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Antimicrobial and antioxidant activity of muscadine (Vitis rotundifolia Michx) extracts as influenced by solvent extraction methods and cultivars

Weiwei Chen

ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF MUSCADINE (*VITIS*

ROTUNDIFOLIA MICHX) EXTRACTS AS INFLUENCED BY SOLVENT

EXTRACTION METHODS AND CULTIVARS

By

Weiwei Chen

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Muscadine seeds from three cultivars ('Carlos', 'Noble' and 'Ison') were processed by 100% v:v methanol, 95% v:v ethanol and 70% v:v acetone. The acetone seed extracts exhibited the highest ($p<0.05$) total phenolics (21.62~24.84mg/g of dw) and antioxidant activity. A strong correlation (R2=0.891, $p<0.01$) was found between total phenolics and antioxidant activity. Methanol and 'Carlos' seed extracts generally showed the highest antimicrobial capacity against pathogen strains tested, which correlated well with tannic acid, catechin, epicatechin and tartaric acid content. Hot water-soluble muscadine skin extracts from 17 muscadine cultivars (6 bronze and 11 dark) showed effective antioxidant properties. Significant variations ($p<0.05$) were observed among the 17 cultivars in total phenolics, organic acids and antioxidant activity. 'Alachua', 'Albermarle', 'Southland', 'Janebell' and 'CA9-37' were the cultivars found to have the highest antioxidant capacity. Skin extracts of dark-skin cultivars showed higher ($p<0.05$) content than bronze-skin cultivars in phenolics, tartaric acid, tannic acid and ellagic acids.

Key words: muscadine, antimicrobial, antioxidant, solvent extraction, cultivar

DEDICATION

To

My parents and my grandmother

With love

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Food preservatives are widely used in food industry to minimize negative changes within food products. Substances that can effectively inhibit bacterial growth and delay or prevent oxidation, while showing no significant toxicity, are ideal candidates for a new-generation of antimicrobials and antioxidants. Currently, instead of chemical preservatives, natural alternatives present in foods and other biological materials are increasingly popular among consumers because most of them are considered safe to consume with unique plant-origin flavor, potential nutritional value, and therapeutic effects (Smid and Gorris, 1999; O¨zkan et al., 2004). Studies have shown that extracts from selected spices, herbs, fruits and vegetables inhibit oxidation and growth of microorganisms (Smid and Gorris, 1999; Gil et al., 2002; Wojdylo et al., 2007).

Muscadines (*Vitis rotundifolia*) are a grapevine species native to the Southeastern United States. They have higher phenolic levels than other *Vitis* genus in addition to possessing some unique compounds (Talcott and Lee, 2002; Pastrana-Bonilla et al., 2003). Studies have shown that muscadines have many bioactive properties: anti-clotting, anticancer, anti-inflammatory and antimicrobial activity (Lee and Talcott, 2002; Pastrana-Bonilla et al. 2003; Greenspan et al., 2005; Yi et al., 2006; Kim et al, 2009; Brown et al, 2009.). Based on the broad range of health benefits, muscadines could be processed and incorporated into food products as natural preservatives.

1

Studies with grapes and berries have shown that phenolics possess antimicrobial capacity (Puupponen-Pimiä et al., 2005a; Nohynek et al., 2006; Kim et al., 2008). Most phenolic compounds of muscadines are located in their seeds and skins (Pastrana-Bonilla et al., 2003). Brown et al. (2009) reported that total phenolic levels in seeds were much higher than in skins, but skins were reported to have stronger antimicrobial activity than seeds against *Helicobacter pylori*. In addition, this report and other studies suggested that antimicrobial properties were associated with not only total phenolic concentrations, but also phenolic types as well as types and concentration of other compounds in muscadines, like some organic acids (Chung et al 1998; Pastrana-Bonilla et al., 2003, Brown et al., 2009). In this study, muscadine seed and skin extracts were used to analyze possible functional compounds and their antimicrobial properties.

More than 300 cultivars of muscadines grow in the Southeastern U.S. varying in color from almost white or bronze to nearly black, and varying in bioactive composition. The concentration of phenolic compounds in muscadines and their antioxidant capacity vary significantly among different muscadine cultivars (Boyle and Hsu, 1990; Mbele et al., 2008). However, studies on variations of antimicrobial ability and related bioactive compounds among different cultivars have not been found.

Extraction is the first step to isolate useful components from plants. Water extraction is a primary and economic method in industry for extraction of plants and adding heat treatment on water-soluble extracts increase individual phenolics compounds as well as antioxidant and antimicrobial activity (Jeong et al., 2004; Stojanovic and Silva, 2007; Kim et al., 2008; Kim et al., 2009). Organic solvents, like ethanol, methanol, acetone, methanol-chloroform-water and ethylene are widely used in extraction of phenolics from herbs, fruits, juice, etc. (Kim et al., 2008). Extraction efficiency increases

2

with increasing polarity of solvents; methanol, widely used in industry, is more efficient at extraction than ethanol, acetone, and chloroform (Economou et al., 1991; Duh et al., 1992). Aqueous solutions of ethanol, methanol or acetone have been reported to perform better than a single-compound solvent for extraction of polyphenols from muscadines (Yilmaz and Toledo, 2006). However, no reports have been found about the effect of the extraction solvents on antimicrobial properties of muscadine extracts.

The objectives of this study were to:

1) investigate the effect of solvent and cultivar on antimicrobial and antioxidant activity of extracts from muscadine seeds,

2) investigate antioxidant activity of muscadine skin aqueous extracts among selected cultivars,

3) discern major phenolics and organic acids by HPLC, and

4) associate the antimicrobial and antioxidant capacities with the concentrations of major phenolics and organic compounds in muscadine extracts

CHAPTER II

LITERATURE REVIEW

Muscadines

Commercial grapes, based on their usage, could be categorized as: table grapes, raisin grapes, wine grapes, sweet juice grapes, and canning grapes (Winkler et al., 1974). Muscadine grapes (*Vitis rotundifo lia* Michx) are mainly used for fresh fruits and production of wine, and are unfamiliar to most people outside the southern United States. They are native to the southern United States, from Delaware south along the Atlantic coast to central Florida, west along the Gulf of Mexico to eastern Texas, north along the Mississippi River to Missouri, and east to Delaware, with the exclusion of the Appalachian Mountain areas and have not attained prominence in the marketplace (Olien, 2001; Leong, 2001).

Not found in other *Vitis* species, like European (*Vitis vin ifera*) and American grapes (*Vitis labrusca*), muscadines (*Vitis rotundifolia*) have many unique characteristics: thick skins, higher concentrations of certain phenolic compounds, inherent resistance to Pierce's disease and various fungal pathogens, as well as their adaptability to the warm, humid conditions of the southeastern U.S.A. (Chen et al., 2001; Olien, 2001). Furthermore, unlike tight bunch grapes, they grow singly or in small, loose clusters of 3-14 grapes (Lee and Talcott, 2002). Muscadine grapes require a long growing season, usually 100 days on the vine to mature the fruit, and in Mississippi, their harvest season are from mid-August to mid-September (Olien, 2001; Coblentz, 2007).

4

Muscadines possess a characteristic fruity aroma with a tough and thick skin. Currently, muscadinea are used as fresh fruits or processed into juice, jelly, wine and nutraceuticals (Leong, 2001). Musacdines are excellent sources of certain phytochemicals associated with many bioactive functions. Phenolics, as an important group of bioactive compounds in most grapes, are present at significant higher levels in muscadines than in other *Vitis* species (Talcott and lee, 2002). Most polyphenolic compounds in muscadines are hydrophobic and located in skins and seeds. The main phenolics found in muscadine seeds are gallic acid, catechin and epicatechin, while the major phenolics in muscadine skins include ellagic acid, myricetin, quercetin, kaempferol and resveratrol (Pastrana-Bonilla et al., 2003). Most organic acids of grapes or muscadines are hydrophilic and located in skins; over 90 percent of the total organic acids in muscadine grapes are tartaric and malic acids (Lamikanra et al., 1995; Pastrana-Bonilla et al., 2003).

Cultivars

Muscadine belongs to the *Vitis* genus. Botanically, *Vitis* includes two subgenera, Euvitis (bunch grapes) and muscadinia. Muscadinia has two American varieties, *Vitis rotundifolia* Michax (Muscadine) and *Vitis munsoniana* Simpson (Basiouny and Himelrick, 2001). Almost all the cultivars in commercial vineyards are *Vitis. rotundifolia* which are commonly called muscadines.

As early as the year 1524 cited in a logbook of French navigator Giovanni de Verrazano, Muscadines were first discovered in North Carolina (Anon, 2000). The first recognized muscadine cultivar was a bronze selection, named "Scuppernong", which was described before the year 1760; with times, the term "Scuppernong" was used to refer to all bronze-fruited varieties, regardless of actual variety names; names for dark-skin

muscadine included Bulls and its variants (bullace, bullet grape, bull grape) (Olien, 1990; Olien, 2001).

In the history of this crop, early cultivars are simply selections from the wild. However, many early cultivars are no longer grown commercially. Scuppernong is now difficult to find because of its inherent deficiency, such as a comparatively low yield and uneven fruit ripening. Development of new cultivars by breeding has been done for nearly 100 years and many improved cultivars with more desirable horticultural characteristics have been released, now widely planted throughout the south (Mortensen, 2001). Cultivars have different and individual-specific characteristics and no muscadine of any cultivar could be perfect in every respect. Some muscadine cultivars could approach perfection in some traits based upon intended end-usage. For example, for fresh market uses, characteristics of high yield, high percentage of dry scars and large berries with high sensory evaluation are important, while for juice or wine production, those of juicy pulp with high sugar content are preferable (Mortensen, 2001). Cultivars preferable to wine productions include 'Carlos', 'Doreen', 'Magnolia', 'Noble', 'Regale', 'Sterling' and 'Welder'; those suitable for fresh market include 'Alachua', 'Black beauty', 'Darlene', 'Fry, Ison', 'Janebell', 'Nesbit', 'Polyanna Sweet Jenny', 'Summit', and 'Tara'; 'Dixie' is a multipurpose cultivar; 'Southern home' is a multipurpose cultivar with ornamental values; 'Eudora' is a new cultivar for fresh-market use (Basiouny and Himelrick, 2001; Stringer et al., 2008). A recent study was conducted at the Mississippi state University Agricultural and Forestry Experiment Station (MAFES) by Stringer et al (2008). They reported some cultivar improvement work as well as indicated that evaluated cultivars performed differently in vigor, resistance to disease, yield, and fruit quality.

Currently, over 300 cultivars of muscadine exist in the Southeastern of U.S.A., varying in color from almost white or bronze to nearly black and over 100 muscadine cultivars available in dooryard planting or on the market (Mortensen, 2001). The phenolic content of grape seeds/ fruits has been demonstrated to be affected by their cultivars and environmental conditions (Silva et al., 1991; Fuleki and Ricardo-da-Silva, 1997; Revilla et al., 1997; Yemis et al., 2008). The characteristics of muscadines vary significantly among cultivars (Mortensen, 2001; Stringer et al., 2008) and very limited studies have focused on muscadine cultivar differences in their bioactive compositions along with their antioxidant (Mbele et al., 2008) and antimicrobial capacities. Antioxidant capacity of muscadines have been indicated to be dependent on phenolic concentration and phenolic concentration as well as their antioxidant capacity varied significantly among muscadine cultivars (Boyle et al., 1990 and Mbele et al., 2008).

Health Benefits

In recent years, nutritional values and health benefits of muscadines have brought an increasing interest among scientists and food manufactures. Muscadines are an excellent source of dietary fibers, essential amino acids, minerals, various vitamins and other functional phytochemicals with high nutritional values and health-promoting properties (Ector, 2001; Hartle et al., 2005; Threlfall et al., 2007). Most protective functions of muscadines are derived from their antioxidant functions, which could prevent or slow destructive oxidation reactions by scavenging "free radicals" (Velioglu et al., 1998; Musingo et al., 2001). A number of components in muscadines contribute to antioxidant ability, which include vitamins, phenolics, carotenoids, and flavonols. Phenolic compounds deserve a special attention because phenolic compounds contributed

much greater to antioxidant activity than vitamin C and carotenoids (Gil et al., 2002). Phenolic content is significantly higher in muscadine than that in other *Vitis* genus (Brown et al., 2009). It plays an important role in defense mechanisms against pathogens and environmental stress (Shahidi and Naczk, 1995; Basiouny, 2001).

Phenolics

Interest in phenolics and consumption of food rich in phenolics are booming recently, due to many positive reports of their disease-preventing functions (Greenspan et al., 2005; Mertens-Talcott et al., 2006; Fattouch et al., 2007). Phenolics are secondary metabolites unique in higher plants, widely existed in fruits, vegetables, wine, tea, chocolate and other cocoa products. Many functional bioactive properties of plant-source materials are associated with the presence, type and content of phenolic compounds, which contribute to the development of color, taste, stability and palatability, as well as the defensive system of plants against pathogens and environmental stress (Payne et al., 1989; Shahidi and Naczk, 1995; Ejechi et al., 1998; Angioni et al., 2004).

Phenolics or polyphenolics are defined as a group of molecules possessing an aromatic ring(s) bearing one or more hydroxyl groups, including functional derivatives, such as esters and glycosides (Shi et al., 2003). Phenolic compounds are subdivided into groups from quite simple to highly complex: 1) the phenolic acids with benzoic acid derivatives such as gallic acid and cinnamic acid, including caffeic, ferulic, etc; 2) flavonoids, a group of phenolics having the skeleton of diphenylpropanes with different oxidation levels an usually represented by flavanols (catechin) and flavonols (kaempferol, quecetin); 3) stilbenes (such as resveratrol); 4) lignans and complex phenolic polymers (polymeric tannins).

8

Total phenolics or individual phenolics found in fruits and vegetables have been demonstrated to have excellent antioxidant capacity (Ames et al., 1993; Hertog et al., 1993; Kähkönen et al., 2001; Musingo et al., 2001; Pastrana-Bonilla et al 2003; Yilmaz and Toledo 2004). Function of antioxidants within plant products include hydrogen donators, oxygen quenchers, free radical scavengers, peroxide decomposers, metal chelators, enzyme inhibitors, and synergists (Wang and Lin, 2000; Shahidi and Naczk, 2004).

Phenolics have demonstrated effective antimicrobial ability in many studies on plant materials (Tranter et al., 1993; Tassou and Nychas, 1994; Puupponen-Pimiä et al., 2001; Fattouch et al., 2007; Esekhiagbe et al., 2009). The inhibition mechanisms of phenolics include absorption and disruption of microbial membranes, interaction with enzymes and substrate and metal ion deprivation (Cowan, 1999; Puupponen-Pimiä et al.; 2005a; Nohynek et al., 2006; Fattouch et al., 2007). In Nohynek et al's study (2006), the mechanisms of microbial inhibitor have been reported as disintegrating the bacterial outer membrane and increasing the permeability of bacterial cells, as observed in cloudberry and raspberry extracts. Some other possible antimicrobial mechanisms could be anti-adhesion and blocking the adherence of bacteria to epithelial cells (Puupponen-Pimiä et al., 2005b).

Grape phenolics can be classified into flavonoids and non- flavonoids. Flavonoids are mainly found in skins, seeds and stems, which include catechin, epicatechin, flavonols, tannin and anthocyanins. Non-flavonoids are mainly found in pulps and comprise hydroxycinnamates and hydroxybenzoates (Kennedy, 2002; Oberholster, 2003).

Recently, nutritional values and health benefits of muscadines have brought an increasingly interest among scientists and food manufactures (Pastrana-Bonilla et al.,

2003). Muscadine grapes are an excellent source of fiber, various vitamins and photochemicals (Ector, 2001; Threlfall et al., 2005). Among all the phytochemicals, phenolics deserve a special attention based on their powerful antioxidant capacity and ability to serve as free radical scavengers (Wang et al., 2000; Shi et al., 2003). Phenolic content is significantly higher in muscadines than that in other *Vitis* genus (Velioglu et al., 1998; Brown et al., 2009).

Most protective attributes of muscadines are derived from their antioxidant functions, which could prevent or slow destructive oxidation reactions by scavenging "free radicals" (Velioglu et al., 1998; Musingo et al., 2001). A number of components in muscadines contribute to their antioxidant capacity, which includes vitamins, phenolics, carotenoids, and flavonols. Among these natural antioxidants, phenolics possess the highest antioxidant capacity (Velioglu et al., 1998; Shi et al., 2003). In Esekhiagbe et al.'s study (2009), a strong correlation (0.93-0.96) was observed between the total phenolic concentration and the antioxidant activity of nectarines, peaches, and plums. Muscadine grapes have a higher concentration of certain phenolic compounds, which are believed to be highly associated with their antioxidant capacity and many health-related bioactive functions (Musingo 2001; Greenspan 2005; Mertens-Talcott 2006; Fattouch 2007; Brown et al., 2009). Muscadines also possess many biological properties: anticlotting properties (Lee and Talcott, 2002; Pastrana-Bonilla et al., 2003), anticancer (Yi et al., 2006), antiinflammatory (Greenspan et al., 2005), antimicrobial activity (Kim et al., 2008; Kim et al. 2009; Brown et al 2009).

Muscadine grapes are approximately 40% skin, 50% pulp and 10% seed (Rizley et al., 1977). Muscadine grape pomace (skin and seeds), which is discarded as by-products from wine and juice industry, account for approximately half of the whole fruit

(Ector, 2001). Most phenolic compounds of muscadines are located in their pumaces (seeds and skins) and muscadine pulps have a very low content of phenolics (Pastrana-Bonilla et al., 2003). The seeds showed higher total phenolic levels, approximately five times higher than the skins among all the cultivars used in Striegler et al.'s study (2005). In Brown et al.'s study (2009), the total phenolic content was reported to be significantly different between muscadine seed extracts (645.5 mg AGE/g dw) and skin extracts (135.0 mg AGE/g dw) and furthermore, skins exhibited stronger antimicrobial ability than seeds against *Helicobacter pylori*. The effective antimicrobial capacity is associated with not only concentrations of total phenolics, but also types of phenolics as well as concentrations and types of some other compounds (such as some organic acids) in muscadines (Chung et al., 1998; Pastrana-Bonilla et al., 2003; Eswaranandam et al., 2004; Brown et al., 2009; Kim et al., 2009;).

Unique among *Vitis* species, muscadine grapes contain ellagic acid and anthocyanins, and their concentration varies among cultivars and methods of extraction (Lee and Talcott, 2002). Anthocyanins, primaily found as 3, 5 -diglucosides in muscadines that affect color of the skin (Flora, 1987; Goldy et al., 1986). Ellagic acid, a dimeric derivative of gallic acid, is formed by hydrolysis from its conjugated forms like ellagic acid glycosides and ellagitannins in muscadines; it has been reported to play an important role in antioxidant activity (Lin and Vine 1990; Boyle and Hsu 1990; Lee and Talcott, 2002).

Organic acids

Organic acids are weak acids widely distributed in fruits and vegetables, which influence the flavor, stability, nutrition, and acceptability of food products (Poyrazoglu et

al., 2002). The type and concentration of organic acids within the same crop are variable largely dependent on varieties, maturity and other growing conditions (Poyrazoglu et al., 2002; Bhandari and Kawabata, 2004). Tartaric and malic acids, the most prominent organic acids in muscadine grapes, decrease during ripening (Lamikanra et al., 1995), while phenolics increase during ripening (Lee and Talcott, 2004; Mbele et al., 2008). Most organic acids of grapes or muscadines are hydrophilic and located in skins; more than 90 percent of the total organic acids in grapes are tartaric and malic acids (Lamikanra et al., 1995).

Organic acids possess antimicrobial effect on bacteria and have been used for decades as food or feed preservatives. The major mode of their antimicrobial ability is penetrating the bacteria cell wall and disrupting the normal physiology of certain types of bacteria. The antimicrobial activity of organic acids towards certain bacteria is pH-dependent (Ricke, 2003). Most organic acids exist in the undissociated forms at low pH (below pKa value of the acid) and those undissociated acids can diffuse through the cell membrane, which usually in microorganisms is negatively charged. By this way, a low pH could alter cell membrane permeability of bacteria, and further change their intracellular physiological status (Puupponen-Pimiä et al., 2005c). Once the pH increases to a certain value (>7), the acids will dissociate causing metabolic uncoupling (Rosen and Kashket 1978). Moreover, individual organic acids at low pH cannot perform significant antimicrobial ability, but works better when incorporated with other antimicrobial agents. Kim et al. (2008) reported that tartaric acid alone, found in high level in muscadine seed extracts, did not inactivate *Escherichia. coli* O157:H7 as much as the whole seed extracts. In their later research, Kim et al., (2009) demonstrated that only the polar fraction (malic, gallic and tannic acids) from water-soluble muscadine seed extracts showed effective

12

antimicrobial activity against *Enterobacter sakazakii*; nevertheless, the inhibition of polar fraction was still not as effective as the original whole seed extracts.

Extraction technology

The extraction effectiveness of phenolic compounds depends on diffusion into the extraction solvent, which is affected by their chemical nature, extraction method, sample size, time and storage conditions as well as the presence of interfering substances (Kallithraka et al., 1995; Cao and Prior, 1999). A valid extraction method should achieve highly effective extractions of the target component groups as completely as possible with limited degradation (ManÉ et al., 2007). It has been summarized by several studies that extraction efficiency was influenced by some main parameters, including solvent composition, extraction temperature and time, the number of extraction stages and solid-to-liquid ratio (Prior and Cao, 1999; Nawaz et al., 2006; Youssef and Adawi, 2006;).

Water extraction is a primary and economic method in food industry for extraction of plants' compounds and adding heat treatment on water-soluble extracts increase individual phenolics compounds as well as antioxidant and antimicrobial activity (Jeong et al., 2004; Stojanovic and Silva, 2007; Kim et al., 2008; Kim et al., 2009). Organic solvents, like ethanol, methanol, acetone, methanol-chloroform-water and methylene, are widely used in the extraction of phenolics from herbs, fruits, juice. etc. (Kim et al., 2008). The phenolic extracts from plant material are a mixture of different classes of phenols, which are selectively soluble in the solvents (Perva-Uzulanic et al., 2006). Youssef and Adawi (2006) reported the addition of organic solvents in water could improve the extraction efficiency of phenolic compounds from grape seeds.

Effect of solvent type on extraction of polyphenols or other natural antioxidants from plants has been studied before. Methanol was reported work well for extraction of lower molecular weight polyphenols, while aqueous acetone was more suitable for higher molecular polyphenols (Foo and Porter, 1981; Hemingway and McGraw, 1983; McMurrough et al., 1996). Aqueous solutions of ethanol, methanol or acetone were reported to work better than pure solvents for extractions of total phenols from muscadine seeds, and optimum percentages of each solvent were summarized and compared as: 50% or 75% (v:v) acetone was more effective than 60% (v:v) of ethanol and 70% (v:v) methanol (Yilmaz and Toledo, 2006). For extractions of antioxidative components from peanut hull, methanolic extracts exhibited the highest yield and strongest antioxidant ability among all the organic solvents (Duh et al.,1992); the extraction efficiency increased with increasing polarity of solvent, which was in the order of methanol > ethanol > acetone > chloroform > n-hexane (Economou et al.1991; Duh et al.,1992). However, Koffi et al (2010) demonstrated that ethanol was the most efficient solvent, compared with water and acetone, for the extraction of polyphenolic components from Ivorian plants.

It has been reported in several studies that heat processing could increase extraction efficiency. Kim et al. (2009) showed that the heat treatment on water-soluble muscadine seed extracts could increase their acidity, organic acids, total phenolics and individual phenolics concentration. Far-infrared radiation on rice hull or simple heat on citrus peels was also reported to increase phenolic contents and antioxidant capacity (Lee et al., 2003; Jeong et al., 2004). The explanation for the increase is possibly that heat treatment could result in the better extraction of organic compounds and may cause thermal degradation of high molecular compounds, releasing the low molecular

14

compounds found in polymeric form (Lee et al., 2004, Jeong et al., 2004; Kim et al., 2008)

Extraction time is another factor determining efficiency. Longer time generally could get a better yield, but may result in some degradation problems (Cork and Krockenerger, 1991). Liquid-to-solid ratio and the number of extraction stages are also the main parameters influencing the extraction conditions.

Usually all those factors interact with each other and optimum conditions may be concluded. In 2006, Nawaz et al. demonstrated the best conditions for polyphenol extraction was 0.2g/ml solid-to liquid ratio and a double- stage extraction with extraction time varying from 5 min to 24h depending on different extraction purpose.

CHAPTER III

ANTIBACTERIAL AND ANTIOXIDANT CAPACITY OF THREE MUSCADINE (*VITIS ROTUNDIFOLIA* MICHX.) SEED EXTRACTS AS AFFECTED BY EXTRACTION SOLVENTS

Abstract

Muscadine seed extracts from three cultivars ('Carlos', 'Noble' and 'Ison') were made by three organic solvents: ethanol (95% v/v), methanol (100% v/v), and acetone (70% v/v). The variation among each extract was screened through the investigation of the antimicrobial, antioxidant activity and bioactive component contents. The acetone extracts exhibited the highest ($p<0.05$) total phenolics (21.62~24.84mg/g of dw) and antioxidant activity; no cultivar differences ($p>0.05$) were found in the antioxidant tests. A strong correlation ($R^2=0.891/p<0.01$) was observed between antioxidant activity and total phenolics. Methanol seed extracts and 'Carlos' seed extracts generally showed the highest antimicrobial capacity against pathogen strains tested. The antimicrobial abilities correlated well with tannin acid, catechin, epicatechin, tartaric acid content.

Introduction

Adding preservatives to foods is an effective method widely used to enhance shelf life of food products by mainly inhibiting oxidative or microbial activities. Natural preservatives, such as plant extracts of fruits, herbs and spices, have an increasing popularity among consumers, since most of them are considered much safer to consume with unique plant-source flavor, potential antioxidant and/or antimicrobial properties,

compared with artificial preservatives of chemical origin, (Smid and Gorris, 1999; O¨zkan, 2004;Theivendran et al., 2006). Grape seeds have been reported to inhibit several human pathogens and used as preservatives in ready-to–eat food products (Jayaprakasha et al., 2003; Theivendran et al., 2006; Kim et al., 2009).

Muscadine grapes (*Vitis roundifolia*) are a grapevine species native to the Southeastern United States, which are used as table fruits, wine, juice or jelly production, possessing a growing and competitive business (Ector et al., 1996; Ector, 2001; Leong, 2001). They are a vigorous vine, well adapted to the hot / humid conditions and tolerant to Pierce's disease (Chen et al., 2001; Olien, 1990; Poling, 1984). Muscadines contain abundant phenolic compounds and other bioactive compounds, which contribute to their health benefits: anti-clotting (Lee and Talcott, 2002; Pastrana-Bonilla et al., 2003), anticancer (Yi et al., 2006), anti-flammatory (Greenspan et al., 2005), antioxidant (Lee and Talcott, 2004; Pastrana-Bonilla et al.,2003; Talcott and Lee, 2002) and antimicrobial properties (Kim et al., 2008; Kim et al., 2009). The phenolics in seeds are significantly higher than that in other parts (skin, pulp and leaves) of muscadines (Pastrana-Bonilla et al., 2003).

Over 300 cultivars of muscadines exist in southern states of U.S.A. varying in color from almost white or bronze to nearly black (Mortensen, 2001). The Georgia Agricultural Experiment Station and the U.S. Department of Agriculture have introduced a number of improved cultivars used as current standard varieties (California Rare Fruit Growers, Inc., 1999). The characteristics of muscadines vary significantly among cultivars (Mortensen, 2001; Stringer et al., 2008), but very limited studies have focused on muscadine cultivar differences in their antioxidant and antimicrobial capacity (Mbele et al., 2008). Antioxidant capacity was reported to be dependent on phenolic

17

concentration; the phenolic content as well as the antioxidant capacity varied significantly among muscadine cultivars (Boyle et al., 1990; Mbele et al., 2008).

Organic solvents, such as ethanol, methanol, acetone, methanol-chloroform-water or methylene, are widely used in extraction of phenolics from herbs, fruits, juices, etc. (Kim et al., 2008). Aqueous solutions of ethanol, methanol or acetone were reported better for extraction of total phenols from muscadine than single-compound solvents (Yilmaz and Toledo, 2006). In Duh et al.'s study (1992) about extraction of antioxidative components from peanut hull, methanolic extracts showed the highest yield and strongest antioxidant ability among all types of solvent extracts. This report also indicated that the extraction efficiency increased with increasing polarity of solvents.

In recent years, several reports have been released about the phenolic compounds and their beneficial properties of muscadine extracts (Esekhiagbe et al., 2009; Pastrana-Bonilla et al., 2003; Mbele et al., 2008; Brown et al., 2009). However, no study has focused on cultivar differences or extraction methods by investigating concentration of phytochemicals, antimicrobial capacity and the correlation of those bioactive properties. The objective of this study was to: 1) determine the antimicrobial and antioxidant capacity among different muscadine cultivars and different extraction solvents, 2) evaluate bioactive compounds responsible for antioxidant or antimicrobial capacities.

Materials and Methods

Materials and seed extractions

Folin-Ciocalteu reagent, sodium carbonate and pure standard of gallic acid (90% purity), (+)-catechin (95% purity), (-)-epicatechin (90% purity), ellagic acid (95% purity), malic acid and tannic acid were purchased from either Fluka (Milwaukee, WI, USA) or

Sigma (St. Louis, MO, USA). Tartaric acid, acetic acid, hydrochloric acid, sulfuric acid, methanol, ethanol, acetone, acetonitrile, and HPLC grade water (high-performance liquid chromatography of HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Muscadine seed powders were provided by a regional muscadine producer, one bronze cultivar ('Carlos') and two purple cultivars ('Noble and 'Ison'). All powders were kept at -20° C in the dark for further analysis. Three extraction solvents were prepared: ethanol (95% v/v), methanol (100% v/v) and acetone (70% v/v). These solvents and concentrations were chosen and designed based on the previous research (Yilmaz and Toledo, 2006; Youssef and Adawi, 2006; ManÉ et al., 2007) and the preliminary work done in our lab (Private communication with Dr. Taejo. Kim, Mississippi State University). In this study, we used the double-stage extraction method to prepare each seed extract (Nawaz et al., 2006). In summary, two grams of powder were mixed with 5ml of the solvent in a screw-capped vial (National Scientific Co., Rockwood, TN) and then incubated in a rotary mixer (Dynal ® Inc., NY) at 67 rpm for three hours at room temperature. After incubation, the sample was centrifuged at 12000 rpm (17,000 g) for 15min and the supernatant was obtained. After the first extraction, 2.5 ml of the same solvent was added to the sediment and extracted once more as in the previous procedure. The supernatants from two extractions were gathered in a new vial as the seed extracts, which were used for evaluation of antioxidant ability, total phenolics, major organic acids and individual phenolic compounds. To get crude extracts for disc diffusion tests used in antimicrobial investigation, the solvent extracts were concentrated under vacuum overnight.

19

Total phenolics

  The concentration of total phenolics in muscadine seed extract was determined according to the Folin-Ciocalteu method (Waterhouse, 2001). Each of the seed extracts was 100-fold diluted with deionized water and then mixed with 100ml of Folin-Ciocalteu's phenol reagent and 300ml of sodium carbonate. After standing for 2 hours at room temperature, the absorbance at 765 nm was measured by a Spectronic Genesys 5 UV-Vis Spectrophotometer (Fisher Scientific Inc, Pittsburgh, PA). The results of total phenolics were expressed as milligrams of gallic acid equivalent (GAE) per milliliter of seed extract. The measurements for each extract were done in triplicate and average values were recorded.

High-performance liquid chromatographic (HPLC) analysis for major phenolics and major organic acids

  Reversed-phase HPLC was used for determination and quantification of major phenolics and organic acids. To separate phenolics from their conjugated forms, each extract sample was mixed with 4N HCL at 1:9 (v/v) and placed in a water bath at 95˚C for one hour (Lee, 2000). After cooling down to room temperature, each acid hydrolyzed sample was centrifuged at 12000 rpm (17,000 g) for 5 min using an Eppendorf model 5414 microcentrifuge (Eppendorf, Brinkmann Instrments, NY, USA), the supernatant was filtered through a 0.45µm syringe filter (Millipore, Bedford, MA) and injected into a Gemini C18 column (250×4.6 mm, Phenomenex®, Torrance, CA) in an Agilent HPLC 1100 series (Agilent Technologies Inc., Santa Clara, CA) with a diode array detector. The injection volume is 25µL. For the determination of individual phenolic compound, the two mobile phases were consisted of solvent A (methanol/acetic acid/water=10:2:88, v/v/v) and solvent B (100% acetonitrile). Individual phenolic compounds were detected at

260nm with a linear gradient used as follows: at 0min, 95% solvent A, 5% solvent B; at 1min, 90% solvent A, 10% solvent B; at 30 min, 30% solvent A, 70% solvent B; at 31min, 90%solvent A, 10% solvent B. The elution was carried out at a flow rate of 0.6 ml/min and individual phenolics were detected at 260nm.

To separate and detect major organic acids (tartaric, malic and tannic acids), muscadine seed extracts were centrifuged at 12,000 rpm (1,700 g) for 5 min, and then each supernatant was filtered and injected into a HPLC system as described above. The mobile phase was 0.01 N H_2SO4 at a flow rate of 0.6 ml/min and individual organic acids were detected at 215nm.

Peaks for those phenolic compounds and organic acids were integrated and analyzed by the ChemStation software (Agilent Technologies Inc). The column was calibrated between injections for 5min with initial mobile phase. Individual compounds were identified based on the retention time of the standards and quantified according to each calibration curve generated from each standard component.

Antimicrobial performance of seed extracts with different treatments

Five bacterial genera, total of 14 strains, were used in this study (A.1), three gram negative: *Salmonella* (t*yphimurium, typhi.)* , *Escherichia coli* O157:H7, *Enterobacter sakazakii*, and two gram positive: *Staphylococcus aureus* , *Listeria* (*inocua, grayii, monocytogenes*). These were provided by the Department of Food Science, Nutrition and Health Promotion, Mississippi State University. The stock cultures were maintained at -65 °C in tryptic soy broth (TSB) (Difco Laboratories, Sparks, MD) containing 10% glycerol. The cultures were separately thawed and reactivated by subculture in tryptic soy

21

broth with shaking at 100 rpm for 16 h at 37 °C (Incubator Shaker 4628, New Brunswick) to reach full-growth.

Antimicrobial activity of each extract was performed by paper disc-diffusion method. Each crude extract from 2 grams of seed powder was mixed with 50µl of dimethyl sulfoxide and 100µl of deionized water in 5-ml vials (National Scientific Co., TN) to obtain the extract mixtures. Acidified deionized water (pH 3.3, °Brix 5.0) was used as control. Each of the freshly full-grown bacteria culture were 100-fold diluted to achieve a density of 5-6 log/ml and then spread evenly on tryptic soy agar (TSA) (Becton Dickinson, Sparks, MD). Paper discs (d=6.7 mm) were placed on the surface of the agar immersed with several drops of the seed extract mixtures (30µl). The inoculated TSA agar plates were incubated at 37°C for 18-24 h. After incubation, zone diameters were read at the point where growth decreased abruptly.

Antioxidant activity of muscadine extracts

Antioxidant ability was determined by using a model 743 Rancimat® (743 Rancimat®, Brinkmann Instruments, Inc., Westbury, NY). 0.208 ml of each seed extract was added into 4.792 ml of vegetable oil (Great Value, marketed by Wal-Mart Stores, Inc.); then the mixture was vibrated for approximately one minute. The control was 0.477ml of deionized water with 4.523 ml of vegetable oil. Then three grams of each mixture were measured and loaded into the reaction vessel. The sample was explored to the airflow circulated at 10± 0.2L/hr, while the heating temperature was kept at 110± 0.2˚C throughout the experiment. Induction periods (IP) for the test samples were recorded. The induction period in this study can be described as the length of time that oil / oily mixture

22

will be stable towards oxidation (Steinbach, 2007), which is the time up to the inflection point of the conductivity vs. time curve (A.2) (Anonymous, 1999).

Statistical analysis

A two-way factorial (extract * solvent) arrangement with at least three replications in a completely randomized design (CRD) was used for measurement of total phenolics, organic acids and major phenolic compounds and antimicrobial activities. PROC GLM or ANOVA was computed to determine mean differences by using the Statistical Analysis System (SAS, 2001). Mean separation was determined by using Fisher's Protected LSD or DUNCAN's test ($P<0.05$). Pearson's correlation was used to examine the relationships between the zone of inhibition and component's concentration and between induction periods (oxidation) and component's concentration. The statistical Analysis System (SAS 9.2, SAS Institute Inc., Cary, NC) was used for all statistical analysis.

Results

Total phenolics, organic acids, and polyphenol compounds in seed extracts

The phytochemical composition of muscadine seed extracts is presented in Table 3.1. Tartaric and tannic acids were detected among all extracts, but no malic acid was detected. Gallic acid, catechin, epicatechin and ellagic acid were the major individual phenolic compounds detected.

Overall, the amount of the organic acids and phenolic compounds varied ($p<0.05$) with the solvents (acetone, ethanol and methanol). In general, acetone (70% v:v), followed by methanol(100% v:v), yielded higher amount of organic acids and phenolic compounds. However, methanol extracts were generally higher in catechin and

epicatechin than acetone extracts, except within 'Carlos' extracts. Acetone and methanol extracts from 'Carlos' showed differences in tannic acid (4.71 and 3.6 mg/ml), gallic acid (3.29 and 2.54 mg/ml), epicatechin (2.63 and 1.67mg/ml) and ellagic acid (1.26 and 0.79mg/ml). The dark cultivar ('Ison' and 'Noble') of methanol extracts were higher (p<0.05) in catechin and epicatechin than bronze cultivar ('Carlos').

Antimicrobial activity of muscadine extracts

The antimicrobial activity of muscadine extracts on 14 strains of pathogenic bacteria are shown in Table A.2. There were significant differences (p<0.05) among seed extracts and significant differences (p<0.05) among bacterial strains. Figure 3.1 showed the susceptibility of bacterial genera to muscadine seed extracts. The gram positive bacteria (*Listeria spp. and Staphylocus spp.*) were affected significantly (p<0.05) by muscadine extracts, while gram negative bacteria (*Cronobacter spp.***,** *Esherichia spp. and Salmonella spp.*) were hardly affected. Generally, All the gram positive bacteria were affected by extracts (Figure 3.2), however methanol extracts and acetone-'Carlos' extracts had higher effect on pathogenic inhibition. Among gram negative bacteria, only methanol-Carlos, methanol-Ison and acetone-Carlos extracts showed antimicrobial effect.

Pearson correlations were used to discern the correlation between muscadine seed extract components and their antimicrobial capacity, and the determination coefficient (R2) and probability (*p*) were presented in Table 3.2. Generally, zone of inhibition correlated well with tannic acid, catechin and epicatecin against most bacteria stains used in this study (Table 3.2). No positive correlation was found between the zone of inhibition and total phenolics.

24

Antioxidant activity of muscadine seed extracts

The antioxidant capacity was expressed as the induction period (IP) detected by the Rancimat® Instrument, with higher values being stronger antioxidant capacity Overall, all extracts showed higher ($p<0.05$) antioxidant capacity than the control (Figure3.3), except ethanol extracts of dark cultivar ('Ison' and 'Noble'). Of the measured organic acids and phenolic compounds, total phenolics ($R^2=0.89$, $p<0.05$), tartaric acid ($R^2=0.87$, $p<0.05$), and gallic acids ($R^2=0.82$, $p<0.05$) were observed correlated ($p<0.05$) with induction period exhibited in Table 3.3.

Discussion and Conclusions

Muscadines contain high levels of certain phenolic compounds, unique among *Vitis* species, and their concentration varies among cultivars and extraction methods (Talcott and Lee 2002; Mbele et al., 2008). Several studies revealed the extraction efficiency among different solvents for plant materials (Duh et al., 1992; Yilmaz and Toledo 2006; Youssef and El-Adawi, 2006; Koffi et al., 2010). Aqueous solutions of ethanol, methanol or acetone were reported to perform better than the pure solvent for total phenol extractions from muscadine (Yilmaz and Toledo, 2006).

In this study, regardless of cultivar, extraction efficiency was in the order of acetone (70% v:v) > methanol(100% v:v) > ethanol (95% v:v), in the determination of organic acids and phenolic compounds. Our results of the solvent efficiency were different from that reported in previous studies (Duh et al., 1992; Koffi et al. 2010). In Duh et al.'s study (1992), methanol was demonstrated to have better extraction efficiency than ethanol, acetone and chloroform for antioxidative components from peanut hulls, with efficiency increasing with increased solvent polarity. However, Koffi et al. (2010) reported ethanol to be more efficient than water, methanol and acetone, for the extraction

of polyphenolic components from selected Ivorian plants. Aqueous solutions of ethanol, methanol or acetone were reported to perform better than the pure solvent for total phenol extractions from muscadine (Yilmaz and Toledo, 2006). One reason for this discrepancy is possibly because the solvents (acetone and ethanol) used in this study were mixed with water (pure solvents used in Duh et al. and Koffi et al.'s study), which would affect the polarity of solvents as well as their extraction efficiency. Another possible reason would be different phenolic compounds existed in between our samples and samples used in their studies, or some other unidentified fruit substance present, since chemical structures of phenolic compounds and interfering substance in fruits can affect the diffusion of phenolics into the extraction solvent so as to affect the extraction efficiency (Kallithraka et al., 1995; Prior & Cao, 1999).

Besides the extraction solvent used in this study, cultivar would be another factor to cause the variation of their component concentrations among seed extracts. The characteristics of muscadines vary significantly among cultivars in phytochemical components. Mbele et al. (2008) reported that phenolic concentrations and their antioxidant capacity of muscadines varied significantly among cultivars during berry development. Kim et al. (2008) concluded that water-soluble muscadine seed extracts of 'Ison'(dark) had higher total phenolics and polar fractions (malic, tartaric and gallic acids) than that of 'Carlos' (bronze). In the current study, for each solvent extraction, there was no cultivar difference in total phenolic concentrations. The possible reasons for this discrepancy could be as follows: 1) Organic solvents, used in current study, have much higher extraction efficiency than water solvents and then possibly result in different amount and types phenolic diffusion. 2) Seasonal (harvest time), geographic origin and agroecological differences likely exist for muscadines between the two studies. Many

26

similar studies have indicated the cultivars, harvest time and environmental conditions affect the phenolic content of grape seeds/fruits (Silva et al., 1991; Prieur et al., 1994; Fuleki and Ricardo-da-Silva, 1997; Revilla et al., 1997).

Phenolic compounds in muscadine play important antioxidant and antimicrobial roles (Musingo et al., 2001; Kähkönen et al., 2001; Pastrana-Bonilla et al., 2003; Yilmaz and Toledo 2004; Esekhiagbe et al., 2009). A strong positive correlation was observed between total phenolic concentrations in fruits and vegetables and their antioxidant activities (Velioglu et al., 1998; Gil et al., 2002; Esekhiagbe et al., 2009). Total phenolic content highly correlated ($p < 0.05$) with the induction period ($R2=0.891, p<0.01$) in this study, consistent with previous reports. However, total phenolics tested in this study showed no correlation ($p>0.05$) with antimicrobial activity on gram negative bacteria, but correlated ($p<0.05$) with some of the gram positive strains: *Staphylococcus aureus, Listeria monocytogenes* ATCC 7694 and *Listeria monocytogenes* (knockout A and B). Previous studies demonstrated phenolics contribute significantly to antimicrobial capacity of spices (Tranter, 1993; Tassou, 1994; Fattouch et al., 2007; Esekhiagbe et al., 2009). However, it has also been suggested that total phenolics do not necessarily determine antimicrobial activity against some bacterial strains, but some other phytochemical might (Brown et al. 2009).

In conclusion, muscadine seed extracts demonstrated an effective antioxidant and antimicrobial property. Tannic, catechin and epicatechin might play an important role in antimicrobial capacity; total phenolics, tartaric and gallic acid might contribute a lot to antioxidant capacity. Certain types of phenolic compounds were possibly diffused by the organic solvents or together with some other possible components that could also contribute to antimicrobial or antioxidant ability. The results suggested that muscadine

27

seeds could be processed by organic solvents and be further used as potential natural antioxidants and/or nature antimicrobials. Further studies are needed to identify some other possible compounds in those organic solvents by High Performance Liquid Chromatography/ Mass Spectrometry (HPLC/MS).

Table 3.1   Total phenolics, organic acids and phenolic compounds (mg/ml) of organic solvent extracts from muscadine seeds

Extracts		Tartaric acid	Tannic acid	Total phenolics	Gallic acid	Catechin	Epicatechin	Ellagic acid
Solvent	Cultivar				mg/ml			
		Organic acid			Phenolic compound			
Acetone	Carlos	1.49ab*	4.71a	7.86ab	3.29ab	1.74c	2.63c	1.26a
70% v:v	Ison	1.91a	1.53e	8.61a	2.56bc	2.78b	3.71b	0.7bc
	Noble	1.37bc	1.82ed	9.03a	4.03a	2.85b	2.70c	1.22a
Ethanol	Carlos	0.25d	1.01f	3.37d	2.07cd	0.67d	0.94ed	0.75bc
95% v:v	Ison	0.31d	1.35ef	3.70d	1.33d	0.93d	0.92ed	0.97ab
	Noble	0.18d	0.94f	3.66d	1.27d	0.86d	0.78ed	0.81bc
Methanol	Carlos	1.13bc	3.6b	5.86c	2.54cd	1.64c	1.67d	0.79bc
100% v:v	Ison	0.93c	2.6c	5.81c	1.56d	4.35a	3.55b	0.59c
	Noble	0.98c	2.05d	6.23bc	1.78cd	5.05a	4.98a	0.97ab
LSD		0.4969	0.5244	1.8493	0.8052	0.7057	0.8213	0.345
CV		30.5064	13.841	17.9214	20.6738	17.7331	19.7027	22.4676

*Mean Values (n=3) within columns having different letters are different (P<0.05).

29

Table 3.2   Pearson correlation coefficients between muscadine extract components and zone of inhibition

Components	Diameters of the inhibition zone (R^2)							Gram negative bacteria
	Gram positive bacteria							
	Lmw☆	Lg	Sa	Li	Lm1	Lm2	Lmk	
Organic acids								
Tartaric acid	0.67 *	0.71*	0.83 *	0.31	0.55	,-0.59	,-0.70*	0.30
Tannic acid	0.71*	0.79*	0.71 *	0.67 *	0.72*	0.15	0.05	0.84*
Phenolic compounds								
Total phenolics	0.59	0.66	0.83*	0.10	0.48	,-0.74*	,-0.84*	0.15
Gallic acid	0.29	0.44	0.62	0.09	0.24	,-0.56	,-0.61	0.08
Catechin	0.77*	0.67 *	0.67 *	0.01	0.72 *	,-0.45	,-0.52	0.18
Epicatechin	0.80*	0.70 *	0.75*	0.06	0.71*	,-0.59	,-0.63	0.15
Ellagic acid	0.03	0.12	0.30	,-0.08	0.04	,-0.04	,-0.35	,-0.15

☆ Lmw:*Listeria monocytogenes* EGD (wild Inl A and B); Lg:*Listeria grayii* ATCC 19120; Sa: *Staphylococcus aureus* ATCC 29213;

Li: Listeria innocua ATCC 19119; Lm:Listeria monocytogenes ATCC 19114; Lm:Listeria monocytogenes ATCC7694; Lmk:Listeria monocytogenes (knokout A and B)

Table 3.3   Pearson correlation analysis performed between the components in the
           muscadine seed extracts and induction period

Components	Induction Period	
	R^2	p
Organic acids		
Tartaric acid	0.87	<0.01
Tannic acid	0.34	>0.05
Phenolic compounds		
Total phenolics	0.89	<0.01
Gallic acid	0.82	<0.01
Catechin	0.41	>0.05
Epicatechin	0.52	>0.05
Ellagic acid	0.18	>0.05
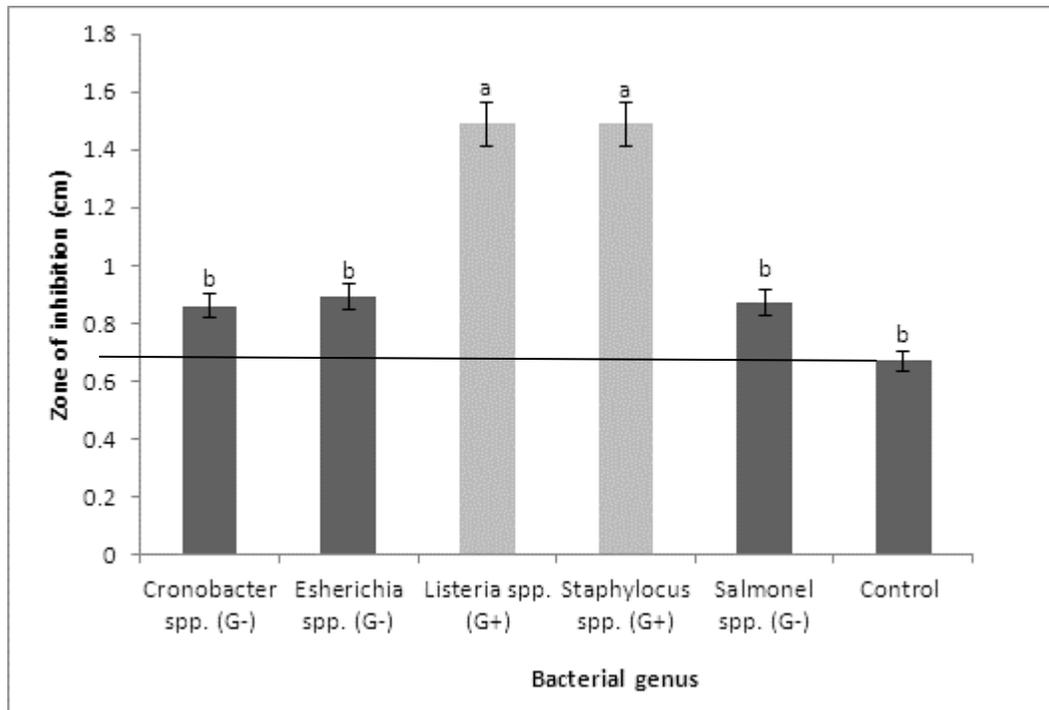
Figure 3.1   Zone of inhibition (diameter: control=0.67cm) for different bacterial genus
           as affected by muscadine extract, regardless of cultivar (overall)
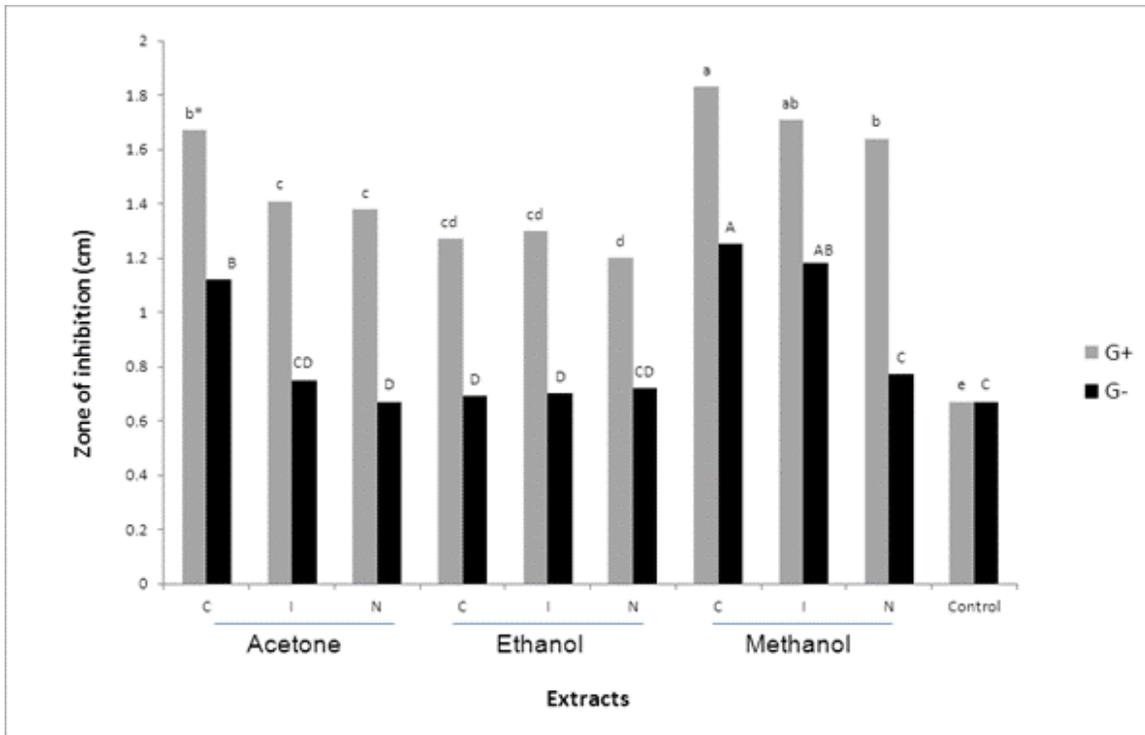
Figure 3.2   Zone of inhibition (diameter: control=0.67cm) for gram positive and gram negative bacteria as affected by all seed extracts.

Extracts: 1) C: Carlos; 2) I: Ison; 3) N: Noble; 4) Control: deionized water.
*Values having the same small letters are not significantly different (P>0.05).
Values having the same capital letters are not significantly different (P>0.05).
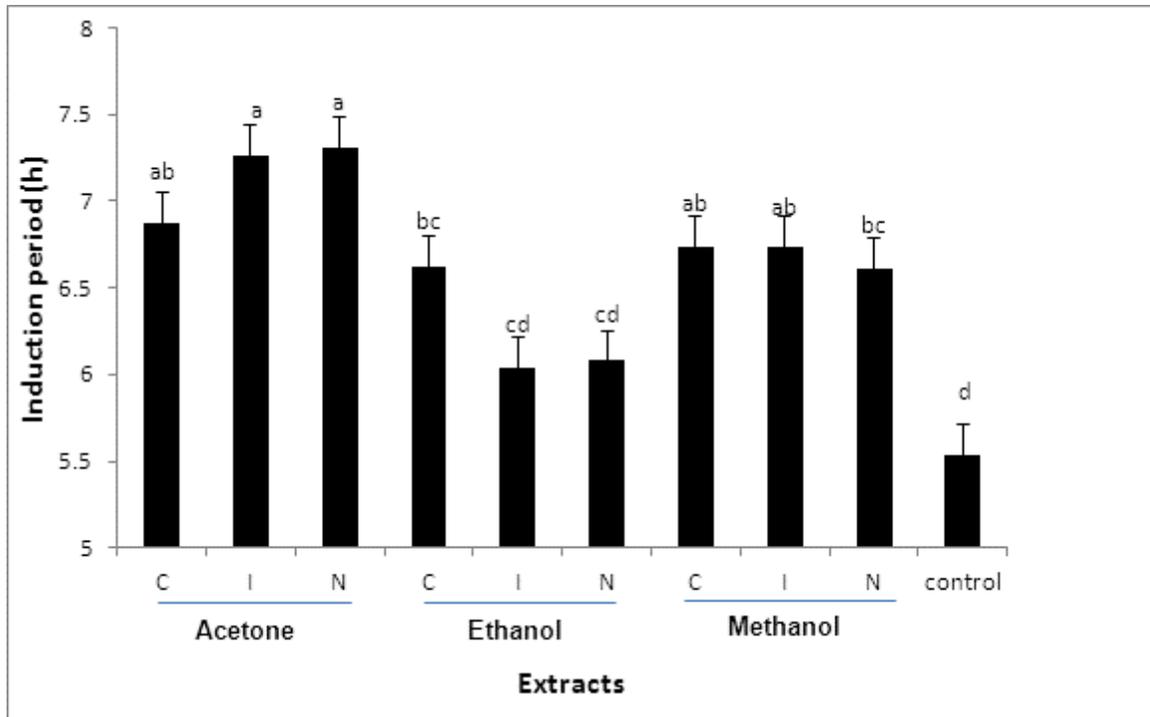
Figure 3.3   Antioxidant capacity (induction period) of muscadine extracts as affected
            by solvent*cultivar

Extracts: 1) C: Carlos; 2) I: Ison; 3) N: Noble; 4) Control: deionized water
*Values having the same small letters are not significantly different (P>0.05).
  Values having the same capital letters are not significantly different (P>0.05).

CHAPTER IV

ANTIOXIDANT CAPACITY AND BIOACTIVE COMPOUNDS OF HOT WATER-SOLUBLE SKIN EXTRACTS FROM 17 MUSCADINE CULTIVARS

Abstract

Muscadine (*Vitis ro tundifolia* Michx.) skins are rich in phenolics and organic acids, which possess strong antioxidant properties. Hot-water-soluble muscadine skin extracts were prepared from 17 cultivars (6 bronze and 11 dark). The antioxidant capacity (Rancimat®), pH, soluble solids, total phenolics, organic acids and individual phenolics of skin extracts were determined. Malic (1.60~6.02 mg/ml) and tartaric acids (2.51~6.55mg/ml) were the prominent aliphatic organic acids, while tannic (0.39~1.21mg/ml), gallic (0.17~0.39mg/ml) and ellagic acids (0.15~1.03mg/ml) were the major phenolics acids. Dark-skin cultivars were higher ($p<0.05$) in total phenolics, tartaric, tannic and ellagic acids. Among dark cultivars, 'Alachua' had the highest ($p<0.05$) antioxidant capacity, similar to 'Albermarle', 'Regale', 'Southland' and 'Nesbit'. The results showed muscadine hot water-soluble extracts exhibited effective antioxidant property and the antioxidant activity were correlated with the content of malic acids and tartaric acids.

Introduction

Natural antioxidants in plant-source materials have attracted considerable interest to the food industry because they are presumed safe with characteristic flavor, potential nutritional values and therapeutic effects (Heinonen et al., 1998; Wang and Lin, 2000).

34

Phenolics, secondary metabolites unique in higher plants, are of great importance due to their powerful antioxidant properties superior to vitamin C, vitamin E, carotenoids and some other well-known natural antioxidants (Gil et al., 2002; Kaur and Kappor, 2002; Shi et al., 2003).

Muscadine (*Vitis rotun difolia* Michx.) grapes are a species native to the Southeastern United States, used as table fruits, wine, juice, jam or jellies (Ector, 2001; Basiouny and Himelrick, 2001). Muscadines are an excellent source of dietary fiber, essential amino acids, minerals, various vitamins and phytochemicals with high nutritional values and health-promoting properties (Ector, 2001; Hartle et al.,; 2005 Threlfall et al., 2007). They are rich in phenolics and certain organic acids, and have been demonstrated to possess antioxidant properties and antimicrobial properties (Lee and Talcott, 2002; Pastrana-Bonilla et al., 2003). The phenolic content in muscadine seeds and skins are significantly higher than in pulp/juice or leaves (Pastrana-Bonilla et al., 2003). There are many cultivars varying in color from almost white or bronze to nearly black and could be generally classified into two groups: purple and bronze cultivars (Ector, 2001). Various studies have explored varying characteristics among muscadine cultivars (Mortensen, 2001; Striegler et al., 2005; Stringer et al.,2008). However, there are limited studies focused on cultivar differences in muscadine phenolics, organic acids and antioxidant properties (Mbele et al., 2008).

The objectives of this study were to: 1) discern the variation on antioxidant activity, major phenolic and organic acids among 17 muscadine cultivars; 2) and correlate the antioxidant capacity with bioactive components.

35

Materials and Methods

Chemicals and reagents

Folin-Ciocalteu reagent, Sodium carbonate and pure standards of gallic acid (90% purity), (+)-catechin (95% purity), (-)-epicatechin (90% purity), ellagic acid (95% purity) ,malic acid and tannic acid were purchased from either Fluka (Milwaukee, WI, USA) or Sigma (St. Louis, MO, USA). Tartaric acid, acetic acid, hydrochloric acid, sulfuric acid, acetonitrile, and HPLC grade water (high-performance liquid chromatography of HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Corn oil were purchased from a local supermarket (Great Value®, marketed by Wal-Mart Stores, Inc., Starkville, MS).

Muscadine extract preparation

Muscadines of Seventeen cultivars, harvested in fall 2009, were provided by the USDA-ARS, Thad Cochran Southern Horticultural Laboratory (Poplarville, MS). They were 11 dark-skin ('Alachua', 'Black Fry', 'Ison', 'Nesbit', 'AD5-103', 'Albermarle', 'Black Beauty', 'Eudora', 'Noble', 'Regale', 'Southland'), and 6 bronze- skin ('Scarlet', 'Carlos', 'Janebell', 'Sweet Jenny', 'Fry' and 'CA9-37') muscadine cultivars. The grapes of each cultivar were separated into a pulp, skin and seed fraction, which had been freeze-dried, labeled and stored in a sealed glass vial at room temperature until further analysis. The skins were grounded into powder for further use in this study.

To prepare hot-water soluble skin extracts, one gram of skin powder was mixed with 5ml of deionized water in a screw-capped vial (National Scientific Co., TN) and vibrated for 1 min at room temperature (~25°C). The mixture was autoclaved (121°C, 103.4 kPa) for 15 min and centrifuged at 12000rpm (17,000 g) for 15 min (Eppendorf

centrifuge 5415C; Brinkmann Instruments, NY). After centrifuging, the supernatant was collected and filtered through a 0.20 μm syringe filter for further use (Millipore, Bedfors, MA).

Total phenolics, pH and Brix

Total phenolic contents of water-soluble skin extracts were measured according to Folin-Ciocalteu procedure (Waterhouse, 2001). Each extract was properly diluted with deionized water and then mixed with 100ml of Folin-Ciocalteu reagent and 300ml of sodium carbonate. After standing for two hours, absorbance at 765 nm was measured with a Spectronic Genesys 5 UV-Vis Spectrophotometer (Fisher scientific Inc., Pittsburgh, PA). The analysis for each extract was done in triplicate and average values were recorded. All the results were expressed as milligrams of gallic acid equivalent (AGE) per milliliter of muscadine extract sample, using a standard curve generated with 100, 250, 500 and 1000 ppm of gallic acid (Figure A.1).

The pH values of muscadine skin extracts were measured at room temperature (about 25 °C) with a Corning Pinnacle 530 pH meter (Corning Inc, St. Liusis, MO). The soluble solids concentration was measured with a Bausch & Lomb Refractometer 33.46.10 (Bauch & Lomb Inc., Rochester, NY). The readings were recorded each time after calibration with deionized water, and expressed as °Brix.

High-performance liquid Chromatographic (HPLC) analysis for major phenolics and organic acids

Each muscadine extract sample (25μl) was analyzed by HPLC using solvent programs for separation and quantification of and organic acids major phenolics.

To separate phenolics in the HPLC system, 4N HCL was added to each sample at 1:9 ratio (v/v) and placed in a water bath at 95°C for one hour for acid hydrolysis of flavonoid glycosides to aglycones. After cooling down to room temperature (about 25°C), each hydrolyzed sample was centrifuged at 12000 rpm (17000g) for 5 min using an Eppendorf model 5414 microcentrifuge (Eppendorf, Brinkmann Instrments, NY, USA), then filtered through a 0.45 μm syringe filter (Millipore, Bedford, MA) and injected into a Gemini C18, 250×4.6 mm, C18 column (Phenomenex Inc., Torrance, CA) in an Agilent HPLC 1100 (Agilent Technologies Inc., Santa Clara, CA) equipped with a diode array detector. The mobile phases were solvent A (methanol/acetic acid/water=10:2:88, v/v/v) and solvent B (100% acetonitile). Individual phenolic compounds were detected at 260nm with a linear gradient used as follows: at 0min, 95% solvent A, 5% solvent B; at 1min, 90% solvent A, 10% solvent B; at 30 min, 30% solvent A, 70% solvent B; at 31min, 90%solvent A, 10% solvent B. The flow rate was 0.6 ml/min and individual phenolic compounds detected at 260nm.

To separate major organic acids (tartaric, malic and tannic acids), mobile phase was 0.01N H_2SO4 at a flow rate of 0.6ml/min. Individual organic acids were detected at 215nm. Peaks for those phenolics and organic acids were intergraded analyzed by ChemStation software (Agilent Technologies). The column was calibrated between injections for 5min with the initial mobile phase. Individual compounds were identified based on the retention time of the corresponding standards and quantified by each standard curve (Figure A.2 ~ A.9)

Antioxidant activity of muscadine extracts

Antioxidant ability was determined by using a model 743 Rancimat® (743 Rancimat®, Brinkmann Instruments, Inc., Westbury, NY). 0.477 ml of each skin extract was added to 4.523 ml of corn oil (Great Value, marketed by Wal-Mart Stores, Inc.); then the mixture was vibrated for around one minute. The control was 0.477ml of deionized water with 4.523 ml of vegetable oil. Then three grams of each mixture were loaded into the reaction vessel. The sample was explored to the airflow circulated at 10 ± 0.2L/hr, while the heating temperature was kept at $110 \pm 0.2°$C throughout the experiment. Induction periods (IP) for the test samples were recorded. The induction period in this study can be described as the length of time that oil / oily mixture will be stable towards oxidation (Steinbach, 2007), which is the time up to the inflection point of the conductivity vs. time curve (Figure A.2) (Anonymous, 1999).

Statistical design and analysis

A one-way factorial arrangement (cultivar) with at least three replications in a completely randomized design was employed to assess the effects of different cultivars on pH, total phenolics, organic acids, phenolics and antioxidant ability. The Statistical Analysis System (SAS 9.2, SAS Institute Inc., Cary, NC) software was used for all statistical analysis. Analysis of variance or GLM procedure was performed to the data. Means separation was performed ($P \leq 0.05$) by using Fisher's protected Least Significant Difference (LSD) or Duncan's test.

Results

Soluble solids, pH, total phenolics, organic acids, and phenolic compounds in muscadine skin extracts from bronze-skin and dark-skin cultivars

The muscadine skin extracts from 17 cultivars in this study were divided into two groups based on their skin color: bronze-skin extracts and dark-skin extracts. The soluble solids (°Brix), pH, total phenolics, organic acids and individual phenolics in both groups are presented in Table 4.1. For all extracts, the concentration of polar fractions (malic, tartaric) was higher than phenolics (tannic, gallic and ellagic acids) and no significant amount of catechin or epicatechin was detected. Malic acids and tartaric acids were the major aliphatic organic acids and had the highest concentration among all the individual components analyzed by HPLC.

There was no significant cultivar difference ($p<0.05$) in soluble solids (°Brix) within either bronze-skin group or dark-skin group. However, there were significant differences ($p<0.05$) among bronze-skin cultivar extracts in pH (3.11~3.66), total phenolics (2.99~4.80mg/ml), malic acid (1.94~4.63mg/ml), tartaric acid (2.51~5.63mg/ml), tannic acid (0.39~0.89mg/ml) and gallic acid (0.18~0.32mg/ml). Among dark-skin cultivars, significant differences were detected in pH (3.03~3.53), total phenolics (2.49~5.97mg/ml), malic acid (1.60~6.77mg/ml), tartaric acid (3.09~6.55mg/ml), gallic acid (0.17~0.39mg/ml) and ellagic acid (0.17~1.03mg/ml).

Antioxidant property of muscadine skin extracts from bronze-skin and dark-skin cultivars

Antioxidant capacity of muscadine skin extracts for corn oil was measured by Rancimat® method expressed as induction period, IP (Figure 4.1). Antioxidant capacity is highest for the largest IP. All 17 cultivars show effective antioxidant capacity,

compared with the control (Fig. 4.1.A). Among the bronze-skin cultivars (Figure 4.1.B), there was no differences (p>0.05) in antioxidant capacity, but they were all higher (p<0.05) than the control. All the dark-skin extracts (Fig. 4.1.C) showed effectiveness but different (p<0.05) antioxidant property against oil oxidation, ranging from 8.69 ('Black Beauty') to 9.66 ('Alachua').

Based on the different antioxidant capacity among cultivars, the skin extracts were classified into three levels (Table 4.2): high-antioxidant group (IP > 9.30 h), medium-antioxidant group (9.00 < IP ≤ 9.30 h) and low-antioxidant group (IP ≤ 9.00) (Table 4.2). Malic acid and tartaric acid were the only compounds that were different among the three groups (p<0.05); they were higher in the higher antioxidant group.

Between dark and bronze skinned cultivars (Table 4.3), the dark-skin cultivars showed significant higher (p<0.05) levels than the bronze-skin cultivars in total phenolics (4.53 and 3.81mg/ml), tartaric acid (4.67 and 4.05mg/ml), tannic acids (0.92 and 0.71mg/ml) and ellagic acid (0.49 and 0.28mg/ml).

Discussion and Conclusions

Muscadine phenolic compounds had been reported to vary among cultivars (Mortensen. 2001;Stringer et al. 2008; Mbele et al., 2008). Pastrona-Bonilla et al. (2003) quantified the phenolic contents in various portions of muscadine grapes from ten cultivars (five dark and five bronze) and a wide variation among cultivars in phenolic compounds was observed. Striegler et al. (2005) showed that total phenolics (3012 ~ 6586 mg/kg fresh weight) and ORAC values (23 ~ 66 _mol of Trolox exuivalents (TE) per gram fresh weight) for whole grape were not different among cultivars. In this study, however, we found significant differences (P<0.001) in total phenolics and antioxidant

capacity (Induction Period) among cultivars. The composition of muscadines may be influenced by various factors such as maturity, variety, growing region and year (Silva et al., 1991; Prieur et al., 1994; Lamikanra, 1995; Revilla et al., 1997). Different extraction methods used could be another possible reason for this discrepancy.

A strong positive correlation between phenolic concentration and antioxidant activity was repeated by several researches (Boyle et al 1990; Gil et al.2002; Pastrana-Bonilla et al. 2003; Mbele et al. 2008). Musacdines are an excellent source of several certain phytochemicals, including phenolic compounds (gallic acid, catechin, epechatachin, ellagic et al.) and organic acids (malic acid, tartaric acid et al.), which are considered to be associated with antimicrobial, antioxidant or disease prevention properties (Ector et al.,1996; Striegler et al., 2005). However, in this study, the variations of total phenolic content and individual phenolic acid content among the different antioxidant levels were not significant ($P<0.05$) (Table 3). Therefore, it is presumable that the phenolic content may not be the main factor that contributes to the antioxidant activity of hot water-soluble skin extracts and that some other antioxidant compounds other than phenolics might be present in higher antioxidant or have stronger antioxidant capacity.

Most polyphenolic compounds are hydrophobic and located in grape skins and seeds; the major phenolics in the skins include ellagic acid, myricetin, quercetin, kaempferol and resveratrol (Pastrana-Bonilla et al., 2003). Most organic acids of grapes or muscadines are hydrophilic and located in skins; more than 90 percent of the total organic acids in grapes are tartaric and malic acids (Lamikanra et al., 1995). This study showed that tartaric and malic acids were the dominant organic acids in the skin extracts with relatively higher levels, while the phenolic acids (tannic, gallic and ellagic acids)

42

were relatively lower. This might be due to their different water soluble abilities. Malic and tartaric acids were prominent organic compounds in grapes and reported to exhibit effective antioxidant activity (Pokorny, 1991; Lamikanra et al., 1995; Gil et al., 2002). In this study, the antioxidant activity might be partially dependent on the concentrations of tartaric and malic acids, which were different ($p<0.05$) among different antioxidant level groups (Table 4.2). Tartaric and malic acids were most prominent among chemical components tested, which could be an important factor that contributes to the antioxidant activity of the hot water-soluble skin extracts.

The color of grape skins is determined by the content of anthocyanins, which are the red pigments in the grapes producing red or purple color and perform strong antioxidant activity (Ichikawa, 2001; Puupponen-Pimiä et al., 2005). Muscadines are usually divided into two groups: dark-skin cultivars and bronze-skin cultivars based on their skin color, which might have different characteristics between each other. In Weng's study (2008), muscadine juice from dark cultivars had lower pH (*P*<0.05) and higher content of tartaric and tannic acids than that from bronze cultivars. Kim et al. (2009) showed seed extracts from 'Ison' (a dark-skin cultivar) had lower pH and higher organic acids, total phenolics, and some major phenolics than seed extracts from 'Carlos' (bronze-skin cultivar). The comparison between dark-skin and bronze-skin cultivars in this study (Table.4.3) was consistent with the above research even though we used a different part (skin) of muscadines. Pastrona-Bonilla et al.(2003) quantified the phenolic contents in muscadine skin extracts from ten cultivars (five dark and five bronze) and dark-skin cultivars showed higher values than bronze-skin only in total anthocyanin within leaves or skins, which differed from our results. Possible reasons for this

43

discrepancy could be different extraction methods and different cultivars used in between our research and the previous research (Pastrona-Bonilla et al., 2003).

In conclusion, hot water-soluble muscadine skin extracts demonstrated an effective antioxidant capacity and considerable differences were found among cultivars in terms of total phenolics, organic acids and antioxidant capacities. High antioxidant activity was determined in some cultivars: Alachua, Albermarle, Southland, Janebell and CA9-37, with relative high levels of malic acids and/or tartaric acids, which might play a role in the antioxidant activity. Such information would help select desirable genotypes for muscadine waste product processing and muscadine skins as well as their organic acid components have the potential to be used as natural antioxidant additives in food industry. In the future, more efforts could be focused on investigating the antioxidant capacity of individual organic acids and phenolics by making systemic substitute.

Table 4.1   Soluble solids (° Brix), pH, total phenolics, major organic acids and phenolic compounds of skin extracts from bronze and dark-skin muscadine cultivars

Skin color	Cultivar	°Brix	pH	Malic acid	Tartaric acid	Tannic acid	Gallic acid	Ellagic acid	Total phenolics
						(mg/ml)			
	Scarlet	14.47[NS]	3.44b*	1.94d	2.51c	0.77ab	0.18c	0.35[NS]	3.01c
	Carlos	13.63	3.15d	3.65bc	5.63a	0.58bc	0.22bc	0.15	4.45a
Bronze	Janebell	13.35	3.11d	4.63a	4.86b	0.74ab	0.32a	0.26	4.80a
	Sweet Jenny	13.93	3.36bc	4.00ab	2.87c	0.89a	0.27ab	0.39	3.13bc
	Fry	14.77	3.66a	2.07d	2.92c	0.39c	0.19bc	0.17	2.99c
	CA9-37	14.17	3.23cd	2.86cd	5.47a	0.88ab	0.26ab	0.33	4.41ab
	LSD	1.3735	0.1477	0.9249	0.5312	0.3033	0.0831	0.2212	1.2931
	Alachua	13.37[NS]	3.37b	6.77a	3.78c	0.81[NS]	0.26bcde	0.43bc	5.21abc
	Black Fry	14.47	3.39ab	2.44cde	3.09e	1.02	0.20ed	0.32cd	4.89abc
	AD5-103	13.70	3.24bc	4.38b	4.36d	0.75	0.22ed	0.41cd	4.43bc
	Albermarle	14.13	3.31bc	3.80bc	6.55a	0.79	0.24ecd	0.67b	4.45bc
	Regale	13.73	3.03e	1.86ed	5.70bc	1.17	0.39a	0.24cd	5.97a
Dark	Southland	13.80	3.21cd	6.02a	5.54c	1.21	0.34ab	0.17d	4.87abc
	Black Beauty	15.00	3.34bc	3.10cbd	3.63ed	0.98	0.17e	0.32cd	2.53d
	Noble	14.40	3.08de	1.69e	5.19c	0.95	0.27bcd	1.03a	5.72ab
	Nesbit	14.80	3.20cd	3.16bcd	6.35ab	1.00	0.31bc	1.02a	5.21abc
	Eudora	14.47	3.32bc	1.60e	3.40e	0.57	0.23ecd	0.34cd	2.49d
	Ison	14.53	3.53a	3.36bc	3.74de	0.86	0.21ed	0.49bc	4.12c
	LSD	1.3945	0.1548	1.386	0.7759	0.5714	0.0863	0.2496	1.3031
	Average			3.37B†	4.45A	0.84C	0.25C	0.42C	

*Within a column and color variety, mean values (n=3) having the different small letters are different ($p < 0.05$).
†Within a row, mean values (n=3) having the different capital letters are different ($p < 0.05$).
NS: no significant different.
LSD: least significant difference

Table 4.2   Comparisons of the extract components among three different antioxidant groups†

	High-antioxidant (>9.3h)	Medium-antioxidant (9.0~9.3h)	Low-antioxidant (<9.0h)
pH	3.26 ± 0.13	3.29 ± 0.20	3.33 ± 0.17
Total phenolics	4.71 ± 0.68	4.26 ± 1.27	3.95 ± 1.44
Malic acid	4.52 ±1.69a ※	3.38±1.48b	2.44±0.95b
Tartaric acid	5.15±1.10a	4.49±1.49ab	3.81±0.84b
Tannic acid	0.81±0.24	0.84±0.36	0.87±0.31
Gallic acid	0.22±0.048	0.27±0.07	0.25±0.08
Ellagic acid	0.42±0.19	0.36±0.29	0.50±0.3

†Values expressed are mean±S.D. of triplicates

※Mean values (n=3) within rows having the different letters are different (p< 0.05)

Table 4.3   pH, Brix, induction period, total phenolics, organic acids and phenolics of water-soluble skin extracts from bronze and dark muscadine†

	Bronze-skin	Dark-skin
Induction period(h)	9.05±0.38	9.18 ±0.31
^{o}Brix	14.08±0.79	14.22±0.84
pH	3.33±0.21	3.27±0.16
Organic acids		
Tartaric acid (mg/ml)	4.05±1.37b	4.67±1.26a
Tannic acid (mg/ml)	0.71±0.23b	0.92±0.33a
Phenolics		
Total phenolics (mg/ml)	3.81±1.01b※	4.53±1.27a
Malic acid (mg/ml)	3.19±1.11	3.47±1.78
Gallic acid (mg/ml)	0.24±0.06	0.26±0.08
Ellagic acid (mg/ml)	0.28±0.14b	0.49±0.31a

†Value expressed are mean ± S.D. of triplicates

※Mean values (n=3) within rows having the different letters are different (p< 0.05)
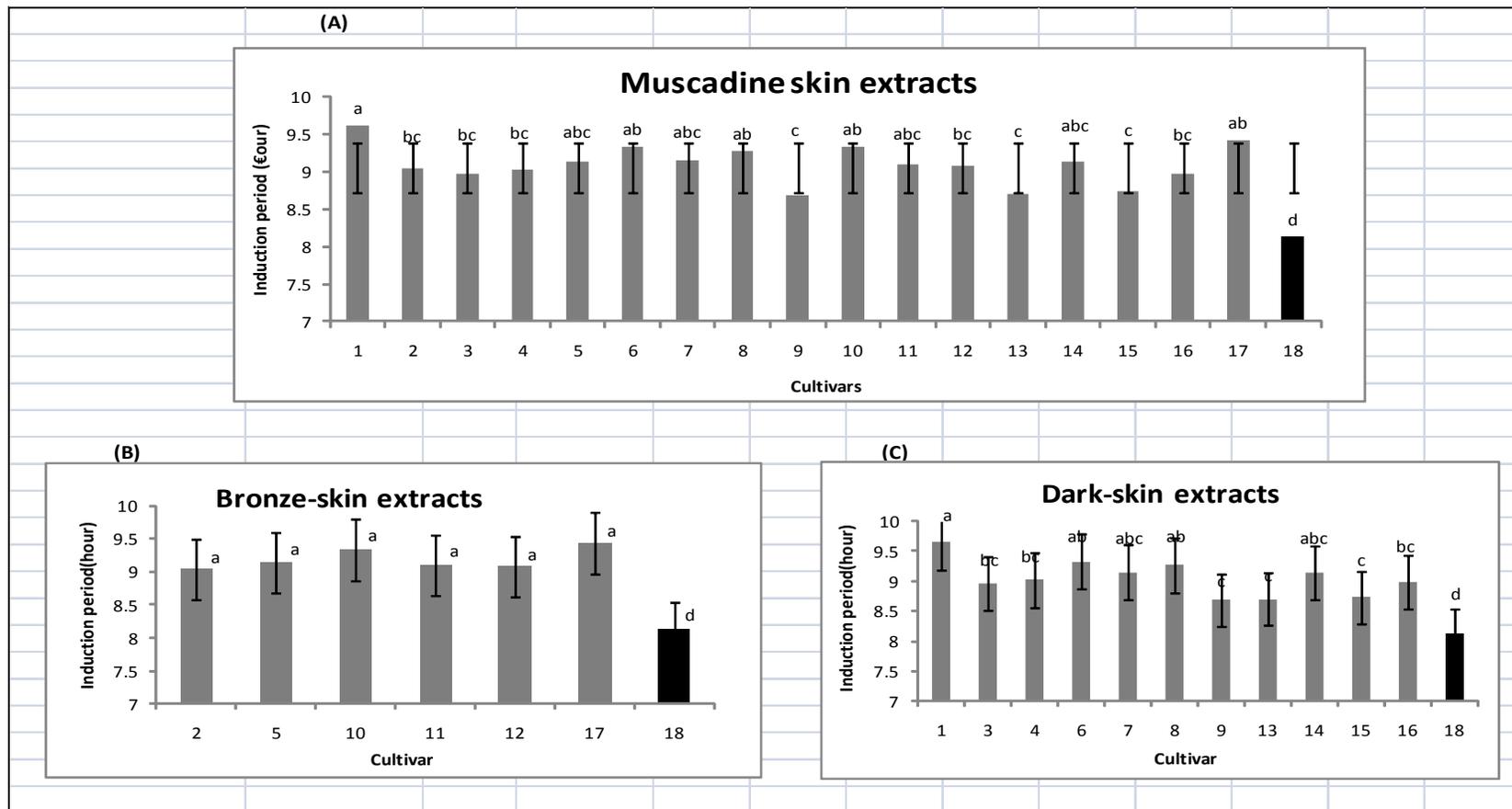
Figure 4.1   Antioxidant capacity of muscadine skin extracts in all (A), bronze-skin (B) and dark-skin (C) cultivars.

Muscadine cultivars: 1: Alachua, 2: Scarlet, 3: Black Fry, 4: AD5-103, 5: Carlos, 6: Albermarle, 7:Regale, 8: Southland, 9: Black Beauty, 10:Janebell, 11:Sweet Jenny, 12: Fry, 13. Noble, 14: Nesbit, 15: Eudora, 16: Ison, 17: CA9-37, 18: Control (deionized water)

CHAPTER V

SUMMARY AND CONCLUSIONS

Muscadine seed extracts were prepared from three cultivars ('Carlos', 'Noble' and 'Ison') by 100% v:v methanol, 95% v:v ethanol and 70% v:v acetone. Antimicrobial activities of muscadine seed extracts were evaluated by disc-diffusion tests with 14 bacterial strains. Methanol seed extracts and 'Carlos' seed extracts generally showed the highest antimicrobial capacity against pathogen strains tested. The content of tannic acid, catechin and epicatechin were positively correlated with their antimicrobial activity. Gram positive bacteria (zone diameter: 1.20~1.83 cm) showed bigger zone of inhibition than gram negative bacteria (zone diameter: 0.67~1.18cm), which means gram positive bacteria are more sensitive to muscadine seed extracts than gram negative bacteria.

Muscadine seed extracts demonstrated an effective antioxidant property. The acetone extracts exhibited the higher yield of phenolics (21.62~24.84mg/g of dw) and better antioxidant activity than methanol and ethanol extracts. Strong correlation (R2=0.891, p<0.01) was found between total phenols and antioxidant activity. Tartaric and gallic acid were also suggested to be correlated to antioxidant capacity. Moreover, other possible phenolic compounds and/or some unknown components from seeds diffused by the organic solvents could also contribute to antimicrobial or antioxidant ability. Results suggested muscadine seeds could be processed by organic solvents and be further used as potential natural antioxidants and/or nature antimicrobials.

Muscadine (*Vitis ro tundifolia* Michx.) skins are rich in phenolics and organic acids, which possess strong antioxidant properties. Hot water-soluble muscadine skin extracts were prepared from 17 cultivars (6 bronze and 11 dark). The antioxidant capacity, pH, soluble solids, total phenolics, organic acids and individual phenolics were determined to investigate the characteristics of the skin extracts among cultivars. Significant variations ($p < 0.05$) were observed among cultivars in total phenolics, organic acids and antioxidant activities of the skin extracts. Malic (1.60~6.02 mg/ml) and tartaric acids (2.51~6.55mg/ml) were prominent aliphatic organic acids in all the extracts, while phenolic acids tannic (0.39~1.21mg/ml), gallic (0.17~0.39mg/ml) and ellagic acids (0.15~1.03mg/ml) showed relatively low levels. The results indicated the antioxidant activities might correlate with the content of malic acids and tartaric acids.

Hot water-soluble muscadine skin extracts demonstrated an effective antioxidant capacity. High antioxidant activity was observed in some cultivars: 'Alachua', 'Albermarle', 'Southland', 'Janebell' and 'CA9-37' with relative high levels of malic acids and/or tartaric acids, which might play a role in the antioxidant activity. Moreover, the dark-skin cultivars showed significant higher ($p < 0.05$) levels of total phenolics (4.53 and 3.81mg/ml), tartaric acid (4.67 and 4.05mg/ml), tannic acids (0.92 and 0.71mg/ml) and ellagic acid (0.49 and 0.28mg/ml) than the bronze-skin cultivars. Therefore, cultivars with different skin color might have different characteristics. Such information would help select desirable genotypes for muscadine waste product processing. Muscadines skins as well as their organic acid components were suggested to have the potential to be used as natural antioxidant additives in food industry.

The results indicated that muscadine extracts could be used as antioxidant and/or antimicrobial agents. Further studies are needed to identify some other possible

compounds from muscadine seed extracts dissolved in those organic solvents by High Performance Liquid Chromatography/Mass Spectrometry (HPLC/MS). Additional efforts could be focused on investigating the antioxidant/antimicrobial capacity of individual organic acids and phenolics by using synthetic compounds, which could further help us to identify the antimicrobial and/or antioxidant contribution of each individual component.

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APPENDIX A

TABLES AND FIGURES

Table A.1   Bacterial strains used in this study

Bacterial #	Name	Source	Abbreviation
1	*Salmonella* typhimurium ATCC 14028	ATCC	ST 14028
2	*Listeria monocytogenes* EGD (wild Inl A and B)	Dr.Cossart*	LM EGD
3	*Listeria grayii* ATCC 19120	ATCC	LG 19120
4	*Staphylococcus aureus* ATCC 29213	ATCC	SA 29213
5	*Escherichia coli* O157:H7 ATCC 43895	ATCC	E. coli 43895
6	*Listeria innocua* ATCC 19119	ATCC	LI 19119
7	*Cronobacter sakazakii (Enterobacter sakazakii)* FEC 39	Dr.Chen**	ES FEC39
8	*Salmonella* typhi ATCC 6539	ATCC	ST 6539
9	*Salmonella* typhimurium ATCC 19585	ATCC	ST 19585
10	*Escherichia coli* O157:H7 ATCC 39150	ATCC	E. coli 39150
11	*Cronobacter sakazakii (Enterobacter sakazakii)* MSDH	Dr.Chen	ES MSDH
12	*Listeria monocytogenes* ATCC 19114	ATCC	LM 19114
13	*Listeria monocytogenes* ATCC 7694	ATCC	LM 7694
14	*Listeria monocytogenes* (knokout A and B)	Dr. Cossart	LM EGD. A. B.

 * Dr. Yoshen Chen (Mississippi State University)
** Pascale Cossart (Pasteur Institute of Paris)

Table A.2   Antimicrobial ability among muscadine seed extracts against each bacterial strain

Extracts		STm1☆	Lmw	Lg	Sa	EC1	Li	Cs1	Sti	STm2	EC2	Cs2	Lm1	Lm2	Lmk
Methonal	Calos	1.147a,E*	2.013a,A	1.763ab,ABC	1.867ab,AB	1.247a,ED	1.887a,AB	1.270a,ED	1.177a,ED	1.367a,EDC	1.277a,ED	1.287a,ED	2.163a,A	1.583a,BDC	1.537a,BDCE
	Noble	0.67b,D	2.12a,AB	1.670ab,BC	1.900ab,ABC	0.763bc,D	1.590bdc,C	0.827c,D	0.67c,D	1.037b,D	0.67d,D	0.780b,D	2.183a,A	0.93edc,D	1.067ab,D
	Ison	1.163a,E	2.117a,AB	1.820a,AB	1.710ab, BDC	1.190a,E	1.730abc,ABC	1.323a,EDC	1.14a,E	1.210ab,E	1.097abc,E	1.170a,E	2.133a,A	1.31abc,ED	1.160ab,E
Ethonal	Calos	0.730b, C	1.350b,B	1.190c,B	0.807c,C	0.67c,C	1.610bdc,A	0.67c,C	0.67c,C	0.67c,C	0.783cd,C	0.67b,C	1.237cd,B	1.300abc,B	1.400a,AB
	Noble	0.78b,D	1.293b,AB	1.090c,BC	0.887c,CD	0.67c,D	1.380d,A	0.670c,D	0.67c,D	0.67c,D	0.907bcd,CD	0.67b,D	1.183d,AB	1.207abc,AB	1.343a,AB
	Ison	0.747b,E	1.327b,CBD	1.177c,D	0.733c,E	0.67c,E	1.750abc,A	0.670c,E	0.67c,E	0.67c,E	0.780dc,E	0.67b,E	1.257cd,CD	1.47ab,B	1.390a,BC
Acertone	Calos	1.153a,C	2.00a, A	1.767ab,B	1.967a, A	1.103a,C	1.770ab, B	1.050b,C	1.183a,C	1.043b	1.190ab,C	1.137a,C	1.946ab, AB	1.093bcd,C	1.113ab,C
	Noble	0.67b,D	1.697ab,B	1.567ab,BC	1.870ab, A	0.67c,D	1.513dc,C	0.670c,D	0.67c,D	0.670c,D	0.67d,D	0.67b,D	1.65bc,BC	0.67e,D	0.67b,D
	Ison	0.72b,C	1.803a, A	1.523b,B	1.657b,AB	0.883b,C	1.660abc, AB	0.670c,C	0.813b,C	0.670c,C	0.793dc,C	0.67b,C	1.603bc,AB	0.797ed,C	0.793b,C
Control		0.67b	0.67c	0.67d	0.67c	0.67c	0.67e	0.67c	0.67c	0.67c	0.67d	0.67b	0.67e	0.67e	0.67b

*Values within columns having the same small letters are not significantly different (P<0.05).

Values within rows having the same capital letters are not significantly different (P<0.05).

☆ STm1 :*Salmonella* Typhimurium ATCC 14028; Lmw:*Listeria monocytogenes* EGD (wild Inl A and B); Lg: *Listeria grayii* ATCC 19120; Sa: *Staphylococcus aureus* ATCC 29213; Ec1: *Escherichia coli* O157:H7 ATCC 43895; Li: *Listeria innocua* ATCC 19119; Cs1: *Cronobacter sakazakii* (Enterobacter sakazakii) FEC 39; STi:*Salmonella* Typhi ATCC 6539; STm2:*Salmonella* Typhimurium ATCC 19585; Ec2: *Escherichia coli* O157:H7 ATCC 39150; Cs2: *Cronobacter sakazakii* (Enterobacter sakazakii) MSDH; Lm1:*Listeria monocytogenes* ATCC 19114; Lm2:*Listeria monocytogenes* ATCC 7694; Lmk:*Listeria monocytogenes* (knokout A and B)

Table A.3   The linear gradient used for phenolic separation in HPLC system

Time (min)	Solvent A (%) Methanol/ acetic acid/water (10:2:88, v/v/v)	Solvent B (%) Acetonitrile
0	95	5
1	90	10
30	30	70
31	90	10
32	95	5

Table A.4   Retention times of organic acids and phenolic compounds in HPLC analysis

Compounds	Retention time (min)
Organic acid at 215 nm	
Tartaric acid	6.9
Malic acid	8.3
Tannic acid	34.9
Phenolic compound at 260 nm	
Gallic acid	8.6
Catechin	12.3
Epicatechin	14.2
Ellagic acid	21.7
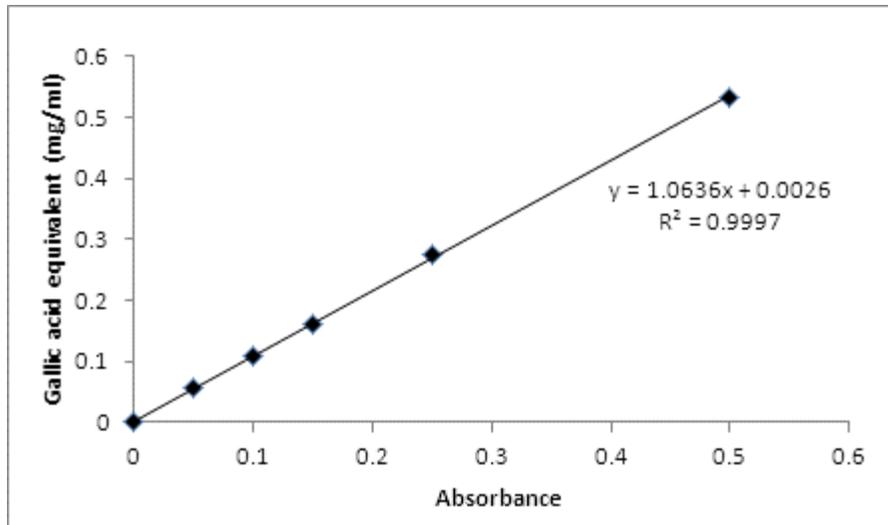
Figure A.1   Standard curve used for total phenolics calculation in Folin-Ciocalteu procedure. Total phenolics were expressed as milligrams of gallic acid equivalents per milliliter.
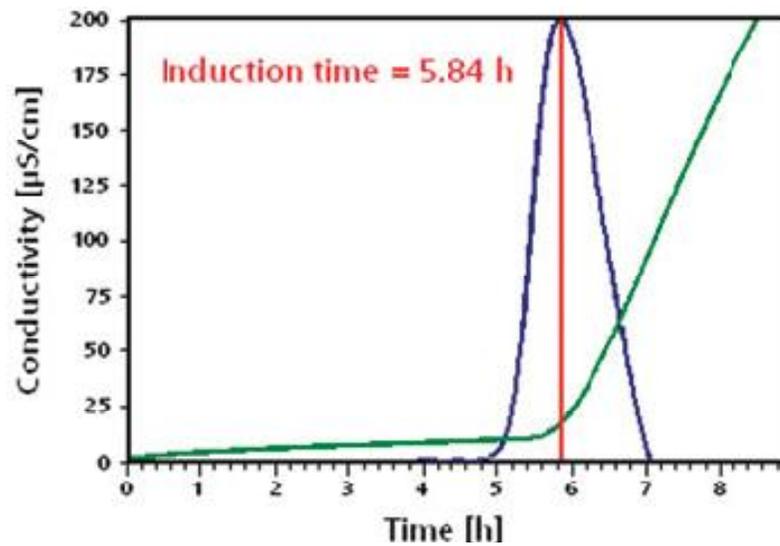
Figure A.2   Calculation of inflection point of the conductivity vs. time curve for oil stability index, OSI (Steinbach, 2007)
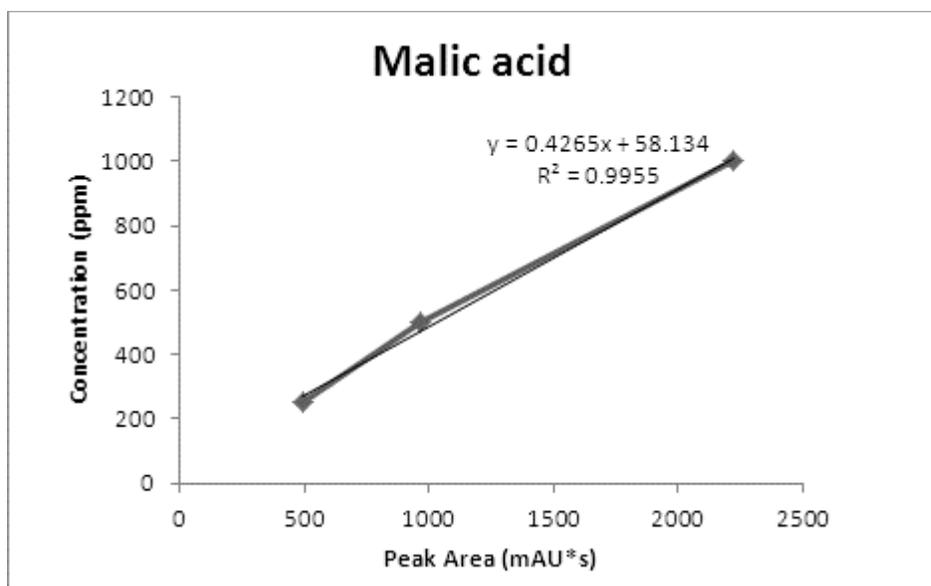
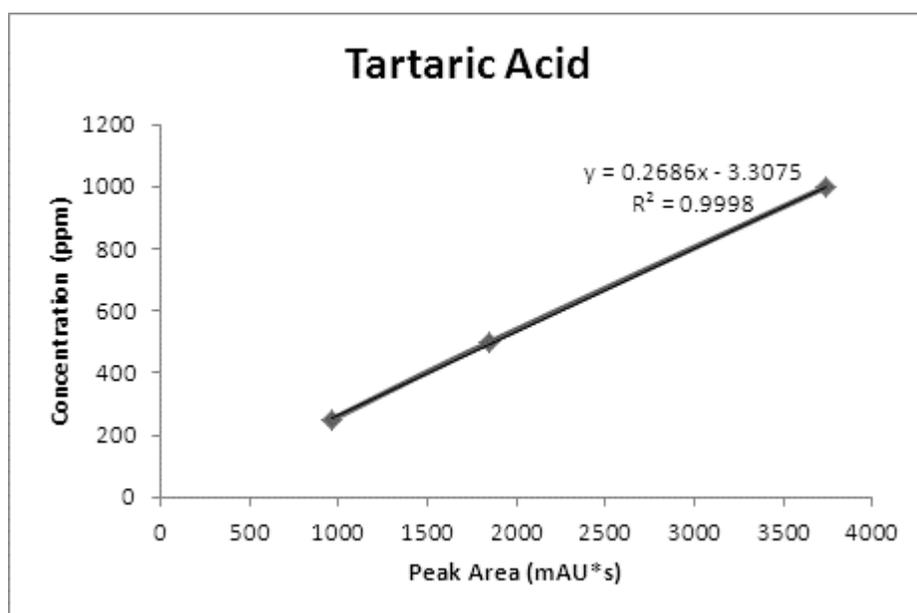Figure A.3   Standard curve used to quantify malic acid in HPLC chromatograms

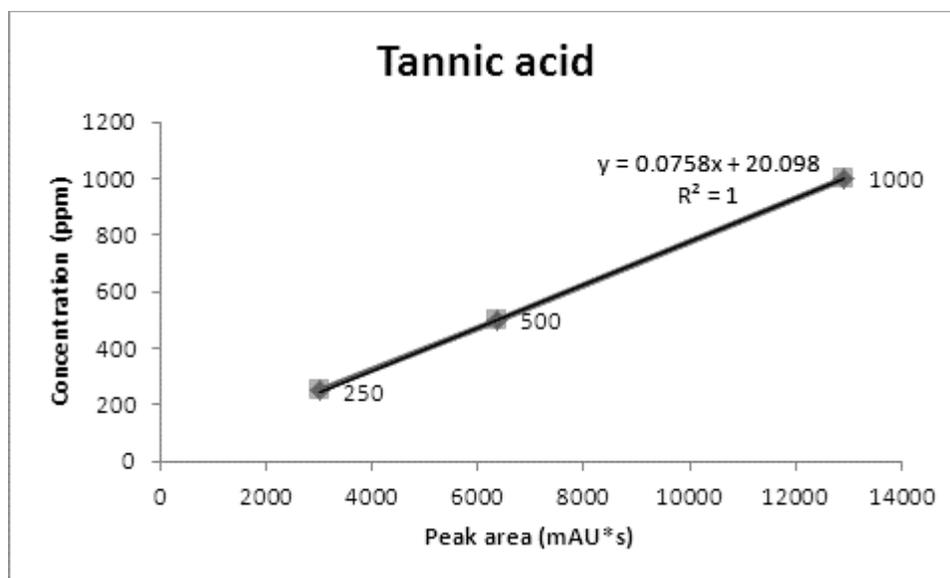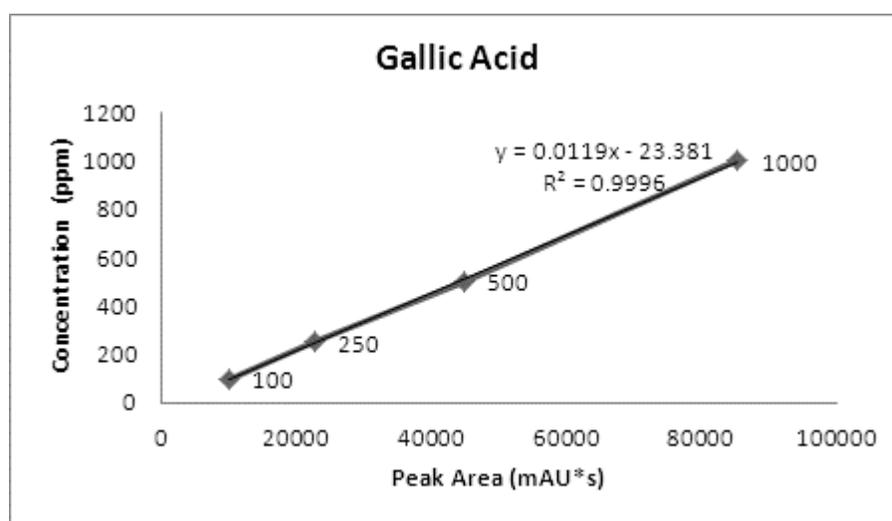Figure A.4   Standard curve used to quantify tartaric acid in HPLC chromatograms

Figure A.5   Standard curve used to quantify tannic acid in HPLC chromatograms

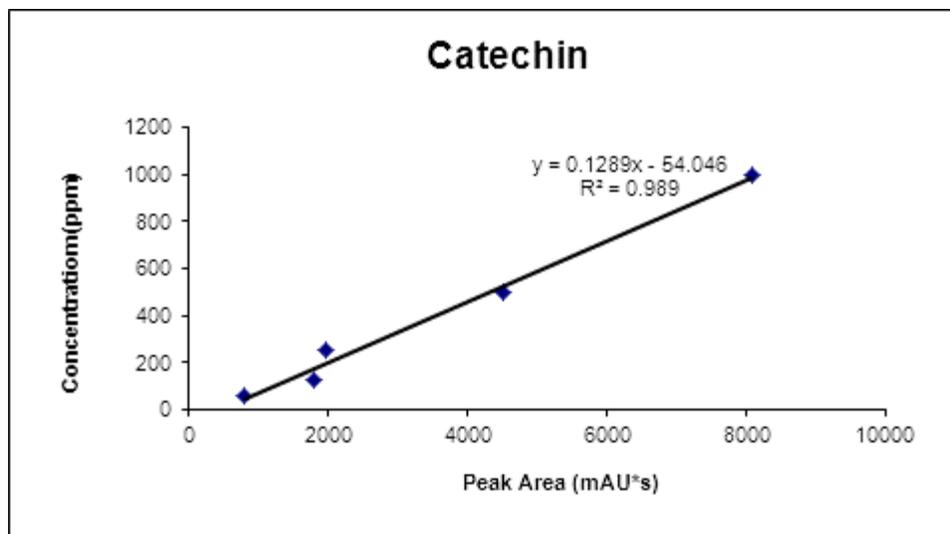Figure A.6   Standard curve used to quantify gallic acid in HPLC chromatograms

Figure A.7   Standard curve used to quantify catechin in HPLC chromatograms
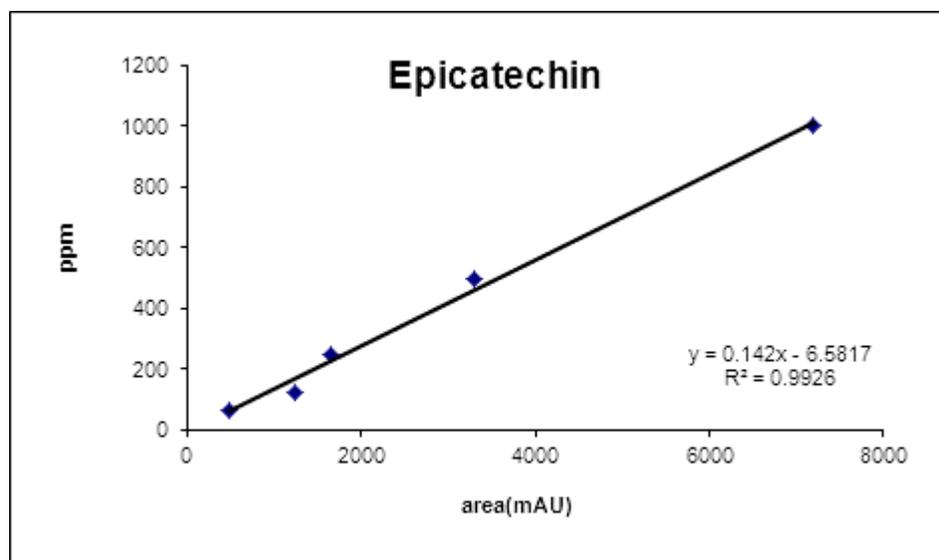

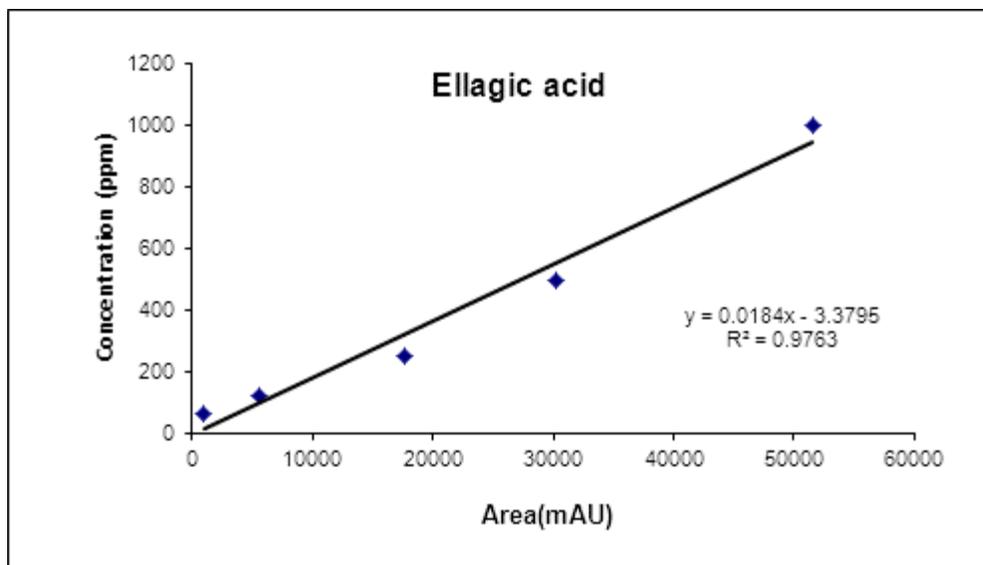Figure A.8   Standard curve used to quantify epicatechin in HPLC chromatograms

Figure A.9   Standard curve used to quantify ellagic acid in HPLC chromatograms