Evaluation of the Efficacy of Anthocyanins as Biologically Active Ingredients in Lipstick Formulations

THESIS

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By

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Abstract

Anthocyanins, a class of flavonoids, have been reported as potent antioxidants, acting as anti-inflammatory, anti-carcinogenic compounds and as natural colorants in the food industry. However, investigations into their application in topical products have been limited, thus far. The attractiveness of using anthocyanins in topical applications is two-fold: their use could potentially provide a source of natural color while concurrently acting as health-promoting active ingredients on the skin. The objective of this study was to evaluate the use of anthocyanins as bioactive colorants in lipstick formulations. The hypothesis was that by targeting anthocyanin sources known for their high stability and reported health benefits, that these properties would also be observed in cosmetics.

Successful formulations showed similar shades to commercial lipsticks, high color stability to accelerated environmental testing; high free radical scavenging antioxidant ability, UV protection, inhibition of melanin production and dermal penetration determined by FTIR spectroscopy and PLS-regression.

Lipsticks were formulated with a standard base of cosmetic-grade waxes and oils and anthocyanin extracts. Sources of non-acylated cyanidin, elderberry, and acylated cyanidin, such as purple carrot; purple corn; and purple sweet potato, were chosen due to their abundance in nature. Red radish, a source of acylated pelargonidin, was investigated due to its reported high stability and color similarity to current certifiable red
colorants. Red grape skin, which contains all six major aglycones, was chosen to better understand the effect of chemical structure on stability. Lipstick formulations were compared to reference samples of commercially available lipsticks and cosmetic colorants. Similar to the reference samples, the anthocyanin-colored lipsticks ranged in color from a vibrant red from red radish to a deep plum from elderberry, highlighting the wide scope of colors obtainable from different anthocyanins.

Shelf stability of all formulations was determined to exceed two years based on spectrophotometric color and monomeric anthocyanin content under accelerated testing conditions. Formulations containing non-acylated and acylated cyanidin, such as elderberry, purple corn and purple sweet potato proved to be the most stable.

All formulas showed increased UV absorption over the lipstick base, with sources acylated with cinnamic acid exhibiting the highest in vitro SPF(UV-B) values. All formulas exhibited high inhibition of DPPH free radicals, and melanin production by tyrosinase, above that of the controls, BHT and kojic acid, respectively. Moreover, their activity was determined to be effective at μg/mg concentrations, which is physiologically relevant for lipstick wearers, who use an average of 24mg per day.

Investigations into the skin permeation of lipsticks formulated with elderberry or red radish revealed their ability to penetrate into the stratum corneum of a porcine ear model, supporting their use in topical applications for skin care. Both the release from the lipstick and the permeation within the skin were dependent on the molecular weight and hydrophilic nature of the compounds, with faster rates observed for the smaller MW and hydrophilic elderberry anthocyanins.
Our results suggest the potential for anthocyanin extracts to act as alternatives to synthetic colorants in lipstick while acting as antioxidants, UV-protection, and anti-aging compounds on the skin.
Acknowledgments

I am extremely grateful for the overwhelming and continuous support I received throughout my graduate research from numerous people. First and foremost, I would like to thank my advisor, Dr. Mónica Giusti, for allowing me the opportunity to become a part of her lab and the endless guidance and knowledge she provided throughout my time there. She inspired me to pursue the research I was passionate about, which truly shaped my experience over the past two years. Without her unwavering encouragement and positivity, the unique idea that sparked into this creative and challenging research project would never have been possible.

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1.1 COLOR

An individual experiences the sensation of color when radiant energy within the visible spectrum range of 380-770nm comes into contact with the retina of the eye (Wrolstad and Smith 2010). Color is especially important in the cosmetic industry because it is used to alter an individuals’ physical appearance and their perception of how they look and feel about their looks (Morante and Diogiovanna 2006).

Color Perception

Color perception is a phenomenon occurring when there is a colored object, light in the visible range, and an observer of said color (Wrolstad and Smith 2010). Color is perceived through a process known as phototransduction, in which photoreceptors in the retina convert wavelengths of light into neural impulses that are then processed by the brain (Figure 1.1). Humans have the ability to perceive up to 10,000 colors (Francis 1999). Color perception between individuals is relatively uniform for those with normal vision (Wrolstad and Smith 2010).
Figure 1.1: Visual observation of color from an object by an observer in the presence of a light source.

Within the retina of the observers’ eye, two components are responsible for vision: the rods and cones (Konica Minolta 2007). The cones are responsible for daylight and color vision, known collectively as “photopic” vision (Wrolstad and Smith 2010). Within the cones are protein receptors sensitive to red, blue, and green light. The rods are responsible for the perception of low-intensity light or night vision, known as “scotopic” vision (Wrolstad and Smith 2010). The light signals from these components are then sent to the brain via the optic nerve, where the brain then interprets the signal as “color”. The “Color Opponent Theory” states that the signal from the red, blue, and green receptors are translated to one brightness signal, which indicates darkness and lightness and two hue signals (Loughrey 2005). The hue signals are red vs. green light and blue vs. yellow light (Wrolstad and Smith 2010).
Although the human eye is extremely sensitive to perceiving color, it is relatively limited in terms of color memory and, therefore, unable to accurately recall colors of objects previously observed (Wrolstad and Smith 2010). The human eye is also less sensitive to minute changes in color (Mokrzycki and Tatol 2012). Due to these limitations, instruments have been developed to precisely interpret and describe colors in space (Konica Minolta 2007).

**Color Measurements**

Colorimetry is the science of color measurement (Wrolstad and Smith 2010). All color systems will describe color in terms of lightness; hue, or the perceived color; and chroma, which is the saturation or intensity of the color (Wrolstad and Smith 2010). There are currently four color systems widely used for colorimetry: Munsell, CIE XYZ, Hunter LAB, and Hunter CIELAB (HunterLab 2012). Three standard illuminants are used by these systems: illuminant A (incandescent light), C (average daylight), and D_65 (average daylight including UV region). There are two observer angles commonly used by color systems, 2° or 10° observer angles; however, the 10° observer angle is the preferred angle utilized (Wrolstad and Smith 2010). The CIE (International Commission on Illumination) developed the first mathematical system for standardization of color measurements. The CIE L*a*b* color space is one of the most commonly used color systems today. In this system, L* is the lightness value, a* is the redness, and b* is the yellowness of the color (Figure 1.2). Similarly, the CIE L*C*h color space describes color in terms of L*, lightness, C*, chroma, and h, or hue angle (Konica Minolta 2007).
Figure 1.2: A. Hunter CIE L*a*b color space and B. CIE L*C*h color spaces (Konica Minolta 2007).

In addition, color differences within the CIE L*a*b color space can be expressed as a numerical value, \( \Delta E \) (Konica Minolta 2007). \( \Delta E \) indicates the size of the color difference and can be calculated by:

\[
\Delta E = \sqrt{[(L_i^* - L_0^*)^2 + (a_i^* - a_0^*)^2 + (b_i^* - b_0^*)^2]}
\]  
(Eq. 1)

The measurement of \( \Delta E \) is especially important in color matching because it can indicate minute variations in color that may not be perceived by the human eye (Mokrzycki and Tatol 2012). These variations are crucial in the cosmetic industry as they allow the company to perform quality assurance testing and color matching of their products for acceptability to the consumer (Morante and Diogiovanna 2006).
1.2 COSMETIC COLORANTS

*Cosmetic Colorants and their history*

Color additives are added to cosmetics for one of two reasons: to act as a color source for the cosmetic and/or to impart color on the skin (Valet et al 2007). They can have either a natural origin (animal, plant, or mineral) or be synthesized chemically. Color additives can be divided into two categories: colorants (dyes), and pigments. Colorants are synthetic organic compounds and are soluble in either water or oil. Pigments, by contrast, are insoluble and remain in a crystalline or particle form after incorporation into the cosmetic (Valet et al 2007). Pigments can be either mineral or organic pigments.

Water-soluble colorants are used to color mixtures in lotions, perfumes, emulsions, and other bath products where concealing is not necessary. They may contain one or more water-soluble groups such as sulfonic (-SO$_3^-$ Na$^+$) or carboxylic (-COO$^-\text{Na}^+$) groups (Morante and DiGiovanna 2006). These colorants are often sensitive to pH, UV radiation, and oxidation/reduction reactions. Examples of water-soluble cosmetic colorants are: carminic acid, caramel, D&C Red No. 33, and FD&C Red No. 40.

Lipid-soluble colorants are used in anhydrous products, such as tanning oils, and do not contain water-soluble groups (Morante and DiGiovanna 2006). They are usually unstable in concentrations higher than a few grams per liter and are sensitive to UV radiation (Valet et al 2007). Examples of lipid-soluble colorants include: β-carotene, and D&C Red No. 17.
Mineral pigments are generally more resistant than organic colorants to degradation; however they tend to give more opaque colors. Iron oxides, yellow (hydrated ferrous oxide), red (ferric oxide), and black (a mixture of ferrous and ferric oxide), are the most widely used mineral pigments in cosmetics. Titanium dioxide ($\text{TiO}_2$) is another example, and is the most commonly used white pigment.

Organic pigments have three types: lakes, toners, and true pigments (Valet et al 2007). Lakes are water-soluble pigments that have been absorbed into insoluble substrates, and have high heat stability. They are commonly used in lipsticks and nail polishes. Toners, by contrast, are water-soluble pigments that are precipitated as metal salts, usually with calcium or barium. Finally, true pigments are those that are insoluble in their natural form and examples include: D&C Red Nos. 30 and 36 (Valet et al 2007).

*Current Regulations of Cosmetic Colorants*

In the United States, cosmetic colorants are known as color additives, which must meet regulations outlined in the U.S. Food, Drug, and Cosmetic Act (FD&C Act) established by the U.S. Food and Drug Administration (FDA). Color additives, in the U.S., are either those that are subject to batch certification or those exempt from certification (Weisz et al 2007). Certified colorants, approved for use in the U.S., can be found in Title 21 of the Code of Federal Regulation (CFR), Part 74 and Part 82 (CFR 2013 Title 21, Chapter 1, Part 74;82). According to the 21 CFR Part 74, there are 36 certified colorants for use in cosmetics, including straight colors and lakes. Lakes are
defined under 21 CFR 70.3 (l) as a straight color that has been included into a substrate by adsorption, coprecipitation, or any chemical combination excluding simple mixing. There are 29 natural and nature identical colorants exempt from certification (21 CFR Part 73).

Most cosmetic ingredients follow the International Nomenclature for Cosmetic Ingredients (INCI) naming system established by the Cosmetic, Toiletries, and Fragrance Association (CTFA); however, this is not the case with color additives. In the European Union (EU), allowable coloring agents are labeled by their Color Index (CI) number, a code assigned by the Society of Dyers and Colourists (SDC) and the American Association of Textile Chemists and Colorists (AATCC) (Valet et al 2007).

While in the United States, acceptable color additives will be labeled based on whether they are exempt from certification or subject to batch certification, as explained above. Those colors that are not exempt will either be listed by their CI name, or if certified will be labeled with a code system adopted by the Food and Drug Administration (FDA). The coding consists of a prefix of either FD&C, certified for use in foods, drugs and cosmetics; D&C, certified for use in drugs and cosmetics; or Ext. D&C, certified for use in external (avoiding lips and mucous membranes) drugs and cosmetics (see Tables 1.1-1.3) (Valet et al 2007). In regards to exempt colors, they will either have simple or chemical names (e.g. titanium dioxide) (See Table 1.4).
Table 1.1: FD&C Color Additives subject to batch certification and approved for use in cosmetics.

<table>
<thead>
<tr>
<th>FD&amp;C Color Additives</th>
<th>Common Name</th>
<th>Color Index #</th>
<th>CAS #</th>
<th>Restrictions</th>
</tr>
</thead>
<tbody>
<tr>
<td>FD&amp;C Blue #1</td>
<td>Brilliant Blue FCF</td>
<td>42090</td>
<td>3844-45-9</td>
<td>Aluminum Lake: Drugs &amp; Cosmetics</td>
</tr>
<tr>
<td>FD&amp;C Green #3</td>
<td>Fast Green FCF</td>
<td>42053</td>
<td>2353-45-9</td>
<td>-</td>
</tr>
<tr>
<td>FD&amp;C Red #40 and Lakes</td>
<td>Allura Red AC</td>
<td>16035</td>
<td>25956-17-6</td>
<td>-</td>
</tr>
<tr>
<td>FD&amp;C Yellow #5</td>
<td>Tartrazine</td>
<td>19140</td>
<td>1934-21-0</td>
<td>Aluminum Lake: Drugs &amp; Cosmetics</td>
</tr>
<tr>
<td>FD&amp;C Yellow #6</td>
<td>Sunset Yellow FCF</td>
<td>15985</td>
<td>2783-94-0</td>
<td>-</td>
</tr>
<tr>
<td>FD&amp;C Lakes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Provisionally Listed</td>
</tr>
</tbody>
</table>

Table 1.2: D&C Color Additives subject to batch certification and approved for use in cosmetics.

<table>
<thead>
<tr>
<th>D&amp;C Color Additives</th>
<th>Common Name</th>
<th>Color Index #</th>
<th>CAS#</th>
<th>Restrictions</th>
</tr>
</thead>
<tbody>
<tr>
<td>D&amp;C Black #3</td>
<td>Bone Black</td>
<td>77267</td>
<td>8021-99-6</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Green #5</td>
<td>Alizarin Cyanine Green F</td>
<td>61570</td>
<td>4403-90-1</td>
<td>Lipsticks (max: 5% of final wt.), external cosmetics</td>
</tr>
<tr>
<td>D&amp;C Orange #5</td>
<td>Dibromofluorescein</td>
<td>45370:1</td>
<td>596-03-2</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Red #6</td>
<td>Lithol Rubin B</td>
<td>15850</td>
<td>4548-53-2</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Red #7</td>
<td>Lithol Rubin B Ca</td>
<td>15850:1</td>
<td>5858-81-1</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Red #21</td>
<td>Tetrabromofluorescein</td>
<td>45380:2</td>
<td>15086-94-9</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Red #22</td>
<td>Eosin</td>
<td>45380</td>
<td>548-26-5</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Red #27</td>
<td>Tetrachlorotetra-Bromofluorescein</td>
<td>45410:1</td>
<td>13473-26-2</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Red #28</td>
<td>Phloxine B</td>
<td>45410</td>
<td>18472-87-2</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Red #30</td>
<td>Helindone Pink CN</td>
<td>73360</td>
<td>2379-74-0</td>
<td>-</td>
</tr>
</tbody>
</table>

(Table 1.2 cont’d below)
### Table 1.2 (cont’d)

<table>
<thead>
<tr>
<th>Color Additives</th>
<th>Common Name</th>
<th>Color Index #</th>
<th>CAS#</th>
<th>Restrictions</th>
</tr>
</thead>
<tbody>
<tr>
<td>D&amp;C Red #33</td>
<td>Acid Fuchsine</td>
<td>17200</td>
<td>3567-66-6</td>
<td>Lipsticks (max: 3% of final wt.), external cosmetics</td>
</tr>
<tr>
<td>D&amp;C Red #36</td>
<td>Flaming Red</td>
<td>12085</td>
<td>2814-77-9</td>
<td>Lipsticks (max: 3% of final wt.), external cosmetics</td>
</tr>
<tr>
<td>D&amp;C Lakes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Provisionally Listed</td>
</tr>
</tbody>
</table>

### Table 1.3: Color Additives subjected to batch certification and approved for use in externally applied cosmetics only.

<table>
<thead>
<tr>
<th>Externally Applied Color Additives</th>
<th>Common Name</th>
<th>Color Index #</th>
<th>CAS#</th>
<th>Restrictions</th>
</tr>
</thead>
<tbody>
<tr>
<td>D&amp;C Brown #1</td>
<td>Resorcin Brown</td>
<td>20170</td>
<td>1320-07-06</td>
<td>-</td>
</tr>
<tr>
<td>FD&amp;C Red #4</td>
<td>Ponceau SX</td>
<td>14700</td>
<td>4548-53-2</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Red #17</td>
<td>Toney Red</td>
<td>26100</td>
<td>85-85-9</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Red #31</td>
<td>Brilliant Lake Red R</td>
<td>15800:1</td>
<td>6371-76-2</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Red #34</td>
<td>Lake Bordeaux B</td>
<td>15800:1</td>
<td>6417-83-0</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Violet #2</td>
<td>Alizurol Purple SS</td>
<td>60725</td>
<td>81-48-1</td>
<td>-</td>
</tr>
<tr>
<td>External D&amp;C Violet #2</td>
<td>Alizarin Violet</td>
<td>60730</td>
<td>4430-18-6</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Blue #4</td>
<td>Alphazurine FG</td>
<td>42090</td>
<td>6371-85-3</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Green #6</td>
<td>Quinizarine Green SS</td>
<td>61565</td>
<td>128-80-3</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Green #8</td>
<td>Pyranine Concentrated</td>
<td>59049</td>
<td>6358-69-6</td>
<td>Max &lt;0.01% of final wt.</td>
</tr>
<tr>
<td>D&amp;C Yellow #7</td>
<td>Fluorescein</td>
<td>45350:1</td>
<td>2321-07-05</td>
<td>-</td>
</tr>
<tr>
<td>External D&amp;C Yellow #7</td>
<td>Napthol Yellow S</td>
<td>10316</td>
<td>846-70-8</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Yellow #8</td>
<td>Uranine</td>
<td>45350</td>
<td>6417-85-2</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Yellow #11</td>
<td>Quinoline Yellow SS</td>
<td>47005</td>
<td>8003-22-3</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Orange #4</td>
<td>Orange II</td>
<td>15510</td>
<td>633-96-5</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Orange #10</td>
<td>Diiodofluorescein</td>
<td>45425:1</td>
<td>3329-19-9</td>
<td>-</td>
</tr>
<tr>
<td>D&amp;C Orange #11</td>
<td>Erythrosine Yellowish Na</td>
<td>45425</td>
<td>38577-97-8</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 1.4: Color Additives exempt from certification and approved for use in cosmetics in the United States.

<table>
<thead>
<tr>
<th>Exempt Color Additive</th>
<th>Color Index #</th>
<th>CAS #</th>
<th>Restrictions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminum Powder</td>
<td>77000</td>
<td>7429-90-5</td>
<td>Externally applied, including eyes</td>
</tr>
<tr>
<td>Annatto</td>
<td>75120</td>
<td>8015-67-6</td>
<td>-</td>
</tr>
<tr>
<td>Beta Carotene</td>
<td>40800</td>
<td>7235-40-7</td>
<td>-</td>
</tr>
<tr>
<td>Bismuth Oxychloride</td>
<td>77163</td>
<td>7787-59-9</td>
<td>-</td>
</tr>
<tr>
<td>Bismuth Citrate</td>
<td>-</td>
<td>-</td>
<td>Limited to hair scalp colorants</td>
</tr>
<tr>
<td>Bronze Powder</td>
<td>77440</td>
<td>7440-50-8</td>
<td>-</td>
</tr>
<tr>
<td>Caramel</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carmine</td>
<td>75470</td>
<td>1390-65-4</td>
<td>-</td>
</tr>
<tr>
<td>Chromium Hydroxide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromium Oxide Greens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromium Oxide Greens</td>
<td>77288</td>
<td>1308-38-9</td>
<td>Externally applied, including eyes</td>
</tr>
<tr>
<td>Copper Powder</td>
<td>77440</td>
<td>7440-50-6</td>
<td>-</td>
</tr>
<tr>
<td>Dihydroxy Acetone</td>
<td>-</td>
<td>62147-49-3</td>
<td>Externally applied</td>
</tr>
<tr>
<td>Ferric Ammonium</td>
<td></td>
<td></td>
<td>Externally applied, including eyes</td>
</tr>
<tr>
<td>Ferrocyanide</td>
<td>77510</td>
<td>14038-43-8</td>
<td>Externally applied, including eyes</td>
</tr>
<tr>
<td>Guaiaculene</td>
<td>-</td>
<td>489-84-9</td>
<td>Externally applied</td>
</tr>
<tr>
<td>Guanine</td>
<td>75170</td>
<td>73-40-5</td>
<td>-</td>
</tr>
<tr>
<td>Henna</td>
<td>75480</td>
<td>83-72-7</td>
<td>Hair colorant only</td>
</tr>
<tr>
<td>Red Iron Oxide,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synthetic</td>
<td>77491</td>
<td>1309-37-1</td>
<td>-</td>
</tr>
<tr>
<td>Yellow Iron Oxide,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synthetic</td>
<td>77492</td>
<td>1345-24-1</td>
<td>-</td>
</tr>
<tr>
<td>Black Iron Oxide,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synthetic</td>
<td>77499</td>
<td>1317-61-9</td>
<td>-</td>
</tr>
<tr>
<td>Lead Acetate</td>
<td>-</td>
<td>6080-56-4</td>
<td>Hair colorant only</td>
</tr>
<tr>
<td>Manganese Violet</td>
<td>77742</td>
<td>10101-66-3</td>
<td>-</td>
</tr>
<tr>
<td>Mica</td>
<td>77019</td>
<td>12001-99-9</td>
<td>-</td>
</tr>
<tr>
<td>Pyrophyllite</td>
<td>44004</td>
<td>8047-76-5</td>
<td>Externally applied</td>
</tr>
<tr>
<td>Silver Powder</td>
<td>77820</td>
<td>7440-22-4</td>
<td>Fingernail polish ≤ 1% by wt.</td>
</tr>
<tr>
<td>Titanium Dioxide</td>
<td>77891</td>
<td>13463-67-7</td>
<td>-</td>
</tr>
<tr>
<td>Ultramarines (All)</td>
<td>77007</td>
<td>1317-97-1; 57455-37-5</td>
<td>Externally applied, including eyes</td>
</tr>
<tr>
<td>Zinc Oxide</td>
<td>77947</td>
<td>1313-13-2</td>
<td>-</td>
</tr>
</tbody>
</table>
Concerns with Cosmetic Colorants

There have been increased public concerns about the safety of synthetic colorants in recent years, and consumer demands are shifting the markets toward more natural alternatives (Duymus et al 2014).

Yellow No. 5, or Tartrazine, gained public scrutiny in the late 1970s. The Public Citizen Health Research Group published a report in 1977 that stated the dye was harmful. The group also tried to push the FDA to ban all certified colors based on the recent banning of colors previously thought to be safe, such as Red No. 2 (Burrows 2009). In a controversial statement, the FDA countered saying the Public Citizen Group was attempting to induce a public scare and overgeneralizing issues; however, admitted that severe allergic reactions in a small group of people had been documented from Yellow No. 5 consumption. Instead of banning the color, in 1979 the FDA made it the first dye required to be listed on the food label’s list of ingredients (Burrows 2009).

FD&C Red #40, is now the most popular food coloring in the U.S. (Stanley 2004). Red #40, or Allura red, is an azo dye (Tsuda et al 2001). Many azo dyes are considered genotoxic, or damaging to DNA, over short periods of time and have shown to be carcinogenic in animals (Tsuda et al 2001). The use of Red #40 is allowed in the U.S. because numerous studies have shown the dye to have nongenotoxic properties; however, some studies conducted in the U.S. and in other countries have reported both genotoxicity and behavioral damage in lab animals (Tsuda et al 2001). Red #40 is absorbed so poorly by humans that many consider it harmless. Azo dyes have the potential to be reduced to metabolically active amines in mammals by azo reductase and therefore may have
cancer-causing risks associated with them; however, this process is not well understand and more research needs to be done to assess the risk (Tsuda et al 2001). It is interesting to note that Red #40 is banned in many EU countries (Griffiths 2005).

Cochineal extract or carmine, a dried extract from pulverized gravid cochineal insects, is currently used as a natural colorant approved for use in food, drugs and cosmetics (Shaw 2009). Due to its origins, public perception of carmine has been low (Shaw 2009). In addition, both carmine and cochineal extract contain residual proteins from the cochineal insect. Cases of contact dermatitis in eye shadows and lipsticks containing carmine have been reported due to the presence of this protein (Shaw 2009).

1.3 LIPSTICK CHEMISTRY AND FORMULATION

Lipstick Formulation

Lipsticks are mixtures of waxes, oils, and pigments, whose concentrations vary depending on the desired properties of the end product (Korichi and Tranchant 2009). Waxes provide the rigid backbone structure to the stick. They can come from vegetative sources, like candellila wax used for brightness or carnauba wax used for hardness. Ozokerite wax, a mineral wax, is used to increase adhesion; whereas, polyethylene, a synthetic wax, is often added because of its compatibility with silicones. Waxes usually account for ~20% of the total formulation and are a carefully chosen blend for desired melting point (Korichi and Tranchant 2009). Oils make up the bulk of the lipstick (40-50%) and give it its characteristic slippery feel and softness. Castor oil is often used to
dispense pigments and oleyl alcohol is used as an emollient and to aid film-formation on the lips.

Color additives are usually present between 2-10% and as a blend of different additives (Barone et al 2002). The majority of colors used are synthetic in origin, but mineral iron oxides and titanium dioxide are also used (Korichi and Tranchant 2009). Manufacturers may choose to add specialized additives for claim substantiation. Specialized additives include organic sunscreens, like methyl p-methoxycinnamate; inorganic sunscreens, such as zinc oxide; antioxidants, such as BHT; preservatives, like methyl p-hydroxybenzoate; chelating agents, such as EDTA; flavors; and fragrances (Barone et al 2002).

Properties of Colorants Used

Colorants impart color onto lips in one of two ways: by staining the skin, which is accomplished by the presence of a dye in the formulation capable of penetrating the outer surface of the skin; or by coloring the lips with a colored film layer, achieved with insoluble dyes and pigments (Gordon 2000).

The most common organic pigments used in lipsticks are D&C Red #7 Calcium Lake, FD&C Yellow #5 Aluminum Lake, D&C Red #6 Barium Lake, D&C Red #28 Aluminum Lake, and FD&C Blue #1 Aluminum Lake (Morante and DiGiovanna 2006). Metal oxides, such as red oxide and titanium dioxide, are used to impart a more natural or earthy undertone to lipsticks (Morante and DiGiovanna 2006). These are known for
having a smoother feel on the lips and better incorporation into lipstick bases than the organic pigments used.

Pearlescent pigments are used to reflect or refract light, which gives lipstick a pearlescent appearance. The original pigment used as a pearl was guanine, a natural pigment responsible for the iridescent appearance of fish scales. This purine base pigment has in recent years been replaced by the synthetic pearl pigment bismuth oxychloride (Barone et al 2002). Mica based pearls are the most popular version used in the lipstick industry; however, their incorporation must be at a low enough concentration as to not affect the feel of the lipstick on the lips (Morante and DiGiovanna 2006). By mixing pearl pigments with organic pigments, such as carmine, or inorganic metal oxides, the cosmetic industry has created many lipsticks with a high shine and lustrous appearance.

Market Trends

In recent years, the cosmetic industry in North America has been largely driven by the demands of an aging population and the desire for multifunctional products (Brandt et al 2011). These two driving forces have aided in the establishment of the ‘cosmeceutical’ product market, which focuses on products intended to reduce and reverse the signs of aging (Brandt et al 2011). The term cosmeceutical was first defined by Dr. Albert Kligman as products bridging the gap between medicine and cosmetics by incorporating ingredients with pharmaceutical benefits (Choi and Berson 2006).
Recent trends in cosmeceuticals point to developing more products intended to help protect the skin from radiation and oxidative damage through the use of nonirritant ingredients, which may also improve the appearance of the skin. Retinoid products intended for topical use are just one example of a drug with proven efficacy against the signs of cutaneous aging (Stratigos and Katsambas 2005).

Antioxidant ingredients have also emerged as the largest sector of active ingredients used in cosmeceutical products (Brandt et al 2011). Topical antioxidants are powerful inhibitors of free radical species. Free radicals cause damage to the skin both at a cellular level through direct oxidation, which damages proteins and cellular membranes, and also through activation of transcription factors for matrix metalloproteases, such as collagenases (Montanari et al 2013). This damage has been shown to greatly increase the visible signs of aging.

Plant-derived ingredients are also increasing in popularity in the cosmeceutical industry due to consumer demands for “more natural” product lines (Draelos 2014).

1.4 ANTHOCYANINS

*Chemical Properties and Structure*

Anthocyanins are a class of water-soluble pigments known as flavonoids, responsible for many of the reds, blues and purples observed in nature (Anderson and Jordheim 2014). They consist of an anthocyanidin aglycone, a sugar moiety, and often an acyl group attachment (Figure 1.3). The aglycone is composed of a C6-C3-C6 carbon...
skeleton, where the first and second benzene rings are referred to as ring A and B, respectively (He and Giusti 2010).

![Anthocyanin skeleton](image.png)

<table>
<thead>
<tr>
<th>Anthocyanidins</th>
<th>Substitutes</th>
<th>( \lambda_{\text{max}} ) (nm) visible spectra</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pelargonidin</td>
<td>H</td>
<td>H</td>
<td>271</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>OH</td>
<td>H</td>
<td>287</td>
</tr>
<tr>
<td>Delphinidin</td>
<td>OH</td>
<td>OH</td>
<td>303</td>
</tr>
<tr>
<td>Peonidin</td>
<td>OCH(_3)</td>
<td>H</td>
<td>301</td>
</tr>
<tr>
<td>Petunidin</td>
<td>OCH(_3)</td>
<td>OH</td>
<td>317</td>
</tr>
<tr>
<td>Malvidin</td>
<td>OCH(_3)</td>
<td>OCH(_3)</td>
<td>331</td>
</tr>
</tbody>
</table>

Figure 1.3: Structure of anthocyanin aglycones commonly found in fruits and vegetables.

The majority of anthocyanins found in nature are based on six aglycone structures, which vary due to substitutions on their B-ring: pelargonidin (Pg), cyanidin (Cy), delphinidin (Dp), peonidin (Pn), petunidin (Pt), and malvidin (Mv) (He and Giusti 2010). The substitutions occurring at the R\(_1\) and R\(_2\) positions on the B-ring are
commonly by H, OH, and OCH₃ groups. These variations in structure are responsible for the slight differences in color of the anthocyanins and their physical properties (Wu 2014). The glycosylation of the aglycone can occur with a variety of mono, di, and tri-saccharides, and commonly occur at the C3, C5, or C7 positions (Wrolstad 2004). The sugar moiety can further be acylated by aliphatic acids, such as malonic, acetic, malic, succinic, and oxalic; or by aromatic acids, such as p-coumaric, caffeic, ferulic, sinapic, gallic, and p-hydroxybenzoic (Giusti and Wrolstad 2003).

**Stability and pH Related Changes**

The stability and color of anthocyanins are affected by many factors including their chemical structure, concentration, pH, light, temperature and interactions with other compounds (Sari *et al* 2011). Depending on the pH, four different species of anthocyanin structure are possible (Castenada-Ovando *et al* 2008). At pH 1, the flavylium cation is the predominant species, and presents as red and purple colors. Between the pH of 2 and 4, the quinoidal blue species is dominant. A colorless species and a pale-yellow species, the carbinol pseudobase and the chalcone, are present at pH 5 and 6, respectively (Figure 1.4). These species can coexist in the pH range of 4-6 (Castenado-Ovando, *et al* 2008).
The stability of the anthocyanin is influenced by substitutions on the B-ring, and the presence of additional hydroxyl or methoxyl groups (Fleschhut et al 2006). The addition of glycosylation and acylation of anthocyanins has shown to greatly improve their stability to pH, light and heat treatments (Giusti and Wrolstad 2003). This increased stability is due to intramolecular and intermolecular copigmentation, self-association, complexation with metals, and inorganic salts present (Giusti and Wrolstad 2003).
Current Uses as Colorants

Currently, anthocyanin extracts are used in varying food products (Wrolstad and Culver 2012). They are used in soft drinks, fruit jams/jellies, canned fruit, confectionary jellies, dairy products like yogurt, dry mixes such as acidic drink and dessert mixes, and some frozen dairy products (Mateus and Freitas 2009). Soft drinks have proven the most effective food matrix for anthocyanins, and resist degradation the best. Use of anthocyanins in cloudy beverages may produce undesirable effects such as a “blueing”. Anthocyanins from grape extracts are the most effective type of anthocyanin used in soft drinks currently because they are more stable in the presence of sulfur dioxide, a common preservative used in food products (Mateus and Freitas 2009).

Anthocyanins, and other natural compounds, are capable of producing color at very low quantities and tend to be more powerful colorants than synthetic forms (Henry 1996). Other than grapes, many anthocyanins have recently been shown to be stable in food-matrixes. Fruit juice concentrates from blackcurrant, elderberry, cranberry, cherry, and plant extracts from oxalis have been used (Pazmino-Duran et al 2001).

Anthocyanin compounds are not limited to fruits, and vegetable extracts are commonly used to restore lost food coloring after processing. Examples of vegetable extracts used are from red cabbage (Dyrby et al 2001), red-skinned potatoes (Rodriguez-Saona et al 1998), radishes (Giusti and Wrolstad 1996), and black carrots (Stingzing et al 2002). Vegetable extracts are extremely high in the acylated forms of anthocyanins, which as previously stated have higher pigment stability through processing (Giusti and Wrolstad 1996). This feature of the vegetable extracts has encouraged research into their
application in food systems. These acylated anthocyanins have also proven to be less susceptible to enzymatic degradation (Rommel et al 1992). They are also more resistant to degradation from light and temperature changes (Inami et al 1996).

1.5 HEALTH BENEFITS OF ANTHOCYANINS

Antioxidant Activity and Free Radical Scavenging

The molecular structure of anthocyanins directly influence their ability to act as antioxidants (Wu 2014). Structurally, anthocyanins are positively charged, have varying arrangements of hydroxyl groups and can have either electron-donor or electron-acceptor substitutions within their B-ring structure, which makes them effective hydrogen-donors. These characteristics of anthocyanins, allow them to donate protons to reactive free radical species, effectively ending formation of new free radicals. It is this property that allows them to be protective against oxidative damage, which has been linked to aging and diseases such as cancer (Bueno et al 2012). They neutralize reactive oxygen species (ROS), such as superoxide and reactive nitrogen species (Bueno et al 2012). The antioxidant capacity of anthocyanins depends largely on the chemical composition, such as the number of hydroxyl groups present, the catechol moiety on the B-ring, the hydroxylation and methylation patterns, and the acylation of the phenolic compounds present.

Early studies of the antioxidant capacity of anthocyanins focused on their direct free radical scavenging ability based on tests performed by the food industry on tert-butylhydroquinone (TBHQ) and butylated hydroxytoluene (BHT) (Wu 2014). These
types of studies investigated the free radical scavenging ability of anthocyanins and anthocyanin-rich extracts in chemical-based assays and in vivo tests.

Anthocyanins are also able to chelate metal ions of Iron (Fe) and Copper (Cu) (Halliwell 2012). These metal ions have the ability to produce reactive radicals, such as superoxide anion radicals and nitric oxide in biological systems. These metals, therefore, are a common target for in vivo antioxidant strategies to minimize their availability for Fenton reaction and other free-radical reactions (Halliwell 2012).

Common chemical-based antioxidant assays employed to evaluate anthocyanins antioxidant ability include ORAC, DPPH, ABTS, TEAC, and TRAP (Wu 2014). Due to anthocyanins chemical structure, they are excellent hydrogen donors and show high antioxidant capacity in the above chemical-based assays, which involve hydrogen transfer reactions (HAT) (Prior et al 2005; Huang et al 2005).

Chemical-based assays have been noted to have some limitations due to inconsistencies with their biological relevance (Wu 2014). Cell-based in vitro assays were developed to better understand the properties of dietary antioxidants, such as anthocyanins. Examples of cell-based in vitro models that have been employed in the testing of anthocyanins include: HepG2, Caco-2, AGS, L-929, Int 407, RAW264.7, MKN-45, MCF-7, HT29, RBC and PMN cells (Cheli and Baldi 2011). Examples of studies conducted on the antioxidant activities of anthocyanins in vitro cell-based models can be seen in Table 1.5.
In addition to cell-based models, studies involving \textit{in vivo} antioxidant activity of anthocyanins have shown increases in antioxidant capacity and changes in biomarkers in biological samples (Wu 2014). More studies need to be performed; however, to fully elucidate the direct role anthocyanins have \textit{in vivo} on ROS and acting as antioxidants on the cellular level. Many of the health benefits associated with anthocyanins are assumed to be directly associated with their antioxidant activity, which is affected by: the quantity

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Table 1.5: Examples from the literature of anthocyanin antioxidant activity confirmed in cell-based \textit{in vitro} models.

<table>
<thead>
<tr>
<th>ANTHOCYANIN SOURCE</th>
<th>CELL LINE</th>
<th>BIOMARKERS</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Grape, Strawberry, Cherry, Sour Cherry</td>
<td>HT29</td>
<td>NF-kB</td>
<td>Kulisic-Bilusic \textit{et al} 2009</td>
</tr>
<tr>
<td>Chokeberry</td>
<td>Caco-2</td>
<td>Cell Proliferation</td>
<td>Stanisavljevic \textit{et al} 2015</td>
</tr>
<tr>
<td>Bilberry, and Blueberry</td>
<td>Caco-2, HepG2, EA.hy926, A7r5</td>
<td>Cellular Antioxidants</td>
<td>Bornsek \textit{et al} 2012</td>
</tr>
<tr>
<td>Cy-3-G</td>
<td>HepG2</td>
<td>AMP-Kinase, Acetyl-CoA carboxylase, Carnitine palmitoyl transferase</td>
<td>Guo \textit{et al} 2012</td>
</tr>
<tr>
<td>Chinese Bayberry</td>
<td>SGC7901, AGS, BGC823</td>
<td>Cell Proliferation, MMP-2</td>
<td>Sun \textit{et al} 2012</td>
</tr>
<tr>
<td>Black Rice</td>
<td>SCC-4, Huh-7, HeLa, SKHep-1</td>
<td>MMPs, urokinase-1 plasminogen</td>
<td>Chen \textit{et al} 2006</td>
</tr>
<tr>
<td>Cherries</td>
<td>HT29, MKN45</td>
<td>Cell proliferation</td>
<td>Serra \textit{et al} 2011</td>
</tr>
<tr>
<td>Red Sorghum Bran</td>
<td>MCF-7</td>
<td>Cell proliferation</td>
<td>Devi \textit{et al} 2011</td>
</tr>
<tr>
<td>Raspberry, Black Raspberry, Red Currant, Black Currant, Bilberry</td>
<td>HeLa, Fem X, LS 174, MCF-7, PC-3</td>
<td>Cell proliferation</td>
<td>Konic-Ristic \textit{et al} 2011</td>
</tr>
</tbody>
</table>
consumed, the metabolized or absorbed amount, and the concentration found in the tissue or plasma (Wu 2014).

The antioxidant properties of anthocyanins may also be beneficial through indirect methods such as regulation of gene expression and signaling pathways that respond to oxidative stress. Anthocyanins have been shown to have a protective effect against oxidative enzymes *in vivo* such as glutathione peroxidase (GSH-Px), superoxide dismutase (SOD1 and SOD2), glutathione S-transferase (GST), glutathione reductase (GSR), thioredoxin reductase, catalase, MnSOD, Gpx1/2, and Gsta1 (Hou *et al* 2010, Hassimoto and Lajolo 2011, Alvarez-Suarez *et al* 2011, Wu *et al* 2010, Murapa *et al* 2010).

Anthocyanins have also been shown to regulate redox-sensitive signaling molecules (Rahman *et al* 2006). A potential target of anthocyanin antioxidant activity on redox molecules is the regulation of nuclear factor-erythroid-2-related factor 2 (Nrf2) (Jung and Kwak 2010). Nrf2 is considered the key regulator of the antioxidant response *in vivo* and it modulates the expression of hundreds of genes that are involved in oxidative signaling pathways (Hyberston *et al* 2011). A recent study investigated the effect of cyanidin on redox-sensitive signaling pathways and found that cyanidin was able to cause a synergistic effect with the PPAR agonist, troglitazone, to deplete the down-regulation of lipid metabolism and H$_2$O$_2$-induced cytotoxicity (Shih *et al* 2012). The study also reported that cyanidin was about to prevent oxidative damage from metabolic dysfunction through the regulation of mitogen-activated protein kinases (MAPKs), as well as Nrf2.
Anthocyanins from purple sweet potato were also shown to attenuate the DMN-induced increases in serum alanine aminotransferase and aspartate aminotransferase activity (Hwang et al 2011). This study suggested that anthocyanins were able to act as antioxidants by activating the Nrf2 signal pathway and to reduce inflammation by NF-kB.

Delphinidin was also recently shown to reduce psoriasiform lesions in mice by inducing caspase-14 expression (Pal et al 2015). The authors reported a reduction in inflammatory cells and mRNA expression of inflammatory cytokines after topical application of delphinidin. The topical treatment also increased expression of activator protein-1 transcription factor proteins and decreased the expression of biomarkers for cell proliferation (Pal et al 2015). This further suggests the anti-inflammatory effects of anthocyanins in skin disorders.

Anti-carcinogenic Properties

Oxidative damage and ROS have been linked to many chronic illnesses including cancer (Wu 2014). Due to this association, anthocyanins have been implicated as chemopreventive compounds for a wide range of cancers. Although the progression of cancer is a complex process, damage to DNA by oxidation is commonly linked to its development (Valko et al 2007). The chemopreventative role of anthocyanins has been extensively documented in vivo and in vitro (Hou 2003, Wang and Stoner 2008). Anthocyanins have been implicated as cancer-preventative for colon cancer, breast
cancer, esophageal cancer, lung cancer, gastric cancers, leukemia, prostate cancer, as well as skin cancer (Jing and Giusti 2011).

In regards to skin cancer, Afaq et al (2007) reported the chemopreventive effect of delphinidin against UVB-induced signs of skin cancer in hairless mice. Topical application of delphinidin to murine skin effectively inhibited apoptosis and biomarkers of DNA damage, including cyclobutane pyrimidine dimers and 8-OHdG (Afaq et al 2007). This study suggested the ability of delphinidin to protect the skin cells against DNA photo-damage due to UVB-radiation.

Cyanidin 3-glucoside treatments were also shown to decrease the number of non-malignant and malignant tumors in the skin caused by 12-O-tetradecanolyphorbol-13-acetate (TPA), a potent tumor promoter, in murine skin (Ding et al 2006). In another similar study, the polyphenolic fraction from grape seeds was shown to inhibit TPA-induced epidermal ornithine decarboxylase (ODC) and myeloperoxidase (MPO) activity, which are both associated with the progression of skin cancer (Bomser et al 1999).

Delphinidin was also shown to inhibit UVB-induced MMP-1 expression in human dermal fibroblasts by targeting NADPH oxidase (Lim et al 2013). This study reported that delphinidin was able to suppress MKK4-JNK1/2, MKK3/6-p38, MEK-ERK1/2 phosphorylation. The authors also reported a higher suppression of NOX activity by delphinidin than apocynin, which is a pharmaceutical NOX inhibitor (Lim et al 2013). These studies further suggest the protective role of anthocyanins against skin cancer through direct ROS scavenging, and indirect modulation of gene expression to oxidative stress.
Photoprotective Role

Skin aging occurs through an intrinsic biological process and causes the appearance of skin wrinkles, dryness, thinning of skin, uneven coloring, and loss of subcutaneous fat (Giacomoni 2008). While the manifestation of skin aging will vary markedly between individuals, many factors can accelerate the skin-aging process. These factors include weakened repair mechanisms of deoxyribonucleic acid (DNA), degradation of mitochondrial functions, slower repair of the extracellular matrix (ECM) of the skin, and alterations in cell cycle regulation (Rojo et al 2014). The most important external factor in acceleration of skin aging is exposure to UV radiation. Exposure to UV radiation, especially UVB radiation (280-320nm), has been shown to trigger inflammatory pathways and oxidative damage in the epidermis and dermis (Rojo et al 2014). UVA radiation (320-400nm), although less powerful than UVB light, can penetrate deeper into the skin (Afaq et al 2005).

Chronic exposure to excessive UV radiation leads to accelerated skin aging (photoaging), hyperkeratosis, and precancerous lesions (Farage et al 2009). One of the major contributors to UV-induced skin damage is an overexpression of metalloproteinases (MMPs), which degrade proteins, especially collagen and elastin, in the ECM (Rojo et al 2014). This damage eventually leads to a loss of skin elasticity and resilience, resulting in skin wrinkles. UV radiation has also been shown to increase redox-sensitive transcription factors, such as nuclear factor kappa-B (NF-kB) and activator protein-1 (AP-1) (Natarjan et al 2014) (Figure 1.5). The visible changes that occur with skin aging, along with the increasingly high diagnoses of skin cancer in the
United States, have placed a high demand on researchers to find active compounds that can help combat UV-induced skin damage (Rojo et al 2014).

In nature, anthocyanins accumulate within the vacuoles of epidermal cells in plant tissues (Silva et al 2012). This accumulation has long been associated with the protection of the plant cells from excessive solar radiation. The flavylium cation form of anthocyanins strongly absorbs UV light and green light, which aids in its photoprotective role (Afaq 2011). The mechanism for the photoprotection occurs by excited-state proton transfer of the absorbed radiation to heat in less than a nanosecond (Silva et al 2012). Anthocyanins absorb strongly in the visible and UV ranges, especially in the 500-550 and

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**Figure 1.5:** Photodamage and stress responses in the skin due to UV light interactions with photosensitizers resulting in ROS and photoaging (Natarajan et al 2014).
280-320nm ranges (Harborne 1958). The ability of anthocyanins to absorb UV light varies with specific aglycones, sugar moieties, and acylation patterns.

In plants, more than a third of anthocyanins occurring are acylated on their sugar moieties attached at the 3-position of the C-ring (Silva et al 2012) (Wu 2014). When the acylation occurring is an aromatic organic acid, such as cinnamic acid structures, absorption in the UV-B range of 280-320nm is greatly increased (Stintzing and Carle 2004). Acylation specifically with coumaric acid, caffeic acid, and ferulic acid has shown increased UVB radiation absorption (Harborne 1958). Due to the photoprotective role of anthocyanins in plant cell tissue, it stands to reason that similar photoprotection could be observed on human skin (Rojo et al 2014).

A recent in vitro study showed that when anthocyanins from purple sweet potato were incorporated into a cosmetic formulation, at a concentration of 0.61mg/100g of cream, they were able to absorb 46% of the UV radiation (Chan et al 2010). This in vitro study suggests that anthocyanins in relatively low doses may reduce the amount of UVB radiation reaching the epidermis; therefore, their topical application may prevent UV-induced skin damage.

Studies investigating anthocyanins preventative role in UV-induced skin damage in cellular and animal models have revealed the pharmacological mechanisms for this behavior. A recent study investigated the potential of pomegranate-derived anthocyanin extracts and juices to prevent UVB-induced damage to the dermis on reconstituted human skin (EpiDerm ™ FT-200) (Afaq et al 2009). In this study, the pomegranate-derived anthocyanins were able to significantly inhibit protein oxidation and oxidative damage to
DNA. The authors suggested the extracts were also able to ameliorate the UVB-induced expression of different MMPs, such as collagenase, gelatinase and elastase (Afaq et al 2009). Similar findings were reported with a study investigating anthocyanins from blueberry extract on the prevention of UVB-induced expression of MMPs and upregulation of UV-induced suppression of collagen formation (Bae et al 2009).

Cimino et al (2006) also reported that cyanidin-3-glucoside (C3G) affectively inhibited translocation of transcription factors NF-kB and AP-1 in human keratinocytes. NF-kB and AP-1 are the main modulators for inflammatory responses in the skin and their inhibition of these signaling pathways could provide multifaceted protoprotection (Rojo et al 2014). This study also found that C3G inhibited overexpression of IL-8, caspase-3 activation, and DNA fragmentation in the human keratinocytes; further suggesting a protective effect of C3G against UV-induced skin damage, as well as prevention of psoriasis (Cimino et al 2006).

Anthocyanins were also shown to reduce UVA-induced reactive oxygen species (ROS) formation and lipid oxidation in human keratinocytes (Svobodova et al 2008). Another research group has reported protective effects of anthocyanins from black soybean coats both in in vitro keratinocytes and in vivo hairless mice (Tsoyi et al 2008). The protective effect of delphinidin, another ubiquitous anthocyanin in nature, has also been determined against UVB-induced oxidative damage and apoptosis in human HaCaT keratinocytes and mouse skin (Afaq et al 2007).

Another important area of photoprotection is prevention of hyperpigmentation (Fisk et al 2014). Pigmentation disorders are one of the highest diagnosed skin disorders
by dermatologist (Halder et al 1983). Hyperpigmentation occurs when melanin is deposited in excess in the skin, which can be exacerbated by UV exposure (Fisk et al 2014). In a clinical setting, hyperpigmentation appears as brown or blue discoloration of the skin depending on whether the melanin formation occurs in the epidermis or dermis (Fisk et al 2014). Inhibition of melanin production or deposition can reduce the clinical manifestation of hyperpigmentation. One of the main mechanisms for inhibiting melanin production is through the inhibition of tyrosinase, which is the key catalase for oxidative reactions in melanin synthesis (Parvez et al 2007). The synthesis of melanin can be seen in Figure 1.6 (Gillbro and Olsson 2011).

![Figure 1.6: Melanin formation pathway (Gillbro and Olsson 2011). Melanin synthesis begins with the formation of L-DOPA via L-Phenylalanine and L-Tyrosine catalyzation by phenylalanine hydroxylase (PAH), tyrosinase, and tyrosinase hydroxylase-1 (TH-1).]
In recent years, the protective role of anthocyanins against hyperpigmentation has been investigated. A study by Hanamura et al (2008) investigated the skin-lightening effects of cyanidin-3-rhamnoside (C3R) and pelargonidin-3-rhamnoside (P3R). The authors reported that C3R and P3R were highly effective at suppressing melanogenesis through inhibition of tyrosinase activity in melanocytes when orally ingested (Hanamura et al 2008). The authors suggested that noncompetitive inhibition was the mechanism of action for the anthocyanins studied.

Another study investigated the anti-tyrosinase activity of crude ethanol extracts of grape seeds and skins (Hsu et al 2012). The authors found similar results reported by Hanamura et al; however, they suggested a mixed-type inhibition as the mechanism for tyrosinase inhibition by the grape extracts (Hsu et al 2012).

Lai et al (2012) reported an anti-tyrosinase activity of anthocyanins from black soybean sprouts that reached 98% inhibition of tyrosinase in vitro. The authors also tested the allergic response of using black soybean extracts on human skin and found no allergic response on human patch tests; indicating a potential use for these extracts in cosmetic formulas (Lai et al 2012).

Another study investigated the protective effects of anthocyanins against melanogenesis in human melanocytes when incorporated into liposomes (Hwang et al 2013). This study reported suppression of melanogenesis by liposome-encapsulated anthocyanins through inhibition of tyrosinase activity, as well as attenuation of the expression of tyrosinase protein and microphthalmia-associated transcription factor (MITF).
The skin-lightening effect of anthocyanins from grapes and blackcurrants were also observed when incorporated into protein-rich matrices for topical delivery (Plundrich et al 2013). This study also found that the extracts had an antimicrobial effect, which again suggests a role for anthocyanins in topical formulas.

The growing body of evidence suggests that anthocyanins may act in a protective role against UV-induced damage on human skin; however, clinical studies need to be utilized to fully elucidate the effectiveness of anthocyanins as photoprotective compounds. One such clinical study reported that a formulation containing anthocyanins and glutathione effectively reduced skin erythema from radiation therapy in breast cancer patients (Enomoto et al 2005). However, more work needs to be done to determine an effective dose and response in clinical settings.

1.6 DERMAL PENETRATION

The Skin Barrier Function

The main role of the skin is to protect the body from the environment (Gordon 2000). The skin has three major layers: the epidermis, dermis, and the hypodermis (Pillai et al 2010) (Figure 1.7). Stratum corneum (SC) is the outermost layer of the epidermis, typically 10-20μm thick, and acts as the primary barrier function for the skin by preventing the loss of water and electrolytes (Darlenksi et al 2014). Previously the SC was considered to be metabolically inert and described as a “brick-and-mortar” compartment system. However, in recent years the SC model has shifted to one that is metabolically active and acts as a biosensor to environmental factors, causing cascade
effects such as proteolytic activity and DNA synthesis (Darlencki et al. 2014). The outermost pH of the SC is in the pH range of 4.5-5.0, and this acidity increases its barrier function by promoting the growth of normal skin microflora (Pillai et al. 2014). The SC is also considered to be the rate-limiting structure in transcutaneous drug delivery.

**Figure 1.7:** Diagram of the different layers of the epidermis and structural composition aiding in the epidermal skin barrier (Pillai et al. 2010).

The SC of the lips is relatively thin when compared to other skin sites (Chan 2011). The SC of the lips is typically between 3-4 layers thick and the SC of the facial skin is usually around 15-16 layers thick (Chan 2011). This makes the lips an attractive site for delivery of xenobiotics into the body.
Due to the presence of a cross-linked protein coat of corneocytes and a lipid-rich intercellular domain, the SC is highly resilient to penetration (Darlenski et al. 2014). Applying drugs topically to the skin can achieve one of three goals (Trommer and Neubert 2006). The first is to have the active compounds stay on the surface of the skin, as is the case with skin disinfectants and colored cosmetics, these are known as epidermal formulations. The second is to have the active compounds designed to allow for dermal penetration into deeper layers of the skin, such as into the viable epidermis and dermis. These compounds are not intended to pass into the systemic circulation and are known as endodermal formulations (Trommer and Neubert 2006). The third is to have the active compounds pass into the circulation and local activity is not desired.

Drug developers in topical or “trans-dermal” drug delivery routes are interested in increasing permeation of compounds into the skin. Drugs can penetrate the skin through multiple routes; via hair follicles, interfollicular sites, or by penetration through corneocytes and lipid bilayer membranes (Schaefer and Redelmeier 1996) (Figure 1.8).
Figure 1.8: Schematic of the drug penetration routes through the SC (Trommer and Neubert 2006).

The ability to penetrate the SC is dependent on the molecular weight, solubility, and molecular configuration of the compound in question. The flux \( J_{ss} \) of a drug through the stratum corneum is often described by Fick’s first law:

\[
J_{ss} = \left( \frac{D_m c_{s,m}}{L} \right) \times \left( \frac{c_v}{c_{s,v}} \right) \tag{Eq. 2}
\]

Where \( D_m \) is the diffusion coefficient of the drug in the membrane, \( c_{s,m} \) its solubility in the membrane, \( L \) is the diffusion path length across the membrane, \( c_v \) the concentration of the drug dissolved in the vehicle, and \( c_{s,v} \) the solubility of the drug in the vehicle (Higuchi 1960).

Based on Fick’s first law, three permeation enhancement strategies can be surmised: (i) increase the diffusion coefficient, (ii) increase the solubility in the membrane by increasing drug partitioning into the membrane, or (iii) increase the ratio of
the dissolved drug to solubility of the drug in the vehicle, which is obtained by increasing the degrees of saturation of the drug in the vehicle (Moser et al 2001).

The third strategy is based on interactions between the drug and the vehicle to enhance the thermodynamic activity of the drug in the formulation. This can be achieved by increasing the drug concentration in the vehicle or by decreasing the solubility of the drug in the vehicle. The first two strategies are based on the vehicle affecting the barrier function of the SC (Moser et al 2001). Penetration enhancers are a way to promote the transportation of active compounds across the skin barrier by these two strategies.

Drug release profiles from cosmetic formulations are often described using Higuchi’s kinetic model (Higuchi 1962):

\[ \frac{Q}{C_0} = 2 \sqrt{\frac{D_v t}{\pi}} \]  
(Eq. 3)

Where \( Q \) is the cumulative amount of permeated drug through a unit of membrane surface, \( C_0 \) is the initial drug concentration within the vehicle, \( D_v \) is the diffusion coefficient within the vehicle over a given time \( t \) (Zillich et al 2013). \( D_v \) values can be derived from the plot of \( Q/C_0 \) against the square root of time by the slope \( (K_h) \) of the linear regression equation:

\[ K_h = 2 \sqrt{\frac{D_v}{\pi}} \]  
(Eq. 4)

Higuchi’s kinetic model is desirable over that of Fick’s first law (Eq. 1) when investigating the cumulative release profiles of drugs from a vehicle rather than the initial release rate (Zillich et al 2013) (Higuchi 1960). \( K_h \) describes the non-initial release rate and can be used to predict the time dependency of the release profile.
In addition, the concentration profile of the drug can be determined as a function of relative position within the SC by fitting the data to Fick’s second law of diffusion (Pirot et al 1997):

\[
c_x = c_{x=0} \left\{ \left(1 - \frac{x}{L} \right) - \frac{2}{\pi} \sum_{n=1}^{\infty} \frac{1}{n} \sin \left( \frac{n\pi x}{L} \right) \exp \left( \frac{-Dn^2\pi^2t}{L^2} \right) \right\} \quad \text{Eq. 5}
\]

Where \(c\) is the concentration of the drug at a relative position (x), the concentration of drug in the outermost layer of the SC \((c_{x=0})\) is divided by its concentration in the applied vehicle \((c_v)\) provides the partition coefficient of the drug between the vehicle and SC \((K)\), and the diffusion parameter \((D/L^2)\). \(D/L^2\) is the first-order rate constant of the drug diffusivity \((D)\) in the stratum corneum to the thickness \((L)\) squared of the barrier (Wiedersberg and Nicoli 2012).

Fick’s second law of diffusion can be applied to drug diffusion assuming that the dose is infinite, the SC is both homogeneous and the rate limiting barrier and that it contains no drug at time=0, and that the drug diffusion by the underlying microcirculation is rapid compared to the SC (Wiedersberg and Nicoli 2012). The final assumption implies a “sink” condition for the permeant at the interface between the stratum corneum and the viable epidermis (Herkenne et al 2007).

Consequently, the derived constants can be applied across the entire thickness of the SC and has been successfully used to compare bioavailability of delivered drug within different vehicles (Herkenne et al 2007).
Properties of Skin Penetrators and Enhancers

Penetration enhancement of active compounds after topical application can be achieved by the addition of several compounds. These compounds have many mechanisms for increased penetration (Trommer and Neubert 2006) (Figure 1.9). One such mechanism is through the interaction of the penetration enhancer with the polar heads of the lipid bilayer. This interaction disrupts the packing order of the lipids, which allows for the diffusion of hydrophilic compounds. This in turn causes an increased flow of free water molecules between the lipid bilayer, thus allowing for the penetration of polar compounds (Williams and Barry 2004). Water and urea are good examples of hydrating penetration enhancers.

The rearranging of the lipid bilayer by polar enhancers can also affect the hydrophobic sections of the lipids, which can lead to increased penetration of lipophilic drugs (Kalbitz et al 1996).

Lipophilic penetration enhancers can also interact with the hydrocarbon chains of the lipid bilayer; facilitating the penetration of lipophilic drugs through packing order disruption of the hydrocarbon chains (Trommer and Neubert 2006). Consequently, this action leads to the disruption of the polar head groups, which can help explain the diffusion of hydrophilic drugs when a lipophilic enhancer is used.
Figure 1.9: Drug penetration pathways for hydrophilic and lipophilic active compounds aided by penetration enhancers (Trommer and Neubert 2006).

Common penetration enhancers categories used are: alcohols, alkyl-N,N-disubstituted aminoacetates, Azone and its derivatives, cyclodextrin, esters, fatty acids, glycols, pyrollidones, terpenes and terpenoids, sulphoxides, surfactants, and urea and its derivatives (Trommer and Neubert 2006).

Fatty acids, Azone, and terpenes/terpenoids all act by the first proposed strategy discussed above, by increasing the diffusion coefficient (Moser et al 2001). Oleic acid is a well-documented example of this type of penetration enhancement, and acts by disrupting lipid arrangement in the lipid bilayer and reducing barrier function. Propylene glycol, ethanol, and N-methyl pyrrolidone are examples of the second proposed strategy discussed above, which encourage penetration by increasing drug solubility in the skin (Hadgraft 1999).
The use of certain penetration enhancers; however, is limited due to increased irritation of the skin with increased concentrations (Moser et al 2001). Therefore, care must be taken in formulation of transdermal drug delivery systems to ensure a balance between enhancement and acceptability. Both in vitro and in vivo percutaneous studies are used to study the influence of topical formulations on drug delivery and skin barrier function (CDER 1998).
CHAPTER 2: ANTHOCYANIN BASED LIPSTICK FORMULATIONS AND STABILITY TESTING

2.1 ABSTRACT
Colored cosmetics, such as lipstick, have a known psychological effect on perception of attractiveness and morale. Consumer concerns with the safety of synthetic colorants has made the need for alternative natural color sources increasingly urgent. Anthocyanins have been proposed as a natural colorant for food applications; however, their uses in cosmetic formulations have not been previously investigated. The aim of this study was to evaluate the feasibility of anthocyanin extracts as colorants in lipstick formulations. Formulations were assessed on the acceptability of the color for this purpose. Accelerated environmental testing commonly used by the cosmetic industry were used to assess the shelf-stability of the products; changes in color were monitored using UV-spectroscopy and CIELab color readings. Samples were incubated at 20, 37, and 45°C for 12 weeks and also underwent six temperature abuse cycles between 20/37°C and six freeze/thaw abuse cycles from -20/20°C. Determination of shelf-stability was based on changes in the color readings and results were analyzed using One-Way ANOVA and regression modeling. Total monomeric anthocyanin content was calculated for each formulation, using the pH-differential method, to better understand the effect of the storage conditions.
on content and color changes. All formulations exhibited comparable color to commercial lipsticks and shelf stability for over two years based on the accelerated testing conditions. Formulations containing cyanidin as their main anthocyanin source proved to be the most stable (elderberry, purple corn, and purple sweet potato). Our results demonstrate the potential for anthocyanins to act as a stable alternative colorant in cosmetic formulations.

**Key Words:** anthocyanins, natural colorants, cosmetics, stability testing

### 2.2 INTRODUCTION

Color is a crucial element for the cosmetic industry because it has a direct and immediate effect on consumer self-perception of attractiveness (Morante and Diogiovanna 2006). The strongest evidence for the psychological influence of colored cosmetics, such as lipstick, is the phenomenon of increased spending by women on attractiveness-enhancing products during times of economic downturn, nicknamed the “lipstick effect” (Hill *et al* 2012). Lipstick has been associated with boosts in morale, as well as increased attractiveness to potential mates, since the Great Depression when lipstick sales skyrocketed unexpectedly (Hill *et al* 2012).

Consumer concerns with the safety of synthetic colorants have been growing in recent years. Eosin and fluorescein based colorants used in cosmetics (D&C Red #21, D&C Red #22, and D&C Red #27) are often associated with photosensitization-induced cheilitis, or inflammation of the mouth (Freeman and Stephens 1999). Azo dyes are frequently associated with contact dermatitis, especially with repeat exposure (Saitta *et al* 2009). Azo dyes, such as D&C Red #6, D&C Red#7, and FD&C Yellow #5, are some of
the most commonly used organic pigments in lipstick formulations (Barone et al 2002). Carmine, a natural source of color in cosmetics, has also been shown to produce contact dermatitis on the lips and skin (Sarkany et al 1967; Shaw 2009).

The combination of concerns with synthetic colorants and an increase in consumer demands for more “natural” ingredients in cosmetics has prompted an increase in botanically-derived products in cosmetics (Draelos 2014).

One such alternative color source is a group of water-soluble flavonoids known as anthocyanins (Wrolstad and Culver 2012). Anthocyanins are responsible for many of the reds, purples, and blues found in fruits and vegetables. While ubiquitous in nature, there are six common aglycones, cyanidin; delphinidin; malvidin; pelargonidin; peonidin; and petunidin, with various types of glycosylation and acylation.

They have been frequently used in the food industry as a natural colorant source (Aberoumand 2011). Their use as replacement for synthetic colorants, such as FD&C Red #40 (Giusti and Wrolstad 2003), FD&C Red #3 and FD&C Blue #2 (Ahmadiani et al 2014) have also been proposed.

The stability and color of anthocyanins is influenced by many factors including pH, temperature, light exposure, and interactions with other compounds (Sari et al 2011). Substitutions on the B-ring of anthocyanins, and the presence of additional hydroxyl or methoxyl groups influence their stability (Fleschhut et al 2006). However, anthocyanin sources with additional glycosylations and acylations have been shown to exhibit high resistance to these degradation factors (Giusti and Wrolstad 2003).
To date, the majority of the research surrounding the use of anthocyanins has been focused on aqueous food systems (Kim et al 2003). The potential of certain anthocyanin sources to match the colors typically used in lipstick formulations, make them an attractive alternative to synthetic colorants.

The aim of this study was to investigate the potential use of anthocyanin extracts from sources known for their high stability, as natural colorants in lipstick formulations by evaluating their ability to produce comparable shades of lipstick to that of commercial brands, as well as their color stability during accelerated environmental testing. Non-acylated and acylated cyanidin based sources, such as elderberry; purple carrot; purple corn; and purple sweet potato, were chosen due to their vast abundance in nature and high stability (Bueno et al 2011). Red radish, a source of acylated pelargonidin, was chosen due to its reported stability and potential as an alternative to synthetic red colorants (Giusti and Wrolstad 1996). Red grape, which contains all six aglycones, was also investigated to better understand the effect of chemical structure on color stability.

2.3 MATERIALS AND METHODS

Materials

Elderberry, purple carrot, purple sweet potato, and red radish dried extract were provided by DD Williamson & Co., Inc. (Louisville, KY), and the purple corn and red grape skin dried extracts were provided by Artemis International (Fort Wayne, IN). The base of the lipstick formulations and the colorants: D&C Red#6 and #7, Mica Red, and Carmine were purchased from MakingCosmetics, Inc. (Snoqualmie, WA). Five
commercial brands of lipstick containing synthetic colorants and one brand containing natural colorants were purchased from a local department store (Columbus, OH). Black lip balm containers were purchased from a local company, Bulk Apothecary (Streetsboro, OH). Glass slides were purchased from Fisher Scientific Inc. (Fair Lawn, NJ). Reagents used were acetone, ethanol, and methanol, and were purchased from Fisher Scientific Inc. (Fair Lawn, NJ).

Methods

HPLC-PDA Analysis

To determine anthocyanin content of the dried extracts, high performance liquid chromatography (HPLC) was used. A reverse-phase high performance liquid chromatograph (HPLC) system (Shimadzu Corporation, Tokyo, Japan) consisted of LC-20AD prominence liquid chromatograph, a SPD-M20A prominence diode array detector. LCMS solution Ver3.30 software was used. A reversed-phase 3.5 μm Symmetry C18 column (4.6x150mm, Waters Corp., MA, USA) fitted with a 4.6 x 150 mm Symmetry 5 microguard column (Waters Corp., MA, USA) was used. Solvents and samples were filtered through 0.45 μm polypropylene membrane filters (Whatman). Separation was achieved by using the following gradient mobile phase: Binary gradient conditions 0 % B 5 min, 5% to 15% B, in 33 min, 15% to 40%B, in 38 min, 40%-0%B in 43 minutes, and 0%B in 53min. Solvent A was 4.5% (v/v) formic acid in water and B was 100% acetonitrile. A 1.0 ml/min flow rate was used and injection volumes were 30 μl. Spectral
information was collected from 260-700 nm, and elution was monitored at 280nm and 520nm.

**Total Monomeric Content**

The total monomeric anthocyanin content for the extracts was measured in 1-cm cuvettes using a spectrophotometer (Shimadzu UV-2450 Spectrophotometer Kyoto, Japan) using the pH-differential method as described by Giusti and Wrolstad (2001). The absorbance at pH 1.0 was determined for the extracts using a potassium chloride buffer with HCl after a 15 minute equilibration time. The absorbance at pH 4.5 was determined for the extracts using a sodium acetate buffer with HCl after a 15 minute equilibration time. The anthocyanin content, expressed as cyanidin-3-glucoside, was determined using the following equation:

\[
\text{Total monomeric anthocyanin content (mg/L)} = \frac{Abs_{pH\ 1.0} - Abs_{pH\ 4.5} \times DF \times 1000}{\epsilon \times d} \quad \text{(Eq. 1)}
\]

Where MW= molecular weight of the major anthocyanin present, DF= dilution factor, \( \epsilon \) = molar absorptivity of the major anthocyanin present, and d=path length (1 cm). Results were then used to determine the anthocyanin content in the dried extract powder based on initial weights and were recorded as mg/g.

**Lipstick Formulation**

Formulations were based on recommendations in the Society of Cosmetic Chemists (SCC) Monograph Number 8: Lipstick Technology (Barone et al 2002). All dried extracts were incorporated as 8% of the final weight (w/w) of each lipstick.
formulation based on preliminary data. Dried extracts were initially weighed out and subjected to a grinding process with a mortar and pestle prior to being added to the lipstick manufacturing. Formulations underwent a wet grinding process in which castor oil was used at a 1:3 ratio (pigment:oil) and silica was included at 1% of the final weight (w/w), to increase uniformity in the final products. Initially, the lipstick base was weighed and placed in a water bath at 70°C with gentle stirring until completely melted. The pre-ground dried extracts were then poured directly into the hot lipstick base and gentle stirred until uniform color was achieved. The lipstick formulas were then poured directly into the lip balm containers and allowed to cool at 4°C until completely solid.

**Accelerated Environmental Testing**

The parameters used for the shelf stability testing were based on the guidelines set by The European Cosmetic, Toiletry and Perfumery Association (COLIPA 2004) and by Cannel (1985). The six formulations tested for shelf stability were formulas using elderberry, purple corn, purple carrot, grape skin, purple sweet potato and red radish as their pigment sources, respectively. Once the formulas were allowed to cool, 0.5 grams of each was secured between two glass slides for use in the stability testing. The formulas were stored in the dark at each temperature in triplicate. The temperatures tested were 20°C, 37°C, and 45°C. Baseline color measurements were taken on day 0. Color measurements were taken again on day 1 to account for any changes that may have taken place in the initial 24 hour period. Color measurements were then taken on a weekly basis until the conclusion of the testing for each temperature at 12 weeks.
In addition, formulas were subjected to two different temperature abuse cycles. The first cycle was 20°C to 37°C. One cycle included being held at 20°C for 24 hours and then subsequently placed in 37°C for 24 hours. The second cycle aimed to test the formulation stability to freeze/thaw abuse. Once cycle included being held at -20°C for 24 hours and then subsequently placed in 20°C for 24 hours. Color measurements were taken after the completion of each 48 hour cycle. The cycles were repeated six times for all samples. The six formulations tested for shelf stability were decided upon preliminary data at 20°C. Following the incubation period at each temperature, samples were collected and placed at -20°C until further analysis.

**Colorimetric Analysis**

Colorimetric analysis was used to determine the similarity of the initial color of the formulations to commercial brands and to monitor color changes during the accelerated environmental testing. Formula color measurements were measured using a reflectance specular included (RSI) mode with a 1cm path length, with a D65 light source and 10° observer angle, on a Color Quest XE Colorimeter (Hunter Associates, Virginia, USA). All readings were replicated (n=3) and reported in the Hunter CIELab system using Easy Match Software Ver3.62 (Hunter Associates, Inc.).

Color measurements were then averaged after each color reading. Changes in L*, a*, b*, C*, h, and ΔE* were recorded against the baseline measurements taken on day 0. L*, is the measurement of the lightness of a sample, with higher values (0-100 scale) indicating a lighter sample. The a* scale is the measurement of red versus green, where a
positive number indicates a red color. Conversely, b* is a measurement of blue versus yellow, where a negative number indicates a blue color. C*, or chroma, is a measurement of intensity or saturation of color. The hue angle, h°, is a measurement of where the sample color falls on the color wheel. Delta E, ΔE*, is a mathematical description of the distance between two samples in the L*a*b* color space. Initial color measurements were used as standards for ΔE* determination based on the following equation:

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

(Eq. 2)

Where \(L_i^*, a_i^*,\) and \(b_i^*\) refer to initial measurements taken on day 0, and \(L_0^*, a_0^*,\) and \(b_0^*\) refer to the color measurement recorded at each time point.

*Extraction of Anthocyanins from Lipstick Formulations*

To understand the effects the stability testing had on the anthocyanins, a method for extracting the pigments out of the formulations was developed. Initially, samples were collected from the microscope slides and weighed. The frozen samples were then powdered using liquid nitrogen and a tissuemeizer (Fisher Scientific, Fair Lawn, NJ). Three extraction solvents were used to extract the anthocyanins from the lipstick base in equal parts: acetone (100% and 70%), acidified ethanol (0.01% HCl) and acidified deionized distilled water.

The powderized samples were then eluted with the extraction solvents and deionized distilled acidified water (0.01% HCl) and vigorously mixed with the tissuemeizer. To cause a phase separation, equal parts (v/v) of chloroform was added to
each extraction and mixed. The extracts were then centrifuged at 10,000rpm for 10 minutes, or until a complete separation was achieved. The aqueous supernatant was then collected in a 250mL-boiling flask and the excess solvent was evaporated off using a Buchii rotary evaporator (Brinkmann Instruments, Inc, Westbury, NY). The pigments were redissolved in acidified deionized distilled water and brought to a known volume. The extracts were then stored at -20°C until further analysis was performed. Recovery efficiency was determined using the pH differential method described previously. Recovered extracts were tested for total monomeric content, the results of which were compared to the initial monomeric content and used to determine recovery rates.

Statistical Analysis

Statistical analysis for anthocyanin content and changes in color measurements were done using one-way ANOVA and linear regressions using Minitab Statistical Software ($\alpha=0.05$) and GraphPad Prism Version 6.

2.4 RESULTS AND DISCUSSION

Anthocyanin Sources and Total Monomeric Content

The sources of anthocyanin were initially chosen based on their aglycone profile and acylation (Figure 2.1). Numbers above peaks correspond to tentative peak assignments (Table 2.1).
Figure 2.1: HPLC chromatograms at 520nm of major anthocyanins in extracts. See Table 2.1 for tentative peak assignments.
Table 2.1: List of main anthocyanins tentatively identified in anthocyanin commercial extracts by HPLC-PDA and literature references.

<table>
<thead>
<tr>
<th>Source</th>
<th>Peak #</th>
<th>Peak %</th>
<th>Retention Time (min)</th>
<th>Tentative Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elderberry</td>
<td>1</td>
<td>62.02</td>
<td>4.56</td>
<td>Cy-3-Sam</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>37.83</td>
<td>6.12</td>
<td>Cy-3-Glu</td>
</tr>
<tr>
<td>Purple Carrot</td>
<td>1</td>
<td>3.40</td>
<td>5.07</td>
<td>Cy-3-Xyl(Glu)Gal</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>16.69</td>
<td>5.52</td>
<td>Cy-3-Xyl-Gal</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.13</td>
<td>6.52</td>
<td>Cy-3-Xyl-(p-hydroxybenzoyl)(Glu)-Gal</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6.66</td>
<td>7.38</td>
<td>Cy-3-Xyl-(Sinapoyl)(Glu)-Gal</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>36.76</td>
<td>8.19</td>
<td>Cy-3-Xyl-(Feruroyl)(Glu)-Gal</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>17.20</td>
<td>9.19</td>
<td>Cy-3-Xyl-(Coumaroyl)(Glu)-Gal</td>
</tr>
<tr>
<td>Purple Corn</td>
<td>1</td>
<td>55.06</td>
<td>6.54</td>
<td>Cy-3-Glu</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.10</td>
<td>8.14</td>
<td>Pg-3-Glu</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>18.43</td>
<td>9.32</td>
<td>Peo-3-Glu</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>10.77</td>
<td>11.26</td>
<td>Cy-3-(6&quot;Malonyl)-Glu</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.38</td>
<td>14.31</td>
<td>Pg-3-(6&quot;Malonyl)-Glu</td>
</tr>
<tr>
<td>Purple Sweet Potato</td>
<td>1</td>
<td>8.96</td>
<td>16.55</td>
<td>Cy-3-(Caffeoyl)-Soph-5-Glu</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>23.52</td>
<td>18.13</td>
<td>Pn-3-(Caffeoyl)-Soph-5-Glu</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>23.86</td>
<td>18.16</td>
<td>Peo-3-(Caffeoyl) (p-hydrozybenzoyl)-Soph-5-Glu</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>26.77</td>
<td>19.00</td>
<td>Peo-3-(Caffeoyl) (Feruroyl)-Soph-5-Glu</td>
</tr>
<tr>
<td>Red Grape Skin</td>
<td>1</td>
<td>2.19</td>
<td>7.48</td>
<td>Del-3-Glu</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.22</td>
<td>11.28</td>
<td>Pet-3-Glu</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.90</td>
<td>12.93</td>
<td>Cy-3-Glu</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6.31</td>
<td>15.00</td>
<td>Peo-3-Glu</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>83.39</td>
<td>16.27</td>
<td>Mal-3-Glu</td>
</tr>
<tr>
<td>Red Radish</td>
<td>1</td>
<td>2.61</td>
<td>22.41</td>
<td>Pel-3-(Caffeoyl)-Soph-5-Glu</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7.10</td>
<td>23.29</td>
<td>Pel-3-(Caffeoyl)-Soph-5-(Malonyl)-Glu</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10.51</td>
<td>24.25</td>
<td>Pel-3-(Coumaroyl)-Soph-5-Glu</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7.38</td>
<td>24.39</td>
<td>Pel-3-(Feruroyl)-Soph-5-Glu</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>35.05</td>
<td>26.09</td>
<td>Pel-3-(Coumaroyl)-Soph-5-(Malonyl)-Glu</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>27.43</td>
<td>26.50</td>
<td>Pel-3-(Feruroyl)-Soph-5-(Malonyl)-Glu</td>
</tr>
</tbody>
</table>

1. (Lee and Finn 2007); 2. (Montilla et al 2011); 3. (Barry 2013); 4. (Ahmadiani 2012); 5. (Liang et al 2008)
Monomeric anthocyanin content was determined to help better illustrate the stability of the dried extracts in their respective lipstick formulas (Nicoue et al 2007). Total monomeric anthocyanin content was expressed as milligrams monomeric anthocyanin per gram of dried extract (Table 2.2). Total monomeric concentration ranged from 32.96 mg anthocyanin/g extract for the elderberry powder to 4.38 mg anthocyanin/g extract for the purple carrot powder.

Table 2.2: Total monomeric anthocyanin content (mg anthocyanin/g extract) as determined by the pH differential method for dried extracts in solution.

<table>
<thead>
<tr>
<th>Dried Extract</th>
<th>Total Anthocyanin Content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elderberry</td>
<td>32.96</td>
</tr>
<tr>
<td>Purple Carrot</td>
<td>4.38</td>
</tr>
<tr>
<td>Purple Corn</td>
<td>22.27</td>
</tr>
<tr>
<td>Purple Sweet Potato</td>
<td>19.20</td>
</tr>
<tr>
<td>Red Grape</td>
<td>29.86</td>
</tr>
<tr>
<td>Red Radish</td>
<td>24.20</td>
</tr>
</tbody>
</table>

**Color Comparison of Formulas with Cosmetic Colorants and Commercial Brands**

Color measurements and visual inspection of the formulations were used to determine suitability of coloration for use in lipsticks when compared to commercially available brands and colorants used in cosmetics (Table 2.3).
Table 2.3: Color and hue angle of preliminary anthocyanin-colored lipsticks compared to commercial lipstick samples and samples colored with non-anthocyanin pigments. \( \Delta E^* \) represents the color change over 6 weeks storage at room temperature as compared to their original color for anthocyanin-lipsticks as part of a preliminary selection process.

<table>
<thead>
<tr>
<th>Reference Samples(^a)</th>
<th>Anthocyanin-Colored Lipsticks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hue</td>
</tr>
<tr>
<td>Sample 1</td>
<td>21.40</td>
</tr>
<tr>
<td>Sample 2(^*)</td>
<td>20.05</td>
</tr>
<tr>
<td>Sample 3</td>
<td>15.83</td>
</tr>
<tr>
<td>Sample 4</td>
<td>15.38</td>
</tr>
<tr>
<td>Sample 5</td>
<td>14.53</td>
</tr>
<tr>
<td>Sample 6(^a)</td>
<td>14.44</td>
</tr>
<tr>
<td>Sample 7</td>
<td>8.03</td>
</tr>
<tr>
<td>Sample 8</td>
<td>6.83</td>
</tr>
<tr>
<td>Sample 9(^c)</td>
<td>352.03</td>
</tr>
</tbody>
</table>

\(^a\) Reference samples were commercial brands or lipstick formulations prepared with non-anthocyanin pigments as follows: \(^*\)Formulated with D&C Red#7; \(^\dagger\)Formulated with Mica Red

Formulations with hue angles similar to those of the reference samples (all except red cabbage) were subjected to preliminary shelf stability testing for six weeks at room temperature and color changes were monitored weekly. Formulations with a \( \Delta E^* \), or total change in color, of \( \leq 1 \) were chosen to continue on with stability testing. Only the hibiscus (\( \Delta E^* = 12.82 \)) and strawberry (\( \Delta E^* = 15.10 \)) formulas showed color changes greater than 1, and the significant changes observed for these were deemed unacceptable for further testing.

All chosen formulations fell within the purple to red spectrum based on their hue angles. Elderberry was the most purple formula (initial hue: 318.64°) and red radish was
the reddest formula (initial hue: 14.34°). The remaining formulas were varying shades of pink. The hue angles were compared to those of commercially available lipsticks and lipstick formulations using traditional colorants: D&C Red #7 lakes, mica red, and carmine at identical concentrations.

The commercial brands chosen were shades of orange/red (Sample 1 and 2), red (Sample 3 and 4), dark red (Samples 5 and 6), and varying shades of pink-red to purple-red (Brand 7-9). The hue angle for the red radish formulation was the most similar to the commercial brands, especially for Samples 4-6.

The hue angle of red radish was closest to those observed with the D&C #7 sample (Sample 2, hue angle: 20.05°) and nearly identical to the mica red sample (Sample 6, hue angle: 14.43°). The hue angles for purple carrot (initial hue: 354.44°) and purple corn (initial hue: 350.45°) were very similar to those observed with the carmine lipstick (Sample 9, hue angle: 352.03°). The hue angles for the red grape (1.42°) and purple sweet potato (1.61°) fell slightly above those seen with the carmine lipstick; however, all three were a reddish-pink hue similar to Samples 7-9. The elderberry formulation was more purple than the commercial samples but was still deemed visually acceptable for use in a lip product.

*Color Changes During Accelerated Environmental Testing*

Accelerated environmental testing conditions were implemented to help predict the shelf stability of the anthocyanin-based lipstick formulations. Color measurements, as well as visual inspections, were used to assess the samples at each condition.
Accelerated environmental testing is commonly used in the cosmetic industry to predict shelf life of new products (Cannell 1985). While each company will have their own set of testing parameters, it is commonly assumed that a product showing stability after 10 weeks at 45°C will have an estimated shelf life of two years at room temperature (Romanowski 2009). Moreover, it is generally recommended that lipsticks should be discarded after two years and “natural” lip products be discarded within 12 months of opening, known as the period after opening (PAO) (Begoun 2014). These recommendations were used as a guideline for interpreting the results of the stability testing.

Initial and final measurements for L*, a*, C*, h°, and dE* for all formulas can be seen in Table 2.4. While individual variations occurred within groups and between formulations, some generalizations can be observed. Overall, increased stability was shown with formulations containing cyanidin (elderbery) and acylated cyanidin (purple corn and purple sweet potato).
Table 2.4: Initial and final CIELab color measurements of ACN-lipstick formulations at accelerated environmental testing conditions for 12 weeks. All results shown are averages (n=9).

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Elderberry</th>
<th>Purple Sweet Potato</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Initial</td>
</tr>
<tr>
<td></td>
<td>-20/20°C</td>
<td>-20/20°C</td>
</tr>
<tr>
<td></td>
<td>20/37°C</td>
<td>20/37°C</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>20°C</td>
</tr>
<tr>
<td></td>
<td>37°C</td>
<td>37°C</td>
</tr>
<tr>
<td></td>
<td>45°C</td>
<td>45°C</td>
</tr>
<tr>
<td>Initial</td>
<td>29.09 1.80 2.41 318.64 0.00</td>
<td>29.98 6.43 6.45 361.61 0.00</td>
</tr>
<tr>
<td>-20/20°C</td>
<td>29.33 2.39 2.92 325.40 0.73</td>
<td>31.98 6.14 6.16 355.90 0.66</td>
</tr>
<tr>
<td>20/37°C</td>
<td>29.18 1.94 2.47 321.98 0.26</td>
<td>30.22 5.53 5.54 359.36 1.09</td>
</tr>
<tr>
<td>20°C</td>
<td>28.78 3.07 3.31 338.45 1.78</td>
<td>31.54 5.85 5.89 354.17 1.99</td>
</tr>
<tr>
<td>37°C</td>
<td>27.40 2.99 3.19 339.29 1.97</td>
<td>30.18 4.28 4.40 346.65 2.80</td>
</tr>
<tr>
<td>45°C</td>
<td>27.99 1.94 2.29 328.24 1.27</td>
<td>28.82 4.52 4.57 351.43 2.36</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Purple Carrot</th>
<th>Red Grape</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Initial</td>
</tr>
<tr>
<td></td>
<td>-20/20°C</td>
<td>-20/20°C</td>
</tr>
<tr>
<td></td>
<td>20/37°C</td>
<td>20/37°C</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>20°C</td>
</tr>
<tr>
<td></td>
<td>37°C</td>
<td>37°C</td>
</tr>
<tr>
<td></td>
<td>45°C</td>
<td>45°C</td>
</tr>
<tr>
<td>Initial</td>
<td>33.63 7.97 8.01 354.44 0.00</td>
<td>35.30 9.66 9.66 1.42 0.00</td>
</tr>
<tr>
<td>-20/20°C</td>
<td>33.95 7.55 7.61 353.27 0.93</td>
<td>34.85 9.47 9.47 0.59 0.41</td>
</tr>
<tr>
<td>20/37°C</td>
<td>33.76 7.22 7.28 352.53 0.44</td>
<td>34.88 9.16 9.16 0.81 0.33</td>
</tr>
<tr>
<td>20°C</td>
<td>32.85 9.25 9.27 356.63 1.28</td>
<td>36.88 9.25 9.27 3.33 0.89</td>
</tr>
<tr>
<td>37°C</td>
<td>33.93 8.87 8.92 355.25 1.07</td>
<td>33.00 8.94 8.96 2.79 1.65</td>
</tr>
<tr>
<td>45°C</td>
<td>32.09 6.29 6.45 347.25 3.21</td>
<td>32.82 6.95 6.96 0.19 3.95</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Purple Corn</th>
<th>Red Radish</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Initial</td>
</tr>
<tr>
<td></td>
<td>-20/20°C</td>
<td>-20/20°C</td>
</tr>
<tr>
<td></td>
<td>20/37°C</td>
<td>20/37°C</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>20°C</td>
</tr>
<tr>
<td></td>
<td>37°C</td>
<td>37°C</td>
</tr>
<tr>
<td></td>
<td>45°C</td>
<td>45°C</td>
</tr>
<tr>
<td>Initial</td>
<td>28.65 3.72 3.77 350.45 0.00</td>
<td>29.37 13.75 14.20 14.34 0.00</td>
</tr>
<tr>
<td>-20/20°C</td>
<td>29.54 4.08 4.13 350.72 0.81</td>
<td>30.54 13.07 13.45 13.51 0.68</td>
</tr>
<tr>
<td>20/37°C</td>
<td>28.83 3.67 3.73 350.34 0.38</td>
<td>29.97 12.15 12.35 10.26 1.76</td>
</tr>
<tr>
<td>20°C</td>
<td>27.42 4.81 4.83 355.92 1.65</td>
<td>29.47 11.62 11.82 10.42 1.80</td>
</tr>
<tr>
<td>37°C</td>
<td>26.75 3.77 3.83 354.52 1.68</td>
<td>28.48 9.57 9.66 7.68 3.83</td>
</tr>
<tr>
<td>45°C</td>
<td>27.09 3.90 3.94 352.35 1.18</td>
<td>28.75 9.22 9.30 7.36 4.22</td>
</tr>
</tbody>
</table>
Changes in lightness (L*) were minute for all formulas acrossed all treatments (≤ 2.5). All samples were slightly darker at the conclusion of the 12 weeks at 45°C. Changes in redness (a*) of the samples were not statistically significant (p value ≤ 0.01) for all treatments, with the exception of the red radish formula at 37°C and 45°C. The elderberry and purple corn formulations showed the smallest changes in a* across treatments, and the red grape and red radish showed the greatest changes. Changes in chroma (C*), followed a similar pattern to changes in a*, with the smallest changes observed with the elderberry and purple corn lipsticks. Interestingly, both elderberry and purple corn formulations showed increased color intensity at 20°C after 12 weeks.

Changes in hue angle varied between formulations, with the greatest changes observed with the elderberry and purple sweet potato formulas. The elderberry lipstick hue angles increased for all conditions, toward a more red hue angle. Conversely, the purple sweet potato lipstick hue angles decreased for all conditions, toward a more purple hue angle. The red radish lipstick hue angles also decreased for all conditions, toward a more red hue angle. Very little changes were observed in hue angle for purple carrot, red grape and purple corn formulations.

Delta E (ΔE*), or the total color difference, can be used to quantify changes in color (Mokrzycki and Tatol 2012). Just noticeable differences (JND) in color can be detected at a Delta E around 2.36; however, unexperienced observers notice differences in color when the Delta E is between 3.5 and 5 (Mokrzycki and Tatol 2012). 10 weeks at 45°C is considered a predictor for shelf stability of two years (Cannel 1985); therefore,
some color changes were expected by week 12. Red radish formulations showed the greatest $\Delta E^*$ after 12 weeks at 45°C ($\Delta E^*$: 4.22) and elderberry and purple corn formulas showed the smallest $\Delta E^*$ after 12 weeks at 45°C ($\Delta E^*$: 1.27 and 1.18, respectively).

Based on the detection limits of color differences, it was determined that the color changes recorded for the formulas at all conditions were within acceptable limits; and therefore, shelf stabilities of at least two years were predicted. In addition, the samples were stored in such a way to mimick an opened container, which increases the assurance of their stability.

**Total Monomeric Anthocyanin Content Changes**

Recovered percentage of monomeric anthocyanin content for the formulations were determined based on the initial monomeric content by weight, following the accelerated environmental testing. In general, the highest recovery was seen for the freeze/thaw cycles for all formulations, with a recovery of 98.91% (± 0.22) for purple corn to 91.72% (± 0.18) for red grape. The recovery from 45°C was the lowest for all formulations, with a recovery of 93.81% (± 0.24) for purple corn to 59.11% (± 1.40) for red grape. The recovery rates for formulations followed the same pattern for all accelerated environmental testing. In order of highest recovery the formulations were: purple corn, purple sweet potato, elderberry, red radish, purple carrot, and finally red grape (See Figure 2.2).
Figure 2.2: Percentage of recovered anthocyanins from formulations after accelerated environmental testing for 12 weeks at 20, 37, and 45°C or six abuse cycles from -20/20°C and 20/37°C. Percentages based on initial total monomeric content by weight determined on day 1 ± S.D. (n=3) by the pH-differential method.

Overall, the percentage of recovered anthocyanins from the formulations followed a similar pattern shown with the color measurements reported above. These results were expected due to the association of changes in color with degradation, or changes in chemical structure, of anthocyanins (Giusti and Wrolstad 2003). It is not surprising then that the formulations that showed the smallest changes in color, such as the purple corn and elderberry, should have the highest recovery of monomeric anthocyanin content, following the storage conditions. It is interesting to note that although the red radish had the greatest change in color, its recovery rate was higher than that of the purple carrot or red grape for all testing conditions. This may be due to the lower initial monomeric
content, as seen with the purple carrot. Another possibility is that some irreversible binding occurred between the anthocyanin and lipstick matrix, as may be the case for the red grape, which had the lowest recovery for all testing conditions.

2.5 CONCLUSION

Anthocyanins incorporated into the matrix of lipstick formulations provided attractive colors similar to reference samples and proved to be stable, even under accelerated environmental testing used to predict a shelf life of at least two years. There were, however, variations in stability noted with the different sources based on colorimetric analysis and percentage of recovered anthocyanins. Sources containing both non-acylated and acylated cyanidin based anthocyanins, such as the elderberry, purple corn and purple sweet potato, were shown to have the greatest stability when compared to the purple carrot, red grape, and red radish formulas. The red radish formulations showed the largest change in color; however, it showed the closest color characteristics to red colorants used in cosmetics (D&C Red 7 and Mica Red). Therefore, it should not be ruled out as a possible natural colorant for the industry. Overall, our results show the potential for anthocyanins to be used as stable alternatives to synthetic colorants in the cosmetic industry.
3.1 ABSTRACT

The growing market demands for health-promoting ingredients, as well as a growing concern over the use of synthetic colorants in consumer products, highlights the need for alternative coloring ingredients in cosmetic products. Anthocyanins, a class of flavonoids, have been reported as potent antioxidants, acting as anti-inflammatory, anti-carcinogenic compounds and as natural colorants in the food industry. However, their application in cosmetic products as active ingredients has scarcely been previously reported. Some of their reported benefits may be seen when applied topically in an appropriate skin-penetrating vesicle, such as liposomes. The objective of this study was to evaluate the use of anthocyanins as bioactive colorants in lipstick formulations. The hypothesis was that by targeting anthocyanin sources known for their high stability and reported health benefits, that these properties would also be observed in cosmetics. Formulations were tested for their ability to absorb UV light, free radical scavenging ability against DPPH, and inhibition of melanin production by tyrosinase. All formulas showed increased UV absorption over the lipstick base, with sources acylated with cinnamic acid exhibiting the highest in vitro SPF(UV-B) values. All formulas exhibited
high inhibition of DPPH free radicals, and melanin production by tyrosinase, above that of the controls, BHT and kojic acid, respectively. Moreover, their activity was determined to be at physiologically relevant levels for lipstick applications. Our results suggest the potential for anthocyanins to be used as biological active ingredients in cosmetic formulations by acting as antioxidants, UV-protection, and anti-aging compounds.

**Key Words:** anthocyanins, UV absorption, antioxidants, tyrosinase, cosmetics

### 3.2 INTRODUCTION

Anthocyanins are water-soluble flavonoids responsible for many of the reds, blues and purple colors found in plants. In nature they occur primarily as one of six aglycones with various glycoside attachments: cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin (Wu 2014). The variations in their chemical structure occur at the 3’ and 5’ positions of the B-ring and acylation of the sugar moiety with various aromatic and aliphatic acids may also occur. The biological activity of anthocyanins is largely dependent on these variations in their chemical structures.

The intense and attractive colors produced by anthocyanins have prompted interest in their uses as colorants for the food industry (Wrolstad and Culver 2012). Their potential to act as powerful antioxidants and use in disease prevention has also been gaining increased attention (Jing and Giusti 2014). They have long been considered as strong antioxidants, with the ability to scavenge free radicals and terminate chain reactions demonstrated in many *in vitro* assays (He and Giusti 2010; Massa 2007). Their protective effects against oxidative stress-induced damage and regulation of redox-
signaling pathways have also been demonstrated (Ramirez-Tortosa et al 2001; Shih et al 2012; Hwang et al 2011).

In addition, recent investigations into anthocyanins potential to prevent oxidative damage to the skin by UV-induced erythema, skin cancer, and photoaging have also demonstrated a protective effect in vitro and in vivo (Chan et al 2010; Bae et al 2009; Tsoyi et al 2008; Hsu et al 2012).

Unabsorbed anthocyanins have also been shown to potentially act as chemopreventive topically in the gastrointestinal tract by preventing oxidative damage to the mucosal lining (Gee and Johnson 2001). This topical activity may also translate to similar benefits when applied to the skin in an appropriate vesicle, such that they are able to react with damaging reactive oxygen species (ROS) (Montanari et al 2013). A recent study demonstrated their ability to trigger a regenerative effect on the skin (Bojanowski 2013). Moreover, anthocyanins have been shown to improve psoriatic lesions in vitro and alleviate atopic dermatitis in vivo (Crisan et al 2013; Kim and Choung 2012).

Few studies have investigated the protective effects of anthocyanins when incorporated into matrices for topical delivery. However, two recent studies positively demonstrated the biological activity of anthocyanins when concentrated onto protein-rich matrices (Plundrich et al 2013) and when incorporated into ultradeformable liposomes (Montanari et al 2013).

The aim of this study was to investigate the biological activity in vitro of anthocyanins when incorporated into lipstick formulations as a source of color. The anthocyanins used were selected based on our previous studies investigating the color
comparisons to commercially available lipsticks and stability of various anthocyanins sources within lipstick emulsions.

3.3 MATERIALS AND METHODS

Materials

Elderberry, purple carrot, purple sweet potato, and red radish dried extract were provided by DD Williamson & Co., Inc. (Louisville, KY), and the purple corn and red grape skin dried extracts were provided by Artemis International (Fort Wayne, IN). The base of the lipstick formulations was purchased from MakingCosmetics, Inc. (Snoqualmie, WA). Black lip balm containers were purchased from a local company, Bulk Apothecary (Streetsboro, OH). Compounds used were gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), mushroom tyrosinase, L-3,4-dihydroxyphenylalanine (L-DOPA), and kojic acid, purchased from Sigma Aldrich (St. Louis, MO). Reagents used were ethanol and methanol, and were purchased from Fisher Scientific Inc. (Fair Lawn, NJ).

Methods

Lipstick Formulations

Formulations were based on recommendations in the Society of Cosmetic Chemists (SCC) Monograph Number 8: Lipstick Technology (Barone et al 2002). All dried extracts were incorporated as 8% of the final weight (w/w) of each lipstick formulation based on preliminary data. Dried extracts were initially weighed out and
subjected to a grinding process with a mortar and pestle prior to being added to the lipstick manufacturing. Formulations underwent a wet grinding process in which castor oil was used at a 1:3 ratio (pigment:oil) and silica was included at 1% of the final weight (w/w), to increase uniformity in the final products. Initially, the lipstick base was weighed and placed in a water bath at 70°C with gentle stirring until completely melted. The pre-ground dried extracts were then poured directly into the hot lipstick base and gentle stirred until uniform color was achieved. The lipstick formulas were then poured directly into the lip balm containers and allowed to cool at 4°C until completely solid.

Total Phenolic Content

The Folin Ciocalteu method was used to estimate total phenolic content of the formulas containing anthocyanins, based on the methods described in the literature (Waterhouse 2000). 25mg of each formula was dissolved in 50mL of methanol, and briefly sonicated. Gallic acid was used as a positive control and gallic acid solutions were made in the following concentrations: 50mg/L, 100mg/L, 250mg/L, and 500mg/L. These concentrations were used to determine a standard curve for the test.

The Folin Ciocalteu reagent was kept at 4°C in the dark until the test was ready to be performed. 20µL of the formula-methanol solutions, the gallic acid solutions, or distilled water were pipetted into 3mL cuvettes, in triplicate. 1.58mL of distilled water was then pipetted into each cuvette. The Folin Ciocalteu reagent was then pipetted at a volume of 100µL into each cuvette and vigorously mixed by pipetting. 300 µL of sodium carbonate solution was then added to each cuvette after a rest period between 1-8 minutes.
The cuvettes were then equilibrated in the dark at room temperature for two hours. After the equilibration time, absorbance readings at 765nm were measured with using a spectrophotometer (Shimadzu UV-2450 Spectrophotometer Kyoto, Japan).

Linear regression was used to determine a standard curve for the absorbance at 765nm of the gallic acid solutions ($R^2 = 0.99$). The standard curve was then used to determine the gallic acid equivalents (GAE) of the average of each sample absorbance. The results were reported as mg polyphenolic/liter of extract solution (GAE).

**In Vitro UV Absorption and SPF Calculation**

*In vitro* sun protection factor for the lipstick formulas were determined based on the methods described by Sayre *et al* (1979) and Dutra *et al* (2004) with modifications based on the COLIPA revised method (Moyal *et al* 2012). Initially, 25mg of each formula was weighed and dissolved in 25mL ethanol and briefly sonicated in a water bath until no color was observed in the lipstick. 25mg of the lipstick base was also dissolved in 25mL ethanol by brief sonication in a heated water bath. 300µL of each solution was then pipetted into an ultraviolet microwell plate, in eight replications. Absorbance readings were carried out by a SpectraMax190 Plate Reader (Molecular Devices, Sunnyvale, CA) across the 290-400nm ultraviolet wavelength range, at 1nm increments and blanked against ethanol.

The absorbance values were then averaged and the standard deviation were calculated for each sample. The *in vitro* SPF values were determined according to the following formula:
\[ SPF_{spectrophotometric} = CF \times \sum EE(\lambda) \times I(\lambda) Abs(\lambda) \] (Eq. 1)

Where CF = correction factor (10), EE(\lambda) = Erythema action spectrum (CIE 1987), I(\lambda) = Spectral irradiance received from the UV source, and Abs(\lambda) = spectrophotometric absorbance values at wavelength \( \lambda \). The EE x I are constants and were determined by Sayre et al. (1979). Results for the ultraviolet absorbance were graphed in 10nm increments between 290 and 400nm and standard deviations were included.

**DPPH Free Radical Scavenging Assay**

The DPPH free radical scavenging assay was used to measure potential antioxidant capacity as described previously (Brand-Williams et al 1995) and (Prior et al 2005), with modifications described by Montanari et al (2013) for use with cosmetics. The methanolic solutions containing the recovered pigments were used for the testing of antioxidant capacity. After the extraction from the formulas, 50mg of lipstick base was dissolved in 50mL methanol by brief sonication in a heated water bath, to be used as a positive control in the assay. BHT was also used as a positive control, and 50mg was weighed out and dissolved in 50mL methanol by stirring. Dried extracts were also weighed out at their respective amounts in each formula (8% w/w) based on their monomeric anthocyanin content. Weighed extracts were dissolved in methanol at a 1mg/mL (w/v) amount, and were used to account for the absorbance of anthocyanins at 515nm. DPPH was kept in the dark and at 4°C until needed in the assay. 2.5mg of DPPH was weighed and dissolved in 250mL methanol, still kept in the dark. Additionally, 5mg
of DPPH was weighed and dissolved in 250mL methanol and subsequent serial dilutions were made to obtain a standard curve for DPPH based on concentration.

The DPPH methanol solution was then pipetted into 5mL plastic cuvettes at a volume ranging from 3.8-3.86mL. Then, 0.2-0.14mL of the methanol solutions of either BHT, the lipstick base, dried extract, or the anthocyanin formulas were pipetted into the cuvettes and mixed thoroughly by pipetting. 300µL of each cuvette was then transferred into a microwell plate, in triplicate. The DPPH methanol solution was also pipetted at 300 µL in triplicate to serve as a positive control. The microwell plate was then placed in a Molecular Devices SpectraMax190 Plate Reader (Molecular Devices, Sunnyvale, CA), and allowed to equilibrate for 30 minutes in the dark at 20°C. Absorbance readings at 515nm were read after the 30-minute equilibration time and blanked against wells containing only methanol.

The average of the absorbance readings were then used to determine the DPPH inhibitory percentage and IC$_{50}$ of each sample. The inhibitory percentage of each sample was determined based on the following equation:

$$I\% = \left( \frac{Abs_{(DPPH)} - Abs_{(Sample)}}{Abs_{(DPPH)}} \right) \times 100$$

(Eq. 2)

The IC$_{50}$ values are defined as the concentration necessary to inhibit 50% of the free radical (Montanari et al 2013). The IC$_{50}$ values of each sample were determined using linear regression of the absorbances at different concentrations (40,60,100 µg/mL). The readings for the anthocyanin formulas were corrected for the absorbance of their respective dried extract in the methanol solution. Samples were tested again after 4
weeks of storage at 4°C and new IC\textsubscript{50} values were calculated and compared to the original values.

\textit{Anti-Tyrosinase Assay}

Tyrosinase inhibition assay was performed as previously reported (Plundrich \textit{et al} 2013). 120\(\mu\)L phosphate buffer (pH 6.5, 50mM), 40\(\mu\)L of formula extract, and 2\(\mu\)L of mushroom tyrosinase (5 U) were gently mixed and incubated for 10 minutes in a 96-well plate. Absorbance was measured at 475nm on a Molecular Devices SpectraMax190 Plate Reader (Molecular Devices, Sunnyvale, CA). Each sample was blanked against a well containing all components except mushroom tyrosinase. Results were compared with the negative control (phosphate buffer) and positive control (kojic acid). The percentage tyrosinase inhibition was calculated as follows:

\[
I\% = \left( \frac{Abs\text{-}Control \cdot Abs\text{-}Sample}{Abs\text{-}Control} \right) \times 100 \quad \text{(Eq. 3)}
\]

Results are presented as the mean (n=8) ± S.D. IC\textsubscript{50} values were predicted using linear regression and are expressed in \(\mu\)g/mL.

\textit{Statistical Analysis}

Results of Folin Ciocalteu, DPPH free radical scavenging assay, and anti-tyrosinase activity were analyzed using two-way ANOVA and regression modeling using Minitab Statistical Software (\(\alpha=0.05\)) and GraphPad Prism Version 6.
3.4 RESULTS AND DISCUSSION

Total Phenolic Content

The amount of total phenolic content in the methanolic extracts was expressed as milligrams of equivalents of gallic acid in one liter (GAE) (Figure 3.1). GAE values were determined based on a calibration curve obtained from gallic acid ($R^2 = 0.99$).

![Figure 3.1: Total Phenolic Content of ACN-lipstick formulations in methanol, expressed as mg Gallic Acid Equivalents (GAE)/L (n=5± SD).](image)

Total phenolic content ranged from $271.6 \pm 6.96$ mg GAE/liter extract for purple sweet potato to $163.8 \pm 16.34$ GAE/liter extract for red grape skin.
UV Absorption and SPF Calculations

The ultraviolet light absorbance of each formula compared to that of the lipstick base alone, from 290-400nm, is shown in Figure 3.2.

![Figure 3.2: UV absorbance of ACN-lipstick formulations compared to lipstick base in ethanol. Results are means (n=8 ± SD).](image)

The formula that showed the highest ultraviolet absorbance was the red radish formula, across the 290-400nm range. The purple sweet potato formula also showed high absorbance across the ultraviolet range. Red radish, purple sweet potato and elderberry showed absorbance values at or above 0.5 for all wavelengths in the UVB (290-320nm) range (Table 3.1). All formulas showed greater absorbance than that of the lipstick base alone along the entire ultraviolet spectrum, although absorption was higher in the UVB range.
range than UVA range for all formulas tested. All formulas showed statistically
significant differences when compared to the base.

Table 3.1: Calculated *in vitro* UVB sun protection factor (SPF) values of ethanolic
extracts of ACN-lipstick formulations compared to the lipstick base. Calculations based
on absorbance values in the ultraviolet wavelength range of 290-320nm.

<table>
<thead>
<tr>
<th>LIPSTICK FORMULATION</th>
<th>CALCULATED UVB SPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base</td>
<td>8.19</td>
</tr>
<tr>
<td>Elderberry</td>
<td>13.85</td>
</tr>
<tr>
<td>Purple Carrot</td>
<td>11.27</td>
</tr>
<tr>
<td>Purple Corn</td>
<td>11.62</td>
</tr>
<tr>
<td>Purple Sweet Potato</td>
<td>14.44</td>
</tr>
<tr>
<td>Red Grape</td>
<td>11.39</td>
</tr>
<tr>
<td>Red Radish</td>
<td>15.84</td>
</tr>
</tbody>
</table>

The calculated *in vitro* UVB sun protection factor (SPF) values of the ethanolic
extracts of the formulas are shown in Table 3.1. Values followed the same pattern as that
of the absorbance values in Figure 3.2. It was found that the calculated SPF values were
highest for red radish, purple sweet potato, and elderberry and lowest for red grape skin,
and purple carrot; however, all formulations showed an increase in absorbance over that
of the base alone. The increased UV absorption by red radish and purple sweet potato
may be due to the addition of acylation by cinnamic acids, which are known to absorb in
the UV range (Crisvert and Salvador 2007). These results may suggest a future role for these extracts as photoprotective ingredients in topical formulas.

**DPPH Free Radical Scavenging Ability**

The inhibitory percentages of each formula extract against DPPH at three concentrations (40, 60, 100 µg/mL) were determined to calculate the IC\textsubscript{50} for each formulation. Inhibitory ability followed the same pattern at all three concentrations. The purple corn and elderberry formulas showed the highest inhibition of DPPH at all three concentrations while the grape skin and purple carrot formulas showed the lowest inhibition of DPPH at all three concentrations. All formula inhibitory values were significantly different than that of the lipstick base alone when analyzed by one-way ANOVA (p value ≤ 0.05). Interestingly, when the inhibitory percentages of the formulas were analyzed against that of their respective extract in methanol no significant differences were found at all three concentrations. These results may be suggesting that the anthocyanins are behaving in a similar manner to their reducing behavior in solution. Free radical scavenging capacity of each formula extract, expressed as IC\textsubscript{50} values is shown in Figure 3.3.

The antioxidant activity in order from highest activity found was: the purple corn formula (4.87 ± 0.28 µg/mL), elderberry formula (13.35 ± 0.21 µg/mL), purple sweet potato (13.44 ± 0.01 µg/mL), red radish (14.49 ± 1.30 µg/mL), purple carrot (28.59 ± 2.65 µg/mL), red grape skin (31.18 ± 0.68 µg/mL), and finally the base lipstick formula (46.72 ± 0.67 µg/mL). All formulations were significantly different when compared to the base
formula. All formulas, except purple carrot and red grape were significantly different when compared to the positive control, BHT (25.78 ± 2.66 μg/mL).

**Figure 3.3**: IC$_{50}$ (μg/mL) of radical scavenging against DPPH for methanolic extracts of ACN-lipstick formulations compared to BHT and lipstick base (n=8 ± SD). Significant differences denoted by different letters above bars.

Overall, the antioxidant capacity values found were in agreement with the amount of total phenolic content discussed above. Purple corn, elderberry and purple sweet potato would be expected to have the highest antioxidant activity due to their higher total phenolic content. Inversely, the grape skin formula and purple carrot would be expected to have lower relative antioxidant activities, based on their phenolic content.

Samples were tested again after four weeks of storage at 4°C and inhibitory percentages and IC$_{50}$ values were compared to the fresh sample values. There was no
statistical significance (p-value ≤ 0.05) between the values of the fresh samples versus the four week old samples.

**Anti-Tyrosinase Activity**

Eluates from the base lipstick and the anthocyanin-lipstick formulations were tested for inhibition of L-DOPA oxidation against mushroom tyrosinase. IC\textsubscript{50} values (μg/mL), or the amount of the extracts necessary to inhibit 50% of the mushroom tyrosinase, were then predicted using linear regression and compared to the positive control, kojic acid (Figure 3.4). In general, all extracts showed the ability to inhibit tyrosinase at similar or lower concentrations than kojic acid (2.41 ± 0.06 μg/mL). The elderberry and purple corn, in particular, exhibited IC\textsubscript{50} values at 11-fold and 8-fold lower concentrations than that of the control. Results were analyzed using one-way ANOVA and all treatments were found to be statistically significant (p ≤ 0.05) when compared to controls.

IC\textsubscript{50} values (Figure 3.4) in order of highest inhibition were: elderberry (0.22 ± 0.06 μg/mL), purple corn (0.31 ± 0.01 μg/mL), purple carrot (0.42 ± 0.03 μg/mL), red grape skin (0.55 ± 0.04 μg/mL), purple sweet potato (0.75 ± 0.06 μg/mL), and red radish (1.78 ± 0.08 μg/mL).
Figure 3.4: IC\textsubscript{50} (µg/mL) against mushroom tyrosinase for ACN-lipstick formulations compared to kojic acid and the lipstick base. Results are presented as means (n=5) ±SD. Significant differences denoted by different letters above bars.

These results are in agreement with expected activity based on the structure of the anthocyanins present. Anthocyanins with a free hydroxyl groups at the 3’ and 4’ position of their B ring (cyanidin and delphinidin) would be expected to have the highest inhibition of tyrosinase (Parvez \textit{et al} 2007). Conversely, it is believed that the substitution of a methoxy group on the 3’ position of the B ring (peonidin, petunidin, and malvidin) causes steric hindrance of the hydroxyl group at the 4’ position, decreasing inhibition potential. Acylation with cinnamic acids may increase inhibition (Parvez \textit{et al} 2007). Acylation with malonic acid may also inhibit tyrosinase through complexation with the copper center of the enzyme. In addition, di-glucoside attachments at the 3 and
5 position of the A ring are believed to decrease inhibition through steric hindrance. Therefore the anthocyanin sources with cyanidin with one sugar attachment at the 3 position (elderberry), as well as their acylated counterparts (purple carrot and purple corn), would be expected to have the highest inhibition of tyrosinase.

It should be noted that the results of IC$_{50}$ values in micrograms in a 1 milligram per milliliter solution are physiologically relevant to lipstick application. The average woman uses 24mg lipstick per day (Loretz et al 2005); therefore, the benefits against tyrosinase activity in cosmetic formulations can be achieved at relatively low concentrations.

3.5 CONCLUSIONS

Anthocyanins incorporated into lipstick formulations were shown to retain their tested biological activity in vitro. All formulations showed the ability to act as photoprotective additives through UV absorption, especially those with cinnamic acid acylation. The anthocyanin formulations showed the capability to act as antioxidants, through scavenging of free radicals, in a lipstick matrix. In addition, the formulas showed proficient tyrosinase inhibition, which is a well-known source of melanin formation in the skin. The concentrations necessary to exhibit these activities were all well within physiologically relevant concentrations based on the average uses of lipsticks within the United States. These results show the potential for anthocyanins to be used as active ingredients within cosmetic formulations for these claim substantiations.
CHAPTER 4: *IN VITRO* ASSESSMENT OF THE PENETRATION OF TOPICALLY APPLIED ANTHOCYANIN BASED COSMETICS BY INFRARED SPECTROSCOPY

4.1 ABSTRACT

The use of beneficial polyphenols in cosmetics has dramatically increased in recent years due to consumer demands for more functional ingredients in products. Anthocyanins are natural colorants with antioxidant properties, which have previously been shown to inhibit some of the reactions that lead to photoaging and diseases of the skin. Despite the evidence of their benefits to the skin, very little is known about their ability to penetrate the stratum corneum, which is a prerequisite for their beneficial activity. The aim of our study was to investigate the penetration of anthocyanin-lipstick formulations through the skin using a porcine ear model. The combined ATR-FTIR and tape-stripping techniques were utilized and results were analyzed using PLS-Regression and permeation kinetic models. For both formulations of elderberry and red radish lipstick, initial release and diffusion within the formula, as well as skin permeation behavior were determined. Consistent with the literature, our results found that the initial release rates and diffusion of the anthocyanins within the formulations were correlated with molecular weight and hydrophilicity of the compounds. Our results show that the lower molecular weight anthocyanins from elderberry were more permeable ($K_p = 3.7 \times 10^{-4} \text{ cm h}^{-1}$) than the red
radish anthocyanins ($K_p = 2.9 \cdot 10^{-4} \text{ cm h}^{-1}$) within the skin; however, both were found at depths, which would support their use as active ingredients in cosmetic formulations.

**Key Words:** anthocyanins, cosmetics, penetration, skin barrier, infrared spectroscopy

### 4.2 INTRODUCTION

In recent years, the health-promoting properties of flavonoids have made them attractive functional ingredients for both the pharmaceutical and cosmetic industries (Zillich *et al* 2015). Cosmetic products with beneficial ingredients, or cosmeceuticals, are a growing sector of the cosmetic industry, and many products containing plant-derived polyphenols have been developed to meet this growth (Draelos 2014; Zillich *et al* 2015). In order for the biological activity of these ingredients to be effective, they must be released from the topically applied formula, reach the skin and overcome the barrier function of the stratum corneum (SC) to penetrate into the epidermis and dermis (Brown *et al* 2012).

The release of active ingredients and skin penetration kinetics are dependent on the molecular properties of the compound, such as molecular weight and lipophilicity, as well as the properties of the vehicle (Moser *et al* 2001).

The skin permeation activity of emulsions containing flavonoids, such as protocatechuic acid; chlorogenic acid; catechin; resversatrol; rutin; quercetin and ECGC, have previously been investigated (Zillich *et al* 2013; Frauen *et al* 2002; Marquele *et al* 2006; Kitagawa *et al* 2011; Dal Belo *et al* 2009; Batchelder *et al* 2004). Although chemically similar, the permeation behavior of each flavonoid varies with differences in their molecular properties, as well as the vehicle formulation.
Conversely, anthocyanins, a group of highly pigmented flavonoids, have only been limitedly investigated for their use in topical formulations or skin-care. The primary focus of the research, thus far, has been on their in vitro biological activity in aqueous solutions or oral administration (Hsu et al 2012; Bae et al 2009; Afaq et al 2005; Calvo-Casto et al 2013; Crisan et al 2013). The few studies that have investigated their activity in matrices for topical usage have shown their ability to act as UV-protectants (Enomoto et al 2005; Afaq et al 2009; Chan et al 2010), antioxidants (Montanari et al 2013), inhibitors of melanin synthesis (Hwang et al 2013; Plundrich et al 2013), chemopreventative (Chamcheu et al 2011; Bojanowski 2013), as well as acting as antimicrobial agents (Plundrich et al 2013). However, the skin permeation activity of these potentially beneficial compounds has not been previously reported.

The objective of this study was to investigate the release of anthocyanin extracts from lipophilic cosmetic formulations, and their in vitro skin permeation behavior using a porcine ear skin model and tape-stripping techniques (Voegeli et al 2007). Attenuated Total Reflectance-Fourier Transform Infrared spectroscopy (ATR-FTIR) coupled with Partial Least Squares Regression (PLS-R) analysis was utilized for analysis of the samples due to the high sensitivity of the technique to subtle chemical variations and minimal sample preparation required (He et al 2007). Elderberry, a source of non-acylated cyanidin, and red radish, a source of acylated pelargonidin, were used in this investigation. These sources were chosen to help elucidate the role varying anthocyanin profiles, including glycosylation and acylation patterns, may have on skin permeation behavior.
4.3 MATERIALS AND METHODS

**Materials**

Elderberry and red radish dried extracts were provided by DD Williamson & Co., Inc. (Louisville, KY). The base of the lipstick formulations was purchased from MakingCosmetics, Inc. (Snoqualmie, WA). The adhesive tape, D-Squame disks (CuDerm Corporation, Dallas, TX) with a diameter of 2.2 cm was used for tape stripping. Bovine serum albumin (BSA) standard was purchased from BioRad (Bio-Rad DC).

**Methods**

**Skin Tissue**

Pig ears were obtained from a local abattoir (Massillon, OH) and stored at -24°C for no more than 6 months prior to use. Initially, the pig ears were defrosted and cleaned with cold water and then gently dried with a soft tissue. Full thickness skin was removed from the cartilage with a scalpel and any hair was removed. Samples were then cut into 2.5 x 7.5 cm pieces and pinned to a Styrofoam board with needles on each corner.

**Formulations**

Lipstick formulations were prepared with the anthocyanin extracts comprising 8% (w/w) of the final formula. This percentage was chosen in accordance with current recommendations for lipstick colorants used by the cosmetic industry (Barone *et al* 2002).
Combined ATR-FTIR and Tape Stripping Experiments

Lipstick was applied on the porcine ear skin sample at 2mg/cm² and incubated at 32°C for one hour. After incubation, the lipstick remaining on the skin surface was removed by blotting with a tissue. Adhesive tape was then applied to the skin sample with a rolling motion of the thumb and removed in one continuous motion. Sequential tape strips were removed in the same manner. Attenuated total reflectance (ATR)-FTIR spectra were collected of the tape strips with the adhesive side facing the ZnSe ATR Crystal of the Cary Eclipse 620 spectrometer (Varian Technologies, Santa Clara, CA, USA). Spectra were collected over the range of 4000-700cm⁻¹ at 4cm⁻¹ resolution. Interferograms of 64 scans were coadded followed by Beer-Norton apodization. The absorbance spectra were ratioed against the blank ATR crystal spectrum collected as background.

In addition to the samples treated with anthocyanin-lipstick, other porcine ear skin samples were incubated with the lipstick base alone. Spectra were also collected of porcine ear skin without application of lipstick in order to eliminate error sources caused by variations in stratum corneum composition between the samples. Experiments were performed in triplicate (n=3). ATR-FTIR spectra were also collected of anthocyanin-lipstick formulations at concentrations ranging from 0-8% anthocyanin, as well as bovine serum albumin (BSA) at concentrations ranging from 0-500μg on the same instrument under the same conditions.
**Protein Quantification**

The amount of corneocytes removed from the individual tape strips after the incubation period was determined by linear regression of the characteristic N-H bending vibrations of Amide I and Amide II absorbance peaks (~1640 cm\(^{-1}\) and ~1540 cm\(^{-1}\)) of the BSA concentration curve (R\(^2\)=0.98). The absorptions were normalized for a tape area of 1 cm\(^2\) and expressed as μg/cm\(^2\). The mean protein density was assumed to be 1g/cm\(^3\) and was used to determine the thickness of the stratum corneum (Lindemann *et al* 2003). Due to the inherent variability between porcine ear skin thicknesses, six parallel (n=6) experiments were performed to ensure the obtained values were in agreement with the literature. The mean stratum corneum thickness of the porcine ear skin was determined to be 8.27 ± 2.4μm. The determined value was in agreement with the assumed thickness of porcine ear stratum corneum of 8.2-8.5± 3.0μm (Herkenne *et al* 2006, Wiedersberg and Nicoli 2012).

**Chemometrics**

Multivariate statistical software was used to collect information from the spectra and to conduct data reduction analysis for statistical analysis purposes. Data preprocessing was conducted by importing the spectra as .spc files into multivariate statistics software (Pirouette 4.2, Infometrix Inc., Woodinville, WA). Spectra were normalized and second derivative transformed using a five-point polynomial-fit Savitsky-Golay function. Preprocessing was performed to mitigate the influence of variations in signal intensity.
For the stratum corneum samples collected, the Amide II absorbance was used as an internal standard to account for variations in sample contact with the ATR crystal (Saad et al 2012).

Partial Least Squares (PLS), a multivariate regression analysis, was used to develop a calibration model of anthocyanin-lipstick formulation at varying concentrations based on FT-IR spectra to predict the relative amounts of anthocyanins present in the tape strips acquired from the porcine skin. Cross validation using the leave-one-out approach was used to obtain the calibration model. Outliers were identified using residual plots in order to avoid the inclusion of data that deviated from normal behavior (Wold 2001). The performance of the regression models was assessed by calculation of the correlation coefficient ($R^2$) and the standard error of cross validation (SECV).

**Determination of Anthocyanin Penetration Profiles**

After determining the thickness of the stratum corneum removed by each tape strip and quantifying the amount of anthocyanin in each tape strip normalized to the area, the diffusivity and solubility of the anthocyanins within the stratum corneum can be determined (Moser et al 2001). Fick’s second law of diffusion is used to predict concentrations of anthocyanin ($c_x$) as a function of position ($x$) within the stratum corneum by applying the following equation (Pirot et al 1997):

$$c_x = c_{x=0}\left(1 - \frac{x}{L}\right) - 2\sum_{n=1}^{\infty} \frac{1}{n} \sin \left(\frac{n\pi x}{L}\right) \exp \left(-\frac{Dn^2\pi^2t}{L^2}\right)$$

(Eq. 1)

The concentration of anthocyanin in the outermost layer of the stratum corneum ($c_{x=0}$) is divided by its concentration in the applied lipstick ($c_v$), which provides the
partition coefficient of the anthocyanin between the lipstick and stratum corneum (K), and the diffusion parameter (D/L^2). D/L^2 is the first-order rate constant of the anthocyanin diffusivity (D) in the stratum corneum to the thickness (L) squared of the barrier (Wiedersberg and Nicoli 2012). To apply Eq. 1 to determine these constants some assumptions are made about the system (Wiedersberg and Nicoli 2012). The assumptions are: an infinite dose is applied, that the stratum corneum is a homogenous barrier and contains no permeant at t=0, and that the diffusivity of the drug within the stratum corneum is slow compared to uptake by the cutaneous microcirculation, or a “sink” condition occurs for the permeant at the interface between the stratum corneum and the viable epidermis (Herkenne et al 2007). This allows for the SC to be considered the rate-limiting step in dermal permeability.

The values for D/L^2 and K, combined with the corresponding SC thickness (L), make it possible to calculate the permeability coefficient (K_p) from each of the anthocyanin-lipstick formulas tested using Equation 2 (Herkenne et al 2006):

$$K_p = K \frac{D}{L^2} L = \frac{KD}{L}$$  \hspace{1cm} (Eq. 2)

In addition, knowing the K_p and the concentration of the anthocyanins in the vehicle (C_v), the steady state flux (J_{ss}) across the SC can be estimated by:

$$J_{ss} = K_p C_v = \frac{KD}{L} C_v$$  \hspace{1cm} (Eq. 3)

The time necessary to reach steady state across the SC is related to the D/L^2 by the classic diffusional lag-time (T_{lag} = L^2/6D) (Roberts et al 1999). Time to reach the steady state is generally regarded as about 2.7T_{lag} (Roberts et al 1999). These equations
were applied for the determination of kinetic parameters of the anthocyanin-lipstick formulations in porcine ear SC.

Fick’s first law can be used to determine initial release rates of the anthocyanins from the formulations:

\[ K_r = \frac{J_{SS}}{C_v} \]  

(Eq. 4)

Where \( K_r \) is the initial release coefficient, \( J_{SS} \) is the steady state flux of the anthocyanin across the formulation and \( C_v \) is the initial concentration of anthocyanin in the formulation (\( \mu g cm^{-3} \)).

Likewise, Higuchi’s kinetic model was used to understand the influence of the anthocyanin non-initial release kinetics from the lipstick formulas on the permeation kinetics within the SC:

\[ \frac{Q}{C_0} = 2 \sqrt{\frac{D_V t}{\pi}} \]  

(Eq. 5)

Where \( Q \) is the cumulative amount of permeated anthocyanin through a unit of membrane surface, \( C_0 \) is the initial anthocyanin concentration within the vehicle, \( D_V \) is the diffusion coefficient within the vehicle over a given time \( t \) (Zillich et al 2013). \( D_V \) values can be derived from the plot of \( Q/C_0 \) against the square root of time by the slope \((K_h)\) of the linear regression equation:

\[ K_h = 2 \sqrt{\frac{D_V}{\pi}} \]  

(Eq. 6)

Higuchi’s kinetic model determines the cumulative release profiles of the anthocyanins from the lipstick formulation (Higuchi 1962). \( K_h \) describes the non-initial release rate and can be used to predict the time dependency of the release profile.
4.4 RESULTS AND DISCUSSION

*ATR-FTIR Spectral Analysis*

Based on the PLSR performed for the anthocyanin-lipsticks, the spectral region from 700-1800 cm\(^{-1}\) was selected for comparison of the samples. Representative ATR-FTIR normalized spectra can be seen in Figure 4.1A of the lipstick formulas and the same formulas on the porcine skin, Figure 4.1B shows representative FTIR spectra of the pig skin alone, as well as the spectra of the porcine skin with the lipstick base or anthocyanin-lipstick formulations applied. The peaks around 1743 cm\(^{-1}\) and 1750 cm\(^{-1}\) were attributed to the characteristic carbonyl (C=O) stretch observed with skin and lipstick, respectively (Hadgraft and Lane 2012, Pasieczna-Patkowska and Olejnik 2013). For quantification of the anthocyanins on the tape strips the spectral region of 900-1160 cm\(^{-1}\) was assigned to the C-O stretching vibration of the sugar moiety of the glycosides present and to a lesser extent to the aromatic C-O stretching (He *et al* 2007). The band around 1445 cm\(^{-1}\) was attributed to the aromatic ring vibrations (He *et al* 2007; Coates 2000). The region of 1150 and 1400 cm\(^{-1}\) showed multiple bands assigned to the C-O stretch and C-O-H bending of phenols, esters, carboxylic acid, and alcohols (Coates 2000).
Figure 4.1: Representative ATR-FTIR spectra of samples from 700-1800cm\(^{-1}\) after normalization to the most intense peak for each spectrum.
Protein Quantification

Quantification of protein removed normalized to the tape area (Figure 4.2) showed differing patterns of protein removed by the different samples. In general, the porcine skin without formula added to it had the highest amount of removed corneocytes. This may be due to decreased hydration of the skin relative to the samples with lipstick applied to them (Saad et al 2012). Another possible reason for the decreased amount of corneocytes removed for the skin samples with lipstick applied could be due to the interaction of the adhesive on the tape strips with the lipstick ingredients, which would decrease the amount of corneocytes removed (Weigmann et al 2001).
Figure 4.2: Mass of corneocytes removed (μg/cm^2) per tape strip of porcine skin incubated for one hour at 32°C with and without applied lipstick base and anthocyanin-lipstick formulas at 2mg/cm^2 (n=6).

Calibration Models For Anthocyanin Concentration

Best performance statistics were obtained for calibration models of the anthocyanin-lipstick formulations by using the FT-IR spectral region from 700 cm\(^{-1}\) to 1700 cm\(^{-1}\) and by using normalization, second derivative and smoothing (Savitsky-Golay) (Table 4.1). Quality of the prediction was greatly improved by selecting specific wavelengths rather than using the entire spectra. The optimum number of factors giving the lowest SECV values for the elderberry and red radish lipsticks were 8 and 6, respectively. Lower SECV values and higher rVAL values (rVAL > 0.8) indicate better
prediction ability and higher accuracy on the prediction of the desired variable (Cavadid 2014). The SECV values obtained with the model for the elderberry and red radish lipstick were 0.048 and 0.028, respectively. The rVAL were also higher than 0.8, thus the calibration models could be used to accurately predict the quantity of anthocyanins in the porcine skin samples.

**Table 4.1**: Performance of the calibration models for elderberry-lipstick (ELD) and red radish-lipstick (RR) formulations from FTIR spectra for the prediction of anthocyanin quantity in samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Optimum Factors</th>
<th>SECV</th>
<th>r Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elderberry</td>
<td>8</td>
<td>0.048</td>
<td>0.96</td>
</tr>
<tr>
<td>Red Radish</td>
<td>6</td>
<td>0.028</td>
<td>0.98</td>
</tr>
</tbody>
</table>

SECV: Standard error of cross validation  
r VAL: correlation coefficient of cross validation

**Anthocyanin Penetration Profiles**

By knowing the cumulative amount of anthocyanin obtained from tape stripping, as well as the concentration within the formulation, the kinetic release parameters could be obtained based on Fick’s first law of diffusion and Higuchi’s kinetic model (Table 4.2).
Table 4.2: Kinetic parameters for the release of elderberry and red radish anthocyanins from lipstick formulations. All values are means ± S.D (n=3). Statistical significance denoted by (×) (p≤ 0.05), determined by Two-Way ANOVA.

<table>
<thead>
<tr>
<th>Release From Lipstick Formulations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Formula</strong></td>
</tr>
<tr>
<td>Elderberry</td>
</tr>
<tr>
<td>Red Radish</td>
</tr>
</tbody>
</table>

$^{\text{a}}K_r$, initial release coefficient, (Eq. 4)
$^{\text{b}}K_H$, Higuchi’s coefficient, (Eq. 5)
$^{\text{c}}D_V$, diffusion coefficient within lipstick, (Eq. 6)

Due to the relatively short steady-state period within the lipsticks, $K_r$ becomes limited to describing initial release rates of the anthocyanins from the formulas (Zillich et al 2013). The further release kinetics are usually determined by the diffusion rate ($D_V$) within the vehicle (Parks et al 1997). Initial release coefficient $K_r$, and the non-initial release coefficient $K_H$ were both higher for elderberry than red radish. Our results support those reported by Zillich et al (2013), who found higher $K_r$ values for more hydrophilic polyphenols, such as catechins ($K_r = 5.18 \pm 0.12$), and lower $K_r$ values for more hydrophobic compounds, such as rutin ($K_r = 1.65 \pm 0.07$). They proposed that release rates increased with decreasing molecular weight and increased hydrophilicity (Zillich et al 2013). These two factors have been described as the most important considerations for release and skin permeation of ingredients from formulations (Fitzpatrick et al 2004). Our results are in agreement with the increased hydrophilicity and decreased MW of
elderberry anthocyanins when compared to red radish anthocyanins, which would also explain its higher diffusion constant across the formulation.

Furthermore, the lipophilic nature of the lipstick base may actually improve the affinity of the anthocyanins to the SC over that of the vehicle as previously shown by Arct et al (2002). They reported a decrease in release rates of catechin, quercetin and rutin from cosmetic formulations when hydrophilic ingredients are included (Arct et al 2002). Suggesting an increase in solubility within the vehicle of the flavonoids and, as a consequence, a decrease in the driving force of the permeation process.

By knowing the quantified amount of anthocyanin per tape strip as well as the area and thickness of the stratum corneum (L), the relative position in the skin (x) of the anthocyanins could be determined using Fick’s second law (Figure 4.3).

**Figure 4.3:** Relative position (depth) within the stratum corneum (SC, x/L) of mean anthocyanin amounts (µg/cm²) as determined by PLSR and linear regression (n=3). The lines are the best-fit for Eq. 1 used to determine permeability and diffusivity of the formulas.
Based on the fitting of Fick’s second law to experimental data obtained from the combined FTIR-tape stripping technique, both the stratum corneum-vehicle partition coefficient (K) and the diffusivity of the anthocyanins as a function of the stratum corneum depth (D/L^2) could be determined. The K values for the elderberry and red radish lipstick formulations were 0.243 ± 0.02 and 0.204 ± 0.01, respectively (Table 4.3). The D/L^2 values (hour^-1) for the elderberry and red radish lipstick were 0.142 ± 0.05 and 0.136 ± 0.07, respectively.

**Table 4.3**: Permeation parameters obtained after application of elderberry and red radish lipsticks to porcine ear skin. All values are means ± S.D (n=3). Statistical significance denoted by (×) (p≤ 0.05), determined by Two-Way ANOVA.

<table>
<thead>
<tr>
<th>Skin Permeation Parameters</th>
<th>Formula</th>
<th>K^* (h^-1)</th>
<th>D/L^2* (cm h^-1)</th>
<th>K_p ∙10^4 (μg cm^-2 h^-1)</th>
<th>t_L[h]</th>
<th>J_{ss} [μg cm^-2 h^-1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elderberry</td>
<td>0.243 ± 0.02</td>
<td>0.142 ± 0.05</td>
<td>3.74 ± 0.59×</td>
<td>1.17 ± 0.13</td>
<td>3.66 ± 0.11×</td>
<td>3.66 ± 0.11×</td>
</tr>
<tr>
<td>Red Radish</td>
<td>0.204 ± 0.01</td>
<td>0.136 ± 0.07</td>
<td>2.92 ± 0.41×</td>
<td>1.23 ± 0.19</td>
<td>2.86 ± 0.04×</td>
<td>2.86 ± 0.04×</td>
</tr>
</tbody>
</table>

*K, Vehicle/SC Partition Constant, D/L^2, diffusivity coefficient within the skin, (Eq. 1), Fig. 18

*K_p, Permeation Coefficient within the skin, (Eq. 2)

^t_L, Lag time for diffusion across the SC

^J_{ss}, Predicted steady-state flux within the skin, (Eq. 3)

The skin permeation coefficient (K_p) for both elderberry and red radish were approximately two orders of magnitude smaller than their respective K_r values. The differences in these two coefficients highlight the difficulty of overcoming the SC barrier (Trommer and Neubert 2006). The partition coefficient between that of the formulation and the SC (K) showed a lower affinity of the anthocyanins for the skin than for the
vehicle. When compared to the steady state flux (Jss), K indicates that the SC barrier must be overcome as a prerequisite for permeation of anthocyanins across the skin. Diffusion through the skin once past the rate-limiting SC should hypothetically arise much quicker and be independent of the vehicle composition (Lane et al 2012).

The work by Bojanowski (2013) supports this hypothesis, specifically for anthocyanins from grape seed extract (GSE). This author reported the hypodermal delivery through a transbuccal membrane of GSE when incorporated into soy phospholipids (Bokanowski 2013). They found GSE was able to permeate into both the dermis and epidermis when applied to the hypodermal side of the skin. Hypodermal delivery is one strategy for getting around the barrier function of the SC and their work further suggests a beneficial role of anthocyanins for skin-care cosmetics.

The passage of compounds through the skin occurs through diffusion; therefore, target molecules for transdermal delivery generally have a molecular weight \( \leq 500 \text{ Da} \) (Lane et al 2012). This is due to the assumption that molecular weight is a direct reflection of molecular size.

Elderberry primarily consists of two major anthocyanins: cyanidin-3-sambubioside (C3S) and cyanidin-3-glucoside (C3G), with MW of 581.5 and 449.2, respectively (Veberic et al 2009). C3S accounted for over 60% and C3G accounted for around 37% of the total anthocyanins present in the extract used. This distribution is consistent with the literature (Veberic et al 2009; Duymus et al 2014). Based on MW, the penetration of elderberry anthocyanins was hypothesized to be feasible and our results indeed support the ability to permeate into the SC.
The primary anthocyanins present in red radish are pelargonidin-sophoroside-5-glucoside (Pg-soph-5-glu) acylated with cinnamic and malonic acid (Giusti and Wrolstad 1996). Pg-soph-5-glu derivatives with MW between 900-950 accounted for around 22%, derivatives with MW between 950-1000 accounted for around 39% and derivatives with MW ≥1000 accounted for around 39% of the total anthocyanins present in the extract used. This distribution is consistent with the literature (Giusti and Wrolstad 1996; Barry 2013). Based on MW, red radish anthocyanins were hypothesized to be unable to overcome the SC barrier; however, our results show that they do indeed permeate into the skin.

The diffusivity (D/L^2) of the red radish lipstick seems to suggest that the molecular weight of these compounds is not a deterrent to their diffusion in the skin. Several studies (Goto 1987; Figuieredo et al 1996a/b; Goto and Kondo 1991) have proposed intramolecular copigmentation with folding of the anthocyanin molecule due to stacking between the planar ring of the aromatic acid and the aromatic nuclei of the anthocyanin through π-π hydrophobic interactions. Intramolecular copigmentation has also been proposed to cause folding of the acyl aromatic rings over the positively charged pyrylium ring, which may account for the increased stability of acylated anthocyanins (Goto and Kondo 1991). More specifically, Giusti et al (1998) reported NMR results for spacial conformations of red radish anthocyanin molecules in which a close proximity was shown between the hydrogens of the acylating groups and the C4 of the aglycone structure, suggesting a sandwich-type folding of the acyl group over the pyrylium ring.
Our work is in agreement with the proposed theory of molecular folding of acylated red radish anthocyanins. Once folded, despite the large red radish anthocyanins molecular weight, the size of the molecules may be more compact than expected, which could account for the permeation of the anthocyanins within the SC. Another possible mechanism for the diffusivity of the red radish anthocyanins is the slightly amphiphilic nature of anthocyanins containing aromatic or aliphatic acylating groups (Anderson and Jordheim 2013, Giusti et al 1999). Moreover, a dataset published by Flynn (1990) confirms that percutaneous absorption of compounds is lower for compounds that are extremely hydrophilic or hydrophobic, suggesting that a slightly amphiphilic nature may be an advantage for diffusion through the inhomogeneous layers of the skin below the SC (Flynn 1990).

Both anthocyanin-lipsticks were found to penetrate the skin and were identified at depths relevant for overcoming the SC. In order for their beneficial properties to be observed, the anthocyanins must also reach the target skin site, such as the epidermis and dermis, and not permeate into the microcirculation (Zillich et al 2015). Catechins, EGCG, and quercetin have all been found to localize in the stratum corneum, viable epidermis and dermis (Zillich et al 2013, Dal Belo et al 2009, Fang et al 2006, Frauen et al 2002), and these findings have been correlated with their health-promoting properties observed on the skin. Based on the evidence in the literature of similar health benefits of anthocyanins, it seems likely that they will also be preferentially located in these layers (Enomoto et al 2005; Afaq et al 2009; Chan et al 2010; Hwang et al 2013; Plundrich et al...
However, studies investigating their quantification in the epidermis and dermis, as well as the IC$_{50}$ values necessary to exhibit these benefits in vivo need to be performed.

4.5 CONCLUSION

Anthocyanins from elderberry and red radish incorporated into lipstick formulations were shown to permeate through the stratum corneum, which is the rate-determining phase for skin penetrating compounds. The combined use of ATR-FTIR and PLS-R analysis was successfully able to quantify the anthocyanins removed by tape stripping of porcine skin with minimal sample preparation. This allowed for the determination of release and skin permeation profiles for the anthocyanins from lipstick formulas. The use of Higuchi’s square root model was used to describe the diffusion of the anthocyanins within the lipsticks. The influence of hydrophilicity and molecular weight of these compounds on their release and skin permeation kinetics was also investigated. Our results are in agreement with the literature that smaller hydrophilic compounds are released and permeate within the skin at a faster rate. However, we found that both elderberry and red radish anthocyanins were able to penetrate into the skin and reach depths relevant for their use as beneficial ingredients in skin care products.
CHAPTER 5: OVERALL CONCLUSIONS

The aim of this study was to evaluate the potential for anthocyanins to be used as both sources of natural color and biologically active ingredients within lipstick formulations.

Successful anthocyanin lipstick formulations exhibited colors ranging from a vibrant red for red radish to a deep plum/purple for elderberry. Lipstick formulations containing acylated pelargonidin based anthocyanins from red radish were the most similar in color when compared to reference samples of commercially available lipsticks and cosmetic colorants providing red color. Formulations from purple carrot, purple sweet potato, purple corn and red grape were very similar to the more pink reference samples.

All formulations showed remarkable stability within the formulations according to accelerated testing of two-year shelf-stability. Non-acylated cyanidin based anthocyanins from elderberry, as well as, acylated cyanidin based anthocyanins from purple corn and purple sweet potato proved to be the most resistant to changes in color at accelerated testing conditions compared to anthocyanins from purple carrot, red grape skin and red radish.

All formulations showed the ability to act as photoprotective additives through UV absorption, especially those with cinnamic acid acylation. The anthocyanin
formulations showed the capability to act as antioxidants, through scavenge free radicals, in a lipstick matrix. In addition, the formulas showed proficient tyrosinase inhibition, which is a well-known source of melanin formation in the skin. The concentrations necessary to exhibit these activities were all well within physiologically relevant concentrations based on the average uses of lipsticks within the United States.

Permeation studies of lipsticks containing elderberry and red radish anthocyanins showed the ability of both cyanidin and pelargonidin based anthocyanins to penetrate the stratum corneum and permeate within the skin when using a porcine skin model. The permeation rates were highest for the lower molecular weight elderberry anthocyanins; however, both anthocyanin sources were found at skin depths relevant for topical benefits to be realized.

Overall, our findings present strong evidence for the beneficial uses of anthocyanins in cosmetics, both to act as alternatives to synthetic colorants, as well as their use as active ingredients for skin care.
REFERENCES


