cultures that were grown in YPD over night at a static 25°C. Cell pellets were collected from the appropriate volume of centrifuged media determined to be necessary after a hemacytometer count. All fermentations were performed in triplicate. The amount of CO_2 lost was monitored by measuring the weight loss of the closed vials. In accordance with the protocol outlined in chapter 11 of "Wine Microbiology: Science and Technology" this value was converted to reflect ethanol concentration (Delfini & Formica, 2001). When between 2% and 3% alcohol was reached, 40 mL of the partially fermented must were transferred to 50-mL conical tubes and centrifuged to remove yeast. Five milliliters of supernatant were added to 20-mL screw cap vials containing 1.5 g NaCl, 500 µL of 0.1% Sodium Azide, 100 µL of 2.13 mg/L 2-octanol.

3.2.3 SPME extraction and GC-MS analysis

A CTC CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland) with a single magnetic mixer (SMM Chromtech) and SPME fiber conditioning station was used to extract the volatiles from the sample vial headspace. A TRACE GC Ultra coupled to a TSQ Quantum triple quadrupole (QqQ) mass spectrometer (Thermo Fisher Scientific) was used. The samples were incubated for 10 min at 40°C under 450 rpm rotation of a magnetic stir bar. Extraction took place for 40 min prior to desorption in the GC inlet for 2 min at 250°C. Helium carrier gas was used with a flow set at 1.2 mL/min and a splitless time of 2.5 min. The GC oven was equipped with a 30 m x 0.25 mm x 0.25 µm VF-WAX column (Agilent Technologies). The GC oven parameters were as follows: initial temperature was 40°C held for 4 min, followed by an increase to 250°C at a rate of 6°C/min, the oven was then held at 250°C for 5 min before returning to the initial temperature (40°C). The total cycle time, was 44 min. The MS detector was operated in scan mode (mass range 40–350 m/z) with a 0.2 sec scan time and the transfer line to the MS system was maintained at 250°C. The aroma compounds were identified by using the NIST library for confirmation and also injection of pure standards where available. As is commonly the case for these types of semi-quantitative analysis, a response factor of 1 with respect to the internal standard was used (Azzolini et al., 2012).

3.2.4 Statistical and network analysis

A Perl program was written to test for significance ($p<0.05$) using t-tests as well as create and annotate the statistical networks. The significant statistical relationships were modeled as networks from a variety of perspectives (species-centric, compound-centric and a combined view).

Correlation networks were built for the compounds as they occurred across samples. Correlations between all compounds were determined with the use of a normalized Czekanowski metric which was subsequently used as the edge weight in the correlation networks. Normalization was achieved by dividing each element in a compound vector by the sum of its vector, thus making it a stochastic matrix. Different networks were created for each of the two musts. A 0.85 threshold was applied and the resulting networks were visualized using Cytoscape 2.8.2(Shannon et al., 2003). Networks from different musts were also merged and the edges colored differently in order to highlight the differences in the two networks.

MultiExperiment viewer was used for hierarchical clustering and heat map visualization (Saeed et al., 2003).

3.3 Results

3.3.1 Ethanol production

As can be seen in Fig. 1, each yeast fermented both musts at approximately the same rate. As expected, *S. cerevisiae* fermented at the fastest rate while the musts inoculated with *Z. kombuchaensis* was the slowest in both musts.

3.3.2 Aroma compound production by specific species as compared to *Saccharomyces cerevisiae*

3.3.2.1 *Saccharomyces cerevisiae*

As can be seen in Fig. 2 the clustering was governed more strongly by yeast rather than by must. This does not however mean that there weren't significant differences between the behaviors of the yeasts in each must. This is true for all of the yeast including *S. cerevisiae* which showed 54 compounds to be significantly different between the musts. The largest difference was seen in linalool which was found to be 10 times higher in concentration in the fermented Syrah must than in the Sauvignon blanc. Many of the other terpenes were also found to be in higher concentration in the Syrah. Meanwhile the fermented Sauvignon blanc must had higher concentrations of 3-methylthio-1-propanol and acetic acid, if only slightly. This demonstrates clearly the matrix effect on metabolic output, a trend which continues for all yeasts studied.

3.3.2.2 *Kazachstania gamospora*

K. gamospora was discovered as a species in 2007 (Imanishi et al., 2007). Since then only one other article has been published in which the species is mentioned. Nisiotou & Nychas, 2008 compared isolates in *Botrytis*-affected fermenting grape juice from Greece to its genetic profile. Here we present, for the first time, the volatile metabolite footprint of *K. gamospora* in grape must and compare it to that of *S. cerevisiae*.

In the *K. gamospora* fermentation, of the 90 compounds observed, 67 in the Sauvignon blanc and 60 in the Syrah musts were found to be significantly different with respect to their *S. cerevisiae* controls (Table 2). Of these, only 23 were positive fold changes (increases in concentration) over the control in both musts. The highest fold change was found in phenethyl propionate in both musts; over 200 times more of this compound was found in the musts fermented with *K. gamospora* compared to *S. cerevisiae* (Fig. 2). This was by far the largest fold change seen in any of the compounds among any of the yeasts. Phenethyl propionate is desirable in wine as it has a floral aroma and is the ester of 2-phenylethanol and propanoic acid. Over all *K. gamospora* produced a higher concentration of esters, than *S. cerevisiae* did.

Another difference between the musts fermented with *K. gamospora* compared to *S. cerevisiae* was observed in 3-methylthio-1-propanol, in the Sauvignon blanc it was only a 1.6 fold increase while in the Syrah must, there was a 14 fold increase. With an aroma of sulfurous onions and relatively low odor threshold this is a particularly undesirable compound in wine. As can be seen in Table 2, the relative amount of this compound produced in the controls was 3.2±0.5 and 0.66±0.06 µg/L in Sauvignon blanc and Syrah, respectively. This means that though there were drastic differences in the fold changes *K. gamospora* only produced approximately twice the amount of 3-methylthio-1-propanol in the Syrah must as it did in the Sauvignon blanc (Table S1). This example illustrates the need to view this data both in the context of fold change and in simple relative concentration since concentration is ultimately responsible for the potential sensory impact of these compounds.

3.3.2.3 *Zygosaccharomyces kombuchaensis*

Similar to *K. gamospora*, *Z. kombuchaensis* is a newly discovered yeast. Since its discovery in 2001, little research has been conducted to establish its metabolic profile beyond its basic comparison to other members of its genera (Kurtzman et al., 2001; Steels et al., 2002). The genus *Zygosaccharomyces* is known for its ability to spoil wine, specifically sweet and sparkling wines (Loureiro, 2003). It is particularly known for its overproduction of acetic acid. However, the acetic acid production of the *Z. kombuchaensis* strain that we tested was not found to be statistically significantly different from the control. Of all the yeast fermentations, the Syrah must fermentation of *Z. kombuchaensis* had the largest number of compounds that were significantly different from the control fermentation (Table 2 and Fig. 2). Seventy-four compounds were produced in concentrations that were statistically significant from the control in the Syrah must and the Sauvignon blanc must fermentations were close with 73 significantly different compounds (Table 2). The majority of the fold changes were negative with only 10 compounds in the Sauvignon blanc and 13 compounds in the Syrah musts showing positive fold changes. A general trend of the fermentations significantly lacking in ester production emerged (Fig. 3). Notably, diethyl succinate, ethyl 9-decenoate, ethyl butyrate, ethyl decanoate, ethyl dodecanoate, ethyl hexanoate, ethyl octanoate occurred in more than 100 times greater concentration in the control fermentations. The *Z. kombuchaensis* fermentations were also notable for their general lack of production of alcohols though it should be noted that of the compounds found to have higher fold changes almost half of them were alcohols in both musts. The yeast also produced significantly less 3-methylthio-1-propanol than in the control in both musts. Of the compounds that did show significant fold increases the largest were benzaldehyde in both musts at 13 and 15 times more in the Sauvignon blanc and Syrah musts, respectively. Benzaldehyde is associated with almond flavor, an aroma that is desirable in wine.

Of the compounds tested, none found to be in higher concentration in the *Z. kombuchaensis* fermentations were known off-flavors. In fact *Z. kombuchaensis* produced the least amount of 3-methylthio-1-propanol of all the yeast.

3.3.2.4 *Lachancea thermotolerans*

L. thermotolerans has long been a yeast associated with winemaking and is currently available commercially as a starter culture. It has been investigated recently for its ability to enhance wine acidity and improve overall wine quality (Gobbi et al., 2013). Here we are able to give a more comprehensive profile of this yeast in two different grape musts.

Sixty-three and 54 compounds were found to be significantly different from *S. cerevisiae* in Sauvignon blanc and Syrah musts fermented with *L. thermotolerans*. Of these, only 11 compounds in the Sauvignon blanc and 20 of these compounds in the Syrah were positive fold changes (Table 2). *L. thermotolerans* showed a significant lack of ester production in both musts (Fig. 3). There were considerable differences in the profiles presented by *L. thermotolerans* in each must there were some notable similarities. Both musts saw roughly the same increases in 2-phenylethanol and phenethyl propionate. Both musts also showed increases in both ethyl salicylate and methyl salicylate as well. The terpenes nerol and terpine-4-ol also exhibited approximately the same fold change in both musts. Another notable significant positive fold increase was seen in 3-methylthio-1-propanol which was found to be six times higher in the Syrah must and 1.7 times higher in the Sauvignon blanc (Table 2). Of the acids we profiled *L. thermotolerans* did not produce more than the control. In fact, it was only second to *Z. kombuchaensis* in lowest total acid production. It did however have a higher overall production of phenols than the control and indeed many of the other species with the exception of *K. gamospora*. It also showed higher terpene production in the Syrah must than the control.

3.3.2.5 *Metschnikowia pulcherrima*

M. pulcherrima is another yeast that has been made available commercially for use in winemaking. A recent study characterized this species in both mono- and co-culture with *S. cerevisiae* by profiling 44 different aroma compounds. Sadoudi et al. 2012 were able to show that the aroma compounds of wine produced via mono-culture were significantly less than those produced in conjunction with *S. cerevisiae*. Our investigation showed that in early fermentation, before *S. cerevisiae* would be added if a sequential inoculation practice was being used, there were 76 significant differences from the control in the Sauvignon blanc and 66 in Syrah musts fermented with *M. pulcherrima*. Of these, 15 and 24 were positive fold increases over the control, respectively (Table 2). Twenty-four is the largest single number of significant positive fold changes seen in any of the musts. The highest fold changes seen in either must were in the phenol 2-methoxy-4-

vinylphenol which was found to be 53 times higher in the Sauvignon blanc must and 80 higher in the Syrah must. The next largest set of fold changes were in the Syrah must: the ester phenethyl propionate, the furan 5-hydroxymethyl-2-furaldehyde, and the ester phenethyl butyrate found to be 18, 13, and 13 times higher in the Syrah but not significantly different in the Sauvignon blanc (Table 2). 3-Methylthio-1-propanol was also found to be 4 times higher in the Syrah while not being significantly different in the Sauvignon blanc. While the total amount of esters in both musts was significantly less than the control (Fig. 3), certain esters showed a positive fold change in both musts. These included hexyl acetate, ethyl 2-hydroxy-4-methylpentanoate, *cis*-3-hexenyl acetate, and isoeugenyl phenylacetate (Fig. 2). The fold changes were slightly higher in the Syrah must than in the Sauvignon blanc but they were all significantly higher than in the control.

3.3.2.6 *Torulaspora delbrueckii*

When the *T. delbrueckii* fermentations were compared to the *S. cerevisiae* fermentations, of the 69 and 62 significant differences seen in Sauvignon blanc and Syrah, only 10 and 14 were positive fold changes, respectively (Table 2). The *T. delbrueckii* fermentations were notably lacking in a significant number of esters (Fig. 3). With the exception of isobornyl acetate, isoeugenyl phenylacetate, and phenethyl propionate, all other esters demonstrated a negative fold change. A further exception was ethyl 2-hydroxy-4-methyl pentanoate which was a negative fold change in the Sauvignon blanc and a positive fold change in the Syrah. Phenethyl propionate was found to be 56 and 53 times higher in the *T. delbrueckii* fermentations of Sauvignon blanc and Syrah, respectively (Table 2). Another noticeable significant increase was in 5-methylfurfural which was found to be 66 fold greater in the *T. delbrueckii* Sauvignon blanc fermentations but not significantly different in the Syrah fermentations. It should also be noted that the sulfur compound 3-methylthio-1-propanol was found to be 5 times higher in the Syrah must but not significantly different in the Sauvignon blanc.

3.3.3 Global must perspective using network analysis

In looking for correlations amongst compound levels across species we constructed a conserved metabolic network. Correlation networks were created comparing the similarity of compounds across samples with a normalized Czekanowski similarity metric. Different networks were created across species in the Syrah and Sauvignon blanc musts and a 0.85 threshold applied to each of them. As can be seen in Fig. 4, the network topology of the resulting networks is quite distinct. The must clearly has an effect on the differential expression of the metabolites as well as many of their correlative relationships. From this, one can infer that the must has an impact on the regulatory framework of the underlying metabolic profiles of each yeast. There are however obviously core compounds that correlate with one another regardless of must. The relationships amongst terpinen-4-ol, linalool, 4-ethyl guiacol, 1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN), a-terpenyl

ethyl ether, 2-penylethanol, Ho-trienol, and beta damascenone are an exaNPmple of a set of relationships that have strong similarities across musts. 1-Propanol, 2-methyl-1-propanol, ethyl-4-hydroxybutyrate and nerol are another group of compounds that have similar correlative relationships across musts. It would appear that there are regulatory networks within and across species that are sensitive to their nutrient environment (must) and adapt accordingly. Meanwhile there are some core areas of metabolism that remain unaltered regardless of nutrient environment.

3.4. Discussion

3.4.1 Impact of non-*Saccharomyces* yeast in early winemaking

Recently, there has been increased interest in the use of non-*Saccharomyces* yeasts in wine fermentations. Several studies have already begun to indicate that certain non-S*accharomyces* yeasts can work in tandem with *S. cerevisiae* to produce desirable aroma and flavor compounds when controlled properly in co-inoculation situations (Andorrà et al., 2012; Ciani & Comitini, 2011; Sadoudi et al., 2012). However, little has been studied about the chemical profiles produced by these yeasts when they are allowed to ferment grape must alone, as they would if they were to be used in a sequential inoculation strategy. Thus, we chose to take a broad approach to evaluate the metabolic footprint of the yeasts: *K. gamospora*, *L. thermotolerans*, *M. pulcherrima*, *T. delbrueckii* and *Z. kombuchaensis* in two different grape musts, Sauvignon blanc and Syrah.

Each yeast had a unique metabolic profile that was different in both musts. As can be seen in Fig. 3, a few overarching trends stood out. The major flavor compounds (alcohols, esters and terpenes) were produced in far lower concentrations by the non-*Saccharomyces* yeasts studied here than *S. cerevisiae.* This confirmed previous results obtained by Viana, Manzanares, & Valle 2011 and many other previously mentioned studies. The exception was *K. gamospora*, which produced slightly higher amounts of esters overall.

Other interesting positive fold increases in esters were seen in specific compounds not commonly found in *S. cerevisiae* fermentations such as isoeugenyl phenylacetate, phenethyl propionate, and isobornyl acetate (Hardy & Ramshaw, 1970; Nykänen, 1986). Isobornyl acetate is described as complexly woody, camphorous, piney and herbal with citrus nuances. Isoeugenyl phenylacetate has a spicy, clove-like aroma while phenethyl propionate, an ester of phenethyl alcohol and propionic acid, has a rose-like aroma. This is just one example of how the differences between non-*Saccharomyces* and *Saccharomyces* yeast metabolism can produce more complex and varied aroma and flavor profiles in wine.

Other complexities included significantly higher amounts of the furans 5-methylfurfural and 5-hydroxymethyl-2-furaldehyde produced in the Sauvignon blanc must fermented by *T. delbrueckii* and the Syrah fermented by *M. pulcherrima.* Both compounds are known to have a spicy, caramel-

like flavor and have recently been studied for their effect on the inhibition of the fermentation process of *S. cerevisiae* (Almeida, Bertilsson, Gorwa-Grauslund, Gorsich, & Lidén, 2009). However, it has also been shown that *S. cerevisiae* can convert furfural and 5-hydroxymethyl-2-furaldehyde to less inhibitory compounds such as furfuryl alcohol which is further degraded to 2-furoic acid (Liu, 2006). The latter has as a sweet, oily, and herbaceous aroma. The presence of these compounds is surprising. Until now, their formation has only been characterized by the acid-catalyzed dehydration of sugars facilitated by acidic and high heat conditions and not reported as products of yeast metabolism. Yeast-yeast inhibition, one of the theorized causes of stuck fermentations where non-*Saccharomyces* yeasts have been found in high amounts, has not been fully explained or characterized (Jolly, Varela, & Pretorius, 2013). The presence of these furans may help explain slow or sluggish 'spontaneous' fermentations and warrants further investigation.

Besides these larger trends, several small but significant differences between the yeasts occurred. To varying degrees, in all but the *Z. kombuchaensis* fermentations of both musts and the Sauvignon blanc/*M. pulcherrima* fermentations, an increase in the concentration of the sulfurous off-flavor 3-methylthio-1-propanol was observed. Thiol compounds are detectable at exceedingly low concentrations in wine compared to other compounds. 3-Methylthio-1-propanol however is only detectable as an off-odor at mg/L ranges, well below levels recorded here (Mestres, Busto, & Guasch, 2000) .

Volatile phenols, when in high concentration, can also impart off-odors to wine. They are generated by microbiologically produced hydroxycinnamate decarboxylase which converts hydroxycinnamic acids naturally present in the wine into vinylphenols which can then be further reduced to ethylphenols by vinylphenol reductase. In low amounts these compounds can add depth and character to wine but above their sensory threshold they impart odors and flavors of 'Band-Aid', medicinal, mousy and horse sweat (Manzanares et al., 2011). Though it was originally thought that only species of *Brettanomyces/Dekkera* were capable of completely degrading hydroxycinnamic acid to ethylphenol compounds, it has been shown that some strains of *Pichia guilliermondii* are able to fully convert it as well. Meanwhile, several other species have proven capable of the first conversion, hydroxycinnamic acid to vinylphenols. These include *Hanseniaspora, Pichia* and *Zygosaccharomyces* as well as some wine strains of *S. cerevisiae* (Chatonnet, Dubourdie, Boidron, & Pons, 1992). For the first time, we report that the species *M. pulcherrima* is capable of producing vinylphenols indicating that at the very least, it possesses the enzyme hydroxycinnamate decarboxylase and that it is active under winemaking conditions. However, since no significant increase in ethylphenols was observed, it is possible that the species does not have vinylphenol reductase activity. Though still below the detection levels and thus potentially beneficial to the organoleptic quality of the final product, these fermentations were stopped

prematurely. It would be worth investigating this strain for its potential impact on a finished wine product to ensure that the volatile phenols it produces remain below the detection threshold.

3.5. Conclusions

The distinct differences in the early fermentation characteristics shown by the data presented here clearly indicate that non-*Saccharomyces* yeasts have the potential to play a positive role in winemaking. We specifically compared the extracellular volatile metabolite profiles of early fermentations in both a red and white grape must inoculated with five different non-*Saccharomyces* yeast genera that are incapable of completing wine fermentations.

Each yeast presented a unique and distinctive profile. *K. gamospora* was able to begin fermentation almost as quickly as *S. cerevisiae* as well as produced a significantly different aroma profile that was actually higher in overall ester production. Given these facts, we conclude that *K. gamospora* could be a good candidate as a yeast that can increase the aromatic complexity of wine.

Conversely, given its slow fermentative capacity and lack of significant positive compound production compared to other yeast species, *Z. kombuchaensis* is most likely ill-suited for use in increasing wine aroma complexity through either sequential or co-inoculation.

The other three yeasts studied are commercial strains already available to wine makers. By determining the relative concentrations of 90 different volatile compounds, we were able to put together a more comprehensive picture of how exactly these yeast function in both red and white grape musts in terms of the kinds of aromas they are capable of producing in the absence of *S. cerevisiae*. This study proved to be a valuable screening tool of these yeasts. The knowledge gained in this study shows the potential aroma contribution of different non-*Saccharomyces* yeasts and further studies will have to be conducted to assess if these difference persist after inoculation with *S. cerevisiae* to complete alcoholic fermentation.

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Tables and figures

Table 1

Starting characteristics of each must.

Must	Fructose (g/L)	Glucose (g/L)	pH	Total acidity (g/L) (as tartaric acid)	Assimilable Nitrogen (mg/L)
Syrah	94	95	3.16	4.6	126
Sauvignon Blanc	105	108	3.02	7.2	121

Table 2

List of compounds and the µg/L concentration* found in the *S. cerevisiae* control next to the relative fold changes of all other samples.

Compound Class	Component Name	CAS Number	Aroma	S.B. with S. cerevisiae µg/L concentration	Syrah with S. cerevisiae µg/L concentration	S.B. with *K. G.*	Syrah with *K.G.*	S.B. with *L.T.*	Syrah with *L.T.*	S.B. with *M.P.*	Syrah with *M.P.*	S.B. with T.*D.*	Syrah with *T.D*	S.B with *Z.K.*	Syrah with *Z.K.*
Alcohols	1-Heptanol[A]	111-70-6	Leafy	2.0±0.04	1.7±0.5	-7.3	NS	-3.3	NS	-6.3	NS	-7.4	-4.0	-6.8	NS
	1-Hexanol[A]	111-27-3	Green	50±0.7	52±0.6	-3.8	-2.9	1.1	1.1	-1.6	-1.5	-1.1	NS	NS	1.5
	1-Nonanol[A]	143-08-8	Fruity	1.2±0.3	1.0±0.03	-2.4	-1.8	NS	-1.4	-2.6	NS	-2.5	NS	-2.8	-2.2
	1-Octanol[A]	111-87-5	Waxy	1.8±0.1	2.3±0.1	-4.1	-2.7	-2.9	-3.9	1.9	3.2	-1.8	NS	-1.7	-1.2
	1-Octen-3-ol[A]	3391-86-4	Mushroom	3.2±0.3	3.1±0.5	-3.4	-3.4	-1.4	NS	-1.3	NS	NS	NS	1.4	1.6
	1-Propanol[A]	71-23-8	Weak fusel	33±5	23±2	-4.5	-4.3	NS	1.6	-8.4	-7.5	-6.2	-4.5	-13	-15
	1-Tetradecanol[A]	112-72-1	Coconut	9.8±0.6	6.5±1	-7.2	-3.7	-1.6	NS	-9.4	-4.2	-9.8	-5.5	-17	-11
	2,3-Butanediol[A]	513-85-9	Sweet	150±20	82±8	-5.7	-5.8	NS	NS	-5.5	-3.8	-15	-13	-59	-59
	2-3-Butandiol_(2)[A]	513-85-9	Sweet	71±16	40±8	-5.4	-5.1	-1.9	-1.9	-7.7	-5.0	-7.6	-4.9	-34	-30
	2-Ethyl-1-hexanol[A]	104-76-7	Citrus	1.4±0.3	2.0±0.4	5.9	3.8	NS	2.7	5.5	4.1	NS	6.4	6.5	6.0
	2-Methyl-1-propanol[A]	78-83-1	Apple	16±3	12±0.9	-4.4	-4.2	1.8	1.8	-3.0	-2.3	-3.2	-3.7	-7.4	-7.6
	2-Nonanol[A]	628-99-9	Cucumber	5.3±0.2	6.8±0.1	NS	-1.4	-1.2	NS	-4.8	-1.7	-4.8	-3.8	NS	-1.8
	2-Propyl-1-pentanol[A]	58175-57-8	Mild Green	1.2±0.2	0.79±0.08	1.7	2.6	-3.4	1.8	1.7	2.8	NS	4.5	2.1	4.3
	3-Methyl-1-pentanol[A]	589-35-5	Fusel	0.67±0.01	0.69±0.06	-2.0	-1.4	-3.2	-4.7	-5.1	-5.4	-9.7	-13	-6.9	-16

	Benzyl alcohol[A]	100-51-6	Fruity	0.32±0.02	1.4±0.04	1.8	NS	-1.2	NS	2.9	1.8	2.6	NS	3.7	3.7
	cis-3-Hexen-1-ol[A]	928-96-1	Green	5.6±1	5.3±0.01	-3.2	NS	NS	1.1	-3.9	NS	-3.4	NS	-1.7	2.0
	trans-3-Hexen-1-ol[A]	928-97-2	Green	0.69±0.02	1.9±0.003	NS	-7.9	NS	NS	1.4	-4.2	1.6	-3.0	2.6	-2.1
Aldehydes	Benzaldehyde[A]	100-52-7	Almond	2.6±0.2	2.4±0.01	6.6	6.9	NS	NS	1.8	2.5	5.2	NS	14	16
	Decanal[A]	112-31-2	Sweet	3.3±0.1	2.3±0.005	2.5	3.3	NS	1.2	2.4	3.6	NS	5.7	2.8	5.3
	Nonanal[A]	124-19-6	Waxy	2.3±0.2	2.0±0.4	-2.5	1.9	NS	NS	-1.5	NS	-1.6	NS	-2.8	NS
	Phenylacetaldehyde[A]	122-78-1	Green	2.5±0.3	1.8±0.009	5.9	16	NS	NS	2.6	6.5	2.7	NS	-1.9	NS
Carboxylic Acids	9-Decenoic acid[B]	14436-32-9	Waxy	38±10	74±26	1.8	NS	-2.9	NS	NS	NS	-7.5	NS	-29	NS
	Acetic acid[A]	64-19-7	Sour	41±3	12±0.6	NS	NS	NS	NS	-1.9	NS	-5.4	NS	NS	NS
	Decanoic acid[A]	334-48-5	Unpleasant	110±20	120±9	-2.2	-2.2	-1.9	-1.9	1.8	2.1	-8.0	NS	-11	-6.2
	Dodecanoic acid[A]	143-07-7	Coconut	12±4	14±5	-3.7	-4.8	-9.3	NS	NS	NS	-5.9	-7.7	-20	-19
	Hexanoic acid[A]	142-62-1	Sour	250±20	200±3	-1.6	NS	-6.8	-3.5	NS	NS	-3.4	-2.2	-6.9	-6.6
	Isovaleric acid[A]	503-74-2	Sweaty Feet	47±5	32±5	NS	1.9	-2.4	-2.5	-6.8	-2.5	-4.4	-2.7	-12	-9.2
	Octanoic acid[A]	124-07-2	Rancid	110±5	110±1	1.5	NS	-5.9	-4.5	NS	NS	-2.5	NS	-7.8	-9.0
Esters	a-Terpenyl ethyl ether[B]	27153-54-4	Fruity	0.62±0.2	0.58±0.01	NS	NS	NS	NS	NS	NS	NS	NS	-2.1	-1.2
	cis-3-Hexenyl acetate[A]	3681-71-8	Green	1.1±0.3	4.0±0.1	6.3	6.3	-15	-42	1.7	1.8	-5.8	-10	-2.8	-2.5
	Diethyl malate[B]	626-11-9	Brown Sugar	3.1±0.4	4.3±0.1	-2.0	-2.9	NS	-1.8	-5.2	-4.2	-2.7	-4.1	-6.8	-14

Diethyl succinate[B]	123-25-1	Fruity	82±9	59±9	-92	-59	-2.6	-2.7	-13	-2.3	-190	-45	-180	-340
Ethyl 2-hydroxy-4-methylpentanoate[B]	10348-47-7	Fresh Blackberry	3.0±0.4	12±0.2	-1.9	NS	-2.4	NS	1.6	1.4	-2.0	1.3	-2.1	NS
Ethyl 2-methylbutyrate[A]	7452-79-1	Fruity	0.41±0.04	0.25±0.02	-2.1	NS	NS	3.0	-9.5	-5.0	-6.0	-7.5	-18	-26
Ethyl 3-hydroxydodecanoate[B]	126679-28-5	?	6.9±0.4	9.2±0.8	-5.3	-11	-12	-11	-3.2	-1.6	-33	-42	-22	-140
Ethyl 4-hydroxybutyrate[B]	999-10-0	Caramel	28±8	13±2	NS	-9.8	2.0	2.8	-11	-2.5	-22	-10	-20	-9.4
Ethyl 9-decenoate[B]	67233-91-4	Fruity	240±20	170±20	-15	-29	-2.3	-2.3	-13	-2.2	-270	-73	-250	-490
Ethyl acetate[A]	141-78-6	Fruity	50±2	36±3	NS	NS	-1.1	NS	-2.1	-3.5	-8.2	-13	-3.4	-3.4
Ethyl butyrate[A]	105-54-4	Fruity	4.5±0.4	4.4±0.2	-1.4	-5.9	-3.3	-3.7	-5.1	-4.9	-4.2	-11	-68	-180
Ethyl decanoate[A]	110-38-3	Sweet	150±20	110±4	-55	-87	-2.6	-5.1	-4.2	-1.6	-190	-79	-290	-190
Ethyl dodecanoate[A]	106-33-2	Waxy	48±10	26±3	-55	-83	-18	-20.0	-5.6	-2.5	-210	-190	-460	-240
Ethyl hexanoate[A]	123-66-0	Fruity	18±10	140±4	-15	-9.4	-2.4	-2.9	-3.1	NS	-8.9	-7.3	-290	-320
Ethyl isovalerate[A]	108-64-5	Fruity	0.14±0.03	0.074±0.006	NS	NS	-2.0	NS	-5.5	-3.9	-4.6	-2.6	-2.8	-5.5
Ethyl octanoate[A]	106-32-1	Apricot	280±20	200±4	-19	-16	-4.4	-4.9	-8.7	-5.5	-110	-97	-810	-610
Ethyl phenacetate[A]	101-97-3	Floral	4.7±0.1	3.5±0.02	1.5	4.3	1.3	2.0	-1.7	NS	-1.4	NS	-4.9	-5.6
Ethyl salicylate[A]	118-61-6	Mint	0.38±0.04	0.27±0.0003	1.7	4.9	1.3	2.1	-1.6	NS	NS	NS	-2.0	-3.8
Geranyl ethyl ether[B]	40267-72-9	Green	1.5±0.04	2.0±0.1	1.5	NS	NS	NS	-2.1	NS	-2.1	-2.3	NS	NS
Hexyl acetate[A]	142-92-7	Banana	15±5	17±1	3.8	4.8	-21	-79	NS	1.5	-44	-81	-12	-9.8

	Isoamyl acetate[A]	123-92-2	Banana	200±10	150±8	1.6	2.1	-2.0	-2.5	-1.6	NS	-140	-160	-19	-5.3
	Isoamyl hexanoate[A]	2198-61-0	Pineapple	17±0.6	14±5	-7.3	-6.5	-3.4	NS	-5.2	NS	-7.5	NS	-7.1	NS
	Isoamyl decanoate[B]	2306-91-4	Waxy	22±6	14±0.2	-6.8	-3.1	-3.8	-7.4	-4.3	NS	-13	-6.8	-25	-25
	Isoamyl octanoate[A]	2035-99-6	Sweet	3.9±0.7	3.8±0.3	-3.0	-7.9	-8.7	-16	-2.2	NS	-47	-79	-71	-62
	Isobornyl acetate[A]	125-12-2	Woody	0.027±0.02	0.023±0.008	2.5	2.8	NS	NS	NS	2.6	NS	2.5	NS	2.4
	Isoeugenyl phenylacetate[A]	120-24-1	Spicy	0.35±0.05	0.21±0.05	NS	NS	NS	NS	NS	6.9	2.5	NS	NS	NS
	Lactic acid, ethyl ester[A]	97-64-3	Butter-scotch	5.3±0.05	3.5±0.1	-5.5	-3.9	-1.4	NS	-3.4	-2.1	NS	NS	NS	1.8
	Ethyl linalyl ether[B]	72845-33-1	Floral	1.3±0.2	1.0±0.009	-7.6	-2.7	-1.3	NS	-9.9	-3.1	-11	-3.0	-6.2	-7.9
	Methyl salicylate[A]	119-36-8	Mint	0.38±0.04	0.27±0.003	1.7	4.9	1.3	2.1	-1.6	NS	NS	NS	-2.0	-3.8
	Neryl ethyl ether[B]	22882-89-9	Clean	1.8±0.3	1.3±0.3	-1.7	NS	-2.6	-2.1	-1.6	NS	-1.8	NS	-1.7	NS
	Phenethyl acetate[A]	103-45-7	Floral, rose	150±6	100±0.9	8.3	8.6	-3.1	-6.4	-3.0	NS	-10	-8.0	-7.8	-4.6
	Phenethyl butyrate[A]	103-52-6	Musty	2.3±0.2	2.7±0.02	NS	2.0	-2.0	-3.2	NS	13	NS	NS	-35	-54
	Phenethyl propionate[B]	122-70-3	Rose	1.8±0.08	1.9±0.1	209	230	1.5	2.5	NS	18	56	49	1.4	NS
	trans-3-Hexenyl acetate[A]	3681-82-1	Fruity	1.5±0.4	4.0±0.2	9.9	6.4	-9.6	-35	1.6	1.8	-7.7	-10	-1.9	-2.5
Furans	2-Pentylfuran[B]	3777-69-3	Green	0.16±0.1	0.047±0.004	NS	NS	NS	NS	NS	NS	NS	NS	NS	-1.7
	5-Hydroxymethyl-2-furaldehyde[A]	67-47-0	Fatty	0.24±0.1	0.23±0.04	NS	NS	NS	NS	NS	13	NS	NS	NS	NS
	5-Methylfurfural[A]	620-02-0	Spicy, Caramel	0.032±0.02	0.018±0.003	NS	NS	NS	NS	NS	NS	66	NS	NS	NS

Ketones	2-Heptanone[A]	110-43-0	Spicy	42±6	26±0.6	-3.8	NS	NS	NS	-20.0	-7.7	-27	-14	-3.1	-3.8
	2-Nonanone[A]	821-55-6	Cheese	1.8±0.1	2.6±0.02	6.3	4.0	-2.8	-3.9	-2.5	NS	4.8	3.4	4.7	2.0
	Acetoin[A]	513-86-0	Sweet, dairy	0.68±0.01	0.55±0.03	NS	NS	NS	-1.3	4.7	3.6	NS	NS	NS	6.4
	Benzophenone[A]	119-61-9	Rose	0.66±0.2	0.48±0.07	NS	NS	NS	NS	-4.9	-2.6	-4.4	NS	-2.7	-4.7
Phenols	2-Methoxy-4-vinylphenol[A]	7786-61-0	Clove	0.063±0.01	0.026±0.02	NS	NS	-6.3	NS	53	80.0	NS	NS	NS	NS
	2-Phenylethanol[A]	60-12-8	Rose	200±3	140±1	-1.2	NS	1.1	1.2	-2.8	NS	-1.6	NS	-7.4	-7.2
	4-Ethylguiacol[A]	2785-89-9	Clove, Spicy	0.086±0.02	0.044±0.003	NS	1.8	NS	NS	NS	1.6	NS	NS	NS	-1.4
	4-Ethylphenol[A]	123-07-9	Smoke	0.14±0.02	0.12±0.03	NS	NS	-2.3	NS	-2.0	NS	NS	NS	-2.1	-2.2
Terpenes	cis-Rose oxide[A]	3033-23-6	Rose	0.10±0.01	0.52±0.009	-3.3	-8.2	-1.6	-5.1	-2.8	-7.4	-2.7	-7.6	-3.3	-13
	trans-Rose oxide[A]	876-18-6	Rose	0.041±0.007	0.21±0.003	NS	-4.7	-1.7	-4.8	NS	-3.7	NS	-3.2	NS	-6.3
	Alpha-terpineol[A]	98-55-5	Lilac	6.8±0.6	5.7±0.8	-7.1	-3.0	-2.5	-3.2	-12	-2.5	-13	-2.1	-19	-4.1
	Beta-citronellol[A]	106-22-9	Citrus	1.5±0.1	1.8±0.1	NS	-2.6	-2.6	-2.0	-9.3	-3.8	-2.7	-2.2	NS	-3.8
	Beta-myrcene[A]	123-35-3	Woody	0.43±0.02	1.4±0.1	-8.2	-9.3	-1.3	NS	-8.1	-5.7	-5.8	-4.9	-6.1	-3.6
	Geraniol[A]	106-24-1	Citrus	1.9±0.5	1.2±0.1	-3.7	NS	-5.4	-2.9	-3.1	-1.5	-4.7	-2.2	-13	-3.8
	Hotrienol[B]	53834-70-1	Fennel	3.8±0.1	5.8±0.3	NS	NS	-1.2	NS	NS	1.3	NS	NS	-1.3	NS
	Lemonene[A]	92-52-4	Green	0.16±0.01	0.47±0.03	-5.9	-7.6	NS	NS	-5.7	-5.0	-3.7	-3.7	-4.3	-4.1
	Linalool[A]	78-70-6	Fruity	2.6±0.03	28±0.3	1.3	NS	-1.2	NS	NS	1.6	1.4	1.6	NS	1.2
	Nerol[A]	106-25-2	Citrus	6.7±2	3.5±0.5	-8.4	-3.3	2.1	2.6	-13	-3.7	-9.9	-3.4	NS	-4.8
	Trans-Nerolidol[A]	142-50-7	Green	6.5±0.5	5.2±0.05	-1.4	NS	NS	1.4	-4.2	-1.7	-1.8	NS	-14	-11

	Terpinen-4-ol[A]	562-74-3	Spicy	0.11±0.02	0.078±0.006	NS	2.1	1.5	1.9	NS	1.4	NS	1.7	NS	NS
	Terpinolene[A]	586-62-9	Woody	0.11±0.006	0.25±0.006	-2.8	NS	-1.2	1.1	-3.1	-1.7	-1.7	NS	-2.8	-2.0
C_{13} norisoprenoids	Beta-damascenone[A]	23696-85-7	Rose	9.5±1	7.1±0.2	2.8	NS	NS	NS	1.8	NS	1.8	NS	1.5	-1.4
	1,1,6-trimethyl-1,2-dihydronaphthalene (TDN)[B]	30364-38-6	Licorice, petrol	0.36±0.03	0.18±0.02	NS	1.4	-1.6	-1.3	NS	NS	NS	NS	-1.6	-1.6
	Vitispirane[B]	65416-59-3	Floral	2.2±0.3	1.0±0.03	-4.5	-2.0	-1.3	1.1	-5.1	-2.1	-4.8	-2.3	-5.9	-4.1
Thiols	3-Methylthio-1-propanol[A]	505-10-2	Sulfurous, Onion	3.2±0.5	0.66±0.06	1.6	14	1.7	6.0	-1.9	4.5	NS	NS	-4.3	-2.3

S.B: Sauvignon Blanc
K.G.: *K. gamospora*
L.T.: *L. thermotolerans*
M.P.: *M. pulcherrima*
T.D.: *T. delbrueckii*
Z.K.: *Z. kombuchaensis*
A: Identification based upon purchased standard references
B: Tentative identification based on mass spectral pattern
***: ug/L equivalent of 2-octanol internal standard**

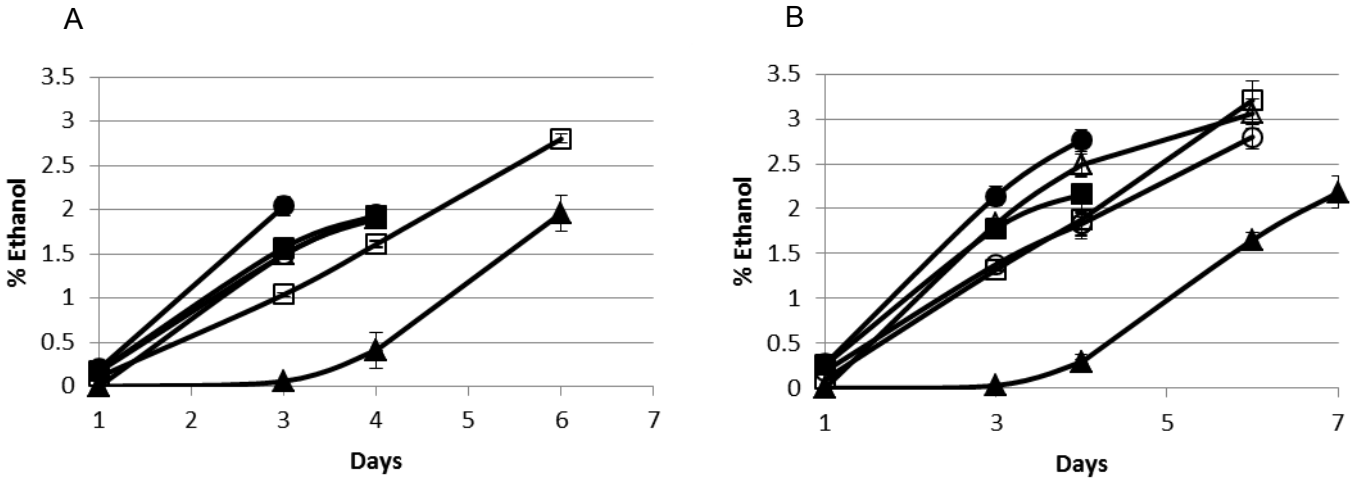

Fig. 1

Growth kinetics as shown by ethanol production over time in Sauvignon Blanc must (A) and Syrah must (B) fermented with single yeast cultures: *S. cerevisiae* (●), *K. gamospora* (■), *Z. kombuchaensis* (▲), *T. delbrueckii* (○), *M. pulcherrima* (□), *L. thermotolerans* (∆)

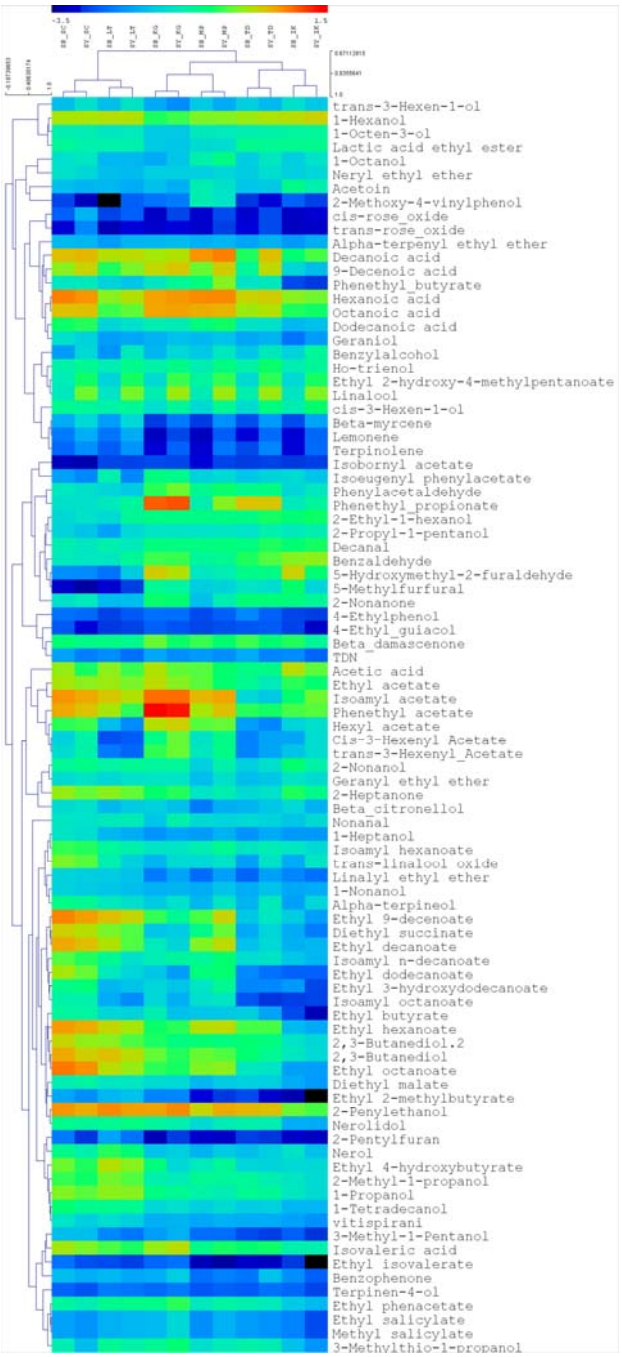

Fig 2.

Relative concentrations of the metabolites in the different musts visualized as a heat map. Normalized peak areas were log transformed for better visualization. The dendrogram represents the hierarchical clustering of the samples. SB indicates Sauvignon blanc must while SY indicates Syrah must. The yeast are indicated by the following abreviations: *S. cerevisiae* (SC), *K. gamospora* (KG), *L. thermotolerans* (LT*), M. pulcherrima* (MP), *T. delbrueckii* (TD), *Z. kombuchaensis* (ZK).

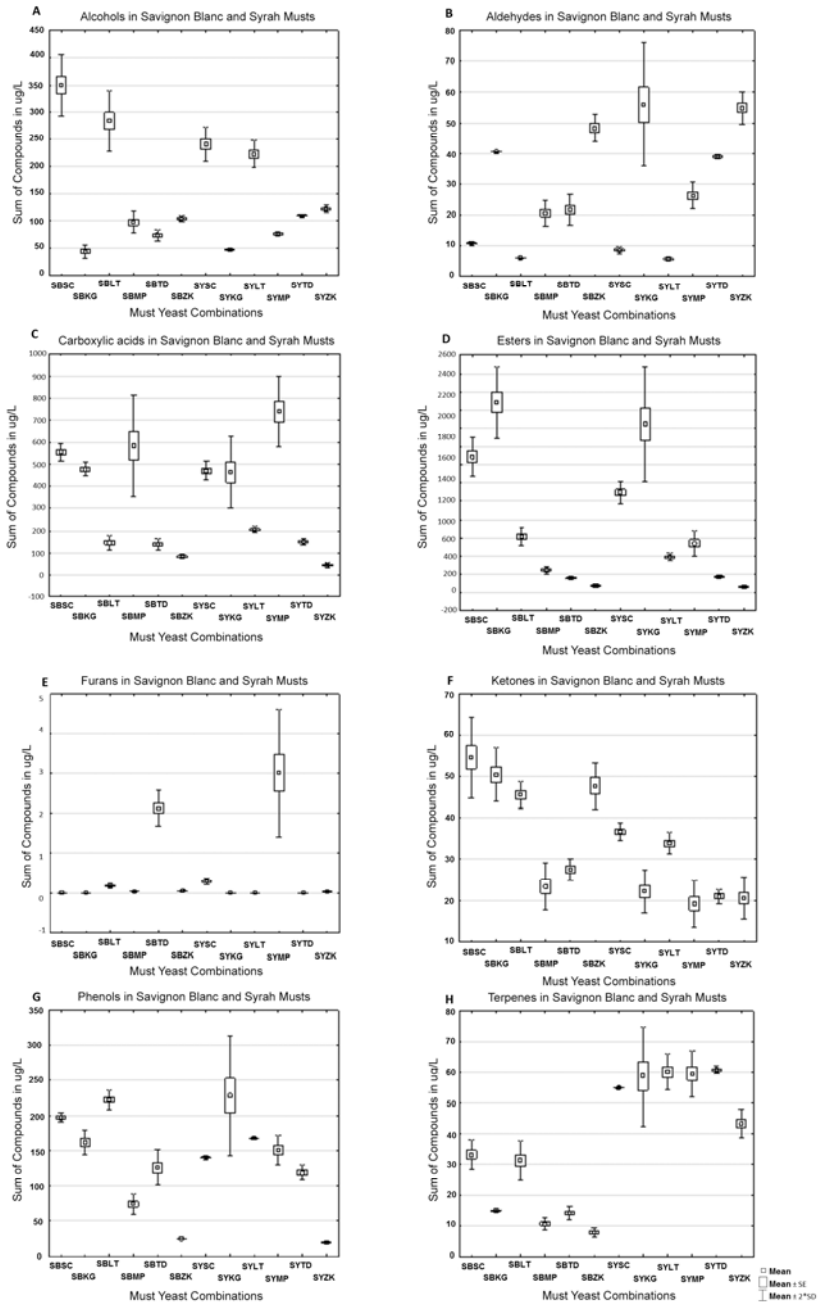

Fig 3.

Box plots expressing the total concentration of all compounds in all fermentations broken down by compound class: A) Alcohols, B) Aldehydes, C) Carboxylic Acids, D) Esters, E) Furans, F) Ketones, G) Ohenols, H) Terpenes. All Sauvignon blanc (SC) fermenations are on the left side of each graph while the Syrah (SY) is on the right. The yeasts are in alphabetical order from left to right fro each must (*S. cerevisiae* (SC), *K. gamospora* (KG), *L. thermotolerans* (LT), *M. pulcherrima* (MP), *T. delbrueckii* (TD), *Z. kombuchaensis* (ZK))

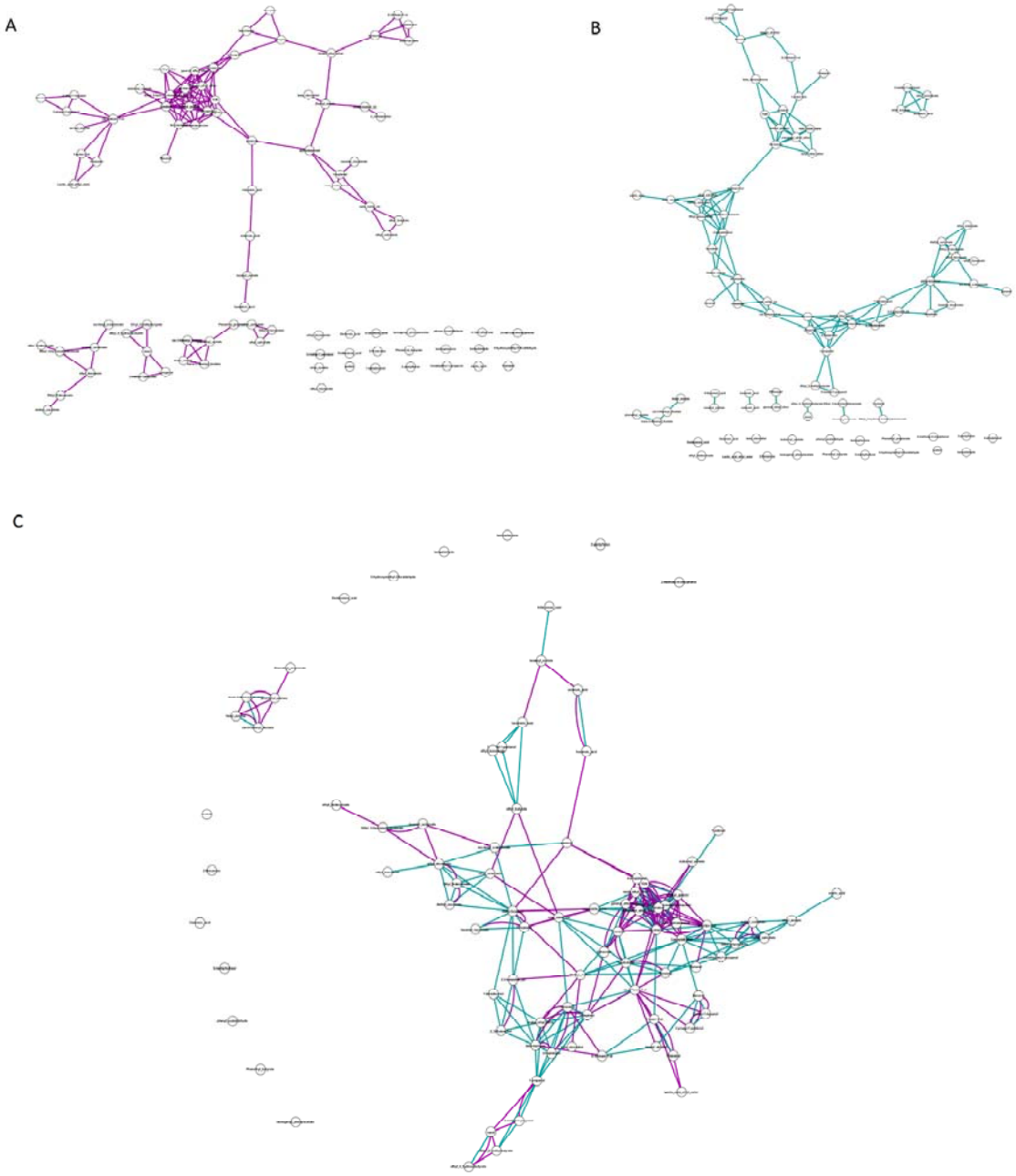

Fig 4.

Correlation networks created comparing the similarity of compounds across samples with a normalized Czekanowski similarity metric. A represents the correlation between compound concentration in Syrah must. B represents the correlation between compound concentration in Sauvignon blanc must and C is an over lap of the two where purple edges are the Syrah and blue edges are the Sauvignon blanc.

Table S1

List of compounds and the µg/L concentrations* found in all the fermentations.

Compound Class	Component Name	Aroma	S.B. with *S.C.*	Syrah with *S.C*	S.B. with *K. G.*	Syrah with *K.G.*	S.B. with *L.T.*	Syrah with *L.T.*	S.B. with *M.P.*	Syrah with *M.P.*	S.B. with T.*D.*	Syrah with *T.D*	S.B with *Z.K.*	Syrah with *Z.K.*
Alcohols	1-Heptanol[A]	Leafy	2.0±0.04	1.7±0.5	0.28±0.04	NS	0.61±0.06	NS	0.32±0.2	NS	0.27±0.01	0.43±0.1	0.30±0.07	NS
	1-Hexanol[A]	Green	50±0.7	52±0.6	13±1	18±1	57±1	57±0.8	32±2	34±1	44±2	NS	NS	78±0.6
	1-Nonanol[A]	Fruity	1.2±0.3	1.0±0.03	0.50±0.1	0.56±0.2	NS	0.70±0.04	0.46±0.08	NS	0.47±0.02	NS	0.42±0.02	0.45±0.02
	1-Octanol[A]	Waxy	1.8±0.1	2.3±0.1	0.44±0.03	0.86±0.1	0.6±0.02	0.59±0.004	3.5±0.3	7.4±0.3	0.98±0.04	NS	1.1±0.1	1.9±0.1
	1-Octen-3-ol[A]	Mushroom	3.2±0.3	3.1±0.5	0.94±0.09	0.92±0.2	2.3±0.1	NS	2.5±0.08	NS	NS	NS	4.5±0.09	5.1±0.2
	1-Propanol[A]	Weak fusel	33±5	23±2	7.4±2	5.4±1	NS	37±1	4.0±0.6	3.1±0.02	5.4±0.2	5.1±0.2	2.5±0.5	1.6±0.2
	1-Tetradecanol[A]	Coconut	9.8±0.6	6.5±1	1.4±0.5	1.7±0.8	6.3±2	NS	1.0±0.6	1.5±0.5	1.0±0.4	1.2±0.2	0.57±0.2	0.57±0.1
	2,3-Butanediol[A]	Sweet	150±20	82±8	25±10	14±5	NS	NS	26±5	21±9	9.8±2	6.1±1	2.5±0.7	1.4±0.2
	2-3-Butandiol_(2)[A]	Sweet	71±16	40.0±8	13±5	7.9±2	37±7	21±5	9.3±1	8.0±3	9.4±2	8.3±3	2.1±0.2	1.4±0.6
	2-Ethyl-1-hexanol[A]	Citrus	1.4±0.3	2.0±0.4	8.06±0.4	7.5±0.6	NS	5.3±0.2	7.6±0.7	8.0±0.3	NS	13±0.8	8.9±0.5	12±0.6
	2-Methyl-1-propanol[A]	Apple	16±3	12±0.9	3.8±0.6	2.8±1	29±1	21±0.8	5.5±0.7	5.1±0.2	5.1±0.7	3.2±1	2.2±0.08	1.6±0.1
	2-Nonanol[A]	Cucumber	5.3±0.2	6.8±0.1	NS	5.0±0.4	4.3±0.4	NS	1.1±0.07	3.9±0.2	1.1±0.03	1.8±0.1	NS	3.9±1
	2-Propyl-1-pentanol[A]	Mild Green	1.2±0.2	0.79±0.08	2.1±0.2	2.0±0.2	0.36±0.03	1.4±0.05	2.0±0.1	2.2±0.1	NS	3.6±0.4	2.5±0.06	3.3±0.1
	3-Methyl-1-pentanol[A]	Fusel	0.67±0.01	0.69±0.06	0.33±0.02	0.50±0.05	0.21±0.02	0.15±0.007	0.13±0.008	0.13±0.007	0.070±0.003	0.050±0.01	0.10±0.03	0.040±0.005

	Benzyl alcohol[A]	Fruity	0.32±0.02	1.4±0.04	0.58±0.02	NS	0.26±0.009	NS	0.92±0.05	2.5±0.2	0.82±0.06	NS	1.2±0.06	5.0±0.9
	cis-3-Hexen-1-ol[A]	Green	5.6±1	5.3±0.01	1.8±0.4	NS	NS	5.6±0.1	1.5±0.3	NS	1.6±0.2	NS	3.3±0.4	11±0.3
	trans-3-Hexen-1-ol[A]	Green	0.69±0.02	1.9±0.003	NS	0.24±0.01	NS	NS	0.97±0.04	0.45±0.03	1.1±0.1	0.62±0.2	1.8±0.1	0.90±0.04
Aldehydes	Benzaldehyde[A]	Almond	2.6±0.2	2.4±0.01	17±0.2	17±2	NS	NS	4.8±0.2	6.1±0.3	14±1	NS	36±2	38±3
	Decanal[A]	Sweet	3.3±0.1	2.3±0.005	8.4±0.4	7.8±0.6	NS	2.7±0.1	8.0±0.7	8.4±0.3	NS	13±0.9	9.4±0.5	12±0.6
	Nonanal[A]	Waxy	2.3±0.2	2.0±0.4	0.9±0.5	3.7±0.2	NS	NS	1.5±0.3	NS	1.4±0.3	NS	0.82±0.5	NS
	Phenylacetaldehyde[A]	Green	2.5±0.3	1.8±0.009	15±0.5	27±5	NS	NS	6.3±0.8	11±2	6.5±0.5	NS	1.3±0.1	NS
Carboxylic Acids	9-Decenoic acid[B]	Waxy	38±10	74±26	68±8	NS	13±2	NS	NS	NS	5.1±1	NS	1.3±0.4	NS
	Acetic acid[A]	Sour	41±3	12±0.6	NS	NS	NS	NS	22±5	NS	7.6±0.7	NS	NS	NS
	Decanoic acid[A]	Unpleasant	110±20	120±9	48±3	56±10	58±7	64±5	200.0±30	260±20	13±5	NS	9.7±3	19±6
	Dodecanoic acid[A]	Coconut	12±4	14±5	3.3±1	2.9±2	1.3±0.3	NS	NS	NS	2.1±0.8	1.9±1	0.6±0.3	0.74±0.9
	Hexanoic acid[A]	Sour	250±20	200±3	160±4	NS	37±20	57±2	NS	NS	74±4	88±20	37±3	30±4
	Isovaleric acid[A]	Sweaty Feet	47±5	32±5	NS	61±4	20.0±3	13±1	6.9±0.2	13±3	11±0.5	12±2	3.8±0.3	3.5±0.4
	Octanoic acid[A]	Rancid	110±5	110±1	160±9	NS	18±2	24±2	NS	NS	43±5	NS	13±2	12±1
Esters	a-Terpenyl ethyl ether[B]	Fruity	0.62±0.2	0.58±0.01	NS	NS	NS	NS	NS	NS	NS	NS	0.30±0.05	0.47±0.03
	cis-3-Hexenyl acetate[A]	Green	1.1±0.3	4.0±0.1	7.2±1	25±2	0.08±0.01	0.10±0.003	2.0±0.2	7.2±0.9	0.2±0.01	0.38±0.04	0.41±0.07	1.6±0.3
	Diethyl malate[B]	Brown Sugar	3.1±0.4	4.3±0.1	1.6±0.2	1.5±0.3	NS	2.3±0.2	0.59±0.1	1.0±0.1	1.1±0.1	1.0±0.5	0.45±0.3	0.30±0.2

Diethyl succinate[B]	Fruity	82±9	59±9	0.89±0.05	0.99±0.2	31±4	21±2	6.5±2	25±6	0.44±0.04	1.3±0.3	0.46±0.3	0.17±0.03
Ethyl 2-hydroxy-4-methylpentanoate[B]	Fresh Blackberry	3.0±0.4	12±0.2	1.5±0.06	NS	1.2±0.06	NS	4.6±0.4	16±0.7	1.5±0.1	15±0.2	1.4±0.2	NS
Ethyl 2-methylbutyrate[A]	Fruity	0.41±0.04	0.25±0.02	0.19±0.03	NS	NS	0.75±0.04	0.040±0.002	0.05±0.003	0.070±0.01	0.030±0.01	0.020±0.007	0.010±0.003
Ethyl 3-hydroxydodecanoate[B]	?	6.9±0.4	9.2±0.8	1.3±0.05	0.82±0.2	0.59±0.09	0.81±0.1	2.1±0.3	5.7±0.8	0.21±0.002	0.22±0.08	0.31±0.4	0.060±0.01
Ethyl 4-hydroxybutyrate[B]	Caramel	28±8	13±2	NS	1.3±0.9	58±10	37±2	2.7±0.3	5.2±0.7	1.3±0.07	1.3±0.3	1.4±0.04	1.4±0.1
Ethyl 9-decenoate[B]	Fruity	240±20	170±20	16±1	5.8±2	110±10	71±7	20.0±5	77±20	0.90±0.3	2.3±1	0.98±0.8	0.34±0.1
Ethyl acetate[A]	Fruity	50.0±2	36±3	NS	NS	44±1	NS	24±2	10±0.4	6.0±0.7	2.7±0.1	15±1	11±5
Ethyl butyrate[A]	Fruity	4.5±0.4	4.4±0.2	3.2±0.4	0.74±0.3	1.4±0.08	1.1±0.1	0.88±0.007	0.89±0.08	1.1±0.3	0.39±0.1	0.070±0.02	0.020±0.002
Ethyl decanoate[A]	Sweet	150±20	110±4	2.6±0.2	1.3±0.4	56±6	22±0.8	34±10	67±20	0.75±0.3	1.4±1	0.51±0.06	0.56±0.05
Ethyl dodecanoate[A]	Waxy	48±10	26±3	0.87±0.07	0.32±0.02	2.7±0.4	1.3±0.1	8.5±2	10.0±2	0.23±0.07	0.13±0.03	0.10±0.01	0.11±0.02
Ethyl hexanoate[A]	Fruity	18±10	140±4	12±2	15±4	73±4	49±6	57±40	NS	20.0±2	19±7	0.62±0.2	0.43±0.06
Ethyl isovalerate[A]	Fruity	0.14±0.03	0.074±0.006	NS	NS	0.07±0.006	NS	0.030±0.006	0.020±0.006	0.030±0.002	0.030±0.004	0.050±0.01	0.010±0.001
Ethyl octanoate[A]	Apricot	280±20	200±4	15±0.4	12±1.4	63±2	42±3	32±10	37±10.1	2.5±0.3	2.1±1.04	0.35±0.02	0.34±0.04
Ethyl phenacetate[A]	Floral	4.7±0.1	3.5±0.02	6.8±0.3	15±3	6.2±0.3	6.9±0.5	2.7±0.3	NS	3.4±0.3	NS	0.95±0.04	0.61±0.06
Ethyl salicylate[A]	Mint	0.38±0.04	0.27±0.003	0.63±0.007	1.3±0.2	0.50±0.05	0.57±0.04	0.24±0.03	NS	NS	NS	0.19±0.008	0.070±0.002
Geranyl ethyl ether[B]	Green	1.5±0.04	2.0±0.1	2.1±0.2	NS	NS	NS	0.69±0.06	NS	0.69±0.1	0.86±0.1	NS	NS

	Hexyl acetate[A]	Banana	15±5	17±1	57±20	84±10	0.70±0.1	0.22±0.04	NS	25±4	0.34±0.003	0.21±0.05	1.2±0.3	1.8±0.5
	Isoamyl acetate[A]	Banana	200±10	150±8	310±30	320±20	97±5	58±5	120±8	NS	1.4±0.3	0.91±0.1	11±3	28±7
	Isoamyl hexanoate[A]	Pineapple	17±0.6	14±5	2.3±0.1	2.1±1	4.9±0.6	NS	3.2±2	NS	2.2±0.2	NS	2.3±0.5	NS
	Isoamyl decanoate[B]	Waxy	22±6	14±0.2	3.2±1	4.5±0.5	5.8±0.7	1.9±0.2	5.1±1	NS	1.7±0.1	2.1±0.5	0.86±0.1	0.57±0.1
	Isoamyl octanoate[A]	Sweet	3.9±0.7	3.8±0.3	1.3±0.03	0.47±0.1	0.44±0.04	0.23±0.02	1.8±0.7	NS	0.080±0.02	0.050±0.008	0.05±0.01	0.060±0.004
	Isobornyl acetate[A]	Woody	0.027±0.02	0.023±0.008	0.068±0.01	0.060±0.008	NS	NS	NS	0.060±0.01	NS	0.060±0.006	NS	0.050±0.001
	Isoeugenyl phenylacetate[A]	Spicy	0.35±0.05	0.21±0.05	NS	NS	NS	NS	NS	1.4±0.1	0.87±0.02	NS	NS	NS
	Lactic acid, ethyl ester[A]	Butter-scotch	5.3±0.05	3.5±0.1	1.0±0.3	0.88±0.07	3.7±0.4	NS	1.5±0.1	1.7±0.06	NS	NS	NS	6.3±0.4
	Ethyl linalyl ether[B]	Floral	1.3±0.2	1.0±0.009	0.17±0.03	0.38±0.04	0.97±0.05	NS	0.13±0.05	0.34±0.1	0.11±0.02	0.35±0.04	0.21±0.04	0.13±0.03
	Methyl salicylate[A]	Mint	0.38±0.04	0.27±0.003	0.63±0.007	1.3±0.2	0.50±0.05	0.57±0.04	0.24±0.03	NS	NS	NS	0.19±0.008	0.070±0.002
	Neryl ethyl ether[B]	Clean	1.8±0.3	1.3±0.3	1.1±0.1	NS	0.70±0.05	0.62±0.09	1.1±0.05	NS	0.99±0.4	NS	1.1±0.1	NS
	Phenethyl acetate[A]	Floral, rose	150±6	100±0.9	1200±100	860±100	49±8	16±1	50.0±7	NS	15±0.4	13±2	19±1	22±4
	Phenethyl butyrate[A]	Musty	2.3±0.2	2.7±0.02	NS	5.4±0.6	1.2±0.1	0.85±0.08	NS	35±8	NS	NS	0.070±0.01	0.050±0.01
	Phenethyl propionate[B]	Rose	1.8±0.08	1.9±0.1	380±30	430±70	2.7±0.2	4.8±0.4	NS	35±2	100.0±8	93±10	2.5±0.3	NS
	trans-3-Hexenyl acetate[A]	Fruity	1.5±0.4	4.0±0.2	15±3	25±2	0.16±0.02	0.11±0.01	2.5±0.2	7.2±0.9	0.20±0.01	0.38±0.04	0.80±0.1	1.6±0.3
Furans	2-Pentylfuran[B]	Green	0.16±0.1	0.047±0.004	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.030±0.002

	5-Hydroxymethyl-2-furaldehyde[A]	Fatty	0.24±0.1	0.23±0.04	NS	NS	NS	NS	NS	3.01±0.7	NS	NS	NS	NS
	5-Methylfurfural[A]	Spicy, Caramel	0.032±0.02	0.018±0.003	NS	NS	NS	NS	NS	NS	2.1±0.2	NS	NS	NS
Ketones	2-Heptanone[A]	Spicy	42±6	26±0.6	11±1	NS	NS	NS	2.1±0.3	3.3±0.4	1.6±1	1.9±0.4	13±3	6.7±1
	2-Nonanone[A]	Cheese	1.8±0.1	2.6±0.02	11±2	10.0±2	0.66±0.09	0.68±0.08	0.73±0.1	NS	8.9±0.9	9.0±0.7	8.7±0.9	5.2±0.7
	Acetoin[A]	Sweet, dairy	0.68±0.01	0.55±0.03	NS	NS	NS	0.43±0.03	3.2±0.03	1.8±0.06	NS	NS	NS	3.6±0.4
	Benzophenone[A]	Rose	0.66±0.2	0.48±0.07	NS	NS	NS	NS	0.14±0.01	0.19±0.02	0.15±0.01	NS	0.24±0.1	0.10±0.03
Phenols	2-Methoxy-4-vinylphenol[A]	Clove	0.063±0.01	0.026±0.02	NS	NS	0.01±0.002	NS	3.4±0.3	2.1±0.3	NS	NS	NS	NS
	2-Phenylethanol[A]	Rose	200±3	140±1	160±7	NS	220±6	170±0.7	71±5	NS	130±10	NS	27±2	19±1
	4-Ethylguiacol[A]	Clove, Spicy	0.086±0.02	0.044±0.003	NS	0.080±0.002	NS	NS	NS	0.07±0.001	NS	NS	NS	0.030±0.001
	4-Ethylphenol[A]	Smoke	0.14±0.02	0.12±0.03	NS	NS	0.06±0.01	NS	0.070±0.01	NS	NS	NS	0.060±0.02	0.060±0.01
Terpenes	cis-Rose oxide[A]	Rose	0.10±0.01	0.52±0.009	0.030±0.008	0.060±0.008	0.06±0.004	0.10±0.02	0.040±0.006	0.070±0.01	0.040±0.006	0.070±0.02	0.030±0.005	0.040±0.009
	trans-Rose oxide[A]	Rose	0.041±0.007	0.21±0.003	NS	0.040±0.004	0.02±0.003	0.040±0.008	NS	0.060±0.006	NS	0.060±0.001	NS	0.030±0.002
	Alpha-terpineol[A]	Lilac	6.8±0.6	5.7±0.8	0.96±0.2	1.9±0.2	2.8±0.6	1.8±0.3	0.59±0.07	2.2±0.3	0.54±0.05	2.6±1	0.36±0.06	1.4±0.05
	Beta-citronellol[A]	Citrus	1.5±0.1	1.8±0.1	NS	0.72±0.5	0.59±0.02	0.93±0.06	0.16±0.03	0.48±0.02	0.56±0.05	0.84±0.1	NS	0.50±0.04
	Beta-myrcene[A]	Woody	0.43±0.02	1.4±0.1	0.052±0.005	0.15±0.04	0.33±0.02	NS	0.050±0.01	0.24±0.02	0.070±0.005	0.29±0.09	0.070±0.01	0.39±0.03
	Geraniol[A]	Citrus	1.9±0.5	1.2±0.1	0.50±0.09	NS	0.34±0.02	0.41±0.02	0.60±0.08	0.79±0.07	0.40±0.04	0.53±0.03	0.14±0.006	0.31±0.05

	Hotrienol[B]	Fennel	3.8±0.1	5.8±0.3	NS	NS	3.1±0.1	NS	NS	7.6±0.8	NS	NS	3.0±0.2	NS
	Lemonene[A]	Green	0.16±0.01	0.47±0.03	0.028±0.01	0.060±0.03	NS	NS	0.030±0.005	0.1±0.006	0.040±0.01	0.13±0.03	0.04±0.02	0.11±0.02
	Linalool[A]	Fruity	2.6±0.03	28±0.3	3.3±0.1	NS	2.2±0.02	NS	NS	43±2	3.6±0.2	43±2	NS	34±1.5
	Nerol[A]	Citrus	6.7±2	3.5±0.5	0.79±0.1	1.1±0.2	14±3	9.2±0.5	0.53±0.02	0.96±0.04	0.68±0.03	1.0±0.05	NS	0.73±0.06
	Trans-Nerolidol[A]	Green	6.5±0.5	5.2±0.05	4.7±0.2	NS	NS	7.4±0.3	1.6±0.3	3.0±0.2	3.7±0.3	NS	0.48±0.06	0.46±0.2
	Terpinen-4-ol[A]	Spicy	0.11±0.02	0.078±0.006	NS	0.16±0.03	0.16±0.02	0.15±0.01	NS	0.11±0.009	NS	0.13±0.01	NS	NS
	Terpinolene[A]	Woody	0.11±0.006	0.25±0.006	0.040±0.02	NS	0.10±0.006	0.27±0.01	0.040±0.002	0.15±0.03	0.070±0.01	NS	0.04±0.03	0.12±0.006
C_{13} norisoprenoids	Beta-damascenone[A]	Rose	9.5±1	7.1±0.2	27±2	NS	NS	NS	17±2	NS	18±0.3	NS	14±0.5	5.1±0.3
	1,1,6-trimethyl-1,2-dihydronaphthalene (TDN)[B]	Licorice, petrol	0.36±0.03	0.18±0.02	NS	0.24±0.03	0.23±0.01	0.13±0.008	NS	NS	NS	NS	0.24±0.03	0.11±0.002
	Vitispirane[B]	Floral	2.2±0.3	1.0±0.03	0.48±0.07	0.53±0.1	1.7±0.05	1.1±0.01	0.42±0.06	0.50±0.05	0.45±0.1	0.45±0.09	0.36±0.06	0.25±0.03
Thiols	3-Methylthio-1-propanol[A]	Sulfurous, Onion	3.2±0.5	0.66±0.06	5.4±0.5	9.5±2	5.6±0.7	4.0±0.4	1.7±0.3	3±0.4	NS	NS	0.75±0.1	0.28±0.08

S.B: Sauvignon Blanc

S.C.: S. cerevisiae
K.G.: K. gamospora
L.T.: L. thermotolerans
M.P.: M. pulcherrima
T.D.: T. delbrueckii
Z.K.: Z. kombuchaensis
A: Identification based upon purchased standard references
B: Tentative identification based on mass spectral pattern
***: ug/L equivalent of 2-octanol internal standard**

Chapter 4

Research results

Untangling the wine metabolome by combining untargeted SPME-GC×GC-TOF-MS and sensory analysis to profile Sauvignon blanc co-fermented with seven different yeasts

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Metabolomics

Untangling the wine metabolome by combining untargeted SPME-GCxGC-TOF-MS and sensory analysis to profile Sauvignon blanc co-fermented with seven different yeasts

Abbreviated title: Sauvignon blanc and the seven yeast, a metabolomics story

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Abstract

***Saccharomyces cerevisiae* (*SC*) is the main driver of alcoholic fermentation however for aroma and flavor formation in wine non-*Saccharomyces* species can have a powerful effect. This study aimed to compare untargeted volatile compound profiles from SPME-GCxGC-TOF-MS and sensory analysis data of Sauvignon blanc wine inoculated with six different non-*Saccharomyces* yeasts followed by SC. *Torulaspora delbrueckii* (*TD*), *Lachancea thermotolerans* (*LT*), *Pichia kluyveri* (*PK*) and *Metschnikowia pulcherrima* (*MP*) were commercial starter strains, while *Candida zemplinina* (*CZ*) and *Kazachstania aerobia* (*KA*), were isolated from wine grape environments. Each fermentation produced a distinct profile both sensorially and chemically. *SC* and *CZ* wines were the most distinct in both of these cases. *SC* wines had guava, grapefruit, banana, and pineapple aromas while *CZ* wines was driven by fermented apple, dried peach/apricot, and stewed fruit as well as sour flavor. Chemically over 300 unique features were identified as significantly different across the fermentations. *SC* wines had the highest number of esters in the highest relative concentration but all the yeasts had distinct ester profiles. *CZ* wines displayed the highest number of terpenes in high concentration but also produced a large amount of acetic acid. *KA* wine was high in ethyl acetate. *TD* wines had fewer esters but three distinctly higher thiol compounds. *LT* wines showed a relatively high number of increased acetate esters and certain terpenes. *PK* wines had some off odor compounds while the *MP* wines had high levels of different methyl butyl-, methyl propyl-, and phenethyl esters. Overall, this study gives a more detailed profile of these yeasts than anything previously reported.**

Keywords: non- *Saccharomyces,* SPME-GCxGC-TOF-MS, Sensory, Sauvignon blanc

4.1 Introduction

Wine has been consumed by humans for thousands of years and for the majority of that time it was produced by crushing grapes and allowing them to ferment using the organisms present on the grapes and in the surrounding environment. There was relatively little a wine maker could do to control the quality of the final product. After Louis Pasteur discovered that yeasts were behind the conversion of sugars into ethanol more than 150 years ago, the wine industry slowly began to move away from some of its somewhat risky and unpredictable production methods however (Hutkins 2006). The use of spontaneous fermentation for example slowly gave way to intentional inoculation of meticulously selected and maintained Saccharomyces cerevisiae starter cultures to ensure a more consistent and predictable product vintage to vintage. We now understand that wine is the result of a complex biological process that takes place between grapes, microorganisms (yeasts, bacteria and fungi), vinification and the wine cellar environment (Fleet 2003). Of all the yeasts found to be associated with the winemaking process, *S. cerevisiae* is indeed by far the most capable and reliable ethanol producer. When it is inoculated at high cell density, it can drastically reduce the chances of stuck fermentation or the production of off-flavors that can come from the unwanted growth of other organisms (Fleet 1993). This simply owes to the fact that it can rapidly outcompete other yeast and bacterial species as well as quickly produce an environment inhospitable to most other organisms primarily through the production of ethanol. However, while this may reduce sources of microbial spoilage, some winemakers feel that this has resulted in a lack of organoleptic complexity. It has been shown in recent years that certain indigenous non-*Saccharomyces* yeasts can contribute to distinct regional and desirable characteristics of wine when inoculated at high concentrations (Jolly et al. 2006).

This has prompted an interest in beginning to understand the specific influences of non-*Saccharomyces* yeasts in winemaking (Andorrà et al. 2010, 2012; Benito et al. 2015; Ciani and Comitini 2010a; Comitini et al. 2011; Dashko et al. 2015; Jolly et al. 2014; Sadoudi et al. 2012; Sun et al. 2014; Zott et al. 2011). Even though the impact of non-*Saccharomyces* yeasts is usually limited because of the fast fermentative metabolism of *S. cerevisiae*, research has shown that this impact may be enhanced when non-*Saccharomyces* yeasts are inoculated at high cell density. However, because most non-*Saccharomyces* yeasts cannot ferment to dryness, *S. cerevisiae* must also be inoculated along with the non-*Saccharomyces* yeast when they are used intentionally. Two modes of inoculation are usually envisaged: staged (sometimes called sequential) and co-inoculations. In co-inoculation, all yeasts are added to the must at the same time while in staged inoculation, the non-*Saccharomyces* yeasts are added first, allowed to ferment for a given amount of time, and the *Saccharomyces* yeasts are added to finish the fermentation. Staged inoculations are of particular interest since they can ostensibly allow for even greater control over the species fermentation progress and thus the aroma and flavor profile of a fermentation. Both strategies have been shown to mimic the results of natural fermentations in

having more complex aromas (Ciani and Maccarelli 1998; Romano, Fiore, et al. 2003). The principal outcomes of fermentations conducted with the aid of non-*Saccharomyces* yeasts have been documented in literature already mentioned here. Nevertheless, the description of the impact of these yeasts is usually restricted to a few specific attributes such as enzyme, acetic acid, glycerol, ethyl acetate, and higher alcohol production (Andorrà et al. 2012; Charoenchai et al. 1997; Clemente-Jimenez et al. 2004; Gobbi et al. 2013; Pina et al. 2004; Rojas et al. 2001; Romano, Granchi, et al. 2003; van Breda et al. 2013; Villena et al. 2007; Wang et al. 2015).

There are between 9 and 15 different yeast genera that are typically reported to be associated with the winemaking process (Johnson and Echavarri-Erasun 2011). Many of these were originally studied in the context of spoilage but this work slowly began to shed light on some potentially beneficial aspects of these yeasts. For example, early work showed that the *Candida, Debaryomyces, Hanseniaspora, Hansenula, Kloeckera, Metschnikowia, Pichia, Saccharomyces* and *Torulaspora* genera isolated from wines could produce extracellular enzymes such as pectinases, amylases, lipases, proteases and glucosidases (Charoenchai et al. 1997). *β*-Glucosidases are of particular interest for their ability to liberate otherwise bound terpenes and thus have a direct impact on wine aroma. This work was expanded on and complemented by investigations of the specific behaviors of certain species in grape must (Ciani and Maccarelli 1998; Esteve-Zarzoso et al. 1998). Studies began to characterize the macronutrient consumption as well as macromolecule production in single and mixed fermentations. This in turn gave way to more targeted studies of the potential impact of specific yeast (Andorrà et al. 2012; Anfang et al. 2009; Azzolini et al. 2012; Ciani et al. 2006; Clemente-Jimenez et al. 2004, 2005; Dias et al. 2003; Gobbi et al. 2013; Pina et al. 2004; Romano, Granchi, et al. 2003; Romano, Fiore, et al. 2003; Wang et al. 2015; Zott et al. 2008). Based on this research, commercial non-*Saccharomyces* starter cultures have recently been developed for use in wine production and are comprised of the following yeast species: *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Pichia kluyveri* and *Metschnikowia pulcherrima*. Nevertheless, compared to *S. cerevisiae*, little research has been conducted that can indicate specifically what metabolic profiles to expect from these yeasts under various fermentation conditions. Indeed, though the mounting evidence supports the use of these yeasts to help improve wine aroma, the majority of the previously mentioned studies are somewhat limited in scope. They focus either on enzyme production or target ester and alcohol production and only Gobbi et al. (2013) complemented their targeted chemical analysis of *L. thermotolerans* and *S. cerevisiae* co-fermentation with sensory work. Therefore there is still a knowledge gap on the impact of these yeasts during wine fermentations.

In this study we specifically compared untargeted volatile compound profiles and sensory analysis data of Sauvignon blanc wine fermented sequentially with six different *non-Saccharomyces* yeasts. Of the six non-*Saccharomyces* species used, four were commercial starter strains, *Torulaspora*

delbrueckii, *Lachancea thermotolerans*, *Pichia kluyveri*, and *Metschnikowia pulcherrima,* while the other two, *Candida zemplinina,* and *Kazachstania aerobia,* are laboratory strains. The goal of this study was to expand on previous work where only the profile of the non-*Saccharomyces* yeasts in single fermentation were characterized (Beckner Whitener et al. 2015). This study completed the wine fermentations through the addition of *S. cerevisiae* in order to gain a better understanding of the aroma compounds present in the final wine following the use of the selected non-*Saccharomyces* yeasts in sequential inoculation. The potential metabolic implications, as well as how these compounds might contribute to the perceived sensory attributes of the finished wine product were assessed.

4.2 Materials and Methods

4.2.1 Grapes, Yeasts, and Chemicals

Sauvignon blanc grapes (vintage 2014) were obtained from the vineyards at Welgevallen Experimental Farm, Stellenbosch University, Stellenbosch, South Africa. The clone was SB 316 and rootstock was R110, the vineyard was planted in 1991. The trellis system used was a seven wire hedge trellis with moveable foliage wires and grapevines were spaced at 2.7 x 1.5 m with a east-west row direction. Grapevines were unilateral cordon-trained and spur pruning was applied. The grapevines were not irrigated and the vineyard was established on a duplex Hutton/Glenrosa soil form according to the 1992 South African Binomial Soil Classification system. *S. cerevisiae* (Enoferm M2®, Lallemand Inc., Quebec, Canada), *T. delbrueckii* (Biodiva®, Lallemand Inc., Quebec, Canada), *M. pulcherrima* (Flavia®, Lallemand Inc., Quebec, Canada), *P. kluyveri* (Viniflora® FROOTZEN™, Chr. Hansen, Horsholm, Denmark), *L. thermotolerans* (Viniflora® CONCERTO™, Chr. Hansen, Horsholm, Denmark), *C. zemplinina* (Institute of Wine Biotechnology (IWBT)-Y1082) and *K. aerobia* (IWBT-Y845) were used. Twenty-milliliter glass screw cap vials, sodium chloride (ACS grade), sodium azide, internal standard 2-octanol, a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coating 50/30 µm, 2-cm length SPME fiber was purchased from Supelco by Sigma-Aldrich S.r.l., Milan, Italy.

4.2.2 Wine-making procedure

Fermentations were carried out using Sauvignon blanc grape must. The must was evaluated for initial sugar (21.7 Brix), titratable acidity (5.8 g/L) and yeast assimilable nitrogen (YAN) (170 mg/L) content, as well as pH (3.39). YAN was adjusted by adding 40 mg/L of diammonium phosphate (DAP) to the must. The yeasts were grown in yeast extract peptone dextrose (YPD) medium (Biolab-Merck, Wadeville, South Africa). They were shaken to ensure aerobic conditions at 30 ^{o}C in successively larger batches using a 1% transfer rate starting from 10 mL and ending at 1 L at which point necessary cell concentrations for wine inoculation were obtained via centrifugation. The 11 L stainless steel fermentation vessels containing 10 L of must were inoculated with a

volume of yeast determined from the pre-culture by plate count and optical density to obtain a level of 10^6 cfu/mL. The inoculation levels were confirmed and yeast growth monitored via plate count on WL Nutrient agar (Fluka, Sigma-Aldrich) which allows for visual differentiation of the yeast strains. Fermentations were carried out in triplicate at 15 ^{o}C. The non-*Saccharomyces* yeasts were allowed to ferment until approximately 2% ethanol concentration was reached. At this point, *S. cerevisiae* was added at 10^6 cfu/mL concentration to finish the fermentations after being grown up in the same manner as the non-*Saccharomyces* yeasts. Samples were taken daily to track fermentation progress via plate count and by Fourier-transform mid-infrared spectroscopy (FOSS WineScan) in accordance with the protocol outlined in Nieuwoudt et al. (2006). The apparatus measured levels of glucose, fructose, titratable acidity, volatile acidity, pH, acetic acid and malic acid. The final wines were bottled after clarification via cold rest for one week at -4 ^{o}C in 750 mL glass bottles with screw caps. Wines were then transported to the laboratory of the Department of Food Quality and Nutrition, Research and Innovation Center, Fondazione Edmund Mach (FEM) for chemical analysis. Sensory analysis was performed at the Department of Viticulture and Oenology, Stellenbosch University.

4.2.3 Sensory Evaluation

General Descriptive Analysis was used as the method to evaluate the experimental wines. A panel of 10 judges was selected; all had moderate to good experience in wine evaluation, in particular Sauvignon blanc. The panel was composed of 8 females ranging in age from 25 to 55; and 2 males (aged about 25). A session was completely dedicated to the taste component of the wines and the panel was trained on sweetness, acidity, bitterness and astringency intensities. For this purpose, a commercial Sauvignon blanc wine was spiked with increasing levels of sugar, tartaric acid, quinine and alum respectively. All were over the counter items purchased as a local grocery store. To score the intensity of the attributes of the experimental wines a 100-mm unstructured scale was used, demarked with ‘None’ and ‘Intense’ at the extreme left and right sides, respectively. Panel performance was evaluated using Panel Check (Tomic et al. 2009). The descriptive study was performed in 2 sessions. Panelists were asked to taste in isolated booths and each treatment was presented to them covered in ISO black glasses and marked with three-digit codes. A complete Block Design was used to randomise the distribution of the wines presented to the panellists (Lawless and Heymann 2010). Each judge evaluated each treatment in triplicate.

4.2.4 SPME extraction and GCxGC-TOF-MS analysis

Vials were prepared as follows- 5 ml of wine and 50 µL of 0.5 mg/L 2-octanol were added to 20 mL screw cap vials containing 1.5 g NaCl. A Gerstel MPS autosampler (GERSTEL GmbH & Co. KG) equipped with the standard sample agitator and SPME fiber conditioning station was used to extract the volatiles from the sample vial headspace. GC×GC-TOF-MS analysis of the extracts was performed using a LECO Pegasus-4D system consisting of an Agilent 6890N (Agilent

Technologies) coupled to a LECO Pegasus 4D detector. The system employed a consumables free modulation system. The samples were incubated for 5 min at 35°C under 500 rpm rotation at 10 s intervals. Extraction took place for either 10 s, 5 min, or 30 min prior to desorption in the inlet for 180 s at 250 °C. Quality control (QC) vials containing an equal mix of all wines were spaced at the beginning and every third sample thereafter within each time batch. Each extraction time consisted of only one batch as all samples and spaced QCs fit into a single cooling tray. Helium carrier gas was used with a flow set at 1.2 mL/min and a splitless time of 180 s. The oven was equipped with a 30 m x 0.25 mm x 0.25 µm VF-WAX MS primary column (Agilent Technologies) and a 1.5 m x 0.15 mm x 0.15 µm RXI 17Sil MS secondary column (Restek Corporation, Bellefonte, PA, USA). The GC oven parameters were as follows: initial temperature was 40 °C held for 2 min, followed by an increase to 250 °C at a rate of 6 °C/min, the oven was then held at 250 °C for 5 min before returning to the initial temperature (40 °C). The total cycle time, was 42 min. The modulation period was set to 7 s with a hot pulse time of 1.4 s. The modulator was offset by 15 ^{o}C. The MS protocol consisted of electron ionization at 70 eV with ion source temperature at 230 ^{o}C, a detector voltage of 1543 V with a voltage offset of 200 V, mass range of *m/z* 35-350, and acquisition rate of 200 spectra per second. There was an acquisition delay of 120 s.

4.2.5 Data processing and Alignment

ChromaTOF software version 4.32 was used to perform baseline correction, deconvolution and peak picking of the raw data. The baseline offset was set to 1, just above the noise level. The first dimension peak width was set to 43 s while the second dimension peak width was set to 0.1 s. A factor of 500 was set as the match required to combine peaks in the second dimension. A signal to noise (S/N) of 10 was used for the 10 s and 5 min extraction times data with a minimum S/N of 6 for sub peak retention. A S/N of 100 was used for the 30 min extraction time data with a minimum S/N of 60 for sub peak retention. Traditional, not adaptive, integration was used. Forward library searching was used with the following parameters: Hits to return were set to 10, minimum molecular weight was set to 40, maximum molecular weight was set to 350, the mass threshold was set to 50 and the minimum similarity match was set to 700. The NIST and Wiley libraries were used to achieve level II identification as a defined by Sumner et al. 2007. For alignment the following parameters were used: a mass threshold of 10, a minimum similarity match of 600, the maximum number of modulation periods matching peaks could be apart was set to 1, a maximum retention time difference was set to 7 s, for peaks not found by initial peak finding the signal to noise ratio was set to 5 for the 10 s and 5 min extractions and to 50 for the 30 min extractions, for analytes to be kept they had to be found in all biological replicates within a class. Each yeast species was given its own class.

4.2.6 Statistical Analysis

Each extraction time, 10 s, 5 min and 30 min, was treated as a separate data set in the following way. First, to avoid underestimation of the variance of the data, zero intensity values (undetected

features) were replaced feature-wise by a random number between the lowest detected intensity and zero. Following this, for each feature, a fixed effects linear model was fitted with yeast strain the as fixed effect. This model was used for pairwise comparisons between all wines without correction for multiple testing. Subsequently, the collection of p values for all comparisons were corrected for multiple testing by controlling the false discovery rate (FDR) and q-values were calculated (Strimmer 2008a, 2008b).

To select the compounds of interest a filter with three requirements was applied to the data. Compounds were selected if their q-values for any of the comparisons between any of the wines were below 0.05 and at least one comparison had a fold change greater than 2.5. In addition, the QC samples were used to calculate the relative coefficient of variance (%CV) for each feature across the whole analysis. Only features with %CV lower than 50% in the QC samples were selected. A Venn diagram was generated to illustrate this filtering process for each extraction time Fig. 1. The features that fell into the center of these diagrams were considered significant compounds of interest (COIs) for each extraction time. The peak area values for each of these compounds were used to generate heat maps and PCA plots to better illustrate the data (Fig. 2 and 3). Unit variance scaling was used for PCA and heat map generation as well as the values seen in Table 1 and Table 1S. Values outside the range of 3 standard deviations were reassigned to 3 in the case of the heat maps. The Pearson correlation coefficient and Ward's minimum variance method were used for hierarchical clustering (Murtagh and Legendre 2014). The PCA bi-plots from the sensory data were generated from the analysis performed using panel check (Fig. 3d).

4.3 Results and Discussion

4.3.1 Fermentation progress and primary metabolite production

All fermentations progressed at slightly different rates. *S. cerevisiae* was the quickest fermenter, reaching 2% ethanol in three days (Fig. 4). The first of the non-*Saccharomyces* fermentations to reach 2% ethanol was *L. thermotolerans,* four days after inoculation. Next was *T. delbrueckii* one day later followed by *C. zemplinina* on day six. *K. aerobia* and *M. pulcherrima* each took seven days and the *P. kluyveri* was the slowest at eight days. The order of fermentation speed is comparable to results in our previous study for *L. thermotolerans, T. delbrueckii,* and *M. pulcherrima* (Beckner Whitener et al. 2015). Once the fermentations reached 2% ethanol, *S. cerevisiae* was added to complete the fermentations. The musts inoculated with only *S. cerevisiae* fermented to dryness in 12 days while the rest of the fermentations took between 19 and 24 days with *L. thermotolerans* again finishing first among the non-*Saccharomyces* fermentations. Almost all of non-*Saccharomyces* fermentations showed a similar pattern of sugar consumption in which glucose was consumed faster than fructose. The *C. zemplinina* fermentation stood out in that it

was the only fermentation in which fructose was consumed more rapidly than glucose even after *S. cerevisiae* addition. This was not surprising since *C. zemplinina* is known to be fructophilic and able to survive to the end of fermentation due to its high ethanol tolerance (Rantsiou et al. 2012). It has also been reported that sequential inoculation of *C. zemplinina* produced a wine lower in acetic acid compared to a *S. cerevisiae* pure culture but this was not the case in our study (Englezos et al. 2015). Of all the fermentations conducted, the *C. zemplinina* fermentations produced the most acetic acid (1.37 g/L while the *T. delbrueckii* produced the least (0.07 g/L) (Fig. 5). Despite the relatively large amount of acetic acid in the *C. zemplinina* fermentations the sensory panel did not note an acetic acid fault in the wine. It is worth mentioning however, that the two fermentations that showed the highest amounts of acetic acid did score the closest to the 'sour' descriptor, those being *C. zemplinina* and *K. aerobia* (Fig. 3d). *L. thermotolerans* fermentations were characterized by the least amount of overall titratable acidity as well as the least amount of malic acid at the end of the fermentation. In fact, all of the co-fermentations had lower overall levels of malic acid than the *S. cerevisiae* control (Fig. 5). This confirms previous findings that *S. cerevisiae* is characterized as a poor metabolizer of L-malate (Salmon 1987). The other yeasts in this study have not been investigated for their L-malate metabolism or their ability to metabolize other TCA cycle intermediates as a sole carbon source (Saayman and Viljoen-Bloom 2006). Given the results in this study however it is likely that all of the non-*Saccharomyces* yeasts used here are able to transport and metabolize L-malate as has been shown for *Candida sphaerica, Candida utilis, Hansenula anomal, Pichia stipitis* and *Kluyveromyces marxianus* (Saayman and Viljoen-Bloom 2006).

Even though there were obvious differences in growth patterns and macro metabolite production, all wines did eventually reach approximately the same ethanol concentration of 14% v/v. The presence of the non-*Saccharomyces* yeasts was monitored during the fermentations and it should be noted that the non-*Saccharomyces* yeast populations began to decline as soon as the *S. cerevisiae* was added but that they remained detectable via plate count for between 6 and 10 days (Fig. 4). This indicates that the non-*Saccharomyces* yeasts remained viable and detectable for over half of the total fermentation time. For *C. zemplinina*, *L. thermotolerans,* and *T. delbrueckii* this is in agreement with literature (Azzolini et al. 2012; Kapsopoulou et al. 2006; Maio et al. 2012). For *M. pulcherrima, P. kluyveri,* and *K. aerobia* this has not been previously reported in a sequential wine fermentation. In all likelihood, the yeasts remained metabolically active and thus able to contribute to the organoleptic profile for even longer than this since the non-*Saccharomyces* colonies became difficult to count due to the overcrowding of the *S. cerevisiae.*

4.3.2 Aroma compound presence in Sauvignon blanc due to specific species: chemistry and sensory analysis

GCxGC-TOF-MS is becoming more common in the field of metabolomics as it has proven to be a powerful tool that can increase separation, detection and identification of a wide variety of metabolic analytes compared to 1D GC (Zhang et al. 2012). When Solid-Phase-Microextraction (SPME) is used as an extraction method it is possible to study, with great chemical selectivity and sensitivity, the volatile profile of samples. The different compounds that make up the headspace of wine samples will be present in a broad concentration range, with varying vapor pressures and have different adsorption binding affinities to the SPME fiber. Therefore, this study employed three separate extraction times to increase compound coverage while limiting chromatographic and detector saturation. The 10 s extraction time proved useful for obtaining peak shapes conducive to consistent integration for the most highly concentrated analytes such as esters and alcohols. The 30 min extraction time was used to characterize the smaller but no less important peaks that represent aroma compounds such as terpenes, volatile phenols, thiols and some of the less concentrated esters and alcohols. The 5 min extraction served as a good middle between compounds found in saturation at 30 min but not detected by the 10 s extraction time. Fig. 1S illustrates this finding by showing two compounds. Compound 1 is in saturation at 30 min (Fig. 1Sc) but measurable in both the 5 min (Fig. 1Sb) and the 10 s (Fig. 1Sa) extraction times. The peak shape is however best for measurement in the 10 s chromatogram. Peak 2 in this figure shows the opposite trend. Some compounds were only measurable at 30 min and too small in 5 min and 10 s to be reliable. However, there were some compounds that were reliably measurable at two or all three extraction times and in this case the data was combined and represented as such in Table 1 and Table 1S. Each extraction time consisted of only one batch and the intra-batch reproducibility was assessed by comparing the peak area of the internal standard in each sample. Fig. 2S shows the normalized mean peak area of the internal standard of each sample from each extraction time batch. Together, these three data sets, along with the sensory analysis, provided a highly detailed volatile compound and aroma profile of Sauvignon blanc wines generated in this study.

It is well known that certain sulfur compounds such as 4-mercapto-4-methylpentan-2-one, 4-mercapto-4-methylpentan-2-ol, 3-mercaptohexanol, and 3-mercaptohexyl acetate give Sauvignon blanc its characteristic tropical and green aromas (Tominaga et al. 1998). Though these compounds were not reliably detectable with the analytical method used in this study, likely due to their relatively low concentration, the compounds have a very low sensory detection threshold and thus are easily distinguished by the human olfactory sense at much lower concentrations, the parts per trillion range, than the SPME fiber is capable of detecting (Dubourdieu et al. 2006). It is for this reason that tropical aromas such as guava and passion fruit were a critical part of the sensory

panel evaluation as can be seen in Table 2S. The sensory analysis not only mirrors the untargeted volatile profile but complements and expands it.

Both the sensory and the analytical methods were able to show a distinct separation of the wines co-fermented sequentially with the different yeasts based on their detectable aroma features; this can be seen clearly in the principal component analyses (PCAs) (Fig. 3). The sensory analysis focused on 16 typical Sauvignon blanc aromas. Only 12 of these (Guava, Passion fruit, Grapefruit, Banana, Apple, Pineapple, Cooked vegetable, Solvent, Sherry, Fermented Apple, Dried peach and Stewed fruit) proved to be consistently evaluated and significantly different across all samples according to ANOVA analysis ($p<0.01$). Thus it is not surprising that, with so few parameters, the sensory PCA is able to account for more than 90 % of the total variance. The first principal component axis is largely defined by a difference in the fruity aroma profiles (Fig. 3d). Esters are primarily responsible for the bulk of fruity aromas and flavors in wine and this result could indicate a significant difference in ester production between the yeast species. In fact, the analytical method showed significantly different ester profiles for each fermentation. The basic flavors of sweet, bitter and sour were also found to be significantly different across all samples and were distributed more along the second component. The SPME method on the other hand was able to detect thousands of volatile aroma compounds which after our feature selection was applied cut the number of features down from over 1000 total identified features to 336 compounds found to be statistically significantly different across the fermentations. The breakdown according to extraction time is as follows: 78 compounds for the 10 s extractions, 196 for the 5 min extractions and 239 for the 30 min extractions. Some compounds were reliably extracted by more than one extraction time and their unit variance scaled values were combined and are shown in Table S1. It is clear from the PCA plots that like the sensory analysis the yeasts showed distinct profiles with strong grouping of the biological replicates. This same result is also confirmed by the hierarchical clustering (Fig. 2a-c) in which it is clearly shown that the yeast replicates grouped with themselves and each grouping had a distinct chemical signature. It should be noted that with so many chemical compounds it was only natural that the yeasts grouped together so well and showed such distinct profiles in the chemical data versus the sensory data. There are two possible explanations for this: either by focusing on only 16 compounds, the tasters “missed” significant odors in the wine, or the compounds produced (or not) by the different yeasts are irrelevant from a sensory point of view, because they remain below detection threshold. In all likelihood the explanation lies somewhere between the two. To put this into perspective the most prominent details of these profiles of each fermentation are discussed below on a yeast by yeast basis.

4.3.2.1 Saccharomyces cerevisiae

The panel associated the *S. cerevisiae* fermentations most closely with guava, passion fruit, grapefruit, banana, pineapple and apple. It was least associated with fermented apple, dried peach

and stewed fruit (Fig 3d). Chemically, the *S. cerevisiae* fermentations were distinguished mostly by a group of 65 compounds found to be in the highest relative concentration across all fermentations. These compounds are seen in red in the heat maps (Fig. 2) meaning they consistently showed the highest relative concentration among those samples. Of these, the majority were alcohols and esters associated with green, fruity, and tropical notes (Table 1). This correlates well with the previously mentioned panel findings, specifically the banana, pineapple and apple aromas. A large portion of the most significant compounds have currently no documented aromas or flavors. Some compounds were wholly unidentified features and all of them represent an area of possible future study. Out of all the fermentations, the *S. cerevisiae* showed the highest number of distinguishing esters, alcohols, and other compounds and this is in agreement with the literature (Dubourdieu et al. 2006; Lambrechts and Pretorius 2000; Majdak and Herjavec 2002; Zalacain et al. 2007). Metabolically speaking, there was nothing out of the ordinary for these fermentations and they served well as a control.

4.3.2.2 Candida zemplinina

The sensory panel found that the *C. zemplinina* fermentations had the most distinct aroma profile next to the *S. cerevisiae* fermentations. They were characterized by the guava, fermented apple, sherry, dried peach/apricot, and stewed fruit descriptors. This is not surprising given its profile of compounds found to be significantly higher, which can be seen in Table 1, and are represented in red in the heat maps in Fig. 3. There were 49 features with statistically significantly larger relative peak areas that separated the *C. zemplinina* fermentations from the rest. Of these, 12 were esters, one of which, 2-methyl-propanoic acid ethyl ester, has a very high odor strength and is characterized as sweet, ethereal and fruity with pungent, alcoholic, fusel and rummy descriptors as well. This is likely one of the main contributors to the ‘fermented apple’ aroma described by the panel. All other yeast fermentations showed almost none of this compound comparatively. Also worth noting is the statistically significant presence of relatively large acetic acid and hydroxyl acetic acid peaks in the SPME-GCxGC-TOF-MS analysis. As previously mentioned, the Fourier-transform mid-infrared spectroscopy analysis revealed a relatively high level of acetic acid (Fig 5), and the sensory panel noted this fermentation to more sour than others. This shows all three analysis methods to be both cohesive and complimentary to one another. It is however, in direct contrast to previously published work which indicates that *C. zemplinina* had the capacity to reduce the amount of acetic acid in a wine fermentation especially when used in conjunction with *S. cerevisiae* (Englezos et al. 2015; Rantsiou et al. 2012; Sadoudi et al. 2012). These differences however, could be due to biological variability between different strains used. Indeed, as noted by Englezos et al. (2015), within this species the strain diversity is significant.

The *C. zemplinina* fermentations were also characterized by the largest number of terpenes and sesquiterpenes. Of the 49 significant compounds, 11 were either a terpene or sesquiterpene

including geraniol, nerol, α-pinene, α-farnesene, ocimene, and linalool (Table 1). In general, these compounds are responsible for floral, pine and citrus aromas. In wine, rather than being produced directly by the yeast through a metabolic pathway, terpenes are released when glycosidases such as *β*-glucosidase free bound glycosylated precursors (Carrau et al. 2005). Two previous studies, Englezos et al. 2015 and Sadoudi et al. (2012), looked specifically at terpene content in single and mixed culture fermentations of *C. zemplinina* and *S. cerevisiae*. Englezos et al. (2015) tested 63 different strains and found that only 5% of the isolates showed *β*-glucosidase activity. Sadoudi et al. (2012) found that, in monoculture, *C. zemplinina* produced more norisoprenoids and terpenols but this trend did not hold in mixed fermentation with *S. cerevisiae*. Our results however, indicate that the strain of *C. zemplinina* used in this study may produce relatively high amounts of *β*-glucosidase even in the presence of *S. cerevisiae* resulting in a wine richer in terpenes. Further screening should be carried out on this strain to confirm and quantitate enzyme production.

In summary, the *C. zemplinina* and *S. cerevisiae* fermentations were both the most sensorially and chemically distinct with *C. zemplinina* displaying the highest number of terpenes and sesquiterpenes as well as some more uncommon esters and presenting more dried fruit rather than fresh fruit aromas. Unfortunately, of all the fermentations it also produced the largest amount of acetic acid.

4.3.2.3 Kazachstania aerobia

The strain of *K. aerobia* used for these fermentations was isolated from Cabernet Sauvignon grape must at the IWBT and here for the first time we outline the chemical and organoleptic properties that this yeast is capable of producing in a finished wine product. Chemically speaking, the *K. aerobia* only showed 30 compounds to be statistically significantly different from the other fermentations. Though less than *C. zemplinina'*s 49, they still provide an interesting picture of what this yeast can bring to a wine fermentation.

The sensory panel agreed that the *K. aerobia* fermentations were driven more by solvent and bitter characteristics and slightly by the dried or stewed fruit aromas than the fresh ones (Fig. 3d). The chemical analysis revealed that the bulk of the compounds, 12 out of the 30, found to be positively different from the other fermentations were ethyl and acetate esters including ethyl acetate. This is most likely the cause of the solvent aroma. This correlates well with the fermentation data which revealed that *K. aerobia* fermentations had the second highest volatile acidity level of which ethyl acetate is a contributor (Fig. 5). 2-Phenethyl acetate and 6-methyl-2-heptanol acetate were two other acetate esters found to be in higher relative concentration. It is interesting to note that the higher alcohols corresponding to the acetate esters in these fermentations were not shown to be significantly higher. The next largest group of compounds found to be significantly positively different was terpenes. α-farnesene, α-terpinene, nerol, *m*-cymene, and terpinolene all showed

only trace peaks in the *S. cerevisiae* fermentations but much more substantial peaks in the *K. aerobia* fermentations. Though not responsible for the majority of a Sauvignon blanc flavor profile, terpenes are beneficial in their ability to provide complexity via subtle earthy, woody, citrus and floral undertones. They enhance and complement the more known fruity and floral notes provided by the esters. Besides a few alcohols, acids, aldehydes, and alkenes the rest of the *K. aerobia's* chemical profile was made up of six compounds which could not be identified based on their mass spectra.

Since *K. aerobia*'s genome has yet to be fully sequenced, it is difficult to point to a specific cause for the abundant presence of these compounds relatively to the other fermentations. However, *Kazachstania*'s nearest genetic relative is the *Saccharomyces* genus. It stands to reason that they share many of the same genes and thus regulatory pathways (Kurtzman 2003).

To recap, the *K. aerobia* fermentations showed relatively high ethyl acetate, ester and terpene production and a few compounds that could not be identified. No major off-flavors were noted either chemically or sensorially.

4.3.2.4 Torulaspora delbrueckii

T. delbrueckii has been used in winemaking for years and is one of a few non-*Saccharomyces* species commercially available for use in wine and beer production. While it may be the best studied species of the genus, like all wine-related non-*Saccharomyces* species, it remains poorly understood. Of the studies that have been conducted, it has been reported that wine fermented with *T. delbrueckii* in co-culture with *S. cerevisiae* were typically characterized by low volatile acidity, higher terpenols, 2-phenylethanol and C6 compound production (Ciani and Maccarelli 1998; Renault et al. 2009; Sadoudi et al. 2012; van Breda et al. 2013). Further metabolic and sensory evaluation of this yeast has yet to be done.

Our study showed that sensorially *T. delbrueckii* fermentations were similar to the *L. thermotolerans*, *P. kluyveri* and *M. pulcherrima* all of which were most significantly characterized by the bitter attribute and equidistant from the fresh and dried fruit aromas (Fig. 3d). Fermentation data confirms previous reports in that *T. delbrueckii* produced the least amount to acetic acid and volatile acidity (Fig. 2) (Sadoudi et al. 2012). Chemically, its unique profile was most closely related to the *L. thermotolerans* across all extraction times (Fig. 2). Where it differed from *L. thermotolerans* and in fact all of the other fermentations was that it showed relatively higher concentrations of the sulfur containing compounds 3-(methylthio)-1-propanol, 3-[(2-hydroxyethyl)thio]-1-propanol, thietane, 3-(methylthio)propanoic acid ethyl ester, and 1,3-oxathiane. Moreira et al. (2002) showed that increased amounts of methionine in grape must lead to increase in 3-(methylthio)-1-propanol and 3-(methylthio) propanoic acid ethyl ester among other unidentified sulfur compounds. They also showed that wines made from must generally low in

amino acids had the highest total amount of sulfur compounds. As such, there are two likely causes of the increased sulfur compounds seen in our *T. delbrueckii* fermentations. Either *T. delbrueckii* itself assimilates and catabolizes methionine more readily than *S. cerevisiae* or *T. delbrueckii* creates an amino acid poor environment and facilitates the formation of these compounds by *S. cerevisiae.* As already stated, sulfur containing compounds have generally very low sensory thresholds and these are no exception. 3-(methylthio)-1-propanol has been described as having a raw potato, sulfurous, onion, soup, vegetable odor and 3-(methylthio)propanoic acid ethyl ester has been described as sulfurous, metallic, pineapple, fruity, and ripe pulpy tomato. They both have very high odor strengths and in too high a concentration would undoubtedly contribute to a wine fault. Given that the sensory panel did not identify a sulfurous fault in the *T. delbrueckii* fermentations, it is likely that though they were identified in the chemical analysis as significantly different these compounds were not in high enough concentration to be detected by the human palate. This however, does indicate the need for further study of amino acid catabolism by non-*Saccharomyces* yeasts with a specific focus on how differences may affect the metabolism and volatile compound production of *S. cerevisiae.* Besides these findings, it should be noted that like the *K. aerobia* fermentations there were two analytes found to be significantly higher in the *T. delbrueckii* fermentations that could not be identified.

To summarize, while *T. delbrueckii* may reduce acetic acid in the final fermentation, it does little else to positively enhance the overall aroma profile. The wine showed higher levels of off-odor causing thiol compounds compared to the other fermentations which, while not noted by the sensory panel, could be detrimental to a final product if concentrations become too high.

4.3.2.5 Lachancea thermotolerans

Various studies have investigated the potential use of *L. thermotolerans* in wine making with regards to acetaldehyde, lactic acid, glycerol, 2-phenylethanol, and polysaccharide production as well as *β*-glucosidase activity. It is well established that strains of this species are capable of producing lactic acid and increasing the pH of wine while reducing its volatile acidity. It has also been shown to increase glycerol and 2-phenylethanol concentrations while being a low acetaldehyde producer (Ciani and Comitini 2010b; Ciani et al. 2006; Comitini et al. 2011; Cordero-Bueso et al. 2012; Kapsopoulou et al. 2006). Gobbi et al. (2013) is the most extensive study of this species in wine to date. They report that even in sequential inoculation, *L. thermotolerans* was the dominant species during fermentation and that these fermentations showed reduced 2-methyl-1-propanol and 3-methyl-1-butanol, higher 2-phenylethanol, reduced acetate esters but higher ethyl acetate. The ethyl acetate was below the sensory threshold, however. Some of this is in direct contrast to our findings where our results indicate that the *L. thermotolerans* population was slowly over taken by *S. cerevisiae* after its addition. Another difference was that over half of the esters found to be higher in the *L. thermotolerans* fermentations in our case were acetate esters. The *L. thermotolerans* fermentations were also characterized in our case by the lowest amount of both

titratable acidity and malic acid out of all the fermentations (Fig. 5). Sensorially, these fermentations were mostly characterized along PC2 in the PCA, specifically the pineapple and bitter descriptor and as previously mentioned grouped closely with *T. delbrueckii, P. kluyveri,* and *M. pulcherrima* (Fig. 3d). Chemically, the *L. thermotolerans* and *T. delbrueckii* showed the most similar profiles according to the PCAs and hierarchical clusters. Of the 34 compounds shown to be significantly higher in the *L. thermotolerans* fermentations, 12 of them had no suitable matches in the NIST library. Many of these were small peaks that were only found in the 30 min extractions. The *L. thermotolerans* fermentations contained the largest number of unknown analytes. Only 8 esters were shown to be higher, 4 of those were acetate esters one of which was citronellol acetate. Farnesol, geraniol, α-ionene, and cosmene were found to be highest in the *L. thermotolerans* fermentations. This is supported by previous research which has shown that certain strains of *L. thermotolerans* can have high *β*-glucosidase activity (Cordero-Bueso et al. 2012).

In short, the *L. thermotolerans* fermentations showed a relatively high number of acetate esters and certain terpenes as well as the lowest amount of both titratable acidity and malic acid out of all the fermentations. There were no notable off-flavors in high relative concentration but there were 12 unidentified compounds, the highest number out of all the fermentations.

4.3.2.6 Pichia kluyveri

Despite the fact that this species is commercially available, comparatively even less research than on the other non-*Saccharomyces* yeasts has been published on its specific contributions to the wine making process. Anfang et al. (2009) co-fermented Sauvignon blanc with a specific *P. kluyveri* isolate from New Zealand and showed that the resulting wines had elevated levels of 3-mercaptohexyl acetate (3MHA), indicating that the specific isolate was capable of releasing more favorable volatile thiols from the Sauvignon blanc must. By contrast, the isolate used in this study did not show a sensorially significant increase in the tropical fruity aromas characterized by 3MHA. In fact, the *P. kluyveri* fermentations fell close to the center of PC1 being equally defined by both fresh and dried fruit aromas (Fig. 3d). Chemically, previous research had shown that *Pichia membraenifaciens* was a good acetate ester producer (Viana et al. 2008). However, this trait does not seem to carry over to *P. kluyveri* when compared to the other yeast in this study. This is unsurprising given the high amount of biodiversity observed in the *Pichia* genus (Domizio et al. 2011). Our study shows for the first time an in depth chemical profile of *P. kluyveri*. In both the PCAs and heat maps the *P. kluyveri* grouped most closely with the *M. pulcherrima* (Fig. 2 and Fig. 3a-c). There were only 23 compounds found to be significantly higher in the *P. kluyveri* over all of the other fermentations. Eight of these were esters with significantly fruity aromas, three of which were 3-methylbutyl esters of three different organic acids (Table 1). 3-Methyl-butanoic acid (isovaleric acid) was also relatively high. This compound is associated with an off-putting sour, sweaty, and cheesy aroma and in too high a concentration is considered a wine fault. It is a

product of L-leucine catabolism and can undergo esterification to create 3-methyl-butanoic acid ethyl ester which has a much more pleasant, fruity aroma. This compound was one of the esters present in relatively high concentration in the *P. kluyveri* fermentations. Another potentially fault inducing compound found to be higher was phenethylamine. Metabolically, there are two enzymes responsible for the conversion of the amino acid phenylalanine to phenethylamine: Aromatic-L-amino-acid decarboxylase and phenylalanine decarboxylase, either of which could have been up-regulated in either the *P. kluyveri* or the *S. cerevisiae*. Ultimately, neither of these potential fault compounds was in high enough concentration to have a sensory impact as the sensory panel did not note an off aroma in the wine. However, given these issues, combined with the lack of notable positive sensory attributes, this particular strain of *P. kluyveri* is conceivably not as good a candidate for Sauvignon blanc production as others covered by this study.

4.3.2.7 Metschnikowia pulcherrima

The *M. pulcherrima* fermentations were, sensorially, closest to the *P. kluyveri* fermentations and similarly not strongly associated with either the fresh or dried fruit aromas but fell closer to the sweet, bitter and solvent traits. Unlike the *P. kluyveri* fermentations however, chemically, there were no discernible off-aromas. A common isolate in vineyards and from grape must, *M. pulcherrima* has long been associated with grapes and wine and early research into the potential of this species showed that certain isolates displayed a high *β*-glucosidase activity (Fernández et al. 2000). Our study indicates that while some terpenes were higher in the *M. pulcherrima* fermentations when compared strictly to the control other yeasts showed higher amounts (Table 1S). Clemente-Jimenez et al. (2004) reported that *M. pulcherrima* produced high amounts of 2-phenyl ethanol and our findings support that as well. Of the thirty compounds found to be relatively higher in the *M. pulcherrima* fermentations, over half were esters most of which being either methyl butyl, methyl propyl, or phenethyl esters. Most of these however, have no recorded aroma. Similarly, there were six compounds that could not be identified, making the *M. pulcherrima* fermentations difficult to characterize both from a sensory and a metabolic standpoint.

Sadoudi et al. (2012) is, to date, the most comprehensive study of *M. pulcherrima* in co-culture with *S. cerevisiae*. They observed that fructose was consumed more slowly over the course of co-culture fermentation. This was not the case in our study but *S. cerevisiae* was added much later in our fermentations than the reported 48 h post *M. pulcherrima* inoculation of Sadoudi et al. (2012). They also reported that the co-cultures showed lower acetic acid production compared to the *S. cerevisiae* mono-culture. In our case, the opposite was true though in the *M. pulcherrima* fermentation the acetic acid level, though higher, remained below the sensory threshold (Fig 5). These differences could be due to any number of variables such as yeast-yeast interactions, or changes in regulation of acetic acid metabolism in one or both species as a result of different

fermentation stresses, to name a few. Like many of the other yeasts in this study, *M. pulcherrima* strain differences might be a possible reason for the discrepancies observed between studies.

4.4 Concluding Remarks

In conclusion, of all the yeasts used in this study, *S. cerevisiae* and *C. zemplinina* had the most distinct and remarkable fermentation profiles. However each of the six non-*Saccharomyces* yeast co-fermentations displayed a unique sensory and metabolic profile. We were able to show that the sensory and chemistry methods complemented each other well and gave a much more detailed profile of these yeasts than any previously published work. Overall, our results would suggest that while the non-*Saccharomyces* yeasts produced wines that were unique, *S. cerevisiae* in single culture produced a product with the strongest positive sensory components thanks to high ester production. While it is true that our results are not fully in line with previously published results, this study was strongly dependent on the wine matrix composition, especially amino acids, terpene and thiol precursors, and thus is not 100% reflective of the non-*Saccharomyces* capabilities. Given how little is currently known about these yeasts in wine and their contribution to wine aroma this study served to greatly increase the body of knowledge and understanding of these yeasts and their metabolism in the wine matrix used.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Compliance with ethical requirements This article does not contain any studies with human or animal subjects.

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4.7 Tables and Figures

Table 1 Metabolites and their associated aromas found to be in highest relative concertation among the treatments across all extraction times. The data presented are the average peak areas after unit variance scaling for each yeast responsible for the start of fermentation.

Yeast	Class	Features	CAS #	Aroma and flavor	Odor strength	Extraction times		
						10seconds	**5 minutes**	**30 minutes**
CZ[a]	Acid	3-Methyl-butanoic acid	503-74-2	Sour stinky feet sweaty cheese tropical	High	NF[g] — —	NF[g] — —	1.54 ± 0.30
	Acid	Cyclopentaneundecanoic acid	6053-49-2	NF[g]	NF[g]	NF[g] — —	NF[g] — —	1.41 ± 0.50
	Aldehyde	Octanal	124-13-0	Aldehydic waxy citrus oran[g]e peel green fatty	High	NF[g] — —	2.05 ± 1.31	NF[g] — —
	Aldehyde	α,4-Dimethyl-3-cyclohexene-1-acetaldehyde	29548-14-9	Spicy herbal	High	NF[g] — —	1.64 ± 0.35	NF[g] — —
	Aldehyde	Decanal	112-31-2	Sweet aldehydic waxy orange peel citrus floral	High	NF[g] — —	1.99 ± 1.76	NF[g] — —
	Aldehyde	Nonanal	124-19-6	Waxy aldehydic rose fresh orris orange peel fatty peely	High	NF[g] — —	2.02 ± 1.49	NF[g] — —
	Amine	Phenethylamine	64-04-0	Fishy	High	NF[g] — —	NF[g] — —	1.60 ± 0.22
	Ester	Ethyl 3-methylbutanoate	108-64-5	Fruity sweet apple pineapple tutti frutti	High	NF[g] — —	NF[g] — —	2.27 ± 0.18
	Ester	3-(Methylthio)propyl acetate	16630-55-0	herbal mushroom cabbage asparagus potato	High	NF[g] — —	NF[g] — —	1.38 ± 0.46
	Ester	Octanoic acid, hexyl ester	1117-55-1	Fruity green waxy berry apple ester	Medium	NF[g] — —	NF[g] — —	1.41 ± 0.16
	Ester	3-Methylbutyl octanoate	2035-99-6	Sweet oily fruity green soapy pineapple coconut	Medium	1.73 ± 0.12	1.78 ± 0.51	1.35 ± 0.28
	Ester	Acetic acid, 2-phenylethyl ester	103-45-7	Sweet, honey, floral, rosy with a slight green nectar fruity body and mouth feel	Medium	1.97 ± 0.54	1.59 ± 0.20	NF[g] — —
	Ester	3-Methylbutyl dodecanoate	6309-51-9	Winey, alcoholic, fatty, creamy, yeasty and fusel	Medium	1.68 ± 1.01	1.66 ± 0.65	NF[g] — —

	Ester	S-Ethyl octanethioate	2432-84-0	NF[g]	NF[g]	1.62	±	0.61	NF[g]	—	—	NF[g]	—	—
	Ester	Ethyl 4-t-butylbenzoate	5406-57-5	NF[g]	NF[g]	NF[g]	—	—	2.09	±	0.77	2.07	±	0.75
	Ester	4-Butyl 1,2-dimethyl 1,2,4-benzenetricarboxylate	54699-35-3	NF[g]	NF[g]	2.00	±	0.17	NF[g]	—	—	NF[g]	—	—
	Ester	2-Methylbutyl decanoate	68067-33-4	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	1.44	±	0.29
	Furan	2,3-Dihydrobenzofuran	496-16-2	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	1.45	±	0.35
	Ketone	3,4-Dihydroxy-3-cyclobutene-1,2-dione	2892-51-5	NF[g]	NF[g]	NF[g]	—	—	1.49	±	0.18	1.41	±	0.47
	Terpene	α-Terpinene	99-86-5	Woody terpene lemon herbal medicinal citrus	Medium	NF[g]	—	—	NF[g]	—	—	1.45	±	0.38
	Unknown	Analyte 1203	NF[g]	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	1.36	±	0.25
	Unknown	Analyte 1053	NF[g]	NF[g]	NF[g]	NF[g]	—	—	1.53	±	0.37	NF[g]	—	—
	Unknown	Analyte 2070	NF[g]	NF[g]	NF[g]	1.42	±	0.33	NF[g]	—	—	NF[g]	—	—
KA[b]	Acid	9-Decenoic acid	14436-32-9	Waxy, green, fatty, soapy with a slight creamy cheese type nuance	Medium	NF[g]	—	—	NF[g]	—	—	1.26	±	1.11
	Alcohol	2-Pentanol	6032-29-7	Alcoholic, fusel, fermented, chocking and musty with sweet white wine top notes with over ripe banana and yellow apple nuances.	Medium	0.84	±	0.34	NF[g]	—	—	NF[g]	—	—
	Alcohol	2-Heptanol, (s)-	6033-23-4	Mushroom oily fatty blue cheese mouldy	Medium	NF[g]	—	—	NF[g]	—	—	1.68	±	0.21
	Aldehyde	1-Cyclohexene-1-carboxaldehyde, 4-(1-methylethenyl)-	2111-75-3	Fresh green herbal grassy sweet mint cumin	High	NF[g]	—	—	NF[g]	—	—	1.52	±	0.68
	Alkene	1-Hexene, 4,5-dimethyl-	16106-59-5	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	1.59	±	0.69
	Alkene	1-Hexene, 4-ethyl-	16746-85-3	NF[g]	NF[g]	NF[g]	—	—	1.34	±	0.49	NF[g]	—	—
	Alkene	Bicyclo[4.2.0]octa-1,3,5-triene	694-87-1	NF[g]	NF[g]	1.83	±	0.86	NF[g]	—	—	NF[g]	—	—
	Ester	Ethyl acetate	141-78-6	Ethereal fruity sweet weedy green	High	1.90	±	0.27	1.88	±	0.75	1.69	±	0.09

Ester	e-11-Hexadecenoic acid, ethyl ester	PubChemCID:5364484	Waxy, fruity, creamy and milky with a balsamic nuance	Low	NF[g] — —	NF[g] — —	1.50 ± 0.53
Ester	10-Undecenoic acid, methyl ester	111-81-9	Fatty waxy citrus earthy fungal rose flora	Medium	NF[g] — —	NF[g] — —	2.17 ± 0.97
Ester	Isoamyl lactate	19329-89-6	Fruity creamy nutty	Medium	NF[g] — —	NF[g] — —	1.68 ± 0.62
Ester	Octyl formate	112-14-1	Fruity rose orange waxy cucumber	Medium	NF[g] — —	NF[g] — —	1.46 ± 0.80
Ester	9-Decenoic acid, ethyl ester	67233-91-4	Fruity, fatty	Medium	1.21 ± 0.45	1.27 ± 0.56	NF[g] — —
Ester	Ethyl e-2-octenoate	7367-82-0	Fruity, pineapple, green with a fatty waxy nuance	Medium	NF[g] — —	NF[g] — —	1.43 ± 0.51
Ester	2-Hexen-1-ol, propanoate, (e)-	53398-80-4	Green, fruity apple and pear pulp with creamy and powdery nuances	Medium	NF[g] — —	1.68 ± 0.69	NF[g] — —
Ester	2-Phenethyl acetate	103-45-7	Sweet, honey, floral, rosy with a slight green nectar fruity body and mouth feel	Medium	NF[g] — —	NF[g] — —	1.34 ± 0.16
Ester	Tridecanoic acid, 3-hydroxy-, ethyl ester	107141-15-1	NF[g]	NF[g]	NF[g] — —	1.24 ± 0.17	NF[g] — —
Ester	6-Methyl-2-heptanol, acetate	67952-57-2	NF[g]	NF[g]	NF[g] — —	NF[g] — —	1.62 ± 0.61
Ether	1,4-dioxane	123-91-1	Faint sweet	Low	NF[g] — —	1.46 ± 0.36	NF[g] — —
Terpene	Benzene, 1-methyl-4-(1-methylethyl)-	99-87-6	Fresh citrus terpene woody spice	High	NF[g] — —	NF[g] — —	1.92 ± 0.89
Terpene	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	99-86-5	Citrusy, woody, terpy with camphoraceous and thymol notes	Medium	NF[g] — —	1.22 ± 0.38	NF[g] — —
Terpene	3,6-Octadien-1-ol, 3,7-dimethyl-, (z)-	106-25-2	Fresh, citrus, floral, green, sweet, lemon/lime and waxy with a spicy depth	Medium	NF[g] — —	NF[g] — —	1.40 ± 0.19
Terpene	1,3,6,10-Dodecatetraene, 3,7,11-trimethyl-, (z,e)-	26560-14-5	Gardinia, floral	Medium	NF[g] — —	NF[g] — —	1.98 ± 0.28
Terpene	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	586-62-9	Sweet, fresh, piney citrus with a woody old lemon peel nuance	Medium	NF[g] — —	1.31 ± 0.19	NF[g] — —

	Unknown	Analyte 551	NF[g]	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	1.73	±	0.45
	Unknown	Analyte 703	NF[g]	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	1.85	±	0.97
	Unknown	Analyte 74	NF[g]	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	2.17	±	0.38
	Unknown	Analyte 1697	NF[g]	NF[g]	NF[g]	NF[g]	—	—	1.47	±	0.32	NF[g]	—	—
	Unknown	Analyte 1935	NF[g]	NF[g]	NF[g]	NF[g]	—	—	1.79	±	0.73	NF[g]	—	—
	Unknown	Analyte 3784	NF[g]	NF[g]	NF[g]	NF[g]	—	—	1.82	±	0.26	NF[g]	—	—
LT[c]	Alcohol	1-Propanol	71-23-8	Alcoholic fermented fusel musty	Medium	NF[g]	—	—	1.80	±	0.18	NF[g]	—	—
	Alcohol	2-Ethyl-1-hexanol	104-76-7	citrus fresh floral oily sweet	Medium	NF[g]	—	—	NF[g]	—	—	1.86	±	0.80
	Alcohol	3-Ethoxy-1-propanol	111-35-3	NF[g]	NF[g]	1.66	±	0.23	1.64	±	0.30	NF[g]	—	—
	Aldehyde	2-Furan carboxaldehyde	98-01-1	Sweet woody almond fragrant baked bread	Medium	NF[g]	—	—	NF[g]	—	—	1.58	±	0.28
	Ester	Isoamyl acetate	123-92-2	Sweet, banana, fruity with a ripe estry nuance	High	NF[g]	—	—	NF[g]	—	—	2.43	±	1.24
	Ester	Citronellol acetate	150-84-5	Floral green rose fruity citrus woody tropical fruit	Medium	NF[g]	—	—	2.01	±	0.40	NF[g]	—	—
	Ester	Isobutyl acetate	110-19-0	Sweet fruity ethereal banana tropical	Medium	NF[g]	—	—	1.79	±	0.40	NF[g]	—	—
	Ester	Ethyl 3-hydroxy tridecanoate	107141-15-1	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	2.05	±	1.47
	Ester	2-Heptenoic acid, ethyl ester, (e)-	54340-72-6	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	2.25	±	0.72
	Ester	3-Methyl heptyl acetate	72218-58-7	NF[g]	NF[g]	2.10	±	1.41	2.13	±	1.36	NF[g]	—	—
	Ester	Ethyl 3,3,4-trimethyl pentanoate	80246-74-8	NF[g]	NF[g]	2.05	±	0.37	NF[g]	—	—	NF[g]	—	—
	Ester	5-chlorooctylacetate	NF[g]	NF[g]	NF[g]	1.83	±	0.06	NF[g]	—	—	NF[g]	—	—
	Ester	Decanoic acid, propyl ester	30673-60-0	Waxy, fruity, fatty, green vegetable, woody, oily, fruity	NF[g]	1.56	±	0.22	2.08	±	0.07	2.05	±	0.65
	Ketone	2-Octanone	111-13-7	Musty, ketonic, bleu and parmesan cheese-like with earthy and dairy nuances	Medium	1.57	±	1.09	NF[g]	—	—	1.61	±	0.97
	Ketone	3-Acetoxy-2-butanone	4906-24-5	Pungent sweet creamy buttery	Medium	NF[g]	—	—	2.39	±	0.63	NF[g]	—	—

	Ketone	2-Undecanone	112-12-9	Waxy, fruity, ketonic with fatty pineapple nuances	Medium	NF[g]	—	—	NF[g]	—	—	1.62	±	0.79
	Ketone	7-Octen-2-one	3664-60-6	NF[g]	NF[g]	NF[g]	—	—	1.64	±	1.32	NF[g]	—	—
	Terpene	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl- (Farnesol)	4602-84-0	Mild fresh sweet floral	Low	NF[g]	—	—	1.77	±	0.23	NF[g]	—	—
	Terpene	2,6-Octadien-1-ol, 3,7-dimethyl-, (e)- (Geraniol)	106-24-1	Sweet floral fruity rose waxy citrus	Medium	NF[g]	—	—	NF[g]	—	—	1.58	±	0.17
	Terpene	Naphthalene, 1,2,3,4-tetrahydro-1,1,6-trimethyl- (alpha ionene)	475-03-6	Violets, floral	Medium	NF[g]	—	—	NF[g]	—	—	2.48	±	0.59
	Terpene/Alkene?	2,6-Dimethyl-1,3,5,7-octatetraene, e,e- (Cosemene)	460-01-5	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	1.81	±	0.32
	Unknown	Analyte 530	NF[g]	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	1.64	±	0.24
	Unknown	Analyte 276	NF[g]	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	1.95	±	0.29
	Unknown	Analyte 925	NF[g]	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	1.96	±	0.92
	Unknown	Analyte 880	NF[g]	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	2.02	±	0.93
	Unknown	Analyte 518	NF[g]	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	2.12	±	0.18
	Unknown	Analyte 586	NF[g]	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	2.26	±	0.13
	Unknown	Analyte 594	NF[g]	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	2.38	±	0.47
	Unknown	Analyte 1895	NF[g]	NF[g]	NF[g]	NF[g]	—	—	1.64	±	0.55	NF[g]	—	—
	Unknown	Analyte 3856	NF[g]	NF[g]	NF[g]	NF[g]	—	—	1.92	±	1.72	NF[g]	—	—
	Unknown	Analyte 1650	NF[g]	NF[g]	NF[g]	NF[g]	—	—	1.97	±	0.04	NF[g]	—	—
	Unknown	Analyte 1780	NF[g]	NF[g]	NF[g]	1.44	±	0.85	NF[g]	—	—	NF[g]	—	—
	Unknown	Analyte 3317	NF[g]	NF[g]	NF[g]	2.31	±	0.72	NF[g]	—	—	NF[g]	—	—
MP[d]	Alcohol	1-Undecanol	112-42-5	Fresh waxy rose soapy clean clothes floral citrus	NF[g]	NF[g]	—	—	NF[g]	—	—	1.76	±	0.75
	Alcohol	Benzeneethanol	60-12-8	Sweet, floral, freeh and bready with a rosey honey nuance	NF[g]	1.54	±	1.36	NF[g]	—	—	NF[g]	—	—
	Alkane	(trans)-3,4-Oxa-2,5-	NF[g]	NF[g]	NF[g]	2.07	±	0.51	NF[g]	—	—	NF[g]	—	—

	dimethylhexane							
Alkene	(4E)-2,3-Dimethyl-1,4-hexadiene	18669-52-8	NF[g]	NF[g]	NF[g] — —	NF[g] — —	1.88 ± 0.30	
Amino Acid	Tyrosine	60-18-4	NF[g]	NF[g]	NF[g] — —	NF[g] — —	1.80 ± 0.61	
Ester	Dibutyl phthalate	88-99-3	Faint odor	Low	NF[g] — —	1.81 ± 0.72	1.96 ± 0.59	
Ester	Butyl octanoate	589-75-3	Butter ether herbal dank	Medium	2.33 ± 0.35	2.22 ± 0.87	1.79 ± 0.52	
Ester	Isoamyl salicylate	PubChemCID:91695386	Floral herbal woody orchid metallic	Medium	1.87 ± 0.76	NF[g] — —	NF[g] — —	
Ester	Isobutyric acid, phenethyl ester	103-48-0	Heavy fruity, honey and yeasty, with balsamic nuances and waxy rosy floral notes on dry out	Medium	NF[g] — —	1.91 ± 1.68	NF[g] — —	
Ester	2-Methylpropyl decanoate	30673-38-2	Oily sweet brandy apricot cognac	Medium	NF[g] — —	2.10 ± 0.81	1.79 ± 1.13	
Ester	2-Methylpropyl benzoate	120-50-3	Sweet fruity musty powdery balsam	Medium	NF[g] — —	2.28 ± 0.41	2.04 ± 0.82	
Ester	2-Phenethyl pentanoate	7460-74-4	Fruity rose leaf	NA	NF[g] — —	NF[g] — —	1.85 ± 1.38	
Ester	2-Methylpropyl hexanoate	105-79-3	NF[g]	NF[g]	2.25 ± 0.75	2.37 ± 0.59	2.43 ± 0.18	
Ester	(E)-Ethyl-3-hexenoate	26553-46-8	NF[g]	NF[g]	2.49 ± 0.68	2.69 ± 0.26	2.64 ± 0.25	
Ester	Methyl 2-aminoacetate	616-34-2	NF[g]	NF[g]	NF[g] — —	1.59 ± 0.11	1.59 ± 0.64	
Ester	Ethyl glutarate	818-38-2	NF[g]	NF[g]	NF[g] — —	1.85 ± 0.67	NF[g] — —	
Ester	2,6-Pyridinedicarboxylic acid, isobutyl phenethyl ester	PubChemCID:91703257	NF[g]	NF[g]	NF[g] — —	NF[g] — —	1.99 ± 1.36	
Ester	Dimethylmalonic acid, ethyl 2-phenethyl ester	PubChemCID:91703267	NF[g]	NF[g]	NF[g] — —	1.86 ± 0.83	NF[g] — —	
Ester	Ethyl 3-methylbutyl butanedioate	PubChemCID:91750109	NF[g]	NF[g]	NF[g] — —	1.95 ± 0.59	1.87 ± 0.68	
Ester	β-Phenylethyl butyrate	103-52-7	Musty sweet floral yeast strawberry	NF[g]	NF[g] — —	NF[g] — —	1.92 ± 1.76	
Ester	3-Methylbutyl dodecanoate	6309-51-9	Winey, alcoholic, fatty, creamy, yeasty and fusel	NF[g]	NF[g] — —	1.96 ± 0.27	NF[g] — —	

	Ketone	3-Ethoxy-2-butanone	1679-38-5	NF[g]	NF[g]	1.84	±	0.79	NF[g]	—	—	NF[g]	—	—
	Lactone	4-(1-Hydroxy-ethyl) γ butanolactone	PubChemCID:12664706	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	1.90	±	0.67
	Unknown	Analyte 1411	NF[g]	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	1.73	±	0.78
	Unknown	Analyte 1030	NF[g]	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	1.97	±	0.99
	Unknown	Analyte 2936	NF[g]	NF[g]	NF[g]	NF[g]	—	—	1.90	±	0.66	NF[g]	—	—
	Unknown	Analyte 3184	NF[g]	NF[g]	NF[g]	NF[g]	—	—	2.12	±	0.29	NF[g]	—	—
	Unknown	Analyte 2651	NF[g]	NF[g]	NF[g]	NF[g]	—	—	2.22	±	0.83	NF[g]	—	—
	Unknown	Analyte 2898	NF[g]	NF[g]	NF[g]	2.26	±	0.28	NF[g]	—	—	NF[g]	—	—
PK[e]	Acid	3-Methyl-butanoic acid	503-74-2	Sour stinky feet sweaty cheese tropical	High	NF[g]	—	—	NF[g]	—	—	1.54	±	0.30
	Acid	Cyclopentaneundecanoic acid	6053-49-2	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	1.41	±	0.50
	Aldehyde	Octanal	124-13-0	Aldehydic waxy citrus orange peel green fatty	High	NF[g]	—	—	2.05	±	1.31	NF[g]	—	—
	Aldehyde	α,4-Dimethyl-3-cyclohexene-1-acetaldehyde	29548-14-9	Spicy herbal	High	NF[g]	—	—	1.64	±	0.35	NF[g]	—	—
	Aldehyde	Decanal	112-31-2	Sweet aldehydic waxy orange peel citrus floral	High	NF[g]	—	—	1.99	±	1.76	NF[g]	—	—
	Aldehyde	Nonanal	124-19-6	Waxy aldehydic rose fresh orris orange peel fatty peely	High	NF[g]	—	—	2.02	±	1.49	NF[g]	—	—
	Amine	Phenethylamine	64-04-0	Fishy	High	NF[g]	—	—	NF[g]	—	—	1.60	±	0.22
	Ester	Ethyl 3-methylbutanoate	108-64-5	Fruity sweet apple pineapple tutti frutti	High	NF[g]	—	—	NF[g]	—	—	2.27	±	0.18
	Ester	3-(Methylthio)propyl acetate	16630-55-0	herbal mushroom cabbage asparagus potato	High	NF[g]	—	—	NF[g]	—	—	1.38	±	0.46
	Ester	Octanoic acid, hexyl ester	1117-55-1	Fruity green waxy berry apple ester	Medium	NF[g]	—	—	NF[g]	—	—	1.41	±	0.16
	Ester	3-Methylbutyl octanoate	2035-99-6	Sweet oily fruity green soapy pineapple coconut	Medium	1.73	±	0.12	1.78	±	0.51	1.35	±	0.28
	Ester	Acetic acid, 2-	103-45-7	Sweet, honey, floral, rosy with a slight green nectar fruity body	Medium	1.97	±	0.54	1.59	±	0.20	NF[g]	—	—

		phenylethyl ester		and mouth feel										
	Ester	3-Methylbutyl dodecanoate	6309-51-9	Winey, alcoholic, fatty, creamy, yeasty and fusel	Medium	1.68	±	1.01	1.66	±	0.65	NF[g]	—	—
	Ester	S-Ethyl octanethioate	2432-84-0	NF[g]	NF[g]	1.62	±	0.61	NF[g]	—	—	NF[g]	—	—
	Ester	Ethyl 4-t-butylbenzoate	5406-57-5	NF[g]	NF[g]	NF[g]	—	—	2.09	±	0.77	2.07	±	0.75
	Ester	4-Butyl 1,2-dimethyl 1,2,4-benzenetricarboxylate	54699-35-3	NF[g]	NF[g]	2.00	±	0.17	NF[g]	—	—	NF[g]	—	—
	Ester	2-Methylbutyl decanoate	68067-33-4	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	1.44	±	0.29
	Furan	2,3-Dihydrobenzofuran	496-16-2	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	1.45	±	0.35
	Ketone	3,4-Dihydroxy-3-cyclobutene-1,2-dione	2892-51-5	NF[g]	NF[g]	NF[g]	—	—	1.49	±	0.18	1.41	±	0.47
	Terpene	α-Terpinene	99-86-5	Woody terpene lemon herbal medicinal citrus	Medium	NF[g]	—	—	NF[g]	—	—	1.45	±	0.38
	Unknown	Analyte 1203	NF[g]	NF[g]	NF[g]	NF[g]	—	—	NF[g]	—	—	1.36	±	0.25
	Unknown	Analyte 1053	NF[g]	NF[g]	NF[g]	NF[g]	—	—	1.53	±	0.37	NF[g]	—	—
	Unknown	Analyte 2070	NF[g]	NF[g]	NF[g]	1.42	±	0.33	NF[g]	—	—	NF[g]	—	—
SC[f]	Acid	Octanoic acid	124-07-2	Fatty waxy rancid oily vegetable cheesy	Medium	NF[g]	—	—	1.02	±	0.20	NF[g]	—	—
	Alcohol	1-Hexanol	111-27-3	Pungent, etherial, fusel oil, fruity and alcoholic, sweet with a green top note	High	1.98	±	0.94	NF[g]	—	—	NF[g]	—	—
	Alcohol	1,2-Propanediol	57-55-6	Faint sweetness	Low	NF[g]	—	—	1.84	±	0.56	NF[g]	—	—
	Alcohol	7-Tridecanol	927-45-7	Musty	Low	NF[g]	—	—	1.88	±	0.99	1.84	±	0.79
	Alcohol	1-Butanol	71-36-3	Fusel oil sweet balsam	Medium	2.56	±	0.25	NF[g]	—	—	NF[g]	—	—
	Alcohol	1-Pentanol, 4-methyl-	626-89-1	Nutty	Medium	1.49	±	0.54	NF[g]	—	—	NF[g]	—	—
	Alcohol	1-Pentanol, 3-methyl-	589-35-5	Pungent, fusel, cognac and wine, cocoa, with green fruity	Medium	2.11	±	0.50	1.64	±	0.43	1.70	±	0.28
	Aldehyde	1-Cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl-	432-25-7	NF[g]	NF[g]	NF[g]	—	—	1.24	±	0.15	NF[g]	—	—

Aldehyde	Pentanal, 4-oxo-	626-96-0	NF[g]	NF[g]	NF[g] — —	2.19 ± 0.31	NF[g] — —
Alkane	Propanal, 2-(acetyloxy)-, (r)-	NF[g]	NF[g]	NF[g]	NF[g] — —	1.42 ± 0.85	NF[g] — —
Alkane	1-Iodotetradecane	19218-94-1	NF[g]	NF[g]	NF[g] — —	NF[g] — —	1.99 ± 0.55
Alkyne	1-Undecyne	2243-98-3	NF[g]	NF[g]	NF[g] — —	NF[g] — —	2.39 ± 0.23
Alkyne	1-Decyne	764-93-2	NF[g]	NF[g]	NF[g] — —	2.32 ± 0.43	NF[g] — —
Ester	Acetic acid, pentyl ester	628-63-7	Ethereal fruity banana pear banana apple	High	2.53 ± 0.56	2.41 ± 0.61	NF[g] — —
Ester	4-Penten-1-ol, acetate	1576-85-8	Green plastic weedy acrylate vegetable metallic cooked meat sulfide	High	2.62 ± 0.32	2.45 ± 0.28	NF[g] — —
Ester	Acetic acid, butyl ester	123-86-4	Sweet, ripe banana, tutti frutti, tropical and candy-like with green	High	NF[g] — —	2.28 ± 0.66	2.52 ± 0.84
Ester	3-Phenyl-1-propanol, acetate	122-72-5	Balsamic, spicy, cinnamic and fruity with honey and hay-like nuances	Medium	NF[g] — —	NF[g] — —	1.99 ± 0.20
Ester	Propyl octanoate	624-13-5	Coconut caco gin	Medium	1.98 ± 0.45	1.53 ± 0.51	NF[g] — —
Ester	Acetic acid, propyl ester	109-60-4	Estry, fruity, etherial, tutti-frutti, banana and honey	Medium	NF[g] — —	2.35 ± 0.52	1.95 ± 0.76
Ester	Undecanoic acid, methyl ester	1731-86-8	Fatty waxy fruity	Medium	1.77 ± 0.32	NF[g] — —	NF[g] — —
Ester	2,4-Octadien-1-ol, acetate, (e,e)-	30361-34-3	Fatty, chicken fat, with a creamy waxy nuance	Medium	NF[g] — —	NF[g] — —	2.16 ± 0.24
Ester	Octanoic acid, ethyl ester	106-32-1	Fruity wine waxy sweet apricot banana brandy pear	Medium	1.69 ± 0.54	NF[g] — —	NF[g] — —
Ester	Acetic acid, phenylmethyl ester	140-11-4	Fruity, sweet, with balsamic and jasmin floral undernote	Medium	NF[g] — —	2.63 ± 0.47	2.24 ± 0.73
Ester	Acetic acid, octyl ester	112-14-1	Green earthy mushroom herbal waxy	Medium	1.50 ± 0.87	NF[g] — —	NF[g] — —
Ester	3-Hepten-1-ol, acetate	3681-71-8	Green tropical banana vegetable fatty	Medium	2.56 ± 0.38	NF[g] — —	NF[g] — —
Ester	Butanoic acid, hexyl ester	2639-63-6	Green, fruity, estry and vegetative with a waxy nuance	Medium	NF[g] — —	2.45 ± 1.12	2.40 ± 1.04

Ester	Acetic acid, hexyl ester	142-92-7	Green, fruity, sweet, fatty, fresh, apple and pear	Medium	2.56 ± 0.47	NF[g] — —	NF[g] — —
Ester	Propanoic acid, hexyl ester	2445-76-3	Pear green fruity musty rotting fruit	Medium	NF[g] — —	NF[g] — —	2.29 ± 0.95
Ester	2-Butenoic acid, ethyl ester, (-z)	6776-19-8	Pungent, sharp, rum- and cognac-like, wth tinny, pineapple, fruity and meaty nuances	Medium	NF[g] — —	1.16 ± 0.43	1.59 ± 0.37
Ester	1-Butanol, 3-methyl-, propanoate	105-68-0	sweet fruity banana pineapple ripe tropical fruit	Medium	NF[g] — —	NF[g] — —	NF[g] — —
Ester	Hexanoic acid, propyl ester	626-77-7	Sweet fruity juicy pineapple green tropical	Medium	1.90 ± 0.20	NF[g] — —	NF[g] — —
Ester	2-Methylbutyl acetate	624-41-9	Sweet, banana, fruity, ripe, estry and tropical with a juicy, fruit-like note	Medium	NF[g] — —	NF[g] — —	2.07 ± 0.68
Ester	Butanoic acid, butyl ester	109-21-7	Sweet, fruity, fresh, diffusive and ripe	Medium	NF[g] — —	2.40 ± 1.14	NF[g] — —
Ester	Octanoic acid, methyl ester	111-11-5	Waxy, green, sweet, orange and aldehydic with vegetative and herbal nuances	Medium	NF[g] — —	1.18 ± 0.40	1.48 ± 0.26
Ester	Butanedioic acid, hydroxy-, diethyl ester, (±)-	626-11-9	Wine fruity apple skin	Medium	NF[g] — —	2.24 ± 0.70	NF[g] — —
Ester	3-Ethoxypropyl acetate	NF[g]	NF[g]	NF[g]	NF[g] — —	1.44 ± 0.31	1.45 ± 0.27
Ester	Diethyle 2-hydroxypentanedioate	NF[g]	NF[g]	NF[g]	NF[g] — —	2.33 ± 0.65	NF[g] — —
Ester	2-Buten-1-ol, 1,4-dimethoxy-, acetate, (e)-	NF[g]	NF[g]	NF[g]	2.70 ± 0.51	NF[g] — —	NF[g] — —
Ester	Hexanoic acid, methyl ester	106-70-7	NF[g]	NF[g]	NF[g] — —	1.03 ± 0.78	NF[g] — —
Ester	1,3-Propanediol, diacetate	628-66-0	NF[g]	NF[g]	NF[g] — —	1.56 ± 1.30	NF[g] — —
Ester	1,4-Butanediol, diacetate	628-67-1	NF[g]	NF[g]	NF[g] — —	NF[g] — —	1.89 ± 0.88
Ester	Ethanol, 2,2'-oxybis-, diacetate	628-68-2	NF[g]	NF[g]	NF[g] — —	NF[g] — —	2.29 ± 0.58

Ester	Butanoic acid, 1-methylpropyl ester	819-97-6	NF[g]	NF[g]	NF[g] — —	NF[g] — —	2.49 ± 1.20
Furan	3-Furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo-	NF[g]	NF[g]	NF[g]	NF[g] — —	NF[g] — —	1.28 ± 0.31
Heterocycle	1,3-Dioxolane, 4,5-dimethyl-2-pentadecyl-	56599-61-2	NF[g]	NF[g]	1.78 ± 0.31	2.13 ± 0.34	2.24 ± 0.41
Ketone	Acetophenone	98-86-2	sweet, cherry pit, marzipan and coumarinic. It has a slight almond nutty and heliotropin-like vanilla nuance	High	NF[g] — —	NF[g] — —	1.31 ± 1.10
Ketone	2,3-Hexanedione	3848-24-6	Sweet, creamy, caramellic, buttery with a fruity jammy nuance	High	NF[g] — —	2.58 ± 0.10	NF[g] — —
Ketone	3-Pentanone, 2,4-dimethyl-	565-80-0	Acetone	Medium	NF[g] — —	NF[g] — —	2.65 ± 0.33
Ketone	2-Butanone, 3-(acetyloxy)-	10150-87-5	NF[g]	NF[g]	NF[g] — —	2.21 ± 0.77	NF[g] — —
Ketone	4-Penten-2-one	13891-87-7	NF[g]	NF[g]	NF[g] — —	NF[g] — —	2.47 ± 0.48
Ketone	4-Hepten-2-one, (e)-	36678-43-0	NF[g]	NF[g]	NF[g] — —	2.45 ± 0.18	NF[g] — —
Ketone	2,5-Furandione, dihydro-3-methyl-	4100-80-5	NF[g]	NF[g]	NF[g] — —	NF[g] — —	1.33 ± 0.16
Ketone	2-Propanone, 1-(1-methylethoxy)-	42781-12-4	NF[g]	NF[g]	1.71 ± 0.21	NF[g] — —	NF[g] — —
Phenol	Phenol, 4-octyl-	1806-26-4	NF[g]	NF[g]	NF[g] — —	NF[g] — —	1.57 ± 0.63
Terpene	2,6-Octadien-1-ol, 3,7-dimethyl-, (e)-	106-24-1	Floral, rosy, waxy, herbal and green with a slight cooling nuance	Medium	NF[g] — —	1.85 ± 0.90	NF[g] — —
Thiol	2-Thiophenecarboxaldehyde	98-03-3	NF[g]	NF[g]	NF[g] — —	1.77 ± 0.18	NF[g] — —
Unknown	Analyte 363	NF[g]	NF[g]	NF[g]	NF[g] — —	NF[g] — —	1.62 ± 0.23
Unknown	Analyte 966	NF[g]	NF[g]	NF[g]	NF[g] — —	NF[g] — —	2.12 ± 0.75
Unknown	Analyte 355	NF[g]	NF[g]	NF[g]	NF[g] — —	NF[g] — —	2.13 ± 0.28
Unknown	Analyte 1158	NF[g]	NF[g]	NF[g]	NF[g] — —	NF[g] — —	2.46 ± 0.06

	Unknown	Analyte 1411	NF[g]	NF[g]	NF[g]	NF[g]	—	—	1.12	±	0.77	NF[g]	—	—
	Unknown	Analyte 1034	NF[g]	NF[g]	NF[g]	NF[g]	—	—	1.61	±	1.51	NF[g]	—	—
	Unknown	Analyte 2547	NF[g]	NF[g]	NF[g]	NF[g]	—	—	2.30	±	0.71	NF[g]	—	—
	Unknown	Analyte 1887	NF[g]	NF[g]	NF[g]	1.63	±	1.61	NF[g]	—	—	NF[g]	—	—
TD[g]	Acetal	1-Ethoxy-1-pentyloxyethane	13442-89-2	NF[g]	NF[g]	1.69	±	1.48	NF[g]	—	—	NF[g]	—	—
	Acid	Benzoylformic acid	611-73-4	NF[g]	NF[g]	NF[g]	—	—	1.74	±	0.83	2.02	±	2.02
	Alcohol	2-Undecanol	1653-30-1	Fresh waxy clean cloth cotton sarsaparilla	Medium	NF[g]	—	—	2.09	±	1.20	2.00	±	2.00
	Alcohol	(2S)-2-Heptanol	6033-23-4	Mushroom oily fatty blue cheese mouldy	Medium	NF[g]	—	—	1.80	±	1.33	NF[g]	—	—
	Alcohol	2-Nonanol	628-99-9	Waxy green creamy citrus orange cheese fruity	Medium	1.95	±	1.39	1.90	±	1.50	2.05	±	2.05
	Alcohol	7-Octen-2-ol	39546-75-3	NF[g]	NF[g]	NF[g]	—	—	2.02	±	1.35	NF[g]	—	—
	Alcohol	1-Chloro-4-hydroxybutane	928-51-8	NF[g]	NF[g]	2.29	±	1.34	NF[g]	—	—	NF[g]	—	—
	Alcohol-Thiol	3-(Methylthio)-1-propanol	505-10-2	Raw potato, sulfurous, onion, vegetable soup	High	2.58	±	0.80	2.50	±	0.95	NF[g]	—	—
	Alcohol-Thiol	3-[(2-Hydroxyethyl)thio]-1-propanol	5323-60-4	Sulfurous onion sweet soup vegetable	High	NF[g]	—	—	1.68	±	0.68	1.61	±	1.61
	Alkane-Thiol	Trimethylene sulfide	287-27-4	NF[g]	NF[g]	NF[g]	—	—	2.00	±	1.42	NF[g]	—	—
	Alkene	4,6,8-Trimethylazulene	941-81-1	NF[g]	NF[g]	NF[g]	—	—	1.84	±	0.20	NF[g]	—	—
	Benzene	1,1'-(1-Methyl-1,2-ethanediyl)bis-benzene	5814-85-7	NF[g]	NF[g]	NF[g]	—	—	1.87	±	1.89	1.69	±	1.69
	Benzene	(1,2,3-Trimethyl-2-cyclopropen-1-yl)-benzene	6393-13-1	NF[g]	NF[g]	NF[g]	—	—	1.64	±	1.21	NF[g]	—	—
	Ester	2-(1-Pentyloxy)-ethyl acetate	NF[g]	NF[g]	NF[g]	NF[g]	—	—	1.88	±	0.52	NF[g]	—	—
	Ester	Butanoic acid, 3-hydroxy-, ethyl ester	5405-41-4	NF[g]	NF[g]	1.52	±	0.77	1.87	±	0.48	2.07	±	2.07
	Ester	Propanoic acid, ethyl	105-37-3	NF[g]	NF[g]	NF[g]	—	—	1.81	±	0.82	NF[g]	—	—

	ester							
Ester	3-Methylbutyl propionate	105-68-0	NF[g]	NF[g]	NF[g] — —	NF[g] — —	1.51 ± 1.51	
Ester	Acetic acid, nonyl ester	143-13-5	NF[g]	NF[g]	NF[g] — —	2.17 ± 1.15	NF[g] — —	
Ester	4-Tert-butylcyclohexyl acetate	5451-55-8	NF[g]	NF[g]	NF[g] — —	2.09 ± 0.79	1.50 ± 1.50	
Ester	Amylpropionate	624-54-4	NF[g]	NF[g]	NF[g] — —	1.87 ± 1.62	NF[g] — —	
Ester	Propanoic acid, 2,2-dimethyl-, 2-phenylethyl ester	67662-96-8	NF[g]	NF[g]	NF[g] — —	2.49 ± 0.33	2.46 ± 2.46	
Ester	β-Phenylethyl butyrate	103-52-6	Musty sweet floral yeast strawberry	NF[g]	NF[g] — —	NF[g] — —	2.06 ± 2.06	
Ester-Thiol	3-(Methylthio)propanoic acid ethyl ester	13327-56-5	Sulfury metallic pineapple fruity ripe pulpy tomato	high	NF[g] — —	2.04 ± 0.50	NF[g] — —	
Ethane	1-Ethoxy-1-pentoxy-ethane	NF[g]	NF[g]	NF[g]	NF[g] — —	1.95 ± 1.92	NF[g] — —	
Ketone	2-Undecanone	112-12-9	NF[g]	NF[g]	NF[g] — —	1.75 ± 1.42	NF[g] — —	
Norisoprenoid	2h-1-Benzopyran, 3,4,4a,5,6,8a-hexahydro-2,5,5,8a-tetramethyl-, (2α,4aα,8aα)-	41678-32-4	NF[g]	NF[g]	NF[g] — —	NF[g] — —	1.86 ± 1.86	
Thiane	1,3-Oxathiane	646-12-8	NF[g]	NF[g]	NF[g] — —	NF[g] — —	2.07 ± 2.07	
Unknown	Analyte 2703	NF[g]	NF[g]	NF[g]	NF[g] — —	1.77 ± 0.71	NF[g] — —	
Unknown	Analyte 4390	NF[g]	NF[g]	NF[g]	NF[g] — —	2.57 ± 1.07	NF[g] — —	

[a] *C. zemplinina*
[b] *K. aerobia*
[c] *L. thermotolerans*
[d] *M. pulcherrima*
[e] *P. kluyveri*
[f] *S. cerevisiae*
[g] *T. delbrueckii*
[h] Not found

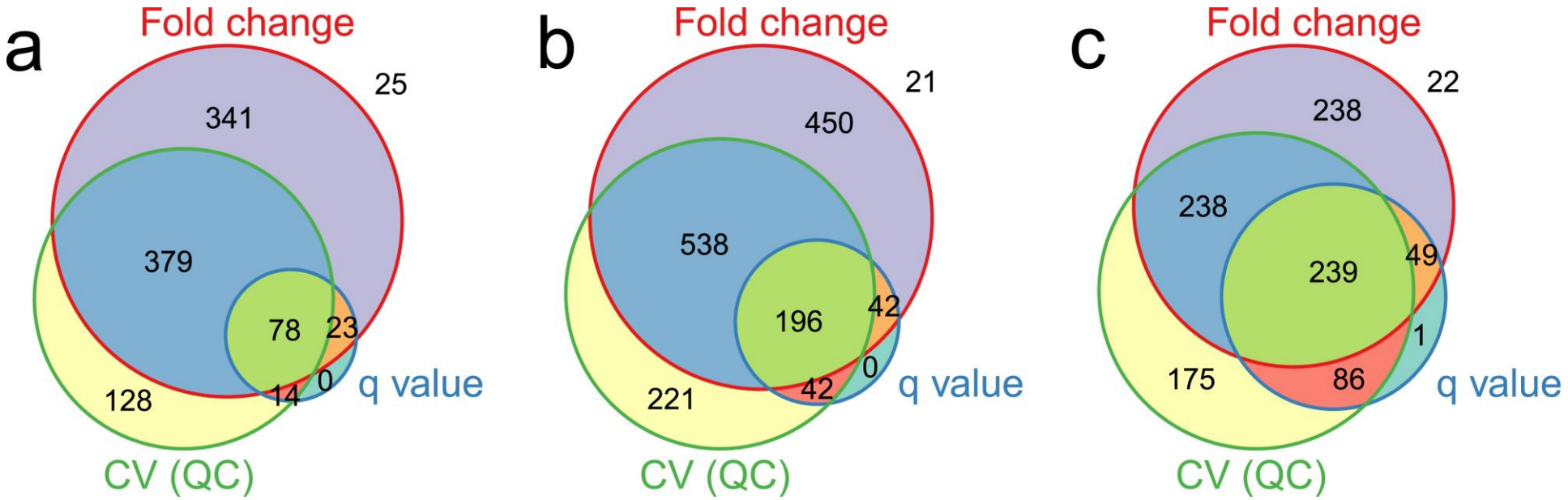

Fig. 1
Venn diagrams in which the center represents significant compounds of interest with q values below 0.05, a fold change of 2.5 or higher and %CV in quality control samples lower than 50%. The data from three extractions times a) 10 seconds, b) 5 minutes, c) 30 minutes are shown.

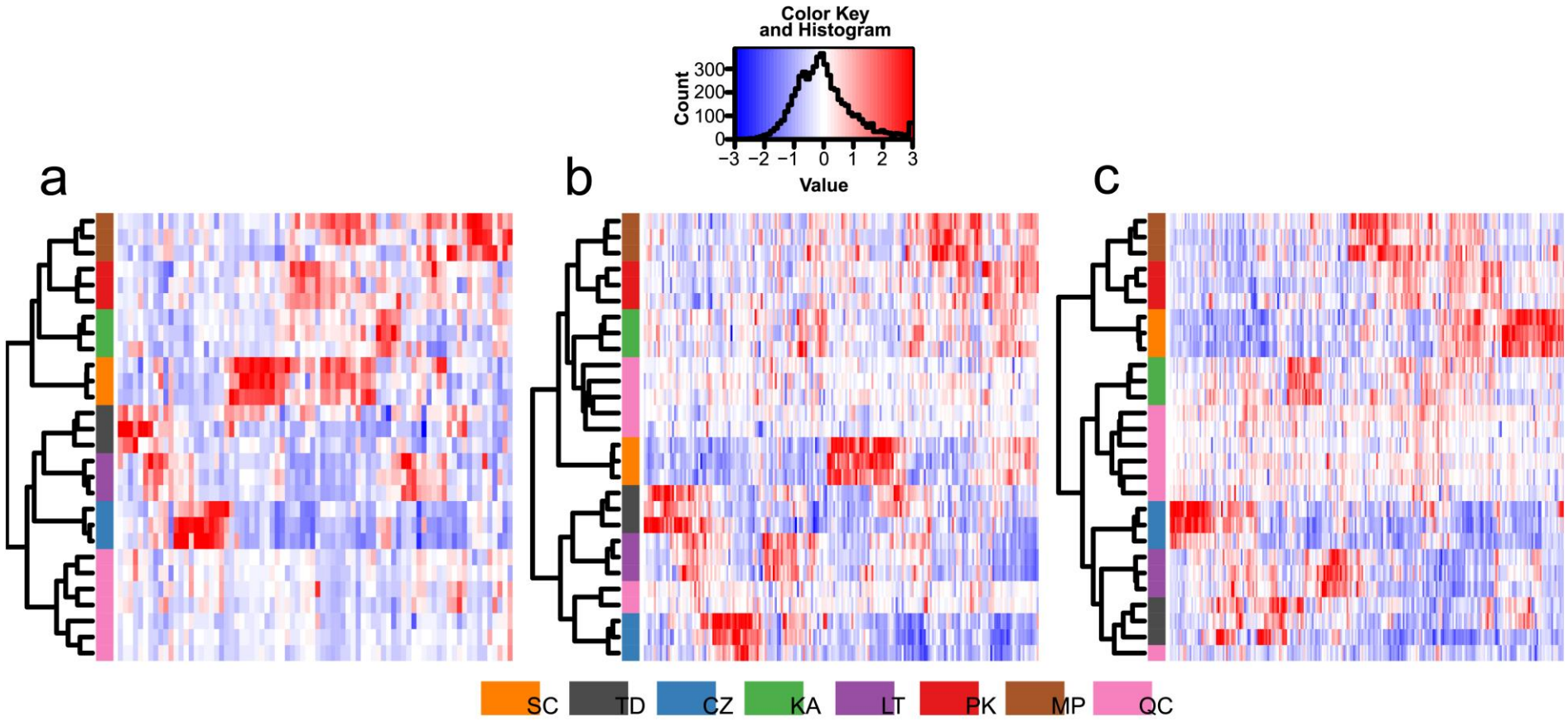

Fig. 2
Heat map of the Venn diagram center features for the a) 10 second, b) 5 minute, and c) 30 minute extraction time data. Ward's minimum variance was used for hierarchical clustering. SC represents *S. cerevisiae* fermentations, TD represents *T. delbrueckii* fermentations, CZ represents *C. zemplinina* fermentations, KA represents *K. aerobia* fermentations, LT represents *L. thermotolerans* fermentations, PK represents *P. kluyveri* fermentations, MP represents *M. pulcherrima* fermentations and QC represents the quality control samples.

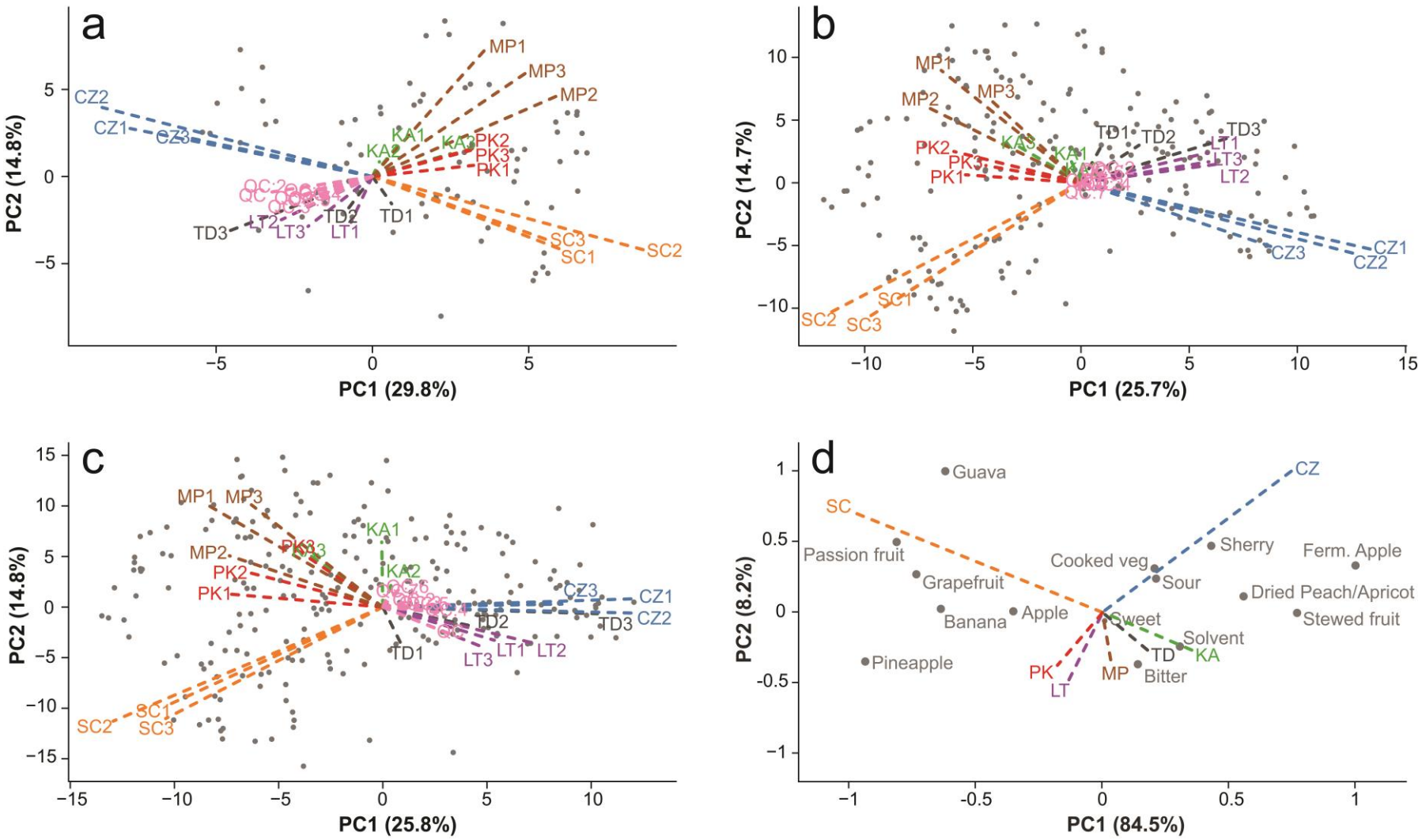

Fig. 3

PCA after unit variance scaling of the Venn diagram center features for the a) 10 second, b) 5 minute, and c) 30 minute extraction time data. d) is the PCA bi-plots of the sensory data. SC represents *S. cerevisiae* fermentations, TD represents *T. delbrueckii* fermentations, CZ represents *C. zemplinina* fermentations, KA represents *K. aerobia* fermentations, LT represents *L. thermotolerans* fermentations, PK represents *P. kluyveri* fermentations, MP represents *M. pulcherrima* fermentations and QC represents the quality control samples.

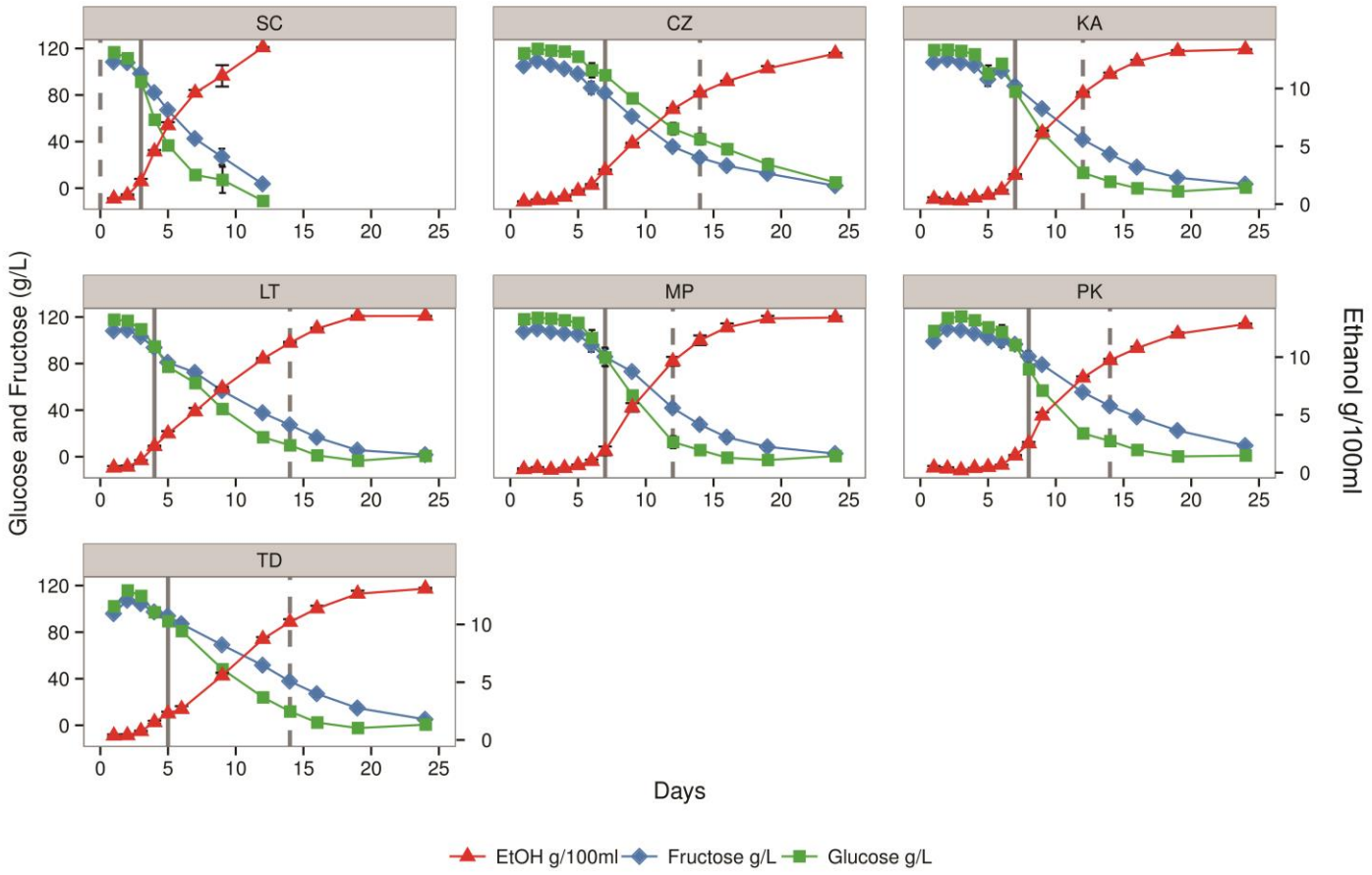

Fig. 4
Each graph indicates the progress of the fermentations by each species. SC: *S. cerevisiae*, CZ: *C. zemplinina*, KA: *K. aerobia*, LT: *L. Thermotolerans*, MP*: M. pulcherrima,* PK*: P. kluyveri,* TD*: T. delbrueckii.* Each graph shows glucose consumption (square shape), fructose consumption (diamond shape), and ethanol production (triangle shape). All of these lines are an average of the three biological replicates and the standard deviation is show by error bars. The solid vertical line indicates where the ethanol concentration reached 2% and in the case of the non-*Saccharomyces* fermentations *S. cerevisiae* was added. The dashed vertical line indicates where the non-*Saccharomyces* yeast was no longer detectable by plate count.

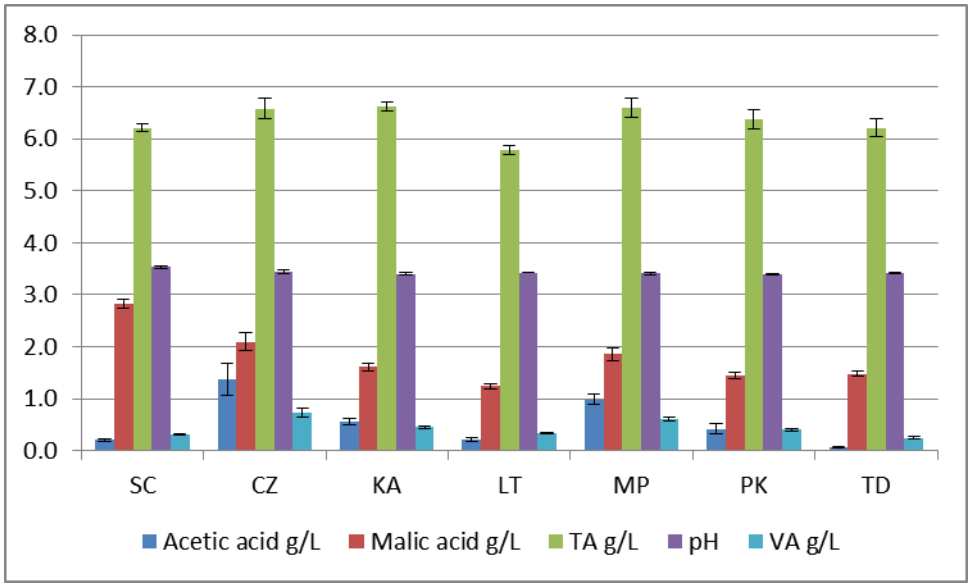

Fig. 5
Bar graph indicating the final average acidity and pH levels of each fermentation. TA indicates titratable acidity while VA indicates volatile acidity. SC: *S. cerevisiae*, CZ: C. *zemplinina*, KA: *K. aerobia*, LT: *L. thermotolerans*, *MP: M. pulcherrima, PK: P. kluyveri, TD:* T. *delbrueckii.*

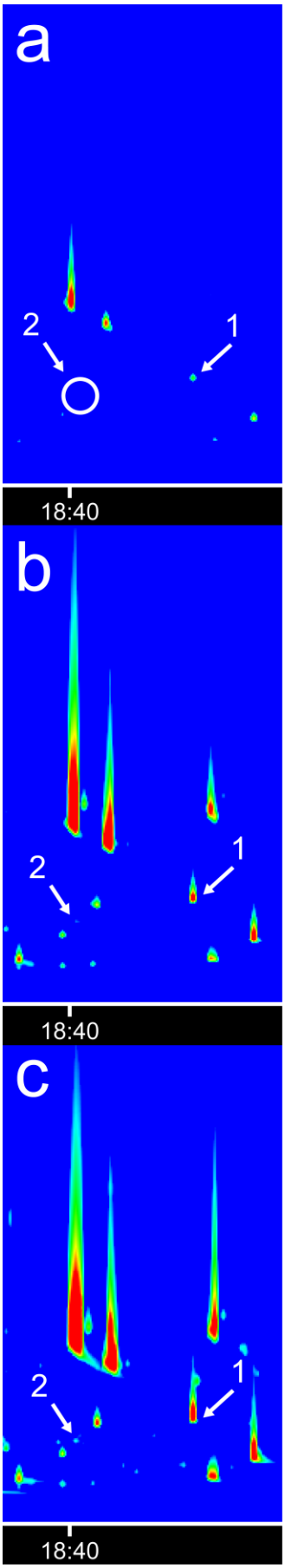

Fig. 1S
2D chromatograms of each extraction time: a) 10 seconds, b) 5 minutes, and c) 30 minutes. Compound 1 is highlighted as an example of a compound that was perfectly measurable in the 10 second extraction but became overly saturated in the 30 minute extraction. Compound 2 represents the revers, it is reliably detectable at 30 minutes, barely detectable at 5 minutes, and non-existent at 10 seconds.

Chapter 5

Research results

The Effect of Non-*Saccharomyces* Yeasts on the Volatile Chemical Profile of Shiraz Wine

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Australian Journal of Grape and Wine Research

Full Title

The Effect of Non-*Saccharomyces* yeasts on the Volatile Chemical Profile of Shiraz Wine

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Short version of title: Chemical profile of Shiraz fermented by Non-*Saccharomyces* yeasts

Abstract

This study evaluated the impact on the volatile chemical profile of non-*Saccharomyces* yeasts used to initiate fermentation in Shiraz grape must. Six different non-*Saccharomyces* yeasts were inoculated and subsequently followed by the addition of *Saccharomyces cerevisiae* (SC). The final wines were assessed using SPME-GCxGC-TOF-MS to produce an untargeted volatile metabolite profile of each treatment. The non-*Saccharomyces* yeasts used were: *Torulaspora delbrueckii* (*TD*), *Lachancea thermotolerans* (*LT*), *Pichia kluyveri* (*PK*), *Metschnikowia pulcherrima* (*MP*), *Candida zemplinina* (*CZ*) and *Kazachstania aerobia* (*KA*). SC in monoculture was used as a reference treatment. Each fermentation produced a unique chemical profile. The *LT-SC* sequential fermentations were the most significantly different primarily in their ester, alcohol and terpene profiles. The *KA-SC* sequential fermentations had the highest amount of volatile acidity. The *PK-SC* sequential fermentations had relatively high amounts of acetaldehyde and a few esters. The *MP-SC* sequential fermentations also showed a few esters to be higher. The *TD-SC* sequential fermentations were notable for their lack of any clearly distinct pattern in comparison to the other fermentations. Given the characteristics, the *LT-SC* sequential fermentations showed the most potential for increased complexity of the Shiraz volatile profile.

Key words

GCxGC-TOFMS, Non-*Saccharomyces,* Sequential Fermentation, Shiraz, Wine Volatiles

5.1 Introduction

Shiraz, also known as Syrah, is an important grape variety grown worldwide. It is typically described as ‘spicy’, ‘jammy’, ‘berry-like’, and can also boast ‘smoky, ‘dark fruit’ and even ‘chocolate’ aromas and flavors (Mayr et al. 2014). The flavor and aroma of a wine is directly related to its chemical composition; the complexity that can be greatly influenced by the growing conditions of the grapes, as well as fermentation and aging practices applied to the wine. Over 1300 volatile compounds have been identified in wine to date (Rapp 1998, Ebeler 2001, Herderich et al. 2012) yet despite its global importance it is only recently that the compounds responsible for the specific aroma of Shiraz have been identified (Parker et al. 2007, Wood et al. 2008, Mayr et al. 2014). Research has shown that Shiraz is separated from other highly prized red grape varietals in that it does not contain the so called ‘green pepper’ compound methyoxypyrazine but it does contain rotundone which is one of the compounds found to be responsible for the wines characteristic ‘spicy’, black pepper aroma (Wood et al. 2008, Koch et al. 2010). The most comprehensive study to date, Mayr et al. (2014), used GC-O and GC-MS to detect and identify 60 primary odorant compounds in Shiraz. The majority of these compounds were fermentation- or yeast-derived compounds such as acids, alcohols, acetate and ethyl esters. Though grape variety certainly has a

significant impact on the final wine product the majority of flavor compounds responsible for the aromas typically associated with wine are produced during the fermentation process by yeast (Ribéreau-Gayon et al. 2006).

Until recently, the majority of research surrounding the influence of yeast on the flavor of wine centered on *Saccharomyces cerevisiae*. The other yeasts, collectively and historically referred to as "non-*Saccharomyces* yeasts" by wine microbiologists, were thought of as spoilage organisms and not given much attention outside of this context. The study of non-*Saccharomyces* yeasts outside of a wine fault context is a fairly new but growing area of research (Andorrà et al. 2010, 2012, Ciani et al. 2010, Zott et al. 2011, Comitini et al. 2011, Sadoudi et al. 2012, Jolly et al. 2014, Sun et al. 2014, Beckner Whitener, Carlin, et al. 2015, Benito et al. 2015, Dashko et al. 2015). To date, the majority of the research has focused on aromatic white wine varieties. However, understanding the effects of these yeasts on the chemical composition of all varieties of wine is of great importance since studies have shown that between nine and twenty different species exist on grape berries and many are capable of at least partial fermentation (Jolly et al. 2006, Kurtzman & Fell 2011). *S. cerevisiae* is typically only found at very low levels on grapes. In natural or spontaneous fermentations, *S. cerevisiae* will eventually dominate and complete the fermentation but it takes time to establish itself. During this time, the other yeasts are actively metabolizing and altering the must/wine environment. For example, many non-*Saccharomyces* species are able to produce extracellular enzymes that can liberate glycosidically bound constituents that *S. cerevisiae* cannot (Charoenchai et al. 1997, Villena et al. 2007). When *S. cerevisiae* is inoculated at high levels however the native yeasts are quickly outcompeted. In an effort to capture some of the characteristics of spontaneous fermentations some winemakers will employ either staged or co-inoculation of non-*Saccharomyces* yeasts. In staged inoculation, the non-*Saccharomyces* yeasts are added first, allowed to ferment for a given amount of time and the *Saccharomyces* yeasts are added to finish the fermentation. This allows the non-*Saccharomyces* yeasts to have the greatest effect on the final product. Early studies showed that both strategies can mimic the results of natural fermentations leading to more complex aromas (Ciani & Maccarelli 1998, Romano, Fiore, et al. 2003). Follow-up studies sought to understand the macronutrient consumption (sugars and amino acids) of various yeasts in grape must and how this can effect macromolecule production (ethanol, acetic acid, glycerol and higher alcohols). These led to results that indicated how some of the species commonly associated with grapes can affect wine through the production of enzymes, acetic acid, glycerol, ethyl acetate, and higher alcohols (Charoenchai et al. 1997, Rojas et al. 2001, Romano, Granchi, et al. 2003, Clemente-Jimenez et al. 2004, Pina et al. 2004, Villena et al. 2007, Andorrà et al. 2012, Gobbi et al. 2013, Van Breda et al. 2013, Wang et al. 2015). In-depth studies of how different yeasts can affect the more complex chemical aspects of wine aroma and flavor, especially with regards to differences between grape varieties have not been carried out. There is

still a lack of understanding with regard to how, specifically, individual species of non-*Saccharomyces* yeasts can alter the organoleptic properties of a wine. There is also little understanding of how these yeasts may affect wines of different cultivars. Broad, untargeted chemical and metabolomic profiling can help fill the gap to enhance the knowledge on Shiraz (Careri et al. 2002, Adahchour et al. 2006, Welke & Alcaraz Zini 2011, Beckner Whitener, Carlin, et al. 2015). Using untargeted SPME-GCxGC-TOF-MS, this study sought to characterize the impact of six different non-*Saccharomyces* yeasts on the volatile chemical profile of Shiraz wine. Wines were fermented sequentially with *S. cerevisiae* serving to complete the fermentations as well as the reference treatment or control. *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Pichia kluyveri*, and *Metschnikowia pulcherrima* were commercial starter strains while *Candida zemplinina* and *Kazachstania aerobia* were laboratory strains chosen on the basis of promising preliminary results obtained for Sauvignon blanc (Beckner Whitener, Stanstrup, et al. 2015).

5.2 Materials and Methods

5.2.1 Grapes, Yeasts, and Chemicals

Shiraz grapes (vintage 2014) were obtained from the vineyards at Welgevallen Experimental Farm, Stellenbosch University, Stellenbosch, South Africa. *S. cerevisiae* (Enoferm M2®, Lallemand Inc., Canada), *T. delbrueckii* (Biodiva®, Lallemand Inc.), *M. pulcherrima* (Flavia®, Lallemand Inc.), *P. kluyveri* (Viniflora® FROOTZEN™, Chr. Hansen, Horsholm, Denmark), *L. thermotolerans* (Viniflora® CONCERTO™, Chr. Hansen), *C. zemplinina* (Institute of Wine Biotechnology (IWBT) Y1082) and *K. aerobia* (IWBT Y845) were used. Twenty-milliliter glass screw cap vials, sodium chloride (ACS grade), sodium azide, internal standard 2-octanol, a divinylbenzene/carboxen/ polydimethylsiloxane (DVB/CAR/PDMS) coating 50/30 µm, 2-cm length SPME fiber was purchased from Supelco by Sigma-Aldrich S.r.l., Milan, Italy.

5.2.2 Wine making procedure

Fermentations were carried out using Shiraz grape must obtained after mechanical crushing of the grapes. The must was evaluated for initial sugar (140 g/L glucose and 140 g/L fructose), titratable acidity (3.17 g/L) and yeast assimilable nitrogen (YAN) (170 mg/L) content, as well as pH (3.8). YAN was adjusted by adding 40 mg/L of diammonium phosphate (DAP). SO_2 (Biolab-Merck, Wadeville, South Africa) was added to inhibit extraneous bacterial or fungal growth. The yeasts were grown in yeast extract peptone dextrose (YPD) medium (Biolab-Merck, Wadeville, South Africa). They were shaken to ensure aerobic conditions at 30°C in successively larger batches using a 1% transfer rate starting from 10 mL and ending at 1 L at which point necessary cell concentrations for inoculation were obtained via centrifugation. The 11-L plastic fermentation vessels containing 10 L of must with skins were inoculated with a volume of yeast determined from the pre-culture by plate count and optical density to obtain a level of 10^6 cfu/mL. The yeast growth

was monitored via plate count on WL Nutrient agar (Fluka, Sigma-Aldrich) which allows for visual differentiation of the yeast strains. Fermentations were carried out in triplicate at 25°C. The red grape must was fermented with the skins until the end of the alcoholic fermentation process, and must aeration and cap management were carried out by punch-down once daily. The non-*Saccharomyces* yeasts were allowed to ferment until approximately 2% ethanol concentration was reached. At this point, *S. cerevisiae* was added at 10^6 cfu/mL concentration to finish the fermentations after being pre-cultured in the same manner as the non-*Saccharomyces* yeasts. Samples were taken daily to track progress via plate count on WL and Fourier-transform mid-infrared spectroscopy (FOSS WineScan, Hillerød, Denmark)in accordance with the protocol outlined in Nieuwoudt et al. (2006). The apparatus measured levels of glucose, fructose, titratable acidity, volatile acidity, pH, acetic acid and malic acid. Upon reaching dryness, the final wines were bottled after press and clarification via cold rest for one week at -4°C in 750-mL glass bottles with screw caps. Wines were then transported to the laboratory of the Department of Food Quality and Nutrition, Research and Innovation Center, Fondazione Edmund Mach (FEM) for chemical analysis.

5.2.3 SPME extraction and GCxGC-TOF-MS analysis

Vials were prepared as follows: 5 ml of a sample from each wine and 50 µL of 0.5 mg/L 2-octanol was added to 20-mL screw cap vials containing 1.5 g NaCl. A Gerstel MPS autosampler (GERSTEL GmbH & Co. KG) equipped with the standard sample agitator and SPME fiber conditioning station was used to extract the volatiles from the sample vial headspace. GC×GC-TOF-MS analysis of the extracts was performed using a LECO Pegasus-4D system consisting of an Agilent 6890N (Agilent Technologies) coupled to a LECO Pegasus 4D detector. The system employed a consumable free modulation system. The vials were incubated for 5 min at 35°C under 500 rpm rotation at 10 s intervals. Extraction took place for either 5 min, or 30 min prior to desorption in the inlet for 180 s at 250 °C. Quality control (QC) vials consisting of an equal mix of all samples were spaced at the beginning and every third vial thereafter. Samples were randomized for both time points and a single SPME fiber was used for both extraction times. The 5 min extraction time samples were run first and the 30 min extractions run immediately after. Helium carrier gas was used with a flow set at 1.2 mL/min and a splitless time of 180sec. The oven was equipped with a 30 m x 0.25 mm x 0.25 µm VF-WAX MS primary column (Agilent Technologies) and a 1.5 m x 0.15 mm x 0.15 µm RXI 17Sil MS secondary column (Restek Corporation, Bellefonte, PA, USA). The GC oven parameters were as follows: initial temperature was 40 °C held for 2 min, followed by an increase to 250°C at a rate of 6°C/min, the oven was then held at 250°C for 5 min before returning to the initial temperature (40°C). The total cycle time, was 42 min. The modulation period was set to 7 s with a hot pulse time of 1.4 s. The modulator was offset by 15°C. The MS protocol consisted of electron ionization at 70 eV with ion source temperature at 230 °C, a

detector voltage of 1543 V with a voltage offset of 200 V, mass range of *m/z* 35-350, and acquisition rate of 200 spectra per second. There was an acquisition delay of 120 s.

5.2.4 Data processing and Alignment

ChromaTOF software version 4.32 was used to perform baseline correction, deconvolution and peak picking of the raw data. The baseline offset was set to 1, just above the noise level. The first dimension peak width was set to 43 s while the second dimension peak width was set to 0.1 s. A factor of 500 was set as the match required to combine peaks in the second dimension. A signal to noise (S/N) of 10 was used for the 5 min extraction time data with a minimum S/N of 6 for sub peak retention. A S/N of 100 was used for the 30 min extraction time data with a minimum S/N of 60 for sub peak retention. Traditional integration was used. Forward library searching was used with the following parameters: Hits to return were set to 10, minimum molecular weight was set to 40, maximum molecular weight was set to 350, the mass threshold was set to 50 and the minimum similarity match was set to 700. The NIST and Wiley libraries were used. For alignment the following parameters were used: a mass threshold of 10, a minimum similarity match of 600, the maximum number of modulation periods matching peaks could be apart was set to 1, a maximum retention time difference was set to 7 s, for peaks not found by initial peak finding the signal to noise ratio was set to 5 for the 5 min extractions and to 50 for the 30 min extractions, for analytes to be kept they had to be found in all biological replicates within a class where each yeast species was its own class.

5.2.5 Statistical Analysis

Each extraction time, 5 min and 30 min, was treated as a separate data set in the following way. First, to avoid underestimation of the variance of the data, zero intensity values (undetected features) were replaced feature-wise by a random number between the lowest detected intensity and zero. Peak areas were then normalized to the internal standard. Following this, for each feature, a fixed effects linear model was fitted with yeast strain as fixed effect. This model was used for multiple comparisons between each strains and the *S. cerevisiae* control without correction for multiple testing. Subsequently, the collection of p values for the comparisons were corrected for multiple testing by controlling the false discovery rate (FDR) and q-values calculated (Strimmer 2008a, 2008b).

To select the compounds of interest a filter with three requirements was applied to the data. Compounds were selected if their q-values were below 0.05 and at least one comparison had a fold change greater than 1. In addition, the QC samples were used to calculate the relative coefficient of variance (% CV) for each feature across the whole analysis. Only features with %CV lower than 50% in the QC samples were selected. The features that met all of these requirements

were considered significant compounds of interest for each extraction time. The peak area values for each of these compounds were used to generate PCA plots to better illustrate the data (Figure 1). Unit variance scaling was used for PCA generation as well as the values seen in Table 1.

5.3 Results and Discussion

5.3.1 Fermentation progress and primary metabolite production

Fermentation progress for all the wines was monitored and Figure 2 summarizes this data. All fermentations progressed at approximately the same rate, finishing 17 days after initial inoculation (Figure 2). The non-*Saccharomyces* yeasts all reached the 2% ethanol mark at approximately the same time, four days post inoculation, as well. All wines reached approximately the same final ethanol percentage of 16% (v/v). This is in contrast to work done by Contreras et al. (2015) which found that Shiraz wines produced via sequential inoculation of *M. pulcherrima* and *S. cerevisiae* were lower in ethanol concentration than the *S. cerevisiae* only control. This contrast can be explained however by a number of differences between inoculation strategies and the fact that the strain of *M. pulcherrima* used in that study was selected from a large strain screening exercise specifically for reduced ethanol potential.

All of the non-*Saccharomyces* sequential fermentations showed a similar pattern of sugar consumption in which glucose was consumed faster than fructose. The *C. zemplinina-S. cerevisiae* (*CZ-SC*) sequential fermentations did however, show fructose being consumed more rapidly than glucose at the beginning, as expected for this fructophilic yeast (Duarte et al. 2012). After the *S. cerevisiae* addition to the fermentations this trend abated (Figure 2). Previous research has shown that *C. zemplinina* is able to survive to the end of fermentation due to its high ethanol tolerance (Rantsiou et al. 2012). While this may have been true in our fermentation it clearly did not govern the sugar consumption after the addition of *S. cerevisiae*. It is also known that *L. thermotolerans* and *T. delbrueckii* can remain viable for some time after *S. cerevisiae* had been added (Kapsopoulou et al. 2007, Azzolini et al. 2012, Maio et al. 2012). *P. kluyveri* and *K. aerobia* have not been previously studied in a sequential red wine fermentation however. Across all sequential fermentations the non-*Saccharomyces* colonies became uncountable shortly after *S. cerevisiae* was added to the fermentations due to the fact that *S. cerevisiae* so quickly outnumbers the non-*Saccharomyces* yeasts on the plates (Figure 2). This, combined with sugar consumption data, shows just how well *S. cerevisiae* was able to dominate the Shiraz fermentations. These findings differ slightly to what is reported for these yeasts in Sauvignon blanc fermentations that used the same inoculation strategy (Beckner Whitener, Stanstrup, et al. 2015). Indeed, most non-*Saccharomyces* yeasts have been shown to survive longer at lower temperatures (Reynolds et al.

2001, Malherbe et al. 2004). Probably in part due to the fact that *Saccharomyces* species grow more slowly than the psychrotrophic nature of other species allows for.

Ethanol production and sugar consumption are not the only primary metabolites of concern in a wine fermentation. Titratable acidity, malic acid and volatile acidity all have a significant impact on the quality of the final product. Figure 3 shows that there were differences in these parameters across the fermentations. The *L. thermotolerans-S. cerevisiae* (*LT-SC*) sequential fermentations were characterized by the least amount of malic acid in the finished product (Figure 3).

S. cerevisiae is known to be a poor metabolizer of L-malate (Salmon 1987, Zelle et al. 2008). The other yeasts in this study have not been investigated for their L-malate metabolism or their ability to metabolize other TCA cycle intermediates as a sole carbon source (Saayman & Viljoen-Bloom 2006). The results in this study indicate that it is likely that most of the non-*Saccharomyces* yeasts used here are able to transport and metabolize L-malate to some extent under wine fermentation conditions. However, the variability seen in the different sequential fermentations obviously indicates a need for further study.

The volatile acidity levels varied even more greatly than the malic acid among the non-*Saccharomyces* fermentations. The *T. delbrueckii* and *S. cerevisiae* sequential inoculations showed the same approximate volatile acid levels as the *S. cerevisiae* control while all other fermentations demonstrated higher amounts. Across all fermentations the volatile acidity level remained below the legal threshold of 1 g/L however.

Even without a more comprehensive chemical analysis, the differences that these yeasts can confer on wine composition are evident. These differences become more pronounced with the addition of the untargeted SPME-GCxGC-TOFMS profiling discussed below.

5.3.2 Differences in the volatile profiles of the finished Shiraz wine

When Solid-Phase-Microextraction (SPME) is used in conjunction with GCxGC-TOF-MS, it is possible to extract and study the different compounds that make up the headspace of wine samples without first altering the samples. The use of GCxGC-TOF-MS allows for unparalleled separation, detection and identification of analytes by first separating them on GC columns of two different phases before passing them to the detector. The result of which is a very clean individual mass spectrum of compounds that would otherwise overlap in a 1D GC set-up (Ong & Marriott 2002). GCxGC-TOF-MS analysis is thus becoming much more common in the fields that regularly analyze complex sample types such as metabolomics, food and wine analysis (Zhang et al. 2012). One of the drawbacks to the increased sensitivity of this system is that a complex matrix such as

wine typically contains a broad concentration range of analytes in its headspace. Compounding this problem is the fact that all of these compounds can have different adsorption rates to the SPME fiber. This can very easily cause saturation of either the columns, the detector or both and so steps must be taken to mitigate these issues in order to extract the most amount of information from a sample.

This can be done in a number of way but our study used two different extraction times to increase compound coverage while limiting chromatographic and detector saturation. The two times, 5 min and 30 min, were used and processed separately to obtain a list of statistically significant compounds of interest for each extraction time. The primary goal was to determine which compounds were responsible for differences between the *S. cerevisiae* control fermentation and the non-*Saccharomyces* sequential fermentations in Shiraz wine.

Table 1 shows the average peak areas after unit variance scaling of each compound found to be significantly different from the control fermentation for both extraction times. Of the 121 compounds found to be significantly different between the non-*Saccharomyces* sequential fermentations and the *S. cerevisiae* control, 43 compounds were, relatively, the highest in the *S. cerevisiae* solo fermentation. This list of compounds represents the main distinguishing factor between the control and the other fermentations. Most of this list is comprised of alcohols and esters. These two classes of compounds are responsible for the majority of the aroma in wine (Ribéreau-Gayon et al. 2006). By contrast, the other non-*Saccharomyces* fermentations, with the exception of the *LT-SC* sequential fermentations, were distinct for their general lack of volatile compounds, a notable exception being terpenes.

Free terpenes in wine are the result of glycosidase enzymes such as β-glucosidase acting on bound glycosylated precursors present in the grape must (Carrau et al. 2005). With descriptors such as ‘floral’, ‘herbal’, ‘rose’ and ‘citrus’ terpene aromas can contribute significantly to the varietal characteristics of wine since different grape varieties have differing levels of bound precursors (Mateo & Jiménez 2000). More than 25 different terpenes have been identified in Shiraz and at least two have been identified as key odor compounds: linalool and α-terpineol (Parker et al. 2007, Mayr et al. 2014). During fermentation yeasts are primarily responsible for the production of the enzymes necessary to liberate bound terpenes. Different yeast species have been shown to have different expression levels and activities of these enzymes (Charoenchai et al. 1997, Fernández et al. 2000, Manzanares Rojas, V., Genoves, S., and Valles, S. 2000, Mendes Ferreira et al. 2001). All of the non-*Saccharomyces* fermentations in this study displayed relatively higher levels of the following terpenes compared to the control: geraniol, trans-β-ocimene, cis-α-ocimene, linalool, and α-terpinene. The typical aroma descriptions of these terpenes are given in Table 1. All are

pleasant and considered positive contributions to wine aroma. As previously stated linalool is a 'key' aroma compound in Shiraz. All of the non-*Saccharomyces* fermentations showed higher amounts of linalool than the control but it was in the highest relative concentration in the *LT-SC* sequential fermentations. Besides the terpenes each of the other non-*Saccharomyces* fermentations had a few characteristics that set it apart from the solo *S. cerevisiae* fermentations. The PCA plots in Figure 1 show the most distinctly different fermentations from the control were the *LT-SC* sequential fermentations and as such warrants the most discussion.

The volatile chemical profile of the *LT-SC* sequential fermentations differed significantly from the *S. cerevisiae* solo fermentations in numerous ways discussed henceforth. As can be seen in Figure 1, the *LT*-SC and *SC* solo fermentations were separated along the X-axis by 28 and 33% in the 5 min and 30 min extraction times, respectively. Chemically, the greatest difference between the two was in the relative peak area of 1-ethyl-1h-pyrrole-2-carboxaldehyde, a pyrrole which has been described as having a burnt, roasted or smoky aroma. This compound has been found in coffee as well as Merlot but not in Shiraz until now (Chin et al. 2011, Welke et al. 2012). A sensory threshold for this compound has not been established but subtle smoky aroma is often a desirable characteristic in Shiraz and other red wines. The *LT-SC* sequential fermentations were also characterized by the relative abundance of 2-methyl propanoic acid and some of its esters. It has a very strong, undesirable odor of rancid butter but it can be esterified with various alcohols to form compounds with much more desirable odors. Propanoic acid, 2-methyl-, ethyl ester and propanoic acid, 2-methyl-, 2-phenylethyl ester have sweet, floral and fruity aromas and were seen in a much higher concentrations in the *LT-SC* sequential fermentations compared to the control. Mayr et al. (2014) found both 2-methyl propanoic acid and its ethyl ester to be a key odorant compound in Shiraz. Pineau et al. (2009) identified propanoic acid, 2-methyl-, ethyl ester as a key black-berry aroma component along with ethyl propanoate and 2-methylbutanoate, neither of which were found to be in relatively higher concentration in any of the yeast treatments compared to the control. In general, ethyl-esters of branched amino acids are produced during wine ageing when branched acids are esterified with ethanol (Díaz-Maroto et al. 2005). Yeasts, however, can also synthesize these compounds through branched amino acid metabolism (Hazelwood et al. 2008). This means that differences in the starting concentration of branched amino acids in the grape must as well as the amino acid metabolic preferences for the yeast have the ability to greatly influence the production of these compounds. Antalick et al. (2015) looked at both Shiraz and Cabernet Sauvignon and found that harvest stage, rather than grape cultivar had the most significant effect on the concentration of branched amino acids in grape must. The differences between the concentration of propanoic acid, 2-methyl-, ethyl ester, 2-phenylethyl ester in this study can be explained by a difference in the activity of the enzymes responsible for catalyzing the conversion of organic acids to esters. *L. thermotolerans* could either be directly responsible for this

in having higher enzyme activity or, more subtly, create an environment in which these genes are upregulated in *S. cerevisiae*. It is well known that fermentation conditions such as temperature, pressure, assimilable nitrogen, pH, and amount of dissolved oxygen can impact ester production (Anderdon & Kirsop 1975, Mallouchos et al. 2002, González-Pombo et al. 2008, Galanakis et al. 2012). Previous studies of *L. thermotolerans* in wine making have shown that it is capable of producing lactic acid and increasing the pH of wine while reducing its volatile acidity as well as increasing glycerol and 2-phenylethanol concentrations while being a low acetaldehyde producer (Ciani et al. 2006, 2010, Kapsopoulou et al. 2007, Comitini et al. 2011, Cordero-Bueso et al. 2012). In contrast, our study showed slightly higher volatile acidity though still well below the acceptable legal threshold of 1 g/L (Figure 3). Gobbi et al. (2013) reported that in sequential inoculation under laboratory conditions, *L. thermotolerans* was the dominant species during fermentation. In industrial wine fermentation conditions, *LT* was less competitive, with limited final biomass amounts. They also reported that these fermentations showed reduced 2-methyl-1-propanol and 3-methyl-1-butanol, reduced acetate esters but higher ethyl acetate and higher 2-phenylethanol. In our case, the ethyl acetate production was approximately equal to that of *S. cerevisiae* and the 2-methyl-1-propanol and 3-methyl-1-butanol concentrations were not found to be statistically significantly different across any of the fermentations. Both of these compounds are produced by yeasts via the Ehrlich pathway, also known as the amino acid catabolism pathway. There are multiple genes that regulate the three major steps in the pathway. A change in the regulation of these due to compounding matrix effect or simply a difference in the starting amount of leucine or valine could explain the differences between our findings and those of Gobbi et al. (2013). Especially since their study used Chianti, not Shiraz. As previously indicated it is well documented that not only do different grape varieties contain different amino acid profiles but that those differences can directly affect the corresponding wine volatile composition (Rapp & Versini 1995, Hernández-Orte et al. 2002, Antalick et al. 2015).

Several other pleasant odor compounds were found to be significantly higher in the *LT-SC* sequential fermentations thus contributing further to the separation with the control (Fig 1.). The majority of these were esters including: isoamyl lactate, acetic acid, butyl ester, butanoic acid, pentyl ester, 3-nonenoic acid, ethyl ester, propanoic acid, and 2-hydroxy-, ethyl ester. The designated aromas for these compounds can be found in Table 1. Not all of the compounds found to be in relatively higher concentrations in the *LT-SC* sequential fermentations had pleasant aromas. Butanethioic acid, 3-methyl-, s-methyl ester is described as having a sharp, ripe cheese, odor which in too high a concentration could have a detrimental effect on any wine but especially Shiraz which is known for its lush and jammy characteristics.

The other non-*Saccharomyces* initiated fermentations did not display nearly the same number of compounds found to be in higher relative concentration compared to the control. This explains the grouping seen in Figure 1 for both extraction times. The few compounds that were different and thus set those fermentations apart are discussed below.

The C*. zemplinina* initiated fermentations were characterized by relatively high levels of δ-valerolactone and pentolactone as well as 2-hexenoic acid and 2-hexanoic acid, ethyl ester (Table 1). The *K. aerobia* initiated fermentations were highest in octanoic acid, ethyl ester, 2-hydroxy-1-methyl ethyl ether, 2-aminoethanol, n,o-diacetyl-, and acetic acid, 2-phenylethyl ester. The *P. kluyveri* initiated fermentations showed relatively high acetaldehyde, methyl acetate and butyl octanoate. Acetaldehyde and methyl acetate both have a strong, hot, solvent-like odor that could contribute to a fault. Butyl octanoate has a nutty and buttery aroma. *M. pulcherrima* initiated fermentations also showed a relatively high amount of butyl octanoate as well as a number of other esters: isobutyl acetate, pentanoic acid, 4-methyl-, ethyl ester, hexanoic acid, 2-methylpropyl ester, 6-octen-1-ol, 3,7-dimethyl-, acetate, acetic acid, methyl ester, hexanoic acid, 2-methylpropyl ester, and 3-hexen-1-ol, acetate, (z). All of which have been associated with sweet, fruity, ethereal, banana, and tropical aromas. *T. delbrueckii* has been used in wine making for years and is one of a few non-*Saccharomyces* species commercially available for use in wine and beer production. Studies that have reported that wine fermented with *T. delbrueckii* in co-culture had lower amounts of volatile acidity, and higher amounts of terpenol, 2-phenylethanol and C6 compound production (Comitini et al. 2011, Azzolini et al. 2012, Van Breda et al. 2013). Our sequential *T. delbrueckii* fermentations showed a low amount of volatile acidity (Figure 3). However, the similarities between this and other studies stop there since the *T. delbrueckii* fermentations were notable for their lack of any strong pattern in comparison to the other fermentations. None of the compounds found to be statistically significantly different among the fermentations were seen to be highest in the *T. delbrueckii* fermentations with the exception of 3-octanol and Analyte 4552, which has not been identified. Overall, *T. delbrueckii* did little to either positively or negatively affect the Shiraz fermentations compared to the other yeasts in this study. These findings are in contrast to results obtained by Renault et al.(2015) who found that there were specific esters produced in only fermentations containing *T. delbrueckii*. This could be explained by the fact that Merlot grape must, a different fermentation temperature, and inoculation strategy were used to carry out their experiments. As previously stated, all of these parameters have the ability to affect ester production.

5.4 Conclusions

Fermentation with non-*Saccharomyces* yeasts in conjunction with *S. cerevisiae* has been used more recently to help improve sensory attributes and the overall complexity of wine. However, relatively little is known about how different non-*Saccharomyces* yeasts can affect the fermentations of different grape cultivars. In this study, seven yeasts, from seven different genera were used to initiate fermentation in Shiraz grape must and *S. cerevisiae* was inoculated to complete fermentation in a typical sequential fermentation strategy. The yeasts chosen were a mix of commercially available and naturally derived strains, all of which have shown promise in wine but have not been evaluated in Shiraz in any great detail. The volatile profiles of the finished wines were evaluated using an untargeted SPME-GCxGC-TOFMS method which offers unparalleled separation efficiency that can greatly enhance the accuracy of compound identification. Overall, each of the non-*Saccharomyces* sequential fermentations showed a distinct volatile profile. The most significantly different profiles were observed in the *S. cerevisiae* control and *LT-SC* sequential fermentations. The majority of these differences were in esters, acids and terpenes. The pyrrole 1-ethyl-1h-pyrrole-2-carboxaldehyde which has never before been reported in Shiraz was relatively much higher in the *LT-SC* sequential fermentations than the control. On top of this, all of the *non-Saccharomyces* sequential fermentations showed higher amounts of terpene compared to the control. This is significant in that terpenes are grape varietal specific and *S. cerevisiae* does not encode the enzymes necessary to liberate them from their bound form. The *K. aerobia* initiated fermentations had the highest amount of volatile acidity. The *P. kluyveri* initiated fermentations had relatively high amounts of acetaldehyde and a few esters. The *M. pulcherrima* initiated fermentations also showed a few esters to be higher. The *T. delbrueckii* fermentations were notable for their lack of any strong pattern in comparison to the other fermentations. Given all of these findings, the *LT-SC* fermentation combination shows the most promise for future study. Quantification and sensory evaluation would go further to establish the usefulness of *L. thermotolerans* in increasing the complexity and varietal characteristics of Shiraz wines.

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5.6 Tables and Figures

Table 1 Metabolites and their associated aromas found to be in highest relative concentration among the treatments across all extraction times. The data presented are the average peak areas after unit variance scaling for each yeast responsible for the start

Class	Compound name	CAS	Aroma	SC		TD		CZ		LT		MP		PK		KA	
Acid	2-hexenoic acid[a]	13419-69-7	powerful fruity sweet warm herbal	-0.66	±0.65	-0.67	±0.4	1.55	±0.56	1.09	±0.39	-0.23	±0.08	0.04	±0.57	-1.08	±1.39
Acid	butanoic anhydride[b]	106-31-0	buttery	2.38	±1.14	-0.26	±0.14	-0.64	±0.09	-0.36	±0.23	-0.41	±0.19	0.25	±1.39	-0.66	±0.05
Acid	methacrylic anhydride[a]	760-93-0	NF	-0.49	±0.79	-0.35	±0.13	-0.95	±0.43	2.56	±0.82	-0.44	±0.45	0.04	±0.18	-0.16	±0.34
Acid	n-decanoic acid[a]	334-48-5	unpleasant rancid sour fatty citrus	2.48	±1.03	-0.25	±0.39	-0.55	±0.34	-0.87	±0.11	0.03	±0.69	0.37	±1	-0.08	±0.2
Acid	octanoic acid[a]	124-07-2	fatty waxy rancid oily vegetable cheesy	2.32	±0.93	0.04	±0.35	-0.85	±0.19	-1.40	±0.19	-0.03	±0.74	0.27	±0.61	-0.35	±0.21
Acid	octanoic acid[b]	124-07-2	fatty waxy rancid oily vegetable cheesy	2.10	±0.84	0.25	±0.54	-0.82	±0.29	-1.71	±0.29	0.18	±0.95	-0.02	±0.31	-0.11	±0.41
Acid	propanoic acid, 2-methyl-[a]	79-31-2	acrid	-0.75	±0.28	-1.02	±0.16	-0.15	±0.3	2.45	±0.46	-0.28	±0.18	-0.66	±0.42	-0.20	±0.77
Acid	propanoic acid, 2-methyl-[b]	79-31-2	acrid	-0.59	±0.15	-0.94	±0.12	-0.27	±0.46	2.64	±0.27	-0.27	±0.4	-0.53	±0.12	-0.17	±0.65
Alcohol	2-aminoethanol, n,o-diacetyl-[a]	NF	NF	-0.89	±0.23	-0.04	±0.28	-0.44	±0.21	-0.92	±0.07	0.36	±0.28	1.00	±0.67	1.99	±1.28
Alcohol	(z)-4-decen-1-ol[a]	57074-37-0	waxy fatty fruity	1.98	±0.47	-1.07	±0.65	0.06	±0.61	-0.91	±0.36	-0.51	±0.37	0.14	±0.83	0.43	±1.34
Alcohol	(z)-4-decen-1-ol[b]	57074-37-0	waxy fatty fruity	2.29	±0.99	-0.82	±0.23	-0.45	±0.63	-0.83	±0.18	-0.50	±0.27	-0.20	±0.34	0.28	±0.78
Alcohol	(z)-4-decen-1-ol[b]	57074-37-1	waxy fatty fruity	2.52	±1.14	-0.41	±0.31	-0.22	±0.39	-0.91	±0.29	-0.55	±0.12	-0.28	±0.11	0.03	±0.55
Alcohol	1-butanol, 3-methyl-[b]	123-51-3	fusel oil alcoholic whiskey fruity banana	-1.67	±0.73	0.37	±0.68	-0.64	±0.41	-0.83	±1.05	0.46	±0.49	0.56	±0.64	-0.52	±0.38
Alcohol	1-butanol[a]	71-36-3	fusel oil sweet balsam	-0.40	±0.36	-0.24	±0.59	-0.13	±0.34	2.26	±1.37	-0.39	±0.21	-0.58	±0.24	-0.36	±0.49
Alcohol	1-butanol[b]	71-36-3	fusel oil sweet balsam	-0.23	±0.24	-0.87	±0.4	-0.84	±0.02	2.32	±0.81	-0.67	±0.54	-0.30	±0.4	-0.13	±0.73
Alcohol	1-decanol[a]	112-30-1	fatty waxy floral orange sweet clean watery	2.50	±1.43	-0.17	±0.51	-0.39	±0.11	-0.90	±0.23	-0.33	±0.15	-0.10	±0.62	0.12	±0.58
Alcohol	1-hexanol, 3-methyl-[b]	13231-81-7	NF	2.35	±0.64	-0.11	±0.26	-0.49	±0.12	-1.41	±0.23	-0.06	±0.34	-0.65	±0.89	0.03	±0.22

Alcohol	1-nonanol[a]	143-08-8	fresh clean fatty floral rose orange dusty wet oily	2.53	±0.93	-0.49	±0.63	-0.33	±0.38	-0.72	±0.53	-0.55	±0.34	-0.31	±0.84	0.02	±0.9
Alcohol	2,5,8, 11-tetraoxatridecan-13-ol[b]	23783-42-8	NF	-0.53	±0.1	-0.64	±0.13	-0.71	±0.01	-0.52	±0.34	1.04	±0.16	-0.63	±0.13	-0.19	±0.8
Alcohol	2-hepten-1-ol, (e)-[a]	33467-76-4	pungent fatty plastic green	2.49	±1.21	-0.53	±0.39	-0.40	±0.08	-0.22	±0.42	-0.49	±0.26	-0.80	±0.71	-0.28	±0.72
Alcohol	2-methyl-3-buten-1,2-diol[b]	115-18-4	herbal earthy oily	2.43	±1.42	-0.85	±0.05	-0.52	±0.24	-0.36	±0.33	-0.50	±0.33	-0.34	±0.53	-0.26	±0.58
Alcohol	2-nonanol[b]	628-99-9	waxy green creamy citrus orange cheese fruity	0.79	±0.32	-0.27	±0.3	-1.04	±0.18	1.84	±1.86	-0.21	±0.82	-0.88	±0.51	-0.14	±0.23
Alcohol	2-pentanol, 4-methyl-[b]	108-11-2	pungent, alcohol	-1.45	±0.18	1.19	±0.74	0.15	±0.91	-1.24	±0.53	1.10	±0.69	-0.18	±0.97	-0.25	±0.57
Alcohol	3-octanol[a]	589-98-0	earthy mushroom herbal melon citrus woody spicy minty	1.31	±0.76	1.26	±0.7	-0.74	±0.61	-0.07	±0.91	0.68	±0.18	-1.17	±1.15	-0.74	±0.83
Alcohol	3-octanol[b]	589-98-0	earthy mushroom herbal melon citrus woody spicy minty	1.23	±0.81	1.29	±0.15	-0.83	±0.32	-0.11	±1.31	0.91	±0.15	-1.15	±0.96	-0.59	±0.82
Alcohol	3-penten-2-ol, 4-methyl-,[a]	2004-67-3	NF	-0.97	±0.81	0.77	±0.28	0.37	±0.8	-0.27	±0.46	1.90	±0.95	-0.55	±0.23	-0.31	±0.17
Alcohol	5-hepten-2-ol, 6-methyl-[b]	1569-60-4	NF	-0.90	±0.86	0.95	±0.66	-0.77	±0.74	-0.39	±0.79	1.09	±1.52	-0.29	±1.76	-0.10	±0.65
Alcohol	5-nonanol[b]	623-93-8	NF	2.15	±1.23	-1.00	±0.2	-0.49	±0.43	-0.14	±0.64	-0.68	±0.3	-0.44	±0.04	0.01	±0.65
Alcohol	cyclooctanemethanol, α,α,-dimethyl-[b]	16624-06-9	NF	2.53	±1.81	-0.39	±0.01	-0.47	±0.28	-0.48	±0.31	-0.27	±0.17	-0.48	±0.22	-0.24	±0.54
Aldehyde	propanal, 2-(acetyloxy)-, (r)-[a]	NF	NF	-1.45	±0.27	-0.79	±0.76	-0.43	±0.37	1.56	±0.58	-0.38	±0.57	-0.28	±0.92	0.95	±1.49
Aldehyde	2-caren-10-al[b]	14595-13-2	NF	2.72	±0.86	-0.70	±0.18	-0.26	±0.68	-0.46	±0.65	-0.11	±0.25	-0.08	±0.62	0.11	±0.5
Aldehyde	acetaldehyde[b]	75-07-0	pungent ethereal aldehydic fruity	-0.38	±0.45	-0.40	±0.44	-0.12	±0.3	-0.05	±1.11	-0.70	±0.35	2.04	±0.54	-0.33	±0.42
Aldehyde	butanal, 3,3-dimethyl-2-oxo-, hemihydrate[a]	4480-47-1	NF	-0.45	±0.32	-1.25	±0.9	0.35	±0.38	2.13	±0.52	-0.88	±0.32	-0.68	±0.38	0.06	±0.7
Alkane	cyclohexane, 1-ethyl-2-methyl-, trans-[b]	3728-54-9	NF	2.61	±0.99	-0.48	±0.21	-0.20	±0.67	0.42	±0.53	-0.46	±0.09	-0.65	±0.31	0.18	±0.68
Alkane	cyclohexane, 1-ethyl-2-methyl-, trans-[b]	3728-54-9	NF	2.49	±1.25	-0.48	±0.08	-0.02	±0.64	0.24	±0.52	-0.14	±0.1	-0.73	±0.42	0.38	±0.43
Alkane	heptane, 4,4-dimethyl-[b]	1068-19-5	NF	2.22	±0.66	-0.52	±0.52	-0.49	±0.13	-0.43	±0.57	0.24	±0.67	-0.32	±0.47	-0.69	±0.06
Alkyne	3,5-dodecadiyne, 2-methyl-[a]	55638-52-3	NF	2.27	±0.87	-0.04	±0.28	-0.03	±0.21	0.34	±0.38	0.11	±0.36	0.22	±0.39	0.48	±0.37

Amide	acetamide, n-(2-methylpropyl)-[b]	1540-94-9	NF	-0.81	±0.66	-0.61	±0.16	0.48	±1.16	1.95	±1.01	0.00	±0.29	-0.95	±0.35	-0.53	±0.5
Amide	acetamide, n-methyl-[b]	79-16-3	NF	-0.86	±0.39	-0.29	±0.38	1.99	±0.29	-1.18	±0.24	0.32	±0.4	-0.56	±0.52	-0.31	±0.51
Amine	2-pyridineethanamine, n-methyl-[a]	5638-76-6	NF	-0.40	±0.13	-0.48	±0.09	-0.53	±0.1	-0.50	±0.13	1.78	±0.35	-0.42	±0.19	0.05	±0.81
Bromoalkane	1-bromo-1-methyl-2-(4,5-hexadienyl) cyclopropane[b]	NF	NF	2.21	±0.71	-1.02	±0.29	-0.25	±0.85	-0.47	±0.45	-0.45	±0.5	-0.38	±0.32	0.71	±1.17
Bromoalkane	butane, 2-bromo-2-methyl-[a]	507-36-8	NF	2.32	±1.01	-0.65	±0.09	-0.49	±0.24	-0.42	±0.12	-0.67	±0.11	0.44	±1.43	-0.47	±0.14
Carboxylate	1,3-dioxolane, 2,2-dimethyl-[b]	695-30-7	NF	-1.15	±0.23	-0.98	±0.34	-0.59	±0.4	1.43	±0.56	0.07	±0.88	-0.32	±0.74	1.28	±1.25
Carboxylate	1,3-dioxolane, 2,4,5-trimethyl-[b]	3299-32-9	NF	2.28	±1.37	-1.14	±0.16	-0.39	±0.57	-0.18	±0.25	-0.59	±0.54	-0.46	±0.23	0.38	±0.98
Diol	1,4-benzenediol, 2,6-bis(1,1-dimethylethyl)-[a]	88-58-4	NF	2.43	±0.94	-0.65	±0.48	-0.66	±0.28	0.07	±0.18	-0.55	±0.21	0.08	±1.01	-0.43	±0.24
Diol	1,4-benzenediol, 2,6-bis(1,1-dimethylethyl)-[b]	88-58-4	NF	2.28	±1	-0.98	±0.23	-0.57	±0.31	-0.28	±0.28	-0.69	±0.44	-0.49	±0.48	-0.22	±0.95
Ester	2-butenedioic acid (e)-, diethyl ester[a]	624-49-7	NF	0.02	±0.62	-0.39	±1.32	2.18	±0.75	-0.62	±0.71	-0.25	±0.67	-0.84	±0.53	-0.21	±0.5
Ester	2-hexenoic acid, ethyl ester[a]	1552-67-6	rum fruity green sweet juicy	-1.45	±0.62	-0.58	±0.33	2.19	±0.26	0.79	±0.41	-0.12	±0.18	-0.32	±0.43	-0.36	±0.32
Ester	2-hexenoic acid, ethyl ester[b]	1552-67-6	rum fruity green sweet juicy	-1.48	±0.72	-0.68	±0.16	2.13	±0.38	0.64	±0.76	-0.08	±0.17	-0.39	±0.58	-0.39	±0.22
Ester	3-hexen-1-ol, acetate, (z)-[a]	3681-71-8	fresh green sweet fruity banana apple grassy	-1.52	±0.84	-0.03	±1.21	-0.01	±0.75	0.89	±0.38	1.27	±0.41	-0.08	±0.52	0.76	±1.18
Ester	3-hexen-1-ol, acetate, (z)-[b]	3681-71-8	fresh green sweet fruity banana apple grassy	-1.24	±0.83	-0.48	±1.18	0.05	±0.34	0.41	±0.77	1.14	±0.52	0.27	±0.34	0.79	±0.67
Ester	3-nonenoic acid, ethyl ester[a]	91213-30-8	NF	0.32	±0.19	0.12	±0.12	0.33	±0.39	2.18	±0.64	-0.09	±0.21	-0.03	±0.63	0.24	±0.15
Ester	3-nonenoic acid, ethyl ester[b]	91213-30-8	NF	0.69	±0.58	-0.16	±0.38	-0.09	±0.8	2.28	±0.48	-0.15	±0.31	-0.32	±0.49	0.14	±0.42
Ester	4-tert-butylcyclohexyl acetate[b]	32210-23-4	NF	2.31	±0.47	1.08	±0.33	-0.84	±0.07	-0.47	±0.39	-0.79	±0.02	-0.18	±1.07	-0.50	±0.74
Ester	5-oxotetrahydrofuran-2-carboxylic acid, ethyl ester[b]	1126-51-8	NF	-0.69	±0.05	0.13	±0.89	1.47	±0.45	-1.33	±0.27	0.61	±0.99	-0.57	±0.51	-0.19	±0.5
Ester	6-octen-1-ol, 3,7-dimethyl-, acetate[b]	150-84-5	floral, rosy, green, fatty, citrus lemon and bois de rose-like.	-0.78	±0.55	-0.04	±0.41	-0.32	±0.73	-0.61	±0.54	1.44	±0.56	1.19	±0.62	1.01	±0.33
Ester	acetic acid, 2-phenylethyl ester[a]	103-45-7	sweet, honey, floral	-1.29	±0.61	-0.49	±1.11	-0.69	±0.45	0.10	±0.86	-0.16	±0.58	1.22	±1.04	0.97	±1.44

			rosy, with a slight yeasty honey note with a cocoa and balsamic nuance														
Ester	acetic acid, 2-phenylethyl ester[b]	103-45-7	sweet, honey, floral rosy, with a slight yeasty honey note with a cocoa and balsamic nuance	-1.08	±0.86	-0.82	±0.45	-0.79	±0.64	-0.36	±0.45	0.21	±0.88	1.11	±0.45	1.51	±1.23
Ester	acetic acid, butyl ester[a]	123-86-4	ethereal solvent fruity banana	-0.72	±0.36	-0.37	±0.33	-0.62	±0.5	2.29	±0.98	0.12	±0.64	0.00	±0.46	-0.28	±0.87
Ester	acetic acid, butyl ester[a]	123-86-4	ethereal solvent fruity banana	-0.88	±0.3	-0.23	±0.61	-0.47	±0.51	2.43	±0.73	-0.64	±0.19	-0.07	±0.46	-0.18	±0.85
Ester	acetic acid, methyl ester[b]	79-20-9	ether sweet fruity	-1.17	±0.02	-1.17	±0	-1.17	±0	0.41	±0.39	1.41	±0.35	1.01	±0.67	-0.72	±0.64
Ester	acetic acid, phenylmethyl ester[a]	140-11-4	sweet, fruity and floral	-0.76	±1.6	-0.69	±0.92	-0.27	±0.49	1.28	±0.72	-0.36	±0.58	-0.37	±1.16	0.55	±1.49
Ester	acetic acid, phenylmethyl ester[b]	140-11-4	sweet, fruity and floral	-1.07	±0.6	-0.69	±0.96	-0.22	±0.61	1.61	±0.59	-0.15	±0.9	-0.30	±0.94	0.81	±1.2
Ester	butanoic acid, pentyl ester[a]	540-18-1	sweet fruity banana pineapple cherry tropical	-0.48	±0.27	-0.29	±0.36	0.07	±0.48	2.36	±1.15	0.50	±0.34	-0.23	±0.18	-0.03	±0.65
Ester	butyl octanoate[a]	589-75-3	butter ether herbal dank	0.23	±0.21	1.03	±0.18	0.63	±0.15	-0.65	±0.16	1.24	±0.57	0.65	±0.21	0.49	±0.13
Ester	butyl octanoate[b]	589-75-3	butter ether herbal dank	0.42	±0.3	0.79	±0.36	0.45	±0.32	-1.16	±0.13	1.30	±0.89	0.72	±0.42	0.57	±0.05
Ester	dodecanoic acid, 3-hydroxy-, ethyl ester[b]	126679-28-5	NF	2.00	±0.42	-0.88	±0.17	-0.89	±0.3	-1.32	±0.38	0.36	±0.6	-0.29	±0.52	0.78	±0.37
Ester	ethyl 3-hydroxytridecanoate[b]	PubChem ID: 575914	NF	2.38	±1.36	-0.32	±0.28	-0.22	±0.37	-0.90	±0.78	-0.02	±0.15	-0.07	±0.19	-0.13	±0.2
Ester	hexanoic acid, 2-methylpropyl ester[a]	105-79-3	sweet, estry, fruity pineapple, green apple, peach and tropical	-0.22	±0.11	0.84	±0.2	0.73	±0.07	-0.13	±0.05	1.30	±0.13	0.52	±0.39	0.59	±0.42
Ester	hexanoic acid, 2-methylpropyl ester[b]	105-79-3	sweet, estry, fruity	-0.49	±0.65	0.74	±0.33	0.59	±0.36	-0.68	±0.31	1.63	±0.45	0.51	±0.57	0.38	±1.12

			pineapple, green apple, peach and tropical														
Ester	hexanoic acid, 3-hydroxy-, ethyl ester[a]	2305-25-1	citrus pineapple grape fruity	2.35	±1.23	-0.55	±0.56	-0.76	±0.34	-1.18	±0.65	0.19	±0.36	0.02	±0.84	0.40	±0.44
Ester	hexanoic acid, 3-hydroxy-, ethyl ester[a]	2305-25-2	citrus pineapple grape fruity	2.42	±1.38	-0.40	±0.22	-0.62	±0.39	-0.91	±0.34	0.00	±0.16	0.05	±0.34	0.14	±0.36
Ester	isoamyl lactate[a]	19329-89-6	fruity creamy nutty	-0.52	±0.19	0.11	±0.66	-0.81	±0.02	2.49	±0.94	-0.35	±0.61	-0.40	±0.64	-0.68	±0.05
Ester	isoamyl lactate[b]	19329-89-6	fruity creamy nutty	-0.56	±0.11	0.13	±0.83	-0.83	±0.01	2.55	±0.45	-0.34	±0.64	-0.43	±0.5	-0.67	±0.01
Ester	isobutyl acetate[b]	141-78-6	ethereal fruity sweet weedy green	-1.31	±0.91	0.46	±0.35	0.00	±0.61	0.06	±0.77	1.96	±0.93	-0.41	±0.36	0.21	±1.15
Ester	octanoic acid, ethyl ester[a]	106-32-1	fruity wine waxy sweet apricot banana brandy pear	-0.50	±0.05	-0.52	±0.04	-0.52	±0.04	-0.56	±0.03	-0.51	±0.04	-0.03	±0.71	2.51	±1.09
Ester	octyl formate[a]	112-32-3	fruity rose orange waxy cucumber	1.96	±0.76	0.38	±0.63	-0.39	±0.36	-2.06	±0.22	-0.16	±0.23	0.19	±0.52	-0.05	±0.42
Ester	octyl formate[b]	112-32-3	fruity rose orange waxy cucumber	2.17	±0.7	0.20	±0.2	-0.55	±0.21	-1.92	±0.23	-0.07	±0.5	0.28	±0.39	0.14	±0.45
Ester	octyl formate[b]	112-32-4	fruity rose orange waxy cucumber	2.59	±0.89	-0.39	±0.11	-0.68	±0.17	-1.16	±0.2	-0.12	±0.41	-0.24	±0.16	0.33	±0.55
Ester	pentanoic acid, 3-methyl-2-oxo-, methylester[a]	30414-53-0	NF	-1.06	±0.14	-0.80	±0.64	0.32	±0.51	2.33	±0.23	-0.66	±0.08	-0.62	±0.51	0.27	±0.82
Ester	pentanoic acid, 3-methyl-2-oxo-, methyl ester[b]	26516-27-8	NF	-1.11	±0.2	-0.77	±0.55	0.33	±0.64	2.35	±0.23	-0.55	±0.23	-0.59	±0.56	0.23	±0.7
Ester	pentanoic acid, 4-methyl-, ethyl ester[a]	25415-67-2	fruity	-0.57	±0.21	-0.06	±0.85	-0.66	±0.09	-0.83	±0.56	1.99	±0.34	0.95	±0.65	-0.44	±0.77
Ester	propanoic acid, 2-hydroxy-, ethyl ester, (s)-[a]	687-47-8	NF	-0.72	±0.18	-0.31	±1.35	-0.85	±0.08	2.03	±0.65	-0.10	±1.02	-0.24	±0.99	-0.75	±0.11
Ester	propanoic acid, 2-hydroxy-, ethyl ester[b]	97-64-3	sharp tart fruity buttery butterscotch	-0.71	±0.18	-0.48	±1.33	-0.98	±0.03	1.92	±0.47	-0.17	±0.99	-0.21	±0.95	-0.73	±0.07
Ester	propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester[a]	74381-40-1	NF	2.38	±1.08	-0.69	±0.3	-0.59	±0.26	-0.33	±0.15	-0.35	±0.25	-0.11	±0.85	-0.55	±0.13
Ester	propanoic acid, 2-methyl-, 2-phenylethyl	103-48-0	heavy fruity, honey	0.17	±0.38	-0.42	±0.78	-0.33	±0.76	2.30	±0.57	0.02	±0.32	-0.21	±0.87	0.23	±0.54

	ester[a]		and yeasty, with balsamic nuances														
Ester	propanoic acid, 2-methyl-, ethyl ester[b]	97-62-1	NF	-0.67	±0.02	-0.40	±0.07	-0.11	±0.37	2.73	±0.88	0.01	±0.08	-0.38	±0.02	-0.28	±0.38
Ester	propanoic acid, 3-ethoxy-, ethyl ester[a]	763-69-9	NF	0.19	±0.07	-0.67	±0.25	-0.87	±0.09	2.44	±1.08	-0.38	±0.2	-0.56	±0.15	0.06	±0.62
Ether	2-hydroxy-1-methyl]ethyl ether[a]	NF	NF	-0.12	±0.04	-0.59	±0.1	-0.37	±0.32	-0.34	±0.25	-0.49	±0.25	-0.34	±0.28	2.46	±0.95
Ether	benzene, 1,1'-[oxybis(methylene)]bis-[a]	103-50-4	sweet fruity cherry earthy mushroom rose plastic	-0.98	±0.34	-0.20	±0.97	1.15	±0.72	0.19	±0.8	-0.69	±0.42	-0.15	±1.2	0.04	±1.3
Ketone	2-octanone[a]	111-13-7	earthy weedy natural woody herbal	2.40	±0.89	-0.92	±0.88	-0.02	±0.11	0.10	±0.17	-0.38	±0.86	0.04	±0.18	-0.02	±0.25
Ketone	3-(2-phenylethyl)pentane-2,4-dione[b]	NF	NF	-0.91	±0.03	-0.93	±0.11	-0.94	±0.14	1.88	±0.5	0.35	±0.71	0.19	±0.77	-0.36	±0.92
Ketone	3-hexanone, 2,5-dimethyl-4-nitro-[a]	59906-54-6	NF	2.14	±0.9	-0.69	±0.08	-0.55	±0.18	-0.48	±0.03	-0.59	±0.19	0.55	±1.62	-0.53	±0.16
Lactone	(δ-valerolactone)2h-pyran-2-one, tetrahydro-[b]	542-28-9	NF	-1.28	±0.29	-0.35	±0.15	2.35	±0.82	-0.05	±0.17	0.10	±0.31	-0.63	±0.58	-0.27	±0.13
Lactone	pantolactone[b]	79-50-5	cotton candy	-0.76	±0.17	-0.39	±0.36	2.26	±0.12	-0.59	±0.25	0.18	±0.24	-0.74	±0.4	-0.54	±0.51
Nitro	cyclopentane, nitro-[b]	2562-38-1	NF	0.79	±0.78	-0.10	±0.08	0.76	±0.75	0.43	±0.35	0.24	±0.69	0.07	±0.34	1.13	±1.39
Norisoprenoid	1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one[b]	23726-93-4	natural sweet fruity rose plum grape raspberry sugar	2.55	±0.94	-0.67	±0.15	-0.59	±0.4	-0.11	±0.81	-0.45	±0.32	-0.51	±0.35	0.21	±0.72
Norisoprenoid	2-buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-[a]	23726-93-4	apple rose honey tobacco sweet	2.44	±1.44	-0.66	±0.42	-0.39	±0.51	0.21	±0.48	-0.46	±0.32	-0.36	±0.71	0.00	±0.7
Norisoprenoid	2-buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-[b]	23726-93-4	apple rose honey tobacco sweet	2.56	±1.17	-0.74	±0.12	-0.43	±0.58	-0.06	±0.67	-0.42	±0.32	-0.52	±0.4	0.12	±0.61
Norisoprenoid	5,9-undecadien-2-one, 6,10-dimethyl-, (e)-[a]	689-67-8	fresh rose leaf floral green magnolia aldehydic fruity	2.34	±0.82	-0.77	±0.56	-0.47	±0.39	-0.35	±0.4	-0.68	±0.04	-0.55	±0.49	-0.32	±0.49
Norisoprenoid	5,9-undecadien-2-one, 6,10-dimethyl-, (e)-[b]	689-67-8	fresh rose leaf floral green magnolia aldehydic fruity	2.62	±0.52	-0.16	±0.29	-0.21	±0.53	-0.88	±0.09	-0.32	±0.18	-0.46	±0.26	-0.15	±0.52
Phenol	phenol, 2,4-bis(1,1-dimethylethyl)-[a]	96-76-4	Phenolic	2.50	±1.26	-0.76	±0.13	-0.69	±0.26	0.01	±0.12	-0.52	±0.26	-0.05	±0.7	-0.23	±0.31
Pyrrole	1h-pyrrole-2-carboxaldehyde, 1-	2167-14-8	burnt roasted smoky	-0.36	±0.13	-0.78	±0.08	-0.44	±0.1	2.76	±0.59	-0.43	±0.08	-0.44	±0.26	-0.27	±0.1

	ethyl-[a]															
Sulfoxide	methane, sulfinylbis-[a]	9008-97-3	None	-0.55	±0.42	0.02	±0.89	1.60	±0.64	0.53	±1.13	0.66	±0.38	0.26	±0.25	0.28	±0.62
Sulfoxide	methane, sulfinylbis-[b]	9008-97-3	None	-0.53	±0.43	0.12	±0.79	1.05	±0.17	0.32	±0.96	0.76	±0.42	0.29	±0.45	1.08	±0.85
Terpene	β-gurjurene (1h-cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1ar-(1aα,7α,7aα,7bα)]-)[b]	17334-55-3	NF	2.62	±0.99	-0.41	±0.17	-0.26	±0.41	-0.02	±0.65	-0.04	±0.39	-0.20	±0.15	0.43	±0.52
Terpene	trans-β-Ocimene (1,3,6-octatriene, 3,7-dimethyl-, (e)-)[b]	3779-61-1	sweet herbal	-0.32	±0.07	0.49	±0.45	1.13	±0.11	0.21	±0.33	0.56	±0.67	0.55	±0.09	0.94	±0.5
Terpene	α-Ocimene (1,3,7-octatriene, 3,7-dimethyl-, (e)-)[a]	502-99-8	fruity floral wet cloth	-0.21	±0.04	0.50	±0.4	0.88	±0.26	0.49	±0.27	0.52	±0.9	0.34	±0.2	0.96	±0.8
Terpene	α-Terpinene (1,3-cyclohexadiene, 1-methyl-4-(1-methylethyl)-)[b]	99-86-5	woody terpene lemon herbal medicinal citrus	1.38	±1.01	-1.35	±0.29	-0.10	±1.02	1.01	±1.28	0.20	±0.83	-0.21	±0.55	0.41	±0.88
Terpene	Linalool (3,7-dimethyl-1,6-octadien-3-ol)[a]	78-70-6	citrus, orange, floral, terpy, waxy and rose	-0.23	±0.23	0.54	±0.09	1.06	±0.18	0.60	±0.06	0.59	±0.3	0.46	±0.38	0.83	±0.56
Terpene	Geraniol (2,6-octadien-1-ol, 3,7-dimethyl-, (e)-)[a]	106-24-1	Floral, sweet, rosey, fruity and citronella-like with a citrus nuance	-1.24	±1.1	-0.75	±0.58	0.45	±0.35	-0.24	±0.57	-0.66	±0.93	-0.29	±1.34	0.32	±1.9
Terpene	Geraniol (2,6-octadien-1-ol, 3,7-dimethyl-, (e)-)[b]	106-24-1	Floral, sweet, rosey, fruity and citronella-like with a citrus nuance	0.03	±0.06	0.37	±0.36	0.92	±0.23	0.64	±0.16	0.41	±0.43	0.43	±0.16	0.82	±0.7
Terpene	Geraniol (2,6-octadien-1-ol, 3,7-dimethyl-, (e)-)[b]	106-24-1	Floral, sweet, rosey, fruity and citronella-like with a citrus nuance	0.82	±0.43	-0.98	±0.39	0.64	±0.64	-0.32	±0.87	-0.28	±1.07	-0.45	±0.19	0.83	±2.13
Terpene	Linalool (3,7-dimethyl-1,6-octadien-3-ol)[b]	78-70-6	citrus, orange, floral, terpy, waxy and rose	-0.36	±0.54	0.38	±0.14	0.90	±0.22	0.42	±0.13	0.55	±0.58	0.44	±0.39	1.23	±0.92
Terpene	cis-α-Ocimene (1,3,7-octatriene-3,7-Dimethyl-, (z)-)[b]	6874-44-8	fruity floral wet cloth	-0.35	±0.8	-0.31	±0.06	1.21	±0.54	0.82	±0.48	0.36	±0.83	0.20	±0.11	1.04	±0.9
Thioester	butanethioic acid, 3-methyl-, s-methyl ester[b]	23747-45-7	sharp ripe cheese sulfury acrid	-0.55	±0.36	-0.99	±0.44	0.17	±0.66	2.03	±0.15	-0.99	±0.19	-0.92	±0.89	0.01	±0.71

			fermented tomato mushroom													
Unknown	analyte 1283[a]	NA	NA	-0.56	±0.15	-0.49	±0.2	-0.24	±0.09	-0.41	±0.13	-0.44	±0.08	0.81	±1.31	2.35	±0.82
Unknown	analyte 2065[b]	NA	NA	2.06	±1.25	-0.41	±0.27	0.27	±0.36	-1.66	±1.32	0.05	±0.15	-0.14	±0.31	0.25	±0.49
Unknown	analyte 2315[b]	NA	NA	-0.04	±0.62	0.22	±0.7	2.11	±0.74	-1.66	±0.14	0.21	±0.17	-0.48	±0.26	-0.23	±0.22
Unknown	analyte 2523[b]	NA	NA	2.56	±1.08	-0.36	±0.24	-0.32	±0.22	-0.18	±0.27	0.39	±0.88	-0.24	±0.05	0.17	±0.36
Unknown	analyte 2532[b]	NA	NA	1.37	±1.07	0.06	±0.38	0.13	±0.17	-0.38	±0.06	1.18	±0.4	-1.35	±0.1	-0.96	±1.14
Unknown	analyte 2720[b]	NA	NA	2.37	±1.19	-1.08	±0.12	-0.66	±0.58	0.34	±0.31	-0.37	±0.3	-0.44	±0.59	-0.24	±0.29
Unknown	analyte 4492[b]	NA	NA	-0.30	±0.38	-0.83	±0.47	-0.24	±0.16	-0.46	±0.31	1.80	±0.62	-0.09	±1.28	-0.27	±0.58
Unknown	analyte 4552[b]	NA	NA	-0.27	±0.12	2.71	±1.2	-0.25	±0.11	-0.18	±0.08	-0.34	±0.19	-0.38	±0.19	-0.40	±0.09
Unknown	analyte 4616[b]	NA	NA	-0.43	±0.06	-0.82	±0.64	-0.54	±0.22	-0.45	±0.05	-0.60	±0.3	2.10	±0.67	-0.39	±0.9

a Compounds found in the 30min extractions
b Compounds found in the 5min extractions
SC *S. cerevisiae*
TD *T. delbrueckii*
CZ *C. zemplinina*
LT *L. thermotolerans*
MP *M. pulcherrima*
PK *P. kluyveri*
KA *K. aerobia*

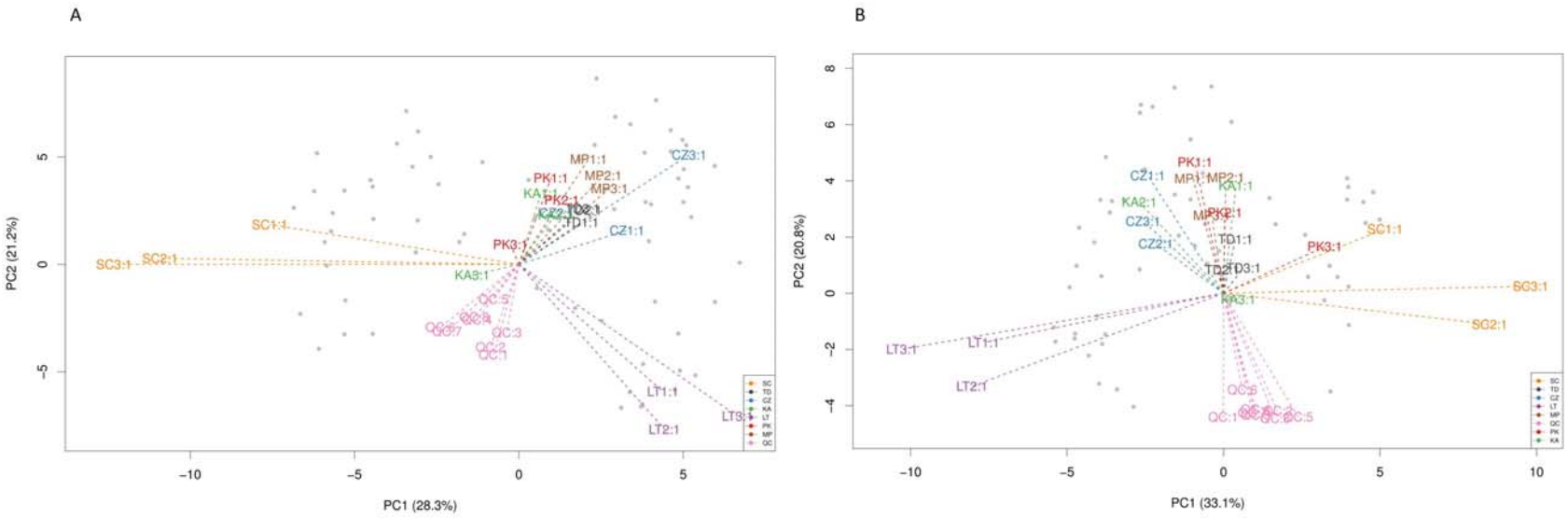

Figure 1

PCA after unit variance scaling of the statistically significant compounds of interest found to be different between the *S. cerevisiae* only control and the non-*Saccharomyces* staged inoculations A) 5 minute, and B) 30 minute extraction time data. SC represents *S. cerevisiae* fermentations, TD represents *T. delbrueckii* fermentations, CZ represents *C. zemplinina* fermentations, KA represents *K. aerobia* fermentations, LT represents *L. thermotolerans* fermentations, PK represents *P. kluyveri* fermentations, MP represents *M. pulcherrima* fermentations and QC represents the quality control samples.

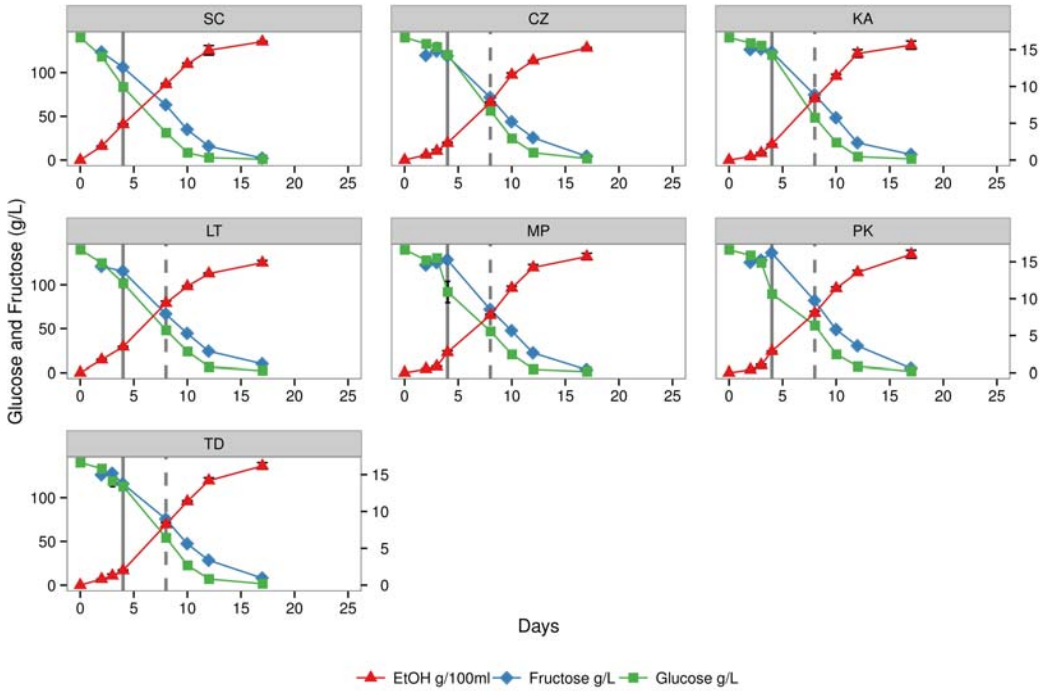

Figure 2

Each graph indicates the progress of the fermentations by each species. SC: *S. cerevisiae*, CZ: *C. zemplinina*, KA: *K. aerobia*, LT: *L. thermotolerans*, MP*: M. pulcherrima,* PK*: P. kluyveri,* TD*: T. delbrueckii.* Each graph shows glucose consumption (square shape), fructose consumption (diamond shape), and ethanol production (triangle shape). All of these lines are an average of the three biological replicates and the standard deviation is show by error bars. The solid vertical line indicates where the ethanol concentration reached 2% and in the case of the non-*Saccharomyces* fermentations *S. cerevisiae* was added. The dashed vertical line indicates where the non-*Saccharomyces* yeast was no longer detectable by plate count.

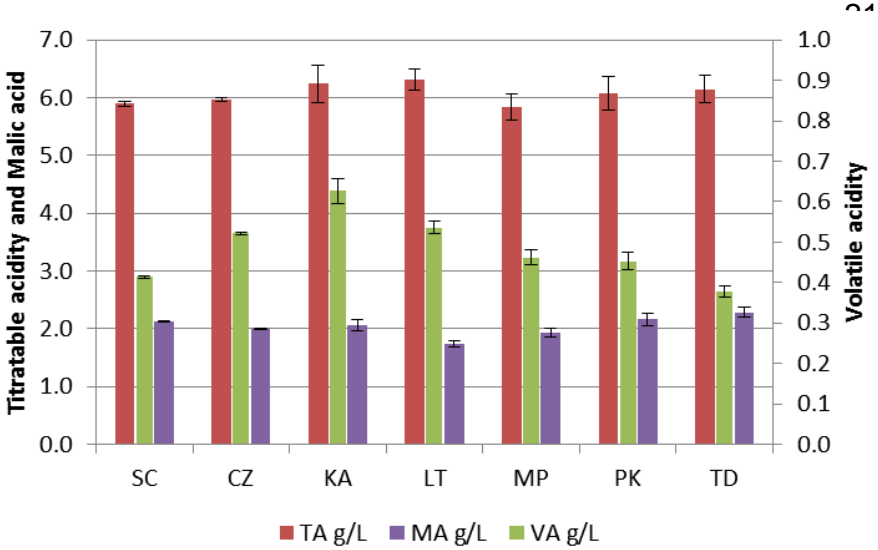

Figure 3

Bar graph indicating the final average acidity and pH levels of each fermentation. TA indicates titratable acidity while VA indicates volatile acidity. SC: *S. cerevisiae*, CZ: *C. zemplinina*, KA: *K. aerobia*, LT: *L. thermotolerans*, MP*: M. pulcherrima,* PK*: P. kluyveri,* TD*: T. delbrueckii.*

Chapter 6

General discussion and conclusions

6.1 General discussion and conclusions

When it comes to alcoholic fermentation *Saccharomyces cerevisiae* is the go-to wine yeast of choice. It is fast and reliable in so far as it can produce a very consistent product. Both are necessary characteristics that are required when running a large, successful, business and wine production is an enormous business that relies heavily on these aspects. In 2013, the world traded 9.7 bn liters of wine worth more than 25.7 bn Euros (International Organisation of Vine and Wine (OIV), 2014). It is no wonder the majority of yeast research to date has been focused on *S. cerevisiae* due to the contribution it makes to the quality, typicality, and style of wine available to the consumer. But what about the other yeasts? The tens of genera and species that have always been present on the grape berries or in the wineries? Those that were very much part of the winemaking process, even if no one knew it, until the discovery and "idolization" of *S. cerevisiae* in the form of strain selection and the production and copious use of starter cultures to quickly outcompete native yeasts.

These so-called 'non-*Saccharomyces*' yeasts were lumped into a category and typically discussed only in the context of spoilage wine organisms. This was in large part due to the fact that they were often isolated from wines that displayed less than desirable organoleptic properties such as high volatile acidity, or high volatile phenol content (Loureiro, 2003). Thus they were identified as detrimental yeasts to be removed as quickly as possible from the fermentation by adding copious amounts of SO_2 and inoculation of high cell numbers of *S. cerevisiae*. Recently however, researchers and winemakers have begun to investigate the potential of these yeasts beyond this previously narrow scope. Of the 1500 known species of yeasts, over 40 have been found in grape must. Though none produce ethanol at nearly the rate of *S. cerevisiae,* studies on spontaneously fermented wines have shown that these wines can have greater aroma, flavor complexity, and even mouthfeel than *S. cerevisiae* inoculated wines. This is slowly leading to a shift in the industry and the intentional use of non-*Saccharomyces* yeasts in wine production to enhance organoleptic properties is catching on. This trend has even led to certain species of yeast being made commercially available and marketed specifically for wine production (Hansen, 2009, 2011; Lallemand, 2012, 2013; Laffort, 2013). Despite their presence in the marketplace however, relatively little is known about how these yeasts interact with different grape musts or with the other yeasts, especially *S. cerevisiae*. Furthermore, how exactly these interactions may influence wine aroma or flavor remains to be determined.

This study sought to begin to answer these questions as well as investigate other non-*Saccharomyces* yeasts not currently commercially available but have in recent years been shown to be part of the wine microbiome. The purpose was to evaluate the organoleptic

potential of these yeasts in wine. In total the following eight non-*Saccharomyces* yeasts were investigated: *Torulaspora delbrueckii, Lachancea thermotolerans, Pichia kluyveri* and *Metschnikowia pulcherrima* were commercial starter strains, while *Candida zemplinina, Kazachstania aerobia, Kazachstania gamospora,* and *Zygosaccharomyces kombuchaensis* were wine environmental isolates. A combination of targeted and untargeted gas chromatography-mass spectrometry (GC-MS) analytical methods were developed specifically to evaluate the volatile chemical profile of grape must and wine fermented with these yeasts.

The first set of experiments assessed the growth characteristics and volatile aroma production of the following five yeasts: *K. gamospora, L. thermotolerans, M. pulcherrima, T. delbrueckii* and *Z. kombuchaensis* in both Shiraz and Sauvignon blanc grape must prior to the addition of *S. cerevisiae* by allowing the yeasts to grow in the musts to an ethanol concentration of 2%. The experiment was performed using small volumes of must at laboratory scale. A solid-phase microextractions (SPME)-GC-MS analysis was then used to analyze the musts. The method targeted 90 different compounds across a wide range of chemical classes. This study clearly showed that some yeasts fermented more quickly than others. The worst was *Z. kombuchaensis* which showed very slow growth kinetics and ethanol production compared to the other yeasts. *K. gamospora* on the other hand fermented almost as quickly as the *S. cerevisiae* control. This study also clearly demonstrated the differences in aroma compound production between the species in the initial stages of alcoholic fermentation. Compared to the *S. cerevisiae* metabolic footprint *K. gamospora* produced relatively more esters and phenols. The other yeasts showed significant differences in individual compound quantities if not differences in all compound classes. Though this method covered a substantial number of compounds there are over 1300 compounds associated with yeast and wine. As such, other methods needed to be developed to gain a more complete picture of the wine yeast metabolome.

Promising results from this preliminary experiment supported the next phase of the project. The experiment had shown that all of the yeasts, with the exception of *Z. kombuchaensis,* did not produce large amounts of off flavors and even produced higher levels of some desirable compounds than the *S. cerevisiae* control. An untargeted SPME-GCxGC-TOF-MS analysis capable of detecting and identifying more than 1000 compounds in the headspace of wine was developed in-house based on literature and trial and error. To study the differences in how these yeasts affected white and red grape must Sauvignon blanc and Shiraz grapes were used to make wine at full scale using a sequential inoculation strategy with *S. cerevisiae.* The Sauvignon blanc wine was also evaluated by a sensory panel and the chemistry data was correlated to those results. A slightly different set of yeasts were used. *Z. kombuchaensis* was dropped and the commercially produced *Pichia kluyveri* was added. A wine environmental

isolate of *C. zemplinina* was added and *K. aerobia* replaced *K. gamospora* as it was not available in South Africa where this set of experiments took place.

In general, it can be said that the yeasts performed very differently in the two grape musts and many of the trends observed in the initial study did not hold to the end of the fermentations completed by the addition of *S. cerevisiae*. In both the chemistry and sensory evaluations of the Sauvignon blanc fermentations the most distinct differences were observed between the control (*S. cerevisiae* only) and the *C. zemplinina-S. cerevisiae* sequential fermentations. In contrast, in the Shiraz fermentations, it was the *L. thermotolerans-S. cerevisiae* sequential fermentations which proved to be the most significantly divergent from the control. These differences were not consistent with the first study which showed a relatively small set of changes from the control fermentation. The *L. thermotolerans-S. cerevisiae* sequential fermentations differed from the control by having the highest relative level of terpenes, especially linalool. In the preliminary study the relative concentration of linalool was not statistically significantly different from that of the control. The possible reasons for the differences seen here are numerous. There is still very little that we know about how yeast interact with one another and affect each other's metabolic processes.

Another compound found to be in high concentration in the completed fermentations was 1-ethyl-1h-pyrrole-2-carboxaldehyde, a pyrrole whose aroma is described as burnt, roasted or smoky. This compound was not part of the targeted analysis used in the first study and highlights the need for both targeted as well as untargeted methods in metabolome analyses to form a more complete profile.

Other differences between the primary and secondary set of experiments was that in the secondary, fully completed wine fermentations, the growth rates, sugar consumption, and ethanol production were also different for the yeasts between the two grape musts. One theory proposed for the reason behind this is the difference in micro-nutrient composition such as amino acid concentrations of the must, combined with a difference in fermentation temperatures. The primary experiment fermented both musts at the same temperature and the must for the second experiments came from a different harvest. Temperature and nutrient availability are two factors known to affect the growth and metabolic behavior of *S. cerevisiae* but which have not been studied closely in the non-*Saccharomyces* yeasts investigated in this study.

All of these experiments shine light on the need for more research into this field. This is also evidenced by the fact that many of the differences seen between the fermentations, regardless of the grape must used, had potential to positively impact the organoleptic properties of the

wine. In general this project was able to take the most in-depth look ever at the chemical composition of two different wines fermented with eight different yeast species. In many ways however, like all good science, these results provided far more questions than they did answers giving a direction for future work.

The technologies we employed were state of the art and were able to separate, detect and identify more than 1000 compounds. Yet, we were still left unable to adequately hypothesize for example, which compound, or even class of compounds, was responsible for the 'fermented apple' aroma that was the sensorial hall mark of the *C. zemplinina-S. cerevisiae* sequential fermentations in Sauvignon blanc. And even though we understand that the starting amino acid composition can drastically affect higher alcohol and ester production, the fact that these weren't the only significant differences seen between the Shiraz and Sauvignon blanc fermentations would indicate that there is more to the overall matrix effect than we currently understand. A significant limitation in the study design was that although we chose conditions that most closely mimicked those in winemaking the change of matrix and conditions between experiments made it difficult to track compounds from early to late stages of fermentation and to clearly identify a metabolic signature of the different strains used. Another limitation was in the data analysis. With such complex data there were obviously many different ways in which the data could have been interpreted. For this reason we made the data sets publically available on the metabolomics repository so that anyone who may be interested can mine the data.

In conclusion, any future studies undertaken should use as complete and comprehensive an approach as possible; incorporating many more stages and types of analysis. Ideally one would start with two or more different grape musts and analyse them quantitatively for their basic chemical makeup including, but not limited to, terpene and amino acid composition. The non-*Saccharomyces* yeasts would then be added and allowed to ferment for a given amount of time before samples were taken and analysed both for volatile and non-volatile constituents. This could begin to answer the questions of how these yeasts are interacting with and using the grape matrix. Samples could also be taken at this stage for genetic analysis to understand gene expression within the yeasts. *S. cerevisiae* would then be added and samples taken periodically to track compound production and consumption over time. All fermentations would need to be conducted in at least triplicate to ensure statistical accuracy.

Even with such an experiment it is entirely possible that a true signature does not actually exist for each yeast and that the yeast footprints vary too much between matrices and growth conditions to formally be able to answer the seemingly simple question of 'if I use yeasts X, Y and Z what, exactly, will my wine taste and smell like?'. That does not mean, however, that attempting to answer this question is not a worthy pursuit, and with the rate of technological

advancement increasing exponentially we may one day very soon be able to generate –and analyse– enough data to fully understand the mystery that is wine.

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