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CHANGES IN ANTHOCYANINS THROUGHOUT THE PROCESSING OF MUSCADINE (*VITIS ROTUNDIFOLIA*) WINE

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Food Science

by
Luke Mumphrey
B.S., Louisiana State University, 2005
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ABSTRACT

Grapes from *Vitis rotundifolia* (Muscadine) are rich sources of different phenolic compounds, (e.g. ellagic acid, myricetin, quercetin, kaempferol, resveratrol, etc.) which are purported to provide health benefits, possibly as antioxidants. Anthocyanins, a pigmented subgroup of the flavonoid group, are responsible for intense pigmentation in the grapes. Maximizing anthocyanins in products is a priority for color, and preservation of potential health benefits.

This research focused on measuring changes in the anthocyanins as wine is produced commercially from a single crop of *Vitis rotundifolia* var. Ison grapes. Samples were taken at points throughout the vintification process, and phenolics and anthocyanins were analyzed. In addition to examining characteristics from the pre-fermented must and finished wine, skins, juice, and press cake were extracted and analyzed to quantify the distribution and changes in the anthocyanins in various fractions during the vinification process. HPLC using a mixed mode C18 column with a diode array detector analysis to detect monomeric glycosylated and polymerized anthocyanins. Potassium metabisulfite bleaching was used to determine polymerized anthocyanins. The study was limited to one crop year from one vineyard which limited annual and spatial variability.

Results identified six anthocyanins: cyanidin, malvidin, petunidin, peonidin, delphinidin, and pelargonidin dihexoses, Concentration of each anthocyanin increased with time, increasing extraction of pigments from the skins until the pressing step. No significant difference was found in anthocyanin concentrations over time when analyzed by HPLC after pressing; however, microplate analysis of total monomeric anthocyanins by pH differential did show a significant

loss in anthocyanins during fermentation, contrasting with concentrations observed by HPLC. Observed declines varied significantly among the identified anthocyanin forms; however, total phenolic levels by Folin-Ciocalteu did not exhibit this decline. Significant levels of polymerized anthocyanins were not detected by either method. These data show, under the study conditions, that anthocyanin forms within Ison variety muscadine grapes are differently resistant to the enological process. Cyanidin and delphinidin forms were most affected, with higher rates of extraction and declines throughout the process. Petunidin and peonidin dihexose forms, showing similar trends, were less drastically affected, malvidin and pelargonidin forms displayed a non-trend, remaining at similar concentrations throughout processing.

CHAPTER 1. INTRODUCTION

Commercially, *Vitis rotundifolia,* muscadine, is consumed in markets proximal to growing areas as fresh grapes, juice, and wine. The Ison variety grapes have the potential to represent a new dietary source of anthocyanins for areas outside of current market areas as a new, unique flavor. *Vitis rotundifolia* species juices are highly susceptible to color degradation during juice production and wine fermentation. Some of this instability may be because the diglucoside forms of anthocyanins are less stable to heat and oxidation than the corresponding monoglucosides.[1] Greater retention of anthocyanin content throughout processing would ensure more stable color during aging and storage. Increased anthocyanin stability would help maintain color quality and possible health benefits, improving marketability of the juice or wine.

The most widely produced variety of red *Vitis rotundifolia* used for wine and juice production is Noble, consequently it has been more extensively studied than the Ison variety.[2] Although the Noble variety is widely used for juice and wine production, other red cultivars: Ison, Paulk, Cowart, and Supreme have been reported to contain more skin anthocyanins, than the Noble variety. The total skin anthocyanins of Noble variety grapes only contained 38% of total skin anthocyanins of Ison variety grapes. [3]

Previous research on anthocyanins within the Ison variety of *Vitis rotundifolia* identified only the anthocyanidin (aglycone) forms of the five anthocyanins present.[4] Specific anthocyanin forms and stability characteristics during processing of this variety have not been fully characterized.

The characterization of changes in the anthocyanins during the commercial vinification process would add to the understanding of the impacts the impacts of processing on the

anthocyanins and the by-products produced. This was accomplished by measuring the distribution changes in anthocyanins in a single harvest and single batch fermentation.

When exposed to heat or oxidative conditions, anthocyanins have a tendency to polymerize. Typical analyses of anthocyanins includes separation by HPLC utilizing standard C18 reversed phase columns; however, the C18 column is not effective for the separation of polymers of anthocyanins in the same chromatographic run. Use of the Primesep B2 mixed-mode HPLC columns enables the researcher to separate monomeric and polymerized anthocyanins concomitantly.[5] The ability to measure the monomeric and polymerized anthocyanins in the same HPLC run allows for a more complete profile of all monomeric anthocyanin values and polymeric anthocyanins in a particular sample.

The sugar content of Ison and other *Vitis rotundifolia* varieties are generally lower than *Vitis vinifera*. Additional sugar is frequently added during the fermentation process of wine to increase fermentable sugars. Some sugar contains residual sulfites, which can bleach the monomeric anthocyanins in the must, resulting in loss of color contributed by monomeric anthocyanin levels.[6] The response of native anthocyanins in Ison variety to the addition of sugar is unknown. Understanding these effects is valuable to the vintner intending to add sugar to the wine must. The color loss can then be properly weighed against the benefit of added sweetness or alcohol content.

The overall goals of this research were: to quantify the impact of commercial wine processing methods on the anthocyanins of Ison variety *Vitis rotundifolia* grapes and to identify the anthocyanin forms contained in Ison variety *Vitis rotundifolia* grapes through mass spectrometry.

CHAPTER 2. LITERATURE REVIEW

2.1 Chemical structure

Anthocyanins, and their aglycones the anthocyanidins, are widely distributed in nature and provide pigmentation in many plant tissues. The anthocyanins are found primarily in plant fruit and flower structures and play an important role in the appreciation of food and beverages, including visual cues to flavor and the ultimate preference or acceptability. The sensitivity of anthocyanins to changes in pH changes in many fruits results in the characteristic hyperchromic shift seen in many ripening fruits. Interest in anthocyanins has evolved from studies in the color of flowers to current interest in health benefits.[7]

The origin of the term anthocyanin comes from two Greek root words: anthos, meaning flower or blossom, and kyanos, which can be translated as blue. With over 500 different structures identified, anthocyanins provide a vast palette of color that includes not only the blue of its namesake, but also orange, red, and purple. Minor shifts in pH can result in significant color changes, as the basic anthocyanin structure can be positively charged, making it sensitive to pH variation.

The aglycone form, an anthocyanidin, is the core of the molecule that can be appended with one or more sugars, including: glucose, rhamnose, galactose, rutinose and many other pentoses and hexoses[8]. The structure of the anthocyanidin molecule is based on the flavilum ion chemically described as 2-phenylbenzopyrilium. The naming convention for the anthocyanidin molecule and anthocyanidin forms (Figure 2.1) are based on the nature of the R-group substitutions on the phenyl ring (Table 2.1).

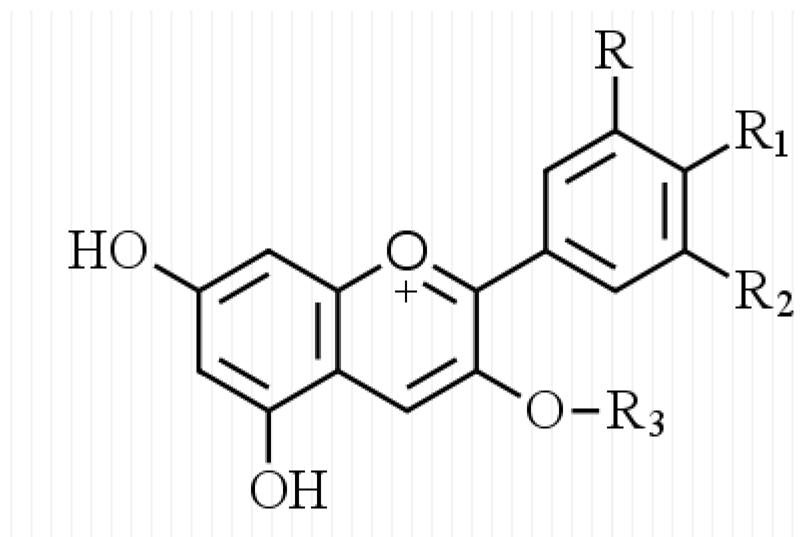

Figure 2.1: Structure of anthocyanidins

Although there are many different forms of anthocyanidins, only six forms are commonly found in food. Three of these forms: cyanidin, pelargonidin, and delphinidin are only hydroxylated. The three methylated anthocyanidins are enzymatically produced from these three hydroxylated forms. Methylation takes place after glycosylation, after the anthocyanins are already formed.[9]

Table 2.1: Anthocyanidin nomenclature and corresponding R groups

Anthocyanidin	**R**	**R1**	**R2**	**R3**
Cyanidin	OH	OH	H	OH
Delphinidin	OH	OH	OH	OH
Pelargonidin	H	OH	H	OH
Malvidin	OCH_3	OH	OCH_3	OH
Peonidin	OCH_3	OH	H	OH
Petunidin	OH	OH	OCH_3	OH

Anthocyanins are synthesized through the flavonoid pathway, following the path through the proanthocyanin step, with a C6-C3-C6 flavonoid skeleton. The three ring structures include: heterocyclic benzopyran ring, fused aromatic ring, and phenyl constituent ring. These ring structures are designated C, A, and B respectively. In the cation form, two double bonds exist within the benzopyran ring creating a positive charge. Several enzymes are involved in the anthocyanin creation process, beginning with anthocyanidin synthase, which catalyzes the final reaction into each aglycon form. From the aglycon, the sugar groups are attached by the enzyme flavonoid glucosyltranferase. The anthocyanins can then be further modified to increase methylation through the enzyme O-methyltransferase, and acylations can be added by the enzyme anthocyanin acyltransferase. The addition of sugars and acylations on the sugars stabilizes the molecules further, allowing the anthocyanins greater solubility in water and alcohol. Increasing methylation, additional glycosylation, and acylations allows the anthocyanins to become more stable than the anthocyanins with less of these added groups.[9]

Anthocyanidin structure is directly responsible for the color produced. Phenyl constituent ring methylation results in increased redness, while increased hydroxylation increases blueness.[9]

The various sugars are bound to the anthocyanidins through a glycosidic bond at the C_3 and C_5 positions. The C_3 bond is the primary bonding site, where monoglycosidic anthocyanins are formed, and C_5 is utilized as a bonding site for diglycosidic anthocyanins.[9]

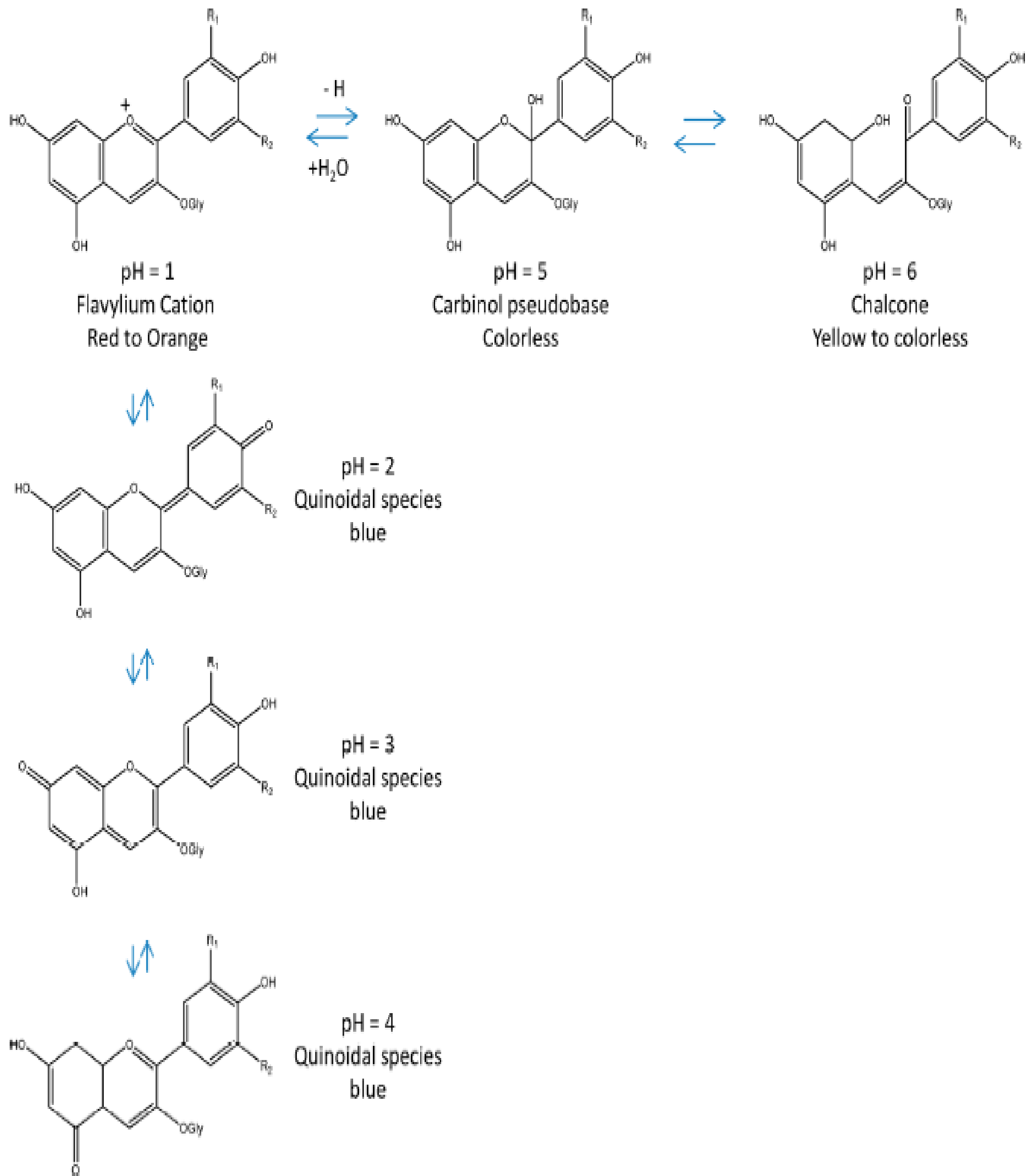

Figure 2.2: Structural influences on colors of anthocyanins[7]

To date, more than 600 different individual forms have been discovered, and are evident in the unique anthocyanin profiles from different plants. The plant kingdom has used a large

number of permutations and combinations of different aglycones being glycosylated with different glycosides. Each anthocyanin profile provides a means of comparison of plant species and cultivars based on the anthocyanins they contain.

Anthocyanins are vulnerable to: pH, light, temperature, metallic ions, oxygen and enzymes like polyphenoloxidase. Anthocyanins have a positive charge at neutral pH, thus their ionic nature causes shifts in form with changes in pH. The anthocyanidin molecule can shift among four forms, depending on the pH: flavilum cation at pH 1, carbinol pseudobase and chalcone forms at pH 4.5, and quinonoidal base at pH 7. The flavilum cation is orange to purple in color, the carbinol and chalcone forms are colorless, and quinonoidal base is blue.[10] Structure also plays a key role in stability, as the anthocyanidin is bonded to additional groups, the stability of the molecule increases. The anthocyanins can be hydrolyzed into the anthocyanidin forms by strong acids.[4]

Monomeric anthocyanins decompose into diphenol and quinone forms, polymerize with other anthocyanins, and/or complex with other phenolic compounds to form co-pigments. These reactions occur in response to stress factors on the monomeric anthocyanins. While some of these reactions result in monomeric color loss, co-pigmentation can preserve color.[11,12,13] Polymerized anthocyanins may retain some antioxidant capacity[14], despite the structural changes to the monomeric anthocyanin form. In the presence of free oxygen, hydrogen ions are removed from the R_1 and R_2 hydroxyl groups. Double bonds are formed once the hydrogen ions are lost, forming an O-quinone. The O-quinone, in the presence of peroxides, will combine with anthocyanins to form an O-diphenol dimer.

2.2 Health benefits

Anthocyanins not only provide pleasing colors, but many studies suggest health benefits such as antioxidant and anti-inflammatory activity.[7, 15] In vitro cell cultures have been used to ascertain the benefits on cells directly.[4,16] Typically skin or pomace extracts are used as the raw material; however, in some cases products containing the anthocyanins, such as juice or wine, are extracted. Crude extracts are produced by solvent extraction and subsequent concentration, while purified extracts are prepared by column chromatography. Interestingly, the use of juice, extracts from skin, pomace, and wine in a single study is rare.

Health benefits to be gained by the ingestion of anthocyanin compounds are limited by bioavailability of the compounds upon ingestion. Bioavailability has been assessed through blood plasma and urine samples, with absorption and excretion of parent compounds and metabolites. Metabolites must also be monitored to prevent an underestimation of absorption. Recently there has been emphasis on the fermentation of anthocyanins by the microbiome in the lower gastrointestinal tract. Since less than 5% of the anthocyanins are absorbed, the changes in the microbiome may be the primary source of health benefits,[7] along with the microbiome in the gastrointestinal tract, which could influence excretion levels through fermentation. The intestinal active distribution of bacteria may be influenced by diet, and changes in this distribution may alter the influence of that microbiome on the anthocyanins. Microbiome, pH, and ambient temperature, have been examined in pig intestines, which are similar in human gut microflora,[17] and culture mediums designed to mimic the conditions of a human distal large intestine. Anthocyanin metabolites that have been identified included: syringic acid, gallic acid, and p-coumaric acid,[18] 3-*O*-methylgallic acid, 2,4,6 –trihydroxybenzaldehyde.[17] Anthocyanins were also found to promote the growth of *Bif idobacterium* spp. and *Lactobacillus-Enterococcus* spp.,

directly influencing the microbiome itself. The anthocyanin metabolites increasing the growth of these intestinal bacteria species represents a positive modification of the bacterial population of the gut.[18]

Anthocyanins have also been shown to have impact on cancer type cells. Anthocyanins derived from *Vitis rotundifolia* grapes have been shown to induce apoptosis within both HT-29 and Caco-2 colon cancer cell lines in vitro, as well as inhibiting cancer cell growth by 50%[4].

Anthocyanins have also been investigated as a possible treatment for spatial learning and memory impairment, induced by d-galactose, in mice. Subcutaneous injection of d-galactose increases oxidative stress in the brain of the mice. The expression of selected synaptic proteins was decreased in the presence of d-galactose, and the anthocyanins derived from purple sweet potatoes were shown to possibly regulate and reverse this decrease, although not to a significant level from the untreated mice.[19]

Oxidative stress factors are also attenuated by anthocyanins. In a mouse model, purple sweet potato anthocyanins reduced oxidative stress induced in the liver by d-galactose and reduced the inflammation caused by this oxidation reaction of d-galactose in the liver as well.[15a] The suppression of hepatocyte apoptosis, triggered by the d-galactose, is accomplished through both inhibition of activation and activity of the enzyme caspase-3. This suppression of this enzyme protects the liver cells.[20] The inhibition of oxidative stress attributed to the sweet potato anthocyanins also reduces insulin resistance within the liver induced by a high fat diet in mice. This is achieved through the suppression of reactive oxygen species production and restoring glutathione content while reactivating antioxidant enzymes. Oxidative stress was also reduced in the endoplasmic reticulum of the liver cells as well.[21]

Dietary anthocyanins have been associated with increasing glutathione levels and which may protect the heart. Maize anthocyanin-mediated increases in glutathione in the hearts of rats increased resistance to regional ischemia and reperfusion insult. [22]

Anthocyanins have significant antioxidant potential, and may inhibit the oxidation of low-density lipoprotein and subsequent endothelial injury. Not only can anthocyanins prevent the oxidation of the low-density lipoprotein[23,24] , but also inhibit the injury caused by the oxidized low-density lipoprotein itself.[25] These effects are dependent on anthocyanin structure

2.3 Anthocyanins in grapes

Grapes have been extensively studied as anthocyanin sources. The grape species that has been most extensively studied is the European wine grape, *Vitis vinifera.* Within that species there are hundreds of cultivars, which express wide variations in color primarily because of variation in type and quantity of anthocyanins present.[26] The volume of published material on anthocyanins in grapes results from the many *V. vinifera* cultivars' and their popularity as wine grapes which are fermented and consumed internationally. North American grapes, *Vitis labrusca* and *Vitis rotundifolia*, are also used to produce wine, but are not as widely consumed or highly regarded as the *V. vinifera* wines. Concord, a *V. labrusca* cultivar, is primarily a juice grape in the United States, where it was first cultivated. Muscadines, as the species *V. rotundifolia* is more commonly known, are native to the southeastern United States and is only cultivated on a local level. These three grape species embody the majority of all grape products consumed in the United States.

In over one hundred selected grape cultivars, 29 distinct anthocyanins were identified. These anthocyanins were mostly malvidin derivatives, and all 29 are glucosides.[26] The other

anthocyanin derivatives present were: petunidin, delphinidin, peonidin, and cyanidin. In these cultivars, total anthocyanins were observed to be higher in wine grapes than table grapes of the same species. Wild and rootstock grapes also contained total anthocyanin levels higher than the interspecific hybrids.[26]

2.4 Environmental effects on anthocyanins

Anthocyanin synthesis is largely controlled by the genetics of the specific cultivar through gene-expression activated enzymatic processes; however, this process is also heavily impacted by environmental factors that influence the vines[27], such as: temperature, water, sunlight, fertilization and organic growing methods. Grapes will contain similar anthocyanins, but the quantity of anthocyanins vary between seasons, due to the changing environmental conditions.

High nighttime air temperature has been shown to result in reduced anthocyanin synthesis in grapes, which has been shown to be associated with reduced expression of genes in the anthocyanin synthetic pathway. These enzymes expressed at lower levels include chalcone synthase, flavanone-3-hydroxylase, dihydroflavonol 4-reductase, leucoanthocyanin dioxygenase, and UDPglucose:flavonoid 3-O-glucosyltransferase, which has been identified as the enzyme most affected by the increased temperature.[28]

Water during grape production has an impact on anthocyanin development through gene expression of synthesis enzymes. Water deficits across four seasons has been found to increase anthocyanin content in merlot grapes, with decreased berry weight and reduced growth.[29] Similar results have been reported for table grapes of the “Red Globe” variety, while opposite results were observed in the table grape, “M. Paliere”.[30]

Light also impacts anthocyanin development, where greater sunlight intensity promotes anthocyanin synthesis; however, excessive light can be detrimental to anthocyanin biosynthesis. High light conditions can result in high grape temperatures, which can exceed the optimal temperature range of the enzymes that promote anthocyanin biosynthesis. Coumarate derivatives of the malvidin-3-glucoside anthocyanin decreased as light intensity increased, suggesting that the enzyme or enzymes responsible for the coumarate pathway were significantly affected by light intensity.[31]

Although directly influencing the grapes and vine by providing essential nutrients for growth, nitrogen fertilization rate does not have any apparent direct effects on anthocyanin levels of table grapes on a short-term basis.[30]

Organic growing practices have been reported to exert an effect on anthocyanin production in eight *Vitis labrusca* juices. Organic grape juices were reported to produce higher concentrations of anthocyanins than conventionally-grown grape juices. The increased anthocyanin content is thought to be a response by the organically-grown grapes to the more difficult growing conditions (e.g. increased pest-induced stress). These results suggest that stress factors during the growth period increase anthocyanin concentrations in response to the absence of pesticides.[32]

Genetic variation has been shown to be responsible for the diversity of anthocyanin composition, while environmental factors can alter gene expression genes and consequently the concentrations of anthocyanins may be affected by a variety of environmental variables (e.g. temperature, water availability, soil conditions, light availability etc.). Environmental variables affect anthocyanin production, while the genetic makeup of the cultivar results in a relatively

consistent distribution of anthocyanins in the plant. [33] This means that identical grape vines will produce the same grape anthocyanins, in both form and distribution in the skin and flesh, year after year. As growth conditions favoring the production of anthocyanins increase, so will the anthocyanins present in the grapes increase; however, the correlation is limited to the specific traits of the vine and thus the cultivar.

Grape harvest time can have a significant impact on anthocyanin levels. Harvest of mature grapes has been shown to increase free anthocyanin levels in wines produced from these later harvested grapes. [34]

2.5 Enological processes

Overall, the process of making red wine can be destructive to the free monomeric anthocyanins in grape juice. Extracting and preserving these pigments in the red wine is not only desirable for the pleasing color and minimal flavor, but also for the potential health benefits.[22,35] As a result, efforts have been made to understand the transitions from grape to wine, and how these transitions affect the characteristics of anthocyanins. Condensation reactions occur throughout the processes; these reactions affect not only anthocyanins, but also catechins and procyanidins. These reactions result in a decrease in these pigments as new polymeric pigments are formed.[36]

From grape to the completion of the fermentation, 3-glucosides of the aglycon anthocyanidins: cyanidin, peonidin, delphinidin and p-coumaroyl glucosides have been reported to decrease throughout all stages of the process. These changes can be triggered by polyphenol oxidase, yeasts, and lactic acid bacteria, which are typically found in the enological process;

however, these potentially damaging compounds may be mitigated by the oxygenation of the anthocyanidin (aglycon) form side rings.[37]

2.5a Storage

Storage of grapes for wine can have a significant effect on anthocyanin content of the finished wine, and is the first step truly in the vintner's control. Control of storage parameters is a valuable step in the process. Storage temperatures up to 20°C have been shown to increase anthocyanins when relative humidity is controlled to increase water loss.[38]

2.5b Juicing and pressing

Before wine can be produced, the grapes must be crushed and juiced. Juicing method and parameters can be controlled to target desired anthocyanin levels. In the production of red wine, the juice is held on the skins for extended periods of time to allow the release of anthocyanins from the skins into the juice. In addition to conducting initial fermentation on the skins, heat can be applied at crushing to increase the release of pigments from the skin. To enhance the liberation of anthocyanins in skins, macerating enzymes (e.g. polygalacturonase, pectinmethylesterase , pectinlyase, etc.) can be added to break the skins down. [39,40]

As a group, pectolytic enzymes have been shown to be effective in increasing the total anthocyanin content of musts, but are not selective to any single anthocyanin form. [40] The efficacy of these enzymes also seems to be dependent on vintage character, only enhancing the native qualities of the grape if those qualities are available.[39]

Hot pressing, or must heating utilizes increased temperature to aid in the extraction of anthocyanins from the skin during the grape pressing stage. Pressing at temperatures of up to

80°C has been shown to increase anthocyanin content in the juice when held for no longer than 45 minutes. These parameters have been observed in *Vitis labrusca*, Campbell Early cultivar.[41] The efficacy of hot pressing also has been noted in another *Vitis labrusca* cultivar, Sunbelt, and a *Vitis rotundifolia* cultivar, Black Beauty. Both cultivars have shown increased anthocyanin concentrations in the juice with hot pressing.[42]

Mash heating has been compared to fermentation on the skins, as a method for increasing available anthocyanins. A combination of both mash heating and fermentation on the skins was found to be most effective, followed by mash heating alone, and finally fermentation on the skins alone.[36]

2.5c Fermentation

Yeasts can affect anthocyanin content during fermentation. During fermentation anthocyanin loss has been observed to be related to the polarity of the anthocyanin molecule, with more polar anthocyanin forms being more vulnerable. Significant loss of color intensity, but not anthocyanins has been linked to certain yeast strains more than others within the fermentation step. [43]

2.5d Cold stabilization

Wines produced from *Vitis rotundifolia* grapes are typically saturated with tartaric acid. When chilled, tartrate crystals form as a precipitate in the wine. To prevent the tartrate crystals from forming in the finished wine, the must is chilled to below 0°C. During this process, anthocyanins can become bound inside the tartrate crystals. As a result, when the tartrate crystals are discarded and the bound anthocyanins are lost.[44]

2.5e Aging

Difficulty maintaining color within the wine as it ages has been well documented. Anthocyanin forms, such as petunidin and delphinidin, have been shown to suffer significant storage-related losses.[1] Pulsed electric field technology has been shown to increase color intensity and phenolic content with a shorter maceration time, but monomeric anthocyanin levels were not significantly affected during aging.[45]

Decreases in monomeric anthocyanins associated with red wine aging have been linked to a rise in anthocyanin derivatives, which add violet hues, maintain the intensity of color, and are formed more readily in grapes from a delayed harvest.[34] These anthocyanin derivatives are responsible for maintaining color intensity in the wine associated with the aging process. Co-pigmentation, which is the process where anthocyanins complex with a cofactor, non-colored compounds, to form new, more intensely colored derivatives. When free anthocyanins complex with a cofactor the newly formed complexes typically become more colored than the anthocyanin alone. The most common wine-related cofactors include: gallic acid, caffeic acid, caftaric acid, catechin, epicatechin, quercetin, kaempferol, and myrecetin. Due to the polarity of water, these phenolic compounds are thought to associate with one another resulting in colored forms. In equilibrium, these anthocyanins are preferentially formed as a result of the less planar nature of the colorless forms, bonds formed are pi-stacking interactions or CH-pi interactions in type as a result of hydrophobic interactions.[11] Available monomeric anthocyanins, along with these cofactors provide the majority of color of wines once bottled.

2.6 Determination of anthocyanins

Numerous methods of analysis exist for anthocyanins. As pigments, anthocyanins can be analyzed in the ultraviolet (UV)/visible(vis) spectrum, and be quantified with Beer's Law. They can also be analyzed as part of the larger group of phenolic compounds. Chromatographic separation is crucial when analyzing specific anthocyanins within complex mixtures like wine or juice.[8]

2.6a High Performance Liquid Chromatography (HPLC) methods

HPLC is the most widely utilized method of anthocyanin analysis. Reversed phase C18 columns are generally used for separation, and 520nm is the wavelength most often used with the UV/visible detector. Separation is generally performed with a gradient between acidified water and an organic solvent. Anthocyanins can be quantified and identified with this method. Quantification can be calculated with a standard for each peak, if these peaks are known and standards exist, or a general standard can be used. The most common standard is malvidin-3-glucoside. When utilizing a general standard, all quantification is expressed in units of the standard. Identification of anthocyanins via HPLC requires a standard for each anthocyanin form which can be difficult and expensive to obtain. The variability of anthocyanin forms can make identification with standards difficult in unknown samples, if standards are available. Mass spectrometry can be used for identification.

Specialized columns for HPLC separation can be employed for specific analytes, one such column, the Primesep B2 mixed mode column, has been shown to group polymerized anthocyanins into a single peak, under certain conditions. This property allows polymerized anthocyanins to be quantified simultaneously with the anthocyanins of interest.[5]

HPLC and UPLC systems can be linked to mass spectrometers, which can aid in the identification of anthocyanins. Mass spectrometers can identify molecular weights of anthocyanins, which can be used to select standards for further identification. Mass spectrometers with fragmentation capabilities can even further identify the anthocyanin form through the fragmentation of the molecular ion, breaking it into the daughter ions. Anthocyanidin types can be identified this way, as well as their basic glycoside structure.

Anthocyanins are especially suited for electrospray ionization mass spectrometry (ESI/MS) because of their charged nature and wide range of variability. Components of the anthocyanins which may vary, such as acylations, bonded sugars, and anthocyanidin forms, can be identified by mass and this data can be used to rapidly identify certain anthocyanin forms which could be otherwise unknown. Anthocyanin forms can also be identified via ESI/MS without extensive purification, which may be required for analysis by other methods.[8]

2.6b Spectrophotometric methods

Similar to the HPLC, spectrophotometers utilize absorbance to quantify concentrations of anthocyanins and other compounds of interest without the need for column separation. Spectrophotometric methods are most commonly used for total anthocyanins and total phenolic content assays. The spectrophotometer allows for more rapid analyses than the HPLC system, which is vital when numerous samples need quantification.

Total anthocyanins can be quantified with the pH differential method [46], which quantifies both monomeric and polymeric forms. This procedure exposes samples to two different pH values. As a result, the monomeric anthocyanins are quantified due to their specific form at each pH value: 1 and 4.5. Results are expressed as cyanidin-3-glucoside units. Polymeric

anthocyanins are determined by bleaching with potassium metabisulfite, which does not bleach polymeric anthocyanins.

Total phenolic concentration assays can also be quantified with the spectrophotometrically. Folin-Ciocalteu reagent is utilized, along with a gallic acid standard curve, to quantify the total phenolic content within a sample.[47] This method cannot quantify individual phenolic compounds, but is useful for noting changes that affect the phenolic compounds as a group.

Microplate detection methods have been developed both for the total anthocyanin[48] and the total phenolic content[47] assays. This method allows many samples to be tested at once, enhancing the assay throughput and resulting in higher efficiency. The microplate methods also allow for minimal sample amounts and reagent for each repetition.

CHAPTER 3. MATERIALS AND METHODS

3.1 Enological process

Ison variety (*Vitis rotundifolia*) grapes were mechanically harvested with a Chisholm Ryder (McConnell Machinery Corporation, 1111 E. 23rd St. Lawrence, Kansas 66046) grape harvester August 24th 2011. Approximately 4762.72kg of grapes were then crushed and destemmed with an Anton Wottle type A2 obertiel crusher/destemmer (WOTTLE Maschinen- und Weinpressenbau, GmbH Schubertstraße 18 – 20 A-2170 Poysdorf, Austria) and pumped into a Mueller 5678.12L stainless steel jacketed tank (Paul Mueller Company 1600 West Phelps Street Springfield, MO 65802) fermentation tank. The pump used was a Kiesel must pump SP5 (G.A. KIESEL GmbH · D-74078 Heilbronn, Germany). Enzyme pectinase used was Zyme-O-color liquid pectic enzyme (American Tartaric Products 1230 Shiloh Road Windsor, CA 95492) which was added at 37mL/ 907.19kg of grapes. The yeast (Lalvin 71B Saccharomyces cerevisiae) was then added (1g/3.79L) and fermentation began. During fermentation the must, immature wine, was kept at 15.56°C, using the Mueller jacketed tank which was cooled with circulation propylene glycol. Headspace in the tanks was filled with argon gas to prevent contamination and to create an anaerobic environment. Must samples were taken weekly, on the same day and time over five weeks, and at two steps in the process: pressing and bottling. Solid samples of skin, pomace, and tartrate crystals were taken at harvest, pressing, and cold stabilization, respectively. All samples were then frozen at -20°C and maintained until analysis.

Pressing occurred in the 2nd week of fermentation, with a Scharfenberger europress, Model EHP 2200 bladder press (Scharfenberger GmbH & Co. KG Maschinenbau Philipp-Krämer-Ring 30 Gewerbegebiet Bruch D-67098 Bad Dürkheim, Germany). The must was pumped from the tank into the press, and then collected in an exposed basin underneath the press

after the grapes were pressed out. Pressing consisted of six cycles, each lasting two minutes, with a spin step after to break up the press cake. Pressure was ascending at the values of 0.2, 0.4,0 .6, 0.8, 1.1 and 1.4 bar. The final volume of must after pressing was approximately 1892.71 liters.

One week after pressing, 181.44 kg of Domino brand refined cane sugar (Domino Foods Inc., 99 Wood Ave. S, Suite 901 Iselin, New Jersey 08830) was added to the must to increase the Brix° to 21. Refined cane sugar can contain up to 10ppm sulfites without labeling quantity (21CFR101.100).

After five weeks of fermentation, must was drawn from the tank into a glass carboy and affixed with a rubber stopper and airlock, all tools and containers for this step were treated with sodium metabisulfite and rinsed before use. This marks the beginning of the laboratory scale portion of the experiment. Must was stored at -4.44°C for 28 days to precipitate tartrates and cold stabilize.

After cold stabilization, the must was filtered with 4g bentonite in 125mL deionized water and then siphoned into bottles with a racking cane. The bottles were corked and then stored at 15.56°C. The tartrate crystals and remaining wine were stored at 4.44°C until filtration through a stainless steel screen. After filtration, the tartrate crystals were centrifuged for two runs of 10 minutes. After each run the supernatant was decanted and stored. The tartrate crystals were then freeze dried. The complete process from harvest to bottling is shown in Figure 3.1.

Samples taken from the processing samples included: the first sample group was a weekly sample collected each of the 5 weeks, a post-pressing must sample and a post bottling wine sample. The post pressing and post bottling samples were collected in Week 2 and Week 9, respectively. The solid samples group included skin and press cake, obtained Week 0 and Week

2, which were extracted for analysis. Also within the solid sample group was the tartrate crystal sample, filtered and collected while bottling the wine in Week 9.

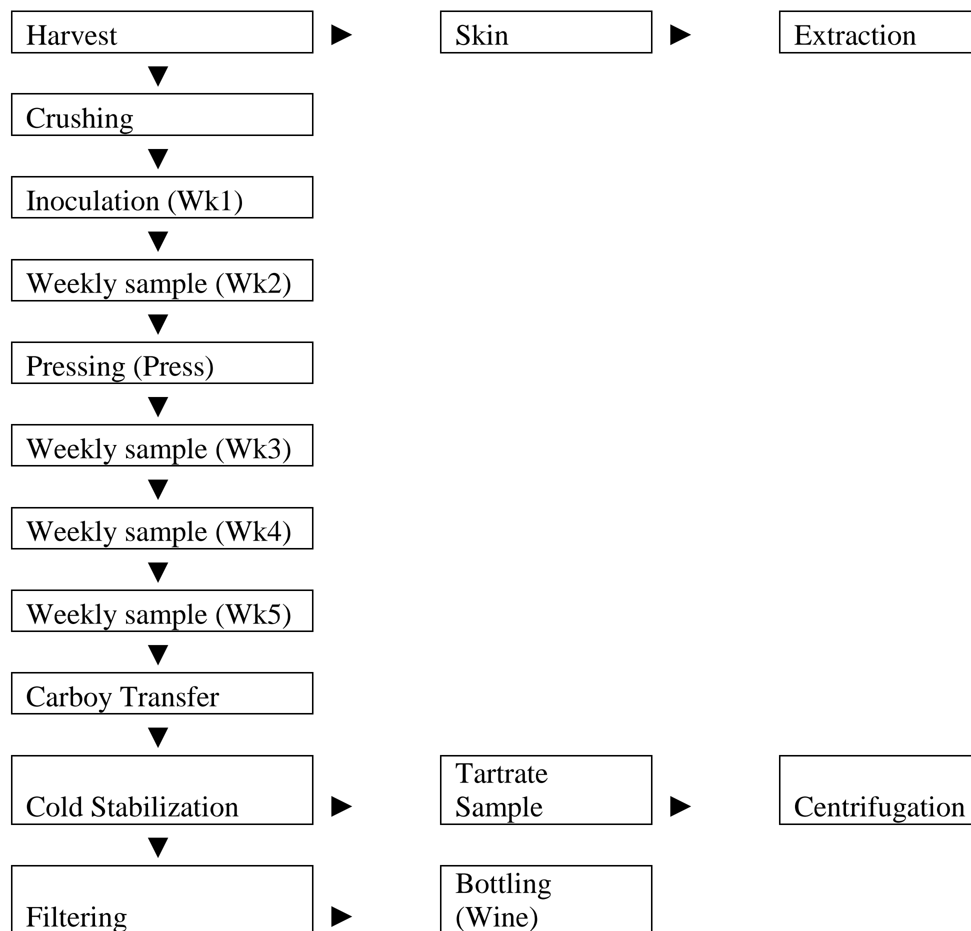

Figure 3.1: Flowchart of enological process and sampling

Table 3.1: Comprehensive sampling list and chronology

Sample Name	Sample Type	Week Sampled	Total Phenolic	Total Anthocyanins	HPLC
Skin	solid	0			X
Wk 1	fluid	1	X	X	X
Wk 2	fluid	2	X	X	X
Press	fluid	2	X	X	X
Press cake	solid	2			X
Wk 3	fluid	3	X	X	X
Wk 4	fluid	4	X	X	X

Table 3.1: Continued

Sample Name	Sample Type	Week Sampled	Total Phenolic	Total Anthocyanins	HPLC
Wk 5	fluid	5	X	X	X
Tartrate crystal	solid	9			X
Wine	fluid	9	X	X	X

3.2 Sample preparation method for grape skin

Whole frozen grapes from harvest were frozen at -87.7°C under liquid nitrogen spray and stored in dry ice until blending. All samples were blended in a Waring (Waring Laboratory 314 Ella T. Grasso Ave. Torrington CT 06790) laboratory blender with a stainless steel pitcher.

Skin was peeled off the grapes by hand while still frozen and then blended in dry ice. The skin powder was transported frozen into a -20°C freezer and double sealed in airtight plastic bags under the cover of the carbon dioxide vapor.

3.3 Skin and press cake extraction

The optimized extraction method was adapted from the literature,[49] with minimal adjustment: formic acid (0.1%) was utilized instead of Triflouroacetic acid (TFA) (0.05%), which was the only adjustment to the extraction solvent. Skin and press cake were extracted with a mixture of acetone, water, and methanol (51:34:15, v/v/v) acidified with formic acid (0.1%). 50mg of solid was added to 7mL of solvent and allowed to extract for 67 minutes at room temperature. After extraction, the samples were centrifuged for 10 minutes and then decanted. Approximately 30% of the supernatant was evaporated under vacuum in a Labconco centrivap (Labconco, 8811 Prospect Avenue Kansas City, MO 64132-2696) at 40°C for 20 minutes, and

then frozen in liquid nitrogen. After freezing, the samples were loaded into a Heto powerdry LC 3000 freeze dryer (Thermo Fisher Scientific 81 Wyman Street Waltham MA 02454 USA). After drying samples were re-dissolved in 225 µl methanol/water (20:80) acidified with 1% HCL, filtered through a 0.22 micron syringe filter and then prepared for HPLC analysis. Tartrate crystal samples were also dried and re-dissolved in this manner.

3.4 Quantification and separation of anthocyanins by high performance liquid chromatography

For HPLC analysis 225 µl of each fluid sample, both must and extracts, was mixed with 25 µl malvidin-3-galactoside chloride standard solution. Sample injections were 10 µl and were repeated five times in HPLC, during which the peaks were manually collected. Collected peaks were analyzed with UPLC ESI mass spectrometry to aid in the identification of the constituent anthocyanin in the peak. Manually collected peaks were frozen at -20°C until analysis.

The HPLC methodology was adapted from literature[5], with the following instrumentation. The chromatographic system consisted of a Waters 616 pump, 600S controller, Waters 2707 autosampler, a Varian Metatherm HPLC column temperature controller (Agilent Technologies 5301 Stevens Creek Boulevard Santa Clara, CA 95051), a Waters 996 Photodiode Assay Detector which were controlled by Waters Empower 2 software (Waters corporation, 34 Maple Street Milford, MA 01757).

The separation was carried on a Primesep B2 mixed-mode column (250 mm x 4,6 mm I.D., 5SIELC Technologies, Prospect Heights, USA) maintained at 35°C by a Varian Metatherm HPLC column temperature controller. The mobile phase consisted of 5% formic acid in DI water(mobile phase A) and 5% formic acid in acetonitrile (mobile phase B) using the following gradients: 4% B (0 min); 9.5% B (25 min); 15% B (45 min); 20% B (60 min); 20% B (70 min);

100% B (75 min); 100% B (80 min); 4% B (85 min); 4% B (90 min). Flow rate was 1.0 ml/min. The detected wavelength was set at 520 nm.

Individual anthocyanins were quantified as malvidin-3-galactoside chloride units based on integrated areas measured 520nm. Each sample was run five times, and means were calculated and arranged according to samples taken over time.

3.5 Determination of total phenolic content

A microplate assay was adapted from literature, [47] with minimal adjustment. All tests were performed at room temperature, approximately 77°C. Either 50 microliters gallic acid standard or sample was added to each well, then 50 microliters of 1:5 Folin-Ciocalteu reagent diluted with water were added. One hundred µl of 0.35m sodium hydroxide solution was then added. Plates were read at 760nm, with no agitation, until a maximum value was reached. The samples were diluted 1:5 with deionized water after the initial absorptions were excessively high. A gallic acid standard curve was created, and concentrations were calculated by plotting sample absorbance on that curve. The plate reader used for this analysis and the total monomeric and polymeric anthocyanin analysis was the Bio-Rad Benchmark Plus microplate spectrophotometer #170-6930.

3.6 Determination of total monomeric and polymeric anthocyanins

A microplate assay was adapted from literature,[48] with minor adjustments. The sole adjustment made was: samples were not evaporated as in the literature prior to analysis and were diluted instead. Samples were diluted as a result of the extremely high absorbance values on the initial test. Samples were diluted with deionized water in a ratio of 1:5, 50 µl of sample was added to each well in triplicate. Nine wells were used per sample with three diluted with sodium

phosphate buffer: pH 1.0, and three more with sodium acetate buffer: pH 4.5. The final three were diluted with potassium metabisulfite, using 100 microliters of each buffer. The plate was read at 520 and 700nm. All experiments were performed at room temperature, ~26°C.

Absorption was calculated by the equation:

$$A = (A_{520nm,\ pH1} - A_{700nm\ ,pH1}) - (A_{520nm\ ,pH4.5} - A_{700nm,pH4.5})$$

Total monomeric anthocyanins were calculated in cyanidin-3-galactoside units with the equation:

$$C(g/L) = \frac{[(A)(MW)(Dilution\ factor)]}{[molar\ absorbance\ (mol/L)][path\ length\ (cm)]}$$

Where MW=493.5, Dilution factor = 5.5(monomeric) 1.5 (polymeric), and Molar absorbance = 28000

Path length is calculated as the height of the cylinder of sample within the wells. In this setting, path length = 8.69 (monomeric).

3.7 Mass spectrometry identification of anthocyanins

Mass spectrometry, especially tandem mass spectrometry with HPLC or UPLC is an effective tool to analyze anthocyanins. Mass spectrometry helps with the identification of the anthocyanins which elute from the UPLC or HPLC columns. When tandem MS is employed, the fragmentation patterns help identify and differentiate both the backbone and glycosides in the chromatogram.

Wine filtered through a 0.2 micron syringe filter was directly infused into the mass spectrometer, rather than separated through a column to allow for all ions present in the wine to be analyzed. The mass spectrometer was tuned for known probable anthocyanin mass weights

collected from literature.[8,4] After tuning for the parent ions, the cone voltage was optimized. The transitions into daughter ions were also tuned to optimize collision voltage. Six different anthocyanin compounds were found based on mass weights of both parent ions and daughter ions. The malvidin-3-galactoside chloride standard was also compared to the unidentified anthocyanins. The system was run in ESI positive ion mode, with a capillary voltage of 2.48kV. The source temperature was 125°C, and the desolvation temperature was 350°C. The desolvation gas was nitrogen with a flow rate of 600L/hr, while the collision gas was argon, with a flow rate of 0.15mL/min.

Ultra performance liquid chromatography (UPLC) was used to separate the collected peaks, once the mass spectrometry system was tuned to the precise anthocyanin weights and daughter transitions. Formic acid (0.1%) in water and acetonitrile were chosen as the mobile phases. The column was a BEH C18 column by Waters, with a 1.7um pore size and dimensions of 2.1 x 50mm. Column temperature was maintained at 25°C and the flow rate was 0.3mL/min. Formic acid (0.1%) in water was designated A, and Acetonitrile was designated B. The gradient for UPLC separation started with an initial ratio of 100% A. The gradient was shifted to 65% A and 35% B over 7 minutes and back to 100% A by 7.10 minutes. The remainder of the ten minute run, 2.9 minutes, was held at 100% A. Full loop injections were used for the sample, with a loop volume of 23.2 µl.

3.8 Data Analysis

Statistical software used was Microsoft Excel 2010 (Microsoft Corporation, One Microsoft Way Redmond, WA 98052-6399). One way Analysis of Variance tests were run at α: 0.05. Groups tested included: HPLC concentrations grouped by anthocyanin form, HPLC

concentrations grouped by sampling time, total phenolic content, total monomeric anthocyanin content, and total polymeric anthocyanin content.

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Total phenolic content throughout the enological process

Analysis of variance confirmed a significant difference between samples taken in different weeks (P=4.58E-14), specifically week 1. Week 1 and 2 contain the crushed grapes as well as the juice. Phenolic compounds extracted from the wine must showed that between the first and second week the total phenolics increased over 50% while on the skins (Figure 4.1); however, after pressing stage the total phenolics remained constant.(Figure 4.1). As expected, removal of the grape pomace halted the extraction of phenolic compounds, resulting in no rise in total phenolic compounds. The phenolic levels observed after pressing (Week 2), remained stable during the three week fermentation process. After one week of fermentation on skins the total phenolic contents were significantly lower than values reported for Noble grapes.[50] This concurs with other research on extracts taken from both Ison and Noble grapes, which showed greater levels of total phenolic compounds in the pulp and seeds in Noble grapes.[3] The skin of Ison grapes, when compared to Noble, contains similar amounts of phenolic compounds.[4] In the wines produced from these fruit crops, phenolics in skin and pulp are most relevant sources of phenolic compounds. The seeds may be removed before the primary fermentation, and are not typically used within the fermentation for flavor.

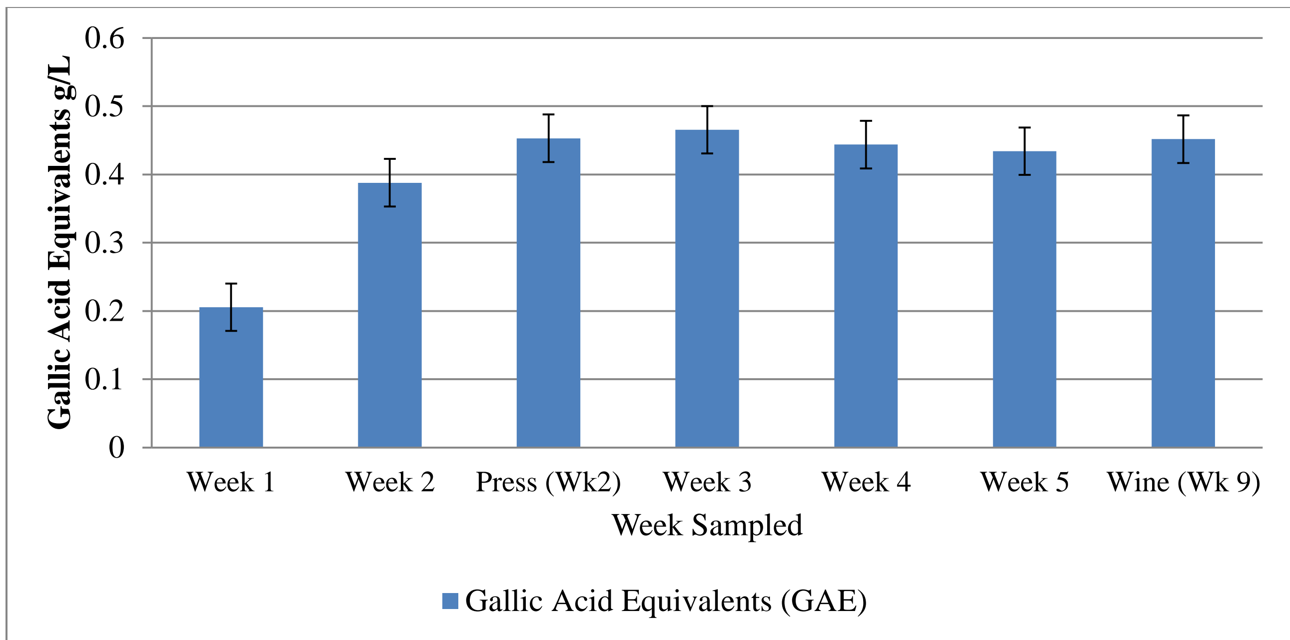

Figure 4.1: Mean total phenolic content expressed in acid equivalents with standard error

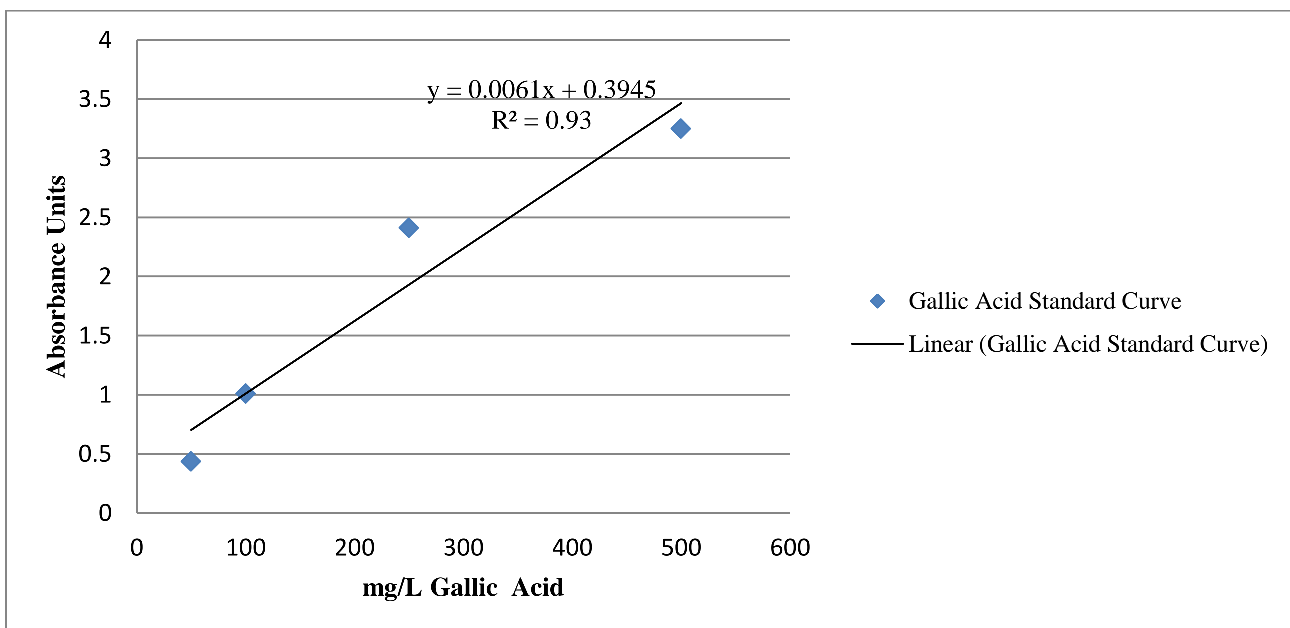

Figure 4.2: Gallic Acid Standard Curve

4.2 Total monomeric anthocyanin levels throughout the enological process

Analysis of variance showed monomeric anthocyanin levels to be significantly different between weeks (P=2.49E-10). Similar to measures total phenolic content during the enological

process, total monomeric anthocyanins peaked at the point of pressing (Figure 4.2). Unlike total phenolic content during the process, the total monomeric anthocyanin levels decline after pressing, and rebound as well at week 5. This decline may be connected to the addition of the sugar in the same week. The cause of the rebound is unknown.

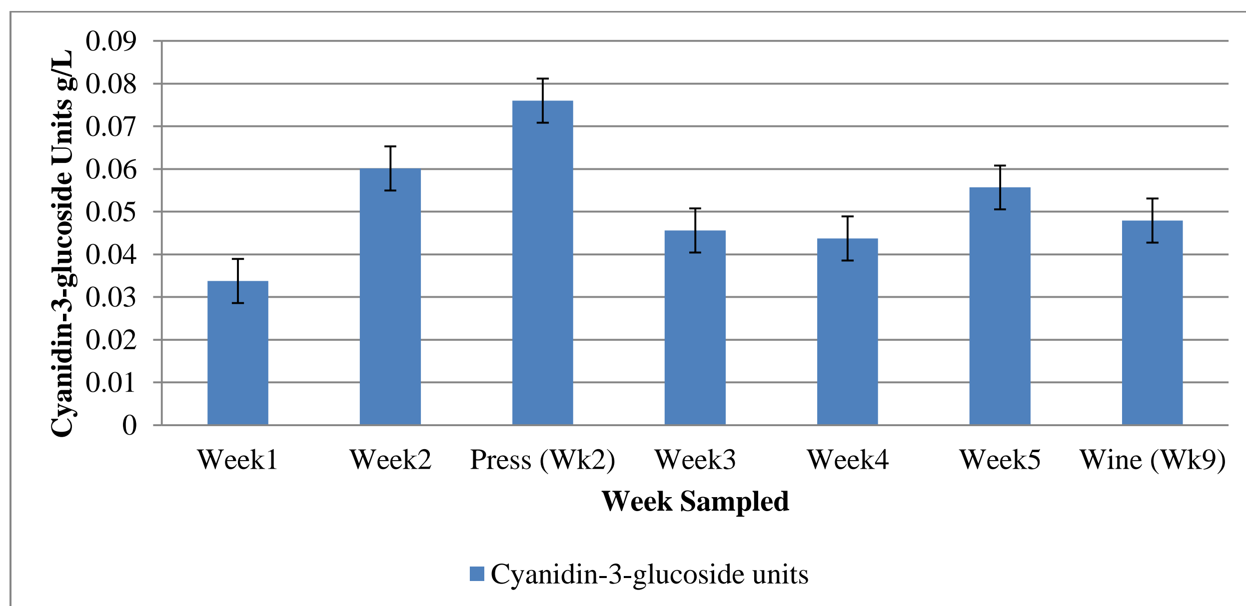

Figure 4.3: Mean total monomeric anthocyanins expressed in cyanidin-3-glucoside units with standard error.

Previous research indicated that total monomeric anthocyanin concentration for Ison grapes was similar[4] or higher than[3] Noble variety levels. Values in this study are lower than previously published studies, which analyzed extracts of skin, pulp, and seed.

4.3 Total polymeric anthocyanin levels throughout the enological process

With the exception of Week 2, polymeric anthocyanins accounted for less than 10% of the total monomeric anthocyanin values from all samples (Figure 4.3). In the case of the weekly samples, there were no significant differences between them (P =0.077). Polymeric anthocyanins were low during Week 1, highest in Week 2 before pressing, and dropped after the press, The

increase between Week 1 and 2 and the decrease between week 2 and 3 were the largest changes. Drops in total monomeric anthocyanins in week 3 do not correspond with a rise in polymeric anthocyanins in week 3,which instead are reduced.

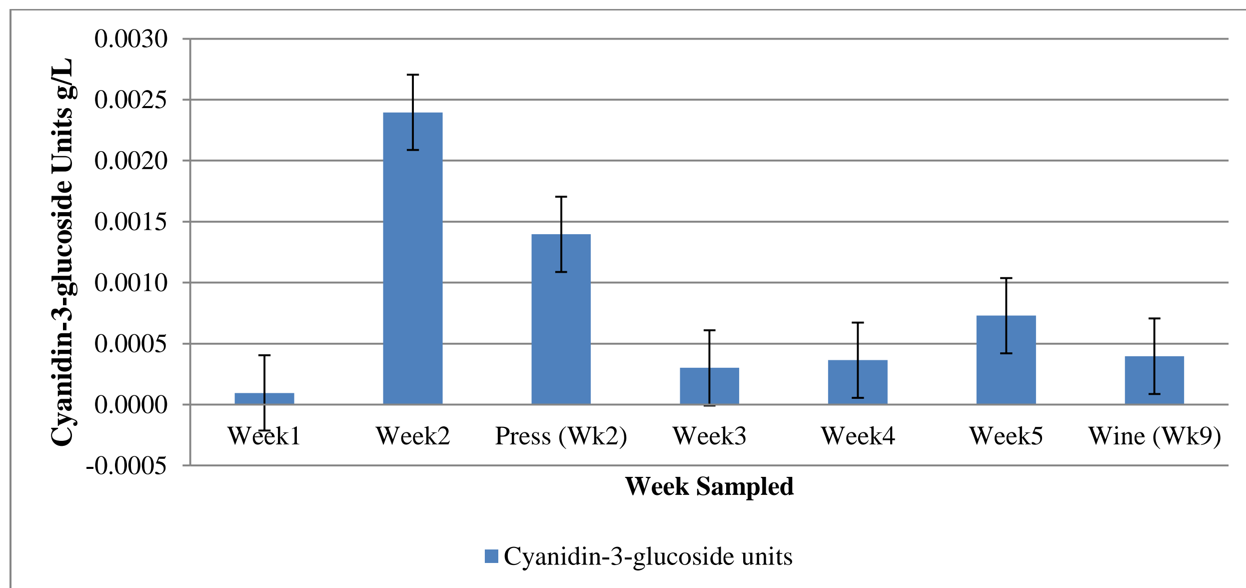

Figure 4.4: Mean total polymeric anthocyanins expressed in cyanidin-3-glucoside units during the enological process with standard error

All chromatograms for each of the ten samples: Weeks 1-5, Press (Wk2), Wine (Wk9), Skin(extract), Press Cake(extract), and Tartrate(filtered crystals), exhibited similar retention times for each peak under the conditions of the runs (Figure 4.4). Regardless of form, peaks remained separated and required no additional treatments. All runs were in succession, with no delays between samples, as a way to minimize variance.

The use of the mixed-mode column allows for the comparison of eluted peaks late in these runs to determine polymeric anthocyanins; however, these chromatograms did not integrate a polymeric anthocyanin peak. This property of the mixed-mode column was previously demonstrated[5] and concurs with the findings of the microplate assay in this study.

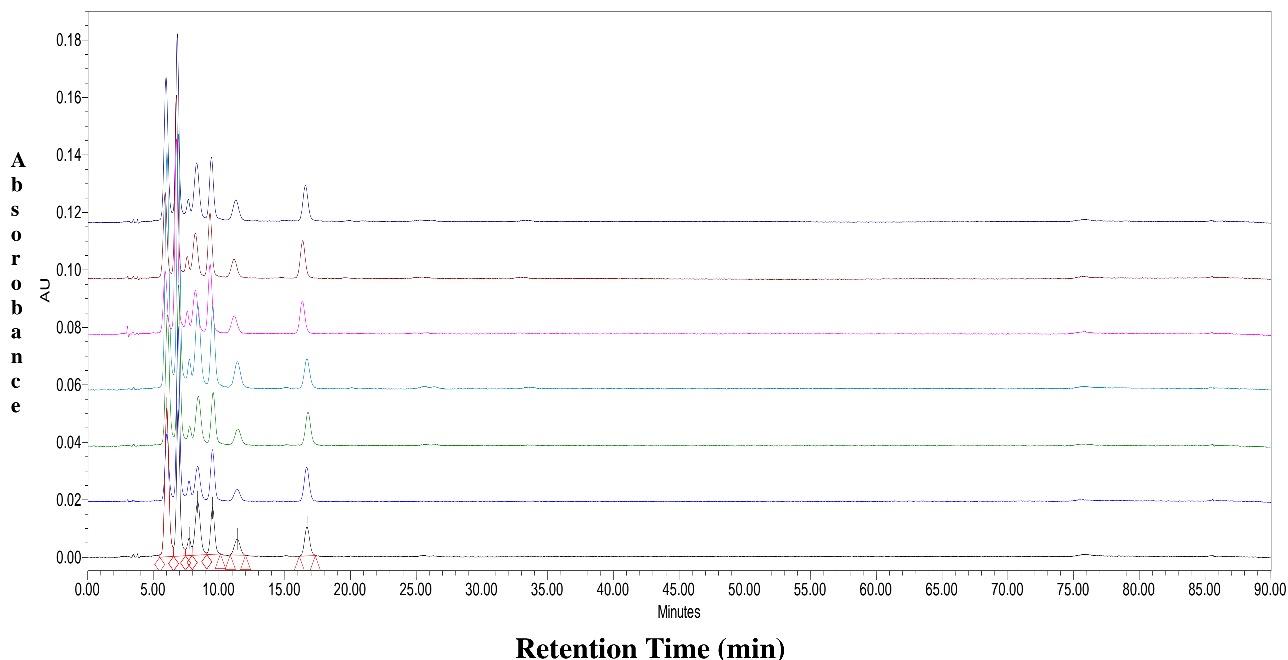

Figure 4.5: Anthocyanin chromatograms with overlap. All integrated peaks are shown. Peaks in the chromatograms are, in order from top to bottom: Tartrate, Press Cake, Skin, Wine, Weeks 5-3, Press, and Weeks 2 and 1.

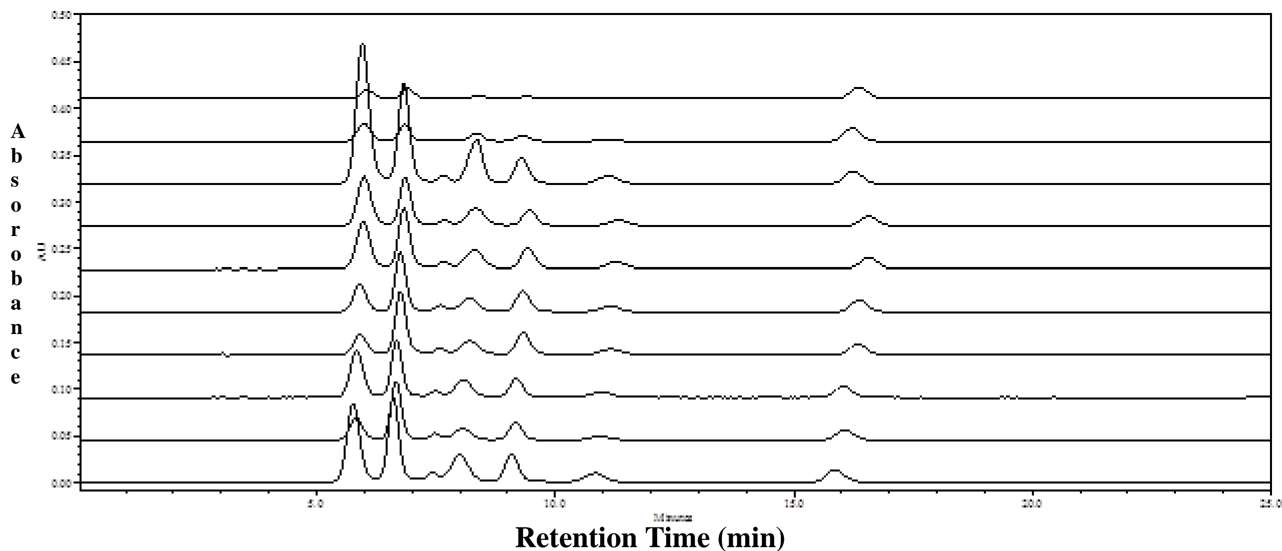

Figure 4.6: Anthocyanin chromatograms with overlap, shortened to 25 minute retention time. All integrated peaks are shown. Peaks in the chromatograms are, in order from top to bottom: Tartrate, Press Cake, Skin, Wine, Weeks 5-3, Press, and Weeks 2 and 1.

4.4 Individual anthocyanin content during enological process

Results were compiled into Figure 4.7, expressed as grams per liter. Analysis of variance did not show significance between weeks in concentration (P=0.85), but did show significant differences between anthocyanin forms (P=2.42E-12).

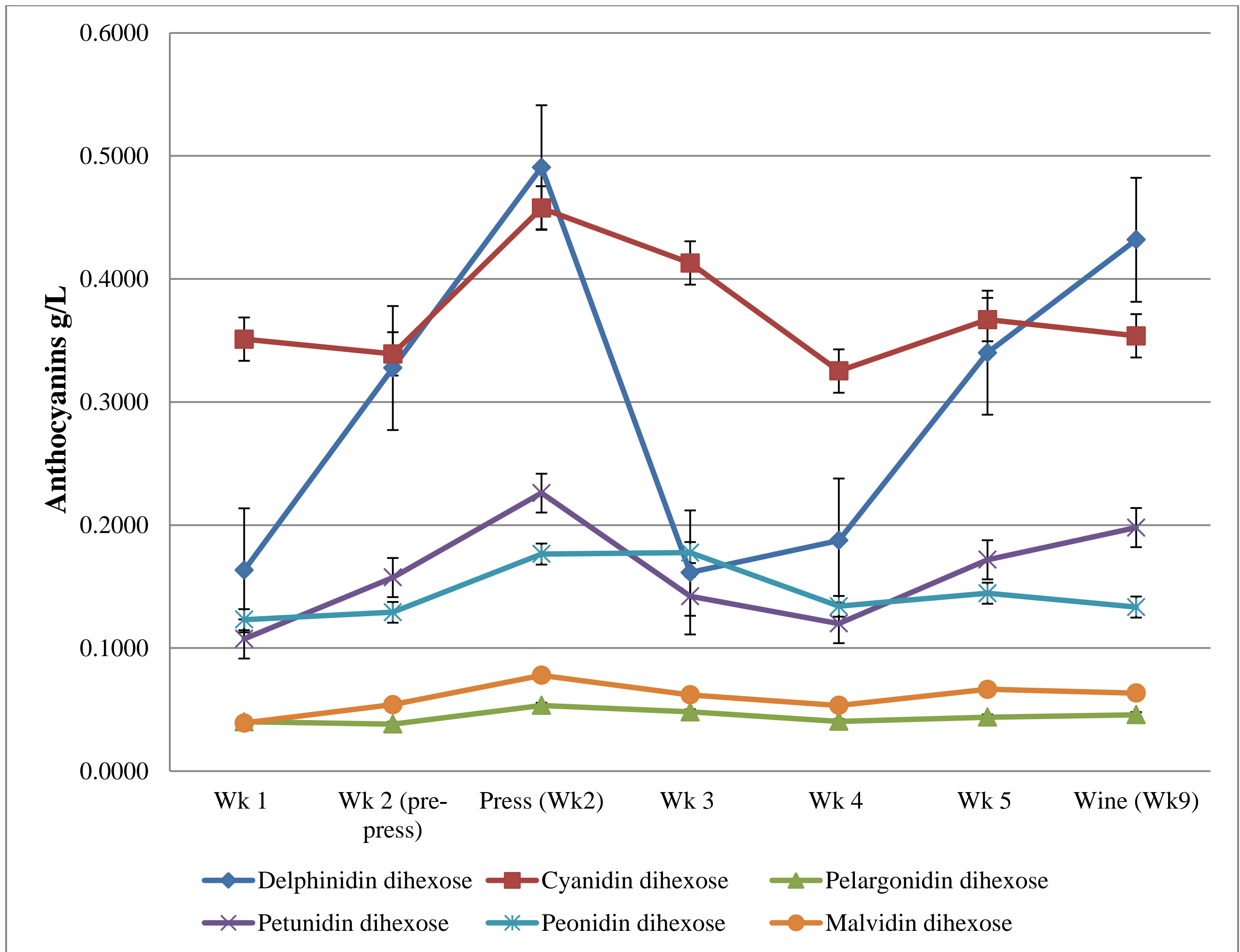

Figure 4.7: Mean concentrations of individual anthocyanins with standard error by sample

Peak concentrations are expressed as malvidin-3-galactoside chloride units. All peak concentrations were at the pressing stage. Delphinidin dihexose (Dpd) showed the most fluctuation over the fermentation process, while cyanidin dihexose (Cyd) was initially the most abundant. Petunidin dihexose (Ptd) and peonidin dihexose (Pnd) had similar increases and decreases to each other, although peak Ptd exhibited more change than Pnd as the weeks passed. Pelargonidin dihexose (Plr) and Malvidin dihexose (Mvd) were also similar in both initial concentration and weekly changes.

When compared to values reported in Noble variety grapes after one week fermentation on the skin, Ison values of each anthocyanidin type were lower than those derivatives within the Noble variety grapes.[50] No other study found at the time of this research tested fermentations weekly.

4.5 Mass spectrometry identification of anthocyanin forms

Anthocyanin peaks can be clearly seen among other mass values in this wine sample which has been filtered with a 0.2 micron syringe filter and no additional purification (Figure 4.8). This demonstrates the ease of analysis of an unpurified sample of this type on an untuned instrument. From this general analysis, the molecular ion masses of interest were be selected for tuning parameters. After tuning, the masses were collided and fragmented for mass confirmation of daughter ions. The daughter ions, broken off of the molecular ions, confirmed the identitiy of the molecular ions, first fragmenting into the monohexose and then anthocyanidin. As a test of a known compound, this fragmentation was also noted in the standard, with the galactose sugar splitting off of the malvidin aglycon.

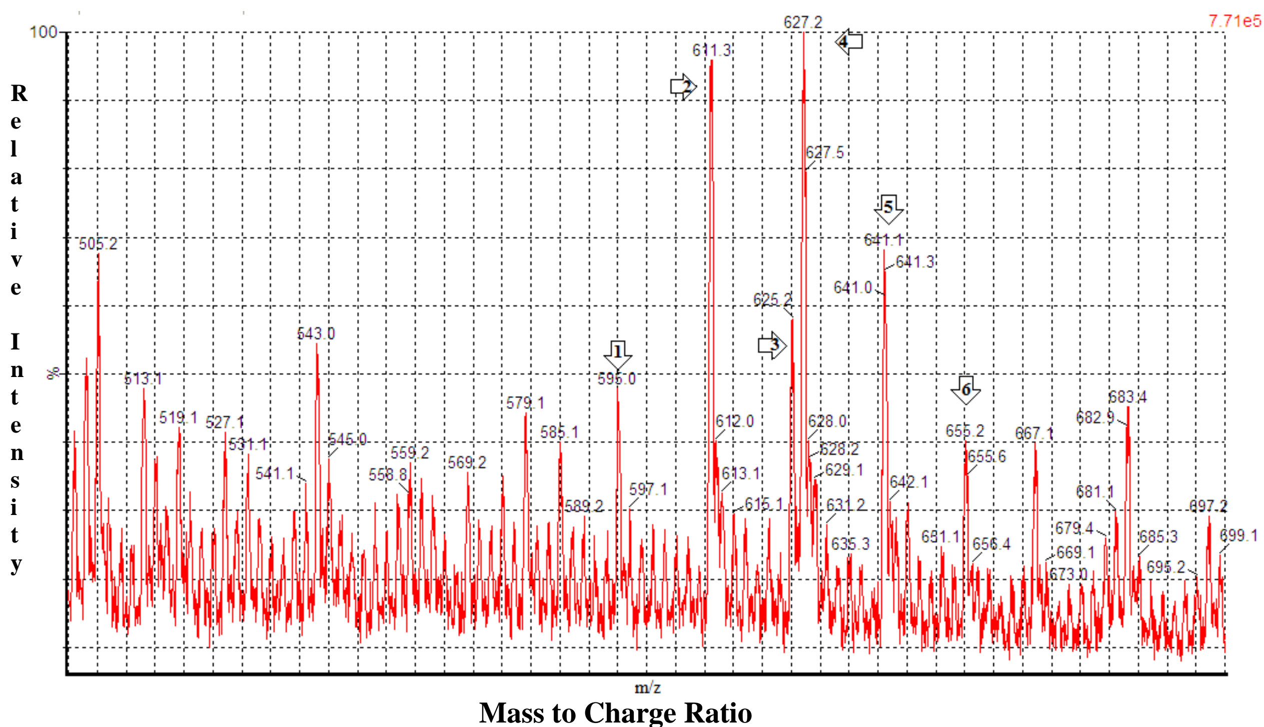

Figure 4.8: Anthocyanin peaks by mass value in finished wine sample: 1. Pelargonidin dihexose, 2. Cyanidin dihexose, 3. Peonidin dihexose 4. Delphinidin dihexose, 5. Petunidin dihexose, 6. Malvidin dihexose

4.6 UPLC identification of collected peaks

The values from tuning yielded the Multiple Reaction Monitoring (MRM) parameters (Table 4.1), which would scan each run of the collected peaks for the constituent anthocyanin. Constant MRM parameters were Dwell and Cone Voltage, which were 1.0 and 175, respectively. Combined with the retention time (RT) data, these parameters allowed for an accurate identification in the collected peaks from HPLC runs to match the forms found in the finished wine. Confirming the separations of the HPLC runs, corresponding peak numbers of different sample runs were identified as the same anthocyanins during the UPLC runs. Mass numbers of each anthocyanin form concur with previous research.[8] Identified forms were consistent with

previous partial identification as well.[4] One exception was the pelargonidin derivative, which was not identified in this previous study.

Table 4.1: MRM parameters, retention times, and ion transitions

RT	Compound	Ion transitions	Collision Voltage
2.33	Delphinidin dihexose> Delphinidin monohexose	627.40>465.10	23
	Delphinidin dihexose> Delphinidin aglycon	627.40>302.90	44
2.66	Cyanidin dihexose> cyanidin monohexose	611.20>449.10	21
	Cyanidin dihexose> cyanidin aglycon	611.20>287.00	44
2.69	Petunidin dihexose> petunidin monohexose	641.10>478.90	23
	Petunidin dihexose> petunidin aglycon	641.10>317.00	44
2.88	Pelargonidin dihexose> pelargonidin monohexose	595.10>433.20	23
	Pelargonidin dihexose> pelargonidin aglycon	595.10>270.80	44
2.94	Malvidin dihexose> malvidin monohexose	654.10>493.00	23
	Malvidin dihexose> malvidin aglycon	654.10>331.00	44
2.98	Peonidin dihexose>peonidin monohexose	625.00>463.00	23
	Peonidin dihexose>peonidin aglycon	625.00>301.00	44
3.58	Malvidin-3-galactoside chloride(standard)> Malvidin aglycon	493.00>331.00	44

All anthocyanins identified were dihexose forms, identified by the loss of mass at each fragmentation. Ion mass lost at each fragmentation was 162.2, the mass of a hexose sugar, with the resulting mass after the second collision identified as the anthocyanidin ion. (Figure 4.9, Table 4.2)

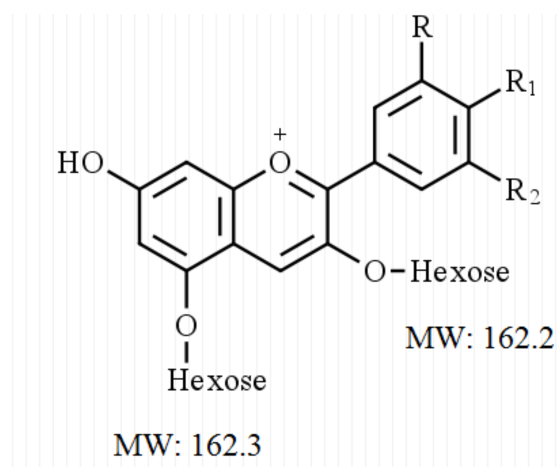

Figure 4.9: Dihexose anthocyanin form

Table 4.2: Molecular ions and daughter ion masses

Anthocyanidin	**Molecular Ion Mass**	**1st Daughter Ion Mass**	**2nd Daughter Ion Mass**
Delphinidin	627.4	465.1	302.9
Cyanidin	611.2	449.1	287
Petunidin	641.1	478.9	317
Pelargonidin	595.1	433.2	270.8
Malvidin	654.1	493	331
Peonidin	625	463	301

Peaks were identified as follows (Table 4.3), in order of elution in HPLC runs. These peaks were identified by UPLC runs after collection during the HPLC runs.

Table 4.3: Peak Identification by UPLC

Peak #	**Identified Anthocyanin**
1	Delphinidin dihexose
2	Cyanidin dihexose
3	Pelargonidin dihexose
4	Petunidin dihexose
5	Peonidin dihexose
6	Malvidin dihexose

Five of these six anthocyanidin forms had been identified in a previous study in the Ison grape variety [4]; however, only the anthocyanidins were identified as a result of the hydrolytic separation involved in their analysis. In addition, pelargonidin was not present in the samples contained in this reference study, even after hydrolysis.

4.7 Skin and press cake extract composition

Skin anthocyanin extracts represent total anthocyanins, while press cake anthocyanin extracts represent total anthocyanins left un-extracted from the wine must. Tartrate anthocyanins represent anthocyanins lost as a result of cold stabilization before finishing and bottling. Total anthocyanin distribution within skin extracts are represented below. (Figure 4.10)

Total anthocyanins extracted from skin into the wine must at the time of pressing totaled 84.74%. Each anthocyanin form was extracted at differing efficiencies, resulting in an increase or decrease in percentage of that form contained within the press cake (Table 4.4).

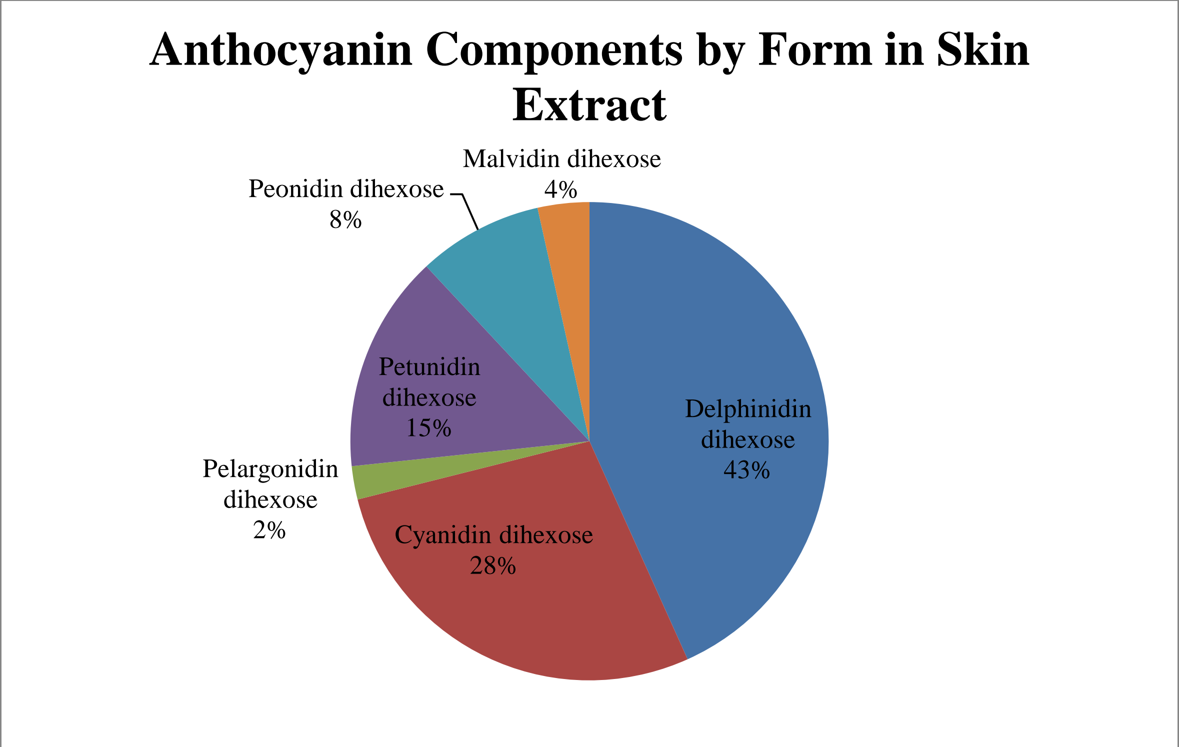

Figure 4.10: Anthocyanin distribution in ison grape skin

The most efficiently extracted anthocyanin form into the must was delphinidin dihexose, while the most inefficiently extracted form into the must was peonidin dihexose. The tartrate crystallization seems to preferentially include cyanidin dihexose over all other forms, while petunidin dihexose is the form most retained in the must when crystallization occurs. Anthocyanin loss as a result of tartrate crystallization is largely dependent on the quantity of tartaric acid within the must. For this fermentation batch, each milligram of tartrate crystal precipitated contained 0.15% of the total anthocyanin content.

Table 4.4: Anthocyanin distribution changes between press cake extract and skin extract and between tartrate crystals and wine must

Anthocyanin Form	Skin Distribution	% Change Skin to Press Cake	% Change Must to Tartrate
Delphinidin dihexose	43.27%	-7.32%	-0.80%
Cyanidin dihexose	27.81%	1.38%	6.79%

Table 4.4: Continued

Anthocyanin Form	Skin Distribution	% Change Skin to Press Cake	% Change Must to Tartrate
Pelargonidin dihexose	2.25%	0.54%	1.02%
Petunidin dihexose	14.72%	1.60%	-4.38%
Peonidin dihexose	8.46%	2.47%	-1.35%
Malvidin dihexose	3.49%	1.33%	-2.54%

Anthocyanin extractions quantified total anthocyanins by form, within the skin and press cake. HPLC quantification, in malvidin-3-galactoside chloride units, allowed each of the newly identified anthocyanin forms to be analyzed within each enological environment.

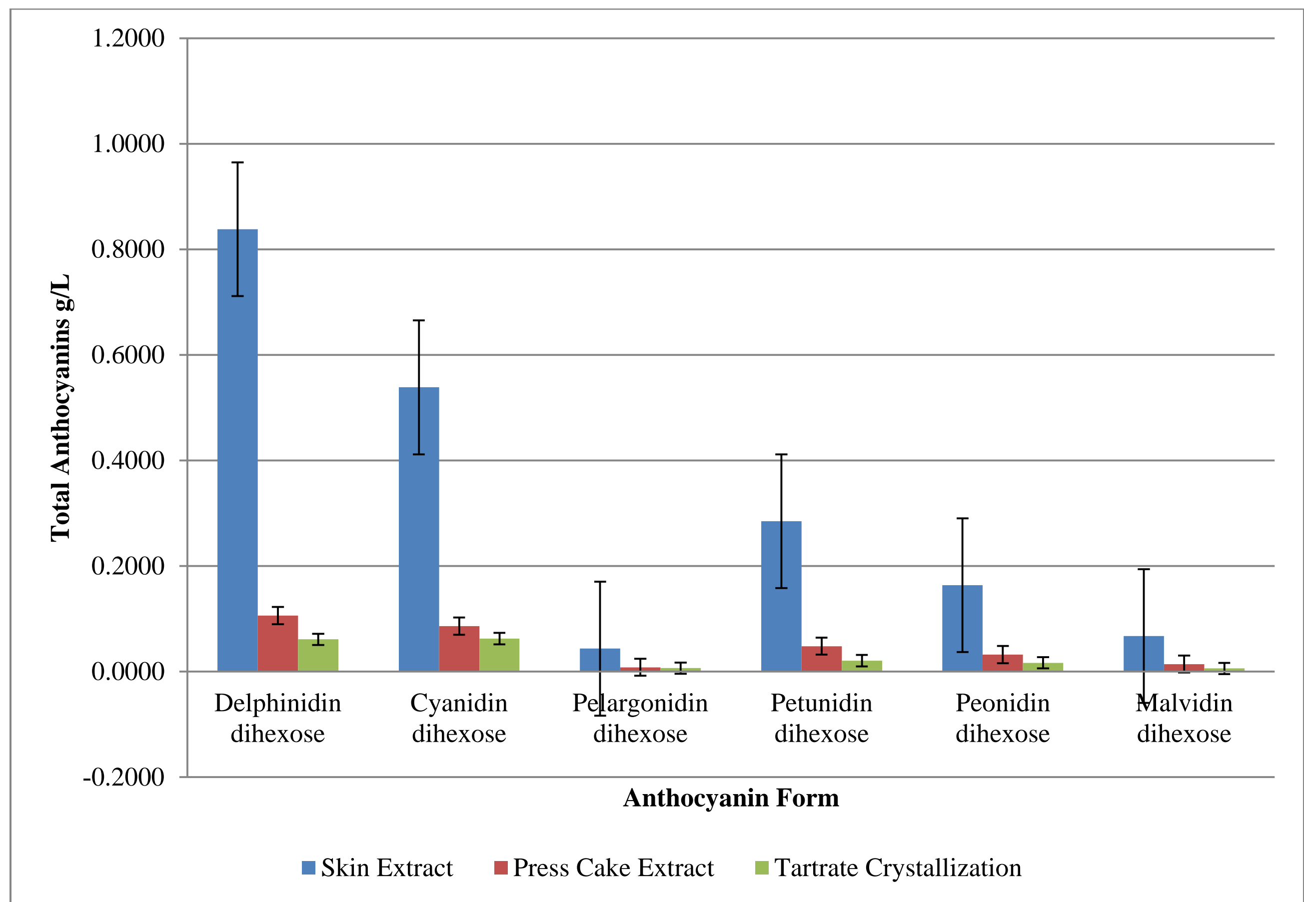

Figure 4.11: Anthocyanin forms and concentrations in extracts

Total anthocyanin levels based on 100g fresh weight extraction were found to be greater than those previously observed for the Ison variety, as well as the Noble variety.[3]

CHAPTER 5: CONCLUSION AND FURTHER RESEARCH

5.1 Conclusion

Total anthocyanin content remained high after the press, not dropping as heavily as the anthocyanin levels in week 3. This suggests that the phenolic compounds in the wine must are less affected by the processes of vintification than the anthocyanins.

Total monomeric anthocyanins exhibited the same drop after pressing noted in the HPLC analysis, confirming the result of the HPLC analysis. Clearly the sample taken immediately after the press experiences such a drastic reduction in monomeric anthocyanins, that for anthocyanin stability in future vintages to be maintained, the cause of this sudden drop must be ascertained. Total polymerized anthocyanins analysis, as well as HPLC analysis, both confirm that the anthocyanin decrease is not due to polymerization. There is no corresponding increase in polymerized anthocyanins as monomeric anthocyanins decrease, suggesting the decrease in monomeric anthocyanins is not caused by anthocyanin polymerization reactions.

One notable influence on the samples marked "week 3" is that sugar was added to the wine at that time. This could explain the large fall in anthocyanins, as white sugar was used to increase the brix. White sugar is bleached with sulfur dioxide. Sulfur dioxide has been shown to degrade anthocyanin compounds, [6] and some residual sulfur dioxide could be present in the white sugar[51]. This sharp drop is noted in the concentrations of the individual anthocyanins by both the HPLC analysis and also the total monomeric anthocyanin analysis.

The increase of delphinidin dihexose in week 5 in the HPLC analysis, and the increase of total monomeric anthocyanins in week 5 in the spectrophotometric method is a concern. With no

skins in the must from which to extract the anthocyanins, and no tissue present to synthesize anthocyanins, the increase in observed anthocyanins remains unexplained.

Possible sources of this increase could include a reversal of the possible sufite bleaching that occurred in week 3. Described in previous research, two anthocyanin forms, pelargonidin 3-glucoside and cyanidin 3-rhamnoglucoside, had exhibited reversible sulfite bleaching at low pH.[52] This reversal of the sulfite bleaching would increase absorbance and, by extension, quantified anthocyanins in both methods used in this study.

Anthocyanin compounds may also be absorbed by yeast cells and retained within the cell walls. A delphinidin compound has been shown to be absorbed in this way in previous research.[53] If the anthocyanins were absorbed, the yeast cells may contain the anthocyanins in the cell walls, which could be extracted later in the fermentation. This is another possible explanation for the increase of week 5 samples.

No research is without error, and while sources of variance were controlled as well as possible, the use of a commercial process and the small scale of the commercial process used, led to variance introduced by the addition of sugar. The small batch process also led to sampling difficulties, since only one fermentation vessel was used and only one sample could be taken per week.

The design of this study allowed a unique look into the changes to monomeric anthocyanins that take place during the enological process. Without the weekly samples taken, the sudden fall in anthocyanins would not have been noticed, as some of the anthocyanin forms rebounded and the total monomeric anthocyanin assay does not show this rebound.

Constituent anthocyanins of the Ison cultivar of *Vitis rotundifolia* have been identified to a more precise level, including an anthocyanin of the pelargonidin type, which was previously thought to not be present in this cultivar. This study illuminates the Ison cultivar itself, and illuminates the cultivar's response to the enological process; furthermore, this experimental design can now be applied to other compounds of interest and other grapes of interest.

5.2 Further Research

These anthocyanins can be further identified by standard identification, the specific hexoses attached to the anthocyanidin groups are not known. Purified standards could be used to match those unknown hexoses, identifying precisely which forms are present in this grape variety.

In addition, more accurate concentration values could be achieved by increasing sampling intervals; daily or even hourly samples could be taken and analyzed, as well as a constant monitoring of tank levels to account for volume loss. The fermentation could also be extended until all of the fermentable sugars were consumed, with samples being taken throughout.

More tests could be done on the white sugar added, to determine the quantity of sulfates, if any, to ascertain the effect on the anthocyanins. Alternatively, the experiment could be repeated without the addition of sugar and monitored for the same drop in anthocyanins that was observed in this study. If the sugar did cause the drop, this could also confirm the rebound.

More accurate representations of the anthocyanin levels could be obtained by decreasing the sampling to analysis times. In this study, samples had to be frozen until analysis to preserve anthocyanins, but if the samples could be analyzed immediately after their removal from the

fermentation tank, there would be less interfering variance within the storage and transport procedures.

More purification and identification of the anthocyanin peaks, especially the delphinidin peak could provide a look at the possibility of a co-eluting compound, as well as a comparison with the current method of purification to account for possible changes in the anthocyanins due to purification.

Separation of the wine batch earlier in the process, to obtain multiple sampling points, could increase the validity of the data as well. This separation should be done in such a way that it mimics the commercial process as closely as possible. Tests on the lees, which contain the yeast cells, should also be done to ascertain the anthocyanin content absorbed.

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