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Abstract

Post-fermentation extended maceration (EM) is a traditional winemaking technique whereby the skins and seeds of red grapes are left in contact with the newly fermented red wine for a period of time beyond the completion of primary fermentation. Winemakers utilize this method to improve the tannin and mouthfeel of their wine, however there has been limited research to support these claims.

This project sought to address some

1. Literature Review

1.1 Introduction

Red wine is characterized by the presence of phenolic compounds that contribute to colour, mouthfeel and astringency (Gawel 1998). These compounds occur naturally in grapes of red *Vitis vinifera* cultivars and winemakers aim to extract them into the wine through various methods of maceration. Post-fermentation extended maceration (EM) is one such method that involves leaving the skins and seeds, or pomace, and wine in contact for a period of time beyond the completion of primary fermentation (Peynaud 1984). Beneficial changes to mouth-feel, tannin concentration and structure are sought by winemakers who employ post-fermentation EM (Pers. Comm. PJ Charteris, Brokenwood Wines, March 2010). However, the traditional use of EM has been based on anecdotal evidence, with little understanding of the chemical consequences and outcomes.

As winemakers primarily employ post-fermentation EM to manipulate phenolic extraction processes and the organoleptic profiles of red wine, this review focuses on phenolic wine compounds specifically in the context of extraction, maceration and organoleptic properties. The existing understanding of post-fermentation EM consequences and their application to commercial winemaking practices is also assessed, along with methods of phenolic measurement and analysis.

1.2 Grape and Wine Phenolics

Red wine contains a complex chemical mix of grape-derived phenolics, along with those modified through maceration and fermentation. The extraction of these phenolics during

vinification is an important process in determining final wine quality (Boulton et al. 1996, Lu and Foo 1999, Kennedy and Peyrot des Gachons 2003). There are several important phenolics that exist in grapes and wine, which contribute variously to colour, astringency and mouth-feel. Importantly, the phenolic composition of grapes will vary depending on variety, growing season, vine health and vineyard management (Auw et al. 1996, Gawel 1998, de Freitas et al. 2000, Romero-Cascales et al. 2005). In addition, under the conditions found in red wine (pH 3.2-3.6, 11-16% v/v ethanol) phenolics will undergo complex reactions and modifications over the course of maturation, resulting in changes in the sensory properties of the wine (Gawel 1998, Guadalupe and Ayestaran 2008).

Grapes phenolics are found in skin and seed tissues, in differing concentrations and forms based on cultivar and season (Sun et al. 1999). Their composition in the grape will also change based on fruit maturity; this has given rise to the concept of "phenolic ripeness" as a criterion for optimum harvest (Ristic and Iland 2005, Adams 2006, Rio Segade et al. 2008).

1.2.1 Monomeric Flavonoids and Non-Flavonoids

The two main classes of phenolics found in grapes and wine are the flavonoids and non-flavonoids. Non-flavonoids such as hydroxycinnamic acid are prevalent in red and white wine grapes, but since they do not contribute to wine colour or astringency in red wines (Verdette et al. 1988), they are of little interest in this study. Flavonoids share a basic C6-C3-C6 structure, consisting of an "A" and a "B" aromatic ring, which are joined by a three carbon chain closed by oxygen, to form a heterocyclic ring, as shown in Figure 1-1. This class of compound includes flavan-3-ols, flavonols and anthocyanins (Gawel 1998).

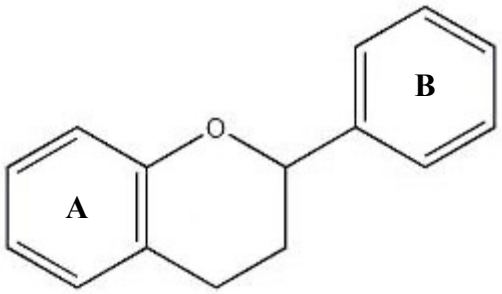
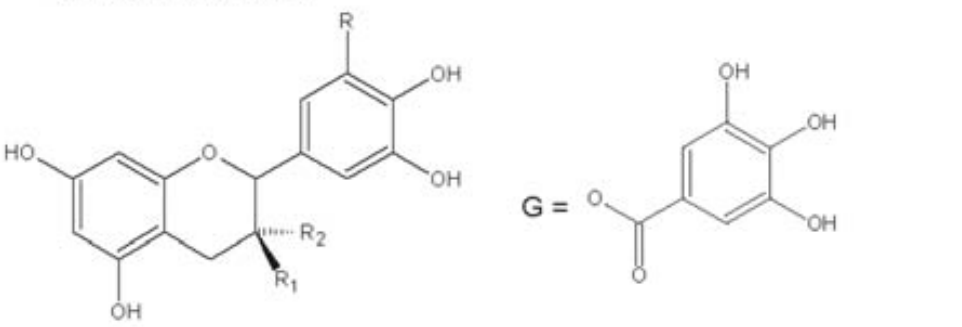


Figure 1.1: Flavonoid C_6-C_3-C_6 structure.

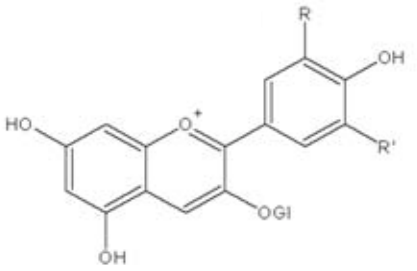
The monomeric flavan-3-ol class is of particular importance in red winemaking, as these molecules contribute to bitterness and astringency (Gawel 1998, Brossaud et al. 2001). They are found in both skins and seeds of grapes and in wine, however Downey et al. (2003) found that seeds contained 15 times the amount of monomeric flavan-3-ols than skins with respect to fresh weight. The molecular structure of several important flavan-3-ols are shown in Figure 1-2.



R=H, R_1=OH, R_2=H : catechin ; R=H, R_1=H, R_2=OH: epicatechin
R=OH, R_1=OH, R_2=H : gallocatechin ; R=OH, R_1=H, R_2=OH: epigallocatechin
R=H, R_1=H, R_2= G: epicatechin 3-gallate
R=OH, R_1=H, R_2= G: epigallocatechin 3-gallate

Figure 1.2: Structure of the main monomeric flavan-3-ol class compounds found in *V. vinifera* cultivars (from Cheynier et al. 2006).

Anthocyanins are a highly reactive class of flavonoids that contribute to the colour of red wine. The expression of anthocyanin colour is highly pH dependent and ranges from red at low pH to blue at higher pH (Eiro and Heinonen 2002). They are also susceptible to bleaching through reactions with bisulphite (Peng et al. 2002). The structure of the main anthocyanin compounds found in red wine is shown below (Figure 1.3). In red *V. vinifera* cultivars these compounds are found as glucosides, bound to glucose at carbon 3 of the centre ring. The sugar residues of anthocyanins may be acylated in some varieties, to give 15 main forms of anthocyanins (Cheynier et al. 2006). Malvidin-3-glucoside is by far the most prevalent form, making up more than 80% of all anthocyanins in Pinot Noir berries (Mazza 2005) and 35% in Shiraz (Ristic et al 2007). Importantly, it has been shown that as monomeric units, anthocyanins do not markedly contribute to astringency or bitterness (Gawel 1998).



R=R'=OH: delphinidin 3-glucoside
R=OH, R'=H : cyanidin 3-glucoside
R=OCH_3, R'=OH:petunidin 3-glucoside
R=OCH_3, R'=H: peonidin 3-glucoside
R=R'=OCH_3: malvidin 3-glucoside

Figure 1.3: Structure of the main classes of anthocyanins as glucosides found in *V. vinifera* cultivars (from Cheynier et al. 2006).

Anthocyanins may also be incorporated into other compounds that contribute to wine colour. These include pigmented polymers formed through reaction with other phenolics (discussed in section 1.2.2) as well as pyranoanthocyanins or "wine pigments", created through reactions with yeast metabolites during fermentation (Bakker and Timberlake 1997, Birse 2007). Examples of

such compounds include; visitin A, formed from reactions with pyruvic acid, and visitin B through reactions with acetaldehyde (Birse 2007). These pigments are more colour stable than free anthocyanins to the effects of pH (Bakker and Timberlake 1997).

1.2.2 Proanthocyanidins and Tannins

This class of compounds includes various polymers of flavan-3-ol monomers linked by C4-C6 or C6-C8 bonds, shown in Figure 1.4 and 1.5. The number of flavan-3-ol monomer subunits that make up a proanthocyanidin molecule is referred to as the degree of polymerisation (Gawel 1998, Kennedy and Jones 2001). While proanthocyanidins contribute significantly to wine astringency (Gawel 1998, Cheynier et al. 2006), it is accepted that they contribute more astringency and less bitterness to wine than monomeric flavon-3-ols, and the perception of astringency increases with the size (polymerisation) of the molecule (Brossaud et al. 2001, Vidal et al. 2002). They occur naturally in grape seeds and skin, however Downey et al. (2003) found that 75% of the extractable proanthocyanidin in Shiraz was from the seeds. It has also been shown that skin proanthocyanidin has a higher degree of polymerisation than seed proanthocyanidin in many varieties, suggesting a potential difference in organoleptic properties between skin- and seed-derived proanthocyanidins (Gawel 1998, Downey et al. 2003, Cheynier et al. 2006).

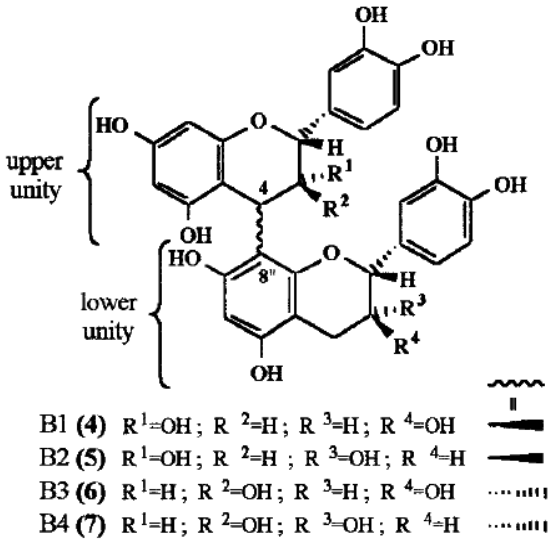


Figure 1.4: C_4-C_8 proanthocyanidin dimers B1-B4 (from de Freitas and Glories 1999).

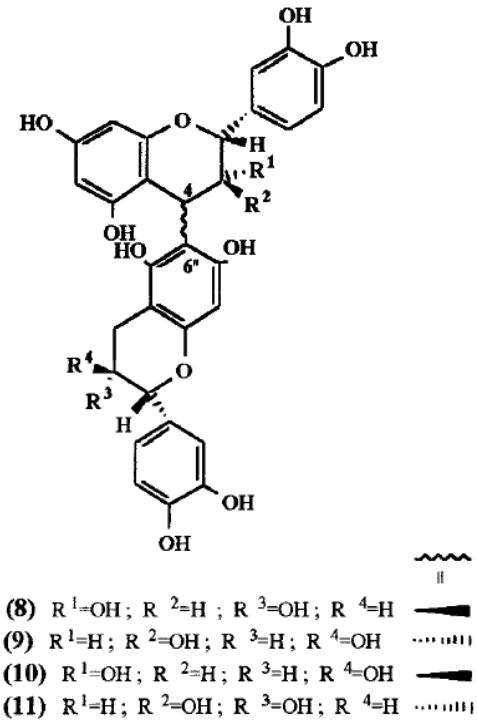


Figure 1.5: C_6-C_8 proanthocyanidin dimers B5-B8 (from de Freitas and Glories 1999).

In wine, proanthocyanidins and monomeric flavan-3-ols undergo condensation reactions to form large polymers, commonly referred to as condensed tannins. These can have high degrees of polymerisation and eventually become insoluble in wine (Gawel 1998, Cheynier et al. 2006).

Anthocyanin units may also be included in the polymerisation of tannins, forming polymeric pigments that contribute significantly to red wine colour (Jurd 1969, Somers 1971, Gawel 1998, Gomez-Plaza et al. 2001, Peng et al. 2002, Cheynier et al. 2006). Harbertson et al. (2002) found only small concentrations of polymeric pigments in Shiraz, Cabernet Sauvignon and Pinot Noir fruit, suggesting that these compounds are primarily formed during the winemaking process. Depending on composition, these pigments variously express red, orange and brown colours (Cheynier et al. 2006), which are more pH stable than monomeric anthocyanins (Somers 1971, Gawel 1998) and are resistant to bisulphite bleaching (Cheynier et al. 2006). In the maturation of young red wines, monomeric anthocyanins are variously bound and converted to different coloured and non-coloured forms (Somers 1971, Birse 2007). Thus, polymeric wine pigments become the prevalent source of colour as the wine ages (Somers 1971, Peng et al. 2002).

1.3 Maceration in Red Winemaking

The extraction of phenolics from grapes is a key aspect of red winemaking, as mentioned previously; phenolic compounds are primarily located in the skins and seeds of *V. vinifera* grapes. The movement of phenolics from these solid grape tissues into the juice must or wine is referred to as maceration and the rate of extraction can be increased through various methods (Robinson 2006). For example, agitation of the must prior to, during, and after primary fermentation has long been known to increase the extraction of phenolics (Ough and Amerine 1961, Kennedy and Peyrot des Gachons 2003). Pre-fermentation maceration, or "cold soaking" is used by winemakers to improve wine colour intensity through increased anthocyanin extraction (Nemanic et al. 2002, Parenti et al. 2004), but without increased tannin extraction from seeds (Peyrot des Gachons and Kennedy 2003). Additionally, winemakers are able to implement various maceration techniques during red wine fermentation, such as plunging or pump-overs

(Boulton et al. 1996). Zoecklein (1991) suggests that these methods of extraction will have different effects. Namely pump-overs may extract less colour due to uneven leaching of the cap but may extract rough, bitter tannins due to the harsh treatment of the must (which increases the level of fine solids in the wine), while plunging results in a more gentle, even extraction of phenolic compounds (Zoeckline 1991).

Importantly, the rate of extraction and types of phenolic compounds extracted by maceration techniques can change depending on the temperature and percentage of alcohol in the must (Kennedy and Peyrot des Gachons 2003, Gonzalez-Manzano et al. 2004, Sacchi et al. 2005). It has been suggested that higher temperatures increase the permeability of hypodermal cells of the skin and seed tissues resulting in greater release of phenolics, while certain phenolic compounds become soluble at different ethanol concentrations, particularly those from seeds (Gonzalez-Manzano et al. 2004, Sacchi et al. 2005). However, one of the most important factors in phenolic extraction has been suggested to be the length of contact time between the phenolic-containing tissues and the juice, must or wine (Singleton and Trousdale 1983, Zoecklein 1991, Auw et al. 1996, Kennedy and Peyrot des Gachons 2003). It is on this principle that winemakers base the use of extended maceration post-fermentation (Zoecklein 1991, Sacchi et al. 2005).

1.4 Post-fermentation Extended Maceration

1.4.1 Practices

Maceration of the skins, seeds and wine beyond the completion of primary fermentation is a traditional practice in many winemaking regions (Peynaud 1984). The length of post-fermentation extended maceration (EM) is generally decided based on regular tasting to determine when the required level of phenolic extraction and modification has occurred, and may last for up to six weeks for Cabernet Sauvignon in California (Peynaud 1984, Zoecklein 1991).

In a survey of Australian winemakers conducted in 2005, Joscelyne (2009, p. 44) found that post-fermentation EM was thought to significantly affect the structure, mouthfeel and palate length of the wine. Anecdotally, this technique is indeed used by winemakers to improve the mouthfeel and tannin profile of a wine through increasing tannin intensity and length, while reducing harsh or bitter, astringent tannins that may be present after primary fermentation (Pers. Comm. PJ Charteris, Brokenwood Wines, March 2010). In a study conducted with Cabernet Sauvignon, Scudamore-Smith et al. (1990) found that post-fermentation EM techniques produced wine that was less fruity but acceptably astringent for longer cellaring. However, Zoecklein (1991) suggests that fruit from young vines (less than 10 years old) or unripe fruit will not benefit from post-fermentation EM, as tannins may be perceived as "green" and "immature" and further extraction by this method will be detrimental to wine quality.

1.4.2 Chemical Implications

As suggested above, many winemakers use post-fermentation EM to improve the organoleptic characteristics of their wine, primarily the mouthfeel and structure. Studies surrounding the chemical implications of such practices have found some effect on the phenolic composition of the wines produced. Zitzlaff (1989), Scudamore-Smith et al. (1990), Kovac et al. (1992), Auw et al. (1996), de Freitas et al. (2000), Gomez-Plaza et al. (2001) and Zimman et al. (2002) all found that post-fermentation EM increased the extraction of phenolic compounds into wine, specifically (+)-catechin, (-)-epicatechin and proanthocyanidins. Scudamore-Smith et al. (1990), Sipiora and Gutierrex Granda (1998) and Mazza (2005) found that these increases were greater with longer maceration times. Due to the much higher concentration of phenolics in the seed tissue of grapes (Sun et al. 1999) and the general inability to extract these in a fermenting must with low alcohol concentration (Zou et al. 2002), the higher concentration of such compounds in post-fermentation EM wines is generally attributed to the increased extraction of seed-bound phenolics (Kovac et

al. 1992, Zimman et al. 2002, Harbertson et al. 2009). In a study with Cabernet Sauvignon wines undergoing 7 days post-fermentation EM, de Freitas et al. (2000) found that seed-derived, low molecular weight proanthocyanidins (specifically dimers B1-B8) contributed up to 66% of the total concentration of such compounds in these wines compared to 11% in wines that were pressed when dry.

Apart from increasing phenolic extraction, post-fermentation EM has been suggested to affect the polymerisation behaviour of phenolic compounds in wine during maturation. In a study on Cabernet Sauvignon wines undergoing post-fermentation EM for 14, 23 and 44 days, Kudo and Sodeyama (2002) found wines with longer pomace contact had an increased molecular weight shift toward larger phenolic molecules when measured by size-exclusion HPLC than those with less pomace contact time. The authors suggest this may be from enhanced extraction of phenolics from the pomace, thus increasing the rate of tannin polymerisation reactions. Gomez-Plaza et al. (2001) also found that extended pomace contact resulted in an increase in proanthocyanidin dimers B2, B4 and B5 over twelve months of maturation in bottle.

Post-fermentation EM has also been shown to influence red wine colour. Scudamore-Smith et al. (1990), Kovac et al. (1992) and Sipiora and Gutierrez-Granada (1998) found that free anthocyanin concentration decreased with increased pomace contact time post-fermentation. However, these studies, along with that of Gomez-Plaza et al. (2001), found that the wine colour density – a spectrophotometric measure of total wine colour (Somers and Evans 1977) – immediately after pressing was greater in wines made with post-fermentation EM. Gomez-Plaza et al. (2001) presented data that suggests that longer pomace contact results in greater anthocyanin extraction, combined with increased stable pigment polymerisation, giving greater colour density. However, Scudamore-Smith et al. (1990) found that after maturation (400 days),

the colour density of post-fermentation EM wines and those pressed at dryness was similar, suggesting that post-fermentation EM will produce wines with greater colour density only at a younger age. Auw et al. (1996) and Gomez-Plaza et al. (2001) found that wine colour hue – the degree of brown colour relative to red colour in the wine (Somers and Evans 1977) – was lower in post-fermentation EM wines up to 12 months after processing, suggesting that this technique results in earlier, and increased production of stable pigmented polymers. Zimman et al. (2002) also found that polymeric pigments provided a greater contribution to wine colour in Cabernet Sauvignon wines with 20 days post-fermentation EM than those pressed at dryness. Harbertson et al. (2009) showed that a 20 day post-fermentation EM treatment increased the concentration of "large polymeric pigments", defined as those that precipitate with protein (Harbertson et al. 2003), at both pressing and 185 days maturation. However, at 185 days, the total polymeric pigment concentration (sum of "small" and "large" polymeric pigments") was similar between control and post-fermentation EM wines. Findings such as these have led to the conclusion that the increased extraction of phenolic compounds through post-fermentation EM provides greater potential for polymerisation between anthocyanins and polymeric phenols to occur, thus conferring improved colour stability to the wine at a younger age (Zoecklein 1991, Auw et al. 1996, Harbertson et al. 2009).

1.5 Previous Research

Studies regarding the effects of post-fermentation extended maceration have generally found that increased pomace contact time resulted in higher phenolic concentration, increased colour stability and (where studied), affected the wine astringency. However, in a review of previous studies into post-fermentation EM, Mazza and Ford (2005) suggested that experiments conducted at a commercial scale without replication indicate a lacked scientific rigour. For example, studies

conducted by Zitzlaff (1989), Kovac et al. (1992), Auw et al. (1996) and Zimman et al. (2002) used no replication, while other studies by Scudamore-Smith et al. (1990) and Reynolds et al. (2001) introduced irreproducible variables through the use of oak barrels. Also, the lack of a "standard" length of post-fermentation EM has made comparison between findings difficult. For example, studies with relatively short periods of EM such as those conducted by Reynolds et al. (2001) of 3 days and de Freitas et al. (2000) of one week may not be comparable to those conducted by Scudamore-Smith et al. (1990), Auw et al. (1996), Kudo and Sodeyama (2002) and Zimman et al. (2002) of at least 3 weeks.

Little research has been published regarding the complete effect of post-fermentation EM on both wine composition and sensory characteristics. In completion of a master's thesis, Mazza (2005) conducted studies on Pinot Noir wines that underwent one or three weeks post-fermentation EM, which were assessed for colour and phenolic composition, along with sensory analysis of bitterness and astringency. HPLC was used to analyse anthocyanin, pigmented polymer and tannin concentrations up to 140 days after fruit processing. Analysis of (+)-catechin and (-)-epicatechin showed that wines with three weeks post-fermentation EM had double the concentration of wines with one week EM, and four times that of conventionally made wine. Gel Permeation Chromatography (GPC) was employed to characterize wine tannin size distribution between treatments. However, due to the absence of standards only relative comparisons between treatment samples could be made. No significant difference in astringency or bitterness between treatments was observed by limited sensory assessment, which differs to the anecdotal findings of Zitzlaff (1989) and Scudamore-Smith et al. (1990). Importantly, the replications of each treatment were discrete fermentations from the initial parcel of fruit, which may have introduced variables from must composition or fermentation dynamics that influenced the sensory assessment of the wines.

Joscelyne (2009) conducted further analysis of these wines 33 months post-crush, whereby colour density and hue were found to be similar between the three treatments by spectral analysis. This reflects the findings by Scudamore-Smith et al. (1990) who demonstrated that differences in colour observed in young wines were lost as the wine matured. However the degree of red pigment colouration and total phenolics measured by spectral analysis, and total tannin measured by the methyl cellulose precipitable tannin (MCPT) assay were found to be significantly higher in the post-fermentation EM wines, suggesting that the increased extraction of phenolic compounds observed in young post-fermentation EM wine is maintained through maturation.

Joscelyne (2009) also produced Grenache and Shiraz wines in 2006 and 2007 that underwent periods of post-fermentation EM (Grenache for three weeks and Shiraz for one and three weeks). Little difference was found in colour between treatments for the Grenache wines (by Somers and CIE *L*a*b** measures), however tannin concentration was shown to increase over time and was highest for post-fermentation EM wines by MCPT assay. However, similar to the findings of Mazza (2005), sensory analysis did not indicate a significant difference in astringency and mouthfeel between the wines. For the Shiraz wines, Joscelyne (2009) found a difference in colour between treatments by CIE *L*a*b** measures, suggesting the 3 week post-fermentation EM wines presented less red colour, and were less intensely coloured than control wines. These findings were also supported by the sensory analysis.

The studies conducted by Mazza (2005) and Joscelyne (2009) were designed to include adequate replication through the use of small-scale vinification. However, in the wider published literature there are few studies that have used this technique which suggests there may be deficiencies in results. This also highlights there are potential gaps in the understanding of post-fermentation EM which indicates the need for further research.

1.6 Analytical Methods

1.6.1 Spectral Analysis

Colour is a very important sensory aspect of red wine, as it is the first property perceived in the glass (Lawless and Heymann 1998). Traditional methods of colour analysis have been based on light absorbance at 420 and 520nm, described by Somers and Evans (1977). While newer methods of colour analysis have been adopted by many wine researchers such as CIE *L*a*b** (Birse 2007), the Somers methods still provide simple, sound and reproducible measurements of wine colour (Iland et al. 1993).

1.6.2 Methyl Cellulose Precipitable Tannin (MCPT) Assay

One characteristic of wine tannin is that it will bind, and precipitate certain polymers, such as methyl cellulose (Mercurio et al. 2007). In the MCPT assay, the formation of an insoluble complex followed by precipitation causes a decrease in the absorbance of the wine at 280 nm, and when compared to a control sample (no methyl cellulose added), the concentration of tannin in the wine can be determined (Mercurio et al. 2007). This is a simple and robust procedure for determining tannin concentration (Sarneckis et al. 2006, Mercurio et al. 2007).

1.6.3 High Performance Liquid Chromatography (HPLC) Analysis

Reverse-phase HPLC is commonly used in the analysis of grape and wine phenolics to provide quantitative measurements of flavan-3-ols, anthocyanins and simple proanthocyanidins (Auw et al. 1996, Downey et al. 2003, Joscelyne 2009). Peng et al. (2002) showed that the pigmented polymer content of red wines may also be analysed by this method but only as an undefined collection of molecules, represented by a "hump" in the HPLC trace. Thus, this method is of use in analysis of simple, monomeric wine phenolics. Extracts of berry tissue phenolics may also be analysed by reverse-phase HPLC.

The subunit composition and mean degree of polymerisation (mDP) of proanthocyanidins can be assessed through acid-catalysed cleavage in the presence of an excess of a nucleophile such as phloroglucinol (Kennedy and Jones 2001). Cleavage of proanthocyanidins releases non-electrophilic terminal units, and electrophilic extension units which form analysable adducts with phloroglucinol. Analysis by reversed-phase HPLC gives information on proanthocyanidin constituents as well as the ratio of terminal units to extension units, thus allowing the mDP to be calculated. This assay may also be used to differentiate the origin of berry derived proanthocyanidins, due to different compositions and mean degrees polymerisation between skin and seed proanthocyanidins (Gawel 1998, Kennedy and Jones 2001, Peyrot des Gachons and Kenney 2003)

However, the phloroglucinol assay does not provide information on size and weight distribution of proanthocyanidins in wines due to the acid-induced catalysis. It can only provide a mean proanthocyanidin size. Gel Permeation Chromatography may be used to separate intact proanthocyanidin samples followed by reverse-phased HPLC to provide a distribution curve based on compound size. The isolated proanthocyanidins may then be analysed by the phloroglucinol assay to determine the molecule size (mDP) (Kennedy and Taylor 2003).

1.7 Conclusion and Proposed Research

Previous research along with surveys and anecdotal evidence from winemakers in the Australian wine industry indicates that post-fermentation EM affects phenolic composition, which may in turn affect the organoleptic characteristics of red wine. An apparent lack of definitive research into different environmental variables (e.g. temperature) encountered during post-fermentation EM and their effect on wine colour and tannin suggests that further study is required in these areas. The survey conducted by Joscelyne (2009) found that 88% of respondents believe that

there is need for further scientific understanding of the consequences of EM strategies in red winemaking.

This study aims to compare the effects of three weeks of post-fermentation EM at different temperatures – 10, 15 and 20°C – to potentially amplify and help identify trends in wine composition. In addition to a control wine pressed at the completion of alcoholic fermentation, four replications of each treatment were made. Additionally, previous studies have made little mention of post-fermentation EM temperature, suggesting that they have been carried out at an ambient cellar temperature. As such, no research into the direct effects of temperature has been made which may be useful when comparing extended maceration between winemaking regions with varying cellar temperature.

In this new study, wine colour, phenolic concentration and composition will be compared to identify distinct chemical markers for the outcome of post-fermentation EM, as well as the effect of temperature at which it is conducted. Any significant differences can between treatments, may provide industry-relevant information to winemakers seeking to employ post-fermentation EM. Along with the chemical analysis conducted in this study, the scientific rigour by which these wines are to be produced will create a set of wines that can be subjected to dedicated sensory assessment in an attempt to further comprehend the effects of post-fermentation EM.

2. Materials and Methods

2.1 Introduction

Previous work has shown the effects of post-fermentation extended maceration (EM) on colour expression and anthocyanin, flavan-3-ol and tannin concentration (Scudamore-Smith et al. 1990, Auw et al. 1996, de Frietas et al. 2000, Kudo and Sodeyama 2002, Zimman et al. 2002, Mazza 2005, Joscelyne 2009). The potential effect of the temperature at which post-fermentation EM is conducted was not examined in these studies. For this project, red wines were produced during the 2010 vintage to examine differences in phenolic extraction due to post-fermentation EM of common duration but different temperatures. A single fruit source was used to produce the wines with appropriate replication to allow comparisons of colour, anthocyanin concentration, monomeric flavan-3-ol concentration and tannin concentration, measured by methods described here.

2.2 Experimental Winemaking

2.2.1 Winemaking Protocol

Fruit used for this project was sourced from *Vitis vinifera* cv. Shiraz from the Virgara Vineyard in the Adelaide Hills, South Australia. 850 kg of fruit was machine harvested on March 26th 2010 and brought to the Hickinbotham Roseworthy Wine Science Laboratory (HRWSL) at the University of Adelaide Waite Campus for processing. Total soluble solids (TSS) expressed as Baumé, pH and TA was analysed for a sample of fruit prior to crushing. A sample of intact berries was frozen for HPLC analysis at a later date.

The total batch of fruit was destemmed and crushed using a Zambelli crusher/destemmer unit and pumped into a suspended 1 ton Potter-type fermenter fitted with a brine cooling jacket and fixed pump-over irrigator. 50 mg/L of SO_2 as potassium metabisulphite (PMS) was added during crushing. The must was inoculated with *Saccharomyces cerevisiae* yeast strain RX60 (Laffort) according to the manufacturer's instructions. The yeast was rehydrated with Dynastart (Laffort). A decrease in the Baumé of the must was noted 12 hours after inoculation, indicating a rapid onset of fermentation.

During alcoholic fermentation, the wine was pumped over twice daily for 5 minutes in the morning and afternoon. The temperature was maintained between 19-22°C with cooling during the first 96 hours of fermentation, until the Baumé reached 5°. At this point, the ferment was inoculated with lactic acid bacteria strain SB3 (Laffort) for malolactic fermentation (MLF). Samples of the liquid and solid (skins and seeds) portions of the ferment were collected daily and frozen for later analysis.

At 115 hours post-inoculation, the ferment was split into 16 replicates. This was achieved by collecting the liquid and solid portions of the ferments separately in pre-weighed vessels. The liquid was drained from the fermenter via gravity for 2 hours. Seeds and skins (the solid portion) were collected and weighed separately. A solids-to-liquid ratio was determined for the original fermentation which allowed the reconstitution of 16 x 30 kg fermentations in 50 L plastic drums with a similar composition. This was done to minimize differences between replicates due to the variable effects of primary fermentation, and to decrease the number of ferments that needed to be managed over the initial stages.

The 16 replicates were randomly allocated into four treatment groups, a control, which was not exposed to post-fermentation EM, and three post-fermentation extended maceration treatments at

10°C, 15°C or 20°C. The post-fermentation EM wines were placed into the corresponding temperature-controlled rooms in the HRWSL for three weeks EM treatment, and the control wines were left at cellar temperature overnight. The following day, control wines were pressed using a 50 L Diemme press (Lugo, Italy), with free run and pressing wines combined and placed back into 50 L containers and placed into the 15°C room to allow MLF to complete. Samples of the wine before and after pressing were collected and frozen. The control wines were racked off gross fermentation lees five days after pressing into 30 L plastic drums with no ullage.

During the three week EM treatment, the wines were gently hand plunged and sampled (liquid and solids) every four days. Dry ice was added every two days to provide protection against oxidation. After three weeks, the EM wines were pressed and racked into 30 L plastic drums with zero ullage after 5 days.

The 20°C EM wines completed MLF six days after pressing and 100 mg/L SO_2 as PMS was added. The control, 10°C and 15°C EM wines were re-inoculated with SB3 bacteria and placed in the 20°C room. These wines had completed MLF when checked two weeks later and 100 mg/L SO_2 as PMS was added. Due to an inappropriately high pH of 4 after MLF in all wines, 1 g/L tartaric acid was added to the control, 10°C and 15°C EM wines and 2 g/L to the 20°C EM wine to bring all to a similar pH (Table 3.1). The wines were placed in the 0°C room for 4 days and racked with the addition of 0.2 mg/L copper ions as $CuSO_4.5H_2O$ to remove minor reductive characters. Each replicate (18 L) was bottled 52 days after crushing and sealed under screw-cap closures. Finished wines were stored at cellar temperature and sampled for analysis as required. A further 9 L of each replicate was bottled with the addition of 25 mg/L oak tannin extract (Quertanin Sweet, Laffort) to represent oak treatment, for use in another project.

2.2.2 Winemaking Analysis

Winemaking analyses were conducted according to methods described in Iland et al. (2000). Sample pH was determined using a pH meter (Radiometer Analytical). Titratable acidity was determined by titration with 0.1M NaOH. Total soluble solids through fermentation and EM were determined by refractometry (fruit samples) and density (Anton Paar hydrometer). The end of fermentation was determined by the Rebelein method and MLF monitored by thin-layer chromatography. Alcohol content was measured by ebulliometry. A combined aspiration still was used to determine SO_2 and volatile acidity levels.

2.3 Analytical Methods

2.3.1 Spectral Measures

Wine colour was measured by the Somers method spectrophotometrically using a Multiskan Spectum spectrophotometer (Thermo, Finland) with a computer running SkanIt Software 2.2. Measurements were made on bottled wines 8 days after bottling (57 days post crush). The method used is described in Iland et al. (1993), with a 1 mm path length quartz cuvette and all samples corrected against a reverse-osmosis water blank. Absorbance was measured at 420, 520 and 280 nm. Path length correction (to 10 mm) was made for measurements taken in 1mm cuvettes.

2.3.2 Methyl Cellulose Precipitable Tannin Assay

The methyl cellulose precipitable tannin (MCPT) assay was used as a measure of tannin concentration in finished wine. Analysis was carried out on the same spectrophotometer system described above in accordance to the method detailed by Mercurio et al. (2007).

2.3.3 Reverse Phase High Performance Liquid Chromatography

A reverse phase high performance liquid chromatography (RP-HPLC) technique was used to quantify anthocyanins, pigmented polymers, monomeric flavan-3-ols and tannins in samples of fermenting must and wine. The method used is described by Peng et al. (2002). A 50 µL sample was injected into a Hewlett-Packard HP1100 HPLC with Agilent ChemStation software for LC 3D systems Rev B.01.03.SR1 [204] (Agilent Technologies 2001-2005) (consisting of a vacuum degasser G1311A Quaternary Pump, G131A Autosampler, G1315B Diode Array Detector, G1316A Column Thermostat). A polystyrene divinylbenzene column (PLRP-d, 250 x 4.6 mm, 5 µm packing, 100 Å) was used, protected by a guard column of the same material (Polymer Laboratories, UK).

Eluting peaks were measured at 280 and 520 nm. Peaks at 280 nm were identified by comparison with standards of (+)-catechin, (-)-epicatechin (Fluka BioChemika), gallic acid and epicatechin gallate (Sigma, Australia), and quantified as (+)-catechin equivalents. A malvidin-3-glucoside standard (Polyphenol Laboratories, Norway) was used to identify anthocyanins and pigmented polymers at 520 nm based on relative retention times, and quantified as malvidin-3-glucoside equivalents.

2.3.4 Isolation of Tannin from Wine and Fermentation Samples

The solid phase extraction (SPE) method for the recovery of polymeric polyphenols from 1 mL of wine and fermentation samples used in this study was described by Jeffery and co-workers (2008) using a 3 mL Oasis HLB mixed polymer cartridge (60 mg, 30 µm) and the collected fraction dried under N_2 at 0.8 psi for 45 minutes using a turbo-vac. However, for this investigation, the F2 and F3 fractions of polymeric polyphenols were collected as one fraction, as

only one measure of total tannins was required. The recovered tannins were divided into three aliquots for further analysis.

2.3.5 Acid-Catalysed Cleavage in the Presence of Excess Phloroglucinol

Polymeric polyphenols were extracted from wine and ferment samples using the solid phase extraction method described by Jeffery and co-workers (Jeffery et al. 2008). A portion of the recovered phenolic material underwent acid-catalysed cleavage in the presence of excess phloroglucinol as described in published literature (Kennedy and Jones 2001, Downey et al. 2003) before analysis by RP-HPLC in a system described by Koerner et al. (2009).

2.3.6 Gel Permeation Chromatography

A sample of the purified wine proanthocyanidin recovered by the SPE method described above (Jeffery et al. 2008) was re-suspended in 100% methanol and a 20 μL sample injected into the Gel Permeation Chromatography (GPC) system described by Kennedy and Taylor (2003). An isocratic method with a mobile phase consisting of N,N-dimethylformamide containing 1% (v/v) glacial acetic acid, 5% (v/v) Milli Q water and 0.15 M lithium chloride was used at 0.75 mL/min and elution monitored at 280 nm.

2.3.7 Statistical Analysis

Data was analysed using GraphPad Prism, and statistical significance between treatments determined using Tukey's method of simultaneous pairwise comparison with 95% confidence intervals unless otherwise indicated.

3. Results and Discussion

3.1 Winemaking and Spectral Analysis

Fruit maturity and initial must analysis data are displayed in Table A.1, Appendix A. A fermentation curve up to 120 hours post inoculation is also shown in Figure A.1, Appendix A. Results from analyses conducted on the wines prior to bottling are displayed in Table 3.1.

Table 3.1: Winemaking analysis conducted prior to bottling. Values are the mean for the four replicates of each treatment. Different letters indicate significant differences for each parameter.

Treatment	pH		TA (g/L H_2T)		Free SO_2 (mg/L)		VA (g/L)		Alcohol (%v/v)	
Control	3.93	bc	6.3	c	32.8	a	0.39	a	13.9	a
10°C EM	3.94	c	5.7	b	29.8	ab	0.41	ab	14.0	a
15°C EM	3.88	b	5.6	b	30.5	a	0.46	b	14.0	a
20°C EM	3.76	a	4.9	a	23.8	b	0.39	a	14.1	a

Significant differences were found between the wines for several parameters. The 20°C EM wine had the lowest pH at bottling followed by the 15°C EM, control and 10°C EM wines. As suggested above, the pH and TA of all the wines at the completion of MLF was unacceptably high for the wines to be considered stable for bottling. As such, the wines were adjusted with an addition of tartaric acid (2 g/L to the 20°C EM wines and 1 g/L to the other treatments). While this left some variation in the bottled wines, this was necessary to ensure a degree of stability in bottle. Furthermore, while the 20°C EM treatment had the lowest pH, it also had the lowest TA. This may have been due to precipitation of unstable bitartrate when these wines were held at 10°C while the other treatments completed MLF. Mazza (2005) found that 21 days post-fermentation EM resulted in an increase in pH in Pinot Noir wines, attributed to an increased exchange of potassium ions from berry skins. While pH can affect the expression of anthocyanins

and other wine pigments (Eiro and Heinonen 2002, Peng et al. 2002, Birse 2007), spectral analysis of wine colour was also conducted with pH-adjusted samples and as such, no further acid adjustments were made prior to bottling. Also, informal sensory tests of further acid additions resulted in undesirable sour/acid tastes becoming present in the wine which may have impeded their use for subsequent sensory analysis.

The spectrophotometric data is displayed in Table 3.2. These results suggested that extended maceration had a significant effect on several wine colour parameters. Colour expression is observed at unmodified pH, and suggested that the control treatment was more strongly coloured than the EM wines (WCD) and displays less brown colouration than the 10°C and 15°C EM wines due to the lower WCH value. This is similar to findings by Zitzlaff (1989). A significant difference in WCD and WCH values for wines with a modified pH of 3.5 was also observed between the control and EM treatments, with the control wine again appearing more intensely coloured and less brown. In addition, total red pigment colouration and total anthocyanins were significantly higher for the control. Coupled with increased WCD, these results suggest that the control wines contained a greater concentration of red pigments such as anthocyanins and pigmented polymers than the wines that underwent post-fermentation EM. This may be explained by oxidation of red coloured phenolic compounds, or association and subsequent removal of pigmented material with solids such as skins, seeds or yeast lees during the extended contact time (Scudamore-Smith et al. 1990, Mazza 2005).

Total phenolic content was greater for the 15°C and 20°C EM wines than both the control and 10° EM wines. Previous research has shown that post-fermentation EM resulted in an increase in total phenolics (Scudamore-Smith et al. 1990, Auw et al. 1996, Zimman et al. 2002), and these results suggest this may be influenced by EM temperature, as the 15°C and 20°C EM treatments

showed a higher total phenolic content than the 10°C EM treatment. It is important to acknowledge that these analyses were carried out on relatively young wines (57 days), and it may be expected that the higher phenolic content of the EM wines may potentially lead to a greater formation of stable pigmented polymers during subsequent aging. However, the EM treatments possessed less SO_2 resistant pigments than the control wine at this time point, with the 20°C EM wine having less than the 10°C or 15°C EM wine. These data suggest that pigmented polymer formation may be negatively affected by post-fermentation EM.

Table 3.2: Spectral measures of wines.

Measurement	Control		10°C EM		15°C EM		20°C EM	
WCD	8.526	a	8.313	ab	7.330	b	8.046	a
WCH (no units)	0.8009	a	0.8699	b	0.8574	b	0.7941	a
Degree of red pigment colouration (%)	10.77	a	12.18	a	10.98	a	12.47	a
Total red pigment colouration	44.00	a	36.77	b	35.23	b	35.98	b
Total Anthocyanins (mg/L)	782.5	a	651.6	b	637.1	b	653.0	b
Total phenolics	61.02	ab	60.01	a	65.20	c	64.22	bc
Modified WCD	16.37	a	12.86	b	12.18	b	13.19	b
Modified WCH (no units)	0.5387	a	0.5767	b	0.5669	b	0.5595	ab
Modified deg. red pigment colouration (%)	24.21	a	22.22	ab	21.62	b	23.51	ab
SO_2 resistant pigment	2.930	a	2.514	b	2.471	b	2.001	c
Modified SO_2 resistant pigment	2.993	a	2.556	b	2.422	b	2.023	c

Conducted in accordance with methods described in Iland et al. (1993) on bottled wines 57 days post-inoculation. Modified samples were adjusted to pH 3.5 before analysis. Values reported as absorbance units (unless indicated otherwise). Different letters indicate significant differences across individual rows for each measurement (n=4).

While spectrophotometric analysis of wines provides data for colour expression in red wines, further quantitative analysis by RP-HPLC provides a more complete understanding of the effects of winemaking treatments on specific phenolic species such as anthocyanins and pigmented polymers (Bakker et al. 1986).

3.2 Quantitative Analysis of Phenolic Compounds

3.2.1 Anthocyanins and Pigmented Polymers

A significant difference in total anthocyanin concentration was observed between the control and all three extended maceration treatments in the finished wines, as shown in Figure 3.1. The highest concentration of anthocyanins occurred at 96 hours post-inoculation, during primary fermentation, followed by a decrease at pressing for the control and much greater decrease through three weeks of extended maceration.

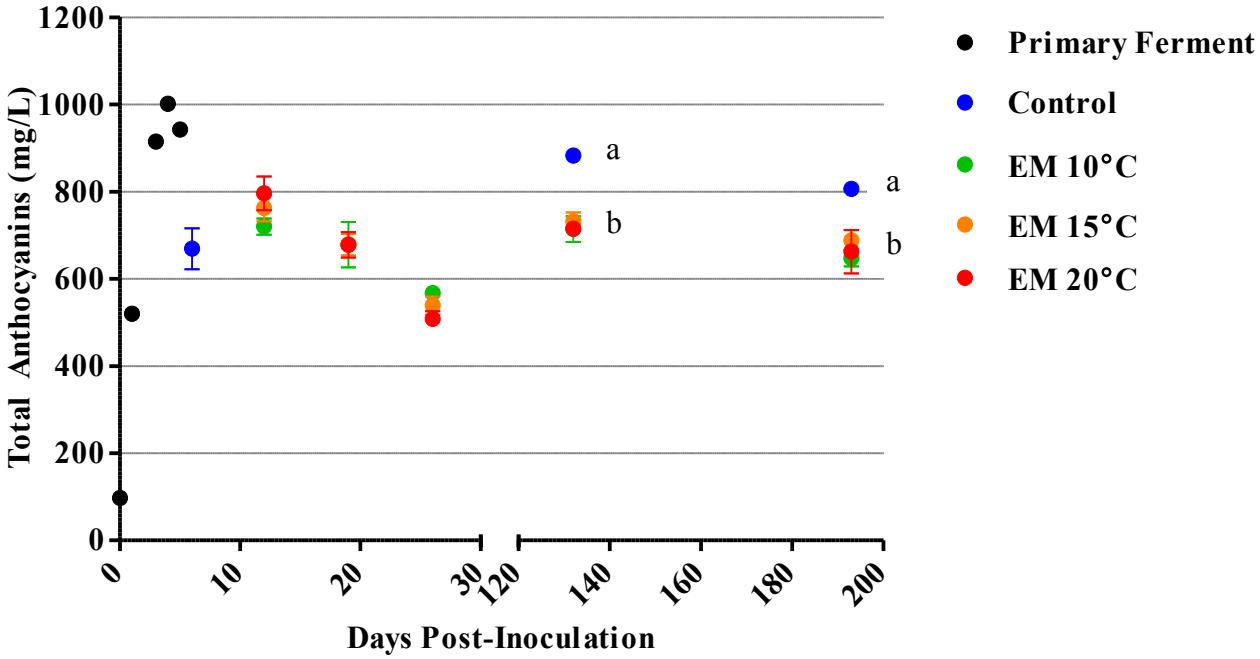


Figure 3.1: RP-HPLC analysis of total anthocyanins in fermentation and wine samples. Expressed as malvidin-3-glucoside equivalents (mg/L). Total anthocyanins were determined as the sum of peak areas of glucoside, acetyl-glucoside and coumaroyl-glucoside forms. Error bars represent ± standard error (n=4). Significance of differences between treatments at the last time points (bottled wines) is indicated by different letters.

This has been widely reported in other studies, and can be ascribed to numerous processes, including adsorption onto yeast lees and grape cell material, incorporation into pigmented polymers and wine pigments such as visitin, or degradation through oxidation or bisulphite

bleaching (Ribereau-Gayon et al. 1970, Nagel and Wulf 1979, Scudamore-Smith et al. 1990, Kennedy and Peyrot des Gachons 2003, Mazza 2005, Balik 2006, Joscelyne 2009). Notably, Figure 3.2 shows that pigmented polymer concentration was significantly lower in two EM wines (10°C and 20°C EM) compared with the control at 195 days post inoculation, which suggests the decrease in anthocyanin may not be due to incorporation into pigmented polymers.

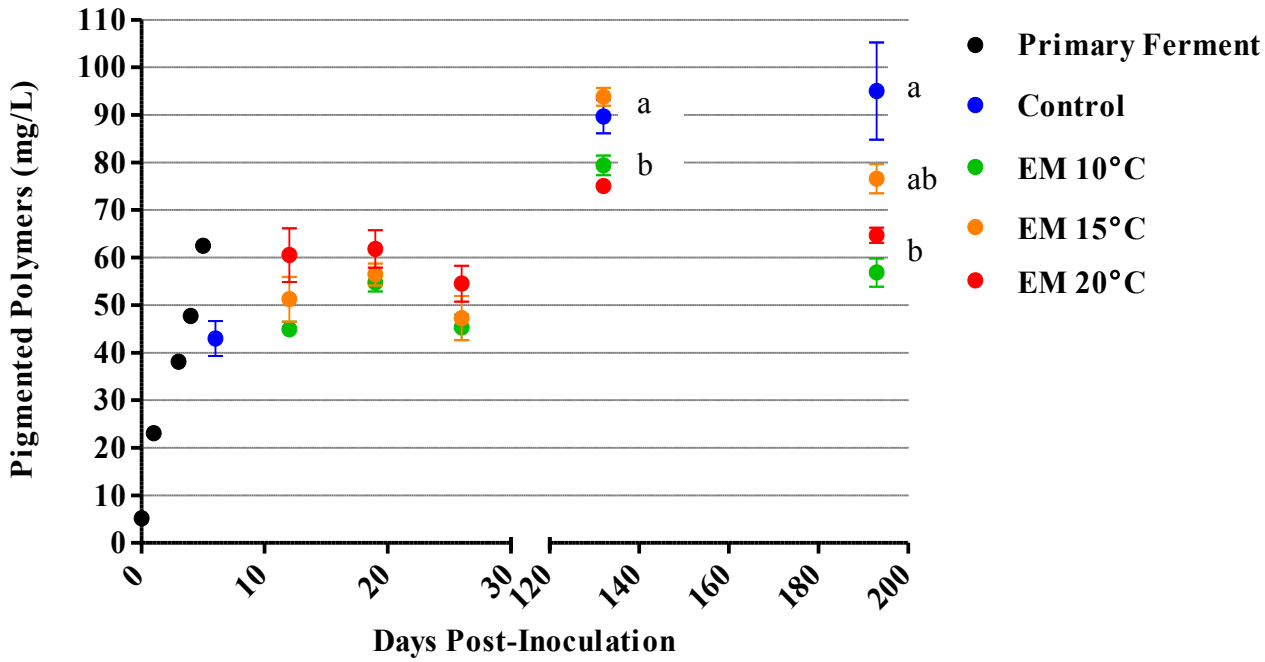


Figure 3.2: RP-HPLC analysis of pigmented polymers in fermentation and wine samples. Expressed as malvidin-3-glucoside equivalents (mg/L). Error bars represent ± standard error (n=4). Significance of differences between treatments at the last time points (bottled wines) is indicated by different letters.

The results also suggested that the decrease in anthocyanins during extended maceration occured irrespective of temperature. Beyond pressing, Mazza (2005) observed a similar increase in total anthocyanins in Pinot Noir wines and attributed this to diffusion of anthocyanins back into the wines from yeast less between two rackings.

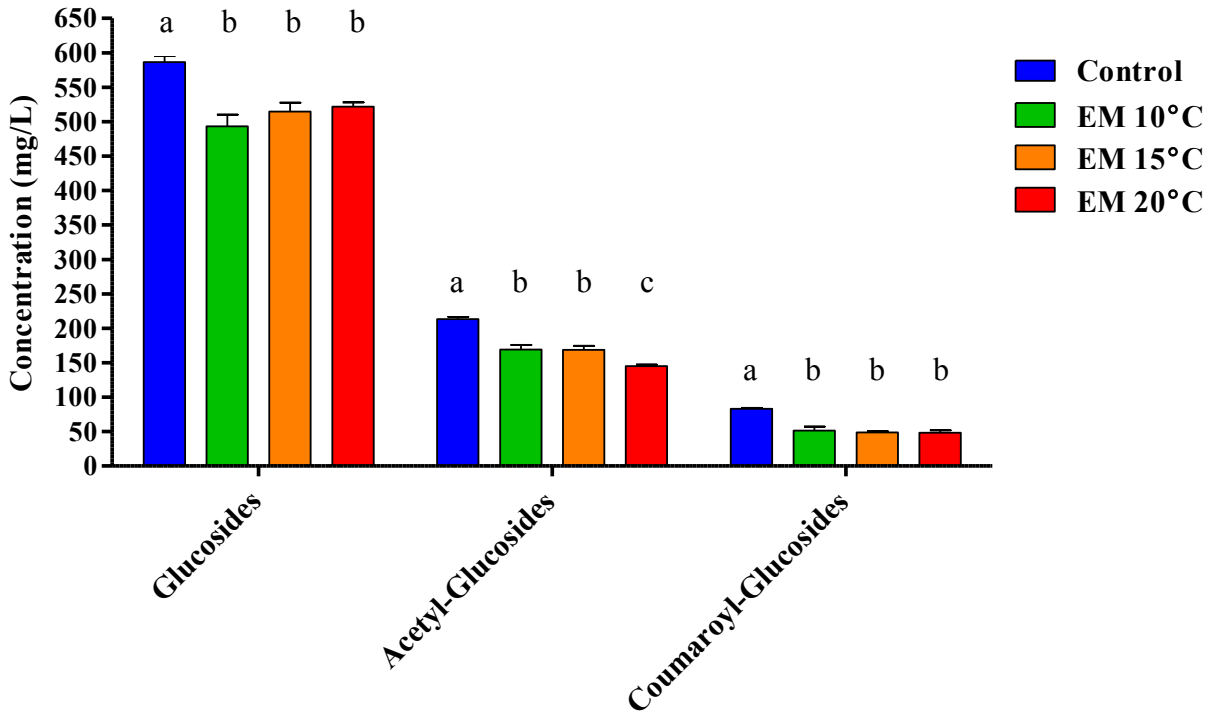


Figure 3.3: Concentration of Glucoside, Acetyl-Glucoside and Coumaroyl-Glucoside forms of anthocyanins in bottled wine. Measured 132 days post-inoculation and expressed as malvidin-3-glucoside equivalents (mg/L). Error bars represent the standard error (n=4). Significance of differences between treatments for each form of anthocyanin is indicated by different letters.

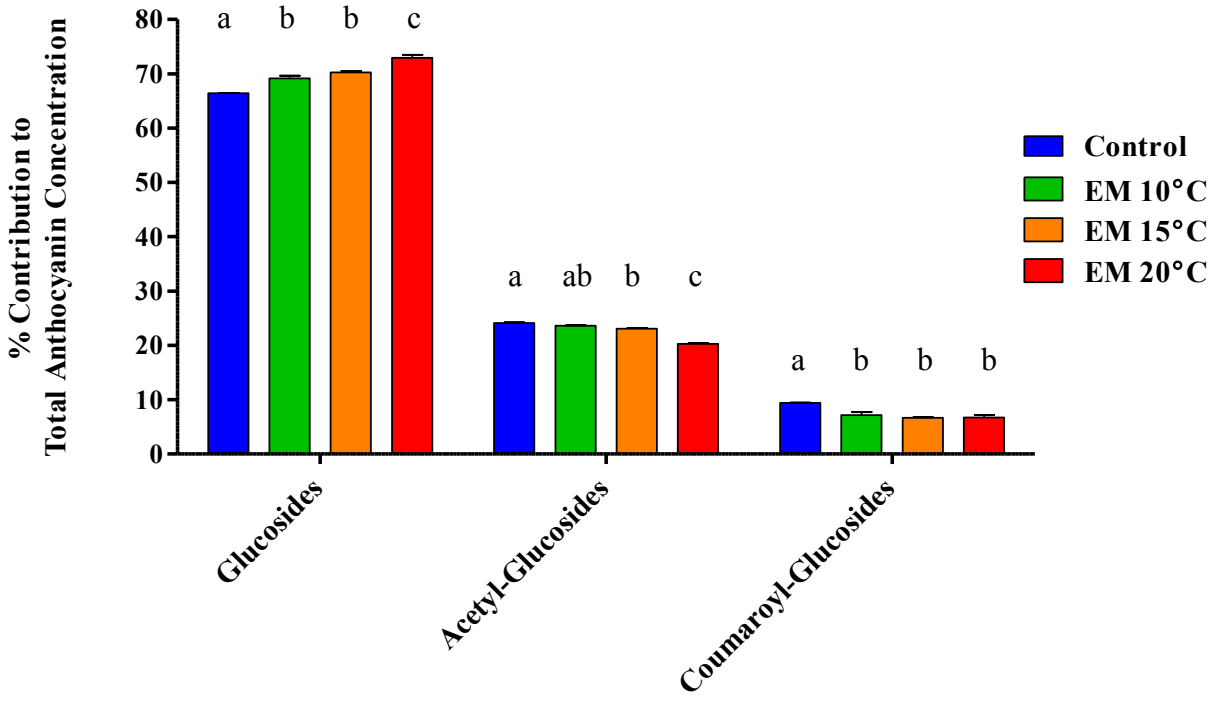


Figure 3.4: Glucoside, Acetyl-Glucoside and Coumaroyl-Glucoside forms of anthocyanins in bottled wine expressed as a percentage of the total anthocyanin concentration for each treatment. Measured 132 days post-inoculation. Error bars represent the standard error (n=4). Significance of differences between treatments for each form of anthocyanin is indicated by different letters.

The increased total anthocyanin concentration of the control wine can be explained by the fact that the extended maceration wines experienced greater time in contact with gross ferment lees and grape cell material, thus allowing more adsorption. The concentration and composition of the three forms of anthocyanins found in Shiraz (glucosides, acetyl-glucosides and coumaroyl-glucosides) in the finished wines is shown in Figure 3.3. All three forms of anthocyanin were in significantly higher concentration in the control wine, however the relative contribution of each class of anthocyanins to the total anthocyanin concentration, shown in Figure 3.3, suggests that extended maceration caused a greater decrease in the acylated forms (acetyl- and coumaroyl-glucoside). It has been indicated that acylated anthocyanins are less stable than non-acylated forms to degradation in red wine during maturation (Dallas et al. 1995, Romero and Bakker 1999,

Mateus and de Freitas 2001), and that they will more readily and more strongly adsorb to yeast lees, potentially explaining the relative decrease in these compounds in the EM wines with respect to the control (Morata et al. 2003, Medina et al. 2005).

3.2.2 Monomeric Flavan-3-ols

Figures 3.4 and 3.5 illustrate the concentrations of (+)-catechin and (-)-epicatechin during primary fermentation, extended maceration, at pressing and for finished wines.

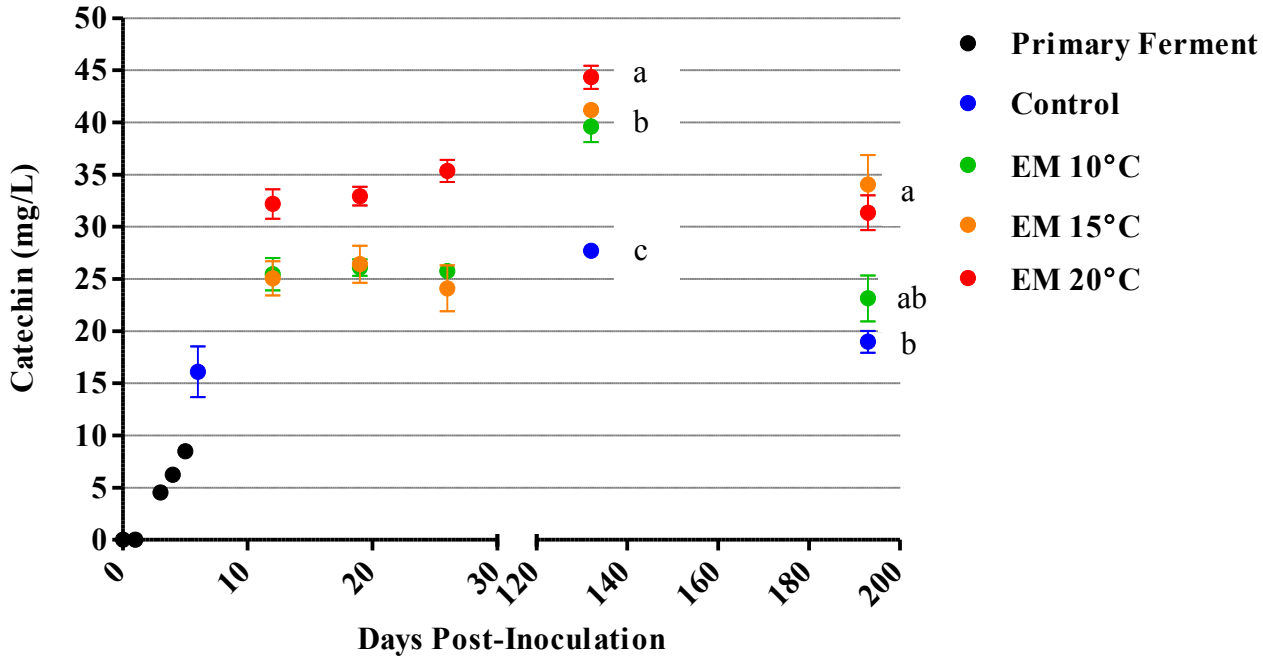


Figure 3.5: RP-HPLC analysis of (+)-catechin in fermentation and wine samples. Error bars represent ± standard error (n=4). Significance of differences between treatments at the last time points (bottled wines) is indicated by different letters.

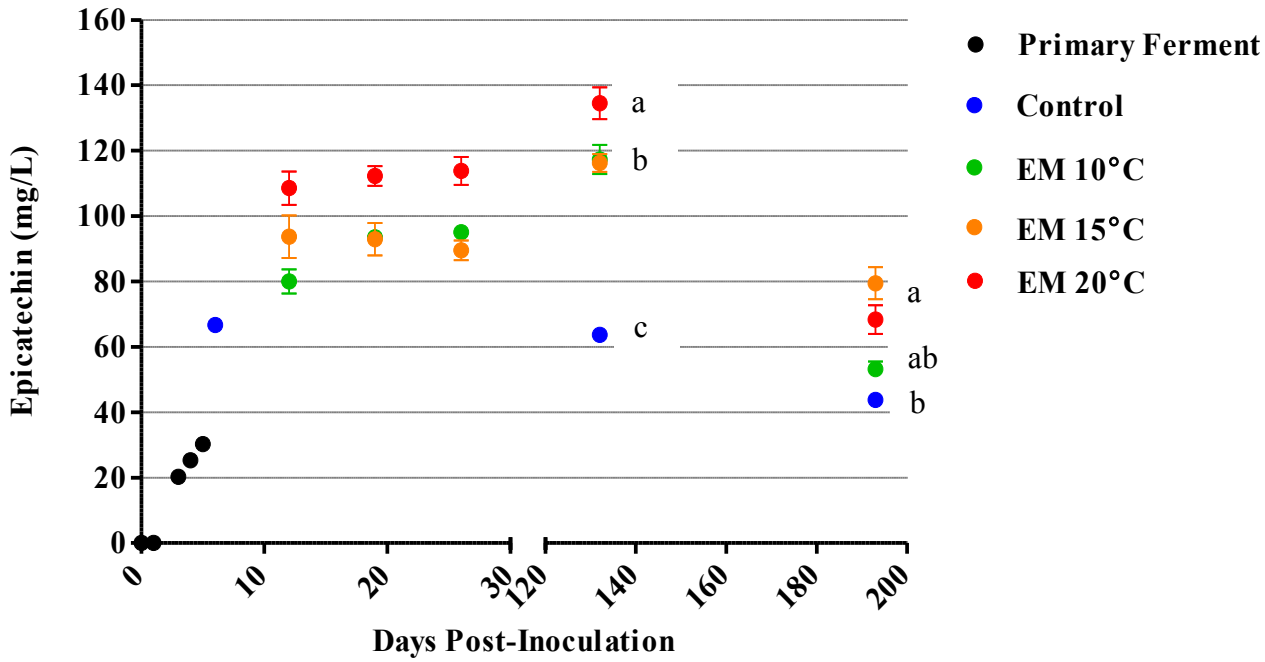


Figure 3.6: RP-HPLC analysis of (-)-epicatechin in fermentation and wine samples. Expressed as (+)-catechin equivalents (mg/L). Error bars represent ± standard error (n=4). Significance of differences between treatments at the last time points (bottled wines) is indicated by different letters.

There was a significant increase in both compounds due to post-fermentation EM when compared with the control, and this was represented in the final wine. Post-fermentation EM at 20°C also appeared to initially result in a greater extraction of both compounds compared with the lower temperatures; however differences appeared to decrease during maturation. The most dramatic increase in concentration occured during the first week of EM for all three treatments, followed by a period of little change for the 10°C and 15°C EM wines, and a gradual increase for the 20°C EM wine until pressing.

An increase in monomeric flavan-3-ols through post-fermentation EM has been observed in several studies (Zitzlaff 1989, Scudamore-Smith et al 1990, Kovac et al. 1992, Zimman et al. 2002, Mazza 2005) and was attributed to the improved permeability of seed tissue cells at higher ethanol concentrations. Singleton and Draper (1964) and Oszimanski et al. (1986) showed that

increasing temperature improved the extraction of monomeric flavan-3-ols in model systems. The results from this study indicated that warmer temperatures (20°C) during post-fermentation EM may improve seed phenolic extraction resulting in a higher (+)-catechin and (-)-epicatechin concentration in the final wine. Thus it is pertinent that winemakers conducting post-fermentation EM monitor both the temperature and sensory characteristics of their wines, as high levels of monomeric flavan-3-ols contribute bitterness to the wine (Gawel 1998, Broussaud et al 2001).

3.2.3 Tannins

Condensed tannins (measured as the tannin "hump") were significantly higher in the control wine compared to the 10°C and 20°C EM treatments as shown in Figure 3.6. The 15°C EM wine was not significantly different to the control or other EM wines.

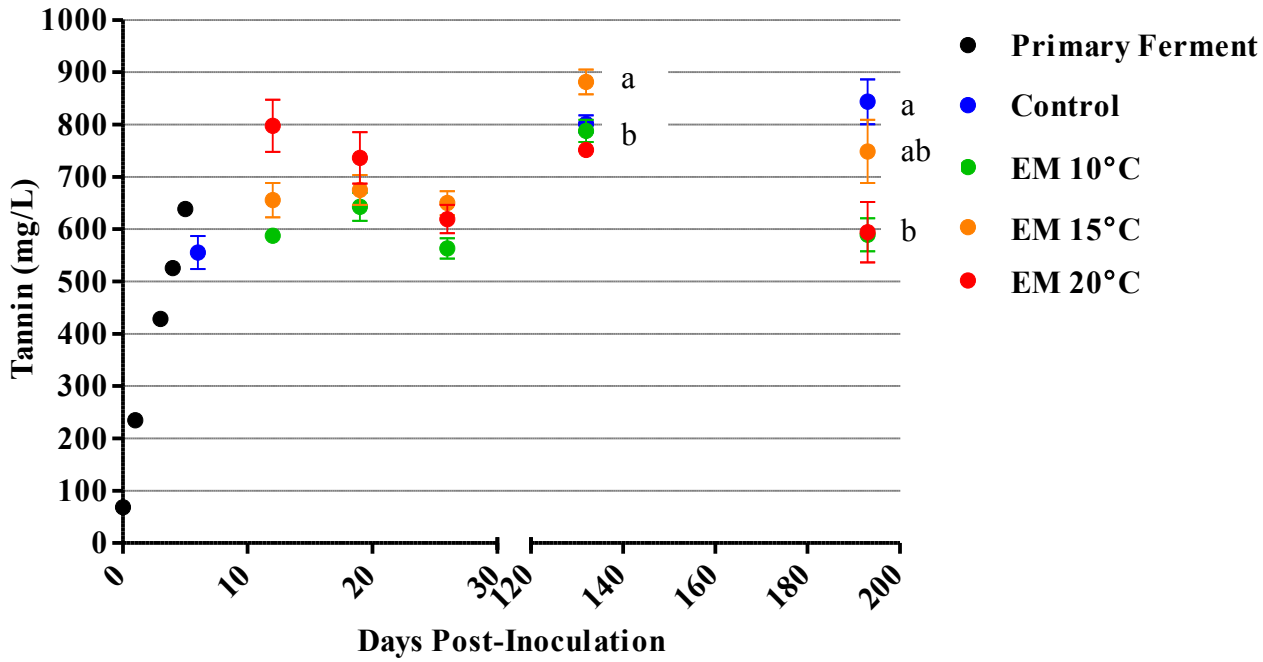


Figure 3.7: RP-HPLC analysis of tannin in fermentation and wine samples. Expressed as (+)-catechin equivalents (mg/L). Error bars represent ± standard error (n=4). Significance of differences between treatments at the last time points (bottled wines) is indicated by different letters.

All EM treatments showed a decrease in tannin at pressing. The 20°C EM wine showed a marked decrease across the three weeks of extended maceration after an initial increase during the first week. A decrease was observed from the second week of EM until pressing for the other two EM treatments. After a period of maturation in bottle, the tannin concentration appeared to increase, however significant differences were observed between the control and 10°C and 20°C EM wines at the last point of measurement. Conversely the methyl cellulose precipitable tannin (MCPT) assay conducted 175 days post-inoculation showed there was no significant difference between the control or EM treatments (Figure B.1, Appendix B). This suggests the differences in tannin concentration observed in the young wines may decrease during maturation, similar to the findings of Zitzlaff (1989) and Scudamore-Smith et al. (1990).

Further analysis of tannin through vinification and in bottle was achieved by phloroglucinol assay and gel permeation chromatography (GPC). Average tannin size, measured by GPC showed no significant differences between treatments through the period of extended maceration (Figure B.2, Appendix B), however differences were observed in the finished wines. Figure 3-7 shows the molecular weight of tannin at 50% tannin elution by GPC, taken to be the average size of tannin in the sample. The 10°C and 15°C EM wines show, on average, larger tannins compared with the control, which is larger again than the 20°C EM wine. Therefore, temperature does not appear to have a continuous scale effect on tannin molecular weight. For this reason, the tannin subunit composition was analysed in response to the various EM treatments.

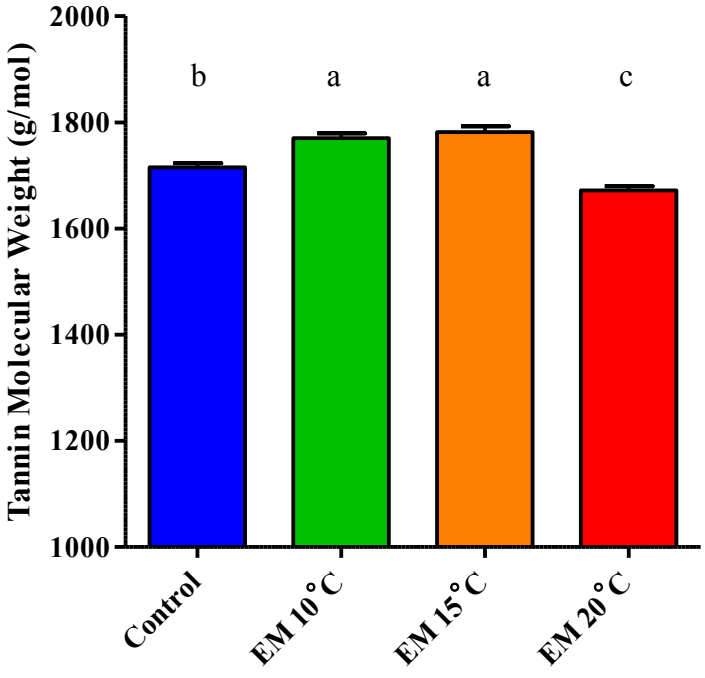


Figure 3.8: Tannin molecular weight (as g/mol) taken at 50% tannin elution of GPC run for bottled wines. Measured at 145 days post-inoculation. Error bars represent the standard error (n=3). Significance of differences between treatments is indicated by different letters.

Table 3.3: Tannin sub-unit composition analysis by phloroglucinol assay for bottled wines. Measured 145 days post-inoculation. Each sub-unit expressed as a percentage of type (extension or terminal). EGC = epigallocatechin, C = catechin, E = epicatechin, ECG = epicatechin gallate. Means and standard errors presented (n=3). Different letters indicate significant differences for each sub-unit.

	Extension sub-units			Terminal sub-units		
	EGC	C + EC	ECG	C	E	ECG
Control	26.91 ± 0.19	68.07 ± 0.32	5.03 ± 0.12	62.42 ± 2.66	31.01 ± 3.09	6.57 ± 0.43
10°C EM	24.26 ± 0.58	69.82 ± 0.71	5.92 ± 0.14	55.84 ± 2.17	36.95 ± 2.75	7.21 ± 0.59
15°C EM	23.23 ± 0.54	71.21 ± 0.46	5.89 ± 0.08	53.66 ± 1.42	39.58 ± 1.87	6.76 ± 0.46
20°C EM	19.18 ± 0.61	74.69 ± 0.66	6.14 ± 0.13	53.91 ± 2.94	39.93 ± 3.34	6.49 ± 0.56

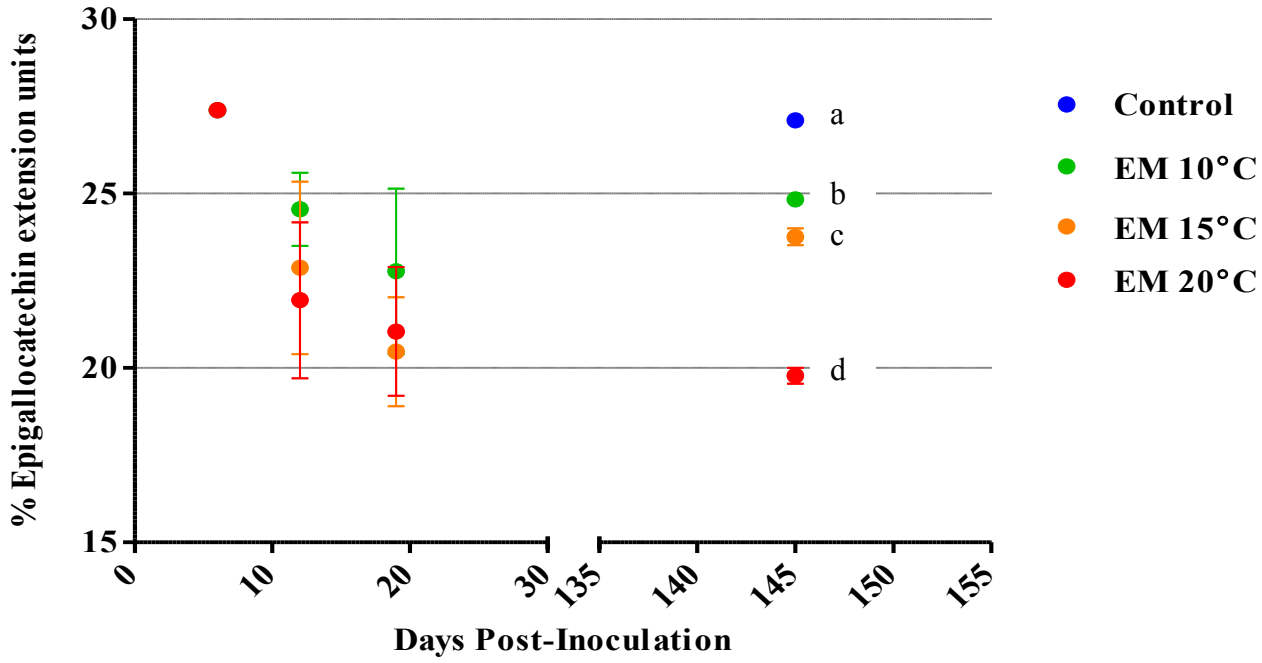


Figure 3.9: % Epigallocatechin extension units in tannins isolated from fermentation and wine samples by phloroglucinol assay. Error bars represent ± standard error (n=3). Significance of differences between treatments at the last time points (bottled wines) is indicated by different letters.

The results of analysis of tannin subunit composition by the phloroglucinol assay for the finished wines are presented in Table 3.3. Of greatest importance, the results showed that the EM wines all contained a lower proportion of epigallocatechin (EGC) extension units than the control, and that there was a decrease in EGC levels as EM temperature increased. EGC is not present in grape seed proanthocyanidins and so may be used as a crude indicator of skin tannin levels in wine (Gawel 1998, Kennedy 2008). This apparent decrease was also observed through extended maceration, as shown in Figure 3.9. While this represents a decrease in the proportion of EGC extension units, it does not appear to be due to a marked increase in seed-derived sub-units. This was shown in the minimal increase in % galloylation through EM (Figure B.3, Appendix B) which indicates a measureable loss of skin tannin due to post-fermentation EM, which may be exacerbated at higher temperatures. Also the apparent decrease in catechin, and increase epicatechin terminal units may be explained by the fact that at the time when the wines were analysed by phloroglucinol assay, the concentration of epicatechin in the EM wines was more than double that of the control (Figure 3.5). However for catechin, it was less than double the concentration of the control in the EM wines (Figure 3.4), thus contributing a lower proportion of tannin terminal units than epicatechin.

While a definitive explanation of the effect of EM temperature on tannin behaviour in wine warrants further investigation, results do suggest that warmer EM temperature may result in less tannin of smaller size in the finished wine. It is well established that tannins in wine are produced from the polymerisation of flavan-3-ol units and proanthocyanidins (Haslam 1980, Boulton et al. 1996, Gawel 1998, Herderich and Smith 2005). Research has shown that this is a dynamic process during the maturation of red wine, suggesting that under the acidic conditions found in wine, polymerisation and depolymerisation reactions will potentially occur until tannin molecules become insoluble and precipitate (Haslam 1980, Vidal et al. 2002, Dallas et al. 2003).

Importantly, Dallas and co-workers (2003) found that the kinetics of tannin modification are greatly influenced by temperature, whereby the half-life of polymeric compounds decreased with increasing temperature. This may explain the smaller tannin size observed here in the warmer EM treatment (Figure 3.8). It has also been shown that grape cell wall debris, present in wine until pressing and racking, will preferentially bind and remove larger molecular weight proanthocyanidins (Bindon et al. 2010). Thus, it is also possible that any larger tannins formed at warmer EM temperatures were readily removed from the wine by grape material present through the EM period, as compared to the control.

Furthermore, the incorporation of large, skin-derived procyanidins as compared with monomeric flavan-3-ols into newly condensed wine tannins would result in the more rapid formation of larger, insoluble molecules. Skin proanthocyanidins are extracted early in red wine fermentations and appear to reach a plateau concentration at around 10 days after crushing (Cerpa-Calderon and Kennedy 2008). Mazauric and Salmon (2005, 2006) showed that yeast lees preferentially bind to polyphenols containing a higher proportion of polar subunits, specifically EGC. Due to the prolonged contact with yeast lees in the EM treatments, this may explain the decrease in EGC, and hence skin tannin, which occured after approximately 10 days of skin contact. Analysis of primary ferment samples prior to extended maceration would be required to confirm this time point as a peak in skin tannin extraction; however it appears that post-fermentation EM may be detrimental to retaining skin tannins in red wine, which has consequences for wine quality.

Limitations in the measurement of tannin by the above methods must also be taken into account. Oxidation of tannins results in a rearrangement of bonding structures within the molecule which stabilises them against depolymerisation in an acidic environment, such as in wine or during the phloroglucinol assay (Herderich and Smith 2005, Zanchi et al. 2007, Zanchi et al. 2008, Poncet-

Legrand et al. 2010). The EM wines have an increased modified wine colour hue (table 3-1), signifying that some oxidation might have occured, and it was likely that a portion of wine tannins were modified to the point that they could not be analysed.

In addition to chemical analyses, the examination of any winemaking technique should consider the sensory impact on the finished wine. While dedicated sensory analysis was not carried out on these wines, an opportunity arose to present the wines (un-masked) to experienced industry representatives at the 14th Australian Wine Industry Technical Conference (AWITC) held in Adelaide, South Australia on the 3rd of July. When asked to rank the wines based on colour, bitterness and astringency, judges found the control to possess the strongest red colour, and the EM 20°C wine was found to be the least astringent and bitter of the treatments (Table B.1, Appendix B). While these results can only provide as an indication of the effect of post-fermentation EM on wine organoleptic characteristics, they do support the findings of the Joscelyne (2009) survey, which demonstrated that winemakers consider EM to beneficially affects astringency and bitterness, but may be detrimental to colour intensity.

4. Conclusion

A key goal of this project was the identification of chemical predictors for the outcomes of post-fermentation EM. Previous research has shown this technique to significantly affect the phenolic content of red wines, (Scudamore-Smith et al. 1990, Auw et al. 1996, de Frietas et al. 2000, Kudo and Sodeyama 2002, Zimman et al. 2002, Mazza 2005, Joscelyne 2009), identifying the need to further examine these classes of wine compounds. The results have indicated several important consequences which winemakers looking to utilize post-fermentation EM should be aware of. First, red wine colour appears be detrimentally affected by post-fermentation EM through the greater loss of acylated anthocyanins and lower production of pigmented polymers, resulting in a lower wine colour density. This has consequences for those winemakers looking to utilize extended maceration with minimal impact on wine colour. Tarara and co-workers (2008) showed that increased berry temperature from veraison to harvest resulted in an increased concentration of acylated anthocyanins, and hence higher total anthocyanin concentration, allowing production of wines with greater anthocyanin concentration and colour intensity. This suggests that pre-harvest quantification of these compounds could provide useful information to winemakers looking to use post-fermentation extended maceration with minimal colour influence, particularly in cooler years. As such, significant further research is necessary to identify appropriate levels of the anthocyanin classes, and the resulting winemaking outcomes through post-fermentation EM.

Second, the observed decrease in skin tannin through extended maceration may have consequences for winemakers looking to beneficially alter the tannin perception of their red wine. A higher proportion of skin tannin has been linked to increased quality in red wine, in terms of ‘riper, softer and longer’ tasting tannins (Vidal et al. 2003, Cortell et al. 2005, Kennedy 2008). Research into viticultural effects has shown that fruit shading and increased vigour can lead to a

decrease in skin tannin concentration and polymerisation (Downey et al. 2004, Cortell et al. 2005, Ristic et al. 2007). Further studies by Hanlin and Downey (2009) have revealed that regional differences may also play a significant role in skin tannin composition. This suggests that the use of post-fermentation EM techniques with fruit containing a low concentration of skin tannin (due to viticultural influences) may lead to a greater risk of decreasing wine quality. Measuring wine "quality" is difficult, however dedicated sensory analysis of the wines produced in this study based on tannin perception would provide a more definitive understanding of the connection of post-fermentation EM and wine outcomes, and of the role of skin tannin in wine quality. Further examination of the connection between viticultural practices and winemaking outcomes through post-fermentation EM is also warranted, due to the complex modification of grape-derived phenolic material during the vinification process. A comparison of wines from different vineyards, made conventionally and with extended maceration, followed by dedicated sensory analysis to identify batches of fruit which appear to have benefitted from post-fermentation EM, and traced back to chemical differences in the fruit and wine, could provide information to address this.

Furthermore, several questions have been raised through this study in regards to understanding the effects of post-fermentation EM on tannin modification, and its eventual effect on sensory perception. The survey of winemakers by Joscelyne (2009) showed that many practiced this technique for much longer periods of time than 3 weeks to achieve desired results. Experimental post-fermentation EM winemaking has generally focused on periods of less than 3-4 weeks (Auw et al. 1996, de Freitas et al. 2000, Zimman et al. 2002, Mazza 2005, Joscelyne 2009), which suggests there is definite scope for dedicated research into the effects of greatly extending the contact time. This is particularly pertinent now that this study has indicated the benefits in terms of increased extraction of phenolic compounds may occur during the first week of EM.

Additionally, this is one of the first studies to utilize specific tannin analysis techniques on post-fermentation EM wines (phloroglucinol assay and GPC), and has indicated that there is very likely an important compositional change in the tannin molecules. Recent research has indicated that wine tannins with increased oxidation effects (or "maturity") interact differently with salivary proteins, thus altering the perception of astringency when tasted (Zanchi et al. 2007). As such, further analysis of the changes in tannin oxidation, shape and size due to post-fermentation EM may provide better understanding of the sensory effects of this technique, as described by industry practitioners (Joscelyne 2009).

From this study, some outcomes of post-fermentation extended maceration have been identified that may be useful to winemakers. The concentration of the various anthocyanin classes, and skin tannin concentration can be measured in the fruit prior to harvest, potentially allowing identification of batches of fruit that could benefit from this technique. Further research into establishing appropriate levels of these compounds in the fruit is required, along with better understanding of the influence of viticultural practices, to potentially produce fruit specifically for post-fermentation EM. The ability to selectively stream batches of fruit from the vineyard greatly improves winemaking efficiency over vintage, which was one of the major issues associated with post-fermentation EM identified by winemakers in Joscelyne's survey (2009). Temperature regulation through EM should also be considered by winemakers, as this study has suggested that the extraction of monomeric flavan-3-ols and tannins, as well as tannin modification may be influenced.

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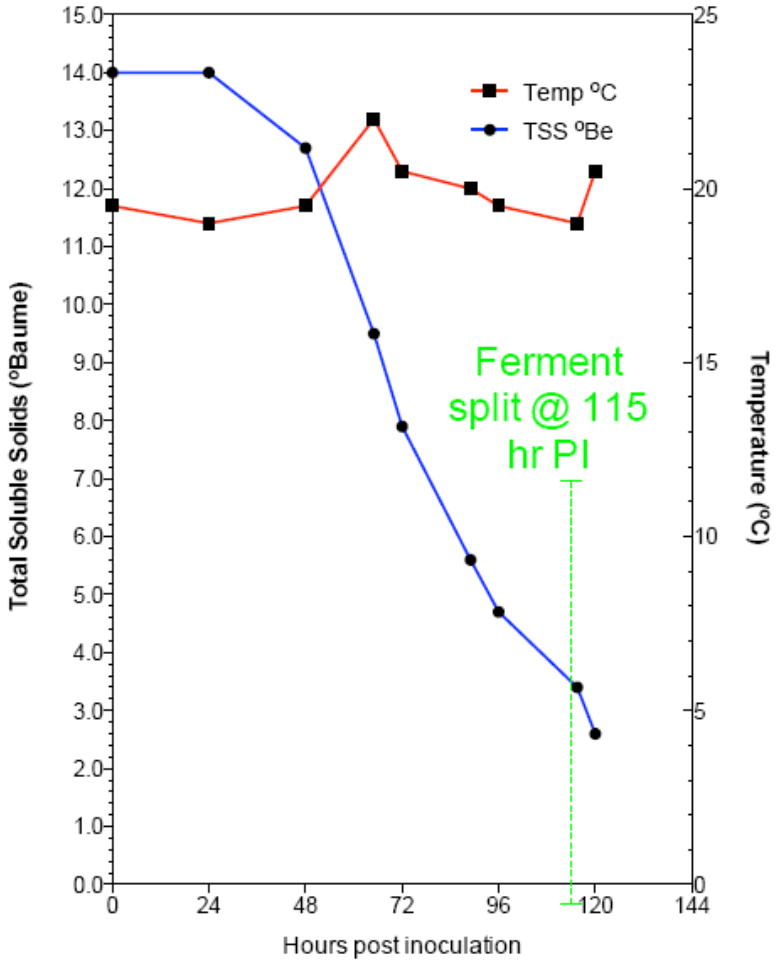
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Appendices

A. *Winemaking*

Table A.1: Analysis of fruit during the growing season and initial must analysis prior to inoculation.

Date	TSS (°Baumé)	pH	TA (g/L H_2T)
15 day pre-harvest (11/3/10	11	3.01	11
7 days pre-harvest (18/3/10)	13.4	3.17	7.8
Post-crushing (26/3/10)	14	3.64	5.6



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Figure A.1: Fermentation curve of the initial batch of fruit up to 120 hours post-inoculation

B. Quantitative Analysis of Phenolic Compounds

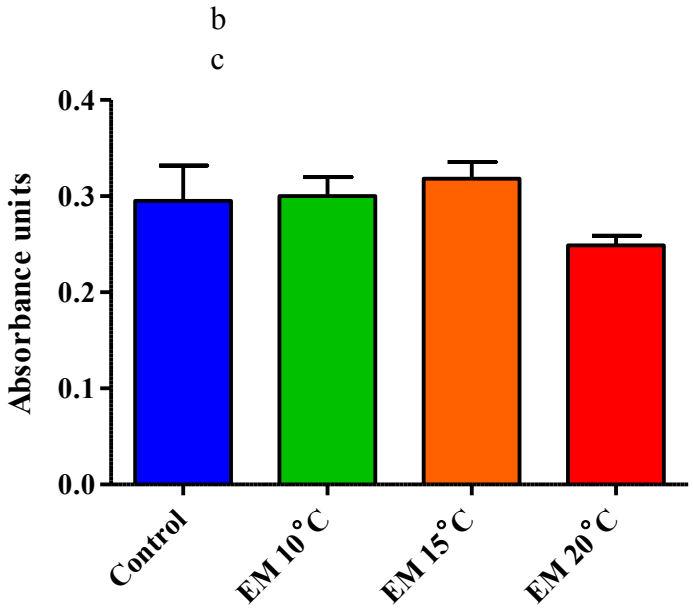


Figure B.1: Analysis of bottled wines 175 days post-inoculation by the methyl cellulose precipitable tannin assay. Error bars represent the standard error (n=4). Significance of differences between treatments is indicated by different letters.

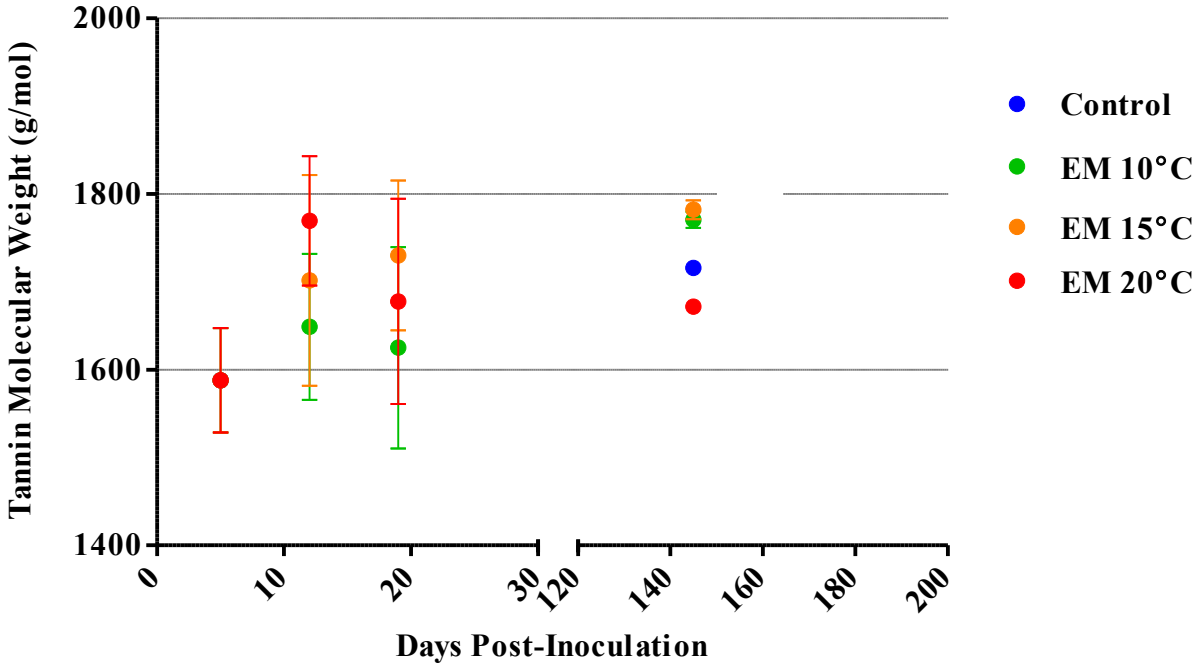


Figure B.2: Changes in tannin molecular weight (as g/mol) over the period of extended maceration taken as 50% tannin elution of GPC run for each sample. Error bars represent ±

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standard error (n=3). Significance of differences between treatments at the last time points (bottled wines) is indicated by different letters.

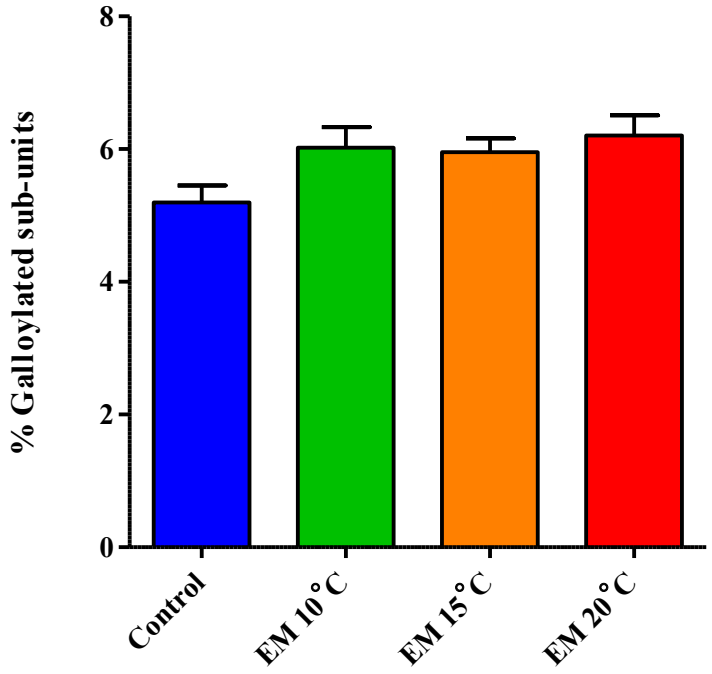


Figure B.3: Galloylated tannin subunits expressed as a percentage of total tannin subunits, measured by the phloroglucinol assay. Measured in bottled wines 145 days post-inoculation. Error bars represent the standard error (n=3). Significance of differences between treatments is indicated by different letters.

Table B.1: Rank scores[¤] for four treatments as tasted by 23 judges at 14th AWITC workshop, 'Managing tannins in the vineyard and winery'.

Treatment	Colour		Astringency		Bitterness	
Control	1.91	a	2.56	ab	2.74	ab
10° EM	2.96	b	2.21	a	2.13	a
15° EM	2.78	b	2.30	ab	2.21	a
20° EM	2.34	ab	2.91	b	2.91	b

Wines were ranked 1 to 4 for three attributes; colour, astringency and bitterness[†]. Letters indicate significant differences based on Dunn's Procedure at 5% significance

[¤] Calculated as rank sum/number of judges

[†]For colour, wines were ranked 1 to 4, with 1 having the strongest red colour and 4 the weakest red colour. For astringency, wines were ranked 1 to 4 with 1 being the most astringent wine and 4 the least astringent. For bitterness, wines were ranked 1 to 4 with 1 being the most bitter wine and 4 the least bitter.