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Study of Chemical Profiles of Volatile and Non-Volatile Bioactive Compounds of Muscadine Grapes (Vitis rotundifolia) During Ripening Stages and Evaluation of Their In Vitro Biological Properties

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STUDY OF CHEMICAL PROFILES OF VOLATILE AND NON-VOLATILE BIOACTIVE COMPOUNDS OF MUSCADINE GRAPES (VITIS ROTUNDIFOLIA) DURING RIPENING STAGES AND EVALUATION OF THEIR IN VITRO BIOLOGICAL PROPERTIES

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In Partial Fulfillment
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by
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ABSTRACT

Muscadine grapes (*Vitis rotundifolia*) have attracted consumers because of their unique flavors and high concentrations of bioactive phytochemicals. The aim of this study was to investigate the effects of ripening stages (i.e., stage I, II, and III) of muscadine on physiochemical properties, volatile organic compounds (VOCs), and phenolic compounds in the grapes, and explore the enzymatic inhibitory effects of the muscadine extracts and some of its inherent phenolics on some important enzymes, such as angiotensin-I-converting enzyme (ACE), pancreatic lipase (PL), tyrosinase, collagenase and elastase, relevant to human chronic diseases.

Physiochemical properties of the muscadine grapes were evaluated in order to understand the biological changes of the grapes during the ripening progress. Significance differences (*p* ≤ 0.05) were observed in regards of the size, weight, pH, total soluble solid, sugar content and titrable acidity of the muscadine grapes during their 3 ripening stages. The berry size, berry weight, and titrable acidity decreased after the stage II, whereas °Brix, pH, sugar content continuously increased through the whole ripening stages up to the stage III.

Twenty eight VOCs were detected by headspace solid phase microextraction (HS-SPME) coupled to a gas chromatography-mass spectrometry (GC-MS). The grapes in the stages I and II had relatively similar flavor patterns, which were different from that in stage III. The principal component analysis (PCA) indicated that butyl-2-butenoate, hexyl acetate, propyl acetate, ethyl *trans*-2-butenoate, hexyl-2-butenoate, ethyl acetate,
butyl acetate, 1-octanol, ethyl hexanoate, and β-citral were present as distinct volatile chemicals in the stage III, which usually result in the fruity, floral, and pleasant aromas.

Phenolic compounds (e.g., epicatechin, epicatechin gallate, resveratrol, and myricetin, etc) at three ripening stages were determined using a high-performance liquid chromatography coupled with diode array detector (HPLC-DAD). In addition, antioxidant activities of the muscadine extracts and identified major phenolics were determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2’-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS). Total phenolic content (TPC) and total flavonoid content (TFC) decreased along with the ripening stages, while the antioxidant activities were enhanced through the ripening stages. With regard to the phenolic compounds, the concentrations of phenolic compounds were significantly different in three ripening stages. Most bioactive phenolic compounds, except myricetin and quercetin, consistently increased along with the ripening stages.

The study of enzymatic inhibitory activities of ACE, pancreatic lipase, tyrosinase, collagenase and elastase demonstrated that the muscadine extracts, regardless of their ripening stages, and the selected phenolics (i.e., epicatechin, epicatechin gallate, resveratrol, and myricetin, etc) exerted their enzymatic inhibitions in a competitive inhibition model against all the aforementioned enzymes. The results indicated that the muscadine grapes possessed the strong anti-hypertension, anti-obesity and anti-skin disease activities.

Furthermore, it was found that the extraction of anthocyanin was the most efficient at pH 3.0, which accompanied with a greater antioxidant activity. To quantify
anthocyanidins in the muscadine grapes, the extracted anthocyanins were hydrolyzed under acidic conditions that were optimized at 100 °C for 60 minutes. The samples extracted from muscadine grapes at pH 3 and pH 5 showed similar anthocyanidin profiles with high concentrations of delphinidin and cyanidin, which are relatively unstable chemicals that are unfavorable for making muscadine juice or wine.
DEDICATION

I would like to dedicate this work to my family for their encouragement to me to pursue my dreams, and their trust and confidence on me to achieve this goal. I also express my sincere appreciation to my grandparents, parents, parents-in-law, and my brother for their love, support, and encouragement, because they made it possible for me to become who I am today. Especially, to my parents, Won-suk Lee and Min-ju Kim, I really appreciate all your love and support to provide me an opportunity for an excellent education and privileged life in US.

Lastly, my deepest appreciation goes to my husband, Dr. Dong Seong Cho, who always stand beside me to offer a tons of helping me up through the most challenging times in my life. This journey would not have been possible without his unconditional love and endless support.
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CHAPTER ONE

LITERATURE REVIEW

Introduction

1.1 Muscadine grapes (*Vitis rotundifolia*)

Muscadine grapes (*Vitis rotundifolia*) are the first native grape to North America and they have been cultivated for over 400 years. They are commonly distributed in the southeastern United States, but unaware to people who live outside of the southeastern United States. Muscadine grapes are adapted to be grown in warm, humid climates and well-drained soil, and are found from Delaware to Central Florida, the Atlantic Ocean to East Texas, and along the Mississippi River to Missouri (Barchenger, et al. 1, Anderson, et al. 2).

All the grapes in specific genus have been divided into two subgenera: *Euvitis* (the European, *Vitis vinifera* L. grapes) and *Muscadinia* (muscadine grapes). Muscadine grapes have their own distinct characteristics compared with the other *Vitis* species, such as European (*Vitis vinifera*) and American grapes (*Vitis labrusca*) (Milholland 3).

Muscadine grapes are regional fruits in the Southeastern United States, which can be grown up to 100 feet in wild vines within a long growing season, but have a short harvest season (4-6 weeks) from August to September. Unlike the other grapes, muscadine berries have a large berry and measured sizes in 1 – 1.5 inches in diameter. The clusters of berries (6 to 24 berries) have thick skin, oblong seed and tough skins that are shown in either light (bronze) or dark (purple to black) skinned color. The bronze-
fruited vines have been of more interest because of their better stability in processing while they are made into wine (Olien and Hegwood, Vashisth, et al., Pastrana-Bonilla, et al.).

Commercial grape products are classified into table grapes, wine grapes, raisin grapes and canning grapes. Similarly, muscadine grapes are eaten as table grapes and processed into wines, juices, jams, jellies, or exist in “U-pick” operations and some functional products that are special food products in the Southeastern United States. (Greenspan, et al.)

Although muscadine is a highly perishable fruit, recently, it has attracted more attention from consumers and researchers because of its inherent bioactive phytochemicals in light of its bioactivities, such as higher antioxidant capacity compared to that of other grapes and fruits, and its noticeable source of several type of bioactive phytochemicals contributing significantly in prevention of human disease, as well as its unique flavor and aroma. Bioactive phytochemicals, often called nutraceuticals, are extracted from the natural products and served as a valuable source to reduce the risks of human chronic diseases. Many natural products, such as fruits and vegetables, have many health benefits such as prevention of heart disease, metabolic disorders, aging, and cancer, etc. Many studies have reported that muscadine grapes have a high concentration of phenolic compounds, which include gallic acid, myricetin, quercetin, kaempferol and resveratrol (Pastrana-Bonilla, et al.).

A worldwide life expectancy has significantly increased in the 21st century, but people are also facing greater risks from chronic diseases such as aging, heart disease,
cancers, etc. In the United States, heart disease and cancer have been ranked the first and second highest mortality rate in 2007 (Jemal, et al. 8), which have prompted scientists and food manufacturers to explore more natural and healthy foods to consumers.

Muscadine grapes are composed of excellent source of dietary fibers, amino acids, minerals, vitamins and high nutritional values and health-promoting phytochemicals (Threlfall, et al. 9). One of the remarkable health benefits of muscadine grapes relies on its high antioxidant capacity, which can prevent destructive oxidation by scavenging biological “free radicals” (Talcott and Lee 10). In the early 1990s, researchers found that one of the major phytochemicals in grapes and wines, (i.e., trans-resveratrol), was shown to act as a strong antioxidant that had many health-promoting effects. Although many other grapes and grape products have a high concentration of resveratrol (Singh, et al. 11), the muscadine grapes have 40 times more resveratrol than the other grapes (Lekli, et al. 12). In addition, a number of other bioactive phytochemicals in muscadine grapes were reported to contribute to antioxidant capacities, block cancer cell from attacking organs, prevent diabetes, etc. Also, it has been reported with an ability to resist Pierce’s bacterial disease (James, et al. 13).

Characterization of volatile compounds

1.2.1 VOCs in muscadine grapes

Food flavor is an important character of many foods and an indicator for consumers to evaluate the food quality. For example, acceptance of a wine heavily relies on its aroma. Food flavors and aromas are typically composed of over thousands of volatile organic compounds (VOCs), which are usually classified into different chemical
groups, such as terpenes, norisoprenoids, carbonyl compounds, esters, alcohols and methoxypyrazines (Williams and Allen 14). However, food flavors normally exist in trace amounts, of which an accurate determination is a big challenge in concern of polarity of solvent, extraction method, sensitivity of instrument, etc (Noguerol-Pato, et al. 15). Muscadine aromas are formed depending on many factors, such as the grape variety, cultivation, and climatic or biological factors (Sánchez-Palomo, et al. 16). Like many other fruits, the volatile chemicals in grapes are accumulated during ripening stages. Particularly, those aromas are characterized by esters and terpenes relevant to the maturity of fruits. However, changes of VOCs of muscadine grapes along with their ripening stages have not been systematically investigated, although a few studies have been conducted about the muscadine flavors and the relationship of its taste to the contents of sugars, acids, and phenolic compounds (Marshall-Shaw, et al. 17, Baek, et al. 18, Breman, et al. 19).

There are three main ripening stages in grapes, refereed as stage I, stage II and stage III that are shown in Figure 1.1. The stage I (lag phase) refers to the beginning period of rapid cell division after fruit-set, which is often signaled by little berry growth with green colors before entry of stage II. After the lag phase, the grapes are moved into stage II which leads to a period with some significant physiochemical changes when the color of grapes changes to pink with little soft textures. The last stage (i.e., stage III) of grape development is related to its textural change into a very softening texture, which is an important stage characterized by a remarkable accumulation of anthocyanins, fructose and glucose. Although there were some studies regarding to the characterization of VOCs
in stage III, they only gave limited and/or indirect information about the aroma precursors and the VOCs synthesized or sequestered prior to this stage (Kalua and Boss 20, Mamede and Pastore 21). Moreover, it is worthy to mention that the changes of VOCs during the ripening stages may be influenced by temperature, water availability and varieties (Palomo, et al. 22).

The aromatic profiles of the muscadine grapes are formed by a complex mixture of VOCs. These VOCs are dependent on muscadine grape species, the geographical locations, climates and harvest conditions. Kepner and Webb 23 initiated the study of constituents of VOCs in muscadine grapes in 1956. Those pioneering researchers isolated VOCs using a reduced pressure pot distilling technique and flash evaporation technique, and identified the following VOCs including ethyl alcohol, n-butyl alcohol, an acetate ester, a laurate ester, an isopropyl ester, methyl alcohol, n-hexanal, 2-hexanal, and acetal. The major identified volatile groups included esters, alcohols and carbonyl compounds. One of the major flavors, (i.e 2-phenylethanol) with a rose-like order, was also identified in muscadine grapes. However, due to the lack of high sensitive detectors, it was difficult to identify the nitrogen, halogen and sulfur containing VOCs at that time.

According to a study by Welch, et al. 24, the VOCs from muscadine grapes were also investigated by gas chromatograph (GC) with flame ionization detector (FID), as well as mass spectrometer and retention index (RI) value. More than 40 VOCs were then identified from the muscadine grapes. The main identified VOCs from muscadine grapes were consistent to the results of Kepner and Webb, including butanol, hexanol, ethyl acetate, ethyl laurate, and 2-phenylethanol, but some of the VOCs including ethanol,
methanol, acetal, 1-hexanal, and 2-hexnal were not detected in this research (Kepner and Webb 23). Welch and co-authors believed that 2-phenylethanol, 3-methyl-1-butanol, hexanol and benzaldehyde were the volatile markers of muscadine grapes. Methyl anthranilate that is one of the nitrogen containing VOCs was not detected in muscadine grapes although it was an important aroma in *Vitis vinifera* (Welch, et al. 24). These VOCs are profiled in Figure 1.2.

1.2.2 Extraction methods for VOCs

Up to now, more than 800 VOCs have been identified from wines. Extraction efficiency of VOCs from grapes depends on many factors, such as chemical polarity, solubility, volatility, pH and concentration. In addition, many VOCs are unstable since they are easily oxidized from air or degraded by heat (Mamede and Pastore 21). Therefore, selection of a proper extraction method is an important and critical step for the subsequent isolation and characterization of the target VOCs in natural products. Therefore, whether to use a solvent extraction method or a solvent-free extraction method depends on the analytical purpose, properties of samples, target chemicals, extraction time, cost and recovery. Many extraction methods have been developed and introduced in order to extract food flavors. Common extraction methods include liquid-liquid extraction (LLE), Soxhlet extraction, solid-phase extraction (SPE), simultaneous distillation and extraction (SDE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE) and headspace solid phase microextraction (HS-SPME). Each method has its own advantages and some disadvantages (Castro, et al. 25). Among them, LLE is the most common and an easy-to-use technique for extracting flavors from samples. This
technique facilitates the separation of the compounds of interest by chemical distribution 
or partition between two immiscible liquid phases, (i.e., a polar phase and a non-polar 
phase), which have significantly different polarity. The more polar hydrophilic 
compounds tend to enter the aqueous phase or polar phase while the more non-polar 
hydrophobic compounds tend to enter the non-polar organic solvent. The most significant 
advantage of the LLE is that all the VOCs can be extracted regardless of their volatilities 
(e.g., the low, medium, and high volatilities), plus advantages in terms of wide selection 
of solvent, high repeatability, and use of a minimum solvent. On the other hand, its 
disadvantages include the time-consuming process, harmfulness to environment, and 
possible contamination of samples during the extraction step (Ivanova, et al. 26). 
Furthermore, the loss and thermal degradation of some of VOCs can be occurred during 
the solvent evaporation step, when it is also possible to form some new volatiles that are 
not in the original samples (Mamede and Pastore 21).

SDE is another popular method for extraction of VOCs. It has become one of the 
most widely used methods among the extraction techniques. It has been widely used and 
applied for extraction of oils, flavors, and some of the volatile products. The greatest 
advantage of SDE is its relatively simple procedure to combine the extraction and 
concentration steps as a one-step procedure, which makes it possible to save time, and 
reduce the solvent consumption since it has a continuous solvent recycling (Teixeira, et 
al. 27). Even though SDE has a low recovery of extraction of most volatile or heat-labile 
compounds, this methods has a higher recovery and repeatability specifically in semi-
volatile and heat-stable VOCs than the other extraction techniques, such as SPME and HS-SPME (Gu, et al. 28).

Despite the aforementioned advantages, the solvent-use extractions have imposed a remarkable environmental burden, which prompts researchers to use a more environmentally friendly extraction method. In addition, there are demands for more safe, rapid, reliable, accurate and less labor-consuming analytical methods in chemistry labs. In this context, the succeeding extraction methods have been developed and operated in one-step combining the isolation and pre-concentration steps in order to overcome the previous drawbacks of the methods which are indispensable to use solvent (Balasubramanian and Panigrahi 29). As a result, the solvent-free extraction technique was introduced as a more promising extraction method. For instance, SPME was developed as one type of the solvent-free extraction techniques. It combines the chemical extraction and concentration steps based on sorption (i.e absorption and/or adsorption, depending on the fiber coating), which is achieved either by submersion in a liquid phase or by exposure to a gaseous phase, or commonly called direct (DI)-SPME and HS-SPME, respectively (See Figure 1.3). The DI-SPME mode involves a coated fiber directly inserted into the liquid sample, where analytes are transferred from the sample matrix to the coated fiber. In order to enhance the extraction efficiency in the aqueous phase, certain auxiliary techniques and/or factors, such as stirring or sonication, vial types, fiber movement, etc., was consequently facilitated (Abdulra’uf, et al. 30). In comparison, the HS-SPME involves a single-step that combines the sample extraction and concentration by the fiber exposed in the headspace of sealed containers under heating. During the
period, there are two states of equilibrium in the headspace extraction. One state is present within the sample matrix and the gaseous phase above it, whereas the other state is present within the headspace and extraction fiber. After the dynamic equilibrium is reached between the concentration of analyte of the sample and the amount of analyte absorbed by the fiber based on the distribution coefficient, the fiber is directly introduced into the injector of GC for determination of VOCs. Being compared to the sample extraction methods, SPME is beneficial owing to its ease of use, economical and rapid extraction, and rapid pre-concentration.

When analyzing VOCs of the samples, it is also important to consider the following factors such as cost, convenience, time, hazardous chemicals, recovery, precision, and accuracy for high throughput analyses. SPME is a microextraction technique that not only uses a relatively small amount of organic solvent compared to the other extraction methods, it is also able to reduce the extraction time while providing a high sensitivity and better analytical reproducibility. As a result, it has been widely applied for a broad range of analyses regarding in food, biological, and environmental sciences (Rocha, et al. 31).

Along with an extensive use of solid-phase microextraction coupled with chromatographic techniques, which has become a common analytical method, the stationary phase (polyacrylate, polydimethyl siloxane, etc.) coated on fused silica fiber for chemical separation has been taken into more serious consideration in light of its significance on effective separation.
There are different types of fibers available in SPME regarding to their different polarities and sorption properties, including: PDMS (polydimethylsiloxane), PA (polyacrylate), PDMS/DVM (divinylbenzene), DVB/CAR (carboxen)/PDMS, CAR/PDMS and CBW (carboxax NiTi-ZrO2-nickel titanium-zirconium oxide)/DVB (Stashenko and Martinez 32). For the evaluation of fragrances and impurities in food products, a fiber with 100 μm thickness of PDMS is most regularly used. For the analysis of pesticides present in food matrices such as fruit, juice, honey and herbs, the following fiber has been employed, PDMS (100 μm), PA, PDMS-DVB. CAR-PDMS and DVB-CAR-PDMS fibers are useful for isolating sulphur compounds, particularly the principal compound which contributes to the strong cheese aroma, methanethiol (Wardencki, et al. 33, Tan and Abdulra'uf 34).

1.2.3 Gas chromatography mass spectrometry (GC-MS)

GC is commonly used to separate a mixture of VOCs into individual chemicals based on their different boiling points and partitioning properties between the carrier gas (mobile phase) and the column (stationary phase). GC often consists of the following components, including an inert mobile phase, an injector port, an oven embedded with a separation column which is considered a stationary phase, and a detector, of which the scheme is shown in Figure 1.4.

The mobile phase is a carrier gas that often adopts an inert gas such as helium, argon and nitrogen, with few exceptions of using hydrogen. Various detectors, including the top two most common detectors, i.e., FID and mass spectrometer (MS), are often connected to GC to detect the separated chemicals.
Similar to the GC-FID, GC-MS is a hyphenated instrumental technique which is composed of a GC and MS. It is an effective combination for separation, identification, and quantification of VOCs. Since the GC-MS appeared in the mid-1950s, it has become an essential tool of a chemical lab. It is mostly used for determining drugs and metabolites in the pharmaceutical area, and for volatile and semi-volatile organic compounds in foods, waters, soils, and other environmental samples. As a result, it has become the foundation of many official methods recognized by Environmental Protection Agency (EPA), Food and Drug Administration (FDA), and United States Department of Agriculture (USDA).

MS is a powerful analytical technique that is highlighted for its high sensitivity and specificity. It is able to characterize chemicals according to their mass to charge (m/s) ratio. A mass spectrometer is often composed of an ionization source, vacuum pumps, and a mass analyzer. When a chemical enters into the MS, the chemical is ionized and separated by a mass analyzer.

There are two common ionization modes available for GC-MS in most labs, i.e., electron ionization (EI) and chemical ionization (CI). The former, which is also called electron impact, is the oldest and the most commonly used ionization technique for MS. It is also a hard ionization process that involves in a relatively high energy than CI. In most cases, mass spectrometer uses an energy of 70 eV for EI, resulting in the gas phase neutral molecules broken down into ion fragments (Hoffmann 35).

EI technique works fine for various gas-phase molecules, but it does have some weakness. Even though the EI technique is recognized with great reproducibility,
sensitivity, and extensively used for construction of spectral libraries, i.e., mass spectra or “fingerprint” of molecules, EI stimulates extensive fragmentations so that the molecular ions may be weak or not present for many compounds (Hoffmann 35, Van Bramer 36).

The chemical ionization (CI) is also called a soft ionization that uses less amount of energy to produce a mass spectrum including a molecular ion with less fragments, of which the characteristic ionization of a chemical is produced by ionic reactions rather than the electron impact. The generated CI mass spectra will probably be of particular interest for determination of molecular ions (MI), or molecular weights (MW) of target chemicals. CI begins when a reagent gas such as methane, isobutene, or ammonia is interacted by electron impact, then reacts with analyte molecules to produces $M+H^+$ ions or $M–H^-$ ions from an ion-molecule reaction. Transferring of an electron, proton or other charged species within a reaction involves in the CI (Munson and Field 37, Hoffmann 35).

**Characterization of phenolic compounds**

1.3.1 Phenolic compounds in muscadine grapes (*Vitis rotundifolia*)

In recent years, there has been an increasing interest of bioactive phytochemicals that are naturally synthesized in plants, and have been reported with various health benefits for reduction of incidence of some chronic diseases such as cancer, diabetes, etc. In most cases, bioactive compounds, along with other chemicals such as antibiotics, mycotoxins, plant growth factors, food pigments, and aroma compounds, are secondary metabolites which are related with physiological and morphological importance in fruits, vegetables and others. (Martins, et al. 38).
Phenolic compounds are one of the secondary metabolite compounds present in plants, such as fruits and vegetables, that contain an aromatic ring bearing one or more hydroxyl (-OH) groups. These compounds that have various chemical structures include from simple phenolic compounds to complex high-molecular compounds. Various complex structures of phenolic compounds are often called “polyphenols”. Phenolic compounds are widely distributed in plants. Many of them are associated with nutritional values and sensory properties. Besides, they have been studied for their antioxidant and antimicrobial properties. Phenolic compounds possess various biological effects, serving as phytoalexins, antifeedants, attractants for pollinators, contributors to plant pigmentation and others (Ignat, et al. 39). More than 8,000 phenolic compounds have been identified from environment (Ignat, et al. 39, Strube, et al. 40), and generally classified into two groups based on their structures, which are flavonoids (anthocyanin, flavan-3-ols condensed tannins, and flavonols) and non-flavonoids that are derived from pentose phosphate, Shikimate, and phenylpropanoid pathways in plants (phenolic acid and stilbenes) (Vattem, et al.41, Ozcan, et al.42).

Particularly, over 4,000 natural phenolic compounds have been identified in blackberries, blueberries, grapes and other fruits. These chemicals are composed of substitution patterns on the fifteen carbons (C6-C3-C6) of the core structures. Flavonoids are the most abundant phenolic compounds in plants, which are the major phytochemicals that contribute to prevent plants against UV light, pathogens and fungal parasites. The major flavonoid groups are divided into the following classes, including flavonols, flavones, flavanones, flavanols, isoflavones, and anthocyanins. Anthocyanins
belong to a family of an extensive class of phenolic compounds that include polyhydroxy glycosides and 2-phenylbenzopyrylium salt’s polymethoxy derivatives (Bkowska-Barczak 41). Anthocyanins (the Greek *anthos* = flower and *kianos* = blue) are water-soluble pigments that possess a variety of colors such as red, purple or blue based on the environmental pH value. There is an increasing interest of anthocyanins due to their natural colors and health benefits such as anti-inflammatory and antioxidant capacities (You, et al. 42). Many studies have reported the antioxidant activities of anthocyanins, which play essential roles in preventing cancer, neuronal, and cardiovascular disease, and diabetes along with others. As a result, many reports have been presented in literature regarding the methodology for the separation and purification of anthocyanins in food and plants, and their effects on the plant stress (Castaneda-Ovando, et al. 43). The anthocyanidins are basic structures of anthocyanins. It is composed of an aromatic ring [A] bounded to a heterocyclic ring [C] that possesses an oxygen, which is also bounded by carbon-carbon bound to a third aromatic ring [B] (Castaneda-Ovando, et al. 43) which is shown in Figure 1.5. There are six anthocyanidins that are commonly available from plants: delphinidin, cyanidin, peonidin, petunidin, pelargonidin and malvidin. Even though they have potential applications in diverse areas such as food, pharmaceutical, and cosmetic industries, their applications are very limited due to their colorant instability which is easily influenced by pH, concentration, and storage condition such as temperature, light, oxygen, solvent, presence of enzymes, and metallic ions (Ignat, et al. 39).
Non-flavonoid phenolic acid is another important chemical class since it accounts for one-third of the dietary phenols, which are abundant in natural existence as free and bound forms in plants. Phenolic compounds can be divided into two subgroups, the hydroxybenzoic acid (C$_1$-C$_6$) and hydroxycinnimic acid (C$_3$-C$_6$). The former includes gallic, $\rho$-hydroxybenzoic, protocatechuic, vanillic and syringic acids, while the latter include caffeic, ferulic $\rho$-coumaric and sinapic acids. Stilbens are associated in non-flavonoids compounds that are presented by 1,2-diphenylethylene nucleus with hydroxyl group substituted on the aromatic rings. One of the major stilbene, resveratrol, has attracted extensive attention because of its biological properties such as anti-carcinogenic and antitumor activities. Stilbenes can be often extracted from dietary sources such as grapes, wine, soy, and peanuts (Han, et al. 44, Delmas, et al. 45).

1.3.2 Extraction methods for phenolic compounds

The sample extraction is an important step to transform analytes of interest to a suitable sample for further separation and characterization. An ideal extraction method should include minimal steps of procedures that, as far as possible, eliminate oxidation and enzymatic reactions, reduce additional sample clean-up steps, minimize using hazardous solvents and reagents, raise a degree of environmental safety, increase a high-throughput capacity, efficiency and selectivity. There are a number of different extraction methods that can efficiently extract and isolate phenolic compounds. The main purposes of extraction and clean-up steps are to remove all the impurities that may interfere with the compounds of interest detection and help to increase the extraction efficiency. However, it is still a challenge to develop and optimize a universal extraction method.
which is impacted by the different chemical properties of the extracts. The most
commonly used method for extraction of phenolic compounds from samples relies on
solvent (Ignat, et al. 39; Armenta, et al. 46), frequently with aids of sample grinding,
vacuum drying and/or lyophilization. LLE is a widely used method to separate and isolate
an organic compound from a mixture based on their relative solubility and density in
mixture with an immiscible solvent such as water and an organic non-polar solvent. It is a
transfer of a solute substance from one liquid phase to another liquid phase according to
their solubility. This extraction method is suitable for sample clean-up step to remove the
unwanted compounds such as chlorophyll and lipids and help to extract the compounds in
a narrower polarity bands.

In order to extract simple phenolic compounds (e.g., benzoic acid, benzoic
aldehydes, cinnamic acids, and catechins) from sample matrix, maceration of the sample
by using organic solvents has been often suggested. Nowadays, LLE is an official
analytical method for extracting phenolic compounds from liquid samples (Garcia-Salas,
et al. 47). However, this technique often needs high cost and dangerous organic solvents
which are unsafe for health, and burdens on environment. SPE is a method combining the
sample extraction and concentration steps. SPE remains analytes on a stationary phase
(sorbent) before they are eluted or desorbed. In contrast with LLE, SPE has several
advantages in terms of its reproducibility, efficiency, accuracy, and cost effectiveness
over LLE. Beside, SPE has a short extraction time. In this context, SPE has been widely
used to extract analyte from the sample. SPE is useful when the interest component in a
sample has a low concentration that is not enough or difficult to be detected by a detector
because it can help concentrate the component to facilitate its detection, and help to remove excess contaminants to minimize the analytical interference, and improve the detective resolution. Four types of SPE are often available, including the reverse phase (RP), normal phase (NP), ion exchange (IE), and adsorption. To extract phenolic compounds, C_{18} (non-polar) SPE is often recommended to be used in an effort to remove sugar, amino acid, organic acids, and some other undesirable chemicals. Phenolic compounds are then eluted with recommended acidified polar solvents (García-Salas, et al. 47, Rodríguez, et al. 48). There are five steps in the SPE experimental procedures, including: (1) activation of the SPE sorbent using a suitable solvent; (2) equilibration using a liquid similar in composition to the sample matrix; (3) sample loading to allow the sorbent to preserve the analytes; (4) washing step to remove the undesirable analytes using a solvent which does not get rid of the interested analytes; (5) using a suitable solvent for the elution of the analytes and keep them for later analysis (Berrueta, et al. 49).

1.3.3 Analytical method – High pressure (performance) liquid chromatography (HPLC)

In biochemistry and analytical chemistry, one of the most powerful analytical tools is HPLC that are frequently used to identify and quantify chemical compounds. HPLC is an efficient analytical technique that has widely been used in measurements of pharmaceuticals, biomolecules and foods. HPLC consists of a column with packing material, a pump to help transport chemical components through the column to the detector, and a detector that identifies each single peak of chemicals (Abia, et al. 50). There are four different types of HPLC columns which are NP, RP, IE, and size-exclusion (SE) columns. The RP-HPLC, which consists of a non-polar stationary phase
and polar solvent, is often used in analyses of natural products and accounts for the
majority of analyses of HPLC. The stationary phases are nonpolar because the backbone
solid particles, such as silica, are often coated with hydrophobic C18 or C8 functional
groups. With an appropriate selection of a HPLC column, various classes of phenolic
compounds can be separated and identified according to their polarity. For example, a
common NP or RP column has 10-30 cm in length, 3.9-4.6 mm ID, and 2.5-10 μm of
particle size. In addition, during the HPLC quantification of phenolics, acetonitrile,
methanol, or their aqueous forms are the dominant mobile phases, and sometimes,
ethanol, tetrahydrofuran (THF), and 2-propanols are also used for phenolic compounds.
Moreover, extra consideration of maintaining the mobile phase at pH 2-4 is suggested so
as to prevent ionization of phenolic compounds during chemical separation and
identification. In this context, acetic acid is often used to acidify the mobile phase.
Formic acid, phosphoric acid or phosphate, citrate, and ammonium acetate buffers are
commonly used to fit the different pH range. On the other hand, as compared to an
isocratic elution system, a gradient elution system is more frequently used to satisfy
various analytical requirements, particularly in regards to analytical resolution and
retention time (Khoddami, et al. 51).

Phenolic acids and anthocyanins have their own characteristics of absorbance. For
instance, hydroxybenzoic acids often have their maximal absorbance wavelength at
around 280 nm, hydroxycinnamic acids at around 320 nm and anthocyanins at around
520 nm. Therefore, these phenolic acids are often detected by ultra-violet (UV) and/or
diode array detectors (DAD).
The most routine detectors for analyses of phenolic compounds include UV-vis, photodiode array (PAD, or DAD), MS, electrochemical (ED), fluorescence (FD), chemiluminescence (CL), refractive index (RI), and evaporative light scattering (ELSD) detectors. Nevertheless, some detectors have less analytical applications owing to their inherent disadvantages in terms of baseline drift, intervention caused by the increased use of certain derivation reagents, or by special restrictions, such as electrochemically inactive compounds (ED), needing complex pretreatment of non-fluorescent analytes (FD), less available chemiluminescence reactions, lack of compatibility of mobile phase with chemiluminescence reactions (CL), and low sensitivity (RI, ELSD). The UV detector and DAD are the two most commonly used detectors. The DAD detector has the ability to scan samples at multiple wavelengths at the same time and offer the information of the spectral features of each identified chemicals, although its sensitivity is less than that of UV detector (Zhang, et al. 52).

MS is a powerful and universal analytical tool that has not only high sensitivity but also specificity for identifying and characterizing chemicals based on their mass spectra, although it is more expensive than other aforementioned detectors. It can provide information regarding to the both qualitative (structure) and quantitative (molecular mass or concentration) analyses of the analytes after their conversion to ions. Initially, when chemicals are introduced into the ionization source of the mass spectrometer, they are firstly ionized to achieve positive and negative charges. Then, based on their mass/charge \((m/z)\) ratio, the ions go through the mass analyzer via different routes or arrive at different times (Ho, et al. 53). HPLC can connect different types of MS, such as MS\(^n\) (e.g., triple
quadrupole) and ion trap MS, or MS in different ionization techniques, including
electrospray ionization (ESI) and atmospheric chemical ionization (APCI), and negative
or positive ion mode (Xia, et al. 54, Sancho, et al. 55). Over the last several decades,
electrospray ionization mass spectrometry (ESI-MS) has been introduced as a significant
technique in analytical chemistry laboratories for study of samples in microliter volumes,
non-volatile, and thermally labile biomolecules that are not desirable for investigation by
other traditional techniques. Due to its remarkable analytical advantages, HPLC/ESI-MS
has emerged as a very influential technique coupled with a HPLC for analyzing small and
large molecules of a variety of complex biological samples.

Transformation of ions from solution into the gaseous phase prior to being
subjected to mass spectrometer is performed by electrical energy in ESI. Therefore, by
using ESI-MS, ionic species in solution can be analyzed with increased sensitivity. From
the process of protonation or cationisation, a neutral compound can be also transformed
to an ionic form in solution or in gaseous phase so that it can be analyzed by ESI-ES as
well. There are three steps for transferring of ionic species from solution into the gas
phase: firstly, scattering of a fine spray of charge droplets; then evaporation of the
solvent; lastly, highly charged droplets ion being injected (Figure 1.6) (Ho, et al. 53).

**Antioxidant assay**

The effect of diet on human health has attracted more and more attention in recent
decades. There has been evidence supported by epidemiological studies that decreased
incidence of chronic diseases, such as coronary heart disease, atherosclerosis, cancer and
aging, is linked with an increased ingestion of phytochemicals from natural products.
Reactive oxygen species (ROS) are harmful intermediates produced by the biological combustion involved in the respiration process. An increased amount of damage to proteins, lipids, and DNA of the body occurs owing to a surplus amount of ROS, or an oxidative stress, resulting in many chronic diseases of human beings. Therefore, for the sustenance of a healthy biological system, the equilibrium between the antioxidants and oxidants is assumed to be a significant perception (Dudonne, et al. 56). The chemicals, which possess antioxidant activities, are partly responsible for these favorable effects against the chronic diseases. For example, there are much evidence to support various epidemiological studies that coronary heart disease and cancer have an inverse relationship with the increased ingestion of fruits and vegetables due to the fruits’ antioxidant activity. Vitamin C and E, carotenoids, and phenolic compounds, particularly flavonoids, are the major antioxidants of fruits and vegetables of which their functionalities are to scavenge free radicals. The first line of defense against oxidative stress are vitamin E and carotenoids, as they extinguish singlet oxygen and then, hinder in the process of both chain propagation and chain breakage (the second defense line). Furthermore, non-phenolic compounds such as ascorbate, α-tocopherol, and β-carotene have also demonstrated to antioxidant activities (Aljadi and Kamaruddin 57).

Previous studies suggested that phenolic compounds, including flavonoids and phenolic acids, are significantly effective antioxidants. In addition, some studies have demonstrated a direct link between the total antioxidant activity of some fruits and their phenolic content (Aljadi and Kamaruddin 57). There is an increasing demand to discover innovative sources of safe and economical antioxidants of natural origin since there is a
lethal and/or carcinogenic health risks of certain synthetic antioxidants, including BHT and BHA. Hence, increasing attention and efforts are being paid for the discovery of natural antioxidants because they are preferred. (Li, et al. 58, Podsędek 59). Moreover, nutraceuticals can be produced by these naturally occurring antioxidants, which can assist to prevent oxidative damages in the body. To evaluate the antioxidant capacities, various assays have been used regardless of the discrepancies of results among the methods and across laboratories. The common in vitro antioxidant tests include the DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonate)), FRAP (ferric reducing antioxidant potential), and ORAC (oxygen radical absorption capacity) assays (Thaipong, et al. 60). In the DPPH assay, the free radical 2,2-diphenyl-1-picrylhydrazyl is reduced by antioxidants, which lightens the purple color of DPPH• that has an absorbance at 515 nm. In this case, the results are given in the form of percentage inhibition, which is the quantity of antioxidant value required for reducing the preliminary DPPH• concentration. Both quercetin and ascorbic acid can be employed in the form of positive controls. The radical scavenging activity is calculated as follows:

\[
\% \text{ Inhibition} = \left( \frac{\text{absorbance (blank)} - \text{absorbance (sample)}}{\text{absorbance (blank)}} \right) \times 100
\]

The DPPH analysis is often used in the process of preliminary antioxidant screening because it requires only a UV-Vis spectrophotometer as the major analytical tool. In addition, the test generally gives consistent results. In contrast, the ABTS assay estimates the relative capacity of an antioxidant in comparison with the Trolox (water soluble vitamin E analogue) standard to scavenge the ABTS produced during the aqueous phase. Initially, the production of ABTS takes place by the reaction of an ABTS salt with
a strong oxidizing agent (e.g., potassium permanganate or potassium persulfate), then a reduction of ABTS radicals is prompted due to addition of hydrogen-donating antioxidant (Shalaby and Shanab 61). Owing to the suppression of the absorption spectrum of its characteristic wavelength (734 nm), an estimation of the antioxidant capacity can be conducted via the measurement of color change of the ABTS radicals. The ferric ion reducing antioxidant power (FRAP) assay estimates the reducing capacity of antioxidants which is based on the reduction of the ferric ions and 2,3,5-triphenyl-1,3,4-triazacyclopenta-1,4-diene chloride (TPTZ) to ferrous (Fe III to Fe II) ion formation. These reaction can be monitored by measuring the change of absorbance, which is directly associated to the “total” reducing power of the electron donating antioxidant activities in the mixture of the samples at 593 nm (Alam, et al. 62). In estimating the antioxidant capability of various biological samples, from pure compounds to complex matrixes, the ORAC assay has been marked as an essential. AAPH produces free radicals, resulting in the consequent oxidation of the fluorescent indicator protein phycoerthrin (β-PE) and loss of its fluorescence, which can be inhibited by antioxidants. With the help of a micro plate fluorescence reader, this process can be monitored. In a phosphate buffer at pH 7.0, some reagents such as Trolox or BHT are prepared to be used as a standard. The use of a protein phycoerthrin as a substrate is the main benefit of this method. It prevents the substrate from producing free radicals and it is also excellent for determining hydrophilic and hydrophobic sample's ability by merely altering the source of generation of radicals and solvent (Alvarez-Suarez, et al. 63).

**Objectives of the Project**
The aim of this project was to investigate the volatile and non-volatile characteristic chemicals of muscadine grapes (*Vitis rotundifolia*). In detail, the research plans to:

1. Identify important VOCs extracted from the muscadine grapes during three different ripening stages using HS-SPME and GC-MS, and compare their aroma profiles using multivariate statistics, such as principle component analysis (PCA) and cluster analysis (*Chapter 2*);

2. Develop, identify and quantify phenolic compounds in muscadine grapes during ripening stages by HPLC-DAD, and investigate the relationship between the phenolic compounds and their antioxidant activities (*Chapter 3*);

3. Study and investigate the effect of various pH values on the anthocyanin extractions; identify and quantify the individual anthocyanidins under different pH values by HPLC-UV and LCQ advantage MAX™ ion trap mass analyzer after the acidic hydrolysis; and compare the chemical patterns of anthocyanidins by statistical analysis (*Chapter 4*);

4. Conduct *in vitro* investigation of the bioactive compounds of three different ripening stages of muscadine (*Vitis rotundifolia*) grape in terms of their inhibitory effects on the angiotensin-converting enzyme, pancreatic lipase, collagenase, elastase, and tyrosinase (*Chapter 5*).
Figure 1.1 Three main ripening stages of Cowart, one species of muscadine grapes (*Vitis rotundifolia*)
Figure 1.2 Identified main VOCs in muscadine grapes \( (Vitis rotundifolia) \) (Kepner and Webb\textsuperscript{23}, Welch, et al.\textsuperscript{24})
Figure 1.3 SPME procedure for HS-SPME and Direct immersion-SPME sampling according to (Vičkačkaitė and Padarauskas).
Figure 1.4 Schematic of a GC
Figure 1.5 Structural identification of anthocyanidins (aglycons)
Figure 1.6 Electrospray ionization (ESI) mechanism (Ho, et al. 53)
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CHAPTER TWO
CHACTERIZATION OF VOLATILE COMPOUNDS IN “COWART” MUSCADINE GRAPE (VITIS ROTUNDIFOLIA) DURING RIPENING STAGES USING GC-MS COMBINED WITH PRINCIPAL COMPONENT ANALYSIS

Abstract

Muscadine grape (Vitis rotundifolia) is a popular fruit in the Southeastern United States because of its unique aroma and strong antioxidant capacity. Volatile organic compounds (VOCs) of a locally cultivated muscadine cultivar “Cowart” were characterized by solid phase micro-extraction (SPME) coupled with gas chromatography-mass spectrometry (GC-MS). Twenty eight VOCs, including fruity short-chain esters, alcohols, terpenes, and carbonyl compounds, were detected based on mass spectra and Kovats indices. There are three main stages during berry development, including stage I (green), stage II (pink), and stage III (black in full maturity), corresponding to the color changes from green to black. Based on principal component analysis (PCA) and hierarchical clustering, the grapes in stages I and II had relatively similar flavor patterns, which were different from that in stage III. Butyl-2-butenoate, hexyl acetate, propyl acetate, ethyl trans-2-butenoate, hexyl-2-butenoate, ethyl acetate, butyl acetate, 1-octanol, ethyl hexanoate, and β-citral were representative volatiles in stage III, nonanal, decanal, and β-citronellol were distinct in stage II, and myrcenol, β-ocimene, and l-limonene were biomarkers in stage I. Understanding VOCs at each stage can assist farmers in choosing the optimal time to harvest muscadine grapes.

Introduction
Muscadine grapes (*Vitis rotundifolia*) were a native American grape species cultivated in the Southeastern United States. They have adapted to a warm–humid climate and well-drained soil. They grow well over a vast area, from Delaware to Central Florida, the Atlantic Ocean to East Texas, and along the Mississippi River to Missouri (Barchenger, et al. 1). For decades, their inherent bioactive phytochemicals have drawn increasing attention owing to their high antioxidant capacity compared to those of other grapes and fruits, as well as their rich content of phytochemicals that contribute significantly to the prevention of human diseases (Kim, et al. 2).

Volatile organic compounds (VOCs) in table grapes, grape juice, and wines have been studied for a long time because aroma is a major factor for consumer acceptance. Those aromas comprise hundreds of VOCs made up of different chemical groups, including alcohols, esters, aldehydes, ketones, monoterpenoids, and C$_{13}$ norisoprenoids. However, the concentration of each VOC varies significantly depending on many factors, such as cultivars, abiotic and biotic stress factors, and ripening stages (Perestrelo, et al. 3, Sánchez-Palomo, et al. 4, López-Tamames, et al. 5, Mamede and Pastore 6). In addition, an accurate determination of unstable VOCs in trace amounts is challenging and often involves the consideration of many factors, such as the solvent’s polarity, extraction method, and instrument sensitivity. Most of the VOCs in grapes are free VOCs that directly contribute to aromas, while the bound glycosidic forms are odorless and considered to be the flavor precursors (Noguerol-Pato, et al. 7, Verzera, et al. 8).

Despite much research on the flavor chemistry of grapes, information on the VOCs of muscadine grapes (*Vitis rotundifolia*) in their ripening stages is limited. In fact,
the production of one muscadine cultivar “Cowart” has decreased in recent years despite its relatively desirable flavor and taste. Therefore, in order to promote the cultivation of Cowart, characterization of its VOCs during its different ripening stages along with the fruit maturity is important. In general, there are three main stages of berry development, i.e., stage I (green), stage II (pink), and stage III (black; fully mature) (Lee and Talcott 9). Stage I includes the period of rapid cell division, but no softening occurs in this stage. In stage II, the berries change colors from green to pink along with cell expansion. Stage III results in the purple and black coloring of mature grapes, which have maximum accumulation of anthocyanins, and present a softened texture (Dokoozlian 10).

During the ripening stages, free and glycosylated VOCs are accumulated (Palomo, et al. 11). The physiological changes of grapes during the ripening stages include not only physical changes such as weight, color, and fruit rigidities, but also chemical changes such as pH, contents of sugar, alcohol, phenolics, and the acidity level (Vilanova, et al. 12, Yang, et al. 13). Thus, it is important to understand the compositional and aromatic changes that occur in fruits during the ripening stages to evaluate the desirable quality and develop a connection between the grape quality and its aromas, which provides a predictive marker that can assist in determining the optimal harvest time.

Methods for extracting VOCs often include LLE, SDE, HS-SPME, and stir bar sportive extraction techniques (SBSE) (Fan, et al. 14). However, the use of solvent extraction methods has some disadvantages, such as possible sample contamination, an environmental burden, and loss of the VOCs owing to their degradation during the
concentration step (Sánchez-Palomo, et al. 4). In comparison, SPME is considered a more desirable technique for many flavor analyses because it is a rapid, inexpensive, and solvent-free technique that combines the extraction and concentration steps in the headspace (Wejnerowska and Gaca 15). Thus, SPME has been widely used in many liquid and fragrance analyses. The aim of this research was to: (1) identify the VOCs extracted from muscadine grapes in the three developmental stages using the HS-SPME technique; and (2) compare the volatile profiles of muscadine grapes using a PCA.

**Materials and Methods**

**Materials, chemicals, and reagents**

A manual SPME holder and mixed coating fiber (DVB/CAR/PDMS, 50/30 μm) were purchased from Supelco (Aldrich, Bellefonte, PA, USA). Glass tubes (40 mL) and polytetrafluoroethylene (PTFE)/silicone septa were purchased from Scientific Specialities Service (Randallstown, MD, USA). HPLC grade dichloromethane (DCM), water, and sodium chloride (NaCl) were obtained from Fisher Scientific (Norcross, GA, USA). Alkane standard chemicals (C8–C20) and an internal standard (6-methyl-5-hepten-2-ol, 99.9% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Sample preparation**

One cultivar of the black-skinned muscadine grape, Cowart, was randomly harvested from a local farm, Happyberries (Seneca, SC) over six weeks (July 24–September 5, 2014) during its growing season (Figure 1.1). The Cowart berries were picked up in three stages. The stage I muscadine grapes were harvested on July 24, 2014.
when the grapes were small green fruits. The stage II grapes were harvested on August 22, 2014 when the grape fruits became soft and translucent and their skin color turned from green to pink/red. The stage III grapes were harvested on September 5, 2014 when their skin color turned from purple to black when harvested. All the samples were assessed based on subjective evaluation of color changes as their skin colors were obviously different in the three ripening stages. After the harvest, all the samples were freshly extracted for volatile chemical analyses or vacuum packed and stored at −20°C for nonvolatile chemical analyses. Three hundred grams of frozen grapes were transferred from plastic bags until they were defrosted at room temperature (20 minutes), then macerated using a commercial blender for 2 minutes.

**Optimization of solid phase microextraction (SPME) method**

First, optimization of the SPME method for volatile extraction from the muscadine grapes was performed based on previously reported methods (Perestrelo, et al. 3). Three hundred grams of fresh samples from each of stages I, II, and III were blended using a commercial blender and transferred into 50 mL plastic centrifuge tubes, and then centrifuged at 1610×g for 30 min at 5°C. The supernatant was transferred to five glass tubes (40 mL) and capped by PTFE/silicone septa. Then, samples were separated into two parts: (a) 20 mL of each sample supernatant were mixed with NaCl at a final concentration 0.2 g/ml of NaCl after moving into a tube (40 mL), and (b) 20 mL of each sample supernatant as a control were not added with NaCl. In addition, the internal standard (6-methyl-5-hepten-2-ol) in a concentration of 4000 ppm was added in both samples. The SPME method was optimized based on three parameters (i.e., extraction
time, extraction temperature, and ion strength of the sample solution). The selected extraction time was at 15, 30, 60, and 90 min, the extraction temperature at 40, 50, 60, and 70°C, and the ion strength of the extraction solution was tested by the blank and unsaturated salt solution (0.2 g/mL of NaCl) in order to obtain the best condition for extraction of muscadine VOCs. The effects of extraction time, temperature, and ion strength on the chromatographic responses of three representative VOCs, i.e., 2-hexanal, 1-octanol, and \textit{trans}-geraniol, were used for the extraction optimization. After preliminary tests, the optimal experimental condition for extracting the VOC of muscadine grapes was set at 60°C with 30 min of extraction time for the solution of grape supernatant with 0.2 g/mL of NaCl.

Moreover, each sample was placed in a water bath at room temperatures for 30 min for headspace equilibrium before the fiber was inserted into the bottle for volatile sampling. After the extraction, the fiber was transferred to the injection port of the GC to desorb the VOCs for 3 min. The SPME fiber was conditioned at 250°C for 5 min in the GC injector, based on the manufacturer’s recommendation. All extraction samples were repeated in triplicate (n=3).

**GC-MS analysis**

A Shimadzu GC-17A GC, which was coupled to a QP5050A MS (Shimadzu, Kyoto, Japan) and equipped with an Agilent J&W (Santa Clara, CA, USA) DB-5 (5% Phenyl, 95% methyl silicone) (60 m length X 0.25 mm ID X 0.25 μm film thickness) capillary column, was used to analyze VOCs. The VOCs were extracted by a SPME fiber (DVB/CAR/PDMS) (Resteck Corporation (Bellefonte, PA USA) in size of 0.77 mm ID
and manually injected into the GC injector port for 3 min to thermally desorb the VOCs in a splitless mode. The ultra-high purity (UHP) helium gas was adopted as the carrier gas in a flow rate at 1.0 mL/min. The initial oven temperature was set at 40°C and held for 5 min, then increased from 40°C to 100°C at 2.5°C/min and held for another 1 min, and then increased from 100°C to 150°C at 3.0°C/min, and finally increased from 150°C to 265°C at 20°C/ min and held for 5 min. The temperatures of the GC injector and interface between GC-MS were set at 250°C and 250°C, respectively. The mass spectrometer was operated in an EI mode at 70 eV, and chromatogram was recorded in a SCAN mode in a mass range of 40–350 m/z from 1.5 to 57 min.

**Volatile chemical identification**

Identification of VOCs was based on matching their mass spectra with the NIST 08 library (National Institute of Standards and Technology, Gaithersburg, MD, USA), Shimadzu Terpene and Terpenoid library (Shimadzu, Tokyo, Japan), and Wiley 08 (Wiley, NY, USA) mass spectral library, as well as the previous data of standards in our lab. VOCs were confirmed to be over 90% with the mass spectral libraries to ensure the searching reliability. Qualification of VOCs was also compared with the Kovats retention index of components and previously published data. The Kovats index was calculated based on the following Van den Dool’s equation:

\[
I = 100z + 100 \frac{T(i) - T(z)}{T(z+1) - T(z)},
\]

where \(T(z)<T(i)<T(z+1)\); \(Z\) is the number of carbon atoms; \(T(i)\) is the retention time of the sample \(i\); and \(T(z)\) and \(T(z+1)\) are the retention times of the \(n\)-alkanes eluted before and after the sample \(i\). Quantification of volatiles was conducted and based on the internal
standard (6-methyl-5-hepten-2-ol). The standard curve was established in five different concentrations (i.e., 5, 10, 15, 20, and 30 ppm) that were prepared using a purified DCM solvent. Then, 1 μL of the extract was injected into the GC and conducted in triplicate (n=3).

**Statistical Analysis**

All the samples, i.e., the extracts of samples from stages I, II, III, were conducted in triplicate (n=3). PCA and hierarchical clustering were performed using OmicsOffice® built in TIBCO® Spotfire®. PCA was run on log2-transformed area of each chemical compound detected by GC-MS with auto scaling. Hierarchical clustering was performed using complete linkage method with correlation.

**Results and Discussion**

**Physiochemical characteristics of muscadine grapes during ripening stages**

According to the previous report (Andjelkovic, et al. 16), the physiochemical characteristics of muscadine grapes were measured in terms of its size, berry weight, pH, °Brix, sugar content, titrable acidity and maturity index, which are shown in Table 2.1. Sugar content can be determined by °Brix and gravity of each samples by hydrometers that is directly related to them. In this study, it was found that the grape had its largest size and heaviest weight at its stage II. The berry size and weight decreased after the stage II due to loss of water content and decreased seed humidity. Similar patterns were reported for red grape of *Vitis vinifera* L. cv. Tannat (Boido, et al. 17). The total soluble
solids (°Brix), pH and sugar content (g/L) increased up to the stage III, while the titrable acidity continuously decreased during the three different ripening stages. The sugar content of the three different samples at different ripening stages ranged from 40.62 to 159.14 g/L.

**Method Optimization**

HS-SPME requires an optimization step of sampling conditions for extracting the most abundant VOCs from muscadine grapes in high recoveries and high efficiencies. Selection of the most efficient HS-SPME depends on the extraction time and extraction temperature. During this research, DVB/CAR/PDMS fiber, which has been introduced as a proper analyzing fiber for semi-VOCs, was used, and all the experiments were performed with the constant magnetic stirring for 30 minutes in order to equilibrate between the headspace and the samples (Sánchez-Palomo, et al. 4).

The groups of VOCs from muscadine grapes are classified into esters, terpenoids, carbonyl compounds, norisoprenoids and alcohols. Three VOCs, (i.e., 2-hexanal, 1-octanol and trans-geraniol, respectively), are picked as the representative VOCs of each group during optimizations. Each VOC was monitored by comparing the response of the peak area from GC-MS during optimization steps.

**Effects of temperature and time on SPME extraction**

HS-SPME sampling relies on the equilibrium between the headspace and sample matrix (Whiton and Zoecklein 18). Temperature employed can strongly affect the sensitivity and efficiency of the extracted VOCs for HS-SPME within reasonable extraction time. High temperature normally increases the diffusion coefficients (Kf) and
avoid Maillard reaction by reducing the equilibrium time (Perestrelo, et al. 19). The optimization of this research was performed based on experiment of (Lin 20) with little modifications. The effects of temperature and time on SPME were monitored from the gas chromatograms of the each extraction carried out at 40, 50, 60 and 70 °C and 15, 30, 60 and 90 min. All the detective responses were determined based on the sum of each chemical peaks from total ion chromatogram (TIC) which are shown in Figure 2.1 and Figure 2.3. There was a significant difference between the detectable responses of VOCs from 40 to 70 °C (p-value = 0.0247 of ANOVA test at α=0.05), but there was no significant differences for 40 to 50 °C, 50 to 60 °C and 40 to 70 °C from the Tukey test (p-value > 0.05). The results revealed that the extraction temperature of 60 °C resulted in the maximal sum of the peak area with lowest standard deviations. At this temperature, more VOCs were absorbed and detected than those in other extraction temperatures. Especially, temperature at 70 °C, less VOCs were absorbed and detected with decreased areas (concentrations) of volatile chemicals. The response area of 2-hexanal, 1-octanol and trans-geraniol with different extraction temperatures and time are shown in Figure 2.2 and Figure 2.4. These chemicals had higher peak areas at 60 °C than other temperatures. Under the same experiment condition, e.g., using the DVB/CAR/PDMS SPME fiber and fixing the extraction temperature at 60°C, optimization of SPME time was conducted at 15, 30, 60 and 90 minutes, and compared based on the peak area of the TIC in triplicate (n=3). The results showed that there was a significant increase of the TIC along with the increase of extraction time from 15 to 90 min (p-value = 0.0003 of ANOVA test at α=0.05). In more detail, there was a significant increase of TIC from 15
to 30 minutes ($p$-value = 0.0002 of Tukey test at $\alpha=0.05$) although there was not a significant increase of TIC from 30 to 60 minutes ($p$-value = 0.3008 of Tukey test at $\alpha=0.05$). Furthermore, the smallest standard deviation and maximum of sum of all the VOCs were observed at 30 minutes followed by 60, 90 and 15 minutes in order. Effects of extraction time and temperature on the chromatographic responses of three representative VOCs, i.e., 2-hexanal, 1-octanol and trans-geraniol, are shown in Figure 2.2 and Figure 2.4, which demonstrated that longer extraction time and higher temperature did not give the positive effects on extracting interest VOCs from muscadine grapes. Hence, the optimal experimental condition of extracting VOCs of muscadine grapes was set at 60 °C for 30 min for further analysis.

**Identification of VOCs in three different ripening stages**

In the three ripening stages (i.e., stages I, II, and III shown in Figure 1.1) of the Cowart muscadine grapes, 28 VOCs were chromatographically separated and identified by HS-SPME and GC-MS. The qualitative and semi-quantitative data are shown in Table 1. The VOCs are classified into different chemical groups and listed according to their semi-quantitative concentrations (Table 2.2). During the period of grape maturity, 17 VOCs were tentatively identified in stage I, 12 in stage II, and 25 in stage III. The identified VOCs were categorized into different chemical groups, including alcohols, esters, carbonyl compounds, terpenoids, and norisoprenoids. In stage I, the 17 VOCs included nine terpenoids, four carbonyl compounds, one norisoprenoid, and three alcohols. The 12 VOCs in stage II included five terpenoids, two carbonyl compounds, one norisoprenoid, and four alcohols. The 25 aromas in stage III included eight esters,
seven terpenoids, four carbonyl compounds, one norisoprenoid, and five alcohols. The observed chromatographic profiles of muscadine grape flavors in the three different ripening stages seemed to be similar or had differences only in their concentrations. However, in a more detailed analysis, fewer VOCs were detected in stage II. Among these chemical groups, the predominant VOC group in the three ripening stages was the terpenoids, which accounted for 86.82%, 75.25%, and 40.40% of the extracted volatiles in stages I, II, and III, respectively. This result was similar to a previous report that the terpenoids was the major volatile group of the grape aromas during ripening (Salinas, et al. 21).

Based on our results, esters were only detected in stage III. Esters are a major chemical group found in the fully ripened grapes, and majority of the detected esters were in their acetate forms, which usually result in the fruity, floral, and pleasant aromas of fruits. These aromas seemed to be accumulated during the ripening stages, and were particularly synthesized during the final maturity stage (Salinas, et al. 21, Kalua and Boss 22). The VOCs in the ester group included ethyl acetate, propyl acetate, butyl acetate, ethyl hexanoate, hexyl acetate, ethyl-trans-2-butenoate, butyl-2-butenoate, and hexyl-2-butenoate. Of these, ethyl acetate was the most abundant ester component, accounting for 24.66% of the VOCs present in stage III.

The volatile carbonyl compounds were detected in all three stages of berry development. Four compounds, hexanal, 2-hexanal, nonanal, and decanal, were herein categorized into the carbonyl compound group. Carbonyl compounds are major volatile group in fruits and wines (Slegers, et al. 23, Garcia, et al. 24). In particular, hexanal and 2-
hexanal were two of the most abundant volatile carbonyl compounds in grapes, and all the carbonyl compounds contribute to the “green” or “grassy” aromas in grape juices (Yang, et al. 25). All four volatile carbonyl compounds were detected in the three stages and their odors were easily recognized and differentiated from other odors because their olfactory threshold values are low. Nonanal and decanal were also detected in the Cowart muscadine grapes at stages I and II in very low concentrations. Similar to the volatile esters in grapes, the carbonyl compounds were accumulated to their highest levels in stage III, the fully ripened fruits. 2-Hexenal and hexanal had similar patterns among the different stages. These two C6 compounds have been suggested to form from the lipoxygenase pathway (El Hadi, et al. 26). The concentrations of carbonyl compounds have been reported to significantly decrease after stage II (Garcia, et al. 24); however, in this study, concentrations of the carbonyl compounds were found to significantly increase until stage III.

As shown in Table 2.2, volatile alcohols were also detected, including (E)-2-hexen-1-ol, 1-hexanol, 1-octanol, (Z)-4-decen-1-ol, and 1-dodecanol. All these chemicals, except 1-octanol and (Z)-4-decen-1-ol, were detected in all the three different ripening stages. 1-Octanol was only detected in stage III, while (Z)-4-decen-1-ol was detected in both stages II and III. The alcohol concentrations, except that of 1-dodecanol that had the highest concentration in stage I, gradually increased until stage III. Owing to the fermentation process that occurs during the grape ripening stages, the alcohol concentrations increased until the harvest time (Jordão, et al. 27). In addition, the 1-hexanol was the second most abundant chemical in the three ripening samples. A
previous study reported that 2-phenylethanol, which gives a “rose” aroma, was abundant in muscadine grapes (Lamikanra, et al. 28), but it was not detected in our study. Such kind of discrepancy was rather difficult to be compared because of many factors, such as different extraction methods (i.e., liquid–liquid extraction vs. SPME), for the determination of the VOCs.

Terpenoid VOCs have been intensively studied because they are important for the sensorial differentiation of wines based on grape variety (Salinas, et al. 21). Ten terpenoids, l-limonene, β-ocimene, α-terpinolene, myrcenol, ocimenol, α-terpineol, nerol, β-citronellol, trans-geraniol, and β-citral, were found in the three different ripening stages of muscadine grapes. These VOCs have very pleasant aromas with low olfactory thresholds. Muscadine grapes in stage I had a high concentration of terpenoids. Among them, α-terpinolene was predominant in this stage and continuously decreased until stage III. On the contrary, trans-geraniol continuously increased during the ripening stages, and finally became the main VOC in the last stage.

During the ripening stage, only one C13 norisoprenoid, β-damascenone, was detected in all three ripening stages and its concentration gradually increased through the ripening stages. The norisoprenoids are often found in low concentrations and exist as glycosidic aromas in fruit. β-Damascenone was found by SBSE-GC-MS throughout the screening of three different natural grapes (Vitis vinifera L.) of “Nebbiolo,” “Dolcetto,” and “Barbara.” These grapes that contained the lowest amount of β-damascenone (48.2±26.1 to 265.2±83.0 μg/kg) also had a lower level of norisoprenoids than Cowart observed in this study (Carlomagno, et al. 29). Ristic et al. (Ristic, et al. 30) who
investigated the C13 norisoprenoid by a solid-phase microextraction GC-MS found that *Vitis vinifera* L. cv. *Shiraz* contained a significantly low concentration of β-damascenone, of which the values ranged from 38.3–71.2 μg/kg. Nevertheless, β-damascenone detected in fruits might be ascribed to the following reason. During the sample preparation steps, the fruits that were crushed in a blender and followed by a moderate thermal heating might induce a pre-fermentative hydrolysis that resulted in the release of free forms of C13 norisoprenoids and facilitate the extraction of VOCs by HS-SPME (Coelho, et al. 31).

**Discrimination of VOCs during the ripening stages**

To examine the similarities of volatile profiles and establish a relationship between the VOCs and three ripening stages of the muscadine grapes, PCA and hierarchical clustering were performed based on the peak areas of VOCs detected by the GC-MS, which are listed in Table 2.2. Figure 2.5 shows the principal component (PC) values, which were based on the log2-transformed data of the peak area values of 28 VOCs, wherein 0 was assigned to the chemicals that were not detected by the GC-MS. According to the PCA analysis, 67.75% and 23.53% of the variance were explained by the first principal component (PC1) and second principal component (PC2), respectively. As over 90% of the PCA variance was covered by the first two principal components (PC1 and PC2), the aroma profiles of the samples under the three maturity stages were considered to be represented by the two components. The differences among the aromatic profiles of the samples were compared, as shown in Figure 2.5. The analyses were performed in triplicate. Figure 2.5 shows that the samples picked in the same ripen stage were closely clustered together, while the samples of different ripen stages were located
in obviously different regions. The average PC1 values of the stage III and I samples had the highest (5.53) and lowest (−4.20) values, respectively. The stage II samples had an intermediate average value of PC1 of (−1.33). However, the stage II samples had the highest PC2 values of (3.32), while the samples in stage I had the lowest PC2 mean value (−2.33) among the three samples. The mean value of PC2 of the stage III samples (−1.00) was closer to that of stage I than stage II.

Moreover, the relationship between the individual VOCs within the three maturity stages is profiled in Figure 2.6. Based on the aforementioned PCA of the three aroma profiles (shown in Figure 2.6), stage III is located in the fourth quadrant (represented by a positive high PC1 value (5.53) and a negative PC2 value (−1.00)), which is reflected by and linked to a group of chemical compounds, such as butyl-2-butenoate, hexyl acetate, propyl acetate, ethyl trans-2-butenoate, hexyl-2-butenoate, ethyl acetate, butyl acetate, 1-octanol, ethyl hexanoate, and β-citral, which exhibited highly similar loading locations of fourth quadrant (positive PC1 (0.22) and negative PC2 (−0.11) values). This indicated that the aroma pattern (or distribution) of the presence and the amounts of these VOCs was highly correlated. They were correlated with the grape maturity in stage III, which was reflected by their concentration levels. This conclusion is also demonstrated and profiled in Figure 2.6, which is a visual representation of the quantification of each VOC during the ripening stages. In addition, Figure 2.7 shows the hierarchical clustering of the VOCs at the three different ripening stages and is useful to further distinguish the VOCs in the three stages, as shown in Figure 2.6. For instance, Figure 2.7 shows that esters, such as ethyl acetate, propyl acetate, butyl acetate, ethyl hexanoate, hexyl acetate, ethyl
trans-2-butenolate, butyl-2-butenolate, and hexyl-2-butenolate, are only produced in stage III. Moreover, three special VOCs, i.e., nonanal, decanal, and β-citronellol, were not detected in the stage II samples (Table 2.2) and possessed in the fourth quadrant of the lowest negative PC2 values (−0.38) and low positive PC1 values (0.04–0.05) (Figure 2.6). These three VOCs can be easily differentiated from other volatiles in the stage II samples that exhibited in the second quadrant with the highest PC2 values, as well as the low negative PC1 values (Figure 2.5). Therefore, these three VOCs are considered the major markers that distinguish the samples in stages I and III from the sample in stage II that lacks these three volatiles. In conclusion, the outcome shown in Table 2.2 is consistent with and confirmed by the aroma profiles presented in Figure 2.5 and Figure 2.6.

There are three terpenoids VOCs, i.e., myrcenol, β-ocimene, and l-limonene, that were detected only in the stage I samples (Table 2.2). These three volatile terpenoids had similar PC loadings, with both low PC1 and PC2 values. As shown in Figure 2.5, the stage I samples, compared with those in stages II and III, had lower average PC1 (−4.20) and PC2 (−2.33) values in third quadrant, which were obviously distinguished from those of other stages. This outcome coincides with the positional loadings of the PC values of the volatile terpenoids in the stage I samples (Figure 2.6). Meanwhile, these terpenoids were lacking in the stage II and III samples. These results suggested that the aforementioned three terpenoids may be major contributors that distinguish the stage I samples from the stage II and III samples.
It was also observed that three alcohols, including \((E)\)-2-hexen-1-ol, \((Z)\)-4-decen-1-ol, and 1-hexanol, located in first quadrant (both high positive PC1 and PC2 values) that were significantly different from those of other chemicals. However, these three volatiles existed in null or relatively low concentrations in stage I, implying that the stage I samples might also be differentiated by these three volatile chemicals, particularly \((Z)\)-4-decen-1-ol, which was clearly different from the chemicals shown in Figure 2.7. Furthermore, the hierarchical clustering (Figure 2.7) was conducted to find the interconnectivity, as well as the closeness of individual volatiles. It revealed the similarities between the individual volatiles and their contributions to the aroma profiles in the three ripening stages. Each VOC, as a log2-transformed value of the peak area from GC-MS, is represented by a color in the heat map. Maximum (24.30), average (12.27), and minimum (0.00) values are represented by red, black, and green, respectively.

Hierarchical clustering (Figure 2.7) shows that butyl-2-butenoate, hexyl acetate, propyl acetate, ethyl \textit{trans}-2-butenoate, hexyl-2-butenoate, ethyl acetate, butyl acetate, 1-octanol, ethyl hexanoate, and \(\beta\)-citral were clustered very closely in one group. However, myrcenol, \(\beta\)-ocimene, and 1-limonene were closely grouped together in the hierarchical clustering. In addition, \((E)\)-2-hexen-2-ol, \((Z)\)-4-decen-1-ol, and 1-hexanol, which were distinct chemicals in the samples of stage I and had relatively low concentrations compared with in samples from the other stages, were found closely clustered. Nonanal, decanal, and \(\beta\)-citronellol were not abundant in stage II. They were also found to be clustered together. These hierarchical clustering classes, which clustered the chemical
compounds based on abundance patterns, were similar to the patterns of the PCA results (Figure 2.5 and Figure 2.6).

**Conclusion**

Overall, muscadine grapes collected in three ripening stages were investigated and 28 VOCs were identified. PCA of these VOCs revealed that grapes in the three different ripening stages had significantly different aroma patterns, particularly in terms of concentrations of the VOCs, although they had subtle differences in their chemical compositions. Butyl-2-butenoate, hexyl acetate, propyl acetate, ethyl trans-2-butenoate, hexyl-2-butenoate, ethyl acetate, butyl acetate, 1-octanol, ethyl hexanoate, and β-citral were distinct VOCs that were only detected in stage III. Nonanal, decanal, and β-citronellol formed another group of distinct chemicals that were absent in the stage II samples; thus, they could be used as chemical markers to differentiate the stage II samples from other samples. Terpenoids, which were accumulated during the ripening stages, are another group of biomarkers that can be used to identify the maturity stage. Although α-terpinolene and trans-geraniol were the predominant VOCs in muscadine grapes, myrcenol, β-ocimene, and l-limonene were detected only in the stage I samples. In contrast with the terpenoids, volatile esters, which are associated with fruity, floral, and pleasant odors were only detected in the fully ripened grapes in stage III. Thus, the grapes in stage III were highly favorable for fresh consumption or made into desirable wines because of their desirable and rich aromas.
<table>
<thead>
<tr>
<th>Stage</th>
<th>(July 24, 2014)</th>
<th>(August 22, 2014)</th>
<th>(September 4, 2014)</th>
<th>p-value $^a$</th>
<th>Significance difference decision$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (in)</td>
<td>0.74 ± 0.03</td>
<td>1.20 ± 0.14</td>
<td>1.35 ± 0.19</td>
<td>0.0105</td>
<td>Yes</td>
</tr>
<tr>
<td>Berry weight (g)</td>
<td>3.40 ± 0.58</td>
<td>8.130 ± 0.63</td>
<td>7.73 ± 0.58</td>
<td>0.004</td>
<td>Yes</td>
</tr>
<tr>
<td>pH</td>
<td>2.76 ± 0.01</td>
<td>3.10 ± 0.01</td>
<td>3.27 ± 0.00</td>
<td>0.0001</td>
<td>Yes</td>
</tr>
<tr>
<td>Total soluble solids (°Bx)</td>
<td>4.00 ± 0.04</td>
<td>9.73 ± 0.05</td>
<td>15.00 ± 0.00</td>
<td>0.0001</td>
<td>Yes</td>
</tr>
<tr>
<td>Sugar content (g/L)</td>
<td>40.62 ± 0.36</td>
<td>101.82 ± 0.36</td>
<td>159.14 ± 0.82</td>
<td>0.0001</td>
<td>Yes</td>
</tr>
<tr>
<td>Titrable acidity (g/L)</td>
<td>16.51 ± 0.06</td>
<td>6.94 ± 0.004</td>
<td>3.45 ± 0.009</td>
<td>0.0001</td>
<td>Yes</td>
</tr>
</tbody>
</table>

$^a$ p-value from hypothesis test $H_0 = \mu_{\text{Stage I}} = \mu_{\text{Stage II}} = \mu_{\text{Stage III}} = 0$, $H_a = \text{Not all are equal.}$

$^b$ The level of significance of ANOVA F-test was $\alpha=0.05$; mean of three replicates ± standard deviation.
**Table 2.2** VOCs in muscadine grapes were extracted by solid-phase microextraction (SPME), and their concentrations were determined by GC-MS

<table>
<thead>
<tr>
<th>No.</th>
<th>Compounds</th>
<th>Kovats index</th>
<th>Semi-quantitative concentration (ppm)(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>KI</td>
<td>STAGE 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KI(^2) (Lit)(^3)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Ethyl acetate</td>
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<td>2</td>
<td>Propyl acetate</td>
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<td>705</td>
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<td>Butyl acetate</td>
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<tr>
<td>4</td>
<td>Ethyl hexanoate</td>
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<td>998</td>
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<td>5</td>
<td>Hexyl acetate</td>
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<td>1014</td>
</tr>
<tr>
<td></td>
<td>Ethyl trans-2-butenoate</td>
<td>842</td>
<td>880</td>
</tr>
<tr>
<td>6</td>
<td>Butyl-2-butenoate</td>
<td>1043</td>
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<tr>
<td>7</td>
<td>Hexyl-2-butenoate</td>
<td>1244</td>
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**Esters**

**Terpenoids**

<p>| 9   | L-limonene           | 1029         | 1029    | 0.76±0.21  | 0±0.00  | 0±0.00  |
| 10  | β-ocimene            | 1046         | 1050    | 0.75±0.21  | 0±0.00  | 0±0.00  |
| 11  | α-terpinolene        | 1100         | 1089    | 61.03±18.14 | 31.88±5.10 | 15.28±1.99 |
| 12  | myrcenol             | 1122         | 1123    | 1.01±0.34  | 0±0.00  | 0±0.00  |
| 13  | ocimenol             | 1166         | 1165    | 4.68±2.65  | 2.94±0.35 | 1.15±0.77 |
| 14  | α-terpineol          | 1197         | 1189    | 33.48±4.86 | 11.84±0.98 | 3.82±1.61 |</p>
<table>
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<tr>
<th>No.</th>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>Concentration 1</th>
<th>Concentration 2</th>
<th>Concentration 3</th>
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<tr>
<td>15</td>
<td>nerol</td>
<td>1225</td>
<td>1230</td>
<td>1.12±0.39</td>
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<td>0.49±0.20</td>
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<tr>
<td>16</td>
<td>β-citronellol</td>
<td>1227</td>
<td>1159</td>
<td>1.62±0.66</td>
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<td>1.07±0.19</td>
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<tr>
<td>17</td>
<td>trans-geraniol</td>
<td>1253</td>
<td>1253</td>
<td>21.05±5.35</td>
<td>37.41±2.64</td>
<td>78.77±4.30</td>
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<tr>
<td>18</td>
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<td>1277</td>
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<td>0.35±0.16</td>
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**Carbonyl compounds**

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<th>Compound</th>
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<th>R2</th>
<th>Concentration 1</th>
<th>Concentration 2</th>
<th>Concentration 3</th>
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<tr>
<td>19</td>
<td>Hexanal</td>
<td>800</td>
<td>801</td>
<td>4.67±1.28</td>
<td>7.17±1.47</td>
<td>11.95±1.52</td>
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<tr>
<td>20</td>
<td>2-hexanal</td>
<td>852</td>
<td>854</td>
<td>9.76±4.56</td>
<td>11.44±2.58</td>
<td>25.23±3.49</td>
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<tr>
<td>21</td>
<td>Nonanal</td>
<td>1105</td>
<td>1101</td>
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<td>0±0.00</td>
<td>0.15±0.00</td>
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<td>22</td>
<td>Decanal</td>
<td>1207</td>
<td>1209</td>
<td>0.1±0.09</td>
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<td>0.08±0.05</td>
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**Norisoprenoids**

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<th>Compound</th>
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<th>R2</th>
<th>Concentration 1</th>
<th>Concentration 2</th>
<th>Concentration 3</th>
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<tbody>
<tr>
<td>23</td>
<td>β-Damascenone</td>
<td>1386</td>
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<td>0.92±0.77</td>
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**Alcohols**

<table>
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<th>R2</th>
<th>Concentration 1</th>
<th>Concentration 2</th>
<th>Concentration 3</th>
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<tr>
<td>24</td>
<td>(E)-2-hexen-1-ol</td>
<td>863</td>
<td>862</td>
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<td>1.89±0.59</td>
<td>2.21±0.24</td>
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<tr>
<td>25</td>
<td>1-hexanol</td>
<td>867</td>
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<td>4.55±0.90</td>
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<tr>
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<td>1068</td>
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<td>0±0.00</td>
<td>3.92±0.45</td>
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<tr>
<td>27</td>
<td>(Z)-4-decen-1-ol</td>
<td>1259</td>
<td>1259</td>
<td>0±0.00</td>
<td>0.57±0.07</td>
<td>18.02±0.73</td>
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<tr>
<td>28</td>
<td>1-dodecanol</td>
<td>1479</td>
<td>1474</td>
<td>1.83±0.43</td>
<td>0.81±0.33</td>
<td>1.23±0.47</td>
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</table>

Values are represented by the mean ± standard deviation of three replicate.

1 Semi-quantitative concentration calculated from peak area/internal standard peak area × internal standard concentration; mean of three replicates ± standard deviation

2 The KI index was calculated based on the DB-5MS capillary column and identified using mas spectra database (90% ≤ matching similarity)
Kovats index values that were previously published in literatures
Figure 2.1 Sum of the total response area of all the VOCs under different temperature treatments by solid-phase microextraction (SPME); mean of three replicates ± standard deviation.
Figure 2.2 Chromatographic responses of the three selected VOCs, e.g., 2-hexanal, 1-octanol, and geraniol, under different temperatures of solid phase microextraction (SPME); mean of three replicates ± standard deviation.
Figure 2.3 Sum of the total response area of all the volatile compounds under different time treatments by solid phase microextraction (SPME); mean of three replicates ± standard deviation.
Figure 2.4 Chromatographic responses of the three selected VOCs, e.g., 2-hexanal, 1-octen-3-ol, and geraniol, under different extraction time of solid phase microextraction (SPME); mean of three replicates ± standard deviation.
Figure 2.5 PCA scatter plots of the VOCs detected in three developmental stages of Muscadine grapes (*Vitis rotundifolia*)
Figure 2.6 PCA scatter plots of the individual VOCs
Figure 2.7 Hierarchical clustering of VOCs at the three different ripening stages. The log2-transformed values of each VOC’s peak areas, which were detected by GC-MS, are represented by colors. The maximum (24.30), average (12.27), and minimum (0.00) values are represented by red, black, and green, respectively.
References


20. Lin, P.-C., Comparison of simultaneous distillation and extraction (SDE) and headspace solid phase microextraction (SPME) for determination of volatiles of muscadine grapes (*Vitis rotundifolia*). **2014**.


23. Slegers, A.; Angers, P.; Ouellet, É.; Truchon, T.; Pedneault, K., Volatile compounds from grape skin, juice and wine from five interspecific hybrid grape cultivars grown in Quebec (Canada) for wine production. *Molecules* **2015**, *20* (6), 10980-11016.


CHAPTER THREE

IDENTIFICATION OF PHENOLIC COMPOUNDS IN ‘COWART’ MUSCADINE GRAPES (*VITIS ROTUNDIFOLIA*) DURING RIPENING STAGES AND THEIR ANTIOXIDANT ACTIVITIES

Abstract

Muscadine grape (*Vitis rotundifolia*) is recognized as a healthy fruit because of its many biological functions, as well as its nutritional value. The phenolic compounds in muscadine grape berries during ripening stages and their corresponding *in vitro* antioxidant activities were investigated in this study. The biosynthesized phenolic compounds at each ripening stage were identified and quantified using a HPLC equipped with a DAD. The concentrations of most identified phenolic compounds consistently increased during the ripening stages, except for myricetin and quercetin, which showed maximal values in the stage I. PCA showed that the chemical profiles of the phenolics in mid-stage and mature stage (stages II and III, respectively) had a similar pattern, but significantly differed from the chemical profiles of the phenolics in stage I. The concentrations of many phenolic compounds were higher in fully ripened grapes at stage III than in immature grapes (stage I) and mid-maturity grapes (stage II). There were low concentrations of gallic acid and protocatechuic acid in stage I, while resveratrol, epicatechin, ellagic acid, catechin, and kaempferol were the major compounds with high concentrations in stage III. In addition, the stage III grapes had higher concentrations of flavan-3-ols, which exhibited strong *in vitro* antioxidant activities by the ABTS and
DPPH assays. The results indicated that there is a direct relationship between the concentration of phenolic compounds and antioxidant activity in muscadine grapes.

**Introduction**

Muscadine grapes (*Vitis rotundifolia*) is a fruit unique to the southeastern United States. It is well-adapted to the warm, humid conditions and well-drained soil, which are unsuitable for the cultivation of other grapes. The main cultivation area of muscadine grapes is from Delaware to central Florida, the Atlantic Ocean to east Texas, and along the Mississippi river to Missouri. Muscadine grape is a highly perishable fruit with a short harvest season (August to mid-October). Muscadine grapes grow and ripen in clusters of 6 to 24 berries with thick and tough skins with colors ranging from bronze to purple or black. The thick skins protect them from ultraviolet radiation, insects, and fungi. Like other grapes and fruits, the skin color of muscadine grapes changes from green to purple or black during ripening. Recently, muscadine grapes have attracted much attention from scientists and consumers because of their unique flavor, aroma, and high concentrations of bioactive phytochemicals such as phenolic compounds, which function as antioxidants (Olien 1, Kim, et al. 2, Anderson, et al. 3, Sandhu and Gu 4). In fact, muscadine grapes are one of the specificity food products in the southeastern United States, which are often consumed in fresh, or processed into wines, juice, jams and jellies, or harvested under “U-pick” operations and made into various functional products (Greenspan, et al. 5).
Due to the recent increase in the health awareness of consumers, researchers have paid more attention to exploring the bioactivity of natural phytochemicals, for example, their antioxidant capacity. Bioactive plant metabolites, often called phytochemicals, are extracted from plants and formulated into supplements that reduce the risks of various diseases. Among such phytochemicals, phenolic compounds, including those in grapes and wines, have been extensively studied due to their health benefits and organoleptic characteristics (Liu, Pérez-Magariño and González-Sanjose, Topalovic, et al., Revilla and Ryan). These chemicals have also received much attention because of their potent antioxidant activities against free radicals. Consumption of fresh and processed foods containing these bioactive compounds has been reported to reduce the risks of some cancers and cardiovascular disease (Navarro, et al.).

Many phenolic compounds are natural phytochemicals and major components in plants, occurring as secondary metabolites that contain one or more -OH groups. Natural phenolic compounds can be classified from simple molecules (e.g., phenolic acids) to highly complex compounds (e.g, tannins) based on their carbon skeleton (Kosar, et al.). Phenolic compounds are often categorized into two groups: flavonoids (including anthocyanins, flavan-3-ols, condensed tannins, and flavonols); and non-flavonoids (including phenolic acids, stilbenes, hydroxyl-cinnamic acids, and benzoic acids). In grapes, these chemicals are distributed in the skin, pulp, and seed, and their compositions are affected by various factors such as grape variety, stage of berry development, geographical factors, and climatic conditions (Hellin, et al.). Factors such as variety, abiotic stress and geography can also affect the ripening stage of grapes, which
determines the physiological, morphological, and biochemical processes of the fruit. On the other hand, changes in physiological and biochemical processes are very important for fruit quality, because they affect aroma, taste, and quality of grapes.

Biochemical processes in fruits include the hydrolysis of starch and the production of phenolic compounds (Lima, et al. 13). In this context, characterization and quantification of phenolic compounds during ripening stages can provide valuable information about the fruit development (maturity) process. Traditionally, berry weight, concentrations and/or ratio of sugar and acids have been determined to decide the best harvest time, but these parameters do not provide direct information about fruit quality and other valuable chemical compounds in the grape berries. Hence, determining the changes of phenolic compounds could be a useful additional test for deciding the best harvest time because phenolic compounds are related to the grape color, berry weight, bitterness, and astringency during ripening (Pérez-Magariño and González-San José 14).

Because of the complex physiological and biochemical changes that occur in grapes during ripening, the ripening process can be divided into three major stages based on fruit skin color changes and texture: stage I (green berries with no softening), stage II (pink berries) and stage III (purple to black berries, with softening texture) (Deluc, et al. 15).

To the best of my knowledge, there have been no previous studies on the changes in terms of the composition and quantity of phenolic compounds in muscadine grapes and their relationships with antioxidant activity during ripening. Therefore, the objective of this research was to investigate the relationship between phenolic compounds and antioxidant capacity during berry development and to identify phenolic compounds
unique to each ripening stages. Understanding the evolution of individual major phenolic compounds during ripening will provide valuable information for predicting the quality of muscadine grapes and products made from them.

**Materials and methods**

**Materials, chemicals and reagents**

Chemical standards of (-)-epicatechin gallate, kaempferol, myricetin, resveratrol were obtained from Chromadex (Irvine, CA). Gallic acid, protocatechuic acid, ellagic acid, epicatechin, ρ-coumaric acid, quercetin, catechin were purchased from Sigma-Aldrich (St. Louis, MO). Hydrochloric acid (HCl), acetic acid, formic acid, HPLC grade methanol, ethyl acetate and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA). HPLC grade water was prepared from a Millipore Synergy UV system (Millipore, Bedford, MA, USA) and filtered through a 0.2 μm polytetrafluoroethylene (PTFE) filter. Sodium carbonate (Na₂CO₃), aluminum chloride hexadrate (CH₃COOK, AlCl₃·6H₂O), potassium chloride (KCl), sodium acetate (CH₃COONa), Folin-Ciocalteu reagent, DPPH, ABTS were bought from Sigma-Aldrich (St. Louis, MO).

**Sample collection**

Fruits of one local variety of muscadine grapes (*Vitis rotundifolia*), Cowart, were randomly collected from the Happyberries Farm (Seneca, SC) during six weeks (July 24 – September 5, 2014) within the harvest season, which covered three different ripening stages. The Stage I muscadine grapes were picked up on July 24, 2014, when the grapes
were in green small berries. The Stage II grapes were picked up on August 22, 2014, when the fruit skin color changed to red and its texture turned into soft. The Stage III muscadine grapes were picked up on September 5, 2014, when the skin color became purple. All samples were kept in a freezer vacuum packaged seal at -20°C until use. The whole berries were freeze-dried using a Freezone 2.5 Labconco, then ground to fine powder. The powder samples were kept in 500 mL glass bottles with sealed lids at refrigerator until analysis.

**Sample preparation and extraction**

Twenty grams of each freeze-dried samples from stage I, II and III were weighted respectively and poured into 500 mL glass bottles with lids. The three weighed samples were also respectively extracted with 200 mL of a methanol/H₂O/acetic acid mixture (methanol/H₂O/acetic acid, 80:19.9/0.01, v/v), under mild vortex for 10 mins and followed by one hour sonication at room temperature. The mixture was filtered after extraction through a 0.45 μm cellulose acetate filter (Costar Corp., Cambridge, MA), then the solvent in the collected extractant was evaporated using a Bunchi Rotavapor R114, R110, and Collegiate Rotavapor (Bunchi, New Castle, DE, USA) to remove all the solvents. The remaining solid after the solvent evaporation was resuspended in methanol into a concentration of 100 mg/mL of the original methanolic extract. All the methanolic extract samples were stored at -20 °C until analysis.

**Quantification of total phenolic content (TPC)**

The total phenolic content (TPC) of muscadine grape was spectrophotometrically determined by a modified Folin-Ciocalteu method with some modifications (You, et al.)
which was used to determine the phenolics in the methanolic extracts obtained from Stage I, II and III grapes. Briefly, aliquots of 100 μL of 5 mM gallic acid in methanol and each sample were dissolved in 6 mL of distilled water and mixed with 500 μL of the Folin-Ciocalteu regent. After 30 seconds, 1.5 mL of 20% (w/v) Na₂CO₃ solution was added into the aforementioned mixture. The reaction mixture was put in dark for 2 hours and its absorbance was measured at 765 nm using a UV-Vis spectrophotometer. Gallic acid was used as the standard and its calibration curve was constructed in a linear regression of \( Y=0.1554X - 0.0092 \), \( R^2 = 1 \). Each sample was conducted in triplicate analyses and their TPC results were expressed as gallic acid equivalent (GAE) in milligrams (mg) per 100 grams of dried whole grapes.

**Quantification of total flavonoid content (TFC)**

As the measurement of total phenolic content (TFC), the TFC was spectrophotometrically determined by the aluminum chloride colorimetric method. Aliquots of 200 μL of each sample were dissolved in 2 mL of methanol before the mixture was mixed with 50 μL of 0.4M aluminum trichloride hydrate and 50 μL of 1M potassium acetate. The reaction was incubated at room temperature for 30 mins, and then absorbance was recorded at 415 nm. The standard calibration curve of quercetin was plotted in a linear regression with \( Y=0.7471X - 0.0186 \), \( R^2 = 0.9748 \). Each sample was run in triplicate and the TFC results were expressed as quercetin equivalents (QE) in milligrams (mg) per 100 grams of dried samples (Chang, et al. 17).

**Determination of antioxidant capacity**

*ABTS cation radical-scavenging activity assay*
The antioxidant method was based on the reduction of ABTS\(^+\) radicals by antioxidants of the plant extract. The ABTS free radicals were prepared into a concentration of 7 mM with distilled water. The ABTS\(^+\) solution was also prepared by mixing the 7 mM ABTS solution and potassium sulfate and get a final concentration of 2.45 mM potassium persulfate, which was placed in the dark for 12 to 16 hours. During the period, the ABTS\(^+\) solution was diluted with methanol with its absorbance within a range of 1–1.5 at 734 nm. Each sample of 100 \(\mu\)L was mixed with 3 mL of the ABTS\(^+\) radical solutions, then placed at room temperature for one hour before its absorbance was measured at 734 nm. The ABTS\(^+\) radical-scavenging-rate (\%) was calculated by the following formula:

\[
\%\text{Inhibition of ABTS} = \left(\frac{Ab - As}{Ab}\right) \times 100
\]

where Ab was the absorbance of blank at 734, while As was the absorbance of the each sample at 734 nm (Li, et al. \(^1^8\)).

**DPPH assay**

Similar to the ABTS assay, another common antioxidant test, DPPH assay, was determined by the DPPH radicals. Each methanolic DPPH solution should be freshly prepared daily. At first, the concentration of the DPPH solution should be adjusted into its initial absorbance at 1.3-1.4 measured at 515 nm. Then, the DPPH solution was diluted by 5-fold before it was mixed with the samples. Finally, the DPPH solution in a volume of 3 mL was mixed with 100 \(\mu\)L of extracts of samples and kept in water bath
covered by parafilm at 37°C for 1 hour, then its absorbance was recorded at 515 nm. The percentage of inhibition of DPPH was calculated by the following formula:

\[
\%\text{Inhibition of DPPH} = \left(\frac{Ab-As}{Ab}\right) \times 100
\]

Where \( Ab \) was the absorbance of blank determined at 515 nm, while \( As \) was the absorbance of the each sample read at 515 nm (Li, et al. 18).

**C\text{18} \text{ SPE}**

A Sep-pak® C\text{18} column packed with 300 mg sorbent (Waters Corp., Milford, MA) was used to concentrate and selectively separate phenolic compounds. All SPE cartridges were conditioned and equilibrated with acidified water. Once the sample was loaded into the cartridge, the phenolic compounds in the sample were eluted by 100% methanol. All the fractions were collected, their solvents were evaporated using a rotary evaporator, then the remaining extract residuals were redissolved with methanol to make a concentration of 100 mg/mL of the dry material.

**Identification of phenolics via HPLC-DAD**

Identification of phenolic compounds in three different ripening stages was performed by HPLC-DAD. The DAD served as the detector of a Shimadzu HPLC system consisting of a LC-20AT pump, CTO-20A column oven, SPD-M20A DAD, CBM-20A communications module. Separation of phenolics was performed on a RP ZORBAX Eclipse XDB - C18 (250 mm x 4.6 mm, 5 μm particle size, Agilent Technologies, Inc., Loveland, CO, USA) with a guard column (12.5 mm x 4.6 mm, 5 μm particle size).

Chromatographic separation was accomplished by a gradient elution with a mobile phase consisting of 0.5% formic acid in distilled water (Solvent A) and 0.5%
formic acid in MeOH (solvent B). The most efficient gradient program was followed: 0 to 3 min, isocratic 90:10 (A:B) (v/v); 3 to 10 min, linear gradient from 90:10 (A:B) (v/v) to 80:20 (A:B) (v/v); 10 to 15 min, isocratic 80:20 (A:B) (v/v); 15 to 15.10 min, linear gradient from 80:20 (A:B) (v/v) to 75:25 (A:B) (v/v); 15.10 to 29 min, isocratic 75:25 (A:B) (v/v); 29 to 30 min, linear gradient from 75:25 (A:B) (v/v) to 70:30 (A:B) (v/v); 30 to 35 min, isocratic 70:30 (A:B) (v/v); 35 to 38 min, linear gradient from 70:30 (A:B) (v/v) to 55:45 (A:B) (v/v); 38 to 55 min isocratic 55:45 (A:B) (v/v); 55 to 56 min, linear gradient from 55:45 (A:B) (v/v) to 0:100 (v/v); 56 to 59 min, isocratic 0:100 (A:B) (v/v); 59 to 59.10 min, linear gradient from 0:100 (A:B) (v/v) to 90:10 (A:B) (v/v); 59.10 to 64 min isocratic 90:10 (A:B) (v/v); 64 to 65 min. Equilibration time for 10 min was required before the next injection. The flow rate was set at 0.7 mL/min and the injection volume was 10 μL. The DAD spectra were recorded in a range of wavelength from 200 to 400 nm. Chemical identification was confirmed by comparison with available standards, retention times and UV spectra.

**HPLC method validation**

* Determination of linearity and range

The detective linearity was evaluated for the mixed standard solutions that were prepared by diluting appropriate amount of the stock solutions in triplicate. The individual calibration curves were prepared in the same day. Then, 10 μL of standard solutions in different levels were injected in to the HPLC-DAD and repeated three times. The calibration curves were plotted between the peak areas versus concentrations of
individual phenolic compounds. The detective linearity of each external phenolic chemicals was evaluated by the correlation coefficient ($R^2$).

**Determination of limits of detection and quantitation**

Limit of Detection (LOD) and Limit of Quantitation (LOQ) of HPLC were measured for the individual phenolic compounds after the measurements of the standard deviation and the slope of the calibration curves. The limits were calculated by the following equations:

\[
LOD = 3.3 \times \frac{s}{S},
\]

\[
LOQ = 10 \times \frac{s}{S},
\]

Where, $s$ is the standard deviation of the response, and $S$ is the slope of the calibration curves.

**Accuracy and Recovery**

The accuracy of the proposed method was evaluated by performing recovery studies at three levels (50, 100, and 150 %) of phenolic standards added into the samples. Then, the amount of the sample recovered was calculated by the following equation:

\[
\% \text{ Recovery} = \left( \frac{\text{Recovered concentration}}{\text{Injected concentration}} \right) \times 100 \%
\]

The results were performed for each concentration level and carried out in triplicate.

**Precision**

The determination of the precision of the injection integration was determined by mixed standard solutions of phenolic compounds on the same day for intraday precision.
and on different days for within day precision by the proposed method. It was expressed as relative standard deviation (RSD).

**Statistical Analysis**

Two multivariate statistical methods, (i.e., PCA and hierarchical clustering), were applied for data analyses based on the OmicsOffice® software built in TIBCO® Spotfire®. PCA was run on a log2-transformed value of the chromatographic area of each chemical detected by HPLC-DAD with auto scaling. Hierarchical clustering was done using a complete linkage method with Euclidean distance. All the samples of the Stage I, II and III grapes were conducted in triplicate. Also, individual samples were used to determine significance of t-test and ANOVA test using JMP 11 (John’s Machintosh Program) with a significance level of $\alpha=0.05$.

**Results and Discussion**

**TPC and TFC at different ripening stages**

The TPC and TFC of muscadine grapes collected at different ripening stages in 2014 are shown in Table 3.1. During the ripening, the color of grape skins changes from green to purple or black, which helps to easily distinguish the maturity stages of the grapes, as shown in Figure 1.1. In the present study, the TPC was measured by the Folin–Ciocalteu colorimetric method, by which the results are expressed as gallic acid equivalents per 100 g dry weight (DW) of sample (mg GAE/100 g DW). The Folin–Ciocalteu method provides a broad estimation of TPC because the reagent not only reacts with total phenols, but also with thiols, vitamins, amino acids, proteins, nucleotide bases,
unsaturated fatty acids, carbohydrates, organic acids, inorganic ions, metal complexes, aldehydes, and ketones (Lu, et al. 19). The TPC of the muscadine grapes during its ripening stages ranged from 143.64 mg to 439.74 mg GAE/100 g. DW. As shown in Table 3.1, the TPC of muscadine grape was the highest at stage I (439.74 mg GAE/100 g DW), and markedly decreased at stage II (147.62 mg GAE/100 g DW) and stage III (143.64 mg GAE/100 g DW). Vasco, et al. 20 classified TPC into three levels such as low (<100 mg GAE/100g DW), medium (100 – 500 mg GAE/100 g DW) and high (>500 mg GAE/100 g DW). Based on this classification, muscadine grapes at stages I, II, and III have medium TPCs. In comparison, Mahmood, et al. 21 reported changes in the TPCs of strawberry and mulberry fruits from Pakistan, and Patel and Rao 22 reported the changes in the TPC in khirni fruits during ripening. Castrejón, et al. 23 studied the changes of TPC in highbush blueberry (Vaccinium corymbosum L.) during ripening, and Amira, et al. 24 profiled the TPC in ripening dates. All of those studies reported that the TPCs in those aforementioned fruits gradually decreased during their ripening stages, and that the highest TPC was in unripe berries, which is consistent with the result of the present study. In contrast, other studies reported that the TPC increased during ripening stages and achieve its peak in fully ripe fruits. The TPC of fully-ripened ‘Cowart’ muscadine grapes detected in this study (143.64 mg GAE/100 g DW) was lower than that reported by Pastrana-Bonilla, et al. 25, who evaluated several cultivars of muscadine grape.

The TFC in muscadine grapes at three ripening stages is shown in Table 3.1. Flavonoids account for a small portion of the TPC, as reported elsewhere (Tang, et al. 26). Similar to the trend in TPC, the TFC gradually decreased during the muscadine ripening.
The total flavonoid contents were expressed as mg quercetin equivalents per 100g DW of sample. The TFC of muscadine at stage I was 83.96 mg QE/100 g DW, and then decreased from stage II (64.50 mg QE/100 g DW) to stage III (47.41 mg QE/100 g DW). The TFC at different maturity stages was shown in the following order: stage I > stage II > stage III. This trend was similar to that observed for TPC. However, the TFC in fully ripened muscadine grapes (47.41 mg QE/100 g DW) was higher than those of ripe red raspberry fruits (9.61 mg CE/100g DW) and strawberry fruits (38.17 mg CE/100g DW) which was reported by de Souza, et al. 27. Our results were consistent with those of previous studies, which reported higher TPC and TFC in unripe grapes than in fully ripened grapes. Many other factors affect the accumulation and degradation of phenolics and flavonoids, including genotype, geographical and environmental conditions, harvest time, experimental conditions, and growth conditions (Amira, et al. 24, Gull, et al. 28).

The TPC and TFC of muscadine grapes are valuable indicators of the health benefits. Both TPC and TFC decreased during the ripening stages of muscadine grape. Some of the possible reasons for these decreases include the oxidation of phenolics by polyphenol oxidase, decreased concentrations of particular classes of phenolics, e.g., tannins, and loss of astringency due to polymerization of leucoanthocyanidins and hydrolysis of the astringent arabinose ester of hexahydrophthalic acid (Gull, et al. 28).

**Validation of the HPLC method for determination of phenolics**

The proposed method for determination of 11 phenolic compounds was evaluated in terms of linearity range, LOD, LOQ, accuracy (recovery), and precision.
The linearity of the method was assessed by constructing standard calibration curves of individual compounds. Peak area (y-axis) was plotted against concentration (x-axis), and then the correlation coefficient ($R^2$) was calculated. The regression equations for 11 phenolic compounds are listed in Table 3.2. The lowest correlation coefficient was 0.93, indicating an excellent linearity of chemical detection by the method. Table 3.2 shows the LOD and LOQ values for each of the phenolic compounds. The LOD values ranged from 0.02 to 3.57 μg/ml and the LOQ values ranged from 0.05 to 10.83 μg/ml. The LOQ values were approximately three times higher than the LOD values, indicating that the method was acceptable to detect these compounds in samples with good precisions.

Method accuracy was evaluated by detecting known amounts of internal standards added to the extracts. The recovery (%) was calculated as follows:

$$\left(\frac{\text{spiked phenolic concentration} - \text{concentration of phenolics naturally present in sample}}{\text{spiked phenolic concentration}}\right) \times 100$$

Each set of samples was analyzed three times with three different phenolic concentrations (50, 100, and 150 %). The average recovery of individual compounds ranged from 94.95 to 117.6 % (Table 3.3). These results demonstrated that the proposed method was accurate since the recovery values were close to 100 %.

For the precision analysis, the retention times of multiple injections of individual phenolic compounds were monitored. Spiked extracts were included in the precision analysis to determine repeatability, which is expressed as a percentage of relative standard deviation (RSD %). The RSD% values obtained for each concentrations are
summarized in **Table 3.4**. The variation in compound retention times was between 0.03 to 0.47 %, and the variation in phenolic concentrations ranged from 1.50 to 10.29 %. These results indicated that the method was sufficiently precise to quantify phenolic compounds in samples.

**Identification and quantification of phenolic compounds at three different ripening stages**

Since the Folin–Ciocalteu method detects total phenolics rather than individual compounds, HPLC has been widely used to identify and quantify individual phenolic compounds of particular interest (Sagdic, et al. 29). In this study, phenolic compounds and their concentrations in extracts of muscadine grapes are determined at three different ripening stages by HPLC-DAD. A total of 11 selected phenolic compounds were identified and quantified based on available reference standards, and by comparison of retention times under identical conditions (**Table 3.2 and Table 3.5**). The HPLC chromatograms showed clear peaks corresponding to gallic acid (peak 1), protocatechuic acid (peak 2), catechin (peak 3), epicatechin (peak 4), epicatechin gallate (peak 5), ρ-coumaric acid (peak 6), resveratrol (peak 7), ellagic acid (peak 8), myricetin (peak 9), quercetin (peak 10), and kaempferol (peak 11) in (**Figure 3.5**). These 11 phenolic compounds were classified into two groups included: non-flavonoids (i.e, gallic acid, protocatechuic acid, ellagic acid, resveratrol and ρ-coumaric acid) and flavonoids (i.e., catechin, epicatechin, epicatechin gallate, myricetin, quercetin and kaempferol).

The HPLC chromatograms of extracts from grapes at stages I, II, and III showed similar patterns, and revealed differences in the amounts of each phenolic compound
among the different ripening stages. The concentrations of individual phenolic compounds were calculated from standard curves with serial dilution (Table 3.2).

As shown in Table 3.5, six flavonoid compounds were detected in muscadine grapes, and all of them were present at all three stages. These findings were consistent with previous reports that that epicatechin, catechin, myricetin, quercetin, and kaempferol are the main phenolic compound in purple-skinned muscadine grape cultivars (‘Paulk’, ‘Cowart’, ‘Supreme’, ‘Ison’, and ‘Noble’) (Pastrana-Bonilla, et al. 25). Epicatechin gallate was previously identified in ‘Noble’ muscadine grape by Sandhu and Gu 4.

Among the phenolic compounds in muscadine grapes during ripening, the main flavan-3-ols were catechin (40.30 ± 4.25 mg/100 g DW in stage III), epicatechin (267.84 ± 15.46 mg/100 g DW in stage III) and epicatechin gallate (112.89 ± 10.16 mg/100 g dried sample in stage III), accounting for 92.14, 80.51, and 82.24% of total flavan-3-ols at stages I, II, and III, respectively. The concentrations of catechin and epicatechin constantly increased throughout the ripening stages, but the epicatechin gallate concentration was the highest at stage I (203.02 ± 12.17 mg/100 g DW), lower at stage II (47.51 ± 0.42 mg/100 g DW), and then higher at stage III (112.89 ± 10.16 mg/100 g DW). Consistent with our findings, Ivanova, et al. 30 also reported that the concentrations of flavan-3-ols (except epicatechin gallate) in red grape skins gradually increased during maturation.

Several studies have shown that there are higher concentrations of catechin and epicatechin in mature grapes than any individual procyanidin in seeds (Santos-Buelga, et al. 31). Also, the amounts of catechin, epicatechin, and epicatechin gallate in grape seeds
were reported to increase rapidly during ripening, with the highest concentrations in mature grapes and slightly lower concentrations in late-harvest grapes (Jordão, et al. 32, Andjelkovic, et al. 33). In contrast, the concentrations of epicatechin gallate decreased during the ripening. In other grapes, the biosynthesis of flavan-3-ols usually starts when the grapes begin to grow, and in some case, the flavan-3-ols concentration decrease from stage II to stage III (Mulinacci, et al. 34).

Flavonols are another important group of flavonoids that are abundant in all grapes and wines. Three flavonol (i.e, myricetin, quercetin and kaempferol) compounds were detected in muscadine grapes. Kaempferol was the most abundant, followed by myricetin, and then quercetin. The concentrations of these compounds changed during the ripening stages. Kaempferol, the main flavonol compounds, was detected at the beginning of stage I (1.33 ± 0.44 mg/100 g DW) and showed the highest concentration at stage III (8.00 ± 0.49 mg/100 g DW). This pattern of accumulation was consistent with that reported in another study (Mahmood, et al. 21). In contrast, the myricetin and quercetin decreased during ripening. In fact, the accumulation of polyphenols during ripening stages of muscadine grape can be affected by extrinsic factors such as fruit type, harvest period, climate, degree of ripeness, and the biosynthesis and degradation of polyphenols by various enzymes (Mahmood, et al. 35, Locatelli, et al. 36).

Gallic acid, protocatechuic acid, and ρ-coumaric acid are widely distributed in fruits, vegetables, and teas, and have been shown to have strong antioxidant and anti-cancer activities (Hogan, et al. 37). These chemicals were also identified in muscadine grapes by You, et al. 16. In muscadine grapes at stage I, the total amount of these three
compounds was 6.29 mg/100 g DW, and it increased to 10.68 and 13.03 mg/100 g DW at stage II and III, respectively. The concentration of the major component, gallic acid, increased significantly during the three ripening stages ($p < 0.05$). Gallic acid is a major phenolic acid in grapes and it is also abundant in wines (Monagas, et al. 38). The only phenolic acid that its concentration decreased during the ripening stages was $\rho$-coumaric acid. Its highest concentration was at stage I (4.26 ± 0.45 mg/100 g DW), consistent with the results reported by Gordon, et al. 39. Overall, the trend of changes in phenolic acids was consistent with those reported in another study (Li, et al. 40). The concentrations of most phenolic acids gradually increased during the berry maturity. Besides, these compounds have received more attention because of their antioxidant capacities (Kubola and Siriamornpun 41).

Among the non-flavonoid phenolic compounds, ellagic acid was the most abundant compound (59.28 ± 2.45 mg/100 g DW) in the ripen muscadine grapes, followed by resveratrol (8.96 ± 0.57 mg/100 g DW). Ellagic acid and resveratrol are natural phenolic compounds found in many plant species, and are the most abundant non-flavonoid phenolic compounds in muscadine grapes (Pastrana-Bonilla, et al. 25). These compounds have been extensively and intensively studied due to their marketable health benefits. For example, ellagic acid has been shown to have potent antioxidant activity, and anti-proliferative properties that are directly related to preventing DNA binding by certain carcinogens (Marshall, et al. 42). Resveratrol is a naturally occurring compound in several plants, including grape, where it is found in fresh skins and seeds, as well as red
wines. It has been widely studied for its health benefits including its estrogenic, antioxidant, anti-inflammatory, and anti-cancer properties (Geana, et al. 43).

The activities of various enzymes can also result in increased concentrations of certain phenolic compounds during ripening (Schulz, et al. 44). The biosynthesis and accumulation of phenolic compounds differs significantly depending on the stage of fruit maturity, climatic factors, plant growing techniques, and cultivar (Obreque-Slier, et al. 45). In the present study, the fully ripe muscadine grapes (stage III) had higher concentrations of flavonoids than non-flavonoid. According to Ivanova, et al. 30, the concentration of each phenolic compound gradually increased in grape berries during ripening. Although the studied grape type in that study was different from that in this study, the results of this study were similar in both studies.

Overall, the TPC and TFC values were different from the HPLC method and the Folin-Ciocalteu method. One of the reasons for such kind of differences include the limitation of availability of the standard phenolic compounds (Sagdic, et al. 29).

**In vitro antioxidants in extracts from muscadine grapes at three ripening stages**

Previous researches (Prior, et al. 46, Velioglu, et al. 47 and Babbar, et al. 48) have studied the relationship between antioxidant capacity and TPC. Some have reported a positive correlation between them, while others could not confirm the existence of such correlation (Ismail, et al. 49). Recently, it has been recommended to use at least two different methods to assay antioxidant activity in vitro, since each method detects different free radical scavengers (Rockenbach, et al. 50). In this study, extracts from muscadine grapes were tested by the DPPH and ABTS methods to evaluate the
antioxidant capacity (Table 3.1). The DPPH and ABTS assays have been widely used to measure antioxidant activities of various foods. Both methods are colorimetric assays in which the colors become lighter when hydrogen ions are donated for scavenging free radicals (Shalaby and Shanab 51). There was a strong correlation between the data obtained using the DPPH and ABTS assays \((r=0.9997)\) in this study. However, the data from the DPPH and ABTS assays showed a negative correlation between their antioxidant capacities \((IC_{50})\) and the TPC. In other word, the antioxidant activity increased as the TPC decreased during the ripening. In the DPPH assay, the \(IC_{50}\) values of extracts from muscadine grapes at stages I, II, and III were 12.86, 11.92 and 6.26 mg/mL, respectively, where a lower value indicates stronger antioxidant activity. Therefore, the strongest antioxidant activity was found at stage III, followed by stage II, and then stage I. A similar trend was detected in the ABTS assay, in which the extract from the stage III grapes showed the strongest antioxidant capacity \((IC_{50} = 5.23 \text{ mg/mL})\), followed by stage II and then stage I \((IC_{50} = 10.88 \text{ and } 12.00 \text{ mg/mL}, \text{ respectively})\). Omoba, et al. 52 reported that fully ripe raspberries had stronger antioxidant activities and higher total amount of anthocyanins than those with the 50% maturity. Our result that the levels of certain phenolic compounds and antioxidant activities increased during ripening is consistent with the findings of Zielinski, et al. 53, who reported that unripe fruits are more susceptible to enzymatic browning because of the low concentration of ascorbic acid and high activity of polyphenoloxidase. Phenolic compounds have many health benefits, but our results showed that they also make an important contribution to antioxidant activity.
Currently, phenolic compounds are receiving much attention because of their health benefits in light of their antioxidant activity (Mousavinejad, et al. 54). The high concentrations of individual flavonoids in the stage III muscadine grapes which were detected by HPLC-DAD explained why this stage had the strongest antioxidant activity. Compared with non-flavonoid compounds, flavonoids showed stronger antioxidant activity in both the ABTS and DPPH assays (Table 3.6). This result is consistent with previous reports that flavonoid compounds have significantly higher antioxidant activities than non-flavonoid compounds because of the number and configuration of phenolic hydroxyl groups, which are affected by glycosylation and the configuration of other moieties (Cai, et al. 55). When flavonoids with and without hydroxyl groups (i.e, trans-chalcone, flavone, flavanone, and isoflavone) were compared, it was clear that the hydroxyl group was essential for radical scavenging activity (Cai, et al. 55).

**Multivariate analyses of phenolic compounds during ripening of muscadine grapes**

A PCA was conducted for the phenolic compounds quantified by HPLC at the different stages of ripening. Since the phenolic compounds varied significantly depending on the ripening stages, the PCA and hierarchical clustering analysis were performed based on log2-transformed values of the peak areas from the HPLC chromatograms to determine similarities and relationships among particular compounds and ripening stages (Table 3.2). The data included in these analyses included the concentrations of gallic acid, protocatechuic acid, catechin, epicatechin, epicatechin gallate, ρ-comediac acid, resveratrol, ellagic acid, myricetin, quercetin, and kaempferol in grapes at stages I, II, and III. In the PCA, up to 73.68 % and 24.63 % of the total variance were represented by the
PC1 and the PC2, respectively. The average values of PC 1 for the stage I, II, and III, were 2.18, 0.09, and -3.32, respectively, and the average PC2 values for the stage I, II, and III were -1.14, 2.19, and -1.04, respectively. All analyses were completed by triplicate and the stage I, II, and III were located in different regions on the PCA scatter plot, as shown in Figure 3.2.

The relationship among individual phenolic compounds at the three different ripening stages are shown in Figure 3.3. As shown in Figure 3.2 and Figure 3.3, gallic acid and protocatechuic acid had relatively low concentrations at stage I, and had low PC1 values (-0.30 to -0.31) and high PC2 values (0.26 to 0.30) in second quadrant. Gallic acid and protocatechuic acid distinguished from stage I which is located in fourth quadrant in Figure 3.2. Resveratrol, epicatechin, ellagic acid, kaempferol, and catechin that are shown in third quadrant have the similar loadings for the low PC1 values (-0.31 to -0.35) and relatively lower PC 2 values (-0.26 to 0.02). This results indicated that the presence and relatively high concentrations of these five phenolic compounds were highly similar and related with stage III which had negative PC1 (-3.3) and negative PC 2 (- 1.04) in third quadrant. The five phenolics, including resveratrol, epicatechin, ellagic acid, catechin, and kaempferol, were indicative of stage III in Figures 3.2 and 3.3. $p$-Coumaric acid and epicatechin gallate had negative PC2 values (-0.56 to -0.57) and their PC1 values were close to zero (0.11 to 0.13) in fourth quadrant. Relatively low concentrations of $p$-coumaric acid and epicatechin gallate can be considered as the major markers that discriminated the samples in stage I and III from the sample in the stage II. In addition, quercetin and myricetin had high PC1 values (0.34) and relatively higher
PC2 (0.12 to 0.15) values in first quadrant. Relatively lower concentrations of these two compounds can easily be differentiated from other phenolic compounds in stage III.

A hierarchical clustering analysis was performed to observe the inter-connectivity and closeness of individual phenolic compounds among the three different ripening stages. This analysis was based on complete linkage, and distances were measured based on correlations. For each VOC, the values of peak areas from HPLC were log²-transformed, and then plotted on the heat map using different colors. Red, black, and green represent maximum (20.58), average (16.28), and minimum (11.27) values, respectively. These results indicated that the stage II and III were relatively similar to each other, and differed from stage I. Cluster 1 (Figure 3.4) contained samples with high concentrations of catechin, kaempferol, resveratrol, ellagic acid, and epicatechin; that is, extracts from muscadine grapes at the fully ripe stage (stage III). Ellagic acid and epicatechin were the most closely grouped compounds in the cluster 1. These two compounds showed dramatically increased concentrations from stage I to II to III. Catechin and kaempferol were also clustered together, because their concentrations increased from stage II to III in a similar pattern. Cluster 2 showed a similar pattern to that of the cluster 1, in that the compounds in this cluster showed high concentrations at stage III. Cluster 3 contained ρ-coumaric acid and epicatechin gallate, which had lower concentrations than those of other phenolic compounds at stage II. Cluster 4 included myricetin and quercetin, which had the highest concentrations at stage I and lower concentrations at stage III. These phenolic compounds were hierarchically clustered with
similar patterns based on their abundance. The results of the PCA and clustering analyses were consistent with those shown in Figures 3.1–3.4.

**Conclusions**

In this chapter, the phenolic compounds in muscadine grapes were investigated. As a result, 11 phenolic compounds were identified and quantified in the extracts prepared from muscadine grapes at three different ripening stages. The results showed that the types of phenolic compounds and antioxidant activities differed significantly among the three ripening stages. Muscadine grape at stage III contained high concentrations of resveratrol, epicatechin, ellagic acid, catechin, and kaempferol. Based on the HPLC-DAD analysis, gallic acid and protocatechuic acid were indicative compounds of stage I, while resveratrol, epicatechin, ellagic acid, catechin, and kaempferol were characteristic compounds of stage III. Low concentrations of ρ-coumaric acid and epicatechin gallate were indicative of stage II. The results of this study exhibited the compositional changes of phenolic compounds during the ripening stages of muscadine grapes. Extracts from stage I (unripe muscadine grapes) and stage II (mid-ripe muscadine grapes) grapes may be of use in nutraceutical industries because of their high concentrations of bioactive compounds, while the muscadine grapes at stage III are more suitable for fresh consumption and/or made into processed foods due to the high concentrations of desirable aromas, which was described in Chapter 2.
Table 3.1 Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activities (reflected by DPPH and ABTS values) of muscadine grapes at different ripening stages.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Date</th>
<th>Fruit color</th>
<th>TPC (mg/100g)</th>
<th>TFC (mg/100g)</th>
<th>DPPH ($IC_{50}$ mg/mL)</th>
<th>ABTS ($IC_{50}$ mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>24-Jul-14</td>
<td>Green</td>
<td>439.74</td>
<td>83.96</td>
<td>12.86</td>
<td>12.00</td>
</tr>
<tr>
<td>Stage II</td>
<td>22-Aug-14</td>
<td>Pink to red</td>
<td>147.62</td>
<td>64.50</td>
<td>11.92</td>
<td>10.88</td>
</tr>
<tr>
<td>Stage III</td>
<td>4-Sep-14</td>
<td>Purple</td>
<td>143.64</td>
<td>47.41</td>
<td>6.26</td>
<td>5.23</td>
</tr>
</tbody>
</table>

*a Expressed in mg gallic acid equivalents (GAE) per 100 g fresh weight (DW)

*b Expressed in mg quercetin equivalents (QE) per 100 g dried weight (DW); mean of three replicates.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Regression equation (y=ax+b)</th>
<th>Calibration range (μg/ml)</th>
<th>Correlation coefficient (R²)</th>
<th>LOD (ug/ml)</th>
<th>LOQ (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>( y=8932.3x+32543 )</td>
<td>20-100</td>
<td>1.00</td>
<td>3.57</td>
<td>10.83</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>( y=9185x-16008 )</td>
<td>10-50</td>
<td>0.95</td>
<td>0.53</td>
<td>1.61</td>
</tr>
<tr>
<td>Catechin</td>
<td>( y=3069.9x-15169 )</td>
<td>20-100</td>
<td>0.95</td>
<td>1.77</td>
<td>5.36</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>( y=5222x+7251.8 )</td>
<td>20-100</td>
<td>0.96</td>
<td>1.47</td>
<td>4.46</td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>( y=7207.1x-23733 )</td>
<td>10-50</td>
<td>0.94</td>
<td>0.15</td>
<td>0.46</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>( y=23579x+2050.4 )</td>
<td>2-10</td>
<td>0.97</td>
<td>0.31</td>
<td>0.93</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>( y=14575x-34317 )</td>
<td>20-100</td>
<td>0.95</td>
<td>1.82</td>
<td>5.53</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>( y=12015x+28278 )</td>
<td>30-150</td>
<td>0.93</td>
<td>2.01</td>
<td>6.09</td>
</tr>
<tr>
<td>Myricetin</td>
<td>( y=7265.2x-6844.2 )</td>
<td>10-50</td>
<td>0.96</td>
<td>0.90</td>
<td>2.73</td>
</tr>
<tr>
<td>Quercetin</td>
<td>( y=177530x+40603 )</td>
<td>5-25</td>
<td>0.98</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>( y=32682x+329566 )</td>
<td>5-25</td>
<td>0.97</td>
<td>0.83</td>
<td>2.51</td>
</tr>
</tbody>
</table>
Table 3.3 Evaluation of phenolic compound recovery at 50%, 100%, and 150% levels.

<table>
<thead>
<tr>
<th>Level 1 (50%)</th>
<th>Compound</th>
<th>Spiked concentration (μg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gallic acid</td>
<td>20</td>
<td>108.70 ± 2.42</td>
</tr>
<tr>
<td></td>
<td>Protocatechuic acid</td>
<td>10</td>
<td>96.16 ± 2.59</td>
</tr>
<tr>
<td></td>
<td>Catechin</td>
<td>20</td>
<td>100.2 ± 6.8</td>
</tr>
<tr>
<td></td>
<td>Epicatechin</td>
<td>20</td>
<td>95.34 ± 2.02</td>
</tr>
<tr>
<td></td>
<td>Epicatechin gallate</td>
<td>10</td>
<td>117.6 ± 3.36</td>
</tr>
<tr>
<td></td>
<td>p-coumaric acid</td>
<td>2</td>
<td>102.2 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>Resveratrol</td>
<td>20</td>
<td>99.39 ± 2.05</td>
</tr>
<tr>
<td></td>
<td>Ellagic acid</td>
<td>30</td>
<td>96.94 ± 2.74</td>
</tr>
<tr>
<td></td>
<td>Myricetin</td>
<td>10</td>
<td>109.83 ± 1.35</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td>5</td>
<td>100.56 ± 1.36</td>
</tr>
<tr>
<td></td>
<td>Kaempferol</td>
<td>5</td>
<td>104.47 ± 3.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Level 2 (100%)</th>
<th>Compound</th>
<th>Spiked concentration (μg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gallic acid</td>
<td>40</td>
<td>99.14 ± 5.55</td>
</tr>
<tr>
<td></td>
<td>Protocatechuic acid</td>
<td>20</td>
<td>102.30 ± 5.99</td>
</tr>
<tr>
<td></td>
<td>Catechin</td>
<td>40</td>
<td>108.19 ± 4.71</td>
</tr>
<tr>
<td></td>
<td>Epicatechin</td>
<td>40</td>
<td>105.57 ± 2.03</td>
</tr>
<tr>
<td></td>
<td>Epicatechin gallate</td>
<td>20</td>
<td>101.76 ± 7.9</td>
</tr>
<tr>
<td></td>
<td>p-coumaric acid</td>
<td>4</td>
<td>105.38 ± 6.87</td>
</tr>
<tr>
<td></td>
<td>Resveratrol</td>
<td>40</td>
<td>99.59 ± 5.49</td>
</tr>
<tr>
<td></td>
<td>Ellagic acid</td>
<td>60</td>
<td>99.23 ± 2.27</td>
</tr>
<tr>
<td></td>
<td>Myricetin</td>
<td>20</td>
<td>109.22 ± 0.41</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td>10</td>
<td>98.55 ± 068</td>
</tr>
<tr>
<td></td>
<td>Kaempferol</td>
<td>10</td>
<td>106.49 ± 1.97</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Level 3 (150%)</th>
<th>Compound</th>
<th>Spiked concentration (μg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gallic acid</td>
<td>60</td>
<td>100.60 ± 0.97</td>
</tr>
<tr>
<td></td>
<td>Protocatechuic acid</td>
<td>30</td>
<td>100.11 ± 2.20</td>
</tr>
<tr>
<td></td>
<td>Catechin</td>
<td>60</td>
<td>97.9 ± 2.67</td>
</tr>
<tr>
<td></td>
<td>Epicatechin</td>
<td>60</td>
<td>94.95 ± 4.18</td>
</tr>
<tr>
<td>Plant Compound</td>
<td>Concentration (mg/L)</td>
<td>IC₅₀ (μM) ± SD</td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td>----------------------</td>
<td>------------------------</td>
<td></td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>30</td>
<td>100.53 ± 4.76</td>
<td></td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>6</td>
<td>101.27 ± 2.83</td>
<td></td>
</tr>
<tr>
<td>Resveratrol</td>
<td>60</td>
<td>102.00 ± 2.22</td>
<td></td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>90</td>
<td>107.43 ± 1.48</td>
<td></td>
</tr>
<tr>
<td>Myricetin</td>
<td>30</td>
<td>95.94 ± 2.13</td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>15</td>
<td>100.87 ± 1.15</td>
<td></td>
</tr>
<tr>
<td>Kaempferol</td>
<td>15</td>
<td>100.88 ± 0.84</td>
<td></td>
</tr>
</tbody>
</table>

Mean of three replicates ± standard deviation.
Table 3.4 Determination of precision based on retention time ($t_R$) and average concentration of phenolic compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$t_R$ min</th>
<th>RSD (%)</th>
<th>Average conc. (μg/ml)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>8.581 ± 0.04</td>
<td>0.47</td>
<td>40.11 ± 1.55</td>
<td>3.86</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>13.909 ± 0.03</td>
<td>0.19</td>
<td>19.83 ± 0.41</td>
<td>2.09</td>
</tr>
<tr>
<td>Catechin</td>
<td>18.656 ± 0.03</td>
<td>0.17</td>
<td>109.59 ± 5.74</td>
<td>5.24</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>26.165 ± 0.04</td>
<td>0.15</td>
<td>142.59 ± 2.15</td>
<td>1.50</td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>31.685 ± 0.04</td>
<td>0.13</td>
<td>257.44 ± 14.89</td>
<td>5.79</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>32.037 ± 0.04</td>
<td>0.12</td>
<td>33.54 ± 0.75</td>
<td>2.24</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>43.947 ± 0.03</td>
<td>0.08</td>
<td>85.43 ± 1.35</td>
<td>1.58</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>45.675 ± 0.02</td>
<td>0.04</td>
<td>21.61 ± 2.22</td>
<td>10.29</td>
</tr>
<tr>
<td>Myricetin</td>
<td>46.827 ± 0.04</td>
<td>0.09</td>
<td>45.02 ± 1.69</td>
<td>3.76</td>
</tr>
<tr>
<td>Quercetin</td>
<td>57.605 ± 0.02</td>
<td>0.04</td>
<td>10.86 ± 1.48</td>
<td>13.64</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>64.048 ± 0.02</td>
<td>0.03</td>
<td>16.74 ± 2.83</td>
<td>16.91</td>
</tr>
</tbody>
</table>

Mean of three replicates ± standard deviation.
Table 3.5 Concentration of selected phenolic compounds in samples extracted at three different ripening stages.

<table>
<thead>
<tr>
<th>Phenolic composition (mg/100g dried samples)</th>
<th>Ripening stages</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compound</strong></td>
<td>Stage I</td>
<td>Stage II</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.00 ± 0.00</td>
<td>7.15 ± 0.35</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>2.03 ± 0.02</td>
<td>2.77 ± 0.06</td>
</tr>
<tr>
<td>Catechin</td>
<td>19.86 ± 0.29</td>
<td>21.23 ± 0.35</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>36.15 ± 2.17</td>
<td>73.45 ± 3.83</td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>203.02 ± 12.17</td>
<td>47.51 ± 0.42</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>4.26 ± 0.45</td>
<td>0.76 ± 0.09</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>4.43 ± 0.17</td>
<td>6.08 ± 0.56</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>6.13 ± 0.11</td>
<td>13.17 ± 0.26</td>
</tr>
<tr>
<td>Myricetin</td>
<td>3.79 ± 0.28</td>
<td>2.83 ± 0.36</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.12 ± 0.01</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>1.33 ± 0.44</td>
<td>1.59 ± 0.13</td>
</tr>
</tbody>
</table>

Mean of three replicates ± standard deviation.
Table 3.6 Concentration of phenolic compounds resulting in 50% inhibition of oxidation in DPPH and ABTS assays (IC$_{50}$).

<table>
<thead>
<tr>
<th>Representative phenolic compounds</th>
<th>DPPH</th>
<th>ABTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>45.16</td>
<td>3.74</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>86.83</td>
<td>7.17</td>
</tr>
<tr>
<td>Catechin</td>
<td>30.25</td>
<td>1.09</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>29.77</td>
<td>0.74</td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>13.68</td>
<td>0.07</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>-</td>
<td>2.21</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>33.40</td>
<td>1.86</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>31.05</td>
<td>1.25</td>
</tr>
<tr>
<td>Myricetin</td>
<td>72.12</td>
<td>6.12</td>
</tr>
<tr>
<td>Quercetin</td>
<td>16.65</td>
<td>0.17</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>37.58</td>
<td>2.68</td>
</tr>
</tbody>
</table>
Figure 3.1 Concentrations of selected phenolic compounds in extracts from muscadine grapes at three ripening stages (I, II, and III). A: Gallic acid, B: Protocatechuic acid, C: Catechin, D: Epicatechin, E: Epicatechin gallate, F: p-coumaric acid, G: Resveratrol, H: Ellagic acid, I: Myricetin, J: Quercetin, K: Kaempferol; mean of three replicates ± standard deviation.
Figure 3.2 PCA scatter plot of three ripening stages (I, II, and III) of muscadine grapes (*Vitis rotundifolia*) based on quantity and type of phenolic compounds; triplicate.
**Figure 3.3** PCA scatter plot of loadings on phenolic compounds of muscadine grapes (*Vitis rotundifolia*) based on quantity and type at different ripening stages; triplicate.
Figure 3.4 Hierarchical clustering of the phenolic compounds at three different ripening stages. Green: minimum concentration, black: average concentration, red: maximum concentration; triplicate.
Figure 3.5 HPLC-DAD chromatogram of phenolic compounds of standard (A) and extracts from muscadine grapes at stage I (B), stage II (C), and stage III (D). Peak show absorbance at 280 nm 1: Gallic acid, 2: Protocatechuic acid, 3: Catechin, 4: Epicatechin, 5: Epicatechin gallate, 6: p-coumaric acid, 7: Resveratrol, 8: Ellagic acid, 9: Myricetin, 10: Quercetin, 11: Kaempferol
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CHAPTER FOUR

EFFECT OF pH ON EXTRACTION AND IDENTIFICATION OF ANTHOCYANINS IN MUSCADINE GRAPES AFTER ACIDIC HYDROLYSIS AND THEIR ANTIOXIDANT ACTIVITIES

Abstract

Muscadine grapes (Vitis rotundifolia) are considered as an important source of dietary phenolic compounds, including anthocyanins that not only contribute to various colors of fruits and vegetables, but also have strong antioxidant, anti-carcinogenic, and anti-inflammatory activities. In this study, anthocyanins were extracted from muscadine grapes using solvents under various pH levels (3, 5, 7, 9, and 11), then quantified and assayed to determine their in vitro antioxidant activities. The extracts were also acid-hydrolyzed to yield individual anthocyanidins that were then analyzed by high performance liquid chromatography and MS for qualitative and quantitative analyses. Four extraction temperatures (70, 80, 90, and 100 °C) and 3 extraction times (30, 60, and 90 minutes) were also evaluated for optimization of the extraction. The results showed that the highest yield of anthocyanins was obtained from extraction at 100 °C for 60 minutes because anthocyanins are relatively stable at high temperatures. In addition, the effect of pH of the extraction solvent on total anthocyanin content and their antioxidant activity was evaluated. The results indicated that extraction with acidic solvents at pH 3 resulted in higher anthocyanin yield and stronger antioxidant activity. This research will help to improve the yield of anthocyanins from muscadine grapes and the utilization of extracted muscadine anthocyanins as antioxidants and natural pigments.
Introduction

Fruits and vegetables contain various bioactive phytochemicals that are associated with significant reduction of the risks of chronic diseases, which has been demonstrated by both epidemiological studies and clinical trials that have been conducted in an effort to investigate healthy dietary habits and evaluate the bioactivities of phytochemicals in natural products (Ju and Howard \(^1\), Nicoue, et al. \(^2\)).

Muscadine grape (\textit{Vitis rotundifolia}) has been cultivated in North America for more than 400 years because it is well-adapted to the warm, humid, and well-drained soil condition of the southeastern United States. As a result, its cultivation area has spread from central Florida to Delaware, the Atlantic Ocean to east Texas, and along the Mississippi River to Missouri (Conner \(^3\)). Muscadine grape is popular in North America because it is considered to be a healthy fruit containing significant amounts of phenolics and flavonoids, which have been linked to many bioactivities. Besides, anthocyanins from muscadine grapes have been shown to inhibit enzymes related to diabetes, obesity, cardiovascular disease, and cancer (You, et al. \(^4\), You, et al. \(^5\)). Huang, et al. \(^6\) reported that muscadine grapes contain five anthocyanins: delphinidin-3,5-diglucoside, cyanidin-3,5-diglucoside, petunidin-3,5-diglucoside, peonidin-3,5-diglucoside and malvidin-3,5-diglucoside.

Anthocyanins are derived from the flavylium or 2-phenylbenzopyrilium cation. They are the largest group of plant secondary metabolites in nature, and responsible for a broad range of colors of fruit, vegetables, and flowers, ranging from red to blue, and purple. Because many synthetic colorants are carcinogenic or have other negative effects
on humans, their use is prohibited in many foods and other products. Therefore, natural pigments such as anthocyanins from fruits, flowers and vegetables are more desirable in light of their less or no negative side-effects that could be used to replace synthetic colorants in the food industry (Assous, et al. 7, Zhang, et al. 8, Türker and Erdogdu 9, Amalia and Afnani 10, Jing and Giusti 11). Besides being colorants, anthocyanins are also bioactive compounds with potential health benefits. Several studies have shown that they have antioxidant activity, and anti-carcinogenic, anti-inflammatory, anti-diabetes (Type II), anti-microbial, and anti-allergic properties (Nicoue, et al. 2, Kyraleou, et al. 12, Flamini, et al. 13, Rojo, et al. 14).

More than 25% of the population prefers foods without artificial ingredients (Sloan 15). Because of their ‘natural products’ status and their health benefits, anthocyanins have become an important commodity in food and beverage industries. To meet this demand, large quantities of anthocyanins have been produced from plant materials. However, poor extraction of anthocyanins can result in weak or unappealing colors even the anthocyanin concentration was sufficient in the source material. In this context, extraction of high-quality anthocyanins in large quantities from grape was reported (Canals, et al. 16). In this case, several types of solvents have been studied in terms of their efficiency in extracting anthocyanins from natural products.

Most anthocyanins present in plants tissues are polar molecules with hydroxyl, carboxyl, methoxyl, and glycosyl groups bound to aromatic rings. Because of these properties, they are soluble in polar solvents such as methanol, ethanol, acetone, and water (Xavier, et al. 17). Small amounts of hydrochloric acid, formic acid, or acetic acid
were often added to facilitate the extraction of anthocyanins since the extraction at low pH is more efficient to prevent oxidation, reduce degradation of the non-acylated anthocyanin pigments, and stabilize anthocyanins (Ruenroengklin, et al. 18, Garcia-Viguera, et al. 19, Laleh, et al. 20). On the other hand, anthocyanins are also relatively unstable and pH-dependent pigments. Therefore, it is important to optimize the extraction condition in light of the parameters, such as pH, temperature, time, type of solvent, to obtain high-quality anthocyanins from muscadine grapes.

Moreover, establishing optimal conditions for extracting anthocyanins from muscadine grapes will be useful for further studies in regards of their health benefits, and their use as nutraceuticals. Hence, the general objective of this study was to investigate the effects of pH of the extraction solvent on the yield of anthocyanins from muscadine grapes. In addition, the relationship between the anthocyanin content and its antioxidant activities in terms of the DPPH and ABTS assays were also evaluated. Furthermore, the samples were acid-hydrolyzed to release anthocyanidins which were analyzed by HPLC. The concentrations of individual anthocyanidins were compared by a multivariate analysis. In general, it was expected that this study will improve the extraction yield of anthocyanins from muscadine grapes and study their antioxidant activities.

**Materials and methods**

**Materials, chemicals and reagents**

HPLC grade methanol, formic acid, hydrochloric acid (HCl) and acetic acid were purchased from Fisher Scientific (Pittsburgh, PA, USA). HPLC grade water was prepared by a Millipore Synergy UV system (Millipore, Bedford, MA, USA) then filtered through
a 0.2 μm polytetrafluoroethylene (PTFE) filter. Anthocyanidin standards, including delphinidin chloride, cyanidin chloride, peonidin chloride and malvidin chloride, were obtained from Chromadex (Irvine, CA, USA).

**Sample preparations**

One cultivar of the Muscadine grapes (*Vitis rotundifolia*), i.e., Cowart, was randomly collected from a local farm, Happyberries (Seneca, SC). Maturity grapes were collected in September 5, 2014 when its skin color became purple. All samples were freeze-dried using a Freezone 2.5 Labconco then kept in a freezer after vacuum seal at -20°C until analyses.

**Sample extraction**

Ten grams of freeze-dried samples were weighted and poured into five glass bottles with lids. Each bottle had a volume of 500 mL. The sample was mixed with 200 ml of 80 % methanol and 20 % distilled water, which was either acidified with HCl to pH value at 3.0 and 5.0, or neutralized to pH 7.0, or go further for basification with sodium hydroxide (NaOH) to pH 9.0 and 11.0 for 1 hour under water bath sonication at room temperature. After the extraction, the mixture was filtered through a 0.45 μm cellulose acetate filter (Costar Corp., Cambridge, MA), then concentrated to 100 mg/ml of original methanolic extract by rotary evaporator.

**Quantitation of total anthocyanin content**

The total anthocyanin content (TAC) was determined using the pH-differential method. Two buffer solutions were freshly prepared and used to dilute the samples. One buffer solution was prepared with 0.025 M KCl buffer solution at pH 1.0 and another
stock solution was prepared by 0.4 M sodium acetate buffer at pH 4.5. Briefly, the concentrated extracts were diluted by the aforementioned buffers for certain times, which was also recorded as a number of a dilution factor (DF). Then, the mixture was allowed to be equilibrated for 15 min. The diluted samples should be measured between 15 min and 1 h at $\lambda_{520\text{nm}}$ and $\lambda_{700\text{nm}}$, respectively, using a spectrophotometer with a blank cell filled with distilled water as a control. The difference of absorbance was calculated from those obtained from the aforementioned two different wavelengths. The total amount of TAC in the original sample was expressed to equivalent to cyanidin-3,5-diglucoside that has a molecular weight of 611 g/mol and molar absorptivity (molar extinction coefficient) of 30,175 L/cm /mol. The TAC is calculated by the following formula:

$$\text{Monomeric anthocyanin pigment } \left( \frac{\text{mg}}{L} \right) = \frac{(A \times MW \times DF \times 1000)}{(\varepsilon \times l)}$$

Where: $A$ = the absorbance of the diluted sample; $DF$ = dilution factor; $MW$ = molecular weight of cyanidin-3,5-diglucoside; $\varepsilon$ = the molar absorptivity of cyanidin-3,5-diglucoside for anthocyanins and the values were converted to mg of total anthocyanin content/ 100g samples.

**Acidic hydrolysis of anthocyanins**

Each sample extracted by different pH values (e.g., pH 3, 5, 7, 9 and 11) in a volume of 2.5 mL was added with equal volume of 2.5 ml of methanol and 1.5 ml of 12M HCl in a 10 mL glass test tube covered with a lid for acid hydrolysis. The mixture was thoroughly mixed and hydrolyzed at 100°C in a water bath for 60 min. Each sample was immediately cooled in an ice bath and concentrated using a rotary evaporator until the evaporation of solvents, and redissolved in 0.05% formic acid acidified water.
**C\textsubscript{18} solid phase extraction (SPE)**

A Sep-pak\textsuperscript{®} C\textsubscript{18} (6 mL, 1g sorbent; Waters Corp., Milford, MA) SPE cartridge was used to concentrate and selectively recover compounds of interest. All cartridges were at first conditioned and equilibrated with the acidified water. After the sample loading, the samples was eluted with 0.05% formic acid in MeOH. All the fractions were collected, the solvent of eluents was evaporated using a rotary evaporator, and then the remaining concentrate was immediately re-dissolved with 0.05% formic acid to make a concentration of 100 mg/ml of anthocyanidin.

**HPLC-UV/Visible**

A HPLC-UV system (Shimadzu Scientific instruments Inc., MD, USA) was equipped with a Shimadzu UFLC Prominence SPD-20A detector, a Shimadzu SIL-10AD auto-injector, a Shimadzu SCL-10A system controller, a Shimadzu Prominence LC-20AT pump, and Shimadzu FRC-10A fraction collector. Separation was performed on a RP ZORBAX Eclipse Plus C\textsubscript{18} (4.6 mm x 250 mm, 5 \textmu m particle size) chromatographic column (Agilent Technologies, Inc., Loveland, CO, USA) for separation of anthocyanidins.

Chromatographic separation was performed by a gradient program with a mixture of two mobile phases: solvent A consists of a mixture of distilled water: formic acid: acetonitrile (ACN) (87:10:3) and solvent B consists of distilled water: formic acid: ACN (40:10:40). The linear gradient program was followed by: 0 to 3 min, isocratic 90:10 A:B (v/v); 3 to 26 min, linear gradient from 90:10 A:B (v/v) to 70:30 A:B (v/v); 26 to 29 min, linear gradient from 70:30 A:B (v/v) to 0:100 A:B (v/v); 28 to 29 min, isocratic 0:100
A:B (v/v); 29 to 29.10 min, linear gradient from 0:100 A:B (v/v) to 90:10 A:B (v/v); 29.10 to 35 min, ended with column rinsing and re-equilibration. The flow rate was 0.8 mL/min and the injection volume was 10 μL. The wavelength of the UV detector was 520 nm. The sample fractions were analyzed by the HPLC, from which each separated peak was identified by comparing their retention times and spectra with the reference standards. The concentrations of anthocyanidins were calculated based on the standard calibration curves (5 to 50 μg/ml) of their corresponding standards, except petunidin due to the lack of its standard.

**Mass analyzer system**

The employed mass analyzer instrumentation was a Thermo Scientific (Waltham, MA, USA) LCQ advantage MAX™ ion trap mass analyzer (operating in the positive ion mode) and the accompanying Xcalibur™ data acquisition software. An isocratic program was applied to the mass analyzer system. The mobile phase contained acetonitrile: distilled water (60:40, v/v with 0.1% TFA) and its flow rate was 40 μL/min. Furthermore, a full scan mode was selected for 200-500 m/z with the spray voltage of 15 V, capillary temperature of 225°C and tube lens offset of 10V.

**HPLC method validation**

*Determining of linearity*

The standard solutions were prepared with five different serial dilutions of internal standards in triplicate. Then, 10 μL of individual standard solutions were injected into HPLC-DAD and repeated three times. The calibration curves were plotted by the
average peak area (y-axis) vs concentration (μg/ml) (x-axis). The linearity of the curves was evaluated by the correlation coefficient.

Accuracy and recovery

The accuracy of an analytical method was determined by the percent recovery (%R) with addition of an internal standard to the sample. The samples were spiked with three different amounts of standard compounds: 1. delphinidin (30, 60, and 90 μg/mL), 2. cyanidin (20, 40, and 60 μg/mL), 3. peonidin (17, 30, and 45 μg/mL) and 4. malvidin (24, 48, and 72 μg/mL). The results were carried out in triplicate and statistically analyzed using the following formula:

\[
\% \text{ Recovery} = \left( \frac{\text{Recovered concentration}}{\text{Injected concentration}} \right) \times 100 \%
\]

Reproducibility

Determination of the repeatability/reproducibility of the injection integration was determined by five replicate injections of each compounds (delphinidin, cyanidin, peonidin, and malvidin). The method was checked by within-day repeatability of response after replicate injections. It was expressed as relative standard deviation (RSD).

\[
\% \text{ RSD} = \left( \frac{\text{standard deviation}}{\text{Mean}} \right) \times 100 \%
\]

LOD and LOQ

Limit of detection and quantification were determined based on the standard deviation and the slope of the calibration curve for individual compounds. The limits were expressed according to following formula:

\[
\text{LOD} = 3.3 \times s/S
\]
\[
\text{LOQ} = 10 \times s/S
\]
Where, s is a standard deviation of the response, and S is the slope of the calibration curve.

**Statistics**

Hierarchical clustering and heatmap were performed using the OmicsOffice® built in TIBCO® Spotfire®. Hierarchical was run on log$_2$-transformed area values of each anthocyanidin chemicals detected by LC-UV with auto scaling. Hierarchical clustering was conducted using a complete linkage with the euclidean distance methods. All the samples were conducted in triplicate, and the statistical significance was determined by the t-test and ANOVA test using JMP 11 (John’s Machintosh Program) with a significance level of $\alpha=0.05$.

**Results and discussion**

**Effect of pH of extraction solvent on total anthocyanin content and antioxidant activity**

Anthocyanins are natural colorants that are responsible for a wide range of colors (red to purple) in natural plants. These pigments have also been shown to have beneficial effects on human health. For example, these phytochemicals were reported to reduce the risks of some chronic diseases such as diabetes, cancer, and heart disease (Steed and Truong). However, there are several factors that have limited their applications. For instance, they are unstable at certain temperatures, and in the presence of oxygen, light, and some enzymes. In addition, pH value of the solution affects the chemical stability and properties of anthocyanins (Gauche, et al., Fossen, et al.).
Muscadine grapes are a rich source of anthocyanins. The aim of this research was to evaluate the effect of the pH of the extraction solvent on the amount and properties of anthocyanins extracted from muscadine grapes. Previous studies have shown that there are five types of anthocyanins in muscadine grapes: delphinidin-3,5-diglucoside, cyanidin-3,5-diglucoside, petunidin-3,5-diglucoside, peonidin-3,5-diglucoside and malvidin-3,5-diglucoside. The anthocyanins of muscadine grapes contain two hexoses that are normally linked at 3- and 5- positions of anthocyanidin molecules (Huang, et al. 6). In muscadine grapes, the sugar moieties linked to anthocyanins are mainly non-acetylated forms of glucose. Hence, the anthocyanin muscadine grapes are the 3,5-diglucoside forms (Flora 24).

The effect of pH on extraction of anthocyanins from muscadine grapes was also investigated. Grape samples were extracted with solvents under the pH values of 3, 5, 7, 9, and 11 at a fixed temperature and time. The anthocyanins extracted from muscadine grapes were quantified by the pH-differential method, and expressed as cyanidin-3,5-diglucoside equivalents. Table 4.1 shows the TAC in all of the extracts.

The TAC differed significantly among the samples extracted under different pH conditions, and ranged between 212.45 mg cyanidin-3,5-diglucoside equivalents/100mg DW (pH = 7) to 272.19 mg cyanidin-3,5-diglucoside equivalents/100 mg DW (pH = 3). The pH of the extraction solvent could be ranked, from the highest TAC of the samples to the lowest, as follows: pH 3 > pH 5 > pH 9 > pH 11 > pH 7. The lowest concentration of anthocyanins in the sample extracted at pH 7 was probably due to an irreversible loss of anthocyanins under neutral conditions (Kalt, et al. 25). The extracted anthocyanins were
highly sensitive to pH, as revealed by the addition of HCl or NaOH. The TAC in the sample extracted at pH 5 was approximately 261.56 mg cyanidin-3,5-diglucoside equivalents/100 mg DW, which was not significantly different from that in the sample extracted at pH 3 ($p > 0.05$). The TAC in the samples extracted at pH 3 and pH 5 were about 1.1 to 1.3 times higher than those in sample extracted at pH 7 and under alkaline conditions at pH 9 and pH 11. The TAC did not have a significant difference ($p > 0.05$) between the samples extracted at pH 3 and pH 5, and between samples extracted at pH 9 and pH 11. However, the TAC values had significant differences among the samples extracted under different pH conditions ranging from 3 to 11, ($p < 0.05$).

In acidified solvents, anthocyanins are in the flavylium cation form, in which they are intensely colored, whereas they are in pale or colorless in the quinoidal pseudobase and chalcone forms under alkaline conditions (Kalt, et al. $^{25}$, Devi, et al. $^{26}$). These pH-dependent ionic forms of anthocyanins have been well characterized (Nicoue, et al. $^2$). Anthocyanins are more stable at low pH, because the enzymatic oxidation is inhibited under these conditions (Ruenroengklin, et al. $^{18}$).

The TACs of the samples extracted under alkaline conditions (pH 9 and 11) were 241.08 and 224.39 mg cyanidin-3,5-diglucoside equivalents/100 mg DW, respectively, higher than that in sample extracted under the neutral condition but lower than those in the samples extracted under the acidic solutions. In the alkaline condition, a rapid loss of proton of flavylium cation occurs to form quinoidal base that tends to become blue or violet colors, hence their absorbance values are higher in the sample extracted at pH 9 and 11. Under high pH values, the amounts of quinoidal bases and carbinol pseudobases
are degraded that could result in a shift in the equilibrium towards colorless chalcone form (He, et al. 27). A previous study showed that anthocyanins are unstable under alkaline conditions because the flavylium cation becomes hydrated during the formation of the quinoidal base, and this reaction results in a change shift in absorbance from 520 to 535 nm (Patil and Datar 28).

Table 4.2 summarizes the scavenging activities of samples extracted from muscadine grapes at different pH levels in the DPPH and ABTS assays. The sample extracted at pH 3 showed the highest activity in the DPPH radical scavenging assay (IC$_{50}$ =12.79 mg/ml), while the sample extracted at pH 7 showed the lowest value (IC$_{50}$ = 15.11 mg/ml). This result was consistent with their TAC values under two pH conditions. A similar trend was also observed in the ABTS assay. The sample extracted at pH 3 and pH 5 showed the highest antioxidant activity. The color intensity of anthocyanins was stronger in the acidified solvents, and correspondingly, the antioxidant activities of the anthocyanins were also stronger at low pH. The results indicated that the antioxidant activities of anthocyanins derived from muscadine grapes were suppressed under the neutral and alkaline conditions. The phenomenon was consistent with the report of Yen and Duh 29, who reported that acidic methanol extracts had higher DPPH radical scavenging activities. These results show that use of acidic extraction could not only result in higher yields, but also higher antioxidant activities of anthocyanins.

**Optimization of acid hydrolysis conditions**

Analysis of anthocyanins by HPLC is useful for assessing a product quality, and for the identification and quantification of these pigments. However, one of the main
disadvantages of HPLC analysis for anthocyanins is that it is difficult to obtain reference standards for all of the individual anthocyanins (Zhang, et al. 30). Because of the sugar conjugation and acylation patterns, there are more than 300 anthocyanins in nature (Nyman and Kumpulainen 31). Fortunately, the glycosides of anthocyanidins can be reduced to six major anthocyanidins (aglycones) by acidic hydrolysis, which can help to remove impurities and simplify the chemical identifications. The major aglycones in nature are delphinidin, cyanidin, peonidin, pelargonidin, malvidin and petunidin. Acid-hydrolyzed samples from muscadine grapes contain five anthocyanidins, i.e., delphinidin, cyanidin, petunidin, peonidin and malvidin (Huang, et al. 6). In our research, reference standards were available for all except petunidin.

Extraction temperature and time are also critical factors in the acidic hydrolysis to increase the extraction efficiency and yield of anthocyanidins from muscadine grapes. According to the method described by Merken, et al. 32, four extraction temperatures, including 70, 80, 90 and 100 °C, and three extraction times, including 30, 60, and 90 minutes, were selected. The yield of total anthocyanidins from muscadine grapes increased as the extraction temperature increased from 70 to 100 °C, indicating that anthocyanidins are relatively stable under high temperature. Thus, the best extraction temperature was chosen as 100 °C. As shown in Figure 4.1, the yield of individual anthocyanidins from muscadine grapes slightly decreased after extraction at 100 °C for 90 minutes, indicating that an extended extraction time have affected the yield as a results of oxidation in presence of light and oxygen (Naczk and Shahidi 33) and decomposition.
(Arnok, et al. 34). Consequently, an extraction condition under 100 °C and 60 minutes was chosen herein as the optimal temperature and time for extraction of anthocyanidins.

Anthocyanidins vary in their hydroxylation and methoxylation patterns, and these variation affect the absorption spectra (Zhang, et al. 30). As shown in Figure 4.2, the peaks on the HPLC chromatograms showed that the anthocyanins were transformed into anthocyanidins to different degrees based on several treatments of extraction time and temperatures. Most anthocyanins were converted to anthocyanidins under an acidic hydrolysis at pH 3 at 100 °C for 30 minutes. After 60 minutes of the hydrolysis, anthocyanin peaks had disappeared and could not be identified or quantified by the HPLC-UV. Therefore, optimization of an extraction method in terms of its temperature and time is an important step to extract the maximal amount of anthocyanidins and retain their structures and bioactivities for maximum health benefits. Overall, acidic hydrolysis was a useful method to simplify characterization of the anthocyanin profiles, which allowed five anthocyanidin aglycones successfully separated from muscadine grapes.

**HPLC method validation**

The HPLC method was evaluated in regards of its analytical linearity, accuracy, precision, and LOD and LOQ for each anthocyanidin. To determine the detective linearity and range, calibration curves were constructed in triplicate for the anthocyanidins, each with five points, using four internal standards of delphinidin, cyanidin, peonidin, and malvidin. The concentration ranges used to test the linearity of standard curve were as the following: 1) 10 - 50 μg/ml delphinidin, 2) 10 – 50 μg/ml cyanidin, 3) 5 – 25 μg/ml peonidin, and 4) 8 – 40 μg/ml malvidin. The regression
equations, calibration ranges, and coefficients of determination are shown in Table 4.3. Linearity was evaluated from the value of the regression coefficient, where a high value means an excellent linearity of the chemical detection of the method. The standard curves of all the anthocyanidins had $R^2$ values greater than 0.95, indicating an excellent linearity. The LOD ranged from 0.13 to 2.01 μg/ml and LOQ ranged from 0.40 to 6.10 μg/ml. These LOD values were approximately one-third of the LOQ values, indicating that the proposed method had an enough sensitivity to detect these compounds in the samples.

To determine the recovery of the HPLC method, the samples were spiked with internal standards (i.e., delphinidin, cyanidin, peonidin, and malvidin) to monitor their recoveries (Table 4.4). The recovery (%) was calculated as follows:

$$\text{Recovery} = \left[ \frac{\text{anthocyanidin concentration in sample after spiking} - \text{concentration of anthocyanidin naturally present in sample}}{\text{spiked anthocyanidin concentration}} \right] \times 100.$$  

The recovery rate was determined from three different anthocyanidin concentrations (50, 100, and 150 %), where the best recovery values were close to 100% with low standard deviations. In addition, the results showed that recovery ranged from 98.74 and 108.04 (Table 4.4).

The intra-day and inter-day precision of the method was also evaluated by monitoring the retention times of multiple injections of different concentrations of each anthocyanidin. Precision is expressed as relative standard deviation (RSD %). The variation in retention time ranged from 0.13 to 0.19 % and the variation in anthocyanidin concentration ranged from 0.08 to 0.42 % (Table 4.5). These low RSD % values
indicated that the method was sufficiently accurate to quantify anthocyanidins in samples. Hence, it was concluded that the HPLC method had a good precision.

**Qualification and quantification of each anthocyanidins after extraction at different pH levels**

The samples were extracted under different pH conditions, then the contents of individual anthocyanidins in the samples were determined by HPLC.

Identification of anthocyanidins in all extracts were based on comparison of their retention times with those of standard compounds, and their elution order as reported in previous studies of published data in earlier studies (Huang, et al. 6). Petunidin was identified only by its retention time and elution order because there was no standard available for this compound. The anthocyanidins in all samples were eluted in a similar order as those in previous reports, but the concentrations of individual anthocyanidins differed significantly under different pH values and extraction temperatures (p < 0.0001). The five anthocyanidins from muscadine grapes were clearly separated on the chromatographic column. The four positively identified anthocyanidins were delphinidin (peak 1), cyanidin (peak 2), peonidin (peak 4) and malvidin (peak 5) (Figure 4.3). The elution order of anthocyanin has been reported as delphinidin glycoside, cyanidin glycoside, petunidin glycoside, pelargonidin glycoside, peonidin glycoside and malvidin glycoside (Wu and Prior 35). Our results were consistent with the results of previous studies on anthocyanidins from muscadine grape (Talcott and Lee 36), where the elution order was delphinidin, followed by cyanidin, peonidin and malvidin, in every sample.
The HPLC chromatograms of anthocyanidins (i.e., delphinidin, cyanidin, peonidin, and malvidin) showed similar patterns regardless of whether the sample was extracted under acidic, neutral, or alkaline conditions.

The concentration of individual anthocyanidins ranged from 4.66 ± 0.11 to 154.11 ± 1.97 mg/100g dried sample, which were significantly affected by the pH value of the extraction solvents (Table 4.6). The pH of the extraction solvent was ranked, from the largest sum of the four anthocyanidins to the smallest, as follows: pH 3 > pH 5 > pH 9 > pH 11 > pH 7. Delphinidin (19.06 ± 0.88 to 154.11 ± 1.97 mg/100g dried sample) and cyanidin (9.24 ± 0.11 to 35.97 ± 0.36 mg/100g dried sample) were the main anthocyanidins in all extracts. The highest concentration of delphinidin was in the sample extracted at pH 3 (154.11 ± 1.97 mg/100g dried samples), and accounted for 68.56% of the TAC in that sample. Conner and MacLean 37 reported that delphinidin was the main anthocyanidin in muscadine grape. Because delphinidin provides a violet/blue color, it is an important marker of ripening and maturity in grapes (Katsumoto, et al. 38). Generally speaking, my results showed that the pH of the extraction buffer strongly affected the yield, quality, and composition of anthocyanins.

To confirm the HPLC data, four major anthocyanidins separated by HPLC (Shimadzu HPLC-UV) were collected in separate fractions using a Shimadzu HPLC fraction collector. Two methods were normally used to collect the fractions: auto peak detection and collection of each peaks based on retention times. By this way, individual compounds were collected and verified against the retention time of their reference standards. Peaks 1, 2, 4 and 5 were collected at 13.55 - 14.25 minutes, 17.00 - 17.50
minutes, 21.50 - 22.00 minutes, and 22.10 – 22.50 minutes, respectively. All the fractions were re-injected into an HPLC-MS to verify the identification of the compounds. Peak 1 was identified as delphinidin with a $m/z$ 303 $[M]^+ \text{ ion}$ and Peak 2 was identified as cyanidin with $m/z$ 287 $[M]^+ \text{ ion}$ (Figure 4.5 and 4.6). Peaks 4 and 5 could not be identified because of their low concentrations.

To evaluate the inter-connectivity and closeness of individual anthocyanidins and identify similarities among samples extracted under different pH conditions, hierarchical clustering (Figure 4.4) was performed on log$_2$ transformed values of peak areas from the HPLC-UV chromatograms for delphinidin, cyanidin, peonidin, and malvidin. Clustering was performed with complete linkage, and correlations were measured based on their relevant distances. In the heat map, the maximum (7.28), average (3.99), and minimum (2.19) values are represented by red, black, and green color, respectively. As shown in Figure 4.4, the concentrations of anthocyanidins in the samples were significantly different depending on the pH conditions during the extraction. In general, the most abundant anthocyanidin was delphinidin followed by cyanidin, malvidin and peonidin. These results were consistent with a previous report that delphinidin-3,5-diglucoside and cyanidin-3,5-diglucoside were the main anthocyanins in ‘Jumbo’ and ‘Cowart’ muscadine grapes (Huang, et al. 6).

According to Talcott and Lee 36, peonidin and malvidin are relatively more stable anthocyanidins in juice and wine, and malvidin is the most important chemical for color stability of wine. In contrast, delphinidin and cyanidin are less stable than peonidin and malvidin. Since muscadine grapes contain relatively low concentrations of peonidin and
malvidin, but relatively high concentrations of delphinidin and cyanidin, muscadine juice is highly susceptible to color degradations, which adversely affects its juice and wine quality. On the contrary, wine grapes (*Vitis vinifera*) contain higher concentrations of malvidin and peonidin and lower concentrations of delphinidin and cyanidin (Goldy, et al. 39). As a result, wine grapes are more favored over muscadine grapes for the wine production, because their anthocyanidins give more stable colors. Furthermore, the number of sugars and their attachment positions could affect the anthocyanin stability. Anthocyanin 3,5-diglucosides, which exist in abundant amounts in muscadine grapes, are more sensitive to heat and oxidation than anthocyanin 3-glucosides, resulting in rapid color loss. Therefore, it is not good for making muscadine wine or juice (Patil and Datar 28).

In the hierarchical clustering analysis (Figure 4.4), cyanidin, malvidin and peonidin were found to be clustered close together, but separated from delphinidin. The extractions at pH 3 and pH 5 produced samples with similar anthocyanidin concentrations, which were higher than those in samples extracted under alkaline conditions. The low-pH extractions also resulted in more strongly colored samples because of the intense color of the flavylium cation. The extractions at pH 9 and pH 11 produced samples with similar total anthocyanin contents and their individual anthocyanidins. The extraction at pH 7 resulted in the lowest concentration of the four anthocyanidins. It is worthy of mention that the extract from pH 7 did neither cluster with the acidic nor the alkaline extraction samples (Figure 4.4). These results highlighted that
the pH condition during the sample extraction strongly affected the quality, composition, and yield of anthocyanins and/or anthocyanidins.

**Conclusions**

pH value of solvents could significantly influence the extraction yield of anthocyanins from muscadine grapes. A solvent at pH 3, which was made of 80% of methanol, 20% of distilled water and adjusted to pH 3 using 1M HCl under room temperature, yielded the highest concentration of anthocyanins in an amount of 272.19 mg cyanidin-3,5-diglucoside equivalents/100g DW from muscadine grapes. This result indicated that the acidic condition was more efficient and suitable for extraction of anthocyanins because those anthocyanins are more stable at low pH. In addition, the extracts obtained from the pH 3 and pH 5 extractions showed relatively high antioxidant activities in the DPPH radical scavenging assay (IC$_{50}$ = 12.79 and 13.81 mg/ml for the pH 3 and pH 5 extracts, respectively) and the ABTS assay (IC$_{50}$ = 12.46 and 12.67 mg/ml for the pH 3 and pH 5 extracts, respectively) due to the high concentrations of anthocyanins. Furthermore, the acidic hydrolysates of the extracted anthocyanins was prepared at 100 °C for 60 minutes, and used for identifications of corresponding anthocyanidins. Four anthocyanidins, i.e., delphinidin, cyanidin, peonidin, and malvidin, from the acidic hydrolysates of the samples extracted under pH 3, 5, 7, 9, and 11, were identified and quantified by HPLC-UV. Based on the clustering analysis, the samples extracted from muscadine grapes at pH 3 and pH 5 showed similar profiles in high concentrations of delphinidin and cyanidin that are relatively unstable chemicals that are
unfavorable for making muscadine juice or wine.
Table 4.1 Total anthocyanin contents in samples extracted at five pH levels

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH 3</th>
<th>pH 5</th>
<th>pH 7</th>
<th>pH 9</th>
<th>pH 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cowart</td>
<td>272.19 ± 3.62</td>
<td>260.56 ± 8.89</td>
<td>212.45 ± 8.29</td>
<td>241.08 ± 0.96</td>
<td>224.39 ± 2.21</td>
</tr>
</tbody>
</table>

Data are Mean ± S.D in triplicate

*Expressed in mg cyanidin-3,5-diglucoside equivalents per 100 g dried weight (DW)*
Table 4.2 $IC_{50}$ values of the DPPH and ABTS assays by the samples extracted from muscadine grape at five pH levels

<table>
<thead>
<tr>
<th>Extraction solvent</th>
<th>DPPH (mg/ml)</th>
<th>ABTS (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 3</td>
<td>12.79</td>
<td>12.46</td>
</tr>
<tr>
<td>pH 5</td>
<td>13.81</td>
<td>12.67</td>
</tr>
<tr>
<td>pH 7</td>
<td>15.11</td>
<td>13.14</td>
</tr>
<tr>
<td>pH 9</td>
<td>14.28</td>
<td>12.85</td>
</tr>
<tr>
<td>pH 11</td>
<td>14.31</td>
<td>12.81</td>
</tr>
</tbody>
</table>

Mean of three replicates
Table 4.3 Standards of four anthocyanidins and their calibration curves for quantitative analyses by HPLC

<table>
<thead>
<tr>
<th>Compound</th>
<th>Regression equation (y=ax+b)</th>
<th>Calibration range (μg/mL)</th>
<th>Correlation coefficient (R²)</th>
<th>LOQ (μg/ml)</th>
<th>LOD (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delphinidin</td>
<td>y=14435x-66433</td>
<td>10-50</td>
<td>0.99</td>
<td>6.10</td>
<td>2.01</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>y=70723x-370717</td>
<td>10-50</td>
<td>0.98</td>
<td>1.10</td>
<td>0.36</td>
</tr>
<tr>
<td>Peonidin</td>
<td>y=61470x-187035</td>
<td>5-25</td>
<td>0.97</td>
<td>0.62</td>
<td>0.20</td>
</tr>
<tr>
<td>Malvidin</td>
<td>y=44755x-229079</td>
<td>8-40</td>
<td>0.95</td>
<td>0.40</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Mean of three replicates
Table 4.4 Evaluation of anthocyanidin recovery at 50%, 100%, and 150% levels.

<table>
<thead>
<tr>
<th>Level 1 (50 %)</th>
<th>Compound</th>
<th>Spiked concentration (μg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delphinidin</td>
<td>30</td>
<td>101.86 ± 7.87</td>
<td></td>
</tr>
<tr>
<td>Cyanidin</td>
<td>20</td>
<td>102.25 ± 2.36</td>
<td></td>
</tr>
<tr>
<td>Peonidin</td>
<td>17</td>
<td>101.88 ± 3.02</td>
<td></td>
</tr>
<tr>
<td>Malvidin</td>
<td>24</td>
<td>102.43 ± 4.94</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Level 2 (100 %)</th>
<th>Compound</th>
<th>Spiked concentration (μg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delphinidin</td>
<td>60</td>
<td>104.34 ± 5.75</td>
<td></td>
</tr>
<tr>
<td>Cyanidin</td>
<td>40</td>
<td>99.23 ± 2.11</td>
<td></td>
</tr>
<tr>
<td>Peonidin</td>
<td>30</td>
<td>98.01 ± 2.31</td>
<td></td>
</tr>
<tr>
<td>Malvidin</td>
<td>48</td>
<td>101.89 ± 2.40</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Level 3 (150 %)</th>
<th>Compound</th>
<th>Spiked concentration (μg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delphinidin</td>
<td>90</td>
<td>108.04 ± 1.35</td>
<td></td>
</tr>
<tr>
<td>Cyanidin</td>
<td>60</td>
<td>98.74 ± 0.80</td>
<td></td>
</tr>
<tr>
<td>Peonidin</td>
<td>45</td>
<td>102.37 ± 1.22</td>
<td></td>
</tr>
<tr>
<td>Malvidin</td>
<td>72</td>
<td>100.40 ± 2.33</td>
<td></td>
</tr>
</tbody>
</table>

Mean of three replicates ± standard deviation.
Table 4.5 Determination of precision based on retention time ($t_R$) and average concentration of anthocyanidins

<table>
<thead>
<tr>
<th>Compound</th>
<th>$t_R$ (min)</th>
<th>RSD (%)</th>
<th>Average conc. (μg/ml)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delphinidin</td>
<td>14.38 ± 0.03</td>
<td>0.19</td>
<td>122.97 ± 0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>17.14 ± 0.03</td>
<td>0.16</td>
<td>39.62 ± 0.21</td>
<td>0.53</td>
</tr>
<tr>
<td>Peonidin</td>
<td>20.96 ± 0.03</td>
<td>0.13</td>
<td>32.75 ± 0.03</td>
<td>0.10</td>
</tr>
<tr>
<td>Malvidin</td>
<td>21.53 ± 0.04</td>
<td>0.17</td>
<td>48.95 ± 0.20</td>
<td>0.42</td>
</tr>
</tbody>
</table>

Mean of three replicates ± standard deviation.
### Table 4.6 Concentration of four anthocyanidins in samples extracted under different pH conditions

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH 3</th>
<th>pH 5</th>
<th>pH 7</th>
<th>pH 9</th>
<th>pH 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delphinidin</td>
<td>154.11 ± 1.97</td>
<td>100.19 ± 0.61</td>
<td>8.87 ± 0.02</td>
<td>35.93 ± 1.31</td>
<td>19.36 ± 0.88</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>35.97 ± 0.36</td>
<td>24.92 ± 0.23</td>
<td>9.24 ± 0.11</td>
<td>17.06 ± 0.25</td>
<td>12.09 ± 0.61</td>
</tr>
<tr>
<td>Peonidin</td>
<td>17.01 ± 0.06</td>
<td>12.07 ± 0.11</td>
<td>4.66 ± 0.11</td>
<td>8.57 ± 0.24</td>
<td>6.20 ± 0.18</td>
</tr>
<tr>
<td>Malvidin</td>
<td>17.71 ± 0.04</td>
<td>13.25 ± 0.09</td>
<td>6.62 ± 0.12</td>
<td>10.06 ± 0.20</td>
<td>7.98 ± 0.26</td>
</tr>
</tbody>
</table>

Mean of three replicates ± standard deviation.
Figure 4.1 Concentration of each anthocyanin (sum of the total area on chromatograms) in samples after extraction for different time and at different temperatures: (A) 70 °C for 30 mins, (B) 80 °C for 30 mins, (C) 90 °C for 30 mins, (D) 100 °C for 30 mins, (E) 100 °C for 60 mins and (F) 100 °C for 90 mins.
Figure 4.2 HPLC chromatograms of samples extracted from muscadine grapes subjected to acid hydrolysis under the following conditions: (A) 70 °C for 30 mins, (B) 80 °C for 30 mins, (C) 90 °C for 30 mins, (D) 100 °C for 30 mins and (E) 100 °C for 60 mins
Figure 4.3 HPLC chromatograms of anthocyanidins in acid-hydrolyzed sample extracted from muscadine grapes at pH 5. 1: Delphinidin, 2: Cyanidin, 4: Peonidin, 5: Malvidin
**Figure 4.4** Hierarchical clustering of anthocyanidins extracted using solvents with different pH conditions.
Figure 4.5 Mass spectrum of compound first faction peak of acid hydrolyzed sample (A), and delphinidin standard (B)
Figure 4.6 Mass spectrum of compound second fraction peak in acid hydrolyzed sample (A), and cyanidin standard (B)
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CHAPTER FIVE

BIOACTIVE COMPOUNDS IN DIFFERENT STAGES OF MUSCADINE GRAPES
(VITIS ROTUNDIFOLIA) WITH THEIR IN VITRO ACTIVITIES OF INHIBITING
THE ANGIOTENSIN I-CONVERTING ENZYME (ACE), PANCREATIC LIPASE,
TYROSINASE, COLLAGENASE AND ELASTASE

Abstract

Phenolic compounds in natural products can act as inhibitors of angiotensin-I-converting enzyme (ACE), pancreatic lipase, tyrosinase, collagenase and elastase, which are linked to some human diseases and/or biological activities. Since muscadine grapes have some potential bioactive compounds, a study was carried out to investigate the inhibitory effects of crude extracts of muscadine grapes during their ripening (i.e., stage I, II, and III) and some selected phenolics found in muscadine, against the ACE using N-[3-(2-furyl) acryloyl]-Phe-Gly-Gly (FAPGG) as a substrate (ACE_{FAPGG}), pancreatic lipase, tyrosinase, collagenase and elastase, which were selected for the enzymatic inhibitory activities determined by continuous spectrophotometric assays, while ACE using hippury-\(L\)-histidyl-\(L\)-leucine (HHL) as a substrate (ACE_{HHL}) was determined by HPLC-DAD. All the enzymatic inhibitory activities were exhibited in dose dependent patterns that were used for calculations of the respective half maximal inhibitory concentrations (\(IC_{50}\)) of the enzymes mentioned above.

The results showed that the extract from the stage III of muscadine grapes had greater inhibitory effects against the ACE for both substrates (i.e., FAPGG and HHL), collagenase and elastase than its counterparts from the stage I and II due to the high
concentrations of inherent bioactive phenolic compounds, while the extract from the stage I had the highest inhibitory effects against the pancreatic lipase and tyrosinase. In detail, the extract from the stage III showed higher enzymatic inhibitory activities on ACE$_{HHL}$, ACE$_{FAPGG}$, collagenase, and elastase ($IC_{50}$ = 1440, 1110, 3.55, and 1.76 μg/ml, respectively), while the extract from the stage I had stronger inhibitory activities against the pancreatic lipase and tyrosinase ($IC_{50}$ = 0.12 and 1.39 mg/ml, respectively). Furthermore, four phenolic compounds, including epicatechin, epicatechin gallate, myricetin, and resveratrol, were tested for their enzymatic inhibitions, with their inhibitive $IC_{50}$ values of 0.81, 6.00, 1.36, and 1.44 μg/ml against the ACE$_{HHL}$, 2.21, 5.45, 3.15, and 1.11 μg/ml against the ACE$_{FAPGG}$, 0.17, 0.25, 0.09, and 0.20 mg/ml against the pancreatic lipase, 4.31, 8.57, 11.78, and 8.79 μg/ml against the tyrosinase, 1.65, 3.35, 1.93, and 2.11 μg/ml against the collagenase, and 0.29, 1.73, 2.13, and 1.63 μg/ml against the elastase, respectively. Moreover, the kinetic models of these enzymatic inhibitions were determined, resulting in the discovery of the competitive inhibitions of all those extracts and phenolics against the enzymes.

In summary, the results demonstrated that the extracts of muscadine grapes, regardless of its ripening stages, were a great source of bioactive compounds to inhibit the aforementioned enzyme, which suggests. Muscadine and its extract can be used as potent nutraceuticals for improving human health.
**Introduction**

Epidemiological studies and clinical trials have revealed an indirect relationship between the dietary intake of flavonoid-rich fruits or vegetables and reduced occurrence of certain illnesses such as cardiovascular disease, cancer, and neurodegenerative disorders (Dong, et al. 1). Those fruits or vegetables also contain various phenolic compounds that are associated with disease prevention in humans. For instance, diverse phenolic compounds that were found in grapes and many other fruits have shown antioxidant activities, as well as inhibitory effects on some enzymes including angiotensin I-converting enzyme (ACE), tyrosinase, collagenase, and elastase (Actis-Goretta, et al. 2, Wittenauer, et al. 3, Zhu, et al. 4, Choi, et al. 5).

The muscadine grape (*Vitis rotundifolia*) has been found containing many health-benefiting phytochemicals such as gallic acid and flavan-3-ol, as well as higher concentrations of anthocyanin and ellagic acid than those in other fruits (Musingo, et al. 6, You, et al. 7). Phenolic compounds in muscadine grapes are well known for their health benefits, including anticancer, antioxidant, antimutagenic, anticarcinogenic, antitopoisonmerase, antiobesity, and hypocholesterolemic activities (Xu, et al. 8, Nakai, et al. 9).

Hypertension is a disorder that gradually worsens in humans, leading to a number of serious chronic diseases including cardiovascular disease, stroke, renal disease, and diabetes. Hypertension is thought to affect a quarter of the world’s adult population, and that proportion is expected to rise to 29% by 2025. Therefore, it is important to treat high blood pressure to prevent the relevant diseases and maintain good human health.
Hypertension has been referred to as the ‘silent killer’ since its symptom is not easily recognized. A patient with hypertension may be asymptomatic for years before suffering a stroke or cardiac arrest. Approximately 90%–95% of patients with high blood pressure will develop serious hypertension. As yet, the cause of this disorder remains unknown (Loizzo, et al. 11). However, it is well known that the renin-angiotensin system (RAS) indirectly controls blood pressure via controlling fluid volume and certain enzymatic reactions. An important component of the RAS is ACE, a zinc-containing enzyme that is distributed in serum and in the endothelial lining of the vasculature of the lungs. This enzyme functions as an exopeptidase to cleave dipeptide bonds at the C-terminus of various lipopeptides (Choi, et al. 12, Massaretto, et al. 13). The ACE plays an important role in maintaining pressure in blood vessels. It converts the histidyl-leucine dipeptide angiotensin I into an active form, angiotensin II, which is an effective vasoconstrictor. Angiotensin II triggers the synthesis of aldosterone, which increases blood pressure by controlling sodium levels in distal or constricted tubules. Captopril, an artificial ACE inhibitor, was shown to be able to effectively control hypertension, and has been used as a therapeutic agent to reduce blood pressure in diabetic and non-diabetic patients (Cheung, et al. 14). Although Captopril can control blood pressure in diabetic and non-diabetic patients, it is not recommended for patients without serious symptoms since 44% of patients reported side effects, such as a persistent dry cough or other minor hypersensitive reactions (Cheung, et al. 14).

Moreover, according to the World Health Organization (WHO), obesity rate has been doubled since 1980. More than 1.9 billion adults, 18 years and older, were in
overweight in 2014 (Organization\textsuperscript{15}). Overweight and obesity is a disease resulted by imbalance between higher calorie intakes and energy expenditure, which is also a strong risk factor for some other chronic diseases, such as hypertension, hyperlipidemia, arteriosclerosis, sleep breathing disorder, cancer and diabetes (Buchholz and Melzig\textsuperscript{16}, Nakai, et al.\textsuperscript{9}). Obesity is ranked in fifth critical disease of global death although it is a preventable disease. As a result, some strategies of managing obesity have been suggested, including effective ways to block fat absorption from intestine or increase metabolic rate and fat oxidation (Nakai, et al.\textsuperscript{9}, Almoosawi, et al.\textsuperscript{17}). The pancreatic lipase (PL) is the critical enzyme for lipid absorption. It was estimated that the enzyme was accountable for digestion of 50-70\% of dietary triglycerides into monoacylglycerides and free fatty acids (Sergent, et al.\textsuperscript{18}). Hence, inhibitors of PL might be one of the solutions to reduce fat absorption and treatment of obesity. For example, Orlistat, a potent pancreatic lipase inhibitor, is a worldwide clinically approved drug for obesity and hyperlipidemia treatment. However, it has several unpleasant side effects such as steatorrhea, abdominal cramping, and leads to have fat-soluble vitamin deficiencies, fecal urgency and flatulence (Lunagariya, et al.\textsuperscript{19}, Birari and Bhutani\textsuperscript{20}). Therefore, researchers have enthusiasms to search natural inhibitors of PL, such as polyphenolic extracts of grape, black or green tea, etc, which can reduce the above adverse effects (Nakai, et al.\textsuperscript{9}, Sergent, et al.\textsuperscript{18}).

Another concern of middle-aged and elderly people is hyper-pigmentation and wrinkling of the human skin, which is a fundamentally important organ for human beings to have a direct contact with the outside environment. Skin aging is influenced by
intrinsic and extrinsic factors. The former includes time, genetic makeup, and hormonal changes, while the latter includes UV radiation, which contributes to photo-aging (Chattuwatthana and Okello 21). Substances that can inhibit tyrosinase, collagenase, and elastase are attracting more attention from both scientists and consumers, since these enzymes are responsible for the skin pigmentation and loss of its elasticity. Phenolic compounds have been found to be able to affect activities of the aforementioned enzymes, and thus, have the potential to treat hyper-pigmentation and skin wrinkling.

Natural products, rather than chemically synthesized inhibitors, are preferred by consumers due to concerns of many side effects, resulting in enthusiasms of exploring safe natural inhibitors. Previous studies have shown that flavonoid-rich natural products can help to reduce blood pressure (Kwon, et al. 22, Afonso, et al. 23), treat obesity and hyperlipidemia (Bustanji, et al. 24), and reduce skin wrinkling and hyper-pigmentation (Moon, et al. 25, Thring, et al. 26).

Therefore, the objectives of this research were to (1) evaluate the inhibitory activities of the muscadine extracts, as well as some phenolics of muscadine, against the ACE, tyrosinase, pancreatic lipase, collagenase, and elastase, and (2) explore their inhibitory enzymatic kinetics.

**Materials and methods**

**Materials, chemicals and reagents**

ACE from rabbit lung (purified ACE), hippury-l-histidyl-l-leucine (HHL), epicatechin, C. histolyticum collagenase type IA (ChC), N-[3-(2-furyl) acryloyl]-Phe-
Gly-Gly (FAPGG), N-[3-2-(furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA), porcine pancrease elastase type III (PPE), N-Succ-Ala-Ala-Ala-p-nitroanilide (AAAPVN), 4-nitrophenyl palmitate (ρNPP), tyrosinase from mushroom and L-tyrosine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hippuric acid, hydrochloric acid (HCl), tris(hydroxymethyl)aminomethane, acetic acid, formic acid, HPLC grade methanol, ethyl acetate, dimethyl sulfoxide (DMSO) and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA). Some chemical standards, including (-)-epicatechin gallate, myricetin, and resveratrol, were obtained from Chromadex (Irvine, CA, USA). HPLC grade water was prepared by a Millipore Synergy UV system (Millipore, Bedford, MA, USA) and filtered through a 0.2 μm polytetrafluoroethylene (PTFE) filter before use.

**Inhibitory assay of angiotensin converting enzyme**

Inhibitory activities against the ACE were determined on two substrates (i.e., HHL and FAPGG). The inhibitory activity of $ACE_{HHL}$ was measured by a modified method (Kwon, et al. 22), which was conducted in 1.0 M NaCl-borate buffer (pH 8.3) while its substrate was also diluted by the same buffer. A mixture consisting of 30 μL of the muscadine extract and 100 μL of 1.0 M NaCl-borate buffer (pH 8.3) containing 20 mU ACE-I solution was incubated at 37°C for 10 minutes in a water bath, then an aliquot of 100 μL of 9.7 mM substrate (HHL) was added into the mixture for incubation at 37°C for another one hour. As a result, hippuric acid was produced in the enzymatic reaction before the reaction was stopped by adding 150 μL of HCl (1M). Then the sample was filtered through disposable 0.45 μm PTFE syringe filters before the HPLC analysis of
hippuric acid, for which a series of standard solutions were used to construct a calibration curve. Separation of hippuric acid was performed on a RP ZORBAX Eclipse XDB-C18 column (250 mm x 4.6 mm, 5 μm particle size, Agilent Technologies, Inc., Loveland, CO, USA) with a guard column (12.5 mm x 4.6 mm, 5 μm particle size). Mobile phase in an isocratic system contained 0.1% (v/v) Trifluoroacetic acid (TFA) in acetonitrile: distilled water (25: 75, v/v). Flow rate was controlled at 1 ml/min. The DAD was used for detecting hippuric acid at 228 nm. In general, the Shimadzu HPLC system consisted of a LC-20AT pump, CTO-20A column oven, SPD-M20A DAD, CBM-20A communications module. The % inhibition was calculated based on the peak area of hippuric acid:

\[
\text{\% Inhibition} = \frac{(\text{hippuric acid)}_{\text{control}} - (\text{hippuric acid)}_{\text{extract}})}{(\text{hippuric acid)}_{\text{control}}} \times 100
\]

The control used 30 μl of buffer instead of the extract, or the phenolic compounds. All analyses were performed in triplicates.

For the FAPGG hydrolysis determined spectrophotometrically, the ACE\textsubscript{FAPGG} inhibitory effects of the muscadine grape extracts at three ripening stages and phenolics were assayed according to the procedure described by Actis-Goretta, et al.\textsuperscript{27} with a few modifications. Briefly, 40 μL of 30 mU ACE solution and 200 μL extracts were mixed and incubated at 37 °C for 15 minutes. After incubations, 300 μL of FAPGG (0.4 to 2 mM) was added, and the absorbance was read at 340 nm for 20 minutes. All other procedures were as same as those described for the ACE\textsubscript{HHL}.

**Inhibitory effects on pancreatic lipase**
Effects of the aforementioned inhibitors on pancreatic lipase were performed according to a previous report (Adisakwattana, et al. 28). Porcine pancreatic lipase (2 units/mL) in 50 mM Tris-HCl buffer solution (pH 8.0), different diluted concentrations (1 to 10 mg/ml) of samples which were dissolved in DMSO, and different concentrations (1 to 5 mM) of the substrate (pNPP) were mixed and incubated at 37°C for 20 mins. The released $p$-nitrophenol from pNPP was monitored immediately at 405 nm every 2 minutes for 30 minutes by a Biotek μQuant 96 micro well plate reader (Bio-tek® Instruments, Inc., Winooski, VT).

**Inhibitory effects on tyrosinase**

The method was modified from a previously reported method (Park, et al. 29). L-Tyrosine was used as the substrate. Briefly, mushroom tryosinase (1200 unit/ml) was mixed with 0.1 M phosphate buffer (pH 6.8) and different concentrations (0.4 to 2 mM) of 1-tyrosine, which were incubated at 25°C for 10 minutes. Then, different concentrations (10 to 50 μg) of the samples were added. Absorbance of the mixture was measured every 2 minutes for 20 minutes at 490 nm by a Biotek μQuant 96 micro well plate reader (Bio-tek® Instruments, Inc., Winooski, VT).

**Inhibitory effects on collagenase and Elastase**

The inhibitory activity of collagenase and elastase were measured according to the method reported by Wittenauer, et al. 3 with minor modifications. In order to measure the inhibitory effects of the aforementioned inhibitors on collagenase, a mixture solution, which contained 0.05 M tricine buffer solution containing 0.4 M NaCl and 0.01 M CaCl$_2$
(pH 7.5), *C. histolyicum* collagenase (ChC) (0.8 U/ml) and FALGPA (1.8 mM), was incubated at 37°C for 20 minutes. Subsequently, different concentrations of FALGPA was added to initiate the enzymatic reaction. Absorbance of the ChC reaction was monitored at 335 nm for 20 minutes by a Biotek μQuant 96 micro well plate reader (Bio-tek® Instruments, Inc., Winooski, VT).

In comparison, the inhibitory activities of the aforementioned inhibitors against the elastase was conducted via a similar procedure to that of collagenase. PPE and AAAPVN were dissolved in 2 mM tris buffer solution (pH 8.0). An aliquot of PPE, tris buffer and different concentrations (10 to 50 μg/ml) of samples were mixed and incubated at 25 °C for 20 minutes. After 20 minutes, different concentrations of the substrate (0.4 – 2 mM) were added, then the absorbance was monitored at 410 nm for 20 minutes by a Biotek μQuant 96 micro well plate reader (Bio-tek® Instruments, Inc., Winooski, VT).

**Statistical Analysis**

Pearson’s correlation coefficient ($r^2$) were calculated from triplicate within the experiments by the JMP 11 (John’s Machinotosh Program). All analyses were performed in triplicate.

**Results and discussions**

**Investigation of anti-hypertension effects of muscadine extracts of Stage I, stage II, and stage III and phenolics on ACE**
Hypertension is a risk factor of some cardiovascular diseases, such as arteriosclerosis, stroke, myocardial infraction and end-stage renal disease, which have affected people all over the world (Carillon, et al. 30). The ACE, a dipeptidylcarboxylpeptidase, is a zinc metallopeptidase and key enzyme of the RAS, which cleaves the angiotensin I to produce the angiotensin II, a powerful vasoconstrictor that is a major factor in high blood pressure (Afonso, et al. 23). ACE inhibitors are able to block the conversion of angiotensin I into the active form, angiotensin II. Hence, reduced production of angiotensin II can result in a reduction of blood pressure, with proven health benefits (Persson, et al. 31). Application of the synthetic ACE inhibitor, captopril, is considered as a powerful therapy for treatment of hypertension. However, there are several adverse side effects of the synthetic ACE inhibitors despite their remarkable dedications for managing blood pressure (Massaretto, et al. 13). Foods that contain high amounts of phenolics were reported to be able to induce reduction in blood pressure, particularly for the pre-hypertensive patients, and provide preventive health benefits while avoiding side effects. Besides, many ACE inhibitory activities have been reported for several flavonoid-rich natural products, such as raw and cooked rice, tea, grape, tomato, and carrot (Massaretto, et al. 13, Persson, et al. 31, Mccue, et al. 32, Eriz, et al. 33).

In the present study, the extracts from muscadine grapes at stage I, stage II, and stage III and four standards (i.e., epicatechin, epicatechin gallate, resveratrol, and myricetin) were evaluated to determine their potential antihypertensive activities, or the ACE inhibitory activities. The inhibitory activity of ACE was measured by two synthetic substrates, HHL and FAPGG. The production of hippuric acid (HA) formed by the
hydrolysis of HHL was separated and determined by HPLC, while FAPGG degradation was determined spectrophotometrically.

The ACE inhibitory activity of the extracts, expressed as $IC_{50}$ values (the concentration required to inhibit the original lipase activity by 50%, where lower $IC_{50}$ values indicate stronger ACE inhibitory activities), are presented in Table 5.1, which clearly shows the differences of the inhibitory capacities among the extracts from the muscadine grapes in stage I, II, and III, as well as the test phenolic standards. The stage III extract exhibited the strongest ACE inhibitory activity ($IC_{50} = 10.48 \, \mu g/ml$ with HHL as the substrate and $IC_{50} = 488.42 \, \mu g/ml$ with FAPGG as the substrate), whereas the $IC_{50}$ values for the stage II extract were $16.82 \, \mu g/ml$ with HHL as the substrate and $1080.00 \, \mu g/ml$ with FAPGG as the substrate, and the stage I extract gave the $IC_{50} = 43.36 \, \mu g/ml$ with HHL as the substrate and $IC_{50} = 2021.23 \, \mu g/ml$ with FAPGG as the substrate. The values obtained from the FAPGG as the substrate were 46 to 68 times higher than those using the HHL as the substrate, which means the former assay had a higher analytical sensitivity.

Based on Chapter three, analyses of phenolic compounds and their concentrations of extracts from muscadine grapes during their ripening stages revealed that the stage III grapes contained high amounts of phenolics that seems to be responsible for the strong inhibitory activity. Persson, et al. and Actis-Goretta, et al. reported that flavonoid-rich food were related to inhibit the angiotensin I-converting enzymes and the inhibitory effects were dependent on the types of phenolic compounds.
In addition, the ACE inhibitory activity of the individual phenolic compounds was investigated. Four phenolic standards (i.e., epicatechin, epicatechin gallate, myricetin and resveratrol), which were found in muscadine grapes (see Chapter three), were selected for their inhibitory activities against the ACE. It was reported that epicatechin, epicatechin gallate and myricetin, except resveratrol, were able to inhibit the ACE (Actis-Goretti, et al. ², Balasuriya and Rupasinghe ¹⁰, Liu, et al. ³⁵, Lee, et al. ³⁶). In contrast with these studies, Kwon, et al. ²² reported that resveratrol which was extracted by Lamiaceae family (Mint family) had a strong ACE inhibitory activity compared to other phenolic compounds.

In this study, the individual phenolic compounds showed significantly different inhibitory activities in terms of the \( IC_{50} \) value ranging from 0.81 to 6.00 \( \mu \text{g/ml} \) using the substrate HHL, when epicatechin exhibited the highest inhibitory activity \( (IC_{50} = 0.81 \mu \text{g/ml}) \), followed by 1.36, 1.44, and 6.00 \( \mu \text{g/ml} \) for myricetin, resveratrol and epicatechin gallate, respectively. Meanwhile, resveratrol showed the highest inhibitory activity \( (IC_{50} = 1.11 \mu \text{g/ml}) \), followed by 2.21, 3.15, 5.45 \( \mu \text{g/ml} \) for epicatechin, myricetin, and epicatechin gallate, respectively, using FAPGG as the substrate. As shown above, the aforementioned four phenolic standards, particularly the epicatechin, showed strong ACE inhibitory activities. It has been reported that presence of the several hydroxyl groups in the phenolic compounds could inhibit the zinc-containing metalloproteinases. In addition, the presence of a catechol group in the B ring (3’4’-dihydroxy) and hydroxylation at the 4’-position of the B ring, like epicatechin, could enhance the ACE inhibitory activities (Nwaji, et al. ³⁷).
To further reveal the enzymatic kinetics of the ACE inhibitors, (i.e., the muscadine grape extracts at stage I, II, and III and four phenolics including epicatechin, epicatechin gallate, myricetin, and resveratrol), the Lineweaver-Burk plots of the enzymatic reaction with and without the ACE inhibitors under different concentrations of the substrate of FAPGG (0.4 to 2 mM) were generated and are shown in Figure 5.1A and 5.1B. As shown in Figure 5.1A, the y-intercept values are nearly same regardless of type of the aforementioned inhibitors, indicating that the extracts of muscadine grapes at their different ripening stages acted as competitive inhibitors with respect to the substrate FAPGG. Similar results were obtained for the four phenolic standards that exhibited in a competitive mode against the enzyme (Figure 5.1B). According to the subsequent Michaelis-Menten equation, values of the inhibitive constant, $K_i$, were calculated as 1.17, 0.63, and 0.028 mg/ml of the extracts of stage I, II, and III, respectively, while epicatechin, epicatechin gallate, myricetin, and resveratrol had their $K_i$ values in 1.28, 3.17, 1.83, 0.65 μg/ml, respectively (Table 5.1).

These results demonstrated that the stage I, II, and III extracts, which showed the significant ACE inhibitory activities that was ascribed to the presence of certain bioactive phenolic compounds in muscadine grapes, had the potential to treat hypertension. This finding was consistent with those reported by Balasuriya and Rupasinghe \(^{10}\), who reported that the amounts of flavan-3-ols and anthocyanins were correlated with ACE-inhibitory activity in an *in vitro* test. However, it is worthy of mention that, although phenolic compounds derived from natural sources such as plants are promising
candidates as the ACE inhibitors without side effects (Afonso, et al. 23), it is necessary to furthermore conduct *in vivo* studies to confirm their antihypertensive activities.

**Investigation of anti-obesity effects of extracts of Stage I, stage II, and stage III and phenolics on the pancreatic lipase**

PL, which splits triglycerides into absorbable glycerol and free fatty acids, is considered as a key enzyme in dietary fat digestion and absorption. Hence, suppression of dietary triglyceride absorption by inhibition of PL could be an effective treatment in the regulation of obesity (Ha, et al. 38). Orlistat, which has been approved as a drug as a lipase inhibitor, has a positive effect on anti-obesity via inhibition of the PL activity, although it also has several adverse side effects, such as fecal incontinence, flatulence, and steatorrhea (Dechakhhamphu and Wongchum 39). Thus, the interest of searching natural PL inhibitors has increased in recent years in light of their safety, potential nutritional, and therapeutic effects (Büyüktuncel, et al. 40). Herein, the effects of the extracts of muscadine grapes during their ripening stages on inhibition of PL activities were investigated using pNPP as the substrate. Different concentrations at 2, 4, 6, 8, and 10 mg/mL of the crude extracts of stage I, II, and III of the muscadine grapes were measured in regards of their *IC*<sub>50</sub> values (*Table 5.2*). The pancreatic lipase activity was effectively inhibited by the polyphenol-rich extracts from the muscadine grapes. All the extracts exhibited the inhibitory effects in dose-dependent manners with the *IC*<sub>50</sub> values at 0.12, 0.35 and 0.8 mg/ml of the extracts from the stage I, II, and III grapes, respectively. The stage I extract exhibited a stronger inhibitory effect with the *IC*<sub>50</sub> of 0.12 mg/mL, which was about one third and one sixth of the *IC*<sub>50</sub> values of those extracted from the
stage II and stage III (i.e., 0.35 and 0.8 mg/mL, respectively). This demonstrated the extract of unripe muscadine grapes (i.e, stage I) possessed a very strong PL inhibitory activity. This is the first time to report that the crude extracts of ‘Cowart’ muscadine grapes had strong anti-lipase activities.

In addition, it was observed that the PL inhibitory effects of the extracts had significantly positive correlations with their total phenolic compounds ($R^2 = 0.958$). Several other studies have also confirmed the positive relationships between the inhibitory activity of PL inhibitors and the total phenolic content (Dechakhamphu and Wongchum 39, Cai, et al. 41).

Besides, four selected phenolics, i.e., epicatechin gallate, epicatechin, myricetin and resveratrol, showed their $IC_{50}$ values at 0.65, 0.44, 0.24, 0.50 mg/ml against the PL, respectively (Table 5.2). Particularly, myricetin was found to be a more potent inhibitor, which existed in a high concentration in the extract of stage I (3.79 ± 0.28 mg/100g dried sample) than that in stage II (2.83 ± 0.36 mg/100g dried sample) and III (1.62 ± 0.10 mg/100g dried sample).

To investigate the dynamic parameters of the PL inhibition, the Lineweaver-Burk linear regression lines, based on the double-reciprocal plot of the M-M equation for the PL inhibition, was constructed to determine the enzymatic kinetics using the ρNPP as the substrate under a series of different concentrations of the selected inhibitors (i.e., extracts from the stage I, II, III, epicatechin gallate, epicatechin, myricetin, and resveratrol). This study has expanded our knowledge from a previous work by You, et al. 42, who demonstrated that muscadine grapes could affect the pancreatic lipase. The results are
profiled in Figure 5.2 and listed in Table 5.2. The former also showed that the inhibitory activities of the extracts had their same $V_{max}$ values in the Y axis, which means the extracts of muscadine grapes (Figure 5.2A) have inhibited the pancreatic lipase in a competitive mode. This result is consistent to a previous report (You, et al. 42). Moreover, the $K_m$ value of control against the PL was determined at 0.64 mg/ml and its $V_{max}$ value was 0.12 mg/ml min$^{-1}$. Also, the inhibition constants ($K_i$) of the extracts were decreased in the following order: stage III > stage II > stage I (with $K_i$ at 0.31 > 0.14 > 0.05 mg/ml, respectively), while the $K_i$ values of the four references were 0.25, 0.20, 0.17, 0.09 mg/mL, corresponding to epicatechin gallate, resveratrol, epicatechin, myricetin, respectively (Figure 5.2B and Table 5.2). The inhibitory activities of the test phenolics against the PL also demonstrated that they were stronger inhibitors than the muscadine extracts. However, compared with previous studies, it was observed that there existed slightly different values of $K_m$ and $V_{max}$, which was ascribed to the differences of the experimental conditions such as composition of buffer solution, pH and temperatures (Brás, et al. 43).

**Investigation of anti-wrinkle formation and anti-hyperpigmentation effects of extracts of Stage I, stage II, and stage III and phenolics on collagenase, elastase, and tyrosinase**

Human skin is an important tissue that protects the internal organs from damage by the outside environment. One of the most dangerous environmental factors is UV radiation, which is a major cause of hyperpigmentation, loss of skin elasticity, and wrinkling (Lee, et al. 44). Inhibiting the activities of enzymes (e.g., tyrosinase,
collagenase, and elastase) involved in these processes can treat and prevent skin aging and block the melanogenesis pathway that leads to skin browning (Wittenauer, et al. 3, Hong, et al. 45).

Tyrosinase is a key enzyme involved in two stages of melanin biosynthesis in melanocytes, including the hydroxylation of monophenols to o-diphenols (monophenolase activity) and the oxidation of the o-diphenols to o-quinones (diphenolase activity). Many forms of hyperpigmentation, for example, dark and discolored spots on the skin, are caused by excess melanin formation. Tyrosinase inhibitors not only block the formation of spots and freckles on skin, but also inhibit enzymatic reactions involved in food deterioration (Hong, et al. 45). Hence, these inhibitors are widely used in the cosmetics and food industries.

In vitro tyrosinase activity was measured to analyze the enzymatic inhibition by the muscadine extracts, as well as the aforementioned phenolics. To determine the $V_{\text{max}}$ and $K_m$ constant, a series of substrate solutions of L-tyrosine (e.g., 0.4, 0.8, 1.2, 1.6, and 2.0 mM) were used in the assay, and the data were used to construct the Lineweaver–Burk plots from the Michaelis–Menten equation (Figure 5.3A). The results showed that all of the extracts had potent the inhibitory activity against the tyrosinase, with $K_i$ values ranging from 0.61 to 1.28 mg/ml. For all of these extracts, the linear regression lines in $1/V$ versus $1/[S]$ were produced with very close y-intercept values but different slopes. The nearly same values of $V_{\text{max}}$ indicated that all of the extracts were competitive inhibitors of the enzyme. That is, the extracts contained compounds that were similar to the substrate and could compete it at the active site of the enzyme. The HPLC analysis
has revealed some bioactive phenolic compounds in the muscadine grape extracts which was discussed in Chapter Three. High concentrations of phenolic compounds such as epicatechin, epicatechin gallate, resveratrol, and myricetin were found in the grape extracts. In this context, the inhibitory effects of those phenolic compounds against the tyrosinase were also analyzed. Similar to the grape extracts, the phenolic compounds had very close points on the y-axis with different slopes of the linear regressions on their Lineweaver–Burk lines (Figure 5.3B), indicating that they were competitive inhibitors of the substrate, and could function as depigmentation agents (Parvez, et al. 46).

The tyrosinase inhibitory activity of the three grape extracts and the four phenolics is summarized in Tables 5.3 and 5.4. All of the extracts inhibited the mushroom tyrosinase activity in a dose-dependent manner (59.54–2920 μg/mL). The IC$_{50}$ values (see Table 5.3 and 5.4) were higher for the grape extracts than for the purified standard phenolic compounds, indicating that the phenolic standards had stronger inhibitory activities. On the other hand, the inhibitory effect of the stage III extract was stronger than those of the stage I and II extracts. This observation was consistent with the results to the inhibition on PL.

Furthermore, the tyrosinase inhibitory activity of the individual phenolic compounds (i.e., epicatechin, epicatechin gallate, myricetin, and resveratrol) were compared. Epicatechin had the strongest inhibitory activity, which was explained that it can penetrate the skin more easily than other chemicals because of its hydrophobic property (Hong, et al. 45). In addition, previous studies reported that the four test phenolics had stronger tyrosinase inhibitory activities than other phenolic compounds,
such as quercetin and kaempferol (Kim and Uyama 47, Karim, et al. 48, Chang 49). The stronger inhibitory activity of the stage III extract against the tyrosinase was likely due to higher concentrations of certain phenolic compounds in the extract. Our results also implied that the tyrosinase inhibitory activity of the extracts were not strongly correlated with its total phenolic content ($R^2=0.40$), but the enzymatic inhibition could be affected by individual bioactive phenolic compounds. This is because the total phenolic content does not necessarily include all of the possible inhibitors, and the inhibitory activity depends not only on the concentrations of individual inhibitors, but on their structures and the interactions among them (Djeridane, et al. 50).

Collagenase and elastase might be responsible for the dehydration and wrinkling of skin. Collagen and elastin are components of the connective tissue of skin. Collagen accounts for 70 – 80% of the skin’s weight and provides structural stability, while elastin accounts for 2 – 4% of the dermis matrix and provides elasticity. Elastin is associated with collagen fibers under the epidermis (Hong, et al. 45). The inhibitory effects of grape extracts on both collagenase and elastase were evaluated using appropriate substrates.

To determine the effects of inhibitors against the collagenase, a serial of concentrations of the grape extracts and the aforementioned phenolic compounds (i.e., epicatechin, epicatechin gallate, resveratrol, and myricetin) were investigated like those for the tyrosinase. The assay monitored the effects of the inhibitors at varying concentrations on the collagenase-catalyzed hydrolysis of the synthetic substrate FALGPA. The inhibition of collagenase by the extracts and the phenolics were determined via the Lineweaver–Burk plots shown in Figure 5.4A and Figure 5.4B. The
kinetic parameters of the enzyme, (i.e., $K_i$ and $V_{max}$), are shown in Table 5.3 and 5.4. As shown in Figure 5.4A and Figure 5.4B, the linear regression lines of all the inhibitors crossed at the same point 0.0513 on the Y axis, indicating that the $V_{max}$ values were as same as that of the control. This indicated that the substrate was competitively inhibited by the aforementioned inhibitors at the active site of the enzyme. The $K_i$ values for the grape extracts in the FALGPA hydrolysis assay ranged from 1.39 to 2.16 μg/ml, and the corresponding $IC_{50}$ values were 5.53, 4.15, and 3.55 μg/ml for the stage I, II, and III, respectively (Table 5.4). All of the extracts and phenolics inhibited the collagenase in a dose-dependent manner. The stage III extract showed the lowest $K_i$ and $IC_{50}$ values, indicating that its inhibitory ability against the collagenase was stronger than its counterparts in the stage I and II. In the same bioassay, the $IC_{50}$ values of the standard phenolic compounds were as follows: 4.21 μg/ml for epicatechin, 8.56 μg/ml for epicatechin gallate, 4.94 μg/ml for myricetin, and 5.39 μg/ml for resveratrol (Table 5.4). These data indicated that these compounds, especially epicatechin, possessed comparable inhibitory activity of the grape extracts.

The inhibitory effects of the muscadine grape extracts and the aforementioned phenolics against the elastase were also evaluated by monitoring the oxidation of AAAPVN (0.4–2 mM). Similarly, the inhibitory model of the inhibitors for elastase was determined by the Lineweaver–Burk plots, which are shown in the Figure 5.5A and 5.5B. The linear regression lines show the values of the maximum velocity ($V_{max}$) of the reactions did not change as the extracts and phenolic compounds changed, indicating that
the aforementioned extracts and phenolic chemicals functioned as competitive inhibitors of the substrate for all three stages. In this study, the $K_i$ values of the extracts and phenolic compounds ranged from 0.18 to 2.13 μg/ml (Tables 5.3 and 5.4). The range of $IC_{50}$ values of the muscadine grape extracts and the selected phenolic compounds showed that they were potent inhibitors of the enzyme, and the stage III extract had the strongest elastase inhibitory activity.

The results of the collagenase and elastase inhibitory assays indicated that all of the grape extracts had significantly inhibitory activities. Since the inhibition of collagenase and elastase by the muscadine grape extract has not been studied previously, our results can not be compared with other studies on V. rotundifolia. Also, it is inappropriate to compare our results with those of other plant species, since our experimental conditions (extraction method, incubation time, type of substrate and enzyme used for analysis) were different from those used in other studies (Wittenauer, et al. 3). However, review of the previous studies on anti-collagenase and anti-elastase activities of other plant extracts is still valuable. Thring, et al. 26 analyzed the phenolic extracts of 21 plants, and found that white tea extract had the strongest inhibitory activities against the collagenase and elastase (87% and 89% inhibition at 25 μg/mL, respectively), which might be due to the highest phenolic content in the white tea extract. Hong, et al. 45 isolated phenolic compounds from green tea that effectively inhibited the collagenase and elastase. A few studies have reported other health benefits of phenolic compounds, including sedative effects on skin, and inhibitory effects against photo-aging and some cancers (Yusuf, et al. 51, Miao, et al. 52). Phenolic extracts have been shown to
be able to inhibit proteases that degrade skin proteins such as collagen and elastin. Polyphenols in the grape pomace from white wine (*Vitis vinifera* L., cv, ‘Weisser Riesling’) showed anti-collagenase and anti-elastase activities (Wittenauer, et al. 3). The ‘natural’ phenolic inhibitors are more preferred by customers since they can avoid some limitations of synthetic inhibitors in concern of their broad activities and toxicity (Hong, et al. 45). Hence, plant extracts are in increasing demands for their applications in many cosmetic products.

Our results showed that the stage III extract had a stronger inhibitory effect on the elastase than on the collagenase. The $K_i$ and $IC_{50}$ values of epicatechin, epicatechin gallate, myricetin, and resveratrol (Table 5.4) confirmed that these natural phenolic compounds have both potent collagenase- and elastase-inhibitory activities. The higher collagenase- and elastase-inhibitory activity of the stage III extract was attributed to the high concentrations of selected phenolic compounds such as resveratrol, epicatechin gallate, and epicatechin. These compounds represent the major functional components with inhibitory activities against both enzymes.

Furthermore, unlike the tyrosinase, there were a certain degree of positive correlations between the total phenolic content in the extracts and the $K_i$ values of the collagenase and elastase assays ($R^2=0.6839$ for collagenase; $R^2=0.6707$ for elastase).

In summary, the stage III extract could strongly inhibited the tyrosinase, collagenase, and elastase activities. These results demonstrated that muscadine grapes are a good source of nutraceutical products that may be beneficial for human health.
Conclusions

The extracts from muscadine grapes at three different ripening stages (stage I, II, and III) contained different concentrations of bioactive compounds. The muscadine grape extracts showed inhibitory activities against the ACE, tyrosinase, collagenase, and elastase. The extract from the ripen grapes (stage III) was more effective on inhibiting the ACE, collagenase, and elastase activities while the stage I extract had higher inhibitory activities on pancreatic lipase and tyrosinase. These properties were partially attributed to the inherent phenolic constituents, such as epicatechin, epicatechin gallate, resveratrol, and myricetin, which are abundant in the extracts of muscadine grapes.

Overall, this study suggested that the extracts of muscadine grapes at stage I, II, and III may be used as alternative treatments for hypertension, obesity, anti-aging, and whitening creams for skin agents. Particularly, the extracts of stage I and stage II grapes also possess potent bioactive compounds, even though the grapes are not suitable for consumption at these stages.
Table 5.1. Inhibitory activity of (a) muscadine grape (*Vitis rotundifolia*) extracts, (b) standards phenolic compounds against the angiotensin I-converting enzyme (ACE), and (c) $K_i$ values using FAPGG as a substrate

(a)

<table>
<thead>
<tr>
<th>Sample</th>
<th>ACE IC$_{50}$ (μg/ml)</th>
<th>HHL</th>
<th>FAPGG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>43.36</td>
<td>2021.23</td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>16.82</td>
<td>1080.00</td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>10.48</td>
<td>488.42</td>
<td></td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Sample</th>
<th>ACE IC$_{50}$ (μg/ml)</th>
<th>HHL</th>
<th>FAPGG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epicatechin</td>
<td>0.81</td>
<td>2.21</td>
<td></td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>6.00</td>
<td>5.45</td>
<td></td>
</tr>
<tr>
<td>Myricetin</td>
<td>1.36</td>
<td>3.15</td>
<td></td>
</tr>
<tr>
<td>Resveratrol</td>
<td>1.44</td>
<td>1.11</td>
<td></td>
</tr>
</tbody>
</table>

(c)

<table>
<thead>
<tr>
<th>Sample</th>
<th>$K_i$ (μg/ml)</th>
<th>FAPGG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>1170</td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>630</td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Epicatechin</td>
<td>1.28</td>
<td></td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>3.17</td>
<td></td>
</tr>
<tr>
<td>Myricetin</td>
<td>1.83</td>
<td></td>
</tr>
<tr>
<td>Resveratrol</td>
<td>0.65</td>
<td></td>
</tr>
</tbody>
</table>

Note: IC$_{50}$ is the concentration of an extract or a compound resulting in 50% inhibition of the enzymatic reaction. Replication of three (n=3)
Table 5.2. $K_i$ and $IC_{50}$ values for (a) muscadine grape extracts at three stages (stage I, II, and III), and (b) standard phenolics against pancreatic lipase

(a)

<table>
<thead>
<tr>
<th>Stages</th>
<th>$K_i$ (mg/ml)</th>
<th>$IC_{50}$ (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>0.05</td>
<td>0.12</td>
</tr>
<tr>
<td>Stage II</td>
<td>0.14</td>
<td>0.35</td>
</tr>
<tr>
<td>Stage III</td>
<td>0.31</td>
<td>0.8</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Standards</th>
<th>$K_i$ (mg/ml)</th>
<th>$IC_{50}$ (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epicatechin</td>
<td>0.17</td>
<td>0.44</td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>0.25</td>
<td>0.65</td>
</tr>
<tr>
<td>Myricetin</td>
<td>0.09</td>
<td>0.24</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>0.20</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Replicates of three (n=3)
Table 5.3. $K_i$ and $IC_{50}$ values for muscadine grape extracts against tyrosinase, collagenase, elastase.

<table>
<thead>
<tr>
<th>Stages</th>
<th>Tyrosinase</th>
<th>Collagenase</th>
<th>Elastase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_i$ (mg/ml)</td>
<td>$IC_{50}$ (mg/ml)</td>
<td>$K_i$ (µg/ml)</td>
</tr>
<tr>
<td>Stage I</td>
<td>0.61</td>
<td>1.39</td>
<td>2.16</td>
</tr>
<tr>
<td>Stage II</td>
<td>1.28</td>
<td>2.92</td>
<td>1.62</td>
</tr>
<tr>
<td>Stage III</td>
<td>0.73</td>
<td>1.67</td>
<td>1.39</td>
</tr>
</tbody>
</table>

Note: $K_i$ is the equilibrium dissociation constant for the inhibitor, calculated from the Michaelis–Menten equation, and $IC_{50}$ is the concentration resulting in 50% inhibition of the enzymatic reaction. Replicates of three (n=3)
Table 5.4. $K_i$ and $IC_{50}$ values for selected reference compounds against tyrosinase, collagenase, and elastase.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Tyrosinase</th>
<th>Collagenase</th>
<th>Elastase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_i$ (μg/ml)</td>
<td>$IC_{50}$ (μg/ml)</td>
<td>$K_i$ (μg/ml)</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>4.31</td>
<td>59.54</td>
<td>1.65</td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>8.57</td>
<td>118.45</td>
<td>3.35</td>
</tr>
<tr>
<td>Myricetin</td>
<td>11.78</td>
<td>162.91</td>
<td>1.93</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>8.79</td>
<td>121.47</td>
<td>2.11</td>
</tr>
</tbody>
</table>

Note: $K_i$ is the equilibrium dissociation constant for the inhibitor, calculated from the Michaelis–Menten equation, and $IC_{50}$ is the concentration resulting in 50% inhibition of the enzymatic reaction. Replicate of three (n=3)
Figure 5.1. Lineweaver-Burk plots of the Angiotensin I-converting enzyme inhibitory activity using the substrate FAPGG, which was inhibited by (A) the extracts from grapes at three stages of ripening and (B) selected phenolic compounds. Replicate of three (n=3)
Figure 5.2. Lineweaver-Burk plots of pancreatic lipase inhibitory activity of (A) the extracts from grapes at three stages of ripening and (B) selected phenolic compounds. Replicate of three (n=3)
Figure 5.3. Lineweaver-Burk plots of tyrosinase inhibitory activity of (A) the extracts from grapes at three stages of ripening and (B) selected phenolic compounds. Replicate of three (n=3)
Figure 5.4. Lineweaver-Burk plots of collagenase inhibitory activity of (A) the extracts from grapes at three stages of ripening and (B) selected phenolic compounds. Replicate of three (n=3)
Figure 5.5. Lineweaver-Burk plots of elastase inhibitory activity of (A) the extracts from grapes at three stages of ripening and (B) selected phenolic compounds. Replicate of three (n=3)
References

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CHAPTER SIX

SUMMARY

Muscadine grape is recognized as a unique native fruit in southeastern United States, in light of its special aroma and high concentration of bioactive phytochemicals, which exhibit anti-cancer, anti-diabetes, anti-inflammatory and antimicrobial activities. Despite much research on characterization of volatile and phenolic compounds in other grapes, information about the VOCs, bioactive phenolic compounds, and health benefits of muscadine grapes (*Vitis rotundifolia*), particularly in their different ripening stages, is limited. In addition, production of one species of muscadine grapes, ‘Cowart’, has decreased in recent years, although it has a desirable flavor and taste. Hence, the general objective of this research was to investigate the effect of ripening stages (i.e., stage I, II, and III) of muscadine on VOCs, and phenolic compounds in the grapes, and explore the inhibitory effects of the muscadine extracts and its inherent phenolic compounds on some enzymes, such as angiotensin-I-converting enzyme (ACE), pancreatic lipase, tyrosinase, collagenase and elastase. The major findings of this study are summarized below:

As described in Chapter 2, VOCs of muscadine grapes in three ripening stages were extracted, identified, and quantified by a HS-SPME coupled with GC-MS. The optimal condition of extracting the volatile muscadine compounds was to use an SPME fiber with a mixed coating (DVB/CAR/PDMS, 50/30 μm) at 60 °C for 30 minutes. Under the optimal condition, 28 aromas, including fruity short-chain esters, alcohols, terpenes and carbonyl compounds, were extracted and characterized based on comparison with mass spectra and Kovats indices. Based on the PCA, the volatile chemicals of muscadine
grapes in three different ripening stages had significantly different aroma patterns in light of their concentrations. Among the volatiles, butyl-2-butenoate, hexyl acetate, propyl acetate, ethyl trans-2-butenoate, hexyl-2-butenoate, ethyl acetate, butyl acetate, 1-octanol, ethyl hexanoate, and β-citral, were found to be the distinct aromas that were only detected in stage III. Nonanal, decanal, and β-citronellol were another group of distinguished chemicals that were absent in the samples of stage II, which made them the chemical markers to differentiate the sample of stage II from other samples. Regarding other volatile chemicals that were accumulated during the ripening stages, terpenoids are another biomarker to indicate the maturity stage. Although α-terpinolene and trans-geraniol were the predominant volatile chemicals in muscadine grapes, myrcenol, β-ocimene, and l-limonene were detected only in the stage I samples. In contrast with the terpenoids, volatile esters that were associated to fruity, floral and pleasant odors were only detected in fully ripen grapes in stage III. In this context, the grapes in stage III were highly favorable to be freshly consumed or made into desirable wines in light of their desirable and rich amount of aromas. This finding is expected to assist farmers to harvest the muscadine grapes in the appropriate time.

The characterization and evaluation of the phenolic compounds and antioxidant activities of the muscadine grapes during their ripening stages were described in Chapter 3. Firstly, muscadine grapes were examined for TPC and TFC, and evaluated for their antioxidant capacities by DPPH and ABTS in vitro assays. Overall, the values of TPC (143.64 – 439.74 mg GAE/100g) and TFC (47.41 – 83.96 mg QE/100g) were in the highest at stage III, when the highest antioxidant capacities in terms of the DPPH (IC$_{50}$
value of 6.26 – 12.86 mg/ml) and ABTS ($IC_{50}$ value of 5.23 – 12.00 mg/ml) radical scavenging capacities showed in the fully matured muscadine grapes (stage III). Besides, the study suggested that there was not a positive correlation between the total phenolic content and antioxidant capacity. This phenomenon could be ascribed to the following factors, such as the presence of different active compounds in muscadine grapes that can affect the antioxidant capacity and their synergistic effects, the experimental conditions and methods used for measuring the antioxidant activities.

Furthermore, biosynthesized phenolic compounds at three different ripening stages were qualified and quantified by a HPLC equipped with a DAD. The eleven phenolic compounds in the muscadine grapes during their ripening stages were characterized. Particularly, the phenolic compounds, including resveratrol, epicatechin, ellagic acid, catechin and kaempferol, were identified as major phenolic compounds in the stage III, while gallic acid and protocatechuic acid were in low concentrations at stage I. Low concentrations of $\rho$-coumaric acid and epicatechin gallate were indicative of the muscadine grapes in stage II. As a the result, the muscadine grapes at stage III are more appropriate for fresh consumption in light of its aroma and taste, while the counterparts in stage I and II can be also be used as a source of nutraceutical products since they contain high amounts of phytochemicals.

Anthocyanins have attracted more attentions recently due to their health benefits and their natural colors responsible for the red, purple and blue colors of many fruits, vegetables and plants. In Chapter 4, the effect of pH values of solvents was investigated on the extraction yield of anthocyanins from muscadine grapes at stage III, which
contains highest anthocyanin content compared to that in the stage I and II. A solvent at pH 3, which was consisted of 80% methanol, 20% of distilled water and small amount of 1M HCl at room temperature, could extracted the highest concentration of anthocyanin in an amount of 272.19 mg cyanidin-3,5-diglucoside equivalent/100g DW from muscadine grapes. This results was ascribed to fact that acidic solvents are more suitable for extraction of anthocyanin because they are more stable and resistant to the chemical degradation at low pH. Additionally, their antioxidant activities in terms of the DPPH radical scavenging assay (IC$_{50}$ = 12.79 and 13.81 mg/ml for the pH 3 and pH 5 extracts, respectively) and ABTS assay (IC$_{50}$ = 12.46 and 12.67 mg/ml for the pH 3 and pH 5 extracts, respectively) were consistent with the high concentrations of anthocyanins. The condition for yielding the highest anthocyanidins content was optimized on an acid hydrolysis at 100 °C for 60 minutes. Furthermore, four anthocyanidins, (i.e., delphinidin, cyanidin, peonidin, and malvidin), were obtained from the acidic hydrolyses under pH 3, 5, 7, 9, 11. Based on the hierarchical clustering, the samples extracted from pH 3 and 5 had similar anthocyanidin patterns that have relatively higher concentrations of delphinidin and cyanidining, which were unstable chemicals that are not good for wine production.

Chapter 5 investigated the enzymatic inhibitory activities of the muscadine grape extracts during their ripening stages, as well as the phenolics (i.e., epicatechin, epicatechin gallate, myricetin and resveratrol) against the five enzymes, (i.e., ACE, PL, tyrosinase, collagenase and elastase). The results of this study clearly indicated that the extracts of muscadine grapes and the selected representative phytochemicals possessed
the potential as nutraceutical agents for their anti-hypertensive, anti-obesity and anti-skin diseases. Furthermore, the lineweaver-burk plots of the enzymatic inhibitory reactions revealed that the aforementioned inhibitors obeyed the competitive mode against all enzymes. According to the values of $K_i$ and $IC_{50}$, crude extracts of stage III was found to have greater ACE, collagenase and elastase inhibitory effects than its counterparts of stage I and III due to their inherent high concentrations of bioactive phenolic compounds, whereas the stage I extract had stronger inhibitory effects on pancreatic lipase and tyrosinase. The results showed TPC was highly correlated with pancreatic lipase and tyrosinase while inhibitory activities of the ACE, collagenase, and elastase were not correlated with the TPC of the extracts. This phenomenon was ascribed to that enzymatic inhibition activities are influenced by the presence of certain bioactive chemicals rather than a total phenolic content.

Overall, the ‘Cowart’ muscadine grapes in all the stages possessed strong antioxidant activities and enzymatic inhibitory activities due to their high concentrations of phenolic compounds, which could be used as nutraceuticals.