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Several laboratories have analyzed the content and stability of anthocyanins from foods and flowering plants ranging from potatoes to petunias. Previous research in the Cech and Lacey laboratories tentatively identified anthocyanins in *Plantago lanceolata* using reversed phase high performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC-ESI-MS). The goal of this research was to gain more information about the identification of these anthocyanins using hydrolysis and nuclear magnetic resonance (NMR) and to test the stability of these anthocyanins when stored for various periods of time at three different temperatures (room temperature, -20, -80 ˚C). Repeatability studies were also conducted to validate the analytical method.

Hydrolysis experiments and analysis by HPLC-ESI-MS confirmed the presence of cyanidin and peonidin glycosides in the original *P. lanceolata* extract. The most abundant anthocyanin was isolated from *P. lanceolata,* but attempts to collect an NMR spectrum of this sample were not successful due to the small yield and apparent low purity of the sample isolated. The method variability from injection to injection on the same day was determined to be 10 – 20 %, variability from day to day on the same extract was 30 % and the variability from extraction to extraction on different days was 30 – 60 % for **cyanidin glycosides a** and **c**, **delphinidin glycoside b**, **peonidin glycoside a**, and **petunidin glycoside b**. Stability studies revealed that there was no degradation that exceeded the variability of the method from day to day in both the -20 and -80 ^{o}C temperatures for any of the anthocyanins. There was however, significant degradation in the room temperature condition for the **cyanidin glycosides a** and **c**.

IDENTIFICATION AND STABILITY OF ANTHOCYANINS IN *PLANTAGO LANCEOLATA*

by

Stacy Dee

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Approved by

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Committee Chair

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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Date of Acceptance by Committee

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Date of Final Oral Examination

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CHAPTER I

INTRODUCTION

Significance of Research

Recently, anthocyanins have become known publicly for their antioxidant properties and proposed role in reducing cancer risks (Montoro 2006, Wrolstad 2004, Shahidi 2000, Meskin 2004). They are also responsible for the vibrant red, blue and purple colors of fruits. These characteristics have launched research within the food industry to utilize anthocyanins as natural food colorants. In doing so, it is important to find inexpensive and readily available sources of anthocyanins. Additionally, these compounds must be stable in the food matrix. Research is currently being conducted on the stability and availability of anthocyanins in foods as well as several plant species (Alcalde-Eon 2004, Montoro 2006, Cortes 2006, Gradinaru 2003, Reyes 2007, Kirca 2007, Janna 2007, Gomez-Plaza 2006).

Anthocyanins

Anthocyanins are a subgroup of the flavonoid family. They are also classified as phytochemicals, which are biologically active compounds found in plants. The most commonly found anthocyanins are cyanidin, malvidin, delphinidin, petunidin, peonidin, and pelargonidin glycosides. Structurally, anthocyanins are glycosylated anthocyanidins (i.e. sugars attached to an anthocyanidin core) (Figure 1) (Shirley 1996).

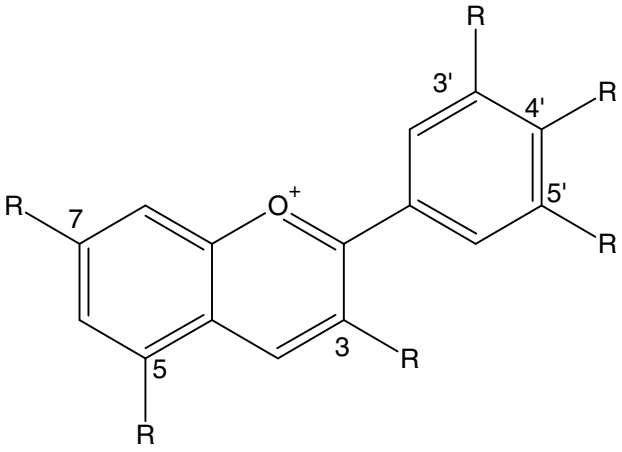

Figure 1. The basic structure of anthocyanidins.

The basic anthocyanidin structure is shown in Figure 1. Anthocyanidins are derived from 2-phenylchromenylium, where substituents may be attached to the 3, 5, 7, 3', 4', and 5' carbons. These R groups can be the same or different functional groups. The identity of the anthocyanidin (aglycone) is determined by the identity of the R groups on the 3', 4' and 5' carbons (Figure 1). These groups are generally H, OH or OCH_3 as shown in Table 1.

Table 1. Anthocyanidin Substituents. These anthocyanidins have hydroxyl groups attached to the 3, 5 and 7 carbons in Figure 1.

Anthocyanidin	**3' R**	**4' R**	**5' R**
Pelargonidin	H	OH	H
Cyanidin	OH	OH	H
Peonidin	OCH_3	OH	H
Delphinidin	OH	OH	OH
Petunidin	OCH_3	OH	OH
Malvidin	OCH_3	OH	OCH_3

All of the anthocyanidins in Table 1 have hydroxyl groups in the 3, 5, and 7 positions, but they differ from each other in terms of how they are substituted in the 3',4' and 5' positio00ns. For example, pelargonidin has no substituents (only a hydrogen) at the 3' and 5' positions and a hydroxyl group at the 4' position, which would be indicative of the structure shown in Figure 2 below.

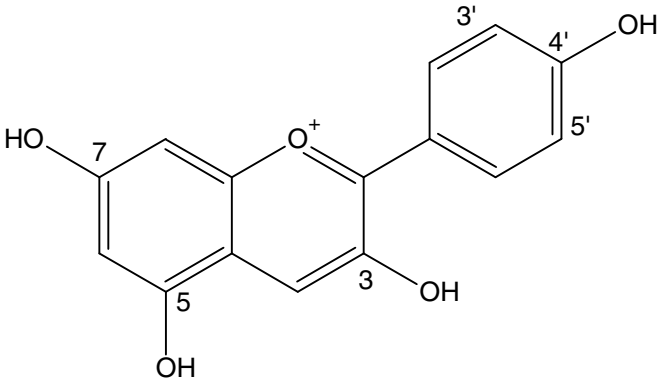

Figure 2. Structure of pelargonidin as indicated by Table 1.

Anthocyanins are anthocyanidins modified by the addition of sugars or acids. For this reason, anthocyanidins and are often referred to as aglycones. Figure 3 illustrates the basic anthocyanin structure where sugars or acids are added to carbons 3, 5 or 7 of the aglycone.

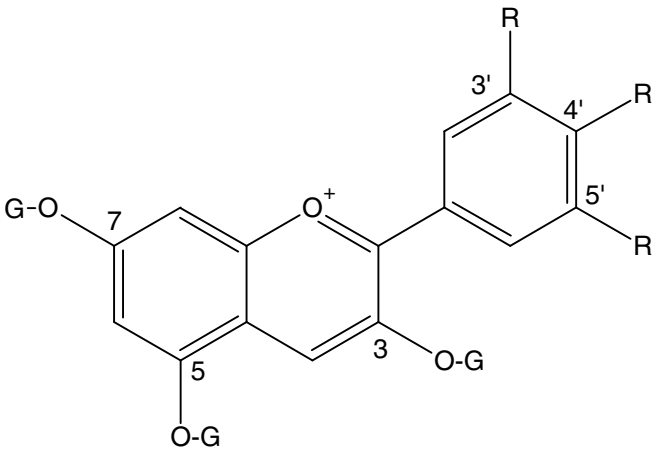

Figure 3. Basic anthocyanin structure. G indicates a position where a sugar or acid may be added.

Some possible sugar or acid substituents include glucose, galactose, rhamnose, xylose, arabinose, malose, malonic acid, caffeic acid, coumaric acid, malic acid and ferulic acid (Meskin 2004, Shi 2002). Caffeic, coumaric and ferulic acids may act as acylating agents to yield acylated anthocyanins (Baublis 1995, Meskin 2004, Shi 2002). The position where these sugars or acids are most commonly found is the 3 carbon, such as in cyanidin-3-glucoside. However, any of the acids or sugars may be found in any combination at carbons 3, 5 and/or 7. There may even be multiple sugars or acids added to one position, for example cyanidin-3-diglucoside.

It is known that anthocyanins are produced biosynthetically within plants (Holton 1995, Shirley 1996, Shirley 2002). As seen in Figure 4, there are three main branches to the anthocyanin biosynthetic pathway. The first produces pelargonidin and its glycosides, the second cyanidin and peonidin and their glycosides, and the third delphinidin, petunidin, malvidin and their glycosides.

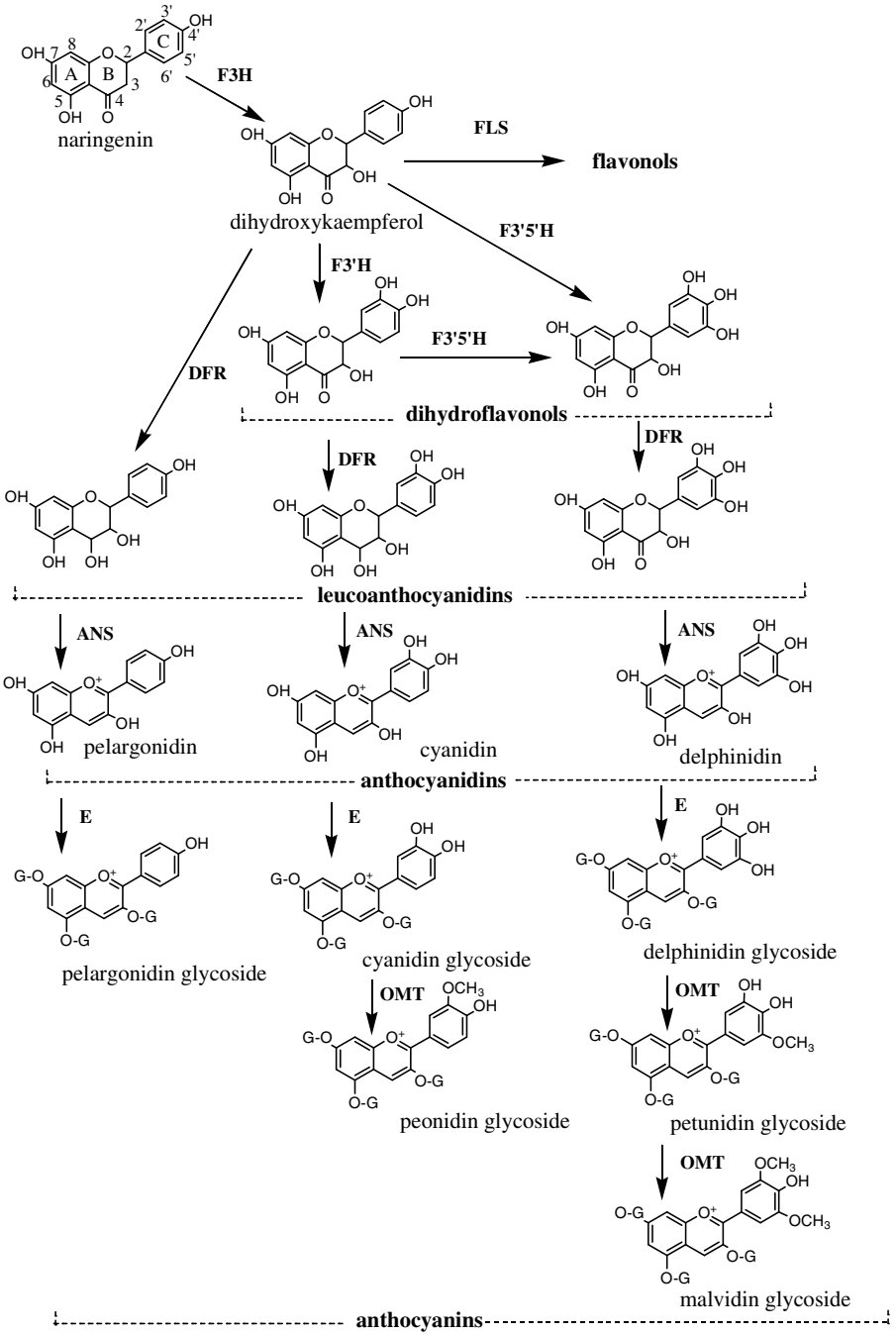

Figure 4. Anthocyanin biosynthetic pathway (Stiles 2007). The enzymes in this pathway are as follows: flavone-3-hydroxylase (F3H), flavonol synthase (FLS), dihydroflavonol-4-reductase (DFR), flavonoid-3'-hydroxylase (F3'H), flavonoid-3',5'-hydroxylase (F3'5'H), anthocyanidin synthase (ANS), and O-methyltransferase (OMT). E represents the enzymes that convert the anthocyanidins to the anthocyanins, which could include but are not limited to OMT, rhamnosyl transferase, UDP flavonoid glucosyl transferase and flavonoid -3-glucosyl transferase.

Plantago lanceolata

A recent collaborative study conducted in the Cech and Lacey laboratories was the first to investigate anthocyanin content of *Plantago lanceolata* (Stiles 2007). *Plantago lanceolata* is a perennial plant known in the South as a hopper weed. Other common names for this plant are ribwort plantain and plantagina. This plant has the ability to modify the color of its floral parts in response to surrounding temperature as a way to regulate temperature of the flowers (Lacey 2005). Floral parts darken (increase concentration of pigments) in cool temperature so they absorb more heat, while in warm temperature they do just the opposite (Shaked-Sachray 2002, Stiles 2007). It is hypothesized that regulation in color improves the ability of the plants to reproduce at various temperatures. The change in *P. lanceolata's* color that is associated with change in temperature is one example of phenotypic plasticity, which is the ability of an organism of a specific genotype to change its phenotype or a phenotypic characteristic in response to an environmental change (Lacey 2005). The pigments responsible for *P. lanceolata's* changes in color have been determined to be anthocyanins (Stiles 2007).

In the Stiles study, the anthocyanins present in *P. lanceolata* were tentatively determined to be glycosides from two of the three branches of the anthocyanin biosynthetic pathway (Table 2).

Table 2. Anthocyanins present in *P. lanceolata* (abbreviations are glu = glucose, gal = galactose,malo = malonic acid, caf = caffeic acid, mal = malose, mali = malic acid , n.i. = not identified , fer = ferulic acid, rha = rhamnose, cou = coumaric acid, xyl = xylose, and arab = arabinose) (Stiles 2007).

Name	Anthocyanin Mass	Aglycone Mass	Glycone Mass and Tentative Assignment
Cyanidin glycoside a	611	287	162 (glu/gal/caf),162 (glu/gal/caf)
Cyanidin glycoside b	449	287	162 (glu/gal/caf)
Cyanidin glycoside c	697	287	162 (glu/gal/caf), 248 (mal+glu/gal)
Cyanidin glycoside d	783	287	599 (n.i.)
Cyanidin glycoside e	463	287	176 (gluc/fer)
Cyanidin glycoside f	449	287	162 (glu/gal/caf)
Cyanidin glycoside g	535	287	248 (malo+glu/gal or mali+xyl/arab)
Cyanidin glycoside h	449	287	162 (glu/gal/caf)
Delphinidin glycoside a	713	303	410 (n.i.)
Delphinidin glycoside b	465	303	162 (glu/gal/caf)
Peonidin glycoside a	477	301	176 (gluc/fer)
Petunidin glycoside a	479	317	162 (glu/gal/caf)
Petunidin glycoside b	625	317	146 (rha/cou), 162 (glu/gal/caf)
Petunidin glycoside c	625	317	146 (rha/cou), 162 (glu/gal/caf)
Petunidin glycoside d	479	317	162 (glu/gal/caf)
Petunidin glycoside e	565	317	248 (malo+glu/gal or mali+xyl/arab)
Petunidin		317	

Each of the anthocyanins listed in Table 2 are shown with tentative assignments of anthocyanidin core (aglycone) and attached sugars or acids (glycone). For purpose of clarity, these tentative anthocyanin labels will be in bold. The assignments of both the anthocyanin and the associated sugars were based on mass spectral studies involving fragmentation of the parent anthocyanins (see Chapter II). However, there are multiple

anthocyanins and glycones that have the same molecular weights and similar fragmentation patterns. Therefore, based on these mass spectrometry studies alone, conclusive identification of anthocyanidins and their substituents is not possible (Stiles 2007).

Objectives of Research

Our objectives in this research are to identify some of the anthocyanins present in *P. lanceolata* using NMR and determine their stability under various storage conditions. To achieve these objectives, methods of extraction, sample preparation, sample concentration, hydrolysis, and separation must be optimized.

CHAPTER II

IDENTIFICATION OF ANTHOCYANINS WITH REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY/ELECTROSPRAY IONIZATION MASS SPECTROMETRY

Introduction

Overview of Instrumentation

Reversed phase high performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC-ESI-MS) is the major technique that will be employed in these studies to investigate anthocyanins. In reversed phase HPLC, a column with a non-polar stationary phase (solid phase bound to the inside of the column) and a polar mobile phase (phase that flows over the stationary phase) is employed. When a sample is injected onto the column, the hydrophobic compounds interact with the non-polar stationary phase to a greater extent than the more polar compounds. As a result, the hydrophobic compounds are retained longer and the polar compounds pass through the column more quickly. The mobile phase solvents can be altered to be increasingly non-polar to elute compounds that are more hydrophobic (Cunico 1998).

As the compounds are eluted from the column, they are directed to an electrospray ionization (ESI) source. Ionization occurs at atmospheric pressure in electrospray, thus, it is known as an atmospheric pressure ionization (API) technique. In ESI, the liquid flows through a fused silica or stainless steel capillary. An applied

voltage and coaxial flow of nitrogen gas cause the liquid to form an aerosol of charged droplets. Generally, ESI is referred to as a soft ionization technique because the ions created by this technique are not fragmented. Ions created by ESI can, however, be broken down into fragments in the mass analyzer through collisions with gas molecules. This process is termed collisionally induced dissociation (CID) or collisionally activated dissociation (CAD) (Watson 1997, Cole 1997).

The ions produced by ESI enter the mass analyzer and are separated based on their mass to charge ratios (m/z). The most common mass analyzers used in combination with ESI are quadrupole mass filters (single or triple) and quadrupole ion traps. For the mass spectrometer used in this research, the analyzer was a quadrupole ion trap. In an ion trap mass analyzer, the ions enter a chamber consisting of a ring electrode and two endcap electrodes. A small negative DC voltage is applied to the endcaps and the ring electrode to keep positively charged ions entering the trap. A radio frequency potential is applied to the ring electrode, causing the ions within the trap to oscillate. As this voltage increases, ion trajectories become increasingly unstable and ions with smaller m/z ratios are ejected, followed by ejection of larger m/z ions. This voltage increase can be controlled to allow the retention of a certain m/z ion (Watson 1997). Once ejected from the trap, the ions enter an ion multiplier detector and produce a current that is related to ion abundance.

The ion trap mass analyzer can be employed to generate fragment spectra through the process of collisionally induced dissociation. In collisionally induced dissociation with an ion trap, an ion of a particular m/z is collided with helium gas within the trap and

the resulting fragments are analyzed in the same way as the precursors. The mass spectrum produced by collisionally induced dissociation of a selected precursor molecule is referred to as an MS-MS spectrum because it has been produced through two stages of mass analysis. The process of isolating and fragmenting precursor ions can be repeated multiple times with an ion trap, generating additional fragmentation spectra from the fragments. These spectra are referred to as MS^3, MS^4, etc. depending on how many stages of fragmentation have been carried out.

Extraction and Analysis of Anthocyanins

One of the first steps in this project was to determine which anthocyanins might be present in *P. lanceolata.* This was achieved by extracting the anthocyanins in a suitable solvent, followed by separation and analysis by HPLC-ESI-MS. Currently, there are a number of techniques that can be used to extract anthocyanins. Many of them utilize acidic solvents as extraction media (Wu 2005, Gao 1994, Fossen 2002, Alonso-Salces 2005). Acidic medium, while widely used, may be unnecessary. It may even hydrolyze some anthocyanins such as malvidin 3-O-acetylglucoside (Revilla 1998).

Preliminary studies in our laboratory showed very little difference in acidic and neutral extraction solvents; thus, in these studies, methanol was used as an extraction solvent without acidification. The first method used for extraction (shown in methods below) was identical to that employed for previous *P. lanceolata* research in the Cech lab (Stiles 2007).

The analysis technique employed was reversed phase HPLC-ESI-MS. The usefulness of HPLC-ESI-MS for analysis of anthocyanins is shown by several

anthocyanin studies that have been conducted using this technique. In 1999, Giusti and coworkers determined that tandem electrospray ionization mass spectroscopy using a triple quadrupole mass spectrometer was an effective technique for the identification of anthocyanins (Giusti 1999). In 2005, Wu and coworkers used HPLC with a diode array detector and with an ion trap mass spectrometer to identify anthocyanins in common nuts, grains and vegetables. The identification was based on comparison of retention times and mass spectral data with that of known anthocyanins (Wu 2005). Similarly, in 2006, Longo and coworkers used an HPLC coupled to a diode array detector and a quadrupole mass spectrometer to identify anthocyanins in *Smilax aspera* berries.

For the research presented, electrospray ionization mass spectrometry coupled to reversed phase HPLC will be used as a method for the analysis of anthocyanins in *Plantago lanceolata*. To our knowledge, our research groups are the first to investigate anthocyanin content of this plant.

Materials

For this and the following chapters the following HPLC grade solvents and standards were used: Chloroform was purchased from Sigma-Aldrich (St. Lewis, MO). Formic acid was purchased from Fisher (Fair Lawn, NJ). Methanol was obtained from Pharmco (Brookfield, CT). Acetonitrile was purchased from Burdick and Jackson (Morristown, NJ). Deuterated methanol was purchased from Acros Organics (NJ). Cyanidin chloride was purchased from Indofine (Hillsborough, NJ). Cyanidin-3,5-diglucoside was purchased from Sequoia Research Products and standards of peonidin

chloride, delphinidin chloride, malvidin chloride, petunidin chloride, pelargonidin chloride and 1R,9S-β-hydrastine were purchased from Sigma-Aldrich (St. Lewis, MO).

The *Plantago lanceolata* plants used were collected by the Lacey laboratory at the University of North Carolina at Greensboro. The genotypes used were high plasticity and were labeled BM12-4, G1-5, G10-3, GD1-4, GH5-1, GH5-4, DU2-4, B7-2 and BM6-5. These plants were stored in convirons with light conditions between 293 and 388 μM with 16 hour days at 15 ^{o}C and 8 hour nights at 10 ^{o}C.

Methods

Flowers (of genotypes BM12-4, G1-5, G10-3, GD1-4, GH5-1, GH5-4, DU2-4, B7-2 and BM6-5) in the pre-flowering stage were removed from the spikes of *P. lanceolata* by cutting the flowers away from the stalk with an Exacto knife. These flowers were then submerged in HPLC grade methanol (10 mL methanol/1 g plant material). Quantities of flowers used in extractions were 1 -10 g depending upon the quantity of spikes produced between collections. The extraction mixture was stored at -20^{o}C for 24 hrs. The solvent was removed by micropipette and stored in a separate container at – 20 ^{o}C. A volume of 50/50 methanol/water equal to the volume of methanol from above was added to the plant material. This mixture was kept at ~4^{o}C for 24 hrs. Finally, the 50/50 methanol/water solvent was removed by micropipette and combined with the methanol extract. This solution was filtered to remove particulate matter, such as bracts that may be transferred by pipette during removal of solvent, and stored at -20^{o}C until needed for study.

Prior to HPLC-ESI-MS analysis, an internal standard, 1R,9S-β-hydrastine in methanol (10^{-4} M), was added to 1.00 mL of extract for a final hydrastine concentration of 10^{-6} M. This sample was then mixed and centrifuged to remove particulate matter. All analyses were performed on the following equipment: a HPLC model HP1100 (pump, degasser and autosampler) (Agilent, Palo Alto, CA) with a 50 x 2.1 mm C18 Column (Prevail Packing, Alltech, Deerfield, IL), and an LCQ Advantage mass spectrometer with ESI source (Thermofinnigan, San Jose, CA). The mass spectrometer settings as used in positive ion mode were as follows: sheath gas pressure 20 arb, spray voltage 4.5 kV, capillary temperature 250 ^{o}C, capillary voltage 3 V, and tube lens offset 60 V. The mass spectrometer was tuned to caffeine at 195 m/z.

An injection volume of 10 μL and mobile phase rate of 0.200 mL/min with a linear gradient were used for the separation. The gradient, where A = 6 % acetic acid and B = acetonitrile, was: 0 - 20 min, 0 – 10 % B; 20 – 35 min, 10 – 15 % B; 35 – 45 min, 15 – 30 % B; 45 – 50 min, 30 – 100 % B; 50 – 60 min, 100 % B; 60 – 60.1 min, 100 – 0 % B; and 60.1 – 70 min, 0 % B

Results and Discussion

The anthocyanins suspected to be present in *P. lanceolata* are glycosides of cyanidin, petunidin, delphinidin and peonidin. These anthocyanins were tentatively identified based on UV absorption spectra and mass spectral fragmentation patterns (Stiles 2007). One way to identify the anthocyanins in *P. lanceolata* is by collisionally induced dissociation in the mass analyzer. The fragmentation spectrum (MS-MS spectrum) can be generated in this way, and the fragments can then be compared to the

known masses of sugar substituents and the mass of the precursor ion. An example of the approach used to identify anthocyanins is shown in Figure 5.

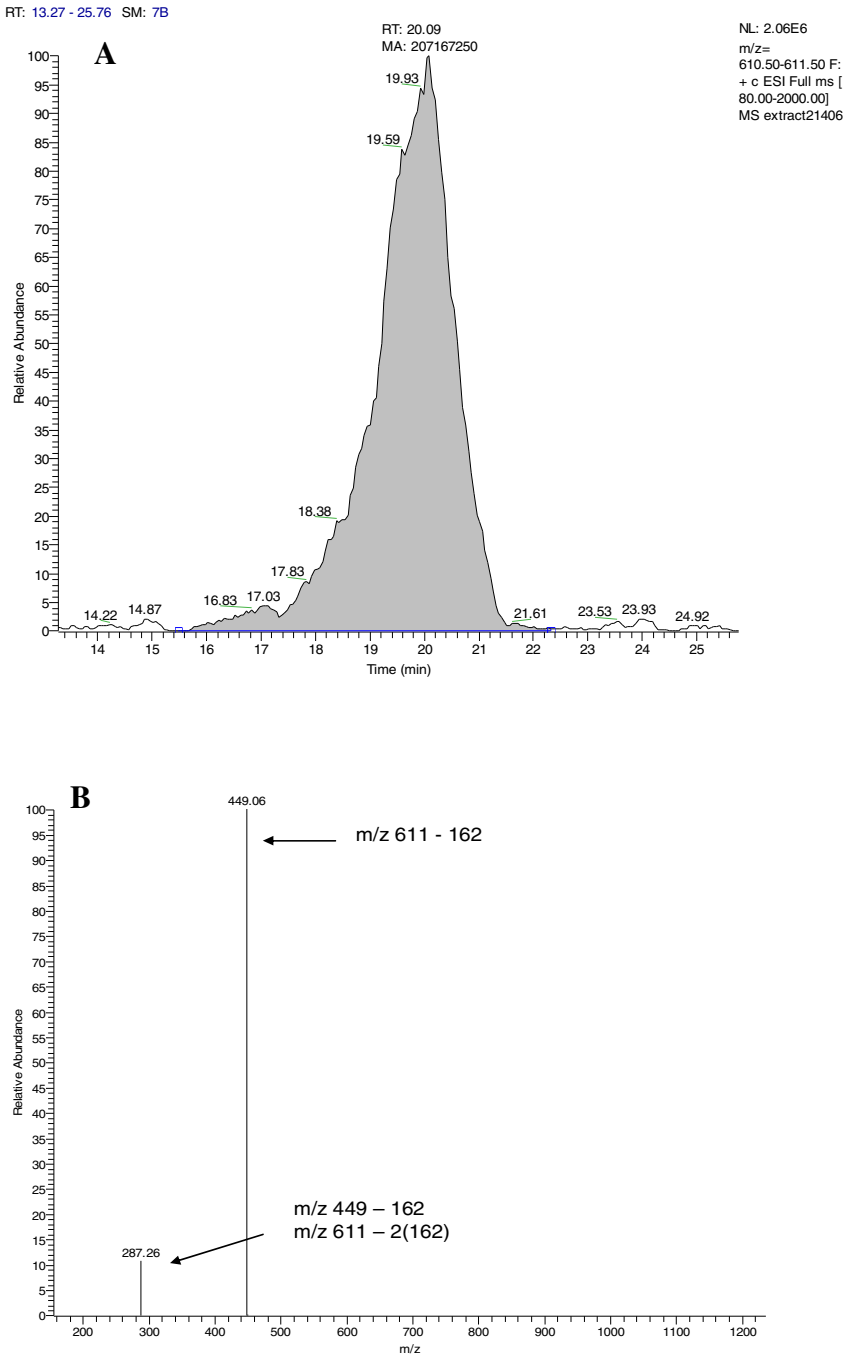

Figure 5. a) Selected ion chromatogram of m/z 611 (cyanidin glycoside a). b) MS-MS fragmentation spectrum of the ion with m/z 611.

Figure 5a shows the selected ion chromatogram of an ion with m/z of 611. The peak at 20.09 min was suspected to be an anthocyanin. To support the identity of this molecule as an anthocyanin, the mass spectrum (Figure 5b) was examined. Fragments at m/z 449.06 and 287.26 were observed. These peaks correspond to the neutral losses of one or two glycones, respectively, both of m/z 162. Based on these masses, the two glycones could be glucose, galactose or caffeic acid or any combination of two. Confirmation that this was an anthocyanidin was given by MS^3, in which the fragmentation of the 287 peak shown in Figure 5b matched that of a cyanidin standard (Stiles 2007). This compound was then labeled **cyanidin glycoside a**, as shown in Table 1. However, based on the mass spectral data alone, the glycoside can only be tentatively identified. The masses of the sugar substituents are known, but there are a couple of different sugars and/or acids that have the same mass. For example, a mass loss of 162 could correspond to glucose or galactose. Also, the positions of these substituents are unknown. Though the substituents usually are positioned at carbon three, they could also be found at carbons five and/or seven (Shi 2002). The substituents and their positions could be determined by comparison of retention times of known anthocyanins. There are several possibilities for the structure of each of the tentatively identified anthocyanins thereby making this method less useful for this research. NMR is another way that one may identify the structure of a pure unknown compound. This method would take several preparation steps as discussed in Chapter VI. Therefore, the names assigned in this and the previous chapter (**cyanidin glycoside a**, etc.) are only used for ease of

reference and, as of yet, we have not conclusively determined the structure *P. lanceolata* anthocyanins.

Implications for Future Research

While the method discussed above is useful for preliminary identification of the aglycone, there are several anthocyanin aglycones with similar mass and potentially similar fragmentation patterns. Thus, aglycone identity cannot be completely confirmed based on mass spectral fragmentation patterns alone. The hydrolysis experiments (Chapter III) will be used for further verification. Hydrolysis of anthocyanins from *P. lanceolata* will reduce them to their aglycone cores, thus allowing for direct comparison of the retention times of the aglycones from the hydrolysis procedure with those of anthocyanidin standards. The ability to match both retention time and mass of the standards will facilitate much more conclusive confirmation of aglycone structure than is possible based on the masses of aglycone fragments alone.

Additionally, a disadvantage of the method described in this chapter is the long 70 min run time. In general, published methods for anthocyanin analysis have relatively long run times, ranging from 30 – 102 min (Wu 2005, Cacace 2002, Revilla 1998, Giusti 1999, Gao 1994, Alonso-Salces 2005, Tsao 2003, Sakakibara 2003, Longo 2006). However, we intend to explore new gradients that may shorten the overall run time for the *P. lanceolata* extracts. A shorter run time would have the advantage of being able to run more samples in one period.

CHAPTER III

IDENTIFICATION OF ANTHOCYANIDIN CORES BY HYDROLYSIS

Introduction

A series of acid hydrolysis reactions were done in order to verify the identity of the anthocyanin aglycones. An acid hydrolysis of a glycoside consists of breaking the glycosidic bonds of the glycoside and adding water to reveal the aglycone and the original sugars (Shi 2002). This reaction is performed under acidic conditions, though basic conditions may be used as well. Since standards for many anthocyanins do not exist, it is not possible to confirm anthocyanin identity based on HPLC retention time comparison. However, standards are available for the most common anthocyanidins that make up the aglycone portions of anthocyanins. By performing hydrolysis prior to HPLC analysis, it is possible to compare retention times of unknown anthocyanidin hydrolysis products from the plant extract with those of standard anthocyanidins, thus facilitating identification of the anthocyanidin portion of the original anthocyanin.

Methods

Several acid hydrolysis methods have been published for anthocyanins (Gao 1994, Ichiyanagi 2001, Pazmino-Duran 2001, Kuwayama 2005). While most acid hydrolysis procedures use 2M HCl followed by 1 to 1.5 hours in a boiling water bath, 1% trifluoroacetic acid (TFA) solution has also been used (Ichiyanagi 2001).

A modification of the procedure used by Pazmino-Duran and coworkers was used for hydrolysis in these experiments. A known volume (1 mL) of methanol/water *P. lanceolata* extract (from genotypes BM12-4, G1-5, G10-3, GD1-4, GH5-1, GH5-4, DU2-4, B7-2 and BM6-5) was dried down using a Savant Speed Vac SC110 with a Savant Refrigerated Vapor Trap RVT 400 and a Savant ValuPump VLP 120. The resulting pellet was then resuspended in 1 mL of 2 M HCl. The samples were placed into a boiling water bath for 45 minutes then were removed and placed on ice to cool for 2 - 3 minutes. The solvent was once again evaporated in the SpeedVac. Finally, the extract was resuspended in 500 μL of 75/25 methanol/water and immediately analyzed by HPLC/ESI-MS using the same methods described in Chapter II. A standard mixture of cyanidin, peonidin, delphinidin and petunidin each at 1 x 10^{-4}M and 1R,9S-β-hydrastine at 1 x 10^{-6} M was also prepared and analyzed at the same time as the hydrolyzed extract.

Variations of this method involving use of the solid phase extraction (SPE) were tried in early hydrolysis attempts as a way to purify the sample before hydrolysis. The SPE steps included preconditioning a 5 g 6 cc tC_{18} Waters SPE cartridge with 6 mL of methanol and 6 mL of 0.01 % HCl in water. Concentrated extract was then added to the column. Water soluble compounds were eluted with 4 mL of 0.01 % HCl in water. The anthocyanins were eluted with 4 mL of 0.01 % HCl in methanol. After SPE, the samples were concentrated into pellets and resuspended in 2 M HCl, then subjected to the hydrolysis procedure above.

Results and Discussion

Anthocyanins with fragment ions at m/z values of 287, 301, 303, 317, and 331 were observed in the non-hydrolyzed *P. lanceolata* extracts, suggesting anthocyanidin cores of cyanidin, peonidin, delphinidin, petunidin, and pelargonidin, respectively. Thus, it was expected that ions with these m/z values would be present in the hydrolyzed extracts. When the hydrolyzed samples were run on the HPLC-ESI-MS, a chromatogram for an ion with a m/z value of 287 was observed, as shown in Figure 6. The peak identified as corresponding to cyanidin in the extract had an adjusted retention time of 31.73 min, in agreement with the adjusted retention time of the cyanidin standard (31.39 min). The retention times shown in Figure 6 were adjusted by subtracting the retention time of the solvent front to correct for poor synchronicity between the start time of the mass spectrometer and the HPLC injection time.

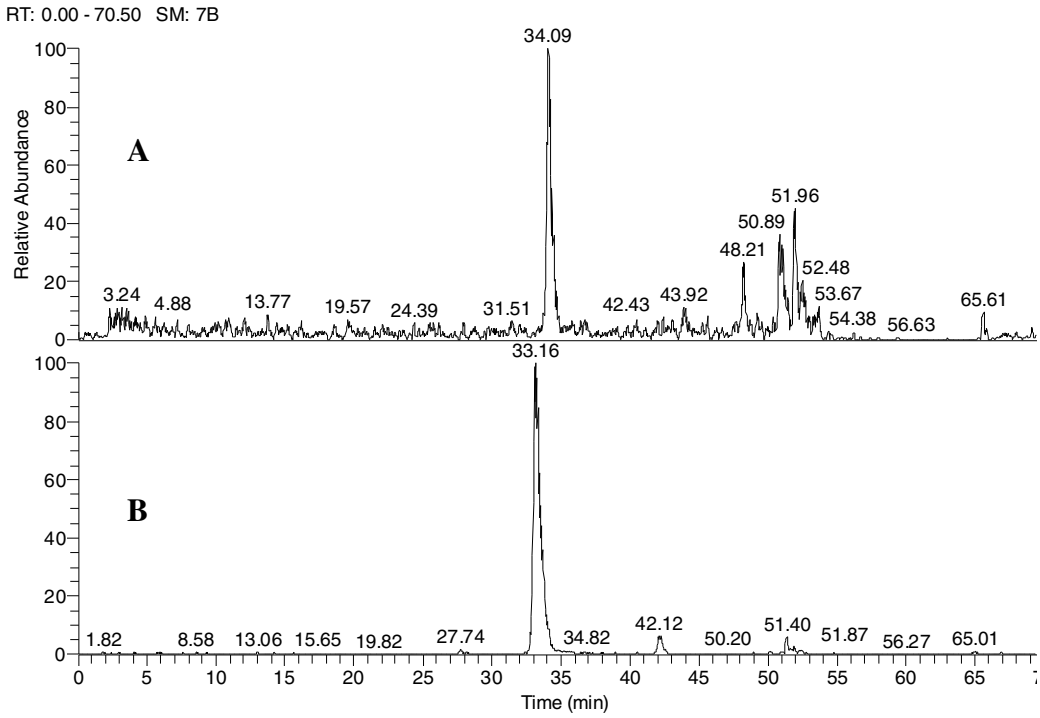

Figure 6. a.) Selected ion chromatogram of m/z 287 in a hydrolyzed extract of *P. lanceolata* and **b.)** for a standard of the anthocyanidin cyanidin of concentration 1 x 10^{-4} M .

The ion at m/z 287 in the hydrolyzed extract and the cyanidin standard had nearly identical MS-MS fragmentation spectra, as shown in Figure 7. The presence of the compound matching the mass and retention time of cyanidin in the hydrolyzed extract (Figure 6) and the match between standard cyanidin fragments and the fragments of the ion with m/z 287 in the hydrolyzed extract (Figure 7) verify the presence of cyanidin glycosides in the original *P. lanceolata* extract.

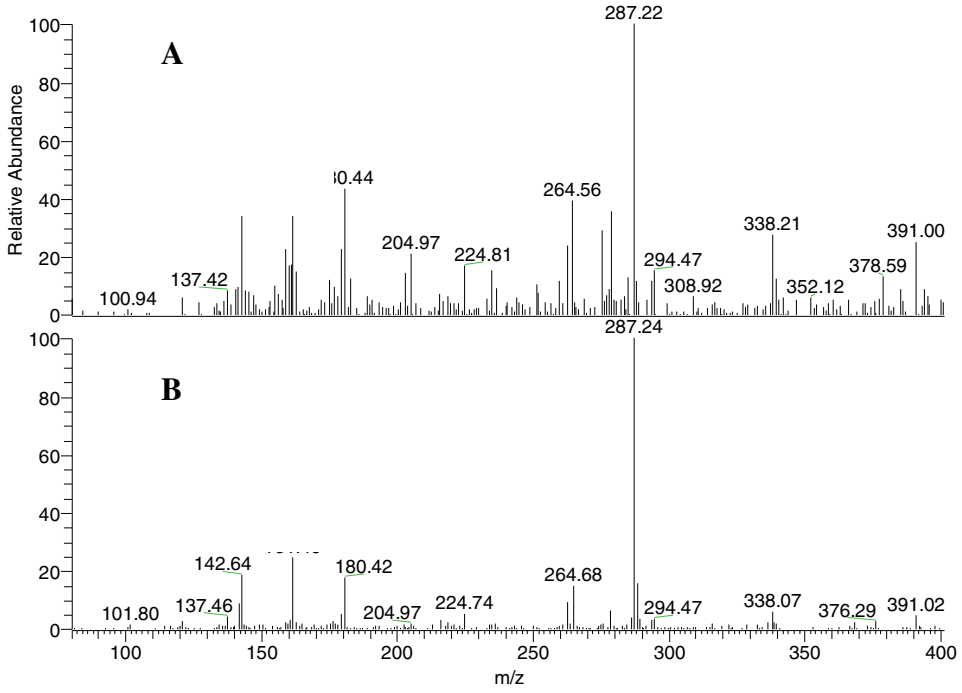

Figure 7. a.) MS-MS fragmentation spectra of precursor ion of m/z 287 for a hydrolyzed *P. lanceolata* extract and b.) a standard of cyanidin .

A small peak at m/z of 301, the mass of peonidin, was observed in the chromatogram obtained from analysis of the hydrolyzed extract (Figure 8). The adjusted retention time of this peak (40.36 min) matched the adjusted retention time of the peonidin standard (40.34 min.)

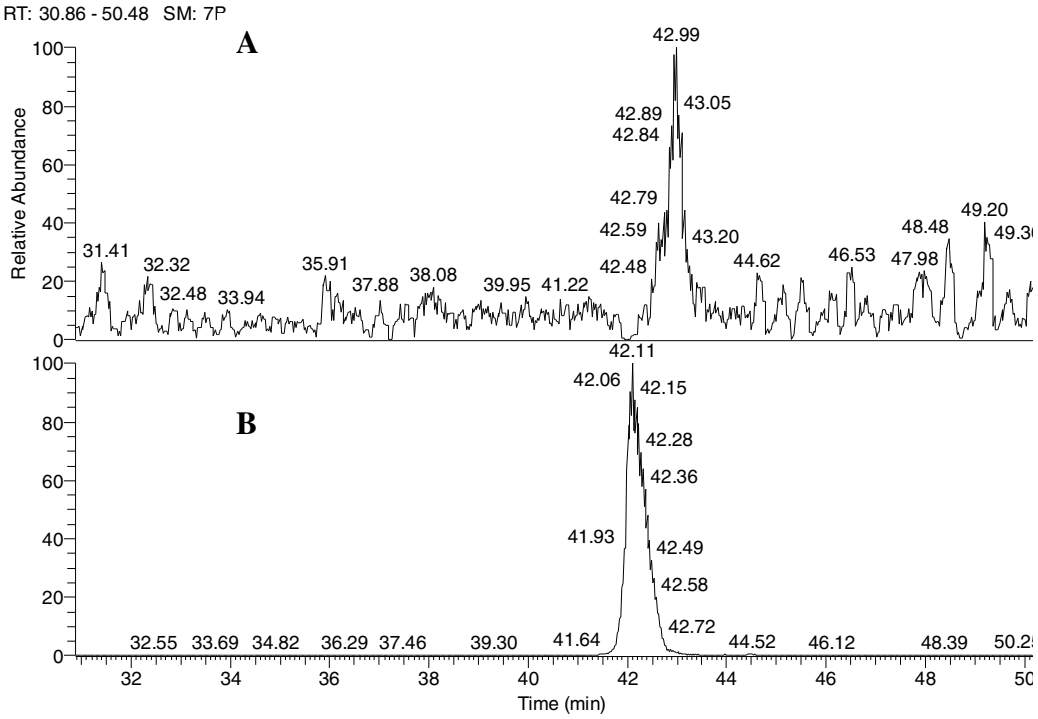

Figure 8. a.) Selected ion chromatogram of m/z 301 in the hydrolyzed extract and b.) for the peonidin standard at concentration of 1 x 10^{-4} M.

Inspection of the mass spectrum of the hydrolyzed extract at a retention time of 43.01 minutes demonstrates that ions of m/z 301 did elute from the column during this time period (Figure 9). The ion with m/z 301 was not, however, the most abundant and hence was not fragmented. Therefore, a repeat of the hydrolysis and analysis experiment was required. In the repeated experiment, the ion trap was set to fragment the ion with m/z 301, and an MS-MS spectrum for this ion was then obtained. Figure 10 demonstrates that there is a match between the fragments produced from the ion at m/z 301 from the extract and the fragments of the peonidin standard.

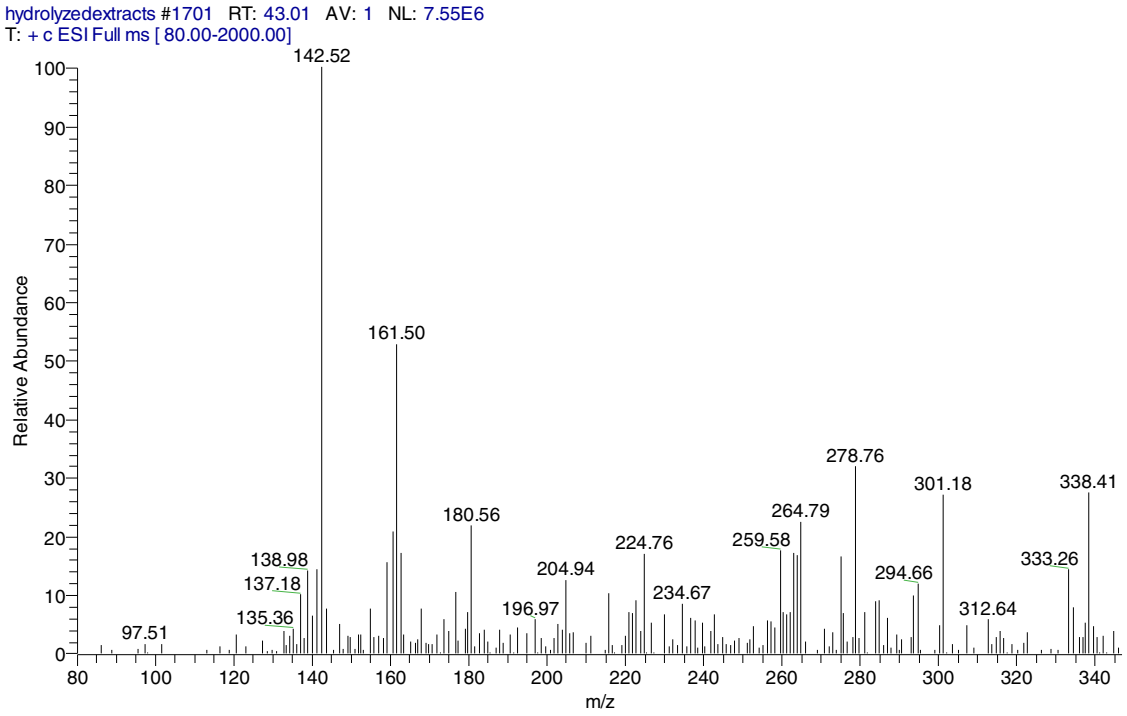

Figure 9. Full MS-MS of hydrolyzed extract at 43.01 min (the retention time of the chromatographic peak corresponding to m/z 301).

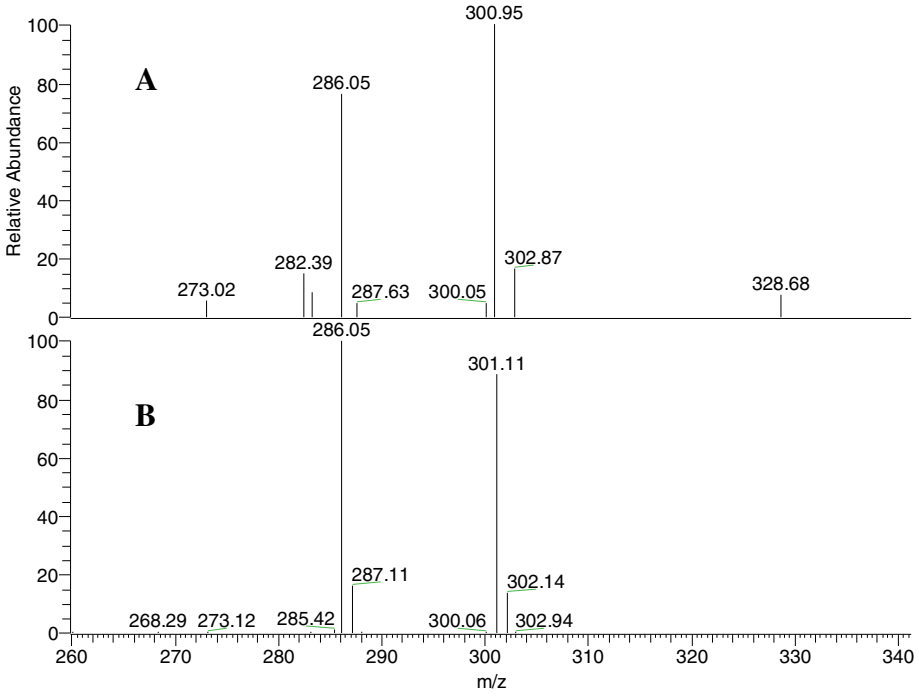

Figure 10. a) MS-MS spectra of the ion at m/z 301 from hydrolyzed extract and b.) the peonidin standard. In both cases, the precursor ion had an m/z of 301.

While peonidin and cyanidin were identified in the hydrolyzed extract, the petunidin and delphinidin were not able to be conclusively identified. Although there were fragments detected at the correct retention time and with the same fragmentation pattern as petunidin, due to the lack of a clear chromatographic peak, the presence of petunidin in the hydrolyzed extract was not verified. Analysis of the hydrolyzed extract generated no chromatographic peak or MS-MS spectrum for an ion with the m/z of delphinidin.

Troubleshooting for Hydrolysis

One reason for the failure to fully detect anthocyanidins other than cyanidin or peonidin in the hydrolyzed extract would be the loss or destruction of the lower abundance anthocyanidins in the some step of the hydrolysis. To verify that all four anthocyanidins could be detected after hydrolysis, an aliquot of anthocyanidin standards was submitted to the same hydrolysis procedure described for the extract. After the hydrolysis procedure, it was still possible to detect all four anthocyanidins in the standard solution. The relative abundances of the anthocyanidins in the solution after hydrolysis was similar to that in the original solution, demonstrating that selective loss of the anthocyanidins did not occur as a consequence of the hydrolysis. There did appear to be a slight decrease in overall anthocyanidin abundance for all of the anthocyanidins after the hydrolysis procedure.

To get some idea of how much loss in anthocyanidin signal resulted from hydrolysis, a comparison was made of the relative peak areas of hydrolyzed and non-hydrolyzed standard solutions and the percent difference was determined (equation 1). The variables are as follows x_i = initial peak area, x_f = final peak area and x_{avg} is the average of x_i and x_f.

$$\%\,Difference = \frac{\left(x_i - x_f\right)}{x_{Avg.}} \times 100\% \qquad \text{(equation 1)}$$

The results of these calculations for all four anthocyanidins are shown in Table 3. The four percentage losses shown in Table 3 are 10, 8, 22, and 8 percent for petunidin, peonidin, delphinidin and cyanidin, respectively. Three out of the four are well within the variability of the instrument from run to run, which was 12 – 16 % as discussed in Chapter IV. Therefore, it is not clear whether the observed decreases are a consequence of instrumental variability or truly a decrease in the amount of anthocyanidins in the sample. Follow up experiments with replicate hydrolysis samples would be required to determine the statistical significance of the loss in anthocyanidin signal, however, it is clear from these experiments that any loss would be small relative to the instrument variability.

Since the cyanidin glycosides were in higher relative abundance than the other glycosides, a small loss in signal as a consequence of the hydrolysis would likely not have prevented them from being detected. Such a loss might, however, have prevented the detection of anthocyanidins from some of the lower abundance anthocyanins. In order for a small loss to be problematic the anthocyanidins would need to be near the limit of detection.

Table 3. Percent differences between hydrolyzed and non-hydrolyzed standard anthocyanidin solutions.

Anthocyanidin	% Difference
Petunidin	10
Peonidin	8
Delphinidin	22
Cyanidin	8

In order to address the possible limit of detection of the method, a calibration curve was run for each anthocyanidin standard at concentrations of 8×10^{-5}, 6×10^{-5}, 4×10^{-5}, 2×10^{-5}, 1×10^{-5}, 5×10^{-6}, 1×10^{-6}, 5×10^{-7} and 1×10^{-7} M. The peak area relative to that of the internal standard (as shown in equation 2 where A_P is the area of the anthocyanin peak of interest, $A_{I.S.}$ is the area of the internal standard, and R.P.A. is the relative peak area) was plotted against the prepared concentrations of cyanidin, peonidin, delphinidin and petunidin standards (Figure 11).

$$RPA = \frac{A_p}{A_{I.S.}} \qquad \text{(equation 2)}$$

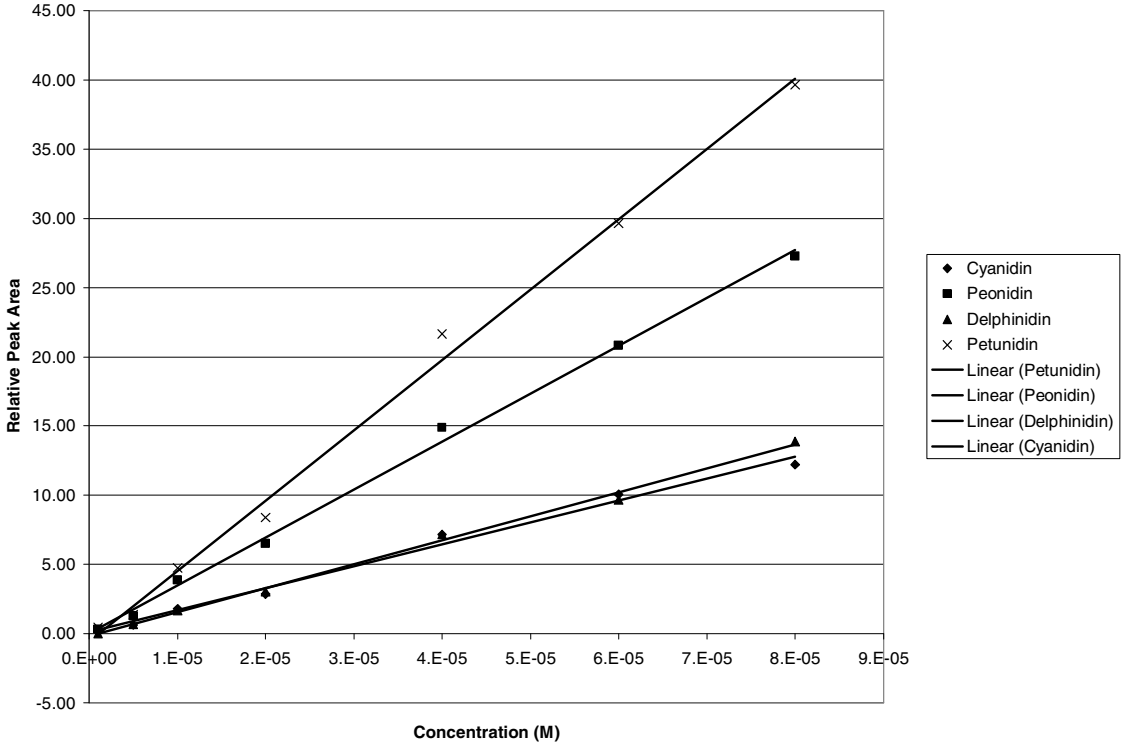

Figure 11. Calibration Curves of Anthocyanidin Standards.

Only peonidin and petunidin were detected at 1 x 10^{-6} M. No anthocyanidins, however were detected for the 5 x 10^{-7} and 1 x 10^{-7} M concentrations. Estimated limits of detection were 1.0 x 10^{-6}, 8.1 x 10^{-7} M, 2.1 x 10^{-6} and 6.2 x 10^{-7} M for cyanidin, peonidin, delphinidin and petunidin, respectively. Limit of detection (LOD) as used in this study was defined as the concentration at which the peak height of the analyte was 3x the height of the noise.

Any loss in anthocyanidins near these concentrations would be detrimental to the analysis even if this loss was within the 12 – 16 % variability of the method. However, the peak areas for the internal standard were lower than expected. This could be due to

limited sensitivity of the mass spectrometer throughout the duration of the experiment. Repeated experiments would be needed to address this issue.

Another possible reason for the failure to detect petunidin and delphinidin in the hydrolyzed extract could be that some of the anthocyanins were not hydrolyzed. This possibility was investigated by inspecting the chromatographic data obtained from the hydrolyzed extracts to determine whether these extracts still contained anthocyanins. It was found that **peonidin glycoside a** and **cyanidin glycoside e** were present in the original extract as well as the hydrolyzed extracts, suggesting these anthocyanins were only partially broken down in the hydrolysis procedure. Partial hydrolysis could explain why there was a very small peak corresponding to the molecular weight of peonidin in the hydrolyzed extract. The larger peak observed for cyanidin could be explained by the fact that there were multiple cyanidin glycosides; thus, some cyanidin could be detected even if not all of the cyanidin glycosides were hydrolyzed completely.

The tentative assignments of the glycones attached to both anthocyanins that were not completely hydrolyzed (**peonidin glycoside a** and **cyanidin glycoside e**) were glucuronic or ferrulic acids, implying that these are acylated anthocyanins (Table 2). Alkaline saponification with potassium hydroxide may be a more useful technique when substituents are conjugated coumaric or caffeic acids or acyl, such as ferrulic acid (Shi 2002). This could prove useful for the identification of peonidin, petunidin and delphinidin as well, since the major substituents for the anthocyanins with these aglycones appear to be ferrulic acid, caffeic acid and/or coumaric acids (Table 2).

Conclusion

It was determined through retention time comparisons of hydrolyzed extract with known anthocyanidins that cyanidin glycosides and peonidin glycosides are present in *P. lanceolata*. Petunidin and delphinidin were not detected in this series of acid hydrolysis experiments, and the reason for the failure to detect these anthocyanidins did not appear to be loss of the compounds during the hydrolysis procedure. It did appear, however, that some of the anthocyanins with these aglycones were not completely hydrolyzed by the acid hydrolysis procedure. Basic saponification may prove to be more useful in breaking the glycosidic bonds for these possibly acylated anthocyanins, and could be a useful avenue of future study.

CHAPTER IV

METHOD VALIDATION

Introduction

In order to address the possible variability or error in those measurements in Chapter II, several sets of data were analyzed for precision. Repeatability is one of the three levels of precision, and is a comparison of how closely data obtained in the same conditions within a short amount of time agree (Swartz 1997). Repeatability can be assessed by analyzing the deviations in measurements made in the same lab under the same conditions in a single day. Variabilty will be assessed in the method used for anthocyanin analysis by performing replicate analyses of the same extract in a single run. Another level of precision that looks at random difference within the lab from the instruments, operators or daily operating conditions over a longer period of time is intermediate precision. For our experiments, intermediate precision will be determined by comparing measurements of anthocyanin peak area of the same extract on different days and different extracts on different days. This will analyze the variability of the instrument from day to day and the variability of the extraction method. The third level of precision is reproducibility, which compares inter-laboratory data. This level will not be addressed in this study.

Methods

To evaluate the repeatability in the analysis process for replicate injections of the same extract on a single day, nine injections were made of a single extract prepared as in the methods section of Chapter II using genotypes G1-5, G10-3, GD1-4, GH5-1, GH5-4, DU2-4, B7-2, BM6-5. The analyses were performed on freshly prepared extract. Intermediate precision was evaluated based on triplicate analyses of the same extract analyzed on eight separate days. Finally, the intermediate precision or variation from extraction to extraction was analyzed by comparing four extracts that were prepared on different days using different sets of spikes from the same plants of one genotype (BM12-4). These extracts were all analyzed within the two days following extraction. Anthocyanins tentatively identified as **cyanidin glycoside a**, **cyanidin glycoside c**, **delphinidin glycoside b**, **peonidin glycoside a** and **petunidin glycoside b** were chosen as representative compounds for these studies.

The following calculations were performed to analyze the data. Relative peak areas were calculated by dividing the area of the peak in the selected ion chromatogram corresponding to the anthocyanin of interest by that of the internal standard. This calculation is demonstrated in Equation 2, where A_P is the area of the anthocyanin peak of interest, $A_{I.S.}$ is the area of the internal standard, and RPA is the relative peak area.

$$RPA = \frac{A_p}{A_{I.S.}} \qquad \text{(equation 2)}$$

The mean, standard deviation (equation 3), relative standard deviation (equation 4), coefficient of variation or percent relative standard deviation (equation 5) and confidence interval (equation 6) were also calculated for these analyses. The following abbreviations will be used in equations 2 – 5: x_i is one individual data point, $\bar{x}$ is the mean or average, s is the sample standard deviation, RSD is relative standard deviation, n is the number of data points, and t is the appropriate Student's t value at the 95 % confidence level (Harris 1999).

$$s = \sqrt{\frac{\sum_{i=1}^{N} (x_i - \bar{x})^2}{n-1}} \qquad \text{(equation 3)}$$

$$RSD = \frac{s}{\bar{x}} \qquad \text{(equation 4)}$$

$$\%RSD = CV = \left(\frac{s}{\bar{x}}\right) \times 100\% \qquad \text{(equation 5)}$$

$$CI = \bar{x} \pm t\left(\frac{s}{\sqrt{n}}\right) \qquad \text{(equation 6)}$$

Results and Discussion

The measures of precision for replicate injections of the same extract on a single day, when n = 9, are shown in Table 4. **Cyanidin glycoside a**, **cyanidin glycoside c**, **delphinidin glycoside b**, **peonidin glycoside a** and **petunidin glycoside b** had

coefficients of variations of 14, 12, 14, 15, and 16 %, respectively, as shown in Table 4. Peak area of each anthocyanin was ratioed to that of the internal standard, hydrastine in Tables 4-6.

Table 4 Repeatability analysis of replicate injections of the same extract on a single day. (n = 9, S.D. = standard deviation, C.V. = coefficient of variation, and C.I. = confidence interval)

Tentative Anthocyanin	Avg. Relative Peak Area	S.D.	C.V.	C.I. (95%)
Cyanidin glycoside a	11	1	14	11 +/- 1.1
Cyanidin glycoside c	2.3	0.3	12	2.3 +/- 0.2
Delphinidin glycoside b	0.9	0.1	14	0.9 +/- 0.1
Peonidin glycoside a	0.9	0.1	15	0.9 +/- 0.1
Petunidin glycoside b	1.6	0.3	16	1.6 +/- 0.2

The coefficient of variation from day to day on the same sample was determined to be 29, 28, 28, 28, and 27 percent for **cyanidin glycoside a** and **c**, **delphinidin glycoside b**, **peonidin glycoside a**, and **petunidin glycoside b** (Table 5) respectively. Here n = 8.

Table 5 Intermediate precision analysis of extract analyzed in triplicate on eight different days. (n = 8. S.D = standard deviation, C.V. = coefficient of variation, and C.I. = confidence interval)

Tentative Anthocyanin	Avg. Relative Peak Area	S.D.	C.V.	C.I. (95%)
Cyanidin glycoside a	9	3	29	9 +/- 1.7
Cyanidin glycoside c	1.8	0.5	28	1.8 +/- 0.4
Delphinidin glycoside b	0.8	0.2	28	0.8 +/- 0.1
Peonidin glycoside a	0.7	0.2	28	0.7 +/- 0.1
Petunidin glycoside b	1.3	0.3	27	1.3 +/- 0.3

The precision of the entire method, including both variability in day to day response of the instrument, and variability in the extraction procedure, is evident from the data reported in Table 6. These data were obtained from four different extracts using spikes collected on different days from the same plants. Each extract was prepared and analyzed separately, so day to day instrument variability in instrument response is included in these results as well. The C.V. values obtained from these analysis for **cyanidin glycoside a**, **cyanidin glycoside c**, **delphinidin glycoside b**, **peonidin glycoside a** and petunidin glycoside b were 12, 30, 61, 21 and 61 % respectively as shown in Table 6.

Table 6 Intermediate precision analysis from extraction to extraction. Analyses were conducted on four different extracts prepared and analyzed on different days. Here n = 4, S.D = standard deviation, R.S.D. = relative standard deviation, % R.S.D. = percent relative standard deviation, and C.I. = confidence interval.

Tentative Anthocyanin	Avg. Relative Peak Area	S.D.	C.V.	C.I. (95%)
Cyanidin glycoside a	8	1	12	8 +/- 1.6
Cyanidin glycoside c	2.0	0.6	30	2.0 +/- 0.9
Delphinidin glycoside b	1.1	0.6	61	1.1 +/- 1.0
Peonidin glycoside a	1.0	0.2	21	1.0 +/- 0.3
Petunidin glycoside b	1.1	0.7	61	1.1 +/- 1.1

In comparing Tables 4 – 6, it is easily seen that the percent relative standard deviations for the replicate injections on the same day were the smallest. These numbers represent the variability that could be due to the problems integrating asymmetrical peaks and to fluctuations in instrument response from run to run. The values for the C.V. for the extract run in triplicate over eight days (Table 5) were higher than the values obtained for

analyses in a single day (Table 4). This suggests that the variability in the overall instrument response over the course of several days is greater than that for a single day, as might be expected. Changing response of the instrument over time could, in part, be due to fouling of the internal lenses, although the use of the internal standard should correct for this problem, provided that the response for the internal standard is the same as that for the sample. Variability in results from day to day could also be caused by changes in temperature or other environmental factors, which could affect the sample as well as instrument response. Since the C.V. values reported in Tables 4 and 5 were fairly consistent for all of the different anthocyanins, it appears that any effect of differences in ambient conditions on the sample was minimal. However, since it is known from the literature that anthocyanins do degrade over time with exposure to light and temperature (Janna 2007) sample degradation may have partially explained the greater C.V. values observed in Table 5 versus Table 4. The possibility of degradation of anthocyanins in our samples under various storage conditions will be addressed in Chapter V.

Finally, the values for C.V. for the intermediate precision study from extraction to extraction were the highest (Table 6). This would be expected given that both variability from the analysis and from the extraction process contribute to the deviations in these data. Unlike the data from Tables 4 and 5, for the data in Table 6, the C.V. values vary greatly from anthocyanin to anthocyanin. These results imply that either there is variation in the extraction efficiency of the various anthocyanins in different extractions, or that different batches of spikes collected from the *P. lanceolata* plants at different

times contained different levels of anthocyanins, even though they were grown under controlled conditions (Stiles 2007). Although all spikes were pre-flowering when collected, there were some color differences between the spikes at various maturity levels. It seemed that the less mature spikes were more darkly colored relative to their size than their more mature counterparts. If the anthocyanin content of the spikes differed in young versus old spikes, these differences could have contributed to the variability observed in the data displayed in Table 5. The large variability from extraction to extraction indicates the importance of using replicate extracts when obtaining quantitative data on the anthocyanin content of plants. In addition, the greater variability in instrument response observed for analyses of the same extract over a several day period (Table 5) versus a single day (Table 4) indicates that erroneous interpretation could result from comparison of the analytical results obtained from two batches of extracts analyzed on two different days. It would be best to perform analyses of all extracts to be compared on a single day, or, alternately, to average multiple analyses of each extract performed on different days.

CHAPTER V

STABILITY OF ANTHOCYANINS IN *P. LANCEOLATA* EXTRACTS

Introduction

Several research groups have investigated anthocyanin stability (Alcalde-Eon 2004, Montoro 2006, Janna 2007). Factors that influence the stability of anthocyanins are temperature, type of anthocyanin, pH, light exposure and matrix. Also, anthocyanins acylated with cinnamic acids are thought to be the more stable, making them better candidates for natural food colorants (George 2001, Cevallos-Casals 2004).

The Montoro laboratory investigated the stability of anthocyanins in *Myrtus communis* berries stored frozen and in dark conditions using HPLC-ESI-MS. The findings of this research suggest that in this condition the anthocyanin content of that plant decreases by half after 6 months (Montoro 2006). The Janna lab determined that the anthocyanins of *Tibouchina semidecandra* were stable for 16 days longer when stored in the dark. Additionally, they found that lower temperatures and pHs were optimal as storage conditions (Janna 2007).

For a number of our investigations, it is necessary to store *P. lanceolata* extracts for long periods of time. We have been performing experiments to compare extracts produced from plants under different conditions, and it is important to ensure that differences in the extracts are due to differences in the plants and not to degradation

during storage. In addition, due to the low concentrations of anthocyanins in *P. lanceolata,* it is necessary for some applications that the extracts be stored and combined with other extracts to generate volumes large enough for analysis. Thus, knowledge of the stability of *P. lanceolata* anthocyanins for various lengths of time under various conditions is very important to this research. The bulk of anthocyanin extracts in our studies have been stored at – 20 ^{o}C. The experiments described here will allow us to determine whether there is any degradation of anthocyanins at room temperature, -20 ^{o}C, and at -80 ^{o}C in the 75/25 (v/v) methanol/water extraction solvent.

Methods

A batch of fresh extract (45.0 mL) was prepared for the study according to the extraction procedure described in Chapter II (genotypes used here were BM12-4, G1-5, G10-3, GD1-4, GH5-1, GH5-4, DU2-4, B7-2 and BM6-5). Hydrastine standard (450 μL) was added to the entire extract for a final concentration of 10^{-6} M. The extract was then separated into 9 plastic screw cap Falcon tubes in 5.0 mL aliquots. Three tubes were immediately stored at each of three different temperatures, -20 ^{o}C, -80 ^{o}C and room temperature. The anthocyanin content of each extract was determined before storage by drawing out 200 μL of sample from each tube and analyzing it using the methods described in Chapter II. The samples were tested for anthocyanin content each week for 4 weeks then every other week for 8 weeks and finally once per month for the duration of the study.

Results and Discussion

There was scatter throughout the duration of the study of the relative peak areas of the anthocyanins investigated (**cyanidin glycoside a** , **cyanidin glycoside c**, **delphinidin glycoside b** , **peonidin glycoside a** and **petunidin glycoside b)** (Figures 12a and b) in the -20 and -80 ^{o}C storage conditions. There was no obvious pattern of degradation, however, for any of the anthocyanins throughout the 140 day study, although statistical analyses would need to be performed to conclusively establish whether there is a trend in the data.

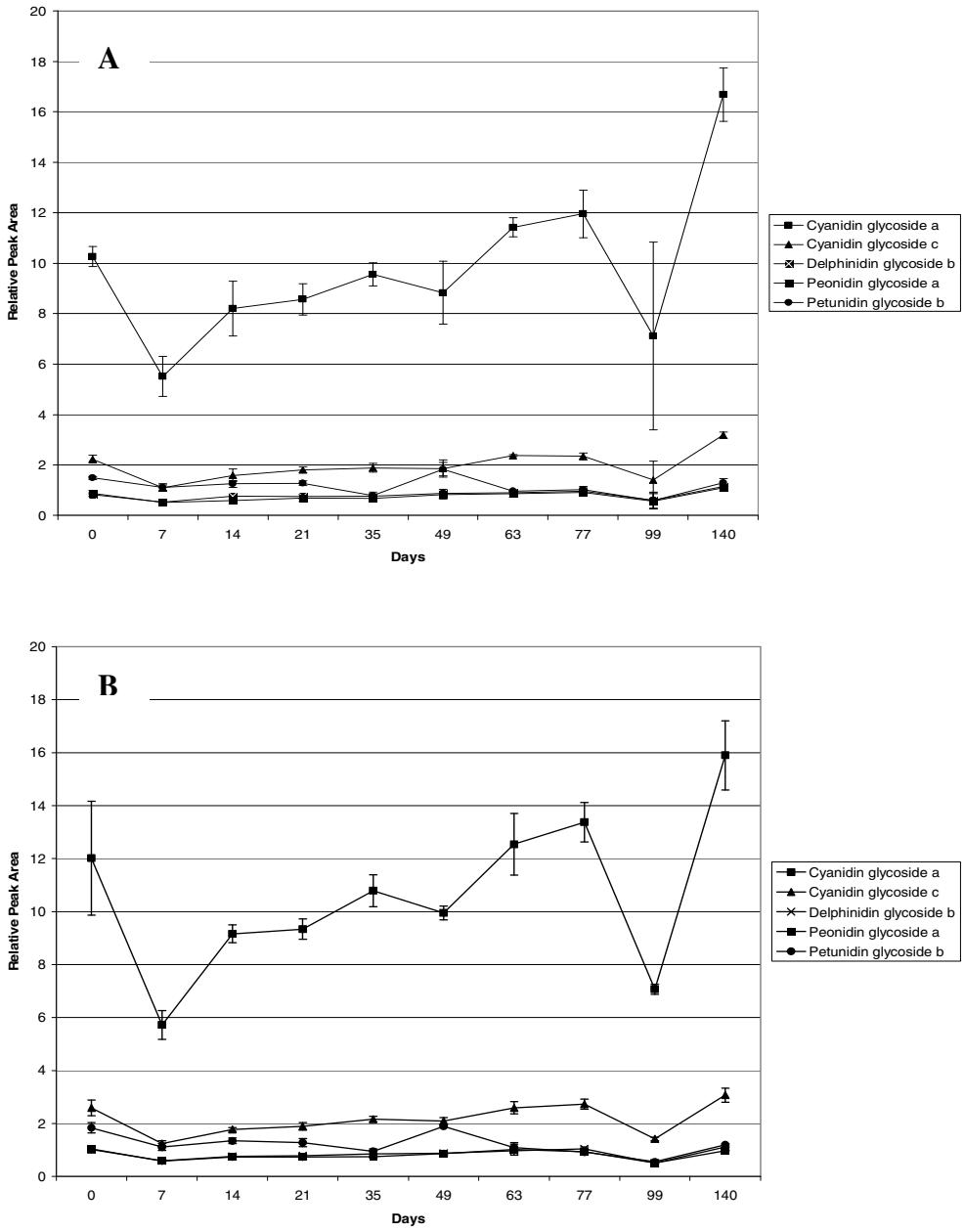

Figure 12. Concentration of anthocyanins in *P. lanceolata* extracts over time stored at -20°C (a) and -80°C (b). Number of replicates = 3. Error bars as shown indicate +/- 1 standard deviation.

It should be noted that the variability study did not take into account any degradation, which was originally postulated as a possible cause of some of the variability from day to day observed in anthocyanin peak area. However, since there was no clear downward trend in this study (Figure 12), it can be assumed that the variability from day to day is mainly due to method variations rather than degradation. As mentioned earlier, statistical analyses of the data could still reveal a trend that is not obvious from the raw results.

Unlike the other conditions, the room temperature samples seemed to show clear degradation in the **cyanidin glycoside a** and **cyanidin glycoside c** (Figure 13). Beginning on day 77 and continuing throughout the duration of the study, the relative peak areas for **cyanidin glycoside a** and **c** were below detection. The data for **delphinidin glycoside b**, **peonidin glycoside a** and **petunidin glycoside b** were somewhat scattered with no clear degradation, thus, it appears that these anthocyanins are more stable than the cyanidin glycosides at room temperature.

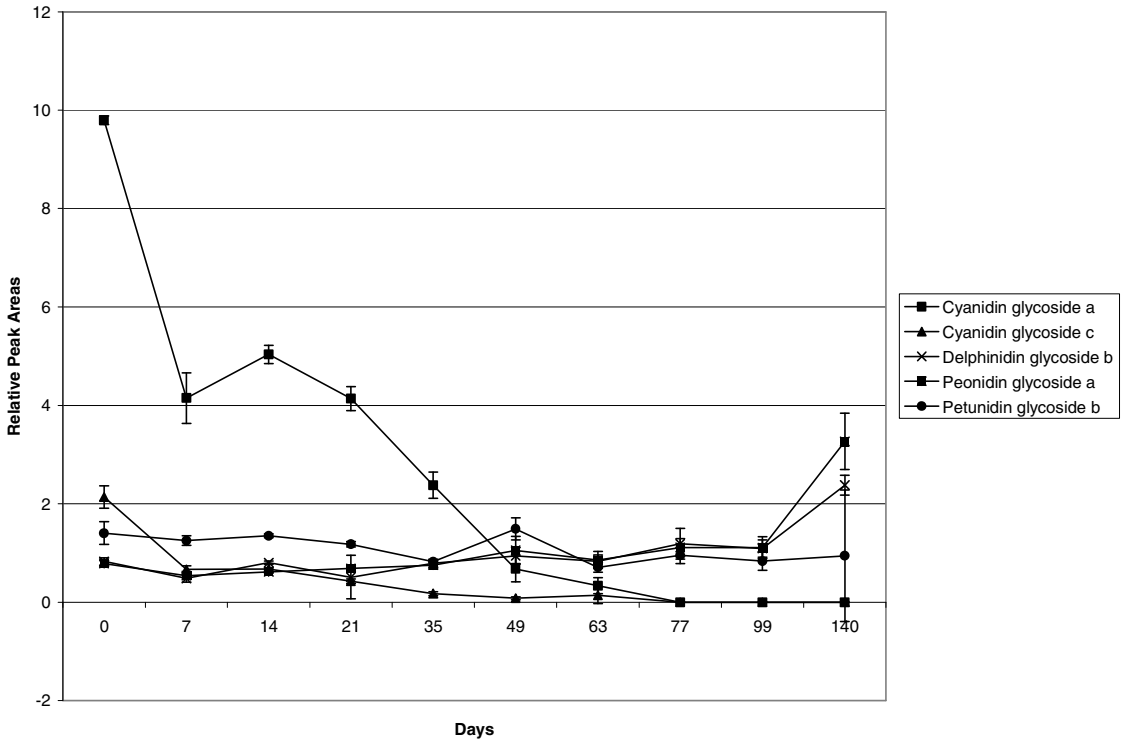

Figure 13. Concentration of anthocyanins in *P. lanceolata* extract as a function of time in samples stored at room temperature. Number of replicates = 3. Error bars as shown indicate +/- 1 standard deviation.

The data in Figures 12 and 13 demonstrate that while some *P. lanceolata* anthocyanins degrade in methanol:water extracts stored at room temperature, storage of these extracts at -20 to -80 ˚C appears to prevent degradation for up to 140 days post extraction. It is possible that slight degradation or even slight increase in anthocyanin concentration due to decomposition of proanthocyanins may occur. However, some anthocyanins (**delphinidin glycoside b**, **peonidin glycoside a** and **petunidin glycoside b**) do appear to be stable at room temperature, which could implicate these compounds as useful in food chemistry applications.

CHAPTER VI

SAMPLE PREPARATION AND NMR

In order to obtain NMR spectra of anthocyanins, milligram quantities of purified samples are needed. Several sample preparation steps are required to obtain such samples. Due to the low relative concentration of anthocyanins in *P. lanceolata*, concentration of the extract is needed. In addition, the *P. lanceolata* extracts are very complex, and separation is required to isolate individual anthocyanins. To meet these goals, concentration steps were performed and experiments with gradients and solid-phase extraction (SPE) were explored to improve separation and yield a pure sample. Due to the long run times of the previously published HPLC method for anthocyanin analysis (Stiles 2007), new gradients were explored in an attempt to achieve enhanced separation of anthocyanins in a shorter time period.

Development of Improved Gradients for Anthocyanin Separation

Several methods for the separation of anthocyanins that were about the same length of time as the original method (as discussed in the methods in Chapter II) were

tried in order to improve separation. Since the currently used separation method for anthocyanins requires 70 min per sample, it would be highly advantageous to shorten the run time. While shorter run time would make analysis by HPLC easier, the more important concern is separation. The current method does not adequately separate **peonidin glycoside a**, **petunidin glycoside d**, and **petunidin glycoside c** which currently coelute. Improving separations would make collection of pure anthocyanins from preparatory HPLC possible. Also any signal suppression resulting from coeluting compounds would be minimized.

Methods

In attempts to improve separation, several other methods similar in length to the original were tried. The first method tried was developed by the Cacace laboratory (Cacace 2002). This method involved the use of 5 % formic acid in water as solvent A and methanol as solvent B. The gradient was 0 – 30 min, 10-25% B; 30 – 50 min, 25 – 45 %; 50 – 55 min, 45 – 100 %; and 60 – 65 min, 100 % B. It is to be noted that the HPLC in our laboratory was setup with A as the organic and B as the aqueous solvents.

The second, third and fourth gradients were gradients employed by the Wu laboratory to separate anthocyanins in vegetables, nuts and grains, and fruits and berries. These gradients utilized 5% formic acid and methanol as A and B, respectively. Wu gradient 1 at 68 minutes in length was 0 – 2 min, 5 %B; 2 – 10 min, 5 – 20 %B; 10 – 15 min, 20 %B; 15 – 30 min, 20 – 30 %B; 30 – 35 min, 30 %B; 35 – 50 min, 30 – 45 %B; 50 – 55 min, 45 %B; 55 – 65 min, 45 – 5 %B; and 65 – 68 min, 5 %B (Wu 2004). Wu gradient 2 at 90 minutes in length was 0 – 2 min, 5 %B; 2 – 10 min, 5 – 20 %B; 10 – 15

min, 20 %B; 15 – 30 min, 20 – 25 %B, 30 – 35 min, 25 %B; 35 – 50 min, 25 – 33 %B; 50 – 55 min, 33 %B, 55 – 65 min, 33 – 36 %B; 65 – 70 min, 36 – 45 %B; 70 – 75 min, 45 – 53 %B; 75 – 80 min, 53 – 55 %B; 80 – 84 min, 55 – 70 %B; 84 – 88 min, 70 – 5 %B; and 88 – 90 min, 5 %B (Wu 2005 a). Wu gradient 2a at 68 minutes in length was 0 – 2 min, 5 %B; 2 – 10 min, 5 – 24 %B; 10 – 15 min, 24 %B; 15 – 30 min, 24 – 35 %B; 30 – 35 min, 35 %B; 35 – 50 min, 35 – 45 %B; 50 – 55 min, 45 %B; 55 – 65 min, 45 -5 %B; 65 – 68 min, 5 %B (Wu 2004).

A new 50 minute isocratic method was run on freshly prepared extract using 10 % B and 90% A (note A is 6 % acetic acid in water and B is acetonitrile). A shortened version of the 70 minute gradient employed by Stiles was also attempted. This gradient was also 50 minutes in length where 0 – 5 min, 5 % B; 5 – 35 min, 5 - 15 %B; 35 – 45 min, 15 – 30 %B; 45 – 45.1 min, 50 %B and 45.1 – 50 min, 50 %B. It is to be noted that the extracts used in all of the above methods in this chapter were from flowers of genotypes BM12-4, G1-5, G10-3, GD1-4, GH5-1, GH5-4, DU2-4, B7-2 and BM6-5.

Results and Discussion

To begin discussing the results of new gradient elutions, it is important to have a reference to refer back to. Therefore, the chromatograms for the Stiles gradient (employed in all of the anthocyanin research in our laboratory up until this point) are shown in Figure 14. In this and the following chromatogram figures, each chromatogram has a normalized (NL) value beside of the m/z label. This indicates what intensity the y-axis was normalized to. For example in Figure 14a, NL = 3.58×10^6, which means that the intensity at 100 % is 3.58×10^6. Additionally, instead of showing a total ion

chromatogram (TIC) in which the y-axis is a plot of the sum of the intensity of all ions detected by the mass spectrometer at any given time, a simpler way to view the chromatographic data is plot the intensity of only an ion with a particular m/z value versus time in a *selected ion chromatogram.* This type of chromatogram also eliminates noise while allowing coeluting ions to be visualized. The selected ion chromatograms for ions 477, 479, 611 and 625 corresponding to **peonidin glycoside a**, **petunidin glycoside d**, **cyanidin glycoside a** and **petunidin glycoside c** are shown in Figure 14. The ions with m/z 477, 479 and 625 were chosen for the demonstration of different gradients because these ions coelute, and one of the goals of the gradient optimization was to resolve coeluting ions. Ion 611 was well separated from other anthocyanins in the original method and, therefore, it was included in the following graphs to show whether the separation of ion 611 (**cyanidin glycoside a)** from other compounds was or was not maintained in each of the following methods. From top to bottom in each of the following figures, the masses selected are 477, 479, 611 and 625. In chromatogram a of Figure 14, at m/z 477 and retention time 45.00 min is a peak representative of **peonidin glycoside a**. Chromatogram b (479 m/z) shows **petunidin glycoside d** at 44.48 min. Chromatogram c (m/z 611) shows a peak at 22.43 min representing **cyanidin glycoside a**. Lastly, chromatogram d (m/z 625) shows **petunidin glycoside c** at 44.48 min. Peaks corresponding to **peonidin glycoside a**, **petunidin glycoside d** and **petunidin glycoside c** elute from the column within 0.52 min (apex to apex) of each other. **Petunidin glycosides c** and **d** elute at the same time. With manual collection from a preparatory HPLC, baseline resolved peaks need to be separated by at least 0.5 minutes to allow for

manual collection. Peak resolution for **peonidin glycoside a**, **petunidin glycoside d** and **petunidin glycoside c** was analyzed visually by inspection of overlaid chromatograms shown in Figure 15. The resolution was calculated using equation 9 below, where Δt_r is the difference in retention time and w_{av} is the average peak width at the baseline (Harris 1999).

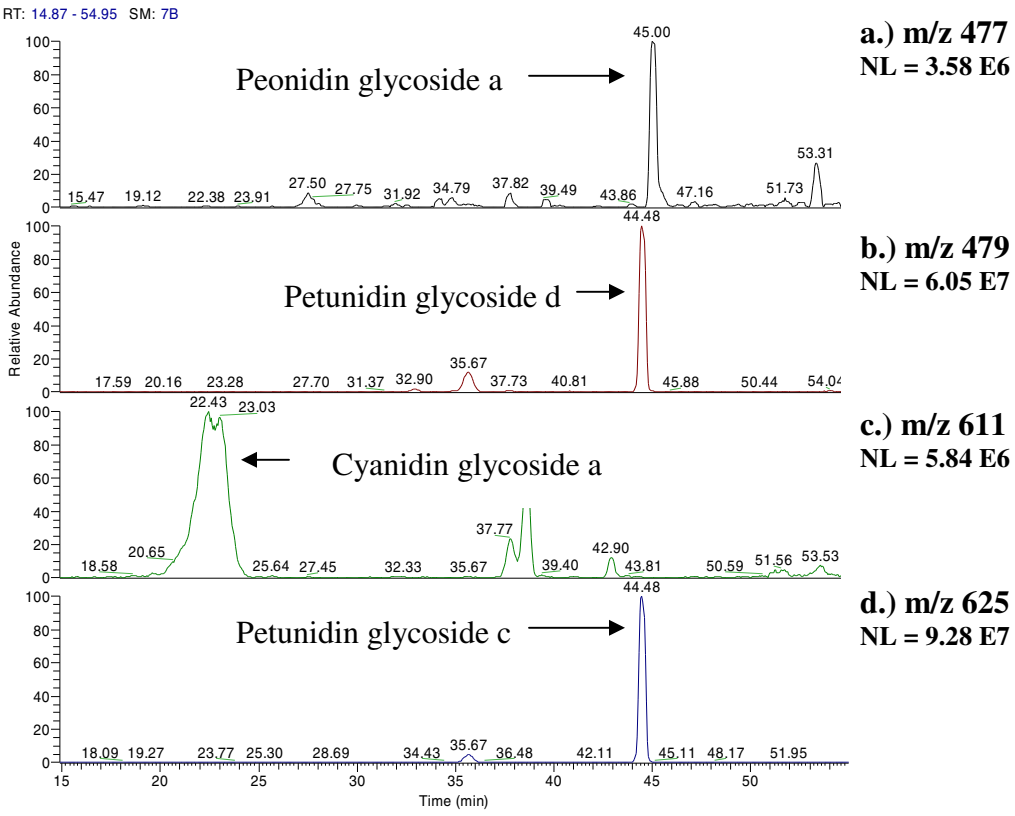

Figure 14. Selected ion chromatograms of four anthocyanins obtained from analysis of a *P. lanceolata* extract using the Stiles gradient (Stiles 2007).

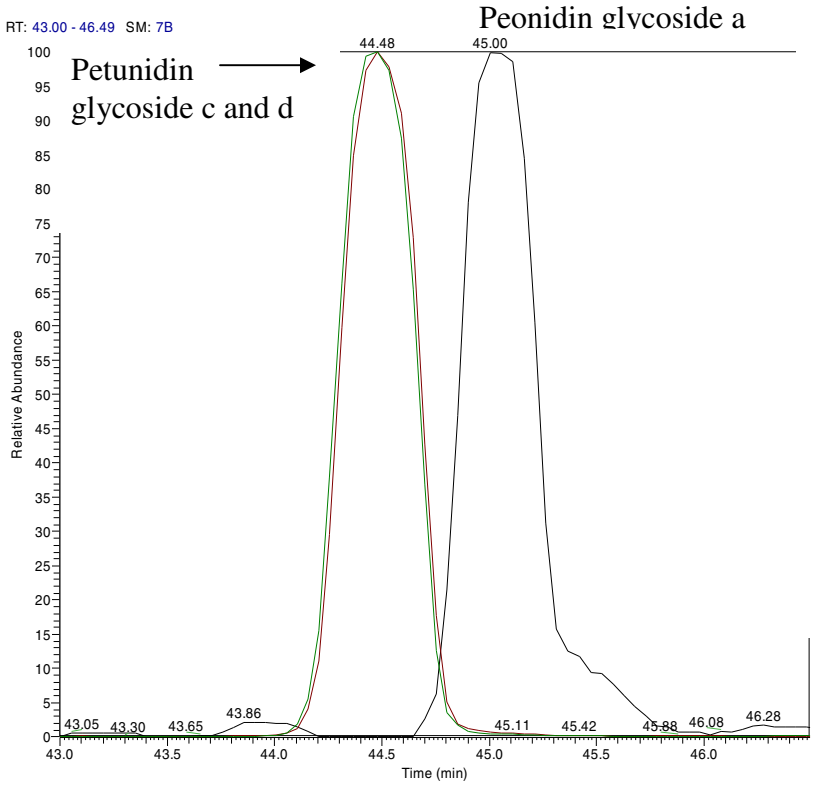

Figure 15. Overlaid HPLC-ESI-MS chromatograms of **peonidin glycoside a**, **petunidin glycoside d** and **petunidin glycoside c** using the Stiles gradient**.**

In Figure 15 it is evident that there is definite coelution of **petunidin glycoside d** and **petunidin glycoside c** with both eluting at 44.48 minutes. Additionally, the

resolution between **peonidin glycoside a** from the petunidin peaks is 0.83. Baseline resolution is said to occur at a resolution of 1.50 (Harris 1999).

$$resolution = \frac{\Delta t_r}{w_{av}}$$

(equation 9)

Since the separation is such a critical step, one 65 minute, two 68 minute gradients and one 90 minute gradient were attempted in order to improve separation. This first gradient was from the Cacace laboratory and is noted in the methods section of this chapter (Cacace 2002). With this new gradient, the peak shapes were similar to those of the original gradient and the closest eluting ions were **peonidin glycoside a** (48.69 min in Figure 16a) and **petunidin glycoside d** (49.02 min in Figure 16b). The separation between these two was 0.33 min with a resolution of 0.37. Additionally, **cyanidin glycoside a** (Figure 16c) coeluted with other compounds as shown in selected ion chromatogram a. There was no improvement in terms of resolution between **peonidin glycoside a** and **petunidin glycoside d** with this method. The separation and resolution for **petunidin glycoside d** and **petunidin glycoside c** was 1.60 and 1.77 respectively, indicating that these two ions would be able to be separated by manual fraction collection. In Figure 16b there seems to be a small peak at the same retention time as **petunidin glycoside c** at 50.62 minutes. This peak is attributed to the loss of a sugar via in-source fragmentation that can occur after the anthocyanin has eluted from the column.

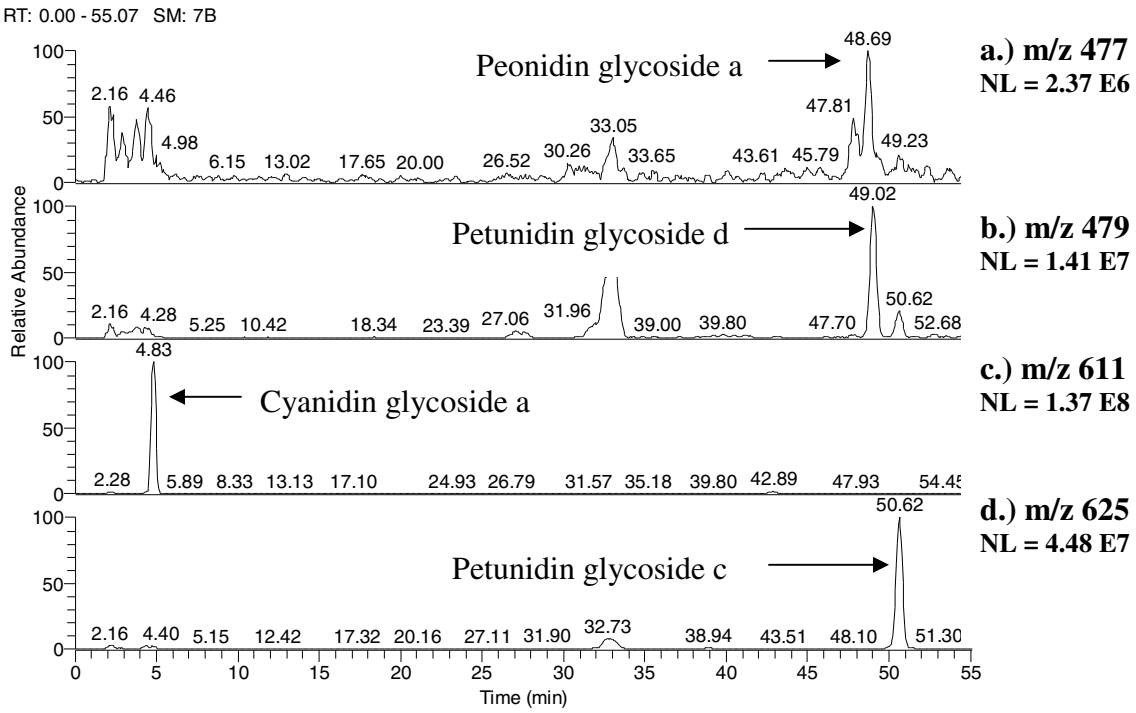

Figure 16. Selected ion chromatograms of four anthocyanins obtained from analysis of a *P. lanceolata* extract using the Cacace method.

Another method tested was one used in the Wu laboratory and is listed as the Wu 1 method in the methods section of this chapter (Wu 2004). With this 70 minute method (Figure 17), the same peaks were examined as in Figure 16. While the peak shapes are mainly Gaussian, the separation between **peonidin glycoside a** (Figure 17a) at 45.26 min and **petunidin glycoside d** (Figure 17b) at 45.48 min was only 0.22 min with a resolution of 0.19. This method exhibited even less separation than the one from the Cacace laboratory for the **peonidin glycoside a** and **petunidin glycoside d**, but **cyanidin glycoside a** separation from coeluting compounds such as that shown in Figure 16a was

improved with the Wu 1 method as compared to the Cacace method. Additionally, the separation and resolution for **petunidin glycosides c** and **d** (Figures 17b and d) were 2.07 and 2.18 respectively. This was an improvement over both of the methods attempted thus far.

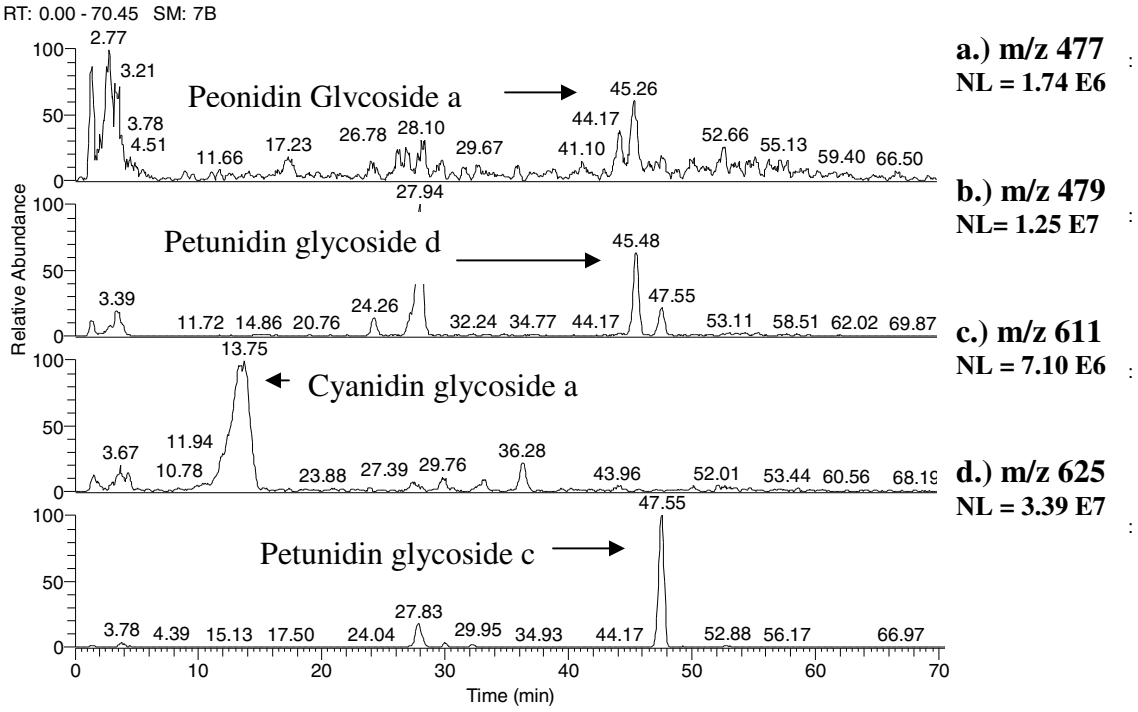

Figure 17. Selected ion chromatograms of four anthocyanins obtained from analysis of a *P. lanceolata* extract using Wu method 1.

The second Wu method tried was 90 minutes in length (see details of method in the methods section of this chapter)(Wu 2005a). As shown in Figure 18a and b below, **peonidin glycoside a** at 55.64 min and **petunidin glycoside d** at 56.02 min had 0.38 minutes of separation and resolution of 0.24. Also, **cyanidin glycoside a** (Figure 18c)

coeluted with other compounds at 4.29 min. **Petunidin glycosides c** and **d** (Figures 18d and b) were found to be separated by 4.10 min and had resolution of 2.05.

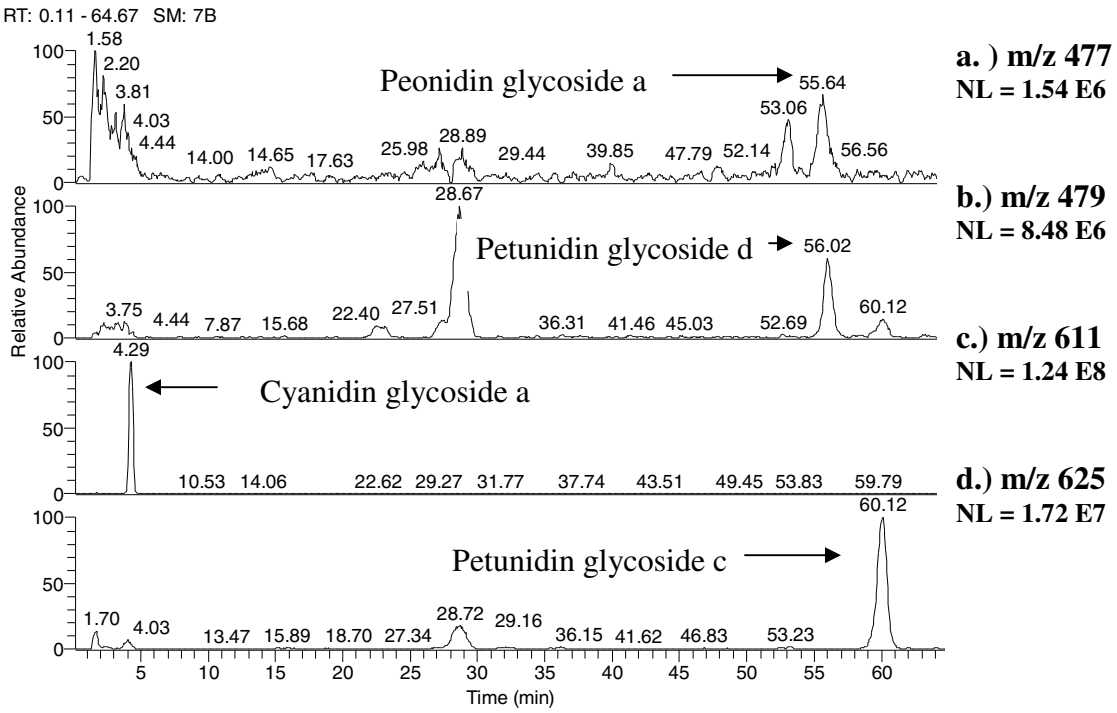

Figure 18. Selected ion chromatograms of four anthocyanins obtained from analysis of a *P. lanceolata* extract using Wu method 2.

Finally Wu method 2a was tried (Wu 2004). The chromatograms from this method are shown in Figure 19. **Peonidin glycoside a** (Figure 19a) at 38.21 min and **petunidin glycoside d** (Figure 19b) at 38.64 min had 0.43 min of separation using this method and had a resolution of 0.33. The Wu 2a method did not give rise to any improvement on the problem of coelution of other compounds with **cyanidin glycoside a** (Figure 19c) at 4.60 min as compared to the Wu 2 and Cacace methods. **Petunidin glycosides c** and **d** (Figures 19d and b) were separated by 2.8 min and had resolution of 2.67, by far the best resolution of any of the methods.

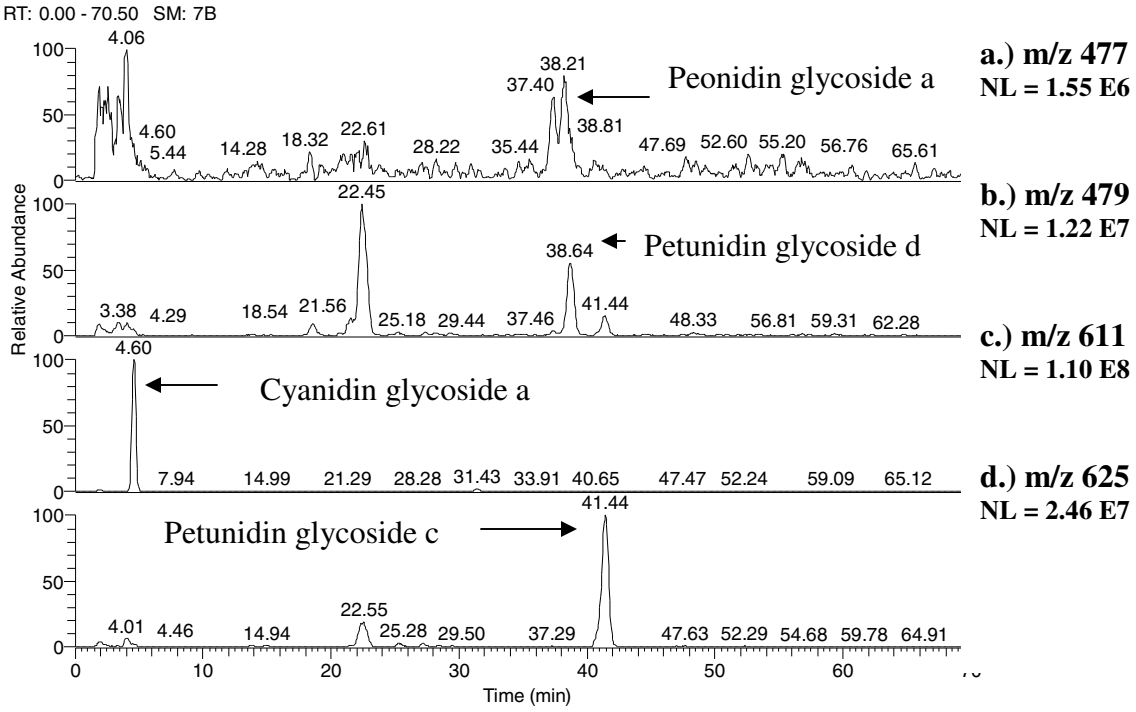

Figure 19. Selected ion chromatograms of four anthocyanins obtained from analysis of a *P. lanceolata* extract using Wu method 2a.

For all of the methods above, the ion with m/z 611 (**cyanidin glycoside a**) eluted early, but coelution of this ion with other compounds was not a problem when the original or Wu 1 methods were employed. Of all the methods, the original resulted in the best separation for **cyanidin glycoside a.** For further comparison Table 7 lists the resolution between the **peonidin glycoside a** peak and the **petunidin glycoside d** as well as the resolution between the two petunidin glycosides. Based on this table it is clear that the best resolution between **peonidin glycoside a** and **petunidin glycoside d** was also with the original method. At a separation of retention time of 0.52 min it is possible to collect **peonidin glycoside a** manually. The petunidin glycosides still coelute with this

method, therefore pure fraction collection of these anthocyanins is not possible with this method.

The best resolution between **petunidin glycosides c** and **d** was with the Wu 2a method. With a retention time separation of 2.8 min it is also possible to collect fractions of **petunidin glycoside c**. Only the **petunidin glycoside c** can be collected with any amount of purity because the **petunidin glycoside d** is not baseline resolved or separated enough from the **petunidin glycoside a** to ensure a pure fraction.

Table 7. Resolutions between **peonidin glycoside a** and **petunidin glycoside d** (column A) and resolution between **petunidin glycosides c** and **d** (column B) for the Stiles, Cacace, Wu 1, 2 and 2a methods.

Method	A.) Resolution between peonidin glycoside a and petunidin glycoside d	B.) Resolution between petunidin glycosides c and d
Original	0.83	0
Cacace	0.37	1.77
Wu 1	0.19	2.18
Wu 2	0.24	2.05
Wu 2a	0.33	2.67

When the gradient was changed to isocratic elution in order to shorten run time (Figure 20), the peaks broadened and while there was more separation in retention time for the 2 petunidin peaks there was still overlap due to the peak broadening. The difference in retention time between **peonidin glycoside a** and **petunidin glycoside d** was 1.07 min with a resolution of 0.39. The difference between retention times for the

two petunidin glycosides was 1.25 min with a resolution of 0.39 (Table 8). This was an improvement in separation of **petunidin glycosides c** and **d** over the original method.

In the 50 min linear gradient (Figure 21), the peaks were narrower than in the isocratic separation, resulting in a greater resolution. The separation and resolution of **peonidin glycoside a** and **petunidin glycoside d** were 1.10 and 0.88 respectively. The separation and resolution for **petunidin glycoside c** and **d** were 0.60 and 0.48. In effect, the time has been reduced and the separation has been slightly improved as compared to the original gradient. Both of these shorter methods fail to achieve baseline resolution thus making pure fraction collection very difficult.

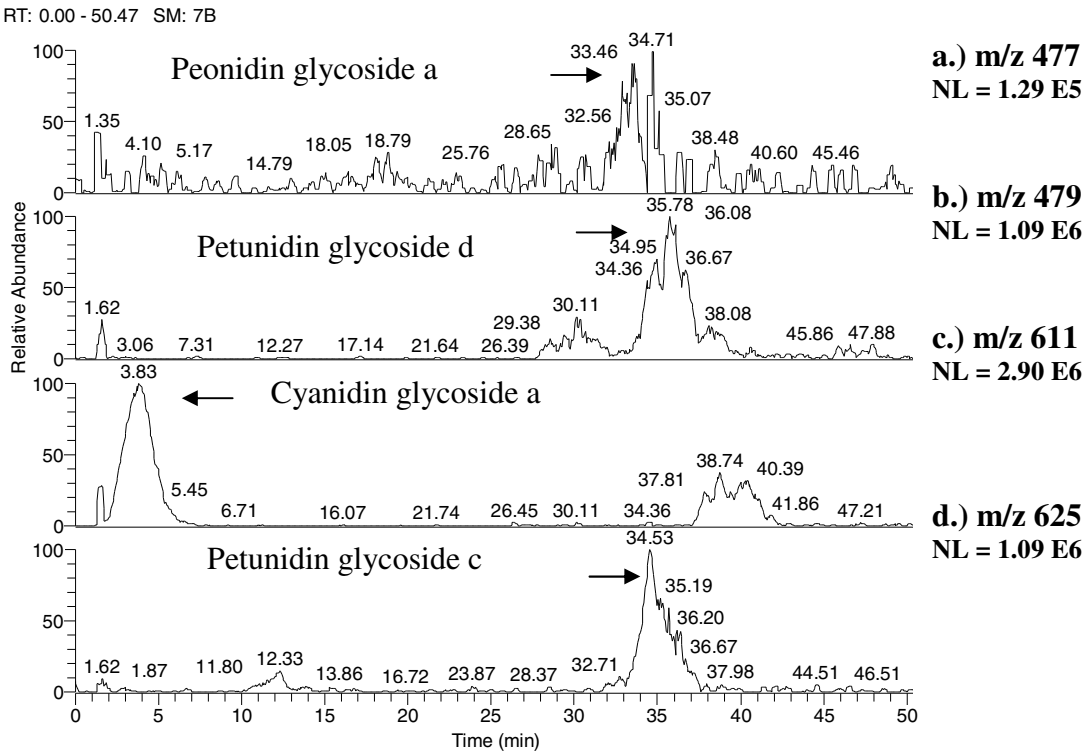

Figure 20. Selected ion chromatograms of four anthocyanins obtained from analysis of a *P. lanceolata* extract using an isocratic separation with 90 % B.

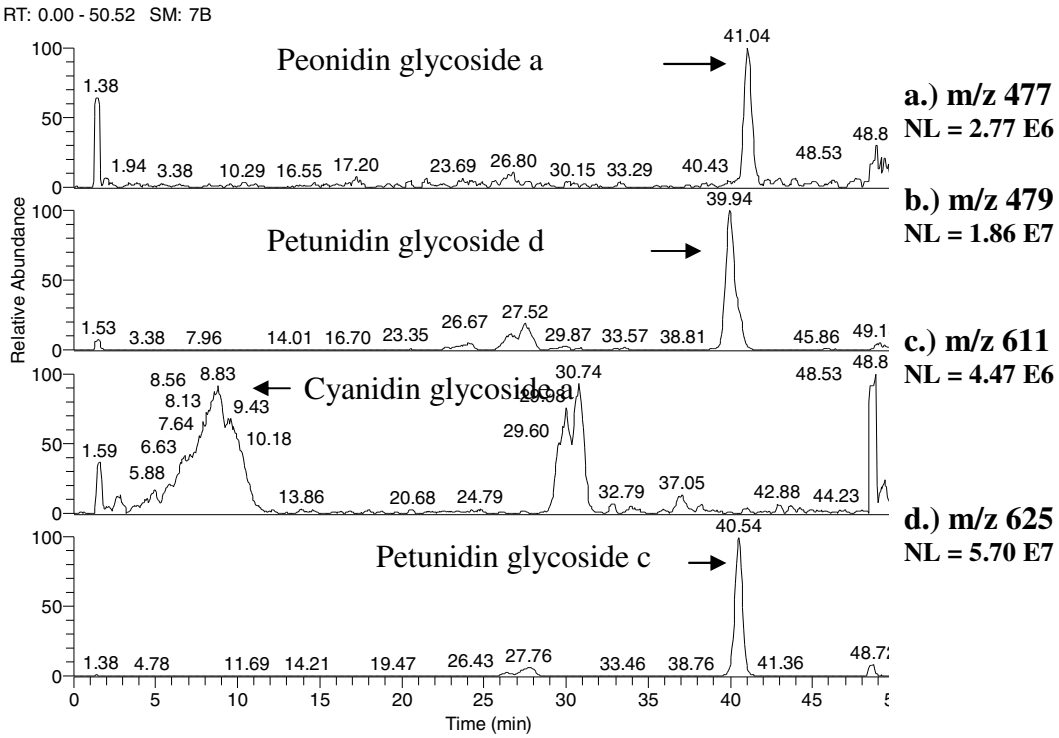

Figure 21. Selected ion chromatograms of four anthocyanins obtained from analysis of a *P. lanceolata* extract using the 50 minute gradient (details in the methods section of this chapter).

Table 8. Resolutions between **peonidin glycoside a** and **petunidin glycoside d** (column A) and resolution between **petunidin glycosides c** and **d** (column B) for the 50 minute isocratic separation and the 50 minute gradient.

Method	Resolution between peonidin glycoside a and petunidin glycoside d	Resolution between petunidin glycosides c and d
50 min isocratic	0.39	0.39
50 min gradient	0.88	0.48

Sample Purification by Solid Phase Extraction

Solid phase extraction (SPE) experiments were explored as a possible method of large scale purification of *P. lanceolata* extracts. With this technique, a solid phase extraction cartridge is used similar to chromatographic column to retain certain compounds and not others. Most SPE cartridges are plastic casings filled with a packing material consisting of graphitized carbon, resin, Florisil, silica or alumina bases that can be modified with different length carbon chains (Supelco bulletin 1998). The choice of packing material determines which type of chromatography will be utilized.

In reversed-phase chromatography, a silica based packing is chemically modified by bonding C-18 chains. These cartridges must be preconditioned before use by running methanol through thereby wetting the silica surface. This step is usually followed by washing with an aqueous based mobile phase to prepare the column for the sample which is dissolved in an aqueous solvent. The sample is then introduced onto the cartridge and analyte molecules interact with the stationary or bonded phase via dispersion or van der Waals forces. Hydrophilic impurities are then removed by another aqueous wash. The compounds of interest can then be eluted with a less polar mobile phase. The polarity of the mobile phase can be adjusted by increasing the percentage of organic in the mobile phase much like HPLC.

Methods

A Waters Sep-Pak Vac 6 cc t-C18 cartridge was coupled to in-house vacuum and preconditioned by washing with 10.00 mL of methanol followed by 10.00 mL of 6% acetic acid. Prior to this experiment, 500 mL of extract (from genotypes BM12-4, G1-5,

G10-3, GD1-4, GH5-1, GH5-4, DU2-4, B7-2 and BM6-5) in 75/25 methanol/water was concentrated to dryness on a Brinkmann Rotovap at 35 ^{o}C with a Chemglass Coldtrap submerged in liquid nitrogen. The dry extract was dissolved in about 20 mL of 75/25 methanol/water and stored in the freezer at – 20 ^{o}C. The sample (500 µL of concentrated extract) was then filtered on the cartridge. The column was then washed with 10.000 mL of 6% acetic acid. **Cyanidin glycoside a**, **petunidin glycoside a**, **cyanidin glycoside c**, **peonidin glycoside a**, and **petunidin glycoside b** were eluted with 10.000 mL of the following percentages of B: 10.15, 12.59, 25.24, 25.81, and 25.98. These percentages were calculated by determining the % B at the adjusted retention times (retention time minus the solvent front). These fractions and the 0 % B wash were collected then analyzed on the HPLC-ESI-MS (see methods section in Chapter II).

Results and Discussion

While the fractions used to elute the anthocyanins were based on the elution from the HPLC column, they were unsuccessful in yielding pure fractions. All of the fractions listed above contained more than one anthocyanin peak. This was not the case with the 0 % B wash however. Based on the chromatographic data it seems that there was only anthocyanin with m/z of 611 in the 0 %B wash. This suggests that if only **cyanidin glycoside a** is needed that using a wash with 0 % B would be useful.

One of the drawbacks of collecting the 0 % B fraction is that the **cyanidin glycoside a** was not the most abundant compound in the chromatograms thus the sample would need to be concentrated. Unfortunately concentrating the anthocyanin would also concentrate the other compounds of higher abundance. Concentration of this aqueous

fraction would require long drying times on the SpeedVac which introduces heat into the sample possibly leading to anthocyanin degradation.

While it seemed that the SPE did not help with the separation, it was found that the SPE cartridge could be used as a method of concentration by running 15 mL of extract through the column. The anthocyanins could then be eluted with 100 % methanol and thus more easily concentrated in the SpeedVac due to the higher volatility of methanol as compared to that of water. Approximately 1.75 hours is required to dry 4 mL of methanolic extract using the SpeedVac, while the same volume of 75:25 methanol:water extract requires more than 3 hours. Therefore, when needing small amounts of concentrated extract, SPE is a useful step.

Fraction Collection of anthocyanins using HPLC with a Diode Array Detector

Several laboratories have used high performance liquid chromatography with diode array detection (HPLC-DAD) to separate and detect anthocyanins in various extracts due to the absorbance of anthocyanins between 520 nm and 530 nm (Wu 2005, Revilla 1998, Giusti 1999, Alonso-Salces 2005). In this technique, eluate from a chromatographic column is directed to a diode array detector where white light from tungsten and deuterium lamps is passed through a flow cell. The light that is transmitted through the sample is reflected onto a polychromator that disperses the light onto the diode array detector. Each diode in the array measures the intensity of a small range of wavelengths (Harris 2001, Cunico 1998). Two types of data may be visualized with a DAD, a chromatogram and a spectrum. A chromatogram is absorbance intensity plotted as a function of time at a particular wavelength. A spectrum is intensity as a function of

wavelength. Since diode array detection is also non-destructive, the anthocyanins detected with this method may be collected intact after detection.

Cyanidin glycoside a was a perfect candidate for fraction collection on the preparatory scale HPLC, because it eluted very quickly and with little interference from other anthocyanins as determined by HPLC-ESI-MS. It also was available in relatively high abundance. The fractions collected will be used to obtain a pure anthocyanin that can be concentrated and analyzed by NMR.

Methods

All analyses were performed on the following equipment: a HPLC model HP1100 (pump, degasser, autosampler and diode array detector) (Agilent, Palo Alto, CA) with a 150 x 10 mm Higgins Analytical Column(5 μm packing size) modified with C-18. The absorbance was monitored at 525 nm. Once the **cyanidin glycoside a** fraction was collected from the HPLC-DAD it was analyzed by HPLC-ESI-MS (see methods section of Chapter II). These collections were stored at – 20 ^{o}C until needed for NMR experiments.

Since **cyanidin glycoside a** eluted so quickly in the original chromatographic method that the run time could be decreased by 20 minutes to a 50 minute gradient. The run parameters were as follows 0 – 20 min, 0 – 10 %B; 20 – 35 min, 10 – 15 %B; 35 – 35.10 min, 15 – 100 %B and 35.10 – 40 min, 100 %B, where A is 6 % acetic acid and B is acetonitrile. An injection volume of 500 μL and a flow rate of 2.00 mL/min were used. All collected **cyanidin glycoside a** fractions were from extracts using flowers from genotypes BM12-4, G1-5, G10-3, GD1-4, GH5-1, GH5-4, DU2-4, B7-2 and BM6-5.

Results and Discussion

Cyanidin glycoside a eluted at approximately 22 minutes in each of the collections, just as shown in Figure 22. This fraction was then analyzed by HPLC-ESI-MS and was verified to be a pure sample of **cyanidin glycoside a**. H^1-NMR analysis was then performed as in the next section of this chapter.

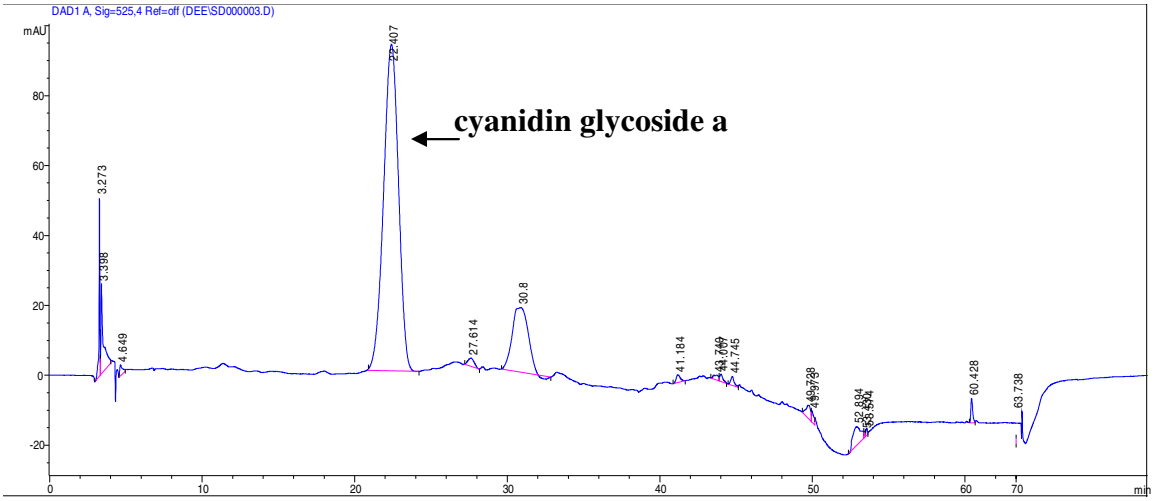

Figure 22. HPLC-DAD chromatogram of *P. lanceolata* extract at 525 nm with **cyanidin glycoside a** eluting at 22.407 min.

Identification of Anthocyanins with NMR Spectroscopy

Nuclear magnetic resonance spectroscopy (NMR) is a technique that can be used to determine the molecular structure and conformation of a compound. ^{1}H- NMR is based on the theory that hydrogen nuclei have spin +/- ½. When a magnetic field is applied, those nuclei spinning against the magnetic field (- ½) are at a higher energy than

those that spin with the magnetic field (+ ½). The lower energy nuclei can absorb energy from a radiofrequency transmitter thus reversing their spin to the higher energy state of – ½ (Carey 2003). This process is sometimes referred to as spin-flipping (Streitwieser 1976).

In a pulsed Fourier-transform NMR, high intensity radiofrequency radiation is applied to the sample over very short periods of time promoting the lower energy hydrogen nuclei to the higher spin energy level. These protons then begin to decay to their original energy levels with the emission of a decaying radiofrequency signal. These signals are then Fourier-transformed into the NMR spectra commonly seen (Carey 2003).

The positions of the signals on these spectra are determined by the absorption of radiation at a particular field strength which is determined by the chemical environment of the nuclei. Electrons create a magnetic field that opposes the external magnetic field thereby causing the effects of the external field to be lessened with respect to the proton, in effect shielding them (Streitwieser 1976). These shielded protons are located upfield towards the lower numbers in chemical shift. Whereas protons close to electronegative substituents on the molecule, are less shielded, because the electron density that could shield the protons is pulled away and are more downfield in shift. Aromatic protons are also deshielded because the applied field is being reinforced by the magnetic field associated with the π electrons (Carey 2003, Streitwieser 1976).

An NMR spectrum is a graphical representation of abundance and chemical shift. Chemical shift is usually represented in parts per million (ppm) instead of Hz making it possible to more easily compare the spectra of one instrument with spectra from another

instrument of different frequency. The equation for converting the position from Hz to ppm is shown below in equation 9 (Carey 2003). The position of the methyl protons of tetramethylsilane (TMS), one of the most shielded organic compounds, is used as a point of reference which allows comparisons of chemical shift of the same compound from different instruments.

$$Chemical\ Shift(\delta) = \frac{position\ of\ signal - position\ of\ TMS\ peak}{spectrometer\ frequency} \times 10^6$$

(equation 9)

Cyanidin glycoside a

Since the most abundant and most easily separated anthocyanin in *P. lanceolata* was **cyanidin glycoside a**, it was a good candidate for the NMR studies. Therefore, the goal of this research is to determine the precise identity of cyanidin glycoside a. Based on the mass spectral data, it was suspected that this anthocyanin may have been cyanidin 3,5-diglucoside.

Methods

Several collections of **cyanidin glycoside a** were made on the preparatory HPLC-DAD and were concentrated to dryness in the SpeedVac. For Sample 1, a pellet of 2-5 mg of dried **cyanidin glycoside a** was resuspended in 1 mL of deuterated methanol and analyzed on JEOL 500 MHz NMR. For Sample 2, the solid 2 – 5 mg of **cyanidin glycoside a** was washed several times with chloroform to remove residual water before

being dissolved in 500 μL of deuterated methanol in a Shigemi advanced NMR microtube, 5mm x 12mm bottom L, matched with CD_3OD. A standard of cyanidin-3,5-diglucoside (3 – 4 mg) (purchased from Sequoia Pacific Research Company, Draper, UT) was dissolved in approximately 1 mL of deuterated methanol and analyzed in the same way as the collected glycoside.

Results and Discussion

The ^{1}H-NMR spectrum of standard of cyanidin-3,5-diglucoside is shown in Figure 23. In this figure, there is a singlet at 9.1, a doublet of doublets at 8.3, a doublet at 8.0 and a doublet of doublets at 7.0 indicative of hydrogens on the 4, 6', 2'and 8 carbons, respectively, of the cyanidin-3,5-diglucoside. The assignments of these peaks correspond well to the literature values, where the H-4 (hydrogen on carbon 4) was found between 8.81 and 9.03 as a singlet, H-6' as a doublet of doublets between 8.06 and 8.35, H-2' as a doublet between 7.83 and 8.12, and H-8 as broad singlet at around 6.98 (Shoji 2002, Byamukama 2005) .

The spectra from the collections of **cyanidin glycoside a** obtained from the *P. lanceolata* extract are shown in Figures 24 and 25. The spectrum of Sample 1 (Figure 24) is suggestive of the presence of an anthocyanin, with aromatic signals in the 6-9 ppm region and complex signals between 3 and 4 ppm. It also contains small signals that correspond to the substance that appears in Sample 2 (Figure 25). The signals in Sample 2 at 7.5 and 8.0 could be indicative of the aromatic protons of an anthocyanidin, but there is no evidence for a peak from the 4-position proton that is typical for these anthocyanins. Since none of the peaks present in the spectra from either Sample 1 or Sample 2 matched

the ones from the standard, it can be concluded that the standard and the collected anthocyanin are two different compounds. Also when the standard and the collected anthocyanin were run on the HPLC-ESI-MS the retention times did not match, which further supports the claim that these compounds are not the same compound as the standard. Additionally, the spectra obtained from Sample 1 and Sample 2 had more noise in the baseline suggestive of minor impurities.

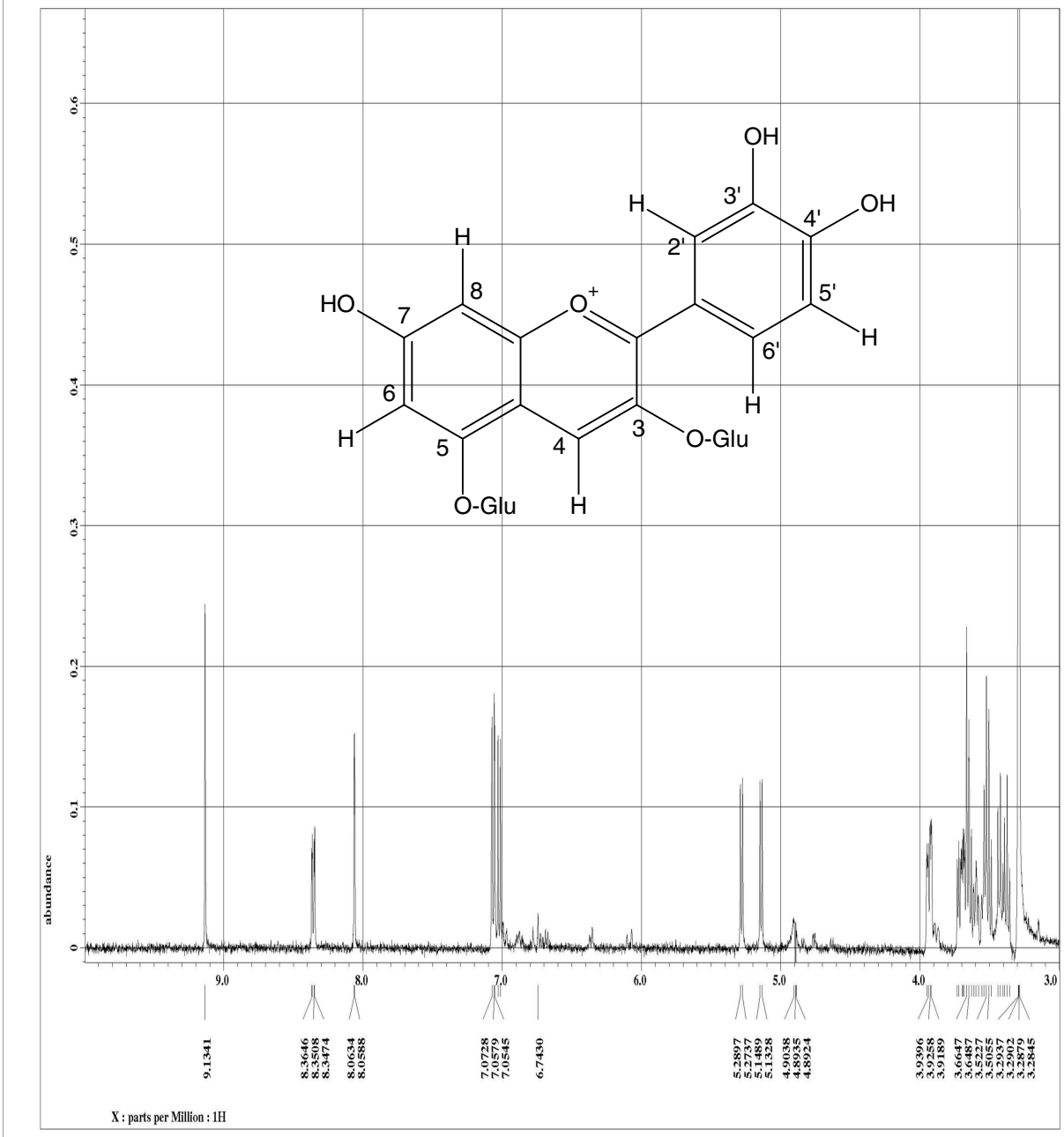

Figure 23. ^{1}H-NMR of Cyanidin-3,5-diglucoside. A singlet at 9.1, a doublet of doublets at 8.3, a doublet at 8.0 and a doublet of doublets at 7.0 indicative of hydrogens on the 4, 6', 2'and 8 carbons

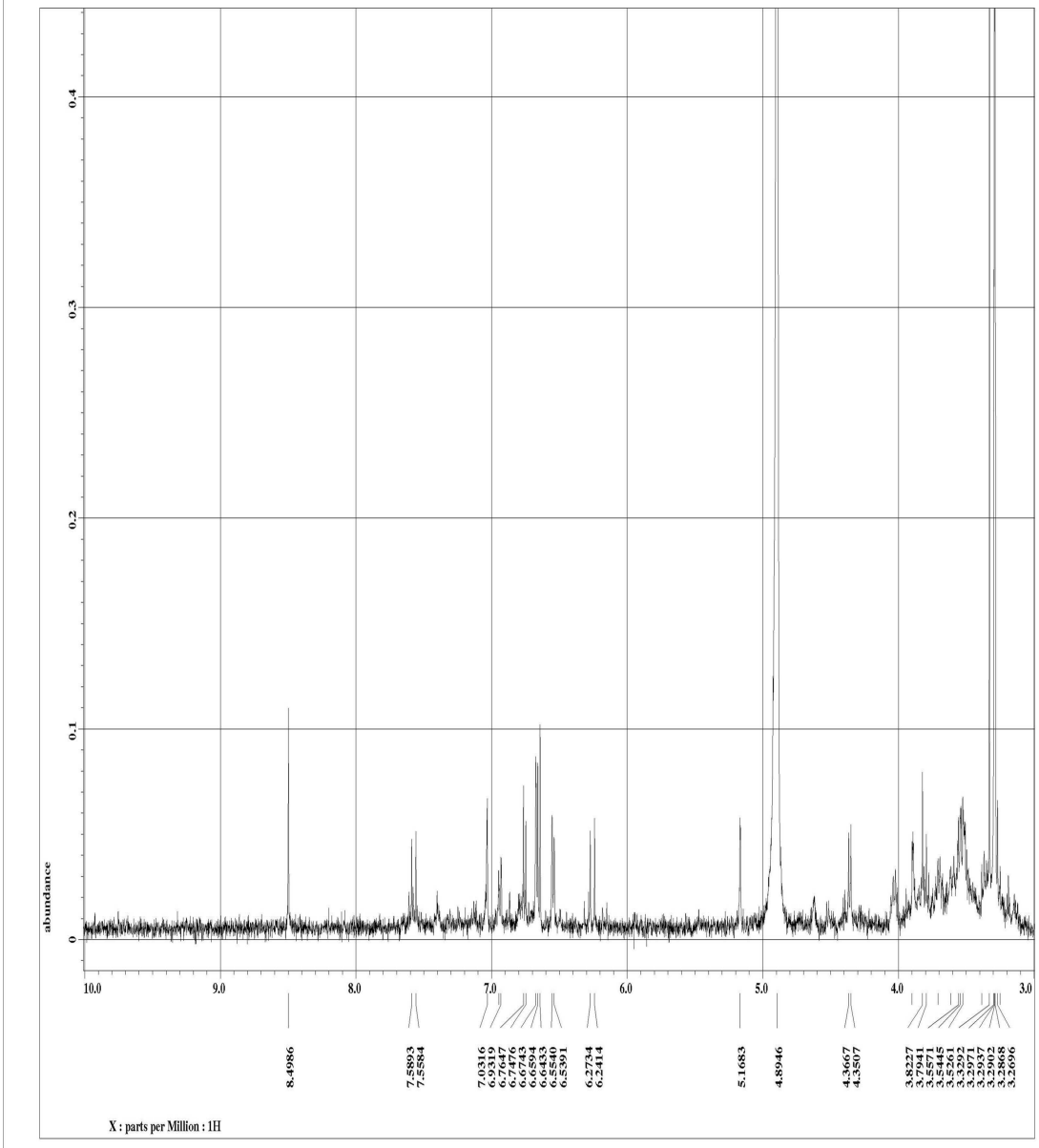

Figure 24. ^{1}H-NMR of Sample 1 which consists of cyanidin glycoside a collected from *P. lanceolata* extracts.

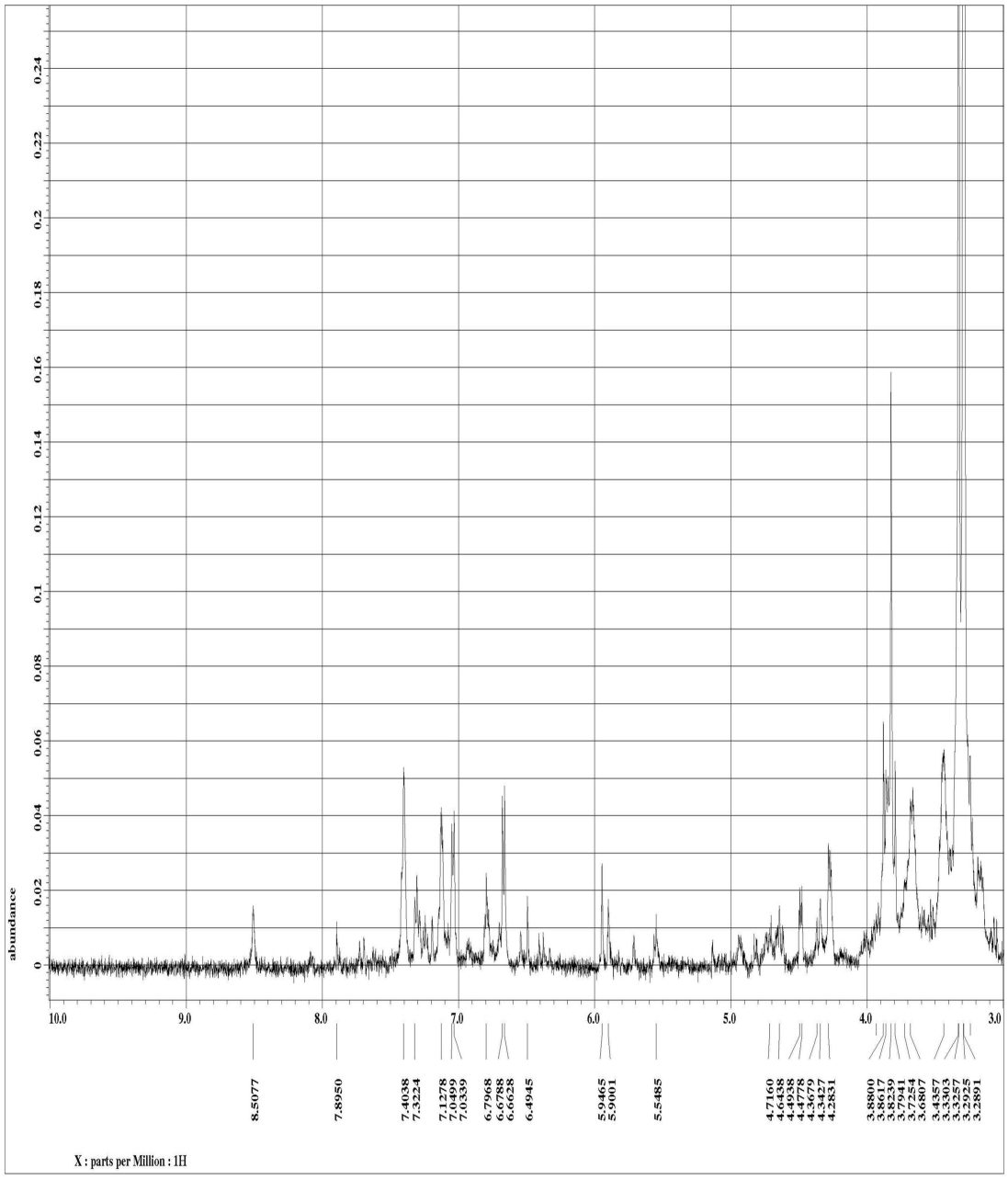

Figure 25. [1]H-NMR of Sample 2, which consists of **cyanidin glycoside a** collected from *P. lanceolata* extracts.

Conclusion

While the NMR spectroscopy analyses did not provide the desired identification of **cyanidin glycoside a**, they did give insight as to future research directions. Since the sample was perhaps not as concentrated or as pure as needed for successful NMR analysis, it would be helpful to try this method on a plant that has higher anthocyanin concentrations to improve the separations method for improved purity and then work back to the lower concentrations in *P. lanceolata*. For successful NMR analysis of anthocyanins from *P. lanceolata,* a more concentrated sample is needed. In order to obtain the most concentrated sample, 25 or more grams of small, darkly-colored spikes would need to be collected at one time to yield an extract that can be concentrated, separated and analyzed with NMR.

CHAPTER VII

CONCLUSIONS

One of the major important outcomes of this research is confirmation of the presence of peonidin and cyanidin glycosides in *P. lanceolata* extract by retention time comparisons of hydrolyzed anthocyanins with standard anthocyanidins. Since it is proposed that the unidentified anthocyanidin cores are from acylated anthocyanins, basic saponification may be a useful technique to identify the anthocyanidin portion of other anthocyanins in the extracts. If total hydrolysis of each of the anthocyanins can be achieved, it could be possible to quantify the total anthocyanin content of *P. lanceolata* for each of the anthocyanidin cores. The ability to make such quantitative analyses would be useful for future studies investigating the effect of environmental conditions and genetic variation on anthocyanin content of *P. lanceolata*.

This research also explored separations and found that it may be possible to separate and collect **petunidin glycoside c** via the Wu 2a method utilizing 5 % formic acid and methanol (as shown in the methods section on pg. 45 of Chapter VI). The reason for needing separation methods for this research was to yield high quantities of purified individual anthocyanins, therefore **cyanidin glycoside a** was explored more exclusively. However, since successful NMR was not achieved, it may be useful in the future to try hydrolysis of individual purified anthocyanins. If each of the anthocyanins could be separated and collected into pure aliquots, then these individual aliquots could

be hydrolyzed and analyzed to determine the sugar substituents (Gao 1994 b). This procedure would not require as large a quantity as the NMR did therefore may be more successful.

Limitations of the method were also explored. It was determined that the CV of the method from injection to injection was between 12 and 16 percent. The CV from day to day was 27 – 29 percent. And the CV from extraction to extraction varied from anthocyanin to anthocyanin with values ranging from 12 to 61 percent. This research points out the importance of having replicate injections when quantification is desired.

Finally, this research showed no clear degradation of anthocyanins in *P. lanceolata* over a period of 140 days when stored at – 20 or – 80 ^{o}C. The anthocyanin and anthocyanidin standards as well as all extracts in this study were stored at -20 ^{o}C. This temperature, as well as -80 ^{o}C may be viable storage conditions. Surprisingly, only cyanidin glycosides **a** and **c** showed clear degradation in the room temperature condition. If other anthocyanins are, indeed, stable at room temperature, they could prove useful in food chemistry applications. This could be an interesting avenue of future study.

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