Selective Use of Winemaking Supplements to Modulate the Chemical Composition and Sensory Properties of Shiraz Wine

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THESIS SUMMARY

A global trend of increasing alcohol strength in table wine has emerged over the past four decades, largely due to advanced grape maturity associated with climate change. Harvesting grapes before they reach full maturity, i.e. at lower total soluble solids, can be an undemanding and effective method to control alcohol levels in wine. However, fruit maturity has a significant influence on wine composition. Wines made from early harvested fruit can be deficient in the desirable organoleptic characters usually associated with wines made from mature fruit, such as aroma and flavour intensity, as well as mouthfeel attributes. The current project therefore aims to improve the quality of Shiraz wines made from early harvested fruit, through selective application of commercial winemaking supplements.

A critical review of literature showed that compared to mature fruit, early harvested fruit has considerably lower tannin and mannoprotein concentrations; i.e. wine constituents that are associated with important mouthfeel attributes, such as astringency and viscosity. To address these deficiencies, three supplements that are legally permitted for use in Australian wine production, i.e. a maceration enzyme, an oenotannin and a mannoprotein product, were selected based on their potential for modifying wine tannin and polysaccharide compositions. These products were added during the vinification process of Shiraz wines produced from early harvested grapes, either individually or in combination. The resultant wines were compared with Shiraz wines made from mature fruit, in terms of both chemical composition and sensory characters. The results showed that modifying tannin and polysaccharide composition could indeed alter the perception of astringency. Furthermore, the combined use of mannoprotein and oenotannin additives resulted in a wine that closely resembled the sensory properties of wines made from mature fruit. The warmer than usual vintage conditions experienced, variation observed in supplement composition, and recovery of additives in treated wines, represented limitations of this study. Thus, three subsequent studies were designed to further explore the effect of additives in more depth.

Fourteen grape based oenotannins and eight mannoproteins were sourced from commercial suppliers in the Australian market. The aim was to understand the compositional variation amongst products, and by extension, the different effects likely to be achieved through product selection. Substantial variation was observed amongst products of both types of supplements. Some products showed good agreement between their composition and the designated material of

origin, whereas others showed significant differences. Based on results from this study, three commercial products, two oenotannins (derived from grape skin and seed respectively) and one mannoprotein were selected, as these products were similar in composition to their counterparts isolated from grape and wine; additives were further characterised in two subsequent studies.

The selected products were introduced into two finished Shiraz wines of 11.5% and 14.5% v/v alcohol content, i.e. wines made from fruit of early and later harvests, respectively. The same supplementation regimes were applied to both wines, and thus established a series of wines comprising different ethanol, tannin and polysaccharide concentrations and/or compositions. The aim was to evaluate, using the sensory analysis techniques, changes in astringency and body (viscosity) mouthfeel characters, attributable to the additives and/or their interactions. However, the judges involved in sensory evaluation could not perceive any variation in astringency resulting from the differences in tannin concentration and composition imparted by the additives. Furthermore, although the judges could perceive differences in wine body between wines of the two harvests, they could not perceive any effects of mannoprotein addition, even at dose rates 2.5 times higher than the level legally permitted in Australia. It was not immediately obvious if the lack of sensory discrimination was due to subtle differences amongst samples or a lack of sensitivity from the judging panel.

Finally, the addition of supplements is expected to influence the colloidal state of wine, which may in turn affect wine stability and sensory characters. To test this hypothesis, two polysaccharides, a mannoprotein and an arabinogalactan, were purified from two commercial products, and combined with a tannin fraction purified from grape seeds, in model wine solutions of 12% and 15% v/v ethanol concentrations. The formation of aggregates between polysaccharides and tannins was explored using a novel technique, nanoparticle tracking analysis (NTA); with results confirmed using dynamic light scattering and UV-visible spectroscopy techniques. The behaviour of the two polysaccharides towards tannin was substantially different. Mannoprotein formed large, highly light scattering aggregates with tannin, while arabinogalactan gave weak interactions with tannin and formed low-intensity light scattering aggregates. The 3% difference in alcohol content was sufficient to modify aggregation between mannoproteins and tannin. The implications for wine colloidal properties are discussed based on these results.

The collective findings of this research offers insights into the compositional variabilities of commercial winemaking supplements, as well as their effects on wine macromolecule

composition, colloidal state and sensory properties. The knowledge gained from these studies can inform winemakers' selection and use of winemaking supplements, especially with regards to improving the quality of red wines made from early harvested grapes.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, 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. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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CHAPTER 1

LITERATURE REVIEW AND RESEARCH AIMS

The bulk of this literature review was prepared in the first 6 months of candidature, i.e. from May 2014 to October 2014. Thus, it mainly covers literature up to 2014. Minor updates were made in late 2017. The relevant literature beyond this review is included in the introduction sections in Chapter 2 to 5.

Chapter 1. Literature Review and Research Aims

1.1 Sensory Studies and Consumer Acceptance of Lower Alcohol Wine

Over the past two decades, mounting evidence has emerged of rising wine alcohol levels in major wine producing countries around the world, including France (Duchêne and Schneider 2005) and America (Meillon Sophie et al. 2010). In Australia, from 1984 to 2008 the average alcohol level in red wine rose steadily from 12.4% to 14.4% (Godden and Muhlack 2010). This trend is said to be attributable to many factors such as hotter climate, healthier vines, more efficient yeast (Saliba et al. 2013), and most importantly winemakers' fondness for riper grapes, which make more aromatic and full bodied wines that are preferred by consumers and wine experts (Wilkinson and Jiranek 2013). It is common in Australia to find white wine with alcohol level at 12 to 13.5% and red wine at 14 to 15% and occasionally in excess of 16% (Wilkinson and Jiranek 2013).

Concurrently, there is growing market interest in reduced alcohol beverages (Bruwer et al. 2014). In the case of wine, this includes de-alcoholised or alcohol free (<0.5% v/v), low alcohol (0.5% - 1.2% v/v), reduced alcohol (1.2 % - 5.5/6.5 % v/v) and lower alcohol wine (5.5% - 10.5% v/v), although categories vary between countries based on legislation (Pickering 2000, Saliba et al. 2013). Aside from these unconventional wine-based low alcohol beverages, Australian wine export also increased between 2008 and 2012, largely for light wine styles such as Moscato and Sauvignon Blanc, as well as lighter style dry red and white wines (between 10 and 12% alcohol) (Wine Australia https://www.wineaustralia.com/market-insights). This trend has been driven by consumer demand. For example, in the UK, Australia's biggest wine export destination, the 2011 volume growth rate for lower alcohol wine was estimated to be 50% more than the previous year (Bruwer et al. 2014). Factors contributing to this trend include lower prices (due to lower tax excise), drink driving concerns, health impacts, reducing adverse effects of alcohol (feeling out of control or hungover), and wine and food pairing (Meillon Sophie et al. 2010, Saliba et al. 2013, Bruwer et al. 2014).

To capture this potential market, it is important for the wine industry to understand the sensory impact of reducing wine alcohol content, as well as consumer perception and liking of lower-alcohol or lighter style wines. Alcohol has substantial sensory impact on wine. High alcohol levels are positively associated with *bitterness* (Fischer and Noble 1994, Vidal, Courcoux, et al. 2004)

and *hotness* (Gawel et al. 2007, Jones et al. 2008), while it has moderate to little effect on *viscosity* (Nurgel and Pickering 2005, Gawel et al. 2007, Runnebaum et al. 2011). Studies on interactions of wine constituents also showed that ethanol can enhance sugar sweetness in wine (Nurgel and Pickering 2006, Zamora et al. 2006), reduce astringency elicited by grape seed tannin (Vidal, Courcoux, et al. 2004, Fontoin et al. 2008), and affect aroma intensity by altering distribution coefficients between the aqueous solution and the headspace of volatile compounds (Escudero et al. 2007, Goldner et al. 2009).

Various studies have demonstrated that inexperienced wine consumers can not readily notice the effect of small decreases in alcohol levels (2 - 3%) on wine attributes. As a result, these consumers do not perceive that the lower alcohol wines are of lower quality relative to standard wines (Masson and Aurier 2008, Meillon Sophie et al. 2010, Meillon S., Viala D., et al. 2010, King and Heymann 2014). In contrast, experienced consumers, trained panellists and wine experts can perceive quality differences due to small reductions in alcohol levels. King and Heymann (2014) found that trained panellists were able to significantly differentiate a wooded Chardonnay with a 1% alcohol reduction from the original wine in triangle tests. Moreover, from descriptive analysis, the *overall aroma intensity* and *hot mouthfeel* were perceived to be significantly different by panellists, when alcohol differences were a mere 0.4%. Similarly, Meillon and colleagues (2010) explored differences in appreciation of two red wines and their de-alcoholised counterparts (by 1.5% and 3%), using both French wine consumers and wine professionals. Results showed that wine professionals and experienced consumers strongly disliked the de-alcoholised wines for the reduction in sensory attributes such as hotness, sweetness, persistence, and balance, compared to the control wines. In comparison, the less experienced consumers preferred de-alcoholised wines. This segmentation of wine experts and connoisseurs versus novice consumers was further illuminated by a recent study in which 203 consumers and 67 winemakers rated their liking of 12 Australian Shiraz and Cabernet Sauvignon wines (with alcohol levels ranging from 12.5% to 14.8%) (Lattey et al. 2010), in which little or no relationship was found between winemakers' scores and consumer preferences. Whilst winemakers preferred wines of higher alcohol content, the wines rated the highest by consumers were at the lower end of alcohol level, with *hotness* negatively correlated with overall consumer liking. Furthermore, wine style can have a significant impact on perceiving changes in alcohol content. Yu and Pickering (2008) found that ethanol difference threshold (EDT) was lower in Chardonnay wines than in Zinfandel wines, and EDT was also lower in wines with a lower initial ethanol content than in those with higher ethanol content. The authors suggested that lower flavour intensity and complexity, such as in Chardonnay

wines and lower alcohol wines, contributed less ‘noise’ that interfered with perceptions elicited by ethanol.

Although some inexperienced consumers preferred wines with lower alcohol levels, ‘low alcohol’ or ‘de-alcoholised wine’, as an information cue could negatively impact consumer’s perception of wine sensory quality. Masson and Aurier (2008) found that although a group of consumers did not rate ‘standard’ and ‘low-alcohol’ wines differently in a blind tasting, despite a 3% difference in alcohol content. However, when informed which wine was “low-alcohol”, the consumers reduced their ratings for that wine. Meillon and colleagues (2010) found similar responses for consumers they studied. However they also identified a group of consumers who increased their ratings of lower-alcohol wine, when given the ‘de-alcoholised wine’ cue. These results highlighted the importance in segmentation in studies concerning consumer acceptance of wines with lower alcohol levels. Saliba and colleagues (2013) showed that although only 15.8% consumers readily accepted low alcohol wines, this number rose to 40.4% when low alcohol wines tasted the same as a standard wine. From these results, it can be presumed that there is a potential market for wines with lower alcohol content, but it depends on the industry’s ability to produce wines with quality similar to or higher than wines with typical alcohol levels.

1.2 Early Harvest as a Means of Reducing Alcohol Content in Wine

Alcohol reduction can be achieved by several methods employed prior, during or post vinification. Prior to fermentation, sugar content can be lowered by using early harvested grapes or by the use of glucose oxidase enzymes; during fermentation, sugar to ethanol conversion can be partly diverted by some yeast strains, while ethanol can also be removed post-vinification by distillation or membrane technologies (as reviewed by Pickering 2000). A recent study compared three strategies, pre-fermentation dilution of must, non-*Saccharomyces* fermentation and post-fermentation membrane contactor techniques, and demonstrated that non-*Saccharomyces* yeast reduced alcohol levels by merely 0.2% – 0.3%, but produced more volatile compounds and anthocyanins, thereby positively contributing to wine quality (Rolle et al. 2017). In comparison, pre- and post-fermentation techniques achieved 1% – 2% alcohol reduction, but had little or negative impact on wine quality. The aim of this study was to devise a strategy to achieve a considerable reduction in ethanol, i.e. > 2%, without compromising wine quality.

Picking grapes at an early ripening stage seems to be the most intuitive approach to making wines with less alcohol. However, red wines with full body and ripe fruit aroma and flavour dominate the market and are currently preferred by both consumers and wine experts. Picking less mature grapes, i.e. fruit with lower sugar content, may affect wine composition besides just alcohol levels. Bindon and colleagues (2013, 2014) harvested Cabernet Sauvignon grapes from different ripening stages and made wines with alcohol concentrations of 11.8%, 12.9%. 13.6%, 14.2% and 15.5% respectively. Descriptive sensory analysis showed that wines made from earlier harvested fruits were rated higher for red fruit (aroma and palate), red colour, fresh green (aroma and palate) and sourness, but lower for overall fruit, purple colour, viscosity, dark fruit, hotness and bitterness. These results were consistent with a similar study (Heymann et al. 2013).

However, in the follow up sensory evaluation of wines made from consecutive harvests (Bindon, et al. 2014), it was found that although a small percentage of consumers (around 20%) preferred red wines with fresh green and red fruit characters, the overall liking score of all consumers was positively correlated with characteristics of wines made from riper grapes, such as purple colour, dark fruit, overall fruit and viscosity. This trend was especially obvious with wine connoisseurs and experts, in agreement with other studies (Lattey et al. 2010, Williamson et al. 2012, Heymann et al. 2013). On the other hand, sensory attributes characteristic of either overripe or under-ripe grapes, such as hotness, bitterness for the former and *sourness* for the latter, could negatively impact consumer liking (Frøst and Noble 2002, Lattey et al. 2010, Bindon, et al. 2014). These results emphasised that wine quality is not necessarily determined by grape maturity, but rather by a balance of wine composition. Therefore, even with early harvested grapes, by modulation of wine sensory attributes through careful addition of wine supplements, winemakers can mask undesirable characters intrinsic of unripe grapes, to generate a more balanced and thus higher quality wine. This is the key aim of this PhD project.

1.3 Identifying the Origins of Undesirable Characters in Wines Made from Early Harvested Grapes

As outlined in the previous section, wines made from early harvested grapes may exhibit undesirable characters, such as prominent ‘green’ characters and diminished purple colour, fruit intensity and viscosity, i.e. attributes which are negatively associated with wine quality or consumer liking. It is important to identify the origins of these characters in order to devise methods to ameliorate them. However, the nature of these characters can be multi-faceted because

wine is a complex matrix where numerous volatile and non-volatile compounds interact with each other to create the sensory profile of each wine. This section provides a summary of literature on the possible origins of negative sensory attributes in wines made from early harvested grapes.

Usually wines made from early harvested grapes display prominent green characters (both as aromas and on the palate), as well as lower fruit intensity, especially of dark fruit notes. The green aroma in red wine, perceived as being vegetal, is associated with high levels of 3-isobutyl-2-methoxypyrazine, a volatile compound that accumulates in grape skins and then diminishes with time during ripening (Roujou de Boubée et al. 2000, Bindon et al. 2013). Green aromas can be masked by red-/dark-berry aromas in wine (Escudero et al. 2007, Hein et al. 2009, Pineau et al. 2009). In fact, green and red-/dark-berry characters are always shown in dichotomy with each other in the sensory analysis of red wine (Preston et al. 2008, Robinson et al. 2011). The red-/black- berry aromas in red wines are associated with higher levels of ethyl esters, such as ethyl propanoate and ethyl butanoate (Pineau et al. 2009), which are produced by yeast during fermentation. Studies have shown that yeast activity is lower in low sugar (20 ^{o}Brix) grape musts compared to high sugar (26 ^{o}Brix) musts, which can result in lower ethyl ester levels in low sugar fermentations (Bindon et al. 2013). This is probably due to the shorter duration of fermentation, since less sugar is needed to be consumed by yeasts before the fermentation is completed. In summary, the green aroma in wine made from early harvested grapes is likely to be a concerted result of high level of volatiles affording vegetal aromas and low level of volatiles affording red-/black-berry aromas.

Conversely, the origin of green characters on the palate is much more complex and less defined than for green aroma. The palate green character is often referred to as ‘green tannins’, which exhibit unpleasant sensations including harsh astringency, high acidity and herbaceous notes (Herderich et al. 2004, Del Barrio-Galán et al. 2012). Astringency is thought to be a result of reduced lubrication in the oral cavity due to precipitation of salivary protein and is perceptually characterised as ‘drying’ and ‘puckering’ sensations (McRae and Kennedy 2011). It is the distinguishing mouthfeel of red wine and is thought to provide ‘structure’ to the beverage (McRae and Kennedy 2011), but can be perceived negatively if it is not balanced by sweetness and ‘body’ (Kennedy 2008). A recent investigation into the green tannin attribute in Shiraz and Cabernet Sauvignon wines revealed that it is not only characterised by vegetal flavours and harsh astringency, but also highly associated with bitterness as well as reduced body and ripe fruit flavours (Capone et al. 2018). It is not surpring that due to the complex nature of green tannin, no compound has been definitively proven to be responsible for this character. However, empirical

evidence suggested that the green tannin characters were related to grapes from young vineyards, unripe grapes and over extraction of skins and seeds during winemaking; albeit no compound has been definitively proven to be responsible for this character (Herderich et al. 2004). Researchers have speculated that green tannins arise from high concentrations of grape seed tannins in wine, since grape seed tannins are more astringent, harsher, coarser and more drying than grape skin tannin when compared at the same concentration and similar average molecular size (Vidal, Francis, et al. 2003). Studies have shown that grape seed tannins are more readily extractable from under-ripe grapes, while the skin tannins become more abundant in wines made from riper grapes, resulting in an increased skin to seed tannin ratio in wines made from more mature grapes (Harbertson et al. 2002, Peyrot des Gachons and Kennedy 2003, Bindon et al. 2013); albeit this trend depends on the grape variety and vintage (Adams 2006). The level of ripeness also affects the size distribution of tannins in wine, which may in turn affect wine mouthfeel. The mean degree of polymerisation (mDP) of skin tannins increases dramatically from veraison to commercial harvest (Kennedy et al. 2001). At harvest, the average mDP of skin tannins is about 30, while the mDP of seed tannins is about 10 (Cheynier et al. 2006). Increases in mDP resulted in an increase in overall astringency as well as related sensations such as ‘drying’, ‘chalky’, ‘adhesive’ and ‘puckering’ in a wine-like medium (Vidal, Francis, et al. 2003). Interestingly, higher molecular weight tannins are not found to be bitter (Vidal, Francis, et al. 2003) while low molecular tannins (monomers to trimers) are (Peleg et al. 1999). Therefore the ratio of skin to seed tannins in wine may potentially affect the organoleptic characters of the wine. In fact, skin tannin was found to be strongly positively correlated to wine quality (Ristic et al. 2010, Kassara and Kennedy 2011), indicating that the quality of wines made from earlier harvested grapes may depend on its natural tannin composition.

The effect of ripeness on tannin concentration is not clear from literature; tannins have been reported to increase, remain unchanged or decrease during ripening (Kennedy et al. 2000, Downey et al. 2003, Bindon et al. 2013, Bindon, Kassara, et al. 2014). On the other hand, the anthocyanin concentration of wine correlates well with ripeness, i.e. riper grapes have higher levels of anthocyanin and as a result, wines made from riper grapes generally have higher colour intensity (Kennedy et al. 2002, Cadot et al. 2012, Bindon et al. 2013). Wine colour intensity was found to be positively correlated with wine quality score, suggesting it as an indicator which consumers and wine professionals rely on when assessing wine quality (Mercurio et al. 2010, Ristic et al. 2010). Therefore wines made from early harvested grapes may be deemed to be of lower quality due to their lighter colour.

The mouthfeel attribute *viscous* was defined as 'an apparent thickness resulting in pressure required to move the wine around the mouth' in the 'mouthfeel wheel' developed for red wine sensory evaluation (Gawel et al. 2000). Ethanol and glycerol were both speculated to contribute to wine viscosity, but most studies investigating these two compounds at typical wine concentration ranges report little or no effect on viscosity (Nurgel and Pickering 2005, Gawel et al. 2007, Jones et al. 2008, Runnebaum et al. 2011). Interestingly, Pickering and colleagues (1998) reported that the perceived viscosity on the palate did not increase as ethanol concentration increased from 7 to 15%, despite significantly higher physical viscosity, measured by a viscometer, at higher ethanol concentrations. Therefore, although wine made from early harvested grapes had lower levels of ethanol and glycerol than those made from riper grapes (Bindon et al. 2013), they might not be responsible for the perception of low viscosity. Polysaccharides have also been associated with increased wine body. Neutral fractions of wine polysaccharides have been found to increase palate fullness in model wine solutions (Vidal, Francis, Williams, et al. 2004). Furthermore, Bindon and colleagues (2013, 2014a) observed that increased viscosity was positively associated with an increase in wine mannoproteins, one of the major classes of wine polysaccharides. Notably, in these studies, it was also observed that the concentration of total polysaccharides decreased in wine as grapes ripened. This led authors to speculate that the change in viscosity was caused by compositional changes, i.e. increased polysaccharide molecular mass, rather than a simple concentration effect. However, viscosity is not simply characterised by palate weight. In wine sensory evaluation, viscosity is not well distinguished from the concept of 'wine body' (Laguna et al. 2017), which is known to be affected by multimodal sensory perceptions. For example, wine body has been associate with higher ratings of flavour in both red and white wines (Gawel et al. 2007, Niimi et al. 2017). Jones and colleagues (2008) investigated the effect of major wine components, namely ethanol, proteins, polysaccharides, glycerol and volatiles, on wine aroma and flavour, taste and mouthfeel. Although none of these compounds significantly affected viscosity on their own, combinations of polysaccharides-proteins-ethanol, and proteins-ethanol-volatiles, significantly increased viscosity, suggesting viscosity might be the result of synergies between multiple wine components. From these studies, it can be inferred that the low viscosity perceived in wines made of early harvested grapes might reflect the combined effect of low levels of ethanol, volatile compounds and polysaccharides (mannoproteins). Therefore, improving viscosity may require modification of several wine components, concurrently.

1.4 Selective Use of Winemaking Supplements to Modulate the Sensory Properties in Wine Made of Early Harvested Grapes

Based on the literature review in the previous section, several wine components associated with the negative sensory properties of wines made from early harvest grapes were identified. In general, wine made from early harvested grapes is characterised by prominent green aromas, increased acidity and a concurrent lack of fruity aroma/flavour and desirable mouthfeel characters, such as viscosity. These sensory properties stem from both the concentration and composition of certain components of wine. The objective of this study is to moderate these qualities through modifying wine composition using commercial wine supplements. There are various winemaking supplements (i.e. additives and processing aids) on the market that winemakers can select to enhance different aspects of wine quality. The full list of approved additives for wine production in Australia is reported in Table 1, adapted from the latest version of the Australia New Zealand Food Standard Code - Standard 4.5.1 – Wine Production Requirements. It can be seen from Table 1 that none of the permitted supplements can result in the direct enhancement of fruit aroma and/or flavour, or targeted removal of green characters. However, some additives can be used to effectively modify the macromolecule composition of wine, which could result in improvements to wine colour and mouthfeel, as well as simultaneously affecting the volatile composition of wine. Three wine supplements, namely pre-fermentation maceration enzymes, oenotannins and mannoproteins, were therefore selected to enhance the tannin and polysaccharide composition of wine made from early harvest. The rationale behind this selection is discussed below.

1.4.1 Pre-fermentation maceration enzyme

Commercial pre-fermentation maceration enzyme preparations (referred to as maceration enzymes below) usually have high pectolytic activity, sometimes with secondary enzymatic activities including degradation of cellulose, hemicellulose and pectin (Guadalupe et al. 2007, Ugliano 2009). These enzyme preparations are added to fermentations immediately after the grapes are crushed, to facilitate degradation of grape cell walls and extraction of cell contents. Most studies concerning maceration enzymes focus on their ability to enhance polyphenolic extraction from grapes, since grape cell wall materials are regarded as a major barrier for the release of these compounds (Sacchi et al. 2005). Previous studies on maceration enzymes are in agreement with each other with regards to the ability of maceration enzymes to enhance tannin

Table 1. Supplements legally permitted to be used in wine production in Australia

Additives	Processing aids	
Ascorbic acid	Activated carbon	Hydrogen peroxide
Carbon dioxide	Agar	Ion exchange resins
Citric acid	Alginates, calcium and potassium salts	Isinglass
Dimethyl dicarbonate	Ammonium phosphates	Lysozyme
Erythorbic acid	Argon	Milk and milk products
Grape juice including concentrated grape juice	Bentonite	Nitrogen
Grape skin extract	Calcium carbonate	Oak
Gum Arabic	Calcium tartrate	Oxygen
Lactic acid	Carbon dioxide	Perlite
Malic acid	Cellulose	Phytates
Metatartaric acid	Collagen	Plant proteins (selected)
Mistelle	Copper sulphate	Polyvinyl polypyrrolidone
Potassium sorbate	Cultures of micro-organisms	Potassium carbonate
Potassium sulphites	Cupric citrate	Potassium ferrocyanide
Sorbic acid	Diatomaceous earth	Potassium hydrogen carbonate
Sulphur dioxide	Dimethylpolysiloxane	Potassium hydrogen tartrate
Tannins	Egg white	Silicon dioxide
Tartaric acid	Enzymes	Thiamin chloride
Yeast mannoproteins	Gelatine	Thiamin hydrochloride

extraction from grapes (Watson et al. 1999, Guerrand and Gervais 2002, Bautista-Ortín et al. 2005, Ducasse et al. 2010). Additionally, the maceration enzymes seem to modify the composition of wine tannin. Ducasse and colleagues (2010) reported higher mDP and higher percentages of epigallocatechin (an extension unit that only exists in grape skin tannin) in enzyme treated wines compared to control wines, indicating enzymes can extract more phenolic compounds from skins than seeds. However another study (Busse-Valverde et al. 2010) found that maceration enzyme had little or no effect on wine tannin mDP and epigallocatechin subunit content. Maceration

enzymes would be expected to increase skin tannin extraction, since it is added at the beginning of fermentation maceration, i.e. when skin polyphenolics are extracted into wine, whereas seed tannin extraction happens towards the end of fermentation, after lipid coating on seeds needs has been eliminated (Romero-Cascales et al. 2012). It is worth studying the effect of maceration enzymes on wine tannin composition because as mentioned above, skin tannin composition is highly correlated to wine quality. Although this correlation may not be causal, higher skin tannin extraction can only benefit the quality of a light wine, through increased colour, tannin and flavour extraction. This is particularly true of flavour compounds which exist in the grape skin, such as β-damacenone, a C_{13}-norisoprenoid compound, which has been shown to enhance fruity aroma and flavour in red wine (Pineau et al. 2007).

Contrary to tannin extraction, the effect of maceration enzymes on anthocyanin extraction seems to be more complicated. Several studies were unable to observe higher concentrations of anthocyanin monomers in enzyme treated wines, compared to control wines, at the end of fermentation. Furthermore, monomeric anthocyanin concentrations continued to decrease during bottle aging, such that enzyme treated wines eventually had lower monomeric anthocyanins than their corresponding control wines (Wightman et al. 1997, Watson et al. 1999, Parley et al. 2001, Bautista-Ortín et al. 2005). These authors also observed that the trend of diminishing monomeric anthocyanin was accompanied by an inverted trend of increasing polymeric anthocyanins, namely, the enzyme treated wines had more polymeric pigment than control wines. This phenomenon may be attributed to a glycosidase impurity in pectolytic enzyme preparations that degraded monomeric anthocyanins to aglycons (Guérin et al. 2009). More importantly, it may be attributed to the higher extraction of tannins which promoted polymerisation of anthocyanins, forming stable red and blue pigments (Parley et al. 2001). Changes in pigment composition were also reflected by higher colour intensity and lower browning hue in enzyme treated wines after several months of bottle aging (Parley et al. 2001, Guerrand and Gervais 2002, Bautista-Ortín et al. 2005, Ducasse et al. 2010). Formation of polymeric pigments not only contributes to colour intensity and stability, it can also modify the perception of astringency in wine (McRae et al. 2012). Incorporation of anthocyanins into tannin polymers has been shown to reduce the astringency of model wine (Vidal, Francis, Noble, et al. 2004), likely due to an interruption of hydrophobic interactions between tannin and salivary proteins (McRae et al. 2010).

Despite near consensus on the ability of maceration enzymes to enhance phenolic extration and colour stability, this effect seems to depend on several factors, including: vintage (Ducasse et al.

2010); grape variety (Wightman et al. 1997); enzyme preparation (brand) (Wightman et al. 1997, Watson et al. 1999, Bautista-Ortín et al. 2005); and dosage (Bautista-Ortín et al. 2005). One interesting phenomenon oberserved by researchers was that the effect of enzyme addition was more prominent in grapes that contain less natural phenolic compounds. For example, Ducasse and colleagues (2010) suggested that the less ripened grapes seemed to benefit from enzyme addition, whereas riper grapes did not. Similarly Wightman et al. (1997) showed that the colour of Pinot Noir wines was modified by enzymes to a greater extent, than for Cabernet Sauvignon wines.

As for the sensory impact of maceration enzymes the existing studies are scarce and with variable findings. Guerrand and Gervais (2002) reported that the enzyme treatment increased in wine the concentrations of norisoterpenoids (especially β-damascenone) and IBMP, both of which originate from grape skins. They also reported that although the enzyme treated wines and control wines were similar at bottling, after a year of aging in bottle, the treated wines were preferred over control wine, for that the control wine was 'green' and 'vegetable' but the enzyme treated wine had more colour, complexity and a stronger aftertaste. In the study conducted by Bautista-Ortín et al. (2005), only one enzyme preparation achieved similar results; other enzyme preparations led to more intese herbaceous notes, astringency and bitterness, compared to control wines, and thus they were deemed to have less 'equilibrium' and 'harmony' than the control. In another study, pectolytic enzymes led to enhanced 'dark fruit' characters and increased astringency in Merlot wine, as well as to more intense fruit characters and 'velvety' mouthfeel perception in Cabernet Sauvignon wine (Canal-Llaubéres and Reynolds 2010).

1.4.2 Oenotannin

Oenotannin is an exogenous tannin that can be added to wine to boost tannin concentrations or to modify tannin composition. The majority of tannins in red wine are extracted from grape skins and seeds during fermentation, with a small proportion derived from oak contact (McRae and Kennedy 2011). Grape derived tannins are condensed polymers of flavan-3-ols, containing subunits of (+)-catechin, (–)-epicatechin, (–)-epigallocatechin and (–)-epicatechin gallate (Adams 2006). In contrast, oak tannins are hydrolysable tannins, i.e. polymers of D-glucose comprising hydroxyl groups esterified with either gallic acid or hexahydroxydiphenic acid (which spontaneously lactonise to ellagic acid), giving rise to gallo- or ellagitannins (Puech et al. 1999). These two types of tannins represent the main forms of oenotannins as well. In addition,

oenotannins can also be derived from other botanical sources, such as chestnut, tara or galla (Versari et al. 2013). Oenotannin is permitted for use in the European Union as a processing aid for clarification and fining of must and wine (Versari et al. 2013), as it can remove haze-forming proteins and metals (Laghi et al. 2010). However, in Australia and New Zealand, oenotannin is permitted as an additive (Table 1). A survey of the Australian wine industry found that winemakers' use of oenotannins depends on the natural tannin content of grapes (Hill and Kaine 2007). The survey further sorted winemakers into four categories based on the primary objectives they sought to achieve by applying oenotannins:

(1) Colour stabilisation. Winemakers in this category mainly use oenotannins to promote colour intensity and stability in wine made from grapes grown in hot regions, which are naturally poor in colour.
(2) Masking faults. Winemakers in this category mainly use oenotannins with grapes from cool regions, in order to mask 'green tannin' characters (e.g. bitter, astringent and/or vegetal characters). In this scenario, oenotannins are thought to 'provide other flavours or soften existing flavours'.
(3) Creating specific wine styles. Winemakers in this category generally use oenotannins in their wines to fine tune the sensory properties, i.e. to make wines 'more complex' or 'richer and more tannic'.
(4) General risk management. Winemakers in this category use oenotannins as a pre-emptive measure to avoid risks in wine quality associated with objectives 1, 2 and 3.

From this survey, it can be inferred that empirically, oenotannin is a good candidate for modulating wine made from early harvested grapes, as it may promote colour stability, enhance mouthfeel characters, and mask green tannin attributes.

However, a recent review summarised the scientific research concerning the ability for oenotannin to stabilise colour, and highlighted conflicting results reported to date (Versari et al. 2013). As with maceration enzymes, the effect of oenotannins on colour stability seems to depend on many factors, including: vintage (Bautista et al. 2007); dosage (Harbertson et al. 2008, Neves et al. 2010); the timing of addition (Neves et al. 2010, Canuti et al. 2012); and the choice of product (Neves et al. 2010, Canuti et al. 2012). Additionally, similar to observations to maceration enzymes, the effect of oenotannins on colour stabilisation is most evident when the amount of native polymerised tannins in grapes is insufficient to stabilise the quantity of anthocyanins present in wine during fermentation (Bautista et al. 2007, Neves et al. 2010). This makes the use

of oenotannin even more applicable in the early harvest fruit scenario. In fact, one study involving the addition of oenotannins to Sangiovese grapes at different levels of maturity found that the best colour stabilisation was achieved for under-ripe grapes (Canuti et al. 2012). In terms of the timing of addition, one study found that oenotannin addition before fermentation achieved better colour stabilisation than addition post-fermentation (Canuti et al. 2012), which was attributed to the high formation rate of polymeric pigments during the fermentation process (Parker et al. 2007). However, another study found that pre-fermentation addition resulted in a loss of oenotannin compared with addition post-fermentation (Neves et al. 2010), which was attributed to loss of oenotannin due to binding with plant cell walls, or other materials such as proteins and yeast cell walls (Sun et al. 1999, Bautista-Ortín et al. 2014). Thus, the optimal timing for addition of oenotannin is not clear and warrants further investigation.

Although the studies reviewed so far do not agree on the extent to which oenotannins can improve colour stability, these studies reported a change in wine phenolic composition due to oenological tannin addition, with only one exception (Parker et al. 2007). Wine astringency is highly associated with tannin concentration (Kennedy et al. 2006) and mDP (Vidal, Francis, et al. 2003), and negatively associated with the degree of incorporation of anthocyanin (Vidal, Francis, Noble, et al. 2004). It is therefore expected that changes in wine tannin composition should impact astringency perception. Many studies have found that supplementing oenotannin in wine at manufacturer recommended dosage (0.2 – 0.4 g/L) results in an increase in overall astringency (Bautista-Ortín et al. 2005, Parker et al. 2007, Harbertson et al. 2008, Chen et al. 2016, Chen et al. 2018). However, it has also been found that oenological tannin can result in bitterness (Bautista-Ortín et al. 2005), especially when it was added an overtly high concentration, i.e. 0.8 g/L (Harbertson et al. 2008). One drawback of oenological tannin supplementation may be that it can promote vegetal flavours in wine (Bautista-Ortín et al. 2005, Harbertson et al. 2008). A potential method for reducing this impact is to use oenological tannin at lower concentrations, since it has been suggested that the astringency associated with oenotannin addition depends on the original matrix of wine; i.e. wines containing lower levels of natural tannin and total phenolics are more affected by the addition (Rinaldi et al. 2010). Oenological tannin was selected for inclusion in the current study given it might modify colour stability and mouthfeel characters, astringency in particular, in wines made from early harvested grapes.

1.4.3 Yeast Autolysis and Mannoproteins

Mannoproteins are one of the major polysaccharides in wine, consisting of mannan, glucan and proteins (Vidal, Williams, et al. 2003, Guadalupe et al. 2010). The concentration of mannoproteins in wine typically ranges from 100 to 150 mg/L (Pérez-Serradilla and de Castro 2008) and may represented 35% of total wine polysaccharides (Vidal, Williams, et al. 2003). Mannoproteins are macromolecules present in yeast cell walls and are released during autolysis (the degradation of cells or tissues by their own enzymes) of dead yeast cells during extended aging in the presence of lees. Wine lees are the debris of winemaking material formed at the bottom of wine fermentation and maturation vessels (tanks, barrels, etc.). It comprises dead yeast and bacteria cells, tartrate crystals and grape cell debris (Salmon et al. 2002). Aging on lees is a traditional practice in France for white wine. For example, production of premium white wines from Burgundy requires aging on lees (Charpentier 2010). It is also a signature of Muscadet wines. In Muscadet, where wines are acidic, bone-dry and neutral, *sur lie* practice was employed to 'enhance fruit' and 'add yeasty-roundness' (Stevenson 2005). Muscadet *sur lie* AOC wines must remain on gross lees for at least a winter or an additional 7 to 8 months if targeting for a fuller style. In recent years, maturation on lees has also been more frequently carried out in red wine production due to its positive effect on wine structure and mouthfeel (Rodríguez et al. 2005).

Yeast autolysis is a slow process that takes months or even years to complete, due to the low pH of wine and cool storage temperatures (Martínez-Rodríguez et al. 2001). Salmon and colleagues (2003) found that in model wine, 48-hour heat treatment of lees released the same level of mannoproteins as that achieved by 6 months of aging. A similar result was also reported for Champagne aging (Martínez-Rodríguez et al. 2001). In order to achieve high mannoprotein levels in wine in a fast and economical way, commercial mannoprotein products prepared from induced fast yeast autolysis can be purchased for addition to wine.

One area that has attracted most researchers' attention is the interaction between mannoprotein and wine polyphenolics. It was observed that yeast mannoproteins can hinder aggregation of seed tannins and consequently prevent their precipitation (Riou et al. 2002). This result led researchers to speculate that mannoprotein addition could prevent formation of highly polymerised phenolic compounds and their eventual precipitation, leading to better colour stability. However, a subsequent study demonstrated that only low molecular weight mannoprotein (average around 50 kD) could prevent seed tannin aggregation, which was attributed to steric hindrance; in contrast, high molecular weight mannoprotein (around 330 kD) had no effect (Poncet-Legrand et al. 2007). However as both studies were performed in model wine for only a short period of time (up to 500

hours), it remains unclear in terms of the interactions between mannoproteins and polyphenolics during extended aging, and how they impact on wine quality. Contradictory results have been reported to date in this area. Some authors observed lower concentrations of both sulphite bleaching resistant pigments (leading to lower colour intensity and stability) and tannin, in wine made with wither mannoprotein addition (Guadalupe et al. 2007, Guadalupe and Ayestarán 2008, Guadalupe et al. 2010) or ageing on lees (Mazauric and Salmon 2005, Rodríguez et al. 2005, Palomero et al. 2007). Conversely, other authors observed increases in polymeric anthocyanin and tannin concentrations in mannoprotein treated wines (Escot et al. 2001, Del Barrio-Galán et al. 2012). However, it is hard to compare the results from these studies, given the substantial variation in the experimental design. The main difference relates to the source of mannoprotein, being either from lees autolysis or commercial products. Even among studies using the same mannoprotein source, there are secondary factors which can affect the outcome of the experiments. For yeast autolysis, factors include oxygen incorporation (Salmon et al. 2002) which can be influenced by storage vessel (tank vs. oak barrel), batônnage regime, duration of aging (Salmon et al. 2003) and the yeast strain used (Escot et al. 2001). For mannoprotein products, the timing of addition and product profile (size distribution and percentage of protein) have substantial impact (Del Barrio-Galán et al. 2012).

Due to interactions with phenolic compounds, mannoproteins have been considered to have significant impact on wine organoleptic characters, especially tannin related characters such as bitterness and astringency. Mannoproteins have been shown to reduce bitterness and enhance palate fullness in model wine (Vidal, Courcoux, et al. 2004, Vidal, Francis, Williams, et al. 2004). Nevertheless, the effect of mannoproteins on mouthfeel in real wine has not been explored in detail. Most researchers speculate that mannoproteins should decrease astringency, either based on a lower total phenolic index (adsorption at 280 nm) or a lower gelatine index (indicating lower tannin content reactive to saliva protein) (Escot et al. 2001, Guadalupe and Ayestarán 2008). Only a handful of studies reported sensory data following mannoprotein addition to wine. Rodríguez et al. (2005) found that mannoprotein addition significantly boosted mouthfeel in a light bodied red wine, but not in a full bodied red wine. As for the effect of mannoprotein on astringency, Del Barrio-Galán et al. (2012) found the addition of commercial mannoproteins reduced the green tannin character on the palate. Additionally, Guadalupe et al. (2007) found that wines made with mannoproteins addition were rated significantly higher for sweet and roundness perception than the corresponding control wines. Mannoprotein addition therefore shows promise for enhancing

wine viscosity and reducing green tannin palate character, which are highly applicable to wines made of early harvested grapes.

Other than interactions with phenolic compounds, mannoproteins were also found to retain a selection of volatile compounds, especially those hydrophobic in nature (Lubbers, Charpentier, et al. 1994, Lubbers, Voilley, et al. 1994, Chalier et al. 2007). Guadalupe and colleagues (2007) found that Tempranillo wines made with mannoprotein addition were dominated by oak, smoky and mineral aromas in contrast to fruit-dominated control wines, which the authors attributed to mannoproteins' ability to retain certain volatiles. Chalier and colleagues (2007) also observed that volatiles were affected differently by mannoprotein fractions which differed in protein and polysaccharide constitution. These results suggest that aroma and flavour modification can be achieved by adding commercial mannoproteins products to wine. Besides affecting wine volatiles, commercial mannoprotein products also contain volatile compounds intrinsic to yeast autolysis, most of which afford 'yeast like' characters such as sweaty and cheesy (Comuzzo et al. 2006). The effect of these volatiles on overall wine perception depends on the dosage of products added. However, an upper limit of 400 mg/L of MP addition is imposed in Australia wines, based on an agreement between Australia and the European Community on Trade in Wine (Food Standards Australia and New Zealand). At or below this dosage, Comuzzo and colleagues (2006) did not find any adverse effect of mannoproteins on red wine aroma and flavour.

As discussed above, three commercial supplements, maceration enzyme, oenotannin and mannoprotein, were chosen because they showed potential to mitigate deficiencies in the compositions and sensory properties of wines made from sub-optimal maturity grapes. The main criteria for selecting these additives were their potential for improving colour stability, enhancing viscous mouthfeel and reducing mouthfeel characters associated with green tannin. In addition, all three products showed potential to modify wine volatile composition as a side effect. The mouthfeel of wine is not limited to tactile sensations, but is likely to be a result of multimodal sensory interactions of touch, flavour and taste. Therefore, the combined application of supplements, to modify several aspects of wine composition concurrently, will also be explored in the current study.

1.5 Summary of Research Aims

This project aims to modify the chemical composition and sensory properties of wines made from sub-optimal maturity Shiraz grapes (obtained through early harvest). In particular, wine colour stability and mouthfeel characters such as astringency and viscosity to be modified by altering wine tannin and polysaccharide composition, through the application of three commercial supplements, a maceration enzyme, an oenotannin and a mannoprotein. A key aim is to make wines from early harvested grapes that resemble those made from mature fruit, in terms of mouthfeel characters and wine colour parameters. This project will provide winemakers with strategies to better manage wine alcohol content, without compromising quality. Project aim will be realised through four individual studies comprising the following objectives:

1. To examine the effect of selected supplements used either individually or in combination, in Shiraz wines made from early harvested grapes;
2. To understand the compositional variation amongst supplements available on the Australian market;
3. To explore the impact of oenotannin, mannoprotein and ethanol on wine sensory properties;
4. To explore interactions between commercial polysaccharide supplements and grape derived tannin, and their impact on the colloidal state of wine.

<u>Objective 1. To examine the effect of selected supplements</u>

Wine quality can be significantly impacted by tannin and polysaccharide composition, which can in turn be influenced by grape maturity and winemaking practices. A study was designed to explore the impact of three commercial wine additives, a maceration enzyme, an oenotannin and a mannoprotein, on the composition and sensory properties of red wine; in particular, in mimicking the mouthfeel associated with wine made from riper grapes. Shiraz grapes were harvested at total soluble solids of 24 °Brix and 28 °Brix, and the former vinified with commercial additives introduced either individually or in combination. The resulting wines were compared with those made from fruit of the later harvest. Compositional analyses of finished wines included tannin and polysaccharide concentration, composition and size distribution by high performance liquid chromatography, while the sensory profiles of wines were assessed by descriptive analysis. This study is reported in Chapter 2. Objective 2, 3 and 4 were derived from the results of this study.

Objective 2. To understand the compositional variability amongst supplements

Oenotannin and mannoprotein additives are used to achieve protein, cold or colour stability in wine, or alternatively, to modify wine sensory properties. In most cases, only basic compositional information and purported effects in wine are provided by the manufacturer. In order to understand the compositional diversity of commercial supplements, 14 grape-based enotannins and 8 mannoproteins were sourced from the suppliers in the Australian market. Their composition and molecular size distribution were determined and compared. This study is summarised in Chapter 3.

Objective 3. To explore the impact of oenotannin, mannoprotein and ethanol on wine sensory

This study is an extension of objective 1 and further explores the effects of oenotannin and mannoprotein additives on wine composition and mouthfeel characters. Three commercial products, two oenotannins (one derived from grape seed and the other from skin) and one mannoprotein, were selected for further trials based on the screening process reported in Chapter 3, i.e. because they showed compositional characters that typically define their counterparts isolated from grapes and wine. Two Shiraz wines, containing 11.5% and 14.5% v/v alcohol respectively, were made. Unlike the study in Chapter 2, same supplementation regimes were introduced into wines made from both unripe and mature grapes. In this way, a series of wines comprising different ethanol, tannin and polysaccharide concentrations and/or compositions were created, which enabled any interactions attributable to these three wine components to be evaluated using sensory analysis techniques. This study is reported in Chapter 4.

Objective 4. To Explore Interactions of Commercial Polysaccharides with Grape Seed Tannin

This study explored the impact of commercial polysaccharide addition on the colloidal state of wine. To this end, a mannoprotein and an arabinogalactan were purified from two commercial additives. Their interactions with a tannin fraction (isolated from grape seed) were characterized in model wine solutions of different ethanol levels, by nanoparticle tracking analysis (NTA) and compared against UV-vis spectroscopy and dynamic light scattering. These analyses measured aggregate formation and particle size evolution, as a result of tannin and polysaccharide interactions. This study is reported in Chapter 5.

Adams DO. 2006. Phenolics and ripening in grape berries. Am. J. Enol. Vitic. 57: 249–256.
Bautista-Ortín AB, Cano-Lechuga M, Ruiz-García Y, and Gómez-Plaza E. 2014. Interactions between grape skin cell wall material and commercial enological tannins. Practical implications. Food Chem. 152: 558–565.
Bautista-Ortín AB, Martínez-Cutillas A, Ros-García JM, López-Roca JM, and Gómez-Plaza E. 2005. Improving colour extraction and stability in red wines: the use of maceration enzymes and enological tannins. Int. J. Food Sci. Technol. 40: 867-878.
Bautista R, Fernández E, and Falqué E. 2007. Effect of the contact with fermentation-lees or commercial-lees on the volatile composition of white wines. Eur. Food Res. Technol. 224: 405-413.
Bindon KA, Holt H, Williamson PO, Varela C, Herderich M, and Francis IL. 2014. Relationships between harvest time and wine composition in *Vitis vinifera* L. cv. Cabernet Sauvignon 2. Wine sensory properties and consumer preference. Food Chem. 154: 90–101.
Bindon KA, Kassara S, Cynkar WU, Robinson EMC, Scrimgeour N, and Smith PA. 2014. Comparison of Extraction Protocols To Determine Differences in Wine-Extractable Tannin and Anthocyanin in *Vitis vinifera* L. cv. Shiraz and Cabernet Sauvignon Grapes. J. Agric. Food Chem. 62: 4558-4570.
Bindon KA, Varela C, Kennedy JA, Holt H, and Herderich M. 2013. Relationships between harvest time and wine composition in *Vitis vinifera* L. cv. Cabernet Sauvignon 1. Grape and wine chemistry. Food Chem. 138: 1696–1705.
Bruwer J, Jiranek V, Halstead L, and Saliba A. 2014. Lower alcohol wines in the UK market: some baseline consumer behaviour metrics. Brit Food J 116: 5-5.
Busse-Valverde N, Gomez-Plaza E, Lopez-Roca JM, Gil-Munoz R, Fernandez-Fernandez JI, and Bautista-Ortin AB. 2010. Effect of different enological practices on skin and seed proanthocyanidins in three varietal wines. J. Agric. Food Chem. 58: 11333–11339.
Cadot Y, Caillé S, Samson A, Barbeau G, and Cheynier V. 2012. Sensory representation of typicality of Cabernet Franc wines related to phenolic composition: Impact of ripening stage and maceration time. Anal. Chim. Acta 732: 91–99.
Canal-Llaubéres R, and Reynolds A. 2010. Enzymes and wine quality. Managing Wine Quality 2: 93 - 132.
Canuti V, Puccioni S, Giovani G, Salmi M, Rosi I, and Bertuccioli M. 2012. Effect of oenotannin addition on the composition of Sangiovese wines from grapes with different characteristics. Am. J. Enol. Vitic. 63: 220 - 231
Capone D, Pearson W, Bindon K, Kassara S, Solomon M, Bey L, Francis L. 2018. What makes a red wine green? Wine & Viticulture Journal 2018(2): 32 - 35
Chalier P, Angot B, Delteil D, Doco T, and Gunata Z. 2007. Interactions between aroma compounds and whole mannoprotein isolated from *Saccharomyces cerevisiae* strains. Food Chem. 100: 22-30.
Charpentier C. 2010. Ageing on lees (sur lie) and the use of speciality inactive yeast during wine fermentation. *In* Managing Wine Quality: Oenology and Wine Quality. AG Reynolds (ed.), pp. 164-187, Cambridge, UK.
Chen K, Escott C, Loira I, del Fresno J, Morata A, Tesfaye W, Calderon F, Benito S, and Suárez-Lepe J. 2016. The effects of pre-fermentative addition of oenological tannins on wine components and sensorial qualities of red wine. Molecules 21: 1445.
Chen K, Escott C, Loira I, del Fresno JM, Morata A, Tesfaye W, Calderon F, Suárez-Lepe JA, Han S, and Benito S. 2018. Use of non-*Saccharomyces* yeasts and oenological tannin in red winemaking: Influence on colour, aroma and sensorial properties of young wines. Food Microbiol. 69: 51-63.

Cheynier V, Dueñas-Paton M, Salas E, Maury C, Souquet J-M, Sarni-Manchado P, and Fulcrand H. 2006. Structure and properties of wine pigments and tannins. Am. J. Enol. Vitic. 57: 298-305.

Comuzzo P, Tat L, Tonizzo A, and Battistutta F. 2006. Yeast derivatives (extracts and autolysates) in winemaking: Release of volatile compounds and effects on wine aroma volatility. Food Chem. 99: 217-230.

Del Barrio-Galán R, Pérez-Magariño S, Ortega-Heras M, Guadalupe Z, and Ayestarán B. 2012. Polysaccharide characterization of commercial dry yeast preparations and their effect on white and red wine composition. LWT - Food Sci. and Technol. 48: 215–223.

Downey MO, Harvey JS, and Robinson SP. 2003. Analysis of tannins in seeds and skins of Shiraz grapes throughout berry development. Aust. J. Grape Wine Res. 9: 15-27.

Ducasse MA, Canal-Llauberes RM, de Lumley M, Williams P, Souquet JM, Fulcrand H, Doco T, and Cheynier V. 2010. Effect of macerating enzyme treatment on the polyphenol and polysaccharide composition of red wines. Food Chem. 118: 369–376.

Duchêne E, and Schneider C. 2005. Grapevine and climatic changes: a glance at the situation in Alsace. Agron. Sustain. Dev. 25: 93-99.

Escot S, Feuillat M, Dulau L, and Charpentier C. 2001. Release of polysaccharides by yeasts and the influence of released polysaccharides on colour stability and wine astringency. Aust. J. Grape Wine Res. 7: 153–159.

Escudero A, Campo E, Farina L, Cacho J, and Ferreira V. 2007. Analytical characterization of the aroma of five premium red wines. Insights into the role of odor families and the concept of fruitiness of wines. J. Agric. Food Chem. 55: 4501-4510.

Fischer U, and Noble AC. 1994. The effect of ethanol, catechin concentration, and pH on sourness and bitterness of wine. Am. J. Enol. Vitic. 45: 6-10.

Fontoin H, Saucier C, Teissedre P-L, and Glories Y. 2008. Effect of pH, ethanol and acidity on astringency and bitterness of grape seed tannin oligomers in model wine solution. Food. Qual. Prefer. 19: 286-291.

Food Standards Australia New Zealand 2008. Final assessment report: A605 – yeast mannoproteins as a food additives for wine. (http://www.foodstandards.gov.au/code/applications/Documents/A605_Mannoproteins_FAR.pdf)

Frøst MB, and Noble AC. 2002. Preliminary study of the effect of knowledge and sensory expertise on liking for red wines. Am. J. Enol. Vitic. 53: 275-284.

Gawel R, Oberholster A, and Francis IL. 2000. A 'Mouth-feel Wheel': terminology for communicating the mouth-feel characteristics of red wine. Aust. J. Grape Wine Res. 6: 203-207.

Gawel R, Sluyter SV, and Waters EJ. 2007. The effects of ethanol and glycerol on the body and other sensory characteristics of Riesling wines. Aust. J. Grape Wine Res. 13: 38-45.

Godden P, and Muhlack R. 2010. Trends in the composition of Australian wine. Aust. N.Z. Grapegrower Winemaker: 47.

Goldner MC, Zamora MC, Di Leo Lira P, Gianninoto H, and Bandoni A. 2009. Effect of ethanol level in the perception of aroma attributes and the detection of volatile compounds in red wine. J. Sens. Stud. 24: 243-257.

Guadalupe Z, and Ayestarán B. 2008. Effect of commercial mannoprotein addition on polysaccharide, polyphenolic, and color composition in red wines. J. Agric. Food Chem. 56: 9022-9029.

Guadalupe Z, Martínez L, and Ayestarán B. 2010. Yeast mannoproteins in red winemaking: effect on polysaccharide, polyphenolic, and color composition. Am. J. Enol. Vitic. 61: 191-200.

Guadalupe Z, Palacios A, and Ayestaran B. 2007. Maceration enzymes and mannoproteins: A possible strategy to increase colloidal stability and color extraction in red wines. J. Agric. Food Chem. 55: 4854-4862.

Guérin L, Sutter D-H, Demois A, Chereau M, and Trandafir G. 2009. Determination of activity profiles of the main commercial enzyme preparations used in winemaking. Am. J. Enol. Vitic. 60: 322-331.

Guerrand D, and Gervais J-P. 2002. Extraction of red wine phenolics during fermentation: A new pectinase preparation. Aust. and N.Z. grapegrower winemaker: 60-64.

Harbertson JF, Hodgins RE, Thurston LN, Schaffer LJ, Reid MS, Landon JL, Ross CF, and Adams DO. 2008. Variability of tannin concentration in red wines. Am. J. Enol. Vitic. 59: 210-214.

Harbertson JF, Kennedy JA, and Adams DO. 2002. Tannin in skins and seeds of Cabernet Sauvignon, Syrah, and Pinot noir berries during ripening. Am. J. Enol. Vitic. 53: 54-59.

Hein K, Ebeler SE, and Heymann H. 2009. Perception of fruity and vegetative aromas in red wine. J. Sens. Stud. 24: 441-455.

Herderich M, Bell S, Holt H, Ristic R, Birchmore W, Thompson K, and Iland P. Grape maturity and tannins: the impact of viticultural treatments on grape and wine tannins. *In* Proceedings of the Proc. 12 th Australian Wine Industry Technical Conference, Melbourne, Victoria. pp. 79-84.

Heymann H, LiCalzi M, Conversano MR, Bauer A, Skogerson K, and Matthews M. 2013. Effects of extended grape ripening with or without must and wine alcohol manipulations on Cabernet Sauvignon wine sensory characteristics. S. Afr. J. Enol. Vitic. 34: 86-99.

Hill M, and Kaine G. 2007. The market potential of tannin related innovations in the wine industry. Victorian Department of Primary Industries, Tatura.

Jones PR, Gawel R, Francis IL, and Waters EJ. 2008. The influence of interactions between major white wine components on the aroma, flavour and texture of model white wine. Food. Qual. Prefer. 19: 596-607.

Kassara S, and Kennedy JA. 2011. Relationship between red wine grade and phenolics. 2. Tannin composition and size. J. Agric. Food Chem. 59: 8409–8412.

Kennedy JA. 2008. Grape and wine phenolics: Observations and recent findings. Ciencia e investigación agraria 35:107 – 120

Kennedy JA, Ferrier J, Harbertson JF, and des Gachons CP. 2006. Analysis of tannins in red wine using multiple methods: correlation with perceived astringency. Am. J. Enol. Vitic. 57: 481-485.

Kennedy JA, Hayasaka Y, Vidal S, Waters EJ, and Jones GP. 2001. Composition of grape skin proanthocyanidins at different stages of berry development. J. Agric. Food Chem. 49: 5348-5355.

Kennedy JA, Matthews MA, and Waterhouse AL. 2000. Changes in grape seed polyphenols during fruit ripening. Phytochemistry 55: 77-85.

Kennedy JA, Matthews MA, and Waterhouse AL. 2002. Effect of maturity and vine water status on grape skin and wine flavonoids. Am. J. Enol. Vitic. 53: 268-274.

Kennedy JA, and McRae JM. 2011. Wine and grape tannin interactions with salivary proteins and their impact on astringency: a review of current research. Molecules. 16: 2348 - 2364

King ES, and Heymann H. 2014. The effect of reduced alcohol on the sensory profiles and consumer preferences of white wine. J. Sens. Stud. 29: 33-42.

Laghi L, Parpinello GP, Rio DD, Calani L, Mattioli AU, and Versari A. 2010. Fingerprint of enological tannins by multiple techniques approach. Food Chem. 121: 783-788.

Laguna L, Bartolomé B, and Moreno-Arribas MV. 2017. Mouthfeel perception of wine: Oral physiology, components and instrumental characterization. Trends Food Sci. Technol. 59: 49-59.

Lattey KA, Bramley BR, and Francis IL. 2010. Consumer acceptability, sensory properties and expert quality judgements of Australian Cabernet Sauvignon and Shiraz wines. Aust. J. Grape Wine Res. 16: 189-202.

Lubbers S, Charpentier C, Feuillat M, and Voilley A. 1994. Influence of yeast walls on the behavior of aroma compounds in a model wine. Am. J. Enol. Vitic. 45: 29-33.

Lubbers S, Voilley A, Feuillat M, and Charpentier C. 1994. Influence of mannoproteins from yeast on the aroma intensity of a model wine. LWT-Food Sci. and Technol. 27: 108-114.

Martínez-Rodríguez AJ, Polo MC, and Carrascosa AV. 2001. Structural and ultrastructural changes in yeast cells during autolysis in a model wine system and in sparkling wines. Int. J. Food Microbiol. 71: 45-51.

Masson J, and Aurier P. 2008. Effects of non-sensory cues on perceived quality: the case of low-alcohol wine. International Journal of Wine Business Research 20: 215-229.

Mazauric J-P, and Salmon J-M. 2005. Interactions between yeast lees and wine polyphenols during simulation of wine aging: I. Analysis of remnant polyphenolic compounds in the resulting wines. J. Agric. Food Chem. 53: 5647-5653.

McRae JM, Dambergs RG, Kassara S, Parker M, Jeffery DW, Herderich MJ, and Smith PA. 2012. Phenolic compositions of 50 and 30 year sequences of Australian red wines: the impact of wine age. J. Agric. Food Chem. 60: 10093-10102.

McRae JM, Falconer RJ, and Kennedy JA. 2010. Thermodynamics of grape and wine tannin interaction with polyproline: implications for red wine astringency. J. Agric. Food Chem. 58: 12510-12518.

McRae JM, and Kennedy JA. 2011. Wine and grape tannin interactions with salivary proteins and their impact on astringency: A review of current research. Molecules 16: 2348–2364.

Meillon S, Dugas V, Urbano C, and Schlich P. 2010. preference and acceptability of partially dealcoholized white and red wines by consumers and professionals. Am. J. Enol. Vitic. 61: 42-52.

Meillon S, Urbano C, Guillot G, and Schlich P. 2010. Acceptability of partially dealcoholized wines – Measuring the impact of sensory and information cues on overall liking in real-life settings. Food. Qual. Prefer. 21: 763-773.

Meillon S, Viala D, Medel M, Urbano C, Guillot G, and Schlich P. 2010. Impact of partial alcohol reduction in Syrah wine on perceived complexity and temporality of sensations and link with preference. Food. Qual. Prefer. 21: 732-740.

Mercurio MD, Dambergs RG, Cozzolino D, Herderich MJ, and Smith PA. 2010. Relationship between red wine grades and phenolics. 1. Tannin and total phenolics concentrations. J. Agric. Food Chem. 58: 12313-9.

Neves AC, Spranger MI, Zhao Y, Leandro MC, and Sun B. 2010. Effect of addition of commercial grape seed tannins on phenolic composition, chromatic characteristics, and antioxidant activity of red wine. J. Agric. Food Chem. 58: 11775-11782.

Niimi J, Danner L, Li L, Bossan H, and Bastian SEP. 2017. Wine consumers' subjective responses to wine mouthfeel and understanding of wine body. Food Res. Int. 99: 115-122.

Nurgel C, and Pickering G. 2005. Contribution of glycerol, ethanol and sugar to the perception of viscosity and density elicited by model white wines. J. Texture Stud. 36: 303-323.

Nurgel C, and Pickering G. 2006. Modeling of sweet, bitter and irritant sensations and their interactions elicited by model ice wines. J. Sens. Stud. 21: 505-519.

Palomero F, Morata A, Benito S, González MC, and Suárez-Lepe JA. 2007. Conventional and enzyme-assisted autolysis during ageing over lees in red wines: Influence on the release of polysaccharides from yeast cell walls and on wine monomeric anthocyanin content. Food Chem. 105: 838-846.

Parker M, Smith PA, Birse M, Francis IL, Kwiatkowski MJ, Lattey KA, Liebich B, and Herderich MJ. 2007. The effect of pre- and post-ferment additions of grape derived tannin on Shiraz wine sensory properties and phenolic composition. Aust. J. Grape Wine Res. 13: 30–37.

Parley A, Vanhanen L, and Heatherbell D. 2001. Effects of pre-fermentation enzyme maceration on extraction and colour stability in Pinot Noir wine. Aust. J. Grape Wine Res. 7: 146-152.

Peleg H, Gacon K, Schlich P, and Noble AC. 1999. Bitterness and astringency of flavan-3-ol monomers, dimers and trimers. J. Sci. Food Agric. 79: 1123-1128.

Pérez-Serradilla J, and de Castro M. 2008. Role of lees in wine production: A review. Food Chem. 111: 447-456.

Peyrot des Gachons C, and Kennedy JA. 2003. Direct method for determining seed and skin proanthocyanidin extraction into red wine. J. Agric. Food Chem. 51: 5877-5881.

Pickering GJ. 2000. Low-and reduced-alcohol wine: a review. J. Wine Res. 11: 129-144.

Pickering GJ, Heatherbell DA, Vanhanen LP, and Barnes MF. 1998. The effect of ethanol concentration on the temporal perception of viscosity and density in white wine. Am. J. Enol. Vitic. 49: 306-318.

Pineau B, Barbe J-C, Van Leeuwen C, and Dubourdieu D. 2007. Which Impact for β-Damascenone on Red Wines Aroma? J. Agric. Food Chem. 55: 4103-4108.

Pineau B, Barbe J-C, Van Leeuwen C, and Dubourdieu D. 2009. Examples of perceptive interactions involved in specific “red-” and “black-berry” aromas in red wines. J. Agric. Food Chem. 57: 3702–3708.

Poncet-Legrand C, Doco T, Williams P, and Vernhet A. 2007. Inhibition of grape seed tannin aggregation by wine mannoproteins: Effect of polysaccharide molecular weight. Am. J. Enol. Vitic. 58: 87-91.

Preston LD, Block DE, Heymann H, Soleas G, Noble AC, and Ebeler SE. 2008. Defining vegetal aromas in Cabernet Sauvignon using sensory and chemical evaluations. Am. J. Enol. Vitic. 59: 137-145.

Puech J-L, Feuillat F, and Mosedale JR. 1999. The tannins of oak heartwood: structure, properties, and their influence on wine flavor. Am. J. Enol. Vitic. 50: 469-478.

Rinaldi A, Gambuti A, Moine-Ledoux V, and Moio L. 2010. Evaluation of the astringency of commercial tannins by means of the SDS–PAGE-based method. Food Chem. 122: 951–956.

Riou V, Vernhet A, Doco T, and Moutounet M. 2002. Aggregation of grape seed tannins in model wine—effect of wine polysaccharides. Food Hydrocoll. 16: 17-23.

Ristic R, Bindon K, Francis LI, Herderich MJ, and Iland PG. 2010. Flavonoids and C13-norisoprenoids in *Vitis vinifera* L. cv. Shiraz: relationships between grape and wine composition, wine colour and wine sensory properties. Aust. J. Grape Wine Res. 16: 369-388.

Robinson AL, Adams DO, Boss PK, Heymann H, Solomon PS, and Trengove RD. 2011. The relationship between sensory attributes and wine composition for Australian Cabernet Sauvignon wines. Aust. J. Grape Wine Res. 17: 327-340.

Rodríguez M, Lezáun J, Canals R, Llaudy MC, Canals JM, and Zamora F. 2005. Influence of the presence of the lees during oak ageing on colour and phenolic compounds Composition of Red Wine. Food Sci. Technol. Int. 11: 289-295.

Rolle L, Englezos V, Torchio F, Cravero F, Río Segade S, Rantsiou K, Giacosa S, Gambuti A, Gerbi V, and Cocolin L. 2017. Alcohol reduction in red wines by technological and microbiological approaches: a comparative study. Aust. J. Grape Wine Res.: (in press).

Romero-Cascales I, Ros-García J, López-Roca J, and Gómez-Plaza E. 2012. The effect of a commercial pectolytic enzyme on grape skin cell wall degradation and colour evolution during the maceration process. Food Chem. 130: 626–631.

Roujou de Boubée D, Van Leeuwen C, and Dubourdieu D. 2000. Organoleptic impact of 2-methoxy-3-isobutylpyrazine on red Bordeaux and Loire wines. Effect of Environmental Conditions on Concentrations in Grapes during Ripening. J. Agric. Food Chem. 48: 4830-4834.

Runnebaum RC, Boulton RB, Powell RL, and Heymann H. 2011. Key constituents affecting wine body - an exploratory study. J. Sens. Stud. 26: 62-70.

Sacchi KL, Bisson LF, and Adams DO. 2005. A Review of the Effect of Winemaking Techniques on Phenolic Extraction in Red Wines. Am. J. Enol. Vitic. 56: 197-206.

Saliba AJ, Ovington LA, and Moran CC. 2013. Consumer demand for low-alcohol wine in an Australian sample. International Journal of Wine Research 5: 1-8.

Salmon J, Fornairon-Bonnefond C, and Mazauric J. 2002. Interactions between wine lees and polyphenols: Influence on oxygen consumption capacity during simulation of wine aging. J. Food Sci. 67: 1604-1609.

Salmon JM, Vuchot P, Doco T, and Moutounet M. 2003. Maintenance and protection of yeast morphology by contact with wine polyphenols during simulation of wine aging on lees. J. Food Sci. 68: 1782-1787.

Stevenson T. 2005. The Sotheby's wine encyclopedia. Dorling Kindersley.

Sun BS, Pinto T, Leandro MC, Ricardo-Da-Silva JM, and Spranger MI. 1999. Transfer of Catechins and Proanthocyanidins From Solid Parts of the Grape Cluster Into Wine. Am. J. Enol. Vitic. 50: 179-184.

Ugliano M. 2009. Enzymes in winemaking. *In* Wine Chemistry and Biochemistry. pp. 103-126. Springer.

Versari A, du Toit W, and Parpinello GP. 2013. Oenological tannins: a review. Aust. J. Grape Wine Res. 19: 1–10.

Vidal S, Courcoux P, Francis L, Kwiatkowski M, Gawel R, Williams P, Waters E, and Cheynier V. 2004. Use of an experimental design approach for evaluation of key wine components on mouth-feel perception. Food. Qual. Prefer. 15: 209–217.

Vidal S, Francis L, Guyot S, Marnet N, Kwiatkowski M, Gawel R, Cheynier V, and Waters EJ. 2003. The mouth-feel properties of grape and apple proanthocyanidins in a wine-like medium. J. Sci. Food Agric. 83: 564–573.

Vidal S, Francis L, Noble A, Kwiatkowski M, Cheynier V, and Waters E. 2004. Taste and mouth-feel properties of different types of tannin-like polyphenolic compounds and anthocyanins in wine. Anal. Chim. Acta 513: 57-65.

Vidal S, Francis L, Williams P, Kwiatkowski M, Gawel R, Cheynier V, and Waters E. 2004. The mouth-feel properties of polysaccharides and anthocyanins in a wine like medium. Food Chem. 85: 519–525.

Vidal S, Williams P, Doco T, Moutounet M, and Pellerin P. 2003. The polysaccharides of red wine: total fractionation and characterization. Carbohydr. Polym. 54: 439–447.

Watson B, Goldberg N, Chen H-P, McDaniel M, and Price S. Effects of macerating pectinase enzymes on color, phenolic profile, and sensory character of Pinot Noir wines. *In* Proceedings of the 2nd Joint Burgundy-California-Oregon Winemaking Symposium. pp. 36-44.

Wightman JD, Price SF, Watson BT, and Wrolstad RE. 1997. Some Effects of Processing Enzymes on Anthocyanins and Phenolics in Pinot Noir and Cabernet Sauvignon Wines. Am. J. Enol. Vitic. 48: 39-48.

Wilkinson K, and Jiranek V. Wine of reduced alcohol content: Consumer and society demand vs industry willingness and ability to deliver. *In* Proceedings of the 1st OENOVITI INTERNATIONAL symposium Pierre-Louis Teissedre (ed.), pp. 98 - 104.

Williamson PO, Robichaud J, and Francis IL. 2012. Comparison of Chinese and Australian consumers' liking responses for red wines. Aust. J. Grape Wine Res. 18: 256-267.

Yu P, and Pickering GJ. 2008. Ethanol Difference Thresholds in Wine and the Influence of Mode of Evaluation and Wine Style. Am. J. Enol. Vitic. 59: 146 - 152

Zamora MC, Goldner MC, and Galmarini MV. 2006. Sourness–sweetness interactions in different media: white wine, ethanol and water. J. Sens. Stud. 21: 601-611.

CHAPTER 2

Use of Winemaking Supplements to Modify the Composition and Sensory Properties of Shiraz Wine

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Use of Winemaking Supplements To Modify the Composition and Sensory Properties of Shiraz Wine

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Supporting Information

ABSTRACT: Wine quality can be significantly affected by tannin and polysaccharide composition, which can in turn be influenced by grape maturity and winemaking practices. This study explored the impact of three commercial wine additives, a maceration enzyme, an enotannin, and a mannoprotein, on the composition and sensory properties of red wine, in particular, in mimicking the mouthfeel associated with wines made from riper grapes. Shiraz grapes were harvested at 24 and 28 °Brix and the former vinified with commercial additives introduced either individually or in combination. Compositional analyses of finished wines included tannin and polysaccharide concentration, composition and size distribution by high performance liquid chromatography, whereas the sensory profiles of wines were assessed by descriptive analysis. As expected, wines made from riper grapes were naturally higher in tannin and mannoprotein than wines made from grapes harvested earlier. Enzyme addition resulted in a significantly higher concentration and average molecular mass of wine tannin, which increased wine astringency. Conversely, mannoprotein addition reduced tannin concentration and astringency. Addition of enotannin did not meaningfully influence wine composition or sensory properties.

KEYWORDS: *maceration enzyme, mannoprotein, mouthfeel, polysaccharide, sensory evaluation, tannin, wine*

INTRODUCTION

Tannin and polysaccharides are the two most abundant macromolecules in red wine. Red wine tannin is predominantly condensed tannin (i.e., condensation polymers of flavan 3 ols), which are extracted from grape skin and seeds during alcoholic fermentation. Tannins are diverse in terms of both size, typically expressed as mean degree of polymerization (mDP), and subunit composition.[1] Tannin is the major contributor to astringent mouthfeel in wine, which is considered central to wine sensory "structure",[2] and this sensory effect depends on concentration, mDP, and subunit composition.[3,4] As such, tannin strongly influences wine quality, evidenced by a recent survey of 73 Australian Shiraz wines, which found a positive relationship between wine quality grades and tannin concen tration, higher mDP, and the molar fraction of epigallocatechin as an extension subunit.[5]

The polysaccharides typically found in wine include those extracted from the pectic network of grape cell walls, that is, rhamnogalacturonan (RG) II, and polysaccharides rich in arabinose and galactose (PRAGs), such as arabinogalactan proteins and arabinans, as well as mannoproteins (MP), which result from the decomposition of yeast cells during fermentation and aging on lees.[6] Polysaccharides interact with polyphenolic compounds and therefore play an important role in modifying the taste and mouthfeel properties of wine.[2] The major wine polysaccharides (RGs, PRAGs, and MPs) have been shown to be negatively associated with bitterness and astringency in red wine.[4,7] Polysaccharides may also directly contribute to wine mouthfeel properties, although this effect has not been extensively studied in red wine. Purified polysaccharides (RGs, PRAGs, and MPs) have been found to significantly enhance the perception of palate fullness, when added to model wine; however, this effect was absent when polysaccharides were combined with grape tannins at levels typical for red wine.[2,8] In this manner, tannins and polysaccharides, individually and through their interactions, may play important roles in determining the sensory (textural) attributes of red wine.

Fruit maturity at harvest has a significant impact on the macromolecule composition of wine. Bindon and colleagues[7,9] showed that wines made from Cabernet Sauvignon grapes harvested earlier, that is, at a lower total soluble solids (TSS) content, had lower tannin and MP concentrations, and less apparent astringency and viscosity, than wines made from grapes harvested later. Whereas the textural attributes associated with wines made from riper grapes might be desirable from a quality standpoint, the implications of taxation imposed at higher alcohol volumes for wine exports could make this unprofitable. To this end, winemaking practices that can affect the concentration and composition of tannins and polysaccharides and in turn mouthfeel, in particular different must processing techniques, duration of maceration, and choice of yeast strain, are of interest.[10−12] However, wine tannin profiles can also be modified through the use of winemaking supplements. Enotannins are commercial tannin extracts, usually prepared from grape materials or oak wood, and are often added during red wine production with the intention of

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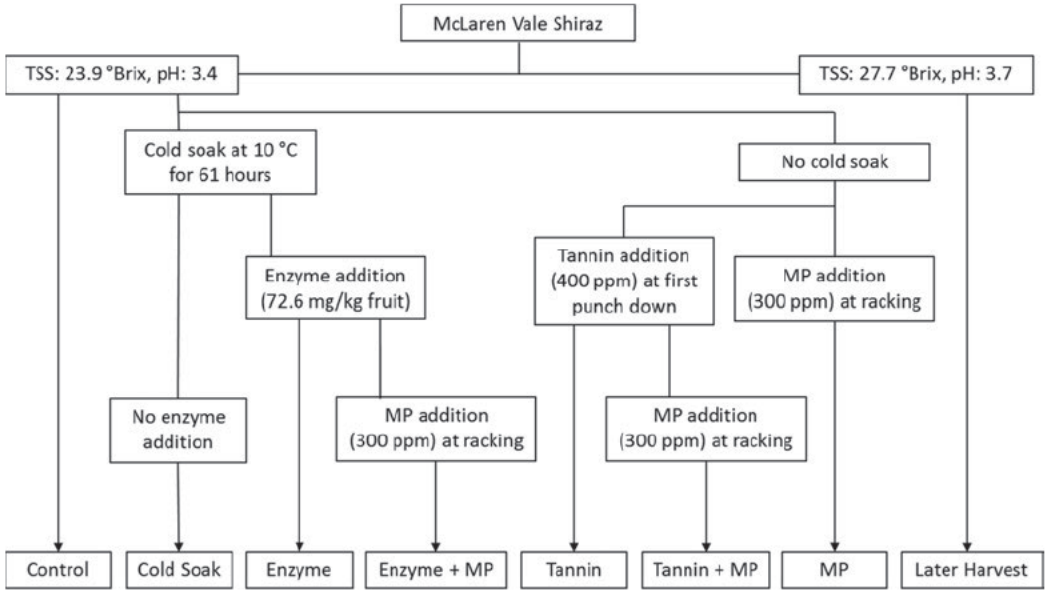

Figure 1. Flowchart of winemaking treatments.

improving wine color and mouthfeel properties.[13] Increased tannin concentration can also be achieved through the application of commercial pectolytic (maceration) enzymes,[14] although the impact on tannin subunit composition and mDP has been reported to vary.[15−19] A further impact of maceration enzyme use is that the degradation of cell wall derived pectin has been found to influence the polysaccharide profiles of red wines, increasing the contribution of PRAGs and RG II.[6,11,20] Another approach to influence the polysaccharide profile of wine is through the addition of yeast derived MPs, which are approved commercial wine additives. Previous studies have suggested MP addition to red wine can influence polyphenol composition and improve color stabilization.[21,22] However, it is interesting to note that the impact of neither maceration enzymes nor MP additions on red wine sensory attributes has been extensively studied.

The aim of this study was therefore to investigate the impact of three commercial winemaking supplements, a maceration enzyme, an enotannin, and an MP, used either individually or in combination, to modify the composition and sensory properties of Shiraz wine. The study also aimed to evaluate whether or not these supplements might be used to mimic the desirable mouthfeel properties typically associated with riper fruit, in wines made from earlier harvested grapes.

■ MATERIALS AND METHODS

Chemicals. Reagents and reference compounds (≥97% purity) used for HPLC analyses were purchased either from Sigma Aldrich (Castle Hill, NSW, Australia). Analytical grade sodium chloride and HPLC solvents were from Chem Supply (Gillman, SA, Australia) and Merck (Kilsyth, VIC, Australia), respectively.

Winemaking Trials. Shiraz grapes were sourced from a commercial vineyard located in the McLaren Vale region of South Australia (35°17′ S, 138°55′ E). The regional climate is considered to be temperate warm; the average mean January temperature (MJT) for the site is 22.9 °C, based on climate data from 2000 to 2016, obtained for Noarlunga (35°16′ S, 138°51′ E) from the Australian Bureau of Meteorology (www.bom.gov.au). In 2015 the MJT was 22.1 °C, with mean maximum temperatures exceeding 30 °C on 8 days (data not shown). Grapes were harvested at two distinct time points: (i) harvest A (February 5, 2015), when the total soluble solids (TSS) and pH of grapes (270 kg) were 23.9 °Brix and 3.4, respectively; and (ii) harvest B (February 11, 2015), when the TSS and pH of grapes (40 kg) were 27.7 °Brix and 3.7, respectively. Berry weights were measured (200 berries, in triplicate) at each time point and were found to be significantly different (p = 0.024), being 0.96 and 0.90 for harvest A and harvest B, respectively, suggesting a small amount of berry shrivel may have occurred. Following harvest, grapes were chilled overnight at 0 °C before being distributed into parcels (comprising 12 kg each). Grape parcels were destemmed and crushed, with the addition of 80 mg/L PMS (potassium metabisulfite, 10% solution, where the addition rate was based on 50% juice yield assumption). Harvest A grape parcels were randomly allocated (in triplicate) to seven different winemaking treatments (Figure 1). Control wines were made by inoculating must with 30 g/hL EC1118 yeast (Lallemand, SA, Australia) immediately after crushing and fermented at 28 °C, with the cap plunged twice daily. Diammonium phosphate (DAP) additions (200 mg/L) were made on the first and third days of fermentation. After 7 days, wines were pressed, transferred into 5 L glass demijohns, and fermented to dryness, that is, to <1 g/L residual sugar, determined enzymatically (D fructose/D glucose assay kit, Megazyme, USA). Wines were racked from gross lees, and 60 mg/L of PMS was added prior to cold stabilization (at 0 °C for 3 months). Wines made with the addition of mannoprotein (MP) were prepared as per the control, but with the addition of 300 mg/L of a commercial MP after racking from gross lees. Cold soak treatment involved must being stored at 10 °C for 61 h prior to inoculation and fermentation as per the control and included wines made without any winemaking supplements (cold soak); wines made with the addition of a commercial maceration enzyme (72.6 mg/kg fruit) prior to inoculation (enzyme); and wines made with the addition of both the maceration enzyme and the MP (as above, enzyme + MP). The cold soak wines serve as an additional control group to wines made with enzymes, accounting for any effects and variations potentially arising from the cold soak process. Wines made with the addition of tannin (tannin), involved must being inoculated and fermented as per the control, but with the addition of 400 mg/L of a commercial enotannin during the first punch down (according to the manufacturer's instructions). Wines were also made with the addition of both tannin and mannoprotein (tannin + MP). Harvest B grape parcels (in triplicate) were inoculated and fermented as per the control to produce wines expected to contain naturally higher levels of tannin and mannoprotein, as well as higher alcohol by volume (abv) content (later harvest). Following cold stabilization, all wines were again racked from lees and 30 mg/L of PMS was added; no wines

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underwent malolactic fermentation. Wines derived from harvest A fruit did not require pH adjustment, but the pH of later harvest wines was adjusted to 3.6 by the addition of 2 g/L tartaric acid. Wines were then bottled (under screw cap closures) and stored at 15 °C for 3 months, prior to chemical and sensory analyses. The commercial additives used in this study were sourced from Laffort Australia. Additive composition (as reported by the manufacturer) was as follows: the enzyme preparation, Lafase HE Grand Cru, contained predominately polygalacturonase activity with arabinose side activity; the enotannin, a 1:1 blend of VR COLOR and VR SUPRA, was extracted solely from grape material; and the mannoprotein, Oenolees MP, comprised a yeast cell wall extract.

Basic Wine Composition. Wine ethanol concentrations were determined using an alcolyzer (Anton Paar, Graz, Austria). The pH and titratable acidity (TA, as g/L equiv of tartaric acid) of wines were measured by the Australian Wine Research Institute's (AWRI) Commercial Services Laboratory using a TitraMaster (Hach, VIC, Australia). Wine color density, total phenolics, and anthocyanins were determined by using a modified Somers color assay.[23] The glucose, fructose, glycerol, and malic acid contents of wines were measured by HPLC (Agilent 1100 HPLC, Agilent Technologies Australia Pty. Ltd., Melbourne, Australia) fitted with an Aminex HPX 87H column (300 mm × 7.8 mm, BioRad, Hercules, CA, USA). Wines were filtered through 0.45 μm PVDF syringe filters, (Merck Millipore, Co. Cork, Ireland) before being injected into the HPLC. The mobile phase was 2.5 mM H_2SO_4, with a flow rate of 0.5 mL/min for a 35 min run time, at 60 °C. Signals were detected with an Agilent G1315B diode array detector and an Agilent G1362A reflective index detector. Instrument control and data analysis were performed with ChemStation software (version B.01.03). Quantitation was achieved using external calibration curves ($R^2 > 0.99$) prepared from authentic standards.

Tannin Analysis. Total wine tannin concentrations were determined using the MCPT assay,[23] and tannins were also isolated by solid phase extraction (SPE)[24] with previously described modifications,[5] reconstituted in methanol (to a final concentration of 10 g/L), and analyzed by phloroglucinolysis[25] to determine subunit composition, mDP, and molecular mass, with modifications described previouly.[26] For both the MCPT assay and phloroglucinolysis, (−) epicatechin (Sigma Aldrich, St. Louis, MO, USA) was used as a standard for quantitation, as described elsewhere.[23,25] Tannin size distribution was determined by gel permeation chromatography (GPC)[26] with an Agilent 1200 HPLC. Tannin fractions isolated by SPE were diluted 1:5 with the HPLC mobile phase prior to injection. The instrumentation, chromatographic conditions, and calibrations for GPC analysis were the same as those described previously.[27]

Polysaccharide Analysis. Wine soluble polysaccharides were precipitated by diluting wine (at a 1:5 ratio) with ethanol and refrigerating overnight at 4 °C. Precipitates were recovered by centrifugation (3273*g* for 5 min) and dialyzed against four changes of Milli Q water using a Pur A Lyzer Midi 3500 dialysis kit (Sigma Aldrich, St. Louis, MO, USA), before being freeze dried. The resulting polysaccharide extracts were dissolved in Milli Q water and hydrolyzed in 2 M trifluoroacetic acid for 3 h at 100 °C. Hydrolysates were dried in vacuo, reconstituted in 0.4 mL of Milli Q water, and mixed 1:1 with an aqueous internal standard solution comprising 0.6 mM ribose and deoxyglucose (Sigma Aldrich, St. Louis, MO, USA). Mixtures were derivatized with 1 phenyl 3 methyl 5 pyrazolone (PMP) and analyzed by HPLC using the method described previouly.[28] For determination of free monosaccharides, 0.06 g of polyvinylpolypyrrolidone (PVPP) was added to a 1 mL aliquot of wine, and the resulting mixture was vortexed and centrifuged (75000*g* for 5 min). The supernatant obtained was then mixed 1:1 with the internal standard and analyzed, as above.

Sensory Analysis. Descriptive analysis (DA) was undertaken approximately 3 months after bottling. The panel comprised four male and seven female judges, with all but one panelist being recruited from a pool of wine science researchers from the University of Adelaide, the majority of whom had extensive DA experience. The DA process followed consensus based methodology.[29] Panelists attended five 2 h training sessions during which they generated appropriate descriptive terms, their definitions, and reference standards, based on a variety of Shiraz wines, including wines from the current study. During the first two training sessions, panelists evaluated wine appearance, aroma, and palate characteristics and as a group discussed the scale for each attribute. Wine appearance terms (color and depth) were subsequently removed because the whole panel agreed that they did not perceive any differences between wines after at least one replicate from each treatment was presented. In subsequent training sessions, panelists were presented with standards for aroma attributes, for *astringency*, *hotness*, and *palate fullness*, and for *palate coarseness* ("touch standards" comprising a range of fabrics and powders). After gaining familiarity with the standards, panelists rated wine sensory properties as a group and compared results. A practice session was then held in sensory booths to evaluate judge performance, using PanelCheck software (v1.4.0). In the subsequent session, feedback was provided to panelists and some nondiscriminating terms were discussed and removed on the basis of panel consensus. The panel eventually decided on an attribute list comprising nine aroma, eight flavor, four taste, and four mouthfeel terms (Supplementary Table 1). During formal evaluation, panelists rated the intensity of these attributes on 15 cm unstructured line scales, with anchors at 10, 50, and 90% of the scale corresponding to "low", "medium", and "high", respectively, with the exception of *palate coarseness*, which was anchored with the terms "talc", "chalk", and "pumice", from low to high.

Two formal evaluation sessions were held, during which panelists were presented with 12 wine samples (50 mL) in ISO standard clear wine glasses, covered with plastic lids. Formal evaluations were held in isolated booths in a dedicated sensory laboratory, maintained at 21 °C. Twenty four wines, that is, three replicates of each treatment, were presented once to each panelist, in a randomized, balanced order. Data were acquired using FIZZ software (version 2.50a, Biosystems, Couternon, France). To avoid sensory fatigue, panelists were required to rest for 1 min after each sample and for 5 min after every 4 samples. Panelists were provided filtered water, 1 g/L pectin solution (pectin from citrus peel, Sigma Aldrich, Castle Hill, NSW, Australia), and plain water crackers to cleanse their palates between samples.

Data Analysis. Chemical data were subjected to analysis of variance (ANOVA) using XLSTAT (version 2015.4.1, VSN International Limited, Herts, UK). Mean comparisons were performed by a least significant difference (LSD) multiple comparison test at $p < 0.05$. PanelCheck (V1.4.2, Nofima Mat) was used to evaluate panel performance during DA; principal component analysis (PCA) of sensory data was performed using SENPAQ (version 6.03, Qi Statistics, Reading, UK).

RESULTS AND DISCUSSION

This study explored to what extent winemaking supplements could modify wine composition, especially tannin and polysaccharide concentrations, and therefore the organoleptic characteristics of Shiraz wine. A later harvest treatment was deliberately included to generate wines naturally higher in tannin and polysaccharide[9] to enable the potential for additives to mimic mouthfeel characters of wine made from riper grapes to be considered.

Impact of Winemaking Supplements on Basic Wine Composition. Significant differences in basic wine composition were not observed among harvest A wines, but, as expected, differences were apparent between harvest A and harvest B wines (Table 1). Notably, ethanol concentrations increased from 14% for harvest A wines to 16.7% for harvest B wines. Wine pH, TA, and malic acid concentrations were similar across all treatments of harvest A, with only MP treatment having slightly higher pH and the enzyme treated wines having a marginally higher TA. Wines made from the later harvest had significantly higher TA than harvest A wines, which could be attributed to the addition of tartaric acid at bottling. Harvest B wines also had higher levels of residual

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Table 1. Basic Composition of Shiraz Wines[a]

	control	cold soak	enzyme	enzyme + MP	tannin	tannin + MP	MP	later harvest	p
ethanol (% v/v)	14.4 b ± 0.08	14.0 d ± 0.11	14.0 d ± 0.11	13.9 d ± 0.10	14.3 bc ± 0.09	14.1 cd ± 0.08	14.0 cd ± 0.08	16.7 a ± 0.05	<0.001
pH	3.69 bc ± <0.01	3.66 cd ± 0.01	3.61 d ± 0.01	3.61 cd ± 0.01	3.68 cd ± 0.01	3.66 cd ± 0.01	3.78 a ± 0.05	3.76 ab ± 0.05	0.002
TA (g/L)	7.4 c ± 0.03	7.4 c ± 0.06	7.8 b ± 0.03	8.0 b ± 0.09	7.4 c ± 0.06	7.5 c ± 0.03	7.4 c ± 0.03	8.1 a ± 0.03	<0.001
glucose (g/L)	0.08 ± <0.01	0.09 ± 0.01	0.07 ± 0.01	0.10 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	0.09 ± 0.00	0.10 ± 0.02	0.118
fructose (g/L)	0.33 b ± 0.03	0.30 b ± 0.02	0.22 b ± 0.01	0.22 b ± 0.01	0.33 b ± 0.03	0.36 b ± 0.01	0.31 b ± 0.02	0.86 a ± 0.28	0.009
glycerol (g/L)	9.79 b ± 0.02	9.18 d ± 0.05	9.50 c ± 0.05	9.40 cd ± 0.09	9.63 bc ± 0.19	9.56 bc ± 0.11	9.56 bc ± 0.04	12.41 a ± 0.04	<0.001
malic acid (g/L)	3.21 ab ± 0.02	3.14 b ± 0.01	3.25 a ± <0.01	3.23 a ± 0.03	3.26 a ± 0.02	3.26 a ± 0.06	3.25 a ± 0.01	3.15 b ± 0.03	0.023

[a]Values are means of three replicates ± standard error. Values followed by different letters within rows are significantly different ($p \leq 0.05$, one-way ANOVA, post hoc Fisher's LSD).

fructose and glycerol, which was expected for wines made from riper grapes.[9]

Impact of Winemaking Supplements on Wine Polysaccharide Composition. At the time of analysis (3 months after bottling) the concentrations of wine soluble polysaccharides ranged from 514 mg/L (for enzyme treatment) to 680 mg/L (for MP treatment), but were not significantly different ($p = 0.057$) between treatments (Table 2). However, in the analysis conducted at the completion of fermentation, similar polysaccharide concentrations were observed in all wines, being 506−716 mg/L, except for wines derived from enzyme treatment that had significantly lower total polysaccharide levels, at 384 mg/L (data not shown). The small increase in polysaccharide levels observed in enzyme treatments from fermentation to 3 months after bottling was due to increased mannoprotein, which will be discussed below.

Despite only minor differences in concentration, polysaccharide composition varied considerably between treatments, as evidenced by monosaccharide residues recovered following acid hydrolysis of polysaccharides (Figure 2). The main monosaccharide residues found were mannose, galacturonic acid, galactose, and arabinose, with minor contributions of rhamnose and glucose, consistent with other studies.[9,11,30] Comparing the control wines with those made from the later harvest grapes, the latter treatment gave higher concentrations of mannose, galactose, and arabinose (Figure 2). Mannose can be attributed to MPs, which are released by yeast during fermentation and aging. One study[9] found the MP concentration increased in wines made from sequentially harvested grapes, which the authors attributed to increased yeast turnover in the higher sugar musts. Galactose and arabinose residues are usually attributed to polysaccharides rich in arabinose and galactose (PRAGs), which are either readily soluble or cleaved from the pectic cell wall fraction. It is generally accepted that during ripening, the PRAG and homogalacturonan (HG) fractions of cell walls are depolymerized and solubilized, although this tends to occur during the early stages of grape development[31] and does not necessarily translate into increased grape derived polysaccharide in wines made from riper grapes.[9] However, in Shiraz grapes, water soluble polysaccharides in skin cell walls have been found to increase steadily over a 10 week period postveraison.[31] In the current study, therefore, the increasing concentration of these polysaccharide residues observed in wines made from the later harvest may indicate a cultivar dependent phenomenon (Table 2).

As was expected, the addition of both enzyme and MP supplements noticeably altered wine polysaccharide composition, whereas tannin additions had no significant impact (Table 2). Commercial enzyme preparations usually contain pectin degrading activity, but some also comprise hemicellulase and potentially glucosidase enzymes;[15,32] hence, their application during red wine fermentation typically results in substantial degradation of the grape cell wall.[14,32] The enzyme supplement employed in this study primarily exhibited polygalacturonase activity and, to a lesser extent, arabinase activity, according to the manufacturer, which was supported by preliminary analysis of its effect on polysaccharides purified from Chardonnay juice (Supplementary Figure 1). Analyses of the monosaccharide composition of enzyme treated wine polysaccharide showed significant (≤60%) decreases in galacturonic acid and arabinose (Figure 2), compared to the control wines, indicating an extensive degradation of pectic polysaccharides of HGs[20,33] and PRAGs[6] (or possibly other arabinose rich polymers) in the

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Table 2. Concentrations (mg/L) of Total Polysaccharides and Selected Free Monosaccharides in Shiraz Wines Made with the Addition of Enzyme, Tannin, and Mannoprotein (MP) Supplements, either Individually or in Combination[a]

	control	cold soak	enzyme	enzyme + MP	tannin	tannin + MP	MP	later harvest	p
total polysaccharides	536 ± 21	560 ± 22	514 ± 57	544 ± 47	571 ± 60	617 ± 33	680 ± 18	674 ± 34	0.057
free monosaccharides									
rhamnose	19.1 ab ± 1.2	19.0 abc ± 0.7	20.7 a ± 0.9	20.7 a ± 1.0	16.8 bc ± 0.9	16.8 bc ± 0.6	16.6 c ± 0.1	16.7 bc ± 0.5	0.005
galacturonic acid	413 bc ± 21	414 bc ± 14	1541 a ± 33	1493 a ± 83	326 c ± 24	322 c ± 16	312 c ± 21	473 b ± 26	<0.001
galactose	60.8 c ± 1.4	61.1 c ± 0.9	95.8 a ± 1.2	91.0 b ± 1.5	61.4 c ± 1.4	60.4 c ± 0.4	59.7 c ± 2.0	63.1 c ± 0.8	<0.001
arabinose	10.3 b ± 1.6	34.8 b ± 1.3	420 a ± 16	423 a ± 24	15.0 b ± 2.6	11.5 b ± 0.5	11.8 b ± 1.5	10.2 b ± 0.7	<0.001

[a]Values are means of three replicates ± standard error. Values followed by different letters within rows are significantly different ($p \leq 0.05$, one-way ANOVA, post hoc Fisher's LSD).

grape cell wall. Consequently, these residues were found as free monosaccharides in wine (Table 2). Notably, the high concentration of free galacturonic acid in enzyme treated wines might account for the higher TA observed (compared to the cold soak control), but with a negligible effect on pH (Table 1). Concomitant with the decrease in galacturonic acid and arabinose as polysaccharide residues, the enzyme treated wines had considerably higher rhamnose as a polysaccharide residue (Figure 2). Numerous studies have also found an increased proportion of rhamnose in wine polysaccharide made with the addition of polygalacturonase enzymes, which has been attributed to the enrichment of RG II.[6,11,20,30,34] RG II is resistant to degradation by commercial enzyme preparations, due to its complex structure; therefore, it is often released into wine intact following enzyme treatment, whereas HGs are largely degraded.[35] This increase of RG II could account for the relatively high level of retained galacturonic acid in the polysaccharides of enzyme treated wines. Interestingly, the enzyme treatments also resulted in a higher concentration of mannose as a polysaccharide residue relative to the control and cold soak treatments (Figure 2). This finding contradicts previous studies[6,11,30,34] and therefore requires further investigation.

Addition of MP supplements gave increased concentrations of polysaccharide derived mannose (Figure 2). However, mannose content did not reflect the initial 400 mg/L MP added, nor were any meaningful changes in total polysaccharide concentrations observed, which was unexpected. The increased mannose concentration as a polysaccharide residue ranged from 10 to 30 mg/L, for enzyme + MP, tannin + MP, and MP treatments compared to the other treatments (Figure 2). We considered that the effect recovery of mannose in treated wines would be foremost dependent on the purity of the product. This was tested by measuring the hydrolysis of the MP product to component monosaccharide residues (in triplicate), and it was found to yield 96.04 mg/g product, which accounted for slightly <10% of the total material. To our surprise, 123.19 and 128.34 mg/g of galactose and arabinose residues were also detected, respectively, which may explain the proportional increases in galactose and arabinose residues as wine polysaccharide in the MP treatments compared to the control. However, these changes were not consistently observed when MP addition was combined with other additives. Nevertheless, on the basis of the results, although marketed as a mannoprotein, the MP product used in this study likely contained a considerable amount of PRAGs, and it was of interest to note that the polysaccharides isolated from the MP treatment were compositionally similar to that of the wine made from grapes harvested later.

Impact of Winemaking Supplements on Wine Tannins and Color. In comparison to the control, wines made from the later harvest grapes were significantly higher in tannin and anthocyanin concentrations and differed substantially in tannin composition, having significantly higher molar proportions of epigallocatechin, together with a higher tannin mDP and molecular mass. On the other hand, lower molar proportions of epicatechin gallate were observed in tannin from these wines (Table 3). These differences in phenolic composition were also reflected in color measurements, namely, higher levels of SO_2 resistant pigments and higher color density. Similar trends in the ripening induced modification of wine tannin composition and color have been observed in Cabernet Sauvignon[9] and Cabernet Franc[10] wines.

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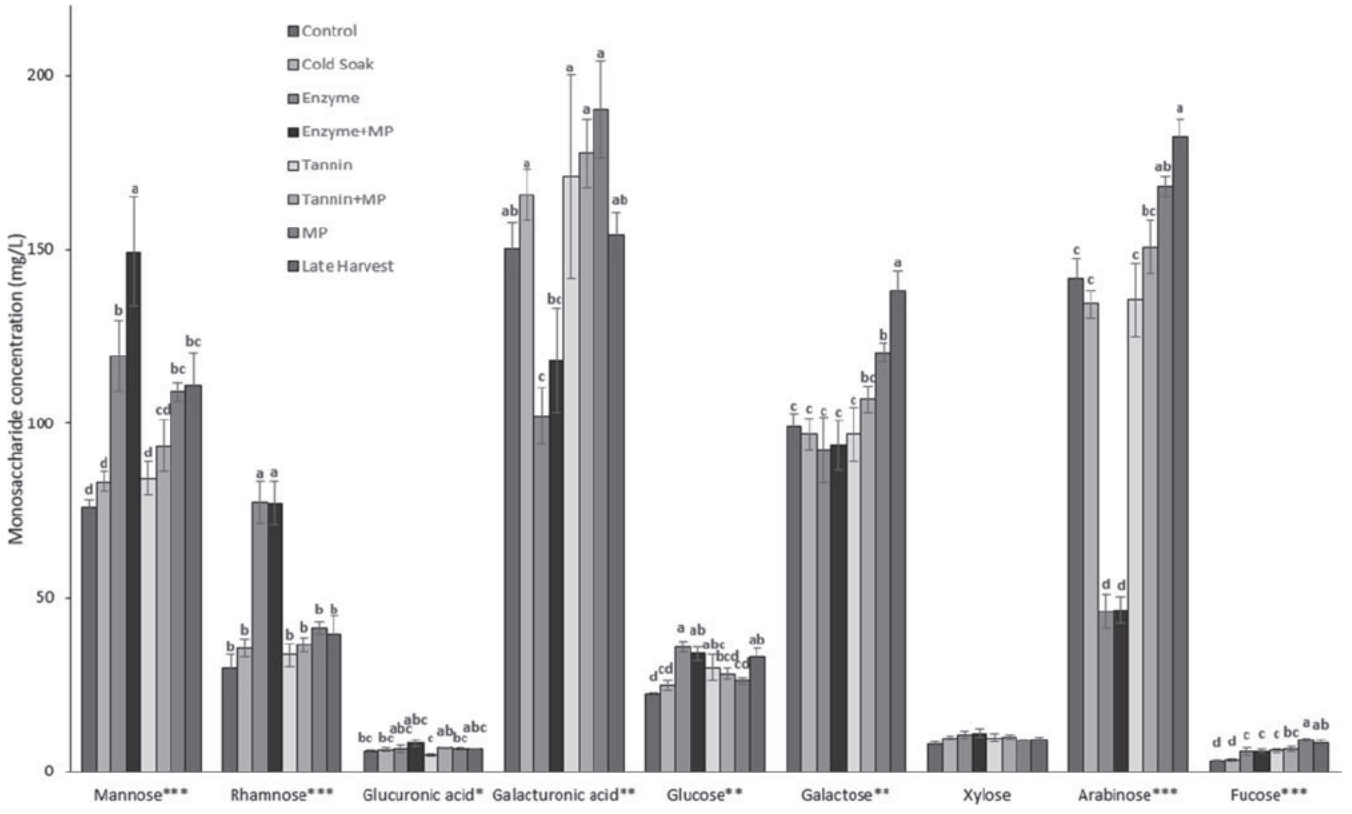

Figure 2. Mean monosaccharide residues following acid hydrolysis of wine soluble polysaccharides in Shiraz wines made with the addition of enzyme, tannin, and mannoprotein (MP), either individually or in combination. Different letters indicate statistical significance determined by ANOVA. Fisher's least significant difference (LSD) was calculated post ANOVA: (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$.

Within the harvest A treatments, enzyme and enzyme + MP had significantly higher tannin concentrations, for example, 1478 and 1419 mg/L respectively, compared to 1168 mg/L for control wines. Similarly, anthocyanin concentration was also higher in wines made with enzyme addition, although SO_2 resistant pigments and color density were unaffected. Enzyme treatment gave higher molar proportions of epigallocatechin as a tannin subunit than for cold soak treatment but not control. The reverse trend was observed for epicatechin gallate in comparison to control and cold soak treatments. In terms of tannin polymerization degree, the enzyme treatment resulted in significantly higher tannin mDP and molecular mass relative to other harvest A treatments, being comparable with later harvest wines (Table 3). To further characterize the effect of enzyme on wine tannin size distribution, tannin extracts were measured by gel permeation chromatography (Figure 3), which showed a notable shift in the elution profile of tannin isolated from enzyme treated wines toward a higher molecular mass (earlier elution time) relative to the other treatments. In comparison, the late harvest wines, which have a mDP similar to that of enzyme wines, showed no obvious change in the early eluted fraction but had less late eluting material. Previous studies have shown maceration enzymes can enhance tannin extraction during vinification,[16−19,34] and their ability to improve extraction from grape seeds has also been confirmed in both a winemaking trial[17] and a model wine like solution.[15] Nevertheless, the effect of enzyme addition on wine tannin composition and mDP is often inconsistent. Ducasse and colleagues[34] found that enzyme treatment increased tannin mDP, but the proportions of epigallocatechin and epicatechin gallate as tannin subunits varied by vintage and the specific enzyme used. Other studies have shown the extent to which enzymes affect tannin composition and mDP may also depend on the geographical origin of fruit[18] as well as grape variety.[16]

Tannin mDP and subunit composition can indicate the origin of tannins in wine, that is, from skin or seed. Skin tannins generally have a higher mDP and are richer in epigallocatechin subunits but low in epicatechin gallate, whereas seed tannins have higher proportions of epicatechin gallate, no epigallocatechin, and lower mDP, despite marginally higher hydrodynamic volumes (size by GPC) than skin tannins at a set molecular mass.[26] In the enzyme treated wines, the proportions of both epigallocatechin and epicatechin gallate increased in line with mDP, suggesting extraction was enhanced from both grape skins and seeds (Table 3). However, because the molar proportion of epicatechin gallate was only significantly higher than in control wines but comparable to cold soak wines (as discussed above), the enhanced seed extraction was likely due to extra soaking time instead of the enzyme effect. The enhanced tannin release observed in wines made with enzyme addition was most likely due to their ability to degrade cell walls. The (homogalacturonan rich) pectic fraction of cell walls is thought to have a higher affinity for binding tannins than hemicellulose and cellulose.[28] Thus, degradation of pectin may enhance tannin extraction in two ways: (i) degradation of skin and seed cell walls during maceration may facilitate diffusion of phenolic compounds into wine by breaking down the major barrier, that is, grape cell walls; and/or (ii) enzymes may reduce adsorption of tannin to grape solids (cell walls),[36] a process by which otherwise soluble tannins are adsorbed to marc or lost as lees, at the end of fermentation.

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Table 3. Phenolic Composition of Shiraz Wines Made with the Addition of Enzyme, Tannin, and Mannoprotein (MP) Supplements, either Individually or in Combination[a]

	control	cold soak	enzyme	enzyme + MP	tannin	tannin + MP	MP	later harvest	p
wine color density (au)	15.3 b ± 0.8	12.7 de ± 0.4	15.3 b ± 0.1	14.8 bc ± 0.4	13.8 bcd ± 0.7	13.5 cde ± 0.4	12.1 e ± 0.5	17.3 a ± 0.6	<0.001
anthocyanin (mg/L)	607 bc ± 16	561 de ± 6	644 a ± 8	635 ab ± 11	613 bc ± 12	588 cd ± 10	555 e ± 7	634 a ± 7	<0.001
SO_2-resistant pigments (au)	2.23 bc ± 0.10	1.73 d ± 0.09	2.32 b ± 0.08	2.17 bc ± 0.08	2.04 bcd ± 0.11	1.97 cd ± 0.12	1.76 d ± 0.06	3.32 a ± 0.17	<0.001
total phenolics (au)	45.6 cd ± 0.7	41.9 e ± 0.4	49.7 ab ± 0.2	47.9 bc ± 1.2	46.7 cd ± 1.4	44.9 d ± 0.8	41.3 e ± 0.9	51.2 a ± 0.9	<0.001
total tannin (mg/L)	1168 b ± 6	1008 bc ± 36	1478 a ± 44	1419 a ± 85	1098 bc ± 27	1033 bc ± 75	949 c ± 91	1492 a ± 69	<0.001
% epigallocatechin[b]	24.6 cd ± 0.2	24.0 d ± 1.2	26.6 bc ± 0.6	27.0 b ± 0.1	27.1 b ± 0.2	26.4 bc ± 0.5	26.9 bc ± 0.2	29.9 a ± 1.2	0.002
% epicatechin gallate[c]	3.7 b ± 0.1	4.1 ab ± 0.1	4.5 a ± 0.1	4.5 a ± 0.1	4.2 ab ± 0.2	3.8 b ± 0.2	3.7 b ± 0.1	3.9 b ± 0.2	0.024
tannin mDP	6.87 d ± 0.03	6.86 d ± 0.21	8.18 ab ± 0.25	8.03 abc ± 0.13	7.34 cd ± 0.01	7.00 d ± 0.29	7.44 bcd ± 0.12	8.45 a ± 0.14	0.001
tannin molecular mass[d] (g/mol)	2059 c ± 8	2061 c ± 64	2464 a ± 77	2442 ab ± 35	2208 c ± 2	2103 c ± 90	2232 bc ± 36	2543 a ± 42	0.001
tannin molecular mass[e] (g/mol)	1451 c ± 37	1482 bc ± 67	1597 a ± 67	1561 ab ± 54	1528 abc ± 13	1489 bc ± 36	1451 c ± 62	1558 ab ± 54	0.025

[a]Values are means of three replicates ± standard error. Values followed by different letters within rows are significantly different ($p \leq 0.05$, one-way ANOVA). [b]Molar proportion of epigallocatechin as extension unit in tannin subunit composition determined by phloroglucinolysis. [c]Molar proportion of epigallocatechin gallate in tannin subunit composition determined by phloroglucinolysis. [d]Determined by phloroglucinolysis. [e]Determined by gel permeation chromatography at 50% elution.

Figure 3. Elution profile of wine tannins following gel permeation chromatography analysis (at 280 nm) of Shiraz wines from control, cold soak, enzyme, and later harvest treatments.

Tannin addition during fermentation did not result in any significant differences in tannin concentration, composition, molecular mass (determined by either phloroglucinosis or GPC), or wine color measurements (Table 3). It was suspected that this was due to the low purity of the tannin product used in the current project. However, when the purity was tested by dissolving the product in model wine and then measuring the concentration by MCPT method, the recovery was 70% w/w, demonstrating the loss of tannin addition was not due to product composition. Nevertheless, other studies[37,38] have also found a lack of effect by tannin addition during fermentation. A recent review[13] on enological tannins highlighted variable implications of their addition on wine color,[12] and it is therefore difficult to conclude whether or not tannin addition is a valid approach for improving color stability because it may depend upon both the product itself and the wine matrix. Factors such as enotannin type, dosage, grape quality, and vintage confound research findings. Furthermore, when enotannin is added during fermentation, they can bind to cell wall material present in wine, leading to a loss of tannin (due to precipitation) shortly after addition. Bautista Ortín and colleagues studied the binding interactions of six commercial tannins with purified grape cell wall material in model wine and found between 13 and 61% of tannins were removed after 90 min, with higher molecular mass fractions of tannin removed preferentially.[39]

In the current study, the addition of MPs in combination with enzyme or tannin supplements did not significantly alter phenolic composition, compared to the addition of enzyme or tannin supplements alone. Wines with MP addition alone had the lowest tannin concentration of all the treatments, being 949 mg/L, but the molar proportions of epigallocatechin and epicatechin gallate, mDP, and molecular mass were not different from those of control wines, enabling the suggestion that some tannin was removed. MP addition significantly reduced both anthocyanins and SO_2 resistant pigments, and as a consequence, color density was significantly lower compared to the control (Table 3). It has been shown that yeast membrane MPs are able to precipitate tannins from red wine in vitro,[40] which may explain the decreased tannin concentrations in the MP treated wines. However, reports on effects of MPs (either commercial preparations or isolates derived from different yeast strains) on wine color and phenolics are inconsistent.[12,21,22] The impacts of addition are seemingly

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Table 4. Mean Intensity Ratings for Sensory Attributes of Shiraz Wines Made with the Addition of Enzyme, Tannin, and Mannoprotein (MP) Supplements, either Individually or in Combination[a]

attribute	control	cold soak	enzyme	enzyme + MP	tannin	tannin + MP	MP	later harvest	LSD
aroma									
red fruit	5.5	6.1	6.3	5.2	7.5	5.4	7.2	5.7	1.5
dark fruit	8.0	8.3	7.8	8.7	6.6	8.8	7.6	9.2	1.3
confectionary	3.6	3.5	3.4	3.2	4.3	2.8	3.5	3.0	0.9
flavor									
flavor intensity	9.4	9.3	9.8	9.3	9.5	9.8	9.6	10.7	0.8
jammy	7.1	7.1	6.4	6.5	6.1	7.4	6.3	8.3	1.3
palate									
sweetness	3.2	3.4	2.5	2.9	3.3	3.3	3.7	5.1	0.7
palate fullness	8.8	8.2	8.3	8.8	7.8	8.6	8.6	9.7	1.0
astringency	9.0	9.0	10.4	9.9	9.1	8.4	8.7	9.0	1.0
surface coarseness	8.1	7.9	9.5	9.1	7.8	7.3	6.8	7.3	1.4
hotness	9.3	8.6	8.2	9.7	8.7	9.4	8.7	11.3	1.2

[a]Values are mean scores from three replicates per treatment, determined by 11 judges. For all attributes, $p \leq 0.05$, one way ANOVA, post hoc Fisher's LSD.

Figure 4. Principal component analysis biplot of (A) sensory data and (B) sensory (◆) and supplementary chemical (▲) data for Shiraz wines made with the addition of enzyme, tannin, and mannoprotein (MP) supplements, either individually or in combination. Abbreviations for chemistry measurements: TA, titratable acidity; GalA res., galacturonic acid residue; Rha res., rhamnose residue; Man res., mannose residue; G+A, sum of galactose and arabinose residues; FrAra, free arabinose; FrGalA, free galacturonic acid; FrRha, free rhamnose; %Epicat gal, %epicatechin gallate; % Tri OH, %epigallocatechin; Tannin MS, tannin molecular mass (determined by GPC).

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influenced by MP characteristics, for example, the percentage of protein and size distribution, but no definitive relationships have been established, and further research is warranted.

Impact of Winemaking Supplements on Sensory Profiles of Wines. Significant differences ($p < 0.05$) were found in intensity ratings of 10 sensory attributes, that is, red fruit, dark fruit, and confectionary aromas, jammy flavor, and flavor intensity, and five palate attributes: sweetness, palate fullness, astringency, surface coarseness, and hotness (Table 4). Principal component analysis (PCA) was performed on sensory data for these attributes, and the first two components accounted for 81.3% of total variance (Figure 4A). Wines were separated along the first principal component (PC) according to positive associations with dark fruit aroma, flavor intensity, jammy flavor, sweetness, palate fullness, and hotness. The later harvest wine was most strongly associated with these attributes and, therefore positioned farthest to the right of the biplot. Red fruit and confectionary aromas were positively correlated with one another ($R^2 = 0.76$), but were negatively correlated with dark fruit aroma ($R^2 = -0.84$ and -0.93, respectively). Wine made with tannin addition was most strongly associated with these characters. Wines with MP addition also showed significantly higher ratings in red fruit compared to control. Although the origin of these changes was not explored in the current study, it has been shown that addition of tannin and mannoprotein products has been observed to interact with wine volatiles and affect wine aroma and flavor.[22,41] A range of volatile compounds known to be important to Australian Shiraz wines[42] were quantified by GC MS as part of this study (Supplementary Table 2), but no significant impact on wine volatiles was detected for tannin and mannoprotein treatments. The second PC separated wines on the basis of astringency and palate coarseness, attributes that were highly correlated with one another. This was not unexpected, given surface smoothness is a subquality of overall astringency and inclusion of this term was intended to further differentiate the impact of treatments on the sensory quality of tannins (smooth vs coarse). Control and cold soak wines were positioned close to the center of the biplot; that is, they were not strongly associated with any particular sensory attributes. In contrast, wines made with enzyme addition exhibited enhanced astringency and coarseness; the addition of MPs to the enzyme treated wines failed to significantly modify the astringent perception. However, the wines made with only MP addition were positioned on the opposing side of PC2, with significantly lower ratings for astringency and surface coarseness. Interest ingly, tannin addition enhanced red fruit and confectionary aromas, whereas in comparison tannin and MP addition gave wines with significantly higher ratings for dark fruit aroma, jammy flavor, palate fullness, and hotness; that is, tannin + MP wines most closely resembled later harvest wines, based on their relative proximity on the PCA biplot (Figure 4A). The modifications of aroma and flavor achieved by combined tannin and MP additions were not observed in other supplement combination (i.e., control vs MP, enzyme, or enzyme + MP).

To elucidate the relationships between the chemical composition and the sensory profile of the wines, some chemical measurements were incorporated as supplementary data into the PCA model built with sensory data (Figure 4B), and the correlation matrices are provided as supporting data (Supplementary Tables 3 and 4). Where measurements were insignificant or highly correlated, for example, tannin size (mDP and molecular mass by phloroglucinosis) or free arabinose and galactose, some data were excluded from PCA. Only the major monosaccharide residues from polysaccharide hydrolysis were plotted to represent the main polysaccharides found in wine (as discussed above). To this end, the sum of arabinose and galactose (arabinose + galactose) residues was used instead of the individual monosaccharides because they were highly correlated and can both be attributed to PRAGs.

The winemaking supplements used in this study had more prominent effects on the macromolecule composition of wines, and therefore palate characteristics, than on aroma and flavor. The PCA model (Figure 4B) suggests free monosaccharides (galactose and arabinose, in particular) and the molar proportion of epicatechin gallate influenced perceptions of astringency and palate coarseness the most. These constituents are indicative of enhanced cell wall degradation and possibly greater seed extraction (as discussed above). Interestingly, mDP and the molar proportion of epicatechin gallate were found to be positively correlated with astringency, whereas the molar proportion of epigallocatechin was not, in agreement with other studies.[3,4] Tannin concentrations were also positively correlated with astringency, as expected. It was of particular interest in this study to explore if and how the composition of wine polysaccharide affects astringent mouth feel. All polysaccharide derived monosaccharide residues incorporated into the PCA model showed correlations with astringency and coarseness; that is, galacturonic acid and arabinose + galactose were highly negatively correlated with these attributes (R^2 between -0.822 and -0.951), whereas rhamnose positively correlated with these two attributes ($R^2 = 0.885$ and 0.816, respectively). However, mannose as a polysaccharide residue contributed only slightly to astringency ($R^2 = 0.623$) with no apparent relationship ($R^2 = 0.486$) to palate coarseness. These results somewhat contradict other studies in which all major wine polysaccharides (MP, PRAG, and RG II) were found to bear no apparent relationship[7] or were negatively correlated[4] with astringency in red wines. These results highlighted the highly complex and interactive effects of the supplements because they modify multiple wine components at once with resultant sensory effects. For example, rhamnose was positively associated with astringency and palate coarseness, probably due to its abundance in the poly saccharides of wines made with enzymes; however, it was more likely that elevated tannin concentration was the main factor associated with this attribute, rather than RG II, due to co correlation of these factors. This is particularly supported by the observation that the effect of polysaccharides on astringency is minor in the presence of tannins in model wine solution, and RG II in fact reduces astringency.[2] Another example would be that wines made with MP treatment were the least astringent, most likely due to their relatively low tannin, but in that instance co correlation with arabinose + galactose (instead of mannose), because the commercial MP product used in this study contained more of these two residues than mannose.

Hotness and palate fullness were highly correlated ($R^2 = 0.9$) and closely associated with ethanol, glycerol, and fructose. Increased concentrations of ethanol and glycerol have been shown to highly correlate with hotness and viscosity in red wine.[7,43] Sweetness was also correlated with hotness and palate fullness and was most closely associated with fructose concentrations, although given the low concentrations present in the current wines (Table 1), this relationship is unlikely to be causal. Sweetness was also, to a lesser extent, associated with higher ethanol, glycerol, pH, and galactose + arabinose residues.

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In the current study, the extent to which selected winemaking supplements could achieve sensory properties that resembled wine made from riper fruit, that is, wine naturally richer in tannin, MPs, and PRAGs with deeper color and more desirable mouthfeel sensations, was evaluated. Harvesting grapes at optimal maturity is critical for achieving targeted wine style; however, it is not always possible to harvest grapes at optimal maturity due to unexpected vintage conditions (e.g., climate) and/or the availability of resources in the vineyard and/or winery or the tax implications of higher wine alcohol content. Furthermore, elevated temperature during the ripening period due to climate change may produce grapes with under developed color and flavor at typical sugar levels for harvesting,[44] forcing winemakers to either pick grapes at high sugar levels for desired sensory characters or pick early to avoid high alcohol levels in wine at a loss of quality. Anecdotally, the commercial harvest of Shiraz grapes from the vineyard involved in the current study was a week after harvest B, which meant the grapes harvested for the "later harvest" treatment (with a TSS of 28 °Brix) were still considered to be slightly under optimal ripeness from an industry perspective for that particular vintage. This study aimed to provide winemakers with more tools to control and modify wine macromolecule composition in the winery when the grapes are of suboptimal quality.

The current study confirmed that harvest time resulted in substantial differences in wine macromolecule composition, especially in terms of polyphenolic compounds. However, although the maceration enzyme treatment enhanced the tannin concentration and average molecular mass, as well as polysaccharide associated mannose (MPs) in harvest A wines to levels comparable to wines made from the later harvest, the two treatments were significantly different in their sensory characteristics; that is, the enzyme treated wines were noticeably more astringent and coarse on the palate (Table 4). This observation indicates that mouthfeel is likely to be affected by other wine sensory components, such as being reduced by fruitiness and sweetness,[45] and these more complex interactions could not be distinguished in this study. However, this result accentuates the importance of the mouthfeel being in balance with other wine components.

Aside from the well defined effects achieved by the maceration enzyme, this study also demonstrated some interesting effects of the use of commercial MP and enotannin products, as well as latent interactions between wine polysaccharide and tannin that can be further explored. MP addition itself decreased the tannin concentration and astringent mouthfeel, but when it was used in combination with other additives, the effects were less clear. The enzyme + MP treatment was similar to enzyme used alone, whereas the tannin + MP treatment had a significant impact on aroma and flavor, but not on mouthfeel, compared to when tannin was used alone. Unexpectedly, tannin additions did not give rise to any changes in tannin concentration and composition, nor were the resultant wines rated more astringent or bitter; yet there was an apparent modification of the wine sensory aroma profile. There is still much yet to be studied in the interactive effects of these three categories of wine supplements, especially considering that other studies also showed that their effectiveness seemed to be significantly affected by the nature of the additive and the natural composition of the wines, both of which can vary considerably from case to case.[21,34,46] Furthermore, in the current study a great loss of added tannin and inconsistent recoveries of the mannoprotein product were observed. This may be due to both the composition of the particular product used and the processes of precipitation and subsequent racking during vinification. Future studies could involve a broader selection of winemaking supplements and investigate their effects in finished wines to minimize some of these effects.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b04505.

Kinetics of galacturonic acid and arabinose release from a Chardonnay juice polysaccharide in the presence of commercial pectolytic enzyme over a 5 day period in citrate buffer, pH 3.4; attributes used for sensory analysis of Shiraz wines; concentration (μg/L) of selected aroma volatiles in Shiraz wines made with the addition of enzyme, tannin, and mannoprotein (MP) supplements, either individually or in combination; correlation matrix of sensory data; correlation matrix of chemical data (PDF)

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Notes

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■ REFERENCES

(1) Smith, P. A.; McRae, J. M.; Bindon, K. A. Impact of winemaking practices on the concentration and composition of tannins in red wine. *Aust. J. Grape Wine Res.* **2015**, *21*, 601−614.

(2) Vidal, S.; Courcoux, P.; Francis, L.; Kwiatkowski, M.; Gawel, R.; Williams, P.; Waters, E.; Cheynier, V. Use of an experimental design approach for evaluation of key wine components on mouth feel perception. *Food. Qual. Prefer.* **2004**, *15*, 209−217.

(3) Vidal, S.; Francis, L.; Guyot, S.; Marnet, N.; Kwiatkowski, M.; Gawel, R.; Cheynier, V.; Waters, E. J. The mouth feel properties of grape and apple proanthocyanidins in a wine like medium. *J. Sci. Food Agric.* **2003**, *83*, 564−573.

(4) Quijada Morín, N.; Williams, P.; Rivas Gonzalo, J. C.; Doco, T.; Escribano Bailón, M. T. Polyphenolic, polysaccharide and oligosaccharide composition of Tempranillo red wines and their relationship with the perceived astringency. *Food Chem.* **2014**, *154*, 44−51.

(5) Kassara, S.; Kennedy, J. A. Relationship between red wine grade and phenolics. 2. Tannin composition and size. *J. Agric. Food Chem.* **2011**, *59*, 8409−8412.
(6) Doco, T.; Williams, P.; Cheynier, V. Effect of flash release and pectinolytic enzyme treatments on wine polysaccharide composition. *J. Agric. Food Chem.* **2007**, *55*, 6643−6649.
(7) Bindon, K.; Holt, H.; Williamson, P. O.; Varela, C.; Herderich, M.; Francis, I. L. Relationships between harvest time and wine composition in *Vitis vinifera* L. cv. Cabernet Sauvignon 2. Wine sensory properties and consumer preference. *Food Chem.* **2014**, *154*, 90−101.
(8) Vidal, S.; Francis, L.; Williams, P.; Kwiatkowski, M.; Gawel, R.; Cheynier, V.; Waters, E. The mouth feel properties of polysaccharides and anthocyanins in a wine like medium. *Food Chem.* **2004**, *85*, 519−525.
(9) Bindon, K.; Varela, C.; Kennedy, J.; Holt, H.; Herderich, M. Relationships between harvest time and wine composition in *Vitis vinifera* L. cv. Cabernet Sauvignon 1. Grape and wine chemistry. *Food Chem.* **2013**, *138*, 1696−1705.
(10) Cadot, Y.; Caillé, S.; Samson, A.; Barbeau, G.; Cheynier, V. Sensory representation of typicality of Cabernet Franc wines related to phenolic composition: impact of ripening stage and maceration time. *Anal. Chim. Acta* **2012**, *732*, 91−99.
(11) Apolinar Valiente, R.; Romero Cascales, I.; Williams, P.; Gómez Plaza, E.; López Roca, J. M.; Ros García, J. M.; Doco, T. Effect of winemaking techniques on polysaccharide composition of Cabernet Sauvignon, Syrah and Monastrell red wines. *Aust. J. Grape Wine Res.* **2014**, *20*, 62−71.
(12) Escot, S.; Feuillat, M.; Dulau, L.; Charpentier, C. Release of polysaccharides by yeasts and the influence of released polysaccharides on colour stability and wine astringency. *Aust. J. Grape Wine Res.* **2001**, *7*, 153−159.
(13) Versari, A.; du Toit, W.; Parpinello, G. P. Oenological tannins: a review. *Aust. J. Grape Wine Res.* **2013**, *19*, 1−10.
(14) Zietsman, A. J. J.; Moore, J. P.; Fangel, J. U.; Willats, W. G. T.; Trygg, J.; Vivier, M. A. Following the compositional changes of fresh grape skin cell walls during the fermentation process in the presence and absence of maceration enzymes. *J. Agric. Food Chem.* **2015**, *63*, 2798−2810.
(15) Bautista Ortín, A.; Jiménez Pascual, E.; Busse Valverde, N.; López Roca, J.; Ros García, J.; Gómez Plaza, E. Effect of wine maceration enzymes on the extraction of grape seed proanthocyani dins. *Food Bioprocess Technol.* **2013**, *6*, 2207−2212.
(16) Busse Valverde, N.; Gomez Plaza, E.; Lopez Roca, J. M.; Gil Munoz, R.; Fernandez Fernandez, J. I.; Bautista Ortin, A. B. Effect of different enological practices on skin and seed proanthocyanidins in three varietal wines. *J. Agric. Food Chem.* **2010**, *58*, 11333−11339.
(17) Busse Valverde, N.; Gómez Plaza, E.; López Roca, J. M.; Gil Muñoz, R.; Bautista Ortín, A. B. The extraction of anthocyanins and proanthocyanidins from grapes to wine during fermentative maceration is affected by the enological technique. *J. Agric. Food Chem.* **2011**, *59*, 5450−5455.
(18) Moreno Pérez, A.; Fernández Fernández, J. I.; Bautista Ortín, A. B.; Gómez Plaza, E.; Martínez Cutillas, A.; Gil Muñoz, R. Influence of winemaking techniques on proanthocyanidin extraction in Monastrell wines from four different areas. *Eur. Food Res. Technol.* **2013**, *236*, 473−481.
(19) Ortega Heras, M.; Pérez Magariño, S.; González Sanjosé, M. L. Comparative study of the use of maceration enzymes and cold pre fermentative maceration on phenolic and anthocyanic composition and colour of a Mencía red wine. *LWT−Food Sci. Technol.* **2012**, *48*, 1−8.
(20) Ayestarán, B.; Guadalupe, Z.; León, D. Quantification of major grape polysaccharides (Tempranillo v.) released by maceration enzymes during the fermentation process. *Anal. Chim. Acta* **2004**, *513*, 29−39.
(21) Rodrigues, A.; Ricardo Da Silva, J. M.; Lucas, C.; Laureano, O. Effect of commercial mannoproteins on wine colour and tannins stability. *Food Chem.* **2012**, *131*, 907−914.
(22) Del Barrio Galán, R.; Pérez Magariño, S.; Ortega Heras, M.; Guadalupe, Z.; Ayestarán, B. Polysaccharide characterization and their effect on white and red wine composition. *LWT−Food Sci. Technol.* **2012**, *48*, 215−223.
(23) Mercurio, M. D.; Dambergs, R. G.; Herderich, M. J.; Smith, P. A. High throughput analysis of red wine and grape phenolics − adaptation and validation of methyl cellulose precipitable tannin assay and modified somers color assay to a rapid 96 well plate format. *J. Agric. Food Chem.* **2007**, *55*, 4651−4657.
(24) Jeffery, D. W.; Mercurio, M. D.; Herderich, M. J.; Hayasaka, Y.; Smith, P. A. Rapid isolation of red wine polymeric polyphenols by solid phase extraction. *J. Agric. Food Chem.* **2008**, *56*, 2571−2580.
(25) Kennedy, J. A.; Jones, G. P. Analysis of proanthocyanidin cleavage products following acid catalysis in the presence of excess phloroglucinol. *J. Agric. Food Chem.* **2001**, *49*, 1740−1746.
(26) Kennedy, J. A.; Taylor, A. W. Analysis of proanthocyanidins by high performance gel permeation chromatography. *J. Chromatogr. A* **2003**, *995*, 99−107.
(27) Bindon, K. A.; Bacic, A.; Kennedy, J. A. Tissue specific and developmental modifications of grape cell walls influence the adsorption of proanthocyanidins. *J. Agric. Food Chem.* **2012**, *60*, 9249−9260.
(28) Ruiz Garcia, Y.; Smith, P. A.; Bindon, K. A. Selective extraction of polysaccharide affects the adsorption of proanthocyanidin by grape cell walls. *Carbohydr. Polym.* **2014**, *114*, 102−114.
(29) Lawless, H. T.; Heymann, H. Descriptive analysis. In *Sensory Evaluation of Food*, 2nd ed.; Springer: New York, 2010; pp 227−257.
(30) Apolinar Valiente, R.; Williams, P.; Romero Cascales, I.; Gómez Plaza, E.; López Roca, J. M.; Ros García, J. M.; Doco, T. Polysaccharide composition of Monastrell red wines from four different spanish terroirs: effect of wine making techniques. *J. Agric. Food Chem.* **2013**, *61*, 2538−2547.
(31) Vicens, A.; Fournand, D.; Williams, P.; Sidhoum, L.; Moutounet, M.; Doco, T. Changes in polysaccharide and protein composition of cell walls in grape berry skin (cv. Shiraz) during ripening and over ripening. *J. Agric. Food Chem.* **2009**, *57*, 2955−2960.
(32) Romero Cascales, I.; Ros García, J.; López Roca, J.; Gómez Plaza, E. The effect of a commercial pectolytic enzyme on grape skin cell wall degradation and colour evolution during the maceration process. *Food Chem.* **2012**, *130*, 626−631.
(33) Bonnin, E.; Dolo, E.; Le Goff, A.; Thibault, J. F. Character isation of pectin subunits released by an optimized combination of enzymes. *Carbohydr. Res.* **2002**, *337*, 1687−1696.
(34) Ducasse, M. A.; Canal Llauberes, R. M.; de Lumley, M.; Williams, P.; Souquet, J. M.; Fulcrand, H.; Doco, T.; Cheynier, V. Effect of macerating enzyme treatment on the polyphenol and polysaccharide composition of red wines. *Food Chem.* **2010**, *118*, 369−376.
(35) Doco, T.; Williams, P.; Vidal, S.; Pellerin, P. Rhamnogalactur onan II, a dominant polysaccharide in juices produced by enzymic liquefaction of fruits and vegetables. *Carbohydr. Res.* **1997**, *297*, 181−186.
(36) Castro López, L. d. R.; Gómez Plaza, E.; Ortega Regules, A.; Lozada, D.; Bautista Ortín, A. B. Role of cell wall deconstructing enzymes in the proanthocyanidin−cell wall adsorption−desorption phenomena. *Food Chem.* **2016**, *196*, 526−532.
(37) Parker, M.; Smith, P. A.; Birse, M.; Francis, I. L.; Kwiatkowski, M. J.; Lattey, K. A.; Liebich, B.; Herderich, M. J. The effect of pre and post ferment additions of grape derived tannin on Shiraz wine sensory properties and phenolic composition. *Aust. J. Grape Wine Res.* **2007**, *13*, 30−37.
(38) Alcalde Eon, C.; García Estévez, I.; Ferreras Charro, R.; Rivas Gonzalo, J. C.; Ferrer Gallego, R.; Escribano Bailón, M. T. Adding oenological tannin vs. overripe grapes: effect on the phenolic composition of red wines. *J. Food Compos. Anal.* **2014**, *34*, 99−113.
(39) Bautista Ortín, A. B.; Cano Lechuga, M.; Ruiz García, Y.; Gómez Plaza, E. Interactions between grape skin cell wall material and commercial enological tannins. Practical implications. *Food Chem.* **2014**, *152*, 558−565.

DOI: 10.1021/acs.jafc.6b04505
J. Agric. Food Chem. 2017, 65, 1353−1364

(40) Rowe, J. D.; Harbertson, J. F.; Osborne, J. P.; Freitag, M.; Lim, J.; Bakalinsky, A. T. Systematic identification of yeast proteins extracted into model wine during aging on the yeast lees. *J. Agric. Food Chem.* **2010**, *58*, 2337−2346.
(41) Mitropoulou, A.; Hatzidimitriou, E.; Paraskevopoulou, A. Aroma release of a model wine solution as influenced by the presence of non volatile components. Effect of commercial tannin extracts, poly saccharides and artificial saliva. *Food Res. Int.* **2011**, *44*, 1561−1570.
(42) Mayr, C. M.; Geue, J. P.; Holt, H. E.; Pearson, W. P.; Jeffery, D. W.; Francis, I. L. Characterization of the key aroma compounds in Shiraz wine by quantitation, aroma reconstitution, and omission studies. *J. Agric. Food Chem.* **2014**, *62*, 4528−4536.
(43) King, E. S.; Dunn, R. L.; Heymann, H. The influence of alcohol on the sensory perception of red wines. *Food. Qual. Prefer.* **2013**, *28*, 235−243.
(44) Webb, L.; Whetton, P.; Barlow, E. IT. *Clim. Res.* **2008**, *36*, 89−98.
(45) Sáenz Navajas, M. P.; Campo, E.; Fernández Zurbano, P.; Valentin, D.; Ferreira, V. An assessment of the effects of wine volatiles on the perception of taste and astringency in wine. *Food Chem.* **2010**, *121*, 1139−1149.
(46) Rinaldi, A.; Gambuti, A.; Moine Ledoux, V.; Moio, L. Evaluation of the astringency of commercial tannins by means of the SDS−PAGE based method. *Food Chem.* **2010**, *122*, 951−956.

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Supplementary Data

Use of Winemaking Supplements to Modify the Composition and Sensory Properties of Shiraz Wine

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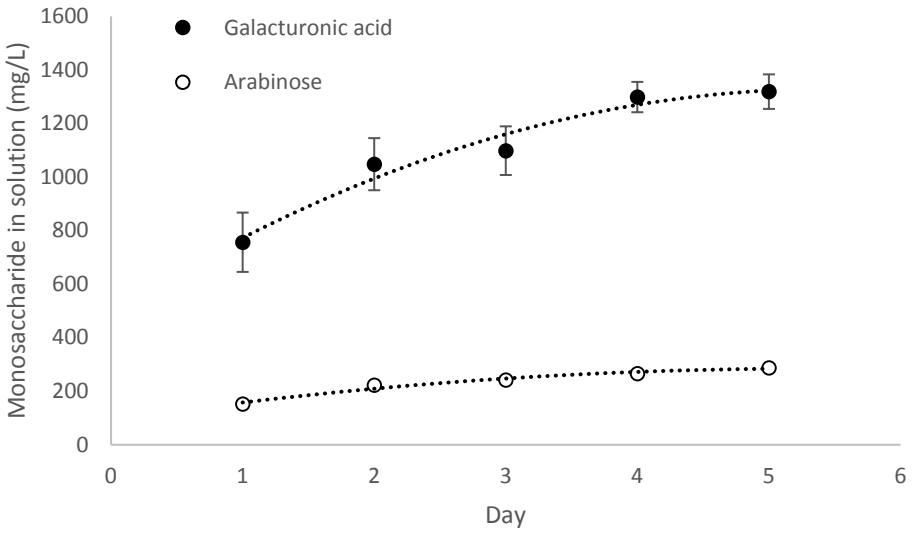
Supplementary Figure 1. Kinetics of galacturonic acid and arabinose release from a Chardonnay juice polysaccharide in the presence of commercial pectolytic enzyme over a 5 day period in citrate buffer, pH 3.4.



Supplementary Table 1. Attributes used for Sensory Analysis of Shiraz Wines.

Attributes	Description
Aroma	
Aroma Intensity	Perception of overall aroma intensity
Red fruit	Perception of fresh raspberry, strawberry, sour cherry and red plum
Dark fruit	Perception of fresh and strewed dark fruit blackberry, blueberry and plum aroma
Jammy	Perception of jam and prune
Vegetal	Perception of cut grass, leaf and fresh herb
Confectionary	Perception of strawberry cream and bubble gum
Earthy	Perception of musty and damp soil and mushroom
Pepper	Perception of black and white pepper
Dark chocolate	Perception of dark chocolate
Flavor	
Flavour Intensity	Perception of overall flavour intensity
Red fruit	Perception of fresh raspberry, strawberry, sour cherry and red plum
Dark fruit	Perception of fresh and strewed dark fruit blackberry, blueberry and plum aroma
Jammy	Perception of jam and prune
Vegetal	Perception of cut grass, leaf and fresh herb
Confectionary	Perception of strawberry cream and bubble gum
Pepper	Perception of black and white pepper
Dark Chocolate	Perception of dark chocolate
Palate	
Acidity	The intensity of acid taste perceived in the mouth or after expectorating
Sweetness	The intensity of sweet taste perceived in the mouth or after expectorating
Bitterness	The intensity of bitter taste perceived in the mouth or after expectorating
Palate fullness	The overall impression of weight or substantiveness of the wine in the mouth
Astringency	Overall level of astringency sensation
Surface coarseness	Texture felt on mouth surfaces
Hotness	The intensity of warmth or heat perceived in the mouth or after expectorating
Aftertaste	Time that wine flavour remains on palate after expectorating

Supplementary Table 2. Correlation Matrix of Sensory Data. (Correlation Efficiencies > ± 0.5 are in Bold and < ± 0.8 are Underlined).

Variables	Red Fruit	Dark Fruit	Confectionary	Flavor Intensity	Jammy	Sweetness	Palate Fullness	Astringency	Surface Coarseness	Hotness
Red Fruit	**1**	**–0.837**	**0.764**	–0.108	**–0.562**	0.045	**–0.573**	–0.088	–0.319	–0.489
Dark Fruit	**–0.837**	**1**	**–0.926**	0.493	**0.804**	0.389	**0.809**	–0.091	–0.021	**0.710**
Confectionary	**0.764**	**–0.926**	**1**	–0.447	**–0.655**	–0.221	**–0.685**	0.121	0.049	**–0.515**
Flavor Intensity	–0.108	0.493	–0.447	**1**	**0.732**	**0.762**	**0.707**	–0.111	–0.305	**0.714**
Jammy	**–0.562**	**0.804**	**–0.655**	**0.732**	**1**	**0.724**	**0.792**	–0.366	–0.338	**0.791**
Sweetness	0.045	0.389	–0.221	**0.762**	**0.724**	**1**	**0.685**	**–0.503**	**–0.692**	**0.769**
Palate Fullness	**–0.573**	**0.809**	**–0.685**	**0.707**	**0.792**	**0.685**	**1**	–0.118	–0.207	**0.900**
Astringency	–0.088	–0.091	0.121	–0.111	–0.366	**–0.503**	–0.118	**1**	**0.919**	–0.217
Surface Coarseness	–0.319	–0.021	0.049	–0.305	–0.338	**–0.692**	–0.207	**0.919**	**1**	–0.275
Hotness	–0.489	**0.710**	**–0.515**	**0.714**	**0.791**	**0.769**	**0.900**	–0.217	–0.275	**1**

Supplementary Table 3. Correlation Matrix of Chemical Data. (Correlation Efficiencies > ± 0.5 are in Bold and < ± 0.8 are Underlined).

Variables	Red Fruit	Dark Fruit	Confectionary	Flavor Intensity	Jammy	Sweetness	Palate Fullness	Astringency	Surface Coarseness	Hotness
Ethanol	–0.145	0.467	–0.262	**0.892**	**0.786**	**0.882**	**0.763**	–0.156	–0.324	**0.855**
pH	0.362	0.032	–0.011	0.498	0.356	**0.814**	0.445	**–0.628**	**–0.857**	0.404
TA	–0.434	0.593	–0.459	0.595	0.404	0.304	**0.683**	0.554	0.399	**0.653**
Color intensity	–0.455	0.452	–0.266	**0.653**	**0.554**	0.356	**0.664**	0.386	0.329	**0.700**
Hue	0.196	0.105	0.034	**0.550**	**0.593**	**0.885**	0.455	**–0.700**	**–0.815**	**0.540**
Total anthocyanin	–0.319	0.211	–0.089	0.412	0.155	–0.007	0.338	**0.678**	**0.625**	0.426
SO_2-resistant pigments	–0.305	0.498	–0.316	**0.851**	**0.672**	**0.629**	**0.764**	0.202	0.054	**0.813**
Malic acid	0.303	**–0.501**	0.222	–0.312	**–0.699**	**–0.551**	–0.439	0.132	0.139	–0.427
Glycerol	–0.155	0.487	–0.309	**0.917**	**0.762**	**0.869**	**0.801**	–0.122	–0.309	**0.868**
Fructose	–0.119	0.492	–0.316	**0.896**	**0.823**	**0.941**	**0.753**	–0.327	–0.483	**0.854**
Tannin concentration	–0.407	0.393	–0.262	0.465	0.254	0.059	0.486	**0.748**	**0.646**	0.459
%Epigal	0.084	0.278	–0.221	**0.785**	0.332	**0.634**	**0.566**	0.084	–0.184	**0.682**
%Epicat-gal	–0.074	–0.112	0.148	–0.196	–0.445	–0.525	–0.230	**0.894**	**0.861**	–0.166
mDP	–0.074	0.275	–0.224	**0.589**	0.127	0.244	0.489	**0.613**	0.354	0.460
Free Galacturonic acid	–0.322	0.138	–0.155	–0.143	–0.303	–0.554	–0.003	**0.937**	**0.914**	–0.133
Free Arabinose	–0.280	0.085	–0.124	–0.222	–0.384	**–0.613**	–0.082	**0.919**	**0.905**	–0.204
Free Rhamnose	–0.436	0.091	–0.048	–0.436	–0.274	**–0.683**	–0.132	**0.813**	**0.927**	–0.302
Galactose + Arabinose residue	0.199	0.173	–0.131	**0.561**	**0.578**	**0.865**	0.439	**–0.822**	**–0.936**	0.493
Rhamnose residue	–0.236	0.135	–0.196	–0.115	–0.349	**–0.513**	–0.003	**0.885**	**0.816**	–0.145
Mannose residue	–0.281	0.353	–0.368	0.080	–0.144	–0.136	0.324	**0.623**	0.486	0.226
Galacturonic acid residue	0.410	–0.170	0.113	0.029	0.119	0.468	–0.077	**–0.936**	**–0.951**	0.029

Supplementary Table 4. Concentration (µg/L) of Aroma Volatiles in Shiraz Wines made with the Addition of Enzyme, Tannin and Mannoprotein (MP) Supplements, either Individually or in Combination.

	Control	Cold Soak	Enzyme	Enzyme + MP	Tannin	Tannin + MP	MP	Later Harvest	*P*
Ethyl acetate	52446 b ± 912	53892 b ± 1676	42995 c ± 1727	51801 b ± 610	51408 b ± 2568	50776 b ± 2920	52917 b ± 1807	71418 a ± 2884	*<0.001*
Ethyl 2-methylpropanoate	102 b ± 5.2	88 c ± 3.7	94 bc ± 0.3	93 bc ± 2.0	101 b ± 2.6	99 b ± 1.5	101 b ± 3.5	121 a ± 4.1	*<0.001*
2-Methylpropyl acetate	56.5 b ± 6.6	42.7 c ± 2.9	44.5 c ± 2.3	41.1 c ± 2.3	45.8 c ± 1.2	42.8 c ± 1.2	46.8 bc ± 3.3	79.3 a ± 4.3	*<0.001*
Ethyl butanoate	181 b ± 7.5	142 de ± 10.3	145 de ± 1.9	134 e ± 2.6	165 bc ± 3.9	153 cde ± 4.1	160 cd ± 6.5	205 a ± 8.6	*<0.001*
Ethyl 2-methylbutanoate	18.1 ± 0.6	17.2 ± 1.1	19.7 ± 0.3	19.3 ± 0.6	18.7 ± 0.4	18.1 ± 0.3	18.5 ± 0.4	18.0 ± 0.6	*0.134*
Ethyl 3-methylbutanoate	27.9 bc ± 0.8	26.9 c ± 1.4	30.5 a ± 0.4	29.8 ab ± 0.3	28.5 abc ± 0.6	27.3 c ± 0.5	28.3 bc ± 0.4	28.0 bc ± 0.8	*0.037*
Ethyl hexanoate	551 a ± 18	483 bc ± 18	454 c ± 10	454 c ± 15	542 a ± 4	517 ab ± 15	544 a ± 7	475 c ± 15	*<0.001*
Ethyl lactate	6075 d ± 77	6687 bcd ± 580	7862 ab ± 550	7545 abc ± 731	6882 acbd ± 204	6487 cd ± 426	6099 d ± 211	8084 a ± 131	*0.022*
Ethyl octanoate	184 a ± 2	170 ab ± 21	144 bc ± 1	131 c ± 8	186 a ± 5	173 a ± 7	170 ab ± 5	188 a ± 4	*0.002*
2-Phenylethyl acetate	75.6 ± 2.3	76.3 ± 3.4	68.1 ± 3.3	81.3 ± 3.0	86.7 ± 3.1	89.7 ± 10.0	76.2 ± 1.8	92.7 ± 8.5	*0.062*
2-Methyl-1-butanol	59523 abc ± 710	53293 cd ± 1695	60431 abc ± 1420	56199 bc ± 5906	65805 ab ± 4239	67727 a ± 1133	61195 abc ± 4229	46169 d ± 2831	*0.006*
3-Methyl-1-butanol	305445 ab ± 2658	292416 c ± 2316	309864 a ± 4922	293285 bc ± 2655	305307 ab ± 3190	297871 abc ± 4050	302330 abc ± 6739	271462 d ± 4507	*<0.001*
1-Hexanol	5377 ± 79	5072 ± 262	5227 ± 93	5002 ± 107	5074 ± 160	5173 ± 166	5240 ± 51	4921 ± 122	*0.445*
2-Phenylethanol	90676 a ± 1175	92565 a ± 2379	92510 a ± 1692	94629 a ± 1495	96132 a ± 2162	94783 a ± 2837	91292 a ± 771	69787 b ± 2584	*<0.001*
Decanoic acid	1254 ± 155	1536 ± 160	1117 ± 57	1293 ± 35	1204 ± 57	1236 ± 168	1461 ± 256	1416 ± 270	*0.649*
Hexanoic acid	1452 ± 97	2690 ± 155	1431 ± 39	1646 ± 142	1469 ± 125	1599 ± 76	1508 ± 14	1547 ± 220	*0.897*

Values are means of 3 replicates ± standard error.
Values followed by different letters within rows are significantly different ($p \leq 0.05$, one way ANOVA, post hoc Fisher's LSD).

CHAPTER 3

Compositional variability in commercial tannin and mannoprotein products

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Overall percentage (%)	70		
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By signing the Statement of Authorship, each author certifies that:

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ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Short version of title: N/A

Abstract: Enotannin and mannoprotein additives are applied in order to achieve protein, cold or color stability in wine, or alternatively to modify wine sensory properties. In most cases, only basic compositional information and a proposed effect in wine are provided by the manufacturer. In this study, 14 grape-based enotannins and 8 mannoproteins were sourced from the Australian market and their composition and molecular size distribution were determined. Diverse product composition was observed for both categories, suggesting a range of effects could potentially be achieved by applying different products. Moreover, some products showed good agreement between product composition and their designated material of origin, while others showed significant differences.

Key words: Enotannin, Mannoprotein, Winemaking additive, Tannin, Polysaccharide

Introduction

Tannin and polysaccharides are key wine macromolecules. Their composition can be affected by both natural variation in grape composition, in particular due to grape maturity (Bindon et al. 2013), as well as deliberate modification by winemakers during vinification, mostly through addition of processing aids and additives (Li et al. 2017).

Enotannins are widely used by the winemakers to modify wine phenolic composition, and in turn wine clarity, color stability and sensory properties. For the clarification of grape juice and wine, enotannins are applied to bind and remove haze-forming proteins and metals (Laghi et al. 2010). In Australia, a survey identified four further objectives winemakers sought to achieve by using enotannins: color stabilization, creating specific wine styles, masking faults and general risk management (Hill and Kaine 2007). In reality, achievement of the desired effects on wine composition and sensory properties through enotannin addition have been shown to be variable, probably due to a range of factors, such as origins, dosage, grape variety and timing of addition

(Versari et al. 2013). In Australia, legally permitted enotannins are derived from two sources: grapes (skins and seeds) and oak wood, and were usually extracted by hot water or steam, and then dried and milled (Versari et al. 2013). A range of claims have been made by manufacturers and winemakers for the benefits of using enotannins, such as color-stabilization, anti-oxidation and mouthfeel improvement (Canuti et al. 2012). It is reasonable to assume that commercial enotannins exhibit a wide range of compositional differences to support these claims, although they are derived from limited types of plant materials.

In comparison to enotannin, the effects of mannoproteins on wine composition and sensory are not as well studied. Mannoprotein is one of the main polysaccharides present in red wine, with one study demonstrating that it accounted for 35% of total wine polysaccharide in a Carignan wine (Vidal et al. 2003). A primary reason for mannoprotein addition is to improve tartrate stability (Marchal and Jeandet 2009). Other research has suggested that mannoprotein can contribute to palate fullness and reduce astringency (Vidal et al. 2004, Quijada-Morín et al. 2014) as well as prevent protein haze formation (Waters et al. 1994). Although mannoproteins are not used as widely as oenotannins, there are still several industry suppliers producing and marketing mannoprotein additives.

In our previous study (Li et al. 2017), it was observed that addition of a grape-derived enotannin at the beginning of fermentation did not result in an increase in tannin concentration or astringent mouthfeel in the final wine, whereas addition of mannoprotein reduced both tannin concentration and coarse mouthfeel. However, we also noted that the effects observed were limited to the characteristics of the particular additives studied and may not extend to other enotannins or mannoproteins. In light of other studies that have yielded contradictory results (Versari et al. 2013), the ongoing survey of commercial enotannin and mannoprotein additives is warranted in order to understand the diversity of product composition and to what degree the choice of product can affect wine composition. This is especially important for the wine industry,

since detailed information on the composition of enotannin and mannoprotein additives is rarely given by manufacturers.

Materials and Methods

Commercial enotannins and mannoproteins

Commercial mannoprotein and enotannin products were sourced from five different manufacturers. At the manufacturers' request, product names have been obscured. Fourteen grape-derived enotannins were sourced, twelve of which were in powdered form while the remaining two were in liquid form. Eight mannoprotein products were obtained, five of which were in powdered form, with the other three in liquid form. Tannin products were labelled as skin, seed, or skin+seed, according to the origin of material reported by supplier, while mannoproteins (MP) were randomly numbered. Groups of products by manufacturer are provided as supplemental data (Table S1).

Analysis of enotannins

Liquid enotannins were freeze-dried and milled into powder prior to analysis. All enotannins were dissolved in model alcohol solution (containing 0.05% trifluoroacetic acid (TFA) and 12% v/v ethanol) at 3 g/L. The tannin concentration of these solutions was measured with the methylcellulose precipitable tannin (MCPT) assay (Mercurio et al. 2007). Based on the concentration, solutions containing approximately 2 mg tannin were loaded onto columns packed with Toyopearl (5 mL bed volume, Tosoh Bioscience, Shiba Minato-ku, Japan) in triplicate, to obtained purified tannin. The loaded columns were washed with 10 mL of 50% aqueous methanol (containing 0.05% TFA), prior to elution with 10 mL of 70% acetone (containing 0.05% TFA). The isolates were dried under nitrogen at 30 °C and reconstituted in methanol to a final concentration of 10 g/L, based on MCPT analysis. These tannin isolates were then analyzed by phloroglucinolysis (Kennedy and Jones 2001), which determined subunit composition, based on

which mean degree of polymerization (mDP) and molecular mass were also derived. Modifications for HPLC analysis were as described previously (Kennedy and Taylor 2003). For both the MCPT assay and phloroglucinolysis, (−)-epicatechin (Sigma Aldrich, St. Louis, MO, USA) was used as a standard for quantitation, as previously described (Kennedy and Jones 2001, Mercurio et al. 2007). Tannin size distribution was determined by gel permeation chromatography (Kennedy and Taylor 2003), with an Agilent 1100 HPLC (Agilent Technologies Australia Pty, Ltd., Melbourne Australia). Tannin fractions were diluted 1:5 with the HPLC mobile phase prior to injection. The instrumentation, chromatographic conditions and calibrations for GPC analysis modified from the original method were as described previously (Bindon and Kennedy 2011).

Freeze-dried, un-purified tannin products were also analyzed by the HPLC method of Mercurio and colleagues (2007) to determine the concentrations of key monomeric phenolics. Concentrations of (+)-catechin, (-)-epicatechin and gallic acid were quantified using analytical grade reference standards (Sigma Aldrich, St. Louis, MO, USA). Anthocyanins (13) were quantified as malvidin-3-glucose equivalents, using a reference standard (Polyphenols AS, Sandnes, Norway). Spectra obtained using Fourier transform mid-infrared spectroscopy (FT-IR) were used to infer the relative contribution of tannin and non-tannin material. Dried tannin products were analyzed using diffuse reflectance MIR on a Spectrum-One (PerkinElmer, Wellesley, MA, USA) FT-IR mid-infrared spectrometer. Comparison of spectra to known reference standards was made and are provided as supplemental information (Table S2).

Analysis of mannoproteins

Powdered MPs were reconstituted in model wine at 1 g/L and liquid products were freeze-dried, then redissolved in model wine at 1 g/L. Analyses were done in triplicate. Polysaccharide composition was determined following hydrolysis with 2 M trifluoroacetic acid for 3 h at 100 °C and monosaccharide residues released were quantified with an Agilent 1100 HPLC, according to a published method (Bindon et al. 2016). Total polysaccharide concentration was calculated by

summing the concentrations of all detected monosaccharide residues. MP solutions were dialyzed against a membrane with 3.5 kD molecular weight cut off (Pur-A-Lyzer Midi 3500 Dialysis kit, Sigma Aldrich, St Louise, USA), freeze-dried, and dissolved in 0.1 M sodium nitrate at a concentration of 2 g/L for size exclusion chromatography, as described in Bindon et al. (2016). Calibration standards of fixed molecular weight ~ 5kDa to ~ 800 kDa (Shodex P-82 Pullulan standards, Phenomenex, Sydney, Australia) were included in the same HPLC run as the samples to generate a standard curve (fitted with a fourth order polynomial function), enabling determination of molecular mass distribution as described previously (Bindon et al. 2016). Total nitrogen content for MPs was measured by the analytical services unit of the Commonwealth Scientific and Industrial Research Organization (CSIRO, Adelaide, Australia), using a TruMAC (Leco Corporation, Saint Joseph, USA). Powdered forms of MPs were combusted in an atmosphere of oxygen and nitrogen was determined as N_2 by thermal conductivity detection. Total protein was estimated by multiplying total nitrogen by a factor of 6.25 (Jones 1941).

Statistical analyses

Principal component analysis (PCA), partial least squares regression (PLS) and Hotelling's T-square (T^2) distribution were performed using either XLSTAT (version 2015.4.1, VSN International limited, Herts, UK). or Unscrambler X 10.5 software (CAMO Software AS, Oslo, Norway).

Results and discussion

Composition of enotannins

For all enotannins, MCPT was used to determine the tannin content in the products, and this value was expressed as a proportion of the gravimetric (dissolved) amount (3 g/L), here presented as percentage (Figure 1). Considerable variation in tannin content was observed amongst the products, from as low as 16% (skin5) to as high as 90% (seed3). These results are in agreement with another study reporting protein-precipitable tannin content in enotannin products, showing

recoveries ranging from 12% to 48% (Harbertson et al. 2012). To account for other possible constituents in the enotannin products, the most abundant monomeric phenolic compounds in grape seed or skin, namely catechin, epicatechin and gallic acid (Yilmaz and Toledo 2004) were quantified (supplemental Table S3). Together, these accounted for between 1% (seed2) and 10% (skin4) of total product weight. Also in skin5 a substantial portion (12% of the product weight) of anthocyanins were detected, but not in any other enotannins. The possibility also exists that dimers such as procyanidin B1 or B2 were not measured by the MCPT assay (Sarneckis et al. 2006), which may be present at various levels in enotannin products (Laghi et al. 2010).

To further characterize the relative similarity or divergence of product composition from grape-derived phenolics, FT-IR spectra analysis was performed. Reference spectra of likely product constituents (condensed tannins, flavonoid monomers) or possible impurities were included. It was found that the products demonstrated the characteristic important absorption bands for condensed tannins (data not shown) described previously (Laghi et al. 2010). PCA of FTIR spectra was performed followed by Hotelling's T^2 (supplemental Figure S1). The analysis indicated that with the exception of skin4 and skin5, all enotannin spectra were grouped similarly to one another and no separation was found based on their expected origin or composition. The enotannins had spectra analogous to the reference spectra for purified wine tannin, ripe skin or seed tannin and seeds from grape marc. The first two PCs of the PCA described 59% of the variance, and 71% in 3 PCs. Divergence of the enotannin spectra from the purified ripe seed tannin standard was indicated by Hotelling's T2 analysis of the PCA scores for PCs 1 and 2. However, for PCs 3 to 5 (data not shown) enotannin spectra were more similar to those of ripe seed tannin as defined by the Hotelling's T2 confidence limits.

For the skin4 and skin5 products, FTIR spectra were significantly related to both the mentioned reference tannins and the most abundant monomeric phenolics reported previously (supplemental Figure S1). It was interesting to note that purified tannin standards of preveraison

tannins (skin or seed) showed poor similarity to the enotannin products, and these reference spectra together with non-tannin reference standards were excluded from the final PCA models. These results indicated that the products showed strong similarity to the expected composition, despite low tannin recovery in some of the products. The nature of the uncharacterized portion of the enotannin products was not investigated further in this study, but was expected to be attributed to an impurity derived from the solubility agent (based on the different solubilities observed for each product). However, it was considered that differences in the tannin extinction coefficient (280 nm) could partially explain the variation in MCPT recovery, and will be discussed below.

In order to investigate the composition of the enotannins, the mDP, subunit composition and molecular size distribution of purified tannin fractions were measured (supplemental Table S4). Tannin mDP, subunit composition and hydrodynamic volume can indicate tannin origin in wine, i.e. from skin or seed (Kennedy and Taylor 2003). These parameters were measured to investigate whether or not (i) enotannins showed the expected composition based on the manufacturer-reported origin of material, i.e. seed and/or skin; and (ii) enotannin compositions were similar by product range for individual manufacturers and between products from different manufacturers; or (iii) whether compositional differences might influence tannin extinction coefficient. To this end, PCA was performed on chemical parameters with the first two principal components (PCs) explaining 69.3% of the total variance (Figure 2). Tannin products were primarily separated in the first dimension which was positively defined by mDP and average molecular mass, and negatively defined by the percentage of terminal epicatechin and catechin subunits. This was expected due to the intrinsic correlation of these parameters. It was not surprising, therefore, that a skin tannin, skin5, was most positively associated with the first PC, while a seed tannin, seed4, showed the opposite. The second PC was positively associated with epicatechin-gallate as both the extension and terminal subunit, as well as higher molecular mass at 50% GPC elution, all three of which were indicative of seed tannins. Seed5 and skin+seed2

were most positively associated with this PC. As expected, the second PC was negatively associated with the epigallocatechin extension subunit, which arises from skin tannin, and skin4 and 5 were the products most closely associated with this factor. Although the phloroglucinolysis and GPC analysis methods cannot determine whether a product was entirely extracted from seed or skin, the information obtained from them can be used to infer this based on their subunit composition and size. A good separation of the products could be observed using PCA, in particular those characteristics which typically define seed and skin tannin, as discussed above. Furthermore, the percentage mass conversion (subunit concentration as a proportion of MCPT concentration) for tannin by phloroglucinolysis analysis was generally higher than 50% (with the exception of skin1, skin6, seed2 and seed4) which indicated that for most products, the measured subunit compositions could be interpreted with confidence. In light of this, several products, both seed and skin tannins, exhibited chemical compositions that strongly agreed with the labeled origin of material. In contrast, for other products, their origin whether from seed or skin, was not immediately discernable by the suite of analytical methods employed. Inclusion of stem derived tannin might explain the indeterminable nature of some of the products, as stem tannins contain the same subunits as skin and seed tannin but to a different proportion and mDP (Vivas et al. 2004). Previous studies have shown that enotannin products could contain components from plant origins that were not specified on the label (Obreque-Slíer et al. 2009), and this amalgamated nature interfered with the identification of origin with chemical methods (Laghi et al. 2010). This has significant implications for commercial practice as skin tannin is generally marketed for at a higher price than seed tannin (Obreque-Slíer et al. 2009) and winemakers may have preferences for different types of tannin (Hill and Kaine 2007). Moreover, a reasonable separation based on PCA could be seen among the range of products, indicating diverse chemical compositions among the commercial enotannin products. However, it is also interesting to note that for certain manufacturers, although products were marketed under different names and with different claims

on effects, their compositions were similar, as indicated by closely clustering on the PCA plot (e.g. skin1, skin2 and seed 2). For certain manufacturers, enotannins showed a high degree of scatter within the PCA plot, suggesting significant compositional differences (e.g. seed 4 and skin 5).

A final objective of the enotannin compositional analysis was to infer the potential contribution of subunit compositional differences to tannin recovery by MCPT, since it is known that the flavan-3-ol types which comprise condensed tannins may have different molar absorptivities (Kennedy and Jones 2001, Pelillo et al. 2004). A PLS model was developed (supplemental Figure S2) showing that a relatively higher proportion of epicatechin-gallate, together with lower or absent epigallocatechin could partially explain tannin recovery differences. This may indicate that underlying extinction coefficient (280 nm) differences were conferred by tannin subunit composition, and warrants ongoing investigation. However, since the bulk of the tannin was represented by the subunits epicatechin and catechin (71.2 to 88.4%) this would be expected to offer only a limited explanation for MCPT recovery differences.

Composition of mannoproteins

Mannoproteins typically consist of mannan, glucan and protein moieties. Thus MP product content was expressed as the percentage recovery of monosaccharide residues and proteins as a function of dry powder mass (Figure 3), with the associated concentration and composition of the polysaccharide fractions reported in Table 1. A lesser degree of variation in percentage recovery by weight was observed for MPs than for enotannins, ranging from 60% for MP4 to nearly 100% for MP7, despite the obvious compositional differences. These recoveries are based on hydrolytically-released monosaccharides only, hence it needs to be considered that differences between products may exist in the proportion of polysaccharide which is hydrolysable. The products were also tested for the presence of monosaccharides, and only mannose and glucose were detected in two products, MP2 and MP4, accounting for 3% and 0.5% of product weight

240 respectively (data not shown). Two types of mannoproteins are typically derived from
241 *Saccharomyces cerevisiae* yeast, namely exocellular mannoprotein which is secreted during
242 fermentation, and membrane-bound mannoprotein which requires disruption of cell walls for
243 extraction to occur. The former has a higher mannan to glucan ratio and a lower contribution of
244 proteins than the latter (Saulnier et al. 1991). Therefore, compositional differences observed
245 amongst MPs may reflect the production methods adopted by individual manufacturers.
246 Interestingly, in MP4, MP5 and MP6, substantial amounts of arabinose and galactose residues
247 were also detected, especially for MP5, where the combined concentration of these two residues
248 exceeded that of mannose and glucose. This indicated the presence of significant amounts of
249 arabinogalactan-proteins (AGP) which are plant-derived (grape), rather than yeast-derived.

250 The protein content of the MP products ranged between 10% and 50% (Figure 3). Despite
251 possible overestimation of protein from total nitrogen value due to the presence of non-protein
252 nitrogen, variations in protein content in polysaccharide products may have significant
253 implications for enological applications through different interactions with tannin. Mekoue
254 Nguela and colleagues (2016) found that yeast protein extracts had much higher affinity to
255 polyphenols than yeast membrane mannoproteins or glucans. In particular, long-chained, linear
256 skin tannin formed irreversible associations with protein, and sedimented spontaneously. One
257 possible explanation is that unfolded proteins and tannins are likely to form compact aggregates
258 leading to precipitation; in contrast, polysaccharides and tannins form loose, microgel-like
259 aggregates that are much likely to stay solvated (Carn et al. 2012). Thus, the use of
260 compositionally different MPs would be expected to impact on wine polyphenolic composition
261 in dissimilar manner based on their colloidal properties.

262 The molecular weight distribution of polysaccharide particles was determined between 5
263 and 800 kDa, within calibration range of the analytical method, and was typical for wine
264 polysaccharide (Guadalupe et al. 2014). Only four characteristic products are presented: MP2 and

MP7 which present the extremes in molecular weight range for products containing only MP, together with MP4 and MP6 which contained both MP and AGPs (Figure 4). The main difference observed between these two groups of products was a lack of material between 5 and 100 kDa, accompanied by a contribution of material larger than 500 kDa in the AGP-containing group. Hence, products containing only MP typically were between 5 and 400 kDa, while the products containing both MP and AGP ranged were between 100 and 600 kDa. This was surprising, given that AGP isolated from wine is reported to be between 50 and 260 kDa while wine mannoproteins were reported to be from 5 to more than 800 kDa (Guadalupe et al. 2014). As discussed previously, these differences may reflect the method of production, and origin of the respective products. In terms of the potential impacts of these products on wine composition, it is relevant to highlight that polysaccharide size may influence the interaction with tannin. Poncet-Legrand and colleagues (2007) showed that for wine-extracted MPs between 30 and 400 kDa, MP fractions of 50 and 60 kDa reduced seed tannin aggregation, while a 300 kDa MP fraction induced flocculation (tannin-MP precipitates). Furthermore, a study showed that a mannoprotein product of approximately 70 kDa could prevent precipitation of anthocyanin adducts, thus contributing to color stability (Alcalde-Eon et al. 2014). Knowledge of the molecular size of an MP product, as well as its composition, are therefore important considerations for a winemaker selecting a commercial MP additive. Further investigation is needed to confirm the effects of various MP products on wine colloidal properties. Our ongoing work will seek to elaborate on the interaction between mannoprotein and tannin, using distinctive products characterized in the current study.

CONCLUSION

This study screened commercial tannin and mannoprotein additives and demonstrated substantial diversity in their compositions. Different effects on wine composition can reasonably be expected to arise from the choice of product. Furthermore, some additives showed good agreement between

290 the origin of product and chemical composition, i.e. skin/seed-derived or mannoprotein-rich,
291 while others did not. It is therefore recommended that winemakers perform bench trials using
292 commercial additives with the wines to be treated, to select products that best suit their objectives.

Literature Cited

Alcalde-Eon C, García-Estévez I, Puente V, Rivas-Gonzalo JC, and Escribano-Bailón MT. 2014. Color Stabilization of Red Wines. A Chemical and Colloidal Approach. J. Agric. Food Chem. 62: 6984-6994.

Bindon KA, and Kennedy JA. 2011. Ripening-induced changes in grape skin proanthocyanidins modify their interaction with cell walls. J. Agric. Food Chem. 59: 2696-2707.

Bindon KA, Li S, Kassara S, and Smith PA. 2016. Retention of proanthocyanidin in wine-like solution is conferred by a dynamic interaction between soluble and insoluble grape cell wall components. J. Agric. Food Chem. 64: 8406-8419.

Bindon KA, Varela C, Kennedy JA, Holt H, and Herderich M. 2013. Relationships between harvest time and wine composition in *Vitis vinifera* L. cv. Cabernet Sauvignon 1. Grape and wine chemistry. Food Chem. 138: 1696–1705.

Canuti V, Puccioni S, Giovani G, Salmi M, Rosi I, and Bertuccioli M. 2012. Effect of Oenotannin Addition on the Composition of Sangiovese Wines from Grapes with Different Characteristics. Am. J. Enol. Vitic.

Carn F, Guyot S, Baron A, Pérez J, Buhler E, and Zanchi D. 2012. Structural properties of colloidal complexes between condensed tannins and polysaccharide hyaluronan. Biomacromolecules 13: 751-759.

Guadalupe Z, Ayestarán B, Williams P, and Doco T. 2014. Determination of must and wine polysaccharides by gas chromatography-mass spectrometry (GC-MS) and size-exclusion chromatography (SEC). *In* Polysaccharides: Bioactivity and Biotechnology. Kishan Gopal Ramawat and Jean-Michel Mérillon (eds.), pp. 1-28. Springer International Publishing, Cham.

Harbertson JF, Parpinello GP, Heymann H, and Downey MO. 2012. Impact of exogenous tannin additions on wine chemistry and wine sensory character. Food Chem. 131: 999-1008.

Hill M, and Kaine G. 2007. The market potential of tannin related innovations in the wine industry. Victorian Department of Primary Industries, Tatura.

Jones DB. 1941. Factors for converting percentages of nitrogen in foods and feeds into percentages of proteins. US Department of Agriculture Washington, DC.

Kennedy JA, and Jones GP. 2001. Analysis of proanthocyanidin cleavage Products following acid-catalysis in the presence of excess phloroglucinol. J. Agric. Food Chem. 49: 1740–1746.

Kennedy JA, and Taylor AW. 2003. Analysis of proanthocyanidins by high-performance gel permeation chromatography. J. Chromatogr. A 995: 99–107.

Laghi L, Parpinello GP, Rio DD, Calani L, Mattioli AU, and Versari A. 2010. Fingerprint of enological tannins by multiple techniques approach. Food Chem. 121: 783-788.

Li S, Bindon KA, Bastian SEP, Jiranek V, and Wilkinson KL. 2017. Use of winemaking supplements to modify the composition and sensory properties of Shiraz wine. J. Agric. Food Chem. 65: 1353-1364.

Marchal R, and Jeandet P. 2009. Use of enological additives for colloid and tartrate salt stabilization in white wines and for improvement of sparkling wine foaming properties. *In* Wine Chemistry and Biochemistry. M. Victoria Moreno-Arribas and M. Carmen Polo (eds.), pp. 127-158. Springer New York, New York, NY.

Mekoue Nguela J, Poncet-Legrand C, Sieczkowski N, and Vernhet A. 2016. Interactions of grape tannins and wine polyphenols with a yeast protein extract, mannoproteins and β-glucan. Food Chem. 210: 671-682.

Mercurio MD, Dambergs RG, Herderich MJ, and Smith PA. 2007. High throughput analysis of red wine and grape phenolics - Adaptation and validation of methyl cellulose precipitable tannin assay and modified somers color assay to a rapid 96 well plate format. J. Agric. Food Chem. 55: 4651–4657.

Obreque-Slíer E, Peña-Neira A, López-Solís R, Ramírez-Escudero C, and Zamora-Marín F. 2009. Phenolic characterization of commercial enological tannins. Eur. Food Res. Technol. 229: 859-866.

Pelillo M, Cuvelier ME, Biguzzi B, Gallina Toschi T, Berset C, and Lercker G. 2004. Calculation of the molar absorptivity of polyphenols by using liquid chromatography with diode array detection: the case of carnosic acid. J. Chromatogr. A 1023: 225-229.

Poncet-Legrand C, Doco T, Williams P, and Vernhet A. 2007. Inhibition of grape seed tannin aggregation by wine mannoproteins: Effect of polysaccharide molecular weight. Am. J. Enol. Vitic. 58: 87-91.

Quijada-Morín N, Williams P, Rivas-Gonzalo JC, Doco T, and Escribano-Bailón MT. 2014. Polyphenolic, polysaccharide and oligosaccharide composition of Tempranillo red wines and their relationship with the perceived astringency. Food Chem. 154: 44–51.

Sarneckis CJ, Dambergs RG, Jones P, Mercurio M, Herderich MJ, and Smith PA. 2006. Quantification of condensed tannins by precipitation with methyl cellulose: development and validation of an optimised tool for grape and wine analysis. Aust. J. Grape Wine Res. 12: 39-49.

Saulnier L, Mercereau T, and Vezinhet F. 1991. Mannoproteins from flocculating and non-flocculating *Saccharomyces cerevisiae* yeasts. J. Sci. Food Agric. 54: 275-286.

Versari A, du Toit W, and Parpinello GP. 2013. Oenological tannins: a review. Aust. J. Grape Wine Res. 19: 1–10.

Vidal S, Francis L, Williams P, Kwiatkowski M, Gawel R, Cheynier V, and Waters E. 2004. The mouth-feel properties of polysaccharides and anthocyanins in a wine like medium. Food Chem. 85: 519–525.

Vidal S, Williams P, Doco T, Moutounet M, and Pellerin P. 2003. The polysaccharides of red wine: total fractionation and characterization. Carbohydr. Polym. 54: 439–447.

Vivas N, Nonier M-F, de Gaulejac NV, Absalon C, Bertrand A, and Mirabel M. 2004. Differentiation of proanthocyanidin tannins from seeds, skins and stems of grapes (Vitis vinifera) and heartwood of Quebracho (Schinopsis balansae) by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and thioacidolysis/liquid chromatography/electrospray ionization mass spectrometry. Anal. Chim. Acta 513: 247-256.

Waters EJ, Pellerin P, and Brillouet J-M. 1994. A Saccharomyces mannoprotein that protects wine from protein haze. Carbohydr. Polym. 23: 185-191.

Yilmaz Y, and Toledo RT. 2004. Major Flavonoids in Grape Seeds and Skins: Antioxidant Capacity of Catechin, Epicatechin, and Gallic Acid. J. Agric. Food Chem. 52: 255-260.

Figure Captions

Figure 1. Methylcellulose precipitable tannin contents in commercial enotannin products, expressed as percentage (w/w). Values are means of three replicates and the error bars represent standard errors.

Figure 2. PCA plot of enotannins based on chemical composition, where product names are italicised and common symbols indicate products were sourced from the same manufacturer; 'ext.', extension subunits, 'ter', terminal subunits.

Figure 3. Proportion of polysaccharides (■) and proteins (▒) in MP additives expressed as a percentage (w/w) of dry power. The values are means of three replicates and the error bars represent standard error.

Figure 4. Molecular mass distribution of polysaccharides between 5 kDa and 800 kDa for products containing only mannoprotein (MP2 —— MP7 - - -) or containing both mannoprotein and arabinogalactan (MP4 —— MP6 - - -), determined by size exclusion chromatography.

Table 1. Monosaccharide composition of mannoprotein products.

Product	Mannose	Glucose	Galactose	Arabinose	Total polysaccharide
	(mg/g)[a]	(mg/g)	(mg/g)	(mg/g)	(mg/g)
MP1	667 ± 30	52 ± 32	n.d.[b]	n.d.	719 ± 33
MP2	442 ± 17	149 ± 2	n.d.	n.d.	590 ± 19
MP3	413 ± 16	4 ± 0	n.d.	n.d.	417 ± 16
MP4	96 ± 3	40 ± 1	123 ± 2	128 ± 3	387 ± 11
MP5	97 ± 2	41 ± 2	39 ± 1	37 ± 1	214 ± 7
MP6	117 ± 10	39 ± 2	37 ± 2	37 ± 1	229 ± 15
MP7	739 ± 13	136 ± 6	n.d.	n.d.	875 ± 7
MP8	641 ± 9	26 ± 15	n.d.	n.d.	667 ± 6

Values are means of 3 replicates ± standard error.
[a] The monosaccharide residue concentrations are expressed in mg per gram dry powder product, the sum of which is used to calculate total polysaccharide.
[b] n.d. = not detected

Figure 1

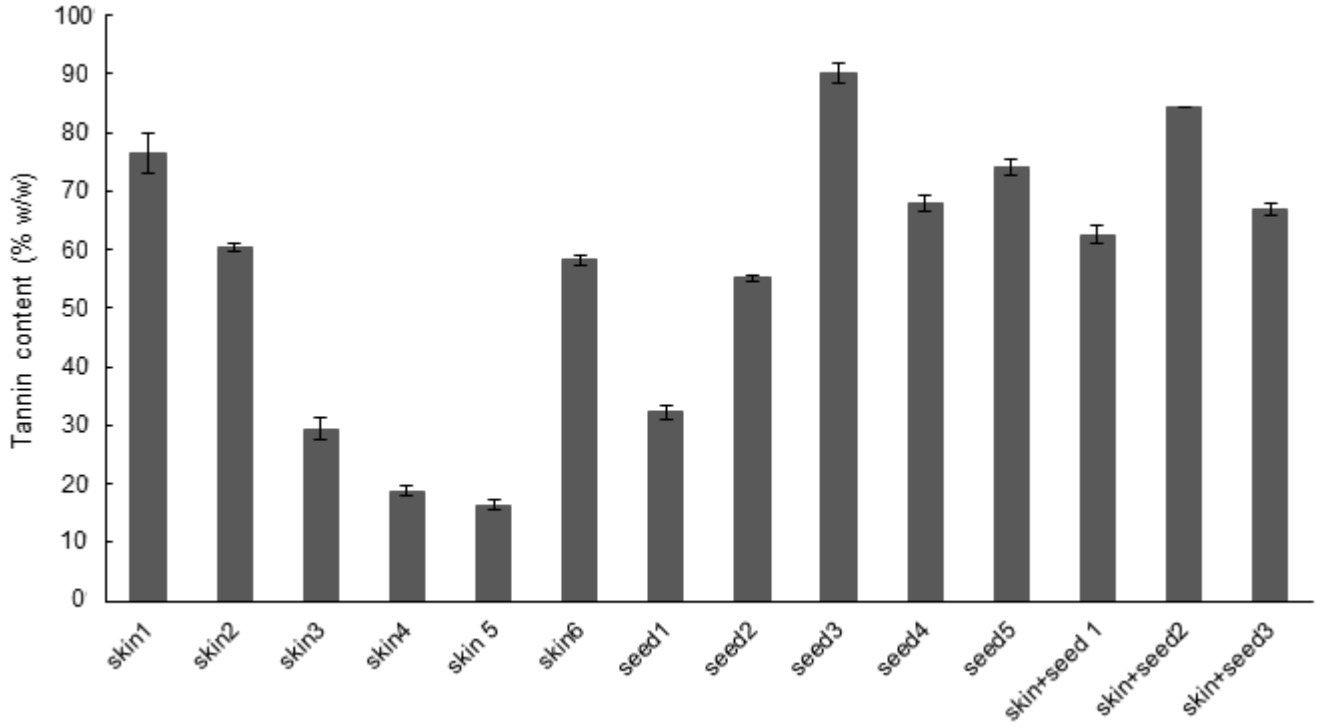

Tannin content (% w/w)
100
90
80
70
60
50
40
30
20
10
0
skin1
skin2
skin3
skin4
skin 5
skin6
seed1
seed2
seed3
seed4
seed5
skin+seed 1
skin+seed2
skin+seed3

Figure 2

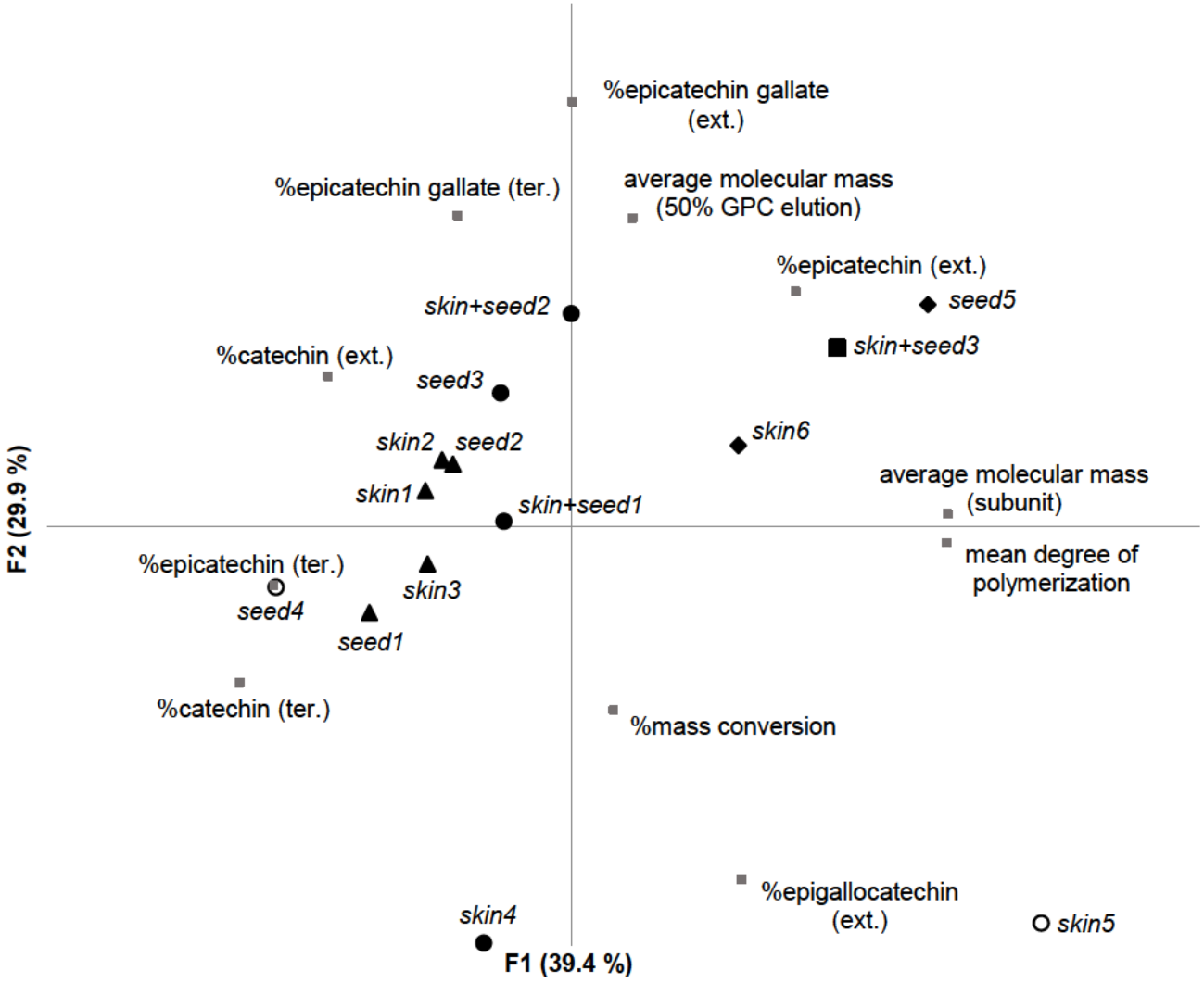

%epicatechin gallate (ext.)
%epicatechin gallate (ter.)
average molecular mass (50% GPC elution)
%epicatechin (ext.)
seed5
skin+seed2
skin+seed3
%catechin (ext.)
seed3
skin6
skin2
seed2
skin1
skin+seed1
average molecular mass (subunit)
mean degree of polymerization
%epicatechin (ter.)
skin3
seed4
seed1
%catechin (ter.)
%mass conversion
%epigallocatechin (ext.)
skin5
skin4
F1 (39.4 %)
F2 (29.9 %)

Figure 3

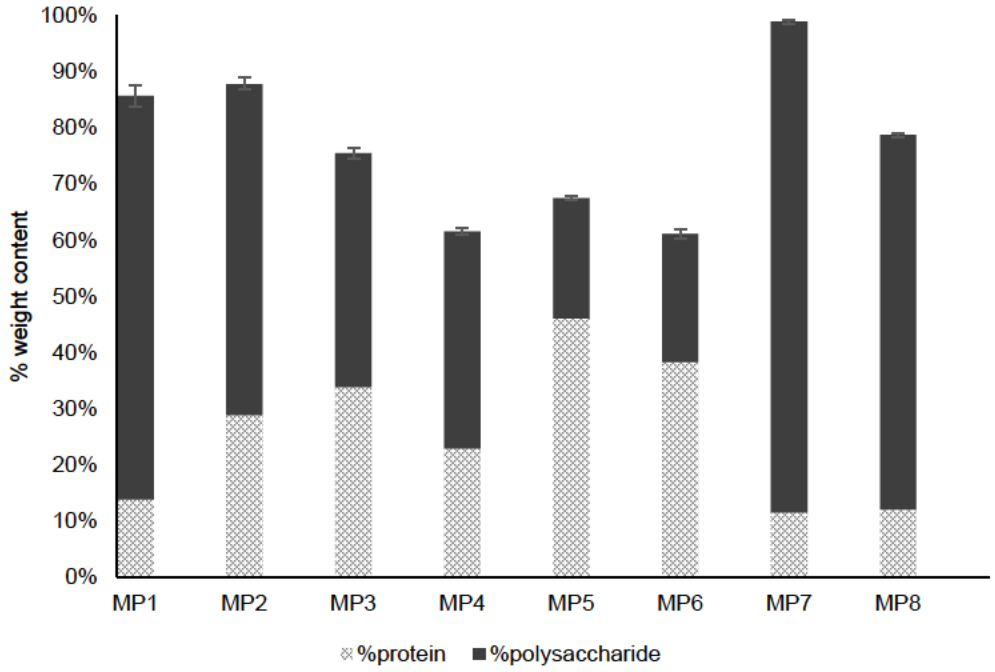
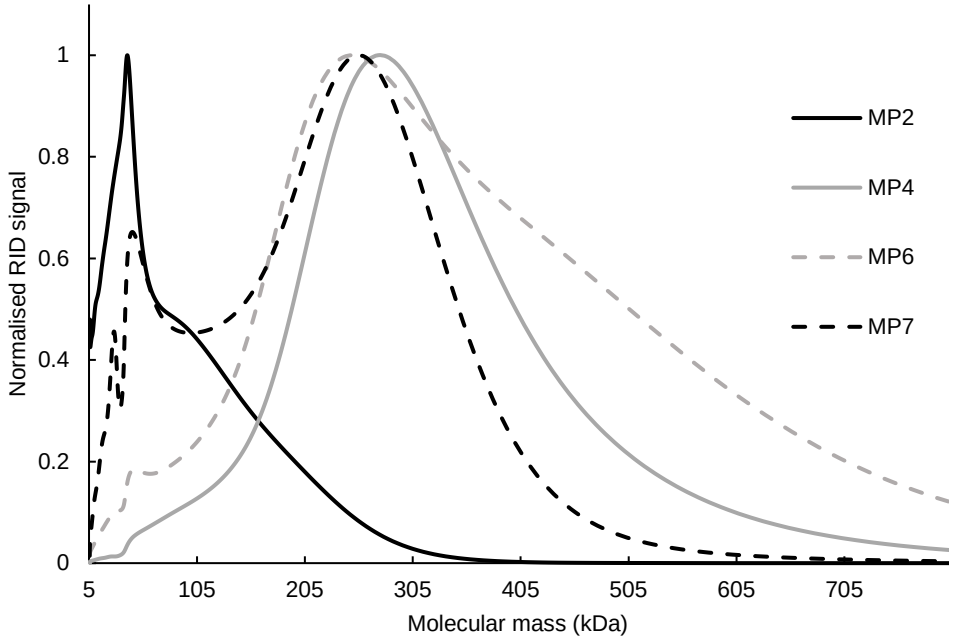

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% weight content
MP1
MP2
MP3
MP4
MP5
MP6
MP7
MP8
%protein
%polysaccharide

Figure 4


Normalised RID signal
1
0.8
0.6
0.4
0.2
0
5
105
205
305
405
505
605
705
Molecular mass (kDa)
MP2
MP4
MP6
MP7

Supplemental Data

Compositional variability in commercial tannin and mannoprotein products

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Table S1. Enotannin and mannoprotein products used in the study showing their codes and grouped according to common manufacturers.

Producer group	Enotannin code	Mannoprotein code
1	skin1	MP2
	skin2	
	skin3	
	seed1	
	seed2	
2	skin+seed3	MP1
		MP4
		MP7
3	skin4	MP3
	seed3	MP5
	skin+seed 1	MP6
	skin+seed2	
4	skin 5	
	seed4	
5	skin6	
	seed5	
6		MP8

Table S2. Reference compounds and their source used for comparison in the Fourier transform mid-infrared spectroscopic analysis of enotannin products.

Reference standard	Supplier / Reference
Arabinogalactan	Sigma Aldrich, St. Louis, MO, USA
Ascorbic acid	Merck, Bayswater, VIC, Australia
Calcium tartrate	Sigma Aldrich, St. Louis, MO, USA
(+)-Catechin hydrate	Sigma Aldrich, St. Louis, MO, USA
Cellulose	Sigma Aldrich, St. Louis, MO, USA
Citric acid	Sigma Aldrich, St. Louis, MO, USA
D-Fructose	Sigma Aldrich, St. Louis, MO, USA
D-glucose	Merck, Bayswater, VIC, Australia
Ellagic acid	Sigma Aldrich, St. Louis, MO, USA
(-)-Epicatechin	Sigma Aldrich, St. Louis, MO, USA
Gallic acid	Sigma Aldrich, St. Louis, MO, USA
Grape marc seeds (white)	Hixson et al. (2015). *Journal of Agricultural and Food Chemistry,* 63(45): 9954-9962.
Malvidin-3-glucoside	Vidal et al. (2004). *Journal of Agricultural and Food Chemistry* , 52(4): 713-719.
Pectin from Apple P-8471 SIGMA	Sigma Aldrich, St. Louis, MO, USA
Polygalacturonic acid Sigma P3889-5G	Sigma Aldrich, St. Louis, MO, USA
Potassium hydrogen tartrate	Sigma Aldrich, St. Louis, MO, USA
Potassium metabisulfite	Sigma Aldrich, St. Louis, MO, USA
Preveraison seed tannin (Tannat)	Hixson et al. (2015). *Journal of Agricultural and Food Chemistry,* 63(45): 9954-9962.
Preveraison skin tannin (Tannat)	Hixson et al. (2015). *Journal of Agricultural and Food Chemistry,* 63(45): 9954-9962.
Purified grape skin cell walls (marc)	Bindon and Smith (2013). *Food Chemistry* , 136(2): 917-928.
Purified yeast lees (red wine)	AWRI database, unpublished
Quercetin	Sigma Aldrich, St. Louis, MO, USA
Red wine tannin	McRae et al, (2013), *61* (47): 11618-11627.
Rhamnogalacturonan (soy)	Megazyme, Irishtown, Bray, Co. Wicklow, Ireland
Seed tannin (ripe)	Cheah et al. (2014). *PLoS One* 9(6) (2014): e98921
Seed tannin (ripe)	Bindon et al. (2010). *Journal of Agricultural and Food Chemistry* , 58(19): 10736-10746
Skin tannin (ripe)	Bindon et al. (2016). J*ournal of Agricultural and Food Chemistry,* 64(44): 8406-8419.
Tannic acid	Sigma Aldrich, St. Louis, MO, USA
Tartaric acid	Sigma Aldrich, St. Louis, MO, USA
Wine protein	Bindon et al. (2016). *Journal of Agricultural and Food Chemistry,* 64(44): 8406-8419.

Table S2. Monomeric phenolic compounds (mg) contained in 1 gram of the enotannin products.

	Catechin (mg/g)	Epicatechin (mg/g)	Gallic acid (mg/g)	Anthocyanin (mg/g)	Total (mg/g)
Skin1	3.4 ± 0.4	22.4 ± 0.3	2.2 ± 0.0	n.d.	28
Skin2	15.0 ± 0.1	13.8 ± 0.2	2.8 ± 0.1	n.d.	31.6
Skin3	22.2 ± 0.2	18.5 ± 0.1	5.5 ± 0.1	n.d.	46.2
Skin4	46.9 ± 0.5	49.0 ± 0.6	7.1 ± 0.1	n.d.	103
Skin5	c n.q.	c n.q.	11.6 ± 0.4	111.6 ± 0.3	123.2
Skin6	15.2 ± 0.2	22.8 ± 1.4	5.4 ± 0.0	n.d.	43.4
Seed1	21.4 ± 0.1	17.8 ± 0.1	5.1 ± 0.1	n.d.	44.3
Seed2	3.3 ± 0.0	c n.q.	7.3 ± 0.0	n.d.	10.6
Seed3	46.8 ± 0.3	30.9 ± 0.3	2.0 ± 0.2	n.d.	79.7
Seed4	22.7 ± 0.4	c n.q.	20.6 ± 0.7	n.d.	43.3
Seed5	39.7 ± 1.0	20.4 ± 0.2	2.5 ± 0.1	n.d.	62.6
Skin+seed1	37.4 ± 0.8	18.0 ± 0.6	12.2 ± 0.1	n.d.	67.6
Skin+seed2	29.6 ± 0.2	25.3 ± 0.1	1.1 ± 0.1	n.d.	56
Skin+seed3	19.3 ± 0.4	15.8 ± 0.3	19.3 ± 1.2	n.d.	54.4

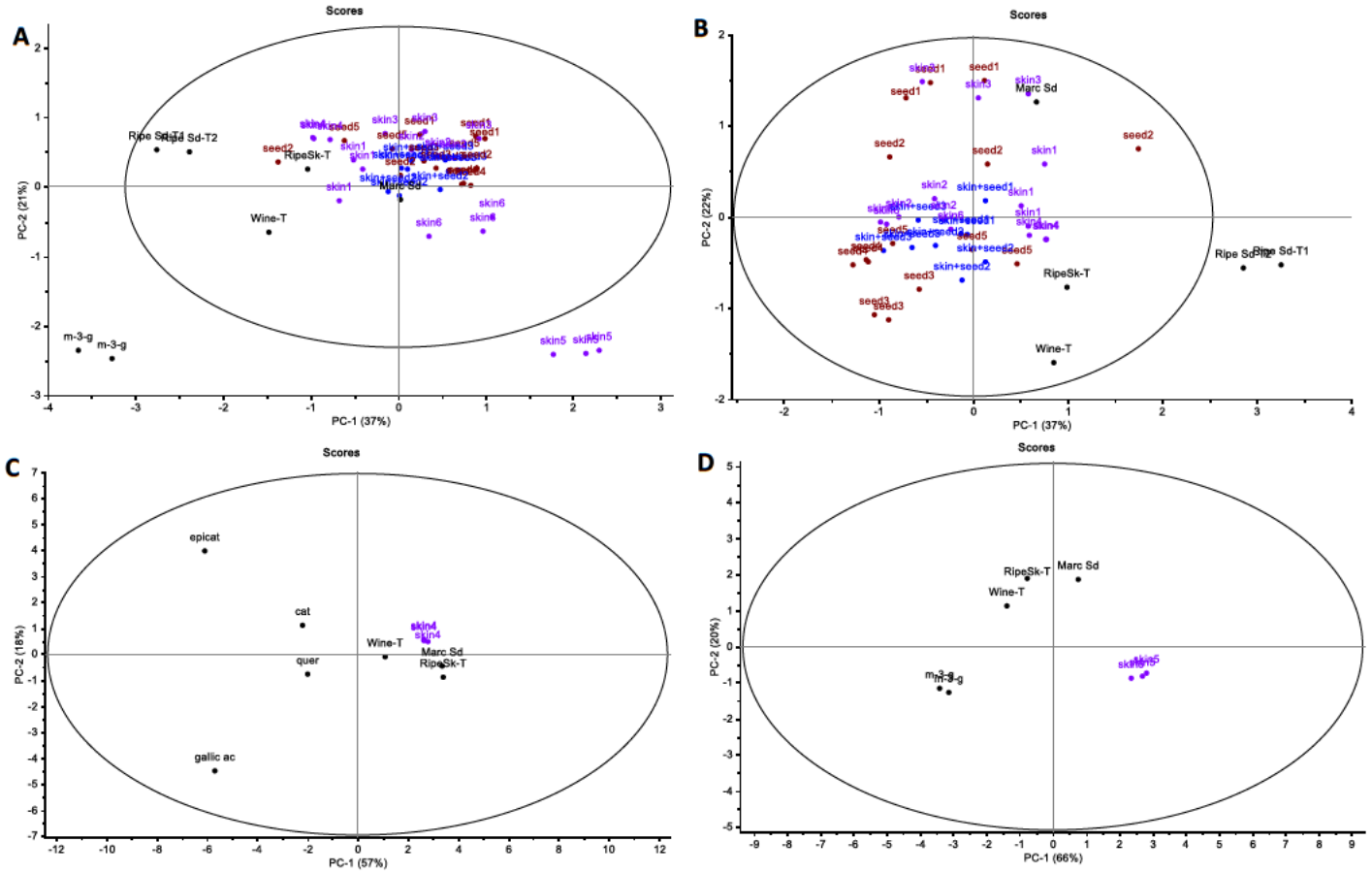
Values are expressed as mg per g of product and are means of 3 replicates ± standard error.
c.n.q.: can not be quantified. For skin5, catechin and epicatechin could not be quantified due to co-eluting with anthocyanins which have high absorbance at 280 nm. In all other cases, compounds could not be quantified because they are present at concentrations lower than quantification threshold.
n.d.: not detected

Table S3. Subunit composition (%), mean degree of polymerization (mDP) and average molecular weight derived from phloroglucinolysis analysis and average molecular weight derived from GPC of commercial enotannin products.

	% epigallo-catechin (ext.)	% catechin (ext.)	% epicatechin (ext.)	% epicatechin-gallate (ext.)	%catehin (ter.)	%epicatechin (ter.)	%epicatechin-gallate (ter.)	mDP	%mass conversion	Molecular weight (g/mol)	50% GPC elution (g/mol)
Skin1	4.0 ± 0.2	9.8 ± 0.3	52.5 ± 1.0	8.1 ± 0.1	13.5 ± 0.3	8.1 ± 0.4	4.1 ± 0.2	3.91 ± 0.12	13.2 ± 0.5	1209 ± 36	1263 ± 4
Skin2	3.5 ± 0.1	10.0 ± 0.2	53.3 ± 0.5	7.6 ± 0.1	11.4 ± 0.5	7.6 ± 0.2	3.5 ± 0.1	3.91 ± 0.09	50.8 ± 1.4	1202 ± 26	1923 ± 17
Skin3	4.3 ± 0.1	9.6 ± 0.1	53.2 ± 0.2	6.5 ± 0.1	12.4 ± 0.1	10.5 ± 0.3	3.3 ± 0.0	3.81 ± 0.06	51.6 ± 1.4	1165 ± 19	1591 ± 10
Skin4	9.5 ± 0.0	7.3 ± 0.0	53.7 ± 0.7	3.7 ± 0.1	13.1 ± 0.9	12.2 ± 0.2	0.5 ± 0.1	3.87 ± 0.10	90.0 ± 4.1	1155 ± 30	998 ± 9
Skin5	24.6 ± 0.5	5.7 ± 0.5	51.1 ± 0.5	3.5 ± 0.1	8.9 ± 0.3	5.5 ± 0.2	0.8 ± 0.2	6.60 ± 0.21	49.1 ± 1.6	1984 ± 62	1413 ± 22
Skin6	3.4 ± 0.1	3.4 ± 0.1	60.3 ± 0.4	8.2 ± 0.2	10.3 ± 0.6	6.7 ± 0.2	2.7 ± 0.3	4.91 ± 0.00	40.6 ± 2.5	1559 ± 60	1599 ± 32
Seed1	4.7 ± 0.4	4.7 ± 0.3	51.6 ± 1.9	6.2 ± 0.3	13.3 ± 1.2	11.1 ± 1.0	3.3 ± 0.1	3.63 ± 0.30	57.8 ± 5.9	1110 ± 92	1539 ± 18
Seed2	1.5 ± 0.0	10.8 ± 0.2	54.6 ± 0.3	8.4 ± 0.1	13.1 ± 0.8	8.3 ± 0.2	3.3 ± 0.2	4.01 ± 0.01	24.6 ± 1.0	1251 ± 21	1360 ± 8
Seed3	1.2 ± 0.1	8.5 ± 0.1	55.0 ± 0.6	10.1 ± 0.1	11.3 ± 0.4	10.0 ± 0.4	4.1 ± 0.2	3.96 ± 0.10	55.4 ± 1.6	1237 ± 32	1741 ± 5
Seed4	6.7 ± 0.2	13.1 ± 0.2	46.0 ± 0.1	7.1 ± 0.1	13.3 ± 0.1	12.6 ± 0.4	1.3 ± 0.1	3.67 ± 0.07	32.5 ± 1.1	1116 ± 20	1960 ± 8
Seed5	2.6 ± 0.2	8.7 ± 0.1	62.5 ± 0.5	9.5 ± 0.1	7.6 ± 0.5	6.1 ± 0.1	3.1 ± 0.1	5.97 ± 0.21	50.8 ± 1.8	1851 ± 65	2029 ± 12
Skin+seed1	2.0 ± 0.1	8.4 ± 0.0	57.3 ± 0.2	7.0 ± 0.2	11.5 ± 0.1	11.1 ± 0.2	2.7 ± 0.0	3.95 ± 0.04	56.4 ± 1.0	1205 ± 13	1728 ± 1
Skin+seed2	2.2 ± 0.2	7.6 ± 0.2	55.2 ± 0.2	10.7 ± 0.2	9.6 ± 0.0	9.8 ± 0.1	4.9 ± 0.2	4.14 ± 0.00	50.0 ± 1.2	1293 ± 12	1973 ± 8
Skin+seed3	2.1 ± 0.3	9.6 ± 0.4	60.8 ± 0.4	9.8 ± 0.3	6.3 ± 0.6	9.7 ± 0.3	1.6 ± 0.2	5.68 ± 0.32	49.2 ± 2.7	1750 ± 98	1963 ± 28

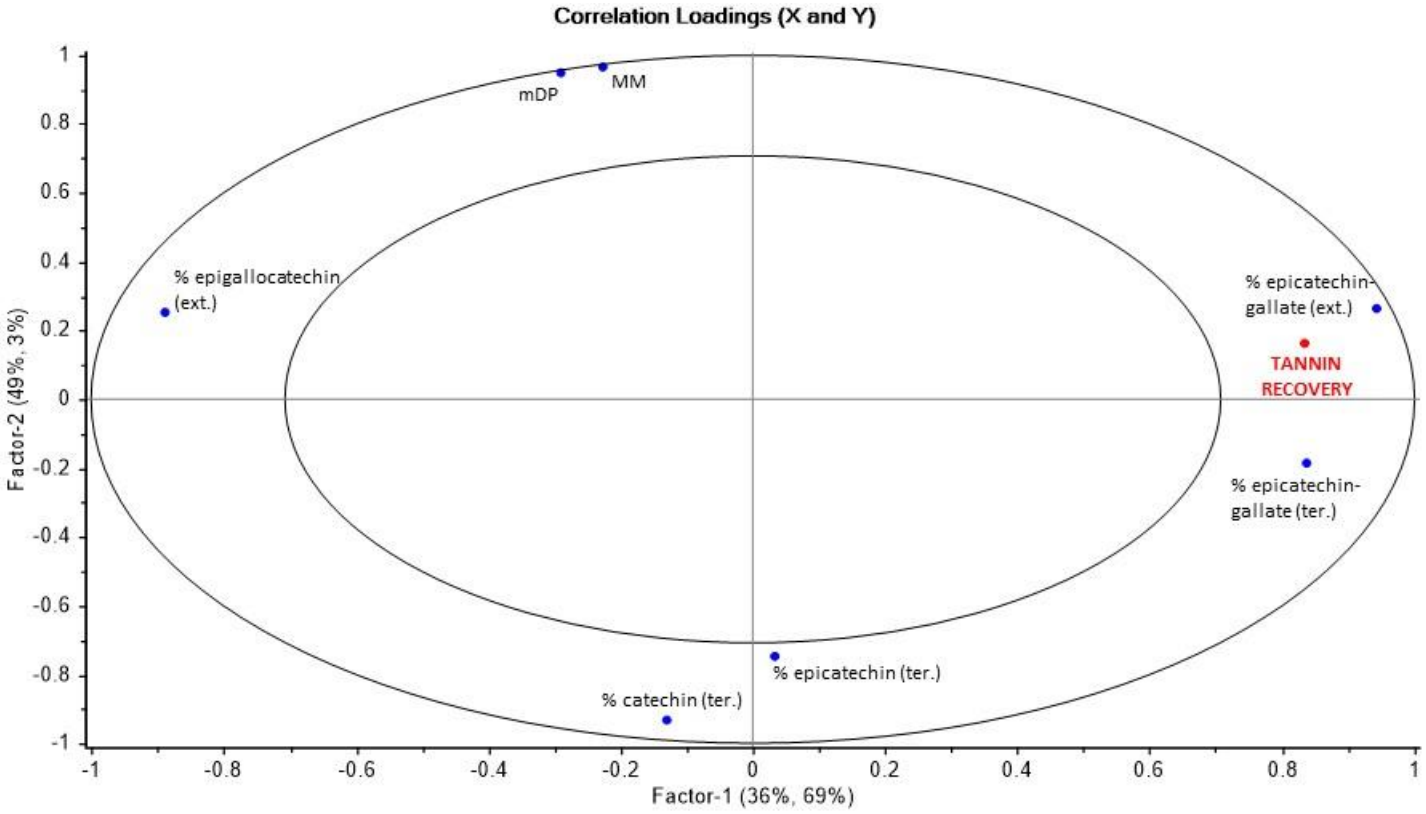
Values are means of 3 replicates ± standard error.
ext. = extension subunit; ter. = terminal subunit

Figure S1. Principal component analysis comparing Fourier transform mid-infrared spectra of dried enotannin products with significant reference compounds, ellipse indicates spectral similarity defined by Hotelling's T-squared distribution (dissimilar or non-significant reference spectra were omitted from the final analysis). **A**. all enotannins and significant reference spectra; **B.** excluding skin 5 and malvidin-3-glucoside; **C.** skin 4 and significant reference spectra; **D.** skin 5 and malvidin-3-glucoside spectra.



Enotannins: seed 1 to seed 5 ; skin 1 to skin 6 ; skin+seed 1 to 3 . Abbreviations for reference spectra: cat = (+)-catechin;epicat = (-)-epicatechin; quer = quercetin; gallic ac = gallic acid; m-3-g = malvidin-3-glucoside; Wine-T = red wine tannin; Ripe Sk-T = ripe skin tannin; Ripe Sd-T = ripe seed tannin (2 reference samples); Marc Sd = ground and dried seeds

Figure S2. Partial least squares regression model (R^2 of calibration 0.86; R^2 of validation 0.69) to predict tannin recovery (% w/w) by MCPT in enotannin products from significant tannin compositional variables.



Chapter 4. Impact of selected oenotannin and mannoprotein products on the sensory properties of Shiraz wines made from fruit harvested at two distinct levels of maturity

4.1 Introduction

Of the various supplement regimes that were evaluated in Chapter 2, the combined use of oenotannin and mannoprotein created an early harvested Shiraz wine that most closely resembled the late harvest Shiraz wine. However, it was also observed that since all supplements were introduced at the beginning of the vinification process, the subsequent settling and racking processes might have contributed to the loss of a portion of each supplement, thereby altering their effects. Three commercial products, two oenotannins (one derived from grape seed and one from skin) and one mannoprotein, were selected for further trials based on the screening process reported in Chapter 3, i.e. because they showed compositional characters that typically define their counterparts isolated from grapes and wine. These products were added to two finished Shiraz wines, to study their impact on wine composition and sensory characters, in particular, mouthfeel characters.

Oenotannin has been shown to increase astringency in wine (Versari et al. 2013). However, sensory evaluation hasn't been applied to compare the effects of skin and seed derived oenotannins. Skin tannins generally have a higher mDP and are richer in epigallocatechin subunits but low in epicatechin-gallate; whereas seed tannins have higher proportions of epicatechin-gallate, no epigallocatechin and lower mDP, despite having marginally higher hydrodynamic volumes (size by GPC) than skin tannins, at set molecular masses (Smith et al. 2015).Together, these attributes can have a significant impact on the perception of astringency (McRae et al. 2010, Quijada-Morín et al. 2012, McRae, Schulkin, et al. 2013). Conversely, mannoprotein has been associated with decreased 'green tannin' characters (harshness, acidity) and enhanced sensations of 'sweetness', 'roundness' and 'fullness' on the palate (Vidal, Francis, et al. 2004, Guadalupe et al. 2007, Del Barrio-Galán et al. 2012). As outlined in Chapter 1, wines made from early harvest grapes can exhibit a range of undesirable characters including pronounced acidity, harshness, and thinness of body. The aim of the study described in this chapter was to explore the sensory consequences of modifying the tannin and polysaccharide contents (both concentration and compositions) of red wine, either individually or in combination; in particular, the impact on mouthfeel properties. Ultimately, the study sought to determine to what extent the mouthfeel

deficiencies perceived in Shiraz wines made from early harvested grapes could be mitigated through selective use of commercial supplements.

As in Chapter 1, this study involved the addition of winemaking supplements to Shiraz wines. However, instead of supplementing only the wines made from early harvested grapes, in this study, the same supplementation regimes were introduced into wines made from both unripe and mature grapes. In this way, a series of wines comprising different ethanol, tannin and polysaccharide concentrations and/or compositions were created, which enabled any interactions attributable to these three wine components to be evaluated using sensory analysis techniques. Jones et al. (2008) investigated the effect of major wine components, namely ethanol, protein, polysaccharide, glycerol and volatiles, on white wine aroma, flavour, taste and mouthfeel, and found that some synergic effects emerged through interactions of wine constituents that affected mouthfeel properties such as viscosity. This study specifically aimed to explore the effect of additives on astringency and viscosity, since ethanol, polysaccharides and tannins, and interactions of these constituents, have previously been shown to affect these two characteristics (Laguna et al. 2017).

4.2 Materials and Methods

4.2.1 Winemaking Trials

Shiraz grapes were sourced from the same commercial vineyard described in Chapter 2. In 2016, the mean January temperature was 23.4 ºC; with the mean maximum temperature exceeding 30 ºC on 10 days. Grapes were harvested at two distinct time points: (i) harvest 1 (H1, February 1, 2016) when the total soluble solids (TSS) content of grapes (400 kg) was 20.8 ºBrix; and (ii) harvest 2 (H2, February 17, 2016) when TSS of grapes (400 kg) was 24.5 ºBrix. The weather was mild between the two harvest points with daily mean maximum temperatures below 30 ºC and 30 mm rainfall in total (climate data from www.bom.gov.au). The average berry weight were 1.06 g and 1.09 g respectively, indicating no berry shrivelling took place between the two harvests.

Winemaking and analysis of grape and fermentation were conducted by the WIC winemaking service (Urrbrae, SA, Australia). Briefly, for each harvest, grapes were divided into two parcels of 200 kg, de-stemmed and crushed. Grape must from each harvest was analysed (Table 1). Upon crushing, 5 g potassium metabisulphite (PMS) and 30 g EC1118 yeast (Lallemand, SA, Australia) were added to initiate each fermentation, and fermentation temperatures were maintained at 15 - 20 ºC, with caps plunged twice daily. After 7 days, wines were pressed, fermented to dryness (< 1 g/L residual sugar) and then racked. Wines were inoculated with Lavin VP41 lactic acid bacteria (Lallemand) at 0.2 g/L. At the completion of malolactic fermentation, wines were racked off lees and free SO2 was

adjusted to 40 mg/L. At this stage, the wines from each parcel were analysed (Table 2) and 2.5 g/L and 4 g/L tartaric acid were added to H1 and H2 wines respectively, to adjust the pH. Wines were then cold stabilised at 0 °C for 21 days. The wines were informally evaluated to ensure no faults were present. Total tannin and polysaccharide levels were measured and found to be similar between replicates from the same harvest (data not shown). Wine replicates were then blended and stored in airtight stainless steel kegs with no ullage, at 0 °C until required (i.e. less than a year).

Table 1. Composition of H1 and H2 grapes.

Harvest	TSS (°Brix)	pH	TA[a] (g/L)	Malic acid (g/L)	YAN[b] (g/L)
H1	20.8	3.47	5.2	3.45	0.21
H2	24.5	3.72	4.4	2.85	0.25

[a]TA, titratable acidity
[b]YAN, yeast assimilable nitrogen

Table 2. Composition of H1 and H2 wines after malolactic fermentation.

Harvest	Replication	Alcohol (% v/v)	pH	TA (g/L)	Malic acid (g/L)	Residual sugar (g/L)	VA (g/L)
H1	1	11.5	3.94	4.7	0.06	0.3	< 0.25
	2	11.5	3.89	5.0	<0.05	0.3	< 0.25
H2	1	14.5	4.25	4.4	0.08	0.1	0.34
	2	14.4	4.23	4.5	0.08	0.1	0.29

4.2.2 Addition of Oenotannin and Mannoprotein Supplements

Two oenotannin and one mannoprotein products were selected for use in the current study, based on results reported in chapter 3. GSeedEx and GSkinEx-A (Tarac Technologies, SA, Australia), being Seed 4 and Skin 5 in Chapter 3, were chosen since they showed compositions typical of grape seed and skin tannin. Mannofeel (Laffort Australia, SA, Australia), MP 7 in Chapter 3, was chosen based on its high product purity and high mannan content. GSeedEx was sourced in dry powder form and was stored at – 20 °C until use; it was subsequently introduced to wines without further purification. GSkinEx-A and Mannofeel were liquid products, and required modification to obtain dry powders, and therefore large quantities were obtained from the manufacturers for this study; i.e. different batches were sourced than those used in the previous chapter. GSkinEx-

A and Mannofeel were both stored at 4 ºC in sealed bottles according to manufacturers' recommendations, prior to modification via the following procedures. To remove non-phenolic material and lower molecular mass phenolics, GSkinEX-A (1L) was mixed with two volumes of AMBERLITE FPX66 polymeric resin (Dow AgroSciences, NSW, Australia), prewashed with 2 L of 0.5% acetic acid (Bindon and Kennedy 2011). The mixture was sealed and stirred at room temperature for 1 hour, then filtered through glass wool. The retained resin was washed with 1 L MilliQ water (containing 0.5% acetic acid) and then 4 L 50% methanol (containing 0.5% acetic acid), with both fractions being discarded. The polymerised phenolic compounds were then eluted with 2 L 70% acetone (containing 0.5% acetic acid). The eluent was filtered through a borosilicate glass microfibre filter (0.5 μm, Advantec, John Morris Australia, SA, Australia). The solvent was removed by rotary evaporation at 34 ºC. The pressure in the evaporator was gradually lowered to 30 mbar and further operated for an hour to ensure complete removal of acetone. The remaining solution was lyophilised. Around 8 g was recovered from purification. The powder was dissolved in model wine solution (12% ethanol v/v) and analysed by HPLC to determine the presence of residual acetic acid, which was not found. Mannofeel was dialysed against MilliQ water using a 7 kDa cut-off membrane (SnakeSkin dialysis tubing, Thermo Scientific, Rockford, USA), with 4 changes of water and then lyophilised. All three supplements as dried powders were stored at –20 ºC until use.

Before supplements were introduced to wines, free SO_2 levels were measured and were found to be 27.2 and 37.3 mg/L for H1 and H2 wines respectively, indicating no spoilage or oxidation had occurred during storage. The free SO_2 content of H1 wine was adjusted to 35 mg/L with PMS. The supplements were introduced into wine based on gravimetric concentration, i.e. mg product per L wine. The treatments were as follows:

(1) No additives (Control)
(2) 300 mg/L GSkinEX-A (Skin)
(3) 300 mg/L GSeedEX (Seed)
(4) 400 mg/L Mannofeel (MP400)
(5) 1000 mg/L Mannofeel (MP1000)
(6) 300 mg/L GSkinEX-A and 1000 mg/L Mannofeel (Skin MP1000)
(7) 300 mg/L GSeedEX and 400 mg/L Mannofeel (Seed MP400)
(8) 300 mg/L GSeedEX and 1000 mg/L Mannofeel (Seed MP1000)

The same treatments were carried out in duplicate in both H1 and H2 wines. The wines were warmed up to room temperature. Supplements were mixed in wine as outlined above, sealed and stirred for at least an hour, until no undissolved power could be visually detected. The wines were

bottled in 375 mL glass bottles, sealed with screw caps and stored for 3 months at 15 ºC, before sensory and chemical analyses. Remaining wine was sealed in airtight stainless steel kegs with no ullage and used as the base wine for the taste and mouthfeel standards used in descriptive analysis.

4.2.3 Chemical Analysis

Wine ethanol concentrations were determined using an alcolyzer (Anton Paar, Graz, Austria). pH and TA were measured with an autotitrator coupled with an autosampler (Mettler Toledo, SA, Australia).

Methylcellulose precipitable tannin (MCPT) in H1 and H2 wines were analysed according to the methods reported in Chapter 2. Based on MCPT results, the tannin fraction was isolated from 3 mL of H1 wine and 2 mL of H2 wine by solid phase extraction, and analysed by phloroglucinolysis and gel permeation chromatography, as described in Chapter 2. The percentage of polymeric pigment in total tannin was estimated by the ratio between GPC peak area under 520 nm and 280 nm. Total wine polysaccharides were isolated from 1 mL of wine and the composition of monosaccharide residues analysed using the method outlined in Chapter 2.

The three supplements were also subject to the same suite of analyses described above. Briefly, the two oenotannins were dissolved at 1 g/L in model wine solution (12% ethanol v/v, pH 3.5) and MCPT was quantified. Subsequently, they were dissolved at 10 g/L in methanol and subjected to phloroglucinolysis and gel permeation chromatography. Mannofeel was dissolved at 1 g/L in the same model wine and its composition was determined as per wine polysaccharides. 4.2.4 Sensory Analysis

Wines were subjected to descriptive analysis (DA) with a panel of ISO screened judges, aged between 54 and 70 years old, comprising three males and six females (n = 9). All judges had previously completed at least 60 hours of wine DA before the current panel and were familiar with descriptive terms often associated with red wine. Seven training sessions were held. In the first two sessions the judges were familiarised with a range of standards that represented common taste and mouthfeel sensations in wine, including sweetness, sourness, bitterness, astringency, hotness and viscosity. In the two subsequent sessions, judges tasted the majority of the additive treatments and were asked to discuss and define the mouthfeel sensations perceived. Four terms were defined based on panel consensus: ‘body’ was defined as the perception of viscosity, weight and density; ‘astringency’ was defined as puckering, grippy (drag of the tongue on the surfaces in the mouth) and rough sensations; ‘texture’ was defined as the sensation of smoothness and coarseness on the surfaces in the mouth; and ‘hotness’ was defined as warm, tingling and numbing sensations. In the remaining sessions, judges rated a selection of treatments using line scales and compared results to improve panel agreement. Two

descriptive terms, astringency and body, were specifically emphasised by presenting the panel with base wines containing different levels of grape seed extract (astringency) and gum arabic (body) (Niimi et al. 2017). After the training sessions, the ability of the panel to discern astringency was assessed by performing directional paired-comparison tests (in duplicate) using H1 bases wines spiked with seed tannin as used in the treatments (at 300 mg/L, 600 mg/L and 1000 mg/L). The same test was performed for 'body' using H1 base wine spiked with gum arabic (at 350 mg/L, 500 mg/L and 650 mg/L). Panel then rated 'astringency' on a line scale on a series of H1 wines spiked seed tannin (300 mg/L, 600 mg/L and 1000 mg/L and 1500 mg/L) in duplicate as well as rated 'body' on a series of H1 base wines spiked with MP at 400 mg/L, 1000 mg/L, 3000 mg/L and 6000 mg/L.

Two formal evaluation sessions were held in a dedicated sensory laboratory (maintained 21 ºC), during which panellists were presented with 16 wine samples (35 mL) in ISO standard black wine glasses. The evaluation protocol established during training sessions was followed. Judges took one sip of wine and swirled it in the mouth for 10 s (controlled by a timer) while rating astringency, sweetness and sourness. The wine was then expectorated and texture was rated. Another sip of wine was taken, swirled for 10 s and bitterness, body, hotness and flavour intensity were rated. The taste and flavour attributes were also included in this instance, to avoid a 'dumping effect', i.e. restricting ratings of perceived attributes could change results on a number of other attributes (Lawless and Heymann 2010). Each attribute was rated on a 10 cm line scale, anchored with 'low', 'medium' and 'high' at 10%, 50% and 90%, with the exception of 'texture', which was anchored with 'corn flour', 'semolina', and 'polenta', to confer smooth to coarse mouthfeel. Data was collected with RedJade software (RedJade, California, USA). A 2 min break was taken between samples and after the 5^{th} and 10^{th} samples, a 10 min break was taken. Judges were provided with pectin solution (1 g/L) and plain crackers to cleanse their palates between samples. 4.2.5 Data Analysis

Chemical and sensory data were subjected to analysis of variance (ANOVA) using XLSTAT (version 2015.4.1, VSN International Limited, Herts, UK) and SENPAQ (version 6.03, Qi Statistics, Reading, UK), respectively. Mean comparisons were performed by Fisher's least significant difference (LSD) multiple-comparison test at 5% level.

Table 3. Phenolic Composition of H1 Shiraz Wines Made with the Addition of Tannin and Mannoprotein (MP) Supplements, either Individually or in Combination.

	Control	Skin	Seed	MP 400	MP 1000	Skin MP 1000	Seed MP 400	Seed MP 1000	*P*
Total tannin at bottling (mg/L)	329 ± 10^{c}	557 ± 9^{a}	608 ± 36^{a}	381 ± 20^{c}	373 ± 4^{c}	476 ± 7^{b}	608 ± 3^{a}	605 ± 2^{a}	*< 0.001*
Total tannin after 3-mth (mg/L)	326 ± 5^{d}	469 ± 56^{bc}	665 ± 22^{a}	356 ± 32^{d}	385 ± 14^{cd}	508 ± 38^{b}	634 ± 36^{a}	631 ± 13^{a}	*0.001*
Extension subunits^{P}									
C	3.7 ± 0.1^{c}	4.3 ± 0.3^{bc}	6.5 ± 0.2^{a}	4.4 ± 0.3^{bc}	4.3 ± 0.1^{bc}	4.5 ± 0.2^{b}	6.8 ± 0.2^{a}	6.6 ± 6.6^{a}	*< 0.001*
EC	47.6 ± 0.0^{bcd}	49.8 ± 0.4^{a}	47.8 ± 0.8^{bc}	48.4 ± 0.9^{ab}	47.1 ± 1.5^{bcd}	48.1 ± 0.3^{bc}	47.5 ± 0.3^{bcd}	46.3 ± 0.4^{d}	*0.013*
EGC	26.3 ± 0.8^{a}	25.5 ± 0.2^{a}	20.4 ± 0.1^{b}	25.2 ±0.2^{a}	26.6 ± 0.9^{a}	26.2 ± 0.4^{a}	20.3 ± 0.4^{b}	21.5 ± 0.2^{b}	*< 0.001*
EC-G	1.7 ± 0.1^{bc}	1.6 ± 0.1^{bc}	3.1 ± 1.8^{a}	1.8 ± 0.0^{b}	1.4 ± 0.1^{c}	1.5 ± 0.1^{bc}	2.8 ± 0.1^{a}	2.8 ± 0.1^{a}	*< 0.001*
Terminal subunits^{P}									
C	16.2 ± 0.5	13.9 ± 0.1	14.4 ± 0.4	15.7 ± 0.7	15.5 ± 0.0	14.5 ± 0.2	14.8 ± 0.2	14.8 ± 0.5	*0.089*
EC	4.8 ± 0.2^{b}	4.7 ± 0.1^{b}	7.2 ± 0.2^{a}	4.4 ± 0.4^{b}	4.7 ± 0.3^{b}	5.1 ± 0.1^{b}	7.2 ± 0.3^{a}	7.6 ± 0.2^{a}	*< 0.001*
EC-G	0.2 ± 0.0^{cd}	0.2 ± 0.0^{c}	0.6 ± 0.0^{a}	0.1 ± 0.0^{cd}	0.2 ± 0.0^{cd}	0.1 ± 0.1^{d}	0.5 ± 0.1^{ab}	0.4 ± 0.1^{b}	*< 0.001*
% Yield^{P}	22.0 ± 0.4^{a}	15.2 ± 0.1^{b}	16.0 ± 0.1^{b}	21.2 ± 2.2^{a}	18.6 ± 1.1^{ab}	16.1 ± 1.5^{b}	17.1 ± 1.3^{b}	16.6 ± 0.8^{b}	*0.021*
mDP	4.84 ± 0.16^{bcd}	5.32 ± 0.03^{a}	4.51 ± 0.12^{cde}	4.96 ± 0.28^{abc}	4.89 ± 0.09^{abcd}	5.08 ± 0.08^{ab}	4.44 ± 0.05^{de}	4.38 ± 0.14^{e}	*0.014*
Tannin molecular mass^{R} (g/mol)	1670 ± 49^{bc}	1745 ± 30^{bc}	1931 ± 81^{a}	1644 ± 58^{c}	1621 ± 32^{c}	1673 ± 49^{bc}	1836 ± 55^{ab}	1815 ± 50^{ab}	*0.027*
% coloured (520:280)^{S}	14.6 ± 0.1	14.7 ± 0.1	11.7 ± 0.0	12.1 ± 2.2	14.3 ± 0.0	14.2 ± 0.3	11.5 ± 0.2	11.7 ± 0.2	*0.05*

Values are means of 2 replicates ± standard error. Values followed by different letters within rows are significantly different ($p \leq 0.05$, one way ANOVA, post hoc Fisher's LSD).

^{P} Molar proportion of subunit composition determined by phloroglucinolysis. C, catechin; EC, epicatechin; EGC, epigallocatechin; and EC-G, epigatechin-gallate. %Yield determined by dividing the total concentration of individual subunits by the concentration of tannin used in phloroglucinolysis reaction.

^{R} Determined by gel permeation chromatography at 50% elution.

^{S} GPC peak area at 520nm as a percentage of peak area of 280 nm.

Table 4. Phenolic Composition of H2 Shiraz Wines Made with the Addition of Tannin and Mannoprotein (MP) Supplements, either Individually or in Combination.

	Control	Skin	Seed	MP 400	MP 1000	Skin MP 1000	Seed MP 400	Seed MP 1000	*P*
Total tannin at bottling (mg/L)	686 ± 21^{d}	824 ± 12^{bc}	903 ± 12^{ab}	733 ± 65^{cd}	761 ± 18^{cd}	825 ± 6^{bc}	1006 ± 52^{a}	976 ± 4^{a}	*0.001*
Total tannin after 3-mth (mg/L)	679 ± 11^{d}	916 ± 10^{c}	995 ± 17^{b}	724 ± 20^{d}	657 ± 17^{d}	886 ± 10^{c}	881 ± 14^{c}	1067 ± 47^{a}	*<0.001*
Extension subunits^{P} (%)									
C	4.0 ± 0.5	4.4 ± 0.3	5.1 ± 0.4	4.2 ± 0.5	4.1 ± 0.5	4.3 ± 0.1	5.2 ± 0.0	5.4 ± 0.3	*0.165*
EC	48.8 ± 1.7^{ab}	49.8 ± 0.1^{a}	50.1 ± 0.0^{a}	49.0 ± 1.1^{ab}	46.7 ± 0.4^{bc}	45.7 ± 0.5^{c}	46.4 ± 0.8^{bc}	46.4 ± 0.9^{bc}	*0.032*
EGC	29.0 ± 2.2^{ab}	28.2 ± 0.6^{b}	23.7 ± 1.3^{c}	28.2 ±1.0^{b}	30.1 ± 0.6^{ab}	32.1 ± 0.1^{a}	26.6 ± 0.0^{bc}	27.6 ± 1.1^{b}	*0.018*
EC-G	2.1 ± 0.0^{cd}	2.1 ± 0.1^{cd}	2.9 ± 0.1^{a}	2.2 ± 0.0^{c}	2.1 ± 0.0^{cd}	1.9 ± 0.0^{d}	2.8 ± 0.1^{a}	2.4 ± 0.1^{b}	*<0.001*
Terminal subunits^{P} (%)									
C	11.2 ± 0.0	10.7 ± 0.2	12.0 ± 0.5	11.3 ± 0.3	11.8 ± 0.4	10.8 ± 0.3	11.9 ± 0.2	11.5 ± 0.1	*0.085*
EC	4.8 ± 0.1^{b}	4.8 ± 0.3^{b}	6.1 ± 0.3^{a}	5.0 ± 0.2^{b}	5.1 ± 0.0^{b}	5.2 ± 0.2^{b}	6.9 ± 0.4^{a}	6.5 ± 0.3^{a}	*0.002*
EC-G	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.2 ± 0.0^{a}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.2 ± 0.0^{a}	0.2 ± 0.0^{b}	*<0.001*
% Yield	19.2 ± 3.9	18.2 ± 1.7	17.4 ± 0.6	21.1 ± 1.2	21.9 ± 1.3	18.1 ± 0.0	23.0 ± 0.9	18.3 ± 1.9	*0.330*
mDP	6.23 ± 0.01^{ab}	6.46 ± 0.20^{a}	5.47 ± 0.24^{cd}	6.12 ± 0.17^{ab}	5.89 ± 0.13^{bc}	6.27 ± 0.20^{ab}	5.28 ± 0.19^{d}	5.50 ± 0.13^{cd}	*0.009*
Tannin molecular mass^{R} (g/mol)	1746 ± 3	1807 ± 26	1889 ± 19	1696 ± 54	1735 ± 41	1746 ± 75	1811 ± 12	1837 ± 52	*0.132*
% coloured (520:280)^{S}	12.9 ± 0.1	13.0 ± 0.1^{a}	11.4 ± 0.1	12.7 ± 0.1	12.8 ± 0.1	13.0 ± 0.0^{a}	11.3 ± 0.0	11.4 ± 0.0	*<0.001*

Values are means of 2 replicates ± standard error. Values followed by different letters within rows are significantly different ($p \leq 0.05$, one way ANOVA, post hoc Fisher's LSD).

^{P} Molar proportion of subunit composition determined by phloroglucinolysis. C, catechin; EC, epicatechin; EGC, epigallocatechin; and EC-G, epigatechin-gallate. %Yield determined by dividing the total concentration of individual subunits by the concentration of tannin used in phloroglucinolysis reaction.

^{R} Determined by gel permeation chromatography at 50% elution.

^{S} GPC peak area at 520nm as a percentage of peak area of 280 nm.

Table 5. Total Polysaccharide and monosaccharide residues (mannan and glucose) following hydrolysis in Shiraz wines made with the Addition of Tannin and Mannoprotein (MP) Supplements, either Individually or in Combination.

	Control	Skin	Seed	MP 400	MP 1000	Skin MP 1000	Seed MP 400	Seed MP 1000	*P*
H1 wines									
Mannose[P]	72 ± 7^{c}	68 ± 1^{c}	69 ± 0^{c}	186 ± 18^{b}	409 ± 11^{a}	378 ± 9^{a}	173 ± 8^{b}	397 ± 10^{a}	*<0.001*
Glucose[P]	20 ± 2^{c}	21 ± 0^{c}	21 ± 0^{c}	40 ± 4^{b}	80 ± 0^{a}	78 ± 5^{a}	37 ± 1^{b}	71 ± 4^{a}	*<0.001*
Total PL[R]	401 ± 45^{cd}	387 ± 3^{d}	398 ± 4^{d}	520 ± 51^{b}	801 ± 15^{a}	755 ± 24^{a}	495 ± 18^{bc}	754 ± 29^{a}	*<0.001*
H2 wines									
Mannose[P]	98 ± 2^{cd}	92 ± 3^{d}	91 ± 6^{d}	214 ± 10^{b}	404 ± 4^{a}	432 ± 36^{a}	210 ± 7^{bc}	452 ± 42^{a}	*<0.001*
Glucose[P]	19 ± 1^{b}	24 ± 3^{b}	20 ± 1^{b}	29 ± 2^{b}	53 ± 4^{a}	60 ± 6^{a}	27 ± 3^{b}	62 ± 2^{a}	*<0.001*
Total PL[R]	403 ± 4^{bcd}	395 ± 9^{cd}	383 ± 23^{d}	518 ± 27^{b}	720 ± 28^{a}	782 ± 67^{a}	504 ± 14^{bc}	820 ± 64^{a}	*<0.001*

Values are means of 2 replicates ± standard error. Values followed by different letters within rows are significantly different ($p \leq 0.05$, one way ANOVA, post hoc Fisher's LSD).

[P] Concentrations of mannose and glucose residues contained in polysaccharide were determined after hydrolysis.

[R] Total polysaccharide was determined as a sum of all monosaccharide residues after hydrolysis.

4.3 Results and Discussion

4.3.1 Effect of Supplements on the Chemical Composition of Wine

MCPT accounted for 33.4% and 74.3% of dry weight of skin and seed oenotannin respectively. The seed tannin MCPT recovery was identical to that reported in Chapter 2 for Seed 4. Therefore, theoretically 300 mg/mL of supplementation should result in 100 and 223 mg/L increases in wine MCPT for skin and seed tannin additives, respectively. However, for both harvest times, the observed increases ranged from 143 to 237 mg/L for skin tannin addition and 202 to 388 mg/L for seed tannin addition, which were higher than the theoretical values (Table 3 and Table 4). These ranges were consistent with those determined immediately after bottling (Table 3 and Table 4), indicating that increases were not derived from the 3 month storage time. Rather, the differences observed between the theoretical and measured values are likely explained by the different matrices, i.e. the model wine used to dissolve oenological tannins and the real Shiraz wines. There is an abundance of phenolic and non-phenolic compounds present in red wine, but not in model wine solutions, that might have influenced the 280 nm absorbance. The MCPT values are derived from absorbance differences at 280 nm before and after precipitation with methylcellulose, and thus the values could be affected. However, the existing investigation into the matrix effect on MCPT was inconclusive (Mercurio and Smith 2008) and warranted study in more depth.

The subunit composition of wine tannin was also determined by phloroglucinolysis. The percentage yield, i.e. the sum of acid labile tannin subunits, ranged between 15.2 and 23.0%, indicating the subunit values reported in Tables 3 and 4 accounted for less than a quarter of the measured MCPT. This result is consistent with previous reports on wine tannin subunit yields (Bindon et al. 2010), as the structure of wine tannin is largely resistant to acid hydrolysis and subsequent nucleophilic addition (Smith et al. 2015). In H1 wines, treatments involving oenotannin addition had lower percentage yields than with other additives. The percentage yield of purified skin oenotannin determined by phloroglucinolysis analysis was only 7% (data not shown), probably due to storage conditions. Before the product was purified, it was stored as a liquid at 4 ºC in a 5 litre PE bottle (with 1 litre head space) for 9 months, and the pH of the product was 2.34. Oxygen ingression and low pH environments have been associated with decreased percentage yields in wine tannin, due to changes in tannin structure through oxidation, intramolecular bond formation and the incorporation of anthocyanins into tannin polymers (McRae, et al. 2013). The low percentage yield obtained for the skin tannin supplement also explained the lack of difference in molar proportions of epigallocatechin observed between the treatments with skin tannin and the control. The skin tannin addition did not result in changes

in tannin molecular mass or the percentage of polymerised pigment in total tannin, as determined by GPC. Compared to the skin tannin, addition of seed tannin resulted in more observable modifications to wine tannin composition. In both H1 and H2 wines, the treatments involving seed tannin addition gave increases in epicatechin-gallate subunits and epicatechin terminal subunits, in agreement with previous reports (Bindon et al. 2010). Seed tannin addition also resulted in a slight decrease in mDP, as well as an increase in tannin molecular mass (determined by GPC), which was consistent with characteristics reported for seed tannin (Kennedy and Taylor 2003). These results were also consistent with previous compositional analyses of this product (Chapter 3, Supplementary table S4).

The mannoprotein used in the current study yielded 500 mg/L of mannose and 100 mg/L of glucose residues, following hydrolysis of 1 g/L of product dissolved in water; which was lower than determined previously (Chapter 3, Table 1). This was attributed to product batch differences and/or different dialysis regimes. Based on these results, theoretically a 400 mg/L addition of supplement should have resulted in an increase of 200 mg/L of mannose residue, following the hydrolysis of wine total polysaccharides. By extension, 500 mg/L should be detected in treatments with 1000 mg/L addition of mannoprotein. However, around 55 and 65% of the theoretical values were detected in treatments comprising 400 mg/L and 1000 mg/L MP supplements, irrespective of the presence of oenotannin or the time of harvest. These differences might originate from interactions between mannoprotein and other wine components that made them resistant to either precipitation by ethanol or hydrolysis by acid.

Despite the unexpected recovery of tannin and polysaccharide in experimental treatments, differences observed in wine tannin and polysaccharide composition between treatments were still significant, especially in relation to total MCPT and polysaccharide concentrations (Table 3, 4 and 5). Across the 16 treatments, tannin concentrations ranged from 326 to 1067 mg/L. At these levels, the increased tannin concentrations have been shown to be positively associated with perceived astringency (Robichaud and Noble 1990, Kallithraka et al. 2011). Mannoprotein has been found to decrease astringency and to contribute to body (viscosity) at concentrations lower than present in the MP1000 wines (Vidal, Francis, et al. 2004, Bindon et al. 2014, Quijada-Morín et al. 2014). Thus it was expected that the mouthfeel characters of wines from different treatments involving MP addition would be perceived differently, based on the range of concentrations generated by the supplementation regimes employed.

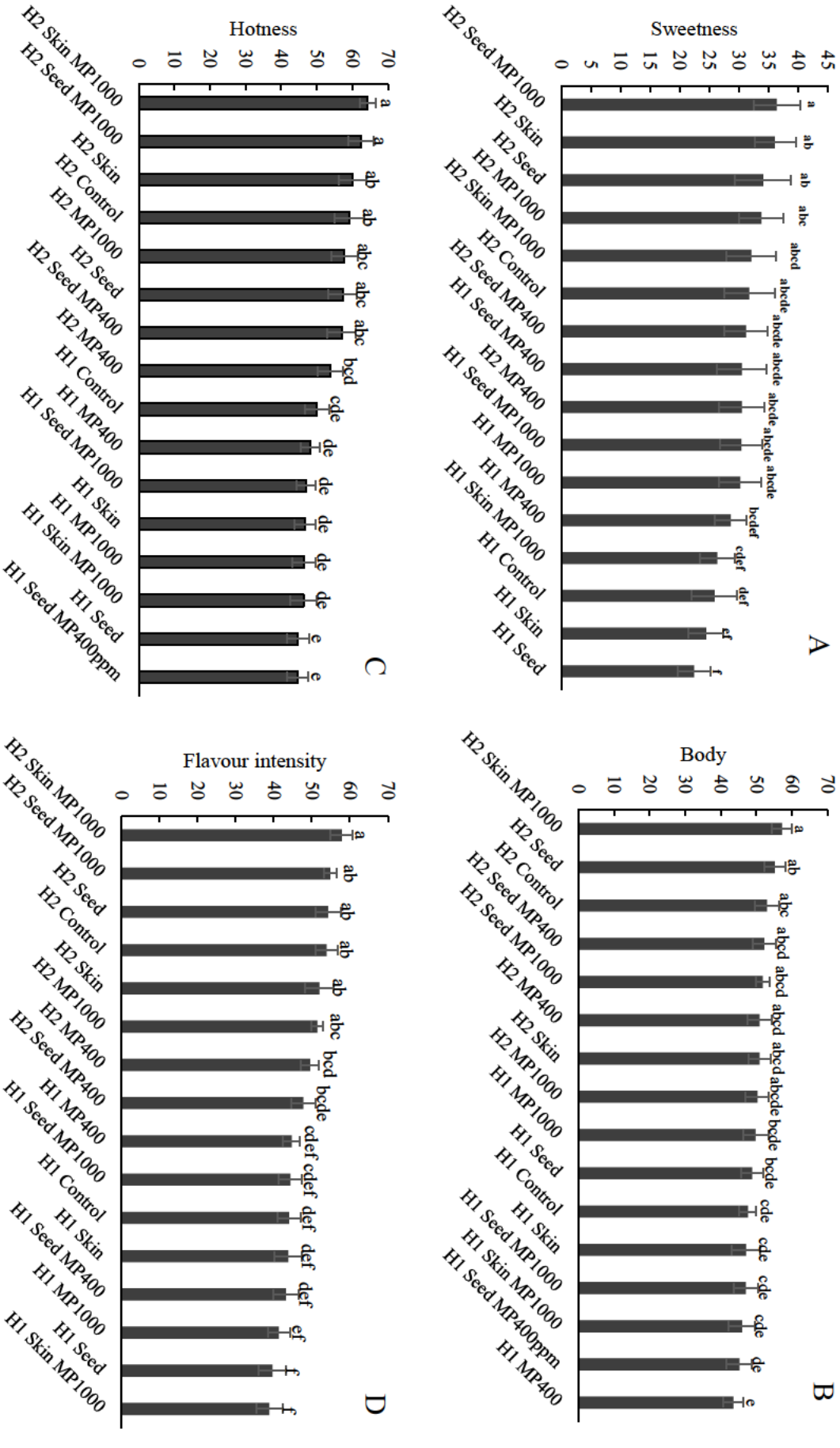


Figure 1. Intensity of selected sensory descriptions of H1 and H2 wines of different treatments. Letters indicate significant differences ($p \leq 0.05$, one way ANOVA, post hoc Fisher's LSD)

4.3.2 Impact of Supplements on Wine Sensory

Four sensory descriptors were found to be significantly different for wines from the two harvest dates, being: 'sweetness', 'body', 'hotness' and 'flavour intensity' (Figure 1). For these four attributes, H2 wines were rated higher than H1 wines, with one exception being the sweetness of H1 Seed MP400 and H2 MP400 (Figure 1A). These results are in agreement with a previous study that explored the impact of harvest time on wine sensory properties (Bindon et al. 2014). However, the effect of supplements were very small. Within each harvest, only 'sweetness' was perceived to be significantly different in H1 wines; with all five treatments involving MP supplementation rated sweeter than wines of control, Skin and Seed (Figure 2). MP has been found to increase the perception of sweetness in a previous study (Guadalupe et al. 2007). However, no relationship was found between sweetness and the different levels of MP added in the current study, or whether MP was used in combination with oenotannin. Oenological tannin addition can enhance the perception of bitterness (Bautista-Ortín et al. 2005, Harbertson et al. 2012), albeit this was not perceived in the current study. However, oenotannin addition could still supress sweetness perception (Keast and Breslin 2003). It is possible that the different levels of sweetness detected in H1 wines could reflect MP addition, compared with the decreased sweetness observed in treatments with only oenotannin addition. No difference in sweetness was found in H2 wines. This could possibly stem from the increased levels of sweetness already present in wines (Figure 1A), negating the potential effects of MP and oenotannin. These results indicated that none of the additive regimes employed could adequately compensate for the mouthfeel differences observed between H1 and H2 wines, due to differences in harvest time.

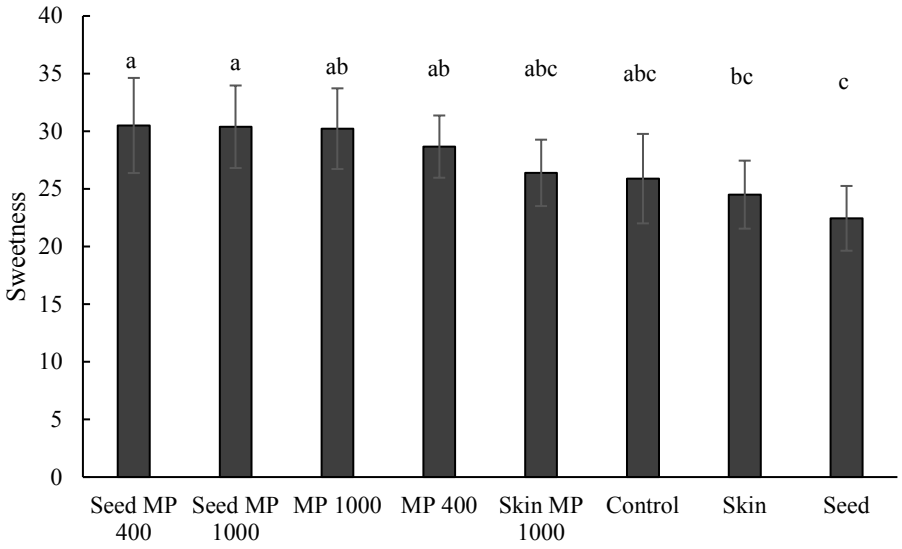


Figure 2. Ratings for sweetness of H1 wines made with oenotannin and mannoprotein, either individually or in combination.

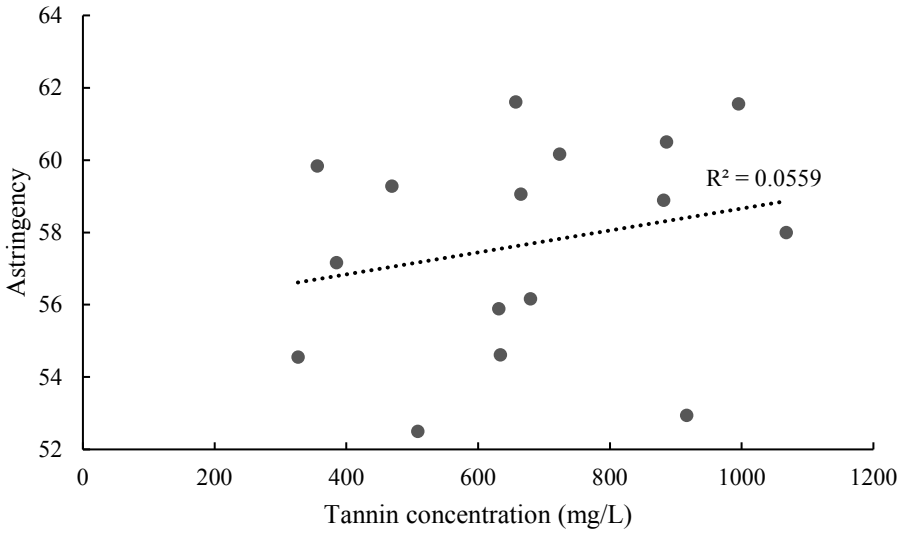


Figure 3. Astringency rating as a dependent variable of tannin concentration. Each dot represents a treatment. A linear trend line was fitted to all data points, with the linear coefficient (R^2) shown.

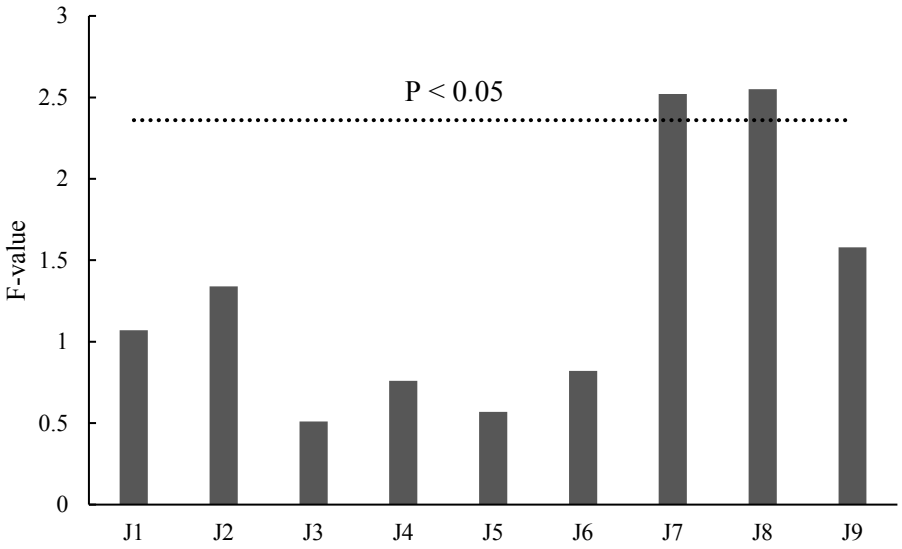


Figure 4. F-values of astringency ratings by individual judge (J1 – J9). The bars exceeding the dotted line indicated that the judge could differentiate astringency levels among samples.

Surprisingly, no relationship was found between tannin concentration and astringency ratings (Figure 3). The replicate effect was not significant for astringency ratings (data not shown). However, out of 9 judges, only 2 could differentiate between samples based on astringency (Figure 4). Prior to commencement of formal DA evaluations, the assessors' ability to differentiate different levels of astringency was tested through directional paired comparison tests, using H1 base wines spiked with seed tannin (at 300 mg/L, 600 mg/L and 1000 mg/L) as reference standards. Only 2 judges gave correct responses at all three levels; 5 judges were corrected with both replicates at 600 mg/L and 1000 mg/L addition, while two additional judges were correct at the 1000 mg/L level only. Panel performance was further assessed by rating astringency in spiked wines using a line scale. The wines spiked with 1000 mg/L and 1500 mg/L seed tannin were perceived to be significantly more astringent than the wines with lower levels of tannin addition, which were not perceived to be different (Figure 5A). Thus, it appeared that the judges were able to perceive different levels of astringency, just not in the concentration range required for wines from the current study. Most previous studies have found that astringency ratings increase with tannin concentrations in wine (Robichaud and Noble 1990, Kennedy et al. 2006, Landon et al. 2008, Mercurio and Smith 2008, Kallithraka et al. 2011). However, some studies observed the perception of astringency had a relatively weak correlation with tannin concentration, and was rather driven by tannin subunit composition, the degree of polymerisation, hydrodynamic volume, structural conformation and less colour incorporation (McRae et al. 2010, McRae et al. 2012, Quijada-Morín et al. 2012, McRae, Schulkin, et al. 2013). The suite of analytical methods employed in the current study revealed only limited variations in tannin composition and hydrodynamic volume (GPC) between treatments (Table 3 and 4) and so it was not clear whether this reflected a lack of treatment effect, or insufficient characterisation of samples. Thus, no definitive conclusion regarding any implications for wine astringency could be drawn from tannin compositional data.

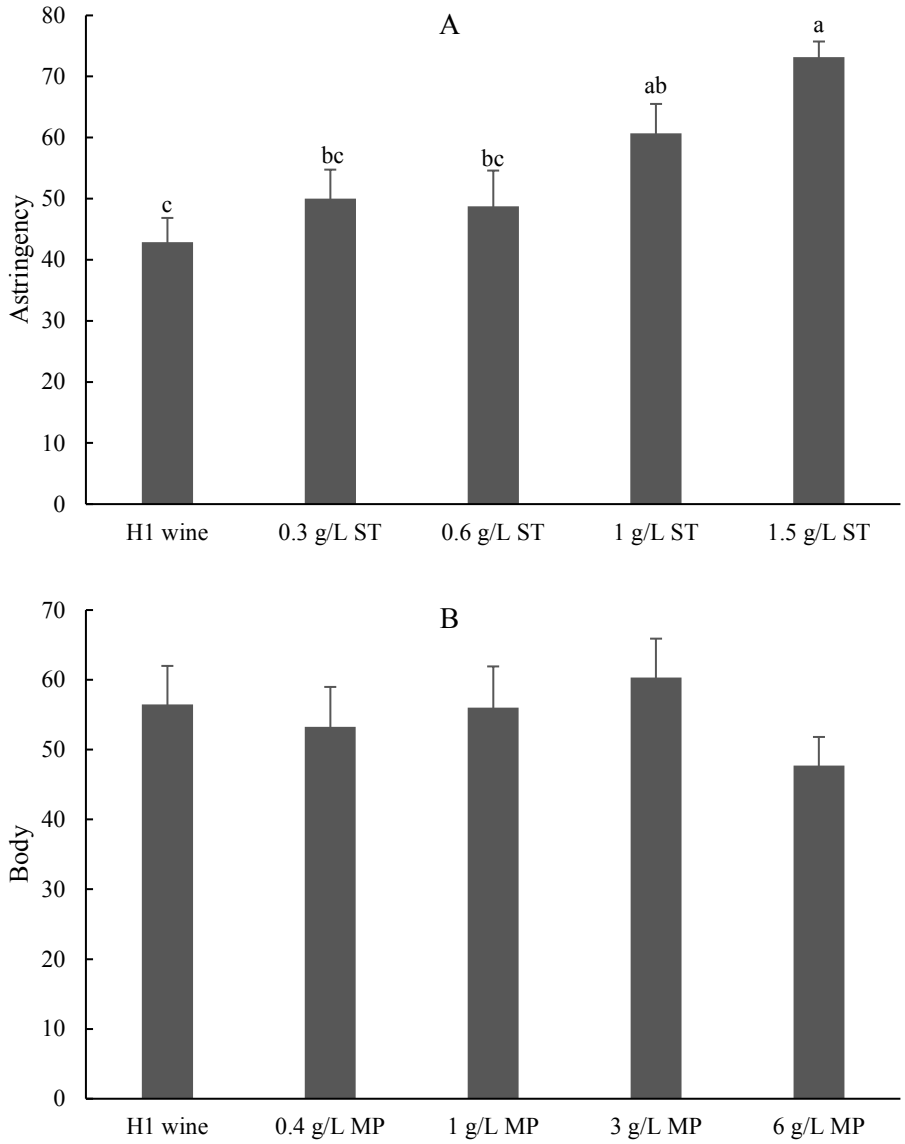


Figure 5. Ratings for 'astringency' and 'body' of H1 wines spiked with (A) 0.3 – 1.5 g/L seed tannin and (B) 0.4 – 6 g/L mannoprotein (MP), respectively. Letters indicate significant differences.

It is also possible that the differences in tannin concentration between treatments (i.e. 200 – 600 mg/L) were simply too low for people to detect perceivable sensory differences. Landon and colleagues (2008) reported astringency differences between wines of low and high concentrations (i.e. 250 vs 1071 mg/L), but medium level (i.e. 631 mg/L) was not significantly different to either low or high levels. Furthermore, although one study showed that astringency increased linearly with red wine tannin concentrations similar to those reported in the current study (Kallithraka et al. 2011), the quantification method used was based on protein precipitation

(BSA). A recent review found that although the results from BSA and MCP were highly correlated, the BSA method gave consistently lower values than MCP (Aleixandre-Tudo et al. 2017). Thus it was likely that the MCPT values of wines used in the aforementioned study were much higher. Where a linear relationship was observed between MCPT and astringency (Mercurio and Smith 2008), the wines studied contained tannin concentrations that exceeded the levels observed in the current study. In fact, since the MCPT method was developed in 2006 (Sarneckis et al. 2006) up to 2015, 33 published studies, involving 281 samples, measured MCPT values ranged between 60 – 3530 mg/L, with an median at 1340 mg/L (Aleixandre-Tudo et al. 2017). This suggests that all wines in this study (including the treatments with high tannin concentrations) only represented red wines with minimal to low tannin concentrations. Therefore the differences in astringency might have been too subtle for the judges to distinguish.

The overall wine matrix is another factor to consider. The largest difference in tannin concentration was found between H1 control and H2 Seed MP1000 wines, which already differed in terms of ethanol content, polysaccharide content and perceived sweetness, all of which could affect astringent perception (Ishikawa and Noble 1995, Vidal, Courcoux, et al. 2004, Quijada-Morín et al. 2014). Notwithstanding the potential interfering factors discussed above, one previous study has demonstrated a significant difference in astringency in Cabernet Sauvignon wines made from fruit harvested at different levels of maturity, with MCPT ranging from 731 to 1088 mg/L (Bindon et al. 2013, Bindon et al. 2014). Thus it is entirely possible that the current panel simply did not possess the sensitivity to distinguish subtle levels of astringency and panellists should either have been rescreened or further trained.

The addition of MP did not decrease the perception of astringency in wine, despite some treatments containing over 400 mg/L mannose residues, i.e. levels far exceeding what is typically observed in red wine, being around 100 mg/L (Quijada-Morín et al. 2014, Watrelot et al. 2017). Mannoprotein has been shown to limit seed tannin aggregation (Poncet-Legrand et al. 2007) and mediate tannin and protein interactions (Rinaldi et al. 2012), thereby having the ability to modulate wine astringency. Reductions in astringency have been inferred through reduction of the Gelatine index following the addition of MP to polyphenols (Escot et al. 2001) or through establishing negative correlations between MP concentrations and astringency ratings using multivariate analysis (Quijada-Morín et al. 2014). Supplementing MP during vinification at much lower levels than used in this study has been demonstrated to reduce astringency and/or harsh tannin characters (Guadalupe and Ayestarán 2007, Del Barrio-Galán et al. 2012). However, in both of these studies, there were racking and/or filtration processes after the addition of MP. Thus, it is not certain that a fining effect achieved the astringency reduction observed in these studies,

i.e. similar to that observed in Chapter 2. Direct addition of MP in model wine containing 250 – 750 mg/L tannin did not result in any reduction in astringency ratings (Vidal, Courcoux, et al. 2004), in agreement with the findings from this study. Clearly the effect of mannoprotein on astringency requires further investigation.

The mouthfeel perception, 'body', was also explored in this study. Directional paired comparison tests using H1 base wine spiked with gum arabic (at 350 mg/L, 500 mg/L and 650 mg/L) were used to test each panellist's ability to perceive body (Niimi et al. 2017). Five judges were correct at all levels, with an additional two correct at both 500 mg/L and 650 mg/L levels. The panel also found differences in body between wines made from fruit from different harvest times (Figure 1). However, no effect on 'body' was found for treatments with MP supplementation, in either H1 or H2 wines. Furthermore, when rating 'body' on a series of H1 base wine spiked with MP at 400 mg/L, 1000 mg/L, 3000 mg/L and 6000 mg/L, no significant differences or any trends were indicated by the judging panel (Figure 5B). The panel could differentiate 'body' as a mouthfeel property, but it was not related to any sensory impact from MP addition. Wine body is usually classified as light, medium or full, but these terms were very loosely defined in wine sensory evaluation (Laguna et al. 2017). The mouthfeel wheel designed for red wine, uses the term 'body' in conjunction with 'flavour' intensity to define 'thin' and 'watery', which were in turn sub-qualities of the 'weight' of mouthfeel (Gawel et al. 2000). Closely linked with 'thin' and 'water' are 'viscosity' and 'full', which describe the pressure on the tongue exerted by wine. Similarly, in practice, 'wine body' was found to be related to viscosity, flavour perception and overall intensity of wine (Gawel et al. 2007, Niimi et al. 2017). The multi-model sensory interactions were supported by observations that the perception of 'body' decreased in wine made from early harvested grapes compared to those from mature fruit (Bindon et al. 2014) or decreased after the wine was dealcoholised (Meillon et al. 2009). Since evaluation of 'body' was concurrent with evaluations of 'sweetness', 'hotness' and 'flavour intensity' (Figure 1), it is possibly that differences were driven by interactions. Spiking MP in H1 wines may well have yielded a sensory impact, but this was not recognised as 'body' by the DA panel. Wine neutral polysaccharides, including arabinogalactan-protein and mannoprotein, have been demonstrated to illicit 'fullness' sensations in model wine (Vidal, Francis, et al. 2004) and to increase the viscosity of white wine (Gawel et al. 2016). However, no direct effect of MP on body in red wine has been reported, and the current study did not find a contribution of MP to red wine body. Further investigations are therefore warranted. However, given that a upper limit of 400 mg/L MP addition is imposed in Australia produced wine, based on an agreement between Australia and the European Community on Trade in Wine (Food Standards Australia and New

Zealand), it is possible that the effect of MP on wine body might not be applicable in winemaking settings.

4.4 Conclusions

Two oenotannins derived from grape seed and skin, as well as a mannoprotein product, were used at different levels and combinations, in Shiraz wines made from fruit corresponding to two harvest dates. This gave rise to a series of wines with significantly different tannin, mannoprotein and ethanol concentrations. The effect of oenotannin on wine tannin composition was more obvious with seed tannin than skin tannin, probably because the former was more susceptible to the phloroglucinolysis and GPC analytical methods used in the current study. The judges could perceive sensory differences between H1 and H2 wines, but could not perceive any effect of supplements on wine sensory properties, except for a minor increase in sweetness induced by mannoprotein in H1 wines. Despite MP being added to wine at a dose that was 2.5 times higher than the legal limit permissible in Australia, neither reduction in astringency nor increase in wine body was observed.

Aleixandre-Tudo JL, Buica A, Nieuwoudt H, Aleixandre JL, and du Toit W. 2017. Spectrophotometric analysis of phenolic compounds in grapes and wines. J. Agric. Food Chem. 65: 4009-4026.

Bautista-Ortín AB, Martínez-Cutillas A, Ros-García JM, López-Roca JM, and Gómez-Plaza E. 2005. Improving colour extraction and stability in red wines: the use of maceration enzymes and enological tannins. Int. J. Food Sci. Technol. 40: 867-878.

Bindon KA, Holt H, Williamson PO, Varela C, Herderich M, and Francis IL. 2014. Relationships between harvest time and wine composition in *Vitis vinifera* L. cv. Cabernet Sauvignon 2. Wine sensory properties and consumer preference. Food Chem. 154: 90–101.

Bindon KA, and Kennedy JA. 2011. Ripening-induced changes in grape skin proanthocyanidins modify their interaction with cell walls. J. Agric. Food Chem. 59: 2696-2707.

Bindon KA, Smith PA, Holt H, and Kennedy JA. 2010. Interaction between grape-derived proanthocyanidins and cell wall material. 2. Implications for vinification. J. Agric. Food Chem. 58: 10736-46.

Bindon KA, Varela C, Kennedy JA, Holt H, and Herderich M. 2013. Relationships between harvest time and wine composition in *Vitis vinifera* L. cv. Cabernet Sauvignon 1. Grape and wine chemistry. Food Chem. 138: 1696–1705.

Del Barrio-Galán R, Pérez-Magariño S, Ortega-Heras M, Guadalupe Z, and Ayestarán B. 2012. Polysaccharide characterization of commercial dry yeast preparations and their effect on white and red wine composition. LWT - Food Science and Technology 48: 215–223.

Escot S, Feuillat M, Dulau L, and Charpentier C. 2001. Release of polysaccharides by yeasts and the influence of released polysaccharides on colour stability and wine astringency. Aust. J. Grape Wine Res. 7: 153–159.

Food Standards Australia New Zealand 2008. Final assessment report: A605 – yeast mannoproteins as a food additives for wine. (http://www.foodstandards.gov.au/code/applications/Documents/A605_Mannoproteins_FAR.pdf)

Gawel R, Oberholster A, and Francis IL. 2000. A 'Mouth-feel Wheel': terminology for communicating the mouth-feel characteristics of red wine. Aust. J. Grape Wine Res. 6: 203-207.

Gawel R, Sluyter SV, and Waters EJ. 2007. The effects of ethanol and glycerol on the body and other sensory characteristics of Riesling wines. Aust. J. Grape Wine Res. 13: 38-45.

Gawel R, Smith PA, and Waters EJ. 2016. Influence of polysaccharides on the taste and mouthfeel of white wine. Aust. J. Grape Wine Res. 22: 350-357.

Guadalupe Z, and Ayestarán B. 2007. Polysaccharide profile and content during the vinification and aging of Tempranillo red wines. J. Agric. Food Chem. 55: 10720-10728.

Guadalupe Z, Palacios A, and Ayestaran B. 2007. Maceration enzymes and mannoproteins: A possible strategy to increase colloidal stability and color extraction in red wines. J. Agric. Food Chem. 55: 4854-4862.

Harbertson JF, Parpinello GP, Heymann H, and Downey MO. 2012. Impact of exogenous tannin additions on wine chemistry and wine sensory character. Food Chem. 131: 999-1008.

Ishikawa T, and Noble AC. 1995. Temporal perception of astringency and sweetness in red wine. Food. Qual. Prefer. 6: 27-33.

Jones PR, Gawel R, Francis IL, and Waters EJ. 2008. The influence of interactions between major white wine components on the aroma, flavour and texture of model white wine. Food. Qual. Prefer. 19: 596-607.

Kallithraka S, Kim D, Tsakiris A, Paraskevopoulos I, and Soleas G. 2011. Sensory assessment and chemical measurement of astringency of Greek wines: Correlations with analytical polyphenolic composition. Food Chem. 126: 1953-1958.

Keast RSJ, and Breslin PAS. 2003. An overview of binary taste–taste interactions. Food. Qual. Prefer. 14: 111-124.

Kennedy JA, Ferrier J, Harbertson JF, and des Gachons CP. 2006. Analysis of tannins in red wine using multiple methods: correlation with perceived astringency. Am. J. Enol. Vitic. 57: 481-485.

Kennedy JA, and Taylor AW. 2003. Analysis of proanthocyanidins by high-performance gel permeation chromatography. J. Chromatogr. A 995: 99–107.

Laguna L, Bartolomé B, and Moreno-Arribas MV. 2017. Mouthfeel perception of wine: Oral physiology, components and instrumental characterization. Trends in Food Science & Technology 59: 49-59.

Landon JL, Weller K, Harbertson JF, and Ross CF. 2008. Chemical and sensory evaluation of astringency in Washington State red wines. Am. J. Enol. Vitic. 59: 153-158.

Lawless HT, and Heymann H. 2010. Discrimination testing. *In* Sensory Evaluation of Food: Principles and Practices. pp. 79-100. Springer New York, New York, NY.

McRae JM, Dambergs RG, Kassara S, Parker M, Jeffery DW, Herderich MJ, and Smith PA. 2012. Phenolic compositions of 50 and 30 year sequences of Australian red wines: the impact of wine age. J. Agric. Food Chem. 60: 10093-10102.

McRae JM, Falconer RJ, and Kennedy JA. 2010. Thermodynamics of grape and wine tannin interaction with polyproline: implications for red wine astringency. J. Agric. Food Chem. 58: 12510-12518.

McRae JM, Kassara S, Kennedy JA, Waters EJ, and Smith PA. 2013. Effect of wine pH and bottle closure on tannins. J. Agric. Food Chem. 61: 11618-11627.

McRae JM, Schulkin A, Kassara S, Holt HE, and Smith PA. 2013. Sensory properties of wine tannin fractions: implications for in-mouth sensory properties. J. Agric. Food Chem. 61: 719-727.

Meillon S, Urbano C, and Schlich P. 2009. Contribution of the Temporal Dominance of Sensations (TDS) method to the sensory description of subtle differences in partially dealcoholized red wines. Food. Qual. Prefer. 20: 490-499.

Mercurio MD, and Smith PA. 2008. Tannin quantification in red grapes and wine: comparison of polysaccharide- and protein-based tannin precipitation techniques and their ability to model wine astringency. J. Agric. Food Chem. 56: 5528-5537.

Niimi J, Danner L, Li L, Bossan H, and Bastian SEP. 2017. Wine consumers' subjective responses to wine mouthfeel and understanding of wine body. Food Res. Int. 99: 115-122.

Poncet-Legrand C, Doco T, Williams P, and Vernhet A. 2007. Inhibition of grape seed tannin aggregation by wine mannoproteins: Effect of polysaccharide molecular weight. Am. J. Enol. Vitic. 58: 87-91.

Quijada-Morín N, Regueiro J, Simal-Gándara J, Tomás E, Rivas-Gonzalo JC, and Escribano-Bailón MT. 2012. Relationship between the sensory-determined astringency and the flavanolic composition of red wines. J. Agric. Food Chem. 60: 12355-12361.

Quijada-Morín N, Williams P, Rivas-Gonzalo JC, Doco T, and Escribano-Bailón MT. 2014. Polyphenolic, polysaccharide and oligosaccharide composition of Tempranillo red wines and their relationship with the perceived astringency. Food Chem. 154: 44–51.

Rinaldi A, Gambuti A, and Moio L. 2012. Precipitation of salivary proteins after the interaction with wine: the effect of ethanol, pH, fructose, and mannoproteins. J. Food Sci. 77: C485-C490.

Robichaud JL, and Noble AC. 1990. Astringency and bitterness of selected phenolics in wine. J. Sci. Food Agric. 53: 343-353.
Sarneckis CJ, Dambergs RG, Jones P, Mercurio M, Herderich MJ, and Smith PA. 2006. Quantification of condensed tannins by precipitation with methyl cellulose: development and validation of an optimised tool for grape and wine analysis. Aust. J. Grape Wine Res. 12: 39-49.
Smith PA, McRae JM, and Bindon KA. 2015. Impact of winemaking practices on the concentration and composition of tannins in red wine. Aust. J. Grape Wine Res. 21: 601–614.
Versari A, du Toit W, and Parpinello GP. 2013. Oenological tannins: a review. Aust. J. Grape Wine Res. 19: 1–10.
Vidal S, Courcoux P, Francis L, Kwiatkowski M, Gawel R, Williams P, Waters E, and Cheynier V. 2004. Use of an experimental design approach for evaluation of key wine components on mouth-feel perception. Food. Qual. Prefer. 15: 209–217.
Vidal S, Francis L, Williams P, Kwiatkowski M, Gawel R, Cheynier V, and Waters E. 2004. The mouth-feel properties of polysaccharides and anthocyanins in a wine like medium. Food Chem. 85: 519–525.
Watrelot AA, Schulz DL, and Kennedy JA. 2017. Wine polysaccharides influence tannin-protein interactions. Food Hydrocoll. 63: 571-579.

CHAPTER 5

Applying Nanoparticle Tracking Analysis to Characterize Interactions between Tannin and Polysaccharide in Wine-like Media

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Name of Principal Author (Candidate)	Sijing Li		
Contribution to the Paper	Conceptualisation of the work / experimental design Experimental work / data collection Data organisation, interpretation and analysis Manuscript preparation and editing		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30/11/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Abstract

Interactions between grape seed tannin and either a mannoprotein or an arabinogalactan in model wine solutions of different ethanol levels were characterized with nanoparticle tracking analysis (NTA) and confirmed with UV-visible spectroscopy and dynamic light scattering. NTA results reflected a shift in particle size distribution due to aggregation. Furthermore, the light scattering intensity of each tracked particle measured by NTA could be used to deduce the presence of aggregates, even when a shift in particle size was not apparent. Mannoprotein and arabinogalactan behaved differently when combined with seed tannin. Mannoprotein formed larger aggregates, while arabinogalactan exhibited only weak interactions and potentially formed small aggregates that were comparable in size to the polysaccharide itself. A 3% difference in alcohol concentration of the model solution (12% vs. 15% v/v) was sufficient to affect the interactions between mannoprotein and tannin when the tannin concentration was high. The implications for wine colloidal properties are discussed based on these results. The current study showed that NTA is a promising tool for measuring polydisperse samples such as grape and wine macromolecules, and their aggregates under wine-like conditions.

Key words: mannoprotein, arabinogalactan, seed tannin, NTA

1. Introduction

Polysaccharides and condensed tannins are two classes of the most abundant macromolecules in red wine. Red wine tannin is predominantly condensed polymers of flavan-3-ols, up to 4 mg/mL (Smith, McRae, & Bindon 2015). Polysaccharides are present in wine from 0.2 to 1.5 mg/mL, and consist predominately of neutral polysaccharides, which are mainly arabinogalactan-protein derived from the grape cell wall and mannoprotein derived from the yeast involved in fermentation (Guadalupe, Ayestarán, Williams, & Doco 2014). Polysaccharides and condensed tannins exist in wine as colloidal dispersions and the particles can associate with each other non-covalently, through hydrogen bonding and hydrophobic interactions (Watrelot, Le Bourvellec, Imberty, & Renard 2014). It has been suggested that this association can compete with protein aggregation, reducing the precipitation of phenolic compounds (Mateus, Carvalho, Luís, & de Freitas 2004). Previous studies have observed the effects whereby polysaccharide mediates interactions between tannins and proteins (Carvalho, et al. 2006; Rinaldi, Gambuti, & Moio 2012), conferring impact on wine mouthfeel (Watrelot, Schulz, & Kennedy 2017), color stabilization (Alcalde-Eon, García-Estévez, Puente, Rivas-Gonzalo, & Escribano-Bailón 2014) and fining (removal) of phenolic compounds (Maury, Sarni-Manchado, Poinsaut, Cheynier, & Moutounet 2016). Polysaccharides have been used by the wine industry to improve wine composition and organoleptic characters. In Australia, two types of commercially manufactured polysaccharide additives are permitted in wine production: yeast mannoprotein and gum arabic, which represent the two most abundant wine neutral polysaccharides (Australia New Zealand Food Standards Code – Standard 4.5.1).

Characterizing interactions between grape- and wine- derived polysaccharides and tannins poses unique challenges, since both materials are very polydisperse. Grape

skin tannins can comprise 3 to 83 flavan-3-ol subunits, while seed tannins are reported to have 2 to 16 subunits (Smith, et al. 2015). Wine polysaccharides are also heterogeneous, with molecular distribution reported to be between 5 and 800 kDa (Guadalupe, et al. 2014). Different fractions of macromolecules isolated from Pinot Noir wines, including tannins, polysaccharides and proteins, were shown to be highly polydisperse, with particle size distributions ranging from 20 to 500 nm (Bindon, et al. 2016). Moreover, the property of the dispersant has a significant impact on the macromolecular interaction, e.g. pH, ethanol concentration and ionic strength (Poncet-Legrand, C., Doco, Williams, & Vernhet 2007). Thus, investigation into these interactions requires non-invasive techniques, so as not to disrupt the non-covalent associations between particles, and at the same time, detect aggregate formation in a wine-like medium. Methods that have been employed to study polysaccharide and condensed tannin interactions include ultraviolet-visible (UV-Vis) spectroscopy, dynamic light scattering (DLS), isothermal titration calorimetry (ITC), small-angle X-ray scattering (SAXS) and transmission electron microscopy (TEM) (Carn, et al. 2012; Mamet, Ge, Zhang, & Li 2017; Poncet-Legrand, C., et al. 2007; Watrelot, Le Bourvellec, Imberty, & Renard 2013).

Nanoparticle particle tracking analysis (NTA) is a relatively new technology (first commercialised in 2006) that derives particle size by analyzing Brownian motion. Although it makes use of the same basic principle as DLS technique, NTA is not an ensemble method based on light scattering of all particles being investigated. Rather, it tracks the movement of individually recognised particles and provides size distribution based on particle concentration. This gives NTA an advantage in characterising polydisperse samples over DLS (Filipe, Hawe, & Jiskoot 2010). NTA has been applied to many food matrices and can handle non-aggressive solvents such as hydroalcoholic

solution (Jarzębski, et al. 2017). However, only one study has employed NTA in sizing wine macromolecules (Bindon, et al. 2016). To date and to our knowledge, it hasn't been used to study tannin and polysaccharide interactions. Thus, the current study aimed to evaluate the suitability of NTA for this type of investigation, corroborated by other techniques that have been successfully applied in this field. To this end, two polysaccharides were purified from two commercial wine additives and a tannin fraction was purified from grape seeds, and combined in model wine solutions containing two wine-like alcohol concentrations. A secondary aim was to investigate to what extent applying these additives would affect the colloidal state of wine, and by inference, the composition and organoleptic characters of wine.

2. Materials and Methods

2.1 Preparation of polysaccharide and tannin materials

Cabernet Sauvignon grapes were obtained at the preveraison (pea size, green) stage from a commercial vineyard in South Australia, and frozen at -80 °C until used. Frozen berries were partially defrosted while kept on ice, and the seeds removed using a scalpel. A sample of 100 g of seeds was extracted overnight in 200 mL of 70% v/v aqueous acetone containing 10 mg/mL ascorbic acid. Extracts were filtered through a 0.5 mm mesh to remove solids and the recovered solution was centrifuged at 1730 x g. Acetone was removed from the supernatant under vacuum at 35 °C and the remaining aqueous solution was lyophilized. The dried extract was reconstituted in 50 mL 60% v/v HPLC grade aqueous methanol containing 0.05% v/v trifluoroacetic acid (TFA) and then applied (~18.3 mL/min) to a glass column (Michel-Miller, 300 x 21 mm, Vineland, NJ, USA) containing Sephadex LH20 chromatography resin (Amersham, Uppsala, Sweden) to an approximate bed volume of 93 mL, previously equilibrated

with the loading solvent. The monomeric phenolics, organic acids and sugars were removed by application of 300 mL 60% v/v aqueous methanol containing 0.05% v/v TFA. Seed tannin (ST) was recovered following application of 250 mL 70% v/v aqueous acetone containing 0.05% v/v TFA. The eluted ST fraction was concentrated under reduced pressure at 35 °C to remove organic solvents and then lyophilized to a dry powder. ST was stored under nitrogen at -20 °C until used. The subunit composition of ST was determined by HPLC following acid catalysis in the presence of excess phloroglucinol (Kennedy & Jones 2001; Kennedy & Taylor 2003). The molar proportion of each subunit, mean degree of polymerization and mass conversion are reported in Supplementary Table S1.

Two polysaccharides were prepared from commercial supplements used in vinification. The mannoprotein (MP) product was a highly pure cell wall extract from *Saccharomyces cerevisiae* (Mannofeel, Laffort Australia, Adelaide, Australia) while the arabinogalactan (AG) was purified from a commercial blend of gum arabic and grape tannin (Surli vitis, Enartis Pacific, Melbourne, Australia) by removing the associated phenolic compounds with three extractions in 70% acetone (monitored by HPLC with UV-vis detector at 280 nm). Both polysaccharides were dialyzed against 4 changes of MilliQ water using a 7 kDa cut-off membrane (SnakeSkin dialysis tubing, Thermo Scientific, Rockford, USA), and then lyophilized. The subunit composition of polysaccharide was determined according to a published method (Bindon, et al. 2016). Briefly, 1 mg/mL polysaccharide solution was hydrolyzed in 2 M TFA for 3 h at 100 °C. Hydrolysates were dried *in vacuo* and reconstituted in 0.4 mL Milli-Q water and mixed 1:1 with an aqueous internal standard solution comprising 0.6 mM ribose and deoxy-glucose (Sigma Aldrich, St. Louis, MO, USA). Mixtures were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) and analyzed by RP-HPLC, using a C18

column (Kinetex, 2.6 µm, 100 Å, 100 x 3mm). The HPLC instrumentation and mobile phase gradient were as reported previously (Bindon, et al. 2016). Total nitrogen content was measured by the analytical services unit of the Commonwealth Scientific and Industrial Research Organization (CSIRO, Adelaide, Australia), using a TruMAC (Leco Corporation, Saint Joseph, USA); powdered polysaccharides were combusted in an atmosphere of oxygen and nitrogen determined as gaseous N_2 by thermal conductivity detection. The composition of the products are reported in Supplementary Table S2.

Two model wine solutions (4 mg/mL tartaric acid, pH 3.4 and ionic strength of 0.02 mol/L) containing ethanol levels at 12% and 15% (v/v) were used in the current study. Solutions were filtered through 0.2 µm membrane (Durapore, Merck Millipore, Cork, Ireland) before use. For all experiments, ST, MP and AG were dissolved in model wine solution at gravimetric concentrations (w/v).

2.2 Particle size characterization

2.2.1 Size exclusion chromatography analysis

The molecular weight distribution of ST, MP and AG were determined by size exclusion chromatography (SEC). ST was analyzed with an HPLC (Agilent 1100, Agilent Technologies Australia Pty. Ltd., Melbourne, Australia), using The gel permeation chromatography (GPC) method originally reported in Kennedy, et al. (2003), with modifications described by Bindon and Kennedy (2011). The calibration curve, which was constructed from preveraison grape seed fractions, was previously reported by Bindon and colleagues (2010). The retention times at 10% and 90% ST elution by volume were compared against the standard curve to derive lower and upper ranges for molecular weight, while the retention time at 50% elution was used to determine mean molecular weight. In addition, the polydispersity index (PdI) was

151 calculated by dividing weight average molecular weight ($M_{w)}$ by number average
152 molecular weight (M_n).

153 The size distribution of polysaccharides was analysed using an Agilent 1260
154 HPLC system fitted with a Yarra SEC-4000 column connected to a Yarra SEC-2000
155 column (silica resin, 3 µm, 300 x 7.8 mm, Phenomenex, California, USA). The mobile
156 phase was 0.1 M $NaNO_3$ with a flow rate of 1.2 mL/min for a 22.5 min run time, at
157 40 °C. Refractive index signals were analyzed with ChemStation GPC data analysis
158 software Rev B.01.01 (Agilent Technologies Australia Pty. Ltd., Melbourne,
159 Australia). Polysaccharide molecular weight was determined by comparing samples to
160 a calibration curve developed with a series of pullulan standards of known molecular
161 weight (Shodex, Showa Denko K.k, Japan): P800 (708 kDa), P400 (344 kDa), P200
162 (200 kDa), P100 (107 kDa), P50 (47.1 kDa), P20 (21.1 kDa), P10 (7.6 kDa) and P5 (5.9
163 kDa). Each standard was run 5 times to check for retention time shift, which was not
164 found (data not shown). A 3rd order polynomial curve was established between elution
165 volume and molecular weight, with an R^2 of 0.9973 (Supplementary Figure S1). The
166 mean and range of molecular weight, as well as PdI of polysaccharides, were
167 determined in the same way as described for ST.

168 **2.2.2 DLS analysis**

169 A Malvern Zeitasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK),
170 equipped with a 633 nm He-Ne laser was used to measure zeta potential and particle
171 size. Instrument control and data analysis were performed with Zetasizer software
172 (version 7.10). For each measurement, the temperature was maintained at 25 °C, and
173 the angle of detection was set at 90°. Measurement position, attenuator level and
174 measurement duration were all set to be automatically optimized by the software.
175 Electrophoretic mobility was measured using disposable folded capillary cells (Malvern

Instruments Ltd, Worcestershire, UK) and zeta potential was derived from Henry's equation:

$$U_E = \frac{2\varepsilon\zeta f(k\alpha)}{3\eta}$$

where U_E is electrophoretic mobility; ε is dielectric constant; ζ is zeta potential; f (kα) is Henry's function and η is dispersant viscosity. Each sample was measured four times.

Particle size (hydrodynamic diameter) was determined using the Stokes-Einstein equation:

$$d(H) = \frac{kT}{3\pi\eta D}$$

Where k is Bolzmann's constant; T is absolute temperature; η is dispersant viscosity and D is diffusion coefficient. D was determined by fitting autocorrelation function to exponential with two different algorithms : (i) cumulants analysis, which determined the mean particle size (Z-ave), polydispersity index (PdI) and (ii) non-negative least squares (NNSL) analysis, which generated intensity weighted size distribution, using the 'general purpose mode' in this instance. Disposable low volume cuvettes with a pathlength of 10 mm were used for measurements.

2.2.3 NTA analysis

Nanoparticle tracking analysis (NTA) was performed on a Nanosight NS300 (Malvern Instruments Ltd, Worcestershire, UK), equipped with a 635 nm laser and a scientific CMOS camera. NTA 3.0 software was used for instrument control and data analysis. The data was collected in the form of 60-second videos captured by the camera. The sample chamber was maintained at 25 °C and a syringe pump was used to keep a continuous flow of sample through the flow cell at 7 µL/min for the duration of measurement.

For each individual sample, settings (screen gain, camera level and focus) were manually adjusted to optimize visualization of the particles and thereafter kept identical for all video repetitions of the same sample. Detection threshold, which determined the minimal brightness of pixels to be considered for tracking, was also adjusted post-acquisition to minimize noise as well as maintain a particle per frame count appropriate for analysis (10 – 100 per frame). Settings were kept consistent for all video repetitions of the same sample. The NTA software measured the mean square displacement from the centre of the particle's scatter as it moved from frame to frame in the collected videos. The hydrodynamic diameter of particles were calculated from the modified Einstein-Stokes equation:

$$\overline{(x,y)^2} = \frac{4kTt}{3\pi d\eta}$$

where $\overline{(x,y)^2}$ is the mean square of displacement; k is Bolzmann's constant; T is absolute temperature; t is time; d is the hydrodynamic diameter and η is dispersant viscosity.

2.2.4 System qualification for NTA and DLS instruments

NIST-traceable polystyrene latex beads standards (100 nm, 200 nm and 400 nm) were supplied by Malvern Instruments Ltd (Worcestershire, UK). The standards were dispersed in 0.01 M KCl. For DLS measurements, all three bead standards were diluted 1:10; for NTA measurements the dilution factors were according to instrument supplier's manual, i.e. 1:1000 for 100 nm, 1:100 dilution for 200 nm and 1:10 dilution for 400 nm. All samples were measured 5 times, by either DLS or NTA. For both systems, the accuracy of measurements of 100 nm and 200 nm beads were within those specified by the International Standardization Organization (ISO 22412:2008 and ISO 19430:2016) and were in good agreement with one another (Supplementary Table S3).

Although the measurements for 400 nm beads deviated more from stated size, accuracy was still within 10% for both methods.

2.2.5 Particle size of tannin and polysaccharide determined by DLS and NTA

Polysaccharides and ST were dissolved in model wine at 0.5 mg/mL and 0.125 mg/mL respectively, for NTA characterization. At these concentrations no excessive scattering was observed while all particles could be clearly visualized under the scientific CMOS camera. Fifteen video repetitions were taken for each sample.

DLS analysis required samples to be much more concentrated. Higher concentrations were trialled on DLS to find a working concentration that was closest to those used for NTA analysis. It was found that 4 mg/mL was the minimal concentration at which sufficient scattered light could be detected by the DLS instrument during a measurement, i.e. a mean count rate higher than 20 kilo counts per second and therefore this concentration was chosen. The same concentrations were used to determine the zeta potential. Each sample was measured five times.

2.3 Characterization of interactions between polysaccharide and tannin

2.3.1 UV-visible spectroscopy analysis

The aggregation between polysaccharides and tannins at various concentrations were measured as absorbance at 650 nm of UV-visible spectrometry. This assay was adapted and modified from a previous study (Watrelot, et al. 2014). ST was dissolved in the two model wine solutions at 10 mg/mL, while MP and AG were dissolved separately at 1 mg/mL. The control samples consisted of 1 mL of diluted ST solution of 0, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5 and 5 mg/mL (w/v), along the columns on a 96-well plate (1.1 mL volume, Axygen, Adelab, Adelaide, Australia). For the treatment samples, 0.5 mg/mL of either MP or AG was added to the ST solutions, while maintaining the same tannin concentrations and volumes as control samples. Both control and treatment

samples were prepared in duplicate. The plates were sealed with a compatible silicone sealing mat, vigorously shaken and stored at 22 °C for 24 hours. Thereafter, 200 µL of each well was then transferred into a clear 96-well cycloolefine plate (Greiner, Sigma-Aldrich, Sydney, Australia) and scattering at 650 nm wavelength was recorded by a SpectraMax M2 Microplate reader (Molecular Devices, Melbourne, Australia). In addition, a 20 µL sample aliquot was diluted with 980 µL 1 M HCl solution and 280 nm absorbance was recorded to determine total phenolics, according to Mercurio, Dambergs, Herderich, and Smith (2007). The plate was then centrifuged at 3273 x g for 5 minutes, and another 20 µL sample diluted with 980 µL 1 M HCl and measured at 280 nm absorbance.

2.3.2 NTA and DLS analyses

ST was dissolved at 2.5 mg/mL or 10 mg/mL, while AG and MP were both dissolved at 1 mg/mL, in both model wine solutions. ST solution was mixed in equal parts (750 µL each) with each polysaccharide solution and all stock solutions were also diluted 1:1 with model wine to create two series of samples with final concentrations of (i) 1.25 mg/mL tannin, 0.5 mg/mL polysaccharide and their mixtures and (ii) 5 mg/mL tannin, 0.5 mg/mL polysaccharide and their mixtures. The solutions were sealed in 1.5 mL Eppendorf tubes and kept at 22 °C for 24 hours and were then centrifuged at 16,100 x g for 5 minutes. The samples were used directly for DLS analysis. However, for NTA, the supernatants containing ST, individually or combined with either polysaccharide type, were diluted 1 in 10 with model wine solutions for low concentration series and 1 in 40 for high concentration series while supernatants containing only polysaccharide were measured undiluted. The samples for NTA and DLS measurements were individually prepared. For all samples, 15 video repetitions were recorded on NTA and 4 replicated measurements were performed by DLS.

3. Results and discussion

3.1 Molecular weight and size of ST, MP and AG

SEC methods have been developed for the rapid analysis of molecular weight distribution of wine polyphenolic and polysaccharide compounds (Kennedy, et al. 2003; Palomero, Morata, Benito, Calderón, & Suárez-Lepe 2009). In the current study, all three polymers had moderate PdIs, from 1.8 to 2.3 (Table 1). However, the molecular weight ranges were substantially different. The molecular weight of ST ranged from 0.5 to 6 kg/mol, with a mean of 1.9 kg/mol, which approximated DP 6 (Bindon, et al. 2010). In contrast, the two polysaccharides had much higher molecular weight ranges, 10 – 98 kg/mol and 48 – 322 kg/mol for MP and AG respectively, which were within the range that is typically observed for wine polysaccharides (Guadalupe, et al. 2014).

For both MP and AG, mean particle size measured by DLS (Z-ave) was much smaller than that measured by NTA, i.e. 24.2 vs. 109.7 nm for MP and 51.6 vs. 151.6 nm for AG (Table 2). Z-ave was not reported for ST in the current study because the error in cumulant fit, the algorithm that derived Z-ave, was higher than 0.005, indicating the correlation function could not be forced to fit to a single exponential curve due to poor quality data or high sample polydispersity. Therefore the Z-ave value was not reliable in this instance (Malvern Instruments, 2014). Nevertheless, all correlation functions could be fitted to a multiple exponential to generate an intensity based particle size distribution (Figure 1), which is more appropriate for polydisperse samples. The two major groups of ST, at 4.1 and 256.6 nm, were comparable to the gyration radii of grape seed tannin measured by small angle neutron scattering under similar experimental conditions (Zanchi, et al. 2007). The size distributions generated by the two methods were compared (Figure 1). DLS detected a peak between 10 and 60 nm

298 for both MP and AG, as well as between 1 and 10 nm for ST, which were not detected
299 by NTA. This was possibly caused by the different detection limits of the two methods;
300 for biological polymers, the lower detection threshold of NTA is 60 nm ± 30%
301 (International Organization of Standardization, 2016), while it is 1 nm for DLS (Filipe,
302 et al. 2010). Both methods detected particles above 60 nm for all samples, although
303 distribution determined by DLS was broader and tended towards higher mean sizes
304 when compared to NTA. This effect has been attributed to Rayleigh scattering, in that
305 the intensity of light scattered by particles is proportional to the sixth power of its
306 diameter; DLS, being an ensemble method, is biased towards higher scattering
307 particles. Li and colleagues (2011) demonstrated that when sizing the aggregates of a
308 lysozyme sample, the mean size determined by DLS was 1.6 times larger than that of
309 NTA.

310 Z-ave and PdI are the most frequently reported parameters derived from DLS.
311 They are determined by analysis of cumulants as defined in the ISO standard
312 (International Organization of Standardization, 2008). Z-ave is calculated using the first
313 cumulant of the decay rate distribution, which was obtained from the initial part of the
314 autocorrelation function and PdI estimates the broadness of the distribution using the
315 first two cumulants. This analysis assumes that the sample only contains a single family
316 of particle size of normal distribution, i.e. monodisperse. It has been reported that,
317 compared to measurements of particle size using more accurate methods, e.g. atomic
318 force microscopy and flow field-flow fractionation, the Z-ave values are only accurate
319 when PdI is less than 0.1, i.e. strictly for monodisperse samples (Baalousha & Lead
320 2012). However, in the current study, all three samples were polydisperse, and as such
321 Z-ave values should not be considered because these did not reflect the average particle
322 size appropriately, even for MP and AG for which the cumulant fit was within range.

Although it is more appropriate to report intensity distribution (Figure 1) for polydisperse samples, it would still be dominated by large particles and so is not recommended when PdI > 0.7 (Malvern Instruments, 2014). A crude isolate from red wine, containing tannins, proteins, polysaccharides and their complexes, gave particle sizes ranging from 20 to 500 nm (Bindon, et al. 2016). Furthermore, studies on tannin self-aggregation, or tannin and polysaccharide aggregation with DLS reported PdI in the range of 0.7 to 1, exceeding the limit (PdI < 0.7) with in which DLS could provide meaningful size information (Mamet, et al. 2017; Pascal, Poncet-Legrand, Cabane, & Vernhet 2008; Poncet-Legrand, Céline, Cartalade, Putaux, Cheynier, & Vernhet 2003). These results indicated that the colloidal dispersions formed in a wine system are very polydisperse and may exceed the limitation of the DLS technique. In comparison, NTA tracked individual particles in the sample and tallied up the number of particles in each size class (every 10 nm), giving a number-weighted hydrodynamic diameter distribution of particles in the sample. This could be considered to give NTA an advantage, compared with DLS, in accurately sizing polydisperse samples. This was demonstrated in a critical evaluation of the two methods, where both approaches were applied to samples constituted of two distinctively-sized NIST-traceable polystyrene beads at different ratios (Filipe, et al. 2010). NTA could accurately discriminate the two different sized particles within these mixtures while DLS could not. Thus, for sizing aggregates formed by grape and wine macromolecules, NTA presents a promising alternative to DLS.

3.2 Interactions between polysaccharides and tannins characterized by UV-visible spectrometry

Formation of aggregates between neutral polysaccharides and ST at a range of concentrations (0.065 mg/mL – 5 mg/mL) were also determined by measuring their

absorbance at 650 nm, since neither of these substances absorb light of this wavelength (Mamet, et al. 2017; Watrelot, et al. 2014). As such, absorbance at this wavelength is dominated by the light scattering intensity of particles. Both polysaccharides had a higher light scattering intensity than tannin, probably due to their larger sizes (Figure 2). In both model wine solutions, the absorbance of ST increased with concentration. This was expected since at higher concentrations, seed tannin self-aggregation is promoted (Poncet-Legrand, Céline, et al. 2003). A sharp increase in 650 nm absorbance was observed at lower ST concentrations, i.e. up to 1.25 mg/mL, followed by a steadier rise to 5 mg/mL. Absorbance of the mixture of ST and AG also followed an identical trend. In contrast to AG, the combination of MP and ST did not result in increases in absorbance at the lower ST concentrations. However, the 650 nm absorbance increased substantially in the MP and ST mixtures at higher tannin concentrations of 2.5 mg/mL and 5 g/mL in 12% model wine, indicating the formation of highly scattering large particles had occurred. Interestingly, in 15% model wine, the absorbance of the MP and ST combination increased evenly across the tannin concentration gradient. Strong increases in 650 nm absorbance have been reported between a protein-rich arabinogalactan-protein (AGP) and procyanidins at high concentrations, although the absorbance reported was much higher than that found in the current study (Watrelot, et al. 2014). This result has since been replicated in our lab (data not shown), confirming the observation in the current experiment. This phenomenon warrants further research, which will be discussed in the following section.

Absorbance at 280 nm was also recorded in order to reflect the impact of polysaccharide addition on phenolic content (retention or precipitation from solution). The 280 nm absorbance increased linearly ($R^2 > 0.99$) with tannin concentrations, but was not affected by the centrifuging step or the alcohol concentration of the model wine

(Figure 3). Although statistical analyses showed some differences between ST and combination of ST and polysaccharides at certain tannin concentrations, there was a lack of consistency in the difference and no general trend could be attributed to the tannin concentration, polysaccharide type, centrifugation or ethanol concentration in the model wine (Supplementary Table S4). It was therefore likely that the addition of polysaccharide did not influence the total phenolic concentration under the conditions of the current study. No loss at 280 nm absorbance was observed in the ST and polysaccharide mixtures before and after centrifugation, indicating that centrifuging did not remove aggregates formed between tannin and polysaccharide. This was consistent with the report that the aggregates formed between tannin and polysaccharide have low density and do not precipitate after ultracentrifugation (Carn, et al. 2012).

3.3 Binding experiment characterised by NTA

Based on UV-vis spectroscopy results, two ST concentration points were further characterized by NTA and DLS: 1.25 and 5 mg/mL ST, combined with 0.5 mg/mL of either MP or AG, in both 12% and 15% model wine solutions.

Number-weighted size distributions of tannin, polysaccharides and their mixtures, were determined by NTA and compared (Figure 4 and Figure 5). Notably, the absolute concentrations (number of particles/mL) between samples were not compared in this instance because the camera settings and detection threshold were optimized for each sample and may have therefore affected particle recognition and count for each size class (and thus affect particle concentration). As a result, comparison of the distribution only aimed to identify shifts in particle sizes, in order to infer the formation of aggregates. NTA also determined particle size at the 10%, 50% and 90% percentiles of the distribution, as well as an overall mean. These numerical data were also reported for ease of comparison (Table 3).

At 1.25 mg/mL, ST particles were smaller than either of the polysaccharides, and when ST was combined with either polysaccharide type, the size distribution of the mixture shifted towards a higher average (Figure 4). The overall particle size of the ST and MP combination was slightly higher than that of MP alone in 12% model wine, but not in 15% model wine (Table 3). On the other hand, at both ethanol levels, the AG and MP combination or AG alone had almost identical size distribution, although AG alone had a slightly higher mean size than the mixture. At 5 mg/mL, ST formed larger particles than at 1.25 mg/mL, which were comparable or slightly larger than MP, but still smaller than AG (Table 3). The trend of particle size evolution between ST, AG and their mixtures observed at lower tannin concentrations generally held true in samples containing 5 mg/mL ST. However, obvious formation of aggregates between ST and MP could be detected at this tannin concentration. In particular, in 12% model wine very large particles between 250 and 400 nm could be found (Figure 5 a). In general, the aggregate formation between AG and ST was relatively unaffected by either tannin or alcohol concentration. In contrast, MP formed significantly larger aggregates at higher tannin concentrations which were further promoted by lower alcohol.

NTA also provided light-scattering intensity data for each tracked particle. In MP and ST mixtures, a range of low-intensity light scattering particles were present in ST and polysaccharide samples were not detected in the mixture, as shown before. The particles in the mixtures had distinctively higher light scattering intensity than the components on their own, especially when ST concentration was high (Figure 6 a, b). Since the light scattering intensity is proportional to the size of the particle, the increases indicated the formation of aggregates in the ST and MP combination. In contrast, no clear difference could be seen in the light scattering intensity of the mixture compared

423 to AG and ST individually, with respect to ST and ethanol concentration (Figure 6 c,
424 d). The observation that smaller particles in ST and MP solutions were almost entirely
425 undetected in the mixtures required further investigation to determine whether it was
426 truly due to tannin and polysaccharide aggregation or the limit of NTA measurement.
427 It needs to be ascertained whether a mixture of two groups of particles of distinct yet
428 similar sizes could be discriminated by NTA; i.e. if particles of larger size could
429 potentially dominate the measurements. As such, a small amount of 100 nm polystyrene
430 beads was mixed with AG in 12% model wine solution and the size distribution
431 measured by NTA (Figure 7). In the size distribution profile of the mixture, both a
432 distinctive peak of approximately 100 nm and a broader shoulder between 150 and 300
433 nm could be identified, representing the beads and the AG particles, respectively. In the
434 ST and polysaccharide binding experiment, the light scattering intensities were fairly
435 similar amongst the tannin, polysaccharides and mixtures (indicated by similar camera
436 settings), with the exception of higher light scattering found MP + 5 mg/mL ST in 12%
437 model wine. Therefore, we concluded that if the smaller ST and MP particles were
438 present in substantial quantities in the mixture, they should not have been entirely
439 obscured by the larger species and should have been detected.

440 The different behaviors between MP and AG towards ST was also explored with
441 DLS, which was more sensitive at detecting aggregates than NTA (Jarzębski, et al.
442 2017). At both 1.25 and 5 mg/mL ST concentrations, the MP and ST combination
443 resulted in a significantly higher light scattering intensity than observed for AG and ST
444 combination (Supplementary Figure S2). In particular, at 5 mg/mL ST concentration in
445 12% model wine solution, the light scattering intensity of MP and ST combination was
446 7 times higher than that of ST and AG combination. The DLS results confirmed those
447 measured by NTA. Furthermore, DLS detected multiple particle size groups (peaks)

448 and high PdI values in the AG and ST mixture (Supplementary Table S5), which were
449 very similar in size to those observed in AG and ST separately (Figure 1). Conversely,
450 MP and ST combination showed only one apparent size group (PdI = 0.2), irrespective
451 of ST and ethanol concentration. These results, together with results from NTA,
452 strongly suggested that MP and ST formed aggregates under the current experimental
453 conditions, while AG and ST had very weak interactions and formed aggregates of low
454 light scattering intensity with no apparent size evolution. These differences could not
455 be explained by the colloidal stability of the materials, since the zeta potential for ST,
456 MP and AG were -4.3 mV, -4.1 mV and -8.6 mV in 12% model wine and -5.9 mV, -
457 4.8 mV and -7.9 mV in 15% model wine. Generally, an absolute value of zeta potential
458 lower than 30 mV indicates instability of the colloidal dispersion (Silva, Cerqueira, &
459 Vicente 2012). Zeta potential is critically affected by pH and ionic strength of the
460 dispersion solution (Mierczynska-Vasilev & Smith 2015), both of which were kept
461 consistent between the two model wine solutions. Thus, under current experimental
462 conditions, the combinations of ST and both polysaccharides were expected to lead to
463 aggregation.

464 The primary aim of this study is to investigate the implementation of NTA in
465 studying macromolecule interactions in wine-like media. NTA was able to provide size
466 information for polydisperse samples and detect different size groups within a sample.
467 The individual particle light scattering intensity data provided more information on
468 aggregation formation when only subtle differences were shown by the size
469 distribution. However, one important feature of NTA, the particle concentration, was
470 not explored in the current study because the measurement and analysis setting was
471 optimised for each sample. Factors need to be critically evaluated include camera
472 shutter and gain, completed tracking numbers (related to number of video repetition)

and sample flow rate (Tian, et al. 2016; Zhou, Krueger, Barnard, Qi, & Carpenter 2015). However, NTA measurements showed excellent repeatability in either intraday comparisons of the same sample or different batches of samples in the same lab (Tian, et al. 2016) or in inter-lab comparisons using the same protocol (Hole, et al. 2013). Therefore the next step is to apply NTA to a wider range of samples and conditions in order to develop a protocol for NTA in analysing grape and wine related macromolecules.

The weak interactions observed between neutral AG and ST were in agreement with previous studies (Carvalho, et al. 2006; Poncet-Legrand, C., et al. 2007; Riou, Vernhet, Doco, & Moutounet 2002; Watrelot, et al. 2014). AG, in both wine and gum arabic, is composed of a ramified (1 → 3)-D-galactose core that is highly branched at the 6 position with (1 → 6) linked D-galactan side chains that are highly substituted with arabinose residues and to a lesser extent, glucuronic acid and rhamnose residues (Mahendran, Williams, Phillips, Al-Assaf, & Baldwin 2008; Pellerin, Vidal, Williams, & Brillouet 1995). This highly branched structure may limit its ability to aggregate with tannin through hydrophobic interactions (Watrelot, et al. 2014). Application of commercial MP in red wine has been observed to either promote tannin aggregation and precipitation (Guadalupe & Ayestarán 2008) or limit the loss of anthocyanin adducts (Alcalde-Eon, et al. 2014). Similarly, in model wine solution, a commercial MP (10 % protein w/w, molecular weight distribution 14 – 500 kDa) has been observed to form large aggregates with grape and wine tannins (Mekoue Nguela, Poncet-Legrand, Sieczkowski, & Vernhet 2016), consistent with the current results. In contrast, MP purified from wine, in particular the low molecular weight fractions (1.6 – 3.5% protein w/w with narrow molecular weight distribution around 51 to 62 kDa), limited seed tannin aggregation through steric hindrance, resulting in a smaller overall particle

size (Poncet-Legrand, C., et al. 2007; Riou, et al. 2002). The MP used in the current study had a molecular weight distribution between 10 and 98 kDa (Table 1) with a protein content at 10% of the dry weight. It appeared that the different behaviours towards ST were more related to protein content than molecular size. It has been shown that mannoproteins have significantly lower affinity to tannin than yeast-derived protein and bovine serum albumin (Mekoue Nguela, et al. 2016; Rowe, et al. 2010). Furthermore, between two wine AGP fractions, only the one with slightly higher protein content (3.6% vs 0.8%) could form aggregates with procyanidins of DP 30. If such small proportion of protein could induce a substantial difference in aggregate formation between polysaccharides and tannins, it might also explain the different behavior between MP and AG in the current study, since MP had more protein than AG (10% vs. 1.4%). This would potentially have significant implications for wine production. This is because native wine polysaccharide composition is highly variable and capable of impacting on tannin composition and subsequently wine astringency (Bindon, et al. 2014; Quijada-Morín, Williams, Rivas-Gonzalo, Doco, & Escribano-Bailón 2014; Watrelot, et al. 2017). Furthermore commercial polysaccharide supplements could also be added to wine, as discussed previously, which adds further unknowns to the system. It has been shown that the protein content of commercial MP products can range from 10 to 50% (Li, Wilkinson, & Bindon 2018). Therefore, the choice of product could have a great impact on the final wine colloidal state, potentially affecting color and organoleptic characters.

Aside from protein content, another hypothesis might also explain the different behaviours between MP and AG towards ST, in that the structure of tannin and polysaccharide may be of importance. Carn and colleagues (2012) demonstrated that different aggregation behaviour between tannin and polysaccharide is dependent on the

523 tannin end-to-end length (L_t). If L_t is less than the persistence length of polysaccharide
524 (L_p), loose oligomeric aggregates with sizes comparable to individual polysaccharide
525 molecules were formed, and the light scattering intensity increased monotonically with
526 tannin concentration, similar to the observations for AG and ST in the current study. In
527 contrast, when $L_t > L_p$, tannin could bridge multiple polysaccharide molecules and form
528 large aggregates, with size increases proportional to tannin concentration, consistent
529 with the current observations between MP and ST mixtures. For semi-flexible
530 polymers, the persistence length is proportional to the intrinsic viscosity of the polymer
531 solution, which is in turn depends on the solvent, as well as polymer molecular weight
532 and conformation (Rushing & Hester 2004). It has recently been shown that the intrinsic
533 viscosity of wine polysaccharides correlates well with mean molecular weight
534 determined by SEC (García, et al. 2017). Therefore, the substantial differences between
535 the molecular weights of MP and AG (Table 1) might have an impact on their
536 persistence length. Furthermore, ST had a DP of 6, which had an estimated L_t of 2 nm,
537 based on $L_t \approx$ DP x 0.34 nm (Carn, et al. 2012). Potentially, the very short tannin chain
538 was sufficient to bridge MP but not AG in this study.

539 Polysaccharide is considered important in mediating tannin and protein
540 aggregation, through one or more mechanisms: (i) polysaccharides form ternary
541 complex with tannin-protein aggregates and thereby increase their solubility; and (ii)
542 polysaccharides bind with tannin and thus limit access of protein (Scollary, Pásti,
543 Kállay, Blackman, & Clark 2012). The current study showed that for certain
544 polysaccharides, the second mechanism is in effect. In the future, different types of
545 protein could be introduced into this system to explore this possibility.

546 Lower ethanol concentration was found to promote the aggregate formation
547 between ST and MP. This effect is attributed to increased tannin solubility (Poncet-

Legrand, Céline, et al. 2003) and is in agreement with previous studies (Poncet-Legrand, C., et al. 2007; Rowe, et al. 2010). However, none of these studies reported an effect when the concentration differences between treatments were as small as used in the current study (3%). Ethanol concentrations between 12% and 15% is typically found in table wine. From a sensory point of view, a 4% increase in alcohol concentration could reduce astringency and enhance bitterness (two mouthfeel characters highly associated with wine polyphenolic composition) in model wine solutions (Fontoin, Saucier, Teissedre, & Glories 2008; Vidal, et al. 2004). Thus the effect of ethanol on the colloidal state of wine macromolecule and its implication on wine sensory characters warrant further investigation.

4. Conclusion

This study presents the first investigation on the application of NTA in the characterization of tannin and polysaccharide interactions in wine-like media. NTA was able to size polydisperse macromolecule samples and their mixtures, and provide detailed insight into aggregate formation. The NTA results were confirmed by DLS and UV-vis analysis. The two polysaccharides, MP and AG, derived from commercial winemaking additives used in wine production, were considerably different in colloidal behaviour towards ST. MP formed larger, highly light scattering aggregates, while AG had only weak interactions with ST, forming low-intensity light scattering aggregates of sizes comparable to AG alone. A 3% ethanol reduction was found to increase aggregate size for MP, but had no impact on AG.

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Alcalde-Eon, C., García-Estévez, I., Puente, V., Rivas-Gonzalo, J. C., & Escribano-Bailón, M. T. (2014). Color stabilization of red wines. a chemical and colloidal approach. *Journal of Agricultural and Food Chemistry, 62*(29), 6984-6994.

Australia New Zealand Food Standards Code – Standard 4.5.1 – Wine Production Requirements (Australia Only), version F2017C01001

Baalousha, M., & Lead, J. R. (2012). Rationalizing nanomaterial sizes measured by atomic force microscopy, flow field-flow fractionation, and dynamic light scattering: sample preparation, polydispersity, and particle structure. *Environmental Science & Technology, 46*(11), 6134-6142.

Bindon, K. A., Carew, A. L., Mierczynska-Vasilev, A., Kassara, S., Kerslake, F., & Smith, P. A. (2016). Characterization of macromolecular complexes in red wine: Composition, molecular mass distribution and particle size. *Food Chemistry, 199*, 838-846.

Bindon, K. A., Holt, H., Williamson, P. O., Varela, C., Herderich, M., & Francis, I. L. (2014). Relationships between harvest time and wine composition in *Vitis vinifera* L. cv. Cabernet Sauvignon 2. Wine sensory properties and consumer preference. *Food Chemistry, 154*(0), 90–101.

Bindon, K. A., & Kennedy, J. A. (2011). Ripening-induced changes in grape skin proanthocyanidins modify their interaction with cell walls. *Journal of Agricultural and Food Chemistry, 59*(6), 2696-2707.

Bindon, K. A., Smith, P. A., & Kennedy, J. A. (2010). Interaction between grape-derived proanthocyanidins and cell wall material. 1. Effect on proanthocyanidin composition and molecular mass. *Journal of Agricultural and Food Chemistry, 58*(4), 2520-2528.

Carn, F., Guyot, S., Baron, A., Pérez, J., Buhler, E., & Zanchi, D. (2012). Structural properties of colloidal complexes between condensed tannins and polysaccharide hyaluronan. *Biomacromolecules, 13*(3), 751-759.

Carvalho, E., Mateus, N., Plet, B., Pianet, I., Dufourc, E., & De Freitas, V. (2006). Influence of wine pectic polysaccharides on the interactions between condensed tannins and salivary proteins. *Journal of Agricultural and Food Chemistry, 54*(23), 8936-8944.

614 Filipe, V., Hawe, A., & Jiskoot, W. (2010). Critical evaluation of nanoparticle
615 tracking analysis (NTA) by NanoSight for the measurement of nanoparticles
616 and protein aggregates. *Pharmaceutical Research, 27*(5), 796-810.
617 Fontoin, H., Saucier, C., Teissedre, P.-L., & Glories, Y. (2008). Effect of pH, ethanol
618 and acidity on astringency and bitterness of grape seed tannin oligomers in
619 model wine solution. *Food Quality and Preference, 19*(3), 286-291.
620 García, M., Apolinar-Valiente, R., Williams, P., Esteve-Zarzoso, B., Arroyo, T.,
621 Crespo, J., & Doco, T. (2017). Polysaccharides and oligosaccharides produced
622 on Malvar wines elaborated with *Torulaspora delbrueckii* CLI 918 and
623 *Saccharomyces cerevisiae* CLI 889 native yeasts from D.O. "Vinos de
624 Madrid". *Journal of Agricultural and Food Chemistry, 65*(31), 6656-6664.
625 Guadalupe, Z., & Ayestarán, B. (2008). Effect of commercial mannoprotein addition
626 on polysaccharide, polyphenolic, and color composition in red wines. *Journal
627 of Agricultural and Food Chemistry, 56*(19), 9022-9029.
628 Guadalupe, Z., Ayestarán, B., Williams, P., & Doco, T. (2014). Determination of
629 must and wine polysaccharides by gas chromatography-mass spectrometry
630 (GC-MS) and size-exclusion chromatography (SEC). In K. G. Ramawat & J.-
631 M. Mérillon (Eds.), *Polysaccharides: Bioactivity and Biotechnology* (pp. 1-
632 28). Cham: Springer International Publishing.
633 Hole, P., Sillence, K., Hannell, C., Maguire, C. M., Roesslein, M., Suarez, G.,
634 Capracotta, S., Magdolenova, Z., Horev-Azaria, L., Dybowska, A., Cooke, L.,
635 Haase, A., Contal, S., Manø, S., Vennemann, A., Sauvain, J.-J., Staunton, K.
636 C., Anguissola, S., Luch, A., Dusinska, M., Korenstein, R., Gutleb, A. C.,
637 Wiemann, M., Prina-Mello, A., Riediker, M., & Wick, P. (2013).
638 Interlaboratory comparison of size measurements on nanoparticles using
639 nanoparticle tracking analysis (NTA). *Journal of Nanoparticle Research,*
640 *15*(12), 2101.
641 International Organization of Standardization (2008). Particle size analysis – Dynamic
642 light scattering (DLS) (ISO 22412:2008)
643 International Organization of Standardization (2016). Particle size analysis – Particle
644 tracking analysis (PTA) method (ISO 19430:2016).
645 Jarzębski, M., Bellich, B., Białopiotrowicz, T., Śliwa, T., Kościński, J., & Cesàro, A.
646 (2017). Particle tracking analysis in food and hydrocolloids investigations.
647 *Food Hydrocolloids, 68*(Supplement C), 90-101.
648 Kennedy, J. A., & Jones, G. P. (2001). Analysis of proanthocyanidin cleavage
649 Products following acid-catalysis in the presence of excess phloroglucinol.
650 *Journal of Agricultural and Food Chemistry, 49*(4), 1740–1746.
651 Kennedy, J. A., & Taylor, A. W. (2003). Analysis of proanthocyanidins by high-
652 performance gel permeation chromatography. *Journal of Chromatography A,*
653 *995*(1–2), 99–107.
654 Li, S., Wilkinson, K.W., & Bindon, K. A. (2018). Compositional variability in
655 commercial tannin and mannoprotein products. *American Journal of Enology*
656 *and Viticulture* (in press).
657 Li, Y., Lubchenko, V., & Vekilov, P. G. (2011). The use of dynamic light scattering
658 and Brownian microscopy to characterize protein aggregation. *Review of*
659 *Scientific Instruments, 82*(5), 053106.
660 Mahendran, T., Williams, P. A., Phillips, G. O., Al-Assaf, S., & Baldwin, T. C.
661 (2008). New insights into the structural characteristics of the
662 arabinogalactan−protein (AGP) fraction of gum arabic. *Journal of*
663 *Agricultural and Food Chemistry, 56*(19), 9269-9276.

Malvern Instruments (2014). Technical note: Size quality report for the Zetasizer Nano. http://www.malvern.com/en/support/resource-center/technical-notes/TN101104SizeQualityReportZetasizerNano.aspx (accessed 03/04/2017).

Mamet, T., Ge, Z.-z., Zhang, Y., & Li, C.-m. (2017). Interactions between highly galloylated persimmon tannins and pectins. *International Journal of Biological Macromolecules*.

Mateus, N., Carvalho, E., Luís, C., & de Freitas, V. (2004). Influence of the tannin structure on the disruption effect of carbohydrates on protein–tannin aggregates. *Analytica Chimica Acta, 513*(1), 135-140.

Maury, C., Sarni-Manchado, P., Poinsaut, P., Cheynier, V., & Moutounet, M. (2016). Influence of polysaccharides and glycerol on proanthocyanidin precipitation by protein fining agents. *Food Hydrocolloids, 60*, 598-605.

Mekoue Nguela, J., Poncet-Legrand, C., Sieczkowski, N., & Vernhet, A. (2016). Interactions of grape tannins and wine polyphenols with a yeast protein extract, mannoproteins and β-glucan. *Food Chemistry, 210*, 671-682.

Mercurio, M. D., Dambergs, R. G., Herderich, M. J., & Smith, P. A. (2007). High throughput analysis of red wine and grape phenolics - Adaptation and validation of methyl cellulose precipitable tannin assay and modified somers color assay to a rapid 96 well plate format. *Journal of Agricultural and Food Chemistry, 55*(12), 4651–4657.

Mierczynska-Vasilev, A., & Smith, P. A. (2015). Current state of knowledge and challenges in wine clarification. *Australian Journal of Grape and Wine Research, 21*, 615-626.

Palomero, F., Morata, A., Benito, S., Calderón, F., & Suárez-Lepe, J. (2009). New genera of yeasts for over-lees aging of red wine. *Food Chemistry, 112*(2), 432-441.

Pascal, C., Poncet-Legrand, C., Cabane, B., & Vernhet, A. (2008). Aggregation of a proline-rich protein induced by epigallocatechin gallate and condensed tannins: effect of protein glycosylation. *Journal of Agricultural and Food Chemistry, 56*(15), 6724-6732.

Pellerin, P., Vidal, S., Williams, P., & Brillouet, J. M. (1995). Characterization of five type II arabinogalactan-protein fractions from red wine of increasing uronic acid content. *Carbohydrate Research, 277*(1), 135-143.

Poncet-Legrand, C., Cartalade, D., Putaux, J.-L., Cheynier, V., & Vernhet, A. (2003). Flavan-3-ol aggregation in model ethanolic solutions: incidence of polyphenol structure, concentration, ethanol content, and ionic strength. *Langmuir, 19*(25), 10563-10572.

Poncet-Legrand, C., Doco, T., Williams, P., & Vernhet, A. (2007). Inhibition of grape seed tannin aggregation by wine mannoproteins: Effect of polysaccharide molecular weight. *American Journal of Enology and Viticulture, 58*(1), 87-91.

Quijada-Morín, N., Williams, P., Rivas-Gonzalo, J. C., Doco, T., & Escribano-Bailón, M. T. (2014). Polyphenolic, polysaccharide and oligosaccharide composition of Tempranillo red wines and their relationship with the perceived astringency. *Food Chemistry, 154*, 44–51.

Rinaldi, A., Gambuti, A., & Moio, L. (2012). Precipitation of salivary proteins after the interaction with wine: the effect of ethanol, pH, fructose, and mannoproteins. *Journal of Food Science, 77*(4), C485-C490.

Riou, V., Vernhet, A., Doco, T., & Moutounet, M. (2002). Aggregation of grape seed tannins in model wine—effect of wine polysaccharides. *Food Hydrocolloids, 16*(1), 17-23.

Rowe, J. D., Harbertson, J. F., Osborne, J. P., Freitag, M., Lim, J., & Bakalinsky, A. T. (2010). Systematic identification of yeast proteins extracted into model wine during aging on the yeast lees. *Journal of Agricultural and Food Chemistry, 58*(4), 2337-2346.

Rushing, T. S., & Hester, R. D. (2004). Semi-empirical model for polyelectrolyte intrinsic viscosity as a function of solution ionic strength and polymer molecular weight. *Polymer, 45*(19), 6587-6594.

Scollary, G. R., Pásti, G., Kállay, M., Blackman, J., & Clark, A. C. (2012). Astringency response of red wines: Potential role of molecular assembly. *Trends in Food Science & Technology, 27*(1), 25-36.

Silva, H. D., Cerqueira, M. Â., & Vicente, A. A. (2012). Nanoemulsions for food applications: development and characterization. *Food and Bioprocess Technology, 5*(3), 854-867.

Smith, P. A., McRae, J. M., & Bindon, K. A. (2015). Impact of winemaking practices on the concentration and composition of tannins in red wine. *Australian Journal of Grape and Wine Research, 21*, 601–614.

Tian, X., Nejadnik, M. R., Baunsgaard, D., Henriksen, A., Rischel, C., & Jiskoot, W. (2016). A comprehensive evaluation of nanoparticle tracking analysis (NanoSight) for characterization of proteinaceous submicron particles. *Journal of Pharmaceutical Sciences, 105*(11), 3366-3375.

Vidal, S., Courcoux, P., Francis, L., Kwiatkowski, M., Gawel, R., Williams, P., Waters, E., & Cheynier, V. (2004). Use of an experimental design approach for evaluation of key wine components on mouth-feel perception. *Food Quality and Preference, 15*(3), 209–217.

Watrelot, A. A., Le Bourvellec, C., Imberty, A., & Renard, C. M. G. C. (2013). Interactions between Pectic Compounds and Procyanidins are Influenced by Methylation Degree and Chain Length. *Biomacromolecules, 14*(3), 709-718.

Watrelot, A. A., Le Bourvellec, C., Imberty, A., & Renard, C. M. G. C. (2014). Neutral sugar side chains of pectins limit interactions with procyanidins. *Carbohydrate Polymers, 99*, 527-536.

Watrelot, A. A., Schulz, D. L., & Kennedy, J. A. (2017). Wine polysaccharides influence tannin-protein interactions. *Food Hydrocolloids, 63*, 571-579.

Zanchi, D., Vernhet, A., Poncet-Legrand, C., Cartalade, D., Tribet, C., Schweins, R., & Cabane, B. (2007). Colloidal dispersions of tannins in water−dthanol solutions. *Langmuir, 23*(20), 9949-9959.

Zhou, C., Krueger, A. B., Barnard, J. G., Qi, W., & Carpenter, J. F. (2015). Characterization of nanoparticle tracking analysis for quantification and sizing of submicron particles of therapeutic proteins. *Journal of Pharmaceutical Sciences, 104*(8), 2441-2450.

Figure Captions

Figure 1. Particle size distribution of (A) MP, (B) AG and (C) ST, measured by dynamic light scattering and nanoparticle tracking analysis.

Figure 2. Absorbance (650 nm) of seed tannin from 0 to 5 mg/mL, with or without addition of polysaccharides in (A) 12% ethanol model wine and (B) 15% ethanol model wine.

Figure 3. Absorbance (280 nm) of seed tannin from 0 to 5 mg/mL, with or without addition of polysaccharides in (A) 12% ethanol model wine and (B) 15% ethanol model wine, before and after centrifuging. Trend lines on each figure were fitted to seed tannin absorbance before and after centrifuging.

Figure 4. Size distribution of binding experiments between 1.25 mg/mL tannin and 0.5 mg/mL polysaccharides determined by nanoparticle tracking analysis. The curves were an average of 15 measurements. (A) ST and MP in 12% ethanol model wine; (B) ST and MP in 15% ethanol model wine; (C) ST and AG in 12% ethanol model wine; and (D) ST and AG in 15% ethanol model wine.

Figure 5. Size distribution of binding experiments between 5 mg/mL tannin and 0.5 mg/mL polysaccharides determined by nanoparticle tracking analysis. The curves were an average of 15 measurements. (A) ST and MP in 12% ethanol model wine; (B) ST and MP in 15% ethanol model wine; (C) ST and AG in 12% ethanol model wine; and (D) ST and AG in 15% ethanol model wine.

Figure 6. Size vs. light scattering intensity (arbitrary unit) for each tracked particle in nanoparticle tracking analysis. Only 1/5 of all tracked particles were included in the figures for clarity. (A) 1.25 mg/mL ST and MP in 12% ethanol model wine; (B) 5 mg/mL ST and MP in 15% ethanol model wine; (C) 1.25 ST and AG in 12% ethanol model wine; and (D) 5 mg/mL ST and AG in 15% ethanol model wine.

Figure 7. Size distribution of 100 nm polystyrene beads, AG and their mixture, determined by nanoparticle tracking analysis.

Table 1. Mean and range of molecular weight (MW) and polydispersity index (PdI) determined by size exclusion chromatography.

Material	Mean MW (kg/mol)	PdI	MW10 (kg/mol)	MW90 (kg/mol)
MP	33.6 ± 1.9	2.25 ± 0.07	9.6 ± 0.3	97.9 ± 8.8
AG	132.5 ± 1.0	1.75 ± 0.01	47.6 ± 0.4	321.6 ± 1.4
ST	1.92 ± 0.02	2.14 ± 0.01	0.5 ± 0.01	6.0 ± 0.09

All values are means of 3 measurements ± standard error. MW10 and MW90 stand for molecular weight at 10 and 90 percentile of elution.

Table 2. Mean size and size distribution of ST, MP and AG, determined by Dynamic light scattering and nanoparticle tracking analysis.

	DLS		NTA	
Material	Z-ave (nm)	PdI	mean (nm)	SD (nm)
MP	24.2 ± 0.3	0.50 ± 0.04	109.8 ± 0.7	30.6 ± 1.6
AG	51.6 ± 0.4	0.91 ± 0.01	151.6 ± 1.3	50.2 ± 1.1
ST	-	-	118.4 ± 1.9	35.7 ± 2.3

Z-ave and PdI are means of 5 measurements ± standard error. Values are missing for ST by DLS due to high fitting error for cumulants analysis.
NTA values are means of 15 measurements ± standard error; SD : standard deviation.

Table 3. Particle size measured by nanoparticle tracking analysis

ST[a] (mg/mL)	Model wine[b]	Treatment	Percentile and mean particle size by NTA(nm)				Treatment	Percentile and mean particle size by NTA (nm)			
			D10	D50	D90	Mean		D10	D50	D90	Mean
1.25	12%	ST	56.4 ± 0.8^{c}	79.1 ± 1.3^{c}	121.9 ± 2.5^{b}	91.4 ± 1.4^{c}	ST	56.4 ± 0.8^{c}	79.1 ± 1.3^{c}	121.9 ± 2.5^{c}	91.4 ± 1.4^{c}
		MP	62.8 ± 0.6^{b}	87.7 ± 0.9^{b}	158.3 ± 5.0^{a}	105.7 ± 1.0^{b}	AG	96.0 ± 0.9^{a}	143.5 ± 0.8^{a}	226.9 ± 2.9^{a}	160.1 ± 1.0^{a}
		ST + MP	80.2 ± 0.8^{a}	104.0 ± 0.9^{a}	152.5 ± 3.3^{a}	116.5 ± 1.0^{a}	ST + AG	85.8 ± 1.3^{b}	133.1 ± 1.2^{b}	214.1 ± 2.8^{b}	147.6 ± 1.4^{b}
	15%	ST	48.3 ± 0.9^{c}	71.4 ± 1.9^{b}	124.0 ± 4.9^{b}	85.7 ± 1.8^{b}	ST	48.3 ± 0.9^{b}	71.4 ± 1.9^{c}	124.0 ± 4.9^{c}	85.7 ± 1.8^{c}
		MP	68.7 ± 1.0^{b}	101.1 ± 1.2^{a}	155.6 ± 1.3^{a}	113.2 ± 0.8^{a}	AG	98.2 ± 1.4^{a}	141.0 ± 2.1^{a}	223.1 ± 4.3^{a}	157.5 ± 1.7^{a}
		ST + MP	73.3 ± 0.9^{a}	99.0 ± 0.8^{a}	160.1 ± 3.3^{a}	113.9 ± 1.0^{a}	ST + AG	97.3 ± 0.9^{a}	134.5 ± 1.0^{b}	209.9 ± 3.4^{b}	151.0 ± 0.9^{b}
5	12%	ST	78.3 ± 1.2^{b}	99.6 ± 1.2^{b}	147.3 ± 4.7^{c}	112.5 ± 1.3^{b}	ST	78.3 ± 1.2^{b}	99.6 ± 1.2^{c}	147.3 ± 4.7^{b}	112.5 ± 1.3^{c}
		MP	71.5 ± 1.3^{c}	100.8 ± 2.1^{b}	160.9 ± 4.9^{b}	113.4 ± 2.0^{b}	AG	93.8 ± 0.7^{a}	132.6 ± 1.8^{a}	193.4 ± 4.1^{a}	144.2 ± 1.9^{a}
		ST + MP	134.5 ± 2.8^{a}	229.2 ± 3.5^{a}	316.2 ± 3.8^{a}	231.2 ± 3.1^{a}	ST + AG	77.3 ± 1.0^{b}	108.6 ± 1.5^{b}	160.9 ± 6.0^{b}	121.4 ± 2.0^{b}
	15%	ST	90.5 ± 1.3^{b}	119.7± 1.7^{b}	170.4 ± 2.9^{b}	131.4 ± 1.5^{b}	ST	90.5 ± 1.3^{b}	119.7± 1.7^{c}	170.4 ± 2.9^{c}	131.4 ± 1.5^{c}
		MP	68.0 ± 1.0^{c}	100.2 ± 4.2^{c}	184.1 ± 7.7^{b}	120.7 ± 3.2^{c}	AG	107.2 ± 1.1^{a}	148.2 ± 2.2^{a}	237.9 ± 5.6^{a}	167.1 ± 2.2^{a}
		ST + MP	117.9 ± 1.5^{a}	153.3 ± 2.5^{a}	249.5 ± 6.2^{a}	176.1 ± 2.5^{a}	ST + AG	105.1 ± 1.0^{a}	132.9 ± 1.0^{b}	192.8 ± 2.6^{b}	147.3 ± 1.3^{b}

Values are means of 15 measurements ± standard error. Values followed by different letters within rows are significantly different ($p \leq 0.05$, one way ANOVA, post hoc Fisher's LSD).

[a]Seed tannin concentration in the solutions.

[b]Ethanol concentrations in the model wine solutions (v/v)

Figure 1

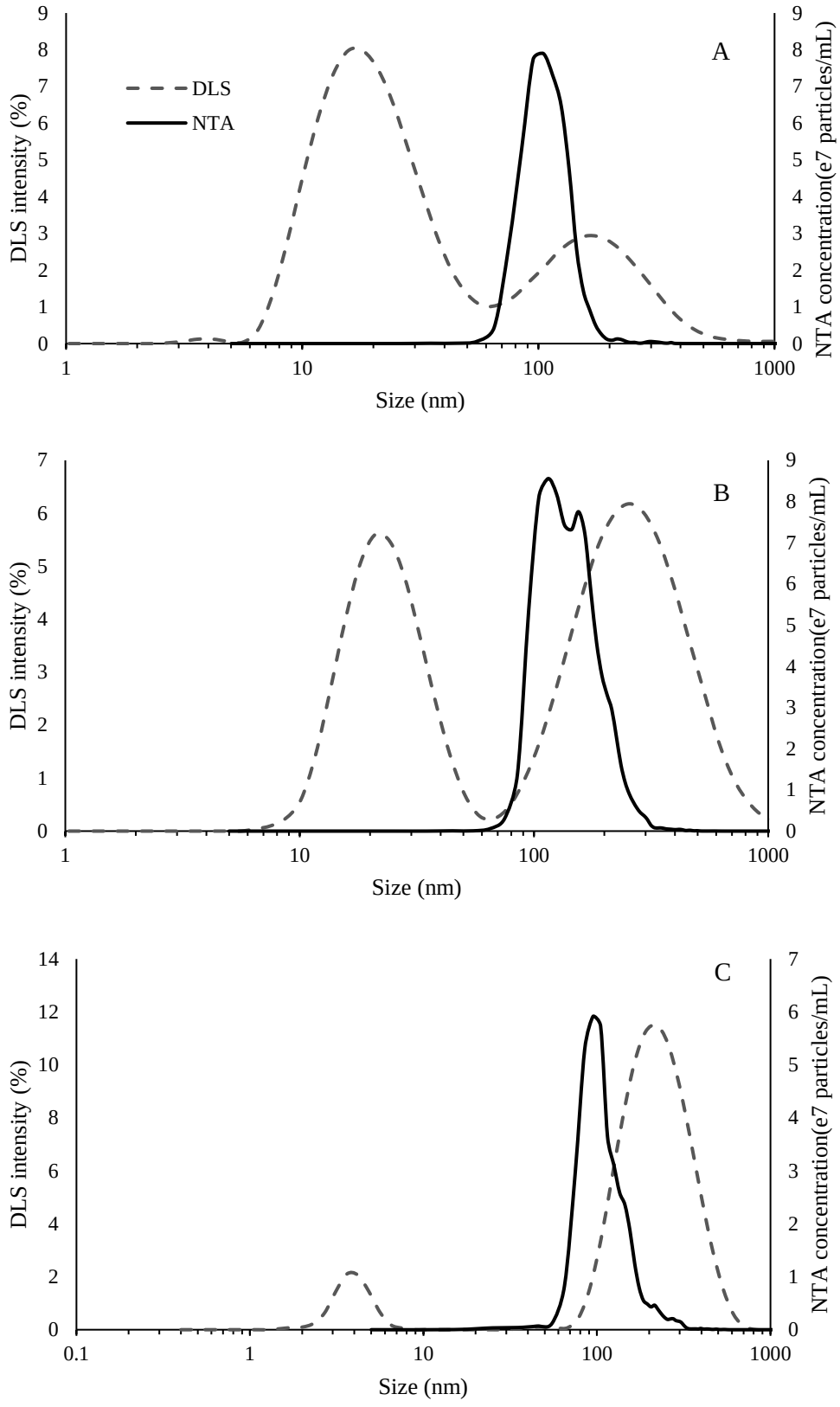

A
DLS
NTA
DLS intensity (%)
NTA concentration(e7 particles/mL)
Size (nm)
B
DLS intensity (%)
NTA concentration(e7 particles/mL)
Size (nm)
C
DLS intensity (%)
NTA concentration(e7 particles/mL)
Size (nm)

Figure 2

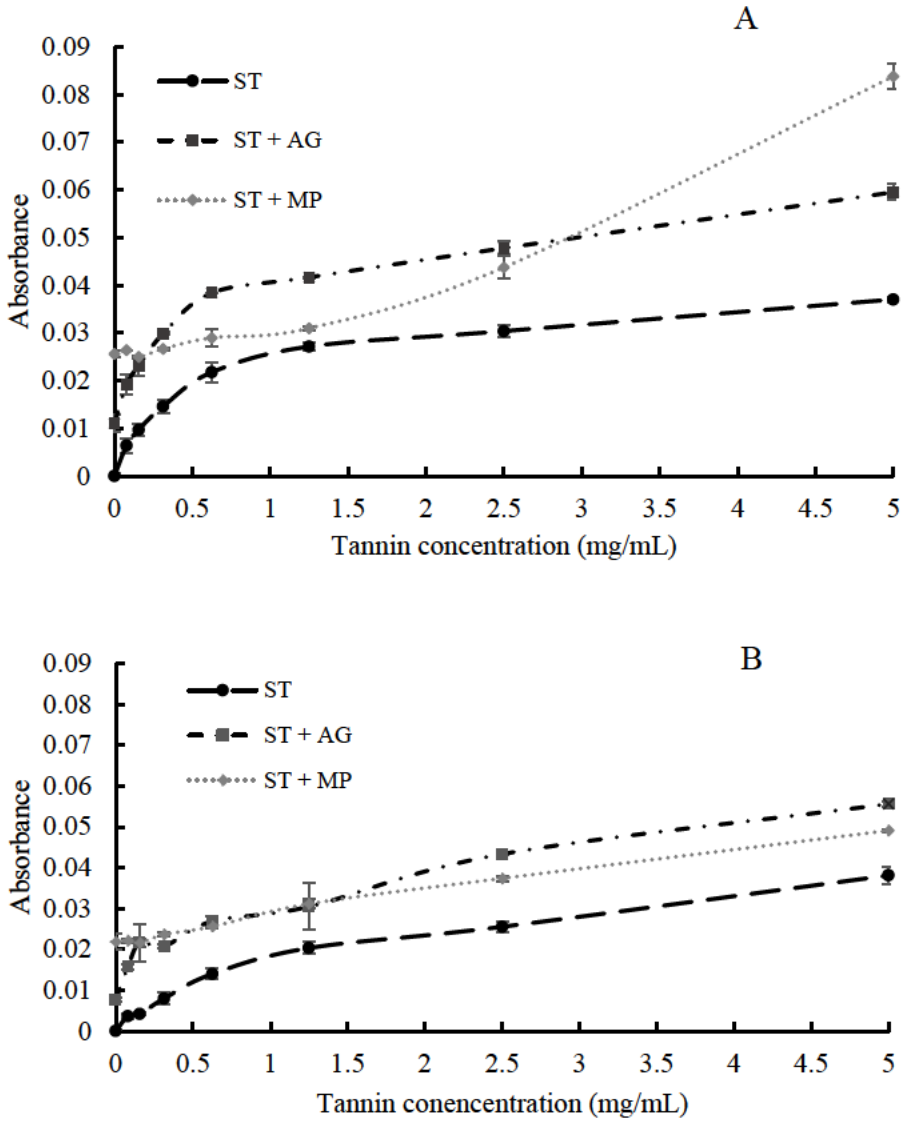
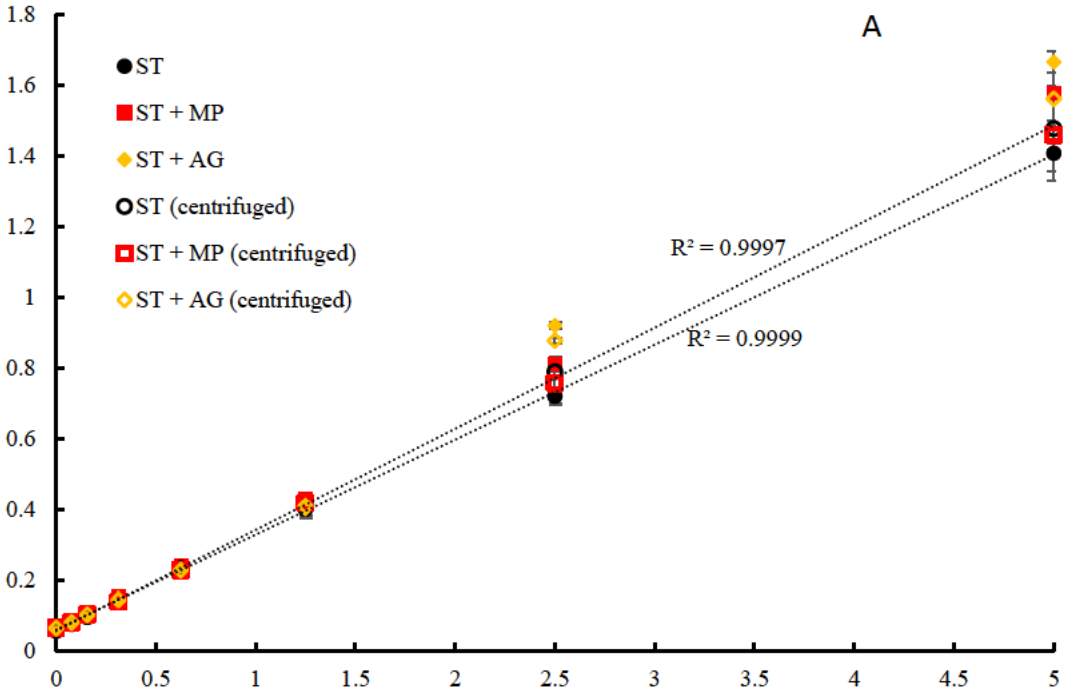

A
ST
ST + AG
ST + MP
Absorbance
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5
Tannin concentration (mg/mL)
B
ST
ST + AG
ST + MP
Absorbance
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5
Tannin conencentration (mg/mL)

Figure 3


A
ST
ST + MP
ST + AG
ST (centrifuged)
ST + MP (centrifuged)
ST + AG (centrifuged)
R² = 0.9997
R² = 0.9999
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5

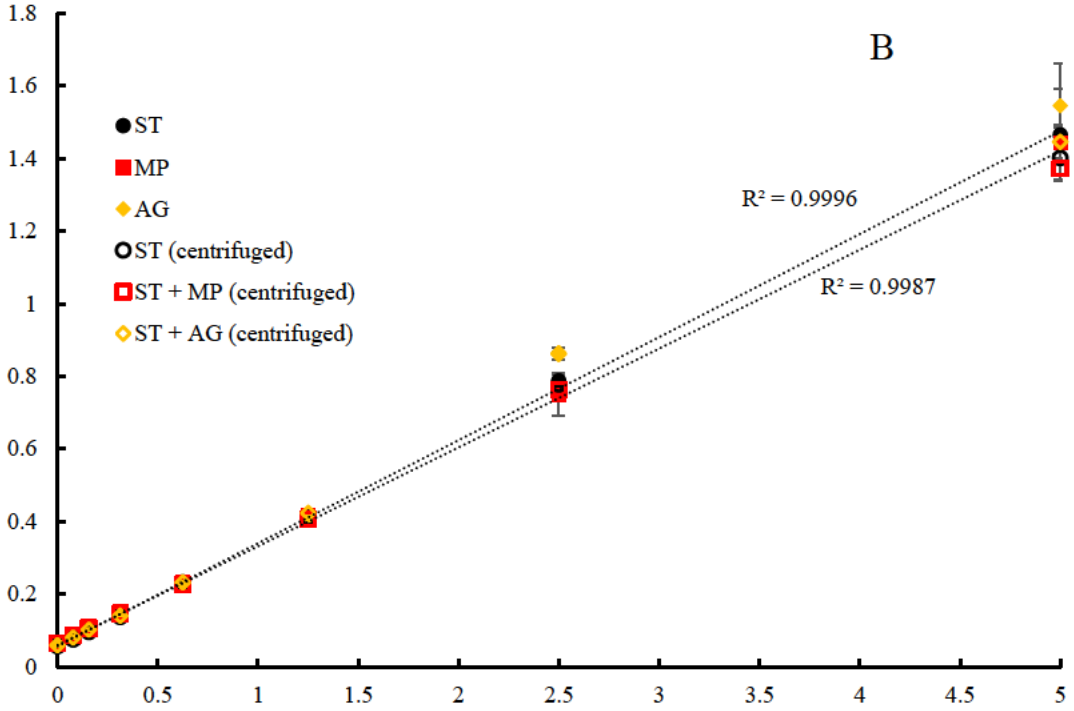

B
ST
MP
AG
ST (centrifuged)
ST + MP (centrifuged)
ST + AG (centrifuged)
R² = 0.9996
R² = 0.9987
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5

Figure 4

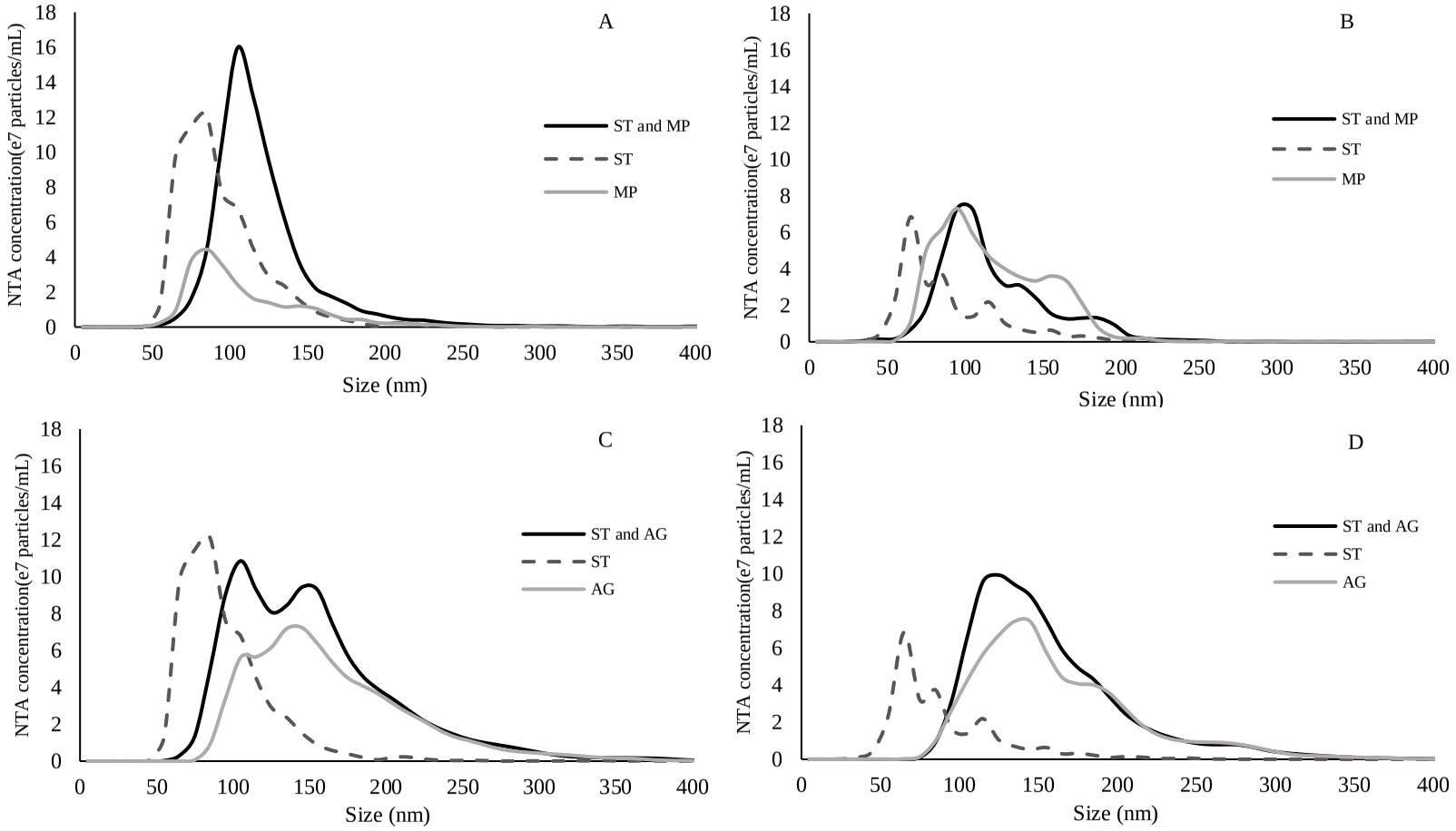

A
B
C
D
NTA concentration(e7 particles/mL)
Size (nm)
ST and MP
ST
MP
ST and AG
AG
0
2
4
6
8
10
12
14
16
18
0
50
100
150
200
250
300
350
400

Figure 5

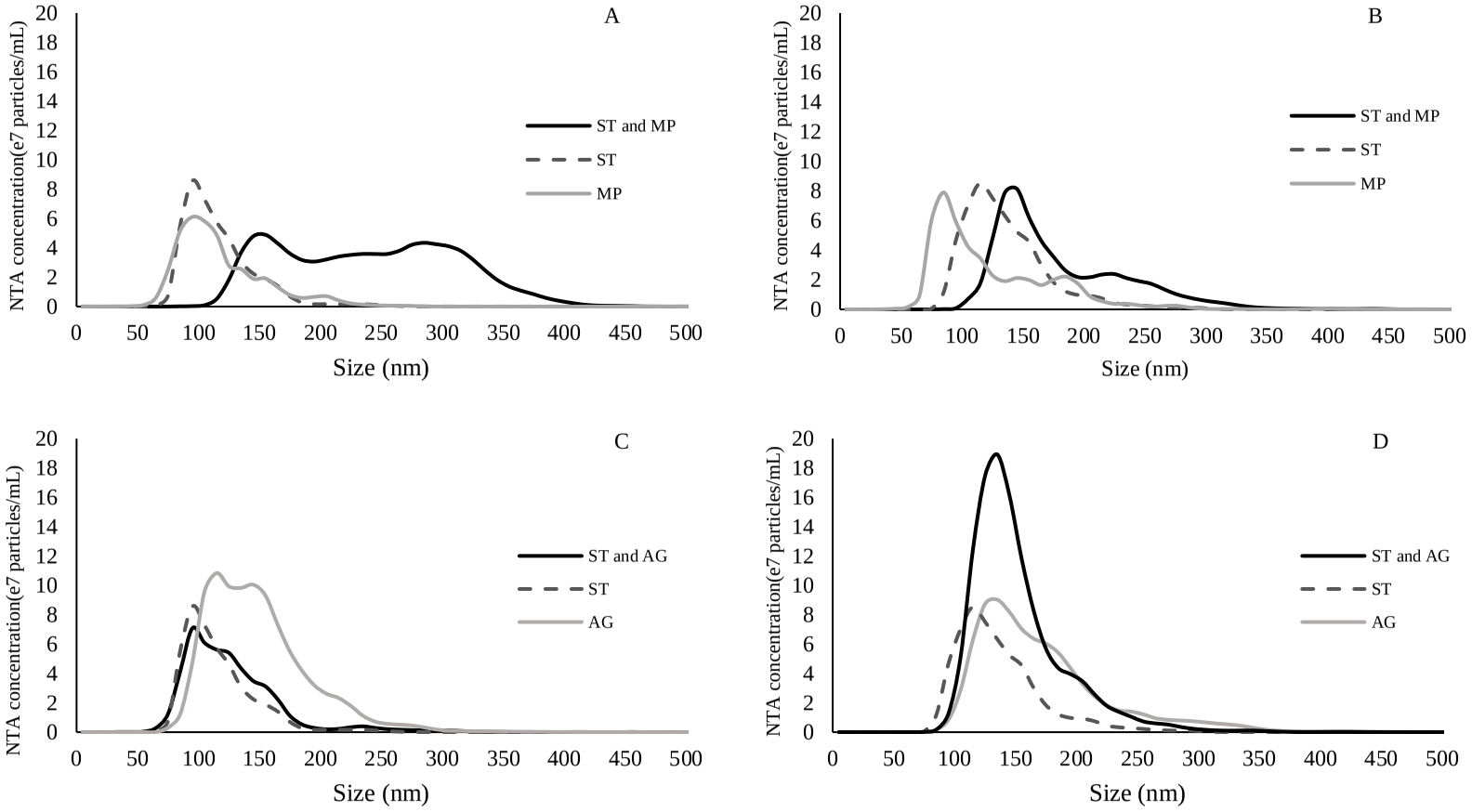

A
B
C
D
NTA concentration(e7 particles/mL)
Size (nm)
ST and MP
ST
MP
ST and AG
AG
0
2
4
6
8
10
12
14
16
18
20
0
50
100
150
200
250
300
350
400
450
500

Figure 6

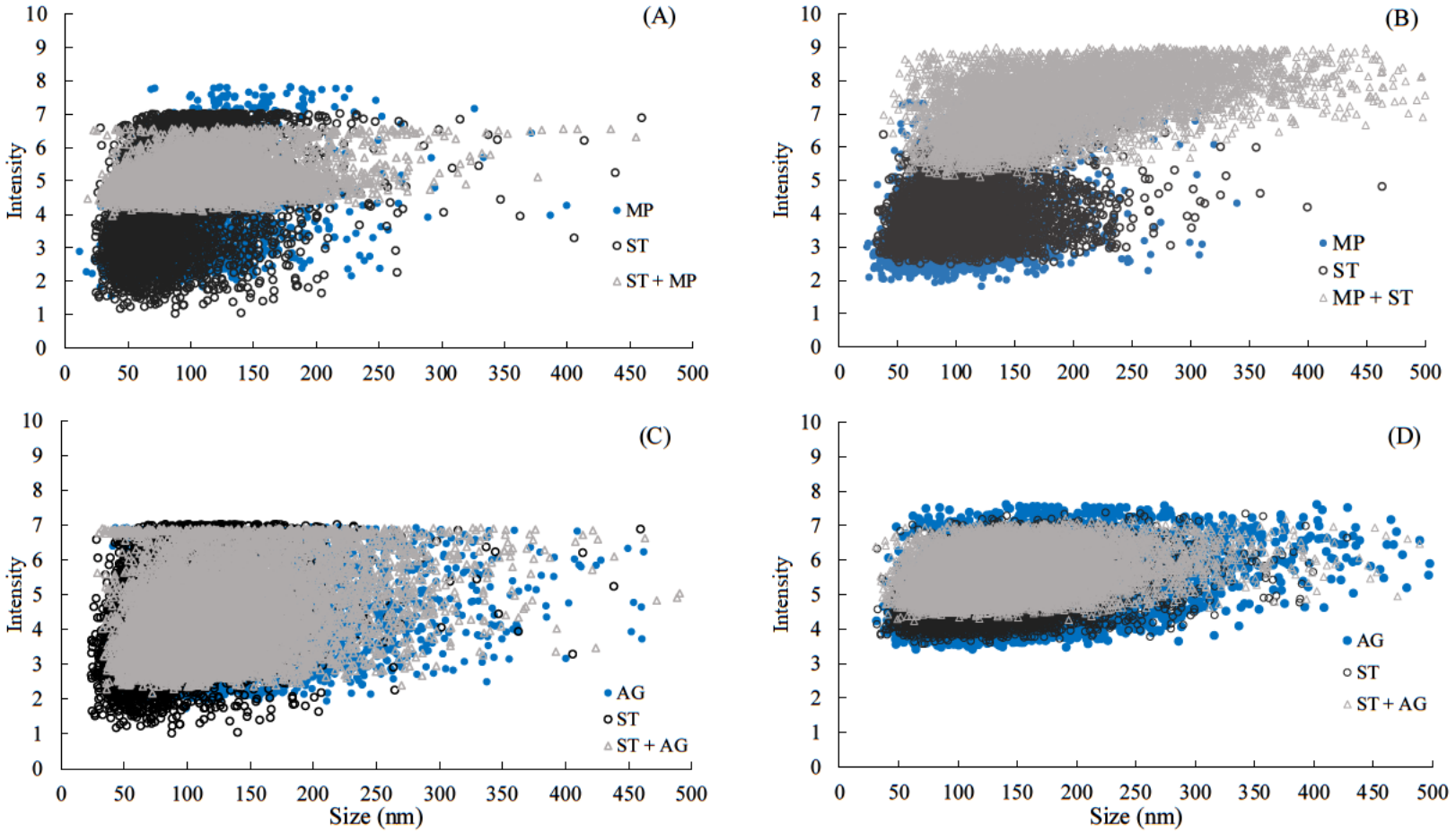
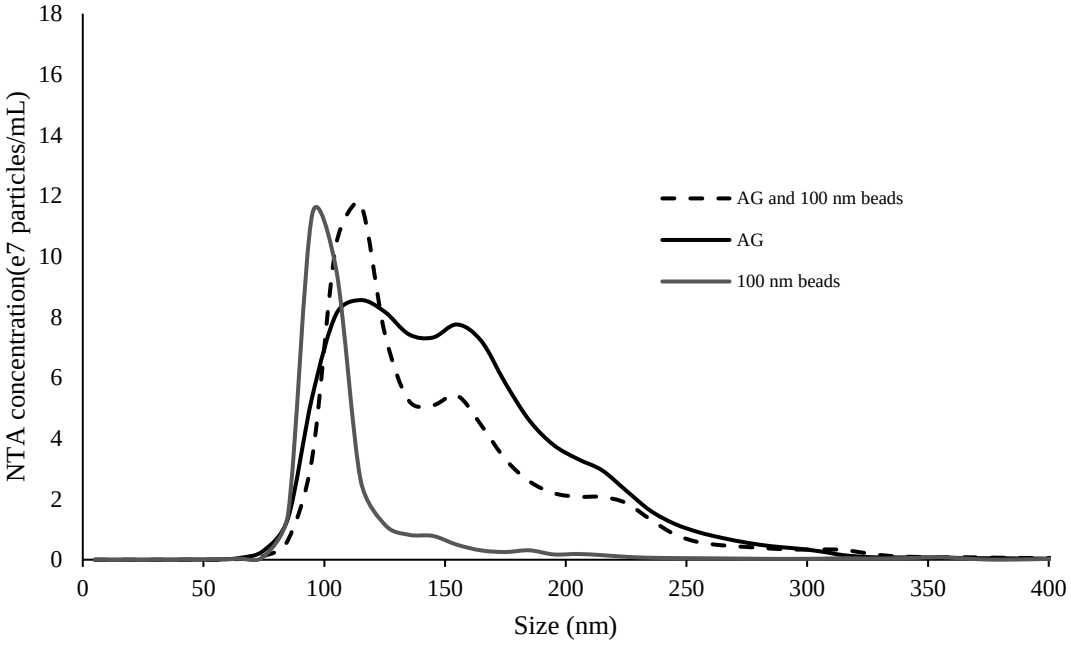

(A)
Intensity
MP
ST
ST + MP
(B)
Intensity
MP
ST
MP + ST
(C)
Intensity
AG
ST
ST + AG
Size (nm)
(D)
Intensity
AG
ST
ST + AG
Size (nm)

Figure 7


NTA concentration(e7 particles/mL)
18
16
14
12
10
8
6
4
2
0
0
50
100
150
200
250
300
350
400
Size (nm)
AG and 100 nm beads
AG
100 nm beads

Supplementary Data

Applying Nanoparticle Tracking Analysis to Characterize Interactions between Tannin and Polysaccharide in Wine-like Media

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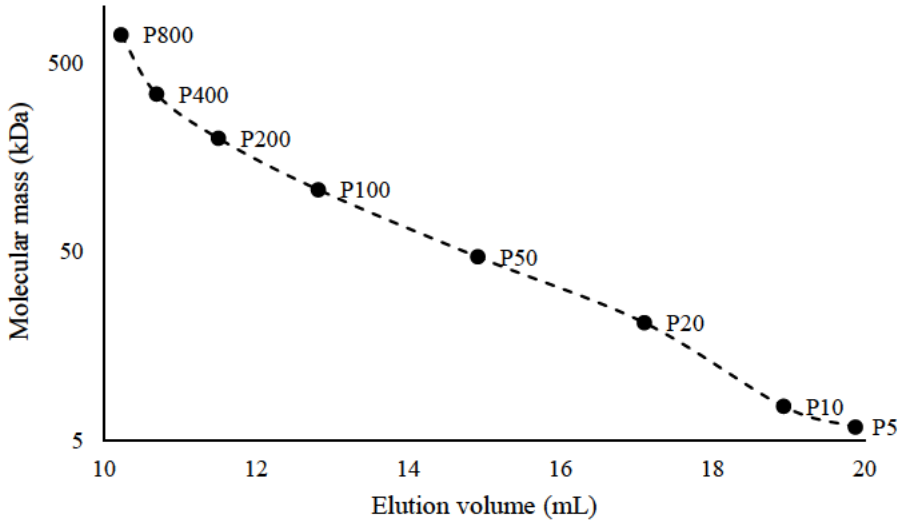

Figure S1. Calibration curve for polysaccharide molecular weight based on size exclusion chromatography.

Table S1. Subunit composition of ST.

Tannin	MC[b] (%)	mDP[c]	Extension subunits[a]				Terminal subunits[a]		
			EGC-P	C-P	E-P	ECG-P	C	E	ECG
ST	67.3	5.18	0.0	7.5	58.9	14.3	7.4	3.0	8.9

[a] Molar fractions (%) of procyanidin subunits with the following abbreviations: (-P) , phloroglucinol adduct of extension subunit; EGC, (–)-epigallocatechin; C, (+)-catechin; EC, (–)-epicatechin; ECG, (–)-epicatechin-3-*O*-gallate.
[b] Mass conversion derived from percent recovery of procyanidin subunits by phloroglucinolysis based on the gravimetric mass.
[c] Mean degree of polymerisation in epicatechin units.

Table S2. Monosaccharide residue composition of polysaccharide following hydrolysis.

Polysaccharide type	PRT[a] (%)	PL MC[b] (%)	Monosaccharide composition[c]						
			Man	Rha	GlcA	GalA	Glu	Gal	Ara
MP	11.3	74.5	85.8	n.d.	n.d.	n.d.	14.2	n.d.	n.d.
AG	1.4	77.7	1.2	3.7	7.4	1.5	0.8	36.4	49.1

[a] Protein as percentage of the gravimetric mass, estimated by multiplying total nitrogen by a factor of 6.25.
[b] Mass conversion derived from the percent recovery of monosaccharide residue based on gravimetric mass.
[c] Molar fraction (%) of monosaccharide residues following hydrolysis, with the following abbreviations: Man, mannose; Rha, rhamnose; GalA, galacturonic acid; GlcA, glucuronic; Gal, galactose; Ara, arabinose.

Table S3. Mean size and size distribution of polystyrene beads determined by dynamic light scattering and nanoparticle tracking analysis.

	DLS		NTA	
Bead size (nm)	Z-ave (nm)	PdI	mean (nm)	SD (nm)
100 nm	100.9 ± 0.3	0.03 ± 0.01	101.2 ± 1.5	13.6 ± 2.9
200 nm	202.8 ± 3.3	0.02 ± 0.01	189.8 ± 0.5	17.0 ± 3.4
400 nm	433.0 ± 3.6	0.05 ± 0.02	371.7 ± 1.3	28.1 ± 3.5

Values are means of 5 measurements ± standard error.

Table S4. Absorbance (280 nm) at each tannin concentration, in 12% and 15% ethanol model wine, before and after centrifugation.

Treatment	Tannin concentration (mg/mL)							
	0	0.078	0.156	0.313	0.625	1.25	2.5	5
12% model wine solution, before centrifugation.								
ST + MP	0.062	0.086 a	0.105	0.154 a	0.239	0.428	0.812 b	1.576
ST + AG	0.061	0.084 a	0.106	0.148 b	0.222	0.402	0.919 a	1.665
ST	0.059	0.079 b	0.099	0.145 c	0.233	0.399	0.719 c	1.407
Pr > F	0.091	0.007	0.094	0.002	0.103	0.563	0.013	0.071
Significant	No	Yes	No	Yes	No	No	Yes	No
12 % model wine solution, after centrifugation								
ST + MP	0.064 a	0.080 a	0.101 a	0.138	0.228	0.418	0.757	1.459
ST + AG	0.063 a	0.080 a	0.101 a	0.143	0.228	0.408	0.877	1.562
ST	0.057 b	0.076 b	0.096 b	0.140	0.233	0.416	0.789	1.477
Pr > F	0.015	0.039	0.014	0.665	0.606	0.624	0.197	0.742
Significant	Yes	Yes	Yes	No	No	No	No	No
15% model wine solution, before centrifugation								
ST + AG	0.059 a	0.078 a	0.100 a	0.142	0.229	0.409	0.865	1.545
ST + MP	0.061 a	0.079 a	0.103 a	0.141	0.227	0.416	0.749	1.443
ST	0.056 b	0.074 b	0.096 b	0.141	0.234	0.416	0.787	1.464
Pr > F	0.018	0.003	0.021	0.959	0.637	0.781	0.210	0.775
Significant	Yes	Yes	Yes	No	No	No	No	No
15% model wine solution, after centrifugation								
ST + AG	0.059 b	0.080 b	0.102 b	0.140 b	0.232	0.423	0.861 a	1.445
ST + MP	0.065 a	0.087 a	0.106 a	0.147 a	0.227	0.407	0.761 b	1.372
ST	0.057 b	0.075 c	0.095 c	0.137 b	0.225	0.409	0.777 b	1.401
Pr > F	0.004	0.002	0.007	0.022	0.194	0.254	0.016	0.378
Significant	Yes	Yes	Yes	Yes	No	No	Yes	No

Values are means of duplicates. Values followed by different letters within column are significantly different ($p \leq 0.05$, one way ANOVA followed by Fisher's LSD).

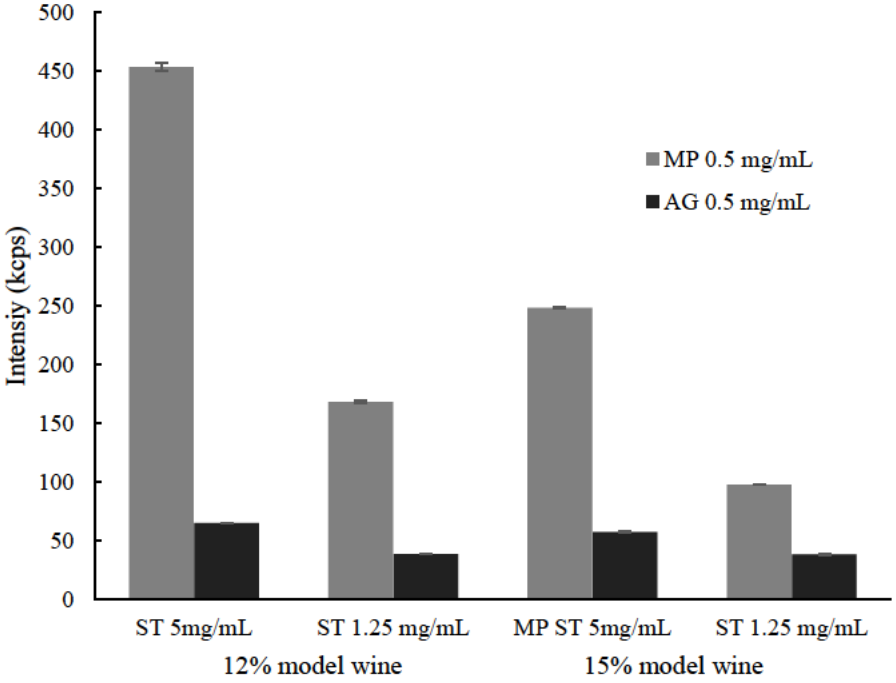

Figure S2. Light scattering intensity measured in derived count of photons (kilo counts per second) in samples containing both tannin and polysaccharide.

Table S5. PdI and intensity weighted mean particle sizes distribution determined by dynamic light scattering.

Treatment	PdI	Peak 1[a] (nm)	Peak 2[a] (nm)	Peak 3[a] (nm)
12% model wine solution				
ST5 + MP	0.20 ± 0.01	193 (100)	-	-
ST5 + AG	0.99 ± 0.01	265 (53)	29 (40)	4.7 (7)
ST1.25 + MP	0.20 ± 0.00	86 (100)	-	-
ST1.25 + AG	0.69 ± 0.07	242 (53)	27 (46)	-
15 % model wine solution				
ST5 + MP	0.25 ± 0.01	131 (100)	-	-
ST5 + AG	1.00 ± 0.00	212 (56)	25 (37)	3.5 (6)
ST1.25 + MP	0.23 ± 0.00	74 (100)	-	-
ST1.25 + AG	0.65 ± 0.08	221 (58)	26 (42)	-

[a]Results are reported as mean particle size of the each peak detected by dynamic light scattering. Values in parentheses are percentage of total scattered light represented by each peak.

Chapter 6. Concluding Remarks and Future Directions

This work aimed to improve the quality of Shiraz wines made from early harvested grapes. In the past two decades, a trend of increasing alcohol levels in wine has been observed globally, largely due to advanced grape maturity associated with climate change. Numerous methods have been devised for controlling the alcohol content of wine; among them, employment of novel harvest regimes, which inevitably involve harvesting grapes at sub-optimal maturity. A range of wine supplements are legally permitted to be used during wine production in Australia. The effects on wine composition and subsequent sensory characters of using grapes of sub-optimal maturity has been discussed in the literature. Tannin and polysaccharide levels were found to be highly influenced and were linked to the loss of mouthfeel characters in wines made from early harvested grapes. Three commercial additives, maceration enzymes, oenological tannin and mannoproteins, were therefore chosen to modify the tannin and polysaccharide composition of these wines, in order to improve mouthfeel.

Macromolecules such as tannin and polysaccharide exist in wine in a colloidal state. Introducing exogenous products into wine can therefore alter the colloidal state of wine, which may have implications for wine stability and organoleptic characters. Thus it was also important to understand the interactions that occur between commercial supplements and native grape and wine macromolecules.

6.1 Conclusions

6.1.1 Use of Winemaking Supplements to Modify the Composition and Sensory Properties of Shiraz Wine

The objective for this winemaking trial was to increase tannin and mannoprotein concentrations in wine made from early harvested sub-optimal maturity Shiraz grapes, and then compare it with a Shiraz wine made from mature grapes. This approach was based on previous reports that wines made from mature fruit had naturally higher levels of tannin and mannoprotein, which are associated with desirable mouthfeel characters, such as wine structure and viscosity. Tannin was manipulated through the addition of either a maceration enzyme or a grape-derived oenotannin, while mannoprotein content was modified through adding a yeast-derived mannoprotein product (MP). Shiraz grapes were harvested at 24 and 28 ºBrix from the same commercial vineyard and the former vinified with commercial additives, introduced either individually or in combination.

All three products were introduced in wine at an early stage of vinification to achieve better integration.

As expected, wines made from riper grapes were naturally higher in tannin and mannoprotein than wines made from grapes harvested earlier. Maceration enzyme had a marked effect on the breaking down of grape cell walls which led to a significantly higher concentration and average molecular mass of wine tannin; i.e. levels were comparable with those of wines made from mature grapes. The enzyme treated wines were rated highest for astringency and palate coarseness, as expected based on the chemical composition. On the other hand, MP addition achieved the lowest tannin concentration and was rated lowest for palate coarseness. However, the increase in MP concentrations in the treated wines was considerably lower than expected. Analyses on the MP product revealed that it only contained 10% mannan, but contained around 25% arabinogalactan (AG). Oenotannin addition did not influence wine tannin composition, colour parameters or mouthfeel properties. However, it increased red fruit and confectionary aromas. When enzyme or oenotannin were applied in combination with MP, the effects were less apparent. The enzyme + MP treatment was similar to when the enzyme was used alone, whereas the tannin + MP treatment had a significant impact on aroma and flavor, but not on mouthfeel, compared to when the tannin was used alone. Principal component analysis revealed that later harvest wines were separated from earlier harvest wines based on more intense aroma and flavor, sweetness, palate fullness and hotness. Furthermore, out of all supplement regimes, tannin + MP most closely resembled the wines made from mature grapes.

This study confirmed the hypothesis that altering tannin concentration, composition and size could affect the perception of astringent mouthfeel. However, although the parameters of wine tannin measured in this study were similar between the enzyme treatment and the later harvest Shiraz, the former was perceived to be astringent while the latter was not. This observation indicated that mouthfeel is likely to be affected by other wine sensory components, such as being reduced by the intensity of fruit characters and/or sweetness. Modifying one factor alone may result in mouthfeel becoming unbalanced with other wine components. Furthermore, the unexpected composition of MP product indicated that there may be a large compositional variation amongst commercial products even within the same types of supplement. Thus the results reported above might be only applied to the three products used in the current study. Also, the current study demonstrated a great loss of added oenotannin, and inconsistent recovery of mannoprotein. This may be due to both the composition of the particular products used and the processes of precipitation and subsequent racking during vinification. Lastly, the vintage conditions of 2015 were warmer than expected, and so the wines made from the two harvests

contained more ethanol than intended. This did not meet the initial aim of this study which was to use wines containing 2% - 3% lower alcohol levels than the average red wine in Australia (14.5%). Thus, future studies (chapter 3 and 4) were designed to (i) use lower alcohol wines, (ii) evaluate a broader selection of winemaking supplements and (iii) investigate the sensory impact of additives in finished wines.

6.1.2 Compositional Variability in Commercial Tannin and Mannoprotein Products

Two of the products used in the previous study were selected for further examination, i.e. grape-derived oenotannin and mannoprotein (MP). The maceration enzyme was not involved in the subsequent studies because it alters both tannin and polysaccharide compositions and could not be applied to a finished wine.

14 grape-based oenotannin products and 8 MP products marketed in Australia were sourced from 6 manufacturers. Their composition and molecular size distribution were determined. In oenotannins, methylcellulose precipitable tannin (MCPT) was measured and was calculated as a percentage of the dry weight to represent the product purity. The MCPT values among products were highly variable, and the contents of major monomeric phenolic compounds were found to be relatively low across all products. Principal component analysis based on tannin composition and size revealed that some products exhibited chemical compositions that strongly agreed with the labelled origin of material (i.e. seed and skin), while others did not. Furthermore, for certain manufacturers, although products were marketed under different names for different oenological purposes, their compositions were actually quite similar, while other manufacturers' products under different labels showed significant compositional differences.

The monosaccharide and protein analyses accounted for 60% to nearly 100% dry weight of MP products. The composition of the polysaccharide fraction of products were also highly variable. All products contained different amounts of mannose and glucose residues. However, some products also contained a considerable amount of arabinose and galactose residues, which indicated presence of arabinogalactans, a polysaccharide not derived from yeast. The protein content of products ranged between 10% and 50%. This is likely to have significant impact on the products' effect on wine, as yeast derived proteins have higher interactions with wine polyphenolics than the polysaccharide fraction. Furthermore, molecular distribution of the products spanned 5 to 800 kD, with products containing arabinogalactan leaning towards higher size averages than products contained only MP.

The impact on mouthfeel of tannin and MP has previously been attributed to concentration, composition (subunit composition for tannin and protein content for MP) and molecular size. It is

therefore reasonable to assume that the choice of products will affect the potential impact on the treated wines. It is therefore crucial for researchers to report product characterisation involved in the study and for winemakers to conduct bench trials using the wines to be treated with different products, in order to make informed decisions regarding the use of supplements.

This study also served as the basis for product selection for the next two studies.

6.1.3 Impact of Selected Oenotannin and Mannoprotein products on the Sensory Properties of Shiraz Wines Made from Fruit Harvested at Two Distinctive Maturity.

This trial addressed some of the shortcomings of the study reported in 6.1.1. Firstly, two oenotannins were selected from screening, based on their compositional characters typical of grape skin and seed derived tannin; one mannoprotein was chosen based on its high purity and compositional similarity to mannoprotein isolated from wine. Secondly, the Shiraz wines used in this trial were of 11.5% and 14.5% ethanol concentration. They were made from two harvests of grapes, H1 representing sub-optimal maturity and H2 representing typical maturity, respectively. Lastly, all additives were introduced into finished wines, without any product loss due to racking processes. The primary aim of this study was the same as 6.1.1, i.e. to explore the impact on mouthfeel characters when both the tannin and mannoprotein compositions of wine are manipulated with commercial additives. However, in contrast to 6.1.1, the same supplementation regimes were applied to wines made from fruit from both harvests. Thus a secondary aim was to investigate the impact of ethanol level on wine mouthfeel, in conjunction with macromolecule composition.

The oenotannins and MP were introduced to wine in different combinations and concentrations. The supplementation regimes created a series of wines with tannin concentrations from 326 to 1067 mg/L, and mannoprotein concentrations of 68 to 452 mg/L. DA revealed judges could perceive that H2 wines had more ‘sweetness’, ‘body’, ‘hotness’ and ‘flavour intensity’ than H1 wines. However, no significant differences were found for astringency across treatments. The sensory panel could not distinguish astringency levels when the differences in tannin concentration were 300 or 600 mg/L between samples, but they could differentiate between samples with 1000 mg/L or more difference in tannin concentration. It was unclear if the difference in tannin levels between treatments was too subtle to be examined by DA or the judges were not sufficiently sensitive and in need of more training. The judges were also unable to perceive an increase in body in wines with higher MP concentrations, even at 6 g/L. It is likely that the increased ‘body’ perception between H1 and H2 wines was due to complex interactions

of flavour, viscosity and other compounding factors. However, increasing mannoprotein concentrations alone could not achieve similar effects.

6.1.4 Applying Nanoparticle Tracking Analysis to Characterise Interactions between Tannin and Polysaccharide in Wine-like Media

This study aimed to explore the interactions between commercial polysaccharide additives and grape derived tannin on the molecular level. Polysaccharides have long been suggested to interact with grape seed tannins, limiting or promoting their size evolution. This theory was tested in the current study using a new technology, nanoparticle tracking analysis (NTA). NTA tracks the movement of individual particles under Brownian motion in a sample and derives number-based particle size distribution. This approach is particularly advantageous in studying samples that are polydisperse in size, since the obscuring effect of large particles on the smaller ones is reduced. NTA has never been applied in studying tannin and polysaccharide interactions before.

One MP and one AG were purified from commercial MP and gum Arabic products respectively, representing the only two types of polysaccharides permitted as additives in wine. Purified tannin was extracted from Cabernet Sauvignon grapes (ST). The two materials were mixed at 2.5 : 1 and 10 : 1 (tannin : polysaccharide, gravimetric concentrations), in model wine solutions of 12% and 15% v/v alcohol, consistent with the real wine samples from 6.1.3. Their size distribution were determined after 24 hrs by NTA.

The two polysaccharides behaved drastically differently towards seed tannin. MP and ST formed highly light scattering aggregates that were larger than particles present in either material. Furthermore, significantly larger particles were formed in 12% ethanol model wine than in 15% model wine, at both ST to MP ratios. In particular, at 10 : 1 in 12 % ethanol model wine, MP and ST formed large aggregates between 250 and 400 nm, exceeding the particle size range observed in all other samples. In comparison, the interactions between AG and ST were very weak, irrespective of the tannin to polysaccharide ratio or ethanol content. Aggregates formed between AG and ST had low light scattering intensity and were comparable in mean size than AG alone. These observations were supported by dynamic light scattering (DLS) and UV-visible analyses of the independently prepared samples that had an identical composition to those used in NTA analyses. Both DLS and UV-visible techniques have previously been successfully applied to study interactions between polysaccharide and tannin. These different behaviour between MP and AG towards tannins were tentatively attributed to the higher protein content of MP. The NTA technique was proven to be suitable for studying interactions between macromolecules in model wine solutions. The ability of NTA to detect two distinctive yet similar sizes co-exist in a mixture

was also demonstrated in this study, which is highly relevant to the characterisation of wine macromolecules and their interactions.

This study implied that certain polysaccharides can aggregate with tannin particles and thus may have an effect on wine molecular assembly and colloidal stability. Furthermore, a 3% ethanol difference can significantly influence aggregation in some instances.

6.2 Future Directions

6.2.1 Sensory Perspective

The impact on wine chemical composition and sensory properties that can be derived from the diverse composition of MP products was not fully explored in the current study. The MP used in chapter 2 contained 23% protein. It is therefore possible that the slight reduction in tannin concentration observed was due to the fining effect by its relatively high protein concentration. This could be confirmed by supplementing red wine with a MP product of low protein content, such as MP8, and comparing with supplementation with the same MP product substituted with yeast invertase to different proportions of product weight. Alternatively, two MP products of low and high protein content, such as MP 3 and MP 8 could be compared. However, in this case the molecular mass distribution of the added MP would be hard to standardise. It would also be interesting to choose a product that contains AG and compare it with a pure MP product, provided that the particle size distribution and protein content between the products were similar.

In chapter 4, no effect on wine body or astringency was observed with MP addition. For a DA panel to characterise wine mouthfeel, the judges would have to be screened specifically for the attributes of interest, i.e. previous experience in wine DA in other contexts may not provide sufficient qualification. Furthermore, the different aspects of wine ‘body’ have to be disentangled. Future MP supplementation could be performed on wines supplemented with MP alone, at different concentrations. Viscometer could then be used to determine if MP can induce changes in physical viscosity. Again, MP of different protein content and size distribution could be compared.

6.2.2 Colloid Perspective

NTA was demonstrated to be a suitable technique for characterisation of wine macromolecules. Future efforts should be devoted to development of protocols for NTA measurement of wine. In particular, particle concentration determination should be further explored for studying macromolecule aggregation.

The MP involved in the study reported in Chapter 5 could interact with seed tannin. Future experiments should therefore explore if MP can interact with other types of tannin, such as wine isolated tannin. If so, protein could be introduced into the system and NTA could be applied for studying polysaccharide-protein, tannin-protein and polysaccharide-tannin interactions, and eventually, interactions of a 3-component system.

The aggregation mechanism between tannin and polysaccharide was not explored in the current study. Isothermal Titration Calorimetry (ITC), which has previously been applied to polysaccharide and tannin research, could be used in the future in conjunction with NTA, to provide information on the affinity, stoichiometry, and reaction enthalpy and entropy, which can be used to deduce the main mechanisms behind aggregation, such as hydrogen-bonding and/or hydrophobic reactions.

Effort should be devoted to elucidating the connections between colloidal properties of macromolecules in wine and the associated mouthfeel sensations. Aside from traditional chemical analyses and sensory studies based on human perceptions, some novel instrumental measurements should be considered. For example, friction on the surfaces of the oral cavity induced by wine tannin may be measured using tribology techniques.

6.3 Summary

This project provided insight into the changes in mouthfeel characters that can be induced by modifying wine tannin and mannoprotein composition, especially in the context of improving the mouthfeel properties of wine made from early harvest grapes. Increasing tannin molecular mass as well as concentration, such as seen in the application of maceration enzymes, produced a wine that was more coarse on the palate. Conversely, the application of oenotannin to finished wine increasing tannin concentration without modifying tannin composition and size, did not result in any change in astringency perception. Similarly, addition of a protein-rich mannoprotein at 400 mg/L during the vinification process could achieve a softer mouthfeel, likely due to fining of wine tannin. However, supplementing a finished red wine with a polysaccharide-rich mannoprotein did not modify either astringency or wine body, even at 1000 g/L addition rate. It was also demonstrated that there is a considerable variation amongst commercial oenotannin and mannoprotein products, which are likely to achieve different effects on wine composition and, by extension, mouthfeel characters. This project also explored interactions between two commercially prepared polysaccharides and a grape seed tannin fraction. The two neutral polysaccharides involved in this study, mannoprotein and arabinogalactan, behaved drastically differently towards tannins, suggesting that modifying the composition of wine neutral

polysaccharide fraction could effectively modify the wine polyphenolic composition. In summary, this project furthered the current level of understanding of commercial wine supplements and showed that their selective and deliberate application can be used to modify the composition and sensory properties of red wine.

APPENDIX

Co-authored publications arising during my candidature:

1. Bindon, K. A., Li, S., Kassara, S., & Smith, P. A. (2016). Retention of Proanthocyanidin in Wine-like Solution Is Conferred by a Dynamic Interaction between Soluble and Insoluble Grape Cell Wall Components. Journal of Agricultural and Food Chemistry, 64(44), 8406–8419. https://doi.org/10.1021/acs.jafc.6b02900

2. Hranilovic, A., Li, S., Boss, P. K., Bindon, K. A., Ristic, R., Grbin, P. R., Van der Westhuizen, T., & Jiranek, V. (2017). Chemical and sensory profiling of Shiraz wines co-fermented with commercial non-Saccharomyces inocula. Australian Journal of Grape and Wine Research, 24(2), 166–180. https://doi.org/10.1111/ajgw.12320