HYDROLYTIC AND ANTIOXIDANT PROPERTIES OF ELLAGIC ACID AND ITS PRECURSORS PRESENT IN MUSCADINE GRAPE

By

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Muscadine grape (*Vitis rotundifolia*) has potential health-promoting benefits and antioxidant properties from characteristic phytochemicals such as ellagic acid, ellagic acid glycosides, and ellagitannins. To provide fundamental information on ellagic acid derivatives in this commodity, typical polyphenolic compounds were characterized in eight cultivars. All polyphenolics generally increased as fruit ripened and the highest concentrations were located in the skins. Hot-pressed juices contained considerably lower polyphenolic concentrations than were present in whole grapes with actual recovery varying widely among cultivars. Antioxidant capacity was appreciably influenced by cultivar, maturity, and location in the fruit with good correlations to soluble phenolics found in both methanolic and ethyl acetate extracts (r=0.83 and 0.92, respectively). Glycosidic forms of ellagic acid and major flavonoids were isolated by a series of solid-phase extractions. By using HPLC-MS/PDA, chemical identities were elucidated as ellagic acid xyloside, ellagic acid rhamnoside, myricetin rhamnoside, quercetin

rhamnoside and kaempferol rhamnoside. Ellagitannins, a major source for hydrolytic free ellagic acid, were present in the C18 non retained fraction and their molecular weights having never been reported were determined.

Because of the lack of data on hydrolytic properties of ellagic acid precursors, central composite design was applied to demonstrate the evolution of hydrolytic ellagic acid depending on various time-temperature combinations in response surface methodology. Hydrolysis of ellagic acid precursors would be more temperature-dependent than time-dependent, and the resultant additional free ellagic acid showed good correlation to antioxidant capacity ($r=0.83$). Using enzymes, β-glucosidase (E.C. 3.2.1.21) or tannase (E.C. 3.1.1.20) is an alternative application to hydrolyze ellagic acid precursors, β-glucosidase showed better reactivity on ellagic acid derivatives compare to tannase.

During storage, ellagic acid glycosides were relatively stable with ellagitannin hydrolysis the major source for evolution of free ellagic acid in a juice system. Free ellagic acid is partially responsible for the formation of insoluble sediments in addition to other juice constituents such as metal ions or insoluble pectins. Ascorbic acid fortification and air sparging did not significantly affect concentrations of ellagic acid derivatives in stored juices. However, a mild heat treatment influenced ellagitannin hydrolysis and was likely associated with activation of natural enzymes present in the juice.

Results provided by these studies suggest beneficial reasons to consume muscadine grapes leading to improve the market value of this crop not only as fresh grapes, juice or wine but also as health promoting components in various types of processed food.

CHAPTER 1
INTRODUCTION

Justification

Muscadine grapes (*Vitis rotundifolia*) are commonly cultivated in the southeastern U.S. as an excellent alternative fruit crop, because many traditional *Vitis* species are impossible to survive due to Pierce's disease and typical climatic characteristics in this region. Muscadine grapes are favorably consumed as fresh fruit, juice, wine or jelly not only for distinguished aroma and flavor characteristics but also for positive health benefits from characteristic phytochemicals including ellagic acid/ellagic acid derivatives and anthocyanins 3,5-diglucosides. Presence of these compounds has been associated with quality defects such as rapid color deterioration of anthocyanin 3,5-diglucosides and the formation of insoluble sediments that may be affected by ellagic acid derivatives during storage. To solve the problem with sediment formation, research has focused on removing free ellagic acid from the matrix using various processing techniques (*1*, *2*). However, none of the trials successfully solved the sedimentation problem due to lack of fundamental information on these compounds in muscadine juice or wine. Therefore, the primary objective of this work is to selectively identify and determine those compounds that impact levels of free ellagic acid in efforts to determine their concentration and stability in fresh and processed muscadine grapes. Mechanisms for their hydrolysis and relative changes are evaluated that may reveal key components affecting their radical scavenging properties and the accumulation of free ellagic acid during storage of muscadine juice. These studies also seek to determine the antioxidant polyphenolics in

muscadine grapes as influenced by their location in the grape, those extracted into juice, and solubility in various fractionations as a function of cultivar and maturity. These evaluations are beneficial for exploring biochemical synthesis and/or fate of pro-ellagic acid compounds. Finally, chemical and physical characteristics of these identified compounds will lead to possible mechanism(s) concerning their hydrolysis and release of free ellagic acid into insoluble sediments. This information will add market value and significantly increase marketability for this under-utilized grape by identification of novel compounds and evaluation of their antioxidant potential.

Objectives

- Objective 1: To quantify the antioxidant polyphenolics in muscadine grapes as influenced by their location in the grape, juice production, and polyphenolic fractionation as a function of cultivar and maturity.
- Objective 2: To isolate and identify ellagic acid glycosides and ellagitannins using advanced HPLC methodologies (PDA, MS) and evaluate their antioxidant capacity.
- Objective 3: To investigate the hydrolytic and oxidative properties of individual ellagic acid derivatives and other polyphenolics affecting concentrations and antioxidant capacity.
- Objective 4: To determine the major source of hydrolytic ellagic acid, and to elucidate the effects of ascorbic acid fortification and oxidation on changes of ellagic acid derivatives in juice system.

CHAPTER 2
LITERATURE REVIEW

Muscadine Grapes

Muscadine grapes are botanically categorized under the genus *Vitis*, which consists of two subgenera: *Euvitis* ("bunch grapes") and *Muscadinia* ("berry grapes") (*3*). *Euvitis* includes diverse species of grapes including *V. vinifera*, which is the most common grape in the world. *Muscadinia* can be represented as an American grape species *V. rotundifolia* Michaux, muscadine grape, and *V. munsoniana* Simpson. When muscadine grapes are grown for commercial harvest, the majority of plantings are *V. rotundifolia* (*4*) and they are indigenously found in the southeastern United States, from Delaware to central Florida and along the Gulf of Mexico to eastern Texas (*3*). In these regions, muscadine grapes are favorably cultivated fruit crops since other grapes are hard to survive in typical climate like humid summers and warm winters (*5*, *6*). Another benefit of growing muscadine grapes is that *V. rotundifolia* has excellent resistance to pests and various diseases, namely Pierce's disease, which is a lethal disease of the grape vine caused by the bacterium *Xylella fastidiosa* and the main obstacle for production of *V. vinifera* in the south and southeastern states (*3*). Unlike bunch grapes, muscadine grape fruits develop in round clusters, containing approximately 4–16 grapes. The fruit are round or oval, larger varieties may reach 26 mm in diameter and weigh from 3-10 g each. Fruits can range from light-skinned (green, pearly white, or bronze) to dark red in color and most have a tough, leathery skin with the pulp inside ranging from meaty, to melting, or juicy (*6*). At present, home gardening or commercial production is provided by over 70

different cultivars, the oldest of which includes Scuppernong (green), Carlos (green), Doreen (green), Noble (red), and Magnolia (green). These cultivars are consumed as fresh fruit, juice, wine, or jelly (*7*). In the production of either fermented or unfermented muscadine grape products, the physicochemical composition of fruit is one of the most important factors affecting overall quality and acceptability. In direct comparisons with *V. vinifera*, muscadine grapes generally have a lower soluble solids concentration (SSC) and titratable acidity (*8*) ranging from 10-18% (13.2% on average) and 0.39-1.5% (0.84% on average) expressed as tartrate equivalents, respectively; resulting in a pH range of 2.9-3.4 (pH 3.14 on average). Muscadine "hulls" (skin plus firmly attached tissue layer) were found to contain significantly higher organic acid concentrations than the pulp on a per weight basis. This helps to explain how juices that are immediately pressed have lower titratable acidity values compared to those for hot-pressed juices or skin fermentation (*9*, *10*).

Phytochemicals in Muscadine Grapes

Ellagic Acid and Its Precursors

Ellagic acid (Figure 2-1) has been found in many woody plants and in diverse fruits and nuts in various concentrations. Interestingly ellagic acid is present only in *Vitis rotundifolia* but not in *Vitis vinifera*. Ellagic acid is believed to be formed by the hydrolytic release from ellagic acid derivatives including ellagic acid glycosides (Figure 2-2, A-C) and ellagitannins (Figure 2-3). The presence of ellagic acid in various fruits and nuts was determined for the purpose of botanical classification, and was identified in strawberries, blackberries, and walnuts (*11*). Concentrations of ellagic acid in various fruits and nuts were determined as total ellagic acid, which was hydrolytic ellagic acid from ellagic acid precursors followed by complete hydrolysis with acid (*12*). This was

done because analyses on individual ellagic acid derivatives were difficult with lower analytical techniques and in the absence of authentic standards. Appreciable concentrations of ellagic acid were detected after acid hydrolysis in strawberries (630 µg/g), raspberries (1500 µg/g), blackberries (1500 µg/g), walnuts (590 µg/g), pecans (330 µg/g), and cranberries (120 µg/g). In strawberries, most ellagic acid (95.7%) was found in pulp, and the remainder was found in seeds. However, the seeds of raspberries contained 87.5% ellagic acid, while the pulp had 12.2%. Amakura et al. (*13*) determined ellagic acid levels in fresh and processed fruits by a simple and rapid high performance liquid chromatographic (HPLC) method that did not involve a hydrolysis step. Values for similar commodities (blackberry, 87.6 µg/g; strawberry, 17.7 µg/g; and raspberry, 5.84 µg/g) were appreciably lower without the hydrolysis step; possibly indicating the presence of ellagic acid precursors including ellagic acid glycosides and ellagitannins.

Ellagic acid glycosides are forms of a single sugar moiety linked to the hydroxyl at the 4-position of ellagic acid, and five or six different ellagic acid glycosides have been separated in raspberry (*14*, *15*): ellagic acid 4-acetylxyloside, ellagic acid 4-arabinoside, and ellagic acid 4-acetylarabinoside (Figure 2-2;). Muscadine grapes also contain two ellagic acid glycosides, which are characterized by 3-7 nm hypsochromic shifts in UV spectra (252 and 360 nm) (*16*). However, free ellagic acid and ellagic acid glycosides

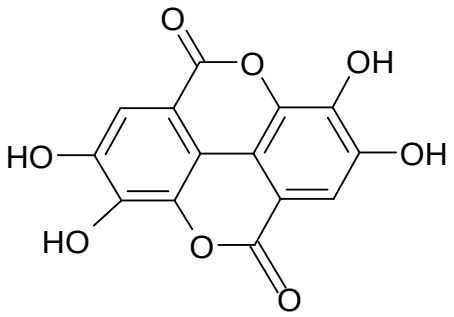

Figure 2-1. Ellagic acid chemical structure

contribute to only part of the total ellagic acid on acid hydrolysis; highly indicating the presence of ellagitannins in muscadine grapes. Ellagitannins (Figure 2-3) are characterized as hydrolysable conjugates containing one or more hexahydroxydiphenoyl (HHDP) groups esterified to a sugar, mainly glucose. HHDP groups are released from the main structure leading to spontaneous conversion into ellagic acid by hydrolysis (Figure 2-4). Specific information on ellagic acid derivatives are lacking for muscadine grapes, but the presence of ellagic acid and its derivatives in muscadine grapes may add value and marketability to the crop due to possible health benefits such as its antioxidant activity (*17, 18*), anti-carcinogenic properties influencing cell cycle arrest and apoptosis (*19*), and inhibition of tumor formation and growth in mammalian models (*20*, *21*). The

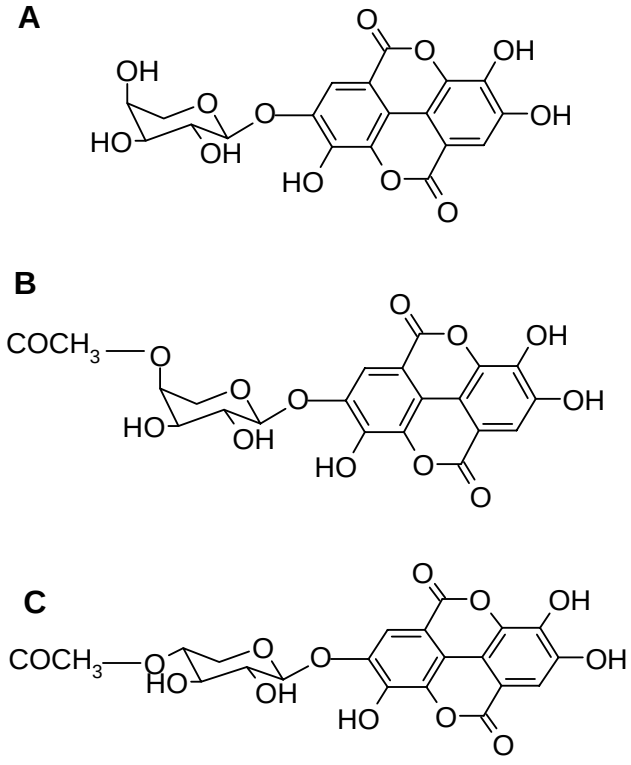

Figure 2-2. Ellagic acid glycosides found in raspberry. A) Ellagic acid 4-arabinoside, B) Ellagic acid 4-acetylarabinoside, C) Ellagic acid 4-acetylxyloside. (Mullen et. al. *Phytochemistry*. **2003**, *64,* 617-624)

types of ellagitannins are varied with approximately 500 different compounds isolated and identified in nature (*22*). Structural diversity in ellagitannins originates from the number of HHDP units, the location of galloyl ester groups participating in biaryl linkage, and the conformation of the glucose ring (*23*). HPLC assisted by mass spectrometry and diode array detections are the most commonly employed to separate and identify various ellagitannins from fruits or plants extracts. Mullen et. al. (*14*)

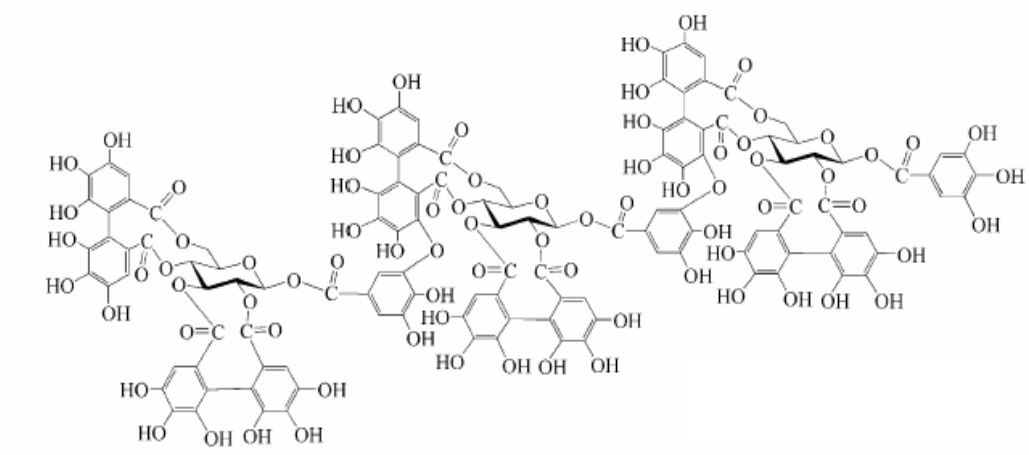

Figure 2-3. Ellagitannins (Lambertianin C) found in raspberry fruits. (Mullen et. al. *Phytochemistry* **2003**, *64*, 617-624)

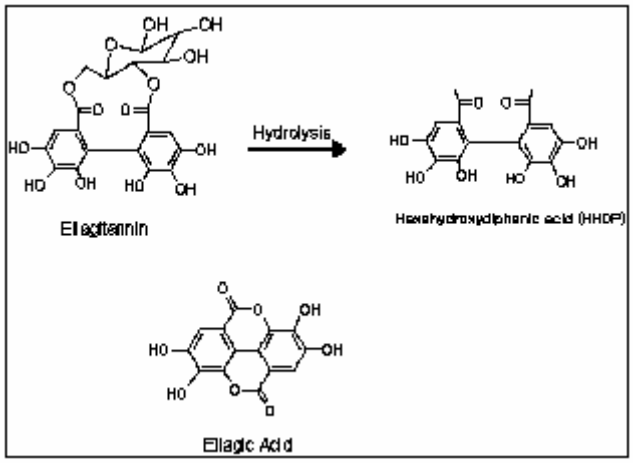

Figure 2-4. Ellagitannins conversion to ellagic acid via hexahydroxydiphenic acid (HHDP). (Quideau and Feldman, *Chem. Rev.* **1996**. *96*, 475-503)

reported phytochemical profiles in raspberry including anthocyanins, quercetin conjugates, ellagic acid glycosides and 3 different ellagitannins, Sanguiin H-10 (3 HHDPs, mass: 1568), Lambertianin C (6 HHDPs, mass: 2804) and Sanguiin H-6 (4 HHDPs, mass: 1870). All of these had observed maximum UV absorbance at 250 nm, but it could be lower than 250 nm since they scanned from 250 to 700 nm. Punicalagins (Figure 2-5) are different types of ellagitannins found in fruits, especially pomegranate (*24*, *25*) and composed of glucose, HHDP and gallagyl acid (ellagic + 2 gallic), which

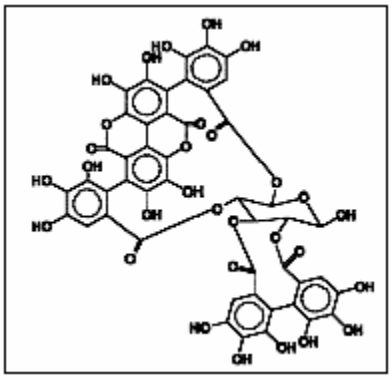

Figure 2-5. Chemical structures of punicalagin found in pomegranate juice as main ellagitannins (Gill et. al. *J. Agric. Food Chem*. **2000**, *48*, 4581-4589).

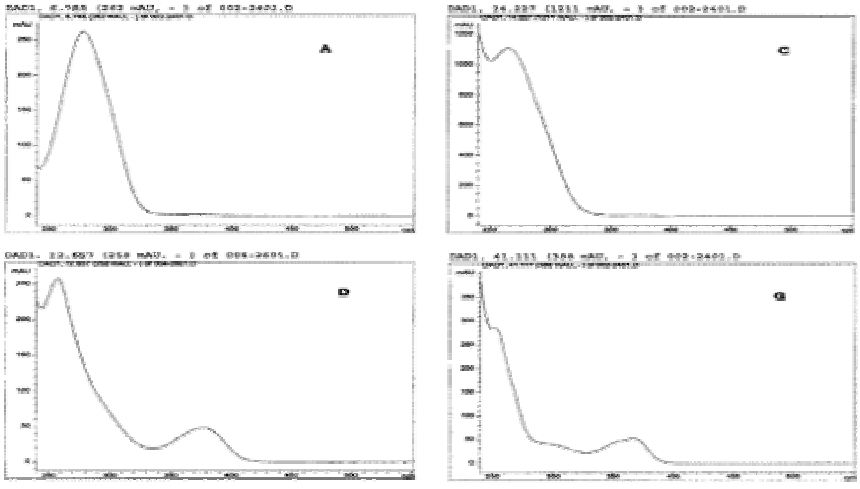

Figure 2-6. UV spectra of pomegranate juice characteristic compounds. A; galloylglucose, C; hydrolyzable tannins, D; punicalagin, G; ellagic acid (Gill et. al. *J. Agric. Food Chem*. **2000**, *48*, 4581-4589).

had a resulting UV spectrum showing maxima around 375 and 265 nm (Figure 2-6, D). Unidentified hydrolysable tannins (ellagic acid + gallic acid + tertgallic acid + etc.) were also observed with characteristic UV spectra, maximum at 266 nm (Figure 2-6, C). Similar UV spectra were reported with birch (*Betula pubescens*) leaves for bis-HHDP-glucopyranose isomers (*26*, Figure 2-7). They also observed that UV spectra of hydrolysable tannins were very similar to gallic acid spectra with 3-7 nm hypsochromic

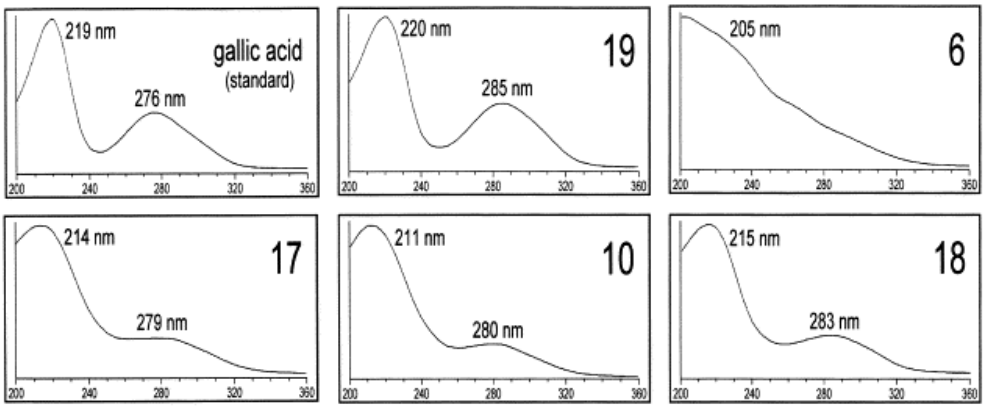

Figure 2-7. Characteristic UV spectra of hydrolysable tannins in birch (*Betula pubescens*) leaves. 19:pentagalloylglucopyranose isomers, 6:bis-HHDP-glucopyranose isomers, 17:galloyl-bis-HHDP-glucopyranose isomers, 10:digalloyl-HHDP-glucopyranose isomers, 18:trigalloyl-HHDP-glucopyranose.(Gill et. al. *J. Agric. Food Chem*. **2000**, 48, 4581-4589).

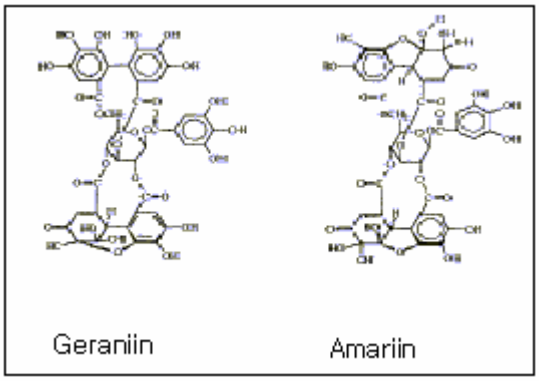

Figure 2-8. Chemical structures of Geraniin and Amariin, containing dehydrohexahydroxydiphenyl (DHHDP) group, found in *Phyllanthus amarus* (Foo. *Phytochemistry*. **1995**, *39*, 217-224).

shifts depending on the number of galloyl groups. In some plants, ellagitannins are found in oxidized form as containing dehydrohexahydroxydi-phenyl (DHHDP) group, and geraniin and amariin have been identified by NMR data (*27*). The released DHHDP units may not be able to convert to ellagic acid because two linked galloyl molecules in HHDP will be inhibited from making a lactone ring by additional linkage (Figure 2-8).

Ellagic Acid and Quality of Muscadine Products

The presence of ellagic acid in muscadine grape and its products are important not only because of its potential health benefits but also because of their possible contribution to form insoluble materials in processed juice and wine. Ellagic acid, hardly soluble in water, results in a significant defective role in the quality perception of wines and juices. In general, *V. vinifera* is not known to contain ellagic acid in its seed, skin or pulp, but small amounts are usually detected following oak barrel storage and aging. Boyle and Hsu (*28*) evaluated ellagic acid concentrations in juices from 11 cultivars of muscadine grapes and found a range from 1.6-23 μg/mL, with concentrations influenced by skin color. Ellagic acid evaluations in muscadine have been frequently conducted with its products like juice or wine with regard to sediment formation during storage. Boyle and Hsu (*28*) reported that ellagic acid is the only compound detectable in sediment, present as yellowish to red crystals; however, recent quantitative analysis of the collected sediments revealed that no more than 12% free ellagic acid by weight was actually present in the sediments. The remaining constituents consist of either unidentified compounds or conjugated forms of ellagic acid (*16*). Many efforts to prevent ellagic acid sedimentation in muscadine juices and wines have been employed, including chemical and physical remediation procedures, but none these were successful in solving the sedimentation problem. Lin and Vine (*1*) treated Magnolia (a white cultivar) juice with

increasing concentrations of fining agents such as polyvinylpolypyrrolidone (PVPP) and gelatin, and found that the highest concentration of PVPP (1.08 g/L) was most effective in reducing ellagic acid. Gelatin (0.4 g/L) also decreased ellagic acid concentration in sediment by 56% in red muscadine juice (*2*). An ultrafiltration technique involving passing juice through a 10,000-30,000 dalton molecular weight membrane demonstrated at most a 50% reduction of ellagic acid in sediment (*2*). However, these chemical and physical remediation procedures only lowered the levels of ellagic acid or sediment and ellagic acid precipitation continued during further storage suggesting that ellagic cid could be hydrolyzed from larger molecules like ellagic acid glycosides or ellagitannins (*16*, *29*). According to the work of Garrido et. al. (*2*), the formation of ellagic acid sediments in white muscadine juice was accelerated by increased storage temperature and following thermal pasteurization (100°C for 10 min), which resulted in more sediment than sterile filtered juices after 8 months storage at 1.5°C. Sims and Bates (*30*) investigated the effect of skin fermentation time on ellagic acid sedimentation of Noble muscadine grape wine. Wines fermented with skins for four and six days had greater amounts of ellagic acid sediment than non-skin and 2-day skin fermented wines. Muscadine juice is normally manufactured by two extraction techniques depending on the final intentions for use. Hot-pressed juices with red cultivars are made following crushing and heating at 70°C prior to pressing, while cold-pressed juices are pressed immediately after crushing white grapes (*8*). The main purpose of the hot-pressing procedure is to increase juice yield and to improve overall juice quality including high intensity of color through extracting more phytochemicals from fruits. Hot-pressing is

likely to influence the extraction of ellagitannins or ellagic acid glycosides resulting in an increase of ellagic acid precipitation in red muscadine juice after 50 days storage (*16*).

Anthocyanins

Other than ellagic acid derivatives, muscadine grapes also contain distinguishable phytochemicals, anthocyanin 3,5-diglucosides, which have been identified as delphinidin, cyanidin, petunidin, peonidin, and malvidin in non-acylated forms (*31*, *32*). Even though anthocyanin stability is influenced by several factors during food processing, the presence of anthocyanin 3,5-diglucosides is the main reason for rapid color loss during storage of muscadine juice or wine due to low stability of diglucosides form compared to corresponding monoglucosides forms of anthocyanins (*11*). It has been found that color loss by oxidation of anthocyanins was correlated to a decrease in radical scavenging activity (*17*). In order to protect the degradation of color in muscadine products, alternative processing schemes have been recently employed to understand the chemical nature of 3,5-diglucoside anthocyanins and to consequently lead the economic growth of this crop (*33*, *34*). Usually anthocyanins are stabilized and develop intense color by chelation with metal ions or binding with colorless polyphenolics and this is known as "copigmentation" reactions. Associated with muscadine grape products, copigmentation can be an alternative strategy to improve quality and market value since it has been reported that incremental addition of rosemary extract (0- 0.4% v/v) affect the hyperchromic shift of anthocyanins corresponding to increased antioxidant activity through copigment complexes with anthocyanins (*33*). Diverse technological improvements have been employed to replace the heating process because heat is a prime source for quality loss during food process. High hydrostatic pressure (HHP), a promising alternative to traditional pasteurization technologies (*35-37*), has been employed for

muscadine grape products in an effort to preserve thermolabile phytonutrients and favorable copigmentation between anthocyanins and plant based polyphenolics such as rosemary and thyme extracts (*33*, 3*4*). However, HHP can hinder improving juice quality by the presence and/or activation of residual enzymes such as polyphenol oxidase (PPO) due to accelerated oxidation of anthocyanins by activated PPO.

CHAPTER 3
FRUIT MATURITY AND JUICE EXTRACTION INFLUENCES ELLAGIC ACID DERIVATIVES AND OTHER ANTIOXIDANT POLYPHENOLICS IN MUSCADINE GRAPES

Introduction

Depending on maturity and availability, it is common to blend grape cultivars for muscadine wine and juice production to obtain the most desirable acidity, color, and flavor. However, little information is available on the phytochemical and antioxidant characteristics among cultivars suitable for wine or juice production.

The phytochemistry of muscadine grapes is distinguishable from most other grape varieties due to its predominance of anthocyanin 3,5-diglucosides and presence of ellagic acid and ellagic acid precursors (*7*). The anthocyanins 3,5-diglucosides, which may be more resistant to degradation during thermal processing compared to monoglucosides, are typically unstable during storage due to a decreased ability to form polymeric pigments and are particularly prone to oxidation and browning reactions (*38*, *39*). The ellagic acid derivatives are the most distinguishing chemical attribute in muscadine grape since these components have not been found in any *Vitis* species. Associated with quality of muscadine juice or wine, ellagic acid has been considered as an undesirable element even though it has potential health benefits because ellagic acid and its precursors are believed to develop insoluble sediments during storage. Phenolic contents in different muscadine cultivars have been reported on only free ellagic acid, resveratrol and other flavonoids including myricetin, quercetin and kaempferol (*17*); however, the current study represents impacts on free ellagic acid as well as ellagic acid glycosides and total

ellagic acid released by all ellagic acid precursors. The objectives of this study were to quantify the antioxidant polyphenolics in muscadine grapes as influenced by their location in the grape, juice production, and polyphenolic fractionation as a function of cultivar and maturity. This information can be used to determine wine or juice blending schemes to produce higher quality muscadine grape products in terms of phytochemical composition and antioxidant potential.

Materials and Methods

Muscadine grapes were donated from local grape growers in central Florida and collected at two maturity stages from the same vines at different time intervals, about 15-20 days apart depending on variety. Varieties included Carlos, Fry, and Doreen, classified as either white or more specifically bronze colored fruit, and the red-skinned varieties Noble, Albemarle, Cowart, Nesbitt, and Georgia Red. Random samplings of 8-15 fruit in duplicate were manually divided between skin and pulp, while whole grapes were processed into juice using a hot-break technique (70°C for 30 min). Polyphenolics were extracted from the skin and pulp by homogenizing with 25 mL of 100% methanol, filtered through Whatman #4 filter paper, and solvent removed at 40°C under a stream of nitrogen. The juice was analyzed directly following centrifugation and filtration. Non-anthocyanin polyphenolics were subsequently partitioned from each isolate into ethyl acetate in three sequential extractions after which the solvents were pooled, removed under reduced pressure at 40°C, and residues redissolved in 50% methanol.

Chemical Analyses

Polyphenolics were separated and quantified by HPLC using solvent programs to identify phenolic acids, free ellagic acid, and ellagic acid derivatives in ethyl acetate extracts, and total ellagic acid and individual anthocyanidins in methanolic extracts

following acid hydrolysis (2N HCl for 60 min at 95°C). Separations were conducted on a Dionex HPLC system using a PDA-100 photodiode array detector and a 250 mm × 4.6 mm Acclaim 120 C_{18} column (Dionex, Sunnyvale, CA) with a C_{18} guard column. Mobile phases consisted of 100% water (phase A) and 60% methanol (phase B) both adjusted to pH 2.4 with *o*-phosphoric acid and run at 1 mL/min according to modified conditions of Lee and Talcott (*16*). Free ellagic acid, ellagic acid glycosides and phenolic acids were separated using a gradient elution program where phase B changed from 0-30% in 3 min; 30-50% in 5 min; 50-70% in 17 min; 70-80% in 5 min; 80-100% in 5 min; and 100% in 9 min for a total run time of 44 min, after which the column was equilibrated to original conditions in 1 min for the next sample injection. Anthocyanidins and total ellagic acid were also separated with a gradient program that ran phase B from 30-50% in 3 min; 50-70% in 2 min; 70-90% in 5 min; and 90-100% in 10 min and returning to original composition in 1 min for column equilibration. Ellagic acid and its derivatives were quantified in ellagic acid equivalents, flavonoid glycosides in equivalents of myricetin (Sigma Chemical, St. Louis, MO), and each anthocyanidin quantified in cyanidin equivalents (Polyphenols Laboratories AS, Sandnes, Norway).

Total soluble phenolics were analyzed using Folin-Ciocalteu assay (*40*) and expressed in gallic acid equivalents (GAE). Antioxidant activity (*41*) was determined using the oxygen radical absorbance capacity (ORAC) assay with fluorescein as modified by Ou et. al. (*42*) from initial protocol by Cao et. al. (*43*). Fluorescence loss by reaction with hydroxyl radical (70 min, 37˚C) was monitored on a Molecular Devices fmax® 96well fluorescent microplate reader (Sunnyvale, CA) following appropriate dilution of

each isolate and data expressed in Trolox equivalents per g of fresh fruit or per mL of juice.

Statistical Analysis

Data represent the mean of duplicate analyses with analysis of variance and Pearson correlations conducted using JMP5 software (*44*); mean separation was conducted using the LSD test ($P<0.05$).

Results and Discussion

Identification of Ellagic Acid and Its Precursors

The free (aglycone) form of ellagic acid and two ellagic acid glycosides were found in all eight muscadine grape cultivars following ethyl acetate extraction and separation by HPLC. The ellagic acid glycosides were tentatively characterized based on UV spectral properties (252 and 360 nm) similar to that of free ellagic acid (252 and 365 nm) as was previously characterized in muscadine grapes (*16*) indicating that these compounds were most likely glycosidic forms at the 4-position of ellagic acid rather than HHDP moieties esterified to glucose (true ellagitannins), with maximum absorption at or near 250 nm (*15*, *45*). Preliminary work to characterize these compounds has identified the presence of glucose, xylose, or rhamnose moieties (data not shown). Similar ellagic acid glycosides were thought to exist in raspberries and were characterized by spectroscopic shifts (4-7 nm hypsochromic) and disappearance of the glycoside after hydrolysis, with a corresponding increase in free ellagic acid (*15, 45- 47*). Similarly, the two ellagic acid glycosides identified in muscadine grapes yielded free ellagic acid upon both acid and enzyme (ß-glucosidase) hydrolysis. True ellagitannins, containing esterified HHDP units to a carbohydrate, were also believed to be present in the grape isolates, but were not separated or detected in muscadine grapes using the HPLC methodology employed.

Evidence of these highly polar compounds was established indirectly by passing an aqueous grape extract through a pre-conditioned Waters C_{18} Sep Pak cartridge and evaluating the non-retained fraction. No peaks analogous to ellagic acid were present in this isolate in the range of 200-400 nm, but following acid hydrolysis free ellagic acid was one of the hydrolytic products, thus providing evidence for their existence. Total ellagic acid was subsequently determined from the methanolic extracts following acid hydrolysis and represented the sum of free ellagic acid and ellagic acid released from both ellagitannins and ellagic acid glycosides.

Ellagic Acid and Its Derivatives

Concentrations of ellagic acid and its derivatives in muscadine grapes were found to significantly vary with ripening, in skin and pulp tissue, among cultivars, and following juice extraction (Table 3-1). Ripening was a critical factor influencing concentrations since appreciable increases in skin and juice during ripening were observed. Since muscadine grapes grow in clusters rather than bunches, inconsistent maturity at harvest is a common occurrence. Changes with ripening were also highly variable among cultivars for free ellagic acid and its glycosidic forms, and ranged from a 0.3 to 13-fold increase in the skins alone. Differences during ripening were less variable for total ellagic acid at a 1.7-fold average increase in the skins. The large increases in ellagic acid and its glycosides observed during ripening may have resulted from various reasons: amplified hydrolysable tannins synthesis during veraison (*8*); a chemoprotective response similar to the formation of resveratrol (*48*); or accelerated hydrolysis of HHDP units from ellagitannins that was observed to produce greater quantities of free ellagic acid in each cultivar. Compared to total ellagic acid, relatively low levels of free ellagic acid and ellagic acid glycosides were present in the grapes, an indication that

Table 3-1. The concentrations (mg/kg, mg/L) of free ellagic acid (EA), two ellagic acid glycosides (EAG 1 and 2) and total ellagic acids on skin, pulp and juice of muscadine grapes as affected by cultivars and ripening stages (U: unripe and R: ripe).

	Cultivars	Color	Free EA		EAG1[1]		EAG2[2]		Total EA[3]	
			U	R	U	R	U	R	U	R
Skin	Carlos	White	32.1 b[4]	8.04 e*	17.4 b	6.76 d*	16.7 d	20.1 d	368 d	879 d*
	Fry	White	31.3 b	87.4 cd*	13.0 cd	90.3 a*	8.81 e	13.6 d	531cd	879 d*
	Doreen	White	10.8 d	138 ab*	3.78 f	93.0 a*	29.7 b	115 a*	918 ab	1620 b*
	Noble	Red	17.5 c	76.4 d*	10.5 de	23.2 c*	31.0 b	41.8 bc	474 d	592 e
	Albemarle	Red	12.7 cd	110 bc*	24.8 a	23.5 c	29.0 b	53.9 b*	1030 a	1090 c
	Cowart	Red	27.5 b	162 a*	13.8 c	95.9 a*	24.5 bc	46.1 bc*	732 bc	1900 a*
	Nesbitt	Red	15.5 cd	136 ab*	7.53 ce	61.7 b*	18.7 cd	39.4 c*	555 cd	1100 c*
	Georgia Red	Red	42.9 a	74.8 d*	12.8 cd	20.5 c*	38.7 a	10.1 d*	996 a	587 e
Pulp	Carlos	White	4.73 e	2.66 c	3.32 a	1.00 c	2.83 cd	2.90 c	159 b	231 b*
	Fry	White	6.44 de	1.01 d*	3.30 a	ND[5] d*	1.57 d	ND e*	189 b	ND c*
	Doreen	White	14.1 a	0.93 d*	1.22 cd	trace d*	12.1 a	0.66 d*	474 a	trace c*
	Noble	Red	3.51 ef	8.69 b*	0.88 d	2.98 b*	2.82 cd	5.79 b*	208 b	168 b
	Albemarle	Red	12.2 ab	24.5 a*	2.06 bc	6.04 a*	9.36 b	12.8 a	203 b	455 a
	Cowart	Red	8.28 cd	1.24 d*	2.12 b	trace d*	5.08 c	trace e	232 b	ND c*
	Nesbitt	Red	10.1 bc	0.54 d*	3.53 a	trace d*	8.63 b	trace e	197 b	ND c*
	Georgia Red	Red	1.13 f	1.00 d	trace[6] d	ND d	1.13 d	ND e	38.2 c	ND c*
Juice[7]	Carlos	White	3.01 cde	4.34 e	1.02 cd	8.60 cd*	4.96 c	5.34 cd*	12.5 e	106 e*
	Fry	White	3.99 bcd	11.2 bcd*	2.63 a	21.7 a*	2.94 e	3.13 d	59.1 c	105 e*
	Doreen	White	3.34 cde	14.1b*	0.56 e	7.68 d*	6.02 b	15.7 b*	12.7 e	172 d*
	Noble	Red	8.75 a	20.5 a*	trace e	5.78 e*	6.70 ab	15.6 b*	10.1 e	257 b*
	Albemarle	Red	5.15 b	23.4 a*	0.77 de	9.68 bc*	6.81 a	20.1 a*	14.0 e	322 a*
	Cowart	Red	4.03 bc	12.5 bc*	1.31 bc	11.2 b*	4.07 d	6.81 c*	81.0 b	219 c*
	Nesbitt	Red	2.17 e	8.82 d*	1.16 cd	5.19 e*	3.20 e	4.85 cd*	26.1 d	187 cd*
	Georgia Red	Red	2.66 de	9.77 cd	1.60 b	ND f*	3.47 de	3.16 d	88.0 a	198 cd*

[1,2]Expressed in ellagic acid equivalents. [3]The sum of free ellagic acid and ellagic acid released following acid hydrolysis. [4]Similar letters within columns for each fruit part are not significantly different (LSD test. $P<0.05$). [5]Concentrations below detection limit. [6]Concentration below 0.5 ppm. [7]Hot-pressed juice. Asterisk (*) indicates significant effects by fruit ripening for each fruit parts (LSD test. $P<0.05$).

ellagitannins were the major source of ellagic acid following hydrolysis. However, the actual concentrations of the ellagic acid glycosides were likely influenced by the use of free ellagic acid as the quantifying standard.

As with most grape varieties, polyphenolic compounds are typically concentrated in epidermal tissues, which are exceptionally thick in muscadine grapes and often hinder efficient juice extraction. On average, the skin and pulp tissue constituted 21 and 69% of the total mass of the grapes respectively, and were similar for both unripe and ripe fruit. Ellagic acid and its derivatives were generally concentrated in the skin, which contained 51-67% of these compounds in unripe fruit on a fresh weight basis. Upon ripening, these compounds were even more localized in the skin and accounted for 82-87% of the total. Doreen and Cowart contained the highest concentrations of ellagic acid and its glycosides among the cultivars, but no meaningful correlation could be made between free ellagic acid and/or ellagic acid glycosides and concentrations of total ellagic acid, an observation that likely reflected the influence of ellagitannins in each isolate. Compared to ellagic acid concentrations present in the skin and pulp, levels present in juice were considerably lower and reflected the low solubility of ellagic acid in aqueous systems. A hot break or "hot-press" technique is commonly used with muscadine grapes to increase juice yields or add pigmentation to wines or juices, and when combined with macerating enzymes (*49*), juice extractions are better facilitated. Additionally, the time and temperature of the heating process will appreciably influence juice yields and phytochemical concentration compared to non-heated fruit juice (*17*) and white or bronze grapes, depending on cultivar, may not be heated to prevent enzymatic and autoxidative browning reactions affecting juice quality (*50*). Textural differences also occur in the grapes during ripening

from action of natural pectinase and may also influence phytochemical solubilization. Typical juice yields may range from 60-75% by weight for hot-pressed muscadine grape juices and is influenced by heating conditions, pressing conditions, the use of pressing aids such as rice hulls, and skin thickness (*33*). The highest concentrations of total ellagic acid were found in the juice of ripe Albemarle (322 mg/L) and Noble (257 mg/L), which reflected a 24% average increase in concentration over juice pressed from unripe grapes. Concentrations of total ellagic acid present in the juice were not necessarily a reflection of levels found in whole grapes, since the juice of unripe fruit contained 2-26% of the amount present in whole grapes compared to 19-78% for ripe fruit. For simplicity, these data were determined based on a 60% juice yield and accounted for the variable contributions from skin and pulp tissue (seeds not included) to the total weight of the grapes. Juice from ripe grapes of Noble, Cowart, Nesbitt, and Georgia Red had the highest total ellagic acid extractions (>58%), while Carlos, Fry, Doreen, and Albemarle were considerably lower (<34%). The low recovery of ellagic acid derivatives in the latter cultivars reflected the difficulty in solubilizing polyphenolics, likely due to physical barriers associated with their thick skins, which left high concentrations of these compounds behind in the skin and pulp material. Free ellagic acid itself, sparingly soluble in water, was also poorly solubilized in all juices, retaining only 27 and 37% on average of the total present in whole grapes for unripe and ripe fruit, respectively. However, the ellagic acid glycosides were considerably more soluble in juices with >56% recovery from whole grapes.

Anthocyanins

Anthocyanidins, quantified only in the red cultivars, were expressed in cyanidin equivalents (Table 3-2) since the predominant anthocyanins in muscadine grapes were

Table 3-2. Concentration (mg/kg, mg/L in cyanidin equivalents) of six anthocyanidins and total anthocyanidins on skin, pulp and juice of red muscadine grapes as affected by ripening stages (U: unripe and R: ripe).

		Delphinidin[1]		Cyanidin		Petunidin		Malvidin + Peonidin		Total[2]	
		U	R[3]	U	R	U	R	U	R	U	R
Skin	Noble	ND[4] b[5]	1450 b	ND b	692 c	159 a	1070 a	ND a	926 a	159 a	4140 b
	Albemarle	44.2 a	424 c	28.5 a	291 d	ND b	ND b	ND a	102 d	72.6 b	817 d
	Cowart	57.6 a	1290 b	37.5 a	1210 a	12.0 b	294 b	ND a	445 c	107 ab	3250 c
	Nesbitt	66.8 a	3550 a	35.1 a	860 b	ND b	ND b	ND a	825 b	102 ab	5230 a
	Georgia Red	72.1 a	300 c	35.4 a	52.5 e	ND b	20.3 b	ND a	17.9 e	108 ab	390 d
Pulp	Noble	ND b	102 a	6.95 a	93.9 a	ND b	78.4 a	ND a	114 a	6.95 a	383 a
	Albemarle	0.84 b	67.1 b	5.85 a	89.0 a	ND b	29.7 b	ND a	21.8 b	6.70 a	212 b
	Cowart	4.54 a	3.75 c	4.00 b	10.6 b	0.90 a	0.63 c	ND a	ND b	9.44 a	15.0 c
	Nesbitt	ND b	19.3 c	ND c	12.4 b	ND b	5.03 c	ND a	ND b	ND b	36.8 c
	Georgia Red	1.17 b	2.52 c	0.980 c	0.76 b	ND b	0.25 c	ND a	ND b	2.15 b	3.53 c
Juice[6]	Noble	ND b	131 a	ND b	125 a	ND b	155 a	ND a	200 a	ND b	610 a
	Albemarle	ND b	52.4 c	ND b	86.1 b	ND b	25.7 c	ND a	18.2 b	ND b	182 b
	Cowart	ND b	48.6 c	ND b	94.0 b	ND b	21.4 c	ND a	16.5 b	ND b	180 b
	Nesbitt	6.98 a	72.5 b	2.83 a	49.3 c	3.04 a	44.3 b	ND a	23.6 b	12.8 a	190 b
	Georgia Red	ND b	10.1 d	ND b	7.16 d	ND b	2.61 a	ND a	ND c	ND b	19.9 c

[1]Cyanidin equivalents. [2]Sum of individual anthocyanidins. [3]All anthocyanins are significantly different at ripening stage. [4.] Concentrations below detection limit. [5]Similar letters within columns for each fruit part are not significantly different (LSD test.P<0.05). [6]Hot-pressed juice. Asterisk (*) indicates significant effects by fruit ripening for each fruit part (LSD test. P<0.05)

previously identified as non-acylated 3,5-diglucosides of six anthocyanidin bases (*17*). In the current study, only three anthocyanidins were positively elucidated following acid hydrolysis using the column and solvent conditions described, due to incomplete separation of peonidin and malvidin and the absence of pelargonidin. As expected, anthocyanins appreciably increased in the skin as the fruit ripened with low concentrations also found in pulp material nearest the skin. Anthocyanidin abundance in ripe fruit were delphinidin > petunidin > malvidin+peonidin > cyanidin with Nesbitt, Noble, and Cowart containing the highest overall concentrations. Color instability of muscadine wine and juice is an established quality defect, and is a consequence of their high concentrations of monomeric 3,5-diglucosides with *o*-diphenolic substituents that include delphinidin, cyanidin, and petunidin (*51*). Among the cultivars evaluated, these three anthocyanidins accounted for 78-96% of the total in fresh grapes and from 67-100% in juice. Ripe Noble grapes, one of the most popular wine and juice cultivars, contained the highest concentration of malvidin+peonidin among the cultivars evaluated. Malvidin is generally considered the most stable anthocyanin form and along with peonidin was present at 22% of the total in the skins compared to 33% in juice. However even with high malvidin+peonidin concentrations, the juice from Noble grapes is considered highly susceptible to color degradation due to likely lack of inter- and intra-molecular copigmentation of 3,5 diglucosidic anthocyanins (*51*) and provides an indication that the remaining cultivars would be even less stable to oxidation or other deteriorative reactions affecting juice or wine pigmentation due to their lower malvidin+peonidin concentrations. These cultivars, as well as the white/bronze varieties, may be more suitable for juice blending to take advantage of their high ellagic acid contents. Noble

grape juice also contained the highest total anthocyanin concentration (610 mg/L), while Georgia Red contained considerably less (20 mg/L) even in relation to the other red varieties that ranged from 180-190 mg/L. Based on a 60% juice yield, only 12% of the total anthocyanins present in grape skins were solubilized into the juice of Nesbitt and Georgia Red, both consumed primarily as table grapes, the former having high anthocyanin content yet poor anthocyanin solubility characteristics during juicing. Juice from the remaining cultivars, commonly consumed either fresh or processed, contained 27-32% of the total anthocyanins present in the each grape. The low anthocyanins recovery values in juice, especially in relation to ellagic derivatives, reflect the degree of processing necessary to solubilize sufficient anthocyanins to produce a suitable red wine or juice.

Total Phenolics and Antioxidant Capacity

Measurements of total phenolics by the Folin-Ciocalteu metal reduction assay and peroxyl radical scavenging activity using the ORAC assay are common index that provide an overall assessment of the content and chemical activity of compounds present in fruits and vegetables. These attributes were quantified in methanolic and ethyl acetate extracts of grape skin, pulp, and juice and following partitioning of phenolic acids and flavonols into ethyl acetate, into which anthocyanins are not soluble, to differentiate between major polyphenolic classes (Tables 3-3 and 4). Values for total phenolics, which varied among cultivars and with fruit ripening, were good predictors of antioxidant capacity in both methanolic and ethyl acetate extracts ($r = 0.83$ and 0.92, respectively). The higher correlation coefficient for ethyl acetate extracts may have reflected the removal of potentially interfering/prooxidant polar compounds or reflected interactions between anthocyanins and other polyphenolics in the methanolic extracts (*52*, *53*). Based

Table 3-3. Concentrations (mg/kg, mg/L) of total soluble phenolics (Folin-Ciocalteu metal reduction assay) in methanolic and ethyl acetate extracts as affected by cultivars and ripening stages (U: unripe and R: ripe).

	Cultivars	Color	Methanolic Extract		Ethyl Acetate Extract	
			U	R	U	R
Skin	Carlos	White	2430 b[1]	2530 e	428 d	706 f*
	Fry	White	1440 c	3360 d*	459 d	987 e*
	Doreen	White	3860 a	3990 c*	1430 a	2280 b*
	Noble	Red	2660 b	3090 d	1020 bc	727 f*
	Albemarle	Red	2580 b	2260 e	1320 ab	756 ef
	Cowart	Red	2660 b	4370 c*	1130 ab	1890 c
	Nesbitt	Red	2480 b	5030 b*	627 cd	1300 d*
	Georgia Red	Red	4220 a	9470 a*	1500 a	2910 a*
Pulp	Carlos	White	405 de	738 b	128 d	258 a
	Fry	White	566 cd	276 d*	138 d	102 cd
	Doreen	White	1210 b	192 d*	1300 a	39.2 de*
	Noble	Red	601 c	848 b*	332 cd	120 bc*
	Albemarle	Red	1410 a	1100 a*	622 b	274 a*
	Cowart	Red	1110 b	200 d*	528 bc	38.2 de*
	Nesbitt	Red	567 c	443 c	502 bc	16.3 e*
	Georgia Red	Red	312 e	467 c*	99.4 d	183 b*
Juice[2]	Carlos	White	1145 c	979 de*	165 a	66.4 b*
	Fry	White	1069 c	1500 cd*	90.1 d	81.0 c
	Doreen	White	1673 a	1293 d	161 a	141 a
	Noble	Red	1630 a	1950 b	139 abc	69.1 c*
	Albemarle	Red	1460 ab	1770 bc	147 ab	120 ab
	Cowart	Red	1200 bc	1360 cd	122 bc	89.4 bc
	Nesbitt	Red	739 d	1210 d*	61.2 e	70.8 c
	Georgia Red	Red	1140 c	2860 a*	118 c	139 a

[1]Similar letters within columns for each fruit part are not significantly different (LSD test. $P<0.05$). [2]Hot-pressed juices. Asterisk (*) indicates significant effects by fruit ripening for each fruit part (LSD test. $P<0.05$).

Table3-4. Antioxidant capacity (μmol trolox equivalents/g or mL) of methanolic and ethyl acetate extracts as affected by cultivars and ripening stages (U: unripe and R: ripe).

	Cutivars	Color	Methanolic Extract		Ethyl Acetate Extract	
			U	R	U	R
Skin	Carlos	White	58.0 d[1]	86.2 cd*	10.2 b	17.5 b
	Fry	White	49.3 d	72.3 d	10.7 b	19.0 b*
	Doreen	White	104 a	90.4 c*	25.2 a	25.5 a
	Noble	Red	97.2 ab	100 c	22.5 a	12.3 c*
	Albermerle	Red	90.8 b	71.1 d	22.6 a	12.0 c*
	Cowart	Red	97.7 ab	119 b	22.6 a	26.3 a*
	Nesbitt	Red	69.7 c	136 a*	12.1 b	25.1 a*
	Georgia Red	Red	89.0 b	128 ab*	25.4 a	29.1 a*
Pulp	Carlos	White	5.95 de	8.75 b*	3.90 e	5.05 b
	Fry	White	6.35 de	7.50 bc	3.90 e	3.70 d
	Doreen	White	34.0 a	2.45 d*	15.0 a	1.60 e*
	Noble	Red	9.40 cd	14.3 a	4.70 e	4.10 cd
	Albermerle	Red	14.8 b	13.1 a	8.05 c	6.45 a*
	Cowart	Red	11.8 bc	2.60 d*	5.70 d	1.55 ef*
	Nesbitt	Red	13.9 b	4.60 cd	9.50 b	1.00 f*
	Georgia Red	Red	4.95 e	6.80 bc	4.10 e	4.45 c
Juice[2]	Carlos	White	20.3 b	15.5 d*	2.37 b	2.06 bc
	Fry	White	14.6 d	20.1 bc	1.61 cd	1.92 bc
	Doreen	White	25.3 a	19.6 bc	2.91 a	2.38 ab
	Noble	Red	24.3 a	26.7 a	2.55 ab	1.51 cd
	Albermerle	Red	19.3 bc	23.3 ab	2.89 a	2.14 b
	Cowart	Red	17.4 cd	21.4 bc*	1.99 c	1.91 bc
	Nesbitt	Red	10.8 e	18.3 cd*	1.27 d	1.20 d
	Georgia Red	Red	20.7 b	26.6 a*	2.50 b	2.96 a*

[1]Similar letters within columns for each fruit part are not significantly different (LSD test. $P<0.05$). [2]Hot-pressed juices. Asterisk (*) indicates significant effects by fruit ripening for each fruit part (LSD test. $P<0.05$).

on abundance, anthocyanins were the major antioxidant compounds present in muscadine grape skin and juice and their concentration was directly related to antioxidant capacity (r= 0.99). Ethyl acetate soluble compounds also contributed to antioxidant capacity and ranged from 12-29%, 22-83%, and 5.7-15% of the total present in methanolic extracts of skin, pulp, and juice, respectively. Other than ellagic acid and its derivatives, many additional compounds were also identified in the ethyl acetate extract including several flavonoids glycosides, phenolic acids, and procyanidins that are all known to possess antioxidant activity (*54*, *55*). In various concentrations, gallic acid, protocatechuic acid, catechin, and epicatechin were identified in ethyl acetate extracts. Flavonoid glycosides were tentatively identified based on their spectroscopic similarities to myricetin, quercetin, and kaempferol with glucose and/or rhamnose moieties. A myricetin glycoside was the predominant flavonoid present in all cultivars and ranged from 8.7-1,350 mg/kg in skin, 0-50 mg/kg in pulp, and 1.6-50 mg/L in juice. Among the cultivars, ripe Georgia Red contained the highest concentrations of total phenolics in both ethyl acetate and methanolic extracts of both skin and juice, in contrast to its low anthocyanin, ellagic acid, and ellagic acid glycoside content, which was primarily attributed to its high flavonoid concentration.

Conclusion

This study demonstrated that ripening, physiology, and juice processing influence phytochemical composition and antioxidant capacity of muscadine grapes. Data suggest a diversity of phytochemical compounds that can be used for novel blending schemes for muscadine grape juice or wine to obtain a desired quality and polyphenolic content relating to their antioxidant capacity.

CHAPTER 4
IDENTIFICATION OF ELLAGITANNINS AND CONJUGATES OF ELLAGIC ACID IN MUSCADINE GRAPES

Introduction

V. rotundifolia are differentiated from *V. vinifera* in several points such as lack of an organized fruit bunch, strong disease resistance, and its unique phytochemical composition primarily due to presence of ellagic acid derivatives. Ellagic acid derivatives are not uncommon in plants and are in abundance in raspberry (*14*, *15*, *46*, *47*), pomegranate (*24*, *66*, *67*), oak (*56*), birch leaves (*68*), or medicinal herbs/plants (*69*, *70*); yet their presence in muscadine grapes is unique among *Vitis* species. Ellagic acid derivatives are a broad classification that includes the free acid state of ellagic acid, these conjugated with various sugars to form simple glycosides or more complex ellagitannins (*46*). Ellagitannins are characterized as hydrolysable conjugates containing one or more hexahydroxydiphenoyl (HHDP) group esterified to a sugar, usually glucose. In raspberries, the predominant ellagitannins were identified as lambertianin C and sanguiin H-6 as well as arabinosides, acetylarbinosides, and acetylxylosides of ellagic acid (*14*) yet similar compounds, if present, have not been determined in the muscadine grape. The objective of this study was to elucidate identities and concentrations of ellagic acid derivatives in muscadine grapes by various extraction and analytical procedures. Investigations on ellagic acid and ellagic acid derivatives in muscadine grapes will add value and marketability to the crop due to beneficial health benefits such as its antioxidant activity (*19*, *20*), anti-carcinogenic properties influencing cell cycle arrest and

apoptosis (*20*), and the inhibition of tumor formation and growth in mammalian models (*21*, *40*) previously attributed to these compounds.

Materials and Methods

Muscadine grapes (cvs. Doreen, Noble, Albemarle) were donated from local grape growers in central Florida and polyphenolics extracted from the skin and pulp with 100% methanol (0.01% HCl). Extracts were filtered through Whatman #4 filter paper, solvent removed at 40°C under reduced pressure, and polyphenolic residue redisolved in water at pH 3.5.

Isolations

Due to the diversity of ellagic acid precursors potentially present, it was necessary to fractionate the polyphenolics based on their affinity to C_{18} and Sephadex LH-20 and partition based on their solubility in various organic solvents (Figure 4-1). Initially, grape extracts were applied to a Sep-Pak C_{18} cartridge and polyphenolics eluted with various

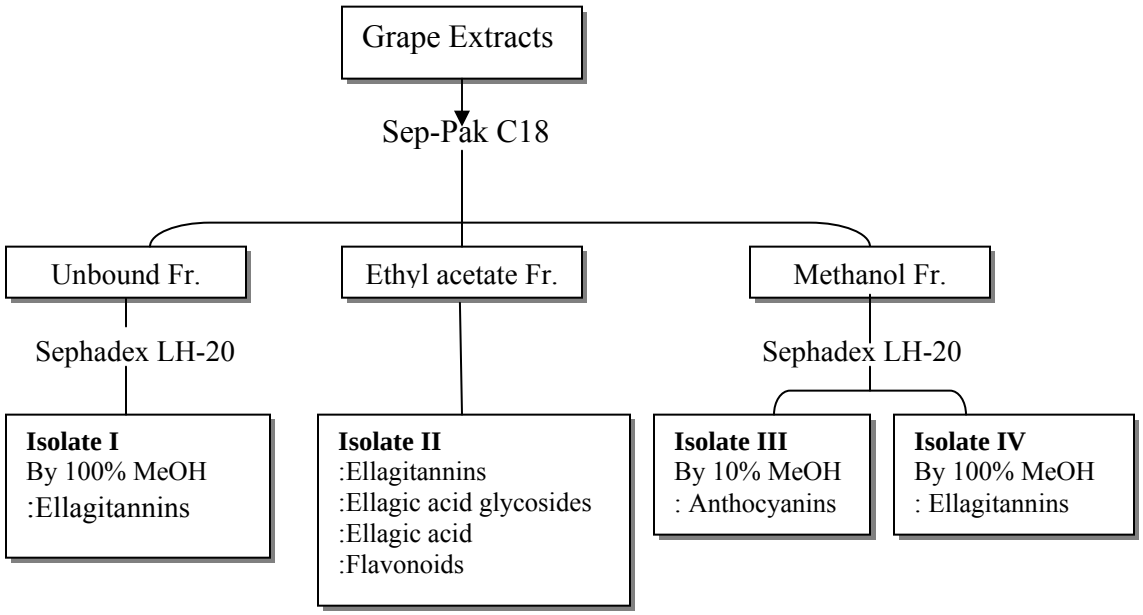

Figure 4-1. Fraction scheme and tentative classification of polyphenolics present in methanolic extracts of muscadine grapes.

solvents in order of water, ethyl acetate, and then methanol (0.01% HCl) to partially isolate compounds of interest. Ethyl acetate was removed under reduced pressure and redissolved in water and methanol (95:5, pH 3.5) and this fraction contained phenolic acids, flavonoids, and ellagic acid derivatives including free ellagic (aglycones), ellagic acid glycosides, and ellagitannins. Ellagic acid derivatives that remained in the unbound (water) fraction and in the final methanol-soluble fraction, following evaporation and solubilization in water, were further concentrated by partitioning from a mini-cartridge of Sephadex LH-20 based on their selective adsorption properties.

Analysis by HPLC-PDA

HPLC-PDA analysis was initially employed to tentatively identify and quantify ellagic acid derivatives in grape extracts and isolated polyphenolic fractions from three muscadine grape cultivars (Doreen, Noble, and Albemarle). Free ellagic acid, ellagic acid glycosides, and total ellagic acid (following acid hydrolysis in 2N HCl for 60 min at 95°C) were evaluated in equivalents of free ellagic acid. Separations were conducted on a Dionex HPLC system using a PDA-100 photodiode array detector and a 250 mm × 4.6 mm Acclaim 120 C_{18} column (Dionex, Sunnyvale, CA) with a C_{18} guard column. Identical mobile phases in Chapter 3 were employed to separate polyphenolics with modified gradient elution program where phase B (60% methanol) changed from 0-60% in 30 min; 60-80% in 10 min; 80-100% in 10 min; and 100% in 10 min for a total run time of 60 min, after which the column was equilibrated to original conditions in 2 min for the next sample injection.

A second HPLC-PDA separation was applied to Isolates I and II in order to obtain UV absorbance spectral data under identical condition with MS^n analysis. Separations were performed using a Waters Alliance 2695 HPLC system and polyphenolics separated

on Phenomenex (Torrace, CA) Synergi 4u Hydro-RP 80A (2 x 150 mm; 4 um; S/N=106273-5) plus C18 guard column (2mm x 4 mm) and a Waters 996 photodiode array detector recorded UV spectra. Mobile phase and gradient program were identical to MS^n analysis stated below.

Analysis by HPLC-MS^n

Mass spectrometric analyses were carried out to achieve structural information based on molecular masses and fragment ions. Only Isolates I and II were evaluated for polyphenolics on an Agilent HPLC system (Palo Alto, CA) using an 1100 series binary pump and separated using the Phenomenex column previously described. Mobile phases consisted of (A) 0.5% formic acid in water (5 mM ammonium formate) and (B) 0.5% formic acid in methanol and run at 0.15 mL/min. Polyphenolic compounds were separated using a gradient elution program where phase B changed from 5-30% in 5 min; 30-65% in 70 min; 65-95% B in 30 min and held for 20 min; and 95-5% B in 10 min to equilibrate the column and whole system to original conditions for 30 min. Effluents from the column were passed through the UV detector (Applied Biosystems Model 785A) and then analyzed by mass spectrometer, ThermoFinnigan (San Jose, CA) LCQ with electrospray ionization (ESI). In order to confirm molecular masses, ionization was conducted in both negative and positive mode.

Total Polyphenols and Antioxidant Capacity

Total soluble phenolics were analyzed using Folin-Ciocalteu assay (*40*) and antioxidant activity was determined using the oxygen radical absorbance capacity (ORAC) assay as described in Chapter 3.

Statistical Analysis

Data represent the mean of duplicate analyses with analysis of variance conducted using JMP5 software (*44*); mean separation was conducted using the LSD test ($P<0.05$).

Results and Discussion

Isolations and Quantification of Polyphenolic Compounds by Solid Phase Extraction

Numerous polyphenolic compounds were present in muscadine grapes when analyzed by HPLC, and their separation and identification were enhanced by preparing fractions using a series of solid phase and liquid-liquid extractions based on polarity and affinity characteristics of each compound (Figure 4-1). Overall, Isolate II represented the majority of non-anthocyanin polyphenolics present in muscadine grapes due to the high affinity of these compounds for ethyl acetate. This fraction was exceptionally high in both phenolic acids and flavonoids. By contrast, Isolate I contained only the most polar compounds, not retained on Sep Pak C_{18} cartridges. This isolate had a strong affinity to Sephadex LH-20, a cross-linked dextran, which is widely used to isolate tannins from plant based sample (*55*). Compounds that remained on the Sep Pak C_{18} cartridges following elution with ethyl acetate were subsequently eluted with acidified methanol and were found to predominantly contain anthocyanins. These compounds also yielded high concentrations of free ellagic acid after acid hydrolysis indicating the presence of ellagic acid derivatives. Sephadex LH-20 was employed to separate anthocyanins from these derivatives, with anthocyanins removed first with up to 10% methanol followed by ellagic acid derivatives with 100% methanol to give Isolates III and IV, respectively. As a result, the initial grape extract and four sub-fractions from three cultivars (Doreen, Noble, and Albemarle) were created for subsequent phytochemical analyses and quantification of total soluble phenolics and antioxidant capacity (Table 4-1 and Figure 4-2).

In each grape cultivar extract, free ellagic acid, three types of ellagic acid glycosides (EAG 1-3) and total ellagic acid were evaluated. Previous studies including Chapter 3 (*16*) have reported only two ellagic acid glycosides, however changes in the HPLC gradient program resulted in the separation of a third ellagic acid glycoside that eluted earlier than the previous two compounds. Extracts from Noble had the highest concentrations of free ellagic acid (49.7 mg/kg) and total ellagic acid glycosides (86.9 mg/kg), yet following acid hydrolysis, total ellagic acid was higher for Albemarle (912 mg/kg) compared to Noble (686 mg/kg). This difference was likely due to the presence of

Table 4-1. The concentrations (mg/kg) of free ellagic acid, ellagic acid glycosides (EAG 1, 2 and 3) and total ellagic acids on each fraction from three different cultivars (Doreen, Noble, Albemarle).

Cultivars	Isolates[1]	Free Ellagic acid	EAG3	EAG1	EAG2	Total Ellagic acid
Doreen	Whole	13.5 a[2]	1.60a	19.5 a	22.5 a	360 a
	I	0.25 b	0.40b	N.A.	N.A.	13.1 c
	II	12.9 a	N.A.	9.15 b	9.70 b	58.9 b
	III	0.15 b	N.D.[3]	N.D.	N.D.	N.D.
	IV	0.80 b	0.55 b	1.95 c	1.80 b	2.95 d
Noble	Whole	49.7 a	6.05 a	31.4 a	49.4 a	686 a
	I	0.35 d	N.D.	N.D.	N.D.	16.8 e
	II	11.5 b	N.D.	N.D.	1.40 d	102 b
	III	1.90 d	2.75 b	9.55 c	14.0 c	32.7 d
	IV	5.55 c	2.20 c	13.5 b	24.0 b	63.2 c
Albemarle	Whole	32.9 a	7.80 a	20.0 a	37.6 a	912 a
	I	0.50 c	N.D.	N.D.	N.D.	53.7 c
	II	27.2 b	N.D.	4.60 b	12.1 b	130 b
	III	1.15 c	N.D.	0.20 c	0.40 c	0.65 c
	IV	2.45 c	4.55 b	6.95 b	12.8 b	33.0 c

[1.] Isolates were prepared by using Sep-Pak C18 and Sephadex LH-20 depending on different behaviors for various solvents. [2.] Similar letters within columns for each cultivar are not significantly different (LSD test. $P<0.05$). [3.] Not Detected.

ellagitannins. The fractionation scheme successfully separated ellagitannins into Isolate I since high levels of total ellagic acid was observed in all three cultivars compared to low amounts (< 1 mg/kg) of either free ellagic acid or ellagic acid glycoside. For example, extracts of Albemarle resulted in >100-fold increase in free ellagic acid after acid hydrolysis. Isolate I of Albemarle was applied to further MS^n analysis to identify the ellagitannins responsible for release of HHDP groups and subsequent conversion to free ellagic acid. Isolate II contained the majority of the free ellagic acid in Doreen (95%) and Albemarle (83%), but only 23% for Noble, which also contained the highest concentration of anthocyanins followed by Albemarle. Although not fully elucidated, these data seem to indicate that anthocyanins may interfere with or inhibit desorption of free ellagic acid with ethyl acetate from C_{18} Sep Pak cartridges. Isolate II was also suspected to contain ellagitannins based on total ellagic acid content following acid hydrolysis; therefore this fraction from Albemarle was subjected to further analysis by MS^n to investigate chemical structures specific to ellagic acid glycosides, ellagitannins, as well as flavonoid glycosides. Isolate III contained predominantly anthocyanins, but low concentrations of free ellagic acid and ellagic acid glycosides were also found. Subsequent recovery of remaining ellagic acid derivatives in Isolate IV was accomplished, representing those that were not solubilized by ethyl acetate earlier in the fractionation process. However, Isolate IV did not show an appreciable increase in total ellagic acid 0.58, 1.4 and 1.2% for Doreen, Noble, and Albemarle respectively. This observation demonstrated that ellagitannins were not retained on C_{18} following elution with ethyl acetate and that the ellagic acid glycosides likely had a similar detector response to free ellagic acid since the sum of free ellagic acid and the three ellagic acid

glycosides were similar before and after acid hydrolysis. This is an important consideration since authentic standards for ellagic acid glycosides are not commercially available.

Folin-Ciocalteu metal reduction assay and oxygen radical absorbance capacity (ORAC) assay were applied to the initial grape extracts and four sub-fractions isolates with the purpose to quantify the total reducing capacity of the samples and to specifically determine the peroxyl radical scavenging properties, respectively (Figure 4-2). Generally, total soluble phenolic concentrations are very well correlated with antioxidant capacity and each fraction showed strong positive relation, at least r=0.93, between the two attributes. As previously noted, Noble muscadine grapes are a commonly utilized cultivar for juice or wine making due to their high anthocyanin content and resulted in both high total soluble phenolic and antioxidant capacity in whole extracts and in Isolate III. The compounds in Isolate I, mainly ellagitannins, also contributed to antioxidant capacity by 17%, 1.5% and 11% of the total present in whole extracts of Doreen, Noble and Albemarle, respectively. Most antioxidant compounds present in Doreen and Albemarle were extracted with ethyl acetate into Isolate II due to relative abundance of non-anthocyanins compounds and resulted in 41% and 48% antioxidant capacity compare to whole extracts. Total soluble phenolics (18%) and antioxidant capacity (6.9%) in Isolate IV of Noble is likely due to residual ellagic acid glycosides and anthocyanins. Due to potential health benefits related with antioxidant activity, data re-emphasize that muscadine grapes are an excellent alternative crop to be utilized as value-added application.

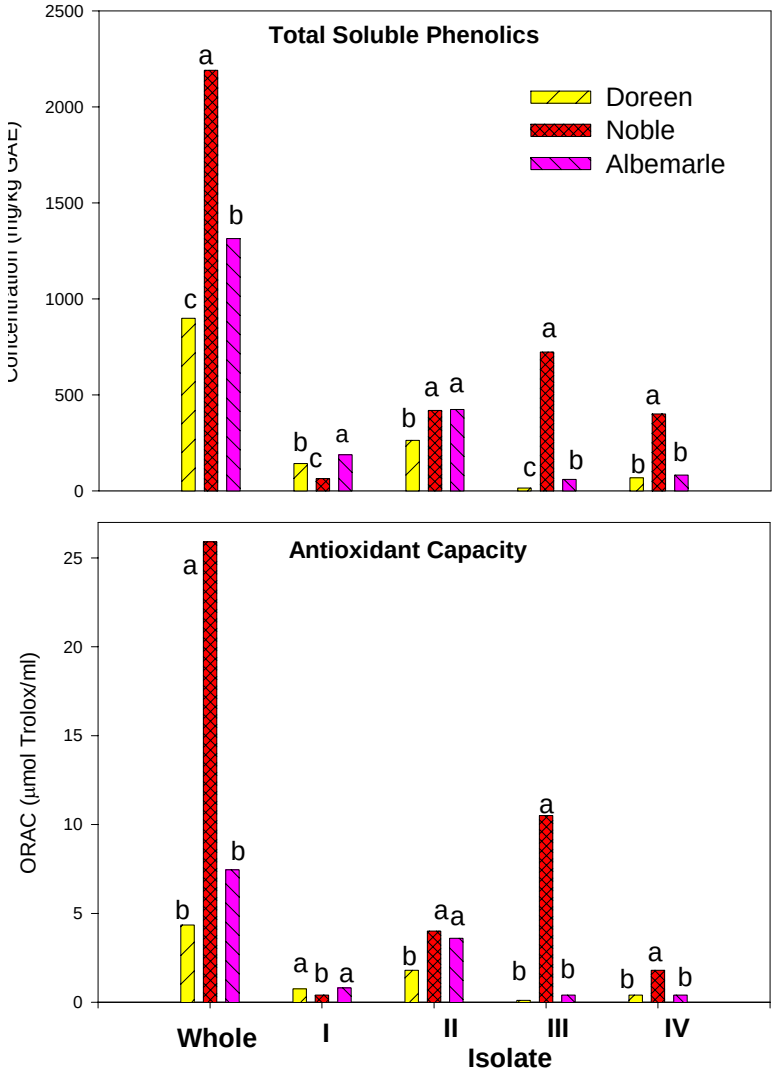

Figure 4-2. Total soluble phenolics and antioxidant capacities of five fractions (Whole, Isolates I, II, III and IV) from three different cultivars (Doreen, Noble, and Albemarle).Same letters within fractions for each attribute are not significantly different (LSD test, $P<0.05$).

Identifications of Ellagic Acid Derivatives and Flavonoids by HPLC-PDA and MS^n

To identify compounds associated with the evolution of free ellagic acid and differentiate these compounds from flavonoids in muscadine grapes, polyphenolic compounds in Isolates I and II from Albemarle were examined for their UV spectroscopic properties and molecular mass/charge ratio by HPLC-PDA and MS^n,

respectively. Only one cultivar was used for these analyses since the phytochemistry among cultivars are similar, and a goal of the analyses was to initially elucidate the identity and concentration of compounds present. HPLC coupled to PDA detector is an effective tool for tentative identification of the polyphenolic classification and can be used in combination with an authentic standard to identify unknown compounds in a plant-based system; however current trends couple this method with HPLC-MS^n to additionally confirm the identity of unknown compounds or compounds where an authentic standard is not available. By combining these methods, a compound can be characterized with greater certainty of its identification.

Ellagic acid derivatives in Isolate I

Isolate I was prepared by sequential solid phase extractions with Sep-Pak C18 and Sephadex LH-20 resulted in a fraction that contained highly polar polyphenolic compounds such as gallic acid, epigallocatechin, and hydrolyzable tannins. As discussed previously, the presence of ellagic acid derivatives was indicated by the release of free ellagic acid after acid hydrolysis. The sample isolate was employed to obtain the HPLC-PDA chromatogram and UV spectroscopic information (Figure 4-3), and also analyzed

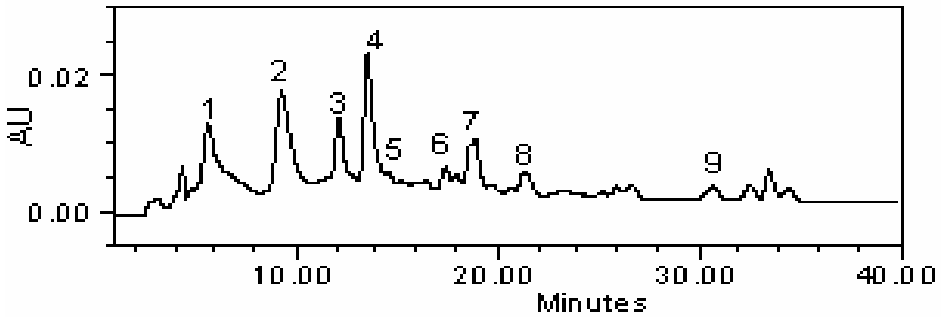

Figure 4-3. HPLC-PDA chromatogram (280 nm) of Isolate I of muscadine grapes.

Table 4-2. UVmax and HPLC-ESI(-)-MS^n analyses of polyphenols in isolate I from muscadine grapes.

Peak No.	Rt (min)	Compound	λ_{max}	MW	MS^1(*m/z*)	MS^2 (*m/z*)	MS^3 (*m/z*)
1	5.7	Ellagitannin 1	228, 262sh	802	801	757, 481	301
2	9.2	HHDP-Galloylglucose	268	634	633	301:$[M-H]^-$ - 332(Gal-Glc)	301
3	12.1	Gallic acid	272	170	169	170, 125	-
4	13.6	HHDP-Galloylglucose	265	634	633	301: $[M-H]^-$ - 332(Gal-Glc)	301
5	15.0	Ellagitannin 2	268	834	835[a]	798, 696, 303	
6	17.5	Epigallocatechin	291	306	305	261, 221, 219, 179	-
7	18.8	Digalloyl glucose	295	484	483	331:$[M-H]^-$ - 152(Gal)	271, 193, 169
8	21.4	Ellagitannin 3	276	832	833[a]	481, 303	-
9	30.7	Digalloyl glucose	272	484	483	331:$[M-H]^-$ - 152(Gal)	271, 193, 169

* $[M+H]^+$

by HPLC- ESI(+/-)-MS^n to determine molecular masses for compound elucidation (Table 4-2).

Peak 1 (t_R =5.7 min; λ_{max} =228, 262sh nm) was tentatively identified as ellagitannin 1 according to UV spectrum (Figure 4-4, A), which agreed with previous studies reported by Zafrilla et al. (*45*). This compound was identified as having at least a single HHDP unit esterified to glucose. The average molecular weight (*m/z*) of peak 1 was 802 by both positive ($[M+H]^+$ = *m/z* 803) and negative ($[M-H]^-$ = *m/z* 801) ion modes. In negative ion mode, the base peak produced fragment ions at *m/z* 481 (M-321), which corresponded to one glucose (179) and one HHDP (302) unit following MS^2. Further fragmentation (MS^3) produced its major ion at *m/z* 301 which corresponds to the ellagic acid precursor HHDP and confirmed by free ellagic acid formed following acid hydrolysis.

Peaks 2 (t_R =9.2 min; λ_{max} =268 nm) and 4 (t_R =13.6 min; λ_{max} =265 nm) had the greatest detector response for the compounds separated and contained similar UV spectroscopic properties (Figure 4-4, B) and (-)ESI-MS mass spectrums. The most abundant ions in their mass spectrum was *m/z* 633 as $[M-H]^-$, which likely corresponded to HHDP-galloylglucose (MW 634) found in birch leaves (*26*). The MS^2 spectrum also had fragments at *m/z* 301 that resulted from the loss of a galloylglucose unit (332) from its parent ion. HHDP-galloylglucose contains a single HHDP and single galloyl group conjugated to a glucose unit and is often referred to as strictinin, sanguiin H4 or sanguiin H5 depending on the location of the HHDP and galloyl group (*56*).

Peak 3 (t_R =12.1 min; λ_{max} =272 nm) was identified as free gallic acid on the basis of its retention time and UV absorbance spectrum in relation to an authentic standard.

Identification was confirmed by MS-MS, which yielded $[M-H]^-$ at *m/z* 169 and a predominant fragment at *m/z* 125.

Peak 5 (t_R =15 min; λ_{max} =268 nm), even at very low UV absorbance, was tentatively identified as an HHDP glucoside (ellagitannin 2) due to the presence of *m/z* 303 ions after serial MS analysis in positive mode. The parent compound MW 834 produced an m/z 835 $[M+H]^+$ ion, which underwent MS/MS to produce *m/z* 303 as its most abundant ion.

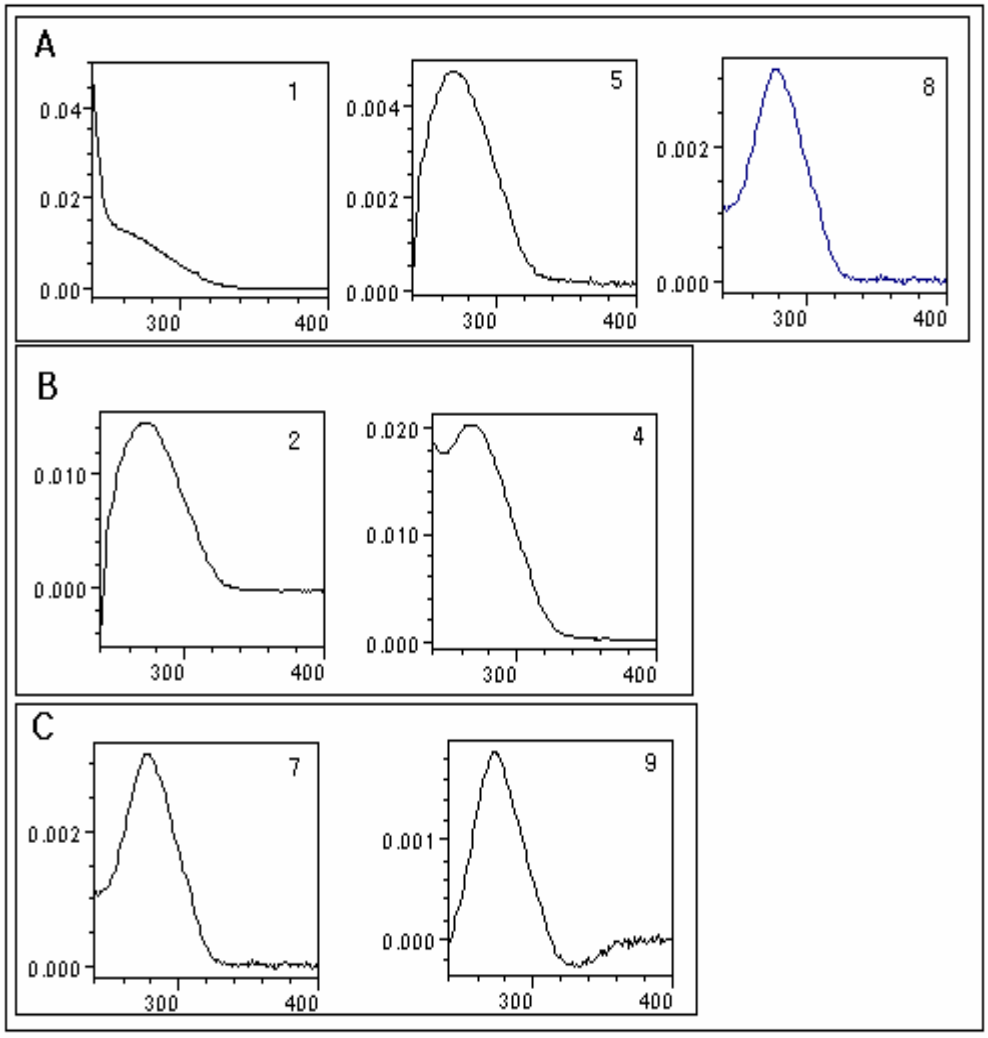

Figure 4-4. UV spectra of ellagic acid derivatives in Isolate I. A, ellagitannins (peaks 1, 5, 8); B, HHDP-galloylglucose (peaks 2, 4); C, digalloyl glucose (peaks 7, 9).

Peak 6 (t_R =17.5 min; λ_{max} =290 nm) was identified as epigallocatechin on the basis of retention time and UV absorbance spectrum in relation to an authentic standard. This was confirmed by the MS-MS, which yielded $[M-H]^-$ at *m/z* 305 and predominant MS^2 ion at *m/z* 261, 221, 219, and 179.

Peaks 7 (t_R =18.8 min; λ_{max} = 295 nm) and 9 (t_R =30.7 min; λ_{max} = 272 nm) were tentatively elucidated as digalloyl glucose (*26*) because $[M-H]^-$ at *m/z* 483 yielded *m/z* 331 by losing a galloyl group (MW 152) from glucose followed by a second galloyl group with subsequent ionization.

Peak 8 (t_R =21.4 min; λ_{max} =276 nm) was thought to contain co-eluting peaks due to the resultant molecular weight indicated from MS^n analysis. One of the compounds was determined as MW 832 due to ions at m/z 833 as $[M+H]^+$ and underwent CID-MS/MS to produce *m/z* 481 and 303, indicating presence of an HHDP unit. Therefore, this compound, ellagitannin 3 had potential to convert into ellagic acid with hydrolysis. Additional compounds (MW 480 and 818) were also detected, but no evidence that either was an ellagic acid precursor.

Ellagic acid derivatives and flavonoid glycosides in isolate II

Isolate II was prepared by ethyl acetate elution through Sep-Pak C_{18} cartridges and resulted in a fraction that was free of anthocyanins yet rich in ellagic acid derivatives and flavonoid glycosides with an aglycone base of myricetin, quercetin, kaempferol, which have been reported as predominant flavonoids in muscadine grape products (*17*). Significant increase in ellagic acid by acid hydrolysis indicated the likely presence of ellagic acid glycosides or ellagitannins in this isolate. In order to elucidate the presence of ellagitannins, two wavelengths (280 and 360 nm) were monitored for HPLC-PDA

application (Figure 4-5). MS^n analysis (Table 4-3) was applied in both (-) and (+) ESI modes to determine molecular weights and compound identification of each peak detected.

Peak 1 (t_R =58~60 min; λ_{maxs} =280sh)was detected only at 280 nm similar to the three ellagitannins observed in Isolate I (Figure 4-4). MS^n analysis, however, yielded single mass spectrum at *m/z* 800 and produced major fragment ions at *m/z* 447 and 303, which matched fragmentation patterns of HHDP-galloylglucose (Peaks 2 and 6) in Table 4-2. Although MS analysis did not clearly explain its molecular identity, this peak was tentatively categorized as an ellagic acid derivative on the basis of its UV absorbance spectrum and presence of an ion at *m/z* 303.

Peak 2 (t_R =86 min; λ_{max} =352 nm) was identified as myricetin-rhamnoside on the basis of its UV absorbance typical for a flavonoid and MS-MS spectrum. The parent compound $[M+H]^+$ at *m/z* 465 ionized to produce the major MS^2 fragment at *m/z* 319, which is indicative of a rhamnosyl unit (146 amu). Further ionization of *m/z* 319 ion

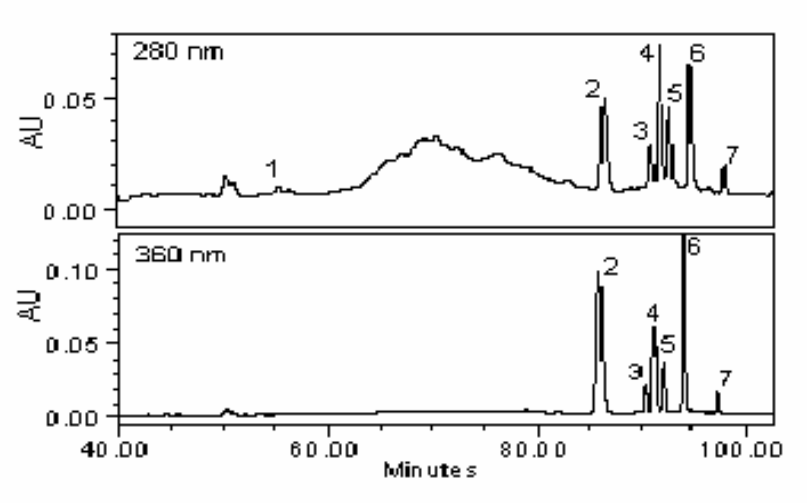

Figure 4-5. HPLC-PDA chromatogram (280 and 360 nm) of Isolate II of muscadine grapes.

yielded typical myricetin fragment at *m/z* 301, 273, 255 and 245 (*57*, *71*). Myricetin was previously identified as the most abundant flavonoid in Noble muscadine grapes (*17*).

Peak 3 (t_R =90.5 min; λ_{max} =360 nm) has been identified as one of ellagic acid glycosides due to its typical UV spectrum. MS-MS analysis revealed that $[M+H]^+$ at *m/z* 435 lost 132 amu, which corresponds to a xylosyl unit, resulting in an ellagic acid ion at *m/z* 303 by MS^2 (*14*, *15*). MS^3 of ellagic acid produced major ions at *m/z* 285 and 257. Consequently, peak 4 is confirmed as ellagic acid xyloside.

Peak 4 (t_R =91.3 min; λ_{max} =361 nm) had a $[M+H]^+$ at *m/z* 449 and MS^2 produced a major fragment at *m/z* 303 (M-146, loss of rhamnosyl group) (*72*). Therefore, this peak was identified as ellagic acid rhamnoside due to presence of major ions at *m/z* 285 and 257 by MS^3.

Peak 5 (t_R =92.3 min; λ_{max} =366 nm) is ellagic acid on the basis of identical retention time and UV spectrum with authentic standard. This was confirmed by the MS-MS, which yielded a $[M-H]^-$ at *m/z* 301 and prominent MS^2 ions at *m/z* 301, 257 and 229 (*14*, *15*).

Peak 6 (t_R =94.2 min; λ_{max} =351 nm) had $[M+H]^+$ at *m/z* 449 same as peak 5, which was identified as ellagic acid rhamnoside and also yield the same aglycone (*m/z* 303) by initial ionization. Further ionization produced major fragments at *m/z* 271, 255, 179, and 151, which is the typical fragmentation pattern for flavonoid compounds. Both ellagic acid and quercetin have identical molecular weights as 302, however, it has been reported that MS^n fragmentation pattern can be used to discriminate these two compounds. Ellagic acid seems to have more rigid structure than quercetin because the former produces bigger ion pieces than the later does with same collision energy (*14*). Also according to

Table 4-3. UVmax and HPLC-ESI(+)/(-)-MS^n analyses of ellagitannins, glycosides of ellagic acid and flavonoids in isolate II from muscadine grapes.

Peak No.	Rt (min)	Compound	λ_{max} [a]	MW	MS^1(m/z)	MS^2 (m/z)	MS^3 (m/z)
1	58-60	Ellagitannins	261 281sh 280sh	799	818[b]	447, 303	277
2	86.0	Myricetin-Rhamnoside	352	464	465	319: $[M+H]^+$ -146(Rhamnose)	301, 273, 255, 245
3	90.5	Ellagic acid-Xyloside	360	434	435	303: $[M+H]^+$ -132(Xylose)	285, 257
4	91.3	Ellagic acid-Rhamnoside	361	448	449	303: $[M+H]^+$ -146(Rhamnose)	285, 257
5	92.3	Ellagic acid[c]	366	302	301	301, 257, 229	-
6	94.2	Quercetin-Rhamnoside	351	448	449	303	257, 229, 165
7	97.5	Kaempferol-Rhamnoside	344	432	433	287: $[M+H]^+$ -146(Rhamnose)	241, 213, 165, 133

[a.] λ_{max} at 2nd band, [b.] $[M+NH4]^+$, [c.] characterized by HPLC-ESI(-)-MS^n.

UV spectra, this peak is a flavonoid glycoside not an ellagic acid glycoside. Consequently, peak 7 was identified as quercetin rhamnoside.

Peak 7 (t_R =97.5 min; λ_{max} =344 nm) was kaempferol rhamnoside because $[M+H]^+$ at *m/z* 432 produced aglycone ion at *m/z* 287 by losing a rhamnosyl (146) unit (*71*, *72*) and MS^3 confirmed kaempferol with fragments at *m/z* 241, 213, 165, 133 and 121 (*73*).

Even though PDA was not able to detect certain compound, while MS revealed trace levels of compounds such as HHDP-galloylglucose ($[M+H]^+$; *m/z* 635), myricetin-glucoside ($[M+H]^+$; *m/z* 481), unknown flavonoid pentosyl conjugate ($[M+H]^+$; *m/z* 467) and unknown compounds containing galloyl and acetylrhamnosyl groups ($[M+H]^+$; *m/z* 923).

Conclusions

Major phytochemicals in muscadine grape were identified by UV spectral properties and mass-charge ratio followed by extraction with a suitable solid phase support. The application of multiple MS analysis discovered fragments consistent with known sugar moieties in ellagic acid glycosides and 4 different ellagitannins in partially purified extracts of muscadine grape. In the case of ellagitannins, these methods were able to assess molecular weights of respective fragments, but not exact chemical identities due to diversity of ellagitannins present with varying functional groups. Additionally, predominant flavonoids, such as myricetin, quercetin and kaempferol, were determined as conjugated forms with rhamnose. All identified phytochemicals were known as excellent antioxidant compounds.

CHAPTER 5
HYDROLYTIC PROPERTIES OF ELLAGIC ACID DERIVATIVES IN MUSCADINE GRAPES

Introduction

Muscadine grapes have been historically used to produce various food products in both small and large-scale operations including juice, wine, jam\jelly, and more recently dried/concentrated products for value-added applications due to their unique and relatively high antioxidant and anticarcinogenic properties. Since no information previously existed on the ellagic acid glycoside and ellagitannin content of muscadine grapes, and likewise, no information is available on ellagic acid conversion from its precursors associate with heating. Therefore, by evaluating hydrolysis time and temperature on the relationship of free ellagic acid from its precursors, the functional properties of these compounds can be evaluated as a result of processing and prolonged storage. Through the use of acid hydrolysis and evaluation of hydrolyase enzymes, specific to certain polyphenolics, the release of free ellagic acid was evaluated in comparison to glycosidic forms for stability characteristics following pasteurization. Response surface methodology (RSM) is a powerful statistical method for modeling and analyzing the response of interest within multiple-interrelated parameters in an effort to optimize this response (58). However in the current study RSM was utilized for a different purpose as a means to continuously monitor the response as affected by two independent variables. The objectives of this study were to determine hydrolytic properties of ellagic acid derivatives and the resultant effects on antioxidant capacity

based on time-temperature combinations using a central composite design for RSM analysis. Additionally, by exploring the characteristics of ellagic acid glycosides and ellagitannins in the presence of ß-glucosidase and tannase the functional properties of these compounds can be evaluated in the absence of a heat-catalzyed hydrolytic reaction.

Materials and Methods

Response Surface Methodology (RSM) and Statistical Analyses

Methanolic extracts from the cultivar Doreen were evaluated using various hydrolytic conditions to monitor changes to ellagic acid derivatives that yield free ellagic acid in a time-temperature dependent manner. Assessment was conducted based on RSM with a central composite design (CCD) including 8 treatment combinations and 3 center points (Figure 5-1). Hydrolysis was performed at pH 3 (control) and at 2N hydrochloric acid (pH <1) with the design evaluated in duplicate. Time-temperature combinations were tested at a range of hydrolysis times from 1 min to 2 hrs and temperatures from 20^{o}C to 100^{o}C in order to generate conditions ranging from partial to complete hydrolysis at each acid concentration. Data were analyzed using JMP5 software (*44*) with analysis of variance and mean separation conducted using the LSD test ($p<0.05$).

Enzymes Preparation

In an effort to evaluate reactions of polyphenolic-active enzymes on ellagic acid derivatives, ß-glucosidase (E.C. 3.2.1.21) and tannase (E.C. 3.1.1.20) were added to Sep Pack C18 unbound and ethyl acetate-soluble fractions, which corresponded to Isolate I and II, respectively from Chapter 4. ß-glucosidase (1.333 units/ml) and tannase (1.738 units/ml) prepared in 0.2M phosphate buffer at pH 5 were added into each fraction at 37˚ C and reactions were stopped by boiling the solution after 3 hr incubation. Enzyme treated samples were analyzed compared to controls with no added enzymes. Controls

were prepared at pH 3 and 5 in order to determine the influence of pH on enzyme reaction and evaluated compounds.

Chemical Analysis

Polyphenolics were quantified with a Waters Alliance 2695 HPLC system connected to Acclaim 120 C18 column (250 mm × 4.6 mm, Dionex, Sunnyvale, CA) with a C18 guard column and to Waters 996 photodiode array detector that recorded UV spectra from 200-400 nm. Identical mobile phases and gradient elution program were employed to quantify polyphenol compounds in Chapter 4. Total soluble phenolics were analyzed using Folin-Ciocalteu assay (40) and antioxidant activity was determined using the oxygen radical absorbance capacity (ORAC) assay as described in Chapter 3.

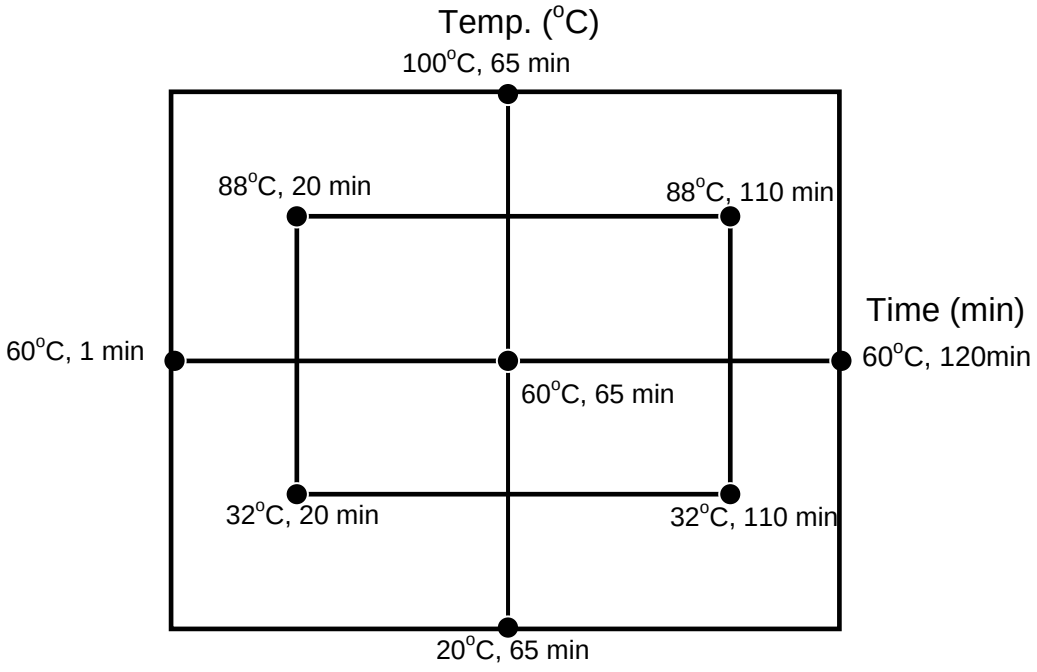

Figure 5-1. Hydrolysis time and temperature combinations included in central composite design (CCD) experiment.

Results and Discussion

Ellagic Acid Derivatives and Flavonoid Glycosides: Effects of Time, Temperature and pHs

Polyphenolic analyses were conducted using the CCD to determine hydrolytic properties of ellagic acid derivatives including free and glycosidic forms of ellagic acid and ellagitannins. Also, resultant changes in antioxidant capacity were measured as an index for functional properties due to changes in chemical composition. The 3-dimensional representations were developed to show continuous changes in ellagic acid derivatives as influenced by two independent variables, time and temperature, during hydrolysis in the presence and absence of hydrochloric acid (Figures 5-2, 3, 4). The data from hydrolysis at pH 3 was utilized to support the limited information on the influence of heating on phytochemical compositions and functional properties during thermal processing of muscadine grape products. Hydrolysis with high acid concentrations (0.5-2N HCl) at various times and temperature is a common way to evaluate polyphenolic aglycones from their respective glycosides (*59*, *60*) and has also been used to assess total ellagic acid following complete hydrolysis of ellagic acid derivatives (*12*, *14*). The present study attempted to provide a picture for alteration of individual phytochemicals by statistical methods during hydrolysis in ranges of 1 to 2 hrs and 20 to 100˚C. Overall, the levels of ellagic acid derivatives were significantly influenced by increased hydrolysis time, temperature, and acid concentration that accelerated conversion to free ellagic acid from ellagic acid precursors. Free ellagic acid (Figure 5-2) changed by 3 and 5-fold with respect to absence and presence of acid respectively, as compared to initial hydrolysis conditions (20˚C and 1 min) through hydrolysis of ellagic acid glycosides and/or ellagitannins. However, when individually monitored, two ellagic acid glycosides

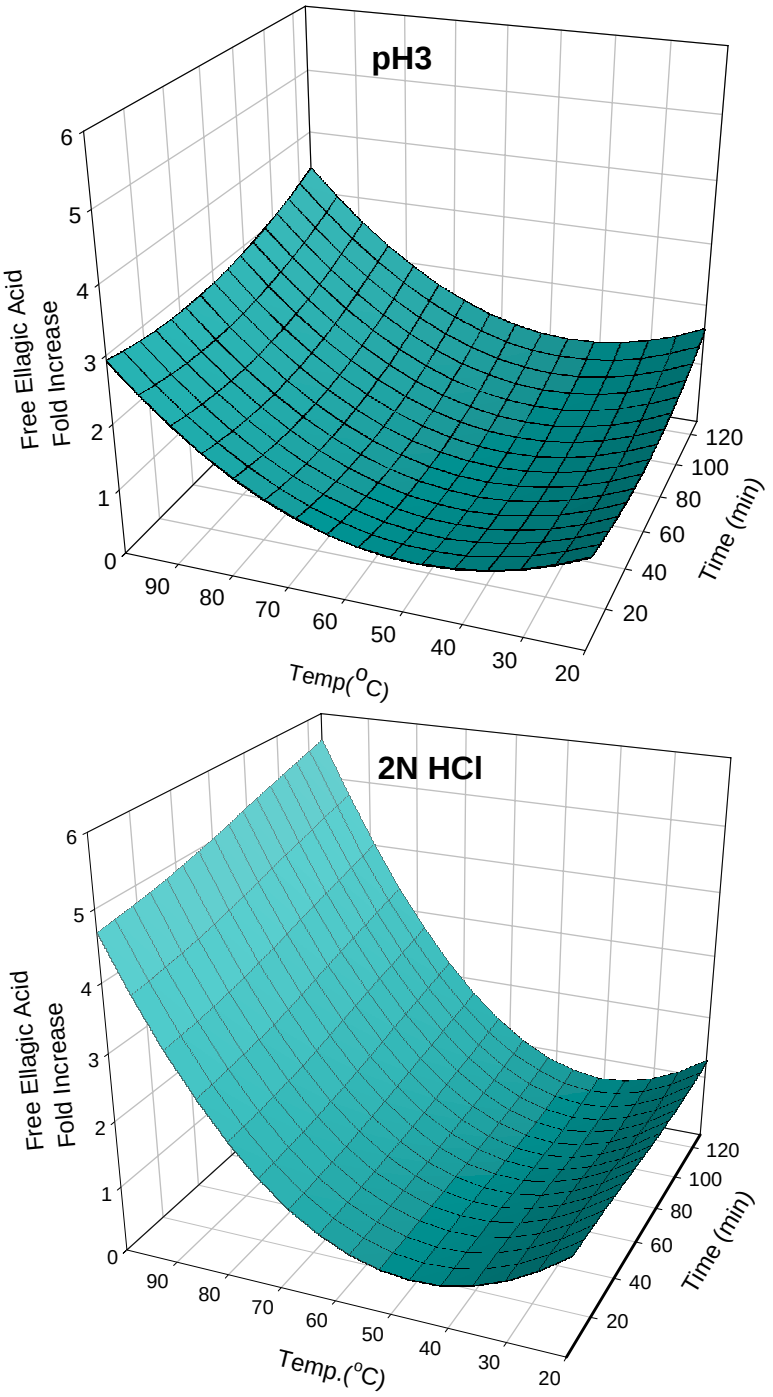

Figure 5-2. Tridimensional representation of free ellagic acid generated using fold increases by response surface model with central composite design experiment in the absence and presence of 2N HCl.

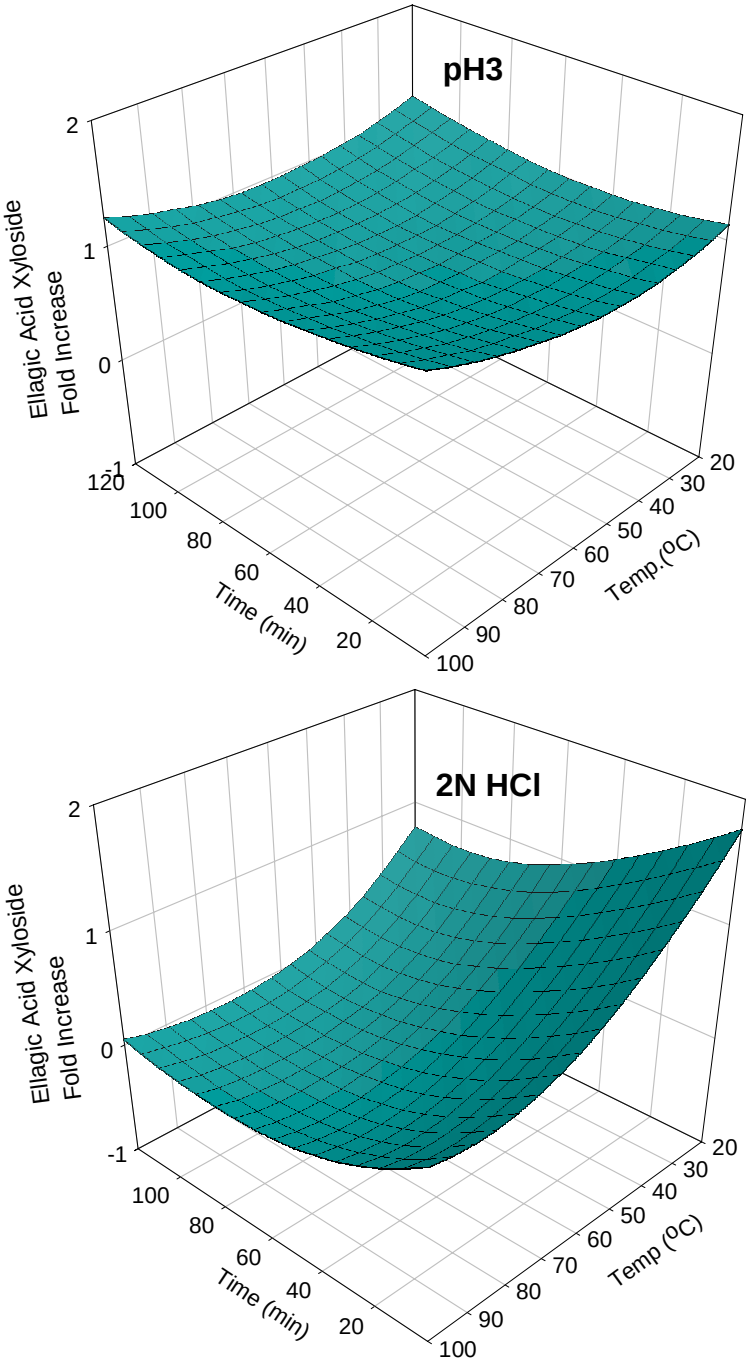

Figure 5-3. Tridimensional representation of ellagic acid xyloside generated using fold increases by response surface model with central composite design experiment in the absence and presence of 2N HCl.

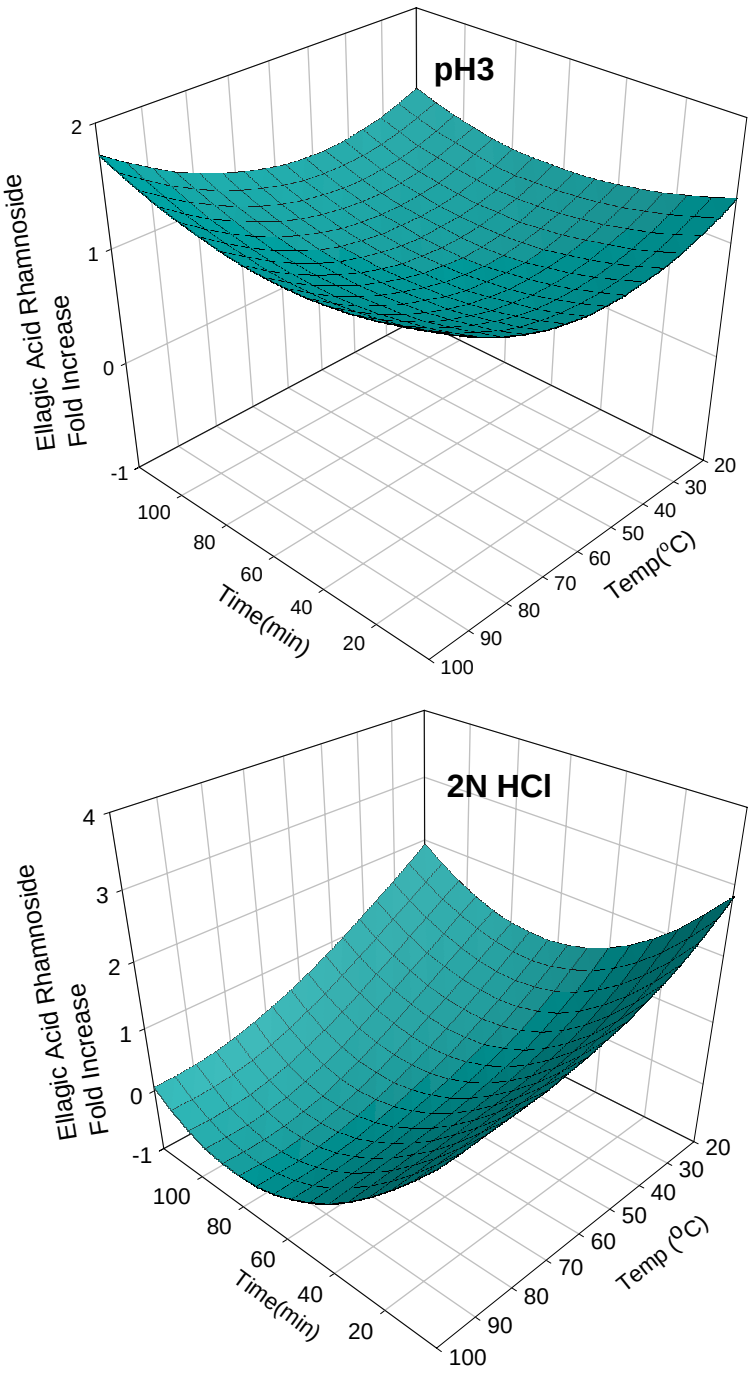

Figure 5-4. Tridimensional representation of ellagic acid rhamnoside generated using fold increases by response surface model with central composite design experiment in the absence and presence of 2N HCl.

(xyloside and rhamnoside) (Figures 5-3, 4) did not decrease in concentration at pH 3 that would indicate glycosidic hydrolysis into free ellagic acid, giving an indication that free ellagic acid was initially derived from ellagitannins in the extract(s) when hydrolysis occurred without acid. These ellagic acid glycosides were not completely converted to free ellagic acid until hydrolysis conditions reached 60°C for 65 min, whereby an appreciable increase in free ellagic acid was observed. Similar observations were made with cyanidin glycosides in blackberry and quercetin glycosides in onion, cleaving the sugar moiety in the first hour of acid hydrolysis at 75°C (*61*). The current data were collected only up to 120 min at fixed acid content (2N HCl), because higher acid contents and prolonged exposure time to acid might lead to degradation of aglycones following hydrolysis (*61*, *62*). Additionally, preliminary data observed that the level of ellagic acid was not significantly changed beyond 120 min at 100°C.

Since flavonoid glycosides also convert into aglycone of flavonoids by liberating a sugar moiety during hydrolysis, major flavonoid glycosides, myricetin, quercetin, and kaempferol rhamnosides, were evaluated in ranges of 1.00~2.06, 0.92~1.82 and 1.89~3.57 mg/kg, respectively, as change in time-temperature combinations for the absence of acid. For 2N HCl hydrolysis, all glycosides were not detected after 65 min. and 60°C, as observed with ellagic acid glycosides. Shown in the 3- dimensional representations (Figures 5-5, 6, and 7), flavonoid glycosides showed less than 50% increase after 2hrs at 100°C compare to before hydrolysis at pH 3 indicating that most flavonoid glycosides might survive through short term heat processing.

Antioxidant Capacity of Polyphenolics as Affected by Aglycone vs Glycosides with Hydrolysis

Phytochemicals contribute to the functional properties of food systems and their

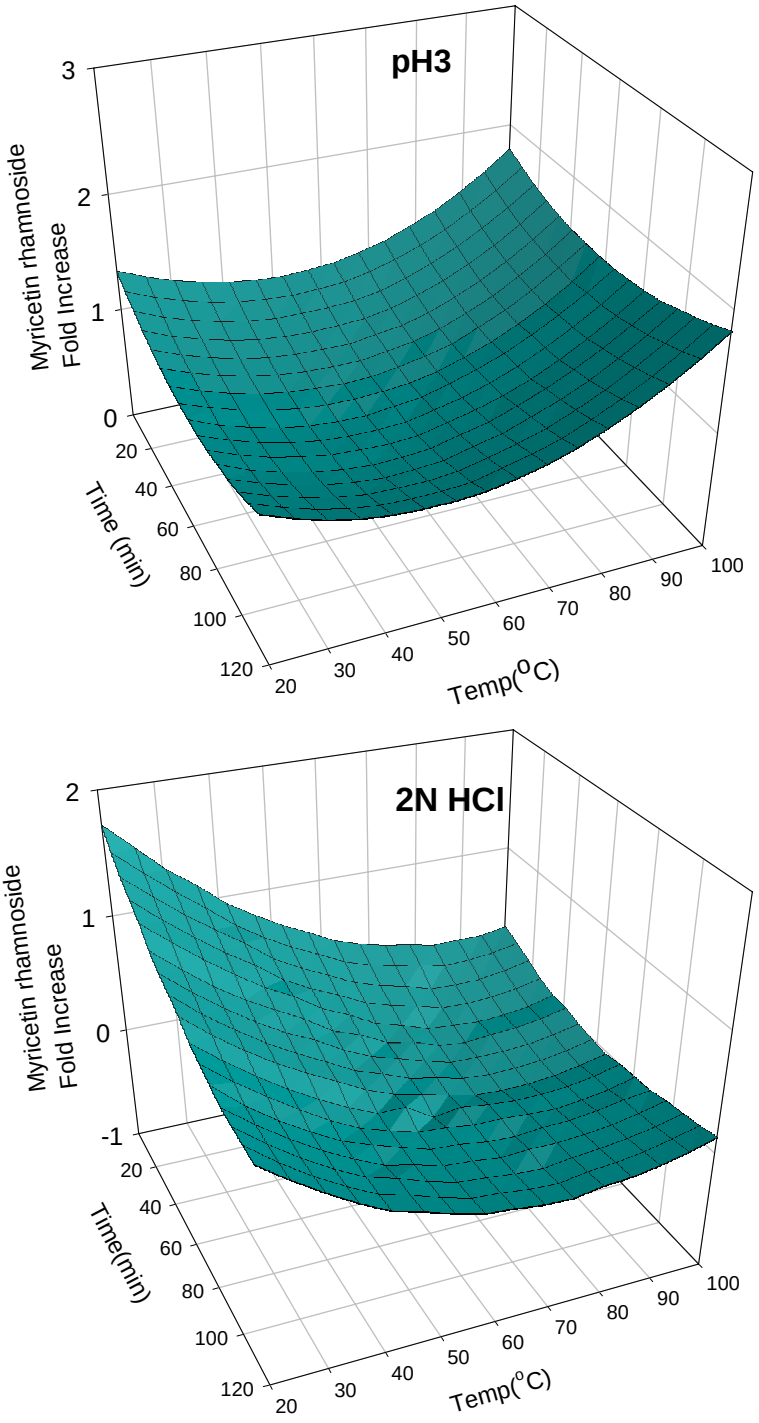

Figure 5-5. Tridimensional representation of myricetin rhamnoside generated using fold increases by response surface model with central composite design experiment in the absence and presence of 2N HCl.

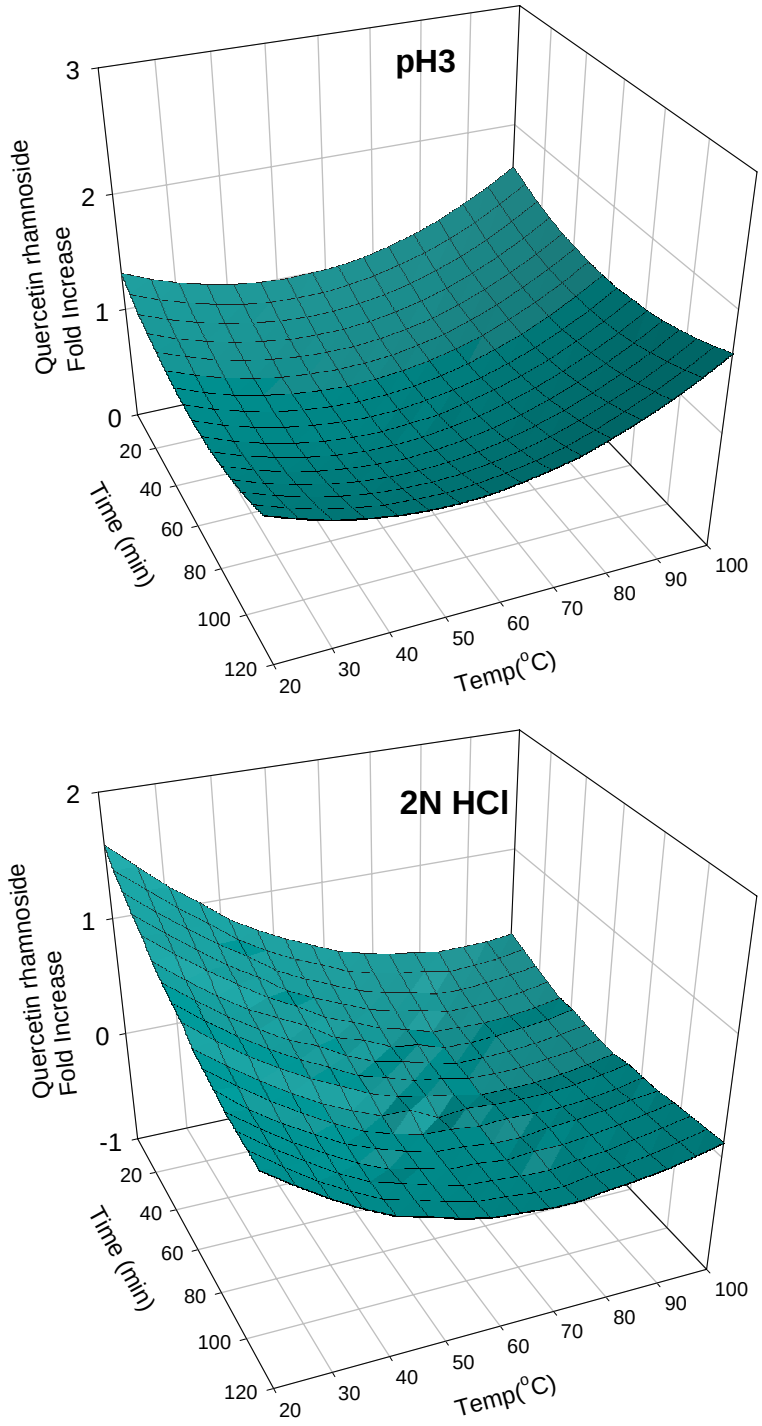

Figure 5-6. Tridimensional representation of quercetin rhamnoside generated using fold increases by response surface model with central composite design experiment in the absence and presence of 2N HCl.

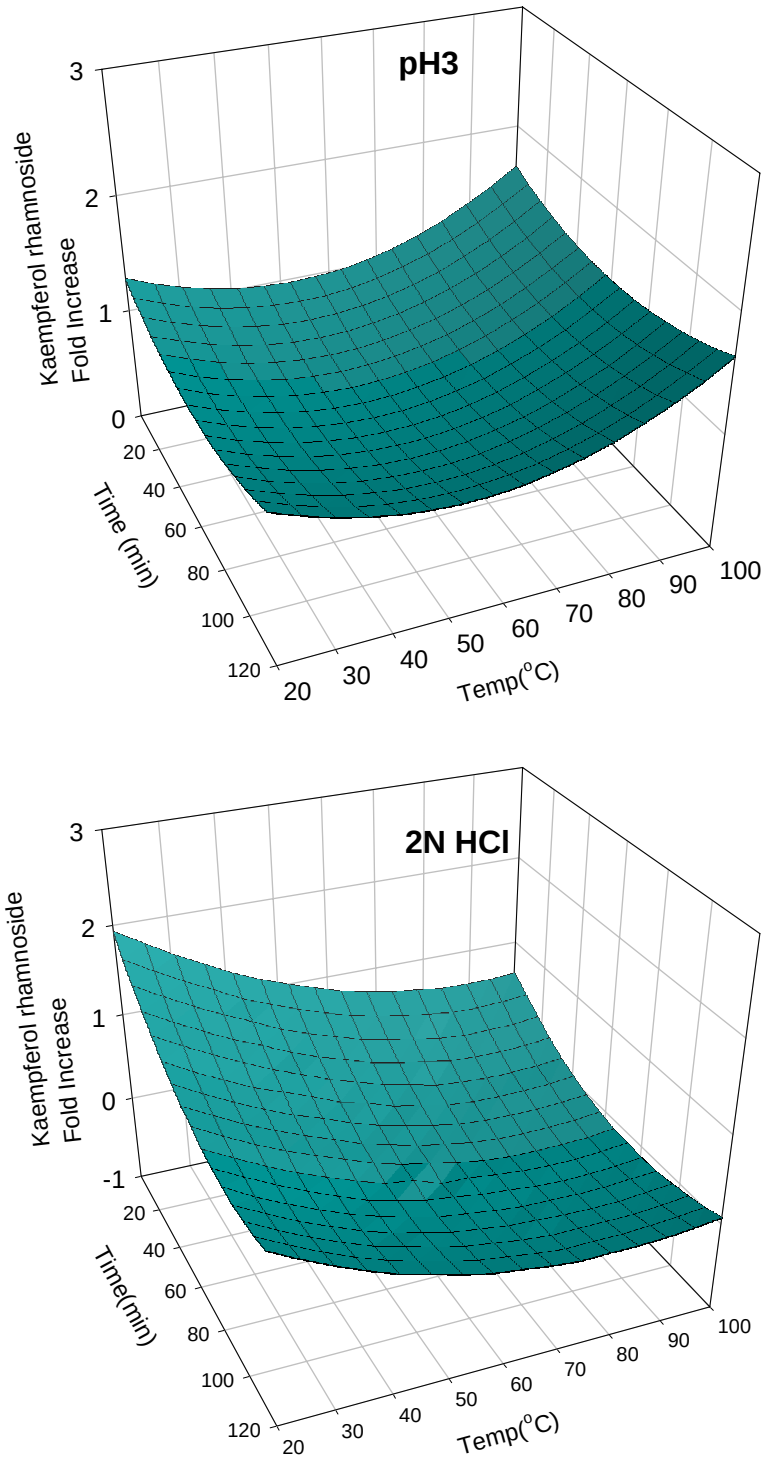

Figure 5-7. Tridimensional representation of kaempferol rhamnoside generated using fold increases by response surface model with central composite design experiment in the absence and presence of 2N HCl.

roles may diverge depending on concentration, structural differences, and synergistic/antagonistic responses in foods and biological systems. Many polyphenolics in fruits and vegetables are found as conjugated forms, with various esterified sugar moieties. Anthocyanins, the most widely distributed class of flavonoids in plants, are commonly investigated to explore antioxidant capacity changes among the various aglycones and glycosidic forms (*62-64*). Anthocyanidins, the aglycone form of anthocyanins, tended to have higher radical scavenging activities than those of corresponding glycosides in ORAC assay (*64*) or DPPH assay (*31*), whereas superior activity was obtained with monoglycosylation of malvidin, pelargonidin, and peonidin in the β-carotene bleaching method (*31*). The current experiment was able to investigate the role of aglycones and sugar conjugated forms including hydrolysable tannins and glycosides of non-anthocyanins polyphenolics in muscadine grapes to affect metal reduction and hydroxyl radical scavenging properties as a result of different stages of hydrolysis. Contour plots were presented for total soluble phenolics (Figure 5-8) and antioxidant capacity (Figure 5-9) by Folin-Ciocalteu assay and oxygen radical absorbance capacity (ORAC) assay, respectively. Data on both attributes support that aglycone polyphenolics have higher ability to reduce metal ions and scavenge hydroxyl radical compared prior to hydrolysis of polyphenolics. Ellagic acid aglycones showed strong correlation with both total soluble phenolics and antioxidant capacity, r=0.89 and r=0.58, respectively, while lower correlations were observed in ellagic acid glycosides, average 52% and 36% for total soluble phenolics and antioxidant capacity, respectivley (Table 5-1). Usually flavonoids glycosides have been evaluated as containing lower antioxidant

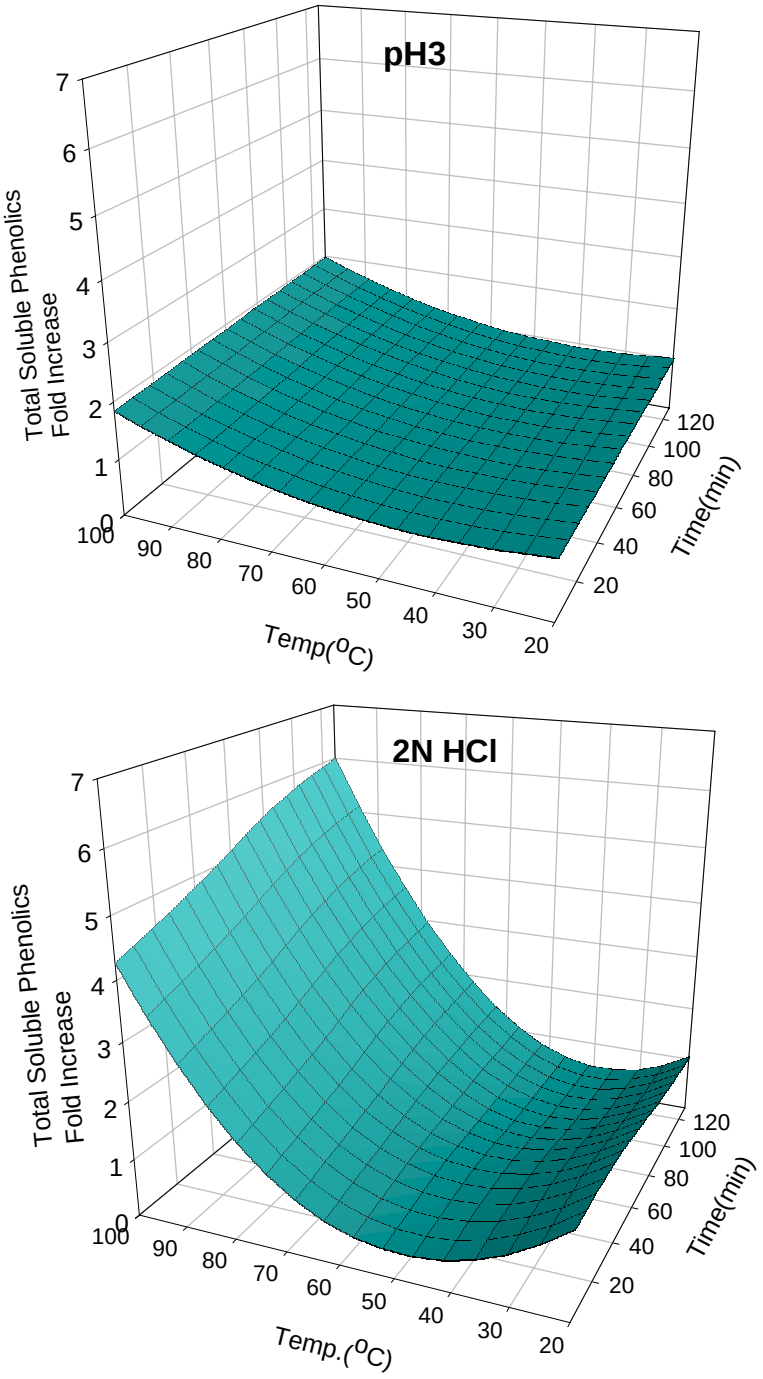

Figure 5-8. Tridimensional representation of total soluble phenolics generated using fold increases of Folin-Ciocalteu measurements by response surface model with central composite design experiment in the absence and presence of 2N HCl.

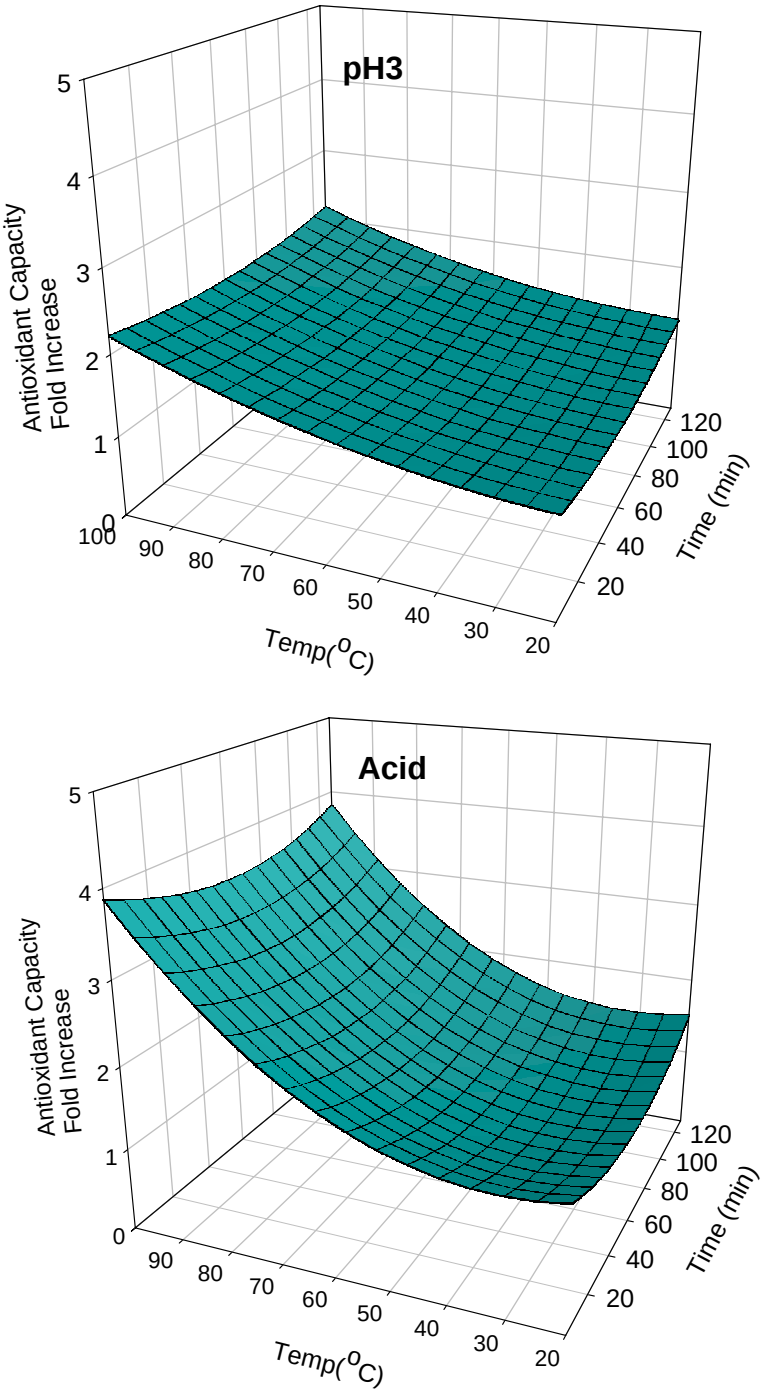

Figure 5-9. Tridimensional representation of antioxidant capacity generated using fold increases of ORAC measurment by response surface model with central composite design experiment in the absence and presence of 2N HCl.

capacity compared to aglycone of flavonoids, since blocking the 3-hydroxyl group in the heterocyclic ring influenced stability of the aroxyl radical of flavonoids and also a decrease in number of free hydroxyl group (–OH) might play an important role in scavenge free radicals (*74*). The structure-activity relationship for hydrolysable tannins, ellagitannins and hydrolytic compounds has not been elucidated with pure compounds; however these data provide evidence that chemical antioxidant capacity increases with higher concentrations of hydrolytic or aglycone forms. Even though HHDP carries 6 hydroxyl groups compared to 4 in free ellagic acid, their electron donating properties are likely inhibited by the presence of the glycosidic moiety. However, due to the low water soluble characteristics of ellagic acid, the tested samples were prepared to contain low amounts of ellagic acid derivatives (<5 mg/mL as total ellagic acid). Therefore, this premise on higher radical scavenging activity of free ellagic acid should be limited to the fact that all free ellagic acid was completely solubilized in solution; however, this is

Table 5-1. Pearson correlations coefficients of individual ellagic acid derivatives contents with total soluble phenolics and antioxidant capacity.

Variable 1	Variable 2	Partial hydrolysis at pH 3	Complete hydrolysis with 2N HCl
Total Soluble Phenolics	Antioxidant Capacity	0.77	0.81
Ellagic acid aglycone	Total Soluble Phenolics	0.89	0.98
	Antioxidant Capacity	0.58	0.83
Ellagic acid-xyloside	Total Soluble Phenolics	0.42	-0.29
	Antioxidant Capacity	0.30	-0.35
Ellagic acid-rhamnoside	Total Soluble Phenolics	0.62	-0.23
	Antioxidant Capacity	0.42	-0.28

the first trial to compare the structure-activity relationship between glycosides or hydrolysable tannins and aglycones in crude plant extracts.

Enzymatic Hydrolysis of Ellagic Acid Derivatives

Though considered a less prevalent technique, hydrolysis by enzymes such as esterase are alternative methods to release aglycones from various polyphenolics through degradation of carbohydrate linkages (*59*). ß-glucosidase (E.C. 3.2.1.21) as well as other glycosidases like arabinofuranosidase were considered important enzymes associated with the quality of foods and beverages and have been used to release aroma components in musts, wine, and fruit juices (*65*). Tannase or tannin acyl hydrolase (E.C. 3.1.1.20) are widely applied in the food industry to produce instant tea, to manufacture gallic acid and propylgallate, which can be utilized as food preservatives, and are commonly used to remove undesirable tannins. However, there are no published data on reactivity of these enzymes on ellagitannins and ellagic acid glycosides in food systems. Muscadine grape extracts were hydrolyzed by ß-glucosidase and tannase in order to provide more information on the properties of ellagic acid derivatives associated with enzyme reactions. Overall, the two enzymes showed distinctive responses in each grape isolate evaluated (Table 5-2) due to their characteristic polyphenolic composition. As described in Chapter 4, an ethyl acetate fraction contained mostly polyphenolic compounds including free ellagic acid, ellagic acid xyloside, ellagic acid rhamnoside, and ellagitannins (MW 799), also there is evidence for the presence of gallotannins in this fraction. Compared to the complex components in the ethyl acetate fraction, the water fraction is relatively simple containing tannin types of ellagic acid precursors such as three different ellagitannins (MW 802, 834, and 832) and HHDP-galloyl glucose. Therefore, enzyme reactivity on ellagic acid glycosides was evaluated in ethyl acetate fraction and any free ellagic acid

evolution in the water fraction was a consequence of enzyme reactions on ellagitannins. Reactions were performed at pH 5 to satisfy optimal conditions for both enzymes, and fortunately this elevated pH did not significantly affect the components as compared to the original pH of grapes. The activities of each enzyme were effectively evaluated by HPLC by monitoring both products and reactants under hydrolysis conditions. ß-glucosidase showed higher reactivity on xylose compared to rhamnose moieties as indicated by a significant decrease in ellagic acid xyloside (3.14 to 1.05 mg/kg), whereas the rhamnoside was unaltered in the ethyl acetate soluble fraction after 3 hrs incubation at 37˚C. Despite the observed decrease in ellagic acid xyloside, a corresponding increase in free ellagic acid was not observed due to the low initial concentrations of the xyloside present. However, when higher concentrations of ß-glucosidase were used in preliminary studies, free ellagic acid significantly increased and was also found in the insoluble sediments. A correspondingly significant decrease in both ellagic acid xyloside and ellagic acid rhamnoside was observed after 2 hrs incubation at 37˚C (data not shown).

Reaction of ß-glucosidase on each ellagic acid glycoside might vary depending on the sugar groups attached to ellagic acid, because ellagic acid glycoside was hydrolyzed with ß-glucosidase in a preliminary study. After incubation of each isolate with tannase, no activity on either ellagic acid glycosides or HHDP units of ellagitannins was detected. However, free gallic acid was significantly affected compared to control as a 17.8-fold increase. Considering the composition of ethyl acetate fraction, additional free gallic acid after incubation with tannase can be a hydrolytic product of both gallotannins and ellagitannins. However, no significant increase in gallic acid for the water fraction suggests that tannase has higher reactivity for gallotannins than ellagitannins. The

reactivity of tannase (from *Aspergillus ficuum*) may not depend on sugar specificity as discussed in ß-glucosidase due to no change in ellagic acid glycosides with tannase incubation. Consequently, tannase seems preferably to cleave off a single gallic acid rather than oxidative coupling of neighboring gallic acid, HHDP unit, from ellagitannins. Various studies on tannase activity investigated various types of hydrolysable tannins such as tannic acid, methylgallate, ethylgallate, or *n*-propylgalltae (*75-77*). Using this form of tannase for muscadine grape extracts did not show promise in hydrolyzing ellagitannins into free ellagic acid, however, other tannase sources may have different reactivities to those compounds present in muscadine juice or wine as a means to alleviate quality defects or to utilize muscadine pomace to produce ellagic acid.

Table 5-2. Concentrations (mg/kg) of ellagic acid, ellagic acid glycosides (xyloside and rhamnoside) and gallic acid affected by enzyme treatment in two different fractions from Doreen (bronze) extracts.

Fractions[1]	pH	Enzyme	Free ellagic acid	Ellagic acid xyloside	Ellagic acid rhamnoside	Gallic acid
Ethyl acetate	3	None	31.9 b[4]	3.92 a	10.5 a	1.80 c
	5	None	39.4 ab	3.14 a	11.3 a	1.80 bc
	5	ß-glucosidase [2]	41.4 ab	1.05 b	10.7 a	2.78 b
	5	Tannase[3]	47.2 a	3.86 a	11.4 a	32.1 a
Water	3	None	1.47 a	N.D.	N.D.	0.589 ab
	5	None	1.27 a	N.D.	N.D.	0.577 ab
	5	ß-glucosidase [2]	1.37 a	N.D.	N.D.	0.505 b
	5	Tannase[3]	1.29 a	N.D.	N.D.	0.789 a

[1.] prepared by Sep-pak C18. [2.] 1.333 units/ml, [3.] 1.738 units/ml in final solution and 3hrs incubation at 37˚C. [4.] Similar letters within columns for each fraction are not significantly different (LSD test. $P<0.05$).

Conclusions

Properties of ellagic acid derivatives were studied as affected by non-enzymatic (time-temperature combinations) and enzymatic (ß-glucosidase and tannase) hydrolysis. Elevated time and temperature without acid created the environment for partial hydrolysis of ellagic acid derivatives including ellagitannins and ellagic acid glycosides; however most glycosidic components (ellagic acid glycosides and flavonoid glycosides) remained after the reaction. Additional 2N HCl completely hydrolyzed ellagitannins and ellagic acid glycosides in 1hr and the free ellagic acid produced was likely to scavenge more hydroxyl radicals than conjugated forms of ellagic acid. This was also indicated by a significant increase in antioxidant capacity with evolution of ellagic acid after heating both in the absence and presence of acid. ß-glucosidase showed the possibility for application on muscadine grape juice or other products to hydrolyze ellagic acid glycosides; however tannase was not a feasible option for ellagic acid precursors.

CHAPTER 6
HYDROLYTIC AND OXIDATIVE PROPERTIES ON ELLAGIC ACID DERIVATIVES DURING STORAGE OF MUSCADINE GRAPES JUICES

Introduction

Free ellagic acid increases with hydrolysis of its precursors including ellagic acid glycosides and ellagitannins and contributes to the development of insoluble sediments in muscadine juice or wine during storage. Prior to investigating chemical or physical processing options to remediate or accelerate sediments in muscadine products, it is important to determine key components affecting relative changes of ellagic acid derivatives during storage.

According to a recent study on phytochemical stability of muscadine grape juice (*33*), phytochemical losses following processing with high hydrostatic pressure (HHP) were presumably due to the activation of residual oxidases after juice extraction and/or autoxidative mechanisms resulting in co-oxidation of anthocyanins and ascorbic acid. Ascorbic acid fortification is common for producing juice with additional oxidative protection while contributing to additional health benefits, quality, and market value. It is hypothesized that ascorbic acid fortification may protect ellagitannins from oxidative degradation in non-anthocyanin containing muscadine grape juices as ascorbic acid is commonly added in plant extracts to protect ellagitannins from oxidation during analysis (*26*). However, few studies have investigated the effects of ascorbic acid fortification on oxidative stability of individual polyphenolics, especially ellagic acid conversion from ellagic acid derivatives, in food products.

The objective of study was to evaluate individual ellagic acid derivatives in whole juice and sub-isolates over time in an effort to reveal the main precursors for producing free ellagic acid during storage of muscadine juice. Additionally, by assessing ellagic acid derivatives as affected by thermal processing, ascorbic acid fortification, and sparging with air, conclusions can be drawn concerning the chemical and oxidative behaviors of ellagic acid derivatives over time in muscadine grape juice.

Materials and Methods

Storage of Red Juices and Isolations

In order to monitor the behaviors of individual ellagic acid derivatives in whole juice and isolations, hot-pressed red muscadine juice was initially prepared by blending Noble and Albemarle (1:1) cultivars, which contain high anthocyanins and ellagic acid derivatives, respectively. Grapes were donated from local grape growers in central Florida and were frozen until processed. Equal portions of each grape were blended and pressed following heating the grapes at 70 °C for 15 min. Juice was filtered and thermally pasteurized (90 °C, 5 min). Isolations were prepared with Sep-Pak C18 cartridge as described in Chapter 4. Resulting isolates including whole juice, water (unbound), ethyl acetate and methanol isolates were stored at 4 and 37˚C for 5 weeks.

Storage of White Juices and Isolations

Cold-pressed white muscadine grape (Doreen) juice was prepared by simply crushing and pressing the fruit in an effort to evaluate the influence on ellagic acid derivatives by thermal pasteurization, ascorbic acid, and air sparging. To compare thermal processing, a portion of juice was thermally pasteurized (90 °C, 5 min) and to the remaining juice sodium azide was added to retard microbiological growth prior to

treatment application. Portions of the remaining juice (unpasteurized) were then fortified with ascorbic acid (1,000 mg/L) and equally distributed into flasks and sparged with air. Air bubbled into samples for 1 hr at room temperature and additional buffer (citric acid buffer, pH 3.2) was added to recover loss of water during the treatment as needed. Equal volumes of non-treated and treated samples were kept in test tubes at 4˚C and 37˚C for 5 weeks.

Chemical Analysis

Samples were collected at 0, 1, 3, and 5 weeks from both temperatures and centrifuged to remove insoluble particles prior to analysis. Ellagic acid derivatives were then analyzed by HPLC, as described in Chapter 5. Total ellagic acid was evaluated following acid hydrolysis (2N HCl for 60 min at 95 °C) and separation was achieved using phase B (60% methanol, pH 2.4) changed from 50-70% in 3 min; 70-80% in 2 min; 80-100% in 20 min; and 100% B in 5 min for a total run time of 30 min.

Results and Discussion

Changes of Ellagic Acid Derivatives during Storage of Red Juice

Muscadine grape products are prone to developing insoluble sediments that are likely created from ellagic acid derived from hydrolysis of its precursors during storage. Unfortunately, various processing and storage regimes have not been successful for reducing sediment formation (1, 2, 28-30) due to lack of understanding the behavior of each ellagic acid derivative during storage. In this study, muscadine juice and 3 isolates from Sep-Pak C18 were stored for 5 weeks at 4 °C and 37 °C, and relative changes in individual ellagic acid derivatives were quantified. Significant changes were observed in each isolate during storage, and were influenced by storage temperature. The whole juice prior to fractionation represented intact juice and quantified chemical attributes were

varied for storage time (Figure 6-1). Variations were more significant at 37°C, especially for free ellagic acid concentration with a 143% increase compared to 58% increase at 4°C. Increases in free ellagic acid were the result of ellagitannin degradation rather than ellagic acid glycosides, since two quantified ellagic acid glycosides decreased only 4% and 9% on average at 4 and 37˚C, respectively. Changes in free ellagic acid concentrations should be considered with formation of sediments, but accurate analyses on sediments were difficult due to the low juice volume used for evaluation. However, free ellagic acid loss via sediments were indirectly determined by evaluating total ellagic acid as sediments removed prior to analysis and resulted in a decrease in total ellagic acid concentrations. Total ellagic acid observed changes over 5 weeks were not significant with an 11% increase at 4˚C and 11% decrease at 37˚C. Considering that whole juice developed the most insoluble components during storage, suggesting free ellagic acid seems to be a minor contributor and supported by a previous study where 12% of sediment by weight was free ellagic acid (*16*).

Water (unbound) isolate was prepared by a Sep-Pak C18 with water to elute polar ellagic acid precursors such as ellagitannins, and the stability of ellagitannins were measured indirectly by evaluating total ellagic acid. Total ellagic acid was considered as hydrolytic ellagic acid from mainly ellagitannins, because only trace levels (<1 mg/L) of ellagic acid glycosides were evaluated. Pro-ellagic acid compounds did not significantly change at 5˚C but at 37˚C total ellagic acid decreased 78% (25 $\rightarrow$ 5 mg/L) (Figure 6-2) indicating significant degradation of ellagitannins. It is interesting to note that free ellagic acid concentrations were not influenced by ellagitannin hydrolysis, indicating that free ellagic acid may precipitate by other components such as metal ions, soluble pectin, or

organic acids isolated in this fraction. It was shown previously that ellagic acid has the ability to bind metal ions (*16*, *78*, *79*).

Ellagic acid glycosides were evaluated in both ethyl acetate and methanol isolates (Figures 6-3, 4) and all ellagic acid glycosides including ellagic acid xylosides and ellagic acid rhamnosides were observed to decrease ranging from 12 to 14% and 13 to 35% at 4°C and 37°C, respectively. Ellagic acid glycosides such as ellagic acid acetyl-xyloside and ellagic acid arabinoside were reported to be stable for up to 6 months in raspberry jam (*45*). Compared to the slow degradation of ellagic acid glycosides in the ethyl acetate isolate, total ellagic acid (↑ 88%) and free ellagic acid (↓ 37%) showed more distinguishable changes during storage at 37°C. Since the presence of ellagitannins in the ethyl acetate isolate was confirmed by HPLC-MS/PDA in Chapter 4, ellagitannins degraded and formed insoluble compounds in solution resulting in a decrease in total ellagic acid. Consequently, elevated storage temperature significantly accelerated hydrolysis of ellagic acid precursors during storage time. Among ellagic acid precursors, ellagitannins are likely to hydrolyze before ellagic acid glycosides, and then stay in solution or contribute to the formation of insoluble compounds with other juice constituents. Additionally, consideration of the possible effects of oxidation on ellagitannins and resultant conversion into free ellagic acid in juice storage was needed, because ellagitannins are very susceptible to oxidation (*27*).

Initial Ellagic Acid Derivatives in White Muscadine Juice as Affected by Ascorbic Acid and Air

Ascorbic acid is commonly fortified into fruit juices or products in efforts to retard the oxidation and add nutritional value; however this is challenge for red grape juice due to mutually destructive properties between anthocyanins and ascorbic acid in presence of

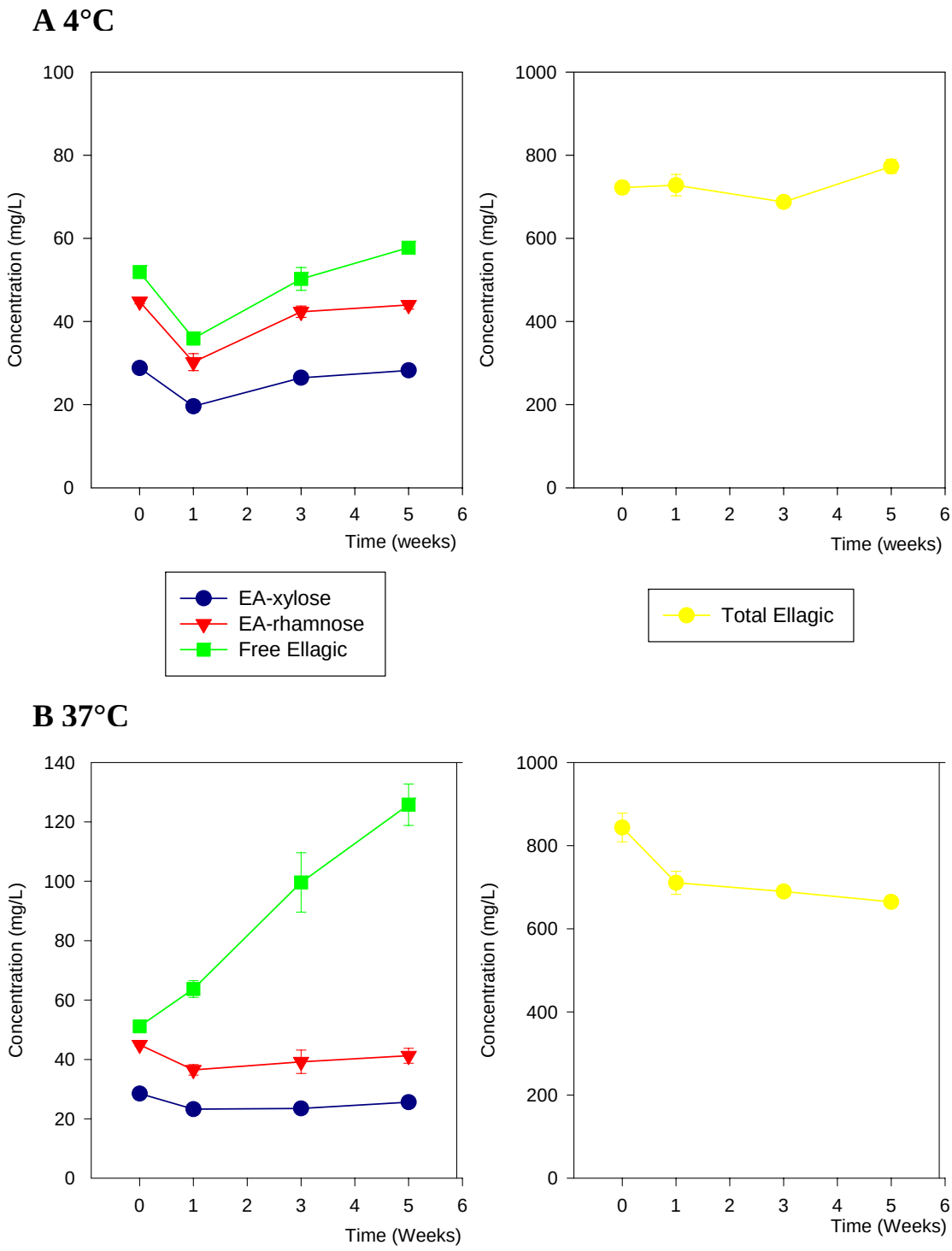

Figure 6-1. Changes in whole red juice for ellagic acid derivatives during storage. A) At 4°C. B) At 37°C.

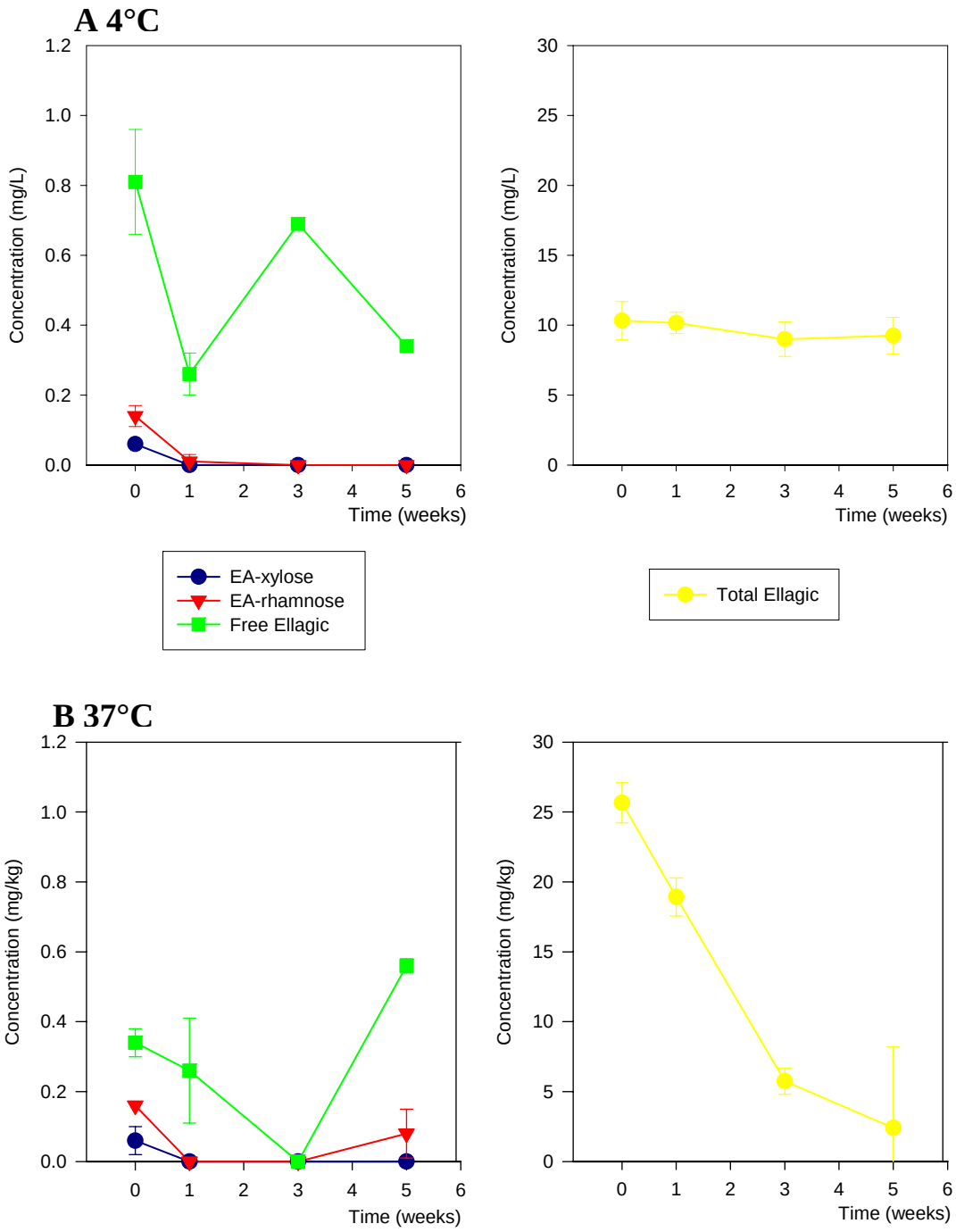

Figure 6-2. Changes in water isolate for ellagic acid derivatives during storage. A) At 4°C. B) At 37°C.

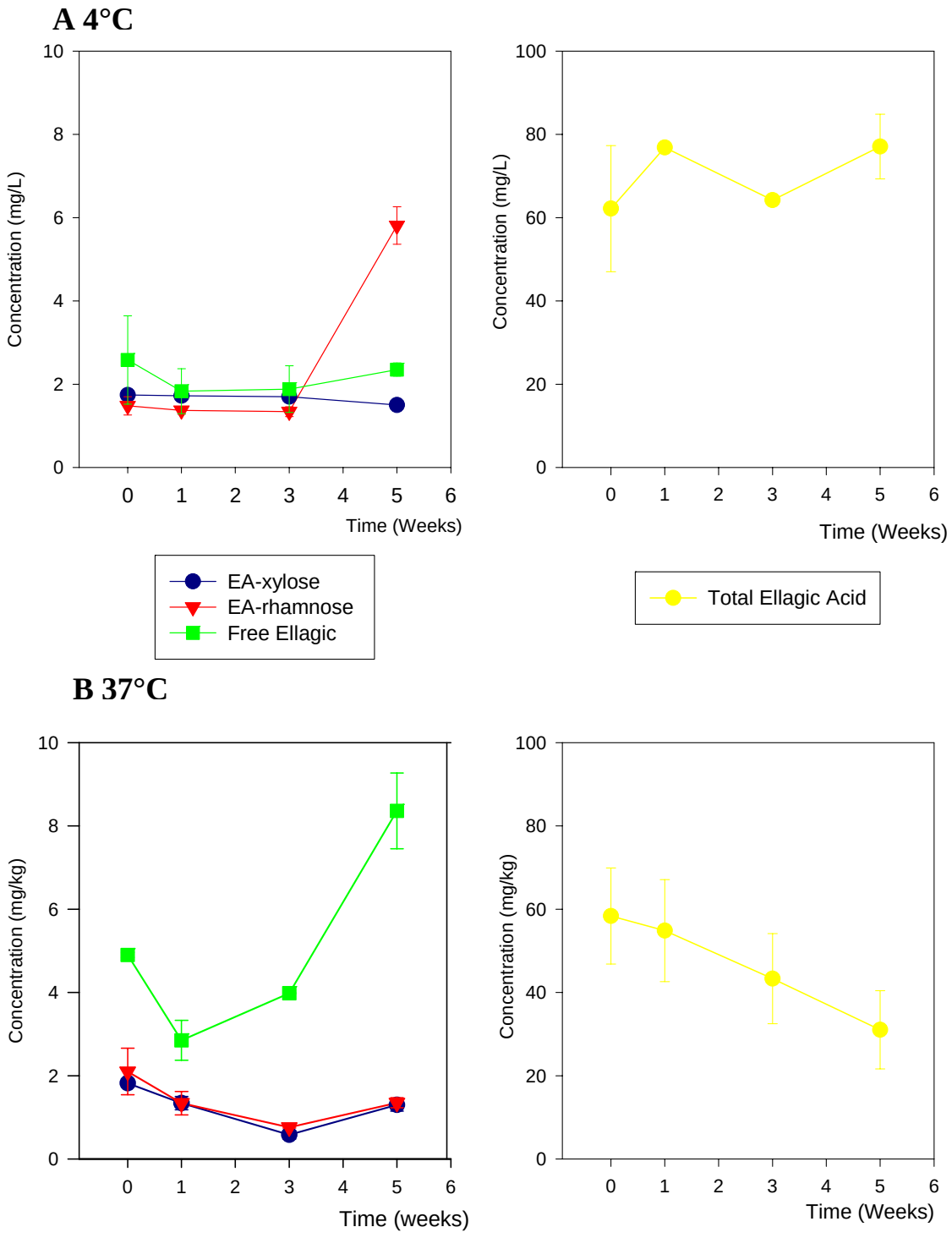

Figure 6-3. Changes in ethyl acetate isolate for ellagic acid derivatives during storage. A) At 4°C. B) At 37°C.

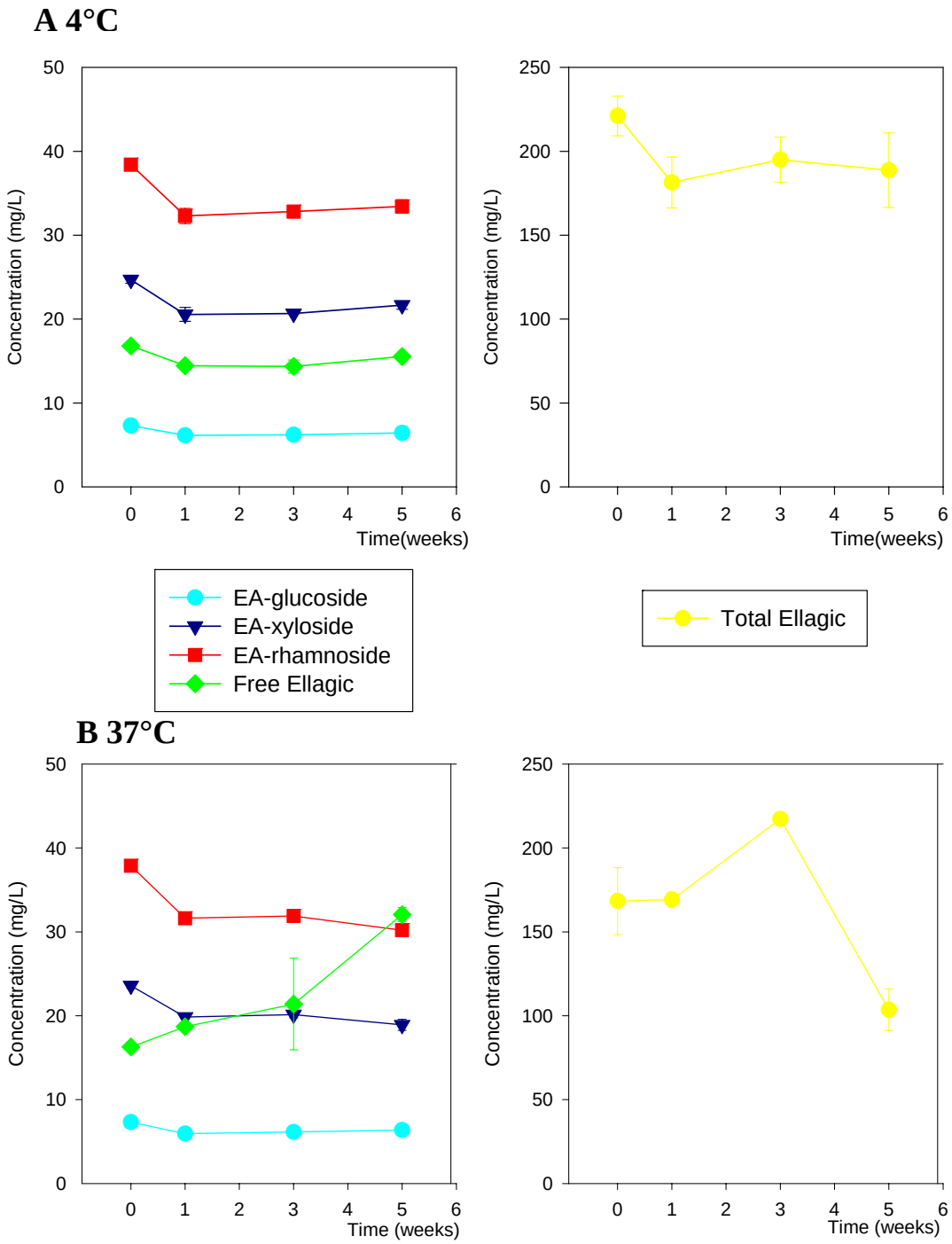

Figure 6-4. Changes in methanol isolate for ellagic acid derivatives during storage. A) At 4°C. B) At 37°C.

oxygen (*84*, *85*). For this reason, the white muscadine grape cultivar Doreen was investigated for fortification as a means to study stability and the effects of oxygen on ellagic acid derivatives during storage. Prior to treatment, juices were either pasteurized or non-pasteurized to evaluate the effect of treatments. Before storage, juices were analyzed for initial levels of ellagic acid derivatives to determine treatment effects (Figure 6-5). A comparison of heated and non-heated juice resulted in a significant increase in free ellagic acid and ellagic acid glycosides, but total ellagic acid remained. This data supported that ellagitannins break down into free ellagic acid by elevated temperature prior to hydrolysis of ellagic acid glycosides, as observed by RSM in Chapter 5. In non-heat treated juices, ascorbic acid seemed to play a role in increasing only free ellagic acid because 2.5 and 3.4 fold increases were observed in samples with ascorbic acid fortification, and the combination of ascorbic acid and air sparging, respectively. Levels of ellagic acid glycosides or total ellagic acid were not significantly changed by ascorbic acid fortification indicating that ascorbic acid may help to retain free ellagic acid in solution or to accelerate ellagitannins hydrolysis. This impact of ascorbic acid on ellagitannins and ellagic acid were likely related to the presence of natural oxidative enzymes such as polyphenol oxidase or peroxidase since ascorbic acid fortification did not influence ellagic acid derivatives in thermally processed juices. Compared to changes by ascorbic acid, excessive amounts of air in the system did not influence the initial level of free ellagic acid. Air sparging at natural juice pH is not likely to be at optimal conditions to induce oxidation of ellagic acid derivatives, since the mode of oxidation is expected to be influenced by the presence of semiquinones that form by the action of phenolate anions with triplet oxygen (*80*). Oxidation of free ellagic by air

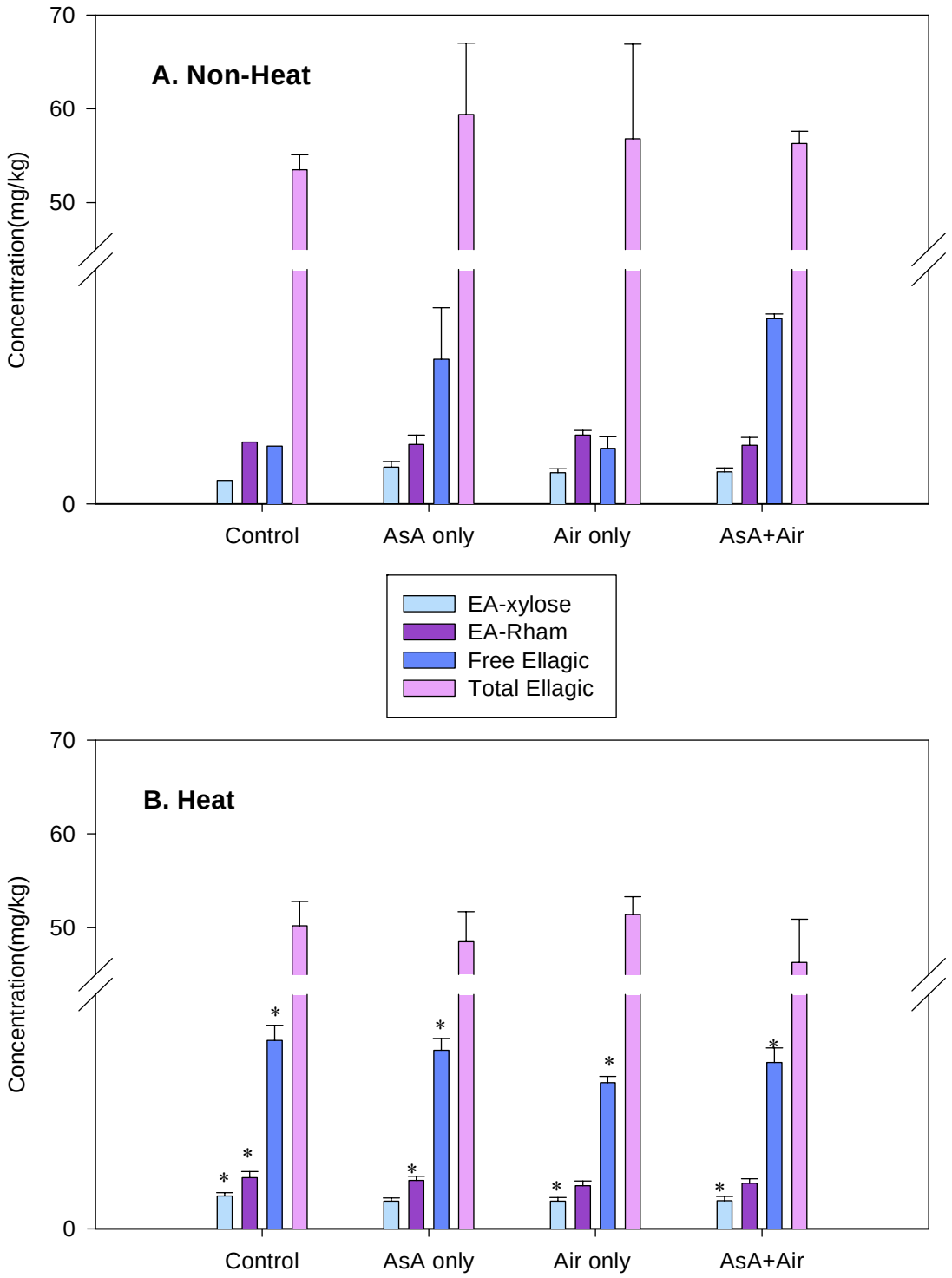

Figure 6-5. Concentrations (mg/L) of ellagic acid derivatives depending on treatments of ascorbic acid (1,000 mg/L) and air at 0day of Doreen juice as affected by thermal pasteurization method (A: Non-heat, B: Heat, * indicates significant differences between non-heat and heat treatments).

sparging could have occurred with an elevated pH due to hydrolysis of its lactone ring or by alkanine hydrolysis of ellagic acid glycosides and ellagitannins.

Post-storage Levels of Ellagic Acid Derivatives in White Muscadine Juice as Affected by Ascorbic Acid and Air

The evolution of free ellagic acid is a consequence of hydrolysis of ellagic acid derivatives during long term storage. In this study, white muscadine juices were stored for 5 weeks at 37°C and relative changes in ellagic acid derivatives quantified to determine the effects of pasteurization process, ascorbic acid fortification and excessive amounts of air on each attribute. Significant changes were observed for each ellagic acid derivatives during storage; however treatments with ascorbic acid fortification and air sparging for juices were not major factors on relative changes of ellagic acid derivatives over time. Among different ellagic acid derivatives, only free ellagic acid showed effects of ascorbic acid fortification (Figure 6-6). Ascorbic acid fortification was initially evaluated to retain higher concentrations of free ellagic acid, but ascorbic acid fortified juice retained greater levels of free ellagic acid through storage in both non-heat and heat treated juices.

Significant changes in chemical composition of stored juices were influenced by thermal processing prior to individual fortification or air sparging. The heat-treated juice without additional treatments had initially higher concentrations of free ellagic acid and changed from 4.02 to 9.35 mg/L during storage, compare to 0.74 to 2.53 mg/L in non-heat treated juice. It is interesting to note that most of the increase in free ellagic acid for non-heat treated juice occurred in the first week of storage and the level decreased but not significantly. However, the heat treated juice increased in the last two weeks (Figure 6-6). The relative stability of ellagic acid glycosides were confirmed again in heat treated

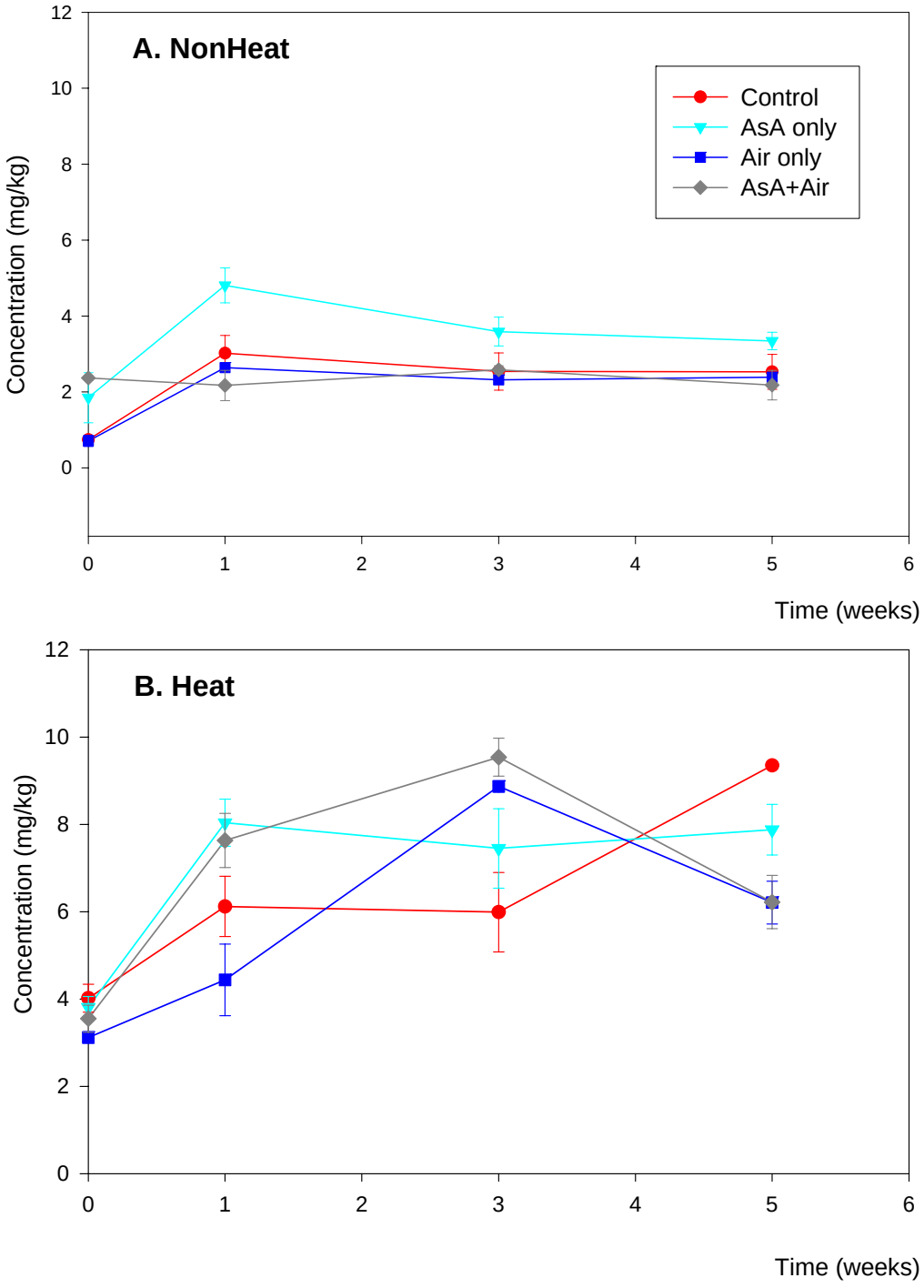

Figure 6-6. Concentrations (mg/L) of free ellagic acid depending on ascorbic acid (1,000 mg/L) and air during storage (5 weeks, 37˚C) of Doreen juice as affected by thermal pasteurization (A: Non-heat treatment, B: Heat treatment).

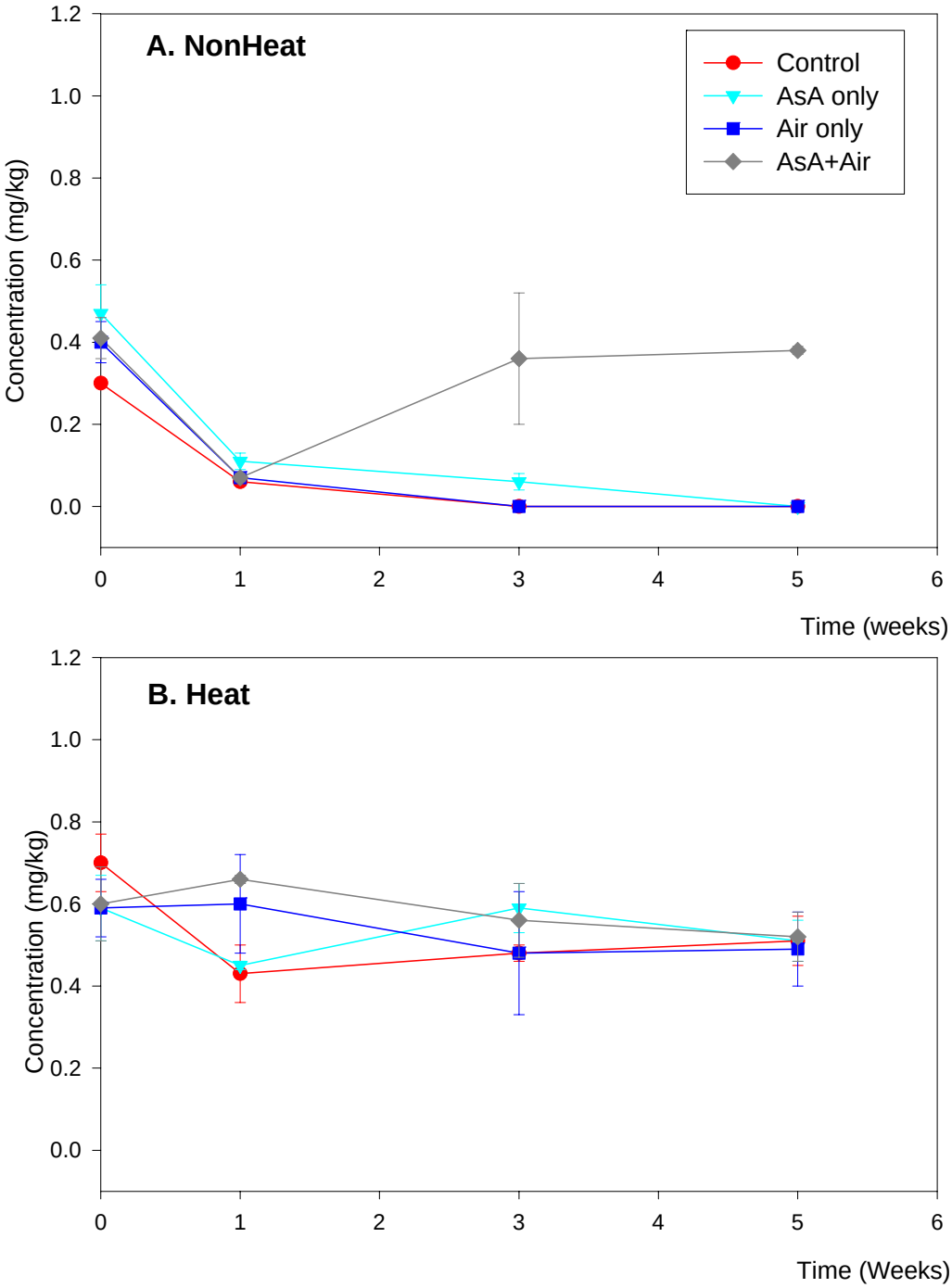

Figure 6-7. Concentrations (mg/L) of ellagic acid xyloside depending on ascorbic acid (1,000 mg/L) and air during storage (5 weeks, 37˚C) of Doreen juice as affected by thermal pasteurization (A: Non-heat treatment, B: Heat treatment).

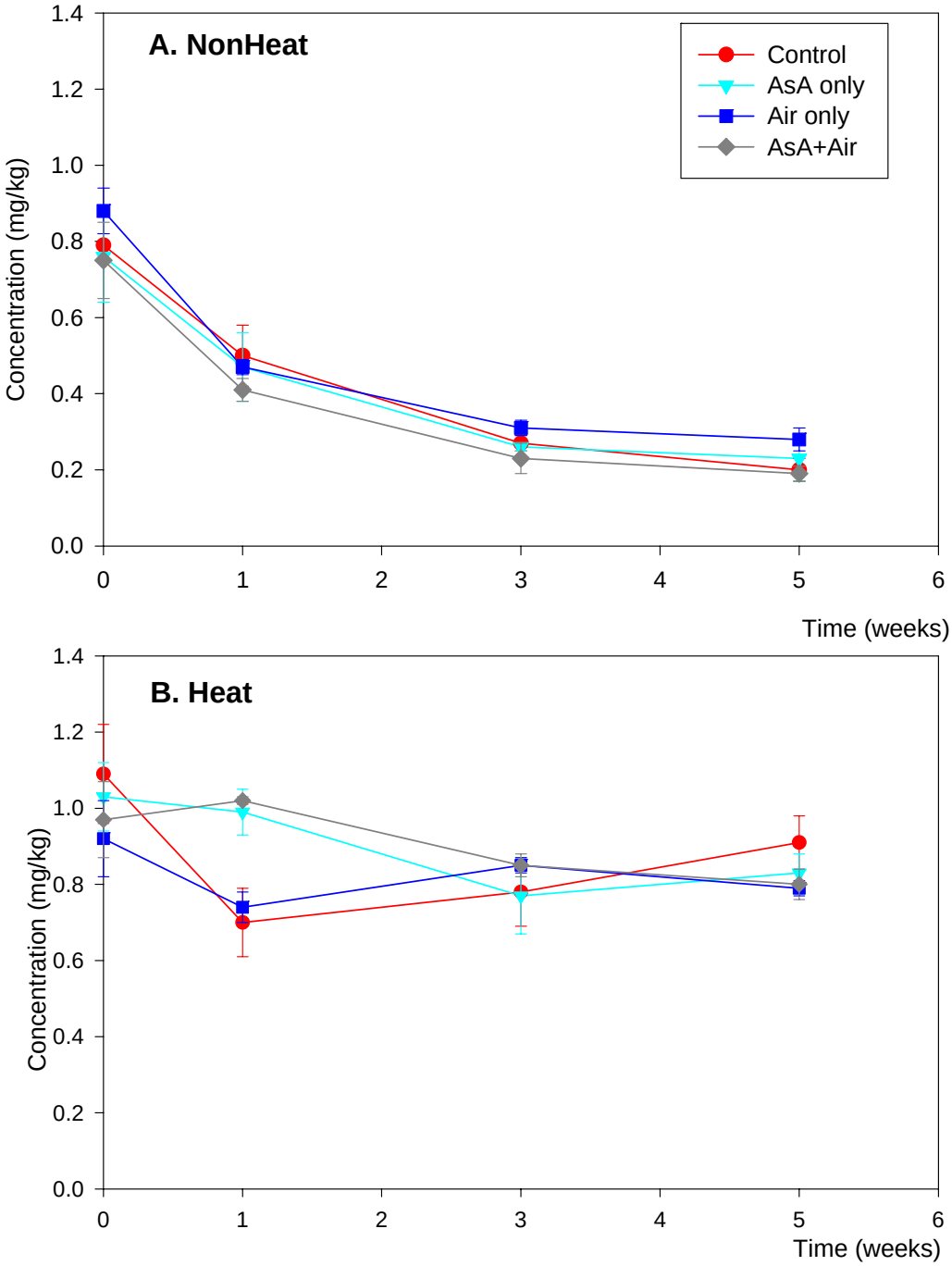

Figure 6-8. Concentrations (mg/L) of ellagic acid rhamnoside depending on ascorbic acid (1,000 mg/L) and air during storage (5 weeks, 37˚C) of Doreen juice as affected by thermal pasteurization (A: Non-heat treatment, B: Heat treatment).

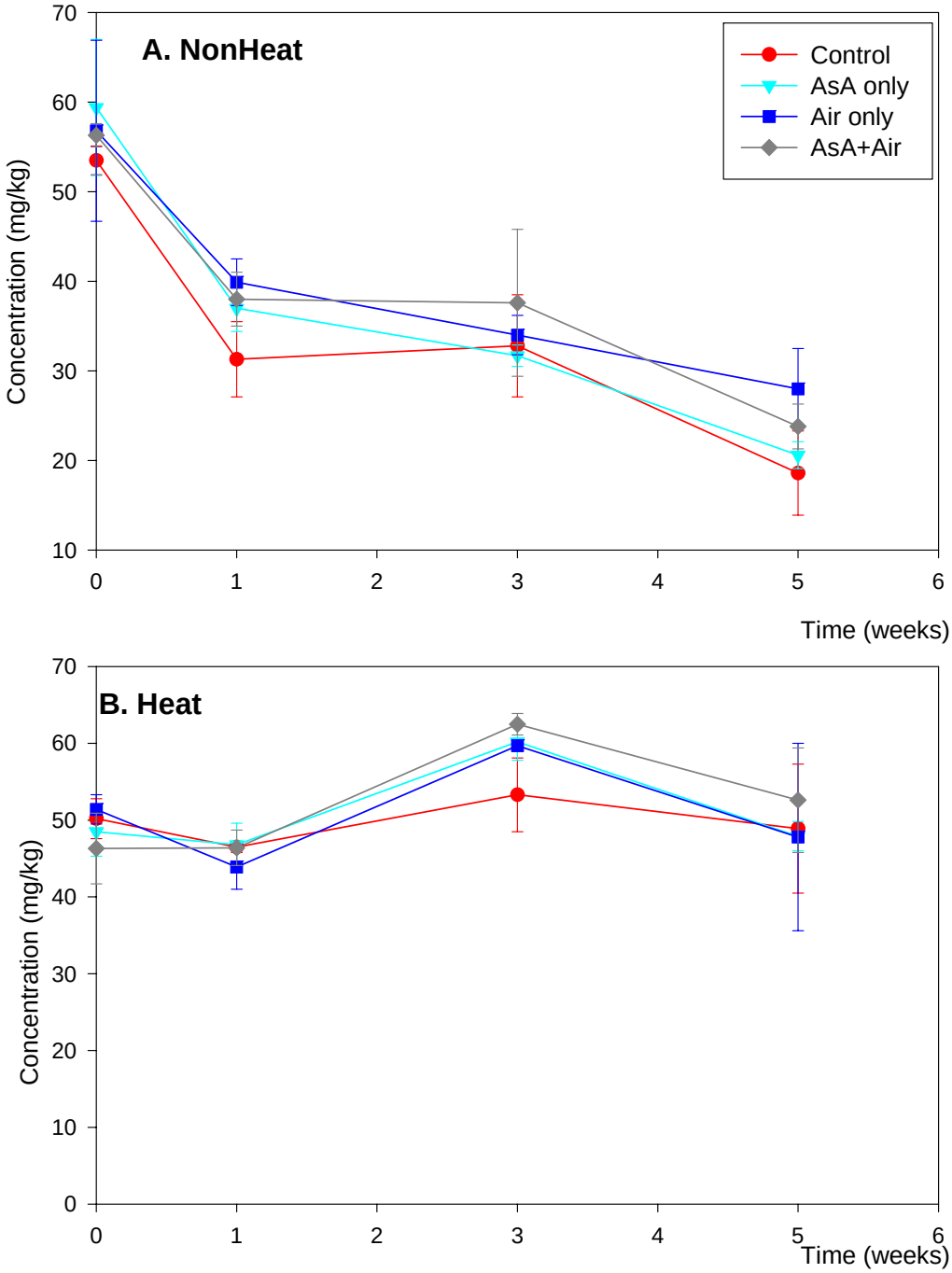

Figure 6-9. Concentrations (mg/L) of total ellagic acid depending on ascorbic acid (1,000 mg/L) and air during storage (5 weeks, 37˚C) of Doreen juice as affected by thermal pasteurization (A: Non-heat treatment, B: Heat treatment).

juices based on only 27% and 17% decrease for ellagic acid xyloside and ellagic acid rhamnoside, respectively. This stability of ellagic acid glycosides in heat-treated juices seems to be affected by thermal processing, since ellagic acid xyloside were no longer detected after 3 week storage and ellagic acid rhamnoside decreased by 74% after 5 weeks in non-heat treated juices (Figures 6-7, 8). The changes of ellagitannins were monitored indirectly as total ellagic acid. Total ellagic acid in non-heat treated juices continuously decreased with storage and retained only 35% of its initial value after 5 weeks (Figure 6-9). This decrease in total ellagic acid was impacts of degradation of mainly ellagitannins, rather than ellagic acid glycosides due to the low amounts of ellagic acid glycosides (< 1 mg/L) in solution. Data on ellagic acid derivatives for control suggested that thermal pasteurization could be important factor on relative changes on ellagic acid precursors during storage through impacts on natural enzymes in juice. Ellagic acid derivatives may not be primer targets for oxidative enzymes due to lack of *o*-dihydroxyl in the structure (33, *81*). However, thermal pasteurization may hinder the developments of *o*-quinones or secondary oxidation products from phenolic acids in grape juice (*82*, *83*), and affect to ellagitannins degradation.

Conclusions

Changes in ellagic acid derivatives of muscadine juices were evaluated initially and after 5 weeks storage to determine primer precursor for free ellagic acid evolution. Through evaluating whole juice and each isolates, ellagitannins seemed to more closely influence on free ellagic acid evolution rather than ellagic acid glycosides. Additionally, other miscellaneous components such as metal ions, soluble pectin or organic acid were likely to accelerate the free ellagic acid decrease via formation of sediments. Ascorbic acid fortification is probably non-harmful options to retain free ellagic acid in white

juices. Thermal pasteurization was a significant factor for relative changes in ellagic acid derivatives during storage likely due to inactivate natural enzymes present in juices, but additional studies are required to directly evaluate the presence of enzymes and their activities in juices prepared by different pasteurization schemes.

CHAPTER 7
SUMMARY AND CONCLUSION

Studies were conducted to provide improved information on ellagic acid derivatives including free ellagic acid, ellagic acid glycosides and ellagitannins in muscadine grape (*Vitis rotundifolia*) to understand the role of these compounds influencing on quality. Overall phytochemicals evaluations in different cultivars of muscadine grapes demonstrated that ellagic acid derivatives were present in a wide range of concentrations and were influenced by ripening, physiology, and juice processing, resulting vary in antioxidant capacity. New blending schemes with Noble and Albemarle can be suggested for red muscadine grape juice or wine to produce high quality products in terms of high color intensity and high contents of ellagic acid derivatives with corresponding high antioxidant capacity.

The main antioxidants were isolated with a series of solid phase extraction and identified by application of advanced chromatographic techniques, PDA and MS detectors connecting to HPLC. Predominant ellagic acid glycosides and flavonoid glycosides were determined their chemical identities; ellagic acid glycoside, ellagic acid xyloside, ellagic acid rhamnoside, myricetin rhamnoside, quercetin rhamnoside, and kaempferol rhamnoside in muscadine grape. In the case of ellagitannins, these methods were able to assess molecular weights of the respective fragments, but not exact chemical identities due to diversity of ellagitannins present with varying functional groups.

Using response surface methodology with two independent variables, time and temperature, successfully demonstrated that evolution of ellagic acid was a result of

temperature dependent hydrolysis of ellagic acid glycosides and ellagitannins in both presence and absence of acid. This additional free ellagic acid in solution was likely to play a significant role for scavenging hydroxyl radical indicated by high correlation ($r=0.83$) between two attributes. Enzymatic application with β-glucosidase (E.C. 3.2.1.21) or tannase (E.C. 3.1.1.20) was tested to suggest options for hydrolysis of ellagic acid glycosides and ellagitannins. ß-glucosidase showed promise as a way to hydrolyze ellagic acid glycosides, resulting in high free ellagic acid content. However, tannase was not a feasible option for break down of ellagic acid precursors.

Through evaluating whole juice and each isolates, ellagitannins seemed to be the main precursor for free ellagic acid evolution since ellagic acid glycosides were evaluated relatively stable during storage. Additionally, it is possible that ellagic acid precipitation may be aided by binding other components forming insoluble precipitates such as proteins, short chain pectins, organic acids, or metal ions. Thermal processing for pasteurization increased free ellagic acid via ellagitannins hydrolysis, and also influenced the kinetic changes of ellagic acid derivatives during storage, possibly due to inactivation of natural enzymes present in juices. Additional studies are required to directly evaluate the presence of enzymes and their activities in juices prepared by different pasteurization schemes.

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BIOGRAPHICAL SKETCH

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