Characterisation of lees and Novel uses for Yeast Lees to Create New Wine Styles.

A thesis presented in fulfilment of the

requirements for the degree of

Doctor of Philosophy

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Abstract

When wine is aged on lees, the process of lees autolysis causes the release of a range of constituents including mannoproteins, polysaccharides, amino acids and fatty acids that interact with the fermented wine leading to changes in the final flavour and sensory aspects of the wine. There is no doubt that ageing wines on lees for extended periods of time, adds beneficial organoleptic qualities to a finished wine. Furthermore, whilst the interactions of wine lees on phenolics, lipids and mannoproteins released have been widely studied, exactly how all these factors and constituents influence final wine quality is not totally clear. It can be said that there is still a poor understanding of how lees impacts on wine aroma. Moreover, the addition of autolytic enzymes to speed up lees breakdown or the addition of inactivated dry yeast (IDY) preparations to enhance the release of mannoproteins and glucans so that the perceived benefits of lees exposure are achieved in a shorter timeframe is only a recent advance over the last decade or so. Consequently, more research is needed in this field in order to further clarify the mechanisms and factors that lead to these perceived changes in a wine upon ageing on lees.

To this end, the Introduction for this PhD thesis provides a complete summary of the current state of play in terms of knowledge as to the scientific and practical potential of ageing wines on lees. It begins with a detailed account into the history of winemaking in Australia and the current state of play in terms of how Australian winemakers perceive utilising lees exposure to prepare their wine styles. Autolysis is a slow process and often is conducted over periods of years. Coupling this with the fact that wines are produced from a range of grape varieties; employ a range of different yeasts and utilise different winemaking protocols, results in a complex field of research study in which it can often be difficult to draw clear conclusions between the research studies and into the mechanisms and factors that lead to these perceived changes of a wine upon ageing on lees. Consequently, the Introduction provides a detailed account of all major research studies completed over the last few decades. The Introduction then concludes with an introduction to the aims of the research to be carried out within this PhD thesis.

Chapter two describes an exploratory study into the effect of lees exposure on both a Pinot Noir wine and a Chardonnay wine over a two-year period. A recent survey into current Australian practices showed that the ageing of Pinot Noir on lees is highly employed in Australia thus leading us to include this variety in this study. The use of lees ageing for the preparation of Chardonnay wines is typical throughout the World. Furthermore, the project was enlarged to include a number of wine and lees treatments (including the addition of commercial enzyme preparations). A range of chemical parameters were measured over the two-year period in order to evaluate further the importance of lees exposure on these wines. Some of the parameters measured, such as levels of polysaccharides or changes in colour, were those that have been shown to alter over time upon lees ageing, thus comparisons were able to be drawn between the work conducted here and that already reported in the literature. Moreover, we also examined other parameters which are not normally associated with lees autolysis such as possible changes in overall viscosity and metal concentrations in the wines to see if further information on how lees ageing impacts on a wines final quality could be gleaned. Unfortunately, the autolytic enzyme utilised in this study was found to be not very active at enhancing the autolysis rate in our wine treatments, thus the results found were not entirely as we hoped, although some new preliminary findings were captured. Clearly further highly focussed research is needed to unravel this complicated field of wine research.

Based on the results of the above study we then found ourselves in a position to devise a new alternative strategy to avoid the uncertainties associated with traditional lengthy lees exposure times, the results of which are described within Chapter three of this thesis. Instead of simply leaving the wines on lees for extended periods of time, we explored the concept that exposure of lees to microwaves for a short period of time may accelerate autolysis and when the treated lees is added back to a base wine for a short time, could the same perceived benefits of lees ageing be observed. This new technological approach for the preparation of new wine styles, coupled with both chemical evaluations and formal sensory trials was evaluated and it was found from our initial studies that there appears to be clear evidence that such a process leads to wines in which there is a perceived difference in the organoleptic properties of the microwaved lees wines when compared to the control wines. Further studies are needed in order to clearly define the key constituents that are altered in terms of their concentrations when such an approach is employed, however, it can be concluded that microwave assisted lysis of yeast lees appears to be a

new method for the accelerating of yeast autolysis and thus allows for a shortening of wine/lees exposure time needed to achieve the perceived organoleptic benefits of lees exposed wines.

Chapter four describes a study on the use of commercial inactivated dry yeast (IDY) preparations and their ability to alter the properties of a fermented Chardonnay wine and a browning model wine system containing (+)-catechin, iron(II), copper(II), and acetaldehyde aover a short period of time. Such yeast derived preparations are now being used as an alternative technique to ageing wines on lees, because they permit a quicker release into the wine of yeast compounds such as mannoproteins and glucans and as such the perceived benefits of lees exposure are achieved in a shorter timeframe. We analysed 10 commercial IDY preparations and evaluated their effectiveness at altering the base wines composition and the browning model wine system by a range of analytical measurements. Importantly, it was found that a range of the IDY preparations were able to substantially inhibit oxidative browning with the amount of soluble proteins being released correlating well with their preventative browning abilities. Such a finding is yet to be reported in the literature. Undoubtedly further scientific research will aid in unlocking the mysteries of IDY preparations and the host of positive effects that they can have on a finished wine.

Finally, Chapter five contains a detailed description of all the experimental methods and analyses utilised throughout these studies.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Yuanyuan Wang

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Abbreviations

μM	Micromolar
11D	Lees racked off on the 11th day after primary fermentation
3D	Lees racked off on the 3rd day after primary fermentation
4-EP	4-Ethylphenol
4-EG	4-ethylguaiacol
5'-GMP:	5'-Guanosine monophosphate
5'-IMP	5'-Inosine monophosphate
AA	Amino acids
Alc	Ethanol concentration
ANOVA	Analysis of variation
AOC	Appellation d'origine contrôlée
AWRI 1503	Yeast strain *Saccharomyces cerevisiae* x *Saccharomyces kudriavzevii*
AWRI Fusion	AWRI 1502: yeast strain *Saccharomyces cerevisiae* x *Saccharomyces cariocanus*
BA	Biogenic amine
Be	Baume
BSO_2	Bound sulphur dioxide
C	Control
CCBG250	Commasie brilliant blue 250
CHAR	Wine made from Chardonnay grape variety
CIE*lab*	Commission Internationale de l'Éclairage
DAD	Diode array detector
DPPH	Diphenylpicrylhydrazyl
E	Enzyme
SEM	Scanning electron microscope
EU	European Union
FLD	Fluorescence detector
FRAP	Ferric reducing ability of plasma
FSO_2	Free sulphur dioxide
g	Gram(s)
GC	Gas chromatography
GRAS	Generally regarded as safe

GSH	Glutathione
h	Hour(s)
hL	Hectolitre(s)
HPLC	High pressure liquid chromatography
IDY	Inactivated dry yeast preparations
kg	Killogram(s)
KHT	Potassium hydrogen tartrate
L	Litre(s)
LAB	Lactic acid bacteria
M	Molar
min	Minutes
mg	Milligram(s)
mL	Millilitre
mmol	Millimoles
MLF	Malolactic fermentation
mM	Millimolar
MS	Mass spectrometer
Mw	Molecular weight
NMR	Nuclear magnetic resonance
non-GMO	Non-genomic modification organisms
OIV	International organisation of wine and vine
OTC	Ochratoxin
PDM	Yeast strain *Saccharomyces cerevisiae* (variety: *bayanus*)
PMS	Potassium metabisulphite
PN	Wine made from Pinot Noir grape variety
ppm	Parts per million(s), mg/L
PPO	Polyphenol oxidase
SD	Standard deviation
SEM	Scanning electron microscopy
SO_2	Sulphur dioxide
TA	Titratable acidity
TEM	Transmission electron microscope
KHT	Potassium hydrogen tartrate
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

TSO_2	Total sulphur dioxide
VA	Volatile acidity
Vc	L-Ascorbic acid or vitamin C
v/v	Volume/volume

Figures, Schemes and Tables

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In Adelaide, I had the darkest experience ever in my life. I wish I could live a healthier and happier life afterwards.

CHAPTER 1: INTRODUCTION.

1.1 General Introduction.

Wine is an especially complex alcoholic beverage that is derived from the fermentation of grapes, a process that dates back many thousands of years to the late Stone Age. The fermentation of wine grapes with yeasts results not only in the consumption of sugar and the production of ethanol and carbon dioxide but also alters the balance of numerous other chemical constituents within the wine. The unique aromas, tastes and oral sensations of wines are attributed to hundreds of volatile and non-volatile organic and inorganic components present in the finished wine.[1] However, the chemical constituents underpinning the unique tastes and aromas of finished wines do not only originate from the grape berry itself nor the primary fermentation process employed but also may originate during other processes utilised during the vinification process. Such other processes may include malolactic fermentation (secondary fermentation) or the addition of additives such as fining agents, sulphur dioxide, tannins and polysaccharides, exposure to oak and in modern wine making times, the addition of commercial autolytic enzymes or inactivated dry yeast products (IDY).

Whilst the importance of how many of these processes change the chemical constituents of wines has been well studied over the last 50 years, the addition of autolytic enzymes to speed up lees breakdown or the addition of inactivated dry yeast preparations to enhance the release of mannoproteins and glucans so that the perceived benefits of lees exposure are achieved in a shorter timeframe is only a recent advance over the last decade or so. Consequently, there is still much research needed to identify the key constituents responsible for these beneficial effects and to determine what the mechanisms are behind the evolution of these effects in a wines medium.

Aging on lees is a classical step in the winemaking process of many wines. It may be applied to both still and sparkling wines and can last for periods of a few months to many years. This timeframe is also controlled by legislation in some countries. Yeast autolysis, which is the breakdown of yeast cells, is a relatively slow process associated with yeast

cell death, and involves the release of cytoplasmic and cell wall components that enrich the finished wine in many desirable ways.

The natural process of autolysis is slow and expensive; both in terms of the equipment required and in terms of the labour involved.[2] To cope with these economic constraints, additives, prepared by autolysis have been developed. For example, yeast autolysates are used to activate alcoholic and malolactic fermentation.[3] The benefits of this method have been studied in terms of interactions between the lees and their environment, notably the macromolecules released from yeasts into the wine during autolysis.[4] However, the experimental methods used vary greatly and it is difficult to extrapolate most of the results to the process of wine aging on lees. Only a few studies have dealt with the physicochemical properties of lees during autolysis, especially concerning oxygen, polyphenols and other wine compounds.[5]

A greater understanding of how lees exposure benefits a wines final organoleptic quality and the development of alternative techniques to the traditional aging of wines on lees, allowing for a quicker release of yeast cell components into a wine, is clearly warranted and forms the basis of the works contained within this thesis.

1.2 The Road to Viticulture and Oenology in Australia.

The production of the first wines has been traced to Western Iran, where jar shards have been dated back to around 3000 BC[6,7] This jar which came from the ancient village of Godin Tepe in the Zagros mountains is believed to have been used for storing wine as chemical analysis identified tartaric acid residues which is considered by scientists to be a marker of wine production.[6] From there the knowledge of wine making is believed to have spread to Egypt, Greece and through Europe by travelling sea merchants.[6] Indeed clear evidence of international winemaking first appeared in the representations of wine presses that date back to the reign of Udima in Egypt.[8] The consumption of wine soon became to be held in high regard in religious circles. In ancient Egypt during the Dynasty period (3150 - 332 BC), the royal family and upper class consumed red wine as it was linked to the blood of Osiris, the God of resurrection and believed suitable for a good afterlife.[7,9] Even in the Christian World, wine is often referred to as the ‘nectar of the

Gods' and is used as a symbol of Christ's blood when taking Holy Communion.[10] Consequently the consumption of wine has been enjoyed for millennia.

Wine is nowadays produced in every corner of the World. The top ten producers, as of 2010, in terms of volume are displayed in *Figure 1.1*. Italy and France lead the way with some 17.5% and 16.2% of total production respectively. Australia is currently the sixth largest producer of wine in the World at some 4.4% of total production.[11] The ideal growing regions for grapevines in terms of their global location generally sit between 30-50 degrees latitude above and below the equator where the average temperature usually sits between 10 - 20 ºC.[12]

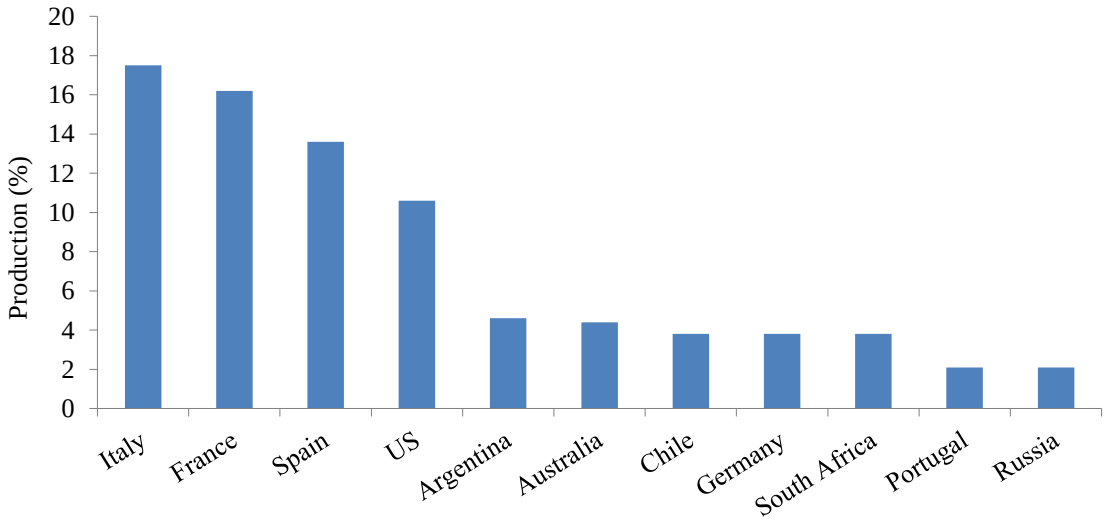


Figure 1.1. The top ten wine producing countries in the World.[11]

Grape growing began in Australia soon after settlement by the first fleet in 1788 when vines were planted by the first settlers.[13] Dry and hot weather conditions limited crops to areas with sufficient rainfall, with many original crops failing.[13,14] However, Gregory Blaxland and Captain John Macarthur grew successful plantations and produced English medal winning wines in 1816 and 1820 respectively. In 1824 the government employed James Busby as a viticulture teacher to establish the Australian wine industry.[13] Some of the main wine regions that came about from this were the Hunter Valley (1830), the North of Adelaide (1837), the Yarra Valley (1840), and the Barossa Valley (1840). From 1880, the introduction of modern irrigation methods led to an expansion of suitable areas

for vineyards to areas with insufficient rainfall, thus Australia's capacity for wine production became a national endeavour.[13]

The Australian wine industry has been through many changes over its 200 years of existence. When the states were joined under federation and Australia became one, the trade barriers were removed, creating interstate competition.[15] The cheaper south Australian wine sales boomed, while the eastern state wineries suffered due to this price competition. The onset of World War I resulted in a great decrease in sales, by temporarily destroying the export industry.[15] After the war, returning soldiers were given newly planted vineyards so they could support themselves through wine production. The great depression caused another low point in sales with more supply than demand resulting in vineyards being ripped out.[15] This wiped out the Yarra Valley at the time. Wineries, such as those in the Barossa, survived by producing mainly fortifieds, which were popular due to their cheap price. After the Second World War, demand and prices increased. More people had more money to spend on luxuries and as such, table wine became increasingly popular.[15]

In more recent years, the Australian wine industry has continued to expand in terms of production scale, unlike most other countries where the demand for their wine is shrinking.[13] The latest data collected by the Australian Bureau of Statistics highlighted that 1.75 million tonnes of grapes were crushed in 2012 - 2013, which is a slight increase on the 1.62 million tonnes picked in the previous 2011 - 2012 harvest.[16] There was an approximately 1.23 million litres of beverage wine produced by wineries with a large enough capacity to crush more than 50 tonnes of grapes per season.[16] By state, South Australia still holds top place in production, sitting around 46% of Australia's total wine production, followed by NSW(ACT), (*ca.* 31 %) and VIC (*ca.* 19%).[16]

Our wine industry and in particular South Australia has the climatic conditions to grow high quality grapes which in turn produce quality wines for the local and overseas markets. Approximately two-thirds of Australia's wine production is exported and production has, since the early 1980's, increased nearly four-fold.[17] It accounts for 10% of all rural exports and 1.5% of Australia's total exports of all goods.[17] In 2010 almost 75% of all Australian wines produced were for export and its production is considered very important to the Australian economy.[16] The top buyers were the UK, USA, Canada, China

and NZ. China has recently added to this growing demand within the export market, which has resulted in a small profit increase to partially combat the decline in exports to countries like the USA, which are suffering from the global financial crisis.[18]

In the last 30 years, there have been numerous technological advancements in the whole-of-chain approach to wine making in Australia and indeed the World. Advancements in vineyard management now provide multiple alternatives for the production of quality grapes. Modern techniques of irrigation,[15,19] trellis,[19] vine clones and grafting (for strength and resistance),[20] mulching,[19] nets (to protect against birds),[19] sprays to protect the vines from diseases (powdery mildew, downy mildew and botrytis),[21] and pesticides,[19] are all aspects needed to be considered when planting modern Australian vineyards. Even weather forecasts help in aiding the time of harvest to avoid rain damaged fruit. These processes are set in place to ensure, excluding any unforseen disaster, the growth and production of top quality grapes.

Furthermore, technological advances in winemaking equipment have been profound in the past few decades with many of them now adopted by the Australian wine industry. Mechanical assistance techniques such as machine harvesting, machine pruners,[22] mechanical transportation systems, sorting machines (for the removal of matter other than grape (MOG)),[23] crushers,[24] presses,[24] automation in fermenting tanks (with cooling and stirring capabilities),[22] refrigeration and bottling lines have all been developed. With this assistance, production can be carried out on a larger scale, resulting in the use of stainless steel fermentation vessels with up to 950,000 litre capability, and large stainless steel storage vessels as an alternative to oak barrels.[22] Maturations can also be carried out with oak chips such that the need to store wine in a barrel is avoided.[25]

Advancements have also been made in relation to controlling the final wines contents. Different fermentation options are available with the introduction of new yeast strains or selectively bred yeasts.[26,27] Wild yeast strains, known as microflora, can be controlled by the use of sulphur dioxide and pH. Introduction of selected yeasts can then become the dominant yeast during primary fermentation although some winemakers still prefer to utilise wild yeast strains. Such tightly controlled processes allow the winemaker to be able to predict with high certainty the types of aromas that will ultimately end up in the finished wine. Additives such as fining agents, sulphur dioxide, tannins and

polysaccharides are also now commonly used to change the mouthfeel, colour and clarity of a finished wine.[28] Furthermore, the addition of autolytic enzymes during lees ageing or the addition of inactivated dry yeast (IDY) preparations are now commonly employed to speed up the process of ageing on lees and/or change the final sensory attributes of the wine.[1] Finally, bottling with a choice of closures can ensure equality in the aging of wine during storage over the years.[29] All these advances have been driven by a greater understanding of the science behind grape growing and winemaking and have led to a tighter control over winemaking protocols needed to produce premium Australian wines.

1.3 The Global History and Significance of Wines Aged on Lees.

Ancient Roman wine makers were the first to observe that wine that was left (or sediment as they called it) exhibited vastly different characteristics than wines that were separated from the lees early on during vinification. While the complex chemical processes, now referred to as autolysis, were not well understood, they were able to perceive differences in finished wines such as enhanced creaminess, unique flavours such as toasty, biscuity, breadlike and floral aromas coupled with a decrease in astringency.[30] From these humble beginnings the process of *sur lie* ageing has evolved to such as extent that winemakers now use it for some of the Worlds best wines. In particular, the perceived benefits of ageing on lees for Champagne production in France is strictly controlled by the Appellation d'origine contrôlée (AOC) regulations, in that wines from Champagne cannot legally be sold until they have been aged on lees in the bottle for at least 15 months. Vintage champagnes must be aged for at least 3 years.[31] Besides Champagne, Cava, Franciacorta from Italy and Cap Classique from South Africa are well known 'bubblies' which employ the *sur lie* method of production.

Lees ageing is common practice for White Burgundy and other Chardonnay wines. The classic white wine example is Muscadet *sur lie* from the Loire valley in France. Today, many other white wines, even aromatic wines such as Sauvignon Blanc spend between three and six months on lees to enhance the structure, mouthfeel and complexity of the wine. Whilst lees contact today for white wines is a common practice after fermentation, red wines are usually racked off their lees earlier and not exposed to prolonged lees contact although this is not always the case.

1.4 Recent Attitudes of the Australian Wine Industry on Utilising Lees Contact during Red Wine Production.

A recent survey of the Australian wine industry highlighted some interesting findings as to the use of lees contact and the techniques employed during the preparation of Australian red wines and importantly also highlighted what the current state of play is in terms of the scientific understanding that winemakers have when employing such a process.[32] The survey was conducted online and was sent to 1658 Australian wineries so that a broad analysis of the industry would be possible. A total of 224 winemakers provided feedback (a response rate of 14%) and were from 49 different wine regions throughout Australia.

Responses from the winemakers indicated that 90% of them use lees contact during the production of white table wines along with a smaller proportion (29%) employing lees contact during white sparkling production, *Figure 1.2.*

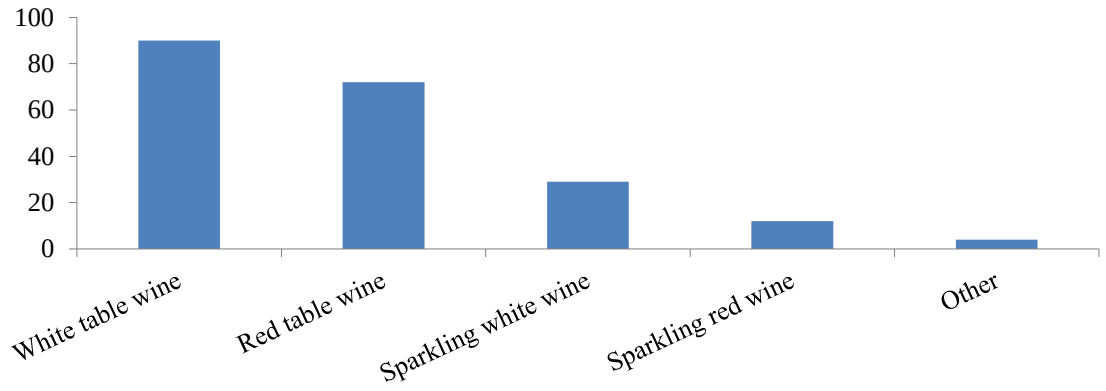


Figure 1.2. The percentage of lees contact used for different wine styles by Australian winemakers.[32]

As highlighted above the use of lees during white wine production is common throughout the World and the high use of lees contact for white table wine production in Australia is not unusual and is most probably related to Chardonnay production, which is the largest white variety produced in Australia.[17] It was, however, interesting to note that 72% of winemakers used lees contact as a red winemaking technique for the production of red table wines, *Figure 1.2*. This finding suggested that Australian winemakers are more

likely to use lees contact during red wine production when compared to the general trend throughout the World.[32]

Another interesting finding was that of the winemakers who used lees contact in red table wine production, 70% used the technique for Shiraz, 55% for Cabernet Sauvignon and 49% for Pinot Noir, *Figure 1.3*. The major use for the first two varieties correlated well with their production levels in Australia.[17] However, there was a disproportionably high percentage of lees contact used for the production of Pinot Noir compared to its relative production in Australia. This finding also bodes well with the fact that Pinot Noir was selected as one of the experimental wines to study for a two-year trial on lees with added autolytic enzymes which is the subject of chapter two of this thesis.

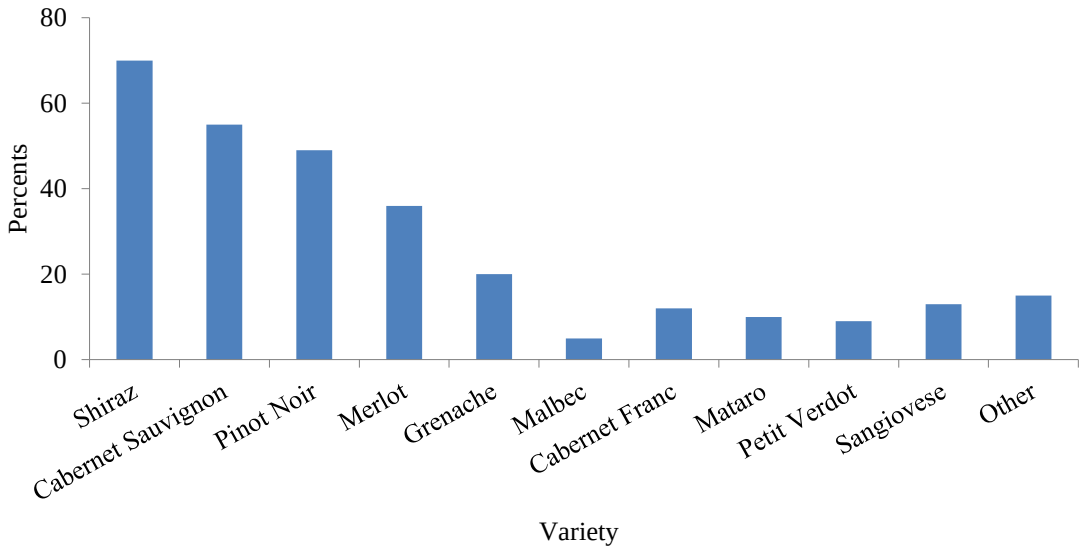


Figure 1.3. The percentage of winemakers employing lees contact on different red grape varieties.[32]

As a red wine making technique, lees contact can be conducted in various ways in order to obtain the desired wine style. It was found that the preferred vessels used to conduct lees contact in Australia were barriques or hogsheads with 76% of winemakers utilising such vessels. This was followed by stainless steel with 17% of winemakers reporting such use. In terms of duration of lees contact Australian winemakers reported that the most common contact period used by winemakers (34%) was for a short period between 0 and 3 months (34%). A further 29% of winemakers left the lees in contact with the red wine

for 3 - 6 months whilst 28% left the lees in contact for 6 - 12 months. Only 8% of winemakers left the lees in contact with their red wine for more than 12 months.[32]

During lees contact, autolysis can be assisted through the constant stirring of the lees and more recently companies have released autolytic enzymes that are reported to accelerate the autolytic process. While these are now readily commercially available, only 8% of winemakers reported that they actually employ such enzymes during lees contact for red winemaking in Australia. When asked, almost all winemakers (88%) suggested that the local wine industry does not know enough about the consequences of lees contact during red winemaking and that the low uptake of the use of autolytic enzymes reflects such attitudes.[32] These latter findings are particularly important to the studies reported in this thesis that focus on the use of autolytic enzymes during lees contacted wines.

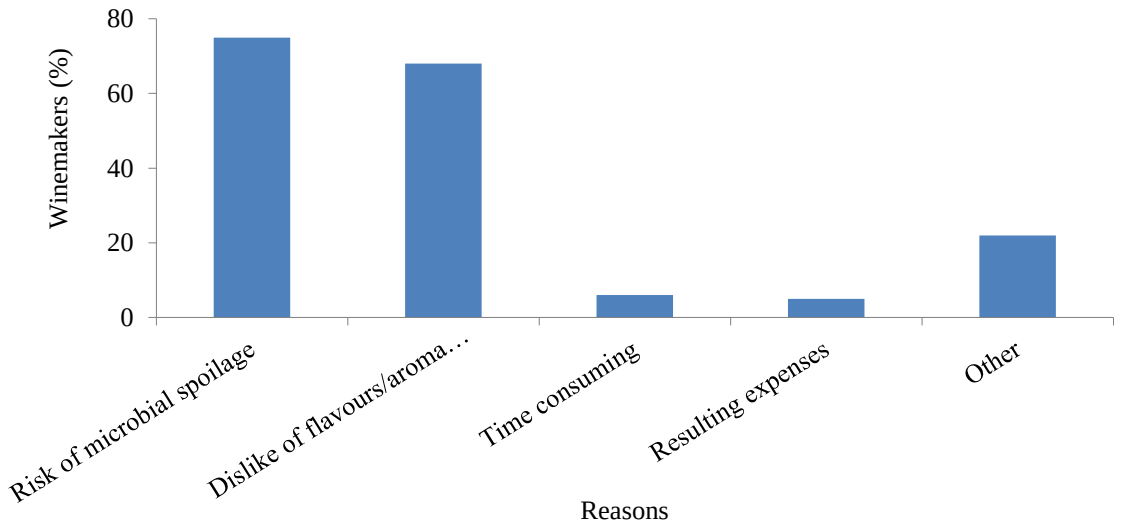


Figure 1.4. The different reasons winemakers don't use lees contact as a red winemaking technique.[32]

The winemakers also cited a range of reasons why some of them were hesitant to employ lees contact during red wine production in Australia. When asked why they chose not to use the technique, 75% believed that the technique increases the chance of microbial spoilage *Figure 1.4*. A further 68% of non-users of lees contact in red winemaking believed that the resulting flavour and aroma produced, was undesirable. Only a small

proportion of winemakers (6% and 5% respectively) believed that it was too time consuming or the resulting expenses outweighed the positives, *Figure 1.4*.

The results of the survey also indicated that Australian winemakers believed that the major consequences that result when a red wine is exposed to extended lees contact were:

- An increase in the structure of the wine
- Has a positive effect on the rate of MLF
- Results in a positive enhancement of aroma and flavour
- Aids in colour stability
- Aids in the reduction of salt levels
- 82% of winemaker believed that there is a positive effect on the sensory properties of the wine

Finally, the survey also explored what winemakers perceived that consumers knew about the use of lees contact in winemaking. Nearly all (99%) of winemakers believe that the general consumer does not understand the consequences of lees contact nor do they appreciate the technique in terms of how it aids in the production of a finished wine.

Overall the survey of winemakers from the Australian wine industry indicated that lees contact as a red winemaking technique is widely practiced with some clear perceived benefits, although there were also some that believed that such lees contact was detrimental to the final wine style that was to be achieved. It was also found that winemakers (88%) believe the industry does not know enough about the consequences of lees contact as a red winemaking technique. This was supported by many comments that stated that many winemakers base the decision to employ lees contact on anecdotal evidence and opinions and that beneficial scientific literature to support these assertions was limited.[32] Many comments were also made concerning where winemakers believe research should be concentrated. While the comments varied there was a strong interest in the sensory outcomes, the effect on ageing potential and how do autolytic enzymes aid in lees contacted wines. Also many respondents were interested in the effects of lees contact on Pinot Noir, thus this particular wine style was chosen as one to be included in the two-year trial discussed in chapter two of this thesis. The overall consensus from winemakers

was to have access to scientific data that could be translated to the industry with practical applicability, which bodes well with the overall themes of this thesis.

1.5 The Production of Lees during Winemaking.

Lees is defined as the dead yeast cells that settle to the bottom of the tank, wooden barrel or even a bottle after fermentation, which is part of the maturation phase. When the sugar food source and the nutrients for yeast become depleted during fermentation or the ethanol concentration reaches such a point where it is toxic to yeast survival, the cells die and sink to the bottom along with other particulates (e.g. grape skins, seed fragments etc) as depicted in *Figure 1.5*.

Figure 1.5. Examples of yeast lees. Lees at the bottom of a white wine in an open tank (left); Lees after fermentation in a bottle for a sparkling wine (right).[33]

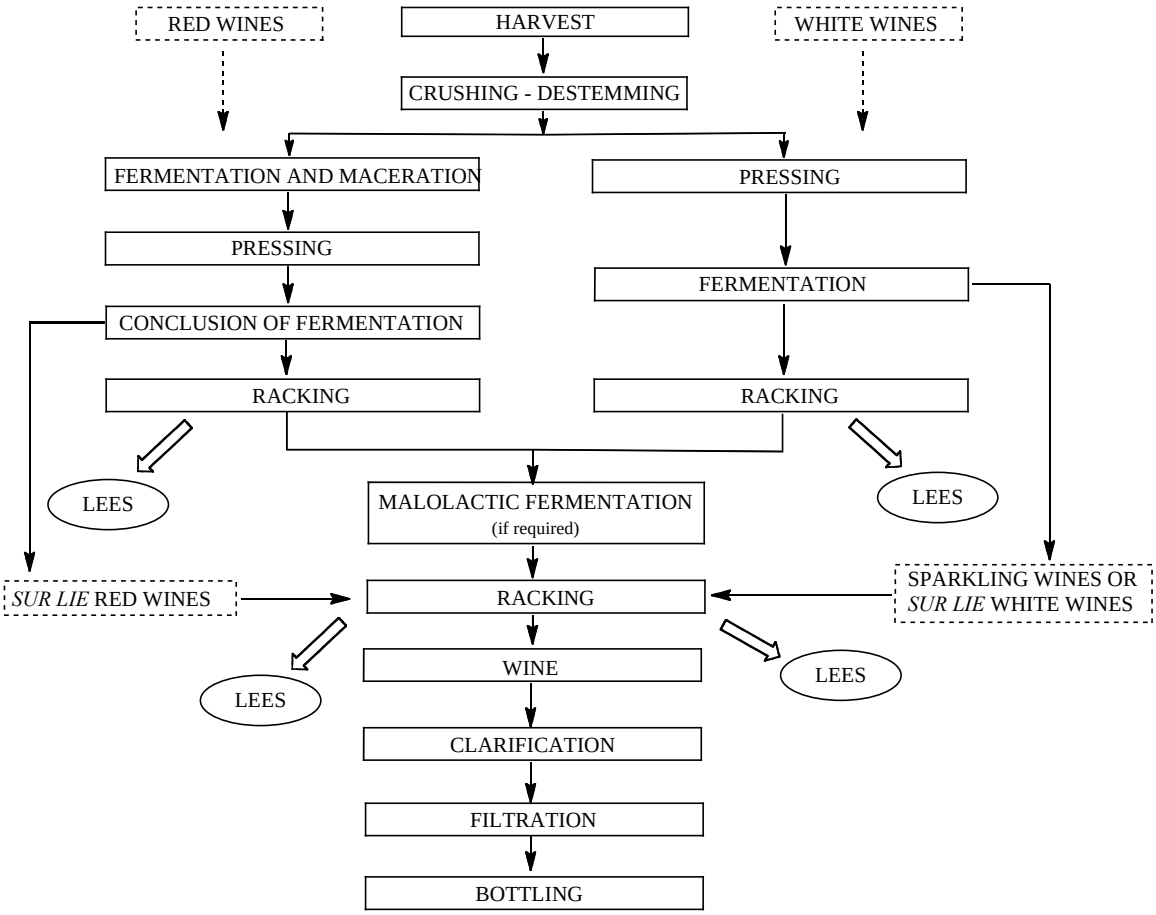
Often the lees is removed by racking the wine into another tank, however, if left in contact with the wine, enzymes begin to break down the cell walls of the dead yeast cells resulting in the release of polysaccharides, mannoproteins and other cellular components.[34,35] This breakdown of dead yeast cells over time is known as autolysis. The process of autolysis in winemaking relates to the complex chemical reactions that take place when a wine spends time in contact with the lees. While not all wines are left on lees for any length of time, many are, and the process is now traditionally known as *sur lie* ageing. It is important to discuss the right lees. Wine is always taken off the *grossier* lees which derives its meaning from the French word for heavy. It is the fine lees, which

is considered to have the best potential during ageing. The fine lees, is that which accumulates after secondary fermentation or that which is collected after the heavier gross lees has first been removed.

The type of lees and quality is considered to be extremely important. Many winemakers prefer to utilise fine lees as apposed to heavy or gross lees. Heavy lees is defined as comprising particles that are typically 100 microns to 2 mm in size and may include the yeast, bacteria, tartaric acid salts, precipitated tannins, colloids and particles formed from fining treatments and typically settle within 24 hours.[36] Light or fine lees comprises particles that are around one micron or smaller in size and typically settle days after alcoholic fermentation has been initiated and comprise yeast and lactic bacteria which is produced towards the end of the primary and secondary fermentations. Whilst there is no clear rule as to whether a winemaker should utilise heavy or fine lees for *sur lie* ageing it is often seen that lighter lees is preferred. Consequently, choosing the timing of heavy lees separation is an important aspect of lees management. Racking after alcoholic fermentation allows one the opportunity to control the amount of fine lees to be employed for *sur lie* ageing. Many winemakers will decide at this point on the amount of lees to include in the bulk wine or whether to utilise it at all based on whether it is clean or has off or dirty aromas.

As highlighted above, it is nowadays common practice to deliberately leave many white wines in contact with the lees for an extended period of time after fermentation whilst red wines are usually racked off their lees earlier although not always. For still wine production, malolactic fermentation usually takes place during ageing on yeast lees, whereas for sparkling wine production, malolactic fermentation, when desired, will be initiated before bottle ageing. In sparkling wine production, a wine is made "sparkling" or "bubbly" as many would refer to it, because the secondary fermentation is conducted when the wine is kept sealed in a bottle on the lees. The development of strong bottles and closures that can withstand the high CO_2 pressures aided in the development of sparkling wine production. Consequently, the carbon dioxide cannot escape and results in a carbonated wine beverage (a fermented sparkling wine) whilst the benefits of lees exposure are also maintained.

During the production of still red, white and sparkling wines lees is produced at several stages. *Scheme 1.1* highlights when this lees is produced in general terms and also highlights options that the winemaker has in terms of leaving a particular wine style on lees during its production. Grapes for red and white wines are harvested, crushed and destemmed. Red wines are left on skins, and primary fermentation and maceration are conducted and then the wine is pressed off skins, *Scheme 1.1*. At this stage there are several options that the winemaker may employ. The wine may be racked off the lees and the lees disposed of or the red wine may be left on the lees for *sur lies* ageing. Furthermore, MLF may or may not be conducted depending on the wine style desired. If the wines were originally racked off gross lees then the process of MLF would naturally produce further amounts of lees and sediments. Again the wine at this stage may be left on this lees for further ageing or immediately racked off and the wine clarified, filtered and bottled.



Scheme 1.1. The steps which produce lees during red, white and sparkling wine production. Adapted from reference 34.

The production of lees during still white wine making follows the same general trend as highlighted in *Scheme 1.1*. Again after primary fermentation has concluded there are several options for the winemaker to either utilise the lees for *sur lie* ageing or not. Furthermore, if a sparkling wine is to be made, then after the primary fermentation is complete, with or without MLF being conducted, the wine is usually racked, clarified and cold stabilised.[37] Because single wines seldom possess all the features that a winemaker desires, wine samples from different base wines and different vintages are blended and a formula for the *cuvée* is developed. At this point a process referred to as tirâge is employed which essentially means the addition of a sucrose solution and nutrients.[37] The *cuvée* is then inoculated and the wine bottled and allowed to undergo fermentation. Lees is thus produced and the wines are allowed to undergo lees ageing for a specified period of time. Finally, the bottles that have been inverted during the ridding process have their necks frozen and the wine is disgorged to remove the lees. The wines are then corked and sent for sale.

1.6 Overall Influences of Lees Ageing on Finished Wines.

When wine is aged on lees, the process of autolysis causes the release of a range of substrates including mannoproteins, polysaccharides, amino acids and fatty acids that interact with the fermented wine leading to changes in the final flavour and sensory aspects of the wine. Wines subjected to autolysis create complexity resulting in an enhanced creamy mouthfeel. In addition, the release of enzymes by the lees which are able to absorb oxygen, inhibit or reduce the risk of oxidation which aids in improving the ageing potential and overall stability of finished wines.[3] The release of mannoproteins have been found to improve the overall stability of the proteins in wines by reducing potential tartrate precipitation. Furthermore, the perception of bitterness or astringency is reduced as the mannoproteins released have been implemented in binding tannins. Moreover, a number of flavours such as biscuits or nuttiness are enhanced in sparkling wines by the increased production of certain amino acids released during autolysis.[38,39] The nutrients released from the dead yeast cells also assist the growth of Lactic Acid Bacteria (LAB). The level of enhanced flavour crudely depends on the length of time the wine is in contact with the lees. Thus, a Cava that has had a minimum nine months on lees will taste more like the original fruit than a vintage Champagne that has had the

compulsory three years on lees. *Sur lies* ageing is also argued to give fineness, greater integration and persistence to bubbles.

It is important to note that many factors influence the quality and quantity of yeast autolysate and include factors such as yeast strain, its conditions of growth, final population density, storage temperature, ethanol content, wine pH and naturally the time of contact employed. Given that the main thrust of this body of research was to further examine the chemical and biochemical changes that occur during *sur lie* ageing and to scope out new ways to utilise lees to develop new wine styles it was deemed appropriate that a detailed summary of all current scientific knowledge including the current biochemical and chemical understanding of how the process of lees ageing and associated autolysis is perceived to alter a wines quality be presented below.

1.7 The Mechanism and Process of Yeast Autolysis.

As highlighted above the mechanism of yeast autolysis begins with the death of the cell. *Figure 1.6* (left) depicts a low temperature SEM of healthy yeast cells 20 days after fermentation. The cells are elongated, ovoid and within the cytoplasm there is a large vacuole. *Figure 1.6* (right) depicts yeast cells after 12 months of ageing in a sparkling wine. The yeast cells are much smaller in size and contain many sperical bodies within the vacuoles which is an indication of cell breakdown.[40]

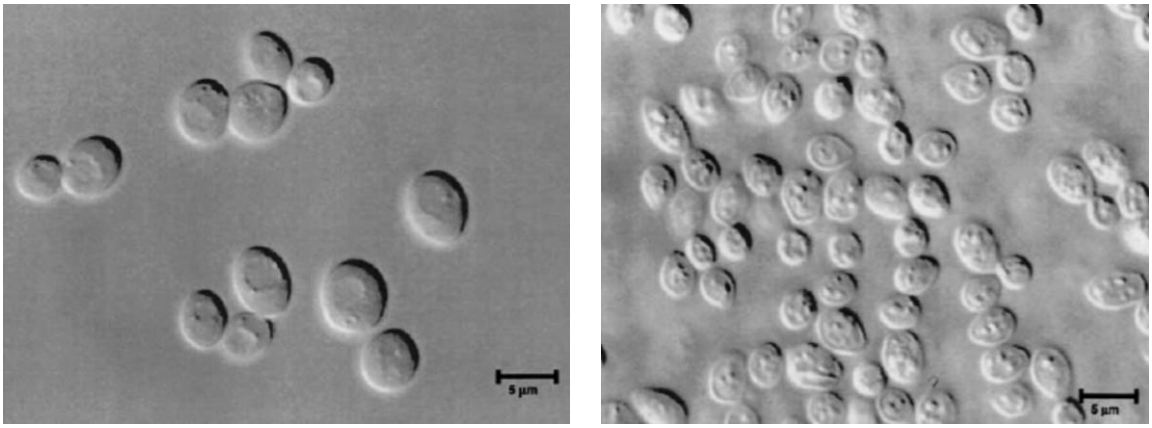


Figure 1.6. (left) Low temperature SEM of yeast cells after 20 days of fermentation (left) and yeast cells after 12 months in a sparkling wine (right). Adapted from reference 40.

In general the following five steps have been postulated to summarise the mechanism of yeast autolysis.[35,41]

- At first, the yeast cell membranous endostructures (cyctoplasmic membrane and other organelles) degrade, releasing vacuolar proteases into the cytoplasm.
- Second, the released proteases are initially inhibited by specific cytoplasmic inhibitors, however, are then activated due to degradation of these inhibitors. At this point the enzymes come in contact with cellular constituents, which are further degraded and become soluble.
- Third, intracellular polymer components (proteins) are attacked by the proteolytic enzyme, protease, which breaks them down into smaller constituent units over time such as peptides and amino acids and then the hydrolysis products initially accumulate in the space restricted by the cell wall. Likewise, nuclease enzymes break down RNA and DNA affording nucleosides, nucleotides and polynucleotides.
- Fourth, the hydrolysed products are released into the wines medium when their molecular masses are low enough to cross pores or penetrate the holes in the cell wall. Furthermore, glucanase and proteinase enzymes further degrade the cell wall by attacking the glucans and mannoproteins causing the cell wall to become extremely porous. The mixture of degraded cellular components, which is often referred to as the autolysate then quickly leaks into the surrounding wine medium.
- Finally, once the autolysate is in the wine medium further processes of degradation occur such as hydrolysis induced by the acidic nature of the wine.

Consequently, the two most important processes for successful yeast autolysis are proteolysis or the breakdown of the cellular proteinaceous substances and degradation of the cell wall.[35] Alexandre and Guilloux-Benatier have beautifully illustrated the key biochemical and morphological changes that occur during autolysis, *Figure 1.7(a-c).*[35] After alcoholic fermentation the yeast cells are smooth with think cell walls and ovoid in shape, *Figure 1.7(a).*

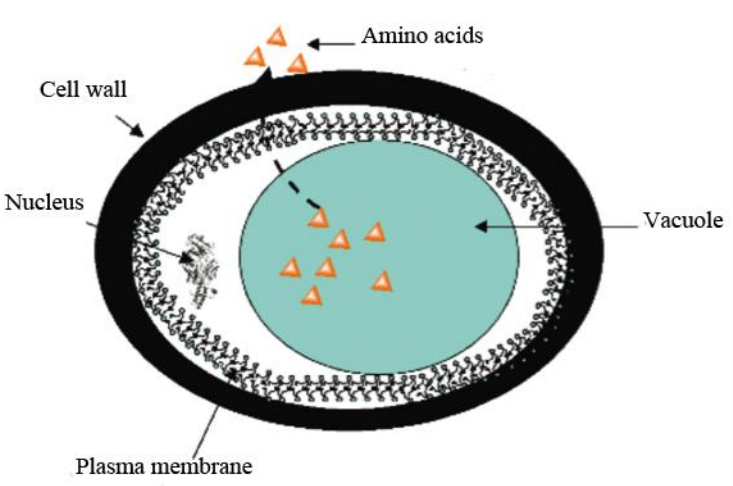


Figure 1.7(a). Schematic representation of the morphological and biochemical changes in yeast during autolysis. Adapted from reference 35.

There is a wide range of protein degrading enzymes located in the vacuoles of the yeast cells. Upon cell death, the cellular matrix becomes disorganised over the first three to six months and the enzymes come in contact with certain cellular substrates. It is at this point that the proteolytic enzymes hydrolyse peptide bonds and afford the breakdown products such as the peptides and amino acids, *Figure 1.7(b).*[35] It should be noted that at the end of alcoholic fermentation, amino acids located within the cells are passively released into the wine in a non-enzymatic process, which leads to an increase in their concentrations and as a consequence, increases in amino acid levels in a wine does not automatically indicate that the process of autolysis has begun.[35]

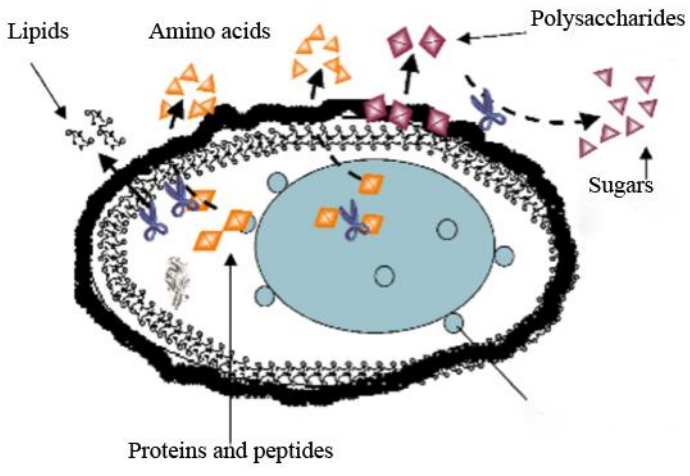


Figure 1.7(b). Schematic representation of the morphological and biochemical changes in yeast during autolysis between 3 - 6 months. Adapted from reference 35.

As depicted in *Figure 1.7(a*) the cell wall is a rigid layer of the cell that confers the overall shape of the yeast cell and envelops the protoplast. The cell wall constitutes some 20% of the cell's weight by dry weight and consists mainly of 80 - 90% polysaccharides along with a small percentage of proteins and lipids.[35] The main polysaccharides are glucans and mannans along with a small amount of chitin. The insoluble glucans aid in cell shape whilst the mannans are linked to proteins. The latter are often referred to as mannoproteins and again aid in the structural integrity of the cell wall. The glucanase and protease enzymes that are released act to degrade the cell wall making it porous and wrinkled resulting in a complex mixture of glucan, mannan, protein and *β*-(1,3)-linked oligosaccharides being released into the wine medium, *Figure 1.7(c*). It should be noted that it is not normal that the cell wall would completely break down.[35] The perceived effect on the organoleptic properties that all the released substituents have on a finished wine is further described in section 1.13 below.

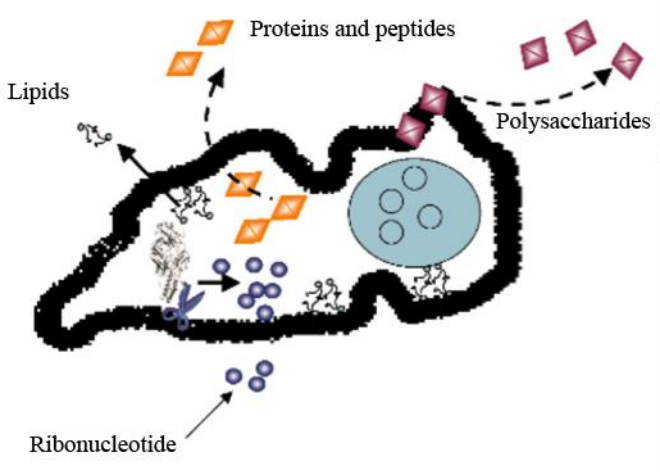


Figure 1.7(c). Schematic representation of the morphological and biochemical changes in yeast during autolysis between 9 - 12 months. Adapted from reference 35.

While the aforementioned mechanism is a summary of the main stages of autolysis it should also be pointed out that autophagy which is a degradation pathway activated by nitrogen and carbon starvation has recently been shown to play a possible role in the release of yeast constituents into a wines medium.[42] This alternative mechanism is characterised by the formation of autophagosomes which contain intracellular structures such as mitochondria which are transported to the vacuole in the yeast cell and degraded.[43]

1.8 Effects of pH and Temperature on Autolysis.

Yeast autolysis is strongly influenced by the pH of the wine medium and also the storage temperature.[35,44,45] The natural pH of wine (3 to 4) and the relatively low temperatures employed during vinification (15 - 18 ^{o}C) along with the ethanol content (12 - 15% v/v) are less than ideal for autolysis due to the slow death rate and low enzymatic reaction rates, and as such winemakers need to leave the wines on lees for a number of years if they want to gain the full effects of yeast lees autolysis. The ideal conditions for facile yeast autolysis have been estimated to be around a temperature of 45 ^{o}C and at a pH of 5.[35,44,45] Naturally, wines are not made under these conditions and as such the prolong timeframes currently needed to be employed add considerable costs to the production of a wine as they need to be constantly monitored for overall health.

1.9 Lees and Oak.

When wines are aged in oak barrels, ellagic tannis are replaced by ellagitannins. Ageing wines on lees can aid in the removal of these harsh oak phenolics and aid in integrating oak flavours as the released polysaccharides bind to the released wood phenolics thus reducing the perceived amount of tannins.[46] Furthermore, substances released by lees may also bind to wood derived chemical constituents such as furfural, vanillin, guaiacol, eugenol and the oak lactones thus modifying the perceived oak aromas present in the finished wine.[47,48] An increase in the release of mannoproteins into red wines has also been noted when the lees is stirred whilst being barrel aged.[5] Consequently, the wood tannins (polyphenols) are adsorbed by the yeast cell walls and mannoproteins released during autolysis and are later removed with the lees. In this manner, lees actually acts as a fining agent lowering the tannin content of a wine.

1.10 Lees and MLF.

Many wines are also taken through malolactic fermentation (MLF) by inoculating with LAB and results in the reduction of a wines acidity by transforming *L*-malic acid into *L*-lactic acid and carbon dioxide.[49] In addition to decreased acidity, this impacts on the organoleptic aspects of the finished wine and is believed to make a wine softer just as

exposure to lees does. However, extreme care must be taken at this point if *sur lie* ageing is also being employed, as none or very little sulphur dioxide will be present, as it will inhibit MLF. Consequently, this provides a window of opportunity for the growth of unwanted bacteria. The most secure approach appears to be that one should inoculate with a LAB culture early on in the ageing process or even inoculate during the primary alcoholic fermentation stage. Sulphur dioxide levels can then be increased to standard levels after MLF completion.

It is now known that conducting MLF in the presence of lees can lead to a lowering of diacetyl concentrations which results in less buttery notes as the bacteria metabolise the diacetyl to compounds such as acetoin and 2,3-butanediol.[49,50] Finally, there have only been a few studies on how MLF is affected by the presence of lees and it is difficult to draw comparisons.[51,52,53] Indeed, there is some debate as to whether exposure to lees results in a predictable change in MLF fermentation rate or is it more likely that increases in micronutrients such as vitamins and amino acids released during yeast autolysis, or consumed during primary fermentation, are the real reasons behind changes in observed MLF fermentation rates. [51,52,53] Lees exposure may exert stimulating, neutral or inhibitory effects on MLF and is widely believed to be associated with the fact that during yeast autolysis, various vitamins and amino acids and other substances are released into the wine.[51] In addition, it has been suggested that leaving wine on yeast lees specifically to maintain a higher level of carbon dioxide may encourage MLF.[54] It is clearly known that lactic acid bacteria (LAB) employed for MLF hydrolyse mannoproteins and given that yeast autolysis provides a source of mannoproteins over time then some observable changes in the organoleptic properties of the final wine is to be expected.[55] Furthermore, it appears that no studies on whether the addition of autolytic enzymes or inactivated dry yeast preparations (in the presence of lees) have an influence on the MLF process have been reported.

1.11 Effect of Yeast Strain, added β-Glucanases and IDY Preparations.

Yeast plays a vital role in the development of a final wines style. Even if the same grape variety is utilised along with the same vinification process, the yeast strain can have dramatic influences on the final wines texture and organoleptic properties.[56] Furthermore,

as highlighted throughout this introduction, choice of yeast strain will determine lees type and thus naturally will influence autolysis speed,[2,51] the amount and type of mannoproteins produced,[57] the type of proteases produced,[58] the amount and types of volatile aroma constituents,[34,35] levels of nitrogen compounds[35,59] and even colour.[60] Indeed selected yeast strains have been explored to speed up the autolysis process. Industrial strains with increased autolysis rates, obtained by classical genetics, have also been employed for the production of sparkling wines.[61,62]

Over the last decade the addition of commercial exogenous enzymes have been found to aid in autolysis during the production of red and white wines. A number of studies have reported on the observable differences found in the concentrations of, for example, polysaccharides, volatiles, proteins and amino acid levels upon exposure to these commercial enzymes.[63,64,65,66] Addition of exogenous enzymes are described as facilitating yeast autolysis by improving the extraction efficiency of yeast cell wall metabolites into the wine medium. The majority of these commercial autolytic enzymatic systems have significant *β*-glucanase activity and are classified as endo- or exo-glucanases and aid to degrade the yeasts cell wall structure.[35,65,67,68] *β*-Glucanases hydrolyse the *β-O*-glycosidic links of the *β*-glucan chains, releasing glucose, oligosaccharides and mannoproteins trapped in the cell wall or covalently bound to the *β*-(1-6) and *β*-(1-3) glucans.[67,68] These commercial available enzymatic products which have been authorised for use in winemaking are produced from the extracts of *Trichoderma harzianum*. Besides the main ingredient being *β*-glucanase, these preparations are often accompanied by several other enzymatic activities, e.g. polygalacturonanase, pectin-esterase, pectin-lyase cinnamoyl and esterases which have the ability to degrade grape derived or bacterial polysaccharides.[68,69] Moreover, besides the obvious acceleration of yeast autolysis, these preparations have also been shown to degrade complex polysaccharides that have a negative influence on wine filtration, to improve both colour extraction and stabilisation in red wines, and to improve certain organoleptic parameters such as roundness and complexity.[68,69]

Finally, a broad range of commercial inactivated dry yeast preparations (IDY) have come on the market and are now being used as an alternative technique to ageing wines on lees, because they permit a quicker release into the wine of yeast compounds such as

mannoproteins and glucans and as such the same perceived benefits of lees exposure are achieved in a shorter timeframe. In essence IDY preparations can be considered as a mannoprotein/glucan supplement to enrich a wines final concentration of these constituents. These products are obtained by thermal or enzymatic inactivation and are classified as inactive yeasts, yeast autolysates, yeast walls and yeast extracts.[70,71,72] These preparations can be utilised throughout the entire vinification process with most being insoluble and readily removed by racking/filtration. Given the infancy of their use, coupled with the diverse range of effects they can have on a finished wine, this is an area of current intensive research in order to try and assemble more information on how such substances may influence a wines final quality and organoleptic attributes.[63,73,74] This will be the subject of studies presented in Chapter 4 of this thesis.

1.12 Influence of Yeast Autolysis on Various Compounds Released into the Wine Medium.

The primary purpose of *sur lie* ageing is to change or enhance the organoleptic properties of a finished wine. Both the taste and smell (aroma) may be altered by prolonged yeast lees contact. The major constituents formed and released into a wine medium during autolysis include various volatile aroma compounds, fatty acids, lipids, vitamins, polysaccharides and nitrogenous compounds including amino acids and nucleic acid components. The effect that lees exposure has on the levels of each of these constituents is described below.

1.12.1 Effect of Lees on Volatiles.

The dead yeast cells liberate esters, particularly fatty acids such as ethyl hexanoate and ethyl octanoate with sweet/spicey fruity aromas. These esters (short chain C_3 - C_4 and medium chain C_6 - C_{13} acyl esters) are the major family of volatile compounds released during autolysis both qualitatively and quantitatively.[35] Interestingly this corresponds to the time when typical fermentation esters such as hexyl acetate and isoamyl acetate are undergoing acid catalysed hydrolysis resulting in a combined sweet/spicey/fruity aroma to the wine. The reason why such volatiles are released upon lees ageing is still unclear with only a few studies being reported over the last few decades.[35] The contact of wine

with lees can also decrease the content of volatile compounds and thus the fruity aroma of wines.[75] The amount of lees and contact time has also been found to be important. Large amounts of lees appear to favour the synthesis of the volatile esters,[76] however, this also leads to the build up of long chain alcohols and volatile fatty acids which are deemed detrimental to a wines overall quality.[77] More recently, Riu-Aumateli et al., studied the development of the volatile compounds in sparkling wine during extended ageing on lees.[78] They found that the concentrations of esters will decrease as the lees contact time is increased during Macabeo wine production. This fact can be explained by the action of esterases which are released by lees within a few days of alcoholic fermentation and have the ability not only to aid in ester synthesis but also the ability to hydrolyse esters back to their alcohols and carboxylic acids.[79] Whilst other studies by Bautista et al.,[80] and Bueno et al.,[81] have found similar findings in Macabeo wines, the study by Bueno et al., for the production of Airen wines showed completely opposite behavior with nearly all esters increasing in concentration as lees contact time extends. It should also be pointed out that a recent study by Chalier et al., found that aroma compounds, such as the acetate esters, hexanol, ethyl hexanoate and β-ionone are absorbed by mannoproteins extracted from *Saccharomyces cerevisiae* yeasts and as such this provides an alternative mechanism for volatile aroma reduction in competition with natural hydrolysis.[82]

Terpenic and higher alcohols such as geraniol, α-terpineol, citronellol and farnesol are also released during autolysis of yeast cells, *Figure 1.8*. These compounds have low perception levels in the range of 100 to 300 μg/L and it has been suggested by both Molnar et al., and Loyaux et al., that such compounds contribute greatly to the aroma quality of sparkling wines.[83,84] Chung also measured and identified approximately 10 aldehydes at concentrations close to or greater than their perception levels found in water alone, during lees ageing trials.[85] 3-Methylbutanal was the most abundant at approximately 40% of total aldehydes present. Such aldehydes are said to have a grassy odour that is considered a negative attribute in terms of wine odour, however, it was also noted that the concentrations of these aldehydes also diminished greatly during ageing on lees.

Several studies over the last decade have identified a range of compounds that may be good ageing markers that can be utilised to discriminate between young and aged

sparking wines.[78,86] Acetates, ethyl and isoamyl esters which are in abundance in young cavas, decrease in concentration over time whilst compounds such as vitispirane, diethyl succinate, TDN, hexenol and ethyl acetate increase over time and ageing on lees. Thus, such levels appear to correlate with lees contact time. It has been suggested that several of these compounds are released from their glycosidic bound precursors such as vitispirane from C_{13} norisoprenoids (megastigmane derivatives) by the action of enzymes released from the yeast lees over time.[78]

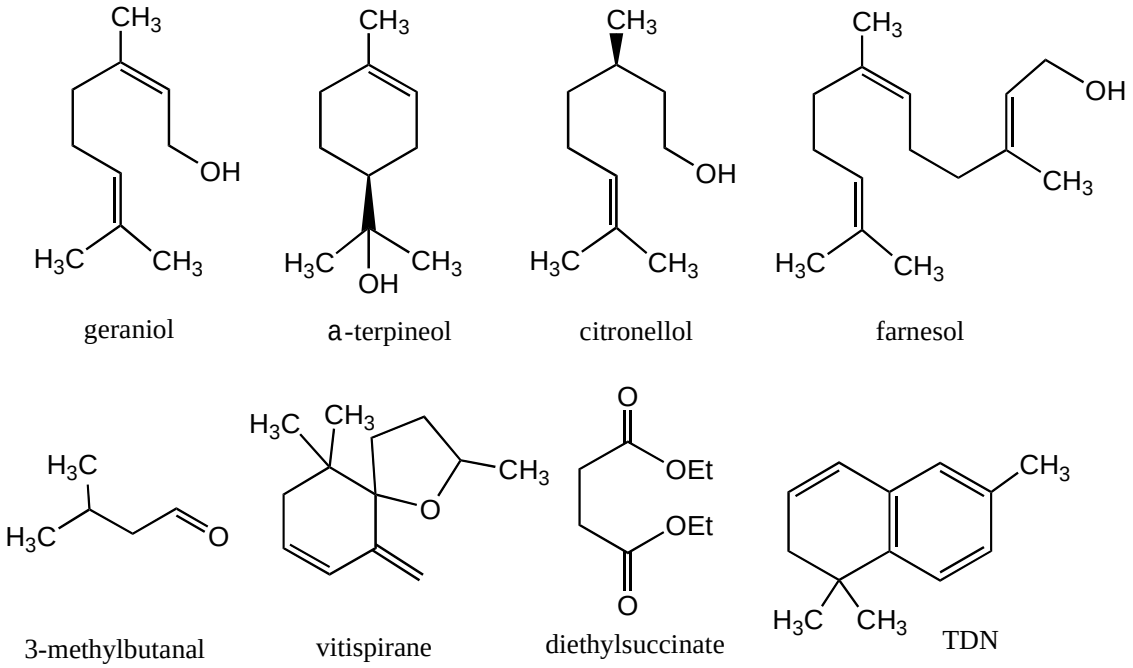


Figure 1.8. A number of key volatile compounds whose concentrations alter upon exposure to lees contact.

Whilst the aforementioned studies clearly indicate that ageing on lees will affect the final organoleptic properties of a wine, it is clear that there is still uncertainty as to which compounds are formed or released during ageing and whether these are the key odour-active compounds needed to observe the perceived aroma benefits of lees exposure. Indeed, a recent review on the effect that yeast autolysis has on sparkling wine production echoes these thoughts.[35]

1.12.2 Lees and Lipid Content.

Lipids are a group of organic compounds, which include the fats, oils, waxes, sterols and triglycerides that are insoluble in aqueous media but soluble in organic solvents and constitute valuable building blocks of living cells. During the pressing of grapes some of the lipids are passed into the must, whilst fermentation of the must also further produces and consumes lipids as yeasts grow.[87] Typical fatty acid lipids from different varieties of grape seeds and musts include palmitic acid (C16:0), steric acid (C18:0) and unstaurated long chain acids such as linoleic acid (C18:2) and linolenic acid (C18:3).[88] Thus, there is a continuous lipid exchange between wine and yeasts during fermentation. Once clarification of the wine has been conducted, there is generally only a low level of lipids in the wine, however, if the wines are kept on lees the lipid content will increase over time.[3,34,89]

It is now well established that lipids may well affect wine flavour, as the fatty acids produced may give rise to volatile breakdown components with low sensory thresholds such as esters, ketones and aldehydes.[44] Lipids have tensioactive properties and a positive relationship between the their presence and the foaming characteristics of sparkling wines has been observed.[90] Pueyo et al., has also reported that experiments in a model wine system showed that the levels of triacylglycerols, 1,3-diacylglycerols, 2-monoacylglycerols, fatty acids and sterols increase in concentration after two days of autolysis and then decrease over time and hypothesised that this may be due to the action of liberated yeast hydrolytic enzymes.[91] There is also conflicting reports in the literature as to which fatty acids contribute to foam stability and foam height. Early studies by Maujean et al.,[92] found that the addition of octanoic acid and decanoic acid reduces foam stability whereas the studies by Pueyo et al., found that linolenic and palmitoleic acids were the best indicators of foam stability.[90] Furthermore, studies by Dussod et al., reported that additional lipid content did not affect foam stability.[93] Finally, a more recent study by Gallart et al., found that C_8 - C_{12} fatty acids were negatively correlated to foam quality whist medium to long chain ethyl esters were positively related.[94] Clearly, further research into the effects of lipid release during lees ageing and its potential pros and cons is warranted.

1.12.3 Importance of Polysaccharides and Mannoproteins Released by Lees.

The main group of macromolecules present in wines are the polysaccharides and either originate from the cell walls of the grapes (arabinans) or are released by yeasts during fermentation or during lees autolysis (mannoproteins). The formation and subsequent release of the mannoproteins correlates with the yeast strain utilised.[95] In addition to the breakdown of mannoproteins into smaller peptidomannans during autolysis, glucanases and proteases result in the release of polysaccharides. These polysaccharides contain mainly glucose (74%) and mannose (26%) with the mannose/glucose ratio increasing during autolysis, which has been suggested to occur due to mannoprotein release after glucan degradation.[35]

The mannoproteins released play a key role in a wines stability and in the final observed organoleptic properties of a wine. They have been shown to reduce haze formation[96] and also aided in preventing the precipitation of tartaric acid salts.[97,98] Moreno-Arribas et al., has also shown the importance of polysaccharides on the foam quality of sparkling wines.[99] Mannoproteins also appear to decrease tannin aggregation and thus prevent precipitation[100] and have been found to interact with phenolic compounds, thus improving colour stability and reducing the astringency of a wine.[34,95,101] Finally, the mannoproteins and polysaccharides are believed to contribute in a positive way to the mouthfeel of a wine with showing that the body index of a wine is increased as the mannoprotein levels are elevated.[102] Doco et al., has also reported that the released polysaccharides can influence the organoleptic quality of a wine and that this relationship increased with increasing lees contact time.[5] Moreover, it was found that the release of mannoproteins is a progressive and linear phenomenon with batonnage significantly increasing the amount of macromolecules extracted into a wine.

1.12.4 Release of Nitrogen Compounds during Lees Exposure.

The process of autolysis leads to subsequent changes in the concentrations of nitrogen containing compounds such as peptides, amino acids, biogenic amines and nucleic acids. These constituents may be utilised by yeast as nutrients during the secondary

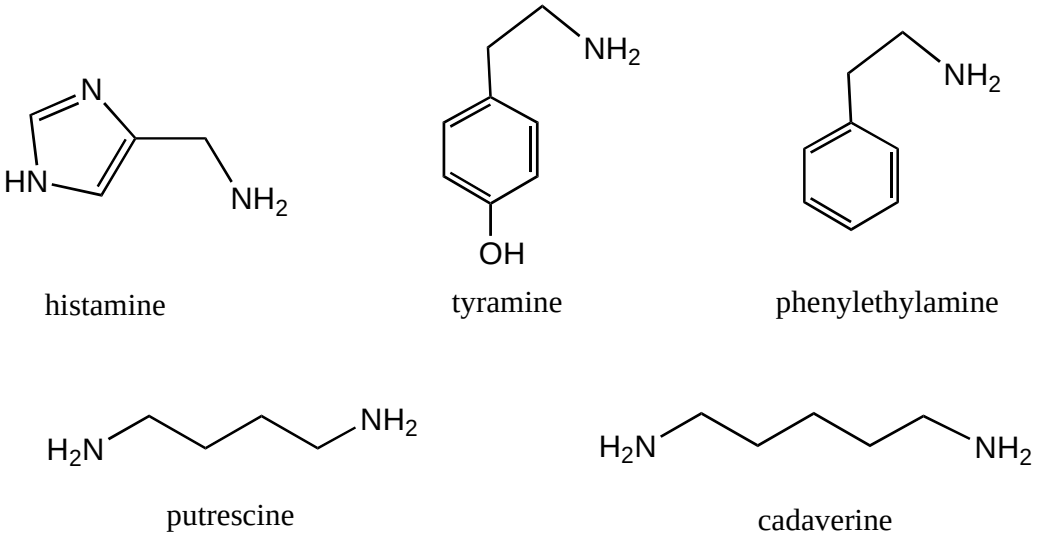
fermentation employed for sparkling wine production or may be released into the wines medium thus altering the final wines organoleptic properties.[35]

Numerous studies agree that the level of total amino acids initially increases before the level of free amino acids increases suggesting that larger peptides are first released into the wines medium during lees ageing and then they undergo further degradation into the free amino acids.[35] Indeed, Moreno-Arribas et al., found no increase in free amino acids levels for the first 9 months during a champenoise study but after this time the levels began to increase significantly indicating the beginning autolysis.[103] These findings have been confirmed in a recent study by Nunex et al.[104] The amount of peptides released is highly variable and depends on grape variety and ageing time.[105] Moreover, this latter study confirmed that the yeast was indeed the origin of the peptides released.

It is believed that amino acid enrichment of a wines medium improves the aroma potential of sparkling wines. It is known that some amino acids are the precursors to certain aroma compounds found in wine.[106] For example, 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone which is also known as sotolon slowly increases in concentration in sparkling wines during ageing on lees.[107] This compound comes from threonine, which is initially transformed into α-ketobutyric acid followed by reaction with acetaldehyde and displays a green nut or curry odour.

The presence of biogenic amines in wines is considered a health risk to some humans as their consumption can lead to undesirable physiological effects such as headache, nausea, hypotension, cardiac palpitations and anaphylactic shock.[108] These simple amines are produced by decarboxylation and deamination of amino acids present in the wine, and result in the formation of histamine, tyramine, putrescine, cadaverine and phenylethylamine, *Figure 1.9*.[109] The microorganisms responsible for such chemical transformations can be the yeast themselves or bacteria if MLF is being conducted. Two recent studies have focused on this issue and they both found that the overall concentration of biogenic amines in wines matured on lees was higher than those produced without lees contact.[110,111] The concentration of putrescine was also found to be the amine which was most affected by the presence of the lees for both studies. Consensus on how the levels of histamine and tyramine changed were contradictory from

these studies as one study found an increase whilst the other a decrease upon lees exposure. It appears that as one study focused of red wines whilst the other utilised white wine, it may well be that skin maceration has a strong influence on the final concentrations of biogenic amines along with lees exposure.



histamine tyramine phenylethylamine

putrescine cadaverine

Figure 1.9. A number of key amines whose concentration alters upon exposure to lees contact.

The degradation of RNA and DNA into nucleic acids during autolysis has only been briefly studied even though they make up 5 to 15% and 0.1 to 1.5% of cell dry weight respectively. It appears that the extent of DNA degradation depends on yeast strain.[112] Zhao and Fleet reported that up to 55% of total DNA was degraded into 3'- and 5'-deoxyribonucleotides during autolysis although complete degradation was never seen.[113] Approximately 95% of total nucleic acid content within yeast cells is tied up as RNA. Zhao and Fleet have also shown that approximately 95% of cell RNA is degraded during autolysis releasing mainly 3'-, 5'- and 2'-ribonucleotides.[114] Moreover, they showed that flavour enhancing monophosphate nucleotides such as 5'-AMP and 5'-GMP could also be produced during autolysis although the conditions were somewhat far from ideal wine making conditions. Nevertheless, these types of monophosphate nucleotides have been detected in Champagne and are believed to contribute to overall Umami taste.[115] A more recent study by Carpentier et al., has identified three flavour enhancing monophosphate mucleotides (5'-UMP, 5'-GMP and 5'-IMP) in Champagne that was aged on lees for some 8 years.[116] Such monophosphate nucleotides are well known flavour compounds in

the food industry, however, further work is needed to ascertain their full impact on wine flavour.

1.13 Overall Sensory Attributes.

Whilst the numerous research articles described within this Introduction clearly highlight that exposure of wine to lees will alter the wines final organoleptic properties mostly in a positive way, it is also apparent that it is difficult to draw precise overall conclusions as to what these benefits are and the associated mechanisms causing these fluctuations in aroma and mouthfeel qualities as the studies in most cases are vastly different. Even as this thesis was being written a report by Rodrigues et al., reported that sensory analysis of wines exposed to newly fermented yeast lees received higher ratings from tasting panels when compared to wines exposed to older fermented yeast lees which were described as having lower wine body, persistence and mouthfeel equilibrium attributes.[117] Consequently, individual unrelated sensory studies will not be described herein unless considered relevant as further research is clearly warranted.

1.14 Lees and Colour.

Phenolic compounds not only contribute to the organoleptic characteristics of a wine but they also are responsible for much of the observable colour of a wine.[118] Colour is an important parameter for critic and consumer when it comes to the evaluation of wine quality**.** It has the ability to affect the consumers perception of a wine; it can bias a consumer to taste or smell something that is, in fact, not there.[119] The overall composition of phenolics and hence colour attributes in a wine, is affected by grape variety[120] and the winemaking procedure.[121] Furthermore, it is well known that lees can both adsorb phenolic compounds and release them into a wines medium.[122] As highlighted above mannoproteins released during autolysis interact with these phenolics aiding in colour stability and diminishing the wines astringency.

Given that anthocyanins and their derivatives are the main compounds responsible for a red wines colour, coupled with the fact that they undergo conversion to complex pigments,[122] numerous studies have focused on their fate during exposure to lees.[122,123]

Indeed, most studies report a decrease in anthocyanin concentration upon lees exposure.[124,125] These latter studies provide no clear picture as to the mechanism for explaining the anthocyanin decrease, however, adsorption appears to be involved to some extent.

Rodriguez et al., conducted an elegant study on how the presence of lees during oak ageing affects colour and phenolic composition.[123] They also found that anthocyanin concentration decreased during ageing on lees and proposed that the permeability of the oak wood allows oxygen in which enhances the formation of ethyl bridged anthocyanins/proanthocyanidins and the adsorption and precipitation of flavanol polymers was behind such changes. These findings correlate well with those of Ibern-Gómez et al., who studies ageing on lees of six sparkling wines and attributed any adverse colour changes (browning) to the oxidation of phenolic compounds.[126]

1.15 Potential Faults Arising from Lees Exposure.

It is important to conduct regular sensory evaluations of wines undergoing *sur lies* ageing to monitor them for potential faults. It is now well known that if the depth of the layer of lees exceeds 10 cm in vats or barrels then there is the potential for faults to develop such as the production of reductive flavours. The use of larger tanks that may have a low oxygen content can be particularly dangerous. This situation may lead to the enzymes that are released during autolysis beginning to digest themselves and promoting the production of hydrogen sulfide (smell of rotten eggs) and mercaptan odors.[34,127]

Production of hydrogen sulfide results from chemical reduction of sulphur dioxide and can be detected at concentrations of 1 ppb. Thus, sensible levels of sulphur dioxide need to be employed and one should avoid fruit that has been exposed to elemental sulphur, which is often employed to control diseases such at botrytis. The production of mercaptans on the other hand is very serious, as the hydroxyl moiety of an alcohol has been replaced with a thiol grouping. Mercaptans such as ethyl mercaptan (burnt rubber) and methyl mercaptan (cooked cabbage) again are detectable at 1 ppb. Consequently, wines on lees are often stirred with a steel rod with a paddle at the end, a practice known as bâtonnage to bring the lees back into suspension and increase the effects of the

influence of the lees on the wine. Such disruption of the settled lees brings the wine back to into a more oxidative and healthier aromatic state. Oxidative stirring increases acetaldehyde concentrations and may increase acetic acid concentrations. The more lees there is the more frequently the stirring should be conducted. This latter technique obviously cannot be done for traditional Champagne although some winemakers practice the art where the bottles are shaken to mix the lees into the wine with examples including the Gosset Champagne and the Recaredo Cava.

1.16 Lees and Its Ability to Remove Unwanted Compounds in Wine.

Exposure of lees to a wines medium is considered to be beneficial in the production of many wines as it aids in the flavour and sensory aspects of the finished premium wines as highlighted above. Whilst this is the major benefit of lees exposure, there have been a number of other studies which show that lees aids in the natural removal of undesirable compounds such as mycotoxins, volatile phenols, pesticides and defoaming agents.[34]

Zimmerli and Dick first reported the presence of the carcinogenic mycotoxin ochratoxin (OTA) in musts and wines, which is produced by various fungal species such as *Aspergillis* and *Penicillium*.[128] Others have also confirmed this finding.[129,130] Various wine fining studies utilising bentonite[131] or activated charcoal[132] have been the most effective way of removing OTA from wines. It has been well demonstrated that yeast cells are able to absorb mycotoxins.[133] Furthermore, García-Moruno et al., added white and red lees to a red wine spiked with OTA and showed that after only 90 minutes of lees-wine contact there was a significant reduction in OTA levels utilising only a small amount of wet lees (20 g/L).[134] Moreover, after several days, OTA levels had been reduced by some 50 - 70% depending on lees type. Consequently, this and other studies have concluded that lees exposure is a natural fining agent for the removal of OTA from wines.[132]

4-Ethylphenol (4-EP) and 4-ethylguaiacol (4-EG) are volatile phenols produced by the contaminating yeast *Brettanomyces* and depart a horsy, medicinal and spicy aroma to wines.[46] Several studies (Guilloux-Benaties et al., and Chassagne et al.,) have shown that there is a decrease in the levels of (4-EP) and (4-EG) in wines exposed to lees as

compared to the same wines aged without lees.[135,136] These findings were, however, highly influenced by variables such as lees age and type, pH, temperature and alcohol content.

Harvested grapes may well contain pesticide residues especially if the agents have been applied late in the growing season due to adverse weather conditions or disease outbreak. A commercial formulation of the fungicide famoxadone was recently applied to wines and the study analysed the fate of this compound during the vinification process.[137] It was found that some of the famoxadone was removed during cake separation whilst the remaining was removed with the lees after fermentation, thus showing that lees is able to remove pesticides by adsorption. Another study has shown that the fungacide chlozolinate and various associated metabolites are also removed during lees racking.[138] Whilst such studies indicate that lees exposure may well be beneficial in aiding the removal of selected pesticides there have also been studies that have shown that not all pesticides are equally well removed upon lees exposure.[139]

Vegetable oil has often been utilised as a defoaming agent when the fermentation of must produces excessive foaming. Whilst the oil is non-toxic it can be considered as an unwanted additive and as such its removal by lees has been studied.[140] It was found that simple stirring of the lees followed by settling overnight followed by filtration resulted in no detectable amounts of the oil residue. Consequently, it was concluded that defoaming agents such as oils are removed by adsorption onto the lees.

1.17 Present and Potential Future Exploitation of Lees.

Yeast lees has an extremely poor nutritional value which makes its use as an animal nutrient unsuitable in most cases.[141] It does, however, have a high content of polyunsaturated fatty acids thus some researchers have considered it as a complement for certain food products for humans.[89] In some countries, lees recovery is performed for the isolation of tartaric acid,[142] which can be reused to correct the pH of musts and wines or for must distillation to obtain ethanol.[143] As highlighted above, lees biomass can also be used as a biosorbent for removal of unwanted compounds from wines.

The growing of earthworms in vermicomposts which contain a mixture of soil and lees has also been studied and showed that vermicomposting is a possible useful tool for the exploitation of wine lees.[144,145] Moreover, recent publications have described the use of wine lees as a unique nutrient to obtain lactic acid from various Lactobacillis strains.[141,146] Overall, it is clear that the scarce current applications of wine lees and their potential exploitation make them an undervalued by-product of the winemaking process.

1.18 Thesis Aims.

As highlighted throughout this introduction and also in an excellent recent review by Pérez-Serradilla and Castro,[34] there is no doubt that ageing wines on lees for extended periods of time, adds beneficial organoleptic qualities to a finished wine. It can be said that there is still a poor understanding of how lees impacts on wine aroma. Furthermore, whilst the interactions of wine lees on phenolics, lipids and mannoproteins released have been widely studied, exactly how all these factors and constituents influence final wine quality is not totally clear. Consequently, more research is needed in this field in order to further clarify the mechanisms and factors that lead to these perceived changes in a wine upon ageing on lees.

To this end, this PhD project was to begin with an exploratory study into the effect of lees exposure on both a Pinot Noir wine and a Chardonnay wine over a two-year period. Pinot Noir and Chardonnay are the leading grape varieties in the Bourgogne area used in making Champagne. A survey into current Australian practices showed that the ageing of Pinot Noir on lees is highly employed in Australia thus leading us to include this variety in this study. The use of lees ageing for the preparation of Chardonnay wines is typical throughout the world. Furthermore, the project was enlarged to include a number of wine and lees treatments (including the addition of commercial enzyme preparations). A range of chemical parameters were to be measured over a two-year period in order to evaluate further the importance of lees exposure on these wines. Some of the parameters to be measured such as levels of polysaccharides or changes in colour were those that have been shown to alter over time upon lees ageing, thus comparisons will be able to be drawn between the work conducted here and that already reported in the literature. Moreover, we also examined other parameters which are not normally associated with

lees autolysis such as possible changes in metal concentrations in the wines to see if further information on how lees ageing impacts on a wines final quality can be gleaned.

Based on the results of the above study we then found ourselves in a position to devise a new alternative strategy to avoid the uncertainties associated with traditional lengthy lees exposure times. Instead of simply leaving the wines on lees for extended periods of time we explored the concept that exposure of lees to microwaves for a short period of time may accelerate autolysis and when the treated lees is added back to a base wine for a short time, could the same perceived benefits of lees ageing be observed. If so, a new technological approach for the preparation of new wine styles could be developed which would be suitable for the wine industry. Both chemical evaluations and formal sensory trials were to be conducted in order to evaluate the effects of such an approach.

It is worth mentioning here that as this thesis was being compiled an excellent publication appeared which detailed attempts to improve the current technologies for wine ageing on lees without compromising the quality of the wine. Martin et al., employed ultrasound to accelerate the lysis of yeasts in lees in a model wine.[36] They found that ultrasound assisted yeast lysis increased the concentrations of proteins and polysaccharides in the model wine with high cell disruption being seen. After 20 hours of treatment virtually no viable yeast cells were found when compared to the control. There is clearly a similarity with what was achieved in this study and that which was proposed within this thesis; that being that microwave assisted lysis of yeast lees may lead to a new method for the accelerating of yeast autolysis and thus allow for a shortening of wine/lees exposure time needed to achieve the perceived organoleptic benefits of lees exposed wines.

Finally, this body of work was to conclude with a study on how a broad range of commercial inactivated dry yeast preparations (IDY) affect a wine over time. Such yeast derived preparations are now being used as an alternative technique to aging wines on lees, because they permit a quicker release into the wine of yeast compounds such as mannoproteins and glucans and as such the perceived benefits of lees exposure are achieved in a shorter timeframe. Whilst numerous studies have reported in recent years on the observed chemical constituent changes observed upon the addition of IDY preparations to a base wine, studies conducted here in a model wine medium shed new light on how some of these IDY preparations may aid in the oxidative protection of wines over time.

CHAPTER 2: TWO-YEAR EXOGENOUS ENZYME ADDITION LEES TRIAL.

As described within Chapter 1 the addition of commercial exogenous *β*-glucanase enzymes are reported to aid in yeast lees autolysis during the production of red and white wines. Whilst a number of studies have reported on the observable differences found in the concentrations of, for example, polysaccharides, volatiles, proteins and amino acid levels upon exposure to these commercial enzymes,[63,64,65,66] there is yet to be any simple diagnostic analytical methods developed to clearly indicate when have the enzymes/lees ceased providing the perceived autolytic benefits. That is to say, whilst exogenous enzymes are described as facilitating yeast autolysis by improving the extraction efficiency of yeast cell wall metabolites and internal constituents into the wines medium, what is the minimum timeframe of exposure necessary to achieve these perceived benefits to the winemaker? The majority of these commercial autolytic enzymatic systems (e.g. Lallzyme MMX, Enovin glucan, VinoTaste® Pro or Optivin® Elevage) are reported to have significant *β*-glucanase activity and are classified as endo- or exo-glucanases and aid to degrade the yeasts cell wall structure.[35,65,67,68] These *β*-glucanases hydrolyse the *β-O*-glycosidic links of the *β*-glucan chains; releases glucose, oligosaccharides and mannoproteins trapped in the cell wall or covalently bound to the *β*-(1-6) and *β*-(1-3) glucans.[67,68] Moreover, besides the obvious acceleration of yeast autolysis, these preparations have also been shown to degrade complex polysaccharides that have a negative influence on wine filtration, to improve both colour extraction and stabilisation in red wines, and to improve certain organoleptic parameters such as roundness and complexity.[68,69]

Consequently, this chapter describes the production of a number of wines and their subsequent exposure to a commercial enzyme that is reported to aid in autolysis or simply exposed to lees contact so that the various treatments can be compared. Various chemical analyses are then conducted in the belief that a simple method that would indicate when the perceived level of autolytic benefits has been reached could be established and that enzyme/lees exposure is no longer warranted.

2.1 Wine Making Protocol for Chardonnay and Pinot Noir, 2010.

Before investigations into the impact that added autolytic enzymes have at promoting yeast autolysis over time could be conducted, it was first necessary to design the appropriate wine making protocols and produce the experimental wines for analysis. As eluded to in chapter one, the use of enzymes in promoting yeast autolysis is not new and a range of scientific studies reporting the impact of such experiments have been reported. However, no studies have been reported on the observable impacts when employing an autolytic enzyme coupled with the use of a range of different fermentation yeast strains. Consequently, we decided to also incorporate the use of several different yeast strains into this study. Moreover, both a white wine (Chardonnay) and a red wine (Pinot Noir) were to be utilised and provided us with the opportunity to examine very different wine styles at the same time.

The autolytic enzyme chosen for use in these trials was commercially available and is marketed as Optivin® Elevage from Enzyme solutions™, Australia. This particular pectic enzyme system is reported to have significant β-glucanase activity and a range of additional benefits including:

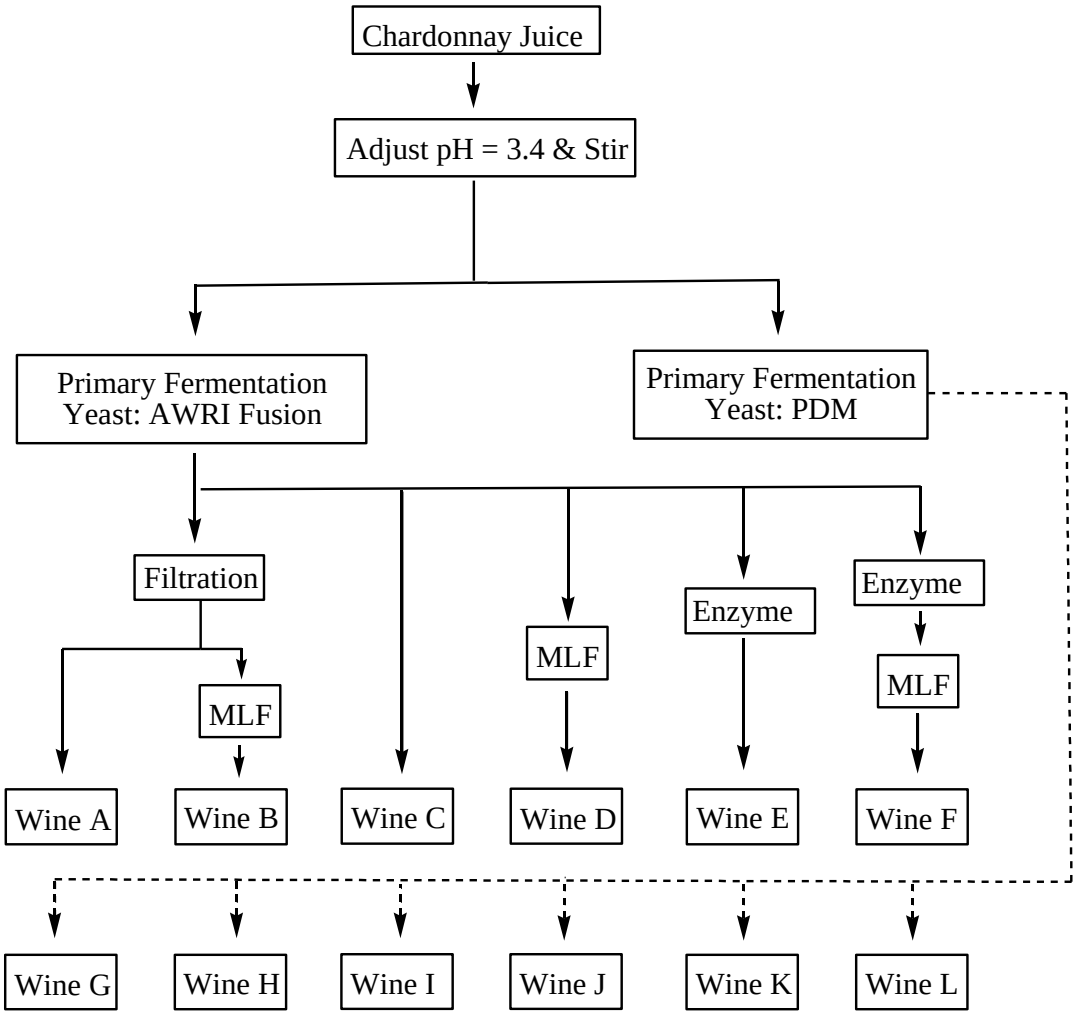
- Accelerates yeast lysis with improved mannoprotein extraction.
- Degrades complex polysaccharides that have a negative influence on wine filtration.
- Improves both colour extraction and stabilisation in red wines, and
- Improves certain organoleptic parameters such as roundness and complexity.

Whilst these reported benefits are collated in the technical information fact sheet provided with this product, there appears to be no mention of the use of this enzyme preparation in any reported scientific study.[147]

The two different yeast strains chosen for the primary fermentations were PDM *Sacchromyces cerevisea* (var. *bayanus*) (AB Maurivin™) and *Sacchromyces cerevisea* x *Sacchromyce Cariocanu* AWRI Fusion. (AB Maurivin™) for the white wine and AWRI 1503 *Saccharomyces cerevisiae* x *Saccharomyces kudriavzevii* and PDM *Sacchromyces*

cerevisea (var *bayanus*) (AB Maurivin™) for the red wine. The wines were to be made at the Hickinbotham Roseworthy Wine Science Laboratory (HRWSL) of the University of Adelaide, Waite campus utilising equipment and machinery suitable for small-scale winemaking. The following sections describe the winemaking protocols employed and the production of the experimental wines in detail.

2.1.1 Production of the Chardonnay Experimental Wines, 2010.



Scheme 2.1. Two sets of six experimental Chardonnay wines produced.

Two sets of six Chardonnay wines were to be produced with *Scheme 2.1* depicting a summary of the different experimental protocols. The wines were coded according to the following: Primary fermentation yeast employed was AWRI Fusion to produce experimental wines (A - F); Wine A: Filtered wine no lees (CHA-FU-C); Wine B: Filtered wine with MLF (CHA-FU-MLF-C); Wine C: Left on lees (CHA-FU-L); Wine D:

Left on lees with MLF (CHA-FU-MLF-L); Wine E: Left on lees, no MLF but with enzyme addition (CHA-FU-E); Wine F: Left on lees, MLF and enzyme addition (CHA-FU-MLF-E). This protocol was also repeated for wines (G - L) with the primary fermentation yeast (PDM) now employed, *Scheme 2.1*. Experimentally each MLF treatment was also split into four batches (replicated four times) and the rates followed to determine if exposure to lees affects MLF fermentation rate. As detailed within Chapter one there is some debate as to whether exposure to lees results in a change of MLF fermentation rate, thus, we had the opportunity here to examine this possibility for our experimental wines.

The Chardonnay grapes (1.5 tonnes) were sourced from the commercial vineyard Schoenth vineyard, Adelaide Hills (34.905^{o}S, 138.89^{o}E). The vines were 12 years old, had vertical shoot position (VSP) and were on their own roots and irrigated. Vine spacing was 2.2 by 2.0 metres. The fruit was machine harvested on 18th March 2010 and crushed and destemmed using a crusher/destemmer (Demoisy, Beaune, France). PMS was added so as to have free SO_2 (20 mg/L) at a concentration of 80 mg/L total (free run, 730 L). The must was then pressed (Willmes air bag press, Germany) utilising a number of different pressure combinations and additions of SO_2 were made so that the juice attained approximately 100 mg/L, *Table 2.1*. Medium and hard pressings were collected together and treated with a PVPP addition of 300 mg/L (90 g) prior to combining with the free run juice to give a final total volume of 1050 L.

Table 2.1. Pressings utilised to attain experimental Chardonnay juice.

Fraction	**Pressing pressure**	**Volume (L)**	**SO_2 added (g)**	**PMS added (g)**
Free run	Free run 0.5 bar press	730	58.4	116
Medium Pressings	0.5 bar press 2	150	15	30
	1.0 bar press	70	7	14
Hard Pressings	1.0 bar press 2	60	6	12
	1.5 bar press	20	2	4
	1.5 bar press 2	10	1	2

Pectinase was added at a rate of 4 mL/hL to the total volume of juice and allowed to cold settle for 3 days at 0 ºC. The cold settled juice was then transferred to two temperature

controlled potters, each containing approximately 500 L of juice. Prior to inoculation, 2 g/L of tartaric acid was added to the juice ensuring a pH of approximately 3.4. Inoculation was initiated on 25th March 2010 with one tank inoculated with *Saccharomyces cerevisiae* strain PDM at the rate of 25 g/hL (150 g) and the other with *Saccharomyces cerevisiae* x *Saccharomyces Cariocanus* strain AWRI Fusion at the same rate according to the manufacturer's instructions. The temperature of the fermentations was kept cool at 15 °C. Primary fermentation of the PDM wine was complete on 16th April 2010 (21 days after inoculation). The fermentation with AWRI Fusion was, however, sluggish and required a further two re-inoculations; 1st May 2010, with 125 g each of Fusion into the corresponding ferment and the second on the 14th May 2010, with UVAferm 43™ (Lallemand, Canada) at the rate of 125 g per ferment to ensure dryness. Each ferment (500 L) was then transferred to temporary holding tanks of 82 L (6 tanks per ferment).

Each holding tank was then treated differently according to the protocol depicted in *Scheme 2.1*. Wine A (no lees, CHA-FU-C) was pad filtered into an 82 L keg. The pad filtering was performed as follows; pads (Z6) were sterilised with sulfur dioxide/citric acid mixture and then washed with copious amounts of water before circulating the wine back to the holding tank prior to transferring the wine into a new sterilised 82 L keg. This procedure was utilised for all pad filtered wines. Wine B (no lees, MLF, CHA-FU-MLF-C) was pad filtered (Z6) to a 82 L holding tank, and lactic acid bacteria (Lallamand VP41, *Oenococcus oeni* strain) was added at a rate of 1 g/hL and then this was split into 4 lots of 18 L kegs. Wine C (lees only, CHA-FU-L) was kept in the 82 L keg. Wine D (Left on lees with MLF, CHA-FU-MLF-L) was transferred into an 82 L keg. MLF culture was added and the total sample then split into 4 lots of 18 L kegs. Wine E (Left on lees, no MLF but with enzyme addition, CHA-FU-MLF-E) was kept in the 82 L keg and the enzyme Optivin® Elevage was added at a concentration of 3 g/hL. Finally, wine F (Left on lees, MLF and enzyme addition, CHA-FU-MLF-E) was transferred into an 82 L keg and both MLF and enzyme additions were performed as previous before being transferred into 4 lots of 18 L kegs. Experimentally each MLF treatment was being replicated four times and the rates followed to determine if exposure to lees affects MLF fermentation rate. All wines undergoing MLF had a final pH adjustment with tartaric acid so that all wines had the same final pH. This protocol was repeated for the PDM yeast ferment and labeled wines G to L as depicted in *Scheme 2.*1.

Table 2.2. Oenological parameters of finished Chardonnay wines.

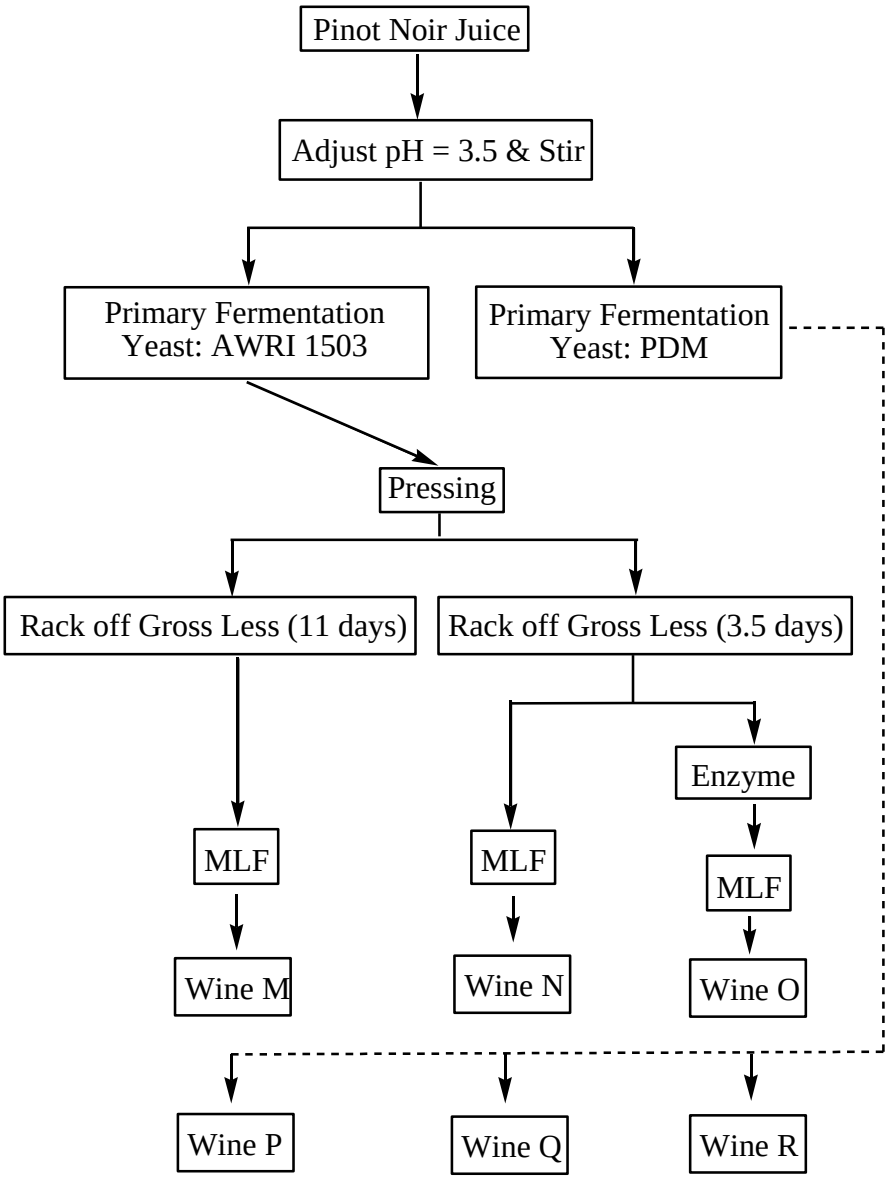
SAMPLES	FSO_2 (mg/L)	BSO_2 (mg/L)	TSO_2 (mg/L)	pH	TA (g/L)	Alc (vol%)	VA (g/L)	SO_2 added (mg/L)
Fu-C	35	75	110	3.53	5.4	13.5	0.39	35
Fu MLF C-T1	24	86	110	3.53	4.9	13.6	0.41	11
Fu MLF C-T2	26	96	122	3.51	5.5	13.4	0.41	9
Fu MLF C-T3	32	96	128	3.53	5.5	13.7	0.41	3
Fu MLF C-T4	29	104	133	3.52	5.5	13.6	0.41	6
Fu-L	35	98	133	3.53	5.4	13.2	0.42	35
Fu MLF L-T1	8	118	126	3.54	5.4	13.2	0.41	27
Fu MLF L-T2	24	96	120	3.54	5.1	13.3	0.41	11
Fu MLF L-T3	30	98	128	3.53	5.6	13.2	0.42	5
Fu MLF L-T4	27	109	136	3.56	5.5	13.3	0.42	8
Fu-E	33	82	115	3.53	5.4	13.5	0.39	33
Fu MLF E-T1	29	117	146	3.53	5.5	13.2	0.41	6
Fu MLF E-T2	35	117	152	3.53	5.4	13.2	0.4	0
Fu MLF E-T3	22	105	127	3.54	5.5	13.2	0.41	13
Fu MLF E-T4	32	93	125	3.54	5.4	13.2	0.38	3
PDM-C	33	112	145	3.51	5.5	13.6	0.37	33
PDM MLF C-T1	34	112	146	3.70	4.6	13.7	0.37	1
PDM MLF C-T2	27	115	142	3.65	4.6	13.6	0.39	8
PDM MLF C-T3	30	123	153	3.64	4.9	13.6	0.38	5
PDM MLF C-T4	34	83	117	3.69	4.8	13.7	0.38	1
PDM-L	35	98	133	3.51	5.5	13.6	0.36	35
PDM MLF L-T1	30	118	148	3.66	4.5	13.5	0.39	5
PDM MLF L-T2	0	96	96	3.67	4.8	13.7	0.37	35
PDM MLF L-T3	34	115	149	3.67	4.8	13.3	0.37	1
PDM MLF L-T4	35	109	144	3.66	4.9	13.6	0.37	0
PDM-E	37	107	144	3.51	5.5	13.7	0.36	37
PDM MLF E-T1	37	120	157	3.68	4.4	13.6	0.37	0
PDM MLF E-T2	35	96	131	3.67	4.3	13.7	0.37	0
PDM MLF E-T3	43	117	160	3.67	5.0	13.6	0.38	0[#]
PDM MLF E-T4	34	112	146	3.67	4.8	13.6	0.37	1

Note: Four replicates for all MLF wines, one treatment for remaining wines; FSO_2 (Free SO_2), BSO_2 (Bound SO_2), TSO_2 (Total SO_2), pH value, titratable acidity (TA), ethanol concentration (Alc), volatile acidity (VA), SO_2 added, [#] 0.1 mL 30% H_2O_2 added, T1 – T4 indicate paralell treatments.

The wines without MLF were bottled 3 weeks after the finish of primary fermentation, whilst the wines that were put through MLF were bottled on completion of all secondary ferments, which was 8 weeks after primary fermentation. All wines were then bottled into 375 mL (half bottles) fitted with a roll on tamper evident closure ROTE (otherwise known as a screw cap with a saranex tin wad). Wines with lees and added enzymes were added to the filler and the wine stirred with the aid of a drill equipped with a stirring bar prior to bottling. Every time the filler contained wine, the filler had solid carbon dioxide added to reduce the risk of oxidation. All experimental wines were analysed for their standard oenological parameters as summarised in *Table 2.2*. To ensure that all wines were protected by sufficient SO_2, various amounts of additional SO_2 were added to each treatment prior to bottling as tabulated. The wines were then stored in a 15 ^{o}C storage room until analysis at various time points (0, 4, 8, 12, 16, 20 and 24 months) and the boxes turned monthly to ensure lees agitation within the bottled wine. C denotes control wines with no lees, L denotes wines on lees and E denotes wines on lees and with added enzyme. All wines coded according to *Scheme 2.1*.

2.1.2 Production of the Pinot Noir Experimental Wines, 2010.

Two sets of three Pinot Noir wines were to be produced with *Scheme 2.2* depicting a summary of the different experimental protocols. The wines were coded according to the following: Primary fermentation yeast employed was AWRI Fusion 1503 to produce experimental wines (M - O); Wine M: Racked off gross lees 11 days after primary fermentation with MLF (PN-1503-11D); Wine N: Racked off gross lees 3.5 days after primary fermentation initiated with MLF (PN-1503-3D); Wine O: Racked off gross lees 3.5 days after primary fermentation initiated with MLF and enzyme addition (PN-1503-3E). This protocol was also repeated for wines (P - R) with the primary fermentation yeast (PDM) now employed, *Scheme 2.2*.



Scheme 2.2. Two sets of three experimental Pinot Noir wines produced.

The Pinot Noir grapes (1.7 tonnes) were sourced from the commercial vineyard Schoenth vineyard, Adelaide Hills (34.905^{o}S, 138.89^{o}E). The vines were 12 years old, had vertical shoot position (VSP), and were on their own roots and irrigated. Vine spacing was 2.2 by 1.5 meters. The fruit was machine harvested on the 16th of March 2010 and crushed and destermmed using a crusher/destemmer (Demoisy, Beaune, France). Addition of SO_2 at 50 mg/L as potassium metabisulphite (PMS) was made at the crusher and the must was split into two receival tanks before transferring into two 1000 L Potters (open fermenters equipped with cooling jacket). Initial parameters were pH 3.75, Be 14.2, TA 7.91 for

PDM innoculation and pH 3.75, Be 14.2, TA 7.05 for AWRI 1503. Prior to inoculation, 1.5 g/L of tartaric acid was added to adjust the pH to approximately 3.5. Inoculations were performed on 17th March 2010; one fermenter with *Saccharomyces cerevisiae* strain PDM (AB Maurivin™, Australia) at a rate of 25 g/hL (130 g) and the other with *Saccharomyces cerevisiae* strain AWRI 1503 (AB Maurivin™, Australia) at rate of 25 g/hL (130 g). During primary fermentation, temperature and Baume were measured every day to monitor the fermentation rate. Cooling jackets on the fermenters were used to keep the ferments at approximately 16 - 18 ºC until the final Baume reached 2.4 for PDM and 4.6 for AWRI 1503 (6 days later) and then discontinued to allow for the fermentations to complete. On 25th, March 2010, the PDM ferment reached 0 Be and the AWRI 1503 ferment 0.7 Be. Both ferments were then pressed (Willmes air bag press, Germany) utilising the following program; pressure press to 1 bar twice then released to zero, followed by a pressure press to 2 bar once. At each pressure release, skins were allowed to break apart by rotation of the press. Each pressed wine was then transferred to 280 L drain down tanks. Wines were racked off according to the following definition: heavy lees after 11 days (one tank) and light lees after 3.5 days (2 tanks).

On 6th April (3 weeks after harvest), the Pinot Noir wines were inoculated with Lactic Acid Bacteria **(**LAB) (Lallemand VP41, *Oenococcus oeni* strains, 1.0 g/hL). Pinot Noir wines labeled O (PN-1503-3E) and R (PN-PDM-3E) had enzyme treatment, Optivin® Elevage (Enzyme Solutions™, Australia) added at the rate of 3 g/hL. All wines finished MLF at the same time, 10th May, 2010. All experimental wines were analysed at this stage for their standard oenological parameters as summarised in *Table 2.3*. SO_2 was added such that free SO_2 was made to approximately 40 mg/L by addition of PMS and the titratable acidity (TA) was adjusted to a value of 6 to 6.5 g/L by adding tartaric acid. It was found the level of free SO_2 for the wines was slightly elevated and was adjusted prior to bottling into 375 mL bottles on the 12th May, 2010, with diluted hydrogen peroxide to a stable free SO_2 level of 40 mg/L. The wines were then stored in a 15 ^{o}C storage room until analysis at various time points (0, 4, 8, 12, 16, 20 and 24 months) and the boxes turned monthly to ensure lees agitation within the bottled wine.

Table 2.3. Final oenological parameters of the Pinot Noir wines prior to bottling.

Sample	FSO_2 (mg/L)	BSO_2 (mg/L)	TSO_2 (mg/L)	pH	TA (g/L)	Alc (vol%)	VA (g/L)
PN 1503 11D	46	66	112	3.36	6.3	14.1	0.39
PN 1503 3D	42	66	108	3.37	6.5	14.1	0.39
PN 1503 3E	48	64	112	3.37	6.2	14.3	0.37
PN PDM 11D	40	50	90	3.39	6.1	14.2	0.39
PN PDM 3D	49	60	109	3.4	6.0	14.0	0.39
PN PDM 3E	43	62	105	3.38	6.2	14.4	0.38

FSO_2 (Free SO_2), BSO_2 (Bound SO_2), TSO_2 (Total SO_2), pH value, titratable acidity (TA), ethanol concentration (Alc), volatile acidity (VA), SO_2 added.

2.2. Analyses of the Exogenous Enzyme Addition Lees Trial Wines.

The β-glucanase enzyme preparation employed for our studies was Optivin® Elevage (Enzyme Solutions™, Australia) and was added at the rate of 3 g/hL. As highlighted above it is reported to accelerate yeast lysis, degrade complex polysaccharides, improve colour extraction and stabilisation in red wines and improve organoleptic parameters such as roundness and complexity. Given these perceived benefits and given that we wished to examine what simple tests could be used to provide information on when this particular β-glucanase enzyme has provided these benefits we decided to examine a number of parameters that would not usually be associated with any reported benefit of autolysis and steer away from the usual things that are measured such as amino acid or polysaccharide levels. The following sections detail our findings.

2.2.1. Effect of Lees on MLF Rate for the Chardonnay Wines.

Malolactic fermentation is an optional fermentation conducted after primary fermentation by inoculating with lactic acid bacteria and results in the reduction of the wines acidity by transforming tart-tasting *L*-malic acid into *L*-lactic acid and carbon dioxide. MLF not only represents a deacidification process but also exerts a significant impact on the organoleptic aspects of the finished wine.[50,148] This is a very common process during red wine production and is also employed for some white wines such as Chardonnay, where it may impart a favourable buttery flavour to the finished wine.[50] As detailed within Chapter one there is some debate as to whether exposure to lees results in a predictable change in

MLF fermentation rate or is it more likely that increases in micronutrients such as vitamins and amino acids released during yeast autolysis, or consumed during primary fermentation, are the real reasons behind changes in observed MLF fermentation rates, although such studies are quite scarce. Furthermore, no studies on whether the addition of autolytic enzymes (in the presence of lees) influences MLF rates have been conducted. In any case, we had here the opportunity to further examine this possibility for our experimental Chardonnay wines. Each Chardonnay MLF treatment was replicated four times, with the rates followed, averaged and presented in *Figure 2.1*.

Malolactic fermentation of the Chardonnay wines that were prepared with the Fusion yeast for the primary fermentation all completed MLF in a much more facile manner (by several weeks) when compared to those prepared utilising PDM as highlighted in *Figure 2.1*. In terms of the Chardonnay wines prepared with the PDM yeast, it appears that exposure to lees results in an overall delay in MLF completion rate by approximately 1 week over the control, and that which had both lees and enzyme added. Consequently, it appears that lees exposure in this case may slow MLF slightly but the presence of the autolytic enzyme in the presence of lees may aid in enhancing the MLF rate. In terms of the Chardonnay wines prepared with the AWRI Fusion yeast, it appears that exposure to lees irrespective of whether enzyme was added (wine with lees hidden by wine with enzyme) results in an overall enhancement in MLF completion rate by approximately 2 weeks over the control. Consequently, lees exposure in this case may enhance MLF significantly.

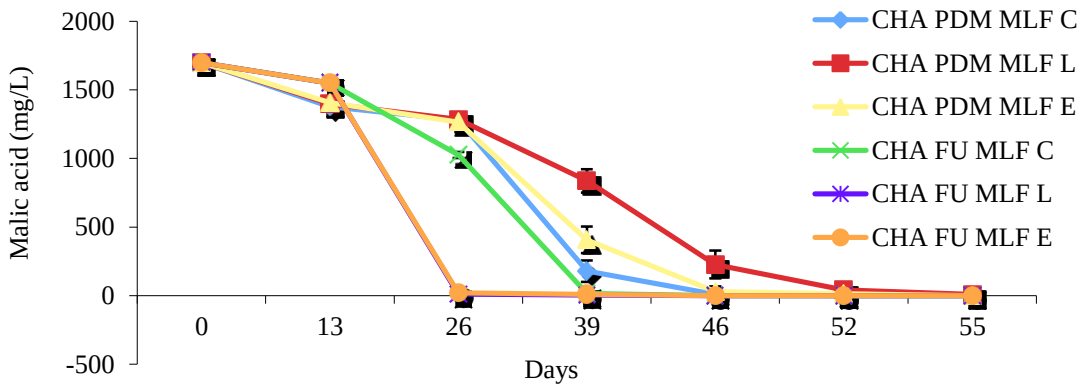


Figure 2.1. For each Chardonnay MLF treatment malic acid levels are derived from four parallel replicates. Note that the MLF rates for CHA-FU-MLF-L and CHA-FU-MLF-E were almost identical with wine with lees obscured by wine with enzyme in the plot.

Given that the wines were prepared from the same bulk juice, it is globally clear that the selection of yeast strain results in wines in which the wines medium is clearly different and as such the observed rates of MLF are also different. This could be due to the fact that the wine medium has been depleted of nutrients by one of the yeasts or due to the production of yeast metabolites that have a negative influence on LAB growth. Such effects have previously been reported.[149,150] In addition, whether lees is present or not also clearly has an effect on MLF rate. Finally, the presence of the autolytic enzymes appears to have no or only a slight effect on MLF rate. Whilst these results indicate that the winemakers choice of yeast for primary fermentation and whether there is the presence of lees (or autolytic enzymes) during subsequent MLF, will clearly result in changes in observed MLF rates and naturally final wine style. Additional studies are needed to further define the key factors involved, however, these findings are important exploratory results given the scarcity of these types of studies thus far reported.

2.2.2. Lees Concentrations and SEM Morphology of Yeast in Finished Bottled Wines.

The concentration of lees produced during standard winemaking is normally considered to be anywhere up to 2% (w/v) according to winemakers. Consequently, for our lees trials it was pertinent to also record the amount of lees that the wines were going to be exposed to over the period of experimentation before any chemical parameters were to be measured. Whilst there is no standard method on how the concentrations of lees should be determined we decided that natural sedimentation over 7 days was an appropriated method as it mimics the natural winemaking process and provided lees concentrations by wet weight that were consistently 10 - 20% in difference between samples. This variability was essentially due to the fact that it was difficult to define a clear separation line between the solid on the bottom and the wine itself. It should be noted that if mild centrifugation was employed to provide a dry cake of lees that the observed trends were also similar.

Wine lees is defined by EEC regulation n 337/79 as “the residue that forms at the bottom of recipients containing wine, after fermentation, during storage or after authorised treatments, as well as the residue obtained following the filtration or centrifugation of this product”. While in our situation, the wines did not go through treatments of bentonite

fining or cold stabilisation. Thus, the main constituents for the white wine are yeasts residues, tartrates and some bacterial residue for the wines that went through MLF. For the Pinot Noir wines, the lees consisted of yeasts residues, tartrates, bacteria residues and grape derived residues. *Table 2.4* contains the results for lees concentrations for the Chardonnay wines that went through MLF and all of the Pinot Noir wines. As highlighted the amount of lees left in the experimental wines averages somewhere between 0 - 2% as expected. In terms of the Chardonnay wines it was found that the wines left on lees only averaged around 1% in total for both yeast strains employed. The wines with lees and added autolytic enzyme furnished a lees content by sedimentation around 0.6%. The lower values returned here are most probably an under estimate as it is well known that the addition of autolytic enzymes of the glucan class as employed here prevent the natural settling out of particles in wines by aiding in there solubility.[151] The Chardonnay control wines (fermented with Fusion or PDM) returned lees concentration values around 0.2 - 0.3%. Whilst these control wines were filtered through a 0.45 μm membrane before bottling it should be noted that they were not subjected to cold stabilisation and it was noted that small amounts of tartrates precipitated out over time. Consequently, it may be concluded that up to 20% of the observed sedimentation lees concentration is actually a result of tartrates.

Table 2.4. Lees concentrations in our finished wines that went through MLF.

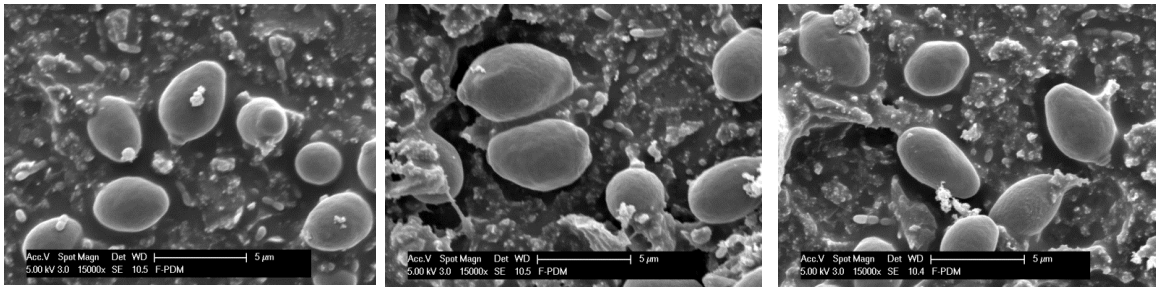
CHA	**FU MLF C**	**FU MLF L**	**FU MLF E**	**PDM MLF C**	**PDM MLF L**	**PDM MLF E**
	0.21 ± 0.01%	0.96 ± 0.03%	0.58 ± 0.18%	0.32 ± 0.12%	0.95 ± 0.26%	0.66 ± 0.16%
PN	**1503 11D**	**1503 3D**	**1503 3E**	**PDM 11D**	**PDM 3D**	**PDM 3E**
	0.89 ± 0.10%	1.62 ± 0.36%	1.75 ± 0.05%	0.67 ± 0.09%	1.85± 0.13%	1.84 ± 0.26%

Lees measured in triplicates from duplicate bottles of the same treatment.

In terms of the Pinot Noir wines it was found that the wines left on lees averaged around 1.6 - 1.8% in total for both yeast strains employed. The wines with lees and added autolytic enzyme furnished a lees content by sedimentation around the same magnitude. It

appears in this case that the addition of autolytic enzymes of the glucan class do not prevent the natural settling out of particles in the Pinot Noir wines as observed for the Chardonnay wines. The Pinot Noir control wines returned lees concentration values around 0.7 - 0.9%. Whilst these control wines were pressed and racked off lees after 11 days when it was expected that all lees would have settled out, it is clear that some fine lees remains in solution and precipitates out over time. Moreover, the wines all went through MLF and as a result some additional sedimentation is expected to occur. Furthermore, the experimental wines were again not subjected to cold stabilisation as would be the case during normal winemaking practices and as such tartaric acid salts again precipitate out over time. Consequently, it is again concluded that up to 30% of the observed sedimentation lees concentration is actually a result of tartaric acid residues, fine lees, MLF residues and small amounts of grape derived residues.

As highlighted within the introduction scanning electron microscopy (SEM) has been utilised to examine the changes in yeast lees morphology during autolysis. Thus we examined several of the Pinot Noir wines to see if the Optivin® Elevage enzyme was enducing any clear changes in morphology. In addition, we also needed to verify that freezing the wines at -20 ^{o}C for an extended period of time followed by de-thawing did not result in damage to the yeasts. *Figure 2.2* displays SEM photos of the Pinot Noir control wine, the wine with added enzyme and the wine that had been frozen at -20 ^{o}C and then de-thawed after 83 days. It can clearly be seen that for the wine with added autolytic enzyme there appears to be no major degradation of the yeast suggesting that the enzyme is not very active at promoting autolysis. Furthermore, freezing followed by de-thawing does not result in rupture of the cells thus such a process does not influence overall autolysis. Finally, the SEM photographs also show LAB bacterial and grape residues which supports the conclusions raised above.



PN-PDM PN-PDM-E PN-PDM-F

Figure 2.2. SEM photos of yeasts morphology in the Pinot Noir wine. PDM: wines fermented with yeast strain PDM; E: with Optivin® Elevage added during winemaking and stored at 15 °C for 83 days in bottle; F: frozen at -20 °C for 83 days immediately after bottling.

2.2.3. Total Glucidic Colloids and Bentonite Fining Tests.

As highlighted within the introduction the yeast cell wall is composed of glucidic colloids made up of essentially β-glucans and mannoproteins which may be release during alcoholic fermentation but which are especially released during ageing on lees.[35] The influence of various types of polysaccharides on a wine is different and therefore different oenological treatments such as enzyme addition should selectively modify their content and are of major importance. As we wished to examine how the addition of β-glucanases influences the ageing of our wines over lees we decided to first evaluate the total glucidic colloid content of our wines over the 24 months of storage to ensure that the lees treated wines (with and without enzyme) were becoming different wine styles over time when compared to the control.

The polysaccharide content of each of the wines was determined colourimetrically according to the method described by Segarra et al., with the results depicted in *Figures 2.3 (a-c).*[152] *Figure 2.3 (a)* displays the data for the Chardonnay wines that were made with either the AWRI Fusion or the PDM yeast strains and which were not taken through MLF. Whilst the total glucidic colloid content of the wines begins at a low level we found a gradual increase in glucidic colloid content with time with the wines containing lees either with or without added enzyme being higher in glucidic colloid content after 24 months over the control wines as would be expected. This was especially noticeable after the 12 month mark. There was, however, only a minor increase in glucidic colloid content

found for the wines containing added enzyme when compared to those only containing lees suggesting that the enzyme addition was making only a minor effect on the degradation of the lees over time.

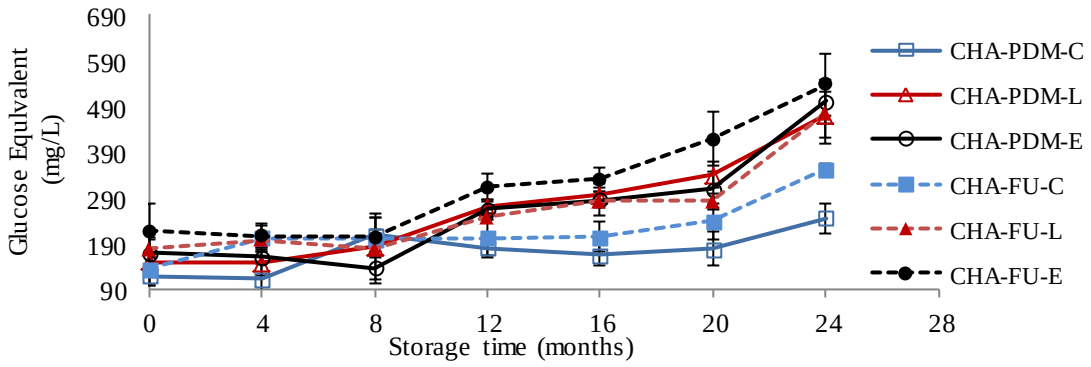


Figure 2.3 (a). Total glucidic colloid content of each Chardonnay wine without MLF. C denotes control wines with no lees, L denotes wines on lees and E denotes wines on lees and with added enzyme.

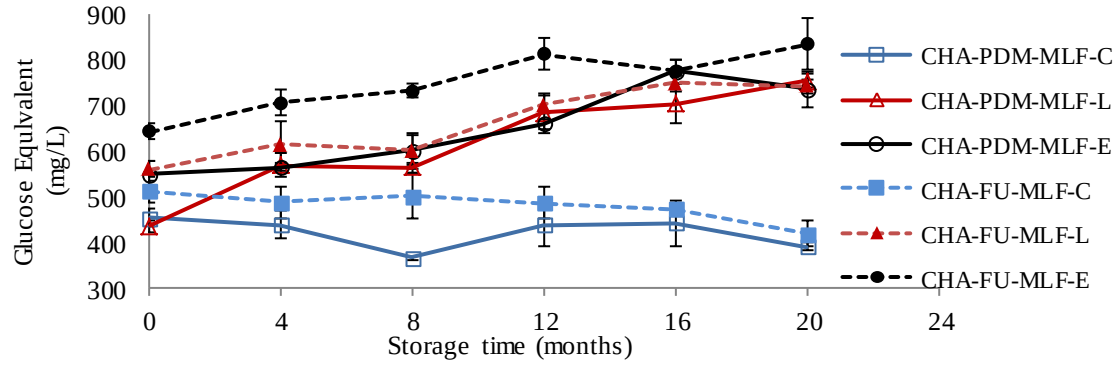


Figure 2.3 (b). Total glucidic colloid content of each Chardonnay wine conducted with MLF. C denotes control wines with no lees, L denotes wines on lees and E denotes wines on lees and with added enzyme.

Figure 2.3 (b) displays the data for the Chardonnay wines that were made with either the AWRI Fusion or the PDM yeast strains and which were also taken through MLF. Again it can be seen that the control wines have a total glucidic colloid content which remains steady over time and much lower than the wines that contain lees or added enzyme. The wines with added enzyme appear to be enhancing the release of total glucidic colloids

over the first 12 months of exposure but fade away by the time 24 months is reached again suggesting that the added enzyme is making only a minor impact to the enhancement of glucidic colloid release. It is apparent that MLF induces an overall increase in total colloid release with initial values ranging from approximately 450 - 650 mg/L when compared to those wines which were no taken through MLF (compare *Figures 2.3(a) and (b)*).

Finally, we also measured the total glucidic colloids released into the Pinot Noir wines over the 24 months, *Figure 2.3 (c).* Interestingly, it appears that the addition of autolytic enzymes to the Pinot Noir wines makes little difference in final glucidic colloid content. Furthermore, little difference was seen in whether the wines were exposed to lees or not suggesting that natural autolysis was not proceeding as expected in these wines.

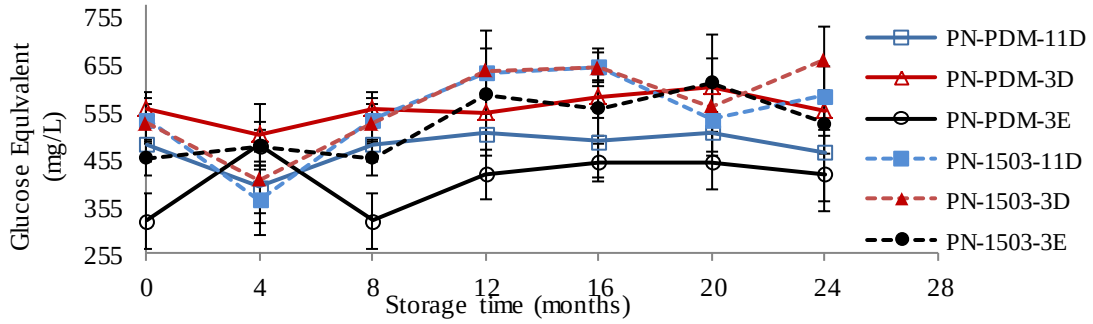


Figure 2.3 (c). Total glucidic colloid content of each Pinot Noir wine. C denotes control wines with no lees, L denotes wines on lees and E denotes wines on lees and with added enzyme.

Ageing on lees not only should result in an increase in glucidic colloids over time but as various proteins are released and degraded it should also result in wines that have lower levels of unstable proteins. Lees ageing will stabilise the wines unstable proteins by, 1) releasing periplasmic enzymes that are invertases to aid in digestion of the unstable proteins, and 2), releasing of mannoproteins.[153] The bentonite test is often employed to evaluate the bentonite required to eliminate unstable proteins before thermal treatments are conducted. Thus, we also measured the bentonite needed to produce a heat stable wine for all the Chardonnay wines over the 24 months exposure time, *Table 2.5*.

Table 2.5. Bentonite (g/L) needed to achieve a heat stable wine for all Chardonnay wines.

Month	PDM			PDM MLF			FU			FU MLF		
	C	L	E	C	L	E	C	L	E	C	L	E
0th	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
4th	0.2	0.2	0.2	0.2	0.1	0.1	0.2	0.2	0.2	0.2	0.1	0.1
8th	0.2	0.1	0.1	0.2	0.1	0.1	0.2	0.1	0.1	0.2	0.1	0.1
12th	0.2	0.1	0.1	0.2	0.1	0.1	0.2	0.1	0.1	0.2	0.1	0.1
16th	0.2	0.1	0.1	0.2	0.1	0.1	0.2	0.1	0.1	0.2	0.1	0.1
20th	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
24th	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Tests were done in triplicate on 2 randomly selected bottles of samples at each time point. PDM wines made with PDM yeast, FU wines made with Fusion yeast. C denotes control wines with no lees, L denotes wines on lees and E denotes wines on lees and with added enzyme.

It was found that only 0.2% bentonite was sufficient to eliminate any unstable proteins from all wines at the zero time point and would result in heat stable wines. This low value is indicative that the levels of unstable proteins within our wines is very low. Over time the amount of bentonite that would be needed reduced to 0.1% for all wines by 24 months indicating that there appears to be no difference between the treatments of lees ageing, enzyme addition and control. Furthermore, there was no difference found irrespective of whether the wines had been put through MLF nor the type of yeast utilised to prepare the wines. Consequently, such tests appear to shed no light on whether the addition of enzymes aid in natural lees autolysis. Whilst the above results were interesting and given that the primary purpose was to examine if we could find a simple analytical measurement which would let us know when natural ageing on lees was apparently complete we decided at this point to look at some other measurements such as total colour change, wine viscosity and the concentrations of various metal ions which have not yet been reported as important changes as an indication of lees autolysis.

2.2.4. Colour Changes of All Lees Treated Wines Over Time.

It is well known that colour stability of red wines may be favoured by the enrichment of the medium in polysaccharides.[71] The polysaccharides act as protective colloids since they interact with the tannins and anthocyanins, preventing their precipitation which leads to an increase in colour stability.[74] Consequently, our Pinot Noir wines which have been exposed to lees over 24 months were expected to be rich in polysaccharides and thus have a greater extent of red wine stability when compared to the control although our aforementioned results indicated that this may not be the case. Furthermore, whist our studies on the Chardonnay wines, which would have low levels of flavonoids and non-flavonoids and no anthocyanins, their differences in colour over time may also be a useful indication that lees is having an effect on the wines over time. Thus, we measured the CIE*lab* values of the Pinot Noir and Chardonnay wines over the two years of lees exposure with the results collated in *Figures 2.4, 2.5 and 2.6 (a-c)*. The *l** parameter affords an indication of the intensity of colour from none or light (100) to maximum or dark (0), *a** gives an indication of red (+*a**) colourisation to green (-*a**) and *b** gives an indication of yellow (+*b**) colourisation to blue (-*b**). Previous studies on utilising CIE*lab* to follow the oxidation of catechin in a model wine system have indicated that a decrease in *l**, an increase in –*a** and an increase in +*b** is to be expected as a wine ages.[154]

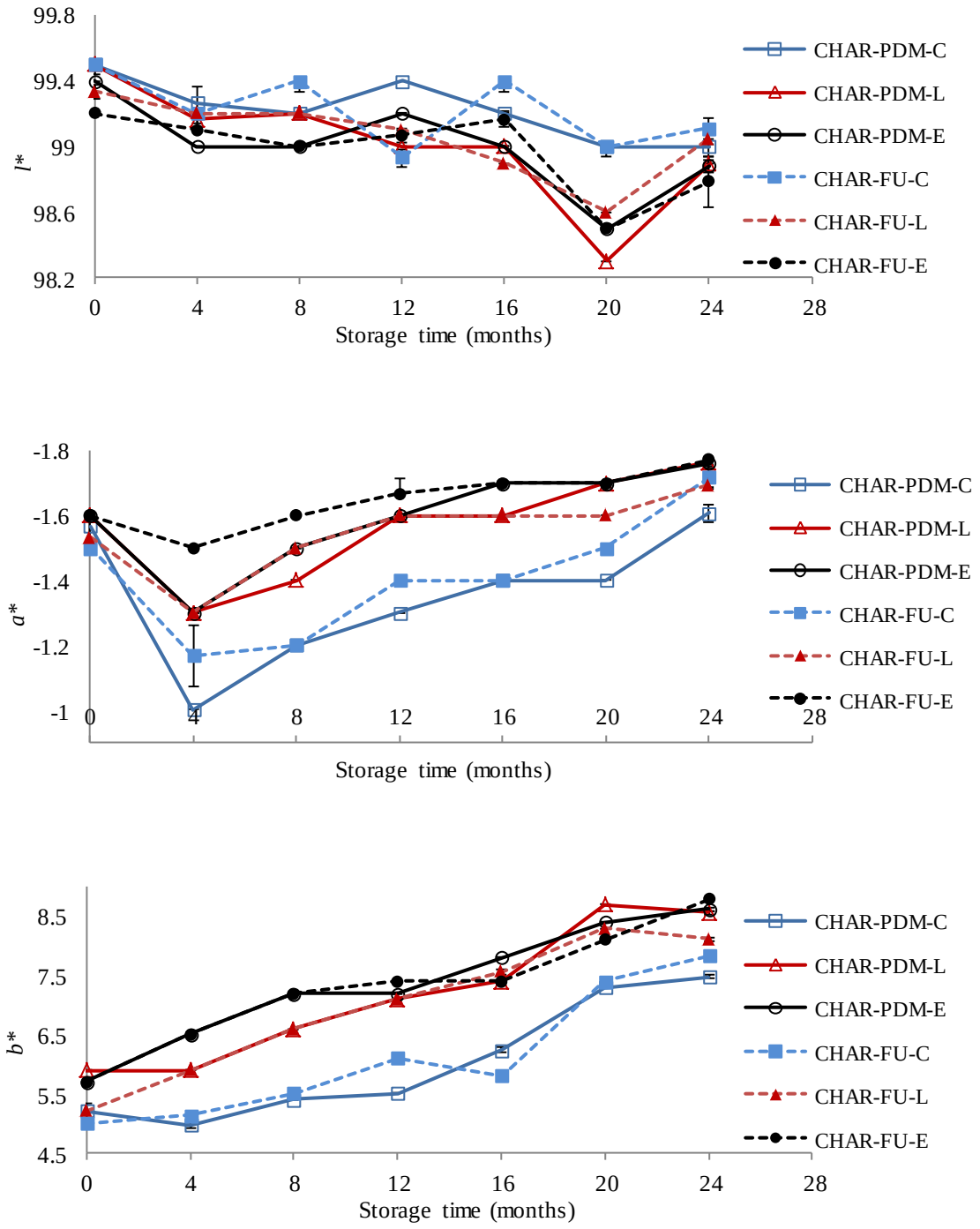


Figure 2.4 (a-c). CIE*lab* values of the Chardonnay wines without MLF over the 24 months of storage at 15 °C. Fu wines made with AWRI Fusion; PDM wines made with PDM yeast.; C: Filtered wine no lees; L: Left on lees; E: Left on lees with enzyme added.

The first wine examined was the Chardonnay wine made without MLF. Inspection of the CIE*lab* values within *Figure 2.4 (a-c)* indicates that there appears to be gradual decrease

in the *l** value indicating that the wines are slowly becoming darker over the 24 months of storage. Control experiments in which additional enzyme was added to the wines at the recommended levels revealed that this colour change was not due to adding the enzyme itself. Comparison of the *a** values indicates that the Chardonnay wines begin with a slight green hue and remain slightly green over time with little difference found after the 24 months of storage. Finally, inspection of the change in *b** values is very revealing and indicated that the wines are continuously shifting towards a yellowish hue over time. Moreover, the wines containing lees and/or added enzyme display a greater increase in *+b** when compared to the control wines for both yeast strains. Consequently, it appears that the wines containing lees are undergoing changes in their chemistries in a similar manner as to what would be found in gradual oxidation of a wine even though this would not be expected when lees is present.

The second set of wines examined were the Chardonnay wines made with MLF. Inspection of the CIE*lab* values within *Figure 2.5 (a-c)* indicates that there appears to be gradual decrease in the *l** value indicating that the wines are slowly becoming more darker over the 24 months of storage. Comparison of the *a** values indicates that the Chardonnay wines again begin with a slight green hue and remain slightly green over time with little difference found after the 24 months of storage. Finally, inspection of the change in *b** values is very revealing and indicated that the wines are again continuously shifting towards a yellowish hue over time. Moreover, the wines containing lees and/or added enzyme display a greater increase in *+b** when compared to the control wines for both yeast strains. Consequently, it appears that these wines are again undergoing changes in their chemistries in a similar manner as to what would be found in gradual oxidation of a wine.

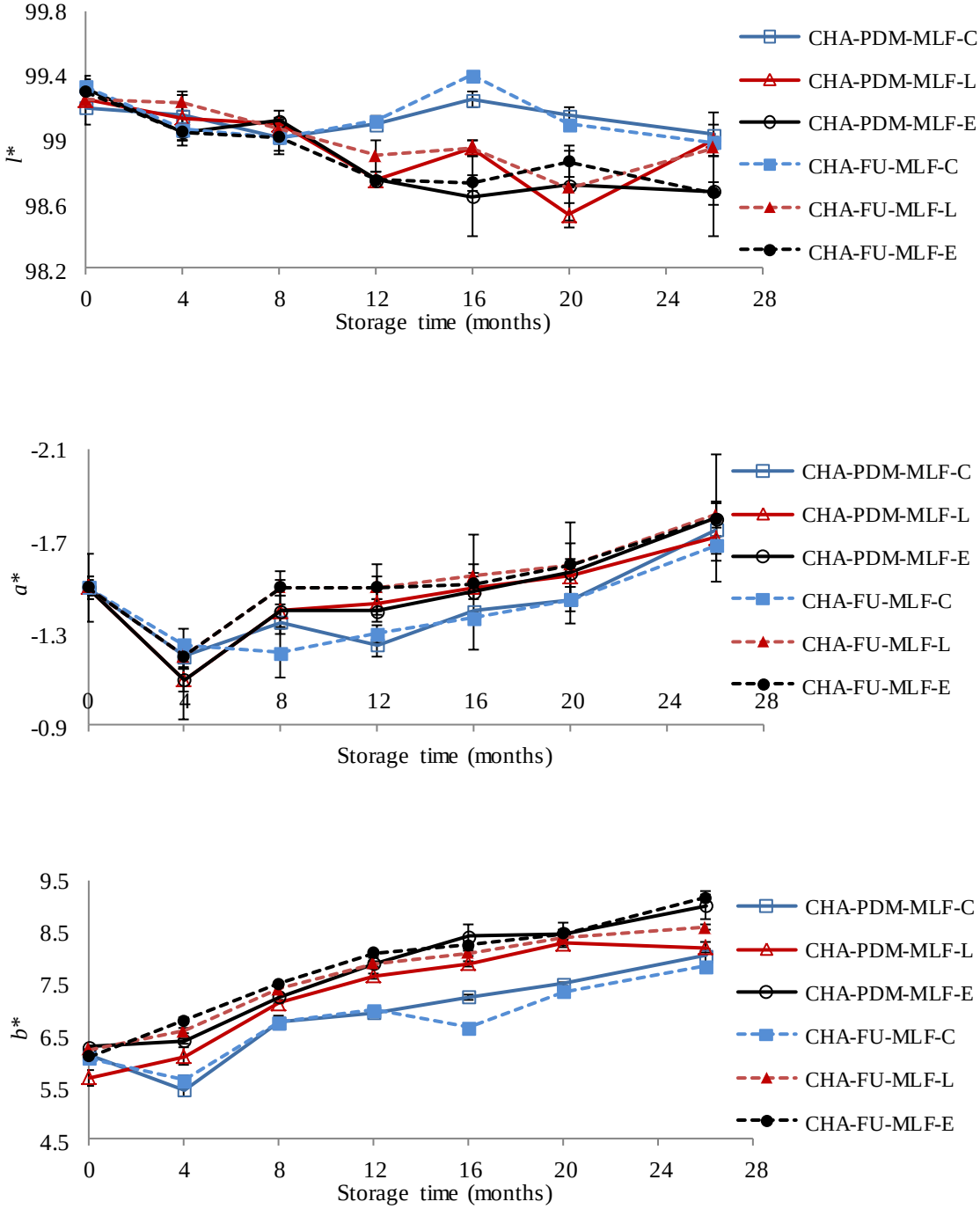


Figure 2.5 (a-c). CIE*lab* values of the Chardonnay wines with MLF over the 24 months of storage at 15 °C. PDM: Wine fermented with PDM yeast strain; FU: Wine fermented with AWRI Fusion yeasts strain; C: Filtered wine no lees; L: Left on lees; E: Left on lees with enzyme added.

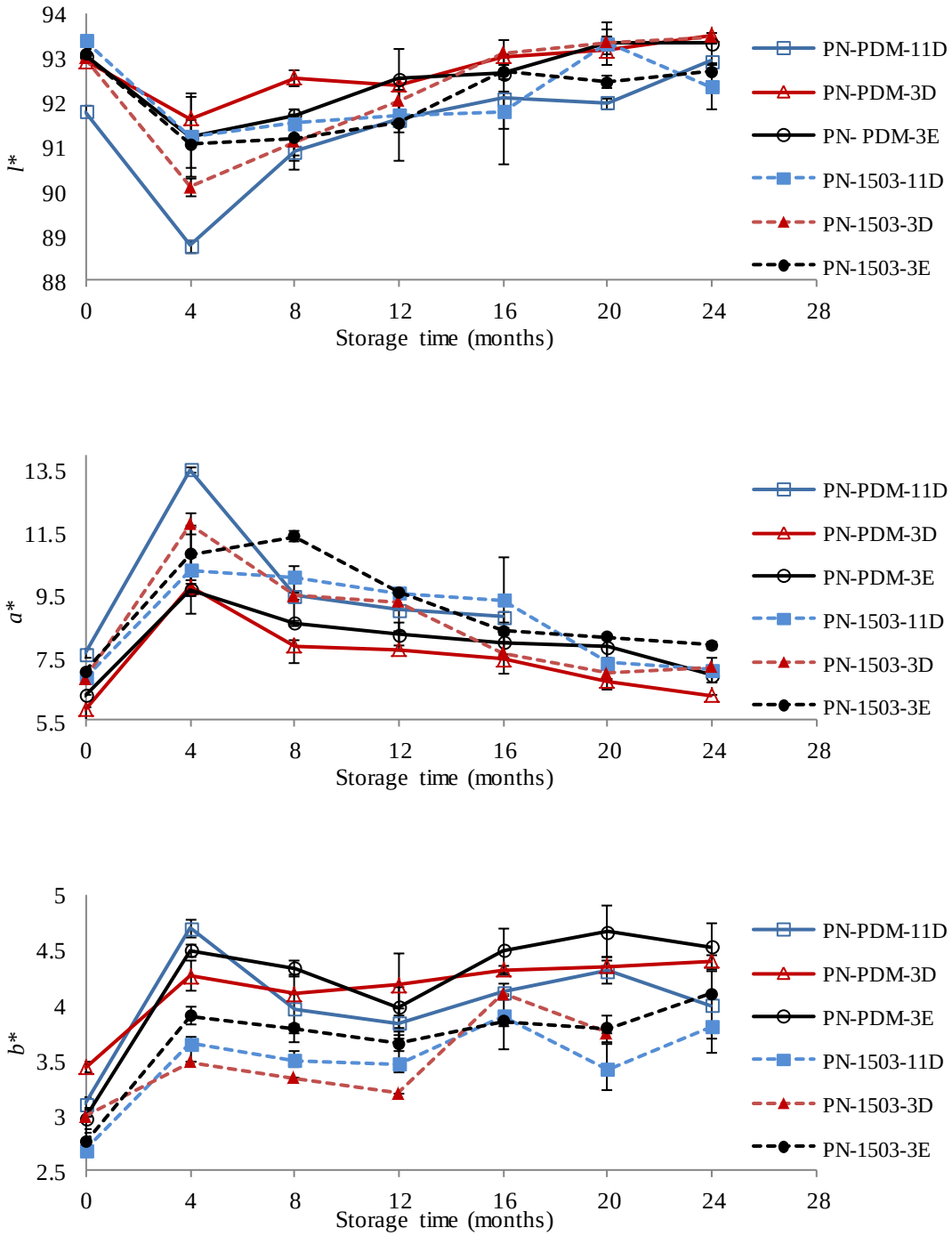


Figure 2.6 (a-c). CIE*lab* values of the Pinot Noir over the 24 months of storage at 15 °C. PDM: Wines made with PDM; 1503: Wines made with AWRI 1503; 11D: Racked off lees 11 days after primary fermentation with MLF, 3D: Racked off gross lees 3.5 days after primary fermentation with MLF, 3E: Racked off gross lees 3.5 days after primary fermentation with MLF with enzyme added.

The final set of wines examined were the Pinot Noir wines. Inspection of the CIE*lab* values within *Figure 2.6 (a-c)* indicates that there appears to be no change in the *l** value after 24 months of storage although an increase in darkness is seen at the 4 month time point and presumably indicates a change in anthocyanin concentration as pigmentation occurs early on with the wines becoming stabilised in terms of colour over time. Comparison of the *a** values also indicates that Pinot Noir wines begin with a significant red hue with little difference found after the 24 months of storage, although there was a substantial increase in redness at the 4 month time point, again most likely linked to anthocyanin polymerisation. Finally, inspection of the change in *b** values indicated that the wines are continuously slowly shifting towards a deepness in yellowish hue over time.

Besides the visual plots of the changes in CIE*lab* highlighted above, another way to analyse the data is to determine the ΔE value, which indicates the difference between two colours, and may be calculated by the formula $\Delta E = (\Delta l^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2}$. *Table 2.6* highlights the ΔE values for all the wine treatments analysed (control vs lees, control vs enzyme and lees addition vs additional enzyme addition) over the period of 24 months. By definition, and in simple terms, a ΔE of 1.0 is the smallest color difference the human eye can see. Thus, any ΔE less than 1.0 is defined as imperceptible and any ΔE greater than 1.0 would be a noticeable change to the human eye. It can be seen from the data in *Table 2.6* that for the Pinot Noir wines there is clear changes in colour that are occurring over time, irrespective of which yeast was employed for the winemaking when comparing the control to those wines which were left on lees or had added enzyme. Thus, it appears again that the autolytic enzyme treatment and the addition of lees itself are causing differences in the colour of the Pinot Noir wines over time when compared to the control.

Inspection of the ΔE values for the Chardonnay wine treatments indicates that the magnitude of any colour changes is much smaller that those seen for the Pinot Noir wines. Indeed only small changes are seen when comparing the control wines to those with added lees or additional enzyme whilst the human eye would not be able to pick up any significant difference between the wines left on lees only when compared to those with the added autolytic enzyme. Thus, it appears again that the autolytic enzyme is not making a significant change to the colour of the Chardonnay wines over time. Independently and in order to prove that the colour difference is detectable when ΔE >1, a

triangle test, concerning the colour of Chardonnay PDM wines stored for 1 year at 15 °C without lees versus with lees (ΔE: *1.17 ± 0.72)*, 11 out of 12 people could pick up the difference ($p > 0.01$).

Table 2.6. Delta E between different treatments at the same time point.

	T	**0th**	**4th**	**8th**	**12th**	**16th**	**20th**	**24th**
CHA-PDM	C&L	0.27±0.29	***1.81±0.20***	***1.47±0.09***	***1.17±0.72***	***1.69±0.73***	***1.53±0.09***	***1.09±0.01***
	C&E	0.24±0.14	***1.88±0.48***	***1.99±0.28***	***1.09±0.92***	0.90±1.00	***1.37±0.17***	***1.21±0.07***
	L&E	0.11±0.04	0.72±0.00	0.64±0.34	0.64±0.59	0.29±0.17	0.23±0.18	0.12±0.09
CHA-FU	C&L	0.48±0.22	***1.67±0.45***	***1.83±0.79***	***1.08±0.06***	***1.41±0.62***	0.86±0.19	0.44±0.22
	C&E	0.54±0.31	***2.74±1.05***	***1.79±0.95***	***1.39±0.08***	***1.55±0.13***	0.82±0.09	***1.11±0.13***
	L&E	0.21±0.05	***1.09±0.59***	0.61±0.29	0.33±0.02	0.42±0.13	0.21±0.04	0.68±0.09
CHA-MLF-PDM	C&L	0.47±0.15	0.66±0.07	0.40±0.12	0.82±0.05	0.73±0.31	***1.01±0.13***	0.18±0.08
	C&E	0.20±0.14	***0.96±0.36***	0.50±0.10	***1.02±0.23***	***1.33±0.48***	***1.09±0.23***	***1.03±0.25***
	L&E	0.60±0.04	0.32±0.31	0.12±0.02	0.27±0.19	0.66±0.18	0.41±0.04	0.88±0.37
CHA-MLF-FU	C&L	0.22±0.06	***0.97±0.06***	0.70±0.20	***0.95±0.03***	***1.52±0.29***	***1.14±0.32***	0.76±0.21
	C&E	0.09±0.02	***1.15±0.07***	***0.79±0.08***	***1.18±0.03***	***1.73±0.35***	***1.15±0.44***	***1.36±0.28***
	L&E	0.15±0.02	0.27±0.02	0.17±0.05	0.28±0.11	0.27±0.05	0.20±0.05	0.68±0.40
PN-PDM	C&L	***2.14±0.03***	***4.74±0.96***	***2.39±1.35***	***2.12±0.64***	***1.71±0.22***	***2.11±0.03***	***2.02±0.05***
	C&E	***1.84±0.20***	***4.57±0.11***	***1.96±1.06***	***1.10±0.21***	***1.24±0.54***	***0.96±0.13***	***1.26±0.32***
	L&E	0.70±0.05	***0.98±0.14***	***1.16±0.40***	***1.43±0.47***	0.70±0.55	***1.47±0.41***	0.87±0.43
PN-1503	C&L	0.51±0.09	0.71±0.04	***0.97±0.23***	0.84±0.09	***1.71±0.22***	***1.55±0.77***	***1.24±0.58***
	C&E	0.41±0.01	0.36±0.10	***1.47±0.01***	0.92±0.41	***2.55±0.80***	***1.70±0.78***	***1.02±0.47***
	L&E	0.37±0.14	0.82±0.10	***2.26±0.09***	***1.06±0.34***	***1.10±0.37***	***1.80±0.06***	***1.22±0.08***

The ΔE > 0.95 are bolded and italicised.

2.2.5. Measuring the Viscosity of Several of the Lees Exposed Wines.

As already highlighted when wine is aged on lees, the process of autolysis causes the release of a range of substrates including mannoproteins, polysaccharides, amino acids and fatty acids that interact with the fermented wine leading to changes in the final flavour and sensory aspects of the wine. Wines subjected to autolysis create complexity resulting in an enhanced creamy mouthfeel. Wine mouthfeel, which is a touch sensation, includes the sensations of astringency, thickness, oiliness and others. The attribute ‘body’ of a wine is a mouthfeel sensation and viscosity has been suggested to be probably the

best property that correlates with the body of a wine and is induced primarily by the presence of alcohol, sugars, glycerol and phenolics.[155,156] Given that our wines on lees were tasted by a expert panel (data not shown) and their findings suggested that the wines were more viscous and rounder on the palate, we considered that an easy measurement as to whether yeast lees autolysis can be considered finished in a certain timeframe may be to simply measure the change in overall dynamic viscosity of a wine.

Consequently, we measured the dynamic viscosity, density and percentage alcohol of several of the Chardonnay and Pinot Noir wines at time points 0 and 24 months that were either exposed to lees or none. The wines chosen were the Chardonnay and Pinot Noir wines made with PDM with and without MLF. *Table 2.7* depicts the viscosities of these wines. In terms of the Chardonnay wines it can be seen that there is a clear difference in the dynamic viscosities of the wines from the standpoint as to whether they contained lees or not even though the overall alcohol contents were identical. For example, whilst the viscosity dropped for the control Chardonnay wine over 24 months exposure, when lees was present the viscosity rose from 1.6448 to 1.6608 perhaps reflecting a greater release of total dry extract or polysaccharides which is to be expected as autolysis proceeds. For the Chardonnay wines that were allowed to go through MLF the viscosities actually fell after 24 months exposure and may be related to the fact that these wines have a reduction of the wines acidity due to the transforming of *L*-malic acid into *L*-lactic acid. Interestingly, the Pinot Noir wines displayed no significant difference in dynamic viscosity over the 24 months on lees although their overall viscosities and alcohol contents were higher that those of the Chardonnay wines.

Whilst we did not measure the viscosities of the wines that had been exposed to additional enzyme additions at the time, in retrospect this would have been interesting to do for our samples to see if changes in viscosity could have been seen although as was found below the choice of enzyme addition for these experiments was perhaps not the best option.

Table 2.7. Viscosities of several of the Chardonnay and Pinot Noir wines at time points 0 and 24 months storage at 15 °C.

Sample	Time point (months)	Dynamic Viscosity* (mPa.s)	Runtime ± Std. Dev.** (s)	Runtime Var. Coeff. (%)	Density (g/cm^3)	Alcohol (Vol %)
CHA PDM C	0	1.6741	17.73 ± 0.0347^{de}	0.1957	0.98745	13.6
CHA PDM L	0	1.6448	17.60 ± 0.0007^{bc}	0.0040	0.98655	13.6
CHA PDM C	24	1.6510	17.66 ± 0.0035^{cd}	0.0200	0.98184	13.6
CHA PDM L	24	1.6608	17.77 ± 0.0018^{e}	0.0099	0.98603	13.6
CHA PDM MLF C	0	1.6481	17.64 ± 0.0032^{bc}	0.0180	0.98662	13.6
CHA PDM MLF L	0	1.6380	17.53 ± 0.0018^{ab}	0.0101	0.98737	13.6
CHA PDM MLF C	24	1.6447	17.60 ± 0.0021^{c}	0.0121	0.98678	13.6
CHA PDM MLF L	24	1.6338	17.49 ± 0.0007^{a}	0.0040	0.98739	13.6
PN PDM 11D	0	1.7262	18.47 ± 0.0021^{f}	0.0115	0.98735	14.2
PN PDM 3D	0	1.7221	18.43 ± 0.0004^{f}	0.0019	0.98844	14.0
PN PDM 11D	24	1.7219	18.43 ± 0.0014^{f}	0.0077	0.98900	14.2
PN PDM 3D	24	1.7233	18.45 ± 0.0067^{f}	0.0364	0.98882	14.0

***** Dyn. Viscosity = constant KI x runtime x (density steel - density sample). The constant KI was calculated as 0.01362 after calibration with deionised H_2O at 20 °C. The density of steel falling ball was 7.85 g/cm^3. *a – f*: samples sharing the same letter are not significantly different (ANOVA by Turkey b, $P < 0.05$).

In summary, although no such studies on how dynamic viscosity changes as natural lees autolysis proceeds have thus far been reported in the literature, it appears from our initial studies here that changes in dynamic viscosities of wines over time may in fact be a possible way to follow yeast lees autolysis although further experiments are clearly needed to verify such possibilities.

2.2.6. Measuring the Concentrations of Certain Metals in the Lees Exposed Wines.

Numerous mineral elements are found in finished wines with the majority originating from the grapes, which in turn absorb these elements from the local soils where the grapes are grown. They play a critical role in wine in terms of binding to certain phenolics aiding the stability of the colour of a wine on the one hand but also causing certain oxidation processes which are considered detrimental to the long-term stability of a wine. Moreover, a recent study has highlighted that levels of certain metals in wine may lead to certain toxicological properties.[157] Some mineral elements are not metabolised or modified during the fermentation process and as such are considered good indicators of the wines geographical origin. For example, Coetzee et al., studied wines from three important wine-producing regions, Stellenbosch, Robertson and Swartland in the Western Cape Province of South Africa.[158] In a stepwise discriminant analysis procedure, functions based on linear combinations of the log-transformed element concentrations of Al, Mn, Rb, Ba, W, and Tl were generated and were able to be utilised to correctly classify wines from each region. More recently, Galgano et al. investigated wines from three important wine-producing Southern Italian regions (Basilicata, Calabria and Campania) and characterised the wines according to their content in macro-, micro-elements and lanthanides.[159] Elemental composition was utilised in multivariate statistical analysis to discriminate the wines according to geographical origin. A total of 61 elements were determined in 120 red wines produced in the years 2000–2002 with precisely 40 samples from each region. Results indicated that element analysis provides a good prospect for discriminating wines by regions, even if the element composition was not dependent on the year of production of wine.

Other studies have shown that the concentrations of some elements do change during the wine making process and is associated with the type of vessel employed for fermentation, whether the wines are stored in oak or when the wines are racked off gross lees which is known to aid in the removal of Pb from wines. Consequently, we considered that during the ageing of our wines on lees that the concentrations of certain metals may change over time due to the breakdown of the lees and associated adsorption and desorption processes and that the changes in the levels of certain metals over time may be able to be utilised as a guide to following lees autolysis. The most common analytical methods employed to accurately determine the concentrations of mineral elements in wines include AAS,[160]

ICP-OES,[161] and ICP-MS,[160,162] We chose AAS as the method of choice to determine the concentrations of certain mineral elements given that it is relatively quick and an easy method to be employed, the results of which are discussed below.

We first examined the Chardonnay wines that were produced with the AWRI Fusion yeast and which had gone through MLF at the time points 0 and 4 months. The concentrations of the metals (Cu, Ca, Na, Fe, K, Zn, Cs, Mg and Rb) were all determined with the results depicted in *Table 2.8.* Comparison of the control wine CHA-FU-C (no lees) with wine CHA-FU-L (on lees) and wine CHA-FU-E (on lees with enzyme) shows some interesting differences in metal concentrations when comparing the 0 and 4 month time points. The wines with added autolytic enzyme appear to have a higher concentration of Cu, Ca, Fe and K at the zero time point which decreases greatly after 4 months when compared to the control wine which was not exposed to lees. On the other hand the concentrations of Zn and Cs appear to increase over time while the remaining metals (Na, Mg and Rb) appear to remain essentially steady when compared to the control wine.

Table 2.8. Metal concentrations of several of the Chardonnay wines which had gone through MLF at time points 0 and after 4 months lees exposure at 15 °C.

Sample	**Time (mths)**	**Cu (μg/L)**	**Ca (mg/L)**	**Na (mg/L)**	**Fe (μg/L)**	**K (mg/L)**	**Zn (mg/L)**	**Cs (μg/L)**	**Mg (mg/L)**	**Rb (mg/L)**
CHA FU MLF C	0	43.5 ± 7.8	2.63 ± 0.27	31.33 ± 0.39	462 ± 24	370.8 ± 55.8	1.22 ± 0.01	16.0 ± 2.0	54.93 ± 1.06	3.58 ± 0.26
CHA FU MLF L	0	51.7 ± 1.2	2.33 ± 0.11	31.97 ± 0.72	391 ± 6	282.2 ± 5.1	1.35 ± 0.01	21.0 ± 4.0	50.46 ± 0.64	3.06 ± 0.00
CHA FU MLF E	0	97.1 ± 3.0	3.29 ± 1.13	27.73 ± 3.49	517 ± 19	446.4 ± 163	1.52 ± 0.03	15.0 ± 1.0	49.61 ± 1.32	3.06 ± 0.04
CHA FU MLF C	4	53.9 ± 0.5	1.57 ± 0.19	28.08 ± 0.05	388 ± 1	506.6 ± 67.3	1.48 ± 0.05	29.0 ± .01	54.75 ± 0.31	4.11 ± 0.04
CHA FU MLF L	4	26.3 ±13.5	1.61 ± 0.01	29.79 ± 0.46	337 ± 6	389.2 ± 56.6	1.46 ± 0.01	30.0 ± 1.0	51.46 ± 1.62	3.65 ± 0.25
CHA FU MLF E	4	18.1 ± 0.5	1.51 ± 0.02	29.37 ± 0.49	325 ± 16	315.1 ± 16.7	1.91 ± 0.07	25.0 ± 2.0	51.06 ± 0.31	3.44 ± 0.03

Primary fermentation yeast employed was AWRI Fusion.

We next examined the Pinot Noir wines that were produced with the AWRI Fusion yeast at the time points 0 and 4 months. The concentrations of the metals (Cu, Ca, Na, Fe, K, Zn, Cs, Mg and Rb) were all determined with the results depicted in *Table 2.9.*

Comparison of the control wine PN-1503-11D (no lees) with wine PN-1503-3D (on lees) and wine PN-1503-3E (on lees with enzyme) again shows some interesting differences in metal concentrations when comparing the 0 and 4 month time points. The concentrations of the metals Ca, Na, K, Cs, Mg appeared to change little between treatments. The changes in the concentrations of the remaining metals (Cu, Fe, Zn and Rb) are difficult to explain as some increased over time while others decrease. Furthermore, whether the wines were on lees or had added autolytic enzymes provided no clear trends in terms of changes in metal concentrations. The Pinot Noir wines do, however, contain a much higher concentration of Fe, Cs and Rb when compared to the Chardonnay wines analysed above and presumably this simply reflects the variability in the origins of where the grapes were grown.

Table 2.9. Metal concentrations of several of the Pinot Noir wines at time points 0 and after 4 months lees exposure at 15 °C.

ID	Time (mths)	Cu (μg/L)	Ca (mg/L)	Na (mg/L)	Fe (μg/L)	K (mg/L)	Zn (mg/L)	Cs (μg/L)	Mg (mg/L)	Rb (mg/L)
PN 1503 11D	0	62.5 ± 9.5	3.61 ± 0.38	25.35 ± 1.12	833 ± 17	478.9 ± 30.7	0.71 ± 0.01	88 ± 5.0	52.38 ± 0.67	11.42 ± 0.13
PN 1503 3D	0	196.0 ± 32.8	3.37 ± 0.30	24.58 ± 1.41	798 ± 89	510.4 ± 19.5	1.93 ± 0.01	83 ± 0.0	49.32 ± 1.91	11.81 ± 0.11
PN 1503 3E	0	57.0 ± 3.0	3.46 ± 0.31	25.41 ± 0.13	519 ± 223	441.7 ± 8.0	0.65 ± 0.02	83 ± 1.0	50.64 ± 1.35	12.91 ± 1.43
PN 1503 11D	4	23.8 ± 0.4	3.18 ± 0.09	23.45 ± 0.43	1021 ± 25	465.2 ± 11.8	0.30 ± 0.02	87 ± 0.0	61.52 ± 10.8	14.06 ± 0.45
PN 1503 3D	4	122.8 ± 26.8	2.79 ± 0.34	23.28 ± 0.01	795 ± 8	474.6 ± 19.7	1.11 ± 0.04	93 ± 0.0	46.98 ± 2.05	14.38 ± 0.19
PN 1503 3E	4	139.6 ± 0.1	3.09 ± 0.27	23.69 ± 0.02	662 ± 15	442.3 ± 7.2	0.28 ± 0.03	95 ± 1.0	49.28 ± 2.05	13.86 ± 0.08

Primary fermentation yeast employed was AWRI 1503.

We also examined the concentrations of the same metals in the other wines produced (data not shown) and found that the concentrations also were highly variable with no clear trend seen between treatments. Given that the results described within this chapter thus far appeared to indicate in many instances that we were having difficulties clearly identifying that the wines made with added autolytic enzymes on lees were clearly undergoing enhanced autolysis as was expected we decided to cease measuring the concentration of the metals over time and to examine the activity of the autolytic enzyme itself to check if it was indeed active at enhancing autolysis in our wine treatments.

2.2.7. Checking Enzyme *β*-Glucanase Activity and Stability.

Given that the wines exposed to the commercial autolytic enzyme Optivin® Elevage over the two year period appeared not to show a dramatic change in the levels of various substrates that were expected to increase or decrease over time we considered the possibility that it was possible that this particular enzyme was slow at enhancing autolysis for our wine systems and that the expected benefits or chemical changes were not being seen in this timeframe. Consequently, it was pertinent that we also assay for the activity of this enzyme employed in both model and real wine systems. Furthermore, for comparison we also included two other commercial enzymes to gauge the potential effect of the different enzymes. These additional enzymes were lallzyme MMX® (lallemand) which is a *β*-glucanase blend which is reported to aid in the release of polysaccharides from yeasts and improves the short maturing of wines on lees[163] and VinoTaste® Pro (Novozymes) which is reported to decrease maturation time by nearly 20% and increases the roundness of the finished wines.[164]

Two different methods were utilised to assay the *β*-glucanase activity of these three enzymes. The first was the *β*-glucanase assay or BGU assay as it is better known as. Model wines of all three enzyme solutions were prepared and analysed for *β*-glucanase activity following the standard procedure (Procedure No. VE 400.42, 1996) as provided by Valley Research, Inc., with little modification.[165] Immediately it was clear that the Optivin® Elevage displayed a much lower level of *β*-glucanase activity when compared to the other two commercial enzymes lallzyme MMX® and VinoTaste® Pro, *Figure 2.7*. Consequently, it appeared that the use of the Optivin® Elevage in our real wine systems may in fact have been a poor choice as it appeared to have a much lower level of *β*-glucanase activity than the remaining two enzymes. Moreover, this may explain further why we often saw only minor changes or differences in the wines that were exposed to lees versus those that were exposed to lees and added enzyme.

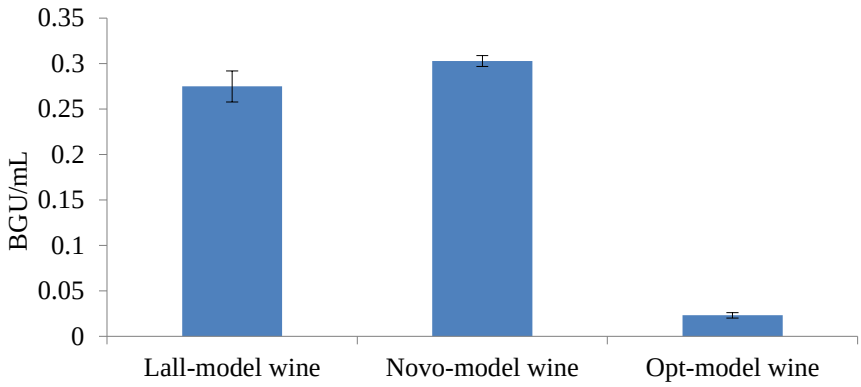


Figure 2.7. β-Glucanase activity of the three commercial autolytic enzymes in model wine as determined by the BGU assay. Lall (lallzyme MMX®), Novo (VinoTaste® Pro) and Opt (Optivin® Elevage).

To further examine if the activity of the Optivin® Elevage was lower that the other two commercial enzymes we also examined the β(1,3)-glucanase activity utilising the method reported by Humbert-Goffard et al., which is a method based on the measurement of the kinetics of glucose release in the presence of laminarin, a β(1,3)-glucan from *Laminarin digitata*.[151] All three enzyme were analysed for β-glucanase activity in both a model wine and in a real wine (Chardonnay) system, *Figure 2.8*. Again it is apparent that when measuring the β-glucanase activity of the three commercial autolytic enzymes in model wine, the Optivin® Elevage displayed lower activity when compared to the other two commercial enzymes. In addition, the β-glucanase activity appeared to decrease by some 30% when measured in the real wine medium for the two commercial enzymes lallzyme MMX® and VinoTaste® Pro and appeared to have neglectable activity for the Optivin® Elevage. This latter finding is consistent with that found by Humbert-Goffard et al.[151] who found significant enzymatic inhibition by wine constituents. Consequently, it appeared again that the use of the Optivin® Elevage in our real wine systems for our two year study may in fact have been a poor choice as it appears to have a much lower level of β-glucanase activity than the other two enzymes. Having said this, it is clear that wine constituents interfere with such assays and it may well be the case that these enzymes do indeed infact perform well as autolytic enzymes as many studies suggest.

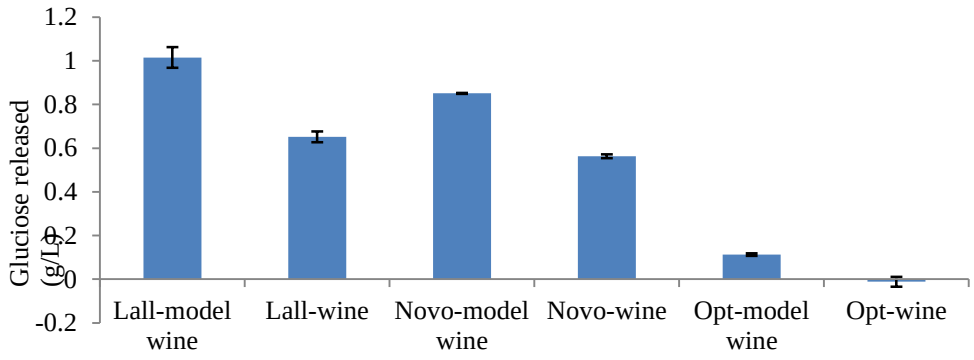


Figure 2.8. β-Glucanase activity of the three commercial autolytic enzymes in model wine and real wine (Chardonnay) systems as determined by the enzymatic assay reported by Humbert-Goffard et al.[131] Lall (lallzyme MMX®), Novo (VinoTaste® Pro) and Opt (Optivin® Elevage).

2.3. Chapter Two Summary.

This Chapter describes the preparation of a range of experimental Chardonnay and Pinot Noir wines that were allowed to be exposed to lees over a two year period and some of which were also exposed to a commercial β-glucanase enzyme preparation reported to enhance lees autolysis. Whilst it is clear that the trials and analyses were not as significant as we first hoped there were some interesting preliminary findings that would be worth pursuing in future research. For example, we found that given that the Chardonnay wines were prepared from the same bulk juice, it was globally clear that the selection of yeast strain results in wines in which the wines medium is clearly different and as such the observed rates of MLF are also different, section 2.2.1. This could have be due to the fact that the wines medium has been depleted of nutrients by one of the yeasts or due to the production of yeast metabolites that have a negative influence on malolactic bacterial growth. In addition, whether lees was present or not also clearly had an effect on MLF rate. Finally, the presence of the autolytic enzymes appears to have no or only a slight effect on MLF rate. Whilst these results indicated that the winemakers choice of yeast for primary fermentation and whether there is the presence of lees (or autolytic enzymes) during subsequent MLF, will clearly result in changes in observed MLF rates and naturally final wine style, although the results found here need to be carefully considered

given that we found that the autolytic enzyme utilised for our trials appeared to be not particularly active. Additional studies are needed to further define the key factors involved here, however, these findings are important exploratory results given the scarcity of these types of studies thus far reported.

Next we examined the amount of lees in our trial wines to ensure that the wines on lees had a significantly greater amount of lees when compared to the control wines, section 2.2.2. Indeed we determined that the trial wines were being exposed to up to 2% lees concentrations by wet weight. Interestingly we also found for the Chardonnay wines that the presence of the autolytic enzyme appeared to prevent the natural settling out of lees particles in the wines by aiding in there solubility, whilst this effect was not see for the Pinot Noir wines and would be worthy of further examination. In conclusion, our trial wines that were being exposed to lees had a significantly greater amount of lees content when compared to the control wines which only contained small amounts of sedimentation which is actually a result of tartaric acid and MLF residues.

As highlighted within the introduction the yeast cell wall is composed of glucidic colloids made up of essentially β-glucans and mannoproteins which may be release during alcoholic fermentation but which are especially released during ageing on lees.[35] As we wished to examine how the addition of β-glucanases influences the ageing of our wines over lees we also evaluated the total glucidic colloid content of our wines over the 24 months of storage to ensure that the lees treated wines (with and without enzyme) were becoming different wine styles over time when compared to the control, section 2.2.3.

Whilst the total glucidic colloid content of the Chardonnay wines which were not taken through MLF begins at a low level, we found a gradual increase in glucidic colloid content with time with the wines containing lees either with or without added enzyme being higher in glucidic colloid content after 24 months over the control wines as would be expected. This was especially noticeable after the 12 month mark. There was, however, only a minor increase in glucidic colloid content found for the wines containing added enzyme when compared to those only containing lees suggesting that the enzyme addition was making only a minor effect on the degradation of the lees over time. Data for the Chardonnay wines that were made with either the AWRI Fusion or the PDM yeast strains

and which were also taken through MLF showed that the control wines have a total glucidic colloid content which remains steady over time and much lower than the wines that contain lees or added enzyme. The wines with added enzyme appear to be enhancing the release of total glucidic colloids over the first 12 months of exposure but fade away by the time 24 months is reached again suggesting that the added enzyme is making only a minor impact to the enhancement of glucidic colloid release. It is apparent that MLF induces an overall increase in total colloid release with initial values ranging from approximately 450 - 650 mg/L when compared to those wines which were no taken through MLF. Finally, we also measured the total glucidic colloids released into the Pinot Noir wines over the 24 months. Interestingly, it appeared that the addition of autolytic enzymes to the Pinot Noir wines makes little difference in final glucidic colloid content. Furthermore, little difference was seen in whether the wines were exposed to lees or not suggesting that natural autolysis was not proceeding as expected in these wines.

Ageing on lees not only should result in an increase in glucidic colloids over time but as various proteins are released and degraded it should also result in wines that have lower levels of unstable proteins. The bentonite test is often employed to evaluate the bentonite required to eliminate unstable proteins before thermal treatments are conducted. Thus, we also measured the bentonite needed to produce a heat stable wine for all the Chardonnay wines over the 24 months exposure time. It was found that only 0.2% bentonite was sufficient to eliminate any unstable proteins from all wines at the zero time point and would result in heat stable wines. This low value is indicative that the levels of unstable proteins within our wines is very low. Over time the amount of bentonite that would be needed reduced to 0.1% for all wines by 24 months indicating that there appears to be no difference between the treatments of lees ageing, enzyme addition and control. Furthermore, there was no difference found irrespective of whether the wines had been put through MLF nor the type of yeast utilised to prepare the wines. Consequently, such tests appear to shed no light on whether the addition of enzymes aid in natural lees autolysis.

Whilst the above results were interesting and given that the primary purpose was to examine if we could find a simple analytical measurement which would let us know when natural ageing on lees was apparently complete we decided at this point to look at some other measurements such as total colour change, wine viscosity and the concentrations of

various metal ions which have not yet been reported as important changes as an indication of lees autolysis.

First we measured the differences in colour over time for all our wines to evaluate if this parameter would be a useful indication that lees is having an effect on the wines over time. Thus, we measured the CIE*lab* values of the Pinot Noir and Chardonnay wines over the two years of lees exposure with the results collated in section 2.2.4. The first wine examined was the Chardonnay wine made without MLF. Inspection of the CIE*lab* values indicated that there appears to be gradual decrease in the *l** value indicating that the wines are slowly becoming more darker over the 24 months of storage. Comparison of the *a** values indicated that the Chardonnay wines begin with a slight green hue and remain slightly green over time with little difference found after the 24 months of storage. Finally, inspection of the change in *b** values was very revealing and indicated that the wines are continuously shifting towards a yellowish hue over time. Moreover, the wines containing lees and/or added enzyme display a greater increase in +*b** when compared to the control wines for both yeast strains. Consequently, it appears that the wines containing lees are undergoing changes in their chemistries in a similar manner as to what would be found in gradual oxidation of a wine even though this would not be expected when lees is present. In a similar manner, inspection of the CIE*lab* of the Chardonnay wines made with MLF revealed the exact same trends.

Inspection of the CIE*lab* values of the Pinot Noir wines indicated that there appears to be no change in the *l** value after 24 months of storage although an increase in darkness was seen at the 4 month time point and presumably indicated a change in anthocyanin concentration as pigmentation occurs early on with the wines becoming stabilised in terms of colour over time. Comparison of the *a** values also indicated that Pinot Noir wines begin with a significant red hue with little difference found after the 24 months of storage, although there was a substantial increase in redness at the 4 month time point, again most likely linked to anthocyanin polymerisation. Finally, inspection of the change in *b** values indicated that the wines are continuously slowly shifting towards a deepness in yellowish hue over time. Overall it can be concluded that the changes in CIE*lab* values of our wines indicates that the wines are indeed changes over time, however, we were unable to clearly identify if these changes were associated with lees or enzyme exposure

when compared to the control wines. Consequently, further more focused studies are recommended for the future.

The attribute 'body' of a wine is a mouthfeel sensation and viscosity has been suggested to be probably the best property that correlates with the body of a wine and is induced primarily by the presence of alcohol, sugars, glycerol and phenolics. Thus a quick and easy measurement as to whether yeast lees autolysis can be considered finished in a certain timeframe may be to simply measure the change in overall dynamic viscosity of a wine. Consequently, we measured the dynamic viscosity, density and percentage alcohol of several of the Chardonnay and Pinot Noir wines at time points 0 and 24 months that were either exposed to lees or none, section 2.2.5. In terms of the Chardonnay wines which were not put through MLF it was found that there was a clear difference in the dynamic viscosities of the wines from the standpoint as to whether they contained lees or not even though the overall alcohol contents were identical and this perhaps reflected a greater release of total dry extract or polysaccharides into the wines over time which is to be expected as autolysis proceeds. For the Chardonnay wines that were allowed to go through MLF the viscosities actually fell after 24 months exposure and may be related to the fact that these wines have a reduction of the wines acidity due to the transforming of *L*-malic acid into *L*-lactic acid. Interestingly, the Pinot Noir wines displayed no significant difference in dynamic viscosity over the 24 months on lees although their overall viscosities and alcohol contents were higher that those of the Chardonnay wines.

Whilst we did not measure the viscosities of the wines that had been exposed to additional enzyme additions at the time, in retrospect this would have been interesting to do. In summary, although no such studies on how dynamic viscosity changes as natural lees autolysis proceeds have thus far been reported in the literature, it appears from our initial studies here that changes in dynamic viscosities of wines over time may in fact be a possible way to follow yeast lees autolysis although further experiments are clearly needed to verify such possibilities.

We also considered that during the ageing of our wines on lees that the concentrations of certain metals may change over time due to the breakdown of the lees and associated adsorption and desorption processes and that the changes in the levels of certain metals over time may be able to be utilised as a guide to following lees autolysis. Consequently

we determined the concentrations of the metals (Cu, Ca, Na, Fe, K, Zn, Cs, Mg and Rb) in a range of our wines by AAS, section 2.2.6. For the Chardonnay wines that were produced with the AWRI Fusion yeast and which had gone through MLF, comparison of the control wine A (no lees) with wine C (on lees) and wine E (on lees with enzyme) shows some interesting differences in metal concentrations when comparing the 0 and 4 month time points. The wines with added autolytic enzyme appeared to have a higher concentration of Cu, Ca, Fe and K at the zero time point which decreases greatly after 4 months when compared to the control wine A which was not exposed to lees. On the other hand the concentrations of Zn and Cs appeared to increase over time while the remaining metals (Na, Mg and Rb) appeared to remain essentially steady when compared to the control wine A. We next examined the Pinot Noir wines that were produced with the AWRI Fusion yeast at the time points 0 and 4 months. Comparison of the control wine M (no lees) with wine N (on lees) and wine O (on lees with enzyme) again showed some interesting differences in metal concentrations when comparing the 0 and 4 month time points. The concentrations of the metals Ca, Na, K, Cs, Mg appeared to change little between treatments. The changes in the concentrations of the remaining metals (Cu, Fe, Zn and Rb) were difficult to explain as some increased over time while others decrease. Furthermore, whether the wines were on lees or had added autolytic enzymes provided no clear trends in terms of changes in metal concentrations. The Pinot Noir wines did, however, contain a much higher concentration of Fe, Cs and Rb when compared to the Chardonnay wines analysed and presumably this simply reflects the variability in the origins of where the grapes were grown.

We also examined the concentrations of the same metals in the other wines produced and found that the concentrations also were highly variable with no clear trend seen between treatments. After careful analysis of all the results thus far determined within this study we became suspicious as to the activity of the added enzyme in our treatments as we were having difficulties clearly identifying that the wines made with added autolytic enzymes on lees were clearly undergoing enhanced autolysis as was expected. Consequently, we decided to cease measuring the concentration of certain constituents in our wines over time and to examine the activity of the autolytic enzyme (Optivin® Elevage) itself to check if it was indeed active at enhancing autolysis in our wine treatments.

For comparison we also included two other commercial enzymes to gauge the potential effect of the different enzymes. These additional enzymes were lallzyme MMX® (lallemand) which is a *β*-glucanase blend which is reported to aid in the release of polysaccharides from yeasts and improves the short maturing of wines on lees and VinoTaste® Pro (Novozymes) which is reported to decrease maturation time by nearly 20% and increases the roundness of the finished wines. Two different methods were utilised to assay the *β*-glucanase activity of these three enzymes. The first was the *β*-glucanase assay or BGU assay and the second was that reported by Humbert-Goffard et al., which is a method based on the measurement of the kinetics of glucose release in the presence of laminarin, a *β*(1,3)-glucan from *Laminarin digitata*. Immediately it became clear that the Optivin® Elevage displayed a much lower level of *β*-glucanase activity when compared to the other two commercial enzymes in both model and real wine systems, section 2.2.7. Consequently, it appeared that the use of the Optivin® Elevage in our real wine systems may in fact have been a poor choice as it appeared to have a much lower level of *β*-glucanase activity in comparison to other commercial enzymes. Moreover, this may explain further why we often sore only minor changes or differences in the wines that were exposed to lees versus those that were exposed to lees and added enzyme. At this stage we decided to leave this study in abeyance and examine the possibility that microwaves could be utilised to enhance lees autolysis or the use of IDY preparations could also be utilised to mimic the effects of enhanced lees autolysis is a short time. These areas of research are the subjects of the research described in the forthcoming Chapters 3 and 4.

CHAPTER 3: MICROWAVES – A POSSIBLE WAY TO ACCELERATE THE EFFECTS OF YEAST AUTOLYSIS.

3.1 General Introduction.

As highlighted in Chapter 1, when wine is aged on lees, the process of autolysis causes the release of a range of substrates including mannoproteins, polysaccharides, amino acids and fatty acids that interact with the fermented wine leading to changes in the final flavour and sensory aspects of the wine.[34,35] Wines subjected to autolysis create complexity resulting in an enhanced creamy mouthfeel. In addition, the release of enzymes by the lees which are able to absorb oxygen, inhibit or reduce the risk of oxidation which aids in improving the ageing potential and overall stability of finished wines.[35] The release of mannoproteins has also been found to improve the overall stability of the proteins in wines by reducing potential tartrate precipitation. Furthermore, the perception of bitterness or astringency is reduced as the mannoproteins released have been implemented in binding tannins. Moreover, a number of flavours such as biscuits or nuttiness are enhanced in sparkling wines by the increased production of certain amino acids released during autolysis.[36] The level of enhanced flavour crudely depends on the length of time the wine is in contact with the lees. However, this timeframe can span many years, thus, much recent work has been conducted in order to find better ways to accelerate the autolytic process or to mimic it in a shorter time. This chapter looks at the use of microwaves to accelerate lees autolysis both from a chemical standpoint and the associated sensory attributes.

3.2 Use of Microwaves to Accelerate Yeast Autolysis.

As already highlighted within the introduction various enzyme assisted autolysis methods have been developed over the last decade or so to speed up and mimic the slow process of autolysis in wine.[64,65] Furthermore, chemical methods such as acidification or mechanical methods such as thermal heating, mechanical disruption and ultrasonics have been employed to aid in lees lysis.[166,167] In fact a very recent study by Cacciola et al., found the ultrasound treatment of lees for just 3 minutes at 90% amplitute was sufficient to allow

for an increase in total colloids, proteins and polysaccharides and was significant when compared to traditional long exposure of wine on the lees.[168] This disruption of the yeast cell walls and associated release on intracellular components is contributed to a process called ultrasonic cavitation which results in the formation of small cavities (100 microns) of high heat (5,000 K) and pressure (1,000 atm) producing shock waves that demolish the cell walls in short time.

Microwaves have been employed as a heat source since the 1940's and are now extensively employed in the food and chemical engineering industries. The food industry is the largest consumer of microwave energy where is may be employed for drying, thawing, tempering, cooking, freeze-drying, sterilisation, pasteurisation, heating and re-heating.[169] Microwaves are an electromagnetic radiation with a wavelength from 0.001 m to 1 m (frequencies between 300 MHz and 300 GHz) which are transmitted as waves.[170] 915 MHz is considered the most useful for industrial applications due to its greater penetration depth while a frequency of 2,450 MHz is usually employed for domestic microwave ovens and for extraction applications for analytical chemistry purposes.[171] When microwaves pass through the medium, its energy is absorbed by solvents with dipoles (water, ethanol etc) and converted to thermal energy thus resulting in sample heating. This heating causes cells to lose moisture through evaporation, thus producing a high pressure on the cell wall. This build up of pressure inside the biomaterial modifies the physical properties of the biological tissues (cell walls and organelles) and will ultimately lead to rupturing of the cells and the release of their components.[170] Whilst the use of microwaves has been employed to extract phenolics from *Vitis vinifera* wastes for example, there have been no reports on the use of microwaves to accelerate yeast lysis.[172] Consequently, we exposed a wine to microwaved lees and evaluated its chemical and sensory attributes in order to examine if such a technique may be employed as a future practical winemaking technique for the preparation of wines that mimic long term natural lees exposure.

3.3 Preparation of Wine Samples with Lees That Had Been Exposed to Microwaves or Thermal Treatment.

Given that we wished to carry out a descriptive sensory analysis of wines that had been exposed to microwave treatment we first needed to decide at what temperature should the

samples be prepared for both the microwave and thermal treatments. In order to do this we decided to expose a lees/water solution to different temperatures between 45 – 95 ^{o}C at 300 W for 3 minutes and measure the total glucidic colloids and soluble proteins released with the theory being that when these levels reach a maximum then we have mimicked normal autolytic lysis of the lees. *Figure 3.1* displays the results of these experiments and clearly shows that when the temperature reaches 85 ^{o}C the levels of total glucidic colloids and soluble proteins released reach a maximum and plateau. Consequently we chose 85 ^{o}C as the desired temperature to be used for the disruption of the lees for the sensory experiments (both microwave and thermal treatments) knowing that there should be a clear effect on the sensorial properties of the wines.

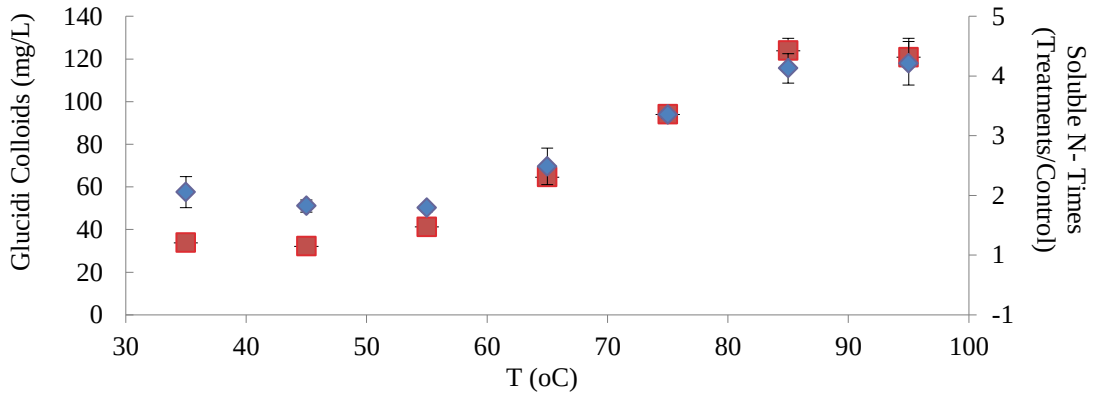


Figure 3.1 Total soluble nitrogen (tan) and glucidic colloid (blue) levels released at various temperatures when microwaving a lees solution at 300 W for 3 minutes.

We also examined the effect of sample size/volume and power level on time to reach 85 ^{o}C. *Figure 3.2* displays the temperature of the sample for 3 different sample sizes of 21 mL, 14 mL and 7 mL in microwave tube sizes 35 mL, 35 mL and 10 mL respectively. It can clearly be seen that a smaller sample size reaches the 85 ^{o}C temperature at a faster rate than larger volumes. Similarly, a higher power level of 300 W also allows the sample to reach 85 ^{o}C in a shorter timeframe. Consequently, we chose a sample size of 7 mL total volume in a 35 mL microwave tube and a power setting of 300 W to be utilised for the preparation of the microwaved lees samples for the sensory study experiments described below. Importantly, a total exposure time of 3 minutes is ample to ensure that all samples reach 85 ^{o}C in less than 3 minutes.

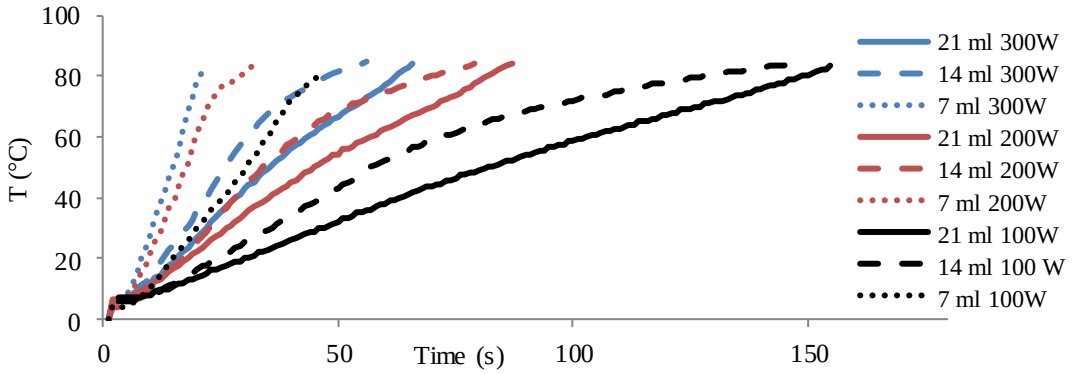


Figure 3.2 Effect of sample size volume and power level on the ability of the sample to reach 85 ^{o}C.

3.4 Descriptive Sensory Assessment of the Wine Samples Exposed to Microwaved or Heated Lees.

Descriptive sensory assessment of beverages such as wines is a powerful tool for elucidating how humans perceive the aroma and texture of a wine both from a nasal point of view and also in terms of the palate and mouthfeel. A panel of experts must first be trained in order to recognise certain attributes such as citrus or creamy at certain levels of detection. After training is complete the panel is then asked to smell and taste the wine and score the intensity of such attributes. In this way the differences between treatments (various styles of wines) can be determined along with the significance from a statistical point of view. Given that we wished to evaluate the differences between wines exposed to microwaved or thermally treated lees over a 24 hour period a formal DA panel was set up and trained.

3.4.1 Training of the DA Panel.

Descriptive sensory analysis of wines consisted of eight training sessions and four formal sessions.[173] Sensory panels comprised 11 (7 males and 4 females) multicultural students studying an oenology or wine marketing degree at The University of Adelaide. Several training sessions were held following the procedure outlined by Lawless and Heymann.[174] During the training period, judges generated appropriate descriptive terms and gained familiarity in recognising and scoring the intensity of selected and defined specific

attributes. The panellists were given a set of reference standards containing typical aromas of Chardonnay wines and lees characters (*Table 3.1*), which were prepared with a small amount of raw material and 30 mL unwooded Chardonnay wines, Morris, vintage 2012.

Table 3.1. Aroma and flavour reference standards employed for DA.

Attribute	**Description**
Tropical fruit [a]	4 g mixed paste of pineapple, mango and passionfruit (ratio 1:1:1) added into 25 mL base wine.
Stone fruit [a]	4 g mixed paste of peach, apricot (ratio 1:1) added into 25 mL base wine.
Pome fruit [af]	4 g mixed paste of ripe pear and red apple (ratio 1:2) added into 25 mL base wine.
Citrus [af]	2 g mixed paste of orange, lime, lemon and grapefruit (ratio 1:1:1:1) added into 25 mL base wine.
Savoury [af]	0.02 g bacon paste added into 25 mL base wine. The solution was diluted at 1:10.
Yeasty [a]	0.2 g mixture of yeast and biscuit powder (ratio 1:1) added into 25 mL base wine.
Vanilla/Butterscotch [af]	0.1 g grounded butterscotch powder and a drop of vanilla essence added into 25 mL wine.
Dairy products [af]	0.1 g mixture of yoghurt and milk (ratio 1:1) added into 25 mL base wine. The solution was diluted at 1:10.
Nutty [af]	0.2 g toasted almond powder added into 25 ml base wine
Green character [f]	4 - 5 pieces of fresh grass leaves chopped and added into 25 mL base wine.

*Base wine was a commercial 2 L Chardonnay wine, [a] is a reference standard for aroma attributes, [f] is a reference standard for flavour attributes.

From the third training session onwards, wine samples were prepared in a drier and less flavoured base wine (Yalumba unwooded Chardonnay 2012 rather than Morris unwooded Chardonnay). Reference standards for aroma and flavour attributes were refined through the training sessions. The gradient standards were prepared to train the assessors on evaluating bitterness, acidity and wine body. The final set consisted of 9 aroma attributes, 7 flavour attributes, and 6 mouthfeel attributes, *Table 3.2.*

Table 3.2. Aroma, palate and mouthfeel sensory attributes used in the sensory assessment.

Attribute	**Description**
Aroma	
Tropical fruit	Aroma of pineapple, mango, passionfruit
Stone fruit	Aroma of peach, apricot
Pome fruit	Aroma of ripe pear, red apple
Citrus	Aroma of orange, lime, lemon, grapefruit
Savoury	Aroma associated with bacon, meat, popcorn
Yeasty	Aroma of yeast, dough, biscuit
Vanilla/Butterscotch	Aroma of butterscotch, vanilla
Dairy products	Aroma associated with yoghurt, milk, butter
Nutty	Aroma of toasted almonds, nuts
Palate	
Pome fruit	Flavour of ripe pear, red apple
Citrus	Flavour of orange, lime, lemon, grape
Green character	Any flavour associated with leafy, grassy, green or unripe fruit
Savoury	Flavour associated with bacon, meat, popcorn
Yeasty	Flavour of yeast, dough, biscuit
Nutty	Flavour of toasted almonds
Dairy products	Flavour associated with yoghurt, milk, butter
Mouthfeel	
Creaminess	Creamy sensation on palate
Acidity	Intensity of sour/acid taste
Length	Time that wine flavour remains on a palate after expectoration
Alcohol	Perception of alcohol intensity
Bitterness	Intensity of bitter taste and/or bitter aftertaste
Body	Mouth filling sensation on palate

Three formal sessions were then conducted in isolated booths at 22 - 23 ^{o}C under sodium lights. Wines were presented in 30 mL samples in 3-digit coded, covered, ISO standard glasses. At each session, all panellists evaluated 18 wines that were in a randomised order across judges. Each fermentation replicate was evaluated in triplicate. Panellists rated aroma attributes and a number of selected mouthfeel attributes while holding the wine in mouth and after expectoration, *Table 3.2*, on a labelled magnitude line scale.[175] The

reference standards were presented at each session, thus panellists could refer to these standards at any time during the session. Panellists rinsed thoroughly with distilled water and had a rest of 1 minute between samples.

3.4.2. Results.

As highlighted in the preamble to this chapter, there have been no reports on the use of microwaves to aid in lees lysis and thus aiding in the possible acceleration of autolysis. Consequently, we examined several of the global chemical parameters such as total soluble protein and glucidic colloid levels in the wines to determine what constituents may have changed in terms of their levels whilst at the same time conducting the formal descriptive analyses of the wines.

3.4.2.1. Changes in Total Soluble Proteins, Glucidic Colloids and Free Radical Scavenging Ability.

Once the samples of the wines were prepared for the sensory tests we also measured the total soluble proteins, glucidic colloids and free radical scavenging ability of the samples to ensure that the microwave and thermally treated samples were significantly different from the control sample itself. *Table 3.3* displays this data and it can be seen that subjecting the lees to the short burst of microwaves leads to samples with a significantly higher level of total soluble proteins and glucidic colloids within the samples. If the lees is untreated and is just exposed to the medium for 24 hours it was found that the glucidic colloid level approximately doubles from 1163 mg//L to 2093 mg/L indicating that both thermal and microwave treatment of the lees results in significant disruption of the lees allowing for the rapid release of soluble proteins and glucidic colloids. It can also be seen that microwave treatment leads to a higher level of released proteins when compared to the thermally treated lees although the levels of released glucidic colloids are approximately the same. The antioxidant capacity of the solutions were also determined by the usual DPPH and FRAP methods and it can be see that there was a slight increase in antioxidant capacity of the solution exposed to thermal treated or microwaved lees. This cannot be associated with release of additional polyphenols as the lees are devoid of such constituents and as such may be a reflection of a greater release of proteins or amino acids which contain oxidisable functional groups such as thiol moieties for example. In any

case, it is clear that from a chemical standpoint, the thermal treatment or microwave treatment of lees results in solutions that are different when compared to simply exposing a solution to lees which has been untreated and simply allowed to undergo slow natural autolysis. Consequently, we were expecting to see substantial differences from a sensorial point of view between the various treatments.

Table 3.3. Total soluble proteins, glucidic colloids and free radical scavenging ability of the microwave and thermally treated samples compared to the control.

	Soluble Protein (mg/L)	**Gluccidic Colloids* (mg/L)**	**Free Radical Scavenge Ability**			
			DPPH		**FRAP**	
			Vc-Eq (µM)	**Trolox-Eq (*µ*M)**	**Vc-Eq (*µ*M)**	**Trolox-Eq (*µ*M)**
Untreated	30.75 ± 3.36	2093.0 ± 88.3	207.46 ± 3.97	193.12 ± 3.12	300.25 ± 12.62	392.11 ± 16.83
Heated	121.61 ± 8.66	7057.3 ± 53.5	238.92 ± 7.25	223.32 ± 6.96	409.09 ± 21.45	537.23 ± 28.61
Microwave	206.88 ± 5.50	6721.3 ± 27.8	219.29 ± 7.94	204.48 ± 7.62	343.65 ± 17.83	449.98 ± 22.85

*Glucidic colloids in base wine: 1162.7 ± 180.0 mg/L. All results based on 2 parallel treatments, except for untreated (one sample), and each treatment was analysed in triplicates. All data were significantly different between treatments by Student-Newman-Keuls one-way ANOVA analysis ($P < 0.5$).

3.4.2.2. TEM Results.

Transmission electron miscroscopy is a powerful tool for evaluating untrastructural changes of biological samples.[176] In short, it essentially involves taking a sample of cells and high-pressure freezing them in a matrix, staining with a dye to enhance image resolution, cutting the material into thin slices and visulisation with a powerful microscope. Recently, Tudela et al., utilised TEM to visulise the ultrostructural changes that occur of sparkling wine lees during long-term ageing under real oenological conditions.[176] They found that TEM could clearly show that the stratified structure of the cell wall disappeared throughout ageing. After 18 months, the microfibrous material of the cell wall appeared more diffuse with the omorphous midzone of the inner wall layer

being progressively degraded. After 30 months the cell wall consisted of a tangled structure of fibers, however, the cell wall itself remained unbroken at 48 months of ageing.

As we had acces to a TEM instrument coupled with the fact that the total soluble proteins and glucidic colloids of our microwaved and thermal treated lees samples increased dramatically when compared to the control, indicating that autolysis was being enhanced, we decided to conduct several TEM analyses to get a visual indication as to what may be occuring during these experiments. It should be poited out that these experiments are very time consuming so only limited samples were prepared for analysis.

Samples of lees that had been microwaved or thermally treated at 85 ^{o}C were collected and prepared for TEM analysis as described by Tudela et al.[176] For the control the lees was freshly harvested from our fermentations and collected directly from the wine. The TEM image of a yeast cell (control) is depicted in *Figure 3.3*. Note that the cell wall is well preserved in its natural stage with the cell membrane, inner and outer cell walls well defined.

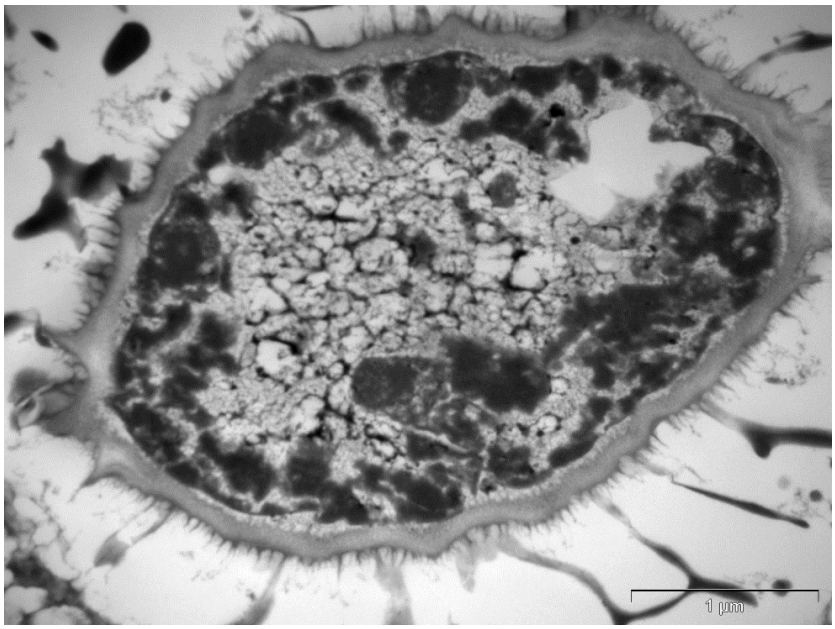


Figure 3.3. TEM image of a *Saccharomyces cerevisiae* cell harvested directly from fermentation.

Figure 3.4 displays a TEM image of a yeast cell that had been heated to 85 ^{o}C for several minutes as described for sample preparation for the sensory experiments. It can clearly be

seen that the cell is no longer ovide in shape and appears to be puckered although the cell wall still appeared to be intact.

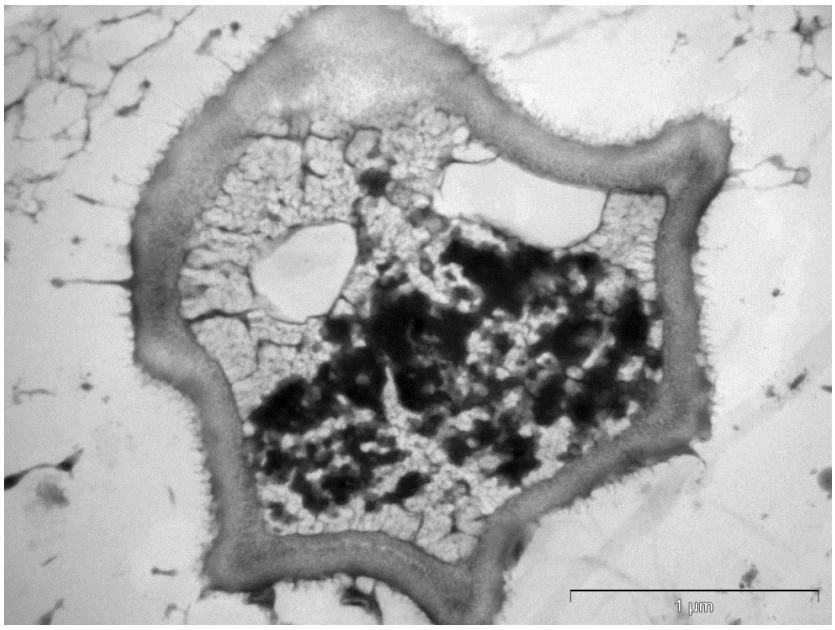


Figure 3.4. TEM image of a *Saccharomyces cerevisiae* cell harvested after thermal treatment at 85 ^{o}C as described for the sensory experiments.

Figure 3.5 displays a TEM image of a yeast cell that had been microwaved at 85 ^{o}C for several minutes as described for sample preparation for the sensory experiments. It can clearly be seen that the cell wall appears thiner when compared to the control. It also appears that the inner cell wall and membrane appear to have been destroyed which would allow for the ready release on cyctoplasmic content. The internal organelles within the cyctoplasm also appear to be greatly disrupted when compared to the control.

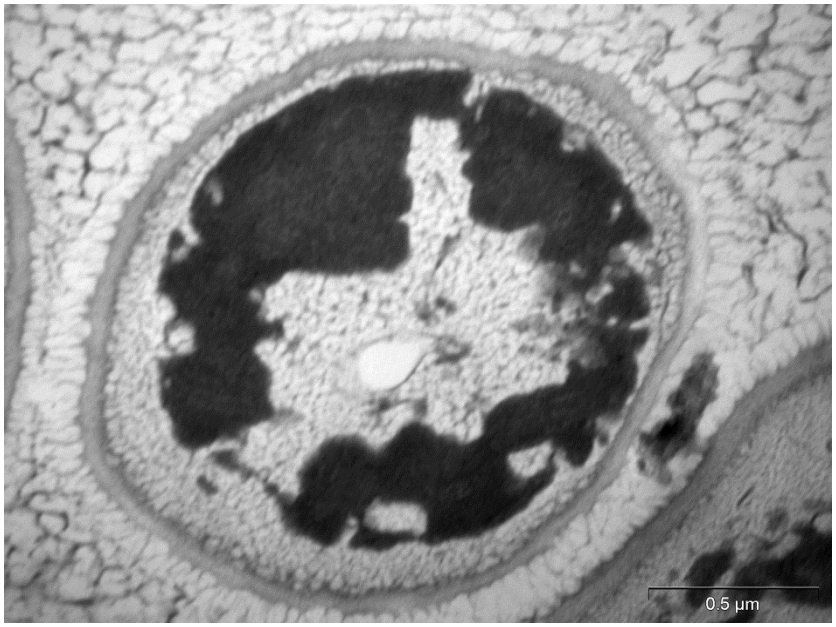


Figure 3.5. TEM image of a *Saccharomyces cerevisiae* cell harvested after microwave treatment at 85 ^{o}C as described for the sensory experiments.

Importantly, these TEM results appear to correlate well with the experimental findings described above, that is, both microwave and thermal treatment lead to an increase in the release of cyctoplasmic content (e.g. soluble proteins etc) and can be considered as an indication of an advanced stage of cell lysis. Consequently, it appears that the samples prepared for sensorial analysis were in a stage of advanced cell lysis, thus suitable to be utilised for the sensory analyses.

3.5. Descriptive Sensory Assessment.

After the initial training was completed three formal sessions were conducted and the panellists were asked to rate each wine for all 9 aroma attributes, 7 flavour attributes, and 6 mouthfeel attributes. As described above the wines were the control (Chardonnay wine), an untreated wine sample (Chardonnay wine with untreated lees), wine exposed to thermally heated lees (at 1%, 2% and 3% reconstitution levels) and the wines exposed to the microwaved lees (at 1%, 2% and 3% reconstitution levels). Scores of each attribute, were rated on a 0 – 15 scale by the 11 panellists and averaged. *Table 3.4* summarises these average scores for all wine samples over the three formal sessions. Data acquisition was carried out using the software Fizz. Each attribute rated by judges was subjected to analysis of variance (ANOVA) to ascertain the effects of treatment, fermentation replicate

nested within treatment, judge and presentation replicate, using a mixed model ANOVA and treating judges in a random manner. The method used to discriminate among the means was Fisher's least significant difference procedure, $P < 0.05$. Consequently, *Table 3.4* contains superscript letter to indicate the level of significant difference.

In order to compare the difference between treatments, the reconstitution addition levels (at 1%, 2% and 3%) were also initially ignored deliberately with the results averaged and recorded in the first 4 columns of *Table 3.4*. Thus, if one ignores the effects that the amount of untreated or treated lees has on scoring the various sensory attributes one finds that there appears to be only small to moderate changes found by the sensory panel for most of the attributes. For example, little difference was seen in the vanilla/butter aroma or the green character of the various wines. There were, however, a few attributes that were significantly different between the treatments. For example, the savoury aroma caused by lees exposure was substantially different to the control Chardonnay wine itself whilst in terms of mouthfeel, the length was also significantly affected. However, whilst the perceived length of the wines exposed to the thermally heated lees was significantly lower than the control, it should be noted that natural lees autolysis normally leads to an increase in observed length. As it was found above that thermal or microwave treatment of the lees results in a greater release of total soluble proteins and glucidic colloids it was also expected that the yeasty/savoury characters of the wines should also be altered. Indeed, one finds that the panel rated differences for savoury/yeasty attributes on the palate although the significant was only moderate.

Table 3.4. Mean ratings for sensory attributes.

	C	**U**	**H**	**MW**	***p***	**C**	**U-1%**	**U-2%**	**U-3%**	***p***	**C**	**H-1%**	**H-2%**	**H-3%**	***p***	**C**	**MW-1%**	**MW-2%**	**MW-3%**	***p***
Aroma																				
Tropical fruit	8.6^{a}	8.6^{ab}	8.6^{ab}	8.1^{a}	*0.47*	8.6^{ab}	8.2^{b}	8.6^{ab}	8.8^{a}	*0.52*	8.6^{ab}	9.0^{a}	8.0^{b}	8.7^{ab}	*0.32*	8.6^{a}	8.2^{a}	7.9^{a}	8.4^{a}	*0.67*
Stone fruit	7.2^{b}	7.9^{ab}	8.1^{a}	7.5^{b}	*0.24*	7.2^{b}	7.3^{b}	7.9^{ab}	8.3^{a}	*0.38*	7.2^{b}	8.3^{a}	7.8^{ab}	8.2^{ab}	*0.47*	7.2^{ab}	7.6^{ab}	7.0^{b}	7.8^{a}	*0.55*
Pome fruit	7.0^{ab}	7.0^{b}	7.5^{ab}	7.8^{a}	*0.18*	7.0^{ab}	6.9^{b}	7.6^{a}	6.7^{b}	*0.15*	7.0^{b}	8.0^{a}	7.5^{ab}	6.9^{b}	*0.21*	7.0^{b}	7.7^{ab}	7.7^{ab}	7.9^{a}	*0.55*
Citrus	7.0^{ab}	7.5^{a}	7.1^{b}	7.0^{b}	*0.17*	7.0^{b}	7.4^{ab}	7.5^{ab}	7.8^{a}	*0.49*	7.0^{a}	7.2^{a}	7.1^{a}	6.9^{a}	*0.94*	7.0^{ab}	6.8^{b}	6.4^{b}	7.8^{a}	*0.06*
Savoury	5.3^{a}	4.6^{b}	4.4^{bc}	4.2^{c}	*0.03*	5.3^{a}	5.1^{a}	4.4^{b}	4.4^{b}	*0.12*	5.3^{a}	4.7^{b}	4.3^{b}	4.3^{b}	*0.11*	5.3^{a}	4.0^{b}	4.3^{b}	4.2^{b}	*0.07*
Yeasty	4.1^{a}	3.7^{a}	3.7^{a}	3.6^{a}	*0.74*	4.1^{a}	3.8^{ab}	3.5^{b}	3.8^{ab}	*0.48*	4.1^{a}	3.5^{b}	4.0^{a}	3.6^{ab}	*0.32*	4.1^{a}	3.7^{a}	3.7^{a}	3.5^{a}	*0.65*
Vanilla/Butter	5.6^{a}	5.7^{a}	5.9^{a}	5.7^{a}	*0.92*	5.6^{ab}	5.6^{b}	5.4^{b}	6.2^{a}	*0.23*	5.6^{a}	5.9^{a}	6.0^{a}	5.6^{a}	*0.76*	5.6^{ab}	5.2^{b}	6.0^{a}	5.8^{ab}	*0.48*
Dairyproducts	4.3^{ab}	4.6^{a}	4.2^{b}	4.5^{ab}	*0.27*	4.3^{bc}	5.1^{a}	3.9^{c}	4.7^{ab}	*0.11*	4.3^{a}	3.7^{b}	4.5^{a}	4.4^{a}	*0.11*	4.3^{ab}	4.5^{ab}	4.8^{a}	4.1^{b}	*0.32*
Nutty	4.3^{a}	4.1^{ab}	4.0^{abc}	3.6^{ac}	*0.29*	4.3^{a}	4.4^{a}	3.7^{a}	4.3^{a}	*0.58*	4.3^{a}	3.8^{ac}	4.3^{ab}	3.8^{ac}	*0.33*	4.3^{a}	3.8^{a}	3.9^{a}	3.2^{b}	*0.19*
Palate																				
Pome fruit	6.7^{b}	7.4^{a}	7.5^{a}	7.3^{a}	*0.25*	6.7^{b}	7.2^{a}	7.6^{a}	7.4^{a}	*0.10*	6.7^{c}	7.8^{a}	7.2^{b}	7.5^{ab}	*0.06*	6.7^{b}	7.1^{ab}	7.6^{a}	7.3^{ab}	*0.32*
Citrus	7.6^{a}	7.7^{a}	7.7^{a}	7.8^{a}	*0.94*	7.6^{a}	7.6^{a}	7.7^{a}	7.7^{a}	*0.98*	7.6^{a}	8.0^{a}	7.5^{a}	7.7^{a}	*0.63*	7.6^{b}	7.4^{b}	7.5^{b}	8.5^{a}	*0.07*
Green	4.1^{a}	4.1^{a}	4.1^{a}	4.2^{a}	*1.00*	4.1^{a}	4.2^{a}	4.0^{a}	4.2^{a}	*0.97*	4.1^{ab}	4.4^{a}	4.0^{ab}	3.9^{b}	*0.44*	4.1^{a}	4.1^{a}	4.0^{a}	4.3^{a}	*0.82*
Savoury	4.5^{a}	4.2^{a}	4.0^{b}	4.3^{a}	*0.24*	4.5^{a}	4.7^{a}	3.8^{b}	4.3^{ab}	*0.17*	4.5^{a}	3.8^{b}	3.8^{b}	4.4^{a}	*0.04*	4.5^{a}	4.3^{a}	4.2^{a}	4.3^{a}	*0.85*
Yeasty	3.2^{ab}	3.4^{a}	3.1^{b}	3.4^{ab}	*0.50*	3.2^{b}	3.4^{ab}	3.2^{b}	3.6^{a}	*0.20*	3.2^{ab}	3.0^{b}	3.4^{a}	3.0^{b}	*0.13*	3.2^{a}	3.5^{a}	3.3^{a}	3.3^{a}	*0.73*
Nutty	4.1^{a}	3.8^{a}	3.8^{a}	4.0^{a}	*0.83*	4.1^{ab}	4.4^{a}	3.4^{c}	3.6^{bc}	*0.08*	4.1^{a}	4.0^{a}	3.7^{a}	3.6^{a}	*0.58*	4.1^{a}	4.2^{a}	4.0^{a}	3.8^{a}	*0.60*
Dairyproducts	3.9^{a}	3.6^{a}	3.6^{a}	3.5^{a}	*0.72*	3.9^{a}	3.9^{a}	3.1^{b}	3.7^{a}	*0.18*	3.9^{a}	3.9^{a}	3.7^{ab}	3.2^{b}	*0.26*	3.9^{a}	3.5^{a}	3.4^{a}	3.5^{a}	*0.65*
Mouthfeel																				
Creaminess	7.1^{a}	6.9^{a}	6.6^{a}	6.9^{a}	*0.79*	7.1^{a}	7.0^{a}	7.1^{a}	6.7^{a}	*0.77*	7.1^{a}	7.1^{a}	6.0^{b}	6.7^{a}	*0.07*	7.1^{a}	7.3^{a}	6.8^{a}	6.6^{a}	*0.65*
Acidity	7.6^{b}	8.6^{a}	8.5^{a}	8.4^{a}	*0.27*	7.6^{b}	8.9^{a}	8.5^{a}	8.4^{ab}	*0.25*	7.6^{b}	8.5^{a}	8.6^{a}	8.2^{ab}	*0.21*	7.6^{b}	8.2^{ab}	8.6^{a}	8.4^{a}	*0.34*
Length	9.4^{a}	8.8^{a}	8.1^{b}	8.8^{a}	*0.07*	9.4^{a}	9.2^{ab}	8.7^{ab}	8.6^{b}	*0.42*	9.4^{a}	8.2^{b}	8.3^{b}	8.0^{b}	*0.15*	9.4^{a}	8.6^{a}	8.7^{a}	9.1^{a}	*0.60*
Alcohol	6.8^{a}	7.5^{a}	7.3^{a}	7.4^{a}	*0.73*	6.8^{b}	7.8^{a}	7.3^{ab}	7.4^{ab}	*0.34*	6.8^{b}	7.6^{a}	7.1^{b}	7.1^{b}	*0.10*	6.8^{b}	7.1^{b}	7.5^{a}	7.5^{a}	*0.20*
Bitterness	6.1^{a}	5.6^{ab}	5.2^{c}	5.5^{bc}	*0.18*	6.1^{a}	5.9^{a}	5.3^{a}	5.7^{a}	*0.56*	6.1^{a}	5.4^{b}	5.0^{b}	5.3^{b}	*0.18*	6.1^{a}	5.3^{a}	5.7^{a}	5.5^{a}	*0.51*
Body	6.8^{a}	7.0^{a}	6.7^{a}	7.0^{a}	*0.80*	6.8^{a}	7.3^{a}	6.9^{a}	6.9^{a}	*0.66*	6.8^{ab}	6.9^{a}	6.4^{b}	6.8^{ab}	*0.58*	6.8^{a}	7.2^{a}	7.0^{a}	7.0^{a}	*0.89*

The DA panel was also able to detect significant differences between the amount of untreated or treated lees added to the base Chardonnay wine, *Table 3.4*. *Figure 3.6* displays the scores for the most significant different attributes as an average and also at their individual reconstitution levels of 1%, 2% and 3%. Simple addition of lees to the base Chardonnay wine at these levels results in no significant difference in the 6 mouthfeel attributes, *Table 3.4*. However, the aroma attributes pome fruit, savoury and dairy products were found to be substantially significant. Both pome fruit and savoury showed a decrease at a 3% level of lees whilst the attribute dairy products increased when compared to the control. The same three attributes were also found to be significantly different on the palate. Analysis of the wines that were exposed to the thermally treated lees at the three different reconstitution levels also indicates that the same three attributes, namely pome fruit, savoury and dairy products are rated as significantly different although there is again some fluctuation seen between the various reconstitution levels, *Figure 3.6*. In terms of mouthfeel the creaminess and alcoholic attributes also were rated as different to the control.

Figure 3,6 also displays the scores for the most significant different attributes at their individual reconstitution levels of 1%, 2% and 3% when the base Chardonnay wine was exposed to the lees that had been subjected to microwaves for 3 minutes. No longer is the attribute pome fruit significantly different, however, the aroma attributes citrus and savoury were very different with $P < 0.1$. It is also noteworthy that the attribute nutty is also somewhat significant. In terms of the palate, citrus was again the only attribute that increased in significance at the 3% reconstitution level whilst there was little difference found for the various mouthfeel attributes.

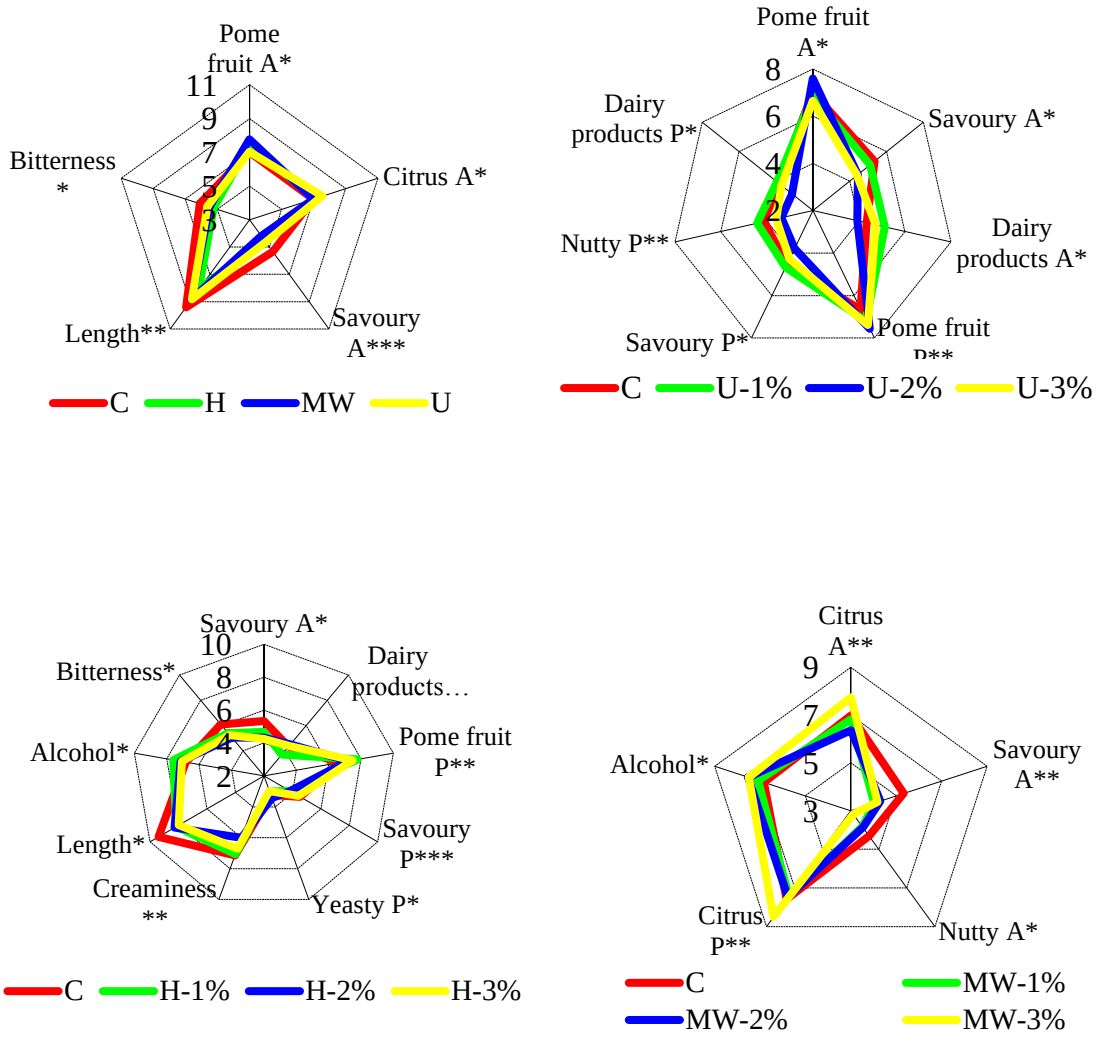


Figure 3.6. Web diagrams of the important differences in sensory attributes for each treatment (untreated lees, heated lees and microwaved lees) along with an overall average. A = aroma attribute and P = palate attribute. ANOVA by Fisher's (* $P < 0.2$, ** $P < 0.1$, *** $P < 0.05$).

The data collated within *Table 3.4* clearly indicates that not only are their subtle differences between the various treatments but also that the reconstitution level plays a significant role on how the various sensory attributes are rated. It can, however, be concluded that exposure of a base wine to lees whether untreated or treated (thermally of microwaved) results in wines that display differences in a number of sensorial attributes and as such can be considered new wine styles.

3.6. Chapter Conclusions.

The primary purpose of conducting these experiments was to ascertain if exposing lees to microwaves for a short period of time, 85 ^{o}C and less than 3 minutes, followed by reconstitution in a base Chardonnay wine would result in significant differences in various chemical and sensorial attributes. Indeed, it was found that exposure of lees to microwaves results in an increase level of release of total soluble proteins and glucidic colloids. Importantly, TEM results also appear to correlate well with these experimental findings. That is, both microwave and thermal treatment lead to yeast cells with thinner cell walls and disrupted cytoplasmic content which can be considered as an indication of an advanced stage of autolysis. Furthermore, certain sensorial attributes were found to be significantly different as depicted within *Table 3.4* and *Figure 3.6* although many of the attributes displayed no significant difference. Many of these attributes such as citrus and nutty are not normally associated with normal lees autolysis over a lengthy period of time and as such this suggests that the origins of these observations requires further research in order to determine the autolytic/chemical mechanisms behind these findings. In terms of future work, it would be worthy to also quantify the differences in aroma compounds by GC/MS in order to evaluate further why there appears to be these detectable differences observed by the DA panel. Furthermore, it would be fruitful to repeat these experiments at a much higher level of reconstitution (e.g. 5% or 10%) to enhance the observed effects so that clearer conclusions can be made through both chemical and descriptive sensorial analysis. Finally, it is clear that prior exposure of lees to microwaves followed by reconstitution into a wine results in different wine styles. As such it would be worthy to conduct large scale wine trials utilising larger industrial microwave reactors to evaluate further whether such an approach is applicable to the wine industry in general.

CHAPTER 4: COMMERCIAL IDY PREPARATIONS - THEIR EFFECTIVENESS AND DIVERSITIES.

4.1 General Introduction.

As highlighted in Chapter 1, when wine is aged on lees, the process of autolysis causes the release of a range of substrates including mannoproteins, polysaccharides, amino acids and fatty acids that interact with the fermented wine leading to changes in the final flavour and sensory aspects of the wine.[34,35] Wines subjected to yeast autolysis create complexity resulting in an enhanced creamy mouthfeel. In addition, the release of unsaturated fatty acids and thiols by the lees which are able to absorb oxygen, inhibit or reduce the risk of oxidation which aids in improving the ageing potential and overall stability of finished wines.[35] The release of mannoproteins has also been found to improve the overall stability of the proteins in wines by reducing potential tartrate precipitation. Furthermore, the perception of bitterness and astringency is reduced as the mannoproteins released have been implemented in binding tannins.[35] Moreover, a number of flavours such as biscuits or nuttiness are enhanced in sparkling wines by the increased production of certain amino acids released during autolysis.[36] The level of enhanced flavour crudely depends on the length of time the wine is in contact with the lees. However, this timeframe can span many years, thus much recent work has been conducted in order to find better ways to accelerate the autolytic process or to mimic it in a shorter time.

Thus, a broad range of commercial inactivated dry yeast (IDY) preparations have come on the market in recent years and are now being used as an alternative technique to ageing wines on lees, because they permit a quicker release into the wine of yeast compounds such as mannoproteins, glucans and amino acids and as such the same perceived benefits of lees exposure may be achieved in a shorter timeframe. These commercial products are obtained from the yeast *Saccharomyces cerevisiae* that are grown on highly concentrated sugar media followed by thermal or enzymatic inactivation.[70,71] Depending on their method of manufacture and main constituents they may be classified into one of four types:

1) Inactive yeast obtained by thermal inactivation followed by drying.

2) Yeast autolysates, which are obtained by thermal inactivation coupled with a incubation step with added β-glucanases which allows degradation of the cell walls and the release of the intracellular cell content.

3) Yeast hulls or yeast walls which are the insoluble yeast walls without cytoplasmic content. They may be subjected to acid/alkaline washing after inactivation.

4) Yeast extracts which is the extract after complete degradation of the cytoplasmic content.

Many different companies now sell a broad range of IDY products with many of them described as improving a wines sensory characteristics in a very specific way or allow for the improvement of the vinification technical process itself. These IDY preparations can be utilised at different stages of the winemaking process depending on their prescribed application. Moreover, many are insoluble and are readily removed by racking/filtration before bottling. Given the infancy of their use, coupled with the diverse range of effects they can have on a finished wine, this is an area of current intensive research in order to try and assemble more information on how such substances may influence a wines final quality and organoleptic attributes.[63,71,73,74] Consequently, given that the focus of this PhD thesis was to further elucidate the mechanisms of how lees can be utilised to enhance a final wines organoleptic properties and overall quality, it was felt appropriate that a study on how a range of ten IDY preparations mimic certain traditional lees ageing over time was warranted. The objective was to further understand their mode of actions and establish criteria for their use during winemaking. The results would also allow comparisons to be drawn between traditional lees ageing compared to the application of IDY preparations to accelerate the perceived benefits of lees ageing. Consequently, this chapter begins with a summary of the main applications of IDY preparations in winemaking with a focus on current mechanistic understanding. A model wine and a Chardonnay base wine was then produced and subjected to ten different IDY preparations. Various analyses are then conducted and described detailing the observed effects on the final wines. The scientific information gleaned is then analysed and compared to that in the current literature.

4.2 Regulation on the use of IDY Preparations in Winemaking.

A number of countries have now adopted legislation permitting the use of inactivated yeasts, yeast autolysates, yeast cell walls and hulls and various yeast extracts including mannoproteins which are all classified under the banner of IDY preparations for specific uses during winemaking. These countries include Argentina, Australia, Chile, European Union, New Zealand, USA and Japan. For example, the International Oenological Codex issued by the International Organisation of Vine and Wine (OIV, 2013 issue) permits the use of yeast mannoproteins (adopted, OENO 26/2004) and yeast protein extracts (OENO 452-2012) for tartaric and/or proteic stabilisation of wines.[177] It appears that most countries have adopted a maximum dosage of 400 mg/L.

4.3 Preparation of IDY Products.

Inactivated dry yeast preparations are commercial products that are produced from a range of wine fermentation yeasts, particularly *Saccharomyces cerevisiae*.[178] They may be produced from single strains or mixtures and contain additional additives such as specific enzymes. The yeasts are produced by a controlled fermentation under aerating conditions. The pure 'mother' culture is grown in a nitrogen-enriched sugar medium that has been fortified with vitamins and minerals for OptiMUM-White® cell activity. After a specific stage of growth has been reached the mature cell mass is separated as a cream and inactivated. Additional additives such as microcrystal micro-cellulose (CMC) are sometimes employed for product stability and dispersion. *Figure 4.1* displays a summary of the key production steps employed to prepare various IDY preparations. The inactivation method employed is often specific to what the aim of the product is expected to achieve.

The most common method of inactivation is thermal inactivation where the yeast is heated to high temperature to produce inactivated dry yeast. At this stage the cytoplasmic cell interiors may be extracted from the inactivated whole yeast cells to afford yeast hulls also known as yeast cell walls. The extracted cytoplasmic material is known as a yeast extract.[178] In order to achieve pure cell walls several purification steps are often required. Yeast autolysates on the other hand are the material left over from yeast autolysis which involves the destruction of the yeast cell by the action of its own enzymes under specific

temperature and pH conditions or by the addition of external enzymes such a proteases or β-glucanases. The latter can include in their composition soluble nitrogen, polysaccharides including mannoproteins and nucleic acids. This process usually involves a thermal inactivation followed by an incubation process as depicted in *Figure 4.1*. Other methods of inactivation or lysis may include plasmolysis (osmotic shock of the cells by adding sodium chloride) or acidic hydrolysis.[167]

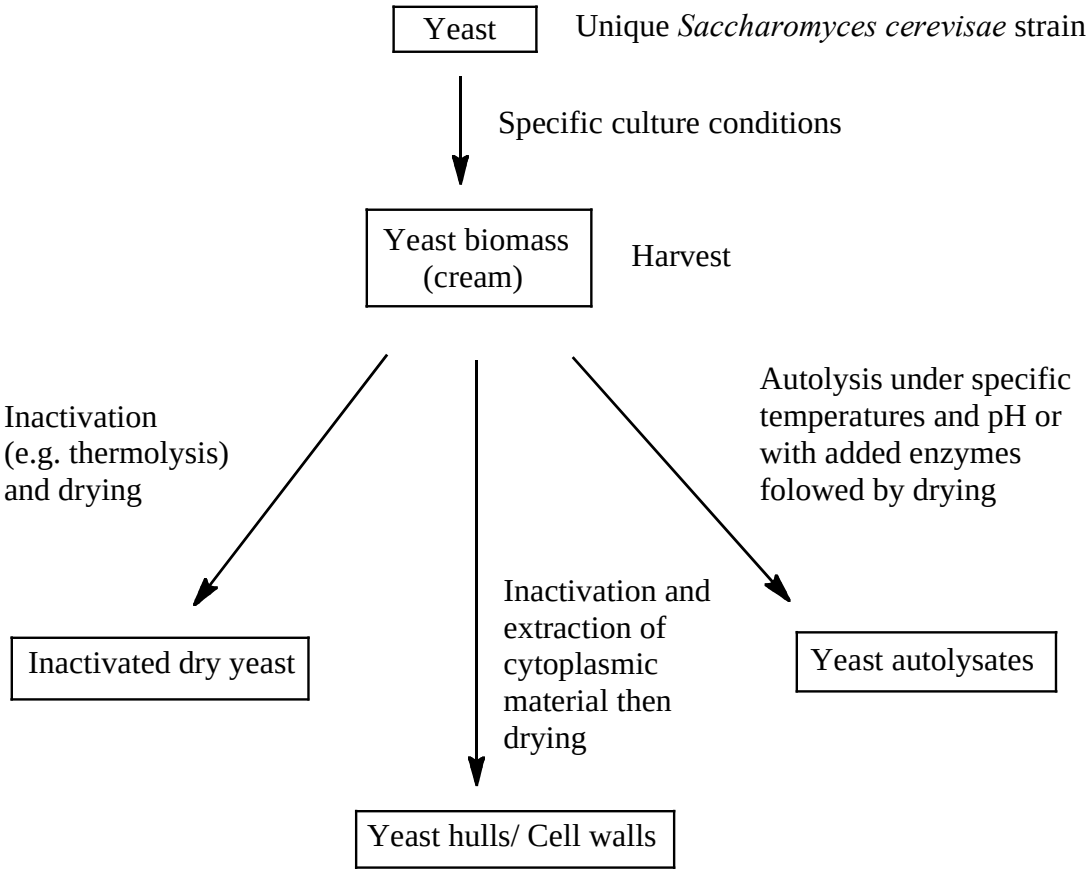


Figure 4.1. General schematic of the typical production steps utilised to prepare IDY preparations.

The final steps in the production process involves the drying of the IDY preparations into a powder by roller drying or spray drying so as to facilitate their easy dose into a wines medium.[178] During this latter step the yeast components may be exposed to high temperatures promoting thermal reactions between the polysaccharides and nitrogen compounds giving rise to different types of Maillard reactions which afford various heterocyclic volatile compounds. These compounds, including pyrazines, may be released

into a wines medium when the IDY is added to a wine.[179] Recently, Pozo-Bayon et al., has shown that supercritical CO_2 may be employed to extract these odorant volatile compounds from IDY preparations, thus leading to cleaner IDY preparations which will not have such a high impact on the sensory attributes of a final wine.[178] It should also be noted that some commercial IDY preparations will also have added other non-yeast derived products such as tannins to also aid in altering a final wines style. Finally, a very recent study by Comuzzo et al. has shown that the method of production of an IDY will have substantial effects on its ability to alter a final wines composition and organoleptic properties highlighting that much research is still needed on further understanding the production processes and their effects of final IDY composition.[167]

4.4 Applications of Specific IDY Preparations in Winemaking and Their Effects on the Wine.

There are now many commercial products formulated with inactive dry yeast preparations used in winemaking. In the main they fall into two categories. Yeast mannoproteins are used as additives for improving either technological winemaking processes or the sensory characteristics of wines. These preparations differ in molecular mass and chemical composition (percentage of proteins, amino acids, glucids and glucosamine), and therefore their effects in a wine can be highly variable.[71] The other category includes all the different types of yeast autolysates which are believed to improve fermentation and a wines organoleptic characteristics. Again their composition if highly variable, however, they all contain metabolites such as amino acids, peptides, proteins, polysaccharides, nucleotides, fatty acids and various vitamins and minerals which are products of yeast autolysis. Furthermore, these yeast autolysate products will vary depending on what yeast culture conditions, growth stage and inactivation method have been employed for their manufacture.[55] The following sections provide further insight on how these products promote such effects.

4.4.1 Effects on Fermentations.

Protection during active dry yeast rehydration: Yeast rehydration of active dry yeast is a critical step in the winemaking process as it prepares the yeast to enter the hostile must environment that has high sugar levels, low pH and added SO_2. Specific vitamins, sterols

and polyunsaturated fatty acids that are extracted from specific inactivated dry yeasts that are enriched in these compounds may be employed for this purpose. Such products are believed to protect and stimulate active dry yeast by stimulating the reactivation of the yeast metabolism and reinforcing the cell membrane at the rehydration stage as it provides OptiMUM-White® levels of micronutrients to be assimilated.[71] At the end of fermentation, the yeast cell membrane is rich in sterols and polyunsaturated fatty acids and therefore able to better resist the high levels of ethanol present at the end of fermentation. Scientifically Dulau et al., showed that rehydration of active dry yeast in the presence of IDY preparations (100 - 200 g/L) increases the fermentation rate with a concomitant decrease in duration.[180] This increase in rate has been further explained by Soubeyrand et al., who showed that the IDY release fragments of yeast cell walls into the rehydration medium that are able to form micelle-like particles that act as bioemulsifiers decreasing the total surface tension of water and solubilises a fraction of the sterols. The incorporation of the micelles into active dry yeasts during the rehydration stage could repair damaged cellular membranes thus increasing overall fermentation rate.[181]

Alcoholic fermentation enhancers: *Saccharomyces* yeasts require certain nitrogen sources such as the ammonium ion and α-amino acids for complete and steady fermentation. Inactivated yeast contain not only these nitrogen sources but also provide other vitamins and minerals to help maintain the integrity and fluidity of the membrane. Low levels of these compounds result in lower fermentation rates and are considered one of the main causes of stuck ferments.[182] In order to avoid such problems, yeast manufacturers have recently started recommending the use of fermentation enhancers based on inactivated dry yeast components. Most of them include a soluble fraction formed by yeast cytoplasm metabolites (proteins, peptides, amino acids, polysaccharides, mannoproteins, sterols and fatty acids) and an insoluble fraction of inactive support which is mainly cellulose.[71] A study by Feuillat and Guerreau utilised four different IDY preparations of different composition as fermentation enhancers in a synthetic medium.[183] All of them showed an improvement in the rate of alcoholic fermentation with a concomitant increase in viable yeast numbers at a dosage rate of 500 mg/L. However, their mechanisms of action were different depending on their composition. For example, the effect of the IDY in mediums rich in assimilable nitrogen was minor whilst their effects in mediums with poor levels of assimilable nitrogen was greatest. Unfortunately,

there is still a lack of literature available that details the effects of these IDY fermentation enhancers in real winemaking conditions.

Malolactic fermentation enhancers: In recent years, the use of IDY as malolactic fermentation enhancers has become very popular. It is often the case that at the end of alcoholic fermentation the wine is depleted of essential nutrients necessary for the successful onset of malolactic fermentation. Moreover, the low turbidity, high alcohol, low pH and high SO_2 content of the wine may also hinder successful MLF. Consequently, specific IDY preparations have been developed to aid MLF. The addition of yeast walls (0.2 g/L) to wine has been shown to stimulate LAB development during MLF or to aid in detoxification since yeast ghosts can adsorb toxic fatty acids.[184] Furthermore, it has been clearly shown that yeast cell wall polysaccharides and proteins and other yeast components such as lipids, vitamins and nucleotides have a stimulating effect on MLF.[71] Given that all these components are now appearing in the commercial IDY preparations it a logical conclusion to draw that such IDY will also enhance MLF although reported scientific studies in real wine systems are still rare. One such study that stands out is that which has been conducted recently by Andújar-Ortiz et al.[185] They studied the role of specific components from six commercial IDY winemaking preparations on the growth of three types of wine LAB. Their results revealed that the extracts that exhibited the most different activity were chemically characterised in amino acids, free monosaccharides, polysaccharides, fatty acids and volatile compounds. In general, specific amino acids and monosaccharides were related to a stimulating effect on LAB whereas fatty acid composition and even some volatiles appeared to display an inhibitory effect on LAB growth. Such information is deemed extremely useful in trying to develop better and more specific IDY formulations for winemakers.

4.4.2 Effects of IDY on Volatile Aroma Compounds.

The ability of IDY derivatives to affect wine aroma perception is related to several aspects. On one hand yeast walls and macromolecules are able to bind volatile compounds in wine-like solutions with the intensity of these interactions being higher for hydrophobic molecules. On the other hand they are also able to release volatile compounds (e.g. cheese-like) into the wines medium.[167] According to Comuzzo et al., the

balance between these opposing trends appeared to be connected to the dosage; low amounts (200 mg/L) increased the volatility of some esters, giving more flowery and fruity notes to the wine while larger amounts (1,000 mg/L) increased fatty acid content leading to yeasty, herbaceous and cheese-like smells.[186]

Another recent study conducted in model wine solutions was designed to compare six commercial IDY preparations for their ability to release soluble compounds and their capacity to interact with seven relevant wine aroma compounds, altering their volatility and perception.[70] Important differences in soluble compounds released into the model wines medium by the IDY were found with free amino acids being among the most released. Moreover, the volatility of most of the aroma compounds was affected by the added IDY preparations at a concentration usually employed during winemaking (0.4 g/L). The extent of this effect was dependent on the physiochemical properties of the aroma compounds and on the length of IDY exposure. Shorter contact times of a few days promoted a salting-out effect whilst longer times of two weeks resulted in a retention effect with a consequent reduction of volatile aroma compounds in the headspace. The different IDY preparations also resulted in different abilities to adsorb these volatiles and is presumably a result of their method of manufacture.[70]

During the manufacture of IDY preparations, yeast components may be subjected to thermal reactions responsible for the production of a large number of aroma compounds. This was the subject of a study by Pozo-Bayón et al., that studied the release of volatile compounds that are originally present in the IDY preparations into a wines medium over time.[179] Some 35 volatile compounds were identified to be released with most of them being heterocyclic-containing nitrogen compounds. Furthermore, it was found that a range of substituted pyrazines increased in their concentrations after 13 days of IDY addition to synthetic wine medium and is believed to be the consequence of Maillard reactions occurring during the preparation of the IDY.

A very recent elegant study by Comuzzo et al., has confirmed that commercial yeast autolysates bind certain volatile compounds in both model wine and a white table wine. Studies on the interaction of certain volatile compounds with yeast walls and released colloids by GC/MS-SPME on a model wine containing a commercial yeast autolysate and five typical wine aroma compounds (ethyl octanoate, linalool, 2-phenylethanol, β-ionone

and octanoic acid) were compared to those of a white table wine also containing the IDY and being spiked with the five volatile chemical constituents.[187] The results confirmed that yeast walls mainly bind less polar molecules with their loss increasing at higher pH values in the synthetic medium. Moreover, temperature and pH affected differently the interactions between yeast colloids and volatile compounds in the wine and model solution.[187] It is clear that more studies are needed to further investigate the types of volatile compounds that will bind to IDY and to further deduce their mode of action on how they will influence the overall aroma of a wine.

4.4.3 Polysaccharides from IDY – Their Effect on Red Wine Colour and Astringency.

It is well known that colour stability of red wines may be favoured by the enrichment of the medium in polysaccharides.[71] The polysaccharides act as protective colloids since they interact with the tannins and anthocyanins, preventing their precipitation which leads to an increase in colour stability.[74] Consequently, IDY formulations rich in polysaccharides may aid in red wine stability. Indeed, it has been found that a wine supplemented with various IDY preparations may lead to a significant increase in polysaccharides in the range of 11 - 20%.[188] Besides a few scientific reports that have recently appeared in the literature, the companies themselves have been conducting research for a number of years in this area and provide some examples of colour stabilisation in their technical notes. For example, Lallemand has one product sold as a Booster Rouge which may be applied to a red wine at the beginning of fermentation and leads to an increased content of high molecular weight polysaccharides which form stable complexes with anthocyanins and tannins, *Figure 4.2*. The results suggest that more stable polyphenols are formed, resulting in wines that have a better colour, increased volume and lower astringency.[189]

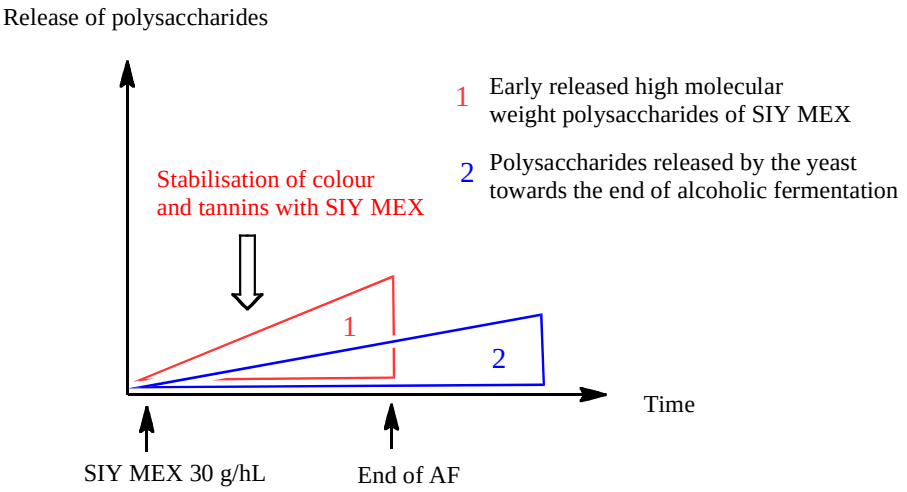


Figure 4.2. Schematic representation of the advantage of adding Booster Rouge at the beginning of alcoholic fermentation.

Whilst early reports appear to suggest that colour stability may be improved by the addition of IDY preparations there are also a number of recent reports that show that there in no improvement in colour stability. A recent study by Rodrigues et al., examined the effects that three commercial mannoprotein enriched preparations have on wine colour stability.[190] It was found that the evolution of colour through time was similar for all IDY supplemented wines suggesting there was no influence on colour stability. Such findings have also been found in several other recent studies.[191,192] Interestingly, a recent study by Fernández et al., found that the presence of lysated lees (with acid treatment) during wine storage in barrels induced greater mannoprotein and glucan release and resulted in wines with more intense colours, increases in sweetness, fullness and mouth length.[166]

There are also some IDY preparations specifically formulated to increase body and mouthfeel of wines and to reduce their astringency. Base on the same mechanism as described above, polysaccharides released by IDY can decrease the aggregation of proanthocyanidins, thus reducing astringency.[71] A recent study by Guadalupe in which mannoprotein enriched IDY preparations were added to a Tempranillo wine supports such conclusions.[191] These current and somewhat contradictory findings suggest that further scientific work is necessary to better understand the relationship between the release of polysaccharides from IDY preparations and their role on colour stability and astringency.

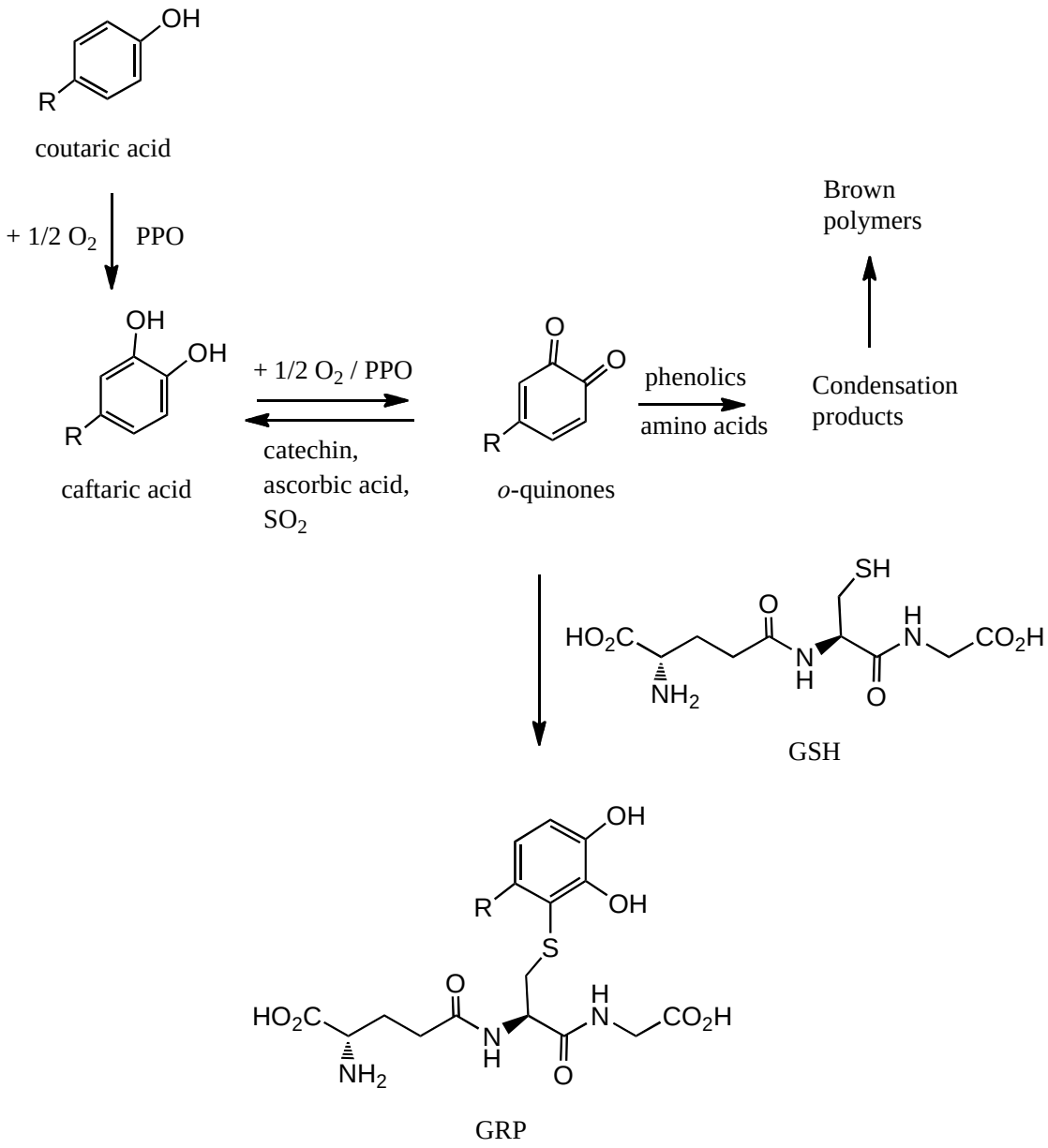
4.4.4 Other Effects of IDY on a Wines Organoleptic Properties.

It is important to remember that the addition of IDY to wines may have some unexpected or undesired effects on the sensory characteristics of wines. Red wine colour may be modified due to the capacity of yeast walls to bind acyl derivatives of anthocyanins.[71] Short chain fatty acids have a great influence on wine aroma, however, they are also know to inhibit alcoholic fermentation. Consequently, IDY which are known to bind and remove such fatty acids will influence overall sensory characteristics.[71] It has also been found that certain aroma precursors such as the glycoconjugated terpenes can interact with yeast walls which could have important consequences on the varietal characteristics of wines supplemented with IDY preparations.[193] Volatile phenols which are responsible for certain wine off-flavours are also believed to be adsorbed by IDY walls. Moreover, depending on the type of wine and its sensory characteristics, the addition of IDY preparations is expected to have a smaller effect on very strong varietal wines but a much greater ability to enhance the aroma of very weakly aromatic wines.[71] Finally, a heat shock protein (Hsp12) has recently been identified from wine yeasts (*saccharomyces cerevisae)* and found to contribute to the sweetness of dry wines.[194] This protein is a 109 residue protein (120kDa) located at the yeast plasma membrane and protects membranes against desiccation and ethanol-induced stress. Hsp12 is essential for the biofilm or 'flor' formation of sherry wines which are another traditional wine type aged on lees.[195] Hsp12 has been isolated from yeast autolysates and sensory studies indicate that it adds sweetness to dry wines.[196]

4.4.5 IDY Rich in Glutathione and Oxidation of White Wines.

Glutathione (GSH) is a tripeptide that has an extremely low redox potential and is an excellent antioxidant that is able to react with quinones to form stable colourless compounds that limit browning in musts and wines.[197] It is now well documented that *o*-diphenols are responsible for oxidative browning of wine.[198] The initiation begins with the conversion of the *o*-catechols (e.g. caftaric or coutaric acids) into *o*-quinones and may be initiated by non-enzymic or enzymic (e.g. PPO) processes. At this stage the *o*-quinones react further with phenolics or amino acids to produce condensation products, which ultimately form brown polymers, *Scheme 4.1*. Given that the *o*-quinones are oxidants themselves they will react with phenols such as catechin, ascorbic acid and SO_2 reverting

back to their initial *o*-diphenols, thus the must or wine can be somewhat protected if it contains high levels of these substrates, *Scheme 4.1*.[198]



Scheme 4.1. Browning in grape must and wines and the effect of GSH and the formation of GRP.

However, perhaps more important is the reaction of the *o*-quinones with GSH to produce 2-*S*-gluthathionyl caftaric acids which are also known as grape reaction product (GRP).[198,71] GRP cannot be oxidised by catecholase and it will aid in limiting oxidative browning of white wines, *Scheme 4.1*. Moreover, the reaction of GSH with *o*-quinones to

form GRP helps conserve the stability of thiol precursors such as 3-MH, a volatile thiol well known for its positive contribution to the aroma of Sauvignon Blanc wines.

Because some wine yeasts are particularly efficient in synthesising and accumulating GSH, commercial companies have now developed processes for the production of GSH-enriched IDY preparations. For example, OptiMUM-White® and OptiWhite® which are specific inactivated dry yeasts with a high GSH content have been developed by Lallemand and can be added to the juice at the onset or at the end of fermentation. These IDY preparations increase the concentration of GSH in the wine and therefore protect the wine from degradation.[199] It should be noted that GSH accounts for approximately 1% of dry weight of *S. cerevisiae* and represents more than 95% of the low molecular weight thiol pool.[71] Finally, a very recent study by Kritzinger et al., developed a method for the determination of glutathione levels in model solutions and grape ferments supplemented with various GSH-IDY preparations.[200] Significant differences in the amount of GSH released into a model wine by different GSH-IDY were observed with ethanol concentration influencing the release under certain conditions. Moreover, it was found that the timing of GSH-IDY additions to grape juice fermentations was particularly important with elevated GSH levels found in wines where the additions were done early during alcoholic fermentation.[200]

4.5 Comparison of Ten Commercial IDY Preparations on a Chardonnay Wine after 1 Month of Exposure.

A total of ten common IDY preparations were chosen for a study on how they may alter certain attributes of a Chardonnay wine after one month of exposure. A summary of each of their reported properties as described within their technical notes is listed below and serves as a base of what might be expected in our studies. The spectrum of IDY formulations utilised includes some which are reported to aid in anti-oxidation and the inhibition of browning which is naturally an important aspect of the ageing potential of any Chardonnay white wine.

OptiMUM-White® is a natural yeast derivative rich in antioxidant properties and polysaccharides and enhances glutathione bioavailability and is recommended for white and rosé wines.[201] It is suggested it should be added after the settling of alcoholic

fermentation and is described as adding enhanced wine complexity and has a smoothing effect to bring more roundness to the wine. A research trial showed the positive effect of glutathione on the preservation of thiols such as 3-mercapto-hexanol (3MH) and its acetate (3MHA) in a Sauvignon Blanc wine. [201]

Opti-LEES has been developed from an oenological yeast strain selected for its autolytic characteristics and polysaccharide release.[202] It is recommended to be utilised during the maturation phase of a finished wine and results in an increase in the palate weight due to the rapid release of polysaccharides. It also results in an increased sweetness perception due to the release of low molecular weight polysaccharides. Several winery scale trials have been conducted with *Figure 4.3* showing a decrease in astringency and an increase in voluptuous descriptors after two months exposure to Opti-LEES.[202]

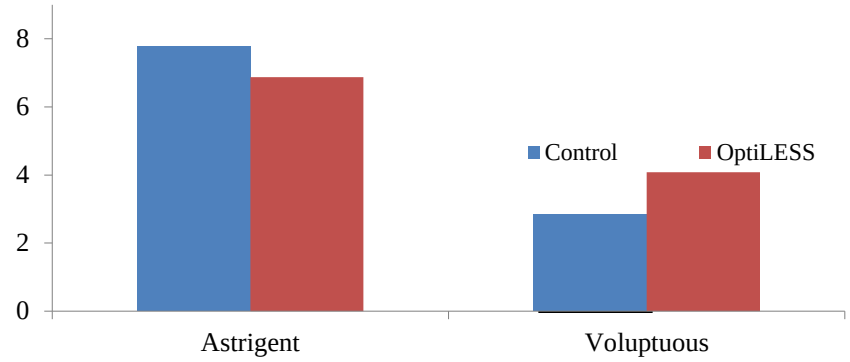


Figure 4.3. Sensory analysis of a trial of Tempranillo, DOC Ribera del Duero, 2010: Comparison of control and addition of Opti-LEES at 20 g/hL. Adapted from reference 202.

Booster Blanc® is also recommended for white and rosé wines and is an inactivated yeast which permits a rapid availability of the soluble fractions of yeast cell walls that leads to an increased mid-palate intensity.[203] It is also utilised for the production of wines with a decreased perception of woody aromas such as sap or sawdust when wines are aged in new barrels. Experimental results also show that it may be employed during botrytised grape fermentations by providing certain nutrients during primary fermentation.

Noblesse® helps modify and stabilise a wines colloidal balance and is comprised of specific inactivated yeast cells from an isolate selected from the ICV.[204] Reported benefits include an increased perception of ripe fruit, more intense structure of the wine, decreased perception of sap/sawdust and harsh, chemical and burning sensations and also stimulates malolactic fermentation.

Mannostab® is rich in a certain mannoprotein extracted from yeast cell walls and is naturally present in wines and has a potassium bitartrate stabilisation property.[205] Called MP40 , this macromolecule is an exciting new tool for inhibiting crystallisation of bitartrate salts in white, rose and red wines, *Figure 4.4.* A trial with a rose wine also indicated that the acid-base balance in conserved and their colour was not modified.[205] It is recommended that it be utilised as the last treatment before bottling.

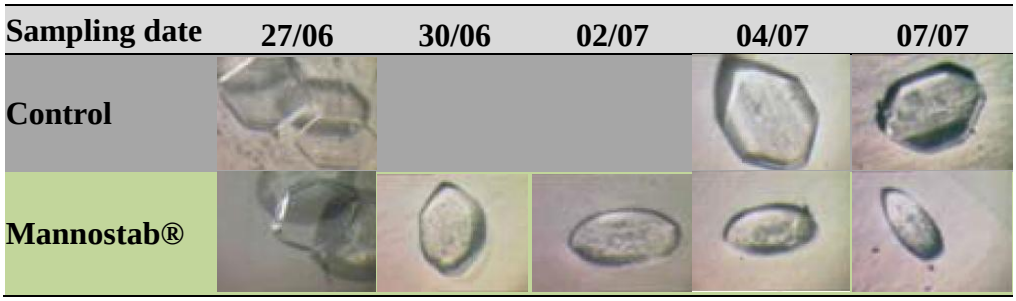


Figure 4.4. Microscopic observation of the development of potassium bitartrate crystals at -4 ^{o}C in a solution with and without Mannostab®. Adapted from reference 205.

Biolees® was developed from a research project, which involved a detailed understanding of the natural phenomena associated with ageing wine on lees. This IDY is a yeast cell wall extract rich in active peptides and polysaccharides and improves the smoothness of both white and red wines. Biolees® is 100% soluble, thus its reported action is immediate and can therefore be added during maturation or as a final treatment before bottling.[206]

Phylia Exel is also a fully soluble fraction obtained from a specific yeast which is rich in mannoproteins. Not only is it reported to decrease the sensation of acidity or harshness in white and rosé wines but it also rapidly softens tannins in red wines when compared to several months of lees ageing.[207] It also is reported to enhance the freshness and fruitiness of wines when added after fermentation.

Phylia Lift contains yeast hulls that slowly release mannoproteins and polysaccharides into a wine. It has been found to protect wines against reduction or oxidation as natural lees will do without the potential risk of mercaptan production.[208] It can be utilised at the end of vinification or at the beginning of ageing of red, white or rosé wines.

Genesis Prime is based on a selection of yeast hulls from a yeast strain that contains a high level of soluble cell wall mannoproteins and glycoproteins which are believed to decrease potential oxidation or reduction of a wine.[209] Moreover, it has been found to release more polysaccharides into a wines medium in a quicker time thus being able to mimic lengthy lees exposure times, *Figure 4.5*. It has also been noted for its ability to reduce sulfur off aromas as well.

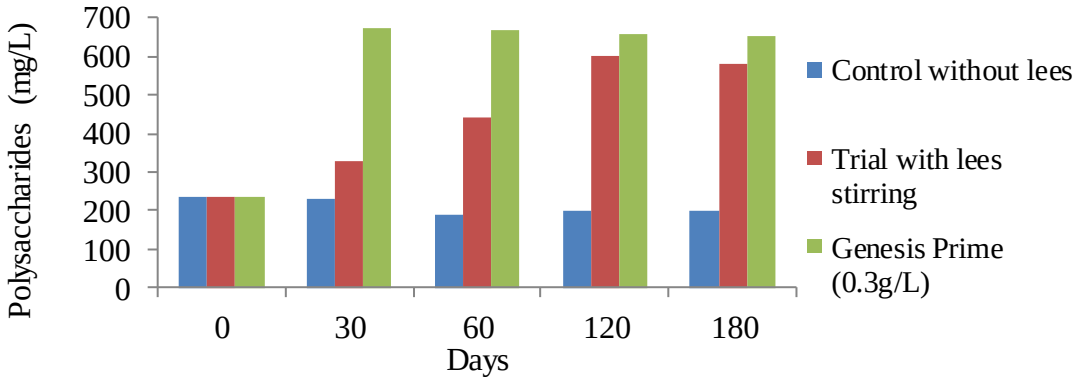


Figure 4.5. Comparison of the effect of Genesis Prime on total polysaccharides when compared to typical lees ageing. Adapted from reference 209.

Genesis Lift is similar to Genesis Prime in that it is also based on a selection of yeast hulls from a yeast strain that contains a high level of cell wall mannoproteins. The perceived benefits include improved colour stabilisation of red wines, improved aromatic complexity of white and rosé wines and an increased persistence and volume in the mouth.[210]

4.6 Preparation of the Chardonnay Wines with Added IDY Preparations.

With the 10 commercial IDY chosen for our studies, it was first necessary to prepare a Chardonnay base wine. The Chardonnay base wine was prepared with PDM (*Saccharomyces cerevisiae* var. *Bayanus*) as described within the experimental chapter and had the following final oenological parameters; pH of 3.30, TA of 0.9 mg/L, VA of 4.08 g/L, free SO_2 (16 ppm) and total SO_2 (174.4 ppm). A total of 10 Chardonnay wines with added IDY were prepared along with the control sample in triplicates. After completion of primary fermentation, the IDY were added to the wines at the maximum recommended levels as depicted in *Table 4.1*. The IDY powders were pre-dissolved or suspended in 5 mL of the base wine and then added into 1 L of the base wine with stirring. In total, 33 bottles (11 treatments x 3 triplicates) were kept in storage for 4 weeks at 15 ^{o}C and shaken once weekly to ensure mixing. After this time the wines were centrifuged with dry ice protection and the wine supernatant removed and bottled in half-bottles (375 mL) ready for analysis.

Table 4.1. The type and amount of IDY utilised in the base Chardonnay wines for analysis.

Abbreviations	IDY utilized	Adding Amount (g/L)
OW	OptiMUM-White®	0.5
OL	Opti-LEES	0.4
BB	Booster Blanc®	0.4
NL	Noblesse®	0.4
MS	Mannostab®	0.3
BL	Biolees®	0.4
PL	Phylia LF	0.3
PE	Phylia Exel	0.3
GP	Genesis Prime	0.2
GL	Genesis Lift	0.2
C	Control	0.0

It should be pointed out that all inactivated dry yeast preparations were mainly insoluble except Mannostab® and Phylia Exel which were totally soluble and Genesis Lift which was mostly soluble as shown in *Figure 4.6.* Naturally, those that have greater solubility

will alter the wines medium immediately while the effects of those that are insoluble will be slower.

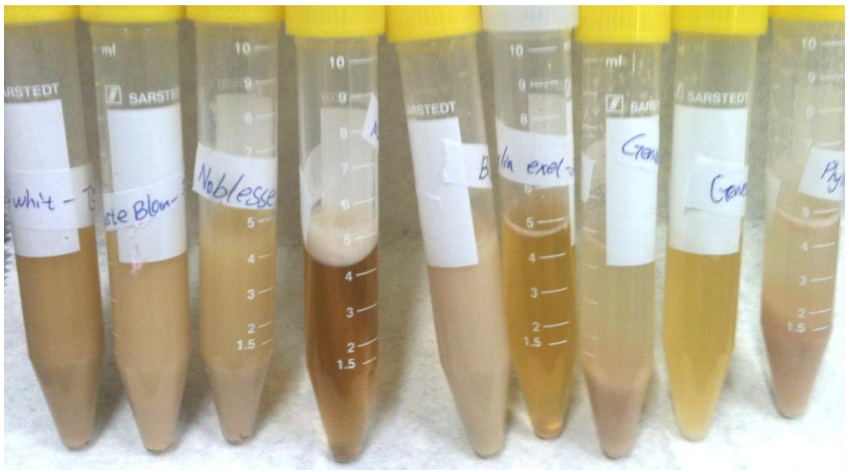


Figure 4.6. Base Chardonnay wine containing the IDY's utilised. From left to right: (OptiMUM-White®), (Opti-LEES, not shown due to delay in delivery, not soluble), (Booster Blanc®), (Noblesse®), (Mannostab®, soluble), (Biolees®), (Phylia Exel, soluble), (Genesis Prime), (Genesis Lift, soluble), (Phylia Lift).

4.7 Analysis of the Change in Polysaccharides, Total Soluble Proteins and Colour of the Chardonnay Wines with Added IDY.

Given that the majority of the commercial IDY preparations report an increase in polysaccharides and total soluble protein content over time or that they have the ability to aid in preventing oxidative effects such as browning we decided that we would first concentrate on measuring these three parameters for the ten IDY Chardonnay wines prepared.

The total polysaccharide content of the Chardonnay wines that had been exposed to the individual IDY preparations for 4 weeks were determined by the colorimetric method described by Segarra et al,. and compared to the control wine.[152] It was found that there were significant changes in the total polysaccharide levels in the Chardonnay wines after 4 weeks of exposure for all the IDY preparations utilised when compared to the control, *Figure 4.7*. This is not surprising as most of the technical sheets for these commercial IDY preparations report that they should release polysaccharides into a wine over time.

Even for those which do not (e.g. Booster Blanc® and Noblesse®) they do report an increase in mouthfeel volume which would be related to polysaccharide release. The increase in polysaccharide concentration was in the range of approximately 30 – 200 mg/L over the control, thus these IDY preparations are able to mimic natural lees autolysis in a much shorter timeframe. Importantly, whether the IDY preparations were initially totally soluble (for Mannostab®, Phylia Exel and Genesis Lift) or insoluble, little difference in the increase in total polysaccharide concentration was seen suggesting that polysaccharide release is facile into the wines medium for all these IDY preparations. Given that Mannostab® is actually a purified mannoprotein naturally present in wine coupled with the fact that it is totally soluble in the wine then it is not surprising that the highest elevated levels of total polysaccharides were observed for this particular trial.

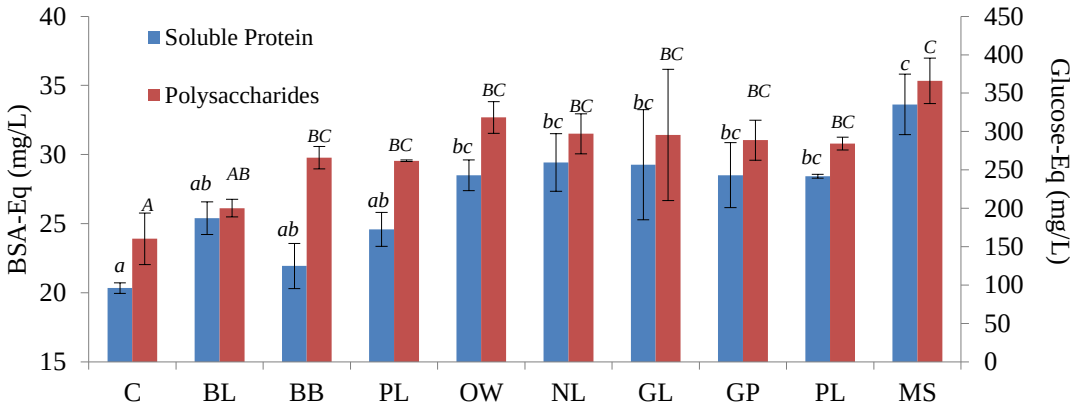


Figure 4.7. The increase in total polysaccharides and soluble protein upon exposure to various IDY preparations for 4 weeks in a Chardonnay wine. Note Phylia Exel was not included in this study as it was not available when the trials were conducted. *a* - *c*: data indicated by different number are significantly different in soluble proteins, and *A* - *C*: data indicated by different number are significantly different in polysaccharides, by Student-Newman-Keuls one-way ANOVA analysis ($P < 0.5$).

As highlighted above, it is well known that colour stability of red wines may be favoured by the enrichment of the medium in polysaccharides.[71] The polysaccharides act as protective colloids since they interact with the tannins and anthocyanins, preventing their precipitation which leads to an increase in colour stability.[74] Consequently, IDY formulations rich in polysaccharides may aid in red wine stability. Thus, whist our studies were conducted on a Chardonnay wine which would have low levels of flavonoids and

non-flavonoids and no anthocyanins, they may also be useful for colour stabilisation of red wines as indeed some of the IDY preparations have been reported to do.

The total soluble protein levels of the Chardonnay wines that had been exposed to the individual IDY preparations for 4 weeks were also determined by the Bradford method as described in Chapter five and compared to the control wine. It was found that there were significant changes in the total soluble protein levels in the Chardonnay wines after 4 weeks of exposure for all the IDY preparations utilised when compared to the control, *Figure 4.7*. This is not surprising as many of the technical sheets for these commercial IDY preparations report that they should release proteins into a wine over time given their methods of preparation. Even for those which do not (e.g. Opti-LEES, Biolees® and Noblesse®) they do report an increase in mouthfeel volume or palate weight, and smoothness which would be related to soluble protein release. The increase in total soluble protein concentration was in the range of approximately 2 – 13 mg/L (BSA equivalents) or 10 - 65% over the control, which had a base protein level of 20 mg/L. Thus these IDY preparations are able to mimic natural lees autolysis in a much shorter timeframe by providing an enrichment of the medium in soluble proteins. Importantly, whether the IDY preparations were initially soluble (for Mannostab®, Phylia Exel and Genesis Lift) or insoluble, little difference in the increase in total soluble protein concentration was seen suggesting that protein release is facile into the wines medium for all these IDY preparations. Given that Mannostab® is actually a purified mannoprotein naturally present in wine coupled with the fact that it is totally soluble in the wine then it is not surprising that the highest elevated levels of total soluble proteins were observed for this particular trial. Finally, *Figure 4.7* clearly indicates that the observed increases in polysaccharides levels also correlate very well with the observed increases in soluble protein levels, thus the origins of observed increases in a wines attributes such as mouthfeel, palate weight and smoothness when exposed to commercial IDY preparations may in fact be due to not just an increase in polysaccharides or soluble proteins alone but in actual fact be due to a concomitant increase in both autolytic constituents.

Given that a number of these commercial IDY preparations are reported to aid in preventing oxidative processes we next turned our attention to measuring the colour of the Chardonnay wines after four weeks of exposure to the IDY preparations. This was to be

done in two ways; the first being simply measuring the absorbance at A_{440} and the second by making CIE*lab* measurements over the range of 380 to 780 nm.

The browning of white wines and the development of brown hues is generally believed to be due to oxidative changes and/or polymerisation of phenolic compounds during wine ageing. A simple measure for the oxidative discolorisation of white wine is the absorbance of light at 420 nm and has often be employed as the industry standard.[211,212,213] There have been several recent studies looking at wine ageing and its relation to colour. In 2005 Skouroumounis *et al*. reported a five year trial looking at a Riesling and Chardonnay wine.[214,215] Both wines were bottled with and without the use of ascorbic acid and involved three different closures.[214,215] Major observations from this trial were that browning in wines did not correlated with absorbance at 420 nm with both wine types.[214,215] In fact, wines with ascorbic acid were found to have a higher absorbance at 420 nm then those without, but when the wines were difference tested visually by a panel, wines without ascorbic acid were considered browner / darker after 3 years of ageing.[215] What was interesting was within a subset of wines, the 420 nm and browning perception did correlate, which highlighted the fact that the absorbance at 420 nm is only a good indicator of ageing within the same wine type, without differing amounts of ascorbic acid.[215] These wines were subjected to partial least squares analysis with the rankings of panellist and the full visible spectrum suggesting that 480 and 500 nm are better values to consider for browning in these cases.[215]

While 480 and 500 nm may be better indicators of browning,[215] absorbance at 420 nm was strongly correlated with total sulphur dioxide depletion and the perceived oxidised character in the wines.[212] More recently it has been reported that bottles of a 1999 Semillon wine with greater 420 nm absorbance also exhibited low free and total SO_2.[29] Those with high 420 nm were rated by a sensory panel as possessing more intense oxidised aromas.[29] So as SO_2 is consumed, a wine develops these oxidative characteristics, and can be measured at 420 nm.

Such studies have highlighted the importance of what wavelength should be utilised to measure oxidative colourisation of white wine. More recently, Clark et al. showed that A_{440} is an excellent absorbance measurement for quantification of oxidative browning of

(+)-catechin in model wine solutions with and without additional phenolics and acids present.[216,154] The pigments responsible are yellow xanthylium cations or brown/red pigments formed from catechin and tartaric acid or other phenolics/acids present in the system. Given that our Chardonnay would typically contain a concentration of catechin in the range of 5 - 100 mg/L[218] we decided to adopt A_{440} as our indicator of browning/oxidation for our Chardonnay/IDY-exposed wines. See below for further discussions on the use of A_{440} as an indicator of oxidation of white wines. Moreover, Clark et al. 2008 also utilised CIE*lab* measurments to follow the impact of ascorbic acid on the oxidative colourisation and associated reactions of a model wine solution containing (+)-catechin, caffeic acid and iron.[154] CIE*lab* provides a more holistic view of perceived colour than a single absorbance measurement at a particular wavelength and as such we also utilised such measurements as an indicator of browning/oxidation for our Chardonnay/IDY-exposed wines

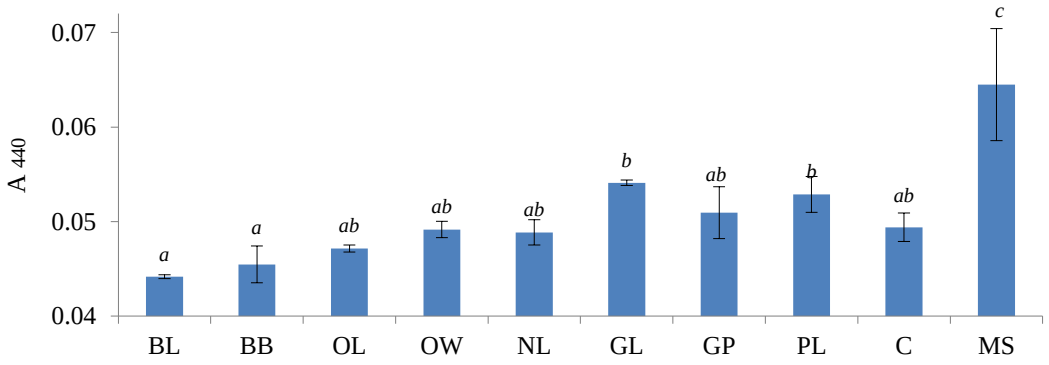


Figure 4.8. A_{440} values of the Chardonnay wines exposed to the IDY preparations for 4 weeks at 15 °C. Note Phylia Exel was not included in this study as it was not available when the trials were conducted. *a* - *c*: data indicated by different number are significantly different by Student-Newman-Keuls one-way ANOVA analysis ($P < 0.5$).

The A_{440} values of the Chardonnay wines exposed to the IDY preparations for 4 weeks at 15 °C were measured immediately after bottling, *Figure 4.8*. As can be seen from the data presented in *Figure 4.8* there appears to be little difference in the absorbance values at 440 nm of the IDY exposed Chardonnay wines when compared to the control except for the wine exposed to the IDY Mannostab®. Consequently, it appears that no substantial oxidation has occurred. This may, however, not be due to any protection provided by the

added IDY preparations but merely reflect that our Chardonnay base wine has sufficient free sulfur to avoid oxidation over the 4 week trial. Interestingly, Mannostab® which is a purified fraction of mannoproteins enzymatically extracted from yeast cell walls[205] and which would be devoid in oxidative protective components such as glutathione appears to have an elevated A_{440} value indicating some oxidation of the wine may have occurred. The reason for this observation is unclear at this stage.

In parallel, we also measured the CIE*lab* values of the Chardonnay wines after 4 weeks of IDY exposure, *Table 4.2*. The *l** parameter affords an indication of the intensity of colour from none or light (100) to maximum or dark (0), *a** gives an indication of red (+*a**) colourisation to green (-*a**) and *b** gives an indication of yellow (+*b**) colourisation to blue (-*b**). Previous studies on utilising CIE*lab* to follow the oxidation of catechin in a model wine system have indicated that a decrease in *l**, an increase in -*a** and an increase in +*b** is to be expected.[154] Inspection of the values within *Table 4.2* indicate that there appears to be little difference in the CIE*lab* values of the IDY exposed Chardonnay wines when compared to the control except for the wine exposed to the IDY Mannostab® in which a small difference was noted. Consequently, it appears that no substantial oxidation has occurred and substantiates the data found above.

Table 4.2. CIE*lab* values of the Chardonnay wines exposed to various IDY preparations for 4 weeks at 15 °C.

CIE*lab*	**C**	**BL**	**BB**	**OL**	**OW**
l*	98.96 ± 0.05	98.96 ± 0.03	98.74 ± 0.05	98.78 ± 0.03	98.77 ± 0.09
a*	-0.74 ± 0.03	-0.74 ± 0.03	-0.49 ± 0.06	-0.69 ± 0.02	-0.74 ± 0.08
b*	5.31 ± 0.13	5.20 ± 0.02	5.54 ± 0.05	5.29 ± 0.02	5.55 ± 0.02
CIE*lab*	**NL**	**GL**	**GP**	**PL**	**MS**
l*	98.72 ± 0.05	98.57 ± 0.02	98.60 ± 0.23	98.65 ± 0.15	98.00 ± 0.31
a*	-0.60 ± 0.04	-0.56 ± 0.04	-0.52 ± 0.10	-0.49 ± 0.10	-0.25 ± 0.10
b*	5.4 3 ± 0.14	5.85 ± 0.06	5.59 ± 0.07	5.74 ± 0.01	6.40 ± 0.38

Interestingly an informal assessment of the aroma of the Chardonnay wines that had been exposed to the various IDY preparations indicated substantial differences in aroma whilst little difference in colour was noted. In order to be able to compare the different IDY preparations for their ability to prevent oxidative degradation of a white wine it would perhaps be more pertinent to repeat the experiments with little of no sulfur dioxide

present, deliberately expose the wines to air or run the experiments for a greater period of time. Unfortunately, realising that these additional experiments were beyond the scope of this PhD we decided to simplify the system to be studied and utilise a model wine system to evaluate the 10 IDY preparations for their ability to prevent browning which is described in the forthcoming sections.

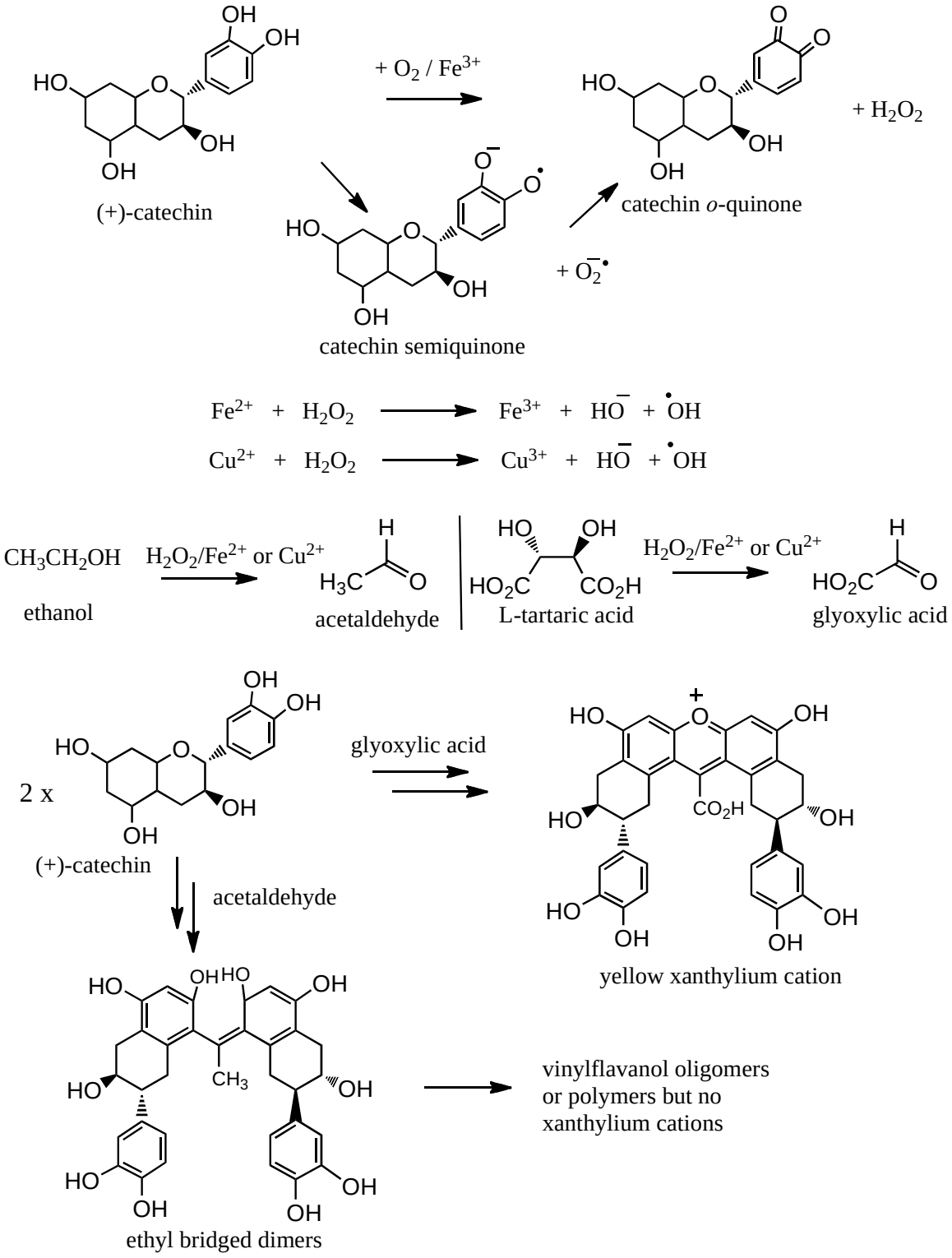
4.8 Preparation of the Model Wines with Added IDY Preparations.

Given that oxidative browning of white wines is a long standing problem in winemaking its not surprising to find that numerous scientific studies have been conducted in order to get a greater understanding of the chemistry of wine browning.[219] In particular, a range of model wine systems have been designed and extensively studied in order to gain insight into non-enzymic oxidation and the formation of the coloured pigments that are behind the appearance of the brown colourisation seen during wine spoilage and ageing. As highlighted above recent studies by Clark et al. have shown that employing (+)-catechin as the sole flavanol in a model wine system under various oxidative conditions results in the solutions turning a yellow colour which can be conveniently measured at an absorbance maximum of A_{440}.[154] The pigments responsible for the yellow colour are xanthylium cations formed from catechin and glyoxylic acid which is the oxidised product of tartaric acid that is employed in the model wine systems. Furthermore, the concentration of metal ions such as Fe(II) and Cu(II) at levels which are typically found in wine has been found to accelerate these autooxidation processes by increasing the rate of reaction between molecular oxygen and the catecholate functional moieties of respective phenolic compounds such as catechin.[216,217] Metal ions such as iron can also participate in Fenton type chemistry or via the Haber-Weiss reaction to hydroxyl radicals, hydrogen peroxide and superoxide all of which may accelerate these oxidative reactions.[219] Finally, the presence of glyoxylic acid or acetaldehyde which are oxidative products of tartaric acid and ethanol respectively in these model wine systems leads to the formation of various coloured vinylflavanol oligomers or xanthylium cations which have been implemented as key pigments behind the observed oxidative browning of white wine.[154,216,217,220]

Given these findings coupled with the fact that we wished to utilise a simplified model wine system to evaluate the 10 IDY preparations for their ability to prevent browning we decided to prepare a browning model wine system which contained (+)-catechin, iron(II), copper(II), and acetaldehyde along with the model wine itself which was 12% v/v ethanol/water buffered with tartaric acid. The pH of this wine-like solution was 3.4. Both the Fe^{2+} and Cu^{2+} concentrations employed (8 mg/L and 0.1 mg/L respectively) are typical of those levels utilised in previous browning studies and are also at levels commonly found in finished wines.[154,217] To this browning model wine system was added the commercial IDY preparations at the recommended dosage as depicted in *Table 4.1* and the samples warmed at 35 ^{o}C for up to 2 weeks after which time their absorbance at A_{440} was again measured to evaluate the extent of yellow/brown colourisation. The model browning model wine systems were also protected from light as this has been found to also contribute to browning of wines and the samples were also prepared under a blanket of CO_2 to limit oxygen exposure. Interestingly, when conducting accelerated browning studies, whether the samples are exposed to excess oxygen via aeration or protected in some way has been found to make no difference in the observed rate of oxidative browning.[216]

Scheme 4.2 highlights the key reactions for this browning model wine system. As (+)-catechin was employed as the sole flavanol it may undergo oxidation in the presence of oxygen and metal ions such as Fe(II) or Cu(II) to form catechin *o*-quinone and hydrogen peroxide.[219] Whilst the samples were protected with a blanket of CO_2 when they were prepared and deliberate aeration was not employed during the experiments, there will still be traces of oxygen present which may participate in this initiation process. The hydrogen peroxide thus formed may also react with these transition metals to produce additional oxidative species such as hydroxyl radicals as depicted in *Scheme 4.2*. These oxidative reactions have been shown to be able to oxidise ethanol to acetaldehyde or tartaric acid to glyoxylic acid. At this stage either the acetaldehyde added to the model

wine browning system or the acetaldehyde produced under the oxidative conditions may react with the catechin to produce coloured vinylflavanol oligomers, which is an indication of browning. Furthermore, any glyoxylic acid formed under the oxidative conditions (accelerated by iron(II) and Cu(II)) may also react with the catechin to form the well known yellow xanthylium cations which are again an indication of browning. The formation of the yellow xanthylium cations is actually through reaction of the A-ring (phloroglucinol-type moiety) of catechin and differs from the typical oxidative mechanism proposed for the formation of brown pigments formed in the presence of PPO or oxygen as highlighted in *Scheme 4.1* above. Alternatively, studies by Clark et al., have shown the importance of the semiquinone as an intermediate in oxidative browning, *Figure 4.2*.[154] This semiquinone which is an intermediate for the formation of the *o*-quinone may be formed by the presence of trace amounts of oxygen or other oxidative species such as hydroxyl radicals or transition metals. It is also important to highlight that ethanol itself will aid to inhibit oxidation as it reacts readily with hydroxyl radicals mopping them up from the system.[154] This however does also lead to further production of acetaldehyde which is able to trap the catechin and result in the formation of various bridged vinylflavanol oligomers which may be coloured or non-coloured. Thus, the browning model wine system employed here has all the key constituents present to cause accelerated oxidative browning at 35 ^{o}C and will allow us to examine the abilities of the IDY preparations to prevent or inhibit browning in a short timeframe by simply monitoring the maximum absorbance at A_{440} over time. Finally, given that the formation of the vinylflavanol oligomers or the yellow xanthylium cations does not require the formation of the *o*-quinone, whether the IDY preparations release GSH or not and aid in the inhibition of browning of these solutions is not directly being tested as described in *Figure 4.1* above.



Scheme 4.2 The key non-enzymic oxidation reactions and the formation of acetaldehyde-mediated and glyoxylic-medicated polymerisation of (+)-catechin in our model wine system.

4.9 Results of the Browning Inhibition Studies for the Ten Commercial IDY Preparations in Model Wine.

The maximum absorbance at A_{440} was measured for all samples of the model wine solutions containing the various IDY preparations after being heated at 35 ^{o}C for 14 days and was found to be essentially zero, thus the IDY preparations do not result in a significant colourisation of the model wines solutions even after being heated at 35 ^{o}C for 14 days. *Figure 4.9* depicts these solutions with the control in the center of the picture.

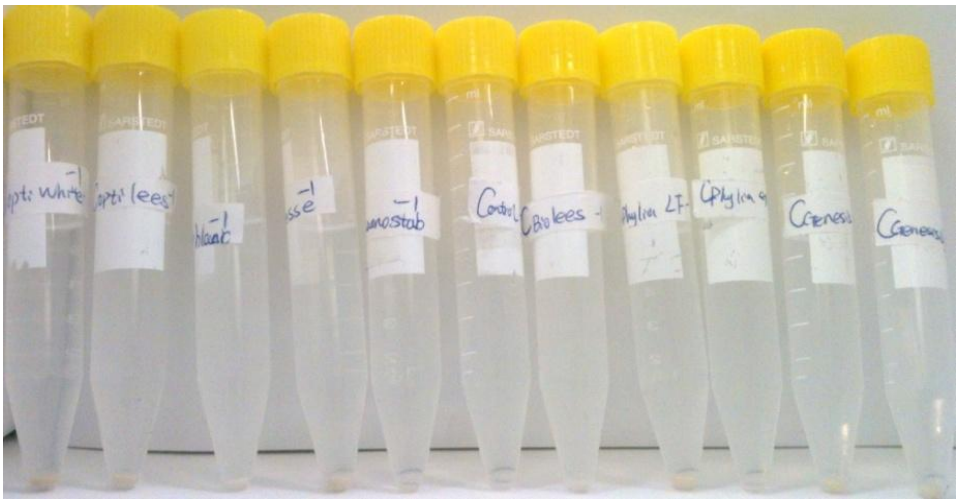


Figure 4.9. Samples of the model wine solutions containing the various IDY preparations after being heated at 35 ^{o}C for 14 days.

The maximum absorbance at A_{440} was then measured for all samples of the browning model wine solutions containing the various IDY preparations, catechin, Fe^{2+}, Cu^{2+} and acetaldehyde after being heated at 35 ^{o}C for 0, 2, 6, 10 and 14 days with the results depicted in *Figure 4.11*. *Figure 4.10* depicts these solutions after 14 days of heating with the control in the center of the picture and it can be clearly seen that some of the IDY preparations retard the browning of these solutions whilst others result in significant browning and appear to provide no resistance to browning when compared to the control.

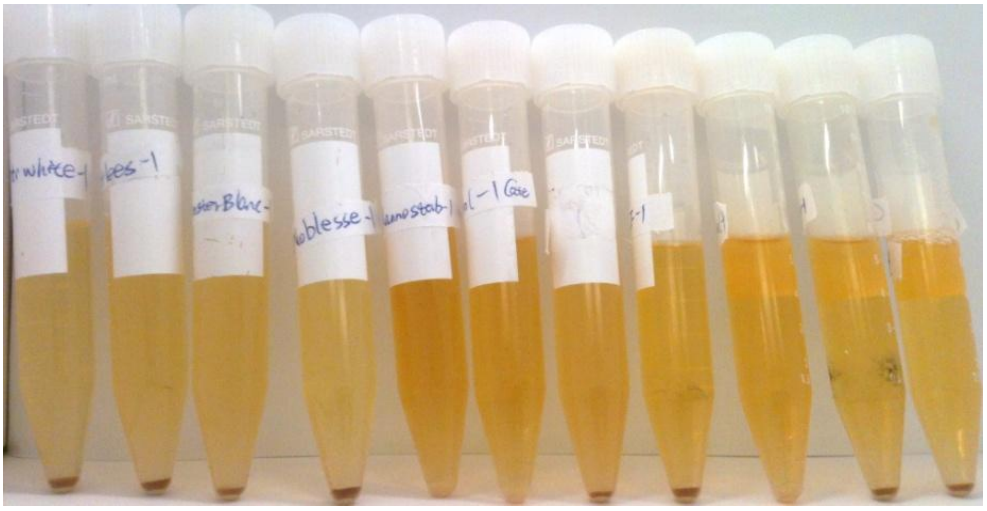


Figure 4.10. Samples of the browning inhibition solutions containing the various IDY preparations after being heated at 35 ^{o}C for 14 days.

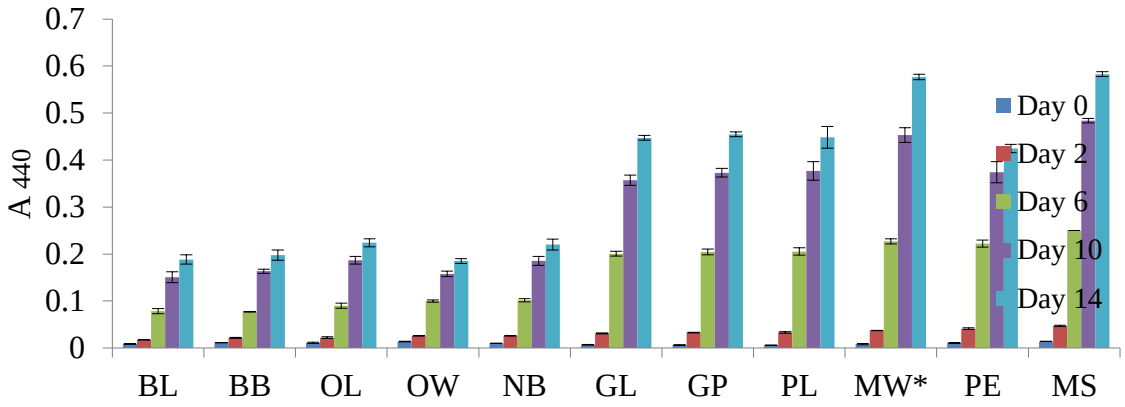


Figure 4.11. Maximum absorbance measurements at A_{440} of the browning inhibition solutions containing the various IDY preparations after being heated at 35 ^{o}C for 0, 2, 6, 10 and 14 days. MW*: catechin in a model wine solution.

Inspection of the results depicted in *Figure 4.11* indicates that all model wine solutions containing the various IDY preparations undergo an increasing in yellowing/browning with time as indicated by their A_{440} absorbance readings. More importantly it appears that the IDY preparations fall into three categories: those which significantly inhibit browning (Biolees, Booster Blanc®, Opti-LEES, OptiMUM-White® and Noblesse®); those which provide s slight protection from browning (Genesis Lift, Genesis Prime, Phylia Lift and Phylia Exel) and one (Mannostab®) which provides no protection when compared to the control. Comparison of the results found here with the stated benefits described within the

technical sheets for these various IDY preparations as highlighted above indicates that for the majority of these IDY preparations it appears that these are new findings that are yet to be pointed out in the technical sheets.

It should also be pointed out that other popular browning indexes namely A_{500} and A_{420} were also measured and gave similar results although the absorbance intensities were lower (data not shown). Thus, this latter finding also supports our decision to utilise A_{440} as a convenient indication of the ability of the IDY preparations to inhibit the browning of the catechin model solutions.

In parallel, we also measured the CIE*lab* values of the browning model wines over the course of the 14 days, *Table 4.12*. As previously discussed, the *l** parameter affords an indication of the intensity of colour from none or light (100) to maximum or dark (0), *a** gives an indication of red (+*a**) colourisation to green (-*a**) and *b** gives an indication of yellow (+*b**) colourisation to blue (-*b**). Previous studies on utilising CIE*lab* to follow the oxidation of catechin in a model wine system have indicated that a decrease in *l**, an increase in –*a** and an increase in +*b** is to be expected.[154] Inspection of the values within *Table 4.12* and comparison of the data above in *Figure 4.11* indicates that the observed changes as measured by CIE*lab* and A_{440} correlate extremely well with the IDY preparations again falling into three categories: those which significantly inhibit browning (Biolees®, Booster Blanc®, Opti-LEES, OptiMUM-White® and Noblesse®) as indicated by an *l** parameter around 95-96 and a +*b** value in the range of 18 - 22 after 14 days; those which provide s slight protection from browning (Genesis Lift, Genesis Prime, Phylia Lift and Phylia Exel) as indicated by an *l** parameter around 90 - 91 and a +*b** value in the range of 37 - 39 after 14 days and one (Mannostab®) which provides no protection when compared to the control (*l** parameter around 88 and a +*b** value around 47 after 14 days), *Table 4.12* Furthermore as found in the study by Clark 2008, the –*a** values begin as slightly negative and increase in magnitude early on and then become positive by the completion of the experiment indicating a redness of the final solutions.[154] These findings fit well with the visual finding depicted in *Figure 4.10* which clearly shows that some of the browning model wine solutions become intensely yellow with hints of brown/redness.

Table 4.3. CIE*lab* values of catechin solution with IDY added at recommended concentrations during storage.

Day	CIE*lab*	BL	BB	OL	OW	NL	GL
0	***l****	99.67 ^{a}	99.64 ^{a}	99.66 ^{a}	99.69 ^{a}	99.79 ^{ab}	99.97 ^{c}
	a*	-0.64 ^{c}	-0.68 ^{bc}	-0.67 ^{bc}	-0.73 ^{ab}	-0.70 ^{bc}	-0.65 ^{bc}
	b*	1.57 ^{a}	1.81 ^{c}	1.86 ^{c}	2.26 ^{e}	1.85 ^{c}	1.69 ^{b}
2	***l****	99.65 ^{d}	99.39 ^{bc}	99.57 ^{cd}	99.35 ^{bc}	99.45 ^{bcd}	99.46 ^{bcd}
	a*	-0.54 ^{b}	-0.82 ^{b}	-0.85 ^{b}	-0.82 ^{b}	-0.84 ^{b}	-0.88 ^{b}
	b*	2.61 ^{a}	2.91 ^{b}	3.20 ^{c}	3.40 ^{d}	3.42 ^{d}	4.22 ^{e}
6	***l****	98.68 ^{e}	98.64 ^{e}	97.98 ^{d}	97.87 ^{d}	98.22 ^{d}	96.10 ^{c}
	a*	-0.66 ^{ab}	-0.76 ^{a}	-0.61 ^{bc}	-0.50 ^{c}	-0.53 ^{c}	0.79 ^{d}
	b*	9.01 ^{a}	8.68 ^{a}	9.80 ^{ab}	10.47 ^{bc}	11.16 ^{c}	20.12 ^{d}
10	***l****	97.22 ^{d}	96.74 ^{cd}	96.03 ^{d}	96.89 ^{cd}	96.56 ^{de}	92.80 ^{c}
	a*	0.06 ^{a}	0.25 ^{a}	0.41 ^{a}	0.24 ^{a}	0.21 ^{a}	3.69 ^{b}
	b*	15.92 ^{a}	16.55 ^{ab}	18.42 ^{bc}	16.50 ^{ab}	19.13 ^{c}	32.69 ^{d}
14	***l****	96.20 ^{e}	96.14 ^{e}	95.37 ^{d}	96.17 ^{e}	95.59 ^{d}	90.89 ^{c}
	a*	0.70 ^{a}	0.88 ^{a}	1.15 ^{b}	0.79 ^{a}	1.27 ^{b}	5.63 ^{e}
	b*	18.78 ^{a}	19.76 ^{a}	21.81 ^{b}	18.50 ^{a}	21.61 ^{b}	38.90 ^{d}
Day	**CIE*lab***	**GP**	**PL**	**C**	**PE**	**MS**	
0	***l****	100.02 ^{c}	100.03 ^{c}	99.84 ^{ab}	99.95 ^{bc}	99.78 ^{a}	
	a*	-0.66 ^{bc}	-0.68 ^{bc}	-0.70 ^{bc}	-0.81 ^{a}	-0.73 ^{ab}	
	b*	1.70 ^{b}	1.79 ^{c}	1.81 ^{c}	2.21 ^{d}	2.40 ^{f}	
2	***l****	99.46 ^{bcd}	99.40 ^{bc}	99.35 ^{bc}	99.26 ^{b}	98.95 ^{a}	
	a*	-0.91 ^{b}	-0.85 ^{b}	-0.99 ^{a}	-0.99 ^{a}	-0.84 ^{b}	
	b*	4.29 ^{e}	4.35 ^{e}	4.77 ^{f}	5.19 ^{g}	5.46 ^{h}	
6	***l****	96.05 ^{c}	96.13 ^{c}	95.68 ^{b}	95.61 ^{b}	94.82 ^{a}	
	a*	0.79 ^{d}	0.64 ^{d}	0.81 ^{d}	0.99 ^{e}	1.56 ^{f}	
	b*	20.39	20.02 ^{d}	22.33 ^{e}	21.85 ^{e}	23.88 ^{f}	
10	***l****	92.57 ^{c}	92.62 ^{c}	91.04 ^{b}	92.10 ^{c}	89.95 ^{a}	
	a*	3.85 ^{b}	5.60 ^{cd}	5.01 ^{c}	3.97 ^{b}	6.32 ^{d}	
	b*	33.66 ^{d}	34.11 ^{d}	39.51 ^{e}	33.52 ^{d}	40.95 ^{e}	
14	***l****	90.76 ^{c}	91.16 ^{c}	88.63 ^{b}	90.94 ^{c}	87.70 ^{a}	
	a*	5.62 ^{e}	5.03 ^{c}	7.74 ^{f}	5.25 ^{d}	8.93 ^{g}	
	b*	39.34 ^{d}	39.17 ^{d}	47.36 ^{e}	37.06 ^{c}	47.18 ^{e}	

All values are the average of triplicate measurements for all samples. *a* - *g*: Values are significantly different by Student-Newman-Keuls one-way ANOVA ($P < 0.05$) from other values within a row as indicated by the superscript letter.

Given these exciting findings on the abilities of the ten commercial IDY preparations to inhibit oxidative browning of a model wine we examined the levels of several other constituents in order to probe possibilities of how these IDY preparations mechanistically may provide this oxidative protection. Consequently, as we found that the IDY formulations were able to release soluble proteins into the Chardonnay wine detailed above, we also measured the amount of soluble protein released into the model wine solution over time, *Figure 4.12*. It should be noted that the IDY preparations were utilised as a 10-fold excess in these experiments when compared to those utilised in the oxidative browning experiments to ensure a significant amount of protein would be released and aids in quantification. The control system (model wine only) returned a soluble protein level of zero as expected.

Inspection of the data in *Figure 4.12* indicates that the IDY preparations fall into three categories: those which significantly release soluble proteins over time (Biolees®, Booster Blanc®, Opti-LEES, OptiMUM-White® and Noblesse®); those which provide only a small level of soluble proteins into the solution and which do no increase over time (Genesis Lift, Genesis Prime, Phylia Lift and Phylia Exel) and one (Mannostab®) which provides a moderate level of soluble protein and again does not increase over time when compared to the control. Interestingly, this trend in soluble protein levels parallels the results found above for the changes in colour associated with oxidative browning. Whilst such a correlation has yet to be seen and reported it appears to suggest that the amount of soluble proteins released by the IDY preparations may aid in inhibiting oxidative browning. Whilst the exact mechanism of how this protection may arise needs further exploration, one possibility is that some of the proteins released have thiol type moieties which would be natural scavengers of oxidative species such as hydrogen peroxide and superoxide thus may aid in oxidative protection.

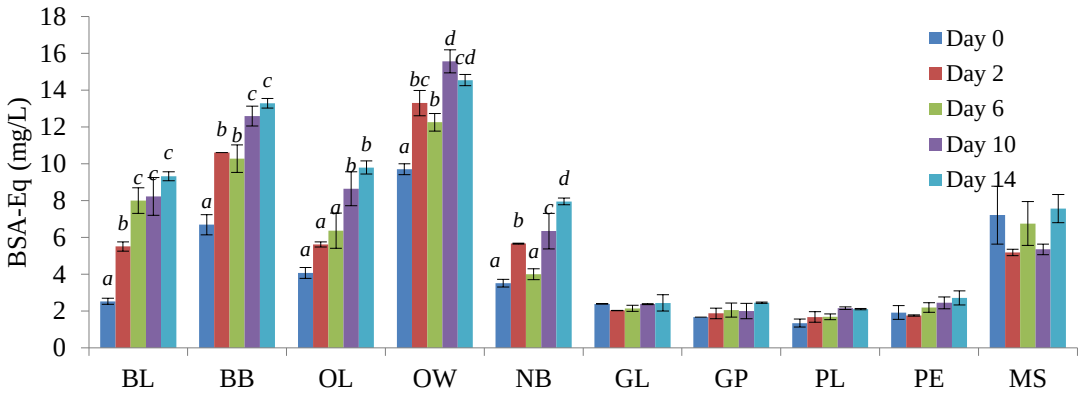


Figure 4.12. The increase in total soluble protein upon exposure to various IDY preparations for 14 days in the browning model wine solutions. *a* - *d*: data indicated by different number are significantly different within each IDY from different time points, by Student-Newman-Keuls one-way ANOVA analysis ($P < 0.5$).

Given that the commercial IDY preparations clearly demonstrated different abilities to protent against oxidative browning in the browning studies detailed above we also examined the overall antioxidant activities of the IDY solutions to investage if there was any correlation between their antioxidant activities and their abilities to prevent browning due to the release of certain chemical antioxidant constituents such as GSH into the medium. Thus, the antioxidant activities of the model wine solutions after being exposed to the various IDY preparations were determined by the well know ferric reducing ability of plasma assay (FRAP) as reported by Benzie and Strain.[221] *Figure 4.13* displays the overall antioxidant capacities of the model wine solutions and comparison with the data in *Figure 4.13* reveals that there appears to be no clear correlation between the antioxidant activities of the IDY exposed model wine solutions and their abilities to prevent oxidative browning except for Booster Blanc® and OptiMUM-White® which display the highest levels of anti-oxidant activity and they also have the highest levels of free GSH (see below) indicating that these two IDY preparations may gain some of their antioxidant capacity to prevent browning from the rapid release of GSH into the wines medium.

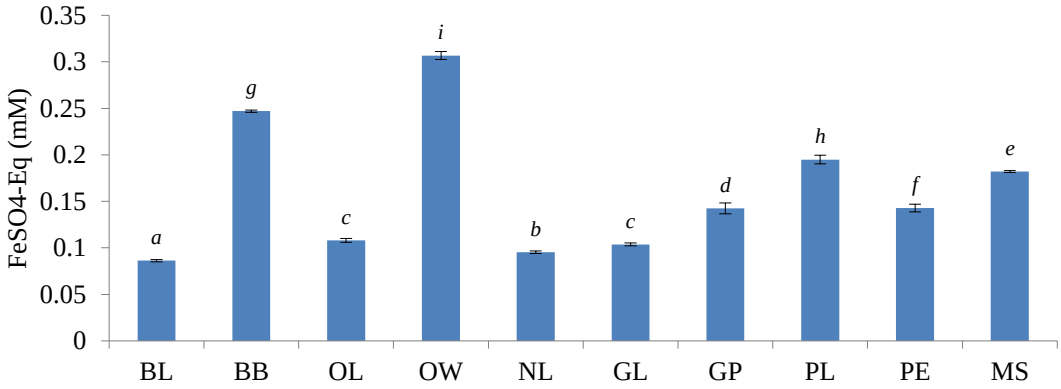


Figure 4.13. The antioxidant activities of the various IDY preparations when dissolved in model wine for 1 day. *a* - *e*: data indicated by different number are significantly different by Student-Newman-Keuls one-way ANOVA analysis ($P < 0.5$).

Table 4.4. Amino acid levels and GSH released into the model wine system after 14 days exposure for all IDY preparations.

(mg/L)	BL	BB	OW	OL	NB	GL	GP	PL	PE	MS
ASP	19.07	9.74	28.31	15.46	34.55	146.63	12.93	21.72	121	-
GLU	102.26	201.41	429.66	425.37	988.78*	568.72	36.95	65.02	510.96*	-
GSH	-	63.33	51.22	24.98	10.37	-	-	-	-	-
ASN*	58.09	105.54	121.7	126.28	138.06	342.29	55.37	81.68	232.97	-
SER	19.64	22.6	32.99	29.69	32.18	184.54	16.28	31.45	131.71	-
GLN*	136.49	238.96	475.26	309.13	556.86	76.75	15.99	16.72	65.4	-
GLY	25.73	14.51	42.44	20.54	39.13	252.61	17.47	28.42	271.55	-
ARG	22.51	30.28	38.71	46.01	45.85	108.33	16.06	25.32	107.19	-
ALA	119.18	-	-	59.89	362.37	451.1	51.13	83.03	461.1	-
TYR	16.02	14.89	27.14	15.08	15.22	47.77	13.34	18.75	46.51	-
CYS2	30.03	43.45	68.23	21.36	40.71	179.97	27.66	33.92	291.54	-
VAL	25.59	32.62	79.38	-	47.08	204.51	22.69	35.4	173.5	-
NOR*	191.44	69.48	105.25	93.54	69.17	-	114.11	118.14	-	37.74
TRP*	69.31	71.03	66.79	65.5	68.8	71.96	124.03	131.02	77.21	61.23
PHE	10.17	-	27.32	13.23	-	163.19	9.47	21.87	144.52	-
ILE	-	-	24.44	-	-	181.07	5.97	19.08	151.37	-
LEU	-	-	69.21	-	-	371.66	31.84	-	375.28	-
PRO	-	209.73	235.42	247.28	283.02	299.97	-	-	415.24	-
YAN	845.5	917.8	1688.0	1266.0	1460.3	3351.0	571.2	731.5	2650.8	98.97

A dash (-) indicates that only traces of amino acid detected at levels < 5 mg/L. * indicates $R > 0.99$ whilst all others $R > 0.999$. YAN, Yeast Assailable Nitrogen.

Furthermore, we also measured the amino acids and GSH released into the model wine medium by the various IDY preparations after 14 days of exposure, *Table 4.4*. It should be noted that these values are determined for solutions containing 10 times the recommended dose into a wine thus the values are significantly higher than what one would normally expect in a real wine system, however, this allows for easy quantification and comparison between IDY preparations.

It was found that the various IDY preparations essentially release all the various amino acids into solution except one (Mannostab®) which is again unique given its unique constitution for which only norvaline, tryptophan are detectable. The levels of individual amino acids released vary substantially although in the main the highest levels released are seen for glutamic acid, glutamine, α-alanine in some cases, norvaline and proline. This compares well with a recent study by Pozo-Bayón et al., which found the major amino acids released into a model wine system for 6 IDY preparations were glutamic acid, α-alanine, γ-aminobutyric acids and ornithine.[70] Furthermore a study by Andújar-Ortiz et al., also found glutamic acid and α-alanine to be two of the major amino acids released by certain IDY preparations.[185] For ease of comparison the IDY preparations are ordered in the table from left to right in their ability to prevent browning in our oxidative experiments described above with Biolees® showing the least amount of oxidation. Consequently it can be seen from the data in *Table 4.4* that there is no clear correlation in an IDY preparation to prevent browning and the levels of individual amino acids released.

Finally, we also measured the amount of GSH released into a model wines medium after 14 days to investigate if the concentration of GSH released is the major factor behind the abilities of the IDY preparations to protect against oxidative browning, *Table 4.4* and *Figure 4.14*. Both Booster Blanc® and OptiMUM-White® display the highest levels of free GSH indicating that these two IDY preparations may gain some of their antioxidant capacity to prevent browning from the rapid release of GSH into the wines medium. Having said this, only small amounts of free GSH released are seen for Biolees®, Opt-lees, Nobelese and Genesis Lift which showed significant ability to prevent oxidative browning in our model wine system thus indicating that there is no strong correlation

between these IDY preparations being able to prevent oxidative browning and the amount of GSH released.

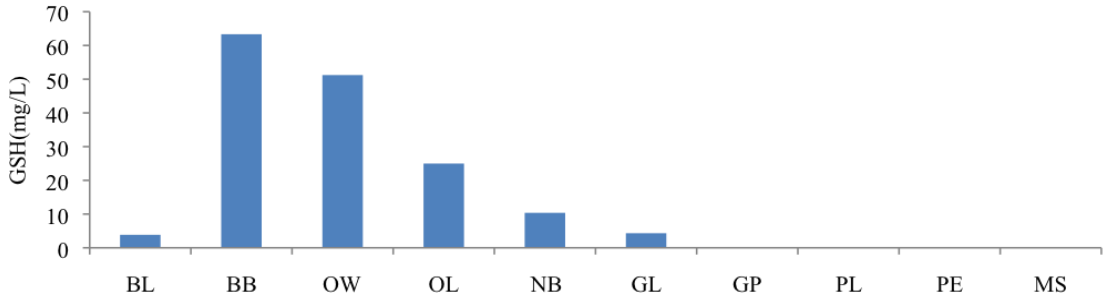


Figure 4.14. Total amount of GSH released into the model wine system after 14 days.

4.10 Chapter Conclusions and Future Directions.

Mechanistically the addition of the IDY preparations to a Chardonnay wine for 4 weeks resulted in significant increases in the total protein and polysaccharide levels, *Figure 4.7*. The A_{440} values of the Chardonnay wines exposed to the IDY preparations for 4 weeks at 15 °C were also measured, *Figure 4.8* and there appeared to be little difference in the absorbance values at 440 nm of the IDY exposed Chardonnay wines when compared to the control except for the wine exposed to the IDY Mannostab®. This latter finding was also seen when measuring CIE*lab* values. Little further information could be gleaned as to how the different IDY preparations are able to prevent oxidative degradation of a white wine. It would perhaps be more pertinent to repeat the experiments with little of no sulfur dioxide present or deliberately expose the wines to air or run the experiments for a greater period of time. At this stage we decided to simplify the system to be studied and utilise a model wine system to evaluate the 10 IDY preparations for their ability to prevent browning.

A browning model wine system which contained (+)-catechin, iron(II), copper(II), and acetaldehyde along with the model wine itself which was 12% v/v ethanol/water buffered with tartaric acid was thus prepared. To this browning model wine system was added the commercial IDY preparations at the recommended dosage and the samples warmed at 35 ^{o}C for up to 2 weeks after which time their absorbance at A_{440} and CIE*lab* values were again measured to evaluate the extent of yellow/brown colourisation. Inspection of the

results indicated that all model wine solutions containing the various IDY preparations undergo an increasing in yellowing/browning with time as indicated by their A_{440} and CIE*lab* readings. More importantly it appeared that the IDY preparations fall into three categories: those which significantly inhibit browning (Biolees®, Booster Blanc®, Opti-LEES, OptiMUM-White® and Noblesse®); those which provide a slight protection from browning (Genesis Lift, Genesis Prime, Phylia Lift and Phylia Exel) and one (Mannostab®) which provides no protection when compared to the control. Comparison of the results found here with the stated benefits described within the technical sheets for these various IDY preparations indicated that for the majority of these IDY preparations it appears that these are new findings that are yet to be pointed out in the technical sheets.

A host of various chemical parameters were then measured in order to probe mechanistically what was behind the ability that some of these IDY preparations are able to provide substantial oxidative protection of catechin in the model wine solution whilst others are not. Results indicated that the order of the IDY preparations abilities to protect against oxidative browning was related to an increase in total soluble protein upon exposure to various IDY preparations for 14 days in the browning model wine solutions. Such findings are yet to be reported in the literature and may indicate that some of these soluble proteins have certain functional substituants such as thiol moieties which are able to act in an antioxidant manner thus protecting the wine from oxidation. Furthermore, the ability of the IDY to release GSH also somewhat correlated with this antioxidant capacity although only two IDY preparations (Booster Blanc® and OptiMUM-White®) displayed the highest levels of free GSH which also correlated well with the overall antioxidant capacity of the wine systems. Analysis of the free amino acids levels did not however correlate with the ability of the IDY preparations to prevent oxidative browning.

Mechanistically, *Scheme 4.2* highlights the key reactions for our browning model wine system. As (+)-catechin was employed as the sole flavanol it may undergo oxidation in the presence of oxygen and metal ions such as Fe(II) or Cu(II) to form catechin *o*-quinone and hydrogen peroxide. Whilst the samples were protected with a blanket of CO_2 when they were prepared and deliberate aeration was not employed during the experiments, there will still be traces of oxygen present which may participate in this initiation process. The hydrogen peroxide thus formed may also react with these transition metals to produce additional oxidative species such as hydroxyl radicals as depicted in *Scheme 4.2*. These

oxidative reactions have been shown to be able to oxidise ethanol to acetaldehyde or tartaric acid to glyoxylic acid. At this stage either the acetaldehyde added to the model wine browning system or the acetaldehyde produced under the oxidative conditions may react with the catechin to produce coloured vinylflavanol oligomers, which is an indication of browning. Furthermore, any glyoxylic acid formed under the oxidative conditions (accelerated by iron(II) and Cu(II)) may also react with the catechin to form the well known yellow xanthylium cations which are again an indication of browning. The formation of the yellow xanthylium cations is actually through reaction of the A-ring (phloroglucinol-type moiety) of catechin and differs from the typical oxidative mechanism proposed for the formation of brown pigments formed in the presence of PPO or oxygen as highlighted in *Scheme 4.1* above. There may in fact be a small contribution to overall oxidative browning via the conversion of catechin to the *o*-diphenol and subsequent polymerisation given that two of the IDY (Booster Blanc® and OptiMUM-White®) displayed the highest levels of free GSH (able to form GRP) and were able to substantially inhibit browning, however, these IDY preparations also result in the release of large amounts of free amino acids which are able to inhibit such processes. Overall, the most likely mechanism for the oxidative browning appears to be the formation of coloured vinylflavanol oligomers including xanthylium cations. The IDY preparations ability to prevent such processes appears to be related to total soluble protein release.

In summary, this chapter provides a host of new results on the effectiveness and diversities of various IDY preparations for their ability to prevent oxidative browning of wines. Undoubtedly further scientific research will aid in unlocking the mysteries of IDY preparations and the host of positive effects that they can have on a finished wine. One such are of research should look at the mode of IDY manufacture and how this influences that final properties of a commercial IDY.

CHAPTER 5: EXPERIMENTAL METHODS.

5.1 General Experimental Methods for Chapter 2.

Oenological parameters.

Standard oenological parameters such as pH, TA, VA and free and total SO_2 were determined as for standard wine making processes according to the methods reported by Iland.[222] A pH meter (Cyberscan pH 510, Eutech Instruments, France) was first calibrated and utilised for all tests of pH, TA and VA. The aspiration method was employed for all evaluations of free and total SO_2 along with VA using a conbo-2-OH VA-SO2-OH still (Glasschem, South Africa).[222]

Measurement of MLF rates for Chardonnay wines.

The amount of *L*-malic acid was determined according to the UV-method described ENZYTEC^{TM} Code N^{o} E1215.[223]

Lees concentration in finished Chardonnay and Pinot Noir wines by natural sedimentation.

Triplicate samples of well mixed wines were utilised. A volume of 50 mL wine was placed into a centrifuge tube (50 mL) and left for sedimentation by gravity for 7 days at which time the upper layer became clear. The supernatant was then carefully removed and the slurry (lees) in the bottom was measured by weight. Lees concentration was estimated by the percentage of lees weight per volume of wine utilised. Errors of standard deviation between samples were <10% in most cases.

Model wine solutions.

A model wine solution was prepared with 14% EtOH in deionised H_2O (v/v), saturated with potassium hydrogen tartaric acid at 4 °C by dissolving the KHT with heating of the solution. The solution was stored at 4 °C overnight and then filtrated through Waterman No 1 filter paper and the pH was adjusted to 3.4 with solid tartaric acid.

SEM.

0.75 Millilitre of yeasts in wine or solution was filtered through a 0.22 μm membrane and the harvested cells fixed to the membrane with aldehyde solution (4% (v/v) paraformaldehyde, 1.25% (v/v) glutaraldehyde and 4% (w/v) sucrose in PBS, pH 7.2) for 20 min. Membranes were washed in buffer (4% (w/v) sucrose in PBS) for 5 min. Cells were dehydrated in an ethanol series (70%, 70%, 90% and 100% (v/v) twice for 10 min.) before using a critical point dryer with liquid CO_2 at 95 bar pressure. Filters were then mounted and coated with platinum (Adelaide Microscopy, South Australia) and examined using a scanning electron microscope (PHILIPS XL30 Field Emission Gun Scanning Electron Microscope).

Total Glucidic Colloid Analysis.

The polysaccharide content was determined colorimetrically according to Segarra et al. with little modification.[152] 100 μL of sample was pipetted into an eppendorf tube. Five volumes of 95% (v/v) acidified (60 mM hydrochloric acid) ethanol was added and the mixture left at 4 °C overnight before before centrifugation (4 °C, 14000 g × 30 min).. The collected pellets were washed twice with 500 μL of the same hydrochloric / ethanol solution and then they were dissolved in milli-Q water. Ultrasonics was employed to aid in dissolving the sample. After drying the pellet at 35 °C for 4 hours, 1 mL fresh 5% phenol (w/w, Fluka) was added to dissolve the pellet. Glucose standards were also prepared in 5% phenol solution. After adding 50 μL sample/standard and 150 μL sulphuric acids into a 96 well palate (Costar® 3956, Corning, USA), absorbance at 490 nm was read by the plate reader (μQuant, Bio-tek instruments, Vermon, America). Linear correlations $R^2 > 0.99$ was achieved with 1 - 100 mg/L glucose standards.

Heat Stability Analysis.

Bentonite tests were conducted to assay the heat stability changes of the Chardonnay wines during storage.[222] All wine samples were filtered through 0.45 μm acetate cellulose filters (Millipore GSE) and degassed by vacuum treatments. Hazes produced by the heat tests were measured spectrophotometrically. Bentonite (Plusgran® Gel, Vason, Italy) stock solution of 5% was added into 50 mL of the filtered wine to make a final

concentration at 0.1 - 0.3% after a preliminary assay. The samples were well mixed and allowed to settle at room temperature for around 4 hours. Then the supernatant was racked off after centrifuging (200 rcf × 5 min.) and placed into an 80 °C oven for 8 hours and then put into 4 °C cold room to cool down. Absorbance at 520 nm in 1 cm path length cells was recorded before and after heating. Evaluation thresholds were set at 0.02 absorbance units (au) between haze and no haze wines and between heated and unheated controls.

CIElab Colour Measurements.

Supernatants were tested after the wine samples were centrifuged at 2000 rpm for 8 minutes. The CIE*lab* parameters *l**, *a** and *b** values, which represent lightness, red-green and yellow-blue characteristics respectively were determined according to the methods described in Method OIV-MA-AS2-11 with little modification.[224] Hence the transmittance of the wine was measured between 380 nm to 680 nm at 0.427 nm intervals, using a spectrophotometer (Cintra 10/20/40, GBC® Scientific Equipment, Australia,) equipped with a D65 illuminate, and slit width 2.0 nm. The wines were contained in a 1 mm quarts cuvette (for red wines) or 10 mm glass cuvette (for white wines) and measured with a 10^{o} standard observer. And the ΔE value, which indicates the difference between two colours, was calculated by the formula as follows.

$$\Delta E = (\Delta l^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2}$$

Viscosity Analysis.

Both the Chardonnay and Pinot Noir wines that were stored at -20 °C for the zero time point or at 15 °C for 2 years had their viscosity and density according to the following method. A falling ball Anton Parr viscometer (DMA 5000) equipped with a density device was utilised as described by Yanniotis et al.[155] Data was averaged over 4 – 6 runs for each sample, and an average value was adopted when the standard deviation was lower than 1%. All runs were taken at 20 °C with the channel angel set to 15°.

Metal Analyses.

Samples of the Chardonnay and Pinot Noir wines for all treatments were utilised at the 0^{th} and 4^{th} time points. β-Glucanase (Optivin® Elevage, Enzyme Solutions™, Australia) was utilised in the winemaking, and tartaric sedimentation occurred after storage of the wine at 4 °C. All wine samples were stored at -20 °C on sampling days, and 2 bottles of wine of each treatment were thawed at 4 °C until they reached equilibrium, and then were vacuum filtrated through pads (Φ 0.8 μm, EKWIP Z6), and then 0.45 μm cellulose acetate membranes. β-Glucanase was dissolved in milli-Q water at a concentration of 3 g/L (100 folds as added in the corresponding wine samples). Tartaric sedimentation was harvested after the filtration of wine storage at 4 °C and dried in a 100 °C oven until constant weight.

1.5% (w/w) of HNO_3 stock solution was made of concentrated HNO_3 of 68.5 - 69.5% (w/w) (AnalaR$^{@}$ Merck Pty, Victoria Australia) and deionised water was employed throughout. All glassware was carefully cleaned before use with 1.5% HNO_3. To 12.5 mL of sample, 1.5 mL of 14 M HNO_3 was added and made up to 15 mL with deionised H_2O. Blank acid digestion was taken when the nitric acid was substituted by deionised water. A general scanning of all metals (except for the transition elements) at typical absorbance wavelengths was done to examine the existence of certain metals prior to quantification. Li, Na, K, Rb, Cs, Mg, Ca, Fe, Cu and Zn were quantified according to the linear built up by 2 batches of multi-elements standard solutions, with one set of Zn, Li, Cs, Mg, and Rb in 1.5% (w/w) HNO_3 and 0.1% KCl added, and the other set with K, Na, Cu Ca, Fe in 1.5% (w/w) of HNO_3. All standards were from Merck (AnalaR$^{@}$ Merck Pty, Germany). 1.5 mL of 14 M HNO_3 was added to 50 mL of wine and diluted to 100 mL. Metals in all samples were tested in triplicate by a high-resolution continuum source electrothermal atomic absorption spectrometer (control AA® 300, Analytic Jena, Germany), with air and acetylene as fuel. The fuel flow rate and burner height were adjusted according to the software built in the "Cook book" developed by Jena. Details of measurements lines are as detailed below.

To accurately quantify the total amount of Cd, Pb, Fe, Cu and Zn, microwave or thermal digestion under acid conditions is required to release the free metals from their complexes in wine.

Table 5.1. Measurement lines and concentration scales for each metal measured.

	Li	**Na**	**K**	**Rb**	**Cs**	**Mg**	**Ca**	**Fe**	**Cu**	**Zn**
Wavelength (nm)	670	330	344**	794*	852	202*	422*	248	324	213
Linear scale (mg/L)	0.002-0.01	5-100	220-2000	2-20	0.04-0.2	16-80	5-100	0.2-2.0	0.1-1	0.8-4

* not using primary measurement line due to stability consideration.

** not among the sensitive measurement lines since CsCl was not added as depression reagent, due to simultaneous Cs test in the same batch of sample.

Measurement of β-glucanase activity.

The three different commercial autolytic enzymes that were assayed for their *β*-glucanase activity were lallzyme MMX® (lallemand, Montreal, Canada), VinoTaste® Pro (Novozyme, Dittingen, Switzerland) and Optivin® Elevage (Enzyme solutions, Melbourne, Australia).

Assay 1: Measurement of glucanase activity employing laminarin.

The activity of three commercial *β*-glucanases was measured according to procedure described by Humbert-Goffard et al. with little modification.[151] Laminarin (Sigma, St. Louis, MO) was utilised as the substrate for testing of endo-*β*-(1,3)-glucanase activity. A stock laminarin solution at 100 mg/mL was freshly prepared in Milli-Q water. The *β*-glucanase stock solutions were prepared at 100 mg/mL in a Chardonnay wine or model wine system. The activities were determined around 3 hours after the solutions were made. The requisite three reaction systems were prepared on a thermostated bath at 23 °C as follows.

Digestion: A mixture of 3.88 mL of the medium (Chardonnay control wine or model wine) was mixed with 20 *μ*L of the enzyme stock solution and 100 *μ*L of the laminarin stock solution. The final concentrations were 0.5 g/L for the three glucanases and 2.5 g/L for laminarin.

• Control 1: A mixture of 3.88 mL of the medium was mixed with 20 *μ*L of water (instead of enzyme stock solution) and 100 *μ*L of laminarin stock solution in order to determine the residual glucose content of laminarin.

• Control 2: A mixture of 3.88 mL of medium was mixed with 20 μL of enzyme stock solution and 100 μL of water (instead of laminarin stock solution) to determine the glucose content of the enzyme solutions.

Reactions were stopped by heating at 100 ºC for 30 minutes on a boiling water bath. Then the glucose released was determined using the hexokinase enzymatic test. The concentration of glucose released by the autolytic enzymes was determined to equal Digestion – (Control 1 + Control 2).

Assay 2: Measurement of glucanase activity employing the BGU assay.

The stability and activity of the commercial β-glucanases was monitored by BGU assay (Procedure No. VE 400.42, 1996) provided by Valley Research, Inc., with little modification.[225] Laminarin (Sigma. St. Louis, MO) was employed instead of lichenan. This assay is based on the enzymatic hydrolysis of lichenan at pH 6.8 and 40 ^{o}C and the reducing sugars are determined by the Nelson-Somogyi method. One BGU unit is the activity which will liberate one micromole of reducing sugar as glucose per minute.

Somogyi's reagent consisted of 52.75 g of disodium hydrogen phosphate heptahydrate ($Na_2HPO_4.7H_2O$) and 40.00 g of sodium potassium tartrate tetrahydrate ($C_4H_4KNaO_6.4H_2O$) in 700 mL deionised water. To this mixture was added 50 mL of a 2 M sodium hydroxide solution. A solution of 8.00 g of copper sulfate pentahydrate ($CuSO_4.5H_2O$) was dissolved in 100 mL water and this solution added to the mixture above. Then 180.0 g of anhydrous disodium sulfate (Na_2SO_4) was added and stirred until dissolved. The final mixture was made up to 1 L in a volumetric flask.

Nelson's reagent consisted of 50.0 g of ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$) in 600 mL of deionised water. The mixture was warmed until full dissolution. Then 50 mL of concentrated sulfuric acid (H_2SO_4) was added. A mixture of 6.78 g of sodium arsenate heptahydrate ($AsHNa_2O_4.7H_2O$) dissolved in 50 mL water was then added and the mixture was made up to 1 L in a volumetric flask. All reagents were kept in the dark at 0 ºC until used.

Laminarin substrate (0.3% w/v) was made by dissolving 0.30 g of finely ground laminarin in 50 mL of hot deionised water (80 – 90 °C) with constant agitation. Then 0.68 g of anhydrous monopotassium phosphate (KH_2PO_4) was added and the pH adjusted to 6.80 with 1 M sodium hydroxide solution. The final mixture was made up to 100 mL in a volumetric flask.

Stock solutions of glucose at 0.2, 0.6, 1.0, 1.4 and 1.8 moles/mL were prepared so that a standard curve could be generated. The commercial enzyme solutions were prepared at various concentrations as described in the reported procedure. The various commercial β-glucanase dilutions and the laminarin substrate solutions were place on a 40 °C water bath for 15 minutes to equilibrate.

At various timed intervals, 50 μL of the equilibrated enzymes was pipetted into the substrate test tubes and mix thoroughly using a vortex mixer. After exactly 15 minutes of reaction, and at the same intervals at which the reactions were started, 200 μL of Somogyi reagent was added to stop the reaction. Blanks were prepared by first combining 50 μL of the appropriate enzyme dilution and 200 μL of Somogyi reagent. Then 100 μL of the substrate was added and mixed thoroughly. The glucose standard curve was generated by pipetting 50 μL of each of the five dilute glucose solutions into 25 mL graduated test tubes containing 200 μL of the Somogyi reagent. All the tubes were sealed (reaction, glucose and blanks) and the reaction stopped by heating them on a boiling water bath for 30 minutes. The samples are then cooled to room temperature and 200 μL of Nelson reagent added to each tube and mixed thoroughly. After 10 minutes of standing the samples were diluted to 2.5 mL with deionised water and mixed thoroughly. Absorbances at 520 nm were then read to quantify the amount of glucose present and recorded in BGU units.

5.2 General Experimental Methods for Chapter 3.

Microwave Reactor.

All reactions performed under microwave conditions were conducted in a closed system, using a CEM Discover microwave reactor (CEM Co., USA). The temperature was

controlled with the use of a Marcus WX801700 air compressor (Campbell Hausfeld, USA) and the pressure set to 200 psi, the stirring level set to high, and cooling was left on.

Microwave Reactor Energy Efficiency Check.

This microwave reactor energy efficiency check was done by heating up 7, 14 or 21 mL samples of deionised water that was pre-cooled to 6 – 8 °C in an ice bath, using a 35 mL glass tube with silica cap to seal (CEM Co., USA). The samples were allowed to heat up to 90 °C, at power inputs of 100, 200 or 300 W, and the temperature change during time recorded.

Wine Sample Preparation and Lees.

Lees for panel training was freshly harvested from an 18 L Chardonnay juice fermentation at 18 °C. The lees was kept at 4 °C for 2 months for continuous weekly use. The Chardonnay juice with a Brix of 15 was inoculated with 300 mg/L PDM (*Saccharomyces cerevisiae* var. *bayanus,* AB Maurivin^{TM}), and left at 18 °C for 48 hours then transferred to 22 °C for robust fermentation. After 44 hours the fermentation vessels were transferred back to an 18 °C room and 200 mg/L DAP (Diammonium phosphate) added when the residual sugar level reached 10.4 Brix. The fermentation was finished in the following 4 days (fructose 0.71 g/L and glucose 0.03 g/L). Lees was harvested after racking off the wine and washed twice with deionised water. Around 163 g wet lees was harvested and reconstituted into 180 mL deionised water giving a final concentration of lees in stock solution of around 0.3912 ± 0.0019 g/mL (wet lees/solution, W /V) or 33.81 ± 0.94 % (wet lees/solution, W /W). All samples prepared thereafter utilised a volume ratio of this stock solution to the final wine volume.

Lees for the formal sensory evaluation trials was freshly harvested from ferments of 54 L Chardonnay juice as above, after racking off, centrifuging and washing with deionised water 3 times until the supernatant was clear. Lees was reconstituted in water at 1/100 volume of fermenting juice afterwards and equilibrated overnight. A total of 264.18 g wet lees was reconstituted into 540 mL deionised water and made up to a final volume volume of 750 mL lees solution. The lees solution was then centrifuged and used for chemical analysis or reconstituted into wine for descriptive sensory analysis according to the following treatments.

Various treatments were prepared as follows:

Microwave treatment of lees to decide the temperature at which the microwave experiments should be conducted: Diluted lees solutions (21 mL, 1:5 stock lees solution/final wine solution) were placed in a 35 mL microwave tube and heated up to 45, 55, 65 75, 85 or 95 °C, at 300 W for 3 minutes and the total glucidic colloids and soluble peptides evaluated.

Microwave: Given that the total glucidic colloids and soluble peptides were at a maximum at 85 ^{o}C this temperature was chosen for the sensory experiments. All samples were conducted with a volume of 7 mL in 10 mL microwave tubes and heated for 3 minutes.

Thermal: A thermal control treatment at 85 °C was prepared as follows. A total of 80 mL well mixed lees solution was placed into a 100 mL beaker and heated up to 85 °C on a boiling water bath for 6 - 8 minutes. The beaker was covered with a plastic cap and a thermometer was placed in the lees solution through a hole in the middle of cap. After 6 - 8 minutes the mixture was allowed to cool to ambient temperature.

Untreated: Control, untreated lees solution only was employed.

Wine Sample Preparation with Added Treated Lees: All treatments were prepared in duplicates (T1 and T2) and each sample was evaluated over three formal sensory sessions. A cask of unwooded Chardonnay wine (Yalumba, Australia, vintage 2012) was adopted as base wine due to its simple wine profile after comparison with several other commercial white wines. All samples of different treatments were added proportionally into this base wine. Reconstituted concentrations were 1%, 2% and 3% (stock solution/wine, v/v) by adding back co-treated stock solution into base wines. Wines were left on the lees for 24 hours at room temperature in Schott bottles and protected with dry ice in the headspace. Wines were centrifuged (963,200 rcf × 20 min., 4 ^{o}C) to remove the lees and bottled in 375 mL green bottles and stored at 15 °C before sensory analysis.

Statistic Data Analysis.

All statistical analyses were performed using SPSS 15.0 (SPSS, Chicago. Illinois, USA) or with Fizz (Version 1.3, Biosystems, Couternon, France). Statistical analyses were performed with SENPAQ verion 5 (Qistatistics, U.K.).

Total Glucidic Colloids.

Measured as for Chapter 2.

Total Soluble Proteins and Peptides.

Measured as described in Chapter 5.

Anti-oxidant Analysis.

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay described by Molyneux[226] and the FRAP (ferric reducing ability of plasma) assay described by Benzie, et al.[221] were utilised to evaluate the antioxidant capacities of the lees solutions. Both results are expressed as mM of Trolox (6-hydroxy-2,5,7,8-tertramethylchroman-2-carboxylic acid) equivalents (TE) or Vc (Ascorbic acid) equivalents (VcE) using an 8-point regression curve (with R^2 > 0.99). Trolox and Vc standard solutions were prepared at 4 mM in methanol as stock solutions. The FRAP assays ustiised Fe^{2+} as standard. To prepare this standard solution, 10 mM ferrous sulfate ($FeSO_4$) prepared in deionised water was employed and all standard solutions are store at -20 °C.

DPPH.

DPPH (2,2-diphenyl-1-picrylhydrazyl) stock solution (4 mM/L) was prepared in MeOH and stored at -20 °C, diluted to 60 μM/L, with Abs_{515} from 1.0 to 1.06 in a 10 mm glass cuvette right before use. 100 μL DPPH stock solution was added into 96 well plate (costar® 3956, Corning, USA), and to this solution, the corresponding wine samples or standards were added, The plate was covered with aluminum foil and after shaking for 15 min, Abs_{515} was read by the plate reader (µQuant, Bio-tek instruments, Vermon, America).

FRAP.

All the stock solutions were prepared with deionised water and kept at 4 °C after preparation as follows, 0.3 M NaAc/HAc buffer at pH 3.6, 10 Mm TPTZ solution in 40

mM HCl and 20 mM $FeCl_3$. The FRAP working reagent was prepared freshly with the stock solutions just before use at volume ratios of acetate buffer/TPTZ solution/$FeCl_3$ solution = 10/1/1, and pre-warmed to 37 °C. This working solution is light sensitive, thus the container was wrapped with aluminium foil and utilised in the dark. 15 μL of the samples was added into the 96 well plates and 150 μL working solution added into this samples. After shaking the plate for 3 minutes the absorbance at 593 nm was read.

TEM.

TEM samples were prepared in a similar manner to that described by Tudela et al.[176] Yeasts solutions were centrifuged (2000 rpm) for 2 minutes to roughly get rid of any moisture and then immediately added to the fixative solution (25% glutaraldehyde in 0.1 M PBS, pH 7.3). After 3 days at 4°C the samples were suspended in 10% agar solution after removal of the fixation solutions by centrifuging (2000 rpm) for 2 minutes. Samples in agar were cut into 230 nm thin slices with a microtome (Reicherd Jung Ultracut-E, Leica, Germany) under a microscope and then the slices were kept at 4 °C in 0.1 M PBS in 1 mL tubes. Lees pellets were prepared and selected utilising a stereomicroscope and transferred to a 1.5 mm diameter and 200 µm deep planchettes with 20% BSA as protection solutions, and immediately cryo-immobilised using a Leica EMPact1 high-pressure freezer (Leica, Vienna, Austria), and stored in liquid nitrogen. After rapid freezing, specimens were transferred to freeze substitution media (anhydrous acetone containing 1% osmium tetroxide, 0.5% uranyl acetate, 2.5% methanol and 5% H_2O) in liquid nitrogen for 3 hours. Afterwards the samples were exerted into 2 ways for substitution, one went through freeze substitution in a Leica AFS high pressure freeze substitution system. The other batch was kept in a foam box until the liquid nitrogen evaporated and gradually allowed to warm up to room temperature in a fume hood without out any temperature control. After several acetone rinses, the samples were embedded in epon resin. The epon was then polymerised for 48 h at 60 °C according to that described by Walther & Ziegler.[227] Ultrathin sections of 70 nm were prepared on a ultramicrotome (EM UC6, Leica, Germany), mounted on copper grids and stained with 4% uranyl acetate in water and followed by lead citrate according to that described by Bozzola & Russell, 1999.[228] The ultrathin sections were observed with TEM (PHILIPS CM100, FEI, Eindhoven) at an accelerating voltage of 80 kV. On average, 40 overview and approximately 8 - 10 detailed electron micrographs were taken for each treatments.

5.3 General Experimental Methods for Chapter 4.

Preparation of the Chardonnay Wines with Added IDY Preparations.

A Chardonnay base wine was prepared from 18 L of grape juice by inoculating with 300 mg/L PDM (*Saccharomyces cerevisiae* var. *Bayanus*, AB Maurivin^{TM}), at 18 ^{o}C for 48 hours. The wine was then transferred to 22 °C for 48 hours and then transferred back to the 18 ^{o}C room after 44 hours. Diammonium phosphate (DAP 200 mg/L was added to the ferment when the residual sugar had reduced to 10.4 ^{o}Brix. The ferment was judged to be complete 4 days later when the fructose was measured at 0.71 g/L and glucose at 0.03 g/L. The lees was harvested by racking for the experiments detailed in Chapter 3 and the based wine utilised for the IDY experiments in Chapter 4. Analysis of the base wine for its standard oenological parameters in duplicate revealed the wine had a pH of 3.30, TA of 0.9 mg/L, VA of 4.08 g/L, free SO_2 (16 ppm) and total SO_2 (174.4 ppm).

A total of 10 Chardonnay wines with added IDY preparations were prepared along with the control sample in triplicates. After completion of primary fermentation, the IDY preparations were added to the wines at the maximum recommended levels as depicted in *Table 4.1*. The IDY powders were pre-dissolved or suspended in 5 mL of the base wine and then added into 1 L of the base wine being stirred in Schott Duran bottles. Dry ice was employed as a blanket for all wine transfers and cling wrap was utilised to seal the neck of the bottles before the lid was screwed tight. In total, 33 bottles (11 x 3 triplicates) were then kept in storage for 4 weeks at 15 ^{o}C and shaken once weekly to ensure mixing. After this time the wines were centrifuged (963,200 rcf × 20 min., 4 ^{o}C) with dry ice protection and the wine supernatant removed and bottled in half-bottles (375 mL). There were a total of 3 half-bottles for each treatment.

Total Polysaccharides of Chardonnay Wines Exposed to the 10 Commercial IDY Preparations for 4 Weeks.

The polysaccharide content was determined by the colorimetric method described by Segarra et al., with little modification.[152] A sample of the wine (100 μL), which had been exposed to an IDY, was pipetted into an Eppendorf tube. Five volumes of acidified (60 mM hydrochloric acid), 95% (v/v) ethanol was added and the sample left at 4 °C

overnight before centrifugation (30 min, 14000 g). The collected pellets were washed twice with 500 μL of ethanol 95% (v/v), dried under a stream of nitrogen and then dissolved in deionised water. Ultrasonic agitation was used to aid in solubilisation. A portion of the solution (1 mL) was then added to 25 μL of 80% phenol (w/w) and 2.5 mL of 96% (w/w) sulphuric acid. Samples were mixed and the reaction allowed to proceed for 30 minutes at room temperature. Absorbance values were measured at 490 nm on a UV/VIS spectrophotometer (Cintra 10/20/40, Australia, GBC® Scientific Equipment). The calibration curve was prepared by using serial dilutions of glucose in water and all analyses were done in triplicate.

Colour Measurements of Chardonnay Wines Exposed to the 10 Commercial IDY Preparations for 4 Weeks.

The CIE*lab* parameters *l**, *a** and *b** values, which represent lightness, red-green and yellow-blue characteristics respectively were evaluated according to the method described described in Method OIV-MA-AS2-11.[224] That is, the transmittance of the wine from 380 nm to 680 nm at 1 nm intervals was measured using a UV/VIS spectrophotometer (Cintra 10/20/40, Australia, GBC® Scientific Equipment) equipped with a D65 illuminate with a 10-degree observer angle. Solutions were contained in a 1 mm quarts curvette or 10 mm acrylic cuvette.

Total Soluble Protein Measurements of Chardonnay Wines Exposed to the 10 Commercial IDY Preparations for 4 Weeks.

The Bradford method with modification was adopted to quantify total soluble proteins in all wine samples with BSA (Bovine Serum Albumin type V) as standard.[229,230] Sedimentation with acetone and 10% trichloroacetic acid was conducted to concentrate the soluble protein in the wine samples as recently described by Smith et al.[231] Coomassie Brilliant Blue G-250 (CBBG250) (100 mg) was dissolved in 50 mL 95% ethanol and ultrasonicated (Ultrasonic Cleaner, Unisonics, Sydney, Australia) to dissolve the mixture. To this solution was added 100 mL 85% (w/v) phosphoric acid. The resulting solution was diluted to a final volume of 1 litre. Final concentrations of the reagents were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric

acid. This stock solution was vacuum filtered through cellulas filterpaper (Whatman No. 1, GE healthcare, UK) and ultrasonicated for 1 minute at room temperature before use. 160 μL CBBG250 solution was added into a 96 well plate (Costar 3596, USA) with 40 μL of the BSA standard solutions at concentrations of 0 - 400 μg/mL for 7 points. The wine samples were added and the mixture shaked on a platform shaker (Ratek MPS1, Australia) to mix well. The absorbance at 450 nm and 590 nm were recorded by a plate reader (μQuant, Bio-tek instruments, Vermon, America). Concentrations of soluble proteins were deduced and expressed as BSA equivalents when the R^2 of the BSA standards was linear with a correlation above 0.99.

Preparation of the Model Wines with Added IDY Preparations.

A model wine solution was prepared with 14% EtOH in deionised H_2O (v/v), saturated with potassium hydrogen tartaric acid at 4 °C by dissolving the KHT with heating of the solution. The solution was stored at 4 °C overnight and then filtrated through Waterman No 1 filter paper and the pH was adjusted to 3.4 with solid tartaric acid.

Model wine (2L) was prepared by adding KHT until saturated in 12% v/v EtOH / deionised H_2O solution at 4 °C. The browning system was prepared (500 mL) with the model wine solution and contained the following constituents: catechin, 500 mg/L; Fe^{2+}, 8 mg/L as $FeSO_4$; Cu^{2+}, 0.1 mg/L as $CuSO_4$ and acetaldehyde at a concentration of 0.02 M. Both Fe^{2+} and Cu^{2+} are at the highest allowed concentration in wines according to the regulations (refers to appendix in the metal part in chapter 2). Ten commercial IDY preparations were selected as depicted in *Table 4.1* Three systems were prepared as follows: system 1; Reconstitution of IDY in model wine (10 mL) at 10 fold recommended adding concentrations as what is described in *Table 4.1*. This system was employed for the determination of total soluble protein as described in chapter 4 and the 10 fold excess was employed so as a significant difference would be observed. All trials were conducted in triplicate thus 150 samples altogether were prepared. Systems 2 and 3 were prepared at the recommended dosage as depicted in *Table 4.1*. After well mixing the IDY stock solutions, 1 in 10 dilutions (1:9 mL) were made into the model wine (system 2) or the browning model solution (system 3). Colour evaluation was done on systems 2 and 3 immediately after sampling. Soluble proteins and antioxidant abilities were evaluated on

samples from system 1 after freezing the supernatant with liquid N_2 and stored at – 20 °C. All systems were maintained anaerobically by filling with a small portion of dry ice and then sealed. All three systems were kept at 35 °C in a constant temperature oven avoiding light and samplings were done at day 0, 2, 6, 10 and 14. The samples were centrifuged (14 000 rpm ×10 min, 4 °C) in 2 mL Eppendorf tubes with any empty space flushed with N_2. The samples for the browning inhibition studies had their supernatant frozen immediately by plunging into liquid N_2 and then stored at – 20 °C until day 14 when all samples were analysed.

Colour Measurements of the Browning Model Wine solutions Exposed to the 10 Commercial IDY Preparations for 14 days.

Simple A_{440} measurements and CIE*lab* measurements were carried out on these solutions as described above for the Chardonnay wine samples.

Antioxidant Activities of the Model Wine solutions Exposed to the 10 Commercial IDY Preparations for 1 day.

The ferric reducing ability of plasma assay (FRAP) was employed to measure the antioxidant activities of the model wine solutions containing the various IDY preparations.[221] The various IDY preparations were placed into the model wine at the recommended commercial rates as detailed in *Table 4.1*. All results are expressed as mM of Trolox equivalents (TE) or Ascorbic acid equivalents (VcE) using an 8-point regression curve (with $R^2 > 0.99$).

Amino Acid and GSH levels of the Model Wine solutions Exposed to the 10 Commercial IDY Preparations for 1 day.

Amino acids were directly assayed from the original wines without prior concentration or dilution, and were analysed by reversed-phase HPLC according to sigma's method with modifications.[232] Hewlett Packard 1100 series, consisting of a G1322A Degasser, G1311A Quart Pump, G1316A column, G1315A DAD and an G13212A fluorescence detector (Angilent Technologies, Germany) were utilised. Chromatographic data were collected and analysed with a Chemistation system (Angilent Technologies, USA).

A mixed amino acids standards (Pierce Amino Acid Standard H) constituted as follows: 2.5 μmol/mL for each amino acid as listed as follows in 0.1 N HCl. Ammonia, *L*-Alanine, *L*-Arginine, *L*-Aspartic Acid, *L*-Cystine (1.25 μmol/mL), *L*-Glutamic Acid, Glycine, *L*-Histidine, *L*-Isoleucine, *L*-leucine, *L*-Lycine, *L*-Methionine, *L*-Phenylalanine, *L*-Proline, *L*-Serine, *L*-Threonine, *L*-Tyrosine, and *L*-Valine. Supplementary AA standards ATG (Asparagine, Tryptophan and Glutamine) (B.D., New York, USA) were prepared as mixture in deionised water each at 2.5 nM/μL, due to their easy degradation in acid environments. Glutathione (Sigma) and Norvaline (B.D.H laboratory, Poole, England) standards were freshly made at 1000 ppm with 0.1 N HCl. Samples/standards were submitted to automatic precolumn derivatisation with OPA (*o*-phthaldialdehyde) in the presence of 3-mercaptoproprionic acid (Agilent Part No. 5061-3335). RP 18 column was utilised Poroshell (2.7 μm, 100 mm x 4.6 mm), with a guard column Poroshell (2.7 μm, 5 mm x 4.6 mm). The two mobile phases were prepared as follows, A: 40 mM phosphate buffer prepared at pH 7.8, by dissolving 5.5 g $NaH_2PO_4 \cdot H2O$ in 1 litre deionised water, pH adjust to 7.8 with NaOH solution (10 N), and the B phase was prepared by mixing, acetonitrile (ACN), methanol (MeOH) and deionised water at the volume ratio of 45:45:10. Separation sequence was run according to *Table 5.2*. LFD parameters were set as depicted in *Table 5.3* with response time > 0.05 min (1s). Quantification of each amino acids derived from five points standards curve at the concentrations from 12.25 pm/μL – 500 pm/μL, with $R > 0.999$.

Table 5.2. Separation programe.

Step	Time (min)	%A	%B	Flow (ml/min)	Max. Press. (Pa.)
1	0.00	100.0	2.0	1.000	400
2	0.35	100.0	2.0	1.000	400
3	13.40	43.0	57.0	1.000	400
4	13.50	0.0	100.0	1.000	400
5	15.70	0.0	100.0	1.000	400
6	15.80	100.0	2.0	1.000	400
7	17.50	100.0	2.0	1.000	400

Table 5.3. Fluorescence detector conditions.

Step	Time (min)	Excitation (nm)	Emission (nm)	PMT-Gain
1	0.00	340	450	10
2	14.00	266	305	9

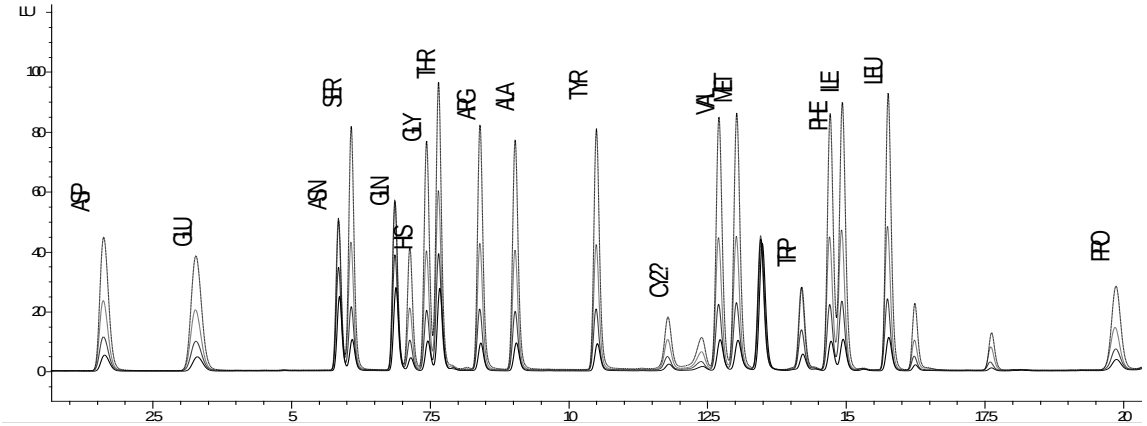


Figure 5.1. Chromatography of amino acids standards.

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