Anthocyanin Based Blue Colorants

THESIS

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By

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ABSTRACT

Anthocyanins are a group of natural pigments extensively found in nature. Two main functions of these compounds are their ability to elicit different colors and to impart health benefits. Color is an essential part of the identity of foods and can affect the products’ success in the market. The food industry is searching for natural alternatives to the use of synthetic dyes. A particularly difficult challenge has been finding a natural blue that can replace synthetic FD&C Blue colorants. Some anthocyanins, under neutral to alkaline conditions can turn blue; however, the shades and stability of the blue color highly depends on the source. The aim of this study was to identify and increase our understanding of anthocyanin-based blue colorants from edible materials that can closely match the color characteristics of synthetic FD&C Blue colorants. To achieve this objective, anthocyanins from edible materials as well as commercial extracts were evaluated in regards to color and stability under a wide range of pH. Antocyanins from Baby Indian, Italian, and American eggplant, known to be delphinidin derivatives, were extracted. Delphinidin has three hydroxyl groups on its B-ring expected to cause a bathochromic shift and yield a blue hue. Eggplant anthocyanins were identified by HPLC-PDA, and quantified by the pH differential method. Color (CIELAB) of the extracts were measured after mixing with buffer with pH 3.5, 5, 6, 7, and 8. Monomeric anthocyanin content varied between 45-83 mg Dp-3-glu /100g skin. The major anthocyanin present was Dp 3-rutinoside. The color of the pigments were red at pH 3.5 with L*, c, and h° values of 79.42, 25.28, and 29.21° respectively; however, at pH 8, these values changed to 70.83, 10.8, and 96.97°
respectively corresponding to a yellow hue. Anthocyanins from eggplant did not produce blue color perhaps due to lack of acylation.

Acylated cyanidin-derivatives were then explored as possible sources of natural blue colors, and compared to sources with different anthocyanin patterns.

Commercial extracts of red cabbage (RC), purple sweet potato (PSP), black carrot (BC), red radish (RR), purple corn (PC), and grape (GE) were mixed with 0.01M potassium phosphate buffer (1:5, v:v) at pH 6, 7 and 8. UV/Vis spectra (400-700nm) and CIELAB values were obtained after 15-20min equilibration and over 20 hours refrigerated storage. HPLC-PDA-MS was used to identify the major anthocyanins in each extract. Highly acylated cyanidin-derivatives were the major pigments in RC, PSP, and BC. Acylated peonidin-derivatives were also abundant in PSP. PC contained ~40% acylated-cyanidin. Acylated pelargonidin-derivatives were predominant in RR. GE contained acylated and non-acylated malvidin, petunidin, delphinidin, and peonidin. RC and PSP produced blue hue (similar to FD&C Blue No.2) with $h^\circ$ and $\lambda_{max}$ of 233°, 612nm and 264°, 605nm respectively at pH 8. The stability of RC, however, was significantly higher than PSP over 20hr refrigeration. The substitution of the B-ring with two hydroxyl or methoxyl groups and acylation with aromatic acids seemed to be critical for the production of blue colors from anthocyanin-rich sources.
توجه به توه مادر...
ACKNOWLEDGEMENTS

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CHAPTER 1: LITERATURE REVIEW

1.1 COLOR

Color is a sensation experienced by an individual when radiant energy within the visible spectrum range of 380-770nm is received by the eye (Wrolstad and Smith 2010). In regards to food, color is one of the three main attributes of the food products along with flavor and texture that affects our taste in the food and provides us with visual clues about the identity of the food (McDougall et al. 2007).

1.1.1 COLOR PERCEPTION

For an observer to perceive a color three factors seems to be important: light in the visible range of spectrum, pigmented object, and an observer (Wrolstad and Smith 2010). When the light illuminates at a colored object, it will be transmitted or reflected within a certain spectral distribution. The observer’s eye receptors, nerves and brain, then, translate that into color response (Konica Minolta 2007).

For color to be perceived there must be a light source within the visible spectrum, which is a small portion of the electromagnetic spectrum that falls in between infrared and ultraviolet (Konica Minolta 2007). The most common light source is daylight which appears to be white. When the light hit an object, it will reflect, diffuse, or get absorbed by the object. Color is the
result of selective absorption of the light. The light gets reflected by opaque samples and transmitted by transparent samples (Wrolstad and Smith 2010). **Figure 1.03** illustrates the interaction between the light and an object.

![Interaction between light and object](image)

**Figure 1.01**- Interaction between light and object (Wrolstad and Smith 2010).

In the observer’s eye, the signals are received by the receptors that are responsible for light and color vision. The signal, then, sent to brain to be perceived. According to “Color Opponent Theory,” the human eye can contrast red against green, and blue against yellow light; which is the base for current color measurement system (Wrolstad and Smith 2010).
1.1.2 COLOR MEASUREMENT

The science of color measurement is defined as Colorimetry (Wrolstad and Smith 2010). Color measurement is important because there should be a way to express a given color accurately; so that, other people be able to reproduce the color we described (Konica Minolta 2007). Different color measurement systems have been developed to help in better visual assessment done by an observer. For food research and quality control, it is essential to use color measurement instruments that provide repeatable data correspond to how the human eye perceives color (Wrolstad and Smith 2010). Three important components of color are hue, lightness, and chroma. Hue component, which form the color wheel, is used for classification of red, yellow, blue, etc. Lightness separated the color into bright and dark categories and changes vertically. Chroma or saturation provides information about the dullness or vividness of the color (Konica Minlota2007).

There are four widely used systems for measuring color: Munsell (an old visual color ordering system), CIE XYZ, Hunter LAB, and Hunter CIELAB. Three standard illuminants used in these systems are illuminant A (incandescent light), C (average daylight), and D65 (average daylight including the UV region). There are also two different observer angle that are commonly used in color measurements: 2° or 10° observer angles. However, 10° standard observer is more preferred. The CIE (International Commission on Illumination) developed in 1931 and was the first mathematical system to quantify and standardize the color measurement. Hunter LAB was first published in 1942 to better relate an observed color to numerical values by applying the color opponent theory of color perception (Figure 1.02). In this system, L indicates lightness (0-100), a, the positive (red) or negative (green) coordinate, and b, the positive (yellow) or negative (blue) coordinate. The Hunter LAB system has been widely used by the food industry since it is very useful for measuring color differences (Wrolstad and Smith 2010).
The Hunter CIELAB system was developed in 1976 which was an improved version of Hunter LAB system. Hunter CIELAB has $L^*$, $a^*$, and $b^*$ components which have the same meaning as the $L$, $a$, and $b$ in the Hunter LAB system; however, the color spacing is different to better correlates with human vision. The hue angle and chroma can be calculated from the $a$ and $b$ (or $a^*$ and $b^*$) values using the equations bellow (Wrolstad and Smith 2010).

\[
\text{Chroma (c)} = \sqrt{a^2 + b^2}
\]

\[
\text{Hue angle (h)} = \text{ArcTan} (b/a)
\]

Figure 1.02: Color solid used by Hunter LAB (Wrolstad and Smith 2010).
1.2 FOOD COLORANTS

1.2.1 WHAT ARE FOOD COLORANTS?

Colorants are compounds added to foods to make up for the color loss during processing, enhance the color and to produce uniform food product (Downham and Collins 2000). According to the US laws, there are two main categories of food colorants: certified colorants and colorants exempt from certification. Table 1.01 summarizes different categories of food colorants assigned by the Food and Drug Administration (FDA). Colorants can be either water soluble (dyes) or fat soluble (lakes) (Giusti, Schwartz et al. 2008).

Table 1.01- Different categories of colorants that the FDA approved for use in food (Giusti, Schwartz et al. 2008).

<table>
<thead>
<tr>
<th>Terms used by FDA</th>
<th>Category</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Certified Colorants</td>
<td>Dye</td>
<td>FD&amp;C Red No.40</td>
</tr>
<tr>
<td></td>
<td>Lake</td>
<td>Lake of FD&amp;C Red No.40</td>
</tr>
<tr>
<td>Exempt from certification</td>
<td>Approved pigments from natural sources</td>
<td>Anthocyanins, Juice concentrate, annatto extract</td>
</tr>
<tr>
<td></td>
<td>Synthetic (natural identical)</td>
<td>Carotene</td>
</tr>
</tbody>
</table>
1.2.2 REGULATIONS OF FOOD COLORANTS

In the U.S. food colorants are overseen by Code Federal Regulations (CFR) title 21, which is assigned to the FDA. There are seven dyes, six lakes, and 26 natural and natural identical colorants listed under parts 70 and 82 of title 21 of the CFR. According to the Code of Federal Regulations (CFR 2011 Title 21, Chapter 1, Part 74) there are 7 color additives approved for use in foods, drugs and cosmetics (FD&C color additives) in addition to other 2 synthetic dyes with very specific applications, citrus red No. 2 (for coloring orange skins) and orange B (for sausage casings). Table 1.02 shows the list of synthetic FD&C colorants that are currently certified in the U.S. and their overall prevalence. There are also 36 natural and natural identical colorants exempt from certification (CFR 2011 Title 21, Chapter 1, Part 73).

Table 1.02- Comparison of quantities of FD&C dyes certified for use in the United States by the FDA in 2007 (Sharma et al. 2011).

<table>
<thead>
<tr>
<th>Dye</th>
<th>Mass, kg</th>
<th>Percentage of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue 1</td>
<td>240,654</td>
<td>4.1</td>
</tr>
<tr>
<td>Blue 2</td>
<td>208,646</td>
<td>3.6</td>
</tr>
<tr>
<td>Green 3</td>
<td>5,739</td>
<td>0.1</td>
</tr>
<tr>
<td>Red 3</td>
<td>70,746</td>
<td>1.4</td>
</tr>
<tr>
<td>Red 40</td>
<td>2,330,576</td>
<td>40</td>
</tr>
<tr>
<td>Yellow 5</td>
<td>1,558,756</td>
<td>26.7</td>
</tr>
<tr>
<td>Yellow 6</td>
<td>1,406,060</td>
<td>24.1</td>
</tr>
<tr>
<td>Totals</td>
<td>5,830,177</td>
<td>100</td>
</tr>
</tbody>
</table>
Labeling of the colorants is also governed by the FDA. If artificial colorants are being used terms such as “artificial color added” or “color added” are being used; however, when the product is being colored by naturally derived colors, terms such as “colored with x” or “x (color)” are used (Downham and Collins 2000).

1.2.3 CONCERNS ABOUT SYNTHETIC COLORANTS

For many decades, synthetic colorants have been criticized because of their potential negative side effects. Through the years many synthetic colorants were banned from foods for toxicity and carcinogenicity. Lately, attention-deficit/ hyperactivity disorder (ADHD), behavioral problems, hyperactivity and allergenicity are among the main concerns related to the use of currently approved synthetic colorants (Andersen and Jordheim 2006, Jacobson 2008; Sharma et al. 2011). These new findings made the European Food Safety Authorities to reevaluate the synthetic colorants used in European market (Sharma et al. 2011). This also triggered FDA’s food advisory committee to establish a meeting on March 2011 to also evaluate the possible association between certified color additives in food and ADHD in children. After a very close vote they decided not to change the labeling requirements at this point but to re-evaluate the safety of all synthetic colorants used in foods (FDA 2011).

1.2.4 COLORANTS FROM NATURAL SOURCES

Color pigments which occur naturally in plant and animal are commonly referred to as natural colors. There are four major types of natural pigments: chlorophylls, carotenoids, anthocyanins, and betalains (Giusti, Schwartz et al. 2008). The usage of naturally derived colorants has significantly increased due to the increase in the consumers’ awareness about the ingredients in
their food. In 2000, the natural colorant market was valued to be as large as $100 million in the United State only (Downham and Collins 2000).

Natural colorants have low stability to light, oxygen, metals, oxidizing agents, temperature, and pH (Giusti, Schwartz et al. 2008). Other difficulties related to the use of these colorants are the presence of undesirable aroma, difficulties in purification in large quantities, and that they do not exactly match the color characteristics of the currently used synthetic colorants (Giusti, Schwartz et al. 2008; Massa and Brouillard 1987). Synthetic colorants, on the other hand, have lower cost, high stability, minimum contribution to the flavor of the food, and pronounced color intensities (Downham and Collins 2000; Mazza and Brouillard 1987).

1.3 ANTHOCYANINS

1.3.1 PROPERTIES AND CHEMICAL STRUCTURE

Anthocyanins are water soluble pigments that occur naturally. In plants, they give protection against the harmful UV irradiations, help as attractants for seed dispersal and pollination; also, they provide antimicrobial and antiviral activities (Wrolstad 2004). Anthocyanins have been used as part of the human diet throughout the history; however, they have gained renewed attention due to their positive health benefits (Bridle and Timberlake 1997).

**Figure 1.03** shows the general molecular structure of anthocyanidins. Anthocyanidins are flavonoids composed of C6-C3-C6 carbon chains; and, their first and the second benzene rings are being referred to as ring A and B respectively (Pereira et al. 2009). Substitution of R1 and R2 groups with H, OH, and OCH3 components in the B ring produces six main anthocyanidins: Pelargonidin, Cyanidin, Peonidin, Delphinidin, Petunidin, and Malvidin (**Figure 1.03**).

Glycosidation of anthocyanidins with any of the mono, di, and tri-saccharides is common at C3,
C₅, and C₇ positions which results in formation of anthocyanins (Andersen and Markham 2006; Wrolstad 2004). The sugar moiety can be acylated with aliphatic (malonic, acetic, malic, succinic, and oxalic) acids or aromatic (p-coumaric, caffeic, ferulic, sinapic, galic, and p-hydroxybenzoic) acids (Giusti and Wrolstad 2003).

![Anthocyanin structure](image)

<table>
<thead>
<tr>
<th>Anthocyanin</th>
<th>Abv</th>
<th>R1</th>
<th>R2</th>
<th>Color</th>
<th>( \lambda_{\text{max}} ) (nm) in HCL acidified MeOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pelargonidin</td>
<td>Pg</td>
<td>H</td>
<td>H</td>
<td>Red</td>
<td>520</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>Cy</td>
<td>OH</td>
<td>H</td>
<td>Magenta</td>
<td>535</td>
</tr>
<tr>
<td>Peonidin</td>
<td>Pn</td>
<td>OCH₃</td>
<td>H</td>
<td>Magenta</td>
<td>532</td>
</tr>
<tr>
<td>Malvidin</td>
<td>Mv</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>Purple</td>
<td>542</td>
</tr>
<tr>
<td>Petunidin</td>
<td>Pt</td>
<td>OCH₃</td>
<td>OH</td>
<td>Purple</td>
<td>543</td>
</tr>
<tr>
<td>Delphinidin</td>
<td>Dp</td>
<td>OH</td>
<td>OH</td>
<td></td>
<td>546</td>
</tr>
</tbody>
</table>

**Figure 1.03** - Basic molecular structure of anthocyanidins (He and Giusti 2010).
1.3.2 HEALTH BENEFITS OF ANTHOCYANINS

Anthocyanins have shown to be good antioxidant compounds due to their effective free radical scavenging properties (Wrolstad 2004). Most of the suggested health benefits of anthocyanins are more or less related to their antioxidant mechanisms (Kong, Chai et al. 2003). In vitro studies of anthocyanins have shown that these compounds possibly have protective effects against chronic disease such as cardiovascular disease, cancers, and viral infections (Heins et al. 2001; He and Giusti 2011). They also have demonstrated some anti-inflammatory activities. In addition, it is also possible that anthocyanins prevent obesity and control diabetes (He and Giusti 2011). Anti-allergic and antimicrobial activities are additionally among many other health benefits of these chemical compounds (Ghosh and Konishi 2007; He and Giusti 2011).

1.3.3 ANTHOCYANINS AS FOOD COLORANTS

Since anthocyanin pigments are capable of producing color from red and orange to purple and blue, they have potential application in coloring of different food products (Giusti, Schwartz et al. 2008). For instance, anthocyanins from red radish and red potatoes could be good alternatives for FD&C Red No.40 (Giusti and Wrolstad 2003). Colorants made of anthocyanins are currently manufactured for food use from horticultural crops and processing wastes (Wrolstad and Smith 2010). Anthocyanins from grape color extract and grape skin extract are the only anthocyanin-based extracts included in the list of colorant additives exempt from certification (Socaciu 2007). However, there are two additional categories of approved colorants that open the doors for the use of a wide variety of anthocyanin-based sources in foods: fruit juice and vegetable juice (CFR, 2011). Currently, red cabbage, black carrot, purple sweet potato, radish, bilberry, elderberry, among others, that are available as juice concentrates for use in foods as colorants. For
anthocyanins to be successfully used as natural alternative for synthetic dyes, several factors are seems to be important: their economic feasibility, their chemical, biochemical, and physical stability during processing, and their appearance at food pH (Stintzing et al. 2002).

1.4 BLUE COLORANTS

1.4.1 SYNTHETIC BLUE COLORANTS

Brilliant Blue (FD&C Blue No.1) and Indigotine (FD&C Blue No.2) are two FDA approved synthetic blue colorants that are currently being used in the food industry. Figure 1.04 illustrates the molecular structures of these two dyes. Brilliant Blue is a water soluble reddish-blue powder which is classified as triphenylmethane dyes (Socaciu 2007). This colorants are created through a chemical reaction of coal tar products, which differentiate them from natural colorants (Maloney et al. 2002). In the food industry, this dye is being used alone or in combination with other dyes such as FD&C Blue No. 2 or FD&C Yellow No. 4 (for making green hue) (Flury and Fluhler 1994). Indigotine blue, on the other hand, is a water soluble dark blue powder which is classified as indigoid dyes. This colorant is widely used as fabric and wool dye. Both of these colorants have good stabilities to light and pH. In addition, the aluminum salt of these dyes are also available as lake (Socaciu 2007). Table 1.02 summarizes some of the chemical and physical properties of these two colorants.
Figure 1.04- Chemical structure of Brilliant Blue (left) and Indigo Blue (right) (Socaciu 2007).

Table 1.03- Some chemical and physical properties of the FDA approved synthetic blue dyes (Socaciu 2007; Flury and Fluhler 1994; Giusti, Schwartz et al 2008).

<table>
<thead>
<tr>
<th>Property</th>
<th>Brilliant Blue</th>
<th>Indigotine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common name</td>
<td>Brilliant Blue FCF, C.I. Acid Blue, FD&amp;C Blue No.1, C.I. Food Blue 1</td>
<td>Indigotine, Indigo Carmine, FD&amp;C Blue No.2, C.I. Food Blue 2</td>
</tr>
<tr>
<td>Classification</td>
<td>Triphenylmethane dye</td>
<td>Indigoid dye</td>
</tr>
<tr>
<td>Color</td>
<td>Greenish blue</td>
<td>Deep blue</td>
</tr>
<tr>
<td>Absorption maximum</td>
<td>630nm</td>
<td>610nm</td>
</tr>
<tr>
<td>Molar mass</td>
<td>792.85 g/mol</td>
<td>262.26 g/mol</td>
</tr>
<tr>
<td>Solubility in water</td>
<td>20g /100mL (25°C)</td>
<td>1.6g /100mL (25°C)</td>
</tr>
<tr>
<td>ADI *</td>
<td>0-10 mg/kg bw</td>
<td>0-5 mg/kg bw</td>
</tr>
<tr>
<td>Toxicity (rat)</td>
<td>Actual oral LD₅₀: &gt;5000 mg/kg</td>
<td>Actual oral LD₅₀: &gt;2000 mg/kg</td>
</tr>
</tbody>
</table>

*ADI: Acceptable Daily Intake; †LD₅₀: Lethal Dose
Currently, there are not many colorants from natural sources that can provide a blue color with acceptable stability for commercial applications and that closely match the color characteristics of the synthetic blue. Moreover, the use of these natural pigments, like other pigments from natural sources, is limited due to the processing temperature, pH, light, their interactions with other food matrices, and their regulatory status (Jespersen et al. 2005).

One alternative is a purplish blue colorant (Huito) obtained from *Genipa Americana* fruit pulp found mainly in central and south America (Echeverry et al. 2011). There is not, however, enough information about the color characteristics and stability of this colorant in food matrices. Other alternatives are Gardenia blue (from *Gardenia Jasminoides* plant), phycocyanins (blue protein complex from blue-green algae of *Spirulinaplatensis*), and natural indigo (from indigo plant or *Isatistinctoria* plant). Gardenia blue color is independent from pH with the visible absorption maximum ($\lambda_{\text{max}}$) of 596nm. Phycocyanin, on the other hand, can have the $\lambda_{\text{max}}$ of 616-620nm; however, it is sensitive to pH, light, and temperature. In addition, there are issues with approving these colorants produced by microorganisms and microalgae due to concerns with them producing toxic compounds (Dufosse et al. 2005). Natural indigo can achieve the $\lambda_{\text{max}}$ of 604nm; but, has shown low stability in beverages (Jespersen et al. 2005). Anthocyanins are also capable of producing blue color. The production of blue color by anthocyanins, however, depends on multiple factors such as pH of the media, molecular structure of the pigment(s), and...
compounds attached to the anthocyanin molecule(s) (Giusti and Wrolstad 2003). Stabilization of the blue color produced by anthocyanins is also another important issue which depends on the interaction of anthocyanins within their molecules and with other compounds in the matrix such as other phenolics or metal ions (Castaneda-Ovando et al. 2009).

1.5 ANTHOCYANINS BLUEING FACTORS

1.5.1 FACTORS AFFECTING THE PRODUCTION OF BLUE COLOR

1.5.1.1 pH

pH of the media is an essential factor in the final color of the anthocyanins solutions (Mazza and Brouillard 1987). Figure 1.05 illustrates the different anthocyanin structures formed at different pH values. Anthocyanins are usually red and carry positive charge at acidic pH when they are in their flavyliumcation structure. When the pH shifts toward neutral, the colorless carbinol pseudo-base (hemiketal) and chalcone forms become more prominent. Blue and purple color, however, appears when quiononoidal base dominates under neutral to alkaline conditions (Giusti, Schwartz et al. 2008).
1.5.1.2 SUBSTITUTION OF THE B-RING

It has been shown that substitution of the B-ring of the anthocyanin molecules affect the color of the pigments. Increase in the hydroxylation and methoxylation on the B-ring results in a bathochromic shift (a shift of $\lambda_{\text{max}}$ toward longer wavelengths) which yield a bluer hue (Mazza and Miniati 1993; Cabrita et al. 2000; Giusti and Wrolstad 2003). Figure 1.06 shows the influence of hydroxyl and methoxyl groups in deepening of the hue in six common anthocyanidins.

Figure 1.05- Different structures of anthocyanin molecules and their colors at various pH environments (Dangles et al. 1993).
Figure 1.06: Substitution effects on six most common anthocyanidins, and their influence on the hue (Giusti, Schwartz et al. 2008).

1.5.1.3 ACYLATION AND GLYCOSYLATION

Table 1.03 shows the effect of acylation and glycosylation on the $\lambda_{\text{max}}$ and hue of two anthocyanin derivatives. Stintzing et al. (2002) studied a series of cyanidin-based anthocyanin pigments to determine how the structural variations affect the color properties of those pigments. In this study, it has been shown that acylation of cyanidin anthocyanins with cinnamic acid
increases the $\lambda_{\text{max}}$ and shift the hue angle to purple color. Giusti et al. (1999) also showed that acylation of pelargonidin with cinnamic acid would shift the maximum absorbance wave length toward higher values. Along with acylation, the position that sugar molecule attaches to the anthocyanin seems to be important. It has also been shown that glycosidic substitution at position 5 of cyanidin and pelargonidin can also change the color toward a bluish hue.

**Table 1.04** The effect of acylation and glycosylation on two anthocyanin aglycone (Giusti et al. 1999; Stintzing et al. 2002).

<table>
<thead>
<tr>
<th>Anthocyanin</th>
<th>$h^\circ$</th>
<th>$\lambda_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 3.5</td>
<td></td>
</tr>
<tr>
<td>Cy-3-glu</td>
<td>8.0</td>
<td>512</td>
</tr>
<tr>
<td>Cy-3-soph-5-glu</td>
<td>355.7</td>
<td>514</td>
</tr>
<tr>
<td>Cyanidin-3-soph-5-glu+ coumaryl &amp; sinapic</td>
<td>333.4</td>
<td>536</td>
</tr>
<tr>
<td></td>
<td>pH 1.0</td>
<td></td>
</tr>
<tr>
<td>Pg-3-glu</td>
<td>44</td>
<td>496</td>
</tr>
<tr>
<td>Pg-3-soph-5-glu</td>
<td>41</td>
<td>497</td>
</tr>
<tr>
<td>Pg-3-glu+ p-coumaric</td>
<td>23.3</td>
<td>506</td>
</tr>
<tr>
<td>Pg-3-glu+ferulic</td>
<td>24.1</td>
<td>506</td>
</tr>
</tbody>
</table>
1.5.2 FACTORS AFFECTING THE STABILITY OF BLUE COLOR

1.5.2.1 CO-PIGMENTATION

Co-pigmentation effect is an important phenomenon which helps in stabilization of anthocyanins colors. There are two main possible co-pigmentation interactions between anthocyanins and other compounds that are rich system in $\pi$-electrons: intramolecular co-pigmentation and intermolecular co-pigmentation (Castaneda-Ovando et al. 2009).

Intramolecular interaction happens when the acyl group is attached to the anthocyanin through a sugar substitute in a certain way that allows for folding of the molecule (Figure 1.07a). Formation of this “sandwich type” complex allows the interaction between the acyl aromatic ring and the planar anthocyanin ring (Giusti and Wrolstad 2003). Color stabilization through this type of molecular stacking according to Brouillard et al could be due to the protection of anthocyanin chromophore against water nucleophilic attack (Francisco 1995). Intermolecular interaction happens between two anthocyanin molecules through self-association (Figure 1.07b); or, between anthocyanin molecules and other colorless co-pigmenting compounds. The colorless compound could be flavonoids, alkaloids, polysaccharides, amino acids, organic acids, nucleotids, and metal ion. These types of interactions could also result in a hyperchromic effect and a bathochromic shift along with color stabilization (Castaneda-Ovando et al. 2009; Asen et al. 1972).
1.5.2.2 METAL COMPLEXATION

The effects of metals in color stabilization of blue flower petals containing anthocyanin pigments have been studied for more than three decades (Yoshida et al. 2009). Anthocyanins with two hydroxyl substitutions on their B-ring, such as Cy, Dp, and Pt, are able to form metal-anthocyanin complex (Boulton 2001). Al, Fe, Cu, Sn, Mg, and Mo are among the metal ions that have shown some abilities to form complex with anthocyanin molecules. Stabilization of anthocyanins blue color could be due to the ability of metals to prevent the oxidation of the blue quinoidal base (Hale et al. 2001). Despite the low interest in the food industry, the addition of metal ion to food matrices for stabilizing anthocyanin colors could be a viable alternative for the production and
stabilization of the blue color; particularly if the metal ions do not impose any health threat or when they are even part of the essential minerals in diet (Hale et al. 2001).

**Figure 1.08** Complexation of delphinidin with a metal ion (M$^{n+}$) (Yoshida et al. 2009).

Overall, color is an important part of the identity of the food product which can affect the product’s success in the market. Food industries often add colorants to enhance the color; and, for many years the main colorant choices have been the synthetic colorants. The safety of these colorants, however, has been questioned by the scientific community, regulatory agencies and consumers. There are currently alternatives for natural red and yellow colorants; however, finding a natural source of blue has remained a challenge. The objective for this study was to find an anthocyanin rich source as an alternative for synthetic blue colorants.
CHAPTER 2: EGGPLANT ANTHOCYANINS AS POTENTIAL SOURCE OF BLUE COLOR

2.1 ABSTRACT

Finding a natural source of blue color as an alternative to the use of synthetic colorants has been a challenge for the food industry. Anthocyanins can provide a wide range of color from red to purple to blue depending on the pH of the media and they could potentially be used as natural sources of blue colorants. Particularly, anthocyanins with more hydroxyl substitutions on their B-ring can provide blue color under alkaline to neutral pH values. Eggplant skin is known to have delphinidin (with three OH groups on its B-ring) as its major anthocyanins. The goal of this study was to evaluate the ability of eggplant anthocyanins for production of blue color in low acid or slightly alkaline pH.

Anthocyanins from three different eggplant varieties including Baby Indian Eggplant, Italian Eggplant, and American Eggplant were extracted and quantified. The anthocyanin pigments were identified using High Pressure Liquid Chromatography (HPLC). Color characteristics of the pigments were measured in a 0.2M sodium phosphate dibasic and 0.1M citric acid solutions at pH of 3.5-8 and expressed in Hunter CIELAB units. Eggplants skins contained 45 to 83 mg Dp-3-glu/100g skin. The major pigments in all three varieties were tentatively identified to be delphinidin 3-rutinoside. The color of the pigments were red at pH 3.5 with \(L^*, C, \) and \(h^*\) values for the extract.
were 79.42, 25.28, and 29.21° respectively; however, at pH 8, these values were then changed to 70.83, 10.8, and 96.97° respectively corresponded to a yellow hue. The pigments, however, were not able to provide a blue hue; perhaps, due to the delphinidin pigments being non-acylated. According to these results, the tested eggplant varieties did not prove to be a suitable source for production of blue color.

2.2 INTRODUCTION

The presence of synthetic colorants in food is becoming less appealing for the consumers; so, the food industry is searching for natural alternatives. Anthocyanin pigments because of their health benefits and their abilities to produce color from red to purple to blue can be good alternatives for synthethic colorants (Giusti, Schwartz et al. 2008). The pigments in the eggplant (Solanum melongena) purple color skin is known to be anthocyanins. The major anthocyanins in non-Japanese eggplant cultivars have been reported to be delphinidin 3-rutinoside (tulipanin, D3R), and the minor anthocyanins to be delphinidin 3-rutinoside-5-glucoside (Azuma et al. 2008). Delphinidin anthocyanidin has three OH groups attached to its B-ring; and, it has been shown that higher number of hydroxylation on the B-ring in anthocyanidins causes the visible absorption maximum ($\lambda_{\text{max}}$) to shift toward higher values (bathochromic shift). Delphinidin has the largest $\lambda_{\text{max}}$ compared to the other five common anthocyanidins (Pg, Cy, Pn, Mv, and Pt) in the acidic condition. This large $\lambda_{\text{max}}$ is the reason for this pigment to have a purple hue even in the acidic condition (He and Giusti 2010).

We hypothesized that since eggplant contains delphinidin, a reported bluing factor for anthocyanin pigments, that they could be a good source of anthocyanin-based natural blue colorant. This study primarily aimed to investigate the potential of the delphinidin anthocyanins from eggplant to be used as blue colorant. For these purpose, we chose three common varieties of
eggplants sold in the U.S. markets, extracted the anthocyanins and study the color variations at a wide range of pH values.

2.3 MATERIALS AND METHODS

2.3.1 PLANT MATERIALS AND REAGENTS

Three different eggplant cultivars including Baby Indian Eggplant, Italian Eggplant, and American Eggplant were purchased from Giant Eagle Market District store (Columbus, Ohio). A commercial grape extract donated by MARS Chocolate NA (Hackettstown, NJ) was used to prepare anthocyanin aglycones standards as a reference material. Reagents used were acetone, tri-fluoroacetic acid (TFA), Chloroform, LC/MS Optima® methanol, LC/MS Optima® water, LC/MS Optima® acetonitrile, purchased from Fisher Scientific (Fair Lawn, NJ).

Buffers pH 3.5, 5, 6, 7, and 8 were made by mixing 0.2M sodium phosphate dibasic and 0.1M citric acid solutions. The pH for the buffers was measured using an Accumet XL-15 pH meter (Fisher Scientific, Fair Lawn, NJ).

2.3.2 EXTRACTION PREPARATION

The eggplants skins were removed using a sharp knife, frozen with liquid nitrogen and kept in a freezer for next day extraction. The frozen skins were then grinded using a stainless steel blender. Liquid nitrogen was gradually added in the grinder throughout the grinding. The frozen eggplant skin powders were used for anthocyanin extraction. The extraction method was adapted from Polit (2009). The frozen powder (≈ 5-10g) was mixed with 150mL of acidified aqueous acetone.
(20:80 v/v) acidified with 0.1% HCl and 0.2% TFA and kept in the fridge for 45 min. The mixture was filtered through Whatman#1 filter paper (Whatman Inc., Florham, NJ) by vacuum suction using a Buchner Funnel. The residual cake was washed with the aqueous acetone (≈150mL) until no more pigment was recovered. The filtrates were combined, transferred to a separatory funnel and mixed with 1 volume of chloroform. Samples were set at room temperature for 4 hours to allow good separation of the phases. The top aqueous phase was then collected and the residual acetone was removed using a Buchii rotary evaporator (Brinkmann Instruments, Inc, Westbury, NY) at 40°C under vacuum. The solution was then taken to 10mL on a volumetric flask with acidified water (0.01% HCl).

2.3.3 SOLID PHASE PURIFICATION

The crude eggplant extract was semi-purified by passing it through a Sep-Pak® C$_{18}$ cartridge (6cc, 1g sorbent; Waters Corp., Milford, MA). The cartridge was activated with 2 volumes of methanol (100%) and washed with 2 volumes of acidified water (0.01% HCl). The crude extract was then loaded into the cartridge and washed with 2 volumes of acidified water (0.01% HCl) to remove the sugars and acids, while phenolic compounds are bound to the column. The eggplant pigments were then recovered with acidified methanol (0.01% HCl). The methanol was evaporated using a Buchii Rotavapor at 40°C under vacuum. The extract was taken to 5mL with acidified water (0.01% HCl). The purified extract, finally, was filtered using a 0.45μm Whatman polypropylene syringe filter and prepared for HPLC analysis.

2.3.4 ACID HYDROLYSIS OF ANTHOCYANINS
Grape is known to have five of the six most common anthocyanin aglycones (Dp, Cy, Pt, Pn, and Mv) (Oh et al. 2008; Anderson et al. 1970); so, it was used as reference for confirmation of identity of the anthocyanin pigments in the eggplants. To prepare the grape extract, the commercial extract (0.5mL) was mixed with 100mL acidified water prior to hydrolysis and treated the same way as the crude eggplant extracts for hydrolysis by following the bellow procedure.

The anthocyanin extract (1mL) was mixed with 10mL of 3N HCl in a test tube. The tube was wrapped with aluminum foil and heated in a boiling water hot bath for 40 min. The tube was then cooled down rapidly in an ice bath for 15 min. The mixture was purified using a Sep-pak® C\textsubscript{18} cartridge. The column was washed with acidified water (0.01% HCl) and hydrolyzed extract recovered by acidified methanol (0.01% HCl). The methanol was evaporated at 40°C under vacuum. The volume was taken to 2mL on a volumetric flask with acidified water (0.01% HCl). The hydrolyzed extract was filtered using a 0.45μm Whatman polypropylene syringe filter for HPLC analysis (Giusti and Wrolstad 2005).

2.3.5 HPLC ANALYSES

Samples were analyzed using a Shimadzu LCMS (Shimadzu Scientific, Inc., Columbia, MD) system coupled with LC-20AD pumps, a SPD-M20A photodiode array detector (PDA), and a SIL-20AC auto sampler at 4°C. For reporting and analyzing the data, an LCMS Solutions Software Ver4.0 (Shimadzu, Columbia, MD) was used. A reversed-phase 3.5μm Symmetry C\textsubscript{18} column (4.6x150 mm, Waters Corp., MA, USA) outfitted with a Symmetry guard column (4.6x2.2 mm, Wat   ers Corp., MA, USA) was used. The mobile phases were A, 4.5% formic acid in LC/MS Optima® water and B, LC/MS Optima® acetonitrile. The chromatographic conditions were set as follows: flow rate: 0.8 mL/min; gradient: 0-1 min, 5% B; 1-20 min, 5-20% B; 20-25
min, 20% B; 25-30 min, 20-5% B; 30-35 min, 5% B. Injection volume varied according to the samples from 20-50 µL. Spectral data was collected during the whole run from 250-700nm. Elution of the anthocyanins was monitored at 510–520 nm wavelengths. The same condition was applied to the hydrolyzed extracts.

2.3.6 MONOMERIC ANTHOCYANIN COUNT

Monomeric anthocyanin concentration was determined by using the pH differential method according to Giusti and Wrolstad (2005) described in Handbook of food analytical chemistry. A diluted factor was determined by diluting each extract in pH 1 buffer (0.025M potassium chloride) up to the point that absorbance was within the appropriate spectrophotometer range (0.2-1.5). Each extract was then diluted using pH 1 and pH 4.5 (0.4 M sodium acetate) buffers according to the dilution factor (DF) obtained from the previous step. The solution was allowed to equilibrate for 15 min in the dark. Absorbance was then read on 1cm pathlength cuvettes using a UV-Visible Spectrophotometer 2450 (Shimadzu, Columbia, MD) at 520nm and 700nm. The equation bellow was used to measure the Monomeric anthocyanin content in each sample.

\[
\text{MAC (mg/L)} = \frac{((A_{520} - A_{700}) \text{ pH1} - (A_{520} - A_{700}) \text{ pH4.5}) \times \text{DF} \times 1000 \times \text{MW}}{(\varepsilon \times \text{P})}
\]

Where MW is the molecular weight (465.2 for Dp-3-glu), \(\varepsilon\) is the molar absorptivity coefficient (13000 cm\(^{-1}\) mg\(^{-1}\) for Dp-3-glu) and P is the cuvette pathlength (Giusti and Wrolstad 2005).

2.3.7 COLOR MEASUREMENTS

To measure the color of delphinidin derivatives from eggplant, 2mL of the crude liquid extract in acidified water (0.01% HCl) was mixed with 8mL of buffer with pH 3.5, 5, 6, 7, and 8. Solutions were allowed to equilibrate for 15-20 min and the color characteristics were measured using a ColorQuestXE (Hunter Associate, Inc) spectrophotometer. The instrument was set up to measure
total transmittance using cuvettes with 1cm pathway, with D65 light source and 10° viewing angle. The colors were reported in Hunter CIELAB system through Easy Match software Ver3.62 (Hunter Associates, Inc).

2.3.8 STATISTICAL ANALYSIS

Statistical analysis for anthocyanin content was done using one-way ANOVA followed by LSD (Least Significant Difference) with $\alpha=0.05$ using SPSS Statistics Ver19 software.

2.4 RESULTS AND DISCUSSIONS

2.4.1 ANTHOCYANIN CONTENT

Anthocyanin contents in the samples varied from 45.27 to 80.75 and 83.32 mg of Dp-3-glu/100g of skin sample for the baby Indian, Italian, and American eggplant (Table 2.01). The anthocyanin contents in Italian and American eggplants with darker skin color were significantly higher than this amount in Baby Indian Eggplant. The large standard deviation between the samples could be due to the natural variability among fruits since each eggplant was analyzed separately from another.

Table 2.01- Average anthocyanin content (mg/100g skin weight) based on monomeric anthocyanin counts (mg dp-3-glu/1) obtained from each cultivar (n=3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean anthocyanin content (mg/100g)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eggplant Variety</td>
<td>Anthocyanin Content</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>----------------------------</td>
<td>---------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Baby Indian Eggplant</td>
<td>45.27 B</td>
<td>±10.81</td>
</tr>
<tr>
<td>Italian Eggplant</td>
<td>80.75 A</td>
<td>±11.69</td>
</tr>
<tr>
<td>American Eggplant</td>
<td>83.32 A</td>
<td>±10.34</td>
</tr>
</tbody>
</table>

2.4.2 ANTHOCYANINS IDENTIFICATION

2.4.2.1 HPLC AND PDA RESULTS

The anthocyanin profiles for the three eggplant varieties evaluated were very similar to each other (Figure 2.01). Two peaks were identified in all three varieties showing the presence of two different anthocyanins in the samples. Peak 1 with shorter retention time was the minor peak and peak 2 with longer retention time was the major one (≈ 90% of the total area at 520nm) in all three samples. Since peak 1 eluted faster than peak 2, the anthocyanins corresponded to this peak should be more polar. The spectrophotometric results obtained by the PDA (Figure 2.01) also confirmed this hypothesis since the spectra corresponded to peak 1 unlike peak 2 had a lower shoulder at $\lambda_{\text{max}}$ of 520nm region. It has been shown by Hong and Wrolstad (1990 a and b) that the ratio between the absorbance at 440nm and the absorbance at the visible $\lambda_{\text{max}}$ is much lower for anthocyanins with glucose atoms attached to both C$_3$ and C$_5$ positions than the ones that only have glucose attached to their C$_3$ position. So, we could conclude that anthocyanin corresponded to peak 1 was di-glycosylated at position 3 and 5; but, anthocyanins corresponded to peak 2 had only one glycosylated position at C$_3$. Relatively short retention time of the peaks could also be due to the anthocyanins being non-acylated.
2.4.2.2 CONFIRMATION OF THE IDENTITY

According to Azuma et al. (2008), the major anthocyanin in the non-Japanese eggplants is Delphinidin 3-rutinoside (tulipanin, D3R); and, the minor one is delphinidin 3-rutinoside-5-glucoside. Wu and Prior (2005) reported dp-3-glu and Dp-3-rutinoside-5-galactoside as two major anthocyanins in the eggplant. To make sure that delphinidn was indeed the major anthocyanins in the tested varieties; acid hydrolysis was done on the eggplant extracts and compared with acid hydrolyzed grape extract. Grape is known to have five major anthocyanins (Dp, Cy, Pt, Pn, and Mv) (Oh et al. 2008; Anderson et al. 1970). **Figure 2.02** shows the chromatographic results for acid hydrolyzed eggplant and grape. Based on the polarity of the anthocyanidin molecules and the literature information, peak 1-5 in the acid hydrolyzed grape
was tentatively identified as Dp, Cy, Pt, Pn, and Mv. All tested eggplant varieties contained only one major anthocyanin after acid hydrolysis (Figure 2.02). The retention time and the $\lambda_{\text{max}}$ (530nm) for this anthocyanin were very well matched the retention time and $\lambda_{\text{max}}$ of delphinidin in the acid hydrolyzed grape.

Based on acid hydrolysis, chromatograms and PDA results; as well as, literature information, we tentatively identified the major anthocyanin (peak 2) as Dp-3-rutinoside and, the minor anthocyanin (peak 1) as Dp-3-rutinoside-5-glucoside in our tested eggplant varieties.

**Figure 2.02**- An example of acid hydrolyzed eggplant compared with acid hydrolyzed grape (peak 1-5: Dp, Cy, Pt, Pn, and Mv).
2.4.3 COLOR VARIATION WITH PH

Since all three varieties had the same anthocyanin profile, the color of American eggplant anthocyanin extract was tested in buffer with pH 3.5, 5, 6, 7, and 8. The pH range was selected mainly in the low acid to alkaline range since anthocyanins are more likely produce blue color in this pH region. **Figure 2.03** and **Table 2.02** illustrate the color change with pH. At pH 3.5, the color was deep red with L*, C, and h° values of 79.42, 25.28, and 29.21°; these values were then changed to 85.39, 15.84, and 62.80° respectively at pH 5 corresponded to a yellow hue. As shown in Figure 2.05, the hue angle did not change considerably between pH 5 and 6. The hue angle was then started to increase up to 96.97° at pH 8 when the solution had a grayish yellow color. The chroma and lightness, however, decreased by increasing the pH. The color change with the pH showed that the anthocyanin pigments in the extract were not able to turn blue even in the alkaline pH range.

![Figure 2.03](image)

**Figure 2.03**- Color change in tested eggplant anthocyanins with pH.
Table 2.02- CIELAB values for liquid eggplant extract in buffer 0.2M sodium phosphate dibasic and 0.1M citric acid at 5 different pH values (n=3).

<table>
<thead>
<tr>
<th>pH</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
<th>C*</th>
<th>h°</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>79.42</td>
<td>22.07</td>
<td>12.32</td>
<td>25.28</td>
<td>29.21</td>
</tr>
<tr>
<td>5</td>
<td>85.39</td>
<td>7.29</td>
<td>14.02</td>
<td>15.84</td>
<td>62.80</td>
</tr>
<tr>
<td>6</td>
<td>81.60</td>
<td>6.10</td>
<td>12.84</td>
<td>14.45</td>
<td>63.14</td>
</tr>
<tr>
<td>7</td>
<td>74.00</td>
<td>1.87</td>
<td>10.12</td>
<td>10.30</td>
<td>79.08</td>
</tr>
<tr>
<td>8</td>
<td>70.83</td>
<td>-1.20</td>
<td>10.72</td>
<td>10.80</td>
<td>96.97</td>
</tr>
</tbody>
</table>

Inability of the eggplant extract to produce a blue color could be due to this fact that the major anthocyanins present in the extract was non-acylated delphinidin derivatives and didn’t have any sugar attached to its carbon 5. Acylation and 5C glycosylation have been shown to move the tonalities of the color toward purple and blue (Stintzing et al. 2002). It is still possible that the carbinol pseudo-base structures (responsible for blue color) were formed in the alkaline conditions in the equilibrium mixture; however, since the pigments were not acylated these species were not stable and pushed the equilibrium toward the formation of chalcone forms. Some Japanese eggplants have been reported to have acylated delphinidin pigments (Azuma et al. 2008), it is possible that those varieties could provide a better blue color. Delphinidin anthocyanin derivatives in the presence of metal ions has shown to be able to produce a blue color in some flowers (Yoshida et al. 2009). It is also possible that the addition of metal ions, which doesn’t impose any threat for human health, to the eggplant anthocyanin extract
helps stabilization of the carbinol pseudo-base structures in the Delphinidin anthocyanin equilibrium mixture at an appropriate pH values and provides a blue color.

2.5 CONCLUSION

Anthocyanins from 3 varieties of eggplant were extracted and identified to be non-acylated delphinidin derivatives, with more than ≈90% of the pigment being Dp-3- rutinoside. Concentrations of the anthocyanin in the tested eggplant varieties ranged from 45 to 83 mg/100g skin. However, eggplant anthocyanin extracts did not produce a blue color under neutral to alkaline pH conditions. This could be due to this fact that the major anthocyanins found in these extracts were non-acylated with no sugar attached to their C5 positions. Additional research would be needed to determine if acylated delphinidin derivatives from other eggplant varieties, or addition of metal ions, could provide a better blue color.
CHAPTER 3: SCREENING FOR ANTHOCYANIN BASED BLUE COLORANTS

3.1 ABSTRACT

Finding a natural source of stable water soluble blue colors for food applications has been a challenge for the food industries. Anthocyanins pigments exert health benefits and can produce a wide range of colors based on the pH of the surrounding food matrices. The aim of this study was to investigate the ability of different anthocyanin extracts with distinctive anthocyanin profiles for the production of blue color. The color stabilities were also studied over 20 hours of refrigerated storage. Commercial extracts of red cabbage, purple sweet potato, black carrot, red radish, purple corn, and grape were diluted in 0.01% acidified water to final concentrations ≈100 mg cy-glu/L, and mixed with 0.01M potassium phosphate buffer (1:5, v:v) at pH 6-8. UV/Vis spectra between 400-700nm and Hunter CIELAB values were obtained after 15-20 min equilibration and after 3, 6, 9, and 20 hours refrigeration storage. HPLC-PDA-MS analyses were performed to obtain information about the pigment profile and spectral characteristics under acidic condition in each extract. Red cabbage, purple sweet potato, and black carrot produced dark purple to blue hues with $\lambda_{\text{max}}$ of ≈612, 605, and 595nm respectively in pH 8. Both red cabbage and purple sweet potato had $\lambda_{\text{max}}$ close to FD&C Blue No.2; however, red cabbage had significantly higher stability than purple sweet potato in the alkaline pH value. The HPLC-MS and PDA data showed that the extracts containing mainly cyanidin and peonidin acylated with aromatic acids could produce blue colors with hue angle between 270° - 225° under alkaline conditions.
The food industry is looking for natural alternatives to the use of synthetic dyes. Finding a blue pigment from a natural source has been challenging. Anthocyanin-based colorants are a possible candidate as they can produce blue colors, depending on their chemical structures and the pH of the surrounding environment.

Different anthocyanin sources usually have different anthocyanin profiles; and, their color behaviors well depend on the major anthocyanins present in their compositions. Red cabbage, purple sweet potato, black carrot, purple corn, red radish, and grape extracts are among the main anthocyanin rich sources from edible plant materials.

Red cabbage mainly contains highly acylated cyanidin-3-sophoroside-5-glucoside derivatives substituted with aromatic (sinapic, coumaric, and ferulic) acids. Cyanidin-3-sophoroside-5-glucoside along with peonidin, both highly acylated with aromatic (caffeic, ferulic, and p-hydroxybenzoic) acids, are the major anthocyanins in purple sweet potato (Stintzing et al. 2002; Hagiwara et al. 2002). Black carrot contains 41% acylated cyanidin-3-xylosyl-glucosyl-galactoside acylated with aromatic (sinapic or ferulic) acids (Kirca et al. 2007; Stintzing et al. 2002). Purple corn mainly has cyanidin-3-glucoside derivative anthocyanin which some are acylated with malonic acid (Yang and Zhai 2010; de Pascual-Teresa et al. 2002). Red radish, however, contains pelargonidin-3-sophoroside-5-glucoside esterified with p-coumaric, ferulic, and caffeic to its sugar substituents (Giusti and Wrolstad 1996). Grape, in addition, is the mixture of acylated and non-acylated malvidin, petunidin, delphinidn, peonidin, and cyanidin derivatives (Anderson et al. 1970; Oh et al. 2008).

It has been well established that hydroxylation of the B-ring in anthocyanins have bathochromic effect in the maximum absorbance spectra of these pigments and acylation of anthocyanin molecules helps in color stabilization (Giusti and Wrolstad 2003). However, most studies have
been conducted with pure compounds and under acidic pH environments. The aim of this study was to investigate the abilities of some acylated cyanidin aglycone rich anthocyanin extracts, such as red cabbage, purple sweet potato, black carrot, and purple corn, in production of blue color in neutral to alkaline pH conditions as well as their stabilities in those pH values. Red radish and grape extract, containing other types of anthocyanin profiles, were also included to better understand the effect of anthocyanins chemical structures on their color behaviors.

3.3 MATERIALS AND METHODS

3.3.1 ANTHOCYANIN SOURCES AND REAGENTS

Commercial anthocyanin extracts of Red Cabbage, Purple Sweet Potato, Black Carrot, Red Radish, Purple Corn and Grape, donated by MARS Chocolate NA (Hackettstown, NJ), were used. Reagents used were LC/MS Optima® methanol, LC/MS Optima® water, LC/MS Optima® acetonitrile. To make buffers pH 6, 7, and 8, 0.01M potassium phosphate buffer was used by mixing 1M K$_2$HPO$_4$ and 1M KH$_2$PO$_4$ according to Table 3.01, and taking the total volume to 1L with distilled H$_2$O.

Table 3.01 - Preparation of 0.1M potassium phosphate buffer.

<table>
<thead>
<tr>
<th>pH</th>
<th>Volume of K$_2$HPO$_4$ (mL)</th>
<th>Volume of KH$_2$PO$_4$ (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>13.2</td>
<td>86.8</td>
</tr>
<tr>
<td>7.0</td>
<td>61.5</td>
<td>38.5</td>
</tr>
<tr>
<td>8.0</td>
<td>94.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>
Another set of buffers pH 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, and 8.0 were also made by mixing 0.2M sodium phosphate dibasic and 0.1M citric acid solutions. The pH for the buffers was measured using an Accumet XL-15 pH meter (Fisher Scientific, Fair Lawn, NJ).

3.3.2 SAMPLE PREPARATION

Colored solutions were prepared from each commercial anthocyanin extract containing about the same concentration of monomeric anthocyanins (~100 mg cy-3-glu/100 mL). Stock solutions were first prepared by mixing a known weight of extract with acidified water (0.01% HCl). Monomeric anthocyanin count was done using the pH differential method according to Giusti and Wrolstad (2005) to estimate the initial monomeric anthocyanin content in stock solution. A dilution factor was determined by diluting each extract in pH 1 buffer (0.025M potassium chloride) up to the point that absorbance was within the appropriate spectrophotometer range (0.2-1.5). Each extract was then diluted using pH 1 and pH 4.5 (0.4 M sodium acetate) buffers according to the dilution factor (DF) obtained from the previous step. The solution was allowed to equilibrate for 15 min in the dark. Absorbance was then read on 1cm pathlength cuvettes using a UV-Visible Spectrophotometer 2450 (Shimadzu, Columbia, MD) at 520nm and 700nm. The equation bellow was used to measure the Monomeric anthocyanin content in each sample.

MAC (mg/L) = \left[ \left( A_{520} - A_{700} \right)_{pH1} - \left( A_{520} - A_{700} \right)_{pH4.5} \right] \times DF \times 1000 \times MW / (\varepsilon \times P)

Molecular weight and molar absorptivity coefficient of Cy-3-glu (MW= 449.2 and \varepsilon = 26900) were used as a reference to calculate the anthocyanin content in all stock solutions.

Several dilutions, using acidified water (0.01%HCl), were necessarily to reach to the target concentration of ≈ 100mg Cy-3-glu/L in all six anthocyanin solutions. Table 3.02 shows the final concentration of anthocyanins in each solution.
Table 3.02 - Final anthocyanin concentrations in diluted solutions based on mg Cy-3-glu/L.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Anthocyanin concentration (mg Cy-3-glu/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Cabbage (RC)</td>
<td>97.19</td>
</tr>
<tr>
<td>Purple Sweet Potato (PSP)</td>
<td>101.28</td>
</tr>
<tr>
<td>Black Carrot (BC)</td>
<td>90.84</td>
</tr>
<tr>
<td>Red Radish (RR)</td>
<td>100.28</td>
</tr>
<tr>
<td>Purple Corn (PC)</td>
<td>95.18</td>
</tr>
<tr>
<td>Grape (GE)</td>
<td>94.18</td>
</tr>
</tbody>
</table>

An additional red cabbage solution with anthocyanin concentration of 40.6 mg cy-3-glu/L was set aside to evaluate the color behavior of this extract at smaller pH increments.

3.3.3 COLOR AND SPECTRAL MEASUREMENTS

Aliquots of each solution (2mL) was mixed with 8mL of 0.1M potassium phosphate buffer solutions with pH 6, 7, and 8 (Table 3.01). In addition, the red cabbage extract with concentration of 40.6 mg cy-3-glu/L was mixed with 0.2M sodium phosphate dibasic and 0.1M citric acid buffers pH 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.8, and 8. The total transmittance was measured after 15-20 min using cuvettes with 1cm pathlength. A ColorQuestXE (Hunter Associates, Inc) with D65 light source and 10° viewing angle was used to measure the color. The colors were reported in CIELAB system through Easy Match software Ver3.62 coupled with the colorimeter. Absorbance was also read from 400nm to 700nm using a UV-Visible Spectrophotometer 2450 (Shimadzu, Columbia, MD) after 15-20min on a 1cm pathlength cuvettes.
3.3.4 COLOR STABILITY

The color stabilities of the solutions after mixing with buffers were determined during refrigerated storage. The Hunter CIELAB values of each sample were recorded using a ColorQuestXE under total transmittance mode with D65 light source and 10° viewing angle. Color measurements were made after 15-20 min, and then 3, 6, 9, and 20 hours of storage. Color stability at each pH value was expressed as ΔE by subtracting L’, a’, and b’ components of color at each time interval from these components at the initial time using the formula bellow.

\[ \Delta E = \sqrt{(L_i^* - L_0^*)^2 + (a_i^* - a_0^*)^2 + (b_i^* - b_0^*)^2} \]

3.3.5 SPE PURIFICATION

To purify the liquid acidified extracts, a Sep-Pak® C₁₈ cartridge (6cc, 1g sorbent) was used. The cartridge was activated with 2 volumes of methanol (100%) and washed with 2 volumes of acidified water (0.01% HCl). The crude extract was then loaded into the cartridge and washed with 2 volumes of acidified water (0.01% HCl) to remove the sugars and acids. The anthocyanin pigments were recovered with acidified methanol (0.01% HCl). The methanol was evaporated using a Buchii Rotavapor (Fisher Scientific, Fair Lawn, NJ) at 40°C under vacuum. The extract was finally taken to 5mL with acidified water (0.01% HCl). The purified extract, was finally filtered using a 0.45μm Whatman polypropylene syringe filter and prepared for HPLC analysis (Wrolstad and Giusti 2005).
3.3.6 HPLC-MS-PDA ANALYSES

Samples were analyzed using a Shimadzu LCMS (Shimadzu Scientific, Inc., Columbia, MD) system coupled with LC-20AD pumps, a SPD-M20A photodiode array detector (PDA), and a SIL-20AC autosampler at 4°C. For reporting and analyzing the data, an LCMS Solutions Software Ver4.0 (Shimadzu, Columbia, MD) was used. A reversed-phase 3.5µm Symmetry C18 column (4.6x150 mm, Waters Corp., MA, USA) outfitted with a Symmetry guard column (4.6x2.2 mm, Water Corp., MA, USA) was used. The mobile phases were A, 4.5% formic acid in LC/MS Optima® water and B, LC/MS Optima® acetonitrile. The chromatographic conditions were set as follows: flow rate: 0.8 mL/min; gradient: 0-40 min, 0-35% B; 40-45 min, 35% B; 45-47 min, 35-0 % B. Injection volume varied according to the concentration of each sample.

Spectral data was collected during the whole run from 250-700nm. Elution of the anthocyanins was monitored at 510–520 mm wavelength.

For MS analyses, a 0.2 mL/min volume was diverted into the MS and ionized under positive ion condition using an electrospray probe. Data was monitored using total ion scan (SCAN) (from m/z 200-1200) and selected ion monitoring at m/z 271 (pelargonidin), m/z 287 (cyanidin), m/z 301 (peonidin), m/z 303 (delphinidin), m/z 317 (petunidin), and m/z 331 (malvidin).

To better understand the color behavior of the samples in the alkaline conditions, the pigment profile of each sample were evaluated. To evaluate the pigments spectral profiles, anthocyanin peaks were grouped based on their $\lambda_{\text{max}}$ according to the data obtained by PDA. The peaks in each samples integrated at 520nm (area under the curve); and, percentage area of each peak (PAP) classified into four groups: $500\text{nm} \leq PAP < 510\text{nm}$, $510\text{nm} \leq PAP < 520\text{nm}$, $520\text{nm} \leq PAP < 530\text{nm}$, and $530\text{nm} \leq PAP < 540\text{nm}$. The proportion distribution of the peaks with
different $\lambda_{\text{max}}$ in acidic condition in each sample was compared to the $\lambda_{\text{max}}$ of the sample at alkaline conditions.

3.3.7 STATISTICAL ANALYSIS

Color stability of the different anthocyanin solutions were compared by using two different approaches.

In the first approach, ANCOVA was used to analyze the relation between $\Delta E$ and time after the second measurement point because $\Delta E$ was zero for the initial point; and, it had a linear trend after the second point. One factor (sample type) was considered for this analysis; and, pH 8 was used since the occurrence of blue color was more possible at this pH. Two parameters summarized the degradation behaviors of the samples. The intercept was an estimation of the amount of $\Delta E$ between the first and the second measurements [$\Delta E$]; and, the slope represented the rate of degradation after the second measurement up to the last measurement point [$\Delta E$/time]. Alpha level of 0.05 was considered as a significant. Adjusted coefficient of determination (Adj-$r^2$) was used as estimator of goodness of fit. The Coefficient of Interval (CI) was used to compare the difference between samples.

In the second approach, ANOVA analysis of variance was used to compare the $\Delta E$ of only the last measurement points for pH 8. One factor (sample type) was used. Alpha level of 0.05 was considered as a significant. Least Significant Difference (LSD) was used to compare the difference between the samples. SPSS software version 19 was used to perform the statistical analysis in both cases.
3.4 RESULTS AND DISCUSSIONS

3.4.1 HPLC-MS RESULTS

Major anthocyanins in each sample were identified by the spectral and MS data and compared with those in the literature. As shown in Figure 3.01 and Table 3.03, cyanidin was the major anthocyanins in the RC, BC, and PC commercial extracts. In the case of PSP sample; however, cyanidin as well as peonidin were the major anthocyanin pigments. As shown in the table, majorities of anthocyanin pigments were acylated in RC, PSP, and BC. PC anthocyanins were mainly non-acylated; except for, cyanidin acylated with malonic acid which is an aliphatic acid.
Figure 3.01: HPLC chromatograms at 520nm of major anthocyanins in RC, PSP, BC, and PC commercial extracts (no peak was eluted after 40 min). See table 3.03 for the labeled peak identification.
Table 3.03 - List of main anthocyanins identified putatively in RC, PSP, BC, and PC commercial extracts by HPLC PDA/MS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak#</th>
<th>Retention Time (min)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt;</th>
<th>M+</th>
<th>Fragment ion</th>
<th>Tentative Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Cabbage</td>
<td>1</td>
<td>12.83</td>
<td>514</td>
<td>773</td>
<td>287</td>
<td>Cy-3-diGlu-5-Glu</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15.34</td>
<td>528</td>
<td>979</td>
<td>287</td>
<td>Cy-3-diGlu-5-Glu+Sinapic</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>23.28</td>
<td>523</td>
<td>919</td>
<td>287</td>
<td>Cy-3-diGlu-5-Glu+p-Coumeric</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>23.5</td>
<td>523</td>
<td>979</td>
<td>287</td>
<td>Cy-3-diGlu-5-Glu+Sinapic</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>24.81</td>
<td>536</td>
<td>1125</td>
<td>287</td>
<td>Cy-3-diGlu-5-Glu+Ferulic&amp;Ferulic</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>25.14</td>
<td>536</td>
<td>1155</td>
<td>287</td>
<td>Cy-3-diGlu-5-Glu+Sinapic&amp;Ferulic</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>25.3</td>
<td>536</td>
<td>1185</td>
<td>287</td>
<td>Cy-3-diGlu-5-Glu+Sinapic&amp;Sinapic</td>
</tr>
<tr>
<td>Purple Sweet Potato</td>
<td>1</td>
<td>21.49</td>
<td>525</td>
<td>581</td>
<td>287</td>
<td>Cy-3-diGlu-5-Glu+ Caffeic</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>23.1</td>
<td>526</td>
<td>949</td>
<td>301</td>
<td>Pn-3-diGlu-5-Glu+Caffeic</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>23.37</td>
<td>526</td>
<td>1069</td>
<td>301</td>
<td>Pn-3-diGlu-5-Glu+Caffeic&amp;p-Hydroxybenzoic</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>24.28</td>
<td>530</td>
<td>1125</td>
<td>301</td>
<td>Pn-3-diGlu-5-Glu+Caffeic&amp;Ferulic</td>
</tr>
<tr>
<td>Black Carrot</td>
<td>1</td>
<td>15.08</td>
<td>517</td>
<td>581</td>
<td>287</td>
<td>Cy-3-diGlu</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>16.58</td>
<td>531</td>
<td>949</td>
<td>287</td>
<td>Cy-3-triGlu+Sinapic</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>17.17</td>
<td>520</td>
<td>919</td>
<td>287</td>
<td>Cy-3-triGlu+Ferrulic</td>
</tr>
<tr>
<td>Purple Corn</td>
<td>1</td>
<td>15.83</td>
<td>520</td>
<td>449</td>
<td>287</td>
<td>Cy-3-Glu</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>18.58</td>
<td>516</td>
<td>463</td>
<td>301</td>
<td>Pn-3-Glu</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>19.99</td>
<td>516</td>
<td>535</td>
<td>287</td>
<td>Cy-3-Glu+Malonic</td>
</tr>
</tbody>
</table>
Figure 3.07 and Table 3.06, also, show the chromatograms and tentative identities of the major peaks in the RR and GE commercial extracts that were used as reference for color comparison. As shown in the table the major anthocyanin peaks identified in the RR extract were acylated pelargonidin compounds. GE, on the other hand, contained mostly non-acylated malvidin, peonidin, petunidin, and delphinidin.

Figure 3.02: HPLC chromatograms at 520nm of major anthocyanins in RR and GE commercial extracts (no peak was eluted after 40 min). See table 3.04 for the labeled peak identification.
**Table 3.04** List of main anthocyanins identified putatively in RR and GE commercial extracts by HPLC PDA/MS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak#</th>
<th>Retention Time (min)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt;</th>
<th>M+</th>
<th>Fragment ion</th>
<th>Tentative Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Radish</td>
<td>1</td>
<td>24.41</td>
<td>520</td>
<td>903</td>
<td>271</td>
<td>Pg-3-diGlu-5-Glu+ p-Coumaric</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24.67</td>
<td>520</td>
<td>933</td>
<td>271</td>
<td>Pg-3-diGlu-5-Glu + p-Coumaric</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>26.46</td>
<td>520</td>
<td>1109</td>
<td>271</td>
<td>Pg-3-diGlu-5-Glu + p-CoumaricSinapic</td>
</tr>
<tr>
<td>Grape</td>
<td>1</td>
<td>12.26</td>
<td>522</td>
<td>627</td>
<td>303</td>
<td>Dp-3-Glu-5-Glu</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>14.51</td>
<td>523</td>
<td>641</td>
<td>317</td>
<td>Pt-3-Glu-5-Glu</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15.87</td>
<td>515</td>
<td>625</td>
<td>301</td>
<td>Pn-3-Glu-5-Glu</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>16.38</td>
<td>525</td>
<td>655</td>
<td>331</td>
<td>Mv-3-Glu-5-Glu</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>26.04</td>
<td>531</td>
<td>801</td>
<td>331</td>
<td>Mv acylated derivatives</td>
</tr>
</tbody>
</table>

### 3.4.2 COLOR VARIATIONS WITH pH

The colors of the different anthocyanin solutions varied greatly with pH as well as anthocyanin composition. **Figure 3.03** shows the actual colors obtained with each sample in buffers with pH 6, 7 and 8. **Figure 3.04** and **Table 3.05**, also, illustrate the changes in the color characteristics of the samples with pH changes.

Anthocyanin pigments in RC, PSP, and BC extracts behaved in a similar manner. They all produced a purple hue color at pH 6; their colors were then turned towards a more intense blue hue by increasing the pH. In the case of RR, the color at pH 6 was a pale purple, with a slight
decrease in the hue angle when the pH increased, resulting on a brighter purple color at the highest pH value of 8, never turning into a blue hue. GE and PC anthocyanin pigments, on the other hand, exhibited completely different color characteristics over the tested pH values. Solutions colored with GE and PC showed a very low chroma, and hues that were closer to grayish brown or even green colors.

Since cyanidin and/or peonidin being the major anthocyanin in RC, PSP, and BC, it is possible that hydroxylation and/or methoxylation of anthocyanins’ B-rings resulted in the bathochromic shifts toward blue hue color. Although cyanidin derivative anthocyanins were the major pigments in the PC extract, it didn’t produce a blue color in the tested pH values; so, the acylation of anthocyanins with aromatic groups for the production of blue color in neutral to alkaline pH range also seems to be important. RR and GE didn’t have high amount of cyanidin or peonidin among its major pigments; this could be the reason that they weren’t able to shift the hue toward blue even at pH 8. Although all the extracts were approximately adjusted to the same concentration, the PC and GE had the lowest chroma in all three pH values. Low number of acylation or acylation with aliphatic acid could be the reason for the low chroma of these samples since acylation is an important factor in color stabilization and formation of blue hue in anthocyanins (Castaneda-Ovando et al. 2009).
**Figure 3.03**- Color variation in six different anthocyanin extracts in buffers pH 6-8 after 15-20 min mixed with buffers.
Figure 3.04: Color change of the extracts with change in the pH after 15-20 min mixed with buffer. See table 3.03 for the $L^*$, $C$, and $h^*$ color components ($n=3$).
Table 3.05- \( L^*, C, \) and \( h^0 \) color components of the samples after 15-20 min mixed with buffers pH 6-8 (n=3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>( pH\ 6 )</th>
<th></th>
<th>( pH\ 7 )</th>
<th></th>
<th>( pH\ 8 )</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( L^* )</td>
<td>( C )</td>
<td>( h^0 )</td>
<td>( L^* )</td>
<td>( C )</td>
<td>( h^0 )</td>
</tr>
<tr>
<td>RC</td>
<td>73.8</td>
<td>23.98</td>
<td>311.34</td>
<td>65.62</td>
<td>26.48</td>
<td>263.67</td>
</tr>
<tr>
<td>PSP</td>
<td>74.9</td>
<td>28.64</td>
<td>323.84</td>
<td>59.19</td>
<td>37.69</td>
<td>299.84</td>
</tr>
<tr>
<td>BC</td>
<td>53.42</td>
<td>37.94</td>
<td>338.4</td>
<td>41.36</td>
<td>34.16</td>
<td>313.52</td>
</tr>
<tr>
<td>RR</td>
<td>83.66</td>
<td>17.31</td>
<td>340.92</td>
<td>74.72</td>
<td>25.94</td>
<td>328.32</td>
</tr>
<tr>
<td>PC</td>
<td>79.3</td>
<td>11.27</td>
<td>6.02</td>
<td>66.89</td>
<td>9.76</td>
<td>1.09</td>
</tr>
<tr>
<td>GE</td>
<td>88.8</td>
<td>4.68</td>
<td>42.73</td>
<td>84.2</td>
<td>1.89</td>
<td>339.85</td>
</tr>
</tbody>
</table>

3.4.3 COLOR CHANGE IN RED CABBAGE WITH 0.1 pH INCREMENTS

Figure 3.05 and Table 3.06 show the color variation in red cabbage extract with the concentration of 40.6 mg cy-3-glu/L when the pH changed from 7-8 by increasing 0.1 increments. By changing the pH only by 0.1 increments in the blue hue region the color of the extract varied noticeably. This sensitivity with the pH in the alkaline condition perhaps could be due to exceptional abilities of some pigments inside this extract that are so sensitive to the neutral to alkaline pH range. Perhaps separation of the red cabbage anthocyanin pigments could help us better understand the exceptional color behavior of these pigments particular at this pH range.
Figure 3.05 - Color change with pH in red cabbage after 15-20 min mixed with buffers pH 7-8.

See table 3.04 for the CIELAB color components.

### Table 3.06 - CIELAB values for the red cabbage anthocyanin mixture in buffers with pH 7 to 8 (n=3).

<table>
<thead>
<tr>
<th>pH</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
<th>C</th>
<th>h</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>72.04</td>
<td>2.99</td>
<td>-16.19</td>
<td>16.47</td>
<td>280.45</td>
</tr>
<tr>
<td>7.1</td>
<td>71.69</td>
<td>0.30</td>
<td>-16.67</td>
<td>16.67</td>
<td>271.01</td>
</tr>
<tr>
<td>7.2</td>
<td>71.41</td>
<td>-0.82</td>
<td>-17.41</td>
<td>17.43</td>
<td>267.31</td>
</tr>
<tr>
<td>7.3</td>
<td>70.56</td>
<td>-2.73</td>
<td>-17.41</td>
<td>17.62</td>
<td>261.11</td>
</tr>
<tr>
<td>7.4</td>
<td>70.55</td>
<td>-3.45</td>
<td>-17.52</td>
<td>17.86</td>
<td>258.86</td>
</tr>
<tr>
<td>7.5</td>
<td>69.94</td>
<td>-7.73</td>
<td>-17.37</td>
<td>19.01</td>
<td>246.01</td>
</tr>
<tr>
<td>7.6</td>
<td>70.31</td>
<td>-10.24</td>
<td>-16.40</td>
<td>19.33</td>
<td>238.02</td>
</tr>
<tr>
<td>7.7</td>
<td>69.99</td>
<td>-11.41</td>
<td>-16.65</td>
<td>20.19</td>
<td>235.59</td>
</tr>
<tr>
<td>7.8</td>
<td>69.24</td>
<td>-14.34</td>
<td>-16.52</td>
<td>21.87</td>
<td>229.03</td>
</tr>
<tr>
<td>8.0</td>
<td>68.89</td>
<td>-17.61</td>
<td>-15.39</td>
<td>23.39</td>
<td>221.15</td>
</tr>
</tbody>
</table>

3.4.4 SPECTRAL DATA AND COMPARISON WITH SYNTHETIC BLUE COLORANTS

To better understand the color behaviors of the anthocyanins in the mixture, it is also important to look at their overall pigment spectral profiles meaning the proportion of pigments with different
\( \lambda_{\text{max}} \) at different wavelength. **Table 3.07** shows the proportion of peaks within the extracts with different maximum absorbance wavelength obtained by the PDA when the 4.5% formic acid in HPLC water was used as the mobile phase. As shown in this table, RC, PSP, and BC had 70% of anthocyanin pigments with \( \lambda_{\text{max}} \) of 520-540nm; so, they were better able to shift the maximum absorbance toward a blue hue when mixed with buffer pH 8. None of the anthocyanin pigments in the RR and PC extracts had \( \lambda_{\text{max}} \) higher than 520nm; so, they had lowest maximum absorbance in the alkaline pH values. Knowing about the pigment profile would give us ideas about how different proportions of pigments with different \( \lambda_{\text{max}} \) within a mixture would change the final color at different pH. This could help us with achieving the target color perhaps by mixing the extracts; or, somehow separating the pigments.

**Table 3.07** - Proportion of peaks in each extract with different \( \lambda_{\text{max}} \) in visible spectra, obtained by PDA using 4.5% formic acid in HPLC water as mobile phase, compared with their \( \lambda_{\text{max}} \) at pH 6, 7, and 8.

<table>
<thead>
<tr>
<th>Extract</th>
<th>( \approx % ) Peak Areas at 500-540nm (pH ≈ 2.5)</th>
<th>( \lambda_{\text{max}} )</th>
<th>pH 6.0</th>
<th>pH 7.0</th>
<th>pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>500-510</td>
<td>510-520</td>
<td>520-530</td>
<td>530-540</td>
<td>554.0</td>
</tr>
<tr>
<td>Red Cabbage</td>
<td>0.00</td>
<td>5.54</td>
<td>56.36</td>
<td>38.10</td>
<td>547.5</td>
</tr>
<tr>
<td>Purple Sweet Potato</td>
<td>0.00</td>
<td>0.64</td>
<td>71.27</td>
<td>28.09</td>
<td>545.4</td>
</tr>
<tr>
<td>Black Carrot</td>
<td>0.00</td>
<td>15.36</td>
<td>75.52</td>
<td>9.11</td>
<td>536.9</td>
</tr>
<tr>
<td>Purple Corn</td>
<td>7.24</td>
<td>92.76</td>
<td>0.00</td>
<td>0.00</td>
<td>533.7</td>
</tr>
<tr>
<td>Red Radish</td>
<td>10.76</td>
<td>89.24</td>
<td>0.00</td>
<td>0.00</td>
<td>493.2</td>
</tr>
<tr>
<td>Grape Extract</td>
<td>0.00</td>
<td>26.42</td>
<td>58.18</td>
<td>15.35</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.06, also shows the spectra of the solutions at pH 8 and compare their $\lambda_{\text{max}}$ with FD&C Blue colorants. The $\lambda_{\text{max}}$ was the highest for RC ($\approx 612\text{nm}$) followed by PSP ($\approx 605\text{nm}$) at pH 8 and were close to the maximum absorbance spectra of FD&C Blue No.2 ($\lambda_{\text{max}}$ of 610nm). The maximum absorbance spectrum of RC at pH 8 was the closest to FD&C Blue No.1 ($\lambda_{\text{max}}$ of 630nm) compare to the other extracts.

**Figure 3.06** - Comparison of the spectra of the six different solutions at pH 8 with absorbance spectra of FD&C Blue No.1 and No.2.
3.4.5 COLOR STABILITIES DURING THE STORAGE

The total color change (ΔE) over time for the tested pH values for all the samples are shown in Figure 3.07. All the extracts, except for RR, were more stable in pH 6 and the least stable at pH 7 at refrigeration storage temperature. RR extract showed more stability in pH 7 than pH 8. All the samples, for the most part, degrade very fast during the first three hours at all three pH values; the rates of degradation, however, slowed down and followed a linear trend after this point. Since pH 8 was the most possible condition for the formation of blue color, the statistical analysis for understanding the degradation behaviors of the samples were done for this pH (Table 3.08).

Because the color changes during time were exponential at the beginning and then followed a linear pattern, to better understand the trend of degradation, color change (ΔE) during time after the 3rd hour was considered as a linear model. The intercept of this line with the Y axe reflects the amount of ΔE between the first two measurement points. The slope shows the change in ΔE during time after the 3rd hour. The ΔEs of the samples at the 20th hour were also compared at pH 8. According to these analyses, the degradation rate was lowest in BC at pH 8. Anthocyanins in black carrot were studied previously by Kirca et al. (2007) and showed a great stability compare to the anthocyanins from other plant. Although red radish had previously shown a very good stability in acidic pH (Giusti and Wrolstad 2003), it had the lowest stability compare to the other sources at pH 8. The second unstable extract at the most alkaline pH tested was GE; this could be due to high number of non-acylated anthocyanin. Between RC and PSP samples that were able to produce an acceptable blue color at pH 8, RC significantly had a higher stability than PSP. Lower stability of PSP at this pH could be due to the different pigment profile of this extract since PSP contains peonidin as well as cyanidin aglycone derivatives.
Figure 3.07- Delta E versus time for six anthocyanin rich extracts at pH 6, 7, and 8 during 20 hours refrigeration temperature (n=3).

Table 3.08- Three main components of the degradation pattern used for statistical analysis (n=3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intercept [ΔE]</th>
<th>Slope [ΔE/time]</th>
<th>Mean ΔE (after 20 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Radish</td>
<td>10.83 A</td>
<td>0.62 A</td>
<td>22.82 A</td>
</tr>
<tr>
<td>Grape</td>
<td>5.28 B</td>
<td>0.23 C</td>
<td>9.79 B</td>
</tr>
<tr>
<td>Purple Sweet Potato</td>
<td>3.33 C</td>
<td>0.32 B</td>
<td>9.62 B</td>
</tr>
<tr>
<td>Red Cabbage</td>
<td>3.97 C</td>
<td>0.21 C</td>
<td>8.01 C</td>
</tr>
<tr>
<td>Purple Corn</td>
<td>3.88 C</td>
<td>0.20 C</td>
<td>7.74 C</td>
</tr>
<tr>
<td>Black Carrot</td>
<td>1.31 D</td>
<td>0.21 C</td>
<td>5.37 D</td>
</tr>
</tbody>
</table>
3.5 CONCLUSION

Different anthocyanin solutions produced very different colors at neutral to slightly alkaline pH. Red cabbage, purple sweet potato, and black carrot could produce a dark purple to blue hue in pH 8; while purple corn and grape under the same concentration and pH couldn’t and had a very low chroma. Although red radish had a high chroma, it produced a reddish purple color at pH 8. The stability of the black carrot followed by red cabbage and purple sweet potato were significantly higher than the other extracts over 20 hours refrigeration storage at pH 8. Red cabbage was identified as a good potential alternative for synthetic FD&C Blue No.2 due to its similar color characteristics to this colorant and its relatively higher stability in alkaline conditions. The HPLC-MS and PDA analyses of the samples illustrated that the extracts containing cyanidin and peonidin esterified with aromatic acids were more capable to produce a blue hue and had significantly higher stabilities under alkaline conditions. Perhaps the presence of hydroxyl and methoxyl groups on only two carbons sites on anthocyanin’s B-ring can facilitates the blue color formation by this pigment.
OVERALL CONCLUSION

Non acylated delphinidin pigments found in three common eggplant varieties in the U.S. market did not produce blue color even in the alkaline pH. Highly acylated anthocyanins of red cabbage, containing mainly cyanidin, and purple sweet potato, containing peonidin and cyanidin, could turn to a blue hue in the pH range between 7 and 8. Cyanidin derivatives anthocyanins in purple corn were not able to generate a blue color even at pH 8.

Three factors seemed to be important in shifting the color toward blue hue in alkaline conditions: substitution of the B-ring with hydroxyl and methoxyl groups at two positions, acylation of anthocyanins with aromatic acids, as well as, glycosidation at C3.

Both red cabbage and purple sweet potato had $\lambda_{\text{max}}$ close to that in FD&C Blue No.2 at pH 8; however, red cabbage showed a better stability than purple sweet potato. Unique color characteristics and pigment profiles of red cabbage anthocyanins showed that these pigments have the potential to further shift the maximum absorbance toward the larger wavelength and produce a blue color more similar to FD&C Blue No.1.
REFERENCES


APPENDIX A: Analysis of variance tables used for stability study (Chapter 3)

Analysis of variance for ANCOVA test (adj-$r^2 = 0.97$) (top) and for ANOVA test (bottom).

<table>
<thead>
<tr>
<th>Source</th>
<th>Type III Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
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</thead>
<tbody>
<tr>
<td>Intercept</td>
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<td>1</td>
<td>473.71</td>
<td>745.62</td>
<td>.000</td>
</tr>
<tr>
<td>Sample</td>
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<td>5</td>
<td>39.27</td>
<td>61.82</td>
<td>.000</td>
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<tr>
<td>Time</td>
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<td>382.06</td>
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<tr>
<td>Sample *</td>
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<td>5</td>
<td>13.10</td>
<td>20.62</td>
<td>.000</td>
</tr>
<tr>
<td>Error</td>
<td>35.58</td>
<td>56</td>
<td>.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5697.41</td>
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<td></td>
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</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>Type III Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1852.60</td>
<td>3857.12</td>
<td>.000</td>
</tr>
<tr>
<td>Sample</td>
<td>570.73</td>
<td>5</td>
<td>114.14</td>
<td>237.65</td>
<td>.000</td>
</tr>
<tr>
<td>Error</td>
<td>5.28</td>
<td>11</td>
<td>.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
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