IDENTIFICATION, ENHANCEMENT AND CONCENTRATION OF PHENOLIC COMPOUNDS IN MUSCADINE GRAPES

By

AMANDEEP K. SANDHU

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To my parents and in-laws for encouraging me at every step; my husband for his unconditional love and support, and my precious son

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
AAPH	2,2’-azobis(2-amidinopropane)
ANOVA	Analysis of variance
BV	Bed volume
CO_2	Carbon dioxide
DAD	Diode array detector
DPPH	2,2-diphenyl-1-picrylhydrazyl
g	Gram
g	Relative centrifugal force
h	Hour (s)
HPLC	High performance liquid chromatography
Kg	Kilogram
L	Liter
m/z	Mass to charge ratio
MS	Mass spectrometer
MeJA	Methyl jasmonate
µg	Microgram
µL	Microliter
µmol	Micromole
mL	Milliliter
mm	Millimeter
min	Minute (s)
nm	Nanometer
nM	Nanomole

ORAC	Oxygen radical absorbance capacity
O_2	Oxygen
psi	Pounds per square inch
PCA	Principal component analysis
PC1	Principal component 1
PC2	Principal component 2
rpm	Revolutions per minute
s	Second (s)
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TSS	Total soluble solids
UV	Ultraviolet
Vis	Visible
v	Volume
w	Weight

Abstract of Dissertation Presented to the Graduate School
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Amandeep K. Sandhu

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The Muscadine grape (*Vitis rotundifolia*) is native to the southeastern United States and possesses a unique phytochemical profile. The phytochemical composition of grapes affects the quality of grapes, such as color and flavor, as well as their health promoting benefits. Therefore, the first objective of this project was to identify various phytochemicals in seeds, skin and pulp of Florida grown muscadine grapes. The second objective was to enhance the phytochemical content by application of pre and post-harvest plant growth regulators abscisic acid (ABA) and methyl jasmonate (MeJA), respectively. Finally, we developed a method to obtain concentrated phytochemical extract from muscadine pomace using resin adsorption technology.

High-performance liquid chromatography equipped with diode array (HPLC-DAD) and electrospray ionization mass spectrometric detection (ESI-MS^n) was used to identify the phenolic compounds in seeds, skin, and pulp of Noble grapes. On average, 87.1, 11.3, and 1.6% of phenolic compounds were present in seeds, skin, and pulp, respectively. A total of 88 phenolic compounds of diverse structures were tentatively identified in Noble, which included 17 in pulp, 28 in skin, and 43 in seeds. Seventeen compounds were identified for the first time in muscadine grapes.

ABA treatment enhanced the antioxidant capacity by 38 and 18% in Noble variety at first and second sampling, respectively. A significant increase in individual anthocyanins was observed in treated Noble grapes at both sampling times. However, increase in the content of ellagic acid and flavonols was observed at first sampling only. No effects of ABA treatment were seen in Alachua grapes. Our results indicate that exogenous application of ABA enhances the antioxidant capacity, anthocyanins and phenolic content of muscadine grapes but these effects may vary depending upon the cultivar. Post harvest MeJA treatment didn't show any effect on the phenolic compounds in muscadine grapes.

The adsorption/desorption characteristics of anthocyanins from muscadine pomace were evaluated on five Amberlite resins (FPX-66, XAD-7HP, XAD-16N, XAD-1180 and XAD-761). FPX-66 and XAD-16N showed highest adsorption and desorption capacities, and ratios. Dynamic testing was done on a column packed with FPX-66 which resulted in a concentrated pomace extract that contained 13% (w/w) anthocyanins with no detectable sugars.

CHAPTER 1
INTRODUCTION

Muscadine Grapes

The Muscadine grape (*Vitis rotundifolia*) is indigenous to the southeastern United States. They differ from other grapes (*Vitis vinefera*) both in morphology and composition. They are either light-skinned (green or bronze) or dark-skinned (red to almost black) [1-2] and are 2.5 – 3.8 cm in diameter with thick, tough skin which protects them from heat, UV radiation, humidity, insects and fungi. They grow in tight small clusters of 3 to 10 berries in contrast to other grape species which grow in bunches. Muscadine grapes are resistant to Pierce's disease, which prevents the commercial production of *Vitis vinifera* grapes [3]. The disease resistance of muscadine grapes has made them uniquely fit for production in the southeastern United States in comparison to traditional bunch grapes.There are more than 300 muscadine cultivars and among them Carlos, Noble, and Magnolia are of commercial importance. Muscadine grapes are consumed as fresh fruit or processed into wine, juice, jam or jelly. Muscadine wines are gaining popularity as consumers begin to appreciate their unique fruity bouquet and the positive health effects.

Phytochemicals in Muscadine Grapes

Phytochemicals originate as a result of secondary plant metabolism and are essential for reproduction, stability and growth processes of plants. They are recognized not only for their health promoting benefits but also for their contribution to color and flavor in different fruits and vegetables. They are categorized into different classes depending upon their structures varying from simple phenolic acids (hydroxybenzoic acid and hydroxycinnamic acid) to complex polyphenols (hydrolysable and condensed

tannins). Several classes of phytochemicals are found in grapes however, structures and contents of these phytochemicals differ in the grapes of different variety or genotype. Muscadine grapes possess unique phytochemical composition that differentiates them from other *Vitis* species such as presence of ellagic acid and its derivatives, and anthocyanin 3, 5-diglucosides [4-5]. Other phytochemicals found in muscadine are flavanols (catechin and epicatechin), condensed tannins (oligomeric flavanols), flavonols (myricetin, quercetin and kaempferol), resveratrol and gallic acid. About 80% and 18% of phytochemicals are located in the muscadine seeds and skin, respectively. The pulp contains very low amount of phytochemicals [6].

Antimicrobial Activity

The high phenolic content not only confers disease resistance in muscadine grapes but also provide antimicrobial activity. Several studies have investigated the effect of muscadine grape components and extracts on pathogenicity. Many of these studies worked with gram negative microorganisms including *Eschericia coli* O157:H7, *Enterobacter sakazakii*, *Cronobacter sakazakii*, *Salmonella enteriditis*, and *Listeria monocytogenes*, pathogens that are watched closely by the food industry. The results from these studies suggest that muscadine grapes and extracts could be used as a natural preservative in beverages and other products [7-9].

Health Benefits

In addition to their disease resistance and antimicrobial activity, muscadine grapes have potential to positively affect a wide variety of health issues including Alzheimer's disease, inflammation, cancer, obesity and diabetes. A study conducted on muscadine wine showed that moderate consumption of muscadine wine attenuates cognitive deterioration in Tg2576 mice by interfering with the oligomerization of amyloid-

β (Aβ) molecules to soluble high-molecular-weight Aβ oligomer species that are responsible for initiating a cascade of cellular events resulting in cognitive decline. Thus, it can be used for the prevention of Alzheimer's disease [10]. Inflammation plays a pivotal role in various immunopathophysiological conditions. Muscadine skin, seed, and combination skin/seed extracts exhibit significant topical anti-inflammatory properties in mice models [11]. Similarly, another study reported the anti-inflammatory properties of muscadine skin powder both *in vitro* and *in vivo* with possible mechanisms including the inhibition of cytokine and superoxide release [12]. Cancer is a rapidly growing health problem that is the biggest challenge to researchers and medical professionals. Muscadine grapes, wines and extracts have shown promising results in preventing various types of cancers. For example, cell culture studies have suggested that polyphenols from muscadine grapes were effective in inhibiting cancer cell viability and inducing apoptosis in a liver cell model (i.e., HepG2 cells) and a human colon cell (i.e., Caco-2 cells), and that the greatest anticancer activities were attributed to muscadine fractions containing ellagic acid and anthocyanins, respectively [13-14]. Muscadine grape skin extract inhibited tumor cell growth in prostate cancer cell lines and exhibited high rates of apoptosis by targeting phosphatidylinositol 3-kinase-Akt and mitogen-activated protein kinase survival pathways [4]. Extracts from red muscadine wines induced cell death in MOLT-4 human leukemia cells [15]. Obesity is a multifactorial condition posing major health problems worldwide that leads to various other metabolic complications. Muscadine grape and wine phytochemicals prevented obesity associated metabolic complications in C57BL/6J mice [16]. Several studies have suggested the anti-diabetic activity of muscadine grapes. A recent study reported that anthocyanins from

muscadine grape extracts inhibited α-glucosidase and pancreatic lipase *in vitro*, the enzymes implicated in diabetes [17]. Extracts of muscadine grape and seeds have also prevented formation of advanced glycation end products *in vitro*, which have been associated with diabetic complications [18]. A human study conducted by Banini et. al., showed that nightly supplementation of 150 mL of muscadine wine and dealcoholized wine both comparably moderated blood glucose, glycated hemoglobin, and insulin in participants with and without Type II diabetes [19].

Research Objectives

This research was conducted to get the comprehensive knowledge of phenolic compounds in muscadine grapes and to investigate the effect of pre- and post-harvest growth regulators on their content. In addition, a method was developed to extract and concentrate phytochemicals from muscadine pomace using resin adsorption technology. The specific objectives were:

1. To evaluate antioxidant capacity, phenolic content and perform an extensive identification of the phenolic compounds in seed, skin and pulp of Florida grown muscadine grapes using HPLC-DAD-ESI-MS^n.
2. To investigate the effects of exogenous abscisic acid on antioxidant capacities, anthocyanins, and flavonol contents of muscadine grape skins.
3. To compare different solvents for the extraction of phenolic compounds from Noble juice and wine pomace.
4. To study adsorption/desorption characteristics and separation of phenolic compounds from muscadine pomace using macroporous adsorbent resins.
5. To investigate the effects of methyl jasmonate on postharvest quality of muscadine grapes at different storage temperatures.

CHAPTER 2
ANTIOXIDANT CAPACITY AND PHENOLIC COMPOSITION IN SEEDS, SKIN AND PULP OF FLORIDA GROWN MUSCADINE GRAPES

Background

Phenolic compounds are a class of phytochemicals that play an important role in the nutritional and sensory properties of various fruits and vegetables. They are categorized into different classes depending upon their structures varying from simple phenolic acids (hydroxybenzoic acid and hydroxycinnamic acid) to complex polyphenols (hydrolysable and condensed tannins) [20-21]. Phenolic compounds have been linked to many positive health benefits including the protective effects against certain diseases such as cancer and cardiovascular diseases [22-24]. The protective effect of phenolic compounds has been attributed in part to their antioxidant capacity [25-26].

Muscadine grapes are commonly grown in the southeastern United States and are well-adapted to warm, humid climates, which are unsuitable for the growth of other grapes (*Vitis vinifera*). They are either light-skinned (green or bronze) or dark-skinned (red to almost black) [1-2, 27] and are 2.5 – 3.8 cm in diameter with thick, tough skin which protects them from heat, UV radiation, humidity, insects and fungi. They grow in tight small clusters of 3 to 10 berries and are marketed in fresh and processed forms such as juice, wine and jam.

Muscadine grapes contain a large variety of antioxidant phytochemicals. They are reported to contain hydroxybenzoic acids, ellagic acid in free and conjugated form, resveratrol and flavonoids including anthocyanins, quercetin, myricetin and kaempferol [1, 5, 28]. Cell culture studies have suggested that polyphenols from muscadine grapes can inhibit proliferation of colon cancer cells and induce apoptosis in them [13-14]. However, the phytochemical profiles of muscadine grapes have been documented by only a few

studies. The phenolic compounds in muscadine grapes have been quantified after acid hydrolysis of the samples, which limits their actual structural identification [27]. Two other studies identified the phenolic compounds in the skin of muscadine grapes [5, 28], however, specific information on the identification of phenolic compounds in seed and pulp of muscadine grapes is lacking. High performance liquid chromatography coupled with diode array detector and a mass spectrometry (HPLC-DAD-MS^n) provides a powerful tool for phytochemical analysis in crude plant extracts. It provides useful structural information and allows for tentative compound identification when standard reference compounds are unavailable and when peaks have similar retention times and UV-absorption spectra [29-30]. The aim of our study was to evaluate the antioxidant capacity and total phenolic content in seed, skin and pulp of eight cultivars of Florida grown muscadine grapes. In addition, an extensive identification of the phenolic compounds in seed, skin and pulp of Noble variety was done using a simple and rapid high performance liquid chromatography and mass spectrometry (HPLC-MS^n) technique. Seventeen different phenolic compounds were identified for the first time in muscadine grapes. The comprehensive knowledge of phenolic compounds in seed, skin and pulp of muscadine grapes can contribute to a better understanding of their influence on the quality of muscadine products especially wine and juice.

Materials and Methods

Chemicals

Ellagic acid, (+) – catechin, (-) – epicatechin, quercetin 3-O-β-glucoside, (-)-catechin gallate, (-)-gallocatechin and (-)-epigallocatechin were purchased from Sigma-Aldrich (St. Louis, MO). Quercetin 3-O-β-rhamnoside was purchased from Indofine (Hillsborough, NJ). (-)-Epicatechin gallate and trans-resveratrol 3-O-β-glucoside were

purchased from Chromadex (Irvine, CA). AAPH (2,2'-azobis(2-amidinopropane)) was a product of Wako Chemicals inc. (Bellwood, RI). Gallic acid, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), HPLC grade methanol, acetic acid, formic acid, Folin-Ciocalteu reagent, Flourescein, 96 well black plate with clear bottom wells and a lid, and sodium carbonate were purchased from Fischer Scientific Co. (Pittsburg, PA).

Sample Preparation

Eight cultivars of muscadine grapes, four bronze (Doreen, Fry, Carlos and Triumph) and four black (Southland, Magoon, Alachua and Noble) were obtained from a local vineyard in central Florida. The grapes were manually separated into seed, skin and pulp within 2 hours of harvest and the separated portions were kept at -20°C until further analysis.

The seed, skin and pulp of grapes were freeze dried and ground to a fine powder using Waring kitchen blender. One gram of freeze dried pulp and 0.5 g of seed or skin were weighed in 20 mL screw capped glass tubes. The weighed samples were extracted with 10 mL of acetone: water: acetic acid (acetone/ H_2O /acetic acid; 70:29.7:0.3, v/v) solvent. The extraction tubes were vortexed for 30 s and sonicated for 5 min, then kept at room temperature for 20 min and sonicated for another 5 min. The tubes were centrifuged at 3000 rpm for 10 min and supernatant collected in separate glass tubes. For identification of phenolic compounds, the same extraction procedure was followed. The collected supernatant was evaporated in a SpeedVac Concentrator (Thermo scientific ISS110, Waltham, MA) under reduced pressure at 25°C to remove the solvent. The solids obtained after evaporation were dissolved in 5 mL of 70% acidified (1% formic acid) methanol and sonicated for 5 min to resuspend the solid

residue. The samples were filtered through 0.45 µm filter prior to injection (20 µL) to the HPLC system. All the prepared samples were kept at -20°C until analysis.

HPLC-DAD-ESI-MS^n Analysis

Chromatographic analyses were performed on an Agilent 1200 series HPLC (Agilent, Palo Alto, CA) equipped with an autosampler/injector and diode array detector. A Zorbax Stablebond Analytical SB-C18 column (4.6 × 250 mm, 5 µm, Agilent Technologies, Rising Sun, MD) was used for separation. Elution was performed using mobile phase A (0.5% formic acid aqueous solution) and mobile phase B (methanol). UV/Vis spectra were scanned from 220 to 600 nm on diode array detector with detection wavelengths at 280, 360 and 520 nm. The flow rate was 1 mL/min and the linear gradient used was: 0-2 min 5% B; 2-10 min 5-20% B; 10-15 min 20-30% B; 15-20 min 30-35% B; 60-65 min 80-85% B; 65-70 min 85-5% B followed by 5 min of re-equilibration of the column for the next run. Electrospray mass spectrometry was performed with a HCT ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). Column effluent was monitored in positive and negative ion mode of the MS in an alternative manner during the same run. Other experimental conditions on the mass spectrometer were as follows: nebulizer, 45 psi; dry gas, 11.0 L/min, dry temperature, 350°C; ion trap, scan from *m/z* 100 to 2200; smart parameter setting (SPS), compound stability, 50%; trap drive level, 60%. The mass spectrometer was operated in Auto MS^3 mode. MS^2 was used to capture and fragment the most abundant ion in full scan mass spectra and MS^3 was used to fragment the most abundant ion in MS^2.

Folin-Ciocalteu Assay

The acetone/ H_2O /acetic acid extracts were diluted to appropriate concentration for analysis. Total phenolic content was determined by using modified method of

Singleton and Rossi [31]. The extracts were mixed with diluted Folin-Ciocalteu reagent and 15% sodium carbonate. Absorption at 765 nm was measured in a microplate reader (SPECTRAmax 190, Sunnyvale, CA) after incubation for 30 minutes at room temperature. The results were expressed as milligrams of gallic acid equivalents per gram of fresh weight (mg of GAE/g) using a standard curve generated with 100-600 mg/L of gallic acid.

Oxygen Radical Absorbance Capacity ($ORAC_{FL}$) Assay

ORAC assay for extracted samples was carried out on a Spectra XMS Gemini plate reader (Molecular Devices, Sunnyvale, CA). Briefly, 50 µL of standard and samples were added to the designated wells of 96 well black plate. This was followed by addition of 100 µL of Flourescein (20 nM). Mixture was incubated at 37°C for 7 minutes before the addition of 50 µL AAPH. Fluorescence was monitored using 485 nm (excitation) and 530 nm (emission) at 1 min interval for 40 minutes. Trolox was used to generate a standard curve. The results of the antioxidant capacity of extracts were expressed as micromoles of Trolox equivalents (TE) per gram fresh weight (µmol of TE/g).

Statistical Analyses

One-way analyses of variance (ANOVA) with Tukey-HSD pair-wise comparison of the means were performed using JMP software (Version 7.0, SAS Institute Inc. Cary, NC). Total phenolic and ORAC values are expressed as mean ± standard deviation of three independent observations. Data points from two samples were omitted as outliers based on the Q-test [32] and the results from those values are expressed as duplicates. A difference of $p \leq 0.05$ is considered as significant.

Results and Discussion

The phytochemical constituents of the muscadine grape (*Vitis rotundifolia*) differ from *Vitis vinifera* in many aspects. The presence of ellagic acid in muscadine grapes is unique and is found in the form of free ellagic acid, ellagic acid glycosides, and ellagitannins [33]. Another unique feature is that the anthocyanins are present as 3, 5-diglucosides (as opposed to 3-glucosides in *Vitis vinifera*) of delphinidin, cyanidin, petunidin, peonidin, pelargonidin and malvidin in nonacylated forms. No condensed tannins were identified in muscadine skin as opposed to *Vinifera* skins [34]. Red muscadine wines are more susceptible to browning and loss of color after ageing. This may be due to slow association of anthocyanin diglucosides and tannins present in muscadine wines [35].

Total Phenolic Content and Antioxidant Capacity

Table 2-1 shows the total phenolic content and antioxidant capacity in eight cultivars of Florida grown muscadine grapes. Based on fresh weight, the total phenolic content was highest in seeds (27.0-81.2 GAE mg/g), followed by skin (4.3-10.2 GAE mg/g) and pulp (0.3-1.2 GAE mg/g). Among the seeds, the total phenolic content was highest in Alachua and lowest in Magoon variety of muscadine grapes. The skin of Carlos variety had highest total phenolic content compared to skin of other varieties. Accordingly, the antioxidant capacity, based on fresh weight was also highest in seeds (276.6-1538.4 μmol TE/g), followed by skin (26.0-77.5 μmol TE /g) and pulp (2.3-4.6 μmol TE/g). The ORAC value was found to be highest in Fry seed, Noble skin and pulp. On average the phenolic content in seeds, skin and pulp was 87.1%, 11.3% and 1.6% of that in whole grapes, respectively. Similarly, the average antioxidant capacity among all the grape cultivars was 93.9% in seeds, 5.6% in skin and 0.5% in pulp. The

correlation coefficient (r) between the total phenolic content and antioxidant capacity in seeds, skin and pulp of eight cultivars of muscadines was 0.87.

Phenolic Identification on HPLC-DAD-ESI-MS^n

Identification of phenolic compounds was done in Noble variety of muscadine grapes. The HPLC-DAD chromatograms of pulp, skin and seed were recorded at 280, 360 and 520 nm (Figures 2-4 to 2-6). Most of the phenolic compounds can be detected at 280 nm. Ellagic acid derivatives and flavonols have maximum absorption at 360 nm. Anthocyanins were detected at 520 nm in the skin. Anthocyanins were also detected in the pulp, however, it could be due to migration of these pigments from skin to the pulp during the separation of fruit into its parts. Mass Spectrometer (MS) was operated in both positive and negative ionization modes in the same HPLC run. Anthocyanins have inherent positive charge so they have maximum sensitivity in the positive modes of MS, however, for most other flavonoids the highest sensitivity was obtained in negative ionization mode. Most flavonoids are present in nature as glycosides and other conjugates [36-37]. Identification of sugar moiety attached to phenolic compounds was based on fragmentation data from MS and previous literature reports [5].

Phenolic compounds in the pulp, skin and seed were identified based on mass spectrum data, chromatography of pure standards and UV/Vis spectra on diode array detector. Seventeen phenolic compounds are reported for the first time in Noble pulp that include caffeic acid hexoside, hydrolysable tannins, mostly gallotannins, epicatechin, epicatechin gallate, ellagic acid and its conjugates, flavonol glycosides and isomeric forms of resveratrol glucoside (Table 2-2). In Noble skin, twenty eight phenolic compounds are reported and among those eight compounds were identified for the first time (Table 2-3). These compounds were - caffeic acid hexoside,

hexahydroxydiphenoic-glucose (HHDP-glucose), monogalloyl glucose, ellagic acid hexoside, kaempferol rutinoside and hexosides of myricetin, quercetin, and kaempferol. No condensed tannins were identified in the skin. Forty three different phenolic compounds identified in Noble seed are outlined in Table 2-4. No previous study on muscadine grapes has identified these compounds. Among the various phenolic compounds in seed, hydrolysable tannins were most prominent. Condensed tannins and flavan-3-ols, ellagic acid conjugates, quercetin rhamnoside and caffeic acid hexoside were also identified in the Noble seed.

Hydroxycinnamic acid derivatives: Compound 1 (Tables 2-2, 2-3 and 2-4, Figure 2-2) had *m/z* 377 $[M + Cl^-]^-$ ion, which indicates a chloride adduct that fragmented to yield *m/z* 341 $[M - H]^-$ as the most intense ion in MS^2. Compound with *m/z* 341 further dissociated to give ion at *m/z* 179 by losing a hexose sugar and was tentatively identified as caffeic acid hexoside. Similar MS fragmentation data was observed in previous studies [38-39].

Hydroxybenzoic acid: The identification of gallic acid (Compound 8, Table 2-4) was confirmed by same retention time and MS data of pure standard which gave *m/z* 169 $[M - H]^-$ ion that dissociated to form *m/z* 125 via loss of CO_2. Gallic acid has been previously identified in muscadine grapes [5].

Hydrolysable tannins: Hydrolysable tannins are categorized into gallotannins and ellagitannins. Gallotannins consist of a glucose molecule in which hydroxyl groups are partly or completely substituted with galloyl groups and ellagitannins are esters of hexahydroxydiphenoyl (HHDP) group consisting of polyol core (glucose or quinic acid). Additionally, galloyl residues may be attached to glucose core via *m*-depside bonds [40-

[42]. Based on MS data the main fragmentation pattern from gallotannins involved the loss of one or more galloyl groups (152 atomic mass unit, *amu*) and/or gallic acid (170 *amu*) from the deprotonated molecule $[M - H]^-$. However, the fragmentation pattern of ellagitannins was less clear than gallotannins as ellagitannins display enormous structural variability because of different linkages of HHDP residues with glucose molecule and their strong tendency to form C – C and C – O – C linkages [41, 43]. The presence of HHDP moiety was confirmed from MS data by the presence of an ion at *m/z* 301 from the deprotonated molecule $[M - H]^-$ as reported in previous studies with fruit and plant material [5, 38, 44-49]. The presence of a compound with same molecular weight at different retention times illustrated isomeric forms of that compound. Different isomeric forms of hydrolysable tannins were observed and have been reported previously in Eucalyptus [45].

Compound 2 (Table 2-4) and Compound 3 (Table 2-3) had a *m/z* 481 $[M - H]^-$ which fragmented to gave an intense product ion at *m/z* 301 $[M - H - 162]^-$ by losing one glucose unit (Figure 2-2). Based on the fragmentation pattern and literature data [45] these compounds were tentatively identified as isomers of HHDP-glucose. Compounds 7, 10 and 16 (Table 2-4) had identical *m/z* 483 $[M - H]^-$ ions which fragmented to form ions at *m/z* 331 $[M - H - 152]^-$ and *m/z* 169 $[M - H - 162]^-$ after sequential removal of galloyl group and glucosyl group. They were tentatively identified as isomers of digalloyl glucose based on fragmentation data and previous literature reports [45, 47, 49-51]. Compound 6 (Tables 2-2 and 2-3, Figure 2-2) gave identical $[M - H]^-$ ions at *m/z* 331, which yielded deprotonated gallic acid residue (*m/z* 169) owing to loss of glucose unit $[M - H - 162]^-$. The gallic acid anion decarboxylates to form fragment at *m/z* 125 (169 –

44, loss of CO_2). This compound was tentatively identified as monogalloyl glucose [51-52]. Compound 4 (Table 2-2), Compound 11 (Table 2-3) and Compounds 5, 9 and 19 (Table 2-4) eluted at different time but gave identical $[M - H]^-$ and product ions as compound 6. These compounds are tentatively identified as isomers of monogalloyl glucose, where gallic acid is attached to different hydroxyl group of the glucose. Compound 13 (Tables 2-2 and 2-4, Figure 2-3) was tentatively identified as monogalloyl diglucose with *m/z* 493 $[M –H]^-$ ion fragmenting to yield ions at *m/z* 331 and *m/z* 169 after sequential removal of two glucosyl groups (162 *amu*). Similar observations were also reported in longan seeds [52]. Compound 22 (Table 2-3) had $[M - H]^-$ ion at *m/z* 625 which dissociated to give intense MS^2 ion at *m/z* 463 $[M - H - 162]^-$ indicating loss of glucose unit. The major ion in MS^3 was at *m/z* 301 $[M - H - 162 - 162]^-$ suggesting loss of another glucose unit. Based on fragmentation data this compound was tentatively identified as HHDP-diglucoside [5].

Compound 12 (Table 2-4) had $[M - H]^-$ ion at *m/z* 633 and major MS^2 fragment at *m/z* 481, indicating presence of HHDP-glucose by losing galloyl unit $[M - H - 152]^-$ and minor fragment at *m/z* 301. Loss of galloyl unit suggested that galloyl units were not directly linked to glucose core but were attached via *m*-depside bond, thus the compound was identified as Galloyl-HHDP-glucose [44, 49]. However, compound 15 (Table 2-3) and compounds 14, 17, 20, 23, 28 and 30 (Table 2-4) gave identical deprotonated ions at *m/z* 633 $[M - H]^-$ but the intense MS^2 fragment was at *m/z* 301 $[M - H - 331]^-$ instead of *m/z* 481 indicating loss of galloylglucose. The fragmentation data suggests that galloyl unit was directly linked to glucose core, thus the compounds were tentatively identified as isomeric forms of HHDP-galloyl-glucose. Compounds 21, 24, 31 (Figure 2-

3), 37, 42 and 45 (Table 2-4) were tentatively identified as isomeric forms of HHDP-digalloyl-glucose. The deprotonated molecule with *m/z* 785 $[M - H]^-$ fragmented to give a major ion at *m/z* 633 $[M - H - 152]^-$ indicating loss of galloyl group and minor ions at *m/z* 483 $[M - H - 301]^-$and *m/z* at 301 $[M - H - 483]^-$ showing the presence of HHDP moiety and loss of digalloyl glucose [5, 47, 49].

Compounds 25, 26 (Figure 2-3), 32, 35, 41 and 43 (Table 2-4) had $[M - H]^-$ at *m/z* 635. The major ions in MS^2 were at *m/z* 483 $[M - H - 152]^-$, 465 $[M - H - 170]^-$ and 423 $[M - H - 212]^-$ indicating loss of galloyl group, gallic acid and loss of another galloyl group along with cross ring fragmentation of glucose [53], respectively. However, MS^3 yielded fragments at *m/z* 331 $[M - H - 152 - 152]^-$, 313 $[M - H - 152 - 152 - 18]^-$, 271 $[M - H - 212 - 152]^-$ and 169 indicating successive loss of galloyl groups. Some other minor fragments were also observed in MS^3. The different retention time and fragmentation pattern suggests presence of isomeric forms of the given molecule. These compounds were tentatively identified as isomers of trigalloyl glucose [45, 47, 49-50]. Compounds 47, 51 and 52 (Table 2-3) were tentatively identified as ellagitannins yielding deprotonated ions at *m/z* at 813, 831 and 817. The presence of HHDP was supported by the formation of *m/z* 301. However, structural elucidation of these compounds was not done due to lack of complete fragmentation data. Ellagitannins have been previously identified in muscadine grapes [5].

Compounds 40, 48 (Figure 2-3) and 49 (Table 2-4) were tentatively identified as isomers of tetragalloyl glucose which dissociated to give identical *m/z* at 787 $[M - H]^-$. The fragmentation of deprotonated ion in MS^1 and MS^2 yielded ions at *m/z* 635 $[M - H - 152]^-$, 617 $[M - H - 152 - 18]^-$, 483 $[M - H - 152 - 152]^-$, 465 $[M - H - 152 - 152 -$

18]$^-$, 313 [M – H – 152 –152 – 18 – 152]$^-$ and 169 [M – H – 152 – 152 – 18 – 152 – 144]$^-$ indicating consecutive losses of galloyl groups and water molecules, and finally loss of glucose from dehydrated galloyl glucose molecule to give deprotonated gallic acid. These findings were confirmed by previous literature reports [45, 47, 49]. Compounds 57 (Figure 2-3) and 58 (Table 2-4) gave identical *m/z* at 939 [M – H]$^-$ dissociating to yield ions at *m/z* 787 [M – H – 152]$^-$, 769 [M – H – 152 – 18]$^-$, 635 [M – H – 152 – 18 – 134]$^-$, 617 [M – H – 152 – 152 – 18]$^-$, 483 [M – H – 152 – 152 – 18 –152]$^-$ and 465 [M – H – 152 – 152 – 18 –152 – 18]$^-$ suggesting loss of galloyl groups and water molecules. Due to lack of complete structural elucidation by fragmentation data in MS^1 and MS^2, these compounds were tentatively identified as isomers of pentagalloyl glucose [49].

Compound 61 (Table 2-4, Figure 2-3) was tentatively identified as hexagalloyl glucose [49] with *m/z* 1091 [M – H]$^-$ that fragmented to give 939 [M – H – 152]$^-$ and 787 [M – H – 152 – 152]$^-$ in MS^2 indicating loss of galloyl groups and presence of pentagalloyl and tetragalloyl glucose residues. The MS^3 fragments were at *m/*z 787 [M – H – 152 – 152]$^-$, 769 [M – H – 152 – 152 – 18]$^-$ and 617 [M – H – 152 – 152 – 18 –152]$^-$ indicating loss of galloyl groups and water molecules. Similar results were reported by Soong et al [52].

Anthocyanins: Six different anthocyanins were identified in Noble grape skin (Compounds 27, 29, 33, 38, 39 and 44 (Table 2-3). The anthocyanins coeluted and represented only three peaks in the chromatogram but they had different retention times. So the peak numbers were marked according to the retention times of individual anthocyanins (Figure 2-5). Although, previous studies have identified and quantified the anthocyanins in muscadine grapes [2, 54], most of the identification and quantification was

done after hydrolysis [2, 28] which does not justify the structure of anthocyanin diglucosides. In the present study, fragmentation pattern of anthocyanins from MS^2 and MS^3 is provided. Similar fragmentation pattern was observed for all anthocyanins indicating loss of glucose residues and formation of aglycone. Anthocyanins in muscadine grapes have been reported to exist in 3, 5-diglucoside forms [54-56].

Compound 27 with *m/z* 627 $[M]^+$ fragmented to two product ions in MS^2 at *m/z* 465 $[M – 162]^+$ and 303 $[M – 162 - 162]^+$, corresponding to delphinidin glucoside and delphindin, respectively. So, this compound was tentatively identified as delphinidin 3, 5-diglucoside. Compound 29 had *m/z* at 611 $[M]^+$ that fragmented to yield two product ions at *m/z* 449 $[M – 162]^+$ and 287 (cyanidin) $[M – 162 - 162]^+$ indicating the compound to be cyanidin 3,5-diglucoside. Compound 33 was tentatively identified as petunidin 3, 5-diglucoside. The molecular ion at *m/z* 641 $[M]^+$ fragmented to yield two product ions in MS^2 at *m/z* 479 $[M – 162]^+$ and 317 (petunidin) $[M – 162 - 162]^+$ indicating two glucose molecules attached to petunidin. Compounds 38 and 39 coeluted having the same retention time but the mass spectrum of the peaks suggested two molecular ions at *m/z* 595 $[M]^+$ and 625 $[M]^+$. The MS^2 spectrum of molecular ion at *m/z* 595 (compound 38) fragmented into two product ions at *m/z* 433 $[M – 162]^+$ and 271 $[M – 162 - 162]^+$, which corresponded to pelargonidin glucoside and pelargonidin, respectively. Compound 38 was tentatively identified as pelargonidin 3, 5-diglucoside. Similarly, the molecular ion at *m/z* 625 (compound 39) had two product ions at *m/z* 463 $[M – 162]^+$ and 301 $[M – 162 - 162]^+$, which corresponded to peonidin glucoside and peonidin, respectively. This compound was tentatively identified as peonidin 3, 5-diglucoside. Compound 44 had molecular ion at *m/z* 655 $[M]^+$ and fragment ions at *m/z* 493 $[M – 162]^+$ and 331 [M –

162 - 162]$^{+}$. Based on mass fragmentation this compound was tentatively identified as malvidin 3, 5-diglucoside. We were able to confirm the presence of pelargonidin 3, 5-diglucoside in muscadine grapes as reported in a previous study [2].

Flavan-3-ols and condensed tannins: The condensed tannins were identified only in Noble seed compared to skin and pulp. Both galloylated and non galloylated flavan-3-ols and condensed tannins were identified (Tables 2-2 to 2-4). Compound 18 (Table 2-3) had deprotonated ion at *m/z* 305 $[M - H]^{-}$ and was identified as gallocatechin. Its identity was confirmed by the same retention time as standard and formation of MS^2 fragment ions at *m/z* 285, 263, 219, 179, 165 and 125 [5, 57]. Compound 34 (Table 2-4, Figure 2-3) with $[M - H]^{-}$ ion at *m/z* 729 generated main MS^2 fragment ion at *m/z* 577 $[M - H - 152]^{-}$ and MS^3 fragment ion at *m/z* 289 $[M - H - 152 - 288]^{-}$ corresponding to the loss of galloyl group and (epi)catechin gallate moiety, respectively. Based on mass spectral data this compound was tentatively identified as galloyl procyanidin dimer [57-58]. Compound 36 (Table 2-4) was tentatively identified as procyanidin dimer with its *m/z* 577 $[M - H]^{-}$ dissociating to yield ions at *m/z* 425 $[M - H - 152]^{-}$ and 289 $[M - H - 288]^{-}$ in MS^2 indicating characteristic loss of 152 *amu* due to Retro-Diels-Alder (RDA) fission [57] and loss of (epi)catechin molecule, respectively. Mass spectral data from MS^3 showed further dissociation of flavanol rings. Compound 46 (Tables 2-2 and 2-4) with identical $[M - H]^{-}$ ion at *m/z* 289 generated the major MS^2 ions at *m/z* 245 (loss of CO_2) and minor ions at *m/z* 205 (cleavage of A-ring of flavan-3-ol), 137 (RDA fission). The major ion in MS^3 was at *m/z* 203 (cleavage of A-ring of flavan-3-ol). Compared with the standard, this compound was identified as epicatechin [30, 38, 44]. Compound 53 (Tables 2-2 and 2-4, Figure 2-3) dissociated at *m/z* 441 $[M - H]^{-}$ to yield product ions in MS^2 at

m/z 289, 169 and 125 corresponding to the deprotonated ion of (epi)catechin and gallic acid, and decarboxylated gallic acid, respectively. The major fragment ion in MS^3 was at *m/z* 245 indicating decarboxylation of epicatechin and minor fragments at *m/z* 205 and 137 suggesting characteristic fragmentation pattern of (epi)catechin [44, 57]. Based on the same retention time with standard and mass spectral data this compound was identified as epicatechin gallate. Compound 60 (Table 2-4, Figure 2-3) gave $[M - H]^-$ ion at *m/z* 593, which yielded major ions at *m/z* 441 $[M - H - 152]^-$ in MS^2 spectra and *m/z* 289 $[M - H - 152 - 152]^-$ in MS^3 spectra, corresponding to loss of successive galloyl groups from (epi)catechin. Based on the mass spectral data this compound was tentatively identified as (epi)catechin digallate [59].

Ellagic acid and conjugates: Ellagic acid had been identified and quantified in muscadine grapes in the previous studies [5, 27]. In this study we identified ellagic acid hexoside in Noble pulp and skin for the first time (Tables 2-2 and 2-3). Compound 55 (Tables 2-2 and 2-3, Figure 2-2) had identical $[M - H]^-$ ion at *m/z* 463 which yielded major ion at *m/z* 301 $[M - H - 162]^-$ and minor ions at *m/z* 284, 257, 229 characteristic of ellagic acid fragmentation [30, 44]. The loss of 162 *amu* corresponded to the loss of hexose sugar and based on the fragmentation pattern this compound was tentatively identified as ellagic acid hexoside [47, 60]. Compound 63 (Tables 2-2, 2-3 and 2-4) was tentatively identified as ellagic acid xyloside with its $[M - H]^-$ ion at *m/z* 433 dissociating to form major ions at *m/z* 301 via loss of xylose (132 *amu*) and minor ions at *m/z* 284, 257 and 185 indicating presence of ellagic acid [5, 48, 52]. The MS spectra of compound 65 (Tables 2-2 and 2-3) gave identical $[M - H]^-$ ion at *m/z* 447 which dissociated to yield major ion at *m/z* 301 $[M - H - 146]^-$ and minor ions at 257, 229 and 185 corresponding

to loss of rhamnose and characteristic fragmentation pattern of ellagic acid. So this compound was tentatively identified as ellagic acid rhamnoside. Compound 67 (Tables 2-2, 2-3 and 2-4) was identified as free ellagic acid based on characteristic fragmentation pattern, standard and previous literature data [5, 30, 38, 44, 52, 60].

Flavonols: The flavonols previously identified in muscadine grapes were glycosides of quercetin, kaempferol and myricetin [5]. In this study myricetin hexoside, kaempferol hexoside, quercetin glucoside and kaempferol rutinoside were identified for the first time (Tables 2-2 and 2-3). Compound 54 (Tables 2-2 and 2-3, Figure 2-2) had identical $[M - H]^-$ ion at *m/z* 593 which fragmented to produce product ions at *m/z* 447 $[M - H - 146]^-$ and at *m/z* 285 $[M - H - 146 - 162]^-$ indicating loss of rhamnosyl group and hexosyl-rhamnosyl group, respectively. The fragment ion at *m/z* 285 corresponded to aglycone of kaempferol, therefore this compound was tentatively identified as kaempferol rutinoside [57]. Compound 59 (Table 2-3, Figure 2-2) with deprotonated ion at *m/z* 479 $[M - H]^-$ gave product ions at *m/z* 317 $[M - H - 162]^-$ and minor ions at *m/z* 271, 179 and 151, suggesting loss of hexose and producing aglycone myricetin. On the basis of the mass spectral data and previously published data [57, 61] this compound was tentatively identified as myricetin hexoside. Compound 62 (Tables 2-2 and 2-3) was tentatively identified as myricetin rhamnoside (464 *amu*) based on MS data that produced major fragment of aglycone myricetin (*m/z* 317) by losing rhamnose (146 *amu*) [5, 39]. Compound 64 (Table 2-3, Figure 2-2) had $[M - H]^-$ ion at *m/z* 463, dissociating to yield fragment ions at *m/z* 301 $[M - H - 162]^-$ and *m/z* 271, 179 and 151, a characteristic of quercetin fragmentation. This compound was identified as quercetin 3-O-β-glucoside based on mass spectral data and standard [30, 61-62]. Compound 68 (Tables 2-2, 2-3 and

2-4) was identified as quercetin 3-O-β-rhamnoside based on standard and mass fragmentation which produced *m/z* 447 $[M - H]^-$ and dissociated to give ions at *m/z* 301 $[M - H - 146]^-$ and *m/z* 179, 151, corresponding to loss of rhamnose and fragmentation of quercetin, respectively [5, 52, 62]. Compound 69 (Tables 2-2 and 2-3, Figure 2-2) gave deprotonated ion at *m/z* 447 which further fragmented to produce major ion at *m/z* 285 $[M - H - 162]^-$ and minor ions at *m/z* 255, 227, the compound was tentatively kaempferol hexoside [44, 62]. Compound 70 (Tables 2-2 and 2-3) had $[M - H]^-$ ion at *m/z* 431, which fragmented to yield major ion at *m/z* 285 $[M - H - 146]^-$ in MS^2. Based on mass spectral data and previous study on muscadine grapes [5], this compound was tentatively identified as kaempferol rhamnoside.

Stilbenes: Compound 50 and 56 (Table 2-2) had $[M - H]^-$ ion at *m/z* 425, due to formation of chloride adduct. The ion at *m/z* 425 $[M+Cl^-]^-$ dissociated to yield two product ions one at *m/z* 389 $[M - H]^-$ and other at *m/z* 227 $[M - H - 162]^-$, corresponding to loss of chloride ion and glucose, respectively. Similarly, Compound 66 (Table 2-3) had $[M - H]^-$ at *m/z* 389. The MS^2 spectrum of the deprotonated ion at *m/z* 389 produced product ion at *m/z* 227 $[M - H - 162]^-$ resulting from loss of glucose unit. Based on mass spectral data and standard, compound 56 was identified as *trans*-resveratrol 3-O-β-glucoside. The other two compounds (compound 50 and 66) having the same mass spectral data were tentatively identified as isomeric forms of resveratrol glucoside [58, 63].

Summary

Our results indicate that muscadine seeds have high phenolic content and antioxidant capacity compared to skin and pulp. The high antioxidant capacity and total phenolic content of the muscadine seeds makes them potentially significant source of

compounds with nutraceutical properties. Additionally, it was confirmed from the results that HPLC – ESI – MS^n, operated under both positive and negative ionization, is a valuable tool for the identification of wide array of known phenolic compounds as well as for the preliminary identification of novel compounds. This method allows simultaneous identification of various phenolic compounds (phenolic acids, anthocyanins, flavonols, flavan-3-ols and condensed tannins, hydrolysable tannins, and stilbenes) under similar chromatographic conditions. The prominent class of phenolic compounds in Noble skin and pulp belong to flavonols compared to seeds in which the majority of compounds belong to hydrolysable and condensed tannins category. The phenolic compounds from the skin and seed get extracted into the wine and juice, and are important quality components that contribute to the color and taste of these products. Thus, the structural elucidation of phenolic compounds in muscadine grapes could provide a better understanding of color and flavor changes occurring in muscadine wine and juice upon storage.

Table 2-1. Total phenolic content and antioxidant capacity of seeds, skin and pulp of different cultivars of muscadine grapes.

Cultivar	Total Phenolic content (GAE mg/g)			Antioxidant capacity (ORAC, µmol TE/g)		
	Seeds	Skin	Pulp	Seeds	Skin	Pulp
Bronze						
Doreen	45.1 ± 5.3^{b}	4.5 ± 0.4^{cd}	1.0 ± 0.0^{b}	797.9 ± 29.8^{c}	26.0 ± 5.3^{d}	4.0 ± 0.3^{ab}
Fry	68.8 ± 5.9^{a}	4.7 ± 0.3^{cd}	0.9 ± 0.0^{bc}	1538.4 ± 41.8^{a}	$^{\#}37.9 \pm 0.3^{bcd}$	4.0 ± 0.3^{ab}
Carlos	37.4 ± 2.0^{bc}	10.2 ± 0.6^{a}	0.8 ± 0.0^{c}	499.6 ± 8.2^{de}	43.1 ± 0.6^{b}	2.4 ± 0.3^{c}
Triumph	40.0 ± 7.7^{bc}	4.3 ± 0.5^{d}	0.3 ± 0.0^{d}	530.8 ± 39.8^{d}	27.1 ± 3.2^{cd}	2.3 ± 0.4^{c}
Black						
Southland	44.4 ± 2.7^{b}	6.2 ± 0.4^{bc}	1.2 ± 0.1^{a}	313.9 ± 2.5^{f}	$^{\#}43.9 \pm 0.2^{b}$	3.6 ± 0.2^{b}
Magoon	27.0 ± 2.2^{c}	5.9 ± 0.6^{bcd}	0.9 ± 0.0^{bc}	432.2 ± 16.5^{e}	37.9 ± 1.0^{bc}	3.4 ± 0.3^{b}
Alachua	81.2 ± 5.8^{a}	6.1 ± 0.7^{bc}	0.9 ± 0.0^{bc}	1105.4 ± 8.8^{b}	42.1 ± 1.8^{b}	3.3 ± 0.3^{b}
Noble	36.6 ± 5.6^{bc}	7.5 ± 0.8^{b}	0.9 ± 0.0^{bc}	276.6 ± 18.3^{f}	77.5 ± 8.1^{a}	4.6 ± 0.1^{a}

Results are mean ± standard deviation of three determinations on fresh weight basis. Values with $^{\#}$ are in duplicates due to rejection of data points based on Q-test. Different superscripts in each column indicate the significant differences in the mean at $p \leq 0.05$.

Table 2-2. Retention time and mass spectrometric data of phenolic compounds in muscadine grape pulp (cv. Noble) determined by HPLC-ESI-MS^n

Compound number	Retention time t_R (min)	Molecular weight	MS^1 (m/z)	MS^2 (m/z)[a]	MS^3 (m/z)[a]	Identified compound
Hydroxycinnamic acid derivatives						
1	3.1	342	377 $[M+Cl]^-$ [341+36] $^-$	**341**, 215	179, **161**, 113, 101	Caffeic acid hexoside*
Hydrolysable tannins						
4	5.7	332	331 $[M-H]^-$	313, 271, **169**, 125		Monogalloyl glucose*
6	7.2	332	331 $[M-H]^-$	271, 211, **169**, 125		Monogalloyl glucose
13	9.4	494	493 $[M-H]^-$	456, 377, **331**, 169, 157		Monogalloyl diglucose*
Flavan-3-ols						
46	20.4	290	289 $[M-H]^-$	**245**, 227, 205, 179, 137, 109	**203**, 188, 161, 123	Epicatechin#
53	24.2	442	441 $[M-H]^-$	**289**, 169, 125	**245**, 230, 203, 179, 107	Epicatechin gallate*#
Ellagic acid and conjugates						
55	25.6	464	463 $[M-H]^-$	**301**, 284, 257, 229, 217		Ellagic acid hexoside*
63	31.7	434	433 $[M-H]^-$	301	**284**, 257	Ellagic acid xyloside
65	32.9	448	447 $[M-H]^-$	**301**, 299, 257	300, **185**	Ellagic acid rhamnoside
67	34.0	302	301 $[M-H]^-$	262, **257**		Ellagic acid#
Flavonols						
54	25.4	594	593 $[M-H]^-$	535, 447, **285**		Kaempferol rutinoside*
62	30.5	464	463 $[M-H]^-$	405, **317**, 316, 271, 179	**287**, 271, 215, 179,126	Myricetin rhamnoside
68	35.6	448	447 $[M-H]^-$	437, 376, 344, 329, 321, **301**, 271, 255, 228, 191, 179, 167, 151	271, **179**	Quercetin 3-O-β rhamnoside**#**
69	36.0	448	447 $[M-H]^-$	327, **285**, 257, 179, 134		Kaempferol hexoside*
70	39.6	432	431 $[M-H]^-$	**285**, 255, 214, 179, 163		Kaempferol rhamnoside
Stilbenes						
50	22.9	390	425 $[M+Cl]^-$ [389+36]	**389**, 227		Resveratrol glucoside
56	25.7	390	425 $[M+Cl]^-$ [389+36]	**389**, 227		*Trans* resveratrol 3-*O*-β-glucoside#

[a] Ions in boldface indicate the most intense product ion (100% relative intensity). Compounds with * were identified for the first time in muscadine grapes. The compounds with # were identified using pure standards. All other compounds were tentatively identified based on mass data.

Table 2-3. Retention time and mass spectrometric data of phenolic compounds in muscadine grape skin (cv. Noble) determined by HPLC-ESI-MS^n

Compound number	Retention time t_R (min)	Molecular weight	MS^1 (m/z)	MS^2 (m/z)[a]	MS^3 (m/z)[a]	Identified compound
Hydroxycinnamic acid derivatives						
1	3.1	342	377 $[M+Cl^-]$ [341+36]	**341**, 215, 179	**179**, 161, 143, 131, 125, 101	Caffeic acid hexoside*
Hydrolysable tannins						
3	4.2	482	481 $[M-H]^-$	421, **301**	258, 201, **185**, 175	HHDP-glucose*
6	7.3	332	331 $[M-H]^-$	271, 211, **169**, 125	125	Monogalloyl glucose*
11	8.8	332	331 $[M-H]^-$	**169**, 125	125	Monogalloyl glucose
15	9.6	634	633 $[M-H]^-$	613, 481, **301**, 275, 250, 230, 178	300, **257**	HHDP-galloyl-glucose
22	12.9	626	625 $[M-H]^-$	623, 481, **463**, 320, 301, 239, 193	355, 319, **301**, 275, 257, 239, 215, 193, 175,164, 147	HHDP-diglucoside
47	21.0	814	813 $[M-H]^-$	781, **763**, 753, 745, 735, 725, 511, 301, 257		Ellagitannin
51	23.5	832	831 $[M-H]^-$	**813**, 795, 787, 769, 752, 741, 723, 707, 697, 680, 664, 611, 578, 451, 365, 301, 291, 254		Ellagitannin
52	23.8	818	817 $[M-H]^-$	**773**, 755, 729, 712, 701, 685, 673, 667, 655, 655, 647, 621, 617, 541, 503, 371, 237	**729**, 712, 701, 685, 655, 617, 577, 531, 465, 407, 301, 237	Ellagitannin
Flavan-3-ols						
18	11.8	306	305 $[M-H]^-$	285, 263, 247, **219**, 198, 179, 165, 151, 137, 125		Gallocatechin#

Table 2-3. contd

Compound number	Retention time t_R (min)	Molecular weight	MS^1 (m/z)	MS^2 (m/z)[a]	MS^3 (m/z)[a]	Identified compound
Anthocyanins						
27	14.7	627	627 $[M]^+$	465, **303**	285, **257**, 229, 149	Delphinidin 3,5-diglucoside
29	16.1	611	611 $[M]^+$	449, **287**	269, 241, 213, **189**, 167, 149, 137, 109	Cyanidin 3,5-diglucoside
33	17.1	641	641 $[M]^+$	479, **317**	**302**, 274, 218	Petunidin 3,5-diglucoside
38	18.2	595	595 $[M]^+$	433, **271**	**225**, 215, 197, 187, 169, 141, 131, 121	Pelargonidin 3,5-diglucoside
39	18.2	625	625 $[M]^+$	463, **301**	**286**	Peonidin 3,5-diglucoside
44	20.0	655	655 $[M]^+$	493, **331**	**315**, 299, 287, 270, 243, 179	Malvidin 3,5-diglucoside
Ellagic acid and conjugates						
55	25.6	464	463 $[M-H]^-$	**301**	**300**, 284, 257, 157	Ellagic acid hexoside*
63	31.8	434	433 $[M-H]^-$	**301**	300, 257, **245**, 229, 188, 145	Ellagic acid xyloside
65	32.9	448	447 $[M-H]^-$	**301**	300, 257, 229, **216**, 160	Ellagic acid rhamnoside
67	34.0	302	301 $[M-H]^-$	284, **257**, 229, 201, 173		Ellagic acid#
Flavonols						
54	25.4	594	593 $[M-H]^-$	534, 431, 333, **285**	**211**	Kaempferol rutinoside*
59	28.2	480	479 $[M-H]^-$	359, **317**, 270, 179	287, **271**, 259, 227, 179, 151, 125, 109	Myricetin hexoside*
62	30.4	464	463 $[M-H]^-$	**317**, 271, 179	287, 271, 242, 193, **179**, 151, 137	Myricetin rhamnoside
64	32.4	464	463 $[M-H]^-$	**301**, 151	299, 271, 255, 230, 212, **179**, 151, 121	Quercetin 3-O-β-glucoside*#
68	35.6	448	447 $[M-H]^-$	**301**	271, 255, 226, 193, 179, **151**	Quercetin 3-O-β-rhamnoside#

Table 2-3. contd

Compound number	Retention time t_R (min)	Molecular weight	MS^1 (m/z)	MS^2 (m/z)[a]	MS^3 (m/z)[a]	Identified compound
69	36.0	448	447 $[M-H]^-$	**285**, 255, 227	267, **255**, 239, 227, 199, 169, 135	Kaempferol hexoside*
70	39.6	432	431 $[M-H]^-$	**285**	267, **255**, 241, 229, 213, 195, 187, 174	Kaempferol rhamnoside
Stilbenes						
66	33.3	390	389 $[M-H]^-$	**227**	**185**, 157, 143	Resveratrol glucoside

[a] Ions in boldface indicate the most intense product ion (100% relative intensity). Compounds with * were identified for the first time in muscadine grapes. The compounds with # were identified using pure standards. All other compounds were tentatively identified based on mass data.

Table 2-4. Retention time and mass spectrometric data of phenolic compounds in muscadine grape seed (cv. Noble) determined by HPLC-ESI-MS^n

Compound number	Retention time t_R (min)	Molecular weight	MS^1 (m/z)	MS^2 (m/z)[a]	MS^3 (m/z)[a]	Identified compound
Hydroxycinnamic acid derivatives						
1	3.0	342	377 [M+Cl$^-$] [341+36]	**341**, 215, 179, 161	**179**, 143, 131, 119, 113	Caffeic acid hexoside*
Hydroxybenzoic acid						
8	7.8	170	169 [M-H]$^-$	**125**		Gallic acid#
Hydrolysable tannins						
2	3.6	482	481 [M-H]$^-$	**301**, 169	**147**	HHDP-glucose*
5	6.7	332	331 [M-H]$^-$	271, 241, 211, **169**, 125	**125**	Monogalloyl glucose*
7	7.4	484	483 [M-H]$^-$	**331**, 313, 169		Digalloylglucose
9	8.3	332	331 [M-H]$^-$	271, **169**, 125	**125**	Monogalloyl glucose
10	8.6	484	483 [M-H]$^-$	**331**, 211, 169	**169**	Digalloylglucose
12	8.8	634	633 [M-H]$^-$	**481**, 301	**185**	Galloyl-HHDP-glucose
13	9.2	494	493 [M-H]$^-$	**331**, 169	**169**	Monogalloyl diglucose*
14	9.2	634	633 [M-H]$^-$	593, 481, **301**	284, **229**	HHDP-galloyl-glucose
16	11.2	484	483 [M-H]$^-$	**331**, 313, 169	271, 241, 211, **169**	Digalloylglucose
17	11.2	634	633 [M-H]$^-$	613, 481, **301**, 185	257, **185**	HHDP-galloyl-glucose
19	12.1	332	331 [M-H]$^-$	**169**, 125	**125**	Monogalloyl glucose
20	12.7	634	633 [M-H]$^-$	615, 481, 421, **301**, 229, 185	300, 257, 201, **187**	HHDP-galloyl-glucose
21	12.7	786	785 [M-H]$^-$	748, **633**, 615, 483, 331, 301, 275		HHDP-digalloyl-glucose*
23	13.2	634	633 [M-H]$^-$	613, 572, 483, **301**, 275, 257, 228, 201	**257**	HHDP-galloyl-glucose
24	13.2	786	785 M-H]$^-$	765, **633**, 301, 275, 231, 223		HHDP-digalloyl-glucose
25	13.7	636	635 [M-H]$^-$	613, **483**, 331, 211	**313**, 207, 169	Trigalloyl glucose*
26	14.2	636	635 [M-H]$^-$	614, **483**, 301, 229	331, **169**	Trigalloylglucose
28	14.8	634	633 [M-H]$^-$	613, 566, 483, **301**, 284, 257, 229, 185		HHDP-galloyl-glucose

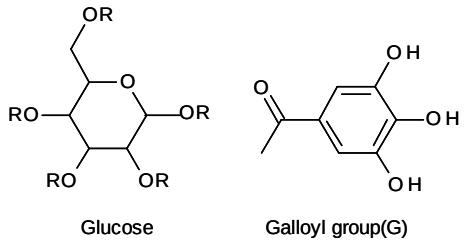
Table 2-4. contd

Compound number	Retention time t_R (min)	Molecular weight	MS^1 (m/z)	MS^2 (m/z)[a]	MS^3 (m/z)[a]	Identified compound
30	16.2	634	633 $[M-H]^-$	465, **301**	**257**, 229	HHDP-galloyl-glucose
31	16.2	786	785 $[M-H]^-$	**633**, 543, 483, 301	482, **301**, 275	HHDP-digalloyl-glucose
32	16.5	636	635 $[M-H]^-$	597, **483**, 465, 420, 313, 193	424, 331, 313, **169**	Trigalloylglucose
35	17.3	636	635 $[M-H]^-$	599, **483**, 423, 406, 332, 235, 194	405, **331**, 313, 271, 211, 169	Trigalloylglucose
37	18.1	786	785 $[M-H]^-$	768, **633**, 615, 596, 465, 419, 301, 285	313, **301**, 275, 214	HHDP-digalloyl-glucose
40	19.4	788	787 $[M-H]^-$	**635**, 483	**617**, 483, 424, 313, 211, 169	Tetragalloyl glucose*
41	19.4	636	635 $[M-H]^-$	617, 545, 483, **465**, 314, 213	313, 249, **169**	Trigalloylglucose
42	19.8	786	785 $[M-H]^-$	765, **633**, 483, 423, 301, 276, 241	**301**, 284, 275	HHDP-digalloyl-glucose
43	19.8	636	635 $[M-H]^-$	617, 483, 466, **423**, 405, 271, 211, 193	**271**, 251, 235, 211, 193, 179, 169	Trigalloylglucose
45	20.4	786	785 $[M-H]^-$	765, **633**, 615, 482, 393, 301	483, 447, **301**, 187	HHDP-digalloyl-glucose
48	22.5	788	787 $[M-H]^-$	635, **617**, 573, 403, 325	573, 529, **465**, 404, 313, 211, 197	Tetragalloyl glucose
49	23.1	788	787 $[M-H]^-$	**635**, 617, 483, 465, 447	617, **483**, 465, 423, 357, 331, 313, 271, 253, 235, 212, 193	Tetragalloyl glucose
57	26.2	940	939 $[M-H]^-$	787, **769**, 617	725, **617**, 601, 573, 465, 431, 387, 295, 260	Pentagalloyl glucose*
58	27.3	940	939 $[M-H]^-$	**787**	769, **635**, 617, 483, 465, 447, 277	Pentagalloyl glucose
61	29.6	1092	1091$[M-H]^-$	**939**, 787	787, **769**, 617	Hexagalloyl glucose*

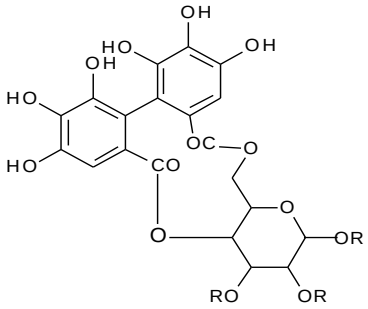
Table 2-4. contd

Compound number	Retention time t_R (min)	Molecular weight	MS^1 (m/z)	MS^2 (m/z)[a]	MS^3 (m/z)[a]	Identified compound
Flavan-3-ols and Condensed Tannins						
34	17.3	730	729 $[M-H]^-$	641, **577**, 407, 299, 211	411, **289**	Galloyl procyanidin dimer*
36	17.6	578	577 $[M-H]^-$	559, 515, **425**, 407, 289, 228, 161	**407**, 299, 257	Procyanidin dimer
46	20.4	290	289 $[M-H]^-$	**245**, 205, 188, 179, 165, 137, 126, 110	227, **203**, 191, 161, 123	Epicatechin#
53	24.2	442	441 $[M-H]^-$	332, **289**, 169, 125	271, **245**, 203, 165, 143	Epicatechin gallate*#
60	28.6	594	593 $[M-H]^-$	**441**, 321, 289, 169	397, 332, **289**, 169	(Epi)catechin digallate*
Ellagic acid and conjugates						
63	31.6	434	433 $[M-H]^-$	**301**	**300**, 259, 228, 213, 201, 185	Ellagic acid xyloside
67	34.0	302	301 $[M-H]^-$	284, **257**, 229, 185		Ellagic acid#
Flavonols						
68	35.6	448	447 $[M-H]^-$	**301**, 273, 151	271, 255, **179**, 164, 151, 121, 107	Quercetin 3-O-β-rhamnoside#

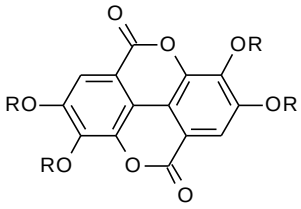
[a] Ions in boldface indicate the most intense product ion (100% relative intensity). Compounds with * were identified for the first time in muscadine grapes. The compounds with # were identified using pure standards. All other compounds were tentatively identified based on mass data.



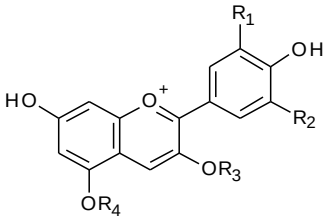
Hydrolysable tannins	R
Monogalloyl glucose	Four R's are H atoms and one R is G group
Digalloyl glucose	Three R's are H atom and two R's are G group
Trigalloyl glucose	Two R's are H atom and three R's are G group
Tetragalloyl glucose	One R is H atom and four R's are G group
Pentagalloyl glucose	All R's are G group
Hexagalloyl glucose	Another G group attaches with pentagalloyl glucose



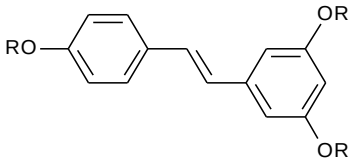
Hydrolysable tannins	R
HHDP-glucose	All R's are H atom
Galloyl-HHDP-glucose	Any R could be G



Ellagic acid and conjugates	R
Ellagic acid	H
Ellagic acid hexoside	Any one R could be hexose
Ellagic acid xyloside	Any one R could be xylose
Ellagic acid rhamnoside	Any one R could be rhamnose



Anthocyanins	R_1	R_2	R_3	R_4
Delphinidin 3,5-diglucoside	OH	OH	glucose	glucose
Cyanidin 3,5-diglucoside	OH	H	glucose	glucose
Petunidin 3,5-diglucoside	OCH_3	OH	glucose	glucose
Pelargonidin 3,5-diglucoside	H	H	glucose	glucose
Peonidin 3,5-diglucoside	OCH_3	H	glucose	glucose
Malvidin 3,5-diglucoside	OCH_3	OCH_3	glucose	glucose



Stilbenes	R
Resveratrol	H
Resveratrol glucoside	Any R could be glucose

Figure 2-1. Proposed structures of phenolic compounds in muscadine grapes.

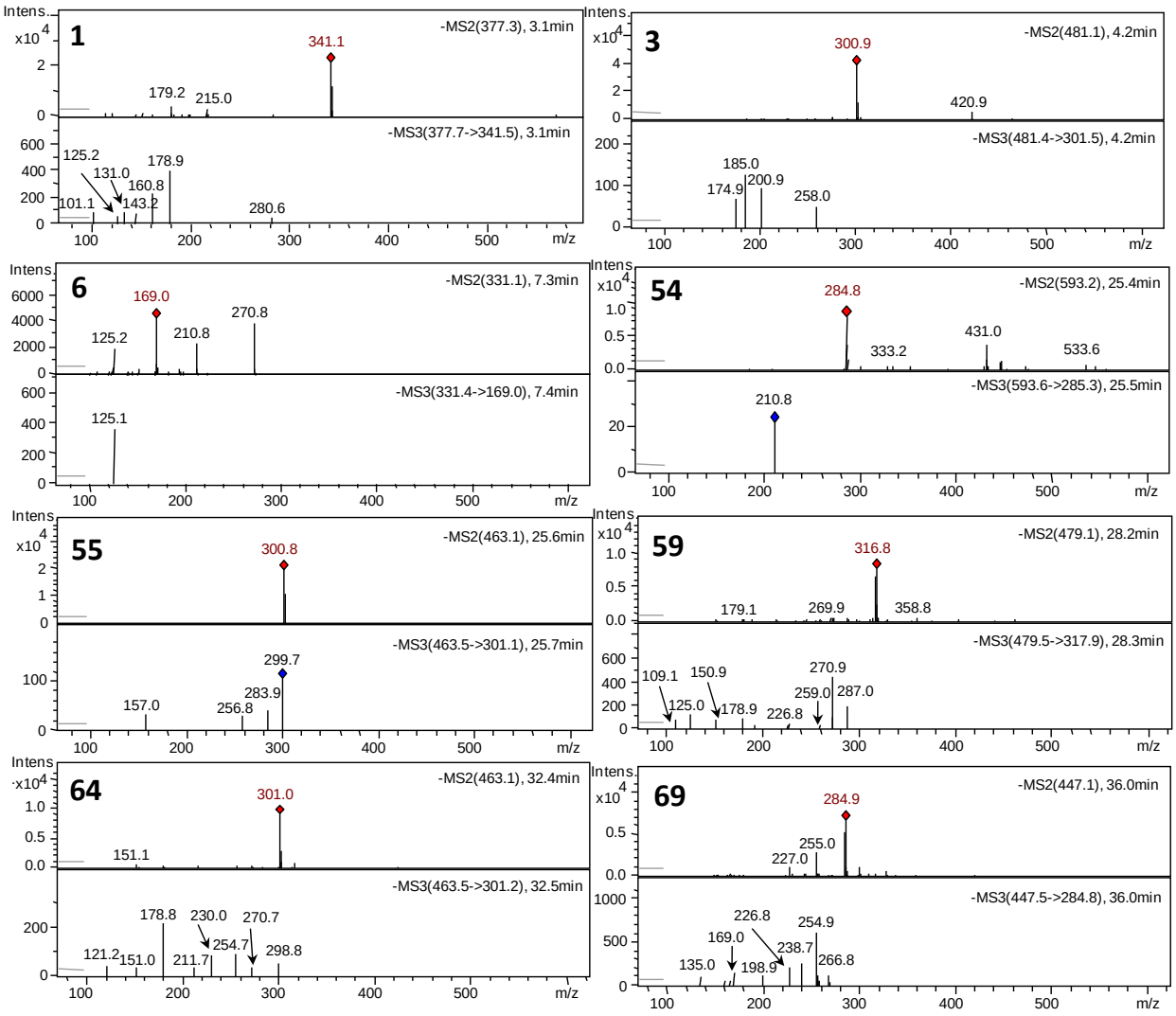


Figure 2-2. Negative ion electrospray product ion mass spectra (MS^2 and MS^3) of phenolic compounds identified for the first time in muscadine (cv. Noble) pulp, skin and seeds. The numbers in bold in each product ion spectra corresponds to the compound number in tables.

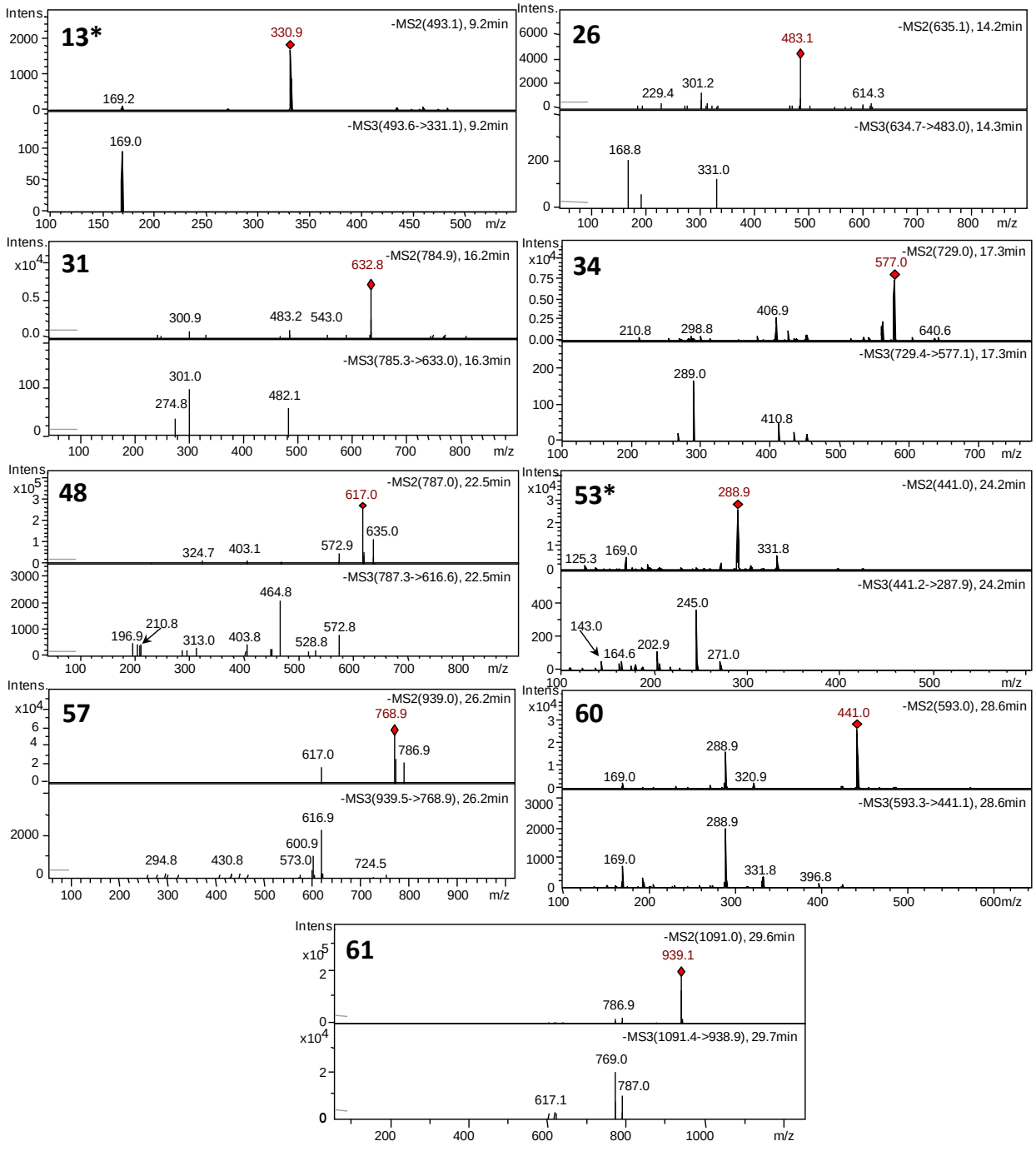


Figure 2-3. Negative ion electrospray product ion mass spectra (MS^2 and MS^3) of phenolic compounds identified for the first time in muscadine (cv. Noble) seeds. The numbers in bold in each product ion spectra corresponds to the compound number in tables. Compound numbers with * also identified in pulp.

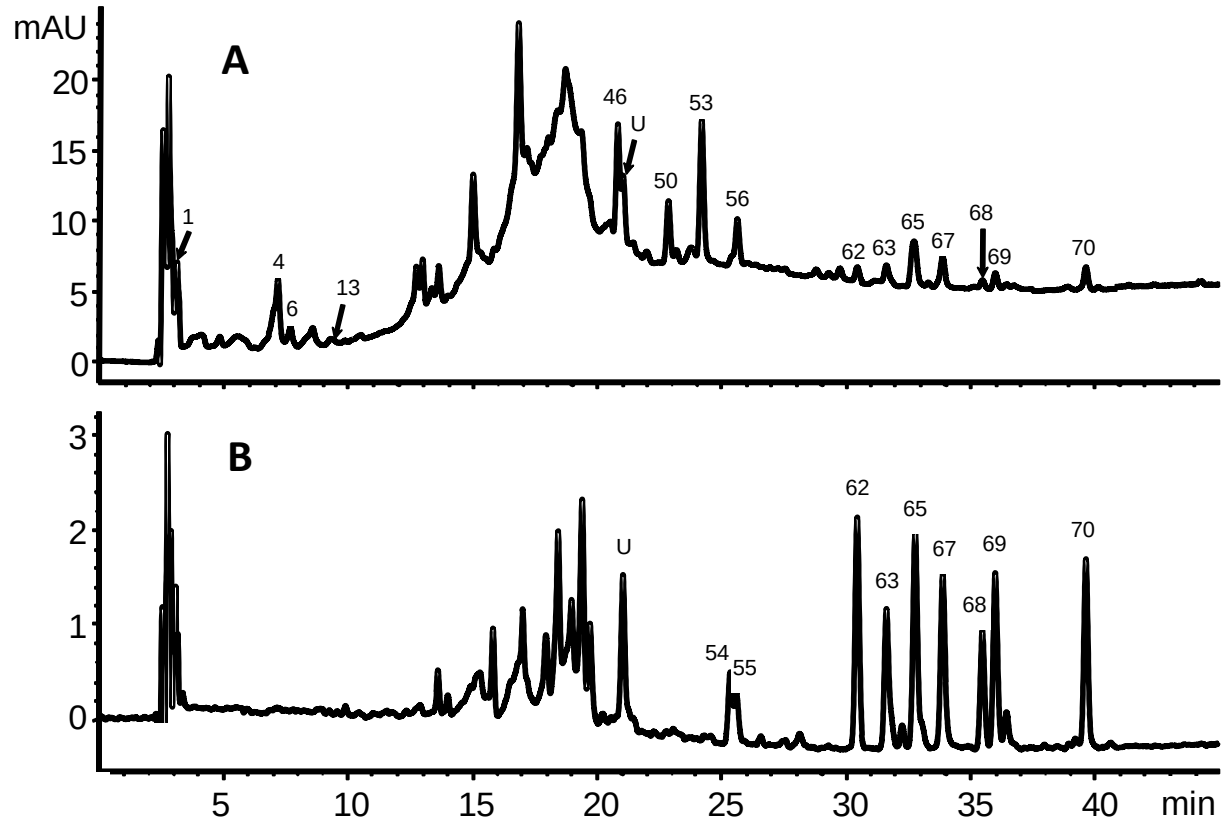


Figure 2-4. HPLC-DAD chromatograms of muscadine (cv. Noble) pulp: (A) 280 nm and (B) 360 nm. Peak with label U was unidentified. Peak numbers correspond to compound numbers in table 2-2.

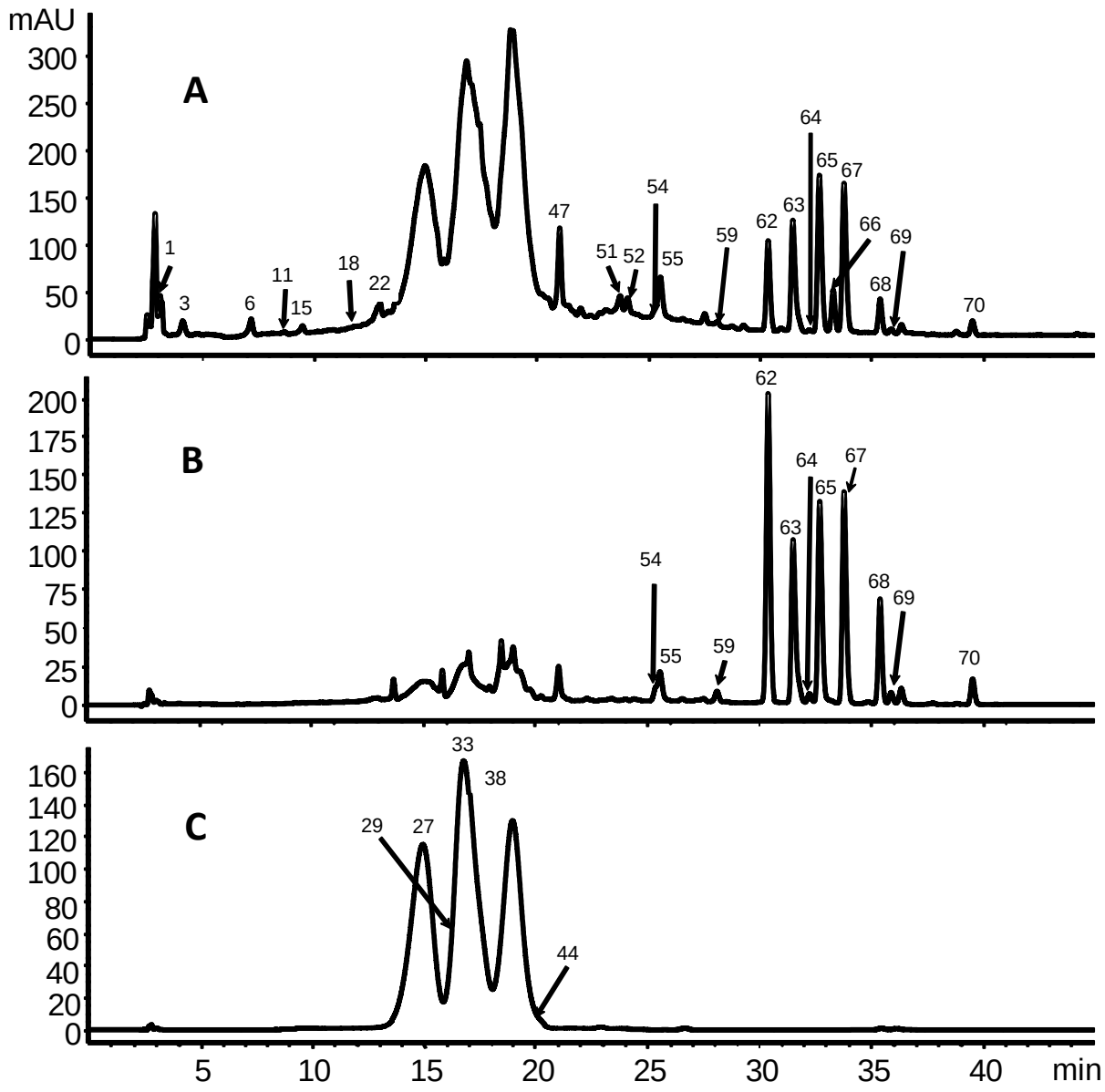


Figure 2-5. HPLC-DAD chromatograms of muscadine (cv. Noble) skin: (A) 280 nm; (B) 360 nm and (C) 520 nm. Peak having two numbers indicate coeluting compounds. Peak numbers correspond to compound numbers in table 2-3.

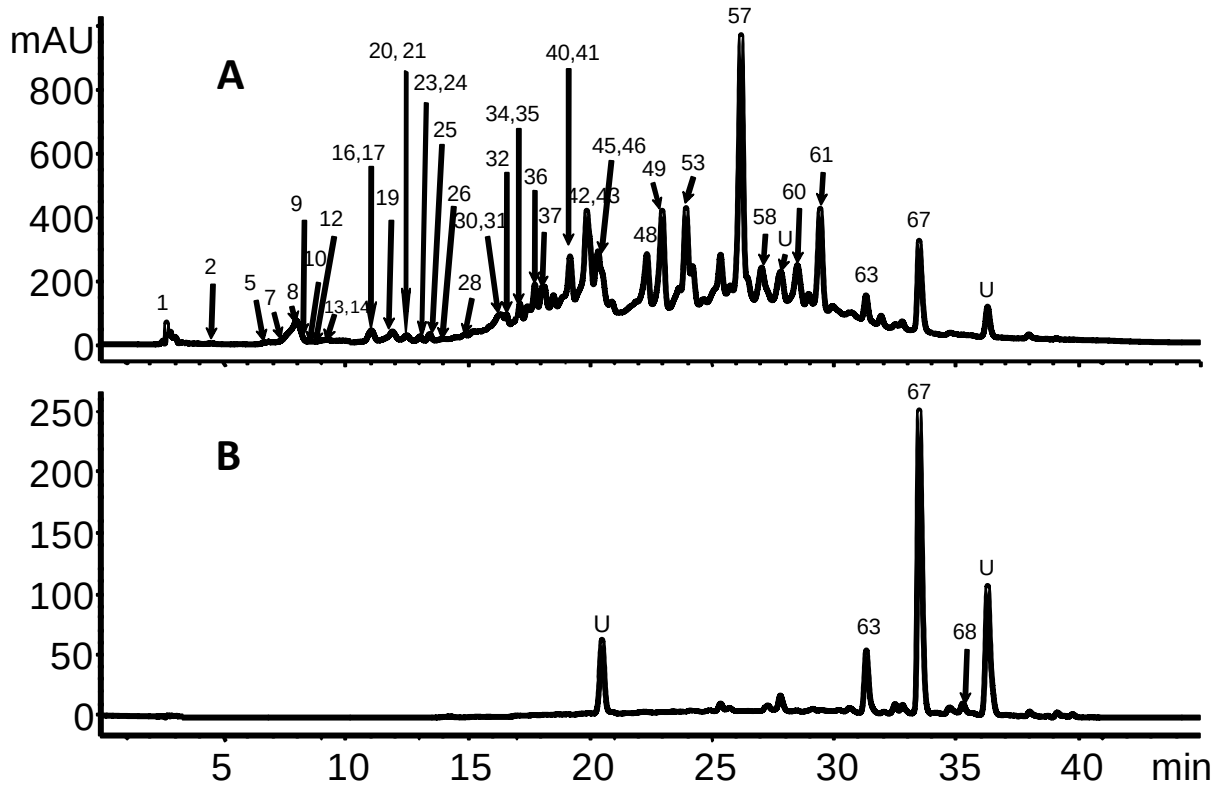


Figure 2-6. HPLC-DAD chromatograms of muscadine (cv. Noble) seed: (A) 280 nm and (B) 360 nm. Peaks with label U were unidentified and peaks having two numbers indicate coeluting compounds. Peak numbers correspond to compound numbers in table 2-4.

CHAPTER 3
EFFECTS OF EXOGENOUS ABSCISIC ACID ON ANTIOXIDANT CAPACITIES, ANTHOCYANINS AND FLAVONOL CONTENTS OF MUSCADINE GRAPE SKINS

Background

Phenolic compounds are secondary metabolites produced by plants as a defense mechanism against various biotic and abiotic stresses. They are categorized into different classes depending upon their structures varying from simple phenolic acids (hydroxybenzoic acid and hydroxycinnamic acid) to complex polyphenols (hydrolysable and condensed tannins) [20-21]. Phenolic compounds play an important role in color and sensory characteristics of fruits and vegetables. The protective effects of these compounds against various chronic diseases such as cancer and cardiovascular diseases are well recognized among researchers and health conscious consumers [22-23].

Muscadine grapes (*Vitis rotundifolia*) are commonly grown in the southeastern United States and are well-adapted to warm, humid climates, which are unsuitable for the growth of *Vitis vinifera*. They are either light-skinned (green or bronze) or dark-skinned (red to almost black) and are 2.5 – 3.8 cm in diameter with thick skin. They grow in tight small clusters of 3 to10 berries and are marketed in fresh and processed forms such as juice, wine, and jam. Muscadine grapes possess unique phytochemical composition that differentiates them from other *Vitis* species such as presence of ellagic acid and its derivatives, and anthocyanin 3, 5-diglucosides [5, 28]. In addition, they also contain hydroxybenzoic acids, resveratrol, quercetin, myricetin, and kaempferol [1, 27]. Cell culture studies have suggested that polyphenols from muscadine grapes can inhibit proliferation of colon cancer cells and induce apoptosis [13-14].

The concentration of phenolic compounds in fruits and vegetables is regulated by genetic, environmental, physiological and chemical factors such as temperature, light,

rainfall, soil, chemicals and plant growth regulators. Various agronomic strategies such as alteration of environmental conditions, water management, grafting of plants, application of elicitors, stimulating agents and plant activators have been employed to enhance the biosynthesis of phenolic compounds in fruits and vegetables [64]. One such strategy is the pre-harvest application of abscisic acid, a plant growth regulator involved in various physiological processes including color development [65].

Endogenous abscisic acid (ABA) plays an important role in plant growth. For example, ABA promotes seed maturation and germination, and serves as a signaling compound when plants are under stresses such as drought, high salinity, cold and microbial infections [66]. In addition, ABA also participates in the initiation of ripening and related changes in grape development [67-69]. It has been demonstrated that exogenous application of ABA increases the anthocyanins in grape skins [70-72] and can improve the color and quality of the grapes [65, 73]. Lacampagne et al., [74] reported that ABA regulates enzymes involved in tannin biosynthesis and thus elevates tannin content of green grapes at veraison. These studies suggest that ABA plays a significant role in triggering the flavonoid biosynthetic pathway. Most studies investigating the effect of exogenous application of ABA have been conducted in *Vitis vinifera* grapes and their hybrids. However, the effects of ABA in muscadine grapes (*Vitis rotundifolia*) have not been investigated. In this study, we hypothesized that exogenous ABA treatment will affect the anthocyanin accumulation, phenolic content and composition of red muscadine grapes.

Materials and Methods

Chemicals

AAPH (2,2'-azobis(2-amidinopropane)) was a product of Wako Chemicals Inc. (Bellwood, RI). Gallic acid, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), HPLC grade methanol, acetic acid, formic acid, hydrochloric acid, Folin-Ciocalteau reagent, Flourescein, Tween 20, and sodium carbonate were purchased from Fischer Scientific Co. (Pittsburg, PA). Standards of the 3-O-β -glucosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (six mixed anthocyanin standard, HPLC grade), were purchased from Polyphenols Laboratories (Sandnes, Norway). Ellagic acid, myricetin, quercetin and kaempferol were obtained from Sigma-Aldrich (St. Louis, MO). *S*-abscisic acid was received as a gift from Valent BioSciences Corporation (Libertyville, IL).

ABA Treatment

ABA treatments were conducted in 2009 on two cultivars of muscadine grapes, Alachua and Noble. Alachua is a red table grape with large berry size, whereas Noble is a red wine grape with much smaller berries. ABA treatment on Alachua grapes was carried out in an experimental vineyard at the Mid-Florida Research and Education Center, Apopka, Florida. For Noble grapes, ABA treatment was carried out in an experimental vineyard at the Center for Viticulture and Small Fruit Research, Florida A&M University, Tallahassee, Florida. The grapevines of the same age, size, and growth conditions were selected and assigned to receive one of the two treatments: control (water only) and 300 ppm of ABA. Tween 20 was used as a wetting agent in both the control and 300 ppm ABA solutions at a concentration of 315 µL/L. The spray aimed directly on the grapes with a hand-held sprayer until run off. For Noble variety 9

vines were used each for ABA treatment and control, however for Alachua 13 vines were used for control and 12 vines for ABA treatment. ABA application dates were chosen to avoid rainfall and wind. The first ABA spray was done at veraison (the onset of ripening, 20% grapes with red color) on both the varieties. The application dates were July 27th for Alachua and August 8th for Noble. The second spray treatment was done 13 and 8 days post veraison (60% of grapes developed red color) for Alachua and Noble, respectively. Two hours before the second spray, 60 grapes were randomly sampled from each vine. Ripe grapes were harvested for second sampling i. e. 10 and 8 days after the second spray for Noble and Alachua, respectively.

Fruit Weight, pH and Total Soluble Solids

The grapes were weighed after each sampling and average berry weight was recorded. During separation, juice was collected, filtered, and analyzed for pH and total soluble solids (°Brix). pH was measured using a pH meter and total soluble solids (°Brix) were measured using a bench top refractometer (Leica Abbe Mark 11, Fisher Scientific, Pittsburg, PA).

Extraction and Sample Preparation

The skin of the grapes was manually separated from seeds and pulp, and freeze dried. Subsequently, it was ground into a fine powder using a Waring kitchen blender and extracted (1 g) with 15 mL of methanol/water/acetic acid (85:15:0.5; v/v) in glass tubes. The samples were then vortexed for 30 s, sonicated for 5 min and kept in the dark at room temperature for 20 min. The tubes were then centrifuged at 1317 *g* for 10 min and the supernatant was removed. The samples were extracted again with 10 mL of methanol/water/acetic acid using the same procedure. The supernatants from two

extractions were pooled and transferred into a 25-mL volumetric flask. Methanol/water/acetic acid was added to make up the final volume to 25 mL.

Folin-Ciocalteu Assay

The extracts were diluted to appropriate concentration for analysis. The total phenolic content was determined as reported previously [6].The results were expressed as milligrams of gallic acid equivalents per gram of fresh grape skins (mg of GAE/ g).

Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay for extracted samples was conducted on a Spectra XMS Gemini plate reader (Molecular Devices, Sunnyvale, CA). Briefly, 50 µL of standard and samples were added to the designated wells of a 96-well black plate. This was followed by the addition of 100 µL of flourescein (20 nM). The mixture was incubated at 37°C for 7 min before the addition of 50 µL of AAPH. Fluorescence was monitored using 485 nm excitation and 530 nm emissions at 1 min intervals for 40 min. Trolox was used to generate a standard curve. The antioxidant capacities of extracts were expressed as micromoles of Trolox equivalents (TE) per gram of fresh grape skins (µmol of TE/g).

DPPH Assay

The DPPH scavenging activities of samples were measured using a previously published method [75]. In summary, DPPH stock solution was prepared by dissolving 20 mg of DPPH in 100 mL methanol and stored at -20°C prior to use. DPPH working solution was freshly prepared by mixing 2.8 mL DPPH stock solution and 7.2 mL methanol. Absorbance at 515 nm was measured on a microplate reader (SPECTRAmax 190, Molecular Devices, Sunnyvale, CA). Diluted extracts (50 µL) were added to 950 µL DPPH working solution and incubated for 60 min in the dark at room temperature. Trolox solutions from 100 to 1000 µM were added to DPPH working

solution as standards. Results of the DPPH scavenging activity of grape skin extracts were expressed as micromoles of Trolox equivalents (TE) per gram of fresh grape skins (μmol of TE/g).

HPLC Analysis of Phenolic Compounds

Chromatographic analysis was performed on an Agilent 1200 HPLC system consisting of an autosampler, a binary pump, a column compartment, a diode array detector and a fluorescent detector (Agilent Technologies, Palo Alto, CA). Reversed phase chromatography was used for the quantification of anthocyanins, ellagic acid and flavonols. An Agilent Zorbax Stablebond SB-C18 column (250 mm × 4.6 mm, 5 μm particle size, Agilent Technologies, Palo Alto, CA) was used for separation of phenolic compounds.

Anthocyanins

The extracts (1 mL) were filtered through 0.45 μm filter units and 5 μL injected directly without any purification. Elution was performed using mobile phase A (5% formic acid aqueous solution) and mobile phase B (methanol). The flow rate was 1 mL/min with the gradient as follows: 0−2 min, 5% B; 2-10 min, 5-15% B; 10-25 min, 15-25% B; 25-30 min, 25-30% B; 30-45 min, 30% B; 45-47 min, 30-70% B; 47-50 min, 70-5% B; followed by 5 min of re-equilibration of the column before the next run. UV-vis spectra were scanned from 220 to 600 nm on a diode array detector and the detection wavelength for the anthocyanins was 520 nm.

Ellagic acid and flavonols

Acid hydrolysis of the samples was done before HPLC analysis. Extract (5 mL) was concentrated in a Speed Vac Concentrator (Thermo scientific ISS110, Waltham, MA) under reduced pressure at 25°C to remove the solvent. The solids were dissolved

in 5 mL of methanol (50%) containing 1.2 N HCl and sonicated for 5 min. Hydrolysis was conducted in a precision water bath (Thermoscientific, Waltham, MA) for 80 min at 90°C and aglycones were separated and quantified using HPLC. The binary mobile phase consisted of (A) 0.5% aqueous formic acid solution and (B) acetonitrile. The flow rate was 1 mL/min and 25 min gradient was used. The gradient is described as follows: 0−5 min, 10-30% B; 5-10 min, 30-40% B; 10-20 min, 40-50% B; 20-23 min, 50-70% B; 23-25 min, 70-10% B; followed by 5 min of re-equilibration of the column before the next run. The column temperature was maintained at 30°C. UV-vis spectra were scanned from 220 to 600 nm on a diode array detector and the detection wavelength for the ellagic acid and flavonols was 360 nm.

Statistical and Multivariate Analysis

Data were expressed as mean ± standard deviation. Student's T-test was performed for the comparison of the means between control and treated groups, and paired comparison T-test was done between the same treatment group at two different sampling times using JMP software (Version 8.0, SAS Institute Inc., Cary, NC). P-values less than 0.05 were considered statistically significant. Principal component analysis (PCA) was done to examine the grouping of samples, outliers and to visualize the relative distribution of the control and treated samples. PCA was done with the JMP software using the content of individual anthocyanins, ellagic acid and flavonols as variables without data transformation or normalization.

Results

Fruit Weight, pH and Total Soluble Solids

ABA treatment had no effect on the average berry weight in both cultivars. Sampling time also had no effect on the average berry weight within ABA treated Noble,

however, a significant decrease was observed in weight of control group of Noble grapes at second sampling (Table 3-1). In case of Alachua, sampling time significantly decreased the average berry weight in control and increased the weight in ABA treated grapes. Application of ABA did not affect the fruit juice composition of either cultivar. No significant differences were observed in soluble solid content (°Brix) and pH of the control and ABA treated juice. Similar observations were reported on *Vitis vinifera* [65, 71, 73]. There was a significant increase in the soluble solids content and pH of control and ABA treated Alachua juice at second sampling. A significant increase in the soluble solid content of ABA treated Noble juice was also observed at second sampling (Table 3-1).

Total Phenolic Content and Antioxidant Capacities

A significant increase in the antioxidant capacities determined by ORAC and DPPH assays in ABA treated Noble was observed at both sampling times, however, effect of ABA treatment on total phenolic content of Noble was observed at first sampling only (Table 3-2). Antioxidant capacity (ORAC) was enhanced by 38% and 18% in treated Noble samples compared to controls at first and second sampling, respectively. Antioxidant capacity determined by DPPH assay also showed a similar effect, however the values were lower than ORAC assay. This is likely caused by the differences in the reaction mechanism of these two methods. ORAC assay applies a competitive reaction scheme, in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo compounds, whereas DPPH assay measures the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced [76]. Overall, the total phenolic content was 30% higher in treated samples than controls after the first sampling. No effects of ABA

treatment were seen on the Alachua. A time-dependent significant increase in total phenolic content and antioxidant capacities in control group of Noble was also observed. There was a significant increase in the total phenolic content and antioxidant capacities of control and ABA treated Alachua at second sampling (Table 3-2).

HPLC Analysis of Anthocyanins and Other Phenolic Compounds

The effect of ABA treatment on the individual anthocyanins (delphinidin, cyanidin, petunidin, peonidin, and malvidin 3, 5-diglucoside) is reported in Table 3-3. ABA treated Noble showed an increase in the concentration of individual anthocyanins throughout the ripening period. The content of delphinidin, cyanidin, and peonidin 3, 5-diglucoside was 50% higher in ABA treated grapes compared to controls at first sampling. Among the anthocyanins, cyanidin 3, 5-diglucoside showed highest increase (63%) in ABA treated grapes compared to controls at first sampling. The concentration remained constant at second sampling. Overall, total anthocyanins were 51% and 39% higher in ABA treated Noble compared to controls at first and second sampling, respectively. ABA treatment did not affect the content of individual anthocyanins in Alachua at the first sampling; however the level of peonidin 3, 5- diglucoside was increased by 20% in ABA treated grapes compared to controls at second sampling. Time related increase in the content of anthocyanins of control and ABA treated grapes was observed in both cultivars, however, this effect was more pronounced in Alachua.

ABA treated Noble grapes had higher concentrations of ellagic acid and flavonols (myricetin, quercetin and kaempferol) compared to controls at first sampling (Table 3-4). Ellagic acid content was enhanced by 47% in treated samples compared to controls. Similarly the levels of myricetin, quercetin and kaempferol were enhanced by 54%, 45% and 48%, respectively, in treated Noble grapes compared to controls. Even

though the effect of ABA treatment on content of ellagic acid and flavonols was not statistically significant at the second sampling, the trend showed an increase in their content. A comparison of control groups from first and second sampling in Noble showed a significant increase in the ellagic acid and kaempferol content. No effect of ABA treatment was seen on the ellagic acid and flavonol content of Alachua grapes. However, the effect of sampling time showed a significant increase in the levels of ellagic acid, myricetin and quercetin in both control and ABA treated Alachua.

Principal Component Analysis (PCA)

The score plots of the first two principal components (PC1 and PC2) for the control and ABA treated muscadine grapes are shown in Figure 3-1. Generally, samples with similar characteristics cluster but samples with different characteristics segregate on score plots. The first three components accounted for 92% of variance in the control and ABA treated Noble samples, where PC1 explains 60%, PC2 26% and PC3 7% of the variance, respectively. Grouping of control samples on left side of the plot and ABA treated samples on the right side was observed (Figure 3-1A). Samples labeled “C” in control group and “T” in treated group clusters are from the same grapevines but sampled at two different times. A change in position of these samples on the score plot showed the effect of time on the phenolic compounds. As the ripening progresses the phenolic content of grapes changes which is depicted in the labeled samples. Contrary to Noble, the score plots of Alachua grapes showed no segregation between the control and ABA treated samples (Figure 3-1B). The contribution of individual phenolic compounds to overall variance in Noble grapes was visualized on the loading plot of PCA. Anthocyanins and flavonols appeared as two clusters on the loading plot. Ellagic acid influenced the variance of Noble grapes similar to flavonols (Figure 3-2).

Discussion

Exogenous ABA has been investigated as a novel strategy to improve the quality of grapes. A few studies have examined the effects of ABA on anthocyanin metabolism and maturity parameters in *Vitis vinifera* grapes and hybrids [65, 70-71, 73, 77]. However, the effects of exogenous ABA on muscadine grapes (*Vitis rotundifolia*) have not been reported. In this study, we investigated the effect of ABA on enhancement of anthocyanins and other phenolic compounds in muscadine grape skin. The concentration of ABA chosen for treatment was based on our previous experiment on lettuce [78]. Similar concentration of ABA has been applied on cabernet sauvignon grapes [79]. No effect of sampling time was seen on berry weight and pH in Noble, which indicates that grapes reached their maximum weight and acidity before the second spray. However, a significant increase in the total soluble solids content of treated Noble was observed with time, suggesting that ABA may positively affect sugar biosynthesis in ripening grapes. Grape berry weight decreased in control Alachua and increased in treated Alachua grapes at two different sampling times. Increase in total soluble solids and pH was observed in Alachua juice at two sampling times. These results suggest that the physiological changes, such as berry expansion and sugar accumulation, occur much faster in Noble than Alachua. The weight of control Noble and Alachua grapes decreased at second sampling. Although the reason for the weight loss is not known, we speculate that it may have been caused by dehydration of the grapes at ripening.

PCA illustrated the patterns found in the data and revealed the relationships between variables. The score plot of Noble showed clustering of control and ABA treated samples with some overlapping. The overlapping of the clusters could be due to large variation in the samples. PC1 variable appears to be strongly associated with the

grouping of control and ABA treated samples and also with the progression of ripening. In contrast, control and ABA treated Alachua samples were randomly scattered on the score plot without any grouping, suggesting no effect of ABA treatment. The loading plot provides a relationship among the variables and how much each variable contributes to each PC. Variables plotted in the same direction from the center are positively correlated, while those on opposite sides are negatively correlated. Loading plot of Noble showed positive correlation in variables 1, 2, 3, 4 and 5 (delphinidin, cyanidin, petunidin, peonidin, and malvidin 3, 5-diglucoside) through PC1. Variables 6, 7, 8 and 9 (ellagic acid, myricetin, quercetin and kaempferol) were positively correlated and contributed equally to PC1 and PC2. Simultaneous interpretation of score and loading plots reveal that samples on right side of the score plot correspond to the direction of variables (anthocyanins, flavonols and ellagic acid) in the loading plot, suggesting higher values of these variables in the respective samples. This could be explained in terms of ABA treatment effect, as grouping of ABA treated samples, which have higher content of phenolic compounds, is observed on the right side of the score plot.

ABA treatment stimulated the accumulation of anthocyanins and total phenolics, as well as enhanced the antioxidant capacities in Noble. This effect paralleled the increase in individual anthocyanins, flavonols and ellagic acid in ABA treated grapes compared to controls. Based on these observations it could be stated that ABA triggers the secondary metabolism of grapes including anthocyanin biosynthesis. Anthocyanins, flavonols and ellagic acid are products of secondary metabolism in plants and generated by common phenylpropanoid pathway [80]. Although these three phenolic compounds share a common biosynthetic pathway, specific enzymes are involved in

their synthesis, which may respond differently to exogenous ABA. Anthocyanins are synthesized from phenylalanine through an anthocyanin biosynthetic pathway regulated by enzyme activities [81] and gene expression [82]. A few examples of anthocyanin biosynthetic pathway genes are—phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), leucoanthocyanidin dioxygenase (LDOX) and UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT). The key enzyme involved in the biosynthesis of flavonols is flavonol synthase (FLS). Ellagic acid is an upstream product of phenylpropanoid pathway and is generated by the hydrolysis of ellagitannins [83]. Previous studies on grapes have reported positive impact of exogenous ABA on color development and anthocyanin biosynthesis [65, 70-73]. The improved accumulation was correlated with an induced expression of a UFGT gene coding for an enzyme specific to the anthocyanin pathway and other genes coding upstream-located enzymes such as PAL, CHI, CHS, DFR, F3H and LDOX [70-71]. ABA treatment enhanced the content of flavonols and ellagic acid at first sampling but there was little effect at ripening. Fujita et al.,[84] reported an increase in the transcript levels of two flavonol synthase genes and enhancement of quercetin content in the skin of Merlot grapes after veraison. Second application of ABA did not change the levels of flavonols and ellagic acid, however increase in the levels of anthocyanins was observed. One possibility could be that accumulation of flavonols occurs in the early stages of development and at ripening but accumulation of anthocyanins begins at ripening and continues until grapes are fully ripe [84].

The increase in antioxidant capacities determined by ORAC and DPPH assays were consistent with the increase in total phenolic content and total anthocyanins of treated Noble grapes. However, total phenolic content in ABA treated grapes was similar to control at second sampling. This was probably caused by the differences in the chemistry and scope of compounds that were measured using these methods.

Contrary to the results in Noble, no effect of ABA treatment was seen in Alachua. This may be due to variation in genetic make-up of two cultivars, thus leading to different ABA responses. The two vineyards used for these trials had similar weather, viticultural practices, and soil conditions. Minor differences in the environmental conditions were less likely to be the major factors that caused different ABA responses in two cultivars.

The mechanism by which ABA gets adsorbed into the grape is not well understood. There are at least two possible hypotheses: either sprayed ABA penetrates through the skin, accumulates inside the grape and enhances the generation of phenolic compounds and ripening related changes or exogenous ABA acts as a signaling agent that triggers the synthesis of endogenous ABA [69]. As small grapes have larger skin surface for the same weight, we speculate that they will adsorb ABA more efficiently than larger grapes and hence be more susceptible to ABA treatment. This may, in part, explain the different effects of ABA on Alachua and Noble grapes.

Summary

In conclusion, exogenous ABA enhanced the antioxidant capacity, anthocyanins and phenolic content of muscadine grapes but these effects varied depending upon the cultivar and were possibly influenced by other environmental factors since the grape varieties were grown in different locations. ABA is ubiquitous in nature and synthesized

by all higher plants. Once production of *S*-ABA for agricultural use becomes cost effective, this plant growth regulator will be a promising tool for the enhancement of phenolic compounds in fruits and vegetables. ABA applied at critical stages of grape development offers opportunities to increase the content of key phytochemicals without affecting the yield. The grapes with enhanced phytochemicals could attract health conscious consumers and also increase the marketability of fresh fruits.

Table 3-1. Average berry weight, total soluble solids (°Brix) and pH of juice from control and ABA treated muscadine grapes.

Variety	Sampling time	Treatment	Average berry weight (g)	Total soluble solids (°Brix)	pH
Noble	First sampling	Control (n=9)	3.81 ± 0.58	9.17 ± 0.59	3.12 ± 0.10
		300 ppm ABA (n=9)	3.25 ± 0.58	8.89 ± 0.72	3.10 ± 0.07
	Second sampling	Control (n=9)	3.09 ± 0.19[a]	9.44 ± 1.64	3.05 ± 0.09
		300 ppm ABA (n=9)	2.96 ± 0.28	9.86 ± 1.26[a]	3.10 ± 0.08
Alachua	First sampling	Control (n=13)	7.16 ± 0.44	10.81 ± 1.31	3.24 ± 0.06
		300 ppm ABA (n=12)	7.17± 0.63	10.76 ± 1.48	3.24 ± 0.05
	Second sampling	Control (n=13)	7.09 ± 0.66[a]	15.40 ± 0.72[a]	3.31 ± 0.06[a]
		300 ppm ABA (n=12)	7.48± 0.90[a]	15.53 ± 0.57[a]	3.38 ± 0.09[a]

Results are mean ± standard deviation on fresh weight basis. [a] indicates significant differences ($p < 0.05$, Paired comparison t-test) between corresponding values during first sampling.

Table 3-2. Total phenolic content and antioxidant capacities (ORAC and DPPH) of control and ABA treated muscadine grape skins.

Variety	Sampling time	Treatment	Total phenolics (GAE mg/g)	ORAC (TE µmol/g)	DPPH (TE µmol/g)
Noble	First sampling	Control (n=9)	3.72 ± 0.56	54.68 ± 8.43	20.49 ± 5.14
		300 ppm ABA (n=9)	4.83 ± 1.04[b]	75.61 ± 13.34[b]	25.15 ± 4.52[b]
	Second sampling	Control (n=9)	4.44 ± 0.73[a]	67.97 ± 12.01[a]	21.71 ± 2.90
		300 ppm ABA (n=9)	5.04 ± 0.66	79.94 ± 9.00[b]	24.64 ± 2.01[b]
Alachua	First sampling	Control (n=13)	3.76 ± 0.64	55.27 ± 10.52	21.51 ± 4.67
		300 ppm ABA (n=12)	3.59 ± 0.94	52.02 ± 12.51	21.43 ± 4.52
	Second sampling	Control (n=13)	4.52 ± 0.52[a]	65.38 ± 8.73[a]	26.60 ± 3.40[a]
		300 ppm ABA (n=12)	4.84 ± 0.77[a]	72.86 ± 13.22[a]	25.07 ± 3.29[a]

Results are mean ± standard deviation on fresh weight basis. [a] indicates significant differences ($p < 0.05$, Paired comparison t-test) between corresponding values during first sampling.[b] Values are significantly different than control ($p < 0.05$, Student's t-test).

Table 3-3. Anthocyanin content of control and ABA treated muscadine grape skins.

Variety	Sampling time	Treatment	Delphinidin 3, 5-diglucoside (mg/g)	Cyanidin 3, 5-diglucoside (mg/g)	Petunidin 3, 5-diglucoside (mg/g)	Peonidin 3, 5-diglucoside (mg/g)	Malvidin 3, 5-diglucoside (mg/g)	Total Anthocyanins (mg/g)
Noble	First sampling	Control (n=9)	0.41 ± 0.17	0.08 ± 0.03	0.17 ± 0.05	0.10 ± 0.02	0.06 ± 0.02	0.82 ± 0.27
		300 ppm ABA (n=9)	0.63 ± 0.21[b]	0.13 ± 0.06[b]	0.24 ± 0.07[b]	0.16 ± 0.05[b]	0.08 ± 0.02[b]	1.24 ± 0.40[b]
	Second sampling	Control (n=9)	0.47 ± 0.13	0.08 ± 0.02	0.21 ± 0.05[a]	0.13 ± 0.02[a]	0.08 ± 0.02[a]	0.97 ± 0.23[a]
		300 ppm ABA (n=9)	0.65 ± 0.15[b]	0.13 ± 0.05[b]	0.27 ± 0.06[b]	0.19 ± 0.05[b]	0.11 ± 0.03[b a]	1.35 ± 0.32[b]
Alachua	First sampling	Control (n=13)	0.92 ± 0.20	0.07 ± 0.02	0.18 ± 0.04	0.04 ± 0.01	0.04 ± 0.01	1.25 ± 0.28
		300 ppm ABA (n=12)	0.82 ± 0.30	0.06 ± 0.03	0.16 ± 0.05	0.04 ± 0.01	0.05 ± 0.01	1.13 ± 0.40
	Second sampling	Control (n=13)	1.27 ± 0.14[a]	0.10 ± 0.02[a]	0.24 ± 0.03[a]	0.05 ± 0.01[a]	0.05 ± 0.01[a]	1.71 ± 0.20[a]
		300 ppm ABA (n=12)	1.29 ± 0.22[a]	0.12 ± 0.03[a]	0.25 ± 0.04[a]	0.06 ± 0.02[a b]	0.06 ± 0.01[a]	1.78 ± 0.32[a]

Results are mean ± standard deviation on fresh weight basis. [a] indicates significant differences ($p < 0.05$, Paired comparison t-test) between corresponding values during first sampling.[b] Values are significantly different than control ($p < 0.05$, Student's t-test).

Table 3-4. Ellagic acid, myricetin, quercetin and kaempferol content of control and ABA treated muscadine grape skins.

Variety	Sampling time	Treatment	Ellagic acid (mg/g)	Myricetin (mg/g)	Quercetin (mg/g)	Kaempferol (mg/g)
Noble	First sampling	Control (n=9)	2.67 ± 0.73	0.28 ± 0.07	0.23 ± 0.08	0.11 ± 0.03
		300 ppm ABA (n=9)	3.92 ± 0.87[b]	0.44 ± 0.10[b]	0.34 ± 0.06[b]	0.16 ± 0.04[b]
	Second sampling	Control (n=9)	3.46 ± 1.32[a]	0.36 ± 0.10	0.27 ± 0.08	0.14 ± 0.05[a]
		300 ppm ABA (n=9)	4.31 ± 1.31	0.38 ± 0.08	0.34 ± 0.08	0.18 ± 0.05
Alachua	First sampling	Control (n=13)	1.56 ± 0.28	0.21 ± 0.05	0.19 ± 0.05	0.08 ± 0.02
		300 ppm ABA (n=12)	1.64 ± 0.36	0.21 ± 0.06	0.20 ± 0.06	0.09 ± 0.02
	Second sampling	Control (n=13)	2.01 ± 0.24[a]	0.28 ± 0.05[a]	0.25 ± 0.05[a]	0.09 ± 0.01
		300 ppm ABA (n=12)	2.00 ± 0.44[a]	0.28 ± 0.09[a]	0.26 ± 0.07[a]	0.10 ± 0.03

Results are mean ± standard deviation on fresh weight basis.[a] indicates significant differences ($p < 0.05$, Paired comparison t-test) between corresponding values during first sampling.
[b] Values are significantly different than control ($p < 0.05$, Student's t-test).

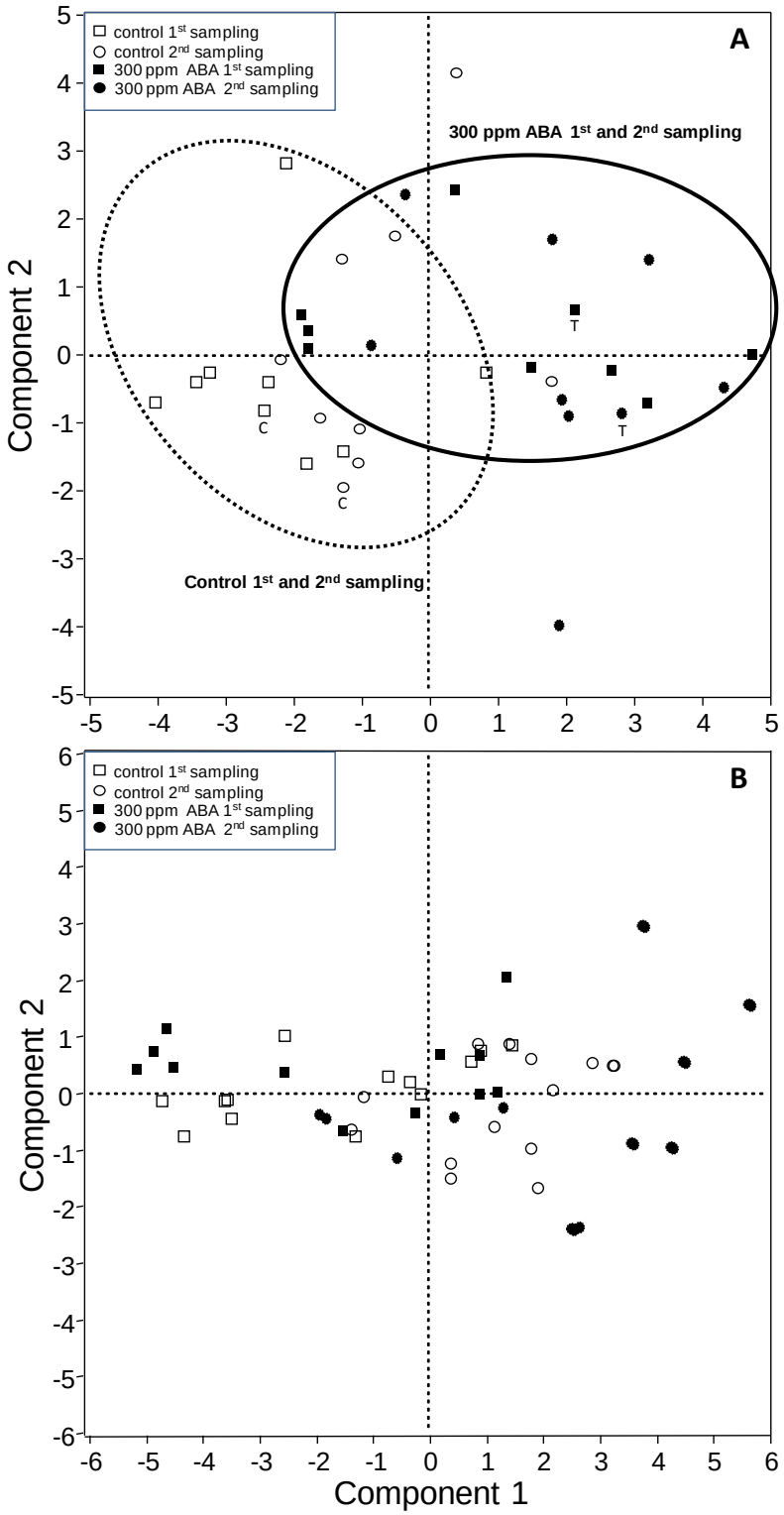


Figure 3-1. Score plots of principal component analysis of control and ABA treated muscadine grapes: A. Noble B. Alachua. Symbols “C” and “T” in Noble score plot refer to control and ABA treated grapes and are the samples from the same grapevines (control and treated) at two different sampling times.

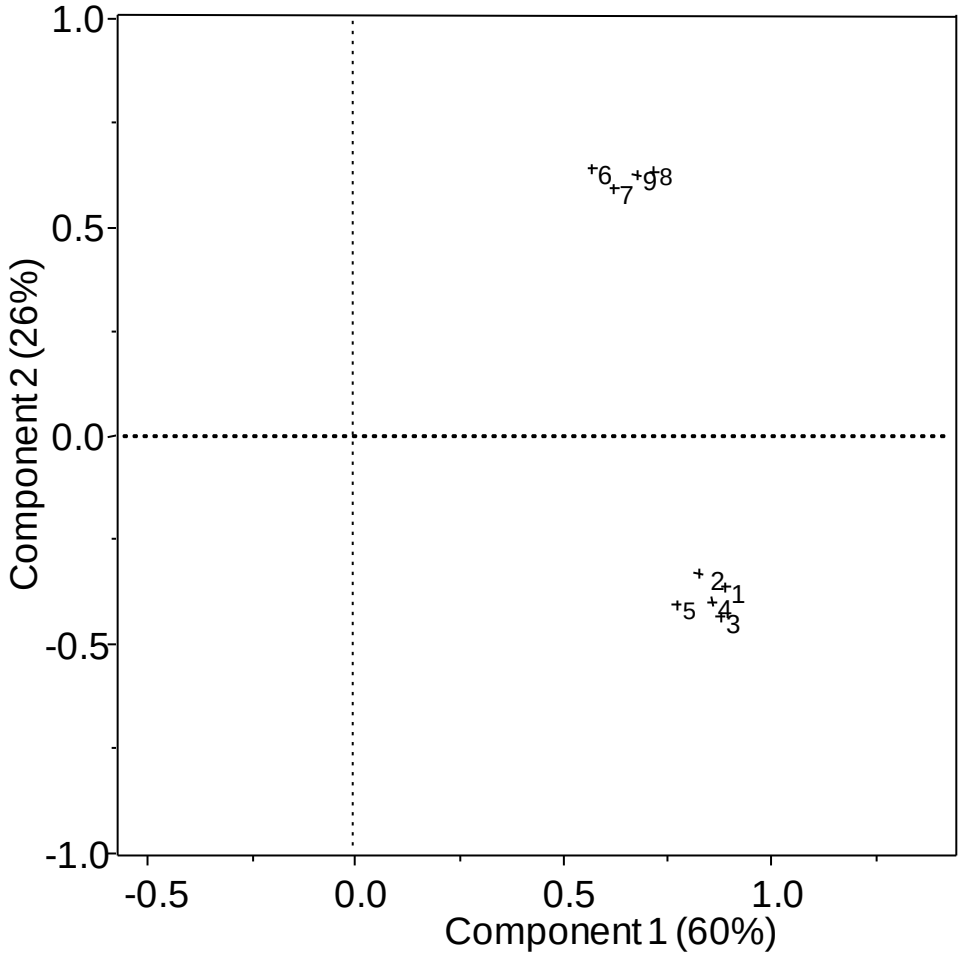


Figure 3-2. Loading plot of principal component analysis of muscadine grapes (cv. Noble) based on principal component 1 and 2: 1. delphinidin 3, 5-diglucoside, 2. cyanidin 3, 5-diglucoside, 3. petunidin 3, 5-diglucoside, 4. peonidin 3, 5-diglucoside, 5. malvidin 3, 5-diglucoside, 6. ellagic acid, 7. myricetin, 8. quercetin, 9. kaempferol

CHAPTER 4
COMPARISON OF DIFFERENT SOLVENTS FOR THE EXTRACTION OF PHENOLIC COMPOUNDS FROM NOBLE JUICE AND WINE POMACE

Background

Muscadines (*Vitis rotundifolia*) are an integral part of agriculture in the southeastern U.S., as they are well adapted to the region's warm and humid conditions. They have a high content of phytochemicals primarily tannins, flavonols, anthocyanins, and resveratrol, that are concentrated in the seeds and skins [6]. Muscadine grapes are mainly processed into juice and wine, thus generating considerable amounts of pomace as a waste. Pomace accounts for 40% of the grape by fresh weight and consists of seeds, skin, pulp and residual solids. Because grape skins and seeds are the predominant constituents in the pomace, this biomass is a rich source of antioxidants. These antixodants may provide protective effects against chronic diseases such as cancer, diabetes and cardiovascular diseases [22]. There has been a considerable interest in utilization of phenolic compounds from grape pomace into value-added products.

Extraction is the first step in obtaining the phenolic compounds from the plant matrix in a highly concentrated form. Different extraction solvents and procedures are used for recovering the phenolic compounds in plant materials [85]. Solid/liquid extraction is a very efficient technique, but its effectiveness depends on several factors such as raw material composition, conditioning, solvent polarity (and phenolic compounds polarity), time, temperature and solid/solvent ratio during the extraction [86]. Another important factor that should be considered is the cost of sample preparation. Aqueous organic solvents are most commonly used for the sample preparation. These solvents can be toxic and the extracts cannot be utilized in food industry. In this study a simple

method using distilled water as a solvent instead of organic solvents was used for the extraction of anthocyanins and other phenolic compounds from muscadine pomace. The objectives of this research were to 1) compare the extraction ability of commonly used solvents (methanol, acetone and distilled water) in terms of phenolic content, antioxidant capacity and anthocyanin content and 2) quantify the phenolic compounds in different extracts of muscadine pomace.

Materials and Methods

Chemicals

Gallic acid, HPLC grade ethanol, acetonitrile, methanol, formic acid, hydrochloric acid, Folin-Ciocalteau reagent, sodium hydroxide and sodium carbonate were purchased from Fischer Scientific Co. (Pittsburg, PA). Standards of the 3-O-β - glucosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (six mixed anthocyanin standard, HPLC grade), were purchased from Polyphenols Laboratories (Sandnes, Norway). Ellagic acid, myricetin, quercetin, kaempferol and cyanidin 3-rutinoside were obtained from Sigma-Aldrich (St. Louis, MO).

Muscadine Juice and Wine Pomace Preparation

Noble grapes were purchased from a local vineyard in central Florida. Grapes were crushed using a manual grape crusher and juice was extracted by hot pressing technique in which the crushed grapes were heated for 30 mins at 60°C. After heating the grapes were pressed by using a stainless steel vertical bladder press to obtain the juice pomace. For wine making, the grapes were crushed using a manual grape crusher, inoculated with yeast, and kept for fermentation at room temperature for 7 days.The fermented must was pressed using a stainless steel vertical bladder press to obtain the wine pomace. Pomace was ground to a fine paste using a mill (Robot Coupe

USA, Inc. Jackson, Mississippi), packaged into gallon ziploc bags and were kept in the freezer (-20°C) until used for extraction.

Extracts Preparation

Three different solvent systems i.e. 1) methanol:acetic acid (99.7:0.3 v/v); 2) acetone:acetic acid (99.7:0.3 v/v); 3) acidified hot water (1% formic acid in 25 mL and 50 mL volume) at three different temperatures a) 40°C, b) 60°C and c) 90°C were used in this study. Fresh pomace (10 g) was extracted twice with each solvent system using a method described by Wu et al. [87]. The first extraction was done with 15 mL of each solvent, than samples were vortexed for 30 s followed by sonication for 5 min. The samples were kept at room temperature for 20 min in darkness, being vortexed and sonicated again before centrifugation. The tubes were centrifuged at 1317 *g* for 10 min and the supernatant removed. The samples were extracted one more time with 10 mL of solvent using the same procedure, and the supernatants were pooled. The combined supernatant was transferred into a 25-mL volumetric flask, and final volume was made up to 25 mL using the respective solvents. In case of hot water apart from similar extraction to other solvents, the volume of water was doubled (50 mL) to make up for the lower extraction efficiency of water. The extracts were used for chemical and HPLC analysis.

Total Anthocyanin Assay

Total anthocyanin content in pomace extracts was measured using the pH differential spectrophotometric method described by Giusti and Wrolstad [88]. The extracts were dissolved in 0.025 mol/L potassium chloride buffer, pH 1.0 and 0.4 mol/L sodium acetate buffer, pH 4.5 with pre determined dilution factor. Absorbance at 520 and 700 nm was measured on a DU 730 Life Science UV/vis spectrophotometer

(Beckman Coulter, Fullerton, CA) after 30 min of incubation at room temperature. The absorbance (A) of the diluted sample was then calculated using $(A_{520} – A_{700})_{pH\ 1.0} – (A_{520} – A_{700})_{pH\ 4.5}$. The monomeric anthocyanin concentration in the original sample was calculated in cyanidin-3, 5-diglucoside equivalents according to this formula: (A × MW × DF × 1000) / (ε × 1), where MW (611) of cyanidin 3, 5-diglucoside is used, the molar absorptivity ε was 30,175; the DF was dilution factor; 1000 is the factor to convert gram to milligram and the A was absorbance. Results for total anthocyanin content were expressed as milligram cyanidin 3, 5-diglucoside equivalent per gram of fresh pomace (mg cyanidin 3, 5-diglucoside/g).

Folin-Ciocalteu Assay

The extracts were diluted to appropriate concentration for analysis. The total phenolic content was determined as reported previously [6].The results were expressed as milligrams of gallic acid equivalents per gram of fresh pomace (mg of GAE/ g).

Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay for extracted samples was conducted on a Spectra XMS Gemini plate reader (Molecular Devices, Sunnyvale, CA). Briefly, 50 µL of standard and samples were added to the designated wells of a 96-well black plate. This was followed by the addition of 100 µL of flourescein (20 nM). The mixture was incubated at 37°C for 7 min before the addition of 50 µL of AAPH. Fluorescence was monitored using 485 nm excitation and 530 nm emissions at 1 min intervals for 40 min. Trolox was used to generate a standard curve. The antioxidant capacities of extracts were expressed as micromoles of Trolox equivalents (TE) per gram of fresh pomace (µmol of TE/g).

HPLC Analysis of Phenolic Compounds

Chromatographic analysis was performed on an Agilent 1200 HPLC system consisting of an autosampler, a binary pump, a column compartment, a diode array detector and a fluorescent detector (Agilent Technologies, Palo Alto, CA). Reversed phase chromatography was used for the quantification of anthocyanins, ellagic acid and flavonols. An Agilent Zorbax Stablebond SB-C18 column (250 mm × 4.6 mm, 5 µm particle size, Agilent Technologies, Palo Alto, CA) was used for separation of phenolic compounds.

Anthocyanins

The extracts (1 mL) were filtered through 0.45 µm filter units and 5 µL injected directly without any purification. Elution was performed using mobile phase A (5% formic acid aqueous solution) and mobile phase B (methanol). The flow rate was 1 mL/min with the gradient as follows: 0−2 min, 5% B; 2-10 min, 5-15% B; 10-25 min, 15-25% B; 25-30 min, 25-30% B; 30-45 min, 30% B; 45-47 min, 30-70% B; 47-50 min, 70-5% B; followed by 5 min of re-equilibration of the column before the next run. UV-vis spectra were scanned from 220 to 600 nm on a diode array detector and the detection wavelength for the anthocyanins was 520 nm.

Ellagic acid and flavonols

Acid hydrolysis of the samples was done before HPLC analysis. Extract (5 mL) was concentrated in a Speed Vac Concentrator (Thermo scientific ISS110, Waltham, MA) under reduced pressure at 25°C to remove the solvent. The solids were dissolved in 5 mL of methanol (50%) containing 1.2 N HCl and sonicated for 5 min. Hydrolysis was conducted in a precision water bath (Thermoscientific, Waltham, MA) for 80 min at 90°C and aglycones were separated and quantified using HPLC. The binary mobile

phase consisted of (A) 0.5% aqueous formic acid solution and (B) acetonitrile. The flow rate was 1 mL/min and 25 min gradient was used. The gradient is described as follows: 0−5 min, 10-30% B; 5-10 min, 30-40% B; 10-20 min, 40-50% B; 20-23 min, 50-70% B; 23-25 min, 70-10% B; followed by 5 min of re-equilibration of the column before the next run. The column temperature was maintained at 30°C. UV-vis spectra were scanned from 220 to 600 nm on a diode array detector and the detection wavelength for the ellagic acid and flavonols was 360 nm.

Statistical Analysis

One-way analyses of variance (ANOVA) with Tukey-HSD pairwise comparison of the means were performed using JMP (version 7.0, SAS Institute Inc., Cary, NC). Data are expressed as means ± standard deviation of three independent observations. A p value ≤ 0.05 is considered significant.

Results and Discussion

The extraction efficiency of phytochemicals depends upon the extraction solvents and conditions used. Therefore, the aim of an extraction process is to provide the extracts with maximum yield and the highest quality of the compounds of interest. Table 4-1 summarizes total phenolics, total anthocyanins and antioxidants extracted from juice and wine pomace using different solvents. Acetone extracted the highest amount of total phenolics followed by methanol extraction in both juice and wine pomace. Water at a volume two times of acetone or methanol extracted more phenolics from pomace. Methanol extracted the highest amount of antioxidants from juice pomace. However, in wine pomace hot water (60°C, 50 mL) yielded the highest amount of antioxidants followed by methanol. The highest amount of total anthocyanin content was obtained in hot water at 90°C (50 mL), followed by methanol and acetone in both juice and wine

pomace. Hot water extracts (25 mL) at different temperatures yielded the lowest amount of total anthocyanins in both juice and wine pomace. The amount of extracted individual anthocyanins was highest in methanol and hot water (50 mL) at different temperatures in juice pomace. Acetone and hot water extracts (25 mL) contained the least amount of individual anthocyanins (Table 4-2). However, for wine pomace the highest amount of individual anthocyanins were extracted in methanol followed by hot water (50 mL) and acetone. Hot water extracts (50 mL) gave the highest yield for cyanidin and peonidin 3, 5-diglucoside. Ellagic acid, myricetin, quercetin and kaempferol were found to be highest in methanol and acetone extracts of both juice and wine pomace (Table 4-3).

An extraction solvent is generally selected according to the purpose of extraction, polarity of the interested components, polarity of undesirable components, overall cost, and safety [89]. To conduct various analyses, organic solvents such as alcohols and acetone, with different levels of water, have been widely used for the extraction of phenolic components from plant materials. Water is a universal solvent which is cheap and safe to be used in food industry. Very few studies have explored the use of water for extraction of phenolic compounds. In this study we compared hot water extraction of anthocyanins from muscadine pomace with organic solvents.

The solubility of phenolics is governed by their chemical structures, as well as the polarity of the solvents used. Phenolic compounds in plant materials vary from simple to highly polymerized substances in different quantities. Therefore, it is difficult to find a universal extraction solvent suitable for extraction of all plant phenolics. Acetone extracts gave much higher yields of total phenolic compounds than methanol and water extracts, which can mainly be attributed to its ability to dissolve both the hydrophilic and

lipophilic components from plant materials. Muscadine pomace contains mixture of polar and non-polar components such as anthocyanins, flavonols, ellagic acid and conjugates, and flavan-3-ols (proanthocyanidins). Total anthocyanins and antioxidant capacity was lower in acetone extracts compared to methanol; therefore it can be assumed that these extracts were rich in proanthocyanidins and other non-polar compounds. A study conducted on extraction of phenolic compounds from barley showed that aqueous acetone was a better mixture for extracting phenolic compounds (which had an overall non-polar character) than aqueous methanol [90]. The high antioxidant capacity for methanol extract in case of juice pomace can be explained by its ability to penetrate the cell wall, thus releasing more phenolic compounds. Hot water extracts (90°C, 50 mL) gave the highest value for total anthocyanins. The solubility of anthocyanins was highest in methanol, a little lower in acetone and the lowest in hot water. Similar results were reported in a study on grape skins [91].However, doubling the volume of hot water resulted in increased extraction efficiency for anthocyanins. Therefore, hot water was as effective as acidified methanol and acetone in extracting anthocyanins from grape pomace.

Extraction is the first step before phenolic compounds can be concentrated. The extraction yield and antioxidant activity of the extracts highly depend on the solvent polarity, which determines both quantitatively and qualitatively the extracted phenolic compounds. Knowing the extraction ability of various solvents will help to select a particular solvent for the extraction of compounds of interest.

Summary

In this study, we found hot water to be the most suitable solvent for the extraction of anthocyanins. Organic solvents work best for the extraction of ellagic acid and

flavonols. The advantages of using water as a solvent outweigh the use of organic solvents as it is non toxic and inexpensive source. In addition, it fits better for the industry scale production because it significantly reduces the overall production cost.

Table 4-1. Total phenolics, antioxidants and total anthocyanins extracted from Noble juice and wine pomace using different solvents.

Extraction Solvent	Total Phenolics extracted (GAE mg/g)	Antioxidant capacity (ORAC, µmol TE/g)	Total Anthocyanins extracted (mg/g)
Juice Pomace			
Methanol:acetic acid (99.7:0.3 v/v)	5.69 ± 0.43^{b}	119.94 ± 19.20^{a}	1.33 ± 0.07^{ab}
Acetone:acetic acid (99.7:0.3 v/v)	9.32 ± 0.69^{a}	91.56 ± 4.59^{b}	1.30 ± 0.04^{b}
Extraction with 25 mL water			
Hot water 40°C	3.86 ± 0.11^{de}	71.66 ± 7.71^{bc}	1.05 ± 0.04^{c}
Hot water 60°C	3.56 ± 0.11^{e}	87.72 ± 8.57^{b}	1.12 ± 0.02^{c}
Hot water 90°C	4.05 ± 0.17^{cde}	75.71 ± 11.11^{bc}	1.09 ± 0.03^{c}
Extraction in 50 mL water			
Hot water 40°C	4.48 ± 0.09^{cd}	61.10 ± 2.07^{c}	1.43 ± 0.05^{a}
Hot water 60°C	4.78 ± 0.28^{c}	57.03 ± 9.25^{c}	1.38 ± 0.04^{ab}
Hot water 90°C	4.60 ± 0.10^{cd}	61.18 ± 6.07^{c}	1.43 ± 0.01^{a}
Wine Pomace			
Methanol:acetic acid (99.7:0.3 v/v)	5.76 ± 0.20^{b}	84.90 ± 10.78^{ab}	0.93 ± 0.00^{a}
Acetone:acetic acid (99.7:0.3 v/v)	8.66 ± 0.32^{a}	73.08 ± 5.24^{bc}	0.89 ± 0.04^{ab}
Extraction in 25 mL water			
Hot water 40°C	2.47 ± 0.08^{d}	61.25 ± 4.80^{cd}	0.58 ± 0.02^{c}
Hot water 60°C	2.71 ± 0.27^{d}	49.93 ± 6.04^{d}	0.61 ± 0.04^{c}
Hot water 90°C	2.71 ± 0.12^{d}	74.24 ± 1.81^{bc}	0.64 ± 0.01^{c}
Extraction in 50 mL water			
Hot water 40°C	3.80 ± 0.15^{c}	68.82 ± 4.91^{bcd}	0.89 ± 0.03^{ab}
Hot water 60°C	3.98 ± 0.08^{c}	96.94± 7.49^{a}	0.83 ± 0.00^{b}
Hot water 90°C	3.98 ± 0.11^{c}	83.32± 14.10^{abc}	0.90 ± 0.01^{a}

Results are mean ± standard deviation of three determinations on fresh weight basis. Different superscripts in each column indicate the significant differences in the mean at $p \leq 0.05$.

Table 4-2. Anthocyanins extracted from Noble juice and wine pomace using different solvents.

Extraction Solvent	Delphinidin 3, 5-diglucoside (mg/g)	Cyanidin 3, 5-diglucoside (mg/g)	Petunidin 3, 5-diglucoside (mg/g)	Peonidin 3, 5-diglucoside (mg/g)	Malvidin 3, 5-diglucoside (mg/g)	Total Anthocyanins (mg/g)
Juice Pomace						
Methanol:acetic acid (99.7:0.3 v/v)	0.26 ± 0.01[a]	0.07 ± 0.00[a]	0.14 ± 0.00[a]	0.09 ± 0.02[a]	0.06 ± 0.00[a]	0.71 ± 0.01[a]
Acetone:acetic acid (99.7:0.3 v/v)	0.22 ± 0.01[b]	0.06 ± 0.00[a]	0.13 ± 0.00[b]	0.14 ± 0.00[a]	0.07 ± 0.02[a]	0.61 ± 0.02[b]
Extraction with 25 mL water						
Hot water 40°C	0.22 ± 0.01[b]	0.06 ± 0.00[a]	0.12 ± 0.00[b]	0.11 ± 0.00[b]	0.07 ± 0.00[a]	0.59 ± 0.01[b]
Hot water 60°C	0.23 ± 0.01[b]	0.06 ± 0.00[a]	0.13 ± 0.00[b]	0.11 ± 0.00[b]	0.07 ± 0.00[a]	0.60 ± 0.02[b]
Hot water 90°C	0.23 ± 0.01[b]	0.06 ± 0.00[a]	0.13 ± 0.00[b]	0.11 ± 0.00[b]	0.06 ± 0.00[a]	0.59 ± 0.01[b]
Extraction with 50 mL water						
Hot water 40°C	0.28 ± 0.00[a]	0.07 ± 0.00[a]	0.16 ± 0.00[a]	0.14 ± 0.00[a]	0.09 ± 0.00[a]	0.73 ± 0.01[a]
Hot water 60°C	0.27 ± 0.00[a]	0.07 ± 0.00[a]	0.16 ± 0.00[a]	0.14 ± 0.00[a]	0.09 ± 0.00[a]	0.72 ± 0.01[a]
Hot water 90°C	0.28 ± 0.00[a]	0.07 ± 0.00[a]	0.16 ± 0.00[a]	0.14 ± 0.00[a]	0.09 ± 0.00[a]	0.74 ± 0.01[a]
Wine Pomace						
Methanol:acetic acid (99.7:0.3 v/v)	0.12 ± 0.00[a]	0.05 ± 0.00[a]	0.11 ± 0.00[a]	0.12 ± 0.00[a]	0.07 ± 0.00[a]	0.47 ± 0.01[a]
Acetone:acetic acid (99.7:0.3 v/v)	0.10 ± 0.00[b]	0.05 ± 0.00[ab]	0.09 ± 0.00[b]	0.12 ± 0.00[a]	0.05 ± 0.00[b]	0.40 ± 0.00[b]
Extraction with 25 mL water						
Hot water 40°C	0.06 ± 0.00[f]	0.04 ± 0.00[b]	0.06 ± 0.00[d]	0.07 ± 0.00[b]	0.04 ± 0.00[b]	0.27 ± 0.01[c]
ot water 60°C	0.06 ± 0.00[f]	0.04 ± 0.00[b]	0.07 ± 0.00[cd]	0.08 ± 0.00[b]	0.05 ± 0.00[b]	0.29 ± 0.03[c]
Hot water 90°C	0.07 ± 0.00[e]	0.04 ± 0.00[b]	0.07 ± 0.00[d]	0.08 ± 0.00[b]	0.04 ± 0.00[b]	0.30 ± 0.00[c]
Extraction with 50 mL water						
Hot water 40°C	0.09 ± 0.00[cd]	0.05 ± 0.00[a]	0.09 ± 0.00[b]	0.11 ± 0.00[a]	0.06 ± 0.00[a]	0.41 ± 0.01[b]
Hot water 60°C	0.08 ± 0.00[d]	0.05 ± 0.00[a]	0.09 ± 0.00[b]	0.11 ± 0.00[a]	0.07 ± 0.00[a]	0.41 ± 0.01[b]
Hot water 90°C	0.09 ± 0.01[bc]	0.05 ± 0.00[a]	0.09 ± 0.00[b]	0.11 ± 0.00[a]	0.07 ± 0.00[a]	0.42 ± 0.02[b]

Results are mean ± standard deviation of three determinations on fresh weight basis. Different superscripts in each column indicate the significant differences in the mean at $p \leq 0.05$.

Table 4-3. Ellagic acid, myricetin, quercetin and kaempferol extracted from Noble juice and wine pomace using different solvents.

Extraction Solvent	Ellagic acid (mg/g)	Myricetin (mg/g)	Quercetin (mg/g)	Kaempferol (mg/g)
Juice Pomace				
Methanol:acetic acid (99.7:0.3 v/v)	2.43 ± 0.05^{a}	0.31 ± 0.01^{a}	0.15 ± 0.00^{a}	0.07 ± 0.00^{a}
Acetone:acetic acid (99.7:0.3 v/v)	2.39 ± 0.10^{a}	0.30 ± 0.01^{a}	0.14 ± 0.01^{a}	0.07 ± 0.00^{a}
Extraction with 25 mL water				
Hot water 40°C	1.15 ± 0.22^{b}	0.18 ± 0.01^{bc}	0.08 ± 0.00^{bc}	0.04 ± 0.00^{bc}
Hot water 60°C	1.27 ± 0.14^{b}	0.18 ± 0.01^{bc}	0.09 ± 0.00^{bc}	0.04 ± 0.00^{bc}
Hot water 90°C	0.95 ± 0.31^{b}	0.15 ± 0.03^{c}	0.07 ± 0.02^{c}	0.03 ± 0.01^{c}
Extraction with 50 mL water				
Hot water 40°C	1.18 ± 0.20^{b}	0.20 ± 0.02^{b}	0.10 ± 0.01^{b}	0.04 ± 0.00^{b}
Hot water 60°C	1.15 ± 0.21^{b}	0.19 ± 0.01^{b}	0.09 ± 0.00^{b}	0.04 ± 0.00^{bc}
Hot water 90°C	0.93 ± 0.07^{b}	0.18 ± 0.00^{bc}	0.09 ± 0.00^{bc}	0.04 ± 0.00^{bc}
Wine Pomace				
Methanol:acetic acid (99.7:0.3 v/v)	0.12 ± 0.00^{a}	0.05 ± 0.00^{a}	0.11 ± 0.00^{a}	0.12 ± 0.00^{a}
Acetone:acetic acid (99.7:0.3 v/v)	0.10 ± 0.00^{ab}	0.05 ± 0.00^{a}	0.09 ± 0.00^{a}	0.12 ± 0.00^{b}
Extraction with 25 mL water				
Hot water 40°C	0.06 ± 0.00^{b}	0.04 ± 0.00^{b}	0.06 ± 0.00^{b}	0.07 ± 0.00^{c}
Hot water 60°C	0.06 ± 0.00^{b}	0.04 ± 0.00^{b}	0.07 ± 0.00^{b}	0.08 ± 0.00^{c}
Hot water 90°C	0.07 ± 0.00^{b}	0.04 ± 0.00^{b}	0.07 ± 0.00^{b}	0.08 ± 0.00^{c}
Extraction with 50 mL water				
Hot water 40°C	0.09 ± 0.00^{b}	0.05 ± 0.00^{b}	0.09 ± 0.00^{b}	0.11 ± 0.00^{c}
Hot water 60°C	0.08 ± 0.00^{b}	0.05 ± 0.00^{b}	0.09 ± 0.00^{b}	0.11 ± 0.00^{c}
Hot water 90°C	0.09 ± 0.01^{b}	0.05 ± 0.00^{b}	0.09 ± 0.00^{b}	0.11 ± 0.00^{c}

Results are mean ± standard deviation of three determinations on fresh weight basis. Different superscripts in each column indicate the significant differences in the mean at $p \leq 0.05$.

CHAPTER 5
ADSORPTION/DESORPTION CHARACTERISTICS AND SEPARATION OF PHYTOCHEMICALS FROM MUSCADINE POMACE USING MACROPOROUS ADSORBENT RESINS

Background

Muscadine grapes are native to the southeastern United States. They contain a wide variety of antioxidants and possess a unique phytochemical profile. They are reported to contain anthocyanins and other polyphenols including ellagic acid, quercetin, myricetin and kaempferol [1, 5, 28]. Cell culture studies have suggested that polyphenols from muscadine grapes can inhibit proliferation of colon cancer cells and induce apoptosis [13-14]. The anthocyanins from muscadine grapes are also reported to be potent inhibitors of α-glucosidase and pancreatic lipase, the two major enzymes involved in diabetes [17].

During juice and wine processing, 40% of the fruit is lost as pomace which consists of seeds, skin, pulp and residual solids. Since phenolic compounds in muscadine grapes are concentrated in seeds and skin [6] considerable quantities are still left in pomace. Therefore, muscadine pomace is an attractive source of bioactive phytochemicals including anthocyanins, ellagic acid, and flavonols [92]. These bioactive compounds, also known as nutraceuticals, possess high antioxidant capacity and may provide protective effects against chronic diseases such as cancer, diabetes and cardiovascular diseases [22, 24]. There is an increasing demand by the grape industry for the extraction of these phenolic compounds from the pomace and utilization for the production of functional foods. Muscadine pomace is currently utilized as compost or animal feed. Utilization of pomace to extract the phenolic compounds will increase the economic value of the muscadine grapes and wines.

Several extraction and separation methods are used for the enrichment of phenolic compounds from the plant based materials such as liquid–liquid extraction, membrane filtration, ion exchange and chromatography. However, these methods have several disadvantages; for example, they are time-consuming, laborious, expensive with poor recovery, and not suitable for large-scale industrial production. Alternatively, great progress has been made in recent years to separate these compounds from the plant materials with macroporous resins. The resins are polar or non-polar polymers having characteristics of good selectivity, different surface properties, high mechanical strength and fast adsorption speed [93]. The advantages of using macroporous resins outweigh other methods of separation and enrichment as they are relatively low cost, easy to use and regenerate, have high efficiency and are suitable for the industrial scale-up. Moreover, some of these resins meet food grade standards of Food and Drug Administration; therefore, the extracted compounds can be used as food ingredients or dietary supplements. Adsorption by macroporous resins has been successfully applied for the recovery of various phenolic compounds such as hesperidin from citrus peels during citrus processing [94], flavonoids from mulberry leaves [95], lycopene from tomato skins [96], vitexin and isovitexin from pigeon pea [97] and polyphenols from apple juice [98-100]. No studies have been done to investigate the use of resins for the separation and enrichment of phenolic compounds from muscadine pomace.

The objectives of this study were to investigate the adsorption/desorption behaviors of anthocyanins and other phenolic compounds from muscadine pomace extracts on different amberlite resins, and optimizing the conditions for the selection of one resin for conducting the dynamic adsorption and desorption.

Materials and Methods

Chemicals

Gallic acid, HPLC grade ethanol, acetonitrile, methanol, formic acid, hydrochloric acid, Folin-Ciocalteau reagent, sodium hydroxide, sodium carbonate, sucrose, glucose and fructose were purchased from Fischer Scientific Co. (Pittsburg, PA). Standards of the 3-O-β -glucosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (six mixed anthocyanin standard, HPLC grade), were purchased from Polyphenols Laboratories (Sandnes, Norway). Ellagic acid, myricetin, quercetin, kaempferol, cyanidin 3-rutinoside, (+)-catechin and (-)-epicatechin were obtained from Sigma-Aldrich (St. Louis, MO). Resins FPX-66, XAD-16N, XAD-1180, XAD-7HP, and XAD-761 were products of Rohm and Haas Co. (Philadelphia, PA).

Muscadine Juice and Wine Pomace Preparation

Noble grapes were purchased from a local vineyard in central Florida. Muscadine juice is normally processed by hot or cold pressing. For this study grapes were crushed using a manual grape crusher and juice was extracted by hot pressing technique in which the crushed grapes were heated for 30 mins at 60°C. After heating the grapes were pressed by using a stainless steel vertical bladder press to obtain the juice pomace. For wine making, the grapes were crushed using a manual grape crusher, inoculated with yeast, and kept for fermentation at room temperature for 7 days.The fermented must was pressed using a stainless steel vertical bladder press to obtain the wine pomace. Pomace was ground to a fine paste using a mill (Robot Coupe USA, Inc. Jackson, Mississippi), packaged into gallon bags and were kept in the freezer (-20°C) until used for extraction.

Preparation of Juice and Wine Pomace Water Extracts

Pomace was extracted twice with acidified hot water (1% formic acid) at 90°C. For the first extraction, pomace (200 g) was mixed with 500 mL of acidified hot water (1% formic acid) at 90°C, sonicated for 15 min and kept in the dark at room temperature for 1 h. The samples were sonicated again for 15 min before filtration using muslin cloth. The residue from the muslin cloth was extracted with 400 mL of acidified hot water (1% formic acid) using the same procedure. The filtered extracts from the two extractions were pooled and transferred into a 1-L volumetric flask. Acidified water (1% formic acid) was added to make up the final volume to 1 L. The final extract was filtered using Whatmann No. 4 filter paper.

Characterization and Phytochemical Analysis of Extracts

The extracts were diluted to appropriate concentration for analysis. Total anthocyanin content in pomace extracts and concentrated extracts obtained after resin adsorption/desorption processes was measured using the pH differential spectrophotometric method described by Giusti and Wrolstad [88]. The extracts were dissolved in 0.025 mol/L potassium chloride buffer, pH 1.0 and 0.4 mol/L sodium acetate buffer, pH 4.5 with pre determined dilution factor. Absorbance at 520 and 700 nm was measured on a DU 730 Life Science UV/vis spectrophotometer (Beckman Coulter, Fullerton, CA) after 30 min of incubation at room temperature. The absorbance (A) of the diluted sample was then calculated using $(A_{520} – A_{700})_{pH\ 1.0} – (A_{520} – A_{700})_{pH\ 4.5}$. The monomeric anthocyanin concentration in the original sample was calculated in cyanidin-3, 5-diglucoside equivalents according to this formula: (A × MW × DF × 1000) / (ε × 1), where MW (611) of cyanidin 3, 5-diglucoside is used, the molar absorptivity ε was 30,175; the DF was dilution factor; 1000 is the factor to convert gram to milligram

and the A was absorbance. Results for total anthocyanin content were expressed as milligram cyanidin 3, 5-diglucoside equivalent per gram of fresh pomace (mg cyanidin 3, 5-diglucoside/g). The total phenolic content was determined as reported previously [6]. The results were expressed as milligrams of gallic acid equivalents per gram of fresh pomace (mg of GAE/ g).

HPLC analysis of anthocyanins, other phenolic phytochemicals, and sugars was performed on an Agilent 1200 HPLC system consisting of an autosampler, a binary pump, a column compartment, a diode array detector, fluorescent detector and a refractive index detector (Agilent Technologies, Palo Alto, CA). Reversed phase chromatography was used for the quantification of anthocyanins, ellagic acid, flavonols, catechin and epicatechin. An Agilent Zorbax Stablebond SB-C18 column (250 mm × 4.6 mm, 5 µm particle size, Agilent Technologies, Palo Alto, CA) was used for the separation of phenolic compounds. For anthocyanin analysis, the extracts (1 mL) were filtered through 0.45 µM filter units and 5 µL injected directly without any purification. Elution was performed using mobile phase A (5% formic acid aqueous solution) and mobile phase B (methanol). The flow rate was 1 mL/min with the gradient as follows: 0−2 min, 5% B; 2-10 min, 5-15% B; 10-25 min, 15-25% B; 25-30 min, 25-30% B; 30-45 min, 30% B; 45-47 min, 30-70% B; 47-50 min, 70-5% B; followed by 5 min of re-equilibration of the column before the next run. UV-vis spectra were scanned from 220 to 600 nm on a diode array detector and the detection wavelength for the anthocyanins was 520 nm. The individual anthocyanins were quantified using standards. The analysis of ellagic acid, flavonols, catechin and epicatechin was done on HPLC after acid hydrolysis of the samples. The solids were dissolved in 5 mL of methanol (50%)

containing 1.2 N HCl and sonicated for 5 min. Hydrolysis was conducted in a precision water bath (Thermoscientific, Waltham, MA) for 80 min at 90°C and aglycones were separated and quantified using HPLC. The binary mobile phase consisted of (A) 0.5% aqueous formic acid solution and (B) acetonitrile. The flow rate was 1 mL/min and 25 min gradient was used. The gradient is described as follows: 0−5 min, 10-30% B; 5-10 min, 30-40% B; 10-20 min, 40-50% B; 20-23 min, 50-70% B; 23-25 min, 70-10% B; followed by 5 min of re-equilibration of the column before the next run. The column temperature was maintained at 30°C. The detection wavelength for the ellagic acid and flavonols was 260 and 360 nm, respectively. Catechin and epicatechin were quantified using fluorescent detection. Excitation and emission of the fluorescent detector were set at 230 and 321 nm, respectively. Sugar analysis was conducted using a Restek ultra amino column (5µm, 250 × 4.6 mm). Acetonitrile:water (65:35 v/v) was used as the mobile phase at a constant flow rate of 1.0 mL/min. The column temperature was maintained at 30°C and a 5 µL of sample was injected. The optical unit temperature was set at 35°C and the refractive index detector signal was monitored in positive polarity. The run time for each sample was 15 min followed by 5 min post time before the next run. Calibration curves were constructed using pure standards of glucose and fructose.

Pretreatment of Macroporous Resins

The physical characteristics of five different resins used in this study are summarized in Table 5-1. Resins were soaked in ethanol overnight and then treated with 2 bed volumes (BV) of 4% HCl and 5% NaOH solutions to remove salts and other impurities trapped inside the pores due to synthesis process. After acid and base wash,

resins were neutralized with distilled water. To determine the moisture content of resins, three samples of each kind of pretreated resin were weighed and dried at 60°C for 24 h.

Static Adsorption and Desorption Tests for Screening of Resins

The static adsorption and desorption experiments were performed as follows: 1 g resin (wet weight) was introduced into a 125 mL Erlenmeyer flask. Then, 50 mL of juice/wine pomace water extract was added to each flask. A control sample was employed to monitor any change in the initial concentration values, to exclude effects on measured absorbance. The flasks were then shaken (120 rpm) for 24 h at room temperature (25°C) in a shaking water bath. After adsorption, resins were filtered and washed with 50 mL of distilled water. For desorption 70 and 95 % acidified (1 % formic acid) ethanol solutions were tested. Fifty milliliters of ethanol–water solution was added to the flasks containing the adsorbate-laden resins. The flasks were kept for shaking (120 rpm) for 24 h at room temperature (25°C) in a shaking water bath. The concentrations of anthocyanins and total phenolics in the liquid phase after adsorption and desorption, were then analyzed by respective assays. Candidate resins were selected in terms of their adsorption capacities, adsorption and desorption ratios and recovery (%).The following equations were used to quantify the capacities of adsorption and desorption as well as their ratios.

Adsorption evaluation

$$Q_e = \frac{(C_0 - C_e)V_0}{m} \quad (5\text{-}1)$$

$$E(\%) = \frac{(C_0 - C_e)}{C_0} \times 100 \quad (5\text{-}2)$$

Where Q_e is adsorption capacity at adsorption equilibrium (mg/g resin), E is the adsorption ratio (%), C_0 and C_e are initial and equilibrium concentrations (mg/L) of

solute in the solution, respectively, V_0 the volume of initial sample solution (mL), and m is the weight of dry resin (g).

Desorption evaluation

$$D = \frac{(C_d V_d)}{(C_0 - C_e)V_0} \times 100 \quad (5\text{-}3)$$

$$R = \frac{C_d V_d}{C_0 V_0} \times 100 \quad (5\text{-}4)$$

Where, D is the desorption ratio (%), C_d concentration of the solute in desorption solution (mg/L), V_d volume of desorption solution (mL) and R is the recovery (%). C_0, C_e and V_0 are the same as described above.

Adsorption Kinetics

The adsorption kinetics was studied on FPX-66, XAD-16N and XAD-1180 resins. The test for adsorption kinetics on the selected resins were conducted by adding 50 mL water extracts of juice/wine pomace with 1g (wet weight) resin, and then shaking (120 rpm) for 8 h at room temperature (25°C). Aliquot (1 mL) of sample solution was taken at equal time intervals until the equilibrium was reached and analyzed for total anthocyanins and total phenolic content. Two mathematical models were applied to simulate the uptake of phenolic compounds on the selected resins with time i. e., pseudo-first order (Eq. 5-5) [101] and pseudo-second kinetic model (Eq. 5-6) [102].

$$ln(Q_e - Q_t) = ln\, Q_e - k_a t \quad (5\text{-}5)$$

$$\frac{t}{Q_t} = \frac{1}{k_b Q_e^2} + \frac{1}{Q_e} t \quad (5\text{-}6)$$

Where, Q_e and Q_t (mg/g) are the amount of phenolic compounds adsorbed per gram of resin at equilibrium and anytime t (min), respectively and k_a (min^{-1}) is the equilibrium rate constant. k_b (g/mg min) is the second-order model rate constant.

Adsorption Isotherms

The tests for adsorption isotherms on the FPX-66, XAD-16N and XAD-1180 resins were conducted at room temperature (25°C), 30 and 35°C for juice pomace extracts. Briefly, 50 mL water extracts of juice pomace at different initial concentrations were mixed with 1g (wet weight) resin, and then kept for shaking (120 rpm) for 8 h at selected temperatures. At equilibrium samples were analyzed for total anthocyanins and total phenolic content. Two standard theoretical models, i.e., Langmuir and Freundlich model, are used to describe the adsorption behavior between adsorbate and adsorbent [103]. Equations describing Langmuir and Freundlich models are given below:

$$\frac{C_e}{Q_e} = \frac{1}{K_L Q_m} + \frac{C_e}{Q_m} t \quad (5\text{-}7)$$

$$ln Q_e = ln K_F + \frac{1}{n} ln C_e \quad (5\text{-}8)$$

Where, Q_m (mg/g) is the maximum amount of adsorption, K_L is the affinity constant in Langmuir model which can be calculated from the slope and intercept of the linear plot of C_e/Q_e versus C_e, respectively. K_F and $1/n$ are the constants as measures of adsorption capacity and adsorption intensity in Freundlich model and can be determined from the intercept and slope of linear plot of ln Q_e versus ln C_e, respectively. The essential characteristics of the Langmuir isotherm can be expressed by means of "R_L" a dimensionless constant, called the separation factor or equilibrium parameter. R_L can be calculated using the following equation:

$$\frac{1}{1+K_L C_o} \quad (5\text{-}9)$$

R_L values indicate the type of isotherm to be irreversible (R_L=0), favorable (0<R_L<1), linear (R_L=1), or unfavorable (R_L>1) [104].

Dynamic Adsorption and Desorption Tests

Dynamic adsorption and desorption tests were carried out on a glass column (22 mm × 350 mm) packed with 28 g (wet weight) of the selected FPX-66 resin. The bed volume (BV) of resin was 30 mL and the packed length of resin bed was 19 cm. The adsorption process was performed by loading juice pomace water extract onto the pretreated resin filled in a glass column. Subsequently, the adsorbate laden column was washed first with 4 BV of distilled water, and then desorbed with 70% acidified (1 % formic acid) ethanol solution. The effect of sample flow rate and ethanol solution flow rate on adsorption and desorption phenomenon were studied. After optimizing conditions for adsorption and desorption, the 70% ethanol eluent was collected and concentrated to dryness in a Speed Vac Concentrator (Thermo scientific ISS110, Waltham, MA) under reduced pressure at 25°C to remove the solvent.

Statistical Analysis

All data are expressed as mean ± standard deviation. Two way analyses of variance (2-way ANOVA) were performed for static desorption tests using Sigma Stat (version 11.0, Systat software Inc., Chicago, IL). One way analyses of variance (ANOVA) with Tukey-HSD pairwise comparison of the means was performed using JMP software (version 8.0, SAS Institute Inc., Cary, NC) for other results. The mean values were considered significantly different at $p \leq 0.05$.

Results and Discussion

Screening of the Resins

Resins differ in chemical structure, polarity, particle size, porosity and surface area. The adsorption of a solute on an adsorbent is a complex process which involves the interactions among three components: the adsorbate (the solute), the adsorbent and

the solvent involving a physical action through Van der Waals force or hydrogen bonding. The adsorption and desorption properties of different resins were tested based on total phenolic and anthocyanin content in juice and wine pomace water extracts.

Total anthocyanin content

Juice pomace. Adsorption capacity describes amount of anthocyanins/phenolic compounds adsorbed on 1 g of resin. Adsorption ratio is the percentage of anthocyanins/phenolic compounds adsorbed by resins from the aqueous extract. Adsorption capacity was highest for FPX-66 and XAD-16N, followed by XAD-1180 and XAD-761 in juice pomace. Similarly, the highest adsorption ratio was observed for FPX-66 and XAD-16N, followed by XAD-1180. However, XAD-7HP showed the lowest adsorption capacity and ratio for juice pomace (Figure 5-1A).

Recovery rate is the % of anthocyanins/phenolic compounds recovered from the resins by the desorbing solvent based on the initial concentration of the extract. The recovery (%) was highest in FPX-66 and XAD-16N, followed by XAD-1180 and 761 (Figure 5-1B). However, recovery (%) of anthocyanins with 95% ethanol was similar for FPX-66, XAD-1180 and 16N. The lowest recovery (%) was seen in XAD-7HP with both 70 and 95% ethanol.

Desorption ratio is the % of anthocyanins/phenolic compounds desorbed from the resins by the desorbing solvent. Significant differences were observed among resins in desorption ratio with 70% ethanol. The highest desorption ratio was observed in FPX-66 followed by XAD-16N using 70% ethanol as desorption solvent (Figure 5-1C). In contrast, the desorption ratio was similar for FPX-66, XAD-1180 and XAD-16N using 95% ethanol. Similar to recovery, XAD-7HP showed the lowest desorption ratio for

anthocyanins with 70 and 95% ethanol. There was a statistically significant interaction between resins and % ethanol ($p < 0.001$).

Wine pomace. Adsorption capacity was highest for FPX-66 and XAD-16N, followed by XAD-1180 and XAD-761 in wine pomace as well. Highest adsorption capacity and ratio was observed in FPX-66, followed by XAD-16N and 1180 while, the lowest adsorption capacity and ratio was observed in XAD-761 and 7HP (Figure 5-2A). The highest recovery (%) and desorption ratio was observed for XAD-16N and FPX-66, followed by XAD-761 and XAD-1180 using 70 and 95% ethanol while, XAD-7HP had lowest recovery (%) and desorption ratio. No significant differences were observed in recovery (%) or desorption ratio in different resins by using 70 and 95% ethanol as desorption solution (Figures 5-2B and C).

The adsorption and desorption of anthocyanins was highest on non-polar resins (FPX-66, XAD-16N and 1180). The pore size of the porous adsorbents and the size of adsorbate molecules play an important role in the process of adsorption. If the pore diameter is too small it can restrict the diffusion of adsorbate molecules. On the other hand, if the pore diameter is too large the molecules adsorbed will be prone to desorption at the same time [105]. In addition, the affinity between different polyphenols for the adsorption sites may vary, with some phenolics being preferentially selected over others. Anthocyanins are small molecules compared to other phenolic compounds present in pomace. They contain both polar hydroxy groups and non-polar phenylallyl groups which could explain the differences in adsorption behaviors of resins with different polarity. Except for XAD-7HP, the amount of anthocyanins adsorbed seems to be proportional to the adsorbent surface area [94, 106]. Resins showing high adsorption

capacity had lower pore diameters which could be another factor for better adsorption of anthocyanins on them. Desorption of anthocyanins was almost complete in case of FPX-66, XAD-16N and XAD-1180 resins. However, the desorption ratio was very low for polar resins i. e., XAD-761 and XAD-7HP, which might be due to strong interaction between polar hydroxy groups of anthocyanins in the solute with the adsorbent material. The large surface area and ideal pore diameter for anthocyanins could be possible explanation for better adsorption and desorption characteristics of FPX-66, XAD-16N and XAD-1180 resins.

Total phenolic content

Juice pomace. Based on total phenolic content adsorption capacity was highest for XAD-7HP followed by FPX-66, XAD-16N and XAD-1180 for juice pomace. Among all the resins XAD-761 had the lowest adsorption capacity and ratio (Figure 5-3A). The recovery (%) based on total phenolic content was found to be highest for FPX-66 resin followed by XAD-16N and XAD-7HP using 70% ethanol in juice pomace while, XAD-1180 and 761 gave lowest recovery (Figure 5-3B). However, with 95% ethanol the recovery (%) was similar for FPX-66, XAD-16N, XAD-7HP and XAD-1180. There was a statistically significant interaction between resins and % ethanol ($p < 0.001$). No significant differences were observed in desorption ratio in different resins at both 70 and 95% ethanol as desorption solution in juice pomace (Figure 5-3C).

Wine pomace. Similar to juice pomace adsorption capacity was highest for XAD-7HP followed by FPX-66, XAD-16N and XAD-1180 while, XAD-761 had the lowest adsorption capacity and ratio (Figure 5-4A). The recovery (%) based on total phenolic content was found to be highest for FPX-66 resin followed by XAD-16N and XAD-7HP using 70% ethanol. XAD-1180 and 761 gave lowest recovery (%) with 70% ethanol. In

contrast to juice pomace, recovery (%) based on total phenolic content in wine pomace was highest in XAD-1180, followed by FPX-66 and XAD-7HP using 95% ethanol. Resin XAD-761 and 16N had lowest recovery (%) (Figure 5-4B). Significant differences were observed in the desorption ratio among different resins with 70% ethanol. However, no differences were observed with 95% ethanol (Figure 5-4C). There was a statistically significant interaction between resins and % ethanol ($p < 0.001$) for recovery (%).

Phenolics in pomace may vary from simple acids to complex flavonoids, hydrolysable tannins and condensed tannins. These compounds vary in their polarity depending upon their structure. XAD-7HP showed highest adsorption capacity for both juice and wine pomace followed by non-polar resins (FPX-66, XAD-16N and XAD-1180). The adsorption of particular species can also depend upon its similarity to the resin on the basis that “like attracts like”, which means polar compounds are attracted more towards polar resins [106]. XAD-7HP, which presented the best results in terms of the adsorptive capacity, is a non-ionic aliphatic acrylic polymer (dipole moment = 1.80), whereas the other adsorbents are made of styrene–divinylbenzene, a hydrophobic polyaromatic polymer (dipole moment = 0.30), making them more efficient for adsorption of compounds of higher hydrophobicity [107]. The adsorption of phenolics seems to be proportional to the surface area of the resins. Recovery (%) and desorption of phenolics was almost complete in both juice and wine pomace however, differences were observed by using 70 or 95% ethanol.

Based on the initial screening three resins (FPX-66, XAD-16N and XAD-1180) were selected for further testing of adsorption kinetics and thermodynamics.

Adsorption Kinetics

The experimental results of the adsorption kinetics of juice and wine pomace based on total anthocyanin and phenolic content on the three selected resins are shown in Figures 5-5 and 5-6. The adsorption of phenolic compounds from the aqueous solution on all the three resins increased quickly in the first 90 mins and then increased slowly until adsorption equilibrium was reached at 490 mins in both the pomaces. Different kinetic models are used to determine the rate of adsorption processes and mechanism of adsorption. The commonly used kinetic models are the pseudo-first order and pseudo-second order model [108]. The pseudo-first order is generally applicable over the initial stage of an adsorption process while pseudo-second order model assumes that rate limiting step is chemisorption and predicts the behavior over the whole range of adsorption [109]. The experimental data were fitted to these two models in order to determine which model best described the adsorption rate of phenolic compounds. Summary of the results are reported in Table 5-2. In the case of FPX-66 and XAD-16N resins, the higher correlation coefficients and agreement between experimental and calculated Qe values conclude that the pseudo-first order model is a more favorable fit for the adsorption of total anthocyanins from juice pomace extracts. However, for XAD-1180, pseudo-second order seems to be a better fit than the pseudo-first order model. For wine pomace pseudo-second order was a better fit for all the three resins based on total anthocyanins (Table 5-2). Except for XAD-16N in wine pomace, the high correlation coefficient ($R^2 > 0.95$) of pseudo-second order model indicates that adsorption of phenolic compounds from muscadine pomace fit better by this model compared to pseudo-first order model (Table 5-3). Similar findings were reported by other studies [106, 110]. Since, the results of wine pomace were comparable to juice

pomace, so only juice pomace was selected for further testing with focus on anthocyanin content.

Adsorption Isotherms

The equilibrium adsorption isotherms of anthocyanins for juice pomace on three selected resins were investigated at three temperatures (room temperature (25°C), 30 and 35°C) as shown in Figures 5-7 A, B and C. The Langmuir and Freundlich isotherms are two of the most commonly used models for describing adsorption isotherms. The Langmuir model describes a monolayer adsorption with energetically identical sorption sites and without mutual interactions between the adsorbed molecules. The Freundlich model assumes adsorption to heterogeneous surfaces which is characterized by sorption sites at different energies. This model can be used to describe the adsorption behavior of monomolecular layer as well as that of the multi-molecular layer [109]. The correlation coefficients for each model on three selected resins at different temperatures are listed in Table 5-4. For all three resins, the Langmuir model was considered as a better model for describing adsorption equilibrium due to higher correlation coefficients (R^2 = 0.9831-0.9994). The values of Q_m (maximum adsorption capacity) increased in the order FPX-66>XAD-16N>XAD-1180. Our results indicate that the adsorption for anthocyanins on the selected resins is favorable and has R_L value between 0 and 1 (Table 5-4). The Freundlich isotherm constant n represents the strength of adsorption process and its value should be greater than 1 and less than 10 for favorable adsorption conditions [109]. The n values obtained from Freundlich plots were greater than 1 for all the three resins indicating favorable adsorption conditions.

Dynamic Breakthrough Curve on FPX-66 Resin

In order to optimize the process of dynamic adsorption and desorption important factors such as feed volume, flow rate of feed and eluent, and volume of eluent were taken into consideration and tested. The process of adsorption involves diffusion of adsorbate, interaction between the adsorbate molecules and the resin including hydrogen bonding, simple stacking or hydrophobic interactions. Resins can easily adsorb the molecules due to their large surface area and a highly porous structure. When the adsorption reaches the breakthrough point (the point of maximum saturation and when the resin cannot hold the adsorbate molecules), the adsorption effect decreases and solute starts to leak from the resin. Thus, it is important to define the breakthrough point in order to calculate the resin quantity, processing volume of sample and proper sample flow rate. The best adsorption performance was observed at the slowest flow rate (2 BV/h) (Figure 5-8). The low flow rate allows more time for the adsorbate molecules to interact with the active sites of the resin at the expense of longer processing time. In contrast, a faster flow rate requires less time but has a negative impact on the adsorption capacity since breakthrough point reaches more quickly [106]. The 5% ratio of exit to the inlet solute was defined as the break through point in this study. Based on the results and taking into consideration the processing time, flow rate of 4 BV/h was selected as the optimum for the adsorption with approximately 17 BV processing volume of the sample solution.

Dynamic Desorption Curve on FPX-66 Resin

These curves were based on the volume of desorption solvent used and the concentration of anthocyanins in the desorption solvent. A 70% acidified (1% formic acid) ethanol solution was used as a desorption solvent. It is important to select the

proper flow rate for better desorption performance. As it can be seen from Figure 5-9, the best desorption performance was observed at slowest flow rate of 1 BV/h. There was no difference in the dynamic desorption at flow rates of 2 and 3 BV/h. Therefore, 2 BV/h was chosen as proper flow rate for dynamic desorption on account of short working time. Approximately, 3 BV of desorption solution was used to completely desorb the anthocyanins from the resin.

The yield of concentrated powder was 5.7 mg/g of fresh juice pomace. The percent recovery of total anthocyanins was 73%, which was comparable with those in some previous studies [96, 111]. Choice of organic solvent and poor stability of anthocyanins may also adversely affect the recovery [112].

Characterization of Concentrated Extracts from Muscadine Juice Pomace

The content of individual phytochemicals in the concentrated extracts obtained after resin adsorption and desorption process is shown in Table 5-5. The values were compared with those in the initial water extract of muscadine juice pomace. The extracts were highly concentrated solutions of anthocyanins, ellagic acid, flavonols, catechin and epicatechin without sugars [113]. Resin adsorption increased the content of peonidin 3, 5-diglucoside by 38 times. Similarly, total anthocyanins and total phenolics were 38 and 42 times higher in the concentrated extract compared to the water extract. A study on muscadine pomace showed increase in the total phenolics and the anthocyanins by 25 times after resin adsorption/desorption process [114]. Similarly, anthocyanins were increased 7 to 20 times from a by-product of blood orange juice processing [113]. The adsorption of phenolic phytochemicals on resin was not completely selective for anthocyanins. In addition to anthocyanins, the content of ellagic acid, and total flavonols (myricetin, quercetin and kaempferol) in the concentrated extract also increased by 6

and 5 times, respectively. Catechin and epicatechin were detected only in the concentrated extract but not in the initial water extract. Muscadine pomace water extract contained significant amount of glucose and fructose (Table 5-5). These sugars were completely removed by water wash during the resin adsorption process.

Summary

In summary, our results indicate that FPX-66 is the most suitable resin among selected commercial adsorbents for the recovery of anthocyanins from muscadine juice pomace. This is due to its high adsorption and desorption capacity, and greater affinity towards these phenolic compounds, with respect to the other tested resins under the same conditions. The optimization of resin adsorption process in the present study sets parameters for the development of pilot-scale separation and concentration of anthocyanins from muscadine pomace. This extract could potentially find application as a natural colorant, dietary supplement, antioxidant ingredient for functional foods, and/or as a raw material in the cosmetic and pharmaceutical industry preparations.

Table 5-1. Physical characteristics of adsorbent resins.

Resin	Chemical nature	Polarity	Surface area (m^2/g)	Average pore diameter (Å)
XAD-7HP	Aliphatic ester	Polar	500	450
XAD-761	Phenol-formaldehyde	Polar	200	600
XAD-16N	Polystyrene-DVB*	Non-polar	800	150
XAD-1180	Polystyrene-DVB*	Non-polar	700	400
FPX-66	Polystyrene-DVB*	Non-polar	700	200-250

*DVB-Divinyl benzene

Table 5-2. Kinetic parameters of muscadine juice and wine pomace on FPX-66, XAD-16N and XAD-1180 resins at room temperature (25°C) based on total anthocyanins.

Resins	Q_e exp (mg/g)	Pseudo-first order			Pseudo-second order		
		Q_e cal (mg/g)	K_a (min^{-1})	R^2	Q_e cal (mg/g)	K_b (g/mg min)	R^2
Juice Pomace							
FPX-66	27.12	25.27	0.0063	0.9883	31.55	2.96×10^{-4}	0.9784
XAD-16N	25.32	23.23	0.0062	0.9907	29.50	4.06×10^{-4}	0.9819
XAD-1180*	16.32	7.57	0.0051	0.8387	16.86	1.64×10^{-3}	0.9920
Wine Pomace							
FPX-66	19.23	16.56	0.0067	0.9159	21.88	8.29×10^{-3}	0.9740
XAD-16N	18.27	15.50	0.0065	0.9018	20.58	5.75×10^{-4}	0.9731
XAD-1180	12.43	6.68	0.0055	0.6151	13.48	1.47×10^{-3}	0.9836

exp-experimental, cal-calculated. Data are based on three replicates. * based on two determinations.

Table 5-3. Kinetic parameters of muscadine juice and wine pomace on FPX-66, XAD-16N and XAD-1180 resins at room temperature (25°C) based on total phenolics.

Resins	Q_e exp (mg/g)	Pseudo-first order			Pseudo-second order		
		Q_e cal (mg/g)	K_a (min^{-1})	R^2	Q_e cal (mg/g)	K_b (g/mg min)	R^2
Juice Pomace							
FPX-66	155.83	187.67	0.0081	0.8745	196.08	5.38×10^{-5}	0.9609
XAD-16N	144.88	176.69	0.0085	0.9045	178.57	4.06×10^{-5}	0.9507
XAD-1180	118.17	113.11	0.0084	0.9587	133.33	1.02×10^{-4}	0.9833
Wine Pomace							
FPX-66	109.13	109.76	0.0087	0.9667	129.87	4.39×10^{-5}	0.9786
XAD-16N	103.71	110.10	0.0093	0.9832	123.46	8.48×10^{-5}	0.9795
XAD-1180	85.29	75.31	0.0076	0.9486	96.15	1.34×10^{-4}	0.9798

exp-experimental, cal-calculated. Data are based on three replicates.

Table 5-4. Langmuir and Freundlich parameters for the adsorption of muscadine juice pomace on FPX-66, XAD-16N and XAD-1180 resins based on total anthocyanins.

Temp (°C)	Langmuir Equation				Freundlich Equation		
	Q_m (mg/g)	K_L (L/mg)	R_L	R^2	n	K_F (L/mg)	R^2
			FPX-66				
25	31.35	0.043	0.09	0.9974	1.94	2.65	0.9654
30	27.70	0.047	0.08	0.9994	1.97	2.49	0.9358
35	30.49	0.039	0.10	0.9979	1.76	2.04	0.9601
			XAD-16N				
25	30.22	0.035	0.10	0.9831	1.90	2.27	0.9627
30	26.81	0.047	0.08	0.9989	2.02	2.51	0.9543
35	27.86	0.040	0.09	0.9987	1.79	1.95	0.9555
			XAD-1180				
25	22.33	0.026	0.13	0.9915	1.94	1.52	0.9478
30	20.84	0.025	0.14	0.9981	1.88	1.29	0.9669
35	20.24	0.023	0.15	0.9987	1.74	1.03	0.9609

Data are based on three replicates.

Table 5-5. Comparison of phytochemical and sugar content of muscadine juice pomace water extract with concentrated extract.

Compounds	Juice pomace water extract (mg/g dry weight)	Concentrated extract (mg/g dry extract)	Enrichment factor
Anthocyanins			
Delphinidin 3,5-diglucoside	1.69 ± 0.01	48.86 ± 0.98	28.9
Cyanidin 3,5-diglucoside	0.44 ± 0.00	12.77 ± 0.23	29.1
Petunidin/Pelargonidin 3,5-diglucoside[#]	0.87 ± 0.01	29.54 ± 0.66	34.1
Peonidin 3,5-diglucoside	0.87 ± 0.00	33.22 ± 0.60	38.2
Malvidin 3,5-diglucoside	0.53 ± 0.00	18.16 ± 0.45	34.1
Total Anthocyanins*	3.41 ± 0.20	130.04 ± 2.73	38.1
Other phenolic compounds			
Ellagic acid	7.23 ± 0.49	42.86 ± 2.19	5.9
Myricetin	1.24 ± 0.35	7.53 ± 0.24	6.1
Quercetin	0.60 ± 0.10	2.79 ± 0.05	4.7
Kaempferol	0.30 ± 0.04	1.33 ± 0.00	4.4
Catechin	ND	17.32 ± 0.24	NA
Epicatechin	ND	10.56 ± 0.29	NA
Total Phenolic Content (mg GAE/g)	16.61 ± 0.43	691.75 ± 65.6	41.6
Sugars			
Fructose	165.66 ± 4.06	ND	NA
Glucose	137.44 ± 4.74	ND	NA

Peaks coeluted on HPLC. Data are mean ± standard deviation for triplicate tests. * determined by pH differential method. ND-Not Detected, NA-Not Applicable.

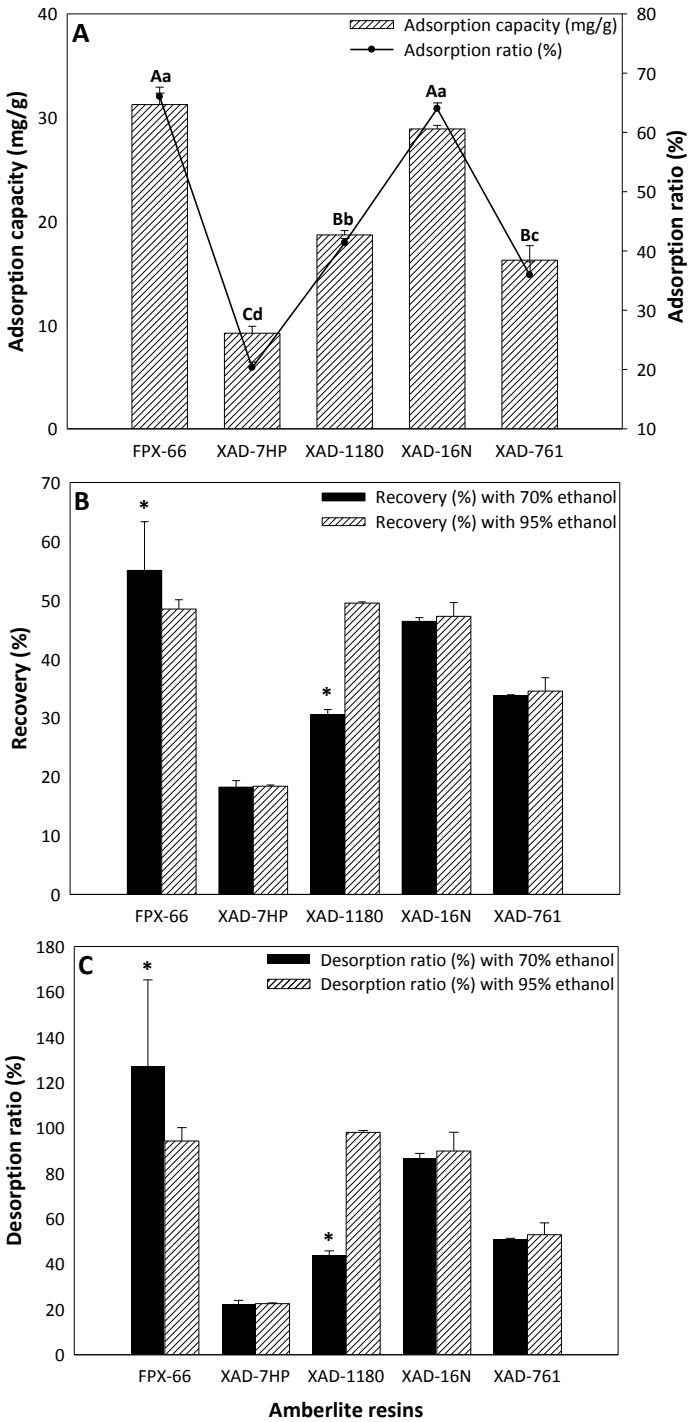


Figure 5-1. Static adsorption results based on total anthocyanin content on different resins in juice pomace: A. Adsorption capacity and ratio. Different upper-case letters indicate significant differences of bars ($p \leq 0.05$). Different lower-case letters indicate significant differences of lines ($p \leq 0.05$). B. Recovery rate (%) using 70 and 95% ethanol. * indicates significant differences ($p \leq 0.05$) in the recovery (%). C. Desorption ratio (%) using 70 and 95% ethanol. * indicates significant differences ($p \leq 0.05$) in the desorption ratio (%). Results are mean of three determinations.

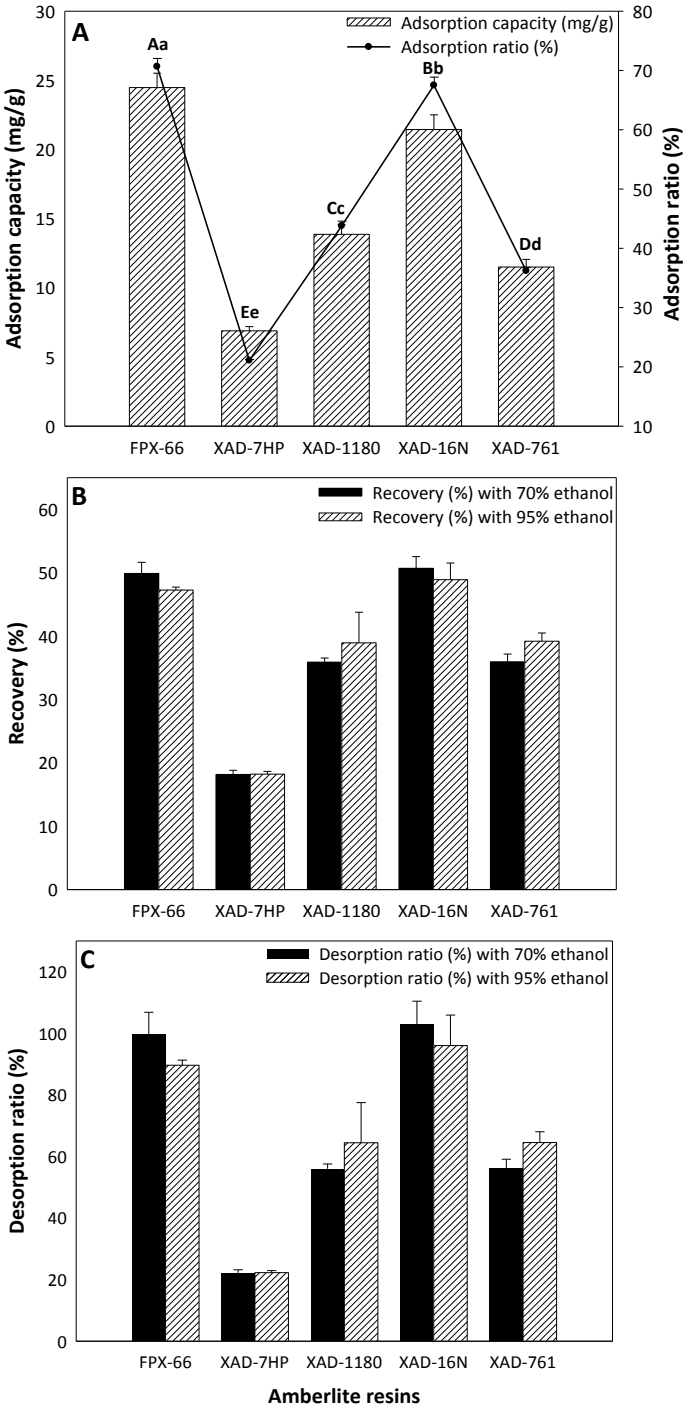


Figure 5-2. Static adsorption results based on total anthocyanin content on different resins in wine pomace: A. Adsorption capacity and ratio. Different upper-case letters indicate significant differences of bars ($p \leq 0.05$). Different lower-case letters indicate significant differences of lines ($p \leq 0.05$). B. Recovery rate (%) using 70 and 95% ethanol. * indicates significant differences ($p \leq 0.05$) in the recovery (%). C. Desorption ratio (%) using 70 and 95% ethanol. * indicates significant differences ($p \leq 0.05$) in the desorption ratio (%). Results are mean of three determinations.

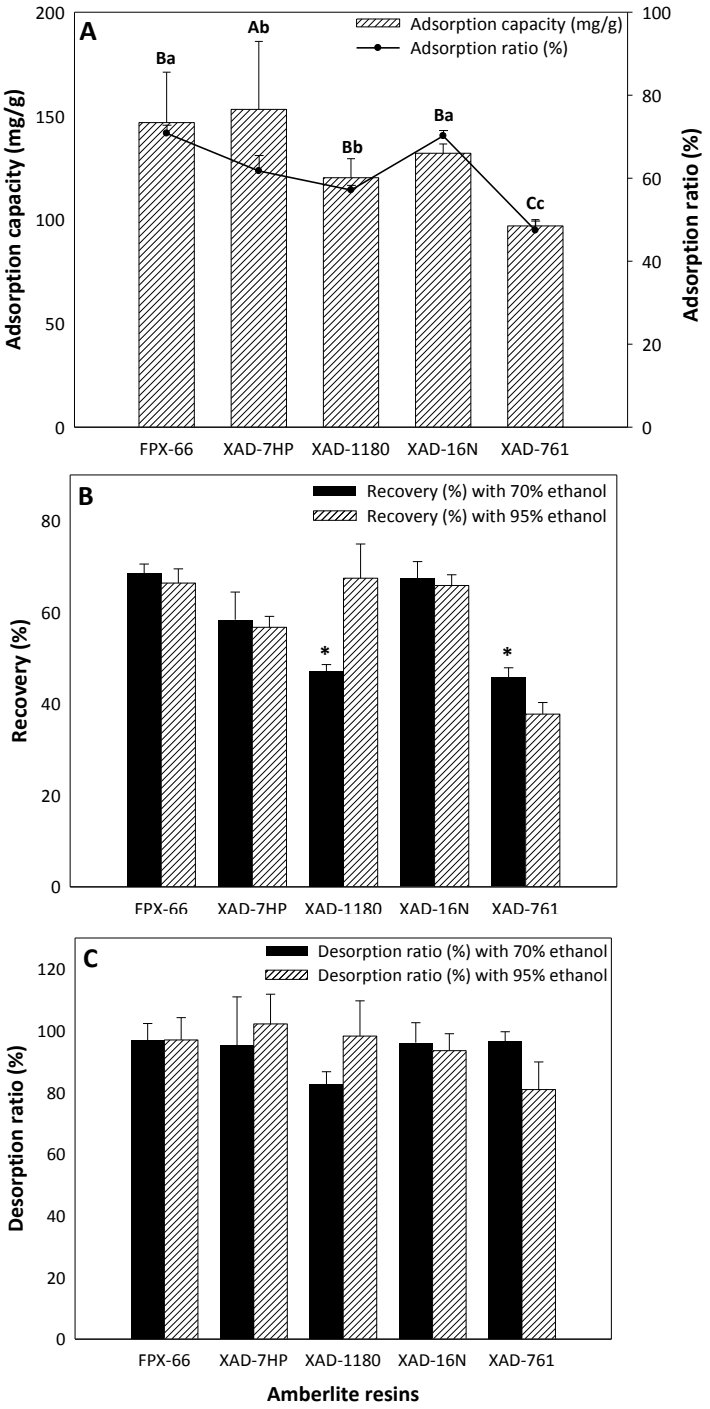


Figure 5-3. Static adsorption results based on total phenolic content on different resins in juice pomace: A. Adsorption capacity and ratio. Different upper-case letters indicate significant differences of bars ($p \leq 0.05$). Different lower-case letters indicate significant differences of lines ($p \leq 0.05$). B. Recovery rate (%) using 70 and 95% ethanol. * indicates significant differences ($p \leq 0.05$) in the recovery (%). C. Desorption ratio (%) using 70 and 95% ethanol. * indicates significant differences ($p \leq 0.05$) in the desorption ratio (%). Results are mean of three determinations.

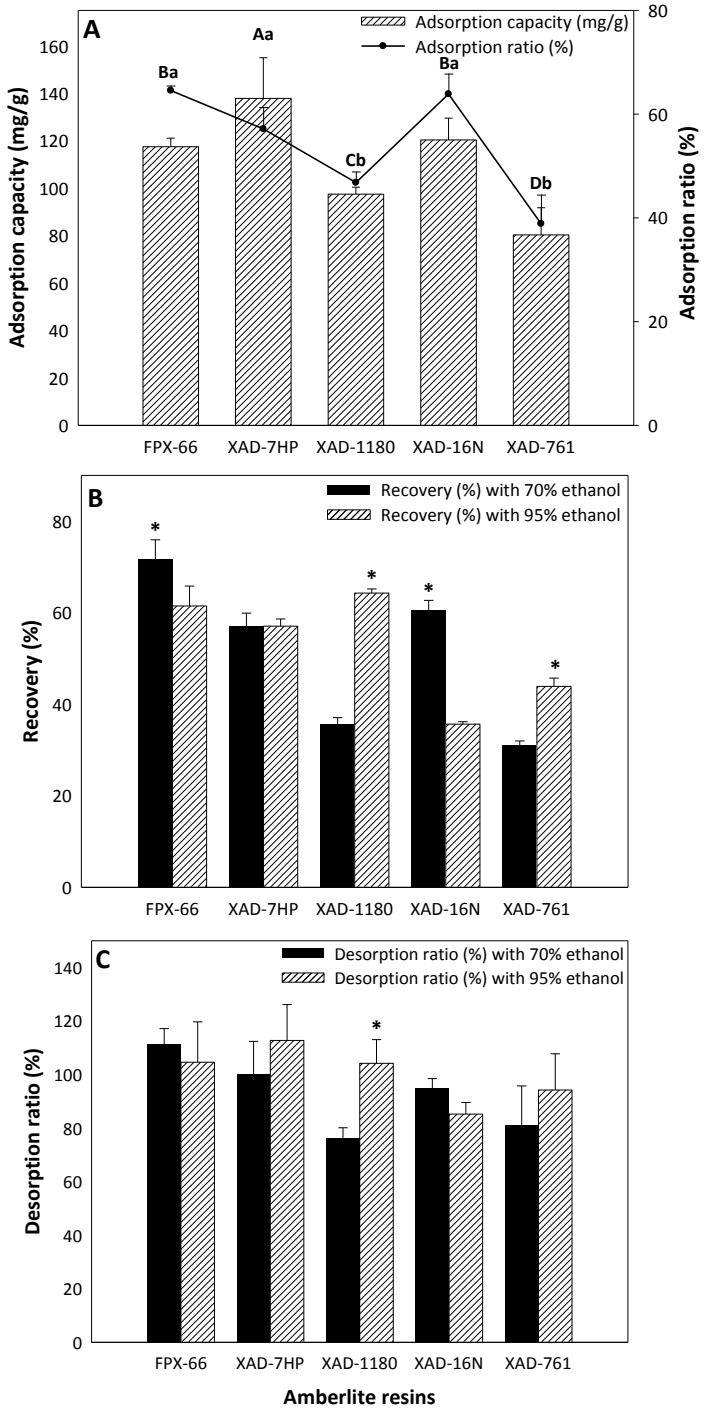


Figure 5-4. Static adsorption results based on total phenolic content on different resins in wine pomace: A. Adsorption capacity and ratio. Different upper-case letters indicate significant differences of bars ($p \leq 0.05$). Different lower-case letters indicate significant differences of lines ($p \leq 0.05$). B. Recovery rate (%) using 70 and 95% ethanol. * indicates significant differences ($p \leq 0.05$) in the recovery (%). C. Desorption ratio (%) using 70 and 95% ethanol. * indicates significant differences ($p \leq 0.05$) in the desorption ratio (%). Results are mean of three determinations.

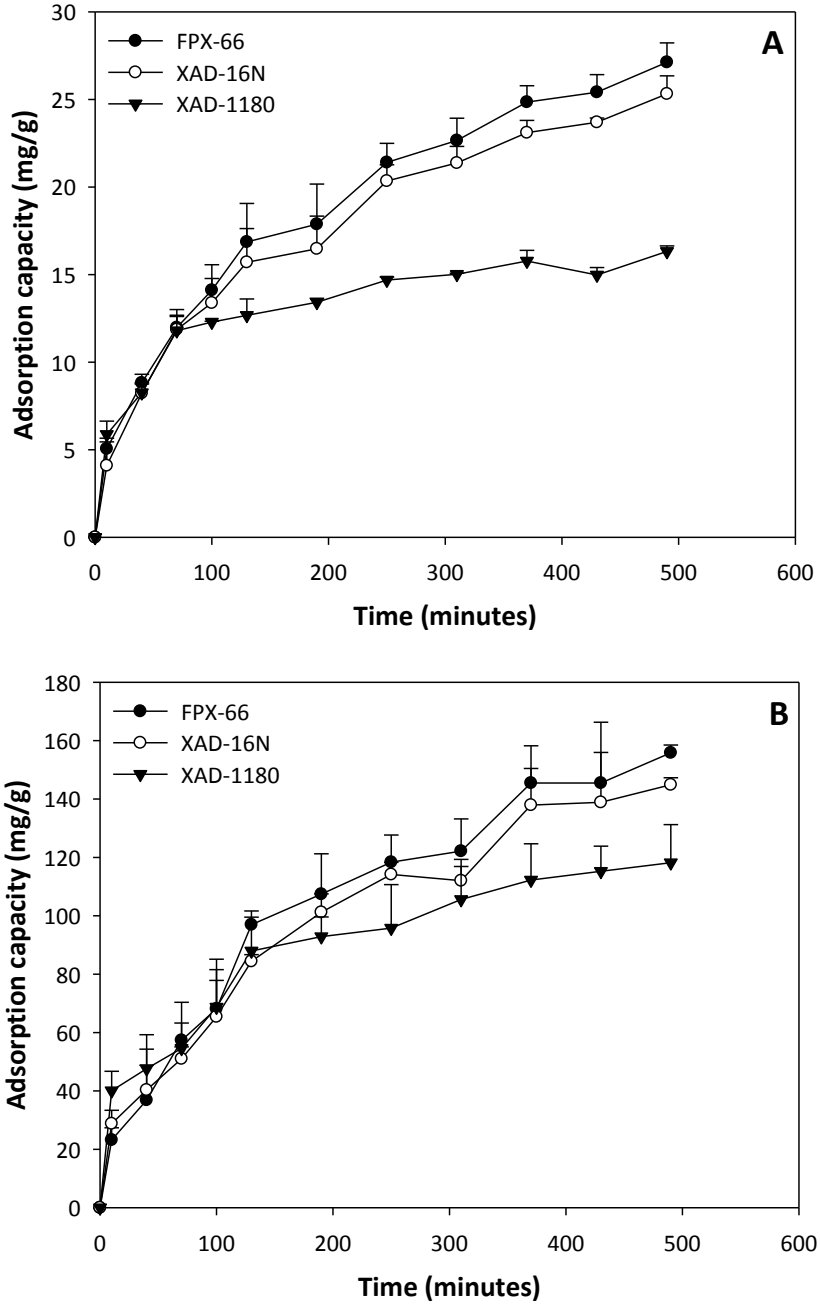


Figure 5-5. Adsorption kinetic curves for juice pomace A. Total anthocyanin content B. Total phenolic content on FPX-66, XAD-16N and XAD-1180. Results are mean of three determinations.

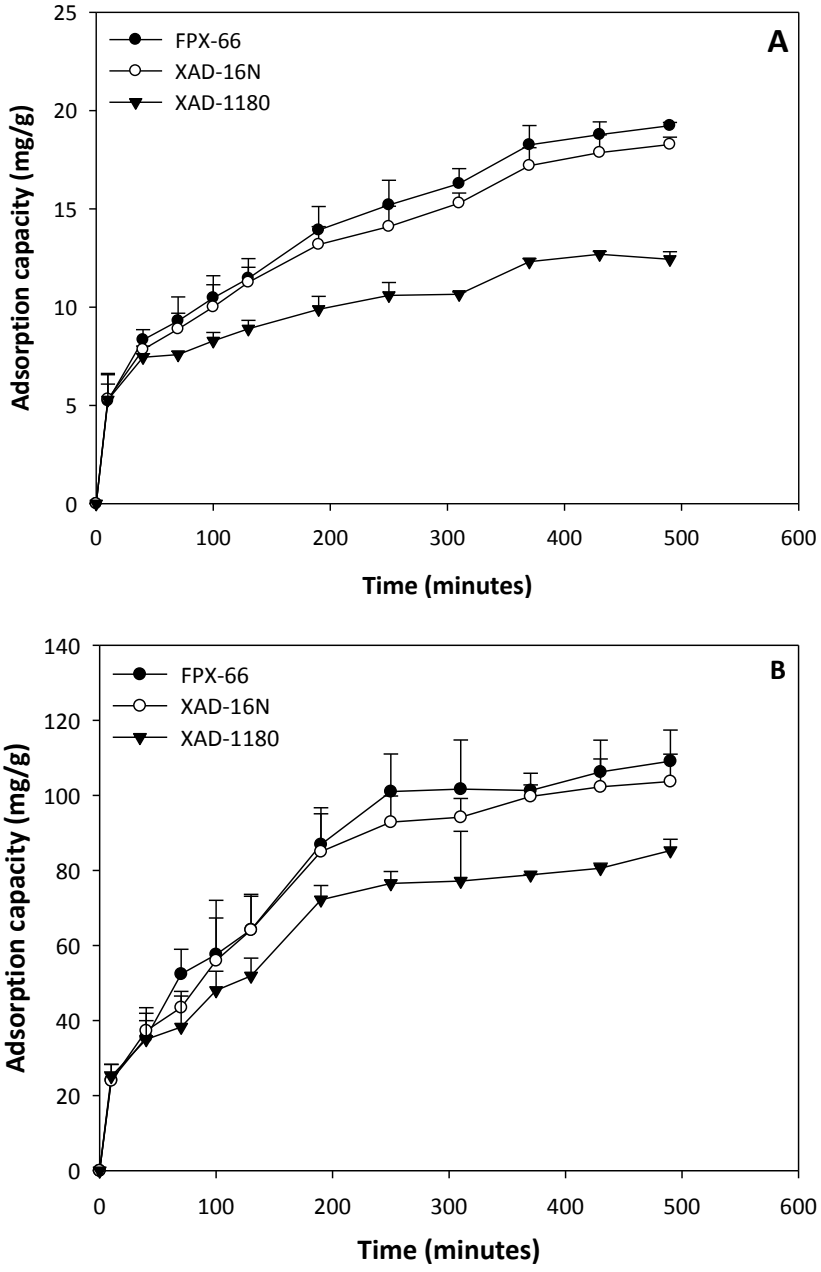


Figure 5-6. Adsorption kinetic curves for wine pomace A. Total anthocyanin content B. Total phenolic content on FPX-66, XAD-16N and XAD-1180. Results are mean of three determinations.

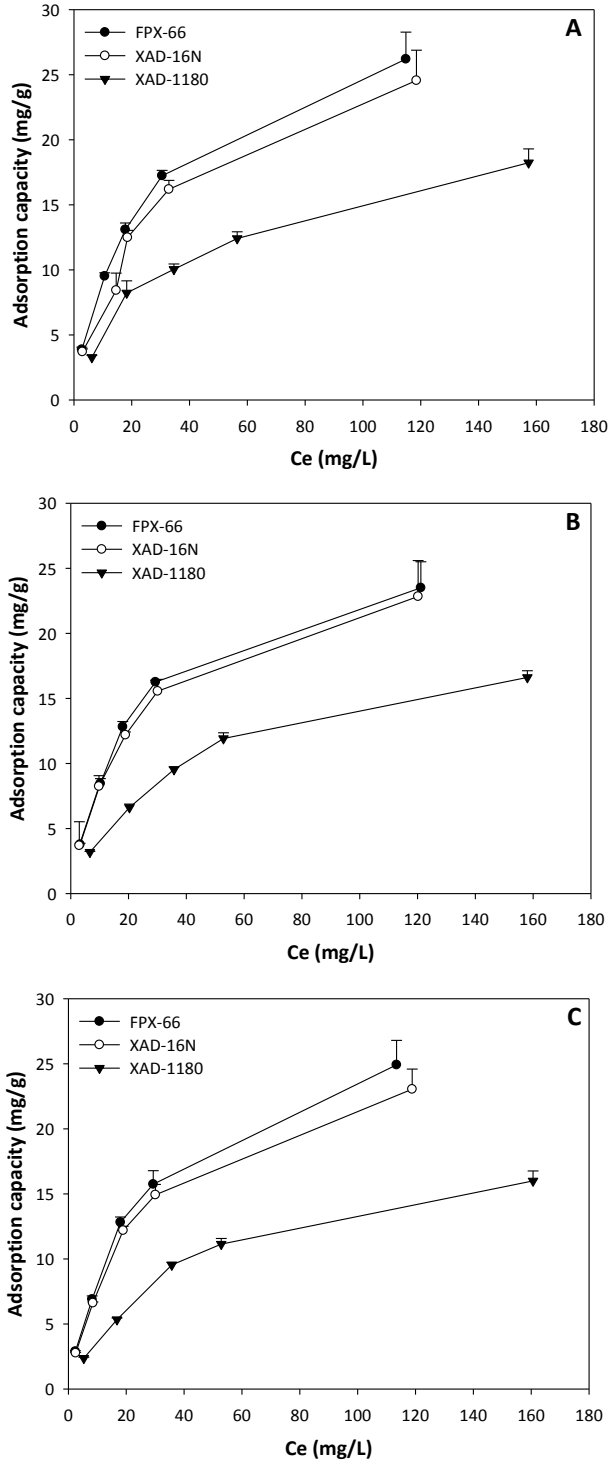


Figure 5-7. Adsorption isotherms for juice pomace based on total anthocyanin content on FPX-66, XAD-16N and XAD-1180 at A. Room temperature (25°C) B. 30°C C. 35°C. Results are mean of three determinations.

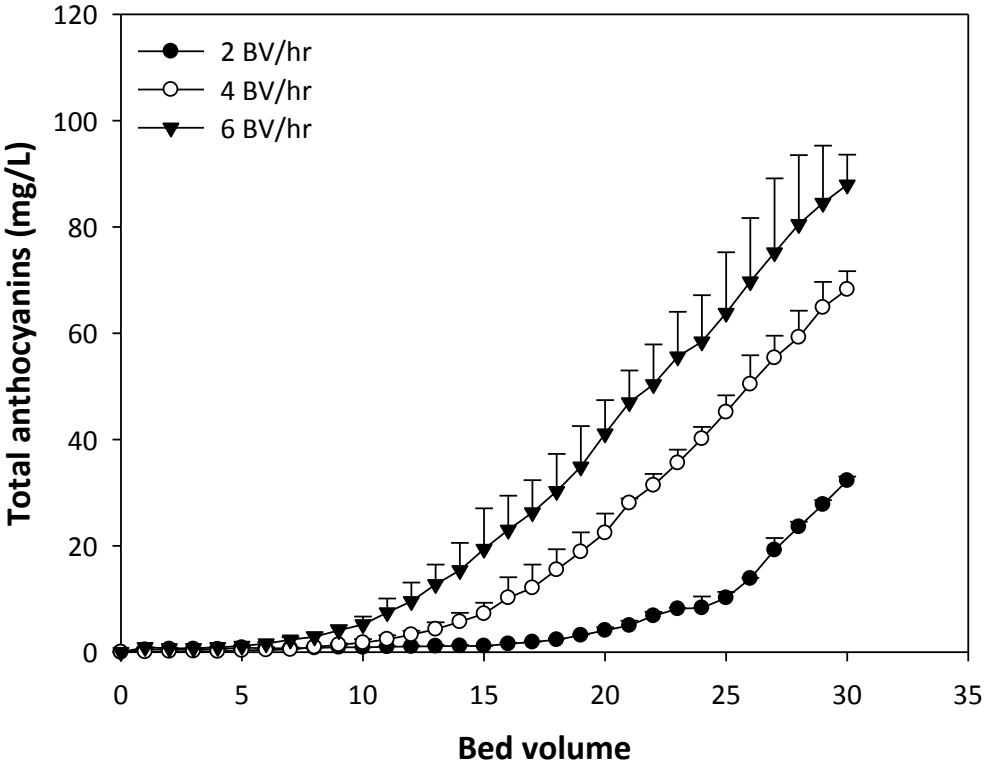


Figure 5-8. Dynamic breakthrough curves of total anthocyanins from muscadine juice pomace on column packed with FPX-66 resin at different flow rates. Results are mean of two determinations.

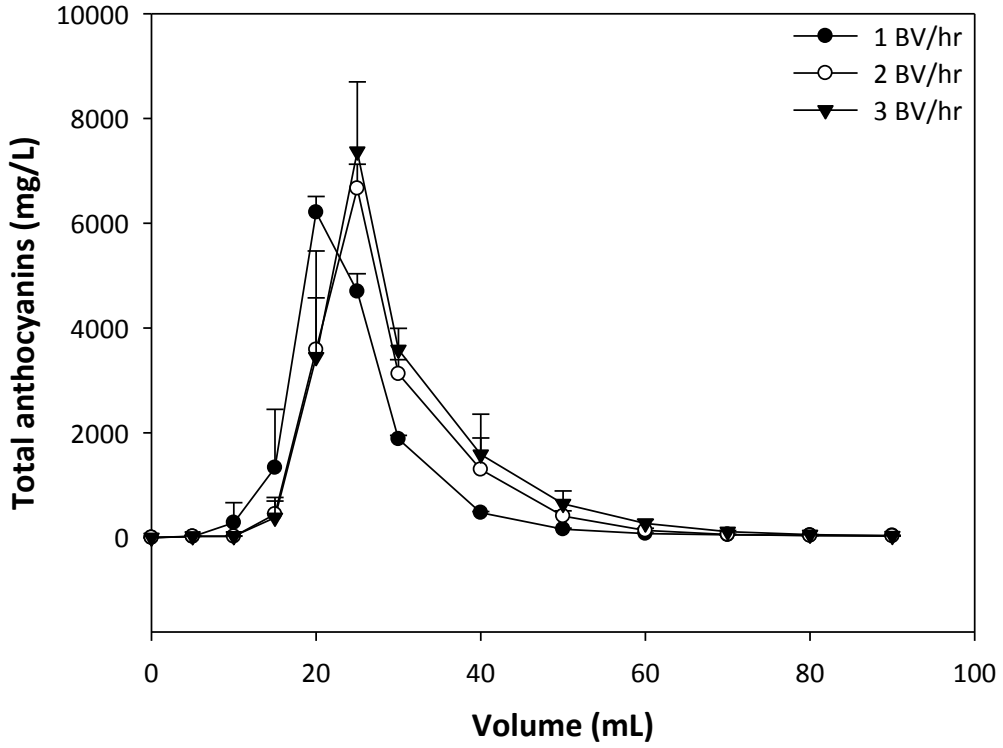


Figure 5-9. Dynamic desorption curves of total anthocyanins from muscadine juice pomace on column packed with FPX-66 resin at different flow rates. Results are mean of two determinations.

CHAPTER 6
EFFECT OF METHYL JASMONATE TREATMENT ON POSTHARVEST QUALITY OF MUSCADINE GRAPES AT DIFFERENT STORAGE TEMPERATURES

Background

Muscadine grapes harvested at optimum maturity should maintain their quality during the time required for storage and marketing. After harvest, the major compositional changes occurring in grapes are loss of sugars and organic acids. In addition, other oxidative changes can occur such as degradation of anthocyanins by enzymatic and non-enzymatic oxidation resulting in dull or brownish colored product [115]. Improper storage of fresh grapes can cause water loss and fruit softening, and favor development of decay [116].

Muscadine grapes have a very short fresh storage life (2 to 3 weeks) compared to other *Vitis* species which can be stored for 6 to 8 weeks [117]. Extending the short storage life of muscadines would eliminate the major problem limiting fresh fruit marketing. Moreover, antioxidant content is becoming an increasingly important parameter with respect to fruit and vegetable quality. Fruits and vegetables have been subjected to various post harvest treatments to prolong their shelf life and maintain quality. Application of postharvest elicitors can trigger distinct changes in the plant's secondary metabolism and can affect the antioxidants in them [118]. Thus, such targeted treatments may be used to obtain fruits and vegetables enriched with phytochemicals for sale as fresh market products or used as raw materials for functional foods and supplements [119]. Postharvest elicitors can be divided into two categories: physical and chemical. The physical elicitors include low temperature, heat treatment, ultraviolet and gamma irradiation, and altered gas composition. Chemical elicitors are primarily plant hormones such as salicylic acid, jasmonic acid, methyl jasmonate (MeJA) and ethylene.

Application of postharvest elicitors had been successfully used in fruits and vegetables with intent of improving their quality and phytochemical content [118].

MeJA is a naturally occurring compound and plays an important role in plant growth and development, fruit ripening and responses to environmental stresses [120]. Because MeJA is already classified by the U.S. Food and Drug Administration as a Generally Recognized As Safe (GRAS) substance, it has the potential for commercial applications in postharvest treatments for quality maintenance by reducing decay and enhancing the phenolic content. It has been reported that MeJA treatment could effectively suppress postharvest diseases of various fruits including sweet cherry [121], loquat [122], peach [123], and grapefruit [124]. In addition, it has been reported that postharvest MeJA treatment maintained higher levels of bioactive compounds and enhanced antioxidant capacity in berry fruits including blackberries, raspberries, and strawberries [125-127]. Postharvest treatment of Golden delicious apples with MeJA promoted β-carotene accumulation [128]. A study conducted on Chinese bayberries showed that the postharvest application of MeJA can effectively reduce fruit decay and improve their antioxidant capacity [129]. However, there is no published data on the effect of postharvest MeJA treatment on the changes in the quality and phenolic content of muscadine grapes upon storage. The objective of this research is to investigate the effect of postharvest MeJA treatment on muscadine grape quality and phytochemical composition.

Materials and Methods

Chemicals

AAPH (2,2'-azobis(2-amidinopropane)) was a product of Wako Chemicals Inc. (Bellwood, RI). Gallic acid, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

(Trolox), HPLC grade methanol, acetic acid, formic acid, Folin-Ciocalteau reagent, Flourescein and sodium carbonate were purchased from Fischer Scientific Co. (Pittsburg, PA). Methyl jasmonate and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich (St. Louis, MO).

MeJA Treatment

Noble and Alachua varieties of muscadine grapes were obtained at commercial maturity (based on full color development, °Brix above 11 and 14 for Noble and Alachua, respectively) from vineyards in Florida. Grapes were selected for uniform size, color and absence of defects and then randomly divided into three lots for each variety. For each lot of Noble and Alachua variety 1.6 and 1.8 kg of grapes were weighed, respectively and placed in 7-L airtight plastic containers for MeJA treatment. An appropriate amount of MeJA or water for control was spotted on a filter paper inside the containers and incubated at 20°C for 24 hrs, allowing MeJA to evaporate. MeJA concentrations of 0, 10 and 100 µmol/l were used [129]. The concentrations of MeJA were chosen based on previous work on other fruits. Three replicates of each treatment were conducted. Before opening the containers, the levels of CO_2/ O_2 were analyzed in each container using an O_2 and CO_2 meter (PBI Dansensor, Checkmate 9900, Ringsted, Denmark). The containers were ventilated in a fume hood and grapes from each variety were further divided into two lots of equal weight for storage at 5 and 20°C for 7 days. Samples were taken at 3, 5 and 7 days during storage for quality parameter analysis. For analyzing fruit decay, 50 (Noble) and 100 (Alachua) grapes were taken from each lot and stored for 2 weeks at 5 and 20°C.

Decay Assessment

Fruit decay was recorded after visually examining the 50 (Noble) and 100 (Alachua) grapes per replication for any defects such as mold growth, shrinkage or overripe grapes. Grapes were monitored for two weeks at 5 and 20°C storage temperatures and results were expressed as % decay.

pH and Total Soluble Solids

pH of the juice was measured using a pH meter and total soluble solids (°Brix) were measured using a bench top refractometer (Leica Abbe Mark 11, Fisher Scientific, Pittsburg, PA).

Extraction and Sample Preparation

The skin of the grapes was manually separated from seeds and pulp, and freeze dried. Subsequently, it was ground into a fine powder using a Waring kitchen blender and extracted (1 g) with 15 mL of methanol/water/acetic acid (85:15:0.5; v/v) in glass tubes. The samples were then vortexed for 30 s, sonicated for 5 min and kept in the dark at room temperature for 20 min. The tubes were then centrifuged at 1317 *g* for 10 min and the supernatant was removed. The samples were extracted again with 10 mL of methanol/water/acetic acid using the same procedure. The supernatants from two extractions were pooled and transferred into a 25-mL volumetric flask. Methanol/water/acetic acid was added to make up the final volume to exactly 25 mL.

Total Anthocyanin Assay

Total anthocyanin content in pomace extracts was measured using the pH differential spectrophotometric method described by Giusti and Wrolstad [88]. The extracts were dissolved in 0.025 mol/L potassium chloride buffer, pH 1.0 and 0.4 mol/L sodium acetate buffer, pH 4.5 with pre determined dilution factor. Absorbance at 520

and 700 nm was measured on a DU 730 Life Science UV/vis spectrophotometer (Beckman Coulter, Fullerton, CA) after 30 min of incubation at room temperature. The absorbance (A) of the diluted sample was then calculated using $(A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5}$. The monomeric anthocyanin concentration in the original sample was calculated in cyanidin-3, 5-diglucoside equivalents according to this formula: (A × MW × DF × 1000) / (ε × 1), where MW (611) of cyanidin 3, 5-diglucoside is used, the molar absorptivity ε was 30,175; the DF was dilution factor; 1000 is the factor to convert gram to milligram and the A was absorbance. Results for total anthocyanin content were expressed as milligram cyanidin 3, 5-diglucoside equivalent per gram of fresh grape skins (mg cyanidin 3, 5-diglucoside/g).

Folin-Ciocalteu Assay

The extracts were diluted to appropriate concentration for analysis. The total phenolic content was determined as reported previously [6].The results were expressed as milligrams of gallic acid equivalents per gram of fresh grape skins (mg of GAE/ g).

Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay for extracted samples was conducted on a Spectra XMS Gemini plate reader (Molecular Devices, Sunnyvale, CA). Briefly, 50 µL of standard and samples were added to the designated wells of a 96-well black plate. This was followed by the addition of 100 µL of flourescein (20 nM). The mixture was incubated at 37°C for 7 min before the addition of 50 µL of AAPH. Fluorescence was monitored using 485 nm excitation and 530 nm emissions at 1 min intervals for 40 min. Trolox was used to generate a standard curve. The antioxidant capacities of extracts were expressed as micromoles of Trolox equivalents (TE) per gram of fresh grape skins (µmol of TE/g).

DPPH Assay

The DPPH scavenging activities of samples were measured using a previously published method [75]. In summary, DPPH stock solution was prepared by dissolving 20 mg of DPPH in 100 mL methanol and stored at -20°C prior to use. DPPH working solution was freshly prepared by mixing 2.8 mL DPPH stock solution and 7.2 mL methanol. Absorbance at 515 nm was measured on a microplate reader (SPECTRAmax 190, Molecular Devices, Sunnyvale, CA). Diluted extracts (50 µL) were added to 950 µL DPPH working solution and incubated for 60 min in the dark at room temperature. Trolox solutions from 100 to 1000 µM were added to DPPH working solution as standards. Results of the DPPH scavenging activity of grape skin extracts were expressed as micromoles of Trolox equivalents (TE) per gram of fresh grape skins (µmol of TE/g).

Statistical Analysis

Three-way analyses of variance (ANOVA) with Tukey-HSD pairwise comparison of the means were performed using Sigmaplot (version 11.0, Systat software Inc., Chicago, IL). Data are expressed as means ± the standard deviation of three independent observations. A p value ≤ 0.05 is considered significant. A summary of the statistical results is shown in Table 6-1.

Results and Discussion

CO_2 and O_2 Analysis

Table 6-2 shows the headspace CO_2 and O_2 concentration in the sealed containers after MeJA treatment of Noble and Alachua grapes. In Noble variety the % of CO_2 and O_2 were not affected by the MeJA treatment, however decrease in headspace O_2 and increase in CO_2 levels were observed in the MeJA treated Alachua grapes

compared to control. This implies that rate of respiration was higher in MeJA treated Alachua grapes and modified atmosphere was created in the containers.

Decay Assessment

There was no effect of the MeJA treatment on grape decay compared to control (Table 6-3). However, temperature had a significant effect ($p<0.05$) on decay with more number of grapes decayed at 20°C compared to 5°C. Similar effect of temperature was observed in strawberries [130].

pH and Total Soluble Solids

The effect of MeJA on pH and total soluble solids of the treated and control juice is shown in Table 6-4. pH of the juice from MeJA treated Noble and Alachua was significantly lower ($p<0.001$) compared to control at all the sampling times and storage temperatures (5 or 20°C). This indicates the amount of organic acids in the MeJA treated grapes were higher than control. The effect was more pronounced at 100 µmol/L MeJA concentration. A number of studies showed increase in organic acid content of MeJA treated fruits [131-132]. There was no effect of storage temperature on pH of the juice from control and MeJA treated grapes. Total soluble solids (TSS) were significantly higher ($p<0.001$) in juice from MeJA treated Alachua; however no effect of the treatment was observed in juice from Noble variety. A study conducted on kiwifruit treated with MeJA showed increase in TSS [133] while another study on raspberry showed no effect of MeJA on pH [115]. A significant decrease in TSS of juice from control and MeJA treated Alachua and Noble grapes was observed with sampling time [115, 130].

Total Anthocyanin Content

There was no significant effect of the treatment on total anthocyanin content of Noble and Alachua (Tables 6-5 and 6-6). The anthocyanin content was affected by

storage time and temperature in both the varieties. In case of Noble grapes, the anthocyanin content increased 5 and 8% in control and 100 µmol/L MeJA treated samples, respectively after 7 days of storage at 5°C. For the same storage time the anthocyanin content at 20°C was increased by 26 and 33% in control and 100 µmol/L MeJA treated samples, respectively. Similar effect of storage time and temperature was observed in Alachua variety. The increase in anthocynain content after storage at 5 or 20°C might be due to ripening related changes going on during storage. In addition, the moisture loss and sugar metabolism during storage can also contribute to increase in anthocyanin content. The continuation of anthocyanin synthesis during postharvest storage has been reported in strawberries, raspberries, highbush blueberries [134] and grapes [135].

Total Phenolic Content and Antioxidant Capacities

There was no significant difference in the phenolic content or antioxidant capacity (measured by ORAC or DPPH) in control and MeJA treated samples of both the varieties (Tables 6-5 ad 6-6). A significant increase in the phenolic content was observed in both varieties with storage time and temperature. The results were consistent with total anthocyanin content. Such an increase could be due to release of phenolic compounds from their complexes with other components like proteins and carbohydrates. Both storage time and temperature significantly increased the antioxidant capacity (ORAC and DPPH) in control and MeJA treated Alachua grapes. However, only storage time affected the antioxidant capacity measured by ORAC in control or MeJA treated Noble grapes and there was no effect of storage time and temperature on the antioxidant capacity measured by DPPH assay. The inconsistency

in the antioxidant capacity results could be attributed to varietal differences or differences in chemistry and mechanisms involved in ORAC and DPPH assays.

Summary

Postharvest MeJA treatment did not affect the total anthocyanins, phenolic content and antioxidant capacity of muscadine grapes. However, there was a significant effect of storage time and temperature on the measured quality parameters. These observations contradicted other studies in which MeJA enhanced the phenolic compounds, anthocyanins, antioxidant capacity, quality and postharvest life of Chinese bayberries [129] and raspberries [115]. However, a study conducted on strawberries showed no effect of postharvest MeJA on flavonol content [136]. Several aspects such as different fruits, cultivars, and influence of other growth regulators such as ethylene and salicylic acid might affect the results. When applied on various fruits, MeJA stimulates the production of phenylalanine ammonia-lyase, a key enzyme of the phenylpropanoid pathway, which is directly involved in the biosynthesis of phenolic compounds, including anthocyanins, stilbenes, and flavonoids [137]. The major effect of MeJA treatment on muscadine grapes appeared to be on pH and TSS which is in agreement with previous studies [131, 133]. In conclusion, we saw no impact of MeJA treatment on decay, anthocyanins, phenolic content and antioxidant capacity of muscadine grapes.

Table 6-1. ANOVA for dependent variables for MeJA treatment, storage time, temperature and their interactions for Noble and Alachua grapes.

Variety	Tests	Time	Temperature	Treatment	Time×Temperature[#]
Noble	pH	*	NS	*	NS
	TSS	*	NS	NS	*
	Total anthocyanins	*	*	NS	*
	Total phenolics	*	*	NS	*
	ORAC	*	NS	NS	*
	DPPH	NS	NS	NS	NS
Alachua	pH	*	*	*	NS
	TSS	*	*	*	NS
	Total anthocyanins	*	*	NS	NS
	Total phenolics	*	*	NS	NS
	ORAC	*	*	NS	*
	DPPH	*	*	NS	NS

* represent significance and NS represents non-significance at $p \leq 0.05$. [#] Interactions with significant results are shown.

Table 6-2. CO_2 and O_2 levels in the headspace of the containers after MeJA treatment of Noble and Alachua grapes.

Variety	MeJA concentration (µmol/L)	% Oxygen	% Carbon dioxide
Noble	0	13.00 ± 0.75[a]	7.33 ± 0.50[a]
	10	12.70 ± 0.30[a]	7.57 ± 0.21[a]
	100	13.10 ± 0.14[a]	7.30 ± 0.14[a]
Alachua	0	14.87 ± 0.59[a]	6.57 ± 0.64[b]
	10	12.93 ± 0.38[b]	8.73 ± 0.40[a]
	100	12.90 ± 0.40[b]	8.73 ± 0.57[a]

Results are mean ± standard deviation of three determinations. Different superscripts for each variety indicate the significant differences in the mean at $p \leq 0.05$.

Table 6-3. Effect of MeJA treatment on decay of Noble and Alachua grapes after 2 weeks of storage at 5 and 20°C.

Cultivar	Storage Temperature (°C)	MeJA concentration (µmol/L)	% grape decay
Noble	5	0	2.67 ± 1.15
		10	2.00 ± 2.00
		100	2.67 ± 1.15
	20	0	14.67 ± 1.15
		10	10.67 ± 1.15
		100	13.33 ± 3.06
Alachua	5	0	1.67 ± 0.58
		10	4.00 ± 2.65
		100	5.00 ± 2.00
	20	0	22.00 ± 3.46
		10	20.33 ± 5.86
		100	25.67 ± 5.03

Table 6-4. pH and total soluble solids (°Brix) of Noble and Alachua juice from control and MeJA treated muscadine grapes.

Cultivar	Storage Temperature (°C)	Days of Storage	MeJA concentration (µmol/L)	pH	Total soluble solids (°Brix)
Noble	5	3	0	3.24 ± 0.04	11.20 ± 0.52
			10	3.24 ± 0.01	11.33 ± 0.23
			100	3.23 ± 0.03	11.73 ± 0.23
		5	0	3.27 ± 0.03	11.53 ± 0.55
			10	3.27 ± 0.02	11.67 ± 0.25
			100	3.22 ± 0.02	11.53 ± 0.32
		7	0	3.60 ± 0.02	11.70 ± 0.17
			10	3.54 ± 0.02	11.27 ± 0.49
			100	3.50 ± 0.04	11.13 ± 0.06
	20	3	0	3.26 ± 0.04	12.07 ± 0.21
			10	3.26 ± 0.02	11.83 ± 0.68
			100	3.19 ± 0.08	11.93 ± 0.29
		5	0	3.24 ± 0.02	11.27 ± 0.32
			10	3.25 ± 0.04	11.47 ± 0.25
			100	3.28 ± 0.05	11.75 ± 0.07
		7	0	3.53 ± 0.02	11.33 ± 0.21
			10	3.55 ± 0.06	11.40 ± 0.36
			100	3.51 ± 0.03	11.23 ± 0.12
Alachua	5	3	0	3.55 ± 0.03	15.13 ± 0.42
			10	3.59 ± 0.06	15.72 ± 0.47
			100	3.51 ± 0.03	15.57 ± 0.21
		5	0	3.62 ± 0.08	15.53 ± 0.67
			10	3.65 ± 0.03	16.10 ± 0.75
			100	3.59 ± 0.04	15.90 ± 0.26
		7	0	3.49 ± 0.05	14.70 ± 0.53
			10	3.54 ± 0.04	15.53 ± 0.23
			100	3.42 ± 0.05	15.03 ± 0.40
	20	3	0	3.55 ± 0.07	14.70 ± 0.36
			10	3.55 ± 0.05	15.20 ± 0.44
			100	3.57 ± 0.06	15.73 ± 0.58
		5	0	3.58 ± 0.02	15.23 ± 0.50
			10	3.58 ± 0.03	15.43 ± 0.32
			100	3.49 ± 0.06	15.53 ± 0.23
		7	0	3.49 ± 0.03	15.07 ± 0.21
			10	3.50 ± 0.05	15.30 ± 0.44
			100	3.39 ± 0.03	14.50 ± 0.14

Table 6-5. Total anthocyanin, total phenolic and antioxidant capacities (ORAC and DPPH) of control and MeJA treated Noble grape skins.

Storage Temp (°C)	Days of Storage	MeJA concentration (µmol/L)	Total anthocyanin content (mg/g)	Total phenolic content (mg/g)	DPPH (TE µmol/g)	ORAC (TE µmol/g)
5	3	0	1.99 ± 0.28	3.73 ± 0.09	21.60 ± 1.24	53.90 ± 6.20
		10	2.01 ± 0.31	3.73 ± 0.53	20.69 ± 2.93	47.87 ± 9.33
		100	1.93 ± 0.21	3.76 ± 0.32	20.80 ± 3.19	53.02 ± 3.79
	5	0	2.13 ± 0.05	3.68 ± 0.08	21.84 ± 1.65	62.91 ± 7.31
		10	2.14 ± 0.26	3.37 ± 0.20	20.28 ± 1.72	52.30 ± 9.72
		100	1.97 ± 0.34	3.60 ± 0.58	20.79 ± 2.48	52.14 ± 5.06
	7	0	2.09 ± 0.13	3.76 ± 0.12	19.99 ± 3.41	43.52 ± 4.50
		10	2.20 ± 0.38	4.09 ± 0.61	22.33 ± 1.18	57.88 ± 5.11
		100	2.10 ± 0.02	3.84 ± 0.28	21.03 ± 2.25	44.74 ± 3.40
20	3	0	2.14 ± 0.04	3.72 ± 0.09	20.75 ± 1.21	50.46 ± 4.08
		10	2.12 ± 0.27	3.51 ± 0.18	20.03 ± 2.00	52.81 ± 4.25
		100	2.04 ± 0.24	3.48 ± 0.17	19.34 ± 2.14	47.50 ± 5.78
	5	0	2.54 ± 0.16	4.16 ± 0.26	23.58 ± 1.12	63.06 ± 3.58
		10	2.48 ± 0.22	4.01 ± 0.33	23.08 ± 1.38	51.60 ± 3.40
		100	2.20 ± 0.42	3.71 ± 0.19	21.49 ± 3.04	53.67 ± 9.90
	7	0	2.91 ± 0.57	4.42 ± 0.67	23.72 ± 2.11	63.11 ± 5.05
		10	2.77 ± 0.16	4.25 ± 0.06	22.33 ± 2.86	48.73 ± 5.06
		100	3.03 ± 0.13	4.68 ± 0.04	24.79 ± 1.85	61.48 ± 2.65

Table 6-6. Total anthocyanin, total phenolic and antioxidant capacities (ORAC and DPPH) of control and MeJA treated Alachua grape skins.

Storage Temp (°C)	Days of Storage	MeJA concentration (µmol/L)	Total anthocyanin content (mg/g)	Total phenolic content (mg/g)	DPPH (TE µmol/g)	ORAC (TE µmol/g)
5	3	0	2.70 ± 0.57	3.73 ± 0.20	19.05 ± 4.77	67.70 ± 4.60
		10	2.75 ± 0.87	3.42 ± 0.84	18.79 ± 2.23	53.06 ± 8.44
		100	2.41 ± 0.92	3.56 ± 1.10	18.62 ± 5.90	68.08 ± 10.24
	5	0	3.83 ± 0.17	6.42 ± 0.50	22.53 ± 1.18	71.65 ± 5.90
		10	3.70 ± 0.35	5.27 ± 0.24	22.54 ± 0.79	64.63 ± 4.85
		100	3.21 ± 0.35	4.81 ± 0.37	20.14 ± 0.26	65.43 ± 5.35
	7	0	3.36 ± 0.75	4.36 ± 0.83	22.92 ± 2.76	55.41 ± 3.12
		10	3.74 ± 0.27	5.22 ± 0.81	23.41 ± 4.10	64.11 ± 2.38
		100	2.82 ± 0.70	4.02 ± 0.79	19.80 ± 4.77	50.86 ± 7.68
20	3	0	3.22 ± 0.23	4.02 ± 0.16	22.16 ± 3.97	64.02 ± 2.35
		10	2.81 ± 0.14	4.10 ± 0.54	22.47 ± 1.75	73.86 ± 11.34
		100	2.70 ± 0.46	3.57 ± 0.18	20.08 ± 2.55	55.57 ± 2.41
	5	0	3.63 ± 0.10	5.98 ± 1.22	24.40 ± 0.56	49.68 ± 2.37
		10	4.09 ± 0.70	5.88 ± 0.33	23.06 ± 2.20	71.13 ± 7.81
		100	3.95 ± 0.66	5.49 ± 0.43	23.94 ± 0.61	83.37 ± 13.91
	7	0	3.72 ± 0.27	5.48 ± 0.03	22.93 ± 2.81	67.78 ± 4.57
		10	4.21 ± 0.44	5.49 ± 0.36	26.09 ± 3.20	42.59 ± 1.17
		100	3.78 ± 0.52	5.99 ± 0.52	25.05 ± 3.22	59.93 ± 14.60

CHAPTER 7
CONCLUSIONS

Muscadine seeds have high phenolic content and antioxidant capacity compared to skin and pulp. A total of 88 phenolic compounds of diverse structures were tentatively identified in Noble variety, including 17 compounds identified for the first time in muscadine grapes. The high antioxidant capacity and total phenolic content of the muscadine seeds make them a potentially significant source of compounds with nutraceutical properties. The structural elucidation of phenolic compounds in muscadine grapes could provide an improved understanding of color and flavor changes occurring in muscadine wine and juice upon storage.

ABA does not affect grape berry weight, pH or total soluble solids (°Brix) of juice. Exogenous ABA enhanced the antioxidant capacity, anthocyanins and phenolic content of muscadine grapes, but these effects varied depending upon the cultivar and possibly environmental factors. Further research may show that ABA applied at critical stages of grape development offer opportunities to increase the content of key phytochemicals without affecting the yield. The grapes with enhanced phytochemicals could attract health conscious consumers and also increase the marketability of fresh fruits.

We found hot water to be the most suitable solvent for the extraction of anthocyanins from muscadine pomace. Organic solvents worked best for the extraction of ellagic acid and flavonols. The advantages of using water as a solvent outweigh the use of organic solvents as it is non toxic and inexpensive. In addition, water fits better for the industry scale because it significantly reduces the overall production cost.

Results from resin adsorption/desorption study indicate that FPX-66 is the most suitable resin among selected commercial adsorbents for the recovery of anthocyanins

from muscadine juice pomace. Resin adsorption caused a tremendous increase in the content of anthocyanins, ellagic acid, and flavonols in the concentrated extract. Using FPX-66 resin, a concentrated pomace extract was produced that contained 13% (w/w) anthocyanins with no detectable sugars. The optimization of the resin adsorption process in the present study sets parameters for the development of pilot-scale separation and concentration of anthocyanins from muscadine pomace. This extract could potentially find application as a natural colorant, dietary supplement, antioxidant ingredient for functional foods, and/or as a raw material in cosmetic and pharmaceutical industry preparations.

Postharvest MeJA treatment did not affect the total anthocyanins, phenolic content and antioxidant capacity of muscadine grapes. However, significant effects of time and temperature of storage on measured quality parameters were observed. Contrary to the ABA study, the major effect of MeJA treatment appeared to be on pH and total soluble solids.

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BIOGRAPHICAL SKETCH

Aman Sandhu was born in Punjab, India. She attended St. Francis School, Tarn Taran and Khalsa College, Amritsar for her high school and senior secondary education, respectively. She obtained her B. S. degree in food science from Khalsa College, Amritsar in 2003 and M. S. degree in food science and technology from the Guru Nanak Dev University, India in 2005. She subsequently worked in food industry as well as a college lecturer before joining the University of Florida. She joined the food science doctoral program at the University of Florida in the spring of 2009. She graduated in May 2013 with PhD in food science.