The effect of grape stem inclusion fermentation on Pinot Noir wine composition

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Whole bunch fermentation is believed by some winemakers to increase complexity, freshness, aromatic expression and textural smoothness in the resultant wine, but may also impart green, herbal and earthy characters. The aim of this study is to investigate the effect of stem inclusion fermentation, including the addition of stems and whole bunches, on the composition of Pinot Noir wines. Standard winemaking protocol was used to prepare five treatments in triplicate: 100% of destemmed and crushed grapes (DS), 100% of destemmed and crushed grapes with 100% of stems added back (DS100), 30% of whole bunch (WB30), 60% of whole bunch (WB60), and 100% of whole bunch (WB100). The resultant wines were analysed for general oenological parameters, the colour parameters by modified Somers assay and CIELab, the phenolic composition by high-performance liquid chromatography (HPLC), and aroma profiling by gas chromatography-mass spectrometry (GC-MS). In comparison to the DS treatment, stem addition caused to increase pH and lower alcohol content in wines. In terms of colour, 100% stem addition (DS100) and high proportions of whole bunch addition (WB60 and WB100) could significantly increase the degree of ionisation of anthocyanin, hue, total phenolics, and SO_2 resistant pigments but decrease total anthocyanin in wines. Cold maceration without adding stems/whole bunches (DS) showed the highest concentration of total anthocyanin. According to the CIELab results, only DS100 had significantly higher luminosity (L*), yellow-blue component (b*), chroma (C*) and tone (H*) values compared to DS treatment. The 100% stem addition (DS100) and the high proportion of whole bunch addition (WB60 and WB100) significantly increased tannin, total phenolics and most of the monomeric phenolics in the resultant wines. Interestingly, the WB100 treatment showed a significantly higher concentration of tannin and total phenolics than the DS100 treatment, indicating whole bunch fermentation is more effective in enhancing the extraction of phenolic compounds into wine. Principle component analysis (PCA) showed stem inclusion treatments significantly increased the concentrations of eugenol, 3-isobutyl-2-methoxypyrazine (IBMP), 3-Isopropyl-2-methoxypyrazine (IPMP) and phenol, which are mostly responsible for spicy, woody and green aromas in wine. Treatments with non-stem addition (DS) and low proportion of whole bunch

addition (WB30) showed higher concentrations of hexyl acetate, 2-methylbutyl acetate and isoamyl acetate, which are responsible for fruity aroma in the wine. This study improves our current understanding of stem inclusion fermentation, which can be used as a winemaking tool to manage the extraction of phenolics and colour in Pinot Noir wine. Green characters imparted from grape stems should be also taken into consideration when stem inclusion fermentation is used for Pinot Noir production.

Keywords: Anthocyanin, Methoxypyrazine, Phenolics, Pinot Noir, Stem, Tannin, Whole bunch.

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"**Wine is the most healthful and most hygienic of beverages**" - **Louis Pasteur**.

I'm truly passionate about wine science since my first degree. Therefore, pursuing a master's research degree from Lincoln University New Zealand is one of my ambitions to pave the path to higher education.

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Chapter 1

Introduction

The extraction of tannin and anthocyanin is essential for winemakers to produce good quality red wine. Pinot noir grapes can be challenging for the winemakers due to the tannin distribution (Carew, Sparrow, Curtin, Close, & Dambergs, 2014). It has low skin to seed tannin ratio compared to other grape varieties (Kennedy, 2008), and seed tannin is more difficult to extract than skin tannin (Dambergs et al., 2012; De Villiers, 1994; Kennedy, 2008; Waterhouse, 2002). Dimitrovska, Bocevska, Dimitrovski, and Murkovic (2011) reported that the anthocyanin concentration in Pinot noir grapes is almost 60% lower than other red grape varieties. Pinot Noir grapes have five types of anthocyanins (malvidin, peonidin, petunidin, delphinidin and cyanidin) in their mono-glucoside form, which is relatively unstable (F. He et al., 2012; Iland, 2013). Hence, Pinot Noir wines tend to have a much lighter colour and a lower concentration of tannin compared to other red wines (Dambergs et al., 2012; Kennedy, 2008). To overcome these issues in Pinot Noir winemaking, some winemakers tend to employ stems as a secondary source of tannin and cold maceration to enhance anthocyanin extraction.

Whole bunch fermentation has become increasingly fashionable in Pinot Noir wine production, particularly for styles with more elegance than power. Several studies have reported that fermentation with the inclusion of grape stem could have an effect on phenolics and colour in the finished wines (Casassa et al., 2019; Hashizume, Kida, & Samuta, 1998; Suriano, Alba, Tarricone, & Di Gennaro, 2015). Casassa et al. (2019) showed that 3% stem addition together with cold maceration (CS + S treatment) or stem addition without cold maceration (control + S) in Pinot Noir winemaking could increase tannin extraction by 60% compared to wine made without adding stems in 2015 vintage, but 20% whole bunch addition could not result in significant variation of the tannin content at the end of the fermentation of Pinot Noir wine (pressing). Suriano et al. (2015) reported that treatment prepared without adding stems had a higher anthocyanin concentration and lower phenolics content compared to whole bunches added treatments (25% and 50%). Hashizume et al. (1998) also reported that stem addition could increase total phenolics and levels of phosphoric acid (phosphorus), potassium, and calcium in the resultant wines. The extraction of potassium lowered TA and increased pH in wines. However, most of the studies were limited to use a lower amount of stems and whole bunches (20-50% whole bunches, or <3% stems) to avoid green aromas and tannic astringency in the finished wines (Hashizume & Samuta, 1997; Sala, Busto, Guasch, & Zamora, 2004). Ultimately, stem inclusion generally increased phenolics, and pH and decreased anthocyanin, titratable acidity in the resultant wines.

Cold maceration facilitates extraction of water-soluble compounds, especially anthocyanin, before starting fermentation in wine (Aleixandre-Tudo & Du Toit, 2018). In cold maceration, breaking cell walls/ membranes and increase cell membrane permeability due to adding sulphur dioxide facilitate internal compounds to leach out during the pre-maceration period (Aleixandre-Tudo & Du Toit, 2018; Sacchi, Bisson, & Adams, 2005). Casassa, Bolcato, Sari, Fanzone, and Jofré (2016) also showed that 100 ppm sulphur dioxide addition could result in higher anthocyanin extraction compared to adding 50 ppm sulphur dioxide in winemaking. Many previous studies reported that cold maceration increases anthocyanin, polymerised anthocyanin, phenolics in wines (Álvarez, Aleixandre, García, & Lizama, 2006; Koyama, Goto-Yamamoto, & Hashizume, 2007).

Several studies have reported that cold maceration and stem inclusion could affect aroma composition of the resultant wines (Álvarez et al., 2006; Cai et al., 2014; Casassa et al., 2019; Hashizume & Samuta, 1997). The stem inclusion could result in more green aromas in wines (Hashizume & Samuta, 1997; Sala et al., 2004). Hashizume and Samuta (1997) showed that these green aromas were mainly due to methoxypyrazines extracted from the stems during fermentation. Cai et al. (2014) reported that cold maceration in pumping over tanks could result in increased some acetate esters, and β-Damascenone, which are mostly contributed to fruity aromas, as well as cold maceration, and lead to a decrease in some higher alcohols specially isobutanol and isopentanol in Cabernet Sauvignon wine.

As discussed above, both stem inclusion and cold maceration can influence wine colour, phenolics and aroma profile of the wine. It is rare to find research articles discussing the combined effect of cold maceration and stem inclusion on wine colour, phenolics and aroma profile in the resultant wines. Some wine manufacturers may use both cold maceration and stem inclusion to produce good quality Pinot Noir wine. Effect of adding higher proportions of stems needs to be further investigated. Thus, to better understand the impact of stem inclusion fermentation together with cold maceration on Pinot Noir wine composition, five treatments have been carried out in this study: 1) treatment prepared with 100% of destemmed and crushed grapes (DS) as control; 2) treatments prepared with whole bunch addition at three levels: 30% (WB30), 60% (WB60) and 100% (WB100); and 3) treatment prepared with 100% of stems added back to destemmed and crushed grapes (DS100). The WB treatments are studied to understand the impact of adding different proportions of whole bunches on Pinot Noir wine composition, and the DS100 treatment was conducted to compare with the WB100 treatment to understand how different forms of stem inclusion could impact the resultant wine composition

Chapter 2

Literature Review

2.1 Phenolic compounds in red wine

Phenolic compounds are important to the wine quality by contributing to colour, taste, and mouthfeel of the wine. They also have strong antioxidant properties which ensure the quality of red wine, which are enhanced during production and maturation through preservation (Dambergs et al., 2012; Kennedy, 2008). Anthocyanins, tannin and tannin-anthocyanin polymers (Pigmented polymers or pigmented tannins) collectively determine the taste and appearance of red wine. The young red wine colour is mainly due to the presence of anthocyanins in their positively charged flavylium state (F. He et al., 2012). Pigmented polymers are important for the long-term stability of red wine colour (Dambergs et al., 2012; Kennedy, 2008). Phenolic substances especially tannin in red wines, contribute to the bitterness and astringency through interactions with salivary proteins and other tactile sensations, which is formally defined as the structure or body of the wine (Waterhouse, Sacks, & Jeffery, 2016). Hence, both visual and flavour perception of red wine depends to a large extent on phenolic compounds extracted from the grape into juice and stabilised in the wine matrix.

The total phenolic content of red wines varies widely, even though generally present around 2000 mg/L of gallic acid equivalents in reds those are ready to drink, and it can rise up to 3500 mg/L if red wine is aged for a long time due to oak derived tannin (Waterhouse et al., 2016). Van Leeuw, Kevers, Pincemail, Defraigne, and Dommes (2014) analysed ten Pinot Noir wines produced between 2006 to 2010 vintages from different countries, total phenolics (gallic acid equivalent) were below 2400 mg/L in most of the wines analysed in the study. Pinot Noir wine contains a relatively low concentration of total phenolics compared to other red wines (Dambergs et al., 2012) and it is mainly due to the lower skin to seed tannin ratio of the grape as previously mentioned.

The majority of phenolic compounds in wine are grape-derived, and some can originate from the oak. Singleton and Esau (1969) reported that the total phenolics distribution in a red grape berry was estimated: 1% in the pulp, 5% in juice, 50% in skin and 44% in the seed. As previously mentioned, Pinot Noir grapes have a higher proportion of tannin in seeds compared to their skin. So, this composition may slightly vary in Pinot Noir grapes. Harbertson, Kennedy, and Adams (2002) also reported that Pinot Noir contained a higher concentration of tannin in seeds compared to skin. There are different classes of phenolic compounds present in different berry components. Grape skin contains mainly, anthocyanins, cinnamic acids and their esters, polymeric tannins, and monomeric flavanols; juice contains cinnamic acids and their esters; seed contains polymeric tannins and monomeric flavanols

(Kennedy, 2008). The main difference of the polymeric tannins found in seed and skin is the size of the molecule. Seed tannins are shorter, with a lower mean degree of polymerisation compared to skin tannin (Bordiga, Travaglia, Locatelli, Coïsson, & Arlorio, 2011; Chira, Schmauch, Saucier, Fabre, & Teissedre, 2009).

Tannins in the stems have similar reactivity to tannins in seeds. The stems and seeds contain high concentrations of polymerised procyanidins and condensed tannin which produce a more marked tannic astringency (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). There are no published studies about the composition of Pinot Noir stems. But there are some studies based on the stems from other red varieties. Souquet, Labarbe, Le Guernevé, Cheynier, and Moutounet (2000) reported that Merlot grape stems contained significant amount of polyphenolic compounds including phenolic acids, flavonols, and flavanonols (astilbin). Those stems contained 200 mg/kg of quercetin 3-glucuronide, 60 mg/kg catechin, 40 mg/kg caftaric acid, 35 mg/kg of astilbin, 18 mg/kg of quercetin 3-glucoside and 4.5 mg/kg of coutaric acid. Epicatechin, engeletin, myricetin 3-glucoside and myricetin 3-glucuronide were present in trace amounts.

Most phenolic compounds are non-volatile, but a few volatile/ odorous phenolics exist, for example, 4-ethylphenol (Waterhouse, 2002). The simplest phenolics found in wine are composed of a single aromatic ring with one or more hydroxyl groups such as guaiacol and caffeic acid. While the majority found in wine are polyphenolics, they are made with multiple phenol rings within a single structure such as resveratrol and epicatechin. Phenolics concentration in red grapes are higher than white grapes due to the presence of anthocyanin in red grape skin. Phenolic compounds found in wine and grapes are complex in nature, but it can be classified into two classes: flavonoids based on a common C6-C3-C6 skeleton and non-flavonoid compounds (Cheynier et al., 2006; Rentzsch, Wilkens, & Winterhalter, 2009; Waterhouse, 2002; Waterhouse et al., 2016). Non-flavonoids are not always derived from grapes and they have slightly more variable structures. Hydroxycinnamates, hydroxybenzoic acids, stilbenes and volatile phenolics are the most well-known classes of non-flavonoids derived in grapes (Rentzsch et al., 2009). There are two main groups of flavonoid phenolics: anthocyanin and flavanols. There are some other classes of flavonoid present in grapes in lower concentrations such as flavonols. Flavanols can exist as catechin monomers, oligomers and polymers, which also called as condensed tannins, or proanthocyanidins (Cheynier et al., 2006).

2.1.1 Non-flavonoid

The nonflavonoid compounds act as co-pigments (hydroxycinnamic and hydroxybenzoic acids) and antioxidants (stilbenes) in wines. But most of the nonflavonoids including hydroxycinnamate, caftaric and coutaric esters do not appear to have a direct impact on the perception of bitterness or astringency, at the concentration levels found in wines (Vérette, Noble, & Somers, 1988; Waterhouse,

2002). Non-flavonoid constituents in wine can be broadly divided into hydroxybenzoic acid (HBA) and hydroxycinnamic acid (HCA), volatile phenolics, stilbenes and miscellaneous compounds including such as lignans and coumarins (Rentzsch et al., 2009).

Hydroxycinnamic acids (HCA) are the major phenols in grape juice and the major class of phenolics in white wine (Waterhouse, 2002). HCAs possess a C6-C3 skeleton and formally belong to the group of phenylpropanoids. There are three commonly found hydroxycinnamic acids namely coumaric, caffeic, and ferulic acids but none of these simple hydroxycinnamic acids is found in the grape pulp in free form because they are conjugated with tartaric acid to form tartaric acid esters namely p-coutaric acid, caftaric acid, and fertaric acid respectively (Rentzsch et al., 2009; Waterhouse et al., 2016). These HCA derivatives can be present in either *cis*- or *trans*- form in wine. More stable *trans*- form is predominantly found in plats and *cis*- form is induced by light. Up to 50% of total HCA is composed of caftaric acid. Grape derived tartrate esters can still be hydrolysed by HCA hydrolase enzyme produced by lactic acid bacteria and other organisms to produce simple HCA (coumaric, caffeic, and ferulic acids) in wine. So, they can be detected in newly fermented wines (Rentzsch, Schwarz, Winterhalter, & Hermosín-Gutiérrez, 2007). HCA concentration in different grape varieties and/or within the same variety are greatly varied in previous publications. Rentzsch et al. (2009) reported that HCA concentration depends on several factors such as grape variety, growing conditions, climate, etc. So, it is the reason to experience more variable concentrations of HCA in wines. Table 2.1 summarizes the most common structures of HCAs and reported concentrations in previous studies.

Hydroxybenzoic acids (HBA) are a minor category of monomeric phenolics derived from benzoic acid in young wines. They are characterized by C6-C1 skeleton. Gallic acid, syringic acid, protocatechuic acid, p-hydroxybenzoic acid, gentisic acid, salicylic acid, and vanillic acid are the most common types of HBA found in wine (Rentzsch et al., 2009; Waterhouse, 2002). HBA is mainly found in their free form in the wine. Gallic acid accounts for the majority of this group. Gallic acid is not found in grapes, but It is formed by the hydrolysis of gallate esters in hydrolysable tannin and condensed tannin in the wine. On long ageing, gallic acid is persistent and can be observed in older wines (Waterhouse et al., 2016). HBA concentration in wine largely depends on the grape variety and growing condition (Rentzsch et al., 2009). Table 2.2 summarizes the most common structures of HBAs and reported concentrations in previous studies.

Stilbenes are also a minor category of phenols found in grapes and wine. The resveratrol is the principle stilbene found in grapes (Waterhouse, 2002). Resveratrol is produced by the grapevine in all tissues as a phytoalexin in response to various stress factors such as UV-radiation, mechanical injuries, and, specifically in vine grapes to Botrytis infection and other fungal attacks (Aaviksaar, Haga, Pussa, Roasto, & Tsoupras, 2003; Waterhouse, 2002). Resveratrol synthesis mainly happened in the skin. So, its

derivatives such as *cis* and *trans* isomers and glucosides of both isomers are found in the skin (Jeandet, Bessis, & Gaugheron, 1991; Waterhouse, 2002). However, cluster stems (rachis) is the richest source of resveratrol in Pinot Noir according to the research conducted by Melzoch, Hanzlíková, Filip, Buckiová, and Šmidrkal (2001). According to his study, resveratrol concentration in different parts of Pinot Noir grapes contained; 2.80 mg/kg in leaves, 13 mg/kg in rachis, and 2.34 mg/kg in berry respectively. The average resveratrol concentration in red wine is about 7mg/L. But, many authors reported that a higher concentration of resveratrol could be found in Pinot noir wines compared to other red wines (Baraboy, 2009; Melzoch et al., 2001).

2.1.2 Flavonoid phenolics

As previously mentioned, a specific three aromatic ring system (C6-C3-C6 skeleton) defines flavonoids structure, the central oxygen-containing pyran ring with the ability to hold different oxidation states is called as C-ring (figure 2.1). It is bonded to an aromatic ring (A Ring) along with one bond and attached to another aromatic ring with a single bond (B ring). The flavonoids found in grapes and wine all have the same hydroxyl substitution groups on ring A, at positions 5 and 7. Differences in the oxidation state and substitution on C ring defines the different classes of flavonoids (Waterhouse, 2002). These compounds are the most important group of compounds in terms of sensory appeal in red wines. However, it can be challenging to produce Pinot Noir due to the unique flavonoid distribution and concentration in grapes (Kennedy, 2008). As previously mentioned, there are mainly two groups, which directly influence wine taste and appearance: Flavanols and anthocyanin. Anthocyanin are the red grape pigments essential in red wine colour.

Flavan-3-ols in red grapes can exist as monomers, oligomers and polymers of catechin and its derivatives such as epicatechin, gallocatechin, epigallocatechin, epicatechin 3-gallate, epigallocatechin 3-gallate (Cheynier et al., 2006). Polymers of flavanols are collectively called as condensed tannin or proanthocyanidin and are available in grape skin, seed and stalks. Oligomers and polymers of flavan-3-ols in a typical young red wine account 25 – 50% of its total phenolics and an even higher proportion in older wines (Waterhouse et al., 2016). Catechin, epigallocatechin (tri-hydroxylated form) are more prevalent in grape skin, and epicatechin 3-gallate (di-hydroxylated form) more prevalent in the seed. (Hayasaka, Birse, Eglinton, & Herderich, 2007). The formation of condensed tannins by the polymerisation of flavanol monomers has been associated with the moderation of the sensory impact of these compounds in wine (McRae, Falconer, & Kennedy, 2010). The condensation occurs to form covalent bonds between flavan-3-ol units, the most common linkages being 4→8 and 4→6 positions (Waterhouse, 2002). Condensation can also happen via bridging by co-factors (acetaldehyde, pyruvate). The monomeric catechins are bitter and astringent compared to polymeric phenolics. In the polymer, the bitterness is minimal, but the astringency remains unchanged (Robichaud & Noble, 1990).

Flavonol is considered as a minor class of flavonoid phenolics in wine. They are found in the berry skin and there are apparently no flavonols in the pulp or seeds. The concentration of flavonols in wine can be affected by processing variables, with factors that increase skin extraction resulting in higher levels (Price, Breen, Valladao, & Watson, 1995). The concentration of flavonols is increased by high sunlight exposure. It appears to function as sunscreen. Price et al. (1995) showed that sunlight exposure of Pinot Noir grape bunches enhances flavonol levels in grape skin.

2.2 Pinot Noir wine colour

The red wine colour is mainly determined by phenolics comprising mainly anthocyanin, tannin and tannin-anthocyanin polymers (Smith, Mercurio, Dambergs, Francis, & Herderich, 2007). Generally, anthocyanin refers to the glycosylated form of anthocyanidins/aglycons. Anthocyanidins are not stable in nature, so they are not found in either grapes or wine. Hence, they are glycosylated to form stable anthocyanin pigments in wine (Waterhouse, 2002; Waterhouse et al., 2016). The glycosylation increases both the chemical stability and the hydrophilicity of anthocyanidins (Durner, 2016; Waterhouse et al., 2016). The young red wine colour is mainly due to the presence of anthocyanins in their positively charged flavylium state. But, only a small percentage of total anthocyanin is present in a coloured form in the original wine conditions. Within the typical wine pH range, only 10–25% of the free anthocyanins occur in the form of the red flavylium cation (Durner, 2016). Anthocyanins are found in the red grape skin, but there are some grape varieties such as “teinturier” grapes, which have anthocyanins in both skin and the pulp (F. He et al., 2012). There are five major types of anthocyanidins present in red grapes: malvidin, peonidin, petunidin, delphinidin and cyanidin. Each of these types of anthocyanidins can exist in three forms: the mono glucoside, the acetyl glucoside and coumaroyl glucoside. The latter two are being referred to as acylated forms because their glucose is further linked through an -o- bond to either acetic acid or coumaric acid. Often the three types of malvidin contribute to the major proportion of the total pool of anthocyanins (Iland, 2013).

The anthocyanin profile of Pinot noir is unique compared to other red grape varieties because it lacks a more stable acylated form of anthocyanins and has a relatively higher proportion of malvidin-3-glucoside (Dimitrovska et al., 2011). Casassa et al. (2019) reported that 78% of total anthocyanin in Pinot Noir wine was composed of malvidin-3-O-glucoside in 2014 vintage. Pinot Noir wine has only five types of anthocyanins in mono glucosides forms: malvidin, peonidin, petunidin, delphinidin and cyanidin (Iland, 2013). Table 2.3 summarises the structure and general concentration ranges of those anthocyanins found in Pinot Noir wine. The concentration ranges in Pinot Noir wine were adopted from the works done by Mazza, Fukumoto, Delaquis, Girard, and Ewert (1999) and Cortell, Halbleib, Gallagher, Righetti, and Kennedy (2007). Mazza et al. (1999) analysed monomeric anthocyanin concentration in Pinot Noir wines from British Columbia using HPLC in 1996 and 1997 vintages

respectively. The winemaking protocol involved a 6-day cold maceration at 4°C. Cortell et al. (2007) also used HPLC technique to analyse monomeric phenol composition in resultant wines from grapes grown in different vine vigour zones in 2003 and 2004 vintages. Winemaking protocol was involved 2.5-day cold maceration at 10°C and malolactic fermentation.

The pH, sulphur dioxide concentration and different interactions of anthocyanin such as Self-association of anthocyanins and co-pigmentation of anthocyanin in wine can influence the colour expression in red wines (F. He et al., 2012; J. He & Giusti, 2010). Anthocyanin structures reversibly undergo a pH-dependent transformation in aqueous solution (Figure 2.2), so they are unique among flavonoids. Four major anthocyanin forms are present in the equilibria: the red flavylium cation, the blue quinonoidal base, the colourless carbinol pseudobase, and the pale yellow chalcone. Low pH can increase the proportion of the flavylium state and retard the hydrolysis of the anthocyanins. As the pH rises, the concentration of the anthocyanins in the flavylium state and the colour density decline rapidly. For example, at the pH of 3.4-3.6, 20-25% anthocyanin is in red flavylium cation form, and at pH 4.0, only 10% of anthocyanins are present at in ionised state (F. He et al., 2012; Jackson, 2008).

An increased amount of free SO_2 will cause "bleaching" of the anthocyanins and decrease the red colour of wine (C. T. Somers & Evans, 1974). SO_2 is bleaching monomeric anthocyanin by nucleophilic addition at position 4 of C ring (Figure 2.2). The red colour of the molecule fades away due to the SP3 hybridisation of the carbon atom, causing interruption of the conjugated double bond system (bisulphite addition). However, this C—S bond is relatively weak. Hence, flavylium cation still can be rearranged if antagonist, such as acetaldehyde, are present in wine (Durner, 2016).

Monomeric anthocyanin molecules in young red wines undergo a wide variety of chemical reactions, especially during the first two years of maturation. These reactions involve the formation of various anthocyanin derived pigments, which is crucial in long term colour stability of red wines (F. He et al., 2012). Even though monomeric anthocyanin concentration of young wines declines rapidly during the first 1-2 years of maceration, the wine still can preserve its red colour. It is due to the reactions and associations involve complex mechanisms, including relatively short-term ones, such as self-association and co-pigmentation, and the relatively long-term ones, such as the formation of polymeric anthocyanins with flavan-3-ols and proanthocyanidins, as well as the formation of new pigments, such as pyranoanthocyanins and their further polymerised products. Anthocyanins in young red wine primarily exist as weak complexes with themselves (self-association) and/or with other compounds (co-factors) to form co-pigments (González-Manzano, Santos-Buelga, Dueñas, Rivas-Gonzalo, & Escribano-Bailón, 2008; F. He et al., 2012)

The co-pigmentation of anthocyanin is an important process in colour development in wines. Variable proportions of colour in red wines are mainly due to the co-pigmentation of anthocyanin with other

molecules in wine (F. He et al., 2012). Co-pigmentation involves forming non-covalent bonds/interactions between the molecules corresponding to the coloured structures (flavylium and quinonoidal base) and other phenolic compounds in a solution. It is also called as intramolecular co-pigmentation (González-Manzano et al., 2008; Waterhouse et al., 2016). It only happens in aqueous solutions, as it is driven by the hydrophobic interactions of anthocyanins (Brouillard, Wigand, & Cheminat, 1990; Goto & Kondo, 1991). Flavonoids including catechin, epicatechin, epigallocatechin, quercetin, rutin, etc., and non-flavonoids, including caffeic acid, p-coumaric acid, ferulic acid, gallic acid, etc. can act as co-factors in this process. As well as arginine and proline like amino acids also can act as co-factors in the reaction (F. He et al., 2012; Waterhouse et al., 2016). Normally, flavan-3-ols, such as (+)-catechin or (-)-epicatechin are recognized as powerful cofactors, which can form coloured complexes most easily and intensely (F. He et al., 2012). Co-pigmentation helps in stabilisation of the pigmented, aromatic forms of anthocyanins as compared to the colourless carbinol pseudo base (Waterhouse et al., 2016). Therefore, co-pigmentation can cause in greater colour intensity of anthocyanin solutions than theoretically could be estimated from the anthocyanin concentration and pH of the media (F. He et al., 2012). These complexes formed during co-pigmentation increase the proportion of red flavylium state of anthocyanin as compared to a solution of anthocyanins alone, in the same manner as decreasing the pH (Figure 2.3). Phloroglucinol is used as the co-factor in the reaction indicated in Figure 2.3 for the simplicity of the reaction even though it is not present in wine.

Self-association phenomena of anthocyanin were first suggested by Asen, Stewart, and Norris (1972) and are demonstrated as a positive deviation from Beer's law, which occurs on increasing the concentration of the anthocyanin (González-Manzano et al., 2008). Self-association is also a special form of the co-pigmentation process as discussed above. However, co-pigmentation can result in greater colour intensity of anthocyanin solutions than theoretically could be expected from the anthocyanin concentration and media pH effects (F. He et al., 2012; Timberlake, 1980). It has been reported previously that the presence of the methoxy group in the B-ring and of the glucose moiety at the C5 or C3 position of anthocyanins influences the self-association and colour appearance of anthocyanins. The hydrophilic interactions between the glucose components (glucose moiety at the C5 or C3 position) of the corresponding anthocyanin molecules and the hydrophobic repulsion that take place between their aromatic nuclei and water, the vertical stacking of anthocyanin molecules in self-association complexes are promoted (Cavalcanti, Santos, & Meireles, 2011; González-Manzano et al., 2008; F. He et al., 2012). This stacking system forms a vertical helical structure composing hydrophobic core surrounded by hydrophilic glucose moieties (Hoshino, 1992). This molecular structure protects anthocyanidins in the core from hydration, which result in ionisation to form colourless pseudo base (Figure 2.4).

2.3 Aroma profile of Pinot Noir wine

Wines contain hundreds of volatile compounds; most of them are not important as they fall well below their respective sensory perception thresholds in wine. Ferreira (2010) classified the wine aroma compounds into six groups according to their role on final wine aroma: genuine impact compounds, major contributor, net contributors, secondary or subtle contributors, aroma enhancers, aroma depressors. Genuine impact compounds in wine present in higher concentrations than their respective sensory threshold levels (Ferreira, 2010). Wine aroma is caused by a complex mixture of volatile aroma compounds present in wine. These aroma compounds are small, nonpolar molecules that readily enter the gas phase from the polar wine matrix. They enter the nasal cavity while we smell or drink a glass of wine, leading to the perception of specific aromas in a wine (Ilc, Werck-Reichhart, & Navrot, 2016).

It is generally accepted that Pinot Noir wine aroma characters are varied with the regional effect of soil, climate, cultural practises, vinification procedures and storage (Girard, Yuksel, Cliff, Delaquis, & Reynolds, 2001). As well as Rita, Leonard, Barney, and McDaniel (1992) reported that the ripening stage of Pinot Noir grapes also influences the final aroma composition of wine. Wines made with Pinot Noir grapes at the end of the ripening period had more odour active peaks than wine from earlier harvested fruit. There are some studies conducted to clarify the effect of doing different maceration techniques on wine aroma. Cai et al. (2014) studied the effect of cold maceration on Cabernet Sauvignon wine aroma using commercial-scale punching down (PD) tanks and pumping over (PO) tanks. The author reported that PO tanks are more effective than PD tanks and when using PO tanks, resultant wine showed decreased higher alcohols and increased levels of some acetate esters in resultant wines. Casassa et al. (2019) examined the effects of cold maceration (CS treatment), whole bunch (WC) fermentation, and stem additions on the sensory and chemical composition of resultant Pinot noir wines. The author reported that control and 20% whole bunch added treatments (control + WC) wines were fruitier than cold macerated wines (CS and CS+S treatments) in 2014 vintage.

Wine aroma can be categorized into three groups based on their origin: primary aroma - grape-derived; secondary aroma – derived via fermentation; and tertiary aroma – derived in ageing (Robinson et al., 2014; Styger, Prior, & Bauer, 2011). However, oak ageing was not used in this study so tertiary aromas are not expected in resultant wines. Reported concentrations of different aroma compounds in Pinot Noir wines from previous studies were summarised in Table A.1 (See Appendix). Identified aromas in this work were categorised into 9 classes based on their chemical structure and biosynthetic origin: acetate esters, ethyl esters, volatile fatty acids, higher alcohols, methoxypyrazines, aldehydes, volatile phenols, norisoprenoids, and monoterpenes.

Varietal aroma compounds in wine reflect the grape variety and the climatic conditions of the grape grown region. Most of these compounds leach into wine due to rupturing the cell walls during

winemaking and harvesting practices. Terpenes, C13 norisoprenoid derivatives, sulphur compounds with thiol function, C6 aldehydes, C6 alcohols, aminobenzoate, phenylpropanoid esters and methoxypyrazines are the major groups of aroma compounds derived from grapes (Ferreira, 2010; Robinson et al., 2014).

2.3.1 Terpenes

There are two types of terpenes in the wine based on the number of isoprene molecules conjugated together to form them: monoterpene and sesquiterpenes. They are made from two and three isoprene units respectively. Monoterpenes in wine mainly contribute to floral and citrus aromas. Formation of monoterpenes in wine involves complex chemical reactions. Most of terpenes are leached into wine as odourless glycosidic precursors such as linalyl, geranyl. Then these odourless compounds are hydrolysed by acids and enzymes derived in yeast and/or grapes into terpenes in the wine. However, many studies reported that their concentration in Pinot Noir wine was below their perception threshold levels (Brander, Kepner, & Webb, 1980; Rutan, Herbst-Johnstone, Pineau, & Kilmartin, 2014; Schreier, Drawert, & Abraham, 1980) but there are still a few studies that have found that the concentration of terpenes was above the threshold level in Pinot Noir wine (Girard et al., 2001; Tomasino et al., 2015).

2.3.2 C13 Norisoprenoid derivatives

C13 norisoprenoids are derived by the cleavage of the carotenoid substrate between C9 and C10 position with carotenoid cleavage dioxygenases enzymes. The cleavage can happen at different positions in the carbon chain. Derived compounds with 13 carbon atoms are called C13 norisoprenoids (Robinson et al., 2014). Mendes-Pinto, Silva Ferreira, Caris-Veyrat, and Guedes de Pinho (2005) reported that β-carotene and lutein constitute 85% of the total carotenoid concentration in Port wine. The aroma of these compounds is responsible for fruity and floral aromas in wine. Chemical diversity of norisoprenoid compounds are mainly due to different non-enzymatic reactions, including photooxygenation, thermal degradation or acid hydrolysis (Ilc et al., 2016). Many studies reported that β-Damascenone had the highest concentration from all detected norisoprenoid in wine. Tomasino et al. (2015) observed the concentration of β-damascenone in 32 New Zealand Pinot Noir wines were range from not detected – 5 µg/L. The perception threshold level of this compound is calculated as 7 µg/L in red wine (Pineau, Barbe, Van Leeuwen, & Dubourdieu, 2007). So reported concentration in Pinot Noir wine was slightly below the perception threshold level of this compound.

2.3.3 C6 alcohols and aldehydes

C6 alcohols and C6 aldehydes in wines are mainly due to the addition of vegetative parts such as stems and whole bunches during the crushing/maceration period. These compounds produce green aromas,

herbaceous odours and fresh-cut grass aromas in wine. C6 alcohols can be formed by reducing the corresponding C6 aldehydes by alcohol dehydrogenase enzyme (Hashizume & Samuta, 1997; Styger et al., 2011). Many factors influence the concentrations of C6 alcohols/aldehydes in wine: the level of enzymatic activity, the degree and form of mechanical injury (crushing), presence of inhibitors, pH, temperature, condition of the leaf (young vs mature), stem ripeness, juice clarification and the amount of oxygen present at the time of crushing (Rutan, 2016). However, the concentration of these compounds in wine is very low compared to grapes due to consumption by yeast during fermentation (Mauricio, Moreno, Zea, Ortega, & Medina, 1997). Casassa et al. (2019) reported that 1-hexanol concentration in Pinot Noir wines was unaffected by the addition of 20% whole bunches in the wine preparation in 2014 vintage and its concentration was below the perception threshold level in all treatments. But, there are some studies reports that 1-hexanol concentration in Pinot Noir wine exceeds the perception threshold level (Table A.1 in Appendix) (Girard et al., 2001; Rutan et al., 2014; Tomasino et al., 2015). This compound can trigger cut-grass aromas in wine, especially when exceed the perception threshold level (1100 μg/L in a model wine) (Peinado, Moreno, Bueno, Moreno, & Mauricio, 2004). However, stem aromatic composition may differ in climatic conditions, which may lead to variations of the stem-derived aromas among different vintages (Casassa et al., 2019).

2.3.4 Phenylpropanoid esters

Most well-known phenylpropanoid esters found in wine are ethyl-3-phenyl-2-propenoate (ethyl cinnamate) and ethyl-3-phenyl-propanoate (ethyl 2,3-dihydrocinnamte or ethyl hydrocinnamate). These compounds are esterification of cinnamic acid which is biosynthesized from phenylalanine, one of the three carbo-aromatic amino acids, from the precursor chorismic acid via phenylalanine ammonia-lyase (Rutan, 2016). Most studies suggested that their concentration in Pinot Noir wine were above their perception threshold levels (Rutan et al., 2014).

2.3.5 Methoxypyrazines

The 3-alkyl-2-methoxypyrazines (MPs) have been found to play an important role in the wine aroma due to their lower sensory perception threshold level (Parr, Green, White, & Sherlock, 2007; Parr et al., 2016; Sala et al., 2004). They produce green aromas, herbaceous odours and fresh-cut grass aromas in wine. The 3-isobutyl-2-methoxypyrazine (IBMP), 3-sec-butyl-2-methoxypyrazine (SBMP), and 3-isopropyl-2-methoxypyrazine (IPMP) are the most important MPs found in wine. Higher concentrations of these compounds can result in undesirable green aromas in the finished wines, made by adding stems and whole bunches (Reynolds, 2010; Sala et al., 2004). Hashizume and Samuta (1997) reported that grape stem is the richest source of MPs compared to berry and leaf of Cabernet Sauvignon and Chardonnay grapes. Hence, there is a risk of ‘green’, ‘grassy’ and ‘herbal’ flavours and aromas when use stems in the winemaking. However, it can be avoided by adding bunches with

lignified stems rather than green and reducing the proportion of whole bunches. Lignification is likely to be lower in cooler climates due to cessation of vine growth as well as lignification tends to be lower in wet conditions specially mid and late season rain fall which stimulate vine growth (Goode, 2016). According to a previous study conducted by Hashizume et al. (1998), reported that Pinot Noir wines made by adding 3% stem caused to result in 2.9 µg/L of IPMP and 16.0 µg/L of IBMP in the resultant wines, and treatment made without adding stems was not contained these compounds.

Volatile aroma groups of fatty acids, esters, higher alcohols and carbonyls in wine are derived from the yeast and bacteria metabolism (Swiegers & Pretorius, 2005). majority of volatile fatty acids, esters, and higher alcohols are absent in grape must and are produced during the fermentation process (Robinson et al., 2014). Wine aroma composition changes rapidly during the fermentation process and immediately after the fermentation. It is due to the production of acetaldehyde, ethanol, fatty acids, esters, higher alcohols and hydrogen sulphide like volatile compounds during sugar metabolism of yeast during fermentation. These chemical reactions typically occur as a result of the enzymatic or acidic hydrolysis of sugars and amino acids during the metabolism of yeast (Rutan, 2016).

2.3.6 Fatty acids

The majority of the fatty acids produced by yeast are long-chain fatty acids (>C12), those are heavier and non-volatile, but they also can produce short (<C6) and medium-chain (C6-C12) volatile fatty acids (Robinson et al., 2014). The total volatile acid concentration in wine usually ranges from 500 to 1000 mg/L, which contributes 10-15% of the total acid content in wine. Acetic acid accounts for more than 90% of total volatile fatty acid (FA) in wine (Swiegers & Pretorius, 2005) but normally concentration of acetic acid in wine is well below its perception threshold level unless there is significant spoilage. Short-chain fatty acids including branched-chain isobutyric and isovaleric acids and straight-chained butyric and propionic acid have been found that they contribute to wine aroma (Robinson et al., 2014; Rutan, 2016). Isobutyric and isovaleric acids cause sweaty, cheese-like aromas in wine. They were identified as markers of *Brettanomyces bruxellensis* spoilage and are thought to be capable of masking the ‘‘Brett character” attributed to 4-ethylphenol and 4-ethylguaiacol (Romano, Perello, Lonvaud-Funel, Sicard, & de Revel, 2009). Many studies reported that concentrations of isovaleric acid, hexanoic acid and octanoic acid in Pinot Noir wines were above the perception threshold level (Girard et al., 2001; Rutan et al., 2014; Tomasino et al., 2015) (Table A.1 in Appendix). Girard et al. (2001) reported that 30°C fermentation temperature caused a slight decrease of hexanoic acid and 20°C fermentation treatment increased the concentration of octanoic acid in Pinot Noir wine.

2.3.7 Esters

Esters are the second largest contributor to the total volatiles, accounting around 36% and is second only to higher alcohols (fusel alcohol) (Sumby, Grbin, & Jiranek, 2010). This group is the primary source of fruity aroma in wines, but more soapy odours can develop when increasing the length of the hydrocarbon chain of the fatty acid (C16 and C18) involve in the formation of esters. Esters can be formed either enzymatically or formed during wine ageing by reacting to alcohol and carboxylic acid functional groups together (Sumby et al., 2010). However, most esters in alcoholic beverages are secondary metabolites produced by yeast during alcoholic fermentation. Their synthesis is linked to the lipid and acetyl-CoA (coenzyme A) metabolism in yeast cell but esters significant to a specific grape cultivar have also been identified. Pinot noir wines are known to exhibit distinct red fruity aromas that particularly evoke the odours of small-stone fruit. Synthesis of acetate esters during fermentation is an energy-requiring process that takes place inside the yeast cell. It requires the important metabolite, acetyl-CoA and occurs in two stages. These reactions require alcohol, fatty acids, CoA, and ester synthesising enzyme (Swiegers & Pretorius, 2005). Though, some variations in ester concentration in different treatments can observe due to the complex nature of wine subjects to continuous changes in composition during storage and even after bottle opening. This may be because of hydrolysis and esterification or could be caused by ester oxidation by hydroxyl radical-related processes. There are many factors that influence the formation of esters in wine such as fermentation temperature, yeast strain, nitrogen levels in must, oxygen availability, and grape variety (Rutan, 2016).

2.3.8 Higher alcohols

Higher alcohols (also called as fusel alcohol) found in wine are the largest group of aroma constituents, accounting around 51% of total volatile constituents found in wine (Sumby et al., 2010). It mostly contributes to the strong, pungent aromas and flavours in wine as well as they act as important precursors for ester production. C6 alcohols also cause vegetal and herbaceous aromas in wine (Ferreira, López, & Cacho, 2000). Higher alcohols are secondary products of yeast alcoholic fermentation (Sumby et al., 2010; Swiegers & Pretorius, 2005). Swiegers and Pretorius (2005) reported that when higher alcohol concentration in wine below 300 mg/L can contribute for wine complexity but if exceeded over 400 mg/L, they have a negative influence on the quality of the wine. There are two forms of higher alcohols in wines: 1) aliphatic alcohols, such as *cis*-3-Hexen-1-ol, *trans*-3-Hexen-1-ol, *trans*-2-Hexen-1-ol, 1-Hexanol, 1-Octanol, 2) aromatic alcohols, such as Phenylethyl alcohol.

2.3.9 Volatile Phenols

Volatile phenols can derive from microbial actions, oak maturation and smoke-taint. The most common volatile phenols found in wines are 4-ethylguaiacol, 4-ethylphenol, guaiacol, eugenol and vanillin (Allen, Bui, Cain, Rose, & Downey, 2013; Chatonnet, Viala, & Dubourdieu, 1997). These

compounds have aroma ranging from the sweaty saddle to cloves. Most significant volatile phenols are originated via microbial actions in wines from the precursors, p-coumaric acid and ferulic acid, both cinnamic acids. *Brettanomyces* yeasts are responsible for the decarboxylation of the precursor acids to vinyl phenols via cinnamate decarboxylase. The vinyl phenols are then converted by vinyl phenol reductase to ethyl phenols (Chatonnet et al., 1997).

The 4-ethylguaiacol, and 4-ethylphenol guaiacol are mainly responsible for "bretty" flavour in wine, and these compounds are considered as indicator compounds for *Brettanomyces* activity. These flavours are often considered as a defect, when present in wines (Fariña, Boido, Carrau, & Dellacassa, 2007; Hornsey, 2007). Feng, Skinkis, and Qian (2017) showed that oak ageing can enhance guaiacol, eugenol, and vanillin in wines. It has been reported that toasting of the oak barrels leads to thermal degradation of lignin and produces the volatile phenols, which could be extracted into the wine. The author reported that American oak could enhance all above three compounds compared to Spanish and French oak used in the study.

Previously reported concentrations of most of the volatile phenols were well below their perception threshold levels in Pinot Noir wine (Rutan et al., 2014; Tomasino et al., 2015). But Eugenol concentration could exceed its perception threshold levels in both of these studies (Table A.1 in Appendix).

2.4 Impact of cold maceration and grape stems on wine composition

A range of winemaking techniques can be used in Pinot Noir wine production to manage the extraction of phenolics and enhance/stabilise colour, for example, cold maceration, whole bunch fermentation, and post-fermentation maceration. Several factors affect the extraction of polyphenolics including tannins and anthocyanins into wine during maceration treatments and fermentation. It includes the duration of the treatment, temperature, alcohol concentration and sulphur dioxide concentration. Anthocyanin concentration typically come into the peak early, while proanthocyanidin extraction usually increases to reach a maximum at pressing. However, the localisation of phenolics in grape berry and interaction of phenolics with other components limit extraction of total grape polyphenolics into wine (Patricia & Kennedy, 2007). Smith, McRae, and Bindon (2015) also reported that extraction of tannins can be influenced by various winemaking interventions, e.g. more tannins can be extracted by crushing grapes, extended maceration, pulsed electric field, enzyme application and microwave maceration.

2.4.1 Adding stem or whole bunch in fermentation

Grape stems contain a significant amount of phenolics (Kosińska-Cagnazzo et al., 2019; Makris, Boskou, Andrikopoulos, & Kefalas, 2008; Ruiz-Moreno et al., 2015; Souquet et al., 2000). Souquet et al. (2000)

reported that merlot grape stems contained significant amounts of polyphenolic compounds, especially phenolic acids, flavonols, and flavanonols comprising: caftaric acid-40 mg/kg, coutaric acid-4.5 mg/kg, quercetin-3-glucuronide-200 mg/kg, quercetin-3-glucoside-18 mg/kg, catechin-60 mg/kg and epicatechin- trace amount. In addition to flavanols and flavonols, Roditis grape stems contain significant amounts of stilbenes and astilbins (Makris et al., 2008). Melzoch et al. (2001) reported that the richest source of resveratrol (main stilbene in wine) is the cluster stems (rachis) of grapes. Resveratrol is a stilbene produced by the grapevine in all tissues as a phytoalexin in response to various stress factors such as UV-radiation, mechanical injuries, and, specifically in vine grapes to Botrytis infection and other fungal attacks (Aaviksaar et al., 2003; Waterhouse, 2002). Resveratrol concentration in different parts of Pinot noir grapes contained; 2.80 mg/kg in leaves, 13 mg/kg in rachis, and 2.34 mg/kg in berry respectively (Melzoch et al., 2001). Pinot Noir wines tend to have a higher concentration of resveratrol compared to other varieties (Baraboy, 2009; Melzoch et al., 2001).

Typically, in the range of 5% to 40% of whole bunch addition is common in practice, but there are many anecdotal pieces of evidence of using 100% whole bunches (Casassa et al., 2019). It has been shown in many studies that maceration in the presence of stems in red winemaking is an important technique to enhance tannins and increase the formation of polymeric pigments, which are useful for long term colour stability in wines (Casassa et al., 2019; Hashizume et al., 1998; Suriano et al., 2015).

Stem addition for all types of varieties is not appropriate because stem may cause significant colour loss, reduced acidity and stemmy flavour to the resultant wine. Most of the time stem addition is common in low tannin grape varieties like Pinot Noir to enhance tannin (Hashizume et al., 1998). The potential drawback of whole bunch fermentation is the risk of ‘green’, ‘grassy’ and ‘herbal’ flavours and aromas, in finished wines. It can be avoided by adding bunches with lignified stems rather than green and reducing the proportion of whole bunches. Lignification is likely to be lower in cooler climates due to cessation of vine growth as well as lignification tends to be lower in wet conditions especially mid and late-season rainfall which stimulates vine growth thus adding vegetal or herbaceous notes to the wines (Goode, 2016). These sensory notes arise from methoxypyrazines, aliphatic carbonyl compounds or C6 higher alcohols such as 1-hexan-ol or 3-hexen-1-ol (Suriano et al., 2015).

2.4.2 Cold Maceration

Cold maceration is a common method used to enhance the total phenolic content in red wine. It is a production tool which may increase the complexity of aroma, colour and colour stability of red wines (Panprivech, Lerno, Brenneman, Block, & Oberholster, 2015). Cold maceration is a maceration practice based on permitting the contact of skins, and seeds just before the beginning of alcoholic fermentation. The absence of fermentative activity is ensured by chilling. They prevent the activity of *Saccharomyces cerevisiae* (Casassa et al., 2019; Dambergs et al., 2012). Absence of ethanol during CS

treatment helps for selective extraction of water-soluble compounds including anthocyanins, free and glycosylated-bound aroma compounds, polysaccharides and low molecular weight tannins (Casassa et al., 2019). Time-temperature combination of this treatment varies widely depending on many factors such as the grape variety used and expected characteristics of resultant wine in the literature.

Previous studies have shown that cold maceration could influence on colour, phenolics and aroma composition of wine. Many authors reported that cold maceration could increase anthocyanin in the resultant wines (Álvarez et al., 2006; Koyama et al., 2007). However, there are some contradictory findings as well Casassa et al. (2019) reported that cold maceration caused to reduce anthocyanin extraction and decrease colour intensity in Pinot Noir wines. In terms of phenolics, most of the previous publications suggest that cold maceration is increased total phenolics in the resultant wines (Álvarez et al., 2006; Casassa et al., 2019; Koyama et al., 2007). Casassa et al. (2019) reported that cold maceration (Dry ice was used to maintain the temperature at 6.7 ± 1.2°C for five days and SO_2 concentration was 80 mg/L) could increase total tannin extraction by 37% in making Pinot Noir wine compared to a control treatment.

Few publications are assessing the effect of cold maceration on wine aroma. Cai et al. (2014) reported that cold maceration in pumping over tanks could result in higher concentrations of β-Damascenone, and acetate esters in Cabernet Sauvignon wines, those are mainly responsible for fruity aromas in wine. But Casassa et al. (2019) reported that cold maceration decreased β-Damascenone concentration in Pinot Noir wine. The author also reported that cold maceration could significantly increase ethyl butanoate, hexyl acetate and hexanol, and cold maceration significantly decreased hexanoic acid and β-Damascenone in cold macerated wines compared to a control treatment (no cold maceration). However, these variations of results may be due to changes in cold macerating parameters such as temperature, duration and method used in reducing the temperatures of the media.

2.5 Project aims

This study aims to investigate the effect of stem inclusion fermentation on the composition of Pinot Noir wines which were fermented with or without the addition of grape stems or whole bunches at different proportions. In total, there are five treatments included in this study: 1) the DS treatment prepared with 100% of destemmed and crushed grapes; 2) the WB30 treatment prepared with addition of 30% of whole bunches; 3) the WB60 treatment prepared with addition of 60% of wholes bunch; 4) the WB100 treatment prepared with addition of 100% of wholes bunch; 5) the DS100 treatment prepared with 100% of stems added back to destemmed and crushed grapes. Analysis of resultant wine composition was carried out to investigate the impact of stem inclusion fermentation on colour parameters, phenolic compounds and aroma compounds of Pinot Noir wine.

2.6 Thesis structure

This thesis contains seven chapters, including the first two chapters of introduction and literature review. Tables and figures used in chapters are given at the end of each chapter. Chapter 3 outlines the general material and methodology used throughout the project. From chapter 4 to 6 are experimental chapters, which are discussing the effect of stem inclusion fermentation on colour parameters, phenolic compounds and aroma profiling of Pinot Noir wines, respectively. Experimental chapters are structured as journal papers containing the abstract, introduction, methodology, results and discussion, and conclusion. The methodology used in experimental chapters discussed briefly to keep the flow and avoid repetition of chapter 3 (general materials and methodology). All the references cited in each experimental chapter are listed in the references at the end of the thesis. Chapter 7 includes the general conclusion of this study and recommendations for future studies.

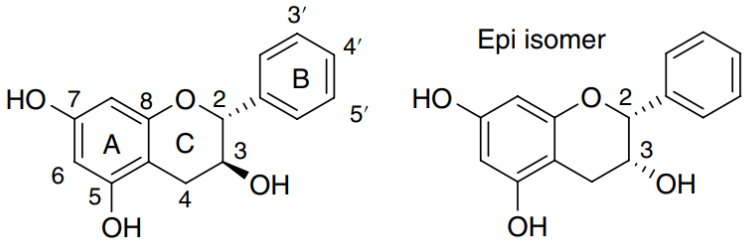


Figure 2.1: Parent ring system for flavan-3-ols

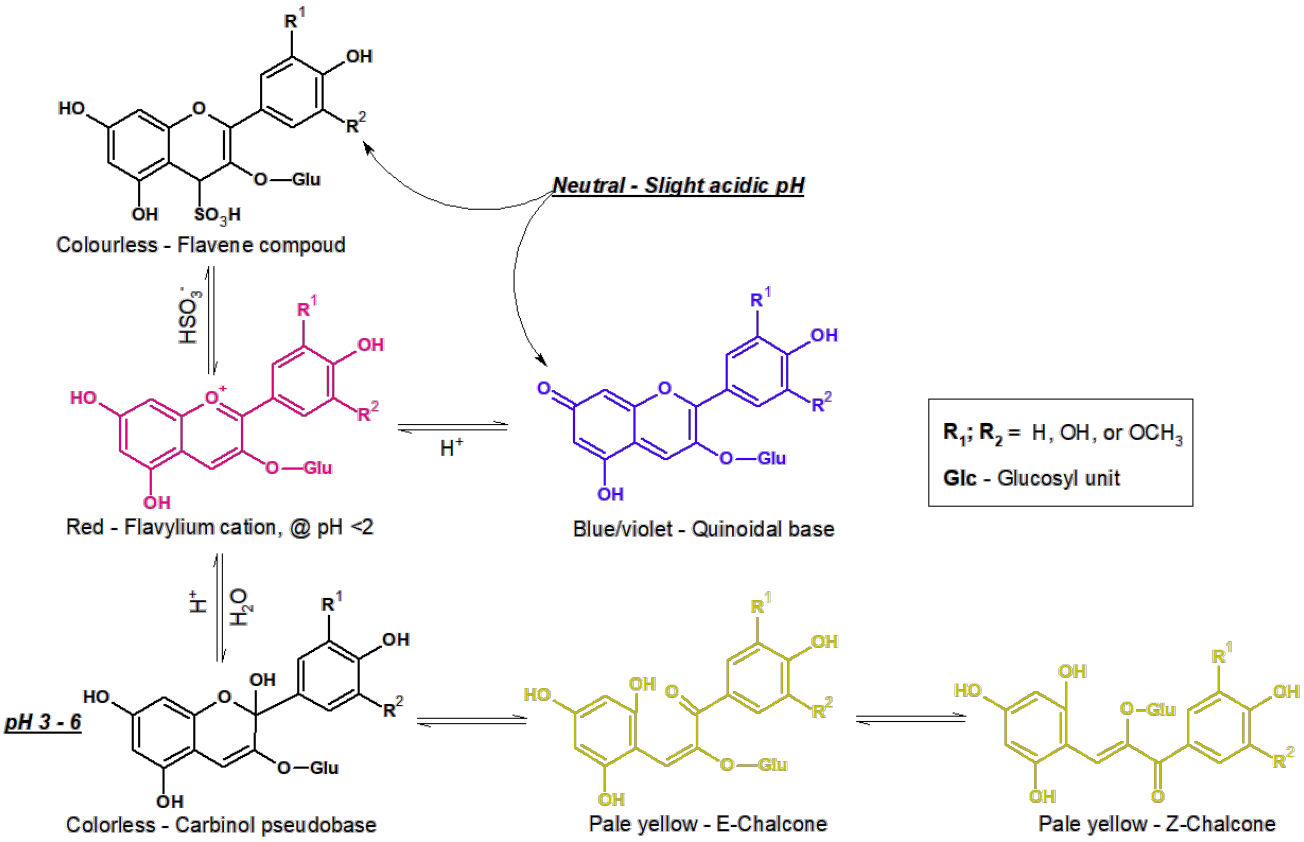


Figure 2.2: The pH-dependant equilibria among the various structural forms of anthocyanins

(F. He et al., 2012; J. He & Giusti, 2010)

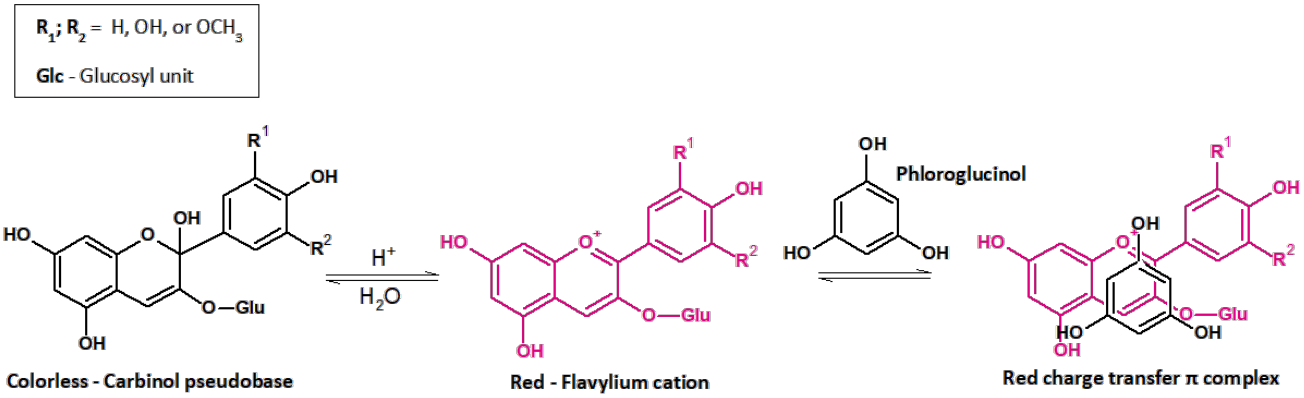


Figure 2.3: Model Co-pigmentation reaction of anthocyanin with a phloroglucinol-electron rich partner (this equilibrium shifts to the right side (red coloured form))

(Waterhouse et al., 2016)

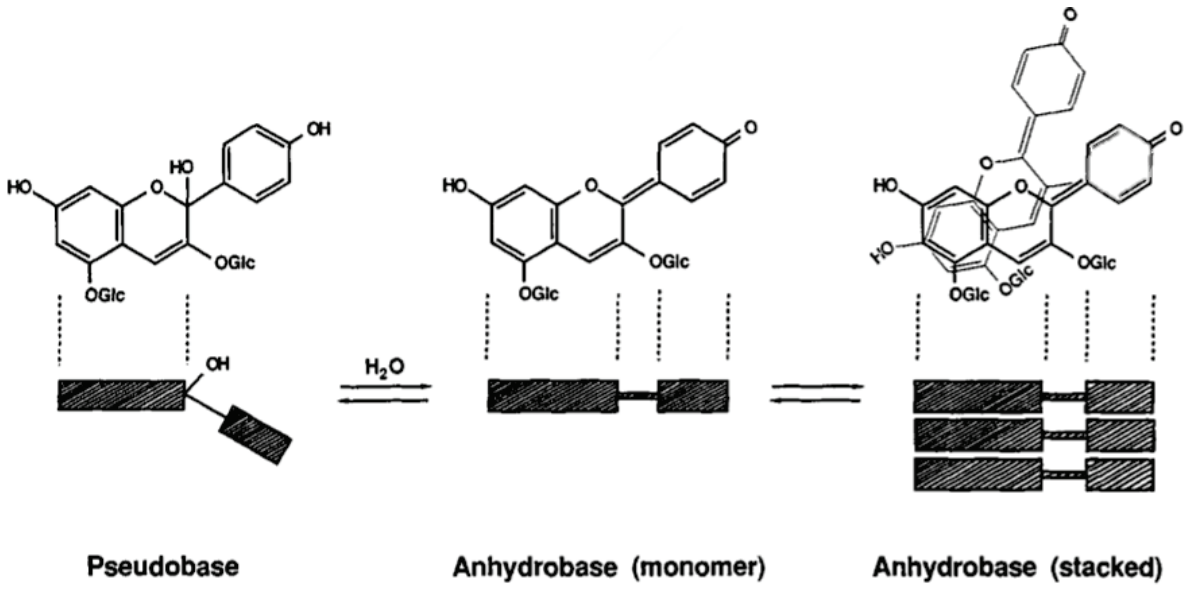
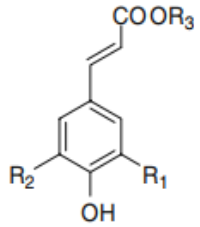


Figure 2.4: Self-association of anthocyanin - stacking of anhydrobase molecules prevent the formation of colourless pseudo base

(Goto & Kondo, 1991)

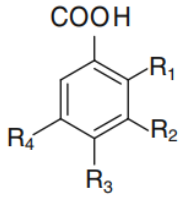
Table 2.1: HCA structures and their concentration in Pinot Noir wine from previous publications.



Non-flavonoid: HCA	R1	R2	R3	Reported concentration range in Pinot noir (mg/L)[1]
Caftaric acid	OH	H	Tartaric Acid	39.91-117.95
cis-coutaric acid	H	H	Tartaric Acid	
trans-coutaric acid	H	H	Tartaric Acid	
caffeic acid	OH	H	H	1.92-7.88
p-coumaric acid	H	H	H	0.49-3.85
ferulic acid	OCH_3	H	H	

[1] (Van Leeuw et al., 2014)

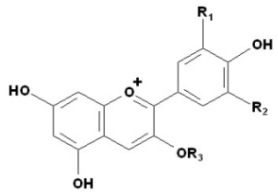
Table 2.2: HBA structures and their concentration in Pinot Noir wine from previous publications



Non-flavonoid: HBA	R1	R2	R3	R4	Reported concentration range in Pinot noir (mg/L)[1]
Gallic acid	H	OH	OH	OH	23.37-105.27
Syringic acid	H	OCH_3	OH	OCH_3	2.36-9.12
Protocatechuic acid	H	OH	OH	H	1.09-6.23
p-Hydroxybenzoic acid	H	H	OH	H	0.09-1.44

[1] (Van Leeuw et al., 2014)

Table 2.3: Anthocyanin structures and their concentration in Pinot Noir wine from previous publications



Monomeric Anthocyanin	R1	R2	R3	Concentration ranges (mg/L)	
				(Cortell et al., 2007)	(Mazza et al., 1999)
Cyanidin-3-O-glucoside	OH	H	Glc	0.49-1.50	3.3-3.5
Delphinidin-3-O-glucoside	OH	OH	Glc	1.48-6.14	10.4-14.5
Peonidin-3-O-glucoside	OCH_3	H	Glc	9.50-22.21	15.7-24.3
Petunidin-3-O-glucoside	OCH_3	OH	Glc	3.26-11.44	11.4-14.4
Malvidin-3-O-glucoside	OCH_3	OCH_3	Glc	79.89-150.32	50.4-52.1

Glc-glucosyl unit

Chapter 3

General Materials and Methodology

3.1 Pinot noir grapes and grape extracts

Two rows of Pinot Noir grapes (clone/rootstock: B777/3309) planted in the Lincoln University vineyard (-43.644970, 172.444760) were selected for the study. The maturity level of the selected two rows of Pinot Noir grapes was monitored by measuring the total soluble solids (TSS), pH and titratable acidity (TA). Maturity level observation started on 18th of February 2019. The pH and TA analysis were started when TSS reached 20° Brix and continued until the 2nd of April 2019 when 23° Brix was achieved prompting manual harvest of the grapes. Three batches of 30 berries were randomly taken at harvest for analysing the TSS, pH, TA, and yeast assimilable nitrogen (YAN). A separate 50 g berry sample was homogenized using a grinder (Breville, BCG200, Australia), and 1 g of the homogenate was extracted into 10 mL of 50% ethanol solution as described by Sarneckis et al. (2006) to analyse tannins and total anthocyanins in grapes at harvest. Any unripe or diseased berries/ bunches were removed, and only healthy grapes were used for winemaking using the protocol described below.

3.2 Experimental design and winemaking

Five treatments were set out according to Table 3.1, each using an initial weight of 5 kg of grape bunches per treatment. Each treatment was carried out in triplicate following the protocol developed in consultation with winemakers, Dom Maxwell at Greystone and Mark Rose at Pegasus bay.

Cold maceration was done in 10 L plastic buckets while in the presence of 30 ppm sulphur dioxide (SO_2). Carbon dioxide gas cover was maintained in the buckets to avoid oxidation and facilitate an anaerobic environment inside the containers during cold maceration. After that, the containers were sealed and incubated at 4°C for 5 days. After CS each treatment bucket was crushed manually by punching down (including whole bunches). Before fermentation, representative samples (50 mL) from each treatment were collected to analyse for TSS, pH, and TA.

Grape must was inoculated with EC1118 commercial yeast (Lalvin, Denmark) and the fermentation was carried out in a room maintained at 26 - 30°C. Superfood^{TM} yeast nutrient was added in two stages during fermentation according to the manufacturer's specifications. Addition charts were developed according to the initial YAN content of grapes. Grapes harvested in this work (YAN: 207 ppm) was categorized in the "Mild Risk" category in the addition chart. Temperatures of the cap and liquid were recorded before cap management. The cap management, manual punch-down, was done twice per day during alcoholic fermentation and reduced to once per day during post-fermentation maceration.

All wines were fermented to dryness, defined as residual sugar less than 4 g/L. Total weight reduction was used to determine the progress of the fermentation. Clinitest™ was used to monitor the endpoint of fermentation. Subsequently, the Rebelein method was used to measure reducing sugar in wine (Iland, 2013). After 4 days post-fermentation maceration, free-run wines were collected, and 50 ppm of sulphur dioxide added. The wines were allowed to settle for 2 days at 1°C before bottling. Three 40 mL aliquots of wine samples were taken into screw cap amber vials, with PTFE/Silicone septa (Supelco Bellefonte, PA, USA, through Sigma- Aldrich, Australia) for HPLC and three 10 mL aliquots of wine samples were taken into screw cap GC vials were for GCMS and kept in the freezer until further analysis. The remaining volume of wine was bottled in 375 mL bottles and sparged for 30 seconds of nitrogen gas flush to headspace before sealing. They were kept in refrigerated conditions.

3.3 General oenological parameters

Total soluble solids (TSS) and pH of harvested grapes and juice after cold maceration were analysed using a refractometer (Model PR-101, Atago Co. Ltd, Japan) and pH meter (Model SP-701, Suntex, Taiwan), according to the methods described in Iland (2013). Titratable acidity (TA) was measured by titrating a known quantity of juice (10 mL) with standardised 0.1 N Sodium hydroxide (NaOH) solution (Fisher Scientific, Waltham, MA, USA) to a pH endpoint of 8.2. Yeast assimilable nitrogen (YAN) and malic acid were determined using commercial enzyme test kits (Vintessential Laboratories, Australia). The alcohol content was determined using the ebulliometer (Dujardin Salleron, France). Rebelein method was used to determine the concentration of residual sugars in wine according to the methods described by Iland (2013).

3.4 Methyl cellulose precipitable (MCP) tannin assay

Tannin in grape extracts and wine was determined using the 1 mL methyl cellulose precipitation (MCP) method described by Sarneckis et al. (2006) as modified by Mercurio, Dambergs, Herderich, and Smith (2007). Epicatechin stock solution (1 g/L) was prepared to develop the calibration curve ranging from 0 to 120 mg/L. Methyl cellulose solution (0.04% of the product; Sigma-Aldrich, M-0387, Sydney, Australia, 1500 cP viscosity at 2%) and saturated ammonium sulphate solution (Sigma-Aldrich A4915, Auckland, New Zealand) were prepared according to the method of Sarneckis et al. (2006). Grape extract samples (100 µL) and 2 times diluted wine samples (25 µL) were used for the analysis. Absorbance readings were taken at 280 nm on a UV-Visible spectrophotometer (Model UV-1800, Shimadzu Corporation, Japan).

3.5 Total anthocyanin measurement

Total anthocyanins content of harvested grape berries was determined based on the method described by Iland, Cynkar, Francis, Williams, and Coombe (1996). Grape extracts (200 µL) were

transferred into a quartz cuvette (10 mm) and 3.8 mL of 1.0 M hydrochloric acid (HCl) added. The cuvette was then covered with parafilm and mixed by inverting a few times. The mixture was kept at room temperature for 3 hours before taking the absorbance readings at 520 nm on a UV-Visible spectrophotometer (Model UV-1800, Shimadzu Corporation, Japan).

3.6 Total phenolic content measurement

Total phenolic content in wines was analysed using the microscale Folin-Ciocalteau colourimetric method as described by Wrolstad (2001). Gallic acid stock solution (5 g/L) was prepared to develop the calibration curve ranging from 0 to 500 mg/L. The concentration of total phenolics was quantified against the gallic acid calibration curve and expressed as mg/L gallic acid equivalent. Absorbance readings were taken at the wavelength of 765 nm on a UV-Visible spectrophotometer (Model UV-1800, Shimadzu Corporation, Japan).

3.7 Analysis of monomeric phenolics by HPLC

Reagents Deionised water was obtained from a Barnstead GenPure system (Thermo Scientific, Germany) for solid-phase extraction (SPE) and HPLC analysis. All reagents used in SPE: methanol (Fisher Chemical, A452, Canada), formic acid (Fisher Chemical, F/1900/PB17, Canada), acetonitrile (Fisher Chemical, A998, Canada) and absolute ethanol (Scharlau, SA, Australia). All standard reference reagents were HPLC grade and purchased from Sigma-Aldrich (Australia).

Monomeric phenolics in wines were separated using the solid phase extraction method described in Jeffery, Mercurio, Herderich, Hayasaka, and Smith (2008). The Oasis HLB cartridge (Waters, Rydalmere, NSW, Australia) was conditioned with 2 mL of methanol followed by 2 mL of water. Wine samples (1.5 mL) were clarified at 8960 g (RCF) for five minutes before the analysis (Model Biofuge 15, Heraeus Sepatech GmbH, Germany). After centrifugation, 1 mL of wine sample was added under gravity to the cartridge. The cartridge was completely dried with a gentle stream of nitrogen once the wine volume was completely absorbed (approximately 25 minutes). Subsequently, the cartridge was eluted with 40 mL of 95% acetonitrile/5% 0.01 M hydrochloric acid to separate the fraction of monomeric phenolics from polymeric phenolics. The eluent was vacuum evaporated (Model CH-9230, Buchi AG, Switzerland) completely at 36°C in a water bath (Model JB2, Smith Biolab Ltd, New Zealand). Solids were dissolved in 1 mL of 10% ethanol/0.1% formic acid and transferred into an HPLC vial for analysis.

HPLC analysis was carried out according to the method described by Gómez-Alonso, García-Romero, and Hermosín-Gutiérrez (2007) with slight modifications. Agilent Technologies 1100 series HPLC machine equipped with quaternary pump and diode array detector (DAD) was used with an ACE 3µ C18-PFP 150X4.6mm (Advanced Chromatography Technologies, Aberdeen, Scotland) as a separation column. The fraction containing monomeric phenolics (10 µl) from each wine sample was injected into

the HPLC column while kept at 20°C temperature. The mobile phase is composed of three solvents: A (0.05M $NH_4H_2PO_4$, pH=2.6), B (100% acetonitrile) and C (0.2M H_3PO_4). The total flow rate was 0.8 mL/min, and the solvents programme is shown in shown Table 3.2. The detection and quantification of monomeric phenolics were recorded at 280, 320, 360 and 520 nm using the photodiode array detector (DAD). The identification of phenolic compounds was carried out by comparing their retention times and the spectra with those of standards (Table 3.3). The quantification of phenolic compounds was calculated by using the individual calibration curve of each standard.

3.8 Aroma profiling of wines

Reagents Deionised water was obtained from a Barnstead GenPure system (Thermo Scientific, Germany). Absolute ethanol was purchased from Scharlau Chemi (SA, Australia), Sodium hydroxide was purchased from Fisher Scientific (Waltham, MA, USA), All pure reference reagents and isotopically labelled compounds were purchased from Sigma-Aldrich (Australia).

3.8.1 Headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME/GC-MS)

Headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME/GC-MS) was used to determine selected aroma compounds in wine samples using the method described by (Tomasino et al., 2015). Three different HS-SPME/GC-MS methods were used to identify and quantify different groups of aroma compounds: esters, higher alcohols, fatty acids and low concentration aromatic compounds. Composite standard solution for each method was prepared from stock standard solutions of aroma compounds, which were selected based on previous studies on Pinot noir wines. Subsamples of the prepared composite standard were stored frozen at -20°C in 4 mL amber colour screw cap vials. A separate internal standard for each method was prepared from the stock solutions containing the isotopically labelled (deuterated) compounds.

To prepare working standards different proportions of previously prepared composite standard subsample (0 - 0.90 mL) (vial thawed to room temperature before use), and acidified (pH 3.5) aqueous 14% (v/v) ethanol solution (0.90 – 0 mL) (“wine matrix”) were mixed and 8.06 mL of acidified (pH 3.5) deionised water, followed by 40 µL of internal standard and 4.5 g of sodium chloride added into a 20 mL amber glass screw cap vial just prior to capping as mentioned in Tomasino et al. (2015). All wine samples were diluted immediately before the analysis. The same working standards preparation procedure applied for the wine sample preparation for the analysis (0.9 mL of wine used instead of composite standard). The total volumes used are equivalent to a 10-fold dilution of the wine sample. All samples were held at 8°C prior to injection in a stack cooler attached to the Combi-Pal autosampler (CTC-Analytics, Zwingen, Switzerland).

The fibre used in this methodology is 2-cm long Stableflex DVB/CAR/PDMS combination SPME fibre (p/n 57348-U, 50/30 µm thickness, 24 gauge). This fibre was conditioned at 250°C for 1 hour in the injection port and it was further conditioned in helium for 10 minutes at 250°C in a fibre conditioning station attached to the Combi-Pal auto-sampler used with the Shimadzu GC-MS instrument (Shimadzu Scientific Instruments, Oceania, NSW, Australia) immediately before each sample analysis.

Method 1: Analysis of 12 esters, 7 alcohols and 1 aromatic aldehyde were determined in this step. Initially, samples were incubated at 60°C while agitating at 500 rpm for 10 minutes. The SPME fibre was then exposed to the headspace of the vial without agitating at 60°C for a 60-minute extraction period. Shimadzu QP2010 GC-MS (Shimadzu Scientific Instruments) equipped with a CTC Combi-Pal autosampler (CTC-Analytics) using Version 5.0 of Shimadzu GC-MS solutions data acquisition software was used for the analysis. The chromatography arrangement contained a short guard column (5 m × 0.25 mm ID, Restek, Bellefonte, PA, USA) connected to dual columns in series: a Rtx-wax column (30 m × 0.25 mm ID × 0.5 µm film thickness, polyethylene glycol, Restek) and a Rxi-1MS column (15 m × 0.25 mm ID × 0.5 µm film thickness, 100% dimethyl polysiloxane, Restek). The helium carrier gas was used at a linear velocity of 33.5 cm/s. The oven of the column was maintained at 35°C for 3 minutes (during desorption of the SPME fibre), after that it was increased to 250°C at 4°C/min and held at this temperature for 10 minutes. The interface and MS source temperature were set at 250°C and 200°C, respectively, with the MS source, operated in electron impact (EI) mode at ionisation energy of 70 eV. The MS acquisition mode was set to full scan for all 20 compounds for the quantification of each aroma compound during post-run data analysis. The reference standards composed of pure compounds and NIST05 (National Institute of Standards and Technology) mass spectral library were used to check the identities for all standards used.

Method 2: Twenty-one low concentration (trace category) compounds were analysed using a similar procedure to method 1 except the data acquisition mode was set to selected ion monitoring (SIM) and altered GC oven temperature ramp to improve separation to ensure the presence of required sensitivity to detect selected compounds. The modified GC oven temperature was held at 35°C for 3 min, increased to 105°C at 3°C /min and held for 10 min, then increased to 140°C at 2°C/min and held for 10 min, then further increased to 250°C at 4°C/min and held for 10 min.

Method 3: A separate method was used to detect and quantify six volatile fatty acids in wine samples. This method also used the same column configuration described in method 1, but HS-SPME extraction conditions were different, as well as the linear velocity of carrier gas flow rate and the GC oven temperature ramp, which was increased to ensure faster analysis. Samples were incubated initially for 10 min at 60°C during which time the vial was agitated at 500 rpm. After 10 min, the SPME fibre was exposed to the headspace of the vial for 30 min at 60°C. The splitless injection was used for the first 3

min of the runtime, after which split mode was used at a 20:1 ratio. The helium carrier gas was set to a constant linear velocity of 46.8 cm/s. The column oven was held at 50°C for 3 min, increased to 240°C at 10°C/min, then further increased to 250°C at 30°C/min and held at this temperature for 5 min. The interface and MS source temperature were set at 250°C and 200°C, respectively, and the MS was operated in EI mode at ionisation energy of 70 eV. Full scan mode was used for all standards

3.8.2 Methoxypyrazine analysis using HS-SPME MD-GC-MS

The automated Headspace solid-phase microextraction with multi-dimensional gas chromatography-mass spectrometry (HS-SPME MD-GC-MS) technique was used to analyse methoxypyrazines including 3-isopropyl-2-methoxypyrazine (IPMP), 3-isobutyl-2-methoxypyrazine (IBMP), and sec-butyl-methoxypyrazine (SBMP) in wine samples. Analysis of IBMP was performed according to the method published by Parr et al. (2016), and some modifications to that method were developed for analysing IPMP and SBMP in wines (Breitmeyer, Field, & Olejar, 2020).

Composite standard solution for each method was prepared from stock standard solutions of above three methoxy compounds. Subsamples of the prepared composite standard were stored frozen at -20°C in 4 mL amber colour screw cap vials. A separate internal standard for each method was prepared from the stock solutions containing the isotopically labelled (deuterated) compounds.

To prepare working standards different proportions of previously prepared composite standard subsample (0 - 3 mL) (vial thawed to room temperature before use), and acidified (pH 3.5) aqueous 14% (v/v) ethanol solution containing 5 g/L tartaric acid (3 – 0 mL) (“wine matrix”) were mixed together and 4.85 mL of acidified (pH 3.5) deionised water, followed by 150 µL of internal standard and 4.5 g of sodium chloride added into a 20 mL amber glass screw cap vial. Immediately after that, 1 ml of 4 M sodium hydroxide was added, and the tube was quickly capped. All wine samples were diluted immediately prior to analysis. The same working standards preparation procedure applied for the wine sample preparation for the analysis (3 mL of wine used instead of composite standard). The total volumes used are equivalent to a 3-fold dilution of the wine sample. All samples were held at 8°C prior to injection in a stacked cooler attached to the Combi-Pal autosampler (CTC-Analytics, Zwingen, Switzerland).

3.8.3 Calculation of odour activity values (OAVs) and aroma series

The odour activity value (OAV) for each aroma compound was calculated by dividing respective aroma compound concentration by the corresponding aroma threshold values obtained from previous studies to determine impacting aroma compounds (OAV>0.1) in resultant wines.

To predict the overall aroma perception in each treatment from the data obtained from GC-MS analysis, aroma active compounds identified in OAV analysis were grouped into eight aroma series (1-Fruity, 2-Floral, 3-Spicy, 4-Chemical, 5-Microbiological/oily/fatty,6-Woody, 7-Vegetative, 8-Nutty) based on their respective odour descriptors mentioned in Table A.1 (See Appendix). The grouping was done according to the aroma wheel developed by Noble et al. (1987) together with previously published research articles (Cai et al., 2014; Zea, Moyano, Moreno, & Medina, 2007). Some odour compounds were categorised into two or more groups due to the high complexity of aroma perception. The aroma compounds in the same aroma category were summed together and plotted in a bar chart.

3.9 Modified Somers Assay

Wine samples were analysed for colour parameters using the modified Somers 10 mL colour assay as described in Mercurio et al. (2007). Wine samples were centrifuged at 4000 rpm for 5 minutes before the preparation of 4 treatments (Model Heraeus Multifuge X1R, Thermo Fisher Scientific, Germany). In treatment A, one in 10 dilutions of wine was added into buffer 1 (model wine, 0.5% w/v tartaric acid in 12% v/v ethanol adjusted to pH 3.4 with 5 M NaOH), and absorbance was read at 420 nm and 520 nm immediately after mixing. In treatment B, one in 10 dilutions of wine was added into buffer 1 plus 0.375% w/v sodium metabisulphite. Samples were mixed and incubated at room temperature for 1 h. Absorbance was read at 520 nm. In treatment C, one in 10 dilutions of wine was added into buffer 1 plus 0.1% v/v acetaldehyde. Samples were mixed and incubated at room temperature for 1 h. Absorbance was read at 420 and 520 nm. In treatment D, one in 50 dilutions of wine was added into 1 M HCl. Samples were mixed and incubated at room temperature in the dark for 3 hours. Absorbance was read at 280 and 520 nm. Absorbance readings at different wavelengths were taken by UV-Visible spectrophotometer (Model UV-1800, Shimadzu Corporation, Japan) and calculations of modified Somers colour parameters were conducted according to formulas described in Mercurio et al. (2007).

3.10 CIELab analysis

The standard procedure published by OIV (2006) was used to calculate the CIELab colour parameters in wine. Transmittance every 5 nm over the visible spectrum range from 380 nm to 780 nm were conducted in a 2 mm path length quartz cuvette against deionised water blank using a UV-Visible spectrophotometer (Model UV-1800, Shimadzu Corporation, Japan). CIELab colour parameters were

calculated for the CIE illuminant D65 and 10° standard observer conditions, according to OIV (2006). The colour parameters include L* (luminosity/lightness), a* (green/red), b* (blue/yellow), C* (chroma) and H* (tone/hue angle).

3.11 Statistical analysis

Data were presented as mean ± SD of three replicates. Data obtained from oenological parameters measurement, HPLC, GC-MS, Somers assay and CIELab were analysed by one-way analysis of variance (ANOVA) with a post-hoc analysis. Post-hoc analysis was carried out by the Tukey HSD test. Analyses were performed at a 0.05 level of significance using Minitab 18 (Minitab, US) software package. Interactions of CIELab coordinates with total phenolics, MCP tannin, total anthocyanin, hue results were analysed according to Pearson Correlation analysis at a 0.05 level of significance using Genstat 19 (Genstat, UK).

All impacting aroma compound's OAV values (OAV>0.1) calculated using GCMS data were presented as mean ± SD of three replicates and analysed by one-way analysis of variance (ANOVA) with post-hoc analysis. Post-hoc analysis was carried out by the Tukey HSD test. The analysis was performed at a 0.05 level of significance by means of Minitab 18 (Minitab, US) software package.

Principle component analysis (PCA) was conducted in this study. Significantly different compounds in GCMS analysis were chosen to perform the PCA analysis at a 0.05 level of significance using Minitab 18 (Minitab, US).

Table 3.1: Treatment Preparation Procedure

Treatment	Grape processing conditions
DS	100% destemmed grapes
DS100	100% destemmed grapes with stems added back
WB100	100% whole bunch
WB60	60% whole bunch + 40% destemmed grapes
WB30	30% whole bunch + 70% destemmed grapes

Table 3.2: Mobile phase solvent gradient table

Time (min)	A (%)	B (%)	C (%)
0	100	0	0
2	100	0	0
5	93.6	6.4	0
17	2.8	11.2	86
22	3.6	14.4	82
29.5	4.2	16.8	79
55	6.6	26.4	67
70	10	40	50
75	10	40	50
78	36	64	0
81	36	64	0
86	100	0	0
90	100	0	0

A: 0.05M $NH_4H_2PO_4$ at pH=2.6; B: 100% Acetonitrile; C: 0.2M H_3PO_4

Table 3.3: Phenolics Standards and Retention time (RT).

#	RT (min)	Quantification wavelength (nm)	Compound name
1	10.407	280nm	Gallic acid
2	14.78	280nm	Protocatechuic acid
3	15.47	280nm	Gallocatchin
4	19.7	320nm	Caftaric acid
5	20.0	280nm	Hydrobenzoric acid
6	21.3	280nm	Epigallocatechin
7	22.9	280nm	Catechin
8	23.3	280nm	Vanilic acid
9	24.89	280nm	Caffeic acid
10	25.29	280nm	Syringic acid
11	27.3	280nm	Epicatechin
12	28.79	520nm	Malvidin-3-O-glucoside
13	31.509	280nm	p-Coumaric acid
14	35.4	280nm	Ferulic acid
15	37.0	360nm	Rutin
16	39.8	280nm	Epicatechin gallate
17	62.3	360nm	Quercetin

Chapter 4

The effect of grape stem inclusion fermentation on colour parameters of Pinot Noir wine

4.1 Abstract

The wine colour is an important sensory attribute of wine quality, which can be influenced by various winemaking techniques, including the use of grape stems. In this study, the basic oenological parameters and colour parameters were measured in wines made from five treatments consisting of different proportions of whole bunches/grape stem. In general, stem inclusion fermentation (by adding stems or using whole bunches) resulted in lower alcohol content and higher pH in the resultant wine. Wine colour measured by modified Somers assay showed that addition of 100% grape stems and high proportions of whole bunches (60% and 100% whole bunches by weight) tend to increase the degree of ionisation of anthocyanin, hue values, total phenolics and SO_2 resistant pigments, and 100% stem inclusion significantly decreased total anthocyanins in resultant wine compared to non-stem added treatment. Wine colour evaluation by CIELab parameters showed that addition of whole bunches regardless of percentage didn't lead to any significant difference in comparison to non-stem addition treatment (DS), but the addition of 100% of stems (DS100) resulted in a significant increase in L*, b*, C*, and H*. These results suggest that grape stem inclusion fermentation may improve wine colour stabilisation by increasing the concentration of SO_2 resistant pigments, and the impact of adding stems into destemmed must is varied from whole bunch fermentation.

Keywords: Anthocyanin, colour, Pinot Noir, stems, whole bunches

4.2 Introduction

Colour is an important quality feature of red wine. The red wine colour is mainly determined by phenolics comprising mainly anthocyanin, tannin and polymeric pigments (formed during ageing) (Smith et al., 2007). The red colour in young red wine is mainly due to monomeric anthocyanins, and polymeric pigments are necessary for the longevity of red wine colour in aged wines (Durner, 2016). Pinot Noir wine colour is much lighter than other red varieties (Morgan & Tresidder, 2015). Because it lacks more stable acylated forms of anthocyanins. Pinot Noir has only five types of anthocyanins in unstable mono glucoside form: malvidin, peonidin, petunidin, delphinidin and cyanidin (Iland, 2013). Malvidin-3-O-glucoside is the main type of anthocyanin found in Pinot Noir wine (Casassa et al., 2019; Iland, 2013). Different winemaking treatments such as cold maceration, direct stem inclusion, and whole bunch addition are being used to enhance/stabilise colour and phenolics, especially when using low-tannin and anthocyanin grape varieties such as Pinot Noir.

Several studies have investigated the effect of stem inclusion on wine colour (Smith et al., 2007). The stem inclusion can provide tannins as a secondary source of tannin in winemaking (Casassa et al., 2019; Suriano et al., 2015) but stem addition may reduce anthocyanins and result in paler colours in wines. Addition of stems/whole bunches can influence the wine colour mainly due to two reasons, (1) formation of stable pigmented tannins in wines by reacting stem extracted tannin with anthocyanin, which can lead to forming protein-precipitable polymeric pigments (Casassa & Harbertson, 2014; Durner, 2016; F. He et al., 2012), and (2) re-adsorption of anthocyanin into stems (Andrew, 2016; Suriano et al., 2015). Suriano et al. (2015) reported that stem inclusion (25% and 50 whole bunches) in Primitivo winemaking caused to decrease total anthocyanin and colour intensity in the resultant wines. Casassa et al. (2019) studied the combined effect of stem inclusion and cold maceration on the colour parameters of Pinot Noir wine. The author reported that anthocyanin concentration was higher in the wines treated with cold maceration together with 3% stems (CS + S treatment), and cold maceration together with 20% whole bunches (CS + WB treatment) compared to wines made without undergoing cold maceration in 2015 vintage. But the opposite was observed in 2014 vintage, whereby cold maceration without adding stems or whole bunches reduced anthocyanin in wines compared to control wine made without undergoing cold maceration.

Hypothetically cold maceration can enhance phenolic compounds, mainly coloured compounds such as anthocyanins, among others, during the pre-maceration period. Many authors have investigated the effect of cold maceration on the resultant wine colour (Álvarez et al., 2006; Gómez-Míguez et al., 2007; Koyama et al., 2007). Álvarez et al. (2006) reported that cold maceration using dry ice was more effective than storing in cold room to increase anthocyanins, especially malvidin-3-glucoside, ionised and polymerised anthocyanin in Monastrell red wines, as well as the maceration time, showed not to

be significant during the process (4 days and 8 days). Koyama et al. (2007) showed that cold maceration could extract phenolics in skins, including anthocyanin, flavonol, and epigallocatechin units within proanthocyanidins during early stages of maceration, and cold maceration has reduced the extraction of phenolics from the seed in Cabernet Sauvignon wines. However, there are some contradictory results have also been observed in literature: G González-Neves, Gil, Barreiro, and Favre (2010) reported that cold maceration was not affected for anthocyanin concentration at 12 months of bottle ageing of Tannat wine, and Casassa et al. (2019) showed that cold maceration decreases anthocyanin extraction and colour intensity in Pinot Noir winemaking.

In most of the previous studies, one maceration treatment was evaluated at once, and there are also some contradictory findings of the effect of cold maceration in wine colour. Hence, evaluating the combined effect of stem inclusion and cold maceration is useful to understand the effect of these treatments on the resultant wine colour. As well as, relatively lower amounts of stems were used in the previous publications (Generally 20-50% whole bunches or <3% stems), and most of the studies used one stem added treatment or cold macerated treatment to evaluate the influence of those treatments, by comparing to a non-treated wine. Therefore, in this work, five treatments were micro-vinified to investigate the combined effect of different proportions of stem inclusion (0-100% whole bunches) and cold maceration on resultant wine colour. Separate treatment was used to evaluate the effect of direct stem addition and whole bunch addition on wine colour.

4.3 Methodology

4.3.1 Wine and juice Samples

Five treatments were examined in this study: 100% destemmed and crushed grapes (DS), 100% destemmed and crushed grapes with stems added back (DS100), 30% whole bunches (WB30), 60% whole bunches (WB60), and 100% whole bunches (WB100). Juice samples immediately after cold maceration were collected. After that triplicate ferments of each treatment were conducted using a standard winemaking protocol, which includes five days cold maceration at 4 $^{\circ}$C and four days post-fermentation maceration at room temperature. Free-run wine samples from each treatment were collected at the end of alcoholic fermentation. Detailed winemaking protocol is described in Chapter 3.2.

4.3.2 General oenological parameters

Grapes after harvesting were analysed for total soluble solids (TSS), pH, yeast assimilable nitrogen (YAN), and total anthocyanin. Total soluble solids (TSS), titratable acidity (TA) and pH of juice after cold maceration were analysed. Ethanol content, residual sugar, pH, titratable acidity (TA), and malic acid were analysed in wine. Detailed procedures of each method are described in chapter 3.3.

4.3.3 Wine colour assessment

Both modified Somers assay and CIELab method were used to evaluate the colour of resultant wines. Modified Somers assay (Mercurio et al., 2007) was used to calculate total anthocyanin, degree of ionization of anthocyanins, colour density, hue, chemical age, sulphur dioxide resistant pigments and total phenolics. The detailed procedure of modified Somers assay is described in chapter 3.9. The CIELab colour parameters were measured according to the standard protocol published by OIV (2006), and details are described in Chapter 3.10.

4.3.4 Statistical analysis

Data are presented as mean ± SD of three replicates. In all cases, ANOVA was performed with the Tukey comparison test to compare means with a 5% level for rejection of the null hypothesis using Minitab 18 (Minitab, US) software package.

Interactions of CIELab coordinates with some modified Somers method parameters were analysed using Pearson Correlation analysis from Genstat 19 (Genstat, UK) software. Five data points (average of each treatment) were used to calculate correlation values (df = n-2). Pearson correlation analysis was conducted at 95% significant level, so if the Pearson correlation coefficient (r) is greater than 0.878, which is considered as a statistically significant relationship according to the Pearson's Correlation Table.

4.4 Results and Discussion

4.4.1 Basic enological parameters of grape, juice and wine

Pinot Noir grapes were harvested with total soluble solids (TSS) of 23.4 °Brix, pH of 3.17, titratable acidity (TA) of 9.33 g/L, and yeast assimilable nitrogen (YAN) of 207 mg/L (Table 4.1). After cold maceration, the total soluble solids (TSS) of juice was significantly decreased in DS100, WB60 and WB100 treatments compared to DS treatment. As grape stems contain more than 70% of water (Hashizume et al., 1998; Rice, 1976), decreased TSS in juice might be due to the dilution effect of water in stems. The differences in TSS of juices after cold maceration were also reflected in alcohol content in wines (12.3%-13.1%). The lower alcohol content was observed in wines made from treatments added with stems (DS100) and a high proportion of whole bunches (WB60 and WB100). The residual sugar in resultant wines was determined with concentration ranging from 0.9 to 1.2 g/L (Table 4.1). Stem inclusion treatments (DS100, WB30, WB60 and WB100) had higher residual sugar in finished wine compared to DS treatment. This may also explain the differences observed in alcohol content between treatments. In this study, 30% whole bunch addition (WB30: approximately 2.2% stems by weight) was not significantly affected to reduce ethanol concentration in resultant wines compared to non-stem added treatment (DS). Casassa et al. (2019) also reported that treatments prepared with

adding 20% whole bunches could not significantly reduce the alcohol content in the resultant Pinot Noir wines in 2015 vintage.

There are significant differences in TA and pH of juice and wine between treatments. After cold maceration, the pH value was significantly increased in DS100 treatment but decreased in treatments with a high proportion of whole bunch addition (WB60 and WB100) compared to non-stem added treatment (DS). A significantly higher concentration of TA was only observed in WB100 treatment compared to DS treatment. These results could be due to a combined effect of cold maceration and inclusion of stems, as both winemaking practises can influence the extraction of potassium ions from grape skin and stems (Aleixandre-Tudo & Du Toit, 2018; Sacchi et al., 2005). Cold maceration in which destemmed and crushed grapes were held at a low temperature has been reported to increase extraction of potassium ions from grape skin, which can consequently increase pH and increase TA in juice. Adding stems could also enhance the extraction of potassium ions from stems during cold maceration. However, when high proportion of whole bunches were used, less potassium ions were extracted from crushed grapes and stems during cold maceration. At the end of alcoholic fermentation, wine pH was increased in all treatments ranging from 3.74 to 3.93, and titratable acidity (TA) was decreased in all treatments ranging from 7.47 to 7.88 g/L respectively. Comparing to DS treatment, the wine pH was significantly increased in all stem inclusion treatments including DS100, WB30, WB60 and WB100, which is likely resulted from gradual extraction of potassium ions from stems during fermentation (Hashizume et al., 1998). Similar to the results observed in titratable acidity of juice after cold maceration, only WB100 treatment showed significantly higher TA compared to DS treatment, which might be due to the least extraction of potassium ions during cold maceration.

The malic acid concentration was significantly different between treatments with a range from 3.73 to 4.20 g/L. As up to 30% of malic acid can be metabolised by yeast during alcoholic fermentation (Moreno & Peinado, 2012), the differences in malic acid between treatments could be due to different rates of malic acid metabolism during fermentation. It seems less malic acid was metabolised in DS100 treatment but no significant difference between DS and WB treatments.

4.4.2 Modified Somers colour assay

The chemical age I, chemical age II, degree of ionisation of anthocyanin, total anthocyanin, colour density, SO_2 corrected colour density, hue, A520-red hue, A420-yellow hue, SO_2 resistant pigments, and total phenolics were measured in resultant wines using modified Somers assay (Table 4.2). As wine samples were collected at the end of alcoholic fermentation, there is no significant difference in the chemical age of wine between treatments. In most cases, 30% whole bunch fermentation (WB30) didn't show significant differences in modified Somers assay parameters (except total anthocyanins) compared to DS treatment.

Degree of ionisation of anthocyanin in wines of all treatments was ranged from 21 % to 23%, which is within the range reported previously in Pinot Noir wine (Dicey, 1996; Durner, 2016). A significantly higher degree of ionisation of anthocyanin was observed in treatments with a high proportion of whole bunches (WB60 and WB100) compared to DS treatment. This is likely due to the variations in wine pH and total anthocyanins in wine with concentrations ranging from 181 to 242 mg/L. The highest concentration of total anthocyanin was observed in non-stem added treatment (DS) as a result of increased extraction of anthocyanins during cold maceration (Álvarez et al., 2006; Gil-Muñoz et al., 2009; Gómez-Míguez et al., 2007; Koyama et al., 2007), and stem addition seems to significantly reduce the concentration of total anthocyanins due to the adsorption of anthocyanins onto stems (Suriano et al., 2015).

Colour density and SO_2 corrected colour density were ranged from 6.0 to 7.3 and from 5.7 to 6.8, respectively. Compared to non-stem added treatment (DS), there was no significant difference in colour density and SO_2 corrected colour density observed in stem inclusion treatments. However, comparing between DS100 and WB100 treatments, significantly higher colour density and SO_2 corrected colour density were observed in WB100 treatment. It seems that cold maceration, together with stems may reduce those two parameters in DS100 treatment due to adsorption of anthocyanins into stems (Suriano et al., 2015).

Hue values in this study were ranged from 0.961 to 1.129. In comparison to non-stem added treatment (DS), both 100% stem inclusion (DS100) and a high proportion of whole bunch addition (WB60 and WB100) significantly increased hue in resultant wines. Hashizume et al. (1998) also reported that Pinot Noir wine made by adding 5% stems resulted in higher hue values compared to wine made without adding stems. Wine hue is defined as the ratio of the yellow hue to red hue, so wines made with addition of stems/whole bunches had higher hue values may be due to 1) the formation of yellow-coloured xanthylium cation after interacting of anthocyanin and stem extracted tannin to form anthocyanin-tannin (A-T) adducts (Durner, 2016); and 2) the decrease of anthocyanins, especially those in red coloured form.

There is no significant difference in SO_2 resistant pigment in stem inclusion treatments comparing to DS treatment, but the higher proportion of whole bunch addition (WB60 and WB100) showed significantly higher SO_2 resistant pigment in resultant wine than that observed in DS100 treatment. This might be because the DS100 treatment had the lowest concentration of anthocyanins and thus less polymeric pigments were formed during fermentation.

Total phenolic content in resultant wines was ranged from 38.9 to 50.8. In comparison to non-stem added treatment (DS), 100% stem addition (DS100) and the high proportion of whole bunch addition (WB60 and WB100) could result in a significantly higher amount of total phenolics in resultant wines,

which is likely due to the additional extraction of total phenolics from stems. When comparing between WB100 and DS100 treatments, WB100 treatment had a significantly higher concentration of total phenolics, which may be due to the precipitation of phenolics in DS100 during cold maceration as a result of the interactions with other macromolecules in juice (*e.g.* proteins and polysaccharides) (Smith et al., 2015).

4.4.3 CIELab colour coordinates

The CIELab colour space was used by several authors to describe wine colour (Bakker et al., 1998; Casassa, Bolcato, & Sari, 2015; Gil-Muñoz et al., 2009; Negueruela, Echavarri, & Perez, 1995). CIELab colour parameters measured in wines of five treatments were shown in Table 4.3. In comparison to non-stem added treatment (DS), only DS100 treatment had significantly different colour coordinates, showing significantly higher luminosity/clarity (L*), yellow-blue colour component (b*), chroma (C*) and tone (H*) values.

In this study, luminosity values (L*) were ranged from 25.9 to 33.0. Directly adding stems (DS100) had significantly higher luminosity value compared to DS treatment. In this study, luminosity has a strong negative correlation with A520 red hue (r=-0.906, p=0.034) and total anthocyanin (r=-0.939, p=0.018) determined by modified Somers assay. So, the higher L* in DS100 treatment is the reflection of the low concentration of anthocyanins and consequent low A520 red hue.

Red-green (a*) colour component was not affected by the winemaking treatment. DS100 treatment had the highest yellow-blue colour component (b*) value and it was significantly high compared to DS treatment. Surprisingly, the lowest yellow-blue colour component (b*) value was recorded in DS treatment. DS treatment had the highest total anthocyanin content in the Modified Somers method. Yellow-blue colour component (b*) from CIELab had a strong negative correlation with total anthocyanin results from modified Somers method (r=-0.958, p=0.010). It seems that decreasing anthocyanin cause to increase b* in wine. This relationship agrees with previous findings Chang-Qing et al. (2008). This same trend was followed in Chroma (C*) parameter also because of Chroma (C*) is derived parameter from a* and b* coordinates.

The highest tonality value was recorded in DS100 treatment. When comparing between WB100 and DS100 treatments, it seems that cold maceration together with stem addition could result in significantly higher tonality values in the resultant wines. Bakker and Arnold (1993) reported that the tonality of wine directly correlates with the perception of brownness in wine. In this work, tonality had a strong negative correlation with A520 red hue and total anthocyanin results from the modified Somers method (r=-0.872, p=0.050, and r=-0.942, p=0.017 respectively). It may be due to the

adsorption of anthocyanin which reduces red hue and promotes brownness in more stem/ whole bunch added treatments.

4.5 Conclusion

Stem inclusion fermentation showed a significant impact on general oenological parameters and wine colour parameters. In general, stem inclusion fermentation can result in lower alcohol content and higher pH in the resultant wines. Cold maceration without adding stems or whole bunches, resulted in the highest total anthocyanin content, which is mainly responsible for the red colour in young red wines. Both direct stem addition and whole bunch addition caused to reduce total anthocyanin content in the resultant wines. Comparing to non-stem added treatment, 100% stem addition and the high proportion of whole bunch addition (at 60% and 100%) significantly increased total phenolic content in the resultant wines. This study shows that whole bunch addition is more efficient to increase the concentration of total phenolic content in wine than adding stems into the destemmed must. A higher amount of phenolics could result in a higher amount of non-bleachable pigments in wine.

Table 4.1: General oenological parameters of grapes at harvest, juice after cold maceration and wine at the end of alcoholic fermentation

Grapes at harvest				
	TSS (°Brix)	**pH**	**TA (g/L)[1]**	**YAN (ppm)**
	23.4 ± 0.3	3.17 ± 0.02	9.33 ± 0.04	207 ± 7

	DS	**DS100**	**WB30**	**WB60**	**WB100**
Juice after cold maceration					
TSS (°Brix)	23.4 ± 0.2a	22.5 ± 0.1b	23.2 ± 0.3a	22.6 ± 0.2b	22.7 ± 0.2b
TA (g/L)[1]	7.88 ± 0.32b	7.72 ± 0.29b	8.43 ± 0.24ab	8.25 ± 0.28ab	8.96 ± 0.33a
pH	3.35 ± 0.03b	3.41 ± 0.02a	3.34 ± 0.02b	3.28 ± 0.02c	3.20 ± 0.02d
Wine					
Alcohol (%)	13.1 ± 0.21a	12.6 ± 0.23bc	12.9 ± 0.10ab	12.5 ± 0.15bc	12.3 ± 0.06c
Residual Sugar (g/L)	0.9 ± 0.03b	1.2 ± 0.06a	1.2 ± 0.06a	1.1 ± 0.05a	1.2 ± 0.06a
pH	3.74 ± 0.01e	3.93 ± 0.02a	3.78 ± 0.02d	3.83 ± 0.01c	3.88 ± 0.01b
TA (g/L)[1]	7.53 ± 0.12bc	7.47 ± 0.08c	7.80 ± 0.08ab	7.48 ± 0.11c	7.88 ± 0.16a
Malic Acid (g/L)	3.76 ± 0.11b	4.20 ± 0.12a	3.87 ± 0.13ab	3.73 ± 0.26b	4.10 ± 0.10ab

Different lowercase letters in rows indicate significant differences among treatments (Tukey'stest, $P<0.05$)
TSS- Total soluble solids; TA-Titratable acidity; YAN- Yeast assimilable nitrogen
[1] Tartaric acid equivalent

Table 4.2: Wine colour parameters measured by modified Somers assay

Parameter	DS	DS100	WB30	WB60	WB100
Chemical age I	0.275 ± 0.02a	0.311 ± 0.01a	0.283 ± 0.01a	0.311 ± 0.02a	0.321 ± 0.03a
Chemical age II	0.068 ± 0.01a	0.080 ± 0.01a	0.073 ± 0.00a	0.084 ± 0.01a	0.084 ± 0.01a
Degree of ionization of anthocyanin (%)	21 ± 0.8c	22 ± 0.4abc	22 ± 0.4bc	23 ± 0.4a	23 ± 0.6ab
Total anthocyanin (mg/L)	242 ± 3a	181 ± 9c	211 ± 7bc	212 ± 12ab	207 ± 19bc
Color Density (Au)	6.8 ± 0.50abc	6.0 ± 0.14c	6.4 ± 0.29bc	7.3 ± 0.28a	7.2 ± 0.21ab
SO_2 Corrected Color Density (Au)	6.5 ± 0.42ab	5.7 ± 0.23b	6.2 ± 0.33ab	6.8 ± 0.32a	6.8 ± 0.30a
Hue	0.961 ± 0.04c	1.129 ± 0.01a	1.004 ± 0.02bc	1.069 ± 0.04ab	1.088 ± 0.05ab
A520-Red hue (Au)	0.347 ± 0.02a	0.282 ± 0.01b	0.318 ± 0.01ab	0.351 ± 0.02a	0.343 ± 0.02a
A420-Yellow hue (Au)	0.334 ± 0.03ab	0.318 ± 0.01b	0.319 ± 0.02b	0.375 ± 0.01a	0.372 ± 0.01a
SO_2 Resistant Pigments (Au)	0.92 ± 0.11ab	0.84 ± 0.05b	0.88 ± 0.04ab	1.04 ± 0.08a	1.06 ± 0.07a
Total Phenolics (Au)	38.9 ± 1.9d	44.2 ± 0.6bc	41.8 ± 0.8cd	46.1 ± 1.6b	50.8 ± 2.2a

Different lowercase letters in rows indicate significant differences among treatments (Tukey'stest, $P<0.05$)

Table 4.3: Wine colour parameters measured by CIELab

Parameter	DS	DS100	WB30	WB60	WB100
Luminosity L*	25.9 ± 1.8b	33.0 ± 0.9a	28.3 ± 2.3ab	28.0 ± 2.3ab	27.8 ± 2.3ab
Red - green a*	53.41 ± 0.4a	53.93 ± 0.2a	54.58 ± 0.9a	54.45 ± 1.0a	54.04 ± 0.7a
Yellow - blue b*	42.12 ± 2.0b	50.83 ± 0.7a	45.11 ± 2.1ab	45.09 ± 2.6ab	45.41 ± 2.8ab
Chroma C*	68.03 ± 1.5b	74.12 ± 0.5a	70.81 ± 2.0ab	70.71 ± 2.2ab	70.60 ± 2.3ab
Tone (°) H*	38.24 ± 1.1b	43.30 ± 0.5a	39.56 ± 0.9b	39.60 ± 1.3b	40.01 ± 1.5b

Different lowercase letters in rows indicate significant differences among treatments (Tukey'stest, $P<0.05$)

Chapter 5

The effect of grape stem inclusion fermentation on phenolic compounds in Pinot Noir wines

5.1 Abstract

The aim of this study is to investigate the impact of adding different proportions of whole bunches (WB30, WB60, and WB100) and stems (DS100) on the concentrations of tannin and phenolics in resultant wines. In comparison to non-stem inclusion treatment (DS), either adding stems (DS100) or whole bunches (except WB30) resulted in a significant increase of tannin, total phenolics and most of monomeric phenolics in resultant wines. Comparing to DS100 treatment, significantly higher concentration of total phenolics and tannin was observed in WB100 treatment. Whole bunch fermentation, including WB30, WB60 and WB100 treatments, didn't show significant impact on the concentration of malvidin-3-O-glucoside, but adding stems into destemmed must (DS100) resulted in a significant decrease of malvidin-3-O-glucoside. These results confirmed that grape stem could be a good source for tannin extraction, but different uses of grape stems (the amount and the form of addition) could have a varied influence on tannin extraction and phenolic composition in resultant wines, which are important to sensory properties of Pinot Noir.

Keywords: Monomeric phenolics, Pinot Noir, stems, tannin, whole bunches

5.2 Introduction

Most of the phenolics found in wine are grape-derived, and they are important constituents in red wines quality, contributing to astringency, bitterness and colour stability of wine (Mercurio, Dambergs, Cozzolino, Herderich, & Smith, 2010; Waterhouse, 2002). The concentration of tannin in grapes depends on several factors such as water status, heat, sunlight and vine vigour (Kennedy, Matthews, & Waterhouse, 2000). The tannins in grapes can be extracted into wine from the skins, seed and stems (if used) during alcoholic fermentation. Extractions rates are mainly depending on time (extended maceration), fermentation temperature and techniques used in winemaking (Sacchi et al., 2005). Tannin concentration in Pinot Noir wine is relatively low compared to other red wines, which is because Pinot Noir grapes have very low skin to seed tannin ratio compared to other red varieties, and seed tannins are more difficult to be extracted during fermentation (Dambergs et al., 2012; De Villiers, 1994; Kennedy, 2008; Waterhouse, 2002). Thus, it could be a difficult challenge for winemakers to enhance extraction of tannins in Pinot Noir wine production.

There are several winemaking practices aimed to improve phenolic composition in wines. Stem inclusion is one of the most famous treatments used to enhance tannin content in wines. In practice, stem inclusion fermentation can be carried out by adding stems into the destemmed must or using whole bunches. As grape stems contain significant amounts of polyphenolic compounds (Makris et al., 2008; Souquet et al., 2000), including stems in the fermentation, have been used to increase tannin concentration in red wines (AWRI, 2018; Casassa et al., 2019; Dambergs et al., 2012; Hashizume et al., 1998). However, most of the previous studies are limited to use 20-50% whole bunches and/or less than 3% stems by weight in winemaking (Casassa et al., 2019; Suriano et al., 2015). Many authors have reported that cold maceration also can enhance total phenolics in wines (Álvarez et al., 2006; Casassa et al., 2019; Gustavo González-Neves, Favre, Gil, Ferrer, & Charamelo, 2015). Due to the water solubility of most phenolics, mainly anthocyanins are extracted during the cold maceration period. Casassa and Harbertson (2014) reported that cold maceration could extract flavonols, flavan-3-ols and proanthocyanidin dimers and trimers at different rates due to their polarity conditions. However, polymeric and oligomeric forms of proanthocyanidins extraction progressed slower due to their hydrophobicity.

Only a few studies are discussing the effect of whole bunch addition on the resultant wine phenolics profile. Suriano et al. (2015) studied the effect of stem contact maceration on phenolics in Primitivo red wines. The author reported that 100% destemmed treatment showed higher anthocyanin concentration, but lower phenolic concentration with respect to the treatments prepared by adding stems 25% and 50% whole bunches. But, Casassa et al. (2019) reported that 20% whole bunch addition together with cold maceration (CS+WC treatment) in Pinot Noir winemaking could not result in a

significant difference in tannin concentration in both vintages (2014 and 2015) compared to a cold macerated wine without adding whole bunches. However, 3% stem addition together with cold maceration (CS+S treatment) could result in a significantly higher concentration of tannins in 2015 vintage.

Stem inclusion, together with cold maceration needs to be further investigated due to lack of previous publications, and there are some contradictions in the previous findings. It is important to find out the level of the whole bunch required to result in a significant increment of phenolics in wine. Hence this study was designed to investigate the influence of adding different proportions of whole bunches and stems together with cold maceration on total phenol, tannin and monomeric phenol composition in the resultant wines. Another separate treatment was used to assess the difference between whole bunches addition and direct stem addition on the phenolic composition of the resultant wines.

5.3 Methodology

5.3.1 Wine samples

Five treatments were examined in this study: 100% destemmed and crushed grapes (DS), 100% destemmed and crushed grapes with stems added back (DS100), 30% whole bunches (WB30), 60% whole bunches (WB60), and 100% whole bunches (WB100). Fermentation in each treatment was carried out in triplicate using a standard winemaking protocol, which includes 5 days of cold maceration at 4°C and 4 days of post-fermentation maceration at room temperature. Detailed winemaking protocol is described in chapter 3.2.

5.3.2 Total phenolics

The concentration of total Phenolics in resultant wines was measured using microscale Folin-ciocalteau colourimetric method (Wrolstad, 2001). Detailed procedures are described in chapter 3.6.

5.3.3 Methyl cellulose precipitable (MCP) tannin

The concentration of tannin in resultant wines was determined using the 1 mL assay of methyl cellulose precipitation (MCP) method (Mercurio et al., 2007). Detailed procedures are described in chapter 3.4.

5.3.4 Solid-phase extraction and HPLC analysis of monomeric phenolics

Deionised water was used in all aspects. All reagents used in solid-phase extraction (SPE) includes methanol (Thermo Fisher Scientific, New Zealand), formic acid (Thermo Fisher Scientific, New Zealand), acetonitrile (Thermo Fisher Scientific, New Zealand) and absolute ethanol (Scharlau, SA, Australia). All standard reference reagents were HPLC grade and purchased from Sigma-Aldrich, Australia.

Optimised solid-phase extraction (SPE) was used to separate monomeric phenolics and polymeric phenols before HPLC analysis (Jeffery et al., 2008). Monomeric phenolics were quantified using HPLC according to the method described by Gómez-Alonso et al. (2007). Detailed procedures are described in chapter 3.7.

5.3.5 Statistical analysis

Data were presented as mean ± SD of three replicates. In all cases, analysis of variance (ANOVA) was performed with the Tukey comparison test using Minitab 18 (Minitab, US) software package. Least significant differences (LSD, 5%) was used to separate means when a significant P-value was obtained.

5.4 Results and Discussion

5.4.1 MCP tannins and total phenolics

The concentration of MCP tannin in resultant wines were ranged from 863 to 1660 mg/L (Table 5.1), which is within the range of tannin concentration in Pinot Noir reported previously (Casassa et al., 2015; Harbertson et al., 2008; Kemp, Harrison, & Creasy, 2011). In comparison to non-stem inclusion treatment (DS), a significantly higher concentration of tannin was observed in wines from DS100, WB60, and WB100 treatments but not in WB30 treatment. The higher percentage of whole bunch added, the higher concentration of tannin was observed in the resultant wine. The increased tannin in wine could be additionally extracted from stems, which has been reported previously (Dambergs et al., 2012; Hashizume & Samuta, 1997). A recent study by Casassa et al. (2019) also reported that a low percentage (20%) of whole bunch addition didn't result in a significant increase of tannin in Pinot Noir wine. In comparison between WB100 and DS100 treatments, WB100 treatment had a significantly higher concentration of tannin. This may be due to: 1) the precipitation of tannins by interacting with other macromolecules (*e.g*. proteins and polysaccharides) during cold maceration in DS100 (Smith et al., 2015), and 2) the adsorption of tannins to stems during cold maceration in DS100, because grape stems contain polysaccharides (González-Centeno et al., 2010) and proteins (R. B. Ferreira et al., 2000); hence tannin molecules (proanthocyanidins) may bound into them (Aleixandre-Tudo & Du Toit, 2018; Casassa & Harbertson, 2014).

The concentration of total phenolics in resultant wines was ranged from 1691 to 2500 mg/L (Table 5.1), which is within the concentration range of total phenolics in Pinot Noir reported previously (Casassa et al., 2015; Dambergs et al., 2012; Kilmartin, Zou, & Waterhouse, 2002). Similar to the results of tannin, in comparison to DS treatment, a higher concentration of total phenolics was observed in wines made from WB60 and WB100 treatment but not for WB30 treatment. Interestingly, in this study adding 100% of stems (DS100) didn't show a significant increase in the concentration of total phenolics compared to DS treatment. As explained for tannin results, it is possible that some phenolic

compounds in DS100 treatment were precipitated due to the interactions with other macromolecules (*e.g.* proteins and polysaccharides) during cold maceration (Smith et al., 2015), and this reduction of total phenolics counteracts the effect of stem addition on increasing total phenolics.

5.4.2 Quantification of monomeric phenolics by HPLC

The same phenolic compounds were found and quantified in Pinot Noir wines from five treatments (Table 5.2). However, the concentrations of these phenolic compounds varied significantly among treatments.

The flavan-3-ols, predominantly catechin, are the major class of phenolics determined in Pinot Noir wines, which represent 61.6 – 67.9% of total monomeric phenolics quantified by HPLC. The concentrations of catechin and epicatechin were determined in the range of 228 mg/L to 315 mg/L and 89 mg/L to 130 mg/L, respectively (Table 5.2). These values are slightly higher than those reported in Pinot Noir wine previously (Cortell et al., 2007; Van Leeuw et al., 2014). This might be because wine samples in this study were collected at the end of alcoholic fermentation at which stage many flavan-3-ols monomers are extracted from skin and seeds but yet to be polymerised into oligomers or tannins. Addition of stems or whole bunches (except WB30) during fermentation significantly increased the concentrations of both catechin and epicatechin in resultant wines, which is likely due to the extraction of flavan-3-ols from stems (Souquet et al., 2000). The changes in flavan-3-ols between treatments are consistent with previous observations for tannin and total phenolics.

Malvidin-3-O-glucoside is the main anthocyanin found in Pinot Noir wine (Dimitrovska et al., 2011; Iland, 2013). The concentration of malvidin-3-O-glucoside was determined in the range from 124 mg/L to 156 mg/L (Table 5.2), which is within the concentration range reported in Pinot Noir wine previously (Casassa et al., 2019; Cortell et al., 2007). There is a significant decrease of malvidin-3-O-glucoside in DS100 treatment comparing to DS treatment, but no significant differences in the concentration of malvidin-3-O-glucoside between DS and WB treatments. It is known that cold maceration could facilitate the extraction of anthocyanins from destemmed and crushed grapes into juice (Álvarez et al., 2006; Gil-Muñoz et al., 2009; Gómez-Míguez et al., 2007; Koyama et al., 2007), but addition of stems might cause the adsorption of anthocyanins to stems and result in lower concentration of anthocyanins (Andrew, 2016; Suriano et al., 2015).

Quercetin and qurcetin-3-O-glucoside were quantified with a concentration range from 1.24 mg/L to 1.90 mg/L and from 3.74 mg/L to 7.08 mg/L respectively, which is within the concentration range reported in Pinot Noir wine previously (Van Leeuw et al., 2014). Flavonols are mainly found in grape skin and stems (Cheynier & Rigaud, 1986; Makris et al., 2008; Souquet et al., 2000). It seems that addition of stems or whole bunches could result in a significant increase of quercetin in Pinot Noir

wine, which might be due to the additional extraction of quercetin from stems or higher degree of hydrolysis of qurcetin-3-O-glucoside during fermentation.

Benzoic acids, including gallic acid, syringic acid, protocatechuic acid and p-hydroxybenzoic acid, were quantified within the concentration range previously reported in Pinot Noir wine (Van Leeuw et al., 2014). In comparison to DS treatment, a significant increase in all types of hydroxybenoiz acids was observed in Pinot Noir wine made from DS100 treatment. Comparing between DS and WB treatments, there was a significant increase of gallic acid in WB60 and WB100 treatments, and a significant increase of syringic acid in WB100 treatment. The significantly higher concentration of hydroxybenoiz acids in DS100, WB60 and WB100 treatments are likely due to the extraction of hydroxybenoiz acids from stems (Waterhouse et al., 2016).

Hydroxycinnamic acids are mainly present in the pulp but also in grape stems (Doshi, Adsule, Banerjee, & Oulkar, 2015; Waterhouse et al., 2016). The same hydroxycinnamic acids (except ferulic acid) were identified in all five treatments and their concentrations were determined within the range previously reported in Pinot Noir wine (Van Leeuw et al., 2014). Ferulic acid was only observed in DS100 treatment (0.08 mg/L) and WB60 treatment (0.08 mg/L). Caftaric acid is the most abundant hydroxycinnamic acids in wine with concentration ranging from 10.4 mg/L to 31.1 mg/L, followed by *cis*-coutaric acid (1.81-2.80 mg/L) and caffeic acid (0.83-2.28 mg/L). Caftaric acid has also been reported previously as major hydroxycinnamic acids in finished wine (Rentzsch et al., 2007). In comparison to DS treatment, the concentrations of caftaric acid, *cis*-coutaric acid and caffeic acid were significantly increased in wines made from DS100, WB60 and WB100 treatments, which is possibly due to the additional extraction of these compounds from stems. There were no significant differences in the concentrations of *trans*-coutaric acid, *p*-coumaric acid, and grape reaction product (GRP) between treatments.

Resveratrol is a stilbene produced by grapevine in all tissues as a phytoalexin in response to various stress factors such as UV-radiation, mechanical injuries, and fungal attacks (Aaviksaar et al., 2003; Waterhouse, 2002). Resveratrol was quantified in all Pinot Noir wines with concentrations ranging from 0.06 mg/L to 0.47 mg/L, which is within the concentration range previously reported in Pinot Noir wine (Baraboy, 2009; Melzoch et al., 2001). In comparison to non-stem inclusion treatment (DS), a significant increase of resveratrol was observed in DS100, WB60 and WB100 treatments but not in WB30 treatment. This is because more resveratrol can be extracted from stems which are the richest source of resveratrol Melzoch et al. (2001).

5.5 Conclusion

Phenolic compounds are important to the quality of wine due to their contribution to wine colour, taste and mouthfeel (Gawel, 1998; Reynolds, 2010). Enhancing the extraction of colour and tannins is

a difficult challenge in light-pigmented Pinot Noir production. This study confirmed that grape stems could be a good resource of tannin extraction, but stem addition during fermentation are also likely to decrease the concentration of malvidin-3-O-glucoside which is the major anthocyanin in Pinot Noir wine. Grape anthocyanins are initially responsible for wine colour, but they will be displaced progressively by more stable polymeric pigments during wine ageing (T. Somers, 1971). Thus, the impact of stem inclusion fermentation on development of wine colour and non-bleachable pigments during ageing should be further studied in the future.

Comparing to DS100 treatment, more tannins and total phenolics are extracted in WB100 treatment. Comparing among the three whole bunch addition treatments (WB30, WB60 and WB100), 30% of whole bunch addition didn't show significant impact on the concentrations of total phenolics, tannin, and most of monomeric phenolics. These results suggest both the way of using stems (whole bunch fermentation versus adding stems into destemmed must) and the amount of stems included in fermentation could have a varied impact on phenolic extraction. Insights into the mean degree of polymerisation (mDP) and size distribution of tannins extracted in wine made from different stem inclusion treatments are worth exploring in the future study.

Table 5.1: Tannin and total phenolics determined in wines from five treatments

Parameter	**DS**	**DS100**	**WB30**	**WB60**	**WB100**
Tannin (mg/L)[1]	863 ± 69c	1270 ± 66b	1073 ± 70bc	1294 ± 79b	1660 ± 143a
Total phenolics (mg/L)[2]	1691 ± 89c	1997 ± 77bc	1900 ± 91bc	2097 ± 149b	2500 ± 147a

[1] Epicatechin equivalent; [2] Gallic acid equivalents; Different lowercase letters in rows indicate significant differences among treatments (Tukey's test, $P<0.05$)

Table 5.2: Monomeric phenolic composition in wines from five treatments

Monomeric phenol (mg/L)	DS	DS100	WB30	WB60	WB100
Flavan-3-ols					
Catechin	228 ± 10c	276 ± 17ab	251 ± 11bc	269 ± 17b	315 ± 19a
Epicatechin	89 ± 4b	111 ± 10a	117 ± 7a	123 ± 3a	130 ± 11a
Subtotal	317 ± 14c	387 ± 27ab	368 ± 17bc	393 ± 18ab	445 ± 29a
Anthocyanins					
Malvidin-3-O-glucoside	156 ± 5a	124 ± 4b	138 ± 10ab	146 ± 4a	140 ± 12ab
Flavonols					
Quercetin	1.24 ± 0.18b	1.85 ± 0.13a	1.33 ± 0.06b	1.90 ± 0.22a	1.58 ± 0.08ab
Quercetin-3-glucoside	7.08 ± 0.70a	3.74 ± 0.30d	4.37 ± 0.08cd	5.67 ± 0.70bc	5.80 ± 0.35ab
Subtotal	8.32 ± 0.62a	5.59 ± 0.35b	5.7 ± 0.02b	7.58 ± 0.91a	7.39 ± 0.39a
Benzoic acids					
Gallic acid	13.8 ± 0.3c	18.1 ± 1.3ab	15.8 ± 0.9bc	17.9 ± 0.4ab	20.0 ± 1.2a
Syringic acid	3.72 ± 0.06a	3.20 ± 0.26b	3.47 ± 0.10ab	3.90 ± 0.30a	2.99 ± 0.10b
Protocatechuic acid	1.84 ± 0.27b	2.75 ± 0.58a	1.87 ± 0.33ab	2.6 ± 0.17ab	2.52 ± 0.06ab
p-Hydroxybenzoic acid	0.46 ± 0.05b	0.81 ± 0.16a	0.47 ± 0.03b	0.51 ± 0.08b	0.50 ± 0.14b
Subtotal	19.8 ± 0.6b	24.9 ± 1a	21.6 ± 1b	24.9 ± 0.9a	26 ± 1.2a
Hydroxycinnamic acids					
Caftaric acid	10.4 ± 1.4c	22.6 ± 1.8b	13.1 ± 2.0c	18.9 ± 1.6b	31.1 ± 3a
cis-Coutaric acid	1.81 ± 0.01c	2.69 ± 0.18a	1.89 ± 0.22bc	2.26 ± 0.10b	2.80 ± 0.17a
trans-Coutaric acid	0.09 ± 0.03a	0.11 ± 0.04a	0.11 ± 0.04a	0.17 ± 0.07a	0.07 ± 0.01a
Caffeic acid	0.83 ± 0.10c	2.28 ± 0.24a	1.14 ± 0.03bc	1.29 ± 0.16b	2.07 ± 0.19a
p-Coumaric acid	0.08 ± 0.01a	0.11 ± 0.01a	0.06 ± 0.01a	0.08 ± 0.04a	0.06 ± 0.03a
Ferulic acid	nd	0.08 ± 0.04a	nd	0.08 ± 0.02a	nd
GRP	0.66 ± 0.22a	0.59 ± 0.18a	0.98 ± 0.25a	1.42 ± 0.96a	0.50 ± 0.34a
Subtotal	13.8 ± 1.7c	28.5 ± 2.2b	17.3 ± 1.9c	24.1 ± 1.8b	36.6 ± 3.6a
Stilbenes					
Resveratrol	0.06 ± 0.04c	0.27 ± 0.06b	0.12 ± 0.02bc	0.28 ± 0.06b	0.47 ± 0.11a

Different lowercase letters in rows indicate significant differences among treatments (Tukey's test, $P<0.05$)

Chapter 6

The effect of grape stem inclusion fermentation on aroma profiling of Pinot Noir wines

6.1 Abstract

This study aims to investigate the impact of adding different proportions of whole bunches (WB30, WB60, and WB100) and stems (DS100) on the aroma composition in the resultant wines. Headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME-GC-MS) was used to determine a total of 51 aroma compounds in Pinot Noir wines, including 3 types of methoxypyrazines: 3-isopropyl-2-methoxypyrazine (IPMP), 3-isobutyl-2-methoxypyrazine (IBMP), and sec-butyl-methoxypyrazine (SBMP). Comparing to non-stem added treatment (DS), 100% stem inclusion and whole bunch addition increased the concentrations of eugenol, IBMP, IPMP, phenol, α-Ionone and ethyl cinnamate in the resultant wines, and those compounds are mainly responsible for green, woody and spicy aromas in wine. When comparing 100% stem added treatments, DS100 treatment had significantly higher concentrations of ethyl octanoate, octyl acetate, ethyl decanoate, ethyl hexanoate, hexanoic acid and octanoic acid compared to WB100 treatment, and those compounds are mainly responsible for fruity, floral and fatty aromas in wine.

Keywords: Aroma, odour activity values, stem inclusion, treatments, whole bunches.

6.2 Introduction

Aroma compounds of wine play a significant role in the perception of wine quality (Reynolds, 2010). The complexity of red wine aroma is high compared to white wines due to the presence of volatile phenolics which exert a suppression effect on fruity notes (Atanasova, Thomas-Danguin, Langlois, Nicklaus, & Etievant, 2004). Several previous studies have investigated the relationship between aroma composition and respective sensory perception in wine (Benkwitz et al., 2012; Escudero, Campo, Farina, Cacho, & Ferreira, 2007; Fang & Qian, 2005; Ferreira et al., 2016; Tomasino et al., 2015). Pinot noir wine has distinctive red fruity aromas evoking particularly odours of small-stone fruits (plum and cherry) (Fang & Qian, 2006). Many authors reported that Pinot noir aroma is a complex formulation of many aroma compounds, and there is no single compound responsible for the characteristic aroma of Pinot noir wine (Fang & Qian, 2005, 2006; Rutan et al., 2014; Tomasino et al., 2015). Fang and Qian (2005) reported that 2-phenyl ethanol, 3-methyl-1-butanol, ethyl 2-methylpropanoate, ethyl butanoate, isoamyl acetate, ethyl hexanoate, and benzaldehyde are the most significant aroma compounds found in Oregon state Pinot Noir wines using aroma extract dilution analysis (AEDA). Tomasino et al. (2015) identified the most significant aroma compounds in New Zealand Pinot Noir wines using canonical correlation analysis. The author showed that ethyl decanoate, ethyl octanoate and 2-phenyl ethanol had a negative correlation with “dark fruit” aromas. While benzaldehyde had a negative correlation with “jam” aroma and a positive correlation with “oak” aromas in wines.

Concentration and the synthesis of aroma compounds depend on several factors, such as grape maturity, weather conditions, soil, irrigation, the winemaking process, geographic region, etc. (Mirandalopez, Libbey, Watson, & McDaniel, 1992). Many authors have studied the impact of different winemaking treatments on resultant wine aroma composition: Zhang, Petersen, Liu, and Toldam-Andersen (2015) analysed the impact of different pre-fermentation treatments including direct press after crushing, whole cluster press, cold maceration, and skin fermentation on the aroma composition of Solaris wine. Cold maceration could enhance apricot and apple aromas, and skin fermentation resulted in flowery aromas in wines. Girard et al. (2001) studied the effect of fermentation temperature on the aroma composition of Pinot Noir wine. There were four treatments in the experimental design: high, ambient, cold and modified cold temperature treatments. Use of high temperatures (30°C) caused to increase vegetal characters in wine. The treatment called modified cold temperature (15°C) had the most tropical fruit and spicy aromas and had the least vegetal characters.

Cold maceration is mainly used to extract water-soluble phenolics, specially anthocyanin before starting the fermentation. However, it was reported that cold maceration could influence on aroma composition of wines. Cai et al. (2014) investigated the effect of cold maceration on the aroma composition of Cabernet Sauvignon wine. The author reported that cold maceration reduced some

higher alcohols and increased some acetate esters and β-Damascenone in the resultant wines. Casassa et al. (2019) reported that cold macerated Pinot Noir wine (CS-treatment) had significantly higher concentrations of hexyl acetate, and 1-hexanol, and significantly lower concentrations of ethyl hexanoate, D-limonene, and β-damascenone compared to a control wine without undergoing cold maceration.

Higher proportions of stem inclusion can result in more green, vegetative aromas in wines, which is mainly due to the extraction of methoxypyrazines and C6 alcohols and C6 aldehydes (Hashizume & Samuta, 1997). Hence, most of the previous studies were used a lower proportion of whole bunches and stems in their studies (Casassa et al., 2019; Suriano et al., 2015). Casassa et al. (2019) reported that 20% whole bunch addition together with cold maceration (CS+WC treatment) in Pinot Noir winemaking could significantly increase ethyl butyrate, ethyl hexanoate, hexyl acetate, and 1-octanol, and significantly lower concentrations of ethyl 2-methylbutyrate, and isobutanol compared to a cold macerated wine without adding whole bunches (CS treatment).

Studying the effect of different winemaking techniques on wine aroma composition is essential to understand the relationship between aroma compounds and their sensory impact on wines. It can help wine producers and manufacturers to make good quality wine. Most of the previous studies involved in finding the effects of a specific maceration practise at once, on aroma composition of wine. As well as, it is rare to find previous studies involved in finding the effect of whole bunch addition on Pinot Noir wine aroma. In this study, both cold maceration and stem inclusion practised for finding the combined effect of these two treatments on the resultant wine aroma composition. Five treatments are involved in the experimental design to facilitate identifying the effect of adding different proportions of stems and evaluate the impact of different stem inclusion methods (direct stem addition and whole bunch addition) on the resultant wine aroma composition.

6.3 Methodology

6.3.1 Wine Samples

Five treatments were examined in this study: 100% destemmed and crushed grapes (DS), 100% destemmed and crushed grapes with stems added back (DS100), 30% whole bunches (WB30), 60% whole bunches (WB60), and 100% whole bunches (WB100). Fermentation in each treatment was carried out in triplicate using a standard winemaking protocol, which includes 5 days of cold maceration at 4°C and 4 days of post-fermentation maceration at room temperature. Detailed winemaking protocol is described in chapter 3.2.

6.3.2 Aroma profiling of wines

The headspace solid-phase microextraction with gas chromatography tandem mass spectrometry (HS-SPME-GC-MS) method (Tomasino et al., 2015) was used to determine a total of 48 aroma compounds in Pinot Noir wines, including 20 esters, 9 alcohols, 7 volatile fatty acids, 1 aldehyde, 4 volatile phenols, 3 norisoprenoids, and 4 monoterpenes. Detailed procedures ware described in chapter 3.8.1.

The headspace solid-phase microextraction with multi-dimensional gas chromatography-tandem mass spectrometry (HS-SPME MD-GC-MS) method (Parr et al., 2016) was used with some modifications to determine 3 types of methoxypyrazines, including 3-isopropyl-2-methoxypyrazine (IPMP), 3-isobutyl-2-methoxypyrazine (IBMP), and 3-sec-butyl-methoxypyrazine (SBMP). Detailed procedures are described in chapter 3.8.2.

6.3.3 Odour activity values (OAVs)

The odour activity value (OAV) for each aroma compound was calculated by using the respective aroma compound concentration divided by the corresponding perception threshold published from previous studies (Table A.1). The complete methodology is described in chapter 3.8.3.

6.3.4 Statistical analysis

Data were presented as mean ± SD of three replicates. In all cases, analysis of variance (ANOVA) was performed with the Tukey comparison test using Minitab 18 (Minitab, US). Least significant differences (LSD, 5%) was used to separate means when a significant P-value was obtained.

Significant aroma attributes were chosen for the principal component analysis (PCA) at a 0.05 level of significance using Minitab 18 (Minitab, US).

6.4 Results and Discussion

A total of 51 aroma compounds were identified and quantified in Pinot noir wines, including 20 esters, 7 volatile fatty acids, 9 higher alcohols, 3 methoxypyrazines, 1 aldehyde, 4 volatile phenols, 3 norisoprenoids, and 4 monoterpenes (Table 6.1). These aroma compounds have also been previously reported in Pinot noir wine (Girard et al., 2001; Rutan et al., 2014; Tomasino et al., 2015). Previously published concentration ranges of identified aroma compounds with the perception threshold levels are summarised in Table A.1. (See the Appendix).

6.4.1 Esters

Esters are the aromatic and fruity compounds in wine, which can be grouped into two classes: acetate esters and ethyl esters. There is no significant difference in the concentrations of total acetate esters

between treatments, but WB100 treatment had significantly lower total ethyl ester concentration compared to DS treatment (Table 6.1). The concentration of total acetate esters was higher than the concentration of total ethyl esters in all Pinot Noir wines, which is in agreement with previous findings (Jiang, Xi, Luo, & Zhang, 2013).

Ethyl acetate showed the highest concentration among all the identified esters, accounting for over 92% of total esters, which agrees with previous findings (Girard et al., 2001; Jiang et al., 2013; Song et al., 2015). Among the six acetate esters identified in this study, there were significant differences in the concentrations of 2-methylbutyl acetate, isoamyl acetate, hexyl acetate and octyl acetate between treatments, but only the concentration of isoamyl acetate, contributing banana aroma into wines, was higher than its perception threshold. Adding stems or whole bunches at 60% resulted in a significant decrease of isoamyl acetate in the resultant wine.

There were significant differences in the concentrations of ethyl 2-methylbutyrate, ethyl hexanoate, ethyl octanoate, diethyl succinate, ethyl cinnamate, and ethyl decanoate between treatments. Among these ethyl esters, only ethyl hexanoate, ethyl octanoate, ethyl cinnamate, and ethyl decanoate had their concentrations above the perception thresholds (Table A.1). The 100% whole bunch fermentation resulted in a significant decrease of ethyl hexanoate and ethyl octanoate in wine. Adding stems (DS100) or a high percentage of whole bunches (WB60 and WB100) led to a significant increase of ethyl cinnamate, contributing spicy and woody aromas into wine.

6.4.2 Volatile fatty acids

Volatile fatty acids are typically described as cheesy and oily aroma in wine (Fang & Qian, 2006). The production of volatile fatty acids has been reported to be dependent on fermentation conditions such as the grape variety, yeast strains, sugar content, pH and temperature (Shinohara, 1986). Acetic acid was the major volatile fatty acid (accounting 89-92%) found in all treatments, and its concentration was below the perception threshold. Adding stems (DS100) showed no significant difference in the concentration of fatty acids compared to the DS treatment. However, there were significant differences in the concentrations of butyric acid, hexanoic acid and octanoic acid between WB treatments with the WB100 treatment showing a significantly lower concentration than in the WB30 and WB60 treatments. Shinohara (1986) reported that lower pH could result in a lower concentration of hexanoic acid and octanoic acid in Cabernet Sauvignon wine. Thus, the decreased concentration of hexanoic acid and octanoic acid in Pinot Noir wine could be due to the high pH in the WB100 treatment.

6.4.3 Higher alcohols

Higher alcohols are secondary products of yeast metabolism during alcoholic fermentation (Sumby et al., 2010; Swiegers & Pretorius, 2005). They are mainly contributing to the vegetal and herbaceous

aromas in wine, and important precursors of ester production (V. Ferreira et al., 2000). In this study, total higher alcohols concentration was ranged from 304 to 336 mg/L. Comparing to the DS treatment, only isobutyl alcohol showed significantly higher concentration in DS100 treatment. There were no significant differences in the concentrations of isoamyl alcohol and 1-heptanol in stem inclusion treatments comparing to DS treatment. However, comparing among stem inclusion treatments (DS100, WB30, WB60 and WB100), the DS100 treatment showed a significantly higher concentration of isobutyl alcohol but a lower concentration of 1-heptanol. Isoamyl alcohol was the major higher alcohol accounting for 70-73% of total higher alcohols found in all treatments.

6.4.4 Methoxypyrazines

Methoxypyrazines, including 3-isopropyl-2-methoxypyrazine (IPMP), 3-sec-butyl-methoxypyrazine (SBMP), and 3-isobutyl-2-methoxypyrazine (IBMP), were analysed in this study as they are the main contributor to vegetative and green characteristics in the wine. Adding stems (DS100) or whole bunches (WB60 and WB100) resulted in a significant increase of the concentration of total methoxypyrazines (Table 6.1), which is likely due to the extraction of methoxypyrazines form grape stems. Hashizume and Samuta (1997) have reported that the concentrations of IPMP, SBMP and IBMP in Cabernet Sauvignon grape stem were 43 ng/kg, 64 ng/kg and 205 ng/kg respectively, and their concentrations in corresponding wines made by adding 100% stems were 2.7 ng/L, 2.8 ng/L and 33.8 ng/L respectively.

The IPMP was not detected in non-stem added treatment (DS) but observed in all stem inclusion treatments, indicating the possible extraction of IPMP from grape stems. The concentration of IPMP was higher in DS100 than in WB100 treatment, indicating the cold maceration with stem addition could even further increase the extraction of IPMP from stems. There was no significant difference in the concentration of SBMP between treatments. The IBMP was the major methoxypyrazines determined in all Pinot Noir wines, and stem inclusion treatments (except WB30) significantly increased the concentration of IBMP with the highest concentration observed in DS100 treatment. Previous studies also reported that IBMP is the main methoxypyrazine found in red wines (Belancic & Agosin, 2007; Botezatu, Kotseridis, Inglis, & Pickering, 2016; Hashizume & Samuta, 1997; Rauhut & Kiene, 2019). These results confirm that inclusion of grape stems in fermentation could significantly increase the extraction of methoxypyrazines, and thus more vegetative/green characteristics in the resultant wine.

6.4.5 Other aroma compounds

Benzaldehyde concentration was ranging from 15.8 to 24.0 µg/L. Its concentration was significantly increased in the DS100 treatment compared to other treatments (Table 6.1). Four volatile phenols, including phenol, guaiacol, 4-ethyl guaiacol and eugenol, were determined in this study, and stem

inclusion treatments showed an increased concentration of phenol and eugenol but a decreased concentration of guaiacol in wine. Phenol and eugenol have been reported present in grape stems (Ruiz-Moreno et al., 2015), so adding stems or whole bunches could increase the extraction of phenol and eugenol from the stem into wine, and consequently increase spicy and woody aromas in the resultant wine. Guaiacol and 4-methyl guaiacol are the products from lignin degradation and commonly found in wine with barrel maturation. These compounds may naturally occur in grapes and leaves (Wirth, Guo, Baumes, & Günata, 2001). Stem inclusion fermentation had no impact on the concentration of 4-ethyl guaiacol, but significantly decreased the concentration of guaiacol in resultant Pinot Noir wines made with the addition of whole bunches.

Three norisoprenoids including β-damascenone, α-Ionone and β-ionone were determined in all Pinot Noir wines. Only β-damascenone and α-Ionone showed significant differences in their concentrations between treatments with the WB100 treatment showing the lowest concentration of β-Damascenone and the DS treatment showing the lowest concentration of α-Ionone. These results indicate that cold maceration could increase the concentration of β-Damascenone (Cai et al., 2014) and stem inclusion fermentation could increase the concentration of α-Ionone.

Four terpenes including geraniol, linalool, nerol and citronellol have been determined in Pinot Noir wines, but none of them showed significant differences in their concentrations between treatments, indicating stem inclusion fermentation had little impact on floral characteristics in the resultant wine.

6.4.6 PCA Analysis

Significant aroma attributes were chosen to conduct the principal component analysis (PCA) representing 66.3% of the variation in the data set with 41.8% and 24.5% explained by PC1 and PC2, respectively (Figure 6.1). Wines were separated very well between treatments on PCA plots. On the PC1 plot, wines made from DS and WB30 treatments were located to the right on the plot and characterised mostly by hexyl acetate and 2-methylbutyl acetate which contribute to fruity aromas into wine; wines made from WB60 treatment were located to the centre on the plot; and wines made from DS100 and WB100 treatments were located to the left on the plot and characterised mostly by eugenol and IBMP which contribute to spicy, woody and vegetative aromas into wine. On the PC2 plot, wines made from DS100 treatment were located to the top of the plot, which separated well from wines made from WB100 treatment that were located to the bottom of the plot. Wines made from these two treatments were mainly characterised by octyl acetate and ethyl octanoate which contribute to fruity aroma into wine.

6.4.7 The odour activity values in different treatments

The OAVs (odour activity values) are commonly used to assess the contribution of volatile compounds on the overall perception of wine aroma (Cai et al., 2014; V. Ferreira et al., 2000; Zea et al., 2007; Zhu, Zhang, Shi, & Duan, 2019). Ferreira, Ortín, Escudero, López, and Cacho (2002) suggested that compounds with OAV between 0.5 and 10 are important on the overall aroma of the wine. However, other studies have considered any aroma compounds with OAV above 0.1 are important contributors to the overall wine aroma due to the synergistic effect of certain aroma compounds (Cai et al., 2014; Zea et al., 2007). Thus, in this study, all aroma compounds with OAV above 0.1 were summarised in Table 6.2. Hence, 36 out of 51 aroma compounds are having their OAV values above 0.1. Those aroma compounds were categorised into 8 aroma series based on their odour descriptors (Table A.1). After that total OAVs (∑OAV) for each aroma series were calculated by adding the OAVs of each individual compound listed in Table 6.2 and showed in Figure 6.2. The ∑OAV of series 8 (Nutty) is not shown in Figure 6.2, due to its low contribution to overall wine aroma (∑OAV<1)

Total OAVs (∑OAV) of Fruity, floral, chemical and fatty/oily aroma series were not significantly affected by the winemaking treatment (Figure 6.2). But it seems that ∑OAVs of fruity and fatty/oily aroma series are decreasing when increasing the whole bunch proportion. The fruity aroma series was dominated by the higher OAV values of ethyl hydrocinnamate, ethyl hexanoate and ethyl acetate in all treatments (Table 6.2). Moio and Etievant (1995) reported that ethyl hydrocinnamate is one of the main aroma compounds found in Burgundy Pinot noir wines. As well as, Fang and Qian (2005) showed that ethyl hexanoate is also important aroma compound in Oregon state Pinot Noir wines. The isovaleric acid, hexanoic acid, and butyric acid were the highest contributors for ∑OAV fatty/oily aroma series in Figure 6.2.

Stem addition (DS100) and whole bunch addition (except WB30) led to significantly higher ∑OAV of spicy, woody and vegetative/green aroma series compared to non-stem added treatment (DS) (Figure 6.2). Ethyl cinnamate and eugenol were the highest contributors for ∑OAV of spicy and woody aromas series in wines, and they were the most significant aroma chemicals to differentiate treatments. In contrast, IBMP, 1-hexanol and IPMP were the highest contributors for ∑OAV of vegetative/green aroma series in the resultant wines. The IBMP and IPMP concentrations were the most significant aroma chemicals to differentiate treatments. However, stem inclusion method (whole bunch addition, and direct stem addition) was not affected on ∑OAV of spicy, woody and vegetative/green aroma series in DS100 and WB100 treatments.

6.5 Conclusion

Stem inclusion fermentation can significantly influence the aroma profile of resultant Pinot Noir wine. Adding 100% grape stems or a high percentage of whole bunches (60% and 100%) in the fermentation could significantly increase the concentrations of eugenol, phenols, IPMP and IBMP responsible for woody, spicy, vegetative and green aromas. Vegetative or green characters in wine, indicating under ripeness of grapes, are normally negatively associated with wine quality. However, 30% of whole bunch addition did not show a significant difference in vegetative/green characters compared to the non-stem inclusion treatment. Thus, a low percentage of whole bunch addition is recommended for winemakers to avoid extraction of green characters from stems.

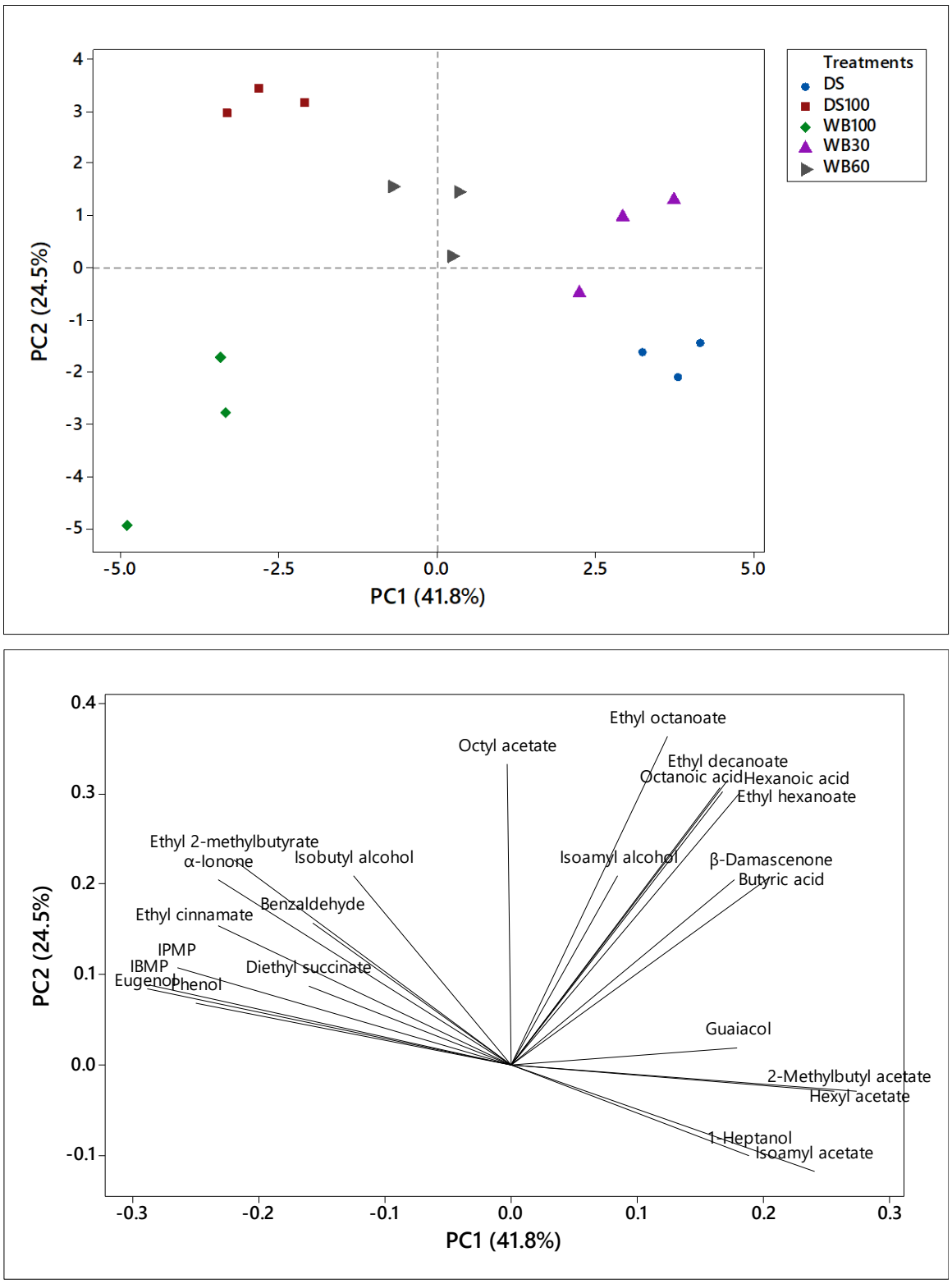


Figure 6.1: Principle component analysis biplots for Pinot Noir wines from five treatments

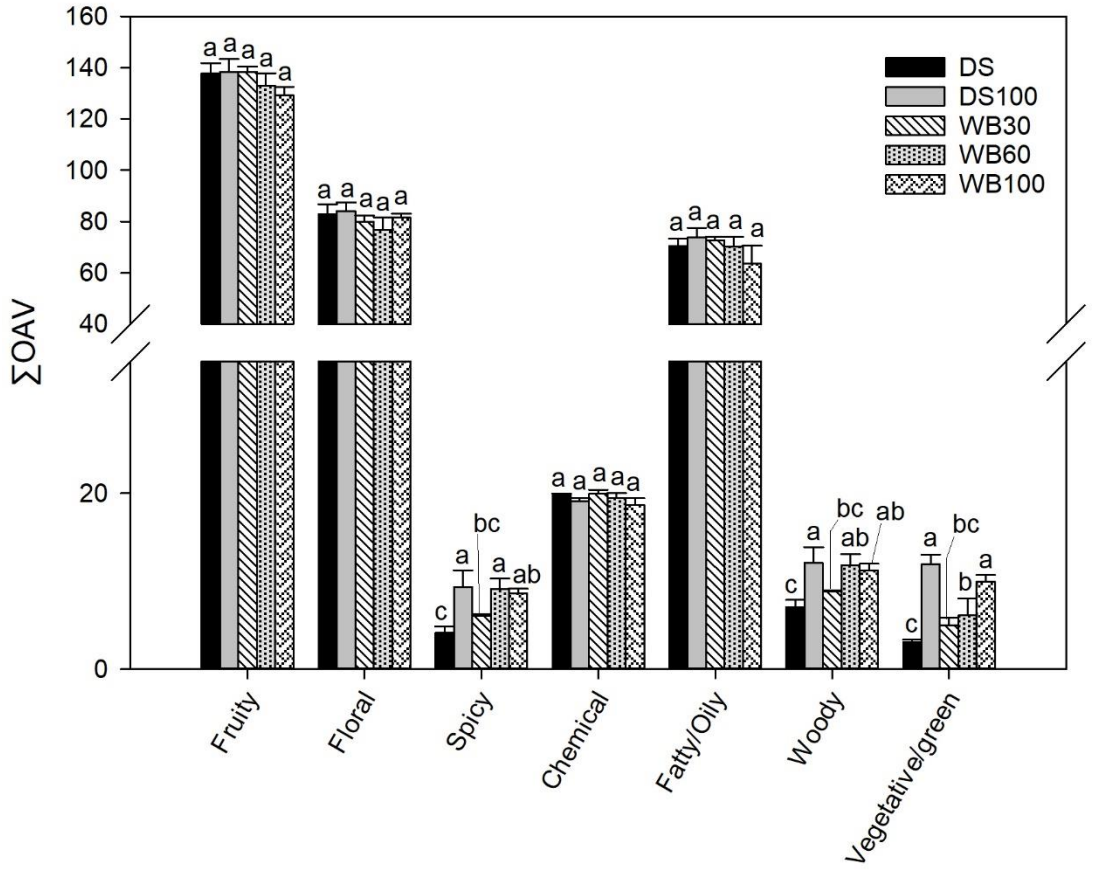


Figure 6.2: Total OAVs (∑OAV) of aroma series in wine from five treatments

Table 6.1: Concentrations of aroma compounds in wine from five treatments (n=3)

Aroma Chemical	DS	DS100	WB30	WB60	WB100
Acetate Esters					
Ethyl acetate (mg/L)	55.8 ± 3.6a	49.0 ± 0.2a	55.5 ± 3.7a	56.9 ± 5.5a	53.7 ± 3a
Isobutyl acetate (µg/L)	30.7 ± 3.3a	28.2 ± 1.8a	32.2 ± 0.7a	28.0 ± 2.4a	29.4 ± 1.5a
2-Methylbutyl acetate (µg/L)*	239 ± 20ab	202 ± 11b	260 ± 12a	219 ± 25ab	204 ± 9b
Isoamyl acetate (µg/L)*	199 ± 5a	165 ± 13b	198 ± 0a	160 ± 15b	171 ± 8ab
Hexyl acetate (µg/L)*	5.62 ± 0.42a	4.32 ± 0.59b	5.96 ± 0.29a	4.91 ± 0.43ab	4.45 ± 0.37b
Octyl acetate (µg/L)*	4.79 ± 0.11ab	6.26 ± 1.41a	5.41 ± 0.46ab	5.85 ± 0.28ab	4.29 ± 0.21b
Subtotal (mg/L)	56.3 ± 3.6a	49.5 ± 0.3a	56 ± 3.7a	57.3 ± 5.5a	54.1 ± 3a
Ethyl Esters					
Ethyl isobutyrate (µg/L)	26.8 ± 1.2a	30.7 ± 3.3a	28.1 ± 0.9a	27.9 ± 0.6a	28.6 ± 2.1a
Ethyl butanoate (µg/L)	213 ± 11a	194 ± 8a	222 ± 13a	220 ± 14a	196 ± 18a
Ethyl lactate (µg/L)	1088 ± 100a	906 ± 64a	976 ± 29a	925 ± 112a	897 ± 106a
Ethyl 2-methylbutyrate (µg/L)*	5.71 ± 0.40c	8.85 ± 0.34a	7.38 ± 0.34b	8.18 ± 0.66ab	7.96 ± 0.61ab
Ethyl isovalerate (µg/L)	6.55 ± 0.47a	6.81 ± 0.74a	6.50 ± 0.23a	6.30 ± 0.4a	5.95 ± 0.48a
Ethyl pentanoate (µg/L)	1.55 ± 0.10a	1.80 ± 0.02a	1.71 ± 0.06a	1.77 ± 0.18a	1.58 ± 0.21a
Ethyl hexanoate (µg/L)*	484 ± 27a	502 ± 18a	528 ± 23a	512 ± 33a	411 ± 18b
Ethyl heptanoate (µg/L)	2.54 ± 0.16a	2.60 ± 0.16a	2.61 ± 0.15a	2.65 ± 0.08a	2.54 ± 0.34a
2-Phenylethyl acetate (µg/L)	21.7 ± 0.6a	20.7 ± 0.8a	20.8 ± 0.9a	20.0 ± 1.6a	21.6 ± 0.7a
Ethyl octanoate (µg/L)*	794 ± 28ab	864 ± 42a	854 ± 18a	836 ± 22a	694 ± 63b
Diethyl succinate (µg/L)*	441 ± 16b	526 ± 52ab	509 ± 42ab	595 ± 41a	551 ± 28a
Ethyl cinnamate (µg/L)*	3.50 ± 0.80b	8.85 ± 2.14a	5.54 ± 0.23ab	8.79 ± 1.41a	8.10 ± 0.90a
Ethyl hydrocinnamate (µg/L)	114 ± 6a	115 ± 5a	110 ± 3a	105 ± 8a	112 ± 1a
Ethyl decanoate (µg/L)*	541 ± 30a	554 ± 21a	556 ± 19a	538 ± 22ab	461 ± 32b
Subtotal (mg/L)	3.74 ± 0.08a	3.74 ± 0.14a	3.83 ± 0.09a	3.81 ± 0.11a	3.40 ± 0.16b
Volatile fatty acids (FA)					
Acetic acid (mg/L)	98 ± 0a	90 ± 13a	102 ± 4a	110 ± 13a	108 ± 2a
Butyric acid (mg/L)*	1.10 ± 0.01ab	1.08 ± 0.07ab	1.14 ± 0.04a	1.17 ± 0.04a	1.00 ± 0.01b
Isobutyric acid (mg/L)	1.90 ± 0.05a	2.09 ± 0.16a	1.95 ± 0.07a	1.96 ± 0.10a	1.91 ± 0.16a
2-Methylbutyric acid (mg/L)	1.45 ± 0.06a	1.55 ± 0.10a	1.49 ± 0.06a	1.43 ± 0.13a	1.32 ± 0.23a
Isovaleric acid (mg/L)	1.67 ± 0.09a	1.77 ± 0.10a	1.72 ± 0.06a	1.64 ± 0.14a	1.52 ± 0.20a
Hexanoic acid (mg/L)*	2.81 ± 0.03a	2.92 ± 0.11a	2.97 ± 0.11a	2.94 ± 0.06a	2.46 ± 0.04b
Octanoic acid (mg/L)*	1.55 ± 0.03ab	1.60 ± 0.11a	1.63 ± 0.05a	1.61 ± 0.02a	1.26 ± 0.25b
Subtotal (mg/L)	109 ± 0a	101 ± 13a	113 ± 4a	121 ± 12a	117 ± 2a
Higher alcohols					
Isobutyl alcohol (mg/L)*	37.2 ± 0.1b	43.0 ± 1.4a	40.2 ± 1.6ab	37.4 ± 1.4b	39.3 ± 2.9ab
Isoamyl alcohol (mg/L)*	229 ± 2abc	238 ± 7a	237 ± 3ab	220 ± 6c	221 ± 9bc
cis-3-Hexen-1-ol (µg/L)	47.2 ± 0.9a	56.1 ± 4.1a	56 ± 2.6a	55.1 ± 6.9a	48.6 ± 6.2a
trans-3-Hexen-1-ol (µg/L)	25.2 ± 1.2a	29 ± 2.7a	28.6 ± 0.5a	29.1 ± 2.8a	26 ± 3.8a
trans-2-Hexen-1-ol (µg/L)	3.73 ± 1.55a	5.65 ± 2.73a	4.17 ± 2.24a	4.71 ± 2.28a	3.70 ± 1.87a
1-Hexanol (mg/L)	2.64 ± 0.12a	2.60 ± 0.19a	2.69 ± 0.08a	2.58 ± 0.10a	2.56 ± 0.21a
1-Heptanol (µg/L)*	51.2 ± 1.7ab	45.5 ± 3.8b	52.5 ± 2.1a	50.7 ± 1.0ab	49.9 ± 2.9ab
Phenylethyl alcohol (mg/L)	45.2 ± 0.3a	52.3 ± 5.3a	46.8 ± 2.0a	44.3 ± 3.6a	51.2 ± 2.2a
1-Octanol (µg/L)	43.6 ± 2.8a	41.4 ± 2.5a	39.4 ± 0.2a	39.5 ± 3.9a	36.5 ± 4.1a

Subtotal (mg/L)	314 ± 2ab	336 ± 14a	326 ± 7ab	304 ± 10b	314 ± 13ab
Methoxypyrazine (MP)					
IPMP (ng/L)	0.00 ± 0.00c	0.97 ± 0.03a	0.01 ± 0.00c	0.06 ± 0.01c	0.63 ± 0.04b
SBMP (ng/L)	0.33 ± 0.37a	1.46 ± 0.47a	1.05 ± 0.36a	0.98 ± 0.59a	1.14 ± 0.29a
IBMP (ng/L)	0.36 ± 0.07c	7.63 ± 0.55a	1.52 ± 0.55bc	2.76 ± 1.30b	6.17 ± 0.32a
Subtotal (ng/L)	0.69 ± 0.38c	10.06 ± 0.98a	2.57 ± 0.81bc	3.80 ± 1.88b	7.94 ± 0.60a
Aldehydes					
Benzaldehyde (µg/L)*	18.8 ± 1.1b	24 ± 1.3a	15.8 ± 1.5b	17.1 ± 2.2b	18.7 ± 0.4b
Volatile phenols					
Phenol (µg/L)*	4.64 ± 0.21b	5.09 ± 0.09a	4.58 ± 0.13b	5.09 ± 0.14a	5.14 ± 0.17a
Guaiacol (µg/L)*	4.55 ± 0.52a	3.52 ± 0.50ab	3.19 ± 0.02b	3.33 ± 0.26ab	2.86 ± 0.54b
4-Ethyl guaiacol (µg/L)	0.20 ± 0.06a	0.23 ± 0.03a	0.19 ± 0.04a	0.17 ± 0.02a	0.23 ± 0.07a
Eugenol (µg/L)*	5.54 ± 0.15b	7.66 ± 0.24a	6.16 ± 0.25b	6.66 ± 0.8ab	7.47 ± 0.35a
Subtotal (µg/L)	14.9 ± 0.51ab	16.5 ± 0.49a	14.1 ± 0.21b	15.2 ± 1.01ab	15.7 ± 0.43a
Norisoprenoids					
β-Damascenone (µg/L)*	20.9 ± 0.4a	19.9 ± 0.8a	19.5 ± 0.7a	19.8 ± 1.0a	17.2 ± 0.6b
α-Ionone (µg/L)*	0.05 ± 0b	0.06 ± 0a	0.06 ± 0a	0.06 ± 0a	0.06 ± 0a
β-Ionone (µg/L)	1.62 ± 0.06a	1.64 ± 0.15a	1.72 ± 0.12a	1.69 ± 0.16a	1.88 ± 0.14a
Subtotal (µg/L)	22.6 ± 0.4a	21.6 ± 0.9a	21.3 ± 0.7a	21.6 ± 0.9a	19.1 ± 0.7b
Monoterpenes					
Geraniol (µg/L)	13.7 ± 0.9a	14.5 ± 1.3a	12.6 ± 0.6a	12.8 ± 0.9a	13.6 ± 1.4a
Linalool (µg/L)	73.9 ± 3.7a	74.7 ± 9.9a	74.1 ± 4.7a	76.8 ± 0.9a	80.4 ± 9.2a
Nerol (µg/L)	6.44 ± 0.83a	6.84 ± 1.67a	7.76 ± 0.84a	6.97 ± 0.49a	7.12 ± 1.21a
Citronellol (µg/L)	17.0 ± 0.5a	18.0 ± 2.7a	16.2 ± 0.5a	16.8 ± 0.3a	18.1 ± 1.6a
Subtotal (µg/L)	111 ± 6a	114 ± 15a	111 ± 6a	113 ± 1a	119 ± 13a

Different lowercase letters in rows indicate significant differences among treatments ($p<0.05$, Tukey comparison).

* Aroma compounds showing significant differences in concentration between treatments and thus selected for the PCA analysis.

Table 6.2: Odour activity values (OAVs) of significant aroma compounds (OAV > 0.1)

Aroma Chemical	DS	DS100	WB30	WB60	WB100
Acetate Esters					
Ethyl acetate	7.45 ± 0.47a	6.54 ± 0.03a	7.40 ± 0.49a	7.59 ± 0.74a	7.16 ± 0.40a
2-Methylbutyl acetate*	0.76 ± 0.06ab	0.65 ± 0.03b	0.83 ± 0.04a	0.7 ± 0.08ab	0.65 ± 0.03b
Isoamyl acetate*	6.64 ± 0.17a	5.51 ± 0.43b	6.59 ± 0.02a	5.34 ± 0.50b	5.69 ± 0.27ab
Ethyl Esters					
Ethyl isobutyrate	1.78 ± 0.08a	2.04 ± 0.22a	1.88 ± 0.06a	1.86 ± 0.04a	1.91 ± 0.14a
Ethyl butyrate	0.53 ± 0.03a	0.49 ± 0.02a	0.56 ± 0.03a	0.55 ± 0.04a	0.49 ± 0.05a
Ethyl 2-methylbutyrate*	0.32 ± 0.02c	0.49 ± 0.02a	0.41 ± 0.02b	0.45 ± 0.04ab	0.44 ± 0.03ab
Ethyl isovalerate	2.18 ± 0.16a	2.27 ± 0.25a	2.17 ± 0.08a	2.10 ± 0.13a	1.98 ± 0.16a
Ethyl pentanoate	0.31 ± 0.02a	0.36 ± 0.00a	0.34 ± 0.01a	0.35 ± 0.04a	0.32 ± 0.04a
Ethyl hexanoate*	34.6 ± 1.9a	35.9 ± 1.3a	37.7 ± 1.7a	36.6 ± 2.3a	29.3 ± 1.3b
Ethyl heptanoate	1.16 ± 0.07a	1.18 ± 0.07a	1.18 ± 0.07a	1.20 ± 0.04a	1.15 ± 0.15a
Ethyl octanoate*	1.37 ± 0.05ab	1.49 ± 0.07a	1.47 ± 0.03a	1.44 ± 0.04a	1.20 ± 0.11b
Ethyl cinnamate*	3.18 ± 0.73b	8.04 ± 1.95a	5.03 ± 0.21ab	7.99 ± 1.29a	7.36 ± 0.82a
Ethyl hydrocinnamate	71.4 ± 3.8a	72.1 ± 3.1a	68.6 ± 2.1a	65.4 ± 4.7a	70.2 ± 0.8a
Ethyl decanoate*	2.7 ± 0.15a	2.77 ± 0.11a	2.78 ± 0.09a	2.69 ± 0.11ab	2.31 ± 0.16b
Volatile fatty acids (FA)					
Acetic acid	0.49 ± 0a	0.45 ± 0.06a	0.51 ± 0.02a	0.55 ± 0.06a	0.54 ± 0.01a
Butyric acid*	6.36 ± 0.08ab	6.23 ± 0.40ab	6.59 ± 0.25a	6.75 ± 0.25a	5.78 ± 0.05b
Isobutyric acid	0.83 ± 0.02a	0.91 ± 0.07a	0.85 ± 0.03a	0.85 ± 0.04a	0.83 ± 0.07a
2-methylbutyric acid	0.48 ± 0.02a	0.52 ± 0.03a	0.50 ± 0.02a	0.48 ± 0.04a	0.44 ± 0.08a
Isovaleric acid	50 ± 2.7a	53.0 ± 2.9a	51.4 ± 1.9a	49.0 ± 4.1a	45.6 ± 6.1a
Hexanoic acid*	6.69 ± 0.07a	6.94 ± 0.27a	7.07 ± 0.26a	7.00 ± 0.15a	5.87 ± 0.09b
Octanoic acid*	3.09 ± 0.05ab	3.19 ± 0.21a	3.26 ± 0.11a	3.21 ± 0.04a	2.51 ± 0.49b
Higher alcohols					
Isobutyl alcohol*	0.93 ± 0.00b	1.07 ± 0.04a	1.00 ± 0.04ab	0.94 ± 0.04b	0.98 ± 0.07ab
Isoamyl alcohol	7.64 ± 0.05abc	7.93 ± 0.22a	7.89 ± 0.10ab	7.32 ± 0.20c	7.37 ± 0.30bc
1-Hexanol	2.4 ± 0.11a	2.36 ± 0.17a	2.44 ± 0.08a	2.35 ± 0.09a	2.33 ± 0.19a
1-Heptanol*	0.26 ± 0.01ab	0.23 ± 0.02b	0.26 ± 0.01a	0.25 ± 0.00ab	0.25 ± 0.01ab
Phenylethyl alcohol	3.23 ± 0.02a	3.74 ± 0.38a	3.34 ± 0.14a	3.17 ± 0.26a	3.66 ± 0.15a
Methoxypyrazines (MP)					
IPMP*	NS	0.49 ± 0.02a	NS	NS	0.32 ± 0.02b
SBMP	0.33 ± 0.37a	1.46 ± 0.47a	1.05 ± 0.36a	0.98 ± 0.59a	1.14 ± 0.29a
IBMP*	0.36 ± 0.07c	7.63 ± 0.55a	1.52 ± 0.55bc	2.76 ± 1.30b	6.17 ± 0.32a
Volatile phenols					
Guaiacol*	0.47 ± 0.03a	0.37 ± 0.05ab	0.34 ± 0.00b	0.35 ± 0.03ab	0.30 ± 0.06b
Eugenol*	0.92 ± 0.02b	1.28 ± 0.04a	1.03 ± 0.04b	1.11 ± 0.13ab	1.24 ± 0.06a
Norisoprenoids					
β-Damascenone*	2.99 ± 0.06a	2.85 ± 0.12a	2.78 ± 0.10a	2.83 ± 0.14a	2.45 ± 0.08b
β-Ionone	0.32 ± 0.01a	0.33 ± 0.03a	0.34 ± 0.02a	0.34 ± 0.03a	0.38 ± 0.03a
Monoterpenes					
Geraniol	0.46 ± 0.03a	0.48 ± 0.04a	0.42 ± 0.02a	0.43 ± 0.03a	0.45 ± 0.05a
Linalool	2.93 ± 0.15a	2.96 ± 0.39a	2.94 ± 0.19a	3.05 ± 0.03a	3.19 ± 0.36a
Citronellol	0.17 ± 0.01a	0.18 ± 0.03a	0.16 ± 0.01a	0.17 ± 0.00a	0.18 ± 0.02a

Different lowercase letters in each row indicate significant differences among treatments ($p<0.05$).

* Aroma compounds showing significant differences in OAV between treatments.

NS – Non significance (< 0.1 OAV)

Chapter 7

General Conclusion and Future Work

In this study, five treatments, including the DS, DS100, WB30, WB60 and WB100 treatments, were prepared to study the effect of stem inclusion fermentation on Pinot Noir wine composition. Either adding stems or whole bunches could result in a decrease of alcohol content attributed to the water content in stems. The inclusion of grape stems also increases the pH of resultant wine due to the extraction of potassium from stems.

Two methods were used to assess the colour of the resultant wines. When concluding the results from Somers method, stem inclusion and whole bunch addition caused a decrease in anthocyanin concentrations, mainly due to adsorption of anthocyanin into stems and formation of polymeric pigments. The inclusion of grape stems also increased the degree of ionisation of anthocyanin, and hue compared to non-stem added treatment. A higher proportion of whole bunch addition (60% and 100% whole bunches) caused to form more unbleachable pigments (SO_2 resistant pigments) in wines. However, in most of the analysis results related to oenological parameters and colour assessment showed that 30% whole bunch addition was not sufficient to result in a significant difference compared to non-stem added treatment. When considering about the CIELab results, only DS100 treatment had significantly different luminosity (L*), tone (H*), yellow-blue (b*), and chroma (C*) coordinate values compared to DS treatment, but red-green (a*) coordinate was not affected by adding stems or whole bunches.

Stem inclusion (direct stem addition and whole bunch addition) increased tannin and total phenolics concentrations due to stem derived tannins. However, 100% stem inclusion led to a significantly lower amount of tannin compared to 100% whole bunch added treatment due to the precipitation of tannins after interacting with proteins and polysaccharides in the media as well as in the stems. It was observed that amount of stem addition and the method of stem addition have varied impacts on the resultant wine phenolics. The concentrations of catechin, gallic acid, caftaric acid, cis-coutaric acid, caffeic acid, and resveratrol in resultant wines were increased with incremental whole bunch addition treatments compared to DS treatment respectively. However, in most cases, WB30 was not enough to result in a significant increase compared to DS treatment.

Both stem addition and whole bunch addition resulted in higher concentrations of eugenol, IBMP, IPMP and phenols attributed for green, woody and spicy aromas in wine. Principle component analysis (PCA) was carried out to find the most significant aroma compounds to categorise treatments, hexyl acetate, 2-methylbutyl acetate, octyl acetate and ethyl octanoate were the most significant aroma

compounds identified in this study to characterise the different treatments. Odour activity value analysis showed that 30% whole bunch addition did not significantly increase green, woody and spicy aroma series values compared to non-stem added treatment. When comparing 100% stem added treatments, DS100 treatment had significantly higher concentrations of ethyl octanoate, ethyl decanoate, octyl acetate, ethyl hexanoate, hexanoic acid and octanoic acid compared to WB100 treatment, which is mostly responsible for fruity and fatty/oily aroma in the wine.

This study has investigated the effect of grape stem inclusion fermentation on the resultant wine composition. Results of this study will be important for winemakers to determine the required quantity and the way of stem inclusion to achieve the desired quality in Pinot Noir wines in terms of colour, phenolics and aroma. However, there are some additional aspects of this study that should be further investigated because they can help to have a clear idea about the impact of stems on resultant wine composition. Potential further investigations include;

- To analyse the composition of Pinot Noir grape stems at different maturity stages.
- Determine the impact of different field practices such as leaf removal on stem composition.
- To standardise the maturity level of stems and evaluate the impact of the maturity stage on resultant wine composition.

Appendix

Table A.1: Information about aroma compounds from previous studies (all concentrations are given in µg/L)

Aroma Chemical (Common name)	Aroma Chemical (IUPAC)	Aroma Description[1, 2, 3, 4, 5, 6]	Perception threshold	Matrix	Aroma Series	(Tomasino et al., 2015)	(Rutan et al., 2014)	(Girard et al., 2001)
Acetate Esters								
Ethyl acetate	Ethyl acetate	Fruity, Pineapple, varnish	7500 [12]	B	4, 1			386.5– 520.5 (×10^3)
Isobutyl acetate	2-Methylpropyl ethanoate	Apple, Banana	1605 [10]	F	1		27-58	10.7-28.2 (×10^3)
2-Methylbutyl acetate	2-Methylbutyl acetate	Banana, fruity	313 [21]	J	1			
Isoamyl acetate	3-Methylbutyl acetate	Banana	30 [12]	B	1	149-378 (×10^3)	189-254	
Hexyl acetate	Hexyl Acetate	Lolly, fruit, Apple, cherry, pear	700 [8]	G	1	2-16	10.6-18.6	3.62-62.7 (×10^3)
Octyl acetate	Octyl acetate	Fruity, neroli, jasmine	50000 [18]	D	1, 2			
Ethyl Esters								
ethyl isobutyrate	Ethyl 2-methylpropanoat	Fruity, strawberry, melon	15 [9]	A	1	104-559	25-54	31.4-94.0 (×10^3)
Ethyl butyrate	ethyl butanoate	Banana, pineapple, strawberry, acid fruits	400 [15]	C	1	116-340	75-153	49-135 (×10^3)
Ethyl lactate	Ethyl 2-hydroxypropanoate	Strawberry, raspberry, buttery	150000 [15]	C	1, 5		134.9-191.7 (×10^3)	
Ethyl 2-methylbutyrate	ethyl 2-methylbutanoate	Sweet fruit, Strawberry	18 [9]	A	1			7.92-28.1 (×10^3)
Ethyl isovalerate	Ethyl 3-methylbutanoate	Fruity, cherry	3 [9]	A	1	12-52	23-54	
Ethyl pentanoate	Ethyl pentanoate	Fruity, strawberry	5 [11]	K	1	1-4		
Ethyl hexanoate	Ethyl hexanoate	Green apple, banana, fruit	14 [9]	A	1	300-593	312-372	142-400 (×10^3)
Ethyl heptanoate	Ethyl heptanoate	Sweet, strawberry, banana	2.2 [16]	K	1	3-9		1.70-6.51 (×10^3)
2-Phenylethyl acetate	2-phenylethyl acetate	Flowery, roses, honey	250 [12]	B	2, 7	-		500-1070
Ethyl octanoate	Ethyl octanoate	Sweet, floral, fruity, banana, pear	580 [14]	C	1, 2	410-874	318-384	213-464 (×10^3)
Diethyl succinate	diethyl butanedioate	Fruity, melon, Over-ripe, lavender	1200000 [14]	C	1, 2		10.9-17.1 (×10^3)	9.96-20.6 (×10^3)

Ethyl cinnamate	ethyl (E)-3-phenylprop-2-enoate	Cinnamon, vanilla	1.1 [9]	A	6, 3		1.6-4.1	
Ethyl hydrocinnamate	ethyl 3-phenylpropanoate	Sweet, pleasant, floral	1.6 [9]	A	1, 2		1.11-2.31	
Ethyl decanoate	Ethyl decanoate	Fruity, fatty, pleasant, soap	200 [9]	A	1, 4, 5	172-971	164-207	73.3-90.6 ($\times10^3$)
Volatile fatty acids(FA)								
Acetic acid	Acetic acid	Volatile acidity, vinegar	200000 [12]	B	4	415-874 ($\times10^3$)		
Butyric acid	butanoic acid	Cheese, rancid	173 [9]	A	5	209-716	1026-1756	1.42-3.47 ($\times10^3$)
Isobutyric acid	2-methylpropanoic acid	Cheese, rancid, butter	2300 [9]	A	5		389-895	3.69-7.46 ($\times10^3$)
2-methylbutyric acid	2-methylbutanoic acid	Cheese, sweaty	3000 [12]	B	5	-		
Isovaleric acid	3-methylbutanoic acid	Cheese, Acid, Rancid	33.4 [9]	A	5	160-591	275-665	
Hexanoic acid	Hexanoic acid	Cheese, fatty, sweaty	420 [9]	A	5	1104-1941	712-1217	1.40-6.22 ($\times10^3$)
Octanoic acid	Octanoic acid	Rancid oily, cheese, fatty	500 [9]	A	5	665-2002	911-1302	1.07-3.07 ($\times10^3$)
Higher alcohols								
Isobutyl alcohol	2-methylpropan-1-ol	Fusel, spiritous	40000 [12]	B	4		10.4-14.7 ($\times10^3$)	16.5-32.1 ($\times10^3$)
Isoamyl alcohol	3-methylbutan-1-ol	Harsh, nail polish	30000 [12]	B	4	136-312 ($\times10^3$)	104.3-150.5 ($\times10^3$)	1861-2579 ($\times10^3$)
cis-3-Hexen-1-ol	(Z)-hex-3-en-1-ol	Herbaceous, green, bitter, fatty	1000 [15]	C	5, 7	24-116	22-43	3.77-6.03 ($\times10^3$)
trans-3-Hexen-1-ol	(E)-hex-3-en-1-ol	Herbaceous, green	1000 [8]	H	7	62-127	18-35	5.6-16.3 ($\times10^3$)
trans-2-Hexen-1-ol	(E)-hex-2-en-1-ol	Herbaceous, green	1000 [18]	D	7	-		
1-Hexanol	Hexan-1-ol	Herbaceous, grass, woody	1100 [15]	C	6, 7	2-5 ($\times10^3$)	809-1272	99-188 ($\times10^3$)
1-Heptanol	Heptan-1-ol	Oily	200 [17]	I	5	12-270		1.64-13.7 ($\times10^3$)
Phenylethyl alcohol	2-phenylethanol	Roses, floral	14000 [9]	A	2	17-101 ($\times10^3$)	68.7-135.0	23.1-26.8 ($\times10^3$)
1-Octanol	octan-1-ol	Jasmine, lemon	800 [14]	C	2			
Methoxypyrazine (MP)								
IPMP	2-methoxy-3-propan-2-ylpyrazine	Earthy, grassy, leafy	0.002[19]	L	7			
SBMP	2-butan-2-yl-3-methoxypyrazine	Green, peas, bell pepper	0.001[20]	K	7			
IBMP	2-methoxy-3-(2-methylpropyl) pyrazine	Herbaceous, earthy	0.001[19]	L	7			
Aldehydes								
Benzaldehyde	Benzaldehyde	Roasted, almond	2000 [15]	C	8	5-66		2.97-5.52 ($\times10^3$)

Volatile phenols								
Phenol	phenol	Phenolic, medicinal	5900 [7]	K	4, 6	Nd-21		
Guaiacol	2-methoxyphenol	Medicinal, smoky	9.5 [9]	A	4, 6		3.1-10.3	
4-Ethyl guaiacol	4-ethyl-2-methoxyphenol	Toasted bread, smoky, clove	33 [9]	A	6	1-46		
Eugenol	2-methoxy-4-prop-2-enylphenol	Cinnamon, clove, wood	6 [9]	A	3, 6	15-80	16.9-25.3	
Norisoprenoids								
β-Damascenone	(E)-1-(2,6,6-trimethylcyclohexa-1,3-dien-1-yl)but-2-en-1-one	Sweet, exotic, flowers, stewed apple, canned peach, dry plum	7 [13]	E	1, 2	1-5	4.02-5.44	
α-Ionone	(E)-4-(2,6,6-trimethylcyclohexen-1-yl)but-3-en-2-one	Sweet fruit	2.6 [9]	M	1		0.28-0.61	180-320
β-Ionone	(E)-4-(2,6,6-trimethylcyclohexen-1-yl)but-3-en-2-one	Dark berries, violet, roses	5 [18]	D	1, 2	Nd-1	0.29-0.42	
Monoterpenes								
Geraniol	(2E)-3,7-dimethylocta-2,6-dien-1-ol	Citrus, Floral	30 [12]	B	1, 2	Nd-5	12.4 – 16.2	600-1020
Linalool	3,7-dimethylocta-1,6-dien-3-ol	Flowery, muscat	25.2 [9]	A	2, 1	41-170	2.25-5.37	
Nerol	(2Z)-3,7-dimethylocta-2,6-dien-1-ol	Violets, floral	300 [18]	D	2		3.79-5.98	
Citronellol	3,7-dimethyloct-6-en-1-ol	Floral, rose	100 [14]	C	2		6.9-11.1	

Aroma description: [1] (Burdock, 2016), [2] (Cai et al., 2014), [3] (Rutan et al., 2014), [4] (Siebert et al., 2005), [5] (Zea et al., 2007), [6] (Zhu et al., 2019)

Perception threshold references: [7] (Baker, 1963), [8] (Benkwitz et al., 2012), [9] (V. Ferreira et al., 2000), [10] (Ferreira et al., 2002), [11] (Flath, Black, Guadagni, McFadden, & Schultz, 1967), [12] (Guth, 1997), [13] (Pineau et al., 2007), [14] (Peinado, Mauricio, & Moreno, 2006), [15] (Peinado et al., 2004), [16] (Takeoka, Flath, Mon, Teranishi, & Guentert, 1990), [17] (Tao & Zhang, 2010), [18] (Zhao, Gao, Qian, & Li, 2017), [19] (Alen, Lacey, Harris, & Brown, 1991), [20] (Sala et al., 2004), [21] (Cameleyre, Lytra, Tempere, & Barbe, 2017)

Matrix used to measure perception thresholds by different authors: A-model wine containing 11% (v/v) ethanol/water solution, 7 g/L glycerine, 5 g/L tartaric acid, and pH at 3.4 (adjusted with 1M NaOH); B-10% (v/v) ethanol/water solution, C-model wine containing 10% (v/v) ethanol/water solution, and pH at 3.5 (adjusted with tartaric acid); D- model wine made with water/ethanol (90 + 10, w/w); E- Thresholds were calculated in red wine; F-model wine containing 10% (v/v), ethanol/water solution, 7 g/L of glycerine, 1 g/L tartaric acid and pH at 3.2; G-model wine containing 12.5% (v/v) ethanol/ water solution at pH 3.2; H-model wine containing 14% (v/v) ethanol/water solution at pH 3.5; I- model wine containing 9.72 g/100 g ethanol/water mixture, 5 g/L tartaric acid and pH adjusted to

3.2; J- model wine containing 12% (v/v) ethanol/water solution, 5g/L of tartaric acid and pH adjusted to 3.5 with NaOH; K- thresholds were calculated in water; L- Thresholds were calculated in white wine; M- No data available

Aroma Series: 1-Fruity, 2-Floral, 3-Spicy, 4-Chemical, 5-Microbiological/oily/fatty, 6-Woody, 7-Vegetative, 8-Nutty

(Tomasino et al., 2015): analysed 34 aroma compounds in the 32 New Zealand regional Pinot Noir wines from solid phase microextraction GC-MS technique; (Rutan et al., 2014): Mean aroma compound concentration range of Estate and Premium Pinot Noir wines in 2009 and 2010 vintages using gas chromatography olfactory analysis (GC/O) technique. Grapes were grown in Bannockburn, Central Otago, New Zealand; (Girard et al., 2001): Mean aroma compound concentration range of four Pinot noir wines made from EC1118 at four different vinification temperatures in British Columbia.

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