Polyphenol Composition of Underutilized Aronia Berries and Changes in Aronia Berry Polyphenol Content Through Ripening

Rod Taheri
rod.taheri@gmail.com

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Polyphenol Composition of Underutilized Aronia Berries and Changes in Aronia Berry Polyphenol Content Through Ripening

Presented by
Rod Taheri, B.S.

Major Advisor
Bradley W. Bolling

Associate Advisor
Ock Chun

Associate Advisor
Mark Brand

University of Connecticut
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Abstract

Chokeberry (*Aronia melanocarpa*, *Aronia prunifolia*, *Aronia arbutifolia*) is notable for its high phytochemical content, particularly anthocyanins and proanthocyanidins. Aronia berries of different genotypes may vary in their polyphenol content, so it is important to characterize these compounds based on species, and to determine whether or not aronia fruit color correlates with phytochemical content. Using normal-phase HPLC, UPLC-MS, and the DMAC and Folin-Ciocalteu assays, polyphenols in twelve different aronia accessions were quantified and characterized based on fruit color. Significant diversity was observed in anthocyanin content among berries of different colors, with black aronia berries (mean = 10.47 mg/g DW) having universally higher levels of anthocyanins than purple aronia berries (mean = 3.24 mg/g DW), and lastly aronia berries (mean = 0.63 mg/g DW) (P = 0.0018). Total phenol values were, however, were higher in red and purple chokeberries than black chokeberries (mean total phenol values were 185, 187, and 167 mg GAE/g DW, respectively). Proanthocyanidin, hydroxycinnamic acid, and flavonol contents did not differ among the three aronia species. Differences in moisture percentage among different accessions did not significantly affect polyphenol content.

Due to the potential of chokeberry as a nutraceutical crop or a source of polyphenols for supplements, we determined the changes in polyphenol content and antioxidant activity of the ‘Viking’ chokeberries (the most readily available commercial variety), throughout fruit ripening. ‘Viking’ chokeberries were harvested once per week for 7 weeks, juiced, and their polyphenol content was quantified using UPLC-MS, and the DMAC and Folin-Ciocalteu assays. Changes in sugar content were analyzed using a refractometer and quantified in degrees brix. Antioxidant
activity was analyzed using the FRAP assay. Anthocyanins increased by 227% between the first and fifth weeks of ripeness, then declined (P < 0.0001). Proanthocyanidins increased by 67% through ripening (P < 0.0001), sugar content increased 36% (P < 0.0001) between the first and last week of harvest, and hydroxycinnamic acids decreased by 33% (P < 0.0001). Total phenols increased by 24% (P < 0.0001) and antioxidant activity via the FRAP assay increased by 53% (P < 0.0003) during ripening in ‘Viking’ chokeberries. No significant trend was observed in flavonol content throughout the growing season. In conclusion, antioxidant capacity correlates most closely with total phenol content, and week 7 is the optimal time to harvest aronia berries with respect to antioxidant capacity.
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List of Abbreviations

AAPH: 2,2’-Azobis(2-amidinopropane) dihydrochloride
ANOVA: Analysis of variance
Anth: Anthocyanins
CE: Catechin equivalents
Cga: Chlorogenic Acid
Cy3A: Cyanidin-3-arabinoside
Cy3Gal: Cyanidin-3-galactoside
Cy3Glu: Cyanidin-3-glucoside
Cy3Sam: Cyanidin-3-sambubioside
Cy3X: Cyanidin-3-xyloside
Del3A: Delphinidin-3-arabinoside
Del3Gal: Delphinidin-3-galactoside
Del3Glu: Delphinidin-3-glucoside
DMAC: 4-(dimethylamino) cinnamaldehyde
DPPH: 2,2-diphenyl-1-picrylhydrazyl
FW: Fresh weight
GAE: Gallic acid equivalents
HCA: Hydroxycinnamic acids
ICAM-1: Intercellular Adhesion Molecule 1
LOD: Limits of detection
LOQ: Limits of quantitation
Mal6Acetyl3Glu: Malvidin-6-acetyl-3-glucoside
Mal3A: Malvidin-3-arabinoside
Mal3Gal: Malvidin-3-galactoside
Mal3Glu: Malvidin-3-galactoside
nCga: Neochlorogenic acid
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NF-κB p65: nuclear factor kappa-light-chain-enhancer of activated B cells protein 65
OC: On column
ORAC: Oxygen Radical Absorbance Capacity
PACs: Proanthocyanidins
PCA: Protocatechuic acid
Pel3A: Pelargonidin-3-arabinoside
Pel3Gal: Pelargonidin-3-galactoside
Peo3Gal: Peonidin-3-galactoside
Peo3Glu: Peonidin-3-glucoside
Pet3A: Petunidin-3-arabinoside
Pet3Gal: Petunidin-3-galactoside
Pet3Glu: Petunidin-3-glucoside
Q3Gal: Quercetin-3-galactoside
Q3Glu: Quercetin-3-glucoside
Q3R: Quercetin-3-rutinoside
RSD: Relative standard deviation
TBHQ: tert-Butylhydroquinone

TE: Trolox equivalents
TNFα: Tumor Necrosis Factor α
TP: Total phenols
VCAM-1: Vascular Cell Adhesion Molecule 1
Introduction

Aronia, also known as chokeberry, is a member of the Rosaceae family and a shrub native to the northeastern United States. Chokeberry shrubs are usually between 2 and 3 meters high, depending on the fertility of the soil that they are grown in [1]. They bear fruit for 15 to 20 years [1]. Aronia has the benefit of cold hardiness [1]. Aronia shrubs begin to flower in May, and fruits are ripe throughout August and September [1].

Chokeberry exists as three major species: *Aronia melanocarpa*, which has mainly black berries, *Aronia prunifolia*, which produces purple berries, and *Aronia arbutifolia*, which bears red fruit [1]. There are however, exceptions as some genotypes will turn red before turning purple. Chokeberries are of particular interest to food scientists as they have exceptionally high levels of phenolic compounds compared to many other foods in the American diet. Anthocyanins and proanthocyanidins are particularly abundant in chokeberries [1]. Anthocyanins are water soluble polyphenols responsible for blue, purple, and some red colors in many plant tissues. Regular consumption of anthocyanin-rich foods has been associated with an 8% decrease in hypertension and a decreased risk of cardiovascular disease in humans [2]. Aronia berry is currently the richest known source of dietary anthocyanins [3]. Chokeberry has mainly been used as a natural food dye, however as information about its phytochemical content and potential health benefits has become more widespread, chokeberry juices like Superberries® Aronia Juice Concentrate, jams, wines like Maple River Winery Aronia Wine, and supplements like Swanson® full spectrum Aronia 400 mg can be found for sale in the United States.
The goals of the research described in this thesis include:

1) Development of methods for quantitation of anthocyanins, proanthocyanins, quercetin derivatives, and hydroxycinnamic acids in chokeberry;

2) Characterization of polyphenol content in red, black and purple chokeberries, as represented by twelve chokeberry genotypes via HPLC, UPLC-MS, and the DMAC and Folin-Ciocalteu assays; and

3) Assessment of changes in antioxidant activity, polyphenol content, and sugar content of *Aronia mitschurini* (*Aronia* cultivar ‘Viking’) throughout fruit ripening, before and after the peak of ripeness.

We compared the polyphenol compositions of twelve different chokeberry accessions, three red (UC021, UC053, and UC057), four black (UC009, ‘Viking’, PI636375, and AMES27010), and five purple (UC007, UC011, UC033, UC047, and PI578096). Chokeberry genotypes were compared on the anthocyanins, proanthocyanidins, flavonols, and hydroxycinnamic acids, since to date these are the most commonly observed polyphenols in chokeberry [4,5]. By characterizing polyphenols in these twelve chokeberry accessions, we can identify trends in phytochemical content on the basis of color, and determine which accession would be best to utilize as source of polyphenols for foods, supplements, or for future studies examining the health benefits of berry consumption.

With ‘Viking’ chokeberry being the most readily available commercially and high in our polyphenols of interest, we elected to test ‘Viking’ chokeberries throughout the harvest season for changes in phytochemical content related to ripeness. Berries were harvested from the onset of ripeness (8/01/2012) until the end of the harvest season, when berries were past peak ripeness
(9/12/2012). They were juiced in a cider press and the seven weekly juice samples were analyzed for changes in polyphenol and sugar content, as well as taste, to determine the true peak of ripeness, and when to harvest chokeberries to maximize polyphenol yield.
Chapter 1:

Literature Review
1.1 HEALTH BENEFITS OF ARONIA

Aronia polyphenols have been marketed for their potential in decreasing the risk of heart disease through their antioxidant and anti-inflammatory activity. Aronia consumption also has a protective effect on some body tissues, including, but not limited to the lung, kidney, and liver. These effects are believed to be related to its high concentrations of phenolic compounds including anthocyanins and proanthocyanidins [1]. Aronia is also known to have a resistance to many pests and microbes, reducing the need for pesticides during cultivation, as well as the risk of consumption of pesticide residues [1]. Flavonols are known to have direct inhibitory activity on some viruses. Quercetin for example, actively inhibits the tomato ring-spot virus by directly interfering with its life cycle [6]. A 5 µg/mL quercetin dose reduced ring-spot virus titre by 89% over a period of 36 weeks in tomato meristematic tip cultures [6]. Many of the health benefits of Aronia consumption are credited to the polyphenols present, including proanthocyanidins, anthocyanins, hydroxycinnamic acids, and flavonols.

1.2 ARONIA POLYPHENOL CLASSES AND THEIR BIOACTIVITY

Proanthocyanidins

Proanthocyanidins are chains of flavan-3-ol units. A-type PACs are linked through C4→C8 or C4→C6 bonds, and have a C2-O7 ether linkage as well. B-type PACs are only linked via C4→C8 or C4→C6 bonds. The primary flavan-3-ols in chokeberry are catechin and epicatechin, which differ in their stereochemistry [7]. Catechin has a trans configuration in its C2-C3 linkage, while epicatechin has a cis configuration at this position [7]. PAC’s consisting of catechin and
epicatechin are called procyanidins, and are abundant in chokeberry [7]. PACs consisting of afzelechin and epiafzelechin are called propelargonidins, and those comprised of epigallocatechin and gallocatechin are called prodelphinidins [7]. The average intake of proanthocyanidins in the United States is about 58 mg per day [8]. Proanthocyanidins are of interest for their antioxidant capacity. Orozco et al. conducted a study on the antioxidant effect of proanthocyanidins involving male Sprague-Dawley rats that were fed diets containing 0%, 0.5%, 1%, or 2% cocoa rich in flavonols and proanthocyanidins for two weeks. They reported that cocoa supplementation was associated with a reduction in testicular 8-hydroxy-2'-deoxyguanosine, a marker for DNA oxidative damage [9]. Further, less oxidized glutathione in erythrocytes was also observed in the cocoa-fed group [9].

A clinical study by Natella et al. administered a meal high in oxidized and oxidizable lipids with or without 300 mg of grape-seed PAC extract to eight healthy volunteers and measured lipid hydroperoxides in their plasma and chylomicrons [10]. After consumption of the meals, chylomicron hydroperoxides were 1.5-fold higher in the control group, and plasma lipid hydroperoxides were only elevated in the control group. Supplementation of grape-seed proanthocyanidin extract limited post-prandial oxidative activity and consequently decreased the rate of LDL oxidation, providing a modest cardio-protective effect [10].

Another notable benefit to consumption of proanthocyanidins is the *E. coli* anti-adhesion effect of cranberry proanthocyanidins to epithelial cells in the urinary tract. Cranberry juice was originally thought to have a bacteriostatic effect by acidifying the urine, [7]. Foo et. al. later identified A-type proanthocyanidins consisting of epicatechin subunits with degrees of polymerization from three to five as the compounds responsible for this anti-adhesion activity.
Effects were observed in epithelial tissues containing $\alpha$-Gal(1→4)$\beta$-Gal receptor sequences [11].

Proanthocyanidins are highly susceptible to degradation and de-polymerization during cooking, drying, and processing. PACs are found in abundance in grapes and fresh plums, while they are not detectable in raisins and prunes (Gu, unpublished data). Prior and Gu found that simmering pinto beans in water for 2 h lead to a considerable loss of PACs with degrees of polymerization >10 [7]. They also found pear infant formula to contain only 13.4 mg/100 g PACs, while fresh pears contained 31.9 mg/100 g, indicating a significant loss during processing [7].

**Anthocyanins**

Anthocyanins are glycosylated pigmented flavonoid compounds found in many plant tissues. Aglycone anthocyanins are referred to as anthocyanidins. The most abundant anthocyanidins include cyanidin, delphinidin, malvidin, peonidin, petunidin, and pelargonidin. Cyanidin exhibits maximal light absorption at 525 nm giving it a purplish-blue hue, while delphinidin glycosides are at 535 nm, making them the closest anthocyanin compounds to the blue spectrum (580 nm) [1]. Blue flowers are known attractors of bee pollinators, so flowers that rely on bee pollination have evolved to contain anthocyanins in their petals, particularly cyanidin and delphinidin glycosides [1].

The average intake of anthocyanins for Americans is about 200 mg per day [12]. Anthocyanins are known mainly for their antioxidant and anti-tumorigenic activity [13]. Kang et al. tested the effects of tart cherry anthocyanins on colon cancer development in APC$^{\text{Min}}$ mice [13]. Tart cherry anthocyanins, like chokeberry, are primarily cyanidin derivatives [15]. The APC gene (adenomatous polyposis coli) is considered a gatekeeper in colon carcinogenesis [14]. APC$^{\text{Min}}$ mice are predisposed to frequent colonic adenomas. Kang et al. fed either a tart cherry
anthocyanin-rich or a control diet to APC\textsuperscript{Min} mice. After 10 weeks, anthocyanin-fed mice were found to have an average of 0.6 colonic adenomas each as opposed to control mice which had a mean of 2.3 adenomas per mouse [15]. Adenoma volume per mouse was 0.7 mm\textsuperscript{3} in the anthocyanin group, as opposed to 3.0 mm\textsuperscript{3} in the control group [15].

Bornsek et al. demonstrated that anthocyanins extracted from blueberry and bilberry have intracellular antioxidant activity, even at concentrations <1µg/L in human colon cancer (Caco-2), human hepatocarcinoma (HepG2), human endothelial (EA.hy926) and rat vascular smooth muscle (A7r5) cells [16]. Karlsen et al. found that anthocyanins from bilberries and black currants suppressed LPS-induced NF-κB activation in cultured monocytes [17]. The same group also conducted a study on 300 mg per day of anthocyanin supplementation for 3 weeks in 120 men and women ages 40 – 74 [17]. They found decreases in multiple pro-inflammatory chemokines including a 60% decrease in IL-4, a 45% decrease in IL-8, a 38% decrease in IL-13, and a 40% decrease in IFNα. Their data shows promise for anthocyanin supplementation in the treatment or prevention of chronic inflammation [17]. Hassellund et al. conducted a study in which they fed pre-hypertensive men either 640 mg of anthocyanins (mostly cyanidin and delphinidin glycosides) or a placebo for 4 weeks after a 4-week washout period [18]. They found that HDL cholesterol was significantly elevated in the anthocyanin group compared to the control, and that triglycerides and lipoprotein A were lowered as well. Their findings indicate potential cardioprotective benefits from anthocyanin consumption [18].

**Hydroxycinnamic Acids**

Hydroxycinnamic acids are derivatives of cinnamic acid, a slightly water-soluble phenolic compound. The most common hydroxycinnamic acid in the diet is chlorogenic acid, which is
caffeic acid linked to quinic acid through an ester bond. The primary source of dietary chlorogenic acid is coffee, and coffee drinkers consume 0.5 to 1 g per day of chlorogenic acid [19]. Other hydroxycinnamic acids commonly found in the diet are caffeic acid, ferulic acid, and p-coumaric acid, and they have been found in many foods including blueberries, grapes, apples, cereal brans, broccoli, spinach, and lettuce [20]. In vitro studies have shown that hydroxycinnamic acids, particularly those containing a second phenolic hydroxyl group can protect against LDL oxidation and spare endogenous antioxidants. Nardini et al. tested the inhibitory effect of 5 µM caffeic acid against LDL oxidation by Cu$^{2+}$ and 2,2$'$-azobis (2-amidinopropane)-hydrochloride (AAPH) [21]. They found that 5 µM caffeic acid efficiently inhibited Cu$^{2+}$ induced LDL oxidation and temporarily inhibited AAPH induced oxidation. A dose of 100 µM caffeic acid almost completely inhibited LDL oxidation by either catalyst [21]. The ability of 5 µM caffeic acid to prevent α-tocopherol and β-carotene oxidation by 5 µM Cu$^{2+}$ were tested as well, since α-tocopherol is the predominant antioxidant to LDL in the human body. Copper-induced oxidation consumed all endogenous α-tocopherol and β-carotene within the first hour in the control group, however the group administered 5 µM caffeic acid showed 40% residual α-tocopherol and 75% residual β-carotene after 1 h. Caffeic acid and α-tocopherol were exhausted after 2 h of incubation, and β-carotene lasted 3 h in the 5 µM caffeic acid model [21].

1.3 ARONIA POLYPHENOL AND SUGAR CONTENT

Anthocyanins
Black chokeberry is currently the richest known source of dietary anthocyanins [1]. Wu et al. determined the anthocyanin and proanthocyanidin content of black chokeberries, black currant, red currant, gooseberry, and elderberries via UPLC-MS [12]. They used black chokeberry fruit, and found cyanidin-3-galactoside (Cy3Gal), cyanidin-3-glucoside (Cy3Glu), cyanidin-3-arabinoside (Cy3Ara), cyanidin-3-xyloside (Cy3Xy), pelargonidin-3-galactoside, pelargonidin-3-arabinoside, cyanidin + rhamnose + pentose, and one non-identified anthocyanin species [12]. Chokeberry had 1,480 mg/100 g FW total anthocyanins, the highest concentration among the berries in the study. Cy3Gal was the predominant anthocyanin, representing 67% of total anthocyanins, followed by Cy3Ara, which represented about 27% of total anthocyanins. Only traces of pelargonidin glycosides were detected [12].

Maata-Riihinen et al. found the anthocyanin content of black chokeberry to be 8,421 mg/kg (aglycone) [22]. The only anthocyanins that they detected were cyanidin derivatives. Chokeberry had the greatest total anthocyanin content of the 18 berries that they tested [22]. Strik et al. found the total anthocyanin content of 5 varieties of black chokeberry to range from 440 to 574 mg/100 g, values which are generally lower than those reported elsewhere [23]. In other fruits, particularly those of pink or red hue, different anthocyanins predominate, for example in strawberry; the predominant anthocyanin is pelargonidin-3-glucoside [24]. Anthocyanins that have been characterized in chokeberry are shown in Figure 1.1.

**Proanthocyanidins**

Prior et al. report that the proanthocyanidin content of chokeberry is about 81.8% polymeric, that they are comprised of catechin sub-units, and that they have B-type linkages [7].
quantified by Wu et al. via normal-phase HPLC [12]. Chokeberries were found to contain 663.7 mg/100 g FW of total proanthocyanidins. This was the highest content of proanthocyanidins among the berries tested, and about 4 times higher than the next highest berry, the Ben Alder cultivar of black currant. Proanthocyanidins in currants, gooseberries, and chokeberries were predominantly >10-mers, however in elderberries, no polymers were detected [12]. Examples of proanthocyanidins are shown in Figure 1.2.

Hydroxycinnamic Acids

Maattaa-Riihinen et al. performed ethyl-acetate extractions of lyophilized black chokeberry and using reverse-phase chromatography, found 892 mg/kg FW of total hydroxycinnamic acids, 93.3% of which were caffeic and ferulic acid derivatives [23]. They found that chlorogenic acid (a caffeic acid derivative) was the predominant hydroxycinnamic acid found in their analysis, followed by neochlorogenic acid. Of the 18 berries that they tested, chokeberry had the greatest hydroxycinnamic acid content by 24% [23]. Hydroxycinnamic acids that have been characterized in chokeberry are shown in Figure 1.3.

Flavonols

The flavonol content of ‘Viking’ chokeberries was characterized by Hakkinen et al. using HPLC and values were compared to 24 other edible berries [25]. Quercetin, myricetin, and kaempferol were the only flavonols quantified in these berries, and of these, quercetin was the only flavonol detected in chokeberry [25]. In this study, flavonols were hydrolyzed via reflux for 2 h in 50% acidified methanol with tert-butylhydroquinone (TBHQ) as an antioxidant. Quercetin was present at 89 mg/kg FW in chokeberry after hydrolysis, the second highest concentration after bog whortleberry (158 mg/kg) [25]. Chokeberry had the seventh highest total flavonol content;
however cranberry and black currant were the only commonly consumed berries with greater
total flavonols than chokeberry [25]. Maata-Riihinen et al. found 348 mg/kg of quercetin in black
chokeberry, which was the fourth highest quercetin value of the 18 berries that they studied, after
three genotypes of bog whortleberry [23]. They also reported traces of kaempferol in chokeberry
[22]. Therefore, berries, and particularly black chokeberries, are rich sources of flavonols.
Flavonols that have been characterized in chokeberry are shown in Figure 1.4.

_Sugars_

Mikulic-Petkovsek et al. measured the sugar profile of 25 different berries and found that the
sugar content of chokeberry was 31% glucose, 24% fructose, 3.5% sucrose, and 40.5% sorbitol
[26]. Chokeberry, rowanberry, and eastern shadbush were the only berries which contained
sorbitol [26]. Chokeberry had 618mmol/kg total sugars, the fourth highest total sugar content
amongst the berries in the study, only surpassed by rowanberry, dog rose, and eastern shadbush.
Chokeberry had a greater concentration of total sugars than those commonly consumed in the
American diet including strawberries, blackberries, blueberries, currants, raspberries, and
cranberries. In spite of the high sugar content, chokeberry maintains considerable astringency
when compared to these other berries and this may be attributed to phenolic content [26]. Sugars
that have been characterized in chokeberry are shown in Figure 1.5.

_Total Phenols_

Total phenols measured in the Mikulic-Petkovsek study were found to be 10,132 mg GAE/kg in
chokeberry, almost twice as high as the wild elderberry (5,149 mg GAE/kg), which had the
second highest total phenol content among 25 species of berries [26]. The Folin-Ciocalteu assay
is a measure of reducing capacity, and can over-estimate the phenolic content of a sample containing amino acids, carbohydrates, or ascorbic acid [27].

1.4 POLYPHENOL EXTRACTION AND QUANTITATION

Proanthocyanidins

Extraction and quantitation of proanthocyanidins can be challenging due to their unstable nature and wide range of molecular sizes. Proanthocyanidins are susceptible to spontaneous oxidation as well as degradation by polyphenol oxidases naturally found in plants. Plant samples should be lyophilized as early as possible, and kept at very low temperatures to slow degradation. Many methods exist for PAC extraction; however an acidified aqueous solvent containing 70% acetone has been shown to help disassociate bonds between PACs and the polar plant matrices they are bound to [29]. Gel permeation, such as Sephadex LH-20 or TSK gel HW-40 is a valuable means of fractionating PACs from extracts [7].

Several methods exist for quantitation of proanthocyanidins; among those are colorimetric and chromatographic methods. Examples of colorimetric methods for PAC quantitation include the HCl/butanol, vanillin, and 4-(dimethylamino) cinnamaldehyde (DMAC) methods. The HCl/butanol assay uses HCl to cleave proanthocyanidins to form carbocations, which are spontaneously converted to anthocyanidins [7]. These anthocyanidins are measured to estimate PACs. The formation of anthocyanidins from cleaved PACs is reportedly low however, and side reactions, PAC structure, and the presence of transition metals can greatly influence the results of the assay [7]. The DMAC reacts with the terminal units of PAC oligomers and polymers, giving
the same molar extinction coefficients for all PACs regardless of their respective number of subunits [30]. The DMAC reagent may also cause precipitation of highly polymeric PACs and for these reasons the DMAC assay may underestimate polymeric PACs [7]. While colorimetric methods are convenient for the purposes of sample comparison, they are empirical in nature, and provide little qualitative data on PACs, such as degree of polymerization, interflavan linkage types, and subunit structure [7].

Chromatographic methods for PAC quantitation provide further data about PACs’ structure. Reversed phase liquid chromatography can be used to quantitate PACs, however is limited in its ability to differentiate oligomers. While monomers, dimers, and trimers separate well, tetramers and above tend to elute together in one broad, unresolved peak, and degree of polymerization becomes indistinguishable [17, 18]. The gel permeation method, while useful for extraction and isolation of PACs, is unfortunately an ineffective method of quantitation, and offers results similar to reversed-phase chromatography: separation of monomers through trimers, and co-elution of all PACs with a degree of polymerization above 4 [7]. PACs with higher degrees of polymerization are generally more abundant in plant tissues than monomers and oligomers. Therefore, effective quantitation methods need improvement to separate PACs with greater degrees of polymerization [33],[34].

In 1993, a method to separate PACs by normal phase HPLC was used to successfully separate PACs in cocoa beans up to pentamers [35]. Optimization of this method and the incorporation of fluorescence detection were used to separate cocoa PACs up to the degree of decamers [35]. Though this method is reliable and reproducible, accurate quantitation of oligomers and polymers remains problematic, as commercial standards for PACs with higher degrees of
polymerization are not readily available. Another complication with PAC quantitation is peak integration. While PACs up to decamers can be resolved by separate peaks, there is a degree of overlap which increases as the degree of polymerization increases, and causes an upward shift in the baseline. Thus, differences in integration methods between laboratories could lead to divergent results. While valley-to-valley integration may underestimate PACs, integration with a flat baseline could yield unreliable values as well. Gu. et al. used a flat baseline to integrate PACs, and checked the method using PAC recovery tests in various food matrices including rice, tomato, and nuts to determine if the quantitation method accurately reflected the PAC content of their samples [36]. They determined that recovery of PACs decreases as degree of polymerization increases, with 99.5% recovery for monomers and 81.2% for polymers [36].

**Anthocyanins**

Several methods have been used to quantify anthocyanins in fruit. Wu et al. used mass spectrometry to quantify anthocyanins in currants, gooseberries, elderberries, and chokeberries [12]. Maatta-Riihinen et al. used reverse-phase HPLC and UV-VIS to quantify anthocyanins in several different species of berries [22]. Kalt and McDonald measured anthocyanins using a pH differential method with malvidin-3-glucoside's extinction coefficient [37]. There is currently no data comparing anthocyanin quantitative method precision. Due to the highly unstable nature of these compounds, extraction may be the critical parameter to standardize assay methods.

Wu. et al compared two different extraction solutions on anthocyanin composition using a number of berry samples [12]. They performed an anthocyanin extraction using a methanol/water/acetic acid (85:14.5:0.5; v/v) extraction solution, as well as a separate solution consisting of acetone/water/acetic acid (85:14.5:0.5; v/v). They found that using acetone as a
solvent for anthocyanin extraction in black currants lead to the formation of artifacts including four pyranoanthocyanins derived from either cyanidin or delphinidin glycosides and two furoanthocyanidins, derived from delphinidin [12]. They reported that after 72 h in 70% aqueous acetone at 40 degrees C, the anthocyanin peaks in their samples had almost completely disappeared while pyranoanthocyanin peaks increased significantly [12]. They reported that the transformation was slower at room temperature but did not report details. They concluded that the use of methanol as a solvent preserved anthocyanin structure better than acetone during extraction [12]. Lu et al. described the pyranoanthocyanin formation mechanism as the nucleophilic addition of an electron-rich enol form of acetone onto the C-4 of a pyrylium salt followed by intramolecular hemi-acetal formation, and oxidation and dehydration reactions delocalizing the charge [38]. Therefore, it is suggested that acetone is not the most effective extraction solvent for anthocyanins, however more research needs to be done in this direction to determine differences in extraction yield between different methods.

Hydroxycinnamic Acids

Various methods have been used to extract hydroxycinnamic acids from plant tissues. Bahri et al. used an extraction solution consisting of ethanol/water/acetic acid (80:15:5, v/v) to extract hydroxycinnamic acids from chicory leaf [39]. They dried their extracts and re-suspended them in 50% methanol for HPLC analysis [39]. Maatta-Riihinen et al. extracted hydroxycinnamic acids, low molecular weight proanthocyanidins, and flavan-3-ols using ethyl acetate [22]. They observed coelution between these compounds however, so they isolated hydroxycinnamic acids by extraction with pH 7.0 sodium acetate buffer and water. Ionizable phenolic acids were removed with the water phase and flavan-3-ols and proanthocyanidins were left in the ethyl
acetate phase [22]. Olsson et al. extracted hydroxycinnamic acids from strawberries using 50% methanol and 1.2 M HCl, a method originally developed by Hakkinen et al. [40],[41].

Wen et al. used HPLC and UV-Vis to quantitate chlorogenic acid in extracts of *Lepidogrammitis drymoglossoides*, a medicinal herb [42]. They found that their mean recovery rates were between 95% and 104% and values were in accordance with previous studies [42]. Olsson et al. also analyzed hydroxycinnamic acids using HPLC with UV-vis detection, using 50 mM acetic acid and 5% acetonitrile in methanol as mobile phases [40]. Maatta-Riihinen et al. used reverse-phase HPLC with diode array detection to quantitate hydroxycinnamic acids in eighteen species of berries [22]. NMR-spectroscopy has been used to quantify chlorogenic acid in blueberry leaves and has shown to be effective even in crude extracts, with LOD and LOQ both calculated at 0.01 mM chlorogenic acid [43]. NMR was also used by del Campo et al. to quantify chlorogenic acid in apples [44].

**Flavonols**

Due to their hydrophilic nature, many of the same extraction protocols used for hydroxycinnamic acids or anthocyanins can be used for flavonols as well. Hakkinen et al. refluxed thawed berries in 50% aqueous methanol containing 1.2 M hydrochloric acid and TBHQ for 2 h at 85°C [25]. This yielded flavonols in their aglycone state. They compared extraction solutions of 25 to 64% methanol in water and found that flavonol yield was 20 to 70% higher when using 50% methanol [25]. The addition of TBHQ as an antioxidant increased flavonol yield by up to 30% [25]. In contrast, Maatta-Riihinen et al. used ethyl acetate as an extraction solvent for flavonols, and reported 0 – 3% loss of hydroxycinnamic acids in all but seabuckthorn, in which 31% of Flavonols were lost [22]. The unresolved flavonols were believed to be bound to cell-walls and
resistant to this extraction method [22]. Acetone has also been used as an extraction solution for flavonols by Heinonen et al. but they did not report extraction yield [45].

Hakkinen et al. used reverse-phase HPLC and UV detection, paired with mass spectrometry and diode array detection to quantify the flavonols: quercetin, myricetin, and kaempferol, in 25 different berries [25]. Their mobile phases for chromatography were 1% formic acid and 100% acetonitrile [25]. Recovery of flavonols ranged from 50 – 106%, and varied largely based on the species of berry tested [25]. They believed that differences in recovery were mainly due to chemical reactions between metal chelators or copigmentation reactions with other phenolic compounds [25]. Diaz-Garcia et al. quantified flavonols in several fruit juices, using HPLC with UV detection and trifluoroacetic acid/acetonitrile/water (0.5/50/49.5, v/v) and trifluoroacetic acid/water (0.5/99.5, v/v) as mobile phases [46]. They did not report on their yield [46].

1.5 POLYPHENOL BIOAVAILABILITY AND METABOLISM

Proanthocyanidins

The degree of polymerization of PACs plays an important role in their metabolic fate. Gonthier et al. compared the metabolism of proanthocyanidin dimers, trimers, and polymers against a catechin control [47]. After 5 days of feeding, 25.7% of consumed catechin was detected in the urine of the control group in the forms of catechin and 3’-O-methyl-catechin. Microbial metabolites of PACs were detected in the urine as well, and 16 derivatives of phenylvaleric, phenylpropionic, phenylacetic, and benzoic acid were identified [47]. Total recoveries after feeding were 10.6% for the monomer-fed group, 6.5% for the dimer-fed group, 0.7% for the
trimer-fed group, and 0.5% for the polymer-fed group [47]. The results indicate that higher
degrees of polymerization limit intestinal absorption of PACs and also inhibit metabolism by
intestinal microflora [47].

**Anthocyanins**

Anthocyanins have reportedly poor bioavailability. Anthocyanins can be degraded to phenolic
acids by intestinal microflora [48]. Food matrix, differences in intestinal microflora, and dosage
may also affect the bioavailability and metabolic fate of anthocyanins [48]. Wiczkowski et al.
gave 13 people chokeberry juice rich in anthocyanins to observe the metabolism of diet relevant
anthocyanin doses [49]. They measured the concentration of parent compounds and metabolites
in the urine and plasma, and found intact anthocyanin glycosides as well as methylated and
glucuronidated metabolites in the plasma [49]. After the three-day washout, no anthocyanins or
metabolites were detected in the plasma and urine. Within the first 8 h after consumption, 96% of
urinary anthocyanins were excreted in the urine, of which 30% were unmetabolized compounds
and 70% were metabolites [49].

A literature survey performed by Manach et al. shows that after an oral dose of 150 to 2000 mg
of total anthocyanins from berries or berry extracts, the range for $C_{\text{max}}$ was 0.75 to 4 h with a
mean of 1.5 h for plasma, and a mean of 2.5 h for urine [50]. Urinary excretions ranged from
0.004% to 0.5% of total intake [50]. They postulate that anthocyanin absorption may be
underestimated however, due to studies that did not characterize anthocyanin metabolites that
could be formed in the GI tract or in the liver, such as protocatechuic acid [50]. Kay et al.
detailed the pharmacokinetics of anthocyanins in the human body by feeding a single 721 mg
oral dose of cyanidin glycosides to subjects fed a strict anthocyanin-free diet for 72 h prior to
testing. Blood was collected hourly for 7 h after consumption and urine was collected throughout the day for 13 subjects. Parent anthocyanins represented about 32% of total anthocyanins detected in the blood, with the remaining 68% being glucuronidated or methylated derivatives, as well as peonidin, an anthocyanin closely related to cyanidin. Likewise, only 32.5% of anthocyanins in the urine were parent compounds, and 67.5% were conjugated metabolites. While Cy3Xyl was detected in chokeberry juice, it was not detected in urine or plasma after consumption. This may be due to low concentrations in the juice, or the metabolism of the xyloside group [51].

Several theories exist concerning the absorption of anthocyanins. Gee et al. suggests that anthocyanin glycosides, like quercetin glycosides, may be transferred through the intestinal brush border and into enterocytes through interaction with SGLT1 [52]. Mulleder et al. conducted a human study on anthocyanin metabolism in elderberry juice [53]. After a washout period, they fed 16 subjects 11 g of elderberry concentrate in water and measured Cy3Glu and cyanidin-3-sambubioside (Cy3Sam), the two major anthocyanins in elderberry, in the urine. They found that both of these glycosylated moieties were excreted intact, along with two metabolites that they were unable to identify. Maximal concentrations of anthocyanin in the urine were between 1 and 3 h after ingestion, and excretion patterns differed between the two anthocyanins, in that Cy3Glu was excreted more gradually than Cy3Sam. This suggested that differences in glycosylation of anthocyanins indeed alter their rate of metabolism [53]. The C_{max} data for Manach et al.’s review suggests that the majority of anthocyanin absorption occurs early in digestion, perhaps as early as in the stomach [50].
The experiment was replicated and elderberry concentrate was also suspended in water with 30 g sucrose to address whether or not the presence of additional sugar in the intestine altered anthocyanin metabolism [53]. Hollman et al. proposed that flavonoids are transported by sodium dependent glucose transporters by means of their sugar moiety, and that the presence of sugars in the intestine may alter glycosylated flavonoid absorption by competing for transporter sites [69]. Mulleder et al. found that the addition of sucrose to the elderberry extract indeed delayed maximal urine concentrations of Cy3Glu by 2 h, and Cy3Sam by 1 h. Without ingestion of sucrose, 0.035% of ingested anthocyanins were excreted in the urine, however with the addition of sucrose, only 0.028% of anthocyanins were excreted [53].

Sabina Passamonti describes several transporters with high affinity for anthocyanins, however, these transporters saturate at low concentrations, limiting absorption of anthocyanins [54]. Bilitranslocase, found in the liver, is an additional transporter with specificity towards anthocyanins, and not flavonols with similar glycosylation [55]. The reason for this is presumed to be because anthocyanins are planar molecules, and flavonols are not [55]. Bilitranslocase draws anthocyanins out of the plasma and into the liver, and anti-bilitranslocase antibody treatment has been shown to increase anthocyanin concentrations in the blood [56]. Anthocyanins with higher bioavailability, like peonidin-3-glucoside and malvidin-3-glucoside are the best bilitranslocase ligands (Ki = 1.8 and 1.4 µM, respectively), while delphinidin-3-glucoside, the anthocyanin with the poorest bioavailability has the lowest affinity for bilitranslocase (Ki = 8.6 µM) [57]. Bilitranslocase is also found in the stomach, both on mucus-secreting cells and acid-secreting parietal cells located deeper in the lining of the stomach [58]. Due to the rapid pharmacokinetics of anthocyanins and the presence of these receptors in the
gastric lining, it is reasonable to assume that a portion of anthocyanin absorption begins in the stomach [50, 58].

Another problem with anthocyanin bioavailability is the lack of efflux from intestinal cells [54]. Human intestinal cells contain bilitranslocase only on their mucosal side, and so anthocyanins can enter intestinal cells freely, but then encounter a barrier which prevents their uptake into the blood [54]. This is demonstrated in a study by Steinert et al. where retention in the cell monolayer was up to 60% after exposure of cultured Caco-2 intestinal epithelial cells to anthocyanins [59].

**Hydroxycinnamic acids**

The bioavailability of hydroxycinnamic acids varies greatly between compounds. Olthof et al. found that esterification of caffeic acid into chlorogenic acid reduces its absorption from 95% to only 33% [60]. Nardini et al. found that while chlorogenic acid is abundant in coffee, it is not observed in plasma after ingestion; and rather caffeic acid is observed [61]. It is believed that intestinal microflora de-esterify chlorogenic acid, forming caffeic acid, the detectable metabolite. In contrast, ferulic acid can be efficiently absorbed in its free form from tomatoes and beer [62], [63]. In cereals, where it is bound to the arabinoxylans of the grain cell walls, ferulic acid is mainly absorbed in the small intestine from the water soluble fraction, and little is absorbed in the colon after hydrolysis [64]. Hydroxycinnamic acids have been shown to increase during storage, for example in white wine, caffeic acid rose from 0.92 to 2.39 mg/L over the course of 12 months [65].

**Flavonols**
Flavonol bioavailability has been shown to vary largely based upon the presence of a sugar moiety. Hollman et al. found that quercetin glucoside had considerably higher bioavailability than quercetin aglycone, and they suggest that sugar carriers in the intestine may play a role in flavonol absorption [66].

Quercetin can undergo metabolism by intestinal microbiota to yield 3,4-dihydroxyphenylacetic, 3-hydroxyphenylacetic, and homovanillic acids [50]. Sawai et al. found that after subjects were given a single oral dose of 75 mg rutin, microbial metabolites accounted for as much as 50% of the ingested dose [67]. Quercetin metabolites are eliminated rather slowly with respect to other polyphenols, with half-lives ranging from 11 to 28 h [50]. Because of this characteristic, regular consumption of quercetin may lead to a small degree of accumulation and increasing plasma concentrations. Conquer et al. administered either 1 g per day of quercetin or a placebo to subjects for 28 days, and found that in those consuming quercetin, average plasma levels were 1.5 µmol/L, however individual variability was very high [68]. In white wines, rutin has been shown to decrease from 0.84 mg/L to undetectable levels over the course of 4 months at 20°C [65].

**Total Phenols**

Total phenols in white wine have been reported to decline from 231 to 217 mg/L over the course of 12 months at room temperature [65]. Begic-Akagic et al. observed the changes in total phenol content of six apple cultivars during 6 months of storage at 1°C and found that total phenol content decreased 10 - 24% in all six cultivars [70]. Phenolic compounds undergo degradation either spontaneously or by means of polyphenol oxidases. In unpeeled and undamaged apples, polyphenols are not in immediate contact with polyphenol oxidases, however cellular injury
exposes polyphenols to oxidation as well as polyphenol oxidases, which causes phenolic compounds to oxidize and convert to ortho-quinones [70]. These compounds in turn polymerize and form brown or black pigments [70].

1.6 ARONIA ANTIOXIDANT, ANTI-INFLAMMATORY, AND ANTI-NEOPLASTIC ACTIVITY

In vitro studies have shown that chokeberry extract has promising anti-inflammatory effects. Zapolska-Downar et al. observed the anti-inflammatory effects of chokeberry extract on human aortic endothelial cells [71]. Cells were pre-treated with various concentrations of aronia extract and then exposed to 10ng/L TNF\(\alpha\). They found significant inhibition of ICAM-1 and VCAM-1, increased NF-\(\kappa\)B p65 and decreased production of intracellular reactive oxygen species in the cells treated with chokeberry extract compared to the control [71].

Jurgonski et al observed the effects of aronia supplementation on prediabetic and hyperlipidemic rats [72]. Rats fed chokeberry had improved antioxidant status in the liver, kidney, and lung, improved cholesterol, and hypoglycemic actions. The hypoglycemic activity was likely due to decreased activity of intestinal disaccharidases, in particular maltase and sucrase [72].

Jakobek et al. tested the contribution of chokeberry polyphenol interactions to radical scavenging in the DPPH assay [73]. They isolated three polyphenol fractions from chokeberry, one containing flavonols and phenolic acids, one containing anthocyanins, and one containing proanthocyanidins and other insoluble phenols. They had a final extract containing all of these polyphenols together to determine the effects of polyphenol interaction on DPPH scavenging
activity. They found that in chokeberry extract, anthocyanins contributed 56% of total antiradical activity ($EC_{50} = 88 \, \mu\text{mol TE/g FW}$). Proanthocyanidins and insoluble phenols contributed 35% of antiradical activity ($EC_{50} = 54 \, \mu\text{mol TE/g FW}$), and flavonols and phenolic acids contributed 9% ($EC_{50} = 14 \, \mu\text{mol TE/g FW}$) [73]. Zheng and Wang performed a similar analysis using only anthocyanins and phenolic acids, and found that anthocyanins contributed 53% to oxygen radical scavenging and phenolic acids contributed 47% [74]. This method yielded significantly different results from the DPPH assay, and merits a note that different polyphenols may perform certain antioxidant functions with greater efficiency [74].

In vitro antioxidant activity may not directly correlate with an ability to prevent oxidative stress in vivo. Rats given chokeberry extract prior to poisoning with the pro-oxidant carbon tetrachloride ($\text{CCl}_4$) showed lesser elevations in hepatic AST, ALT, and MDA levels [75]. While it is unknown which compounds are directly responsible for this phenomenon, chokeberry extract appears to have a hepatoprotective effect against carbon tetrachloride, partially through an antioxidant mechanism [75].

Zhao et al. tested the effects of grape, bilberry, and chokeberry anthocyanin-rich extracts for chemopreventive activity in colon cells [76]. They conducted a sulforhodamine B assay, and exposed colon-cancer-derived HT-29 and nontumorigenic NCM460 cells to 10-75 µg/mL anthocyanin-rich extracts for up to 72 h. They found that anthocyanin-rich extracts from all three fruits inhibited HT-29 growth, with chokeberry extract being the most effective. Exposure to 25 µg/mL chokeberry extract for 48 h inhibited HT-29 growth by 50%. At lower concentrations, the extracts inhibited HT-29 growth without inhibiting the growth of NCM460, indicating that the anti-hyperplastic effect of anthocyanins had a preference for tumorigenic cells [76].
1.7 POLYPHENOL INTERACTIONS

Jakobek et al. combined chokeberry flavonol and phenolic acid, anthocyanin, and proanthocyanidin/insoluble phenol fractions in various ratios and performed DPPH analyses to determine whether or not interaction between chokeberry polyphenols had a synergistic antioxidant effect [73]. They found that a 10:9:81 ratio of flavonols, phenolic acids, and anthocyanins resulted in the greatest decrease in DPPH inhibition, while some combinations of polyphenols showed an increase in DPPH inhibition [73]. This phenomenon has been referred to as negative synergism, or antagonism, and has also been observed by Pinelo et al. in an experiment on interaction between catechin, resveratrol, and quercetin, and by Meyer et al. whom observed an antagonistic effect when combining ellagic acid with catechin [77], [78].

Pignatelli et al. found that combining quercetin and catechin contributed to a synergistic radical scavenging effect [79]. Neither polyphenol had a significant inhibitory effect on platelet adhesion to collagen, but the combination of quercetin and catechin greatly inhibited adhesion, collagen-induced hydrogen peroxide production, calcium mobilization, and 1,3,4-inositol triphosphate formation [79].

Rossetto et al. tested the effects of (+)-catechin, malvidin-3-glucoside, and peonidin-3-glucoside on peroxidation of linoleic acid by 2,2(‘)-azobis[2-(2-imidaxolin-2-yl)propane]. They found that malvidin-3-glucoside and peonidin-3-glucoside inhibited linoleic acid peroxidation and (+)-catechin did not [80]. However, when catechin and either malvidin-3-glucoside or peonidin-3-
glucoside were combined, they had a synergistic effect on preventing linoleic acid peroxidation, due to the tendency of catechin to regenerate the more efficient antioxidants [80].

Hidalgo et al. performed DPPH and FRAP (Ferric Reducing Ability of Plasma) assays using (+)-catechin, (-)-epicatechin, various anthocyanins, kaempferol, myricetin, and quercetin, and found several interactions when compounds were combined. The DPPH assay is a measure of radical scavenging ability, while the FRAP assay measures ferric reduction potential. Cyanidin-3-glucoside and myricetin-3-glucoside had a synergistic effect on DPPH values [81]. Among the compounds tested, kaempferol was found to have nearly the lowest antioxidant activity, however when combined with myricetin, quercetin, or quercetin-3-glucoside, a significant increase in radical scavenging was observed [81]. Pairing pelargonidin-3-glucoside with catechin, epicatechin, quercetin, quercetin-3-glucoside, or kaempferol created an antagonistic effect on DPPH scavenging activity [81]. Differences in the structure of pelargonidin-3-glucoside and kaempferol may be responsible for their opposing effects. Kaempferol has a 4-oxo functional group and a 2,3-double bond on the C ring, while pelargonidin has a glucoside residue at C3 and an oxonium ion on the C-ring [81]. Hidalgo et al. found that most combinations of the polyphenols that they tested had antagonistic effects on radical scavenging, however the strongest antagonistic effect was observed when anthocyanins were combined with quercetin-3-glucoside [81]. They postulate that this effect may be due to hydrogen-bonding between hydroxyl groups in the two respective molecules, decreasing their availability for interaction with DPPH [81].

FRAP analyses performed by Hidalgo et al. yielded somewhat different results with respect to polyphenol interaction. Polyphenol interactions were synergistic in the FRAP assay [81].
Quercetin and quercetin-3-glucoside acted synergistically with other flavonoids to enhance ferric reduction. Quercetin was not synergistic with myricetin, and decreased FRAP capacity by 9% at a 1:1 ratio [81]. Peonidin-3-glucoside had an antagonistic effect when combined with malvidin-3-glucoside or delphinidin-3-glucoside [81].

Chokeberry contains quercetin, hydroxycinnamic acids, cyanidin-derived anthocyanins, and proanthocyanidins. While the combination of anthocyanins and quercetin-3-glucoside has been shown to inhibit radical scavenging [81], the presence of flavonols, phenolic acids, and anthocyanins has shown an increase in radical scavenging via DPPH [73]. The combination of catechin and quercetin, also present in chokeberry, has shown a myriad of radical scavenging effects [79]. Thus, polyphenol content alone may not predict antioxidant capacity, and studies of chokeberry extract should be used to determine bioactivity.

1.8 CHANGES IN POLYPHENOL CONTENT OF FRUITS DURING RIPENING

A variety of chemical changes occur during fruit ripening. For example, physical appearance, texture, sweetness, tartness, and astringency change in fruits. Sweetness changes are caused by sugars including glucose, fructose, and sucrose, while tartness is the result of a decrease in fruit pH, with malic acid being the primary contributor [82]. Astringency, also known as a drying sensation in the mouth, is often caused by phenolic compounds [82]. Sugar content of fruits generally increases throughout ripening, and acidity tends to decrease [82]. Some fruits however, like lemons, tend to increase in acidity through ripening [82]. There is some variation in changes in phytochemicals during ripening and this appears to vary based on the species observed [44].
Campo et al. observed changes in sugar, polyphenol, and organic acid content in four apple varieties throughout ripening [44]. They collected apples once per week for 6 weeks between September and October, and juiced them for analysis. Sugars were tested using a series of enzymatic assays, chlorogenic acid and (-)-epicatechin were calculated using proton nuclear magnetic resonance, and organic acids were determined by HPLC. They found that total sugars, as well as individual values for glucose, fructose, and sucrose increased throughout ripening for all four apple varieties tested [44]. Succinic acid increased, while citric acid decreased in all apple varieties [44]. Lactic acid decreased in two apple varieties and increased in one, and malic acid decreased in all but one apple variety [44]. Chlorogenic acid decreased between 32 and 58% and epicatechin decreased between 1 and 68% in the four apple varieties [44]. Glucose/fructose ratio remained steady throughout ripening for all apple varieties [44].

In strawberries, between the 50% red stage and the peak of ripeness, total phenol values steadily decreased, however ORAC values and total anthocyanin content consistently increased [83]. Olsson et al. found that chlorogenic acid, p-coumaric acid, quercetin, and kaempferol all increased with ripening in Honeoye and Senga Sengana cultivar strawberries [40]. In lowbush blueberries, cinnamic acid derivatives have been shown to decrease during ripening; however chlorogenic acid did not change from the slightly unripe stage to the over-ripe stage [84]. Blueberry total phenols steadily increased during ripening, as did anthocyanins, which rose from 0 to 11 mg/g DW [37].

In cranberries, Vvedenskysya and Vorsa found that during ripening, proanthocyanidin content in ‘Stevens’ and ‘Ben Lear’ cultivars increased by 10% and 31%, respectively [85]. Anthocyanin
levels greatly increased in both cultivars of cranberry as well. Flavonol content fluctuated, however there was no significant change in levels throughout ripening [85]

Antioxidant capacity can vary greatly at different stages of fruit maturity. Blackberries, raspberries, and strawberries tend to have higher total phenol content and ORAC values in their green stage, and lower values in their pink stage [83]. Following the pink stage, and into ripeness, ORAC values of these berries again begin to rise [83]. High ORAC values in the green stage are believed to be due to proanthocyanidins, while the rise in ORAC values during ripeness are believed to be due to anthocyanin content [86]. Wang and Lin observed a nearly 50% increase in ORAC values in ‘Marion’ and ‘Evergreen’ blackberries during ripening [87].

A variety of factors can influence aronia fruit quality and polyphenol content, including cultivar, maturity, harvesting, season, site, soil type, fertilizers, and the use of pesticides. Anthocyanin content has been shown to vary by up to 110% from a good year to a bad year [88]. Soil mineral content can also dramatically alter anthocyanin content of berries. Jeppsson reported that anthocyanin content of aronia berries can be up to 50% higher when fertilizers are used sparingly as opposed to growth in high fertilizer soil [89]. Anthocyanin content as well as soluble solid content of black chokeberry has been shown to increase with aronia berry maturation, while titratable acids decreased [90].

Jeppsson et al. harvested chokeberries in Sweden at nine different points throughout the harvest season, beginning on August 14th, and ending on September 12th [88]. They found that the mass of a 100 berry sample increased by about 30% for ‘Viking’, Nero, and Aron cultivars [88]. Anthocyanin content increased close to 200% in these cultivars as well; however values peaked on September 7th and then declined [88]. They found total acidity to decrease from 10 g kg$^{-1}$ to
8.2 g kg\(^{-1}\), an 18% decrease [88]. Kaack and Kuhn found that in a comparable time period, total acidity in aronia berries decreased from 12.2 to 9.6 g kg\(^{-1}\), a 21% decrease [90]. Malic acid is the predominant organic acid in black chokeberry. Jeppsson and Johansson found that levels of malic acid remained constant over this time period [88].

**1.9 BIOACTIVITY OF ARONIA IN CLINICAL TRIALS**

With polyphenols exhibiting a myriad of health benefits in vitro and in animal models, great interest has developed for aronia for bioactivity in humans. Factors which have shown improvement from polyphenol administration include biomarkers of heart disease [10, 18, 21], inflammation [17], cancer [13, 15, 16], and oxidative stress [21].

Sikora et al. tested the short-term effects of chokeberry extract on blood lipids and coagulation parameters in patients with metabolic syndrome [91]. Significant decreases in total cholesterol, LDL-C, and triglycerides were observed, as well as significant decreases in platelet aggregation, clot formation, and fibrinolysis after 1 month of supplementation with *Aronia melanocarpa* extract [91].

Naruszewicz et al. observed the effects of supplementation of 255 mg of chokeberry polyphenol extract per day on myocardial infarction survivors using statins. The group supplementing chokeberry had an average of 29% reduction in ox-LDL and MCP-1 levels, 23% reduction in hsCRP, a 38% reduction in serum 8-isoprostanes, and an average of 11 and 7.8 mmHg reduction in systolic and diastolic blood pressure, respectively, compared to the placebo group [92].
Skoczynska et al. observed the effects of chokeberry juice consumption on various indices of glucose tolerance and heart disease [93]. They observed an 8% decrease in fasting blood glucose and a 12% increase in high sensitive C-reactive protein in the study group [93]. Uric acid was found to decrease from 5.3 mg/dl to 5.14 mg/dl, and homocysteine decreased from 9.4 to 8.8 µM/L. Total cholesterol decreased by 12%, LDL decreased by 17%, and triglycerides decreased from 188 to 139 mg/dL, a 26% decrease in the supplemented group [93]. Systolic blood pressure decreased from 138 to 125 mmHg (a 9% decrease), and diastolic blood pressure decreased from 89 to 82 mmHg (an 8% decrease) in the supplemented group [93].

The Skoczynska et al. study included 6 weeks of chokeberry juice consumption, followed by 6 weeks without consumption, and then an additional 6 weeks of chokeberry juice consumption. A few biomarkers increased between weeks 6 and 12 when no juice was consumed. These included systolic and diastolic blood pressure, fasting blood glucose, uric acid, and C-reactive protein (decreased) [93]. Therefore the beneficial effect of chokeberry juice on these biomarkers appears to be temporary. With respect to total cholesterol, LDL, and triglycerides however, values continued to decline between weeks 6 and 12, indicating a potentially lasting protective effect [93].

1.10 CONCLUSIONS

Aronia is a rich source of polyphenols including anthocyanins, proanthocyanidins, hydroxycinnamic acids, and flavonols. These compounds have been shown to prevent tumor formation [13, 15, 16, 76], improve risk factors for heart disease [10, 18, 21], and mitigate oxidative stress and inflammation [17, 21]. Their bioavailability is important to consider, as many mechanisms come into play with respect to polyphenol absorption and transport, and many
interactions take place between compounds either improving or decreasing bioavailability [73, 78-81].

For accurate quantitation of polyphenols and measurement of bioactivity, it is essential to develop methods of extraction and quantitation that provide high polyphenol yields. Methods should be developed that can quantify proanthocyanidins to >10mers [35]. A standard method for HPLC integration of proanthocyanidin oligomers does not currently exist, and some utilize a flat baseline while other use valley-to-valley integration, which produce drastically different results [36]. Different methods may be needed for different polyphenols, for example anthocyanins, which may be converted to pyranoanthocyanins under certain extraction conditions [12].

With respect to chokeberry, many questions remain unanswered. Chokeberry preparations for research are typically produced from Viking, Aron, and Nero cultivars, as well as an extract from Artemis International Inc. [12, 25, 88]. These are all black chokeberry preparations and little information exists about the polyphenol content and bioactivity of purple and red chokeberries. Polyphenol content of chokeberries is also greatly dependent on harvest timing, as changes in polyphenol content through ripening have been observed in several species of fruit [44, 82-87]. It is essential to time harvest with respect to polyphenol content because different polyphenols perform certain antioxidant functions with greater efficiency [74].
Figure 1.1 Anthocyanins in chokeberry.

Cyanidin

Cyanidin-3-arabinoside

Cyanidin-3-galactoside

Cyanidin-3-xyloside

Pelargonidin-3-galactoside

Pelargonidin-3-arabinoside

Cyanidin-3-glucoside
Figure 1.2 Proanthocyanidin monomers and polymers previously identified in foods.

(+)-catechin

(-)-epicatechin

Proanthocyanidin B3
Figure 1.3 Hydroxycinnamic acids in chokeberry.

- Caffeic acid
- Chlorogenic acid
- Ferulic acid
- Neochlorogenic acid
Figure 1.4 Flavonols in chokeberry.
Figure 1.5 Sugars in chokeberry.
1.11 REFERENCES


Chapter 2:
Method Development and Polyphenol Quantitation in 12 Aronia Accessions
2.1 INTRODUCTION

Chokeberry consists of three species; *Aronia melanocarpa*, which bears black fruit, *Aronia prunifolia*, which bears purple fruit, and *Aronia arbutifolia*, which yields red berries. Among these species are a variety of different genotypes and cultivars. The most common commercial variety of chokeberry is ‘Viking’, a black chokeberry; however this variety differs greatly from most in that it is an allotetraploid, genetically bred as a mix between *Sorbus aucuparia* (Mountain ash, or rowanberry) and *Aronia melanocarpa*. It bears larger, moister, and more palatable berries than most chokeberry genotypes, and is even considered by some to be a separate species [1].

*Aronia melanocarpa* is the most widely studied of the chokeberry species, and is known for its abundance of anthocyanins and proanthocyanidins. Chokeberry is among the most concentrated sources of dietary anthocyanins and currently the richest known source of proanthocyanidins among berries [2] [3]. As the list of health benefits of polyphenol consumption grows, scientists are giving greater attention to the chokeberry. *Aronia arbutifolia*, the red chokeberry, while currently only used as an ornamental crop, has recently become a crop of interest to scientists due to its biological similarity to the black chokeberry. Little research has been conducted on purple and red chokeberries and their polyphenol content remains uncharacterized. By investigating the polyphenol content of red chokeberry, we may be able to determine whether or not it could be utilized as a nutraceutical crop or polyphenol-rich supplement, as is the black chokeberry.

Fruit and flowers that exhibit blue or purple colors typically do so because of their abundance of anthocyanins. Blueberries for example, are rich in cyanidin, malvidin, and delphinidin
glycosides, all of which contribute to their deep blue fruit color [4]. Previous studies in black chokeberry have revealed an abundance of cyanidin glycosides [3-5]. These anthocyanins exhibit maximal light absorption at 525 nm, giving them a strong blue-purple color [6]. Literature has yet to reveal whether or not red chokeberries are given their color by a different subset of anthocyanins, for example pelargonidin glycosides, which are known to give strawberries their red color [7], or petunidin glycosides, which give the petunia flower its red and pink colors [6]. Proanthocyanidins, the long chains of flavan-3-ols that are believed to be responsible for the astringency of chokeberry, are also known to yield pink to red hues, and so these compounds may contribute to the pigmentation of red chokeberry [6]. We hypothesize that black, purple, and red aronia berries differ in either their anthocyanin composition, or their total anthocyanin content.

Twelve chokeberry accessions originating from various locations on the east coast of the United States were analyzed in this study; three were red (UC021, UC053, and UC057), five were purple (UC007, UC011, UC033, UC047, and PI578096), and four were black (UC009, ‘Viking’, PI636375, and AMES271010). Berries were grown in test plot in Storrs, CT, and harvested between 8/13/2011 and 9/15/2011. These chokeberries were analyzed for their anthocyanin, flavonol, hydroxycinnamic acid, proanthocyanidin, total phenol, and moisture content using mass spectrometry, UPLC-UV, HPLC-FLD, and the DMAC and Folin-Ciocalteu assays. The goals of this study were:

1) To develop accurate and reproducible methods of quantitation for anthocyanins in chokeberry extracts using UPLC and UV-Vis detection.
2) To Quantitate polyphenols in the twelve chokeberry accessions to determine whether or not differences in phytochemical content of chokeberries can be attributed to their fruit color.

3) To determine whether or not differences in moisture content contribute to the polyphenol concentration of chokeberries, and whether or not comparison of chokeberries on a dry weight basis is applicable.

2.2 MATERIALS AND METHODS

2.2.1 Preliminary HPLC Analysis of Blueberry and Chokeberry Anthocyanins

Method development for anthocyanin extraction began with blueberries, since like the chokeberry; blueberry has significant anthocyanin content and diversity. Blueberry anthocyanins have been extensively researched, and we used a method by Wu et al. that produced reproducible results similar to those observed in previous blueberry studies in order to accurately quantify chokeberry anthocyanins [3, 8]. Blueberries used were Wyman’s of Maine frozen blueberries obtained from a local supermarket. Thawed blueberries (2 g) were suspended in 30 mL of an acetone/water/acetic acid mixture (70/29.5/0.5), stirred for 5 min, centrifuged at 290 x g for 15 min, and stored in 1 mL aliquots at -80°C. 20 µL of thawed sample were injected into the HPLC on an Agela Venusil C18 column (250 x 4.6 mm). The mobile phases were 5% formic acid in water (mobile phase A), and 100% methanol (mobile phase B). The gradient ran from 5 – 35% B over the course of 40 min, and then was returned to 5% and held for 10 min. Anthocyanins were determined via UV detection at 520 nm.

It was determined that the extraction method used may be unreliable due to the fact that moist, thawed berries were simply mashed and suspended in extract solution, leaving much of the skin
intact. For a more thorough extraction, blueberries were frozen at -80°C, weighed, and lyophilized for 24 to 48 h in a Labconco freezone 4.5 lyophilizer (Kansas City, MO). Blueberry powder was suspended in acetone/water/acetic acid (70/29.5/0.5) or 100% methanol at a concentration of 100 mg/mL, centrifuged, and 1 mL was aliquoted for analysis. In order to elute peaks earlier and shorten the method, the gradient was raised to 20 – 35% B and held at 20% at the end. The injection volume was decreased to reduce baseline noise, and a 5 min hold period was added to the beginning to sharpen and separate peaks. Chokeberry anthocyanin analysis began with reverse-phase HPLC analysis of a commercial black chokeberry extract standardized to 15% anthocyanins from Artemis Inc. (Fort Wayne, IN). Chokeberry extraction and quantitation followed the same method as the blueberry.

2.2.2 UPLC-MS Analysis of Anthocyanins and other Polyphenols

Chokeberry accessions (n = 12) originating from various locations throughout the United States were grown by Dr. Mark Brand in a test plot in Storrs, CT and harvested when deemed ripe. Information about plant origins, color, moisture content, and harvest date are shown in Table 2.1. Immediately after harvest, berries were stored on ice, and refrigerated at 13°C for up to 48 h. Intact, edible berries were separated from stems, leaves, debris, and defective fruit, and held at -80°C until further processing. Chokeberries were lyophilized in a Labconco freezone 4.5 lyophilizer (Kansas City, MO) for 24 to 48 h. The masses of lyophilized berries were used to determine moisture content, and equivalent portions of lyophilized berries from each plant were pooled to create composite samples for each chokeberry variety. Lyophilized berries were ground into a fine powder using an IKA A11 Basic Grinder (St. Louis, MO), and all powders were stored at -80°C and analyzed by UPLC-MS within 6 mo.
Anthocyanins, flavonols and hydroxycinnamic acids were extracted from our chokeberry accessions using a modified method by Prior et al. [9]. Chokeberry powder (2 g) of chokeberry from each accession was suspended in 70% acetone, 29.5% ultrapure water, and 0.5% acetic acid, sonicated for 5 min in a Fisher Scientific FS30D sonicator, and centrifuged at 290 x g for 10 min. The pellet was re-extracted twice, and supernatants were stored at -80°C. The final pellet extraction was in 40 mL acidified acetone water as above, and the extract was agitated in a test tube rocker for 12 h in darkness at 23°C, and centrifuged as above. The supernatants were combined and dried at 40°C in a Buchi R210 rotavapor (Flawil, Switzerland) for approximately 1 h. Dried extract was reconstituted in 12 mL of 20% methanol in water, and divided into 250 µL aliquots. Aliquots were dried under nitrogen gas at 23°C and stored at -80°C.

UPLC-MS analysis was performed on a Shimadzu Nexera UPLC equipped with a DGU-20A5 solvent degasser, SIL-30AC autosampler, CTO-30A column oven, SPD-M20A diode array detector, and LCMS-2020 mass spectrometer with a Dual Ionization Spray source (DUIS) (Columbia, MD). Compounds of interest in UPLC-MS analysis included cyanidin-3-galactoside (MW: 448) (Cy3Gal), cyanidin-3-glucoside (MW: 448) (Cy3Glu), cyanidin-3-arabinoside (MW: 418) (Cy3Ara), cyanidin-3-xyloside (MW: 418) (Cy3Xy), pelargonidin-3-galactoside (MW: 432) (Pel3Gal), pelargonidin-3-arabinoside (MW: 402) (Pel3Ara), chlorogenic acid (MW: 354) (Cga), neochlorogenic acid (MW: 354) (nCga), resveratrol (MW: 228), polydatin (MW: 390), caffeic acid (MW: 180), protocatechuic acid (MW: 154), quercetin (MW: 302), quercetin-3-galactoside (MW: 464) (Q3Gal), quercetin-3-glucoside (MW: 464) (Q3Glu), and quercetin-3-rutinoside (MW: 610) (Q3R). Prior to analysis, dried chokeberry extract aliquots were reconstituted in 1 mL of methanol. Ionization information is shown on Table 2.2.
Mixed standard solutions were prepared consisting of Cga, nCga, resveratrol, polydatin, caffeic acid, protocatechuic acid, Q3Gal, rutin trihydrate, quercetin, and Cy3Gal. External standard concentrations ranged from 1 to 100 µg/mL and contained 1 mM daidzein as an internal standard. Injections of 1 µL or 5 µL of each standard solution were made on a Kinetex PFP (2.1 x 50 mm), 1.7 µm, 100A column (Phenomenex Inc., Torrance, CA) to create standard curves. Linear regression of standard curves had correlation coefficients of >0.999 for Cy3Gal, and >0.996 for all other polyphenols. The injection volume for reconstituted chokeberry extract was 1 µL. The mobile phases used for chromatography were 5% formic acid in water (A) and 100% methanol (B) at a total flow rate of 0.2 mL/min. The gradient consisted of 20% B from 0 to 6 min followed by a linear gradient to 50% B at 12 min, descending to 20% B over 2 min, with a 4 min hold period at 20% B, for a total runtime of 20 min. Anthocyanins were detected by MS and quantitated via UV at 520 nm. Quantitation was performed with Cy3Gal as a standard.

Non-anthocyanin polyphenols were quantified by MS in DUIS mode, with a detector voltage of 1.5 kv, interface temperature of 35°C, DL temperature of 250°C, Heat block temperature of 400°C, and drying gas flow rate of 15 L/min. Compounds were quantified based on standard curve values and were normalized to the internal standard. Limits of detection (LOD) were determined by injecting our standards at increasing concentrations until the signal to noise ratio of their peaks approached 3. Limits of quantitation (LOQ) were defined as signal to noise ratios of 10 [10]. LOD and LOQ are shown in Table 2.2. A representative MS chromatogram of black chokeberry extract is presented in Figure 2.2.

2.2.3 HPLC Analysis of Proanthocyanidins
Proanthocyanidin analysis was performed using a method developed by Gu et al. [11], and began with a Sephadex LH-20 extraction of freeze-dried blueberry (Wyman’s of Maine) and chokeberry extract (Artemis, Inc). Sephadex LH-20 (11 g) was mixed in 150 mL of 20% methanol in water, stirred for 3 min, and left to swell for 4 h. Sephadex columns 1 cm in diameter were rinsed with 20% methanol and then packed with ~4 g of Sephadex LH-20 and eluted with 20% methanol in water for 2 h. Freeze-dried blueberry or chokeberry extract (600 mg) were suspended in 6 mL of 20% methanol and centrifuged at 290 x g for 5 minutes. Supernatants were removed and 5 mL were applied to columns and then flushed with 40 mL of 20% methanol to elute sugars, and then 70 mL of 40% methanol to elute flavonoids and anthocyanins. To remove proanthocyanidins, 40 mL of 90% methanol was then used; however some pink coloring remained persistent in the column indicative of persistent proanthocyanidin residues. Therefore, 100% acetone was used to remove the last of the proanthocyanidins and the columns were re-equilibrated with 20% methanol for 2 h.

Sephadex extraction of proanthocyanidins in the twelve chokeberry varieties was modified based on additional work with almond proanthocyanidins [12]. Sephadex columns were equilibrated similarly; however 2.5 cm diameter columns were used, and were packed with ~6 grams of Sephadex LH-20. Each column was applied two aliquots of dried chokeberry extract after reconstitution in 2 mL of 30% methanol. Columns were eluted with 50 mL of 30% methanol followed by 50 mL of 50% ethanol to remove sugars, neutral phenolics, and anthocyanins. Lastly, 100 mL of 90% acetone in water was used to elute proanthocyanidins. The proanthocyanidin fractions were dried by rotary evaporation, reconstituted with 10 mL acetone, and dried under nitrogen gas and stored at -20°C until HPLC analysis.
Proanthocyanidins were quantified via normal phase HPLC with fluorescence detection on a Dionex Ultimate 3000 UHPLC equipped with a Hypersil silica (250 x 4.6 mm) column. Detector excitation and emission wavelengths were 276 nm and 316 nm, respectively. The mobile phases used were methanol (A), dichloromethane (B), and 50% acetic acid in water (C). Mobile phase C was held at 4% for the duration of the method. The gradient began at 82% B and descended to 67.6% over the course of 30 min, continued to 56.4% B at 45 min, and then descended to 1% B at 55 min, with a 5 min hold period at 1% B, followed by a 5 min increase to 82% B, and a 5 minute hold period at 82% B for a total runtime of 75 min.

A catechin standard curve was prepared for quantitation of proanthocyanidins. The standard curve ranged from 1.25 to 25 µg/mL in methanol. Chokeberry proanthocyanidin fractions were reconstituted in 1 mL methanol to make a solution equivalent to 41.66 mg lyophilized chokeberry/mL. Proanthocyanidin monomers through nonamers were quantified by 5 µL injections, while polymers were calculated with 1 µL injections to prevent detector overload. LOD and LOQ were determined as described for UPLC analysis.

2.2.4 DMAC Analysis of Proanthocyanidins

The DMAC (4-(Dimethylamino) cinnamaldehyde) assay was performed on the 12 chokeberry accessions to determine total proanthocyanidin content using a validated method by Prior et al. [13]. A catechin standard curve was created with (+)-catechin in 91% ethanol in water ranging from 1.56 to 100 µg/mL. Dried chokeberry extract aliquots were re-suspended in 250 µL of an acetone/water/acetic acid solution (70/29.5/0.5, (v/v)) and diluted 100-fold with 91% ethanol in water. Standard solutions and chokeberry samples (70 µL each) were pipetted into microtiter wells. Next, 280 µL of DMAC solution was added to each well and immediately placed into a
Biotek Synergy HT (Winooski, VT) plate reader. Absorbance values at 640 nm were recorded for 15 min and chokeberry proanthocyanidin values were calculated based on the standard curve.

2.2.5 Folin-Ciocalteu Analysis of Total Phenols

The Folin-Ciocalteu total phenol assay was performed on the 12 chokeberry samples following a method by Singleton et al. [14]. A standard curve was prepared ranging from 16.125 µg to 2 mg of gallic acid/mL in distilled water. Chokeberry extract aliquots were diluted 100-fold in distilled water. The assay was performed by adding 10 µL of standards or extract to each microtiter well, followed by 173 µL of distilled water and 15 µL of Folin-Ciocalteu reagent. The plate incubated for 5 min at room temperature, and then 45 µL of saturated sodium carbonate in water and 57 µL of distilled water were added to each well. The microtiter plate was incubated in darkness for 1 h at 23ºC and then absorbance was measured at 765 nm in a microplate spectrophotometer.

2.2.6 Statistical Analysis

Results were expressed as mean ± standard deviation of three replicates. Results were analyzed using one-way or two-way ANOVA, and significance was defined as $P < 0.05$. Tukey’s multiple comparison test was performed when data were deemed significant by ANOVA. Principal components analysis was performed using Minitab 16 statistical software (Minitab Inc., State College, PA) using anthocyanin, flavonol, phenolic acid, total phenol, and proanthocyanidin content as variables.

2.3 RESULTS AND DISCUSSION

2.3.1 Method Development
We encountered several problems with our initial reverse-phase HPLC trials with blueberry extract. Several peaks were missing, and we decided that this was due to the fact that much of the skin was left intact during extraction. We corrected for this by lyophilizing and grinding berries prior to extraction. To reduce baseline noise, we decreased injection volume from 20 µL to 10 µL and added a 5 minute hold period at the beginning of the method. This successfully sharpened and separated peaks, however our peaks were eluting late and our method was very long. To correct for this, we increased the methanol in our gradient and this successfully eluted peaks earlier. Our chromatography was finally clean and reproducible. Sample chromatograms from before and after our modifications are shown in Figure 2.1.

Among our compounds of interest, resveratrol, polydatin, caffeic acid, and protocatechuic acid were not observed in the extracts prepared from the 12 chokeberry accessions. Trans-resveratrol has been observed in chokeberry wine at a concentration of 8.67 µg/mL, however has not been observed in intact chokeberry to date [15]. Intraday variation was < 5.6% for all polyphenols quantified by MS, with an average of 2.45%, while inter-day variation was < 17%, with the exception of Q3R which was 34% (Table 2.2). Thus, this method was reproducible and an efficient means of quantifying Aronia ssp. polyphenols. Results were expressed as mean ± standard deviation of three replicates.

### 2.3.2 Anthocyanins

Between chokeberry accessions, a great deal of variety in phytochemical content was observed. We detected four anthocyanins in our chokeberry extract, Cy3Gal, Cy3Glu, Cy3A, and Cy3X. Identification was on the basis of molecular weights, daughter ions, and expected retention times. The two pelargonidin glycosides found in the Artemis extract were not found in our LCMS
analysis of chokeberry varieties. Wu et al. also observed traces of Pel3Gal and Pel3A in Artemis chokeberry extract [3]. Quantitative values for anthocyanins were expressed as Cy3Gal equivalents [9]. Anthocyanins quantified by UPLC are shown in Table 2.3. Anthocyanin content was dependent on chokeberry color. Cy3Ga, Cy3Glu, and Cy3Ara were identified in all twelve chokeberry genotypes tested, while Cy3X was only observed in black and some purple berries. Black chokeberries, particularly UC009 and ‘Viking’, had the highest levels of total anthocyanin, with a range of 6.23 to 14.84 mg/g DW, followed by purple chokeberries which ranged from 2.4 to 3.99 mg/g DW, and lastly the red, which contained between 0.48 and 0.82 mg/g DW. Maata-Riihinen et al. detected 8,421 mg/kg of anthocyanins in black chokeberry, which is within our range of black chokeberry values [5]. Wu et al. found 1,480 mg/100 g FW of total anthocyanins in their Artemis extract, which also falls within our range of values [3].

At first, we were unsure as to whether or not red chokeberries contained additional anthocyanin pigments, for example, petunidin or peonidin glycosides, which are known to yield shades between purple and red, and are found in red fruits like strawberry, as well as red flowers like petunia [6], [7]. We did not however, detect these in any of our chokeberry extracts. Cyanidin glycosides give fruit a dark purple color, and cyanidin glycosides were the only anthocyanins that we detected in chokeberries of any color.

Despite differing levels of anthocyanin content between the 12 chokeberry genotypes, the order of abundance for anthocyanins always followed the same pattern: Cy3Gal > Cy3A > Cy3Glu > Cy3X. Differences in relative levels were observed however, as UC009 had the most total anthocyanin with 14.84 mg cyanidin-3-galactoside equivalents/g, however ‘Viking’ had higher levels of Cy3A and Cy3Glu. Sample chromatograms of black, purple, and red chokeberry
anthocyanins are shown in Figure 2.3. Cy3Gal represents the large peak at roughly 2.2 min, Cy3Glu represents the shoulder at roughly 2.8 min, Cy3Ara represents the peak at 3.5 min, and Cy3X represents the peak at 6.7 min, which is only visible in the red and purple chokeberry. The LOD and LOQ for anthocyanins were 1.2 ng and 1.4 ng on column (OC), respectively. Our method was highly reproducible, with < 1.65% intra-day variation and < 8.89% inter-day variation for anthocyanins (Table 2.2).

2.3.3 Flavonols

No trend was observed between chokeberry color and total flavonol content. However UC047, a purple chokeberry genotype, had the highest level of flavonols by a 64% margin (Table 2.4). Total flavonol values ranged from 0.47 to 1.33 mg/g DW. Hakkinen et al. observed 89 mg/kg FW of total flavonols in ‘Viking’ chokeberry after removal of sugars [16]. Assuming similar recovery, estimated flavonol aglycone values for our ‘Viking’ sample is about 598 mg/kg, over six-fold greater. Their results did however fall within our range of total flavonol values for the different chokeberry accessions, and difference in flavonol content may have been due to extraction method, differences in response between flavonol aglycones and glycosides or differences in ripeness between berries in their study and ours. Flavonol content of other common berries in the Hakkinen study includes 157 mg/kg for cranberry, 40 mg/kg for blueberry, 15 mg/kg for strawberry, and 8 mg/kg for raspberry [16]. Maata-Riihinen et al. found 348 mg/kg of quercetin aglycone in black chokeberry, which is within our range of flavonol values, however lower than any black chokeberry value that we detected [5]. They also found traces of kaempferol in chokeberry, which we did not screen for [5].
UC047, UC033 and UC057 had considerably higher levels of rutin than any of the other chokeberry varieties, with 0.64, 0.46 and 0.48 mg/g, respectively. Q3Gal was present in greater quantity than Q3Glu in all but the PI578096 accession. Quercetin-3-vicianoside and quercetin-3-robinobioside have previously been observed but not quantified in black chokeberry [17]. In some injections of our ‘Viking’ chokeberry extract, we observed two peaks that we were unable to identify, both of which eluted before Q3Gal and Q3Glu, and had retention times of 3.57 and 6.77 (Figure 2.2). LC-MS/MS of these peaks are required to test this hypothesis. LOQ for Q3Gal, Q3Glu, and rutin were 0.5, 0.5, and 0.4 ng OC respectively. LOD for flavonols was 0.4 ng OC. Intra-day variation for flavonols was < 5.55%, and inter-day variation was 16.62%, 14.18%, and 34.0% for Q3Gal, Q3Glu, and Q3R, respectively (Table 2.2).

2.3.4 Hydroxycinnamic Acids

Hydroxycinnamic acid content was greatest in two purple accessions, and one particular red accession (UC057), but was otherwise similar across Aronia ssp., ranging from 5.19 to 17.28 mg/g DW. UC047 had 17.3 mg/g DW, the highest content of hydroxycinnamic acid among the genotypes that we tested. Cga represented 78% of its total hydroxycinnamic acid content. Cga was present in all Aronia ssp. accessions at 3.11 to 13.5 mg/g DW. Maata-Riihinen et al. detected only 0.892 mg/g FW of hydroxycinnamic acids in chokeberry, about 1/3 of the lowest value that we obtained [5]. Their protocol differed in that they used ethyl-acetate to extract water-soluble phenolics as opposed to our acetone/water/acetic acid solution. They also used reverse-phase HPLC as opposed to our UPLC-MS [5]. nCga was present in purple and black accessions, but only one red accession, at a level at least 50% less than black and purple berries. nCga content of purple and black berries were similar; however PI636375 had at least 71% more
nCga than other accessions. While we did not observe any caffeic acid in our chokeberry accessions, caffeic acid has been observed in wild chokeberries at 1.411 µg/g FW [4]. Hydroxycinnamic acids are shown in Table 2.3. We may not have detected caffeic acid in our samples for a variety of reasons. Our chokeberries were grown on test plots under controlled conditions, while the Zheng et al. used wild chokeberries. We used an acetone/water/acetic acid as our extraction solution, while Zheng et al. used an acidified water extraction, followed by a methanol/water/formic acid extraction [4]. Caffeic acid in their samples may have just been a degradation product of Cga or nCga. LOD for hydroxycinnamic acids was 0.5 ng OC. LOQ for Cga and nCga were 0.7 and 0.6 ng OC respectively. Intra-day variation for hydroxycinnamic acids was < 2.97%, and inter-day variation was < 16.13% (Table 2.2).

2.3.5 Proanthocyanidins

Proanthocyanidin content ranged from 4.25 to 18.6 mg CE/g DW from the DMAC analysis, and from 1.1 to 3.37 mg CE/g DW in the HPLC analysis. While the quantitative values differ greatly between the DMAC and HPLC, the genotypes are still ranked the same way, with UC021 having the highest level of proanthocyanidins and UC053 having the lowest. Proanthocyanidin values were not dependent on color (P = 0.6776), and UC021 and UC053 are both red accessions. Proanthocyanidins were expected to be highest in red chokeberries due to the pinkish hue of proanthocyanidin moieties; however there appeared to be no correlation between chokeberry color and proanthocyanidin content, as determined by the DMAC and HPLC analyses (Table 2.5 and 2.6).

The DMAC assay is known to underestimate polymeric proanthocyanidins, however in our study the proanthocyanidin content determined by the DMAC assay was up to three-fold higher than
the HPLC data [18]. A potential cause for this discrepancy may be due the usage of a catechin standard curve for both DMAC and HPLC analysis. While catechin is a monomer, the majority of proanthocyanidins in chokeberry are polymeric. We found that at least 99.6% of our proanthocyanidins in our study were polymers. A study by Wu et al. (2004) found the PAC concentration of Artemis chokeberries to be 6.64 mg/g fw in Aronia melanocarpa [3], which is within our range of values for DMAC analysis, however greater than any value that we obtained through HPLC analysis [3]. Wu et al. did not specify whether they used valley-to-valley integration or a flat baseline, and did not specify whether or not they used a correction factor. We used a valley-to-valley integration and no correction factor for differences in response factors of oligomers and polymers for fluorescence detection, and this may have caused us to underestimate proanthocyanidin content, particularly that of oligomers. HPLC chromatograms for proanthocyanidins are shown in Figure 2.4. Figure 2.4A represents a 1 µL injection of chokeberry extract and a broad peak for polymers. Figure 2.4B shows oligomers with degrees of polymerization up to 9, from a 5 µL injection of chokeberry extract.

### 2.3.6 Total Phenols

Total phenol values did not appear to differ significantly based on color, however mean values for total phenols were higher in red and purple chokeberries than black by about 10%, due to the fact that the UC033 (purple) and UC021 (red) accessions had nearly 20% higher total phenol values than the next highest chokeberry variety. Total phenols ranged from 127 mg GAE/g DW in AMES27010 to 250 mg GAE/g DW in UC021. UC033 was a particularly interesting genotype with respect to the total phenols assay because it did not have remarkably high levels of any particular phenolic compound that we measured; however it had the second highest total phenols.
value by 1.6%, indicating that there may be other uncharacterized polyphenols in chokeberry. Total phenol values are shown in Table 2.6. Using the Folin-Ciocalteu assay, Kahkonen et al. found the total phenol content of black chokeberry to be 40.1 mg GAE/g DW, less than 1/3 of the value in our lowest sample [19]. Total phenol values for other common berries in the Kahkonen study include 23.7 mg GAE/g DW for strawberry, 23.9 mg GAE/g DW for raspberry, and 21.2 mg GAE/g DW for cranberry [19].

2.3.7 Moisture Content

Moisture content of each chokeberry accession was calculated through mass difference before and after lyophilization (Table 2.1). Moisture percentage ranged from 63.6% (UC007) to 76% (Viking). Differences in moisture content of berries generally made little difference in their comparative polyphenol values. The only value of interest that differed between the dry weight and fresh weight data was Q3Gal, which was highest in UC007 when observed on a fresh weight basis, but higher in ‘Viking’ when observed on a dry weight basis. Unfortunately, berries from different genotypes were picked on different dates and ideal harvest timing was not predetermined. Differences in moisture content may be greater at different stages of ripening, and may contribute to differences in polyphenol content if all berries were harvested at their peak. Also, ‘Viking’, the genotype with the greatest moisture content that we observed, differs from the other chokeberries that we tested in that it is an allotetraploid, and is believed to be an intergeneric hybrid between Sorbus aucuparia and Aronia melanocarpa [1]. Some even designate ‘Viking’ and other moist, large-fruited black chokeberries as a separate species, Aronia mitschurinii, named after Ivan Mitschurin, a Russian breeder whom was known to experiment
with chokeberries [1]. Nonetheless, fresh weight and dry weight analysis are both accurate means of measuring phytochemical content in chokeberries.

### 2.3.8 Polyphenol Correlations

The twelve chokeberry accessions had great variety in their polyphenol content, some of which can be predicted by their color, such as anthocyanin content, since those compounds are strongly pigmented. We performed 1-way ANOVA analyses of each polyphenol class to determine whether or not their content was correlated with chokeberry color. Only anthocyanins were found to be dependent on chokeberry color (P = 0.0018).

For more detailed characterization of compounds in each individual genotype, we performed a principal components analysis, shown in Figure 2.5. A summary of compound class and means and ranges between different colored chokeberries are presented in Table 2.7. Compounds in each genotype were generally comparable, with some deviations. Notably, the percentage of phenolic compounds represented by proanthocyanidins in UC021 is considerably higher than any other chokeberry variety. UC047 had an abundance of phenolic acids compared to the other chokeberry genotypes. The black chokeberries were expectedly differentiated by the anthocyanin vector.

### 2.4 CONCLUSIONS

Great variation is observed across chokeberry genotypes with respect to polyphenol content. While anthocyanin content in the twelve varieties that we observed is restricted to cyanidin glycosides, total anthocyanin content is strongly correlated with chokeberry color. Black chokeberries contain the most anthocyanins, followed by purple, and lastly the red accessions.
Flavonol, hydroxycinnamic acid, proanthocyanidin, and total phenol values however are not dependent on chokeberry color. Differences in moisture content of different chokeberry accessions in minor, and we found that dry weight and fresh weight data had no significant difference. Different methods of extraction and analysis for polyphenols can yield dramatically different results, for example Sephadex LH-20 extraction or the use of DMAC and HPLC analyses. Different integration methods for HPLC analysis, for example valley-to-valley integration and the usage of a flat baseline can dramatically affect results as well. While our anthocyanin analysis was highly reproducible, it may not have been entirely accurate, due to the fact that we used an acetone-based extraction which is known to cause conversion of anthocyanins to other compounds [20]. Additional, more reproducible methods for flavonol and hydroxycinnamic acid analysis should be explored, and methods which can quantify proanthocyanidins to degrees of polymerization > 10 are also warranted. Additional compounds which have not been screened for may be present in chokeberry, as indicated by the fact that UC033 had an abundance of total phenols in spite of unremarkable levels of the polyphenols which we observed.
Table 2.1. Color, origin, harvest date and moisture content of *Aronia ssp.* used for subsequent polyphenol analysis.

<table>
<thead>
<tr>
<th>Color</th>
<th>Accession</th>
<th>Origin of Accession</th>
<th>Date of Harvest</th>
<th>Number of plants harvested</th>
<th>Moisture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td>UC021</td>
<td>cultivar ‘Erecta’</td>
<td>9/15/2011</td>
<td>2</td>
<td>72.2</td>
</tr>
<tr>
<td>Red</td>
<td>UC057</td>
<td>Manahawkin, NJ</td>
<td>9/15/2011</td>
<td>1</td>
<td>73.4</td>
</tr>
<tr>
<td>Red</td>
<td>UC053</td>
<td>Millville, NJ</td>
<td>9/15/2011</td>
<td>3</td>
<td>70.6</td>
</tr>
<tr>
<td>Purple</td>
<td>UC047</td>
<td>Plainfield, MA</td>
<td>8/23/2011</td>
<td>2</td>
<td>65.1</td>
</tr>
<tr>
<td>Purple</td>
<td>UC011</td>
<td>Nobleboro, ME</td>
<td>8/23/2011</td>
<td>1</td>
<td>64.7</td>
</tr>
<tr>
<td>Purple</td>
<td>UC033</td>
<td>Groton, CT</td>
<td>8/23/2011</td>
<td>1</td>
<td>69.2</td>
</tr>
<tr>
<td>Purple</td>
<td>PI578096</td>
<td>USDA collection VA</td>
<td>9/15/2011</td>
<td>1</td>
<td>74.5</td>
</tr>
<tr>
<td>Purple</td>
<td>UC007</td>
<td>Chaplin, CT</td>
<td>8/23/2011</td>
<td>1</td>
<td>63.6</td>
</tr>
<tr>
<td>Black</td>
<td>‘Viking’</td>
<td>commercial cultivar</td>
<td>8/13/2011</td>
<td>3</td>
<td>76.0</td>
</tr>
<tr>
<td>Black</td>
<td>UC009</td>
<td>Nobleboro, ME</td>
<td>8/13/2011</td>
<td>3</td>
<td>66.8</td>
</tr>
<tr>
<td>Black</td>
<td>PI636375</td>
<td>USDA collection Russian Federation</td>
<td>8/13/2011</td>
<td>3</td>
<td>67.5</td>
</tr>
<tr>
<td>Black</td>
<td>AMES27010</td>
<td>USDA collection MI</td>
<td>8/13/2011</td>
<td>3</td>
<td>67.0</td>
</tr>
</tbody>
</table>
Table 2.2. Analytical parameters, reproducibility, characteristic ions, and limits of quantitation (LOQ) and detection (LOD) for UPLC-MS analysis of chokeberry polyphenols.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time</th>
<th>m/z</th>
<th>Quantitation</th>
<th>% RSD</th>
<th>LOQ</th>
<th>LOD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(min)</td>
<td>(mode)</td>
<td></td>
<td>interday</td>
<td>intraday</td>
<td>ng OC</td>
</tr>
<tr>
<td>nCga</td>
<td>1.2</td>
<td>354 (-)</td>
<td>M-H</td>
<td>16.13</td>
<td>2.97</td>
<td>0.6</td>
</tr>
<tr>
<td>Cga</td>
<td>1.9</td>
<td>354 (-)</td>
<td>M-H</td>
<td>11.09</td>
<td>2.49</td>
<td>0.7</td>
</tr>
<tr>
<td>Cy3Gal</td>
<td>2.2</td>
<td>449 (+)</td>
<td>UV</td>
<td>0.34</td>
<td>0.52</td>
<td>1.4</td>
</tr>
<tr>
<td>Cy3Glu</td>
<td>2.6</td>
<td>449 (+)</td>
<td>UV</td>
<td>8.89</td>
<td>1.65</td>
<td>1.4</td>
</tr>
<tr>
<td>Cy3Ara</td>
<td>3.4</td>
<td>419 (+)</td>
<td>UV</td>
<td>0.75</td>
<td>0.45</td>
<td>1.4</td>
</tr>
<tr>
<td>Cy3Xy</td>
<td>6.6</td>
<td>419 (+)</td>
<td>UV</td>
<td>5.22</td>
<td>0.94</td>
<td>1.4</td>
</tr>
<tr>
<td>Q3Gal</td>
<td>9.3</td>
<td>464 (-)</td>
<td>M-H</td>
<td>16.62</td>
<td>5.36</td>
<td>0.5</td>
</tr>
<tr>
<td>Q3Glu</td>
<td>9.8</td>
<td>464 (-)</td>
<td>M-H</td>
<td>14.18</td>
<td>5.55</td>
<td>0.5</td>
</tr>
<tr>
<td>Q3R</td>
<td>9.9</td>
<td>610 (-)</td>
<td>M+H</td>
<td>34.00</td>
<td>2.06</td>
<td>0.4</td>
</tr>
<tr>
<td>Quercetin</td>
<td>14.4</td>
<td>303 (+)</td>
<td>M+H</td>
<td>19.90</td>
<td>3.07</td>
<td>0.5</td>
</tr>
</tbody>
</table>

RSD, relative standard deviation; OC, on column; DW, dry weight.
**Table 2.3.** Flavonol and hydroxycinnamic acid content of chokeberry accessions determined by UPLC-MS as mg/g dry weight chokeberry.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Cga</th>
<th>nCga</th>
<th>Q3Gal</th>
<th>Q3Glu</th>
<th>Q3R</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC021 [R]</td>
<td>8.45 ± 0.17</td>
<td>nd</td>
<td>0.184 ± 0.01</td>
<td>0.126 ± 0.01</td>
<td>0.159 ± 0.00</td>
</tr>
<tr>
<td>UC057 [R]</td>
<td>11.2 ± 0.30</td>
<td>nd</td>
<td>0.494 ± 0.01</td>
<td>0.211 ± 0.00</td>
<td>0.482 ± 0.01</td>
</tr>
<tr>
<td>UC053 [R]</td>
<td>4.10 ± 0.07</td>
<td>1.09 ± 0.02</td>
<td>0.257 ± 0.00</td>
<td>0.121 ± 0.00</td>
<td>0.350 ± 0.00</td>
</tr>
<tr>
<td>UC047 [P]</td>
<td>13.5 ± 0.00</td>
<td>3.82 ± 0.02</td>
<td>0.411 ± 0.00</td>
<td>0.285 ± 0.01</td>
<td>0.640 ± 0.01</td>
</tr>
<tr>
<td>UC011 [P]</td>
<td>6.68 ± 0.05</td>
<td>3.38 ± 0.00</td>
<td>0.347 ± 0.00</td>
<td>0.233 ± 0.01</td>
<td>0.180 ± 0.00</td>
</tr>
<tr>
<td>UC033 [P]</td>
<td>7.16 ± 0.05</td>
<td>3.76 ± 0.02</td>
<td>0.403 ± 0.01</td>
<td>0.240 ± 0.00</td>
<td>0.460 ± 0.01</td>
</tr>
<tr>
<td>PI578096 [P]</td>
<td>3.11 ± 0.02</td>
<td>2.37 ± 0.01</td>
<td>0.150 ± 0.00</td>
<td>0.170 ± 0.00</td>
<td>0.176 ± 0.00</td>
</tr>
<tr>
<td>UC007 [P]</td>
<td>6.42 ± 0.10</td>
<td>3.22 ± 0.05</td>
<td>0.320 ± 0.01</td>
<td>0.239 ± 0.01</td>
<td>0.181 ± 0.01</td>
</tr>
<tr>
<td>‘Viking’ [B]</td>
<td>3.88 ± 0.01</td>
<td>2.50 ± 0.02</td>
<td>0.461 ± 0.00</td>
<td>0.389 ± 0.00</td>
<td>0.159 ± 0.00</td>
</tr>
<tr>
<td>UC009 [B]</td>
<td>3.87 ± 0.06</td>
<td>3.14 ± 0.06</td>
<td>0.468 ± 0.01</td>
<td>0.371 ± 0.00</td>
<td>0.189 ± 0.00</td>
</tr>
<tr>
<td>PI636375 [B]</td>
<td>3.32 ± 0.02</td>
<td>6.54 ± 0.04</td>
<td>0.558 ± 0.00</td>
<td>0.424 ± 0.00</td>
<td>0.177 ± 0.00</td>
</tr>
<tr>
<td>AMES27010 [B]</td>
<td>5.06 ± 0.15</td>
<td>2.16 ± 0.04</td>
<td>0.526 ± 0.01</td>
<td>0.302 ± 0.01</td>
<td>0.158 ± 0.00</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation, n = 3/cultivar. Statistical significance levels among polyphenols by two-way ANOVA were P < 0.0001 for polyphenol, P < 0.0001 for genotype, and P = 0.0001 for their interaction. ANOVA includes results from Table 2.4.

A[R]: Red aronia accession

B[P]: Purple aronia accession

C[B]: Black aronia accession
Table 2.4. Anthocyanin content of chokeberry accessions determined by UPLC-MS as mg/g dry weight chokeberry.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Cy3Gal</th>
<th>Cy3Glu</th>
<th>Cy3A</th>
<th>Cy3X</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC021 [R]</td>
<td>0.577 ± 0.00</td>
<td>nd</td>
<td>0.016 ± 0.00</td>
<td>nd</td>
</tr>
<tr>
<td>UC057 [R]</td>
<td>0.730 ± 0.00</td>
<td>0.013 ± 0.00</td>
<td>0.080 ± 0.00</td>
<td>nd</td>
</tr>
<tr>
<td>UC053 [R]</td>
<td>0.410 ± 0.00</td>
<td>0.008 ± 0.00</td>
<td>0.060 ± 0.00</td>
<td>nd</td>
</tr>
<tr>
<td>UC047 [P]</td>
<td>3.88 ± 0.01</td>
<td>0.050 ± 0.00</td>
<td>0.059 ± 0.00</td>
<td>nd</td>
</tr>
<tr>
<td>UC011 [P]</td>
<td>2.08 ± 0.04</td>
<td>0.045 ± 0.00</td>
<td>1.01 ± 0.02</td>
<td>0.055 ± 0.00</td>
</tr>
<tr>
<td>UC033 [P]</td>
<td>1.53 ± 0.01</td>
<td>0.038 ± 0.00</td>
<td>0.817 ± 0.01</td>
<td>0.015 ± 0.00</td>
</tr>
<tr>
<td>PI578096 [P]</td>
<td>2.49 ± 0.01</td>
<td>0.041 ± 0.00</td>
<td>0.711 ± 0.00</td>
<td>nd</td>
</tr>
<tr>
<td>UC007 [P]</td>
<td>2.21 ± 0.01</td>
<td>0.049 ± 0.00</td>
<td>1.05 ± 0.01</td>
<td>0.057 ± 0.00</td>
</tr>
<tr>
<td>‘Viking’ [B]</td>
<td>9.00 ± 0.05</td>
<td>0.469 ± 0.01</td>
<td>4.06 ± 0.02</td>
<td>0.391 ± 0.00</td>
</tr>
<tr>
<td>UC009 [B]</td>
<td>14.5 ± 0.10</td>
<td>0.191 ± 0.00</td>
<td>0.184 ± 0.00</td>
<td>nd</td>
</tr>
<tr>
<td>PI636375 [B]</td>
<td>4.50 ± 0.05</td>
<td>0.109 ± 0.01</td>
<td>2.16 ± 0.02</td>
<td>0.126 ± 0.00</td>
</tr>
<tr>
<td>AMES27010 [B]</td>
<td>3.95 ± 0.01</td>
<td>0.098 ± 0.00</td>
<td>2.04 ± 0.01</td>
<td>0.150 ± 0.01</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation, n = 3/cultivar. Anthocyanins are expressed as Cyanidin-3-Galactoside equivalents. Statistical significance levels among anthocyanins by two-way ANOVA were P < 0.0001 for anthocyanins, P < 0.0001 for genotype, and P = 0.0001 for their interaction. "Below limit of detection. ANOVA includes results from Table 2.3."
Data are expressed as mean ± standard deviation, n = 3/cultivar. PACs are expressed as catechin equivalents. Statistical significance levels among PACs by two-way ANOVA were P < 0.0001 for PACs, P < 0.0001 for genotype, and P = 0.0001 for their interaction. Significance was assessed by considering each oligomer separately (e.g. monomers to nonamers).

Table 2.5. Normal Phase HPLC Analysis of Proanthocyanidins (PACs), expressed in µg/g DW.

<table>
<thead>
<tr>
<th></th>
<th>Monomers</th>
<th>Dimers</th>
<th>Trimers</th>
<th>4-6mers</th>
<th>7-9mers</th>
<th>Polymers</th>
<th>Total (Sum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC021 [R]</td>
<td>0.04 ± 0</td>
<td>1.32 ± 0.15</td>
<td>3.16 ± 0.26</td>
<td>6.69 ± 0.52</td>
<td>1.41 ± 0.15</td>
<td>3658.5 ± 277.15</td>
<td>3671.12 ± 278.24</td>
</tr>
<tr>
<td>UC057 [R]</td>
<td>0.05 ± 0</td>
<td>0.48 ± 0.04</td>
<td>0.78 ± 0.06</td>
<td>1.65 ± 0.14</td>
<td>0.27 ± 0.03</td>
<td>1096.01 ± 36.16</td>
<td>1099.23 ± 36.44</td>
</tr>
<tr>
<td>UC053 [R]</td>
<td>0.01 ± 0</td>
<td>0.14 ± 0.02</td>
<td>0.75 ± 0.13</td>
<td>2.24 ± 0.54</td>
<td>0.5 ± 0.15</td>
<td>1727.14 ± 24.51</td>
<td>1730.76 ± 25.35</td>
</tr>
<tr>
<td>UC047 [P]</td>
<td>0.09 ± 0</td>
<td>1.24 ± 0.09</td>
<td>1.89 ± 0.09</td>
<td>3.75 ± 0.19</td>
<td>0.71 ± 0.03</td>
<td>2009.32 ± 41.56</td>
<td>2017.01 ± 41.96</td>
</tr>
<tr>
<td>UC011 [P]</td>
<td>0.19 ± 0.01</td>
<td>0.69 ± 0.09</td>
<td>0.87 ± 0.09</td>
<td>1.99 ± 0.21</td>
<td>0.45 ± 0.06</td>
<td>1967.77 ± 66.19</td>
<td>1971.96 ± 66.67</td>
</tr>
<tr>
<td>UC033 [P]</td>
<td>0.02 ± 0</td>
<td>0.55 ± 0.07</td>
<td>1.77 ± 0.12</td>
<td>3.63 ± 0.36</td>
<td>0.75 ± 0.11</td>
<td>1987.1 ± 136.09</td>
<td>1993.82 ± 136.75</td>
</tr>
<tr>
<td>PI578096 [P]</td>
<td>0.01 ± 0</td>
<td>0.14 ± 0.04</td>
<td>0.45 ± 0.06</td>
<td>1.58 ± 0.25</td>
<td>0.47 ± 0.09</td>
<td>1721.01 ± 42.22</td>
<td>1723.67 ± 42.67</td>
</tr>
<tr>
<td>UC007 [P]</td>
<td>0.01 ± 0</td>
<td>0.25 ± 0.04</td>
<td>0.74 ± 0.07</td>
<td>1.7 ± 0.21</td>
<td>0.36 ± 0.08</td>
<td>2330.88 ± 1.20</td>
<td>2333.94 ± 1.59</td>
</tr>
<tr>
<td>‘Viking’ [B]</td>
<td>0.01 ± 0</td>
<td>0.1 ± 0.03</td>
<td>0.22 ± 0.02</td>
<td>0.48 ± 0.04</td>
<td>0.1 ± 0.01</td>
<td>3257.85 ± 209.98</td>
<td>3258.76 ± 210.09</td>
</tr>
<tr>
<td>UC009 [B]</td>
<td>0.01 ± 0</td>
<td>0.39 ± 0.04</td>
<td>0.86 ± 0.07</td>
<td>1.75 ± 0.19</td>
<td>0.36 ± 0.05</td>
<td>2289.61 ± 76.52</td>
<td>2292.99 ± 76.88</td>
</tr>
<tr>
<td>PI636375 [B]</td>
<td>0.01 ± 0</td>
<td>0.22 ± 0.04</td>
<td>0.49 ± 0.03</td>
<td>1.02 ± 0.05</td>
<td>0.21 ± 0.01</td>
<td>1561.80 ± 40.66</td>
<td>1563.76 ± 40.79</td>
</tr>
<tr>
<td>AMES27010 [B]</td>
<td>0.02 ± 0</td>
<td>0.28 ± 0.07</td>
<td>0.63 ± 0.09</td>
<td>1.32 ± 0.17</td>
<td>0.24 ± 0.03</td>
<td>2057.34 ± 111.94</td>
<td>2059.81 ± 112.3</td>
</tr>
</tbody>
</table>
Table 2.6. Total phenols and proanthocyanidins by DMAC of *Aronia* ssp. accessions.

<table>
<thead>
<tr>
<th>Berry Color</th>
<th>Accession</th>
<th>Proanthocyanidins (mg CE/g DW)</th>
<th>Total phenols (mg GAE/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>red</td>
<td>UC021</td>
<td>18.6 ± 1.10</td>
<td>250 ± 7</td>
</tr>
<tr>
<td>red</td>
<td>UC057</td>
<td>4.25 ± 0.09</td>
<td>154 ± 11</td>
</tr>
<tr>
<td>red</td>
<td>UC053</td>
<td>13.6 ± 0.30</td>
<td>151 ± 12</td>
</tr>
<tr>
<td>purple</td>
<td>UC047</td>
<td>5.24 ± 0.03</td>
<td>208 ± 7</td>
</tr>
<tr>
<td>purple</td>
<td>UC011</td>
<td>12.8 ± 0.40</td>
<td>148 ± 14</td>
</tr>
<tr>
<td>purple</td>
<td>UC033</td>
<td>5.56 ± 0.10</td>
<td>246 ± 12</td>
</tr>
<tr>
<td>purple</td>
<td>PI578096</td>
<td>12.9 ± 0.70</td>
<td>193 ± 11</td>
</tr>
<tr>
<td>purple</td>
<td>UC007</td>
<td>9.25 ± 0.89</td>
<td>138 ± 8</td>
</tr>
<tr>
<td>black</td>
<td>‘Viking’</td>
<td>9.79 ± 0.42</td>
<td>163 ± 12</td>
</tr>
<tr>
<td>black</td>
<td>UC009</td>
<td>9.27 ± 0.32</td>
<td>197 ± 15</td>
</tr>
<tr>
<td>black</td>
<td>PI636375</td>
<td>13.5 ± 0.60</td>
<td>182 ± 11</td>
</tr>
<tr>
<td>black</td>
<td>AMES27010</td>
<td>10.5 ± 0.40</td>
<td>127 ± 3</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation of n = 3 determinations. P < 0.0001 for total phenols and P < 0.0001 for proanthocyanidins by one-way ANOVA. Total phenols are expressed as gallic acid equivalents and PACs are expressed as catechin equivalents.

^A Catechin equivalents

^B Gallic acid equivalents
Table 2.7. Polyphenol composition of *Aronia* ssp. as sum of classes on a dry weight basis.

<table>
<thead>
<tr>
<th>Polyphenol (units)</th>
<th>Value</th>
<th>Aronia Color</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>range</td>
<td>red</td>
<td>purple</td>
<td>black</td>
<td></td>
</tr>
<tr>
<td>Anthocyanins (mg/g DW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>0.63</td>
<td>3.24</td>
<td>10.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxycinnamic acids (mg/g DW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>8.27</td>
<td>10.68</td>
<td>7.62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavonols (mg/g DW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>0.79</td>
<td>0.89</td>
<td>1.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proanthocyanidins, HPLC (mg/g DW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>2.17</td>
<td>2.01</td>
<td>2.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proanthocyanidins, DMAC (mg CE/g DW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>12.15</td>
<td>9.15</td>
<td>10.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total phenols (mg GAE/g DW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>185</td>
<td>187</td>
<td>167</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are for n = 3/red, n = 5/purple, and n= 4/black *Aronia* ssp.
Figure 2.1. A. Preliminary and B. Optimized reverse-phase HPLC chromatograms of blueberry anthocyanins.
Figure 2.2. Representative UPLC-MS chromatograms of ‘Viking’ chokeberry extract.
Figure 2.3. UV chromatograms of A. black (‘Viking’), B. purple (UC011), and C. red (UC057) chokeberry anthocyanins from 1 μL injections.

A.  

Viking

B.  

UCO11

C.  

UCO57
Figure 2.4. Representative normal-phase HPLC Chromatogram of UC021 ssp. red chokeberry proanthocyanidins from A. 1 µL and B. 5 µL injections.

A.

B.
Figure 2.5. A. Biplot and B. loading plot of first two principle components of *Aronia* ssp. accession polyphenol content.
2.5 REFERENCES


Chapter 3:

Influence of Harvest Timing on Aronia Juice Polyphenol Content
3.1 INTRODUCTION

A variety of factors beyond genetics can affect the phytonutrient content of chokeberries. These include but are not limited to climate, soil conditions, season, and harvest timing. Many changes occur throughout fruit ripening. Apples have been shown to increase in total sugar content and maintain their respective glucose/fructose ratio throughout ripening [1]. Apple organic acids including succinic, malic, lactic, and citric acid either increase or decrease depending on variety [1]. Chlorogenic acid can decrease by as much as 58%, and epicatechin has been shown to decrease by up to 68% during apple ripening [1]. Polyphenols also change in berry ripening. For example, chlorogenic acid and anthocyanins have been shown to increase in strawberries during ripening [2]. Hydroxycinnamic acids, anthocyanins, and total phenols have been shown to increase throughout ripening in lowbush blueberries [3].

Chokeberries differ from apples in terms of polyphenol changes during ripening, and appear to follow a pattern similar to strawberries and blueberries with respect to hydroxycinnamic acids, anthocyanins, and total phenols. Previous studies have shown that the average chokeberry mass can increase by up to 30% and anthocyanin content can increase by up to 200% throughout the growing season [4]. Acidity also decreases by 18-21% in chokeberry throughout ripening as well [4, 5]. Malic acid content of black chokeberry has been shown to fluctuate throughout ripening, but no visible trend has been observed to date [6].

Due to the ongoing use of ‘Viking’ chokeberry as a nutraceutical crop or as a polyphenol-rich supplement, optimization of harvest timing with respect to polyphenol content is essential for maximizing product yield. Previous studies on chokeberry ripening have employed a limited number of plants with limited polyphenol analysis. The Jeppsson and Johansson study only used...
3 plants and only reported on berry weight, anthocyanin content, and browning [6]. Changes in hydroxycinnamic acids, proanthocyanidins, and flavonols have not been characterized in black chokeberry. The objectives of this study were to perform a well-controlled, randomized harvest collection and quantitate polyphenols in chokeberry juice prepared from ‘Viking’ ssp. berries. This data will provide further information needed to determine the optimal harvest dates to maximize phytochemical yield and antioxidant function of chokeberry juice.

3.2 MATERIALS AND METHODS

3.2.1 Berry Harvesting and Juicing

‘Viking’ ssp. plants (~600) (3 years old) were grown on a single test plot in Storrs, CT maintained by Dr. Mark Brand of the University of Connecticut. Berries were grown in the absence of fertilizer or pesticides. Prior to harvest, 10 blocks of 5 plants each were randomized. Beginning at the first signs of ripening, 5 blocks were harvested weekly beginning on 7/10/2012 and ending on 9/12/2012. We considered the beginning of ripeness to be 8/01/2012, and berries picked prior to this date were typically small and green, while those picked in the last 7 weeks were considered to be within the window of harvesting edible berries. We considered week 7 (9/12/2012) to be the last week of ripeness, as berries were beginning to shrivel. Only ripe berries were juiced and analyzed for phytochemical content.

Berries were prepared for juicing by washing in cold water, and drying on paper towels for 1 h. Dried berries were frozen at -21°C. At the end of the harvest season, berries were thawed, macerated, and squeezed in a cider press. Juice was separated into 20 mL aliquots and stored at -
21°C. Juice samples were centrifuged at 290 x g and their supernatants were removed and stored at -80°C for further analysis.

### 3.2.2 Folin-Ciocalteu Analysis of Total Phenols

The Folin-Ciocalteu total phenol assay was used to determine changes in total phenol content of chokeberry juices throughout the growing season following a method by Singleton et al. [7]. Briefly, 10 µL of standards or extract were pipetted into each microtiter well, followed by 173 µL of distilled water and 15 µL of Folin-Ciocalteu reagent. The plate was incubated for 5 minutes at room temperature, and then 45 µL of saturated sodium carbonate and 57 µL of distilled water were added to each well. The plate was left in the dark for 1 h and then absorbance was measured at 765 nm in a microplate spectrophotometer. Juices were diluted in ultrapure water and tested at various dilutions ranging from 10- to 200-fold against a standard curve ranging from 16.125 to 2000 µg gallic acid/mL in distilled water. The 10-fold dilution of juice was suitable for determination of total phenols.

### 3.2.3 DMAC Analysis of Proanthocyanidins

The DMAC (4-(dimethylamino) cinnamaldehyde) assay was performed on the juices to determine changes in total proanthocyanidin content throughout the growing season using a method by Prior et al. that was validated for cranberry proanthocyanidin analysis [8]. A catechin standard curve of 1.56 to 100 µg (+)-catechin per mL of 91% ethanol in water was used to quantify proanthocyanidin equivalents. Juices were diluted in an acetone/water/acetic acid solution (70/29.5/0.5) and a trial run was performed with UC021 to determine an appropriate dilution factor for chokeberry samples in DMAC analysis. A 100-fold dilution factor was suitable for analysis. The assay was performed by pipetting 70 µL of standards or chokeberry
juice into microtiter wells. The DMAC solution (280 µL) was added to each well and immediately placed into a plate reader. Absorbance at 15 min (640 nm) was used to determine proanthocyanidin content.

3.2.4 Brix Analysis of Sugar Content

Using a portable refractometer, measures of relative sugar content in the 7 chokeberry juices were determined. Drops of juice were placed onto the refractometer slide, and the refractometer was held at 30º towards a light source to measure °brix. Juices were analyzed in triplicate to determine an average brix value for each juice sample.

3.2.5 FRAP Analysis

The FRAP (Ferric Reducing Ability of Plasma) assay is a common method used to measure antioxidant capacity. The FRAP assay quantifies the reduction of ferric to ferrous iron, by measuring the ferrous-tripypyridyltriazine complex, a blue indicator, in a spectrophotometer [9]. A Molecular Devices SpectraMax M2 spectrophotometer (Sunnyvale, CA) was used to monitor the reaction. Chokeberry juices were diluted 100-fold prior to analysis. Cuvettes were loaded with 90 µL distilled water and 30 µL of either samples, standards, or water (blank). A 900 µL aliquot of cocktail solution consisting of: 70 mL 300 mM acetate buffer standardized to pH 3.6, 7 mL TPTZ (2,4,6-Tripyridyl-S-Triazine) in 40mM HCl, and 7 mL ferric chloride in distilled water, was quickly added to each cuvette and mixtures were left to incubate for 60 min at room temperature in the dark. Following incubation, the absorbance was determined at 593 nm. Data was plotted against a Trolox standard curve ranging from 15.62 to 250 µg/mL.
3.2.6 UPLC-MS Analysis of Anthocyanins and Flavonoids

Chokeberry juice was centrifuged at 290 x g for 10 min and diluted 10-fold with 30% methanol in water prior to UPLC-MS analysis. Compounds of interest in UPLC-MS analysis included cyanidin-3-galactoside (MW: 448), cyanidin-3-glucoside (MW: 448), cyanidin-3-arabinoside (MW: 418), cyanidin-3-xyloside (MW: 418), chlorogenic acid (MW: 354), neochlorogenic acid (MW: 354), quercetin-3-galactoside (MW: 464), quercetin-3-glucoside (MW: 464), and quercetin-3-rutinoside (MW: 610). A standard curve of prepared consisting of chlorogenic acid (Cga), neochlorogenic acid (nCga), quercetin-3-galactoside (Q3Gal), quercetin-3-glucoside (Q3Glu), rutin (Q3R), and cyanidin-3-galactoside (Cy3Gal) was prepared in 30% methanol. Standard concentrations ranged from 0.001 to 0.1 mg/mL. Standards or samples were injected with a Shimadzu Nexera UPLC-MS (as described in chapter 3) onto a Kinetex PFP 2.1 x 50 mm, 1.7 µm, 100A column (Phenomenex Inc., Torrance, CA) in 1 µL volumes. Mobile phases were 0.5% formic acid in water (A) and 100% methanol (B) at a total flow rate of 0.2 mL/min. The gradient consisted of 30% B from 0 to 6 min, a linear gradient to 55% B at 12 min, descending to 30% B over 2 min, with a 4 min hold period at 30% B, for a total runtime of 20 min. The concentration of formic acid in mobile phase A was reduced from the previous study (Chapter 3) because of instrument corrosion concerns.

Anthocyanins were quantified by UV at 520 nm using Cy3Gal as a standard. Anthocyanin content was expressed as Cy3Gal equivalents. The limit of detection (LOD) for anthocyanins was 1.2 ng on column and the limit of quantitation (LOQ) was 1.4 ng on column.

Non-anthocyanin flavonoids were analyzed with MS in DUIS mode, following the same instrument settings and method as described in Chapter 3. The detector voltage was 1.5 kv,
interface temperature was 35°C, DL temperature was 250°C, Heat block temperature was 400°C, drying gas flow rate was 15 L/min, mobile phase flow rate was 0.2 mL/min, and maximum pressure was 10,000 Psi. Flavonoids and phenolic acids of interest in this study included Cga, nCga, Q3Gal, Q3Glu, and Q3R, all of which were observed in the chokeberry juices. LOD and LOQ were determined using the method in the previous study. LOQ for Q3Gal, Q3Glu, and rutin were 0.5, 0.5, and 0.4 ng OC respectively. LOD for flavonols was 0.4 ng OC. LOD for hydroxycinnamic acids was 0.5 ng OC. LOQ for Cga and nCga were 0.7 and 0.6 ng OC respectively.

3.2.7 Statistical Analysis

To determine whether harvest timing had a significant effect on polyphenol content, sugar content, and antioxidant activity, a one-way analysis of variance (ANOVA) and a Tukey's multiple comparison test were utilized. A Pearson's correlation analysis was used to determine whether any significant correlations existed between polyphenols of different classes. Significance level for all statistical analyses was $p = 0.05$. Statistical analyses were performed using Graphpad Prism software.

3.3 RESULTS AND DISCUSSION

3.3.1 Total Phenol Content

Total phenol values increased from 4.03 to 5.03 mg gallic acid equivalents (GAE)/mL between weeks 1 and 7 (Table 3.1). Total phenols decreased from previous weeks in weeks 2 and 6 (Figure 3.1). This represents a 24% increase in total phenol content during ripening (Table 3.2). A Pearson’s correlation analysis revealed that total phenol values for the chokeberry juices had
strong positive correlations with DMAC (p = 0.004) and FRAP (p = 0.030) values, as well as Brix (p = 0.026). The correlation between DMAC and total phenols is indicative of the relatively high proanthocyanidin content of chokeberry with respect to other polyphenols. The strong correlation between total phenols and FRAP values reflects the collective reducing power of polyphenols in chokeberry. A one-way ANOVA revealed that P < 0.0001 for total phenols.

Total phenol values for berries of different species change differently during ripening. For example, strawberry total phenols are highest in the unripe stage and steadily decrease throughout ripening, while blueberries increase through ripening [3, 10]. Thus, the total phenol content of blueberries is similar to chokeberry during the ripening process.

3.3.2 Proanthocyanidin content

Proanthocyanidin content assessed by the DMAC assay increased from 214 to 352 µg catechin equivalents (CE)/mL between weeks 1 and 7, and decreased at week 2 (Table 3.1 and Figure 3.1). This represents a 67% increase in proanthocyanidin content of the berry juices over the course of 7 weeks (Table 3.2). A one-way ANOVA revealed that P < 0.0001 for proanthocyanidins. These results are similar to those observed in cranberry, but in contrast to those typically observed in blackberries, raspberries, and strawberries [11, 12]. The DMAC assay provides no data about proanthocyanidin composition or degree of polymerization, and so HPLC analysis should be performed in the future to further characterize these changes throughout ripening.

3.3.3 Brix values
Brix analysis was used to determine the changes in soluble solids, mainly sugars throughout the chokeberry harvest season. While the content of individual sugars in chokeberry juice was not measured in this study, the relative sugar contents of samples harvested at each week were determined through the brix analysis and a trend was observed showing a gradual increase from 10.5 ± 0 to 14.33 ± 0.3 degrees brix between weeks 1 and 7, with a decrease from the previous week at weeks 2 and 5 (P < 0.0001) (Table 3.1 and Figure 3.1). This represents a 36% increase in sugar content of the berry juices over the course of 7 weeks (Table 3.2). Kaack and Kuhn observed an increase in soluble solids in black chokeberry throughout ripening, similar to the present study [5]. These data are also similar to gains in apple sugar content throughout ripening [1]. Chokeberry has a greater concentration of sugars than most sweet commercial berries including strawberries, blueberries, blackberries, currants, and raspberries [13]. The sweetness of chokeberry is masked by astringency however, which is likely due to its high polyphenol content. Unlike most berries, chokeberry contains appreciable levels of sorbitol. Sorbitol represents 40.5% of the sugar in chokeberry [13].

3.3.4 Flavonol content

Q3Gal, Q3Glu, and Q3R were observed in chokeberry juice (Table 3.3). Total flavonol values began at 52.65 ± 0.15 at week 1 and ended at 45.06 ± 0.05 µg/mL, however values fluctuated as much as 24% between some weeks, indicating no real trend in total flavonol content from the beginning to the end of the harvest season (Table 3.2 and Figure 3.2). A one way ANOVA revealed that P <0.0001 for Q3R, Q3Gal, and total flavonols; and P = 0.0001 for Q3Glu. This is similar to the observations made by Vvedenskysya and Vorsa in Ben Lear and Stevens cranberries, but contrary to observations made in strawberry [2, 11]. The order of abundance for
flavonols began as Q3Gal > Q3Glu > Q3R at week 1, and changed to Q3Glu > Q3Gal > Q3R at week two. All three values decreased within the first week, however the greatest decrease was observed in Q3Gal content. Q3Glu remained the predominant flavonol in the rest of the chokeberry juice samples, and by week 6 the order of abundance was Q3Glu > Q3R > Q3Gal.

In the previous study (Chapter 3), we found that Q3Gal represented 46% of total flavonols and Q3R only represented 16% of total flavonols in lyophilized ‘Viking’ berries. ‘Viking’ juices contained significantly lower levels of Q3Gal and higher levels of Q3R however, as juice from week 1 is 37% Q3Gal and 29% Q3R, and juice from week 7 is 31% Q3Gal and 31% Q3R. This may be due to preferential extraction of Q3Gal with the acetone/water/acetic acid solution used in chapter 3 as compared to juice expression. The content of Q3Gal may also be higher in chokeberry skins, which were only extracted in Chapter 3.

3.3.5 Hydroxycinnamic acid content

The hydroxycinnamic acids observed in ‘Viking’ juices were Cga and nCga (Table 3.4). Total hydroxycinnamic acid content gradually declined from 1,248 ± 0 to 835 ± 24 µg/mL between weeks 1 and 7, a 33% decrease (Table 3.2 and Figure 3.2). A one-way ANOVA revealed that P < 0.0001 for Cga, nCga, and total hydroxycinnamic acids. While minor fluctuations in hydroxycinnamic acid content were observed throughout the season, hydroxycinnamic acid profile remained fairly constant. The hydroxycinnamic acid profile of lyophilized ‘Viking’ berries sampled in 2011 was 60.8% Cga, as opposed to an average of 56.8% observed in ‘Viking’ juice. This may indicate a greater presence of Cga retained in the berry skins following juicing, or may simply be due to yearly variation. The hydroxycinnamic acid profile remained fairly constant throughout the growing season. At week 1, hydroxycinnamic acid content was
56.9% nCga and 43.1% Cga. At week 7, nCga represented 56.7% and Cga represented 43.3% of total hydroxycinnamic acids. Caffeic acid was not detected in any of the juice samples.

Hydroxycinnamic acids in apples have also been shown to decrease in apples during ripening, but as much as 58% [1]. Cinnamic acid derivatives in blueberry have been shown to decrease during ripening, however chlorogenic acid values (the primary cinnamic acid in chokeberry) did not change between the slightly unripe and over-ripe stage [14].

Cga represented 61% of total hydroxycinnamic acids in lyophilized ‘Viking’ berries (Chapter 3). ‘Viking’ juices however, contained a greater proportion of nCga, nearly 57% in both week 1 and week 7. This may be due to greater retention of Cga in chokeberry skins or poor recovery of nCga with the acetone/water/acetic acid solution used in chapter 3 as opposed to the amount extracted by juicing.

### 3.3.6 Anthocyanin content

The anthocyanin analysis of chokeberry juices differed slightly from the whole berry study (Chapter 3) in that Cy3Glu was not separated from Cy3Gal in juices. This is likely due to the co-elution of Cy3Gal and Cy3Glu in our UPLC-MS analysis, a result of reducing the formic acid content of the mobile phase. Initial total anthocyanin content was 366 ± 3 µg/mL in week 1 and peaked at 1200 ± 39 µg/mL at week 5, and then declined to 817 ± 13 µg/mL (P < 0.0001) (Table 3.5 and Figure 3.2). This represents a 227% increase between week 1 and week 5 followed by a 32% decrease in the next two weeks (Table 3.2). The anthocyanin profile of ‘Viking’ juice at week 1 was about 75.4% Cy3Gal + Cy3Glu and 24.6% Cy3Ara. Cy3X was not detected until week two. By week 5, Cy3Gal + Cy3Glu represented 74.2% of total anthocyanins, Cy3Ara represented 22.3%, and Cy3X was 3.5%. At week 7, Cy3Gal + Cy3Glu represented 74.9%,
21.6% for Cy3Ara, and 3.5% for Cy3X. While the total anthocyanin content of the berries varied by as much as 327% between weeks 1 and 5, the anthocyanin profile remained nearly identical between weekly samples; the only exception being week 1 when Cy3X was undetectable. Unlike the other polyphenols quantified in this study, anthocyanins peak at week 5, and not week 7. In the Jeppsson 2000 study, anthocyanin content peaked on September 7th, which was closest to our week 6. While the timing was not identical, anthocyanin content in their study also peaked before the end of the growing season, then declined [4]. Anthocyanins in strawberry and lowbush blueberry have been shown to increase throughout ripening, however these fruits do not peak and then decline in anthocyanin content before senescence [10], [3].

3.3.7 FRAP values

FRAP values for chokeberry juices increased from 6.79 mg/mL Trolox equivalents (TE)/mL at week 1 to a high of 10.41 mg/mL week 7, with a peak of 9.93 mg/mL week 4 (Table 3.1 and Figure 3.3). This represents a 53% increase in antioxidant activity (Table 3.2). A one-way ANOVA revealed that P < 0.0003 for FRAP values. In blackberries, ORAC values have been shown to increase from 43.0 µmol TE/g FW to 62.7 µmol TE/g FW in the Marion cultivar, and from 46.1 µmol TE/g FW to 64.4 µmol/g FW in Evergreen during ripening [15]. Upon visual inspection of FRAP values, the trend did not appear to follow the pattern similar to other polyphenols. However, Pearson’s correlation analysis revealed that FRAP values correlated with total phenols. The correlation between Total phenols and FRAP had a coefficient of 0.80 with P = 0.030 (Table 3.7). Our data indicates that of the polyphenol content analyses conducted, total phenol analysis was the best indicator of antioxidant capacity, and no single polyphenol class predominantly affected FRAP values.
Therefore, maximum polyphenol content and antioxidant capacity (as determined by the FRAP assay) of chokeberries was observed at week 7 of harvest. In contrast, if it is desirable to obtain the maximum anthocyanin content of chokeberries, week 5 is the ideal collection time. While we did not collect a sample from week 8, the shriveled and dried nature of the week 7 berries indicated that berries may not be edible at that point.

3.3.8 Moisture data

Moisture values were calculated for berries harvested at each week, as well as one sample harvested a week before the onset of ripeness. One week before ripeness, berries were mostly green and were 70% water. In the next week, berries turned purple and moisture increased to 83% (P < 0.0001). Moisture steadily declined to 80% at week 7, a 3% decrease overall (Table 3.8). ‘Viking’ berries harvested in 2011 were only 76% water (Chapter 3), indicating that moisture percentage may vary year to year between chokeberries of the same genotype.

3.4 CONCLUSIONS

Anthocyanins increased by 227% between the first and fifth weeks of ripeness then declined. Proanthocyanidins increased by 67%, sugar content increased 36%, total phenols increased by 24%, antioxidant activity via the FRAP assay increased by 53%, and hydroxycinnamic acids decreased by 33% during ripening in ‘Viking’ chokeberries. No significant trend was observed in flavonol content throughout the growing season. Antioxidant capacity had a stronger correlation with total phenols (P = 0.030) than any single polyphenol value.

The effect of storage conditions on polyphenol content should also be considered. All berry samples were frozen and thawed in this study; however previous studies have reported major
changes in flavonol and hydroxycinnamic acid content during storage, as well as a negative correlation between storage time and total polyphenol content [16, 17]. We assume that storage at -21°C for less than two months will minimally impact phytochemical content in chokeberries. It is important however to consider that activity of polyphenol oxidases may change when berries are stored at different temperature ranges and this may alter polyphenol content to some extent [16].

Polyphenol content is known to vary between harvest seasons [18], so this study should be repeated at later dates to determine whether trends remain the same between years. Differences are observed in polyphenol content of chokeberries of different genotypes as well as those of different color (Chapter 3), and repetition of this study with a different genotype of chokeberry, for example UC021, could yield different trends as well.

Additional changes through ripening should be assessed, for example polymerized anthocyanins, hydrolysable tannins, titratable acidity, and vitamin and mineral content. Due to the low palatability of chokeberry with respect to other fruit, sensory analysis should be conducted to determine changes in astringency, sweetness, and bitterness that occur through ripening. This information can shed light on the association between various polyphenols and sensory characteristics of the chokeberry, as well as reveal optimal harvest timing for chokeberries that are grown for consumption.
Table 3.1. Total phenols, proanthocyanidins by the DMAC\(^A\) assay, sugar content, and antioxidant capacity by the FRAP\(^B\) assay for ‘Viking’ juices from berries harvested at different time points..

<table>
<thead>
<tr>
<th>Week</th>
<th>Total Phenols</th>
<th>Proanthocyanidins</th>
<th>Brix</th>
<th>FRAP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg GAE(^C)/mL</td>
<td>mg CE(^D)/mL</td>
<td>°Brix</td>
<td>mg TE(^E)</td>
</tr>
<tr>
<td>1</td>
<td>4.03 ± 0.06</td>
<td>4.03 ± 0.06</td>
<td>10.5 ± 0.00</td>
<td>6.80 ± 0.34</td>
</tr>
<tr>
<td>2</td>
<td>3.79 ± 0.12</td>
<td>3.79 ± 0.12</td>
<td>7.80 ± 0.60</td>
<td>8.46 ± 0.67</td>
</tr>
<tr>
<td>3</td>
<td>4.35 ± 0.06</td>
<td>4.35 ± 0.06</td>
<td>11.2 ± 0.30</td>
<td>8.19 ± 0.06</td>
</tr>
<tr>
<td>4</td>
<td>4.68 ± 0.02</td>
<td>4.68 ± 0.02</td>
<td>12.7 ± 0.60</td>
<td>9.93 ± 0.28</td>
</tr>
<tr>
<td>5</td>
<td>4.66 ± 0.07</td>
<td>4.66 ± 0.07</td>
<td>11.00 ± 0.50</td>
<td>9.18 ± 0.11</td>
</tr>
<tr>
<td>6</td>
<td>4.29 ± 0.14</td>
<td>4.29 ± 0.14</td>
<td>13.3 ± 0.30</td>
<td>8.70 ± 0.00</td>
</tr>
<tr>
<td>7</td>
<td>5.03 ± 0.08</td>
<td>5.03 ± 0.08</td>
<td>14.3 ± 0.30</td>
<td>10.41 ± 0.51</td>
</tr>
</tbody>
</table>

Data is expressed as mean ± standard deviation, n = 3 for total phenols, proanthocyanidins, and brix; n = 2 for FRAP. Statistical significance for weekly values by one-way ANOVA were P < 0.0001 for total phenols, proanthocyanidins, and brix; P < 0.0003 for FRAP.

\(^A\)DMAC: 4-(dimethylamino) cinnamaldehyde assay

\(^B\)FRAP: Ferric Reducing Ability of Plasma

\(^C\)GAE: Gallic acid equivalents

\(^D\)CE: Catechin equivalents

\(^E\)TE: Trolox equivalents
Table 3.2. Polyphenol composition, antioxidant capacity, sugar content, and ∆% of week 1 of ‘Viking’ chokeberry juices from 4 representative weekly samples.

<table>
<thead>
<tr>
<th>Polyphenol (units)</th>
<th>Mean values per week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value 1</td>
</tr>
<tr>
<td>Anthocyanins (µg/mL)</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>∆% of week 1</td>
</tr>
<tr>
<td>Hydroxycinnamic acids (µg/mL)</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>∆% of week 1</td>
</tr>
<tr>
<td>Flavonols (µg/mL)</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>∆% of week 1</td>
</tr>
<tr>
<td>Proanthocyanidins (mg CE/mL)</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>∆% of week 1</td>
</tr>
<tr>
<td>Total phenols (mg GAE/mL)</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>∆% of week 1</td>
</tr>
<tr>
<td>FRAP (mg TE/mL)</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>∆% of week 1</td>
</tr>
<tr>
<td>°Brix</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>∆% of week 1</td>
</tr>
</tbody>
</table>
Table 3.3. Flavonol content of 7 weekly ‘Viking’ juices quantified via UPLC-MS and expressed as µg/mL juice.

<table>
<thead>
<tr>
<th>Week</th>
<th>Q3R</th>
<th>Q3Gal</th>
<th>Q3Glu</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.64 ± 0.30</td>
<td>19.54 ± 0.24</td>
<td>17.47 ± 0.18</td>
</tr>
<tr>
<td>2</td>
<td>12.74 ± 0.23</td>
<td>15.59 ± 0.24</td>
<td>16.2 ± 0.47</td>
</tr>
<tr>
<td>3</td>
<td>12.43 ± 0.30</td>
<td>13.59 ± 0.12</td>
<td>14.87 ± 0.40</td>
</tr>
<tr>
<td>4</td>
<td>13.32 ± 0.30</td>
<td>13.84 ± 0.56</td>
<td>16.32 ± 0.30</td>
</tr>
<tr>
<td>5</td>
<td>15.15 ± 0.49</td>
<td>15.91 ± 0.81</td>
<td>19.11 ± 1.04</td>
</tr>
<tr>
<td>6</td>
<td>11.1 ± 0.11</td>
<td>10.92 ± 0.24</td>
<td>13.08 ± 0.40</td>
</tr>
<tr>
<td>7</td>
<td>14.27 ± 0.08</td>
<td>14.09 ± 0.02</td>
<td>16.7 ± 0.01</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation, n = 3 for Q3R, Q3Gal, and Q3Glu. P < 0.0001 for Q3R, and Q3Gal, P = 0.0001 for Q3Glu by one-way ANOVA analysis.
Table 3.4. Hydroxycinnamic acid content of 7 weekly ‘Viking’ juices quantified via U PLC-MS and expressed as µg/mL juice.

<table>
<thead>
<tr>
<th>Week</th>
<th>Cga</th>
<th>nCga</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>538.05 ± 0.97</td>
<td>710.09 ± 1.77</td>
</tr>
<tr>
<td>2</td>
<td>408.78 ± 2.01</td>
<td>595.33 ± 5.14</td>
</tr>
<tr>
<td>3</td>
<td>370.38 ± 6.69</td>
<td>421.65 ± 0.15</td>
</tr>
<tr>
<td>4</td>
<td>353.69 ± 4.47</td>
<td>528.29 ± 9.15</td>
</tr>
<tr>
<td>5</td>
<td>385.79 ± 33.38</td>
<td>522.98 ± 41.82</td>
</tr>
<tr>
<td>6</td>
<td>347.24 ± 10.49</td>
<td>449.26 ± 6.41</td>
</tr>
<tr>
<td>7</td>
<td>361.51 ± 10.55</td>
<td>473.81 ± 14.03</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation, n = 3 for Cga and nCga, P < 0.0001 for Cga and nCga by one-way ANOVA analysis.
Table 3.5. Anthocyanin content of 7 weekly ‘Viking’ juices quantified via UPLC-UV and expressed as μg/mL juice.

<table>
<thead>
<tr>
<th>Week</th>
<th>Cy3Gal</th>
<th>Cy3A</th>
<th>Cy3X</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>276.37 ± 2.09</td>
<td>90.04 ± 0.54</td>
<td>nd</td>
</tr>
<tr>
<td>2</td>
<td>455.52 ± 10.31</td>
<td>144.09 ± 10.31</td>
<td>8.98 ± 3.47</td>
</tr>
<tr>
<td>3</td>
<td>572.9 ± 0.77</td>
<td>180.51 ± 0.77</td>
<td>24.99 ± 0.52</td>
</tr>
<tr>
<td>4</td>
<td>755.77 ± 0.45</td>
<td>225.94 ± 0.45</td>
<td>35.19 ± 1.53</td>
</tr>
<tr>
<td>5</td>
<td>890.39 ± 12.35</td>
<td>268.05 ± 12.35</td>
<td>41.12 ± 2.65</td>
</tr>
<tr>
<td>6</td>
<td>524.31 ± 1.53</td>
<td>157.86 ± 1.53</td>
<td>27.01 ± 0.73</td>
</tr>
<tr>
<td>7</td>
<td>612.07 ± 2.89</td>
<td>176.38 ± 2.89</td>
<td>28.51 ± 0.04</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation, n = 2 for CyGal, Cy3A, and Cy3X, P < 0.0001 for Cy3Gal, Cy3A, and Cy3X by one-way ANOVA analysis.
Table 3.6. Pearson’s correlation coefficients and P-values for tests performed on 7 weekly juice samples.

<table>
<thead>
<tr>
<th>P-values</th>
<th>FRAP</th>
<th>Anth(^A)</th>
<th>HCA(^B)</th>
<th>Flav(^C)</th>
<th>DMAC</th>
<th>TP(^D)</th>
<th>Brix</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRAP</td>
<td>0.071</td>
<td>0.094</td>
<td>0.553</td>
<td>0.071</td>
<td>0.030</td>
<td>0.168</td>
<td></td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>0.071</td>
<td>0.149</td>
<td>0.900</td>
<td>0.222</td>
<td>0.094</td>
<td>0.497</td>
<td></td>
</tr>
<tr>
<td>Hydroxycinnamic Acids</td>
<td>0.094</td>
<td>0.149</td>
<td>0.049</td>
<td>0.080</td>
<td>0.208</td>
<td>0.219</td>
<td></td>
</tr>
<tr>
<td>Flavonols</td>
<td>0.553</td>
<td>0.900</td>
<td>0.049</td>
<td>0.535</td>
<td>0.961</td>
<td>0.429</td>
<td></td>
</tr>
<tr>
<td>DMAC</td>
<td>0.071</td>
<td>0.222</td>
<td>0.080</td>
<td>0.535</td>
<td>0.004</td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td>Total Phenols</td>
<td>0.030</td>
<td>0.094</td>
<td>0.208</td>
<td>0.961</td>
<td>0.004</td>
<td>0.026</td>
<td></td>
</tr>
<tr>
<td>Brix</td>
<td>0.168</td>
<td>0.497</td>
<td>0.219</td>
<td>0.429</td>
<td>0.010</td>
<td>0.026</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Correlation Coefficients</th>
<th>FRAP</th>
<th>Anth</th>
<th>HCA</th>
<th>Flav</th>
<th>DMAC</th>
<th>TP</th>
<th>Brix</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRAP</td>
<td>0.715</td>
<td>-0.679</td>
<td>-0.274</td>
<td>0.714</td>
<td>0.802</td>
<td>0.584</td>
<td></td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>0.715</td>
<td>-0.606</td>
<td>-0.059</td>
<td>0.529</td>
<td>0.679</td>
<td>0.311</td>
<td></td>
</tr>
<tr>
<td>Hydroxycinnamic Acids</td>
<td>-0.679</td>
<td>-0.606</td>
<td>0.757</td>
<td>-0.701</td>
<td>-0.543</td>
<td>-0.532</td>
<td></td>
</tr>
<tr>
<td>Flavonols</td>
<td>-0.274</td>
<td>-0.059</td>
<td>0.757</td>
<td>-0.286</td>
<td>-0.023</td>
<td>-0.359</td>
<td></td>
</tr>
<tr>
<td>DMAC</td>
<td>0.714</td>
<td>0.529</td>
<td>-0.701</td>
<td>-0.286</td>
<td>0.913</td>
<td>0.877</td>
<td></td>
</tr>
<tr>
<td>Total Phenols</td>
<td>0.802</td>
<td>0.679</td>
<td>-0.543</td>
<td>-0.023</td>
<td>0.913</td>
<td>0.814</td>
<td></td>
</tr>
<tr>
<td>Brix</td>
<td>0.584</td>
<td>0.311</td>
<td>-0.532</td>
<td>-0.359</td>
<td>0.877</td>
<td>0.814</td>
<td></td>
</tr>
</tbody>
</table>

\(^A\)Anth: Anthocyanins  
\(^B\)HCA: Hydroxycinnamic acids  
\(^C\)Flav: Flavonols  
\(^D\)TP: Total phenols
Table 3.7. Moisture percentages of chokeberries harvested at different stages of ripeness.

<table>
<thead>
<tr>
<th>Week #</th>
<th>Harvest date</th>
<th>Average Moisture %</th>
</tr>
</thead>
<tbody>
<tr>
<td>(unripe)</td>
<td>7/26/2012</td>
<td>69.8</td>
</tr>
<tr>
<td>1</td>
<td>8/1/2012</td>
<td>82.6</td>
</tr>
<tr>
<td>2</td>
<td>8/8/2012</td>
<td>81.6</td>
</tr>
<tr>
<td>3</td>
<td>8/15/2012</td>
<td>81.6</td>
</tr>
<tr>
<td>4</td>
<td>8/22/2012</td>
<td>80.5</td>
</tr>
<tr>
<td>5</td>
<td>8/29/2012</td>
<td>78.8</td>
</tr>
<tr>
<td>6</td>
<td>9/6/2012</td>
<td>79.9</td>
</tr>
<tr>
<td>7</td>
<td>9/12/2012</td>
<td>79.6</td>
</tr>
</tbody>
</table>

n = 5 for moisture values. P < 0.0001 for weekly changes in moisture % by one-way ANOVA analysis.
Figure 3.1. A. Total phenols, B. Brix, and C. Proanthocyanidin values for ‘Viking’ chokeberry juices from 7 weekly samples.

A.

![Total Phenols Graph](image)

B.

![Brix Graph](image)

C.

![DMAC Graph](image)

Data are means ± standard deviation of n = 3. P < 0.0001 for total phenols, Brix, and DMAC by one-way ANOVA analysis.
Figure 3.2. A. Flavonols, B. Hydroxycinnamic acids, and C. Anthocyanins for ‘Viking’ chokeberry juices from 7 weekly samples calculated via UPLC-MS and expressed as µg/mL juice.

A.

![Flavonols graph]

B.

![Hydroxycinnamic Acids graph]

C.

![Anthocyanins graph]

Data are means ± standard deviation for n = 3 for flavonols and hydroxycinnamic acids and n = 2 for anthocyanins. P < 0.0001 for flavonols, hydroxycinnamic acids, and anthocyanins by one-way ANOVA analysis.
**Figure 3.3.** FRAP values for ‘Viking’ chokeberry juices from 7 weekly samples.

Data are means ± standard deviation of n = 3. P < 0.0001 by one-way ANOVA analysis.
3.5 REFERENCES


Chapter 4:

Conclusions and Future Directions
4.1 SUMMARY OF KEY FINDINGS

- Anthocyanin content of chokeberries was strongly correlated with berry color. Black chokeberries have greatest abundance of anthocyanins, followed by purple chokeberries, and lastly red.

- Flavonols, proanthocyanidins, hydroxycinnamic acids, and total phenols did not differ significantly based on chokeberry color.

- Regardless of color or genotype, the order of abundance for anthocyanins in chokeberry was cyanidin-3-galactoside (Cy3Gal) > cyanidin-3-arabinoside (Cy3A) > cyanidin-3-glucoside (Cy3Glu) > cyanidin-3-xyloside (Cy3X).

- Quercetin and its glycosides are the only flavonols that we detected in chokeberry. This is consistent with other studies [1, 2].

- Chlorogenic acid (Cga) and neochlorogenic acid (nCga) are the only hydroxycinnamic acids that we detected in chokeberry; however caffeic acid has been previously detected in wild chokeberries [3]. This may be a product of microbial breakdown or the extraction process.

- While moisture content ranged from 63.6% to 76% in our twelve chokeberry accessions, little difference was observed between fresh weight and dry weight data when ranking them based on polyphenol content.
• Chokeberry total phenol, proanthocyanidin, anthocyanin, and brix values all increased through ripening, while hydroxycinnamic acids decreased and flavonols did not change significantly.

• Anthocyanin content of ‘Viking’ chokeberries peaks at the fifth week of harvest (8/29/12 in our study) and then begins to decline.

• Juice from chokeberries picked later into ripening have greater antioxidant activity, as evaluated by the FRAP assay.

• Data for DMAC and HPLC analyses of proanthocyanidin content did not correlate in our 12 genotypes study, and HPLC data showed consistently lower levels of proanthocyanidins. This may be due to poor recovery with Sephadex LH-20 extraction, or our valley-to-valley integration as opposed to the use of a flat baseline.

The polyphenol content of aronia berries differs based on a variety of factors including genotype, growing conditions, and seasonal differences. By measuring the polyphenol content of 12 chokeberry accessions including *A. melanocarpa*, *A. arbutifolia*, and *A. prunifolia*, we found that anthocyanin content is stratified based on berry color. Black accessions had more anthocyanins than purple, which had more than red. Other polyphenol classes however did not differ significantly based on chokeberry color.

We observed considerable differences in the polyphenol content of ‘Viking’ juices through the harvest season, with proanthocyanidins and total phenols increasing significantly from the onset of ripening to the last week at which the berries were considered edible, and hydroxycinnamic acids decreasing concurrently. Total phenol content of chokeberry juice was maximized at week
7 of harvest. Anthocyanins increased rapidly at the onset of ripening, but peaked at week 5, indicating that the ideal harvest timing for chokeberries with respect to anthocyanin content is about 5 weeks of ripeness. Maximum hydroxycinnamic acid occurred at week 1, however antioxidant capacity determined by the FRAP assay was greatest at the end of the harvest season.

**4.2 FUTURE DIRECTIONS**

These new data about the polyphenol content of aronia berry raise more questions. Normal-phase HPLC quantitation of polymeric proanthocyanidins can only estimate proanthocyanidin content. Colorimetric methods for quantifying tannins, such as the DMAC assay, are largely empirical and known to underestimate proanthocyanidins through precipitation or misrepresentation of molar extinction coefficients [4]. Normal-phase HPLC is capable of separating proanthocyanidins with a degree of polymerization up to decamers, however according to our data, over 99.6% of chokeberry proanthocyanidins have degrees of polymerization >10, and polymers in this range are represented as a large broad peak. Thiolysis is needed to determine the sub-units of proanthocyanidin oligomers and polymers. Separation of oligomers by preparative HPLC, thiolysis of these compounds into monomers, and quantitation of flavan-3-ol monomers should be the next step in characterizing chokeberry proanthocyanidins. A factor currently limiting the accuracy of polyphenol quantitation is the lack of availability of specific standards, particularly in the case of oligomeric or polymeric compounds. Chokeberry, as a rich source of polyphenols, could be developed as a source of polyphenol standards, particularly oligomeric proanthocyanidin standards if these compounds can be successfully isolated.

Improving methods for extraction of polyphenols, particularly anthocyanins, could improve recovery rates. Wu et al. found that acetone reacts with cyanidin and delphinidin glycosides to
yield pyranoanthocyanins and furoanthocyanidins [5]. Acetone is frequently used as an extraction medium for hydrophilic flavonoids [5], [2], [3]. Other commonly used solvents may have similar effects on other flavonoids, and these phenomena should be explored in greater detail.

The ripening of chokeberry should be investigated in greater detail. Chokeberry is known for its astringency and this may be the main obstacle in its popularization. A sensory analysis of different varieties of chokeberry, or of chokeberry harvest at different times throughout ripening is needed. Timing the harvest of chokeberry to reduce bitterness may increase its palatability and improve its commercial potential. We measured changes in total proanthocyanidins through ripening, however we did not use HPLC to determine whether proanthocyanidins with different degrees of polymerization increased or decreased throughout ripening. Such information combined with sensory analysis would help us better understand the contribution of tannin profile to chokeberry astringency. While Heppsson and Johansson have quantified malic acid in chokeberries and measured changes through ripening [6], the identification of other organic acids and changes in their content through ripening is also needed.

Different postharvest processing and storage conditions should be tested to observe their effects on polyphenol content in chokeberries. Variations in temperature, light, and humidity may increase or decrease the levels of various polyphenols either through spontaneous conversion or up-regulation/down-regulation of polyphenol-degrading enzymes.

While chokeberries are currently used in jams, dyes, juices, wines, and supplements; the market for additional chokeberry products may increase as the health benefits of the berry become better known to the public. Chokeberry ice cream, sorbet, smoothies, chokeberry flavored yogurt, soft
drinks, and many other products could be commercialized. Additional research into methods of reducing or masking astringency in products may also increase the tolerability of chokeberry for general consumption. Aside from ornamental use, the red chokeberry is scarcely used for food or beverages, but additional research and marketing of red chokeberries with high polyphenol content such as UC021 may lead to a new nutraceutical crop. Additional chokeberry genotypes should be investigated for their polyphenol content and bioactivity, as the 12 that we have investigated show major differences in polyphenol content and profile.

Many major polyphenol classes have not been screened for in chokeberry, including flavanones, isoflavonoids, and hydrolyzable tannins. Information about the content of these compounds could improve the strategic use of chokeberry for improving health.
4.3 REFERENCES


