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CHARACTERIZATION OF ANTHOCYANINS IN *ARONIA*

BY

THEODORE MOHR

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Biological Sciences

Specialization in Food Science

South Dakota State University

2018

CHARACTERIZATION OF ANTHOCYANINS IN *ARONIA*

THEODORE MOHR

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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## ABBREVIATIONS

cm	centimeter
C3-Gal	Cyanidin 3-Galactoside
Fig	Figure
g	gram
HCL	Hydrochloric Acid
μm	micrometer
mg	milligram
mL	milliliter
mm	millimeter
nm	nanometer
N	Normality



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ABSTRACT  
CHARACTERIZATION OF ANTHOCYANINS IN *ARONIA*

THEODORE MOHR

2018

Anthocyanins function as antioxidants and offer numerous other health benefits, such as anti-atherogenic and anti-inflammation protection, and may aid in the prevention of degenerative diseases. *Aronia* berries, uniquely high in anthocyanins, have a great potential in improving human wellness. The objectives of this study were: (1) to develop a rapid and reproducible analytical method for estimating the anthocyanin amount in *Aronia* berries; and (2) to determine the effect of growing conditions on anthocyanin concentration. A standard curve method was established based on anthocyanin absorbance in the visible range. The results reveal that the developed protocol could yield rapid and reproducible values. Subsequently, a total of 51 *Aronia* samples of Viking, McKenzie, and Galicjanka varieties grown at nine locations (Ogle County, IL, Minnehaha County, SD, Harrison County, MO, Clay County, IA, Plymouth County, IA, Charles Mix County, SD, Buchanan County, IA, Webster County, IA, and Woodbury County, IA) in the Midwest region of the United States have been analyzed. In general, the anthocyanin amount increased throughout the harvest season. It is not statistically ( $p < 0.05$ ) dependent on the mean maximum temperature, mean temperature, mean minimum temperature, precipitation, and latitude. However, berries grown in the southeastern locations tend to have higher anthocyanins than those from the northwestern region.

## CHAPTER 1. INTRODUCTION

Recent consumer health interests have led to a renewed focus on food products that offer more nutritional benefits. Lately, these foods have been given a nickname “superfood,” to demonstrate their value. *Aronia* berry is regarded as a “superfood” because of its high antioxidant content.

The *Aronia* berry (*Aronia Melanocarpa*), commonly known as the chokeberry, is native of eastern North America. It shows high resistance to frost and therefore can be grown under most northern climates. The berries are dark purple with astringent taste, and gained consumer interest recently due to their healthful properties. In addition, the berries are resilient to transportation and storage conditions, thus making mass production possible. While *Aronia* has not seen an increase in popularity to the point of becoming a household fruit, it is used in many food products such as juices, jams, wines, preserves, and herbal teas. It is also used as a natural colorant. In order to further expand utilization of *Aronia* berries, it is important to characterize the amount and nature of anthocyanins. Currently, HPLC and pH Differential methods are used for anthocyanin quantification. However, these are expensive and time-consuming protocols, thus there is a need to develop simple and less expensive alternatives. The objectives of this study were: (1) to develop a rapid and reproducible analytical method for estimating the anthocyanin amount in *Aronia* berries; and (2) to determine the effect of growing conditions on anthocyanin concentration.

## CHAPTER 2. LITERATURE REVIEW

### Origination

*Aronia* berries originate from eastern North America.<sup>1</sup> They are from a perennial shrub within the *Rosaceae* family that is indigenous to this region.<sup>2</sup> *Aronia prunifolia*, *Aronia arbutifolia*, and *Aronia melanocarpa* are the three species.<sup>2</sup> Species can be identified based on fruit color, with *Aronia arbutifolia* red, *Aronia prunifolia* purple, and *Aronia melanocarpa* black, the latter is the most common specie cultivated.<sup>2</sup> The shrub can quickly grow to 2-3 meters tall depending on the quality of soil and is hearty. It has the ability to survive harsh winters and can be cultivated up to 15 or 20 years.<sup>2</sup> The most productive twigs on the shrub are between 2 and 6 years old.<sup>2</sup> Pests are not a significant problem for *Aronia* cultivation.<sup>1</sup> The shrubs are not very selective on soil type, but ample sunlight and air circulation are required. It flowers in late spring, an advantage for survival during spring frost, and over the course of the summer the berries will ripen from green to dark purple. They are harvested in late summer and early fall. Harvesting is typically done with specialized equipment or by hand. One bunch on the shrub can produce up to 15 berries that weigh from 1 to 1.5 grams apiece, and each shrub has the capacity to yield 20-30 pounds.<sup>2</sup> Common *Aronia* varieties grown include: Viking, McKenzie, Galicjanka, Hugin, Nero, and Rubina.<sup>3,4</sup>

Although native to North America, *Aronia* was first cultivated in Europe. It made its way to Russia, Norway, and Poland near the beginning of the 20<sup>th</sup> century. Later in the 1930s, Russian botanist, Ivan Mitchurin, found *Aronia* was nutritious and shrubs could survive the cold climate.<sup>2</sup> Since then, its cultivation started in Russia and then spread to other European nations with similar climatic conditions.<sup>5</sup> However, Sweden was the first

to initiate commercial cultivation of *Aronia* in the late 1980's.<sup>5</sup> In 1988, a Polish company, Agropharm S.A., in Tuszyn first manufactured the red pigment from *Aronia* to replace artificial color in foods.<sup>2</sup> A few years later another company, Aronia S.A., in Leczyca, Poland introduced *Aronia* juice to the Polish market. The juice caught on quickly and saw an increase in demand. This could be attributed to new scientific studies on the role of antioxidants in human health.<sup>2</sup> A few studies suggest that *Aronia* can offer anti-atherogenic, anti-cancer, and anti-inflammation protection, and help in the prevention of degenerative diseases.<sup>1,2</sup>

### **Oxidative Stress**

The increase in *Aronia* cultivation and consumption is due to its health benefits. The high antioxidant capacity of *Aronia* is an important reason.<sup>2</sup> Studies have shown an inverse correlation between consumption of fruits and vegetables and disease risks of cardiovascular disease, diabetes, and cancer.<sup>6</sup> These degenerative diseases are linked to oxidative stress.<sup>6</sup> Free radicals are responsible for oxidative stress in the body. Dietary sources of antioxidants mainly from fruits, vegetables, teas, and wines, aid in maintaining a normative antioxidant status, and improving the plasma antioxidant capacity.<sup>7</sup> Incidentally, *Aronia* consumption increases antioxidant capacity.<sup>8</sup>

The unpaired electron of a molecule is very reactive and tries to pair with another electron. Often times, there are no other radicals in the vicinity and a reaction takes place between the free radical and a nearby molecule.<sup>2</sup> When the body is incapable of neutralizing free radical production from these reactive species, oxidative stress occurs. Oxidative stress contributes to neurodegenerative and cardiovascular disease, and age-



related cognitive decline, to name a few.<sup>9</sup> Over time, this oxidative stress can lead to the damaging of organelles and macromolecules in the body.<sup>9</sup> This occurs through the depletion of the reserves of antioxidants in the system and results in damage to the DNA.<sup>2</sup> Radiation, stress, poor nutrition, pollution, and use of tobacco or drugs often lead to oxidative stress.<sup>8</sup> Oxidative damage takes place naturally, and increases as the body ages.<sup>7</sup>

### **Health Benefits**

To combat oxidative stress in the human body, antioxidants from dietary sources are helpful. Antioxidants in plants serve a protective purpose. They play crucial roles in securing plants from excessive ultraviolet radiation exposure. For example, they reduce damage to DNA by free radicals.<sup>2</sup> They scavenge harmful radicals or oxygen species, reducing the oxidative stress experienced by the plant.<sup>2</sup>

Two groups of antioxidants are defined based on their mechanism of action: preventative and chain-breaking.<sup>8</sup> Preventative antioxidants work towards suppressing the formation of reactive oxygen species. This leads to less damage to biologically essential molecules. On the other hand, chain-breaking antioxidants work by retarding the chain oxidative process.

Phenolic compounds in olive oil have been shown to protect endogenous antioxidant mechanism during the postprandial lipemia state.<sup>10</sup> Cigarette smoking aggravates reactive oxygen species and increases oxidative stress.<sup>11</sup> Smokers also have a lower antioxidant capacity in the blood than non-smokers.<sup>11</sup> Lower antioxidant capacity in the blood is one biomarker of elevated oxidative stress that may contribute to

cardiovascular disease.<sup>11</sup> Drinks with high antioxidants increase plasma antioxidant capacity and decrease oxidative stress biomarkers.<sup>10</sup> Tea and red wine enhance the plasma antioxidant status, two products that can be developed using *Aronia*.

### ***Polyphenolic Compounds***

Polyphenolic compounds from fruits and vegetables are important antioxidants. They are the phytochemicals with the ability to scavenge free radicals and oxidants.<sup>8</sup> They act as reducing agents that donate hydrogen, trap free radicals, and satisfy singlet oxygen.<sup>7</sup> Structurally, they have one or more OH groups that are linked to an aromatic ring. Gallic acid, catechins, myricetin, quercetin, and resveratrol are known examples.<sup>7</sup> They are found in fruits, vegetables, teas, and wines. Flavonoids account for over half of the more than eight thousand known polyphenolic compounds.<sup>12</sup> Flavonoids are compounds that consist of fifteen carbons with two aromatic rings joined by a three carbon linkage.<sup>12</sup> They include flavonols, flavanones, flavones, isoflavones, flavanonols, catechins, and anthocyanins.

*Aronia* is rich in polyphenolic compounds, but variations may exist by variety. *Aronia* can have as much as 10-20 grams of polyphenols per kilogram.<sup>2</sup> The concentration can vary depending on growing conditions. It tends to grow better in a northern climate that is slightly cooler and has a shorter growing season.<sup>2</sup> Exposure to strong ultra-violet (UV) radiation promotes the development of phenolic compounds in *Aronia*.<sup>2</sup> The use of fertilizers, herbicides, and pesticides has not been shown to impact phenolic content.<sup>2</sup>

### **Anthocyanins in *Aronia***

*Aronia* has the strongest antioxidant activity among all other berries and fruits.<sup>13</sup> This can be attributed to larger amounts of anthocyanins.<sup>13</sup> Anthocyanins are responsible for the purple, blue, and red colors of fruits, vegetables, flowers, herbs, and grains.<sup>14</sup> They are particularly high in dark fruits and berries. The average anthocyanin consumption in the United States is estimated to be around 180-215 milligrams daily, compared to the intake of flavonols which is only about 23 milligrams<sup>15</sup> Anthocyanin color is highly influenced by light, pH, oxygen, temperature, and chemical structure.<sup>14</sup> A pH of 3.0 or below is required for them to stay as the flavylium cation. At higher pH conditions, they change conformation leading to a colorless pseudo-base.<sup>14</sup> Cool and dark storage conditions are needed to prevent the degradation of anthocyanins.

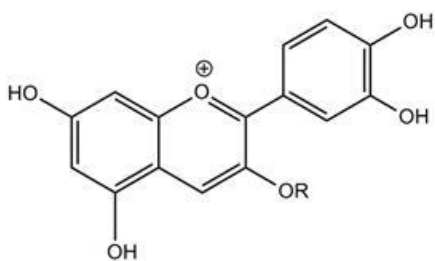
Total anthocyanin content varies from differing fruit species, with *Aronia* having a greater amount. As highlighted in Table 1, one such study found *Aronia* to contain about 4300 mg/kg anthocyanins compared to 230, 240, and 1100 mg/kg from strawberry, red raspberry, and blackberry, respectively.<sup>16</sup>

**Table 1: Concentration of total anthocyanins (TA) (mg Cyanidin 3-glucoside Equivalents kg<sup>-1</sup>) in Berries. Values are means  $\pm$  SD (n=3)<sup>16</sup>**

<b>Berry</b>	<b>Total anthocyanins (mg/kg)</b>
<b>Red Raspberry</b>	242.90 $\pm$ 3
<b>Blackberry</b>	1108.87 $\pm$ 6
<b>Strawberry</b>	232.16 $\pm$ 10
<i><b>Aronia</b></i>	4341.06 $\pm$ 22

In *Aronia*, the four major anthocyanins are 3-galactoside, 3-arabinoside, 3-glucoside, and 3-xyloside of cyanidin. Their chemical structure is shown in Fig. 1.

Cyanidin-3-galactoside and cyanidin-3-arabinoside account for about 69% and 24% of total anthocyanin content in *Aronia*, while cyanidin-3-xyloside and cyanidin-3-glucoside account for 4% and 3% respectively.<sup>16</sup> One study claims that *Aronia* contains an anthocyanin concentration of 4600 mg/1000g fresh weight, while another has anthocyanin content at 6192 mg/1000g fresh weight, both in terms of cyanidin-3-glucoside equivalents.<sup>17,18</sup>



1. R = galactose: Cyanidin 3-galactoside
2. R = glucose: Cyanidin 3-glucoside
3. R = arabinose: Cyanidin 3-arabinoside
4. R = xylose: Cyanidin 3-xyloside

**Fig 1: Structure of the anthocyanins found in *Aronia*.**<sup>13</sup>

### ***Effect of Growing Location on Anthocyanins***

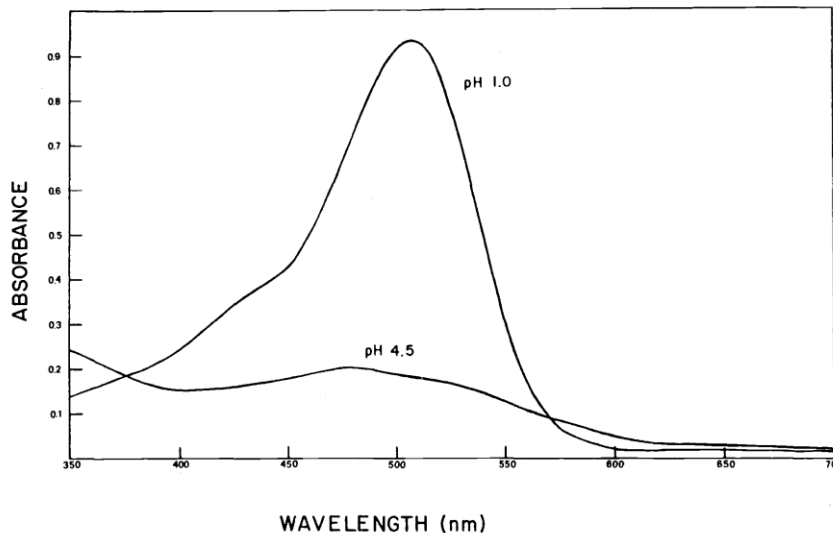
Anthocyanins are compounds that are rich in the *Aronia* berry. However, the total amount present can vary due to numerous factors. One such factor is the harvest time of *Aronia*. A study from University of Connecticut tracked the anthocyanin content over a 7-week harvest period in the Northeastern United States. The study found that anthocyanin content doubled at the fifth week of harvest and then declined the following two weeks.<sup>19</sup> While this data was specific for one region, we can see that anthocyanin content in *Aronia* is not constant during the growing season. The study of this change will enable growers to harvest *Aronia* berries when they contain highest anthocyanin content.

*Aronia* berries can remain ripe for over a month, giving plenty of time for anthocyanins to change.<sup>19</sup> It is also important to know what factors impact anthocyanin changes. Weather characteristics such as air temperature, rainfall, and plant maturity may affect the quantity of anthocyanin content. The degree of fruit maturity is a large factor in anthocyanin development.<sup>20</sup> By giving information to growers regarding the optimal time for harvest, they can produce *Aronia* that maximize antioxidant potential in the berries.

### ***Anthocyanin Profile Over Time with Differences in pH***

Anthocyanins are affected by pH. A pH of 3 or below is required for anthocyanins to stay as the flavylium cation form. As the pH is raised, the anthocyanin changes conformation leading to a colorless pseudo-base.<sup>14</sup> Because anthocyanins are easily oxidized, they are susceptible to degradation over time leading to difficulties with storage. The effect of time and pH with respect to anthocyanin degradation is not yet fully understood. Buffers within a range of pH's 1.0 through 5.0 will be prepared to create a range of anthocyanin solutions. Through spectrophotometry, the absorption of these solutions can be recorded in five nanometer intervals in the visible spectrum. Each profile can then be compared to deliver an anthocyanin profile analysis.<sup>21</sup>

For spectrophotometric methods, anthocyanins must be quantified in a pH 1.0 solution. However, it is not known the complete effect pH has on the absorption characteristics of anthocyanin. Studies have compared the profile of anthocyanins at a pH of 1.0 and 4.5 within the visible spectrum.<sup>22</sup> The change in anthocyanin configuration at a pH of 4.5 alters the absorbance spectra, as seen below in Fig. 2.



**Fig 2: Visible absorbance spectrum of anthocyanins in pH 1.0 and pH 4.5 buffer.**<sup>22</sup>

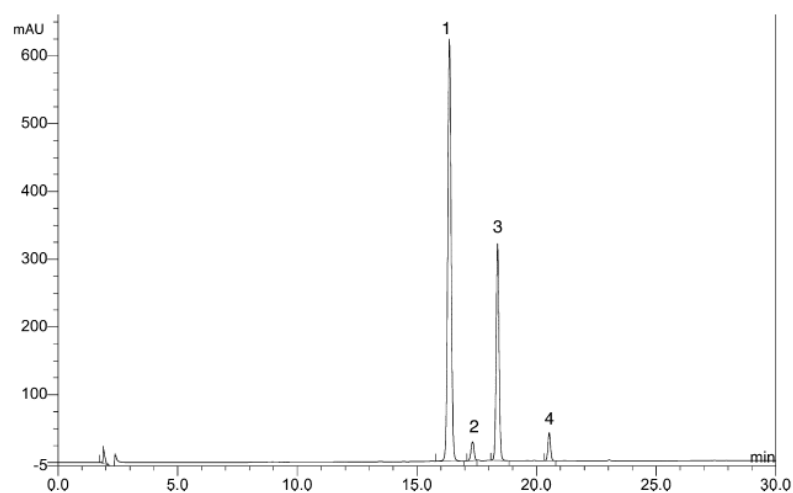
Questions remain about the visible spectrum of purified anthocyanin aside from a pH of 1.0 and 4.5. These pH's have been studied in prior work due to use in the pH Differential Method, a spectrophotometric method that uses the difference in absorbance at an optimal wavelength to determine anthocyanin content.<sup>23</sup> Therefore, research further showing the effect that pH has on anthocyanins is relevant in this area. Data from the absorbance spectrum from an acidic pH such to a more neutral pH would show this effect in greater detail.

The effect of time on the anthocyanin profile is also relevant in this research area. Even when altered by pH, the effect of time on the stability of anthocyanin is not yet known. Because exposure to light, oxygen, and heat are capable of degrading anthocyanin, the absorbance profile may change over time.<sup>24</sup> An anthocyanin solution can be created with Cyanidin 3-galactoside standard and used for this experiment. Absorbance of the anthocyanin solution can be measured using a spectrophotometer with

wavelength measurements at five nanometer intervals. The solution will be measured and re-measured on the following days: 0, 2, 4, 6, and 14. This will create numerous curves that can be analyzed. At a peak absorbance of 510 nanometers, changes in absorbance can be plotted by time and pH.

### ***Anthocyanin Quantification***

Methods for anthocyanin quantification typically include HPLC or pH Differential method.<sup>23,25</sup> Both methods have advantages and disadvantages. HPLC is generally regarded as an excellent method to quantify anthocyanins, as individual anthocyanins may be quantified. An external standard of Cyanidin 3-galactoside will be used with HPLC to create a standard curve from which *Aronia* samples can be compared. Cyanidin 3-galactoside is the external standard because it comprises the highest percentage of anthocyanin present in *Aronia*, typically between 65% - 70% of total anthocyanin.<sup>16</sup> The validity of this approach is due to the assumption that all anthocyanins have the same UV response to that of Cyanidin 3-galactoside.<sup>25</sup> At different concentrations, the total peak area is used to create this curve. HPLC offers advantages such as the ability to separate compounds such as anthocyanins based on characteristics that differentiate such as polarity.<sup>25</sup> There are four anthocyanins present in *Aronia*: cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside, and cyanidin 3-xyloside.<sup>26</sup> A typical chromatogram for an *Aronia* sample can be seen below:



**Fig 3: Typical Chromatogram of anthocyanins at 520 nm. The peaks represent: (1) Cyanidin 3-galactoside, (2) Cyanidin 3-glucoside, (3) Cyanidin 3-arabinoside, and (4) Cyanidin 3-xyloside.<sup>27</sup>**

HPLC offers other advantages such as repeatability as well as speed, sensitivity, and accuracy. HPLC can be used on a wide variety of samples as different columns, modules, and mobile phases can be used to achieve desired results. However, it also has drawbacks, such as high costs, use of organic solvents, and complexity. Depending on the requirements of the sampler, HPLC may or may not be the best tool for anthocyanin quantification.

pH Differential method is another way to quantify anthocyanins. This method relies on the difference in absorbance at two different pH's caused by reversible structural transformations undergone by anthocyanins.<sup>23</sup> The absorbance of a solution is measured at 510 nm and 700 nm using both pH 1.0 and pH 4.5 buffers. Using these four absorbance readings, a formula can be used to calculate total anthocyanin content. This formula can be seen in Fig. 4.



$$\text{Anthocyanin pigment (C3Glucoside Equivalents, } \frac{\text{mg}}{\text{L}}) = \frac{A * MW * DF * 10^3}{\epsilon * 1}$$

**Fig 4: pH Differential formula.  $A = (A_{510\text{nm}} - A_{700\text{nm}})_{\text{pH 1.0}} - (A_{510\text{nm}} - A_{700\text{nm}})_{\text{pH 4.5}}$ , MW = molecular weight, DF = Dilution Factor, and  $\epsilon = 26,900$  molar extinction coefficient, in  $\text{L} * \text{mol}^{-1} * \text{cm}^{-1}$ , for cyanidin 3-glucoside.<sup>23</sup>**

The molecular weight value used (449.2 g/mol) corresponds to the predominant anthocyanin present in *Aronia*, and the molar extinction coefficient is predetermined based on prior research in the area.<sup>23</sup> The pH Differential method is capable of getting an accurate measurement of total anthocyanin in an *Aronia* sample. It is relatively quick and simple and requires very little equipment. Only a spectrophotometer, cuvettes, mortar and pestle, acidified organic solvent, pipette, and pH 1.0 and pH 4.5 buffer are required. This method is much more cost efficient than HPLC, as a spectrophotometer is not nearly as expensive to purchase or maintain as an HPLC system is. There is also no software required to operate a spectrophotometer. A drawback of the pH Differential method is that numerous measurements must be made to determine total anthocyanin content. Because of this, a Standard Curve using Cyanidin 3-galactoside at different concentrations will be created to compare to the pH Differential method and HPLC. A Standard Curve requires fewer measurements and less buffer and involves simpler calculations for total anthocyanin determination. Absorbance measurements of *Aronia* at an optimal wavelength of 510 nm will be determined using the Standard Curve and compared to previous method results.

### CHAPTER 3. MATERIALS AND METHODS

#### Materials

*Aronia* samples were provided by nine growers of different regions belonging to National Aronia Growers, LLC. Eight growers provided Viking *Aronia* samples. One grower also provided McKenzie *Aronia* in addition to Viking *Aronia*, and one provided Galicjanka *Aronia* alone. Growers began harvesting *Aronia* when a Brix reading of 12% to 15% was reached for the berries. All samples were frozen immediately from harvest to their reception in the laboratory. Table 2 shows the growing locations of each sample provided.

**Table 2: Grower locations and *Aronia* sample variety provided for analysis. Viking was the most common variety supplied.**

Grower	Location (County)	Variety
Grower 1	Ogle, IL	Viking
Grower 2	Minnehaha, SD	Viking
Grower 3	Harrison, MO	Viking
Grower 4	Clay, IA	Viking
Grower 5	Plymouth, IA	Galicjanka
Grower 6	Charles Mix, SD	Viking, McKenzie
Grower 7	Buchanan, IA	Viking
Grower 8	Webster, IA	Viking
Grower 9	Woodbury, IA	Viking

HPLC grade methanol (100%) for extractions was purchased from Fisher Scientific. Distilled water, hydrochloric acid, potassium chloride, sodium acetate, and sodium dihydrogen phosphate were ingredients for buffers. A VWR 1000  $\mu$ l pipette was used to measure and deliver solutions. A Genesys 20 spectrophotometer from Thermo

Scientific (Waltham, MA) with plastic one-centimeter cuvettes from Fisher Scientific was used to gather spectrophotometric data.

### **New Analytical Method**

It was proposed that anthocyanin content could be determined in *Aronia* by multiple regression models developed based on spectra data of *Aronia* extracts using calibration and validation sample sets. However, many issues must be studied before this can be done, including the absorption spectrum profiles of anthocyanins and how pH and time will impact these properties.

### ***Standard Extraction Method***

The anthocyanin extraction procedure was slightly modified maceration method as used by Jakobek.<sup>16</sup> Preparation of sample included: weighing, blending, extraction, and dilution. To create a representative sample, 50 grams of frozen *Aronia* berry sample was weighed out in a plastic weigh boat. The 50-gram sample was added to the small sample blender cup. After blending for 10 seconds, the sample was removed from the cup with a spatula. Five grams of blended *Aronia* sample was placed in a mortar and ground with a pestle with 20 milliliters of acidified methanol (0.1%, v/v).

Using the pestle, five grams of the blended *Aronia* berry sample was macerated for exactly one minute and transferred to a 25-mL covered beaker to sit in a dark area for 15 minutes. Then the liquid was decanted, and the macerated sample was placed back into the mortar. This procedure was repeated twice more, as previous research and our own test have shown no further repetition was required.<sup>16</sup> The extracts were combined,

covered, and stored in the dark until further analysis. This process for each *Aronia* sample was done in triplicate.

### ***Light Absorption Spectrum of Anthocyanins***

Using both the cyanidin 3-galactoside and cyanidin 3-arabinoside standards, separate solutions in 100% methanol were created at a concentration of 0.001 mg/mL. Two milliliters of sample were placed in plastic 1 cm cuvettes. Using the spectrophotometer, absorbance measurements were made from 350 nanometers to 710 nanometers in 5 nanometer intervals using a methanol blank. The spectrophotometer was allowed to warm up for 30 minutes before use.

### ***Effects of pH on the Spectrum Profile***

The effect of pH on the anthocyanin spectrum profile was studied. Five buffers were prepared: pH 1.0, 2.0, 3.0, 4.0, and 5.0. Buffers were required due to the pH sensitivity of the anthocyanin molecule. Preparation of each buffer is shown below in Table 3. pH measurements for each buffer were ensured to be  $\pm 0.05$  the target pH.

**Table 3: Preparation of buffer solutions used in determination of pH effect on spectrum profile.**

<b>Buffer Solutions</b>	
<i>pH</i>	<i>Preparation</i>
<b>1.0</b>	0.66 g potassium chloride in 99.9 ml Distilled Water with 0.1 ml of 12N HCl
<b>2.0</b>	0.66 g potassium chloride in 98 ml Distilled Water with 2 ml of 12N HCl
<b>3.0</b>	1.20 g sodium dihydrogen phosphate in 99.9 ml Distilled Water + 0.1 ml 85% phosphoric acid
<b>4.0</b>	5.44 g sodium acetate in 95.7 ml Distilled Water + 4.3 ml HCl
<b>5.0</b>	5.44 g sodium acetate in 98.3 ml Distilled Water + 1.7 ml HCl

For the cyanidin 3-galactoside standard, 0.5 mg was weighed and added to a 10-mL volumetric flask. Methanol (100%) was added to bring the solution to 10 milliliters. One milliliter of this solution was added to nine milliliters of each of the five buffers in five separate beakers. This created five 0.005 mg/mL solutions of cyanidin 3-galactoside for measurement. Two milliliters of sample were placed in separate plastic 1 cm cuvettes. Absorbance measurements were made from 350 nanometers to 710 nanometers in 5 nanometer intervals using a methanol blank using the spectrophotometer.

### ***Spectrum Stability Over Time***

The effect of time on anthocyanin stability was studied at five different pH's. Five buffers were prepared: pH 1.0, 2.0, 3.0, 4.0, and 5.0. Preparation of each buffer is shown in Table 3. Measurements were ensured to be  $\pm 0.05$  the target pH. Anthocyanins were extracted from the *Aronia* sample using the Standard Extraction Method as previously described. Half a milliliter of the extract was added to each of the 24.5 mL buffers from pH 1.0 to 5.0. Using plastic 1 cm cuvettes and the spectrophotometer, the absorbance was

measured at 510 nm for each of the five solutions. To observe the impact of time on peak anthocyanin content, measurements were taken on the first day (Day 0) as well as Days 2, 4, 6, and 14. After spectrophotometric measurement, each buffered solution was transferred back from the cuvette to its original beaker. Beakers were then covered with Parafilm and stored in a dark location at room temperature.

### ***Equation Development***

Using the cyanidin 3-galactoside standard, a concentration curve was created from which anthocyanin content of *Aronia* samples could be calculated spectrophotometrically. To create the curve, five different concentrations were created using pH 1.0 buffer and measured using the spectrophotometer: 0.005 mg/mL, 0.01 mg/mL, 0.015 mg/mL, 0.02 mg/mL, and 0.025 mg/mL. Two milliliters of each concentration were transferred to a plastic cuvette and measured at 510 nm by the spectrophotometer. Absorbance readings were then plotted in Microsoft Excel and a linear regression formula was created. This was used for calculations of *Aronia* anthocyanin content using the developed method. Three *Aronia* samples were tested by three quantification techniques: the developed method, the pH Differential method, and HPLC.

The anthocyanin extraction technique for the developed method followed the Standard Extraction Method as previously described. The extract was filtered through a 0.2  $\mu\text{m}$  luer-lock syringe filter. Of the filtered extract, 0.1 mL was added to 4.9 mL of pH 1.0 buffer as designed in Table 3. This was done to dilute the sample to keep absorbance readings within an acceptable range of the spectrophotometer. The spectrophotometer

was zeroed with a blank cuvette of methanol and absorbance readings at 510 nm were recorded. With output data collected from the spectrophotometer, anthocyanin content in each sample was calculated using the cyanidin 3-galactoside concentration curve and reported as cyanidin 3-galactoside equivalents (C3Gal Equivalents).

Comparison of the developed method to an established method was of interest. The pH Differential method was carried out according to Lee et al.<sup>28</sup> A buffer with a pH of 1.0 was created as designed in Table 3. Another buffer with a pH of 4.5 was created using 27.25 grams of sodium acetate in 490 mL of distilled water with 10 mL of 12 N HCl. The standard *Aronia* extraction procedure was conducted as described in the previous section. Upon completion of extraction, the combined extract was filtered through a 0.2  $\mu\text{m}$  luer-lock syringe filter. Of the filtered extract, 0.1 mL of aliquot was added to both 4.9 mL of pH 1.0 buffer and 4.9 mL of pH 4.5 buffer. This was done to dilute the sample to keep absorbance readings within an acceptable range of the spectrophotometer. The spectrophotometer was zeroed with a blank cuvette of methanol and absorbance readings at 510 nm and 700 nm were recorded. The pH Differential equation could then be used to determine anthocyanin content.<sup>28</sup> Each sample was tested in triplicate.

An HPLC method for anthocyanin determination in *Aronia* was also completed. This method used a Waters High Performance Liquid Chromatography system. It was done for comparison to the developed method and pH Differential method. The Waters HPLC consisted of a 1525 Binary pump, 717plus Autosampler, and 2487 Dual Absorbance Detector. The column used was a Luna® Omega 5  $\mu\text{m}$  Polar C18 100Å LC Column 250 x 4.6 mm. The standard *Aronia* extraction procedure was conducted as

described in the previous section. The combined aliquot was then filtered with 0.2  $\mu\text{m}$  luer-lock syringe filter and 1 milliliter was transferred to the test vial.

For the HPLC mobile phase, a gradient elution required 2 different solutions to be used with the binary pump. A 0.5% phosphoric acid water solution (mobile phase A) along with 50% acetonitrile, 48% water, 1.5% acetic acid, 0.5% phosphoric acid solution (mobile phase B) were used. Both mobile phases were placed in a Sonicator bath for 20 minutes to ensure no bubbles were present. A flow rate of 1 ml/min was used with following elution conditions: 0-10% B 0-1 minutes; 10-50% B 1-18 minutes; 50-90% B 18-22 minutes; 90-10% B 22-22.1 minutes; 10% B 22.1-25 minutes.

A 10-minute re-equilibration period with mobile phase B ensured sample was fully flushed from the system. These three *Aronia* samples were tested in triplicate. Detection with dual-absorbance detector was completed at 510 nm. Anthocyanin content was quantified with Empower software provided with the HPLC system. Quantification was completed using linear regression analysis of cyanidin 3-galactoside standard, the primary anthocyanin in *Aronia*. Standard curves for both HPLC and the developed method were created using the same cyanidin 3-galactoside standard solution.

## **Factors Impacting Anthocyanin Content**

### ***Effects of Harvesting Time on Anthocyanin Content***

Anthocyanin content in *Aronia* was determined using the developed method as previously described. Each grower provided samples that were collected over the course of the harvest season. Every sample was tested in triplicate using the developed method and plotted to express the change in anthocyanin content over time. After measurement,



sample groupings were determined to identify trends in anthocyanin development. Based upon these groupings, further analysis was carried out considering average and standard deviation.

### ***Impact of Location***

The impact of growing location on maximum anthocyanin content in Viking *Aronia* was of interest. Of the nine growers that submitted *Aronia* samples, seven submitted a common variety, Viking. Using the Standard Extraction Method as previously described, the developed method was used to quantify anthocyanin content in these berries. The maximum anthocyanin content was then plotted by growing location via Microsoft Excel Maps. This was done to study the effect of location on maximum anthocyanin content.

### ***Difference Between Variety***

There were twelve total samples provided from Grower 6 containing two *Aronia* varieties: Viking and McKenzie. Due to the common location from which these berries were grown, a comparison on variety could be attempted. The Standard Extraction Method was used to extract anthocyanin from these samples. Anthocyanin content was then calculated using the developed method. Samples from both varieties were then plotted to show differences between Viking and McKenzie variety *Aronia* for Grower 6. Standard deviation was included through error bars to show variability.

### ***Effects of Weather Conditions***

Polyphenolic content in *Aronia* has been shown to be affected by weather conditions.<sup>29</sup> Over the course of the growing season, the impact of five different factors on anthocyanin content were considered: mean temperature, mean maximum temperature, mean minimum temperature, precipitation, and latitude. To determine the impact of each, weather data was collected from weather.com. Accumulating data for mean temperature, mean maximum temperature, and mean minimum temperature was collected 90 days prior to when maximum anthocyanin content in Viking *Aronia* was reached. Precipitation data in the one week prior to each sample harvest was compiled. Latitude data was also collected for each growing location. This data was used to discover trends in anthocyanin content and the effect of weather conditions in each region.

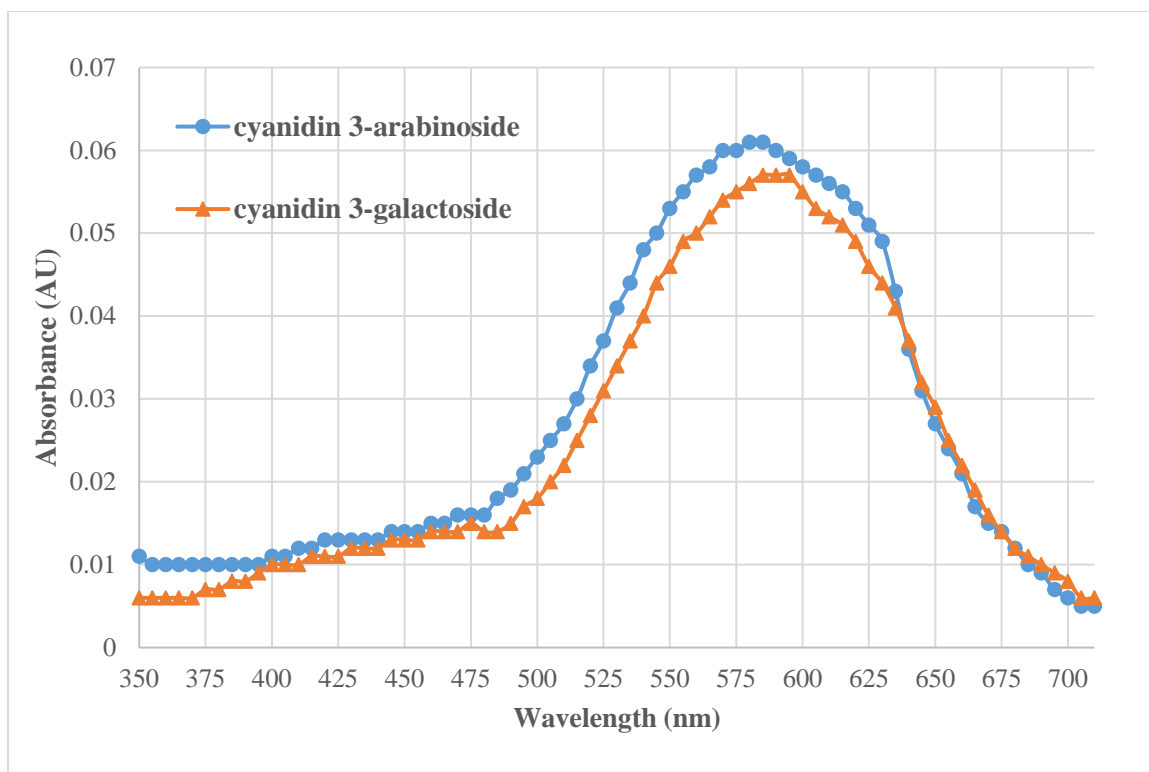
## CHAPTER 4. RESULTS AND DISCUSSION

### **New Analytical Method**

In order to develop a new analytical method based on the light absorption properties of anthocyanins, spectra from two anthocyanin standards, cyanidin 3-galactoside and cyanidin 3-arabinoside, were recorded. The effects of pH on the spectra were also studied. Later, stability of the standards over time was investigated. Finally, a simple linear equation was developed to estimate the anthocyanin content in *Aronia*.

### ***Light Absorption Spectrum of Anthocyanins***

A wavelength scan of cyanidin 3-galactoside and cyanidin 3-arabinoside standards in 100% methanol was performed. Fig. 5 depicts typical spectra from the concentration of 0.001 mg/mL. The cyanidin 3-arabinoside peaked at 580-585 nm, and the cyanidin 3-galactoside at 585-595 nm. However, absorbance readings were inconsistent, and the spectra were not reproducible. In addition, the color of the solution varied with each preparation. This is all due to the pH variability of methanol. Therefore, the effect of pH on the spectra had to be studied for further development.



**Fig. 5: Absorbance spectra of cyanidin 3-galactoside and cyanidin 3-arabinoside in (0.001 mg/mL) 100% methanol in the range of 350 to 710 nm.**

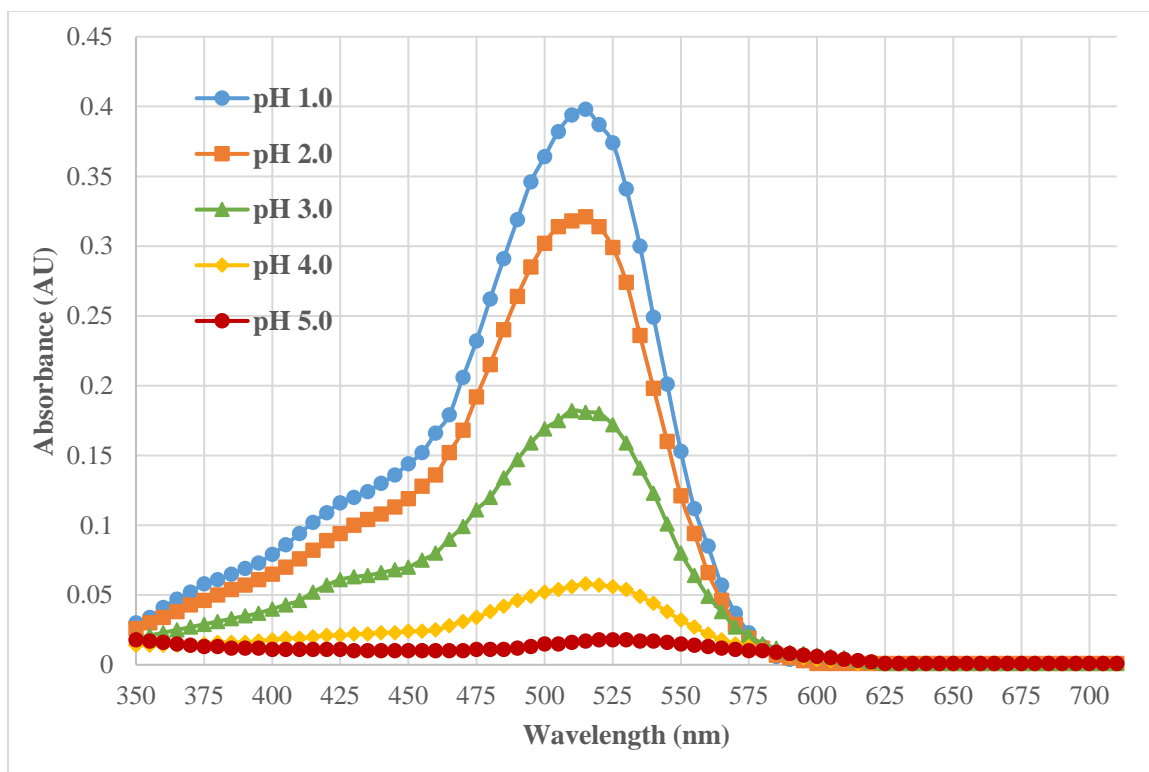
### *Effects of pH on the Spectrum Profile*

The effect of pH, in the range of 1.0 to 5.0, on the cyanidin 3-galactoside absorbance is shown in Fig. 6. Each pH had a maximum absorbance in the same wavelength range. Peak absorbance was highest at the pH of 1.0 but declined as pH increased. There was a noticeable difference between the overall spectrum at pH 1.0 and pH 2.0, with a difference in peak absorbance. At the pH of 1.0 a peak of 0.394 AU was observed at 510 nm, compared to a peak of 0.318 AU for pH 2.0. Initially for pH 1.0, absorbance increased slightly from 350 nm to 450 nm before beginning to rapidly increase towards 510 nm. The absorbance then fell very rapidly until a wavelength of

about 600 nm (0.002 AU). At this point, low absorbance was observed to be stable until 710 nm. A similar trend was noticed for the pH 2.0. Absorbance increased slightly from 350 nm to 450 nm before a rapid increase to the peak of 510 nm. After peaking, absorbance then quickly fell to 0.001 AU at 600 nm.

This overall trend was also observed in the cyanidin 3-galactoside sample at a pH 3.0, however, the peak absorbance at 510 nm was observed to be lower at 0.182 AU. Initially, absorbance increased from 350 nm (0.019 AU) to 450 nm (0.070 AU) and then increased rapidly up to the peak absorbance. At this point, a quick decline was observed until 590 nm (0.009 AU). Absorbance then stabilized until 710 nm, reaching a minimum of 0.001 AU. At the pH 4.0, maximum absorbance was lower than the pH 3.0, with an observed reading of 0.056 AU at 510 nm. At this pH, the absorbance gradually increased from 350 nm to about 400 nm. A greater increase was noticed at 460 nm until the peak at 510 nm. The absorbance then fell from this wavelength to 590 nm where a slow decline in absorbance was observed until 710 nm (0.01 AU). Finally, at a pH of 5.0, absorbance declined only slightly from 350 nm (0.018 AU) to 400 nm (0.011 AU) and then remained stable from 400 nm to 500 nm (0.015 AU). The peak absorbance at this pH was essentially non-existent, with a peak range of 520 nm to 530 nm observed (0.018 AU).

In general, absorbance trends were the same at each pH measured; even though differences in peak absorbance were discovered they could presumably be due to anthocyanin conformation changes. This could be the impact of pH on the anthocyanin molecular structure. As the solvent becomes more basic, the anthocyanin moves to the colorless pseudo-base.<sup>14</sup>



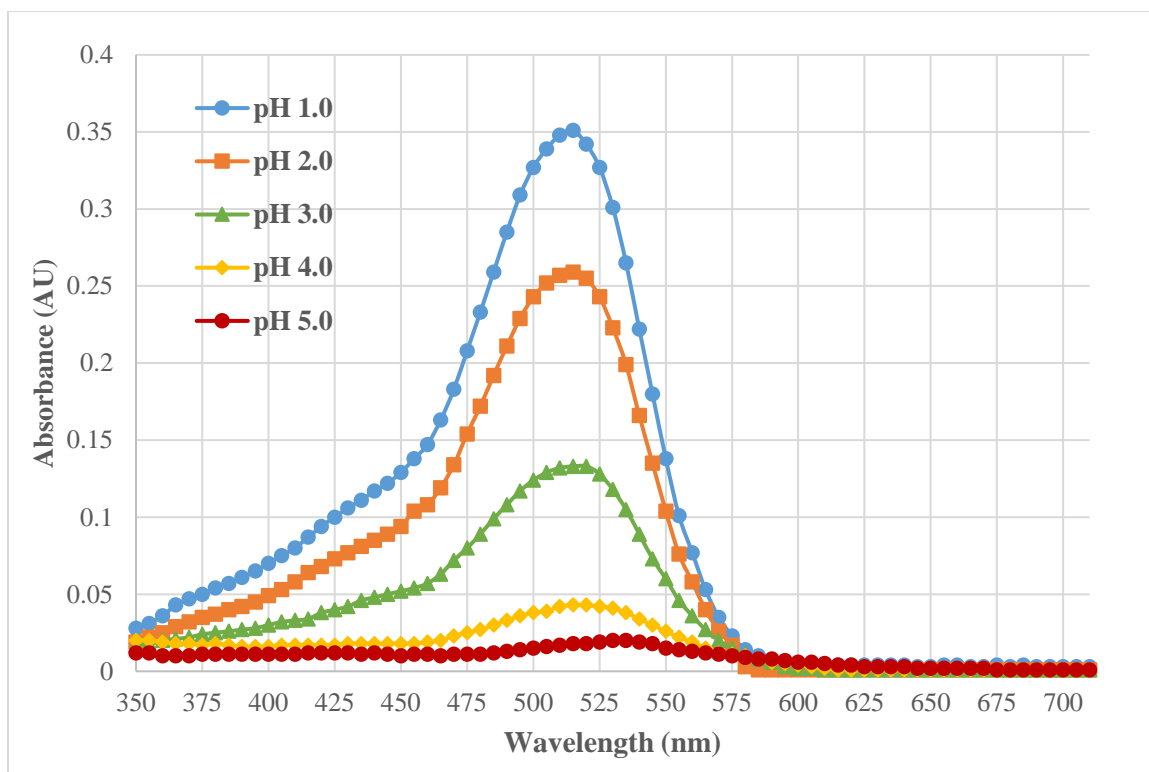
**Fig. 6: Effect of pH on the cyanidin 3-galactoside spectra at pH 1.0, 2.0, 3.0, 4.0, and 5.0. Peak absorbance was higher at more acidic pH conditions.**

There were clear visual differences among the solutions at each pH (Fig. 7). The pH 1.0 sample was bright red, but as the pH became more basic the color changed to a dull purple. The fading of color is due to the loss of the flavylium cation form of anthocyanin leading to the pseudo-base.<sup>14</sup> Thus, it appears that anthocyanins must be held in an acidic buffer for proper spectrophotometric quantification.



**Fig. 7: Effect of pH on anthocyanins. From left to right: pH 1.0, 2.0, 3.0, 4.0, and 5.0. Color fades as pH becomes less acidic.**

The cyanidin 3-galactoside and cyanidin 3-arabinoside had an identical wavelength of maximum absorbance (Fig. 6 and Fig. 8). It appears that the sugar type does not influence the absorbance properties of the anthocyanins. Though there are four different anthocyanins present in *Aronia*, the major two compounds had the same absorbance peaks. Therefore, the rest of the investigation was focused on cyanidin 3-galactoside. Other research also substantiates this evidence.<sup>23</sup>



**Fig. 8: Effect of pH on cyanidin 3-arabinoside spectra at pH 1.0, 2.0, 3.0, 4.0, and 5.0. The peak absorbance is inversely correlated with pH.**

### *Spectrum Stability over Time*

The *Aronia* solution was created in buffers of pH 1.0, 2.0, 3.0, 4.0, and 5.0.

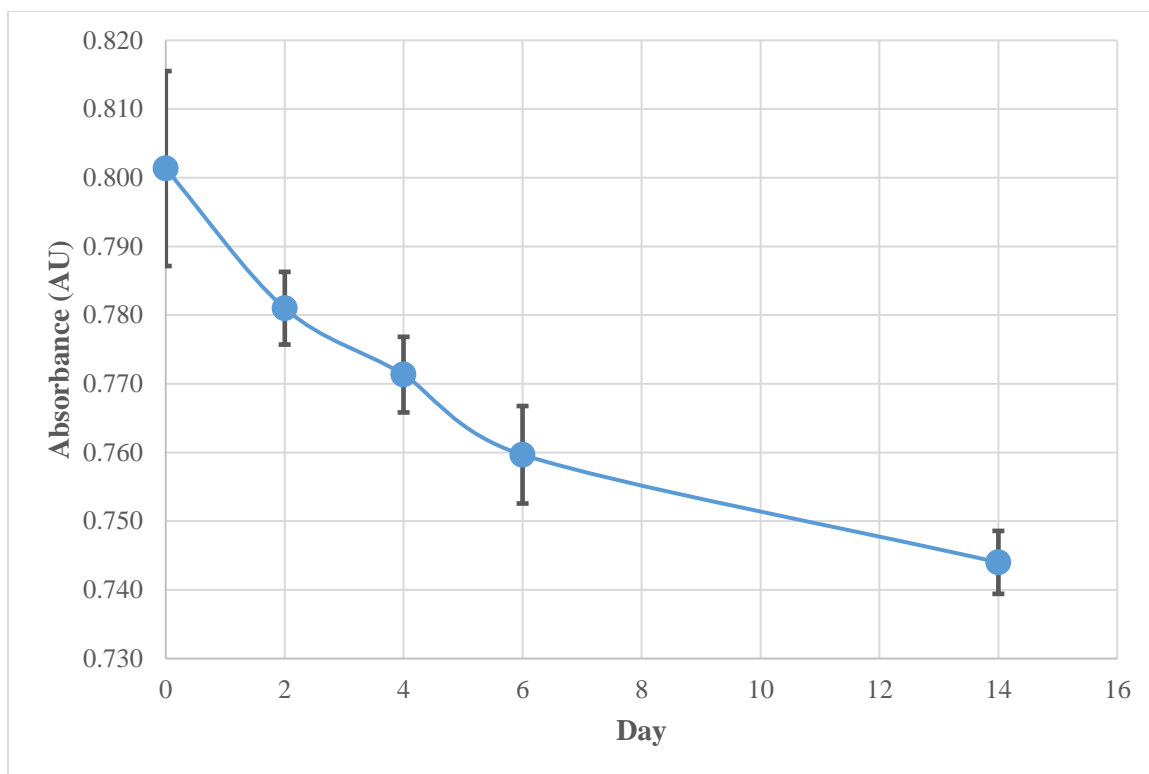
Because the anthocyanin molecule is not stable, the effects of time and pH were studied.

The stability over a 14-day period is shown in Fig. 9 through Fig. 14.

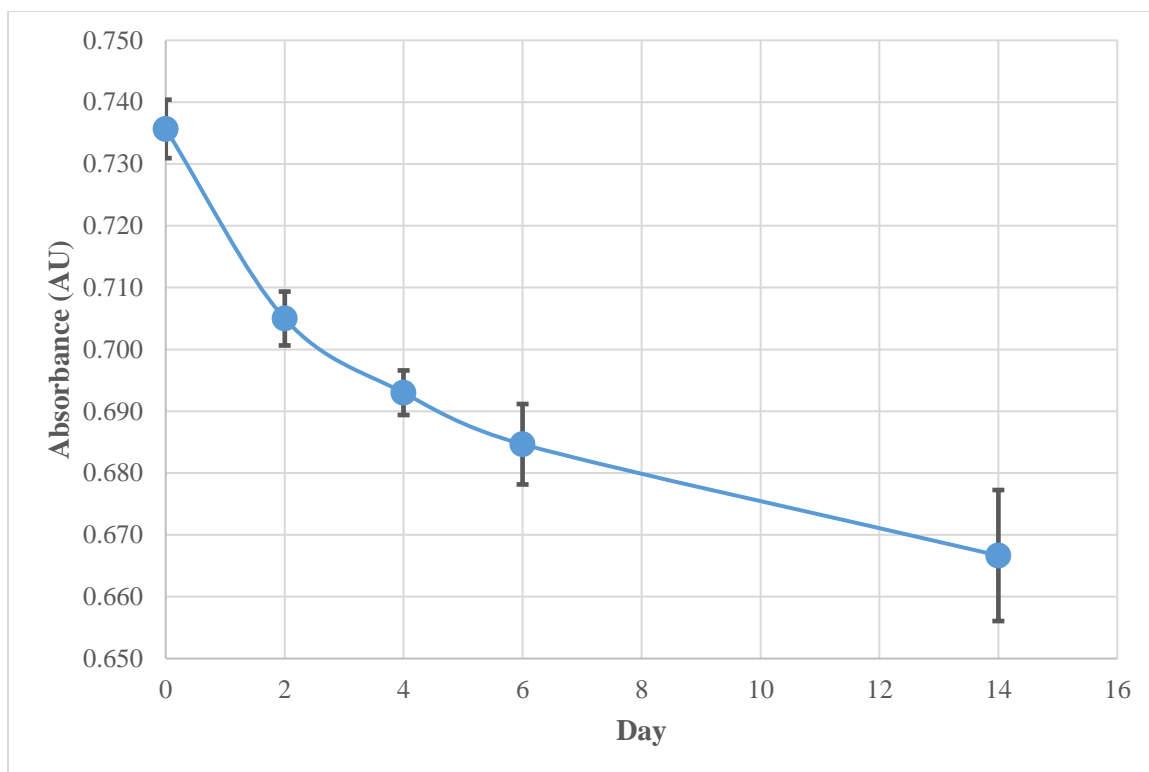
Change in peak absorbance at 510 nm for each pH was recorded in triplicate on Day 0, Day 2, Day 4, Day 6, and Day 14. The average values are 0.801, 0.781, 0.771, 0.760, and 0.744 AU, respectively, for the pH 1.0 *Aronia* solution. The absorbance decreased drastically from Day 0 through Day 2, but decreased steadily on later days. A similar trend is observed at the pH 2.0. The average measured values are 0.736, 0.705,



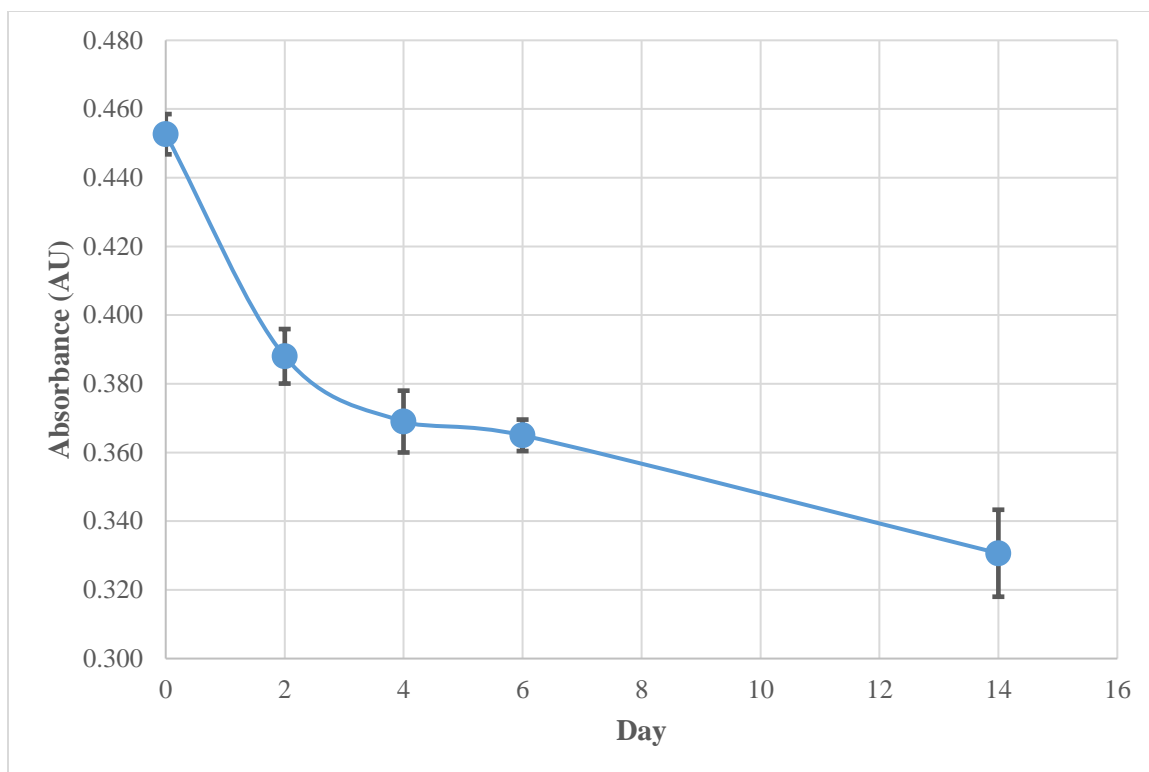
0.693, 0.685, and 0.667 AU, in the same sample order. For the pH 3.0, there was slightly larger change in the absorbance. The measured values for peak absorbance are 0.453, 0.388, 0.369, 0.365, and 0.331 AU. For pH 4.0, they were 0.183, 0.151, 0.141, 0.135, and 0.124 AU at each day, respectively. Only a moderate decline in the peak absorbance over time was observed. Finally, at the pH 5.0, the observed peak absorbance measurements were 0.101, 0.081, 0.075, 0.068, and 0.068, respectively. A subtle decrease in peak absorbance was observed at the pH 5.0, and by Day 14 the absorbance reading remained unchanged. In Fig. 9 through 14, standard deviations are shown through error bars.



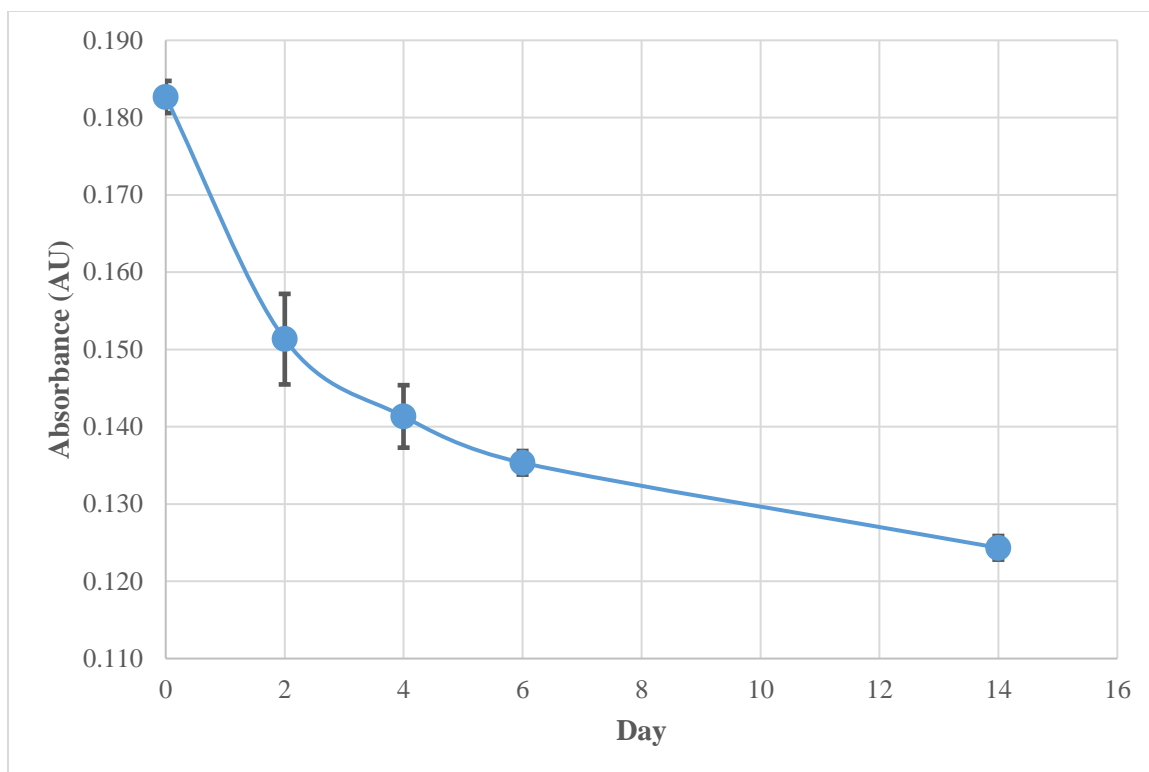
**Fig. 9: Change in the maximum absorbance over time at 510 nm at pH 1.0.  
Maximum absorbance declined steadily from Day 0 to Day 14.**



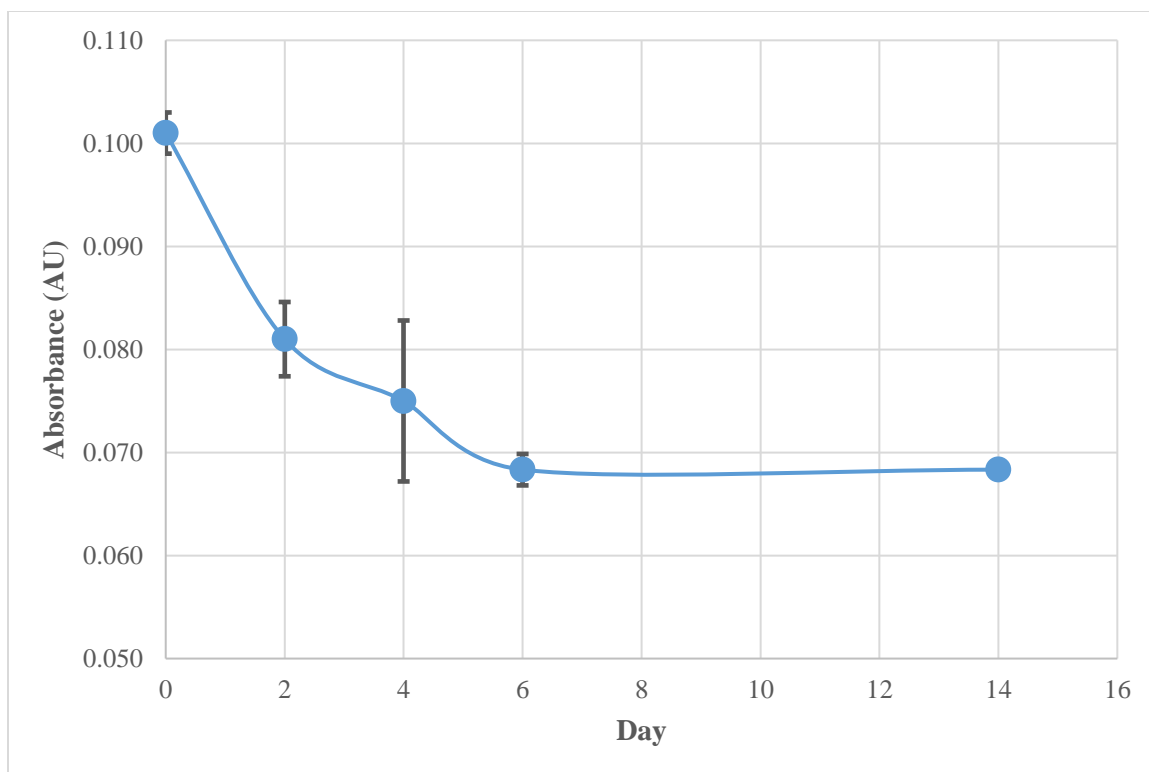
**Fig. 10: Change in the maximum absorbance over time at 510 nm at pH 2.0. There was a rapid decline from Day 0 to Day 2, followed by a slow decrease thereafter.**



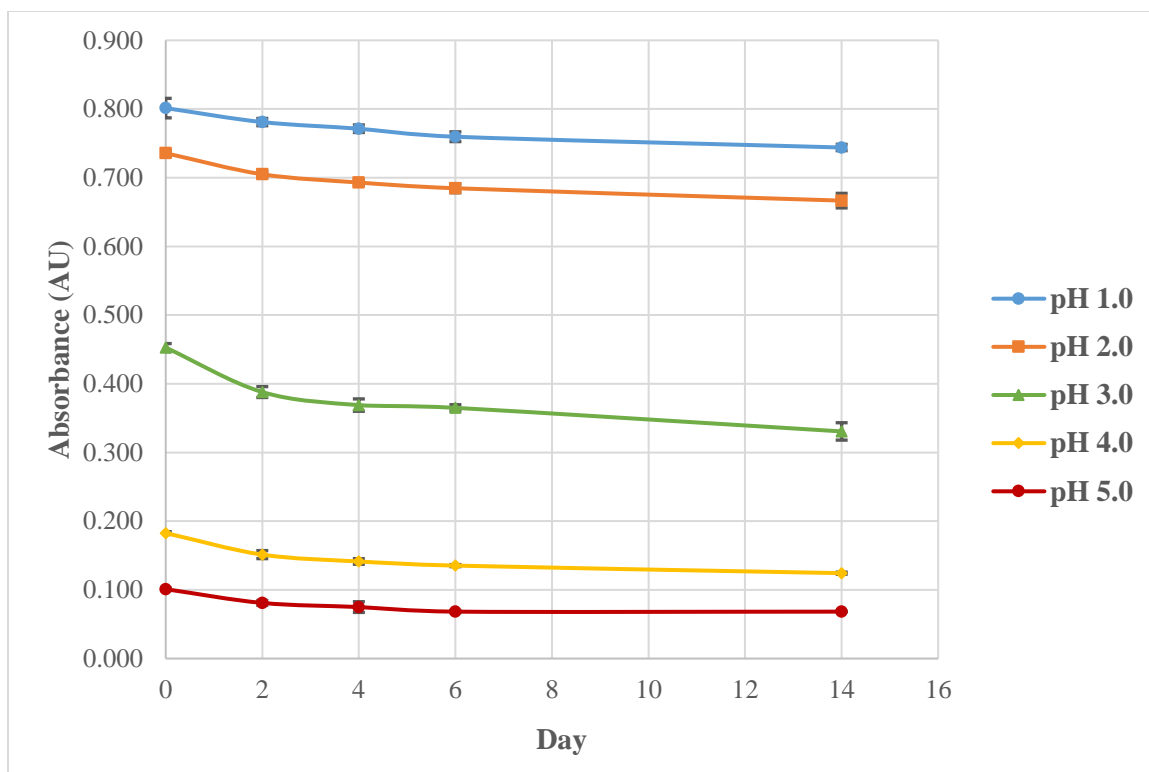
**Fig. 11: Change in the maximum absorbance over time at 510 nm at pH 3.0. A quick decline from Day 0 to Day 2 followed by a steady decline from Day 2 to Day 14 thereafter.**



**Fig. 12: Change in the maximum absorbance over time at 510 nm at pH 4.0. A rapid decline was first noticed on Day 2, followed by a slow decline thereafter.**



**Fig. 13: Change in the maximum absorbance over time at 510 nm at pH 5.0. After an initial decrease from Day 0 and Day 6, the absorbance then remained stable until Day 14.**



**Fig. 14: Change in the average maximum absorbance at each pH solution as a function of time. Lower pH's gave higher maximum absorbances.**

Maximum absorbance fell steadily over the course of the trial. This effect shows the anthocyanin profile in *Aronia* is not stable and changes over time in solution. Therefore, anthocyanin solutions might not accurately represent the total anthocyanin content beyond Day 0. The anthocyanins in *Aronia* appear to degrade in similar fashion over time regardless of pH. Table 4 shows the percent decrease in the absorbance at each pH and time. Degradation as a function of percent occurred most rapidly from Day 0 to Day 2 in each buffered solution. A larger decrease in absorbance by percent also occurred at less acidic pH's. This can be explained by a similar decrease in the total absorbance for each buffered solution, as well as lower initial absorbances measured on Day 0 at these pH's.

**Table 4: Percent decrease in the absorbance (AU) at 510 nm between days. The largest percent decrease was observed between Day 0 and Day 2, regardless of pH.**

<b>Percent Decrease in Absorbance by Day</b>					
<i>Day</i>	<i>pH 1.0</i>	<i>pH 2.0</i>	<i>pH 3.0</i>	<i>pH 4.0</i>	<i>pH 5.0</i>
<b>Day 0-2</b>	2.5	4.2	14.3	17.2	19.8
<b>Day 2-4</b>	1.2	1.7	4.9	6.6	7.4
<b>Day 4-6</b>	1.5	1.2	1.1	4.2	8.9
<b>Day 6-14</b>	2.1	2.6	9.4	8.1	0.0

Table 4 highlights the cumulative percent decrease in absorbance from Day 0 to Day 14. Over the course of the trial, there was a 7.2, 9.4, 27.0, 31.9, and 32.3% cumulative decrease in the absorbance at pH 1.0, 2.0, 3.0, 4.0, and 5.0, respectively. Greater stability over time was observed with a decrease in the pH. Research on the stability of anthocyanin is mostly limited to neutral pH's, however, one study reported



similar findings with cyanidin 3-glucoside.<sup>30</sup> In this study, it was discovered that there was a greater percent decrease in maximum absorbance at higher pH over time.<sup>30</sup> This suggests there is an effect of oxidation on anthocyanins, and an effect of pH on anthocyanin stability. Overall, it appears that anthocyanins in *Aronia* could be best preserved at more acidic pH conditions, giving insight to storage limitations of *Aronia* products stored at room temperature.

**Table 5: Percent absorbance (AU) loss of *Aronia* solution from Day 0 to Day 14. Higher pH solutions experienced a greater percentage loss.**

<b>Cumulative Percent Decrease: Day 0 – Day 14</b>				
<i>pH 1.0</i>	<i>pH 2.0</i>	<i>pH 3.0</i>	<i>pH 4.0</i>	<i>pH 5.0</i>
7.2	9.4	27.0	31.9	32.3

### ***Equation Development***

As shown in Fig. 5, the two major anthocyanins (cyanidin 3-galactoside and cyanidin 3-arabinoside) had identical absorption profiles. This revelation enabled us to quantify the total anthocyanins using one single wavelength instead of the multiple regression approach. A simple equation was developed to calculate total anthocyanin:  $y = 47.923x + 0.037$ .

Table 6 shows the comparison between these three anthocyanin quantification methods: HPLC, pH Differential, and the developed method for three *Aronia* samples. HPLC gave the highest results of the methods tested and the pH Differential method gave the lowest results of the methods tested. Tukey's multiple comparison test determined that there was a significant difference between anthocyanin results concluded by each

method for the three samples tested ( $p < 0.05$ ). The developed method was beneficial due to simplicity, speed, reproducibility, and cost compared to the HPLC and pH Differential methods.

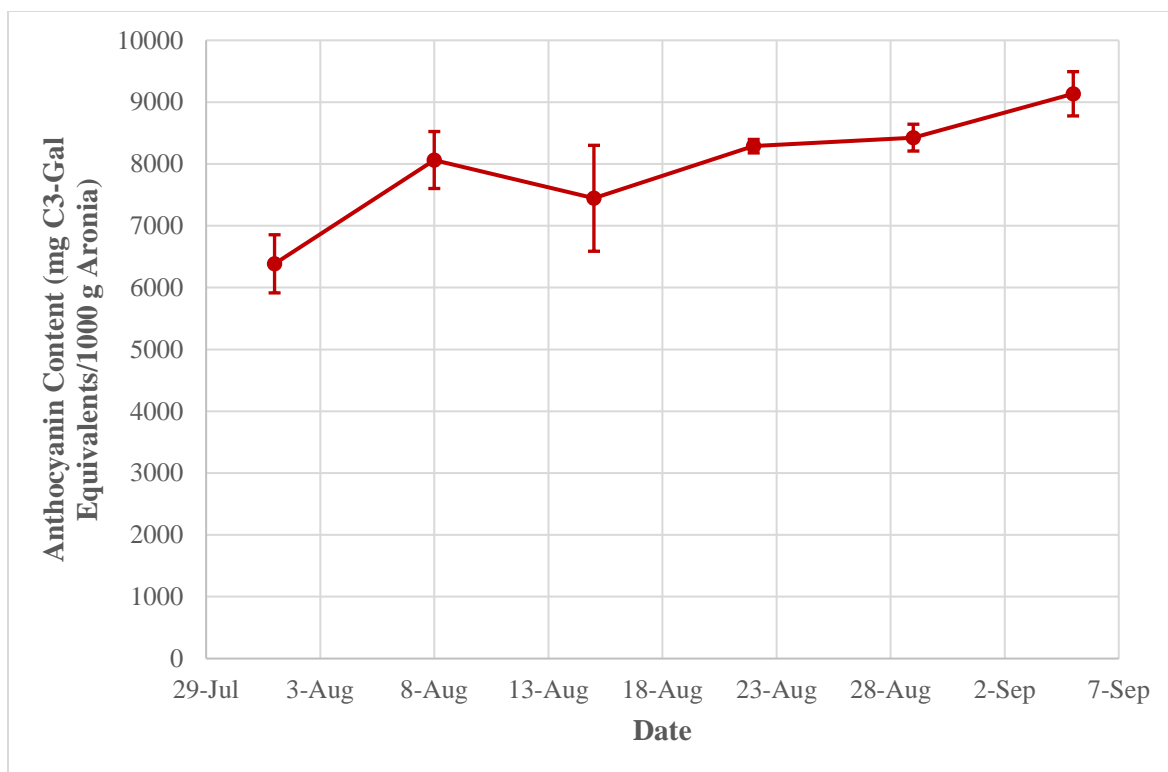
**Table 6: Difference in anthocyanin amounts (C3Gal Equivalents) based on the method of quantification. HPLC gave consistently higher amounts than the developed Standard Curve Method and pH Differential protocol. The letters a, b, and c from the Tukey's multiple comparison test suggest the significant differences among the three methods.**

	HPLC	Standard Curve	pH Differential
<b>Sample 1</b>	4517 ± 102 a	3280 ± 219 b	2588 ± 145 c
<b>Sample 2</b>	10713 ± 342 a	8535 ± 366 b	6372 ± 245 c
<b>Sample 3</b>	9859 ± 596 a	8756 ± 89 b	6486 ± 38 c

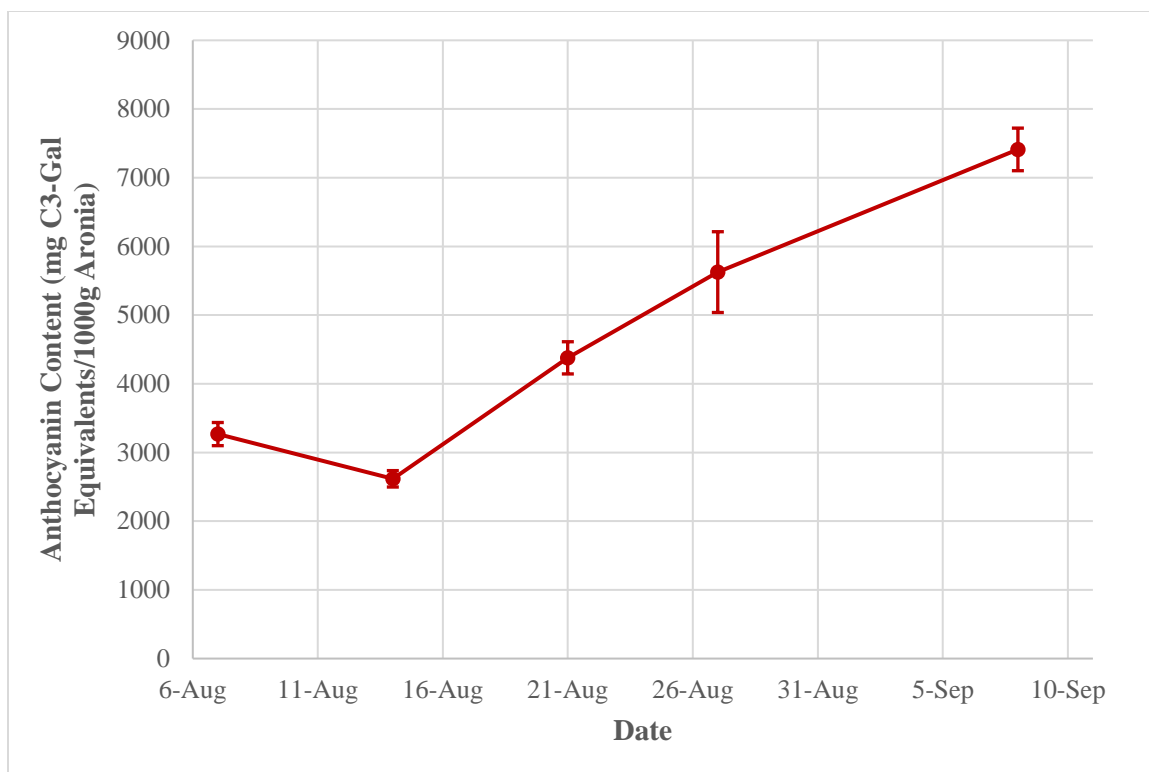
## Impact of Growing Conditions on Anthocyanin Content

### *Effects of Harvesting Time on Anthocyanin Content*

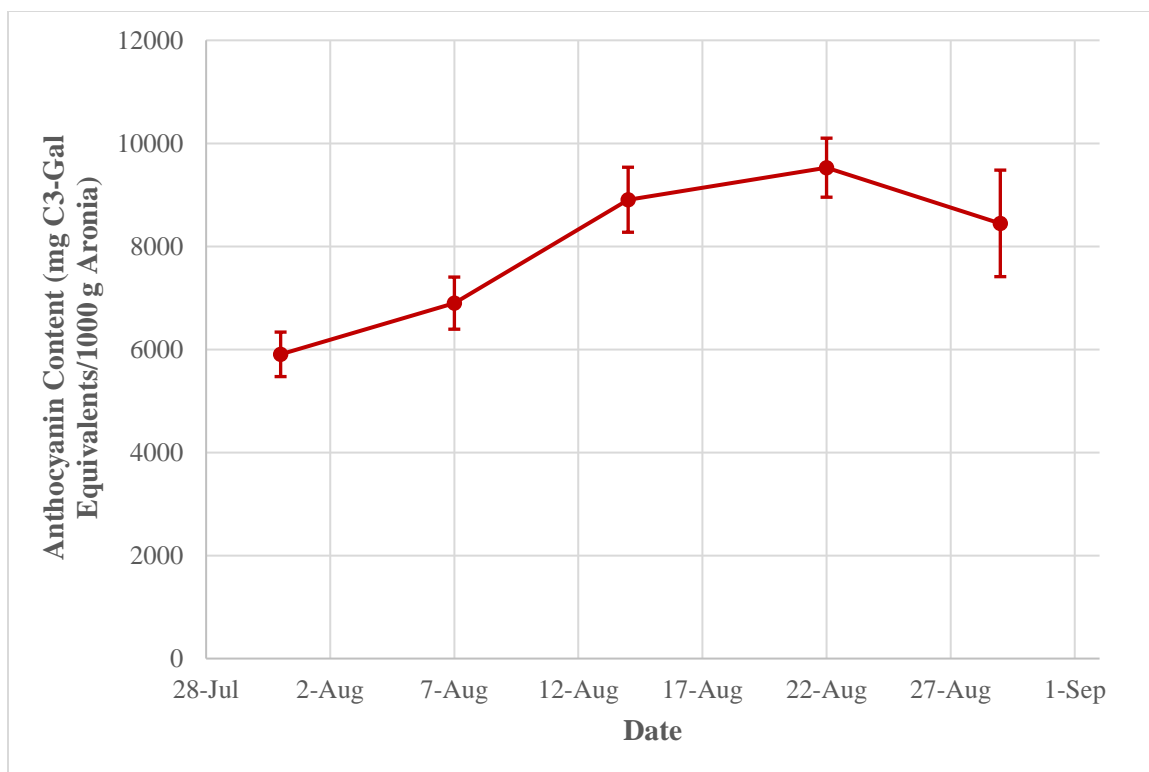
Differences in the anthocyanin amounts in *Aronia* over the growing season were discovered. Fig. 15 through Fig. 25 portray the changes in samples from nine growers. The anthocyanin amount was calculated using the developed standard curve method. Standard deviation is shown through error bars.



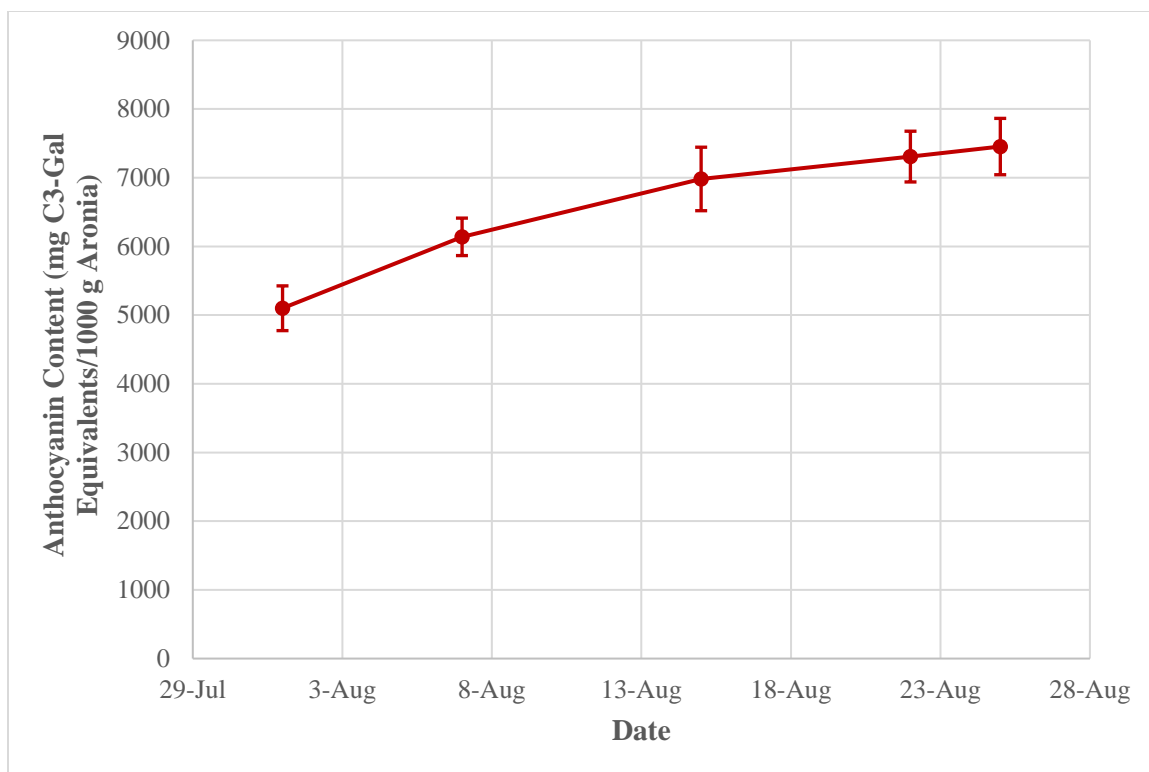
**Fig. 15:** Change in the anthocyanin content over time from the Viking *Aronia* variety (Grower 1).



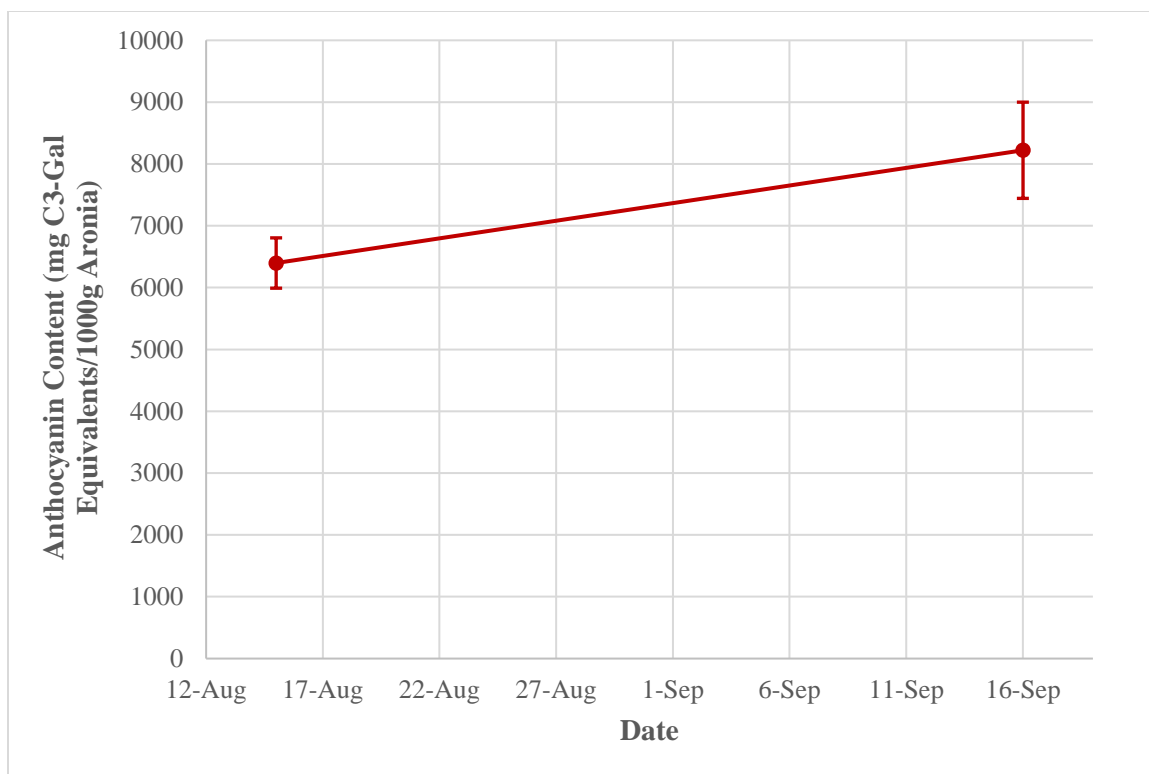
**Fig. 16:** Change in the anthocyanin content over time from the Viking *Aronia* variety (Grower 2).



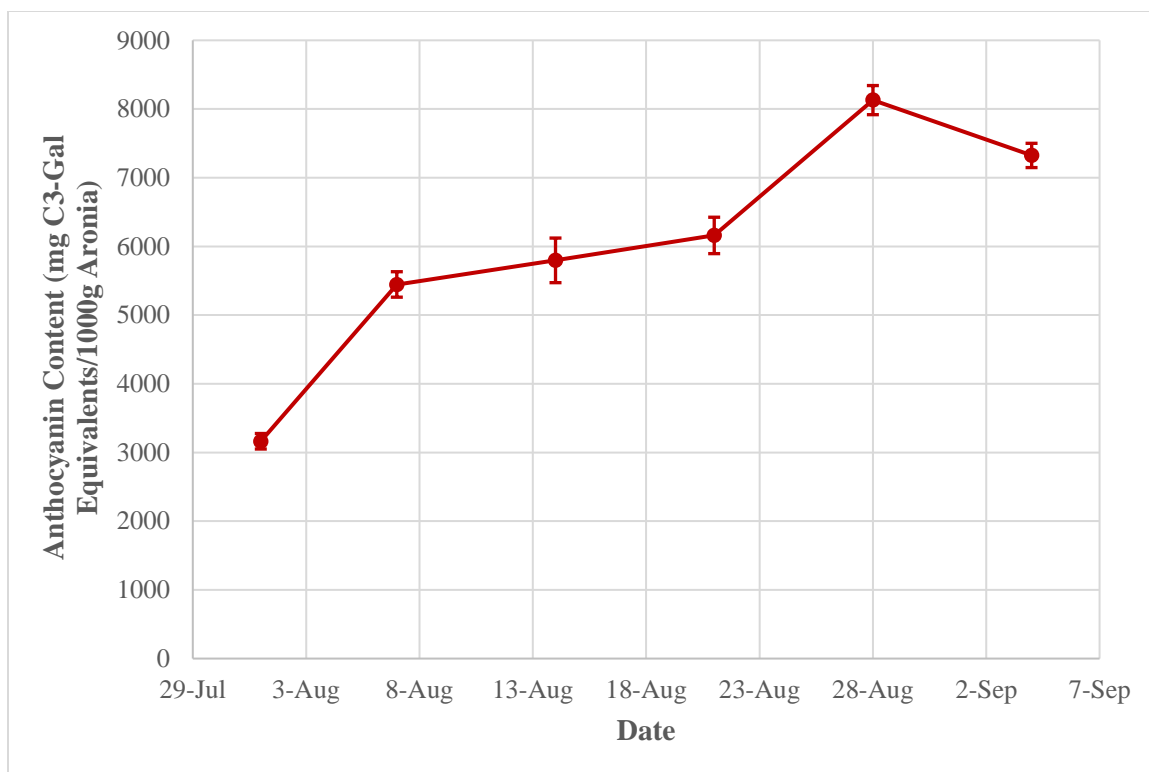
**Fig. 17: Change in the anthocyanin content over time from the Viking *Aronia* variety (Grower 3).**



**Fig. 18:** Change in the anthocyanin content over time from the Viking *Aronia* variety (Grower 4).

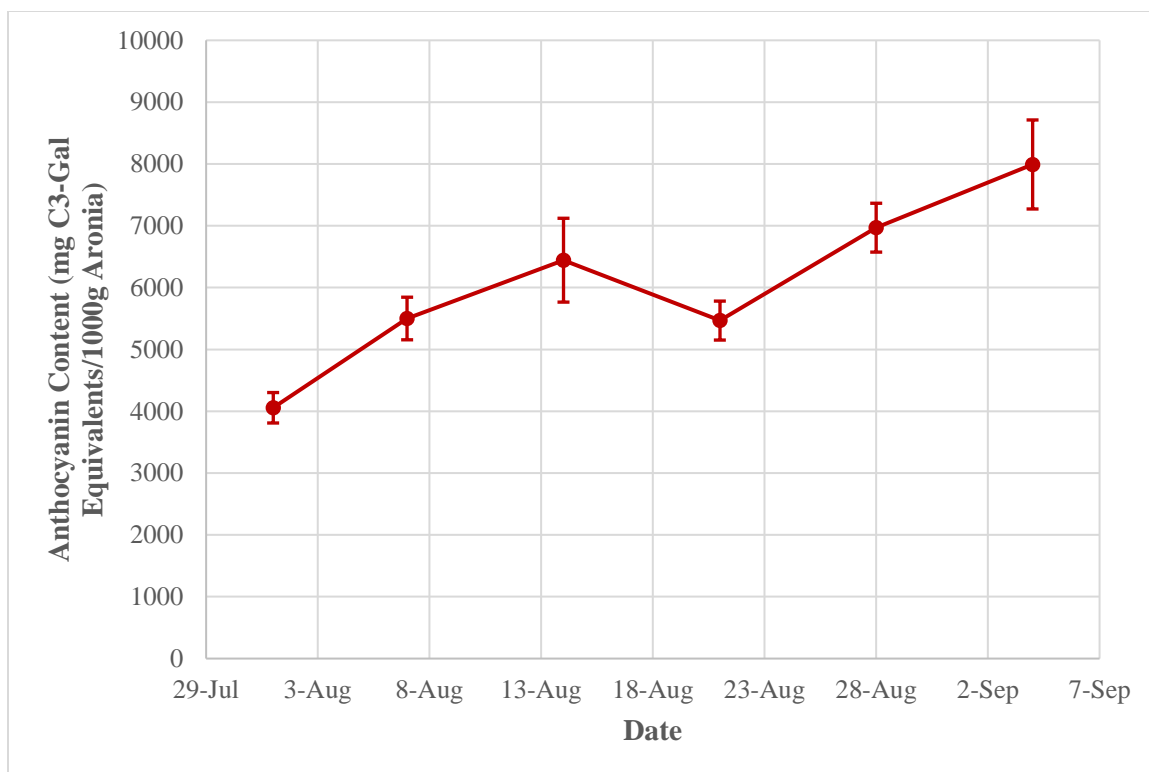


**Fig. 19: Change in the anthocyanin content over time from the Galicjanka *Aronia* variety (Grower 5).**

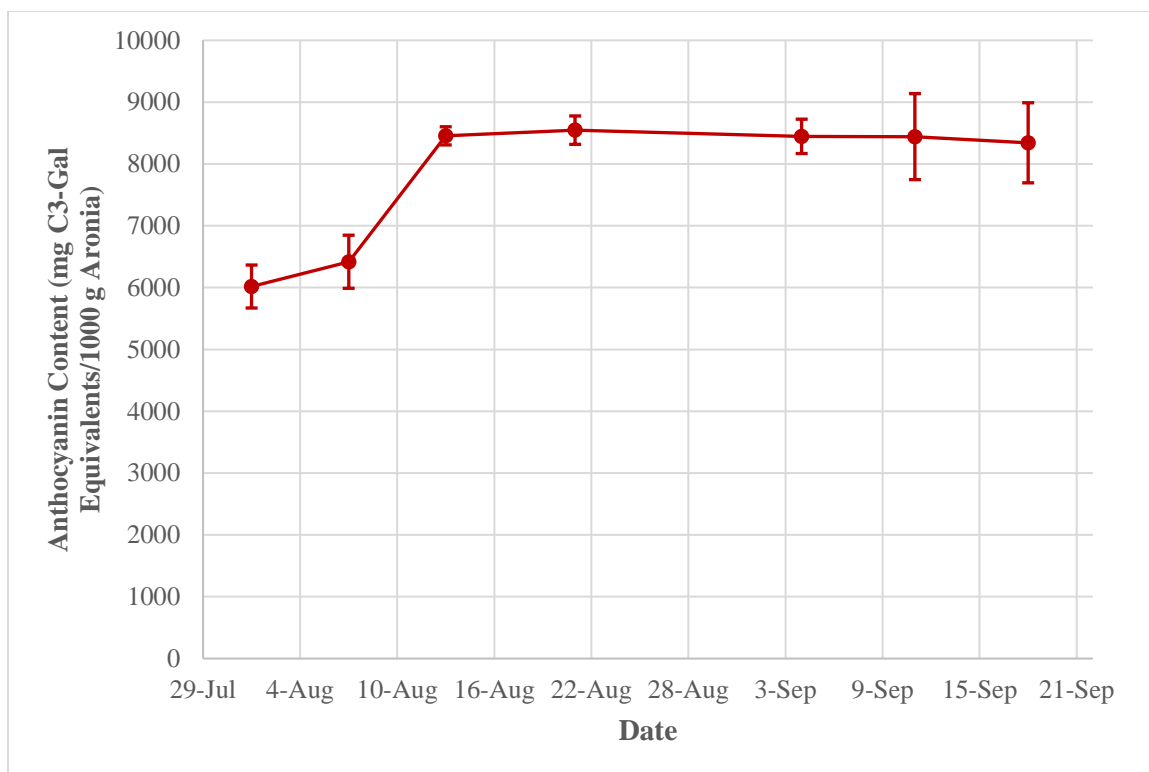


**Fig. 20:** Change in the anthocyanin content over time from the Viking *Aronia* variety (Grower 6).

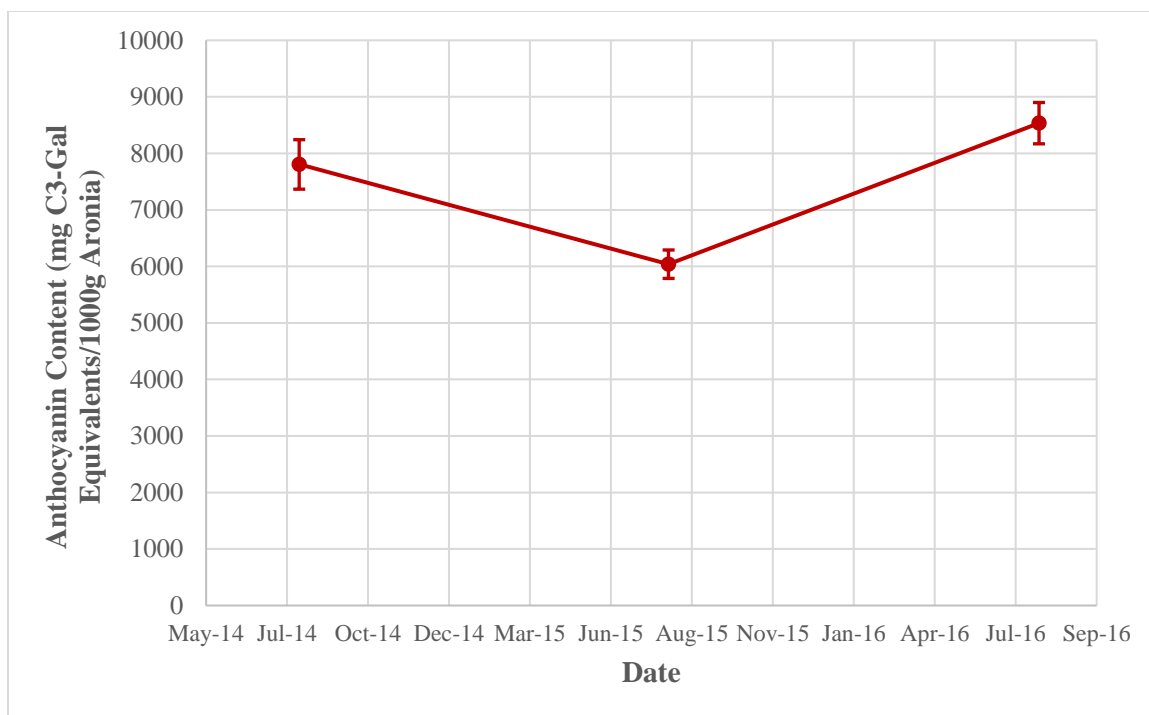




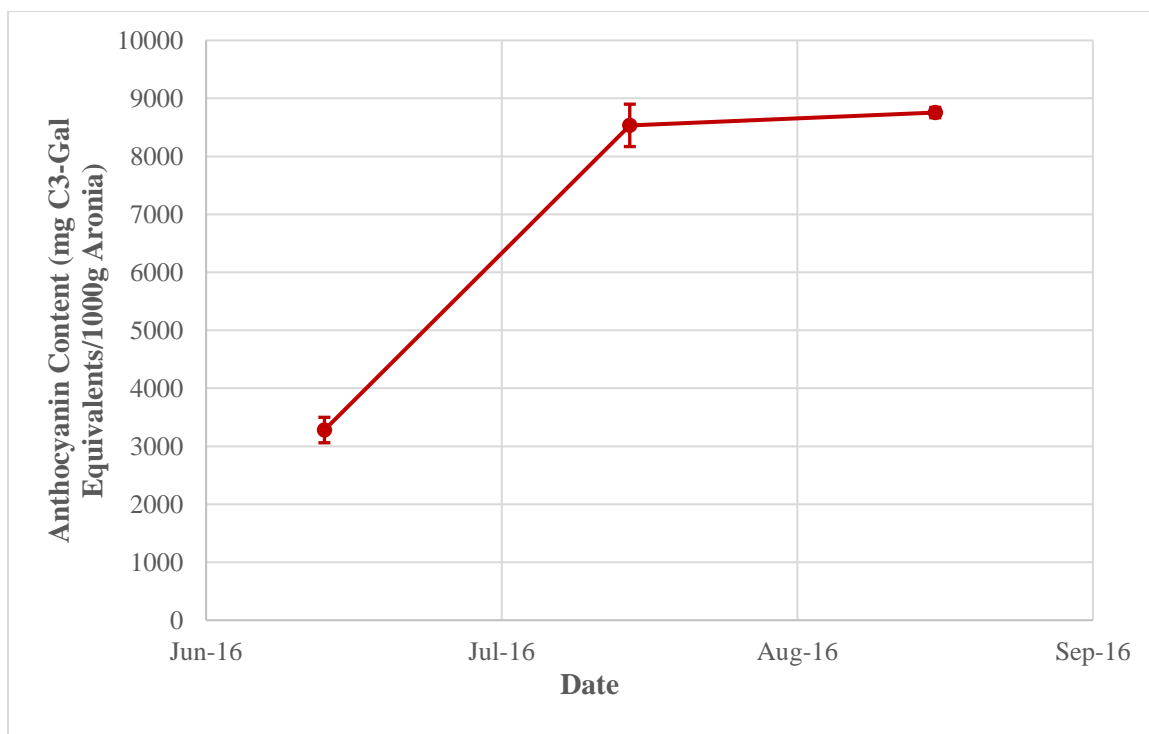
**Fig. 21:** Change in the anthocyanin content over time from the McKenzie Aronia variety (Grower 6).



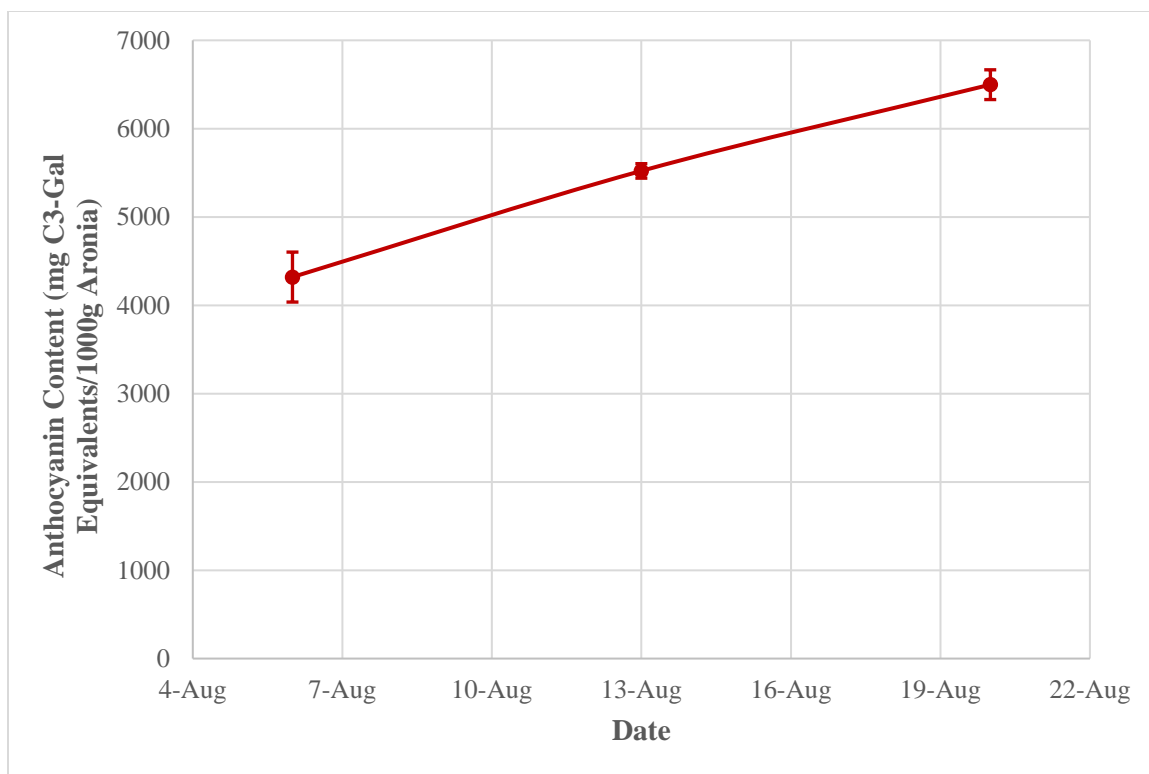
**Fig. 22:** Change in the anthocyanin content over time from the Viking *Aronia* variety (Grower 7).



**Fig. 23: Change in the anthocyanin content by year (August 2014, August 2015, and August 2016) from the Viking *Aronia* variety (Grower 8).**

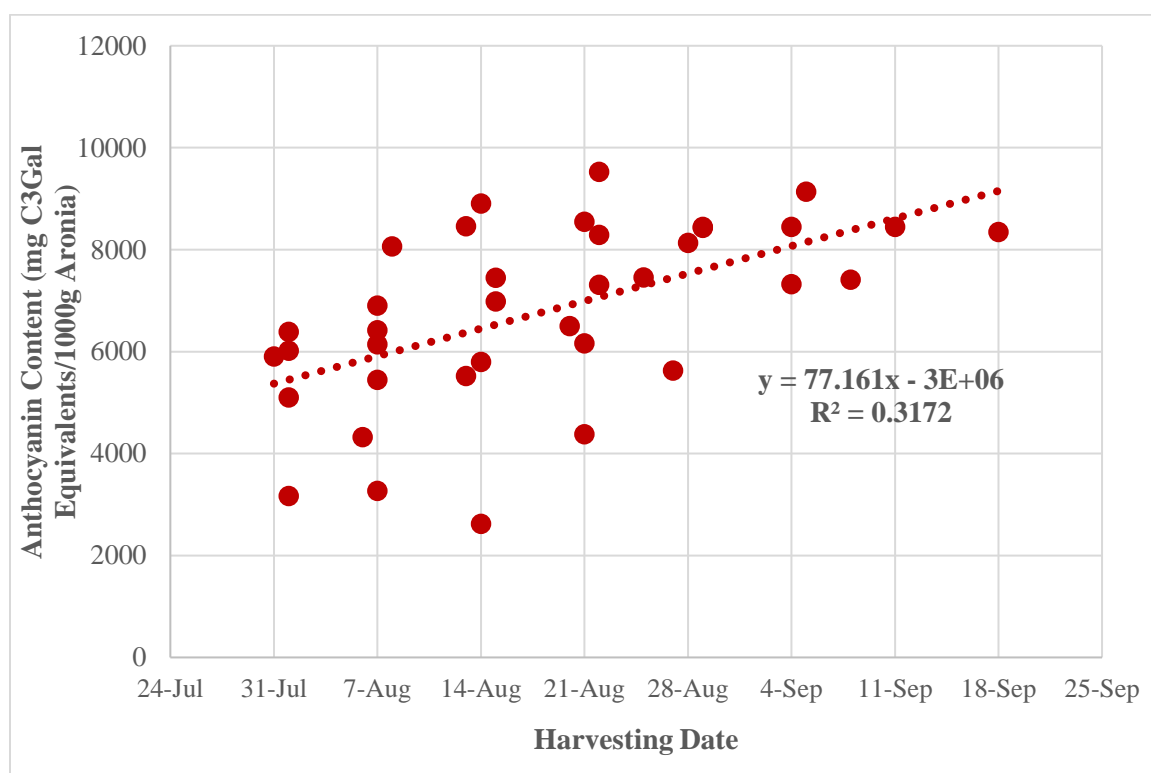


**Fig. 24: Change in the anthocyanin content by month (July, August, and September 2016) from the Viking *Aronia* variety (Grower 8).**



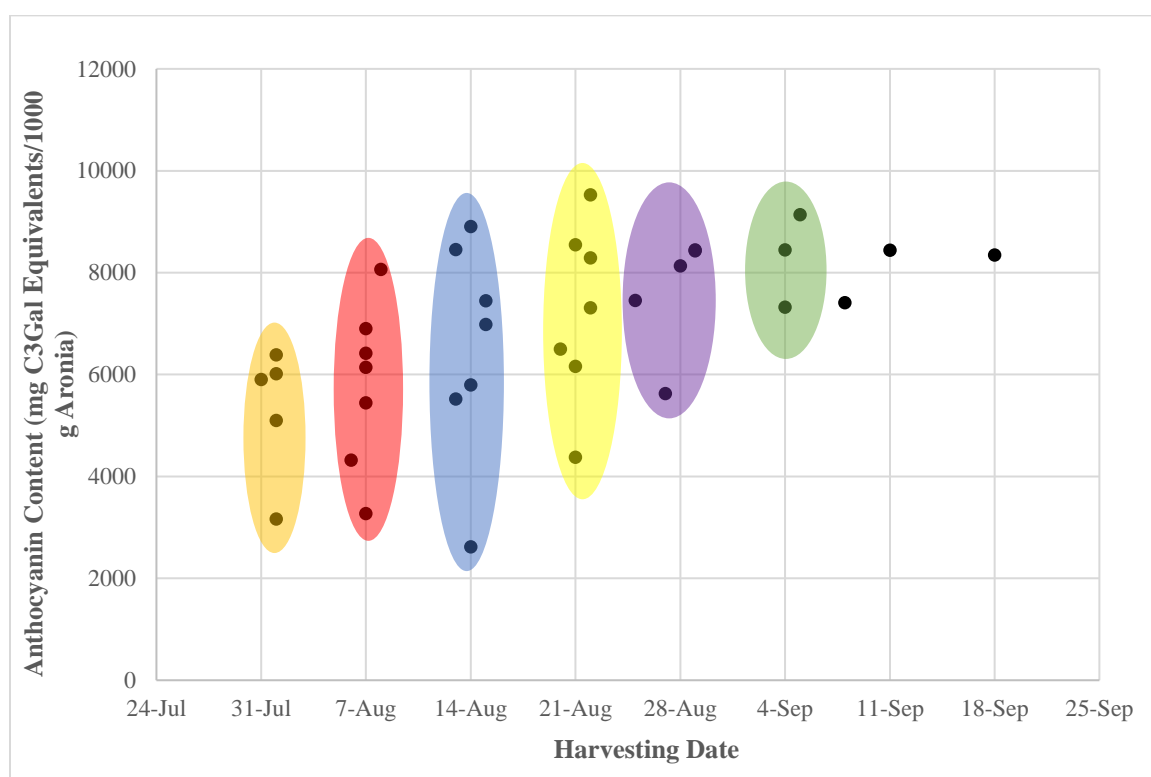
**Fig. 25: Change in the anthocyanin content over time from the Viking *Aronia* variety (Grower 9).**

The *Aronia* variety “Viking” was the majority of sample that was procured during this research. Fig. 26 highlights the amount of anthocyanin as a function of harvesting date. More interestingly, a positive correlation noticed but the  $R^2$  was around 0.32. Such low correlation could be due to the wide variety of samples sourced from different locations across the Midwest. The general increasing trend of anthocyanins amount with harvesting time clearly suggest the appropriate time for harvesting the *Aronia* berries. However, more research is needed to gain further insight.



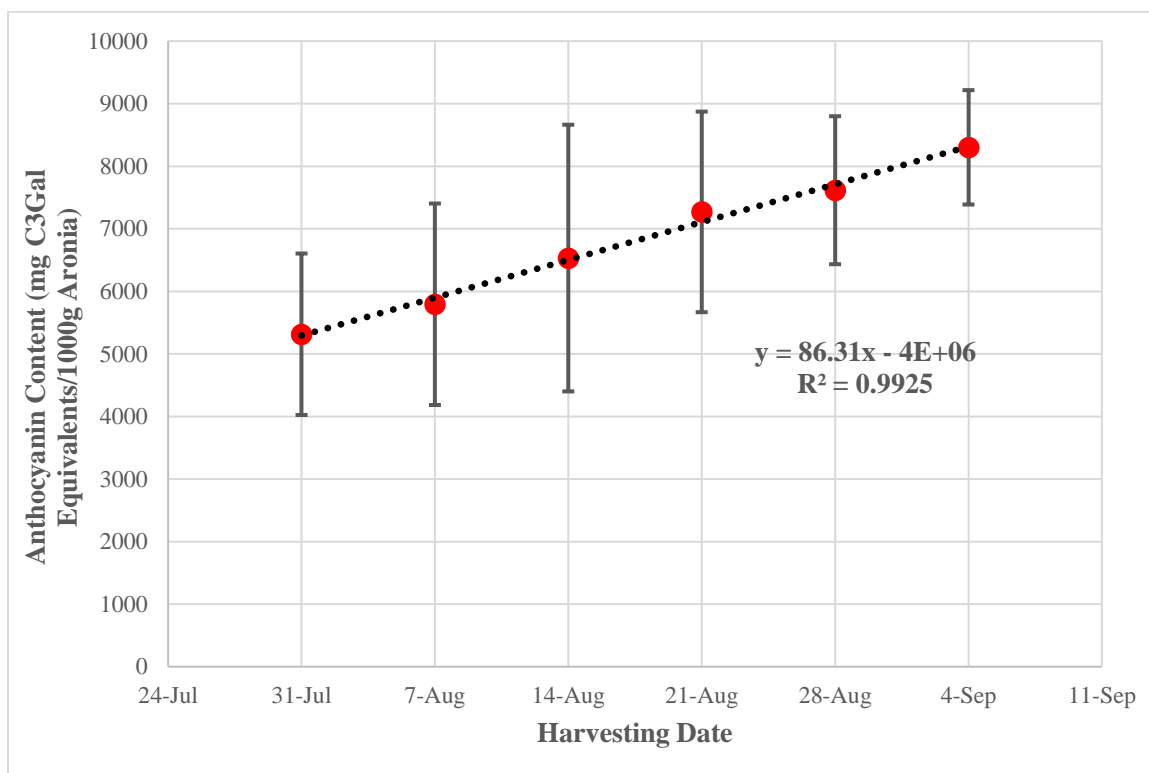
**Fig. 26: Scatter plot of anthocyanin amount as a function of harvesting date from the Viking *Aronia* samples collected in this study. In general, a positive correlation between the harvesting date and anthocyanin content is noticed.**

Due to a large available set of Viking variety *Aronia*, further analysis through groupings could be conducted. Six groupings were assessed based on harvest time in cluster intervals (Fig. 27). Group one through six were samples harvested from July 31st through August 1st, August 7th through August 8th, August 13th through August 15th, August 21st through August 22nd, August 25th through August 29th, and September 4th through September 5th, respectively. Fringe outliers at the end of the harvest season were not considered in these groups.



**Fig. 27: Groupings of each Viking *Aronia* samples based on the harvesting period: July 31<sup>st</sup> through August 1<sup>st</sup>, August 7<sup>th</sup> through August 8<sup>th</sup>, August 13<sup>th</sup> through August 15<sup>th</sup>, August 21<sup>st</sup> through August 22<sup>nd</sup>, August 25<sup>th</sup> through August 29<sup>th</sup>, and September 4<sup>th</sup> through September 5<sup>th</sup>.**

Based upon these groupings, further analysis was carried out considering average and standard deviation. The results of each six groupings are shown in Fig. 28.



**Fig. 28: Average anthocyanin content of Viking *Aronia* samples from six harvesting periods: July 31<sup>st</sup> through August 1<sup>st</sup>, August 7<sup>th</sup> through August 8<sup>th</sup>, August 13<sup>th</sup> through August 15<sup>th</sup>, August 21<sup>st</sup> through August 22<sup>nd</sup>, August 25<sup>th</sup> through August 29<sup>th</sup>, and September 4<sup>th</sup> through September 5<sup>th</sup>. The error bars indicate standard deviation.**

A positive correlation with an  $R^2$  of 0.99 was discovered for the average anthocyanin content during the harvest season for Viking *Aronia*. It appears the first week of September is the optimal harvesting time for attaining maximum anthocyanin content in Viking *Aronia* berries. It is believed that, like other fruits and berries, *Aronia* chemical composition could very well depend on numerous environmental factors such as climate and soil conditions, berry maturity, and harvest methods.<sup>29</sup> Prior studies

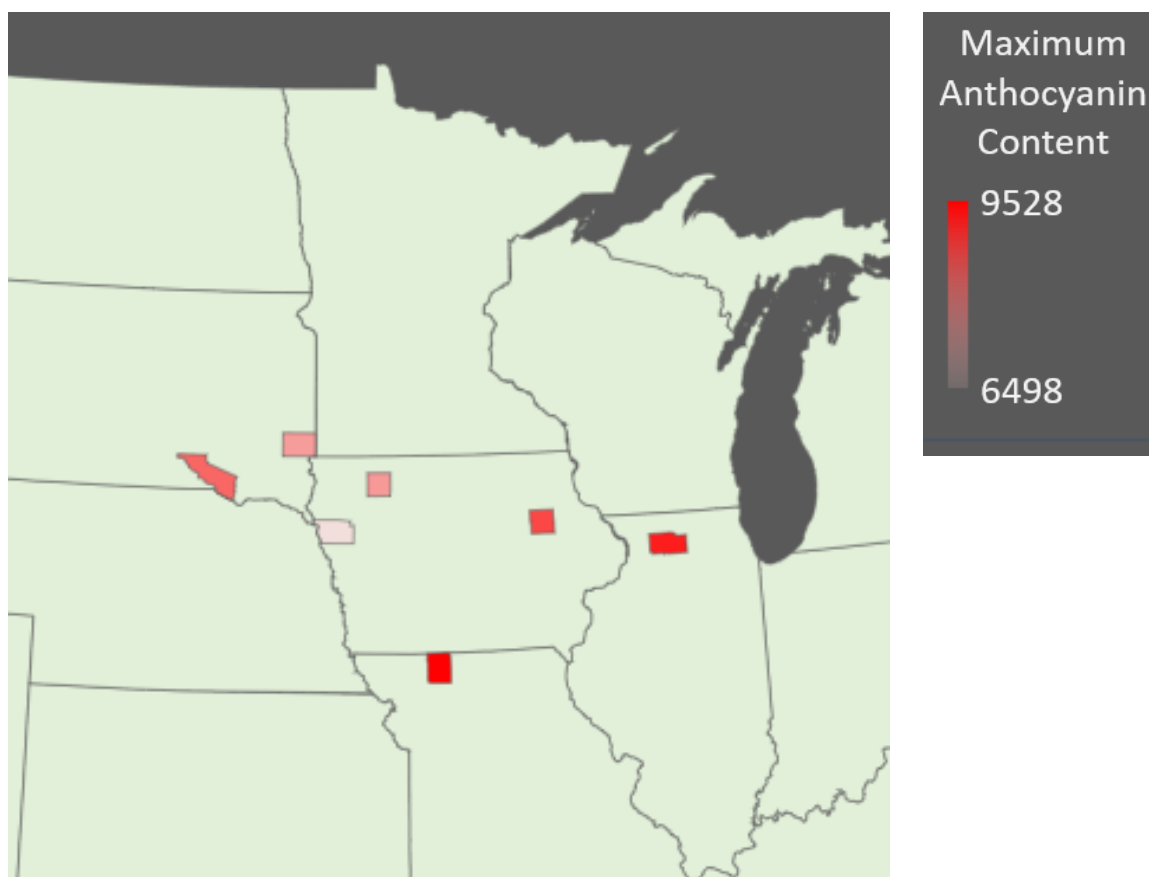
indicate that anthocyanins and hydroxycinnamic acids in *Aronia* experience the greatest changes by harvest date in comparison to other polyphenols.<sup>19</sup> Thus, an in-depth study is needed for further establishing the positive correlation obtained in this study.

Research is scarce for the effect of harvesting time on *Aronia*, however published data from other berries offer the required insights. Barberries have been found to have an increase in anthocyanin content over the course of the harvest season.<sup>31</sup> Anthocyanin concentration in *B. buxifolia* has been found to increase when fruit growth phase ends. This is due to a shift in carbon allocation from biomass-increase to the synthesis of secondary metabolites such as anthocyanins.<sup>31</sup> Thus, it appears that the anthocyanin content increases as the harvest season progresses; a similar phenomenon might be occurring with Viking *Aronia* and certainly warrants further study.

### ***Impact of Location***

Fig. 29 depicts the maximum anthocyanin content by location tested; only Viking *Aronia* was considered for consistency.





**Fig. 29: Maximum anthocyanin content by grower location. Maximum content of 9528 mg Cyanidin 3-galactoside equivalents/1000g *Aronia* was observed at the southernmost region.**

These results suggest that maximum anthocyanin content was higher in regions further south and east such as Missouri, Illinois, and Eastern Iowa. While data on the effect of location on *Aronia* is not available, research on grapes has shown that environmental conditions clearly influence the anthocyanin development. Higher average daily temperatures and an earlier accumulation of growing degree days may be related with higher fruit biomass, as well as higher soluble solids and anthocyanin content.<sup>32</sup> It has been established that temperature is a limiting factor of the rate of photosynthesis; at higher temperatures compounds produced by photosynthesis are produced at a more rapid

rate.<sup>32</sup> Thus, an increase in translocation of photoassimilates from the leaves of the plant to the berries is possible.<sup>32</sup>

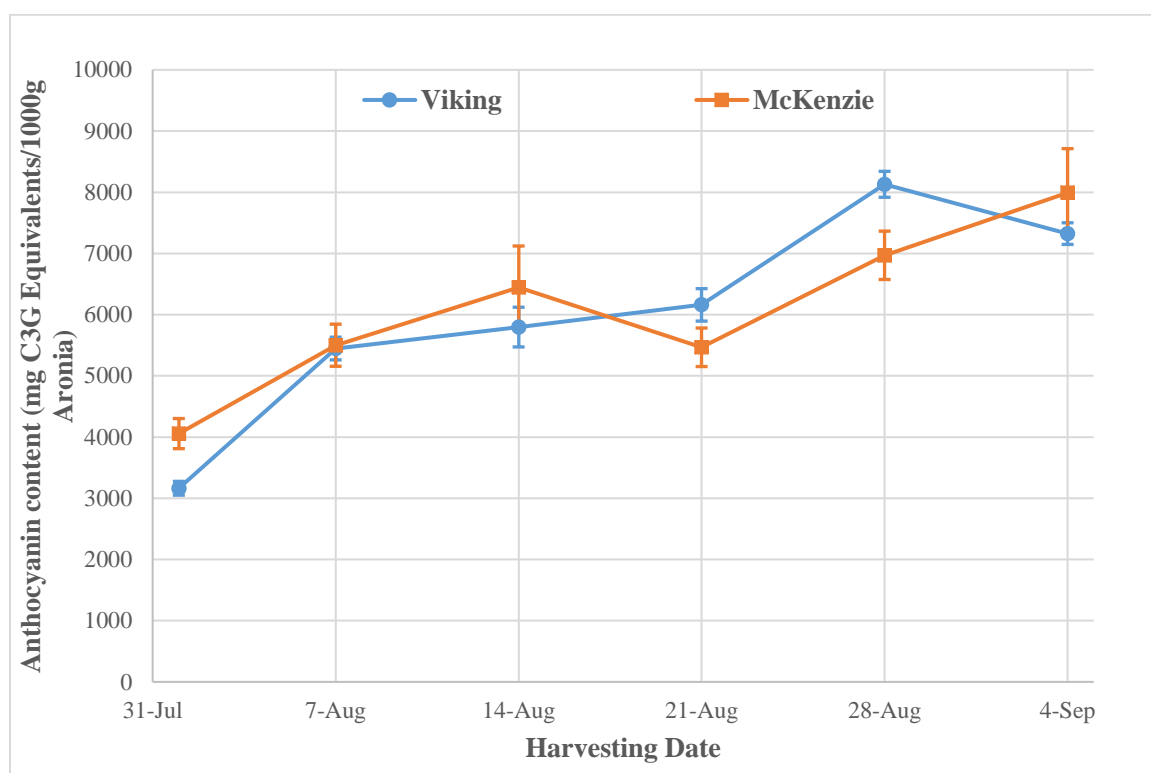
Anthocyanins are produced in the berry as protection from ultraviolet radiation. In red wine grape cultivars, anthocyanin production has been shown to increase linearly in relation to sunlight exposure.<sup>33</sup> In general, fruits exposed to high sunlight conditions typically have greater anthocyanin and polyphenolic compounds while having decreased berry weight.<sup>33</sup> Anthocyanin development likely is also be affected by temperature parameters, as seen in grapes.<sup>33</sup>

While there are theories as to what impacts anthocyanin content, some research has shown weak correlations between anthocyanin content in *Aronia* and environmental factors.<sup>29</sup> Upon parameters studied, the average seasonal temperature has been shown to have the largest correlation with anthocyanin content, with average seasonal bright sunshine also showing slight correlation.<sup>29</sup> This information indeed offers some insights as to why anthocyanin content is not static during the growing and harvesting season in *Aronia*.

### ***Differences Between Variety***

Differences in anthocyanin content by *Aronia* varieties Viking and McKenzie can be seen in Fig. 30. These samples were each provided by Grower 6. Initially, Viking *Aronia* increased significantly from Week 1 to Week 2 (3163 to 5446 mg), and then increased slightly between Week 2 and Week 3 (5797 mg) followed by moderate increase at Week 4 (6160 mg). Finally, a drastic increase was noticed between Week 4 and Week 5 (8130 mg) with a decrease at Week 6 (7324 mg).

McKenzie variety *Aronia* followed a slightly different pattern as compared with Viking variety. Initially, anthocyanin content increased from Week 1 to Week 2 (4057 mg to 5500 mg), and then again at Week 3 (6444 mg). Subsequently, a decrease in anthocyanin content was observed at Week 4 (5467 mg). However, an increase was then seen at Week 5 (6970 mg) that further continued to Week 6 with 7992 mg during the last week of harvesting.

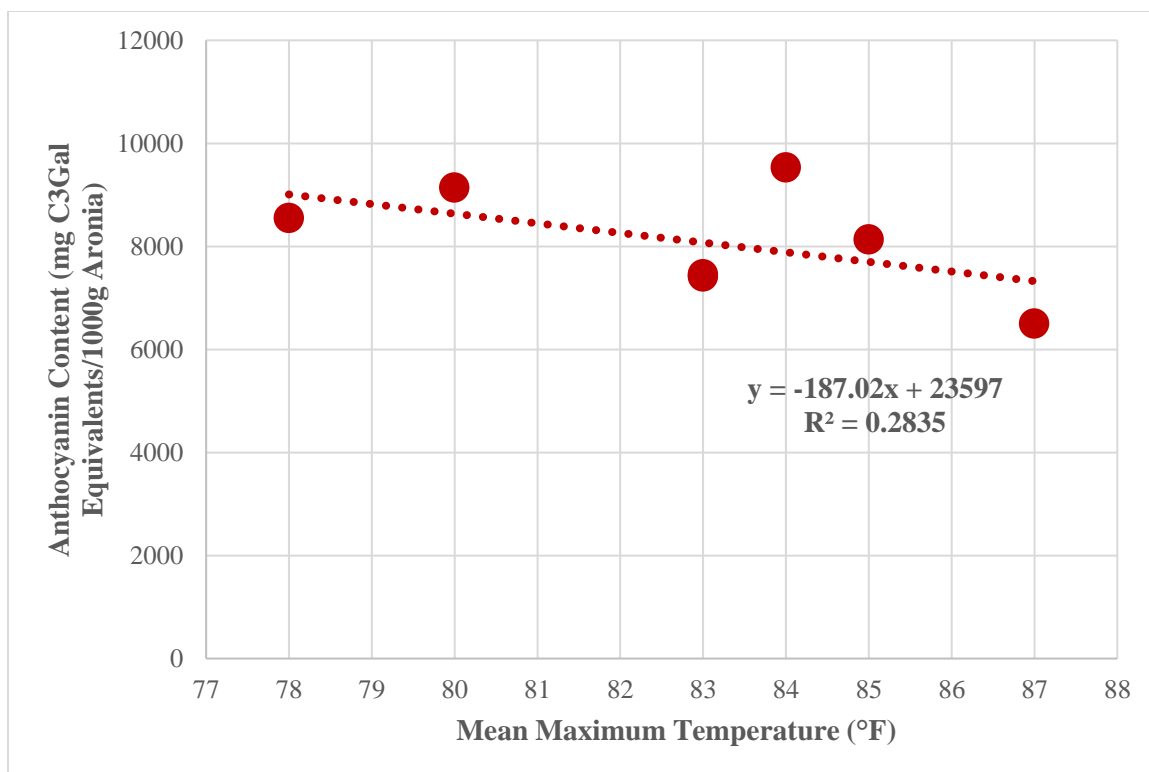


**Fig. 30: Comparison of *Aronia* varieties Viking and McKenzie in anthocyanin content by harvesting date. Both varieties were grown and harvested by Grower 6.**

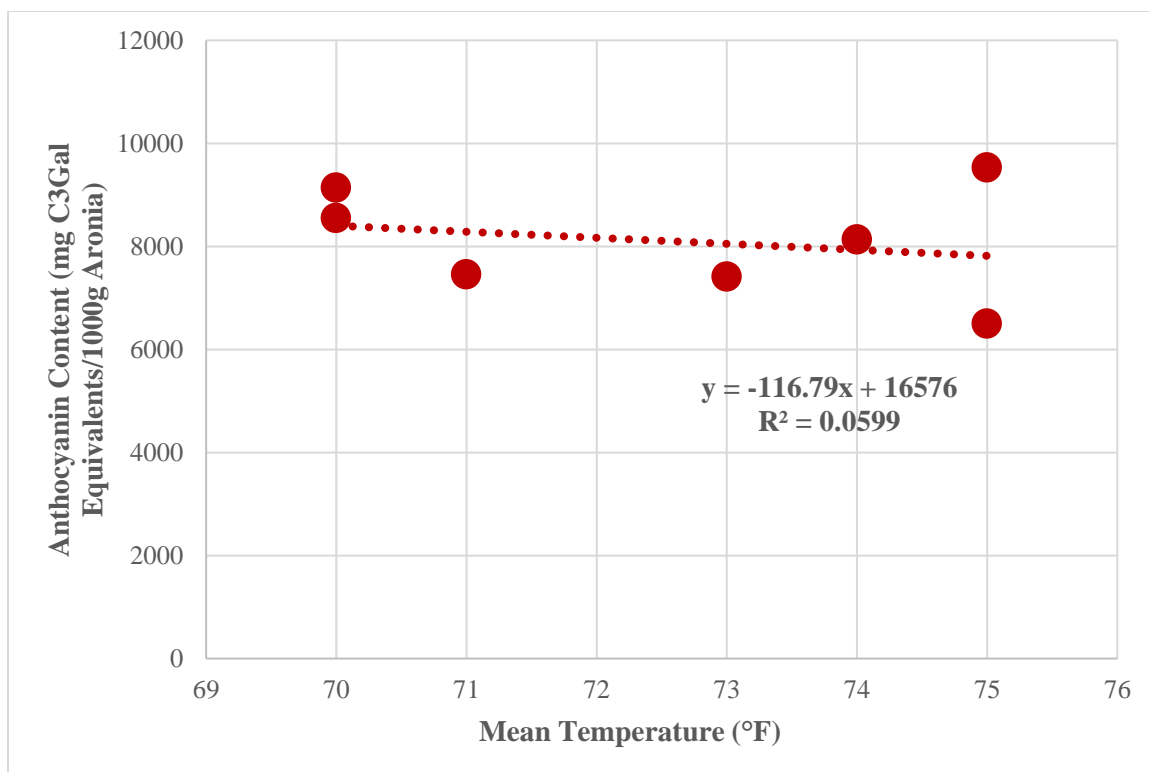
Published research on common varieties of *Aronia* has shown a significant difference in total phenolic compounds between the Viking, Galicjanka, Nero, and Hugin varieties.<sup>4</sup> Furthermore, Duncan's Test ( $p < 0.05$ ) showed Hugin variety to have significantly higher total phenolic compounds than Galicjanka variety. Galicjanka variety in turn had a significantly higher amount of total phenolic compounds than both Viking and Nero varieties.<sup>4</sup> Other research has concluded that there is limited variation among Aron, Nero, and Viking varieties, with no statistically significant difference in anthocyanin content ( $p < 0.05$ ).<sup>34</sup> Because data on the McKenzie variety is so scarce, Fig. 30 is pertinent to future understanding its differences with other common varieties.

### ***Effects of Weather Conditions***

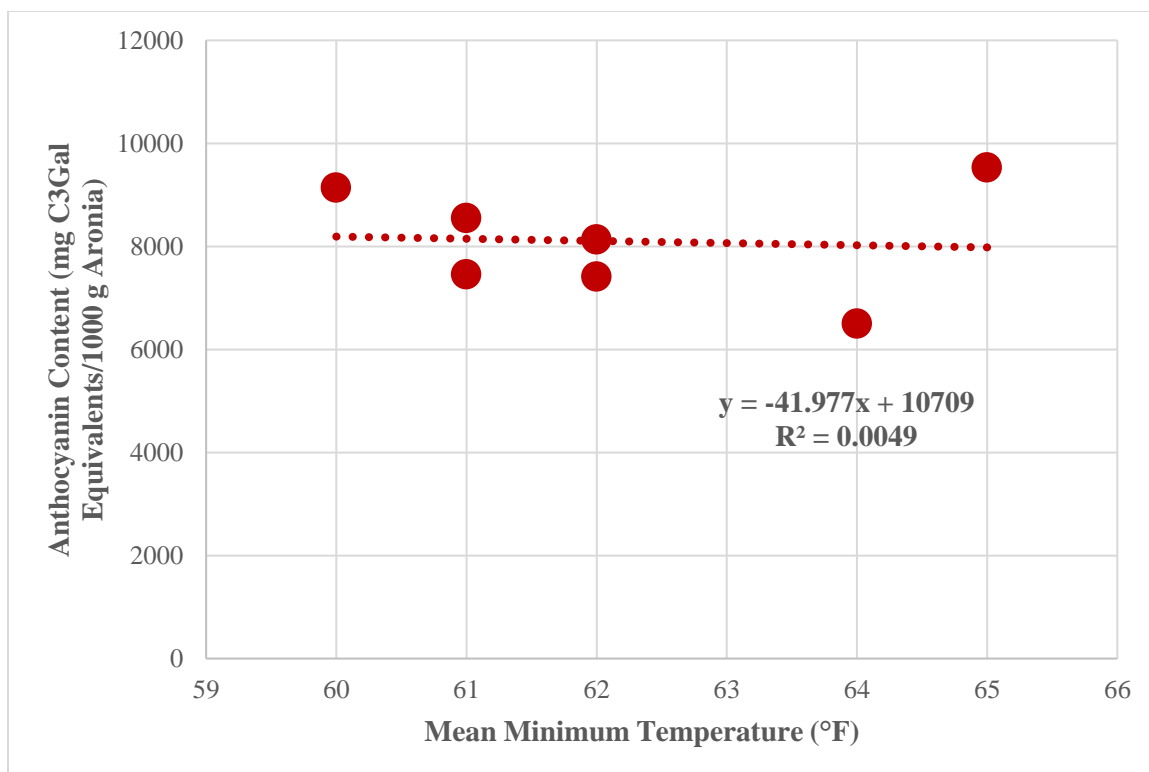
In Fig. 31 through Fig. 35, maximum anthocyanin content was analyzed as a function of the weather factors: mean temperature, mean maximum temperature, mean minimum temperature, precipitation, and latitude. This analysis was aimed at determining the influence of weather conditions on anthocyanin amounts in *Aronia* berries grown in the Midwestern region.



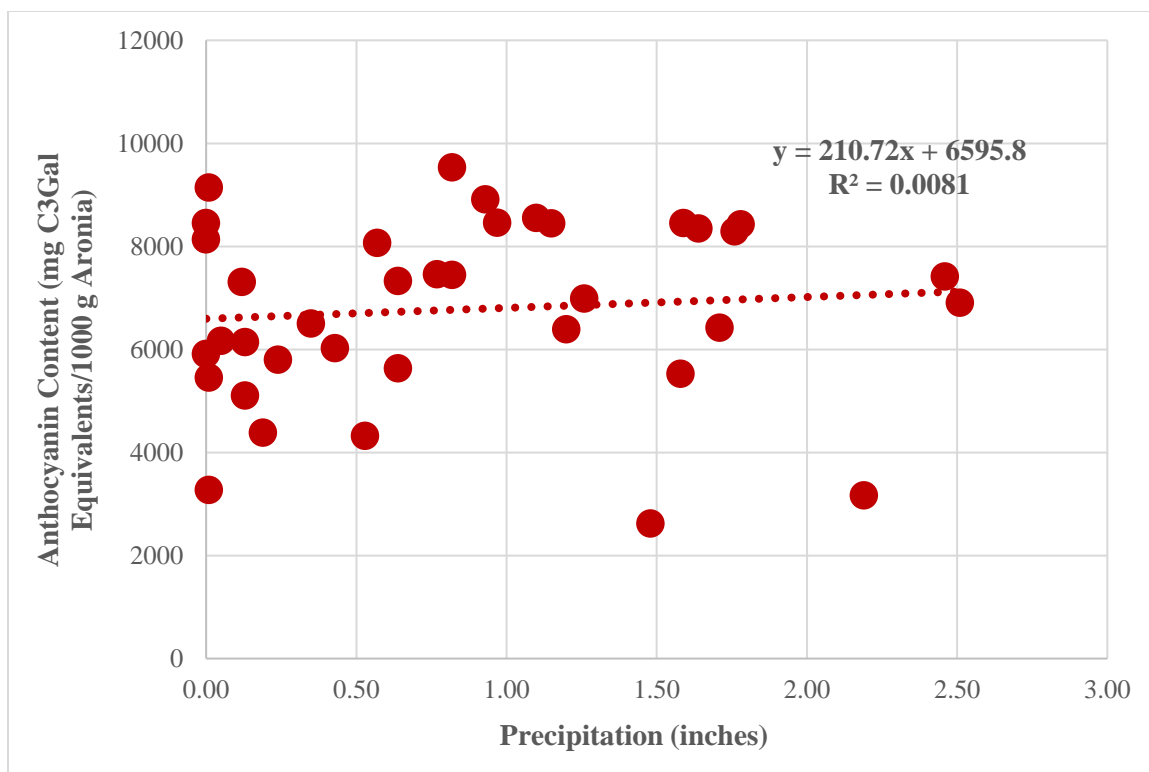
**Fig. 31: Peak anthocyanin content for each Viking *Aronia* grower as a function of the mean maximum temperature 90 days prior to harvest.**



**Fig. 32: Peak anthocyanin content for each Viking *Aronia* grower as a function of the mean temperature 90 days prior to harvest.**

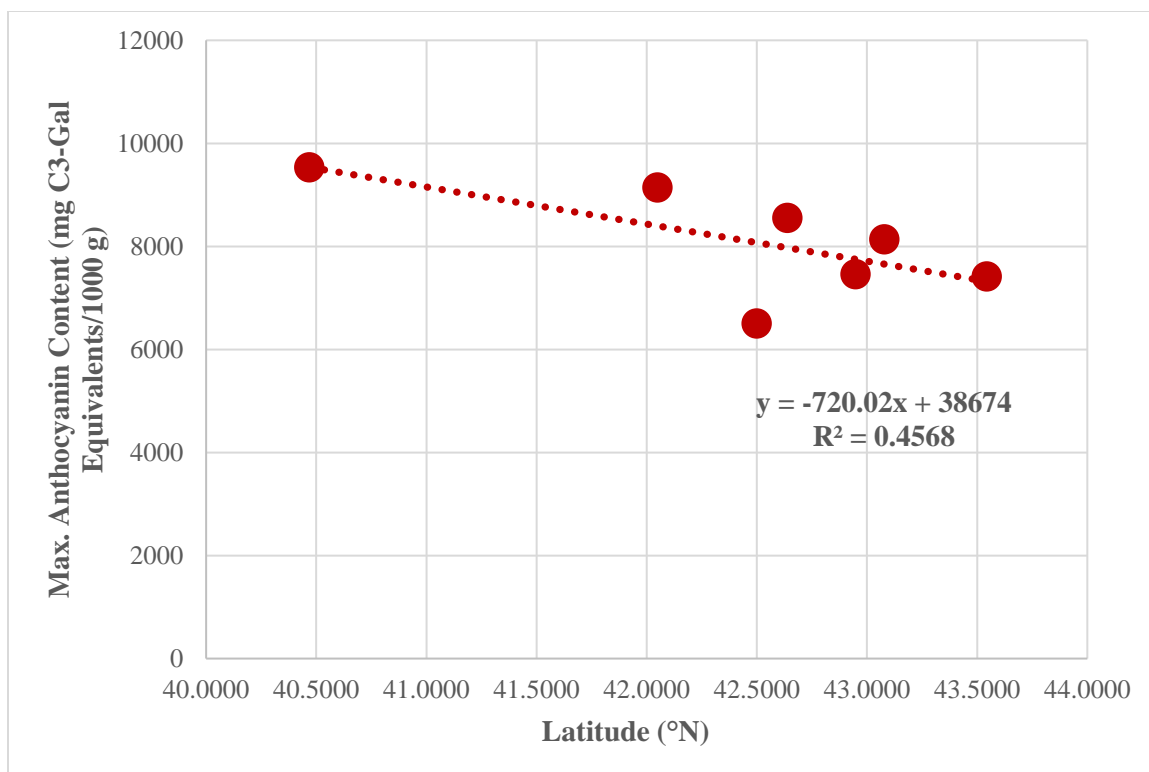


**Fig. 33: Peak anthocyanin content for each Viking *Aronia* grower as a function of the mean minimum temperature 90 days prior to harvest.**



**Fig. 34: Anthocyanin content in Viking *Aronia* as a function of total precipitation one week prior to harvest for every sample collected.**





**Fig. 35: Maximum anthocyanin content as a function of latitude for each of the seven growers producing Viking *Aronia*.**

A negative correlation ( $R^2$  of 0.46) between latitude and maximum anthocyanin content seemed to indicate that locations of lower latitude tend to produce *Aronia* with higher anthocyanin content. This impact may be explained by better growing conditions at lower latitudes.<sup>29</sup> UV exposure and soil conditions may also vary by region affecting the production of anthocyanin in *Aronia*. However, analysis suggests that the impact of latitude on maximum anthocyanin content was statistically insignificant ( $p < 0.05$ ). A wider range of latitudes could give a better representation of this variation. More data points may be required to show the evidence of a trend between latitude and maximum anthocyanin content. A slight negative correlation between mean maximum temperature and maximum anthocyanin content was also discovered. Here, an  $R^2$  value of 0.28 was discovered. However, again this was statistically insignificant ( $p < 0.05$ ).

Other factors showed much weaker  $R^2$  values, indicating large variations from a linear model. A small correlation between mean temperature and maximum anthocyanin content is observed. Likewise, no correlation was discovered between mean the minimum temperature and maximum anthocyanin content. Precipitation data collected one week prior to sample harvest was also had no strong impact on anthocyanin content.

## CHAPTER 5. CONCLUSION

pH affected the absorbance spectra of anthocyanins, as a buffer with a pH of 1.0 was required to keep them in the flavylium cation form. Improper pH conditions led to inaccurate anthocyanin quantification, and the spectra of anthocyanins held in methanol was inconsistent. Time also affected the absorbance spectra of anthocyanins, as degradation occurred over a 14-day period. These effects were discovered with both cyanidin 3-galactoside and cyanidin 3-arabinoside. However, the sugar group attached to the anthocyanin did not affect absorbance spectra.

HPLC was found to give the highest anthocyanin results when compared to the pH Differential method and the developed method. The Standard Curve method gave reproducible results that were below HPLC yet above pH Differential quantification. Because the developed Standard Curve method was simple, rapid, and low-cost in comparison to these other methods, it indeed has potential to quantify anthocyanins from *Aronia* berries.

For Viking variety *Aronia*, a positive correlation was discovered between the time and anthocyanin content. Late harvest is preferred to maximize the anthocyanin content for growers in the Midwest region. Of the samples tested, maximum anthocyanin content was highest in samples grown further south. This could be attributed to location parameters such as sunlight, seasonal temperature, rainfall, and soil conditions of the region. However, there was no statistically significant effect of the mean maximum temperature, mean temperature, mean minimum temperature, precipitation, and latitude discovered on maximum anthocyanin content in Viking *Aronia*. This information is useful to growers as they try to produce berries with high anthocyanin potential.

## CHAPTER 6. LIMITATIONS

This research gives greater insight to anthocyanins in *Aronia*. However, limitations exist in the scope of this work. Growers were entrusted to harvest a representative sample from their fields. Samples were not controlled for conditions such as plant age, soil, and sunlight hours. Research has shown that plants beyond 16 years of age may produce *Aronia* that has different polyphenolic characteristics than younger plants, such as lower anthocyanins and vitamin C, and higher dry matter.<sup>20</sup> Research data concerning influence of climate and soil conditions with anthocyanins in *Aronia* is scarce. However, similar research on grapes has concluded both factors are capable of influencing anthocyanin development.<sup>35</sup> These limitations could add unexplained variability in anthocyanin content beyond the scope of this research.

While weather parameter data collected for mean maximum temperature, mean temperature, mean minimum temperature, and precipitation was assumed to be accurate, there may exist slight variations from actual on-site data and the data collected via weather.com. Trends in anthocyanin content over the harvesting season should also be considered for only Viking variety *Aronia*, as the scope of this research considered only this variety. While Viking and McKenzie variety samples were shown to contain comparable anthocyanin content when provided by the same grower, differences may become apparent as more samples are analyzed. With more data in this regard, both varieties may not necessarily be significantly similar in anthocyanin content.

## CHAPTER 7. FUTURE WORK AND RECOMMENDATIONS

While *Aronia* is valuable as a raw material, it is often further processed into a different product. Heat treatment is one common processing method used, however because of the poor thermostability of anthocyanins, they are readily destroyed through processing. Therefore, the effect of heat treatment on *Aronia* is of interest to minimize anthocyanin loss. Anthocyanins also degrade over time, creating storage and transportation challenges for *Aronia* based products. Studying and understanding this issue may lead to techniques to prevent anthocyanin loss and developing novel products rich in *Aronia* anthocyanins.

Product development may also be beneficial in future *Aronia* research. While *Aronia* has numerous health benefits that encourage consumption, taste and astringency of the berry may require the development of new products which are desirable to the consumer. The effects of processing also encourage future research on product development to further create healthy, tasteful products which minimize anthocyanin losses in the berry.

To gain a better understanding on the effects of time on anthocyanins in *Aronia*, nine growers collected samples each week beginning when a Brix reading of 11% was reached. Because of this, sample collection date varied by grower. Further research in this area may prefer that samples are collected on the exact same day to reduce sources of variability. More data may also help in this instance, therefore a larger group of growers collecting samples could give better data. More samples collected in different regions may also help determine the effect of weather conditions on maximum anthocyanin content in *Aronia*. Statistical analysis determined that there was no statistical significance

between each weather parameter and maximum anthocyanin content, however, more data points may show a correlation in this regard.

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## APPENDIX

### *Effect of Microwave Extraction on Anthocyanins*

A newer method for anthocyanin extraction is Microwave Assisted Extraction. This extraction technique is applied during the extraction stage, before the HPLC or Spectrophotometric method is utilized for anthocyanin quantification. Typically, an organic solvent is used with a physical method such as maceration to bring anthocyanins from *Aronia* into solution. A physical method breaks down the cellular structures of the plant sample which enhances anthocyanin yield. However, physical methods may not be enough to completely remove anthocyanins from the skin of *Aronia*. Physical methods can also lead to long extraction times which are not compatible with analytical needs. For this reason, extraction techniques which can speed up extraction are of interest.

There are numerous methods which have been developed that can speed up extraction times, such as pressurized liquid extraction and sonication assisted extraction.<sup>36</sup> However, other methods that are rapid and require little equipment are also beneficial. One such method involves the addition of heat to anthocyanin extraction. One study has used a microwave oven to extraction anthocyanins of grape skins, which offered a rapid method of increased extraction in addition to maceration methods.<sup>37</sup> This study determined that different conditions during extraction could influence the amount of anthocyanin removed from the skin. Temperatures as high as 100°C were shown to be effective for extracting anthocyanins from grape skins.<sup>37</sup>

The selection of extraction method depends on the type of flavonoid that is to be extracted. Typically, an acidified organic solvent such as methanol is used for extraction of anthocyanins in *Aronia*.<sup>23</sup> This solvent is acidified to lower the pH of the solution, in

order to prevent the formation of the colorless hemiketal form of anthocyanin. However, there are limitations to using an organic solvent for extraction. The boiling point of methanol is 64.7°C, leading to bubbling and, eventually, evaporation. In some cases, a combination of organic solvent with water has been used to increase the boiling point of the solution.<sup>36</sup>

When thermolabile compounds are being extracted, it may be desirable to use a solvent combination with lower dielectric properties. The temperature of the solvent will then remain cooler while the microwave energy interacts with the plant matrix, leading to a release of these compounds into the cooler solvent.<sup>36</sup> Microwave power can also effect the interactions that occur for extraction. One such study found that with an increase in microwave power from 200 to 1,000 Watts, the yield of the flavonoids from *Radix astagali*, an herb, was increased.<sup>36</sup> However, high microwave temperatures caused by microwave power may decrease extraction yield through compound breakdown over time, requiring a short extraction time.<sup>36</sup>

With microwave extraction, additional anthocyanins in *Aronia* may be extracted and quantified. Microwave extraction is an additional step that is rapid when compared to other heat additive methods. One such heat based method that can be used for comparison is the use of a water bath.<sup>36</sup> A water bath method can reach the same temperatures as Microwave extraction; however, extraction times are longer. A water bath is not nearly as rapid in reaching a desired temperature as a microwave assisted extraction is, which may affect total anthocyanin.<sup>36</sup>

### ***Microwave Extraction Methodology***

In addition to the study of traditional extraction methods using maceration, a microwave-based extraction method was also completed. This followed the same procedure as previously described in the Standard Extraction Method section, however an additional microwave step was added following anthocyanin extraction. Three *Aronia* samples were tested in this trial. Following a 1-minute maceration of 5 grams of blended sample with 20 milliliters of acidified methanol (0.1%, v/v), the slurry was transferred to a beaker and a microwave was used to heat the sample for 15 seconds to about 130°F. This temperature was chosen to prevent boiling of the acidified methanol used in extraction. The extraction procedure continued following the sample protocol as described previously with the developed method for anthocyanin quantification.

A water bath extraction was also completed using the same *Aronia* samples for comparison as a heat-addition method. This followed the same procedure as the traditional maceration extraction method described previously. Following a 1-minute maceration of 5 grams of sample with 20 milliliters of acidified methanol (0.1%, v/v), the slurry was transferred to a beaker and placed in a hot water bath to sit for eight minutes. This was enough time for the temperature of the slurry to reach temperatures achieved by microwave extraction.

### ***Microwave Extraction Results***

The objective of microwave extraction was to determine if anthocyanin would be more completely extracted from the pomace of the *Aronia* berry. Table 7 shows the effect of an additional microwave extraction step on total anthocyanin extraction when

compared to a water bath and the developed method alone. The microwave extraction method had anthocyanin contents of 4132, 5672, and 7825 mg cyanidin 3-galactoside equivalents/1000 g *Aronia* for Samples 1, 2, and 3 respectively. This was compared to the developed method alone which had 2825, 4303, and 5154 mg cyanidin 3-galactoside equivalents/1000 g *Aronia* respectively. The microwave extraction method also had higher anthocyanin values than the developed method with water bath treatment, however, more variability existed as shown with standard deviation.

**Table 7: Comparison of anthocyanin quantification between the microwave and water bath steps. Microwave extraction yielded the highest results with the highest variability.**

	<b>Developed Method</b>	<b>Developed Method with microwave extraction</b>	<b>Developed Method with water bath extraction</b>
<b>Sample 1</b>	2825 ± 315	4132 ± 656	3718 ± 213
<b>Sample 2</b>	4303 ± 89	5672 ± 686	5054 ± 194
<b>Sample 3</b>	5154 ± 63	7825 ± 970	6126 ± 32

It may be difficult to completely remove anthocyanin from the skin of the berry with a maceration method alone. Microwave extraction was of interest in anthocyanin quantification due to the possibility for more complete extractions at a more rapid rate in *Aronia*. Microwave extraction has been used to obtain more complete anthocyanin extractions from grape skins.<sup>37</sup> Depending on the extraction solvent, microwave temperatures at a lower level did not negatively affect anthocyanin stability in grapes.<sup>37</sup> This extraction technique has potential for increasing anthocyanin extraction because of



fruit tissue disruption in the skin.<sup>38</sup> Microwave extraction offers promising results, however, more research is needed to gain further insight.