LC-MS/MS METHOD DEVELOPMENT FOR QUANTIFICATION OF BIOACTIVE COMPOUNDS IN ELDERBERRY AND GARLIC BOTANICALS

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Doctor of Philosophy

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

LC-MS/MS METHOD DEVELOPMENT FOR QUANTIFICATION OF BIOACTIVE COMPOUNDS IN ELDERBERRY AND GARLIC BOTANICALS

Presented by Mitch Johnson,

A candidate for the degree of doctor of philosophy,

And hereby certify that, in their opinion, it is worthy of acceptance.

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Abstract

Elderberry (*Sambucus nigra* spp.) juice contains a variety of polyphenols mostly anthocyanins. In order to understand the variation of polyphenol levels by genotype, various elderberry juice samples were analyzed for total phenolics (TP), total monomeric anthocyanins (TMA) and individual anthocyanin content (IAC). The Folin-Ciocalteu total phenolic method and pH differential method were used to measure the TP and TMA content, respectively. In addition, ultra-performance liquid chromatography coupled with triple quadrupole mass spectrometry was used to separate and detect individual anthocyanins from samples prepared by solid phase extraction. Multiple-reaction-monitoring was used to process data for the reduction of false positives, maximizing selectivity, and reliable quantification. The quantitative performance of the method was validated, and a detection limit of 0.3 ng/mL for cyanidin 3-*O*-glucoside was determined. This newly developed method may serve to characterize and profile various anthocyanins in elderberry juices for quality control, assessment of dietary intake, and anthocyanin-based biomedical studies.

The effects of frozen storage on the anthocyanin and polyphenol content of elderberry fruit juice are investigated. Juice from three genotypes of American elderberry

(Adams II, Bob Gordon, and Wyldewood) was screened for total phenolic and total monomeric anthocyanin content with spectrophotometric methods. The individual anthocyanin content of the juice was tested by coupling solid phase extraction with ultra-performance liquid chromatography/tandem mass spectrometry. Juice samples were tested initially upon harvest, then again after 3, 6, and 9 months of frozen storage. The three different genotypes of juice had significantly different TP, TMA, and IAC profiles initially ($p<0.05$). The TP, TMA, and IAC content of different genotypes were significantly affected ($p<0.05$) by the frozen storage time, suggesting that both genotype and length of frozen storage time can affect the anthocyanin content of elderberry fruit juice.

Garlic is a prevalent plant botanical, and aged garlic extract (AGE) has been used as a nutritional supplement and implied to promote health benefits by exhibiting anti-oxidant and anti-inflammatory activity, as well as hypolipidemic and antiplatelet effects. It has recently been discovered that N-α-(1-deoxy-D-fructos-1-yl)-L-arginine (FruArg) is a major contributor to the bioactivity of AGE and exerts significant ability in regulation of Nrf2-mediated antioxidant response. A very sensitive analytical method was developed and optimized for quantitation of FruArg in rat plasma and brain tissue samples. Phree™ phospholipid removal solution was used to separate samples spiked with FruArg from potentially interfering compounds present in biological fluids. Eluates were collected and analyzed using ultra-performance liquid chromatography-tandem mass spectrometry. A full method validation was conducted including analysis of the limit of quantitation, selectivity, linearity, range, recovery, matrix effect, inter-day precision and intra-day precision for both plasma and brain tissue. This method was

applied to pharmacokinetic study where mice plasma and tissue samples from four regions of the brain were analyzed for FruArg concentration at 15, 30, 60, and 180 min after being injected intraperitoneally with FruArg. It was determined that FruArg is well absorbed into the bloodstream and detected in four sub-regions of the brain suggesting it crosses the blood-brain barrier (BBB).

Cyanogenic glycosides (CG) are present in a variety of plants and can rapidly breakdown to hydrogen cyanide, which is very toxic to human beings. The potential CG content of all parts of elderberry plant is not well understood. In order to investigate the occurrence of CGs in elderberry ultra-performance liquid chromatography-tandem mass spectrometry methods were developed in order to quantitate several individual CGs at very low concentration levels, which is a large improvement in the current methodology. Upon harvest, the seeds, skin, juice, pulp and stems of elderberry will be analyzed for the presence of CGs.

Chapter 1: Introduction

1.1 American Elderberry (*Sambucus nigra subsp. canadensis*)

Elderberry (*Sambucus nigra* spp.) (Figure 1.1.) is a widespread genus that is native or naturalized in many parts of Europe, Asia, North Africa, and North America. Elderberry products are consumed as dietary supplements for their potential health benefits.[1] Researchers have linked elderberry products to anti-influenza, anti-oxidant, anti-carcinogenic, anti-viral, and antibacterial activities[2-5] Elderberry products are also suggested to manifest an array of health promoting benefits such as oxidative stress protection, anti-inflammatory effects and inhibition of some human tumor cells.[6-10] Anthocyanins, among other polyphenols present in plants, have been hypothesized to be responsible for these effects based on their ability to quench free radicals. They are also responsible for the rich colors observed in plants and fruits.[11]

Figure 1.1 Elderberry plant.

1.2 Dietary Supplements

The Food and Drug Administration (FDA) defines a dietary supplement as a product that is intended to supplement the diet and contains either a macronutrient, vitamin, mineral, amino acid, herb or botanical.[12] Dietary supplements are intended to be ingested, not represented as an entire meal and cannot make a drug claim. In 2011, dietary supplement sales reached $30 billion and juice-based supplements were responsible for $828 million; which was a top seller among herbal supplements.[13] Forty-nine percent of adults over 20 years old regularly consume at least one type of dietary supplement.[14]

1.2.1 Dietary Supplement Health and Education Act (DSHEA)

In 1994, dietary supplements were defined for the first time as their own specific entity. The Dietary Supplement Health and Education Act (DSHEA) was a big step forward to increase regulation of dietary supplements.[15] Section 7 of DSHEA discusses the requirements for proper labeling practices of ingredients and nutritional information. Section 8 says if a novel dietary ingredient is included in a dietary supplement, the FDA must be notified of the ingredient 75 days before the product is commercially available. DSHEA also granted the FDA the right to promote good manufacturing practices (GMPs) for the production of dietary supplements under section 9.

1.2.2 Problems With The Dietary Supplement Industry

Although DSHEA has helped with regulation, there are still a lot of problems associated with the dietary supplement industry. For instance, there is no pre-market approval required to launch a dietary supplement, nor are there formulation standards or proof of safety requirements in place. This means the effectiveness and safety of the supplement does not need to be proven. Therefore, a majority of regulation from the Food and Drug Administration will occur after problems with a dietary supplement arise.

1.2.3 Elderberry Dietary Supplements

One of the primary reasons elderberries are cultivated are for their use in the dietary supplement industry. In 2014, sales of elderberry dietary supplements totaled $9.9 million in the United States alone, which was a 64% increase from 2013.[16] Elderberry supplements generated enough revenue to make it the 20th top-selling herbal supplement. In the U.S. Sambucol® (Figure 1.2) is an example of a popular elderberry supplement used to treat symptoms of the common cold and flu. In a clinical study, participants given 60 mL of Sambucol® per day for 5 days saw cold and flu symptoms relieved by and recovered an average of four days quicker than the placebo group.[17]

1.3 Bioactive Compounds

Fruits are a dense source of bioactive compounds that possess an array of potential health benefits.[18] There is increasing evidence from scientific studies that these bioactive compounds have several health benefits, such as antioxidant, anticancer,

antimutagenic, anti-inflammatory and neuroprotective effects.[19-26]

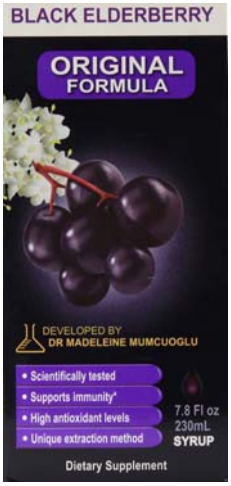


Figure 1.2 Elderberry dietary supplement.

1.3.1 Polyphenols

One class of bioactive compounds are polyphenols, which are present in high concentrations in a variety of plants. Some common types of polyphneols are flavonoids, catechins, anthocyanins, proanthocyanidins, and flavanols. Polyphenols can exhibit antioxidant activity by their ability to neutralize free radicals.[27] This can happen either through electron donation from one of the several hydroxyl groups or from a delocalized electron that is part of the conjugated π-system of the molecule (Figure 1.3). A proposed mechanism is demonstrated in Figure 1.4. After a polyphenol (POH) neutralizes a free radical (R•) it becomes a phenol radical (PO•), which can further neutralize other radicals.

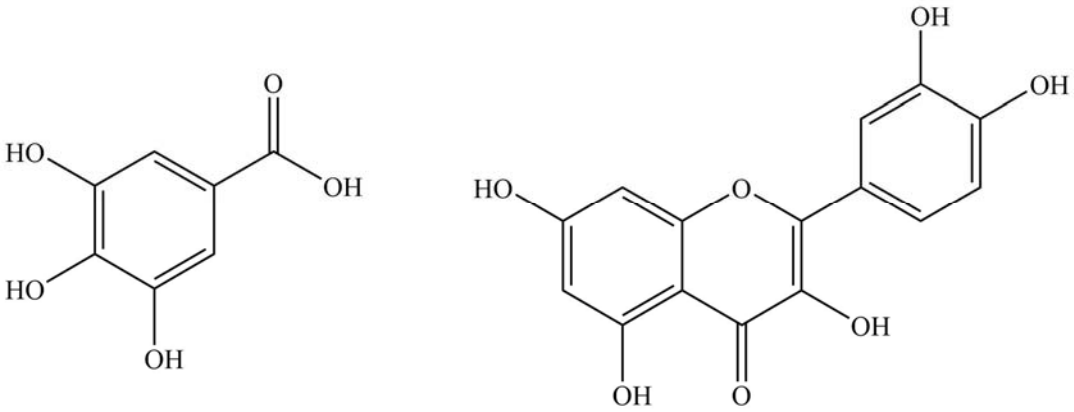


Figure 1.3 Structures of gallic acid (left) and quercetin (right).

An issue surrounding polyphenols is their bioavailability. Due to the variety of polyphenols, there are varied chemistries that can influence whether a polyphenol is absorbed and how well it is metabolized. For example, the aglycone form of polyphenols are readily absorbed in the small intestine and they commonly exist in nature in other forms (i.e. glycosides) that are not readily absorbed.[28] This can lead to inconsistent bioavailability among polyphenols, which increases the importance for research about the effects of specific polyphenolic compounds *in vivo*.

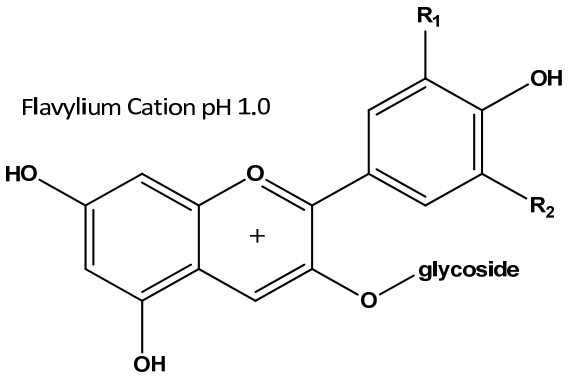
$$R\bullet + POH \rightarrow RH + PO\bullet$$

$$PO\bullet + R\bullet \rightarrow POR$$

Figure 1.4 Antioxidant mechanism of polyphenols.

1.3.2 Anthocyanins

Anthocyanins are a subclass of polyphenols that are responsible for the rich color of the pigment. Six major anthocyanins have been identified in plants: cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin (Figure 1.5).



Anthocyanin	R_1	R_2
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OCH_3	OCH_3
Pelargonidin	H	H
Peonidin	OCH_3	H
Petunidin	OCH_3	OH

Figure 1.5 Structure of six different anthocyanins.

Anthocyanins exhibit a structural transformation as a function of pH change (Figure 1.6), making them a natural indicator. At a low pH, anthocyanins are present in a flavylium cation form, and have a bright pink color. Anthocyanins are colorless and neutral from a pH range of roughly 4-10. They become bluish in the quinoidal base form at a pH greater than 10.

1.3.3 Previous Studies With Anthocyanins

Similar to polyphenols, there is concern surrounding the bioavailability of anthocyanins and what forms of the compounds are bioavailable. In a previous study, adults were dosed with 2.7 mg of cyanidin-3-*O*-glucoside per kg body weight and their plasma was analyzed. It was discovered that cyanidin-3-*O*-glucoside was present in the plasma, while the aglycone forms or metabolites were not identified.[29] This result

suggests that the glycosidic form of anthocyanins is responsible for the bioactivity associated with consumption.

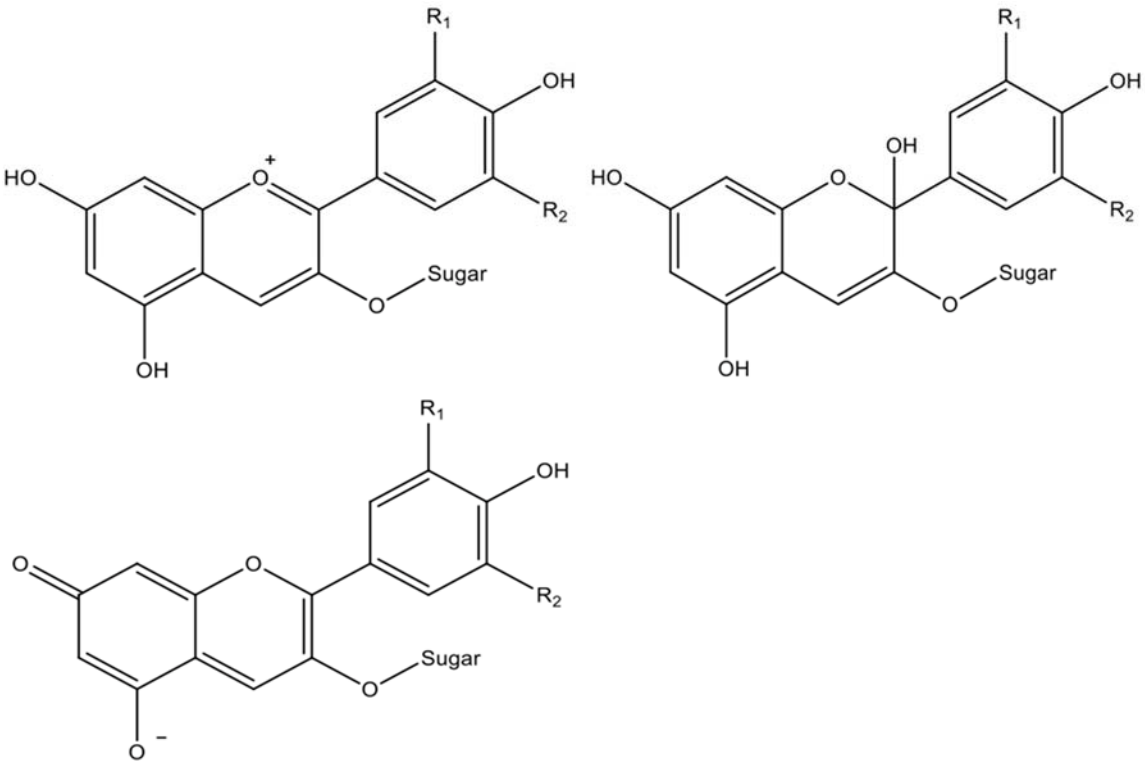
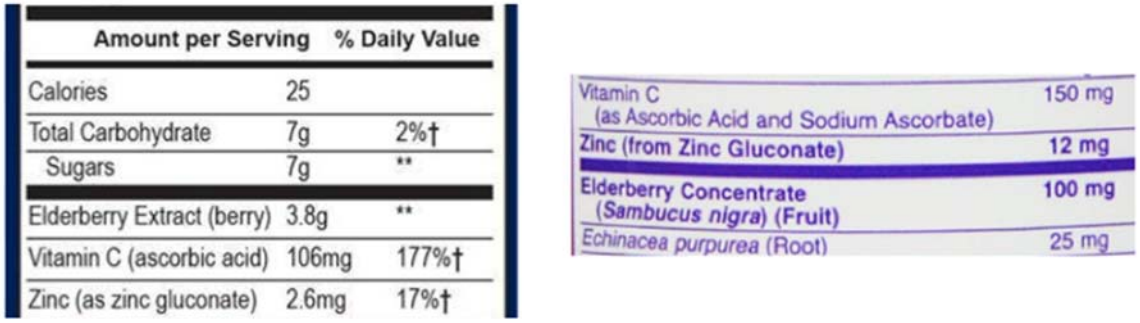


Figure 1.6 Anthocyanin structures at various pHs; flavylium cation (top left), chalcone (top right), quinodal base (bottom left).

Although polyphenols and anthocyanins are purported for the health benefits associated with elderberry consumption and supplementation, these compounds rarely measured or explicitly stated on dietary supplement labels (Figure 1.7). This is problematic because different subspecies of elderberry can have an extremely different profiles of anthocyanins. European elderberry contains mainly cyanidin-3-*O*-glucoside, whereas American elderberry is concentrated with acylated anthocyanins.[30] The anthocyanin profile of different genotypes within the same subspecies of elderberry can

also vary highly. This means that various elderberry extracts across several different product lines could differ highly in the amount of anthocyanins and polyphenols present.



Amount per Serving		% Daily Value
Calories	25	
Total Carbohydrate	7g	2%†
Sugars	7g	**
Elderberry Extract (berry)	3.8g	**
Vitamin C (ascorbic acid)	106mg	177%†
Zinc (as zinc gluconate)	2.6mg	17%†

Vitamin C (as Ascorbic Acid and Sodium Ascorbate)	150 mg
Zinc (from Zinc Gluconate)	12 mg
Elderberry Concentrate (*Sambucus nigra*) (Fruit)	100 mg
Echinacea purpurea (Root)	25 mg

Figure 1.7 Supplement fact labels from Sambucol® (left) and Now® elderberry extract (right).

1.4 Chromatography

1.4.1 Chromatography Overview

Chromatography is the process of separating or isolating compounds between two phases – a stationary phase that is immobile and a mobile phase that passes through the stationary phase. Differences in polarities between the stationary and mobile phases is the most common separation mechanism used in chromatography, although a variety of other modes of separation are available. Mikhail Tswett, a Russian botanist, is considered to be the father of chromatography. His pioneering work in chromatography demonstrated the separation of two pigments in chloroplast− chlorophyll a and chlorophyll b using ambient pressure column liquid chromatography.[31] Advancements in chromatography over the years have provided a variety of new stationary phase chemistries (ion-exchange, size-exclusion, hydrophilic interaction, etc.), which have increased the number of chromatographic scientific applications.

1.4.2 High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC), also known as high-pressure liquid chromatography, was first introduced in the 1960s.[32] An HPLC column has much smaller particles than traditional low-pressure columns and are packed tightly into the column.[33] Thus, high pressures are required to push the mobile phase through the column. HPLC is advantageous because the pressure of the column is stabilized and the flow rate of the mobile phase can be held constant. This leads to improved reproducibility and a more powerful analytical technique.

1.4.3 Ultra-Performance Liquid Chromatography (UPLC)

Jorgensen first described ultra high-pressure liquid chromatography (UHPLC) in 1997.[34] Then, Waters® released an ultra-performance liquid chromatography (UPLC) commercial system for the first time in 2004. UPLC utilizes sub 2 micron particles which allows for a more efficient separation compared to traditional 5 micron particles used in HPLC columns. The increase in separation efficiency leads to shorter column lengths, thus reducing the overall run time anywhere from 3−10 fold and does not sacrifice kinetic performance.[35] Faster run times allow for more rapid method development and adjusting the mobile phase composition (pH, buffers, modifiers, gradient parameters, etc.) to determine optimal run conditions. This also lowers the costs associated with analysis and reduces the total amount of mobile phase used, making it a greener technique.

1.5 Mass Spectrometry

Mass spectrometry was first developed as an analytical technique in 1912 by J.J. Thomson.[36] Since its inception, mass spectrometry has evolved tremendously to be used for a wide variety of applications. Mass spectrometry measures the mass-to-charge ratio of a compound (m/z), hence requiring ionization of the analyte in the gas phase. Mass spectral data can reveal information about the molecular weight of a compound, and also structural information pertaining to functional groups lost during fragmentation. A general layout for a mass spectrometer includes sample introduction from a inlet, ionization source, mass analyzer(s), detector and a data acquisition system (Figure 1.8).

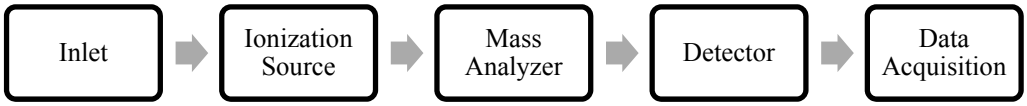


Figure 1.8 Schematic of mass spectrometry instrument layout.

1.5.1 Ionization Sources

1.5.1.1 Electron Ionization (EI)

Electron ionization (EI) was first described as in 1918 by A.J. Dempster.[37] When an electron present possesses the same energy as a transition in a molecule, the electron can be lost creating an ion. EI is known as a "hard ionization" because it causes extensive fragmentation of the parent ion, or the analyte of interest that has been ionized. Fragmentation can be beneficial because it yields information about functional groups in

the molecule. EI also requires that the ionization occurs in a vacuum which can limit its utility. However, because of the energetics involved, this ionization technique is mainly suitable for volatile molecules.

1.5.1.2 Electrospray Ionization (ESI)

Electrospray Ionization (ESI) was developed in 1988 by J. Fenn.[38] ESI is a favorable ionization technique for a variety of compounds such as proteins and other less volatile species. ESI is considered a "soft ionization" because minimal fragmentation occurs and an intense protonated parent ion peak is usually observed. Because of this, mass spectra generally feature a strong peak for the molecular ion which yields more confidence in the presence of an analyte. Figure 1.9 is a mass spectrum of lidocaine generated using ESI positive mode and a large peak can be seen at 235.1 Da. The monoisotopic mass of lidocaine is 234.173 Da, therefore this peak is lidocaine plus a proton, or $[M + H]^+$.

Molecules are introduced in the liquid phase at atmospheric pressure through a heated capillary. A strong electric field is produced between the capillary and a counter electrode at the tip of the capillary, causing a charge accumulation. Larger molecules, like proteins can form multiply charged ions, but smaller compounds will generally only have a single charge. Ions produced are then desolvated by a sheet of nitrogen gas and accelerated into the mass spectrometer. Since ionization is performed at atmospheric pressure, ESI is very compatible with liquid chromatography. A differential pumping system is used to gradually lower the pressure to achieve an acceptable operating level. This ensures a large enough mean free path that prevents unwanted collisions between ions and other gas phase particles, which could convolute the mass spectrum.

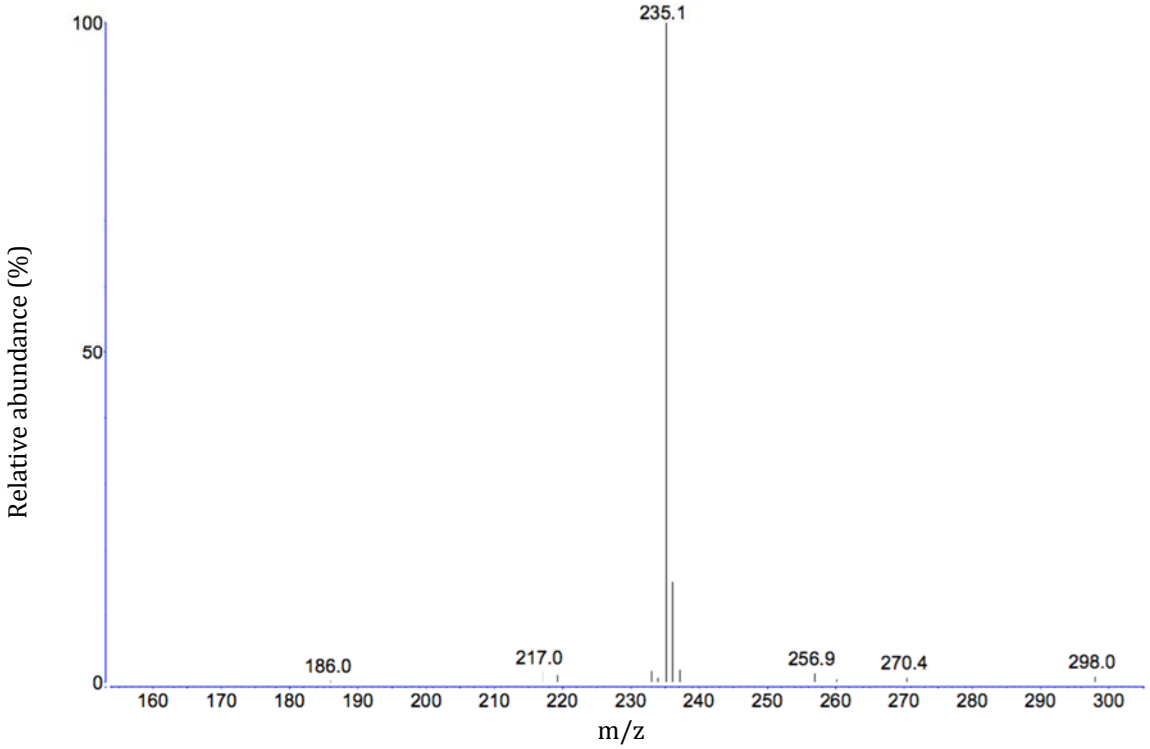


Figure 1.9 ESI mass spectrum of lidocaine in positive mode.

1.5.2 Mass Analyzers

Once an ion is produced, it is analyzed based on its mass-to-charge ratio (m/z) by the mass analyzer. There are a variety of mass analyzers commercially available today that are suitable for different applications. Some mass analyzers, such as an Orbitrap, feature high mass accuracy. This allows for more confidence during unknown compound identification. A time-of-flight (TOF) mass analyzer has essentially an infinite upper mass range limit, which is an advantage over some mass analyzers that have an upper mass limit of a few thousand Daltons.

1.5.2.1 Quadrupole

Quadrupole mass analyzers are comprised of four perfectly cylindrical rods that are equidistant from one another (Figure 1.10). All four rods have either a positive or negative potential, with opposing rods having the same potential.[39] As an ion enters the quadrupole it will be attracted to the rod with the opposing charge. The presence of an additional RF potential on the rods allows an ion to have a stable trajectory all the way through the quadrupole and reach the detector.

Compared to other mass analyzers, quadrupoles are low resolution instruments. This means they cannot disseminate ions with similar mass-to-charge ratios as well as some other analyzers. Generally, quadrupoles have at least unit mass resolution, allowing for identification of ions that are one atomic mass unit apart. The mass accuracy is the difference between the instruments measurement and theoretical mass of an ion. Quadrupoles have a mass accuracy of roughly 100 ppm.[39]

The advantage of quadrupoles is their continuous scanning nature and versatility. A variety of different acquisition modes are available to provide an array of information. As discussed later, quadrupoles can be used in tandem for quantitation, structural elucidation, or compound verification.

Figure 1.10 Quadrupole mass analyzer from the side (left) and front (right).

1.5.3 Detectors

1.5.3.1 Photomultipliers

Photomultipliers (PM) have emerged as a popular mass spectrometry detector due to their long lifetimes and low background noise. The premise for PM detection is conversion of an ion to a photon, which occurs in two steps. Ions that make it through the mass analyzers will first strike a conversion dynode, which causes secondary electrons to be released. These electrons collide with a phosphorescent plate that causes conversion to photons, which are then detected by the PM. The signal is then amplified and converted to a digital signal.

1.5.4 Tandem Mass Spectrometry (MS/MS)

1.5.4.1 Triple Quadrupole (QqQ)

Triple quadrupole mass spectrometry is a powerful and versatile analytical tool used for many different types of analysis. Several acquisition modes, such as multiple-reaction monitoring (MRM), single ion monitoring (SIM), and neutral loss, are readily available depending on the type of information desired. Qualitative information is available by identifying precursor ions from targeted fragments, or identifying fragmented ions from selected precursor ions. Quantitative information is generated when a quadrupole is set in static mode, allowing only ions of a specific m/z to reach the detector.

1.5.4.2 Multiple Reaction Monitoring (MRM)

Multiple reaction monitoring (MRM) is a type of targeted MS/MS scan (Figure 1.11). A parent or precursor ion m/z is selected in the first quadrupole. Only ions with

this specific m/z will have a stable trajectory along the z-axis of the quadrupole. Ions that successfully make it through will enter the second quadrupole, which is a collision cell. These ions collide with inert gas present inside the collision cell, which is usually argon. A portion of the kinetic energy from the collisions will be converted to internal energy, causing the parent ion to fragment. The fragments will be accelerated to a third quadrupole, where a daughter or product ion m/z will be selected. Only the fragments with this specific m/z will be stable along the z-axis of the third quadrupole and reach the detector. When a daughter ion successfully reaches the detector it will create an ion current that is converted into the signal. The area under the curve of this peak (counts versus time) can be integrated and used for quantification. The signal in this form is referred to as an ion chromatogram. MRM does not produce a mass spectrum because neither of the quadrupoles are scanning, rather both are set at a static m/z. Because of this, MRM is an extremely selective and sensitive mass spectrometry mode.

Figure 1.11 Tandem quadrupole mass spectrometry using MRM scans.

1.5.5 Mass Spectrometry Coupled With Liquid Chromatography (LC-MS)

Traditional direct infusion mass spectrometry will provide a mass spectrum with all of the ions present relative to the most abundant species. LC-MS is a very powerful tool

for mass analysis of a complex mixture by separating each analyte into an individual component before mass spectrometric analysis. This is very applicable for analysis of pharmaceuticals, foods, and plants, for example. Atmospheric pressure ionization sources, such as ESI, are very compatible with LC because of the ease of transfer from separation to ionization at the same pressure.

1.6 References

(1) Netzel, M., Strass, G., Herbst, M., Dietrich, H., Bitsch, R., Bitsch, I. and Frank, T. (2005) The excretion and biological antioxidant activity of elderberry antioxidants in healthy humans. *Food Res. Int.* 38, 905–910.

(2) Roschek, B. Jr., Fink, R. C., McMichael, M. D., Li, D. and Alberte, R. S. (2009) Elderberry flavonoids bind to and prevent H1N1 infection in vitro. *Phytochemistry*. 70, 1255–1261.

(3) Werlein H. D., Kutemeyer C., Schatton G. (2005) Influence of elderberry and blackcurrant concentrates on the growth of microorganisms. Food Control 16, 729-733.

(4) Milbury, P.E., Cao, G., H. Prior, R.L. and Blumberg, J. (2002) Bioavailablility of elderberry anthocyanins. *Mech. Ageing Dev.* 123, 997–1006.

(5) Tarascou, I., Mazauric, J. P., Meudec, E., Souquet, J. M., Cunningham, D., Nojeim, S., Cheynier, V. and Fulcrand, H. (2011) Characterisation of genuine and derived cranberry proanthocyanidins by LC–ESI-MS. *Food Chem.* 128, 802–810.

(6) Rodrigo, N. C., Diego, T. S., and Maria, A. A. (2011) Non-thermal stabilization mechanisms of anthocyanins in model and food systems—An overview. *Food Res. Int.* 44, 499–509.

(7) Kong, J. M., Chia, L. S., Goh, N. K., Chia, T. F. and Brouillard, R. (2003) Analysis and biological activities of anthocyanins. *Phytochemistry* 64, 923-933.

(8) Elisia, I., Hu, C., Popovich, D. G. and Kitts, D. D. (2007) Antioxidant assessment of an anthocyanin-enriched blackberry extract. *Food Chem.* 101, 1052–1058.

(9) Bagchi, D., Roy, S., Patel, V., He, G., Khanna, S., Ojha, N., Phillips, C., Ghosh, S., Bagchi, M. and Sen, C. K. (2006) Safety and whole-body antioxidant potential of a novel anthocyanin-rich formulation of edible berries. *Mol. Cell. Biochem.* 281, 197-209.

(10) Dreiseitel, A., Schreier, P., Oehme, A., Lochner, S., Rogler, G., Piberger, H., Hajak, G. and Sand, P. G. (2008) Inhibition of proteasome activity by anthocyanins and anthocyanidins. *Biochem. Biophys. Res. Commun.* 372, 57–61.

(11) Zhang, Y. J., Vareed, S. K., and Nair, M. G. (2005) Human tumor cell growth inhibition by nontoxic anthocyanidins, the pigments in fruits and vegetables. *Life Sci*. 76, 1465–1472.

(12) Dietary Supplements. [updated 2015 June 15; accessed 2016 July 15]. http://www.fda.gov.

(13) Nutrition Business Journal. [updated 2012 July 1; accessed 2014 June 6]. http://www.newhope.com/dietary-supplements.com

(14) Bailey, R. L., Gahche, J. J., Miller, P. E., Thomas, P. R., and Dwyer, J. T. (2013). Why US Adults Use Dietary Supplements. *JAMA Intern. Med.* 173, 355–361.

(15) "Dietary Supplement Health and Education Act of 1994."

(16) Smith, T., Lynch, M. E., Johnson, J., Kawa, K., Bauman, H., and Blumenthal, M. (2015) Herbal Dietary Supplement Sales In US Increase 6.8% in 2014. *Herbalgram.* 107, 52–59.

(17) Zakay-Rones, Z., Thom, E., Wollan, T., Wadstein, J. (2004) Randomized study of the efficacy and safety of oral elderberry extract in the treatment of influenza A and B virus infections. *J. Int. Med. Res.* 32, 132–140.

(18) Nile, S. H., and Park, W.P., (2014) Edible berries: Bioactive components and their effect on human health. *Nutr*. 30, 134–144.

(19) Satue-Garcia, M. T., Heinonen, and M., Frankel, F. N. (1997) Anthocyanins as antioxidants on human low-density lipoprotein and lecithinliposome systems. *J. Agric. Food Chem*. 45, 3362–3367.

(20) Constantino, L., Rastelli, G., Rossi, T., Beroldi, M., and Albasini, A. (1994) Composition superoxide radicals scavenging and antilipoperoxidant activity of some edible fruits. *Fitoterapia*, 65, 44–47.

(21) Vant-Veer, P., Jansen, M. C., Klerk, M., and Kok, F. J. (2000) Fruits and vegetables in the prevention of cancer and cardiovascular diseases. *Public Health Nutr.* 3, 103–107.

(22) Surh, Y. J., Na, H. K., Lee, J. Y., and Keum, Y. S. (2001). Molecular mechanisms underlying antitumor promoting activities of heat-processed Panax ginseng CA Meyer. *J Korean Med Sci,* 16, S38–S41.

(23) Katsube, N., Iwashita, K., Tsushida, T., Yamaki, K., Kobori, M. (2003) Induction of apopstosis in cancer cells by bilberry (Vaccinium myrtillus) and the anthocyanins. *J. Agric. Food Chem.* 51, 68–75.

(24) Meyer, A. S., Heinonen, M., and Frankel, E.N. (1998). Antioxidant interactions of catechin cyanidin caffeic acid and quercetin and ellagic acid on human LDL oxidation. *Food Chem*. 61, 71–75.

(25) Ramassamy, C. (2006) Emerging role of polyphenolic compounds in the treatment of neurodegenerative diseases: a review of the their intracellular targets. *Euro. J. Pharmacol*. 545, 51–64.

(26) Vauzor, D., Vafeiadou, K., Rodriguez-Mateos, A., Rendeiro, C., and Spencer, J. P. (2008) The neuroprotective potential of flavonoids: a multiplicity of effects Genes. *Nutr.* 3, 115–126.

(27) Dai, J., and Mumper, R. J. (2010) Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules*. 15, 7313–7352.

(28) Manach, C., Scalbert, A., Morand, C., Rémésy, C., and Jimenéz, L. (2004). Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.* 79, 727–747.

(29) Miyazawa, M., Nakagawa, K., Kudo, M., Muraishi, K., and Someya, K. (1999) Direct intestinal absorption of red fruit anthocyanins, cyanidin-3-glucoside and cyanidin-3,5-diglucoside, into rats and humans. *J. Agric. Food. Chem.* 47, 1083–1091.

(30) Lee, J., and Finn, C. E. (2007) Anthocyanins and other polyphenols in American elderberry (*Sambucus canadensis*) and European elderberry (*S. Nigra*) cultivars. *J. Sci. Food Agric.* 87, 2665–2675.

(31) Tswett, M. S. (1906) Physikalisch-chemische studien uber das chlorophyll. Die adsorptionen. *Ber. Bot. Ges.* 24, 316–332.

(32) Unger, K. K., Ditz, R., Machtejevas, E., and Skudas, R. (2010) Liquid chromatography—its development and key role in life science applications. *Agnew. Chem. Int. Ed. Engl.* 49, 2300–2312.

(33) Miller, J. M. (2005) Chromatography Concepts And Contrasts. Second Edition. Hoboken, NJ: Wiley and Sons.

(34) MacNair, J. E., Lewis, K. C., and Jorgenson, J. W. (1997) Ultra high-pressure reversed-phase liquid chromatography in packed capillary columns. *Anal. Chem.* 69, 983–989.

(35) Nguyen, D. T. T, Guillarme, D., Rudaz, S., and Veuthey, J.L. (2006) Fast analysis in liquid chromatography in pharmaceutical analysis. *J. Pharm. Biomed. Anal.* 87, 105–119.

(36) Thomson, J. J. (1913) Rays of positive electricity and their application to chemical analysis. Longmans Green, London.

(37) Dempster, *A. J. (1918)* A new method for positive ray analysis. *Phys. Rev*. 11, 316–325.

(38) Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., and Whitehouse, C. M. (1989) Electrospray ionization for mass spectrometry of large biomolecules. *Science* 246, 64–71.

(39) de Hoffmann, E. and Stroobant, V. Mass Analyzers. Mass Spectrometry Principles and Applications, Third Ed; John Wiley & Sons; Hoboken, NJ, 2007, 85–98.

Chapter 2: Frozen Storage Study

2.1 Introduction

Fruit consumption is a vital part of the human diet. Fruits are a rich source of vitamins, minerals, and polyphenols. Elderberry is a small, dark purple fruit rich in anthocyanins, a subclass of polyphenols that are responsible for the colored pigments of fruits. The European elderberry (*Sambucus nigra* subsp. *nigra*) has been extensively studied[1] but little research has been conducted concerning the stability of anthocyanins and other polyphenols in American elderberry (*Sambucus nigra* subsp. *canadensis*). The American elderberry grows wild throughout much of eastern North America, but is being increasingly cultivated in the United States for use in the dietary supplement market.

Elderberries have a variety of potential health benefits associated with their consumption.[2,3] Research supports that anthocyanins and other polyphenols found in elderberry juice can help the body protect against oxidative stress through their ability to quench free radicals[4] and their immunomodulatory and anti-inflammatory properties.[5] Several in vitro and in vivo animal studies have successfully demonstrated antioxidant, anti-viral[6] and chemoprotective[7] activity of anthocyanins; although evidence in human trials is still limited. However, anthocyanin consumption through fruit has been associated with aiding in ailments such as certain cancers[8,9], heart disease,[3] and neurodegenerative disease.[5] The proposed mechanisms of effects of polyphenols include enzyme function and the regulation of gene and protein expression.[3] Several previous studies have focused on profiling and quantifying the anthocyanin and other polyphenol content in several different fruits.[10-12]

2.1.1 Previous Elderberry Studies

The total phenolic (TP) and total monomeric anthocyanin (TMA) spectrophotometric methods are commonly utilized to estimate their respective content in fruit. Studies have suggested that the cultivar or genotype, growing season, location, and storage affect the anthocyanin content of elderberry and other fruits.[10,13-15] The effects of storage on the phytochemical profile of fruit are important because of the seasonal availability of fresh fruit, thus the need to adequately preserve vital nutraceuticals. One study showed that the anthocyanin content in several blackberry (*Rubus spp.*) fruit cultivars was unaffected during long term freezer storage, including both fast freezing using liquid nitrogen, and traditional slow freezing in the freezer.[13] Most individual anthocyanins were cyanidin based, and cyanidin-3-*O*-glucoside was present at the highest concentrations amongst all the cultivars. However, measurements were only taken initially and after 7 months of frozen storage, and concerned the fruit. Additional studies have been conducted concerning anthocyanin stability in other small fruits during storage.[13,16-18] Elderberry juice is a rich source of cyanidin-based anthocyanins, but research regarding the effects of frozen storage on the individual anthocyanin content (IAC) of American elderberry fruit juice is lacking. This study also includes screening for TP, TMA, and IAC periodically to understand the effects frozen storage has on anthocyanins of elderberry fruit juice.

2.2 Experimental

2.2.1 Materials

2.2.1.1 Plant Material

American elderberry fruit grown at the University of Missouri's Southwest Research Center near Mt. Vernon (37.072073 N, -93.880829 W, 378 m alt.) was used. The soil is an upland Creldon silt loam that is deep, moderately well drained, and with a fragipan at 50 - 90 cm. Soil chemistry was evaluated prior to planting, with the analysis indicating a good soil for elderberry: pH 6.8, cation exchange capacity 13.2 meq/100g, organic matter 2.6%, and high to very high levels of P, K, Ca, and Mg based on recommendations for berry crops.

Three commercially available elderberry genotypes (Adams II, Bob Gordon, and Wyldewood) were used. These three genotypes have widely disparate geographic origins, originating from wild genetic material in New York, Missouri, and Oklahoma, respectively. Phenotypically, they behave differently when cultivated side-by-side, suggesting that there are inherent genetic differences among them.[19] Experimental plots contained four plants of the same genotype, planted 1.2 m apart. Forty-eight plots were established in four rows, with plots separated 2.4 m within and 3.1 m between rows. The three genotypes were assigned to 16 of the 48 plots in a completely randomized manner. The total number of plants was 192 and the site covered 0.10 ha. The site was prepared by killing existing vegetation in the planting rows with glyphosate herbicide prior to planting, and alleyways of mixed grasses were maintained and mowed during the study. Throughout the study, plants were irrigated via drip lines to provide 2.5 to 4.0 cm water

per week when rainfall was lacking. Weeds were managed with mulch, hand weeding, and herbicides (glyphosate, clethodim); no insecticides or fungicides were used.

For this study, fresh ripe fruit was harvested from 39 of the 48 plots on August 26-27, 2013, promptly refrigerated, and transported under refrigeration to the laboratory on August 28 for analysis, and stored at -80° C for one week. Elderberry samples were then thawed, de-stemmed and hand pressed into juice. All other debris, pulp, and seeds were discarded. The juice obtained was filtered through a nylon filter (0.22 μm), and 1.5 mL of each replicate was aliquoted into 1.5 mL micro centrifuge tubes and stored at -20° C The samples were screened for TP, TMA, and IAC initially, and after 3, 6, and 9 months of frozen storage. Samples were thawed, an aliquot removed for analysis, and then re-frozen for future analysis. The number of independent juice samples ranged from 11 to 14 for each of the three genotypes for all assays conducted.

2.2.1.2 Chemicals

Cyanidin 3-*O*-glucoside chloride analytical standard (≥95%), Folin-Ciocalteu's phenol reagent, sodium acetate trihydrate (≥99.0%), and gallic acid (97.5-102.5%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium hydroxide (Optima), sodium carbonate anhydrous (certified ACS powder), potassium chloride (certified ACS crystalline), hydrochloric acid (Optima), water (HPLC-grade), formic acid (HPLC-grade) and acetonitrile (HPLC-grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Trifluoroacetic acid (99%) used for acidifying solid phase extraction (SPE) mobile phase, was purchased from Acros Organics (NJ, USA).

2.2.2 Total Phenolic Content (TP)

Folin-Ciocalteu's method was used with slight modifications.[20] Juice samples were initially diluted 100-fold with water, then 0.5 mL of Folin-Cicoalteu's phenol reagent (diluted 10-fold) was added, followed by 0.4 mL of sodium carbonate (75 g/L) after 30 seconds, but before eight minutes. Samples equilibrated at room temperature, in absence of light, for two hours. The workflow for the TP measurement is shown schematically as Figure 2.1. Absorbance measurements were taken in triplicate at wavelength of 760 nm with a PerkinElmer Enspire 2300 multimode plate reader. Five gallic acid standards of 25, 50, 100, 200, and 400 μg/mL were prepared in the same manner as the samples. The TP concentrations are expressed as gallic acid equivalents (mg GAE/mL).

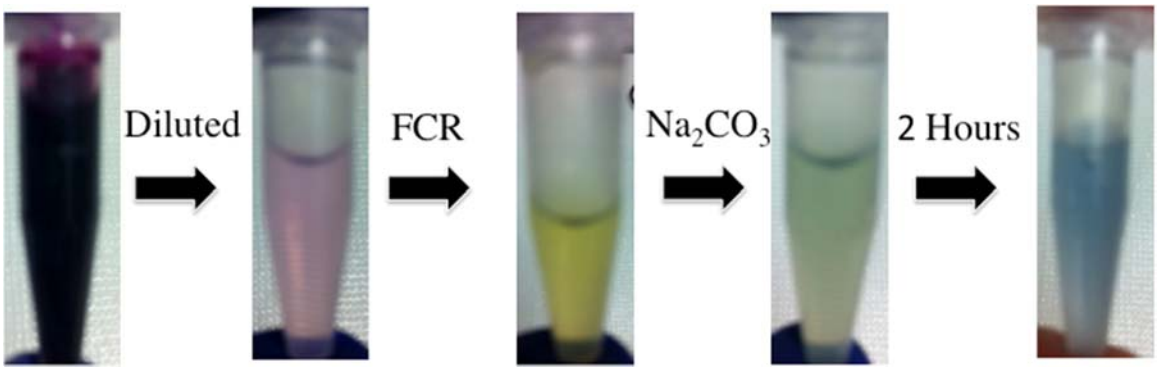


Figure 2.1 Schematic of Folin-Ciocalteu method to measure TP.

2.2.3 Total Monomeric Anthocyanin (TMA)

The TMA content of elderberry juice was measured utilizing the pH-differential method.[21] Elderberry juice samples were diluted 200-fold in potassium chloride (pH 1.0) and sodium acetate (pH 4.5) solutions, and equilibrated for 60 minutes at room

temperature. The workflow of the method is shown schematically as Figure 2.2. Five cyanidin 3-*O*-glucoside standards of 2, 4, 6, 8, and 10 μg/mL were prepared in the same manner as the samples. Absorbance measurements were taken in triplicate at a wavelength of 520 nm and 700 nm in each solution. Equation 2.1 shows how the final absorbance values were obtained. TMA concentrations were quantitated using a standard curve and are expressed as cyanidin 3-*O*-glucoside equivalents (mg C3GE /mL). The standard solution concentrations were verified using the molar extinction coefficient of cyanidin 3-*O*-glucoside (ε= 26900 $M^{-1}cm$, λ = 520 nm).[22]

$$A = (A_{520} - A_{700})_{pH1.0} - (A_{520} - A_{700})_{pH4.5} \quad (2.1)$$

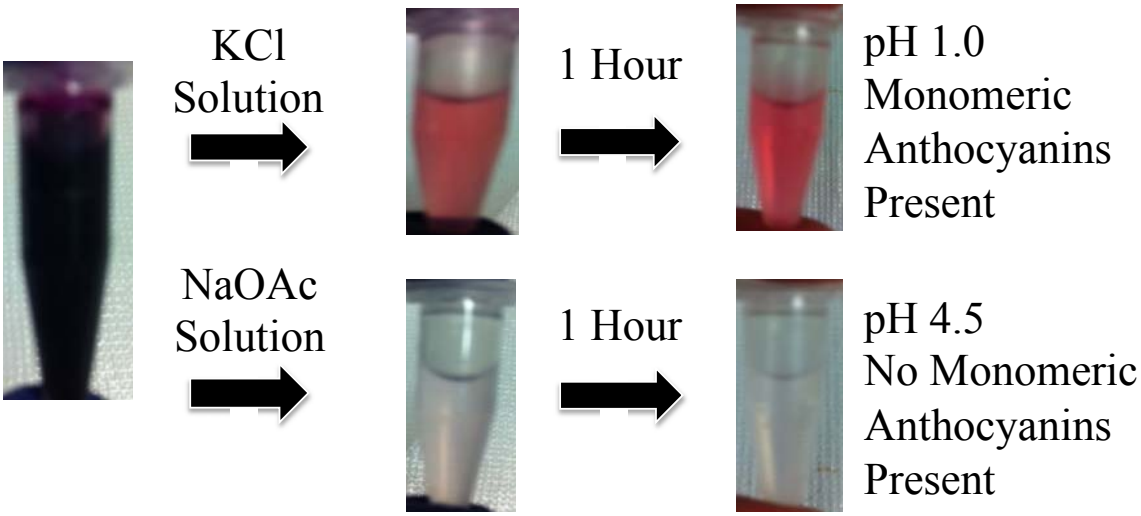


Figure 2.2 Schematic of pH differential method to measure TMA.

2.2.4 Solid-Phase Extraction (SPE)

Mixed mode cation-exchange chromatography was employed to isolate the anthocyanins from the other potentially interfering compounds in the juice matrix.[4] Oasis MCX 3cc (60mg) extraction cartridges were used with a Supelco Visiprep SPE vacuum manifold, operated at 10 ± 2 in. Hg (Figure 2.3). Columns were initially conditioned with 2 mL of acidified water (0.01% trifluoroacetic acid, TFA). Figure 2.4 demonstrates a work flow for SPE sample preparation. After conditioning, 50 µL of juice was added, followed by an additional 2 mL of water (0.01% TFA), and 2 mL methanol (0.01% TFA). Then 1 mL of methanol (1% NH_4OH) and 1 mL 40:60 water/methanol (1% NH_4OH) was added, and these two eluates were combined for analysis. 10 µL of formic acid was added to re-acidify the solution. The solvent was evaporated with a constant flow of nitrogen gas. Dry samples were re-dissolved in 1 mL of water (4.5% formic acid) and refrigerated until analysis.

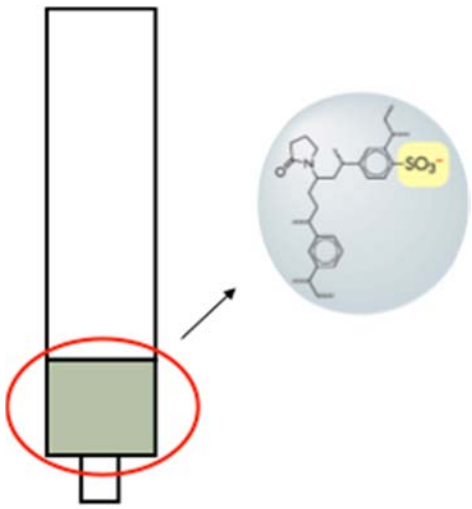


Figure 2.3 Mixed-mode cation exchange SPE cartridge.

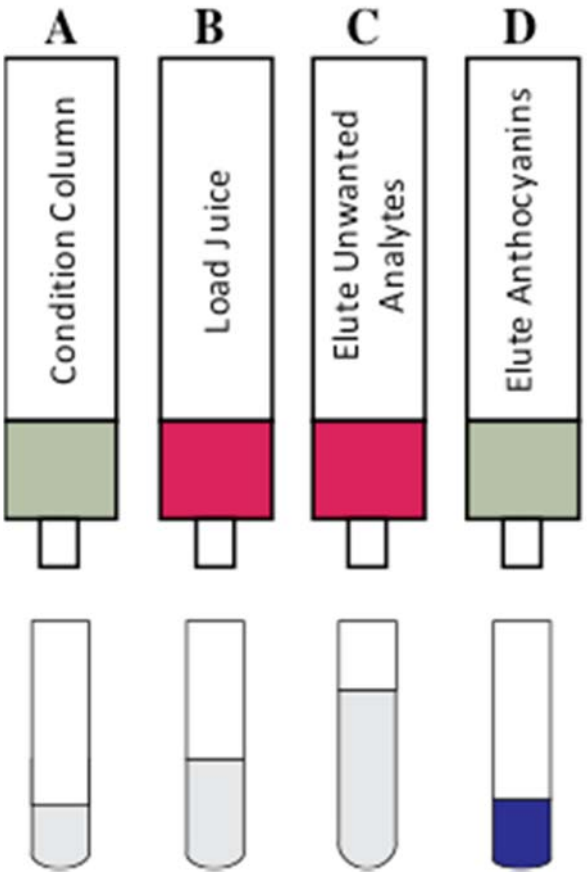


Figure 2.4 Stepwise isolation of anthocyanins using SPE.

2.2.5 SPE Recovery

Juice samples were spiked with three concentrations of cyanidin 3-*O*-glucoside standard before the extraction step to assess the overall recovery rate of the SPE procedure. The cyanidin 3-*O*-glucoside concentration of the spiked juice sample was compared to a control juice sample. The percent recovery was calculated using equation 2.2.

$$\frac{Concentration\ Spiked\ Sample - Concentration\ Control\ Sample}{Concentration\ Standard\ Added}\ x\ 100\% \qquad (2.2)$$

The average SPE recovery of cyanidin-3-*O*-glucoside ranged from 94-107% (Table 2.1). Three different volumes of cyanidin-3-*O*-glucoside standard spiking solution were used to analyze recovery over a range of concentrations. Acceptable recovery was seen for all volumes of concentrations added.

	Average Response	C3GE (ng/mL)	C3GE added (ng/mL)	Average Recovery (%)
Control	220,956	525.2	-	
2 µL spike	340,669	808.3	298.4	95 ± 1
3 µL spike	395,931	938.0	439.2	94 ± 1
5 µL spike	539,214	1,277.8	705.5	107 ± 1

Table 2.1 Average recovery of elderberry juice spiked with three different cyanidin-3-*O*-glucoside concentrations before SPE. Three independent samples were tested for each spike concentration. Concentrations were measured using UPLC-MS/MS and are represented as C3GE (ng/mL). Concentrations of the spiked juice sample are compared to the concentration of the control juice sample. Recoveries are represented as a percentage ± the standard error of the mean (n=3).

2.2.6 Freeze-Thaw Analysis

The TMA content of juice samples was tested to determine if sample degradation occurs by repeated freeze-thaw cycles. Samples were initially thawed and analyzed for TMA content, frozen overnight (-20° C) and tested each day for four days (Table 2.2). Each genotype included seven independent samples (n=7). The TMA content of the juice did not have a significant difference between any of the freeze-thaw cycles ($p>0.05$). This was true for all three genotypes over the course of all freeze-thaw cycles.

	Adams II (mg/mL) ± SE	Bob Gordon (mg/mL) ± SE	Wyldewood (mg/mL) ± SE
Day 1	4.6 ± 0.5	4.8 ± 0.2	3.9 ± 0.2
Day 2	4.7 ± 0.2	4.7 ± 0.1	4.0 ± 0.2
Day 3	4.7 ± 0.4	4.9 ± 0.2	3.7 ± 0.3
Day 4	4.4 ± 0.4	4.7 ± 0.4	3.9 ± 0.1

Table 2.2 TMA content of the Adams II, Bob Gordon, and Wyldewood genotypes over the course of four days, which included three freeze-thaw cycles. The data are represented as the mean (mg C3GE/mL) ± the standard error of the mean (n=7).

2.2.7 UPLC Gradient

A fully validated UPLC-MS/MS method was followed for anthocyanin quantification.[23] A Waters Acquity ultra-performance liquid chromatograph (UPLC), with a quaternary solvent manager was used. A C18 column (Acquity BEH, 1.7-µm, 50-mm x 2.1-mm, Waters, Milford, MA, USA) was selected to separate anthocyanins. The mobile phases used were water **A** (4.5% formic acid) and acetonitrile **B** (0.01% formic acid). The gradient used was 95% **A** at 0 min, 91% **A** at 2.5 min, 85% **A** at 3.5 min, 5% **A** at 4 min, 95% **A** at 4.8 min. The flow rate was 400 µL/min and the sample injection volume was 10 µL in full loop mode. The column and sample chamber were kept at ambient temperature.

2.2.8 Mass Spectrometry Conditions

A Waters Xevo TQ-S triple quadrupole mass spectrometer with electrospray ionization (ESI) in the positive ion mode was used. Multiple reaction monitoring scans were conducted by selecting the parent and daughter ion masses of the anthocyanins. A desolvation temperature of 350 °C and source temperature of 150 °C were used. A capillary voltage of 2.0 kV and cone voltage of 20 V were used, and the nebulizer gas flow rate was 500 L N_2/hour. Individual collision energies were manually optimized.

MassLynx software (version 4.1, Waters, Milford, MA, USA) was used for all data acquisition. Six cyanidin 3-*O*-glucoside external standards of 1, 10, 100, 200, 500, and 1,000 ng/mL were prepared and used to quantitate IAC. Samples were measured in triplicate and concentrations were determined based on their ion chromatographic peak areas. The resulting standard curve is shown as Figure 2.5. Individual anthocyanin concentrations are expressed as (µg C3GE /mL).

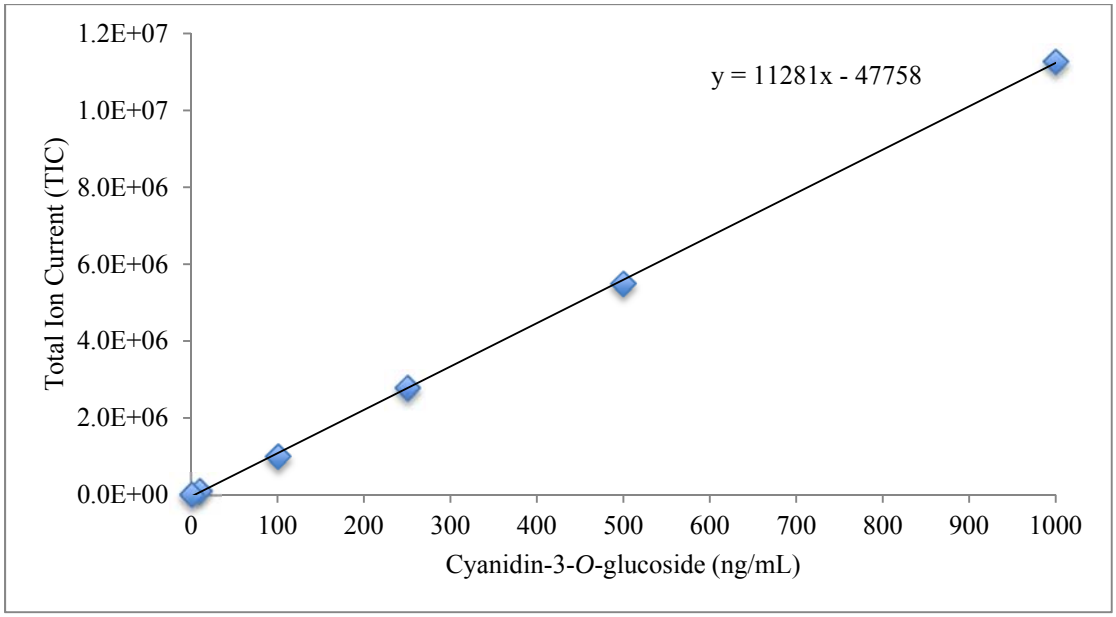


Figure 2.5 Standard curve of cyanidin-3-*0*-glucoside.

Nine individual anthocyanin compounds were monitored during the experiment. Information regarding their parent and daughter ion mass-to-charge ratios (m/z) and retention times (R_t) are listed in Table 2.3.

Anthocyanin	Parent Ion (m/z)	Daughter Ion (m/z)	R_t (min)
A) cyanidin-3-*O*-coumaroyl-sambubioside-5-glucoside	889.4	287.1	4.61
B) cyanidin based anthocyanin	785.3	287.1	4.45
C) cyanidin-3-*O*-sambubioside-5-glucoside	743.2	287.1	2.47
D) cyanidin-3-*O*-coumaroyl-sambubioside	727.4	287.1	4.67
E) cyanidin-3-*O*-sophoroside	611.1	287.1	2.37
F) cyanidin-3-*O*-rutinoside	595.1	287.1	4.63
G) cyanidin-3-*O*-sambubioside	581.0	287.1	3.78
H) cyanidin-3-*O*-galactoside	449.1	287.1	2.41
H) cyanidin-3-*O*-glucoside	449.1	287.1	3.66

Table 2.3 Parent and daughter ion mass-to-charge ratios (m/z) of each individual anthocyanin monitored, along with the retention time (Rt) of each ion chromatographic peak.

2.2.9 Statistical Analysis

Data analysis was carried out in Matlab, version 8.3 (Mathworks, Natick, MA). One-way ANOVA was performed to compare the effects of frozen storage on the TP and TMA content of the elderberry juice samples. The Tukey honestly significant difference (hsd) test was conducted to compare the TP and TMA values of the same genotype at different time points during long-term frozen storage, and also to compare the TP and TMA values of the three genotypes at the same time points, both done at the 95% confidence interval. Two-way t-tests were performed at the 95% confidence interval to analyze the difference in IAC during frozen storage.

2.3 Results and Discussion

2.3.1 TP Content

The TP content was significantly different among all three genotypes initially ($p<0.05$), suggesting that genotype plays a role in TP content of elderberry fruit juice, as previously reported.[14-15] TP content was measured by making absorbance measurements

at 760 nm after the juice samples reacted with FCR reagent for 2 h (Figure 2.6). Bob Gordon contained the highest TP content initially (8.1 ± 0.3 mg GAE/ mL) followed by Wyldewood (6.7 ± 0.1 mg GAE/ mL) and Adams II (5.6 ± 0.3 mg GAE/ mL) (Figure 2.7). After three months of frozen storage the Bob Gordon had a significantly higher TP content compared with Adams II and Wyldewood, however the latter two genotypes were not significantly different.

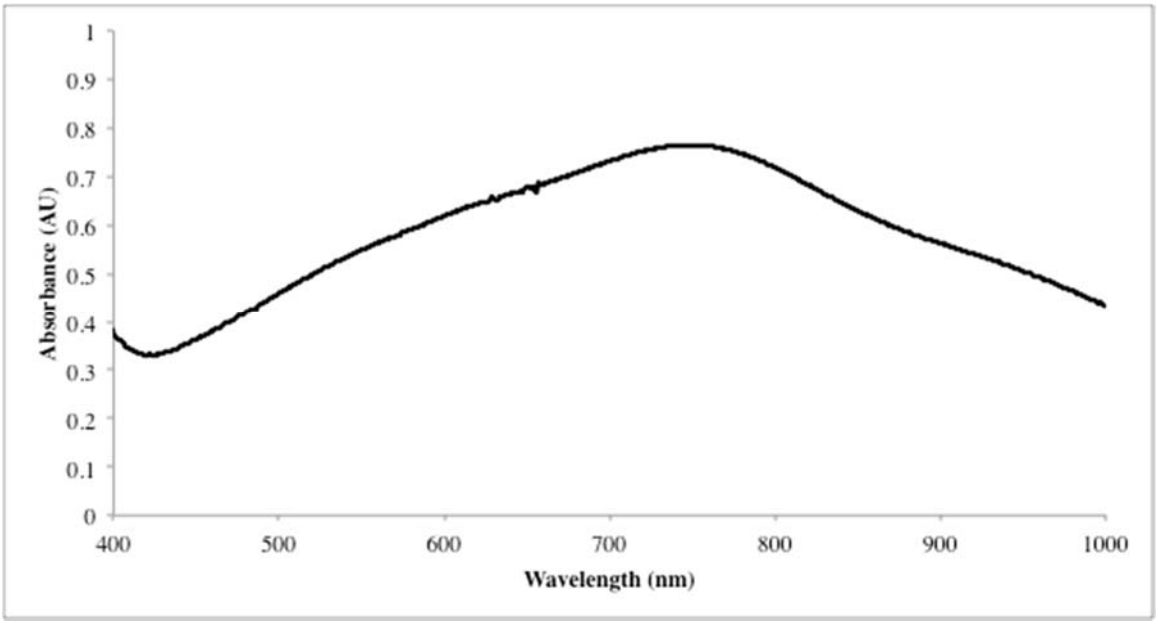


Figure 2.6 UV-VIS absorption spectrum of elderberry juice diluted in FCR reagent with a maximum wavelength of absorption at 760 nm.

Each genotype displayed the same trend of losing a statistically significant ($p<0.05$) amount of polyphenols during the first 3 months of storage (Figure 2.7) and then remained rather stable for the course of the storage study. By the end of 9 months of frozen storage, all of the genotypes still had at least 72% of their initial polyphenol content. Some of this TP loss can be attributed to anthocyanin degradation, which is

discussed further below. Therefore, the other non-anthocyanin polyphenols in elderberry fruit juice exhibited greater stability during long term frozen storage.

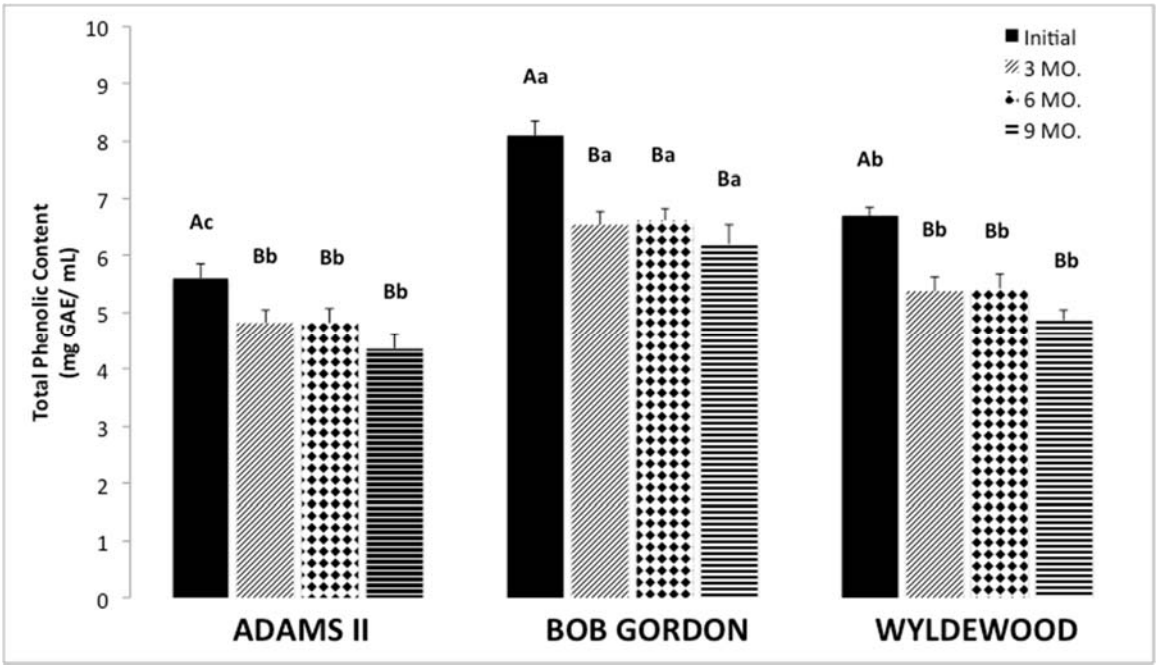


Figure 2.7 Total Phenolic (TP) content of Adams II, Bob Gordon, and Wyldewood genotypes during frozen storage. The data are represented as the mean ± the standard error of the mean (n=11-14). Each column indicates the time period of testing, starting with the initial test, 3 month, 6 month, and 9 month (left to right). Significant differences ($p < 0.05$) between TP content of frozen storage periods are represented by different letters (A is significantly different than B, but AB is equivalent to both A and B groups, for example), according to Tukey's honest significance test. Significant differences ($p < 0.05$) between TP content between each genotype during the same time periods are indicated by different lower case letters.

2.3.2 TMA Content

The initial TMA content of the Bob Gordon juice was also significantly different than the Adams II and Wyldewood juice ($p<0.05$), suggesting that the genetics of the elderberry fruit is one factor that influences the anthocyanin content of the juice (Figure 2.9). The TMA content was estimated by taking absorbance measurements at 520nm,

which is the maxiumum wavelength of absorption for cyanidin-3-*O*-glucoside (Figure 2.8). Once again, Bob Gordon had the highest concentration initially, (3.8 ± 0.2 mg C3GE/ mL) followed by Wyldewood (2.4 ± 0.2 mg C3GE/ mL) and Adams II (1.8 ± 0.2 mg C3GE/ mL) (Figure 2.9). However, after three and six months of frozen storage the three genotypes possessed significantly different TMA contents from each other (Figure 2.9). Not only did the Bob Gordon genotype have the highest TMA content, but it also had the highest anthocyanin percentage of the TP content at 47% anthocyanin, followed by 36% and 32% for Wyldewood and Adams II, respectively. In another study involving American elderberry fruit, the TMA content of 14 different elderberry fruit accessions varied highly (37.5%), which was much more than the 14.4% TP variance amongst accessions.[10] This suggests that the anthocyanin content is more susceptible to changes than other polyphenols based on the genotype, growing conditions, location, year, and other factors, which is in good agreement with our results. Future studies investigating these parameters would be helpful to understand how to optimize and the preservation of anthocyanins in elderberry fruit.

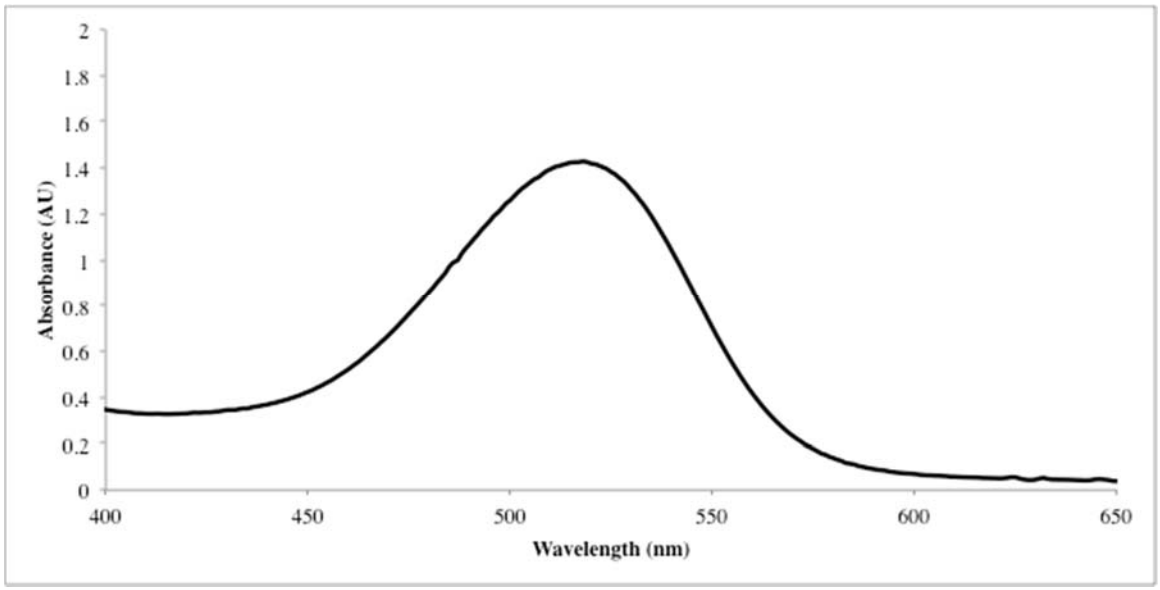


Figure 2.8 UV-VIS absorption spectrum of elderberry juice diluted in pH 1.0 KCl solution with a maximum wavelength of absorption at 520nm.

There were large differences in the stability of the anthocyanins during frozen storage among genotypes. Bob Gordon showed the best TMA stability during the first three months of frozen storage (Figure 2.9), while the other two genotypes saw a significant decline in TMA content during this time. All three genotypes saw a significant and relatively similar decline in TMA content from three months to six months, and also from six months to nine months of frozen storage. The Bob Gordon retained 99%, 76%, and 58% of its initial anthocyanins during 3, 6, and 9 months of storage, respectively. Wyldewood retained 72%, 44%, and 28% and Adams II 42%, 30%, and 18% of their initial anthocyanins during 3, 6, and 9 months of storage, respectively. These large differences during frozen storage suggest that the genetics of the fruit also influence the stability of anthocyanins in the juice matrix, with an increase in storage time adversely affecting the TMA content of the juice.

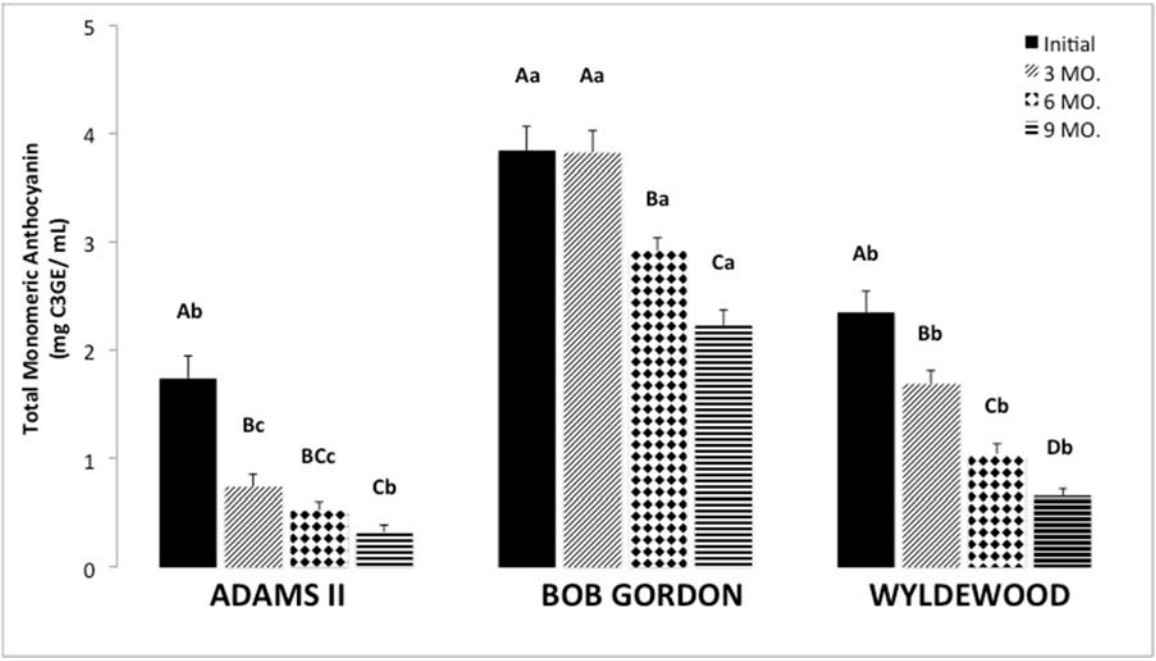


Figure 2.9 Total Monomeric anthocyanin (TMA) content of Adams II, Bob Gordon, and Wyldewood genotypes during frozen storage. The data are represented as the mean ± the standard error of the mean (n=11-14). Each column indicates the time period of testing, starting with the initial test, 3 month, 6 month, and 9 month (left to right). Significant differences ($p < 0.05$) between TMA content of frozen storage periods are represented by different letters (A is significantly different than B, but AB is equivalent to both A and B groups, for example), according to Tukey's honest significance test. Significant differences ($p < 0.05$) between TMA content between each genotype during the same time periods are indicated by different lower case letters.

Thomas et al., tested elderberry fruit for TMA and included the same three genotypes in this study.[14] The TMA content of their samples are in good agreement with the values obtained from this study, showing that Bob Gordon was more concentrated in anthocyanins than Wyldewood and Adams II, strengthening the hypothesis that the genotype of elderberry is a primary factor influencing the anthocyanin composition of the berry.

There are a variety of processes that could cause a decrease in the TMA content of the juice during frozen storage. For instance, enzymatic degradation could have

contributed to anthocyanin loss, especially since the juice samples were not heated during processing. During the study, the same samples were thawed and re-frozen every three months for analysis. Reactivation of enzymes upon repeated thawing could possibly cause anthocyanin degradation or polymerization. To explore the role of enzymes, juice samples were tested for their freeze-thaw integrity over multiple cycles to ensure any anthocyanin loss was due to long term frozen storage and not another cause. During this test, there were no significant differences ($p>0.05$) in TMA content of any of the genotypes of juice during the initial testing and after four days of daily freeze-thaw cycles, strengthening the theory that long term frozen storage contributes to anthocyanin loss in elderberry juice.

2.3.3 Individual Anthocyanin Content (IAC)

Three main individual anthocyanin compounds, among all three genotypes, were identified using SPE coupled with UPLC-MS/MS: cyanidin-3-*O*-coumaroyl-sambubioside-5-glucoside, cyanidin-3-*O*-sambubioside-5-glucoside, and cyanidin-3-*O*-sophoroside (Figure 2.11, 2.12, 2.13). Six other cyanidin based anthocyanins were also present in the juice samples in smaller quantities, including: cyanidin-3-*O*-coumaroyl-sambubioside, cyanidin-3-*O*-rutinoside, cyanidin-3-*O*-sambubioside, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, and a cyanidin based anthocyanin with a tentative identification. An example of one MRM ion channel chromatogram of cyanidin-3-*O*-coumaroyl-sambubioside-5-glucoside of a Wyldewood juice sample is presented in Figure 2.10. The integrated ion signal listed under the retention time indicated in the figure is used to quantitate IAC. Four chromatograms, one for each test period, are

presented and a decrease in ion signal over time was seen. A continual decrease in concentration of this particular individual anthocyanin occurs as a function of increased frozen storage time, since the area under the chromatographic peak decreases over time.

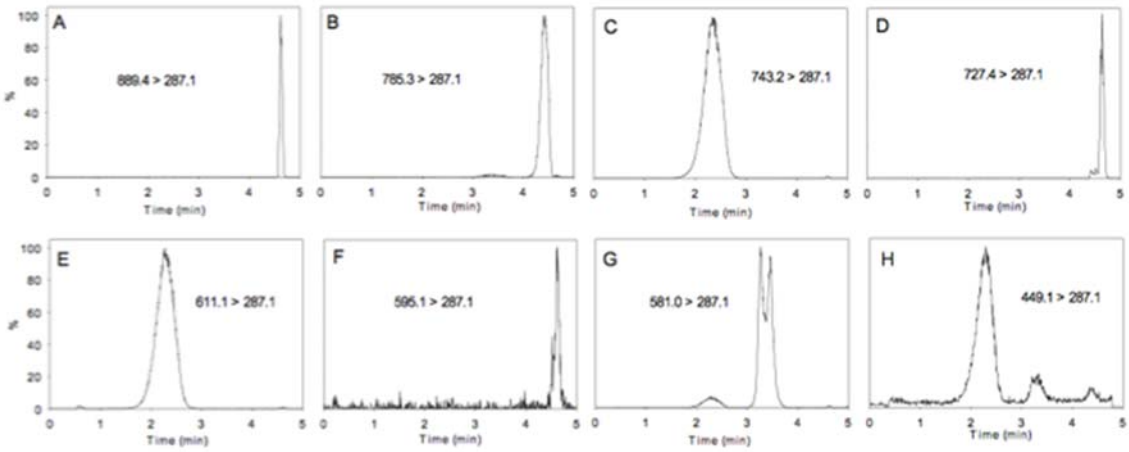


Figure 2.10 MRM chromatograms (A-H) of nine individual anthocyanins analyzed. Parent-daughter ion transitions for each anthocyanin are listed in Table 2.3.

Lee and Finn showed that individual anthocyanin profile varies dramatically between *Sambucus nigra* subsp. *canadensis* and subsp. *nigra*.[24] Cyanidin 3-coumaroyl-sambubioside-5-glucoside and cyanidin-3-sambubioside-5-glucoside were identified as the major individual anthocyanin components in subsp. *canadensis* in their study, and are also two of the three main individual anthocyanins found in the three subsp. *canadensis* genotypes in the present study. Most of the prior work analyzing the anthocyanin content of elderberry involves subsp. *nigra* and identifies cyanidin-3-*O*-glucoside as a major individual anthocyanin.[1] Cyanidin-3-*O*-glucoside is present in subsp. *canadensis,* but in much smaller quantities, which agrees well with Lee and Finn, who tested both subspecies for IAC.[23] Although the magnitudes of IAC were different initially among the three genotypes, the relative composition, or contribution of each individual anthocyanin

to the total IAC were extremely similar, especially between Adams II and Wyldewood. Bob Gordon also had a similar relative composition, except the cyanidin-3-*O*-sophoroside content was almost two-fold higher than the other two genotypes. This could have relevance in future biochemical studies. A prior study showed that the structure of the anthocyanin affects the stability of the molecule in the human oral cavity, and some anthocyanins are more readily available for uptake by the buccal cells, while others degrade quickly in the mouth.[25] Between their different stabilities in vivo and during storage, anthocyanins do not interact equally. This information regarding higher levels of certain anthocyanins in different genotypes underscores the importance of developing new cultivars of fruit with different anthocyanin profiles.

Bob Gordon (198 μg C3GE/ mL) had the highest IAC, followed by Wyldewood (84 μg C3GE/ mL) and Adams II (78 μg C3GE/ mL), respectively (Figure 2.11, 2.12, 2.13). A large decline in IAC in the Adams II genotype is seen after 3 months of frozen storage, and although there is a statistical difference between the Bob Gordon and Wyldewood IAC after 3 months of frozen storage, the anthocyanins are much more stable in these two genotypes during long term frozen storage. This trend is seen in both the cyanidin-3-*O*-coumaroyl-sambubioside-5-glucoside and cyanidin-3-*O*-sambubioside-5

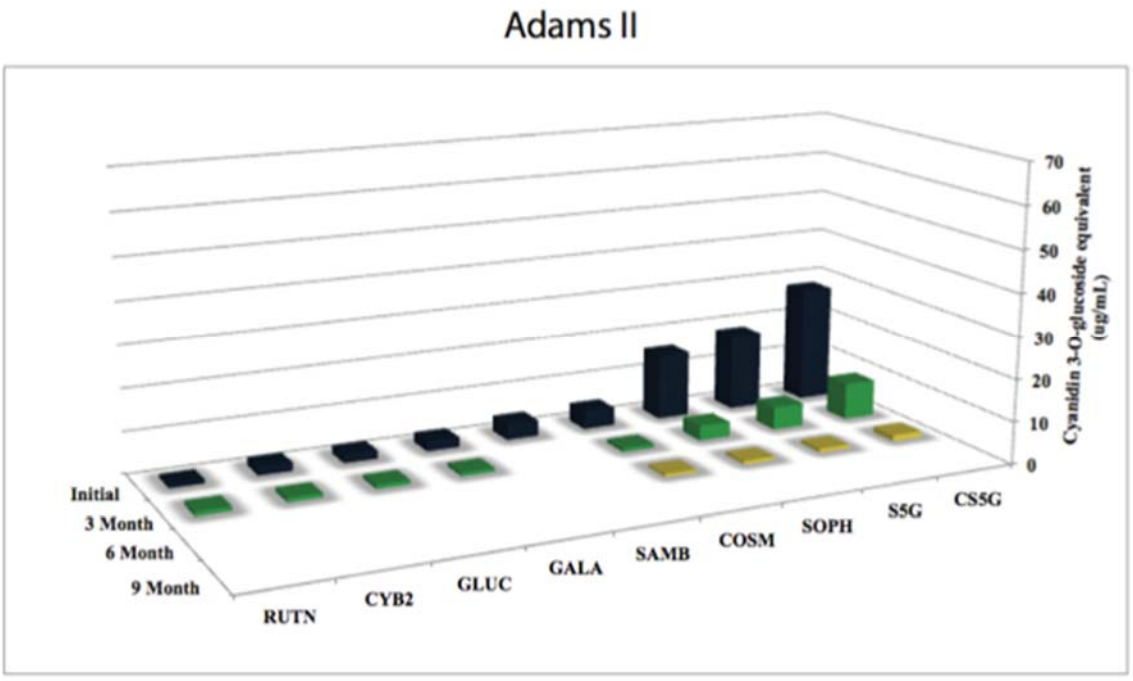


Figure 2.11 Individual anthocyanin content (IAC) of Adams II during frozen storage.

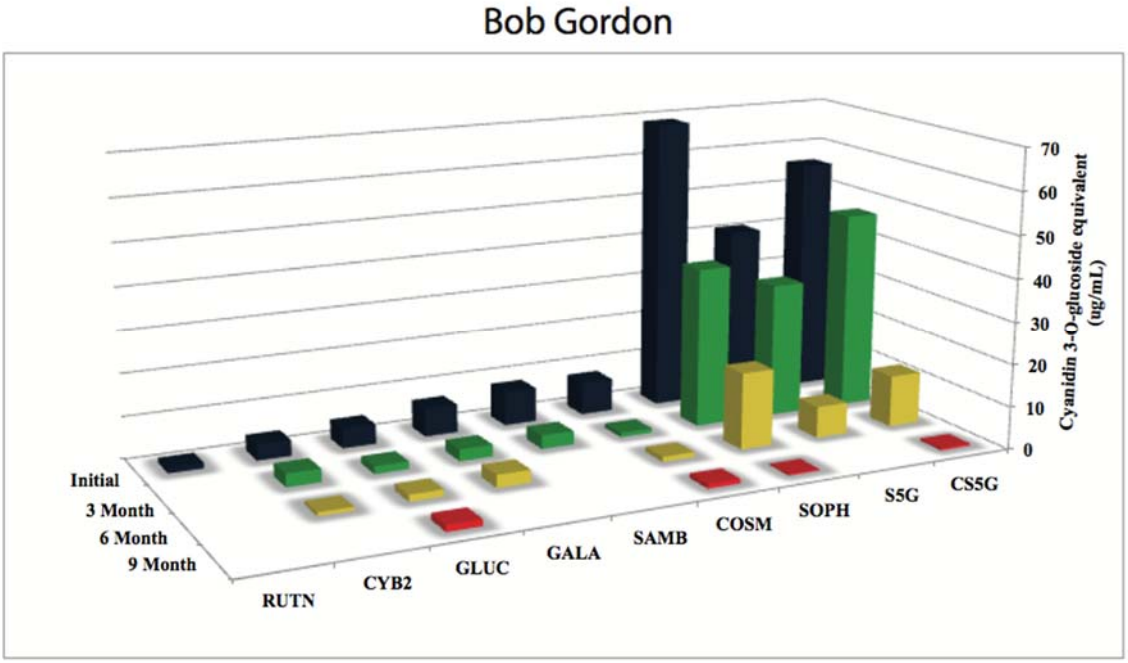


Figure 2.12 Individual anthocyanin content (IAC) of Bob Gordon during frozen storage.

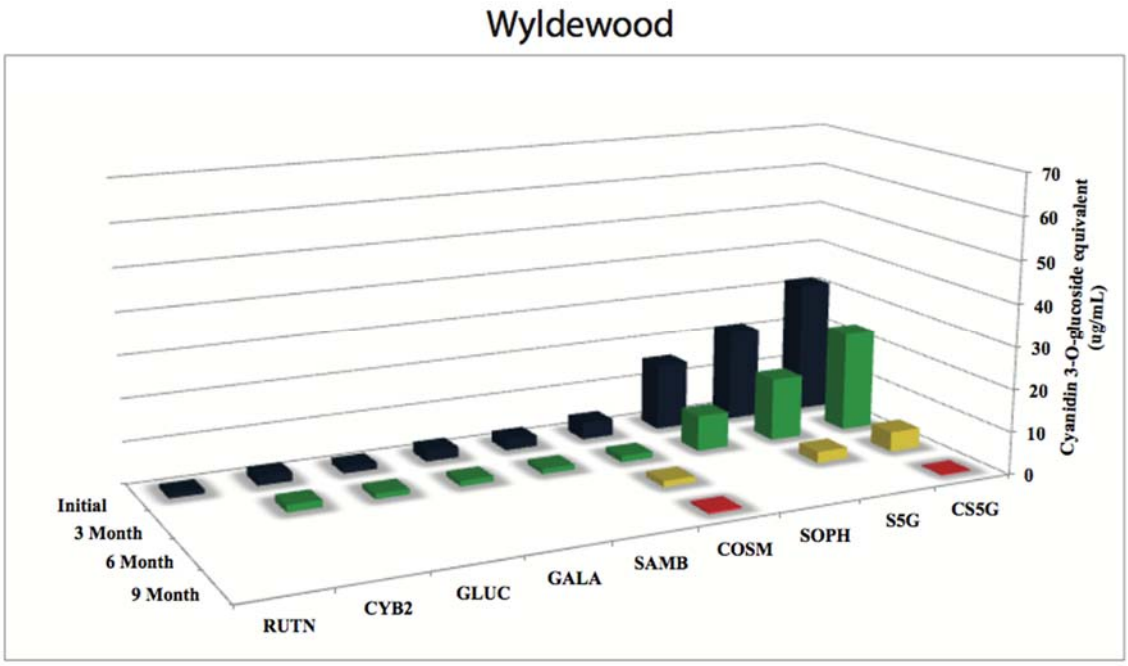


Figure 2.13 Individual anthocyanin content (IAC) of Wyldewood during frozen storage.

glucoside anthocyanins. It is also interesting to note that cyanidin-3-*O*-sophoroside content varies highly between the genotypes initially.

Anthocyanins can exist as acylated species in fruit juice. The sugar moieties can be acylated by a variety of acids, such as gallic, caffeic, p-coumaric, acetic, as well as several others.[26,27] It is widely accepted that the acylated anthocyanins are more stable during processing and storage than unacylated anthocyanins, due to intermolecular copigmentation.[28] The acyl group of one acylated anthocyanin can stack over top of the ring possessing the flavylium cation.[27] This reduces the likelihood of nucleophilic attack, which would cause a structural transformation of the anthocyanin. Depending on the acyl substitution pattern, the acylated anthocyanin can possess the same UV-Vis absorption pattern as unacylated anthocyanins (520nm).[21] Therefore, acylated anthocyanins would contribute to an absorption signal during the TMA assay. However, due to acyl

substitution, these compounds have an increase in mass-to-charge ratio compared to the unacylated derivatives that were measured for IAC. The results of this study show a higher TMA content and the sum of the IAC. Also, there is a small difference in the stabilities of the anthocyanins between the two assays. This is likely attributed to the presence of acylated anthocyanins present in the elderberry fruit juice.

The individual anthocyanins in elderberry juice are less stable during frozen storage compared to the monomeric anthocyanins. Bob Gordon retained 65%, 22%, and 2% of its total IAC during 3, 6, and 9 month of frozen storage, respectively (Figure 2.11, 2.12, 2.13). Wyldewood and Adams II retained 65%, 10%, <1%, and 29%, 10%, and 0% of its IAC during 3, 6, and 9 months of frozen storage, respectively. These results suggest that prolonged frozen storage has a significant impact on the anthocyanin content of elderberry fruit juice. The different sugar-containing moieties may play a role in the stability of the anthocyanins during storage. It is difficult to identify a trend in the individual anthocyanins with a very small contribution since a majority fall below the detection limit after 3 months of frozen storage. Cyanidin-3-*O*-coumaroyl-sambubioside was the least stable during storage amongst all three genotypes, retaining less than 38% IAC in all three genotypes after 3 months of frozen storage. Cyanidin-3-*O*-coumaroyl-sambubioside-5-glucoside and cyanidin-3-*O*-sambubioside-5-glucoside showed much better stability in the Bob Gordon with 84% and 78% anthocyanin retention, respectively, and Wyldewood with 75% and 69% anthocyanin retention, respectively.

Individual anthocyanins also displayed different stabilities between genotypes. Cyanidin-3-*O*-sophorisde had very poor stability (23% retention) in Adams II, but >53% retention in both Bob Gordon and Wyldewood after 3 months of frozen storage.

Cyanidin-3-*O*-sambubioside-5-glucoside displayed a very similar trend. Adams II once again had very poor stability (30%), while Bob Gordon (78%) and Wyldewood (68%) showed much higher anthocyanin stability after 3 months of frozen storage. Cyanidin-3-*O*-coumaroyl-sambubioside-5-glucoside had the best stability in all three genotypes during the first three months of frozen storage. Bob Gordon saw 84% anthocyanin retention, and Wyldewood and Adams II had 75% and 31% retention, respectively. Cyanidin-3-*O*-coumaroyl-sambubioside had the worst stability of the individual anthocyanins tested, with all genotypes having <38% of their IAC after 3 months of frozen storage. Further exploration of the juice matrix of different genotypes of elderberry fruit juice could provide insight into which compounds may contribute to increased anthocyanin stability and degradation.

2.4 Conclusions

It has been shown that individual anthocyanin concentrations in elderberry fruit juice can vary during frozen storage and the UPLC-MS/MS method used here yields important information that is not obtained by commonly used TP and TMA testing methods. By conducting extensive sample clean up, potentially interfering juice matrix compounds were removed, allowing for more accurate anthocyanin quantification. More information was gathered regarding the stability of individual anthocyanins in three genotypes of elderberry juice. The TP, TMA, and IAC content of elderberry juice varied with genotype.

2.5 References

(1) Ulbricht, C., Basch, E., Cheung, L., Goldberg, H., Hammerness, P., Isaac, R., Khalsa, K. P., Romm, A., Rychlik, I., Varghese, M., Weissner, W., Windsor, R. C., and Wortley, J. (2014) An evidence-based systematic review of elderberry and elderflower (*Sambucus nigra)* by the natural standard research collaboration. *J. Diet. Suppl.* 11, 80−120.

(2) Del Rio, D., Borges, G., and Crozier, A. (2010) Berry flavonoids and phenolics: bioavailability and evidence of protective effects. *Br. J. Nutr.* 104, S67−S90.

(3) González-Gallego, J., García-Mediavilla, M. V., Sánchez-Campos, S., and Tuñón, M. J. (2010) Fruit polyphenols, immunity and inflammation. *Br. J. Nutr.* 104, S15−S27.

(4) He, J., and Giusti, M. M. (2011) High-purity isolation of anthocyanins mixtures from fruits and vegetables – a novel solid-phase extraction method using mixed mode cation-exchange chromatography. *J. Chromatogr. A* 1218, 7914−7922.

(5) Tuñón, M. J., García-Mediavilla, M. V., Sánchez-Campos, S., and González-Gallego, J. (2009) Potential of flavonoids as anti-inflammatory agents: modulation of pro-inflammatory gene expression and signal transduction pathways. *Curr. Drug Metab.* 10, 256−271.

(6) Kinoshita, E., Hayashi, K., Katayama, H., Hayashi, T., and Obata, A. (2012) Anti-influenza virus effects of elderberry juice and its fractions. *Biosci. Biotechnol. Biochem.* 76, 1633−1638.

(7) Yao, L. H., Jiang, Y. M., Shi, J., Tomás-Barberán, F. A., Datta, N., Singanusong, R., and Chen, S. S. (2004) Flavonoids in food and their health benefits. *Plant Foods Hum. Nutr.* 59, 113−122.

(8) Knekt, P., Järvinen, R., Seppänen, R., Heliövaara, M., Teppo, L., Pukkala, E., and Aromaa, A. (1997) Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. *Am. J. Epidemiol.* 146, 223−230.

(9) Bosetti, C., Rossi, M., McLaughlin, J. K., Negri, E., Talamini, R., Lagiou, P. Montella M., Ramazotti, V., Franceshi, S., and LaVecchia, C. (2007) Flavonoids and the risk of renal cell carcinoma. *Cancer Epidemiol. Biomarkers Prev.* 16, 98−101.

(10) Ozgen, M., Scheerens, J. C., Reese, R. N., and Miller, R. A. (2010) Total phenolic, anthocyanin contents and antioxidant capacity of selected elderberry (*Sambucus canadensis L.*) accessions. *Pharmacogn. Mag.* 6, 198−203.

(11) Plaza, M., Kariuki, J., and Turner, C. (2014) Quantification of individual phenolic compounds' contribution to antioxidant capacity in apple: a novel analytical tool based on liquid chromatography with diode array, electrochemical, and charged aerosol detection. *J. Agric. Food Chem.* 62, 409−418.

(12) Tian, Q., Giusti, M. M., Stoner, G. D., and Schwartz, S. J. (2005) Screening for anthocyanins using high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry with precursor-ion analysis, product-ion analysis, common-neutral-loss analysis, and selected reaction monitoring. *J. Chromatogr. A* 1091, 72−82.

(13) Veberic, R., Stampar, F., Schmitzer, V., Cunja, V., Zupan, A., Koron, D., and Mikulic-Petkovsek, M. (2014) Changes in the contents of anthocyanins and other compounds in blackberry fruits due to freezing and long-term frozen storage. *J. Agric. Food Chem*. 62, 6926−6935.

(14) Thomas, A. L., Veazie, P. P., Beyers, P. L., Finn, C. E., and Lee, J. (2013) A comparison of fruit characteristics among diverse elderberry genotypes grown in Missouri and Oregon. *J. Berry Res.* 3, 159−168.

(15) Thomas, A.L., Byers, P.L., Gu, S., Avery, Jr. J. D., Kaps, M., Datta, A., Fernando, L., Grossi, P., and Rottinghaus, G. E. (2015) Occurrence of polyphenols, organic acids, and sugars among diverse elderberry genotypes grown in three Missouri (USA) locations. *Acta Hort*. 1061, 147−154.

(16) Wilkes, K., Howard, L. R., Brownmiller, C., and Prior, R. L. (2014) Changes in chokeberry (*Aronia melanocarpa* L.) polyphenols during juice processing and storage. *J. Agric. Food Chem*. 62, 4018−4025.

(17) Hager, A., Howard, L. R., Prior, R. L., and Brownmiller, C. (2008) Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of processed black raspberry products. *J. Food Sci.* 73, H134−H140.

(18) Brownmiller, C., Howard, L. R., and Prior, R. L. (2009) Processing and storage effects on procyanidin composition and concentration of processed blueberry products. *J. Agric. Food Chem*. 57, 1896−1902.

(19) Finn, C. E., Thomas, A. L., Byers, P.L., and Serce, S. (2008) Evaluation of American (*Sambucus canadensis*) and European (*S. nigra*) Elderberry Genotypes Grown in Diverse Environments and Implications for Cultivar Development. *Hort. Sci*. 43, 1385−1391.

(20) Singelton, V. L., and Rossi, J. J. (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* 16, 144−158.

(21) Giusti, M., and Wrolstad, R. E. (2001) Characterization and measurement of anthocyanins by UV-Visible spectroscopy. In *Curr. Protoc. Food Analyt. Chem.*,

John Wiley & Sons, Inc.: Hoboken, NJ, F1.2.1−F1.2.6.

(22) Jurd, L., and Asen, S. (1966) The formation of metal and "co-pigment" complexes of cyanidin 3-glucoside. *Phytochemistry.* 5, 1263−1271.

(23) Wu, H., Johnson, M. C., Lu, P., Fritsche, K. L., Thomas, A. L., Cai, Z., and Greenlief, C. M. (2015) Determination of anthocyanins and total polyphenols in a variety of elderberry juices by UPLC/MS and other methods. *Acta Hort*. 1061, 43−51.

(24) Lee, J., and Finn, C. E. (2007) Anthocyanins and other polyphenolics in American elderberry (*Sambucus canadensis*) and European elderberry (*S. nigra*) cultivars. *J. Agric. Food Chem*. 87, 2665−2675.

(25) Kamonpatana, K., Failla, M. L., Kumar, P. S., and Giusti, M. M. (2014) Anthocyanin structure determines susceptibility to microbial degradation and bioavailability to the buccal mucosa. *J. Agric. Food Chem*. 62, 6903−6910.

(26) Giusti, M., and Wrolstad, R. E. (2003) Acylated anthocyanins from edible sources and their applications in food systems. *Biochem. Eng. J.* 14, 217−225.

(27) Bakowska-Barczak, A. (2005) Acylated anthocyanins as stable, natural food colorants−A review. *Pol. J. Food Nutr. Sci.* 14, 107−116.

(28) Inami O., Tamura, I., Kikuzaki, H., and Nakatani, N. (1996) Stability of anthocyanins of *Sambuskus canadensis* and *Sambuskus nigra*. *J. Agric. Food Chem.* 44, 3090−3096.

Chapter 3: Multi Year Harvest Study

3.1 Introduction

American elderberry [*Sambucus nigra* L. subsp. *canadensis* (L.) Bolli] has emerged as a popular crop for producing dietary supplements, natural food colorants, wines, and other commercial products from both its fruit and flowers.[1] Although the European elderberry (*Sambucus nigra* L. subsp. *nigra*) has been extensively studied in terms of its bioactive components and human health benefits, less is known about the American elderberry; especially its bioactive components.[2-4] Elderberries, including the American subspecies, are a very rich source of anthocyanins and polyphenols which are responsible for some of the purported health benefits associated with their antioxidant activity.[5] Antioxidants have the ability to scavenge free radicals, reducing the amount of oxidative stress that can cause cardiovascular diseases, cancer, and neurological disorders over a long period of time.[6]

Although there is extensive scientific literature supporting the proposed health benefits of elderberry consumption, some studies suggest that the bioavailability of some polyphenols can be dramatically different based on the aglycone, or even sugar moiety substitution.[7-8] Another study showed that the anthocyanins with the same aglycone but different sugar moieties have different stabilities in the mouth.[9] Anthocyanins have powerful antioxidant properties, but are relatively unstable molecules that are susceptible to degradation and polymerization. Studies have shown that anthocyanins are prone to degradation during thermal processing, frozen storage, and pasteurization.[9-14] This phenomenon could be overlooked by producers or consumers of dietary supplements, and

in clinic trials, where the amounts of specific anthocyanins are not normalized and the bioavailability of the active ingredient is not taken into consideration.

Several studies have investigated the phytochemical profile of elderberry fruits and fruit juice, elderberry extracts, and elderberry based dietary supplements.[5,15-18] Previous studies on American elderberry have found significant differences in polyphenol content within vegetative tissues and within fruit and fruit juice based on environmental and crop management factors.[16,19-21] This confirms that, in addition to genetics, environmental variations can potentially influence the phytochemical profile of American elderberry. This work investigates the potential effect genotype, growing location and year-to-year factors have on the individual anthocyanin and polyphenol content of American elderberry juice.

Multivariate statistical methods have been used previously to develop a chemical profile of ginseng and to classify commercial ginseng products.[22] Ultra-performance liquid chromatography – quadrupole time of flight (UPLC-QTOF) mass spectrometry analysis revealed ten compounds that were unique among three ginseng species. Yuk et al., also used this method to determine which species of ginseng were found in various commercial herbal supplements. In a different study, Italian saffron (*Crocus sativus* L.) samples from five growing locations were analyzed for various flavonoids and several other bioactive compounds using high-performance liquid-chromatography (HPLC).[23] Classification to the actual saffron growing location using a discriminant analysis was performed with 88% accuracy. Multivariate techniques, like discriminant analysis, are providing new information, such as chemical profiles, location identification, and sample authentication, which is important in the dietary supplement industry.

3.2 Experimental

3.2.1 Materials

3.2.1.1 Plant Material

American elderberry fruit for this study was grown at the University of Missouri's Southwest Research Center near Mt. Vernon, and Missouri State University's State Fruit Experiment Station at Mountain Grove, both in southern Missouri. Three commercially available elderberry cultivars ('Adams II', 'Bob Gordon', and 'Wyldewood') were used. Plants were propagated from our own mother plants in late winter, 2011, and transplanted to the sites in June, 2011. Experimental plots contained four plants of the same cultivar, planted 1.2 m apart. At each site, 48 plots were established in four rows, with plots separated 2.4 m within and 3.1 m between rows. The three cultivars were each assigned to 16 of the 48 plots in a completely randomized manner. During the growing seasons, plants were irrigated via drip lines to provide 2.5 to 4.0 cm water per week when rainfall was lacking. Weeds and pests were managed using standard horticultural practices.[24] During the establishment year (2011), inflorescences were removed to encourage development of healthy roots and structure.

Ripe fruit for this study was collected during four consecutive growing seasons (2012–2015). The fruit was transported to lab under refrigeration, where it was stored at -20 °C. The fruit was later thawed, de-stemmed, and hand-pressed into individual aliquots of juice, then re-frozen and stored at -20 °C until analysis.

3.2.1.2 Chemicals

Water, acetonitrile, and formic acid were all HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA). Triflouroacetic acid (TFA; 99%) was obtained from Acros Organics (Morris Plains, NJ, USA). Cyanidin-3-*O*-glucoside analytical standard (≥ 95%) was purchased from Sigma Aldrich (St. Louis, MO, USA).

3.2.2 SPE

Juice samples were thawed in an oven at 60 °C for 5 min. A modified SPE protocol was used for polyphenol separation.[25] 200 µL of juice was diluted with 200 µL of water (0.01% TFA) and vortexed. An Oasis C18 SPE cartridge was washed with 5 mL of water (0.01% TFA) before adding the diluted juice samples and adding an additional 5 mL of water (0.01% TFA) to wash away unwanted analytes. 10 mL of methanol (0.01% TFA) was ran through the column and fractions were collected and dried using a forced air evaporator at 50 °C. Samples were reconstituted in water (2% acetic acid) for HPLC analysis. Anthocyanins were separated from the juice using a previously developed method (Wu et al., 2015). Briefly, mixed mode cation-exchange SPE was used (Oasis MCX 3cc, 60mg) and extractions performed using a Supelco Visiprep vacuum manifold.

3.2.3 HPLC UV-Vis Analysis of Polyphenols

The HPLC system consisted of a Hitachi L-7100 pump, a Hitachi L-7200 autosampler (20 µL injection), and a Hitachi L-7400 UV detector (detection wavelength 320 nm) with a Synergi™ 4 µm Hydro-RP 80Å (2.0 x 150 mm) column (Phenomenex, Torrance, CA, USA) fitted with a SecurityGuard C18 (ODS) 4.0 x 3.0 mm guard column

(Phenomenex). The mobile phase used was: (A) water (2% acetic acid) and (B) 50:50 water/acetonitrile (0.5% acetic acid). The gradient was 2% B initially until 2 min, then linearly ramped to 40% B from 2-52 min, 100% B from 53-58min, and 2% B from 59-63 min. The mobile phase flow rate was 0.6 mL/min and system was run at room temperature. Data were recorded and processed by a Hitachi D-7000 data acquisition package with ConcertChrom software on a microcomputer. Standards of chlorogenic acid and rutin were prepared at 0, 10, 25, 50, 100 and 200 ppm and quantification was done by comparing the area under the chromatographic peaks of elderberry samples to the calibration curve. Polyphenols monitored were neo-chlorogenic acid, chlorogenic acid, crypto-chlorogenic acid, quercetin 3-rutinoside, quercetin 3-glucoside, kaempferol 3-rutinoside, isorhamnetin 3-rutinoside, and isorhamnetin 3-glucoside.

3.2.4 UPLC-MS/MS Analysis of Anthocyanins

A previously described ESI-UPLC-MS/MS method was used for anthocyanin analysis and cyanidin-3-*O*-glucoside standards were prepared at 1, 10, 50, 100, 250, 500, and 1,000 ng/mL.[26] Quantification was performed using the area under the chromatographic peaks for individual multiple reaction monitoring ion channels for each anthocyanin and comparing it to a standard curve. Individual anthocyanins monitored were cyanidin 3-*O*-coumaroyl-sambubioside-5-glucoside, cyanidin based anthocyanin, cyanidin 3-*O*-sambubioside-5-glucoside, cyanidin 3-*O*-coumaroyl-sambubioside, cyanidin 3-*O*-sophoroside, cyanidin 3-*O*-rutinoside, cyanidin 3-*O*-sambubioside, and cyanidin 3-*O*-glucoside. Multiple reaction monitoring ion chromatograms of each anthocyanin are shown in Figure 2.10.

3.2.5 Discriminant Analysis

Anthocyanin and polyphenol raw data was normalized to the same concentration units and represented as individual aliquots. Discriminant analysis was performed using the XLSTAT data analysis software in Microsoft Excel, version 14.5.3 (Microsoft, Santa Rosa, CA, USA) at a tolerance of 0.0001, p-value <0.05. Box's and Wilks' Lambda tests were used to asses if the covariance matrices and means vectors between the genotypes were significantly different, respectively. A confusion matrix, which is routinely constructed as part of DA, using cross-validation was used to test the ability of the method to properly group the samples based on genotype.

3.3 Results and Discussion

3.3.1 Individual Polyphenol Content

Although the European elderberry has been extensively analyzed, studies regarding the phytochemical profile of the American elderberry are limited. The three main polyphenols identified in the three genotypes of American elderberry juice were quercetin 3-rutinoside, isorhamnetin 3-rutinoside, and chlorogenic acid (Table 3.1). Quercetin 3-rutinoside had the highest average content among polyphenols for each genotype, year and growing location, except 2012 Bob Gordon at Mountain Grove.

3.3.2 Individual Anthocyanin Content

The main anthocyanins identified were cyanidin 3-*O*-coumaroyl-sambubioside-5-glucoside, cyanidin 3-*O*-sambubioside-5-glucoside, and cyanidin 3-*O*-sophoroside (Table 3.2). These findings agree well with a previous study investigating the individual

polyphenol and anthocyanin content of ten American elderberry fruit cultivars.[5] 3-*O*-coumaroyl-sambubioside-5-glucoside was present in relatively higher concentrations in the Bob Gordon compared to the Wyldewood and Adams II at both locations over the course of the study. Bob Gordon was also a much richer source of cyanidin 3-*O*-sophoroside than the other two genotypes. Polyphenol and anthocyanin concentration ranges in this study were similar to analytes tested by Lee and Finn.[5]

3.3.3 Influence of Growing Year

Changes in polyphenol and anthocyanin content among the three cultivars during the four different growing years were very apparent. At Mt. Vernon, anthocyanin content was significantly higher in 2013 than 2014 for all genotypes. For example, Bob Gordon had almost triple the content of cyanidin 3-*O*-coumaroyl-sambubioside-5-glucoside and roughly eight times the cyanidin 3-*O*-sambubioside-5-glucoside and cyanidin 3-*O*-sophoroside content in 2013 compared to 2014. The same trend was observed at Mountain Grove between growing years, where anthocyanin content was significantly lower in 2015 than 2014. The cyanidin 3-*O*-coumaroyl-sambubioside-5-glucoside, cyanidin 3-*O*-sambubioside-5-glucoside and cyanidin 3-*O*-sophoroside content were all approximately five fold lower in the Wyldewood genotype in 2015 than 2014 at Mountain Grove. The quercetin 3-rutinoside content of Adams II elderberry juice ranged from 47−792 ppm and the isorhamnetin 3-rutinoside concentration ranged from 35−250 ppm. This suggests that environmental and management variables can have a significant impact on the anthocyanin and polyphenol content of American elderberry juice

Table 3.1 Average polyphenol content of three elderberry genotypes at two different Missouri growing locations from 2012, 2013 and 2014 (ppm ± standard error).

	Neo-chlorogenic acid	Chlorogenic acid	Crytpto-chlorogenic acid	Quercetin 3-rutinoside	Isoquercetin	Kaempferol 3-rutinoside	Isorhamnetin3-rutinoside	Isorhamnetin 3-glucoside
Adams II								
2012 MG	16 ± 3	26 ± 8	4 ± 1	289 ± 48	39 ± 3	14 ± 2	250 ± 31	15 ± 5
2013 MG	76 ± 11	181 ± 25	2.0 ± 0.3	792 ± 143	26 ± 4	49 ± 7	220 ± 41	11 ± 3
2014 MG	7 ± 2	28 ± 4	3.0±0.2	47 ± 13	13 ± 3	6 ± 1	35 ± 9	9 ±1
2012 MV	27 ± 5	47 ± 11	3.0 ± 0.8	244 ± 35	25 ± 2	13 ± 2	199 ± 21	8 ± 1
2013 MV	50 ± 6	138 ± 20	1.3 ± 0.2	212 ± 38	13 ± 2	17 ± 2	87 ± 19	7 ± 2
2014 MV	116 ± 11	309 ± 36	10 ± 1	650 ± 44	54 ± 4	49 ± 4	191 ± 12	14 ± 1
Bob Gordon								
2012 MG	16 ± 3	21 ± 4	1.6 ± 0.4	129 ± 22	25 ± 3	15 ± 2	143 ± 22	7 ± 1
2013 MG	81 ± 10	129 ± 14	2.1 ± 0.4	699 ± 116	24 ± 3	75 ± 12	272 ± 49	10 ± 4
2014 MG	79 ± 18	98 ± 24	4 ± 1	214 ± 90	16 ± 4	21 ± 8	72 ± 22	4.6 ± 0.5
2012 MV	43 ± 5	56 ± 8	2.4 ± 0.5	256 ± 24	27 ± 3	24 ± 3	144 ± 18	5.1 ± 0.6
2013 MV	51 ± 5	61 ± 8	1.9 ± 0.2	272 ± 44	12 ± 1	30 ± 4	73 ± 17	8 ± 3
2014 MV	66 ± 5	70 ± 7	9 ± 1	501 ± 32	36 ± 2	45 ± 3	145 ± 12	13 ± 1
Wyldewood								
2012 MG	8 ± 3	18 ± 5	3.3 ± 0.5	235 ± 30	37 ± 3	17 ± 2	190 ± 22	12 ± 3
2013 MG	71 ± 8	152 ± 17	3.9 ± 0.5	692 ± 142	20 ± 3	40 ± 7	176 ± 39	9 ± 2
2014 MG	74 ± 10	137 ± 24	6 ± 1	208 ± 108	18 ± 6	15 ± 5	107 ± 38	6 ± 1
2012 MV	32 ± 6	75 ± 17	1.6 ± 0.3	619 ± 80	27 ± 2	29 ± 3	127 ± 14	8 ± 2
2013 MV	59 ± 12	142 ± 30	3.0 ± 0.5	563 ± 83	15.7 ± 1	37 ± 4	100 ± 14	8 ± 3
2014 MV	71 ± 9	183 ± 22	11 ± 1	1,140 ± 107	53 ± 4	62 ± 5	300 ± 24	20 ± 2

Table 3.2 Average IAC of three elderberry genotypes at two different Missouri growing locations from 2013, 2014 and 2015 in μg/mL cyanidin 3-O-glucoside equivalents ± standard error (parent ion m/z → daughter ion m/z).

	cyanidin 3-*O*-coumaroyl-sambubioside-5-glucoside (889.4→ 287.1)	cyanidin based anthocyanin (785.3→ 287.1)	cyanidin 3-*O*-sambubioside-5-glucoside (743.2→287.1)	cyanidin 3-*O*-coumaroyl-sambubioside (727.4→ 287.1)	cyanidin 3-*O*-sophoroside (611.1→ 287.1)	cyanidin 3-*O*-rutinoside (595.1→ 287.1)	cyanidin 3-*O*-sambubioside (581.0→ 287.1)	cyanidin 3-*O*-glucoside (449.1→ 287.1)
Adams II								
2014 MG	8 ± 1	0.10 ± 0.04	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.2	0.06 ± 0.01	0.10 ± 0.04	0.29 ± 0.05
2015 MG	0.44 ± 0.07	nd	0.14 ± 0.03	0.29 ± 0.06	0.13 ± 0.03	nd*	nd	nd
2013 MV	28 ± 1	2.05 ± 0.05	18.5 ± 0.1	4.4 ± 0.4	15.9 ± 0.2	0.97 ± 0.02	3.9 ± 0.3	2.1 ± 0.1
2014 MV	21 ± 1	0.88 ± 0.06	5.4 ± 0.4	3.1 ± 0.2	5.8 ± 0.5	0.30 ± 0.02	1.9± 0.2	1.06 ± 0.08
2015 MV	3.5 ± 0.8	0.21 ± 0.08	1.3 ± 0.3	1.1 ± 0.3	1.2 ± 0.3	nd	nd	nd
Bob Gordon								
2014 MG	30 ± 1	0.87 ± 0.08	8.4 ± 0.8	2.9 ± 0.3	14 ± 1	0.57 ± 0.09	3.0 ± 0.6	2.5 ± 0.2
2015 MG	5.8 ± 0.5	0.35 ± 0.04	3.6 ± 0.5	3.2 ± 0.5	5.7 ± 0.7	0.15 ± 0.02	0.28 ± 0.5	nd
2013 MV	56 ± 3	3.8 ± 0.3	41 ± 4	7.8 ± 0.4	69 ± 7	1.28 ± 0.04	8.1 ± 0.7	4.7 ± 0.3
2014 MV	19.2 ± 0.8	0.74 ± 0.04	5.1 ± 0.3	2.2 ± 0.2	8.9 ± 0.6	0.30 ± 0.02	1.4 ± 0.11	0.89 ± 0.04
2015 MV	10.5 ± 0.6	0.7 ± 0.3	6.2 ± 0.3	4.8 ± 0.4	14.7 ± 0.7	0.29 ± 0.05	0.9 ± 0.3	nd
Wyldewood								
2014 MG	21 ± 1	0.69 ± 0.06	7.3 ± 0.5	2.1 ± 0.2	8.5 ± 0.8	0.37 ± 0.03	2.3 ± 0.3	2.2 ± 0.3
2015 MG	3.7 ± 0.5	0.19 ± 0.03	1.6 ± 0.3	2.3 ± 0.4	1.8 ± 0.4	nd	nd	nd
2013 MV	32 ± 3	2.2 ± 0.2	22 ± 2	4.0 ± 0.3	16 ± 1	1.01 ± 0.04	2.6 ± 0.2	1.54 ± 0.06
2014 MV	17.0 ±0.7	0.67 ±0.04	5.8 ± 0.4	1.9 ± 0.1	5.4 ± 0.5	0.27 ±0.2	1.5 ±0.1	0.99 ±0.08
2015 MV	n/a**	n/a	n/a	n/a	n/a	n/a	n/a	n/a

* nd: concentration was below the detection limit, **n/a: samples were unavailable for analysis

3.3.4 Effects of Genotype and Growing Location

The average concentration of several anthocyanins and polyphenols varied highly between genotypes at the same location, during the same season. For instance, Bob Gordon had about double the concentration cyanidin 3-*O*-coumaroyl-sambubioside-5-glucoside (56 ppm) than Adams (28 ppm) at Mt. Vernon in 2013 (Table 3.2). In 2014 at Mt. Vernon, Wyldewood had and average quercetin 3-rutinoside concentration of 1,140 ppm which was roughly double that of Adams (650 ppm) and Bob Gordon (501 ppm) that year (Table 3.1). In a previous study, it was shown that the total phenolic and total monomeric anthocyanin content were dramatically affected by the genotype, growing location, and year.[20] Although these are different measurements, this study also suggests that climactic conditions play a large role in the anthocyanin and polyphenol content of American elderberry juice.

It appears that all of the genotypes have similarities in their polyphenol and anthocyanin profiles, with the same analytes generally being the most abundant among the genotypes. However, we observed substantial differences in the concentrations of anthocyanins and polyphenols between year, genotype and growing location. Other elderberry field studies have reached similar conclusions, but the precise roles of specific climate, soil, and crop management factors in producing consistent levels and profiles of specific polyphenols and anthocyanins have not been elucidated.[16,20,27] A better understanding of these factors is extremely important as the popularity of elderberry as a dietary supplement continues to grow. Elderberry dietary supplements generally include a quantity of elderberry extract, but do not factor in the concentrations of specific phytochemicals present. This may result in different amounts of specific anthocyanins

and polyphenols in dietary supplement products. Furthermore, in order to investigate the efficacy and function of specific phytochemicals in clinical trials, the content of specific anthocyanins being administered should be included in the analysis.

3.3.5 Discriminant Analysis

A common statistical analysis approach would be to perform ANOVA between the averages of the different groups and identify significant differences. Although this can provide information, with such a large data set it would be difficult to propose conclusions from this analysis, other than to point out where significant differences appear. The goal of the discriminant analysis is to be able to perform grouping based on a set of traits, in this case the anthocyanin and polyphenol contents of individual elderberry juice samples. It appears that random differences in polyphenol and anthocyanin content are observed for the different genotypes between the locations and years based on their average values. However, it would advantageous to be able to distinguish elderberry based on its phytochemical profile. Thus, a discriminant analysis was performed for all genotypes in 2014 at the Mt. Vernon location (Fig. 3.1). The reason this data set was chosen is two-fold. First, it isolates growing location and year, which have shown to significantly impact the phytochemical content. Second, it appears to have the smallest difference in average values between the same anthocyanins between genotypes. Significant differences ($p<0.01$) were identified for cyanidin 3-*O*-coumaroyl-sambubioside-5-glucoside, cyanidin 3-*O*-sophoroside, quercetin 3-rutinoside, isorhamnetin 3-rutinoside, and chlorogenic acid. The discriminant analysis successfully shows the ability to group the individual juice samples by genotype.

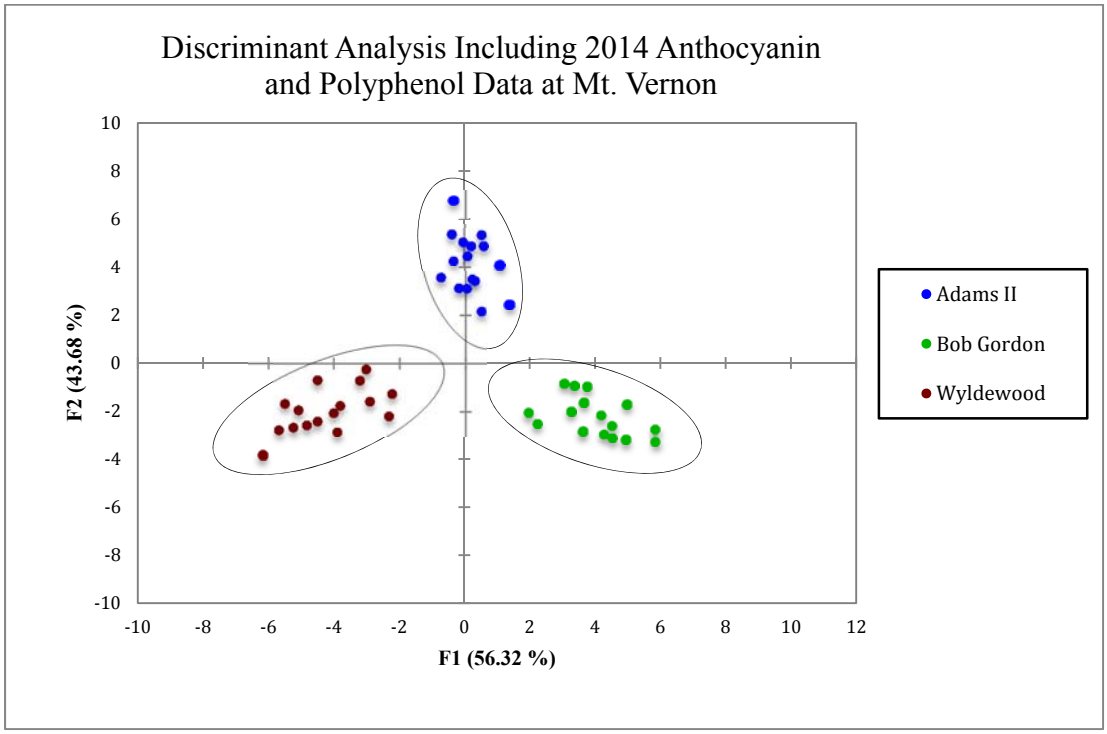


Figure 3.1 Discriminant analysis of 48 American elderberry juice samples (n=16 from each genotype) from Mt. Vernon in 2014 based on the anthocyanin and polyphenol content of each individual juice aliquot.

A cross-validation was performed to further test the ability of this statistical method to discriminate the juice samples and this method was able to correctly identify 45 out of 48 samples (94%) based on their individual anthocyanin and polyphenol profile (Table 3.3). All 16 of the juice samples from Wyldewood genotype were correctly identified. Fourteen of the juice samples from the Adams II genotype were correctly identified, while two samples were incorrectly identified as Wyldewood. Fifteen Bob Gordon samples were properly identified and only one was incorrectly identified as Adams II. This illustrates that although the average concentrations of anthocyanins and polyphenols can be similar, it appears that the genotype has a specific and statistically

distinguishable profile when combining all the polyphenol and anthocyanin concentrations of individual juice aliquots.

From\To	Adams II	Bob Gordon	Wyldewood	Total	% correct
Adams II	14	0	2	16	87.50%
Bob Gordon	1	15	0	16	93.75%
Wyldewood	0	0	16	16	100.00%
Total	15	15	18	48	93.75%

Table 3.3 Cross validation results from confusion matrix from the 2014 discriminant analysis at Mt. Vernon.

Discriminant analyses were also performed on the juice data from Mt. Vernon in 2013 and Mountain Grove in 2014 (Appendix C). Individual anthocyanin data was not obtained for either locations in 2012, or Mountain Grove juice samples in 2013. Polyphenol data was not obtained for either location in 2015. Although each of these groups had a smaller sample population, the discriminant analysis was successfully able to group the juice samples by genotype based on the anthocyanin and polyphenol content of the juice for both of Mt. Vernon in 2013 and Mountain Grove in 2014. However, the confidence ellipses surrounding the groups are larger and the confusion matrix cross-validation results are slightly lower at approximately 86% for both data sets. Therefore, the 2014 Mt. Vernon data set is the most comprehensive and thus is the best representation of the discriminant analysis. Although the other discriminant analyses are not as conclusive as the 2014 Mt. Vernon, they illustrate reproducibility in the method over the course of growing seasons and locations.

3.4 Conclusion

There are large deviations present when measuring a set of juice samples from the same genotype, location and year. This suggests that factors between plots, such as ripeness, sun exposure, and soil variability can have an influence on the overall magnitude of the anthocyanins and polyphenols present. However, when grouping the samples individually based on the anthocyanin and polyphenol concentration, the discriminant analysis can distinguish the genotype of a juice. It is important to gain insight into the phytochemical profile as elderberry continues to become more popular in the dietary supplement and commercial industry. This type of analysis could be crucial to determine elderberry based dietary supplements that have been adulterated with other fruits or fillers. It could also help demonstrate consistency between lots and batches of dietary supplements with a proposed amount of specific anthocyanins. The three genotypes studied herein are commercially available American elderberry cultivars. Adams II, Bob Gordon, and Wyldewood were derived from indigenous germplasm originating in New York, Missouri, and Oklahoma, respectively.[27] The disparate geographical origins of these cultivars may have generated subtle but unique genetic factors that influence metabolite composition, unique enough to be distinguished by discriminate analysis. Cultivars and genotypes with more similar origins may or may not be distinguishable.

American elderberry juice is a rich source of anthocyanins and polyphenols. Different genotypes within this subspecies appear to demonstrate a similar phytochemical profile. The average anthocyanin and polyphenol content of the elderberry juice varied highly between growing location and year. For the first time, elderberry juice from

different genotypes was successfully grouped by a discriminant analysis based on the anthocyanin and polyphenol profile of individual juice aliquots. This suggests that although the magnitude of anthocyanins and polyphenols may change from year-to-year and between locations, the genotype of juice can properly identified by the anthocyanin and polyphenol content of individual juice samples.

3.5 References

(1) Charlebois, D., Byers, P.L., Finn, C.E., and Thomas, A.L. (2010) Elderberry: Botany, horticulture, potential. *Hort. Rev.* 37, 213−280. Wiley-Blackwell.

(2) Barak, V., Halperin, T., and Kalickman, I. (2001) The effect of Sambucol, a black elderberry-based, natural product, on the production of human cytokines: I. Inflammatory cytokines. *Eur. Cytokine Netw.* 12, 290−296.

(3) Veberic, R., Jakopic, J., Stampar, F., and Schmitzer, V. (2009) European elderberry (*Sambucus nigra* L.) rich in sugars, organic acids, anthocyanins, and selected polyphenols. *Food Chem*. 114, 511−515.

(4) Zakay-Rones, Z., Thom, E., Wollan, T., and Wadstein, J. (2004) Randomized study of the efficacy and safety of oral elderberry extract in the treatment of influenza A and B virus infections. *J. Int. Med. Res.* 32, 132-140.

(5) Lee, J., and Finn, C.E. (2007) Anthocyanins and other polyphenolics in american elderberry (*Sambucus canadensis*) and european elderberry (*S. Nigra*) cultivars. *J. Sci. Food Agric*. 87, 2665−2675.

(6) Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993) Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. U.S.A*. 90, 7915−7922.

(7) Sidor, A., and Gramza-Michałowska, A. (2015) Advanced research on the antioxidant and health benefit of elderberry (*Sambucus nigra*) in food – a review. *J. Funct. Foods* 18, 941−958.

(8) Novotny, J. A. (2012). Anthocyanin bioavailability: past progress and current challenges. In B. S. Patil, G. K. Jayaprakasha, K. N. Chidambara Murthy & N. P. Seeram (Eds.), Emerging trends in dietary components for preventing and combating disease, Vol. 1093: In vitro and in vivo evidences of dietary components for health benefits (1st ed) (pp.559-568). Oxford, UK: Oxford University Press, Inc.

(9) Kamonpatana, K., Failla, M. L., Kumar, P. S., and Giustu, M. M. (2014) Anthocyanin structure determines susceptibility to microbial degradation and bioavailability to the buccal mucosa. *J. Agric. Food Chem.* 62, 6903−6910.

(10) Patras, A., Brunton, N. P, O'Donnell, C., and Tiwari, B. K. (2010) Effect of thermal processing on anthocyanin stability in foods; mechanisms and kinetics of degradation. *Trends Food Sci. Tech*. 21, 3−11.

(11) Sadilova, E., Carle, R., and Stintzing, F. C. (2007) Thermal degradation of anthocyanins and its impact on color and in vitro antioxidant capacity. *Mol. Nutr. Food Res*. 51, 1461−1471.

(12) Sui, X., Bary, S., and Zhou, W. (2016) Changes in color, chemical stability and antioxidant capacity of thermally treated anthocyanin aqueous solution over storage. *Food Chem.* 192, 516−524.

(13) Johnson, M. C., Thomas, A. L., and Greenlief, C. M. (2015) Impact of frozen storage on the anthocyanin and polyphenol contents of american elderberry fruit juice. *J. Agric. Food Chem*. 63, 5653−5659.

(14) Turker, N., Aksay, S., and Ekiz, H. I., (2004) Effects of storage temperature on the stability of anthocyanins of a fermented black carrot (Daucus carota var. L.) beverage: shalgam. *J. Agric. Food Chem*. 52, 3807−3813.

(15) Mudge, E., Applequist, W. L., Finley, J., Lister, P., Townesmith, A. K., Walker, K. M., and Brown P. N. (2016) Validation of select flavonols and chlorogenic acid content of elderberry collected throughout the eastern United States. *J. Food Compost. Anal*. 47, 52−59.

(16) Thomas, A.L., Byers, P.L., Gu, S., Avery, Jr., J.D., Kaps, M., Datta, A., Fernando, L., Grossi, P., and Rottinghaus, G.E. (2015) Occurrence of polyphenols, organic acids, and sugars among diverse elderberry genotypes grown in three Missouri (USA) locations. *Acta Hort.* 1061, 47−154.

(17) Duymuş, H. G., Göger, F., and Hüsner Can Başer, K. (2014) *In vitro* antioxidant properties and anthocyanin compositions of elderberry extracts. *Food Chem.* 155, 112−119.

(18) Vlachojannis, C., Zimmermann, B. F., and Chrubasik-Hausmann, S. (2015) Quantification of anthocyanins in elderberry and chokeberry dietary supplements. *Phytother. Res.* 29, 561−565.

(19) Thomas, A.L., Byers, P.L., Finn, C.E., Chen, Y.C., Rottinghaus, G.E., Malone, A.M., and Applequist, W. L. (2008) Occurrence of rutin and chlorogenic acid in elderberry leaf, flower, and stem in response to genotype, environment, and season. *Acta Hort.* 765, 197−206.

(20) Thomas, A.L., Perkins-Veazie, P., Byers, P.L., Finn, C.E., Lee, J. (2013) A comparison of fruit characteristics among diverse elderberry genotypes grown in Missouri and Oregon. *J. Berry Res.* 3, 159−168.

(21) Finn, C.E., Thomas, A.L., Byers, P.L., and Serçe, S. (2008) Evaluation of American (*Sambucus canadensis*) and European (*S. nigra*) elderberry genotypes grown in diverse environments and implications for cultivar development. *HortScience* 43, 1385−1391.

(22) Yuk, J., Patel, D.N., Isaac, G., Smith, K., Wrona, M., Olivos, H.J., and Yu, K. (2016) Chemical profiling of ginseng species and ginseng herbal products using UPLC/QTOF-MS. J. Braz. Chem. Soc. 27, 1476−1483.

(23) D'Archivio, A.A., Giannitto, A., Maggi, M.A., and Ruggieri, F. (2016) Geographical classification of Italian saffron (Crocus sativus L.) based on chemical constituents determined by high-performance liquid-chromatography and by using linear-discriminant analysis. Food Chem. 212, 110−116.

(24) Byers, P.L., Thomas, A.L., Cernusca, M.M., Godsey, L.D., and Gold, M.A. (2014) Growing and marketing elderberries in Missouri. Agroforestry in Action Publication # AF1016−2014. University of Missouri Center for Agroforestry.

(25) He, J., and Giusti, M. M. (2011) High-purity isolation of anthocyanins mixtures from fruits and vegetables – A novel solid-phase extraction method using mixed mode cation-exchange chromatography. *J. Chromatogr. A* 1218, 7914−7922.

(26) Wu, H., Johnson, M. C., Lu, P., Fritsche, K. L., Thomas, A. L., Cai, Z., and Greenlief, C. M. (2015) Determination of anthocyanins and total polyphenols in a variety of elderberry juices by UPLC/MS and other methods. *Acta Hort.* 1061, 43−51.

(27) Finn, C.E., Thomas, A.L., Byers, P.L., and Serçe, S. (2008) Evaluation of American (Sambucus canadensis) and European (S. nigra) elderberry genotypes grown in diverse environments and implications for cultivar development. HortScience 43, 1385−1391.

Chapter 4: Method Development and Validation For Quantification of *N*-α-(1-deoxy-D-fructos-1-yl)-L-arginine (FruArg)

4.1 Introduction

4.1.1 Background of Aged Garlic Extract (AGE)

The medicinal properties of garlic (*Allium sativum L.*) have long been recognized and, more recently, subjected to a large number of studies in detail. For example, garlic was shown to protect the cardiovascular system by lowering total cholesterol in serum, by reducing supine systolic blood pressure, and by modulating platelet aggregation through upregulation of cyclic adenosine monophosphate (cAMP) in blood [1-3]. Dietary garlic may have cancer preventive effects, as well.[4,5] Thus, epidemiological studies in both Asian and Western populations established that gastric and colon cancer risks are inversely associated with garlic consumption.[6,7] One of the popular garlic nutritional supplements is aged garlic extract (AGE), an odorless product prepared by a prolonged (10-12 months) soaking of fresh garlic in 15-20% aqueous ethanol at room temperature. Health-promoting properties of AGE have been documented and the list keeps expanding. For instance, AGE can act as a superoxide radical scavenger[8-10] and it was shown to offer a potent antioxidant protection to cells by stimulating the activity of cellular antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase, and by increasing intracellular glutathione.[11,12] In humans, AGE, when delivered as a dietary supplement, reduced total serum cholesterol, low-density lipoprotein, and systolic pressure in hypercholesterolemic patients.[13] Other effects of AGE, such as inhibition of platelet aggregation and pro-circulatory effects, have been demonstrated both in animal and human subjects.[14,15]

4.1.2 FruArg

While the antioxidant and anti-inflammatory effects of whole AGE and its sulfur-containing constituents are reasonably well understood, the non-sulfur nutraceuticals from AGE, such as fructose-amino acids (FAs), have not been investigated as thoroughly. The most abundant FA found in aged garlic extract is *N*-α-(1-deoxy-D-fructos-1-yl)-L-arginine (FruArg), belongs to the family of "amino sugars" resulting from a non-enzymatic condensation reaction between free glucose and amino groups of free amino acids and proteins in processed dairy products, dehydrated fruits, vegetables and medicinal preparations such as extracts from Korean red ginseng or garlic.[16,17] It was estimated that dietary consumption of FAs averages about 1 g per day. In addition, fructose-amino acids form *in vivo*, largely as a result of the interaction between free glucose and *N*-terminal or lysine amino groups in proteins. These modified (glycated) proteins, hemoglobin A_{1c}, for example, have been used as a diagnostic marker for circulating glucose levels in metabolic diseases such as diabetes, for over three decades.[17,22,23] In spite of an overwhelming evidence for constant exposure to FAs, physiological roles or significance of these compounds in humans are largely unknown.

FAs are known to possess antioxidant properties, either by acting as reducing agents (e.g., electrophiles) or as chelators of redox-active metals such as copper and iron.[18-21,27,28] Reports have also demonstrated the effects of FAs as immune stimulants and the ability of FAs to prevent tumorigenesis and metastasis, due to their ability to inhibit cancer cell proliferation and adhesion.[17,29,30]

FruArg is a typical Amadori rearrangement product arising from the condensation reaction between free glucose and arginine during early stages of the Maillard reaction,

which is responsible for the specific color and, to a large extent, the antioxidant properties of AGE.[25,26] FruArg has been shown to exhibit antioxidant activity and hydrogen peroxide scavenging capacity that is comparable to the potent hydrogen peroxide scavenging compound ascorbic acid.[31,32] In our previous study, both AGE and FruArg exhibited a capability to modulate neuroinflammatory regulation by suppressing nitric oxide (NO) production, in a concentration-dependent manner, in LPS-induced mouse BV-2 microglial cells without affecting the cell viability.[33] This result has thus created a pretext for further studies aiming to evaluate the potential of AGE and FruArg in targeting neuroinflammation *in vivo*.

4.1.3 The Blood-Brain Barrier (BBB)

A major issue in the development of drug therapy for neurological diseases is the blood-brain barrier (BBB) since more than 98% of all small molecule drugs failed to pass through the BBB to enter the brain.[36] There are certain properties a molecule must possess in order to be able to cross the BBB. Typically, these include: a molecular weight under 500 Da and having less than 10 atoms that form hydrogen bonds.[37] As the surface area of the molecule increases, there is also a decreased likelihood that it will be able to penetrate the tight junctions formed between endothelial cells in the BBB. Several amino acids and sugars have the ability to cross the BBB.[38,39] FruArg molecular weight is 336 and it has 11 heteroatoms theoretically capable of participating in hydrogen bonding. However, available crystallographic data consistently demonstrated a propensity of 3-4 heteroatoms in fructose-amino acids to form strong intramolecular hydrogen bonds, while the furanose oxygen has not been involved in any.[40] We therefore have hypothesized that

FruArg's low molecular weight and structural characteristics may allow it to cross the BBB and enter the brain.

4.1.4 Research Aims

To enable pharmacokinetic and pharmacodynamics studies of FruArg *in vivo*, a reliable method for its quantification at very low concentrations is needed. Ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) is a very powerful tool used for reliable and sensitive quantification in metabolomics. Multiple reaction monitoring (MRM) allows for the rapid analysis of a selected parent and daughter ion pair of a specific analyte, which increases sensitivity and reduces false positives. Herein, we report on a successful application of the UPLC-ESI-MS/MS method to quantify FruArg in mouse plasma and brain tissue using a modified approach combining an ion-pairing reagent and a solid phase column for the enrichment of FruArg. This method was evaluated for limit of quantification, linearity and range, recovery, selectivity, matrix effect, intra-day precision, and inter-day precision.

4.2 Experimental

4.2.1 Materials

4.2.1.1 Chemicals

Water (Chromasolv®), Acetonitrile (Chromasolv®), L-Lysine-3,3,4,4,5,5,6,6-d_8 hydrochloride (98 atom %D) and perfluoropentanoic acid (97%) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

4.2.1.2 Synthesis of FruArg

N-α-(1-deoxy-D-fructos-1-yl)-L-arginine (FruArg) was synthesized via a reflux reaction between L-arginine and D-glucose (Figure 4.1).[41] Identification and purity of FruArg were measured by ^{1}H and ^{13}C NMR and electrospray ionization mass spectrometry. A batch of purified FruArg, in form of hydroacetate salt, was sterile-filtered and kept frozen at the stock concentration of 200 mM in double-distilled water. The stock solution at 4ºC is stable for at least 6 months.

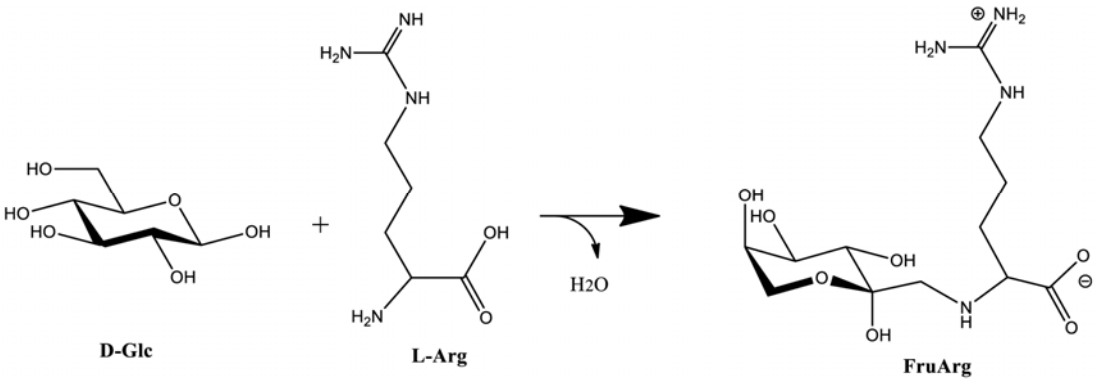


Figure 4.1 Synthesis of FruArg.

4.2.2 IntelliStart® Optimization

Waters IntelliStart® software was used to identify transitions used for multiple reaction monitoring scans. A 10 μM FruArg standard solution was directly infused in the mass spectrometer at 20 μL/min. Intellistart also finds the optimal collision energy and cone voltage that produces the highest number of ions. Two fragment ions of FruArg were identified; the more abundant used for quantification and the less abundant ion as a qualifier

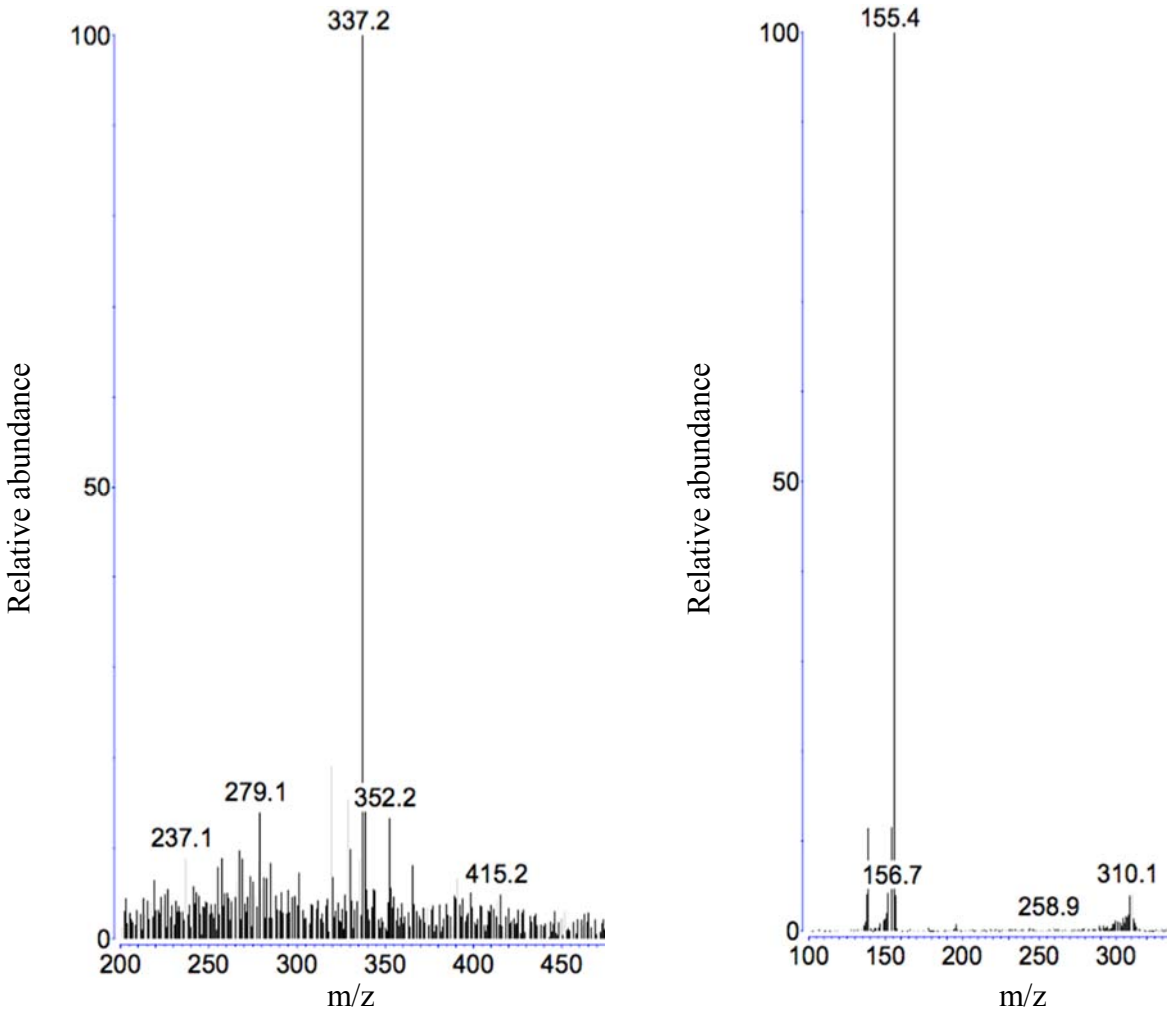


Figure 4.2 Direct infusion mass spectrum of FruArg (left) and L-Lysine-d_8 (right) using ESI positive mode.

4.2.3 L-Lysine d-3,3,4,4,5,5,6,6 Internal Standard

L-Lysine-d_8 was used as an internal standard throughout the experiment. Identification of L-Lysine-d_8 was verified using electrospray ionization mass spectrometry (Figure 4.2). A stock solution of 2,000 ng/mL was made and 25 µL of the stock was spiked with 25 µL of plasma or 50 µL of brain tissue initially before any sample preparation. Intellistart was used to identify transitions for MRM scans of L-

Lysine-d_8 in the same manner as listed above. All quantitative measurements of FruArg were normalized to the peak areas of L-Lysine-d_8 during the same LC-MS/MS run.

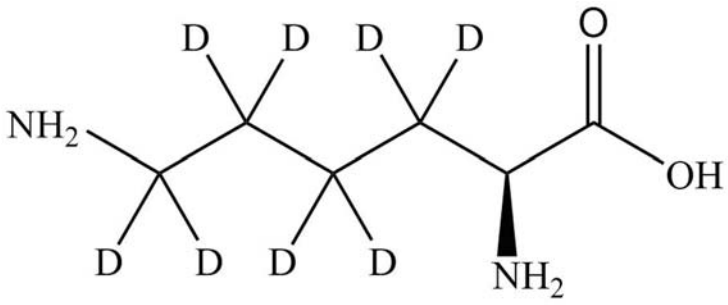


Figure 4.3 Structure of L-Lysine d-3,3,4,4,5,5,6,6 (L-Lysine d_8).

4.2.4 UPLC Gradient Optimization

4.2.4.1 Aqueous and Acetonitrile Gradient

An attempt to separate FruArg was made using a typical gradient with a high aqueous percentage initially, gradually ramped up to a high organic composition. The high hydrophilic character of FruArg was observed when it eluted from the traditional octadecyl (C18) column at approximately 0.32 min (Figure 4.4). There are two problems associated with this. First, several endogenous compounds still included after sample preparation elute during this time frame and could potentially cause a signal suppression. Second, the poor peak shape associated with FruArg MRM chromatogram led to an inconsistent peak area between injections and poor sensitivity. Therefore, an alternated methodology was needed to increase the sensitivity of the method, along with the reproducibility. Incorporating an ion-pairing agent into the mobile phases helped alleviate both of these problems. The interaction between the FruArg ion and ion-pairing agent

increased the retention time to 5.9 min, increased the signal approximately 5-fold, and decreased the full width at half maximum of the peak.

Figure 4.4 MRM chromatogram of FruArg using a water and acetonitirile gradient.

4.2.4.2 Ion Pairing Agent

A Waters Acquity ultra performance liquid chromatograph (UPLC) with a quaternary solvent manager was used with a C18 column (Acquity BEH, 1.7 µm, 50 mm × 2.1 mm, Waters, Milford, MA, USA). A previously described 15 min UPLC method was used for FruArg separation.[42] The mobile phases used were 5 mM perfluoropentanoic acid solution in water (A) and 5 mM perfluoropentanoic acid solution in acetonitrile (B). The gradient elution was 98% A at 0 min, 98% A at 2 min, 50% A at 5 min, 50% A at 7 min, 98% A at 9 min, 98% A at 15 min. The flow rate was 0.2 mL/min, and the injection volume was 10 µL in full loop mode. The column was heated to 40 °C and the sample chamber was cooled to 10 °C.

4.2.5 Mass Spectrometry Conditions

A Waters Xevo TQ-S triple-quadrupole mass spectrometer with electrospray ionization (ESI) in positive ionization mode was used. Multiple reaction monitoring (MRM) scans were conducted by selecting the parent and daughter ion m/z transitions of FruArg and L-Lysine d_8 which were optimized using IntelliStart® software. The area under the chromatographic peak (AUC) of ion m/z transitions of 337.1 → 70.1 and 155.1 → 92.1 were monitored for FruArg and L-Lysine-d_8, respectively, and the ratio of AUCs was used for quantification. A desolvation temperature of 350 °C and a source temperature of 150 °C were used. A capillary voltage of 2.0 kV, cone voltage of 20 V, and collsion energy of 24 eV were used, and the nebulizer gas rate was 500 L N_2/h. MassLynx software (version 4.1, Waters) was used for all data acquisition.

4.2.6 Phree Sample Preparation

Solid phase extraction (SPE) was facilitated using Phree™ phospholipid removal solution 1-mL columns (Phenomenex®)(Figure 4.5). The columns were used to separate FruArg from mice plasma and brain tissue samples. The samples, 25 µL of plasma and 50 µL of brain tissue, were spiked with 25 µL of L-Lysine-d_8 used as an internal standard (I.S.) and added directly on to the Phree™ column. 500 µL of methanol (1% formic acid) was added to the column and samples were vortexed at maximum velocity for 2 min. Vacuum pressure (5-7 mmHg) was applied and the eluent was collected and dried under a steady stream of N_2 gas and reconstituted in 500 µL of mobile phase A.

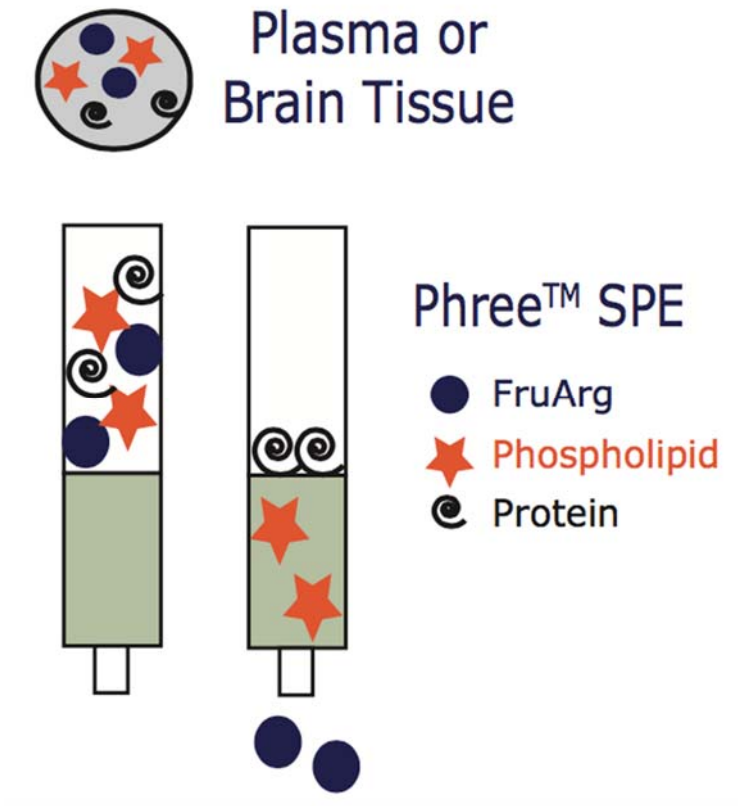


Figure 4.5 Isolation of FruArg from plasma and brain tissue using Phree.

4.2.7 Method Validation

An analytical method validation was performed including analysis of selectivity, sensitivity, linearity, range, recovery, matrix effect, precision and accuracy.

4.2.7.1 Selectivity

Aliquots of blank plasma and brain tissue from six different mice were prepared to test the selectivity of the method. 25 µL of blank plasma and 50 µL of blank brain tissue were prepared as mentioned in section 4.2.6 and analyzed to ensure that any potentially interfering endogenous compounds did not affect the quanitification of FruArg. The signal observed for all samples was below the limit of quantitation (LOQ).

4.2.7.2 Sensitivity

The LOQ was determined by performing serial dilutions on FruArg stock until the signal-to-noise ratio of MRM chromatogram was approximately 10:1. Subsequent injections (n=7) at this predicted LOQ concentration were performed and the CV% was determined and needed to be < 20%.

4.2.7.3 Linearity and Range

FruArg standards were prepared at 400, 200, 100, 50, 25, 10, 5, and 1μM and analyzed for AUC using the UPLC-MS/MS method discussed above in order to analyze the linearity and range of the analytical method.

4.2.7.4 Recovery

The recovery of the analytical method was analyzed for both biological matrices. 25 μL of blank mice plasma (n=4) was spiked with 25 μL of FruArg 100 μM and 25 μL L-Lysine-d_8 (2 μg/mL) before SPE. 25 μL of blank plasma (n=4) was spiked with equal amounts of FruArg and L-Lysine-d_8 after SPE. Recovery was assessed by comparing the ratio of peak areas of FruArg/ L-Lysine-d_8 of the samples spiked before SPE to the ratio of peak areas of FruArg/ L-Lysine-d_8 spiked after SPE (equation 4.1). The recovery of FruArg in the tissue samples was measured in the same way by spiking 50 μL of blank tissue with 25 μL of FruArg and 25 μL L-Lysine-d_8 before and after SPE.

$$\text{Recovery (\%)} = \frac{\text{Average Spike Before } \frac{\text{AUC FruArg}}{\text{AUC L-Lysine d8}}}{\text{Average Spike After } \frac{\text{AUC FruArg}}{\text{AUC L-Lysine d8}}} \text{ x } 100\% \qquad (4.1)$$

4.2.7.5 Matrix Effect

The potential matrix effect of the plasma and brain tissue were analyzed by comparing the signal of ratio of peak areas of FruArg/ L-Lysine-d_8 spiked after SPE to the ratio of peak areas of FruArg/ L-Lysine-d_8 of neat standards prepared at the same concentration, but diluted from the initial FruArg stock solution in mobile phase A (equation 4.2). Average values used for recovery and matrix effect calculations are listed in Table S2.

$$\text{Matrix Effect (\%)} = 100 - \left(\frac{\text{Average Spike After } \frac{\text{AUC FruArg}}{\text{AUC L-Lysine d8}}}{\text{Average External Standard } \frac{\text{AUC FruArg}}{\text{AUC L-Lysine d8}}}\right) \text{ x } 100\% \qquad (4.2)$$

4.2.7.6 Precision and Accuracy

The precision and accuracy of this method was analyzed by performing analysis on three different batches of quality control (QC) samples over the course of three different days. The precision was assessed by the coefficient of variation (%) between samples (n=3, intra-day; n=9, inter-day) using one-way ANOVA and the threshold of acceptance was <15%, except at the LLOQ where <20% is acceptable. The accuracy was assessed by calculating the mean concentration obtained for each QC level (n=3, intra-day; n=9, inter-day) and the threshold of acceptance was within 15% of the calculated concentration, except at the LLOQ where within 20% of the calculated value was acceptable. The initial FruArg stock (20 mM) was prepared in water. Serial dilutions of the initial stock were performed to obtain 200, 150, 100, 50, 25, 10, and 5 µM FruArg working stock solutions for the calibration curve. 25 µL of blank plasma was spiked with 25 µL of each concentration of working stock solution and 25 µL L-Lysine-d_8 (5 µg/mL)

and prepared the same way as mentioned above. A calibration curve was generated using IntelliStart® software utilizing a $1/x^2$ weighting factor.

4.2.7.7 Calibration Curve

The initial FruArg stock (20 mM) was prepared in water. Serial dilutions of the initial stock were performed to obtain 200, 150, 100, 50, 25, 10, and 5 μM FruArg working stock solutions for the calibration curve. 25 μL of blank plasma was spiked with 25 μL of each concentration of working stock solution and 25 μL L-Lysine-d_8 (5 μg/mL) and prepared the same way as mentioned above. A calibration curve was generated using IntelliStart® software utilizing a $1/x^2$ weighting factor.

4.2.8 Mice Study

Adult male C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, ME) at age 8 weeks were housed 4/cage and maintained on a 12-hour light/dark cycle (lights on at 7:00 AM) with unrestricted access to food and water. Approved animal protocols were obtained in accordance with University of Missouri and the National Institutes of Health guidelines for the Care and Use of Laboratory Animals. Mice were injected intraperitoneally with 2.5 μL/g body weight FruArg (40 mM). Terminal blood samples were collected at various time points (15 min, 30 min, 1 hr, and 3 hr) by cardiac puncture following CO_2 asphyxiation, using heparin as an anticoagulant. The blood was centrifuged to obtain plasma and frozen at -80 °C until use. Whole brain samples were harvested and dissected into different regions (cortex, striatum, hippocampus, and cerebellum) after transcardiac perfusion with saline and immediately flash frozen in liquid nitrogen and stored at -80 °C until analysis. Brain samples were weighed and homogenized for 5 minutes in 3 volume equivalent of Milli-Q water using a bullet

blender (Next Advance, Inc., Averill Park, NY). The homogenates were centrifuged at 12,000 rpm for 20 minutes at 4°C and the supernatants were collected and analyzed.

4.3 Results and Discussion

4.3.1 Intellistart Optimization

MRM transitions were identified at 337.1 → 70.1 and 337.1→ 114.1 as the quantifier and qualifier for FruArg, respectively (Figure 4.6). An optimized collision energy of 24 eV and an optimized cone voltage of 20 V were found. An MRM transition was identified at 155.1 → 92.1 and used for quantification of L-Lysine d_8 (Figure 4.7). An optimized collision energy of 13 eV and an optimized cone voltage of 24 V were found, as well.

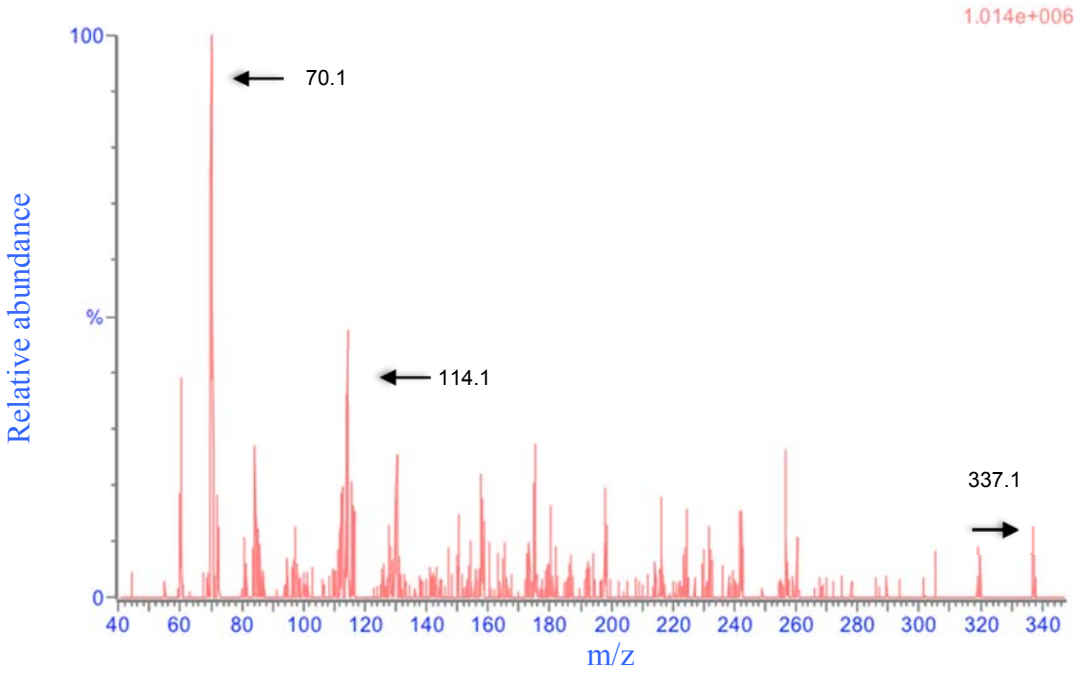


Figure 4.6 Product ion mass spectrum (MS/MS) for FruArg. Parent m/z of FruArg is 337.3 Da andMRM transitions of FruArg were identified at m/z = 337.1 → 70.1 and 114.1 Da. The most abundant daughter ions were used for quantification.

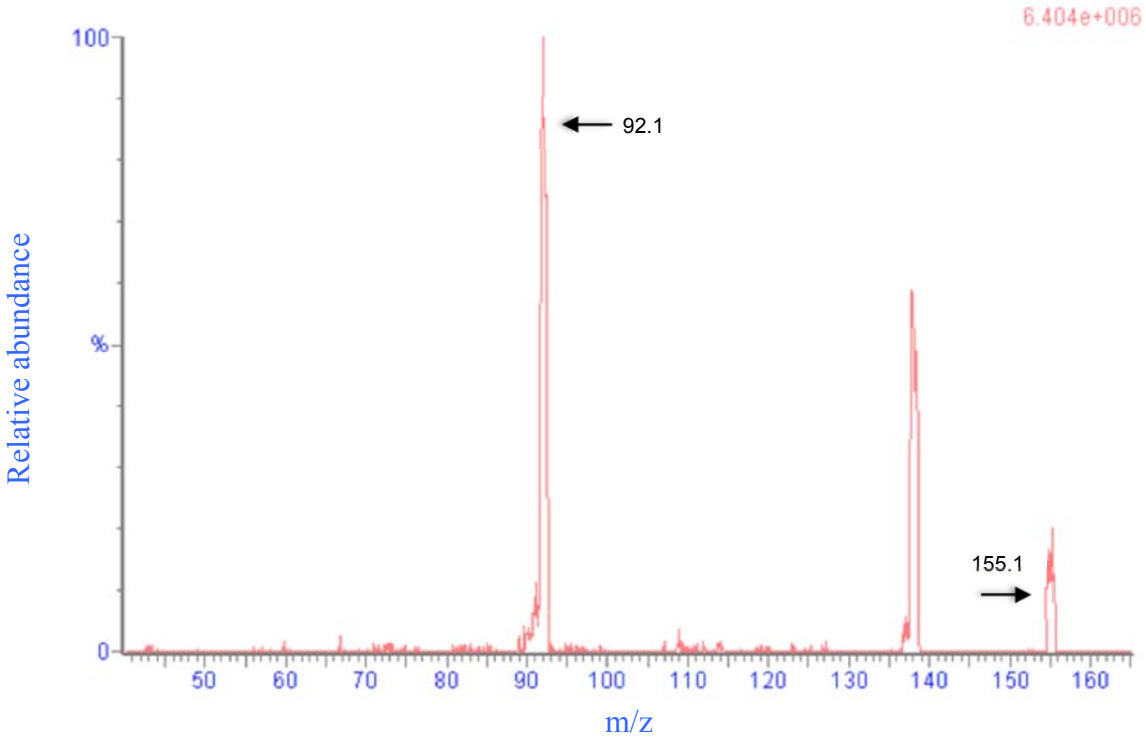


Figure 4.7 Product ion mass spectrum (MS/MS) for L-Lysine-d_8. Parent m/z of L-Lysine-d_8 is 155.1 Da and MRM transitions of m/z = 155.1 → 92.1 Da were identified for L-Lysine-d_8. The most abundant daughter ions were used for quantification.

4.3.2 UPLC Gradient Performance

Successful separation was achieved with a UPLC gradient using perfluoropentanoic acid as an ion-pairing agent. FruArg eluted from the column at 5.9 min and L-Lysine-d_8 eluted at 6.1 min (Figure 4.9). Excellent peak shape and reproducible ion chromatographic peak areas and retention times were observed throughout the study. The performance of the analytical method was verified by performing a method validation. This included tests for limit of quantitation, selectivity, linearity of the signal, range, recovery, matrix effect, and inter-day and intra-day precision. Values for these parameters are listed in Table 4.1.

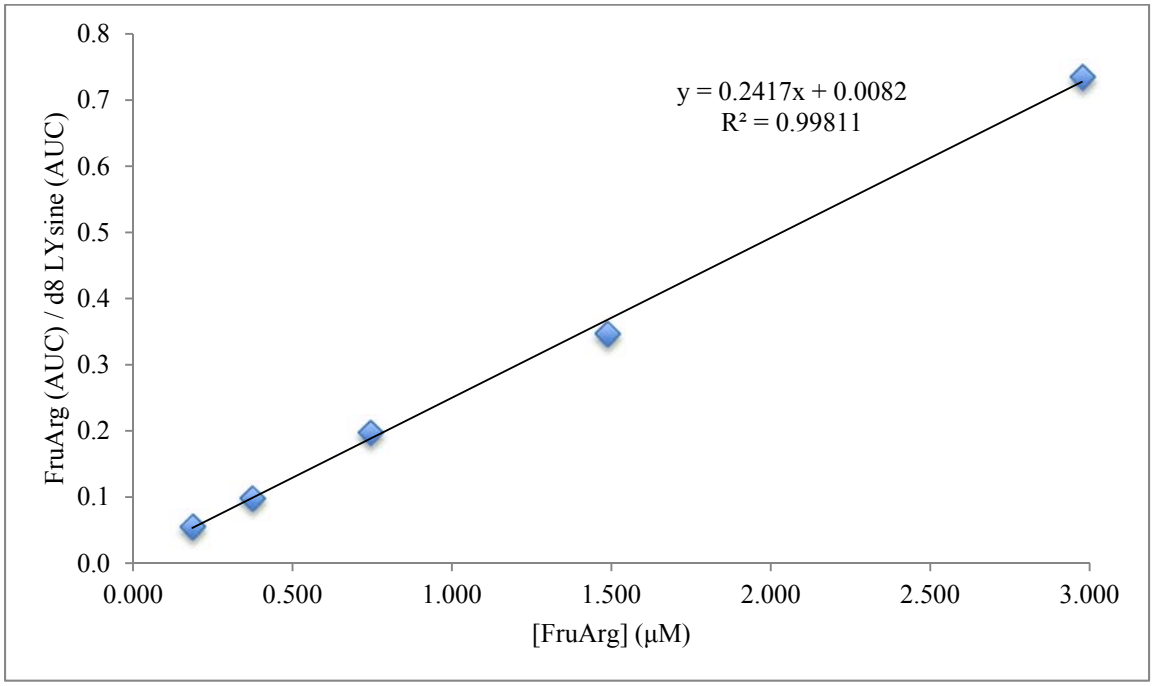


Figure 4.8 Example standard curve used for FruArg quantification.

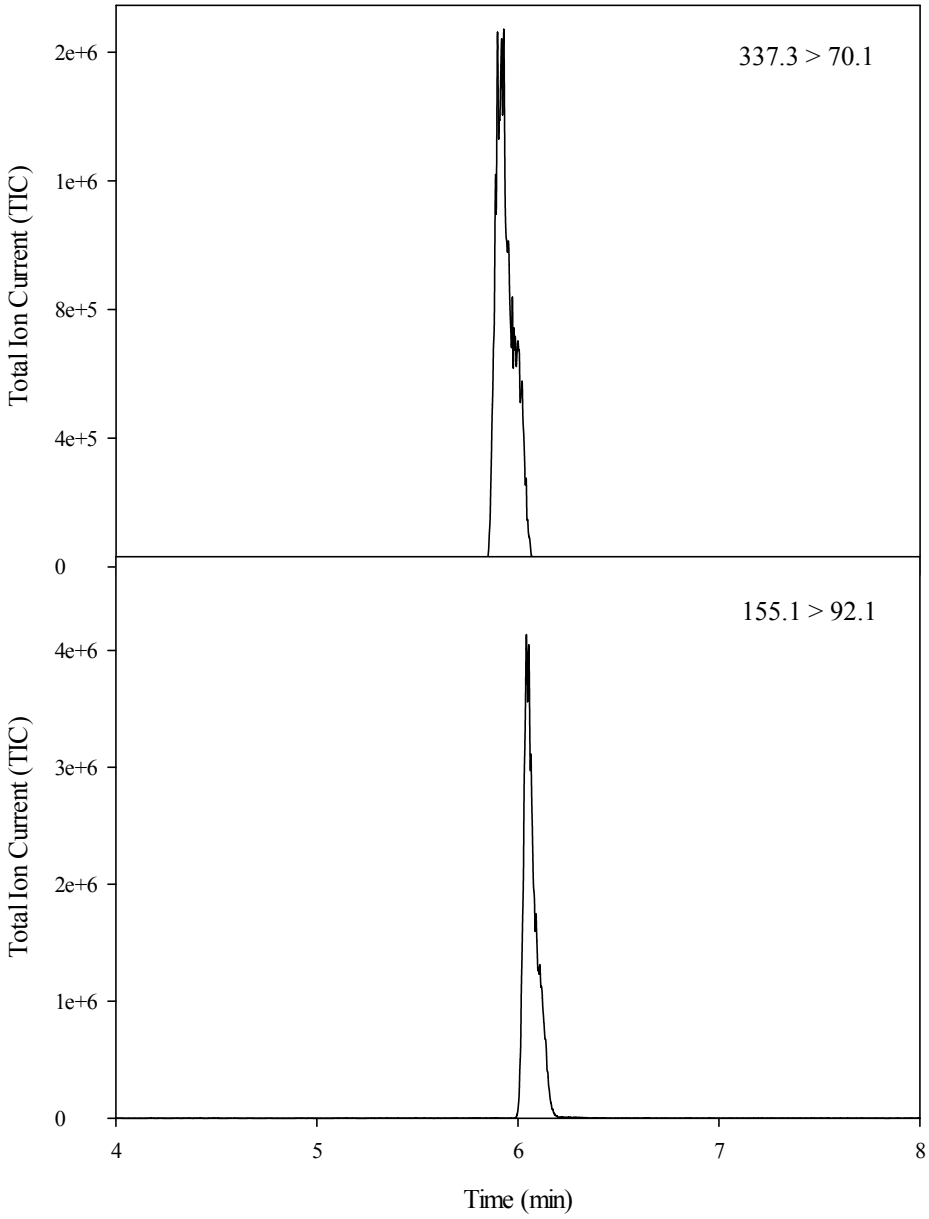


Figure 4.9 MRM chromatograms for FruArg and L-Lysine-d8. FruArg (top) and L-Lysine-d8 (bottom) were eluted from the column at 5.9 and 6.1 min, respectively. Their parent daughter ion pairs were monitored as 337.3 → 70.1 Da and 155.1→ 92.1 Da, respectively. The peak areas of the MRM chromatograms were integrated and used for quantification. All FruArg concentration calculations were relative to L-Lysine-d8 internal standard.

4.3.3 Method Validation

The performance of the analytical method was verified by performing a method validation. This included tests for limit of quantitation (LOQ), linearity of the signal, range, recovery, matrix effect, inter-day and intra-day precision. Values for these parameters are listed in Table 4.1. The dynamic working range was identified for each matrix and excellent linearity was also observed for both (Figure 4.8). Recovery was much better in the plasma (99.6%) than the brain tissue (63.3%) although both matrices exhibited consistent recovery (Table 4.2). The matrix effect was negligible in the plasma (0.29%) and very minimal in the brain tissue (4.3%). Reproducibility and accuracy were well within the threshold of 15% CV for each matrix within batches, as well as between days.

Validation Parameters		
	Plasma	Brain Tissue
Limit of Quantitation	1 μM	0.4 μM
Linearity	$r^2 > 0.998$	$r^2 > 0.997$
Range	1 μM − 400 μM	0.4 μM − 200 μM
Recovery	99.6%	63.3%
Matrix Effect	0.29%	4.3%
Inter-day precision	CV= 6.4%	CV = 2.4%
Intra-day precision	CV= 4.9%	CV = 2.6%

CV: coefficient of variance

Table 4.1 Method validation parameters for plasma and brain tissue.

	Plasma $\frac{\text{AUC FruArg}}{\text{AUC L−Lysine d8}} \pm$ SE	**Brain Tissue** $\frac{\text{AUC FruArg}}{\text{AUC L−Lysine d8}} \pm$ SE
FruArg spike before Phree	2.43 ± 0.01	2.89 ± 0.04
FruArg spike after Phree	2.44 ± 0.02	4.6 ± 0.2
FruArg external standard	2.447 ± 0.001	4.41 ± 0.02

Table 4.2 Average values (n=4) for AUC FruArg/L-Lysine d_8 spiked before Phree, after Phree and external standards.

4.3.4 Plasma Analysis

FruArg (40 mM) was administered intraperitoneally (2.5 μL/g BW) to three mice. The plasma was extracted at 15, 30, 60 and 180 min and analyzed for FruArg concentration. At 15 min, an average FruArg concentration of 111 ± 3 μM was observed (Figure 4.10). The FruArg concentration rapidly decreased to 33 ± 9 μM and 5 ± 2 μM at 30 min and 60 min, respectively. At 180 min the concentration of FruArg fell below the LOQ. This illustrates that FruArg is rapidly absorbed into the blood stream, and then distributed to other parts of the body.

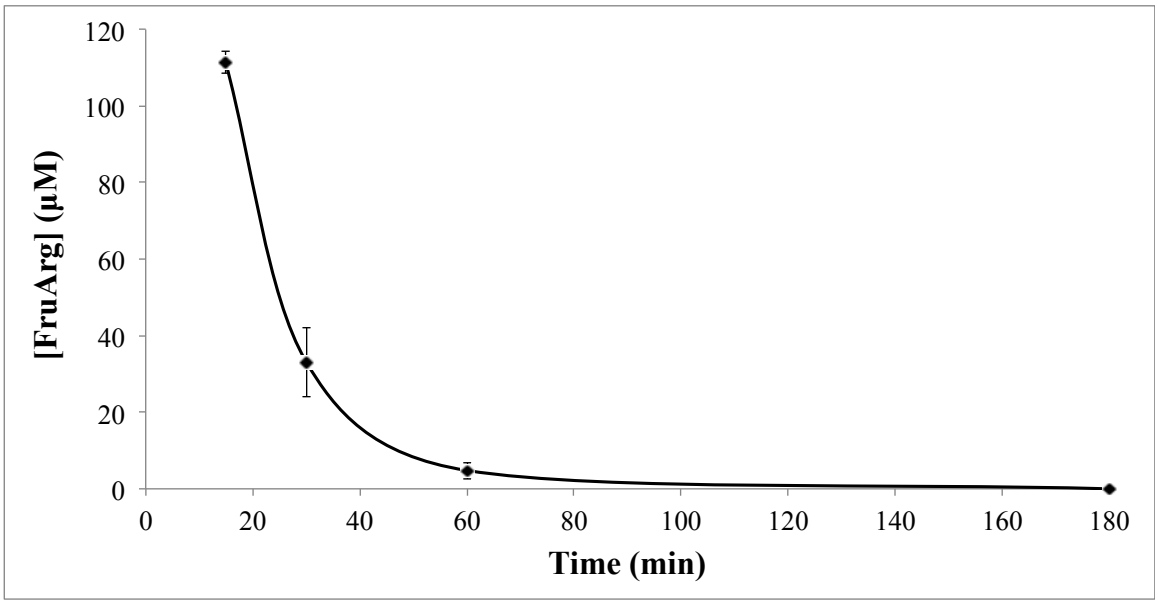


Figure 4.10 Plasma concentration time curve of FruArg. Concentration of FruArg in mice plasma as a function of time at 15, 30, 60 and 180 minutes. Individual data points are represented as the average (n=3) FruArg concentration (μM) ± SE. The curve shown is the results from the fit of the pharmacokinetic data from Table 4.3.

4.3.5 Pharmacokinetic Parameters (PK)

Pharmacokinetic (PK) parameters were calculated to describe how FruArg progresses in plasma and are listed in Table 4.3. The initial concentration (C_0) of FruArg 292 μM and was measured by extrapolating the PK curve to 0 min. The area under the curve (AUC) measures the systemic exposure of FruArg. The ($AUC_{0\rightarrow last}$) was 4.86×10^3 μM·min, which measured the AUC from the initial concentration to the last quantifiable time point. The ($AUC_{0\rightarrow\infty}$) was 4.93×10^3 μM·min and extrapolates from the initial concentration to infinity. The elimination rate (k) is the fraction of FruArg that is removed from the body per unit time and was 6.91×10^{-2} min^{-1}. The half-life ($t_{1/2}$) of FruArg was 10.03 min, clearance (Cl) 1.8×10^{-4} L·min, and volume distribution (VD) 2.63×10^{-3} L. The line shown in Figure 2 is the fit based on the PK parameters.

4.3.6 Brain Tissue Analysis

Brain tissue samples were collected from the cerebellum and cortex brain sub-regions at 15, 30, 60 and 180 min and at 15 min for the striatum and hippocampus from mice (n=3) injected intraperitoneally (2.5 μL/g BW) with FruArg (40 mM). A concentration of FruArg as a function of time in different types of brain tissue is shown in Figure 4.11. FruArg was detected in all four subregions of the brain investigated, with concentrations ranging from 4-7 pmol/mg tissue at 15 min, showing non-specific and time-dependent character of FruArg accumulation in brain (Table 4.4). This provides, for the first time, solid evidence in favor of the ability of FruArg and, possibly, other fructose-amino acids, to pass through the BBB from the circulation to enter the brain. Although the FruArg concentration detected in the brain is low compared to the original injection concentration, similar results have been obtained in previous BBB studies. Gooyit et al., detected SB-3CT, a selective inhibitor of matrix metalloproteinase-2 and-9, present in similar concentrations in the brain tissue (5.0 ± 0.8 pmol/mg at 10 min), after administration of comparable dosages to this study (25 mg/kg).[43] The dose detected in the brain may not represent the effective dose for FruArg and further analysis of the dose range could be explored in the future.

Pharmacokinetic Parameters	
Original concentration	292 μM
Elimination rate (k)	6.91×10^{-2} min^{-1}
Volume distribution (VD)	2.63×10^{-3} L
Half-life ($t_{1/2}$)	10.03 min
Clearance (Cl)	1.8×10^{-4} $L \cdot min^{-1}$
$AUC_{0 \rightarrow last}$	4.86×10^{3} μM·min
$AUC_{0 \rightarrow \infty}$	4.93×10^{3} μM·min

Table 4.3 Calculated pharmacokinetic parameters of FruArg in plasma.

	[FruArg] pmol/mg tissue ± SE
cerebellum	6 ± 2
cortex	4 ± 2
hippocampus	4 ± 2
striatum	7 ± 3

Table 4.4 Average [FruArg] (n=3) at 15 min after intraperitoneal injection in cerebellum, cortex, hippocampus and striatum subregions of the brain.

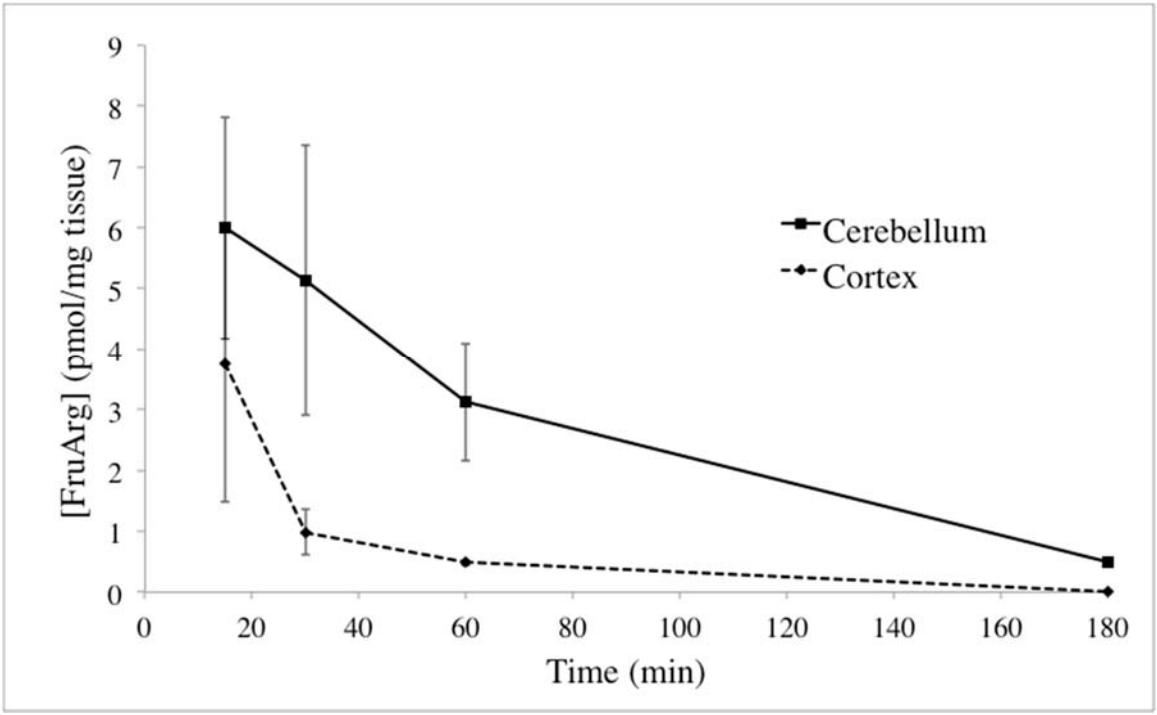


Figure 4.11 Brain tissue concentration time curve of FruArg in cerebellum and cortex. Concentration of FruArg in cerebellum (solid line) and cortex (dotted line) brain sub-regions as a function of time at 15, 30, 60 and 180 minutes. Individual data points are represented as the average (n=3) FruArg concentration (pmol/mg tissue) ± SE.

4.4 Conclusion

Current strategies for drug delivery to the brain to overcome the BBB include trans-cranial, trans-nasal, BBB disruption and lipidization of small molecules, among a few others. Most of these methods present major drawbacks, such as having an invasive trans-cranial surgery for drug delivery. The workflow of identifying molecules that can be distributed to the brain without any type of assistance could help promote an alternative form of treatment via long-term prevention. As treatments for neurological diseases advance, it is critical to consider the BBB's effect of drug delivery. FruArg has exhibited excellent anti-inflammatory and inhibited nitric oxide production in an *in vitro* model.[33] Here, we have demonstrated that FruArg is rapidly absorbed in blood, crossed over the BBB and reached all four sub-regions of the brain. This is somewhat unexpected, since a very limited number of highly hydrophilic molecules have such ability. Since passive penetration of BBB by FruArg is highly unlikely, this molecule may use one of the active carbohydrate transporters, such as GLUT5. Regardless of the mechanism, our data suggest further testing of FruArg in therapeutic models aiming at prevention or treatment neurological diseases.

4.5 References

(1) Ried, K., Toben, C., and Fakler, P. (2013) Effect of garlic on serum lipids: an updated meta analysis. *Nutr. Rev.* 71, 282−299.

(2) Stabler, S. N., Tejani, A. M., Huynh, F., and Fowkes, C. (2012) Garlic for the prevention of cardiovascular morbidity and mortality in hypertensive patients. *Cochrane Database Syst. Rev.* 8, CD007653.

(3) Rahman, K. (2007) Effects of garlic on platelet biochemistry and physiology. *Mol. Nutr. Food. Res*. 51, 1335−1344.

(4) Amagase, H., Schaffer, E. M., and Milner, J. A. (1996) Dietary components modify the ability of garlic to suppress 7, 12-dimethylbenz (a) anthracene-induced mammary DNA adducts. *J. Nutr.* 126, 817−824.

(5) Amagase, H., and Milner, J. A. (1993) Impact of various sources of garlic and their constituents on 7, 12-dimethylbenz [α] anthracene binding to mammary cell DNA. *Carcinogenesis* 14, 1627−1631.

(6) Woo, H.D., Park, S., Oh, K., Kim, H. J., Shin, H. R., Moon, H. K., and Kim, J. (2014) Diet and cancer risk in the Korean population: a meta- analysis. *Asian Pac. J. Cancer Prev.* 15, 8509−8519.

(7) Steinmetz, K.A., Kushi, L. H., Bostick, R. M., Folsom, A. R., and Potter, J. D. (1994) Vegetables, fruit, and colon cancer in the Iowa Women's Health Study. *Am. J. Epidemiol*. 139, 1−15.

(8) Moriguchi, T., Saito, H. and Nishiyama, N. (1997) Anti-ageing effect of aged garlic extract in the inbred brain atrophy mouse model. *Clin. Exp. Pharmacol. Physiol.* 24, 235−242.

(9) Kyo, E., Uda, N., Kasuga, S., and Itsakura, Y. (2001) Immunomodulatory effects of aged garlic extract. *J. Nutr.* 131, 1075S−1079S.

(10) Morihara, N., Hayama, M. and Fujii, H. (2011) Aged garlic extract scavenges superoxide radicals. *Plant Foods Hum. Nutr.* 66, 17−21.

(11) Imai, J., Ide, N., Nagae, S., Moriguchi, T., Matsuura, H., and Itakura, Y. (1994) Antioxidant and radical scavenging effects of aged garlic extract and its constituents. *Planta. Med.* 60, 417−420.

(12) Borek, C. (2001). Antioxidant health effects of aged garlic extract. *J. Nutr.* 131, 1010S−1015S.

(13) Steiner, M., Khan, A. H., Holbert, D., and Lin, R. I. (1996) A double-blind crossover study in moderately hypercholesterolemic men that compared the effect of aged

garlic extract and placebo administration on blood lipids. *Am. J. Clin. Nutr.* 64, 866−870.

(14) Rahman, K., and Billington, D. (2000) Dietary supplementation with aged garlic extract inhibits ADP-induced platelet aggregation in humans. *J. Nutr.* 130, 2662−2665.

(15) Steiner, M., and Li, W. (2001) Aged garlic extract, a modulator of cardiovascular risk factors: a dose-finding study on the effects of AGE on platelet functions. *J. Nutr.* 131, 980S−984S.

(16) Amagase, H. (2006) Clarifying the real bioactive constituents of garlic. *J. Nutr.* 136, 716S−725S.

(17) Mossine, V. V., and Mawhinney, T. P. (2010) 1-Amino-1-deoxy-D-fructose ("fructosamine") and its derivatives. *Adv. Carbohydr. Chem. Biochem.* 64, 291−402.

(18) Dittrich, R., Dragonas, C., Kannenkeril, D., Hoffmann, I., Mueller, A., Beckmann, M. W., and Pischetsrieder, M. (2009) A diet rich in Maillard reaction products protects LDL against copper induced oxidation ex vivo, a human intervention trial, *Food Res. Int.* 42, 1315−1322.

(19) Gyurcsik, B., and Nagy, L. (2000) Carbohydrates as ligands: coordination equilibria and structure of the metal complexes, *Coordin. Chem. Rev.* 203, 81−149.

(20) Ide, N., Lau, B. H., Ryu, K., Matsuura, H., and Itakura, Y. (1999) Antioxidant effects of fructosyl arginine, a Maillard reaction product in aged garlic extract, *J. Nutr. Biochem.* 10, 372−376.

(21) Vhangani, L. N., and Van Wyk, J. (2013) Antioxidant activity of Maillard reaction products (MRPs) derived from fructose-lysine and ribose-lysine model systems, *Food Chem.* 137, 92−98.

(22) Arif, B., Ashraf, J. M., Moinuddin, Ahmad, J., Arif, Z., and Alam, K. (2012) Structural and immunological characterization of Amadori-rich human serum albumin: role in diabetes mellitus, *Arch. Biochem. Biophys.* 522, 17−25.

(23) Danese, E., Montagnana, M., Nouvenne, A., and Lippi, G. (2015) Advantages and pitfalls of fructosamine and glycated albumin in the diagnosis and treatment of diabetes, *J. Diabetes Sci. Technol.* 9, 169−176.

(24) Bayan, L., Koulivand, P.H., Gorji, A. (2014) Garlic: a review of potential therapeutic effects, *Avicenna J. Phytomed.* 4, 1−14.

(25) Ledl, F. (1990) Chemical pathways of the Maillard reaction. The Maillard reaction in food processing, human nutrition and physiology, 1990. 19−42.

(26) Martins, S.I., Jongen, W. M., and Van Boekel, M. A. (2000) A review of Maillard reaction in food and implications to kinetic modelling. *Trends Food Sci. Tech.* 11, 364−373.

(27) Sompong, W., Meeprom, A., Cheng, H., and Adisakwattana, S. (2013) A comparative study of ferulic acid on different monosaccharide-mediated protein glycation and oxidative damage in bovine serum albumin. Molecules 18, 13886−13903.

(28) Wijewickreme, A., and Kitts, D. (1999) Hydroxyl scavenging activity of glucose, fructose, and ribose-lysine model Maillard products. *J. Food Sci.* 64, 457−461.

(29) Mossine, V.V., Glinsky, V. V., and Mawhinney, T. P. (2010) Antitumor effects of the early Maillard reaction products, in The Maillard Reaction: Interface between Aging, Nutrition and Metabolism, M.C. Thomas and J. Forbes, Editors. 2010, Royal Society of Chemistry. 170−179.

(30) Mossine, V.V., Chopra, P. and Mawhinney, T. P. (2008) Interaction of tomato lycopene and ketosamine against rat prostate tumorigenesis. *Cancer Res.* 68, 4384−4391.

(31) Ryu, K., Ide, N., Matsuura, H., and Itakura, Y. (2001) N-α-(1-deoxy-D-fructos-1-yl)-L-arginine, an antioxidant compound identified in aged garlic extract. *J. Nutr.* 131, 972S−976S.

(32) Ide, N., Lau, B. H., Ryu, K., Matsuura, H., and Itakura, Y. (1999) Antioxidant effects of fructosyl arginine, a Maillard reaction product in aged garlic extract. *J. Nutr. Biochem.* 6, 372−376.

(33) Zhou, H., Qu, Z., Mossine, V. V., Nknolise, D. L., Li, J., Chen, Z., Cheng, J., Greenlief, C. M., Mawhinney, T. P., Brown, P. N., Fritsche, K. L., Hannink, M., Lubahn, D. B., Sun, G.Y ., and Gu, Z. (2014) Proteomic analysis of the effects of aged garlic extract and its FruArg component on lipopolysaccharide-induced neuroinflammatory response in microglial cells. *PLoS One* 9, e113531.

(34) Corea, G., Fattorusso, E., Lanzotti, V., Capasso, R., and Izzo, A.A. (2005) Antispasmodic saponins from bulbs of red onion, Allium cepa L. var. Tropea. *J. Agric. Food Chem.* 53, 935−940.

(35) Matsuura, H. (2001) Saponins in garlic as modifiers of the risk of cardiovascular disease *J. Nutr.* 131, 1000S−1005S.

(36) Pardridge, W.M. (2005) The blood-brain barrier: Bottleneck in brain drug development. *NeuroRx.* 2, 3–14.

(37) Pardridge, W.M. (2007) Blood-brain barrier delivery. *Drug Discov. Today*. 12, 54−61.

(38) Abbott, N. J., Rönnbäck, L., and Hansson, E. (2006) Astrocyte-endothelial interactions at the blood-brain barrier. *Nat. Rev. Neurosci.* 7, 41−53.

(39) Cecchelli, R., Berezowski, V., Lundquist, S., Culot, M., Renftel, M., Dehouck, M. P., and Fenart, L. (2007) Modelling of the blood-brain barrier in drug discovery and development. *Nat. Rev. Drug Discov*. 6, 650−661.

(41) Mossine, Valeri V., Barnes, Charles L. and Mawhinney, Thomas P. , 'The Structure of N-(1-Deoxy-β-D-fructopyranos-1-yl)-L-proline Monohydrate ("D-Fructose-L-proline") and N-(1,6-Dideoxy-α-L-fructofuranos-1-yl)-L-proline ("L-Rhamnulose-L-proline")', *J. Carbohyd. Chem.* 26, 249 – 266.

(41) Lowy, P., and Borsook, H. (1956). Preparation of N-substituted 1-amino-1-deoxy-darabinohexuloses of arginine, histidine, and lysine. *J. Am. Chem. Soc.* 78, 3175−3176.

(42) Troise, A. D., Fiore, A., Roviello, G., Monti, S. M., and Fogliano., V. (2015) Simultaneous quantification of amino acids and Amadori products in foods through ion-pairing liquid chromatography high-resolution mass spectrometry. *Amino Acids*. 47, 111−124.

(43) Gooyit, M., Suckow, M. A., Schroeder, V. A., Wolter, W. R., Mobashery, S., and Chang, M. (2012) Selective gelitinase inhibitor neuroprotective agents cross the blood-brain barrier. *ACS Chem. Neurosci*. 3, 730−736.

Chapter 5: Determining the Potential Occurrence of Cyanide in Elderberries

5.1 Introduction

5.1.1 Background on Cyanide in Elderberry

An extremely important issue that has been uncomfortably ignored by all parties in the production and marketing chain of elderberry is the possible occurrence of cyanide, a class of toxic compounds, in raw or processed elderberries. "Conventional wisdom" says that raw ripe elderberries contain cyanide, but that heat treatment or fermentation destroys or removes the cyanide. A 1983 poisoning incident involving several people in California is attributed to consumption of raw elderberries along with leaves and stems of the plant.[1] Cyanide is often implicated in that incident, but this was not definitively proven. Nevertheless, hearsay and uncertainty regarding the occurrence of cyanide in elderberry continues to foster hesitancy among both producers and consumers, which is most likely significantly hindering the full development of this potentially-lucrative agricultural specialty crop in Missouri.

The fact is that we simply do not know for certain what forms of cyanide may occur in ripe or unripe elderberry fruits, and if so, specifically where these compounds occur (pulp, juice, skin, seed, and/or pedicel). Furthermore, while heat treatment and fermentation are used to remove cyanide in some food products, no scientifically-based guidelines on elderberry processing for that purpose, including temperature regimens, have been published. In addition to juice products, dried whole elderberries (which contain intact seeds) are also being increasingly used in trail mixes and energy bars. Protocols for safe preparation of such minimally-processed elderberry products need to be studied. Exhaustive searches of the scientific literature have not found a definitive

study or publication on the occurrence of cyanide in elderberry fruits, nor how processing might ameliorate such occurrence for human consumption.

In addition to the concern about consumption of cyanide in raw or inadequately-processed elderberry fruits, the processing environment for elderberry products may also be of concern. If, in fact, ripe elderberry fruits contain cyanide, and heat treatment indeed removes or denatures it, cyanide is likely evolving into the atmosphere as hydrogen cyanide gas, which would be toxic to workers in the processing facility. We simply must develop a better understanding of the potential occurrence of cyanide and its dynamics within all aspects of the elderberry production and processing chain.

5.1.2 Previous Cyanide Studies

It is widely accepted that some plants contain specific cyanogenic glycosides. Several studies have determined the linamarin content of cassava and the amygdalin content of apple seeds. However, amygdalin has also been detected in the seeds of several other fruits related to the apple species, such as apricot, cherry, plum, and peach.[2] Amygdalin has also been detected in the seeds of other non-related fruits, such as cucumber, melon and acorn squash. Although the processing is thought to destroy cyanogenic compounds, it was detected in several processed apple products, albeit in much smaller quantities.

Research on cyanogenic glycoside content of berries is very sparse. One study detected prunasin and amygdalin in the seeds of several cultivars of Saskatoon berries at several stages of ripeness.[3] Interestingly, the prunasin and amygdalin content was high in the seeds an unripe berry in two of the cultivars, but was not detected in the unripe berries

of the other three cultivars. The cyanogenic glycoside content was generally higher in the ripe berries. Another study analyzed the leaves of elderberry plants for sambunigirin and it was detected in two of nine plants tested.[4]

5.1.3 Current Methods For Cyanide Analysis

A variety of spectrophotometric methods have been developed for the analysis of cyanide. Picrate paper methods[5,6] and acid hydrolysis methods[7-8] are very commonly used that provide a quick, easy and cheap estimate of cyanide content. These methods require three components: extraction of cyanogens from the plant, breakdown of cyanogen to hydrogen cyanide and measurement of the formation of hydrogen cyanide.[9] The limitations of these methods are the lack of selectivity and sensitivity. Information about the specific cyanogens present is not available when using these types of methods. Although they provide quick analysis and can even be used outside of the laboratory, more powerful methods that reveal information about individual cyanogen levels are available.

5.2 Method Development

Amygdalin (≥ 99%), dhurrin (≥ 95%), linamarin (≥ 98%), and prunasin (≥ 90%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of 1 μM were prepared for each standard in HPLC grade water (0.1% formic acid). The stock solutions were directly infused into the Xevo TQ-S mass spectrometer at flow rate of 5 μL/min. IntelliStart software was used to generate the optimal cone voltages and collision energies that produce daughter ions with the highest intensity. The parent-daughter ion

transition of 248.1 → 85.9 and 248.1 → 97.1 were identified for linamarin and will be used as part of a multiple reaction monitoring protocol for quantification (Figure 5.1). The parent-daughter ion transition of 458.2 → 296.1 and 458.2 → 158.1 were identified for amygdalin and will be used as part of a multiple reaction monitoring protocol for quantification (Figure 5.2).

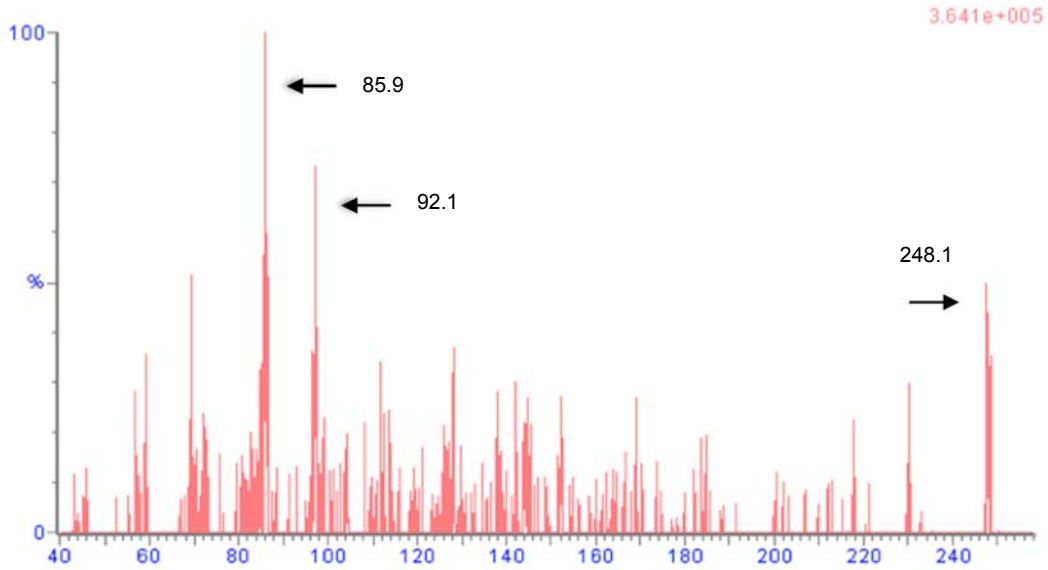


Figure 5.1 Product ion mass spectrum of linamarin.

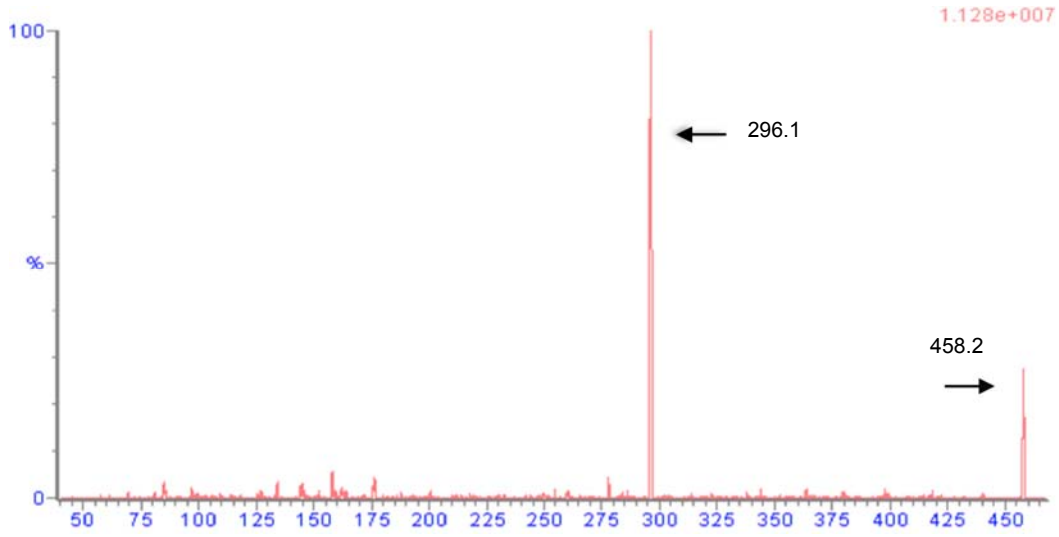


Figure 5.2 Product ion mass spectrum of amygdalin.

Unfortunately, Intellistart was unable to identify parent-daughter ion transitions for dhurrin or prunasin compounds using the $[M + H]^+$ or $[M - H]^-$ parent ions. After further analysis, it was discovered that these two compounds exist primarily as sodium adducts, resulting in an increase in m/z of 23 and also formed dimers (Table 5.1). IntelliStart optimization was attempted using $[M + Na]^+$ as the parent ion for dhurrin and prunasin, however, these adducts are very stable and the energy applied in the collision cell is not sufficient to cause ample fragmentation. Daughter ions were found, but their

Compound	Monoisotopic mass (amu)	Observed Peaks (m/z)
Amygdalin	457.2	458.2
Dhurrin	311.1	333.9, 360.0
Linamarin	247.1	269.9, 517.0, 248.1
Prunasin	295.1	318.1

Table 5.1 Mass spectral peaks observed when performing direct infusion analysis.

intensities were very low (roughly 2% relative to the parent) which is unsuitable for proper MRM quantitation. Atmospheric pressure chemical ionization (APCI) is an alternative to (ESI) that was used to create the ions in this experiment. APCI also failed to produce parent ions that are more suitable for fragmentation.

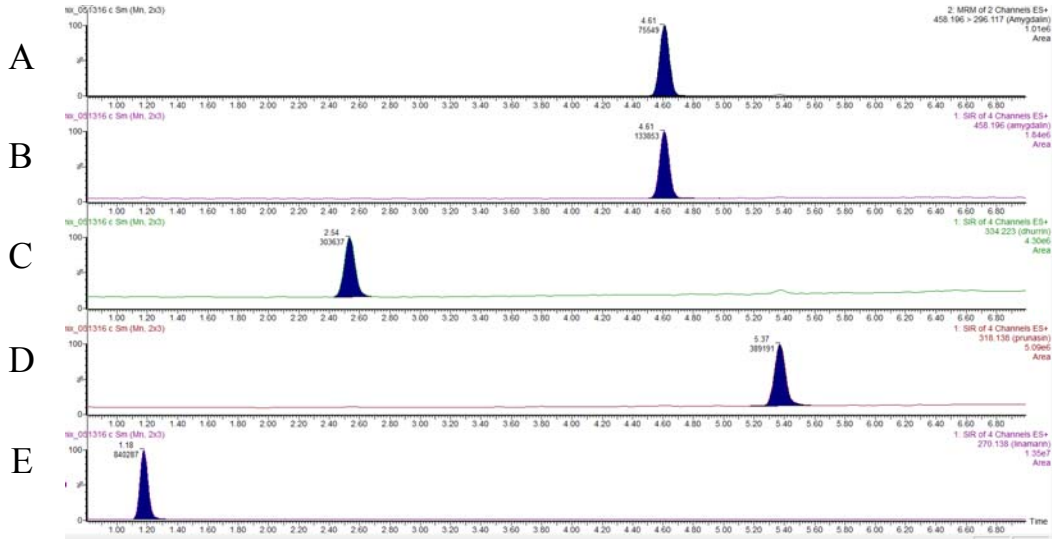


Figure 5.3 MRM chromatrogram of amygdalin (A) and SIR chromatograms of amygdalin (B), dhurrin (C), prunasin (D) and linamarin (E). The top number above each peak indicates the retention time for that ion and the number below is the area under the chromatographic peak.

This problem can be overcome by investigating alternate mass spectrometry scans. Quantification can also be performed using selected ion recording (SIR), which only allows particles of a specific mass-to-charge ratio through the third quadrupole mass analyzer. SIR mode, like MRM is also very sensitive but it is not as selective as MRM. This is because only one ion is being monitored, instead of a parent and daughter ion pair. However, given the fact the cyanogenic glycosides don't form typical $[M+H]^+$ or $[M-H]^-$ parent ions, we feel this is the best option for quantitative analysis moving forward. Thus, Intellistart was used to optimize cone voltages for $[M+Na]^+$ ions for all four standards.

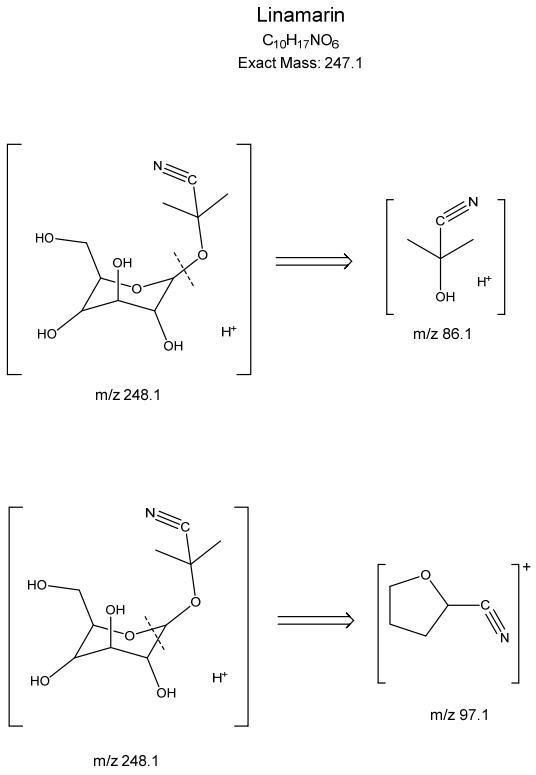


Figure 5.4 Proposed fragmentation pathway of linamarin.

A UPLC gradient was optimized for the separation of four cyanogenic glycoside standards. A previously published gradient for the quantification of amygdalin in almonds was reduced from 20 min down to 10 min.[10] Mobile phases used were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient used was 95% A, 0−1 min; 95−80% A, 1−3 min; 80−40% B, 3−7 min; 40% B, 7−8 min; 95% B 8.1−10 min. This method displays excellent separation of the four standards (Figure 5.3) and exhibited retention time repeatability and good peak shape. Proposed fragmentation pathway of the linamarin and amygdalin parent ions to their daughter ions are shown in Figure 5.4 and 5.5, respectively.

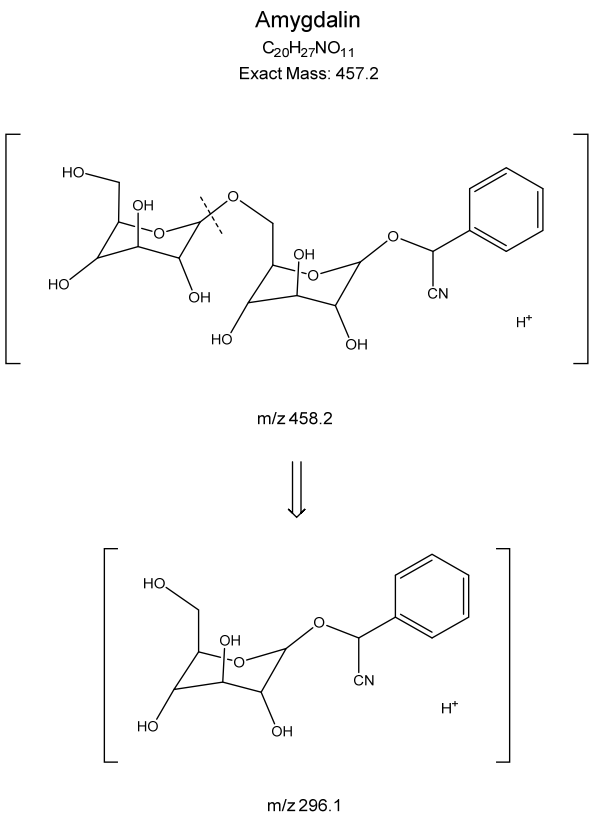


Figure 5.5 Proposed fragmentation pathway of amygdalin.

5.3 Future Work

Now that an LC-MS/MS method has been developed and optimized, it will be validated for analytical performance. Several different parts of the elderberry plant will be tested for potential cyanogenic glycoside content. Therefore, extraction techniques for each part will need to be developed. Elderberry samples will be tested after harvest in 2016 and 2017 using the described method. If cyanogenic compounds are identified then subsequent processing studies will be performed to understand ideal conditions to eliminate the presence of cyanide. A quick, easy and cheap paper method is being adapted from previously established methods to complement the LC-MS/MS method. This paper method has been used frequently to test for the presence of any cyanogen

compound. This type of test does not reveal information about the specific cyanogenic glycoside present, but can be useful as a quick screen. It can be very useful in the field and by someone without a lot of laboratory experience.

5.4 References

(1) Centers for Disease Control and Prevention. (1984). Poisoning from Elderberry Juice – California. Retrieved from www.cdc.gov/mmwr.

(2) Bolarinwa, I. F., Orfila, C., and Morgan M. R. A. (2014) Amygdalin content of seeds, kernels and food products commercially-available in the UK. *Food Chem.* 152, 133−139.

(3) Mazza, G., and Cottrell, T. (2008) Carotenoids and cyanogenic glucosides in Saskatoon berries (*Amelanchier alnifola Nutt.) J. Food Comp. Anal.* 21, 249−254.

(4) Buhrmester, R. A, Ebinger, J. E., and Seigler, D. S. (2000) Sambunigrin and cyanogenic variability in populations of Sambucuc canadensis L. (Caprifoliceae). *Biochem. Syst. Ecol.* 28, 689−695.

(5) Egan, S. V., Yeoh, H. H., and Bradbury, J. H. (1998) Simple picrate paper kit for determination of the cyanogenic potential of cassava flour. *J. Sci. Food Agric.* 76, 39−48.

(6) Bradbury, M. G., Egan, S. V., and Bradbury J. H. (1999). Picrate paper kits for determination of total cyanogens in cassava roots and all forms of cyanogens in cassava products. *J. Sci. Food Agric.* 79, 593−601.

(7) Cooke, R. D. (1978) An enzymatic assay for the total cyanide content of cassava. *J. Sci. Food Agric.* 29, 345−352.

(8) Bradbury, J. H., Egan, S. V., and Lynch, M. J. (1991). Analysis of cyanide in cassava using acid hydrolysis of cyanogenic glycosides. *J. Sci. Food Agric.* 55, 277−290.

(9) Bradbury, J. H., Bradbury, M. G., and Egan, S. V. (1994) Comparison of methods of analysis of cyanogens in cassava. *Acta Hort.* 375, 87−96.

(10) Lee, J., Zhang, G., Wood, E., Castillo, C. R., and Mitchell A. E. (2013) Quantification of amygdaline in nonbitter, semibitter, and bitter almonds (*Prunus dulcis)* by UHPLC-(ESI)QqQ MS/MS. *J. Food Agric. Chem.* 61, 7754−7759.

Appendix A: List of Abbreviations

AGE: Aged garlic extract

ANOVA: Analysis of variance

APCI: Atmospheric pressure chemical ionization

AUC: Area under curve

BBB: Blood-brain barrier

BW: Body weight

C3GE: Cyanidin-3-O-glucoside equivalent

CG: Cyanogenic glycoside

Cl: Clearance

COSM: cyanidin-3-O-coumaroyl-sambubioside

CS5G: Cyanidin 3-coumaroyl-sambubioside-5-glucoside

CV%: Coefficient of variation

C0: Initial concentration

C18: Octadecyl

CYB2: cyanidin based anthocyanin

Da: Dalton

DSHEA: Dietary Supplement Health and Education Act

EI: Electron ionization

ESI: Electrospray ionization

eV: electron volt

FCR: Folin-Ciocalteu Reagent

FDA: Food and Drug Administration

FruArg: N-α-(1-deoxy-D-fructos-1-yl)-L-arginine

GAE: Gallic acid equivalent

GALA: = cyanidin-3-O-galactoside

GLUC: = cyanidin-3-O-glucoside

GMP: Good manufacturing process

ha: hectacre

HPLC: High-performance liquid chromatrography

IAC: Individual anthocyanin content

IS: Internal standard

k: elimination rate

kV: kilovolt

LC-MS/MS: Liquid chromatography tandem mass spectrometry

L-Lysine-d8: L-Lysine d-3,3,4,4,5,5,6,6

LOQ: Limit of quantitation

LPS: Lipopolysaccharide

m/z: Mass-to-charge ratio

meq: millequivalents

MCX: Mixed-mode cation exchange

MG: Mountain Grove

mmHg: millimeters mercury

MRM: Mutliple reaction monitoring

MV: Mount Vernon

NMR: Nuclear magnetic resonance

NO: Nitric oxide

PK: Pharmacokinetic

PM: Photomultiplier

(PO·): Phenol radical

POH: Polyphenol

ppm: part per million

QC: Quality control

QqQ: Triple quadrupole

(R·): Free radical

Rt: Retention time

RUTN: cyanidin-3-O-rutinoside

S5G: cyanidin-3-sambubioside-5-glucoside

SAMB: cyanidin-3-O-sambubioside

SOPH: cyanidin-3-O-sophoroside

SPE: Solid phase extraction

SIM: Single ion monitoring

SIR: Single ion recording

$t_{1/2}$: half-life

TFA: Trifluoro acetic acid

TIC: Total ion current

TOF: Time-of-flight

TMA: Total monomeric anthocyanin

TP: Total phenolic

UHPLC: Ultra high pressure liquid chromatography

UPLC: Ultra-performance liquid chromatography

UV-Vis: Ultraviolet-visible

VD: Volume distribution

Appendix B: Frozen Storage Study Anthocyanin Data

	Coum-samb-5-glc[1]	Samb-5-glc[2]	Coum-samb[3]	Sophor[4]	Rutin[5]	Samb[6]	Galact[7]	Glc[8]	Based-2[9]
Adams II									
Initial	28 ± 1	18.47 ± 0.07	4.4 ± 0.4	15.9 ± 0.2	0.97 ± 0.02	3.9 ± 0.3	2.46 ± 0.01	2.1 ± 0.1	2.05 ± 0.05
3 Month	8.8 ± 0.2**	5.5 ± 0.5**	0.95 ± 0.03**	3.6 ± 0.2**	1.03 ± 0.01	nd[10]	0.86 ± 0.01**	0.97 ± 0.03**	1.01 ± 0.01**
6 Month	1.39 ± 0.06**	0.9 ± 0.1**	0.66 ± 0.01**	0.83 ± 0.07**	nd	nd	nd	nd	nd
9 Month	nd	nd	nd	nd	nd	nd	nd	nd	nd
Bob Gordon									
Initial	56 ± 3	41 ± 4	7.8 ± 0.4	69 ± 7	1.28 ± 0.04	8.1 ± 0.7	6.7 ± 0.6	4.7 ± 0.3	3.8 ± 0.3
3 Month	47 ± 4*	32 ± 3*	1.04 ± 0.05**	38 ± 5**	nd	3.2 ± 0.3**	2.8 ± 0.3*	1.8 ± 0.2*	3.1 ± 0.3
6 Month	12.2 ± 0.9**	7.4 ± 0.9**	1.2 ± 0.3**	18 ± 3**	nd	nd	3.0 ± 0.5*	1.6 ± 0.1*	0.85 ± 0.07*
9 Month	0.49 ± 0.03**	nd	1.04 ± 0.06**	0.28 ± 0.04**	nd	nd	nd	1.5 ± 0.2*	nd
Wyldewood									
Initial	32 ± 3	22 ±2	4.0 ± 0.3	16 ± 1	1.01 ± 0.04	2.6 ± 0.2	2.6 ± 0.1	1.54 ± 0.06	2.2 ± 0.2
3 Month	24 ± 2*	15 ± 1*	1.5 ± 0.1*	8.5 ± 0.9*	nd	1.22 ± 0.08*	1.29 ± 0.05*	1.26 ± 0.06*	1.7 ± 0.1
6 Month	4.7 ± 0.3**	2.64 ± 0.09**	1.4 ± 0.1*	nd	nd	nd	nd	nd	nd
9 Month	0.21 ± 0.01**	nd	0.45 ± 0.02**	nd	nd	nd	nd	nd	nd

Table A1. Individual anthocyanin content (IAC) of Adams II, Bob Gordon, and Wyldewood genotypes during different durations of frozen storage. Data are represented as mean ± standard error of the mean as µg C3GE/ mL (n=11-14). Significant differences between the IAC of samples during frozen storage compared to the initial IAC are represented with asterisks ($p<0.05$= *, $p<0.01$= **) according to two-sample mean t-test. Coum-samb-5-glc[1] = cyanidin-3-O-coumaroyl-sambubioside-5-glucoside; Samb-5-glc[2] = cyanidin-3-O-sambubioside-5-glucoside; Coum-samb[3] = cyanidin-3-O-coumaroyl-sambubioside; Sophor[4] = cyanidin-3-O-sophoroside; Rutin[5]= cyanidin-3-O-rutinoside; Samb[6] = cyanidin-3-O-sambubioside; Galact[7] = cyanidin-3-O-galactoside; Glc[8] = cyanidin-3-O-glucoside; Based-2[9] = cyanidin based anthocyanin; nd [10]= not detected. Limit of detection (0.3 ng/mL).

Appendix C: Discriminant Analysis of Other Elderberry Harvests

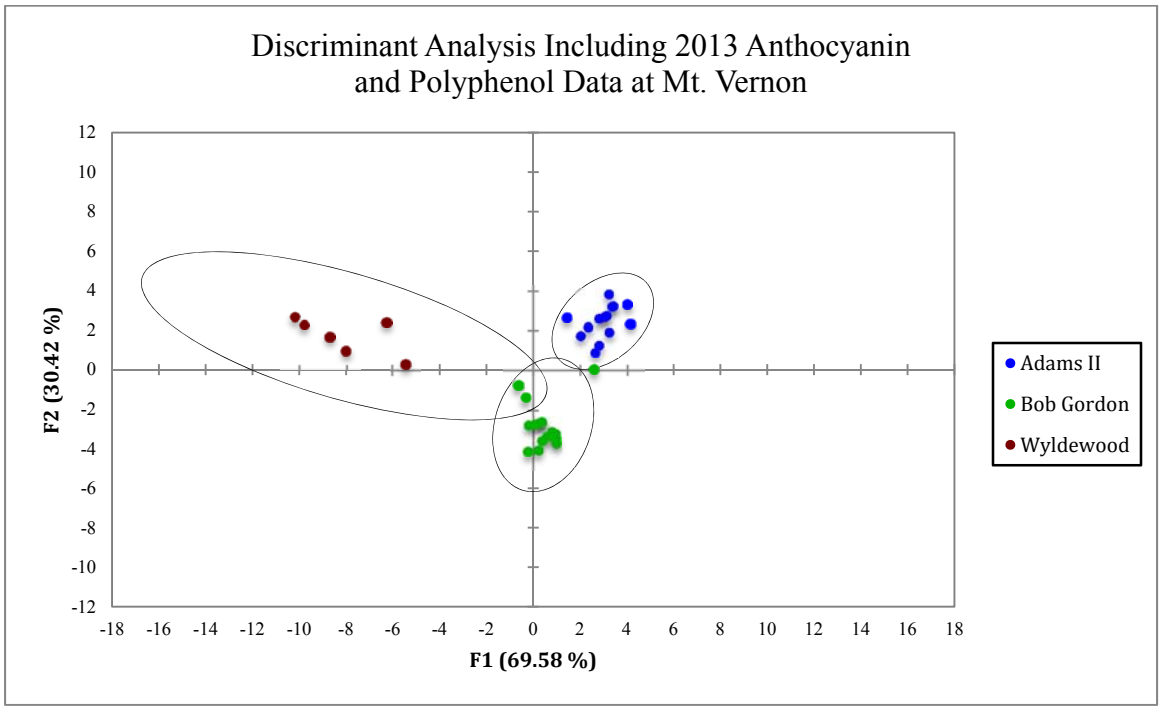


Figure B1 Discriminant analysis of American elderberry juice samples from Mt. Vernon in 2013 based on the anthocyanin and polyphenol content of each individual juice aliquot.

From \ To	Adams II	Bob Gordon	Wyldewood	Total	% correct
Adams II	14	0	0	14	100.00%
Bob Gordon	1	13	0	14	92.85%
Wyldewood	0	2	4	6	66.67%
Total	15	15	4	34	86.51%

Table B1 Cross validation results from confusion matrix from the 2013 discriminant analysis at Mt. Vernon.

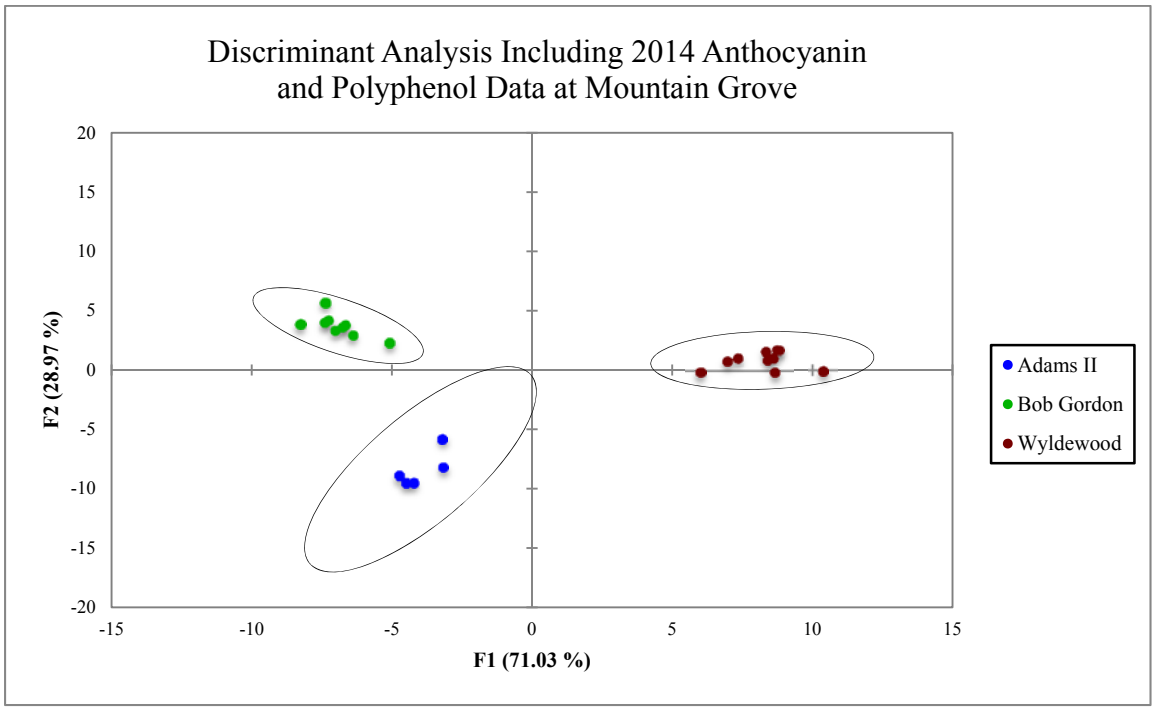


Figure B2 Discriminant analysis of American elderberry juice samples from Mountain Grove in 2014 based on the anthocyanin and polyphenol content of each individual juice aliquot.

From \ To	Adams II	Bob Gordon	Wyldewood	Total	% correct
Adams II	4	0	1	5	80.00%
Bob Gordon	0	9	0	9	100.00%
Wyldewood	0	2	8	10	80.00%
Total	4	11	9	24	86.67%

Table B2 Cross validation results from confusion matrix from the 2014 discriminant analysis at Mountain Grove.

Appendix D: Individual Anthocyanin Data By Aliquot

ADAMS II

Year	Location	Coum-samb-5-glc[1]	Based-2[2]	Samb-5-glc[3]	Coum-samb[4]	Sophor[5]	Rutin[6]	Samb[7]	Glc[8]
2013	MV	30.91	2.21	18.26	5.49	16.51	1.03	4.60	2.41
2013	MV	24.36	1.91	18.68	3.35	15.25	0.91	3.09	1.82
2013	MV	27.91	2.04	16.79	4.69	17.90	1.43	3.96	1.90
2013	MV	36.33	2.33	19.36	4.32	14.30	1.35	4.26	2.33
2013	MV	32.10	2.41	17.96	5.03	19.65	1.11	4.05	2.01
2013	MV	24.04	1.86	18.90	3.02	11.62	0.84	3.01	2.27
2013	MV	19.66	1.80	18.33	2.98	14.32	0.80	2.86	2.25
2013	MV	25.96	1.87	18.03	4.66	17.32	0.88	4.35	2.50
2013	MV	31.52	2.03	19.54	5.07	20.96	0.87	4.70	2.21
2013	MV	27.34	2.14	18.93	5.30	14.32	0.80	3.96	1.63
2013	MV	29.86	2.00	18.37	4.78	13.02	0.75	4.03	1.88
2014	MV	25.67	0.94	6.82	3.18	8.51	0.38	1.89	1.06
2014	MV	29.33	1.30	7.16	4.54	6.80	0.38	2.66	1.32
2014	MV	25.76	1.07	6.32	4.46	6.43	0.31	2.39	1.12
2014	MV	29.68	1.26	7.33	4.71	8.15	0.48	3.22	1.92
2014	MV	22.24	0.98	6.94	3.34	9.38	0.35	2.72	1.35
2014	MV	15.26	0.51	3.39	1.30	3.94	0.22	0.83	0.76
2014	MV	27.23	1.16	7.54	4.77	8.07	0.44	3.48	1.52
2014	MV	24.01	1.06	6.32	3.73	7.02	0.36	2.63	1.25
2014	MV	21.34	1.13	8.24	3.65	8.15	0.33	2.81	1.72
2014	MV	14.85	0.61	3.48	1.55	3.32	0.20	1.02	0.75
2014	MV	20.14	0.78	4.52	3.10	4.70	0.36	1.69	0.89
2014	MV	15.52	0.58	3.51	2.53	3.77	0.18	1.25	0.70

ADAMS II

Year	Location	Coum-samb-5-glc[1]	Based-2[2]	Samb-5-glc[3]	Coum-samb[4]	Sophor[5]	Rutin[6]	Samb[7]	Glc[8]
2014	MV	21.24	0.84	4.15	3.27	3.82	0.25	1.29	0.67
2014	MV	15.18	0.57	3.30	2.52	3.27	0.14	1.04	0.82
2014	MV	17.24	0.66	3.91	1.97	3.78	0.25	1.17	0.98
2014	MV	21.47	0.96	5.54	3.57	5.36	0.32	1.81	0.92
2014	MG	11.77	0.23	1.46	1.09	1.23	0.08	0.19	0.38
2014	MG	4.30	0.02	0.18	0.37	0.15	0.05	0.01	0.14
2014	MG	7.82	0.05	0.42	0.52	0.40	0.08	0.05	0.28
2014	MG	11.25	0.16	1.19	0.79	1.09	0.07	0.21	0.39
2014	MG	6.73	0.05	0.41	0.51	0.42	0.06	0.07	0.25
2015	MV	3.57	0.29	1.80	1.30	1.86	nd	nd	0.63
2015	MV	5.45	0.39	1.72	1.86	1.56	nd	nd	0.57
2015	MV	3.22	0.18	1.01	1.03	0.96	nd	nd	0.37
2015	MV	1.80	nd	0.55	0.36	0.44	nd	nd	nd
2015	MG	0.56	0.04	0.30	0.15	0.19	0.02	0.01	0.01
2015	MG	0.26	0.02	0.10	0.09	0.08	0.02	nd	0.02
2015	MG	0.51	0.03	0.15	0.16	0.12	0.02	0.01	0.02
2015	MG	0.29	0.01	0.11	0.09	0.08	0.01	0.01	nd
2015	MG	0.27	0.02	0.12	0.08	0.10	0.01	0.01	nd
2015	MG	0.76	0.02	0.17	0.64	0.21	0.02	nd	0.02
2015	MG	0.23	0.01	0.02	0.22	0.03	0.02	nd	nd
2015	MG	0.23	0.01	0.03	0.18	0.02	nd	nd	nd
2015	MG	0.15	0.01	0.06	0.10	0.07	nd	nd	nd
2015	MG	0.80	0.03	0.17	0.67	0.23	0.04	0.01	0.04

ADAMS II

Year	Location	Coum-samb-5-glc[1]	Based-2[2]	Samb-5-glc[3]	Coum-samb[4]	Sophor[5]	Rutin[6]	Samb[7]	Glc[8]
2015	MG	0.21	0.01	0.03	0.15	0.03	nd	nd	nd
2015	MG	0.06	nd	0.02	0.04	0.02	nd	nd	nd
2015	MG	0.91	0.05	0.38	0.32	0.38	0.03	0.02	0.02
2015	MG	0.64	0.03	0.20	0.65	0.15	0.02	0.01	0.03
2015	MG	0.79	0.04	0.22	0.73	0.21	0.02	nd	0.01

BOB GORDON

Year	Location	Coum-samb-5-glc[1]	Based-2[2]	Samb-5-glc[3]	Coum-samb[4]	Sophor[5]	Rutin[6]	Samb[7]	Glc[8]
2013	MV	67.82	5.02	58.59	9.50	109.19	1.49	11.74	6.10
2013	MV	45.90	2.96	40.33	7.33	40.56	1.33	7.10	4.33
2013	MV	48.36	5.16	32.69	7.56	84.03	1.47	6.77	4.80
2013	MV	63.48	5.33	53.72	9.67	77.80	0.86	7.03	4.46
2013	MV	38.48	4.11	30.16	5.51	61.53	1.38	7.49	4.83
2013	MV	57.33	4.96	42.63	8.92	44.90	1.30	8.93	3.37
2013	MV	61.20	2.34	23.80	8.69	38.22	1.08	5.22	3.22
2013	MV	58.02	3.65	39.27	7.62	67.85	1.17	7.79	4.72
2013	MV	43.29	2.99	31.47	6.03	80.21	1.32	6.35	3.20
2013	MV	64.90	2.67	49.42	7.51	77.35	1.60	9.93	5.96
2013	MV	55.91	2.98	39.63	7.43	91.36	1.62	9.06	6.03
2013	MV	63.12	4.04	51.44	8.90	88.21	1.33	8.75	6.33
2013	MV	60.85	3.37	53.07	8.77	70.54	1.29	9.84	4.91
2013	MV	49.92	3.07	38.54	5.40	36.93	0.94	6.58	3.55
2014	MV	19.09	0.67	4.51	2.11	7.72	0.22	0.87	0.75
2014	MV	22.71	0.78	5.10	2.64	8.84	0.29	1.23	0.92
2014	MV	21.47	0.68	4.39	2.46	7.25	0.34	1.03	0.98
2014	MV	18.30	0.64	4.45	2.04	6.72	0.17	0.96	0.73
2014	MV	25.66	0.99	7.29	3.75	13.53	0.49	2.16	1.25
2014	MV	17.88	0.67	5.03	1.74	7.33	0.33	1.55	0.85
2014	MV	18.45	0.79	5.27	1.06	9.17	0.23	1.47	0.75
2014	MV	15.92	0.66	5.00	1.87	10.39	0.19	1.54	0.94
2014	MV	24.37	1.20	8.13	3.14	13.65	0.45	2.35	1.16

BOB GORDON

Year	Location	Coum-samb-5-glc[1]	Based-2[2]	Samb-5-glc[3]	Coum-samb[4]	Sophor[5]	Rutin[6]	Samb[7]	Glc[8]
2014	MV	16.03	0.59	4.21	1.93	7.62	0.36	0.98	0.83
2014	MV	20.75	0.87	5.82	2.66	9.32	0.35	1.52	0.83
2014	MV	18.51	0.68	5.14	1.91	9.74	0.33	1.46	0.89
2014	MV	14.68	0.52	3.57	1.70	5.94	0.28	0.91	0.65
2014	MV	16.25	0.62	4.09	1.43	6.63	0.26	0.96	0.82
2014	MV	18.64	0.67	4.70	2.34	7.21	0.26	1.06	1.05
2014	MV	20.85	0.70	5.14	2.89	9.25	0.34	1.38	0.88
2014	MG	32.66	0.75	7.38	4.06	12.81	0.48	1.98	1.76
2014	MG	32.61	0.77	7.64	4.43	13.28	0.44	2.88	2.68
2014	MG	27.40	0.67	6.48	2.03	10.68	0.45	1.52	1.89
2014	MG	23.68	0.57	5.08	2.02	8.79	0.23	0.90	1.61
2014	MG	26.50	0.63	6.25	2.86	11.18	0.43	1.60	2.04
2014	MG	37.69	1.17	11.01	2.88	19.74	1.19	5.53	3.69
2014	MG	32.84	1.13	10.81	2.32	15.68	0.65	4.62	2.84
2014	MG	30.97	1.13	10.46	2.17	14.89	0.73	4.89	2.96
2014	MG	31.28	1.04	10.33	3.06	17.42	0.59	3.02	2.77
2015	MV	9.16	0.62	5.71	3.95	15.23	0.27	0.71	3.77
2015	MV	10.57	0.64	6.60	4.75	16.09	0.22	0.63	3.80
2015	MV	10.85	0.73	5.99	4.74	14.16	0.41	0.64	3.71
2015	MV	9.53	0.61	5.29	4.19	13.04	0.23	0.48	3.03
2015	MV	7.05	0.46	4.27	2.95	8.93	0.29	0.41	2.42
2015	MV	9.05	0.65	5.91	3.48	12.76	0.19	0.44	2.86
2015	MV	8.69	0.66	5.65	3.22	11.50	nd	0.43	2.75
2015	MV	13.92	0.78	7.22	6.92	20.04	0.45	0.76	4.16

BOB GORDON

Year	Location	Coum-samb-5-glc[1]	Based-2[2]	Samb-5-glc[3]	Coum-samb[4]	Sophor[5]	Rutin[6]	Samb[7]	Glc[8]
2015	MV	11.62	0.80	5.76	4.78	13.59	0.26	0.53	3.37
2015	MV	15.60	0.93	7.97	9.29	18.37	nd	1.10	4.95
2015	MV	11.54	0.77	6.58	5.76	15.95	0.30	0.78	3.98
2015	MV	9.99	0.77	7.07	4.79	16.98	0.26	0.76	4.05
2015	MV	10.03	0.68	6.26	4.86	16.09	0.43	0.74	4.19
2015	MV	8.12	0.62	4.83	2.85	12.39	0.18	0.34	2.58
2015	MV	12.26	0.79	7.75	5.69	16.10	0.82	4.59	3.96
2015	MG	10.84	0.80	8.93	4.96	12.43	0.28	1.17	0.49
2015	MG	6.12	0.38	4.01	2.21	7.55	0.21	0.29	nd
2015	MG	4.36	0.29	3.20	1.35	4.96	0.17	0.24	0.07
2015	MG	7.07	0.31	3.69	2.76	7.15	0.22	0.28	0.25
2015	MG	6.26	0.30	3.41	2.31	6.63	0.18	0.33	0.20
2015	MG	4.67	0.22	2.69	1.52	4.95	0.13	0.26	nd
2015	MG	3.89	0.25	2.86	1.23	3.99	0.12	0.21	nd
2015	MG	5.95	0.36	4.17	2.20	6.20	0.17	0.32	nd
2015	MG	4.02	0.27	2.20	3.65	3.54	0.07	0.07	nd
2015	MG	3.83	0.24	1.89	3.28	3.47	0.10	0.09	nd
2015	MG	4.27	0.34	3.01	4.10	3.41	0.06	0.12	0.35
2015	MG	6.34	0.32	2.54	6.56	4.75	0.09	0.11	nd
2015	MG	7.16	0.49	4.00	5.35	5.16	0.10	0.10	nd

WYLDEWOOD

Year	Location	Coum-samb-5-glc[1]	Based-2[2]	Samb-5-glc[3]	Coum-samb[4]	Sophor[5]	Rutin[6]	Samb[7]	Glc[8]
2013	MV	26.88	2.41	19.88	4.08	11.31	1.20	1.69	1.60
2013	MV	37.18	2.58	26.49	4.96	16.42	1.33	2.63	1.87
2013	MV	16.73	1.38	12.60	2.28	9.15	0.83	1.45	1.27
2013	MV	30.93	2.12	23.35	3.70	18.77	0.92	2.83	1.72
2013	MV	35.92	2.44	23.99	3.62	16.98	0.99	2.54	1.90
2013	MV	40.54	2.38	24.70	4.03	18.35	0.87	2.90	1.55
2013	MV	41.30	2.60	25.68	4.88	17.01	1.13	2.52	1.65
2013	MV	37.96	2.31	24.54	4.43	19.66	1.05	2.75	1.63
2013	MV	15.70	0.98	14.04	2.31	8.51	0.43	1.44	1.03
2013	MV	18.90	0.95	17.66	2.57	9.58	0.67	1.90	1.01
2013	MV	31.63	2.66	20.94	4.39	20.44	1.53	3.84	1.67
2013	MV	38.60	2.53	23.41	5.00	18.70	1.44	3.13	1.69
2013	MV	36.56	2.26	21.15	4.73	15.41	1.19	2.98	1.71
2013	MV	44.19	2.93	28.97	5.31	19.08	1.10	3.16	1.44
2014	MV	22.15	0.90	6.96	2.50	7.26	0.29	2.10	1.18
2014	MV	17.08	0.62	4.70	1.84	4.38	0.30	1.04	0.99
2014	MV	22.28	0.80	6.41	2.26	5.80	0.38	1.76	1.00
2014	MV	19.19	0.82	7.97	3.02	8.26	0.29	2.45	1.29
2014	MV	18.93	0.72	6.63	2.79	6.07	0.29	2.10	1.14
2014	MV	20.38	0.83	7.55	2.85	7.22	0.33	2.02	1.13
2014	MV	13.83	0.56	4.73	1.58	4.46	0.13	0.98	0.75
2014	MV	17.29	0.88	9.58	1.33	8.46	0.36	1.59	1.87
2014	MV	17.32	0.81	7.33	1.65	6.93	0.25	1.84	0.81
2014	MV	15.92	0.60	5.62	2.04	5.00	0.33	1.22	1.01

WYLDEWOOD

Year	Location	Coum-samb-5-glc[1]	Based-2[2]	Samb-5-glc[3]	Coum-samb[4]	Sophor[5]	Rutin[6]	Samb[7]	Glc[8]
2014	MV	14.36	0.61	5.14	1.30	5.04	0.26	1.33	0.83
2014	MV	15.23	0.45	4.10	1.89	3.95	0.17	1.10	0.82
2014	MV	13.98	0.52	3.78	1.75	2.95	0.23	0.95	0.67
2014	MV	15.00	0.48	3.70	1.37	2.85	0.22	0.81	0.84
2014	MV	16.63	0.63	5.36	2.11	4.79	0.28	1.40	0.88
2014	MV	12.51	0.42	3.46	0.75	3.15	0.20	0.85	0.63
2014	MG	25.50	0.80	6.97	2.35	7.18	0.45	2.16	1.49
2014	MG	20.33	0.63	6.98	2.51	9.94	0.36	2.51	3.42
2014	MG	16.58	0.45	4.28	1.40	4.89	0.24	1.19	1.70
2014	MG	15.79	0.49	6.02	1.78	6.00	0.19	0.90	1.16
2014	MG	19.91	0.59	6.84	2.90	7.58	0.36	2.20	2.17
2014	MG	17.64	0.48	6.10	1.83	6.14	0.30	0.97	1.37
2014	MG	26.02	1.03	8.76	1.54	12.44	0.49	4.17	3.82
2014	MG	22.64	0.80	8.45	2.37	9.63	0.48	3.07	2.23
2014	MG	24.40	0.81	8.91	1.56	10.61	0.42	3.25	2.39
2014	MG	24.16	0.81	9.50	2.35	10.14	0.41	2.90	2.45
2015	MV	5.98	0.36	2.97	1.88	2.44	nd	nd	1.03
2015	MG	1.70	0.09	0.69	0.90	0.92	nd	nd	nd
2015	MG	5.70	0.30	2.84	2.06	3.63	0.12	0.27	0.09
2015	MG	2.17	0.13	1.18	1.42	nd	nd	nd	nd
2015	MG	2.83	0.16	1.15	2.47	1.10	0.04	0.03	nd
2015	MG	2.58	0.15	1.43	1.85	1.44	0.04	0.03	nd
2015	MG	5.31	0.26	2.28	4.20	2.87	0.07	0.09	nd
2015	MG	3.69	0.15	0.83	2.31	0.76	nd	nd	nd

WYLDEWOOD

Year	Location	Coum-samb-5-glc[1]	Based-2[2]	Samb-5-glc[3]	Coum-samb[4]	Sophor[5]	Rutin[6]	Samb[7]	Glc[8]
2015	MG	6.01	0.36	3.14	4.12	3.63	nd	nd	nd
2015	MG	3.03	0.17	1.14	1.68	1.68	nd	nd	nd

Coum-samb-5-glc[1] = cyanidin-3-O-coumaroyl-sambubioside-5-glucoside; Based-2[9] = cyanidin based anthocyanin ; Samb-5-glc[3] = cyanidin-3-O-sambubioside-5-glucoside; Coum-samb[4] = cyanidin-3-O-coumaroyl-sambubioside; Sophor[5] = cyanidin-3-O-sophoroside; Rutin[6]= cyanidin-3-O-rutinoside; Samb[7]= cyanidin-3-O-sambubioside; Glc[8] = cyanidin-3-O-glucoside; MV = Mt. Vernon; MG = Mountain Grove; nd = not detected.

VITA

Mitch Curtiss Johnson was born on September 29th, 1989 in Ottawa, IL. He graduated from Ottawa Township High School in 2008. After high school he enrolled at Monmouth College located in Monmouth, IL. In May 2012, he earned a Bachelor's of Arts degree in chemistry.

Mitch's graduate school research focused on developing sensitive analytical methods, mainly using liquid chromatography-tandem mass spectrometry. These methods were then applied to study the chemical composition of elderberry and garlic botanicals. He graduated from the University of Missouri-Columbia with a Ph.D. in analytical chemistry in December 2016.