ANTIOXIDANT CAPACITY, PHENOLIC AND VOLATILE COMPOUND COMPOSITION OF BLACKBERRY WINES PRODUCED USING KOREAN TRADITIONAL WINEMAKING TECHNIQUES

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Abstract:

This research project was designed to evaluate the winemaking potential of 'Natchez' and 'Triple Crown' blackberries grown in Oklahoma as well as to examine the measurable basic physical properties and potential health-benefits of phenolic compounds in blackberry wines made using traditional Korean winemaking techniques with variations in fermentation temperature (21.6 °C vs. 26.6 °C), and fermentation type (yeast inoculation vs. wild-type fermentation). Korean traditional wine processing methods, which typically employ wild-type microorganisms for fermentation, may provide different types and levels of health-related compounds than common wine production methods. The pH, titratable acidity, soluble solids, and percent alcohol of blackberry juice and wine samples were measured as basic physical properties. The Harbertson-Adams assay, oxygen radical absorbance capacity (ORAC), high performance liquid chromatography (HPLC), and gas chromatography (GC) of blackberry juice and wine samples were investigated as indicators of health benefits of phenolic compounds, primarily antioxidant potential. Total percent fat of whole blackberries and pomace was also analyzed.

Among physical properties, 'Triple Crown' wines made at the higher fermentation temperature were sweeter and had higher alcohol concentrations than 'Natchez' wines. 'Natchez' berries had higher percent total fat than 'Triple Crown' berries. 'Natchez' juices and wines generally had higher concentrations of total phenolics, tannins, and anthocyanins while 'Triple Crown' juices and wines generally had higher concentrations of polymeric pigments, phenolic acids, and free volatile compounds. 'Triple Crown' wines also generally exhibited higher antioxidant activity. Examining the two fermentation temperatures, 26.6 °C increased the initial concentration of phenolics in the wines. However, wines fermented at 21.6 °C showed less loss of phenolics during aging. Between two fermentation types, wild-type fermentation had higher phenolic concentrations than yeast inoculated wines.

Overall, the traditional Korean winemaking technique examined in this study provided good production and retention of phenolic compound and volatile aroma compounds in the wines and that the wines produced were high in antioxidant capacity.

TABLE OF CONTENTS

Chapter Page

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

CHAPTER I

INTRODUCTION

Blackberries are an aggregate fruit made up of several duplets that each contains seeds. Blackberries are mostly consumed fresh but can be processed as frozen, pureed, freeze dried, or juice or concentrate. In the food and pharmaceutical industries, blackberries are used for ice cream, jelly, jam, tea, cake and ink (Kaume and others 2012, Milosevic and others 2012, Nile and others 2014, Veberic and others 2014). Blackberries are receiving greater interest from consumers due to their valuable nutrient content as well as their bioactive compounds, including many classes of phenolic compounds. Phenolic compounds are produced and synthesized during plant development and act as a plant defense system against ultraviolet radiation, infection or wounding by pathogens, parasites and predators (Stalikas 2007, Haminiuk and others 2012). Some of the most important types of phenolic compounds in blackberries are the anthocyanins, which give red, blue, and purple colors to many fruits. The most common types of anthocyanin are cyanidin-based compounds in fruits and procyanidins in seeds (Mertz and others 2007, Lee and others 2012).

Blackberries are also high in antioxidant capacity due to relatively high levels of carotenoids, vitamins, phenolic compounds, flavonoids, dietary glutathione and endogenous metabolites (Thomas and others 2012). Phenolic compounds found in blackberries include ellagic acid, tannins, ellagitannins, gallic acid, anthocyanins, and cyanidins (Huang and others 2012). The health benefits of phenolic compounds found in blackberries have been shown to include anti-carcinogenic, anti-inflammatory, anti-mutagenic, antiviral, antimicrobial, anti-atherosclerotic, anti-proliferative, anti-tumorigenic, and anti-neurodegenerative activities (Turkben and others 2010, Dimic and others 2012, Huang and others 2012, Lee and others 2012, Nile and others 2014).

Fermentation functions in part as an extraction process that increases the phenolic concentration of blackberry wines by extracting these compounds from berry skins and seeds. The beverage fermentation process relies on the ability to yeast to convert sugar to alcohol, esters and other volatile and non-volatile compounds (Duarte and others 2010). Fermentation processes increase the level of antioxidant activity by facilitating the extraction of anthocyanins and other phenolic compounds as well as by forming other new polymerized pigments and other polyphenols (Johnson and others 2013). The fermentation process differs for different types of fruits or vegetables. A byproduct of fermentation, pomace, which is the skins and seed material filtered from the wine, has received attention recently as a potential source of antioxidants and bioactive compounds. Berry seed oil has become gourmet food item known to contain high amounts of essential fatty acids along with omega-3-fatty acids (Dulf and others 2012).

Koreans are very interested in fermented products and have a long tradition of consuming fermented food such as Kimchi, soy paste, and chili paste as everyday food items. Production of these items is still commonly done at the household level. Recently, Koreans have become more interested in healthy lifestyles and the possible uses of functional foods to promote wellbeing.

Many functional foods are now available for purchase in the market as a result. One class of favorite functional foods commonly consumed is fermented beverage made from fresh fruits, vegetables, and mixture of herbs. These beverages are made using a very simple and natural process which involves mixing the fruits or vegetables with sugar and allowing them to ferment for a couple of weeks. Blackberries are one of the popular fruits eaten in Korea and consuming fermented blackberry drink could increase their health benefits.

There have been a number of research studies undertaken to investigate blackberry phenolic compounds and the antioxidant activity of blackberries and blackberry juices and wines. Blackberry phenolic composition has been shown to vary on the basis of variety, growing temperature, growing season, geographic location, maturity at harvest, environmental stress, soil type, UV light exposure, hydrophobicity of compounds, and processing storage conditions (Koka and others 2009, Sariburun and others 2010, Clark and others 2011, Huang and others 2012, Kaume and others 2012, Zhang and others 2012, Souza and others 2014). Different varieties of blackberries clearly may have different chemical compositions. Relatively little research has been done on 'Natchez' and 'Triple Crown' blackberries, cultivars that are suitable for growing in the Midwest section of the United States. The suitability of these blackberries for winemaking and the health benefits of phenolic compounds of wines made from these berries have not previously been studied.

Wine making and wine consumption are becoming more popular as they are known to provide products that are high in antioxidants. Blackberry wines are good sources of antioxidants because they contain relatively high concentrations of anthocyanins and other phenolic compounds. Regular consumption of modest amounts of grape red wine has been correlated with a reduced risk of coronary heart disease and some cancers. Phenolic compounds found in berries should provide

health benefits similar to those linked to the consumption of grape red wines. However, cultivar, genetics, growing location, environmental factors, and different wine-making techniques are all known to play an important role in the phenolic composition of wine (Mitic and others 2013). The Korean traditional wine processing method, which typically employs wild-type microorganisms for fermentation, may provide different types and levels of health related compounds than common grape wine production methods.

This research project was designed to evaluate the winemaking potential of 'Natchez' and 'Triple Crown' blackberries grown in Oklahoma as well as to examine the physical properties and health benefits of phenolic compounds in blackberry wines made using variations on traditional Korean winemaking techniques. This was accomplished by investigating the chemical composition of berries and wines based on measurable basic physical properties such as pH, soluble solid, titratable acidity, alcohol, and total sulfur dioxide concentration. Those basic physical properties are important since different cultivars, fruit growing temperatures and maturity change the bioactive phenolic compositions and levels found in blackberry juice and wines. These basic physical properties influence the stability of anthocyanins found in blackberry juice and wines. Also, the presumptive health benefits of berries and wines were assessed by measuring concentrations of total and specific phenolic compounds including anthocyanins, percentage of extractable lipids, and antioxidant capacity. Perceived physical properties was also estimated by measuring certain specific volatile flavor and aroma compounds. Phenolic compound content of blackberries and wines, especially anthocyanins, is important since it is known to change with different cultivars, temperature, and extraction methods and phenolic compound content is linked to antioxidant activity and thus to presumed health benefits. Some of these presumed health benefits of blackberry juice and wines linked to antioxidant activity include reduced fatigue, enhanced immunity, decreased serum cholesterol, and the prevention of cardiovascular disease and some cancers.

The following specific study objectives were tested:

1. There are significant differences in the physical properties and health benefits of phenolic compounds in blackberry juices and wines made from ‘Natchez’ versus ‘Triple Crown’ blackberries.

2. Higher and lower fermentation temperatures, i.e. 21.6°C and 26.6°C, influence the physical properties and health benefits of phenolic compounds in blackberry juices and wines made from the ‘Natchez’ and ‘Triple Crown’ blackberries.

3. Yeast inoculation fermentation and wild-type fermentations influence the physical properties and health benefits of phenolic compounds in blackberry juices and wines made from the ‘Natchez’ and ‘Triple Crown’ blackberries.

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CHAPTER II

REVIEW OF LITERATURE

History of blackberry cultivation

Blackberries are native across Eurasia and North America. Traditionally blackberries were consumed wild; cultivation first began in the mid to late 1800s. Fresh fruit production began to be more common for local sale in the 1900s. The development of the raspberry/blackberry hybrid 'Logan' in the 1880s served as the basis for the canning industry and freezing technology in the Pacific Northwest. In the late 1950s, the first commercial machine harvester was produced and more people began to consume blackberries as a result of their greater availability.

Blackberries have many similar horticultural characteristics to raspberries, but have lower production costs than raspberries due to their more vigorous nature, greater disease tolerance and therefore longer-lived plantings. The taste of blackberries varies according to growing locations: The southern species tend to be sweeter with a slightly grassy and occasionally quite bitter flavor with crunchy seeds, while western species tend to have an intense, aromatic flavor with good sweetness/acid balance. In recent years blackberries have

become more popular due to a greater recognition of their health benefits, for example their antioxidant content, which is in part a function of the anthocyanins they contain that give the fruit its intense purple color (Finn and others 2011).

Plant cycle of blackberries

Traditional blackberry plants are perennial with biennial canes. They have two different kinds of cane: primocane and floricane. The primocanes are produced the first year and only bear leaves and do not produce flowers. These canes will produce flowers and bear fruit their second year. At this time they are termed floricanes. Once the floricane has produced fruit, it will die after that season. Recently, floricane fruiting varieties of blackberry have been developed. These varieties produce canes that bear fruit their first season and produce new canes every year. (Clark and others 2011).

Types of blackberries

Blackberries have three different types of cane structure. The 'Erect' types of blackberries produce primocanes from buds at the base of the floricanes at the crown of the plant, or from buds that grow from the roots. The 'Semi-erect' and 'Trailing' types of blackberries produce new primocanes from buds on the crown. Most of blackberry cultivars are the semi-erect type (Strik and others 2007).

'Triple Crown' blackberries are a 'semi-erect' type of blackberry that was developed by the USDA-ARS Beltsville program. This berry is thornless, with vigorous, erect canes that grow

from 4 to 6 meters long from a crown and arch to the ground. They also may be trellised (Anderson and others 2011, Clark and others 2011).

'Natchez' blackberries are an 'erect' type of blackberry that was developed at the University of Arkansas, Clarksville, AR. This berry is also thornless and is fully upright in stature. In this variety, many of the new canes, or 'suckers', grow from beneath the soil line. This results in less of a crown being formed and more continuous of row of canes. With this type of blackberry, canes grow upward to a height of near 1 meter before arching to the horizontal orientation (Anderson and others 2011, Clark and others 2011).

Blackberry production

Blackberry production has increased in recent decades due to new cultivars with improved shipping characteristics, improved handling facilities along with enhanced marketing and promotion, greater interest by consumers in new crops plus interest in crops with high antioxidant levels, as well as the recognition that blackberries were more profitable to grow due to longer-lived plantings that some of their *Rubus* relatives such as red raspberry (Clark and others 2011).

Blackberries can be widely adapted to many temperature regions. Blackberries are well adapted to high summer temperatures. However, in regions with intense sunlight and low humidity blackberry fruit is susceptible to sunburn. A key climate factor is winter temperatures, since blackberries cannot adapt to extreme cold temperature. To prevent cold injury, tunnels are used to protect against adverse climate, some insects, diseases, and to facilitate higher yields and better shelf life (Strik and others 2007). The best field conditions in which to grow

blackberries are those which provide well-drained, fertile soils with adequate moisture. Compared to red raspberries, blackberries in general are more heat tolerant, less winter cold tolerant and more tolerant of heavy soils (Clark and others 2011).

Harvesting blackberries

The fruiting season for blackberries depends to some extent on the cane types: 'erect' type blackberries are typically harvested from mid-May to August, the 'semi-erect' type blackberries are usually harvested from July to October, and the 'tailing' type blackberries are generally harvested from late June to August (Strik and others 2007).

Blackberries can be harvested by hand or by machine. For the hand harvesting, blackberries may be picked every second or third day. The ideal time for hand picking blackberries is in the early morning after the dew has evaporated since the temperature is cool and the berries are easy to remove. However, blackberries should never be picked during wet and rainy weather since the moisture from rain facilitates the growth of spoilage microorganisms. All harvested fruits should be stored in the shade to prevent sunburn. Hand picking the berries allows to harvester to evaluate the color of the fruit and ensure uniform ripeness. It is costly and labor intensive, however. Therefore, hand harvesting is typically used only for relatively small planting of blackberries.

Machine harvesting of blackberries allows the grower to pick at night; this facilitates berry removal and helps to maximize fruit maturity, aroma, flavor, and sugar levels. Machine harvesters utilize a shaking motion to selectively harvest fully ripened blackberries. Machine

harvesting is the most efficient method of harvesting large quantities of blackberries (Strik and others 2007).

Blackberry quality attributes

The level and composition of bioactive phenolics in berries vary according to genetics, climate factors, fertilization, and other cultural practices. The genotype has a major role in the level and composition of different compounds such as flavonoids. The climate factors such as seasonal variation, light intensity and fruit growing temperature (day/light) also influence the quality of berries. Cultivation factors such as soil type, the use of composts, mulching and fertilization influence the water and nutrient availability and uptake by the plants and thus affect the nutritional composition and antioxidant activity of berry fruit. Applying high amounts of fertilizer often results in increased vegetative growth and yield, but it may negatively affect some elements of fruit quality. An excessive supply of nitrogen has been shown to decrease quality in some berries and fruits. Excess potassium also induces antagonistic interactions, with other nutrients such as calcium and magnesium in bramble cultivars (Ali and others 2012).

In general, the flavor and other quality attributes of fruits depend on ripeness, maturity, cultivar, irrigation and soil fertility (Thomas and others 2012). From a marketability perspective, the most important blackberry fruit quality parameters are flavor, which is strongly influenced by sugar, acids, volatile component contents, size, appearance, and nutrient content. The antioxidant concentration of blackberries has been observed to differ according to cultivar, growing year, growing location, and ripeness. Sweetness is most important in the fresh market whereas flavor intensity is more important for processed blackberries. High acidity is important for anthocyanin stability. A soluble solid concentration of at least 10% provides for a sweet taste

for fresh berries. The sweetest berries are those that are very mature to the stage of dull black after loss of fruit glossiness. Volatiles are much higher in dull black compared to shiny-black fruits. However, shiny-black is better for shipping than dull-black due to berry firmness. The firmness is influenced by environment, particularly rainfall near the time of harvest. Fruits exposed to rainfall within four days of harvest exhibit greatly reduced postharvest storage potential (Clark and others 2011).

Blackberry aroma and volatiles

Aroma compounds in fruits may be generated from fatty acids, amino acids and/or carbohydrates. Different climates and growing sites can influence flavor development since they can affect the levels of precursor compounds and also the activities of related enzymes. Acid or enzymatic hydrolysis of glycosides release odor-active aglycones, which can be potent odorants in fruits (Meret and others 2011). Glycosidically-bound volatiles can be released during fruit processing and consumption and contribute to the overall quality of fruits (Du and others 2010).

Blackberry flavor is mainly formed during a ripening period and is influenced by internal and external factors. Internal factors are based on plant characteristics such as metabolism and genotype, while external factors are related to fruit growth and cultivation factors such as climate, soil, fertilization and harvest date (Qian and other 2005). In general, the major blackberry volatiles are 2-heptanol, ρ-cymen-8-ol, 2-heptanone, 1-hexanol, α-terpineol, pulegone, 1-octanol, isoborneol, myrtenol, 4-terpineol, carvone, elemicine, furaneol and nonanal. The major aroma compounds of blackberries are ethyl 2-methylbutanoate, 2,5-dimethyl-4-hydroxy-3-furanone, 2-ethyl-4-hydroxy-5-methyl-3-furanone, 4-hydroxy-5-methyl-3-furanone, 4,5-dimethyl-3-hydroxy-2-furanone, dimethyl trisulfide, linalool and methional (Nile

and others 2014). Studies have shown that the most important aroma compounds in 'Marion' blackberries are 2,3-butanedione, 2-heptanol, linalool, dimethyltrisulfide, 1-penten-3-one, methional, ethyl-2-methylbutanoate, benzaldehyde and hexanal. The most important aroma compounds in 'Thornless Evergreen' blackberries are 2,3-butanedione, 2-heptanol, λ-carvone, β-pinene, methional, ethyl-2-methyl-propanoate, thiophene, dimethyl disulfide, and furanone (Wang and others 2005).

The quality of wine has been reported to be highly dependent on the aroma constituents of fresh fruits, and volatile compounds are well known to play an important role in wine quality. It is well known that during wine maturation and ageing, there are many chemical changes in the volatile composition of the wine. This reaction depends on wine composition, pH, storage time and temperature (Wang and others 2012). Wine processing techniques influence the amount of phenolic compounds in wines, as well as their level of antioxidant activity and their aroma (Gao and others 2012). Among the compounds responsible for wine aroma are terpineols, norisoprenoids, alcohols, esters, acids and volatile phenols (Vilanova and others 2009).

Plant phenolics: Types and their functions

Phenolics are well known as antioxidant compounds; their signature characteristic is the presence of one or more aromatic rings with one or more hydroxyl substituents. Polyphenols are known to participate in protection against the harmful actions of reactive oxygen species. Phenolic compounds are hypothesized to scavenge free radicals from cells. Thus, a diet rich in phenolic compounds may mitigate oxidative damage to cells that leads to ageing and age-related diseases.

The concentration of phenolic compounds in berries are not only affected by genetic differences i.e. genus/species, cultivar/genotype and pre-harvest environmental conditions i.e. ripeness, plant age, climate, soil composition, geographic location, storage conditions, seasonal variations but also by the degree of solar radiation, temperature, virus status and other biotic and abiotic stresses (Dai and others 2010, Turkben and others 2010, Haminiuk and others 2012, Lee and others 2012). For example, a study of 'Triple Crown' blackberries showed that total phenolic concentrations significantly decreased as the fruit matured from the green to ripe stages. However, the opposite results were shown in tropical highland blackberries (Kaume and others 2012). Fast freezing followed by frozen storage did not affect the concentration of phenolic acids in blackberry fruit, but slow freezing resulted in a higher extraction of phenolic acid, flavonoids such as anthocyanins, as well as flavonols when compared to other post-harvest treatments. Presumable the slow freezing allowed a more complete extraction of the phenolic compounds (Veberic and others 2014).

Plant phenolics are synthesized during development and constitute major fruit pigments. Also, plant phenolics are produced as a defense against ultraviolet radiation, infection, or wounding by pathogens, parasites and predators (Stalikas 2007, Haminiuk and others 2012). Phenolics contribute to the bitterness and astringency of fruit and fruit juices, because of the interaction between phenolics and glycoproteins in saliva (Haminiuk and others 2012). Plant phenolics encompasses simple phenols, phenolic acids, coumarins, flavonoids, stillbenes, up to hydrolysable and condensed tannins, liganans, and lignins. The water-soluble compounds are phenolic acids, phenyl propanoids, flavonoids, and quinones. The water-insoluble compounds are condensed tannins, liganins and cell-wall bound hydroxycinnamic acids.

Flavonoids that have a C_6-C_3-C_6 basic carbon structure are the most common plant pigments after chlorophyll and carotenoids (Stalikas 2007). In plants, flavonoids act as a defense system against insects, catalysts in the light phase of photosynthesis and/or regulators of ion channels involved on phosphorylation. They also function as stress protectants in plant cells by scavenging reactive oxygen species produced by the photosynthetic electron transport system. In addition, they protect plants from the ultraviolet radiation of the sun and scavenge ultraviolet generated reactive oxygen species (Haminiuk and others 2012).

Flavonoids are widely distributed in fruits and they are recognized as being natural antioxidants (Appendices D). Fruits are a particularly rich source of flavonoids, especially flavonols, flavan-3-ols, anthocyanins, hydroxycinnamic acid, and hydroxybenzoic acid. When the flavonoid group is linked to one or more sugar molecules they are known as flavonoid glycosides and when they are not connected to a sugar molecule they are called aglycones. Flavonoids mostly occur in plants as glycosylated derivatives. Flavonoid glycosides are poorly absorbed until they have undergone hydrolysis by bacterial enzymes in the intestine, whereupon the aglycone form can be readily absorbed (Stalikas 2007).

The second most important group of phenolic phytochemicals are the phenolic acids (Appendices A), which have a carboxylic acid group attached to a phenol ring. They have two subgroups: The first subgroup is hydroxycinnamic acid, which has a C_6-C_3 skeleton and usually presents as derivatives. Examples of this group are ferulic acid, ρ-coumaric acid, and caffeic acid. The second subgroup is hydroxybenzoic acid, which found in various fruits, has a C_6-C_1 skeleton, and mostly occurs as esters. Examples of this group are gallic, vanillic, ellagic and syringic acids.

In plants, phenolic acids are involved in nutrient uptake, protein synthesis, enzyme activity, photosynthesis, forming structural components, and allelopathy (Haminiuk and others

2012). According to Kaume and others (2012), phenolic acids in blackberries are composed mainly of hydroxybenzoic acids (gallic, vanillic, and salicylic acids) and hydroxycinnamic acids (caffeic, p-coumaric and ferulic acids).

Tannins are astringent and bitter substances that are present as phenolic polymers. Tannins have the ability to precipitate proteins. They mainly occur as two types: Gallotannin or tannic acid is a type of hydrolysable tannin found in fruits. Condensed tannins or proanthocyanidins are the major phenolic compounds found in grapes (Haminiuk and others 2012).

Stillbenes are a group of phenylpropanoid-derived compounds with C_6-C_2-C_6 backbone (Appendices B). Resveratrol is an example of this class of compounds, and is mainly produced in grapes in response to injury and fungal infection. Resveratrol is found concentrated in grape skins. It is believed that the continuous and moderate ingestion of this grape-derived compound, especially in red wine, may play a key role in preventing heart disease (Haminiuk and others 2012).

The lignans are the one of the major classes of phytoestrogens and consist of two phenyl propanoid moieties connected via their side chain C_8 carbon (Appendices B). Fruit are not the main dietary source of lignans in food and low concentrations are found in strawberries and cranberries. The highest concentrations are found in flaxseeds (Haminiuk and others 2012).

Important properties of anthocyanins

Anthocyanins are a class of flavonoids that are water-soluble and function as pigments responsible for the orange, red, purple and blue colors of many fruits, vegetables, flowers and

roots (Nile and others 2014, Veberic and others 2014). The aglycone forms of anthocyanins are called anthocyanidins (Appendices C); these are structurally based on the flavilium ion or 2-phenylbenzopyrilium and possess hydroxyl and methoxyl groups in different positions. Anthocyanins was found in fruits are linked to one or more glycoside units. The most common anthocyanidins in higher plants are: delphinidin (12%), cyanidin (50%), petunidin (7%), pelagonidin (12%), peonidin (12%), and malvidin (7%). The most common anthocyanin in most fruit is cyanidin-3-glucoside (Pascual-Teresa and others 2008, Haminiuk and 2012).

Anthocyanin pigments are normally found dissolved uniformly in the vacuolar solution of epidermal cells. Anthocyanins are highly soluble in water and alcohol solution. When in water they are more stable at low pH. At low pH, the predominant form of the anthocyanin is the flavylium cation which is red in color. Thus, lower pH value in the fruit tissues stabilize the anthocyanin structure and lead to a darker appearance of the fruit (Vebric and others 2014). As pH increases, most of the flavylium ions change into other anthocyanin forms: some of them shift to quinonoidal forms i.e. blue in color, and others shift to pseudo bases and chalcones, which are colorless (Pascual-Teresa and others 2008). Refrigerated storage at 2°C has been observed to cause an increase of blackberry fruit pH value, and this could be associated with lower titratable acidity values. Higher pH values were also seen to cause a shift to a more orange color after frozen storage (Vebric and others 2014).

Anthocyanin concentration may vary from fruit to fruit even among the same species, variety, and/or cultivar due to different external and internal factors, such as genetics, and agronomic factors including intensity and type of light, temperature, and other environmental factors (Pascual-Teresa and others 2008, Azofeira and others 2013). In many fruits, anthocyanin synthesis is enhanced by sunlight and the position of the fruit on the plant affects anthocyanin

accumulation due to sunlight intensity. In addition, low temperatures have long been considered to promote and high temperatures to reduce anthocyanin synthesis. Habitats with cool weather were associated with a rapid anthocyanin accumulation in the skin whereas the warm weather was associated with slower anthocyanin accumulation. Nutrient deficiencies, especially phosphorus (P) and nitrogen (N), commonly induce the accumulation of anthocyanin in many plant species.

Anthocyanin synthesis continues after harvest and during long term cold storage, but it is inhibited in fruits stored under high carbon dioxide concentrations. Treatment of harvested fruit with carbon dioxide applied as controlled atmosphere storage (CA) or modified atmosphere packaging (MAP) inhibits the increase of anthocyanin by affecting its biosynthesis (Pascual-Teresa and others 2008). In the absence of elevated levels of carbon dioxide, anthocyanin concentration in blackberries decreased during room temperature storage but increased when berries were stored at low temperatures postharvest (Veberic and others 2014). In another study, anthocyanin concentration was also seen to increase more during refrigerated storage than storage at room temperature (Yuksel and others 2008).

Anthocyanins are a good source of natural antioxidants and provide color but are unstable during processing and storage. In a study by Veberic and others (2014), late-ripening cultivars were characterized by a significant decrease in total anthocyanin concentration. Cyanidin 3-glucoside in particular was most easily degraded during processing and during the storage period. Product storage temperature, pH, light, oxygen, water activity and heat processing are considered to be important factors influencing anthocyanin stability (Zhang and others 2012).

Anthocyanins are also affected by the presence in the medium of sulfur dioxide, ascorbic acid, or metal ions among other food components and additives. Anthocyanin may react to chelate polyvalent metal ion such as Fe, Cu, Al and Sn if they are present in the media or packaging. This is generally considered to be undesirable since it results in a change of pigment color. According to Pascual-Teresa and others (2008), higher pH tend to increase anthocyanin destruction. Even so, anthocyanins are commonly used as food additives in beverages, fruit fillings, snacks, and dairy products (Pascual-Teresa and others 2008).

Anthocyanin degradation is primarily caused by oxidation, cleavage of covalent bonds, or enhanced oxidation reactions due to thermal processing (Zhang and others 2012). During storage, anthocyanins gradually disappear as monomeric compounds and are transformed into polymeric forms. Anthocyanin loss can be easily determined by color analysis (Yuksel and others 2008).

Co-pigmentation is a well-known mechanism that has been suggested as a main color stabilizing mechanism in plants that protects the colored flavylium cation from nucleophilic attack by water molecules. This attack by water converts the flavylium ion to a colorless pseudobase, resulting in color loss. Formation of a complex between the pigment and a co-pigment causes a hyperchromic effect (Δ A) and a bathochromic shift (Δ λ). While the hyperchromic effect causes an increase in color intensity, the bathochromic shift alters the maximum absorbance wavelength. This article was test on co-pigmentation of blackberry juice with olive leaf, green tea, pine bark, red wine and bioflavonoid. The samples with bioflavonoid and red wine had high influence on co-pigmentation. The structure of the anthocyanin aglycones seems to significantly affect the rate and degree of co-pigmentation, probably by influencing the degree of intramolecular co-pigmentation, as well as the hydration efficiency of

the pyranic ring. Other factors that influence the degree of co-pigmentation include pH, ionic strength of solution, temperature, and pigment to co-pigment molar ratio (Kopjar and others 2011).

Polyphenols are determinant factors in several wine properties such as color, astringency, and bitterness (Ortiz and others 2013). The antioxidant potential of wines is likely due to phenolics and anthocyanins (Johnson and others 2012). The color characteristics of wine and color stability during ageing and storage depends on the absolute and relative concentration of anthocyanins in the fruit, the wine production method, and the multiple chemical reactions taking place during fermentation and ageing such as the reactions between anthocyanins and various wine compounds, including other phenolics (Ortiz and others 2013). During red wine ageing, there is a loss of anthocyanins and other pigments are formed through various mechanisms. These newly-formed molecules function to stabilize the red wine color and contribute to the changes in color characteristics seen during red wine ageing. It has been shown that alcoholic extraction at medium to high temperatures may accelerate the formation of new pigments by acylation of the original anthocyanins (Pascual-Teresa and others 2008). These reactions are responsible for the generation of new, more stable pigments related to the natural evolution of the red wine color from red to orange nuances (Arozarena and others 2012, Mitic and others 2013).

Anthocyanins in blackberries

Rubus fruits are considered a healthy and nutritious food that contains valuable nutrients and nutraceutical compounds. Nutrients include vitamin C, B-vitamins, dietary fiber, α-tocopherol, tocotrienol, calcium, potassium, magnesium, carotenoids, linoleic acid and

linolenic acid (Lee and others 2012). Bioactive compounds include phenolics such as ellagic acid and anthocyanins (Hassimotto and others 2008).

It is important to note that the antioxidant properties of fruits may vary considerably. Many factors such as genotype, growing temperature, growing season, maturity at harvest, environmental stress, soli type, light, extraction solvent, the hydrophobicity of compounds affect measured anthocyanin composition in plants (Reyas-Carmona and others 2005, Koka and others 2009, Sariburun and others 2010, Clark and others 2011, Hwang and others 2012). Nevertheless, blackberries are in general a rich source of anthocyanins, mostly cyanidin–based compounds in the non-acylated form (Mertz and others 2007, Lee and others 2012). The predominant anthocyanins in blackberry have been reported to be cyanidin-3-o-glucoside, cyanidin-3-o-arabinoside cyanidin-3-arabinoside, cyanidin 3-xyloside, cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin 3-galactoside, cyanidin-3-malonylglucoside, cyanidin 3-dioxalylglucoside, pelagonidin-3-glucoside, lambertianin C, and sanguiin H-6. (Siriwoharn and others 2004, Sariburun and others 2010, Azofeira and others 2013, Veberic and others 2014). The flavonols, primarily quercetin glycosides, were the major phenolics in blackberry fruit tissue whereas procyanidins such as catechin and epicatechin based and ellagic acid derivatives predominated in the seeds (Siriwoharn and others 2004).

As with other fruits, the measured anthocyanin concentration of blackberry varies due to differences in variety, environmental conditions, cultivation site, maturity, UV light exposure, harvesting method, extraction solvent and processing (Siriwoharn and others 2004, Sariburun and others 2010, Kaume and others 2012, Zhang and others 2012, Souza and others 2014). According to Kaume and others (2012), total anthocyanins increase markedly as the fruit ripens from pink to dark bluish purple stages. The higher extraction of total organic acids was achieved

after frozen storage. Blackberry fruits maintained their characteristic sour-sweet taste even after long-term storage regardless of the initial freezing method (Veberic and others 2014). Anthocyanins in frozen fruit become more easily extractable due to degradation of cell structures in berries. Storage of frozen fruits for 6 months slowed down anthocyanin degradation but after 10 months there was an accelerated degradation of anthocyanins. At a storage temperature of -18°C, the storage life of frozen fruits packed in polyethylene bags or plastic boxes for up to 6 months was seen as optimal for obtaining products with superior antioxidant properties and minimal loss of color (Poiana and others 2010, Veberic and others 2014).

Blackberry wine has been shown to be a rich source of anthocyanins and other phenolics, with an even higher in-vitro antioxidant activity than grape wines (Mudnic and others 2012).

Anti-oxidant activity of polyphenolic compounds

Antioxidants are substances that are able to delay the oxidation process by inhibiting the polymerization chain reaction initiated by free radicals and by preventing other substituent oxidizing reactions (Dai and others 2010, Haminiuk and others 2012). The antioxidant properties of phenolic compounds can be mediated by their ability to scavenge radical species such as ROS/RNS, to suppress ROS/RNS formation by inhibiting certain enzymes, by chelating trace minerals involved in free radical production, and by up-regulating or protecting cellular antioxidant defenses. Phenolic compounds possess an ideal chemical structure for free radical scavenging activities because they have phenolic hydroxyl groups that are prone to donate a

hydrogen atom or an electron to a free radical as well as an extended conjugated aromatic system to delocalize an unpaired electron (Dai and others 2010).

The antioxidant activity of a phenolic compound is determined by its basic structural orientation, which influences how easily a hydrogen atom from a hydroxyl group can be donated to a free radical, and the compound's ability to support an unpaired electron. The position of hydroxyl groups seems to play a greater role in influenced a phenolic compound's antioxidant capacity than the number of such groups, i.e. hydroxyl groups in the ortho position of the B ring can greatly enhance the antioxidant capacity of compounds such as catechins. Phenolic acids also have greater or lesser antioxidant capacity depending on the number of free hydroxyl group in the molecule. For example, the antioxidant capacity of the following phenolic acids is seen to decrease in relation to the number of free hydroxyl goups such that the antioxidant capacity of protocatechuic acid> caffeic acid> ρ-hydroxybenzoic acid> ferulic acid >vanilic acid >ρ-coumaric acid (Dai and others 2010, Hwang and others 2012). Dietary phenolics have been observed to be multifunctional antioxidants that can act as reducing agents, hydrogen-donating antioxidants, and/or single oxygen quenchers (Dai and others 2009).

Measuring anti-oxidant activity

There are two major mechanisms hypothesized to explain antioxidant activity: the hydrogen-atom transfer (HAT) method and electron transfer (ET) method. Assays based on the ET method measure the capacity of an antioxidant to reduce an oxidant probe, which changes color when reduced. Examples of ET-based assays include the Trolox Equivalent Antioxidant Capacity (TEAC) assay, the Ferric Reducing Ability of Plasma (FRAP) assay, and the DPPH Radical Scavenging Activity assay. The TEAC method can measure both hydrophilic and hydrophobic

antioxidant capacity of plant extracts (Huang and others 2005, Dai and others 2010, Haminiuk and others 2012).

Assays based on the HAT method measure the capacity of an antioxidant to quench free radicals by hydrogen atom donation. Examples of HAT-based assays include the Oxygen Radical Absorbance Capacity (ORAC) assay, the Total Radical-Trapping Antioxidant Parameter (TRAP) assay, the ABTS Radical Scavenging assay, and the Lipid Peroxidation Inhibition Capacity assay. The ORAC assay was used in this study.

The ORAC assay employs a fluorescent probe to compete with a sample antioxidant for peroxyl radicals generated by AAPH. The very complicated reaction among free radicals, substrates and antioxidants makes it impossible to use a fixed equation to express the kinetic order. The accurate measurement of antioxidant capacity requires both inhibition degree and inhibition time to be taken into account. The oxygen radical absorbance capacity (ORAC) is the only method so far to measure both inhibition time and inhibition degree (Huang and others 2002).

Health benefits of polyphenols

Phenolic compounds such as those found in berries have been shown to have potentially beneficial effects on health, primarily as a function of their antioxidant activity. These benefits include, to greater or less extents, anti-cancer, anti-inflammatory, anti-mutagenic, antiviral, antimicrobial, anti-atherosclerotic, anti-proliferative, anti-tumor, and anti-neurodegenerative properties (Reyas-Carmona and others 2005, Stalikas 2007, Turkben and others 2010, Dimic and others 2012, Hwang and others 2012, Lee and others 2012, Nile and others 2014). Adverse environmental conditions, such as smog and ultraviolet radiation in

addition to diets rich in saturated fatty acids and carbohydrates, are believed to increase oxidative damage in the body (Cespedes and others 2007). One of the mechanisms that appears to be important for the development of many degenerative diseases is oxidative stress, which is induced by free radical attack on cells by active oxygen species. Antioxidants, which prevent oxidative stress, are considered to be important in reducing the initiation and progression of degenerative diseases (Connor and others 2005). Antioxidants are presumed to prevent or delay the not only the onset of degenerative illnesses such as certain cancers, cardiovascular and neurodegenerative diseases, but also conditions related to oxidative stress dysfunction, such as cataracts and other age-related deterioration (Thomas and others 2012, Nile and others 2014).

Anthocyanins are not only colorful pigments but they are also bioactive compounds with functional benefits such as protection against liver injury and DNA damage (Ogawa and others 2008, Gancel and others 2011). Anthocyanins have been shown to significantly reduce blood pressure and contribute to a smoother blood flow, which leads to a lower risk of high blood pressure and helps to maintain heart health (Gancel and others 2011, Lute and others 2012). Anthocyanins have also been applied in the prevention and treatment of glaucoma and other eyesight disorders (Denev and others 2010). In addition, anthocyanins have demonstrated the potential to serve as radiation protective agents, vasotonic agents, and chemo-protective agents as well as exhibiting anti-proliferative effects (Koka and others 2009, Gancel and others 2011). In model biological systems, anthocyanin extract has been shown to prevent cardiovascular disease and some cancers due to inhibition of low density lipoprotein oxidation (Denev and others 2010).Other potential benefits of anthocyanins that have been researched include sustaining memory function, strengthening the immune system, enhancing responses against pollutants, preventing several skin diseases, and functioning as anti-obesity and anti-diabetes

agents (Ogawa and others 2008, Ali and others 2012, Lute and others 2012, Nile and others 2014).

Wines contain many different phenolic compounds; as a result the antioxidant and other in-vivo activities of wines are a function of the mix and synergistic activities of these compounds. Recent studies indicate that the consumption of small amounts of red wine on a regular basis reduces the risk of coronary heart diseases and atherosclerosis. This benefit is ascribed to the antioxidant properties of the polyphenolic compounds in the wine. The genetic, agronomic or environmental factors and different wine-making techniques play an important role in the phenolic composition of wines. Commercial blackberry wine samples containing a high total phenolic concentration have been observed to have higher antioxidant activity than that of raspberry and sour cherry wines (Mitic and others 2013). Phenolic compounds found in berries have many of the same potential health benefits as grape wine compounds with respect to inhibiting cardiovascular disease and improving endothelial function and heart health (Johnson and others 2011).

Antioxidant activity and other health benefits of blackberries

Since ancient times, blackberries have been recognized for their uses in folk or herbal medicine. For example, blackberries were traditionally used as an anti-diarrheic and during pregnancy to shorten labor and to make it easier (Ali and others 2012). Modern studies have indeed shown that tea made from blackberry leaves can be effective for reducing the risk of dysentery and diarrhea (Agaoglu and other 2006). We better understand now how these beneficial health properties are related to blackberry chemical composition. Blackberries possess a relatively high anthocyanin concentration compared to many other fruits. These

compounds have potent antioxidant activity and have been shown, among other things, to reduce capillary blood vessel fragility and increase capillary blood vessel permeability. Also, anthocyanins have been shown to take part in collagen reticulation and to inhibit the enzyme degradation of collagen during inflammation (Denev and others 2010).

Blackberries are known to be an excellent source of antioxidants including carotenoids, vitamins, phenolic compounds, flavonoids, dietary glutathione and endogenous metabolites (Thomas and others 2012). Blackberry extracts have been shown to have various bioactivities including protecting against endothelial dysfunction and vascular failure in vitro. They have been demonstrated to attenuate the injury caused by lipo-polysaccharide (LPS)-induced endotoxic shock in rats and to exhibit cytotoxic effects on human oral, prostate, lung cancer cells.

The measured phenolic compound concentration and antioxidant activity of blackberries have been observed to vary between cultivars, and different extraction methods (Dai and others 2009). Even so, blackberries have been shown to have antioxidant activity as radical scavengers; this suggests they may have possible health benefits that include reducing the risk of cancer, cardiovascular disease and other pathologies (Mertz and others 2007). Blackberries also rank high among fruits for antioxidant activity due to their high phenolic compound concentration. These compounds include ellagic acid, tannins, ellagitannins, quecetin, gallic acid, anthocyanins and cyanidins (Hwang and others 2012). According to Kaume and others (2012), based on ORAC assay analyses, the antioxidant activity of blackberries is the third highest among commonly-consumed berries, trailing only strawberries and black raspberries. This high antioxidant activity can be attributed to the high concentrations of acylated anthocyanins and cyanidin-3-glucoside found in blackberries. Studies have shown that

cyanidin-3-glucoside is ranked high in ORAC activity and is reported to be 3.5 times stronger than Trolox whereas pelagonidin was reported to have antioxidant activity equivalent to that of Trolox (Kaume and others 2012).

Berries are well known as 'super fruits' in the nutraceutical and functional food markets for their potential health benefits. Berry consumption has been associated with decreased LDL oxidation, lipid peroxidation, serum glucose, and total cholesterol levels and an increase in HDL cholesterol (Azofeira and others 2013). It has been reported that blackberries reduce fatigue, enhance immunity, decrease cholesterol, and prevent cardiovascular disease as well as cancers (Wang and others 2012). Blackberries are also notable for their more conventional health benefits based on a high concentrations of nutrients such as dietary fiber, vitamin C, vitamin K, folic acid and manganese.

Effect of blackberry processing on potential health benefits

Blackberry is an aggregate fruit made up of several duplets each containing a seed. Blackberries are mostly consumed fresh but can be processed and sold as individually quick frozen pack, bulk frozen, as seedless or seeded puree, freeze-dried, or as juice or juice concentrate. In the food and pharmaceutical industries, blackberries are used for the production of dietary supplements, ice cream, jam, jelly, marmalade, wine, tea, cake, ink, dyes, fruit leather and medicine (Agaoglu and others 2006, Kaume and other 2012, Milosevic and others 2012, Nile and others 2014, Veberic and others 2014). Previous studies on blackberries indicated that genetic and environmental factors such as cultivar, maturity, ultraviolet light exposure, soil, cultural practices and harvesting methods play an important role in berry composition

(Milosevic and others 2012). Color is important issue for the food industry but the color of foods can be altered through the action of light, temperature, oxygen, presence of ascorbic acid, sugars, sulphite salts, metal ions, co-pigments and endogenous enzymes during processing and storage (Kopjar and others 2012).

Fruit processing affects polyphenol concentrations and alters fruit microstructure, resulting in the loss or enrichment of some polyphenols and influencing their bioavailability. Industrial fruit juices usually present low flavonoid concentrations, because flavonoids are frequently removed during clarification or stabilization processes. For example, tannins from grape red wines are mainly extracted during wine making by pressing the fruit and during fermentation. The fermentation process in winemaking can result in the transformation of polyphenols from grapes or the formation of new structures and the presence of ethanol in wine may contribute to their bioavailability. Approximately, 48 % of dietary polyphenols are bioaccessible in the small intestine and 42 % become bioaccessible in the large intestine (Haminiuk and others 2012).

Potential health benefits of berry seed oil

Beverage and juice industry wastes, mainly consisting of seed and peels, have attracted considerable attention as potential sources of natural antioxidants and other bioactive compounds such as fatty acids and sterols. Berry seed oils contained significant amounts of essential fatty acids with a good balance between ω-6 and ω-3 fatty acids. Diets rich in ω-3 fatty acids can prevent coronary heart disease and certain forms of cancer (Dulf and others 2012).

The source of berry seed oils are red raspberry, black raspberry, boysenberry, Marion blackberry, Evergreen blackberry, blueberry, strawberry and cranberry. Seeds constitute 9-12% of berry weight on a wet basis for different types of Rubus, and seeds typically are 10-23% oil on a dry basis. All berry seed oils have a high concentration of polyunsaturated fatty acids in common, which provide essential fatty acids in the diet. The major fatty acids found in the oils are linoleic (41-70%), linolenic (13-36%), and oleic (11-19%), all of which have excellent anti-inflammatory activity. These oils are also rich in antioxidants that may protect against cardiovascular lipid oxidation and may also provide antitumor activities. For example, Marion berry, Boysenberry, red raspberry and blueberry seed oils and been shown to contain substantial amounts of α-linoleic acid (19.6-34.2g/100g oil), tocopherol (260-2277 μmol/kg oil), polyphenols (0.09-2 mg GAE/g oil) and carotenoids (12.5-30 μmol/kg oil) (Van Hoed and others 2009).

High oil content in the berry seeds, the unique fatty acid composition of the oils, high tocol contents, and excellent oxidative stability and anti-inflammatory activity indicate that berries are an excellent source of specialty oils (Oh and others 2007). Blackberry seed oil in particular has been observed to have a dark brown or greenish color with an orange hued layer. The total oil present in dry blackberry pomace has been measured at about 14%, with about 3.48% of the oil consisting of oleic acid (Dimic and others 2012).

Effect of fruit fermentation on potential fruit health benefits

The beverage fermentation process relies mainly on the ability of yeasts to convert sugars into alcohol, esters and other volatile and non-volatile compounds. Due to the differences in fruit composition, yeast strains used for fermentation have to adapt to different

environments e.g. sugar composition and the concentration of various organic acids, etc. The use of selected yeast strains can significantly affect a wine's composition and sensory profile and can consequently affect the wine's quality (Duarte and others 2010).

Fermentation is known to be able to increase the phenolic concentration of berry juice products by increasing phenolic compound extraction from the skin. These compounds include anthocyanins, and therefore fermentation may increase the antioxidant capacity of a beverage and thus its potential health benefits. Fermentation causes an absolute and relative change in anthocyanin concentration, allowing for new pigments to be generated or formed during the fermentation process or during wine maturation (Johnson and others 2013). Fruit wines may offer health benefits unique and distinct from those offered by grape wines because of different components present in the fruit initially and different components formed during the fermentation process. For example, fermentation may facilitate the conversion monomeric phenolics to polymeric forms such as polymeric proanthocyanidins. This may boost antioxidant capacity in the fermented product, even though total phenolics and total anthocyanin concentration may have been higher in the original juice. In addition, it is not clear whether the monomeric forms of these compounds have the same absorption and bioavailability as the compounds in the wines. For these reasons, fermented fruit products may offer health-beneficial attributes not present in the starting fruit juices (Johnson and others 2011).

Korean fermented drink

As with most cultures, Koreans are very interested in their health and prefer a life style that promotes wellbeing. As a result, many different kinds of health products and foods are popular. Blackberries are one such popular berry fruit in Korea as Koreans believe that

consumption of blackberries can improves eye sight, ease urination, enhance sexual function, and prevent ageing (Oh and others 2007).

In Korea, blackberries are mostly eaten raw as fresh fruits or consumed as juice, but Koreans also enjoy consuming blackberries as a fermented beverage. Since Korea has many fermented products dating from ancient times such as Kimchi, soy sauce, and chili paste, it is common to apply basic fermentation techniques to almost all kinds of foods. Nowadays, fermented drinks are very popular in Korea, so many fermented drinks from fruits, vegetables and mixed herbs are made. The traditional process is fairly simple; it consists of adding brown sugar to the fruits and/or vegetables and then allowing them to ferment for about two weeks or so. Many people consume such drinks to maintain their health, but many of these supposed health benefits have not yet been scientifically proven (Park 2009).

Korean earthenware jars and their breathability

The Korean earthenware jar (‘onggi’) has been a popular container from ancient times that is used to make fermented foods and also for the long-term storage of fruits, vegetables and grains. Korean earthenware jars are similar to German fermenting crocks that are used in biomass plant methanol production. Many Koreans still use these earthenware jars at home to make Kimchi, soy sauce, soybean paste, chili paste, and pickles.

The ‘onggi’ is made of a mixture of clay and sand. The surface of the jar is glazed with a natural paint made of a mixture of dried leaves, grasses and their ashes. The earthenware jars are baked in a kiln at 1200°C; these high temperatures form leucites. Leucites have many small holes of about 1-20μm in diameter, and this makes the ‘onggi’ more permeable or “breathable”

than glass or metal. Air can move through holes of about 0.0022µm in diameter and thus can pass through the walls of the earthenware jar without great difficulty. However, due primarily to electrostatic forces, water molecules do not pass easily through the walls of earthenware jar. Additional evidence that 'onggi' are "breathable" is produced when these jars are used to make fermented products such as plum extract, soybean paste, and chili paste with high salt or sugar contents. The 'onggies' that contained those products have been shown to possess thin layers of sugars or salts on the outer surface of the jars after fermentation due to osmosis. The pores in the earthenware jars were big enough to transfer salt molecules (0.00056µm) and/or sugar molecules (0.00096µm) and this demonstrated that the 'onggies' are not only "breathable" but also influence the osmotic balance in fermented products (Onggi Folk Museum 2007, Kim 2009).

Many believe that the 'onggi' jar is the best container to make fermented products. However, not many people have studied how these containers might influence fermentation. According to Sim (2009), four different types of containers i.e. plastic, stainless still, glass and 'onggi' were tested for breathability. Testing involved placing cut apples in each container type at room temperature in order to observe the rate and degree of browning, which is an oxygen-mediated reaction. Results showed that the 'onggi' jar had the fastest color change after 1.5 hours followed by plastic container (2 hours), stainless steel (1 day) and glass (>3days). This demonstrated that the 'onggi' jars were more oxygen permeable than other materials commonly used for fermentation vessels.

Some studies have shown that fermented food products were judged to have better taste and quality made using 'onggi' jars than when made using other types of fermentation vessels. For example, kimchi made using earthenware jars had higher numbers of lactic acid bacteria than kimchi made using other containers. The lactic acid produced by these bacteria

helps to prevent the growth of other microorganisms and contributes to a desirable flavor and texture. Research was conducted to observe the extent to which different containers affected the levels of lactic acid bacteria during fermentation at 15°C. The kimchi produced in the earthenware jars had a higher concentration of lactic acid bacteria on the sixth day of fermentation (1.42×10^{10} CFU/ml) compared to kimchi produced in plastic vessels (4.93×10^{9} CFU/ml). The following species were the dominant microorganisms found in the kimchi: Bacillus subtulis, B. licheniformis, B. safensis, Lactobacillus brevis and B. pumilus. All have been associated with the production of high quality kimchi (Han and others 2013).

Differences between fermentation containers have also been shown to affect the taste and quality of soybean paste. In one study, soybean paste samples made with earthenware jars were determined to be less acidic, less salty and to have more aroma and better taste than samples made using stainless steel, glass, and plastic containers. Also, the soybean paste made using earthenware jars was found to contain fewer spoilage bacteria but greater numbers of yeast and other fermentative microorganisms. Furthermore, earthenware jars had the advantage of maintaining a desired temperature better than the other containers tested, which helped support the growth of microorganisms related to fermentation (Jung 2012).

In addition, different types of fermentation vessels have been shown to affect the volatile organic compounds found in traditionally-fermented soy sauce. In one study, the concentration of acetic acid was found to be higher in samples made using earthenware jars than in samples made using glass containers. The authors concluded that because acetic acid was produced in these samples by the aerobic bacterium *Bacillus subtilus*, therefore the earthenware jars better facilitated oxygen transfer into the product during fermentation than the glass vessels (Park 1996).

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CHAPTER III

MATERIALS AND METHODS

Blackberry collection and storage

Two blackberry cultivars, 'Natchez' and 'Triple Crown', were collected from the Cimarron Valley Research station (Perkins, OK). Blackberries were collected after they turned fully purplish black. Blackberries were collected over a period of two years, 2011 and 2012. All blackberries were hand harvested starting from the third week of May and ending about the third week of July. During the harvest period, the berries were collected every Monday, Wednesday, and Friday morning. Blackberries were placed into polyethylene bags and placed into the freezer (-15°C) within one hour of harvest for storage and subsequent experimental use. All blackberry samples remained stored at -15 °C after collection and until further processing or analysis.

Figure 1. Overview of blackberry field at Cimarron Valley Research Station in Perkins, OK (A) and harvesting 'Triple Crown' blackberries (B).

Physical properties for whole blackberry, juice and wine

General physical properties of whole blackberries

The frozen blackberries were stored in the refrigerator for 2 days and then put them in a room temperature for 3 hours. Ten thawed blackberries were randomly selected from each cultivar. Berries were weighted an analytical balance (Denver Instrumental Company A-160, Bohemia, NY) and length was measured using a ruler. Weights and lengths were averaged to obtain mean values.

Preparation of juice samples

Frozen whole blackberries were placed in a refrigerator at about 4° C for 2 days until thawed, and then held at room temperature until they came to temperature equilibrium in

about 4-6 hours. Fresh juice samples were collected by manually pressing 100-150 blackberries against a mesh screen, which helped to separate skins and seeds. Inside the mesh, take 20-30 berries at a time and then applied enough pressure to squeeze and break down berries. Once done collecting the juice, the skins and seed inside the mesh was removed and then repeat this until we collect enough juice. The 100mL of juice samples were collected into 120 mL brown amber bottles for future analysis.

pH

The pH of the blackberry juice was measured using an Accumet AB 15 pH meter (Buffalo, NY).

Soluble solids

Blackberry juice sugar concentration was estimated as percent soluble solids using a Leica Auto ABBE refractometer (Buffalo, NY).

Titratable acidity

The titratable acidity of blackberry juice and wine samples were measured manually. Briefly, 5 mL of blackberry juice or wine was mixed with 125 mL of deionized water in a 250 mL beaker. The sample was stirred using a magnetic stir bar and the initial pH was measured using an Accumet AB 15 pH meter (Buffalo, NY) The sample was titrated with 0.1 N sodium hydroxide

(Arcos Organics, Fair Lawn, NJ) until it reached pH 8.2 and the volume of titrant used was recorded. The titratable acidity was then calculated as % malic acid using the following formula:

% malic acid = [(mL NaOH x N NaOH x mili-equivalent wt. of malic acid)/ mL sample added] x 100 = [(mL NaOH x 0.1 x 0.067)/5] x 100

Two duplicate readings were taken from each blackberry juice and wine sample and averaged.

Percent alcohol

The percent alcohol (w/w) of wine samples was measured using an Alcoholizer Wine M (Anton Paar, Ashland, VA). Samples of the aged wines were collected into 60 mL brown glass bottles. Approximately 30mL of wine per sample were used in the analysis. Two duplicate readings were taken from each wine sample and averaged.

Sulfur dioxide test

Blackberry wine sulfur dioxide concentration was measured by the aeration/oxidation method from Enartis Vinqury using custom aeration/ oxidation glassware (Adams and Chittenden Scientific Glass, Berkeley, CA). This method employed an impingement flask to quantify sulfur dioxide by monitoring a color change of the solution in the flask as describe below. The method relied on transferring gaseous sulfur dioxide from an acidified wine sample to the indicator solution in the impingement flask. As the sulfur dioxide dissolved in the indicator solution, it changed the color of the solution from greyish-green to purple (Figure 2-C and D). Sulfur dioxide concentration was calculated from the amount of sodium hydroxide used to titrate the solution back to the original gray-green color.

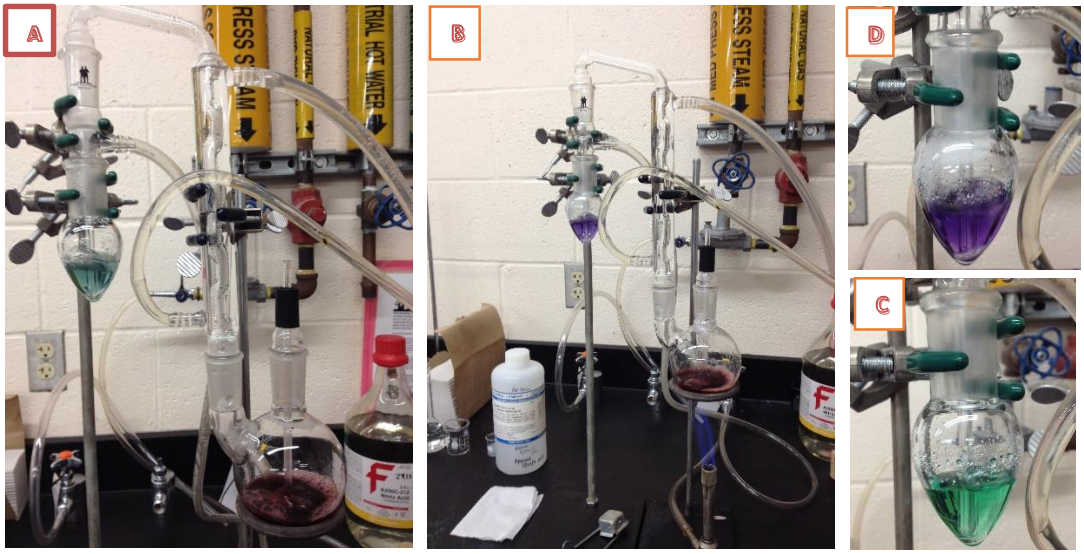

Figure 2. Sulfur dioxide test apparatus with free (A) and bound (B) form. The starting indicator solution with grey-green color (C) and end point of indicator solution with purple color (D).

Briefly, the impingement flask contained about 10 ml of a 0.3 % solution of hydrogen peroxide (Fisher Scientific, Pittsburg, PA) to which had been added 3 drops of an indicator solution- consisting of a pre-made mixture of 0.1% methyl red and 0.05% methylene blue (Presque Isle Wine Cellars, North East, PA). A round-bottomed flask containing the blackberry wine sample was connected to the impingement flask by a condenser tube. The round-bottomed flask contained 20 mL of blackberry wine and 10mL of 25% phosphoric acid (Ricca Chemical Company, Arlington, TX). Air was pulled through the acidified wine sample and bubbled through the impingement flask by the use of vacuum. Free sulfur dioxide (Figure 2-A) was measured by pulling air through the acidified wine sample and impingement flask for 10 minutes, after which the impingement flask was removed and titrated with 0.01 N sodium hydroxide (Arcos Organic, Fair Lawn, NJ). A fresh impingement flask containing 0.3 % hydrogen peroxide solution and indicator solution was then prepared. Bound sulfur dioxide (Figure 2-B) was measured by heating the acidified wine sample around 100°C and pulling air through the wine sample and impingement flask for 15 minutes, after which the impingement flask was

removed and titrated with 0.01N sodium hydroxide. The following formulas were used to calculate final sulfur dioxide concentrations:

Free and Bound sulfur dioxide (ppm) = N NaOH x mL NaOH x 1600 = mL NaOH x 16

Total sulfur dioxide (ppm) = free dioxide (ppm) + bound dioxide (ppm)

All wine samples were measured in duplicate.

Korean traditional blackberry wine-making process

Sample preparation

Frozen blackberry samples harvested in 2012 were placed into a refrigerator at 4°C to thaw for 3-4 days for subsequent processing and analysis. Thawed berries were then equilibrated at room temperature for 3 hours. Figure 3 shows an overview of the blackberry sample preparation for fermented juice and wine-making.

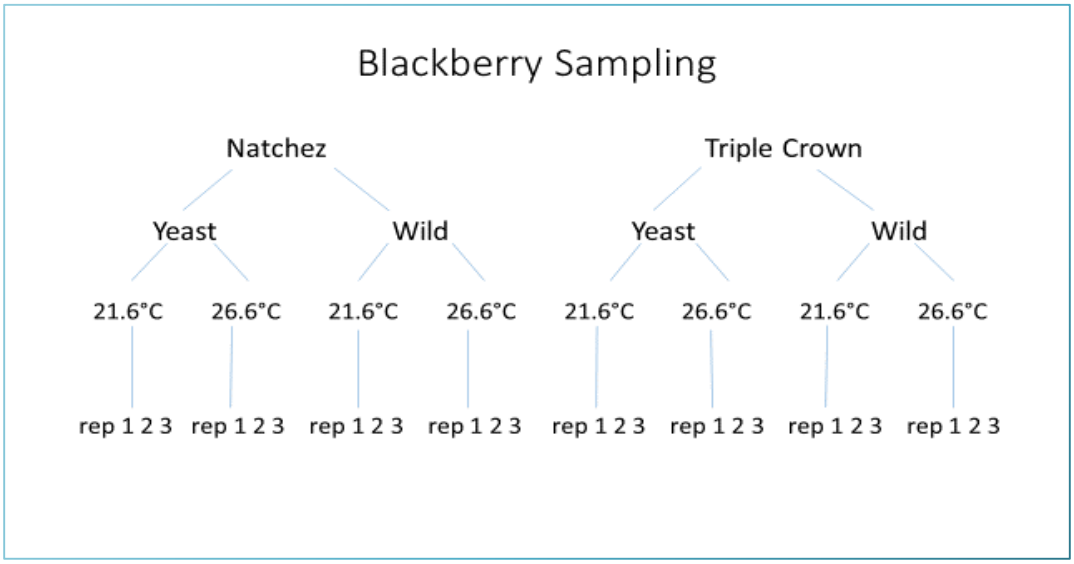

Figure 3. Overview of blackberry sample preparation.

Wine-processing

A modified combination of Korean traditional winemaking techniques taken from Park (2009, 2010) and Yun (2007) was used in this research. The figure 4 showed overview of winemaking process which demonstrated that the comparison between western standard winemaking process and Korean traditional winemaking process. Before starting the Korean traditional winemaking process, a 500mL sample of fresh blackberry juice was measured into a beaker and % soluble solids and pH were determined as described previously. For the best results using traditional Korean winemaking techniques, the authorities cited above recommend that the starting sugar levels in the wine be in the range of 22-24 % soluble solids in order to make a finished wine containing 12-13% alcohol (Yun 2007, Park 2009).

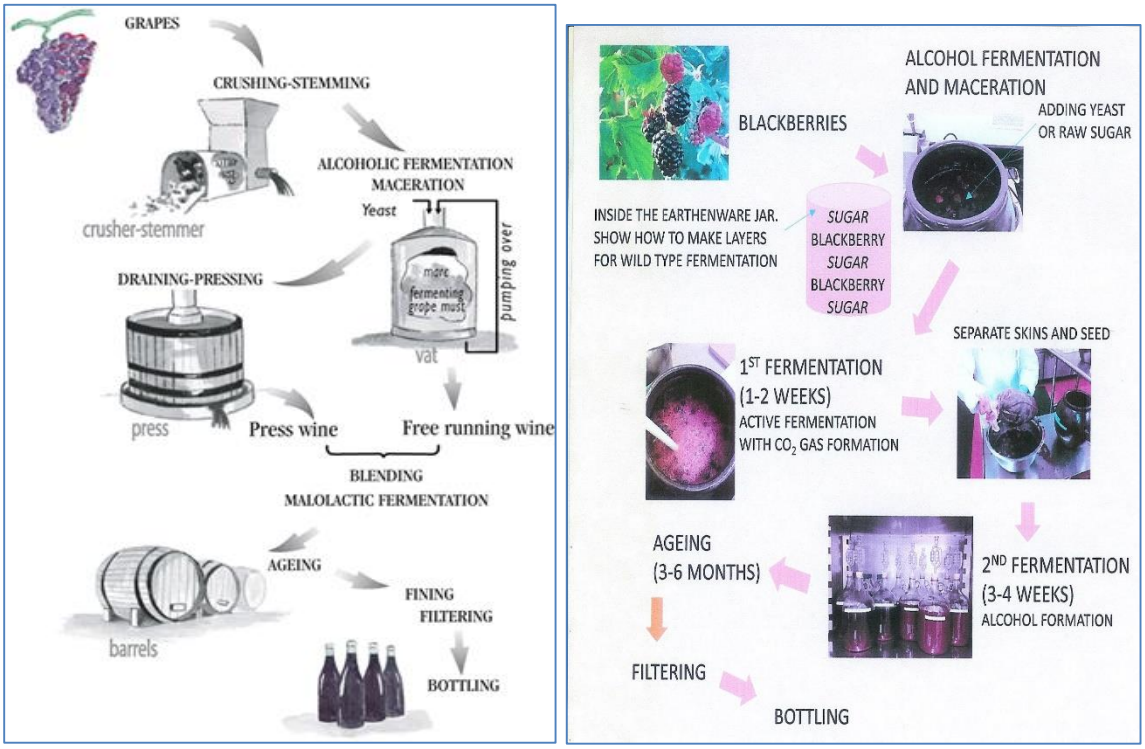

Figure 4. Overview of winemaking process: The western standard red winemaking process (left) and Korean traditional winemaking process (right).

For this project, sugar levels were adjusted to 25 % soluble solids with addition of raw sugar (Cumberland Packing Co, Brooklyn, NY). The using raw sugar instead of white sugar since raw sugar have relatively more micronutrients than white sugar which could help produce more microorganisms in wild-type fermentation. Prewashed 12L Korean traditional earthenware jars (Sin-Il Earthenware, Korea) were used as fermentation vessels. The earthenware jars were purchased from a local Asian market (H mart, Dallas, TX). The figure 5 showed the basic ingredients for Korean winemaking process.

Figure 5. Preparation of Korean traditional blackberry winemaking with whole blackberries (A); raw sugars and yeast (B); Korean earthenware jar (C).

In each Korean earthenware jar, approximately 4.5kg of blackberries were used and about 20 % raw brown sugar by blackberry weight was added. Thin layers of blackberries and sugar were laid down in the jar until the jar was about 2/3 full by volume. Figure 6 illustrates how the layers were made inside the Korean traditional jars.

Figure 6. Korean traditional blackberry winemaking technique. How the layers were made inside the Korean earthenware jar (A); actual pictures of adding sugar (B); the final product (C).

After filling the blackberries and sugar into the Korean earthenware jars, the jars were covered with breathable paper secured around the neck of the jar with a string. The treatment factors applied were two cultivars, two fermentation temperatures (26.6°C and 21.6°C) and two fermentation microflora (no added yeast, and added yeast). The best temperature for starting fermentation was 25°C (Yun 2007). According to Park (2009), a fermentation temperature of 20°C should take longer, be less active, and produce off flavors. If the temperature was 28°C or higher, then the fermentation time would be shorter but there is an increased chance of growing spoilage bacteria. In order to observe the effects of fermentation temperature on the wine, we choose two temperatures; one at the lower end of the recommended range and one at the higher end.

The yeast added was Enoferm L2226 (Scott Laboratories Inc., Petaluma, CA). Because Korean traditional winemaking uses natural microorganisms rather than added yeast, we choose to compare a wild-type fermentation to yeast inoculation. Three Korean earthenware jars were used for each treatment combination; each jar was considered an experimental unit for purposes of statistical analyses. Thus, each treatment combination was replicated three times. An environmental chamber (Ultimate Hot Pack Inc, Lander, WY) was used for temperature control.

Fermentation

A two-part fermentation process was used for all samples. The first fermentation took 1-2 weeks. During the first fermentation period (Figure 7), samples were mixed with a spatula every morning and evening to help insure sufficient aeration. The Korean earthenware jar facilitated this process since the container is breathable and allows relatively more oxygen

transfer to the samples than plastic or glass jars. According to Korean literature, the earthenware jars has many small size of pores (1-20 µm) which makes the air and gas inside the jar but keep the rain outside. While active fermentations were under way, the blackberries stated to change color from purple to pink and the individual berries lost their structural integrity as well. The end of the first fermentation stage was determined via a two-part test. First the rate of fermentation was observed by visually evaluating the rate of CO_2 release. When the rate had slowed, then the soluble solids concentration was tested daily using a handheld refractometer (Atago USA Inc., Bellevue, WA). When the soluble solids concentration dropped below 10 degrees brix, then the sample was determined to be ready for the second stage of fermentation.

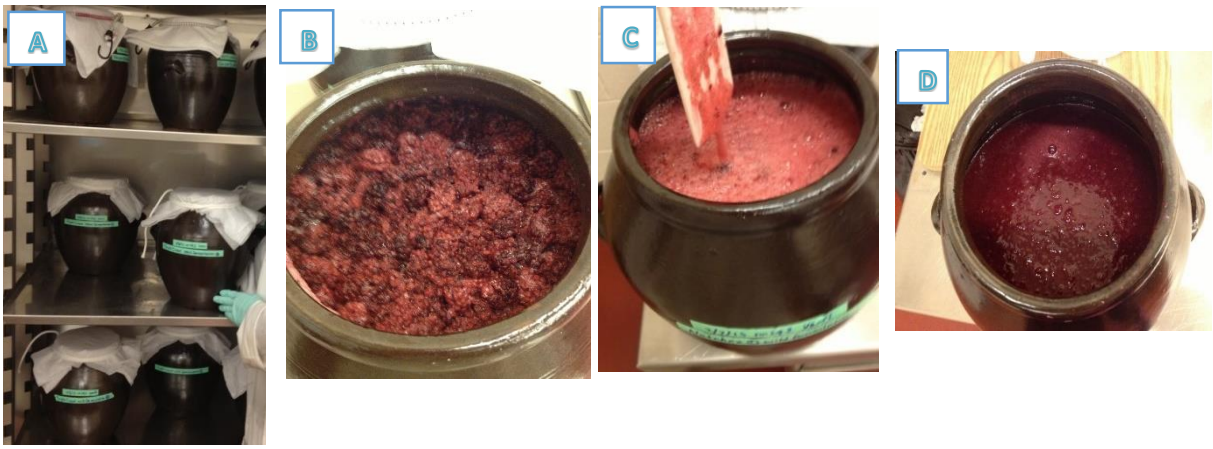

Figure 7. Blackberry 1st fermentation in environmental chamber (A); blackberries floating on the surface (B); bubbles produced during the mixing of the berries (C); appearance of the mixture near the end of the 1st fermentation (D).

For the second fermentation stage (Figure 8), blackberry skins and seeds were removed by using a nylon straining bag (small fine size, 10" x 23", LD Carlson, Kent OH). The strained pomace was collected into polyethylene bags and stored in a -18°C freezer for further analysis. The strained wine was then transferred into a second type of fermentation vessel.

During the second stage of fermentation, the goal was to limit oxygen contact. For this reason, 1 gallon glass jars or 1.5 L plastic water bottles with lids were used as needed in order to fill each vessel to around 4/5 full (Yun, 2007). Two types of bottles were used since different volume were shown in different treatments. Using water bottles helps to make small head space with less amount of fermented juice samples. This was done to control oxygen concentration in the headspace of the vessel and thus minimize wine oxidation. In the case of the plastic water bottles, the vessels were loosely capped after filling and then put into the environmental chamber at 21.6°C or 26.6°C. For the 1 gallon glass jars, the vessels were corked with fermentation locks and put into the environmental chamber as well. The second fermentation stage took 3-4 weeks. The finishing point of the second fermentation stage was determined by visually examining gas production on both glass jars and plastic bottles. When no further production of CO_2 bubble was noted, the fermentation was deemed complete. Triplicate samples were collected at the end of 1st and 2nd fermentations for subsequent further analyses. All samples were collected in 120 mL brown bottles and stored in a -15°C freezer for further analysis.

Figure 8. Preparation of blackberry 2nd fermentation. Blackberry skins and seeds were separated from juice (A and B). Blackberry wine undergoing the 2nd fermentation stage in the environmental chamber (C).

Ageing wine

After the second fermentation stage was complete, the wine was racked (off the sediment decanted) using plastic tubing and a Master Flex L/S™ wash-down modular controller high performance peristaltic pump (Cole Parmer Instrument Co., Vernon Hills, IL). The racked wine was filled into 950mL brown amber glass bottles to the top and the bottles were tightly sealed with screw caps. The wine was stored at 12.8°C according to the recommendation of Yun (2007) and 100mL samples were collected once a month in 120mL brown bottles for three months for further analyses.

Blackberry extraction

Hydrophilic extraction for antioxidant analysis

A 500 gram sample of blackberries was thawed in a refrigerator at 4°C for two days and then allowed to equilibrate at room temperature for 3 hours. The blackberry samples were then blended for one minute with a coffee grinder (Hamilton Beach, Washington, NC). The pureed blackberry samples were then collected into polyethylene bags for extraction.

The blackberry purees were extracted as follows with an extraction solvent (v/v) that consisted of 40 % acetone, 40 % methanol, 20 % deionized water and 0.1 % acetic acid. This was done by making mixture of solvent extract in 300mL volumetric flask. Mix with 120 mL acetone, 120 mL methanol, 60 mL deionized water and 300 µl of acetic acid into 300mL volumetric flask. Twenty grams of blackberry puree were weighted into a 100mL volumetric flask. The flask was filled to volume with the extraction solvent described above and the flask was mixed using a vortex agitator for about 10 seconds. The flask was then placed in a reciprocal shaking water

bath model 50 (Precision Scientific, Winchester, VA) and held at 60°C for an hour with an agitation rate of 60 rpm. Each flask was mixed 2-3 times during the first 10 minutes of extraction to remove excess gas bubbles. After an hour in the water bath, the flask was removed and allowed to cool for 40 minutes.

Cooled extracts were then filtered through a funnel lined with miracloth and collected into 120mL brown glass bottles. The bottles were capped, the caps were wrapped with plastic film and the sealed bottles were stored in freezer -15°C for subsequent analyses. All the whole blackberries and pomace samples were extracted in triplicate.

Diethyl ether extraction for total fat percent

The diethyl ether extraction measured total fat percentage of whole blackberries and pomace. For this analysis, 500 gram samples of whole blackberries or pomace were dried in a forced air drying oven (Proctor and Schwartz Inc., Horsham, PA) at 60°C for three days. After drying, the samples were ground in a coffee grinder (Magic Bullet, Emson Inc., New York, NY) for 2 minutes to make a powdered form. The powder was collected into polyethylene bags and stored in a freezer at -15°C until needed.

For extraction, 0.5 gram samples of dried blackberry or blackberry pomace were measured into 2 dram glass vials with screw cap. For each sample, four vials were prepared and the fourth vial was spiked with 0.010 gram of added canola oil. The diethyl ether extractions were conducted in a fume hood. For each vial, 4 mL of diethyl ether was added and stirred with a magnetic stir bars to mix for 20 minutes. After mixing, samples were centrifuged for 20 minutes under Speed Vac concentrator (Savant Inc., Midland, MI). The supernatant was then transferred to a new vial and the volatile components were evaporated in a Speed Vac

concentrator with refrigerated vapor trap for 20 minutes. This extraction procedure was repeated on each sample three times to ensure complete extraction and the resulting extracts were combined. The extracts were filtered through a nylon membrane syringe filter (nylon 66, 0.45 micron, Grace, Deerfield, IL) in new, tarred vials. The extract was then dried in the speed vacuum concentrator for 55 minutes until all either was removed. After samples were dried, the final weight of each vial was recorded. These weights were used as wet weight basis fat percentages.

To calculate percent fat on a dry weight basis, the moisture concentration of the powdered samples were measured as follows. A 2 gram sample of powdered whole blackberry or pomace was weighed into pre-weighted aluminum weight boat trays. The trays were pre-dried at 70 °C for 2 hours and then allowed to cool inside a desiccator (Boekel, Philadelphia, PA) for 30 minutes. The samples were dried in forced air oven (Hotpack, Watlow Series 922, Philadelphia PA) at 70°C for 24 hours to make equilibrium weight and then placed into a desiccator to cool for 30 minutes. The weights of the dried samples were recorded and the percent moisture was calculated based on following formula:

% moisture = (weight of wet sample-weight of dry sample)/ weight of wet sample x 100

To calculated dry weight basis total fat percent, the following formula for correcting sample weights from wet weight basis to dry weight basis was used:

Corrected sample wt. = original wet sample weight-(wet sample weight x percent moisture)

All powdered blackberries and pomace samples were measured in triplicate.

Antioxidant activity analyses

Modified Harbertson-Adams assay

The Harbertson-Adams assay was developed and modified at the University of California at Davis by Dr. Harbertson and Dr. Adams. This assay determined multiple classes of phenolic compounds that are considered important components of wine: anthocyanins, tannins, pigmented polymers, and non-tannin iron-reactive phenols (Viticulture and Enology at University of California Davis, 2005). The assay involved using a spectrophotometer to measure the absorbance of samples following several chemical reactions.

Chemical reagents and buffers

All the chemical reagents and buffers were mixed and diluted with deionized water.

- Model wine: potassium bitartrate (Sigma Aldrich, St. Louis, MO) + 95% ethanol (Pharmaco AAPER, Brookfield, CT) and adjust to pH 3.3
- Bleach solution: potassium metabisulfite (Presque Isle Wine Cellars, North East, PA)
- Ferric chloride reagent: ferric chloride (Sigma Aldrich, St. Louis, MO) + 12.1 N concentrated hydrogen chloride (VWR, Render, PA)
- Washing buffer: sodium chloride (EMD, Gibbstown, NJ) + glacial acetic acid (Pharmaco AAPER, Brookfield, CT) and adjust to pH 4.9
- Resuspension buffer: sodium dodecyl sulphate (Sigma Aldrich, St. Louis, MO) + triethanolamine (Sigma Aldrich, St. Louis, MO) and adjust to pH 9.4
- Anthocyanin buffer: maleic acid (Arcos Organics, Fair Lawn, NJ) + sodium chloride (EMD, Gibbstown, NJ) and adjust to pH 1.8 with sodium hydroxide

- Protein stock: bovine serum albumin (Sigma Aldrich, St. Louis, MO), stored -80°C

Procedures

The Harbertson-Adams assay consisted of four major tests. The first test measured total phenolic concentration calculated as mg/L catechin equivalent. This test also provided dilution factors for subsequent tannin and polymeric pigments tests. The second test measured non-tannin polymeric pigments and provided total polymeric pigments in the wine samples. The third test measured tannins and two classes of polymeric pigments using a bovine serum albumin protein solution. The results were reported as small or large polymeric pigments and mg/L catechin equivalents for tannins. The fourth and the final test measured total anthocyanins calculated as mg/L malvidin-3-monoglucoside. A detailed description of the test procedures may be found in Appendix F. All samples were measured in duplicate.

Oxygen radical absorbance capacity (ORAC) assay

The ORAC assays were conducted using the method as modified by Huang and others (2002).

This method measured antioxidant capacity of blackberry juice, wine and pomace samples.

Chemicals and buffers

A 75 nM phosphate buffer at pH 7.0 was made using potassium phosphate monobasic (Fisher Scientific, Pittsburg, PA) and sodium phosphate (Amresco, Solon, OH).

The water-soluble tocopherol Trolox (6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was purchased from Fluka (St. Louis, MO) and a 100µM solution of Trolox was made using the phosphate buffer. The Trolox was diluted with phosphate buffer to a concentration of 50µM as a working solution.

Fluorescein was purchased from Sigma-Aldrich (St. Louis, MO) and 376µM of fluorescein stock solutions was made using the phosphate buffer. The fluorescein stock solution was diluted at a ratio of 1:1000 to make a working solution.

The radical generator AAPH (2,2'-azobis[2-amidino-propane] dihydrochloride) was purchased from Arcos Organics (Fair Lawn, NJ). A 306mM solution of AAPH was made daily using the phosphate buffer.

Sample Preparation

All blackberry juice, wine, and pomace samples were diluted at a ratio of 1:2000 with phosphate buffer prior to being tested in the ORAC assay. This dilution factor was found through trial and error to give the appropriate chemical kinetics during the assay. The best decay curve in ORAC results were between blank as phosphate buffer and Trolox standard.

Procedures

The ORAC assays were conducted on samples using a 96-well flat bottom tissue culture plate with low evaporation lid (BD Falcon™, Durham, NC). Microplates were loaded using a Bio Tek® Precision™ Microplate Pipetting System which was controlled by Precision Power™ software version V2, 03.2. The system was pre-programed to load each plate well with Fluorescein and either buffer, Trolox, or test sample as follows: Each well was filled with 160 µL of fluorescein. For 'blank' wells, 20µL phosphate buffer were added. For the 'standard' wells,

20µL of Trolox were added. The pipetting system was programmed to automatically dilute the first Trolox concentration (50µL) to 25µL and 12.5µL so overall three different concentrations of Trolox were made. For the 'sample' wells, 20µL of the blackberry sample were added. All reagents were added automatically under the pipetting system.

After the initial plate loading, plates were placed into a Bio Tek® Synergy™ 2 Microplate Reader (Bio Tek Instruments, Inc., Winooski, VT) which was controlled by Gen 5 software (version 5.1) and the plates were incubated for 10 minutes at 37°C to allow them to come to the assay temperature. After the incubation period was finished, 20µL of AAPH, a free peroxy radical generator, was added manually with multiple pipeter to each well. The microplate reader was programmed to record fluorescein every minute and until it reached 35 minutes. The chemical kinetics of the test were deemed acceptable if at least 90 percent degradation of the relative fluorescence occurred during that 35 minute period for each sample. Raw ORAC results were obtained by calculating the area under the fluorescein decay curve (AUC) for each of the blank, Trolox, and sample wells as follows:

$$AUC = f_1/f_0 + f_i/f_0 + \ldots + f_{34}/f_0 + f_{35}/f_0$$

Where f_0 = initial fluorescence reading at 0 min and f_i = fluorescence reading at time i. Subtraction of the blank area allowed us to directly compare the under the areas of Trolox to the sample wells. The final results of the ORAC assay were calculated as µmol Trolox equivalent per gram of blackberry juice, wine or pomace. All samples were measured in duplicate.

Chemical Analyses

<u>High Performance Liquid Chromatography (HPLC) with PhotoDiode Array (PDA) detector</u>

Chemicals and reagents

HPLC grade acetonitrile and formic acid (>99%) were purchased from Acros Organics (Fair Lawn, NJ) and HPLC-grade methanol was purchased from Pharmco-AAPER (Brookfield, CT). Deionized water was produced by Mili-Q-system (EMD Milipore, Billerica, MA). Phenolic standards such as gallic acid, caffeic acid, p-coumaric acid, catechin hydrate, epicatechin and delphinidin chloride were purchased from Sigma-Aldrich (St. Louis, MO). The ferulic acid that was used as an internal standard and the anthocyanin standards such as kuromanin chloride and keracyanin chloride were purchased from Fluka (St. Louis, MO).

Phenolic standards preparation

All nine standards were made into a stock solution with HPLC-grade methanol. The gallic acid, caffeic acid and p-coumaric acid were mixed to a concentration of 75 ppm. The catechin hydrate and epicatechin were mixed to a concentration of 250 ppm. All of the three anthocyanin standards, kuromanin chloride, keracyanin chloride and delphinidin chloride, were mixed to a concentration of 150 ppm. The internal standard of ferulic acid was mixed to a concentration of 300 ppm. The various standards were mixed in a vial and the mixture was used to determine order of elution, approximate retention times, and to correlate compound concentrations to peak areas.

Sample preparation

Blackberry juice and wine samples were prepared for HPLC analysis as follows. Briefly, a 5mL Sep-pak syringe filter (Waters Corporations, Milford, MA) was conditioned with 4mL of acidified HPLC-grade methanol (0.1% v/v hydrochloric acid, VWR, Render, PA) followed by 4mL

of deionized water. A 4mL sample of juice or wine was filtered through the Sep-pak cartridge using a 5 mL syringe (Cadense Inc., Micro Mate Interchangeable syringe, Staunton, VA). The cartridge was then washed with 8mL of deionized water to remove sugars and other water-soluble components. The cartridge was then washed again with 8 mL of acidified methanol and the methanol-soluble components were collected in a test tube. The extract was then dried using a SpeedVac evaporator (ThermoSavant, Model SPD 121P, Waltham, MA) for 3 hours without heat. The concentrated blackberry juice/wine sample was then transferred into a 4mL vial and the weight was recorded. For the injection sample, a mixture of 500µL of concentrated extract and 200µL of ferulic acid as an internal standard were placed into a 2mL injection vial and capped. Our experiment measured only free form of anthocyanins.

Procedures

The modified reverse-phase HPLC method used by Thimothe and others (2007) was used for the analysis of phenolic compounds. The HPLC system used 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 Chromelon software version 6.80. Separation was achieved by a gradient elution at 40°C with a SunFire™C18 column (4.6mm x 250mm x 5µm), including a SunFire™C18 guard column (4.6mm x 20mm), both of them were from Waters Corporations (Milford, MA). The flow rate was at 0.8mL/min. The two mobile phases were 10 % formic acid for the 'A' solution, and a mixture (v/v) of 10 % formic acid, 22.5 % acetonitrile and 22.5 % methanol for the 'B' solution. The elution parameters were: 0 min, 94% A; 5min, 70% A; 30min, 20% A; 35 min, 40% A; 45 min, 94% A; and 75min, 94% A. Data were acquired for 75 minutes and chromatograms were drawn using absorbance at wavelengths of 280, 320, 370 and

520 nm. Maximum absorbance for the phenolic acids and flavanols were measured at 280nm while maximum absorbance for anthocyanins were measured at 520 nm. Phenolic compounds were identified and quantified based on the retention times and peak areas observed with the standard solutions described previously. Samples were measured in duplicate.

Gas Chromatography (GC) with Flame ionized detector (FID)

Chemicals and standards

Dichloromethane (HPLC grade, >99%) was purchased from Arcos organics (Fair Lawn, NJ). Hexane was purchased from Fisher Scientific (Pittsburgh, PA). Ethanol was purchased from Pharmco-AAPER (Brookfield, CT). The following pure standards (>98%) were purchased from Arcos organics (Fair Lawn, NJ): 2-heptanol; methyl salicylate; hexanoic acid; 2-heptanone; cinnamyl alcohol; and trans-2-hexenal. The following standards (purity of >95%) were obtained from Sigma-Aldrich (St. Louis, MO): : Heptanol; α-pinene; fenchone; linalool; α-terpineol; geraniol; eugenol; hexanal; p-cymene; and theaspirane A. Furaneol was purchased from TCI America (Portland, OR). Ethyl acetate was purchased from EMS (Hatfield, PA).

Volatile standards preparation

A standard solution of each of the 19 standards was prepared by dividing the molecular weight of each stock compound by its declared purity, then calculating the weight of the standard needed to create a working stock solution of 10 mL at 100ppm for eugenol; 200 ppm for α-pinene; 350 ppm for geraniol and cinnamyl alcohol; 400 ppm for p-cymene, ethanol and α-terpineol; 500 ppm for fenchone, 2-heptanone, ethyl acetate and methyl salicylate; 650 ppm for

trans-2-hexenal, hexanal, hexanol,2-heptanol, and linalool; 750 ppm for theaspirane; 900 ppm for hexanoic acid and furaneol. For each standard, the corresponding amount of the compound was then weighed into a 10 mL volumetric flask and each flask was filled to volume with hexane. After making the stock solutions, working solutions of the standards were made by diluting the stock solutions 1:10 with additional hexane. The 100µL aliquots of all 19 of these working standard solutions were then mixed in an injection vial and this mixture was used to identify standard retention times and to correlate relative sample responses to standard concentrations.

Sample preparation

A method modified from Oliveira and others (2006) and Vilanova and others (2009) was used for free volatile analysis via GC. Dichloromethane micro-extraction was used to measure free volatiles in blackberry juice and wine samples. Briefly, 32mL of blackberry juice or wine was mixed with 120µL of extraction internal standard, p-cymene, and 1.6mL of dichloromethane in a 40mL test tube. The mixture was then stirred with a magnetic stir bar for 15 minutes. The tubes containing the samples were then cooled in 0°C water for 10 minutes. The tubes were then centrifuged using a Sorvall® RC 5c Plus (DuPont, Wilmington, DE) at 5110 rpm for 5 minutes at 4°C. The clear bottom of the dichloromethane layer was then collected and filtered with a syringe filter (17mm PVDF, 0.2 UM, Thermo Science, Rockwood, TN) into a 4 mL injection vial. A volume of 665µL of sample was filtered into the vial, then 35µl of fenchone was added as an analytical internal standard and the vial was capped.

Procedure

A gas chromatography (GC) system with a flame ionization detector was used to analyze free volatile compounds. The GC system was manufactured by Agilent technologies (Santa Clara,

CA) and consisted of a 6890N networked GC system, a 7683B series injector, a 7683 series auto sampler and an all pure purifier from Alltech (Portland, ME). The GC system was operated by HP Agilent Chemstation software. The three gases used by the system were purchased from Airgas (Radnor, PA). Hydrogen (model UHP300) was used as carrier gas, helium (model UHP300) was used as makeup gas, and air (model Z300) was used as the flame igniter. The injector temperature was 40°C and detector temperature was 300°C. The DB-Wax fused silica capillary GC column (30m length x 0.25mm narrow bore I.D. x 0.25 µm film thickness) on which the separations were carried out was purchased from J and W Scientific Inc. (Folsom, CA). The oven temperature was maintained at 55°C for 2minutes, raised at 0.5°C/min up to 75°C, then raised at 5°C/min up to 145°C, and finally raised at 20°C/min to 250°C and held for 10 minutes. The front Inlet was set as splitless mode with hydrogen as a carrier gas, along with 290°C for the heater, a pressure of 11.38 psi, and a total flow of 48.6 mL/min. The sample injection volume was 2µL.

The extraction recovery was estimated by p-cymene as internal standard. All volatile compounds were identified and quantified by comparisons with the retention times and peak areas obtained using the pure standard solutions. All samples were measured in duplicate.

Statistical Analysis

Statistical Analyses were performed using SAS 9.3 (SAS institution, Cary, NC). For all analyses, an analysis of variance (ANOVA) for each set of data was conducted using a three factor factorial treatment scheme in a completely randomized design with repeated measures. Means were separated using least significant differences (LSD) with a 95% confidence interval

($p<0.05$). Pearson correlation coefficients were created to assess the relationships between chemical constituents as measured by the Harbertson-Adams Assay and ORAC values.

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CHAPTER IV

RESULTS AND DISCUSSIONS

Physical properties for whole blackberry Juice and wine

General physical properties of whole blackberries

Table 1 shows the size and weight of whole 'Natchez' and 'Triple Crown' blackberries after freezing. The 'Natchez' berries were bigger and heavier than 'Triple Crown' blackberries. The 'Natchez' berries were more oval in shape while 'Triple Crown' berries were more rounded. When comparing over two years, the berries collected in 2012 were bigger and heavier than the berries collected in 2011. Compared to 2012, the weather in 2011 was hotter and drier, thus it is possible that berries were sunburned and became dried out and therefore smaller and lighter.

Table 1. Average mean values of whole blackberry weight and size (n=10).

Cultivar	**Natchez**		**Triple Crown**	
Harvest Year	*2011*	*2012*	*2011*	*2012*
Weight (g)	6.14	6.87	5.78	6.51
Size (cm)	3.3	3.5	2.7	3.0

pH

The mean pH values of blackberry juice samples are shown in table 2. In general, the blackberries collected in 2011 were more acidic than blackberries collected in 2012. Between the cultivars, 'Natchez' juice possessed a slightly lower pH value than 'Triple Crown' juice. It seems likely that harvest year had a greater effect on pH value than cultivars. Overall, 'Triple Crown' juice from 2012 had the highest mean pH value of 3.15 and 'Natchez' juice from 2011 had the lowest mean pH value of 2.88. However, the only statistically significant difference was found in 'Triple Crown' juice between the two years (p=0.0465).

Table 2. Average mean pH values of pressed blackberry juice (n=2)

Cultivars	**Natchez**		**Triple Crown**	
Harvest Year	*2011*	*2012*	*2011*	*2012*
pH	2.88	3.01	2.92†	3.15†

† Denotes a statistically significant difference between the two harvest years within cultivar (p=0.0465).

Table 3 shows the average mean pH values of blackberry wine samples made from berries harvested in 2011 and 2012. Comparing the two cultivars, 'Triple Crown' wine showed a slightly higher pH value than 'Natchez' wine. Since the pH of 'Triple Crown' whole berries were higher than 'Natchez' berries this could be applied to the wine as well. Significant differences ($p<0.05$) were found in two cultivars for pH at the higher fermentation temperature. Specifically, 'Triple Crown' wines with wild-type fermentation had statistically significantly higher pH than 'Natchez' wines with wild-type fermentation ($p<0.0001$). The 'Triple Crown' wines with yeast inoculation were also statistically significantly higher in pH than 'Natchez' wines with yeast inoculation ($p<0.0001$).

Table 3. Mean pH values in blackberry wines by inoculation type and fermentation temperature (n=3).

Cultivar	**Natchez**				**Triple Crown**			
Inoculation Treatment	*Yeast*		*Wild-type fermentation*		*Yeast*		*Wild-type fermentation*	
Fermentation Temperature (°C)	*21.6*	*26.6*	*21.6*	*26.6*	*21.6*	*26.6*	*21.6*	*26.6*
pH	3.1$^{\Psi}$	2.6$^{\Psi *}$	3.08$^{\Psi}$	2.64$^{\Psi *}$	3.12$^{\Psi \dagger}$	2.87$^{\Psi * \dagger}$	3.05$^{\Psi \dagger}$	2.76$^{\Psi * \dagger}$

$^{\Psi}$ Denotes a statistically significant difference between the two fermentation temperatures within cultivar and inoculation treatment ($p<0.0001$).

* Denotes a statistically significant difference between the two cultivars within inoculation treatment and fermentation temperature ($p<0.0001$).

† Denotes a statistically significant difference between inoculation treatments within cultivar and fermentation temperature ($p=0.0015$ for low temperature, $p<0.0001$ for high temperature).

Comparing inoculation treatments, yeast-inoculated wines generally had slightly higher pH values than wild-type fermentation. Yeast-inoculated 'Triple Crown' wines had statistically significantly higher pH values at both fermentation temperatures ($p=0.0015$ for low temperature, $p<0.0001$ for high temperature). No significant differences in pH were seen between inoculation treatments for the 'Natchez' wines, however.

Comparing fermentation temperature treatments, the low fermentation temperature treatments generally had higher pH values than wines fermented at the higher temperature. Statistically significant differences were found between fermentation temperature treatments for both inoculation treatments and cultivars ($p<0.0001$). Overall, 'Triple Crown' blackberry wine with yeast inoculation and with the low fermentation temperature had the highest pH value of 3.12, while 'Natchez' blackberry wine with yeast inoculation and with the high fermentation temperature had the lowest pH value of 2.60.

The range of pH values in blackberry juice and wine was from 2.6 to 3.15. This result matches that found by Milosevic and others (2012), which was a pH range between 2.6 and 3.2. Also, similar pH value were found by Rutz and others (2012), Arozarena and others (2012), and

Johnson and others (2013). However, some researcher found higher pH values, from 3.2 to 4.2, due to differences observed among cultivars and growing locations (Hassimotto and others 2008, Johnson and others 2011, Ali and others 2012, Gao and others 2012, Thomas and others 2012).

Environmental conditions such as weather and temperatures along with ripeness affects the berry pH. Wet and cloudy weather during harvest, for example, can lead to under-ripened berries that are relatively high in acid. Cooler temperatures can also cause berries to take a relatively longer time to fully ripen. In this study, 'Triple Crown' berries were generally smaller and had relatively less surface area than 'Natchez' berries. This could have allowed 'Triple Crown' berries to over ripen than 'Natchez' berries in general. It could be possible that 'Natchez' berries could be less ripen than that of 'Triple Crown' berries. 'Triple Crown' berries had relatively small surface area than 'Natchez' berries, it was easy to ripen all the way to inside but 'Natchez' berries may need longer time to fully ripen. Given that acid concentration tends to drop and pH values tend to rise as berries ripen, this may help explain why 'Triple Crown' berries tended to have higher pH values than 'Natchez' berries in this study. In addition, basic genetic tendencies could vary between the two cultivars and this could have affected the acidity of berries.

Titratable acidity

Titratable acidity of blackberry juice was expressed as % malic acid and the mean values are shown in table 4. Overall, there were no significant differences in titratable acidity observed between cultivars in the same harvest year. Juice from berries harvested in 2012 did have higher average titratable acidity values than that juice from berries harvested in 2011. 'Triple Crown'

berries from 2012 had the highest titratable acidity at 0.422 % malic acid while 'Natchez' berries from 2011 had the lowest titratable acidity at 0.386 % malic acid. However, the only statistically significant difference observed was between 'Triple Crown' blackberries harvested in 2011 and those harvested in 2012 (p=0.0465).

Table 4. Mean values of titratable acidity in blackberry juice (n=2).

Cultivars	**Natchez**		**Triple Crown**	
Harvest Year	*2011*	*2012*	*2011*	*2012*
% Malic Acid	0.386	0.403	0.391†	0.422†

† Denotes a statistically significant difference between the two years within cultivar ($p<0.05$).

The mean titratable acidity values of blackberry wines, expressed as % malic acid, are shown in table 5. Between two cultivars, 'Natchez' wines had higher titratable acidity values than 'Triple Crown' wines. The fact that 'Natchez' berries were observed to have slightly lower pH values than 'Triple Crown' berries may reflect a somewhat higher starting acid concentration in the 'Natchez' berries as opposed to the 'Triple Crown' berries. However, fermentation likely played a role in the differences observed as well.

Table 5. Mean values for titratable acidity of blackberry wines (n=3).

Cultivar	**Natchez**				**Triple Crown**			
Inoculation Treatment	*Yeast*		*Wild-type fermentation*		*Yeast*		*Wild-type fermentation*	
Fermentation Temp. (°C)	*21.6*	*26.6*	*21.6*	*26.6*	*21.6*	*26.6*	*21.6*	*26.6*
% Malic Acid	0.415ᴪ	0.349ᴪ*	0.412ᴪ	0.353ᴪ*	0.419ᴪ†	0.385ᴪ*†	0.409ᴪ†	0.370ᴪ*†

ᴪ Denotes a statistically significant difference between fermentation temperatures within cultivar and inoculation treatment ($p<0.0001$).

* Denotes a statistically significant difference between the two cultivars within inoculation treatment and fermentation temperature ($p<0.0001$).

† Denotes a statistically significant difference between inoculation treatments within cultivar and fermentation temperature ($p=0.0015$ for low temperature, $p<0.0001$ for high temperature).

Significant differences were found between the two cultivars at the higher fermentation temperature. Specifically, wine made from 'Triple Crown' berries with both yeast inoculation

and wild-type fermentations had statistically significantly higher titratable acidity values than wine made from 'Natchez' berries ($p<0.0001$).

Comparing inoculation treatments, overall wild-type fermentations showed higher titratable acidity values than yeast inoculations. This corresponds well to the lower pH values observed for wild-type fermentation treatments compared to yeast inoculation treatments. However, the only statistically significant difference observed in relation to inoculation treatment was for wines made from 'Triple Crown' berries. At both fermentation temperatures, wines with a yeast inoculation had significantly higher titratable acidity values than wines made with a wild-type fermentation ($p=0.0015$ for low fermentation temperature, $p<0.0001$ for high fermentation temperature).

Comparing the two fermentation temperatures, overall the low fermentation temperature showed higher titratable acidity values than the high fermentation temperature. Statistically significant differences were found between fermentation temperatures over both cultivars and inoculation treatments ($p<0.0001$). Overall, the highest titratable acidity of 2.122 % was found in 'Natchez' wine with wild-type fermentation at high fermentation temperature while the lowest level of titratable acidity of 1.184 % was seen in 'Triple Crown' wine with yeast inoculation at high fermentation temperature.

The titratable acidity values of blackberry juice and wine samples ranged from 0.349 to 0.419 % malic acid. This range was close to the higher ends of Thomas and others (2012), which was 0.17 to 0.36 %. Most researchers have recorded somewhat higher titratable acidity values than those observed in the current study (Hassimotto and others 2008, Wang and others 2008, Du and others 2010, Ali and others 2012, Arozarena and others 2012, Rutz and others 2012, Ortiz and others 2013). Titratable acidity can be influenced by the weather conditions: lack of

sunshine, low temperature, or high rainfall. The relatively low titratable acidity values measured in this study suggest that the berries were sweeter and less tart than some.

Soluble solids

The mean sugar concentrations of blackberry juice samples, expressed as % soluble solids are shown in table 6. In general, higher soluble solid values indicate higher sugar concentration and thus a sweeter taste. Overall, 'Triple Crown' berries had higher average soluble solid concentrations than 'Natchez' berries. A statistically significant difference in soluble solids concentration was observed in 'Natchez' juice between the two harvest years (p=0.0225). To compare with the two years, there were no clear trends but more variation was seen in 2012. For 'Natchez' berries, juice from 2011 was higher in soluble solids than juice from 2012. The opposite case was seen for juice from 'Triple Crown' berries. A statistically significant difference was seen between cultivars for berries harvested in 2012: Specifically, 'Triple Crown' juice was significantly higher in soluble solids than 'Natchez' juice (p=0.0075). Overall, 'Triple Crown' juice from 2012 had the highest soluble solids concentration of 12.06% solid and 'Natchez' juice from 2012 had the lowest soluble solids concentration of 10.04%.

Table 6. Mean concentration of soluble solids in blackberry juice (n=2).

Cultivars	**Natchez**		**Triple Crown**	
Harvest Year	2011	2012	2011	2012
% Soluble Solids	11.64†	10.04†*	11.08	12.06*

† Denotes a statistically significant difference between the two cultivars within year (p=0.0225).
* Denotes a statistically significant difference between the two years within cultivar (p=0.0075).

The soluble solids concentration of blackberry juice observed in this study was about 10-12%, which is close to the range seen by Thomas and others (2012). However, many previous researchers have reported slightly lower soluble solid concentrations of below 10% (Wang ant others 2008, Ali and others 2012). It is well known that environmental condition such as

weather and planting location can affect the sugar level of blackberries. More sunshine and less rain or clouds during berry development would help to increase blackberry sugar concentration as well as formation of good sugar/acid balance.

Percent alcohol

The average percent alcohol of blackberry wines are shown in table 7. In general, 'Triple Crown' blackberry wine showed higher % alcohol than 'Natchez' blackberry wine. To compare the two cultivars, for blackberry wine samples with wild-type fermentation at the high fermentation temperature, 'Triple Crown' wine was statistically significantly higher than 'Natchez' wine ($p=0.0395$). This observed difference is minimal, however, and of debatable practical significance. Yeast-inoculated 'Triple Crown' wines were also significantly higher than yeast-inoculated 'Natchez' wines at both fermentation temperatures ($p<0.0001$).

Table 7. The average mean % alcohol found in blackberry wine (n=3).

Cultivars	**Natchez**				**Triple Crown**			
Inoculation Treatment	*Yeast*		*Wild-type fermentation*		*Yeast*		*Wild-type fermentation*	
Fermentation Temp. (°C)	*21.6*	*26.6*	*21.6*	*26.6*	*21.6*	*26.6*	*21.6*	*26.6*
% Alcohol	13.91*†	13.57*	13.46†	13.26*	15.76ψ*†	15.01ψ*†	13.68†	13.67*†

ψ Denotes a statistically significant difference between fermentation temperatures within cultivar and inoculation treatment ($p=0.0009$).

* Denotes a statistically significant difference between two cultivars ($p<0.0001$ for yeast inoculations, $p=0.0395$ for wild-type fermentation at high temperature).

† Denotes a statistically significant difference between inoculation treatments within cultivar and fermentation temperature ($p<0.0001$ for 'Triple Crown' berries, $p=0.0247$ for 'Natchez' berries).

Comparing inoculation treatments, blackberry wine samples with yeast inoculations had a higher alcohol % than wild-type fermentations. Yeast-inoculated 'Triple Crown' wine samples were statistically significantly higher in % alcohol, about 1.5 %, than wild-type fermentations at

both fermentation temperatures ($p<0.0001$). At the low fermentation temperature, alcohol concentration in 'Natchez' wine with yeast inoculation was also significantly higher than alcohol concentration in wines made with wild-type fermentation ($p=0.0247$) although this difference was within half a percent.

To compare among fermentation temperatures, overall the wines prepared at the low fermentation temperature had higher average % alcohol than wines prepared at the high fermentation temperature. However, the only statistically significant difference was seen in yeast-inoculated 'Triple Crown' wines where the lower fermentation temperature wine samples had a higher alcohol concentration, about 0.75 % higher, than the wine fermented at the higher temperature ($p=0.0009$). Among the blackberry wine samples, yeast-inoculated 'Triple Crown' fermented at the low fermentation temperature had the highest % alcohol (15.76 %) and 'Natchez' wine made with wild-type fermentation at the high fermentation temperature showed the lowest % alcohol (13.26 %).

The alcohol concentrations measured in the blackberry wines ranged from 13.26 % to 15.76 %. These alcohol percentages were higher than those recorded by Gao and others (2012) and previous research by Joh (2010). Because sugar was added while processing the wine, it is not surprising that a relatively high alcohol concentration was seen in the wines. According to Johnson and others (2011), the reported alcohol concentration of commercial blackberry wines is about 9-15 % and the wines involved in their research was measured at 7-24 % alcohol. The wines made in this study were well within this range.

Alcohol content in wines is a function of the sugar concentration in the starting material, up to the alcohol tolerance of the yeast doing the fermentation, presuming that the fermentation goes to completion. Other factors may affect fermentation efficiency, however,

such as available fermentable nitrogen and other yeast nutrients. In this study 'Triple Crown' berries produced more alcohol with yeast inoculation than 'Natchez' wines. Since the same ratio of sugar was added based on the total volume of berries used, alcohol should have been produced at a similar rate. The 'Triple Crown' berries had somewhat higher starting soluble solids concentration than 'Natchez' berries, but not high enough to account for the observed final difference in wine alcohol concentration. This suggests that other fermentation factors may account for the differences observed. The wild-type fermentation was more consistent in terms of final alcohol concentration than the yeast-inoculated samples, indicating that the wild-type microflora may have been less sensitive to particular fermentation conditions.

Sulfur dioxide testing

The results of sulfur dioxide tests of blackberry wines are shown in table 8. Between the cultivars, 'Natchez' blackberry wines showed slightly higher total sulfur dioxide concentrations than 'Triple Crown' blackberry wines. However, only yeast-inoculated wine sample fermented at the higher temperature showed a statistically significant difference (p=0.0259) between 'Triple Crown' and 'Natchez' wines, 'Triple Crown' having a higher sulfur dioxide concentration.

Table 8. Mean values of total sulfur dioxide concentration in blackberry wines (n=3).

Cultivars	**Natchez**				**Triple Crown**			
Inoculation Treatment	*Yeast*		*Wild-type fermentation*		*Yeast*		*Wild-type fermentation*	
Fermentation Temp. (°C)	*21.6*	*26.6*	*21.6*	*26.6*	*21.6*	*26.6*	*21.6*	*26.6*
Total Sulfur Dioxide (ppm)	10.7†	16.8*†	22.4Ψ†	6.9Ψ†	16.0Ψ	6.9Ψ*	24.0Ψ	6.4Ψ

Ψ Denotes a statistically significant difference between the fermentation temperatures within cultivar and inoculation treatment (p=0.0014 for 'Natchez' wild-type fermentation, p=0.0005 for 'Triple Crown' wild-type fermentation, p=0.0385 for 'Triple Crown' yeast inoculation).

* Denotes a statistically significant difference between the two cultivars within inoculation treatment and fermentation temperature (p=0.0259).

† Denotes a statistically significant difference between inoculation treatments within cultivar and fermentation temperature (p=0.01 for low fermentation temperature, p=0.0259 for high fermentation temperature).

Comparing inoculation treatments, blackberry wine with wild-type fermentation had slightly higher sulfur dioxide concentrations than yeast-inoculated wines. Looking at the combination of inoculation treatment and fermentation temperature, wines made from 'Natchez' berries showed statistically significant differences between inoculation treatments at both fermentation temperatures (p=0.01 for low fermentation temperature; p =0.0259 for high fermentation temperature). The results were not consistent, however. Wild-type fermented 'Natchez' wine was higher in sulfur dioxide at the lower fermentation temperature whereas at the higher fermentation temperature the yeast-inoculated 'Natchez' wine was higher. This same basic pattern was seen in the 'Triple Crown' berries, although the difference between yeast-inoculated and wild-type fermented wines was not significant at the higher fermentation temperature.

Comparing the two fermentation temperatures, the low fermentation temperature wines had almost double amount of sulfur dioxide concentration than the wines fermented at the high fermentation temperature except for yeast-inoculated wines made from 'Natchez' berries. The average sulfur dioxide concentration measured in yeast-inoculated wines made from 'Natchez' berries and fermented at the lower fermentation temperature was about half that seen in the same wine fermented at the higher fermentation temperature, but this difference was not statistically significant. All of the other differences in sulfur dioxide concentration seen among fermentation temperatures were statistically significant (p=0.0014 for 'Natchez' wild-type fermentation; p=0.0005 for 'Triple Crown' wild-type fermentation; p=0.0385 'Triple Crown' yeast inoculation). Thus, fermentation temperature appeared to have the greatest effect of all the attributes measured on sulfur dioxide concentration, which is not

surprising given that sulfur dioxide is a volatile that can be lost through forming complexes with other molecules and through vaporization over time.

Overall, 'Triple Crown' blackberry wine made with wild-type fermentation at the lower fermentation temperature had the highest total sulfur dioxide concentration at 24 ppm while 'Triple Crown' blackberry wine made with wild-type fermentation at the higher fermentation temperature showed the lowest total sulfur dioxide concentration at 6.4 ppm.

The sulfur dioxide concentration of blackberry wines measured in the study ranged from about 6 ppm to 24 ppm. Sulfur dioxide is important in maintaining wine quality and stability because it acts as an antioxidant as well as an antimicrobial to prevent growth of spoilage yeast and some unwanted bacteria. A recommended sulfur dioxide concentration for wines at a pH of about pH 3.0 is around 10-30 ppm. Some factors that have been shown to influence sulfur dioxide concentration of blackberry wine are fermentation temperature, yeast strain and the sulfur concentration of the starting blackberries (Jackson 2008). The samples with wild-type fermentation had more variation in sulfur dioxide concentration due to fermentation temperature than yeast inoculated samples. It seems like that wild-type fermentation at higher fermentation temperature might affect the stability of the wine, resulting in lower retention of sulfur dioxide.

Blackberry extraction

Diethyl ether extraction for total fat percent

The mean values for total % fat on a dry weight basis of whole blackberries over two harvest years are shown in table 9. To compare within cultivars, in 2012 'Natchez' berries had

significantly a higher oil concentration than 'Triple Crown' blackberries (p=0.0373). No statistically significant difference was seen between cultivars in berries harvested in 2011. Statistically significant differences between harvest years were seen in 'Triple Crown' berries (p=0.0017). The 'Triple Crown' blackberries had more variability in % fat between years whereas 'Natchez' berries had a more consistent % fat over the two years. Overall, 'Triple Crown' blackberries harvested in 2011 had the highest oil concentration at 4.23 % and 'Triple Crown' blackberries harvested in 2012 had the lowest oil concentration at 2.94 %.

Table 9. Mean total extractable lipid percentage of whole blackberry over two years (n=3).

Cultivars	**Natchez**		**Triple Crown**	
Harvest Year	2011	2012	2011	2012
% Oil	3.47	3.78*	4.23†	2.94†*

† Denotes a statistically significant difference between two years within cultivar (p=0.0017).
* Denotes a statistically significant difference between the two cultivars within year (p=0.0373).

The mean values for total fat % on a dry weight basis of blackberry pomace are shown in table 10. Comparing cultivars, in general, pomace from 'Natchez' berries had a slightly higher % oil than pomace from 'Triple Crown' berries. Statistically significant differences were seen in pomace derived from berries that had undergone a wild-type fermentation at the low fermentation temperature, in which 'Natchez' pomace contained significantly more oil than 'Triple Crown' pomace (p =0.0203). Similarly, 'Natchez' pomace derived from yeast-inoculated berries fermented at the high fermentation temperature contained significantly more oil than pomace from 'Triple Crown' berries receiving the same treatments (p=0.0015).

Table 10. Mean total extractable lipid concentration (%) of blackberry pomace (n=3).

Cultivars	**Natchez**				**Triple Crown**			
Inoculation Treatment	*Yeast*		*Wild-type fermentation*		*Yeast*		*Wild-type fermentation*	
Fermentation Temp. (°C)	*21.6*	*26.6*	*21.6*	*26.6*	*21.6*	*26.6*	*21.6*	*26.6*
% Oil	2.78$^{\psi}$	3.7$^{\psi *}$	2.54*	3.34	2.96†	3.18*	1.92$^{\psi *\dagger}$	3.23$^{\psi}$

$^{\psi}$ Denotes a statistically significant difference between fermentation temperatures within cultivar and inoculation treatment (p=0.0064 for 'Natchez' yeast inoculation, p=0.0024 for 'Triple Crown' wild-type fermentation).

* Denotes a statistically significant difference between the two cultivars within inoculation treatment and fermentation temperature (p=0.0203 for wild-type fermentation at low temperature, p=0.0015 for yeast inoculation at high temperature).

† Denotes a statistically significant difference between inoculation treatments within cultivar and fermentation temperature (p=0.0003).

Comparing inoculation treatments, at a given fermentation temperature pomace derived from yeast-inoculated berries generally similar or higher average % oil values than pomace derived from wild-type fermentations. However, the only statistically significant difference observed was for pomace derived from 'Triple Crown' berries that were fermented at the lower fermentation temperature (p=0.0003), in which pomace from yeast-inoculated berries had a significantly higher oil concentration than pomace derived from berries that had undergone a wild-type fermentation.

Comparing the two fermentation temperatures, within cultivar and inoculation treatment the pomace from berries fermented at the higher fermentation temperature always had higher average oil concentration than pomace from berries fermented at the lower fermentation temperature. The only treatment combination that did not show a statistically significant difference between fermentation temperatures was pomace from 'Triple Crown' yeast-inoculated berries. Oil concentration in pomace from 'Triple Crown' berries that had undergone a wild-type fermentation at the higher fermentation temperature was significantly higher than oil concentration in 'Triple Crown' pomace that had undergone a wild-type

fermentation at the lower fermentation temperature (p=0.0024). Extractable lipid content in 'Natchez' pomace from berries that had undergone yeast-inoculated at the higher fermentation temperature were higher than extractable lipid contents in pomace fermented at the lower fermentation temperature (p=0.0064).

The total lipid concentration seen in whole blackberries and blackberry pomace ranged from 1.5 to 4.5 % in this study. This range was within the range reported in literature for fresh blackberries (1-2 %) and pomace (2-6 % oil) (Zhao 2007, Natural Sourcing 2013, Blackberries Nutrition Facts 2014). In this study, the pomace samples were from berries harvested in 2012; these samples had extractable lipid concentrations of 3.78 % for 'Natchez' whole berries and 2.94 % for 'Triple Crown' whole berries as seen in table 10. Comparing pomace to whole berries, the 'Natchez' berries had higher lipid percentages in the whole berries than in the pomace, whereas the opposite relationship was seen with 'Triple Crown' berries i.e. the percent lipids seen in pomace samples was higher than the percent lipids measured in whole berries except for 'Triple Crown' pomace that were fermented at the lower fermentation temperature. For the pomace samples, the higher fermentation temperature treatment yielded higher apparent lipid concentrations in the pomace after fermentation almost without exception. The effects of cultivar and inoculation treatment were not consistent. It is possible that higher fermentation temperatures could have helped in seed separation and general tissue maceration, which would have increased the percentage of seed material in the final pomace and thus increased the concentration of lipids in the final pomace. These potential effects may have also helped facilitate lipid extraction from the pomace that had been subjected to the higher temperature fermentation treatment. During wine processing, it was noted that the pomace from the 'Natchez' berries had relatively more and larger seeds than 'Triple Crown' pomace; this could help account for the observed increase in percentage of extractable lipids seen in 'Natchez'

pomace. For pomace from 'Triple Crown' berries, the samples had a higher percentage of extractable lipids than that of whole berries. This is very likely due to the concentration of relatively lipid-rich skin and seed material that occurred in the production of the pomace.

In addition, the variation seen in lipid concentration could have arisen as a result of limitations in the extraction procedure. For example perhaps the pomace samples were not homogeneous and some samples contained a higher percentage of seeds and therefore lipids simply by chance. The physical characteristics of the pomace may have varied from sample to sample as well, which may have impacted the efficacy of the grinding process and therefore the efficiency of the extraction process. More study would be needed to clarify these possibilities.

Antioxidant activity analyses

Modified Harbertson-Adams assay

1. Total phenolic content

The total phenolic concentration in blackberry juice and wine, expressed as mg/L catechin equivalents, are shown in figure 9 (for juices and wines made from 'Natchez' berries) and figure 10 (for juices and wines made from 'Triple Crown' berries). Overall, the total phenolic concentrations of blackberry juice and wine tended to decrease over time. Between the two cultivars, 'Natchez' berries showed slightly higher total phenolic concentration than 'Triple Crown' berries. Interestingly, the lower fermentation temperature was associated with higher phenolic concentration in the 'Natchez' wines whereas higher phenolic concentration was associated with higher fermentation temperature in 'Triple Crown' wines.

Overall, Total phenolic concentrations ranged from 440 to 1420 mg/L catechin equivalents (CE). Our results were similar to Arozarena and others (2012) which showed between 601 to1624 mg/L CE. These researchers also noted that different locations of the fruit possessed different total phenolic values. According to Kopjar and others (2011) blackberry wine showed a similar lower end for total phenolics with values ranging from 380 to 520 mg/L CE. This article examined the effect of storage on total phenolics; the levels of total phenolic decreased over time. Other blackberry wine articles (Johnson and others 2011, Ortiz and others 2013) showed that range of total phenolics was between 955 to 1400 mg/L GAE which were close to the higher end of our results. However, some blackberry wine research (Mudnic and others 2012, Mitic and others 2013) had total phenolic concentrations between 1608 and 2836 mg CE/L which were higher than our result. Since different wine-making technique and different cultivars were applied during processing berries the total phenolic concentration values were varied.

Samples with wild-type fermentations generally showed higher total phenolic concentrations than yeast inoculations. It is possible that wild berry microorganisms created a fermentation environment that was more conducive to the preservation of phenolic compounds – perhaps by inhibiting phenolic polymerization and complex formation – or perhaps that yeast-inoculation facilitated loss of phenolics.

Fermentation temperature also affected total phenolic concentrations. Higher fermentation temperatures were generally correlated with higher total phenolics concentrations. The one exception to this rule was ‘Natchez’ wild-type fermentation samples. It is possible that phenolics in the ‘Natchez’ berries were more sensitive to heat than those from ‘Triple Crown’ berries and, because fermentation took longer at the lower fermentation

temperature and blackberry skins and seeds spent more time in the wine at the lower fermentation temperature, more phenolic compounds were extracted into the wines made from 'Natchez' berries. Overall, total phenolics concentration in wines made from 'Triple Crown' berries was more consistent values at the higher fermentation temperature and more phenolics were extracted than at the lower fermentation temperature.

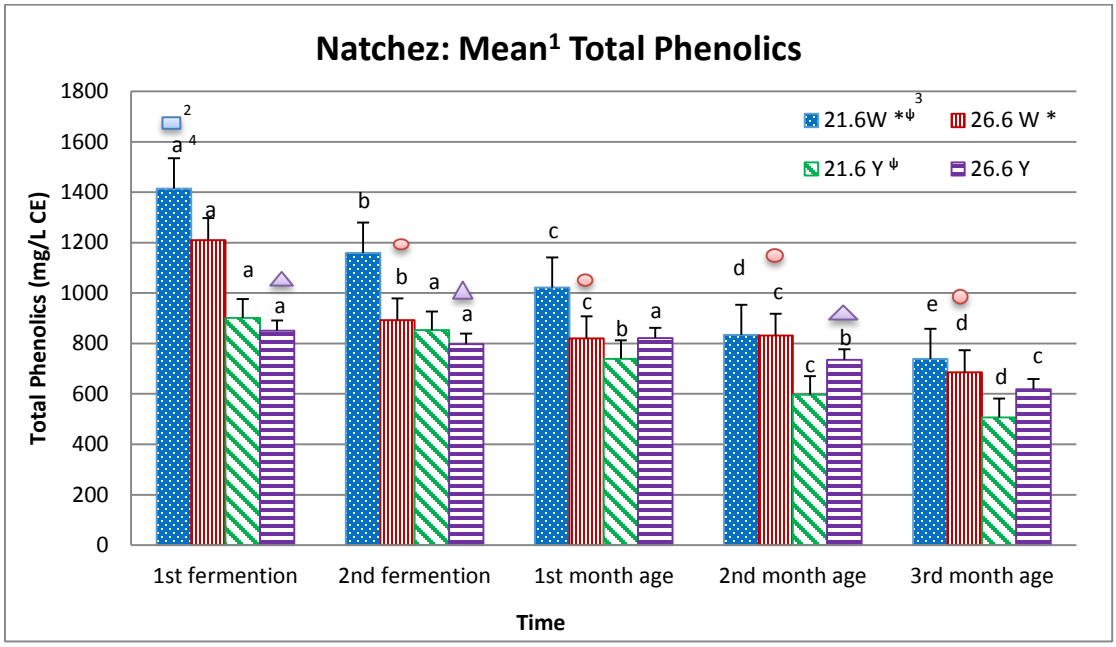

Figure 9. Mean total phenolic concentration of 'Natchez' blackberry fermented juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

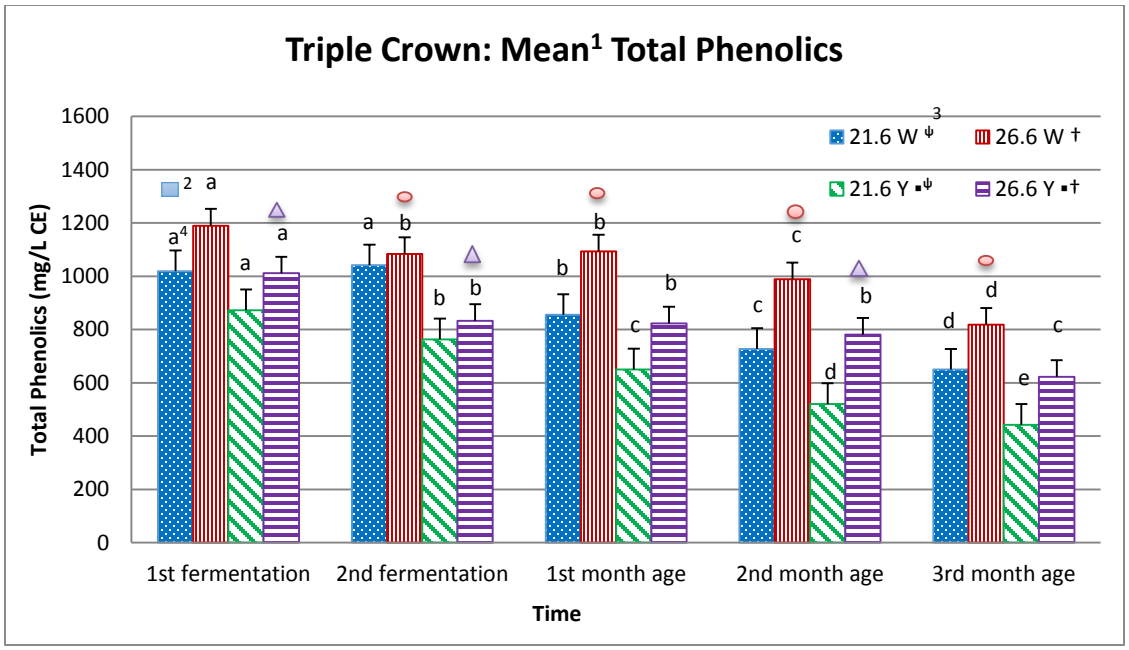

Figure 10. Mean total phenolic concentrations of 'Triple Crown' blackberry fermented juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

Comparing samples over time, statistically significant differences were found among all samples ($p<0.001$). Comparing between cultivars, all samples with wild-type fermentations showed higher total phenolic concentrations than yeast-inoculated wines. For the wild-type fermentation with low fermentation temperature, there was a statistically significant differences between the two cultivars at the 1st fermentation time ($p<0.0001$). For the wild-type fermentation with high fermentation temperature samples, there were statistically significant

differences between the two cultivars for all treatments except for the 1st fermentation time ($p<0.0001$). In the case of yeast inoculation with low fermentation temperatures, there were no statistically significant differences between cultivars ($p>0.05$). However, for yeast-inoculated wines with the high fermentation temperature, there were statistically significant differences between the two cultivars at the stage of 1st fermentation ($p<0.0001$), 2nd fermentation ($p=0.009$), and 2nd month ageing ($p=0.0046$).

Comparing fermentation temperatures within cultivar at a given time, statistically significant differences were seen at all times between samples fermented at high and low temperatures for all wines made from 'Natchez' berries with wild-type fermentations ($p=0.0004$ for 2nd month ageing; $p<0.0001$ for the rest). For 'Natchez' berries with yeast inoculation, statistically significant difference between the two fermentation temperatures were observed at the stage of 1st fermentation ($p=0.0002$), and 2nd fermentation ($p=0.0001$). In the case of wines made from 'Triple Crown' berries with wild-type fermentations, statistically significant differences between the two inoculation temperatures were seen at the 2nd fermentation stage ($p=0.0029$). For wines made from 'Triple Crown' berries with yeast inoculation, there were statistically significant differences between the two fermentation temperatures on all of the samples during ageing ($p= 0.0113$ for 1st month ageing; $p<0.001$ for 2nd month ageing; $p=0.0072$ for 3rd month ageing).

Comparing inoculation levels at a given time, significant differences were seen between both cultivars at the lower fermentation temperature ($p<0.0001$). In the case of higher fermentation temperatures, only wines made from 'Triple Crown' berries showed significant differences between inoculation treatments over time ($p=0.0007$ for 1st fermentation; $p=0.0002$ for 3rd month age; $p<0.0001$ for the rest).

2. Non-tannin polymeric pigments

The non-tannin polymeric pigments are expressed as mg/L CE. Results for wines made from 'Natchez' berries are shown in figure 11 and for wines made from 'Triple Crown' berries in figure 12. Overall, the graphs showed a trend toward decreasing values over the time period in this study. Wines made from 'Natchez' berries with a wild-type fermentation had higher levels of non-tannin pigments than wines made with yeast inoculation. For wines made from 'Natchez' berries with a wild-type fermentation, the lower fermentation temperature had more non-pigment tannins. Between samples of yeast-inoculated wines made from 'Natchez' berries, samples made using the higher fermentation temperature had more non-tannin pigments. Looking at wines made from 'Triple Crown' berries with both inoculation treatments, samples made using the higher fermentation temperature had more non-tannin pigments than those made using the lower fermentation temperature.

Overall, the range of non-tannin pigment levels observed was from 0 to 1337 mg/L CE. This result was very similar to that seen in the previous test i.e. total phenolic concentration. Both cultivars showed higher non-tannin pigments concentrations with a wild-type fermentation than with yeast inoculation. Again, it appears that either a wild-type fermentation acts to preserve phenolic compound concentrations or yeast inoculated acts to decrease the retention of the same compounds.

Fermentation temperature also affected non-tannin pigments. In general, the higher fermentation temperatures seemed to favor higher non-tannin pigment concentrations, perhaps because the berries were better broken down and the pigments were better extracted during the fermentation process. Wines made from 'Natchez' berries showed higher non-tannin pigment concentrations at the lower fermentation temperature during fermentation but

showed better retention of these compounds at the higher storage temperature during ageing. Lower fermentation temperatures may have favored pigment extraction during the initial fermentation process since that process took a relatively longer time to finish at the lower temperature. Regardless, the pigments were not especially stable during ageing as the total non-tannin pigment concentrations dramatically decreased and dropped to close to zero at the end of 3rd month of ageing.

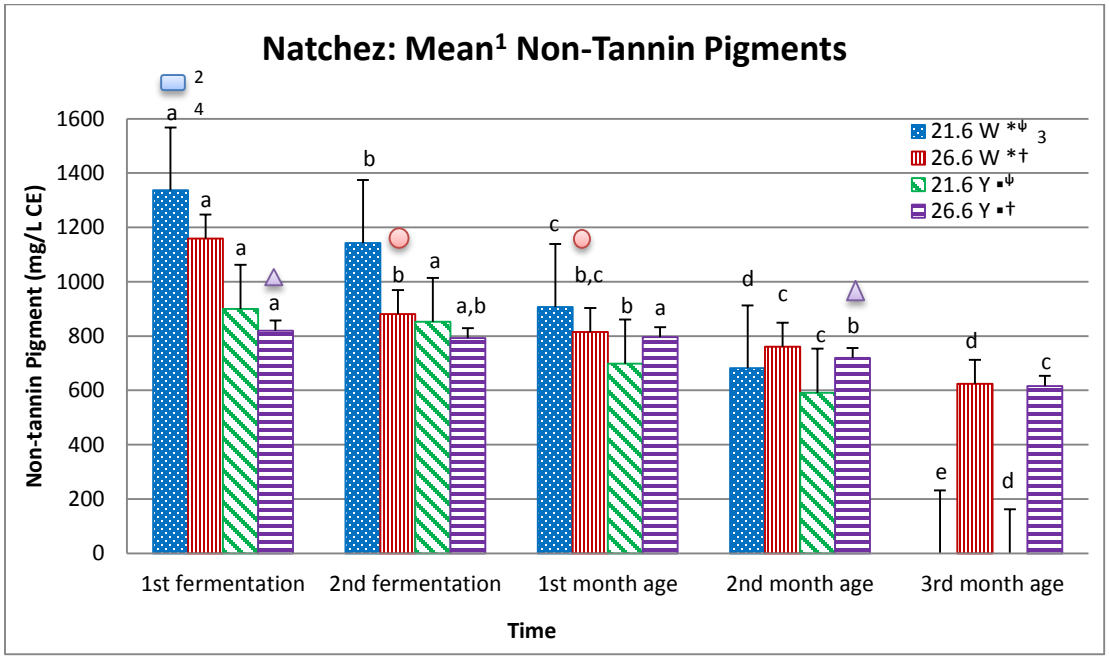

Figure 11. Mean non-tannin pigments of 'Natchez' blackberry fermented juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

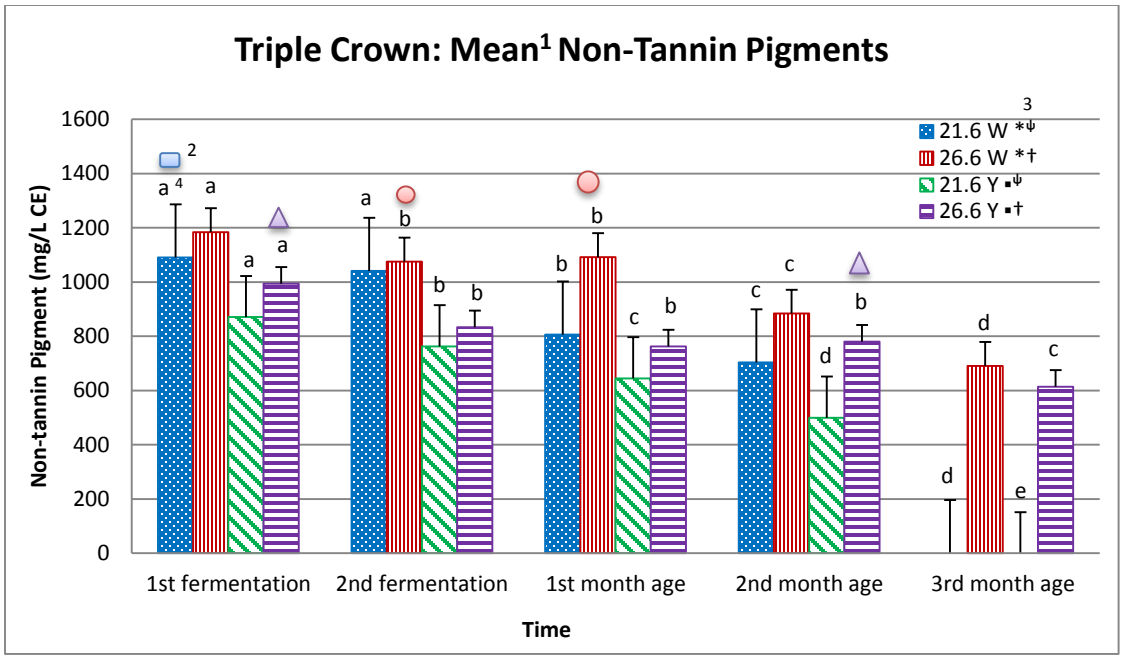

Figure 12. Mean non-tannin polymeric pigments of 'Triple Crown' berries fermented juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

Comparing samples over time, statistically significant differences were found among all samples ($p<0.0001$). For example, between cultivars samples made with a wild-type fermentation and at the lower fermentation temperature were statistically significantly different at the stage of 1st fermentation ($p=0.004$). Also, wild-type fermentation with high fermentation temperature showed statistically significant differences between the two cultivars at the stage of 2nd fermentation ($p=0.0028$) and 1st month ageing ($p<0.0001$). In the case of yeast inoculation

with low fermentation temperatures, there were no statistically significant differences between the two cultivars ($p>0.05$). However, with yeast inoculation and high fermentation temperatures, there were statistically significant differences between the two cultivars observed at the stage of 1st fermentation ($p=0.0002$) and 2nd month ageing ($p=0.432$).

Comparing the two fermentation temperatures within cultivar at a given time, for wines made from 'Natchez' berries with a wild-type fermentation, statistically significant differences between the two fermentation temperatures were seen at the stages of 1st fermentation ($p=0.0002$), 2nd fermentation ($p<0.0001$), 1st month ageing ($p=0.0193$), and 3rd month ageing ($p<0.0001$). For wines made from 'Natchez' berries with yeast fermentation, there were statistically significant differences seen between fermentation temperatures at the stage of 1st fermentation ($p=0.0017$), 2nd fermentation ($p=0.0047$), and 3rd month ageing ($p<0.0001$). In the case of wines made from 'Triple Crown' berries with a wild-type fermentation, there were statistically significant differences seen between the two fermentation temperatures at the stage of 1st month ageing ($p=0.0109$) and 3rd month ageing ($p<0.0001$). For wines made from 'Triple Crown' berries with yeast inoculation, there were statistically significant differences observed between the two fermentation temperatures at the stage of 2nd month ageing ($p=0.0002$) and 3rd month ageing ($p<0.0001$).

To compare inoculation treatments within cultivar at a given time, for wines made from 'Natchez' berries with low fermentation temperature, there were statistically significant differences seen between wild-type and yeast-inoculated wins at the stage of 1st fermentation ($p<0.0001$), 2nd fermentation ($p<0.0001$) and 1st month ageing ($p=0.004$). For wines made from 'Natchez' berries at the higher fermentation temperature, there were statistically significant differences seen between the two inoculation treatments at the stage of 1st fermentation

($p<0.0001$) and 2nd fermentation ($p=0.0483$). For wines made from 'Triple Crown' berries at the lower fermentation temperature, there were statistically significant differences observed between the two inoculation levels at the stage of 1st fermentation ($p<0.0001$), 2nd fermentation ($p<0.0001$), 1st month ageing ($p=0.0009$), and 2nd month ageing ($p<0.0001$). Finally, for wines made from 'Triple Crown' berries at the higher fermentation temperature, there were statistically significant differences noted between the two inoculation treatments at the stage of 1st fermentation ($p=0.0153$), 2nd fermentation ($p=0.0011$) and 1st month ageing ($p<0.0001$).

3. Polymeric pigments and tannin contents

3-1. Small polymeric pigments

Polymeric pigments were divided up by their size in the Adams-Harberston assay. The small polymeric pigments found in wines made from 'Natchez' berries are shown in figure 13 while those found in wines made from 'Triple Crown' berries are shown in figure 14. Overall, samples made from both 'Natchez' berries and 'Triple Crown' berries had slightly different trends over the observed time period. Wines made from 'Natchez' berries showed values that were closer to each other and more uniform than those seen in wines made from 'Triple Crown' berries. In the case of wild-type fermentation using 'Natchez' berries, small polymeric pigments were seen to be higher at the lower fermentation temperature.

Overall, the range of small polymeric pigments measured in this study was from 0.64 to 2.10 mg/L. The concentration of small polymeric pigments is assumed to be a function of anthocyanin concentrations and stability in the juices and wines and were considered to be part

of the total color pigments found in the juice and wines and to be derived mostly from monomeric anthocyanins present in the starting juice.

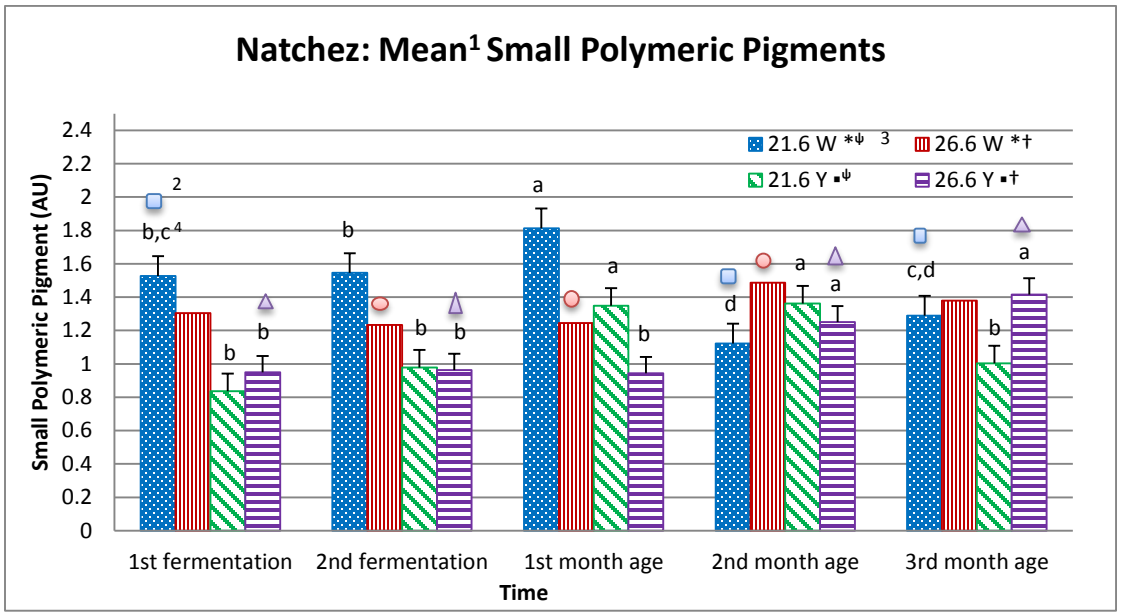

Figure 13. Mean small polymeric pigments of 'Natchez' blackberry fermented juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

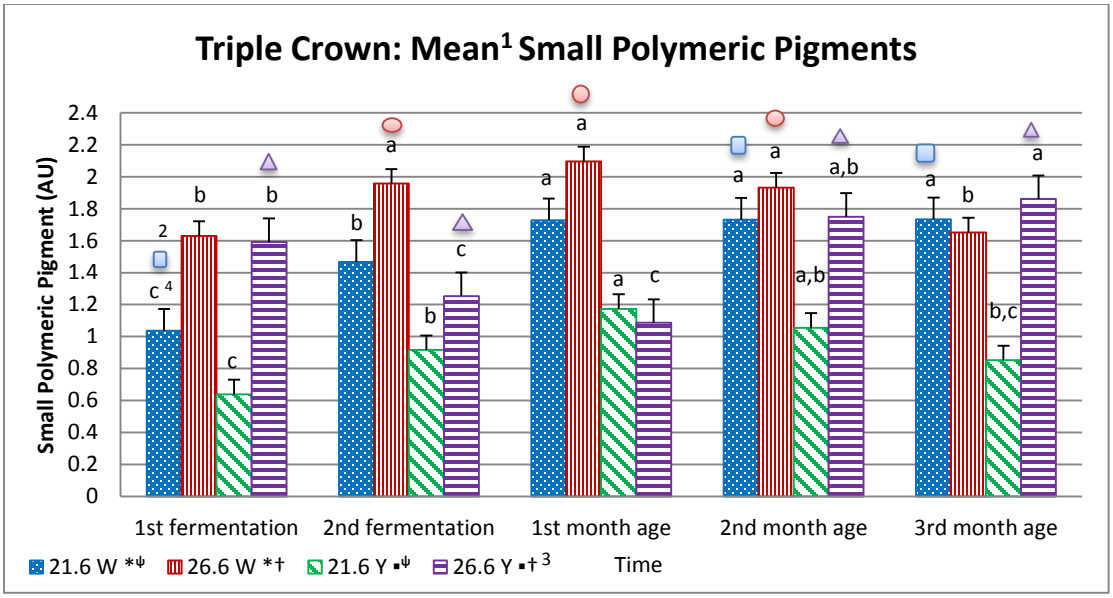

Figure 14. Mean small polymeric pigments of 'Triple Crown' blackberry fermented juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

Comparing samples over time, all of the wines made from 'Natchez' berries at the lower fermentation temperature showed statistically significant differences ($P<0.0001$) regardless of inoculation treatment. At the higher fermentation temperature, wines made from 'Natchez' berries with yeast inoculation showed statistically significant differences over time ($p=0.0003$ for high fermentation temperature). For wines made from 'Triple Crown' berries, statistically significant differences were seen among inoculation treatments and fermentation temperature treatments over time ($p<0.0001$ for wild-type fermentation with low fermentation temperature

and yeast inoculation with high fermentation temperature; p=0.0008 for wild-type fermentation with high fermentation temperature and yeast inoculation with low fermentation temperature).

Comparing samples between cultivars at a given time, statistically significant differences were seen between wines made from the two cultivars using a wild-type fermentation and the lower fermentation temperature at the stage of 1st fermentation (p=0.0389), 2nd month ageing (p=0.0001) and 3rd month ageing (p=0.0023). For samples made using a wild-type fermentation and the higher fermentation temperature, there were statistically significant differences seen between two cultivars at the stage of 2nd fermentation (p=0.0003), 1st month ageing (P<0.0001) and 2nd month ageing (p=0.0299). For wines made with yeast inoculation at the lower fermentation temperature, none showed statistically significant differences whereas at the higher fermentation temperature statistically significant differences were seen between the two cultivars at the stage of 1st fermentation (p=0.0002), 2nd fermentation (p=0.0455), 2nd month ageing (p=0.002) and 3rd month ageing (p=0.0049).

Comparing fermentation temperature treatments within cultivar at a given time, wines made from 'Natchez' berries using a wild-type fermentation were statistically significantly different based on fermentation temperature at the stage of 1st month ageing (p=0.0073), and 2nd month ageing (p=0.0147). For wines made from 'Natchez' berries with yeast inoculation, statistically significant difference were observed between the two fermentation temperatures at 1st month ageing (p=0.0014). For wines made from 'Triple Crown' blackberries using a wild-type fermentation, there were statistically significant differences seen between the two fermentation temperatures at the stage of 1st fermentation (p=0.006) and 2nd fermentation (p=0.0269). Finally, for wines made from 'Triple Crown' blackberries using yeast inoculation, there were statistically significant differences seen between the two fermentation temperatures at the

stage of 1st fermentation ($p<0.0001$), 2nd month ageing ($p= 0.0056$), and 3rd month ageing ($p<0.0001$).

Compare inoculation treatments within cultivar at a given time, wines made at the higher fermentation temperature were shown more statistically significant differences between wild-type and yeast-inoculated samples than wines made at the lower fermentation temperature. For wines made from 'Natchez' berries at the lower fermentation temperature, statistically significant differences were seen between the two inoculation treatments at the stage of 1st fermentation ($p=0.0022$), 2nd fermentation ($p=0.0151$), and 2nd month ageing ($p=0.0437$). For wines made from 'Natchez' berries at the higher fermentation temperature, statistically significant differences were observed between the two inoculation treatments at the stage of 1st fermentation ($p=0.0067$), 2nd fermentation ($p=0.023$), 1st month ageing ($p=0.0155$), and 2nd month ageing ($p=0.0363$). For wines made from 'Triple Crown' berries using the lower fermentation temperature, there were statistically significant differences seen between the two inoculation levels at the stage of 2nd fermentation ($p=0.0096$), 1st month ageing ($p=0.0089$), 2nd month ageing ($p=0.0012$) and 3rd month ageing ($p<0.0001$). Lastly for wines made from 'Triple Crown' berries using the high fermentation temperature, there were significant differences observed between the two inoculation levels at the stage of 2nd fermentation ($p=0.0001$) and 1st month ageing ($p<0.0001$).

3-2. Large polymeric pigments

The mean values for large polymeric pigment concentrations are shown in table 11. First of all, we should note that wines made from 'Natchez' berries using a wild-type fermentation at the higher fermentation temperature presented a value that likely was an outlier at the stage of

1st fermentation. This could have happened due to sampling error or simply inherent sample instability. That sample aside, wines made from values for most samples tended to decrease over time and to show fairly large variability. This could be due to natural fluctuations in the concentration of large polymeric pigments at any given point during fermentation and aging as polymers formed and then precipitated out of solution.

Overall, the large polymeric pigments were ranged from 0 to 0.46 mg/L in juice, exclude the value described as an outlier. Large polymeric pigments concentration generally decreased during the winemaking process. Large polymeric pigments were generally present at lower levels in blackberry juice and wine samples than small polymeric pigments, likely due to the fact that larger polymers would more readily precipitate out of solution. Wines made with lower the fermentation temperature generally showed higher levels of large polymeric pigments than wines made at the higher fermentation temperature. This could simply be a function of slower rates of polymerization and precipitation at the lower fermentation temperature.

Table 11. Mean values of large polymeric pigments of 'Natchez' blackberry and 'Triple Crown' blackberry juice and wine (mg/L).

	'Natchez' Blackberries				'Triple Crown' Blackberries			
	21.6 W	**26.6 W**	**21.6 Y**	**26.6 Y**	**21.6 W**	**26.6 W**	**21.6 Y**	**26.6 Y**
1st fermentation	$1.218^{\Psi *\dagger}$	$4.604^{a*\dagger}$	$0^{\dagger}$	$0^{\Psi\dagger}$	0.501^{Ψ}	1.036	0.613	0.492^{Ψ}
2nd fermentation	1.317	$0.158^{b\Psi}$	1.201	0^{Ψ}	1.192	0.013^{Ψ}	0.308	0.02^{Ψ}
1st month age	1.137	$0.202^{b\Psi}$	1.123	0.173	0.15	0.212^{Ψ}	0.268	0.325
2nd month age	0.426^{Ψ}	$0.354^{b\Psi}$	0	0^{Ψ}	0.176^{Ψ}	0.259^{Ψ}	0.144	0^{Ψ}
3rd month age	0.455^{Ψ}	0.36^{b}	0.338	0.075^{Ψ}	0.302^{Ψ}	0.631	0.4	0^{Ψ}

n= 6

$^{\Psi}$ Denotes a statistically significant difference between the two cultivars within inoculation treatment and fermentation temperature ($p<0.05$).

* Denotes a statistically significant difference between fermentation temperatures within cultivar and inoculation treatment ($p<0.0001$).

† Denotes a statistically significant difference between inoculation treatments within cultivar and fermentation temperature ($p=0.0474$ for wild-type fermentation at low temperature, $p<0.0001$ for high temperature).

Looking at the samples over time, the only statistically significant difference among times observed was in wines made from 'Natchez' berries using a wild-type fermentation at the higher fermentation temperature ($p<0.0001$). Since this sample showed such variability, it is difficult to attach practical significance to the statistical difference observed.

Comparing between cultivars at a given time, wild-type fermentations made at the lower fermentation temperature showed statistically significant differences at the stage of 1st fermentation ($p=0.0389$), 2nd month ageing ($p=0.0001$), and 3rd month ageing ($p=0.0023$). For wild-type fermentations made using the higher fermentation temperature, there were statistically significant differences seen between the two cultivars at the stage of 2nd fermentation ($p=0.0003$), 1st month ageing ($p<0.0001$), and 2nd month ageing ($p=0.0299$). In the case of yeast-inoculated samples, there were no significant differences observed between the two cultivars at the lower fermentation temperature. However, at the higher fermentation temperature, yeast-inoculated samples did show statistically significant differences between two cultivars at the stage of 1st fermentation ($P=0.0002$), 2nd fermentation ($p=0.0455$), 2nd month ageing ($p=0.002$) and 3rd month ageing ($p=0.0049$).

Comparing fermentation temperatures within cultivar at a given time, wines made from 'Natchez' berries at high and low fermentation temperatures using a wild-type fermentation were statistically significantly different at the stage of 1st fermentation ($p<0.0001$).

Comparing inoculation treatments within cultivar at a given time, wild-type fermented wines made from 'Natchez' berries using the lower fermentation temperature were statistically significant different from yeast-inoculated wines at the stage of 1st fermentation ($p=0.0474$). Also, for wines made from 'Natchez' berries using the higher fermentation temperature, there was statically significant differences between the two inoculation treatments at the stage of 1st

fermentation ($p<0.0001$). There were no significant differences seen in wines made from 'Triple Crown' berries based on fermentation temperature and inoculation treatment ($p>0.05$).

3-3. Tannin content

The tannin concentrations of fermented blackberry juice and wines were expressed as mg/L catechin equivalents and are shown in table 12. As with the large polymeric pigments – chemically closely related to tannins – a fair amount of variability was seen in tannin concentrations over time and among treatments.

Wines made from 'Natchez' berries showed level of tannins that decreased the most at the 2nd fermentation stage, then increased during the initial portion of the aging period, but decreased again by the 3rd month of ageing. Tannin concentrations in all of the wines made at the lower fermentation temperature decreased to zero by the 3rd month ageing for both cultivars and inoculation treatments. For wines made using a wild-type fermentation, tannin concentrations were s higher at low fermentation temperature but the opposite was observed with yeast-inoculated wines.

Overall, the range of tannin concentrations seen in these experiments was from 0 to 300 mg/L CE. As with the previous results for large polymeric pigments, the tannin concentrations had the same sample ('Natchez', wild-type fermentation, higher fermentation temperature) that was identified as an outlier. The same possible causes for the variability seen in large polymeric pigment concentrations noted early could easily be applied to the chemically close-related tannin compounds.

Table 12. Mean concentration of tannins in 'Natchez' blackberry and 'Triple Crown' blackberry juice and wine (mg/L Catechin Equivalents).

	'Natchez' Blackberries				'Triple Crown' Blackberries			
	21.6 W	**26.6 W**	**21.6 Y**	**26.6 Y**	**21.6 W**	**26.6 W**	**21.6 Y**	**26.6 Y**
1st fermentation	$77.386^{bc\dagger}$	299.869	0.75†	30.143	0	6.347^{b}	2.178	17.036
2nd fermentation	16.193^{c}	10.623	0	5.333	0	7.802^{b}	0	0
1st month age	$114.427^{ab*\dagger}$	5.095*	39.828†	25.393	49.25	1.544^{b}	5.345	60.56
2nd month age	$151.81^{a\psi*\dagger}$	$69.917^{\psi*}$	4.679†	16.375	23.149^{ψ}	$105.542^{a\psi\dagger}$	21.161	$1.119^{\dagger}$
3rd month age	0^{c}	61.167^{ψ}	0	1.893	0*	$128.387^{a\psi*\dagger}$	0	$9.589^{\dagger}$

n= 6

$^{\psi}$ Denotes a statistically significant difference between the two cultivars within inoculation treatment and fermentation temperature ($p<0.05$).

* Denotes a statistically significant difference between fermentation temperatures within cultivar and inoculation treatment ($p<0.05$).

† Denotes a statistically significant difference between inoculation treatments within cultivar and fermentation temperature ($p<0.05$).

Looking at wild-type fermentations over time, statistically significant differences were found in wines made from 'Natchez' berries using the lower fermentation temperature ($p<0.0001$) and in wines made from 'Triple Crown' berries using the higher fermentation temperature ($p=0.0001$).

Comparing cultivars within fermentation temperature and inoculation treatment at given times, wines made using a wild-type fermentation at the lower fermentation temperature from 'Natchez' berries were seen to be statistically significantly different from wines made from 'Triple Crown' berries at the stage of 2nd month ageing ($p=0.0099$). For wines made using a wild-type fermentation at the higher fermentation temperature, there were statistically significant differences observed between the two cultivars at the stage of 2nd month ageing ($p=0.04$) and 3rd month ageing ($p=0.0075$).

Comparing the two inoculation treatments within fermentation temperature and cultivar at given times, wild-type fermented wines made from the 'Natchez' berries with low fermentation temperature were statistically significantly different at the stage of 1st

fermentation (p=0.0392), 1st month ageing (p=0.0432), and 2nd month ageing (p=0.0007) than yeast-inoculated wines. In the case of wines made from 'Triple Crown' berries using the higher fermentation temperature, there were statistically significant differences seen between the two inoculation treatments at the stage of 2nd month ageing (p=0.0144) and 3rd month ageing (p=0.0064).

Comparing the two fermentation temperatures within cultivar and inoculation treatment at given times, higher-fermentation temperature wines made from 'Natchez' berries using a wild-type fermentation were statistically significantly different at the stage of 1st month ageing (p=0.0005) and 2nd month ageing (p=0.0028) than wild-type fermented wines made at the lower fermentation temperature. Wines made from 'Triple Crown' berries with a wild-type fermentation showed statistically significant differences between fermentation temperatures at the stage of 3rd month ageing (p=0.0114).

4. Anthocyanin contents

Anthocyanin concentration of 'Natchez' berries is shown in figure 15 and anthocyanin concentration of 'Triple Crown' berries is shown in figure 16. Anthocyanin concentrations are expressed as mg/L malvidin 3-monoglucoside.

All of the berry juice and wine samples showed decreasing monomeric anthocyanin concentrations over time. Within cultivars, wine samples made with yeast inoculation tended to have higher anthocyanin levels than wines made with wild-type fermentations. Interestingly, wines made from 'Natchez' berries showed higher anthocyanin concentrations at the higher

fermentation temperature whereas wines made from ‘Triple Crown’ berries showed higher levels at the lower fermentation temperature.

Overall, the anthocyanin concentrations were range from 85 to 320 mg/L malvidin 3-monoglucoside. Our Adams-Harbertson assay results showed somewhat higher anthocyanin concentration values than some other reports. According to Mudnic and others (2012), the range of anthocyanin were from 12 to 167 mg/L malvidin 3-glucoside in blackberry wine, which was two-fold lower than our result. Also, other wine research articles (Johnson and others 2011, Arozarena and others 2012) reported that the range of anthocyanin was between 17 to 192 mg/L cyanidin 3-glucose which was also a lower value than our results. It is useful to note that these wines were prepared using different methods than the wines processing in the current study. It is possible that the fermentation method used in the current study affected anthocyanin concentrations of blackberry juice and wine. The blackberry cultivars used were different as well.

In this study, wines made from ‘Natchez’ berries showed higher anthocyanin concentrations than wines made from ‘Triple Crown’ berries. Since ‘Natchez’ berries were relatively bigger than ‘Triple Crown’ berries and contained a higher proportion of anthocyanin-rich skin, this might have led to increased anthocyanin concentrations. They also may have undergone a more complete maceration process than the ‘Triple Crown’ berries, being softer and thinner skinned.

Wines made from both cultivars showed higher concentrations of anthocyanins associated with yeast-inoculation treatments. Adding yeast accelerated the speed of the fermentation process; this may have favored the process of co-pigmentation and thereby slowed the degradation of anthocyanin during fermentation and aging. Further, both cultivars

showed relatively high anthocyanin concentrations at the stage of 1st fermentation. Concentrations then tended to slightly decrease or stay nearly the same over time. This indicates that it is important to extract anthocyanin concentrations as completely as possible at the beginning of fermentation in order to maximize color density and antioxidant activity.

Fermentation temperature influenced the anthocyanin concentrations the most, but not consistently: 'Natchez' berries showed higher anthocyanin concentrations at the higher fermentation temperature while 'Triple Crown' berries showed higher anthocyanin concentrations at the lower fermentation temperature as noted above. It may be that higher fermentation temperatures serve to better extract anthocyanins from fruit tissue for some cultivars while lower temperatures are more effective for others. In support of this hypothesis, 'Natchez' berries were relatively large and soft-skinned. They may have broken down more easily at the higher fermentation temperature and this may have led to a more complete extraction of anthocyanins. On the other hand, 'Triple Crown' berries were relatively small and firm. They may not have broken down as well and thus the longer fermentation time seen at the lower fermentation temperature may have allowed a more complete extraction.

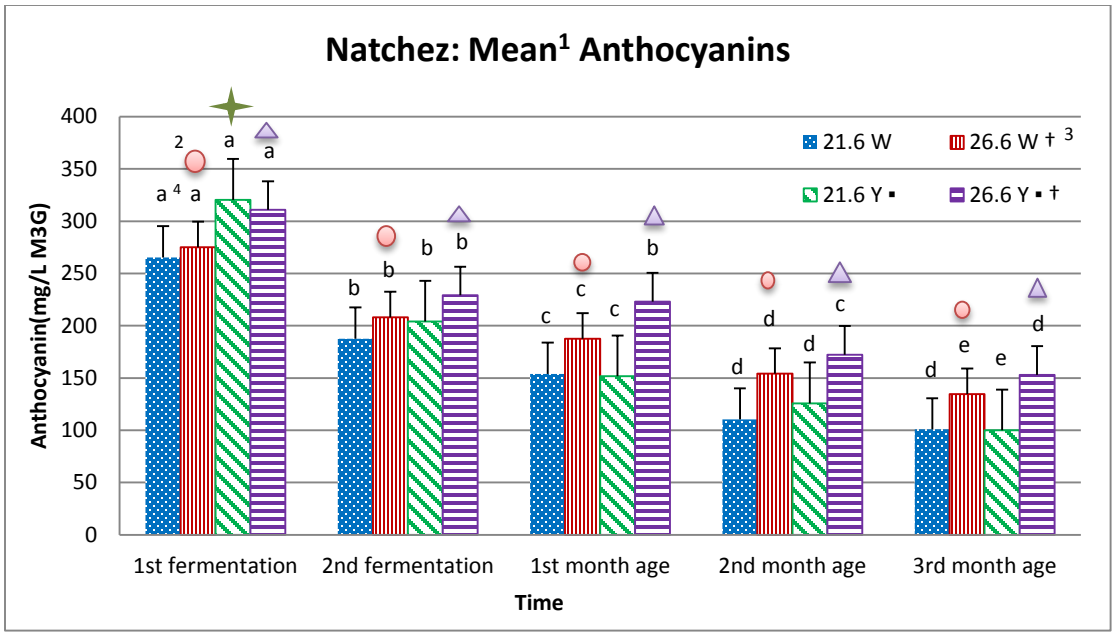

Figure 15. Mean total anthocyanin concentrations of 'Natchez' blackberry fermented juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

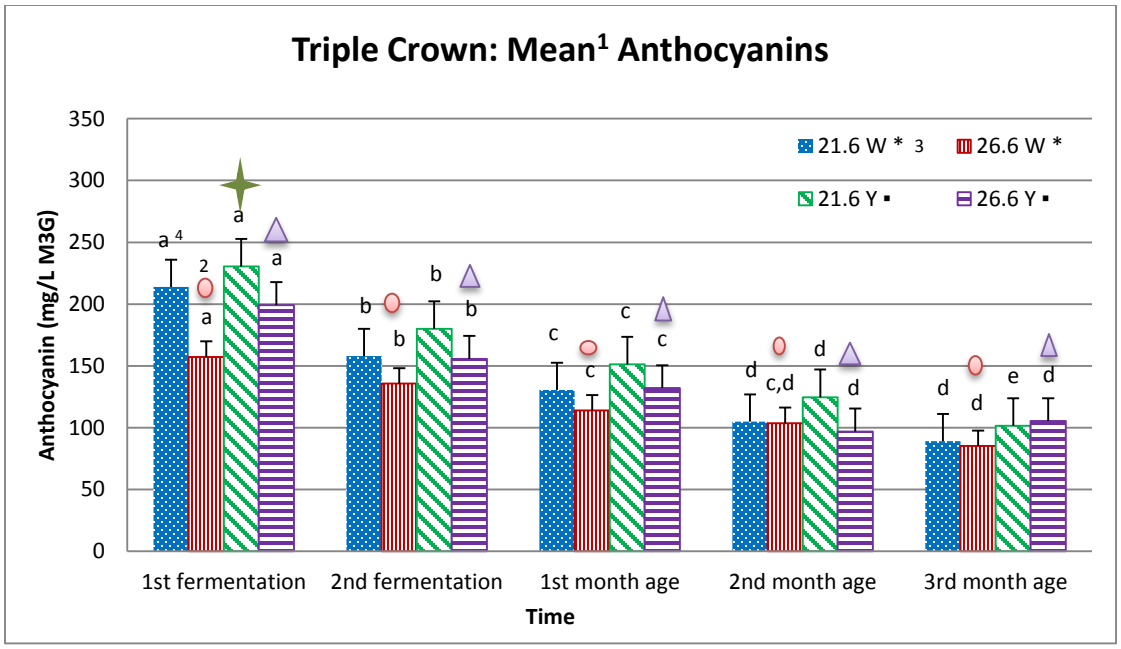

Figure 16. Mean total anthocyanin concentrations of 'Triple Crown' blackberry fermented juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

Comparing samples over time and within treatments, all of samples showed statistically significant decreases in anthocyanin concentration regardless of cultivar, inoculation treatment, and fermentation temperature ($p<0.0001$).

Comparing wines made from the two cultivars within inoculation treatments and fermentation temperatures at given times, 'Natchez' wines made using a wild-type fermentation at the higher fermentation temperature were significantly different from 'Triple Crown' wines at all 5 stages ($p<0.0001$ for 1st fermentation, 2nd fermentation and 1st month ageing; $p=0.0049$ for

2nd month ageing; and p=0.006 for 3rd month ageing). However, none of the samples made using a wild-type fermentation at the lower fermentation temperature showed statistically significant differences (p>0.05) between cultivars. For yeast-inoculated wines made using the lower fermentation temperature, the only statistically significant difference between cultivars was found at the stage of 1st fermentation (p<0.0001). For yeast-inoculated wines made at the higher fermentation temperature, there were statistically significant differences between two cultivars seen at all 5 stages (p<0.0001 for 1st fermentation, 1st month ageing and 2nd month ageing; p=0.0002 for 2nd fermentation; and p=0.0356 for 3rd month ageing).

Comparing fermentation temperatures within cultivar and inoculation treatment at given times, wines made from 'Natchez' berries using a wild-type fermentation and a higher fermentation temperature were only significant different from wines made at the lower fermentation temperature at the stage of 2nd month ageing (p=0.0179). For wines made from 'Natchez' berries using yeast inoculation, there were statistically significant differences between the two fermentation temperatures at all the stage of ageing wines (p<0.0001 for 1st month ageing; p=0.0016 for 2nd month ageing; and p=0.0003 for 3rd month ageing). In the case of wines made from 'Triple Crown' berries using a wild-type fermentation, there were statistically significant differences seen between the two fermentation temperatures at all of the 5 stages (p<0.0001 for 1st fermentation; p=0.0002 for 2nd fermentation; p=0.0007 for 1st month ageing; p=0.0159 for 2nd month ageing; and p=0.01 for 3rd month ageing). For wines made from 'Triple Crown' berries with yeast inoculation, there were statistically significant difference seen between the two fermentation temperatures at the stage of 1st fermentation (p=0.0134), 2nd fermentation (p=0.0456), and 2nd month ageing (p=0.0258).

Comparing inoculation treatments within cultivars and fermentation temperatures at given times, wild-type fermented wines made from 'Natchez' berries at the lower fermentation temperature were only significantly different from yeast-inoculated wines at the stage of 1st fermentation (p=0.0032). For wines made from 'Natchez' berries using the higher fermentation temperature, there were statistically significant differences seen between the two inoculation treatments at the stage of 1st fermentation (p=0.0242) and 1st month ageing (p=0.0244). For wines made from 'Triple Crown' berries using the lower fermentation temperature, there were no statistically significant differences found between the two inoculation levels (p>0.05). However, a statistically significant difference between inoculation treatments was seen for wines made from 'Triple Crown' berries using the higher fermentation temperature – but only at the stage of 1st fermentation (p=0.0014).

Oxygen radical absorbance capacity (ORAC)

ORAC values were expressed as μmol Trolox equivalents/ gram of juice. The higher the ORAC value, the stronger the antioxidant capacity found in the sample. The ORAC values for blackberry fermented juice and wines are shown in figure 17 for 'Natchez' berries and in figure 18 for 'Triple Crown' berries.

The antioxidant capacity of blackberry juices and wines deceased over the time period during which they were sampled. Wines made from 'Natchez' berries all showed statistically significant differences over time regardless of cultivar, inoculation treatment, and fermentation temperature (p=0.0008 for yeast inoculation with low fermentation temperature, p<0.0001 for all the rest. As well, all of the wines samples made from 'Triple Crown' berries except for yeast-inoculated wines made at the higher fermentation temperature showed significant differences

over time between inoculation treatments and fermentation temperatures ($p=0.0188$ for wild-type fermentation with high fermentation temperature, $p<0.0001$ for low fermentation temperatures).

Overall, the range of ORAC values observed were between 9776 and 37845 µmol Trolox equivalents (TE)/ gram of sample. As with the anthocyanin concentrations previously reported, the ORAC values recorded in this study were somewhat higher than those reported in other literature. According to Cespedes and others (2007), the range of ORAC values observed in Chilean blackberry juices were from 7200 to 29600 µmol TE/ gram of sample. This range was close to our result but slightly lower. Other wine articles showed that the ORAC value varied a lot depending on variety, geographic growing location, and extraction method (Siriwoharn and others 2004, Reyes-Carmona and others 2005, Denev and others 2010, Thomas and others 2012). As fermentation techniques and extraction methods are known to influence ORAC values, these results may indicate that the Korean fermentation style used in this study was more efficient at extracting and/or preserving antioxidant activity in the final wine than other fermentation methods.

Fermentation temperature influenced the antioxidant capacity in blackberry juice and wine in this study, but the results were not consistent over time. At times, 'Natchez' wines showed higher antioxidant activity at the lower low fermentation temperature and 'Triple Crown' wines showed higher antioxidant activities associate with the higher fermentation temperature. At other times the reverse was true. By the end of the three month aging process, higher antioxidant activity was generally correlated with higher fermentation temperatures for both cultivars and inoculation treatments.

For ‘Natchez’ wines, the highest antioxidant activity was found during fermentation and it decreased during aging. However, ‘Triple Crown’ wines showed the highest antioxidant activity at the beginning of ageing. This supports the idea that the antioxidant compounds were more easily extracted from the ‘Natchez’ berries.

In the case of pomace samples, blackberry seeds and skin residue had higher antioxidant activity than that of ageing wine samples, indicating that the pomace retains substantial amounts of antioxidant compounds even after wine processing.

Genetic differences can also affect the antioxidant capacity of blackberry wine: ‘Triple Crown’ berries are known to better tolerate higher growing temperatures than ‘Natchez’ berries.

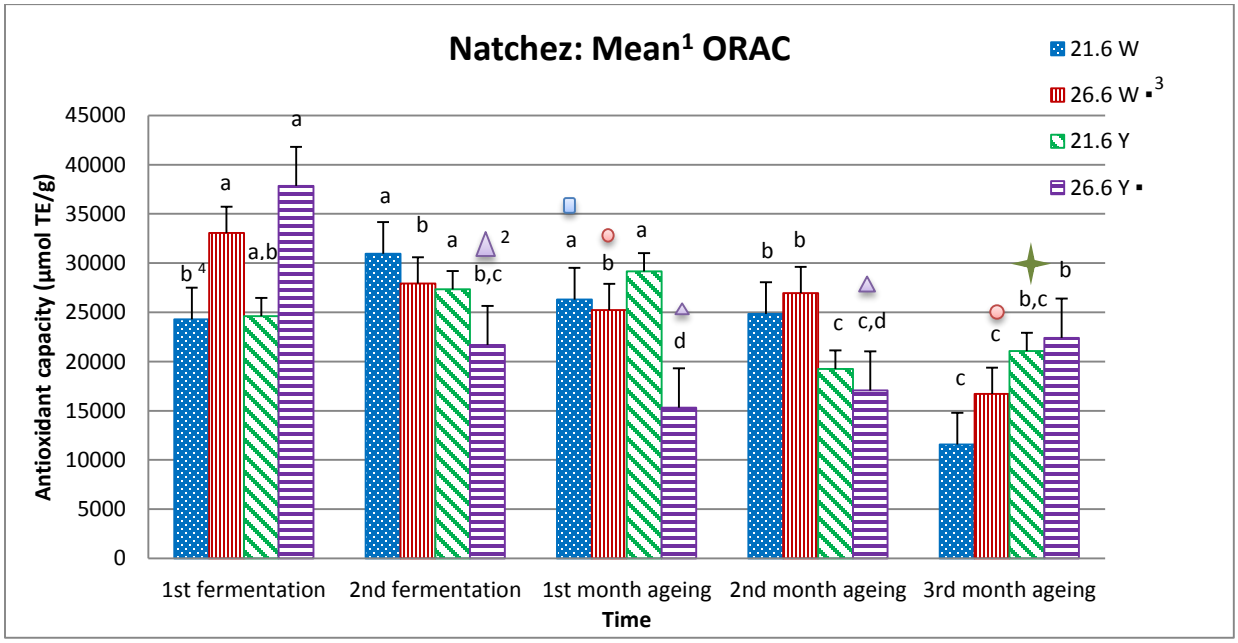

Figure 17. Mean oxygen radical absorbance capacity of ‘Natchez’ blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).

3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

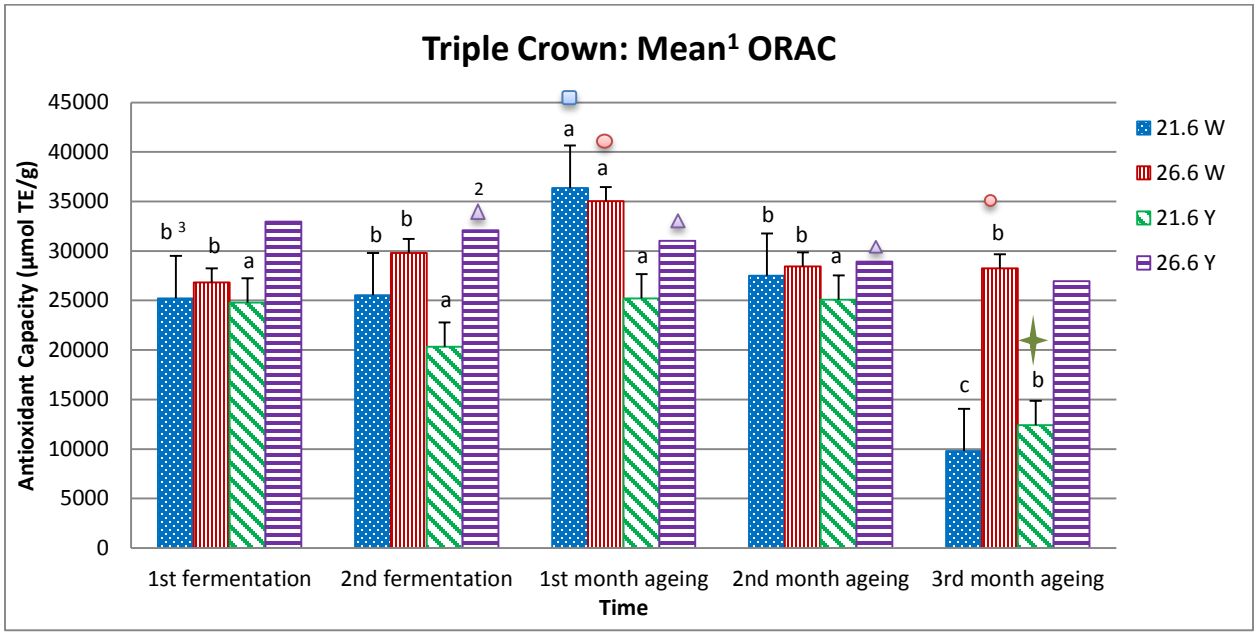

Figure 18. Mean oxygen radical absorbance capacity of 'Triple Crown' blackberry fermented juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

Comparing the two cultivars within inoculation treatment and fermentation temperature at given times, 'Triple Crown' wine samples showed generally higher antioxidant capacities than wines made from 'Natchez' berries. For wines made using a wild-type fermentation at the lower fermentation temperature, statistically significant differences were only seen between the two cultivars at the stage of 1st month ageing ($p=0.003$). For wines made

using a wild-type fermentation at the higher fermentation temperature, statistically significant differences were observed between the two cultivars at the stage of 1st month ageing (p=0.0107), and 3rd month ageing (p=0.0032). In yeast-inoculated wines made at the lower fermentation temperature, statistically significant differences were observed between the two cultivars at the stage of 3rd month ageing (p=0.0382) only. For yeast-inoculated wines made at the higher high fermentation temperature, there were statistically significant differences seen between the two cultivars at the stage of 2nd fermentation (p=0.0093), 1st month ageing (p=0.0002), and 2nd month ageing (p=0.0034).

Comparing the two fermentation temperatures within cultivar and inoculation treatment at given times, wines made from 'Natchez' berries generally showed more variability than wines made from 'Triple Crown' berries. The 'Natchez' wines made with a wild-type fermentation showed statistically significant differences between the two fermentation temperatures at the stage of 1st fermentation (p=0.0035). The 'Natchez' wines made with yeast inoculation showed statistically significant differences between the two fermentation temperatures at the stage of 1st fermentation (p=0.0011), and 1st month ageing (p=0.0003). In the case of 'Triple Crown' wines made using a wild-type fermentation, there were statistically significant differences seen between the two fermentation temperatures at the stage of 3rd month ageing (p<0.0001). For 'Triple Crown' wines made using a yeast inoculation, there were statistically significant differences observed between the two fermentation temperatures at the stage of 2nd fermentation (p=0.0121), and 3rd month ageing (p=0.0017).

Comparing the two inoculation treatments within cultivar and fermentation temperature at given times, wild-type fermented Natchez wines made at the lower fermentation temperature were significantly different than yeast-inoculated wines at the stage

of 3rd month ageing (p=0.0026). Wild-type fermented 'Natchez' wines made at the higher fermentation temperature were significantly different than yeast-inoculated wines at the stage of 2nd fermentation (p=0.047), 1st month ageing (p=0.042), and 2nd month ageing (p=0.042). In the case of 'Triple Crown' wines made at the lower fermentation temperature, significant differences between the two inoculation treatments were only seen at the stage of 1st month ageing (p=0.0124). There were no statistically significant differences seen between wild-type fermented and yeast-inoculated 'Triple Crown' wines made at the higher fermentation temperature.

The average mean ORAC values of blackberry pomace are shown in table 13. As for general trends, 'Triple Crown' pomace showed higher ORAC values than 'Natchez' pomace. For both cultivars, wild-type fermentation at the higher fermentation temperature showed the highest ORAC values.

Table 13. Mean ORAC values for blackberry pomace (n=3).

Cultivars	**Natchez**				**Triple Crown**			
Inoculation Treatment	*Yeast*		*Wild-type fermentation*		*Yeast*		*Wild-type fermentation*	
Fermentation Temp. (°C)	*21.6*	*26.6*	*21.6*	*26.6*	*21.6*	*26.6*	*21.6*	*26.6*
ORAC (µmol TE/g)	16920	18628 *	16625	19055 *	17209 ψ	12481 ψ*†	19428	23200 *†

N=24

ψ Denotes a statistically significant difference between fermentation temperatures within cultivar and inoculation treatment (p=0.0183)

* Denotes a statistically significant difference between the two cultivars within inoculation treatment and fermentation temperature (p<0.05).

† Denotes a statistically significant difference between inoculation treatments within cultivar and fermentation temperature (p<0.0001).

To compare between the two cultivars, statistically significant differences were found in wines made at the higher fermentation temperature for both inoculation treatments. Also at the higher fermentation temperature, inoculation treatments were significantly different within

both cultivars. ‘Triple Crown’ pomace taken from the higher temperature, wild-type fermentation process showed higher ORAC value than did ‘Natchez’ pomace from the same process ($p=0.0351$). On the other hand, ‘Natchez’ pomace taken from the higher temperature, yeast-inoculated process showed higher ORAC values than that of ‘Triple Crown’ pomace ($p=0.0036$).

Within the yeast-inoculated ‘Triple Crown’ pomace samples, there were statistically significant differences found between fermentation temperatures ($p=0.0183$). Within high-temperature ‘Triple Crown’ pomace, there were significant differences seen between fermentation treatments ($p<0.0001$).

Correlation between modified Harbertson-Adams assay values and ORAC values

Correlations between ORAC values for blackberry fermented juice and wines and total phenolics, anthocyanins, small polymeric pigments, large polymeric pigments, tannins and non-tannin pigments were analyzed. In the case of ORAC, there were a statistically significant positive correlations with total phenolics [correlation coefficient=0.43401($p<0.0001$)], anthocyanins [correlation coefficient= 0.2186 ($p=0.0165$)], small polymeric pigment [correlation coefficient= 0.25269 ($p=0.0054$)], and non-tannin pigments [correlation coefficient=0.59384 ($p<0.0001$)]. Large polymeric pigments were not significantly correlated with ORAC values, so these compounds did not greatly affect the antioxidant activity of our samples.

ORAC values are related to the concentration of compounds with antioxidant activity in the wines, so ORAC values were expected to be correlated closely connected to the concentrations of total phenolics, anthocyanins, small polymeric pigments as well as non-tannin

pigments in our samples. The strongest correlation was seen between ORAC values and non-tannin pigments such as catechin, epicatechin and phenolic acids.

Chemical analyses

High performance liquid chromatography (HPLC) with photodiode array detector

Results from the high performance liquid chromatography analysis of blackberry phenolic compounds were expressed as µg/L of juice or wine. Table 14 shows the retention times of phenolic compounds measured as pure standards and as injected into blackberry wine samples. It is important to note that the retention times for the standards shifted slightly in the wine as compared to times of pure standards in methanol/water, with retention times being longer in the wines by anywhere from a few seconds to nearly a minute. The retention times as measured in the wines were used to identify unknown peaks in sample chromatograms.

Table 14. Average retention time of pure HPLC standards (n=10) and blackberry samples (N=240).

Phenolic Compounds	Standard Retention Time (min)	Sample Retention Time (min)
Gallic acid	5.32	5.35
Catechin hydrate	14.18	14.46
Caffeic acid	20.72	21.01
Epicatechin	25.58	25.73
Kuromanin chloride	29.05	29.45
p-coumaric acid	33.52	33.63
Keracyanidin chloride	34.18	34.55
Delphinidin chloride	35.87	35.96
Ferulic acid (IS)	52.86	53.72

Eight major and minor phenolic compounds of blackberry juice and wine samples were identified in this HPLC analysis. The major compounds found in this research were kuromanin

chloride (=cyanidin 3-o-glucoside), keracyanin chloride (=cyanidin 3-o-rutinoside), and delphinidin chloride. The minor compounds were epicatechin, gallic acid, catechin hydrate, caffeic acid, and ρ-coumaric acid. As expected, all of the anthocyanin compounds were found in relatively high concentrations. Kuromanin (cyanidin 3-o-glucoside) was found in the highest concentration in this research.

1. Major phenolic compounds

1-1. Kuromanin (Cyanidin 3-o-glucoside)

Three major anthocyanin compounds were found in blackberry juice and wine. The first and the most concentrated compound identified was kuromanin; these results are shown in figure 19 for 'Natchez' juices and wines and figure 20 for 'Triple Crown' juices and wines. Overall, the concentration of kuromanin chloride in blackberry juice and wine decreased over time. In general, 'Natchez' juices and wines had higher kuromanin concentration than 'Triple Crown' juices and wines.

In this study, the kuromanin (cyanidin 3-o-glucoside) concentrations of blackberry juices and wines ranged from 2777 to 11108 µg/L of juice. Most of articles used cyanidin 3-glucoside as a standard instead of cyanidin 3-o-glucoside. According to the literature, cyanidin 3-glucoside is typically found in higher concentration in blackberries than cyanidin 3-o-glucoside, which has variously been reported to comprise from 64.7 to 830 mg/100g of sample (Hassimotto and others 2008, Acosta-Montoya and others 2010, Du and others 2010, Meret and others 2011, Gao and others 2012). However, other studies have shown that cyanidin 3-glucoside has been found in concentrations from 5000 to18000 µg/L of blackberry juice (Wang and others 2008,

Ortiz and others 2013). Our results were similar to those reported by Johnson and others (2013), which listed kuromanin concentration at 10100 µg/L of juice with a retention time of 29.69 minutes using the same HPLC analysis protocol.

Overall, 'Natchez' juices and wines showed higher kuromanin concentration than 'Triple Crown' juices and wines. This corresponds well to the general anthocyanin results seen in the Adams-Harbertson assay and the explanations offered for those results could explain these results as well.

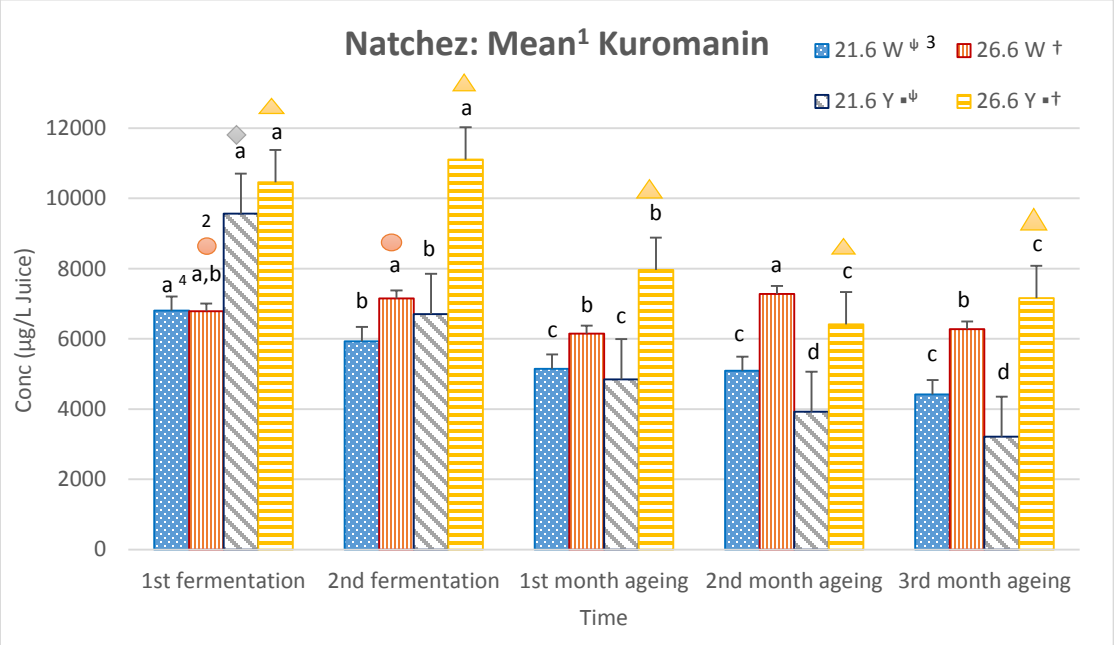

Figure 19. Mean kuromanin chloride concentration of 'Natchez' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

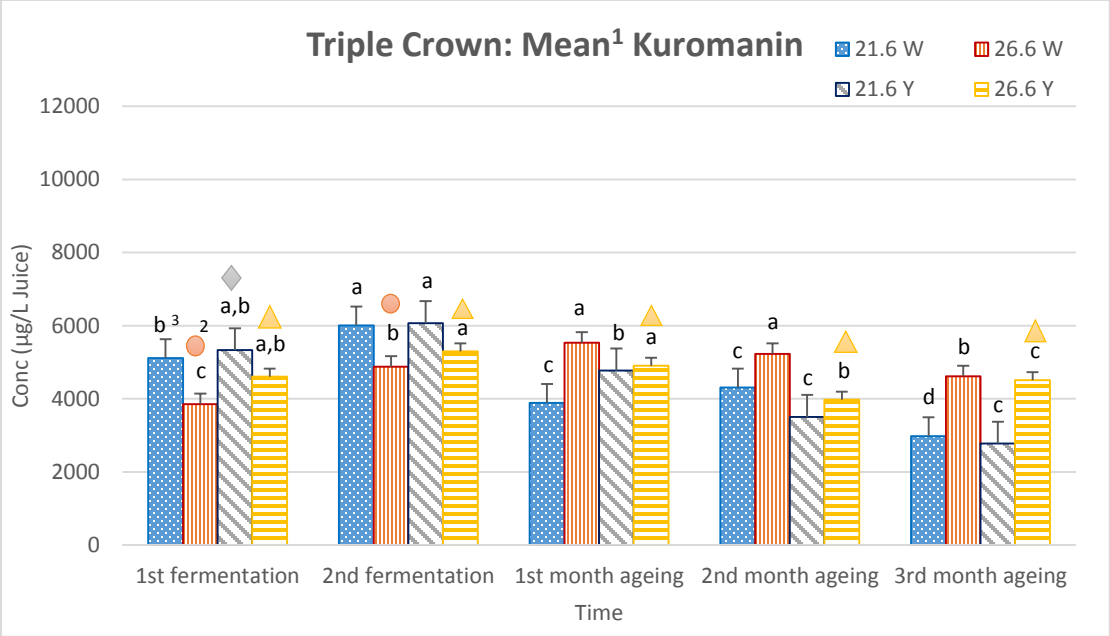

Figure 20. Mean kuromanin chloride concentration of 'Triple Crown' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

Comparing samples over time, all yeast-inoculated 'Natchez' juices and wines showed statistically significant differences in kuromanin concentration ($p<0.0001$), as did wild-type fermented wines made at the lower fermentation temperature ($p<0.001$) and wild-type fermented wines made at the higher fermentation temperature ($p=0.01$). All 'Triple Crown' juices and wines showed statistically significant decreases in kuromanin concentration over time regardless of inoculation treatment and fermentation temperature ($p<0.0001$ for both fermentation temperatures, $p=0.0004$ for wild-type fermentation, $p=0.0145$ for yeast-inoculated samples).

Comparing the two cultivars within inoculation treatments and fermentation temperatures at given times, yeast-inoculated 'Natchez' juices and wines made at the higher fermentation temperature were significantly different than 'Triple Crown' juices and wines ($p<0.0001$ for 1st and 2nd fermentation; $p=0.0006$ for 1st month ageing; $p=0.0172$ for 2nd month ageing; $p=0.006$ for 3rd month ageing). For yeast-inoculated samples made at the lower fermentation temperature, 'Natchez' samples were significantly different than 'Triple Crown' samples only at the stage of 1st fermentation ($p<0.0001$). For wild-type fermentations, only samples fermented at the higher temperature showed significant differences between the two cultivars: 'Natchez' wines sampled at 1st fermentation ($p=0.0006$) and at 2nd fermentation ($p=0.0204$) were significantly higher in kuromanin than 'Triple Crown' wines.

Comparing inoculation treatments within cultivar and fermentation temperatures at given times, at the stage of 1st fermentation, significant differences in kuromanin concentration were seen in 'Natchez' wines between yeast-inoculated and wild-type fermented samples at both high and low fermentation temperatures ($p<0.0001$). For 'Natchez' wines at the stages of 2nd fermentation and 1st month ageing, significant differences between yeast-inoculated and wild-type fermented samples were seen only at the higher fermentation temperature ($p<0.0001$ for 2nd fermentation; $p=0.0078$ for 1st month ageing). For 'Natchez' wines at the stage of 2nd month ageing, significant differences were once again seen between yeast-inoculated and wild-type fermented samples at both fermentation temperatures ($p=0.0102$ for low fermentation temperatures; $p=0.0383$ for high fermentation temperature). For 'Natchez' wines at the stage of 3rd month ageing, only wines made at the lower fermentation temperature showed statistically significant differences between inoculation treatments ($p=0.0084$). In the case of 'Triple Crown' wines, significant differences in kuromanin concentration between the two inoculation treatments were only seen at the stage of 2nd month ageing: differences were seen for both low-

temperature and high-temperature fermented samples (p=0.0073 for low fermentation temperature; p=0.0125 for high fermentation temperature).

Comparing fermentation temperatures within cultivar and inoculation treatment at given times, yeast-inoculated 'Natchez' wines in general showed more statistically significant differences than other samples. For yeast-inoculated 'Natchez' wines, all samples except for those taken at the stage of 1st fermentation showed statistically significant differences between the two fermentation temperatures at the various sampling times ($p<0.0001$ for 2nd fermentation and 3rd month ageing; p=0.0005 for 1st month ageing; p=0.0133 for 2nd month ageing). For wild-type fermented 'Natchez' wines, however, the only statistically significant difference seen between the two fermentation temperatures occurred at the stage of 2nd month ageing (p=0.0484). In the case of yeast-inoculated 'Triple Crown' wines, the only statistically significant difference in kuromanin concentration between the two fermentation temperatures was found at the stage of 3rd month ageing (p=0.0108). For wild-type fermented 'Triple Crown' wines, significant differences between the two fermentation temperatures were seen at the stage of 1st fermentation (p=0.0003) and 2nd fermentation (p=0.0007).

1-2. Keracyanin (cyanidin 3-o-rutinoside)

The second major compound we identified in our blackberry samples was keracyanin; results are shown in figure 21 for 'Natchez' wines and figure 22 for 'Triple Crown' wines. In general, the keracyanin concentration decreased over time. However, wild-type fermented 'Triple Crown' wines actually showed an apparent increase in keracyanin concentration by the end of the study. This may simply reflect sample variability. In general, the keracyanin concentration of 'Natchez' wines was higher than 'Triple Crown' wines, similar to the general

trend for anthocyanins noted previously. All 'Natchez' and 'Triple Crown' wine samples showed statistically significant changes in keracyanin concentrations over time ($p=0.0027$ for 'Natchez' wild-type fermentation 26.6°C; $p=0.0037$ for 'Triple Crown' yeast 26.6°C; $p<0.0001$ for the rest).

The range of keracyanin (cyanidin 3-o-rutinoside) concentrations in blackberry juices and wines seen in this study was from 642 to 1886 µg/L of juice. In the study of Ortiz and others (2013), the concentration of cyanidin 3-rutinoside ranged from 25000 to 69000 µg/L of juice. Our results were lower, but relatively few studies have previously quantified concentrations of keracyanin in blackberry juice or wine. It seems plausible that our results would fall into a normal range.

Overall, the 'Natchez' berries had higher concentration of keracyanin than that of 'Triple Crown' berries. Again, this fits the pattern previously described for anthocyanin concentration. It was interesting to note that at the end of 3rd month of ageing, the concentration of keracyanin appeared to increase at the lower fermentation temperature. As noted above, this could have been an artifact of sampling variability. But it may also reflect enhanced anthocyanin stability at the lower fermentation and storage temperature.

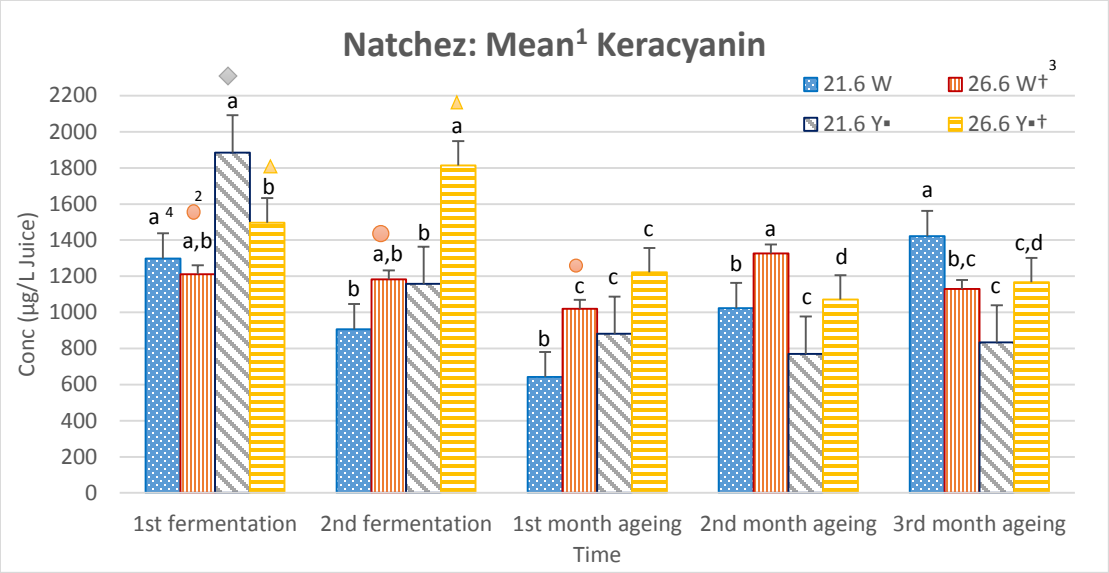

Figure 21. Mean keracyanin chloride concentration of 'Natchez' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

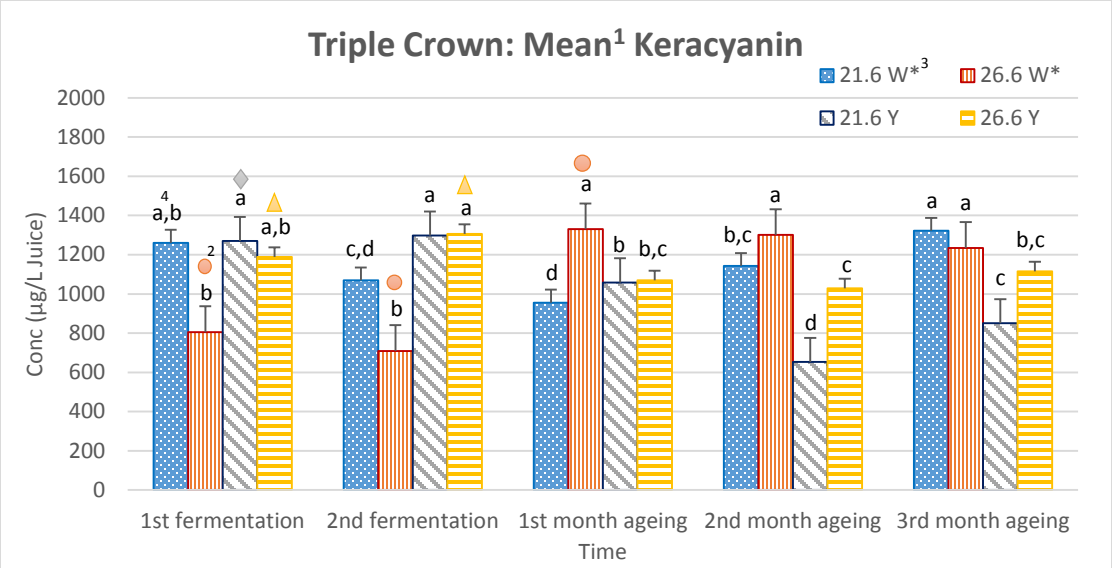

Figure 22. Mean keracyanin chloride concentration of 'Triple Crown' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

Comparing the two cultivars within inoculation treatment and fermentation temperature at given times, high fermentation temperatures generally showed more significant differences than low fermentation temperatures. For wild-type fermentations, there were statistically significant differences between the two cultivars for samples made using the higher fermentation temperatures ($p=0.0007$ for 1st fermentation; $p<0.0001$ for 2nd fermentation; $p=0.003$ for 1st month ageing). For yeast-inoculated samples, there were also statistically significant differences seen between the two cultivars for samples made using the lower fermentation temperatures at most of the same sampling times ($p<0.0001$ for 1st fermentation

and 2nd fermentation). For yeast-inoculated wines made using the higher fermentation temperature, the only statistically significant difference between two cultivars was seen at the stage of 1st fermentation ($p=0.0058$).

Comparing inoculation treatments within cultivars and fermentation temperatures at given times, the wines made using the higher fermentation temperature generally showed more significant differences than those made using the lower fermentation temperature. For 'Natchez' wines at the stage of 1st fermentation, yeast-inoculated samples were significantly different from wild-type fermented samples for both fermentation temperatures ($p<0.0001$ for low fermentation temperature; $p=0.0182$ for high fermentation temperature). For 'Natchez' wines at the stage of 2nd fermentation, the only significant difference observed between inoculation treatments was in wines made using the higher fermentation temperature ($p<0.0001$). For 'Natchez' wines at the stage of 2nd month ageing, significant differences were seen between inoculation treatments for both fermentation temperatures ($p=0.0081$ for low fermentation temperature, $p=0.0129$ for high fermentation temperature). For 'Natchez' wines at the stage of 3rd month ageing, the only significant difference seen between inoculation treatments was in samples made using the higher fermentation temperature ($p<0.0001$). In the case of 'Triple Crown' wines, there were statistically significant differences seen between inoculation treatments in samples made using the higher fermentation temperatures at most of the time intervals tested ($p=0.0028$ for 1st fermentation; $p<0.0001$ for 2nd fermentation; $p=0.0061$ for 1st month ageing). For 'Triple Crown' wines at the stage of 2nd month ageing, statistically significant differences were seen between inoculation treatments for both fermentation temperatures ($p<0.0001$ for low fermentation temperature; $p=0.0044$ for high fermentation temperature). For 'Triple Crown' wines after 3 months ageing, a statistically

significant difference was seen between the two inoculation treatments only in samples made using the lower fermentation temperature ($p<0.0001$).

Comparing fermentation temperatures within cultivar and inoculation treatment at given times, there were generally more significant differences seen in 'Natchez' wines than in 'Triple Crown' wines. For yeast-inoculated 'Natchez' wines, there were statistically significant differences between fermentation temperatures seen at all 5 stages ($p<0.0001$ for 1st fermentation, 2nd fermentation; $p=0.0162$ for 1st month ageing; $p=0.039$ for 2nd month ageing; $p=0.0193$ for 3rd month ageing). For wild-type fermented 'Natchez' wines, significant difference between fermentation temperatures were only seen at 3 months ageing ($p=0.0007$). In the case of yeast-inoculated 'Triple Crown' wines, there were statistically significant differences seen between the two fermentation temperatures only at the stage of 2nd month ageing ($p=0.0121$). For wild-type fermented 'Triple Crown' wines, there were statistically significant differences observed between the two fermentation temperatures at the stages of 1st fermentation ($p<0.0001$), 2nd fermentation ($p<0.0001$) and 1st month ageing ($p=0.0116$).

1-3. Delphinidin

The third and last major anthocyanin compound identified in our blackberry wine samples was delphinidin; results are shown in figure 23 for 'Natchez' wines and figure 24 for 'Triple Crown' wines. In general, the measured levels of delphinidin tended to slightly increase over time in terms of numerical values. While some of these changes were statistically significant, given that there is no source for additional monomeric anthocyanins after fermentation is complete and skins and seeds are filtered out of the wines, is it possible if not likely that the apparent increases seen represent sampling variation more than actual increases in the concentration of delphinidin. These results do suggest, however, that delphinidin was

relatively stable in the finished wines compared to other anthocyanin pigments. Interestingly, 'Triple Crown' wines had higher delphinidin concentrations than 'Natchez' wines in general. This is in contrast to results seen for other anthocyanins.

Overall, the range of delphinidin concentrations observed in this study was from 824 to 2521 µg/L of juice. One relatively recent article from Cho and others (2004) described delphinidin concentrations in blackberry samples; they reported 60000 µg/kg in whole berries. This value is substantially larger than what was measured in the wines in this study. However, as it is known that extraction method, cultivar, climate, and wine-making method can all influence the delphinidin concentration of blackberry wines, it seems likely that our results are within a normal range.

As noted above, nominally higher delphinidin concentrations were found in 'Triple Crown' berries than in 'Natchez' berries and delphinidin concentrations seemed relatively stable over time. It is also interesting to note that higher concentrations of delphinidin were found in wild-type fermentations than in yeast inoculations in the Triple Crown wines. It is possible that the differing chemistry of the wild-type fermentations caused by the larger variety of microorganisms present may have acted to help facilitate extraction and stabilization of this anthocyanin in this wine type.

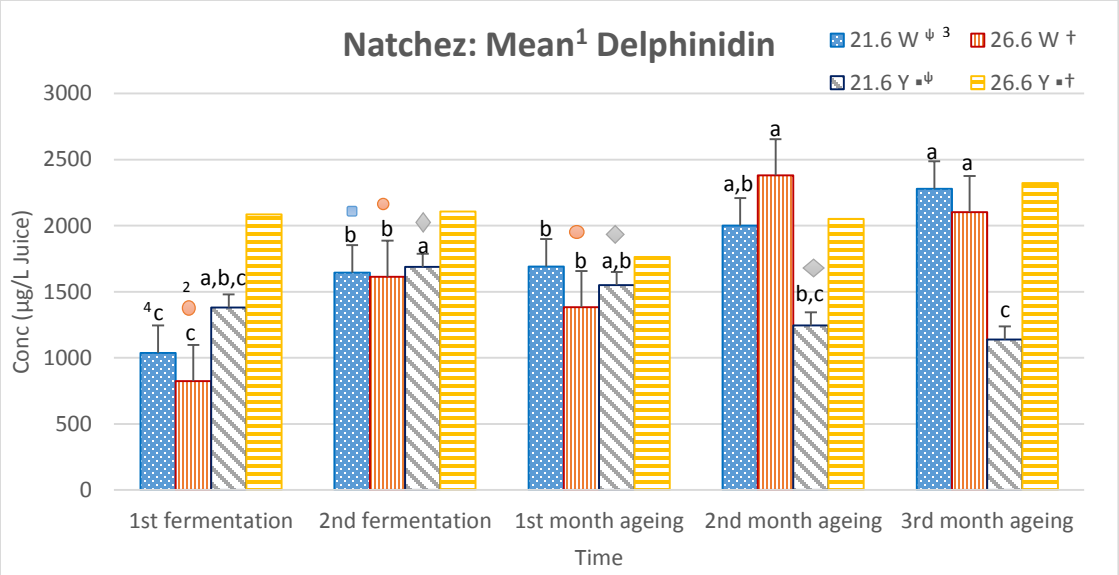

Figure 23. Mean delphinidin chloride concentration of 'Natchez' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

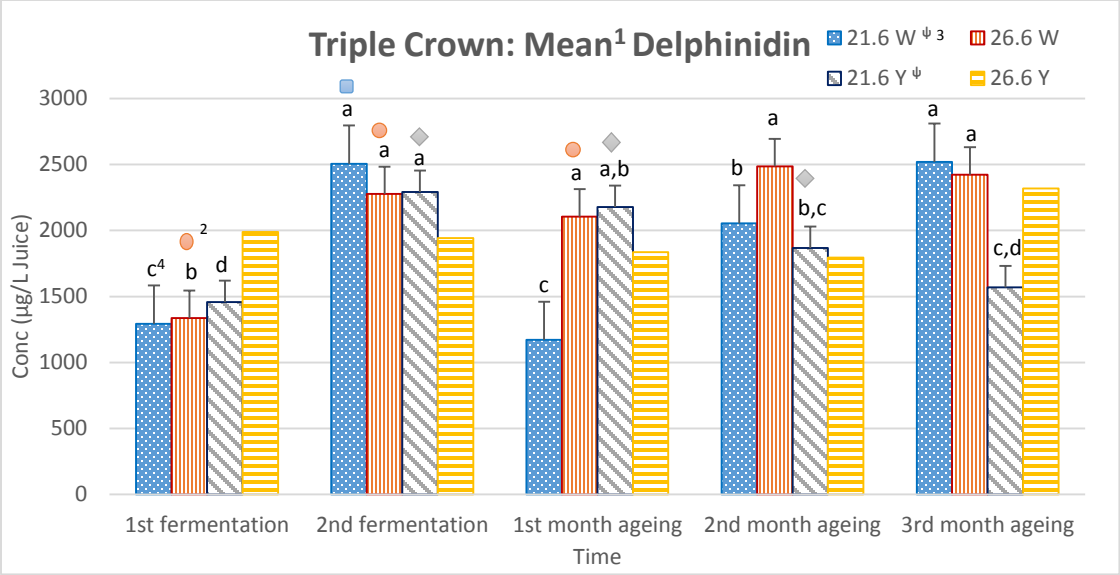

Figure 24. Mean delphinidin chloride concentration of 'Triple Crown' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

Looking at changes in delphinidin concentrations over time, all samples except for the yeast-inoculated wines made at the higher fermentation temperature showed statistically significant differences over the time period evaluated. All wild-type fermentation samples made with 'Natchez' and 'Triple Crown' berries at the higher fermentation temperature showed statistically significant differences over time ($p<0.0001$). Wild-type fermentations at the lower fermentation temperature also showed statistically significant differences over time ($p=0.0398$ for 'Natchez' berries; $p<0.0001$ for 'Triple Crown' berries).

Comparing the two cultivars within inoculation treatment and fermentation temperature at given times, wild-type fermentations made at the higher fermentation temperature generally showed more significant differences while yeast-inoculated samples made at the lower fermentation temperature generally exhibited more significant differences. Significant differences between cultivars were seen in wines made with wild-type fermentation at the higher fermentation temperature at several times ($p=0.0164$ for 1st fermentation; $p=0.0036$ for 2nd fermentation; $p=0.0019$ for 1st month ageing). For wines made using a wild-type fermentation at the lower fermentation temperature, the only statistically significant difference seen between the two cultivars was at the stage of 2nd fermentation ($p=0.0012$). For yeast-inoculated wines, there were statistically significant differences seen between the two cultivars only in wines made at the lower fermentation temperature ($p=0.0243$ for 2nd fermentation; $p=0.0198$ for 1st month ageing; $p=0.0205$ for 2nd month ageing).

Comparing inoculation treatments within cultivar and fermentation temperature at given times, wines made from 'Natchez' berries at the lower fermentation temperature generally showed more significant differences than did wines made from 'Triple Crown' berries at the higher fermentation temperature. For 'Natchez' wines made using the higher fermentation temperature, statistically significant differences between the two inoculation treatments were seen at the stage of 1st fermentation ($p<0.0001$) and 2nd fermentation ($p=0.0224$). For 'Natchez' wines made using the lower fermentation temperature, there were statistically significant differences seen between the two inoculation treatments after the 2nd month of ageing ($p=0.0037$) and the 3rd month of ageing ($p<0.0001$). In the case of 'Triple Crown' wines made using the higher fermentation temperature, the only statistically significant difference seen occurred after the 2nd month of ageing ($p=0.0045$). For 'Triple Crown' wines made using the lower fermentation temperature, statistically significant differences between

the two inoculation treatments were seen after the 1st month of ageing (p=0.0019) and the 3rd month of ageing (p=0.0003).

Comparing fermentation temperatures within cultivar and inoculation treatment, 'Natchez' wine usually exhibited more significant differences than 'Triple Crown' wines. Also, yeast-inoculated wines showed more significant differences than wild-type fermented wines. In the case of yeast-inoculated 'Natchez' wines, there were statistically significant differences between the two fermentation temperatures seen at the stages of 1st fermentation (p=0.0118), 2nd month ageing (p=0.0042) and 3rd month ageing (p<0.0001). There were no statistically significant differences between fermentation temperatures seen for wild-type fermented 'Natchez' wines. For yeast-inoculated 'Triple Crown' wines, there was a statistically significant difference between the two fermentation temperatures observed for 3-month aged wines (p=0.0274). For wild-type fermented 'Triple Crown' wines, a statistically significant difference between the two fermentation temperatures was seen only after the 1st month of ageing (p=0.0043).

2. Minor phenolic compounds

2-1. Gallic acid

One of the minor phenolic compounds identified in our blackberry juices and wines was gallic acid; results for gallic acid concentrations are presented in figure 25 for 'Natchez' wines and juices and in figure 26 for 'Triple Crown' juices and wines. In general, the numbers for gallic acid concentration were stable or nominally increased over time – again this apparent increase after fermentation was complete and wines had been separated from pomace may be function of sampling variability. Several significant increases in gallic acid concentration were seen during

the fermentation period; this would be consistent with ongoing extraction of phenolic compounds during skin contact time. Statistically significant differences were observed during aging in some cases, but the practical significant of those changes is debatable.

Overall, the range of gallic acid concentrations seen in this study was from 47 to 322 µg/L. Our results for gallic acid concentration were low compared to reports for whole berries in the literature. According to Rutz and others (2012), gallic acid was found in whole blackberries in a range from 40000 to 60000 µg/kg. This suggest that substantial amounts of gallic acid were lost during our wine processing, perhaps due to polymerization. Our results showed that 'Triple Crown' wines contained more gallic acid overall than 'Natchez' wines. This may be related to inherent differences in the berries or to differences in extraction discussed previously. Interestingly, as with delphinidin concentration, gallic acid levels were generally higher in the wild-type fermentations. This suggests the possibility that the differing chemistry of the wild-type fermentations caused by the larger variety of microorganisms present may have acted to help facilitate extraction and stabilization more than one type of phenolic compound in this wine type.

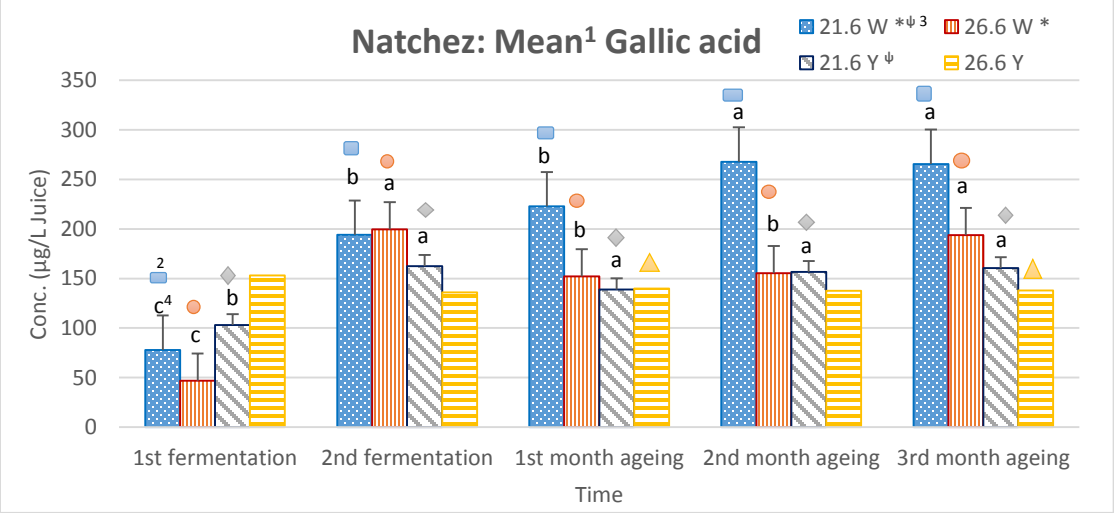

Figure 25. Mean gallic acid concentration of 'Natchez' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature (p<0.05).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time (p<0.05).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time (p<0.05).

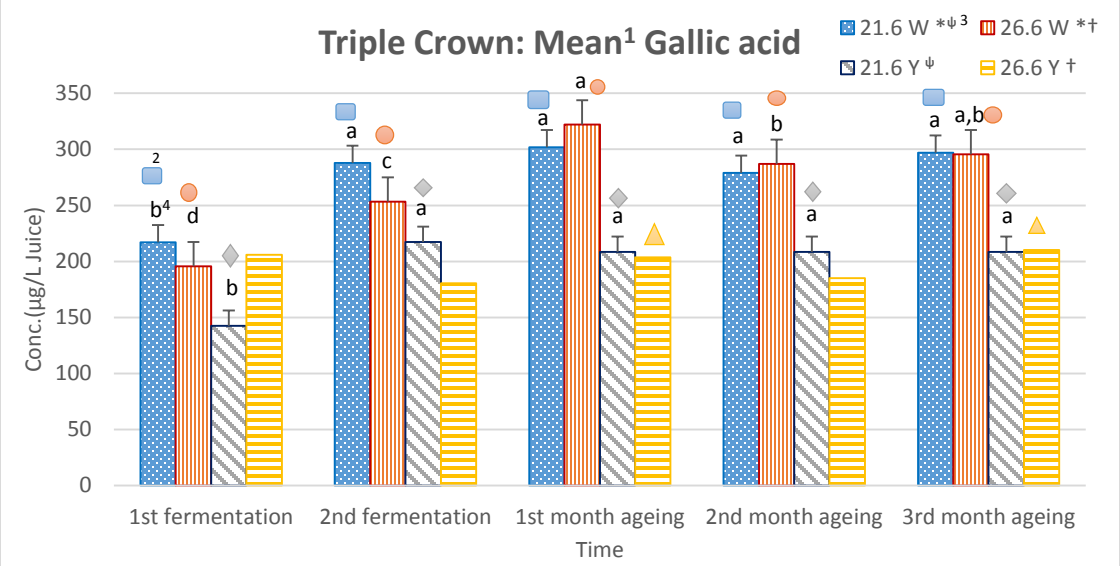

Figure 26. Mean gallic acid concentration of 'Triple Crown' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature (p<0.05).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time (p<0.05).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time (p<0.05).

Comparing cultivars within inoculation treatments and fermentation temperatures at given times, 'Triple Crown' wines were generally higher in gallic acid concentration than

'Natchez' wines. Statistically significant differences were seen for both inoculation treatments and fermentation temperatures at most time intervals. In particular, wild-type fermented 'Triple Crown' wines made at the higher fermentation temperature generally showed higher relative levels of gallic acid compared to the same treatment combination in 'Natchez' wines.

Comparing inoculation treatment within cultivar and fermentation temperatures, 'Triple Crown' wines showed more significant differences than 'Natchez' wines and lower fermentation temperatures showed more significant differences than higher fermentation temperatures. Overall, wild-type fermentations generally showed higher levels of gallic acid concentration than yeast-inoculated fermentations.

Comparing fermentation temperatures within cultivar and inoculation treatment at given times, again wild-type fermented wines exhibited more significant differences than yeast-inoculated wines for both cultivars. Differences in gallic acid concentration between fermentation temperatures were not pronounced after aging in 'Triple Crown' wines. Larger differences were seen in 'Natchez' wines produced using the lower fermentation temperature.

2-2. Catechin

The catechin concentrations of our blackberry juices and wine are shown in figure 27 for 'Natchez' juices and wines and in figure 28 for 'Triple Crown' juices and wines. Similar to the results seen for gallic acid, catechin concentrations generally increased during fermentation – the time period of skin contact – and then fluctuated slightly or dropped during aging. Overall, all of the treatments except for yeast-inoculated 'Natchez' wines made using the higher fermentation temperature showed statistically significant changes in catechin concentrations

over time (p=0.0064 for 'Triple Crown' yeast with high fermentation temperature; p<0.0001 for the rest of treatment).

Overall, the range of catechin found in this study ranged from 141 to 445 µg/L. This value was considerably lower than that reported by Gao and others (2012), which was about 12870 µg/L of wine. This may have been due to inherent differences in the catechin concentration of the starting berry juice as well as differences due to wine making techniques employed.

Again, wild-type fermentations seemed to be associated with higher levels of catechin in the finished wines.

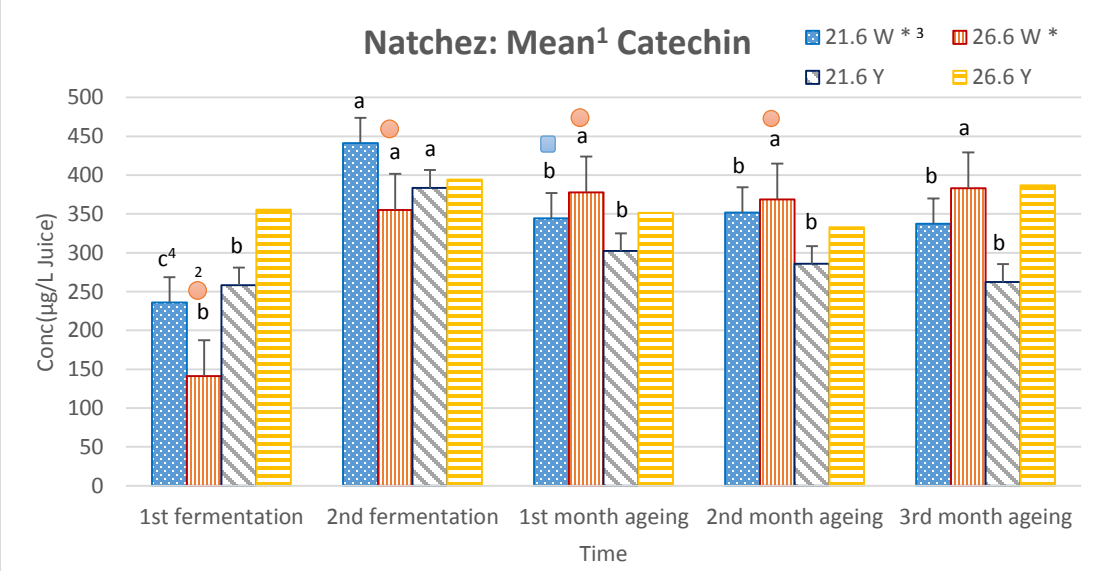

Figure 27. Mean catechin concentration of 'Natchez' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature (p<0.05).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time (p<0.05).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.

4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time (p<0.05).

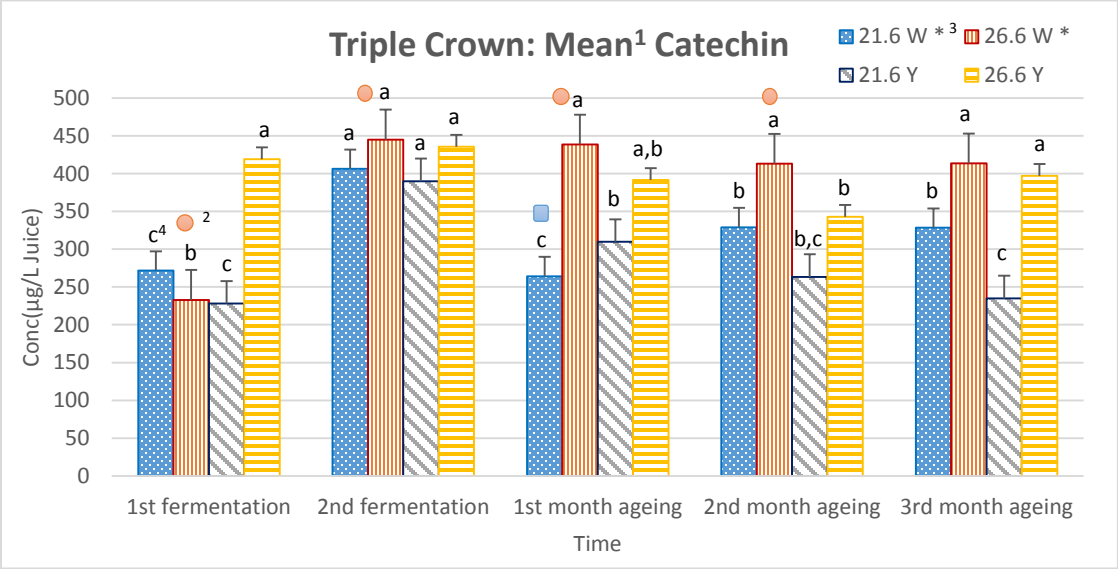

Figure 28. Mean catechin concentration of 'Triple Crown' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature (p<0.05).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time (p<0.05).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time (p<0.05).

Comparing the two cultivars within inoculation treatments and fermentation temperatures at given times, only wild-type fermentations showed any statistically significant differences between cultivars (p<0.05). Catechin concentrations of wild-type fermented wines made using the higher fermentation temperature were fairly consistently higher for 'Triple Crown' wines than 'Natchez' wines. The same trend held true at the lower fermentation

temperature, but the only statistically significant difference was seen after one month aging in these samples.

Comparing inoculation treatments within cultivar and fermentation temperatures at given times, we saw that wild-type fermented wines again tended to have higher levels of catechin than yeast-inoculated wines. This pattern generally held after fermentation and during aging for both fermentation temperatures and cultivars.

Comparing fermentation temperatures within cultivar and inoculation treatments at given times, we saw wines made using the higher fermentation temperature generally had catechin concentrations that were higher than or equal to wines made using the lower fermentation temperature during most stages of aging. This pattern generally held for both inoculation treatments and cultivars.

2-3. Caffeic acid

Caffeic acid concentrations of blackberry juice and wine samples are shown in figure 29 for 'Natchez' juices and wines and in figure 30 for 'Triple Crown' juices and wines. As with the other phenolic acids quantified, caffeic acid levels generally increased during the fermentation process and then stayed the same or showed slight variations during aging. Overall, all of the treatments showed statistically significant differences over time ($p<0.0001$).

Overall, the range of caffeic acid concentrations seen in this study was from 75 to 241 µg/L. This value was again lower than some reported in the literature (Gao and others 2012, Mitic and others 2013), which mentioned caffeic acid levels in the range of 3830 to 9860 µg/L of wine sample. Again, this may have been due to inherent differences in the caffeic acid

concentration of the starting berry juice as well as differences due to wine making techniques employed.

The relationships seen between caffeic acid concentration and cultivar, inoculation treatment, and fermentation temperature were generally consistent with those seen for the other non-anthocyanin phenolic compounds previously discussed.

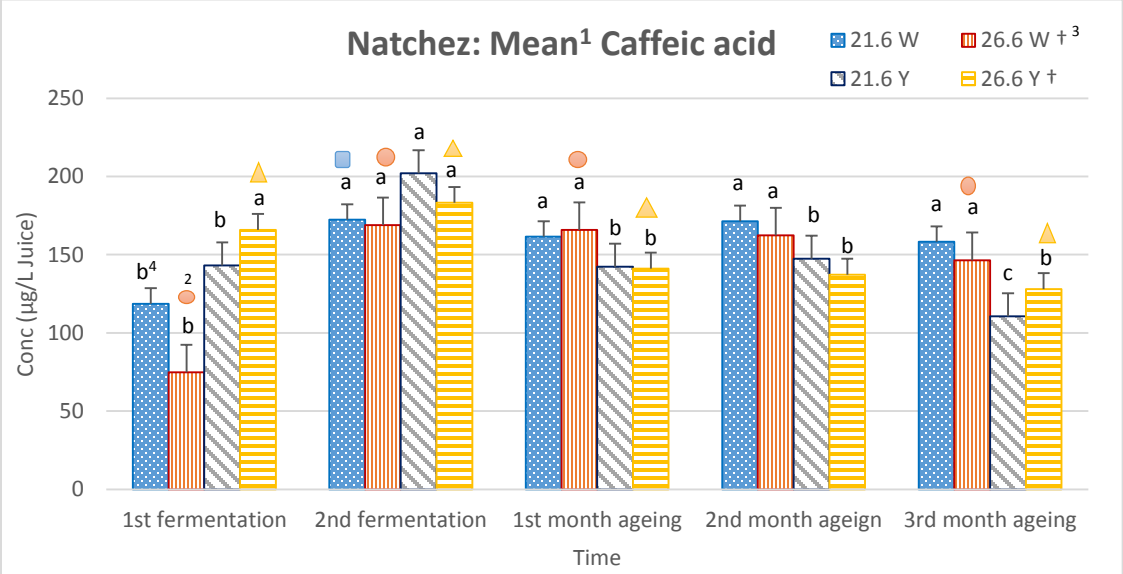

Figure 29. Mean caffeic acid concentration of 'Natchez' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

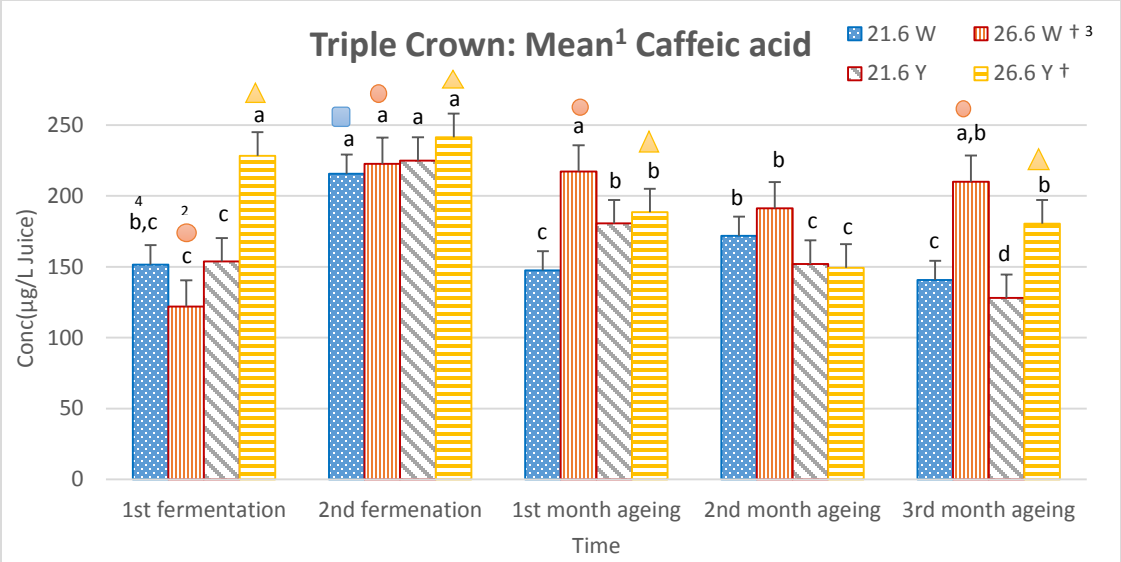

Figure 30. Mean caffeic acid concentration of 'Triple Crown' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

Comparing the two cultivars within inoculation treatment and fermentation temperature at given times, high fermentation temperatures generally demonstrated more significant differences between cultivars than that low fermentation temperatures. Where the differences were significant, 'Triple Crown' wines typically had higher levels of caffeic acid than 'Natchez' wines.

Comparing the two inoculation treatments within cultivar and fermentation temperatures, wines produced using the higher fermentation temperature again tended to show more statistically significant differences than wines produced using the lower

fermentation temperature for both cultivars. Where significant differences occurred, wines made using the wild-type fermentation again had higher caffeic acid concentrations than wines made using the yeast inoculation.

Comparing the two fermentation temperatures within cultivar and inoculation treatment at given times, 'Triple Crown' wines generally showed more significant differences than 'Natchez' wines. Not a lot of significant differences were observed after fermentation, but where they did occur the higher fermentation temperature was associated with higher levels of caffeic acid.

2-4. Epicatechin

The epicatechin concentrations of our blackberry juices and wines are shown in figure 31 for 'Natchez' juices and wines and in figure 32 for 'Triple Crown' juices and wines. In general, 'Natchez' wines had higher epicatechin concentrations than 'Triple Crown' wines. For both cultivars, yeast-inoculated wines made using the higher fermentation temperature showed relatively higher epicatechin concentrations than yeast-inoculated wines made using the lower fermentation temperature. Overall, almost all treatment combinations showed statistically significant changes in epicatechin concentrations over time. Quite a bit of variability in epicatechin concentrations was observed between and among treatments over time. This suggests that epicatechin was not as stable in the wines as some of the other phenolic compounds that were quantified.

Overall, the range of epicatechin measured in this study was from 306 to 1173 µg/L. This value was lower than the values measured in fresh berries by Gancel and others (2011). Their

study showed a range of epicatechin concentrations from 200 to 630mg/Kg of fresh berries, but again we would expect to see lower values in a finished wine than in fresh fruit.

Our results showed 'Natchez' wines in general had higher epicatechin concentrations than 'Triple Crown' wines. Both cultivars generally showed that the higher fermentation temperature generated higher levels of epicatechin in the final wines than lower temperature and that wild-type fermentations generated higher concentrations of epicatechin than yeast-inoculated fermentations in those cases where significant differences based on inoculation treatment were observed. Other than the fact that higher levels of epicatechin were associated with 'Natchez' rather than 'Triple Crown' wines, these results are in line with those observed for the other non-anthocyanin phenolic compounds assayed in this study.

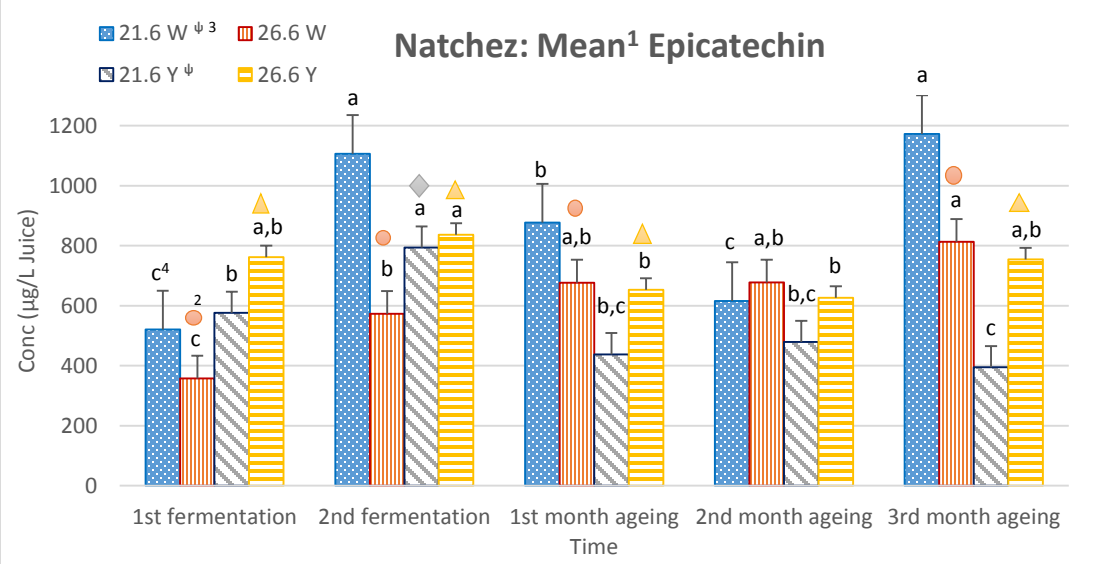

Figure 31. Mean epicatechin concentration of 'Natchez' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different.

Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.

4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

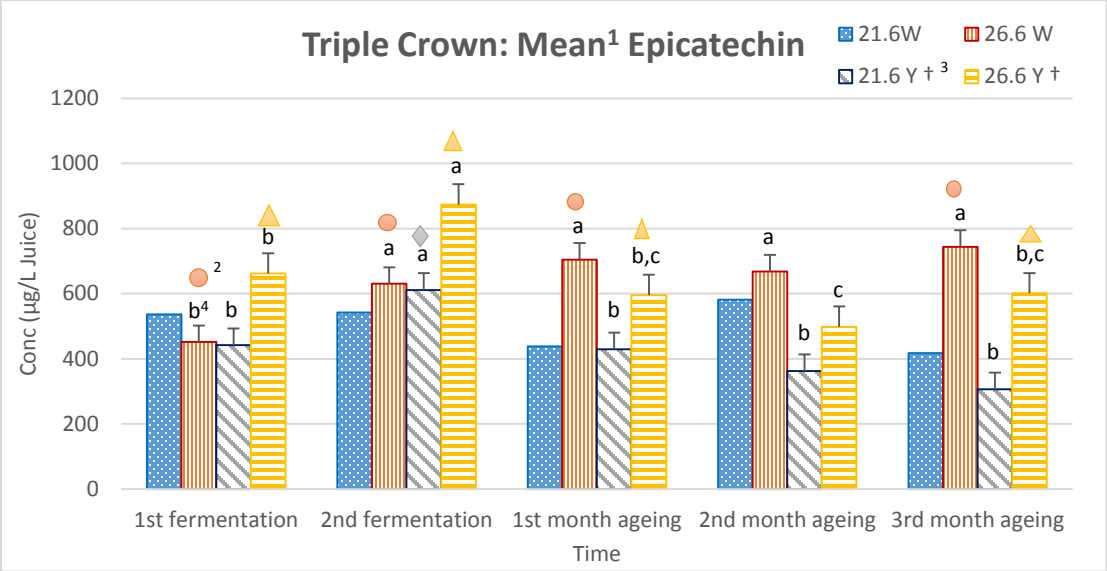

Figure 32. Mean epicatechin concentration of 'Triple Crown' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

Comparing the two cultivars within inoculation treatment and fermentation temperatures at given times, wines produced at the higher fermentation temperature again tended to show more significant differences than wines produced at the lower fermentation temperature for both inoculation treatments. Where significant difference occurred, 'Natchez'

wines had higher epicatechin concentrations than 'Triple Crown' wines. This is in contrast to the other non-anthocyanin phenolic compounds discussed previously.

Comparing inoculation treatments within cultivar and fermentation temperature at given times, 'Natchez' wines showed more significant differences than 'Triple Crown' wines. Where differences occurred, wild-type inoculated wines were higher in epicatechin concentration than yeast-inoculated wines and this was true for both cultivars and fermentation temperatures.

Comparing fermentation temperatures within cultivar and inoculation treatments at given times, all treatment showed significant differences between fermentation temperatures after the 3rd month of aging, but the results were not consistent. For 'Triple Crown' wines, higher fermentation temperatures were associated with higher epicatechin concentrations for both inoculation treatments. For 'Natchez' wines, higher fermentation temperatures were associated with higher epicatechin concentrations in yeast-inoculated wines, but the opposite was true in wild-type fermented wines. Further research would be necessary to clarify this discrepancy.

2-5. ρ-coumaric acid

The ρ-coumaric concentration of our blackberry juice and wine samples are shown in figure 33 for 'Natchez' juices and wines and in figure 34 for 'Triple Crown' juices and wines. In general, 'Triple Crown' wines showed higher concentrations of ρ-coumaric acid than 'Natchez' wines. In a pattern similar to that seen for epicatechin, ρ-coumaric acid levels tended to fluctuate for some treatment combinations during aging. Again, this may indicate that both

cultivars, wild-type fermentation at low fermentation temperature, the ρ-coumaric acid was not as stable under all treatment conditions as some other phenolic compounds quantified in this study.

Overall, the concentrations of ρ-coumaric acid measured in this study ranged from 0 to 1208 µg/L. The higher values we measured were within the range reported for blackberry wines by Mitic and others (2013). They reported concentrations of ρ-coumaric acid ranging from 800 to 3010 µg/L of wine.

Our results for ρ-coumaric acid concentrations were generally in line with the results for the other non-anthocyanin phenolic compounds assayed apart from epicatechin. Higher levels were generally associated with 'Triple Crown' wines, wild-type fermentations, and the higher fermentation temperature.

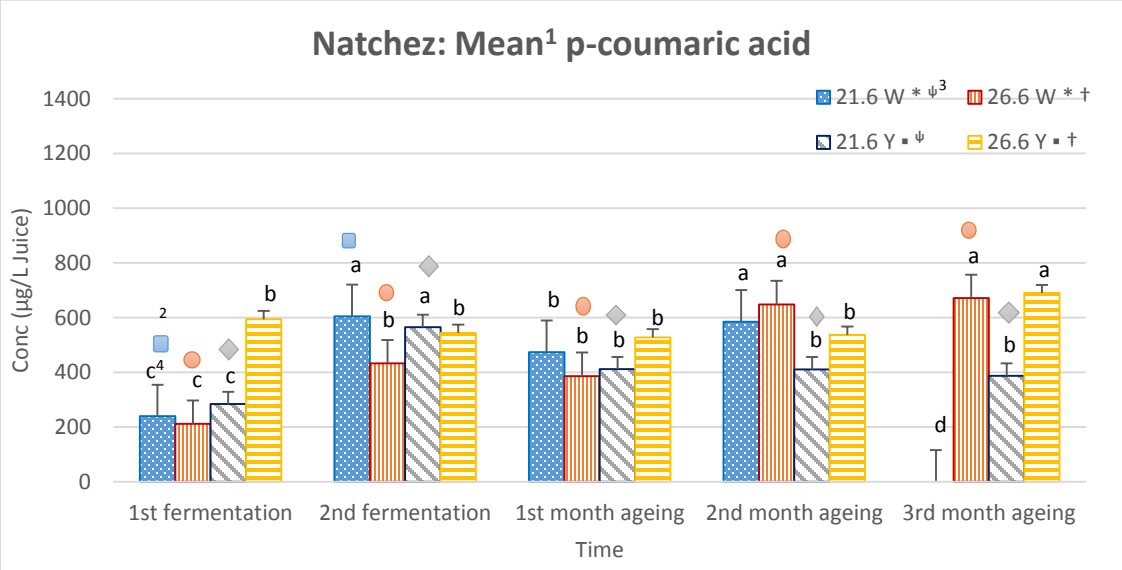

Figure 33. Mean ρ-coumaric acid concentration of 'Natchez' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).

3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

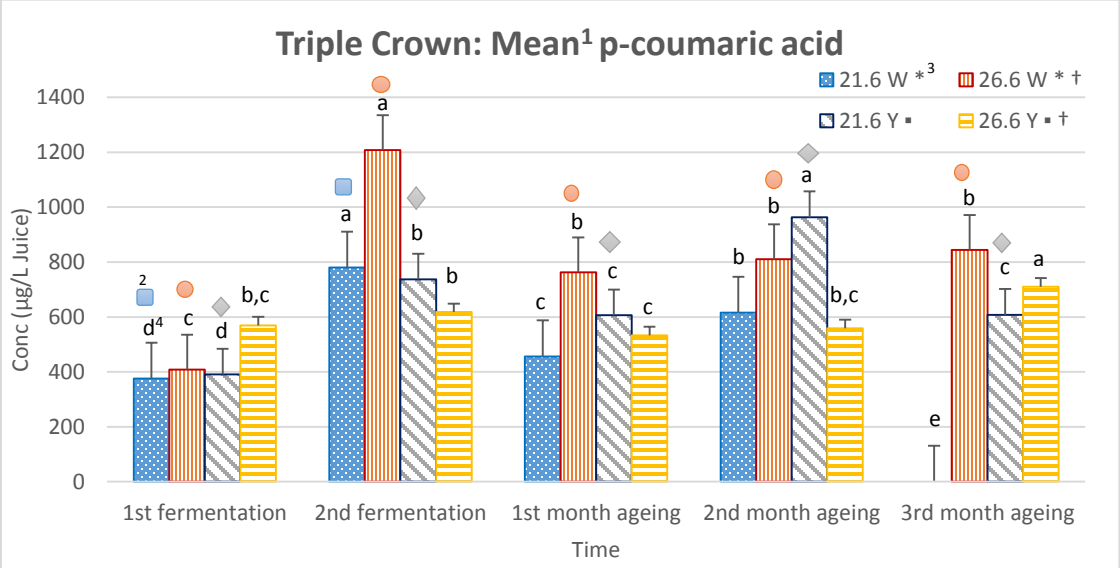

Figure 34. Mean ρ-coumaric acid concentration of 'Triple Crown' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$).). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

Note that for wild-type fermentations conducted at the lower fermentation temperature, ρ-coumaric acid levels drop below the range of measurement for both cultivars at the end of the 3rd month of aging. Overall, all samples showed statistically significant differences

over the time period of this study ($p=0.0004$ for yeast-inoculated 'Natchez' wines produced at the higher fermentation temperature; $p=0.0002$ for yeast-inoculated 'Triple Crown' wines produced at the higher fermentation temperature; $p<0.0001$ for the rest).

Comparing the two cultivars within inoculation treatment and fermentation temperature at given times, wines produced using the wild-type fermentation showed slightly more significant differences than wines produced using yeast inoculation at both fermentation temperatures. In those cases where statistically significant differences were observed, 'Triple Crown' wines were higher in ρ-coumaric acid concentrations than 'Natchez' wines.

Comparing inoculation treatments within cultivar and fermentation temperature at given times, 'Triple Crown' wines showed more significant differences between inoculation treatments than 'Natchez' wines at both fermentation temperatures. Where significant differences were observed, wild-type fermentations were generally associated higher ρ-coumaric acid concentrations than yeast-inoculated fermentations. This was not always the case however. In 'Triple Crown' wines after two months of aging, the yeast-inoculated wine made at the lower fermentation temperature had significantly higher levels of ρ-coumaric acid than the wild-type fermented wine. Further research would be necessary to account for this anomaly.

Comparing fermentation temperatures within cultivar and inoculation treatment at given times, where significant differences were observed the higher fermentation temperatures were generally associated with higher ρ-coumaric acid levels than the lower fermentation temperatures. The exception was again found in 'Triple Crown' wines after two months of aging.

Gas Chromatography (GC) with flame ionized detector

Results for the gas chromatography assays of blackberry volatile compounds were expressed as µg/L of juice or wine. The retention times of the volatile compounds used as standards are shown in Table 15; these were selected as the major volatile compounds found in blackberry juice and wine. Standard Retention Time refers to the retention times observed for pure standards and Sample Retention Time refers to the retention times observed for standards assayed after being recovered from samples of blackberry wine. Note that the Sample Retention Times were slightly earlier than the Standard Retention Times. The Sample Retention Times were used to identify compounds of interest in the sample juices and wines.

Table 15. Average retention time of pure GC standards (n=10) and blackberry samples (N=240)

Compounds	Standard Retention Time (min)	Sample Retention Time (min)
Hexanal	3.961	3.769
2-heptanone	6.356	6.019
Trans-2-hexenal	7.639	7.547
Furaneol	8.557	8.532
p-cymene (IS)	10.067	10.017
2-heptanol	14.629	14.565
Hexanol	17.332	17.115
Fenchone(IS)	18.893	18.709
Theaspirane A	30.523	30.354
Linalool	42.547	42.294
α-terpineol	50.900	50.814
Methyl salicylate	52.633	52.485
Geraniol	55.639	55.398

A total of 11 major, minor and trace volatile compounds were found by GC analysis of our blackberry juice and wine samples. The major compounds found in both cultivars were hexenal and trans-2-hexenal. The minor compounds found in both cultivars were methyl salicylate and geraniol. Minor amounts of the compound 2-heptanol were found in 'Natchez' juices and wines and trace amounts were found in 'Triple Crown' juices and wines. Trace

amounts of the volatile compounds 2-heptanone, furaneol, hexanol, theaspirane A, linalool and α-terpineol were found in juices and wines made from both cultivars.

1. Major volatile compounds

1-1. Trans-2-hexenal

One of the major volatile compounds that was found in blackberry juices and wines was trans-2-hexenal. The concentrations of trans-2-hexenal are shown in figure 35 for 'Natchez' juices and wines and figure 36 for 'Triple Crown' juices and wines. This volatile compound generally was found in higher concentration in 'Triple Crown' juices and wines than in 'Natchez' juices and wines, although results were variable and not a lot of statistically significant differences were observed in the finished wines. Overall, the inoculation treatment appeared to have the greatest influence on the concentration of trans-2-hexenal. Yeast-inoculation was associated with higher starting concentrations of trans-2-hexenal that generally decreased over time, particularly in 'Triple Crown' wines. Samples with wild-type fermentation were more stable over time in trans-2-hexenal levels.

Overall, the range of trans-2-hexenal was from 15234 to 104013 µg/L. These results were high compared to other reported values. According to Ogawa and others (2008), the concentration of trans-2-hexenal was 70 µg/L for Marion juice and 250 µg/L for Evergreen juice. It is difficult to extrapolate too much from these values given that different cultivars, extraction methods, fermentation techniques, and analytical methods were used in the current study, but these results may indicate that the fermentation technique used in this study was better at retaining volatile compounds than some other methods.

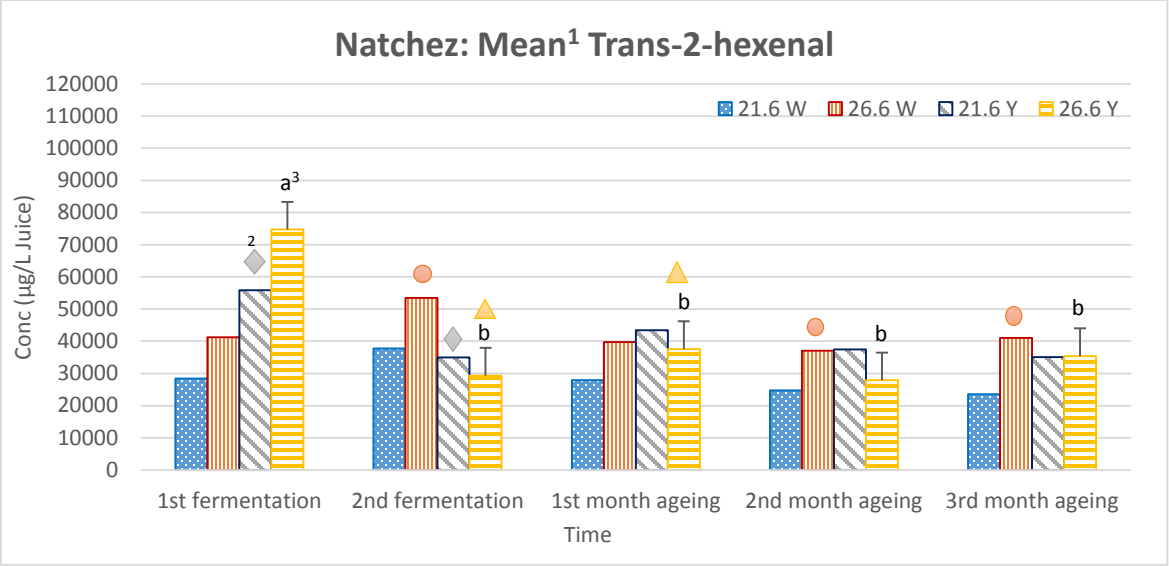

Figure 35. Mean trans-2-hexenal concentration of 'Natchez' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

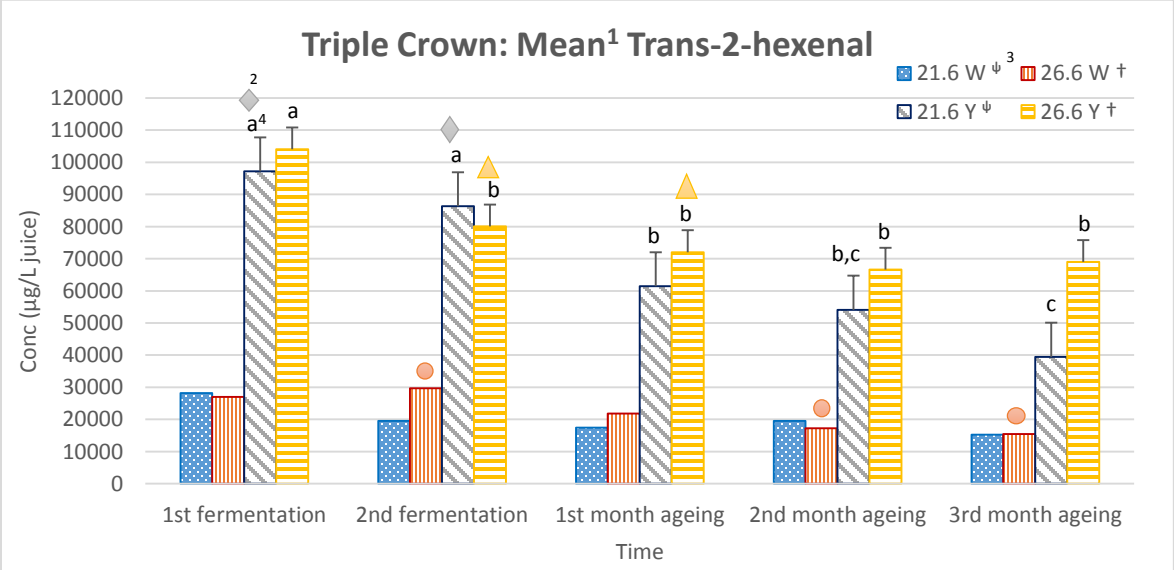

Figure 36. Mean trans-2-hexenal concentration of 'Triple Crown' blackberry juices and wines.

1. n= 6

2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

Comparing cultivars within inoculation treatment and fermentation temperature, more statistically significant differences between cultivars were seen in wines produced using the higher fermentation temperature. Where significant differences were observed, results varied and for some treatments 'Triple Crown' wines had higher levels of trans-2-hexenal while for other the levels were higher in 'Natchez' wines.

Comparing fermentation temperatures within cultivar and inoculation treatment at given times, few significant differences were found. Where differences were seen, higher fermentation temperature was associated with higher trans-2-hexenal levels.

Comparing inoculation treatments within cultivar and fermentation temperatures at given times, no significant differences between inoculation treatments were seen in 'Natchez' wines after the first fermentation period, but significant differences were seen in almost all of the 'Triple Crown' wines, where yeast inoculation was associated with higher trans-2-hexenal concentrations.

1-2. Hexanal

The second major volatile compound identified in our blackberry juices and wines was hexanal; concentrations are shown in figure 37 for 'Natchez' juices and wines and in figure 38

for 'Triple Crown' juices and wines. As a general trend, the concentration of hexanal decreased over time for most samples after the fermentation period was complete.

Overall, the range of hexanal we observed was from 1831 to 32281 µg/L. As with trans-2-hexenal, these values were higher than those reported elsewhere in the literature. According to Du and others (2010), the concentration of hexanal was 60 µg/L in Marion berry and Evergreen berry juice, for example. Another article reported a hexanal concentration of 373 µg/kg for fresh Marion berries and 58 µg/kg for fresh Black Diamond berries (Ogawa and others 2008).

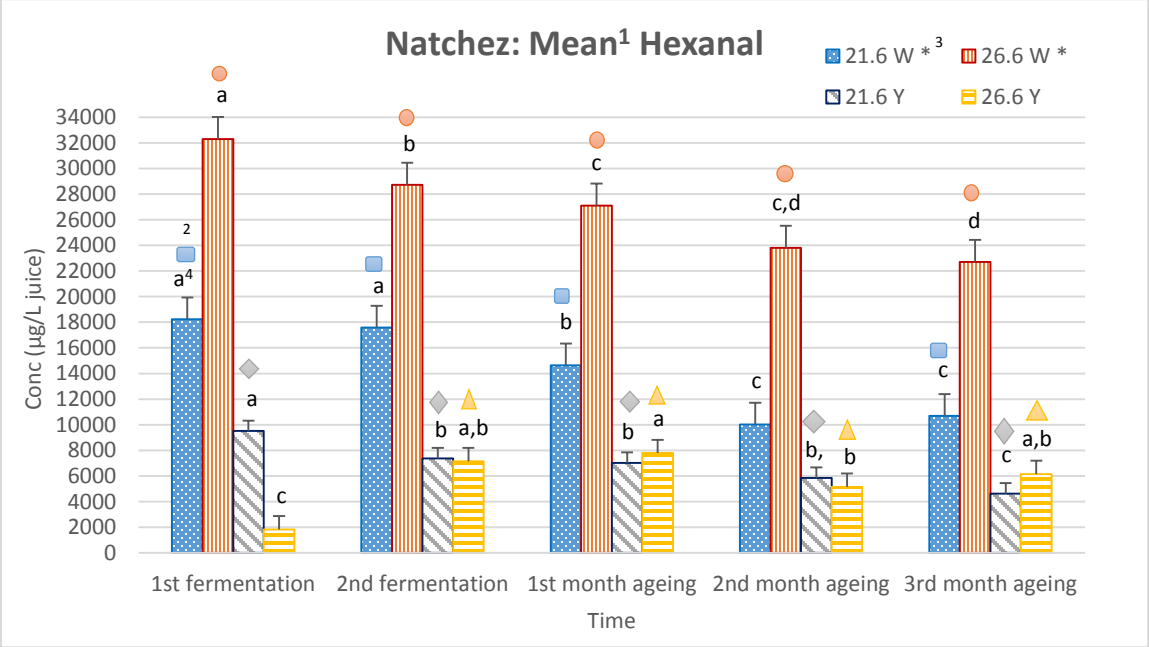

Figure 37. Mean hexanal concentration of 'Natchez' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time ($p<0.05$). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.

4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time (p<0.05).

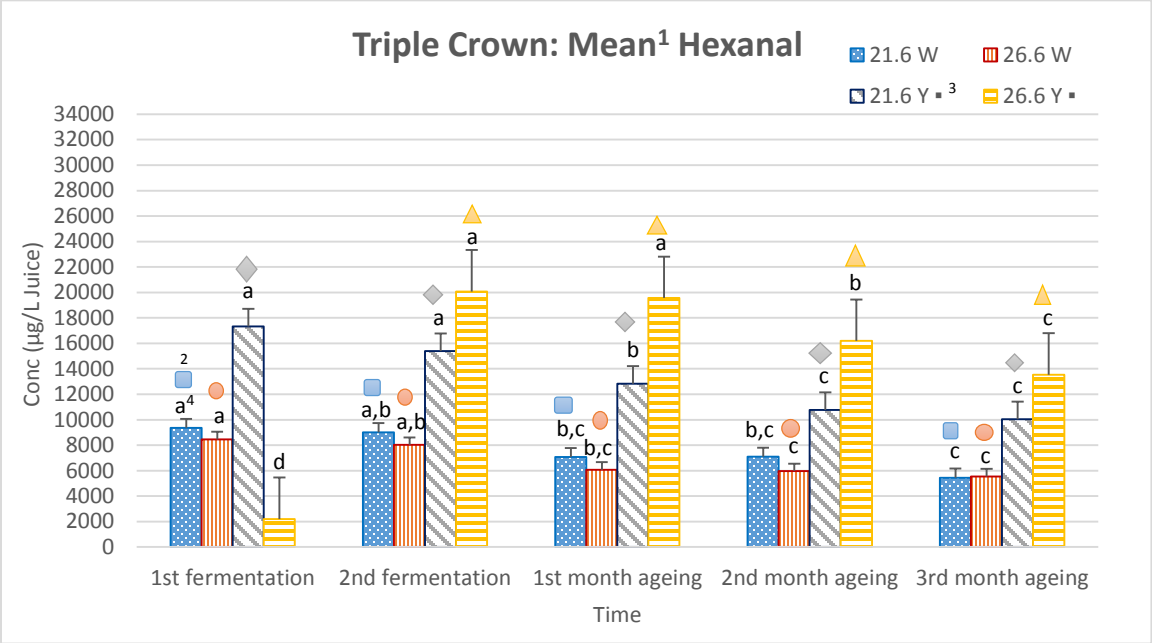

Figure 38. Mean hexanal concentration of 'Triple Crown' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature (p<0.05).
3. Symbols on the legend denote significant differences within a cultivar between fermentation temperatures and inoculation treatments over time (p<0.05). Note that these symbols were only shown if more than three values were significantly different. Significant differences between one or two means were only discussed in the text and not noted on the graph in order to enhance clarity.
4. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time (p<0.05).

Comparing the two cultivars within inoculation treatment and fermentation temperature at given times, almost all of the samples showed statistically significant differences, but these differences were not consistent between cultivars. The 'Natchez' wines were generally higher for wild-type fermentations, whereas 'Triple Crown' wines were typically higher for yeast-inoculated fermentations.

Comparing fermentation temperatures within cultivar and inoculation treatment at given times, all 'Natchez' wines made at the higher fermentation temperature using a wild-type fermentation were significantly higher in hexanal than wines made at the lower fermentation temperature, but this was not true for 'Triple Crown' wines. The opposite was seen for yeast-inoculated wines in which wines made using the higher fermentation temperature were higher in hexanal than wines made at the lower fermentation temperature for 'Triple Crown' wines but not for 'Natchez' wines. Overall it seems that higher fermentation temperatures may be associated with higher hexanal levels, but the effect was not consistent across cultivars and inoculation treatments.

Comparing inoculation treatments within cultivar and fermentation temperatures at given times, almost all samples showed statistically significant differences. As noted above, wild-type fermentations were associated with higher hexanal levels in 'Natchez' wines whereas yeast-inoculated fermentations were associated with higher hexanal levels in 'Triple Crown' wines.

2. Minor volatile compounds

2-1. Geraniol

One of the minor volatile compounds identified in our blackberry juices and wines was geraniol. The measured concentrations of geraniol for 'Natchez' juices and wines are shown in figure 39 and for 'Triple Crown' juices and wines in figure 40. For the most part, geraniol concentrations fluctuated over time during the aging process. Concentrations decreased in some treatments but not in others.

Overall, the range of geraniol measured in this study was from 237 to 1375 µg/L. Again, this was higher than the values reported elsewhere: the concentration of geraniol was found to be between 7 to 420 µg/kg of fresh berries by Koka and others (2009) and Du and others (2010).

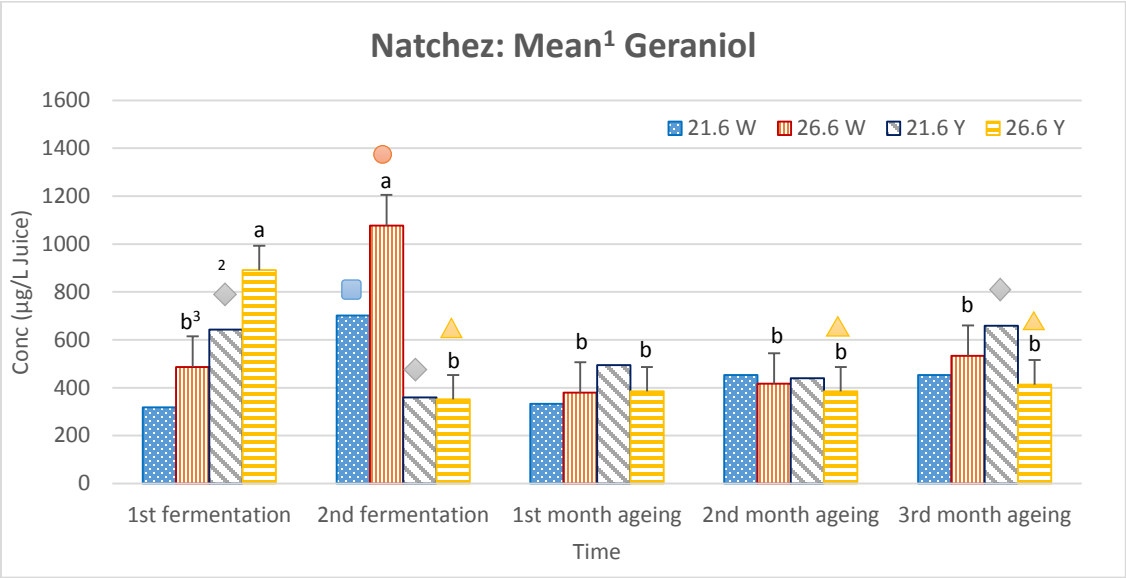

Figure 39. Mean geraniol concentration of 'Natchez' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

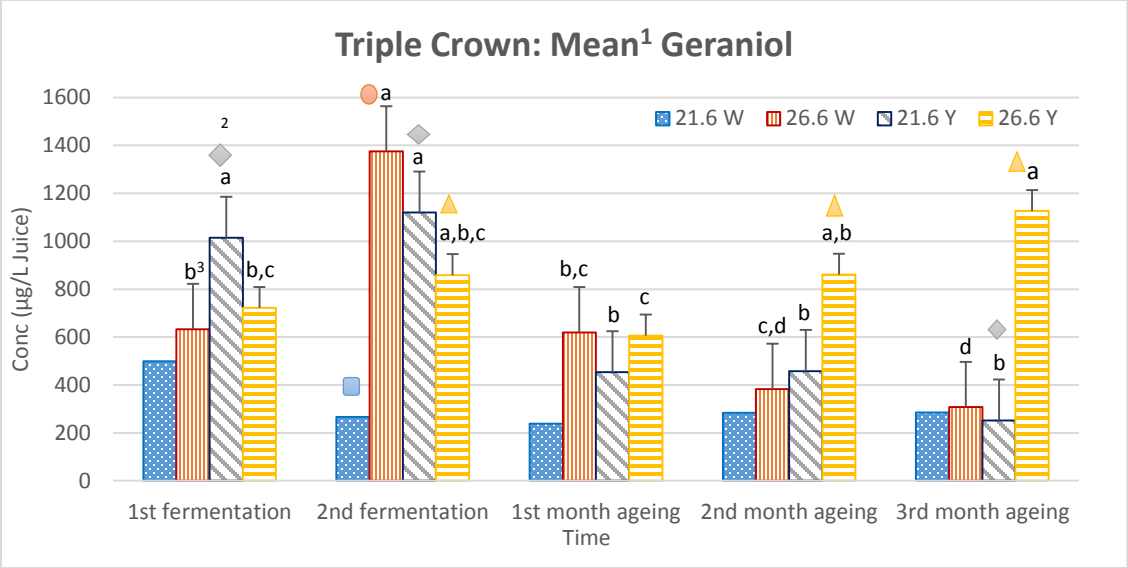

Figure 40. Mean geraniol concentration of 'Triple Crown' blackberry juices and wines.

1. n= 6
2. Different shapes above the bars indicate significant differences between cultivars at a given time for a given combination inoculation treatment and fermentation temperature ($p<0.05$).
3. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

Comparing cultivars within inoculation treatments and fermentation temperatures at given times, as a general trend 'Triple Crown' wines possessed higher concentrations of geraniol than 'Natchez' wines, particularly early in fermentation and aging. This trend did not uniformly extend to the finished wines, however. In the finished wines, yeast-inoculated 'Triple Crown' wines produced using the higher fermentation temperature were higher in geraniol than the same 'Natchez' wines. On the other hand, at the lower fermentation temperature, yeast-inoculated 'Natchez' wines were higher in geraniol than the same 'Triple Crown' wines.

Comparing fermentation temperatures within cultivar and inoculation treatment at given times, 'Triple Crown' wines generally showed more significant differences than 'Natchez' wines. Where significant differences were observed, higher fermentation temperatures were associated with higher concentrations of geraniol.

Comparing inoculation treatments within cultivar and fermentation temperatures at given times, wines made using the higher fermentation temperature showed slightly more significant differences between wild-type and yeast-inoculated fermentations than wines made using the lower fermentation temperature. Where significant differences were seen, higher levels of geraniol were generally associated with wild-type fermentations.

2-2. Methyl salicylate

The second minor compound identified in our blackberry juice and wine samples was methyl salicylate. The concentrations of methyl salicylate found in 'Natchez' juices and wines are shown in figure 41 and in figure 42 for 'Triple Crown' juices and wines. Note that this compound was not found in all of the treatment samples over time. As a general trend, the concentration of methyl salicylate decreased over time for most treatments. However, few statistically significant differences were seen in samples as a function of time.

Overall, the range of methyl salicylate concentration was from 0 to 271 µg/L of blackberry juice. These values were again higher than those reported in other studies. For example, the concentration of methyl salicylate in whole blackberries has been reported to range from 6 to 97µg/kg (Du and others 2010, Koka and others 2009). In another study, the concentration of methyl salicylate in Marion blackberry juice was reported to be 60µg/L and in Evergreen blackberry juice was reported to be 130µg/L (Ogawa and others 2008). Again, these results may indicate that the fermentation technique used in this study was better at retaining volatile compounds than some other fermentation methods.

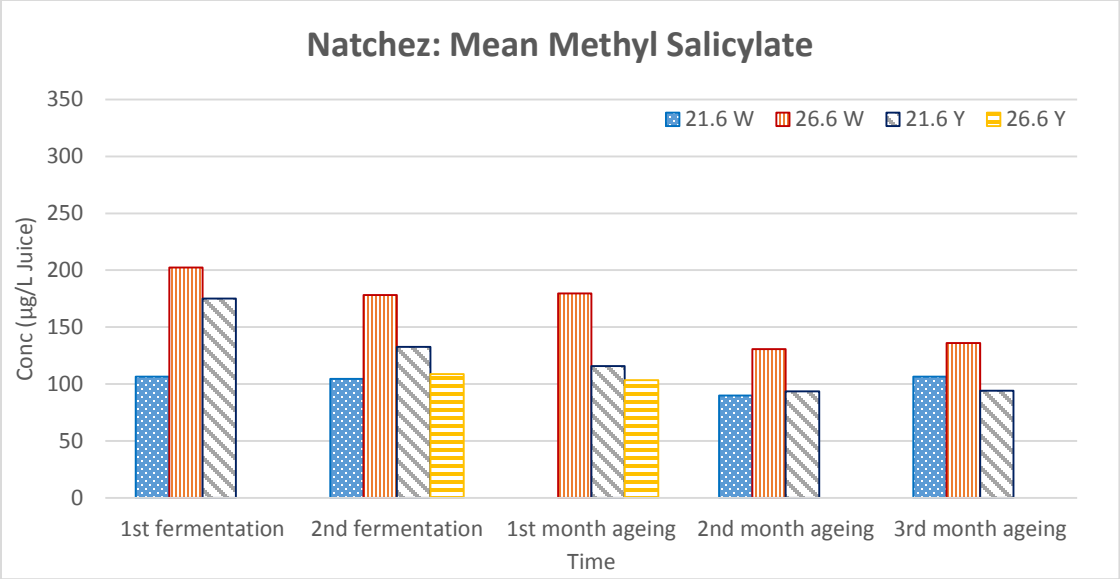

Figure 41. Mean (n=6) methyl salicylate concentration of 'Natchez' blackberry juices and wines. None of samples were significant differences ($p>0.05$).

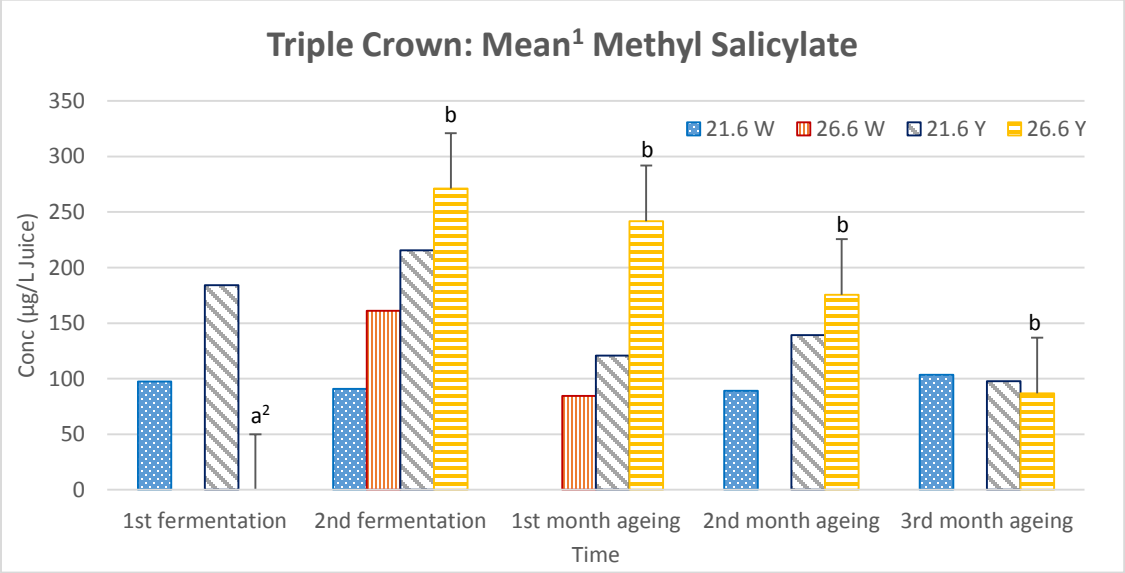

Figure 42. Mean methyl salicylate concentration of 'Triple Crown' blackberry juices and wines.

1. n= 6
2. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

Comparing cultivars, no statistically significant differences were seen. Similarly no significant differences were seen between inoculation treatments. Comparing fermentation temperatures within cultivar and inoculation treatment at given times, the only statistically significant difference was found in yeast-inoculated 'Triple Crown' wines after the 1st fermentation (p=0.0027) where the higher fermentation temperature was associated with a higher methyl salicylate level.

2-3. 2-heptanol

The amounts of minor volatile compounds detected varied between the two with cultivars. One volatile compound, 2-heptanol, was found mostly in 'Natchez' juices and wines and the concentrations found are shown in figure 43. Concentrations fluctuated over time, dropping for some treatments but not for others. No clear pattern was seen for inoculation treatment or fermentation temperature only at the time of 2nd fermentation sampling was that a significant difference seen between fermentation temperatures.

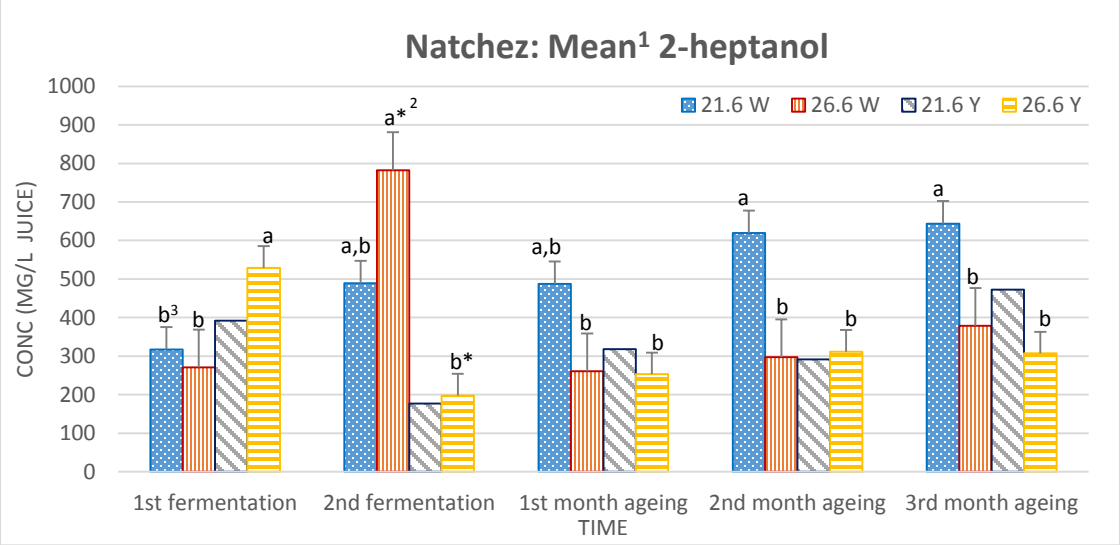

Figure 43. Mean 2-Heptanol concentration of 'Natchez' blackberry juices and wines.

1. n= 6
2. Symbols next to letters denote significant differences between fermentation temperatures and inoculation treatments over time ($p<0.05$).
3. Letters indicate significant differences among fermentation temperatures and inoculation treatments within time ($p<0.05$).

Overall, the range of 2-heptanol found was from 176 to 644 µg/L in our juices and wines. Values have been reported for 2-heptanol concentration in blackberry whole berries, juice, and wine. These values vary quite a bit from one study to another. Our values were lower than the amount found by Fan-Chiang and others (2005). They reported 397608-835648 µg/L of 2-heptanol in blackberry wine and stated that the differences they found were due to different fermentation methods. Other researchers showed 2-heptanol concentrations of 270 µg/L for Marion blackberry juice and 4050 µg/L for Evergreen blackberry juice (Ogawa and others 2008). Another study stated that the 2-heptanol concentration of blackberries was 197 to 5597µg/kg (Du and others 2010, Koka and others 2009). Clearly fermentation methods, extraction and analytical methods, and cultivar can all influence the concentration of 2-heptanol in blackberry juice and wine. Overall, our results for 2-heptanol in 'Natchez' wines falls into the lower range of values reported by others.

3. Trace volatile compounds

Trace amounts of other volatile compounds were found in a few of the samples. No pattern was seen for these compounds related to the treatments applied of the cultivars tested and no comparative statistical analysis was possible. Thus, we describe them as trace compounds and simply report the amounts found.

Hexanol was one such compound, and the average of concentration of hexanol found in 'Natchez' juices and wines was 138 µg/L and in 'Triple Crown' juices and wines was 125 µg/L. According to other studies found in the literature, hexanol has been quantified in whole blackberries and in juice samples. The range of hexanol concentration in whole blackberries was from 14 to 1705 µg/kg (Du and others 2010, Koka and others 2009). The range of hexanol concentration in blackberry juice was 190 µg/L for Marion and 1920 µg/L for Evergreen. Comparing our results to those found in the literatures, hexanol concentrations were within the reported range.

Furaneol was another trace volatile compound detected. The average concentration of furaneol in 'Natchez' juices and wines was 1769 µg/L while the average in 'Triple Crown' juices and wines was 1962.36 µg/L. Furaneol concentrations have been previously reported for whole blackberries. According to Du and others (2010) and Koka and others (2009), the concentration of furaneol was 50 to 4835 µg/kg in fresh blackberries; our result was within this range.

Some trace volatile compounds were detected in only cultivar. One compound, α-terpineol, was detected only in some 'Triple Crown' wine samples; the average concentration was about 181 µg/L. Other studies reported α-terpineol concentration in whole blackberries and the range was from 7 to 495 µg/kg (Du and others 2010, Koka and others 2009). Our result was within this range.

Linalool was another volatile compound that was found only in some samples of 'Triple Crown' wines in this study. The average concentration of linalool found was 246 µg/L. Some other studies have reported that linalool was found in whole blackberries and blackberry juice. The range of linalool in whole blackberry was reported to be between 17 and 3870 µg/kg (Du and others 2010, Koka and others 2009); blackberry juice was reported to be 530 µg/L for

Marion blackberry juice and 170 µg/L for Evergreen blackberry juice (Ogawa and others 2008). Thus, our results for linalool concentrations were within the range reported in the literature.

A couple of trace volatile compounds were found in wine samples only after the fermentation process was complete. Specifically, these were theaspirane A and 2-heptanone. These compounds were found only in 'Natchez' wines produced at the higher fermentation temperature. The average concentration of theaspirane A was 585 µg/L and the average concentration of 2-heptanone was 163 µg/L. According to Du and others (2010) and Koka and others (2009), in whole blackberries the range of theaspirane A was 2 to 46 µg/kg and the range of 2-heptanone was 16 to 10 0 µg/kg. Both of these reported values were lower than our results. The compound 2-heptanone was also found in Marion blackberry juice at a concentration of 50 µg/L and in Evergreen blackberry juice with concentration of 460 µg/L (Ogawa and others 2008). Our results fell into this range.

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CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

This research was focused on how cultivar, yeast inoculation, and fermentation temperatures could affect phenolic concentrations, volatile compounds and antioxidant activity of Korean-style blackberry fermented juices and wines. Overall, our results showed that both fermentation temperature and inoculation treatment had effects with the higher fermentation temperature and wild-type fermentation generally being associated with higher levels of phenolic compounds, volatile compounds and antioxidant activity than the lower fermentation temperature and yeast-inoculated fermentations. The effects were variable, however.

First, differences in physical properties and health benefits of phenolic compounds were seen between blackberry juices and wines made from 'Natchez' and 'Triple Crown' blackberries. The two cultivars used in this study, 'Natchez' and 'Triple Crown', were collected over two years. For both cultivars the berries collected in 2012 were higher in pH, titratable acidity, and soluble solid concentrations than those collected in 2011. The weather clearly affected the physical properties attributes of berries: 2012 had more sunshine, less rain and milder temperature than 2011. Between the two cultivars, 'Triple Crown' juices and wines had higher pH, titratable

acidity, soluble solid values, and percent alcohol than 'Natchez' juices and wines. This is probably attributable to some interaction between growing conditions and inherent genetic differences between the cultivars. More study would be necessary to elucidate these influences.

The results for the total percentage of extractable lipids in whole blackberries and blackberry pomace showed that 'Natchez' berries contained more extractable lipids than 'Triple Crown' berries. As the 'Natchez' berries were bigger than 'Triple Crown' berries and had relatively more seeds, this result makes intuitive sense. The treatments applied did appear to affect the efficiency of lipid extraction somewhat in ways that depended on the physical characteristics of the blackberry cultivar. Thus, anyone wishing to capture oils from the blackberry pomace may be well advised to adapt their processing methods to the cultivar being used in winemaking and the winemaking technique being used.

The health benefits of phenolic compounds such as modified Harbertson-Adams assay, high performance liquid chromatography, gas chromatography of blackberry juices and wines showed that the results were variable depending on treatment and berry physical attributes, thus specific wine-making technique may need to be adapted to specific cultivars for best results. However, for antioxidant capacity i.e. the oxygen radical absorbance capacity, results showed that overall 'Triple Crown' juices and wines had higher antioxidant capacity than 'Natchez' juices and wines.

Second, fermentation temperatures did influence the physical properties and presumptive health benefits related to phenolic compound content of blackberry juices and wines made from 'Natchez' and 'Triple Crown' blackberries. With respect to the tested basic physical properties of the blackberry wine samples, fermentation temperatures affected the

basic physical properties such as pH, and titratable acidity between the two cultivars. The 'Triple Crown' wine samples fermented at the higher temperature were sweeter and less acidic than 'Natchez' wine samples. In the case of percent alcohol, higher fermentation temperature and yeast inoculation resulted in higher percent alcohol than lower fermentation temperature and wild-type fermentation.

The results from the modified Harbertson-Adams assay for several classes of phenolic compounds generally indicated that the higher fermentation temperature helped to extract phenolic compounds into the wines and that 'Natchez' wines tended to be higher in several classes of phenolic compounds than 'Triple Crown' wines, but these effects were not consistent among all classes of phenolic compounds. In some cases the compounds proved to be more stable in the wines at the lower fermentation/storage temperature, particularly in those wines made using the wild-type fermentation, even if the starting levels were lower than those found in the wines made at the higher fermentation temperature. Our results indicate that winemaking techniques may need to be optimized for different cultivars in order to obtain maximum phenolic extraction and stability over time. Our results also show that phenolic extraction and stability may possibly be enhanced by using a higher fermentation temperature at the start of the winemaking process and moving to a lower fermentation/storage temperature as fermentation is completed. Higher extraction and formation of complex phenolic compounds such as tannins in blackberry wine samples could increase wine storage stability.

The antioxidant activities of blackberry juice, wine and pomace were analyzed using the Oxygen Radical Absorbance Capacity (ORAC) assay. In general, our ORAC results supported the idea that the higher fermentation temperature helped to extract polyphenol compounds more

efficiently than the lower fermentation temperature and that ORAC values are typically correlated with phenolic concentration in blackberry juices and wines, particularly non-tannin pigments. No clear effect of inoculation treatment was seen. The ORAC values obtained were generally high, however, suggesting that the winemaking method used in this study was effective for creating high antioxidant-activity wines.

The results for the HPLC analyses of individual phenolic compounds tended to reinforce the results obtained by the modified Harbertson-Adams assay. The anthocyanin compounds identified -- kuromanin, keracyanin, and delphinidin were present in amounts consistent with concentrations reported in other studies, 'Natchez' wines tended to be higher in these compounds than 'Triple Crown' wines, higher fermentation temperature was associated with higher levels and the lower fermentation temperature and wild-type fermentations were associated with enhanced retention of these compounds over time.

Third, yeast inoculation fermentation and wild-type fermentation also influenced the basic physical properties and presumptive health benefits related to phenolic compound content of blackberry juices and wines made from 'Natchez' and 'Triple Crown' blackberries. Wild-type fermentations were generally associated with higher phenolic compound contents. Wild-type fermentation produced relatively more acids and less ethanol than yeast inoculation, probably due to the mixture of different kinds of wild-type microorganisms present in the wild-type fermentation.

Yeast-inoculated 'Triple Crown' wine fermented at the lower fermentation temperature was the sweetest and least acidic wine. Wild-type fermented 'Natchez' wine fermented at the higher fermentation temperature was the most sour and acidic wine. Yeast-inoculated 'Triple Crown' wines made using the lower fermentation temperature had the highest percent alcohol

while wild-type fermented 'Natchez' wines made using the higher fermentation temperature had the lowest percent alcohol. Results for sulfur dioxide stability in the wine samples generally showed that yeast-inoculated fermentations consumed less sulfur dioxide than wild-type fermentations and that fermentation temperature impacted sulfur dioxide stability more in the wild-type fermentations. Thus, winemakers employing this type of fermentation should monitor the sulfur dioxide levels in their wines with particular diligence.

A wild-type fermentation is by definition a fermentation driven by a mixed-microflora. Thus, it seems likely that the wild-type fermentation would produce organic acids in addition to ethanol. This would explain the higher acidity and lower ethanol in the wild-type fermented samples. Higher temperatures would also favor more rapid and possibly more complete fermentations and may favor some species in a mixed fermentation more than others. This could explain the increased variability seen in the samples fermented at the higher fermentation temperature.

Our HPLC analyses of phenolic compounds of blackberry juices and wines showed that for some phenolic compounds identified in the wines -- catechin, epicatechin, gallic acid, caffeic acid and p-coumaric acid – higher levels of these compounds in the final wines were associated with wild-type fermentations and higher fermentation temperature. Further studies would be necessary to demonstrate the mechanism by which wild-type fermentations may have enhanced phenolic compound extraction and/or stability in blackberry wines. However, given that these compounds are linked to antioxidant activity and other health benefits, our results indicate that wild-type fermentation and a higher initial fermentation temperature may be useful for enhancing the health benefits of phenolic compounds in blackberry wines.

The results from the GC analysis of volatile compounds in our blackberry juice and wine samples indicated that the effects of fermentation temperature and inoculation treatment were variable and difficult to predict. The effect of cultivar was also variable and no clear patterns emerged. In general the results showed that concentrations of volatile compounds often decreased during wine aging. However, the compounds that were detected were typically present in concentrations that were normal to very high compared to the concentrations reported in other studies. Given that the concentrations reported elsewhere in many cases had a very large range, it is difficult to draw firm conclusions. Nevertheless, it seems safe to say that the Korean style winemaking process employed in this study was effective at retaining high levels of many volatile compounds associated with fresh blackberries. This could translate into wines with preferred flavors and good consumer acceptance.

Overall, this research showed that the traditional Korean style of winemaking can be successfully applied to blackberry cultivars suitable for growing in the Midwestern United States, particularly soft-skinned cultivars such as 'Natchez'. This project also demonstrated the potential for ancillary co-products such as blackberry seed oil. The blackberry wines made in this study were relatively high in polyphenolic compounds as well as antioxidant capacity, and they also possessed distinct fruity flavor and aroma compounds. As the Korean wine making method is relatively easy to adapt to small-scale production, it may be especially well suited to helping small local companies add value to the blackberry crop in Oklahoma.

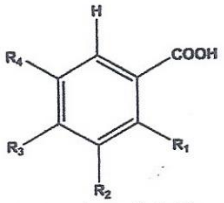
Suggestions for future research include examining wines held at different storage temperatures, wines made with different starting sugar levels, and wines inoculated with different types and levels of added yeast. Assaying the populations of microflora involved in the

wild-type fermentations would also be useful. All of this would help to optimize the fermentation and aging process.

Along with blackberry wine making, more research could be directed toward the blackberry pomace generated as a byproduct of winemaking. For example, an analysis of the fatty-acid concentrations of the oil extracted from the pomace could be applied toward developing functional products that could be used in the nutraceutical and pharmaceutical industries.

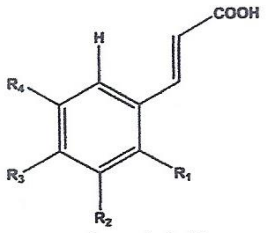
APPENDICES

A. Structure of the phenolic acids



Hydroxybenzoic Acids

Name	R_1	R_2	R_3	R_4
Benzoic acid	H	H	H	H
p-Hydroxybenzoic acid	H	H	OH	H
Vanillic acid	H	OCH_3	OH	H
Gallic acid	H	OH	OH	OH
Protocatechuic acid	H	OH	OH	H
Syringic acid	H	OCH_3	OH	OCH_3
Gentisic acid	OH	H	H	OH
Veratric acid	H	OCH_3	OCH_3	H
Salicylic acid	OH	H	H	H

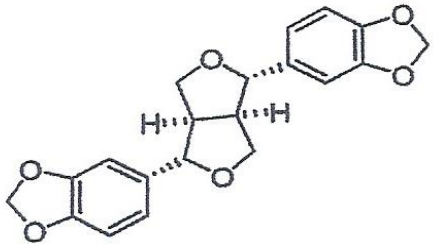


Hydroxycinnamic Acids

Name	R_1	R_2	R_3	R_4
Cinnamic acid	H	H	H	H
o-Coumaric acid	OH	H	H	H
m-Coumaric acid	H	OH	H	H
p-Coumaric acid	H	H	OH	H
Ferulic acid	H	OCH_3	OH	H
Sinapic acid	H	OCH_3	OH	OCH_3
Caffeic acid	H	OH	OH	H

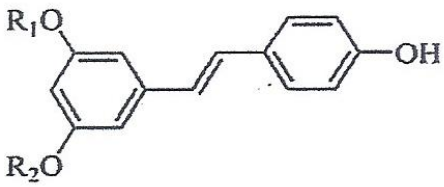
B. Structure of lignin and stilbene

Lignan



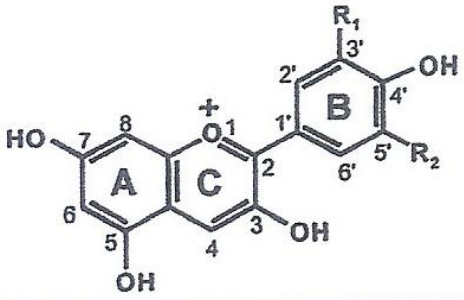
Sesamin

Stilbene

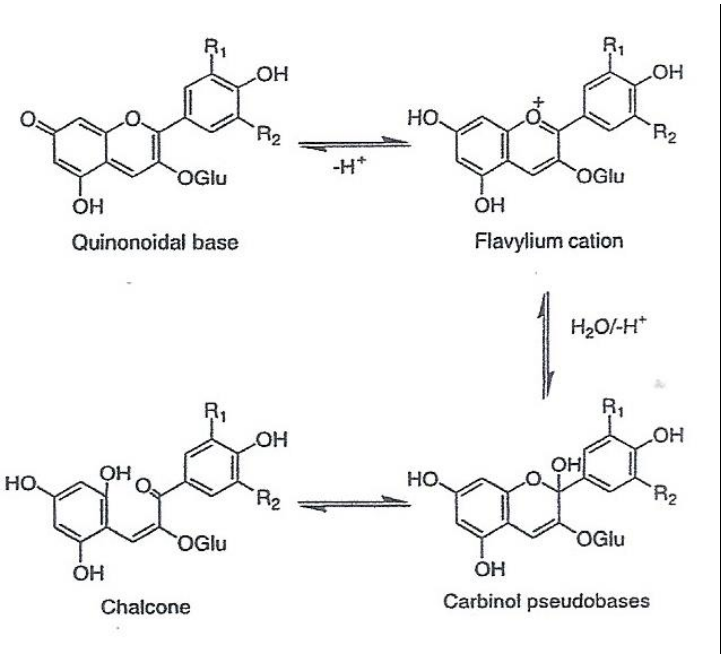


Resveratrol: $R_1 = R_2 = H$

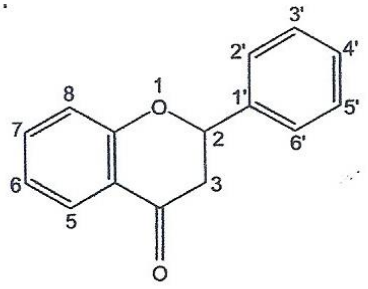
C. Structure of anthocyanidin (top) and chemical transformations of anthocyanins (bottom).



	R_1	R_2
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OCH_3	OCH_3
Pelargonidin	H	H
Peonidin	OCH_3	H
Petunidin	OCH_3	OH

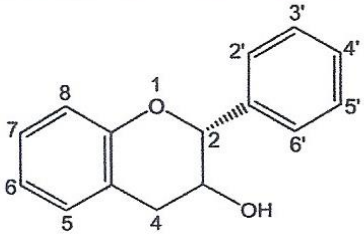


D. Different classes of flavonoids and their substitution patterns.



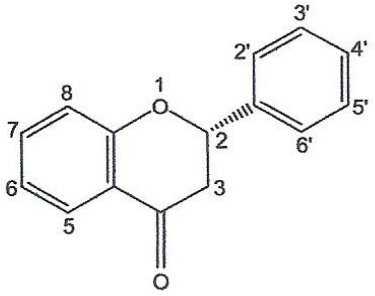
Flavones

Position Compound	5	7	3′	4′
Apigenin	OH	OH	-	OH
Luteolin	OH	OH	OH	OH
Chrysin	OH	OH	–	–



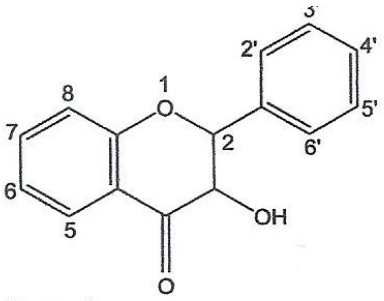
Flavan-3-ols

Position Compound	3	5	7	3′	4′	5′
(+)-Catechin	βOH	OH	OH	OH	OH	–
(−)-Epicatechin	αOH	OH	OH	OH	OH	-
(−)-Epigallocatechin	αOH	OH	OH	OH	OH	OH



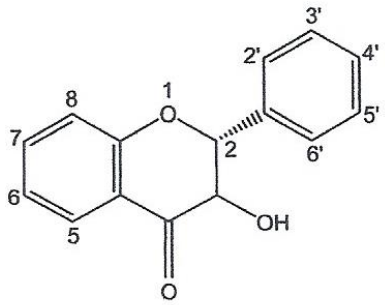
Flavanones

Position Compound	5	7	3′	4′
Naringenin	OH	OH	-	OH
Naringin	OH	*O*-Rha-Glu	–	OH
Hesperetin	OH	OH	OH	OCH_3
Hesperidin	OH	*O*-Rha-Glu	OH	OCH_3



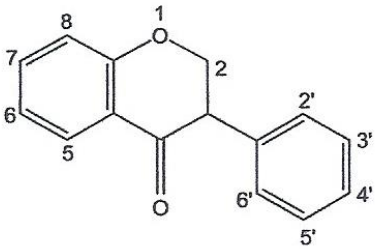
Flavonols

Position Compound	5	7	3′	4′	5′
Quercetin	OH	OH	OH	OH	–
Kaempferol	OH	OH	–	OH	–
Galangin	OH	OH	–	–	–
Fisetin	–	OH	OH	OH	–
Myricetin	OH	OH	OH	OH	OH



Flavanonol

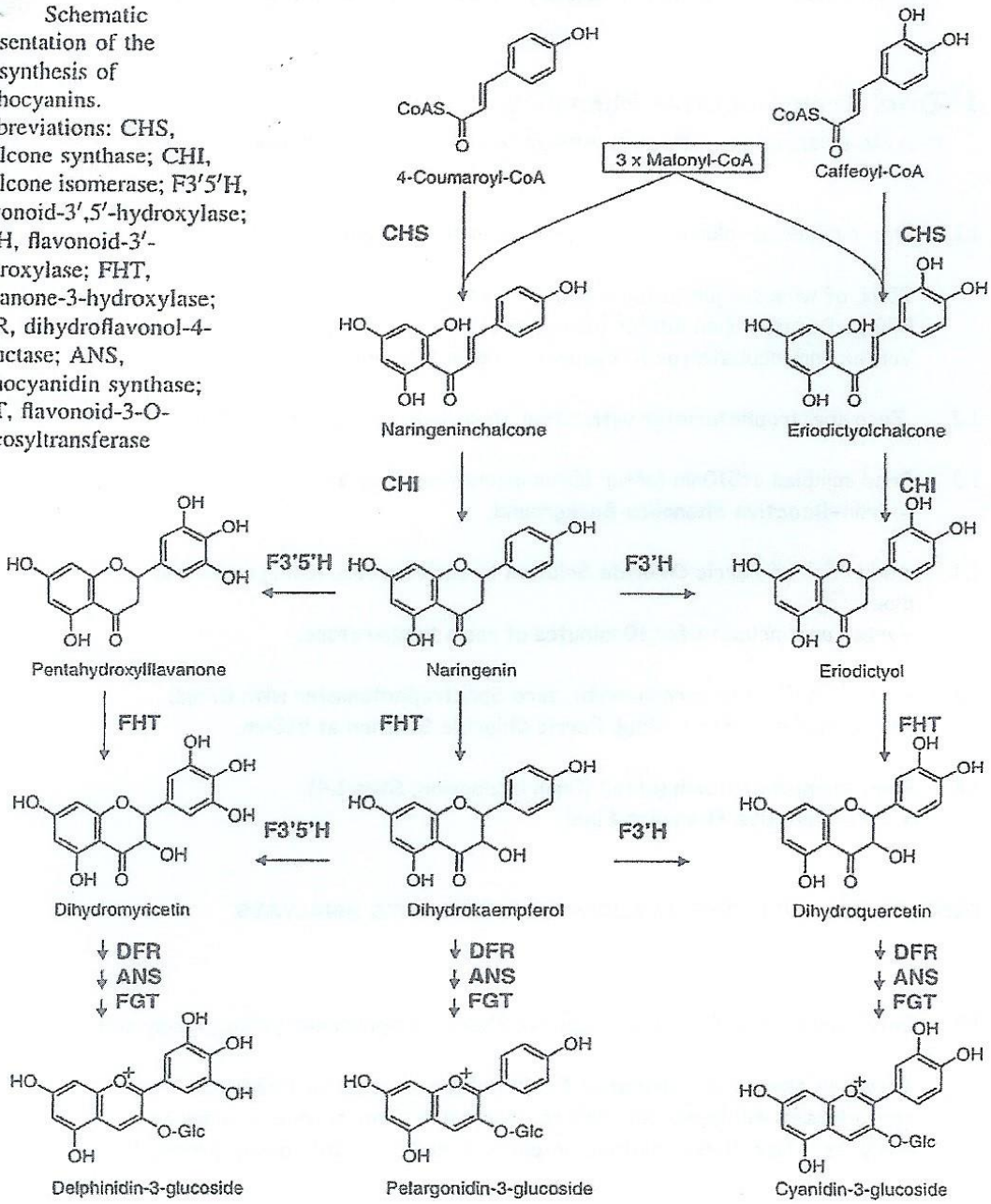
Position Compound	5	7	3′	4′
Taxifolin	OH	OH	OH	OH



Isoflavones

Position Compound	5	7	4′
Genistein	OH	OH	OH
Genistin	OH	*O*-Glu	OH
Daidzein	–	OH	OH
Daidzin	–	*O*-Glu	OH
Ononin	OH	*O*-Glu	CH_3

E. Schematic presentation of the biosynthesis of anthocyanins



Schematic presentation of the biosynthesis of anthocyanins. Abbreviations: CHS, chalcone synthase; CHI, chalcone isomerase; F3′5′H, flavonoid-3′,5′-hydroxylase; F3′H, flavonoid-3′-hydroxylase; FHT, flavanone-3-hydroxylase; DFR, dihydroflavonol-4-reductase; ANS, anthocyanidin synthase; FGT, flavonoid-3-O-glucosyltransferase

F. Adams Assay for phenolics in wine- show all reagents and buffers

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

1.2 Zero spectrophotometer with 875µL Resuspension Buffer at 510nm

1.3 Read samples at510nm (after 10min incubation, Step 1.2).
= **Iron-Reactive Phenolics Background.**

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

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

1.6 Read samples at510nm (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 = 500µL

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

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

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

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

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

2.7 Read samples (Step 2.5) at 520nm.
= **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 = 500µL

1.0mL 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:

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

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

3.5 Read absorbance of samples (step 3.3) at 520nm
= **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 500µL 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 875µL 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 1mL pipette). Incubate for 10 minutes at room temperature.

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

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

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

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

3.18 Read absorbance of samples at 510nm (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).

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).
1.0mL Anthocyanin Buffer (using repeating pipettor).
Vortex and incubate for 5 minutes at room temperature.

4.2 Zero Spectrophotometer with Anthocyanin Buffer at 520nm.

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 800μL 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 (40mg/mL). Transfer protein stock solution to 50mL Falcon tube, add 39mL of Washing Buffer & mix well. Final concentration 1mg/mL → sufficient quantity for 40 assays.

VITA

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