EFFECTS OF CONCENTRATION PRIOR TO COLD-STABILIZATION ON THE COLOR OF CONCORD GRAPE JUICE

by Kristin Suzanne Alongi

EFFECTS OF CONCENTRATION PRIOR TO COLD-STABILIZATION ON THE COLOR OF CONCORD GRAPE JUICE

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ABSTRACT

Anthocyanins are ubiquitous in nature, found in many fruits and vegetables. Concerning Concord grape juice, anthocyanins are the prominent color pigment, giving the juice its characteristic purple hue. They contribute to color as both a free, unbound species, and also through reactions with other compounds, forming copigmented complexes or polymeric pigments. Color is often a defining factor of consumer acceptance; therefore, understanding the effect of processing on the color of the juice is extremely pertinent to the success of the industry. Recently, there has been anecdotal evidence that concentration prior to cold storage may significantly impact the overall color of Concord grape juice produced form concentrate.

The color of Concord grape juice produced by concentration before cold-stabilization/detartration (direct-to-concentrate, DTC) was compared to juice produced via cold stabilization prior to concentration (standard concentrate, SC). Following reconstitution, DTC juice had a 63% greater absorbance at 520 nm than SC juice. A significant loss of anthocyanins was observed using a paired t-test during cold-stabilization of single-strength juice during SC processing (averaging 79 mg/L as cyanidin-3-glucoside, 23% of total anthocyanins), while no significant loss of anthocyanins or color was observed during cold stabilization of DTC concentrate. The concentration of anthocyanins in the SC bitartrate crystals was 0.80% w/w compared to 0.13% w/w in the DTC bitartrate crystals. Based on changes in titratable acidity during processing, the loss of anthocyanins in SC juice due to coprecipiation was estimated to be 64 mg/L. The decrease in coprecipitation of anthocyanins with bitartrate crystals during DTC cold-stabilization may be due to lower water concentration and decreased pH, hindering the adherence of colored flavylium ions to the bitartrate crystal.

BIOGRAPHICAL SKETCH

Kristin Alongi was born in Chittenango, a small town outside of Syracuse, NY. She attended Hamilton College and was the 2008 Valedictorian, graduating with a B.A. in Chemistry. After discovering a passion for food science, she continued on to Cornell University to work under Dr. Gavin Sacks, studying the color of Concord grape juice. She enjoys hiking, kayaking, and spending time in upstate New York. She is most grateful for her family, friends, and long-term boyfriend, Bill. They have all shaped the person she has become and continue to bring joy into her life. Her success is not merely a measure of her professional accolades, but rather a combination of these accomplishments with the strength of her relationships and treasured memories made throughout the years.

To my wonderful family and friends for their support, guidance, and laughs.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

BACKGROUND

Anthocyanins

Flavonoids

Anthocyanins are natural colorants that are found in many fruits and vegetables. Associated with many health benefits, they play a prominent role in the food and beverage industries (Castaneda-Ovando and others 2009). Anthocyanins are a type of flavonoid, which are phenolic compounds with a C6-C3-C6 skeleton. They are the main color pigment in red grapes, although grapes also contain other types of flavonoids, such as flavan-3-ols and flavonols. The main groups of flavonoids are outlined below in Figure 1, adopted from Liu's 2004 publication (Liu 2004).

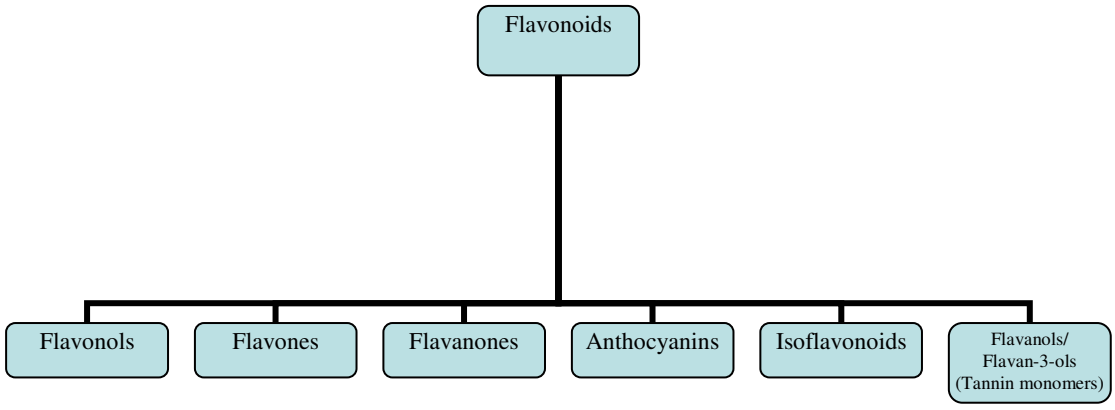


Figure 1: Classification of flavonoids

Flavan-3-ols, which are present in the grapes as condensed tannins (proanthocyanidins or catechin monomers), serve to stabilize color, and provide astringency in grape juice. When heated in acid, proanthocyanidins release red anthocyanidins, hence their name,

and also precipitate proteins in solution (Cheynier and others 2006). Flavonols act as co-pigmentation cofactors in the juice and can intensify color (Boulton 2001).

Anthocyanins are the glycosylated forms of anthocyanidins and are more prevalent in fruits and vegetables than their aglycone counterparts. Their C6-C3-C6 skeleton consists of an aromatic ring [A], heterocyclic ring [C], and aromatic ring [B], respectively, see Figure 2 (Mazza and Miniati 1993).

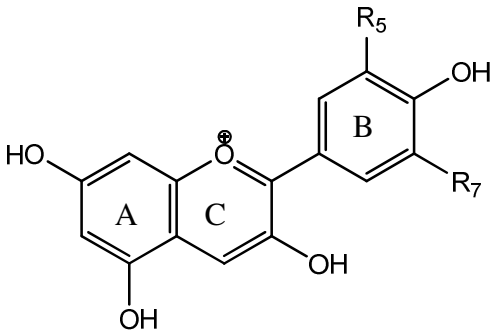


Figure 2: The Chemical Structure of Anthocyanidins (Mazza and Miniati 1993).

Table 1: The Five Most Common Anthocyanidin Structures and Their Characteristic Colors (Castaneda-Ovando et al. 2009)

Name	R_5	R_7	Color
Cyanidin	OH	H	Orange-red
Delphinidin	OH	OH	Blue-red
Malvidin	OCH_3	OCH_3	Blue-red
Peonidin	OCH_3	H	Orange-red
Petunidin	OCH_3	OH	Blue-red

The positively charged cation structure, shown in Figure 2, is commonly referred to as a flavylium ion. It contains a system of conjugated double bonds that give the pigment enhanced stability. This chemistry is also responsible for its color, which is a result of the resonance structure of the conjugated double bonds and the delocalized system of π electrons in the aromatic ring (Pauling 1939). Consequently, any reactions that disrupt this aromatic ring will cause a loss of color.

The most common anthocyanins in grapes are the glycosylated derivatives of cyanidin, peonidin, petunidin, delphinidin, and malvindin (Cheynier et al. 2006; Monagas and Bartolomé 2009). These compounds have different hydroxyl and methoxyl patterning on the 3 and 5 positions on the B ring, reference Figure 2 and Table 1 (Castaneda-Ovando et al. 2009). The concentrations of these compounds within the fruit vary based on the grape variety (Monagas and Bartolomé 2009; Bates and others 2001). The sugar substituent usually attaches to the 3 (C ring), 5 (A ring), or 7 (A ring) position on the anthocyanin, with the most common being the 3 position. On the sugar, the linkage is usually at the C1 position (Mazza and Miniati 1993). The type of sugar can vary, however, and be either glucose, galactose, rhamnose, xylose, or arabinose, although mostly glycosides are found in concord (Lee and others 2008). Furthermore, these sugars may undergo other modifications, greatly increasing the number of possible anthocyanin species. The anthocyanidin pigment itself also has vast diversity and can have different patterns of hydroxyl groups, methylation, or acylation (Mazza and Miniati 1993). Both the glycosylation and acylation of the pigment can affect its color, detection threshold, and antioxidant capability, as described by Stintzing et al. concerning cyanidin aglycones (Stintzing and others 2002).

The grape's main pigments are located in the solid parts of the cluster. Specifically, anthocyanins are found in the grape skins and are released upon maceration (Cheynier et al. 2006; Monagas and Bartolomé 2009). The concentration of anthocyanins initially increases during ripening, beginning at veraison, as does the pH (Hrazdina and others 1984).

Concord grapes

Concord grapes are of the interspecific species *Vitis labruscana (labrusca x vinifera)* and have thicker skins than the grapes of *Vitis vinifera*, the most common grape used in wine making (Mullen and others 2007). Concord grapes are rich in phenolics and anthocyanins, containing acylated and nonacylated glucosides of the five most common anthocyanidins previously listed (Mazza and Miniati 1993). Cyanidin 3-monoglucoside and delphinidin 3-monoglucoside are the two most prevalent anthocyanins in Concord (Munoz-Espada and others 2004).

In a 2004 study, Munoz-Espada and colleagues found that the skins of the Concord grapes contained 326 ± 5.9 mg of total anthocyanins per 100g of skin, with an average total anthocyanin concentration of 95 mg per100g of grapes. Additionally, they used mass spectrometry to determine the relative concentrations of these anthocyanin species. The most abundant compounds were the cyanidin and delphinidin aglycons. The second group of most abundant compounds were: petunidin aglycon, malvidin aglycon, malvidin diglucoside, and cyanidin coumaroyldiglucoside. Lastly, peonidin aglycon, cyanidin monoglucoside, peonidin monoglucoside, delphinidin acetylglucoside, cyanidin coumarolyglucoside, petunidin coumaroyl glycoside, and malvidin coumaroyl glucoside were also found. Results showed a large number of aglycons, which may have been inflated due to the use of an acid in the extraction, possibly cleaving the glycosidic linkages (Munoz-Espada et al. 2004).

Health Benefits

In recent years, consumers have become more health conscious. Consequently, functional foods, foods that have additional health benefits beyond adequate nutrition, have become increasingly popular. These types of food often contain antioxidants that may prevent diseases caused by oxidative stress or the abundance of dangerous

oxygen radical species in the body (Kaur and Kapoor 2001). These radical compounds react with molecules in the body, stealing their electrons and causing more dangerous free radicals, which can oxidize proteins, DNA, or lipids, destructing cells (Halliwell 1992). Many illnesses, such as cardiovascular problems, cancer, cataracts, rheumatism, and other auto-immune diseases, are believed to be caused by these unstable free oxygen radicals. Antioxidants react with radicals before they oxidize other compounds in the body, preventing harmful side effects. The anthocyanins found in grapes react as antioxidants, thereby scavenging these dangerous free radicals. More specifically, the phenolic hydroxyl groups donate a hydrogen to free electrons and turn into stable compounds, which prevent the formation of additional free radicals throughout the body (Kaur and Kapoor 2001). Numerous studies have indicated that the consumption of food containing antioxidants have had preventative health benefits, such as reducing the risk of cancer or neurological diseases (Joseph and others 1999). This is discussed in the Steinmetz and Potter 1996 review article on vegetable/fruit consumption and cancer risk (Steinmetz and Potter 1996).

Out of all commercial fruit juices, grape juice has the highest antioxidant capability and, therefore, has great potential health benefits (Wang and others 1996). For example, the consumption of grape juice has been shown to inhibit low density lipoprotein oxidation (Day and others 1997). In a 1998 study, Concord grape juice decreased LDL oxidation by 67%. Furthermore, all of its antioxidant potential was related to the concentration of anthocyanins, opposed to other compounds within the juice (Frankel and others 1998). The antioxidant activity of these compounds in the juice, however, may be influenced by their oxidation state and impacted by storage and processing conditions. (Kaur and Kapoor 2001).

Pertaining specifically to Concord grapes, Munoz-Espana and colleagues determined that cyanidin is a better antioxidant than malvidin. This is a result of the

extra methylation on malvidin's B ring, which hinders the loss of electrons and the molecule's antioxidant ability. Conversely, cyanidin's hydroxylation on the B ring increases its ability to be oxidized in a redox environment (Munoz-Espada et al. 2004).

Forms

Monomeric pigments

Monomeric pigments are free anthocyanins, unbound to other species. One of their defining characteristics is that they exhibit structural changes, producing different colors, in various pHs. The four structures in equilibrium are: the red flavylium cation, blue quinoidal base, and colorless carbinol pseudo-base or colorless/slightly yellow chalcone (Mazza and Miniati 1993). Figure 3 outlines this structural equilibrium.

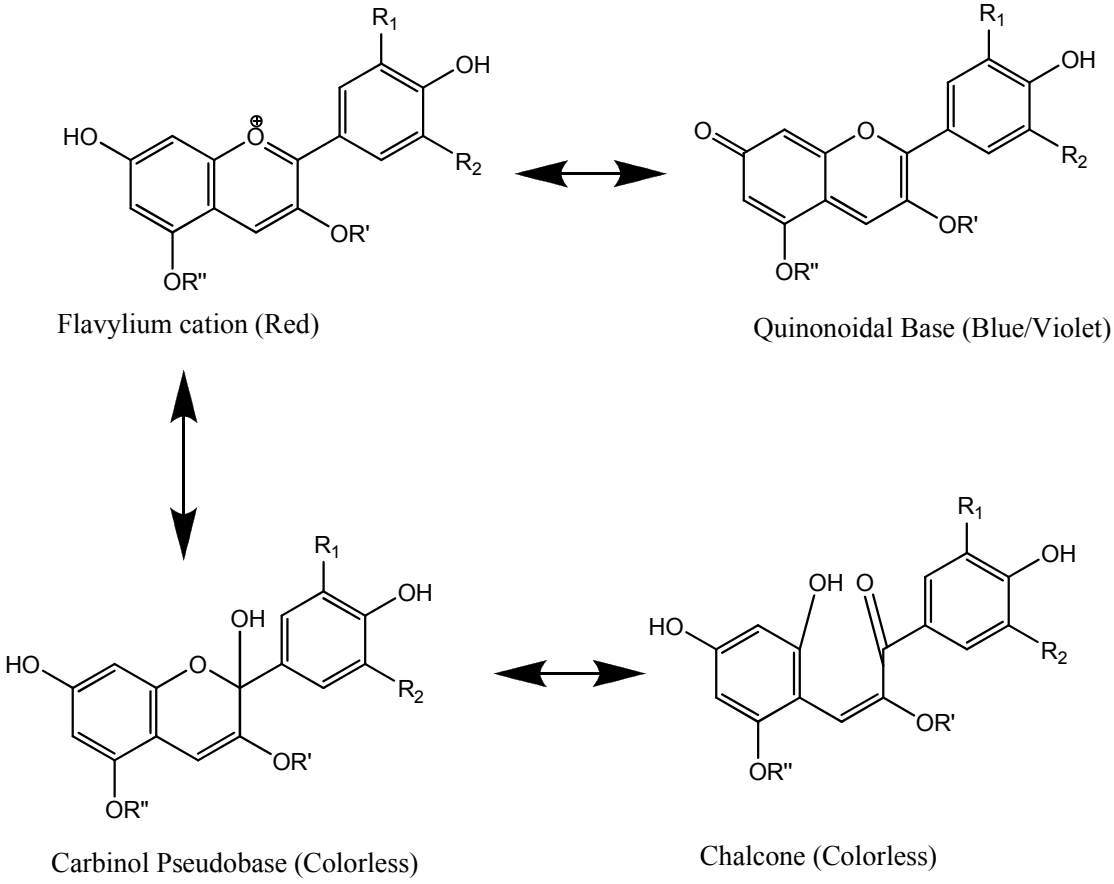


Figure 3: Equilibria of Anthocyanidin structures (Brouillard and Markakis 1982).

At pH < 2, the flavylium cation will dominate, exhibiting a red color. When pH increases to the range of the pKa, approximately 4-6, the flavylium cation will lose a proton and establish an equilibrium with the blue quinodial base form (Brouillard and Markakis 1982). As pH increases, however, the flavylium cation, will also be hydrated, taking up an OH group, to form the colorless carbinol species, which then also equilibrates with the colorless chalcone species. Therefore, a mixture of these anthocyanins will appear in solutions that will vary in concentration based on the monomeric anthocyanin species and the pH. For example, concerning cyanidin 3,5-diglucoside, an equilibrium between the red flavylium cation and colorless carbinol exists at pK_h=3.38. Aside from these two compounds, however, some of the solution will consist of the blue base and colorless chalcone. Overall, as pH increases the cation is converted to the quinoidal base (pk_a=2.23) and carbinol form, with an equilibrium dependent upon the substitution on the flavylium ring. For cyanidin 3,5 diglucoside, the carbinol form is favored, with some converted to the chalcone form. Reference Figure 4 for an approximate distribution of anthocyanin structures over various pHs, adopted from the work of Mazza and Brouillard (Mazza and Brouillard 1987).

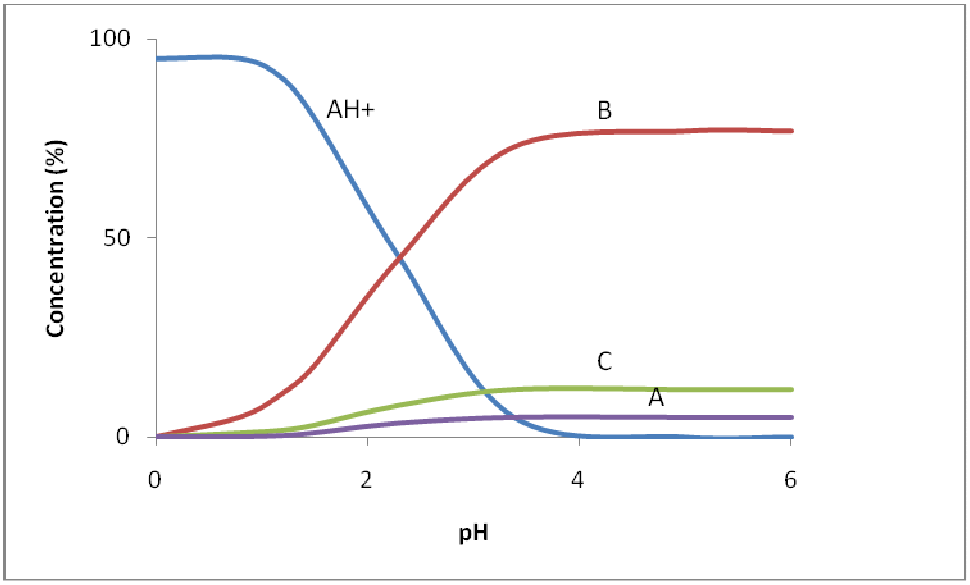


Figure 4: Approximate distribution of AH^+ (red flavylium cation), A (blue quinoidal base), B (colorless carbinol pseudo-base), and C (colorless/slightly yellow chalcone) of cyanidin 3,5-diglucoside as a function of pH(Mazza and Brouillard 1987).

In other anthocyanidin species, however, the blue quinonodial form dominates at high pHs over 6. (Mazza and Miniati 1993).

Since the anthocyanin structure and color changes with varying pHs, its concentration can be determined by comparing absorbance at pH=1, where it is red, compared to pH=4.5, where it is colorless, using Eqs.1-2:

$$\text{Total Anthocyanins (mg/L)} = (A \times MW \times D \times 10^3)/(\varepsilon \times l) \qquad \text{Eq. 1}$$

$$A = (A_{max} - A_{700nm})pH_{1.0} - (A_{max} - A_{700nm})pH_{4.5} \qquad \text{Eq. 2}$$

MW= molecular weight of the major anthocyanin
D=dilution factor
ε=molar extinction coefficient of prominent anthocyanin
l=path length, normally 1 cm

Normally the absorption (A) measurement has an A_{max} around 510-520 nm (Wrolstad and others 2005).

Aside from being affected by pH, monomeric pigments are also bleached by bisulfites, which attach to the C4 or C2 positions on the anthocyanin to form a stable complex (Berke 1998). The addition destroys the aromaticity of ring, thereby causing a loss of color.

The various structures of monomeric anthocyanins also have different reactivities. At low pHs, when the flavylium ion exists, the anthocyanin can act as an electrophile at the C2 or C4 positions. At higher pHs, however, the carbinol pseudo-base can act as a nucleophile at its C6 and C8 positions on the A ring (Monagas and Bartolomé 2009).

To stabilize color, monomeric pigments can react with a variety of compounds through copigmentation or the formation of polymeric pigments and other adducts. Table 2 summarizes the various reactions that occur during winemaking (Monagas and Bartolomé 2009):

Table 2: Reactions of Anthocyanins in Winemaking (Monagas and Bartolomé 2009)

Non-Anthocyanin Reactant	Product	Details
Flavanols	Flavanol-anthocyanin adduct	Colored, formed in direct condensation reaction, colorless dimer is formed that is dehydrated to red flavylium ion form
Flavanols	Anthocyanin-flavanol adduct	Colorless, formed in direct condensation reaction
Flavanols, Acetaldehyde	Flavanol-ethyl-anthocyanin adducts, Anthocyanin-ethyl anthocyanin dimmers	Polymeric Pigments Condensation Reactions
o-quinones of caftaric acid	CA-anthocyanin adducts	
Vinylflavanols (derived from tannins)	Flavanyl-pyranoanthocyanins	Polymeric pigments, Condensation Reactions
Acetaldehyde	Vitisin B pyranoanthocyanins	Polymeric Pigments

Table 2 (Continued)

Pyruvic Acid	Vitisin A pyranoanthocyanins	Polymeric Pigments
Pyruvic acid and Vinylphenols	Hydroxyphenyl-vinylpyranoanthocyanins	Polymeric Pigments
Pyruvic acid and vinylflavanols	Portisins	Polymeric Pigments, exhibit blue shift
Vinylphenols, hydroxycinnamic acids (caffeic acid, *p*-coumaric acid)	Hydroxyphenyl pyranoanthocyanins	Condensation reaction, Polymeric pigments
Small, planar, aromatic species	Non-covalent interactions	Copigmentation

Through these reactions, both colorless and color stabilizing polymers are formed. Potentially, many of the products listed in Table 2 could also affect the appearance of grape juice, except those reactions involving ethanol or acetaldehydes (Mazza and Miniati 1993). Pyruvic acid is also significantly lower, as there is not yeast addition.

Polymeric Pigments

Polymeric pigments are formed from a variety of anthocyanin reactions: condensation with an aldehyde group, reaction with a hydroxycinnamic acid, ethyl bridging with aldehydes, or condensation with other flavonoids (Monagas and Bartolomé 2009). These compounds have an additional ring formed from the cyclization of the hydroxyl group at C5 position to the C4 position. It is believed that they are also more stable than the original anthocyanins due to the presence of this fourth ring (Castaneda-Ovando et al. 2009). The name polymeric pigment in this sense is somewhat of a misnomer, as they are not long chains of repeated units but rather large complexes of covalently bonded molecules (Harbertson and Spayd 2006).

These polymeric pigments are more stable color compounds than their monomeric counterparts and exist as a variety of structures. Polymeric pigments are

not as affected by changes in pH, as they have a stable aromatic structure and prevent the formation of the colorless base because the C4 site is blocked (Mazza and Miniati 1993; Lee and others 2005). Moreover, this describes why polymeric pigments are not bleached by bisulfites, since bisulfites cannot bind to the C4 position as they do with monomeric anthocyanins (Berke 1998). This stability is often utilized as a means of measuring the concentration of polymeric color, as bleaching will eliminate color form monomeric anthocyanins and copigmented complexes (Wrolstad et al. 2005; Somers and Evans 1974; Somers and Evans 1977). Recently, however, Versari et al. found that polymeric pigments are partly bleached by SO_2, but still to a much lesser extent than monomeric compounds (Versari 2008).

Tannins are often involved in the formation of polymeric pigments and are thought to enhance color in wine (Boulton 2001; Somers 1971). Since tannins can be precipitated by proteins, these complexes can also be precipitated out with protein assays (Harbertson and others 2002). In 2002, Harberston et al. found that this method only precipitated some of the bisulfate resistant polymeric pigments, called large polymeric pigments (LPP). The polymeric pigments that did not precipitate out but were still resistant to bisulfate bleaching were classified as small polymeric pigments (SPP) (Harbertson et al. 2002).

Pyranoanthocyanins are another type of polymeric pigment formed from the complexion of anthocyanins with low molecular weight species (Rentzsch and others 2007). Many SPP are pyranoanthocyanins but not all, as pyranoanthocyanins have a specific structure of a pyran ring and SPP are based on more of an operational definition. Some common pyranoanthocyanins are hydroxyphenyl-pyranoanthocyanins, vitisins, vinylflavanol-pyranoanthocyanins, portisins, and rosacyanin B. The specific colors of these compounds vary based on their structure, and range from orange-red to blue (Rentzsch et al. 2007).

Copigmentation

Monomeric anthocyanins, in the aromatic flavylium and quinodial form, can also contribute to color in the form of copigmentation. Copigmentation occurs when anthocyanins interact with organic non-anthocyanin molecules to form a complex of noncovalent interactions. The complex is held together by molecular associations and usually results in a color shift or more intense, stabilized color of the solution (Boulton 2001).

The possible copigment compounds often involved in copigmentation reactions are phenolic acids, flavonoids, and derivatives of flavonol and flavone subgroups (Boulton 2001). Such complexes play a prominent role in the color of young wines, creating a bathochromic shift, yielding a blue purple color opposed to a red node in the wine, as the solution absorbs at a longer wavelength. Copigmented complexes also display hyperchromicity, an increase in absorption, enhancing the existing color (Asen and others 1972). These characteristics are affected by the concentration of the pigment, pH, molar ratios of the copigment compound to pigment, or anions in the solution (Boulton 2001). Overall, it is believed that there needs to be a concentration of at least 35 μM of the anthocyanin for copigmentation be significantly detectable (Boulton 2001; Jurd and Asen 1966). Pertaining specifically to Concord grapes, it is also believed that grape seed extract, sugars, and zinc ions have no copigmentating effects and do not affect the color of the juice in any way (Scheffeldt and Hrazdina 1978).

The most widely accepted explanation for the formation of copigmented complexes is that hydrophobic, π-π interactions between the pigment and the aromatic ring of a cofactor cause planar stacks and an association between the two molecules (Boulton 2001; Hoshino and others 1981b). The stacking stabilizes the aromaticity of

the anthocyanin, thereby, stabilizing its color. Only flavylium and quinonoidal bases are capable of this interaction, as their planar, hydrophobic, and aromatic structures allow the complexion (Asen et al. 1972; Terrier and others 2009). Moreover, cofactors conducive to this stacking arrangement are small, planar, and aromatic, which cause less steric hindrance (Terrier et al. 2009). Overall, flavonoid derivatives make good cofactors, especially those that allow face to face stacking and have electron withdrawing groups on their rings (Boulton 2001).

The effects of copigmentation on color enhancement vary with the pH of the solution and it is unknown if all anthocyanin forms are involved in copigmentation. Various beliefs exist in an attempt to explain this phenomenon. One belief is that the different anthocyanin species at each pH account for this observable effect. For example, an anionic cofactor would complex with a flavylium cation at acidic pHs vs. the interaction of an uncharged species with the quinodial species at more neutral pHs. Conversely, color dependence may instead depend on the stability of flavyium ions in the stack, which could vary with pH, or other cofactors (Boulton 2001). Overall, maximum copigmentation occurs over a pH range of 3-5, depending on the cofactors involved, and has a bell shape distribution (Davies and Mazza 1993).

Similar to free anthocyanins, copigmented complexes can be bleached with bisulfites, which must be accounted for in anthocyanin analysis methods (Boulton 2001; Levengood 1996).

Within the juice, there is an equilibrium between the copigmented complexes (C) and the free anthocyanin (A):

$$[A\text{-}C] + [B - C] = [C] \qquad \text{Eq.3}$$

Where A symbolizes the moles of the free anthocyanin in solution, B, the moles of the cofactors, and C, the moles of copigmented anthocyanins. With an equilibrium constant of:

$$K_{eq} = ([C])/([A\text{-}C][B\text{-}C]) \qquad \text{Eq. 4}$$

Along with the concentrations of the compounds, dilution, even at a constant pH, can shift the equilibrium to non-copigmented forms. As observed from the equations above, the reaction is second order. Therefore, as viewed from Eq. 4, a 1 fold dilution and decrease in concentration of A and B will cause a 4 fold decrease in the concentration of the complexed form. Changes in temperature can also affect copigmentation, as a high temperature favors the dissociation of copigmented anthocyanins but also increases the solubility of many compounds.

The color of the solution due to copigmentation can be calculated using the molar extinction coefficients of the anthocyanins (E_a) and copigmented anthocyanins (E_c):

$$A_{520} = (Ec*[C] + Ea*[A\text{-}C])*f) \qquad \text{Eq. 5}$$

In Eq. 5, *f* is the fraction of anthocyanins in the flavylium form at the pH of the solution. The color enhancement from the copigmentation can also be calculated comparing the absorption of a solution with the addition of cofactors to the initial absorption of the solution (Boulton 2001).

As wine ages, the amount of polymeric pigments increased, decreasing the number of monomeric anthocyanins and cofactors available for copigmentation (Somers 1971). Since copigmentation only occurs with monomeric anthocyanins, its rate of formation is a significant component of the color of young wine but its role decreases as the wine ages (Harbertson and Spayd 2006). This change in the

prominent pigments causes the shift in color as wine matures, from a bright red/purple color, resulting from copigmentation, to a darker red (Somers 1971).

In interactions very similar to copigmentation, anthocyanins can also self associate and form complexes to enhance the color of the solution (Asen et al. 1972). These associations result in hypsochromic, hyperchromic, or bathochromic shifts depending on the two anthocyanins, as self associations with malvidin and cyanin quinonoidal base yield different chromic shifts (Hoshino and others 1981a). The self association forms as a result of π-π interactions, causing the stacking of anthocyanins. It is believed to be a hydrophobic interaction, where the attached hydrophilic sugars surround the association (Hoshino et al. 1981a; Hoshino and others 1982). Intramolecular copigmentation may also occur, but involves just one anthocyanins that has two or more aromatic acyl groups (Mazza and Miniati 1993).

Additionally, anthocyanins can form complexes with metal ions, which may contribute to the characteristic color of Concord grapes (Ingalsbe and others 1963). Both the flavylium ion and quinonoidal forms are capable of metal complexion, which prevent hydration and formation of the colorless pseudo-bases, thereby stabilizing color. This effect was observed when the anthocyanins were in an aqueous solution of neutral salts (Goto and others 1976).

Stability

As previously discussed, anthocyanin stability varies based on the other compounds in the solution. Isolated anthocyanins are fairly unstable and degrade easily, as they are affected by many processing and storage factors, such as pH and temperature (Giusti and Wrolstad 2003).

Monomeric compounds degrade when exposed to sunlight (Iacobucci and Sweeny 1983). Consequently, antioxidants are the most stable in dry, dark environments (Wrolstad et al. 2005). Calvi and Francis found anthocyanins extracted from Concord grapes remained stable over a pH range of 2.8-3.6 in juice model systems. The presence of oxygen in the solution, however, was found to negatively impact stability (Calvi and Francis 1978).

Aside from the reactions already discussed, the addition of other compounds to juice may affect its color. A Talcott et al. study showed that ascorbic acid degraded anthocyanins in juice, having negative impacts on the overall color (Talcott and others 2003). Concerning Concord grape juice, however, Vitamin C addition was shown to have no affect on the antioxidant capability of the beverage (Frankel et al. 1998).

The addition of specific copigmentation co-factors, though, has been shown to increase color stability in juice and wine. Talcott showed that isoflavonoids from red clover leaves exhibited this effect (Talcott and others 2005). In a different study, rosemary extract had a similar effect and increased copigmentation in the solution. It caused hyperchromic and bathochromic shifts (Talcott et al. 2003; Brenes and others 2005). Furthermore, anthocyanins with a greater degree of acylation are capable of intramolecular copigmentation, and therefore exhibit greater stability in solution (Giusti and Wrolstad 2003; Malien-Aubert and others 2001). Overall, increasing copigmentation is an effective strategy to help maintain grape juice color (Del Pozo-Insfran and others 2006).

Methods of Analysis

Extraction and Purification

Aqueous mixtures of ethanol, methanol, or acetone may be used to extract anthocyanins, due to their polar nature (Castaneda-Ovando et al. 2009). These methods, however, are not selective because other compounds can be extracted from

the solution as well. This was observed by Coutinho et al. when sugar was extracted along with anthocyanins in red cabbage using common extraction techniques (Coutinho and others 2004). Consequently, purification is necessary when implementing extraction methods (Castaneda-Ovando et al. 2009).

CIELAB

CIELAB is a method to accurately measure color. Its indices include L* corresponding to lightness, C* for chroma, and h* as a measure of hue angle. Values a* and b* are coordinates used to measure hues and chroma. A positive value of a* indicates the relation of the color to redness, while a negative value corresponds to the amount of green in the color. In contrast, positive b* values represent color in the direction of yellow and negative indicates an association with blue. These values are not simple a measure of redness, or greenness but rather a vector indicating the direction of a color towards these components. Using these two values, h* or the hue angle is determined to represent the overall hue of the color, where:

$$h^*=\arctan(b^*/a^*) \qquad \text{Eq. 6}$$

Hue angle values range from 0˚ to 360˚, representing a span of colors. To fully characterize the color quantitatively, chroma is needed to convey its intensity, which is expressed as:

$$C^*=(a^*+b^*)^{1/2} \qquad \text{Eq.7}$$

The CIELAB method incorporates the three measurements of L*, C*, and h* to represent color in a universal manner (Wrolstad et al. 2005).

Spectrophotometeric Assays

A spectrophotometer is often used in assays to determine anthocyanin content. The pH Differential Method uses spectroscopy to measure the concentration of

anthocyanins in solution. The method exploits free anthocyanins' structural changes over the pH range of 1.0 to 4.5, as they change from colored to colorless, respectively. The concentration of anthocyanin pigments can, therefore, be made by comparing the difference in $\lambda_{vis\text{-}max}$ at 520nm at pH=1 and pH=4.5. Lee, et. al. reported values in terms of cyanidin-3-glucoside, as it is one of the more common anthocyanins, particularly in Concord grapes**.** The Lee et al. paper was a collaborative study. It showed that two labs could use this method for determining monomeric anthocyanin content and receive results that are in excellent agreement, with a relative standard deviation of 1.06-4.16%. Overall, the method proved to be simple, quick, and accurate (Lee et al. 2005).

In a 2008 study, Lee, Rannaker, and Wrolstad reaffirmed the reliability of the pH differential method but also discovered the importance of the standards used to express anthocyanin content, as they found varying results whether total anthocyanin content was expressed in terms of malvidin-3-glucoside or cyanidin-3-glucoside (Lee et al. 2008).

The pH Differential method often is combined with an assay similar to that of Somers and Evans, in which the addition of SO_2 is used to determine the concentration of polymeric pigments (Somers and Evans 1974; Somers and Evans 1977; Giusti 2001). The Somers and Evans assay adjusts the pH of the solution to 1, where the flavylium ions are a vibrant red color. The absorbance is then taken and compared to that of a sample with a bisulfate addition, which bleaches the monomeric pigments and leaves only polymeric pigment. The assay, however, has been shown to overestimate the amount of polymeric pigments. If corrected, though, the method shows a good correlation with HPLC analyses in determining the concentration of unbleached pigmented polymers (Versari 2008).

One drawback of the pH Differential assay is that it does not account for copigmentation, which will not contribute color when the solution is diluted (Levengood and Boulton 2004). Furthermore, the assay requires a large pH adjustment, which can be time consuming (Boulton 2001; Harbertson and Spayd 2006).

Boulton's copigmentation assay expands on the Somers' assay to include copigmentation effects (Boulton 2001; Levengood and Boulton 2004). The assay compares diluted and undiluted samples to determine the color due to copigmentation in the solution, as the decrease in anthocyanin concentration disrupts the complexes and shifts the equilibrium. To begin, the solution is adjusted to pH 3.6. The concentration is calculated according to the following absorptions (Levengood and Boulton 2004; Harbertson and Spayd 2006):

Color due to
Copigmentation: A_{520nm}(Acetaldehyde solution)-A_{520nm}(diluted 1/20) x 20 Eq. 8

When the solution is diluted, some of the anthocyanins that were previously colorless and bound to bisulfites become unbound. To account for this, some acetaldehyde is added to the undiluted solution, which reacts with bisulfites. This frees some of the previous bound anthocyanins to compensate for a similar freeing of bound anthocyanins in the diluted sample (Harbertson and Spayd 2006). The acetaldehyde addition is only needed for wine samples, as there are not high bisulfite levels in the juice.

Bisulfite addition can also be used to determine the color due to monomeric and polymeric anthocyanins, for similar reasons previously discussed in the Somers' Assay (Harbertson and Spayd 2006; Levengood and Boulton 2004). In 2008, Versari et al. found the method in good correlation with HPLC methods (Versari 2008). One

drawback of this assay is that it does not measure the total concentration of anthocyanins in the solution (Harbertson and Spayd 2006).

HPLC

In 2008, Lee, Rannaker, and Wrolstad used HPLC to determine the total monomeric anthocyanin concentration of various juices. Reversed phase HPLC with photodiode array detection was used to identify and calculate total monomeric anthocyanin concentration. In HPLC, anthocyanins are eluded at different rates due to their various polarities. The values are then quantified using an external standard. This, however, can pose a problem, as solutions with a mixture of anthocyanins can often cause an underestimate of the monomeric anthocyanin concentration when only one external standard is used. Lee, Rannaker, and Wrolstad used both malvidin-glucoside and cyanidin-glucoside as an external standard. They found that malvidin-glucoside produce consistently higher results by 5.2%. Overall, they found that HPLC is accurate and correlated with the pH differential method of calculating monomeric anthocyanin concentration. Unlike pH differential method, however, HPLC can also identify and determine individual anthocyanin concentrations (Lee et al. 2008).

Versari, Boulton, and Parpinello also used HPLC, but with silica-based reversed-phase columns, polymeric-based reversed-phase columns, and an addition of SO_2 to the mobile phase to determine the concentrations of monomeric and polymeric pigments in wine. They found that the polymeric-based reverse-phase columns provided anthocyanin concentrations that were in good agreement with the silicone-based reverse phase columns. When using reverse-phase columns, however, it can often be hard to distinguish between polymeric pigments because they cause a broad peak in the spectrum (Versari 2008).

HPLC can also be coupled with other analysis methods. In 2003, Wang et al. determined the anthocyanins in Concord grape juice using HPLC with a diode array

spectrophotometer and ion trap mass spectrometer. Wang et al. found that in reversed column chromatography the anthocyanins elude in the following order: delphinidin, cyanidin, petunidin, pelargonidin, peoidin, and malvidin (Wang and others 2003).

Recently, Bonerz et al. developed a method of detecting anthocyanins and phenolics in wine. The protocol is simple and efficient, where wines only need to be filtered through a 0.45 µm filter before being injected onto a C_{18} reverse phase column. Anthocyanins are detected at 520nm and are easily quantified using external standards (Bonerz and others 2008).

Other Methods

In addition to those listed, there are various other methods of analyzing anthocyanins, including other forms of chromatography, mass spectroscopy, NMR, capillary electrophoresis (CE), solid phase extraction, or membrane ultrafiltration (Castaneda-Ovando et al. 2009; Lee et al. 2008; Lee et al. 2005; Kalbasi and Cisneros-Zevallos 2007).

Grape Juice Processing

According to the Food and Agricultural Organization of the United Nations, the United States is the largest consumer of grape juice, indicating that it is an integral component of our society. Furthermore, the quantity of grapes harvested for the production of juice exceeds the volume of any other fruit worldwide. Overall, grape juice consists of few ingredients and its distinctive flavor profile results from the whole grape (excluding the oils and crude fiber), and include sugars, acids, methyl anthranilate, volatile esters, alcohols, and aldehydes. These compounds along with the color of the juice determine the overall quality (Bates et al. 2001).

Grape juice processing is constantly evolving and changing with new technology. In the Eastern United States, grapes typically are harvested, destemmed,

crushed, and then hot pressed (Morris 2005). During a hot press, the crushed grapes are heated to 60˚C in a steam-jacketed vacuum preheater or heat exchanger and then passed into holding tanks. Within these tanks, the grapes are mixed with pectolytic enzyme and purified paper pulp, a pressing aid, for 30-60 minutes. The enzyme serves to break down the pectin in the juice and extract color from the skins. A similar method to hot press is hot break, which is often implemented on the West Coast of the United States. During a hot break, the grapes are heated to 82˚C, instead of 60˚C. Once the temperature is reached, the juice is then cooled to 60˚C and the pectinase and paper press aid are added. Typically, both of these methods result in juices with higher total solids and color extraction than cold press methods. In cold press, the grapes are not heated at all. This results in juice with less color and astringency. It is also more susceptible to browning because polyphenol oxidase is not deactivated by the initial heat treatment (Morris 2005; Bates et al. 2001).

After the grapes are heated by one of the following methods, they are either put through a screw press or decanter. The screw press acts as a normal press, yielding a juice mixture and leaving behind solids. The decanter acts like a centrifuge and the supernatant juice is collected. The juice from each method is then pasteurized at about 185˚C for 1 minute. It leaves the pasteurizer cold, at room temperature, and is stored under refrigeration to let the tartrates precipitate out. After detartration, the juice is filtered and pasteurized again with a hot fill into bottles, creating a vacuum seal (Morris 2005). If the juice is to be concentrated, it is put through an evaporator after detartration and then stored until subsequently reconstituted and hot filled (Bates et al. 2001).

Overall, processing can greatly impact anthocyanin concentration and the color of the juice. Polyphenol oxidase (PPO) indirectly causes the degradation of anthocyanins, causing browning. The enzyme's activity is influenced by temperature,

hence, extraction temperatures may greatly affect the color of the juice. If the grapes are heated before the pectolytic enzyme is added, however, PPO will be inactivated, preventing anthocyanin degradation (Bates et al. 2001). In a 1982 study, Montgomery et al. found that when crushed grapes were heated to 88˚C and 99˚C the enzyme was deactivated (Montgomery and others 1982). (Yokotsuka and Singleton 1997). A 2010 publication by Iyer et al. indicated the heating juices to 60˚C (hot press) vs. 82˚C (hot break) produced no significant differences in the overall color of the final juice, indicating the enzyme is also activated at these lower temperatures (Iyer 2010).

During detartration, there is about a 20-40% color loss due to the precipitation of various anthocyanin pigments. Specifically, Ingalsbe, Neubert, and Carter isolated 14 different anthocyanin species from this precipitate, including anthocyanin-metal complexes and acylated anthocyanins. The *p*-coumaric acid esters of delphinidin 3-monoglucoside and cyanidin 3-monoglucosides, in particular, were identified. Furthermore, it was shown that these compounds, and the metal complexes, produced a blue solution when in an aqueous media in Concord grape juice's characteristic pH range. Consequently, these pigments are expected to contribute to the blue color of Concord grape juice (Ingalsbe et al. 1963). Copigmentation complexes, which enhance the color of juice, are also affected by temperature. When juice is cooled these copigmented forms may precipitate out, affecting the color of the juice (Boulton 2001).

Storage and Anthocyanin Content

During storage, oxidation of the juice may occur, producing brown pigments. Large tanks with low temperature, especially, can lead to this color degradation within the juice (Sistrunk and Gascoigne 1983). The rate of copigmentation in the juice, however, may affect this phenomenon. Free anthocyanins and polyphenols are

involved in oxidation reactions. If these compounds are copigmented together, there will be less available to be oxidized. Therefore, it is believed that the more copigmentation within the juice, the slower the rate of oxidation (Boulton 2001).

The temperature of storage can also influence the browning of wine. Morris observed that higher storage temperatures, a comparison of 40˚C, 30˚C, 20˚C, resulted in browner wines and an increased absorbance at 430 nm. There was also a decrease in the total amount of anthocyanins, with larger decreases correlated to higher storage temperatures. This was believed to be a result of the formation of more polymeric pigments and also more overall degradation of anthocyanins (Sims and Morris 1984). Atanasova et al. confirmed this increase in polymeric compounds, as he saw oxidation increased pyranoanthocyanins and ethyl-bridged compounds in wine (Atanasova and others 2002). In a 2004 publication, Tsai, Huang, and Haung found similar results, as monomeric anthocyanins in mulberry wine decreased with storage, while copigmented and polymeric complexes increased. Overall, they detected a color change from red to brown as the wine was stored. (Tsai and others 2004).

In contrast to the study on mulberry wine, wine from grapes has been shown to decrease in copigmented anthocyanins and increase in pigmented polymers as the wine aged. This trend is more common than observing increases in both copigmented and polymeric pigments. The changes in these compounds produced a wine of orange-red color (Boido and others 2006).

Compounds such as antioxidants, chelating agents, and acid neutralizers may help stabilize color pigments in stored juices. In a 1983 study, Sistrunk and Gascoigne studied the effect of additives on color retention on stored Concord juice. Anthocyanin concentration, browning index, a/L ration, and L, a, b values proved to be the best methods of defining color changes in the juice. They found that color changed quickly after the 3 month mark and that by 18 months the juice was decidedly brown.

Exceptions to the initial rapid change were juices treated with $CaSO_4$ and $SnCl_2$, which stabilized the color through the formation of metal complexes and changes in pH. Ascorbic acid, however, had an adverse effect and accelerated the change in color to red, opposed to purple, of the samples (Sistrunk and Gascoigne 1983).

Although many factors affect the stability of anthocyanins during storage, Sistrunk and Cash found that the length of time had the most significant impact on anthocyanin degradation when compared to ascorbic acid, PPO, Cu^{+}, and Fe^{2+} additions, which have all been shown to decrease anthocyanin concentration (Sistrunk and Cash 1974).

Current Color Profile Studies on Concord Grape Juice

Most studies simply identify the most prominent anthocyanins in juice and not the relative distribution of polymeric, monomeric, and copigmented anthocyanins. Moreover, the literature mainly focuses the color profile of wines, opposed to juice.

Some literature, however, does discuss the different components of Concord grape juice. Mullen, Marks, and Crozier studied the various phenolic species in Concord grape juice, which consisted of mainly flavan-3-ols, anthocyanins, and hydroxycinnamates (Mullen et al. 2007). Additionally, Hong and Wrolstad used HPLC/Photodiode array detection to determine the color profile of grape extract from Concord grapes. They found the most prevalent anthocyanin to be delphinidin-3-glucoside followed by cyanidin-3-glucoside (Hong and Wrolstad 1990b). This data correlates to that found by Munoz-Espada et al. in their 2004 study (Munoz-Espada et al. 2004). Using pH differential method, Hong et al. also found that most of the color was attributed to acylated monomeric pigments while a small percentage consisted of

polymeric pigments. This was determined using bisulfate addition and attributing the non-bleachable color to polymeric pigments (Hong and Wrolstad 1990a).

Concerning wine, it has been shown that the color components significantly differ based on numerous factors, such as cultivar. Concerning polymeric pigments, especially, levels are influenced by vintage, the grape, and fermentation/storage conditions (Versari and others 2007).

In 2006, Biodo et al. observed changes in the types of anthocyanin concentration of Tannat wines as the wine aged, and noted the color changes using CIELAB parameters. HPLC-DAD-MS and UV-vis spectroscopy were used to analyze the changes in anthocyanin concentration. The forms of anthocyanins studied were divided into four categories: anthocyanins, pyranoanthocyanins, direct and acetaldehyde-mediated flavanol-anthocyanin condensation products. The specific pyranoanthocyanins included were A and B type vitisins, 4-vinylphenol adducts, 4-vinylcatechol adducts, and vinylflavanol adducts. The role of copigmented complexes was not mentioned in the study. Results indicated that during aging the amount of color attributed to anthocyanin and flavanol-anthocyanin condensation products decreased, while that caused from pyranoanthocyains and direct condensation products increased. The individual concentrations, however, varied, as the type A and B vitisins, and direct condensation products decreased as the wine aged but their percentage of the overall color pigments increased. Color, in general, changed from a purple to red-orange. Despite this hue change, however, chroma and lightness remained the same (Boido et al. 2006). A 2006 study by Alcalde-Eon et al. partially confirmed this trend but also disputed various aspects. They found that while pyranoanthocyanins, characteristic of orange hues, increased both direct and acetaldehyde mediated flavanol-anthocyanin condensation products, causing blue hues, decreased. The publication also consisted of a comprehensive analysis of the

changes during wine aging, analyzing 129 different pigments involved in the process (Alcalde-Eon and others 2006).

REFERENCES

Alcalde-Eon C, Escribano-Bailon MT, Santos-Buelga C & Rivas-Gonzalo JnC. 2006. Changes in the detailed pigment composition of red wine during maturity and ageing: A comprehensive study. Papers presented at the 4th SYMPOSIUM IN VINO ANALYTICA SCIENTIA - In Vino 2005 563(1-2):238-254.

Asen S, Stewart RN & Norris KH. 1972. Co-pigmentation of anthocyanins in plant tissues and its effect on color. Phytochemistry 11(3):1139-1144.

Atanasova V, Fulcrand H, Cheynier V & Moutounet M. 2002. Effect of oxygenation on polyphenol changes occurring in the course of wine-making. Analytica Chimica Acta 458(1):15-27.

Bates RP, Morris JR & Crandall PG. 2001. Chapter 12: Grape Juice. In: Anonymous, editor). Principles and practices of small - and medium - scale fruit juice processing. FAO Agricultural Services Bulletin 146 , Food Science and Human Nutrition Department, University of Florida. p. 135.

Berke B,Cheze C, Vercauteren J, Deffieux G. 1998. Bisulfite addition to anthocyanins: revisited structures of colourless adducts. Tetrahedron letters 39(32):5771-5774.

Boido E, Alcalde-Eon C, Carrau F, Dellacassa E & Rivas-Gonzalo J. 2006. Aging Effect on the Pigment Composition and Color of Vitis vinifera L. Cv. Tannat Wines. Contribution of the Main Pigment Families to Wine Color. Journal of Agricultural and Food Chemistry 54(18):6692-6704.

Bonerz DPM, Nikfardjam MSP & Creasy GL. 2008. A New RP-HPLC Method for Analysis of Polyphenols, Anthocyanins, and Indole-3-Acetic Acid in Wine. Am. J. Enol. Vitic. 59(1):106-109.

Boulton R. 2001. The Copigmentation of Anthocyanins and Its Role in the Color of Red Wine: A Critical Review. Am. J. Enol. Vitic. 52(2):67-87.

Brenes CH, Del Pozo-Insfran D & Talcott ST. 2005. Stability of Copigmented Anthocyanins and Ascorbic Acid in a Grape Juice Model System. Journal of Agricultural and Food Chemistry 53(1):49-56.

Brouillard R & Markakis P. 1982. Chemical Structure of Anthocyanins. In: Anonymous, editor). Anthocyanins as Food Colors. New York: Academic Press. p. 1-38.

Calvi JP & Francis FJ. 1978. Stability of Concord Grape (V Labrusca) Anthocyanins in Model Systems. Journal of Food Science 43(5):1448-1456.

Castaneda-Ovando A, de Lourdes Pacheco-Hernandez M, Elena Paez-Hernandez M, Rodriguez JA & Andres Galan-Vidal C. 2009. Chemical studies of anthocyanins: A review. Food Chemistry 113(4):859-871.

Cheynier V, Duenas-Paton M, Salas E, Maury C, Souquet J-M, Sarni-Manchado P & Fulcrand H. 2006. Structure and properties of wine pigments and tannins. American Journal of Enology and Viticulture 57(3):298-305.

Coutinho MR, Quadri MB, Moreira RFPM & Quadri MGN. 2004. Partial purification of anthocyanins from Brassica oleracea (red cabbage). Separation Science and Technology 39(16):3769-3782.

Davies AJ & Mazza G. 1993. Copigmentation of simple and acylated anthocyanins with colorless phenolic compounds. Journal of Agricultural and Food Chemistry 41(5):716-720.

Day AP, Kemp HJ, Bolton C, Hartog M & Stansbie D. 1997. Effect of concentrated red grape juice consumption on serum antioxidant capacity and low-density lipoprotein oxidation. Annals of Nutrition and Metabolism 41(6):353-357.

Del Pozo-Insfran D, Balaban MO & Talcott ST. 2006. Enhancing the Retention of Phytochemicals and Organoleptic Attributes in Muscadine Grape Juice through a Combined Approach between Dense Phase CO2 Processing and Copigmentation. Journal of Agricultural and Food Chemistry 54(18):6705-6712.

Frankel EN, Bosanek CA, Meyer AS, Silliman K & Kirk LL. 1998. Commercial Grape Juices Inhibit the in Vitro Oxidation of Human Low-Density Lipoproteins. Journal of Agricultural and Food Chemistry 46(3):834-838.

Giusti MM & Wrolstad RE. 2003. Acylated anthocyanins from edible sources and their applications in food systems. Advance in Plant Anthocyanin Research and Development 14(3):217-225.

Giusti MM, Wrolstad RE. 2001. Characterization and measurement of anthocyanins by UV-Visible spectroscopy. In: Wrolstad, R. E., editor). Current protocols in food analytical chemistry. New York: John Wiley & Sons, Inc. p. F1.2.1, F1.2.13.

Goto T, Hoshino T & Ohba M. 1976. Studies on anthocyanin complexes .1. stabilization effect of neutral salts on anthocyanins - flavylium salts,

anhydrobases and genuine anthocyanins. Agricultural and Biological Chemistry 40(8):1593-1596.

Halliwell B. 1992. Reactive Oxygen Species and the Central Nervous System. Journal of Neurochemistry 59(5):1609-1623.

Harbertson JF, Kennedy JA & Adams DO. 2002. Tannin in Skins and Seeds of Cabernet Sauvignon, Syrah, and Pinot noir Berries during Ripening. American Journal of Enology and Viticulture 53(1):54-59.

Harbertson JF & Spayd S. 2006. Measuring Phenolics in the Winery. American Journal of Enology and Viticulture 57(3):280-288.

Hong V & Wrolstad RE. 1990a. Characterization of anthocyanin-containing colorants and fruit juices by HPLC photodiode array detection. Journal of Agricultural and Food Chemistry 38(3):698-708.

Hong V & Wrolstad RE. 1990b. Use of HPLC separation/photodiode array detection for characterization of anthocyanins. Journal of Agricultural and Food Chemistry 38(3):708-715.

Hoshino T, Matsumoto U & Goto T. 1981a. Self-association of some anthocyanins in neutral aqueous solution. Phytochemistry 20(8):1971-1976.

Hoshino T, Matsumoto U, Goto T & Harada N. 1982. Evidence for the self-association of anthocyanins IV. PMR spectroscopic evidence for the vertical stacking of anthocyanin molecules. Tetrahedron letters 23(4):433-436.

Hoshino T, Matsumoto U, Harada N & Goto T. 1981b. Chiral exciton coupled stacking of anthocyanins: interpretation of the origin of anomalous CD induced by anthocyanin association. Tetrahedron letters 22(37):3621-3624.

Hrazdina G, Parsons GF & Mattick LR. 1984. Physiological and Biochemical Events During Development and Maturation of Grape Berries. American Journal of Enology and Viticulture 35(4):220-227.

Iacobucci GA & Sweeny JG. 1983. The chemistry of anthocyanins, anthocyanidins and related flavylium salts. Tetrahedron 39(19):3005-3038.

Ingalsbe DW, Neubert AM & Carter GH. 1963. Concord Grape Pigments. Journal of Agricultural and Food Chemistry 11(3):263-&.

Iyer M, Sacks, GL; Padilla-Zakour, OI. 2010. Impact of harvesting and processing conditions on green leaf volatile development and phenolics in Concord grape juice. Journal of Food Science 75(3):C297-C304.

Joseph JA, Shukitt-Hale B, Denisova NA, Bielinski D, Martin A, McEwen JJ & Bickford PC. 1999. Reversals of Age-Related Declines in Neuronal Signal Transduction, Cognitive, and Motor Behavioral Deficits with Blueberry, Spinach, or Strawberry Dietary Supplementation. Journal of Neuroscience 19(18):8114-8121.

Jurd L & Asen S. 1966. The formation of metal and â€œco-pigmentâ€ complexes of cyanidin 3-glucoside. Phytochemistry 5(6):1263-1271.

Kalbasi A & Cisneros-Zevallos L. 2007. Fractionation of Monomeric and Polymeric Anthocyanins from Concord Grape (Vitis labrusca L.) Juice by Membrane Ultrafiltration. Journal of Agricultural and Food Chemistry 55(17):7036-7042.

Kaur C & Kapoor HC. 2001. Antioxidants in fruits and vegetables - the millennium's health. International Journal of Food Science and Technology 36(7):703-725.

Lee J, Durst RW & Wrolstad RE. 2005. Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: Collaborative study. Journal of AOAC International 88(5):1269-1278.

Lee J, Rennacker C & Wrolstad RE. 2008. Correlation of two anthocyanin quantification methods: HPLC and spectrophotometric methods. Food Chemistry 110:782-786.

Levengood J. 1996. A survey of copigmentation in Cabernet Sauvignon wines. University of California, Davis.

Levengood J & Boulton R. 2004. The variation in the color due to copigmentation in young Cabernet Sauvignon wines. In: Waterhouse, A. J. & Kennedey, J. A., editors. Red Wine Color: Revealing the Mysteries. American Chemical Society. p. 35-52.

Liu RH. 2004. Potential Synergy of Phytochemicals in Cancer Prevention: Mechanism of Action1. Journal of Nutrition 134(12S):3479S-3485S.

Malien-Aubert C, Dangles O & Amiot MJ. 2001. Color Stability of Commercial Anthocyanin-Based Extracts in Relation to the Phenolic Composition. Protective Effects by Intra- and Intermolecular Copigmentation. Journal of Agricultural and Food Chemistry 49(1):170-176.

Mazza G & Brouillard R. 1987. Color stability and structural transformations of cyanidin 3,5-diglucoside and four 3-deoxyanthocyanins in aqueous solutions. Journal of Agricultural and Food Chemistry 35(3):422-426.

Mazza G & Miniati E. 1993. Anthocyanins in Fruits, Vegetables, and Grains. Boca Raton: CRC Press.

Monagas M, Bartolomé B, Moreno-Arribas MV & Polo MC. 2009. Anthocyanins and Anthocyanin-Derived Compounds. In: Anonymous, editor). Wine Chemistry and Biochemistry. Springer. p. 439.

Montgomery MW, Reyes FGR, Cornwell C & Beavers DV. 1982. Sugars and Acid Analysis and Effect of Heating on Color Stability of Northwest Concord Grape Juice. Journal of Food Science 47(6):1883-1891.

Morris JR, Striegler KR. 2005. Grape Juice: Factors That Influence Quality, Processing Technology, and Economics In: Processing fruits: science and technology 2nd ed. Barrett, D. S., L; Ramaswamy, H, editors. Boca Raton, Fl: CRC press.

Mullen W, Marks SC & Crozier A. 2007. Evaluation of Phenolic Compounds in Commercial Fruit Juices and Fruit Drinks. Journal of Agricultural and Food Chemistry 55(8):3148-3157.

Munoz-Espada AC, Wood KV, Bordelon B & Watkins BA. 2004. Anthocyanin quantification and radical scavenging capacity of Concord, Norton, and Marechal Foch grapes and wines. Journal of Agricultural and Food Chemistry 52(22):6779-6786.

Pauling L. 1939. Recent work on the configuration and electronic structure of molecules. Fortschritte der Chemie Organischer Naturstoffe 3:203-235.

Rentzsch M, Schwarz M & Winterhalter P. 2007. Pyranoanthocyanins â€“ an overview on structures, occurrence, and pathways of formation. 4th International Congress on Pigments in Food 18(10):526-534.

Scheffeldt P & Hrazdina G. 1978. Co-pigmentation of anthocyanins under physiological conditions. Journal of Food Science 43(2):517-520.

Sims CA & Morris JR. 1984. Variables affecting color and stability of Muscadine Wine. Arkansas Farm Research 33(4):10.

Sistrunk WA & Cash JN. 1974. Processing Factors Affecting Quality and Storage Stability of Concord Grape Juice. Journal of Food Science 39(6):1120-1123.

Sistrunk WA & Gascoigne HL. 1983. Stability of Color in Concord Grape Juice and Expression of Color. Journal of Food Science 48(2):430-&.

Somers TC. 1971. The polymeric nature of wine pigments. Phytochemistry 10(9):2175-2186.

Somers TC & Evans ME. 1974. Wine quality - correlations with color density and anthocyanin equilibria in a group of young red wines. Journal of the science of food and agriculture 25(11):1369-1379.

Somers TC & Evans ME. 1977. Spectral evaluation of young red wines - anthocyanin equilibria, total phenolics, free and molecular so2, chemical age. Journal of the science of food and agriculture 28(3):279-287.

Steinmetz KA & Potter JD. 1996. Vegetables, Fruit, and Cancer Prevention: A Review. Journal of the American Dietetic Association 96(10):1027-1039.

Stintzing FC, Stintzing AS, Carle R, Frei B & Wrolstad RE. 2002. Color and Antioxidant Properties of Cyanidin-Based Anthocyanin Pigments. Journal of Agricultural and Food Chemistry 50(21):6172-6181.

Talcott ST, Brenes CH, Pires DM & Del Pozo-Insfran D. 2003. Phytochemical Stability and Color Retention of Copigmented and Processed Muscadine Grape Juice. Journal of Agricultural and Food Chemistry 51(4):957-963.

Talcott ST, Peele JE & Brenes CH. 2005. Red clover isoflavonoids as anthocyanin color enhancing agents in muscadine wine and juice. Food Research International 38(10):1205-1212.

Terrier N, Poncet-Legrand C, Cheynier V, Moreno-Arribas MV & Polo MC. 2009. Flavanols, Flavonols and Dihydroflavonols. In: Anonymous, editor). Wine Chemistry and Biochemistry. New York: Springer. p. 463-507.

Tsai P, Huang H & Huang T. 2004. Relationship between anthocyanin patterns and antioxidant capacity in mulberry wine during storage. Journal of Food Quality 27:497-505.

Versari A, Parpinello GP & Mattioli AU. 2007. Characterisation of Colour Components and Polymeric Pigments of Commercial Red Wines by Using UV-vis Spectrophotometric Methods. S. Afr. J. Enol. VItic. 28(1):6-10.

Versari A, Boulton RB, Parpinello GP 2008. A comparison of analytical methods for measuring the color components of red wines. Food Chemistry 106(1):397-402.

Wang H, Cao G & Prior RL. 1996. Total Antioxidant Capacity of Fruits. Journal of Agricultural and Food Chemistry 44(3):701-705.

Wang H, Race EJ & Shrikhande AJ. 2003. Characterization of Anthocyanins in Grape Juices by Ion Trap Liquid Chromatographyâˆ’Mass Spectrometry. Journal of Agricultural and Food Chemistry 51(7):1839-1844.

Wrolstad RE, Durst RW & Lee J. 2005. Tracking color and pigment changes in anthocyanin products. Trends in Food Science & Technology 16(9):423-428.

Yokotsuka K & Singleton VL. 1997. Disapperance of Anthocyanins as Grape Juice Is Prepared and Oxidized with PPO and PPO Substrates. Am. J. Enol. Vitic. 48(1):13-25.

CHAPTER 2

EFFECTS OF CONCENTRATION PRIOR TO COLD-STABILIZATION ON THE COLOR OF CONCORD GRAPE JUICE

Abstract

The color of Concord grape juice produced by concentration before cold-stabilization and detartration (direct-to-concentrate, DTC) was compared to juice produced via cold stabilization prior to concentration (standard concentrate, SC). Using the Boulton Copigmentation Assay, the majority of color in bottled SC juice (72%) was due to monomeric anthocyanins. Following reconstitution, DTC juice had a 63% greater absorbance at 520 nm than SC juice. A significant loss of anthocyanins was observed using a paired t-test during cold-stabilization of single-strength juice during SC processing (mean loss: 79 mg/L as cyanidin-3-glucoside, 23% of total anthocyanins), while no significant loss of anthocyanins or color was observed during cold stabilization of DTC concentrate. The concentration of anthocyanins in the SC bitartrate crystals was 0.80% w/w compared to 0.13% w/w in the DTC bitartrate crystals. Between DTC and SC, no difference in copigmentation was observed in cold-stabilized concentrate or reconstituted juice, indicating that the increased color stability could not be credited to greater copigmentation in DTC during detartration. HPLC analyses indicated that anthocyanin species with higher pK_h and thus proportionally greater flavylium ion concentration at juice pH are preferentially lost during SC processing. The proportional color loss during shelf-life stability testing (0-16 weeks, 2-30°C) was not significantly different between SC and DTC juices.

Introduction

In the US, the primary cultivar used for purple grape juice is Concord (*Vitis labruscana*). Concord juice is typically produced by the hot press method in the Eastern United States and the hot break method in Washington State (Morris 2005). In the hot press method, grapes are heated to 60°C before enzyme addition. In hot break, they are initially heated to temperatures >75°C, cooled to 60°C, and then undergo depectinization (Morris 2005).

Grapes are uniquely high in tartaric acid, and fresh grape juice will precipitate potassium bitartrate crystals during cold storage. To prevent this bitartrate instability from occurring in bottled juice, a cold-stabilization is usually performed on single-strength juice, which can cause a loss of anthocyanin pigments (Morris 2005). In Concord grape juice, a loss of 20-40% of the initial color was reported to occur following detartration (Ingalsbe et al. 1963). Losses have also been observed during cold-stabilization in wine production, and bitartrate crystals from Carignan wines are reported to contain 0.2-0.3 % w/w anthocyanins on a dry weight basis (Vernhet and others 1999). Since bitartrate crystals from grape juice are typically smaller and less pure than those from wine, comparable or greater amounts of anthocyanin loss would be expected during cold-stabilization of grape juice (McLellan 1990).

The mechanism for the loss of anthocyanins during detartration is not well understood. During cold storage, anthocyanins adhere to the surface of a growing bitartrate crystal and are lost from solution. Occlusion of anthocyanins within the crystal lattice does not appear to occur (Correa-Gorospe and others 1991; Balakian and Berg 1968). The attractive forces responsible for this adsorbance are variously proposed to be ionic, hydrogen-bonding, or charge-transfer in nature (Rodriguez-Clemente and Correa-Gorospe 1988; Celotti and others 1999).

The pigments in grape juice may exist in several forms, which for simplicity have been categorized by previous authors as one of three pigment classes: monomeric anthocyanins, polymeric pigments, and copigmented complexes (Boulton 2001). The stability of each of these classes during cold-stabilization is unknown. Monomeric or "free" anthocyanins are anthocyanidin glucosides. The molar absorptivity of monomeric anthocyanins is highly pH dependent, resulting in a range of colors from red to colorless with increasing pH, and are readily bleachable by bisulfite (Mazza and Miniati 1993). Polymeric pigments represent the fraction of color that is not bleached by bisulfite, and are formed via covalent reactions of anthocyanins with other juice components (Monagas and Bartolomé 2009). Copigmented complexes in juices are formed through non-covalent interactions of anthocyanins with other compounds, such as flavonols (Boulton 2001), or other anthocyanins ("self association") (Scheffeldt and Hrazdina 1978). Such complexes play a prominent role in the color of young wines (Boulton 2001) and result in a hyperchromic shift (Asen et al. 1972).

Traditional juice processing methods (standard concentration, SC) involve a concentration step following cold stabilization (Bates et al. 2001). Alternatively, the order of these two steps can be switched such that concentration precedes cold storage ("direct-to-concentrate", DTC), and detartration is performed on the concentrate. Anecdotally, DTC production has been reported to improve color as compared to SC practices, but the impact of this practice has not been characterized in the literature. Assuming anecdotal accounts were correct, we hypothesized that monitoring changes in the contributions of monomeric anthocyanins, polymeric pigments, and copigmented complexes to Concord juice color throughout processing and storage could provide insight into the mechanism behind color differences of DTC and SC. In

this study, we analyzed changes of these color components produced via hot press DTC in comparison to hot press and hot break juice processed by SC methods.

Materials and Methods

Grapes

Concord grapes were hand harvested from a nearby vineyard (Penn Yan, N.Y., U.S.A.) and received at the New York State Agricultural Experiment Station (Geneva, N.Y., U.S.A.) in the fall of 2009. The grapes were grown using the standard cultivar practices of the region. Prior to processing, grapes were stored at 2˚C, for no more than 7 days. Grapes varied in maturity from 14-16° Brix, measured using a Leica Auto Abbe refractometer (Buffalo, N.Y. U.S.A.).

Samples

Samples for the juice were collected at six time points throughout processing, outlined in Table 3. Bitartrates were collected after cold storage, time point 3 for hot break standard concentration (BSC) and hot press standard concentration (PSC) and time point 5 for direct-to-concentrate methods (DTC).

Table 3: Sample points throughout processing of standard concentrate hot press and hot break (PSC, BSC) and direct to concentrate (DTC).

Sample Point	PSC	BSC	DTC
1	Juice after heat treatment	Juice after heat treatment	Juice after heat treatment
2	Juice before cold storage	Juice before cold storage	Not applicable
3	Juice before concentration	Juice before concentration	Juice before concentration
4	Concentrate before storage	Concentrate before storage	Concentrate before storage
5	Concentrate after storage	Concentrate after storage	Concentrate after storage
6	Reconstituted juice	Reconstituted juice	Reconstituted juice

Standard Concentrate Hot Break and Hot Press Processing

PSC and BSC processing was performed on the grapes in 230 kg batches, according to industry standards (Morris 2005). A schematic summarizing the processing steps is shown in Figure 5. Two replicates of standard concentrate processing with both hot break and hot press treatments were performed. On October 15th 2009, the first replicates of hot break standard concentrate and hot press standard concentrate were conducted. The second replicates of each were performed a week later on October 22nd 2009.

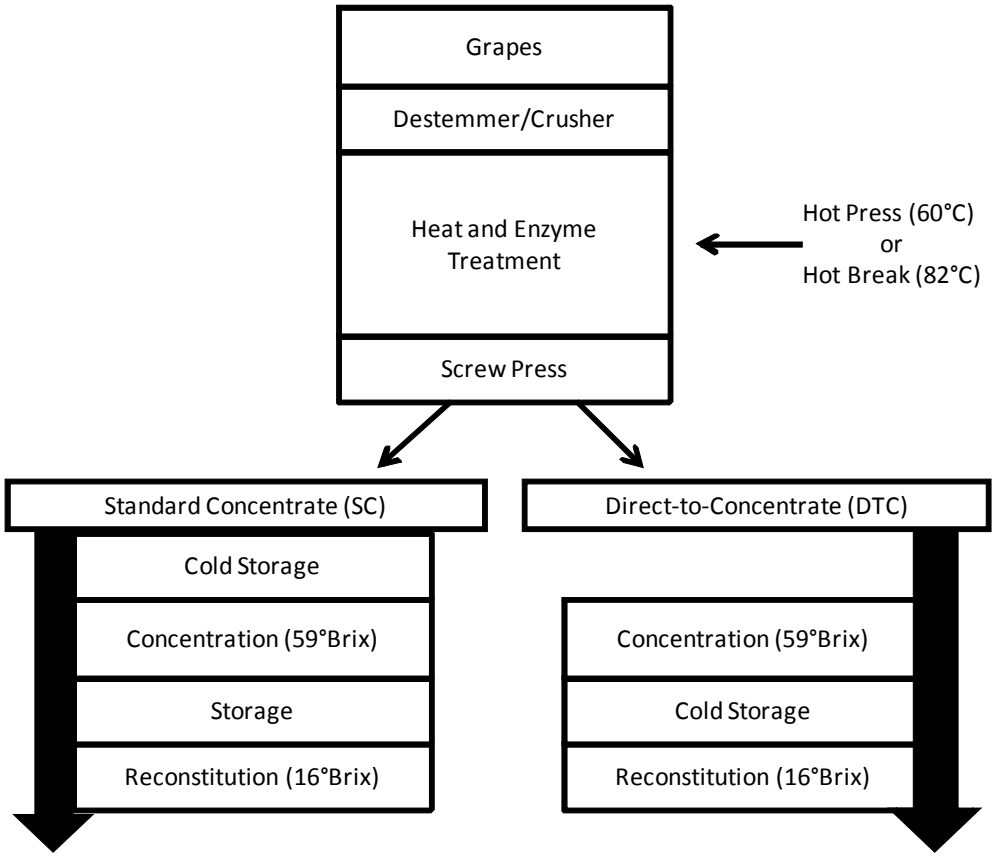


Figure 5: Methods of grape juice production with variations in heat treatments (hot press or hot break) and concentrate processing (standard concentration or direct-to-concentrate).

Both hot break and hot press standard processes began with destemming and crushing grapes in a Mori (Florence, Italy) Eno 20 destemmer-cusher. The hot break grapes were then heated to 82˚C in a steam-jacketed kettle and subsequently cooled to 60˚C. Adex G® depectinizing enzyme (DSM, Parsippany, NJ) was then added at 0.03 ml per kg of grapes along with Pressanier-J® paper as a press-aid at 7.5 g per kg of grapes (supplied by Welch Foods Inc., Westfield, N.Y, U.S.A) during agitation. The must was then held at 60°C for 30 minutes. The hot press standard concentrate

processing followed the same protocol but was initially heated to 60˚C, not 82˚C. Depectinizing enzyme and paper press aid were added when 60˚C was reached.

After the 60°C hold, both hot break and hot press standard concentrate juices were pressed in a Buffalo Hammer Mill press (Buffalo, N.Y., U.S.A.), then pasteurized at 85˚C for 1 minute in a MicroThermics® (Raleigh, N.C. U.S.A.) tubular pasteurizer. A clarifying enzyme, K201 (DSM, Parsippany, NJ), was then added at 150 mg/L and the juices were stored at 2˚C for 2 weeks.

Following cold storage, the juice was siphoned off of the bitartrates and the turbidity was measured on a HACH 2100P turbidimeter (Loveland, CO, U.S.A) to ensure that the juice was under 100 NTU. Juices were concentrated with a Unipektin AG® falling film two-effect evaporator at 50-55°C and -0.9 atm (Zürich, Switzerland) to 59° Brix. Following concentration, juice was stored at 2˚C for two weeks. After storage, the hot break and hot press standard concentrates were reconstituted with water to 16° Brix then hot filled (MicroThermics® tubular pasteurizer, Raleigh, N.C. U.S.A.) at 85˚C with a 1 minute hold prior to filling and 1 minute hold in the bottle before cooling. Juice was packed into 240 ml Ball® PET bottles (Broomfield, C.O, U.S.A.) for use in shelf life studies.

Direct-To-Concentrate (DTC) Processing

DTC processing is summarized in the schematic shown in Figure 5. Two replicates were performed on October 19th and 26th 2009 at the New York State Experiment Station (Geneva, N.Y., U.S.A.) with grapes sourced from Grindley Vineyard (Penn Yan, N.Y., U.S.A.). Grapes were processed in approximately 230 kg batches. DTC processing was similar to PSC processing, with the only variation in processing occurring following pressing. A second pectinase enzyme treatment, K201 (DSM, Parsippany, NJ) was then added at 300 mg/L to 57˚C juice. The 2nd enzyme

treatment required 1 hour until a negative pectin level by alcohol test was observed. The juice was then put through a plate and frame filter with Celite 503 Diatomaceous earth (DE) and concentrated with a Unipektin AG® falling film 2 effect evaporator (Zürich, Switzerland) to 59˚ Brix. The concentrate was then heated to 85˚C in a steam jacketed kettle and subsequently stored at 2˚C for two weeks.

After cold-storage and detartration, DTC concentrate was reconstituted to 16˚ Brix with water, and hot filled (MicroThermics® tubular pasteurizer, Raleigh, N.C. U.S.A.) at 85˚C with a 1 minute hold in the machine and 1 minute hold in the bottle before cooling. Juice was packed into Ball® PET bottles (Broomfield, C.O, U.S.A.), which were then used for shelf life studies.

Samples were taken throughout processing, reference Table 3.

Color Analysis

The total color intensity was measured as the absorbance at 520 nm and determined on a Pharmacia LKB Novaspec II spectrometer (Uppsala, Sweden) using a 1.0 mm pathlength cuvette for juice and a 0.25 mm pathlength cuvette for concentrate, (Aline, Inc. Specvette™ Redondo Beach, CA, U.S.A) to give a reading in the linear range of the spectrometer.

A modified version of the Boulton Assay (Levengood and Boulton 2004) was used to measure the absorbance at 520 nm due to copigmentation, polymeric pigment, and monomeric anthocyanins. The modification was that assays were conducted at the pH of the sample rather than adjusting all samples to pH 3.6 as suggested by Boulton. The pH was taken prior to the analysis (Cole-Parmer Accumet Basic pH Meter, Vernon Hills, IL U.S.A). Model solutions of the juice and concentrate were made

with corresponding levels of glucose (Sigma Aldrich, Milwaukee, WI, U.S.A.), fructose (Sigma Aldrich, Milwaukee, WI U.S.A.), and tartaric acid (Fisher Scientific, Fair Lawn, NJ, U.S.A.), and the pH of the model solution was adjusted with NaOH (Fischer Scientific, Fair Lawn, NJ, U.S.A). Potassium metabisulfite (Sigma Aldrich, Milwaukee, WI. U.S.A.) was used in the polymeric pigment analysis. Absorbance at 520 nm was determined on a Barnstead Turner Spectrophotometer (Fischer Scientific, Fair Lawn, N.J., U.S.A.).

The pH Differential method (Giusti 2001; Lee et al. 2005) was used to determine several metrics, including total anthocyanins (mg/L as cyanidin-3-glucoside), color density, polymeric pigment, and the percentage of polymeric pigment color in the juices. Potassium metabisulfite bleaching was used to determine the amount of polymeric pigment.

There was significant variability in grape color among treatment replicates, since the grapes were harvested at different maturities for each replicate, to account for this all absorbance values were normalized to the initial Time Point 1 to facilitate statistical comparisons across treatments:

Normalized Absorbance at Time Point N (Norm-AU) =
(Absorbance at time point N)/(Absorbance at time point 1) Eq. 9

Time point 1, the sample after heat treatment, was used as the denominator because it occurred prior to the divergence of processing strategies. All color analyses were performed in analytical duplicates.

Anthocyanins in the final, reconstituted PSC and DTC juices were also evaluated on a HP 1100 HPLC system (Agilent, Santa Clara, CA) by a previously described method (Bonerz et al. 2008). Briefly, juices were filtered through a 0.2 µm

filter and 20 µL were injected directly onto a C_{18} reverse phase column (250 mm X 4.6 mm ID, 5 µm particle size). Solvent A was water/phosphoric acid (99.5/0.5;v/v) and Solvent B was acetonitrile/water/phosphoric acid (50/40.5/0.5; v/v/v). Following injection with 100% A and a 2 min hold with, B was ramped from 0% to 100% over 40 min. Column eluent was monitored by a diode array detector, and the signal at 520nm used for peak detection and quantification. Delphidin-3-glucoside, cyanidin-3-glucoside, malvidin-3-glucoside, and delphidin-3-*p*-coumaryl-glucoside (gift from Dr. Justine Vanden Heuvel, originally from Dr. Geza Hrazdina, Cornell University) were used for identification.

Color Stability Analysis

Shelf life studies of bottled juices were performed at three different temperatures: 30˚C, 18˚C, and 2˚C. Samples were taken at 0, 2, 9 and 16 weeks. Samples were centrifuged on an Eppendorf Microcentrifuge 5417 C at 140000 RPM for 15 minutes to remove turbidity at time points 9 and 16 weeks. Color was assessed using the previously described methods: absorbance at 520 nm, modified Levengood-Boulton Assay (Levengood and Boulton 2004) and pH Differential Method (Giusti 2001; Lee et al. 2005).

Anthocyanin Content and Light Microscopy Analysis of Bitartrate Crystals

The bitartrate crystals from PSC and DTC processing were analyzed for total anthocyanin concentration. The bitartrate crystals from cold storage were dissolved in 0.1N HCl, as described by (Vernhet et al. 1999) and the solution assessed by pH Differential Method. DTC crystals were also washed with ethanol. The amount of anthocyanins was reported on a w/w % basis of the bitartrate crystal.

Light microscopy was performed on a MEIJI Techno Microscope (Saitama, Japan) with phase contrast. A 100x magnification was used on bitartrate crystals from PSC processing and 400x magnification from bitartrate crystals from DTC processing.

Statistical Analysis

All processes were performed in duplicate, with two additional analytical replicates for each sample point. Means and standard error were calculated using Microsoft Excel® software (Redmond, W.A., U.S.A). Data treated with analysis of variance (ANOVA) using JMP® 8.0 (SAS Inst. Inc., Cary, N.C., U.S.A.) and means were compared with Tukey-Kramer HSD at a 95% confidence interval.

Results and Discussion

Color Composition of Concord Grape Juice

The contribution of monomeric anthocyanins, copigmented complexes, and polymeric pigments to overall color in the final juice produced from hot press standard concentrate methods (PSC) was calculated using the Boulton Copigmentation Assay (Levengood and Boulton 2004) and shown in Figure 6.

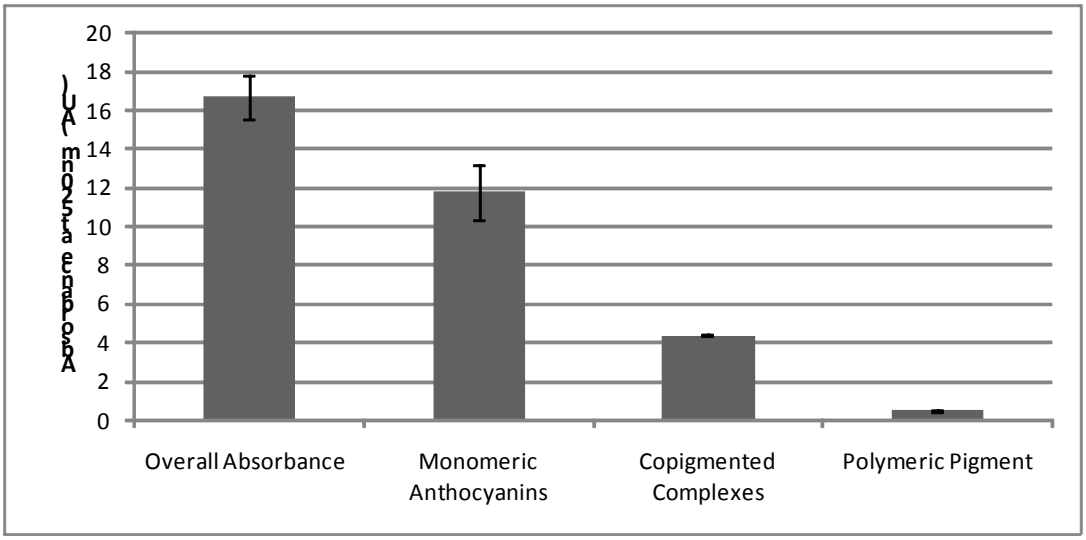


Figure 6: Color profile of Concord grape juice produced from the standard concentrate hot press (PSC) method: overall absorbance along with the color contribution due to monomeric anthocyanins, copigmented complexes, and polymeric pigment, measured by Boulton Copigmentation Assay. Error bars represent one standard error.

The total absorption of the juice at 520 nm was 16.4 AU, with the majority (11.8 AU, 72%) of the color assigned to the monomeric anthocyanin fraction. Hong and Wrolstad similarly reported that the majority of color in Concord grape colorant was due to monomeric anthocyanins in their 1990 publication (Hong and Wrolstad 1990a), although copigmentation was not considered.

Copigmention contributed to 26% of the overall color of standard PSC juice. It is not clear if this copigmented color is primarily due to π-π stacking with other small molecules vs. self-association. The Boulton copigmentation assay only measures the increase in color compared to that predicted from Beer's Law and does not provide further chemical information about the copigmented species. The contribution of copigmentation to PSC juice in our work was comparable to results from earlier work on Muscadine, which indicated that the removal of natural cofactors from Muscadine grape juice resulted in a loss of about 25% of the overall color (Talcott and Lee 2002).

The color due to copigmentation was also within the range previously reported in young red wines, 8-46%, (Main and Morris 2008; Versari 2008; Jensen and others 2008).

Polymeric pigments contributed little to the overall color of the final reconstituted juice (0.5 AU). Concord grape juice is relatively low in tannin and the final PSC juice was relatively young, which likely explains the limited role of polymeric pigments in overall color. The low contribution of polymeric pigment is in concordance with previous reports on Concord grape extract (Hong and Wrolstad 1990).

Effect of Heat Treatments on Concord Grape Juice Color

To determine the effect of hot break vs. hot press heat treatments on Concord grape juice color, we observed the overall absorption at 520 nm of hot press standard concentrate (PSC) and hot break standard concentrate (BSC) throughout processing (Figure 7).

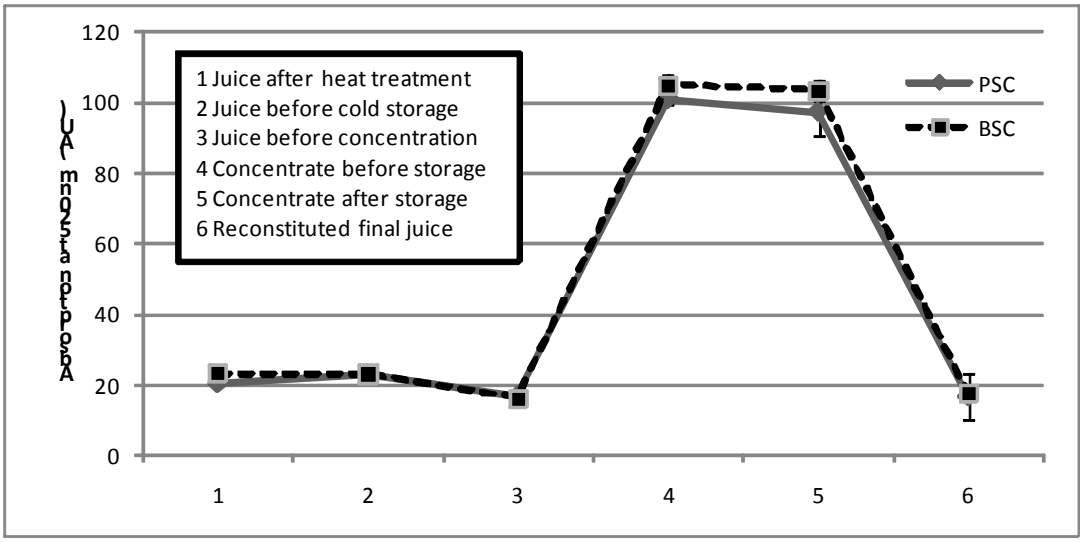


Figure 7: Absorption at 520nm of standard concentrate from hot press (PSC) and standard concentrate from hot break (BSC) throughout processing. Error bars represent one standard error.

Final reconstituted grape juice from PSC and BSC both had overall absorbencies of 16.7 and 17.7 AU, respectively. There were no significant differences in color between the hot press and hot break treatments at any time point during processing, in concordance with previous work by our group on New York State – grown Concord (Iyer 2010).

Effect of Concentrate Parameters on Concord Grape Juice Color and Bitartrate Crystal Composition

A comparison of Abs 520 of the juice during cold storage (juice before cold storage to juice before concentration) and the final reconstituted juice of PSC, DTC, and BSC is shown in Figure 8. There was significant variability in grape color among treatment replicates, since the grapes were harvested at different maturities for each replicate. To account for this variability, all absorbance values were normalized with respect to their color after depectinization (Time Point 1), as described in Materials and Methods, and reported as normalized absorption units, Norm-AU.

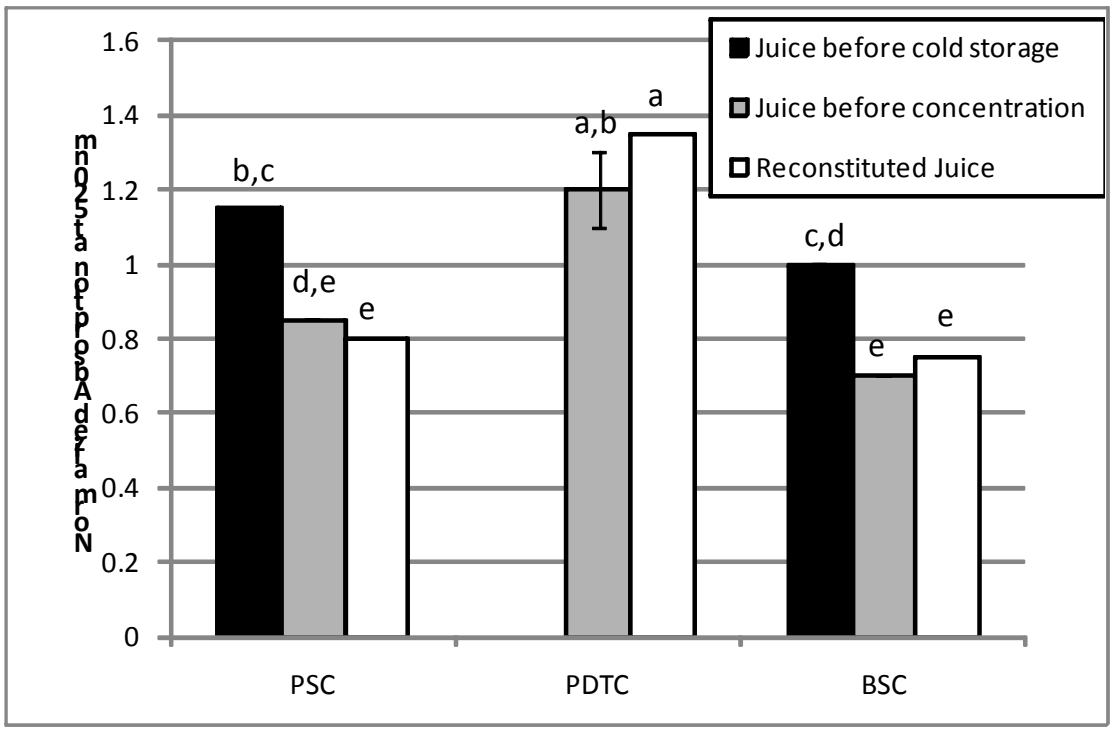


Figure 8: Comparison of single-strength juice color from hot press direct-to-concentrate (DTC), standard concentrate from hot press (PSC), and standard concentrate from hot break (BSC) at different processing stages. Values are reported as the absorption at 520nm at each step, normalized to the juice after heat treatment (Norm-AU) as described in the text. Error bars represent one standard error. Columns not connected by the same letter are significantly different, p value < 0.05.

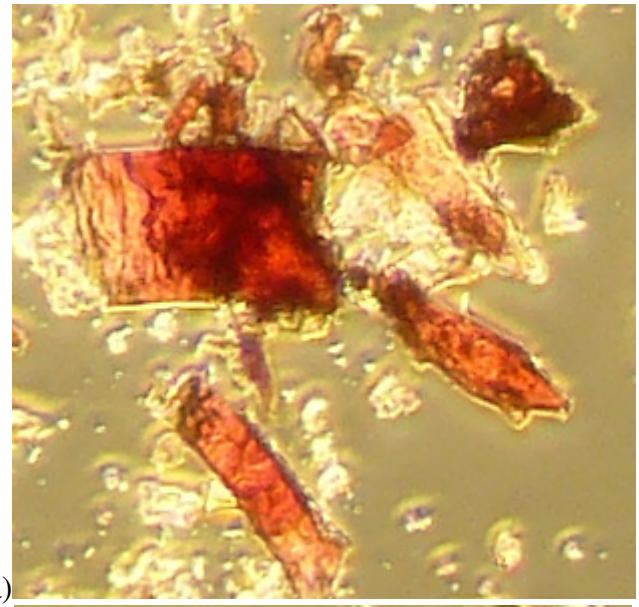
In the standard concentrate methods, BSC and PSC, the final reconstituted juices had normalized absorbencies of 0.8 Norm-AU, or a 20% decrease in color in the final juice compared to the initial juice following depectinization (Figure 8). The decrease in color in the final juice was attributable solely to the cold-storage step, with no significant change in color observed in the intermediate steps, i.e. concentration, concentrate storage, and reconstitution. A comparable loss in color during cold stabilization and detatration has been previously reported (Ingalsbe et al. 1963).

The color of the DTC juice following reconstitution (1.35 Norm-AU) was not significantly different than the normalized absorbance prior to concentration and cold storage. The color of the reconstituted DTC juice was also significantly higher than the color in both PSC and BSC juices. The absorbance of DTC final juice was 63% greater than that of PSC, confirming anecdotal evidence that DTC produces juices with enhanced color in comparison to traditional SC methods.

The DTC and SC methods differed in three respects. In DTC, the second pectinase treatment, plate and frame filter step, and concentration occur prior to cold storage. The timing of the 2nd pectinase enzyme treatment and additional filtering step did not appear to be critical; DTC juice sampled after these steps but prior to concentration, then cold stabilized as single strength, showed a similar decrease in color to SC juice (data not shown). Therefore, the difference in final color between SC and DTC methods could be assigned solely to differences in color loss occurring during cold-stabilization of single strength vs. cold-stabilization of concentrate.

The bitartrate crystals formed by DTC and SC processing were visibly different (Figure 9). Crystals formed during cold-storage of SC juices were approximately 3-4x larger than the DTC crystals, more irregularly shaped, and purplish-black, with the color likely due to coprecipitation of anthocyanins with the crystals. Anthocyanins reportedly adhere to the bitartrate crystal surface during crystal growth (Balakian and Berg 1968; Correa-Gorospe et al. 1991), and bitartrate crystals sampled from wine during cold-storage are reported to contain 0.2-0.3% w/w anthocyanin (Vernhet et al. 1999). By comparison, the DTC crystals were smaller and colorless, see Figure 9. DTC crystals also suggest more isotropic growth than those of SC.

(a)



(b)

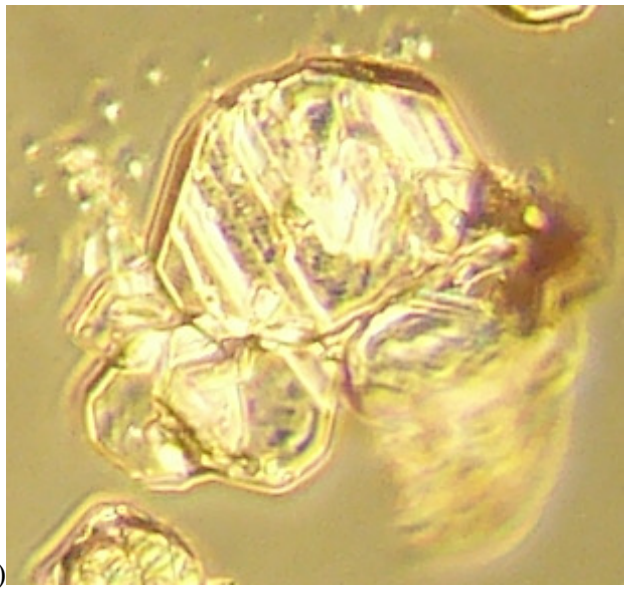


Figure 9: Light microscopy images using phase contrast of bitartrate crystals from a) PSC processing, 100x magnification and b) DTC processing, 400x magnification.

There was no significant decrease in the concentration of total anthocyanins (mg/L as cyanidin-3-glucoside by pH differential) during detartration of the DTC concentrate. In contrast, during each replicate of SC, there was a significant loss (mean = 79 ± 15 mg/L). To determine if the difference in anthocyanin loss between the DTC and SC methods could be explained by coprecipitation with bitartrate

crystals, we analyzed the composition of the bitartrate crystals collected from each method. The crystals were dissolved in 0.1 N HCl and anthocyanins quantified by the pH differential method. The concentration of anthocyanins in PSC crystals was 0.8% w/w. By comparison, the anthocyanin concentration of bitartrate crystals from the DTC method was 0.13% w/w. The concentration of potassium bitartrate lost during PSC and DTC cold storage was estimated from the difference in titratable acidity between the non-detartrated juice and final juice. Similar decreases in titratable acidity, 3.2 g/L as tartaric acid, were observed in PSC and DTC, resulting in similar estimated potassium bitartrate losses of 8.03 g/L. Assuming the sampled crystals contained negligible concentrations of other impurities, the estimated anthocyanin loss due to coprecipiation can be calculated (Table 4).

Table 4: Anthocyanin loss during cold-stabilization of juice from hot press standard concentrate (PSC) and concentration from direct-to-concentrate (DTC), and anthocyanin content of respective bitartrate crystals. Observed and estimated anthocyanin loss from juice (SC) or concentrate (DTC) reported. Anthocyanins in DTC bitartrate crystals were calculated using analytical replicates. Anthocyanin concentrations are calculated as cyanidin-3-glucoside equivalents.

	Process	
	PSC	DTC
Anthocyanins in bitartrate crystals (% w/w basis)	0.8	0.13
Estimated bitartrate loss(g/L)	8.03	8.03
Estimated anthocyanin loss with bitartrate crystals (mg/L)	64	10
Observed change in anthocyanins during detartration (mg/L)	-79 ± 15 (juice)	+5 ± 160 (concentrate)
Observed change in anthocyanins during detartration (%)	-23 ± 4	+ 1 ± 13

The calculations outlined in Table 4 illustrate that the majority of observed anthocyanin loss in PSC (79 mg/L) can be accounted for by anthocyanins coprecipitationg with bitartrate crystals (64 mg/L). Additionally, this latter number may be underestimated due to potential impurities in the crystal, as has been reported in wine (Vernhet et al. 1999).

Changes in Copigmentation During Processing

During PSC, we observed a significant overall color loss ($p < 0.05$) during the detartration of single strength juice, as outlined in Figure 8. The normalized absorbance decreased from 1.1 Norm-AU before cold storage to 0.8 Norm-AU after the two week cold stabilization. There was no significant color loss when DTC concentrate underwent this detartration step. To better characterize the differences between the processes, we evaluated changes in copigmented complexes during DTC and PSC processing (Figure 10). Polymeric pigment was not considered due to its low contribution to total color.

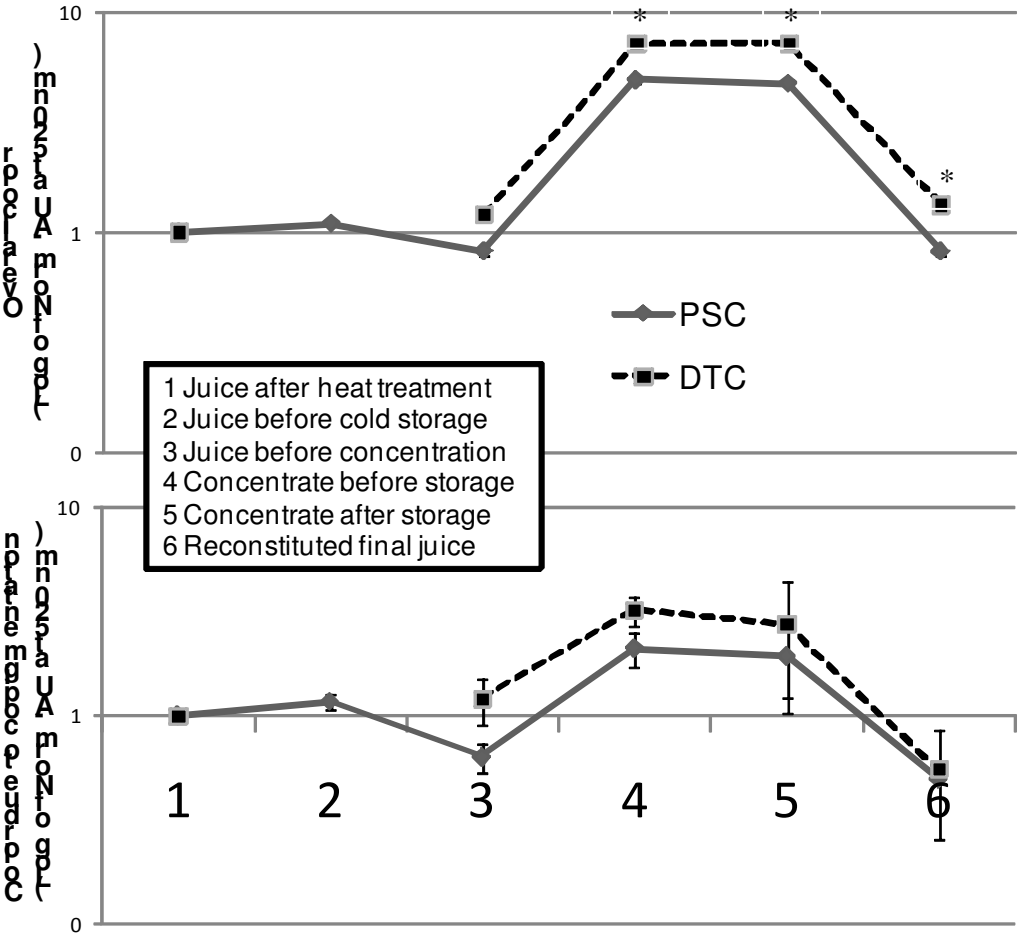


Figure 10: Changes in the total absorbance (520 nm) and copigmented complexes of direct-to-concentrate (DTC) and hot press standard concentrate (PSC) Concord grape juice throughout processing, reported in the log of the normalized absorbance at 520 nm. Error bars represent one standard error. * symbolizes significantly (p value < 0.05) different values between DTC and PSC.

Copigmentation has been reported to enhance anthocyanin stability in aqueous solutions (Talcott et al. 2003; Talcott et al. 2005). Since the degree of copigmentation is dependent on both the anthocyanin and cofactor concentration, i.e. 2nd order, we expected a proportionally greater contribution of copigmentation to color in concentrate as compared to juice. We initially hypothesized that the DTC process would result in reduced color loss because copigmentation would increase the anthocyanin stability or solubility and prevent anthocyanin coprecipitation with

bitartrate crystals. This hypothesis appears to be incorrect. Figure 10, bottom, illustrates that for both DTC and PSC the normalized color due to copigmentation decreases by 50% in the final reconstituted juice as compared to initial juice. This loss is consistent with color analyses of wine during aging which show that copigmentation decreases as a function of time (Harbertson and Spayd 2006; Somers 1971). The 50% drop in copigmentation color following cold-stabilization of PSC is greater than the 20% loss in total absorbance (Figure 6, top), possibly because of the simultaneous coprecipitation of cofactors like flavonols and hydroxycinnamic acids along with anthocyanins (Vernhet et al. 1999). Since color due to copigmention in both SC and DTC following concentration and in the final, reconstituted juices is not significantly different, copigmentation does not directly or indirectly account for the enhanced color of DTC.

Interestingly, we observe only a 3-4 fold increase in the amount of color due to copigmentation in concentrate as compared to the initial single strength juice. Because copigmentation is 2nd order, we had expected to see an approximately [(59 Brix) / (16 Brix)]^2 = 13.5-fold increase in copigmented color during the concentration stage. Copigmentation effects are reported to diminish at lower pH (Asen et al. 1972), and the proportionally lower contribution of copigmentation than expected may be because of the lower pH of concentrate compared to the pH of juice (2.5 vs. 3.1).

As previously discussed, there was no significant decrease in the concentration of total anthocyanins (mg/L as cyanidin-3-glucoside) during detartration of the DTC concentrate but a significant loss during each PSC replicate. Similarly, based on the Boulton assay, we observed significantly higher color due to monomeric anthocyanins in DTC final juice, 2.1 ± 0.7 Norm-AU, as compared to the PSC/BSC treatments, 1.0 ± 0.1 Norm-AU. ‘Monomeric anthocyanins’ and ‘anthocyanins by pH differential’

measure similar components, except that the former will be dependent on the pH of the juice, which changes during processing. In summary, there is a reduction in coprecipitation of monomeric anthocyanins with bitartrates during DTC processing, but this phenomenon is not mediated by copigmentation.

HPLC Analysis of Anthocyanins in Finished Juices

To better understand the mechanism behind monomeric anthocyanin loss in PSC but not DTC during cold storage, anthocyanins in PSC and DTC reconstituted juices were analyzed by HPLC, see Figure 11 and Table 5.

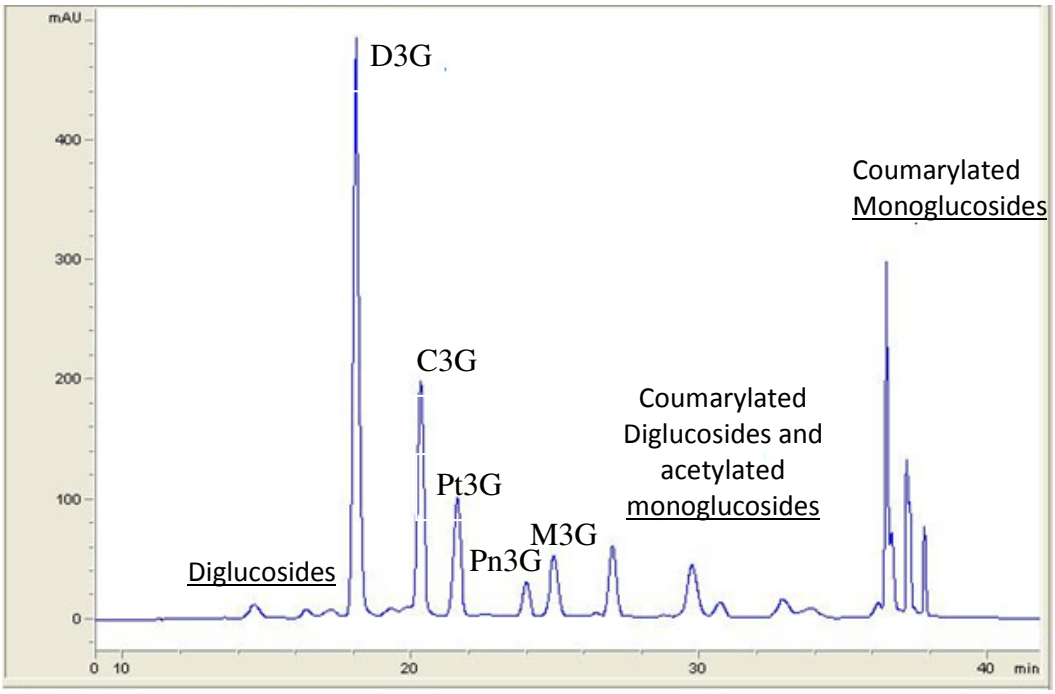


Figure 11: HPLC Chromatogram of Standard Concentrate reconstituted Concord grape juice at 520 nm.

Table 5: Retention time, areas, and peak assignments from Standard Concentrate and Direct-to-Concentrate juices by HPLC analysis. Percent decrease of SC compared to DTC is reported. * indicates that decrease was significant, $p < 0.05$. Assignments are based on external standards (normal font) or tentatively identified based on previous work (*italicized*)

RT (min)	Average area SC juice	Average area DTC juice	% Decrease	Assignment
15.4	290	280	-3*	*Diglucoside*
16.4	166	180	9*	*Diglucoside*
17.1	205	203	-1	*Diglucoside*
18.1	7430	7673	3*	Delphidin-3-glucoside
20.3	3063	3528	15*	Cyanidin-3-glucoside
21.6	1694	1921	13*	*Petunidin-3-glucoside*
24.0	477	579	21*	*Peonidin-3-glucoside*
24.9	1005	1153	15*	Malvidin-3-glucoside
27	974	839	-14*	*Coumarylated diglucoside or acetylated monoglucoside anthocyanin*
29.7	1135	1184	4*	*Coumarylated diglucoside or acetylated monoglucoside anthocyanin*
36.4	3170	4247	34*	Coumarylated monoglucoside anthocyanins (Delphidin and *Cyanidin*)
37.2	1469	2091	42*	*Coumarylated monoglucoside anthocyanins (Petunidin and Peonidin)*
37.8	534	713	34*	*Coumarylated monoglucoside anthocyanin (Malvidin)*

Delphidin, malvidin, and cyanidin 3-glucosides were identified by comparison with authentic standards, and eluted in the range 18-25 min. Peonidin and petunidin 3-glucosides were assigned based literature values (Durst 2000). The *p*-coumaryl derivative of delphinidin-3-glucoside (RT = 36.4 min) was also identified by comparison to an authentic standard. We tentatively identified peaks eluting around this peak (RT = 36-38 min) as other anthocyanin-3-coumarylglucosides. Based on previous studies of Concord anthocyanins by HPLC, we tentatively identified peaks eluting prior to the monoglucosides at RT = 15-17 min as anthocyanin-3,5-diglucosides and peaks eluting after the monoglucosides at RT = 27-32 min as anthocyanin-3-coumarylglucoside-5-glucosides and anthocyanin-3-acetylglucosides (McCallum and others 2007). Coumarylated species are the most common acylated anthocyanins in Concord grapes (Hrazdina 1975), so the majority of the late eluting species were not thought to derive from other acylated anthocyanins, e.g. acetylated anthocyanins.

Significant smaller peaks were observed for several anthocyanins in the PSC juice in comparison to the DTC juice. The largest decreases were observed for the acylated monoglucosides, i.e. anthocyanin-3-coumarylglucosides (34-42%), with modest decreases also observed for anthocyanin-3-glucosides (3-21%), indicating that these species were preferentially lost during cold storage. Peaks tentatively identified as diglucosides, acetylated monoglucosides and coumarylated diglucosides showed negligible differences, and in some cases were slightly higher in the PSC juice. Interestingly, delphinidin-3-glucoside showed the smallest decrease of the five anthocyanins-3-glucosides during detatration, even though it is widely reported to be most rapidly hydrolyzed during storage in juice-like conditions (Figueiredo and others 1996).

These results are comparable to those of Vernhet et al., who showed that coumarylated species are more likely to be lost from solution than monomeric anthocyanins during detartration. In this previous work, coumaric acid derivatives represented a higher percentage of the total anthocyanins in bitartrate crystals than in their corresponding wines (Vernhet et al. 1999). Vernhet, et.al., attempted to explain the preferential loss of coumarylated anthocyanins as due to lower solubility of these compounds in comparison to anthocyanin-3-glucosides. This hypothesis would also explain why diglucosides only experienced negligible losses. However, it is not clear with this explanation why DTC should yield no significant co-precipitation of anthocyanins with bitartrate crystals. Concentration results in a decrease in pH and an increase in the flavylium ion form, as described below, which is expected to increase solubility. The pH of our single-strength Concord juice (3.1), however, is already well below the pK_h of coumarylated anthocyanins (~4.0), so no large change in solubility is expected.

An alternative explanation for differential losses among species is that the stability of an anthocyanin species during detartration is related to its pK_h. The pK_h value of the monoglucosides decrease with electron withdrawing substitutes at the 3' and 5' positions of the B-ring, with the order $OH>OCH_3>H$. Based on these principles and published pK_h values, we observed that anthocyanin-3-glucosides with higher pK_h values had a larger percent decrease in PSC reconstituted juice: delphinidin-3-glucoside (3% decrease, pK_h=2.36), petunidin-3-glucoside (13%, predicted $2.36<pK_h<2.6$), malvidin-3-glucoside (15%, pK_h=2.6), cyanidin-3-glucoside (15%, pK_h=3.01), peonidin (21%, predicted $pK_h>3.01$) (Figueiredo et al. 1996; Mazza 1987; Stintzing et al. 2002). Additionally, coumarylated anthocyanins-3-glucosides, which reportedly have higher pK_h values (Wrolstad 2004), were lost to a greater extent (34-42%) than other anthocyanin species in the juice. Conversely, 3, 5-diglucosides are

reported to have lower pK_h values than monoglucosides, which may explain their negligible losses (Stintzing et al. 2002; Wrolstad 2004).

Our alternative hypothesis, in which the likelihood of co-precipitation is related to higher pK_hs, suggests that the flavylium ion is more likely to co-precipitate with bitartrate crystals, as based on the K_h equilibrium:

$$K_h = [BH\text{-}OH][H^+] / [AH^+][H_2O] \qquad \text{Eq. 10}$$

B = the carbinol base, AH^+= flavylium ion

This suggests that interactions occurring during co-precipitation are between the flavylium form of the anthocyanins and the deprotonated sites of the bitartrate crystals, although as previously mentioned co-precipitation does not involve incorporation of the anthocyanin into the crystal lattice (Rodriguez-Clemente and Correa-Gorospe 1988). Higher pH will result in a more negative surface charge on the bitartrate crystals (Celotti et al. 1999) , which would in turn increase the likelihood of coprecipitation with flavylium forms. The pH of single strength Concord juice from SC was 3.1 prior to cold stabilization, while the pH of the DTC concentrate was 2.5. Celotti et al. suggest that at pH=2.5 there is a neutral surface charge on the bitartrate crystal, as opposed to a negative overall charge at pH=3.1. While lower pH should also increase the flavylium ion concentration of all anthocyanin species, this may be less important than the availability of negatively charged bitartrate sites. Finally, we would also expect that DTC should have higher ionic strength, and thus a shorter Debye length for charged bitartrate crystals, which may further reduce the likelihood of coprecipitation.

Color Stability in DTC and SC During Shelf-Life Studies

The overall color (absorption at 520nm) of DTC and PSC final juices at 30˚C, 18˚C, and 2˚C was analyzed for stability during storage, reference Figure 12.

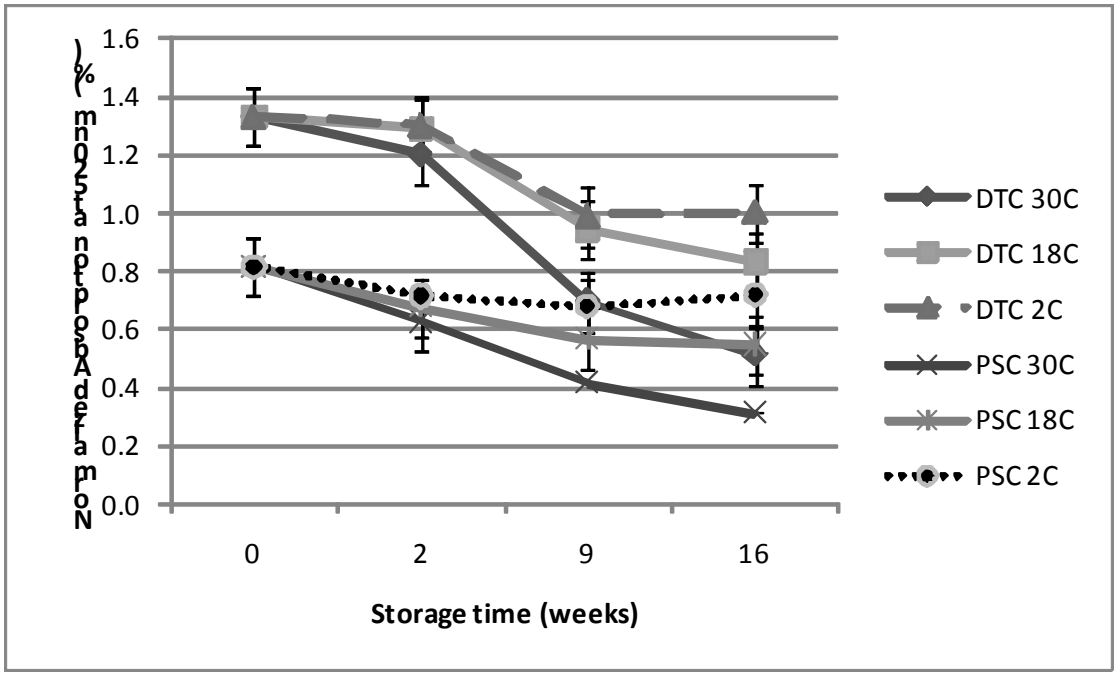


Figure 12: Shelf life study of Concord grape juice reconstituted to 16 Brix from DTC and PSC concentrates at 30°C, 18°C, and 2°C for 16 weeks. Normalized absorbance at 520nm is reported. Error bars represent one standard deviation.

Abs 520 of all samples decreased over time. All DTC juices had consistently greater 520 nm absorbencies than the PSC juices at the same temperature. DTC juices also had higher turbidity, and all samples were centrifuged after the 9 week time point. The reason for increased turbidity with DTC samples was unknown. The percent color loss after 16 weeks storage is shown in Table 6

Table 6: Percentage of color loss during storage for DTC and PSC reconstituted Concord grape juice at 30°C, 18°C, and 2°C. Calculated by comparing the normalized absorption (520nm) at the final storage time point of 16 weeks to the initial absorption at the start of the shelf life study.

	Percentage decrease in color following 16 weeks of storage	
Temperature	PSC (%)	DTC (%)
30°C	61.6 ± 8.3	61.7 ± 7.6
18°C	32.9 ± 16	37.5 ± 13
2°C	13.5 ± 11	24.9 ± 14

The percent color loss was not significantly different between PSC and DTC processing for any storage temperature. The DTC juices have a significantly greater absorbance at 520nm at all time points, indicating that the increased color associated with DTC processing will still be present throughout juice storage.

Conclusion

Direct to concentrate (DTC) methods yielded greater overall absorbance at 520 nm in final Concord juice, as compared to traditional hot press (PSC) and hot break (BSC) processing methods. The discrepancy is linked to the preferential loss of monomeric anthocyanins during the cold storage and detartration of single strength juice in standard concentrate methods, while no anthocyanin losses were observed during cold stabilization of concentrate. We hypothesize that this difference is due to the lower pH of concentrate, which raises the surface charge of the bitartrate crystals, preventing anthocyanin adherence. If our hypothesis is correct, we expect that the fraction of anthocyanins that coprecipitate with potassium bitartrate will be pH and ionic strength dependent, a hypothesis which could be validated with model systems. Finally, these finding may have implications to the wine industry for red wines undergoing cold stabilization, as it may be possible to modify wine properties to minimize losses during cold stabilization.

REFERENCES

Asen S, Stewart RN & Norris KH. 1972. Co-pigmentation of anthocyanins in plant tissues and its effect on color. Phytochemistry 11(3):1139-1144.

Balakian S & Berg HW. 1968. The Role of Polyphenols in the Behavior of Potassium Bitartrate in Red Wines. American Journal of Enology and Viticulture 19(2):91-100.

Bates RP, Morris JR & Crandall PG. 2001. Chapter 12: Grape Juice. In: Anonymous, editor). Principles and practices of small - and medium - scale fruit juice processing. FAO Agricultural Services Bulletin 146 , Food Science and Human Nutrition Department, University of Florida. p. 135.

Bonerz DPM, Nikfardjam MSP & Creasy GL. 2008. A New RP-HPLC Method for Analysis of Polyphenols, Anthocyanins, and Indole-3-Acetic Acid in Wine. *American Journal of Enology and Viticulture* 59(1):106-109.

Boulton R. 2001. The Copigmentation of Anthocyanins and Its Role in the Color of Red Wine: A Critical Review. *American Journal of Enology and Viticulture* 52(2):67-87.

Celotti E, Bornia L & Zoccolan E. 1999. Evaluation of the electrical properties of some products used in the tartaric stabilization of wines. American Journal of Enology and Viticulture 50(3):343-350.

Correa-Gorospe I, Polo MC & Hernandez T. 1991. Characterization of the proteic and the phenolic fraction in tartaric sediments from wines. Food Chemistry 41(2):135-146.

Durst R, Wrolstad, RE 2000. Separation and Characterization of Anthcyanins by HPLC. In: Wrolstad, R. E., editor). Handbook of Food Analytical Chemistry. Hoboken: John Wiley & Sons.

Figueiredo P, Elhabiri M, Toki K, Saito N, Dangles O & Brouillard R. 1996. New aspects of anthocyanin complexation. Intramolecular copigmentation as a means for colour loss? Phytochemistry 41(1):301-308.

Giusti M, Wrolstad RE. 2001. Characterization and measurement of anthocyanins by UV-Visible spectroscopy. In: Wrolstad, R. E., editor). Current protocols in food analytical chemistry. New York: John Wiley & Sons, Inc. p. F1.2.1, F1.2.13.

Harbertson JF & Spayd S. 2006. Measuring Phenolics in the Winery. American Journal of Enology and Viticulture 57(3):280-288.

Hong V & Wrolstad RE. 1990. Characterization of anthocyanin-containing colorants and fruit juices by HPLC photodiode array detection. Journal of Agricultural and Food Chemistry 38(3):698-708.

Hrazdina G. 1975. Anthocyanin Composition of Concord Grapes. Food Science and Technology 8.

Ingalsbe DW, Neubert AM & Carter GH. 1963. Concord Grape Pigments. Journal of Agricultural and Food Chemistry 11(3):263-&.

Iyer M, Sacks, GL; Padilla-Zakour, OI. 2010. Impact of harvesting and processing conditions on green leaf volatile development and phenolics in Concord grape juice. Journal of Food Science 75(3):C297-C304.

Jensen JS, Demiray S, Egebo M & Meyer AS. 2008. Prediction of Wine Color Attributes from the Phenolic Profiles of Red Grapes (Vitis vinifera). Journal of Agricultural and Food Chemistry 56(3):1105-1115.

Lee J, Durst RW & Wrolstad RE. 2005. Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: Collaborative study. Journal of AOAC International 88(5):1269-1278.

Levengood J & Boulton R. 2004. The variation in the color due to copigmentation in young Cabernet Sauvignon wines. In: Waterhouse, A. J. & Kennedey, J. A., editors. Red Wine Color: Revealing the Mysteries. American Chemical Society. p. 35-52.

Main GL & Morris JR. 2008. Impact of Pruning Methods on Yield Components and Juice and Wine Composition of Cynthiana Grapes. *American Journal of Enology and Viticulture* 59(2):179-187.

Mazza G, Brouillard, R. 1987. Recent Developments in the Stabilization of Anthocyanins in Food-Products. Food Chemistry 25(3):207-225.

Mazza G & Miniati E. 1993. Anthocyanins in Fruits, Vegetables, and Grains. Boca Raton: CRC Press.

McCallum JL, Yang R, Young JC, Strommer JN & Tsao R. 2007. Improved high performance liquid chromatographic separation of anthocyanin compounds from grapes using a novel mixed-mode ion-exchange reversed-phase column. Journal of Chromatography A 1148(1):38-45.

McLellan M, Rau, EJ. 1990. Grape juice processing. In: Hicks, D., editor). Production and Packaging of Non-Carbonated Fruit Juices and Beverages. Glasgow, UK: Blackie and Sons. p. 226-242.

Monagas M & Bartolomé B. 2009. Anthocyanins and Anthocyanin-Derived Compounds. In: Moreno-Arribas, M. V. & Polo, M. C., editors. Wine Chemistry and Biochemistry. Springer. p. 439.

Morris J, Striegler, KR. 2005. Grape Juice: Factors That Influence Quality, Processing Technology, and Economics In: Barrett, D. S., L; Ramaswamy, H, editor). Processing fruits: science and technology 2nd ed. ed. Boca Raton, Fl: CRC press.

Rodriguez-Clemente R & Correa-Gorospe I. 1988. Structural, Morphological, and Kinetic Aspects of Potassium Hydrogen Tartrate Precipitation from Wines and Ethanolic Solutions. *American Journal of Enology and Viticulture* 39(2):169-179.

Scheffeldt P & Hrazdina G. 1978. Co-pigmentation of anthocyanins under physiological conditions. Journal of Food Science 43(2):517-520.

Somers TC. 1971. The polymeric nature of wine pigments. Phytochemistry 10(9):2175-2186.

Stintzing FC, Stintzing AS, Carle R, Frei B & Wrolstad RE. 2002. Color and Antioxidant Properties of Cyanidin-Based Anthocyanin Pigments. Journal of Agricultural and Food Chemistry 50(21):6172-6181.

Talcott ST, Brenes CH, Pires DM & Del Pozo-Insfran D. 2003. Phytochemical Stability and Color Retention of Copigmented and Processed Muscadine Grape Juice. Journal of Agricultural and Food Chemistry 51(4):957-963.

Talcott ST & Lee JH. 2002. Ellagic acid and flavonoid antioxidant content of muscadine wine and juice. Journal of Agricultural and Food Chemistry 50(11):3186-3192.

Talcott ST, Peele JE & Brenes CH. 2005. Red clover isoflavonoids as anthocyanin color enhancing agents in muscadine wine and juice. Food Research International 38(10):1205-1212.

Vernhet A, Dupre K, Boulange-Petermann L, Cheynier V, Pellerin P & Moutounet M. 1999. Composition of tartrate precipitates deposited on stainless steel tanks during the cold stabilization of wines. Part II. Red wines. American Journal of Enology and Viticulture 50(4):398-403.

Versari A, Boulton, RB, Parpinello, G.P. 2008. A comparison of analytical methods for measuring the color components of red wines. Food Chemistry 106(1):397-402.

Wrolstad RE. 2004. Symposium 12: Interaction of natural colors with other ingredients - Anthocyanin pigments - Bioactivity and coloring properties. Journal of Food Science 69(5):C419-C421.