Characterization and quantification of anthocyanins and other phenolics in the fruit of Beautyberry species

Thesis

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Abstract

Beautyberry plants are native to the United States and Asia, depending on the species. The most common species grown are the American beautyberry (*Callicarpa americana*), Japanese beautyberry (*Callicarpa japonica*), Bodinier Beautyberry (*Callicarpa bodinieri*), and Purple beautyberry (*Callicarpa dichotoma*). Some species have been bred to showcase white or pink fruit, but the most popular and original fruit colorations are the purple varieties. The vibrant purple of the purple varieties is rarely found in nature, but little is known about the fruit and the color. The objective of this study was to identify the compounds contributing to the color of the purple-colored beautyberry fruit. It was hypothesized that the color and pigmentation were from anthocyanins.

For this study, cultivated beautyberry fruit were analyzed and compared to determine color-contributing compounds and characteristics. Three species of beautyberry (americana, dichotoma, and bodinieri beautyberry) grown in triplicate in Piketon, Ohio were analyzed comparatively to determine their quality attributes including individual fruit weight, total harvest weights, pH, color(CIE L*a*b* C_{ab}*h_{ab}˚), total soluble solids (˚Brix), in addition to color-contributing components using assays for total phenolics (Folin-Ciocalteu assay for gallic acid equivalents), flavonoids (aluminum chloride colorimetric assay for catechin equivalents) and total monomeric anthocyanins

(pH differential method for cyanidin-3-glucoside equivalents) in addition to analyses using uHPLC PDA MS/MS chromatography on a C18 column.

Results showed that the American beautyberry had significantly larger individual fruit weights than Bodinier and Purple beautyberry in addition to significantly larger fruit diameters, while Purple beautyberry had significantly larger fruit yields than American and Bodinier beautyberry fruit (138 g compared to 28 g and 25 g, respectively). The total soluble solids and fruit pH were not significant between plant species. The results showed that the anthocyanin content was not significantly different between American (196 $\pm$ 220 cyanidin-3-glu equivalents mg/100 g FW), Purple (140 $\pm$85 cyanidin-3-glu equivalents mg/100 g FW), and Bodinier beautyberry fruit (112 $\pm$6 cyanidin-3-glu equivalents mg/100 g FW), despite the larger size of the American beautyberry fruit. This suggests that the color should show no significant different between species, as anthocyanins are major color contributing components in plants. But a difference could be detected by the common observer ($\Delta E \geq 5$) of the Purple beautyberry fruit extract compared to American or Bodinier beautyberry fruit extract. The total flavonoids were also not significant between American, Purple, and Bodinier beautyberry fruit. However, the total phenolics were significantly different between Purple beautyberry (477 $\pm$ 124 gallic acid equivalents mg/100 FW) and Bodinier beautyberry (2165 $\pm$ 311 gallic acid equivalents mg/100 FW), but American beautyberry fruit total phenolics (862 $\pm$603 gallic acid equivalents mg/100 FW) was not significantly different from Purple or Bodinier beautyberry. This was consistent with the ΔE values of the initial extract. The chroma of the extract decreased with the removal of non-anthocyanin phenolics with

ethyl acetate on a C18 cartridge, which suggests that phenolics have an important contribution to the color of the anthocyanins through copigmentation.

Color-contributing compounds were tentatively identified through *m*/*z*, fragmentation patterns, retention times, and spectra compared to literature. Purple beautyberry fruit analysis led to the tentative identification of two major anthocyanins , specificallypeonidin 3,5 diglucoside non-acylated and acylated with malonic acid. InAmerican beautyberry fruit the anthocyanins were tentatively identified as cyanidin 3,5 diglucoside non-acylated and acylated with malonic acid. Bodinier beautyberry fruit had four major anthocyanins from both Purple and American beautyberry fruit. The major phenolics present that may be contributing to copigmentation but removed with ethyl acetate were tentatively identified as verbascoside, 3-caffeoylquinic acid, apigenin-7-O gluconuoride, apigenin 7,4' diglucunoride, luteolin 7-O-gluconuoride, and quercetin-3-gluconuroide.

The color in American beautyberry, Bodinier beautyberry, and Purple beautyberry is a result of the composition containing anthocyanins and other flavonoids. Understanding the structural characteristics of the color-contributing compound in beautyberry fruit will allow for a better understanding of its potential food applications.

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Publications

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Allan, M.., Chamberlain, M., Grush, E., Owens, B., & Mauer, L. (2018). Deliquescence and anhydrate-hydrate RH-temperature phase diagrams of three hydrate-forming crystalline ingredients: Lactose, trehalose, and caffeine. *IFT*.

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Fields of Study

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Chapter 1. Introduction

Beautyberry plants, or *Callicarpa*, are deciduous shrubs that were named for their gorgeous fruit, with the name originating in Greek from the words “callos” and “carpos”, which translates to “beauty” and “fruit” (Atkins, 1999). The fruit has traditionally been used to make wine, tea, and jelly in non-commercial settings, but the plant is underutilized in comparison to the potential opportunities for use (Ullah *et al*, 2020). Beautyberry plants are most known for their edible purple fruit, which is vibrant and has a coloration that is rarely found in nature. While there have been several studies conducted on the leaves of the plant, there is a deficit in information about the fruit and the unique coloration.

Beautyberry is plant in the Lamiaceae family, with over 140 known species (Hung, 2020). American beautyberry (*Callicarpa americana*), Bodinier beautyberry (*Callicarpa bodinieri*), and Purple beautyberry (*Callicarpa dichotoma*) are commonly grown in the United States in landscaping. While some varieties have been bred to have white or pink fruit, these species are known for producing vibrant purple fruit with a mild taste. The American beautyberry is native to the southeastern United States. Bodinier and Purple beautyberry (*Callicarpa bodinieri* and *dichotoma*, respectively) have increased hardiness against the colder temperatures of the Midwest compared to American beautyberry, so these two were also chosen for the increased potential to favor the

growing conditions of Ohio used in this study and their availability from plant growers (Martin & Sick, 1995; Graves & Thomas, 2011; Reed *et al,* 2011).

Callicarpa bodinieri fruit has been found to contain anthocyanins with cyanidin and peonidin aglycones, which are types of pigments know as anthocyanins (Jones and Kinghorn, 2008). However, Purple beautyberry and American beautyberry are also commonly grown in the US, and their fruit compositions have not been studied extensively. In addition, reports of the aglycones in Bodinier beautyberry were found but information on substitutions, which play an important role in anthocyanin color expression.

Anthocyanins are currently used in food applications due to their unique ability to contribute a range of colors from red to blue depending on the pH and are derived from fruits and vegetables (Wallace & Giusti, 2019). Vegetable sources of anthocyanins like red cabbage can have bright colorations and increased stability due to the tendacey to be acylated, but also impart undesirable off-flavors (Sigurdson *et al,* 2017). Fruit sources generally have a mild taste that can be masked, but many are dark in color or are used for food consumption rather than pigment extractions. New sources of anthocyanins that have bright colorations, are under-utilized, and have a mild flavor are desirable to replace artificial or synthetic colors with naturally derived colors.

Colors from pigments found naturally in plant sources are of increased interest as consumers move towards clean-label, natural-based products and away from synthetic ingredients, over associations between synthetic food colorants and hyperactivity in kids and general distrust of synthetic ingredients (McCann et al., 2007). The current supply of

naturally derived colors is not sufficient to fully replace artificial colors, with the Institute of Food Technologists finding that "the supply of natural raw materials could be a concern if demand continues to grow" and Sensient reporting that they "have exhausted the current colors from natural sources available at this time (Nachay, 2019; Poinski, 2021). Therefore, investigations into additional sources of color are needed to meet the demand for naturally-derived colors.

Many of the plants containing naturally derived pigments that are used in food applications also contain compounds that have led to their usage for medicinal purposes that could provide value in food color applications while imparting other compounds that are beneficial to health (Khoo, 2017). In recent years, compounds from American beautyberry plant leaves have been studied and it has been suggested that there are compounds present that could be effective against MRSA and exhibit insect-repelling capabilities comparable to DEET (Dettweiler *et al*, 2020; Bryson, 2006; Core *et al*, 2005). These characteristics could potentially be contributed to phenolics, which are compounds found in plants. Phenolic compounds can be beneficial in human health, including potentially functioning as antioxidants that can reduce cell damage from free radicals (Soto-Vaca, 2012). With the increased interest in the production of American beautyberry for the functional properties of the leaves, the fruit could become a byproduct or secondary product from the same crop, so compositional studies are needed to optimize the usage of the plant.

The purpose of this study was to evaluate the composition of American, Bodinier, and Purple beautyberry by investigating major compounds that impart color to the fruit.

The quality attributes were investigated first, including fruit weights, pH, and total soluble solids. The composition analyses included total phenolics and monomeric anthocyanins, in addition to uHPLC PDA MS/MS analyses. From this research, the results could increase understanding of the color from beautyberry species commonly grown in the United States and increase the utilization of the plants beyond the leaves through further investigation of the fruit for naturally-derived colorant food applications.

Chapter 2. Literature Review

2.1 Beautyberry

2.1.1 General Botanical Information

Beautyberry plants are perennial, deciduous shrubs from the Lamiaceae family that are native to Asia, Central America, and southeastern North America. Beautyberry, or *Callicarpa* species, are named after the beautiful fruit they bear, with the name originating from the Greek words “callos” and “carpos”, which translates to “beauty” and “fruit”(Atkins, 1999). Of the over 140 species of beautyberry, some of the most common species are reported in Table 1 (Hung, 2020). A few cultivars have been bred for white fruit (*Callicarpa dichotoma* f. albifructa ‘Duet’, *Callicarpa americana* var. *lactea*, etc.), but the plant was originally named for the vibrant coloration of the purple fruits (*Callicarpa americana*, *Callicarpa dichotoma*, and *Callicarpa bodinieri*)(Hodges et al, 2015). The hues of the purple fruit are unique and rarely found in nature, which exhibits a beautiful contrast against the bright green leaves of the plant. Beautyberry plants are currently abundant in nature and are utilized as ornamental plants in landscaping that add visual appeal. Additionally, the fruit has a history of consumption and has been used in home recipes that include teas and jellies (Ullah *et al*, 2020).

***Callicarpa* species**	**Native Growing Locations**
Callicarpa americana	United States, Cuba, Bermuda, Bahamas
Callicarpa bodinieri	China
Callicarpa dichotoma	China, Japan, Korea, Vietnam
Callicarpa japonica	China, Japan, Korea, Taiwan
Callicarpa kwangtungensis	China
Callicarpa longifolia	China, New Guinea, Christmas Island
Callicarpa marcophylla	China, Himalayas, India, New Guinea
Callicarpa nudiflora (Hook & Arn)	China, Himalayas, India

Table 1. Common *Callicarpa* species and native growing regions.

The fruit belongs to the drupe category, which contains 2-4 seeds encased in the endocarp (North Carolina State University Extension). The plants are drought-resistant and grow best in full sun to partial sun with well-drained soil, varying from clay floodplains to sandy and salty soil. Beautyberry spp. can grow up to 2-12 feet tall and 3-6 feet wide (Martin & Mott, 1997). The leaves have an aromatic and medicinal scent, which is common to plants in the Lamiaceae family. Beautyberry plants are not susceptible to significant damage from insects or diseases, and require low maintenance, easily undergoing propagation and growth (Andrews, 2013). They are self-compatible but can have increased fruit production if multiple plants are grown together. Flowering occurs in the summer, which aids in survival against spring frost, and the fruit ripens from green to vibrant purple from summer to autumn. The fruits are harvested in late summer and early fall, by hand or with pruners (Graves & Thomas, 2011). Beautyberry

species that are commonly grown in the United States include: *americana* (American), *dichotoma* (Purple), *bodinieri* (Bodinier), and *japonica* (Japonica) beautyberry. American, Purple, and Bodinier beautyberry were chosen for this study (Figure 1) due to the availability of plants for the 2021 growing season in Ohio. Additionally, there has been quite a few research publications on the composition of Japanese beautyberry, whereas there is still many unknowns regarding the other three species (Atkins, 1999; Jones & Kinghorn, 2008) American beautyberry is native and could be a low-cost plant to utilize by harvesting naturally growing plant fruits, but an additional two varieties were chosen for their ability to withstand cold temperatures more effectively (Martin & Sick, 1995). Comparing several species of beautyberry, despite their close visual similarities, was also chosen due to the findings by that found that there were noticeable differences in the volatile compounds of *Callicarpa japonica* and *Callicarpa americana* leaves (Tu *et al,* 2013).

Figure 1. Pictures of beautyberry plants. American beautyberry (Top), Bodinier beautyberry (Bottom), and Purple beautyberry (Right).

American beautyberry (*Callicarpa americana)* is native to the southeastern United States (Figure 2) while Purple beautyberry (*Callicarpa dichotoma*), and Bodinier beautyberry(*Callicarpa bodinieri)* are indigenous to Eastern Asia. The plant characteristics and required growth conditions are important when considering a cultivation location. The USDA Plant Hardiness Growing Zones are a determinant that gardeners and plant growers use to determine where plants are most likely to thrive (Figure 3). American beautyberry grows in USDA zones 6-11, Purple beautyberry (*Callicarpa dichotoma*), and Bodinier beautyberry(*Callicarpa bodinieri)* are indigenous to China and are hardy in USDA growing zones 5-8. Piketon, Ohio was chosen as the growing location and is located in growing zone 6 so American, Purple, and Bodinier beautyberry plants were expected to thrive.

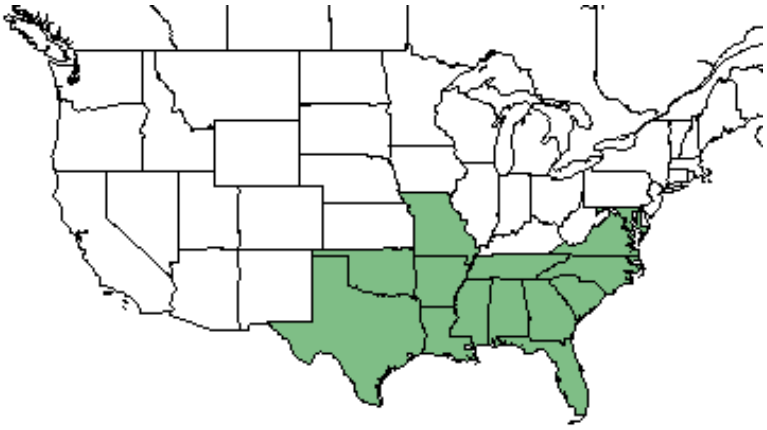

Figure 2. Map of United States where American beautyberry is native. (USDA-Natural Resources Conservation Service).

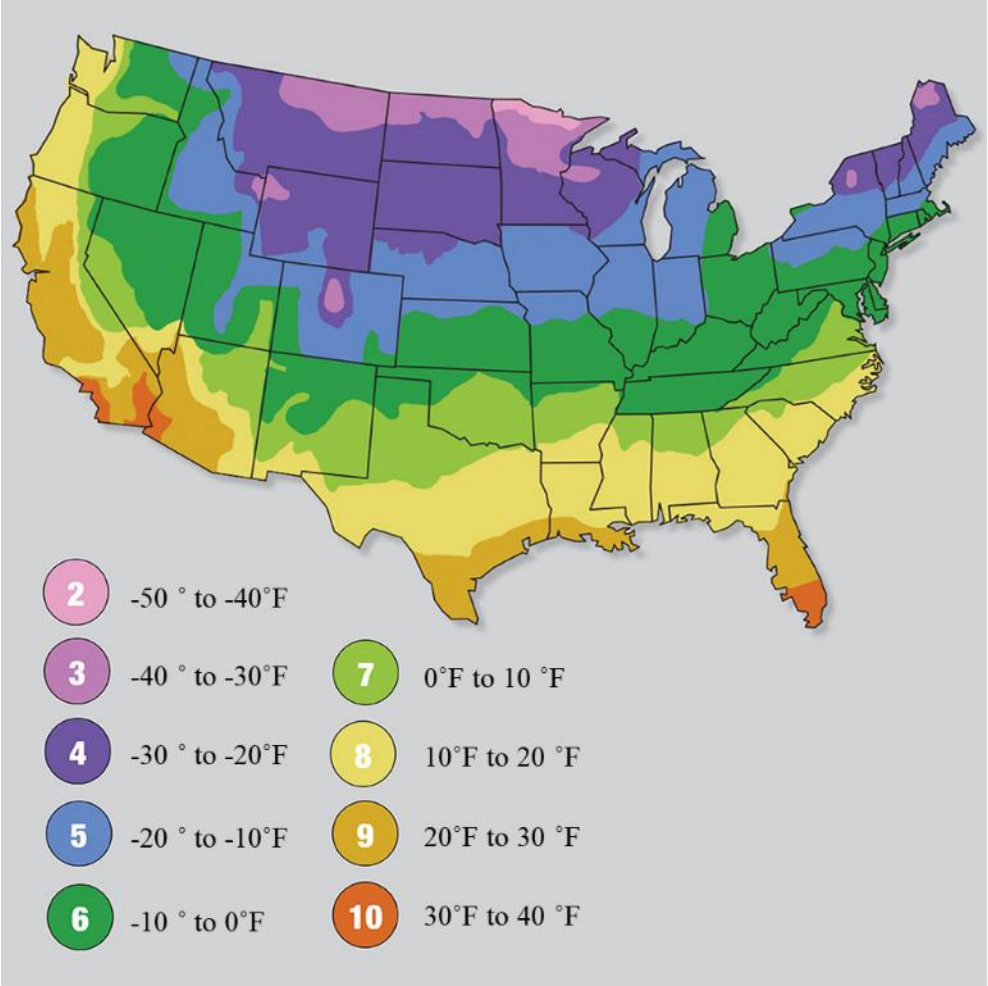

Figure 3. Map of USDA hardiness growing zones. American beautyberry hardiness zones: 6-10. Bodinier and Purple beautyberry hardiness zones: 5-8.

When distinguishing between American, Bodinier, and Purple beautyberry plants, the fruit and the leaves are key components that have some noticeable differences shown in Figure 4. American beautyberry fruit grows in tight clusters close to the branch, while Bodinier and Purple Beautyberry fruit grow in loose clusters along the branches. American beautyberry has slightly larger fruit than the non-native species and larger plant leaves that are wide and rounded. Purple beautyberry leaves are smaller and oblong while

Bodinier beautyberry leaves are rounded but smaller than American beautyberry leaves (Clemson University, 2015).

Figure 4. Picture of ripe beautyberry fruit. American beautyberry (left), Purple beautyberry (center), and Bodinier beautyberry (right).

2.1.2 American Beautyberry

Although there are over 140 species of beautyberry, American beautyberry is the only one native to the United States (Hung *et al*, 2020). American beautyberry is native to the central and southeastern United States and is also native to northern Mexico, Cuba, and Bermuda. The purple fruit grows in tight clusters and have a mild flavor. American beautyberry produce fruit once they are two years old with optimum fruit production at five years of age with over 1500 g of fruit produced but following years decrease to around 100 g when the plant is 10 years old. The serrated leaves are twice as long as they are wide with a pointed tip and rounded tapered base that can grow 3-6 inches long and 1-3 inches wide with a medicinal fragrance emitted (Halls, 1973). Beautyberry fruit is consumed in the late fall through early winter by small mammals, including armadillos, foxes, opossum, raccoons, and squirrels, in addition to songbirds, including the American Robin, Brown Thrasher, Purple Finch, and Eastern Towhee. White-tailed deer feed on the

leaves during the summer months and consume the fruit after the leaves drop in the fall (North Carolina State University Extension).

2.1.3 Purple Beautyberry

Purple beautyberry, or *Callicarpa dichotoma,* is native to Asian countries including China, Japan, Korea, and Vietnam. Although native to Asia, is non-invasive and seedlings spread very slowly after dropping (Vogelpohl, 2016). It is hardy in USDA zones 5-8, which includes most of the Midwestern and southern states, from Illinois as far south as Alabama. However, *Callicarpa dichotoma* is on the invasive species list in Alabama so should not be grown there (Alabama Invasive Plant Council, 2007). The species name dichotoma means forked in pairs, which is descriptive of the branched fruit clusters (North Carolina State University Extension). It is one of the most common species of beautyberry sold in the United States for landscaping and is more hardy to cold temperatures than the native *Callicarpa americana* (Martin & Sick, 1995). It is a deciduous, woody shrub with purple or white fruit. *Callicarpa dichotoma* has beautiful purple fruit while *Callicarpa dichotoma* forma *albifruita* is grown for abundant white fruit clusters (North Carolina State University Extension). The cultivar *Callicarpa dichotoma* 'Issai' is a cultivar bred for shorter branches that produce more fruit, and produce fruit at an earlier age. Issai means fruiting in early years, which is what gave the cultivar its name ((Missouri Botanical Garden,n.d.). *Callicarpa dichotoma* "Early Amethyst" is a cultivar that was bred to bloom earlier than other cultivars, hence the inclusion of early and amethyst describes the purple fruit (Laushman *et al*, 2019). The fruit is edible and has a mild flavor. It can grow in a variety of soil conditions, from

acidic to neutral pH soil that is composed of clay, loam(silt), or sand, with a variety of moisture compositions, including well-drained, moist, or occasionally dry soil. It grows best in full sun or partial-shade. Purple beautyberry is a low maintenance plant that is resistant to deer browsing and drought, and experiences low susceptibility to pests and diseases. Bees and butterflies are attracted to the flowers in the spring and summer and provide pollination for fruit production (Janoski & Yisela, n.d.). The fruit ripen in September and can last on the branch through early winter. The fruit is a wildlife food source that is consumed by birds and small mammals. The plant can grow 2-4 feet tall and wide and has serrated leaves that range from 1-3 inches long, that are twice as long as they are wide (North Carolina State University Extension).

2.1.4 Bodinier Beautyberry

Bodinier beautyberry, *Callicarpa bodinieri*, is native to China and produces fruit in September and October. The fruit will stay into early winter after leaves had dropped. The plant was named after Emile Marie Bodinier, who was a French missionary, who created an extensive plant collection of more than 3000 samples and had 200 plants named after her. The species was named by Augustin Abel Hector Léveillé who was a French botanist and a priest (Natural History Museum, 2013). It is hardy in zones 5-8 and can grow best in sets of pairs but are also self-compatible. Pollinators such as bees, butterflies, and birds are attracted to the shrub and birds feed on the fruit when other sources are depleted in late fall and early winter. The strong aroma from the leaves deter deer from foraging on the plant, making it resistant to deer damage (North Carolina State University Extension). The cultivar “Profusion” or *Callicarpa bodinier v. giraldii* is the

most grown cultivar of Bodinier beautyberry and was bred to have an increased fruit production (Breen, 2022).

2.1.5 Health Benefits and Uses

The National Institute of Health described American Beautyberry and its usage by Native Americans for a variety of traditional medicine purposes (Jones & Kinghorn, 2008). The bark was used to treat fever, malaria, and rheumatism (joint and muscle inflammation). The roots were used to treat colic, skin cancer, and dysentery (bloody diarrhea). The fruit was shown to also be used for colic. Bodinier beautyberry was also used for medicinal purposes in in Traditional Chinese medicine for wounds, blood in excreted body fluids, bleeding after surgical procedures, and bruises (Jones & Kinghorn, 2008).

Historically, American beautyberry plant leaves were used as an insect repellent by placing the leaves under the harnesses of horses and mules. The friction of the harness against the animal crushed the leaves and created a paste that repelled biting insects (Cantrell & Klun, 2006). The United States Department of Agriculture- Agricultural Research Service (USDA-ARS) did a study (Core *et al*, 2005) on the effectiveness of the leaves as an insect repellent, and found intermedeol and callicarpenal, compounds that were as effective at repelling insects as SS220. SS220 is an insect repellant synthesized and patented by the USDA-ARS, that when used at 20 percent, is as effective as 33 percent N,N-diethyl-meta-toluamide (DEET) in protecting against the major species of disease-carrying mosquitoes, including *Aedes aegypti*, the yellow fever mosquito, and *Anopheles stephensi*, a Malaria-carrying specie of mosquito. Additionally, intermedeol

and callicarpenal were also effective at deterring ticks. DEET is the major insect repellent on the market for personal application and use. However, alternatives to DEET are of interest, due to some consumer health concerns over skin irritation in addition to the capability of DEET to melt some plastics (Legeay, S., 2018; Core *et al*, 2005).

In addition to the insect-repelling capabilities, an ethanol-extracted clerodane diterpene from American beautyberry leaves have also shown potent synergistic effectiveness against Methicillin-resistant Staphylococcus infections, or MRSA, when used in conjunction with oxacillin, a β-lactam antibiotic that is commonly used against MRSA. The clerodane diterpene in a recent study conducted by Notre Dame University and Emory University (2020) was identified as 12(*S*),16ξ-dihydroxycleroda-3,13-dien-15,16-olide, and also displayed moderate activity in decreasing MRSA when used alone (Dettweiler, 2020). MRSA is a major problem in the healthcare community because of the hard-to-treat infections that can spread in hospital settings during invasive procedures or from skin-to-skin contact. It is considered a superbug, due to the resistance to antibiotics. MRSA can start as a pimple and stay confined to the skin or it can penetrate bones, joints, the bloodstream, heart valves, and lungs, which can result in deadly infections (Vestergaard *et al*, 2019). Therefore, additional aids in the treatment of MRSA are extremely needed.

American beautyberry has also been shown to inhibit the growth of the Gram-positive bacterium *Cutibacterium acnes*, which is attributed to the occurrence of acne from the irritation of skin follicles (Pineau *et al*, 2019). Acne is a major skin impurity that affects hundreds of thousands of people and different skin types can react to treatments

differently, so a variety of available solutions is of value. Biofilms from C.acnes can also form on medical equipment, which can be difficult to remove due to the binding tendencies (Varin-Simon, 2021). There is an increased risk of infection from surgical procedures due to the durability and strength of some bacteria to form biofilms on medical equipment used in and around exposed areas on the body.

2.1.6 Past Compositional Research

Many species of *Callicarpa* have been studied for the identification of components in the plant leaves because of their role in traditional medicine. A compilation of compounds identified in various beautyberry species is shown in Table 2. Phenylpropanoid glycosides along with the flavonoids luteolin and apigenin derivatives have been semi-quantified and identified in *Callicarpa nudiflora Hook et. Arn, macrophylla, Vahl.* and *kwangtungensis Chun* (Shi *et al,* 2013). In Bodinier beautyberry leaves, diterpenoids, oxygenated sesquiterpenoids, sesquiterpene hydrocarbons, and oxygenated monoterpenoids were identified in the leaves (Ren *et al,* 2003).

Compound	Compound Type	Plant Part	Usage	Species
callicarpenal	Diterpenenoid	Leaves	Insect repellent	*C.americana, C. japonica*
intermedeol	Sesquiterpenoid	Leaves	Insect repellent	*C. americana, C. japonica*
apigenin-7-O-glucuronide	Flavonoid	Leaves	Antioxidant, anti-inflammatory	*C. longifolia, C. macrophylla, C. americana*
luteolin-7-O-glucuronide	Flavonoid	Leaves	Antioxidant, anti-inflammatory	*C. longifolia, C. marcophylla*
verbascoside (aceteoside)	Phenylethanoid	Leaves	Antimicrobial, anti-inflammatory, neuroprotective	*C. bodinieri, C. tomentosa*
camphor	Sesquiterpenoid	Leaves	Analgesic, cough reducer, antifungal	*C. japonica*
cyanidin	Flavonoid	Fruit	Antioxidant, anti-inflammatory	C. bodinieri
Cynaroside (Luteolin-7-O-glucoside)	Flavonoid	Entire plant	Anti-inflammatory, antioxidant	*C. bodinieri*
peonidin	Flavonoid	Fruit	Antioxidant, anti-inflammatory	*C. bodinieri*

Table 2. Common compounds reported in *Callicarpa* species and their characteristics. (Jones & Kinghorn, 2009; Jones *et al*, 2007).

American beautyberry fruit and leaves have been shown to contain clerodane diterpenes in addition to genkwanin (Dettweiler *et al*, 2020; Porras *et al*, 2019). Additionally, anthocyanins have been found in the reproductive parts of American beautyberry plants, which include the flowers and fruit, but studies on the aglycone and

substitutions were limited (Lee & Collins, 2001). Luteolin derivatives have been found in Bodinier beautyberry, in addition to the flavonoids peonidin and cyanidin (Jones & Kinghorn, 2009). However, there is limited knowledge on the color and color-contributing compounds in the fruit, specifically for the concentration of anthocyanins and the identification of specific anthocyanins structures.

2.2 Phenolics

Phenols, or phenolics, are secondary metabolites that are innately present in many plants. They are a class of chemical compounds that have one or more hydroxyl groups (-OH) bonded to an aromatic hydrocarbon group. These can be simple or complex from polymerizations, depending on the number of hydroxyl groups. Phenolics are often referred to as polyphenols and there are thousands of phenolic compounds found in nature (Schwartz *et al*, 2015).

Plants synthesize phenolics throughout growth, and concentrations can be impacted by environmental conditions including climate as the plant responds and adapts to external conditions. Phenolic compounds function as a natural defense for plants against overcrowding from other plants, microorganisms, insects, and ultraviolet radiation from the sun (Kumar *et al*, 2020). Phenolics can also act as selective attractants for insects and birds for pollination, and seed distribution via flavor and aromatic compounds. One way this is expressed is through specific levels of astringency or acidity that is characteristic of specific plant sources (Zhang, 2016). Phenolic content in food sources can also vary due to the degree of ripeness and processing conditions (Duodu, 2011).

In addition to potentially contributing benefits to the plant, phenolics can also impart color. Phenolics can undergo enzymatic oxidation by polyphenol oxidase (PPO), which can lead to black, brown, or red pigments (Mijangos *et al*, 2006). Phenolic oxidation can be seen in the browning of freshly cut apple slices when exposed to oxygen. Phenols are generally stable against processing conditions if there is minimal oxygen contact and PPO activity. While the color compounds of phenolic oxidation is undesirable, some phenolics contribute characteristic colors that are attractive and beneficial. For example, anthocyanins act as natural pigments that are important in food acceptance while also potentially contributing beneficial health benefits (Shahidi & Naczk, 2003). Other phenolics in the prescence of anthocyanins can also provide copigmentation that increases the intensity of the absorbance of light at 520 nm. (Pangestu *et al*, 2020). Out of the thousands of phenolics found in nature, flavonoids, which includes anthocyanins, are a main source of phenolic compounds in the diet (Prior & Cao, 2000).

2.2.2 Flavonoids

Flavonoids are a class of phenolics and there are over 6,000 flavonoids found in nature in plants (Schwartz *et al*, 2015). Flavonoids are found in the epidermis of plant leaves and the skin of fruits (Agati *et al*, 2013). The structure of flavonoids is composed of $C_6C_3C_6$ with three aromatic rings, A, B, and C (Figure 5). Flavonoids that are commonly found in nature are flavanols, flavonols, flavones, isoflavones, flavanones, and anthocyanins. Compounds in these classes are identified and grouped according to their hydroxyl, methoxyl, and other substitutions such as oxygenation, alkylation, glycosylation,

acylation, and sulfonation, including the number and positioning (Schwartz *et al*, 2015). Flavonoids typically occur as glycosylated or esterified forms, which are more stable than without these attachments. Common positions for substitutions are shown in Figure 5. Glycosylations and hydroxyl groups increase the solubility of the compounds in water, while other groups such as methyl groups can cause lipophilic behaviors (Plaza *et al*, 2014). Glycosylations can attach glucuronic acids or sugars, such as glucose, rhamnose, or galactose, to increase molecule solubility. A comparison of glucose and gluconic acid is shown in Figure 6. Flavonoids are known for their antioxidant properties through their redox potential and ability to chelate metals, which can contribute color, such as yellow and white in their non-oxidized state and brown and black oxidation products, in addition to contributions to red and blue colors (Steyn *et al*, 2014).

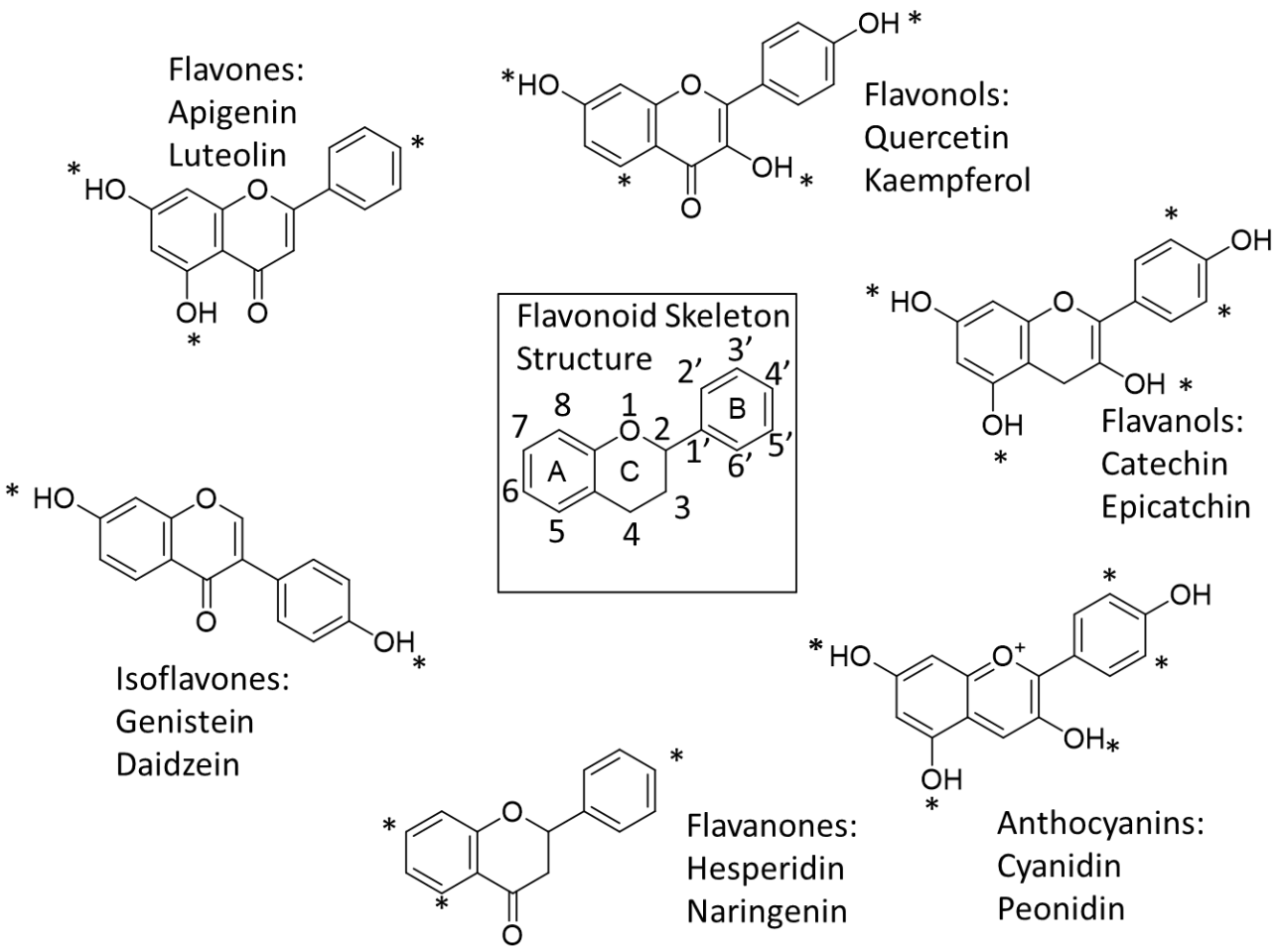

Figure 5. Skeleton structure of major flavonoid classes. Common positions available for substitution are designated with "*".

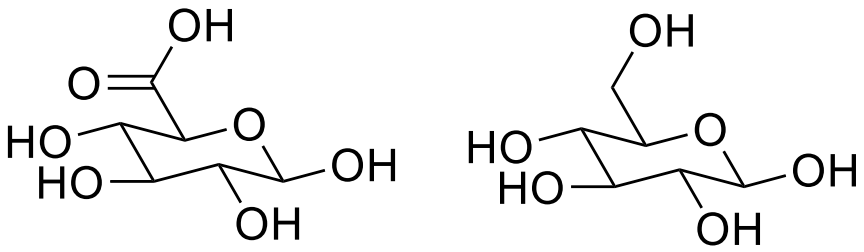

Figure 6. Comparison of structures of glycosylations. Glucuronide (left) and glucose (right).

2.2.3 Anthocyanins

Anthocyanins are water-soluble phenolic flavonoids with a $C_6C_3C_6$ structure and express a range of colors from red to purple to blue. Anthocyanins are the largest and most diverse group of plant pigments, with over 700 anthocyanins found in nature. They are naturally found in fruits, vegetables, and flowers (Schwartz *et al*, 2015).

Anthocyanins are typically composed of three main components in nature: an aglycone, sugar group, and acyl group. The base, core structure of anthocyanins are aglycones, or anthocyanidins (Figure 7). There are six major aglycones found in nature: cyanidin, delphinidin, malvidin, petunidin, peonidin, and pelargonidin (Table 3) that have unique characteristics due to differences in hydroxyl and methyl groups on the B ring, including stability and color expression. Anthocyanidins rarely occur in nature due to their limited stability (He & Giusti, 2010). Because of this, anthocyanidins typically have sugar attachments with glycosidic bonds, commonly at position 3' or 5'. Acyl groups are sometimes attached to sugars via ester bonds, and the anthocyanin is referred to as acylated. Acylation contributes to intermolecular and/or intermolecular stacking to increase anthocyanin stability and water solubility (Schwartz *et al*, 2015). Acylated

anthocyanins are not very common in fruits and are more prevalent in vegetable sources. They are more color-stable and soluble than the non-acylated anthocyanins, and they can impart a deeper color (He Giusti, 2010).

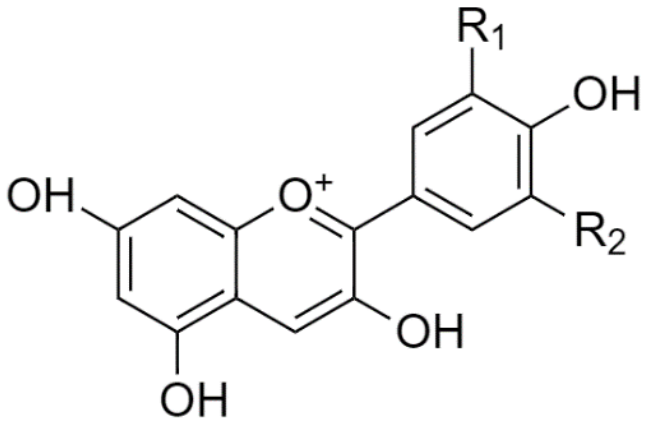

Figure 7. Chemical structure of an anthocyanidin.

Name	**R_1**	**R_2**
delphindin (dp)	OH	OH
petunidin (pt)	OCH_3	H
cyanidin (cy)	OH	H
pelargonidin (pg)	H	H
peonidin (pn)	OCH_3	H
malvidin (mv)	OCH_3	OCH

Table 3. Structure of the six most common anthocyanin aglycones.

Anthocyanins accumulate in plant cell vacuoles and formation increases when plants are under stress, such as when deciduous trees experience less sunlight in the fall and vibrant reds, oranges, and yellows can be seen in autumn leaves, in addition to the

loss of chlorophyll from decreased photosynthesis (Panagiota, 2006). Anthocyanins are found in in anthocyanoplasts (aggregations of anthocyanins), epidermal tissue of plants (the outermost layer of cells of flowers, stems, leaves, fruit, and roots), mesophyll (internal cells of leaves situated between two epidermal layers), and the flesh of fruits and vegetables (Steyn *et al*, 2002).

In addition to providing benefits to plants, anthocyanins could have some beneficial properties for human health. Anthocyanins have been suggested to have a variety of potential health benefits, including functioning as an antibacterial, antiviral, anti-inflammatory, and antioxidant (Wallace & Giusti, 2019). Anthocyanins are an alternative to artificial color usage in food due to the wide range of colors that can be produced by adjustments in pH (He & Giusti, 2010).

2.3 Color

Color is all around, and it impacts our experiences with the world. Color is the reception of energy waves by the eye that falls in the visible spectrum, about 380-780 nm (Schwartz *et al*, 2015). It has played an important part in food for thousands of years, such as serving as an indicator of ripeness in produce or as a warning against the consumption of poisonous animals (Zhou *et al*, 2020; Mallet & Joron, 1999). Foods with inherent color contain pigments, substances that are commonly found in the cells and tissues of plants and animals. Many of the compounds that cause pigmentation and color expression are biologically active, extending color beyond a visual characteristic to a bioactive one. (Solymosi *et al*, 2015) The ability of a specific food to reflect or emit

energy wavelengths within the visible wavelength range determines the color expression and overall perception of a food.

Studies have shown that color contributes to the perceived flavor and quality of food, which majorly impacts consumer decisions (Wallace & Giusti, 2019). For example, green strawberries are less likely to be selected for consumption compared to red strawberries due to perceived tartness, since color of fruit can be connected to sugar content (Maleyski *et al*, 1977) Color hues also can help in differentiation of flavors, such as a grape or berry flavored food could be purple, while a red or pink one could be strawberry flavored. Color lightness, or value, can also be important for flavor, such as the perception of a dark blue food perceived as blueberry while a vibrant blue could be perceived as blue raspberry. Therefore, the color of food is important for consumer perception and acceptability.

2.3.1 Color Additives

Color associations are an important component of consuming food. To standardize color of food products, colors are often added to enhance foods that may have lost color during processing or have naturally-occurring color variations. According to the U.S. Food and Drug Administration, food colorants can only be used to enhance and correct colors already present, provide color identity to colorless foods, or replenish color that was lost during storage. Color additives are described as “any dye, pigment, or other substance made or obtained from a vegetable, animal, mineral, or other source capable of coloring a food, drug, or cosmetic or any part of the human body” Ingredients that are added to a food for colorant purposes cannot be added for additional purposes, otherwise

they are not considered a color additive. Food colorants can never be used to hide product defects or with the intent to deceive consumers, and must be approved for use (21 C.F.R . § 70).

2.3.2 Certified Colorants

Studies have shown that color influences consumer decisions, a theme consistent throughout history, and has motivated the addition of color to maintain the appeal of products (Sigurdson *et al*, 2017). With an increased interest in colors in food, chemists and food producers turned to artificial colors. Substances that are added for color enhancing purposes are called dyes (21 C.F.R . § 70). Dyes used in food are food-grade, water-soluble, certified by the U.S. Food and Drug Administration in the United States, and given a FD&C number to express that it is acceptable for use in food, drug, and cosmetic products. (Schwartz *et al*, 2015). Colorants that are currently subject to certification in food and batch certification include FD &C Red No. 3, Red No. 4, Red No. 40, Blue No. 1, Blue No. 2, Green No. 3, Yellow No. 5, and Yellow No. 6, in addition to Citrus Red No. 2 and Orange B (21 C.F.R . § 70.4).

2.3.3 Consumer Concerns for Synthetic Colorant Safety

However, there has been a growing concern over the usage of artificial colors in food. In 2007, the South Hampton Study was released, which suggested a possible link of hyperactivity in children with ADHD to certain dyes (McCann *et al*, 2007). Although research is still ongoing to investigate the findings further, the consumer responses and increased regulations have been aggressive, with the implementation of warning labels on foods containing artificial colors. In Europe, the response was more severe, with a ban on

artificial colorants in food (Stich, 2016). In addition, the potential of food allergies and hypersensitivities to artificial food colorants has become a consumer concern as well (Vojdani & Vojdani, 2015).

2.3.4 Colors Exempt from Certification

Naturally-derived colors are an alternative to artificial colors and are exempt from certification, but must still be approved for use in the United States. Naturally-derived colors include compounds and extracts that are obtained from natural sources, fruit and vegetable juice concentrates, and synthetic compounds that are "nature identical". Foods with inherent color contain pigments, substances that are commonly found in the cells and tissues of plants and animals and can be extracted for use. There are 27 non-certified, naturally-derived pigments currently approved for food use, including carmine, annatto, turmeric, saffron, paprika, carrot oil, beetroot powder, butterfly pea flower extract, cottonseed oil, and grape skin and seed extract (21 C.F.R . § 70.3). In order to gain exemption status from certification, the source must meet the standard of showing a history of consumption and undergo scrutinization of the extraction methods to be deemed acceptable for use (21 C.F.R . § 70.3).

2.3.5 The Need for Additional Naturally-Derived Colorants

With consumers becoming more conscious of the ingredients in food, they are gravitating towards foods containing familiar ingredients without artificial ingredients. Therefore, there is an increased demand for the removal of artificial colors. However, compared to naturally derived colorants, artificial colors are more durable against most food processing methods, affordable, highly pigmented, and encompass a vast array of

color that could be difficult to fully replace (Galaffu *et al*, 2015. But there is an expectation from consumers that the food they know and love will not change in appearance with the removal of artificial colors. Natural colorants currently have a limited portfolio, and it can be challenging to replicate the intensity and hues of artificial colors (He & Giusti, 2010). Thus, viable replacements are needed.

With the increased usage of natural colors, there have been some reports of food sensitivities, specifically with carmine, annatto, turmeric, saffron, and paprika. However, there are currently no sensitivities reported for anthocyanins, betalains, or carotenoids, which make them preferable for the replacement of artificial colors (Hayder *et al*, 2011).

Betalains are most abundant in beets, but are also present in prickly pear and dragon fruit, and contribute red-violet or yellow colorations. Carotenoids are found in tomatoes, carrots, oranges, mangos, watermelon, and bell peppers, and give red, orange, and yellow colorations. Anthocyanins are found in flowers, fruits, and vegetables, namely berries, grapes, pomegranate, purple corn, red radishes, and purple sweet potatoes. Anthocyanins are red, blue, pink, orange, yellow, green, and purple, with color expression dependent upon pH (Sigurdson *et al*, 2017). The large variations of colors from anthocyanins make them an especially interesting chemical class for naturally-derived colorants.

2.3.6 Purple Colorants

Purple is connotated with symbolizing royalty due to the historical scarcity of purple dyes for the coloring of textiles (Anderson, 1989). Purple colorants have become

an attractive choice for coloring food, often enhancing the sensory characteristics of fruit-flavored foods and beverages, and has been correlated with sweetness (Spence, 2019).

Artificial colors are able to create a range of purples through combinations of FD&C Red 40, Red 4, Red 3, Blue 1, and Blue 2, but natural sources typically have duller shades.

With the demand for artificial color replacement with natural colors, purple hues that are vibrant and have blue undertones can be difficult to replicate (Sigurdson *et al*, 2017). Some sources where deep hues of purple can be found in are red cabbage, purple carrot, beets, dragon fruit, prickly pear, eggplant, red onion, and purple sweet potato.

In order to replace artificial colors with naturally-derived pigments, considerations on flavor and cultivation methods are important. Vegetable sources of naturally-derived colors can impart negative off-flavors to food, such as red cabbage (Zhou *et al*, 2020). While dragon fruit and prickly pear do not have strong off-flavors, the growing conditions are not optimal for the United States. Dragon fruit is not well suited to growing in the United States outside of Hawaii, California, and Florida, so a large portion of the supply is from international sources (Paull & Chen, 2019). Prickly pears grow very slowly and require three to four years of plant growth before fruit can be produced and harvested (Irish, 2001).

Chapter 3. Materials and Methods

3.1 Fruit Material and Fruit Sample Collection

Purple beautyberry (*Callicarpa dichotoma* 'Early Amethyst') (Shrub Bucket, Ithaca, NY), American beautyberry (*Callicarpa americana*) (Wilson Bros Gardens, McDonough, GA), and Bodinier beautyberry (*Callicarpa bodinieri*) (Straders Garden Center, Columbus, OH) were purchased in triplicate as 3-gallon sizes. The plants were planted in Piketon, OH (USDA Hardiness zone 6) in the spring of 2021. The plants were grown in full sun, with a 17mm drip line irrigation system with 0.42 gallons per hour emitters spaced 12" apart, with water applied one-three hours a week depending on dry conditions. The beds were raised 6' above the surrounding area to promote drainage. The beds were amended with Growers Select Soil Amendment (screen pine bark media) and grown in soil with a pH of 6.8. Fruit will remain on the plant into early winter but the fruits were harvested in September and October during the peak ripe season. Fully ripe fruit samples were harvested from three plants of each species by hand during morning hours and placed in polyethylene bags, labeled, and transported to the lab. Fruit was collected in triplicates for each species of beautyberry (n=9). All ripe fruits were collected and determined to be ripe with the absence of green coloration. Fruit that was damaged or showing signs of being over-ripe (shriveled with brown hues) were discarded from analysis. Fruits were stored at -18 ◦C until further analysis.

3.2 Fruit Quality Characteristics (Brix, pH, Weight, Diameter)

The initial chemical state of the whole fruits was measured with a handheld refractometer (Fisher, Waltham, MA) to determine the total soluble solids (degrees Brix) that was standardized with deionized water and expressed as °brix. Three replications were conducted. The pH was measured in triplicate with a micro pH meter (Mettler Toledo, Columbus, OH) to determine the pH of the fruit. The average fresh weight of the fruit was determined by the quotient between the weight of the whole berry samples and number of berries per sample for 4 replicates from each species (n=12), and the size was measured with a ruler with 10 replicates that were averaged.

3.3 Extraction

The extraction followed the procedure described by Giusti and Wrolstad (1996). Whole frozen fruits samples were weighed in 5.0 g quantities and extracted in triplicates (n=9). Liquid nitrogen was added before homogenizing with a micro homogenizer to make a fruit puree. The pH and Brix of the puree were recorded, with analyzed material captured in a container using a DI water rinse. The puree was blended with 100 % acetone and then filtered through a 70mm Whatman #4 filter under vacuum. Subsequent rinses of 70 % acetone were applied to the filter cake until the filtrate was clear. The extract was partitioned with chloroform in a separatory funnel with a 2:1 ratio of chloroform to acetone and mixed by inverting a few times. The mixture was incubated at refrigerated temperatures (4˚C) overnight. After solvent separation, the organic layer was removed. The aqueous layer was captured, and the remaining organic solvents were

removed in a Buchi rotory evaporator at 40˚C. The crude extract was brought to a known volume with acidified water (0.01 % HCL) before storing in the freezer at -18˚C until analysis.

3.4 Color of Crude Extracts

Initial Crude Extract Color

An aliquot of 300 µL of each crude extract (pH 2±0.05) was added to the wells of a 96-well plate and the spectra of the extract was measured with the SpectraMax M2 Plate Reader in triplicates (n=9). The spectra were converted to CIE L*a*b* and L*c*h˚ values using ColorbySpectra software developed by Farr and Giusti (2017). The extract color of the fruit was reported as Hunter CIE L*, C*, and h˚. The L* value corresponds to lightness, with 0 being the darkest black and 100 being bright white. The C* value corresponds to the chroma with higher numbers indicating more intense colorations and the h˚ value corresponds to the hue angle with 0 degrees representing red, 90 degrees representing yellow, 180 degrees representing green, 270 degrees representing Blue, and 360 degrees representing red-purple.

Crude Extract Color Expression Across pH

Buffers at pHs 1-9 were used and include the following: pH 1-2 0.025M KCl, pH 3-7 citric acid and Na_2HPO_4, pH 8 Na_2HPO_4+ Na_2HPO_4, and pH 9 Na_2HPO_4+ Na_2HPO_4+$NaHCO_3$, according to the Sigma-Aldrich's Buffer Reference Center (2022) with pH ±0.05. An aliquot of 60 µL of the crude extract was added to 240 µL of buffer directly into the well of a 300 µL well of a 96 well plate and mixed with sample uptake and release with a pipet into the well several times. Three replications per pH were plated

(n=9). The plate was allowed to equilibrate for 15 minutes at room temperature on the bench before reading on the SpectraMax M2 Plate Reader to analyze absorbance changes from 400-700 nm. Once spectral data was collected, it was translated into CIE $L^*a^*b^*C_{ab}^*h_{ab}$ color values using ColorbySpectra software (Farr and Giusti 2017).

The difference in color between samples was calculated according to the CIELAB color space equation:

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

where L* is lightness, a* is redness (+)/greenness (–), b* is yellowness (+)/blueness (–), d ≥ 5 is a color difference noticeable to the common observer (Konica Minolta, 2007).

3.5 Determination of Total Monomeric Anthocyanin Content

Concentrations of total monomeric anthocyanins in the fruit were measured using the pH differential method as described by Giusti & Wrolstad (2001). Sample aliquots of 200 µL were mixed with 800 µL of 0.025 M potassium chloride buffer (pH 1.0) and repeated with 800 uL 0.4 M sodium acetate buffer (pH 4.5) in triplicates (n=9). The absorbance was measured at each sample $\lambda_{vis\text{-}max}$ and 700 nm against a blank cuvette filled with distilled water on a SpectraMax M2 Plate Reader. The absorbance was calculated as follows:

$A = [(A_{\lambda\ vis\text{-}max} - A_{700nm})\ pH\ 1.0] - [(A_{\lambda\ vis\text{-}max} - A_{700nm})\ pH\ 4.5]$

The concentration of the monomeric anthocyanin pigment concentration in the original sample was calculated with the following equation:

Monomeric anthocyanin pigment (mg/L) = (*A* x MW x DF x 1000)/(Ɛ x 1*)

MW: 449.2 g/mol (molecular weight of cy-3-glu)

DF: Dilution Factor of 5

Ɛ: 29,000 L/cm * mol (molar absorptivity of cy-3-glu)

* pathlength of 1 cm

The total monomeric anthocyanin concentration was calculated as mg/L and expressed as mg cy-3-glu per 100 g of fruit fresh weight.

3.6 Determination of Total Phenolic Content

Concentrations of total phenolics in the fruit were measured in triplicates (n=9) according to the Folin-Ciocalteu (FC) method described by Slinkard and Singleton (1977) and modified by Waterhouse(2002). A gallic acid stock solution was prepared by dissolving 0.5 g of gallic acid in 10 mL of ethanol before bringing to 100 mL volume with distilled water. The stock was diluted to make standards of 50, 100, 250, and 500 mg/L by diluting 10, 20, 50, and 100 uL to 1 mL with distilled water.

An aliquot of 20 uL of sample, gallic acid standard, or blank, were added to a 1-cm, 2.0 mL plastic cuvette with 1.58 mL of distilled water before adding 100 uL of FC reagent and mixed by pipetting up and down several times. Samples were incubated at room temperature for 1 to 8 minutes before adding 300 uL of 20% sodium carbonate solution, mixed by pipetting up and down, and incubated at room temperature for 2 hours. The sample absorbance was measured at 765 nm on SpectraMax M2 Plate Reader. The absorbance of a water blank was subtracted from all readings and a calibration curve

was created from the standards. The slope equation from the standard curve was used to calculate the concentration of total phenols in gallic acid equivalents (GAE) mg/L.

3.7 Determination of Total Flavonoid Content

Concentrations of total flavonoids in the fruit were measured in triplicates (n=9) according to the Aluminum Chloride Assay using catechin as a quantification standard (Zhishen *et al*, 1999; Dewanto *et al*, 2002). Catechin concentrations to build a standard curve at 510 nm was produced as follows. A catechin standard was made by dissolving 50 mg Catechin in 1 mL ethanol and 9 mL of water 5 g/L stock solution. The stock was added as 5,10,20,50,100, and 200 uL brought to 1 mL with deionized water to create standards 25, 50, 100, 250, 500, and 1,000 mg Catechin/L. Of the samples and standards, 250 uL were added to 1250 uL deionized water and 75 uL of 5% $NaNO_2$ solution and allowed to rest for 6 minutes before the addition of 150 uL of 10% $AlCl_3$ $6H_2O$. The sample rested for another 5 minutes before the addition of 500 uL 1 M NaOH and 275 uL deionized water and then read on SpectraMax M2 Plate Reader at 510 nm.

3.8 Anthocyanin Acidified Water and Methanol C18 Semi-Purification

The crude extract was semi-purified as previously described (Wrolstad *et* al, 1990). The crude extract was passed through a C-18 cartridge, previously activated with two cartridge volumes of acidified methanol (0.01% HCl) followed by 0.01 % HCl acidified water. Anthocyanins and other phenolics were temporarily bound to the column while sugars, acids, and other water-soluble compounds were eluted with two volumes of 0.01 % aqueous HCl. Anthocyanins were captured in a round bottom flask with two

cartridge volumes of methanol containing 0.01 % HCl (v/v). The methanolic extract was then concentrated using a Buchi rotovapor at 45˚C and brought to a known volume with deionized water containing 0.01% HCl.

3.9 Alkaline Hydrolysis

Alkaline hydrolysis was conducted in triplicates (n=9) to determine acylation characteristics of the anthocyanins, as the procedure cleaves ester bonds of molecules. Sample semi-purified extracts that underwent acidified water and methanol on a C-18 (as previously described) was aliquoted (~ 2 mg monomeric anthocyanins) and hydrolyzed (saponified) in a glass screwtop tube with 2 mL 10% KOH for 8 minutes at room temperature (25 degrees C) in the dark, as described by Hong and Wrolstad (1990). The solution was neutralized with 2N HCl and the hydroslyte was purified on a C-18 SepPak cartridge (Wrolstad *et al*, 1990) as previously described.

3.10 Partial and Complete Acid Hydrolysis

Acid hydrolysis was conducted in triplicates (n=9) for exploration for shared aglycone verification and the positioning of sugar attachments, as the procedure cleaves glycosidic bonds of molecules. Ten mL of 2 N HCl was added to 1.0 mL of extract that went alkaline hydrolysis (as previously described) in a glass test tube that was capped and covered in foil. The tube was placed in boiling water (100˚C) for 15 minutes (partial acid hydrolysis) and 45 minutes (complete acid hydrolysis) then cooled in an ice bath. The hydrolysates was purified on a C-18 Sep-Pak cartridge as previously described.

3.11 Sample Preparation for Color Expression of Crude Extract after Ethyl Acetate Rinse

To determine influence of non-anthocyanin phenolics, the crude extract was semi-purified with a C18 cartridge as previously described but with an additional ethyl acetate rinse. Ethyl acetate removes polyphenolic compounds such as phenolic acids and flavonols, while the anthocyanins remain bound to the C18 column. The same procedure was followed as described in triplicate with an additional washing step using two volumes of ethyl acetate rinses of the column that was discarded before to cartridge volumes of 0.01% HCl methanol was added to the cartridge to elute the anthocyanins that were captured in a round bottom flask that was then concentrated using a Buchi rotory evaporator at 45˚C (Wrolstad *et* al, 1990).

3.12 Color Expression Across pH of Semi-purified Extracts Before and After Phenolics Removed by Additional Ethyl Acetate Rinse

The color of the extract semi-purified with acidified water and methanol (0.01% HCl) and the extract semi-purified with the additional ethyl acetate rinses were comparably analyzed to determine the impact of non-anthocyanin phenolics on color expression of the crude extracts. The color of the extract in buffers of increasing pH were prepared and measured as previously described in triplicate. Once spectral data was collected, it was translated into CIE L*a*b*C_{ab}*h_{ab} color values using ColorbySpectra software (Farr and Giusti 2017).

3.13 uHPLC PDA MS/MS

Anthocyanin Aglycone Identification

Anthocyanin identification was conducted using a Shimadzu ultra-high-pressure Liquid Chromatography (uHPLC) system equipped with LC_2040C pumps coupled to a tripe-quadrupole Shimadzu LCMS-8040 mass spectrometer using LC-2040C PDA detector (Shimadzu, Columbia, MD, USA). A Restek reverse phase C-18 column (50 x 2.1 mm) with 1.9 µm particle size was used (Restek Corporation, Bellefonte, PA, USA). Samples were filtered through a 0.2 µm RC membrane filter (Phenomenex, Torrance, CA, USA) before injection (5 µL). Samples were run using a flow rate of 0.3 mL/min and solvent A: 4.5% formic acid in HPLC water and solvent B: 100% acetonitrile, at 30 °C. Anthocyanin separation was achieved using a linear gradient from 3% -20 % B in 15 min and 20-40% in 3 min with a 5 minute hold at 40% before decreasing B to 3% for 5 min with 200–700 nm spectra collected.

Mass spectrometry analysis was performed using nitrogen ionization under positive ion mode with the following settings: nebulizing gas flow: 2.5L/min, interface bias: +4.5kV. A total ion scan in Q1 from 100–1000 m/z and precursor ion scans at -20V at 271(pelargonidin), 287 (cyanidin), 301(peonidin), 303(delphinidin), 317(petunidin), and 331(malvidin) m/z was used to search for common anthocyanidins. MS data, order of elution, and comparison to literature were used for the anthocyanin identification.

Anthocyanin Characterization

For anthocyanin characterization of specific anthocyanins, the same MS nebulizing gas flow rate, MS interface bias, and uHPLC PDA conditions were used as in “aglycone identification”. The mass spectrometry analysis was performed in triplicate under positive ion mode with product ion scans with -20V for the *m/z* values of 611, 625,

697, 711(the m/z of the major anthocyanins found in the aglycone identification scans). Precursor ion scans were also conducted for the partial acid hydrolysis samples at -20V for the m/z values of 287(cyanidin) and 301(peonidin), in addition to 449 (cyanidin-3-glucoside) and 463 (peonidin-3-glucoside) to support identification through fragmentation patterns. A reference material of organic 100% cranberry juice (R.W. Knudsen, Knudsen & Sons, Inc. Chico, CA, USA) was used to compared monohexosides generated from partial acid hydrolysis for determining if glycoslations were from glucose or galactose attachments.

Phenolic and Flavonoid Characterization

Phenolic identification was conducted using a Shimadzu ultra-high-pressure Liquid Chromatography (uHPLC) system equipped with LC_2040C pumps coupled to a tripe-quadrupole Shimadzu LCMS-8040 mass spectrometer using LC-2040C PDA detector (Shimadzu, Columbia, MD, USA). A Restek reverse phase C-18 column (50 x 2.1 mm) with 1.9 µm particle size was used (Restek Corporation, Bellefonte, PA, USA). Samples were filtered through a 0.2 µm RC membrane filter (Phenomenex, Torrance, CA, USA) before injection (5 µL). Samples were run in triplicate using a flow rate of 0.3 mL/min and solvent A: 0.1% formic acid in HPLC water and solvent B: 100% acetonitrile, at 30 °C. Separation was achieved using a linear gradient from 10% -20 % B in 10 min, 20%-40% in 6 min, with a 3 minute hold at 40% before decreasing B to 3% over 5 min with a 3 min hold at 3% with 270–700 nm spectra collected.

Mass spectrometry analysis was performed under positive and negative ion mode with the following settings: nebulizing gas flow: 2.5L/min, interface bias: +4.5 kV. A total ion

scan in Q1 from 200–1500 m/z in positive and negative mode, in addition to product ion scans at -20V at 463, 447, 477, 788, 642, and 623 *m/z* was used to after prominent phenolic and flavonoid m/z values were selected. Positive and negative mode neutral ion losses of 176, 162, and 146 were also scanned for. A precursor ion scan in positive mode was also selected for the m/z of 623. MS data, order of elution, and comparison to literature were used for the phenolic and flavonoid identification.

3.14 Statistical Analysis

The statistical calculations were performed with R studio to execute R programming (Rstudio, Boston, MA). The distribution of the fruit weights, total monomeric anthocyanins, total flavonoids, total phenolics, pH, and total soluble solids and significance was determined by Kruskal-Wallis rank sum tests with a 95% confidence level when the significance level is $p \leq 0.05$. For values with a significant different between species, a Wilcox rank-sum test was conducted with continuity correction to determine if the population mean was shared between two species with a family-wise error rate of 0.05.

Chapter 4. Results and Discussion

4.1 Fruit Quality Characteristics

Fruit Weight

Means for individual weight for American, Bodinier, and Purple beautyberry were presented in Table 4. The individual weight of beautyberry fruits ranged from 0.27 to 0.80 g. The highest individual fruit weights were from American Beautyberry and weighed significantly more than the Purple and Bodinier beautyberry individual fruits. The American beautyberry had one plant arrive damaged and the replacement plant was planted in late June, which had an average fruit size of 0.31 ± 0.03 g compared to the two American plants planted in April that had an average fruit size of 0.76 ± 0.03 g, so it was removed from the data set for the Kruskal-Wallis test as an outlier due to the delayed growing period and is not included in the data below.

Beautyberry Sample	**Individual weight**	**Individual diameter**	**pH**	**TTS (Brix°)**
American	0.54 ± 0.03	0.46± 0.06	5.77 ± 0	15
Bodinier	0.41 ± 0.07	0.41± 0.05	5.80 ± 0	15
Purple	0.39 ± 0.05	0.39± 0.06	5.76 ± 0	15

Table 4. Comparison of quality attribute means and standard deviations of beautyberry species.

In the harvest year of 2021, Purple beautyberry produced the most fruit, with an average total harvest weight of 138 g ± 15 g. American and Bodinier beautyberry had smaller yields, averaging 28 ± 21 g and 25 ± 17 g, respectively. However, the total harvest weights were not significantly different (Kruskal Wallis test, p-value=0.4911). Fruit weight and size could change depending on plant age. A study conducted by the USDA (Halls, 1974) found that fruit production did not occur in American beautyberry until the plant was 1-2 years old, reached optimum production yields at age 5 (around 1500 g/ plant), and fruit production decreased around age 10. The plants were about a year old when planted and fruit production could increase in subsequent years are the plants grow larger and have larger root systems and branches to allow for increased fruit production.

Fruit Diameter

The fruit diameters (Table 4) were significantly different between species (p-value=4.961e-09). The largest diameters were from American beautyberry fruit (0.46± 0.06) followed by Bodinier beautyberry (0.41± 0.05) and Purple(0.39± 0.06). Purple and Bodinier beautyberry diameters were subjected to a Wilcox rank-sum test and were not significantly different (p-value=0.3499) but American beautyberry was significantly different than Purple (p-value= 4.443e-08) and American beautyberry was significantly different than Bodinier (p-value=2.699e-08). The previously mentioned American beautyberry plant that was planted later than the others was removed as an outlier in the data due to the delayed growing period having an effect as significantly smaller fruit.

With the outlier included, the fruit diameters the significance was not as pronounced (p-value=0.006).

Total Soluble Solids and pH

The Total Soluble Solids (˚Brix) and pH are shown in Table 4. The average brix for beautyberry fruit was 15 to 15.005. The Brix was not significantly different (p = 1.0), which meant that species showed no effect on total sugar content under the conditions of this study.

The pH of the fresh fruit ranged from 5.7 – 5.8. The pH was not significantly different (p=0.49), which meant that species showed no effect on fruit pH.

4.2 Color Comparison of Crude Extracts

The color of the initial extract was measured and shown in Figure 9. The color at pH ~2 was a medium pink in all three species. The ΔE value for the difference between American and Bodinier beautyberry was 4, meaning that the common observer would not be able to distinguish between the two. However, the ΔE between Bodinier beautyberry and Purple beautyberry was 7, and between American and Purple was 5. Untrained observers would be able to distinguish Purple Beautyberry extract from Bodinier and American beautyberry. This suggests that the American and Bodinier beautyberry extracts may share similar pigments due to the similar color expression at pH 2 that could not be distinguished by color by the common observer.

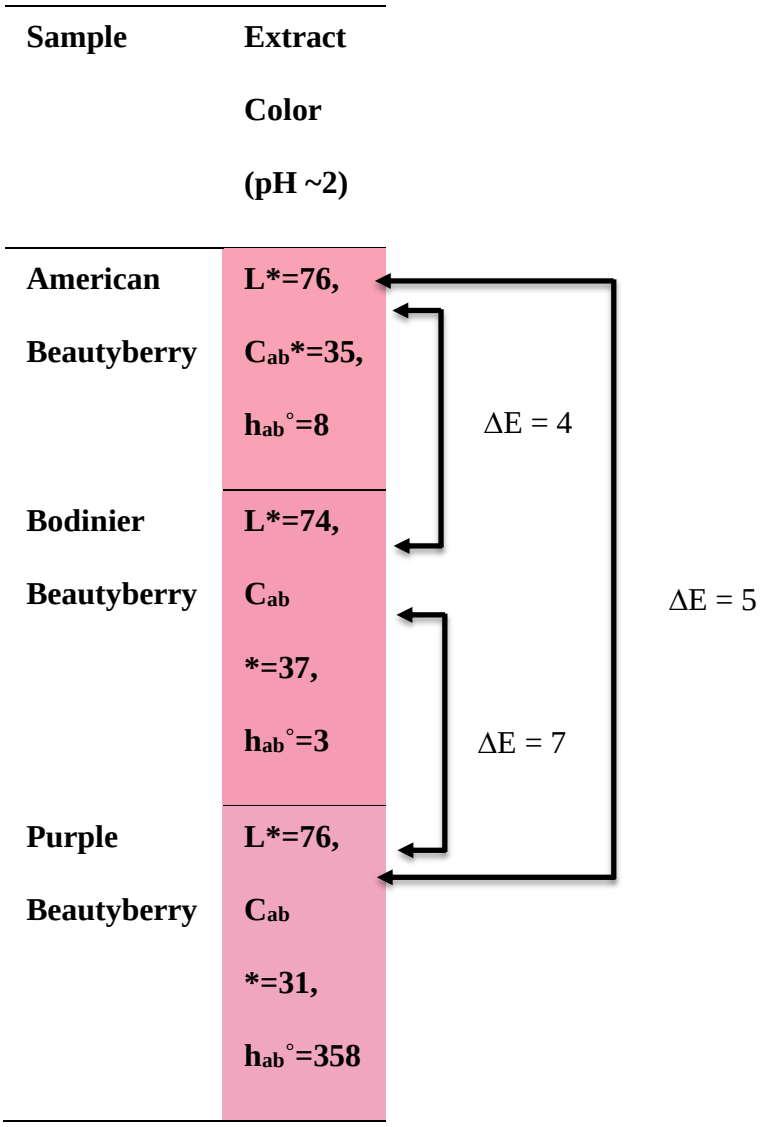

Figure 8. Color values and ΔE values for the crude extracts of beautyberry fruits at pH 2. A ΔE value ≥ 5 indicates that the two samples could be distinguishable to the common observer.

4.3 Total Monomeric Anthocyanins, Phenolics, and Flavonoids

The comparison of total phenolics, and two subclasses, total flavonoids and total monomeric anthocyanins in American, Bodinier, and Purple beautyberry are presented in Table 5. Bodinier beautyberry had significantly more phenolics than Purple beautyberry (p-value ≤ 0.05) but was not significantly different from American beautyberry total

phenolics. The total flavonoids and total monomeric anthocyanins were not significantly different (p-value $\geq$ 0.05).

The highest anthocyanin content of the three species was found in American beautyberry of 196 mg/100 g FW, followed by 140 $\pm$ 85 mg/ 100 g FW in Purple beautyberry, and 112 $\pm$ 6 mg/ 100 g FW in Bodinier beautyberry fruit. American beautyberry fruit may have had the highest content of monomeric anthocyanins due to the significantly larger diameter and fruit size attributed a larger skin surface area for anthocyanins to be expressed than in the other two varieties (Kruskal-Wallis rank sum test, p-value=0.9146). But the influence of size did not have a significant difference on the total monomeric anthocyanin content.

Bodinier beautyberry had the average phenolic concentration of 2165 $\pm$ 311 while American and Purple beautyberry averaged 862 $\pm$ 604 and 477 $\pm$ 124 mg GAE/100 g FW, respectively. There was a significant different of total phenolics between species(Kruskal-Wallis rank sum test, p-value=0.0094). With the Wilcox rank-sum test, Bodinier total phenolics were significantly different than Purple beautyberry (p-value=0.009). However, American beautyberry was not significantly different than Bodinier (p-value=0.1135). Additionally, American beautyberry was not significantly different than Purple beautyberry (p-value=0.1).

Flavonoids are a type of phenolic and were found to be about 25-50% of the total phenolics in the extracts, and anthocyanins are a type of flavonoid, that comprised 5-30% of the total phenolics present in the extracts and 10-90% of the total flavonoids present depending on the plant species. The average total flavonoid content in American

beautyberry was 213 mg /100 g FW, in Purple beautyberry was 222 mg / 100 g FW, and in Bodinier was 913 mg /100 g FW. The Bodinier beautyberry fruit had a much higher concentration of total flavonoids content than American or Purple beautyberry but there was not significant difference in total flavonoids between species (p-value=0.066). In American and Purple Beautyberry, anthocyanins accounted for more than 50% of the total flavonoids, while in Bodinier beautyberry, they were less than 10% of the total flavonoids. However, all three species produced similar measured color values of the extracts at pH 2. This could be due to copigmentation from the phenolics, specifically flavonoids, that caused an increased stability of the anthocyanins and a bathochromic and hyperchromic shift that provided enhanced color expression.

Beautyberry species	Total phenolics (mg GAE/100g FW)	Total flavonoids (mg CAT/100g FW)	Total monomeric anthocyanins (Cy-3-glu mg/100g FW)
American	862 ± 604^{ab}	213 ± 58^{a}	196 ± 220^{a}
Bodinier	2165 ± 311^{a}	913 ± 86^{a}	112 ± 6^{a}
Purple	477 ± 124^{b}	222 ± 35^{a}	140 ± 85^{a}

Table 5. Concentration of phenolics, flavonoids, and monomeric anthocyanins means and standard deviations in beautyberry fruit (n=9)

4.4 Major Anthocyanin identification

The beautyberry fruits were previously reported as containing anthocyanins with the aglycone of cyanidin and peonidin in Bodinier beautyberry fruit but the substitutions

were not reported and limited information was found on the anthocyanins in American and Purple beautyberry fruit (Jones & Kinghorn, 2009). In addition, reports of anthocyanin concentrations were limited.

Additional knowledge into the specific anthocyanins were investigated through HPLC analysis of anthocyanins that allowed the anthocyanins comprising the total monomeric anthocyanins to be tentatively identified in Figure 10 in Table 6.

The three species showed different anthocyanin compositions, as shown in Figure 11. Bodinier had four major anthocyanins while American and Purple beautyberry had two. The anthocyanins were tentatively identified as cyanidin dihexoside, peonidin dihexoside, cyanidin dihexoside with malonic acid, and peonidin dihexoside with malonic acid from the absorbance at 520 nm in addition to the *m/z* of parent ions and the MS/MS ionization that produced fragment ions that allowed for tentative identification of the anthocyanin structures. Cyanidin dihexosides were the prominent anthocyanins in American beautyberry and peonidin dihexosides were the prominent anthocyanins in Purple beautyberry.

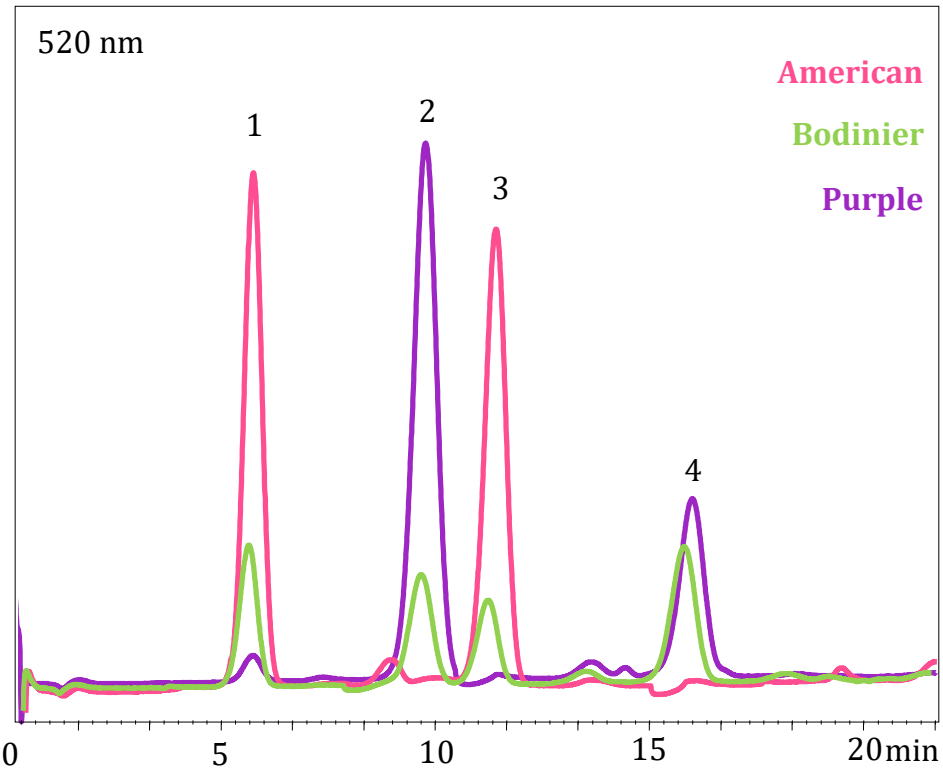

Figure 9.Chromatogram of anthocyanins in crude American, Bodinier, and Purple beautyberry at 520 nm.

Peak Number	Tentative Identification	Precursor Ion *m/z* [M+H]	Fragment Ion *m/z* [M+H]	Major Species
1	cyanidin dihexoside	611	287	**AB**, **BB**
2	peonidin dihexoside	625	301	**PB**, **BB**
3	cyanidin dihexoside + malonic acid	697	287	**AB**, **BB**
4	peonidin dihexoside + malonic acid	711	301	**PB**, **BB**

Table 6. Tentative identification of major anthocyanins in American(AB), Bodinier(BB), and Purple(PB) beautyberry fruit crude extracts.

Alkaline Hydrolysis for Acylation Confirmation

Anthocyanins with acylations can be saponified to cleave ester bonds and break acylations. The resulting anthocyanins can be isolated, separated by HPLC, and characterized according to specific spectra and retention behavior.

To add support to the preliminary tentative identification of cyanidin and peonidin dihexose acylated with malonic acid, the extract was saponified and the chromatograms were shown in Figure 11 with tentative identification in Table 7, and Figure 12 with tentative identification in Table 8 (Bodinier beautyberry was not included since there was not sufficient extract and to provide simplified Figures with only one aglycone).

The original chromatograms had two major peaks for the two major anthocyanins. After alkaline hydrolysis, the second peaks converged into the first peaks which showed the concentration of the tentatively identified dihexoside, nonacylated anthocyanins increased and the tentatively identified acylated anthocyanins decreased. The pattern of the two peaks becoming one is strong evidence that the two major anthocyanins share the same structure with the difference being acylations. The m/z, spectra, and retention time of the peak present after alkaline hydrolysis matched that of the first major peaks, which occurred in both American and Purple beautyberry chromatograms, indicated that both species have acylated anthocyanins. While some acids are give an absorbance reading, malonic acid is not able to be detected by absorbance since it does not have a characteristic spectra (Wrolstad et al, 2002). Therefore, the *m/z* gives important information about the presence of malonic acid

acylations through characteristic m/z values. The anthocyanins were tentatively identified in Table 7 for American beautyberry and Table 8 for Purple beautyberry.

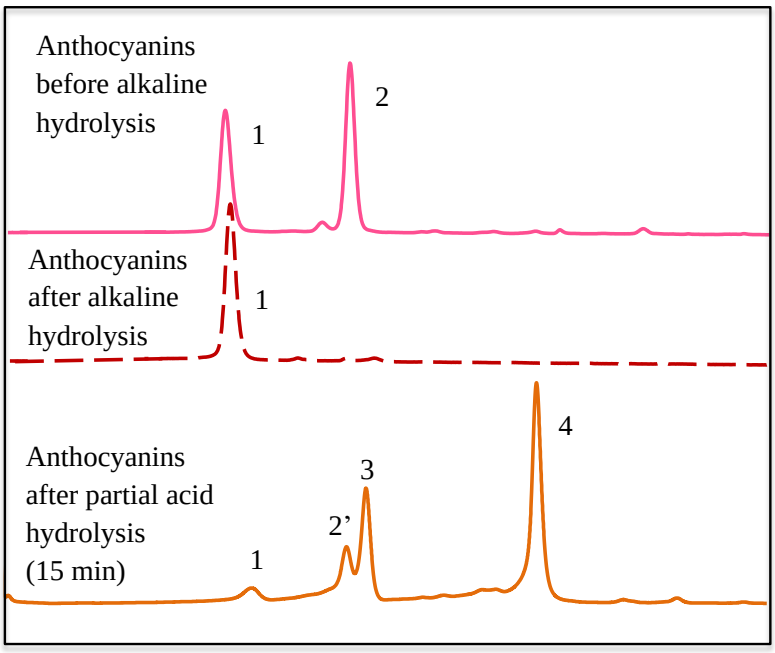

Figure 10. Anthocyanins in American beautyberry transformed by alkaline and acid Hydrolysis.

Peak Number	Tentative Identification	Precursor Ion *m/z* [M+H]	Product Ion *m/z* [M+H]
1	cyanidin dihexoside	611	449, 287
2	cyanidin dihexoside + malonic acid	697	449, 287
2'	cyanidin-3-hexoside	449	287
3	cyanidin-5-hexoside	449	287
4	cyanidin	287	-

Table 7. Tentative identification of anthocyanins in American beautyberry after alkaline and acid hydrolysis.

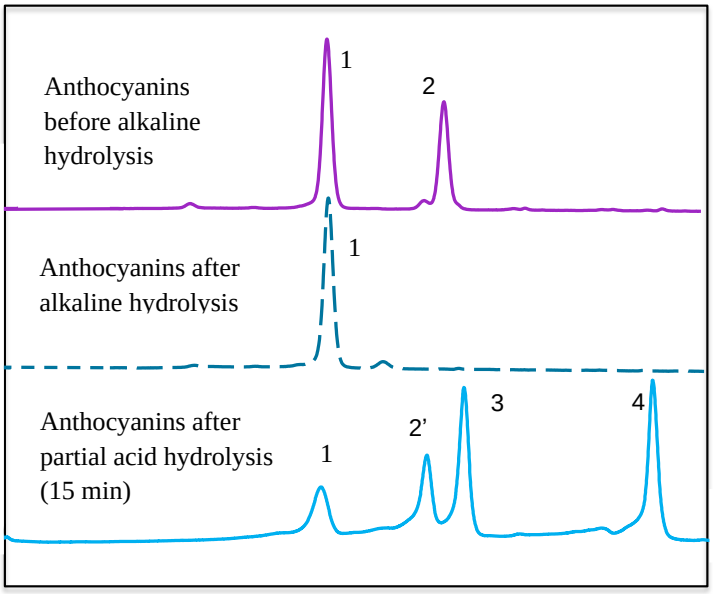

Figure 11. Anthocyanins in Purple beautyberry transformed by alkaline and acid hydrolysis.

Peak Number	Tentative Identification	Precursor Ion *m/z* [M+H]	Product Ion *m/z* [M+H]
1	peonidin dihexoside	625	463, 301
2	peondin dihexoside + malonic acid	711	463, 301
2’	peonidin-3-hexoside	463	301
3	peonidin-5-hexoside	463	301
4	peonidin	301	-

Table 8. Tentative identification of anthocyanins in purple beautyberry after alkaline and acid hydrolysis.

Partial and Complete Acid Hydrolysis for Glycosidic Bond Characterization

Anthocyanins with glycosidic bonds can be hydrolyzed to cleave the structure into the aglycone structures. Complete acid hydrolysis converts anthocyanins with glycosidic bonds to aglycones, while partial acid hydrolysis can be helpful in determining the intermediates between the original anthocyanins and the acid hydrolyzed anthocyanin aglycones. Partial acid hydrolysis was conducted to give more information about the positioning of hexoside attachments. Glycosidic bonds on anthocyanins are typically at position 3 or 5, and can occur simultaneously on both. The glycosidic bonds between sugars and anthocyanins are the most susceptible to acid hydrolysis compared to two sugars linked together with a glycosidic bond. When determining the positioning of sugar attachments, a single anthocyanin with two sugar attachments occurring at the 3' or 5' position would produce peaks on a chromatogram the original anthocyanin, one intermediate with one sugar attached, and the aglycone after partial acid hydrolysis. However, a single anthocyanin with two sugar attachments with one on the 3' position and one on the 5' position will show peaks on a chromatogram of the original anthocyanins, one intermediate with a sugar on the 3' position, another intermediate with a sugar on the 5' position, and the aglycone.

In American beautyberry, the timepoint at 15 minutes of partial acid hydrolysis after alkaline hydrolysis transformed the single peak to 4 peaks. The first peak m/z and spectra matched the original saponified dihexoside, peak 2 and 3 shared the same m/z, and peak 4 had a m/z and spectra that was consistent with a cyanidin aglycone.

In addition to the partial acid hydrolysis fragmentation, the spectra of peak 2 had a slight shoulder, while peak 3 did not. This suggests that peak 2 was a cyanidin with a sugar attachment on the 3' position, as the presence of a slight shoulder was reported to be characteristic and consistent in literature (Wrolstad et al, 2002). The ratio between the absorbance at 440 nm and the absorbance at the $\lambda_{vis\text{-}max}$ is almost twice as much in anthocyanins with glycosidic substitutions only at position 3 compared to anthocyanins with substitutions at 3 and 5 or just the 5 position (Wrolstad et al, 2002). This was consistent with the ratio calculations in Table 9 and seen in Figure 13. Peak 3 was designated as a cyanidin with a sugar attachment at position 5'.

A similar occurrence was seen in Purple beautyberry, shown in Figure 14 and Table 10. The timepoint at 15 minutes of partial acid hydrolysis Partial acid hydrolysis after alkaline hydrolysis transformed the single peak to 4 peaks. Peak 1 had a m/z and spectra that was consistent with the original saponified dihexoside, peak 2 and 3 shared the same m/z of 463, and peak 4 spectra and m/z was consistent with reported values in literature for the peonidin aglycone (Wrolstad et al, 2002). Peak 2 in Purple beautyberry had a slight shoulder, similar to peak 2 in American beautyberry, which suggests that peak 2 is a peonidin with a sugar attachment on the 3' position while peak three was consistent with literature of peonidin with a sugar attachment at the 5' position. The ratio between the absorbance at 440 nm and the absorbance at the $\lambda_{vis\text{-}max}$ is almost twice as much in Peak 2 as Peak 1 and 3, which suggests that the parent compound is a peonidin with 3,5 glycosidic substitutions.

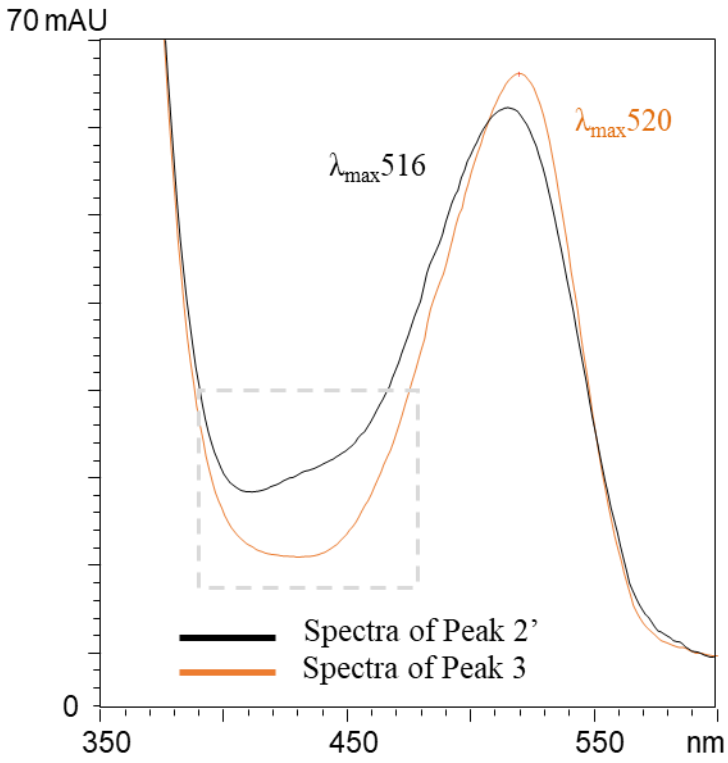

Figure 12. Shoulder present in American beautyberry peak 2' tentatively identified as cyanidin-3-hexoside compared to peak 3 tentatively identified as cyanidin- 5-hexoside.

Peak Number	Tentative Identification	Absorbance of 440 nm/ $\lambda_{vis\text{-}max}$
1	cyanidin dihexoside	0.24
2'	cyanidin-3-hexoside	0.34
3	cyanidin-5-hexoside	0.17

Table 9. Comparison of ratios of absorbance at 440 nm and λvis-max values in American beautyberry partial acid hydrolysis extracts.

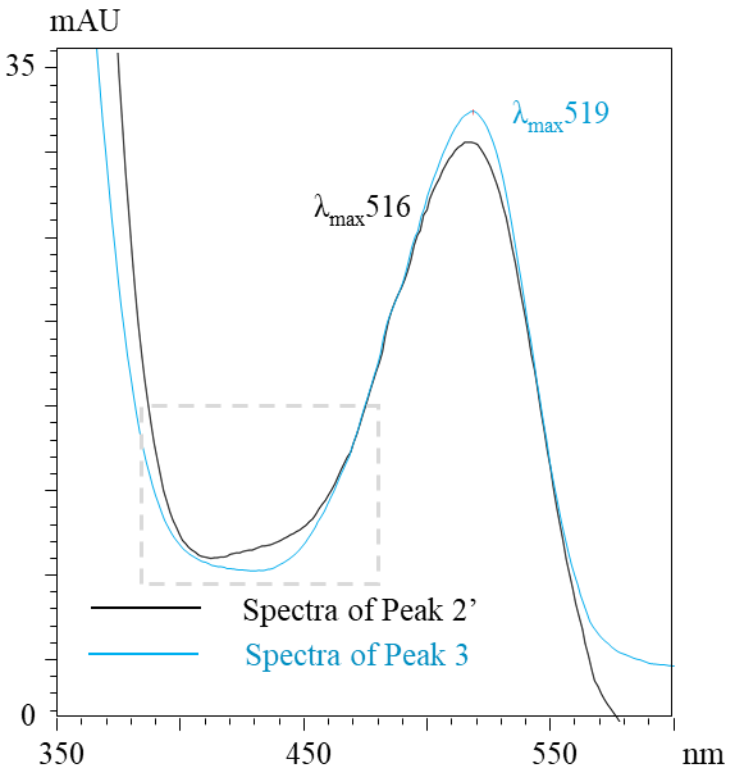

Figure 13. Shoulder present in Purple beautyberry peak 2' tentatively identified as peonidin 3-hexoside compared to peak 3 tentatively identified as peonidin 5-hexoside.

Peak Number	**Tentative Identification**	**Absorbance of 440 nm/ $\lambda_{vis\text{-}max}$**
1	peonidin dihexoside	0.24
2'	peonidin-3-hexoside	0.34
3	peonidin-5-hexoside	0.17

Table 10. Comparison of ratios of absorbance at 440 nm and λvis-max values in Purple beautyberry partial acid hydrolysis extracts.

Comparison of beautyberry fruit anthocyanins to anthocyanins in reference materials

To identify the hexose attachments on the anthocyanins, beautyberry fruit extracts were compared to reference materials, cranberry juice and muscadine grape juice. Pure standards of anthocyanins can be difficult to obtain. Although purified reference standards are becoming more widely available, the current offerings are still limited and they can be expensive. However, there are natural sources of identified anthocyanins that are vastly available. Cranberry was chosen as a reference sample due to the numerous studies with consistent reports of the anthocyanin profile and the composition of only cyanidin and peonidin derivatives, with both monohexosides of galactose and glucose attachments (Wrolstad et al, 2002). Galactosides elute faster than glucoside since hydrophobicity of molecules are seen in more polar compounds elute faster than less polar compounds (Hong & Wrolstad, 1990). Muscadine grape juice was also chosen due to containing five of the major six anthocyanins and containing glucose substitutions on positions 3 and 5. This was chosen to determine if the two hexose substitutions were on positions 3 and 5.

In American beautyberry, the saponified and partial acid hydrolysis extract was compared to the anthocyanins in cranberry juice to determine if the sugar moiety was consistent with cyanidin 3 glucoside or cyanidin 3 galactoside. In Figure 15, Peak 2 of cranberry and American beautyberry were consistent. Cyanidin-5-glucoside is not very stable and so is not prevalent in plant sources. Based on the data from the alkaline hydrolysis, partial acid hydrolysis, and consistency with the cranberry reference material, we theorized that that American beautyberry contains two major anthocyanins: cyanidin

3,5 diglucoside and cyanidin-3,5-diglucoside acylated with malonic acid. To provide additional supporting evidence, the crude American beautyberry fruit extract anthocyanins were compared to the anthocyanins in muscadine grape juice (Figure 16). The retention time, lambda max (513), spectra, and m/z (611, [M+H]) were consistent for peak 2 in muscadine grape juice and American beautyberry. Based on the consistency of characteristics of anthocyanins in muscadine grape juice with American beautyberry, we proposed a tentative identification of the two major anthocyanins in American beautyberry fruit as cyanidin-3,5-diglucoside and cyanidin-3,5-diglucoside acylated with malonic acid as cyanidin 3-malonylglucoside-5-glucoside (Table 11). The positioning of malonic acid at position 3 was proposed because acylations commonly on position 3 in plants.

In Purple beautyberry, the saponified and partial acid hydrolysis extract was also compared to the anthocyanins in cranberry juice to determine if the sugar moiety was consistent with peonidin-3-glucoside or peonidin-3-galactoside. In Figure 15, Peak 5 of cranberry was consistent with Peak 2 of the saponified and partial acid hydrolysis anthocyanin extract. Peonidin-5-glucoside is not very stable and so is not prevalent in plant sources. Based on the data from the alkaline hydrolysis, partial acid hydrolysis, and consistency with the cranberry reference material, we theorized that Purple beautyberry contains two major anthocyanins: peonidin 3,5 diglucoside and peonidin 3,5 diglucoside acylated with malonic acid. To provide additional supporting evidence, the crude Purple beautyberry fruit extract anthocyanins were compared to the anthocyanins in muscadine grape juice (Figure 16). The retention time, lambda max (514), spectra, and m/z(625,

[M+H]) were consistent for peak 4 in muscadine grape juice and Purple beautyberry. Based on the consistency of characteristics of anthocyanins in muscadine grape juice with Purple beautyberry, we proposed a tentative identification of the two major anthocyanins in Purple beautyberry fruit as peonidin-3,5-diglucoside and peonidin-3,5-diglucoside acylated with malonic acid as peonidin 3-malonylglucoside-5-glucoside (Table 11). The positioning of malonic acid at position 3 was proposed because acylations commonly on position 3 in plants.

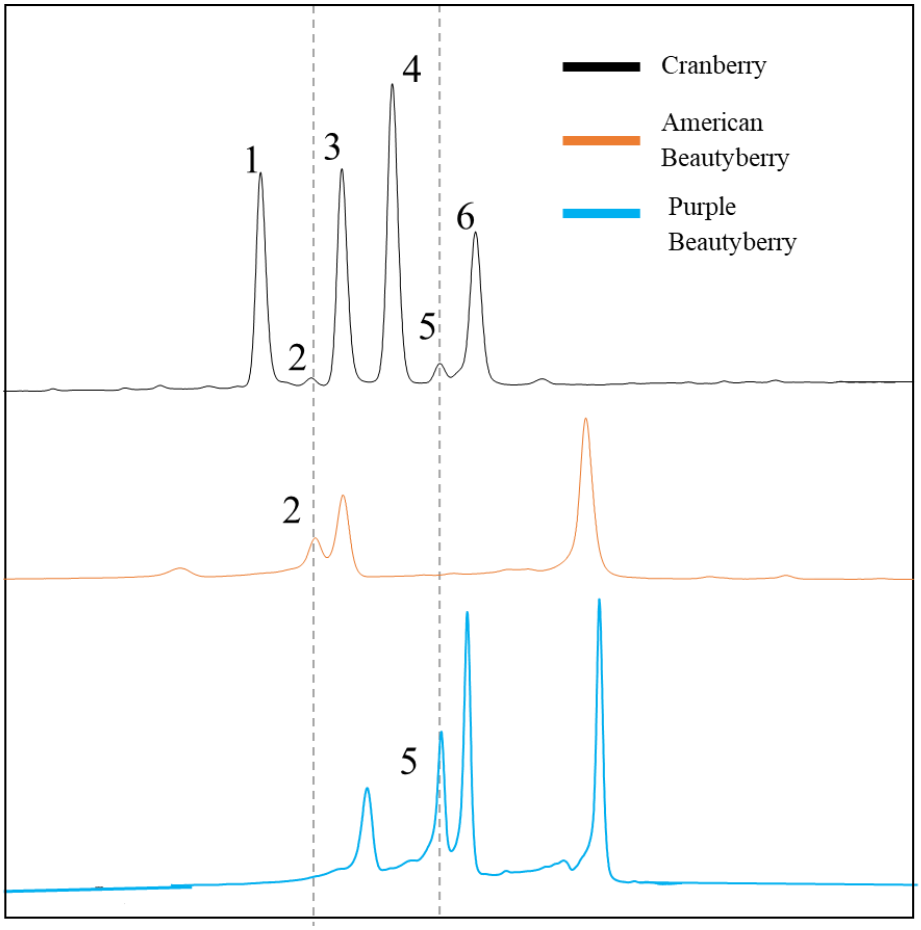

Figure 14. Chromatogram of cranberry anthocyanins, American beautyberry, and Purple beautyberry saponified and partial hydrolysis anthocyanins detected at 520 nm. Peak identification: 1. Cy-3-galactoside; 2 Cy-3-glucoside; 3. Cy-3-arabinoside; 4. Pn-3-galactoside; 5. Pn-3-glucoside; 6. Pn-3-arabinoside.

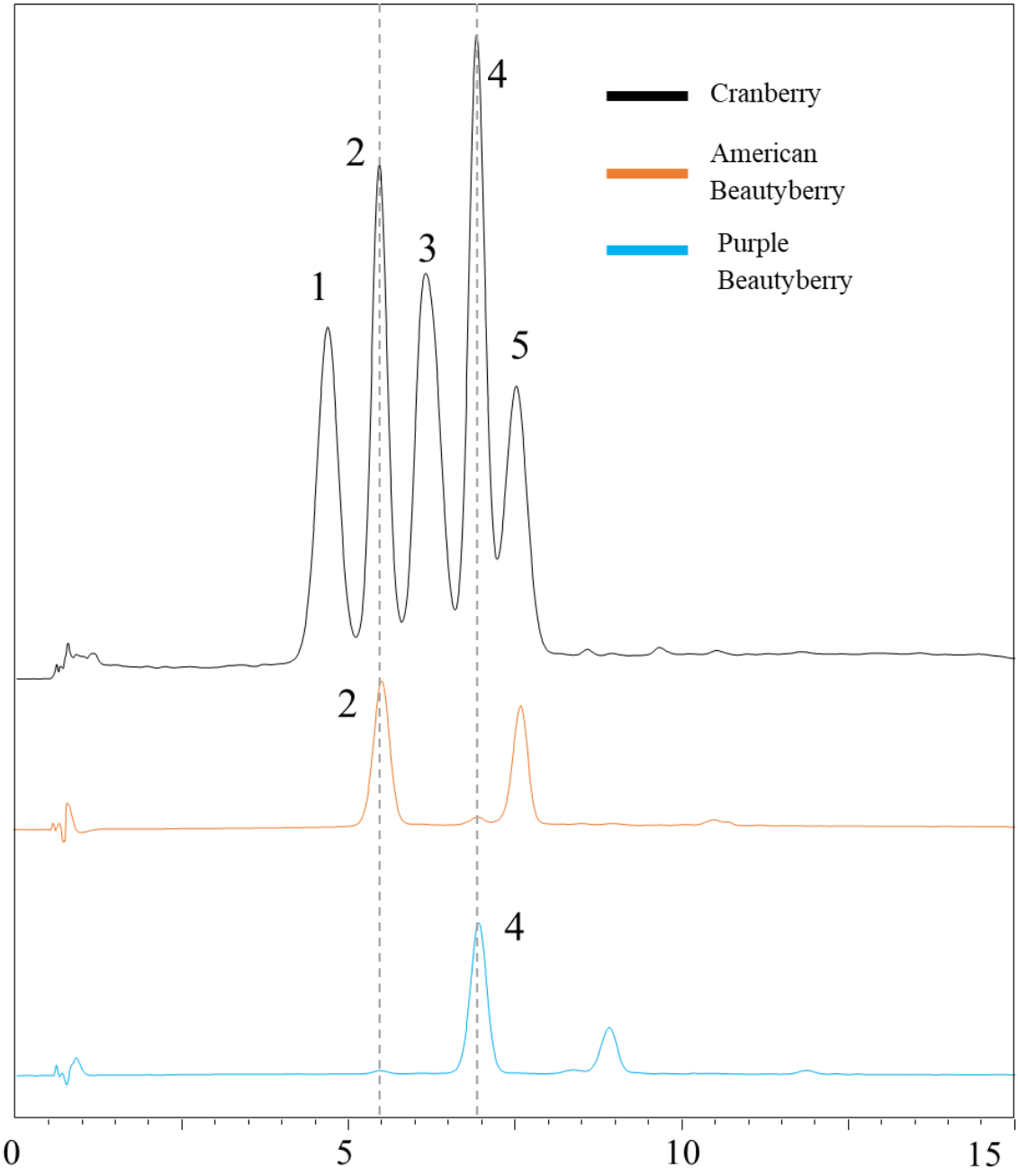

Figure 15. Chromatogram of Muscadine grape juice anthocyanins and anthocyanins of American and Purple beautyberry fruit crude extracts at 520nm. Peak identification: 1. Dp-3,5-diglucoside; 2. Cy-3,5-diglucoside; 3. Pt-3,5-diglucoside; 4. Pn-3,5-diglucoside; 5. Mv-3,5-diglucoside

Peak	Tentative Identification	Precursor Ion *m/z* $[M]^+$	Fragment *m/z* $[M]^+$	Species
1	cyanidin 3,5 diglucoside	611	449, 287	**AB, BB**
2	peonidin 3,5 diglucoside	625	463, 301	**PB, BB**
3	cyanidin 3-malonylglucoside-5-glucoside	697	449, 287	**AB, BB**
4	peonidin 3-malonylglucoside-5-glucoside	711	463, 301	**PB, BB**

Table 11. Tentative identification of anthocyanins (Figure 10) with additional information from alkaline and acid hydrolysis and reference materials.

The tentative structures are shown in Figure 17. For confirmation of identity, NMR analysis would be beneficial. However, for this project, the extract volumes were limited so purification and NMR analysis were not able to be conducted.

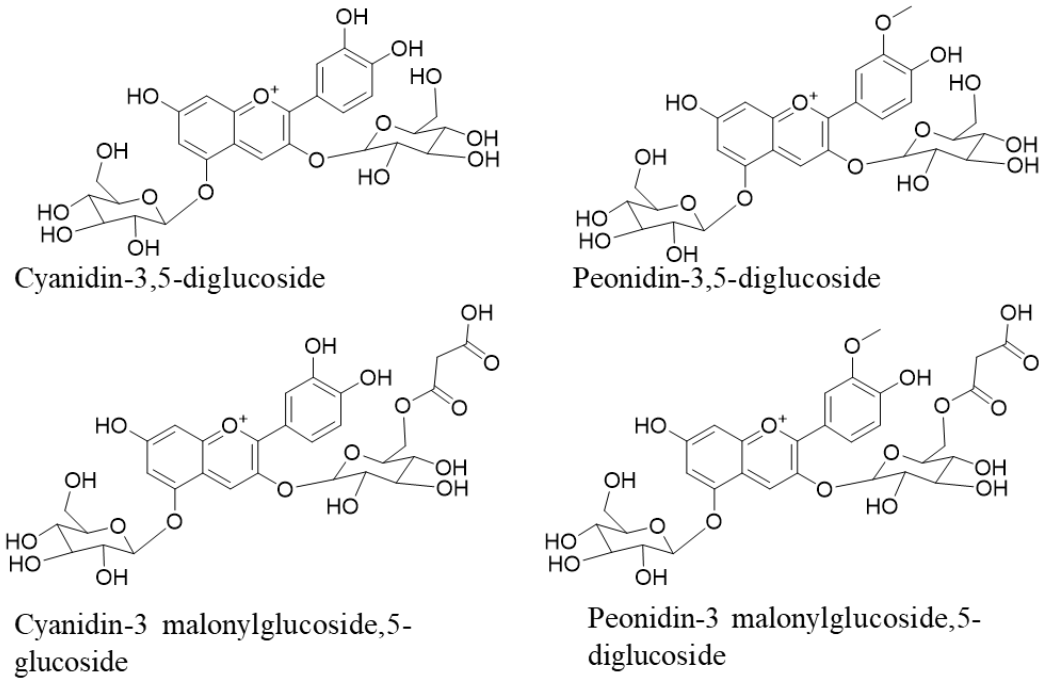

Figure 16. Proposed structures for tentative identification of major anthocyanins in American, Purple, and Bodinier beautyberry fruit.

Anthocyanin Botanical Possible Linkages

Bodinier Beautyberry was assigned the tentative identification all four of the prominent cyanidin and peonidin-3,5 -diglucosides found in American and Purple beautyberry. The presence of all four anthocyanins could suggest that Bodinier beautyberry shares both American and Purple Beautyberry linage. However, Bodinier beautyberry was well-documented as a native plant in Eastern Asia before international travel was very prevalent, whereas American Beautyberry is native to the United States. However, there could be other species of beautyberry out of the over 140 identified, that are also native to the Eastern Asia regions, that may have had cyanidin derivatives that provided natural cross breeding with Purple beautyberry to create Bodinier beautyberry. However, genotyping would need to be conducted to investigate potential plant ancestry.

Another potential link to the similar compounds is the presence of methylation enzymes in the plant. The difference between the aglycone cyanidin and peonidin is a one methyl group, shown in Figure 18. The presence of the methylation enzyme in Bodinier and Purple beautyberry could explain the presence of peonidin in the fruit.

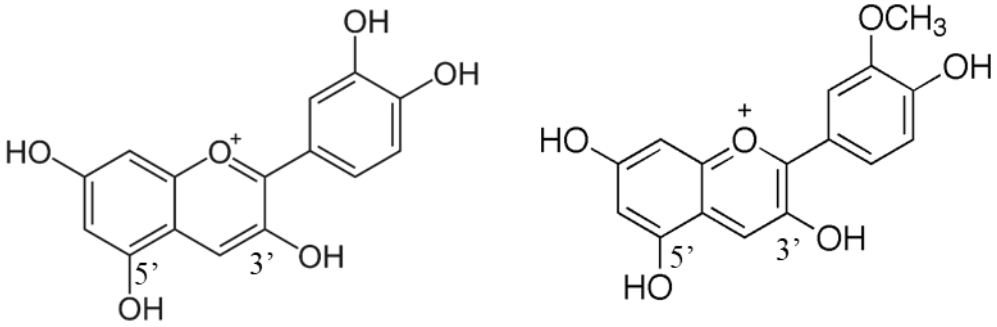

Figure 17. Similarity in cyanidin and peonidin structures.

4.5 Impact of Composition on Color

The anthocyanin content of the fruit extracts was modest compared to expressed color and an investigation into other contributing factors was conducted. The concentration of anthocyanins were low compared to the broad class of phenolics. Phenolics can impart co-pigmentation effects that can cause the intensity of color expression from anthocyanins increases. To measure the impact of the phenolics on the color expression, the semi-purified anthocyanin extract was compared to extracts that underwent an additional 2 washes of ethyl acetate to remove non-anthocyanin phenolics following a solid-phase extraction procedure on a C18 column to obtain an anthocyanin-enriched extract. Bodinier beautyberry was not included due to insufficient sample volume. The color expression decreased on both the American and Purple beautyberry

extracts after the ethyl acetate rinses (Figure 19). The spectra of expressed wavelengths was measured from 400-700 nm and shown in Figure 20. In American beautyberry, the color at all pH's had $\Delta E \geq 5$, which shows that the removal of phenolics and flavonoids could be seen in a decrease in color by the common observer. This shows that the phenolics increased the color expression of the anthocyanins found in the fruit.

In Purple beautyberry fruit, the extracts from pH 1-3 and pH 7-9 had $\Delta E \geq 5$, while pH 4-6 had $\Delta E \leq 5$ (2.9, 1.9, 1.7, in order of increasing values). At pH 4.5, anthocyanins will bleach due to the conversion of the flavylium cation to the chalcone form. This indicates that the anthocyanins in purple beautyberry at pH 4-6 were not as impacted by co-pigmentation with other phenolics and flavonoids than at other pH's.

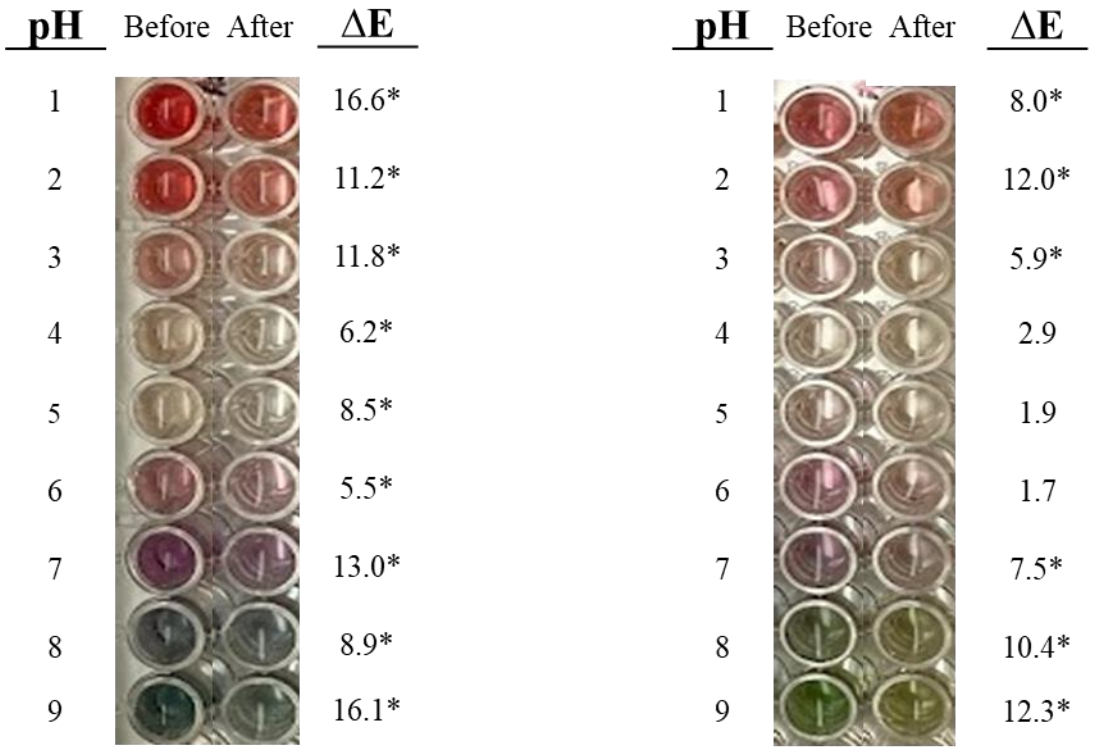

Figure 18. Color comparison of American beautyberry(left) and Purple Beautyberry(right) SPE extract before ethyl acetate rinse and after ethyl acetate rinses. * indicates the difference in color could be detected by the common observer ($\Delta E \geq 5$).

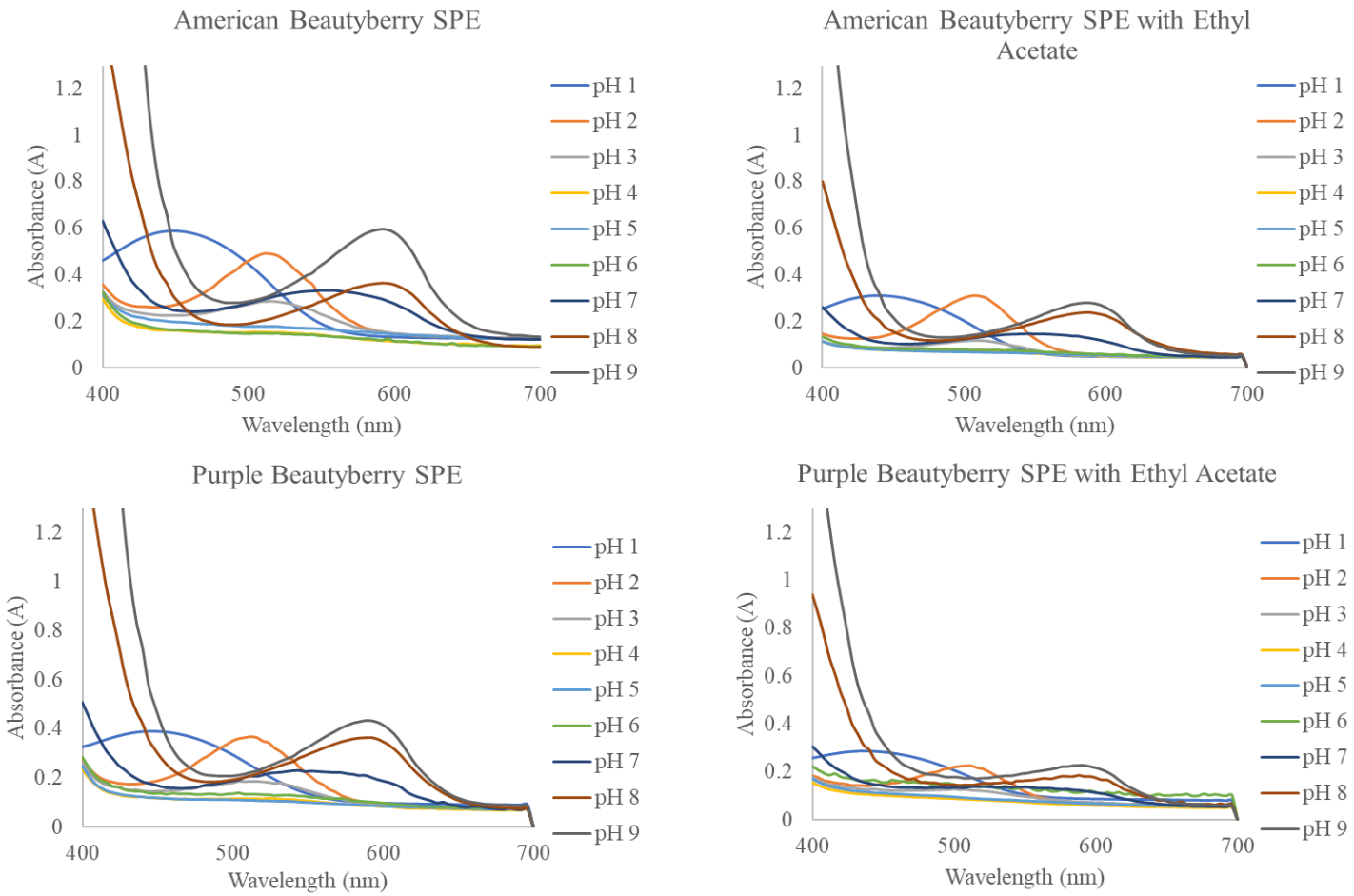

Figure 19. Absorbance at 400-700 nm of 60ul sample in 240ul of pH 1-9 buffers after 15 minutes of equilibration. Absorbance values are averages of sample replicates (n=3).

4.6 Major Phenolic and Flavonoid identification

The extracts of American, Bodinier, and Purple beautyberry that underwent semi-purification without ethyl acetate were run with 0.1% formic acid on the uHPLC PDA MS/MS. Less acid was used since phenolics and flavonoids typically give a stronger signal with low acidity, while anthocyanins give a stronger signal with more acid. The chromatogram of American beautyberry, Bodinier beautyberry, and Purple beautyberry are presented in Figure 21. Phenolic acids and flavonoids show maximum absorbance values at 260 nm and 320 nm, respectively. A plot of absorbances from 280-700 was utilized to view phenolic and flavonoids that absorb strongest outside the visual wavelengths (280-380nm) to the wavelengths that are in the visible range (380-700nm). The area within the rectangle are where anthocyanins eluted, which showed that anthocyanins are not the major compounds in the extracts, despite the color contributions. The major phenolics that were identified are shown in Table 12.

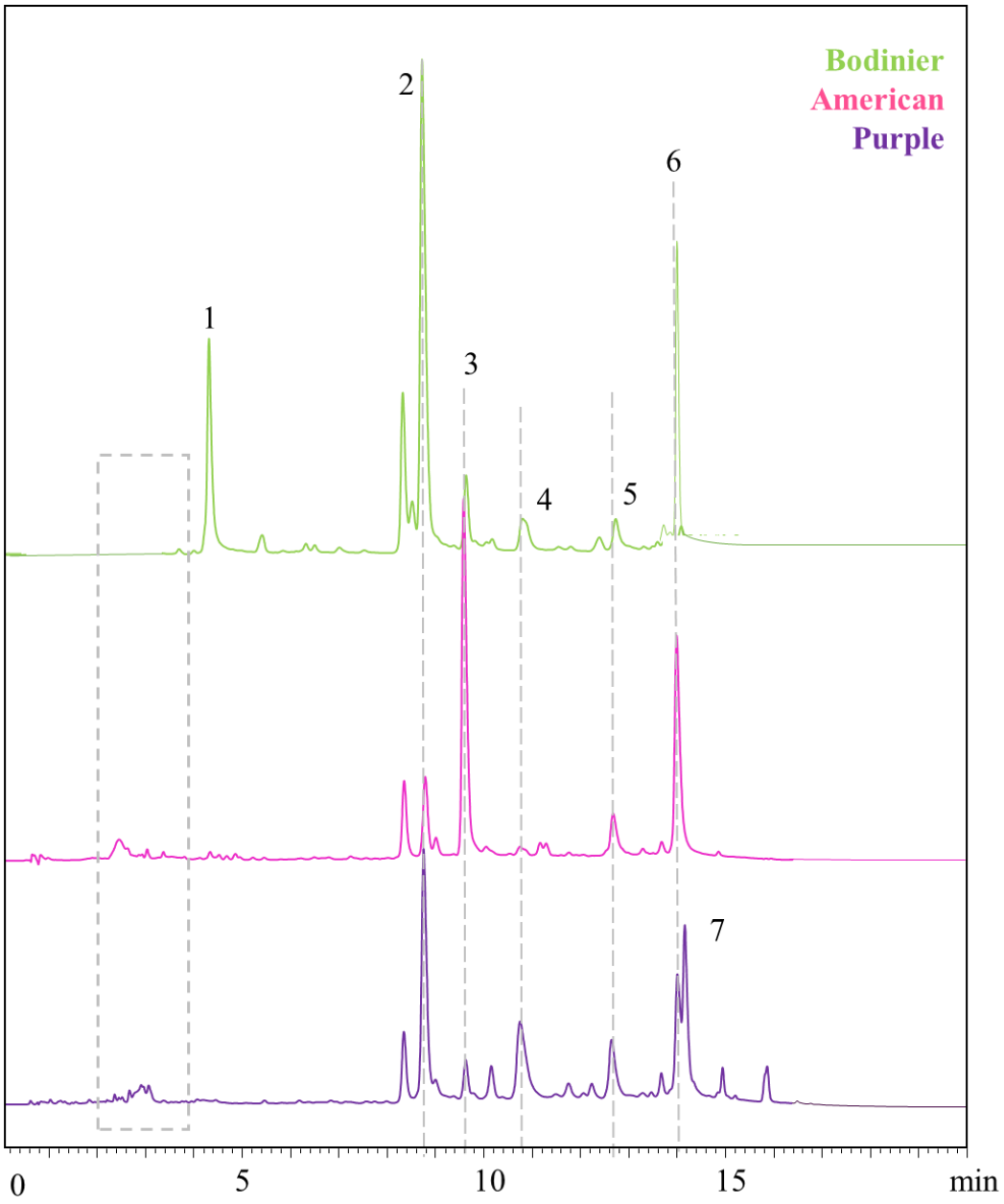

Figure 20. Chromatogram of beautyberry fruit from 260-700 nm. Numbers designate major phenolics tentatively identified in Table 12. Dotted lines indicate shared retention times. Areas within rectangle are where anthocyanins elute.

Peak	Tentative Identification	Precursor Ion *m/z* $[M]^+/[M]^-$	Fragment *m/z* $[M]^+/[M]^-$
1	chlorogenic acid[a]	355/353	191(-)
2	verbascoside	642/623	471, 325,309,163
3	verbascoside derivative	788	471, 325, 163
4	apigenin-7,4'-diglucuronide	623/621	447,271/445(-)
5	luteolin-7-O-glucuronide	463/461	287/285
6	apigenin-7-O-glucuronide	447/445	271/269
7	quercetin-3-glucuronide[b]	477/475	301/299

Table 12. Tentative identification of major compounds in American, Bodinier, and Purple beautyberry fruit. All phenolics were found in all three species except for 'a'(Bodinier only) and 'b'(Purple only).

Peak 1 (Figure 21) was present in Bodinier beautyberry but not in American or Purple beautyberry. The spectra had a lambda max at 325 nm, which indicated that it was a flavonoid. The MS total ion scan produced the largest signal of 355 m/z [M+H] and 353 m/z [M-H], with a higher intensity of ionization in negative mode. Since peak 2 had a loss of a hexoside, peak 1 was also run under a neutral loss scan. Botanically, plants use similar substitutions on related molecules for conservation of energy and maximum efficiency. Peak 1 showed a loss of 162 *m/z* from 353 *m/z*[M-H], that fragmented into 191 *m/z* (Wu & Prior, 2005; Giusti *et al*, 1999). The parent ion and fragmentation in negative mode was consistent with chlorogenic acid (Simirgoitis *et al*, 2015), hence peak 1 was tentatively identified chlorogenic acid (Figure 22). A similar process was used for the other peaks tentatively identified.

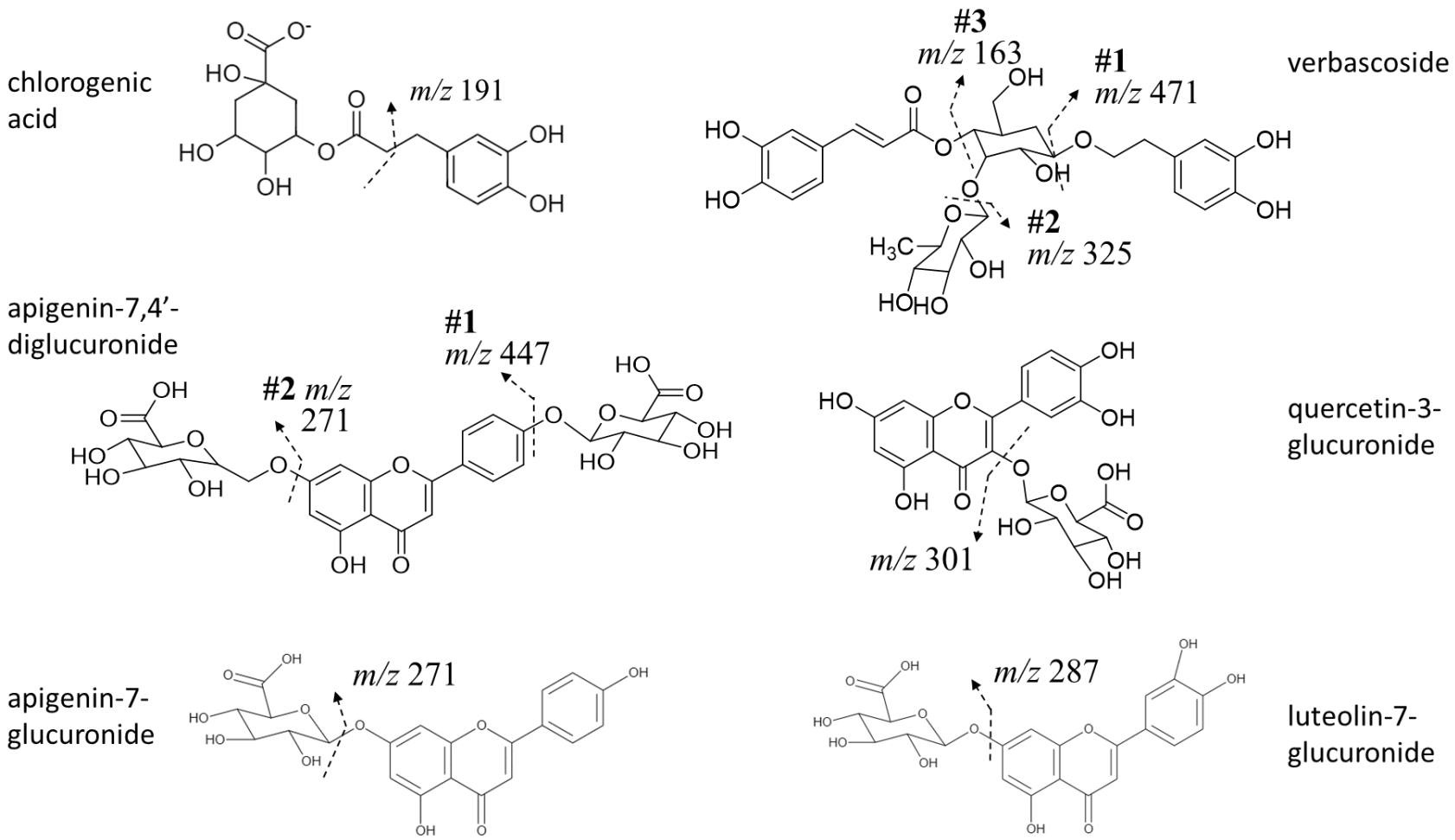

Figure 21. Proposed structure and fragmentation of major compounds in American, Bodinier, and Purple beautyberry fruit.

The largest peak from 260-700 nm was peak 2 (Figure 21), which was a major compound in all three species. Upon looking at the spectra, the lambda max of 331 indicated that it was a flavonoid. The MS total ion scan at peak two had the largest signal of 642 *m/z* [M+H] and 623 *m/z* [M-H]. The product ion scan for the fragments of 642 [M+H] were 471, 325, 309 and for 623 [M-H] was 325 and 309. The intensity of signal was twice as much in positive mode as in negative mode. The neutral loss scan for a loss of a hexoside (162) showed that 325 [M-H] loss 162 to produce 163 (Wu & Prior, 2005; Giusti *et al*, 1999). The fragmentation pattern was consistent with values reported in literature for verbascoside, shown in Figure 22(Attia *et al,* 2018).

Peak 3 (Figure 21) was a major compound in American beautyberry and present in smaller concentrations in Purple and Bodinier beautyberry fruit. The lambda max at 331 indicated that the compound was a flavonoid. The precursor ion scan in positive mode produced 788 m/z. A product ion scan of 788 produced 479, 471, 325, and 309. To verify the presence of the fragments, a neutral loss scan for a hexoside (162 m/z) was run. In addition, a neutral loss of 176, which correlates to a glucuronide, was run to determine if 479 fragmented into 309, as the difference between 479 and 309 is 176 (Wu & Prior, 2005; Giusti *et al*, 1999).. The fragment of 325 had a neutral loss of 162, which indicated a hexose loss similar Peak 2. The ion of 479 had a loss of 176 under the neutral loss scan and confirmed that 479 fragmented into 309. The consistent fragmentation pattern to peak 2 tentatively identified as verbascoside led to the tentative identification of peak 3 as a verbascoside derivative (Attia *et al,* 2018). The ion of verbascoside (642) did not appear in the product ion scan. However, the difference between the prominent signal of 788 in

peak 3 and 642 in peak 2 was 146, which is consistent with a rhamnose (Wu & Prior, 2005; Giusti *et al*, 1999). A neutral loss scan of 146 m/z was added but there was no signal produced for ions with a neutral loss of 146 from peak 3. This suggests that if a rhamnose is present, it is attached to another substitution with a weaker bond than a glycosidic bond joining the rhamnose to the structure that would cause both substitutions to fragment at the same time as the weaker bond breaks. Additional analyses such as NMR could be helpful in identification of peak 3.

Peak 4 (Figure 21) was a moderate peak in Purple beautyberry and low in American and Bodinier beautyberry fruit. The lambda max of 323 indicate that it is a flavonoid. The total ion scan produced a strong signal at 623 [M+H] and 621 [M-H]. This showed that the compound was able to be ionized in both positive and negative mode, which is common in flavonoids. The signal was stronger in intensity in positive ionization, so a product ion scan of 623 in positive mode was conducted. The resulting fragment ions were 447 and 271. Scans for a neutral loss of rhamnose (146), hexose (162), and glucuronide (176) were conducted and the loss of 176 from 623 was observed, which confirmed the fragment of 447 (Wu & Prior, 2005; Giusti *et al*, 1999). There was also the observation of a loss of 176 from 621 in negative mode, which supports this fragmentation pattern. The loss of 176 from 447 was not observed but the difference of mass of 471 and 271 is 176. The ionization energy may not have been high enough to see this fragmentation pattern. The fragmentation was compared to literature and tentatively identified as Apigenin-7,4’-diglucuronide, shown in Figure 22 (Xie *et al*, 2007).

Peak 5 (Figure 21) was a moderate peak in Purple beautyberry and also low in American and Bodinier beautyberry fruit. The lambda max at 345 indicated that it was a flavonoid. The total ion scan produced a strong signal at 463 [M+H] and 461 [M-H] which indicted the presence of a flavonoid due to the capability to ionize in positive and negative mode and absorb between 280-360 nm. A product ion scan was conducted and 287 was produced from 463 [M+H]. To confirm the fragmentation, a neutral loss of 176, which would correlate to the mass difference and loss of a glucuronide, was conducted (Wu & Prior, 2005; Giusti *et al*, 1999). A loss of 176 was seen in positive and negative mode as 463 fragmenting into 287 and 461 would fragment into 285. The compound was tentatively identified as Luteolin-7-O-glucuronide when compared to fragmentation in literature and shown in Figure 22 (Wang *et al*, 2017). Botanically, this is also possible as Peak 4 was tentatively identified as apigenin with 2 glucuronide, which would be more polar and elute faster than a compound with 1 glucuronide. Luteolin and apigenin are also very similar in structures, with the difference of one hydroxide group.

Peak 6 (Figure 21) was a major compound in all three species. The lambda max was 332 suggest that it is also a flavonoid. The total ion scan produced *m/z* of 447 [M+H] and 445 [M-H] as major signals. To gather more information about the compound, a product ion scan was conducted in positive mode, since the intensity of signal was stronger. A fragment of 271 was detected, which has a difference of 176 m/z. A neutral loss for 176 was run to confirm the fragmentation pattern and a neutral loss of 176 was detected for both 447 [M+H] and 445 [M-H]. The fragmentation patterns of Peak 6 was similar to Peak 4 and the literature reported the same fragmentation pattern for Apigenin-

7-O-glucuronide and is shown in Figure 22 (Zhang *et al*, 2020; Wu & Prior, 2005; Giusti *et al*, 1999). The tentative identify of apigenin with 1 gluconuroide for peak 6 and apigenin with 2 glucuronides is consistent with polarity characteristics and botantical patterns for the production of phenolics.

Purple beautyberry also had peak 7(Figure 21), which was not present in significant amounts in the other species. Peak 7 coeluted with Peak 6 but the lambda max was 347. The total ion scan produced ions of 477 [M+H] and 475 [M-H]. A product ion scan was conducted and 301 m/z was produced from 477 [M+H]. To confirm the presence of this fragment, a neutral loss scan of 176 was conducted, due to the consistent trend of glucuronide substitutions and the difference of 176 between 477 and 301 m/z [M+H]. The neutral loss scan did show a loss of 176 m/z in both 477 [M+H] and 475 [M-H]. When the fragmentation patterns, mass, and spectral characteristics were compared to literature, peak 7 was consistent with and tentatively identified as Quercetin-3-glucuronide, shown in Figure 22 (Li *et al*, 2016).

The phenolics, specifically flavonoids, were tentatively identified in American beautyberry as, verbascoside, luteolin-7-O-glucuronide, and apigenin-7-O-glucuronide. In Bodinier beautyberry, the major phenolics, specifically flavonoids, were 3-caffeoylquinic acid, verbascoside, and apigenin-7-O-glucuronide**.** In Purple beautyberry, the major phenolics, specifically flavonoids, were identified as verbascoside, apigenin-7,4'-diglucuronide**,** luteolin-7-O-glucuronide**,** apigenin-7-O-glucuronide**,** and quercetin-3-glucuronide.

Verbascoside has been previously reported in other species of beautyberry and has a history of use in traditional medicine. Verbascoside is most commonly identified in Lemon Verbana which suggests some biochemistry similarities with beautyberry fruit. Apigenin and luteolin are both flavones with the difference being one hydroxide group on the B ring. Luteolin and quercetin are structurally the same except for an extra hydroxide group on position 3' of quercetin. An apigenin 7,4' diglucoside has an extra glucuronic acid compared to apigenin-7- glucoside. When plants produce polyphenols, there is a biological tendacy for commonalities in core structure of compounds with variations in attachments that can be achieved through enzymes such as methoxylation enzymes (Cuycken & Claeys, 2004; Arrango *et al*, 2015). It is efficient for the plant to create compounds structurally similar, and this was seen in American, Purple, and Bodinier beautyberry. The presence of glucuronide acid attachments that are consistent across the major phenolics is also an example of how plants generate structurally similar compounds that are easily exchanged depending on the needs of the plant to interact with the environment and monitor plant cellular function (Koes *et al*, 1994).

Chapter 5. Conclusions and Future Work

The quality characteristics of American, Bodinier, and Purple beautyberry fruit were comparable, with some differences emerging in the specific compounds present. The total concentration of flavonoids, and anthocyanins overall were not significantly different, nor were the pH or total soluble solid values. Acylated and non-acylated anthocyanins were tentatively identified in American beautyberry as cyanidin- 3,5-diglucoside and cyanidin-3-malonylglucoside-5-glucoside, and in Purple beautyberry as peonidin-3,5-diglucoside and peonidin-3-malonylglucoside-5-glucoside. Bodinier beautyberry showed botanical commonality with both American and Purple beautyberry due to the composition of anthocyanins from both species. There were also similarities in the phenolics, with chlorogenic acid found exclusively in Bodinier beautyberry fruit and quercetin-7-gluconuride in Purple beautyberry fruit. Despite the slight compositional differences, the presence of phenolics was important to the color expression of anthocyanin due to copigmentation. Phenolics that participated in copigmentation that were tentatively identified in the fruit of selected beautyberry species were also previously identified in the plant leaves. This is interesting because compounds are often found in both but fruits often contain additional unique compounds not present in the leaves. The simplicity of sharing major fruit phenolics with plant leaves and having simple anthocyanin profiles with only two potential aglycones indicate that other components of the plant would have similar compositions that are also simple and related.

Further studies on beautyberry could include increasing the replications and study the effect of the environment on the fruit composition. The scope of this study was limited to one growing year in one location, but additional growing years can account for the difference in weather patterns year after year. Additional locations can also provide more information on the composition of the fruit when subjected to different growing environments, including a variety of climates. Future studies could also include the investigation of the specific anthocyanins and phenolics could continue in the form of Nuclear Magnetic Resonance (NMR) to determine exact position and constitutes of these compounds. For this study, the fruit samples were not sufficient in quantity to produce isolates needed for NMR. The presence of other phenolics contributed to co-pigmentation the intensified the anthocyanin color expression. Anthocyanins and other phenolics interact to produce the vibrant coloration of American, Purple, and Bodinier beautyberry fruit.

However, the presence of other phenolics with anthocyanins in the extract still did not produce the unique coloration exhibited in the fruit. The extract included the fruit skin, flesh, and seeds. The small fruit size made separation of fruit components difficult. But if the anthocyanins of the color are primarily in the skin, the compounds included from the flesh and seeds may have affected the color expression. It is also possible that the cell structure is important to the color expression. When fruit is frozen and thawed, this disrupts cell membranes that previously separated compounds in different compartments. The interaction these compounds that were previously separated could also have an effect on the color expression. Additional research is needed to determine if

an extract from beatuyberry fruit could produce a color similar to the fresh fruit coloration. Future work could also investigate optimizing the extraction methods. Recent research was limited on the color of beautyberry specie fruits, and additional experiments are needed to optimize the color expression and extraction of color-contributing compounds in order to replicate the unique coloration of the fruit as extracts for applications.

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