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Method Development for the Analysis of Anthocyanins in Aronio Berries via HPLC

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METHOD DEVELOPMENT FOR THE ANALYSIS OF ANTHOCYANINS IN ARONIA BERRIES VIA HPLC

by

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Abstract

Aronia is a deciduous shrub native to the northeastern United States, with many varieties of berry colors. They are colloquially known as chokeberries and they are typically utilized to make wine, jam, juice, tea and extracts. These berries are known to contain many polyphenols, and this research examined a subclass called anthocyanins. Anthocyanins are pigment molecules common to all higher plants which change color from red to purple to blue depending on pH. It is believed that these pigments are used to attract animals to the fruit for seed dispersal and they are known to protect cells from light damage by absorbing UV rays. Anthocyanins are also antioxidants and have analgesic, neuroprotective and anti-inflammatory effects. There is little data published on the relative concentrations of anthocyanins found in these berries. The aim of this research was to establish a working procedure to identify and quantify anthocyanins found in Aronia using high performance liquid chromatography (HPLC) via modified methods in the peer reviewed literature. The compounds were extracted using methanol acidified with citric acid. These samples were then compared to four analytical standards, which were cyanidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-xyloside and pelargondin-3-glucoside. A multi-step gradient was utilized with the eluents A: acetonitrile and B: 0.1% trifluouroacetic acid and detected compounds at a wavelength of 520 nm. The data produced from this research will be useful for identifying and quantifying anthocyanins in berries and value added product

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Introduction

Aronia melanocarpa

Aronia is a genus of deciduous shrubs that belong to the Rosaceae family [1]. They are native to northeastern North America and now grow throughout Europe [2]. Wild shrubs are usually found growing in wet woods or swamps. They are often used as ornamental plants or for commercial production of berries [1,2]. Aronia is hardy up to USDA zone 4, which means it can survive temperatures as low as -40 °F [3]. Commonly called chokeberries, there are multiple species of Aronia. Red Aronia is known as *Aronia arbutifolia*, purple Aronia is known as *Aronia prunifolia* and black Aronia is known as *Aronia melanocarpa* [3]. These distinctions are mostly made by the color of the shrubs fruits, but have variations in fruit size (black Aronia being the largest), time of harvest, foliage and growth patterns [3]. The berries are known to contain many polyphenolic compounds, especially anthocyanins, have a moderate sugar level and a pH between 3.3 and 3.7 [3]. They have an astringent flavor due to the high amount of tannins present and are tart due to their acidic nature [3].

They are often consumed for their health benefits which can be attributed to their high phenolic content; higher than many other berries including blueberries, raspberries and blackberries [4]. In the past, Native Americans have used them as both a food source and as a cold medicine, though their health benefits have been shown to include much more than a simple cold remedy [1].

Aronia has been found to have anti-tumoral effects on undifferentiated cancer stem cells in the form of a juice [5]. Sharif et al. found that *Aronia melanocarpa* juice (AMJ) had chemopreventive effects on mouse P19 embryonal carcinoma (EC) cells versus normal NIH/3T3 fibroblasts [5]. AMJ selectively inhibited cell proliferation and lowered viability in p19 EC cells in a concentration dependent manner, whereas no effects were found in NIH/3T3 fibroblasts [5]. The p19 EC cells were found to arrest their cell development in the S phase and, therefore, prevented them from entering G_2 phase where cell division would occur [5]. This also caused a significant, concentration dependent increase in apoptosis in p19 EC cells that were exposed to AMJ [5].

Some molecular targets related to the cell cycle and apoptosis were also examined. The expression of the tumor suppressor protein p53 (induces cell cycle arrest and apoptosis) and a functional homolog, p73, were found to be undetectable in control cells, but had significant increases in expression after exposure to AMJ [5]. An increased expression in cleaved caspases-3, an important marker and executor of apoptosis, was found in parallel with p53 and p73 expression [5]. The expression of cyclin B1 was found to decrease at higher concentrations of AMJ and was likely a main contributor in the arrest of the cell cycle because of its role in transitioning between phases [5]. There was also a decrease in expression found in the epigenetic regulator UHRF1 (Ubiquitin-like, containing PHD and RING Finger Domains, 1) which is linked to cancer cell proliferation and survival capacity [5]. The results show that AMJ selectively affects p19 EC cells through regulation of proteins that play important roles in the cell cycle and apoptosis.

Aronia has been shown to reduce risks of cardiovascular disease (CVD) through various mechanisms. Kim et al. found that Aronia extract decreased expression of genes for cholesterol synthesis, uptake and efflux in a dose-dependent manner in humans [6]. These genes included sterol regulatory element-binding protein 2, scavenger receptor class B Type 1, and ATP-binding cassette transporter A1 (ABCA1) [6]. Aronia extract showed decreased expression of genes involved in lipid metabolism and lipoprotein assembly, which include fatty acid synthase and acyl-CoA oxidase 1 [6]. There was a significant increase in levels of LDL receptors and cellular LDL uptake, meaning that cholesterol was taken into the cell, degraded and used in processes like membrane synthesis, steroid production or bile production [6]. The overall effect showed a lowered plasma cholesterol level due to increased cellular uptake and decreased chylomicron formation [6].

Park et al. found that Aronia has anti-viral activity due to its high polyphenol content [7]. A virucidal assay was used to test Aronia's anti-influenza efficacy against 5 different strains of seasonal and oseltamivir-resistant influenza virus [7]. The seasonal strains examined were H1/K09, H3/PE16, B/BR60 and the oseltamivir-resistant strains examined were H1/K2785 and HPAI rH5/IS06 [7]. Aronia was tested at concentrations ranging from 0.0625 mg to 0.5 mg and showed anti-viral activity at all concentrations. At low concentrations (0.0625 mg), Aronia was able to inhibit almost 70% of viral plaques from the H1 and H3 virus as well as the oseltamivir-resistant strain H1/K2785 [7]. Aronia exhibited a concentration dependent increase in efficacy, with greater than 60% efficacy against all tested viruses at concentrations of 0.125 mg [7]. These findings demonstrate Aronia's antiviral activity against a wide variety of influenza strains. With

many beneficial health effects and their high polyphenolic content, Aronia is an attractive fruit to study for health benefits and commercial production.

Aronia is underutilized in the United States, but is popular in Eastern Europe and Russia where the fruits are processed into juices, teas, wines, jellies, jams, soft spreads and flavoring for ice cream and yogurt [3]. There are many nutraceutical products that contain Aronia, which include: juice concentrates, gummy chews, energy gels and fresh frozen berries. The fruits are edible when fresh, but, due to their astringent taste, are more appealing when processed or mixed with other fruits [3]. The University of Wisconsin-Madison Center for Integrated Agricultural Systems found Aronia to have one of the highest potentials for sustainability in the United States due to high nutraceutical value, high adaptability, long harvest season, substantial crop production and high level of disease and pest resistance [3, 8]. Currently, there are few products in the U.S. that contain Aronia and they are mostly restricted to fresh or frozen berries or juice concentrates. Some small farms such as Bellbrook Berry Farm (Brooklyn, WI), Blazerfarmz (Easton, MO) and Sawmill Hollow Family Farm (Missouri Valley, IA) sell Aronia products. These products include fresh or frozen Aronia berries that sell for $5 to $14 a pound, Aronia extracts, pressed Aronia juice, Aronia jams and freeze-dried Aronia capsules. These Aronia products are advertised for their high antioxidant content and their accompanied health benefits. There are also companies such as Superberries (Omaha, NE) and Orenda International (Tempe, AZ) that sell various Aronia products. Superberries sell Aronia juice concentrates, fresh frozen Aronia and Aronia gummy chews. Orenda International has an Aronia juice that is combined with other fruits and

botanicals. Outside of these businesses, Aronia is still underutilized and has much more potential use for product development.

Anthocyanins

Anthocyanins are natural water-soluble pigments found in vascular plant vacuoles [9]. They are responsible for the colors orange, pink, red, violet and blue found in many flowers and fruits [9]. There are many roles for anthocyanins in plants. Their color and strong UV absorbance help attract insects and animals to pollinate plants [10]. The strong UV absorbance of the pigments also acts to protect the plant from UV damage to DNA [10]. They have also been shown to play a role in plant pest resistance and have anti-bacterial properties [11]. Anthocyanins act as antioxidants and are of great interest due to their range of biological activities. These activities include prevention of neuronal decline, prevention of cardiovascular disease and diabetes, anti-tumor activity, anti-inflammatory activity and much more [9, 11]. Their biological activity, as well as their use as natural dyes and food additives, makes anthocyanins an attractive area of investigation.

Anthocyanins are in a subclass of polyphenols called flavonoids. These are a class of plant secondary metabolites that have a shared three ring structure (refer to figure 1) [12]. The major classes of flavonoids are anthocyanins, flavonols, flavanols and proanthocyanidins [12]. They produce a variety of colors, from red to purple for anthocyanins, colorless to yellow for flavonols and colorless flavanols that turn brown after oxidation [12]. There are differing concentrations of flavonoids depending on the plant species, developmental stage, tissue type and growth conditions [12]. The

flavonoids have a range of functions that include visual signals, antioxidant activity, UV light protection and protection from plant pathogens [12].

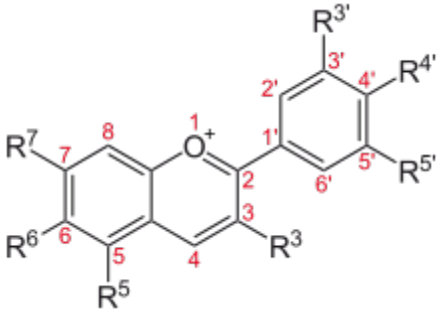

Fig. 1 General Anthocyanin Structure [11]

There have been over 500 different anthocyanins identified and 23 anthocyanidins [9]. Anthocyanins are the glycoside (sugar-linked) versions of anthocyanidins, which are characterized by their unique chemical structure [9]. Anthocyanidins have an aromatic ring bonded to a heterocyclic ring with one oxygen atom, creating the flavylium ion [9]. These two bonded rings are connected by a carbon to carbon bond to another aromatic ring. The rings can have multiple side chains with each giving them different characteristics. Commonly these side chains are made up of hydrogen, hydroxyl or methoxy groups [9].

Isolated anthocyanins are very unstable and degrade easily due to a variety of factors including: pH, storage temperature, light, oxygen and the presence of enzymes, proteins and/or metallic ions [9]. Hellstrom et al. demonstrated the importance of storage temperature on anthocyanin stability in various juices. They measured the anthocyanin contents of various juices before and after storage for 11 weeks and 22 weeks at temperatures of 25°C (room temperature), 9°C and 4°C. The results of this study show

that anthocyanin degradation rate is related to storage temperature, where lower storage temperatures will cause less degradation of anthocyanins. There was over a three-fold increase in anthocyanin half-life in juices stored at 9°C compared to room temperature and the half-life increased even further for juices stored at 4°C [13]. The juice matrix can cause co-pigmentation to happen and have effects on anthocyanin stability. Co-pigmentation can help stabilize anthocyanins by forming complexes with metal ions, other phenolics or self-association [13]. Chlorogenic acid and cinnamic acid have been shown to readily form co-pigments with anthocyanins and contribute to the stability in solution [13].

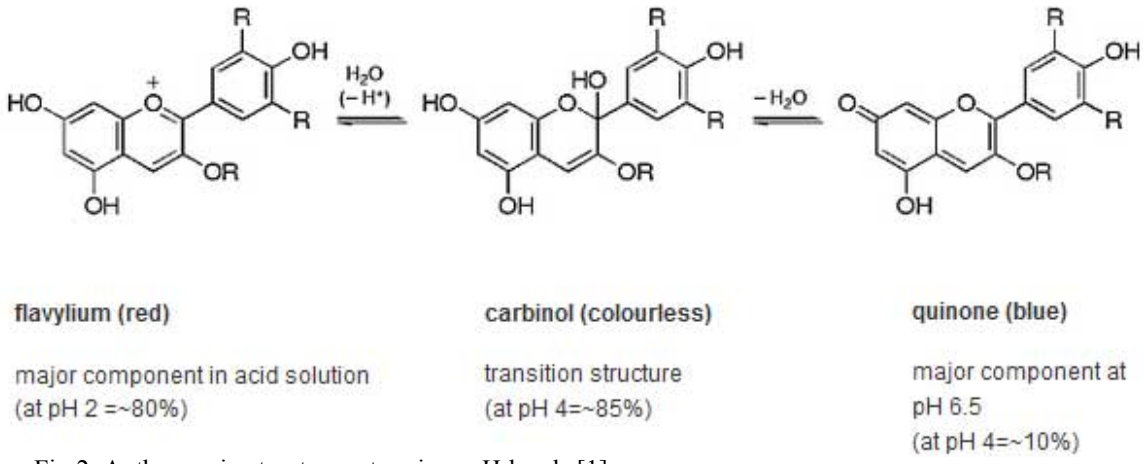

Fig 2. Anthocyanin structures at various pH levels [1]

The effect of pH is also important to anthocyanin stability in solution. Depending on the pH of the solution, the color of anthocyanins can change from red to blue to colorless. This color change is induced by reaction of the flavylium ion, which is stable in acidic conditions (refer to figure 2) [14]. When the pH is raised, the structures of the bonds around the flavylium ion change to form a colorless carbinol at around pH 4 and a purple/blue quinine compound at pH 6.5 and higher [14].

The anthocyanins cyanidin, delphinidin and pelargonidin are the most common ones found in nature. They are found in 80% of pigmented leaves, 69% of fruits and 50% of flowers [9]. The distribution of the six most common anthocyanidins in fruits and vegetables are as follows: cyanidin 50%, delphinidin 12%, pelargonidin 12%, peonidin 12%, petunidin 7% and malvidin 7% [9]. The most common glycosides found in nature are 3-monosides, 3-biosides, 3,5- and 3,7-diglucosides, with cyanidin-3-glucoside being the most common anthocyanin overall [9]. In Aronia, there are four major anthocyanins that are responsible for the dark red and purple colors. These are cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-xyloside and cyanidin-3-arabinoside [15].

Colorimetric Assays

A colorimetric assay was utilized to analyze the total monomeric anthocyanin content found in Aronia berries. Colorimetric assays are a method to determine the concentration of a compound in a solution using its absorbance as a proportional measurement. This is accomplished due to Beer's Law, which states that a substance's concentration is directly proportional to the amount of light it absorbs [16]. The absorbance of the compound is read with a spectrophotometer or a microplate reader, depending on the sample size needed to be analyzed. If large numbers of small samples need to be analyzed, a microplate reader is a more efficient means of obtaining data due to large plate capacity (96-well format).

This research utilized the pH differential method to find the total monomeric anthocyanin content of five Aronia berry ascensions. This assay is based on the

reversible structural change in the chromophore of monomeric anthocyanins between pH 1.0 and 4.5, which goes from a max absorbance of around 520nm to nearly colorless, respectively [1]. The difference in absorbance between pH 1.0 and 4.5 at 520nm is proportional to the anthocyanin concentration [1]. Polymeric anthocyanins are resistant to color change with pH and, therefore, absorb light at both pH values and are not measured by this method [1]. The max absorbance is chosen as 520 nm because it is the midrange absorbance value for the anthocyanins being observed and resulting weights are expressed as cyanidin-3-glucoside equivalents because it is the most common anthocyanin pigment found in nature [1].

Five different Aronia samples were analyzed, including two purple Aronia, two black Aronia and Viking, a commercial black cultivar. Several dilutions were made with potassium chloride buffer, 0.025M, pH 1.0, in order to find the absorbance range for the samples. Then, extracts made with acidified (1% citric acid) methanol were pipetted into 2 mL centrifuge tubes and diluted with two buffers. The two buffers used were potassium chloride (0.025M) at pH 1.0 and sodium acetate (0.4M) at pH 4.5. These solutions were made in triplicate and analyzed with a microplate reader reading at 515nm and 690nm. The 690nm reading is used to correct for haze in the samples [1]. The values 515nm and 690nm were used instead of the typical 520nm and 700nm values because those are the filters that were used in our microplate reader.

HPLC

High-performance liquid chromatography (HPLC) was the technique used to analyze the anthocyanin compounds in this research. HPLC has applications other than

research that include medical purposes, such as testing for metabolites in blood samples; legal purposes, such as urine drug analysis; and manufacturing purposes, such as analyzing pharmaceutical products for impurities [17]. It can be used for both quantitative and qualitative analyses. Quantitative analyses use measurable data, whereas qualitative analyses use observable and descriptive data that aren't measurable. This gives HPLC an advantage over traditional techniques of liquid chromatography that are used primarily in identification of compounds. HPLC is a specific type of liquid chromatography that uses a computer system with a sampler, mobile phase reservoirs, pumps, a column and a detector. The sampler injects small amounts of samples onto the column and the compounds separate based on their polarity. An UV-Vis detector then records the presence of compounds in the sample based on a certain wavelength.

Reverse-phase partition chromatography was used in this research. Liquid chromatography is an analytical technique that separates analytes in a sample, such as ions or molecules, by interaction with a liquid solvent in a column that holds a stationary phase [17]. Each analyte interacts uniquely with the liquid and stationary phase and moves through a column at different rates based on separating behavior which can be physical or chemical in nature (i.e. ion-exchange, adsorption or size separation) [17, 18].

Partition chromatography refers to the use of a column that contains a stationary phase that is bonded to inert particles with a diameter ranging from 3-10 μm [19]. The particles are commonly made of either silica or alumina and have bonded ions, polar, or non-polar compounds [17]. Particles that range from 3-5 μm are typically used in analytical applications, whereas larger particles are used in preparative scale applications [19].

The column determines what type of chromatography is being used. In reverse-phase chromatography, a polar mobile phase and non-polar column are used. The mobile phase is usually water and a mixture of other polar solvents such as acetonitrile or methanol [17]. The column consists of a non-polar stationary phase that usually contains hydrocarbon chains such as n-octyldecyl (C_{18}) chains or phenyl groups [19]. On such a column, less polar molecules will elute slower and therefore have a greater retention time, whereas highly polar molecules will elute quickly from the column [17]. The retention time can be modified with the mobile phase, also. The less polar the mobile phase is, the shorter the retention time [17]. Often, a gradient is used to introduce a less polar solvent (i.e. acetonitrile or methanol) to a water based mobile phase over the course of analysis to reduce retention time [17]. This is important for fast and high resolution analyses of compounds, because some compounds will take over an hour to resolve with an isocratic condition, but can be reduced to around 30 minutes with a gradient [17].

There are five major components to an HPLC system. The first component is the pump which pumps the liquid mobile phase through the column at a specific flow rate and pressure. This flow rate is expressed in mL/min and is typically between 1 to 2 mL/min [17]. The pressure can range anywhere from 800-9000 psi (60-600 bar) depending on the type of system and column used [17]. Another important function of the pump is its ability to vary the mobile phase composition. An isocratic or constant mobile phase can be used for simple separations, or a gradient that changes the mobile phase can be used, as described earlier, in analyses of complex samples [17].

The major component of an HPLC system is the injector. The injector is used to inject the liquid sample into the stream of the mobile phase for analysis. There are two

different types of injectors: manual and automatic. A manual injector is where a user injects the sample into the flowing mobile phase using a syringe which transports the sample to the column for analysis [17]. An automatic injector uses vial trays that the user loads and can then set the autosampler to automatically measure the proper injection volume, inject the sample and flush the injector to be ready for the next sample [17]. This technique allows for automatic, unattended use of the system and is more efficient for large sample sets [17].

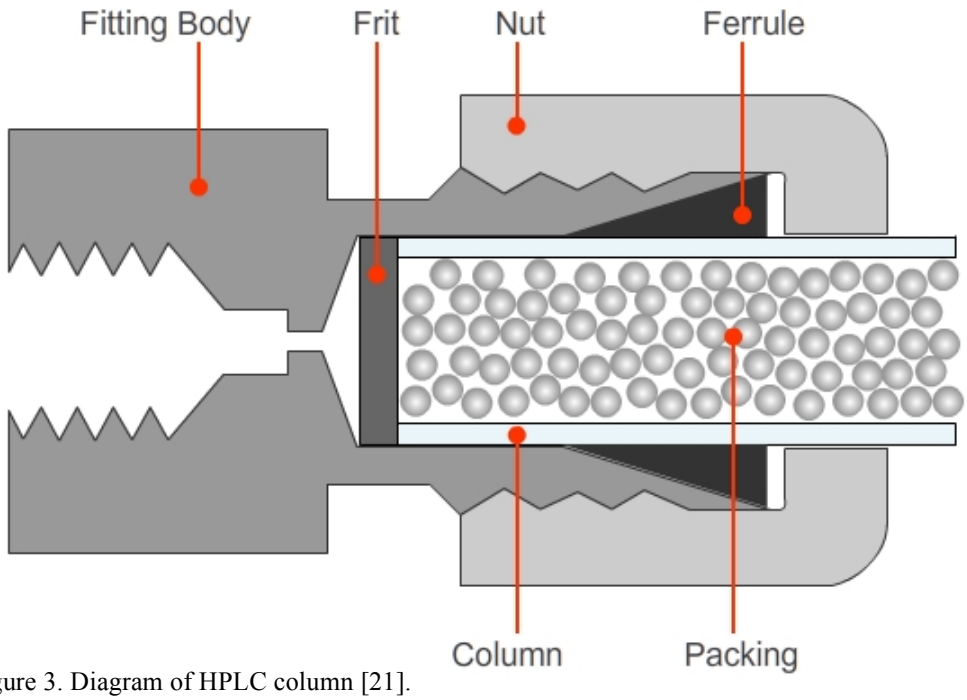

Figure 3. Diagram of HPLC column [21].

The column is one of the most important components of the HPLC because it is where the sample analytes separate due to the column's stationary phase [17]. The small particles in the column are what cause the high pressures that the system must overcome and make the required use of a pump. This is because the particles take up most of the

room in the column and when a solvent is pushed through it, there needs to be a lot of pressure to keep a constant and steady flow through the column (refer to figure 3). Commonly, silica particles are used in the column and have side chains attached to them to form the column matrix (refer to figure 4). This research utilized a C-18 and a C-6 Phenyl column, which have an 18 carbon chain and 6 carbon chain with a phenyl group attached, respectively. Columns can be made of many different materials and different sizes for their various applications. Typically column housings are made of stainless steel, glass or polyether ether ketone (PEEK) polymers [17]. Stainless steel is most commonly used because it allows for applications of high pressure. The various applications of columns are analytical, preparative, capillary and nano scale. Analytical columns generally have an internal diameter of 1.0 to 4.6 mm and have lengths ranging from 15 to 250 mm [17, 19]. Preparative columns are typically larger than analytical columns. They have internal diameters greater than 4.6mm and lengths that range from 50 to 250 mm [17, 19]. Capillary columns have small internal diameters ranging from 0.1 to 1 mm and can have various lengths depending on their application [17, 19]. Nano scale columns are the smallest, with internal diameters smaller than 0.1 mm and lengths less than 100 mm [17, 19]. As columns get smaller in diameter and length, they can detect smaller quantities and produce larger chromatographic peaks for sensitive detection of compounds.

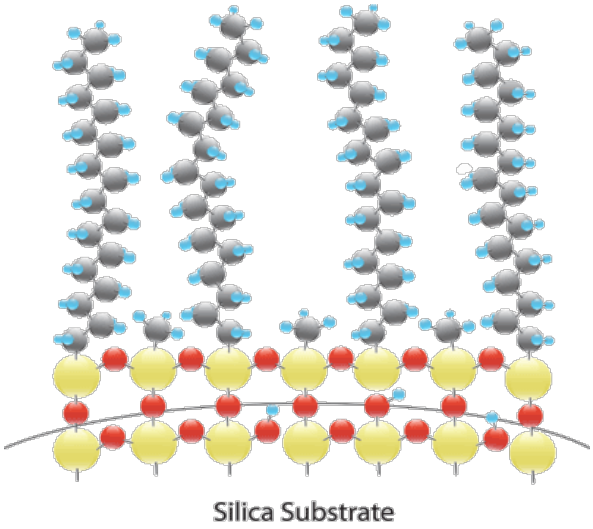

Figure 4. Diagram of column matrix [22].

Column temperature is another important consideration for making reproducible analyses. Controlling the temperature at a constant rate is important because retention times are temperature-dependent and, therefore, require a constant temperature to elute compounds consistently and reliably [17]. Another aspect to take into consideration is compound solubility. Some compounds may have low solubility in the mobile phase and require higher temperatures to prevent precipitates from entering the column [17]. Also, when examining some compounds, such as biological compounds like proteins or enzymes, the temperature needs to be kept low in order to prevent degradation and destabilization [17].

Another vital component of an HPLC system is the detector which detects the compounds that elute from the column. The detector measures the amount of analyte eluting from the column and allows for a quantitative analysis of all compounds of interest in the sample [17]. A chromatogram is used to represent the data from the UV-Vis detector and plots the absorbance against the time of elution from the column,

allowing for identification and quantification of analytes. Compounds can be identified by their elution time, which is unique to individual compounds. The compound can then be quantified by using the total peak area to calculate the concentration in the original product. There are multiple detection techniques that can be applied, of which spectrographic, refractive index and fluorescence detection are most common [17].

There are two types of spectrographic techniques most commonly used in HPLC. These include UV-Vis spectrometry and mass spectrometry, of which our research made use of UV-Vis spectrometry at a single wavelength. UV-Vis detection is one technique that employs a UV and visible light beam through a flow cell and passes on to a sensor that detects changes in light absorbance as an analyte passes through the column [17]. This technique can use two different technologies depending on the application necessary. A variable wavelength detector can record at one wavelength of light, whereas a diode array detector can detect at multiple wavelengths of light [20]. A diode array detector is useful when retention times of analytes are close and an examination of their spectrum may be needed to determine their identity [20]. Mass spectrometry is another common technique used to identify compounds of interest. A mass spectrometer ionizes or breaks up a compound into smaller parts and then measures their masses [17]. This leaves a mass spectrum that is unique to a compound, much like a fingerprint, and allows for easy identification of analytes based on their chromatographs [17].

The last component of an HPLC system is the computer which integrates and controls all of the other components as well as records the data into a chromatograph [17]. The computer uses software to control the varying aspects of each component (i.e. flow rate, column temperature, detection, etc.) and allows for the programming of mobile

phase gradients. The chromatograph, as stated earlier, plots the detection technique against the time of elution from the column, allowing for identification and quantification of analytes. In our research, an UV-Vis spectrometer was used and the milli-absorbance units (mAU) are plotted against time in minutes. As the data is collected, there are chromatographic peaks on the graph that represent each separated compound [17]. Once all the data is collected, it can be used to identify and quantify compounds in a sample. Compounds are most commonly identified by their elution times but can also be based on chemical structure or molecular weight depending on the detector used [17]. Compound quantification is used to find the concentration of a compound in the sample. This is mainly done by determining the area of a sample peak and comparing it to a standard with a known concentration [17].

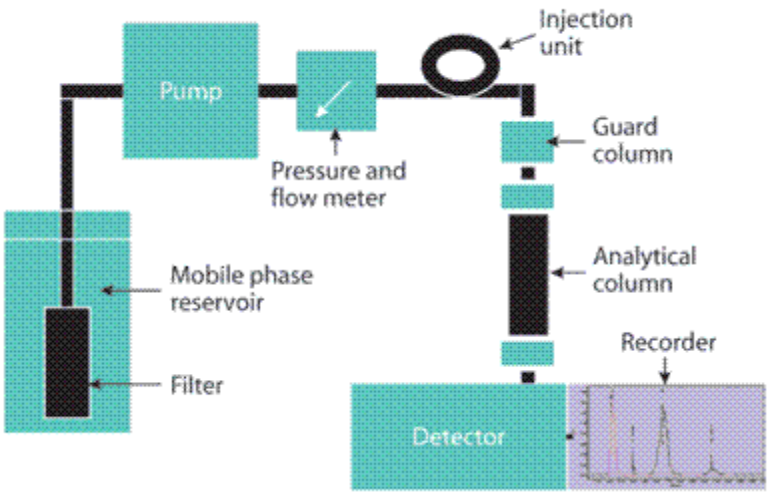

Figure 5. HPLC Diagram [23].

HPLC and Colorimetric Assays

HPLC and colorimetric assays both use the same principles in analyzing anthocyanins. They use the UV-visible spectrum absorption of the sample to detect the presence of compounds of interest. The absorption of UV or visible light by compounds is caused by excitation of electrons [24, 25]. The most common excitation of organic compounds occurs in bonds of certain functional groups called chromophores, which contain valence electrons with low energy [24]. When these electrons are hit by light, they are excited to a higher energy level and absorb the wavelength of light associated with the amount of energy absorbed [24, 25]. This relationship can be explained by the equation $E=hv$, where E is the energy of light, h is Planck's constant and v is frequency [24]. This shows that as energy of the light increases, so does the frequency and, therefore, the wavelength of light decreases because of the inverse relationship between frequency and wavelength ($\lambda=c/v$) [24].

UV-Vis spectrometry uses this principle of absorbance and Beer's law to find the concentration of a compound in solution. Beer's law creates a directly proportional relationship between absorbance and concentration of a sample [25]. The equation for Beer's law is $A=\varepsilon cl$, where A is absorbance, ε is the molar extinction coefficient, c is sample concentration and l is the path length [25]. Absorbance is measured as transmitted light which is represented as a ratio of intensity of incident light (I_o) to the intensity of transmitted light (I) [25]. Beer's law uses this ratio and correlates it with sample concentration which is related to the path length of light that travels through the sample. At fixed path lengths, the absorbance is directly proportional to the concentration of the absorbing compound [25]. The molar extinction coefficient is

dependent on the nature of the compound, the solvent, wavelength and pH of the solution if the compound has different ionization states [25]. The units for the molar extinction coefficient are L mol^{-1} cm^{-1} and it is a measurement based on how strongly a certain wavelength of light is absorbed by a compound [25].

Methods and Materials

Chemicals and Materials

Methanol (HPLC Grade), acetonitrile (HPLC Grade), water (HPLC Grade), trifluoroacetic acid (HPLC Grade), formic acid (>99%), citric acid, potassium chloride and sodium acetate were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ethanol (ACS/USP Grade, 200 proof) was purchased from Pharmco Products (Brookfield, CT, USA). Purple Aronia #1 (*Prunifolia virginia*, P1603107), purple Aronia #2 (*Prunifolia virginia*, UC085), Black Aronia #4 (*Aronia melanocarpa*, P1603106) and black Aronia #5 (*Aronia melanocarpa*, UC024) and Viking Aronia, a commercial variety, were donated by Dr. Mark Brand from the University of Connecticut, Storrs, CT.

Extraction Methods

A sample of 25g of Aronia berries were added to 100 mL of solvent (acidified methanol or ethanol). The berries were homogenized in a commercial blender (Magic Bullet, Emson, NY, USA) for 30 seconds. The homogenized sample was then transferred into 50 mL centrifuge tubes and was centrifuged (fixed-angle) for five minutes at 7197 RCF to remove particulate matter. A 1 mL sample of the supernatant was transferred to a 1.5 mL centrifuge tube and centrifuged again at 20817 RCF to further remove particulate

matter. The supernatant was then pipetted into HPLC vials for analysis. Extra samples were stored at -20°C.

Colorimetric Assays

A Thermo Scientific (Pittsburgh, PA, USA) 96-well microplate was used for the pH differential assay of anthocyanins. Samples were diluted by a factor of 20 by adding 50 µL of sample and 950 µL of buffer. The blank of D.I. water was placed in the first well. The samples were set up in triplicate and there were two columns for each sample at pH 1.0 and pH 4.5. The microplate was analyzed at 515nm and 690nm by a Biotek ELx808 microplate reader (Winooski, VT, USA).

HPLC Conditions

A flow rate of 1.0 mL/min was used. The injection volume was 5 µL for each sample. The column temperature was 35 ± 5°C. UV-Vis detection was at 520 nm. A solvent gradient was utilized. For 3 minutes, 95% B (0.1% TFA) flowed through the column. From 3 to 25 minutes, a linear gradient went to 5% B and from 25 to 30 minutes the column was re-equilibrated to 95% B.

Equipment

A Dionex 3000 HPLC system (Pittsburgh, PA, USA) was utilized for anthocyanin analysis. Three HPLC columns were used: a Phenomenex Gemini C6 Phenyl, 250 x 4.6mm, 5 µm (Torrance, CA, USA), a Phenomenex Jupiter C-18, 250 x 4.6mm, 5µm (Torrance, CA, USA) and a Phenomenex Gemini-NX C18 110A, 250 x 4.6mm, 5 µm (Torrance, CA, USA). An Eppendorf centrifuge 5430 (Hamburg, Germany) was utilized

for the extraction process with two different rotors: an Eppendorf 5430/5430R rotor F-35-6-30 HL037 and an Eppendorf 5430/5430R rotor FA-45-30-11 HL097. A Biotek ELx808 microplate reader (Winooski, VT, USA) was utilized for colorimetric assays. A Magic Bullet blender (Emson, NY, USA) was utilized in the extraction process.

Results

HPLC and Colorimetric Assays

Total monomeric anthocyanin content as expressed in cyanidin-3-glucoside equivalents for HPLC was 6.85 mg anthocyanin per gram of berries and 3.27 mg/g for the total monomeric anthocyanin assay (See figure 6).

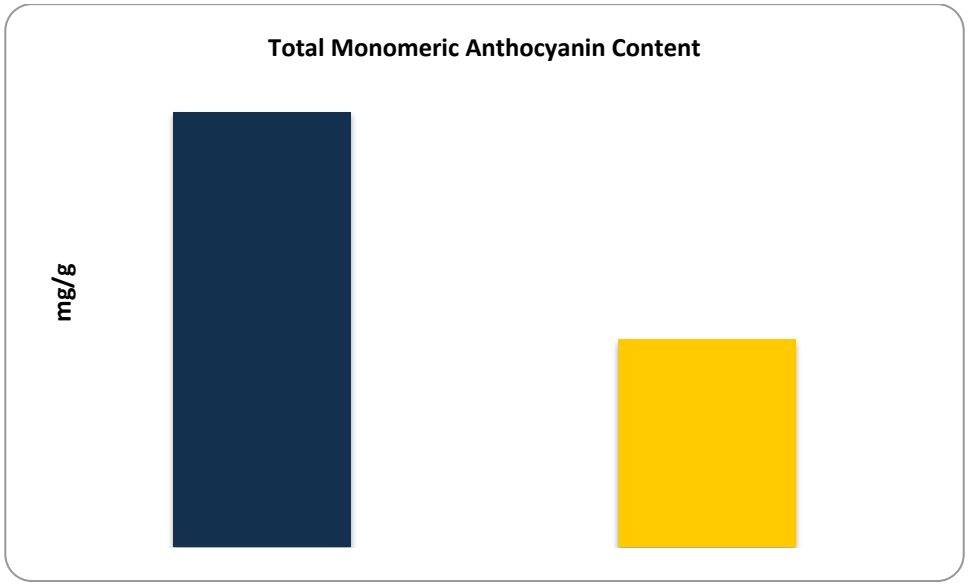

Fig 6. Total monomeric anthocyanin content expressed as Cy-3-Glu equivalents

Ethanol vs. Methanol Extracts

There were a total of three extractions done for each solvent and they were analyzed using HPLC. The total relative anthocyanin concentrations were expressed as average chromatographic peak area with units of mAU*min. Figure 7 shows that acidified methanol extracted more anthocyanins than acidified ethanol. The average peak area for acidified methanol was 221.25 mAU*min and 188.64 mAU*min for acidified ethanol. Methanol extracted 15.9% more anthocyanins than did ethanol with this method.

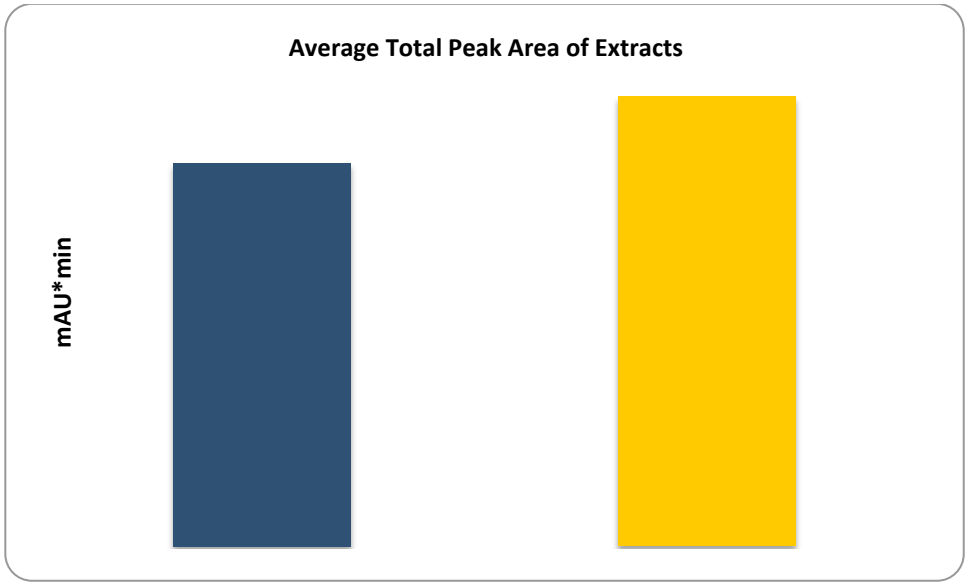

Fig. 7 Comparison of average total peak area of extracts

The anthocyanin ratios (AR) from the extracts were also compared to see if there were changes in extraction of certain anthocyanins between the two solvents in figure 8. Cyanidin-3-Galactoside was the largest fraction of the extraction and represented 65.11% of the AR in acidified methanol and 65.29% in acidified ethanol. The unknown chromatographic peak represented the second largest fraction of each extraction and represented 28.67% of the AR in acidified methanol and 29.04% in acidified ethanol.

Cyanidin-3-xyloside had an AR of 4.02% in acidified methanol and 3.92% in acidified ethanol. Cyanidin-3-glucoside had an AR of 2.19% and 1.75% in acidified methanol and ethanol, respectively. The values do not appear different, showing that the extracts contained nearly equal fractions of each anthocyanin.

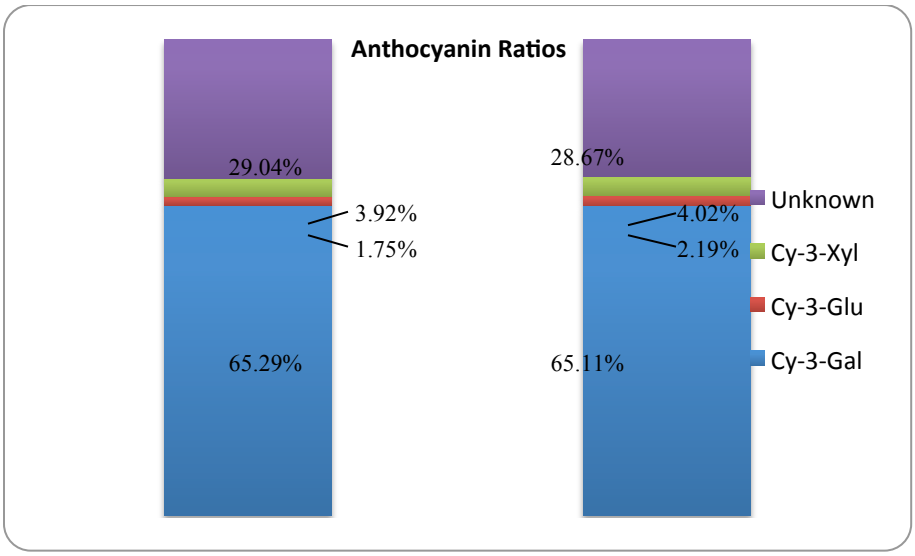

Fig. 8 Anthocyanin ratios in acidified methanol and ethanol extracts

Sonication

The effectiveness of a 10 minute sonication step on total anthocyanin yield in the extraction process was also examined. There was a single extraction done for each solvent. The sonication step was added after homogenization in order to further disrupt cellular compartments in hopes of extracting more anthocyanins.

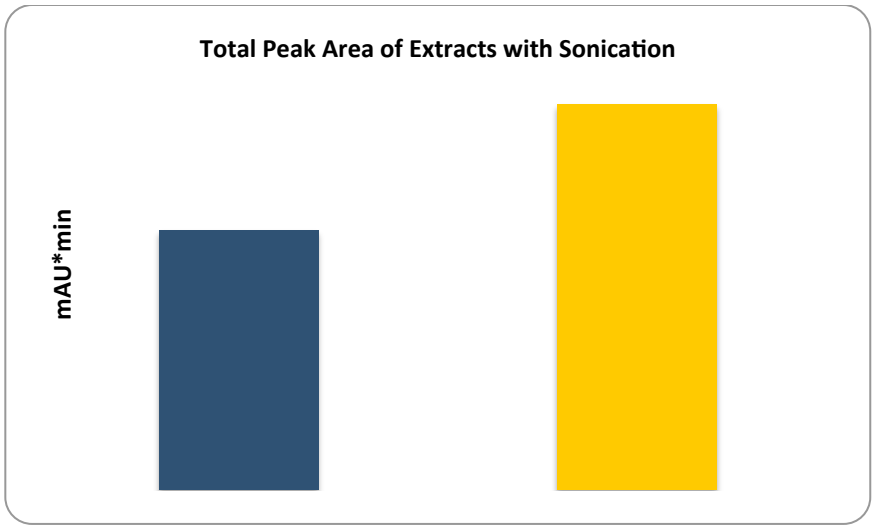

Fig. 9 Comparison of average total peak area of extracts with sonication

The total relative anthocyanin concentrations were expressed as average chromatographic peak area with units of mAU*min. Figure 9 demonstrates that acidified methanol with sonication yielded higher amounts of anthocyanins than acidified ethanol with sonication. Acidified methanol extract had a peak area of 342.89 mAU*min and acidified ethanol had a peak area of 230.72 mAU*min. Using this method, acidified methanol extracted 39.1% more anthocyanins than acidified ethanol. The sonication step showed a 43.1% increase in yield with acidified methanol and a 20.1% increase in yield with acidified ethanol.

HPLC Optimization

The first mobile phase used was 0.01% formic acid, 22.5% methanol and 50% acetonitrile under isocratic conditions. These conditions were tested with purified anthocyanin standards. The resulting peaks from the initial analysis were wide and one had extra peaks, showing poor resolution (See figure 10). Peaks fully eluted between 4 to

8 minutes and were broad, except for cyanidin-3-glucoside which had an extra peak and should not be the case with purified standards. The mobile phase was changed to 90% acetonitrile and 0.01% TFA for the next trial. Peaks were still broad, but did not show any shouldering or extra peaks in the sample. The elution time was reduced to 3 to 4 minutes per peak, which was still too long for a useful analysis. A multi-step gradient was then utilized with the previously mentioned mobile phase. Mobile phase A was 90% acetonitrile and 0.01% TFA and mobile phase B was 0.01% formic acid, 22.5% methanol and 50% acetonitrile. For three minutes, an isocratic condition of 95% B was used. Then, the gradient went to 5% B between 3 to 25 minutes in a linear fashion. The column was then re-equilibrated for 5 minutes back to 95% B. This reduced the peak elution times to about 0.2 minutes per standard; a 20-fold increase in resolution over the previous isocratic conditions. The mobile phases, acetonitrile and 0.1% TFA, were then used under multi-step conditions and had similar results to the previous multi-step gradient conditions (See figure 11). These mobile phases were chosen to simplify the preparation process and proved equally effective. All of the samples eluted within 15 minutes, regardless of mobile phase used.

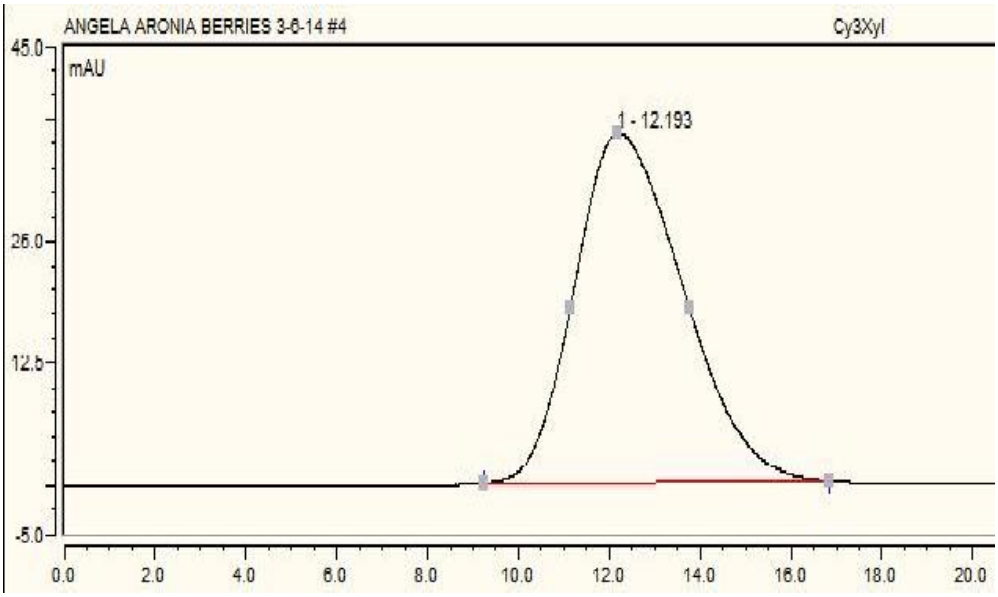

Figure 10. HPLC chromatogram with isocratic solvent 0.01% formic acid, 22.5% methanol, 50% acetonitrile. Peak elution was ~6 minutes. (Cyanidin-3-Xyloside)

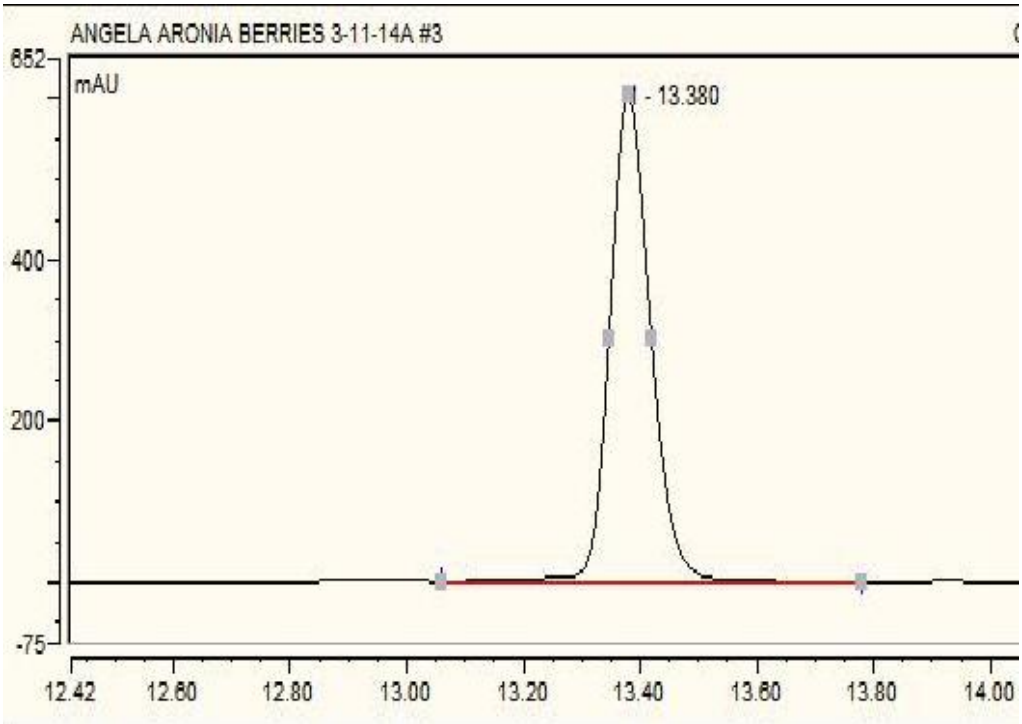

Figure 11. HPLC chromatogram of gradient conditions with 0.1% TFA (A) and acetonitrile (B). Peak resolution took ~0.2 minutes. (Cyanidin-3-Glucoside)

The first column tested was a C-6 phenyl column due to its non-polar phase. The peaks took 4 to 8 minutes to elute and were round without shoulders. The column was changed to a Jupiter C-18 column and the resulting peaks took 4 to 8 minutes to elute and had large shoulders (See figure 12). This suggests that anthocyanins were staying on the column for too long and could be due to the mobile phase used with the column. Jupiter columns are also designed for protein and peptide characterization, which could have created this effect on anthocyanins. Due to time restraints and in an effort to expedite the analysis of anthocyanins, the last column, a Gemini-NX C-18, was used in conjunction with a switch to the multi-step gradient mobile phase. The results showed a reduction in peak elution time to about 0.2 minutes per peak.

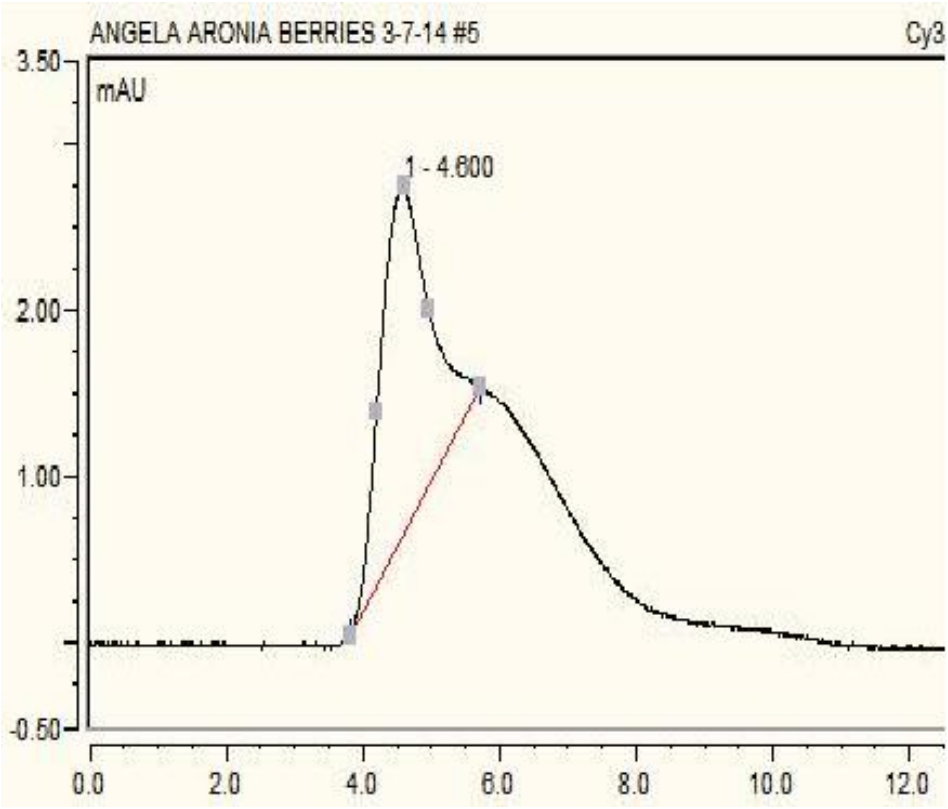

Figure 12. HPLC chromatogram of Jupiter C-18 column under isocratic conditions. An example of shouldering.

Discussion

HPLC and Colorimetric Assays

HPLC and total monomeric anthocyanin assays both use similar principles for detection but have differences in analysis time, compound specificity and detection sensitivity. There are two main factors in the analysis time between the two techniques. One is sample preparation and the other is sample analysis. Sample preparation for HPLC is a straight forward procedure that requires the extraction of anthocyanins from Aronia berries. This is done by adding acidified methanol to the berries, homogenizing them, and then centrifuging to remove particulate matter from the sample. To ensure high extraction yield of anthocyanins, the berry solids should be extracted three times with acidified methanol. The sample then can be used for analysis on HPLC, usually without dilution because of the small injection volumes used. The total monomeric anthocyanin assay requires the same extraction procedure and uses the same extract as the HPLC samples, but requires setting up a microplate to add buffer. The assay uses two microplate columns per sample for the pH 1.0 and pH 4.5 buffers, plus additional rows for duplicates. Setting up the microplate takes time to dispense the buffers to all of the wells for each sample. The total monomeric anthocyanin assay requires a much longer preparation time than HPLC sample preparation.

The analysis time difference between HPLC and total monomeric anthocyanin assay is also considerable. HPLC analyzes a single sample at a time and for our research we had a total run time of 30 minutes for each sample. The total monomeric anthocyanin assay takes about one minute to read a microplate. There is a considerable difference in

analysis time between analyzing samples using a microplate reader when compared to analyzing a single set of samples on HPLC.

There have been reported differences in detection sensitivity between HPLC and total monomeric anthocyanin assays. Lee et al. found that samples analyzed on HPLC gave values higher than total monomeric anthocyanin assays, with elderberry samples showing a difference of 2.0 to 2.3 times greater concentration in HPLC. The values between HPLC and total monomeric anthocyanin assay analysis in the study were highly correlated suggesting that they all had similar differences in concentration ratios [15]. Our research found similar differences between HPLC and total monomeric anthocyanin assay concentrations of anthocyanins. Figure 6 demonstrates this difference with samples of Viking Aronia berries. The total monomeric anthocyanin content as expressed in cyanidin-3-glucoside equivalents for HPLC was 6.85 mg anthocyanin per gram of berries and 3.27 mg/g for total monomeric anthocyanin assays. This is a 2.1-fold increase in anthocyanin concentration when using HPLC, similar to the values in Lee et al.'s study. These data suggest that total anthocyanin values are dependent on the method used to analyze them. This data would need to repeated several times to verify the results in figure 6, though, because it was only from one sample comparison due to time restraints.

A major difference between the analyses is that HPLC analyzes individual anthocyanins in a sample, whereas a total monomeric anthocyanin assay analyzes the total anthocyanin content in a sample. The detection of single compounds is unique to HPLC. A total monomeric anthocyanin assay measures all of the anthocyanins in a sample at a single wavelength and cannot differentiate them. HPLC sends samples through a liquid mobile phase where they are separated by polarity and travel through the

column at different times, allowing for identification of individual anthocyanins by their retention time. Therefore, HPLC is better suited for finding and separating compounds in a sample so single anthocyanins can be analyzed as well as total anthocyanins. HPLC is able to obtain more detailed analyses of compounds than a total monomeric anthocyanin assay.

Extraction Optimization

This research sought to optimize the extraction of anthocyanins from Aronia berries. An extraction is a separation process that removes compounds of interest from a material matrix. Most commonly, solvent extractions are used to extract a multitude of compounds from fruits, including anthocyanins. This process is accomplished by first grinding up the fruit to disrupt the cellular compartments where the anthocyanins are stored [9]. Then, due to the polar nature of anthocyanins, a polar solvent is used to extract the anthocyanins into the liquid phase where they can be analyzed through various techniques [9]. Often further purification is required because many other polar compounds are extracted along with anthocyanins and there can be particulate matter in the solvent [9]. Centrifugation can be utilized to remove particulate matter and further extraction with techniques such as solid phase extraction can be used to remove other polar substances.

Common solvents for anthocyanins include methanol, ethanol or acetone [9]. These, especially methanol and ethanol, are usually acidified to increase anthocyanin stability in solution and prevent degradation [9]. Acids that are commonly used in extractions include: acetic, citric, formic, tartaric and hydrochloric [26]. The solvents are

usually kept weakly acidic because hydrolysis of acylated and glycosidic anthocyanins can occur in strongly acidic conditions, therefore, degrading them [9]. Some studies have looked into the use of sulphur compounds as extractants. Cacae et al. used 80% ethanol with SO_2 and found that variations in sulphur concentration, temperature and solvent to solid ratio affected yields. Maximum yields were obtained at SO_2 concentrations between 1000-1200 ppm and 19 L of solvent per kg berry [27]. Increasing the temperature of the extraction increased the extraction speed, but temperatures above 35°C will degrade anthocyanins in the sample [27].

Many studies have found acidified methanol to yield the highest amount of anthocyanins, therefore, being the best solvent for anthocyanin extractions and the most commonly used [9]. Metivier et al. found that anthocyanin extraction from grape pomace was most effective with acidified methanol. It was 20% more effective than with ethanol and 73% more effective than water alone [28]. Awika et al. found that acidified (0.1% HCl) methanol was twice as efficient as 70% aqueous acetone at extracting anthocyanins from black sorghum [29]. The aqueous acetone extract was also found to make structural modifications in anthocyanins by oxidative addition which made them unable to be identified via HPLC [29].

This research examined the effects of different solvents and a 10 minute sonication step on the extraction yield. From reviewing the literature, acidified (0.1% Citric Acid) methanol and ethanol were chosen as solvents due to their high extraction efficiencies. The solvents were compared by examining their total yields as expressed in peak areas from HPLC chromatograms. The procedure was repeated with an added sonication step and results were compared between the trials.

HPLC Optimization

This research sought to optimize HPLC conditions for short analysis times and clear peak resolution. We used three different columns: a Gemini C6 Phenyl, 250 x 4.6mm, 5 µm; a Phenomenex C-18 Jupiter, 250 x 4.6mm, 5µm; and a Gemini NX C18 110A, 250 x 4.6mm, 5 µm. The columns all had non-polar stationary phases. Multiple solvents were used, including: 0.01% formic acid, methanol, acetonitrile and 0.1% trifluoroacetic acid (TFA). These were used in varying concentrations and differences between isocratic and gradient conditions were explored.

The type of mobile used in HPLC is important for the type of separation to be accomplished. In the case of anthocyanins, they are polar molecules and a polar mobile phase is used to elute them. This type of chromatography is called reverse-phase and uses a polar mobile phase and a non-polar column. Commonly, a water mobile phase and a less polar organic mobile phase such as acetonitrile or methanol are used [17]. This technique is useful for fast elutions of polar compounds such as anthocyanins, because the more polar a molecule is, the faster it will elute from the system [17]. Also, the less polar a mobile phase is, the faster polar molecules will elute [17]. The use of a multi-step gradient can be used to introduce less polar organic mobile phase to the water mobile phase and elute compounds faster and with higher resolution. As the organic mobile phase is introduced, it increases the solvent strength and improves elution of compounds that are retained on the column [17]. This gives a considerable advantage over isocratic conditions, where compounds that are not very miscible in the mobile phase can be retained by the column and cause peak broadening. The multi-step gradient will give better resolution and elution times over an isocratic condition.

The type of column used in HPLC is an important consideration when analyzing anthocyanins. Due to their polar nature, anthocyanins should be used with a non-polar column to reduce retention time on the column. These columns work on the principle of hydrophobicity, where hydrophobic interactions between the non-polar stationary phase and non-polar segments of compounds of interest occur [17]. This effect causes hydrophobic compounds to be more attracted to the column as more water is added, creating a hydrophilic mobile phase [17]. The more hydrophobic a mobile phase becomes (i.e. by adding more organic mobile phase) the less attracted hydrophobic compounds are to the column, therefore decreasing retention time. It is important to choose a non-polar, hydrophobic column, such as a C-18 column, when analyzing anthocyanins due to their polar nature.

This research found that using a Gemini-NX C-18 column in combination with the eluents 0.1% TFA and acetonitrile worked best for HPLC analysis of anthocyanins. The eluents were used in a multi-step gradient and had significantly better resolution over isocratic conditions. For analysis of anthocyanins on HPLC it is recommended to use a highly hydrophobic column like the C-18 column, along with a water based mobile phase and an organic based eluent in a multi-step gradient that introduces more organic mobile phase over time. This allows for faster retention times and quicker analyses with good resolution. Further experimentation is needed to resolve shouldered peaks that were found in this research. Possible solutions could be to change the flow rate, change the column chemistry, or change the eluent compositions. Changing the flow rate would decrease the retention time of every compound and could change the difference in retention time between the peaks enough to become two separate peaks. Changing the

column chemistry would change the interactions with each compound and could change retention times between compounds. Changing the eluent composition would change retention time by changing the polar interactions of each compound. The less polar the eluent is, the faster polar compounds like anthocyanins will elute.

Conclusions

This research has found that total anthocyanin concentrations vary between method of analysis (HPLC or total monomeric anthocyanin assay) used, with HPLC showing the highest values of total anthocyanin content. The data show that HPLC reported a 2-fold increase in total anthocyanin content over total monomeric anthocyanin assays. HPLC is most useful when identifying and quantifying individual anthocyanins in samples. Samples can also be stored reliably for weeks at a time without much degradation. The total monomeric anthocyanin assay is a simpler and cost efficient method for analyzing total anthocyanin content. It can analyze large sample sizes rapidly and a microplate reader or spectrophotometer is less costly than an HPLC system. Therefore, it is recommended to use HPLC when looking to identify and quantify individual anthocyanins. When large numbers of samples need to be analyzed, or there is not access to an HPLC system, a total monomeric anthocyanin assay is a good alternative method of analysis.

Acidified methanol was found to be a superior solvent over acidified ethanol in the extraction of Aronia berries because of its higher yield. The extraction was also more effective with an added sonication for 10 minutes after homogenizing the berries. As

only two solvents were tested, this brings up the question of what other solvents could be more effective than acidified methanol. Some possible solvents that could be looked into in the future are acetonitrile, acetone or water due to their polar properties. These would all have to be acidified in order to keep the anthocyanins within the solution stable. Acetone has been found to cause structural changes in anthocyanins via oxidative addition and should, therefore, be used with caution [9]. Other methods for extraction could be looked into further, such as freeze drying of the berries, which could help in keeping anthocyanins from degrading while being stored, allowing for a greater extraction yield. Letting the homogenized solution stand for greater periods of time (an hour or greater) would allow for greater extraction yields of anthocyanins, also. The pH of the solution is important in the extraction and stability of anthocyanins in solution. Keeping pH lower than 3 is necessary to prevent the formation of the colorless hemiketal form of anthocyanins. The solid to solvent ratio is also an important consideration in the extraction as too much solvent will lead to more dilute anthocyanin concentrations in the final extract.

This research focused on protocol development and therefore had limitations when it comes to statistical analysis. There was a limited amount of berries to work with and there could only be so many extractions done for each cultivar. This limited the number of extractions and the types of solvents that could be used. Time was also of concern for this research. It took a few weeks to train with the HPLC equipment and software. This left a short amount of time to dedicate towards research, data analysis and writing. If there was more time and more berries, this research could have looked into

other extraction techniques and HPLC set ups. Hopefully, this will be explored in further research and this research will be the starting point for further optimization.

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Author's Bio

Kyle Rousseau was born and raised in Lewiston, ME. He graduated from Lewiston High School in 2010 and will graduate from the University of Maine in 2014 with a B.S. in Biochemistry. Kyle also has a minor in chemistry and interests in neuroscience, food science and nutrition. He is a brother of Sigma Phi Epsilon fraternity and has received a Maine Scholars Achievement Award and a Center for Undergraduate Research Fellowship for thesis research.

After graduating, Kyle has secured a 10 week internship to do research with ion-mobility mass spectrometry at Baylor University in Waco, Texas. He then plans to find an industry job in analytical chemistry research before working on an advanced degree.