THE EFFECTS OF MICRO-OXYGENATION ON PHENOLIC COMPOUNDS IN CHAMBOURCIN AND RUBY CABERNET WINES

By

EECHIN NG

Bachelor of Science in Chemical Engineering
Oklahoma State University
Stillwater, Oklahoma
2003

Master of Science in Food Science
Oklahoma State University
Stillwater, Oklahoma
2007

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THE EFFECTS OF MICRO-OXYGENATION ON PHENOLIC COMPOUNDS IN CHAMBOURCIN AND RUBY CABERNET WINES

Dissertation Approved:

Dr. William McGlynn

Dissertation Adviser

Dr. Timothy Bowser

Dr. Carla Goad

Dr. Edralin Lucas

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The effect of micro-oxygenation on phenolic compounds was evaluated in wines made from a traditional vinifera variety, Ruby Cabernet, and a French-American hybrid variety, Chambourcin. Skin contact times (SCT) prior to pressing were 6 and 12 days. Injection of oxygen commenced after pressing and occurred daily for 16 weeks. Three levels of oxygen were applied: control, low (2.1 mL O_2 per fermentation vessel per day) and high (21 mL O_2 per vessel per day). Samples were collected weekly over 16 weeks and an additional sample was collected after approximately 18 months of storage.

Our analyses showed that in both wines oxygenation treatment generally did not significantly affect the content of most of the phenolic compounds analyzed during the initial 16 weeks of treatment. After 18 months, oxygenated Chambourcin wines had lower monomeric anthocyanins compared to controls. Oxygenated 12-day SCT Ruby Cabernet wines had lower concentrations of monomeric anthocyanins than controls from 16 weeks on and after 18 months storage. Oxygenated 12-day SCT Ruby Cabernet wines also had lower concentrations of long polymeric pigments (LPP) than controls after 18 months of storage. Tannins in 12-day SCT Chambourcin wines showed significant oxygen effect for the 16-week experimental period, but none after storage. In terms of antioxidant capacity, oxygenation effect was also insignificant. Liquid chromatography analysis of individual phenolic compounds also showed that oxygenation had an insignificant impact on most of the phenolics content and composition after 16 weeks. Catechin, myricetin and quercetin were the major phenolics identified.

Although the observed effects of micro-oxygenation were generally consistent with an accelerated ageing process, quality-related effects on the phenolic compounds in both wines were not readily apparent from the chemical testing performed. It is possible that an experimental design with a lone SCT treatment and with varying oxygenation levels could better demonstrate the effects of the oxygenation treatment. As chemical analyses alone cannot fully describe the quality of a wine, sensory testing may be beneficial in detecting differences.

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CHAPTER I

INTRODUCTION

Phenolic compounds are a prominent component in grapes and grape wines. Different classes of phenolics are found in the skin, flesh and seed of the grape berry. The type of phenolic compounds found in grapes, grape juices, and grape wines can vary depending on a multitude of factors. For instance, variety, geographical origin, soil type, environmental conditions and grape processing/handling techniques can all effect phenolic compound composition (Thimothe and others 2007; Russo and others 2008). The compositional differences of these phenolics in turns influence the taste and overall organoleptic characteristics of the wine produced. Apart from environmental and post-harvest handling factors, vinification practices also can have a huge influence on the phenolics content of the finished wine. The skin-contact time, or the duration at which the skins and seeds of the grapes stay in contact with the must (a mixture of grape juice and partially pressed grape berries) can directly impact the amount of phenolics extracted into solution. In general, the longer the duration of skin contact, the higher the concentration of phenolics extracted. Another vinification practice, the ageing process, probably the will significantly alter the phenolics structure of the wine. Most of these changes during

ageing bring about positive contributions, resulting in “smoothness” and “balance", the characteristics synonymous with a good red wine.

Currently, plant phenolics have attracted much attention due to recent findings concerning their antioxidant, anticancer and antimicrobial properties. For instance, grape phenolic extract had been shown to be highly effective against specific virulence traits of *Streptococcus mutans*, a known dental pathogen (Thimothe and others 2007) and certain phenolic fractions extracted from a Petite Syrah wine were found to be effective in inhibiting the oxidation of low-density lipoprotein *in vitro* (Teissedre and others 1996). Bilberry extract containing phenolic compounds, including anthocyanins, have been shown to inhibit growth of human colon carcinoma cells and human leukemia cells *in vitro.* From this bilberry extract, pure malvidin and delphinidin glucosides (anthocyanins) were isolated and have displayed apoptotic effects in human leukemia cells (Katsube and others 2003).

Oxygen has been recognized as an important player in the course of the life of a wine. Oxygen participates in numerous microbiological and biochemical processes that ultimately affect the organoleptic properties of the finished wine (Parish and others 2000). One of the recent innovations in enology is the introduction of minute quantities of oxygen during the ageing process; this is termed micro-oxygenation. This process introduces controlled amounts of oxygen into the wine to induce favorable changes such as improved palatability, enhanced color stability, increased oxidative stability and decreased vegetative aromas and reductive characters (Parish and others 2000). Micro-oxygenation is supposed to mimic the diffusion of oxygen into wine during oak barrel

ageing, where air escapes into the wine through the permeable wooden staves. This is an obvious contrast to the periodic aerated racking process, where large doses of oxygen are added to the wine instead (Paul 2002).

The practice of micro-oxygenation has been shown to be beneficial. Oxygen participates in the polymerization of polyphenolic compounds that produces stable forms of anthocyanins that resist discoloration by sulfur dioxide, and therefore helps to provide color stability in red wines across a range of wine pH values. Periodic racking processes have also been instrumental in decreasing green, herbaceous aromas. However, oxygen also has its destructive effects. Too much oxygen can lead to over-polymerization where the large molecules are unable to remain suspended in solution and this results in the precipitation of polymeric materials and a loss of color intensity (Paul 2002; Cano-López and others 2006).

Color is one the factors used in the quality evaluation of red wine. Anthocyanins, a major group of phenolics in grapes, play a vital role in the color of young red wines. Anthocyanins are instable and they react with other phenolic compounds, mainly flavanols, to form more stable, colored compounds during wine maturation (Atanasova and others 2002; Cano-Lopez and others 2008). Therefore, the quantities of free anthocyanins decrease during ageing, dropping to about 20% of the initial number. Tannins are the other major phenolics that contribute to bitterness and astringency sensations, two important components of the overall mouthfeel of red wines. Astringency is an important factor as it gives a certain bite to red wine. The composition of these phenolic compounds can be estimated by various chemical methods; however these analyses are insufficient in providing a comprehensive picture of the overall quality of a

wine. Therefore, sensory evaluation can be instrumental in giving the researcher a glimpse of a different dimension that cannot be readily observable with chemical analysis. With the outcomes of chemical testing and evaluation by trained sensory panelists in hand, a winemaker is better able to form an educated conclusion regarding which techniques succeed in improving wine quality and which do not.

Chambourcin is a French-American hybrid that was first cultivated in France in the regions of Loire Valley. Its exact parentage is unclear; however, the National Grape Registry has this variety listed under the Seyve-Villard 12-417 x Seibel 7053 parentage (Iowa State University Viticulture 2008). In the U.S., Chambourcin is currently grown in Pennsylvania, New Jersey, and Virginia (Hudson Valley Wine Magazine) as well as in various Midwestern states and is typically used to produce dry red wine that is deeply colored (Iowa State University Viticulture 2008) and rich in fruity flavors (Hudson Valley Wine Magazine).

Ruby Cabernet is a traditional *Vitis vinifera* variety popular in California. This variety is commonly used in a blend, as it lacks complexity in its varietal form (Wine Searcher 2012). Chambourcin and Ruby Cabernet are two of grape varieties cultivated in Oklahoma. They have shown to be resistant to environmental elements, and therefore have potential to be made into wine. The purpose of this research projects is to test the ,effect of micro-oxygenation on the phenolic compounds of Chambourcin and Ruby Cabernet wines. The specific objectives of this project are as follows:

- To determine the effect of micro-oxygenation on the phenolics content of Chambourcin and Ruby Cabernet wines by spectrophotometric method.

- To determine the effect of micro-oxygenation on the individual phenolic compounds of Chambourcin and Ruby Cabernet wines by high-performance liquid chromatography.
- To determine the effect of micro-oxygenation on the antioxidant capacity of Chambourcin and Ruby Cabernet wines.

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CHAPTER II

REVIEW OF LITERATURE

CHEMICAL COMPOSITION OF GRAPES AND WINES

Sugars and Organic Acids

The most abundant sugars present in grapes are glucose and fructose. These are essential substrates for the fermentation process in winemaking, where they are converted to ethanol and carbon dioxide by yeast. Sucrose is also present in grapes in minor quantities. Together glucose, fructose and sucrose comprised over 90% of total sugars in grapes (Johnson and Carroll 1973). Even in wines fermented to absolute dryness still contain between 0.1-0.2% of unfermented sugars (Vine and others 2002).

Minerals

Potassium is the most abundant cation in grape berries. While potassium is essential for grapevine growth and development, in excess it can cause a decrease in free acid levels. Potassium also combines with tartaric acid to form potassium bitartrate in wines. Potassium bitartrate is largely insoluble in wines and it precipitates out of solution during winemaking and storage, which leads to an increase in wine pH. Elevated pH in

turn causes deleterious effects in wine quality, such as greater susceptibility to microbial spoilage, decrease in color stability and possibly unsatisfactory sensory attributes (Davies and others 2006). Besides potassium, calcium, sodium, magnesium and iron are also present in grape berries (Vine and others 2002).

Pectic Substances

Pectic substances are a group of closely related polysaccharides that can be classified into two groups: (1) neutral pectic substances (arabans, 1-4 galactans and 1-4 arabinogalactans) and (2) acidic pectic substances, or pectins, which are exclusively made of galacturonic acids. Pectin is a component of the cell wall of grape berry and calcium chelation of the pectic components is essential in maintaining the cell wall stability (Chardonnet and others 1997). According to Silacci and Morrison (1990), the total pectin concentration in Cabernet Sauvignon grapes increased during the period of rapid berry growth following veraison (the onset of ripening), but decreased during ripening. In winemaking, commercial pectic enzymes preparations are sometimes added to wine prior to pressing to enhance juice release (Lea and Piggott 1995).

Nitrogenous Compounds

Amino acids, peptides and proteins are some of the nitrogenous compounds found in grapes and they made up less than 1% of grape composition. The content of these compounds vary depending on grape variety, vineyard locale, climate and other factors. During fermentation, amino acids are required as a catalyst in synthesizing nitrogen into the free ammonium state that is required by yeasts. Red wines, which have a higher

phenolics content, tend to precipitate protein complexes much more that the white variety (Vine and others 2002).

Phenolic Compounds

The most fundamental phenolic compound is the phenol, which is a benzene ring with a single hydroxyl group (OH). Phenol is not found naturally in grapes or wine, but various substitution patterns of this basic structure form the many phenolic compounds found in wine. Phenolic compounds contribute significantly to the overall quality of wine and they can be divided into two major groups -- flavonoid and non-flavonoid. Flavonoids consist mainly of anthocyanins, flavanols (catechin, epicatechin, epicatechin gallate) and flavonols (quercetin, kaempferol, myricetin) (Waterhouse 2002; Pè rez-Magariño and others 2008). The non-flavonoid subgroup includes the phenolic alcohols, aldehydes, acids and their derivatives, and other related compounds such as stilbenes (Pè rez-Magariño and others 2008) (Figure 1).

Many factors can influence the content of phenolics in wine and that includes the grape characteristics and winemaking practices, such as length of maceration and frequency of pumping over (Cano-López and others 2008). Maceration time is the time where the grape skins stay in contact with the juices for a specific amount of time. Pumping over is the practice of pumping the fermented wine over the cap (i.e. the layer of skins and seeds that floated to the surface of the liquid).

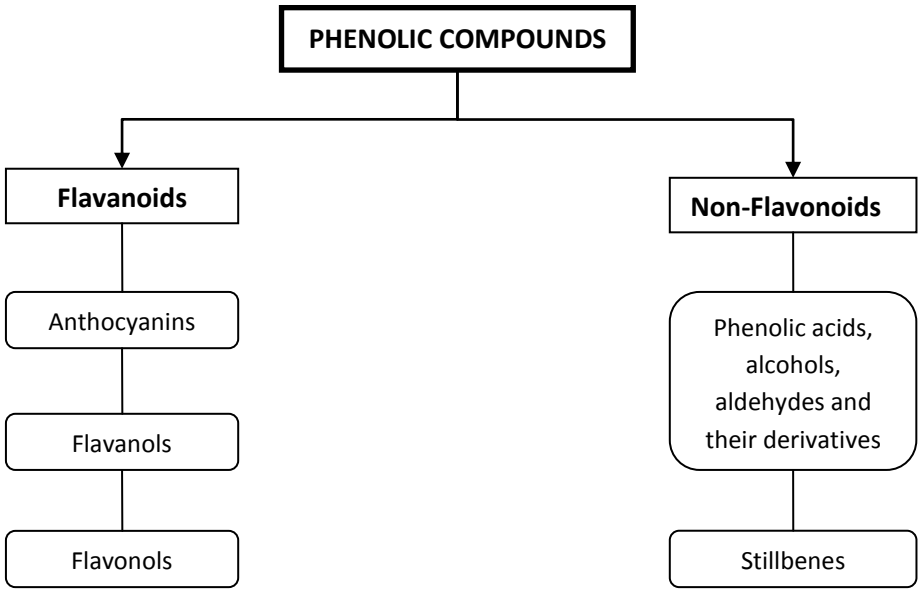

Figure 1. Classes of phenolics

FLAVONOIDS

The flavonoids in grapes and wine have the same ring system as shown in Figure 2, and all have the same hydroxyl substitution groups on ring A, at position 5 and 7. The differences in the oxidation state and substitution on ring C define the different classes of flavonoids. Flavonoids are the major phenolics in red wine and they are mostly derived from the skins and seeds of grapes during the fermentation process (Waterhouse 2002).

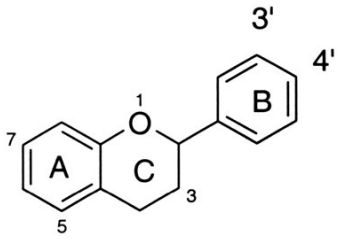

Figure 2. The flavonoid ring system (Reproduced from Waterhouse 2002)

Anthocyanins

In red wine, the signature deep maroon hue provides visual cues about the wine's quality and character. In fact, one of the many criteria of wine tasting is the evaluation of color. The major contributors of the color of young red wine, the anthocyanins (Revilla and others 1999; Cano-López and others 2008; Pè rez-Magariño and others 2008), are one of the most studied phenolics in wines. There are almost exclusively located in the outer layers of the grape skins and are sensitive to pH (Jensen and others 2008). The term "anthocyanin" implies a glycoside. Its non-glycoside counterpart is the anthocyanidin, which is never found in grapes or wine, except in trace quantities (Waterhouse 2002) (Figure 3).

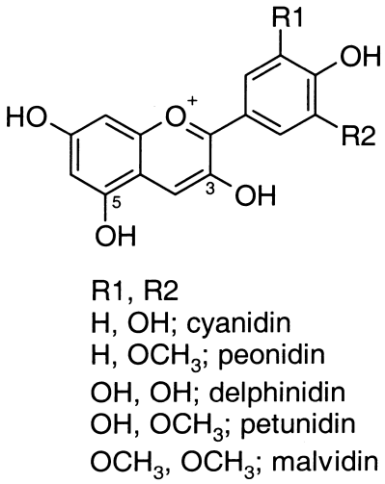

Figure 3. Anthocyanidin structures (Reproduced from Waterhouse 2002)

In red wine, some of the monomeric anthocyanins present include delphinidin-3-monoglucoside, cyanidin-3-monoglucoside, petunidin-3-monoglucoside, peonidin-3-monoglucoside, and malvidin-3-monoglucoside, with malvidin-3-monoglucoside being the dominant species (Mazza 1995) (Figure 4).

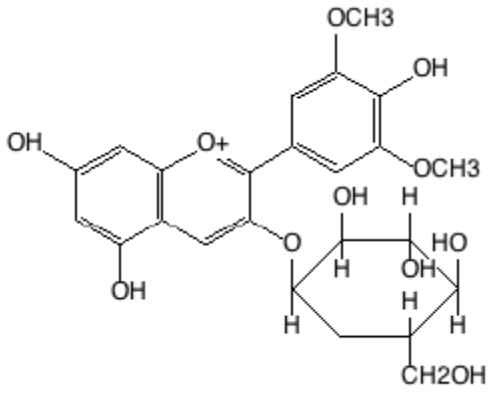

Figure 4. Malvidin-3-monoglucoside (Reproduced from WSU Viticulture & Enology 2012)

Anthocyanins are highly unstable (Cano-López and others 2006) and their interactions with other phenolics results in the color changes observed in maturing wines (Cano-López and others 2008). These changes of color in maturing wines are due to the reaction of anthocyanins with other phenolics in wines, resulting in more stable polymeric pigments. The anthocyanin monomers and polymeric pigments are distinguishable on the basis of their behavior at different pHs and their susceptibility to bleaching by bisulfite (SO_2) (Somers and Evans 1977). According to Cabrita and others (2000), at 520 nm, monomeric anthocyanins showed a large decrease in absorbance when pH is raised from 1 to 5, whereas the absorbance due to polymeric pigments are stable under those conditions. Additionally, the red color of monomeric anthocyanins is easily bleached by excess bisulfite addition, whereas the polymeric pigments continued to show absorbance at 520 nm.

Flavanols

Flavanols or flavan-3-ols are the most abundant class of flavonoids in grapes and wine and include simple monomeric catechins (Figure 5). They are mainly located in the

seed and skin of berry and are bitter and astringent (Waterhouse 2002). Tannins are polymeric flavanols containing catechin, epicatechin, epicatechin gallate or epigallocatechin (Harbertson and others 2003). They contribute to the astringency that is essential to the overall mouthfeel of red wine (Parish and others 2000). Astringency is considered to be a tactile sensation which is caused by a reaction between salivary proteins and flavanols (Parish and others 2000). It is supposed to add a certain bite to the wine. The delicate balance of astringency is hard to achieve: if the wine is too astringent, it will be judged unfavorably harsh; on the other hand if the astringency is too low, the wine is considered flat (Gonzáléz-Sanjosé and others 2008).

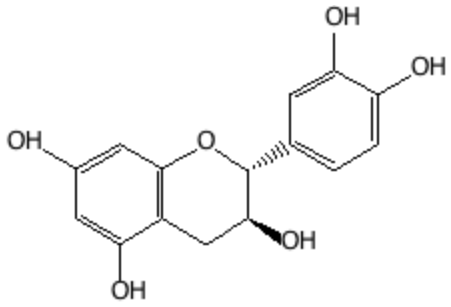

Figure 5. Catechin (Reproduced from WSU Viticulture & Enology 2012)

Flavonols

Flavonols are found in plants in glycoside form and in grapes, are mainly located in the skin. Three forms of simple flavonol aglycones in grapes include quercetin (Figure 6), myricetin and kaempferol, and they occur with a diverse combination of glycosidic forms (Waterhouse 2002), with D-glucose being the most common sugar residue (Häkkinen 2000). Other sugar residues include D-galactose, L-rhamnose, L-arabinose, D-

xylose and D-glucuronic acid (Hakkinen 2000). Other flavonols identified in grapes include isorhamnetin, laricitrin and syringetin (Castillo-Muñoz and others 2007).

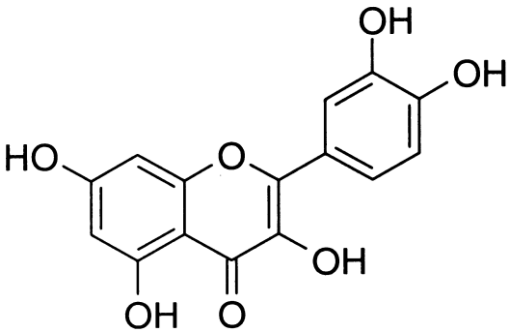

Figure 6. Quercetin (Reproduced from Waterhouse 2002)

NON-FLAVONOIDS

Phenolic Acids

Phenolic acids can be divided into two categories: hydroxycinnamic acids and hydroxybenzoic acids. The four most common hydroxycinnamic acids include caffeic, ferulic, sinapic and *p*-coumaric acids (Häkkinen 2000). Hydroxybenzoic acids are derived directly from benzoic acid. Variations in the structures of the individual hydrobenzoic acids depend on the methylations and hydroxylations of the aromatic ring. These acids include p-hydroxybenzoic, gallic, vanillic, syringic and protocatechuic acids (Häkkinen 2000). In red wines, gallic, vanillic, syringic, *p*-coumaric, caffeic and ferulic acids have been identified (Buiarelli and others 1995).

Stillbenes

Stillbenes appear in trace quantities in grapes and wine. Even so, they have drawn considerable attention due to their potential anti-carcinogenic properties as well as their

possible role in preventing heart disease. The principle stillbene in grapes is resveratrol (Figure 7), and it appears in both *cis* and *trans* isomers, including the glucosides of both isomers (Figure 8). All forms are found in wine, but *cis*-resveratrol is absent in grapes. Derivatives of resveratrol are found only in grape skins, especially in red grapes (Waterhouse 2002).

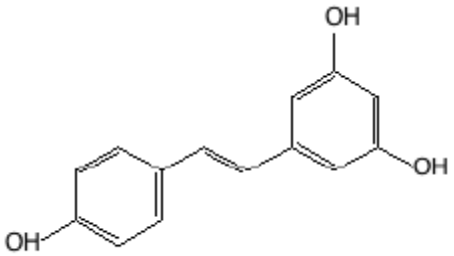

Figure 7. Resveratrol (Reproduced from WSU Viticulture & Enology 2012)

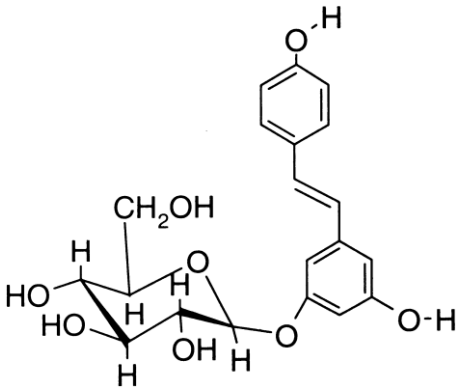

Figure 8. *Trans*-piceid, the resveratrol glucoside (Reproduced from Waterhouse 2002)

EFFECT OF MICRO-OXYGENATION ON PHENOLIC COMPOUNDS IN WINE

Oxygen is an important player in many of the reactions that occur during winemaking (Cejudo-Bastante and others 2011). In oak barrels, oxygen permeates the

wine through the bungholes or staves, and also through diffusion of air found in the headspace. Wine is also exposed to air during the filtration or racking processes (Parish and others 2000; Kelly and Wollan 2003). However, the introduction of oxygen into the wine through these processes is difficult to control. Moreover, according to Kelly and Wollan (2003), the barrel's own diffusion rate is less than 2.5 mL/L/month, thus supplementation with additional oxygen could be beneficial.

Since the 1990s, wineries have been introducing small amount of oxygen continuously into wine in a controlled way -- a process referred to as micro-oxygenation. This process is aimed at manipulating the oxygen-requiring processes that occur in wine to bring about desirable changes in aroma and texture (Paul 2002). Micro-oxygenation has been shown to stabilize wine color, soften the astringent tannins (Parish and others 2000), and decrease unpleasant green, herbaceous notes (Parish and others 2000; Gonzáléz-Sanjosé and others 2008). However, too much oxygen can lead to adverse effects. For instance, oxygen can cause polymerization where the large molecules formed are unable to remain solubilized, causing precipitation and loss of color intensity. Also, excess oxygen may also contribute to oxidation of phenolics, which effects are often detrimental and irreversible (Cano-López and others 2006).

The effect of micro-oxygenation on the phenolics profile of red wines has been explored in multiple studies. A few studies have indicated that the addition of oxygen to red wines leads to an increase in color density as more polymeric pigments are formed (Cano-López and others 2006, 2008; du Toit and others 2006). As mentioned earlier, anthocyanins are the major phenolics contributing to the color of young red wines, but they are highly unstable (Cano-López and others 2006). Anthocyanins participate in

many reactions during fermentation and maturation to form more stable colored compounds (Cano-Lopez and others 2006). This perhaps explains the conversion of the red-bluish color of young red wines to the red-brownish color of aged wines (Atanasova and others 2002). A few mechanisms for the formation of new pigments from anthocyanins have been proposed:

1. ***Direct reactions between anthocyanins and flavanols***

Using LC/MS, Remy and others (2000) showed the formation of two covalent structures between tannin and native pigments in red wines. These structures differ in the linkage position of the anthocyanin moiety. One of these structures, denoted T-A, was formed when malvidin-3-glucoside was linked by its C-6 or C-8 top as a terminal unit in the original derived pigment. The second structure, A-T, was formed from direct reaction between malvidin-3-glucoside and catechin. Similarly Cano-López and others (2006) also identified the same compound as Remy and others (2000), that is malvidin-3-glucoside-(epi)catechin.

2. ***Condensation reaction between anthocyanins and flavanols mediated by acetaldehyde***

Acetaldehyde is produced as a byproduct of yeast metabolism. It can also form when ethanol is oxidized in the presence of oxygen. The condensation process between anthocyanins and flavanols in the presence of acetaldehyde yields ethyl-bridged pigments which are expected to be favored by the presence of oxygen (Atanasova and others 2002). According to Dallas and others (1996), reaction between cyanidin-3-glucoside and procyanidins in the presence of acetaldehyde resulted in the formation of two polymeric pigments that disappeared after 12 days. In another model solution containing peonidin-

3-glucoside in place of cyanidin-3-glucoside, two colored compounds were developed, but they became undetectable after 10 days. The researchers attributed the disappearance of these colored compounds to polymerization to higher molecular weight compounds, as evidenced by the presence of precipitation in both of the model solutions.

In a different study (Atanasova and others 2002), new ethyl-linked pigments were formed due to the condensation of dephinidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside and malvidin-3-glucoside with epicatechin. Cano-López and others (2006) also identified some ethyl-linked compounds formed from malvidin-3-glucoside with epicatechin: malvidin-3-glucoside-ethyl-dicatechin, malvidin-3-glucoside-ethyl-catechin and malvidin-3-coumarylglucoside-ethyl-catechin. These compounds are present in both the control and micro-oxygenated wines, but are higher in micro-oxygenated wines. In another study, also by Cano-López and others (2008), ethyl-linked compounds were also found in greater concentration in micro-oxygenated wines. These ethyl-linked compounds are purple in color and are less sensitive to bleaching by SO_2 than monomeric anthocyanins.

3. Reaction between anthocyanins and compounds with polarisable double bonds such as vinyl phenols or pyruvic acid

Vinyl phenols and pyruvic acid are some of the byproducts of yeast metabolism. These compounds have polarisable double bonds and have been shown to react with anthocyanins to form pyranoanthocyanins (Atanasova and others 2002; Cano-López and others 2006). Pyranoanthocyanins are important to the color of red wines as they are very stable and resistant to oxidation (Cano-López and others 2006).

In the study by Fulcrand and others (1996a), two malvidin-derived pigments, A and B, formed from major anthocyanins [malvidin 3-monoglucoside and malvidin-3-(6-p-coumaroyl) monoglucoside] with 4-vinylphenol were identified. In another study by Atanasova and others (2002), an oxygenated red wine that had been stored for 7 months was shown to have pyranoanthocyanins adducts. These pyranoanthocyanins adducts were formed from the reactions of pyruvic acid with delphinidin-3-glucoside, petunidin-3-glucoside, malvidin-3-glucoside, malvidin-3-acetylglucoside and malvidin-3-*p*-coumaroyglucoside.

Similar results were reported by Cano-López and others (2006). In this study, multiple pyranoathocyanins were detected in the micro-oxygenated wines: petunidin-3-glucoside pyruvate, vitisin A (malvidin-3-glucoside pyruvate), acetyl vitisin A (malvidin-3-(acetylglucoside) pyruvate) and coumaryl vitisin A (malvidin-3-(coumarylglucoside) pyruvate). At the end of the study, Cano-López and others (2006) reported the concentration of vitisin A-like compounds (petunidin-3-glucoside pyruvate, vitisin A and coumaryl vitisin A) was increased in the micro-oxygenated wines and the greatest increase was observed in the wines receiving the highest dose of oxygen. Meanwhile, these compounds had lower concentrations in control wines. Besides pyranoanthocyanins, Cano-López and others (2006) also detected a different group of anthocyanins, referred to as the hydroxyphenyl-pyranoanthocyanins, which were formed from the reactions between anthocyanins with vinyl derivatives. The compounds detected included malvidin-3-glucoside-4-vinylphenol, pinotin A (malvidin-3-glucoside-4-vinyl-catechol) and malvidin-3-glucoside-4-vinylguaiacol.

The success of micro-oxygenation on the promotion of color density depends on several factors: age of the wine, timing of oxygenation, initial phenolics contents, and dosage of oxygen. Oxygenation is more effective in young red wines when it is performed after malolactic fermentation. Malolactic fermentation is the process where tart-tasting malic acid, which occurs naturally in grape must, is converted to the softer-flavored lactic acid. Micro-oxygenation is also more effective when the red wine has a higher initial phenolics content (Cano-López and others 2008), probably because oxygen-mediated color-stabilizing reactions in the wine require substantial beginning concentrations of appropriate substrates. This observation was supported by results observed by du Toit and others (2006), where a decrease in total phenolics content in micro-oxygenated red wines was found. The dosage of oxygen is also crucial as oxygen oxidizes ethanol to acetaldehyde, and acetaldehyde participates in many reactions that contribute to the formation of new pigments (Atanasova and others 2002; Cano-López and others 2006; Dallas and others 1996; Fulcrand and others 1996b). However, as mentioned earlier, too high a dosage may yield the opposite effect. It should be pointed out that oxygenation doesn't always increases the color intensity of red wine, as observed by du Toit and others (2006).

EFFECT OF SKIN CONTACT TIME ON AROMA CONTENT IN WINE

Skin contact or maceration time is the period when the macerated grapes and its juices (collectively referred to as '*must'*) are held in contact for a period ranging from 3 to 14 days in order to extract the compounds that contribute to flavor and color (Schmidt

and Noble 1983). Extended skin contact time usually results in greater extraction of tannins and anthocyanins. Anthocyanins, which are mainly found in grape skin, are extracted during this time and they give rise to the vivid maroon hue that is commonly associated with red wine. However, some grape species are not suitable for extended skin fermentation, as in muscadine grapes, which resulted in greater astringency (Gómez-Plaza and others 2002). Studies focusing on the effect of skin contact time on the phenolics profile of red wines are scarce. The few papers found on this subject were on the aroma profile or free volatiles of wines subjected to different duration of skin contact.

In a study by Schmidt and Noble (1983), the researchers analyzed two Cabernet Sauvignon wines by descriptive analysis. There were two vintages, 1977 and 1978, and both were subjected to skin contact time (SCT) of 2 to 7 days. For the 1977 vintage (SCT 2, 3, 3.5, 4, 5, and 6 days), they found the major change in aroma character (canned green bean/canned asparagus aroma and berry aroma) occurred between 2 and 3 days of skin contact. Vegetative notes as defined by "canned green bean/canned asparagus" decreased with extended SCT but the berry aroma increased. In the 1978 vintage (SCT 2, 3, 4, 5, 6 and 7 days), only those wines made with 2 and 7 SCT were significantly different in terms of aroma and astringency increased with extended SCT. Overall, this study concluded that the differences of aroma due to SCTs between 2 and 7 days were very small.

In another study (Maggu and others 2007), evaluated whether skin contact time and the pressure applied during pressing could impact the composition of aroma compounds in the juice and ultimately the finished wine. In this study, the compound pivotal to the varietal characteristics of Sauvignon Blanc wines, 2-methoxy-3-

isobutylpyrazine (IBMP), was evaluated along with *S*-(3-hexan-1-ol)cysteine (3MH-*S*-sys), which was the pre-cursor to the passion fruit-like aroma of thiol 3-mercapto-hexanol (3MH). IBMP and 3MH are both volatile and IBMP is located largely in the skin (95%). In this study, they found that longer skin contact time (32h) and increasing pressure resulted in greater concentration of 3MH-*S*-sys and IBMP in the juice during laboratory trials using a grape crusher/destemmer. This study evaluated these compounds in the must, but not in the finished wine.

ANTIOXIDANT CAPACITY

Antioxidants are substances that can reduce oxidative stress in the human body by scavenging free radicals. Oxidative stress is characterized by an imbalance between free radical production and antioxidant capacity, causing the accumulation of oxidative products such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). Free radicals are generated due to the stress imposed on the body, for instance a high fat diet, obesity, hyperglycemia, and smoking, to name a few. The increase and accumulation of free radicals can lead to various bodily disorders, such as DNA damage, LDL (low-density-lipoprotein) oxidation and protein oxidation. In time these disorders can lead to the development of chronic illnesses such as atherosclerosis, cancer, and diabetes mellitus. Antioxidants, with their ability to neutralize free radicals, can exert protective effects in human bodies that can ultimately lead to lower risk of chronic diseases and better health. The effectiveness of antioxidants to neutralize free radicals is termed antioxidant capacity. The higher the antioxidant capacity, the higher capacity the

compound has to quench free radicals (Vizzotto and others 2007). Antioxidant capacity can be measured using methods such as Trolox Equivalent Antioxidant Capacity (TEAC), Ferric Reducing Antioxidant Power (FRAP), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay and Oxygen Radical Absorbance Capacity (ORAC). The summary of each of these methods is as follows:

1. ***Trolox Equivalent Antioxidant Capacity (TEAC)***

In this assay, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) is oxidized by peroxyl radicals or other oxidants into its radical cation $ABTS^{\cdot+}$, which is an intense-colored compound. The antioxidant capacity is measured as the ability of the antioxidant to decrease the color-forming reaction, which is measured using a spectrophotometer. The radical cation $ABTS^{\cdot+}$ has several maximum-absorption wavelengths: 415, 645, 734 and 815 nm. Wavelengths of 415 and 734 nm are commonly used (Prior and others 2005).

2. ***Ferric Reducing Antioxidant Power (FRAP)***

This assay measures the reduction of ferric-tripyridyltriazine (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ), which is an intense blue compound. Absorbance is read at 593 or 595 nm (Gil and others 2002; Prior and others 2005). This assay is very similar to TEAC. Unlike TEAC which is conducted at neutral pH, the FRAP assay requires acidic pH at 3.6 to maintain iron solubility (Prior and others 2005).

3. ***2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay***

The DPPH· radical forms a deep purple color in solution; the DPPH assay is based on the ability of antioxidants to reduce the DPPH· radical into the pale yellow

nonradical form (Seeram and others 2008). The radical scavenging activity is followed spectrophotometrically by the loss of absorbance at 515 nm (Huang and others 2002; Prior and others 2005) or 517 nm (Seeram and others 2008).

4. Oxygen Radical Absorbance Capacity (ORAC)

ORAC measures the inhibition of peroxyl radical ($ROO^{\cdot}$) by antioxidants. Of all the methods mentioned above, ORAC is widely considered to be the standard method for measuring antioxidant capacity in the nutraceutical, pharmaceutical and food industries (Huang and others 2002). In this assay, the peroxyl radical reacts with a fluorescent probe and results in the loss of fluorescence over time. Currently, the ORAC assay employs fluorescein (3',6'-dihyroxy-spriro [iso-benzofuran-1[3H], 9'[9H]-xanthen]-3-one) as the fluorescent probe (Huang and others 2002; Prior and others 2005). The blank, sample and Trolox standard (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) are mixed together with the fluorescein solution in a 96-well microplate and incubated at a constant temperature (37°C) before the addition of AAPH (2,2'-azobis(2-amidinopropane)dichloride, the peroxyl radical generator) to initiate the reaction. The fluorescence intensity (excitation: 485 nm, emission: 530 nm) is measured every minute for 35 minutes in the microplate reader (Huang and others 2002) (Figure 9). Some of the advantages of the ORAC assay are as follows (Prior and others 2005):

1. It provides a controllable source of peroxyl radicals that models the reactions of antioxidants with lipids in food and biological system.
2. The original ORAC assay was configured to measure only hydrophilic antioxidants. Now, this assay can be altered to measure lipophilic antioxidants as well by modifying the radical source and solvent.

3. Traditional antioxidant methods measure the extension of the lag phase only. The ORAC assay however, measures the oxidation reaction for an extended time (about 35 min), and therefore can prevent underestimation of antioxidant activity and account for potential effects of secondary antioxidant products. The ORAC assay uses the AUC (Area Under the Fluorescence Decay Curve) method to calculate the protective effect of an antioxidant and this method accounts for lag time, initial rate and total extent of inhibition in a single value.

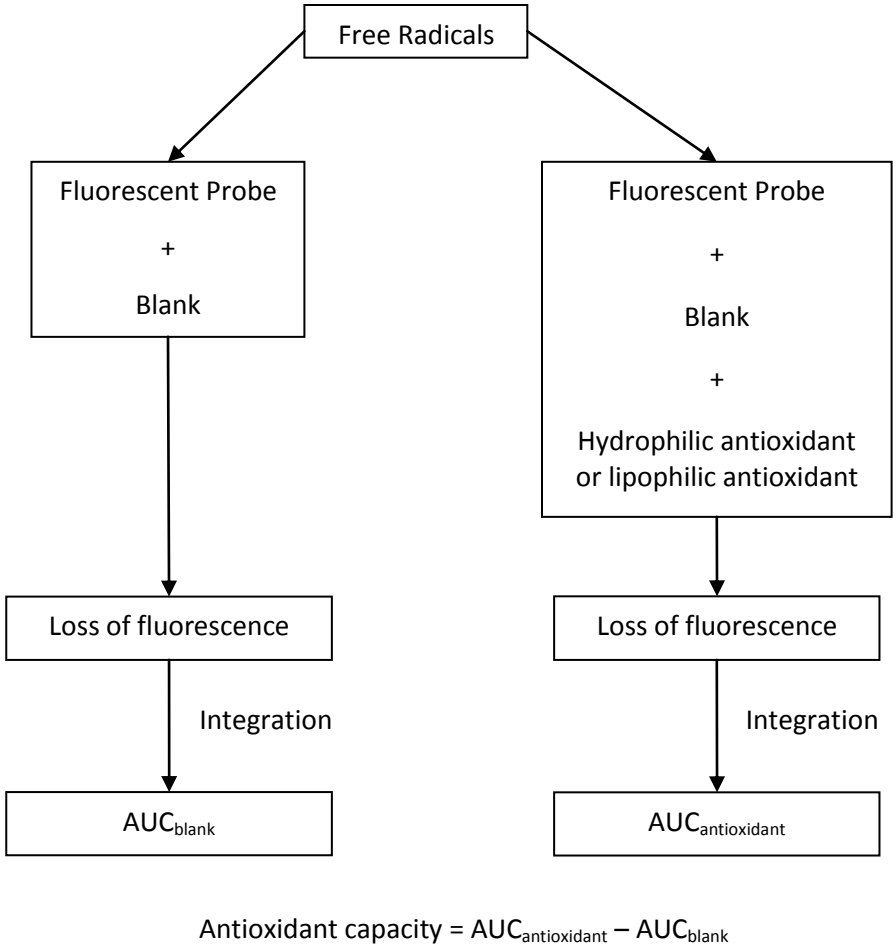

Figure 9. Schematic of the principle of ORAC (Reproduced from Huang and others 2002)

4. The ORAC assay is readily automated. The availability of microplate pipetting systems helps to minimize error associated with manual pipetting, and also allows multiple analyses to be completed at a single time.

However, the ORAC assay also has some disadvantages. For instance, small temperature variations in the wells can lead to decreased reproducibility. Also, the analysis time is relatively long compared to other available methods. Lastly, not all instruments are readily available at some laboratories (Prior and others 2005).

In wine, the common opinion is that the radical scavenging capacity is related to its phenolic content. This fact was demonstrated by Scalzo and others (2012). In this study, three fractions were obtained from all red and white wines studied. The first fraction was the unfractionated portion (UND), where the dried wine aliquot was brought back to its original volume with phosphate buffer and diluted with cold water. The second fraction was the water-eluted portion from a C-18 column (FR1) and the third fraction (FR2) was the methanol-eluted portion of compounds retained by the C-18 column. FR2 contained the phenolic compounds and FR1 essentially had all the non-phenolics such as the hydroxy acids (tartaric, malic, lactic and succinic), glucose, fructose and glycerin. According to this study, ORAC activity was detected in all fractions, but it was found almost exclusively in all FR2 fractions of both red and white wines, with a small fraction in FR1. This result suggests that the phenolics are the compound that contributes to the ORAC activity. However, this study also stressed the importance of considering the use of peroxyl (ORAC), superoxide anion, and hydroxyl radical assays collectively to determine the total antioxidant profile of a food product. The antioxidant capacity by ORAC of some red wines is summarized in Table 1 below.

Table 1. ORAC values of some red wines

Wine Type	*ORAC Value (µmol TE/mL wine)**	*Source*
Cabernet Sauvignon	6.0 – 87.0	Lee and Rennaker, 2007
Cabernet Sauvignon	8.9 – 24.4	Li and others, 2009
Cabernet Gernischet	9.6 – 18.0	Li and others, 2009
Merlot	19.0 – 21.0	Li and others, 2009
Merlot	3.1 – 82.8	Lee and Rennaker, 2007
Blend	14.5 – 22.8	Li and others, 2009
Muscat Hamburg	15.2	Li and others, 2009
Rose Honey	20.0	Li and others, 2009

* TE – Trolox equivalent

The red wines analyzed by Li and others (2009) were from different geographical origins in China. They were 2003 to 2006 vintages. On the other hand, the wines from Lee and Rennaker (2007) were made from grapes cultivated in the Snake River Valley of Idaho and vintages were from 2000 to 2003. Information on the winemaking process was not available for either study.

The effect of different enological practices on the antioxidant capacity of red of wines was evaluated by Villaño and others (2006). A total of 27 monovarietal samples were used: 8 Cabernet Sauvignon, 9 Tempranillo and 10 Syrah wines. During the maceration and fermentation processes, wine samples were collected on different days for analysis. This study found maceration time to have a positive effect on antioxidant capacity. Even though each of the three types of wine had different maceration times, at the end of the process, the final ORAC value was at least 2-fold the initial. This study also examined the effect of clarification processes using albumin or gelatin and membrane filtration on antioxidant capacity. There was a decrease in antioxidant capacity for wines clarified with both albumin and gelatin, as compared to non-clarified wines; statistical analysis showed no significant differences ($p<0.7731$) between the two fining

agents. As for filtration, there were no significant differences in antioxidant capacity due to the filtration process ($p<0.3514$).

PHENOLIC COMPOUNDS AND THEIR HEALTH BENEFITS

Consumption of fresh fruits and vegetables has long been associated with the prevention, delay or onset of chronic degenerative diseases, including cancer. These products contain relatively large quantities of phytochemicals, which may work synergistically to incur disease-preventive action (Zafra-Stone and others 2007).

Some of these phytochemicals in plants are the phenolics and they have shown to promote cardiovascular health. In general, higher consumption of saturated fats and cholesterol leads to higher mortality rate from cardiovascular heart disease (CHD) (Frankel and others 1993). An epidemiological study conducted in France in 1992 revealed a shocking finding -- the French population exhibited a lower incidence of CHD compared to other industrialized nations despite consuming a diet high in saturated fat. This anomaly came to be known as "The French Paradox. A solution was proposed to explain this paradox that related the decrease in CHD to a relatively high consumption of red wine. Subsequent studies have indeed shown that moderate consumption of beer, wine and spirits are all inversely related to CHD; particularly the consumption to wine and beer. For example, Renaud and de Lorgeril (1992) concluded that the intake of red wine led to a reduction in CHD. On a related note, phenolic compounds are also found to inhibit the oxidation of low density lipoprotein (LDL), whose effect is implicated in the development of atherosclerosis (Kerry and Abbey 1997). Kerry and Abbey (1997) found

that red wine separated into catechins, monomeric anthocyanidins and phenolic acids fractions all inhibited LDL oxidation as well as red wine as a whole. Similar results were also obtained from other studies using Petite Syrah (Sirrah) wines (Frankel and others 1993; Teissedre and others 1996).

Besides CHD, phenolics have also demonstrated protective effect against cancers. Phenolics, especially anthocyanins are capable of inhibiting the growth of multiple types of tumors such as human colon cancer cells (Kang and others 2003; Zhao and others 2004) and esophageal tumors in rats (Wang and others 2009). Anthocyanins are also shown to be apoptotic against human leukemia cells (Katsube and others 2003) and helped to decrease the incidence of type-2 diabetes (Ghosh and Konishi 2007).

QUANTIFICATION METHODS OF PHENOLICS

Spectrophotometric Method

1. Total Phenolics Assay

The quantification of phenolics can be achieved spectrophotometrically using the Total Phenols (or Phenolics) Assay by Folin-Ciocalteu reagent. In this assay, phenolics react with the Folin-Ciocalteu reagent only under basic conditions. The sample to be tested is adjusted to the required basic condition by the addition of a sodium carbonate solution. During the reaction, the phenolic proton is dissociated to form the phenolate anion, which is capable of reducing the Folin-Ciocalteu reagent. The original intense yellow solution of the Folin-Ciocalteu reagent is reduced to blue. One of the drawbacks of this assay is that the Folin-Ciocalteu reagent is non-specific to phenolics as it can be

reduced by a number of non-phenolic compounds such as vitamin C and copper iodide [Cu(I)] (Huang and others 2005).

2. pH Differential Method

Anthocyanins undergo structural transformations that are reversible with pH change. At pH 1.0, anthocyanins appear as a colored oxonium form and at pH 4.5, they are a colorless hemiketal form. Using this knowledge, the content of total monomeric anthocyanins can be determined by measuring the absorbance at two different pH values. The sample to be tested is prepared using two different buffers and absorbance is taken at 520 nm and 700 nm, to correct for haze. The final absorbance value is calculated using a formula. This absorbance value is then used in a second formula to calculate the total monomeric anthocyanins content, expressed as cyanidin-3-glucoside. This method is more accurate as it corrects for the interferences of anthocyanins degradation products and other interfering compounds (Giusti and Wrolstad 2000).

3. Harbertson-Adams Assay

This assay was developed by Drs. Harbertson and Adams at the University of California at Davis. It has the ability to quantify multiple phenolics that are considered important in wines such as anthocyanins, tannins, short and long polymeric pigments and non-tannin iron-reactive phenols (Viticulture & Enology University of California Davis 2005). This assay has multiple steps where each step requires the addition of at least one buffer. One of the biggest advantages of this assay is that the reaction occurs in the micro-cuvette itself, and therefore require very small amount of sample. After vortexing and incubation at room temperature for a fixed amount of time, the sample is read at a specific wavelength.

This assay operates on the ability of protein (Bovine Serum Albumin - BSA) to precipitate tannins and some of the red pigments. The pigments that bind to BSA are not released by washing and are stable in the presence of bisulfite. These observations suggest that these pigments are polymeric. However, the pigments precipitated by BSA using centrifugation do not account for all the pigments present in the wine. Some of the pigments are still suspended in the supernatant fraction. Thus, BSA is able to fractionate the polymeric pigments into two distinguishable classes: short polymeric pigments (SPP) that are still suspended in the supernatant fraction and long polymeric pigments (LPP) that precipitate along with the tannins.

Liquid Chromatography Method

Reverse-phase high-performance liquid chromatography (RP-HPLC) has been the instrument of choice for the purpose of detecting phenolic compounds in grape and grape products such as wine. This instrument is normally used with a photodiode array detector (PDA). Due to recent developments in separation science, HPLC-PDA is now used in conjunction with a mass spectrometry (MS) detector equipped with an electrospray ionization source (ESI-MS) to confirm peak identification. Since there are a significant number of phenolic compounds in each phenolic class, where each of them has different absorption maximum, it is common to quantify each of these classes at their maximum absorption wavelength. Most phenolics absorb at 280 nm, so this is a good wavelength to evaluate overall sample complexity. Hydroxycinnamates like caffeic acid absorb at 320 nm, and flavonols such as quercetin have a maximum at about 365 nm. Anthocyanins have absorption maxima at 520 nm (Waterhouse and others 1999).

In a study by Nicoletti and others (2008), HPLC-PDA-ESI-MS was used to identify and quantify the phenolics in grapes. In order to develop a library containing retention times and UV-visible and mass spectra, stock solutions of major phenolics in grapes were prepared and subjected to analysis using the RP-HPLC and both PDA and ESI-MS detection. The software product "Class VP" was employed to compare the closeness of spectra of the standards and the corresponding phenolic compounds separated from the grape extracts. A similarity index (SI) was calculated using the software and an SI value closer to unity was considered to be indicative of greater similarity. In this study, the calibration graphs for all 15 standards showed correlation coefficient above 0.99. Detection wavelength was set at 520 nm for anthocyanins and 280, 306, 320 and 370 nm for other analytes. Mobile phase used was acetonitrile in water with 5% formic acid.

In a similar study, Gomez-Alonso and others (2007) separated phenolic compounds from grape seed and skin extracts and also from wine prepared from the *Vitis vinifera* Cencibel using HPLC-PDA and fluorescence detection. The wavelengths chosen were nearly identical to Nicoletti and others (2008): Anthocyanins at 520 nm and others at 280, 320 and 360 nm. However, the mobile phases used were drastically different than Nicoletti and others (2008): (A) ammonium phosphate, 50 mM, pH=2.6, (B) 20% A and 80% acetonitrile and (C) phosphoric acid, 200 mM, pH=1.5. As in the study by Montealegre and others (2006), the most noticeable difference was the choice of mobile phases -- water/acetic acid (97.5/2/5) and acetonitrile/solvent A (80/20), where the detection wavelengths only varied slightly. The summary of compounds detected and their respective wavelengths are shown in Table 2 below:

Table 2. Detection wavelengths of phenolics by HPLC/PDA

Phenolics	***Wavelength used (nm)***
Gallic acid	280[a,b]
Catechin	280[a,b], 275[c]
Epicatechin	280[a,b], 275[c]
Epicatechin gallate	275[c]
Protocatechuic acid	280[b], 275[c]
Caftaric acid	320[a,b]
Caffeic acid	320[a,c]
Coutaric acid	320[a]
Coumaric acid	320[a,c]
Fertaric acid	320[a]
Ferulic acid	320[a,c]
Cyanidin-3-glucoside	520[a, b]
Delphinidin-3-glucoside	520[a, b]
Peonidin-3-glucoside	520[a,b]
Petunidin-3-glucoside	520[a,b]
Malvidin-3-glucoside	520[a,b]
Myricetin-3-glucoside	360[a], 365[c]
Quercetin-3-glucoside	360[a], 370[b], 365[c]
Kaempferol-3-glucoside	360[a], 370[b], 365[c]
Isorhamnetin-3-glucoside	360[a], 365[c]
Rutin	370[b]
Trans-resveratrol	320[a], 306[b]
Trans-piceid	306[b]
Procyanidin B1	280[a],275[c]
Procyanidin B2	280[a],275[c]
Procyanidin B3	280[a],275[c]
Procyanidin B4	275[c]

[a]Gomez-Alonso and others (2007)
[b]Nicoletti and others (2008)
[c]Montealegre and others (2006)

Capillary Electrophoresis Method

Capillary electrophoresis or capillary zone electrophoresis (CZE) is analytical method used to separate ions under the influence of an electric field. CZE operates under the basic principle of *opposites attract*, that is negatively-charged ions will migrate

towards the positively-charged electrode and vice-versa. The strength and expediency of CZE lies in the fact that ions move at different rates and the velocity of migration is dependent upon the electrophoretic mobility of the ions and the electro-osmotic mobility of the buffer in the capillary (European Pharmacopoeia 2005). CZE has been used to separate anthocyanins in blackcurrant juice within the time range expected from liquid chromatography analysis (da Costa and others 2002). In that study, four anthocyanins (cyanidin and dephinidin-3-glucosides and 3-rutinosides) were separated using uncoated fused-silica capillary under very acidic condition (pH 1.8).

TITRATIBLE ACIDITY, PH AND TOTAL ALCOHOL OF WINE

In grape berries, the dominant organic acids are malic and tartaric acids. The acidity in wine helps to balance out the alcohol and residual sugars. The presence of acids also aids in other capacities such as helping in the selection of desirable micro-organisms, enhancing the fruity character, increasing microbial protection of SO_2 and promoting a desirable color hue and color stability. Titratible acidity (TA) is often confused with pH. The pH value measures the strength of the acids in solution, meanwhile TA is the approximation of the solution's total acid content. The TA method involves titrating the wine to the phenolphthalein endpoint or pH=8.2 with a diluted sodium hydroxide solution. TA is expressed as g tartaric acid equivalent/100 mL.

According to the Code of Federal Regulations, Title 27 Part 24 (GPO Access 2010b), *the fixed acid level of the juice or wine may not be less than 5.0 gram per liter after the addition of ameliorating material.* This states that the acid content in wine may

not be less than 0.5% after amelioration. For alcohol content, the Code of Federal Regulations, Title 27 Part 4 (GPO Access 2010a) states: *table wine is grape wine having an alcohol content not in excess of 14% by volume and can be designated as "light wine," "red table wine," "light white wine," "sweet table wine," etc., as the case may be.* The majority of red wines sold in the USA is labeled as table wine and hence, should have less than 14% alcohol by volume.

In a study performed by Lee and Rennaker (2007), TA was determined by titration to an endpoint of pH=8.1. In that study, Cabernet Sauvignon wines had TA values ranging from 5.70-6.83 g tartaric acid/L, and Merlot wines from 4.97-6.90 g tartaric acid/L. In the same study, these red wines had pH values ranging from 3.3-3.8. These represent very typical pH and TA values for red wines made from V. *vinifera* grapes.

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CHAPTER III

MATERIALS AND METHODS

WHOLE RAW GRAPE

Grape collection and storage

Grapes were obtained from the Oklahoma State University Fruit Research Station in Perkins, Oklahoma and transferred to Robert M. Kerr Food and Agricultural Products Center at Oklahoma State University in Stillwater, Oklahoma. They were stored in the freezer at -20°C until further processing. For raw grape analysis, approximately 450 g of each variety was collected randomly and stored at -20°C until homogenization.

Homogenization

Grape berries were submerged in liquid nitrogen prior to homogenization. Using a Waring® blender (Woodbridge, ON), berries were pulverized until a powdered consistency was reached. Each variety was separated into three bags, vacuum-sealed and stored at -20°C until extraction.

Extraction of phenolic compounds

Twenty grams of homogenized grape were weighed into a 100 mL volumetric flask. The flask was filled with extraction solvent consisting of 40% acetone (Fisher Scientific, Fair Lawn, NJ), 40% methanol (Pharmco-AAPER, Brookfield, CT), 20% deionized water (EMD Millipore, Billerica, MA) and 0.1% glacial acetic acid (Pharmco-AAPER, Brookfield, CT) by volume and vortexed for 10 to 20 seconds. The sample was then incubated in a 60°C reciprocal shaking water bath (Precision Scientific) for 60 min at 60 rpm. After 60 min, the sample was cooled down to room temperature and homogenized using a PowerGen 700 homogenizer (Fisher Scientific, Fair Lawn, NJ) for 30 s. After homogenization, the sample was crudely filtered using Miracloth® (Calbiochem, La Jolla, CA) into an amber bottle and froze at -20°C until further analyses.

Soluble solids and pH

Percent soluble solids or % sugar was measured using a bench-top refractometer (Leica Auto ABBE, Buffalo, NY) with sample temperature compensation. This procedure was performed on the day of winemaking on freshly pressed grape juice.

Grape must pH was measured using a bench-top Accumet AB 15 pH meter (Cole-Parmer, Vernon Hills, IL) that had been calibrated prior to use. Measurements were taken on freshly pressed grape juice. This procedure was performed on the day of winemaking.

Total phenolics content (TPC)

Total phenolics content in the grape extracts was determined using a modification of the method of Singleton and Rossi (1965). Results were expressed as gallic acid

equivalents (GAE) per 100 g tissue. Equivalent gallic acid concentration in each sample was calculated using a standard curve prepared from gallic acid (Sigma-Aldrich, St. Louis, MO). All grape extracts and gallic acid standard solutions were treated in the same manner as listed below. Briefly, 0.5 mL of extract or gallic acid solution was added into 25 mL volumetric flask. Next, 1 mL Folin-Ciocalteu solution (Fluka Chemica, St. Louis, MO) was added, followed by 5 mL of deionized water (EMD Millipore, Billerica, MA). The contents were mixed and allowed to stand at room temperature for 5-8 min. After 5-8 min, 10 mL of 7% (w/v) of sodium carbonate solution (Sigma-Aldrich, St. Louis, MO) were added and deionized water was used to fill the flask to volume. The prepared solution was left at room temperature for a total of 2 hours, after which absorbance was read at 765 nm by a spectrophotometer (Beckman DU® 520, Brea, CA).

Total anthocyanins content

Total anthocyanins were measured using the pH differential method first reported by Giusti and Wrolstad (2000). Absorbance was taken at 520 nm and 700 nm and a formula was used to calculate the total anthocyanins content expressed as mg cyanidin-3-glucoside/100 g tissue. For this assay, one mL grape extract was added to a 25 mL volumetric flask, which was then brought to volume using potassium chloride buffer, pH 1.0 (Sigma-Aldrich, St. Louis, MO) and mixed well. One mL of grape extract was added to another 25 mL volumetric flask and sodium acetate buffer, pH 4.5 (Fisher Scientific, Fair Lawn, NJ) was used to fill the flask to volume and mixed well. These solutions were allowed to stand at room temperature for 15 min to equilibrate. After 15 min, absorbance was taken for all solutions at 520 and 700 nm (Beckman DU® 520 spectrophotometer, Brea, CA). The overall absorbance value was calculated using the formula below:

$$A = (A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5}$$

Using the A value above, the total anthocyanins content (expressed as mg cyanidin-3-glucoside/100 g tissue) was then calculated using the following formula:

$$\text{Total anthocyanins} = A \times MW \times [1/\varepsilon] \times DF \times 100$$

Where A = Absorbance

MW = Molecular weight of cyanidin-3-glucoside, 457.16 mol/g

ε = Molar extinction of cyanidin-3-glucoside, 29600

DF = Dilution factor of sample

100 = Conversion factor to per 100 g tissue or 100 mL juice basis

RED WINE

Fermentation vessels preparation

Food-grade plastic fermentation vessels (5 gal) and their accompanying lids were purchased from a plastic products retailer (U.S. Plastic Corp., Lima, OH). Using a spherical cardboard template with three holes 120° apart, the bottom of each vessel was marked for drilling. Drilling was performed using a 19.05 mm (0.75 inch) drill bit and the holes were then plugged with plastic tube fittings. These tube fittings were essentially a hollow tube with the ends fitted with two caps with holes in them. One end of the open-ended tube was fitted with a PTFE/silicone septum (adjusted to fit using a cork borer) before being secured with the accompanying cap. The cap held the septum in place so wine would not leak from the vessel and the needle from the syringe could penetrate

through to deliver oxygen. The cap on the opposite end of the tube fitting was also secured to hold the apparatus in place.

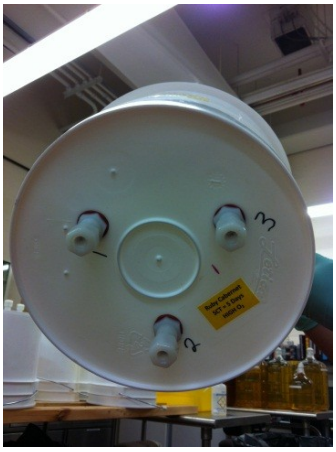


Figure 10. Bottom of fermentation vessel

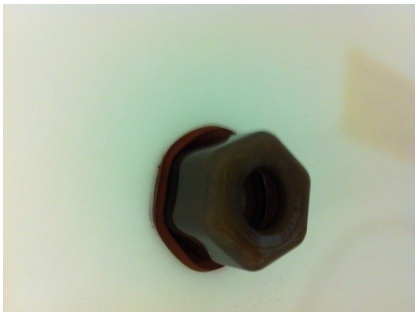


Figure 11. End of tube inside fermentation vessel

General preparation

Grape berries were retrieved from the freezer and left to thaw in the cooler for 48 hours prior to wine-making. On the day of winemaking, all grape berries were weighed and their weights recorded.

Crushing and destemming

Grape berries were crushed and destemmed using a small scale commercial crusher/destemmer (Model Jolly 60, St. Patrick's of Texas, Austin, TX). Must was collected in clean plastic totes and weighed. Small samples of the must were collected to be analyzed for pH and % soluble solids. All must was pooled together in a large metal vessel where wine yeast *Saccharomyces cerevisiae* (Lalvin Bourgovin RC 212, Montreal, CAN), yeast nutrients (Fermaid), potassium metabisulfite (an antimicrobial agent,

Presque Isle Wine Cellars, North East, PA), acid (a blend of malic, tartaric and citric acids) and table sugar (Great Value, Bentonville, AR) were added and mixed together. Acid and sugar were added to adjust the pH of the must to approximately 3.6 and the sugar content to 24%. After the additions, 25 kg of must was weighed and placed in each fermentation vessel. For each grape variety, 12 vessels were used. A total of 24 vessels were filled and the leftover must was discarded. All 24 vessels were then placed on elevated shelves to allow subsequent access for oxygen introduction.

Fermentation and pressing

Must was allowed to undergo fermentation. Every other day, the cap (i.e. the layer of skins and seeds that floated to the surface of the liquid) was punched down using a kitchen-style whisk until pressing. On the 6th day, 6 vessels of each variety were pressed using a small-scale water-powered bladder press (Zampelli Enotech JRL, Italy), where the wine and pomace were separated. The wine was poured back into the vessel and the pomace discarded. A total of 12 vessels were pressed and these were labeled as the 6-Days Skin Contact Time treatment. On the 12th day, the remaining 12 vessels were pressed and these were the 12-Days Skin Contact Time treatment. All vessels were placed on the elevated shelves where oxygen injection through the bottom of the vessel commenced the day after pressing.

Oxygen injection and sample collection

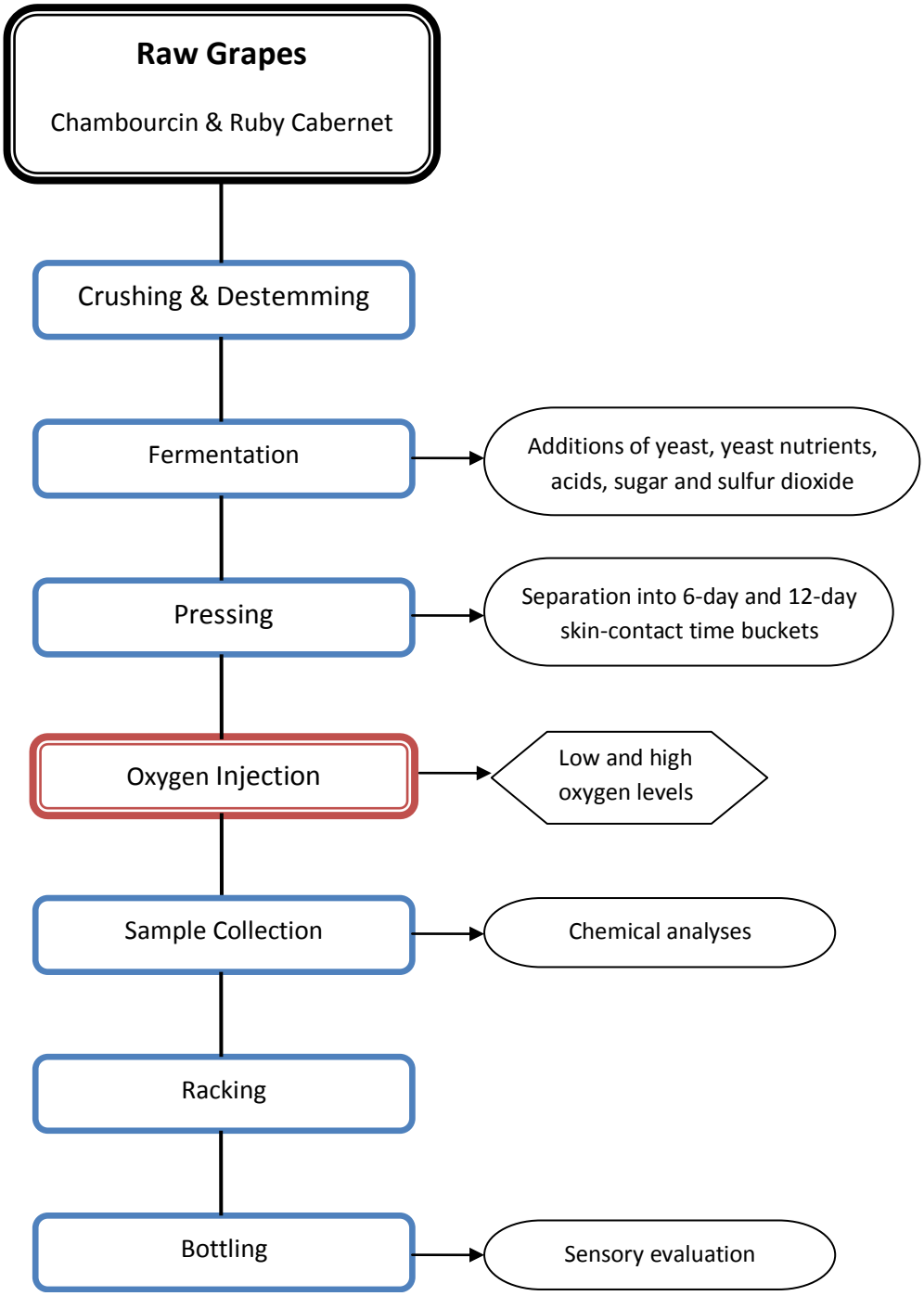
Oxygen injection commenced immediately the day after pressing. Food-grade oxygen was used. A syringe adapter apparatus (Supelco, St. Louis, MO) was affixed to the output of the gas regulator attached to the oxygen tank. The adapter enabled a

syringe (1 and 10 mL Pressure-Lok® Series A, Supelco, St. Louis, MO) to be inserted in order to withdraw oxygen. Oxygen levels were assigned as control, low and high. For the low oxygen level, 2.1 mL/day/vessel were delivered and for high level, 21 mL/day/vessel. The oxygen was injected slowly and distributed evenly among the three holes. Every week, for a total of sixteen weeks, a small amount of wine was collected from each vessel for analysis before beginning oxygen injection. Samples were drawn from the top of the vessel and the headspace was displaced with food-grade nitrogen before capping and securing with parafilm.

Racking and bottling

All vessels were racked at about week 10 to remove sediments from the wine. This process was accomplished by manually siphoning the wine into another vessel while leaving the sediments behind. The vessel containing the sediments was rinsed out and the wine was poured back into it. Headspace was displaced with food-grade nitrogen before capping and securing with parafilm. At the end of oxygen injection, all wines in the vessels were bottled in 1L plastic soda bottles (U.S. Plastic Corp., Lima, OH) and capped. Bottled wine was stored for further analyses at a later date. The entire winemaking process is illustrated in Figure 12.

Figure 12. Winemaking process



Oxygen radical absorbance capacity (ORAC) assay

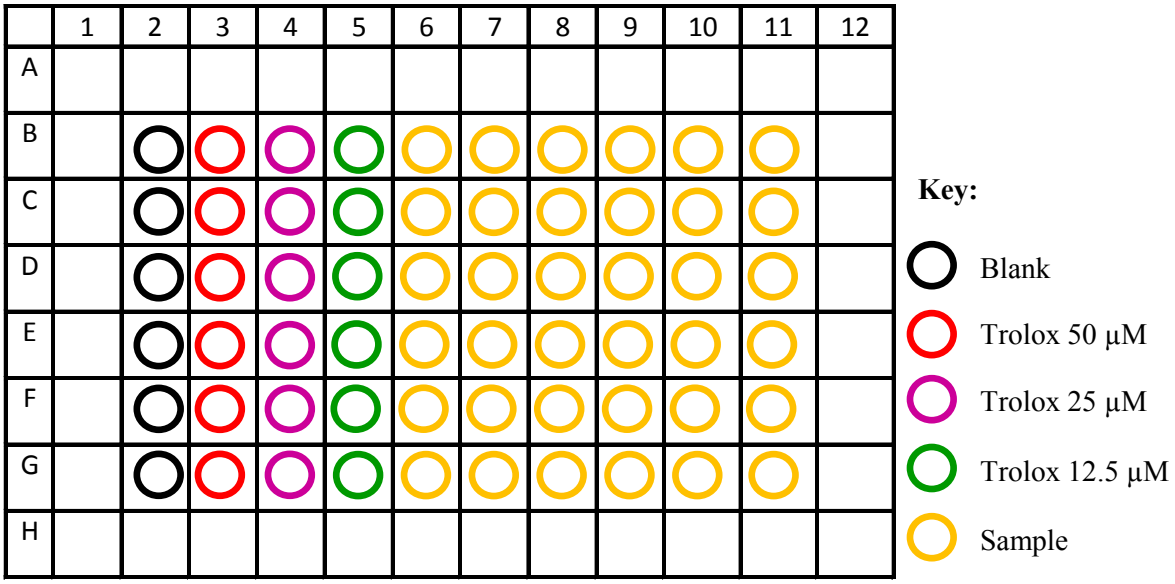
Chemical and reagents

Potassium phosphate dibasic anhydrous was purchased from Fisher Scientific (Fair Lawn, NJ), sodium phosphate monobasic anhydrous was available from Amresco (Solon, OH). Other chemicals include fluorescein (3',6'-dihyroxy-spriro [iso-benzofuran-1[3H], 9'[9H]-xanthen]-3-one) from Sigma-Aldrich (St. Louis, MO), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standard from Fluka (St. Louis, MO) and AAPH [2,2'-azobis(2-amidinopropane)dichloride] from Acros Organics (Fair Lawn, NJ).

Sample preparation and procedures

Wine sample was diluted 1000-fold with phosphate buffer prior to usage. No other preparation was performed.

Figure 13. Layout of ORAC microplate



Antioxidant capacity by ORAC was performed using a modified method of Huang and others (2002). ORAC values were obtained using the BioTek® Synergy™ 2 microplate reader (BioTek Instruments, Inc., Winooski, VT). To ensure consistency, all reagents except AAPH were added to the microplate using the BioTek® Precision™ Microplate Pipetting System. This system was fully-automated using the Precision Power™ software (version V2.03.2).

Trolox standard (100 µM), fluorescein stock (376 µM) and phosphate buffer (pH 7.0) were prepared in advance. For our purpose, Trolox was diluted 1:1 to generate a 50 µM working concentration and fluorescein was diluted 1000-fold. AAPH (306 mM), the peroxide radical generator was made fresh daily. All reagents including diluted wine samples were prepared using the phosphate buffer. First, 160 µL of fluorescein was added to all wells, followed by 20 µL of phosphate buffer (blank) in the second row. Subsequently, 20 µL of 50 µM Trolox was added to the adjacent row. The pipetting system was programmed to dilute the Trolox standard into two additional working concentrations, resulting in a total of 3 Trolox concentrations (50, 25 and 12.5 µM). Lastly, 6 rows of the plate were filled with 20 µL of diluted sample. The first and last columns, including the top and bottom rows of the plate were left unused (Figure 13).

After pipetting, the microplate was loaded onto the BioTek® Synergy™ 2 microplate reader, which is controlled by the Gen5 software (version 5.1) where the plate was incubated for 10 min at 37°C prior to AAPH addition, which was performed manually. The reader was programmed to record the fluorescence of all working wells every minute for 35 minutes, after which greater than 90% degradation of fluorescence

was expected. The total area for each well was calculated using the Area under the Fluorescence Decay Curve (AUC) method according to Eq. 1 below:

$$AUC = f_1/f_0 + \ldots. f_i/f_0 + \ldots. \ f_{34}/f_0 + f_{35}/f_0 \qquad \text{Eq. 1}$$

where f_o is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i. For all wells containing Trolox and sample, the net area for these wells were obtained by subtracting the average blank area from the total area. Taking into account the dilution factor and sample volume, the final ORAC value was calculated and expressed as μmol Trolox equivalents (TE)/100 mL of wine.

High-performance liquid chromatography (HPLC)

Chemicals and reagents

HPLC-grade acetonitrile was purchased from Acros Organics (Fair Lawn, NJ) and HPLC-grade methanol was from Pharmco-AAPER (Brookfield, CT). Formic acid (>99%) and sodium acetate trihydrate were available from Fisher Scientific (Fair Lawn, NJ). The type-HP 2 β-glucuronidase enzyme was purchased from Sigma-Aldrich (St. Louis, MO). Deionized water was produced using the Milli-Q system (EMD Millipore, Billerica, MA). Individual phenolic standards (11 total), anthocyanins standards (4 total) and an internal standard (7-ethoxycoumarin) were obtained from various retailers as follows:

- From Sigma-Adrich (St. Louis, MO) – Gallic acid, caffeic acid, p-coumaric acid, catechin hydrate, epicatechin gallate, resveratrol, quercetin hydrate, kaempferol,

cyanidin chloride, delphinidin chloride, pelargonidin chloride, and malvidin chloride.

- From Indofine Chemical Company, Inc. (Hillsborough, NJ) – Myricetin and 7-ethoxycoumarin.
- Ferulic acid was purchased from Fluka (St. Louis, MO).

Phenolic standards preparation

All individual phenolic and anthocyanins standards, including the internal standard, were made into stock solutions using HPLC-grade methanol (500 ppm for anthocyanins, 250 ppm for isorhamnetin and 750 ppm for others). They were subsequently mixed together to form a standard mixture of 37.5 ppm for most compounds, except isorhamnetin at 12.5 ppm and anthocyanins at 25 ppm.

Enzymatic hydrolysis of wine

Wine was subjected to enzymatic hydrolysis prior to HPLC analysis. An aliquot of 4 mL wine was filtered through a pre-conditioned Sep-Pak filter (Waters Corporations, Milford, MA) and eluted with 8 mL of acidified methanol (0.1% v/v hydrochloric acid). An internal standard, 7-ethoxycoumarin, was added in the amount of approximately 37.5 ppm in total solution. The eluent was dried completely in a SpeedVac evaporator (ThermoSavant, Model SPD 121P), where the heat setting was turned off. After drying, the wine solid was reconstituted with 500 µl 50% HPLC-grade methanol. After that, 110 µL 0.78M acetate buffer, 100 µL 0.3M vitamin C solution and 50 µL β-glucuronidase enzyme were added. The solution was vortexed to mix and incubated at 37°C for 17 hours in a reciprocal shaking bath (Precision Scientific, Model 50, Waltham, MA). After

incubation, 250 µL of cold HPLC-grade methanol was added and the solution was centrifuged (Fisher Scientific Centrific™) for 25 min at 4000 rpm. The supernatant was then transferred to a HPLC vial for injection.

Procedures

Phenolics analysis on the HPLC was carried out using modified methods of Thimothe and others (2007) and Perati and others (2012). The HPLC system was from Dionex Corporation (Sunnyvale, CA) and consisted of a P680 pump, a TCC-100 temperature-controlled column compartment, an ASI-100 automated sample injector and an Ultimate 3000 photodiode array detector. The HPLC system operated on Chromeleon software version 6.80. Separation was achieved by a gradient elution at 40°C with a SunFire™ C18 column (4.6 mm x 250 mm x 5 µm), including a SunFire™ C18 guard column (4.6 mm x 20 mm), both from Waters Corporations (Milford, MA). Flow rate was set at 0.80 mL/min. The gradient elution employed two mobile phases: (A) 10% formic acid, and (B) 10% formic acid, with 22.5% methanol and 22.5% acetonitrile. The elution parameters were as follows: 0 min 94% A, 5 min 70% A, 30 min 20% A, 42 min 40% A, 50 min 94% A, and 65 min 94% A. Data acquisition was applied for 65 min and chromatograms were acquired at 280, 320 and 370 nm for phenolic acids, flavanols, flavanols and stillbenes, and also at 520 nm for anthocyanins. Phenolic compounds were identified by comparing their retention times with those of pure standards and by occasional spiking using standard stock solutions.

Titratible acidity (TA)

Five mL of grape wine was added to a 250 mL Erlenmeyer flask and diluted with 100 mL distilled water. Sample was titrated to endpoint of pH 8.2 using 0.1 N NaOH (Acros Organics, Fair Lawn, NJ). Volume of NaOH used was recorded and result was calculated as % tartaric acid using the equation below:

$$TA = \frac{(\text{mL NaOH}) \times (\text{N of NaOH}) \times (\text{milliequivalent weight of tartaric acid})}{\text{Sample size (g or mL)}} \times 100$$

$$TA = [\text{mL NaOH} \times 0.1 \times 0.075 \times 100] / 5$$

Free and bound SO_2

The SO_2 test was performed using the oxidation/aeration apparatus. A sample of 20 mL wine was measured into a round bottom flask and 10 mL of 25% phosphoric acid (Ricca Chemical Company, Arlington, TX) and some boiling beads were added to it.

The impinger was filled with 10 mL 3% hydrogen peroxide (VWR, West Chester, PA) and three drops of indicator (50/50 mix of methyl red and methyl blue indicator solutions). The indicator changed the peroxide solution in the impinger bright purple, but was adjusted to a light gray-green color using a diluted 0.01 N sodium hydroxide (Fisher Scientific, Fair Lawn, NJ). By applying vacuum, the SO_2 in its gaseous form was released from the wine and captured in the peroxide solution, causing it to turn bright pink. The pink solution was then titrated with 0.01 N NaOH until the initial light gray-green color was achieved. The volume of NaOH used was recorded and the free SO_2 was calculated as:

Free SO_2 = normality of NaOH x mL NaOH x 1600 = mL NaOH x 16

For bound SO_2, the solution in the impinger was discarded and replaced with fresh one. The same procedure was repeated, except that heat was applied to the sample while under vacuum. Similarly, the titration was performed until a light gray-green color was obtained. The same equation above was used to calculate the bound SO_2. Total SO_2 was calculated by the addition of free and bound SO_2.

Total SO_2 = Free SO_2 + Bound SO_2

Total alcohol

Total alcohol was determined using a boiling point differential method by using an ebulliometer. Since alcohol boils at a lower temperature than water, the boiling point of water-alcohol mixtures changes as a function of their concentrations. Prior to analyzing wine, the boiling point of water was determined to set the "zero" point where the alcohol content of samples would be measured against. After filling the ebuilliometer with wine, it was allowed to boil and the thermometer reading was taken after the mercury level had stabilized. The boiling temperature of the wine was referred to the reference chart to determine % alcohol. This method is adequate only if the wine has less than 0.5% sugar.

Harbertson-Adams assay

This assay was developed by Drs. Harbertson and Adams at the University of California, Davis. It has the ability to quantify multiple phenolics that are considered important in wines such as anthocyanins, tannins, pigments and non-tannin iron-reactive

phenols (Viticulture & Enology University of California Davis 2005). This assay operates on the ability of protein (bovine serum albumin - BSA) to precipitate tannins and some of the red pigments. The pigments that bind to BSA are not released by washing and are stable under the presence of bisulfite. These observations suggest that these pigments are polymeric. However, the pigments precipitated by BSA using centrifugation do not account for all the pigments present in the wine. Some of the pigments are still suspended in the supernatant fraction. Thus, BSA is able to fractionate the polymeric pigments into two distinguishable classes: short polymeric pigments (SPP) that are still suspended in the supernatant fraction and long polymeric pigments (LPP) that precipitate along with the tannins.

Chemicals and reagents

This assay requires preparation of multiple solutions such as model wine, washing buffer, resuspension buffer, anthocyanin buffer, ferric chloride reagent, bleach solution and BSA solution. Deionized water was produced using the Milli-Q system (EMD Millipore, Billerica, MA). The make-up of all the reagents is as follows:

- Model wine – Potassium bitartrate (Sigma-Aldrich, St. Louis, MO), 95% ethanol (Pharmco-AAPER, Brookfield, CT), hydrochloric acid to adjust pH (VWR, Radnor, PA) and deionized water.
- Washing buffer – Sodium chloride (Gibbstown, NJ), glacial acetic acid (Pharmco-AAPER, Brookfield, CT), sodium hydroxide to adjust pH (Acros Organics, Fair Lawn, NJ) and deionized water.

- Resuspension buffer – Sodium dodecyl sulfate (Sigma-Aldrich, St. Louis, MO), triethanolamine (Sigma-Aldrich, St. Louis, MO), hydrochloric acid to adjust pH (VWR, Radnor, PA) and deionized water.
- Anthocyanin buffer – Maleic acid (Acros Organics, Fair Lawn, NJ), sodium chloride (EMD, Gibbstown, NJ), sodium hydroxide to adjust pH (Acros Organics, Fair Lawn, NJ) and deionized water.
- Ferric chloride reagent – Ferric chloride (Sigma-Aldrich, St. Louis, MO), hydrochloric acid to adjust pH (VWR, Radnor, PA) and deionized water.
- Bleach solution – Potassium metabilsulfite (Presque Isle Wine Cellars, North East, PA) and deionized water.
- BSA stock solution – Bovine serum albumin (Sigma-Aldrich, St. Louis, MO) and deionized water.

The very first step of this assay determines the total phenolics content which is measured as mg/L catechin equivalents (CE). The second step is two-fold: total polymeric pigments are determined first. Then, by using BSA, the SPP is quantified next and subtraction is performed to obtain the LPP. The precipitated pellets are then washed and re-suspended to measure the content of tannins. The last step quantifies total anthocyanins, which is expressed as mg/L malvidin-3-monoglucoside, instead of mg/L CE. The procedures are too long to be reproduced here, but are presented in Appendix B.

Statistical Analysis

For each of the variety of grapes, three levels of oxygenation (control, low=2.1 mL O_2/bucket/day and high=21 mL O_2/bucket/day) and two SCTs (6 and 12 days) were

examined. Two independent wine preparations (bucket) were evaluated for each of the six (3 x 2 factorial) oxygen/SCT combination. Five repeated measures (week = 1, 4, 8, 12 and 16) were recorded for each bucket. Data were analyzed using mixed model methods for repeated measure experiments. The level of significance for all tests was set at $\alpha = 0.05$. The output for this project was generated using SAS software, Version 9.3 of the SAS System. Copyright © 2012 SAS Institute Inc. SAS and all SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA.

REFERENCE

Viticulture & Enology University of California Davis: Harbertson-Adams Assay [Internet]. Davis, CA: Department of Viticulture and Enology, University of California-Davis; 2005 [Assessed 2012 Jan 31]. Available from http://boulton.ucdavis.edu/uv-vis/adamsassay.htm.

CHAPTER IV

RESULTS AND DISCUSSIONS

Harbertson-Adams Assay

Total Phenolics Content

Statistical analysis showed that for Chambourcin wines, oxygenation treatment had no significant effect ($p = 0.7263$) on the total phenolics analyzed. However, the SCT by week interaction was significant ($p = 0.0251$) and this is illustrated in Figure 14. For all weeks, the 12-day SCT treatment yielded significantly higher total phenolics means than the 6-day treatment ($p < 0.0007$); however, this difference was not consistent across weeks.

For Ruby Cabernet wines, oxygenation treatment also showed insignificant effect ($p = 0.4337$) in total phenolics. As with the Chambourcin, the interaction between SCT and week was significant ($p < 0.0001$, Figure 15). At weeks 1, 4, 8 and 12, the 12-day SCT treatment yielded significantly higher total phenolics means than the 6-day SCT treatment ($p < 0.0001$); however, at week 16, there was no SCT effect ($p = 0.1056$) (Figure 15).

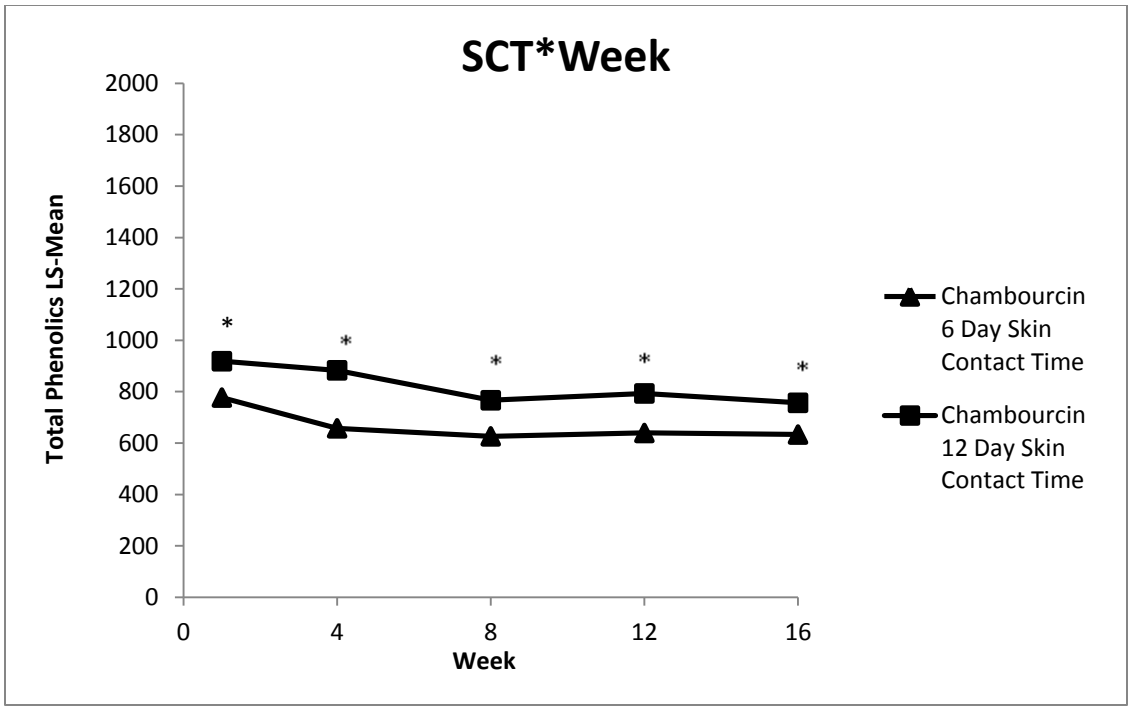


Figure 14. Least square means of skin contact time (SCT) by week combinations for total phenolics content of Chambourcin wines (asterisk [*] denotes week where significant SCT effect was observed)

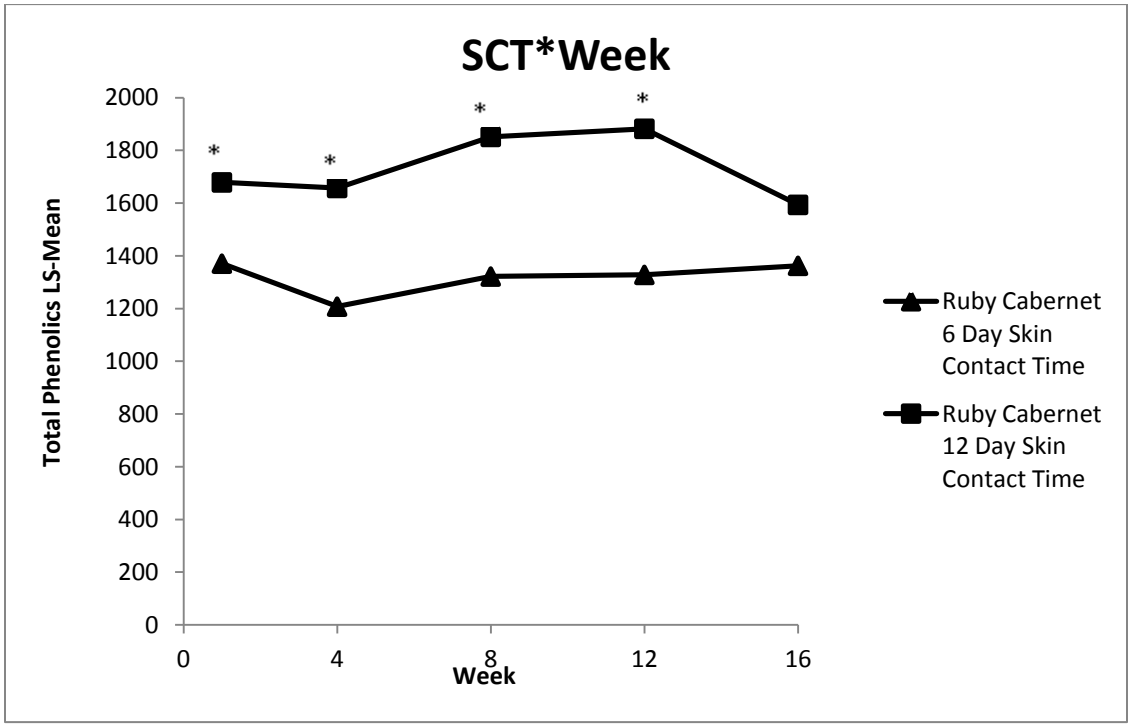


Figure 15. Least square means of skin contact time (SCT) by week combinations for total phenolics content of Ruby Cabernet wines (asterisk [*] denotes week where significant SCT effect was observed)

While statistical analyses were not performed to verify statistically significant compositional differences between the two varieties, the Chambourcin wines were observed to have lower total phenolics than the Ruby Cabernet, with the latter having approximately twice as much (Figures 14 and 15). The skin contact period, also known as maceration time, helps to extract phenolic compounds from the grapes into solution and as a result, longer time will translate to higher phenolics content. The data obtained in this study was consistent with this model. Overall, the total phenolics content in both wines fluctuated slightly over the course of 16 weeks in both SCT treatments (Figures 14 and 15).

Statistical analysis performed on samples from week 16 and week 94 (~18-month) storage revealed significant SCT by week interaction for total phenolics content of both wines ($p < 0$ .0231). In Chambourcin wines, at weeks 16 and 94, 12-day SCT treatment yielded significantly higher means than 6-day SCT ($p < 0.0001$), with greater difference observed at week 16. This result is consistent with previous findings (Figure 14). For the Ruby Cabernet wines, the difference between the two SCT treatments at weeks 16 and 94 was insignificant ($p > 0.1211$). However, there was a significant drop of total phenolics content in the bottled samples (week 94) for both of the SCT treatments, where the largest drop was observed in the 12-day SCT wines. Looking at the individual SCT treatment, the total phenolics content of both wines dropped after storage of 18 months (Tables 9a and 10b, Appendix A). Again, this is expected as phenolics are known to polymerize and/or bind with other constituents in the wine and precipitate out of solution during extended storage. Indeed, this is a desirable part of the normal ageing process for red wines.

Tannins Content

For Chambourcin wines, there was significant SCT by week interaction ($p = 0.0006$) for tannins content, as shown in Figure 16. At weeks 1, 4, 8, 12 and 16, the 12-day SCT yielded significantly higher tannins content than the 6-day SCT treatment ($p < 0.0238$). Longer maceration time will yield higher tannins contents, as tannins are mainly located in the seed and skin of grape berry (Waterhouse 2002).

The SCT by oxygen interaction was also significant ($p = 0.0299$) for the Chambourcin wines. It was revealed at 6-day SCT, there was no oxygen effect ($p = 0.3631$). At 12-day SCT, the oxygen effect was significant ($p = 0.0218$), with the tannins content of the control wines being higher than the high-oxygenated wines ($p = 0.0074$).

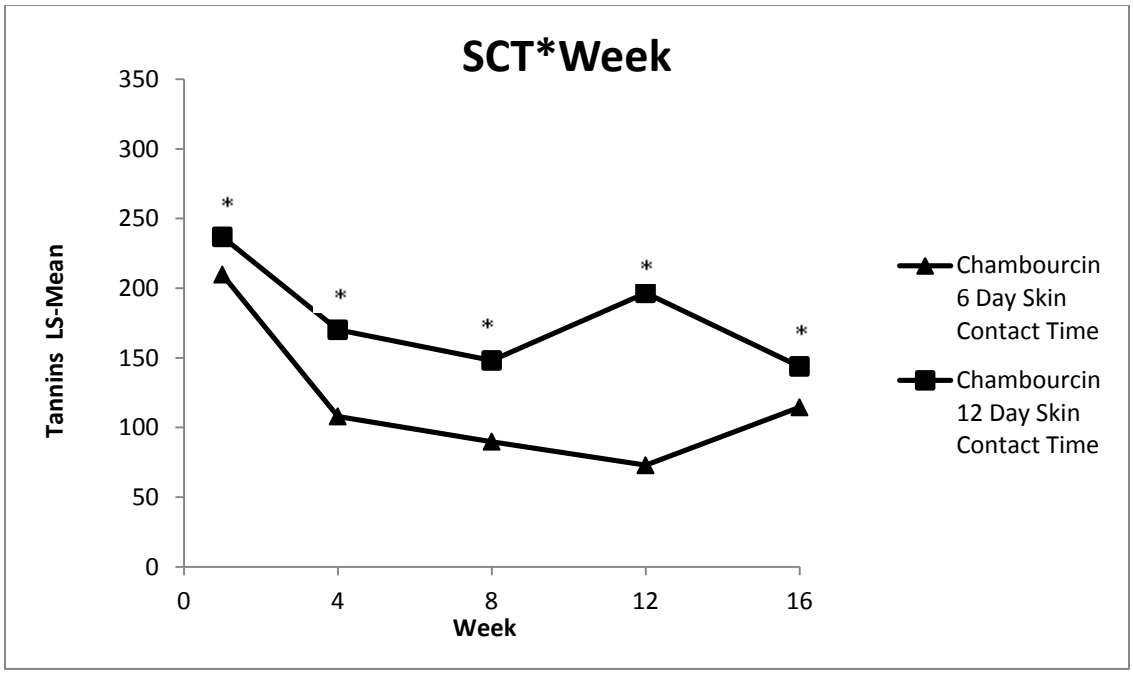


Figure 16. Least square means of skin contact time (SCT) by week combinations for tannins content of Chambourcin wines (asterisk [*] denotes week where significant SCT effect was observed)

For Ruby Cabernet wines, the only significant effect on tannins content was the SCT effect ($p < 0.0001$) -- 12-day SCT treatment yielded significantly higher tannins concentration than the 6-day SCT treatment (899 vs. 644 mg CE/L) (Table 8b, Appendix A).

Tannins, which are made of polymers containing catechin, epicatechin, epicatechin gallate or epigallocatechin (Harbertson and others 2003) are one of the major phenolics in wine. The lower total phenolics in Chambourcin grapes also contributed to a lower tannins content, where the 12-day SCT treatment showed a higher amount (Figure 16). In the Ruby Cabernet, the tannins content alone accounted for approximately half of the total phenolics. In fact, throughout the 16 weeks treatment period, the tannins content of the Ruby Cabernet stayed relatively constant: 6-day SCT at approximately 600 mg CE/L and 12-day SCT at 900 mg CE/L (results not shown).

The tannins content of the Chambourcin and Ruby Cabernet wines were analyzed after approximately 18 months of storage (week 94). The tannins content of the Chambourcin showed highly significant SCT by week interaction ($p = 0.0029$). At week 16, the tannins of 12-day SCT treatment were significantly higher than the 6-day SCT ($p = 0.0159$); by week 94, this difference was much larger. For both SCT treatments the tannins content of Chambourcin wine declined noticeably after storage (Tables 9a, Appendix A). For Ruby Cabernet however, the only significant effect was the week effect ($p = 0.0471$), where the tannins amount decreased after storage (Table 10a, Appendix A) and no difference was seen between skin contact times after 94 weeks storage.

Anthocyanins Content

Anthocyanins are the glycosides of anthocyanidins, which are typically the second most abundant class of phenolics in grapes (Harbertson and others 2003). They are responsible for the red hue of red grape berries and are the predominant color pigments of young red wines. The five major anthocyanins found in red wine are the monoglucosides of malvidin, cyanidin, peonidin, pelargonidin and petunidin. Due to the acylation of the sugar residues, these five anthocyanins can be found as ten or more chemically unique forms (Harbertson and others 2003). In the Harbertson-Adams assay, the anthocyanins content was quantified as a whole and expressed in terms of milligram malvidin-3-glucoside per liter wine, where malvidin-3-glucoside is the predominant species.

Statistical analysis showed that for Chambourcin wines, oxygenation treatment had no significant effect ($p = 0.6710$) on the content of monomeric anthocyanins. However, the SCT by week interaction was significant ($p = 0.0008$) and this is illustrated in Figure 17. At week 1, the 6-day SCT treatment yielded significantly higher monomeric anthocyanins means than the 12-day treatment ($p = 0.0035$). This trend was also observed in weeks 8 and 12 ($p < 0.0075$). At week 4 and 16 however, there was no SCT effect ($p > 0.1797$). Contrary to total phenolics and tannins, the monomeric anthocyanins did not seem to follow the trend where longer maceration time would yield greater concentration of phenolics. This suggests that the bulk of extractable anthocyanins in Chambourcin grapes are extracted by the sixth day of maceration; it should be noted that the anthocyanins content of both the 6- and 12-day SCT treatments were indeed very similar in values across the weeks (Table 7b, Appendix A) and the statistically significant

differences in anthocyanins content observed for weeks 1, 8, and 12 may not represent practically-observable differences in wine quality.

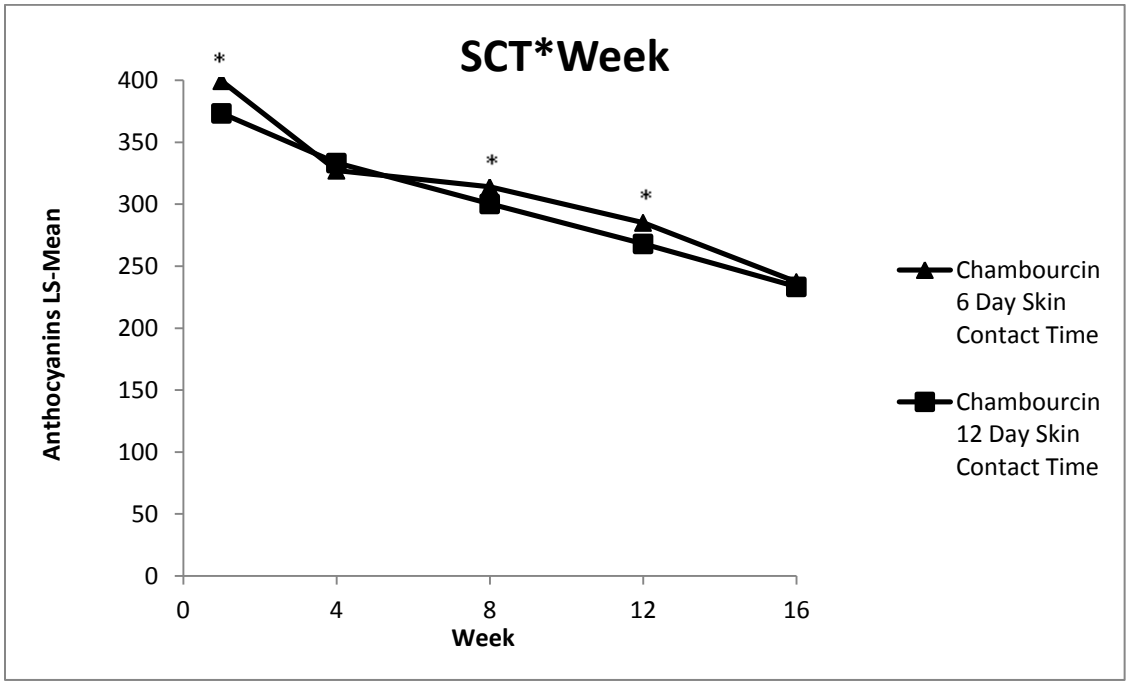


Figure 17. Least square means of skin contact time (SCT) by week combinations for monomeric anthocyanins content of Chambourcin wines (asterisk [*] denotes week where significant SCT effect was observed)

In the Ruby Cabernet wines, the three-way interaction of SCT by oxygen by week was significant ($p = 0.0157$) (Figure 18). According to Figure 18, the content of anthocyanins of the Ruby Cabernet wines decreased over time for both SCT treatments, similar to what was observed for Chambourcin wines (Figure 17). For the 6-day SCT wines, from week 1 till 16, the decline seemed to be consistent across all the oxygenation treatments. This observation was supported by statistical analysis, where no difference in anthocyanins content was observed in regard to oxygen treatment ($p > 0.4581$) for all weeks. For the 12-day SCT treatment, from week 1 till 12, no oxygen effect ($p > 0.1434$)

was observed. At week 16 however, the declining trend reached a break point where the oxygenated wines (both low and high) suffered a notable decrease. At week 16, the control wines had significantly higher anthocyanins content than the low and high oxygen-treated wines ($p < 0.0008$).

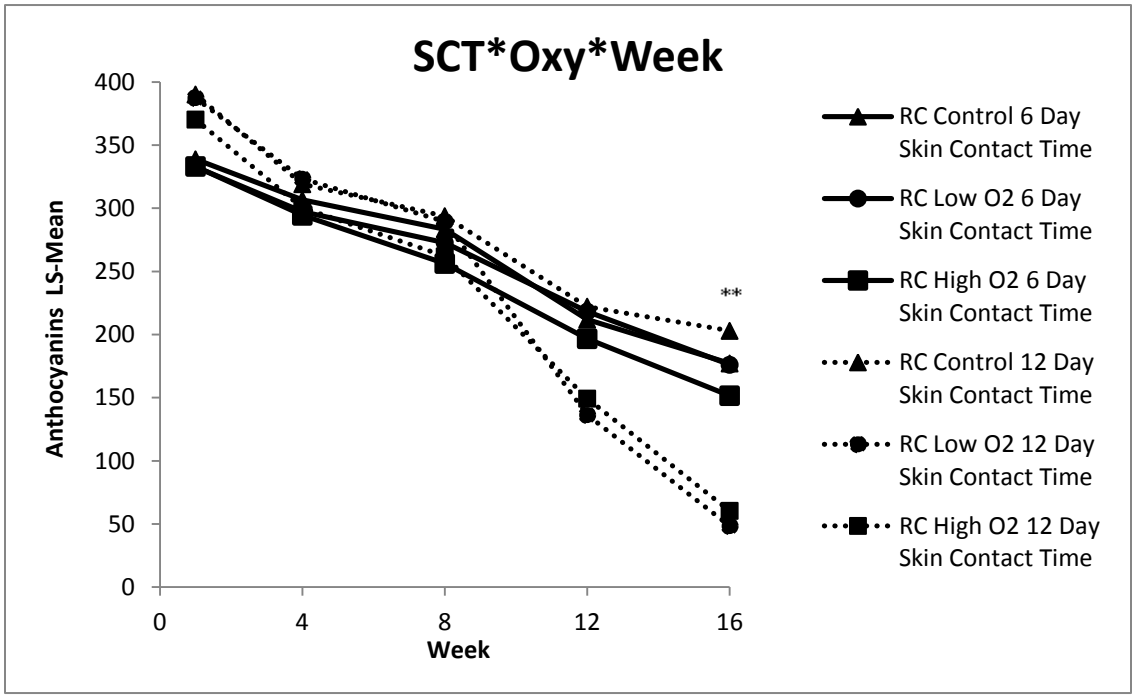


Figure 18. Least square means of skin contact time (SCT) by oxygen by week combinations for monomeric anthocyanins content of Ruby Cabernet wines (RC = Ruby Cabernet, O2 = oxygen, double asterisk [**] denotes week where significant oxygen effect was observed)

For both SCT treatments, the monomeric anthocyanins were observed to decrease over the course of 16 weeks in both wines (Figures 17 and 18). This decreasing trend was expected since these anthocyanins are highly unstable. In fact, according to Mazza and others (1999), the content of monomeric anthocyanins was observed to increase at the beginning of alcoholic fermentation and reached a maximum level 2 to 3 days after beginning fermentation. The level then decreased during subsequent storage. The loss of

monomeric anthocyanins can be attributed to the formation of polymeric pigments, as shown by many research studies done to date (Dallas and others 1996; Es-Safi and others 1999; Cano-López and others 2006). Polymeric pigments are composed of anthocyanins and flavan-3-ols.

Of the two wines, the Ruby Cabernet seemed to exhibit a larger decrease in anthocyanins content in the 16-week timeframe, particularly the samples with the higher SCT treatment (Figure 18). One possible explanation could be that the Ruby Cabernet wines were more "sensitive" to oxygenation treatments, possibly due to having higher concentrations of oxygen-sensitive substrates, and therefore underwent a much more extensive polymerization process during ageing. This polymerization effect in turn, seemed to be propagated further by the higher SCT treatment.

The analysis of the Ruby Cabernet wines after 18 months of storage (week 94) in comparison to samples from week 16 revealed significant SCT by oxygen by week interaction ($p = 0.0055$), which was similar to our previous finding (Figure 18). At week 94, in the control and low-oxygenated wines, the 12-day SCT treatment yielded significantly lower anthocyanins content that the 6-day SCT treatment ($p < 0.0118$). This further reiterates the speculation that the decrease in the concentration of monomeric anthocyanins in the Ruby Cabernet wine was due to a compounded effect of oxygen and high SCT treatments.

Similarly, statistical analysis was also performed on the bottled Chambourcin wines as above after storage. In Chambourcin wines, significant oxygen by week interaction ($p = 0.0401$) was detected. The bottled control wines had significantly higher

monomeric anthocyanins content than the bottled low-oxygenated wines ($p = 0.0457$), and partially so for the bottled high-oxygenated wines ($p = 0.0535$). This is a complete reversal of what was observed previously, where the only significant effect was the SCT by week interaction ($p = 0.0008$, Figure 17). It appears as if oxygenation treatment had a greater impact on the loss of monomeric anthocyanins than skin contact treatment over time. The decline in the concentration of these pigments during ageing is expected as monomeric anthocyanins polymerize and form complexes with other compounds. Overall, the changes observed appeared to be consistent with the kind of effect expected in micro-oxygenation application.

Polymeric Pigments Content

Polymeric pigments are the stable forms of color compounds in red wines. As red wine ages, the monomeric anthocyanins form polymeric pigments with flavonols, either by direct reaction or indirectly through cross-linking of individual units (Harbertson and others 2003). These polymeric pigments are categorized into short (SPP) and long (LPP) forms, and are less sensitive to pH changes than monomeric anthocyanins (Gao and others 1997).

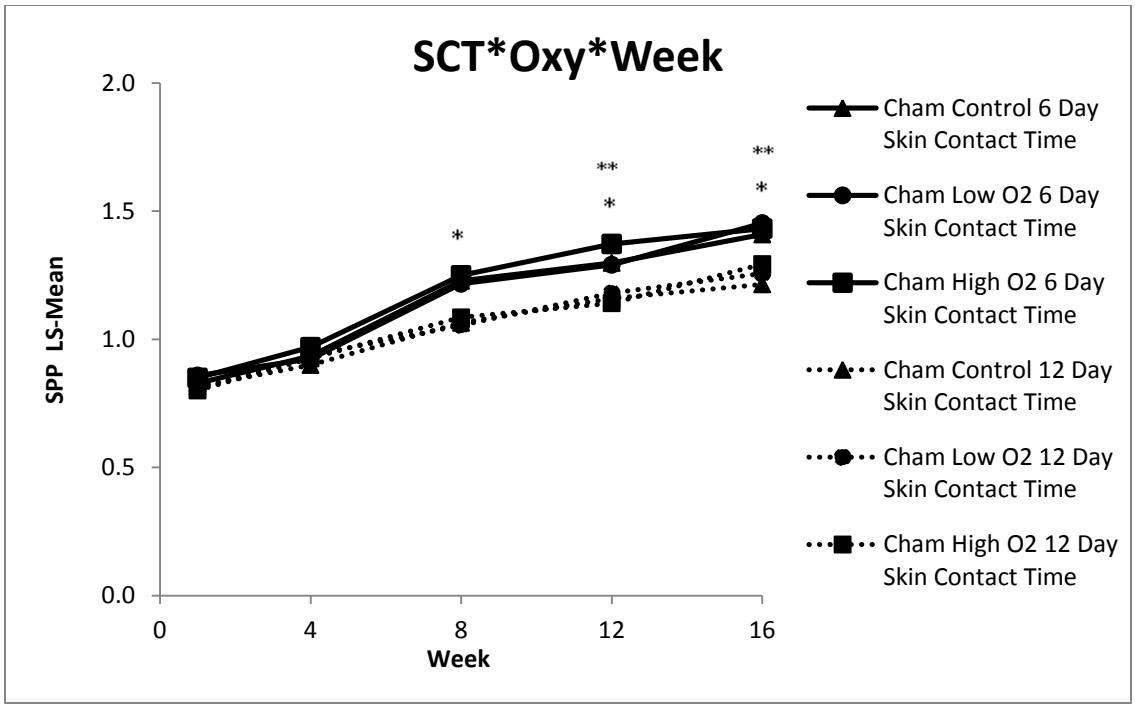


Figure 19. Least square means of skin contact time (SCT) by oxygen by week combinations for SPP content of Chambourcin wines (Cham = Chambourcin, O2 = oxygen, asterisk [*] denotes week where significant SCT effect was observed, double asterisk [**] denotes week where significant oxygen effect was observed)

In Chambourcin wines, the three-way interaction of SCT by oxygen by week ($p = 0.0295$) was significant for SPP content, as illustrated in Figure 19. Overall, there was a rising trend in the SPP content of Chambourcin wines. The 6-day SCT wines seemed to have higher mean SPP content than the other treatment throughout the weeks, but according to statistical analysis, only at week 8, 12 and 16 was the SPP content of 6-day SCT treatment significantly higher than the 12-day SCT treatment ($p < 0.0053$). The effect of oxygen on the SPP content was neither readily apparent nor consistent. There were only a few instances where this effect could be seen -- at week 12, for 6-day SCT, the SPP content of the high-oxygenated wines was significantly higher than the control and low-oxygenated wines ($p < 0.0252$); at week 16, for 12-day SCT, the high-

oxygenated wines showed higher SPP content than the control wines ($p = 0.0302$), but not the low-oxygenated ones ($p = 0.2478$).

In the Ruby Cabernet wines, the only significant effect on SPP content was the week effect ($p < 0.0001$), where there was a steady increase until week 12, then the content became level (Table 8a, Appendix A).

In the Chambourcin wines, the higher SPP content of the 6-day SCT treatment (Figure 19) again challenged the notion of longer maceration time giving rise to higher phenolics content. It should be noted that the higher SPP content also coincided with the higher monomeric anthocyanins content in these wines (Figure 17). According to Atanasova and others (2002), ethyl-bridged pigments were formed as anthocyanins glycosides condensed with epicatechin. These ethyl-linked products fit the description of SPP, where Harbertson and others (2003) expected to contain low molecular weight compounds such as malvidin-3-glucoside-catechin adducts. We speculate that the higher SPP content of the 6-day SCT wines were due to more monomeric anthocyanins being available to form complexes with catechin or epicatechin; higher levels of oxygenation may have facilitated this process.

Statistical analysis of samples from weeks 16 and 94 of the Chambourcin wines was revealed to have significant SCT by oxygen by week interaction ($p = 0.0054$) for the SPP content. For week 16, the 6-day SCT wines had significantly higher SPP means than the other treatment ($p < 0.0052$), which was the trend noted previously (Figure 19). For the bottled samples (week 94), only the oxygenated wines (low and high) had significantly higher SPP content in the 6-day SCT treatment ($p < 0.0081$). In terms of

oxygenation effects, there were a few instances where it was significant -- in the bottled samples of 12-day SCT treatment, the high-oxygenated wines has significantly lower SPP content than both the control ($p = 0.0065$) and low-oxygenated wines ($p = 0.0092$). The Ruby Cabernet wines, however, only had significant week effect ($p = 0.0005$). The SPP content of Ruby Cabernet wines dropped after storage (Table 10a, Appendix A).

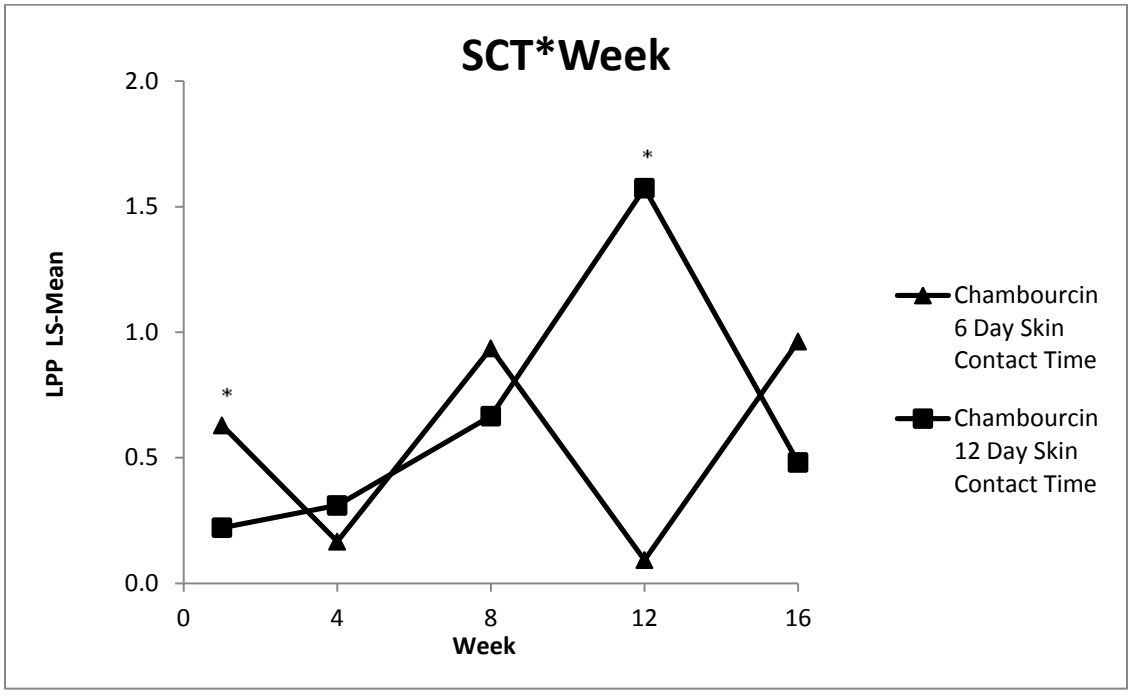


Figure 20. Least square means of skin contact time (SCT) by week combinations for LPP content of Chambourcin wines (asterisk [*] denotes week where significant SCT effect was observed)

For Chambourcin wines, the LPP content was not affected by oxygenation ($p = 0.2253$), but SCT by week interaction was significant ($p < 0.0001$). Figure 20 shows the SCT by week interaction effect of LPP in Chambourcin wines. At week 1, 6-day SCT treatment had a significantly higher LPP means than 12-day SCT ($p < 0.0001$). At week 4 and 8, SCT effect was insignificant ($p > 0.0914$). At week 12, 12-day SCT yielded significantly higher LPP means than 6-day SCT ($p = 0.0002$). At week 16, the observed

p-value for the difference in LPP content between skin contact times was 0.0836. This difference is insignificant at the 95% confidence level ($p < 0.05$) but significant at the 90% confidence level ($p < 0 .10$). Henceforth we will refer to such differences as being marginally significant.

In Ruby Cabernet, the effect of oxygen treatment was also insignificant ($p = 0.0632$) for the LPP content. The SCT by week interaction was highly significant ($p < 0.0001$) and is illustrated in Figure 21. At week 1, LPP for 12-day SCT treatment was significantly lower than 6-day SCT ($p < 0.0001$) and the reverse was true at week 4 ($p < 0.0001$). No SCT effect was seen at

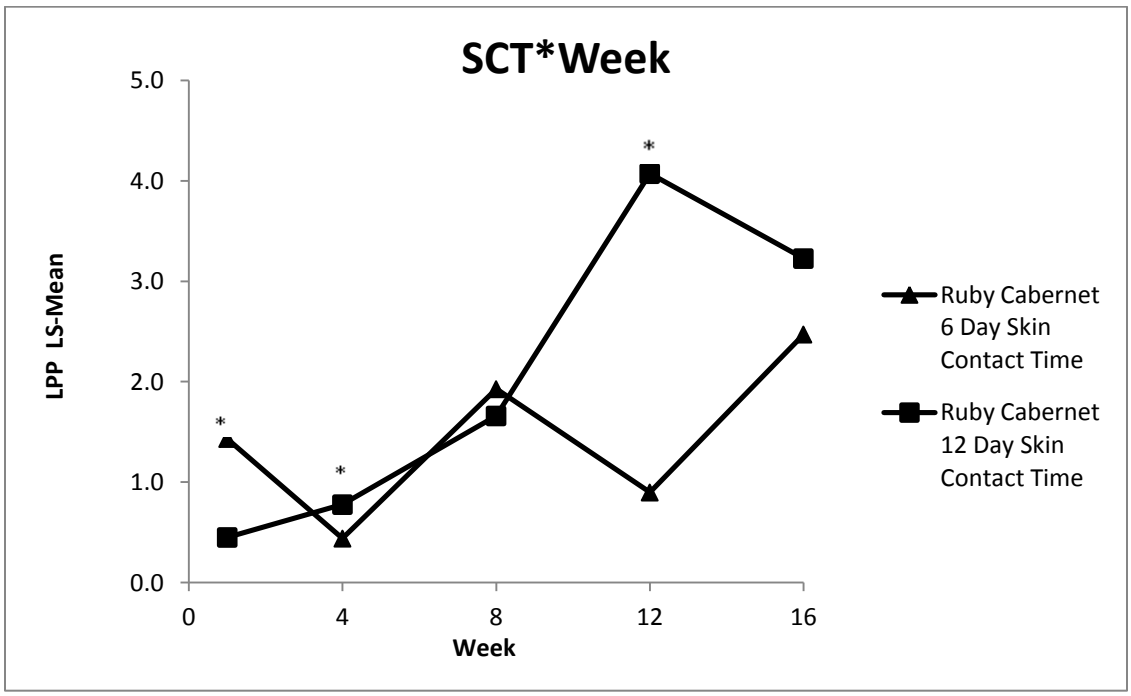


Figure 21. Least square means of skin contact time (SCT) by week combinations for LPP content of Ruby Cabernet wines (asterisk [*] denotes week where significant SCT effect was observed)

week 8 ($p = 0.3938$). But, at week 12, 12-day SCT yielded higher LPP means than 6-day SCT ($p = 0.0010$) and at week 16, this difference was only marginally significant ($p = 0.0749$).

In general, the LPP content showed an erratic fluctuation pattern in the 6-day SCT treatment in both wines (Figures 20 and 21). The 12-day SCT showed an increase from week 1 till 12, at which the level then started to decline (Figures 20 and 21). The inconsistent pattern of the LPP level could be due to the formation-and-breakdown cycle of pigment polymers -- while heavy polymers precipitate out of solution, new ones are constantly being formed.

After 18 months storage (week 94), the LPP content of Chambourcin and Ruby Cabernet wines was analyzed in comparison with week 16 samples. The SCT by week interaction was significant ($p = 0.0133$) for Chambourcin wines as previously noted (Figure 20). The bottled samples (week 94) had significantly higher LPP content in the 12-day SCT treatment ($p = 0.0006$), but at week 16, the LPP means between the two SCT treatments was insignificant ($p = 0.0797$). This is not surprising considering the LPP level had been seen to fluctuate during the 16-week experimental period.

As for the Ruby Cabernet wines, the three-way effect of SCT by oxygen by week was significant ($p = 0.0389$). It should be noted that the only significant effect prior was the SCT by week interaction (Figure 21). A notable oxygen effect was that for the 12-day SCT bottled samples (week 94), the control treatment had significantly higher LPP content than both the oxygenated wines ($p < 0.0156$). The reverse was observed for the

week 16 samples, where both the low- and high-oxygenated wines had significantly higher LPP amount than the control ($p < 0.0203$).

Information on the potential use of LPP and SPP values is scarce. However, there is one direct application where the ratio of LPP to SPP can be used to predict a wine's response to fining agents. Common commercial fining agents like gelatin or casein tend to remove the LPP fraction while leaving the SPP fraction undisturbed. Hence, wine with high LPP/SPP will be more susceptible to fining agents (Harbertson and others 2003). Harbertson and others (2003) measured the ratio of LPP to SPP in a limited number of wines from 1998 vintage and they found that even wines made from the same grape variety (Cabernet Sauvignon) had very different LPP/SPP values. This seems to suggest that the formation of polymeric pigments is influenced by many factors and the LPP/SPP value is perhaps not an adequate parameter to predict the quality of a red wine.

Individual phenolics by HPLC

Red grape has a wide array of phenolics compounds. These compounds contribute to sensory characteristics of red wines, especially the color and astringency (Mazza and others 1999). Phenolic acids such as gallic, ferulic, caffeic, ρ-coumaric, caftaric and protocatechuic acids have been identified in wines. Flavan-3-ols including catechin and epicatechin and flavonols such as quercetin, myricetin and kaempferol have also been identified (Sartini and others 2007; Nicoletti and others 2008). The glucoside form of flavonols is commonly found in red wines; however those in galactoside, rutinoside and

glucuronide forms have also been detected in varying ratios (Castillo-Muñoz and others 2007; Gómez-Alonso and others 2007).

In the present study, nine non-anthocyanin phenolics were identified: catechin, myricetin, quercetin, gallic acid, caffeic acid, ρ-coumaric acid, along with traces of ferulic acid, resveratrol and kaempferol. The major non-anthocyanin compounds detected in all wines were catechin, quercetin and myricetin. Since ferulic acid, resveratrol and kaempferol only appeared in a small number of wines and in trace quantities, they were not used in the statistical analysis.

Catechin

Catechin is one of the major flavan-3-ols found in wines, along with the phenolic acid, gallic (Waterhouse 2002). Statistical analysis showed that in Chambourcin wines, oxygenation treatment had no significant effect ($p = 0.3234$) on the catechin content analyzed. However, the SCT by week interaction was significant ($p = 0.0040$) and this is illustrated in Figure 22. At week 1, 8, 12 and 16, the 12-day SCT treatment yielded significantly higher catechin means than the 6-day treatment ($p < 0.0303$). However, there was no SCT effect at week 4 ($p = 0.1866$).

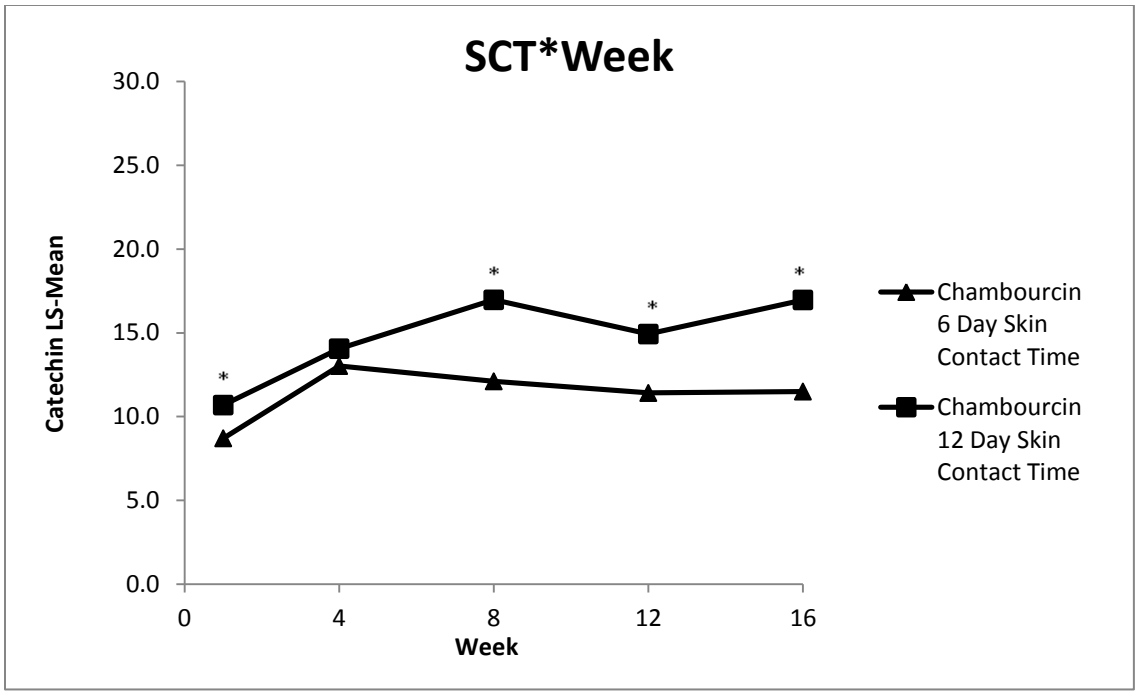


Figure 22. Least square means of skin contact time (SCT) by week combinations for catechin content of Chambourcin wines (asterisk [*] denotes week where significant SCT effect was observed)

Oxygenation treatment also had no significant effect on the catechin content of the Ruby Cabernet wines ($p = 0.2021$). The SCT by week interaction was significant however ($p = 0.0034$) (Figure 23). According to Figure 23, for week 1, the catechin content of SCT 6 treatment was significantly higher than SCT 12 ($p = 0.0462$). At week 4, 12 and 16, the reverse was true ($p < 0.0068$), with week 8 being marginally significant ($p = 0.0742$). In general, the 12-day SCT treatment yielded higher catechin content.

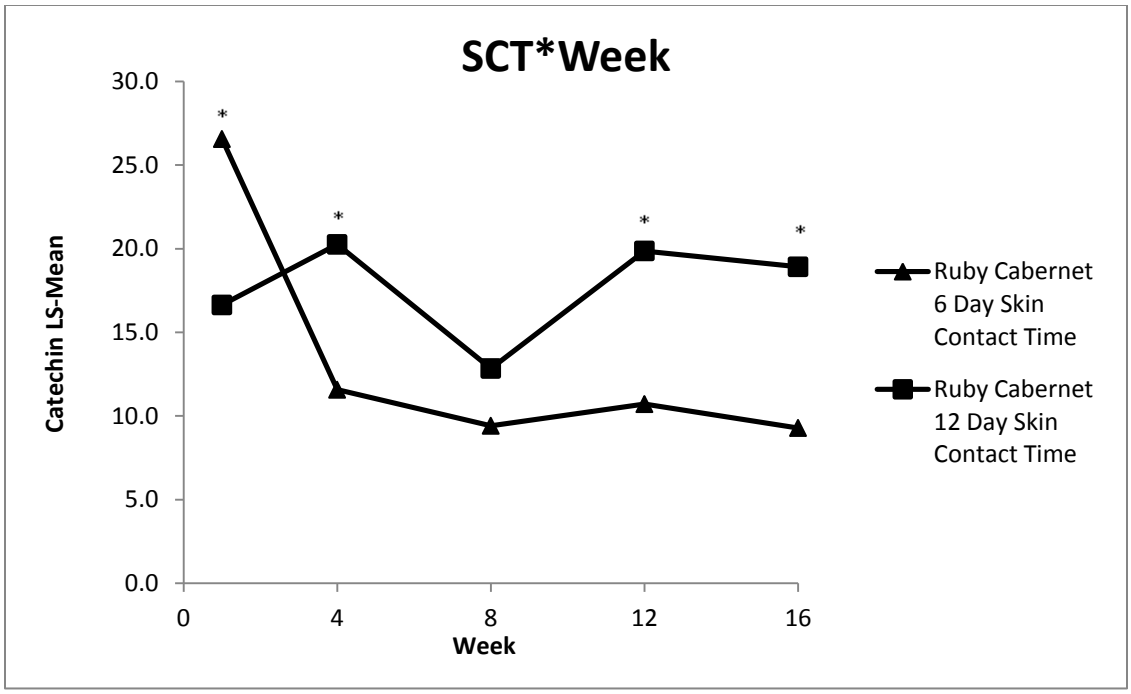


Figure 23. Least square means of skin contact time (SCT) by week combinations for catechin content of Ruby Cabernet wines (asterisk [*] denotes week where significant SCT effect was observed)

In a study by de Villiers and others (2005), the catechin content in 5 types of red wines (Cabernet Sauvignon, Merlot, Pinotage, Ruby Cabernet and Shiraz) ranged from 32-58 ppm (Table 3). Meanwhile, Gómez-Alonso and others (2007) obtained an average of 31 ppm catechin from 10 Cencibel wines. Both of these studies utilized wines that were not subjected to any kind of manipulation, and hence may be used as a comparison to the current study. Comparing to the research of de Villiers (2005), our study found lower amounts of catechin, especially the Chambourcin wines, whose range was from 9-17 ppm (Table 7c, Appendix A). The Ruby Cabernet wines in this current study had approximately 9-27 ppm of catechin (Table 8e, Appendix A). The catechin content in our Ruby Cabernet wines was considered low compared to the study of de Villiers and others (2005), where this particular variety was also tested (Table 4).

Table 3. Average phenolic content of 5 red wines (de Villiers and others, 2005)

Compound	*Concentration (ppm)(n=13)*
Gallic acid	20.6 - 36.7
Catechin	31.8 - 57.5
Caffeic acid	7.7 - 33.1
ρ-Coumaric acid	5.7 - 7.1
Myricetin	4.0 - 8.0
Quercetin	7.4 – 15.0

Table 4. Comparison of phenolic content of Ruby Cabernet wines from two studies

Compound	*de Villiers (2005) (n=13)(ppm)*	*Present study (ppm)*	
		Lowest level detected	*Highest level detected*
Gallic acid	20.6	1.1	4.4
Catechin	31.8	9.3	26.6
Caffeic acid	7.7	0.4	1.5
ρ-Coumaric acid	7.1	0.4	2.0
Myricetin	8.0	2.1	5.4
Quercetin	8.3	0.7	5.1

Gallic acid

Both Chambourcin and Ruby Cabernet wines had significant skin contact by oxygen by week interaction ($p < 0.0359$) for gallic acid content. This three-way interaction for Chambourcin wines is shown in Figure 24. From Figure 24, the level of gallic acid over the 16-week timeframe showed significant fluctuations. At a glance, it appears the gallic acid content of the 12-day SCT treatment was higher than the 6-day SCT in all weeks. However, according to statistical analysis, this effect was only significant at week 8 ($p < 0.0349$) and week 16 ($p < 0.0009$). There were some significant oxygen effects at week 1 and 12, but they didn't contribute any meaningful insight in terms of which oxygenation treatment was better overall.

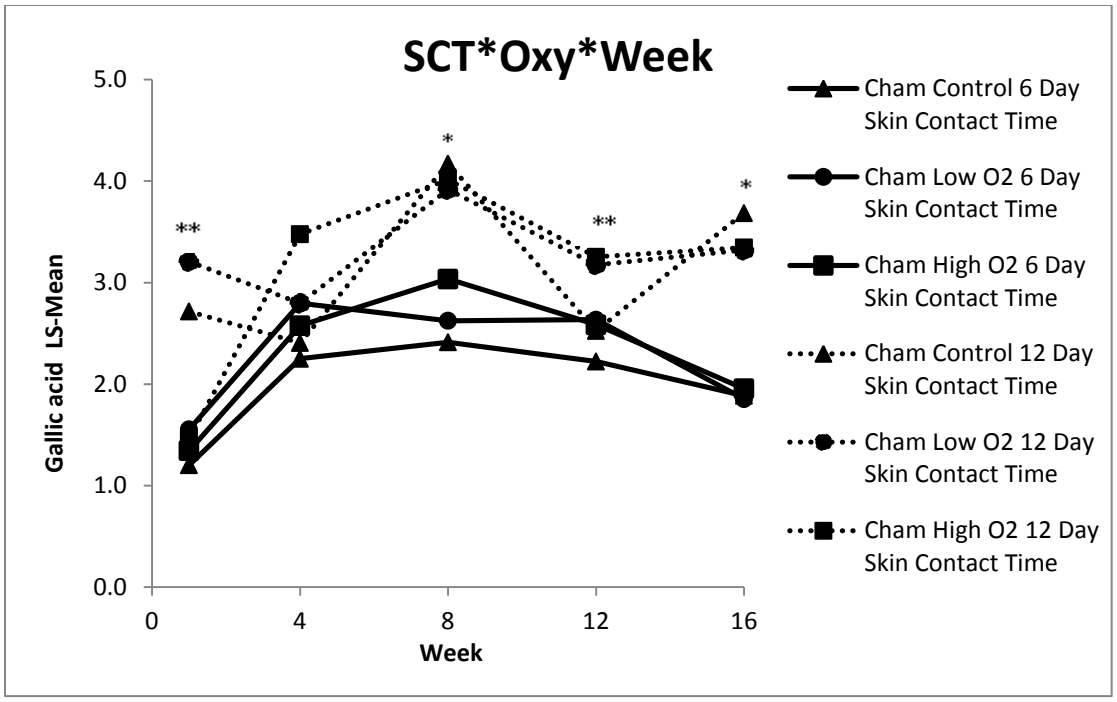


Figure 24. Least square means of skin contact time (SCT) by oxygen by week combinations for gallic acid content of Chambourcin wines (Cham = Chambourcin, O2 = oxygen, asterisk [*] denotes week where significant SCT effect was observed, double asterisk [**] denotes week where significant oxygen effect was observed)

The three-way interaction effect of the Ruby Cabernet wines is illustrated in Figure 25. As with the case of Chambourcin wines, the gallic acid content of the Ruby Cabernet also demonstrated erratic fluctuation patterns. Again, statistical analyses didn't reveal any clear conclusions.

Gallic acid content ranged from 21-37 ppm in the study of de Villiers and others (2005) (Table 3). Similarly, Gómez-Alonso and others (2007) quantified 20 ppm of gallic acid in some Cencibel wines (n=10). Comparing those findings to the present study, the amount of gallic acid detected in our study was much lower, at less than 4.5 ppm (Tables 7e and 8f, Appendix A). The relatively low amount of gallic acid may be attributable to

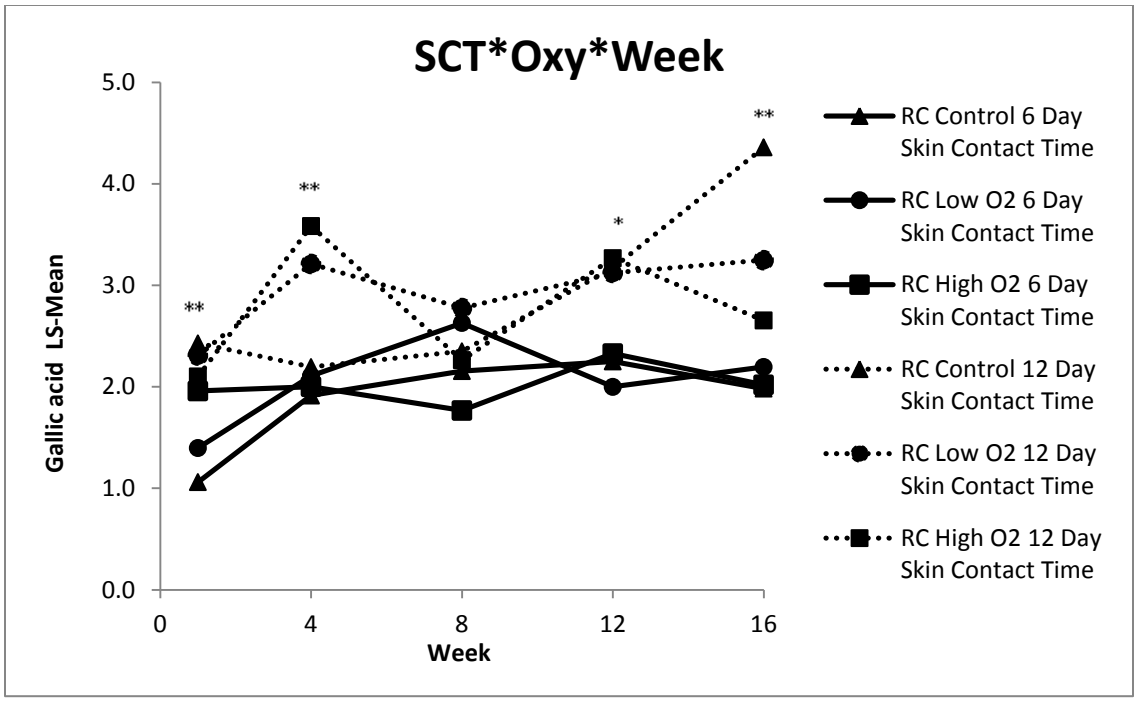


Figure 25. Least square means of skin contact time (SCT) by oxygen by week combinations for gallic acid content of Ruby Cabernet wines (RC = Ruby Cabernet, O2 = oxygen, asterisk [*] denotes week where significant SCT effect was observed, double asterisk [**] denotes week where significant oxygen effect was observed)

the use of C18 cartridges. These cartridges are a popular choice to isolate phenolic compounds in wines and have been postulated to clean up a sample by removing sugars and organic acids. Sugars will interfere with chromatographic analysis, so their removal is beneficial. But, the cartridge can cause of a low recovery of phenolic acids such as gallic, caffeic, ferulic and ρ-coumaric, due to the alcohol reducing the retention of some phenolics by the sorbent. Gallic acid is especially susceptible to this loss (Pérez-Magariño and others 2008). In addition, it is also possible that factors related to climate and/or growing season could be responsible for the relatively low gallic acid concentrations observed in the tested wines. Comparative data would need to be collected over time to test this hypothesis.

Caffeic and ρ-coumaric acids

In terms of caffeic acid, both wines showed insignificant oxygen effect ($p > 0.1099$). SCT by week interaction was significant however for both wines: Chambourcin, $p = 0.0032$ and Ruby Cabernet, $p = 0.0338$. For Chambourcin wines, the SCT by week interaction is illustrated in Figure 26. Only at week 16 that12-day SCT treatment yielded significantly higher caffeic acid means than 6-day SCT ($p = 0.0008$).

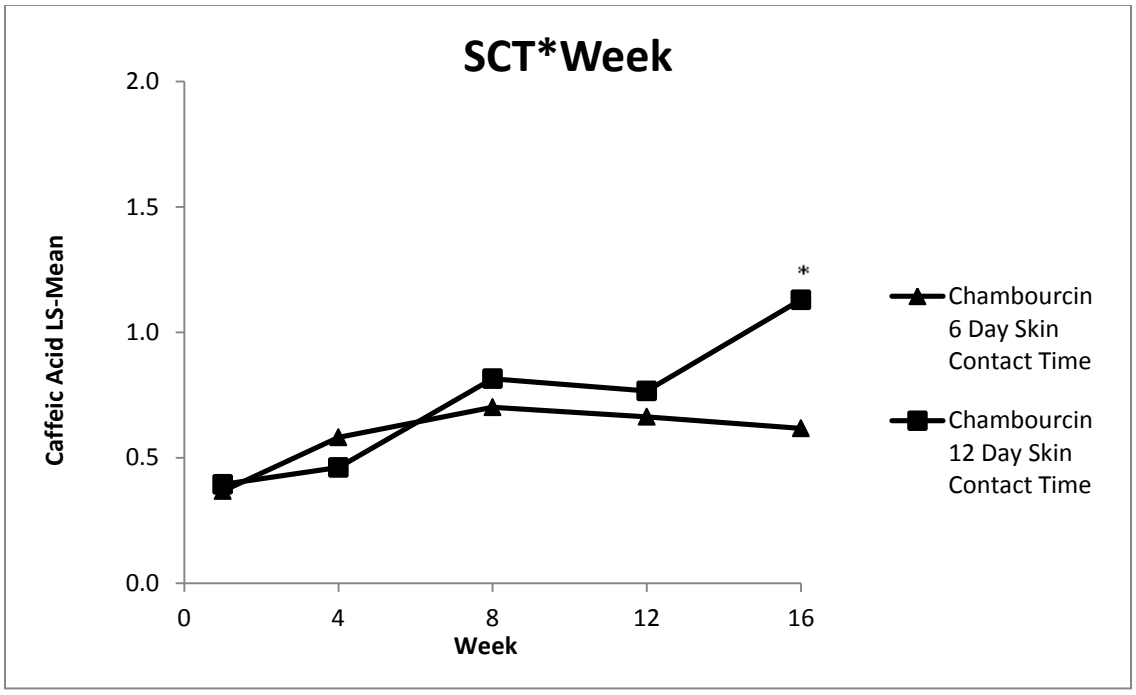


Figure 26. Least square means of skin contact time (SCT) by week combinations for caffeic acid content of Chambourcin wines (asterisk [*] denotes week where significant SCT effect was observed)

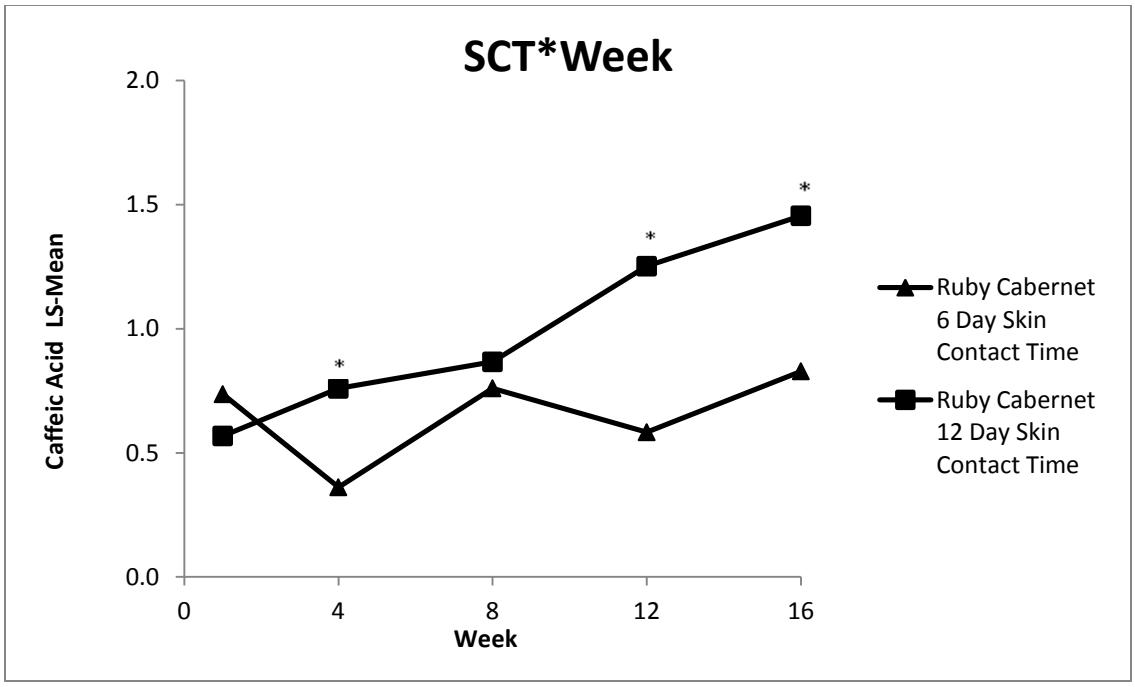


Figure 27. Least square means of skin contact time (SCT) by week combinations for caffeic acid content of Ruby Cabernet wines (asterisk [*] denotes week where significant SCT effect was observed)

For the Ruby Cabernet wines, the SCT by week interaction is illustrated in Figure 27. At week 1 and 8, no SCT effect was observed ($p > 0.2721$). At week 4, 12-day SCT yielded significantly higher caffeic acid means ($p = 0.0002$) than 6-day SCT. The same was also observed at week 12 ($p = 0.0101$) and week 16 ($p = 0.0462$).

For Chambourcin wines, the only significant effect on ρ-coumaric acid content was the week effect ($p = 0.0029$). The content of ρ-coumaric acid seemed to fluctuate across the weeks with no discernible trend (Table 7a, Appendix A).

For Ruby Cabernet wines, three significant effects were observed on ρ-coumaric acid content: oxygen ($p = 0.0190$), SCT ($p = 0.0009$), and week ($p < 0.0001$). For oxygenation treatment, the low oxygenated wines had significantly higher ρ-coumaric acid means than the high oxygenated ones ($p = 0.0068$) (Table 8c, Appendix A). For the

SCT effect, the 12-day SCT treatment had significantly higher ρ-coumaric acid means than 6-day SCT (Table 8b, Appendix A). In terms of week effect, it was revealed the content of ρ-coumaric acid increased steadily from week 1 till week 8, and at week 12, the level dipped and rose again (Table 8a, Appendix A).

Looking at the present study, the quantity of caffeic and ρ-coumaric acids in both of the wines was low. The amount detected for these phenolic acids was less or approximately 2 ppm, which were considerably lower than those reported by de Villiers and others (2005) (Table 3). The low recovery of these phenolic acids could be due to the use of C18 cartridges as noted above in the discussion of gallic acid contents. Again, it is also possible that factors related to climate and growing season could be responsible for the relatively low values observed.

Myricetin and quercetin

For Chambourcin wines, the concentrations of the flavonol myricetin had significant SCT by week interaction ($p = 0.0003$). The SCT by week interaction for myricetin is illustrated in Figure 28, where for all weeks 1, 4, 8, 12 and 16, the means of 6-day SCT treatment were significantly higher than the 12-day SCT ($p < 0.0122$), with greater difference occurring early in the experiment.

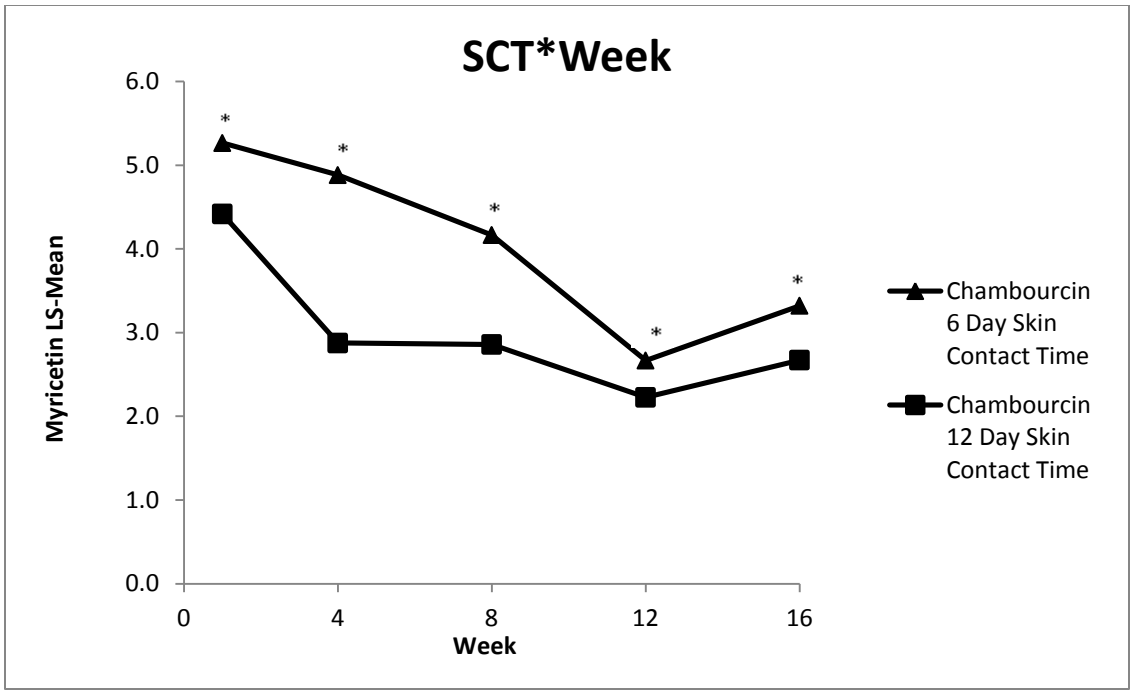


Figure 28. Least square means of skin contact time (SCT) by week combinations for myricetin content of Chambourcin wines (asterisk [*] denotes week where significant SCT effect was observed)

For Ruby Cabernet wines, myricetin showed insignificant oxygenation effect ($p = 0.1680$) but significant SCT by week interaction effect ($p = 0.0021$), which is shown in Figure 29. At weeks 1 and 12, there were no SCT effect ($p > 0.6502$). At week 4, the difference was marginally significant ($p = 0.0578$). At week 8, the myricetin content of SCT 6 was higher than SCT 12 ($p = 0.0030$), as well as week 16 ($p = 0.0005$).

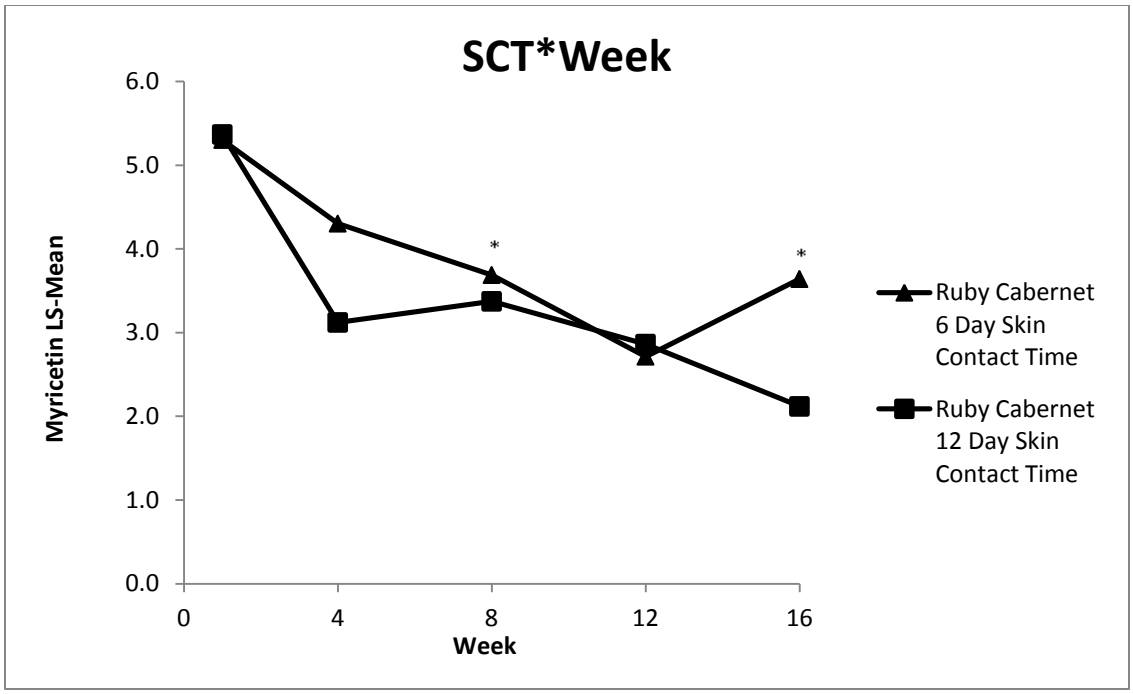


Figure 29. Least square means of skin contact time (SCT) by week combinations for myricetin content of Ruby Cabernet wines (asterisk [*] denotes week where significant SCT effect was observed)

The content of myricetin seemed to hover in the 5 ppm range for both of the Chambourcin and Ruby Cabernet wines (Tables 7c and 8e, Appendix A), and this coincided with the median range as reported by de Villiers and others (2005) in Table 3.

As with the case of myricetin in Chambourcin wines, the significant effect observed for quercetin was the SCT by week interaction ($p = 0.0001$) (Figure 30). As demonstrated in Figure 30, for all weeks (1, 4, 8, 12 and 16), the average of quercetin content in the 6-day SCT treatment was significantly higher than the 12-day SCT ($p < 0.0185$), with greater difference observed early in the study.

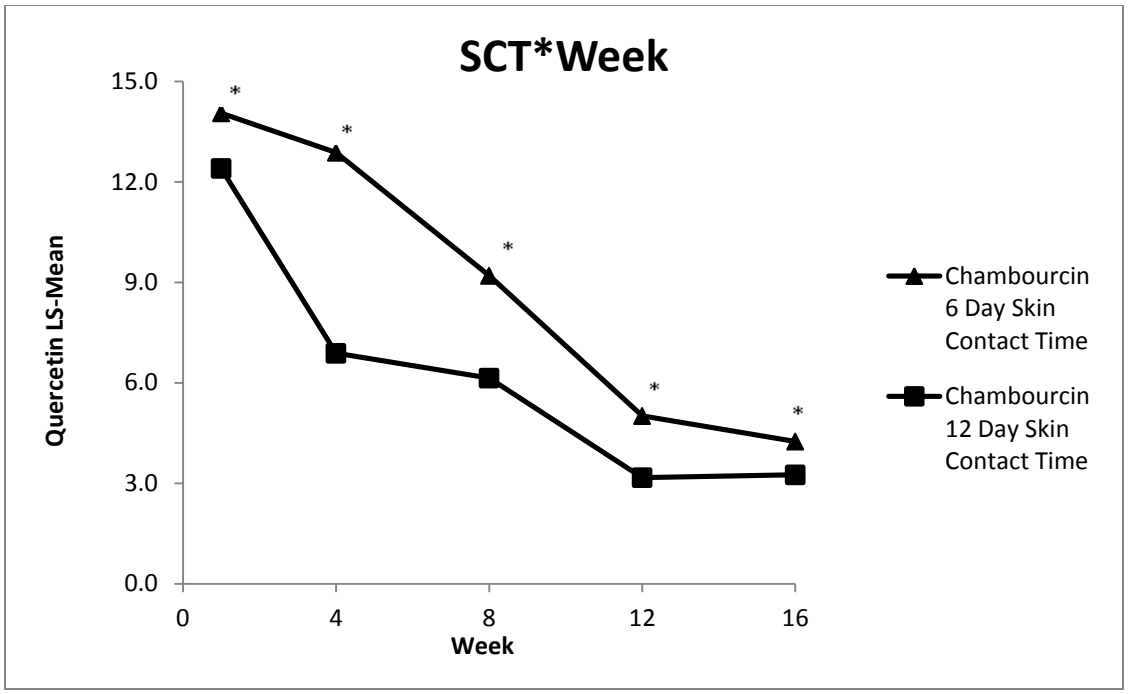


Figure 30. Least square means of skin contact time (SCT) by week combinations for quercetin content of Chambourcin wines (asterisk [*] denotes week where significant SCT effect was observed)

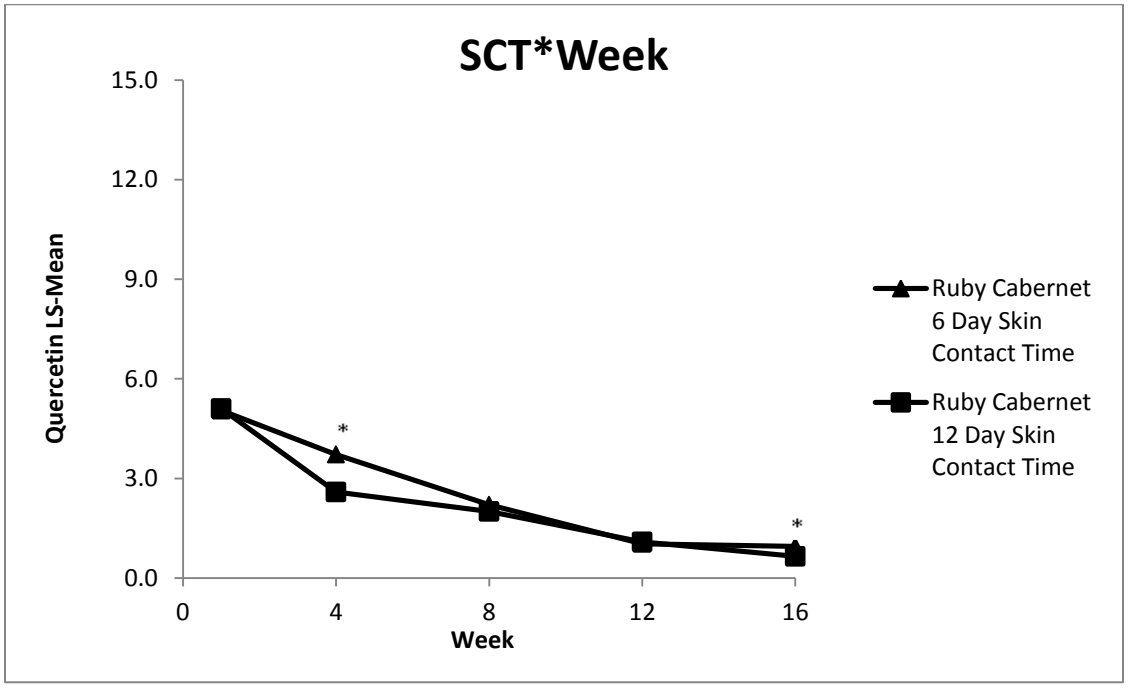


Figure 31. Least square means of skin contact time (SCT) by week combinations for quercetin content of Ruby Cabernet wines (asterisk [*] denotes week where significant SCT effect was observed)

For Ruby Cabernet wines, oxygenation treatment had no impact on quercetin content ($p = 0.5302$). The significant SCT by week interaction is shown in Figure 31 ($p = 0.0303$). From Figure 31, at week 1, 8 and 12, no SCT effect was observed ($p > 0.4043$). At week 4, the quercetin average of 6-day SCT was significantly higher than the 12-day SCT ($p = 0.0162$), as well as for week 16 ($p = 0.0197$).

From Tables 7c and 8e in Appendix A, we see that the concentration of quercetin declined over the course of 16 weeks in all wines. While statistical analysis was not performed to detect differences between varieties, Chambourcin wines had a higher observed quercetin content than the Ruby Cabernet (Tables 7c and 8e, Appendix A). It should be noted that the highest concentration detected (14 ppm) came from a 6-day SCT treatment wine. In general, a longer SCT treatment will be expected to yield higher concentration of phenolics in wine. However, in the current study, this was not always the case. Per statistical analysis, both myricetin and quercetin seemed to be more concentrated in the 6-day SCT wines than the 12-day SCT ones (Tables 7c and 8e, Appendix A).

The content of flavonols (myricetin, quercetin and kaempferol) is of particular interest since they are indicative of co-pigmentation potential. This co-pigmentation association involves the anthocyanins glycosides and their "cofactors" such as certain flavonoids and phenolic acids; quercetin in particular has been shown to contribute significant color enhancement (Boulton 2001). A correlation was observed at present study in the Chambourcin wines, in which the quercetin concentration was declining consistently as the SPP content increased (Figure 19). This is by no means a scientific

proof that co-pigmentation has taken place; it was simply a hypothesis that would require further examination to prove.

Monomeric anthocyanins

The anthocyanins profile varied between the two wines. Chambourcin wines had a major anthocyanin eluting at approximately 11.5 min and the Ruby Cabernet had one at 13.7 min (Figures 32 and 33). The specific identities of these peaks were not been positively confirmed. Nevertheless, the peak areas were substantial and there was no evidence that those peaks were polymeric pigments. According to Ginjom and others (2011), polymeric pigments eluted at 520 nm as a distinct hump below the completely separated monomeric anthocyanins. In this current study, the peaks at 520 nm separated

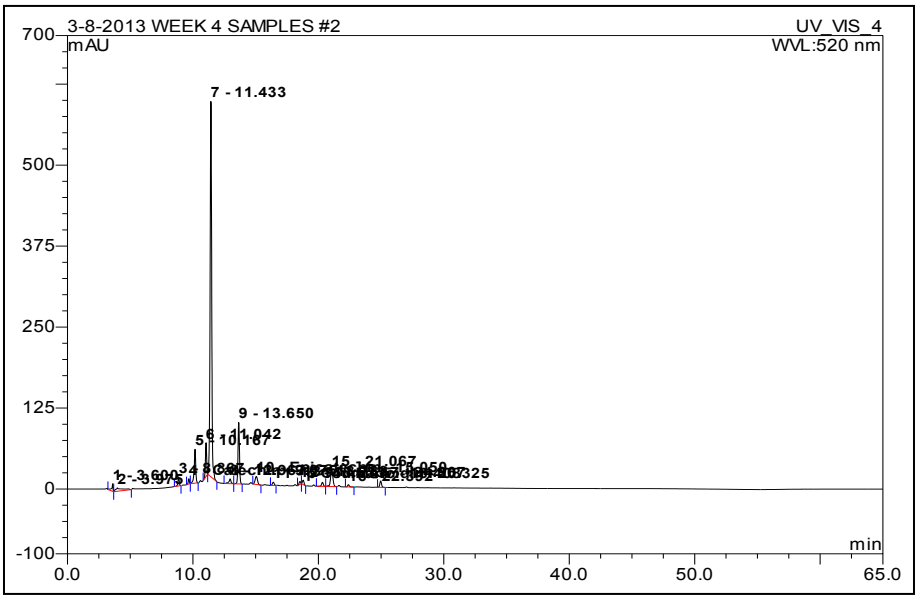


Figure 32. Sample chromatogram of a Chambourcin wine at 520 nm

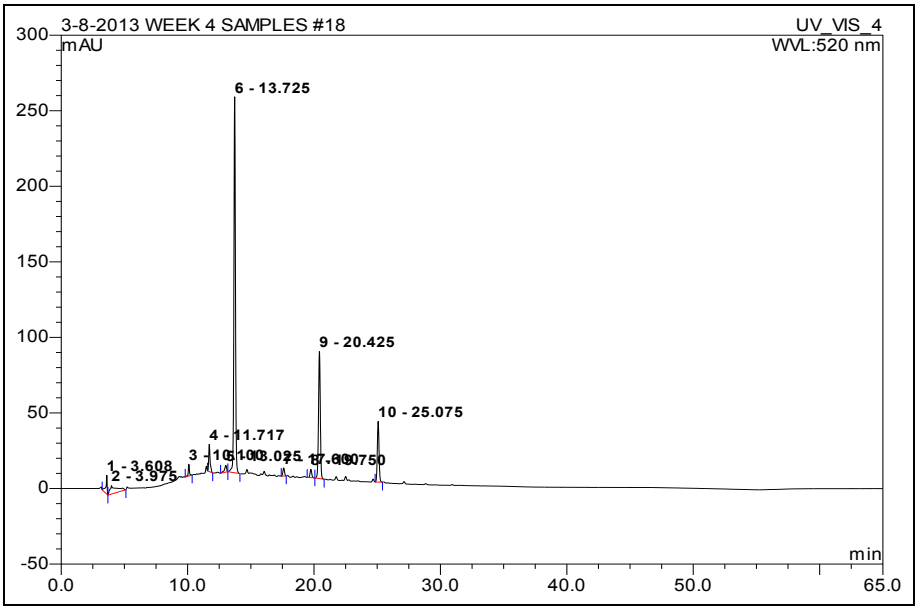


Figure 33. Sample chromatogram of a Ruby Cabernet wine at 520 nm

completely and distinctively, with no evidence of humps or condensation, which further indicates that those peaks were most probably monomeric anthocyanins. Since definitive anthocyanin identification was not possible due to the absence of appropriate standards, statistical analysis was not performed. But we assume that the monomeric anthocyanins would decline over time as expected.

A number of publications have detailed the types of monomeric anthocyanins present in red wines. In *vitis vinifera*, only five monoglucosides of anthocyanidins exist (Mazza and others 1995): malvidin, delphinidin, cyanidin, petunidin and peonidin. Meanwhile, some diglucosides of anthocyanidin are found in some other grape species (Hebrebo and others 1989). Of all the glucosides, malvidin-3-monoglucoside is the most abundant anthocyanin in grapes from the *Vitis vinifera* L. species (Mazza and others 1995; Gómez-Alonso and others 2007). Hybrid grapes are speculated to contain different anthocyanins and this is demonstrated in the study of Thimothe and others (2007). In this

study, the extracts from some interspecific hybrid grapes (Baco Noir and Noiret) had very different anthocyanins fingerprint than those of the *Vitis vinifera* (Pinot Noir and Cabernet Franc) extracts. These hybrids had a much higher concentration of delphinidin and petunidin, where the Noiret extract contained up to 30 times more. It perhaps would be fair to assume the Chambourcin grape used in this current study to also contain a different anthocyanins profile than the Ruby Cabernet. If the retention times of the two major peaks in both of the wines were any indication (11.5 min and 13.7 min), these two varieties of grape did indeed have two very different major anthocyanins.

A few publications have stated that red wines required no preparation step prior to HPLC analysis (Revilla and others 1999; García-Beneytez and others 2003), even though in some cases a filtration process was performed (Waterhouse and others 1999; Pérez-Magariño and others 2008). In the present study, the wine sample underwent a filtration process by C18 cartridge to remove the sugars and organic acids, and was then processed further by enzymatic hydrolysis. The hydrolysis step was meant to simplify the chromatographic data and should not cause a huge difference in the concentration of the anthocyanins compounds present initially in the sample. However, the use of the C18 cartridge may have contributed to the loss of phenolic acids as noted previously.

Antioxidant capacity by ORAC

In wine, the common opinion is that the radical scavenging capacity is related to its phenolic content. For a substance to impart antioxidant power, it has to be readily

oxidizable. Wine contains high levels of substances with the catechin group molecular structure, which is very reactive with oxidants (Waterhouse 2002).

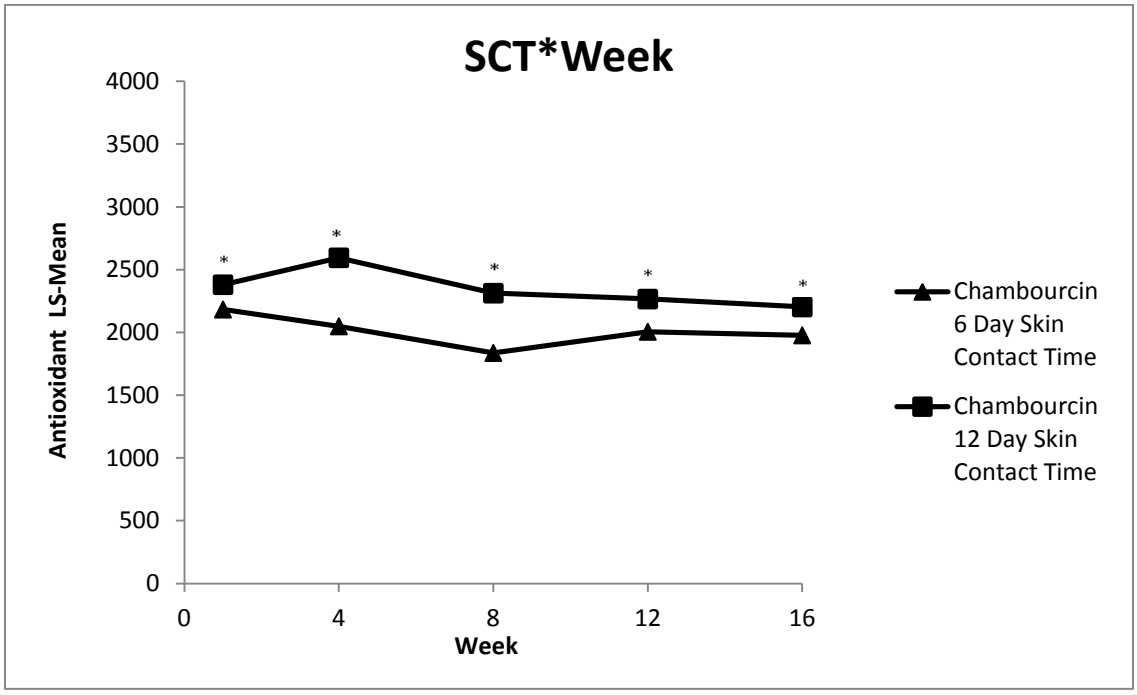


Figure 34. Least square means of skin contact time (SCT) by week combinations for antioxidant content of Chambourcin wines (asterisk [*] denotes week where significant SCT effect was observed)

For the Chambourcin wines, the SCT by week interaction was significant ($p = 0.0020$) for the antioxidant content, as was the SCT by oxygen interaction ($p = 0.0208$). The SCT by week interaction is shown in Figure 34, where at week 1, 4, 8, 12 and 16, the 12-day SCT treatment produced significantly higher antioxidants means than 6-day SCT ($p < 0.0184$). This is not surprising considering higher SCT treatment will yield higher total phenolics, which translates to higher antioxidant capacity. In terms of the SCT by oxygen interaction effect, there was significant oxygenation treatment ($p = 0.0015$) for the 12-day SCT treatment. At 12-day SCT, the control wines had significantly higher

antioxidant content than the high oxygenated wines (p = 0.0004), and the low oxygenated wines had greater antioxidant content than the high oxygenated ones (p = 0.0115) (Table 7d, Appendix A).

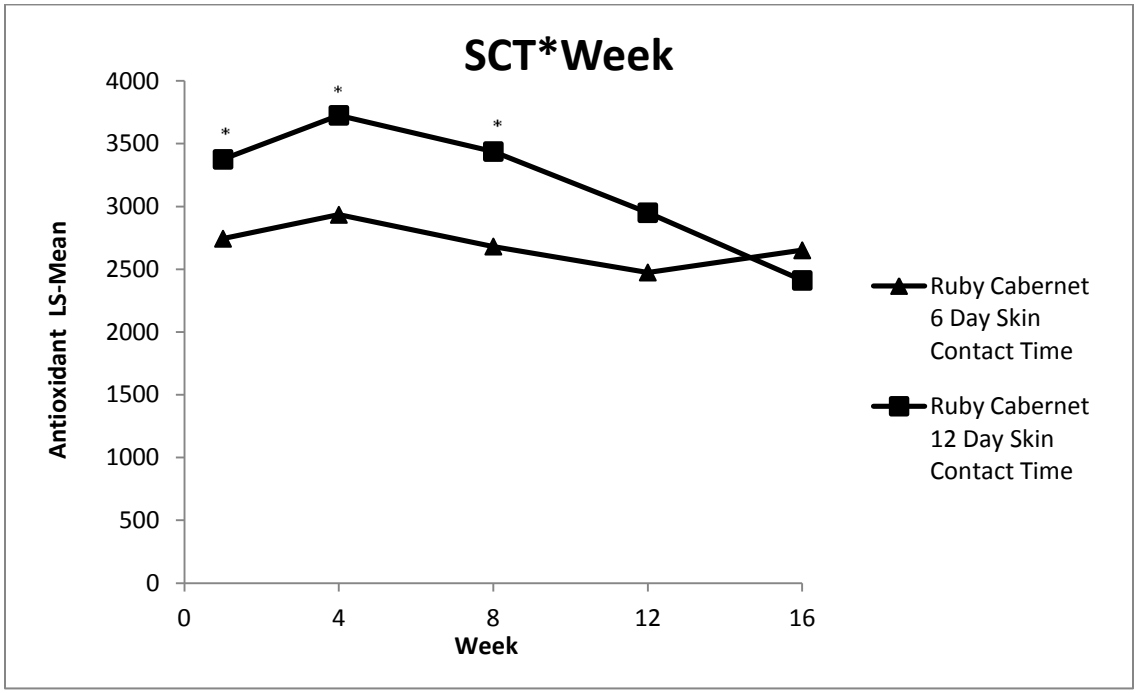


Figure 35. Least square means of skin contact time (SCT) by week combinations for antioxidant content of Ruby Cabernet wines (asterisk [*] denotes week where significant SCT effect was observed)

For Ruby Cabernet wines, oxygenation has no significant impact on the antioxidants content (p = 0.7172). As with the Chambourcin wines, the SCT by week interaction was significant (p = 0.0220), as shown in Figure 35. At week 1, 4, and 8, the 12-day SCT treatment yielded significantly higher means than the 6-day SCT treatment (p < 0.0001). However, at week 12 and 16, there were no SCT effect (p > 0.0797).

Chambourcin wines were observed to have noticeably lower total phenolics content than the Ruby Cabernet, and also possessed much lower ORAC values (Tables 7b

and 8d, Appendix A). In both wines, the 12-day SCT treatment had higher antioxidant activity. No discernible trend was observed for the antioxidant activity of the Chambourcin wines (Figure 34). Nevertheless, in the Ruby Cabernet wines there seemed to be a notable drop in antioxidant activity over time for the 12-day SCT wines (Figure 35). As mentioned previously, the Ruby Cabernet wines perhaps were more "sensitive" to oxygenation treatments and therefore polymerized more, causing the loss of phenolics due to precipitation. Perhaps if oxygenation treatments had been continued, we might have seen that over time, oxygen would have accelerated the polymerization process even more, causing the loss of phenolics due to precipitation and a subsequent decrease in antioxidant capacities.

Antioxidant capacity of some red wines from China was quantified by Li and others (2009), whose amounts ranged from 960-2440 μmol Trolox equivalents (TE)/100 mL. In this current study, the values obtained were from 1800-3700 μmol TE/100 mL. As mentioned previously, the Ruby Cabernet wines, for both SCT treatments, had higher ORAC values due to higher total phenolics (Table 8d, Appendix A). Scalzo and others (2012) reported that wine fractions containing phenolic compounds showed the highest ORAC activity, which suggested that the phenolics were the components responsible. Meyer and others (1997) tested the inhibition of human LDL oxidation *in vitro* by using 14 grape phenolic extracts. The study found that the level of inhibition was comparable to those previously found for wines; however, a pure catechin standard used as a comparative measure rated consistently higher. More examination is necessary to determine the synergistic or antagonistic effects of grape phenolics on antioxidant activity (Meyer and others 1997). The information obtained could be beneficial in

developing the most ideal enological practices to achieve the maximum antioxidant capacity possible.

Total alcohol content of wine

For red wine, the Code of Federal Regulations (CFR) Title 21 states that it should contain not in excess of 14% alcohol by volume in order to be labeled as "Table Wine". The wines tested in this study ranged from 12.9-14.3% alcohol, where some wines were slightly above the FDA's specification. For both wines, the alcohol content was in the same range and no obvious distinction was observed in the oxygenation treatments (Table 11, Appendix A).

pH and titratible acidity of wine

The pH of the Chambourcin wines ranged from 3.4-3.7 and the Ruby Cabernet ones were higher at 3.7-3.9 (Tables 12 and 13, Appendix A). The desirable range of red wine should be between 3.4 and 3.7 (MoreWine!). Higher pH will negatively impact the color intensity and wine with higher pH is more likely to lose its quality quickly (Acuvin 2012).

The titratible acidity (TA) measurement is used to quantify tartness in juice or must. Wines with less than 0.5 g tartaric acid/100 mL are considered bland and levels exceeding 0.8 g tartaric acid/100 mL are categorized as sharp (Vine and others 2002). The wines analyzed in this study were in the range of 0.6-0.7 g tartaric acid/100 mL,

which could be considered to have *medium* tartness (Table 14, Appendix A). In the study of Lee and others (2007), the Cabernet Sauvignon wines had TA values ranging from 0.6-0.7 g tartaric acid/100 mL, and Merlot wines from 0.5-0.7 g tartaric acid/100 mL.

Total phenolics and monomeric anthocyanins of raw grape

Total phenolics content of raw grapes is expressed as mg of gallic acid equivalents (GAE) per gram of fresh weight (FW). Chambourcin and Ruby Cabernet had 3.8 mg GAE/g FW and 5.5 mg GAE/g FW, respectively (Table 15, Appendix A). Du and others (2012) reported the total phenolics content of seven dark grape varieties and the value ranged from 1.2-2.2 mg GAE/g FW, which was lower than those reported in the current study. Oikonomakos and others (2009) reported the total phenolics content of Cabernet Franc whole grapes to have 5.3 mg GAE/g FW, which was comparable to the amount quantified in this study.

Total monomeric anthocyanins in raw grapes are quantified as cyanidin-3-glucoside (C3G) per gram of fresh weight (FW). In Chambourcin grape, the total monomeric anthocyanins content was 0.4 mg C3G/g FW and Ruby Cabernet grape had 0.6 mg C3G/ g FW (Table 15, Appendix A). Compared to the values by Bu and others (2012), which was in the range of 0.05-1.7 mg C3G/g FW, the result obtained in this current study was approximately in the median range.

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Boulton R. 2001. The copigmentation of anthocyanins and its role in the color of red wine: A critical review. Am J Enol Vitic 52(2):67-87.

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CHAPTER V

CONCLUSIONS

Wine is a complex chemical system. One component of particular interest in wines is the phenolic compounds, which have a large impact on the overall sensory perception of a given wine. One group of phenolics, anthocyanins and anthocyanin polymers, gives the red wine its signature color. Another group of phenolics, which may loosely be described as tannins, provides the astringency and body that add a certain amount of complexity and appeal to a red wine. Bitterness, although it should be minimal, is another desirable character of red wine provided by phenolic compounds.

The phenolics in red grapes and red wines have been researched extensively. Many methods exist for the identification and quantification of the different kinds of phenolics in grapes and wines. In fact, there has been effort to use anthocyanins composition to predict grape cultivar (Ryan and Revilla 2003). The content and composition of phenolics in wines are dependent on the grape berry, on which genetic and environmental conditions can have a huge impact (Thimothe and others 2007), and also on vinification practices such as skin contact time and the pressure applied during pressing (Maggu and others 2007). Ageing of wine is solely for the purpose of improving

wine quality by allowing flavors and aromas to develop. Micro-oxygenation is a relatively new method first introduced in early 1990s as an alternative to barrel ageing (Carlton and others 2007). Similar to traditional barrel ageing, micro-oxygenation is purported to bring about desirable changes in wine, but in a shorter time.

Based on statistical findings, the effect of micro-oxygenation on the content of most phenolic compounds, both the major and individual components, was not readily apparent in the wines tested. However, in general, the changes in phenolics content and composition were consistent with the kind of accelerated ageing processes expected to occur with the application of micro-oxygenation. In terms of sensory attributes, it is nevertheless possible that the micro-oxygenation treatments did have a detectable impact on final wine quality. Sensory testing will be required to evaluate this question. Aside from the phenolics content, the effect of oxygenation on the antioxidant capacity was also not significant. This suggests that the possible advantages of micro-oxygenation need not incur a significant loss in antioxidant activity in red wines.

The graphical presentation of the total phenolics, anthocyanins and tannins contents of the Ruby Cabernet wines tended to indicate that this variety was more "sensitive" to oxygenation treatments as opposed to the Chambourcin variety in the sense that the changes seen in the concentrations of these compounds were larger in magnitude over the duration of the study in the oxygenated Ruby Cabernet wines. This was perhaps due to the higher initial total phenolics content measured in the Ruby Cabernet wines, which provided a higher concentration of substrates available to participate in oxygen-mediated chemical reactions. Thus, Ruby Cabernet wines might possibly benefit more than Chambourcin wines from the practice of micro-oxygenation, particularly if the

oxygen treatments were adjusted to accommodate the change in phenolics content over time. However, this practice would necessarily be time consuming, as samples would need to be collected and analyzed at a frequent interval, perhaps including sensory analysis, in order to justify changes in oxygen treatment.

The results of this study might indicate that wines made from traditional *vinifera* grapes might benefit more from micro-oxygenation than wines made from hybrid grapes such as Chambourcin. However, given that the initial total phenolics content of Chambourcin is relatively low, more studies with additional hybrid varieties would need to be done to substantiate this hypothesis.

Examining the possibilities for future research, a number of possible improvements in the experimental design could be considered. For example, it is possible that an experimental design with a lone SCT treatment and with varying oxygenation levels could better demonstrate the effects of the oxygenation treatment. Perhaps, it would also be beneficial to introduce the oxygen in small aliquots throughout the day so as to not overwhelm the system. Since the oxygen bubbles are supposed to dissolve into the wine solution as they travels to the top of the vessel, the design of the fermentation vessel should allow for adequate distance to facilitate full gas exchange and dissolution (Cano-López and others 2006). Using a larger volume of wine and having more replications per treatment would also be beneficial.

In all, predicating wine quality simply by chemical analyses is difficult. We anticipate that sensory analysis will be able to detect subtle differences that are not

readily apparent from chemical testing. We plan to conduct sensory evaluation of some of the wines from this research project at a later date.

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APPENDICES

APPENDIX A: Tables 5 – 15

APPENDIX B: Modified Adams Assay for Phenolics in Wine

APPENDIX A

Table 5. [1]p-Values of all statistical analysis of Chambourcin wines

a. 16-Week Samples: Harbertson-Adams and Antioxidant (ORAC) Assays

Effects	Total Phenolics	Monomeric Anthocyanins	SPP	LPP	Tannins	Antioxidant
SCT	<0.0001	0.0380	<0.0001	0.1652	<0.0001	< 0.0001
OXY	0.7263	0.6710	0.2103	0.2253	0.1923	0.0115
SCT*OXY	0.2830	0.3965	0.6780	0.7888	**0.0299**	**0.0208**
WEEK	<0.0001	<0.0001	<0.0001	0.0004	<0.0001	< 0.0001
SCT*WEEK	**0.0251**	**0.0008**	0.0002	**<0.0001**	**0.0006**	**0.0020**
OXY*WEEK	0.2126	0.1152	0.5770	0.4143	0.6618	0.3788
SCT*OXY*WEEK	0.7055	0.8784	**0.0295**	0.6033	0.9187	0.6121

b. 16-Week Samples: HPLC Analysis

Effects	Gallic	Catechin	Caffeic	ρ-Coumaric	Myricetin	Quercetin
SCT	<0.0001	<0.0001	0.0046	0.1615	<0.0001	<0.0001
OXY	0.0850	0.3234	0.1099	0.2291	0.0423	0.0041
SCT*OXY	0.3462	0.3982	0.2022	0.1167	0.1802	0.0939
WEEK	<0.0001	<0.0001	<0.0001	**0.0029**	<0.0001	<0.0001
SCT*WEEK	0.0001	**0.0040**	**0.0032**	0.4439	**0.0003**	**0.0001**
OXY*WEEK	0.0002	0.3324	0.1700	0.7378	0.3679	0.2828
SCT*OXY*WEEK	**0.0017**	0.6220	0.1429	0.5051	0.5708	0.2375

c. 18-Month Storage Samples: Harbertson-Adams Assay

Effects	Total Phenolics	Monomeric Anthocyanins	SPP	LPP	Tannins
SCT	<0.0001	0.1779	<0.0001	0.7355	0.0002
OXY	0.8964	0.0834	0.2257	0.3354	0.4298
SCT*OXY	0.0837	0.1725	0.0482	0.9232	0.3333
WEEK	<0.0001	<0.0001	<0.0001	0.0178	<0.0001
SCT*WEEK	**0.0142**	0.5167	0.0023	**0.0133**	**0.0029**
OXY*WEEK	0.3958	**0.0401**	0.0567	0.2583	0.0733
SCT*OXY*WEEK	0.9517	0.3162	**0.0054**	0.1673	0.0790

[1]Numbers in bold denotes significant effects ($\alpha = 0.05$)
SCT – Skin contact time effect
OXY – Oxygen effect

Table 6. [1]p-Values of all statistical analysis of Ruby Cabernet wines

a. 16-Week Samples: Harbertson-Adams and Antioxidant (ORAC) Assays

Effects	Total Phenolics	Monomeric Anthocyanins	SPP	LPP	Tannins	Antioxidant
SCT	<0.0001	0.6024	0.0845	0.0027	**<0.0001**	0.0001
OXY	0.4337	0.0795	0.9035	0.0632	0.4757	0.7172
SCT*OXY	0.4511	0.2729	0.6201	0.0591	0.8179	0.3511
WEEK	<0.0001	<0.0001	**<0.0001**	<0.0001	0.1296	0.0001
SCT*WEEK	**<0.0001**	<0.0001	0.2041	**<0.0001**	0.2317	**0.0220**
OXY*WEEK	0.1179	0.0125	0.4073	0.2561	0.3823	0.2933
SCT*OXY*WEEK	0.2453	**0.0157**	0.9448	0.2480	0.3982	0.5007

b. 16-Week Samples: HPLC Analysis

Effects	Gallic	Catechin	Caffeic	ρ-Coumaric	Myricetin	Quercetin
SCT	<0.0001	0.0128	0.0012	**0.0009**	0.0147	0.0792
OXY	0.6516	0.2021	0.1904	**0.0190**	0.1680	0.5302
SCT*OXY	0.6118	0.3224	0.8156	0.2452	0.2977	0.1949
WEEK	<0.0001	0.0006	0.0043	**<0.0001**	<0.0001	<0.0001
SCT*WEEK	0.0830	**0.0034**	**0.0338**	0.1482	**0.0021**	**0.0303**
OXY*WEEK	0.0368	0.2027	0.3949	0.2008	0.7843	0.8820
SCT*OXY*WEEK	**0.0359**	0.0714	0.7946	0.4873	0.0838	0.1698

c. 18-Month Storage Samples: Harbertson-Adams Assay

Effects	Total Phenolics	Monomeric Anthocyanins	SPP	LPP	Tannins
SCT	0.4923	0.0017	0.2880	0.3102	0.6452
OXY	0.0620	0.0049	0.3142	0.5702	0.1520
SCT*OXY	0.0608	0.0145	0.1741	0.6527	0.2249
WEEK	<0.0001	<0.0001	**0.0005**	0.0023	**0.0471**
SCT*WEEK	**0.0231**	0.0083	0.2247	0.0824	0.1170
OXY*WEEK	0.4248	0.0036	0.9080	0.0440	0.2150
SCT*OXY*WEEK	0.1523	**0.0055**	0.9636	**0.0389**	0.2491

[1]Numbers in bold denotes significant effects ($\alpha = 0.05$)
SCT – Skin contact time effect
OXY – Oxygen effect

Table 7. [1] LS-means of Chambourcin wines (16-week samples)

7a. WEEK (Week effect)

Week	*ρ-Coumaric (ppm)*
1	1.5±0.1[b,c]
4	1.3±0.1[c]
8	1.9±0.1[a]
12	1.6±0.0[b]
16	2.1±0.1[a]

7b. SCT*WEEK (Skin contact time by week interaction effect)

SCT	*Week*	*Total Phenolics (mg CE/L)*	*Anthocyanins (mg M3G/L)*	*LPP (Au)*	*Tannins (mg CE/L)*	*Antioxidant (µmol TE/100 mL)*
6	1	777.1±12.4[b,c]	399.4±3.8[a]	0.6±0.0[b,c]	209.8±3.9[b]	2182.7±31.3[d,e]
6	4	657.5±23.4[d]	327.1±10.2[c]	0.2±0.0[d]	108.0±5.6[e]	2049.0±54.9[e,f]
6	8	626.2±8.2[d]	313.9±2.7[c]	0.9±0.2[b]	89.9±4.6[f]	1837.0±33.5[g]
6	12	639.3±9.1[d]	285.1±2.1[e]	0.1±0.1[d]	72.9±9.5[f]	2005.6±39.3[f]
6	16	633.3±9.4[d]	237.2±1.9[g]	1.0±0.2[b]	114.5±6.6[e]	1977.0±49.6[f]
12	1	918.6±12.4[a]	373.4±3.8[b]	0.2±0.0[d]	236.8±3.9[a]	2380.1±31.3[b]
12	4	883.1±23.4[a]	333.3±10.2[c]	0.3±0.0[c,d]	170.1±5.6[c]	2594.7± 54.9[a]
12	8	766.7±8.2[c]	300.3±2.7[d]	0.7±0.2[b,c]	148.1±4.6[d]	2312.9±33.5[b,c]
12	12	793.2±9.1[b]	268.0±2.1[f]	1.6±0.1[a]	196.6±9.5[b]	2266.2±39.3[c,d]
12	16	756.7±9.4[c]	233.4±1.9[g]	0.5±0.2[b,c,d]	143.8±6.6[d]	2202.8±49.6[c,d,e]

7c. SCT*WEEK (Skin contact time by week interaction effect)

SCT	*Week*	*Catechin (ppm)*	*Caffeic (ppm)*	*Myricetin (ppm)*	*Quercetin (ppm)*
6	1	8.7±0.3[e]	0.4±0.0[d]	5.3±0.1[a]	14.1±0.4[a]
6	4	13.0±0.5[b,c]	0.6±0.0[b,c]	4.9±0.1[a]	12.9±0.3[b]
6	8	12.1±1.1[b,c,d]	0.7±0.1[b,c]	4.2±0.1[b]	9.2±0.4[c]
6	12	11.4±0.3[d]	0.7±0.0[b,c]	2.7±0.1[d]	5.0±0.2[e]
6	16	11.5±0.5[c,d]	0.6±0.1[b,c]	3.3±0.1[c]	4.2±0.2[f]
12	1	10.7±0.3[d]	0.4±0.0[d]	4.4±0.1[b]	12.4±0.4[b]
12	4	14.1±0.5[a,b]	0.5±0.0[c,d]	2.9±0.1[d]	6.9±0.3[d]
12	8	17.0±1.1[a]	0.8±0.1[b]	2.9±0.1[d]	6.1±0.4[d]
12	12	14.9±0.3[a,b]	0.8±0.0[b]	2.2±0.1[e]	3.2±0.2[g]
12	16	17.0±0.5[a]	1.1±0.1[a]	2.7±0.1[d]	3.3±0.2[g]

7d. SCT*OXY (Skin contact time by oxygen interaction effect)

Oxy	*SCT*	*Tannins (mg CE/L)*	*Antioxidant (µmol TE/100 mL)*
Control	6	117.1±5.6^{c}	2042.4±40.5^{c}
Low	6	114.1±5.6^{c}	1970.4±40.5^{c}
High	6	125.8±5.6^{c}	2018.0±40.5^{c}
Control	12	194.3±5.6^{a}	2468.0±40.5^{a}
Low	12	178.2±5.6^{a,b}	2375.4±40.5^{a}
High	12	164.9±5.6^{b}	2210.6±40.5^{b}

7e. SCT*OXY*WEEK (Skin contact time by oxygen by week interaction effect)

Oxygen	*SCT*	*Week*	*SPP (Au)*	*Gallic (ppm)*
Control	6	1	0.8±0.0^{m,n}	1.2±0.1^{l}
Control	6	4	0.9±0.0^{k}	2.3±0.3^{e,f,g,h}
Control	6	8	1.2±0.0^{d,e,f,g}	2.4±0.3^{e,f,g,h}
Control	6	12	1.3±0.0^{c}	2.2±0.1^{f,g,h}
Control	6	16	1.4±0.0^{a,b}	1.9±0.2^{g,h,i}
Low	6	1	0.9±0.0^{l,m}	1.6±0.1^{i,j,k}
Low	6	4	0.9±0.0^{k}	2.8±0.3^{d,e,f}
Low	6	8	1.2±0.0^{e,f,g}	2.6±0.3$^{d,e.f}$
Low	6	12	1.3±0.0^{c,d}	2.6±0.1^{d,e,f}
Low	6	16	1.5±0.0^{a}	1.9±0.2^{h,i,j}
High	6	1	0.8±0.0^{l,m}	1.3±0.1^{k,l}
High	6	4	1.0±0.0^{k}	2.6±0.3^{d,e,f,g}
High	6	8	1.2±0.0^{c,d,e,f}	3.0±0.3^{c,d,e}
High	6	12	1.4±0.0^{b}	2.6±0.1^{d,e,f}
High	6	16	1.4±0.0^{a}	2.0±0.2^{g,h,i}
Control	12	1	0.8±0.0^{m,n}	2.7±0.1^{d,e,f}
Control	12	4	0.9±0.0^{k,l}	2.4±0.3^{e,f,g,h}
Control	12	8	1.1±0.0^{j}	4.2±0.3^{a}
Control	12	12	1.2±0.0^{g,h}	2.5±0.1^{e,f,g}
Control	12	16	1.2±0.0^{e,f,g}	3.7±0.2^{a,b,c}
Low	12	1	0.8±0.0^{n}	3.2±0.1^{c,d}
Low	12	4	0.9±0.0^{k}	2.8±0.3^{d,e,f}
Low	12	8	1.1±0.0^{j}	3.9±0.3^{a,b,c}
Low	12	12	1.2±0.0^{f,g,h}	3.2±0.1^{c,d}
Low	12	16	1.3±0.0^{c,d,e}	3.3±0.2^{c,d}
High	12	1	0.8±0.0^{n}	1.5±0.1^{j,k,l}

High	12	4	0.9 ± 0.0^{k}	$3.5\pm0.3^{a,b,c,d}$
High	12	8	$1.1\pm0.0^{i,j}$	$4.0\pm0.3^{a,b}$
High	12	12	$1.1\pm0.0^{h,i}$	$3.3\pm0.1^{c,d}$
High	12	16	$1.3\pm0.0^{c,d}$	$3.3\pm0.2^{b,c,d}$

[1]LS-mean ± standard error
[a - n] LS-means with the same letter are not significantly different ($\alpha = 0.05$)

CE – Catechin equivalent
M3G – Malvidin-3-glucoside
SPP – Short polymeric pigments
LPP – Long polymeric pigments
Au – Absorbance unit
TE – Trolox equivalent
ppm – part per million

Table 8. [1]LS-means of Ruby Cabernet wines (16-week samples)

8a. WEEK (Week effect)

Week	*SPP (Au)*	*ρ-Coumaric (ppm)*
1	0.9±0.0[d]	0.4±0.0[d]
4	1.1±0.0[c]	1.0±0.1[c]
8	1.3±0.0[b]	1.6±0.2[a,b]
12	1.4±0.0[a]	1.5±0.1[b]
16	1.5±0.1[a]	2.0±0.2[a]

8b. SCT (Skin contact time effect)

SCT	*Tannins (mg CE/L)*	*Coumaric (ppm)*
6	644.3±20.5[b]	1.0±0.1[b]
12	898.7±20.5[a]	1.5±0.1[a]

8c. OXY (Oxygen effect)

SCT	*Coumaric (ppm)*
Control	1.3±0.1[a]
Low	1.5±0.1[a]
High	1.0±0.1[b]

8d. SCT*WEEK (Skin contact time by week interaction effect)

SCT	*Week*	*Total Phenolics (mg CE/L)*	*LPP (Au)*	*Antioxidant (µmol TE/100 mL)*
6	1	1370.6±20.0[c,d]	1.4±0.0[d,e]	2743.6±60.9[c,d]
6	4	1207.5±47.4[e]	0.4±0.0[f]	2934.1±104.9[c]
6	8	1321.7±39.4[d,e]	1.9±0.2[c,d]	2681.4±66.1[c,d]
6	12	1328.0±40.7[d,e]	0.9±0.4[e,f]	2474.7±185.4[c,d]
6	16	1362.5±98.0[c,d,e]	2.5±0.2[b,c]	2651.1±196.1[c,d]
12	1	1678.8±20.0[b]	0.5±0.0[f]	3375.5±60.9[b]
12	4	1657.8±47.4[b]	0.8±0.0[f]	3725.9±104.9[a]
12	8	1851.5±39.4[a]	1.7±0.2[d,e]	3437.8±66.1[b]
12	12	1882.3±40.7[a]	4.1±0.4[a]	2950.4±185.4[c]
12	16	1593.7±98.0[b,c]	3.2±0.2[a,b]	2411.4±196.1[d]

8e. SCT*WEEK (Skin contact time by week interaction effect)

SCT	*Week*	*Catechin (ppm)*	*Caffeic (ppm)*	*Myricetin (ppm)*	*Quercetin (ppm)*
6	1	26.6±2.8^{a}	0.7±0.1^{c,d}	5.3±0.2^{a}	5.0±0.3^{a}
6	4	11.6±0.4^{c,d}	0.4±0.0^{d}	4.3±0.3^{b}	3.7±0.2^{b}
6	8	9.4±1.1^{d}	0.8±0.2^{c,d}	3.7±0.0^{b}	2.2±0.2^{c,d}
6	12	10.7±1.7^{c,d}	0.6±0.1^{c,d}	2.7±0.2^{c,d}	1.0±0.1^{e}
6	16	9.3±1.2^{d}	0.8±0.2^{b,c}	3.6±0.2^{b}	0.9±0.1^{e}
12	1	16.6±2.8^{b,c}	0.6±0.1^{c,d}	5.4±0.2^{a}	5.1±0.3^{a}
12	4	20.3±0.4^{a,b}	0.8±0.0^{c,d}	3.1±0.3^{b,c}	2.6±0.2^{c}
12	8	12.8±1.1^{c,d}	0.9±0.2^{b,c}	3.4±0.0^{b}	2.0±0.2^{d}
12	12	19.9±1.7^{a,b}	1.3±0.1^{a,b}	2.9±0.2^{c}	1.1±0.1^{e}
12	16	18.9±1.2^{b}	1.5±0.2^{a}	2.1±0.2^{d}	0.7±0.1^{f}

8f. SCT*OXY*WEEK (Skin contact time by oxygen by week interaction effect)

Oxygen	*SCT*	*Week*	*Anthocyanins (mg M3G/L)*	*Gallic (ppm)*
Control	6	1	338.8±10.2^{b,c}	1.1±0.1^{g}
Control	6	4	306.6±10.8^{d,e,f}	1.9±0.3^{e,f}
Control	6	8	283.4±14.7^{e,f,g,h}	2.2±0.3^{d,e,f}
Control	6	12	211.8±27.5^{i,j,k}	2.3±0.2^{d,e}
Control	6	16	177.1±16.5^{k}	2.0±0.2^{e,f}
Low	6	1	332.7±10.2^{c,d}	1.4±0.1^{f,g}
Low	6	4	297.3±10.8^{d,e,f,g}	2.1±0.3^{d,e,f}
Low	6	8	272.7±14.7^{f,g,h,i}	2.6±0.3^{b,c,d,e}
Low	6	12	218.1±27.5^{h,i,j,k}	2.0±0.2^{e,f}
Low	6	16	176.1±16.5^{k}	2.2±0.2^{d,e}
High	6	1	333.1±10.2^{c,d}	2.0±0.1^{e,f}
High	6	4	294.4±10.8^{d,e,f,g}	2.0±0.3^{e,f}
High	6	8	256.2±14.7^{g,h,i,j}	1.8±0.3^{e,f,g}
High	6	12	196.7±27.5^{k,j}	2.3±0.2^{d,e}
High	6	16	151.6±16.5^{k}	2.0±0.2^{e,f}
Control	12	1	390.1±10.2^{a}	2.4±0.1^{c,d,e}
Control	12	4	319.1±10.8^{c,d,e}	2.2±0.3^{d,e,f}
Control	12	8	293.8±14.7^{d,e,f,g}	2.4±0.3^{c,d,e}
Control	12	12	222.1±27.5^{h,i,j,k}	3.2±0.2^{b,c,d}
Control	12	16	202.9±16.5^{k,j}	4.4±0.2^{a}
Low	12	1	387.3±10.2^{a}	2.3±0.1^{d,e}
Low	12	4	323.4±10.8^{c,d,e}	3.2±0.3^{b,c,d}
Low	12	8	289.4±14.7^{e,f,g,h}	2.8±0.3^{b,c,d,e}

Low	12	12	136.3±27.5[k,l]	3.1±0.2[b,c,d]
Low	12	16	48.4±16.5[m]	3.2±0.2[b,c]
High	12	1	370.3±10.2[a,b]	2.1±0.1[e,f]
High	12	4	300.3±10.8 [d,e,f]	3.6±0.3[a,b]
High	12	8	262.0±14.7 [g,h,i,j]	2.3±0.3[d,e]
High	12	12	149.3±27.5[k]	3.3±0.2[b]
High	12	16	60.4±16.5[l,m]	2.7±0.2[b,c,d,e]

[1]LS-mean ± standard error
[a - m] LS-means with the same letter are not significantly different (α = 0.05)

CE – Catechin equivalent
M3G – Malvidin-3-glucoside
SPP – Short polymeric pigments
LPP – Long polymeric pigments
Au – Absorbance unit
TE – Trolox equivalent
ppm – part per million

Table 9. [1]LS-means of Chambourcin wines (18-month storage samples)

9a. SCT*WEEK (Skin contact time by week interaction effect)

SCT	*Week*	*Total Phenolics (mg CE/L)*	*LPP (Au)*	*Tannins (mg CE/L)*
6	16	633.3±8.8[b]	1.0±0.2[a]	114.5±6.2[b]
6	94	430.2±10.9[c]	0.1±0.0[b]	35.8±6.6[c]
12	16	756.7 ±8.8[a]	0.5±0.2[b]	143.8±6.2[a]
12	94	614.6±10.9[b]	0.5±0.0[a]	118.2±6.6[b]

9b. OXY*WEEK (Oxygen by week interaction effect)

Oxygen	*Week*	*Anthocyanins (mg M3G/L)*
Control	16	236.6±2.5[a,b]
Control	94	64.2±5.1[c]
Low	16	228.3±2.5[b]
Low	94	46.8±5.1[c,d]
High	16	241.0±2.5[a]
High	94	46.0±5.1[d]

9c. SCT*OXY*WEEK (Skin contact time by oxygen by week interaction effect)

Oxygen	*SCT*	*Week*	*SPP (Au)*
Control	6	16	1.4±0.0[d]
Control	6	94	2.3±0.1[a,b]
Low	6	16	1.4±0.0[d]
Low	6	94	2.5±0.1[a]
High	6	16	1.4±0.0[d]
High	6	94	2.5±0.1[a]
Control	12	16	1.2±0.0[f]
Control	12	94	2.2±0.1[b]
Low	12	16	1.3±0.0[e,f]
Low	12	94	2.1±0.1[b]
High	12	16	1.3±0.0[e]
High	12	94	1.8±0.1[c]

[1]LS-mean ± standard error
[a - f]LS-means with the same letter are not significantly different ($\alpha = 0.05$)

CE – Catechin equivalent
M3G – Malvidin-3-glucoside
SPP – Short polymeric pigments
LPP – Long polymeric pigments
Au – Absorbance unit

Table 10. [1]LS-means of Ruby Cabernet wines (18-month storage samples)

10a. WEEK (Week effect)

Week	*SPP (Au)*	*Tannins (mg CE/L)*
16	1.5±0.1[a]	758.3±34.1[a]
94	0.7±0.0[b]	415.3±40.1[b]

10b. SCT*WEEK (Skin contact time by week interaction effect)

SCT	*Week*	*Total Phenolics (mg CE/L)*
6	16	1362.5±98.0[a]
6	94	727.6±81.4[b]
12	16	1593.7 ±98.0[a]
12	94	661.6±81.4[b]

10c. SCT*OXY*WEEK (Skin contact time by oxygen by week interaction effect)

Oxygen	*SCT*	*Week*	*Anthocyanins (mg M3G/L)*	*LPP (Au)*
Control	6	16	177.1±16.5[a]	2.6±0.4[a,b,c]
Control	6	94	19.1±3.4[c]	1.7±0.3[c,d]
Low	6	16	176.1±16.5[a]	2.6±0.4[b,c]
Low	6	94	17.3±3.4[c]	1.6±0.3[c,d,e]
High	6	16	151.6±16.5[a]	2.2±0.4[c]
High	6	94	8.6±3.4[c,d]	1.7±0.3[c,d,e]
Control	12	16	202.9±16.5[a]	1.8±0.4[c,d]
Control	12	94	9.6E-14±3.4[d]	2.5±0.3[c]
Low	12	16	48.4±16.5[b,c]	4.1±0.4[a]
Low	12	94	8.9E-14±3.4[d]	0.8±0.3[d,e]
High	12	16	60.4±16.5[b]	3.8±0.4[a,b]
High	12	94	9.3E-14±3.4[d]	0.5±0.3[e]

[1]LS-mean ± standard error
[a - e] LS-means with the same letter are not significantly different ($\alpha = 0.05$)

CE – Catechin equivalent
M3G – Malvidin-3-glucoside
SPP – Short polymeric pigments
LPP – Long polymeric pigments
Au – Absorbance unit

Table 11. [1]Average alcohol content of Chambourcin and Ruby Cabernet wines

Variety	*Week*	*SCT*	*Oxygen Level*	*Alcohol Content (%)*
Chambourcin	16	6	Control	12.9±0.3
			Low	14.3±0.5
			High	13.9±0.0
Chambourcin	16	12	Control	14.0±0.1
			Low	13.9±0.3
			High	13.9±0.3
Ruby Cabernet	16	6	Control	14.2±0.6
			Low	14.3±0.8
			High	14.1±0.3
Ruby Cabernet	16	12	Control	13.8±0.1
			Low	14.1±0.3
			High	13.5±0.3

[1]Arithmetic mean ± standard deviation (n = 2)

Table 12. [1]Average pH of Chambourcin wines

Variety	*Week*	*SCT*	*Oxygen Level*	*pH*
Chambourcin	4	6	Control	3.4
			Low	3.4
			High	3.5
Chambourcin	8	6	Control	3.5
			Low	3.5
			High	3.5
Chambourcin	12	6	Control	3.5
			Low	3.5
			High	3.5
Chambourcin	16	6	Control	3.5
			Low	3.5
			High	3.5
Chambourcin	4	12	Control	3.6
			Low	3.6
			High	3.7
Chambourcin	8	12	Control	3.5
			Low	3.5
			High	3.6
Chambourcin	12	12	Control	3.5
			Low	3.5
			High	3.5
Chambourcin	16	12	Control	3.5
			Low	3.5
			High	3.5

[1]Arithmetic mean (n = 2), standard deviation was zero

Table 13. [1]Average pH of Ruby Cabernet wines

Variety	*Week*	*SCT*	*Oxygen Level*	*pH*
Ruby Cabernet	4	6	Control	3.7
			Low	3.7
			High	3.7
Ruby Cabernet	8	6	Control	3.7
			Low	3.7
			High	3.7
Ruby Cabernet	12	6	Control	3.7
			Low	3.7
			High	3.7
Ruby Cabernet	16	6	Control	3.7
			Low	3.7
			High	3.8
Ruby Cabernet	4	12	Control	3.8
			Low	3.8
			High	3.9
Ruby Cabernet	8	12	Control	3.8
			Low	3.8
			High	3.8
Ruby Cabernet	12	12	Control	3.7
			Low	3.7
			High	3.7
Ruby Cabernet	16	12	Control	3.7
			Low	3.7
			High	3.7

[1]Arithmetic mean (n = 2), standard deviation was zero

Table 14. [1]Average titratible acidity (TA) of Chambourcin and Ruby Cabernet wines

Variety	*Week*	*SCT*	*Oxygen Level*	*TA (g tartaric acid/100 mL)*
Chambourcin	16	6	Control	0.7
			Low	0.7
			High	0.7
Chambourcin	16	12	Control	0.6
			Low	0.6
			High	0.6
Ruby Cabernet	16	6	Control	0.7
			Low	0.7
			High	0.7
Ruby Cabernet	16	12	Control	0.7
			Low	0.6
			High	0.6

[1]Arithmetic mean (n = 2), standard deviation was zero

Table 15. [1]Average total phenolics and anthocyanins of raw grapes

Variety	*Total Phenolics (mg GAE/g FW)*[2]	*Total Anthocyanins (mg C3G/g FW)*[3]
Chambourcin	3.8± 0.0	0.4±0.0
Ruby Cabernet	5.5± 0.4	0.6±0.0

[1]Arithmetic mean ± standard deviation (n = 2)
[2]Milligram gallic acid equivalents per gram fresh weight
[3]Milligram cyanidin-3-glucoside per gram fresh weight

APPENDIX B

Modified Adams Assay for Phenolics in Wine

1. Total Iron-Reactive Phenolics

THIS VALUE WILL DETERMINE DILUTIONS FOR TANNIN & POLYMERIC PIGMENT ANALYSES

1.1 Into a reduced volume cuvette, pipette in the following order:

75 µL of wine sample (using a 200 µL pipette).
800 µL Resuspension Buffer (using repeating pipettor). Vortex and incubate for 10 minutes at room temperature.

1.2 Zero spectrophotometer with 875 µL Resuspension Buffer at 510 nm

1.3 Read samples at 510 nm (after 10min incubation, Step 1.2).
= Iron-Reactive Phenolics Background.

1.4 Add 125 µL of Ferric Chloride Solution to each cuvette (using repeating pipettor). Vortex and incubate for 10 minutes at room temperature.

1.5 Add 125 µl FeCl to zero cuvette, zero Spectrophotometer with 875µL resuspension buffer + 125 µL Ferric Chloride Solution at 510nm.

1.6 Read samples at 510 nm (after 10min incubation, Step 1.4).
= Iron-Reactive Phenolics Final.

DISCARD ALL CUVETTES ASSOCIATED WITH THIS ANALYSIS

1.7 Enter values into Total Iron-Reactive Phenolics worksheet (Wine_Assay.xls)

Based on the value calculated for Total Iron-Reactive Phenolics, the spreadsheet will generate dilutions for tannin and polymeric pigment analyses. Use these dilutions in parts 2 and 3 of this assay protocol.

2. Polymeric Pigment – Measures "A" and "B"

Use the Wine volume and Model Wine volume generated in the Total Iron-Reactive Phenolics worksheet (Wine_Assay.xls) in step 2.1.

2.1 Into a reduced volume cuvette, pipette in the following order:

____µL Wine Sample – see above
____µL Model Wine – see above
} Total volume = 500 µL

1.0 mL Washing Buffer (using repeating pipettor).
Vortex and incubate for 10 minutes at room temperature.

2.3 Zero Spectrophotometer with 1.0 mL Washing Buffer at 520nm.

2.4 Read samples (Step 2.1) at 520 nm.
= MEASUREMENT "A"

2.5 To each cuvette add 120 µL Bleaching Reagent (using repeating pipettor). Vortex and incubate for 10 minutes at room temperature.

2.6 Zero Spectrophotometer with 1.0 mL Washing Buffer at 520 nm.

2.7 Read samples (Step 2.5) at 520 nm.
= MEASUREMENT "B"

DISCARD ALL CUVETTES ASSOCIATED WITH THIS ANALYSIS

2.8 Enter values for MEASUREMENT "A" and MEASUREMENT "B" into the Wine Phenolics Worksheet (Wine_Assay.xls).

3. Tannin & Polymeric pigment Measurement "C"

Use the Wine volume and Model Wine volume generated in the Total Iron-Reactive Phenolics worksheet (Wine_Assay.xls) in step 3.1.

3.1 Into a 1.5mL Eppendorf tube, pipette the following:

____µL Wine Sample – see above
____µL Model Wine – see above
} Total volume = 500 µL

1.0 mL Protein Solution (using repeating pipettor)
Incubate for 15 minutes at room temperature with occasional inversion

3.2 Centrifuge at maximum speed for 5 minutes to form a pellet.

Part I

3.3 Into a reduced volume cuvette, pipette the following:

1.0 mL supernatant (from step 3.2) (using 1ml pipette)
80 µL bleaching reagent (using repeating pipettor)
Vortex and incubate for 10 minutes at room temperature.

3.4 Zero Spectrophotometer with 1.0 mL Washing Buffer at 520 nm.

3.5 Read absorbance of samples (step 3.3) at 520 nm
= MEASUREMENT "C".

DISCARD ALL CUVETTES ASSOCIATED WITH THIS ANALYSIS

3.6 Enter values for MEASUREMENT "C" into the Wine Phenolics Worksheet (Wine_Assay.xls).

Part II

3.7 Carefully aspirate remaining supernatant from pellet (step 3.2).

3.8 Add 500 µL Washing Buffer (using repeating pipettor), close the lid and gently invert the tube.

3.9 Centrifuge at maximum speed for 5 minutes.

3.10 Carefully aspirate the supernatant.

3.11 Add 875 µL of Resuspension Buffer to the pellet (step 3.9) (repeating pipettor). Incubate for 20 minutes at room temperature WITHOUT mixing.

3.12 After 20 minutes, vortex sample to resuspend pellet.

3.13 Transfer resuspended pellets to cuvettes (using 1 mL pipette). Incubate for 10 minutes at room temperature.

3.14 Zero Spectrophotometer with 875µL Resuspension Buffer at 510 nm.

3.15 Read samples at 510 nm (Step 3.13).
= BACKGROUND TANNIN

3.16 Add 125 µL Ferric Chloride solution to each cuvette. Vortex and incubate for 10 minutes at room temperature.

3.17 Zero Spectrophotometer with 875 µL Resuspension Buffer + 125 µL of Ferric Chloride solution at 510nm.

3.18 Read absorbance of samples at 510 nm (Step 3.16).
= FINAL TANNIN

DISCARD ALL CUVETTES ASSOCIATED WITH THIS ANALYSIS

3.19 Enter values for BACKGROUND TANNIN and FINAL TANNIN into the Wine Phenolics Worksheet (Wine_Assay.xls).

4. Anthocyanin, measurement "D"

4.1 Into a reduced volume cuvette, pipette in the following order:

400 µL Model Wine (using repeating pipettor).
100 µL wine sample (using 200 µL pipette).
1.0 mL Anthocyanin Buffer (using repeating pipettor).
Vortex and incubate for 5 minutes at room temperature.

4.2 Zero Spectrophotometer with Anthocyanin Buffer at 520 nm.

4.3 Read samples at 520nm (step 4.1).
= MEASUREMENT "D"

DISCARD ALL CUVETTES ASSOCIATED WITH THIS ANALYSIS

4.4 Enter values for MEASUREMENT "D" into the Wine Phenolics Worksheet (Wine_Assay.xls).

SOLUTION RECIPES

Model Wine

In 1.0L Schott bottle dissolve 5.0g potassium bitartrate in 800mL de-ionized (DI) water (magnetic heater/stirrer). Cool to room temperature, add 120mL of 96% Ethanol, stir 5 minutes (without heating), adjust to pH3.3 with hydrochloric acid (HCl), & make volume up 1.0L with distilled water. Store at room temperature.

Washing Buffer

In 1.0L Schott bottle dissolve 9.86g sodium chloride (NaCl) in 500mL DI water, add 12mL glacial acetic acid, & adjust to pH4.9 with sodium hydroxide (NaOH). Make volume to 1.0 L with DI water. Store @ room temp.

Resuspension Buffer

In 1.0L beaker, dissolve 50g SDS in 800mL of DI water, add 50mL triethanolamine, stir gently (magnetic stirrer) to dissolve SDS. When pH stabilises adjust to pH9.4 with HCl. Transfer to 1.0L Schott bottle, rinse beaker with 100mL of DI water & add to bottle. Make volume to 1.0 L with DI water. Store @ room temp.

Anthocyanin Buffer

In 1.0L Schott bottle, dissolve 23g of maleic acid & 9.93g NaCl in 800mL DI water. Adjust to pH1.8 with NaOH & make to 1.0L with DI water. Store @ room temp.

Ferric Chloride Reagent

In 1.0L Schott bottle, dissolve 2.7g ferric chloride in 800mL DI water, add 800<L conc. HCl (12.1 N; 33-37%) & make to 1.0L with DI water. Store @ room temp.

Bleach Solution

In 50mL Falcon tube, dissolve 2.0g of potassium metabisulfite in 25mL DI water, prepare fresh as required. Discard unused solution.

Preparing Protein Stock Solution for storage

In 500mL glass beaker, dissolve 10g of BSA (Bovine Serum Albumin) granules into 250mL of DI water to max. soluble concentration of 40mg/mL. Aliquot 1.0mL of concentrated (40mg/mL) BSA solution into screw cap vials. Store at –80°C.

Preparing Stored Stock Protein Solution for use

Thaw frozen aliquot of protein stock solution (40 mg/mL). Transfer protein stock solution to 50 mL Falcon tube, add 39mL of Washing Buffer & mix well. Final concentration 1 mg/mL → sufficient quantity for 40 assays.

VITA

Eechin Ng

Candidate for the Degree of

Doctor of Philosophy

Thesis: THE EFFECTS OF MICRO-OXYGENATION ON PHENOLIC COMPOUNDS IN CHAMBOURCIN AND RUBY CABERNET WINES

Major Field: Food Science

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy in Food Science at Oklahoma State University, Stillwater, Oklahoma in July, 2013.

Completed the requirements for the Master of Science in Food Science at Oklahoma State University, Stillwater, Oklahoma in 2007.

Completed the requirements for the Bachelor of Science in Chemical Engineering at Oklahoma State University, Stillwater, Oklahoma in 2003.

Experience:

Analytical chemistry
Wine and grape analyses

Professional Memberships:

Institute of Food Technologists (IFT)
Golden Key International Honour Society