THE CONTRIBUTION OF MONO- AND DIGLUCOSIDIC ANTHOCYANINS TO RED WINE COLOR

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

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May 2016

ABSTRACT

Non-*vinifera* grapes and wines, including interspecific hybrids, have unique anthocyanin and color profiles, most notably high concentrations of anthocyanin diglucosides. While there are many studies on the anthocyanin profiles and color composition of traditional European *Vitis vinifera* grapes and wine, there are few studies on non-*vinifera* grapes and wines. Red wine color is an important quality parameter for consumers, who associate deeper color with higher quality. Anecdotal evidence suggests that the color of red hybrid wines is unstable, and does not undergo the transition to a brick-red color upon aging like *V. vinifera* wines. In this study, the individual reaction kinetics of the conversion of monomeric anthocyanin mono- and diglucosides to polymeric pigment (stable color) in the presence of acetaldehyde were measured using high performance liquid chromatography (HPLC). The impact of anthocyanin competition, or a more complex matrix, on reaction kinetics was measured by preparing reactions that contained both the mono- and diglucosidic forms of each anthocyanidin base. Changes in color that occurred as a result of chemical reactivity were recorded with colorimetry. It was found that anthocyanin diglucosides react significantly slower than monoglucosides, indicating slower formation of polymeric pigment and less stable color. When the mono- and diglucosidic forms of one anthocyanidin base were placed in competition with each other, the rate of the monoglucosides decreased significantly, indicating that the competition of the diglucosides impacted the reaction rates of the monoglucosides. In each trial, the color either remained red, or transitioned from red to red-orange or orange. These results indicate that non-*vinifera* and hybrid wines containing high concentrations of anthocyanin diglucosides are likely to have less polymeric pigment formation over time compared to *V. vinifera* wines that contain only monoglucosides. This lack of polymeric pigment will significantly affect the color and acceptability of hybrid wines.

Because there are few studies on the compositions of hybrid grapes and wines, a second study was completed to provide further analytical information on five interspecific hybrid grape cultivars: Frontenac, La Crescent, Marquette, MN 1200, and St. Croix. Anthocyanin concentration was measured via HPLC, protein concentration with a modified version of the Amido Black protein assay, and tannins with the Adams-Harbertson assay. Hybrid grapes were found to contain relatively high levels of total anthocyanins, and anthocyanin profiles were dominated by diglucosides, which impacts the ability to form polymeric pigment. Protein concentrations were found to be lower than in other studies on hybrid grapes, while tannin concentration was similar to that found in the scientific literature and lower than that of *V. vinifera* wines; low tannin concentrations also impact the formation of polymeric pigment. This information will give winemakers using hybrid grapes baseline measurements, which will allow them to choose processing methods to reach desired wine composition.

BIOGRAPHICAL SKETCH

Claire Burtch is from Rockford, Ohio and graduated from Purdue University in West Lafayette, Indiana in 2014 with a B.S. degree in Food Science and a minor in English Literature. She studied anthocyanins in model beverage systems and wine as an undergraduate and continued this study at Cornell University in August of 2014. At Cornell, Claire studied the impact of mono- and diglucosidic anthocyanins on red wine color in the Cornell Enology Extension Lab with Dr. Anna Katharine Mansfield. She minored in Applied Economics and Management and was advised by Drs. Miguel Gómez and Bradley Rickard.

ACKNOWLEDGEMENTS

I would like to thank Dr. Anna Katharine Mansfield for serving as my advisor and guiding me throughout the completion of my master's degree. I am thankful for her support and kind encouragement throughout my time at Cornell. I would like to thank both Drs. Bradley Rickard and Miguel Gómez for acting as my minor advisors at various points in my degree program and helping me to choose coursework that I found very interesting and applicable to my future.

I would like to thank Dr. David C. Manns for teaching me more about chemistry and experimental design, answering all of my questions, and for encouraging me in my academic and professional pursuits. I would like to thank Dr. Lindsay Springer for her help in performing grape protein analysis and Jillian Jastrzembski for her help in performing mass spectrometry.

I thank my lab mates, Alex Fredrickson, Diane Schmitt, Camila Tahim, Meaghan Frank, Seth Urbanek, Marie Guido-Minor, Dani Friedenberg, and Mark Skoglund for teaching me more about wine, endless hours of conversation, and reminding me to have fun. Their support is truly appreciated.

This work would not have been possible without the generous funding from the USDA. The Northern Grapes Project is funded by the USDA's Specialty Crops Research Initiative Program of the National Institute for Food and Agriculture, Project #2011-51181-30850.

I thank my family and friends for their support of my academic career at all stages. Your love and encouragement mean the world to me. I would especially like to thank my parents, Ed Burtch and Melissa Kaufman, for believing in me always and giving me the independence to discover life on my own.

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LITERATURE REVIEW

1. Interspecific Hybrid Wine Color

Interspecific hybrid grapes are obtained by crossing the grapes of two different *Vitis* species. In winemaking, crosses of *V. vinifera*, the European winegrape, and non-*vinifera* species such as *V. riparia*, *V. rupestris*, *V. labrusca,* and others are frequently used to produce wine.[1] Hybrid grapes are bred to be disease resistant, cold hardy, or desirable for some other specialized characteristic. While hybrid grapes were first developed approximately 200 years ago, modern grape breeding is attributed to the introduction of North American grape diseases (powdery mildew, downy mildew, black rot) and insects (phylloxera, *Daktulosphaira vitifoliae*) to Europe in the mid-19th century.[2] When these diseases and insects destroyed *V. vinifera* grapes, interspecific hybrids were bred for disease resistance. Today, grape breeders continue to explore cultivars that are disease resistant, while in wine regions with cool climates, breeding for cold hardiness is also common. Interspecific hybrid grapes are widely grown in northern North America, including New York State, due to minimum winter temperatures of -35°C.[2]

Anecdotal evidence has shown that the color of wines made from hybrid grapes differs from that of *V. vinifera* wines. These differences are attributed to several factors, including differences in the anthocyanin profiles and concentrations of condensed tannins. Hybrid wines tend to have higher concentrations of blue and purple anthocyanins, including delphinidin, petunidin, and malvidin, than *V. vinifera* wines.[3] This leads to young hybrid wines being more blue and purple compared to young *V. vinifera* wines, which are more red. Also, hybrid wines often contain a much higher concentration of anthocyanin-3,5-diglucosides, which have two attached glucose units, compared to the anthocyanin-3-monoglucosides (with one glucose unit) found in *V. vinifera* wines. In surveying the anthocyanin profiles of 151 wines produced from

hybrid grapes, Van Buren et al.[1] found anthocyanin profile composition ranging from all monoglucosides to all diglucosides. In this study,[1] malvidin diglucosides were found to be the most prominent anthocyanin, followed by malvidin monoglucoside, peonidin diglucoside and petunidin diglucoside. In contrast, the most common anthocyanin in *V. vinifera* wines is malvidin-3-glucoside,[4] and *V. vinifera* wines contain only trace amounts of diglucosides, if any. Historically, it was thought that anthocyanin diglucosides were not present at all in *V. vinifera* grapes and their wines. However, with technological advances granting greater sensitivity, anthocyanin-3,5-diglucosides have been identified at low concentrations in some *V. vinifera* grapes and wines.[5,6] Alcalde-Eon et al.[5] also identified the 3,7-diglucosides of delphinidin, petunidin, peonidin and malvidin in a red wine made from Tempranillo grapes, a *V. vinifera* cultivar.

The additional glucose in the anthocyanin diglucoside structure affects its reactivity with other compounds, products of which are critical to wine color evolution. For example, anthocyanin-3,5-diglucosides cannot form pyranoanthocyanins, which are major contributors to the stable color in *V. vinifera* wines. Pyranoanthocyanins are orange in color and 100% colored at wine pH. Alternatively, monomeric anthocyanins, whether they are monoglucosides or diglucosides, are only 20-25% colored at wine pH.[7] While the extent of pyranoanthocyanin contribution to stable color in *V. vinifera* red wines is not completely understood, it is clear that the lack of pyranoanthocyanins in hybrid red wines will produce a noticeable color difference, especially during aging. Even in hybrid wines made from juice dominated by monoglucosides, the dominance shifts to the diglucosides after fermentation, which suggests that the monoglucosides have reacted to form other pigments, while the diglucosides have not.[3]

Hybrid wine color is also affected by the relatively low tannin concentrations of hybrid grapes compared to *V. vinifera* grapes.[3] A study of Maréchal Foch, a French-American hybrid (Goldriesling [Riesling x Courtiller Musque] x 101-14 Millardet et de Grasset [*V. riparia x V. rupestris*], found that the berry not only had very low levels of tannins, but also that the extractability of these tannins into the wine was very low.[8] Polymeric pigment, known to increase over time and contribute to stable wine color, is a result of the reaction of anthocyanins with tannins. In wines with low tannin concentrations, like hybrid wines, there is likely to be less polymeric pigment and less stable color. This means the color of hybrid wines is dependent not on new pigment formation, but on copigmentation reactions and on monomeric anthocyanins, which degrade over time.

Few studies have been published about the color of interspecific hybrid grapes and wines. In contrast, there have been many studies and reviews published about the color of *V. vinifera* grapes and wines[4,5,7,9,10] and the anthocyanins that contribute their color.[11] While the differences in the anthocyanin profiles of hybrid and other non-*vinifera* wines, including the American species, have been noted, the effect this difference has on color has not been explored. By combining the understanding of *V. vinifera* grape and wine color, the reactions that drive the evolution in color of *V. vinifera* wines as they age, and the differences in the pigment profile of hybrid grapes and wines compared to *V. vinifera* wines, hybrid wine color can be better understood. This paper will first consider the pigmented compound responsible for grape and wine color: the anthocyanin. *V. vinifera* wine color through the contribution from the grapes, the winemaking process, and the chemical reactions that occur during and post-fermentation will then be discussed. Hybrid grapes and wines will be compared and contrasted to the *V. vinifera* grapes and wines throughout.

2. V. vinifera Color

Vitis vinifera is the primary wine grape species and produces some of the most sought after wines in the world. *V. vinifera* species include Cabernet Sauvignon, Cabernet franc, Pinot noir, Riesling, and Chardonnay, among others. Anthocyanins are responsible for the color of red grapes, juices, and wines.

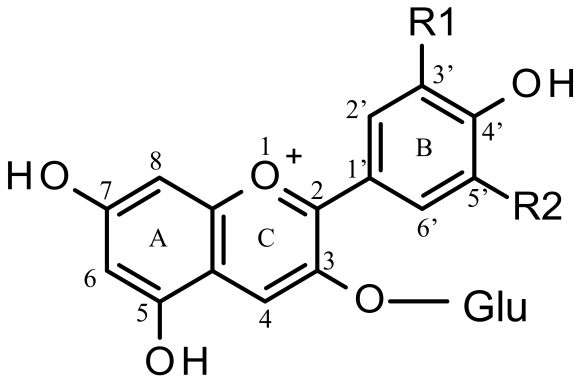
2.1 Anthocyanins

Anthocyanins are a class of phenolic pigments found in nature, which contribute orange, pink, red, violet, and blue colors to a wide variety of plants including fruits, vegetables, cereal grains and flowers. Anthocyanin structure ranges from very simple to extremely complex, depending on the degree of glycosylation and acylation, with over 600 identified structures in nature.[12]

2.1.1 Anthocyanin Structure

The anthocyanidin is the backbone of the anthocyanin molecule. Anthocyanidin structure is composed of an aromatic ring [A] bound to a heterocyclic oxygen-containing ring [C], which is bound to a second aromatic ring [B] (Figure 1.1).[9] As seen in Figure 1.1, anthocyanidins are differentiated by the hydroxyl and methoxy substitution patterns found on the B-ring.[9] At least 17 different anthocyanidins have been identified in nature.[13]

Figure 1.1 Monoglucoside form of the primary wine anthocyanins.



Name	R1	R2
Cyanidin-3-glucoside	OH	H
Delphinidin-3-glucoside	OH	OH
Malvidin-3-glucoside	OCH_3	OCH_3
Peonidin-3-glucoside	OCH_3	H
Petunidin-3-glucoside	OCH_3	OH
Pelargonidin-3-glucoside	H	H

A glycosylated anthocyanidin is an anthocyanin. The most common sugars among anthocyanins are glucose, galactose, arabinose, xylose, and rhamnose, though sophorose, rutinose, and sambubiose have also been observed on anthocyanin glycosides.[13] Anthocyanins are usually glycosylated with a single glucose unit at the 3-OH site on the C-ring;[13] these monoglycosides are anthocyanin 3-glycosides. Anthocyanins can also be glycosylated at the 5-OH position on the A-ring in addition to the 3-OH position on the C-ring, forming a diglycoside.

Anthocyanins can be further differentiated by the presence and positioning of aromatic and/or aliphatic acylations of the sugar moieties.[14] Acylations of anthocyanins are typically positioned at the C6' position of the glycose moiety.[10] Common aromatic acyl groups include *p*-coumaric acid, caffeic acid, ferulic acid, and *p*-hydroxybenzoic acid.[13] Common aliphatic acyl groups include malonic acid and acetic acid.[13] Acylations can be in the form of *cis*- and *trans*-

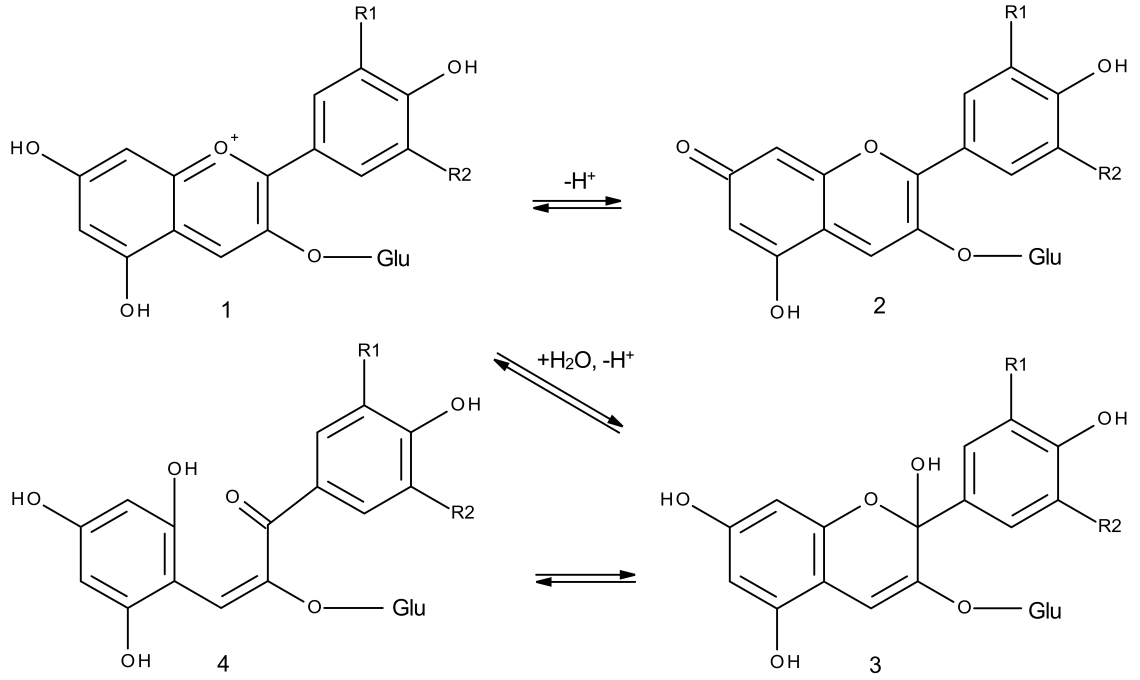
isomers.[10] Recent studies have shown anthocyanins with lactic acid acylations in trace amounts.[5] It is hypothesized that these form in an esterification reaction of anthocyanins with lactic acid produced during malolactic fermentation.

2.1.2 Anthocyanin Color

The color of monomeric anthocyanins is dependent on anthocyanin structure and electron delocalization, which varies by the number of hydroxyl and methoxy groups, the presence, type, and location of sugar molecules, and the presence of aromatic acylations.[7,15] Anthocyanin conformation will change with pH.

In an aqueous system, four different anthocyanin conformations are present in equilibrium, with conformation proportions dictated by pH and temperature (Figure 1.2).[10] The flavylium cation (1) is the center of this equilibrium. It appears red, and is dominant at a low pH (< 2). If the flavylium cation is deprotonated, as with increasing pH, a quinonoidal base forms (2). The quinonoidal base appears blue and is predominant in the pH range of 2-4.[11] If, however, the flavylium cation is both hydrated and deprotonated, a carbinol (or hemiketal) pseudobase forms (3). This conformation is colorless and typically occurs at a pH of 5.[11] The structure of the carbinol pseudobase can open and rearrange to form a pale yellow chalcone, typically at a pH of 6 (4).[11] At wine pH of 2-4, anthocyanins exist in an equilibrium in which only two of the four conformations are truly colored.[10]

Figure 1.2. Influence of pH on anthocyanin conformation.



The identity of the R groups on the B-ring also influences anthocyanin color. Hydroxyl groups promote blueness, while methoxy groups promote redness.[10] Given this fact, it is intuitive that as the number of hydroxyl groups on the B-ring increases, the color of the molecule changes from red to violet.[9] For example, delphinidin (with hydroxyl groups on R1 and R2) appears purple in color, while pelargonidin (which lacks hydroxyl groups) appears orange. This change in color reflects an increase in the wavelength of maximum absorption, known as a bathochromic shift.

The additional glucose moiety of the diglucoside influences the color of the anthocyanin. For example, one study found that glucosidic substitution at the 5-OH position resulted in a large decrease of the hue angle (H°), a measure of visual color, in alkaline solutions. At a pH of 8.0, cyanidin-3-glucoside appeared red-orange, while an acylated cyanidin-3,5-diglucoside appeared bluer, likely due to copigmentation.[16] The same study found that anthocyanin diglucosides had

significantly higher chroma (C_{ab}) values, indicative of color saturation, in the pH region of 6.6-8.0. In contrast, monoglucosides had lower color saturation. While these pH values are much higher than any found in wine, the findings suggest that anthocyanin monoglucosides and diglucosides affect wine color differently.

The addition of acylations influences anthocyanin color as well. For example, Torskangerpoll and Anderson[16] observed more purple or bluish tones in cyanidin-3-(2”-(2”-sinapoylglucosyl)-6”-sinapoylglucoside)-5-glucoside when compared to cyanidin-3-glucoside and cyanidin-3-(2”-glucosylglucoside)-5-glucoside. This was attributed to the two aromatic acyl groups found in this compound, compared to the lack of acylations in cyanidin-3-glucoside, and only one additional glucose acylation in cyanidin-3-(2”-glucosylglucoside)-5-glucoside. The same study[16] also found that acylated anthocyanins had much higher color saturation than their non-acylated counterparts in the pH range of 3.0-6.6. Wine pH can fall within this range, so this characteristic of acylated anthocyanins is relevant to wine color. Overall, Torskangerpoll and Anderson[16] found that pigments with aromatic acylations showed increased color stability compared to non-acylated anthocyanins, as changes in pH did not affect the color of acylated anthocyanins as much as non-acylated anthocyanins.

2.1.3 Anthocyanin Reactivity

Like color, anthocyanin chemical stability and reactivity are functions of anthocyanin structure. Acylated anthocyanins are less likely to react with other compounds than their non-acylated counterparts due to intra- and intermolecular copigmentation and self-association reactions.[16] Torskangerpoll and Andersen[16] specifically showed this increased stability with aromatic acylations. In addition to creating the potential for more copigmentation reactions,

aromatic acylations are thought to protect the anthocyanidin backbone against hydration in the C-2 and C-4 positions.[16]

Structural differences aside, anthocyanin-3-glucosides are very unstable in general and tend to react with other phenolic compounds once released from the berry.[10] These reactions may occur with a variety of compounds in wine, including other anthocyanins, flavanols, flavonols, tannins, hydroxycinnamic acids, and phenolic acids, to form more stable and complex compounds. The C-4 position on the C-ring, and the free hydroxyl group at the C-5 position on the A-ring, are significant reaction sites for the formation of stable color. Bonds at these sites differentiate the reactivity of anthocyanin monoglucosides from diglucosides, since diglucosides lack the aforementioned free hydroxyl group.

Anthocyanin-3,5-diglucosides are less reactive than their monoglucoside counterparts,[17] mostly due to the lack of a 5-OH group. A second cause is the steric hindrance of the C-4 position created by the second glucose moiety. These positions are pivotal in the reactivity of the anthocyanin-3-glucoside.

2.2 Grapes

Anthocyanins are located in the hypodermal cells of grape skins, and also in the flesh of a few so-called teinturier cultivars.[7] Total anthocyanin content of red grapes ranges from about 30 to 750 mg/100 g of ripe berries,[4] and anthocyanin concentration and profile depend on grape species, variety, maturity, seasonal conditions, production area, and fruit yield. For example, the same variety grown in a warm vineyard, as compared to a cooler one, is expected to accumulate fewer anthocyanins.[18] In support of this, Mateus et al.[19] found that when comparing berries grown at different altitudes at the same site, those grown in the lower altitude with a maximum temperature of approximately 33°C (and lower relative humidity) had less anthocyanin

accumulation than those grown at a higher altitude with a maximum temperature of approximately 28°C (and higher relative humidity). An excess of water or irrigation may decrease anthocyanin accumulation.[19] Light exposure also impacts anthocyanin accumulation, with increased exposure resulting in higher levels of anthocyanins.[20]

The onset of veraison, or grape ripening, is noted as the point at which anthocyanin accumulation begins.[18] In the early stages of veraison, anthocyanin concentration increases until reaching a maximal concentration, dependent on environmental conditions. Anthocyanin concentration then decreases until harvest.[19] Somers[21] found that maximum anthocyanin concentration occurred 20-30 days after veraison in South Australia. Through day 50, while the pigment held constant, the anthocyanin concentration decreased, indicating that the anthocyanins may have been participating in copigmentation reactions.[21] Alternatively, Mateus et al.[19] found that anthocyanins reached a maximum concentration 40-60 days after veraison in northern Portugal. The extractability of anthocyanins decreases as the berry shrinks towards the end of ripening.[21]

The 3-glucosides of six major anthocyanidins, delphinidin, petunidin, malvidin, cyanidin, peonidin, pelargonidin, and their acylated esters (acetyl, coumaryl, and caffeoyl), are found in *V. vinifera* grapes.[9,19] Malvidin-3-glucoside is most often the dominating anthocyanin in *V. vinifera* grapes, usually representing at least 40% of total anthocyanins.[18] Pelargonidin-3-glucoside is the least common and was only recently identified in *V. vinifera* grapes.[22] Pinot noir is the only *V. vinifera* red grape noted for a complete lack of acylated anthocyanins,[23] though certain non-*vinifera* grapes including Muscadine are also known to be non-acylated. *V. vinifera* anthocyanin profiles are comprised almost exclusively of anthocyanin-3-glucosides, while anthocyanin-3,5-diglucosides play an important role in the anthocyanin profiles of hybrid grapes.[23] Though

backcrossing with *V. vinifera* has produced some hybrid grapes without anthocyanin-3,5-diglucosides, they dominate the anthocyanin profiles of many hybrid cultivars. In some cases, the anthocyanin profile of a grape can be used for identification or to group specific cultivars.[18]

2.3 Extraction

Anthocyanin extraction is influenced by several factors, including degree of crushing, type and duration of maceration, fermentation conditions, time of pressing, and other winemaking parameters. Despite an emphasis on producing deeply colored wines, only 20-30% of the anthocyanins and total phenols found in grapes are typically extracted.[18]

Crushing puts the juice, skin, pulp and seed into mutual contact and releases anthocyanins from the grape hypodermal cells. While total crush is typically the goal for red wine production, partial crushing with the transfer of some proportion of whole berries is also an option. For example, in carbonic maceration crushing is completely avoided. Rather, anthocyanins are extracted from the skins into the pulp during intracellular fermentation and are only released when the berries later burst.[24] The anthocyanin concentrations of these wines are typically lower than those produced by traditional vinification, and light colored wines are produced.[24] Whether or not the stems are separated from the must during crushing will also affect color, as stems adsorb anthocyanins.[25]

Maceration type will influence anthocyanin extraction as well, though it has a greater affect on the rate, than the extent, of extraction.[25] Three maceration types will be discussed here: cold maceration or cold soaking, traditional maceration, and extended maceration post-fermentation. In cold maceration, must is held between 15 and 20°C for one to two days before it is fermented.[18] Soto Vazquez et al.[26] found that, although cold soaking produced a wine with a higher concentration of anthocyanins, the color intensity was not as high as that of a wine

produced traditionally. This emphasizes the fact that anthocyanin concentration is not always directly related to color intensity. In conventional maceration, must is inoculated in a fermentor typically held between 25 and 30°C. Because skins rise to the top of the fermentor, a punch-down or pump-over operation occurs, usually multiple times per day, to increase contact between skin and juice and enhance extraction.[27] A third method, extended maceration, continues skin contact beyond the completion of fermentation with the goal of increased extraction. However, peak anthocyanin concentration occurs around the second or third day of fermentation, after which time it slowly decreases.[25] As anthocyanins are extracted, they begin to associate with each other and with other small colorless phenolic compounds in copigmentation reactions, which may explain the decrease in anthocyanin concentration after the third day of fermentation. Copigmentation produces increased color relative to what would be expected from the anthocyanin concentration alone. As fermentation continues and ethanol concentration increases, however, these copigmentation associations are disrupted and some color intensity is lost.[26]

Winemakers often attempt to extract anthocyanins by manipulating temperature at different points in the winemaking process. For example, freezing the must before fermentation may increase extraction due to the breakage of cell membranes and the release of anthocyanins.[27] Others use heat. Thermovinification, the process of heating must to between 60 and 70°C for 10 to 30 minutes, can be used to increase extraction from grapes that are low in pigmentation.[18] As with freezing the must, thermovinification damages cell membranes, releasing anthocyanins in the process.[27] Increased fermentation temperature has been found to increase the solubility of most phenolic species, but also to disrupt copigmentation complexes, decreasing the color of a young red wine.[28]

Following fermentation, the time of must pressing also influences anthocyanin extraction. Determining the optimum time to press is a challenge. It is difficult to optimize extraction for both peak color and an acceptable tannin level, since anthocyanin concentration peaks early in fermentation and tannins continue to be extracted exponentially.[25]

Other treatments that will affect anthocyanin extraction and color include additions of sulfur dioxide, enzymes, and tannin. Sulfur dioxide is regularly added at various points in the winemaking process to decrease the effects of oxidation and microbial contamination. However, sulfur dioxide bleaches anthocyanins by binding to the C-4 position on the C-ring,[7] so it is important to keep wine color in mind with adding sulfur dioxide. Pectolytic enzymes are added to must to break down cell walls. One effect of this is the release of anthocyanins with the intent of improving color. Studies have found that this increases extraction of other phenolics, but not of anthocyanins. However, pectolytic enzymes have been found to increase polymeric pigment formation.[27] Soto Vazquez et al.[26] found that the addition of pectolytic enzymes at the beginning of fermentation, and the addition of exogenous tannins during fermentation, produced a wine with greater color intensity than a control. Anthocyanin concentration was also found to be greater than the control once bottled.[26] Overall, the effects of pectolytic enzymes on anthocyanin extraction are variable.

Above all, different methods of extraction will lead to differing anthocyanin physiochemical states,[29] which explains the lack of consistent correlation between anthocyanin concentration and color intensity. For example, a wine produced by thermovinification had greater color than wines produced by the traditional method or carbonic maceration, despite having a lower anthocyanin concentration.[29]

Anthocyanin extraction in hybrid grapes may behave differently than in *V. vinifera* grapes due to their unique anthocyanin profile, notably the presence of anthocyanin-3,5-diglucosides. One study on hybrid grape cultivars Maréchal Foch, Corot noir, and Marquette showed dramatic increases in anthocyanin concentration in thermovinified musts, and only modest increases in anthocyanin concentrations after cold-soaking, compared to the control extraction. However, increased anthocyanin levels did not carry over into the subsequent wines after fermentation,[3] suggesting that anthocyanins were lost during fermentation. The same study found that pectolytic enzymes and exogenous tannins made a minimal impact on anthocyanin extraction.[3]

2.4 Wine

Monomeric anthocyanins that have yet to react with other compounds or polymerize are responsible for the deep red-purple hue of young red wines. The six major anthocyanins in grapes, the 3-glucosides of cyanidin, malvidin, delphinidin, petunidin, peonidin, and pelargonidin, are also those found in red wine.[23] In *V. vinifera* wines, malvidin-3-glucoside is found in the highest concentrations and is responsible for the majority of color.[10]

In young *V. vinifera* wines, monomeric anthocyanin monoglucosides are quick to associate with small colorless compounds to form copigmentation complexes, resulting in a decrease in anthocyanins. This decrease begins after the second or third day of fermentation and continues throughout the aging process.[10] However, as fermentation ends and aging progresses, reactions between anthocyanins and other compounds create new anthocyanin-derived pigments, which further the decrease in monomeric anthocyanins and increase color stability. As many as 129 anthocyanin or anthocyanin-based compounds have been identified in aged wine.[5] Anthocyanin derivatives will absorb light at different wavelengths than their original monomeric

anthocyanins, causing the color transition to a red-orange hue during aging.[5] Anthocyanin diglucosides are less likely to participate in these reactions due to their structure. Rather than react, they may form non-covalent associations with other compounds or degrade over time. This degradation may explain why anthocyanin diglucosides are more likely to brown during aging.[17] The differences in the reactivity of monoglucosides and diglucosides suggest some reasons for the differences in *V. vinifera* and hybrid wine color.

2.4.1 Copigmentation

Copigmentation is the molecular association of anthocyanins with other monomeric organic molecules in young red wine. It is responsible for a non-linear increase in color, and stabilizes anthocyanins in their colored forms, causing more color to be exhibited by anthocyanins than would be expected by concentration alone.[28] Copigmentation is responsible for up to 50% of the color in young red wines.[28] As anthocyanins are extracted during fermentation, some portion of them will bind into their copigmentation forms, depending on the concentration of copigmentation cofactors. Copigmentation shifts the equilibrium between anthocyanins in the skin and anthocyanins in the wine, and allows more anthocyanins to be extracted from the skins. Therefore, grapes that have high concentrations of copigmentation cofactors extracted during fermentation will also have greater concentrations of extracted anthocyanins and a greater copigmentation effect.[28] Copigmentation may cause underestimation of anthocyanin concentration with analysis methods utilizing a pH shift, but may cause an overestimation of anthocyanin concentration when a bleaching method is used.[28]

Intermolecular copigmentation occurs when anthocyanins in either the flavylium cation form or quinonoidal base form (both colored) associate in stacks with other planar molecules in wine.[7] Anthocyanin glucosides, phenolic acids, flavonoids, and derivatives of the flavonol and

flavone subgroups can all act as copigments.[28] The strongest known copigments are the C-6 and C-8-glucosyl apigenins, and the flavonols, which include myricetin, kaempferol, and quercetin.[28] These molecular stacks of anthocyanins and copigments are situated so that water molecules are unable to hydrate the flavylium form of the anthocyanin to the colorless hemiketal form.[7] Instead, the anthocyanin molecule remains in the flavylium cation form and the color is intensified (hyperchromic effect) and becomes purple, which is evident in a bathochromic shift to a longer wavelength of maxima absorption.[7]

The extent of the copigmentation effect depends on type and concentration of anthocyanin, type and concentration of copigmentation cofactor, pH, temperature, and the ionic strength of the solution.[15] The copigmentation effect increases with increasing concentrations of anthocyanins and copigmentation cofactors, though the increase is dependent on type of anthocyanin and copigmentation cofactor.[15] Mazza and Brouillard[15] found that the copigmentation effect increased with degree of methoxylation and glycosylation, and that both increased temperature and increased ionic strength decreased the copigmentation effect.

When an anthocyanin reacts with an anthocyanin of a different type (i.e. malvidin-3-glucoside with cyanidin-3-glucoside), this is considered copigmentation. However, the interaction between anthocyanins of the same type (i.e. malvidin-3-glucoside with malvidin-3-glucoside) is known as self-association, which is different than copigmentation and only occurs in relatively concentrated solutions.[28] Like copigmentation, self-association causes a non-linear increase in color due to molecular stacking of anthocyanins. However, unlike copigmentation, self-association induces a slight hypsochromic shift in the wavelength of maximum absorption.[30] Anthocyanins self-associate in the flayvilium cation form (red), quinonoidal base form (blue-violet), or pseudobase form (colorless), with the quinonoidal base form forming the strongest

self-association.[31] Due to the necessity of high anthocyanin concentrations (>1 mM), self-association is not common in wine and is unlikely to make a large contribution to wine color.[28]

2.4.2 Chemical Reactions During Aging

Many studies have shown that monomeric anthocyanins decrease in a first-order reaction throughout wine aging.[32–35] This decrease is the result of chemical reactions between anthocyanins and other compounds including flavanol monomers, dimers, trimers, and polymers (tannins), other phenolic compounds, acids, and aldehydes. Evidence of these reactions is observed in the changing color of a wine from purple-red to brick-red. Reactions can also be monitored by measuring anthocyanin concentration.

As ethanol increases, copigmentation complexes are disrupted and anthocyanins are free to react with other compounds. In general, anthocyanin reactions begin with the formation of small compounds, some of which will be covalently bound. As these compounds continue to react, some will polymerize and form polymeric pigments. The following sections will briefly describe common reactions that take place between anthocyanins and other wine compounds, the products they create, and the effect this has on wine color.

2.4.2.1 Pyranoanthocyanins

Pyranoanthocyanins form through the cycloaddition of pyruvic acid, acetaldehyde, acetone, 4-vinylcatechol, 4-vinylphenol, 4-vinylguaiacol or vinylcatechin with monomeric anthocyanins.[5] Only anthocyanin-3-glucosides can form pyranoanthocyanins, as a free hydroxyl group at the C-5 position of the A-ring is necessary for their formation,[9] and anthocyanin diglucosides lack this group. The addition of another molecule at the C-4 position makes the pigment more stable by protecting the ion from hydration. While hydration of the ion would yield a colorless compound, pyranoanthocyanins are 100% colored at wine pH.[7,36]

Pyranoanthocyanins are more resistant to pH changes and will maintain color over a wider pH range than monomeric anthocyanins, up to a pH of 7.0.[9] Pyranoanthocyanins induce a hypsochromic shift in the maxima absorption compared to anthocyanins, which creates red-orange pigments. Pyranoanthocyanins have a pronounced effect on the color of wine as it ages, and participate in the transition from purplish-red to brick-red in aging wines. Pyranoanthocyanins are also not bleached by sulfur dioxide, further differentiating them from monomeric anthocyanins.

2.4.2.2 Pyranoanthocyanin-Flavanols

Pyranoanthocyanin-flavanol pigments have been identified in port wines[19] and are structurally identical to pyranoanthocyanins, with the addition of a flavanol. They are hypothesized to form when a flavanol with a vinyl residue reacts with a monomeric anthocyanin. The flavanol is bound to the vinyl residue in one of two ways: 1) through the cleavage of ethyl-linked flavanol oligomers or 2) by direct dehydration of the flavanol-ethanol adduct formed after reaction with acetaldehyde.[19] Pyranoanthocyanin-flavanol compounds cause a hypsochromic shift in the wavelength of maximum absorption, leading to a tawny red color and a resistance to sulfite bleaching, like that found in pyranoanthocyanins.[7] The extent of the decrease in maxima absorption is dependent on the degree of polymerization of the flavanol moiety (e.g. monomer, dimer, etc.).[9] Like pyranoanthocyanins, these compounds are stable due to the ring formed at the C-4 and 5-OH sites of the anthocyanin, and are resistant to bleaching.

2.4.2.3 Vinylpyranoanthocyanin-Flavanols

Portisins are pyranoanthocyanins linked to flavanols by a vinyl group.[9] The reaction between the pyranoanthocyanin and flavanol is mediated by acetaldehyde, a compound formed in wine as a byproduct of yeast metabolism or through ethanol oxidation. It is hypothesized that

acetaldehyde first reacts with a flavanol to form a vinylflavanol adduct; a formic acid group is then lost, and an oxidation reaction creates the vinyl bridge.[37] Portisins can also be formed directly through the reaction of a pyranoanthocyanin and a flavanol-ethyl-flavanol adduct. Unlike the pyranoanthocyanins and pyranoanthocyanin-flavanol compounds, portisins cause a bathochromic shift, which creates a blue hue.[9] Like pyranoanthocyanins and pyranoanthocyanin-flavanol pigments, portisins retain their color over a broader pH range than monomeric anthocyanins due to protection from hydration, and for the same reason have greater resistance of discoloration by sulfur dioxide than monomeric anthocyanins. Portisins have a relatively high molar extinction coefficient value compared to that of monomeric anthocyanins, so their color is more intense at the same concentration.[9]

2.4.2.4 Anthocyanin-Anthocyanin Pigments

Direct condensation reactions between anthocyanins are possible, though uncommon. Anthocyanin dimers and trimers have been identified in the skin extract of Shiraz (*V. vinifera*) grapes. There are two possible mechanisms by which anthocyanins can form dimers and trimers. The first, known as A-type, is bound at C2-O-C7 and C4-C8, and the second, or B-type, is bound at C4-C8. In both cases, the terminal unit is in the flavylium cation form, while the extension units are in the flavan or flavene forms for A-type and B-type, respectively.[38] Due to the single anthocyanin in the flavylium form, the dimers and trimers are colored in the same way as a monomeric anthocyanin. It is unknown whether or not these anthocyanin dimers and trimers are stable against bleaching by sodium dioxide. Anthocyanin-anthocyanin reactions can also be mediated by acetaldehyde, forming anthocyanin-ethyl-anthocyanin adducts.[39] Atanasova et al.[39] identified malvidin-3-O-glucoside-ethyl-malvidin-3-O-glucoside in model wines in three forms: 1) with both anthocyanins in the cationic form, 2) with one anthocyanin in the cationic form and

the other in the hemiketal form, and 3) with one anthocyanin in the cationic form and the other in the quinonoidal base form. Of these, the second form was the most common. This study also found ethyl-linked anthocyanin trimers and tetramers with the anthocyanins occurring in their various forms as mentioned above with the dimers. The color of anthocyanin-anthocyanin adducts depends on the form of the anthocyanins, as the cationic moieties will be red, the quinonoidal will be blue, and the hemiketal forms are colorless.

2.4.2.5 Anthocyanin-Flavanols

Anthocyanin-flavanol and anthocyanin-tannin pigments in wine have been widely studied.[5,7,9,19,40] Flavan-3-ols (flavanols) are catechin and epicatechin monomers. Polymers of flavanols are known as proanthocyanidins or condensed tannins.[7] Proanthocyanidins built from catechin, epicatechin, and epicatechin 3-gallate are called procyanidins, and are primarily found in the seed. Prodelphinidins also contain epigallocatechin, and are mostly found in the skin.[7]

Anthocyanin-flavanol pigments can form as a result of an acetaldehyde-mediated reaction or through direct condensation.[40] Aldehyde-mediated reactions of anthocyanins and flavanols produce an ethyl linkage and have been extensively studied.[29,32–34] Ethyl-linked anthocyanins are 50% or more colored at wine pH and add a purple tint to wine.[7] However, the ethyl bridge of these compounds is highly unstable, and these compounds typically break down and form more stable pyranoanthocyanin derivatives, which produce an orange color.[7]

There are two proposed methods for direct condensation reactions between anthocyanins and flavanols. In the first, the anthocyanin, in the electrophilic flavylium form, is attacked by the nucleophilic flavanol yielding an anthocyanin-tannin (A-T). The A-T is colorless unless oxidized to A+-T, where the anthocyanin is in the flayvilium cation form and is red. In the second proposed method, acid-catalyzation of the interflavanic bond of a procyanidin, leads to a

carbocation. The carbocation acts as an electrophile, while the anthocyanin, in the colorless hemiketal form, is the nucleophile. This yields a tannin-anthocyanin (T-AOH), which can dehydrate to the red T-A+ form.[41] This pigment will only be colored if the anthocyanin is in the flavylium cation form.

2.4.2.6 Polymeric Pigment

Reaction products of anthocyanins and condensed tannins are known as polymeric pigment. Little is known about the composition of polymeric pigment, but it is thought to be responsible for the stable color of aged wines, so there is great interest in studying it.[7,42] Polymeric pigment is at least partially responsible for the stable color in red wine, as its color is not influenced by pH. Like pyranoanthocyanins, polymeric pigment is responsible for a red-orange color in wines, and is generally believed to be un-bleachable by sulfites. It has been reported, however, that this criteria does not apply to all polymeric pigments, and depends on the specific pigment structure.[7]

Anthocyanins can react directly with tannins to form polymeric pigment in reactions similar to those between anthocyanins and flavanols, as described above, or may first react with flavanol monomers or other phenolic compounds and form polymeric pigment precursors. As wine ages, these precursors and monomeric anthocyanins can continue to polymerize and form polymeric pigment. Increasingly larger polymers are not always synonymous with aging, however, as some compounds become large enough to precipitate from solution, and polymeric pigment will also degrade.[7] Wines produced from hybrid grapes tend to have relatively low tannin concentrations, which may explain a lower concentration of polymeric pigment.[8,43]

2.4.3 Wine Color Summary

Copigmentation is the driving force of the purplish-red color of young red wines. As ethanol concentration increases, copigmentation associations disassociate, and reactions between anthocyanins and other wine compounds take place. These reactions tend to move the wine towards a brick-red color produced by pyranoanthocyanins and polymeric pigment. The pyranoanthocyanins, pyranoanthocyanin-flavanol pigments, vinylpyranoanthocyanin-flavanol pigments, anthocyanin-anthocyanin pigments, and anthocyanin-flavanol pigments described above may all form in *V. vinifera* wines. The likelihood of the formation of each of these compounds, however, is not well understood.

In non-*vinifera* wines with high diglucoside concentrations, pyranoanthocyanins cannot form, which leads to a lack of formation of other compounds (some types of polymeric pigment, pyranoanthocyanin-flavanol pigments, vinylpyranoanthocyanin-flavanol pigments, etc.). This affects non-*vinifera* wine color, and greatly reduces the tendency to form a brick-red color. Low tannin concentrations in hybrid wines also influence the lack of polymeric pigment formation.[43]

3. Analyses

The accurate quantification and identification of anthocyanins is significant to grape and wine research, as anthocyanin concentration is indicative of wine color and anthocyanin profiles can be used to monitor wine adulteration.[44] There are multiple ways in which both anthocyanins and the color they produce can be measured.

3.1 Quantifying Anthocyanins

Different anthocyanin properties can be exploited to measure pigment concentration. Simple spectrophotometric readings and the pH differential method rely on the absorptivity of

anthocyanins for quantification. Alternatively, HPLC exploits anthocyanin polarity to separate anthocyanins from other compounds.

3.1.1 pH Differential Method

Anthocyanins absorb light between 490 and 520 nm, with a maximum absorbance at 520 nm. Methods utilizing a pH differential measure the absorbance of an anthocyanin-containing sample at two pH values, and use the difference in anthocyanin conformation at these two pH values to measure anthocyanin concentration. This method has been used to measure anthocyanin concentration in wine.[20]

The dependency of anthocyanin structure on pH was first proposed to quantify anthocyanins in 1948, and since then, various pH values have been explored.[13] The pH differential method published by Giusti and Wrolstad[44] utilizes pH values of 1.0 and 4.5. A pH differential method created by Lee, Durst, and Wrolstad has been the official AOAC method for quantifying total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines since 2006.

In the pH differential method proposed by Giusti and Wrolstad,[44] the pH of samples is manipulated by diluting two aliquots of a sample with buffers of pH 1.0 and 4.5. The absorbance of each of these samples is measured spectrophotometrically at the wavelength of maximum absorbance, 520 nm, and at a wavelength that allows for haze correction, 700 nm. The overall absorbance of the sample is calculated with the following equation:

$$A = (A_{\lambda vis\text{-}max} - A_{700})_{pH1.0} - (A_{\lambda vis\text{-}max} - A_{700})_{pH4.5}$$

This absorbance is converted to the monomeric anthocyanin concentration using the following equation:

$$\text{Monomeric anthocyanin pigment (mg/L)} = (A \times MW \times DF \times 1000)/(\varepsilon \times 1)$$

where the molar weight (MW) and molar absorptivity (ε) correspond to the predominant anthocyanin in the sample, DF = dilution factor, and the path length is assumed to be 1 cm.

The pH differential method is fast and easy for quantifying free monomeric anthocyanins,[44] but has the potential to underestimate anthocyanin concentration when there are a large proportion of highly acylated anthocyanins. Acylated anthocyanins tend to maintain some degree of color at a pH of 4.5 because they are more stable than non-acylated anthocyanins; as a result, the anthocyanins contributing to this color are essentially subtracted from the total anthocyanin content when the absorbance at pH 4.5 is subtracted from the absorbance at pH 1.0.

3.1.2 HPLC

Reverse-phase high performance liquid chromatography (RP-HPLC) coupled with an ultraviolet-visible (UV-Vis) or diode-array detector (DAD) is the most common chromatography analysis applied to anthocyanins, and has been used extensively to quantify anthocyanins in wine.[3,5,13,36,37,41]

In this method, the column is hydrophobic, while the mobile phase is hydrophilic and becomes more hydrophobic over time. The order of anthocyanin elution is a function of the polarity of the mobile phase compared to that of the anthocyanin. Compounds are visualized as peaks, and the peak area correlated to the concentration of the compound. The acidic mobile phase usually consists of an aqueous solvent and an organic solvent; mobile phase acidity is

important, as the anthocyanins are most stable in the flavylium cation form. Weaknesses of HPLC include lack of structural information and misidentification of peaks.

3.2 Identifying Anthocyanins

Diode array detection, mass spectrometry (MS) and nuclear magnetic resonance (NMR) can be used to identify anthocyanins.[11] MS and NMR are the preferred techniques due to their specificity.

3.2.1 Diode Array Detection

Diode array detection is often coupled with HPLC to identify anthocyanins, and is commonly used to identify them in wine.[5,40,41] After separating a wine sample into individual components on a chromatography column, a beam of light passes through each compound in a flow cell. When the compound absorbs light, a peak is formed, creating a chromatogram as separated compounds move successively through. After the flow cell, the light is dispersed so that many diodes, each measuring a narrow band of the spectrum, can record the entire absorption spectrum for any part of the chromatogram.[45] Although the diode array detector (DAD) can produce spectra for all wavelengths within its range (typically 190-680 nm), a set number of wavelengths is specified, such as 280 nm for phenolic compounds or 520 nm for anthocyanins. Drawbacks of using DAD for identification include the difficulty in securing pure anthocyanin standards, the similarities in the spectra of different anthocyanins, and a lack of structural evidence.[11]

3.2.2 Mass Spectrometry

Mass spectrometry (MS) is the most selective method of anthocyanin identification, and along with NMR is a preferred method of analysis.[13] MS has been used extensively to determine

the structure of a compound, monitor changes in wine during aging, and study new compounds formed between anthocyanins and other phenolic compounds in wine.[5,19,38,40]

Mass spectrometry consists of fractionating and ionizing the compounds that have been separated, often by HPLC. These ions are then separated by mass and are read as electrical signals. By examining the mass fractions, the structure of compounds can be understood. If multiple compounds produce identical mass spectra, their chromatograms can be compared to help identify the compound. For example, glucose and galactose, having the same molar mass, produce the same mass spectra, but will elute at different times from the column.

Several types of MS have been developed to improve methodology for sensitive compounds like anthocyanins and to reduce the time of analysis. These methods each supply energy to the compound in different ways, with the goal of forming gaseous ions directly, avoiding volatilization and ionization.[11] Some of these include electron impact MS (EI-MS), electrospray ionization MS (ESI-MS), atmospheric pressure chemical ionization MS (APCI-MS), matrix-assisted laser desorption/ionization MS (MALDI-MS), MALDI-Time of Flight (MALDI-ToF), and fast atom bombardment MS (FAB-MS).[11,13]

3.2.3 NMR

NMR is also used to elucidate anthocyanin structures and to identify the reaction products of anthocyanins and other compounds.[11] NMR is more specific than MS, as it can determine the position of sugar attachment and angle of glycosidic linkages.[46][47] NMR is used extensively to identify wine anthocyanins.[36,37,39]

In NMR, an external magnetic field is applied to the compound in question. The local magnetic fields of each compounds' nuclei can align with or against the magnetic field. Aligning with the magnetic field requires less energy, so an excess of nuclei orient in one way, producing

a net magnetism, which is then manipulated to identify the structure. The nuclei involved in the net magnetism are excited by a pulse of radio-frequency electromagnetic energy, moving the net magnetism into a new plane, and separating it into individual components. This produces an NMR signal specific to the compound in question, which can be used for identification.

3.3 Measuring Color

Red wine color has been extensively studied. The lightness, chroma, and hue angle components of wine can be measured by the CIEL*a*b* system, while various methods for the measurement of polymeric pigment have been tested. Browning of wine can also be measured.

*3.3.1 CIEL*a*b* System*

The CIEL*a*b* system is used to measure the visual color of food samples including juice and wine, and is best used as a way to track color changes, rather than to accurately determine color.[46] Because the analysis is rapid and requires no sample prep, it is frequently used to measure wine color.[24,26,32]

In the CIEL*a*b* system, L* is defined as a 'lightness' scale ranging from 0-100, with 0 being absolute black and 100 being absolute white. The a* value ranges from positive to negative, denoting redness to greenness, and positive to negative b* values denote yellowness to blueness. The hue angle is determined as $\tan^{-1}$ b*/a* and describes three-dimensional color on a 360° grid. Hue angles of 0° = bluish-red, 90° = yellow, 180° = green and 270° = blue. On this scale, a difference of 1° is visually perceivable by the human eye. Chroma is defined as the intensity or saturation of color and is derived from a* and b* values as $(a^*+b^*)^{1/2}$.[32] Chroma increases with pigment concentration to a point and then decreases.[46]

3.3.2 Polymeric Pigment

Like anthocyanin concentration, polymeric pigment concentration is measured either spectrophotometrically or chromatographically.

3.3.2.1 Spectrophotometrically

Unlike monomeric anthocyanins, polymeric pigment is not bleachable by sulfur dioxide due to the lack of a free C-4 position.[44,47] If this characteristic is exploited, polymeric pigment can be measured spectrophotometrically. Because pyranoanthocyanins also lack a free C-4 position, they are measured as polymeric pigment by this method.

A bisulfite bleaching method for wine was first developed by Somers in 1977.[46] This method consists of first measuring the color density of the sample, which is the sum of absorbances at the λ_{max} and at 420 nm.[44] Next, a sample is mixed with a sodium bisulfite solution to bleach the monomeric anthocyanins. This sample is also read at the λ_{max} and 420 nm, and the polymeric color calculated as the sum of these absorbance values. Once any dilution factors and haze are accounted for, the percent polymeric color is defined with the following equation:

$$\% \text{ polymeric color} = (\text{polymeric color}/\text{color density}) \times 100$$

The measurement of the bisulfite-treated sample at 420 nm serves as an index for browning. As this absorbance increases, the sample becomes browner, denoting anthocyanin degradation.[44] The measurements of polymeric pigment and browning are useful in tracking changes in the wine, as well as assessing the wine quality.

3.3.2.2 HPLC

When analyzing polymeric pigment by HPLC on a hydrophobic column, the pigment typically elutes as one broad peak, making it impossible to identify the components composing the polymeric pigment. New developments in columns may improve polymeric pigment chromatography analyses.

Versari et al.[48] compared two HPLC methods: one utilizing a C-18 reverse phase column and one utilizing a polystyrene-divinylbenzene reversed-phase (PLRP) column. Polymeric pigment eluted as a broad envelope peak on the C-18 column and as a single large peak resolved at baseline on the PLRP column, and the two methods had a correlation coefficient of 0.97. A comparison of the data from the PLRP column and the results of polymeric pigment content from Somers' assay showed a correlation coefficient of 0.99. The higher values returned from Somers' assay were attributed to the inclusion of compounds other than monomeric anthocyanins that are not bleachable by bisulfite.[48]

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CHAPTER 2
REACTION KINETICS OF MONOMERIC ANTHOCYANIN CONVERSION TO POLYMERIC PIGMENT IN MODEL WINE

Abstract

The objective of the current study was to quantify the rate of decrease of monomeric anthocyanins in the presence of catechin and acetaldehyde in model wine. High performance liquid chromatography was used to monitor the decrease in monomeric anthocyanin concentration. Colorimetry was used to measure the L*, a*, and b* values. Hue angle and the change in color (ΔE) were calculated. Results indicate that diglucosides decrease more slowly than monoglucosides. When monoglucosides and diglucosides exist in the same solution, the reaction rate of the monoglucosides is slower than that of the monoglucosides alone. All samples became darker throughout the experiment, and hue angles described transitions from red to red-orange, orange, or orange-yellow. The evolution in anthocyanin color was indicative of the chemical reactions taking place between the anthocyanins, catechin, and acetaldehyde. Results suggest that wine containing high concentrations of anthocyanin diglucosides will form less polymeric pigment.

Keywords: polymeric pigment, colorimetry, anthocyanins, diglucosides, monoglucosides, hybrid cultivars

Introduction

Interspecific hybrid grapes, bred for disease resistance and cold tolerance,[1] are obtained by crossing two different *Vitis* species. Those used for wine production are typically crosses of the European winegrape, *Vitis vinifera,* and non-*vinifera* species such as *V. riparia*, *V. rupestris*, *V. labrusca*.[2] Anecdotal evidence has shown that non-*vinifera* and hybrid wines do not undergo the same color evolution from purple-red to brick-red,[3] as wines made from *V. vinifera*.[4] This lack of color evolution has multiple contributing factors, including unique anthocyanin types and concentrations and lower concentrations of condensed tannins.[4,5]

Anthocyanins are pigmented phenolic compounds that color red wine. *V. vinifera* grapes contain almost exclusively anthocyanin monoglucosides, while non-*vinifera* and hybrid grapes often contain high concentrations of anthocyanin diglucosides in addition to monoglucosides.[6] Anthocyanin monoglucosides are very reactive and unstable.[7] Their color and structure is pH-dependent, and they are bleachable by bisulfites.[6] Upon extraction from the grape, monoglucosides react with other wine compounds like aldehydes, acids, and phenolic compounds including flavanols, and polymerized flavanols, or tannins. When monoglucosides react, they form more stable compounds that are less affected by pH and are no longer bleachable by bisulfites. These reactions also tend to change the visible color of the pigment, often from purple-red to orange or tawny red, which gives aged red wines their characteristic brick-red color.[7] When anthocyanins react with tannins, they form stable pigments called polymeric pigment.[7] Polymeric pigment is less likely to change over time and the anthocyanin chromophore has less risk of discoloration.

Other, non-polymeric stable pigments include pyranoanthocyanins, very stable orange-colored compounds that are important contributors to the tawny, brick-red color of aged *V.*

vinifera wines. Pyranoanthocyanins form when a small compound such as pyruvic acid, acetaldehyde, or acetone reacts with an anthocyanin and forms a new ring between the carbon at the C-4 position and a free hydroxyl group at the C-5 position.[3] Pyranoanthocyanins cannot be formed with diglucosides, as the second glucose is located at the C-5 position, blocking the reaction.[8]

At least one study has shown that hybrid grapes and wines form fewer polymeric pigments than *V. vinifera* wines, which is one reason the color evolution from purple-red to brick-red is not observed.[9] A second reason is the lack of pyranoanthocyanin formation.[8] The high concentration of diglucosides likely contributes to the lower concentration of polymeric pigment, as they are less reactive than monoglucosides and have a greater tendency to brown.[9-10] Hybrid wines also have lower concentrations of condensed tannins, which are key components of polymeric pigment.[5]

In the current experiment, the rate of decrease of monomeric anthocyanins of both the mono- and diglucosidic forms of the primary wine anthocyanidins were measured as polymeric pigment concentration increased. Polymeric pigment composition consisted of (+)-catechin and anthocyanins. Reactions took place in the presence of excess acetaldehyde, a byproduct of yeast fermentation, which is also formed through the oxidation of ethanol.[11] Acetaldehyde increases the rate of polymeric pigment formation.[12] The mono- and diglucosidic forms of each anthocyanidin were then paired in coupled reactions to observe how competition affects reaction rate. It is hypothesized that diglucosides react more slowly and form less polymeric pigment than monoglucosides, contributing to less stable color in hybrid wines. The color evolution was also monitored as the reactions proceeded.

Materials And Methods

Materials. Petunidin-3,5-diglucoside chloride and peonidin-3,5-diglucoside chloride were purchased from Polyphenols (Sandnes, Norway). Petunidin-3-glucoside chloride was procured from the lab of Dr. Justine Vanden Heuvel of Cornell University (Ithaca, NY, USA). The 3-glucosides and 3,5-diglucosides of cyanidin, delphinidin, and malvidin, and peonidin-3-glucoside were purchased from Extrasynthese (Genay, France). Potassium bitartrate (KHT) and (+)-catechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetaldehyde, acetonitrile, phosphoric acid, and HCl were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ethanol was purchased from Pharmco-AAPER (Brookfield, CT, USA). Type 1 water was generated on an Arium® 611 ultrapure water system (Sartorius Stedim Biotech, Edgewood, NY, USA). All solvents were HPLC grade.

Model wine. Model wine was composed of 12% ethanol and 2 g/L potassium hydrogen tartrate dissolved in water, and adjusted to pH 3.6 with 1M HCl.

Polymeric Pigment Formation. Reactions containing one anthocyanin will be referred to as 'single', and reactions containing two anthocyanins will be referred to as 'coupled'. The ten individual anthocyanins were each dissolved in 20 ml of model wine to a final concentration of 0.1 mM; these were used for the 'single' reactions. For 'coupled' reactions, the mono- and diglucoside forms of each anthocyanidin base were dissolved to a final concentration of 0.05 mM in 20 ml of model wine, giving a total anthocyanin concentration of 0.1 mM. In both cases, (+)-catechin was added at molar ratio of 50:1 (catechin: anthocyanin). After the initial (0 day) time point sample was removed from the reaction flask, acetaldehyde was added at molar ratio of 400:1 (acetaldehyde: anthocyanin). This solution was continuously stirred on a Scilogex model SK-0330 Pro orbital shaker (LPS Inc., Rochester, NY, USA) at 150 rpm at 22°C and shielded

from light for the duration of the experiment. The first sample was taken immediately upon dissolution of all reagents before the addition of acetaldehyde, and subsequent samples were taken at 1, 2, 3, 4, 5, 6, 7, 14, 21, and 28 days. Each reaction flask was blanketed with ultra-pure nitrogen (Airgas, Radnor, PA, USA) throughout the experiment.

Color Analysis. Color parameters, including L*, a*, and b* values and the spectral data from 360-780 nm, were measured on a HunterLab UltraScan VIS colorimeter, and data was collected with HunterLab EasyMatch QC color software version 4.60 (Reston, VA, USA). Chroma (C^*_{ab}), hue angle (H°), and the change in color as described by the distance from the initial time point (ΔE), were calculated using the following equations:

[1] $$C*_{ab} = \sqrt{a*^2 + b*^2}$$

[2] $$H^\circ = \tan^{-1}\frac{b*}{a*}$$

[3] $$\Delta E = \sqrt{\Delta L*^2 + \Delta a*^2 + \Delta b*^2}$$

Any ΔE value greater than 1.0 is considered visually perceivable by the human eye.[13]

Anthocyanin Analysis. Samples were filtered through a 0.22 µL polyethersulfone (PES) filter (Krackeler Scientific, Albany, NY, USA) into a tinted glass HPLC sample vial (Agilent Technologies, Santa Clara, CA, USA). Samples were then injected onto an Agilent 1260 Infinity series HPLC system equipped with a Kinetex® core–shell C_{18} 100 mm x 4.6 mm column fitted with an inline Krud Katcher® pre-filter (Phenomenex, Torrance, CA, USA). The method parameters are the same as those previously described by Manns and Mansfield[14], with the exception that the post-run time was increased from 3 minutes to 8.5 minutes in order to allow kinetic analysis in real time. Anthocyanins and polymeric pigments were observed at 520 nm. Anthocyanins were identified based on spectral characteristics and previously determined

retention times.[14] The collected absorbances of the homogenous population of polymeric pigment was aggregated and treated as a single measurement, defined subsequently as 'polymeric pigment.' A Finnigan TSQ Quantum Discovery Max mass spectrometer (MS) coupled to a Finnigan Surveyor HPLC system with an autosampler and MS pump (Thermo Scientific, Waltham, MA, USA) was used to identify specific peaks.

Kinetic Models. While previous studies have described anthocyanin degradation as a first-order rate reaction,[15-18] several models (zero-, first-, and second-order) were trialed in this study to determine the best fit. The first-order reaction had the best fit, and reaction rate constants (k) and the half-lives ($t_{1/2}$) were determined with the following equations:

[4] $$k = \frac{\ln remaining\ anthocyanin\ (\%)}{time\ (days)}$$

[5] $$t_{1/2} = \frac{\ln 2}{k}$$

It is assumed that all monomeric anthocyanins were converted into polymeric pigment.

Experimental Design and Statistical Analysis. All experiments were performed in triplicate. All results are reported as the mean of the three replicates with standard deviations in parentheses. Differences between reaction rate constants and differences in color parameters were determined by one- and two-way ANOVA, and paired comparisons of least-squares means (α=0.05) using R version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria).

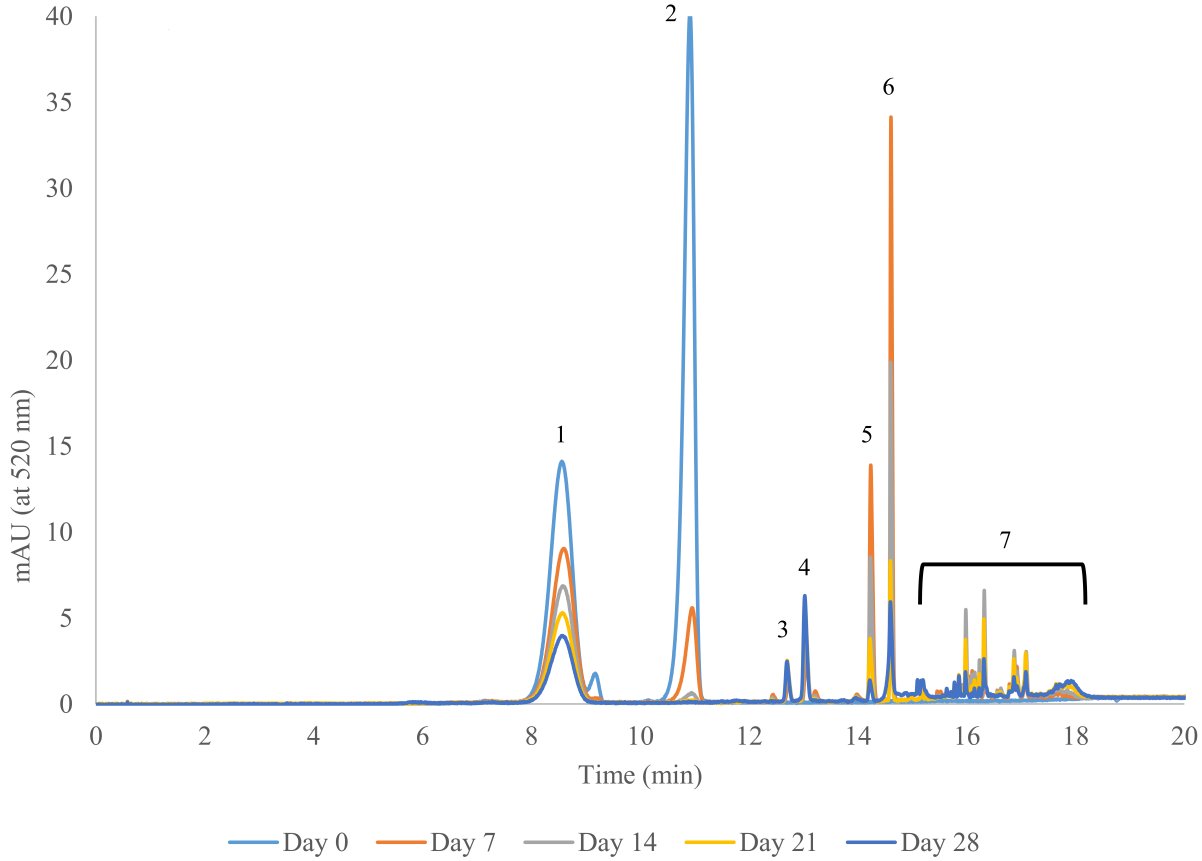
Results and Discussion

Chromatographically, monomeric anthocyanins eluted first, and the complex collection of compounds making up the newly formed polymer fraction was observed as an unresolved hump at the end of each chromatogram (Figure 2.1). For each monomeric anthocyanin in a solution, two peaks eluted after the monomeric anthocyanin, but before the polymeric pigment (peaks 3, 4, 5, and 6 in Figure 2.1). Further analysis of a single malvidin-3-glucoside trial by

HPLC-MSMS showed these peaks to be diastereoisomers of malvidin-3-glucoside linked to catechin by an ethyl bridge, a polymeric pigment precursor that has been reported in other studies.[3] The intact molecular masses and fragmentation ions for the single malvidin-3-glucoside trial matched previously reported m/z values.[3] The identity of the analogous peaks in the diglucosidic reactions could not be confirmed, likely due to slower formation. Diglucosidic anthocyanins ethyl-linked to catechin were presumed to form, however, based on extensive evidence in the literature for the formation of monoglucosidic forms.[3,19] Furthermore, the formation patterns of the peaks of the presumed diglucosidic forms are identical to that of the monoglucosidic forms in the current study with the exception of retention time. Ethyl-linked compounds produce a blue-purple hue, which was observed in the model wines.[7,16] While these compounds are unstable, they tend to break down and form more stable pigments,[3,7] which likely occurred.

As polymeric pigment formed, precipitates formed and fell out of solution. This is common to other studies that used acetaldehyde to produce polymeric pigment.[16,17] It is hypothesized that some of the precipitate is uncolored polymeric catechin (tannin) due to the large excess of catechin in the model wines. Colored precipitates were also formed, and it is hypothesized that these are large ethyl-linked polymers of anthocyanin and catechin, as described in other studies.[16,17] This is similar to the natural phenomenon encountered in an aging bottle of wine, where the formation of colored precipitates can also be observed.[20] This portion of the polymeric fraction was not measured, as all samples were filtered before HPLC and color analysis.

Figure 2.1. Chromatograms of the coupled malvidin reaction at 0, 7, 14, 21, and 28 days.[a]



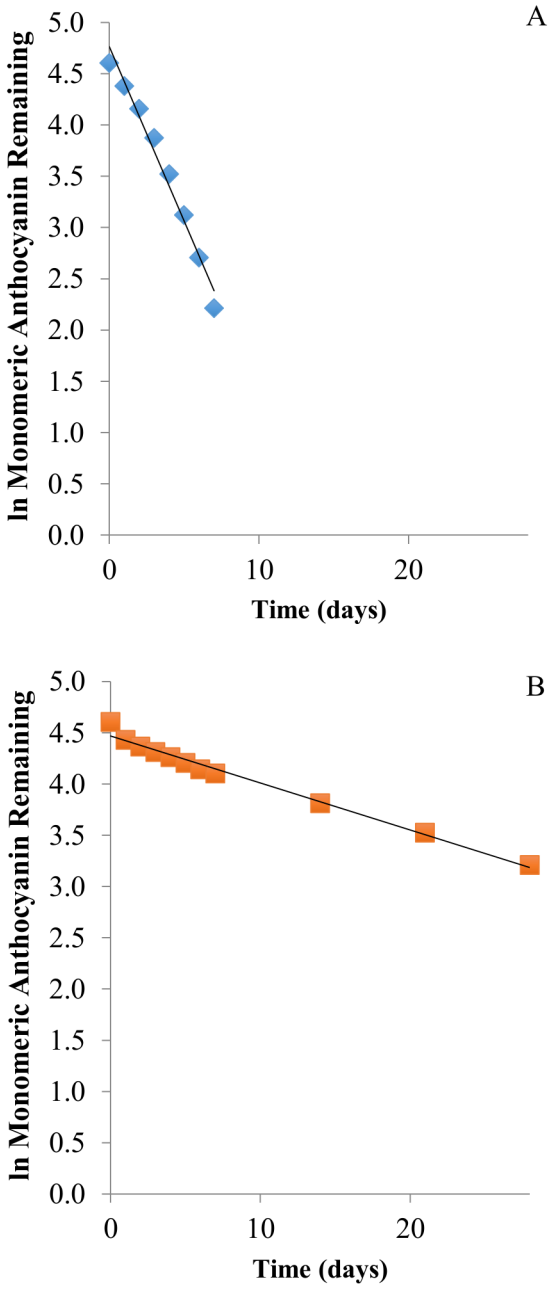
[a]Peak 1 = malvidin-3,5-diglucoside; peak 2 = malvidin-3-glucoside; peaks 3 and 4 = unidentified; peaks 4 and 5 = malvidin-3-glucoside-ethyl-catechin; peak area 7 = polymeric pigment.

Anthocyanins

Single Reactions

All monoglucosides in the single reactions reached non-detectable levels in the first 14 days, indicating complete conversion to polymeric pigment. In contrast, after 28 days, monomeric diglucosides were still present at between 12 and 31% of their original concentrations in all experiments, indicating incomplete conversion. Figure 2.2 depicts the decreases of malvidin-3-glucoside (2.2a) and malvidin-3,5-diglucoside (2.2b) in the single reactions.

Figure 2.2. First-order rate of decrease of malvidin-3-glucoside (2.2a) and malvidin-3,5-diglucoside (2.2b) in single-anthocyanin reactions. Reaction rate constants (k) are calculated as the slope of the line (k_{mono} = 0.34 $days^{-1}$; k_{di} = 0.046 $days^{-1}$).



The reaction rates of monoglucosides were statistically the same among single reactions, but among diglucosides, delphinidin reaction rates were different than cyanidin, malvidin and peonidin (Table 2.1). Monoglucosides were also converted to polymeric pigment around 7.5

times faster than diglucosides, as demonstrated by the average reaction rates of monoglucosides and diglucosides of 0.370 d^{-1} and 0.053 d^{-1}, respectively (Table 2.1). Each monoglucoside reacted more quickly than its related diglucoside. The average half-life ($t_{1/2}$), or time required for anthocyanins to decrease to half of their original concentration, was 1.90 d for monoglucosides and 14.3 d for diglucosides.

Table 2.1. First-order rate constants (k) and half-lives ($t_{1/2}$) of the decrease in monomeric anthocyanin concentration in the single anthocyanidin reactions.[a]

	Monoglucosides		**Diglucosides**		
Anthocyanidin	**k[b] ($days^{-1}$)**	**$t_{1/2}$[c] (days)**	**k ($days^{-1}$)**	**$t_{1/2}$ (days)**	**k_{mono}:k_{di}**
Cyanidin	0.35 (0.02) a	2.0 (0.1)	0.046 (0.003) a	15.1 (0.98)	7.6
Delphinidin	0.35 (0.02) a	2.0 (0.1)	0.075 (0.01) b	9.6 (2.5)	4.6
Malvidin	0.34 (0.07) a	2.1 (0.4)	0.046 (0.01) a	15.8 (3.6)	7.5
Peonidin	0.40 (0.01) a	1.7 (0.05)	0.037 (0.007) a	19.0 (3.7)	10.8
Petunidin	0.40 (0.02) a	1.7 (0.07)	0.059 (0.0009) ab	11.8 (0.18)	6.9
Average	**0.37 (0.03)**	**1.9 (0.2)**	**0.053 (0.02)**	**14.3 (3.7)**	**7.5**

[a] Values are mean (n = 3) with standard deviation in parentheses. Statistical differences in rate of anthocyanin decrease (by column) at $\alpha = 0.05$ are denoted by different letters using paired comparisons [b]k = first-order rate constant in $days^{-1}$. [c]$t_{1/2}$ = half-life in days.

Diglucosides remained in their monomeric form longer than monoglucosides, in agreement with previous studies that found monoglucosides to be less stable and more reactive than diglucosides.[10] This instability allows monoglucosides to readily react and polymerize, leading to the stabilization of the chromophore. The slower rates of conversion for diglucosides result in slower polymer formation, and lower concentrations of polymeric pigment, as well as less protection of the chromophore. This 7.5-fold difference in reaction rates, coupled with low concentrations of condensed tannins,[5] supports the hypothesis of slower or lower polymeric pigment formation in interspecific hybrid wines, where up to 75% of the anthocyanin concentration is in diglucoside form. This preponderance of diglucoside anthocyanins is the key difference between hybrid and *V. vinifera* wine color.[2,21]

Coupled Reactions

Because hybrid wines also contain some proportion of monoglucosidic anthocyanins, the competitive interactions of mono- and diglucosidic forms of each anthocyanidin base are also likely to affect hybrid wine color. As in the single reactions, monoglucosides in coupled reactions had reached non-detectable concentrations within 14 days, indicating complete conversion to polymeric pigment (Figure 2.3). With the exception of malvidin-3-glucoside, the reaction rates of monoglucosides in coupled trials were significantly lower than in single trials (Table 2.2). The decrease in monoglucoside reaction rate has multiple potential causes. It is possible that the acetaldehyde preferentially reacts with diglucosides, decreasing the amount of acetaldehyde available to react with the monoglucosides. A second possibility is that mono- and diglucosidic anthocyanins self-associated rather than formed covalent bonds with catechin or each other, though this is unlikely, as high anthocyanin concentration is typically required for self-association.[6] It should be noted that there was a lower concentration of the monoglucoside in the coupled reaction (0.05mM compared to 0.1mM in the single reaction), though this should not have affected the rate of decrease, as the total anthocyanin concentration was kept constant.

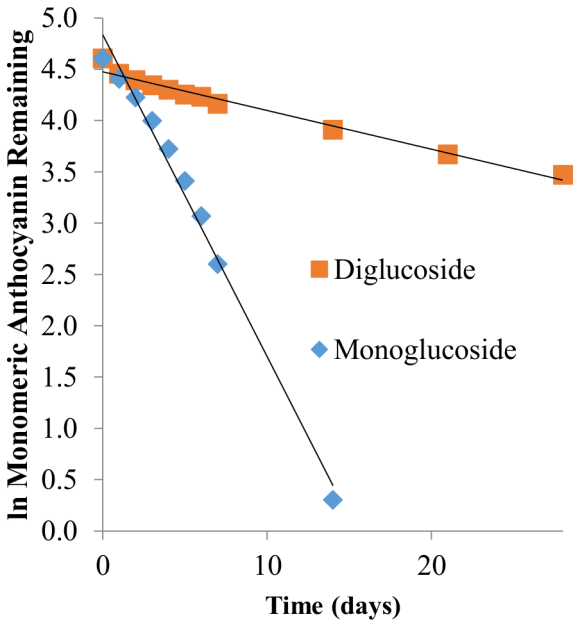
As in the single reactions, diglucosides in coupled reactions never achieved complete conversion to polymeric pigment (Figure 2.3). Among diglucosides, the rates of cyanidin, malvidin, peonidin, and petunidin remained the same in single and coupled reactions, while the rate of delphinidin-3,5-diglucoside was notably slower in the coupled reaction. While delphinidin-3,5-diglucoside had an exceptionally high reaction rate in the single reactions, the decrease of the reaction rate in the coupled reactions made it more similar to the other diglucosides. The cause of this large decrease is not understood.

Table 2.2. First-order reaction rate constants (k) and half-lives ($t_{1/2}$) of monomeric anthocyanin decrease in coupled reactions.

	Monoglucosides		Diglucosides		
Anthocyanidin	**k (days-^{1})**	**$t_{1/2}$ (days)**	**k (days^{-1})**	**$t_{1/2}$ (days)**	**k_{mono}:k_{di}**
Cyanidin	0.23 (0.004) bc	3.0 (0.05)	0.041 (0.006) ab	17.3 (2.6)	5.7
Delphinidin	0.22 (0.006) c	3.1 (0.08)	0.041 (0.003) ab	16.7 (0.11)	5.3
Malvidin	0.31(0.005) a	2.2 (0.04)	0.038 (0.003) b	18.4 (1.3)	8.3
Peonidin	0.25 (0.04) bc	2.8 (0.4)	0.036 (0.009) b	20.1 (4.4)	6.9
Petunidin	0.27 (0.01) ab	2.6 (0.09)	0.052 (0.002) a	13.2 (0.50)	5.2
Average	**0.26 (0.04)**	**2.7 (0.4)**	**0.042 (0.006)**	**17.1 (2.5)**	**6.2**

[a] Values are mean (n = 3) with standard deviation in parentheses. Statistical differences in rate of anthocyanin decrease (by column) at α = 0.05 are denoted by different letters using paired comparisons [b]k = first-order rate constant in days^{-1}. [c]$t_{1/2}$ = half-life in days.

Figure 2.3. First-order rates of decrease of malvidin-3-glucoside and malvidin-3,5-diglucoside in a coupled reaction. Reaction rate constants (k) are calculated as the slope of the line (k_{mono} = 0.31 days^{-1}; k_{di} = 0.038 days^{-1}).



Application to Wine

Wines containing high concentrations of diglucosides are not likely to evolve from purple-red to brick-red like *V. vinifera* wines. The primary reasons for this are three-fold: first, the diglucosides have slower rates of polymeric pigment formation and second, the competition from diglucosides slows down the formation of polymeric pigment by monoglucosides. Finally,

as discussed previously, the evolution of hybrid wine color is also affected by the inability of diglucosides to form stable red-orange pyranoanthocyanins.

The competitive reactions in this work represent a simplified, controlled version of the complex matrix of anthocyanins and other phenolic compounds found in wine, but it is clear that the addition of even a second anthocyanin results in an increase in system complexity. The decrease observed in the reaction rates of four monoglucosides and one diglucoside in the coupled reactions is indicative of the impact that increasing phenolic complexity can have on phenolic reaction kinetics and wine color.

Colorimetry

Change in Color (ΔE): Single Reactions

The evolution of wine color is intricately linked with anthocyanin systems. At the same molar concentration, each anthocyanin had a distinct initial color, and followed an anthocyanin-specific evolution over the duration of the experiment. This evolution or color change can be quantified by the ΔE, which is based on the L*, a*, and b* values. The ΔE is the distance a color has moved from an initial starting point (day 0 in this study) and values greater than 1.0 are visually perceivable by the human eye. In all trials performed in this study, single and coupled, ΔE values were greater than 1.0 after one day (Tables 2.3 and 2.4). These distances increased over time, in part because all samples became darker over the duration of the experiment (note the decreasing L* values) (Table 2.5). It is important to keep in mind that similar ΔE values do not necessarily indicate similar colors, as each anthocyanin had a specific set of L*, a*, and b* values initially. In other words, two samples with the same measured change had both different initial and final values.

Table 2.3. Anthocyanin color change (ΔE) in single reactions at 1, 7, 14, 21, and 28 days.[a]

	ΔE				
Anthocyanin	**Day 1**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Cyanidin 3-glucoside	3.29 (0.1) a	18.88 (0.7) a	18.65 (1.0) ab	22.07 (1.2) a	31.60 (7.6) a
Cyanidin 3,5-diglucoside	3.39 (0.7) a	4.09 (1.7) d	10.80 (2.0) bc	15.74 (6.4) a	20.13 (10.6) a
Delphinidin 3-glucoside	3.18 (0.6) a	14.44 (2.1) bc	15.18 (0.8) abc	19.93 (2.0) a	25.05 (3.5) a
Delphinidin 3,5-diglucoside	3.03 (0.9) a	5.67 (0.9) d	14.93 (5.3) abc	21.92 (11.2) a	28.07 (16.6) a
Malvidin 3-glucoside	3.75 (3.0) a	19.45 (2.6) a	21.88 (3.2) a	24.45 (9.8) a	31.20 (2.9) a
Malvidin 3,5-diglucoside	2.81 (0.1) a	5.29 (0.7) d	13.48 (1.2) bc	19.33 (8.7) a	22.78 (13.3) a
Peonidin 3-glucoside	2.48 (0.8) a	17.58 (0.8) ab	14.79 (0.7) abc	18.69 (0.7) a	21.59 (0.6) a
Peonidin 3,5-diglucoside	3.62 (0.5) a	4.49 (0.4) d	3.08 (3.8) c	15.90 (5.4) a	18.83 (10.0) a
Petunidin 3-glucoside	2.69 (0.2) a	11.78 (1.5) c	13.70 (4.1) bc	17.97 (9.2) a	24.81 (10.6) a
Petunidin 3,5-diglucoside	12.48 (1.9) b	10.63 (0.4) c	15.66 (0.2) abc	22.28 (3.4) a	29.16 (11.3) a

[a] Values are mean (n=3) with standard deviation in parentheses. Statistical differences by column at α = 0.05 are denoted by different letters.

Table 2.4. Anthocyanin color change (ΔE) in coupled reactions at 1, 7, 14, 21, and 28 days.

	ΔE				
Anthocyanin	**Day 1**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Cyanidin	4.68 (1.1) a	12.93 (1.7) a	12.55 (0.7) a	15.52 (3.4) a	18.08 (7.2) a
Delphinidin	3.47 (0.8) a	9.15 (0.4) a	10.95 (1.3) a	9.15 (2.7) a	9.44 (0.7) a
Malvidin	2.88 (0.6) ab	14.52 (3.6) ab	12.01 (2.7) ab	10.32 (1.3) a	12.59 (3.4) a
Peonidin	5.40 (0.9) ac	12.49 (3.6) ac	9.68 (0.5) ac	6.49 (2.5) a	15.31 (4.5) a
Petunidin	4.88 (0.4) a	4.44 (0.5) a	7.38 (0.8) a	5.93 (5.5) a	13.87 (9.2) a

[a] Values are mean (n=3) with standard deviation in parentheses. Statistical differences by column at α = 0.05 are denoted by different letters.

The size of the ΔE did not correlate to faster reaction kinetics. Among monoglucosides, peonidin and petunidin had the highest rates of decrease (k), meaning they reacted the most quickly (Table 2.1), however, they had the smallest ΔE after 28 days compared to the other monoglucosides (Table 2.3). The colors of the compounds formed by the monoglucosides of peonidin and petunidin did not differ greatly from their original colors, despite forming quickly. Diglucosides with the highest rates of decrease (k), delphinidin and petunidin, had the largest ΔE as well (Tables 2.1 and 2.3); in other words, the compounds formed upon by the decrease of the diglucosides of delphinidin and petunidin were very different in color than the monomeric anthocyanins of which they were partially composed.

While ΔE will not describe how quickly an anthocyanin has reacted, it can communicate the impact specific anthocyanins will have on wine color through aging, and whether that impact is small or large. In the single anthocyanin experiments, cyanidin-3-glucoside had the greatest ΔE after 28 days, followed by petunidin-3,5-diglucoside, malvidin-3-glucoside, delphinidin-3,5-diglucoside, and delphinidin-3-glucoside, respectively (Table 2.3). This suggests that wines containing high concentrations of cyanidin-3-glucoside will see greater changes in color over time than wines containing high concentrations of peonidin-3,5-diglucoside, which had the lowest ΔE value.

After 7 days, all monoglucosides in the single anthocyanin trials showed greater changes in color than their diglucoside counterparts, with the exception of petunidin (Table 2.3). This indicates that monoglucosides had a greater capacity to change solution color, and that wines containing higher concentrations of monoglucosides, like *V. vinifera* wines, may see greater color changes through aging. After 14 days, the monoglucosides of only cyanidin, malvidin, and peonidin had greater changes in color than their diglucoside counterparts, but all of the monoglucosides had polymerized completely. Consequently, any further changes in the monoglucosidic color were due to pigment reorganization, break down, and reformation. After 21 days, the lack of difference among anthocyanidin pairs suggests that both types of anthocyanins had reached their maximum capacity for color change.

Change in Color (ΔE): Coupled Reactions

The ΔE of the coupled reactions showed a similar trend to that of the anthocyanin conversion to polymeric pigment (Table 2.4). When the mono- and diglucosidic forms of the same anthocyanidin were combined, the ΔE decreased compared to the changes in mono- and diglucosidic color of the single trials (Tables 2.3 and 2.4). Comparatively, the rates of decrease

of the monoglucosides in the coupled reactions were significantly reduced when placed in a competitive environment with a diglucoside. Overall, regardless of the exact size of the ΔE, at the completion of the experiment (day 28), all ΔE values had greatly increased and changes in color were perceived visually.

Figure 2.4. Conversion of L*, a*, and b* values to RGB for visualization of anthocyanin color.

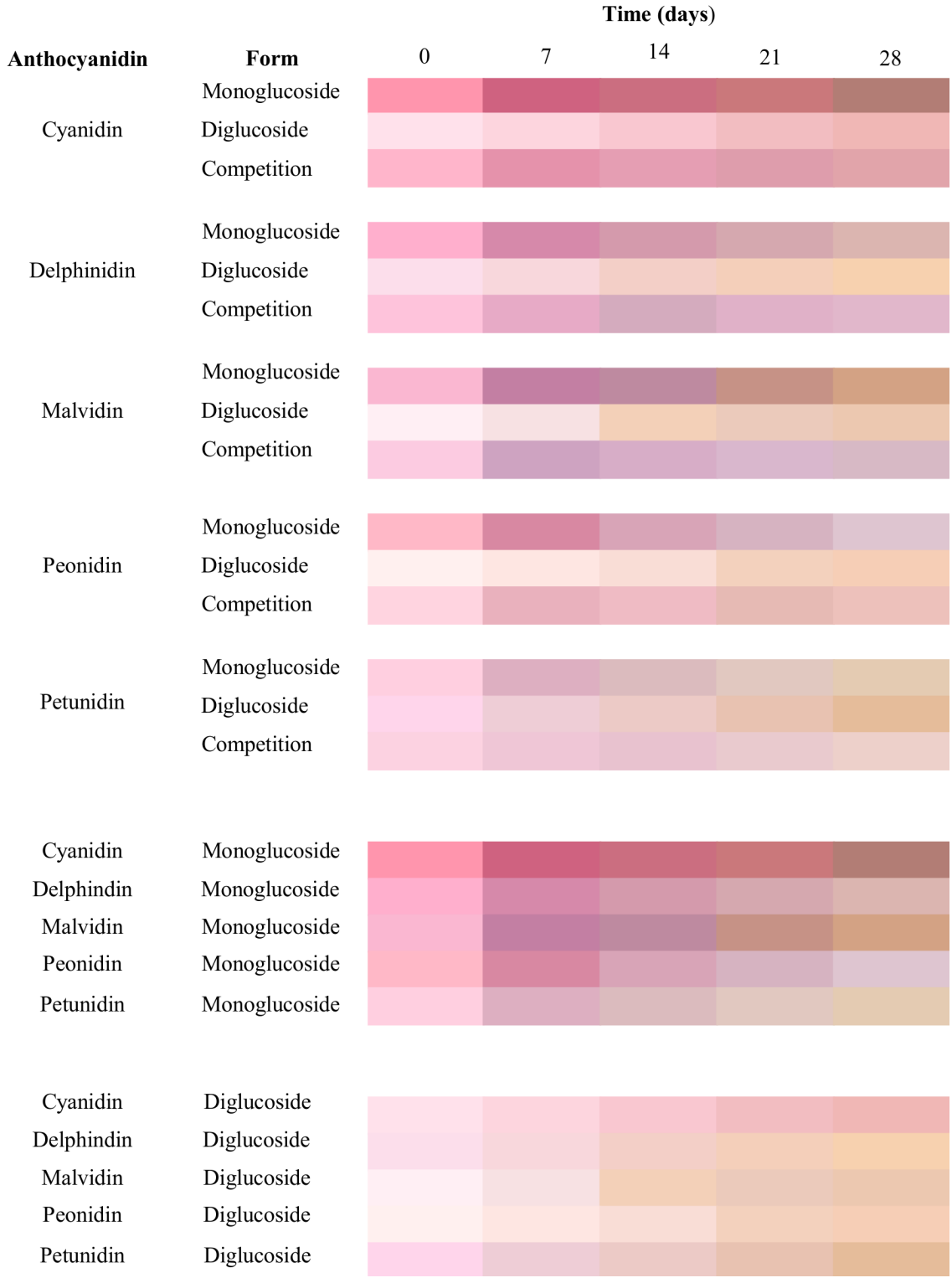


Table 2.5. Color parameters of each anthocyanin in single and coupled experiments at day 0 (initial) and day 28 (final).[a]

		L*		a*		b*		Hue Angle		Hue Angle Color Descriptor	
Anthocyanin	**Form**	**Initial**	**Final**	**Initial**	**Final**	**Initial**	**Final**	**Initial**	**Final**	**Initial**	**Final**
Cyanidin	Mono-	75.6 (1.5)	63.7 (11)*	45.4 (2.6)	18.4 (7.7)*	5.8 (0.98)	6.4 (11)	7.2 (0.85)	15.4 (28.5)	Red	Red-orange
Delphinidin	Mono-	79.9 (1.7)	76.8 (1.2)	34.0 (2.7)	13.6 (3.1)*	-4.0 (0.23)	6.86 (12)	353 (0.33)	23.4 (41.7)	Red	Red-orange
Malvidin	Mono-	80.1 (1.0)	68.3 (3.9)*	29.3 (1.5)	15.6 (3.6)*	-4.8 (0.46)	15.1 (17)*	351 (0.54)	36.0 (30.4)	Red	Orange
Peonidin	Mono-	82.5 (0.11)	81.7 (1.5)	31.6 (0.86)	11.0 (0.76)*	2.9 (0.23)	-3.05 (1.1)	5.3 (0.54)	344 (5.7)	Red	Red-purple
Petunidin	Mono-	87.6 (0.20)	83.0 (2.6)	20.2 (0.37)	5.4 (0.79)*	-2.8 (0.31)	14.6 (14)*	352 (0.92)	54.3 (34)*	Red	Orange
Cyanidin	Di-	92.7 (0.50)	79.5 (3.8)*	14.7 (0.44)	20.8 (1.1)*	-0.51 (0.09)	8.2 (16.7)	358 (0.32)	15.6 (34.7)	Red	Red-orange
Delphinidin	Di-	91.1 (0.72)	86.0 (2.6)*	12.9 (1.6)	8.4 (7.8)	-3.1 (0.67)	21.1 (21.9)*	347 (1.3)	51.3 (55.8)*	Red	Orange
Malvidin	Di-	96.1 (0.62)	83.0 (4.9)*	8.52 (0.21)	9.1 (1.5)	-0.8 (0.038)	15.6 (16.3)	355 (0.21)	37.9 (3.74)	Red	Orange
Peonidin	Di-	96.9 (0.67)	85.5 (4.6)*	9.15 (0.18)	10.2 (0.66)	2.7 (0.025)	16.6 (11.3)	16.3 (0.20)	51.7 (23)	Red-orange	Orange
Petunidin	Di-	90.3 (0.85)	79.0 (2.2)*	22.2 (1.6)	10.2 (0.44)*	-4.0 (0.57)	22.3 (3.7)*	350 (0.68)	65.2 (3.7)	Red	Orange-yellow
Cyanidin	Comp-	82.3 (1.2)	73.3 (3.6)*	34.8 (0.45)	23.9 (0.63)*	1.1 (0.51)	5.08 (14)	1.8 (0.85)	9.6 (28.8)	Red	Red
Delphinidin	Comp-	84.7 (1.6)	78.6 (0.97)*	25.1 (0.19)	18.3 (1.0)*	-4.6 (0.19)	-4.9 (2.1)	350 (0.45)	345 (5.7)	Red	Red-purple
Malvidin	Comp-	86.4 (1.5)	77.9 (2.5)*	21.3 (0.91)	13.0 (0.17)*	-5.2 (0.095)	-3.0 (4.8)	346 (0.80)	348 (19.6)	Red	Red
Peonidin	Comp-	89.8 (0.42)	81.8 (6.4)*	21.6 (0.70)	14.9 (2.8)*	1.1 (0.14)	8.3 (8.9)	2.9 (0.32)	23.0 (27.6)	Red	Red-orange
Petunidin	Comp-	88.0 (0.38)	85.7 (1.5)	17.7 (0.74)	9.5 (0.62)*	-3.0 (0.46)	5.8 (13)	350 (1.1)	16.4 (43.2)	Red	Red-orange

[a]Values are mean (n=3) with standard deviation in parentheses. Asterisks (*) indicate a statistical difference at $\alpha = 0.05$ between final and initial values for each parameter.

Hue Angle (H°)

Increasing ΔE values and illustrated conversions of the L, a*, and b* values to RGB values (Figure 2.4) demonstrate the chemical reactions occurring between the anthocyanins, acetaldehyde, and catechin. Hue angle, however, provides a better measurement of color change. Presented as a 0 to 360° scale, H° values around 0° represent red colors, 45° represent orange colors, 90° represent yellow colors, 180° represent green colors, 270° represent blue colors, and 315° represent lilac colors.[22] A 1° difference in hue angle is perceivable by the human eye.[23]

Single Reactions

While only petunidin-3-glucoside and delphinidin-3,5-diglucoside in the single reactions had statistically different H° values after 28 days, the hues of all anthocyanins were different by more than 1°, indicating visually perceivable transitions in color from day 0 to day 28 (Table 2.5). At day 0, H° values of all anthocyanins indicated red colors with the exception of peonidin-3,5-diglucoside, which was red-orange (Table 2.5). Over time, H° values transitioned, with new hues reflecting the presence of the polymerized anthocyanin forms. As discussed previously, monoglucoside concentrations were non-detectable after 14 days indicating their complete conversion to polymeric pigment. This means that the final H° value of each monoglucoside anthocyanin is the contribution it would make to overall wine color, though these color transitions are not exactly what would be observed in a real wine system, as polymeric pigment involves more than a single type of anthocyanin and a tannin composed of only one type of flavanol. Within this framework, however, the transition of the monoglucosides indicated a transition to red-orange and orange hues, with the exception of peonidin-3-glucoside, which became more purple.

Alternatively, as discussed previously, there were measurable diglucoside anthocyanins at day 28, indicating incomplete conversion to polymeric pigment. This indicates that the color contribution from diglucoside anthocyanins has the potential for further changes, as more monomeric anthocyanins can be converted to polymeric pigment. Diglucosides transitioned from red to red-orange, orange, and orange-yellow hues.

Coupled Reactions

In the coupled reactions, cyanidin and malvidin maintained their initial red hue, while peonidin and petunidin transitioned to red-orange and delphinidin transitioned to purple. The color stability of cyanidin and malvidin is in line with the reduced changes observed in the reaction rates of monoglucosides in the coupled reactions and the reduced ΔE values of the coupled reactions versus the single reactions.

Color Evolution

In all trials visually perceived color evolved, though some anthocyanins transitioned more than others. Most anthocyanins approached the orange-red color typical of an aged wine. This study supports the idea that wine color will depend on the specific anthocyanin profile and the extent to which anthocyanins have decreased and polymeric pigment has formed.

Conclusion

Anthocyanin diglucosides reacted more slowly than anthocyanin monoglucosides to form polymeric pigment, suggesting that wines with high concentrations of anthocyanin diglucosides, like those made from hybrid grapes, will form less polymeric pigment during maturation and aging. Rather, monomeric diglucosides will brown or degrade. As model wine matrices became more complex, anthocyanins interacted with each other, and diglucosides reduced the conversion rate of monoglucosides to polymeric pigment. The 30 or more monomeric anthocyanins and

other phenolic compounds in a wine matrix present far greater opportunities for such interactions, complicating the overall impact on the formation of polymeric pigment. Anthocyanins all became darker with time, as seen through decreasing L* values, and their color changed perceptibly as early as day 1. The color changes increased throughout the experiment, and though the final hue angles of most were not significant compared to their initial values, the visible differences were observed. Further studies of the impact various anthocyanins have on polymeric pigment formation will allow better prediction of color evolution and stability.

Appendix

Table A1. Average color parameters for cyanidin-3-glucoside over time.

Time (days)	L*	a*	b*	ΔL*	Δa*	Δb*	C*	H	ΔC*	ΔE	ΔH*
0	75.58	45.39	5.77				45.75	7.21			
1	74.88	42.53	4.39	-0.70	-2.86	-1.37	42.76	5.86	-3.00	3.29	1.04
2	71.00	43.76	3.81	-4.58	-1.62	-1.95	43.93	4.94	-1.82	5.28	1.77
3	66.36	44.98	3.26	-9.21	-0.41	-2.51	45.10	4.11	-0.66	9.57	2.46
4	62.12	45.84	2.79	-13.46	0.46	-2.98	45.94	3.42	0.19	13.81	3.01
5	56.62	47.75	4.18	-18.96	2.36	-1.59	47.93	5.01	2.18	19.28	1.83
6	58.74	46.21	2.42	-16.84	0.82	-3.35	46.30	2.90	0.54	17.23	3.41
7	57.07	45.36	2.27	-18.51	-0.03	-3.49	45.45	2.78	-0.30	18.88	3.47
14	60.25	36.53	2.78	-15.33	-8.86	-2.99	36.94	3.59	-8.82	18.65	3.53
21	63.02	29.39	5.73	-12.55	-16.00	-0.03	30.88	8.20	-14.87	22.07	8.99
28	63.71	18.41	6.41	-11.87	-26.98	0.65	20.98	15.36	-24.78	31.60	12.69

Table A2. Average color parameters for delphinidin-3-glucoside over time.

Time (days)	L*	a*	b*	ΔL*	Δa*	Δb*	C*	H	ΔC*	ΔE	ΔH*
0	79.88	33.98	-4.03				34.22	353.22			
1	79.94	31.28	-3.96	0.07	-2.96	-0.52	34.98	352.78	-3.02	3.18	0.65
2	76.35	32.36	-4.35	-3.53	-1.62	-0.32	32.65	352.36	-1.56	3.96	0.52
3	71.84	34.07	-4.98	-8.04	0.10	-0.95	34.44	351.70	0.22	8.14	0.92
4	68.86	34.98	-5.62	-11.02	1.01	-1.58	35.43	350.86	1.22	11.20	2.20
5	66.52	35.11	-5.50	-13.36	1.14	-1.47	35.54	351.11	1.33	13.55	1.29
6	65.58	35.18	-5.64	-14.30	1.20	-1.61	35.64	350.93	1.42	14.58	1.41
7	65.71	34.40	-5.87	-14.17	0.42	-1.84	34.91	350.35	0.70	14.44	1.74
14	69.60	24.53	-1.43	-10.28	-9.45	2.60	25.02	358.17	-9.19	15.18	5.88
21	73.09	17.67	2.01	-6.79	-16.31	6.04	19.09	8.24	-15.13	19.93	10.76
28	76.77	13.61	6.86	-3.21	-20.20	17.67	18.94	23.36	-14.48	25.05	22.59

Table A3. Average color parameters for malvidin-3-glucoside over time.

Time (days)	L*	a*	b*	ΔL*	Δa*	Δb*	C*	H	ΔC*	ΔE	ΔH*
0	81.18	28.50	-4.54				28.86	350.70			
1	79.92	27.03	-4.71	-1.26	-1.47	-0.18	27.44	349.14	-1.42	2.00	0.42
2	74.63	29.49	-5.96	-6.55	0.99	-1.43	30.09	348.07	1.23	6.80	1.23
3	68.93	31.15	-7.00	-12.25	2.65	-2.46	31.94	346.79	3.08	12.81	1.91
4	64.91	32.68	-7.97	-16.27	4.18	-3.43	33.67	346.06	4.82	17.23	7.95
5	62.07	33.29	-8.51	-19.11	4.79	-3.98	34.39	345.22	5.53	20.14	2.89
6	61.47	32.88	-8.88	-19.71	4.38	-4.35	34.09	344.23	5.23	20.71	3.30
7	61.10	31.83	-9.15	-20.08	3.33	-4.61	33.14	343.95	4.28	20.92	3.74
14	63.08	23.68	-4.28	-19.82	-4.61	7.03	24.95	358.08	-3.91	22.36	7.30
21	65.24	18.12	13.62	-15.94	-10.38	18.15	24.20	16.76	-4.66	27.66	18.49
28	70.38	14.33	22.15	-10.80	-14.17	26.69	26.78	35.99	-2.08	29.16	28.69

Table A4. Average color parameters for peonidin-3-glucoside over time.

Time (days)	L*	a*	b*	ΔL*	Δa*	Δb*	C*	H	ΔC*	ΔE	ΔH*
0	82.49	31.62	2.94				31.76	5.31			
1	81.61	29.59	2.15	-0.87	-2.03	-0.79	29.67	4.15	-2.09	2.48	0.62
2	76.57	30.40	1.49	-5.91	-1.22	-1.44	30.44	2.82	-1.32	6.29	1.35
3	73.44	32.94	0.74	-9.05	1.32	-2.19	32.95	1.29	1.19	9.43	2.27
4	69.66	34.00	-0.19	-12.80	2.01	-2.96	34.07	359.68	1.88	13.30	9.60
5	67.30	34.77	-0.68	-15.18	3.15	-3.61	34.78	358.88	3.02	15.95	3.73
6	66.57	34.47	-1.17	-15.92	2.85	-4.11	34.49	358.05	2.73	16.71	4.19
7	65.65	34.06	-1.43	-16.83	2.44	-4.36	34.09	357.60	2.33	17.58	4.42
14	72.62	22.69	-3.24	-9.87	-8.93	-6.17	22.96	351.95	-8.80	14.79	6.28
21	76.26	15.22	-3.31	-6.23	-16.40	-6.24	15.62	347.83	-16.14	18.69	6.76
28	81.70	10.96	-3.05	-0.78	-20.66	-5.99	11.42	344.41	-20.34	21.59	6.87

Table A5. Average color parameters for petunidin-3-glucoside over time.

Time (days)	L*	a*	b*	ΔL*	Δa*	Δb*	C*	H	ΔC*	ΔE	ΔH*
0	87.59	20.24	-2.81				20.44	352.08			
1	91.09	12.10	0.72	-1.59	-2.60	1.22	12.12	352.97	-2.58	3.39	1.25
2	85.00	19.03	-2.93	-2.60	-1.21	-0.11	19.26	351.28	-1.18	2.93	0.63
3	81.06	20.39	-3.35	-6.54	0.15	-0.54	20.67	350.67	0.23	6.59	0.51
4	78.11	21.40	-4.02	-9.48	1.16	-1.21	21.78	349.39	1.34	9.67	1.00
5	73.08	20.85	-4.06	-14.51	0.61	-1.25	21.26	348.97	0.82	14.68	1.29
6	76.79	21.05	-4.29	-10.80	0.81	-1.48	21.51	348.64	1.07	11.05	1.69
7	75.99	20.21	-3.61	-11.60	-0.03	-0.79	20.58	350.10	0.14	11.78	1.79
14	78.66	12.32	1.82	-8.94	-7.92	4.64	13.70	7.95	-6.74	13.70	5.32
21	82.49	7.95	6.43	-5.11	-12.29	9.24	12.90	20.01	-7.54	17.97	9.45
28	83.02	5.44	14.61	-4.57	-14.80	17.42	16.48	54.26	-3.96	24.81	19.71

Table A6. Average color parameters for cyanidin-3,5-diglucoside over time.

Time (days)	L*	a*	b*	ΔL*	Δa*	Δb*	C*	H	ΔC*	ΔE	ΔH*
0	92.68	14.69	-0.51				14.70	358.03			
1	91.09	12.10	0.72	-1.59	-2.60	1.22	12.12	3.43	-2.58	3.39	1.25
2	92.37	12.28	0.23	-0.31	-2.41	0.74	12.29	1.12	-2.41	2.61	0.72
3	86.58	12.22	0.44	-6.10	-2.47	0.94	12.23	2.08	-2.47	7.33	0.94
4	86.46	13.65	0.88	-6.22	-1.04	1.38	13.73	3.48	-0.97	6.52	1.39
5	89.53	14.12	0.42	-3.15	-0.57	0.92	14.17	1.75	-0.53	3.52	0.97
6	89.44	14.90	0.23	-3.24	0.21	0.73	14.92	0.97	0.22	3.42	0.74
7	88.91	15.74	0.08	-3.77	1.05	0.58	15.79	0.44	1.08	4.09	0.80
14	84.71	19.30	1.37	-7.97	4.60	1.88	20.16	4.56	5.46	10.80	3.79
21	81.33	20.05	4.40	-11.35	5.35	4.91	22.54	10.64	7.84	15.74	6.31
28	79.50	20.78	8.16	-13.18	6.09	8.66	25.59	15.56	10.89	20.13	9.02

Table A7. Average color parameters for delphinidin-3,5-diglucoside over time.

Time (days)	L*	a*	b*	ΔL*	Δa*	Δb*	C*	H	ΔC*	ΔE	ΔH*
0	91.14	12.88	-3.08				13.24	346.67			
1	92.51	11.01	-1.37	1.37	-1.87	1.71	11.10	352.92	-2.15	3.03	1.32
2	89.87	10.88	-1.19	-1.27	-2.00	1.89	10.96	353.83	-2.29	4.73	1.49
3	90.87	11.22	-0.21	-0.27	-1.66	2.87	11.23	358.93	-2.02	3.82	2.61
4	90.72	11.37	-0.27	-0.42	-1.51	2.81	11.42	359.07	-1.82	3.34	2.58
5	88.14	11.25	0.58	-3.00	-1.63	3.66	11.40	4.20	-1.85	5.20	3.58
6	89.43	11.98	0.61	-1.71	-0.90	3.68	12.17	4.12	-1.07	4.72	3.65
7	88.76	12.00	1.00	-2.38	-0.88	4.07	12.30	6.72	-0.95	5.67	4.13
14	85.81	11.72	7.95	-5.33	-1.16	11.03	17.28	35.09	4.03	14.93	11.38
21	85.77	9.86	14.46	-5.37	-3.02	17.54	23.09	46.39	9.84	21.92	16.82
28	86.05	8.36	21.06	-5.09	-4.52	24.14	28.52	51.26	15.28	28.07	20.64

Table A8. Average color parameters for malvidin-3,5-diglucoside over time.

Time (days)	L*	a*	b*	ΔL*	Δa*	Δb*	C*	H	ΔC*	ΔE	ΔH*
0	96.07	8.52	-0.77				8.55	354.81			
1	94.50	6.38	-0.01	-1.58	-2.14	0.77	6.38	359.94	-2.17	2.81	0.66
2	93.52	6.48	-0.24	-2.55	-2.03	0.54	6.50	357.95	-2.05	3.33	0.47
3	93.48	6.84	-0.12	-2.59	-1.68	0.66	6.85	359.13	-1.70	3.18	0.57
4	86.81	6.86	0.31	-9.27	-1.66	1.08	6.91	2.95	-1.64	9.55	1.14
5	89.89	7.01	-0.12	-6.19	-1.50	0.65	7.12	359.41	-1.44	6.57	1.41
6	91.26	7.72	0.18	-4.81	-0.80	0.96	7.85	1.81	-0.70	5.19	1.65
7	91.19	8.08	0.43	-4.88	-0.44	1.20	8.23	3.77	-0.32	5.29	1.81
14	85.72	9.21	5.40	-10.36	0.69	6.17	12.18	26.91	3.63	13.48	6.82
21	83.56	9.43	11.05	-12.51	0.91	11.83	17.49	34.48	8.94	19.33	11.08
28	82.95	9.11	15.55	-13.13	0.59	16.33	20.66	37.87	12.11	22.78	12.98

Table A9. Average color parameters for peonidin-3,5-diglucoside over time.

Time (days)	L*	a*	b*	ΔL*	Δa*	Δb*	C*	H	ΔC*	ΔE	ΔH*
0	96.91	9.15	2.67				9.54	16.28			
1	94.92	6.16	2.93	-1.99	-2.99	0.26	6.83	25.41	-2.71	3.62	1.29
2	94.39	6.70	3.54	-2.52	-2.46	0.86	7.58	27.62	-1.96	3.83	1.70
3	93.91	6.71	3.72	-3.00	-2.44	1.05	7.68	29.02	-1.86	4.05	1.90
4	93.20	6.99	3.86	-3.71	-2.16	1.19	8.00	28.80	-1.53	4.50	1.92
5	93.69	7.14	3.73	-3.22	-2.02	1.05	8.07	27.49	-1.47	3.98	1.72
6	92.18	7.50	4.24	-4.73	-1.65	1.57	8.65	29.24	-0.88	5.40	2.07
7	93.11	7.70	4.18	-3.80	-1.46	1.51	8.81	28.27	-0.73	4.49	1.94
14	90.05	8.94	6.12	8.54	-6.86	-0.21	5.87	12.61	41.93	3.08	9.25
21	86.17	9.56	13.30	-10.74	0.41	10.62	16.88	49.89	7.34	15.90	7.69
28	85.53	10.19	16.61	-11.38	1.04	13.94	20.27	51.72	10.73	18.83	9.03

Table A10. Average color parameters for petunidin-3,5-diglucoside over time.

Time (days)	L*	a*	b*	ΔL*	Δa*	Δb*	C*	H	ΔC*	ΔE	ΔH*
0	90.28	22.21	-4.01				22.57	349.79			
1	88.98	10.93	-1.21	-1.30	-11.28	2.80	11.01	353.65	-11.56	12.48	1.08
2	90.63	11.64	-1.30	0.35	-10.57	2.71	11.71	353.62	-10.86	10.96	1.09
3	88.47	12.42	-1.28	-1.81	-9.78	2.73	12.50	354.08	-10.07	10.44	1.25
4	86.21	12.67	-1.81	-4.07	-9.53	2.20	12.80	351.87	-9.76	10.76	0.62
5	87.30	13.26	-1.68	-2.98	-8.95	2.34	13.37	352.81	-9.20	9.73	0.91
6	84.50	13.42	-1.63	-5.78	-8.79	2.38	13.53	353.13	-9.04	11.31	1.01
7	85.26	13.61	-1.37	-5.02	-8.60	2.65	13.71	354.36	-8.86	10.63	1.36
14	83.92	11.76	5.62	-6.36	-10.45	9.63	13.08	25.46	-9.49	15.66	10.48
21	81.29	11.16	12.84	-8.99	-11.05	16.86	17.40	47.00	-5.17	22.28	18.95
28	79.05	10.15	22.32	-11.24	-12.06	26.33	24.55	65.21	1.98	31.20	28.70

Table 11A. Average color parameters for cyanidin competition trial over time.

Time (days)	L*	a*	b*	ΔL*	Δa*	Δb*	C*	H	ΔC*	ΔE	ΔH*
0	82.34	34.79	1.09				34.81	1.80			
1	84.19	30.65	0.54	1.85	-4.14	-0.56	30.66	1.01	-4.15	4.68	0.45
2	78.09	30.96	0.41	-4.25	-3.83	-0.68	30.96	0.76	-3.84	5.85	0.70
3	79.36	32.51	-0.17	-2.98	-2.28	-1.27	32.51	359.70	-2.30	4.19	1.24
4	76.93	33.62	-0.28	-5.41	-1.17	-1.37	33.62	359.53	-1.18	5.76	1.36
5	75.92	34.60	-0.67	-6.42	-0.19	-1.76	34.61	358.90	-0.20	6.79	1.77
6	72.44	34.92	-0.88	-9.90	0.13	-1.97	34.93	358.57	0.13	10.13	1.97
7	69.63	34.85	-0.82	-12.71	0.07	-1.91	34.87	358.68	0.06	12.93	1.91
14	72.47	29.40	-0.84	-9.87	-5.39	-1.94	29.67	358.74	-5.14	12.55	4.91
21	71.25	26.76	0.88	-11.09	-8.03	-0.22	27.75	2.03	-7.06	15.52	8.01
28	73.27	23.87	5.08	-9.07	-10.92	3.99	26.75	9.55	-8.06	18.08	10.63

Table 12A. Average color parameters for delphinidin competition trial over time.

Time (days)	L*	a*	b*	ΔL*	Δa*	Δb*	C*	H	ΔC*	ΔE	ΔH*
0	84.69	25.10	-4.59				25.52	349.63			
1	86.35	22.42	-3.89	1.66	-2.68	0.70	22.76	350.15	-2.76	3.47	0.34
2	84.94	23.44	-4.41	0.25	-1.66	0.18	23.86	349.35	-1.66	1.91	0.32
3	82.24	24.26	-4.82	-2.45	-0.84	-0.23	24.74	348.76	-0.78	2.65	0.38
4	82.26	25.32	-5.19	-2.43	0.22	-0.60	25.84	348.42	0.33	2.75	0.55
5	78.49	26.38	-5.40	-6.20	1.28	-0.81	26.93	348.44	1.41	6.39	0.54
6	77.96	26.75	-5.76	-6.73	1.65	-1.17	27.36	347.83	1.84	7.11	0.83
7	75.90	27.03	-6.06	-8.79	1.93	-1.47	27.70	347.37	2.19	9.15	1.06
14	74.03	26.08	-6.79	-10.66	0.98	-2.20	26.96	345.42	1.44	10.95	1.93
21	76.89	21.23	-5.96	-7.80	-3.87	-1.36	22.08	344.51	-3.44	9.15	2.16
28	78.64	18.30	-4.85	-6.05	-6.80	-0.26	18.99	345.45	-6.52	9.44	2.21

Table 13A. Average color parameters for malvidin competition trial over time.

Time (days)	L*	a*	b*	ΔL*	Δa*	Δb*	C*	H	ΔC*	ΔE	ΔH*
0	86.37	21.33	-5.16				21.95	346.38			
1	87.45	19.09	-4.37	1.08	-2.24	0.79	19.59	347.10	-2.36	2.88	0.26
2	85.45	19.83	-5.45	-0.92	-1.50	-0.29	20.57	344.64	-1.38	2.50	0.66
3	81.69	21.45	-6.33	-4.68	0.12	-1.17	22.37	343.56	0.42	4.87	1.10
4	80.63	22.34	-7.17	-5.74	1.01	-2.01	23.46	342.22	1.51	6.30	1.65
5	77.56	24.01	-7.72	-8.81	2.68	-2.56	25.22	342.17	3.27	9.69	1.73
6	74.61	24.50	-8.37	-11.76	3.17	-3.21	25.89	341.15	3.94	12.61	2.19
7	72.64	24.47	-8.60	-13.73	3.14	-3.44	25.94	340.63	3.99	14.52	2.40
14	75.06	19.98	-8.49	-11.31	-1.35	-3.33	21.72	336.96	-0.23	12.01	3.59
21	77.94	16.18	-7.40	-8.43	-5.16	-2.24	17.87	335.74	-4.08	10.32	3.77
28	77.94	13.04	-3.04	-8.43	-8.29	2.12	13.93	347.84	-8.02	12.59	4.07

Table 14A. Average color parameters for peonidin competition trial over time.

Time (days)	L*	a*	b*	ΔL*	Δa*	Δb*	C*	H	ΔC*	ΔE	ΔH*
0	89.82	21.61	1.11				21.64	2.93			
1	85.72	18.32	1.36	-4.10	-3.29	0.25	18.38	4.26	-3.26	5.40	0.77
2	85.70	19.36	0.79	-4.12	-2.26	-0.31	19.38	2.37	-2.27	4.80	0.32
3	80.60	20.15	0.36	-9.22	-1.46	-0.75	20.17	0.95	-1.47	9.45	0.74
4	83.34	21.45	0.59	-6.48	-0.16	-0.52	21.46	1.58	-0.18	6.51	0.51
5	82.28	21.69	0.50	-7.54	0.08	-0.61	21.70	1.33	0.06	7.62	0.71
6	82.57	22.18	0.22	-7.25	0.56	-0.88	22.19	0.53	0.55	7.46	0.92
7	77.52	22.15	1.28	-12.30	0.53	0.17	22.25	3.27	0.60	12.49	1.51
14	80.82	20.29	2.04	-9.00	-1.33	0.93	20.59	5.49	-1.05	9.68	3.16
21	79.23	17.10	5.14	-10.59	-4.52	4.04	18.45	14.79	-3.20	13.34	6.49
28	81.78	14.88	8.31	-8.04	-6.73	7.20	18.10	23.02	-3.54	15.31	9.90

Table 15A. Average color parameters for petunidin competition trial over time.

Time (days)	L*	a*	b*	ΔL*	Δa*	Δb*	C*	H	ΔC*	ΔE	ΔH*
0	88.04	17.68	-3.01				17.93	350.38			
1	91.31	14.15	-2.29	3.27	-3.53	0.72	14.33	350.81	-3.60	4.88	0.17
2	87.91	14.90	-2.49	-0.14	-2.78	0.51	15.11	350.52	-2.82	3.68	0.37
3	86.54	15.76	-2.76	-1.50	-1.92	0.24	16.00	352.80	-1.93	2.61	0.13
4	87.07	16.73	-3.44	-0.98	-0.95	-0.44	17.09	348.41	-0.84	1.69	0.61
5	84.52	17.36	-2.96	-3.52	-0.32	0.05	17.62	350.37	-0.31	3.56	0.36
6	85.24	16.67	-3.34	-2.80	-1.01	-0.33	17.02	348.71	-0.92	3.17	0.58
7	83.70	17.34	-3.55	-4.34	-0.34	-0.54	17.71	348.53	-0.22	4.44	0.60
14	82.09	15.75	-1.83	-5.95	-1.93	1.18	16.29	354.52	-1.64	7.38	3.03
21	83.89	12.07	1.34	-4.15	-5.61	4.35	13.99	5.15	-3.94	9.63	5.93
28	85.73	9.52	5.76	-2.32	-8.15	8.77	13.99	16.35	-3.94	13.87	8.16

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CHAPTER 3
ANTHOCYANINS, PROTEINS, AND TANNINS IN HYBRID GRAPES FOR THE NORTHERN GRAPE PROJECT

Abstract

In this study, eighteen samples of hybrid grapes, including cultivars Frontenac, La Crescent, Marquette, MN 1200, and St. Croix, were profiled for anthocyanin type and concentration, protein concentration, and tannin concentration. High performance liquid chromatography (HPLC) for anthocyanins, a modified version of the Amido Black assay for proteins, and the Adams-Harbertson method for tannins were used for analysis. Anthocyanin profiles were dominated by delphinidin-3,5-diglucoside and malvidin-3,5-diglucoside and contained very high anthocyanin concentrations, up to 6100 mg/L. Protein concentrations were found to be low compared to other values reported for hybrids in the literature, between 37 and 119 mg/L. Tannin concentrations were similar to those reported in the literature and lower than those found in *V. vinifera* grapes at 8 to 91 mg/L catechin equivalents. High anthocyanin concentration and low tannin concentration will inhibit the formation of polymeric pigment, which will result in poor color quality.

Keywords: anthocyanins, diglucosides, hybrid, tannins

Introduction

Interspecific hybrid grapes, developed for their disease resistance and cold hardiness, are crosses of *Vitis vinifera* and other *Vitis* species, such as the North American varieties of *V. riparia, V. rupestris, or V. labrusca*.[1] Interspecific hybrids are often grown in the northern United States where temperatures can reach winter minimums of -35°C.[2]

Wines made from hybrid grapes are often viewed as lower quality than those produced from their *V. vinifera* parents.[2] This perception is due in part to differences in color quality. Color has been shown to greatly influence the consumer's preference of a wine, and deep red wines are rated more highly than less deeply colored wines.[3] Red wine color is produced by anthocyanins, phenolic compounds found mainly in the skins of red grapes. There are five major grape and wine anthocyanins, and the profile of a specific grape or wine is dependent on cultivar, growing region and conditions, winemaking procedures and vinification methods.[4]

The anthocyanin profile, and therefore the color profile, of hybrid wines differs significantly from that of *V. vinifera* wines. Hybrid grapes tend to have more blue and purple anthocyanins like delphinidin, peonidin, and malvidin, while *V. vinifera* wines tend to have more red anthocyanins.[5] Also, hybrid grapes can have large proportions of anthocyanin diglucosides, unlike *V. vinifera* grapes, which contain primarily anthocyanin monoglucosides.[1,5] The additional glucose on the anthocyanin diglucosides makes the formation of pyranoanthocyanins, one type of stable color, impossible due to steric hindrance and the lack of a free hydroxyl group at the C-5 position on the A-ring.[6] Pyranoanthocyanins are very stable, contribute to the brick-red hue of wine formed over time, and are resistant to bleaching by sulfites.[7] Polymeric pigment, another form of stable color, is the result of reactions between monomeric anthocyanins and tannins, which are polymers of flavanols including catechin, epicatechin, epicatechin-3-*O*-gallate, and

epigallocatechin.[8] As time passes, monomeric anthocyanin concentration decreases, while polymeric pigment concentration increases.[7] While this conversion has been documented in *V. vinifera* wines,[3] it is less common in hybrid wines, likely due to the high concentration of anthocyanin diglucosides.

Hybrid grapes have been shown to have lower concentrations of extractable condensed tannins compared to *V. vinifera* grapes.[8] This lack also contributes to lower wine quality, as tannins provide astringency and mouthfeel to wines by binding with proteins in the oral cavity.[8] Lower tannin concentrations may also contribute to the lower concentrations of polymeric pigment observed in hybrid wines, as tannins are a major constituent of polymeric pigment. Like pyranoanthocyanins, polymeric pigment is stable, contributes to the brick-red hue formed over time, and is mostly resistant to sulfite bleaching. The low condensed tannin concentrations in hybrid grapes means there is less stable color in the wines they produce.

Other studies have shown that high tannin binding by grape cell wall material may cause the low tannin concentrations observed in hybrid wines. Protein in the cell wall is one contributing factor to tannin binding. Differences in the protein concentrations of *V. vinifera* and hybrid grapes have been recorded, with hybrid grapes having higher protein. This provides one explanation for the lower concentrations of extractable tannins in hybrid grapes, and the lower tannin concentrations found in hybrid wines compared to *V. vinifera* wines.[8]

Unlike *V. vinifera* grapes and wines, there are few studies on the composition of hybrid grapes and wines. This study was conducted to record baseline measurements of extractable anthocyanins, proteins, and condensed tannins in 18 hybrid grape samples representing five cultivars in two growing seasons, 2014 and 2015, at six different growing sites.

Materials And Methods

Reagents. Acetic acid, acetonitrile, catechin hydrate, ethylenediaminetetraacetic acid (EDTA), ferric chloride, HCl, and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Bovine serum albumin (BSA), HPLC grade water, sodium chloride (NaCl), and sodium hydroxide (NaOH) were purchased from VWR Analytical (Radnor, PA, USA). Phosphoric acid was purchased from EMD Chemicals (Bellerica, MA, USA). Malvidin 3-glucoside and malvidin 3,5-diglucoside standards were purchased from Extrasynthese (Genay, France). Triethanolamine was purchased from Avantor Performance Materials (Center Valley, PA, USA). Ethanol, Amido black, sodium dodecyl sulfate (SDS), sodium hydroxide (NaOH), trichloroacetic acid (TCA), and Tris (hydroxymethyl)aminomethane, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Grape Sample Collection. Eighteen grape samples of five *Vitis* spp. hybrids were hand harvested across six vineyards (the Connecticut Agricultural Experiment Station in New Haven, CT; Willsboro, NY; Black Diamond Farm in Trumansburg, NY, USA; the University of Vermont in Burlington, VT; the University of Minnesota Horticultural Research Center in Excelsior, MN; and the New York State Agricultural Experiment Station in Geneva, NY) in September of 2014 and September of 2015. Of the eighteen samples, eight were Marquette, five Frontenac, three St. Croix, one MN1200, and one La Crescent. There were 13 samples from 2014 and five samples from 2015 (Table 3.3). Between 230-665 grams of fruit were collected in 2014, and between 127-148 grams were collected in 2015. Intact berries were stored at -20°C until analysis.

Sample Preparation. Berry samples were thawed and hand-crushed in plastic bags. Juice was strained through cheesecloth and frozen in 2 ml centrifuge tubes (Eppendorf NA, Hauppauge, NY, USA) until analysis.

Solid Phase Extraction. Prior to high performance liquid chromatography (HPLC) analysis, juice samples underwent solid phase extraction (SPE). Individual 3 cm^3, 60 mg Oasis HLB solid phase extraction cartridges (Waters, Milford, MA, USA) were preconditioned with 3 ml of methanol, followed by 3 ml of 0.01 N HCl. Between 200-500 μL of sample, depending on darkness of sample color, was diluted by 1 ml 0.01 N HCl and applied to the cartridge. Once eluted, the cartridge was rinsed with 1 ml 0.01 N HCl and dried under vacuum for 5 minutes. Anthocyanins were eluted with 15 mL of an acetonitrile:0.01 N HCl (95:5) solution. The fraction was concentrated to dryness at 35°C under nitrogen and resuspended in 1 mL of 0.1 N HCl. All samples were then filtered through a 0.22 μm polyethersulfone (PES) membrane (CELLTREAT, Shirley, MA, USA).

Anthocyanin Analysis. HPLC was performed using an Agilent 1260 Infinity series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a 100 mm x 2.1 mm pentaflurophenyl (PFP) column packed with 2.6 μm diameter particles with a 100 Å pore size. The system also consisted of an inline degassing unit, binary pump, autosampler thermostated column compartment, and a diode array detector fitted with a 1 μL volume Max-Light cartridge flow cell.

The method used was identical to that of Manns and Mansfield.[9] In this method, analytes were resolved using a biphasic gradient with a flow rate of 0.2 mL/min. Mobile phase A consisted of 0.5% phosphoric acid in water and mobile phase B consisted of 0.5% phosphoric acid in methanol. At 0, 15, 25, 27, and 30 minutes the solvent composition was 15, 30, 60, 60,

and 15% mobile phase B, respectively. The temperature was controlled at 45°C. The diode array detector monitored the absorbance at 520 nm with a reference of 630 nm. Anthocyanin monoglucosides and modified anthocyanins (acetylated, acylated, etc.) were quantified as malvidin-3-glucoside equivalents. Anthocyanin diglucosides were quantified as malvidin-3,5-diglucoside equivalents. The quantification was performed via the use of external standard curves ($R^2 > 0.9999$).

Protein Analysis. A modified version of the Amido Black assay described by Springer et al.[10] was used. A Multiscreen® HTS Polyvinylidene (PVDF) 96 well filter plate with 0.45 μm pores (EMD Millipore, Billerica, MA, USA) was wetted with 70% ethanol for one minute. Ethanol was drawn through the membrane by vacuum, and the plate was rinsed with filtered water (Millipore Corp., Billerica, MA, USA), which was also drawn through by vacuum. Standards and juice samples were prepared in 200 μL aliquots in triplicate in PCR tubes. Standards consisted of 0, 5, 10, 15, 20, 25, and 30 μg BSA in filtered water. 20 μL of a Tris-SDS solution (1 M Tris, 100 g/L SDS) and 80 μL of a 500 g/L TCA solution were added to each PCR tube and vortexed. Standards (or samples) were applied to the filter plate and pulled through by vacuum. PCR tubes were rinsed with 200 μL of 60 g/L TCA. This rinse was loaded onto its respective sample well and pulled through by vacuum. 50 μL of Amido Black staining solution (1 g/L amido black in 9:2:9 methanol:acetic acid:water) was added to each well and allowed to incubate 22°C for 10 minutes.

Excess stain was dumped from the plate, which was then rinsed with filtered water. The entire plate was then placed in a bath of the destaining solution (90:2:8 methanol:acetic acid:water). Destaining solution was replaced approximately every three minutes and the plate soaked for a total of 10 minutes. Excess solution was poured from the plate. The filter plate was

placed on top of a 96 well cell culture plate and 150 µL of elution solution (25 mM NaOH, 500 mL/L EtOH, 0.05 mM EDTA) was added to each well and allowed to incubate 22°C for 10 minutes. The plate was then placed in a Spinchron bucket centrifuge (GMI, Ramsey, MN, USA) at 2000 rpm for 10 minutes. A second addition of 150 µL of elution solution was added to each well and the plate was centrifuged a second time. Absorbance at 630 nm was recorded. The quantification of protein concentration in samples was performed via the use of the standard curve ($R^2 > 0.99$).

Tannin Analysis. The Adams-Harbertson Tannin assay was used to measure tannins.[11] Briefly, a bovine serum albumin (BSA) solution was added to a juice sample. After incubation and gentle agitation, samples were centrifuged at 14000 x RCF for five minutes. The supernatant was removed and discarded. Buffer A (~200 mM glacial acetic acid, ~170 mM NaCl in water, adjusted to a pH of 4.9 with NaOH) was added and the tube was centrifuged for one minute at 14000 x RCF. Again, the supernatant was removed and discarded. Buffer C (5% (v/v) triethanolamine, 5% (v/v) sodium dodecyl sulfate (SDS) in water, adjusted to a pH of 9.4 with HCl) was added to each tube, followed by a 10 minute incubation at 22°C. The tube was vortexed until the pellet was completely dissolved, followed by a second 10 minute incubation 22°C. Background absorbance was read at 510 nm on a Spectronic Genesys 2 spectrophotometer (Leeds, UK). After the addition of a ferric chloride solution and a 10 minute incubation at 22°C, the final absorbance was read at 510 nm. Tannin concentration as catechin equivalents (CE) was calculated using a standard curve of catechin at 0, 50, 100, 150, 200, 250, and 300 µL brought to a total volume of 1 ml by Buffer C ($R > 0.999$). Standards and samples were run in duplicate.

Results and Discussion

Anthocyanin Analysis

There were no trends within vintage or growing site for total anthocyanins. However, there was a clear varietal trend (Tables 3.1 and 3.2); Frontenac grapes had the greatest anthocyanin concentration, followed by MN1200, Marquette, and St. Croix. Previous studies have shown that *V. vinifera* grapes and wines contain lower concentrations of anthocyanins than hybrid grapes and wines. [12] In one study, six hours after crushing *V. vinifera* berries and leaving them on the skins, the anthocyanin concentration of the juice was approximately 100 mg/L.[13] In the current study, berries were crushed and left on the skins for less than one hour, which means there was less extraction time, and the extracted anthocyanin concentration was much higher than that of the anthocyanin concentration of *V. vinifera* grapes.

While the anthocyanin profile of *V. vinifera* grapes consists primarily of monoglucosides, hybrid grapes can have diglucoside concentrations of up to 85%.[14] This was clearly illustrated in the current work, as the anthocyanin profiles of all red cultivars were dominated by diglucosides (Table 3.1). High concentrations of diglucosides can result in low formation of stable color as wines age, and can cause color instability.[15] Delphinidin-3,5-diglucoside was the dominant anthocyanin in all Frontenacs, one Marquette, and one St. Croix, comprising between 40 and 69% of these samples. Malvidin-3,5-diglucoside dominated the profiles of the ten remaining samples, comprising between 32 and 50% of these samples. This is in agreement with several studies that have found malvidin-3,5-diglucoside to be the primary anthocyanin in non-*vinifera* anthocyanin profiles,[1,5,16] while studies on *V. vinifera* grapes have identified malvidin-3-glucoside as the primary anthocyanin in these cultivars.[4,16] While St. Croix contained the lowest

concentrations of total anthocyanins, the average diglucoside to monoglucoside ratio was 17.34, the highest of any variety (Table 3.2).

The varietal trends of anthocyanin monoglucosides and diglucosides were similar to that of total anthocyanins, with Frontenac containing the highest concentrations of mono- and diglucosides followed by MN1200, Marquette, and St. Croix. The dominant monoglucoside was malvidin-3-glucoside for all grapes with the exception of three Frontenacs, which had higher levels of delphinidin-3-glucoside. Delphinidin is purple in color, and other studies have shown that hybrid wines contain higher concentrations of delphinidin than *V. vinifera* wines, which may explain the blue-ish hue that has been observed anecdotally.[5] As discussed previously, of the diglucosides, malvidin was found in the highest concentrations.

Table 3.1. Anthocyanin concentrations in hybrid grapes.[a]

		Final [Antho], mg/L (variable equivalents)														
Variety	**Vintage**	**Del di-**	**Cy di-**	**Pt di-**	**Pn di-**	**Mlv di-**	**Del mono-**	**Cy mono-**	**Pt mono-**	**Pn mono-**	**Mlv mono-**	**Total mono-**	**Total di-**	**Total Modified ACNs**	**Ratio of di- to mono-**	**Total ACNs**
Frontenac	2014	4580.69	83.78	400.27	96.94	1135.72	128.98	24.48	86.58	6.63	71.36	318.03	6297.40	16.23	19.80	6631.66
Frontenac	2014	3653.69	121.29	378.44	190.05	1300.98	172.59	54.64	138.35	13.94	124.77	504.28	5644.45	21.97	11.19	6170.70
Frontenac	2014	1733.78	36.69	207.00	80.25	877.51	79.95	20.99	80.55	8.44	95.40	285.33	2935.22	10.05	10.29	3230.60
Marquette	2014	530.32	16.58	51.15	50.55	349.15	25.41	10.04	26.00	5.47	49.96	116.87	997.75	28.68	8.54	1143.31
Marquette	2014	234.37	9.67	23.56	61.58	332.81	47.99	27.02	60.63	28.25	158.77	322.66	661.99	51.90	2.05	1036.54
Marquette	2014	289.96	21.00	38.09	96.49	410.60	39.36	32.00	57.74	27.70	145.92	302.71	856.15	44.76	2.83	1203.62
Marquette	2014	290.17	14.65	42.67	79.58	517.08	24.93	14.98	37.41	11.25	103.30	191.87	944.15	47.30	4.92	1183.32
Marquette	2014	133.77	6.31	19.05	51.25	379.56	25.62	12.90	41.87	14.74	136.90	232.03	589.94	41.87	2.54	863.84
MN 1200	2014	782.85	13.08	132.30	73.26	1365.57	49.99	9.56	76.64	7.01	144.00	287.21	2367.06	59.58	8.24	2713.85
St. Croix	2014	243.96	20.33	20.38	49.24	275.81	3.25	7.36	4.45	2.37	12.20	29.64	609.73	11.61	20.57	650.97
St. Croix	2014	227.03	30.13	13.20	48.62	199.16	2.52	9.20	3.67	3.07	16.08	34.54	518.13	10.30	15.00	562.97
St. Croix	2014	166.41	20.72	10.81	44.21	215.76	2.42	6.72	3.06	2.40	13.24	27.84	457.92	13.17	16.45	498.93
Frontenac	2015	2285.92	114.54	264.46	175.99	1086.85	106.99	52.04	115.89	16.23	126.80	417.96	3927.76	9.86	9.40	4355.58
Frontenac	2015	3139.65	99.98	315.97	124.60	971.78	113.59	35.62	105.64	10.13	100.23	365.22	4651.98	10.94	12.74	5028.14
Marquette	2015	153.48	9.25	19.61	56.54	262.41	18.65	20.35	34.35	21.23	117.91	212.51	501.29	38.16	2.36	751.96
Marquette	2015	235.31	27.92	40.29	88.51	417.72	16.91	22.07	29.73	15.46	98.31	182.47	809.74	21.88	4.44	1014.09
Marquette	2015	102.46	10.31	17.19	53.44	338.31	7.75	11.48	17.76	10.01	92.96	139.96	521.72	29.53	3.73	691.22

[a]Del = delphinidin, Cy = cyanidin, Pt = petunidin, Pn = peonidin, Mlv = malvidin, mono- = monoglucoside, di- = diglucoside, ACN = anthocyanin.

Table 3.2 . Average anthocyanin concentrations of Frontenac, Marquette, and St. Croix samples.[a]

	Final [Antho], mg/L (variable equivalents)														
Variety	**Del di-**	**Cy di-**	**Pt di-**	**Pn di-**	**Mlv di-**	**Del mono-**	**Cy mono-**	**Pt mono-**	**Pn mono-**	**Mlv mono-**	**Total mono-**	**Total di-**	**Total Modified ACNs**	**Ratio of di- to mono-**	**Total ACNs**
Frontenac	3078.7 (1121.1)	91.26 (33.7)	313.23 (79.8)	133.57 (48.1)	1074.57 (161.7)	120.42 (34.1)	37.55 (15.4)	105.4 (23.3)	11.07 (4.0)	103.71 (22.9)	378.16 (86.4)	4691.36 (1337.6)	13.81 (5.3)	12.68 (4.2)	5083.34 (1373.4)
Marquette	246.23 (134.6)	14.46 (7.2)	31.45 (13.1)	67.24 (18.3)	375.95 (75.4)	25.83 (12.7)	18.85 (7.9)	38.19 (14.9)	16.76 (8.3)	113 (34.8)	212.64 (72.1)	735.34 (192.6)	38.01 (10.4)	3.93 (2.1)	985.99 (196.8)
St. Croix	212.47 (40.8)	23.73 (5.5)	14.8 (5.0)	47.36 (2.7)	230.24 (40.3)	2.73 (0.5)	7.76 (1.3)	3.73 (0.7)	2.61 (0.4)	13.84 (2.0)	30.67 (3.5)	528.59 (76.4)	11.69 (1.4)	17.34 (2.9)	570.96 (76.3)

[a]Del = delphinidin, Cy = cyanidin, Pt = petunidin, Pn = peonidin, Mlv = malvidin, mono- = monoglucoside, di- = diglucoside, ACN = anthocyanin. Results are expressed as mean (n=5 for Frontenac; n=8 for Marquette; n=3 for St. Croix) with standard deviation in parentheses.

Protein Analysis

Protein is a minor component of the total berry composition and subsequent wines. Springer et al.[8] found that protein represented between 8 and 14% of berry weight in six *V. vinifera* cultivars and six interspecific hybrids, while in a separate study it was reported that protein concentration in Maréchal Foch wine was 0.02% of total weight.[17] High protein concentrations have been correlated with high cell wall binding of tannin.[8] This may result in lack of astringency in hybrid wines, as well as the slower rate of formation of polymeric pigment compared to *V. vinifera* wines. Springer et al.[17] reported that juice from Native *Vitis* species contained higher protein concentrations than interspecific hybrid juice, which contained higher protein concentrations than *V. vinifera* wines. Higher protein concentrations are likely linked to greater disease resistance.

In this study, protein concentrations ranged from 37 to 119 mg/L (Table 3.3), which is lower than the average for interspecific hybrids (175.8 mg/L) reported in the previously discussed study. One possible reason for lower values is that all cultivars analyzed in each study were different.

Table 3.3. Tannin and protein concentration of hybrid grapes.[a]

Variety	Vintage	Location	Tannin (mg/L CE)	Protein (mg/L)
Frontenac	2014	New Haven, CT	28.5 (3.4)	83.2 (18.6)
Frontenac	2014	Excelsior, MN	39.7 (0.4)	73.3 (1.4)
Frontenac	2014	Willsboro, NY	8.1 (5.1)	74.0 (4.5)
La Crescent	2014	New Haven, CT	15.0 (6.1)	116.7 (27.2)
Marquette	2014	New Haven, CT	62.4 (0.3)	42.7 (1.4)
Marquette	2014	Trumansburg, NY	15.7 (6.7)	77.6 (5.4)
Marquette	2014	Geneva, NY	22.8 (5.9)	37.4 (5.6)
Marquette	2014	Burlington, VT	30.1 (2.9)	37.7 (1.5)
Marquette	2014	Willsboro, NY	15.7 (3.1)	46.5 (3.5)
MN 1200	2014	Willsboro, NY	24.7 (4.1)	118.8 (9.7)
St. Croix	2014	New Haven, CT	86.2 (5.8)	61.8 (2.9)
St. Croix	2014	Burlington, VT	28.6 (0.9)	36.6 (6.3)
St. Croix	2014	Willsboro, NY	37.6 (0.4)	87.0 (16.5)
Frontenac	2015	Burlington, VT	37.3 (11.3)	78.5 (7.3)
Frontenac	2015	Willsboro, NY	85.5 (31.3)	57.9 (3.0)
Marquette	2015	Trumansburg, NY	35.0 (1.1)	59.6 (1.0)
Marquette	2015	Burlington, VT	91.3 (62.1)	52.1 (2.2)
Marquette	2015	Willsboro, NY	30.7 (8.9)	49.3 (1.0)

[a]Results are expressed as mean (n=2 for tannins; n=3 for protein) with standard deviation in parentheses.

Tannin Analysis

Hybrid grapes contain lower concentrations of condensed tannins relative to *V. vinifera* grapes.[8] This is in agreement with the current study. While there did not appear to be any varietal, vintage, or geographical trends, the range of tannin concentration was between 8 and 91 mg/L CE for all samples. Alternatively, Springer and Sacks found that *V. vinifera* wines from the Finger Lakes region of New York State, which is an environment similar to the growing location of all samples, had an average of 248 mg/L CE, much higher than hybrid wines.[8] Studies of *V. vinifera* wines from warmer regions have found concentrations as high as 500 mg/L CE.[8] The lower concentration of tannin in hybrid wines likely contributes to a lack of stable color formation in finished wines, making it difficult to attain the brick-red color associated with aged

V. vinifera wines. Wines made from hybrid grapes are also likely to be less astringent, as astringency is characteristic of tannins.

Conclusion

Of the eighteen hybrid grape samples analyzed in this study, the anthocyanin profiles were dominated by anthocyanin-3,5-diglucosides, primarily delphinidin-3,5-diglucoside, which is known to give a blue tint to red wines produced from hybrid grapes, and malvidin-3,5-diglucoside. The anthocyanin concentration of all grapes was very high in comparison to concentrations found in *V. vinifera* grapes. The high concentration of anthocyanin diglucosides makes the formation of stable color as polymeric pigment occur more slowly than in wines with high concentrations of anthocyanin monoglucosides. As has been noted in other studies, tannin concentrations for these hybrid grapes were low (<100 mg/L) in comparison with *V. vinifera* grapes. Grapes with low tannin concentrations will produce wines with low astringency, and with less polymeric pigment. Finally, protein concentrations were found to be lower than those reported for other hybrids, possibly due to the fact that the cultivars analyzed in this study were not analyzed in other literature. Overall, producing high quality wines with hybrid grapes poses several challenges, and future studies should address processing steps that will enhance extraction of tannin, so that higher concentrations of polymeric pigment can form in hybrid wines.

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CHAPTER 4
FUTURE WORK

Anthocyanins, their chemical reactivity, and the effect of these reactions on *V. vinifera* wine color have been studied from a variety of perspectives, but there has been little research on the color of non-*vinifera* and hybrid wines and their unique differing anthocyanin profiles. Suggestions for future study include a focus on non-*vinifera* and hybrid wines and their ability, or lack thereof, to form polymeric pigment. The research discussed in chapter 2 of this thesis studied the effect of the reaction rates of anthocyanin diglucosides in model wine systems under specific conditions of excess acetaldehyde and catechin. Future studies might address different concentrations of anthocyanin, acetaldehyde, and catechin. It will also be important to look at the effect of different flavanols, such as epicatechin. Model solutions might be made more complex, with multiple anthocyanin types and multiple flavanol types. The addition of condensed tannin, rather than monomeric flavanols, might also be considered.

It is important to continue the analysis of real hybrid red wines, such as Frontenac, Marquette, Corot noir, and others, for polymeric pigment concentration in the future. Ways in which to increase polymeric pigment formation in hybrid wines should also be pursued. Current work has identified specific yeasts strains as promoting more polymeric pigment formation than other yeast strains, likely due to an increase in acetaldehyde production.[1] This, and other processing changes, may offer solutions to improving polymeric pigment formation in hybrid wines.

There is anecdotal evidence of differences in hybrid wine color as compared to *vinifera*, especially when considering the evolution of wine color throughout aging. However, there is a gap in the scientific literature supporting these claims. Future studies should consider monitoring

the color of hybrid wines over time with colorimetry. HPLC analysis of these wines would offer supplemental evidence as well, such as how the anthocyanin profiles of hybrid wines change throughout aging.

The influence of pyranoanthocyanins to aged wine color in hybrid wines is another interesting area of research. Pyranoanthocyanins influence wine color of aged *V. vinifera* wines by promoting the transition to brick-red and tawny red. Anthocyanin diglucosides cannot form pyranoanthocyanins, and there is evidence that diglucosides decrease the ability of anthocyanin monoglucosides to react with other compounds. Therefore, the formation of pyranoanthocyanins in hybrid wines and their contribution to wine color warrants further exploration.

Further work defining the composition of polymeric pigment would improve understanding of the reactions of polymeric pigment formation and degradation. Currently, the literature reports that polymeric pigment is composed of anthocyanins bound to tannins. However, these tannins have multiple subunits, and variable length and composition. There are other ways to form polymeric pigments, such as pyranoanthocyanins that react with flavanols to form polymers. Also, there are hundreds of permutations of anthocyanins, with different anthocyanidin cores, glucose attachments and acylations. Polymeric pigment composition is clearly very complex. Today, chromatography methods usually show polymeric pigment eluting as a broad, unresolved hump at the end of the chromatogram. Improving this technology would improve the understanding of polymeric pigment composition and its influence on wine color. Relating polymeric pigment composition to visible color would be helpful in understanding how polymeric pigment influences the color of aged wines.

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