DEVELOPMENT OF TECHNOLOGIES FOR THE PRODUCTION OF POLYPHENOLIC NUTRACEUTICALS FROM MUSCADINE GRAPES AND RABBITEYE BLUEBERRIES

by

RONITA BISWAS

(Under the Direction of Dr. Robert D. Phillips)

ABSTRACT

Muscadines grapes and Rabbiteye blueberries are a good source of polyphenolic compounds, which may reduce the occurrence of degenerative diseases caused by free radicals – cancer, ageing, and Alzheimer's. The overall objective of the research was to develop technologies to produce polyphenolic rich, shelf stable, non-sticky powders from these berries. Response surface methodology (RSM) was employed to optimize extraction and stability of total phenolics and anthocyanins. Aqueous extraction consisted of preheating slurries at 25, 45, and 60C for 0.5, 1, and 2 h respectively followed by pectinase incubation at the same temperatures for 1, 2, 3 h. The optimum region was ~60 C, and 1 h of incubation time. Pectinase treated slurries were extracted with aqueous ethanol (10 – 70% w/w) at an extraction temperature of 25, 45, and 60 C for 1, 2, and 3 H. The optimum regions were 30-40% ethanol for muscadines and 35-40% for blueberries at 60 C for 1 h. The fermentation parameters were 25 C, solid to solvent ratio 1:2, and ethanol concentration 11 % v/v. Total phenolics (g/K dry matter) extracted by aqueous, ethanolic, and fermentation approaches were 21.3, 20.6, and 25.0

for muscadines and 18.1, 28.4, and 17.0 for muscadines and blueberries, respectively. The stability of the total phenolics and anthocyanins in ethanolic extracts corresponded to removing ethanol under reduced pressure (T<65C). Purification and concentration of the extracts were conducted by adsorption/desorption onto neutral styrene-divinyl benzene resins (Sepabeads 700 and Sepabeads 70). Eighty to ninety percent of total phenolics and 100% of anthocyanins were recovered from the resin. Sepabeads 700 retained (mg/g wet resin) 54.5 total phenolics and 10.1 of anthocyanins compared to 13.4 of total phenolics and 2.26 of anthocyanins by Sepabeads 70. All the three extractions, followed by filtration, purification, and removal of ethanol from the extracts repeated at pilot scale with 20 G (76 L) of extracts. The concentrated extracts were spray dried to generate non-sticky powders. The total phenolics and the anthocyanins in the powders had a 22-52% recovery from original sources, which indicated the potential for commercial production of these materials.

INDEX WORDS: Muscadine grapes, Rabbiteye blueberries, Total phenolics, Anthocyanins, Antioxidant activity, Response surface methodology, Resin, Freeze drying, Spray Drying

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DEDICATION

To ma, baba, and dada.

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CHAPTER 1

INTRODUCTION

Muscadine grapes and Rabbiteye blueberries are native to the South Eastern United States. They are known to be a rich source of polyphenolic compounds (Talcott et al., 2002; Sellapan et al., 2002; Pastrana-Bonilla et al., 2003; Talcott et al., 2003; Yilmaz et al., 2004; Lee et al., 2004). The polyphenolic compounds are shown to exhibit numerous health benefits such as inhibiting platelet aggregation, protecting low-density lipoprotein, and ameliorating age-related decline in neuronal and cognitive function (Kris-Etherton et al., 2002). The above attribute of the polyphenolic compounds had been attributed to its free radical scavenging activity (Rice-Evans et al., 1996; 1997). These compounds are also used to retard rancidity in foods or as natural coloring agents (Bonilla, 1999; Stintzing, 2004).

The market for dietary supplement in the United States is rising sharply. The third National Health and Nutriion Examination Survey (NHANES III) (1988 – 1994) found that 40 % of the US population was consuming dietary supplements (Ervin, 2000). Other surveys done for the years 1996 – 1999 showed that 48 % of the US population was using dietary supplements (Blendon, 2001). According to Burdock, Carabins, and Griffith, 2006 one hundred and fifty million Americans spend over $20.5 billion on dietary supplements and neutraceuticals in 2004 which was twice the amount spent in 1994. This leads to a development of new market for the neutraceuticals and also help in expansion of the existing market. The popularity and increased use of dietary supplements have led to concerns about their safety and possible health risks.

Dietary supplements are either consumed in the form of powders in capsules or as an ingredient in the food or as liquid. Production of dietary supplements from plant sources consists of extraction, purification, concentration, and drying. To ensure safety

of the dietary supplement, use of non-toxic extraction solvents (ethanol and water) is highly recommended. Most researchers have used methanol, ethyl acetate, and acetone for extraction of polyphenolic compounds from plant sources (Guendez, 2005; Orak, 2007; Giuseppe, 2007; Yilmaz, 2006; Giorgia, 2007). The shelf life of the dietary supplements is increased when they are produced in the form of non-hygroscopic powders. These health concerns, and increased shelf life of these dietary supplements invoked interest in research that would reduce the use of toxic solvents and manufacture of dietary supplements in the form of powders.

Georgia is one of the largest producers of Muscadine grapes and Rabbiteye blueberries. Both the fruits are marketed as directly for fruit consumption, juices, jams, jellies, wines and others. In the process of making wines, the manufacturers generate large amount of byproducts (pomaces and seeds), which are mostly used as dietary fibre for animal consumptions. The use of byproducts to manufacture neutraceutical preparations from Georgia grown Muscadines grapes and Rabbiteye blueberries will strengthen both the production of the commodities by providing new markets and/or by adding value to byproducts. It will expand existing, or initiate new businesses to conduct such manufacturing and marketing. Finally, it will provide reliable nutraceuticals for consumers, while connecting these products to Georgia and its industries in the consciousness of consumers.

The overall objective of this research is to develop optimum technologies for production of polyphenolic rich powder from Georgia grown Muscadine grapes and Rabbiteye blueberries at pilot scale. This research will facilitate a marketing chain from producers of Georgia Muscadines and Rabbiteye blueberries to manufacturers and

marketers of nutraceuticals. The specific objectives of this thesis were to: (1) to optimize the effect of pectinase incubation time temperature combination on the aqueous extraction of the polyphenolic compounds from muscadine grapes and blueberries, (2) to optimize the effect of ethanol concentration, extaction temperature and extraction time on pectinase trested extracts of muscadine grapes and blueberries, (3) to optimize the fermentation temperature, ethanol concentration, and the solid solvent ratio of the pectinase treated extracts of muscadine grapes and blueberries, (4) to optimize the stability of the extracts of muscadine pomace upon distillation of ethanol from the extracts at different ethanol concentration, temperatures for different times, (5) to study the recovery of the total phenolics and the anthocyanins from the extracts of muscadine pomaces upon adsorption/desorption onto styrene divinyl benzene resins (Sepabeads 70 and Sepabeads 700), and (6) to spray dry the concentrated extracts of muscadine pomaces and blueberry and study the recovery of total phenolics and anthocyanins in the powders.

REFERENCE

Pastrana-Bonilla, E., Akoh, C. C., Sellappan, S., & Krewer, G. (2003). Phenolic Content and Antioxidant Capacity of Muscadine Grapes. *Journal of Agricultural and Food Chemistry*, 51(18), 5497-5503.

Sellapan, S., Akoh, C.C., & Krewer, G. (2002). Phenolic compounds and antioxidant capacity of Georgia-Grown Blueberries and Blackberries. *Journal of Agricultural and Food Chemistry*, 50, 2432-2438.

Yilmaz, Y., & Toledo, R.T. (2006). Oxygen radical absorbance capacities of grape/wine industry byproducts and effect of solvent type on extraction of grape seed polyphenols. *Journal of Food Composition and Analysis*, 19(1), 41-48.

Giorgio, S., Tramelli, L., & Faveri, D.M.D. (2007). Effects of extraction time, temperature and solvent on concentration, and antioxidant activity of grape marc phenolics. *Journal Of Food Engineering*, 81(1), 200-208.

Giuseppe, R., Renda, A., Amigo, V., Daquino, C., Tringali, C., Spatafora, C., & Tomnasi, N.D. (2007). Polyphenol constituents and antioxidant activity of grape pomace extracts from five Sicilian red grape cultivars. *Food Chemistry*, 100(1), 203-210.

Orak, H.H. (2007). Total antioxidant activities, phenolics, anthocyanins, polyphenoloxidase activities of selectd red grape cultivars and their correlations. *Scientia Horticulturae*, 111(3), 235-241.

Kris-Etherton, P.M., hecker, K.D., Bonanome, A., Coval, S.M., Binkoski, A.E., Hilpert, K.F., Griel, A.E., & Etherton, T.D. (2002). Bioactive Compounds in Foods: Their Role in the prevention of cardiovascular disease and Cancer. *The American Journal of Medicine*, 113(9), 71-88.

Lee, J.-H., & Talcott, S. T. (2004) Fruit Maturity and Juice Extraction Influences Ellagic Acid Derivatives and Other Antioxidant Polyphenolics in Muscadine Grapes *Journal of agricultural and food chemistry*, 52(2), 361-366.

Talcott, S. T., & Lee, J.-H. (2002). Ellagic Acid and Flavonoid Antioxidant Content of Muscadine Wine and Juice *Journal of agricultural and food chemistry*, 55(2), 255 – 260.

Talcott, S. T., Brenes, C. H., Pires, D. M., & Del Pozo-Insfran, D. (2003). Phytochemical Stability and Color Retention of Copigmented and Processed Muscadine Grape Juice *Journal of agricultural and food chemistry*, 51(4), 957-963

Rice-Evans, C.A., Miller, N.J., & Paganga, G. (1996). Structure-Antioxidant activity relationships of flavonoids and phenolic acids. *Free radical biology and medicine*, 20(7), 933 – 956.

Rice-Evans, C.A., Miller, N.J., & Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trends in plant science*, 2(4), 152 – 159.

Bonilla, F., Mayen, M., Merida J., Medina, M. Extraction of phenolic compounds from red grape marc for use as food lipid antioxidants. *Food Chemistry*. 1999, 66, 209-215.

Stintzing, F.C., Carle, R. Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. *Trends in Food Science and Technology*. 2004, 15(1), 19-38.

Burdock, G.A., Carabin, I.G., and Griffiths, J.C. (2006).The importance of GRAS to the functional food and nutraceutical industries. *Toxicology*, 221(1), 17-27.

Ervin, R.B., Wright, J.D., Kennedy-Stephenson, J. Reed-Gillette, D. (2000). Prevalence of Leading Types of Dietary Supplements Used in the Third National Health and Nutrition Examination, 1988-94. Hyattsville, MD: National Center for Health Statistics; 2000. Advance Data From Vital and Health Statistics, No. 349.

Blendon, R.J., DesRoches, C.M., Benson, J.M., Brodie, M., and Altman, D.E. (2001). Americans' views on the use and regulation of dietary supplements, *Archives of Internal Medicine,* 161, 805–810.

CHAPTER 2

REVIEW OF LITERATURE

Muscadine grapes (*Vitis rotundifolia* Michx.)

Grapes are the world's largest temperate fruit crop with approximate annual production of 65 million metric tons. About 80 % of the total crop is used in wine making, 13 % is used as table grapes, and the rest is used for making raisins, juice, jam, and other products. The main growing region of grapes is in Europe, with Italy, France and Spain accounting to 40 % of the world's 9,000,000 hectares of grapevines. California produces about a fifth of the world's raisins and 10 % of the world's table grapes.

There are two major types of grapes: European and North American. European grapes belong to the species *Vitis vinifera* L and the grapes are characterized by a relatively thick skin, which adheres to a firm pulp that is sweet throughout. North American grapes belong to two main species: *Vitis labrusca* and *Vitis rotundifolia*. The *labrusca* grapes are grown primarily in the lower Great Lakes region of the United States and Canada. The *rotundifolia* grapes are grown through out the southeastern United states (Girard et al., 1998).

The common names for the rotundifolia grapes (Figure 2.1) are Muscadine, Bullace, Scuppernong, and Southern Fox. The muscadine grape is native to the South Eastern United States (California Rare Fruit Growers, 1997, USDA, 1999), extending from Delaware to the Gulf Of Mexico and westward to Missouri, Kansas, Oklahama, and Texas (Figure 2.2). Georgia is one of the largest produces of muscadines covering 1200 acres annually (Figure 2.3).

Muscadines are suited to grow to the warm, humid conditions of the South Eastern United States. The vines are deciduous and vigorous growing 60 to 100 ft. in the

wild. The fruits are round shaped, 1 to 1-1/2 inch and have a thick, tough skin. They may contain upto 5 hard, oblong seeds. The color of the fruits vary from greenish bronze to bronze pinkish red, purple and almost black. Several cultivated varieties of muscadine cultivars are available from various sources such as Black Beauty, Black Fry, Darlene, Fry, Higgins, Jumbo, Scuppernong, Sugargate, Summit, Supreme, Sweet Jenny, Carlos, Cowart, Dixieland, Dixie Red, Fry Seedless, Magnolia, Nesbitt, Noble, Redgate, Regale, and Sterling. The muscadine grapes are processed into wine, juice, jam, raisins, brandy, grapeseed oil, grape pomace, hydrocolloids, and anthocyanins.

Rabbiteye Blueberries (*Vaccinium ashei*)

Blueberries are grown wild around the world with US being the largest producer (55 %). The annual production of blueberries in the United States in the years 2000 – 2002 was estimated as 273 million pounds. However there are three commercial and popular varieties of blueberries grown across the world, namely *Vaccinium corymbosun* (Northern Highbush), *Vaccinium ashei* (Southern Rabbiteye), and *Vaccinium angustifolium* (Lowbush or wild blueberries).

The highbush varieties survive in the cooler climates and native to the North American regions. The rabbiteye varieties can tolerate warmer climates and are grown mostly in the Southern United States. The lowbush varieties are grown in the northeastern United States and the eastern provinces of Canada (USDA, 2003).

The rabbiteye blueberry (Figure 2.4) is native to Southern Georgia, Northern Florida and Alabama. Georgia's blueberry industry has experienced remarkable growth from zero in 1955 to 4600 acres in 2000. More than 90 % of the blueberry acreage is

contributed by rabbiteye blueberries (Scherm et al., 2003). The blueberries are mostly grown in the South Eastern and South Central parts of Georgia (Figure 2.5).

The fruits are characterized by bluish black color. Some of the varieties of blueberries are Woodard, Tifblue, Garden Blue, Delite, Briteblue, Climax, Brightwell, and Sharpblue. Blueberries are used either as fresh or processed. Processed blueberries are marketed as canned, frozen, dried or in liquid forms.

Chemistry of flavonoids and phenolic acids

The polyphenolic flavonoids consists of the diphenylpropane ($C_6C_3C_6$) skeleton consisting of 15 carbon atoms. The basic structure of flavonoids consists of two benzene rings (A, B) connected by a six membered ring (C). The ring A is condensed with a six-member ring (C), which has a phenyl benzyl ring (B) in the 2 position as a substituent (Figure 2.6). The ring C could be a heterocyclic pyran, yielding flavanols (catechins), and anthocyanidins, or pyrone, yielding flavonols, flavones, and flavanones as indicated in the Figure 2.7. Flavonoids can be monomeric, dimeric, or oligomeric. Monomers vary greatly in size, for example flavones have a molecular weight of 222, while blue anthocyanin has a molecular weight of 1759. Flavonoids are classified into flavones, flavanols, flavanones, catechins, anthocyanidins, and isoflavones.

The basic structure of phenolic acids consists of benzoic acid. Phenolic acids are classified into hydroxy benzoic acids and hydroxycinnamic acids (C_6C_3). Both the structure of phenolic acids and flavonoids varies within the major classification depending on the nature of substituents such as hydrogenation, hydroxylation, methylation, malonylation, sulphation and glucosylation. Most of the flavonoids and

phenolic acids occur as glycosides (Cook et al., 1996, Aherne et al., 2002, Rice Evans et al., 1996). Different structures of flavonoids and phenolic acids are shown in Figure 2.8.

Synthesis of flavonoids and phenolic acids

The flavonoids and the phenolic acids are synthesized through shikimate pathway, which follows from the pentose phosphate, and glycolytic pathways. In oxidative pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PDH) catalyses the conversion of glucose-6-phosphate to 6-phosphoglucono lactone and the concurrent reduction of NADP to $NADPH_{2,}$ which in turn is converted to ribulose 5 phosphate by 6 – phospho gluconate dehydrogenase. The shikimic pathway gets its substrates erythrose-4-phosphate from pentose phosphate pathway and phosphoenol pyruvate from glycolysis to produce phenylalanine. The shikimate pathway converts these simple carbohydrates to aromatic amino acids like phenylalanine, which is the starting material for phenylpropanoid pathway for the synthesis of various secondary metabolites such as rosmarinic acid, flavonoids, phenolic acids, phytoalexins, and lignins.

In the phenylpropanoid pathway, phenylalanine ammonia lyase catalyses the conversion of phenylalanine to cinnamic acid, the precursor of phenolics. The cinnamic acid is hydrolyzed to p-coumaric acid. Para coumaric acid is converted to caffeic acid, which in turn is converted to ferulic acid. Another condensation reaction occurs between one molecule of p-coumaric acid and three molecules of malonyl-CoA results in the formation of chalcone. Ring closure and hydration of chalcone gives rise to such compounds as catechins and flavonoids. The biosynthetic steps are illustrated in the Figure 2.9. (Aherne et at., 2002, Shetty, 2004, Treutter, 2001).

Muscadines and Rabbiteye Blueberries as source of polyphenolic compounds

Muscadines (*Vitis rotundifolia)* are shown to be a good source of polyphenolic compounds. Bonilla-Pastrana et al., (2003) analyzed muscadine fruits of ten different cultivars and found that the average total phenolic contents in whole fruits, seed, skin, pulp, and leaves were 0.25, 2.18, 0.37, 0.02, 0.35 g/100 g gallic acid equivalent fresh weight respectively. The average of total anthocyanin content in whole fruits, skin, seed, and pulp was determined as 28.4, 67.1, 4.3, and 4.6 mg/100 g fresh weight respectively. Bonilla-Pastrana et al., (2003) also analyzed individual phenolic compounds present in muscadine grapes. They found the major phenolics in seeds as gallic acid, catechin, and epicatechin and in skins as ellagic acid, myrecetin, quercetin, kaempferol, and trans-resveratrol. Yilmaz et al., (2004) studied two different species of grapes on the content of phenolic compounds in the seeds and skins of grapes. They found the concentrations of gallic acid, monomeric catechin, and epicatechin concentrations as 99, 12, and 96 mg/100 g of dry matter (dm) in Muscadine seeds respectively.

Talcott et al., (2003) analyzed total phenolics and anthocyanins in unprocessed, thermal pasteurized, and high hydrostatic pressure processed muscadine juice and found them as 1.75, 1.77, 1.66 g/L and 1.29, 1.13, and 1.10 g/L respectively. They also analyzed individual anthocyanins and found that the major anthocyanins present in muscadine juices were delphinidin 3,5-diglucoside, cyanidin 3,5-diglucoside, petunidin 3,5-diglucoside, pelargonin 3,5-diglucoside, peonidin 3,5-diglucoside, and malvidin 3,5-diglucoside.

Lee et al., (2004) evaluated the effects of fruit maturity and juice extraction on the content of ellagic acid derivatives and other antioxidant polyphenolics in eight varieties

of muscadine grapes and found that free ellagic acid, ellagic acid glycosides, and total ellagic acid ranged from 8 - 162, 7 - 115, and 587 - 1900 mg/kg, respectively, in the skin of ripe grapes. They found that hot pressed juice had a lower content of phenolic compounds than that present in whole grapes. They also identified five anthocyanins (delphinidin, petunidin, malvidin, peonidin, and cyaniding) in variable amounts in the different cultivars of muscadine grapes. Lee et al., (2005) did a study on the identification of ellagic acid conjugates and other polyphenolics by using high pressue liquid chromatography-electrospray ionization-mass spectrophotometry in three cultivars of muscadine grapes and identified three ellagic acid glycosides (rhamnoside, xyloside, and glucoside) and rhamnosides of quercetin, myricetin, and kaempferol in addition to at least four different ellagitannins.

Research conducted by Lamikanra et al., (1996) on the content of hydroxylated stilbenes in muscadine grapes and muscadine table wines showed that the trans-resveratrol was the dominant hydroxystilbenic compound, with 5.6 mg/100 g of the fresh berry weight. *cis*-Resveratrol and tetrahydroxystilbene contents are 0.08 and 0.05 mg/100 g of fresh fruit, respectively. Ector et al., (1996) conducted a study on the content of resveratrol in muscadine berries, juice, pomace, purees, seeds, and wines and found that the seeds had higher concentration of resveratrol than other parts of the grapes. The concentration of resveratrol varied from 2.7 – 62.2 μg/g. Magee et al., (2002) did an experiment on the effect of fungicides on the resveratrol content in five muscadine cultivars. They found the average amounts of resveratrol in the skins and the seeds of muscadines as 2.84 and 0.83 μg/g respectively. Talcott et al., (2002) analyzed eight wines and juices produced by different methodologies from red and white muscadine

grape varieties for total phenolics and total anthocyanins and found that they vary from 212 – 1860 and 40.3 – 1440 mg/L of juice. They also analyzed individual phenolics and anthocyanins and indicated the presence of ellagic acid, myricetin, quercetin, kaempferol, delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin.

Studies conducted by Auw et al., (1996) on the effect of processing on the total phenol content in muscadine grapes showed that the total phenolics ranged from 162 – 1096 mg/L gallic acid equivalents and also identified inidividual phenolic compounds as gallic acid, caftaric acid, coutaric acid, ellagic acid, catechin, and epicatechin. Boyle et al., (1990) conducted a study on the polyphenolic content of eleven varieties of muscadine juices and found that ellagic acid ranged from 1.6-23.1 μg/mL of the juice. Musingo et al, (2001) did a study on the changes in the total phenolic content of the muscadine juices upon storage and found that there was a decrease in ellagic acid due to the formation of ellagic acid sediment. Other phenolic compounds like gallic acid, catechin, epicatechin, and epicatechin gallate showed some devioation from their original amounts as present in the fresh juice. Research done by Flora, (1978) on 11 cultivars of muscadine grapes showed that total anthocyanins ranged from 40 to 403 mg/100 g of fresh fruit. They found that delphinidin was the major anthocyanin present in the fruit with small amounts of cyaniding, petunidin, peonidin, and malvidin.

Rabbiteye blueberries (*Vaccinium ashei*) have been shown to be good source of polyphenolic compounds. Sellapan et al., (2002) did research on the polyphenolic content of twelve cultivars of rabbiteye blueberries. They found the total phenolics and total anthocyanins varied from 270.0 – 929.6 and 12.7 – 197.3 mg/100 g fw respectively. They also identified individual phenolic compounds as gallic acid, p- hydroxy benzoic

acid, caffeic acid, p- coumaric acid, ferulic acid, ellagic acid, catechin, epicatechin, myricetin, quercetin, and kaempferol. Phenolic acids and flavonoids ranged from 0.19 – 258.9 and 2.51 – 387.48 mg/100 g fw respectively.

Prior et al., (1998) did a study on the phenolic and the anthocyanin content of *Vaccinium* species. They studied four different cultivrs of rabbiteye blueberries and found that the total phenolic and the total anthocyanin contents varied from 230 – 457 and61.8 – 187.2 mg/100g fw respectively. Prior et al., (2001) identified procyanidins and anthocyanins in blueberries and cranberries using high performance liquid chromatography/Mass Spectrometry. They found that the total phenolic, and total anthocyanin of rabbiteye blueberries as 8 mg/g, and 2.8 mg/g fresh weight respectively.

Moyer et al., (2002) did a study on the total phenolic content, total anthocyanin content, and antioxidant capacity of diverse small fruits. They studied three different cultivars of rabbiteye blueberries and found that the total phenolic and total anthocyanin content varied from 717 – 961 and 242 – 515 mg/100 g respectively. Stojanovich et al., (2007) investigated the effects of osmotic concentration, continuous high pressure ultrasound, and dehydration on antioxidants, color, and chemical properties of rabbiteye blueberries. They found that the total phenolics and the total anthocyanins were 551 and 135 mg/100 g fw respectively. Su et al., (2006) conducted an experiment on the effect of fermentation on antioxidant activity, anthocyanins, and phenolics of rabbiteye blueberry by products. They found the total phenolics and the total anthocyanins in juice pomace, wine pomace, and vinegar pomace as 29.2, 27.8, and 20.1 and 11.9, 10.9, and 2.3 mg/g respectively.

Research showed that both the fruits muscadine and rabbiteye blueberries are potential sources of isolated polyphenolic compounds. Polyphenolic compounds are shown to exhibit health benefits (Cook, 1996). To extract these compounds, to apply them in the form of nutraceuticals and to sell them to the consumers, we need to understand the distribution of these polyphenolic compounds in the fruit tissues.

Distribution of polyphenolic compounds in the plant tissues

Distribution of polyphenolic compounds at the tissues, cellular, and subcellular level is non-uniform. At the tissue level, the soluble phenolics are found mostly in the outer tissues (epidermal and subepidermal layers) with few in the inner tissues (mesocarp and pulp) of the fruit. Anthocyanins are located in the peel of the fruits. In the inner cells of the skins they are present as neutral quinoidal base forms, while in the outer cells they are present as flavylium cationic forms.

At the subcellular level, phenolic compounds are accumulated in vacuoles with trace amounts in the free space and none in the cytoplasm. The reason for this the concentration of H+ ion is high enough to maintain the hydroxyl groups of phenolic compounds in a non-ionized reduced state within the cell vacuoles than that in the cytoplasm. At a higher pH, the phenolic compounds remain in a free state and are most likely to get oxidized and polymerized. In some cases, it is found that the flavonoids and ferulic acids are present in the cell wall, whereas the soluble phenolics are present in the cell vacuole (Antolovich et al., 2000). Ferulic acids are found to be crosslinked to the pectins and arbinoxylans or to cell wall polysaccharides. Cell wall phenolics, lignins and hydroxycinnamic acids are linked to various cell components contributing to mechanical

strength of cell walls and henceforth playing a regulatory role in plant growth, morphogenesis, and cell response to stress and pathogens (Naczk et al., 2004).

Phenolic compounds are stored mainly as glucosides, in a form, which is not toxic to plant cells. Glycosidases are present in large amounts in the cells, which strip the phenolic compounds of their glucose moiety. The aglycones are highly reactive and toxic to the cell protoplast. This also indicates that this process must be rigorously controlled for normal plant functioning. The control is achieved mostly by compartmentalization (Beckman, 2000).

Polyphenolic compounds exhibit their health benefits by counteracting the free radicals (source of may diseases). To have a complete understanding of the dieases caused by the action of free radicals, and the preventive action of the polyphenolic compounds we need to understand the structure of free radicals, their mechanistic action and the preventive actions of polyphenolic compounds.

Free Radicals

Free radicals are defined as molecules that have an unpaired electron in their outer shell. They are highly unstable and very reactive. Free radicals include oxygen free radicals (superoxide, hydroxyl, peroxyl, alkoxyl, and hydroperoxyl radicals), and nitrogen free radicals (nitric oxide and nitrogen dioxide). Oxygen and nitrogen free radicals are converted to non-radical reactive species such as hydrogen peroxide, hypochlorous acid, hypobromous acid, and peroxy nitrite.

Free radicals are produced in two different pathways, one is useful and deliberate for proper functioning of the body, and the other is accidental and unavoidable. Free radicals (reactive oxygen species, ROS) in the body are produced i) during respiratory

burst of activated phagocytic white cells to kill microbial cells, ii) during passage of electrons along the mitochondrial electron transport chain, iii) during body exercise, iv) during metabolism of drugs and pollutants by the mixed function cytochrome P-450 oxidase (phase I) detoxifying system, iv) during autooxidation of ascorbic acid, catecholamines, dopamine, hemoglobin, flavins and thiol compounds catalyzed by iron, v) by pathological processes and agents like chronic inflammation, infection, ionizing radiation, and cigarette smoke, and vi) breathing oxygen rich air. Reactive nitrogen species (nitric oxide), being a vasodilator is important for maintaining normal blood pressure, reduces aggregation of platelate. Hydrogen peroxide is formed upon enzymatic action of superoxide dismutase on superoxide and plays an important role in cell signaling and gene activation.

Production of free radicals in the body can be beneficial as they serve as signaling and regulatory molecules and can be dangerous by inducing oxidation of protein, amino acids, lipids and DNA, which leads to pathogenesis of many chronic diseases. The beneficial effects include signal transduction, gene transcription, regulation of soluble guanylate cyclase activity in cells, regulation of contraction and proliferation of vascular smooth muscles, leukocyte adhesion, platelet aggregation, angiogenesis, thrombosis, vascular tone, hemodynamics, neurotransmitter, mediator of immune response, and inhibitor of enzyme contaning an iron-sulphur center. The harmful effects of free radicals include oxidation of biomolecules that leads to cell injury and death, altering the physical, chemical, and immunological properties of superoxide dismutase that results in increased oxidative damage to cells. Although molecular oxygen is required for aerobic

respiration, it is also toxic under certain circumstances. This phenomenon is termed as the oxygen paradox.

A sudden increase of ROS in the body overcomes antioxidant defense system in the body and results in onset of several diseases. The sites of high load of ROS in the human body are the mitochondria, the eyes, the skin, areas of damaged cell, inflammation, and the post-ischemic reperfusion, the liver, the lungs, and the brain. The initial events include DNA breakage and mutation, oncogene activation, tumor-suppresor gene activation, peroxidation of unsaturated fatty acids, loss of membrane fluidity, formation of low density lipoprotein, activation/inactivation of enzymes, crosslinking and fragmentaion of protein, changes in immunogenecity, crosslinking of carbohydrate, and disturbance of receptor. These changes lead to chronic diseases such as coronary heart disease, cancer, cataract, dementia, stroke, aging and cancer. Removal of excess free radicals from the body by scavenging action of antioxidants is a powerful means of delaying the harmful effects of ageing, decreasing the risk of chronic diseases, and improving functional longevity (Benzie et al., 2005; Fang et al., 2002).

An example of free radical initiated oxidation and the inhibitor reactions are shown as below. (where R_2N_2 = azo compound, LH = substrate, AH = antioxidant).

Initiation

$$R_2N_2 \rightarrow 2R^{\bullet} + N_2$$

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + LH \rightarrow ROOH + L^{\bullet}$$

Propagation

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$$

Inhibition

$$LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet}$$

Termination

$$A^{\bullet} + (n\text{-}1)LOO^{\bullet} \rightarrow \text{nonradical products}$$

$$LOO^{\bullet} + LOO^{\bullet} \rightarrow \text{nonradical products}$$

Antioxidants

An antioxidant can be defined as anything that can delay or prevent oxidation of a susceptible substrate initiated by oxygen or peroxides. A more biological definition of antioxidants is synthetic or natural substances added to products to prevent or delay the onset of oxidation by oxygen of air and on interaction with reactive oxygen species (ROS) should get transformed to a weaker reactive species than the original ROS.

In biochemistry and medicine, antioxidants are referred to as enzymes or other organic substances that are capable of counter affecting the damaging effects of ROS. In the chemical industry, antioxidants are referred to compounds that hinder the oxidation of rubber and plastics. In the food industry, antioxidants are referred to as compounds to

prevent fat from getting rancid and maintain normal physiological function in humans (Huang et al., 2005).

The various parts of human body are exposed to a variety of ROS, but have developed a defense system to protect itself from them. Human plasma and other biological fluids contain Vitamin C and Vitamin E. Bilirubin, uric acid, glutathione, flavonoids, and carotenoids also have antioxidant activity, and are present in cells and / or plasma. In addition to these there are cellular antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. There are protective proteins like transferrin, ferritin, and caeruplasmin which bind transition metals such as iron and copper involved in reacting with ROS and leading to the formation of highly reactive ROS. Heptaglobin, hemopixin, and albumin can also be considered as antioxidants as they take part in binding the transition metals as part of their function (Benzie et al., 2005).

Dietary intakes of plant based antioxidants are recommended to complete the antioxidant system in human body. It is generally recommended that minimum of five servings of fruits and vegetables to be eaten daily. The recommendation was based on epidemiological evidence, which indicated prevention of 30 – 40 % cancers (Benzie et al., 2005). Plant based antioxidants refer to Vitamin C, Vitamin E, folic acid, flavonoids, and others. Dietary antioxidants are important as they take part in boosting the antioxidant network (Packer., 1999; Benzie et al., 2005; Awad et al., 2003).

Polyphenolic compounds such as flavonoids and phenolic acids have the capability of scavenging free radicals due to their inherent structural characteristics, which will be discussed later. Anthocyanins were determined as the efficient antioxidant

among other polyphenolic compounds. Research indicated that a high intake of dietary antioxidants is associated with low incidence of disease and reduced oxidation of protein, lipid and DNA.

Phenolic Compounds as Antioxidants

The ability of polyphenolic compounds to act as hydrogen or electron donating agents will determine their antioxidant property. The desirable properties are reactivity as a hydrogen or electron donating agent, the nature of resulting antioxidant radical (which is determined by its ability to delocalize the unpaired electron), reactivity with other antioxidants, and metal chelating properties (Schmidl et al., 2000). In human body free radicals are produced as part of their normal functioning. Excessive production of free radicals is dangerous to human system leading to theonset of several diseases as stated above in the "free radical" section. Free radicals are very unstable due to the presence of an unpaired electron. Neutralization of free radical in human system is necessary to terminate the chain reactions initiated by free radicals leading to damage of fresh cells. Polyphenols have ideal structural chemistry for free radical scavenging activities. The ortho 3', 4'-dihydroxy moiety in the B ring which contributes higher stability to the radical form and helps in electron delocalization, meta 5, 7-dihydroxy arrangements in the A ring, and or 2, 3-double bond with both the 4-keto group and 3-hydroxyl group in the C ring takes part in delocalization of electrons of the polyphenolic compounds impart these compounds ideal for free radical scavenging activity. Quercetin satisfies the above requirements and is found to be more effective antioxidant than the other flavanols (catechin, epicatechin which lack the 2,3- double bond with the 4-keto group in the C ring). Other scientific studies indicated that position and degree of

hydroxylation is fundamental to antioxidant potential. Myrecetin due to its extra 5' hydroxyl group in the B ring has made it a more powerful antioxidant than quercetin. The antioxidant activity of the anthocyanins is attributed to the reducing power of the ortho 3', 4'-dihydroxy structure in the B ring as in cyanidin. Glycosylation of the flavonoids in the 3 position in the C ring dimishes the antioxidant activity. The above findings were true when the reaction takes place in aqueous phase.

In the lipophilic phase, the inhibitory effect of these flavonoids on the oxidation of the lipid caused is not as definitive as in the aqueous phase. Studies showed that the flavonoid may react by i) chelating copper ions by ortho dihydroxy phenolic structure, ii) acting as chain breaking antioxidants by scavenging lipid alkoxyl and peroxyl radicals, and iii) regenerating a-tocopherol by reduction of a-tocopherol radical. Interestingly the 2,3 double bond in the C-ring was not found to be as important as in the aqueous phase to destabilize the lipid alkoxyl and peroxyl radicals.

The number of hydroxyl groups present in the molecules determines the intensity of the antioxidant activity of the phenolic acids. For example gallic acid (3,4,5 trihydroxy benzoic acid) is a stronger antioxidant than the dihydroxy benzoic acids. However, the electron withdrawing carboxylate group in the benzoic acids has a negative effect on the hydrogen donating capacity of these molecules. The presence of hydroxyl groups in the meta position to the carboxyl group in the dihydroxy benzoic acids make them effective hydrogen donors compared to others where the hydroxyl groups are located in the ortho or para positions. Hydroxy cinnamic acids (coumaric, caffeic, and ferulic), are found to be better antioxidants than the benzoate counterparts because of the presence of the –CH=CH-COOH group, which facilitates the hydrogen donating ability

of the molecule. Substitution of 3-hydroxyl group in caffeic acid with 3-methoxy group in ferulic acid has increased the antioxidant potential of ferulic acid compared to that of the caffeic acid. The electron donating methoxyl group helps in increased stabilization of the aryloxyl radical (formed after donation of hydrogen) by electron delocalization (Rice-Evans et al.,1996).

Health effects of polyphenolic compounds

Research in the past twenty years has shown that consumption of fresh fruits are associated with reduced risk of cardiovascular diseases, ischemic stroke, cancer, and others. Studies done by Verlangieri et al., 1985 on the American population from 1964 – 1978 showed a negative correlation with the consumption of fresh fruits with cardiovascular diseases (r = -0.802), diabetes milletus (r = -0.412), and cystic fibrosis (r = -0.567). They proposed that the content of Vitamin C in fruits was responsible for the negative association with the above diseases. Joshipura et al., 1999 did prospective cohort studies on 75, 986 women (age group 34 – 59) and 38,683 men (age group 40 – 75) to investigate the effect of consumption of fruits on the incidence of ischemic stroke and found a relatively lower risk of stroke in the individuals who had a higher intake of fruits. A meta analysis of cohort studies on 91,379 men, 129,701 women done by Dauchet et al., (2006) to access the strength of association between consumption of fruits and coronary heart disease (CHD) showed that the risk of CHD was decreased by 7 % for each additional portion of fruit intake daily.

Epidemiological evidence of cardioprotective effects of polyphenolic compounds

Knekt et al., (2002) investigated the effects of flavonoid intake on 10, 054 men and women on the coronary heart disease. They found that people with higher intake of

quercetin had lower incidence of ischemic heart disease and a negative correlation between higher intake of kaempferol and heart disease. The Zutphen Study (Keli et al., 1996), conducted a study on a cohort of 552 men aged from to 50 to 69 years, to examine the association between flavonoid intake and incidence of stroke found that the relative risk (RR) of coronary heart disease mortality in the highest versus the lowest quartile of flavonoid intake (> or = 28.6 mg/d vs <18.3 mg/d) was 0.27. They also concluded that quercetin was found to be the most effective among the flavonoids to lower the risk of stroke. Hertog et al., 1993 conducted the Zutphen Elderly Study on 805 men aged 65-84 years to examine the effects of flavonoid intake and coronary heart disease. They found that the daily intake of flavonoids was 25.9 mg and was associated with a negative incidence of coronary heart disease and myocardial infarction. Research indicated inverse relationship of consumption of red wine with coronary heart diseases (Sun et al., 2002). This was found to be true for French people which lead to the French paradox. Ingestion of ethanol leads to oxidative damage by enhancing the production of free radicals. The low incidences of heart diseases within the French people despite high consumption of high fat diet was attributed to the polyphenolic content of non-alcoholic fraction of the red wine.

Retardation of low-density lipoprotein (LDL) oxidation in vitro by polyphenolic compounds

Most of the flavonoids have the ability to reduce the oxidation of LDL in vitro by macrophages or by copper ions. Research done by Steffen et al., (2006) showed the inhibitory effects of epicatechin on oxidation of LDL and counter effects on the deleterious effects of oxidized LDL on vascular endothelial cells. Schewe et al., (2005),

reported the same observation. Cirico et al., (2006) reported the synergistic effects of antioxidants (catechin, hesperidin, quercetin, and ferulic acid) to prevent the copper mediated LDL oxidation.

Hou et al., (2004) studied the antioxidant effects of quercetin, kaempferol, myrecetin and others on free radical or copper initiated LDL oxidation and found them as effective antioxidants in preventing the oxidation process. They also concluded that the presence of ortho-dihydroxy groups in the flavonoid molecules exhibited the highest antioxidant capacity. Safari et al., (2003) studied the antioxidative effects of quercetin, pelargonidin, and others on free radical or Copper mediated oxidation of LDL and reported that quercetin was the most effective one in preventing oxidation of LDL among others.

Anticarcinogenic effects of polyphenolic compounds

Several studies on inhibitory effects of flavonoids on different types of cancer have shown positive results. Rossi et al., (2006) conducted a case control study on 304 cases (275 men, 29 women) with a first diagnosis of squamous-cell carcinoma of the esophagus and 743 controls (593 men, 150 women) with no history of cancer to investigate the effects of flavanones (hesperitin and narigerin), flavan-3-ols (epicatechin and catechin), flavonols (quercetin, myricetin and kaempferol), flavones (apigenin and luteolin), and anthocyanidines (cyanidin and malvidin) on the risk of squamous cell esophageal cancer. They concluded that there was inverse correlation between intake of flavanones and flavonols and the risk of cancer. The other compounds did not have a significant influence on the risk of cancer. Friedman et al., (2006) did a study on the structure activity relationships of tea flavonoids against human cancer cells and

concluded that catechins and other flavonoids reduced the numbers of the following human cancer cell lines: breast (MCF-7), colon (HT-29), hepatoma (liver) (HepG2), and prostate (PC-3).

Fink et al., (2006) did a study on the anticarcinogenic effects of flavonols, flavones, flavan-3-ols, and lignans on breast cancer risk on 1434 breast cancer cases and 1440 controls and they concluded that all the groups of flavonoids decreased the risk of postmenopausal breast cancer. Kang et al., (2003) did a study on the anticarcinogenic effects of anthocyanins on human colon cancer cell lines and inhibitory effects of tumor development in mice. They reported that there was a reduction in the volume of cecal adenomas in the mice which ingested anthocyanin than the ones which did have such diet. They also found that the anthocyanins reduced the cell growth of human colon cancer cell lines. Singletary et al., (2003) conducted a study on the effects of anthocyanin rich concord grape juice on the growth of rat mammary adenocarcinoma cell line and reported that there was a significant decrease in tumor development in the group of mice which were given the diet containing grape juice than that of the controls.

Greenspan et al., (2005) conducted a study on the anti-inflammatory activity of muscadine extracts. They reported that the extracts of muscadine at 1:100 dilutions inhibited the release of superoxides by 60 % and tumor necrosis factor, interleukin-1 at 1:200 dilutions by 15 and 90 % respectively.

Antiageing effects of polyphenolic compounds

Neurodegenerative diseases such as Parkinson's disease and Alzeihmer's disease are influenced by various genetic and nongenetic factors. Engelhardt et al., (2002) conducted a study on the association between intake of flavonoids and risk of

Alzeihmer's disease. The Rotterdam study included 5395 participants (aged ~55 years) showed that the relative risk of Alzeihmer's disease decreased with intake of flavonoids.

Studies done by Joseph et al., (1999, 2005) showed an improvement in age related declines in neuronal and cognitive function of rats upon intake of flavonoid rich extracts of blueberries and other fruits. Some of the neuronal and behavioral parameters included motor behavioral performance on the rod walking and accelerod tasks, Morris water maze performance and others. Lindsay et al., (2002) did a study on the risk factors of Alzeihmer's disease and reported that wine consumption among other factors was associated with reduced risk of Alzeihmer's disease. Truelsen et al., (2002) investigated the effects of alcohol consumption on the risk of dementia on 83 subjects (diagnosed with dementia) and 1629 non-demented subjects. They reported that monthly and weekly intake of red wine was associated with reduced risk of dementia.

Antimicrobial effects of polyphenolic compounds

Researchers studied the antimicrobial effects of polyphenolic compounds and reported positive effect. Ikigai et al., (1993) did a study on the mode of antibacterial action of epicatechin and other flavonoids in green tea. They reported that the bactericidal catechins primarily act on the bacterial membranes and damage them. Rauha et al., (2000) did a study on the antimicrobial effects of polyphenolic compounds derived from Finnish plant extracts. They found that quercetin, and kaempferol showed moderate antimicrobial activity against *Stapphylococcus aureus*, *Stapphylococcus epidermitis*, and *Bacillus subtilis*. Basile et al., (2000) studied the antibacterial activity of extracts rich in quercetin, kaempferol and others and found that quercetin was the most effective

antimicrobial agent against S. *aerogenes*, *S. aureus*, *P. vulgaris*, *P. aeruginosa*, *E. cloacae*, *E. coli*, and *K. pneumoniae*.

Puupponen-Pimia et al., (2001) investigated the effects of flavonoids from berries on the growth of probiotic bacteria and other intestinal bacteria. They reported that myrecetin was the only potent flavonoid, which showed inhibitory effect on lactobacillus organisms. They concluded that the phenolic acids (cinnamic acid, 3-coumaric acid, caffeic acid, ferulic acid and chlorogenic acid) showed activity only against Gram-negative bacteria at high concentrations. They also found that the anthocyanidins pelargonidin, delphinidin and cyanidin, as well as cyanidin-3-glucoside, only inhibited growth of *E. coli* CM 871 and had no effect on other bacterial strains.

Research indicated several beneficial and protective effects of the polyphenolic compounds. To ensure safety of polyphenolic compounds in the human body we had to understand the recommended dietary intakes of these compounds, their adsorption, and metabolism in the human body. We need to know the body's limitations to the intake of polyphenolic compounds as they may lead to adverse affects discussed below.

Dietary Intake of polyphenolic compounds

According to Kuhnan, (1976) the average intake of flavonoids in USA is 1g/day. Several other authors also reported the different daily intake of different classes of flavonoids. Sesso et al., (2003) reported that the overall daily intake of flavonoids (flavonols and flavones) was 24.6 ± 18.5 mg/day (main contributor was quercetin 70.2 %) within women population of USA. According to Geleijnse et al., (2002), Hertog et al., (1993), and Knekt et al., (2002), the mean intake of flavonoids (including flavonols, flavones, and flavanones) was estimated to be 24.2 ± 26.7, 28.6 ± 12.3, and 25.9 mg/day

in the populations of Finland, Denmark, and the Netherlands, respectively. Prior et al., (2005) estimated the daily intake of flavan-3-ols monomers from tea to be in the range of 12.7–34.2 mg/day/person for adults in the United States, which is less than that in Netherlands (50 ± 56 mg/day/person). They also estimated the mean intake of proanthocyanindins for all ages (>2 yr old) as 53.6 mg/day/person.

Human Adsorption, metabolism and bioavailibility of polyphenolic compounds

The antioxidant levels in plasma determine adsorption of polyphenlic compounds in human body. Scalbert et al., 92002) did a study on the adsorption of polyphenolic compounds in the gut. They reported that small molecules like phenolic acids (caffeic acids and others) were easily adsorbed through the gut barrier whereas large molecules like proanthocyanidins were difficult to adsorb. Among flavonoids the recovery of quercetin in the plasma was very poor (0.3 – 1.4 %), whereas for tea catechins it was 3 – 27 %. They stated that adsorption of flavonoids were facilitated when they were glycolysated due to the presence of lactase phlorizin hydrolase or the cytosolic β-glycosidase in the intestinal epithelial cells (enterocyte). After absortion they are converted to glucoronides or sulfates in the liver. The polyphenolic compounds, which are not absorbed in the small intestine, are extensively metabolized by the gut microflora into simple molecules of phenolic acids (Aherne et al., 2002).

The conjugates of the flavonoids were shown to retain some of the biological activity. However the biological acitivities of the conjugates are dependent on the site of conjugation. Reports indicated that quercetin was more biologically active than the conjugates (Walle, 2004). During their process of passage through gut, the polyphenols

protect the gut mucosa from oxidative stress and also react with gut nutrients (Iron and others) and reduce its absorption in the gut.

Adverse effects of polyphenolic compounds

Exposure of human body to increased levels of polyphenolic compounds may have adverse effects by generating free radicals leading to DNA damage. Reports showed that doses of 1 – 1.5 g of flavonoids /day leads to acute renal failure, hemolytic anemia, thrombocytopenia, hepatitis, fever, and skin reactions (Cook et al., 1996). Research indicated tha tea polyphenols (catechin and epicatechin) had prooxidant effect on human body generating superoxide anion and hydroxyl radicals and leads to oxidative DNA cleavage (Azam et al., 2004). Sakihama et al., (2002) conducted a study on the prooxidant activities of polyphenolic compounds and reported that interaction of phenoxyl radical with DNA associated Copper may result in breakage of DNA strands.

Research demonstrated that polyphenolic compounds when taken in recommended doses leads to prevention of life threatening diseases. This necessitates the maximum extraction of the polyphenolic compounds from the fruit matrices and development of a pure polyphenolic rich extract either in liquid form or in powder form.

Extraction of polyphenolic compounds

Extraction of polyphenolic compounds from plant materials is dependent on various factors such as chemical nature, extraction method, sample particle size, storage time and conditions, and interfering substances. Polyphenolic compounds vary from simple phenolic acids to complex proanthocyanidins. The chemical structure of these compounds determines their solubility in various solvents.

Enzyme assisted extraction

The grape skin wall provides a limiting barrier to the release of polyphenolic compounds. Polyphenol extraction requires the degradation of middle lamella walls, release of cells, and lysis of cell walls to extract and leach the contents of the cells in the solvent. Researchers have used enzyme assisted extraction procedures to extract polyphenolic compounds. Meyer et al., (1998) conducted a study on the enzymatic release of polyphenolic compounds from grape pomace. They reported that the Grindamyl pectinase catalyzed degradation of grape pomace polysaccharides, which facilitated the release of polyphenolic compounds. Kammerer et al., (2005) did a study on the enzymatic release of polyphenolic compounds from winery by-products in pilot scale level and they found significant improvement in extraction with 5000 ppm (based on dry matter) of a pectinolytic and 2500 ppm of a cellulolytic enzyme preparation, respectively, at 50 C than without enzyme preparations. They also reported that application of enzyme assisted in cell wall breakdown which released polyphenolic compounds and increased the yield of these compounds. Pardo et al., (1999) investigated the effects of various pectinase enzyme preprarations (Endozyme contact pelliculatire, Biopectinasa, Ultrazyme 100G, Rapidase Excolor)on the yield of anthocyanins and total polyphenolic copounds from grapes. They reported a significant improvement in the yield with the first two enzyme preparations. Ortin et al., (2005) did a study on the effect of macerating enzymes (pectinase preparation with a secondary hemicellulase, and pectinase preparations with hemicellulase and cellulase secondary activities) on color extraction and stability in red wines and showed that there was a significant improvement in the yield of total anthocyanins in wine treated with the second enzyme preparations.

Landbo et al ., (2001) studied the effects of Grindamyl pectinase, Macer8 FJ, Macer8 R, and Pectinex BE, as well as treatment with Novozym 89 protease on the release of polyphenolic compounds from black currant juice press residues and found significant improvement in the yield of polyphenolic compounds with all the enzymes except Grindamyl pectinase.

Solvent assisted extraction

Talcott et al., (2004) extracted polyphenolics from muscadine skin and pulp by homogenizing with 100 % methanol and non-anthocyanin phenolics by ethyl acetate three times. Methanolic extracts (400 – 4220 mg/Kg) gave better yield of total phenolics (TPH) than the ethyl acetate extracts (60 – 1500 mg/Kg). However, the antioxidant activity correlated well with the ethyl acetate extracts. Sims et al., (1996) compared the yield of TPH and anthocyanins (ACY) from muscadine grapes as influenced by the extraction process (immediate press for wine and juice, hot press for wine and juice, and skin fermentations). They found that TPH was the highest in skin fermentation (1269 mg GAE/L, 14 days) than the other extraction procedures (162 – 1096 mg GAE/L). Anthocyanins were extracted more by 14 day fermentation on skins than by simply juicing or shorter term fermentation. Sims et al., (1994) followed the immediate press wines and skin fermentation approach to improve the yield of TPH and total anthocyanins (ACY) from muscadine grapes. They found that with increase in days (0 to 6) of skin fermentation TPH and ACY had increased from 152 to 1155 and 21 to 92 mg/L respectively. Yilmaz et al., (2004) extracted some phenolic compounds from deoiled muscadine seed and skin by 70% methanol/water mixtures in a sonicator. They found that the concentration of gallic acid was 99 mg/L in the extracts prepared from the seeds.

Talcott et al., (2002) extracted TPH and ACY from two varieties of muscadine grapes by cold press, hot press, and fermentation methods. TPH and ACY varied from 248- 1860 and 194 -1440 mg/L respectively with maximum being extracted in 7 day fermentation of noble variety and minimum being extracted cold pressed juice from Carlos variety. Pezet et al., (1996) extracted resveratrol from skin of grapes by fermentation and found an increase in its content from that of juice (46%, 46 days). Baydar et al., (2004) extracted powdered grape seeds and bagasse with petroleum ether to defat them and then reextracted with acetone: water: acetic acid (90:9.5:0.5) at 60°C / ethylacetate: methanol: water (60:30:10)/ethanol: water (95:5, for bagasse only). The concentration of TPH extracted with acetone: water: acetic acid from seeds was found to be more than that from the other solvents. However, in bagasse, ethylacetate: methanol: water gave better yield of TPH than the other solvent. Fuleki et al., (2003) evaluated different extraction methods (cold pressed, hot pressed, pasteurization) on the yield of TPH and found that hot pressed juice gave better yields compared to others. Mayen et al., (1995) extracted TPH from grapes by fermentation method and found that fermentation had improved the yield of TPH. Taylor et al., (1999) extracted TPH from grape seeds by using supercritical carbon dioxide method by using different percent of ethanol/methanol as modifier at different temperatures. They got the best results with 0.95 g/mL CO_2, 10 % methanol, and 55°C. Bonilla et al., (1999) extracted TPH from crushed and uncrushed grape marc by using 50 % ethyl acetate and water for 5, 10, 15, and 20 mins. They found that ethyl acetate phase contained more TPH than that of the aqueous phase. The crushed grape marc gave more yield of TPH from the uncrushed ones.

Kader at al., (1996) extracted TPH and ACY by ethyl acetate (4 times) from macerated blueberries and methanol (two times, two hours, room temperature) from blueberry skins respectively and got good yields of both of them. Ehlenfeldt et al., (2001) extracted polyphenolics from fruit and leaf tissues of highbush blueberry by homogenizing the tissues with acetonitrile/acetic acid. Total phenolics (TPH) and anthocyanins (ACY) in the fruit ranged from 0.60 – 1.99 mg GAE/g fw and 1.11 – 3.31 mg c3g/g fw respectively. Lyons et al., (2003) extracted resveratrol from raw and baked blueberries, bilberries by sonicating the berries with methanol (three times), followed by β-D-glucosidase digestion, then subsequently extracting it with ethyl acetate three times. Resveratrol ranged from 50 to 140 pmol/g of the sample. Kahkonen et al., (1999) homogenized grounded freeze dried berries and fruit extracts with 70% aqueous acetone and found that TPH ranged from 12.4 to 50.8 mg GAE/g. Prior et al., (2001) extracted freeze dried blueberry and cranberry with acetone: water: acetic acid (70:29.5:0.5) and found that TPH and ACY ranged from 0.3 to 6.3 and 0.012 to 3.6 mg/g fw respectively. Lee et al., (2004) extracted TPH and ACY from blueberry processing waste by enzyme, citric acid, and sulphur dioxide at two different temperatures. They found higher levels of temperature (80 °C) and sulphur dioxide (100 ppm) increased the extraction of TPH (978 mg GAE/L as compared to 55.9 mg GAE/L) and ACY (446.1 mg/L as compared to 27.5 mg/L) respectively. Howard et al., (2003) extracted homogenized blueberry with ethanol: acetone: water: acetic acid (40:40:20:0.1) for 60 mins at 60°C. The TPH and ACY ranged from 2.00 to 6.00 g/Kg and 0.5 to 2.4 g/Kg respectively. Rossi et al., (2003) compared the yield of ACY in blueberry as affected by pasteurization (90°C) and steam blanching. The blanching treatment demonstrated better yield (44.8 mg/100 g) of ACY

than the non-blanched ones (22.0 mg/g). Taruscio et al., (2004) extracted TPH and ACY from frozen ground berries with acetone: water (70:30). TPH and ACY ranged from 0.81 – 2.84 mg GAE/g fw and 0.1 – 1.35 mg cyanidin 3 glucoside equivalents/g fw respectively. Iwai et al., (2003) extracted anthocyanins from fruits of *Viburnum dilatum* thumb by passing the juice through Sephadex LH-20 column and eluting it with water, 20, 60, and 100 % methanol stepwise. They got maximum yields of ACH in the 20 and 60 % methanol fractions. Keinan (1993) did a comparative extraction study of flavonoids from birch leaves. He used homogenization, refluxing and Soxhlet extraction using 100 and 80% (v/v) aqueous methanol, ethanol and acetone. He found that Soxhlet and refluxing were the efficient extraction methods. Methanol was the most efficient solvent for extraction. Liyana-Pathirana et al., (2005) extracted phenolic compounds from wheat by using water/ethanol (0 to 100 %v/v), varying temperature (15 to 95°C) and time (15 to 105 min). They found the optimal conditions for extractions are 54% ethanol, 61 °C, 64 min. Douglas et al., (2004) extracted catechin from ground tamarind seed coat using supercritical CO_2/ supercritical CO_2 with 10 % ethanol and with ethanol/ethylacetate at ambient temperature, achieving yields of 336 µg/100g of seed coat as compared to extractions with supercritical CO_2 (22 µg/100g of seed coat). They also found that ethanol extractions gave yield of epicatechin as 32 mg/100 g of seed coat as compared to ethylacetate extractions (25 mg/100g of seed coat). Lee et al., (2003) did polyphenolic phytochemical extractions of powdered freeze dried plums and Gala apples using 80% aqueous methanol with ultrasound assistance. The total phenolic contents of various cultivars of plums and Gala apples were in a range of 174 to 375 mg/100 g and 118±1.4 mg/100 g, expressed as gallic acid equivalents (GAE), on a fresh weight basis. Cacace et

al (2003) optimized the extraction of ACY from grounded black currants using ethanol concentrations (39 to 95%), temperature (6 to 74°C) and solvent/solid ratio (6 to 74 mL/g) and got maximum extractions at 60 % ethanol concentrations and 30 to 35 C temperatures.

The review of literature showed that various solvents such as methanol, ethanol, propanol, acetone, ethyl acetate, dimethyl formamide, and their combinations with various proportions of water had been used. Methanol appeared to be the most commonly used solvent, however if the compounds are extracted for human consumption, ethanol and water are the most recommended solvents. The polyphenolic compounds were found to be thermally unstable, so an extraction temperature below 60 C was highly preferred to prevent degradation of these compounds. Extraction time varied with the different nature of grapes and berries. The higher the solvent to solid ratio, the better it is for extraction, however the use of solvent is limited from a practical point involving the time and cost of removal of solvent from the extracts.

The extracts contain polyphenolic compounds in a highly concentrated form, which could give rise to several complex reactions within the polyphenolic compounds. One of such phenomenon is called copigmentation effect.

Copigmentation effect of anthocyanins

Copigmentation is explained as molecular associations between anthocyanins and other organic molecules in solution. These associations result in exhibition of greater color than that of the original pigments. The copigment factors can be phenolic acids, flavonoids, and derivatives of flavonol and flavone subgroups. These associations result in higher absorbance values, providing a blue-purple tone instead of red color. The

enhancement of the color is dependent on the concentration of pigment, molar ratio of cofactor to pigment, pH, the extent of non-aqueous conditions, and the anions in the solution. The minimum concentration of anthocyanins appears to 18.5 mg/L as malvidin-3-glucoside for the copigmentation effect to occur. In relatively concentrated solutions of anthocyanins, self-association takes place causing a hypsochromic shift. The copigment complexes are structurally favored by hydrophobic and $\pi-\pi$ interactions causing planar stacks of aromatic molecules. The stronger cofactors are flavonoids with 3 to 6 hydroxyl groups. The electron withdrawing groups favor face-to-face stacking arrangements. Due to this arrangement, the copigment expels few water molecules from the pigment solvation shells into the bulk water and thus prevents the attack of nucleophilic water molecule on flavylium chromophore. Intramolecular copigmentation also occurs when the pigment and the copigment are the parts of the same molecule. Copigmentation is best at neutral pH to an acidic pH of 3.5. It is found that an increase in temperature partially disrupts these associations, therby releasing flavylium ions in bulk water (Boulton, 2001, and Brouillard et al., 1994).

Gris et al., (2007) conducted a study on the copigmentation between caffeic acid and anthocyanins in model systems. They reported that addition of caffeic acid to the anthocyanins from grape extracts at 1: 1 ratio significantly stabilized the anthocyanins. The copigment complex was stable at temperature of 4 $\pm$ 1 C. They found that addition of caffeic acid increased an hyperchromic and bathochromic shift in the absorbance. Talcott et al., (2005) used red clover isoflavonoids as anthocyanins color enhancing agents and found that the maximum color absorption was at a ratio of 1:8 (isoflavonoids: anthocyanins). Prodanov et al., (2005) discussed some limitations in the

spectrophotometric analysis of anthocyanins in anthocyanins rich extracts due to the effect of copigmentation and other possible reactions. Kowska et al., (2004) studied the effects of UV irradiation, temperature, and storage on the stability of anthocyanins copigment complexes. They used copigments as quercetin-5′-sulphonic acid (QSA), sodium salt of morin-5′-sulphonic acid (NaMSA), rutin, quercetin, chlorogenic acid, tannic acid, and unknown flavones from roots of the Chinese origin herb *Scutellaria baicalensis* Georgi. They reported an increase in copigmentation with copigment content, and a decrease in stability of copigment complexes when exposed to UV irradiation (greater than heating at 80 C). Direct sunlight has a negative effect on the copigment complexes.

The polyphenolic compounds in the extracts can be quantified by several spectrophotometric methods. Since the extracts are not pure and have interfering substances, they need to be acid hydrolysed to remove the interfering substances and also to hydrolyse the glycosides to aglycones before quantifying individual phenolic compounds by high pressure liquid chromatography.

Quantification of polyphenolic compounds

Numerous spectrophotometric methods are used to quantify polyphenolic compounds. These assays are based on different principles and are specific to different functional groups.

Spectrophotometric assays

The Folin-Denis Assay is the most widely used assay for total phenolics. The principle of this assay is reduction of phosphomolybdic-phosphotungstic acid (Folin-Denis) to a blue colored complex in alkaline solution by polyphenolic compounds. Most

of the researchers used Folin-Ciocalteaux method (Singleton et al., 1965) to determine total phenolics working on the same principle as above. The Folin-Ciocalteaux reagent is more sensitive to reduction by polyphenolic compounds than the Folin-Denis reagent by increasing the molybdate content and by addition of lithium sulphate to prevent precipitation of sodium complex salts. Singleton, (1965) modified it further by using alkali time temperature conditions and galic acid as reference standard. The absorbance of the blue colored solutions was measured at 765 nm. The concentrations of phenolic compounds are mostly reported as gallic acid equivalents.

The commonly used method for determination of total anthocyanins is the pH differential method (Giusti et al., 2001). The principle of this method is dependent on the structural transformations of anthocyanins due to pH change. It is observed that the colored oxonium form and the colorless hemiketal form dominate at pH of 1.0 and 4.5 respectively. The absorbance of the solution is measured at two different wavelengths (510 and 700 nm). The monomeric anthocyanins pigment is calculated as follows

$Absdiff = (A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}$.

Monomeric anthocyanins pigment (mg/L) = $(A \times MW \times DF \times 1000)/(e \times 1)$

Where MW = molecular weight of cyanidin – 3 – glucoside (449.2).

E = molar absoptivity (26,900)

DF = dilution factor

A = Absdiff

The principle of the antioxidant assays is dependent on their ability to reduce the substrate either by transferring a hydrogen atom or an eletron. The antioxidant activities of the extracts are determined by various methods. All the methods are classified in two

different types (electron transfer and hydrogen atom transfer). The assays involving electron transfer methods include Trolox, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid equivalent antioxidant capacity (TEAC), ferric ion reducing antioxidant parameter (FRAP), and 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) copper (II) reduction capacity. The assays involving hydrogen atom transfer are oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP), Crocin bleaching assay, Inhibited oxygen uptake (IOU), inhibition of linoleic acid oxidation, and inhibition of LDL oxidation. These assays consist of a free radical, oxidizable molecular probe, and an antioxidant.

Some of them used ferric reducing absorbance capacity (Benzie et al., 1999). The principle of this method is based on the reduction of herein (ferric salt) by antioxidant. In this method, the oxidant in the FRAP assay is prepared by mixing 2,4,6-tripyridyl-s-. triazine (TPTZ, 2.5 mL, 10 mM in 40 mM HCl), 25 mL of acetate buffer, and 2.5 mL of $FeCl_3 \cdot H_2O$ (20 mM). The conglomerate is referred to as "FRAP reagent". To measure FRAP value, 300 L of freshly prepared FRAP reagent is warmed to 37 C and a reagent blank reading is taken at 593 nm; then 10 L of sample and 30 L of water are added. Absorbance readings are taken after 0.5 s and every 15 s until 4 min. The change of absorbance ($A = A_{4\text{min}} - A_{0\text{min}}$) is calculated and related to A of an Fe (II) standard solution. A is linearly proportional to the concentration of antioxidant.

Researchers have used oxygen radical absorbance capacity to determine total antioxidant activity (Cao et al., 1999). In this method the oxidant probe, free radical generator, and antioxidant are carboxy-fluorescein, 2,2*-Azobis(2-Amidinopropane) Dihydrochloride (AAPH) and TROLOX respectively. In general, samples, controls, and

standard (Trolox of four or five different concentrations for construction of a standard curve) are mixed with fluorescein solution and incubated at constant temperature of 37 C before AAPH solution is then added to initiate the reaction. The fluorescence intensity [485 nm (ex)/525 nm (em)] is measured every minute for 35 min at ambient conditions (pH 7.4, 37 C). With time, fluorescein is consumed and its intensity decreases. In the presence of antioxidant, the FL decay is inhibited (Huang et al., 2005).

Chromatographic methods

Individual phenolic compounds were quantified by adsorption and desorption to a reverse phase columns. The order of the compounds eluted from the column is based on the polarily with most polar molecules to elute first. Hakkinen et al., (1999) used ODS-Hypersil with a guard column and a ternary solvent system to separate the flavonoids and phenolic acids in nineteen berries. The solvents were ammonium dihydrogen phosphate (A), ortho-phosphoric acid (B), and 20 % of A in acetonitrile (C). They found reasonable results with their method. Keinanen (1993) did fractionation of flavonoids from extracts of birch leaves by running the extracts through HO Hypersil ODS II using aqueous tetrahydrofuran + phosphoric acid and methanol. They were able to fractionate many flavonoids. Hertog et al ., (1992) did an optimization study on the high pressure liquid chromatographic (HPLC) determination of flavonoids from fruits and vegetables. They performed their chromatographic separations on a Nova-Pak C18 equipped with a guard column using two mobile phases. The first mobile phase used 25% acetonitrile in dihydrogen potassium phosphate and the second mobile phase used 45 % methanol. They found that each of these two mobile phases gave very different results. Mobile phase 1 resulted in less peak interference, however, quercetin and luteolin cannot be

separated by this phase. Prior et al., (2001) separated anthocyanins from extracts of blueberries and cranberries on a Zorbax C 18 column with a gradient of 5% formate and 100 % methanol and successfully detected a series of oligomers. Taruscio et al., (2004) analyzed the different flavonoids in *Vaccinium* species by running the extracts on waters Xterra MS C18 equipped with a guard column and following a gradient elution with trifluoroacetic acid and acetonitrile as two main solvents. Hakkinen et al., (2000) used two HPLC methods to fractionate flavonols and phenolic acid from extracts of strawberries and Vaccinium species. They used LiChroCART column protected with a guard column, followed a gradient comprising of 1 % formic acid and acetonitrile. They separated the phenolic acids using a different gradient with these solvents and the recovery of the compounds was 70 – 90%. Aaby et al., (2004) used HPLC coupled with coulometric array detector to characterize the electrochemical behavior of seventeen flavonoids and three cinnamic acid derivatives. Chromatographic separations were done on Luna C18 protected with a guard column. The mobile phase consisted of acetonitrile, methanol, and dihydrogen potassium phosphate following a linear gradient. They achieved a good separation of the compounds. Zhang et al., (2004) did a comparison of HPLC methods for determinations of anthocyanins and anthocyanidins in bilberry extracts. They used Beckman Ultrasphere ODS column using a gradient elution with 0.4 % trifluoroacetic acid in water and 0.4 % trifluoroacetic acid in acetonitrile. The average recovery for the compounds was achieved at 102 ± 2 (%). Chinnici et al., (2004) conducted a study on the improvement of HPLC method to determine phenolic compounds from apples, and they found that using a reverse phase monolithic column assays can be completed within 21 mins. They used acetonitrile and methanol as the

eluting solvents. They found that methanol allowed very rapid separation of the compounds whereas acetonitrile gave the best resolution.

Purification of polyphenolic compounds

It is necessary to purify the extracts containing polyphenolic compounds to eliminate the interfering substances, which might create problems in handling of these extracts. The extracts had to be in a pure edible form (liquid, or powder) before selling to the consumers, which leads to the purification step. Presence of sugars interferes with handling the TPH of the foods due to their hygroscopy, so it is desirable to eliminate sugars from isolated phenolic preparations. Researchers have either used adsorption column technology or fermentation to eliminate sugars. In the first case, phenolic compounds are adsorbed on to hydrophobic resins while sugars and other more polar compounds pass through the column or are eluted with polar solvents. Prior et al., (2001) used twenty milliliter columns filled with hydrated Sephadex LH-20 and eluted with 20 % methanol/water to remove sugars and phenolic acids. Heinonen et al., (1998) used solid phase extraction (SPE) to remove sugars. They used SPE tubes (C18 Sep-Pak Vac 200 mg) and eluted the extracts with water followed by methanol. Kahkonen et al., (1999) also used same method as Heinonen et al., (1998) to remove sugars from the plant extracts. Kennelly et al., (2004) used dianion HP-20SS resin to remove sugars and ascorbic acid from fruits extracts. The resin was conditioned sequentially with methanol and water. Then the extracts were allowed to adsorb on the resin for 20 mins and eluted sequentially with water, water: methanol (1:1), methanol, methanol: acetone (1:1), and acetone. They found that sugars and ascorbic acid were eluted in the water fractions, whereas the phenolic compounds were eluted in methanolic and aqueous methanolic

fractions. Rouseff et al., (1987) purified the flavonoids from sugars in citrus juice by passing them through conditioned Sep-PaK C-18 cartridge and the column was washed with 10 % methanol to get rid of sugars followed by 100% methanol to get the flavonoids fractions.

Maccarone et al., (2004) used different resin materials to purify cyanidine-3-glucoside. All of these resins were made up of styrene-divinyl benzene copolymers. The resins were characterized by wide range of surface area, pore radii, and porosities. They used citric acid/potassium citrate monohydrate buffer for the absorption experiments. They found that EXA-118, having the highest surface area gave the best yields of cyanidine-3-glucoside. Kin et al., (2005) conducted a purification of rutin study from whole plant of buckwheat. They used styrene-based adsorption resin based column. The samples were loaded onto the column and washed with water, 20% aqueous ethanol, and finally eluted with 30% ethanol. They found that 20 % aqueous ethanol preferentially dissolved the highest proportion of hydrophilic fraction relative to rutin, eluting with 30 % aqueous ethanol yielded about 98% rutin with 51% purity, where eluting with water didn't give promising yields. Scordino et al., 2004 used thirteen commercial resins with different hydrophobicity, surface areas (330 – 1200 m2/g), and pore areas (20 – 260 A) to study the absorption of cyanidin-3-glucoside. They found thar the styrene – divinylbenzene EXA – 118 resin was the most effective one. They used the same resin to adsorb the anthocyanins from orange juie and found a recovery of 80 % after eluting it with ethanol. A second valuable effect of adsorbing phenolics on to resins from dilute extracts is that they may be eluted by smaller volumes of aqueous ethanol or other solvent, resulting in much more concentrated solutions for subsequent drying.

Fermentation removes sugars by metabolizing them to alcohol and CO_2, but does not concentrate the desirable compounds.

Spray drying of these compounds

Drying is an important part of processing operations. Drying of polyphenolic rich purified extracts to a non-sticky powder would increase the shelf life of the powders and reduce the problems associated with handling and tranport. The common drying techniques are hot air (fixed and fluidized bed), freeze and spray drying. Both TPH and ACY present in muscadines and blueberries are sensitive to temperature, oxygen, light, and water activity. Freeze drying is one of the best ways to dry these compounds. However, spray drying is more practical and economical method of producing dry powders (processing cost is about 30 -50 times less than freeze drying) (Cai et al., (2000). Spray drying involves atomization of feed in the form of spray with the drying medium with moisture removal caused by hot air in the chamber. It is difficult to spray dry sugar rich products like muscadine and blueberry extracts. The problem of stickiness is contributed to by very low glass transition temperatures (Tg) of glucose, fructose, tartaric acid, citric acid etc. It is necessary that the particle temperature should be much below Tg to get a dry, non-sticky powder. As a consequence, high molecular weight compounds (having a high Tg) are added to the spray drying feed to have a successful drying under practical conditions. There is also an added advantage of employing carrier materials, as they provide a protective wall around the feed particle preventing it from oxidation. Recommended solid concentration in the feed and particle size (produced by the atomizer) are 25%, and 5-30 □m respectively to ensure optimal drying. Common outlet drying temperature for sticky materials ranges from 65 – 90°C.

Adhikari et al., (2005) conducted spray drying experiments with sticky materials and found that in the drop surface if the drop surface layerTg is greater than the drop temperature (Td) by 10 C, then the drop surface becomes completely non-sticky. They also introduced a dimensionless time ψ (ratio of time required to enter the safe drying regime to the time needed to achieve the final moisture content) and relate it to powder recovery (np) [$\psi < 1$, np >50%; $\psi = 1$, np=50%; $\psi > 1$, np<50%]. They validated the above fact by spray drying experiments with mixture of carriers and sugars/sugar and acid solutions. They found that pineapple juice (DE 6) (60:40) gave np > 50%. Truong et al., (2004) used a steady state mathematical model to find the optimal spray drying conditions for sugar rich foods. They found that stickiness of sugar-rich foods can be decreased by choosing proper inlet variable with respect to glass transition temperature. Cai et al., (2000) studied the production and properties of spray dried amaranthus betacyanin pigments. They used a range of M (10 – 25DE) and starch as coating agent to spray dry and found that inlet/outlet temp (165 – 180°C/ 92 – 96°C), 20 – 40 % feed content, M 25 and 10 DE gave highest product recovery and stability. Main et al., (1978) conducted experiments on production of spray dried anthocyanin powders using a carrier. They found outlet air temperature < 90°C gave the best yield. They used inlet air temperature of 175°C. Adamopoulus et al., (2005) spray dried tomato pulp by varying inlet air temperature (110 – 140°C), air flow rate (17.50 – 22.75 m^3/h) and compressed air flow rate (500 – 800 L/h). They found that there is an increase in product recovery with increase in the above variables. They also studied the properties of the spray dried tomato pulp and found that powder moisture content decreases with increase in air flow rate and reverse is true for bulk density and solubility.

Figure 2.1. Muscadine grapes

(*adopted from www.crfg.org/pubs/ff/muscadinegrape.html*)

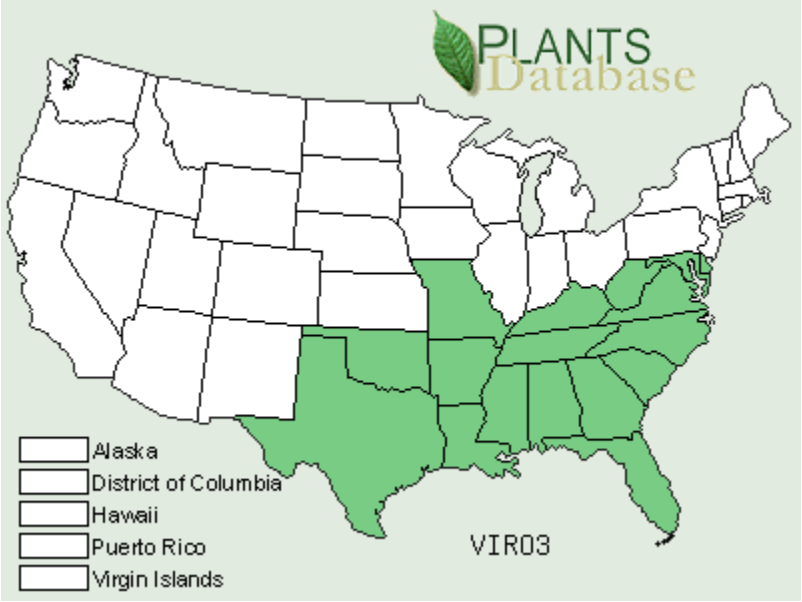


Figure 2.2. The South Eastern growing regions of Muscadine grapes in the USA.

(*adopted from USDA Natural Resources Conservation Service*)

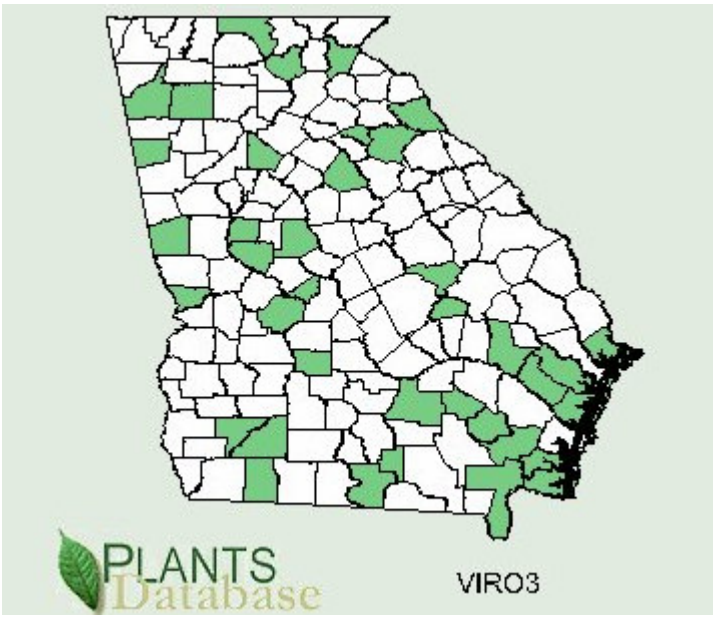


Figure 2.3.The growing regions of Muscadine grapes in the State Of Georgia (*adopted from USDA Natural Resources Conservation Service*)

Figure 2.4. Rabbiteye blueberries

(adapted from http://www.paradisenursery.com/mirbl.html)

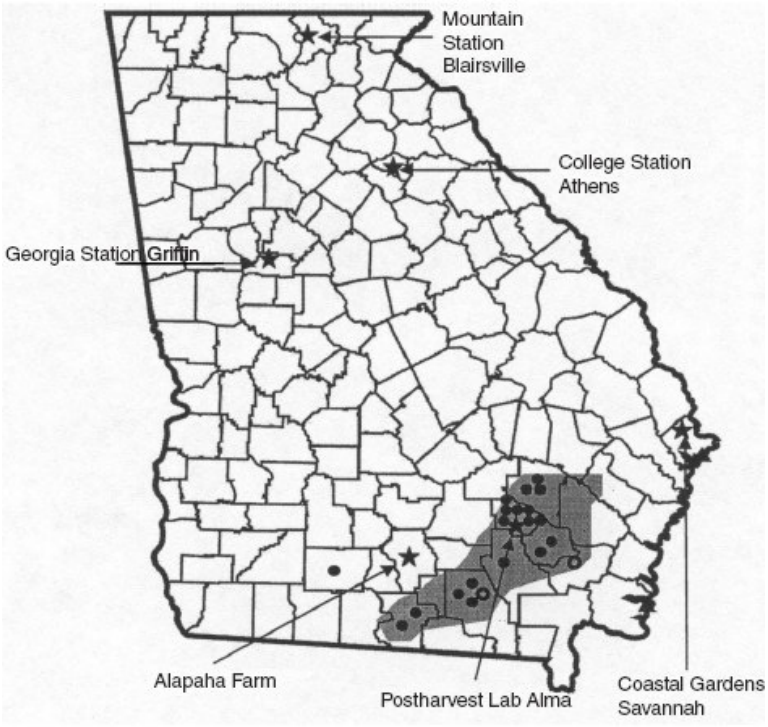


Figure 2.5. Location of the main blueberry growing regions in Georgia (Scherm et al., 2003)

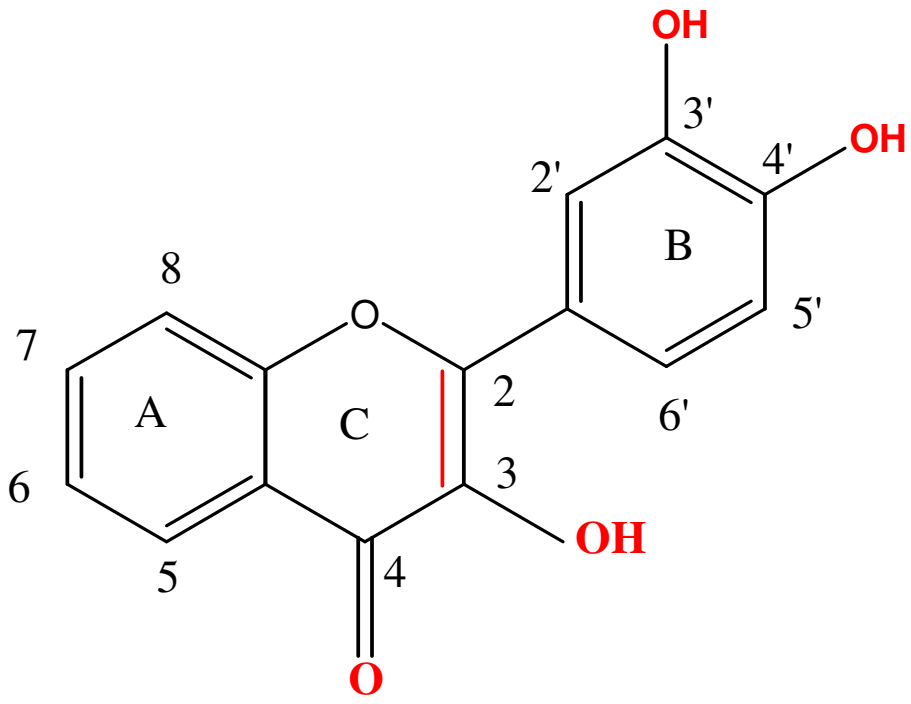
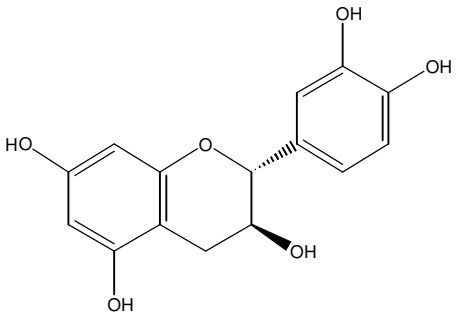
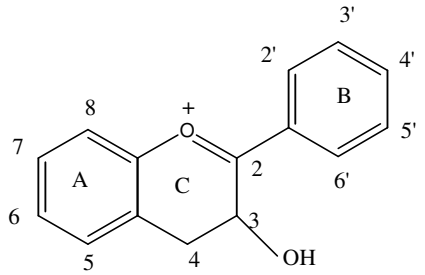


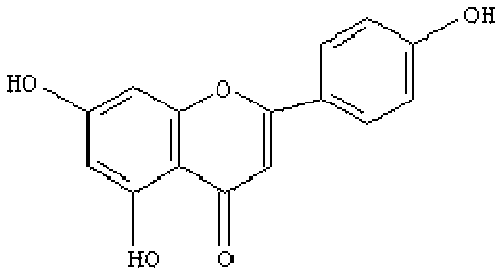
Figure 2.6. Structure of flavan nucleus



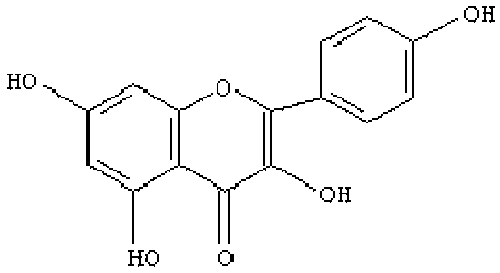
Flavanols (Catechin, Epicatechin)



Anthocyanidin

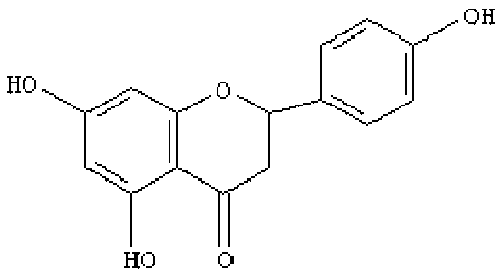


Flavone (Luteolin, Apigenin)



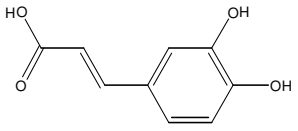
Flavonol (Quercetin, Kaempferol)

Figure 2.7 Structure of different classes of flavonoids

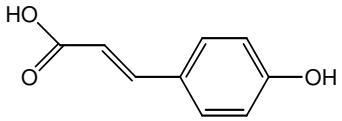


Flavanone (Hesperetin, Naringenin)

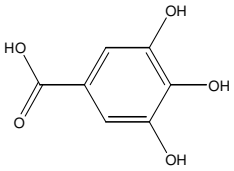
Figure 2.7 (contd.) Structure of different classes of flavonoids



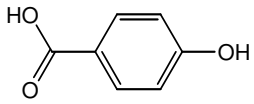
Caffeic acid



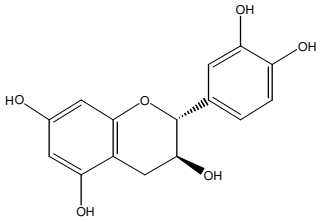
p-coumaric acid



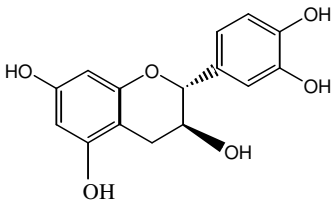
Gallic acid



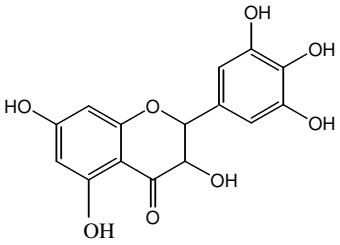
4-hydroxy benzoic acid



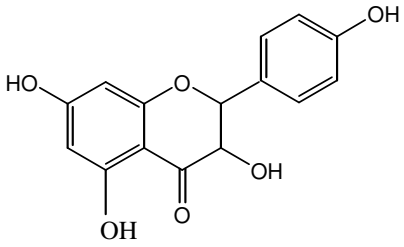
Catechin



Epicatechin



Myrecetin



Kaempferol

Figure 2.8. Structures of some of the phenolic compounds assayed in the study.

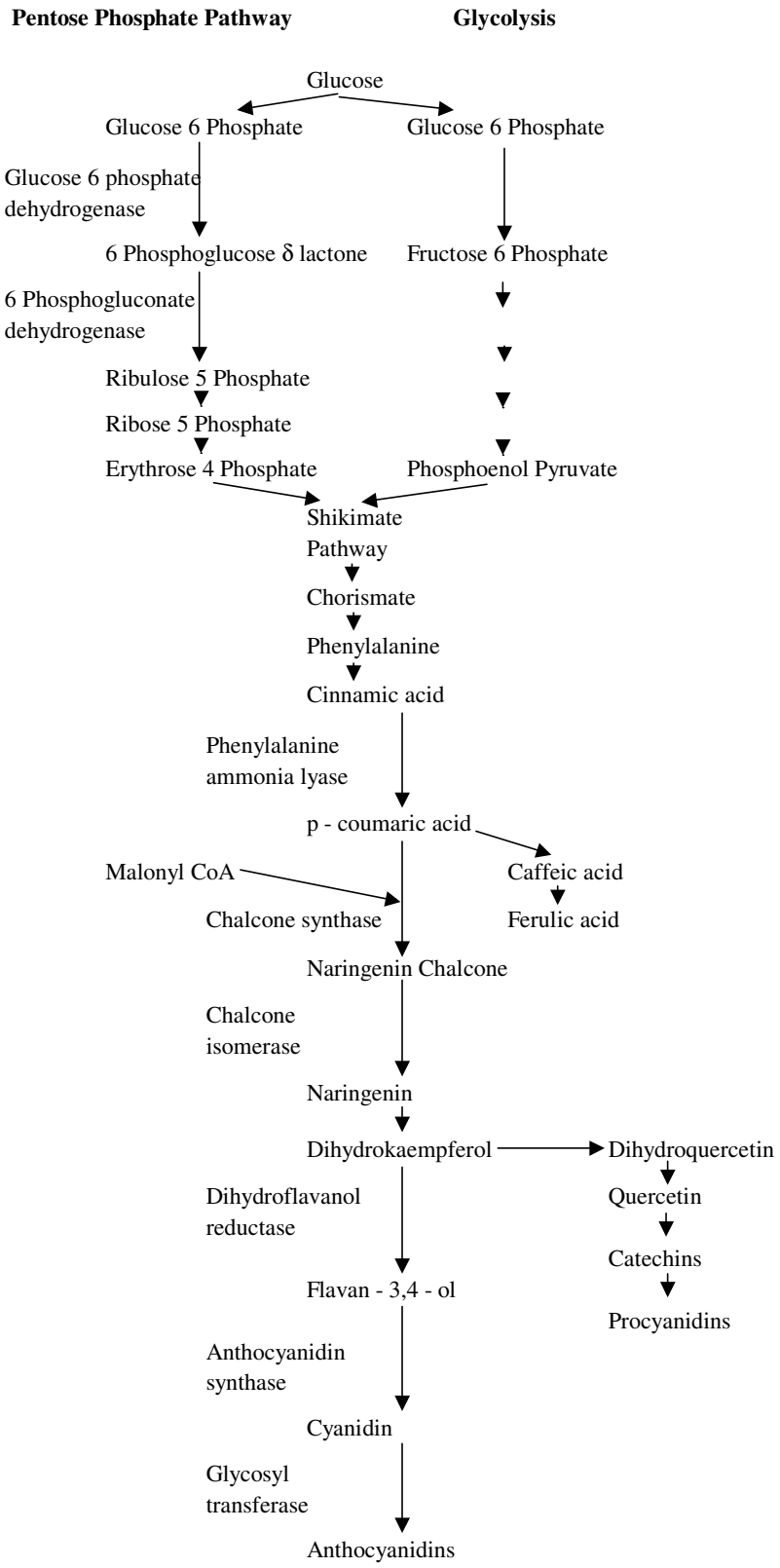


Figure 2.9. Biosynthetic pathways of phenolic compounds

References

Aaby, K., Hvattum, E., and Skrede, G. 2004. Analysis of flavonoids and other phenolic compounds using high-performance liquid chromatography with coulometric array detection: relationship to antioxidant activity. *J. Agric. Food Chem.* **52**:4595-4603.

Adhikari, B., Howes, T., Lecomte, D., and Bhandari, B. R. 2005. A glass transition temperature approach for the prediction of the surface stickiness of a drying droplet during spray drying. *Powder Technol*. **149**:168-179.

Aherne, S.A., Brien, N.M. 2002. Dietary Flavonols: Chemistry, Food Content, and Metabolism. *Nutrition*. **18**:75 – 81.

Antolovich, M., Prenzler, P., Robards, K., and Ryan, D. 2000. Sample preparation in the determination of phenolic compounds in fruits. *Analyst*. **125**:989 – 1009.

Auw, J.M., Blanco, V., O'Keefe, S.F., and Sims, C.A. 1996. Effect of processing on the phenolics and color of cabernet sauvignon, chambourcin, and noble wines and juices. *Am. J. Enol.Vitic* **47**(3):279 – 286.

Awad, M.A., and Jager, A.D. 2003. Influences of air and controlled atmosphere storage on the concentration of potentially healthful phenolics in apples and other fruits. *Postharvest Biol. Technol*. **27**(1):53-58.

Azam, S., Hadi, N., Khan, N.U., and Khadi, S.M. 2004. Prooxidant properties of green tea polyphenols: epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. *Toxicol. In. Vitro*. **18**(5):555 – 561.

Basile, A., Sorbo, S., Giordano, S., Ricciardi, L., Ferrara, S., Montesano, D., Castaldo Cobianchi, R., Vuotto, M. L., and Ferrara, L. 2000 Antibacterial and

allelopathic activity of extract from Castanea sativa leaves. *Fitoterapia*. **71**:S110–S116.

Bautista-Ortín, A.B., Martínez-Cutillas, A., Ros-García, J.M., López-Roca, J.M., and Gómez-Plaza, E. 2005. Improving colour extraction and stability in red wines: the use of maceration enzymes and enological tannins. *Int. J. Food Sci. Tech*. **40**(8):867–878.

Baydar, N. G., Ozkan, G., and Sagdic, O. 2004. Total phenolic contents and antibacterial activities of grape (Vitis vinifera L.) extracts. *Food Control*. **15**:335-339.

Beckman, C.H. 2000. Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defense responses in plants? *Physiol. Mol. Plant Pathol*. **57**:101 – 110.

Benzie, F.F.I., and Strain, J.J. 1999. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. **In** *Methods Enzymol*. **299**:15-27.

Benzie, I.F.F., and Strain, J.J. 2005. Diet and antioxidant defense. *Antioxidants*. 117 – 131.

Bonilla-Pastrana, E., Akoh, C.C., Sellapan, S., and Krewer, G. 2003 Phenolic content and antioxidant capacity of muscadine grapes. *J. Agric. Food. Chem*. **51**:5497 – 5503.

Boulton, R. 2001. The copigmentation of anthocyanins and its role in the color of red wine: A critical review. *Am. J. Enol. Vitic*. **52**(2):67–87.

Boyle, J. A., and Hsu, L. 1990. Identification and quantification of ellagic acid in Muscadine grape juice. *Am. J. Enol. Vitic*. **41**:43-47.

Brouillard, R., and Dangles, O. 1994. Anthocyanin molecular interactions: the first step in the formation of new pigments during wine ageing? *Food Chem*. **51**:365–371.

Cacace, J. E., and Mazza, G. 2003. Optimization of extraction of anthocyanins from black currants with aqueous ethanol. *J. Food Sci*. **68**(1):240-248.

Cai, Y. Z., and Corke, H. 2000. Production and properties of spray-dried Amaranthus betacyanin pigments. *J. Food Sci*. **65**(6):1248-1252.

Cai, Y. Z.,and Corke, H. 2000. Production and properties of spray-dried Amaranthus betacyanin pigments. *J. Food Sci*. **65**(6):1248-1252.

Cao, G., and R. Prior. 1999. Measurement of oxygen radical absorbance capacity in biological samples. In *Methods Enzymol.***299**:50-62.

Chinnici, F., Gaiani, A., Natali, N., Riponi, C., and Galassi, S. 2004. Improved HPLC determination of phenolic compounds in Cv. Golden delicious apples using a monolithic column. *J. Agric. Food Chem* **52**:3-7.

Cirico, T.L., and Omaye, S.T. 2006. Additive or synergetic effects of phenolic compounds on human low density lipoprotein oxidation. *Food Chem. Toxicol*. **44**(4):510 – 516.

Cook, N.C., and Samman, S. 1996. Flavonoids – Chemistry, metabolism, cardioprotective effects, and dietary sources. *Nutr. Biochem*. **7**:66 – 76.

Dauchet, L., Amouyel, P., Hercberg,S., and Dallongeville,J. 2006. Fruit and Vegetable Consumption and Risk of Coronary Heart Disease: A Meta-Analysis of Cohort Studies. *J. Nutr*. **136**:2588-2593.

Douglas, P.L., Luengthanaphol, S., Mongkholkhajornslip, D., Douglas, S., Pengsopa, L., and Pongamphai, S. 2004. Extraction of antioxidants from sweet Thai tamarind seed coat-preliminary experiments. *J. Food Eng*. **63**:247-252.

Ector, B.J., Magee, J.B., Hegwood, C.P., and Coign, M.J. 1996. Resveratrol concentration in muscadine berries, juice, pomace, purees, seeds, and wines. *Am. J. Enol. Vitic*. **47**(1):57 – 62.

Ehlenfeldt, M. K., and Prior, R. L. 2001. Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf tissues of highbush blueberry. *J. Agric. Food Chem*. **49**(5):2222-2227.

Engelhart, M.J., Geerlings, M.I., Ruitenberg, A., Van Swieten, J.C., Hofman, A., Witteman, J.C., and Breteler, M.M. 2002. Dietary intake of antioxidants and risk of Alzheimer disease. *JAMA*. **287**:3223–3229.

Fang, Y-Z., Yang, S., and Wu, G. 2002 Free radicals, Antioxidants, and Nutrition. *Nutrition*. **18**(10):872 – 879.

Fink,B.N., Steck, S.E., Wolff, M.S., Britton, J.A., Kabat, G.C., Gaudet, M.M., Abrahamson, P.E., Bell, P., Schroeder, J.C., Teitelbaum, S.L., Neugut, A.I., Gammon, M.D. 2007. Dietary Flavonoid Intake and Breast Cancer Risk among Women on Long Island. *Am. J. Epidemiol*. **165**(5):514-523.

Flora, L. F. 1978. Influence of heat, cultivar and maturity on the anthocyanin-3,5-diglucosides of muscadine grapes. *J. Food Sci*. **43**:1819-1821.

Friedman, M., Mackey, B. E., Kim, H.-J., Lee, I.-S., Lee, K.-R., Lee, S.-U., Kozukue, E., and Kozukue, N. 2007. Structure-Activity Relationships of Tea Compounds against Human Cancer Cells. *J. Agric. Food Chem*. **55** (2):243 –253.

Fruits and Tree Nuts Outlook/FTS-305/July 30, 2003, Economic Research Service, USDA.

Fuleki, T., and Ricardo-Da-Silva, J. M. 2003. Effects of cultivar and processing method on the contents of catechins and procyanidins in grape juice. *J. Agric. Food Chem*. **51**(3):640-646.

Geleijnse, J.M., Launer, L.J., Van der Kuip, D.A.M., Hofman, A., and Witteman, J.C.M. 2002. Inverse association of tea and flavonoid intakes with incident myocardial infarction: the Rotterdam study. *Amer. J. Clin. Nutr*. **75**:880–886.

Girard, B., and Mazza, G. 1998. Functional Grape and Citrus Products. **In**: Functional Foods: Biochemical and Processing aspects. pp. 139-140. Technomic Publishing Company, Inc., Lancaster, Cambridge.

Giusti, M.M and R.E.Wrolstad. 2001. Characterization and measurement of anthocyanins by UV-visible spectroscopy. **In** current protocols in food analytical chemistry. pp F1.2.1-F1.2.13. Wrolstad, R.E., Acree, T.E., An, H., Decker, E.A., Penner, M. H., Reid, D.S., Schwartz, S.J., Shoemaker, C.F., Sporns, P., Eds. Wiley: New york, NY.

Goula, A. M., and Adamopoulos, K. G. 2005. Spray drying of tomato pulp in dehumidified air: II. The effect on powder properties. *J. Food Eng*. **66**:35-42.

Goula, A. M., and Adamopoulos, K. G. 2005.Spray drying of tomato pulp in dehumidified air: I. The effect on product recovery. *J. Food Eng*. **66**:25-34.

Greenspan, P.; Bauer, J. D.; Pollock, S. H.; Gangemi, J. D.; Mayer, E. P.; Ghaffar, A.; Hargrove, J. L.; Hartle, D. K. 2005. Antiinflammatory Properties of the Muscadine Grape (Vitis rotundifolia).*J. Agric. Food Chem.* **53**(22):8481-8484.

Gris, E.F., Ferreira, E.A., Falcão, L.D., and Bordignon-Luiz, M.T. 2007. Caffeic acid copigmentation of anthocyanins from Cabernet Sauvignon grape extracts in model systems. *Food Chem.* **100**(3):1289–1296.

Hakkinen, S. H., and Torronen, A. R. 2000. Content of flavonols and selected phenolic acids in strawberries and Vaccinium species: influence of cultivar, cultivation site and technique. *Food Res. Int*. **33**:517-524.

Hakkinen, S., Heinonen, M., Karenlampi, S., Mykkanen, H., Ruuskanen, J.; and Torronen, R. 1999. Screening of selected flavonoids and phenolic acids in 19 berries. *Food Res. Int.* **32**:345-353.

Heinonen, I. M., Lehtonen, P. J., and Hopia, A. I. 1998. Antioxidant activity of berry and fruit wines and liquors. *J. Agric. Food Chem*. **46**:25-31

Hertog, M. G. L., Hollman, P. C. H., and Venema, D. P. 1992. Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits. . *J. Agric. Food Chem.* **40**:1591-1598.

Hertog, M.G., Feskens, E.J., Hollman, P.C., Katan, M.B., and Kromhout, D. 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet.* **342**:1007–1011.

Hou, L., Zhou, B., Yang, L., and Liu, Z.L. 2004. Inhibition of human low-density lipoprotein by flavonols and their glycosides. *Chem. Phys. Lipids*. **129**(2):209 – 219.

Howard, L.R., Clark, J.R., and Brownmiller, C. 2003. Antioxidant capacity and phenolic content in blueberries as affected by genotype and growing season. *J. Sci. Food Agric*. **83**(12):1238-1247.

Huang, D., Ou, B., and Prior, R.L. 2005 The chemistry behind antioxidant capacity assays. *J. Agric. Food. Chem.* **53**:1841 – 1856.

Ikigai, H., Nakae, T., Hara, Y., and Shimamura, T. 1993. Bactericidal catechins damage the lipid bilayer, *Biochimica et Biophyshica Acta - biomembranes* . **1147**:132–136.

Joseph, J.A., Shukitt-Hale, B., Denisova, N.A., Bielinski, D., Martin, A., McEwen, J.J., and Bickford, P.C. 1999. Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. *J. Neurosci*. **19**:8114–8121.

Joseph, J.A., Shukitt-Hale, B., and Casadesus, G. 2005. Reversing the deleterious effects of aging on neuronal communication and behavior: beneficial properties of fruit polyphenolic compounds. *Am. J. Clin. Nutr*. **81**:313S–316S.

Joshipura, K.J., Ascherio, A., Manson, J.E., Stampfer, M.J., Rimm, E.B., Speizer, F.E., Hennekens, C.H., Spiegelman, D., and Willett, W.C. 1999. Fruit and vegetable intake in relation to risk of ischemic stroke. *JAMA*. **282**:1233–1239.

Kahkonen, M. P., Hopia, A. I., Vuorela, H. J., Rauha, J.-P., Pihlaja, K., Kujala, T. S., and Heinonen, M. 1999. Antioxidant acitivity of plant extracts containing phenolic compounds. *J. Agric. Food Chem*. **47**(10):3954-3962.

Kammerer, D., Claus, A., Schieber, A., and Carle, R. 2005. A novel process for the recovery of polyphenols from grape (Vitis vinifera L.) pomace. *J. Food Sci.* **70**:157–163.

Kang, S.Y., Seeram, N.P., Nair, M.G., and Bourquin, L.D. 2003. Tart cherry anthocyanins inhibit tumor development in Apc (Min) mice and reduce proliferation of human colon cancer cells. *Cancer Lett* **194**:13–19.

Keinanen, M. 1993. Comparison of methods for the extraction of flavonoids from birch leaves (Betula pendula Roth.) carried out using high-performance liquid chromatography. *J. Agric. Food Chem.* **41**(11):1986-1990.

Keli, S.O., Hertog, M.G., Feskens, E.J. and Kromhout, D. 1996. Dietary flavonoids, antioxidant vitamins, and incidence of stroke: the Zutphen study. *Arch. Intern. Med.* **156**:637–642.

Kennelly, E.J., Einbond, L.S., Reynertson, K.A., Luo, X., and Basile, M.J. 2004. Anthocyanin antioxidants from edible fruits. *Food Chem*. **84**:23-28.

Kidder, F., Rovel, B., Girardin, M., and Metche, M. 1996. Fractionation and identification of the phenolic compounds of highbush blueberries (Vaccinium corymbosum, L.). *Food Chem*. **55**(1):35-40.

Kim, K.H., Lee, K.W., Kim, D.Y., Park, H.H., Kwon, I.B., and Lee, H.J. 2005.Optimal recovery of high purity rutin crystals from the whole plant of Fagopyrum

esculentum Moench (buckwheat) by extraction, fractionation, and recrystallization, Bioresour. Technol. 96(15):1709-1712.

Kim, M.-Y.; Iwai, K.; Onodera, A.; Matsue, H. 2003. Identification and Antiradical Properties of Anthocyanins in Fruits of Viburnum dilatatum Thunb. J. Agric. Food Chem. 51(21): 6173-6177.

Knekt, P., Kumpulainen, J., Jarvinen, R., Rissanen, H., Heliovaara, M., Reunanen, A., Hakulinen, T., and Aromaa, A. 2002. Flavonoid intake and risk of chronic diseases. *Am. J. Clin.Nutr*.. **76**:560–568.

Kowska, A.B., Kucharska, A.Z., and Ski, J.O. 2004. The effects of heating, UV irradiation, and storage on stability of the anthocyanin–polyphenol copigment complex. *Food Chem*.**81**(3):349–355.

Kuhnan, J. 1976. The flavonoids, a class of semi-essential food components: their role in human nutrition. *World Rev. Nutr. Diet*.. **24**:117 – 191.

Lamikanra, O., Grimm, C.C., Rodin, J.B., and Inyang, D.I. 1996. Hydroxylated stilbenes in selected American wines. *J. Agric. Food Chem*. **44**(4):1111 –1115.

Landbo, A.K., and Meyer, A.S. 2001. Enzyme assisted extraction of antioxidative phenols from black currant juice press residues (Ribes nigrum). *J. Agric. Food Chem*. **49**:3169–3177.

Lee, C. Y., Jeong, S.W., and Kim, D. 2003. Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. *Food Chem*. **81**:321-326.

Lee, J., and Wrolstad, R.E. 2004. Extraction of anthocyanins and polyphenolics from blueberry processing waste. *J. Food Sci*. **69**(7):C564-C573.

Lee, J.-H. Talcott, S. T.2004. Fruit Maturity and Juice Extraction Influences Ellagic Acid Derivatives and Other Antioxidant Polyphenolics in Muscadine Grapes. *J. Agric. Food Chem.* **52**(2):361-366

Lee, J-H., Johnson, J.V., and Talcott, S.T. 2005. Identification of Ellagic Acid Conjugates and Other Polyphenolics in Muscadine Grapes by HPLC-ESI-MS. *J. Agric. Food Chem.* **53**(15):6003 –6010.

Lindsay, J., Laurin, D., Verreault, R., Hebert, R., Helliwell, B., Hill, G.B., and McDowell, I. 2002. Risk factors for Alzheimer's disease: a prospective analysis from the Canadian Study of Health and Aging. *Am. J. Epidemiol*. **156**:445–453.

Liyana-Pathiriana, C., and Shahidi, F. 2005. Optimization of extraction of phenolic compounds from wheat using response surface methodology. *Food Chem.* **93**:47 -56.

Lyons, M. M., Yu, C., Toma, R. B., Cho, S. Y., Reiboldt, W., Lee, J., and Van Breemen, R. B. 2003. Resveratrol in raw and baked blueberries and bilberries. *J. Agric. Food Chem.* **51**(20):5867-5870.

Magee, J.B., and Smith, B.J. 2002. Resveratrol Content of muscadine berries is affected by disease control spray program., *HortScience*. **37**(2):358 – 361.

Main, J.H., Clydesdale, F.M., and Francis, F.J. 1978. Spray drying anthocyanin concentrates for use as food colorants. *J. Food Sci*. **43**:1693-1697.

Mayen, M., Merida, J., and Medina, M. 1995. Flavonoid and non-flavonoid compounds during fermentation and post-fermentation standing of musts from cabernet sauvignon and tempranillo grapes. *Am. J. Enol.Vitic*. **46**(2):255-261.

Meyer, A.S., Jepsen, S.M., and Sørensen, N.S. 1998. Enzymatic release of antioxidants for human low-density lipoprotein from grape pomace. *J. Agric. Food Chem.* **46**:2439–2446.

Moyer, A., Hummer, K.E., Finn, C.E., Frei, B., and Wrolstad, R.E. 2002. Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: Vaccinium, Rubus, and Ribes. *J. Agric. Food. Chem.***50**:519–525.

Muscadine grape. 1997. www.crfg.org/pubs/ff/muscadinegrape.html

Musingo, M. N., Sims, C. A., Bates, R. P., O'Keefe, S. F., Lamikanra, O. 2001. Changes in ellagic acid and other phenols in muscadine grape (Vitis rotundifolia) juices and wines. *Am. J. Enol. Vitic*. **52**:109-114.

Naczk, M., and Shahidi, F. 2006. Phenolics in cereals, fruits, and vegetables: Occurrence, extraction and analysis. *J. Pharm. Biomed. Anal*. **41**:1523-1542.

Packer, L. 1999. The Flavonoids: The Healing Power of Plants—Ginkgo Biloba and Pycnogenol **In** The Antioxidant Miracle. Pp. 117-132. John Wiley & Sons Inc., New York, NY.

Pardo, F., Salinas, M. R., Alonso, G. L., Navarro, G., & Huerta, M. D. 1999. Effect of diverse enzyme preparations on the extraction and evolution of phenolic compounds in red wines. *Food Chem*. **67**:135–142.

Pezet, R., Perret, C., Jean-Denis, J. B., Tabacchi, R., Gindro, K., and Viret, O. 2003. Viniferin, a resveratrol dehydrodimer: one of the major stilbenes synthesized by stressed grapevine leaves.. *J. Agric. Food Chem*. **51**(18):5488-5492.

Plants Profile for Vitis rotundifolia (muscadine grapes) In USDA: Natural Resources Conservation Service. 1999. http://plants.usda.gov/java/profile?symbol=VIRO3

–polyphenol copigment complex. *Food Chem*.**81**(3):349–355.

Prior, R., and Gu, L. 2005. Occurrence and biological significance of proanthocyanidins in the American diet. *Phytochem*. **66**(18):2264 – 2280.

Prior, R.L., Cao, G., Martin, A., Sofic, E., McEwen, J., O'Brien, C., Lischner, N., Ehlenfeldt, M., Kalt, W., Krewer, G., and Mainland, C.M. 1998. Antioxidant capacity as influenced by total phenolics and anthocyanins content, maturity, and variety of vaccinium species. *J. Agric. Food. Chem*. **46**: 2686–2693.

Prior, R.L., Lazarus, S.A., Cao, G., Muccitelli, H., and Hammerstone, J.F. 2001. Identification of procyanidins and anthocyanins in blueberries and cranberries (Vaccinium Spp.) using high performance liquid chromatography/mass spectrophotometry. *J. Agric. Food. Chem*. **49**:1270 – 1276.

Prodanov, M.P., Domínguez, J.A., Blázquez, I., Salinas, M.R., and Alonso, G.L. 2005. Some aspects of the quantitative/qualitative assessment of commercial anthocyanin -rich extracts. *Food Chem*. **90**(4):585–596.

Puupponen-Pimia, R., Nohynek, L., Meier, C., Kahkonen, M., Heinonen, M., Hopia, A., and Oksman-Caldentey, K.M. 2001. Antimicrobial properties of phenolic compounds from berries. *J. Appl. Microbiol*.. **90**(4):494-507.

Rauha, J.P., Remes, S., Heinonen, M., Hopia A., Kahkonen M., Kujala T., Pihlaja K., Vuorela H., and Vuorela P. 2000. Antimicrobial effects of Finnish plant

extracts containing flavonoids and other phenolic compounds. *Int. J. Food Microbiol.* **56**:3–12.

Rice-Evans, C.A., Miller, N.J., and Paganda, G. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids, *Free Radical biol. Med.* **20**(7):933 – 956.

Rossi, M., Garavello, W., Talamini, R., La Vecchia, C., Franceschi, S., Lagiou, P., Zambon, P., Maso, L.D., Bosetti, C., and Negri, E. 2006. Flavonoids and risk of squamous cell esophageal cancer. *Int.J. Cancer*. **120**(7):1560-1564.

Rossi, M., Giussani, E., Morelli, R., Scalzo, R. L., Nani, R. C., and Torreggiani, D. 2003. Effect of fruit blanching on phenolics and radical scavenging activity of highbush blueberry juice. *Food Res. Inter*.**36**:999-1005.

Rouseff, R.L., Martin, S.F., and Youtsey, C.O. 1987. Quantitative survey of narirutin, naringin, hesperidin, and neohesperidin in citrus. *J. Agric. Food Chem*.**35**(6): 1027-1030.

Safari, M.R., and Sheikh, N. 2003. Effects of flavonoids on the susceptibility of low density lipoprotein to oxidative modification. *Prostaglandins Leukot. Essent. Fatty Acids.* **69**(1):73 – 77.

Scalbert, A., Morand, C., Manach, C., and Remesy, C. 2002. Absorption and metabolism of polyphenols in the gut and impact on health. *Biomed Pharmacother*. **56**(6):276 – 282.

Scherm, H., and Krewer, G. 2003. Blueberry production in Georgia: Historical Overview and Recent Trends. *Small Fruits Review*. **2**(4):83 – 91.

Schewe, T., and Sies, H. 2005. Myeloperoxidase-induced lipid peroxidation of LDL in the presence of nitrite. Protection by cocoa flavanols. *Biofactors*. **24**(1-4):49 – 58.

Schmidl, M.K., and Labuza, T. 2000. Antioxidants and their effect on health **In** Essentials of functional foods. Pp. 303-317. Aspen Publishers Inc.

Scordino, M., Mauro, A.D., Passerini, A., and Maccarone, E. 2004. Adsorption of flavonoids on resins: Cyanidin – 3 – gluoside. *J. Agric. Food Chem*. **52**:1965–1972.

Sellapan, S., Akoh, C.C., and Krewer, G. 2002. Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. *J. Agric. Food. Chem*. **50**:2432 – 2438.

Sesso, H.D., Gaziano, J.M., Liu, S., and Buring, J.E. 2003. Flavonoid intake and the risk of cardiovascular disease in women. *Amer. J. Clin. Nutr*. **77**:1400–1408.

Shetty, K. 2004. Role of proline-linked pentose phosphate pathway in biosynthesis of plant phenolics for functional food and environmental applications: a review. *Process Biochem.* **39**(7):789-804.

Sims, C. A., and Bates, R. P. 1994. Effects of skin fermentation time on the phenols, anthocyanins, ellagic acid sediment, and sensory characteristics of a red Vitis rotundifolia wine. *Am. J. Enol. Vitic*. **45**(1):56-62.

Singletary, K.W., Stansbury, M.J., Giusti, M., Van Breeman, R., Wallig, M., and Rimando, A. 2003. Inhibition of rat mammary tumorigenesis by concord grape juice constituents. *J Agric Food Chem*. **51**:7280–7286.

Singleton, V.L., and J.A. Rossi. Jr. 1965. Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. *Am. J. Enol. Vitic*. **16**:144-158.

Steffen, Y., Schewe, T., and Sies, H. 2006. Myeloperoxidase-mediated LDL oxidation and endothelial cell toxicity of oxidized LDL: attenuation by (-)-epicatechin. *Free Radic. Res*. **40**(10):076-85.

Stojanovich, J., and Silva, J.L. 2007. Influence of osmotic concentration, continuous high frequency ultrasound and dehydration on antioxidants, color, and chemical properties of rabbiteye blueberries. *Food Chem*. **101**(3):898 – 906.

Su, M-S., and Silva, J.L. 2006. Antioxidant activity, anthocyanins, and phenolics of rabbiteye blueberry (Vaccinium ashei) by-products as affected by fermentation. *Food Chem*. **97**(3):447 – 451.

Sun, A.Y., Simonyi, A., and Sun, G.Y. 2002. The "French Paradox" and beyond: neuroprotective effects of polyphenols. *Free Radic. Biol. Med*. **32**(4):314-318.

Talcott, S. T. Brenes, C. H. Pires, D. M. Del Pozo-Insfran, D. 2003. Phytochemical Stability and Color Retention of Copigmented and Processed Muscadine Grape Juice. *J. Agric. Food Chem.* **51**(4); 957-963

Talcott, S.T., and Lee, J-H. 2002. Ellagic acid and Flavonoid Antioxidant Content of Muscadine Wine and Juice. *J. Agric. Food Chem*. **50**:3186 – 3192.

Talcott, S.T., Brenes, C.H., Pires, D.M., and Pozo-Insfran, D.D. 2003. Phytochemical stability and color retention of copigmented and processed muscadine grape juice. *J. Agric. Food. Chem*.**51**:957 – 963.

Talcott, S.T., Peele, J.E., and Brenes, C.H. 2005. Red clover isoflavonoids as anthocyanin color enhancing agents in muscadine wine and juice. *Food Res. Int.* **38**(10):1205-1212.

Taruscio, T. G., Barney, D. L., and Exon, J. 2004. Content and profile of flavanoid and phenolic acid compounds in conjunction with the antioxidant capacity for a variety of northwest Vaccinium berries. *J. Agric. Food Chem*. **52**:3169-3176.

Taylor, D.,and Larick, D. 1999. Investigations into the effect of supercritical carbon dioxide extraction on the fatty acid and volatile profiles of cooked chicken. *J. Agric. Food Chem*. **43**:2369-2374.

Treutter, D. 2001. Biosynthesis of phenolic compounds and its regulation in apple. *Plant growth Regul*. **34**:71-89.

Truelsen, T., Thudium, D., and Gronbaek, M. Amount and type of alcohol and risk of dementia: the Copenhagen City Heart Study. *Neurology*. **59**:1313–1319.

Truong, V., Bhandari, B. R., and Howes, T. 2005. Optimization of cocurrent spray drying process for sugar-rich foods. Part II--Optimization of spray drying process based on glass transition concept. *J. Food Eng*. **71**(1):66-72.

Verlangieri, A.J., Kapeghian, J.C., el-Dean, S., and Bush, M. 1985. Fruit and vegetable consumption and cardiovascular mortality. *Med Hypotheses*. **16** :7–15.

Walle, T. 2004. Absorption and metabolism of flavonoids. *Free Radic. Biol. Med.* **36**(7):829 – 837.

Yilmaz, Y.; and Toledo, R.T. 2004. Major flavonoids in grape seeds and skins: Antioxidant capacity of catechin, epicatechin, and gallic acid. *J. Agric. Food. Chem*. **52** (2), 255 – 260.

Zhang, Z., Kou, X., Fugal, K., and McLaughlin, J. 2004.Comparison of HPLC methods for determination of anthocyanins and anthocyanidins in bilberry extracts. *J. Agric. Food Chem*. **52**:688-691.

CHAPTER 3

EFFECT OF PECTINASE, HEAT TREATMENT, AND FREEZING ON YIELD OF TOTAL PHENOLICS, TOTAL ANTHOCYANINS, AND ANTIOXIDANT POTENTIAL IN AQUEOUS EXTRACTS FROM GEORGIA-GROWN MUSCADINE GRAPES AND RABBITEYE BLUEBERRIES[1]

[1]Biswas, R., Saalia, F.K., and Phillips, R.D. To be submitted in *LWT-Food Science and Technology*

Abstract

Muscadine grapes and rabbiteye blueberries, grown in Georgia, were analyzed for the effect of pectinase treatment conditions, heat treatment, and freezing on the yield of total phenolics, total anthocyanins, and total antioxidant potential (Ferric reducing/antioxidant power assay, and Oxygen radical absorbance capacity). The total phenolics and total anthocyanins ranged from 17.3 to 21.3 g gallic acid equivalents/Kg db of starting material and 3.91 to 5.57 g cyanidin 3-glucoside equivalents/Kg db of starting material for muscadine grapes and 8.5 to 18.1 g gallic acid equivalents/Kg db of starting material and 0.3 to 2.53 g cyanidin 3-glucoside equivalents/Kg db of starting material for blueberries respectively. The FRAP and ORAC values ranged from 79.7 to 105.1 and 145 to 340.3 mmol TROLOX equivalents/Kg db for muscadine grapes and 47.8 to 86.7 and 56.1 and 231.3 3 mmol TROLOX equivalents/Kg db for blueberries respectively. The maximum yield of total phenolics in both the aqueous extracts was found when they were incubated at 60 C. Whereas, the maximum yield of total anthocyanins were found when the muscadine grape and blueberry extracts were incubated at 25 C and 60 C respectively. The maximum FRAP and ORAC values for both aqueous extracts were obtained when the extracts were incubated at 60 C. Both FRAP and ORAC values were well correlated with total phenolics, but not with total anthocyanins. Freezing didn't improve the yields of polyphenolic compounds over pectinase treatment.

3.1. Introduction

Muscadine grapes (*Vitis rotundifolia* Michx.) are native to the southeastern United States, where the hot, humid climate favors their growth. The berries are larger than most grapes; grow individually rather than in bunchs and have a thick tough skin. The color of the fruit ranges from greenish bronze to bronze, pinkish red, purple, and almost black (California rare fruit growers, Inc, 1999). Georgia now has about 900 - 1,200 acres of commercial muscadine grapes. Typical production is 3-4 tons per acre and may be as much as 8-9 tons per acre under ideal conditions.

Rabbiteye blueberries (*Vaccinium ashei*) are popular all across the South. They vary from small to large sizes and are shiny blue to black in color, and are one of the easiest fruit plantings to grow (Home gardening Rabbiteye blueberries, 1998). In 2004, the Georgia blueberry crop was about 21 million pounds harvested from 4,800 acres (Georgia Crop Estimates, 2005). Muscadine grapes and blueberries are marketed as fresh fruits, juice, jellies, jams, preserves, syrups, dessert toppings, and in the case of muscadine grapes, as wine.

The health benefits of consuming fruits, especially highly colored fruits are well established (Cao et al., 1996; Youdim et al., 2000; Prior et al., 2001; Sun et al., 2002; Jayaprakash et al., 2003; Auroma et al., 2003; Schmidt et al., 2005). Both muscadine grapes and Rabbiteye blueberries are known to be excellent sources of polyphenolic compounds (Sellapan et al., 2002; Pastrana-Bonilla et al., 2003; Talcott et al., 2003; Talcott et al., 2003; Lee et al., 2004; Yilmaz et al., 2004). These secondary metabolites are biologically active compounds (Beckman, 2000) and are derived from the shikimate pathway and phenylpropanoid metabolism. The ability of these compounds to inhibit

platelet aggregation, protect low-density lipoprotein, and ameliorate age-related decline in neuronal and cognitive function have been reported (Kris-Etherton et al., 2002). The health benefits of these compounds are attributed to their unique antioxidant free radical scavenging properties (Rice-Evans et al., 1996; 1997). Polyphenolic compounds ameliorate a broad range of diseases in adult populations including cardiovascular disease by inhibiting platelet aggregation, cancers by preventing damage to DNA, and neuronal and cognitive function loss by preventing inflammation in the brain.

Because polyphenolic antioxidants are efficacious when consumed either as part of the fruit matrix or as isolated compounds, the possibility exists to produce concentrates useful for marketing them as nutraceutical preparations and food ingredients. The first step in such a process would be the efficient extraction of the polyphenolics from their matrix.

The goal of most procedures for extracting polyphenolics from fruits has been as part of an analytical scheme to quantify them or their contribution to antioxidant capacity. Such methods are practiced on a few grams to a few kg of fruits and have used a range of processes such as ultrasonic cell disruption and various solvents including toxic or hazardous ones like methanol (Sellapan et al., 2002; Pastrana-Bonilla et al., 2003; Talcott et al., 2003; Talcott et al., 2003; Yilmaz et al., 2004; Lee et al., 2004; Naczk et al., 2004). The overall goal of this research project is to develop practical technologies for isolating commercial quantities of antioxidants from appropriate grades or byproducts of blueberries and muscadine grapes and ultimately for the preparation of stable, highly concentrated nutraceutical powders. For this reason, we have chosen to use approaches

that should be adoptable by small and medium processors at minimal expense and maximum safety.

The non uniform distribution of the polyphenolic compounds in different parts of the cell complicates the extraction process. Soluble phenolic compounds are located in the cell vacuole whereas lignin, ferulic acid, and flavonoids are located in the cell wall. In many colored fruits, most polyphenolics are located in the outer cells of the skin (Naczk et al., 2004; Antolovich et al., 2000). Juice and wine producers routinely use pectinolytic enzymes to break down cell walls allowing release of phenolics covalently bound to pectin and other cell wall polysaccharides and access by extracting solvents. Reports by Kammerer et al. (2005) showed that optimal extraction of polyphenolic compounds from grape pomace was obtained by using 5000 ppm of a commercial pectinolytic enzyme preparation. Studies by Meyer et al. (1998) showed that solvent extraction of polyphenolic compounds from grape pomace after enzymatic treatments gave higher yields of phenol than without any enzymatic treatment. There have been no such reports of enzymatic treatment of muscadine grapes and rabbiteye blueberries to improve the yields of these compounds from the cell matrix. However, in the light of the above findings, it is hypothesized that there would be a significant effect of pectinolytic enzymatic treatment on the yield of polyphenolic compounds from the muscadine grapes and rabbiteye blueberries. Specifically, we have sought to optimize the pectinolytic enzyme treatment of muscadine grape and rabbiteye blueberry, to examine the effect of freezing and also to investigate the effect of processing on concentrations of polyphenolic compounds from these sources.

3.2 Materials and methods

3.2.1. Fruits

Muscadine grapes (Supreme variety) were obtained as a gift from Paulk vineyards. Blueberries (Brightwell variety) were obtained from a commercial grower in Florida.

3.2.2 Chemicals

Gallic acid, L-ascorbic acid, 6-hydroxy-2, 5, 7, 8-tetramethychroman-2-carboxylic acid (TROLOX); 2, 4, 6-tripyridyl-s-triazine (TPTZ), 6-carboxy fluorescein, and 2,2'-Azobis (2-methylpropionamidine) dihyrochloride (AAPH) were purchased from Sigma (St. Louis, MO). Moisture analysis of the berries was done by a vacumn oven method at 70 C and 25mm Hg for 8 H (AOAC 934.06). Pectinex BE XXL® (Novozyme North America, Inc., Franklinton, NC) was chosen because it was stable at high temperatures.

3.2.3. Experimental design

The Box-Behnken design was used to select the aqueous extraction experiments. The processing parameters for the enzyme assisted aqueous extraction were temperature (25, 45, and 60 C), preheating time (0.5, 1, 2 h) and enzyme incubation time (1, 2, 3 h). A factorial design (3 x 2 x 2 x2) was done to investigate the effects of freezing with factors as incubation time (1, 3, 6 h), amount of enzyme (50, 250 μL), and incubation temperature (25, 60 C) for fresh and frozen blueberries.

3.2.4. Enzyme treatment and aqueous extraction

One hundred grams of frozen berries were thawed under a N_2 (Airgas South Inc. Marietta, GA, USA) atmosphere and macerated intermittently along with 0.2 g ascorbic

acid in a kitchen blender. The macerated berries were pureed for 3 mins, for muscadine grapes and 30 s for Rabbiteye blueberries. The pulp was transferred from the blender to 250 mL screw capped glass bottles by washing with 100 mL of deionized water. The bottles were flushed with N2 gas and preheated for 0.5, 1, and 2 h at 25, 45, and 60 C. An aliquot of 100 μL of (Pectinex BE®) was added to the preheated pulp with stirring, the contents flushed once again with N_2 gas, and then incubated at 25, 45, and 60 C for 1, 2, and 3 h. A wrist action shaker (Model 75, Burrell Corporation, Pitsburg, PA, USA) was used to shake the pulp in the bottles at 25 C, and a reciprocating water bath, at 45, and 60 C. Pectinase-treated pulp samples were cooled to room temperature (25 C) and made up to 250 mL with deionized water in 250 mL volumetric flasks. About 40 mL of the processed pulp in duplicate were transferred to 50 mL round bottom tubes and centrifuged for 15 mins at 20,000 rpm and 25 C. The clear supernatants were collected in vials and stored at -20 C before analysis. The entire experiment was conducted under subdued light.

The factorial design was employed to study the effects of freezing, the amount of pectinase, the incubation time and extraction temperature. Same procedure as above was followed.

3.2.5. Total phenolics

Total phenolics were estimated colorimetrically using the Folin-Ciocalteaux method (Singleton, and Rossi, 1965).A sample aliquot of 200 mL was added to 800 mL of deionized water, 5 mL of Folin-Ciocalteaux reagent, and 4 mL of saturated sodium carbonate solution (75g/L) and mixed on a vortex mixer. The absorbance was measured at 765 nm with a Hewlett Packard 8451A diode array spectrophotometer (Avondale, PA,

USA) after incubation for two hours at room temperature (~25 C). Quantification was based on the standard curve generated with 100, 200, 300, 400, and 500 mg/L of gallic acid. The final concentration of phenolics was calculated based on total volume of extract and initial weight of berries; and expressed as mg/Kg dry weight. The experiment was conducted under subdued light.

3.2.6. Total anthocyanins

Total anthocyanins were measured by the pH differential method (Giusti, and Wrolstad, 2001) using two buffer systems - potassium chloride buffer, pH 1.0 (0.025 M) and sodium acetate buffer, pH 4.5 (0.4 M). Extracts were diluted with water to give Absorbance in the range of 0.1 - 1.2 at 510 nm. Aliquots of 0.2 ml of diluted extracts were mixed with 1.8 mL of each buffer, vortexed and read against a blank at 510 and 700 nm with a Hewlett Packard 8451A diode array spectrophotometer (Avondale, PA, USA). Absorbance was calculated as

$A = (A_{510}\text{ nm} - A_{700}\text{ nm})\text{ pH 1.0} - (A_{510}\text{ nm} - A_{700}\text{ nm})\text{ pH 4.5}$

Monomeric anthocyanin pigment concentration in the extract was calculated as cyanidine-3-glucoside

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

Where A = absorbance, MW = molecular weight (449.2), DF = dilution factor, ε = molar absortivity (29,600), l = path length (1 cm).

The final concentration of anthocyanins was calculated based on total volume of extract and weight of starting fruit, and expressed as mg/Kg dry weight.

3.2.7. Assay of antioxidant capacity

The antioxidant capacity was assayed by Ferric reducing/antioxidant power assay (FRAP) and Oxygen radical-scavenging activity (ORAC).

Ferric reducing/antioxidant power assay (Benzie, and Strain, 1996). FRAP reagent was made by mixing 2.5 mL of 10 mM TPTZ in 40mM HCl , 25 mL of 300 mM acetate buffer, pH 3.6, and 2.5 ml of 20 mM $FeCl_3$.6H20. One hundred mL of the diluted sample was added to 3 mL of the working FRAP reagent, vortexed, and allowed to stand for 10mins. Absorbance was measured at 593 nm with a Hewlett Packard 8451A diode array spectrophotometer (Avondale, PA, USA). Quantification was based on the standard curve generated with 100, 200, 300, 400, and 500 mM of TROLOX. The final concentration was expressed as umol of TROLOX equivalents per Kg dry basis. .

Oxygen radical absorbance capacity (Cao, and Prior, 1999). A stock solution of 6-carboxy fluorescein (1.5 × 10-3 M) was prepared in 75 mM phosphate buffer, pH 7.0. Working solution was prepared by diluting it with 75 mM phosphate buffer, pH 7.0, to a concentration of 1.5 × 10-4 M. AAPH (320 mM, held at 0 C) was prepared in 75 mM phosphate buffer. The reaction mixture containing, 2.625 mL 75 mM phosphate buffer, pH 7.0, 150 mL fluorescein and 150 mL diluted sample, was incubated at 37 °C for 15 mins. To initiate the reaction, 75 mL of AAPH was added to the reaction mixture, vortexed, transferred to thermostated cuvette (37 C) in a spectroflourometer (RF 5301PC Shimadzu scientific instruments Inc., Columbia, MD,USA),and decay of fluorescence measured over time. Optimal excitation (~325 nm) and emission (~520 nm) wavelengths of standard fluorescein solution (1.5 x 10-5 M) were selected daily using

RF-5301PC software. Quantification was based on the standard curve generated with 50, 100, 200, and 300mM of TROLOX. The final concentration was expressed as mmol of TROLOX equivalents per Kg dry basis. The experiment was conducted under subdued light.

3.2.8. Hydrolysis

In order to decrease the number of compounds to be determined, resulting in better resolution and improved characterization of the phenolic compounds, the extracts were acid hydrolysed to completely remove the glycosides. This also eliminated the risk of procuring expensive standards. The hydrolysis method of filtered aqueous extracts was modified from the method described by Hertog, 1992. Two milliliters of the extract were mixed with 10 mL of 6N HCl and 38 mL of methanol and the mixture was flushed with nitrogen, then refluxed at 95 C for two hours. The extract was allowed to cool and was subsequently made up to 100 mL with methanol. Approximately 2 mL of the extract was filtered through 0.45 μm prior to injection.

3.2.9. High pressure liquid chromatography

Twelve individual phenolic compounds were separated and detected by high pressure liquid chromatography. Analysis was done by using Waters (Waters Corporation, Milford, MA) HPLC system comprised of a Waters 717 sample injector, Waters 2695 separations module, and Waters 996 photodiode array detector (PDA) set to monitor the UV spectrum from 240 to 400 nm. The analytical column was heated using a column heater module (Waters Corporation, Milford, MA) to 40 C equipped with a temperature control module (Waters Corporation, Milford, MA). The Waters Millenium[32] software, version 3.05 (Waters Corporation, Milford, MA) was used to

control the HPLC auto sampler, gradient settings, PDA and data acquisition. The column used was a 3.9 by 300 mm μBondapak™ RP C18 (Waters Corporation, Milford, MA). The solvents used were (A) 88% water, 10% HPLC grade methanol, 2% HPLC acetic acid; (B) 50% HPLC grade acetonitrile, 20% HPLC grade methanol, 30% water, adjusted to1% in acetic acid; and (C) 100% HPLC acetonitrile. Water used was double deionized and filtered, by vacuum, through a 0.2 μm nylon filter (Millipore Corporation, Bedford, MA). A stock solution of all the standards was made in HPLC grade methanol. The carousel temperature was maintained at 20 C. The detection wavelengths used were 280 and 320 nm. The gradient used was shown in Table 2.1.

3.2.10. Data Analysis

The response surface regression (RSM) procedure was followed using Statistica® (Statistica ® 2001) Version 6.0 (Statsoft, Inc., Tulsa OK). Experimental data were used to fit a quadratic polynomial equation to obtain the regression coefficients of the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X^2_i + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$$

where β_0, β_i, β_{ii}, β_{ij} are the regression coefficients for intercept, linear, quadratic, and interaction terms respectively. The independent variables and response variables are symbolically represented as Xi, Xj, and Y respectively. The independent variables were temperature; preheat time, and enzyme incubation time subscripted as 1, 2, and 3 respectively. The dependent variables were total phenolics, total anthocyanins, total

antioxidant activity (FRAP), and ORAC. Reduced models were developed using SAS version 8. Statistica software was used to generate 3-D surface and contour plots of dependent variables as function of two independent variables. Correlation studies were also conducted to correlate FRAP and ORAC with TPH and ACY respectively.

3.2.11. Model verification

Optimal conditions were obtained from the predictive equations of RSM. Nine combinations of the independent variables which include points of optimum and non-optimum yields of total phenolics, total anthocyanins, and total antioxidant activity were chosen for the verification experiments. A paired t-test was done to compare the observed and predicted values using Statistica.

3.3. Results and discussions

3.3.1. Total phenolics

Total phenolics (TPH) in pectinase treated muscadine grape extracts ranged from 17.3 to 21.3 g gallic acid equivalents/Kg db of starting material. Moisture content of whole muscadine grapes was determined as 83 %. Research by Pastrana-Bonilla et al. (2003) showed that the average total phenolics in the whole fruit of muscadine grapes of different varieties was 169-310 mg/100g fresh weight for bronze muscadine grapes and 185-426 mg/100g fresh weight (11-25 g/Kg dry matter) for purple varieties. Their results for the Supreme variety (11g/Kg dry matter) were lower than our findings. Research done by Kammerer et al. 2005 showed that pectinolytic and cellulolytic enzyme treatment of comminuted grape pomace increased the yield of phenolic acids by 5 fold and flavonoids and stilbenes 1.7- fold more than the non-enzymatic aqueous extraction. They also found that the yields of anthocyanins improved slightly upon enzyme

treatment. Meyer et al. 1998 showed that there was a significant effect ($p<0.01$) of Grindamyl® pectinase on the yield of phenolics from grape pomace. In the present study, maceration and pectinase treatment were expected to enhance the breakdown of cell walls, and help release polyphenolic compounds from the cell components. Ferulic and p- coumaric acids exist as esters of pectins or may be crosslinked with cell wall polysaccharides. Pectinase treatment may have helped in release of these acids and other polyphenolic compounds from cell vacuole. In the experimental design, the maximum amount (21.3 g/Kg gallic acid equivalents db) of polyphenolics were found to be extracted when pulps were incubated with pectinase at 60 C for three hours (Table 3.2). In the reduced model developed by response surface regression analysis (Table 3.3), enzyme incubation time tested had some linear effect ($p = 0.2001$) on the yield of TPH (Figure 3.1a.). The preheat time also had a linear ($p = 0.2500$) effect on the yield of TPH. There is an interaction effect of enzyme incubation time and preheat time on the yield of TPH. The effect of temperature was also analyzed and the p values for the linear ($p = 0.1012$) and quadratic ($p = 0.0661$) effects of temperature on the yield of TPH was found to be the highest among the other respective components. Increase in the concentrations of the polyphenolic compounds at high temperatures can be due to increase in their solubility, diffusivity coefficients, inactivation of polyphenol oxidase enzyme, and/or decrease in solubility of oxygen. The model has a coefficient of multiple determination as $R^2 = 0.5751$. Overall the model was not significant at 5 % level, indicating that effects of incubation time were weak, having little effect on yield of TPH. From a processors perspective, this is a positive finding, since it provides considerable leeway in designing the process and required equipment.

Total phenolics in pectinase treated blueberry extracts ranged from 8.5 to 18.1 g gallic acid equivalents /Kg db of starting material. The moisture content of Rabbiteye blueberries was determined as 80 % wb. The amounts of total phenolics were found to be within the range reported in the literature (Howard et al., 2003; Kalt et al., 2001; Kalt et al., 2000; Lee et al., 2004;; Su et al., 2005; Taruscio et al., 2004; and Zheng et al., 2003). In the optimized study, we found that TPH was extracted maximally at enzyme incubation temperature of 60 C (Table 3.4). Similar results were found in previously reported literature (Kalt, McDonald, & Donner, 2000). However the enzyme incubation time didn't have any influence on the maximum yield of TPH (Figure 3.2a.). A response surface model was developed ($R^2 = 0.793707$). Then a reduced model was developed incorporating the terms, which were significant ($P<0.5$) in the full model (Table 3.5). The model was affected by enzyme incubation time ($p = 0.2061$). There was a quadratic effect ($p = 0.2840$) of incubation temperature, an interaction effect of incubation temperature and preheat time ($p = 0.2164$), and a significant interaction effect of preheat time and enzyme incubation time ($p<0.05$) on the yield of total phenolics ($p < 0.05$). Enzyme assisted extraction had similar yields of TPH as compared to previously reported literature where there is use of mixture or polar and non-polar solvents (Sellapan et al. 2002). We also studied the effect of freezing on the yield of total phenolics from Rabbiteye blueberries. We hypothesized that the process of freezing and thawing would facilitate the extraction of polyphenolic compounds by breakdown of the cell walls. Our results showed that there is very little difference between fresh and frozen berries as indicated in the Figure 3.2b. The total phenolics amounted to 24.5 and 25.6 g/Kg db for fresh and frozen blueberries respectively, when incubated at 60 C for an hour. Increasing

the amount of pectinase by five times the original amount didn't change the yield of total phenolics significantly as indicated in the Table 3.6.

3.3.2. Total anthocyanins

Total anthocyanins (ACY) in pectinase treated muscadine grape extracts ranged from 3.91 - 5.57 g cyanidin 3- glucoside equivalents/ Kg db of the starting material (Table 3.2). The results were found to be comparable to those previously reported (Pastrana-Bonilla et al., 2003). Optimization experiments were done and a model was developed. The maximum amounts of total anthocyanins were extracted at lower temperatures. However, enzyme incubation time of three hours improved the yield of total anthocyanins (Figure 3.1b.). Anthocyanins are unstable molecules and their stability is increased by copigmentation. Increase in temperatures increase the dissociation of copigmented forms, which subsequently results in loss of anthocyanins. Anthocyanins are located in the skins of muscadine grapes. Increased contact time of pectinase enzyme with macerated fruit improved the breakdown of cell walls, which subsequently exposed the cells to the extracting solvent. Since anthocyanins exist in ionic forms, they readily diffuse into water. Full and reduced ($R^2 = 0.7957$) models were developed for anthocyanins. There were linear effects of incubation temperature, preheat time, and enzyme incubation time on the yield of anthocyanins. Additionally, there was a significant quadratic effect of enzyme incubation time ($p < 0.05$) as well as interaction effects of incubation temperature and enzyme incubation time, and enzyme incubation time and preheat time on the yield of total anthocyanins.

Total anthocyanins in the enzyme assisted aqueous extracts of Rabbiteye blueberries varied from 0.3 to 2.53 g cyanidin 3- glucoside equivalents/ Kg db of the starting material

(Table 3.4). The values were slightly lower than previously reported in the literature (Kalt et al., 2000; Kalt et al., 2001; Howard et al., 2003; Zheng et al., 2003; Lee et al., 2004; Su et al., 2005; Taruscio et al., 2004). Optimization studies were conducted and a response surface model was developed ($R^2 = 0.5419$). Even though the model was not significant, it has a significant lack of fit ($p<0.001$). A reduced model was developed with the parameters (Table 3.5). Contrary to muscadine grape extracts, the maximum amounts of total anthocyanins were extracted at enzyme incubation temperature of 60 C for three hours (Figure 3.2b). High temperature had assisted in the extraction of these compounds. A similar trend was reported for a different variety of blueberry (Kalt et al., 2000). One possible reason could be due to the anthocyanins present in blueberries are thermally stable. Sellappan et al., 2002 reported ACY content for Brightwell variety of Rabbiteye blueberries of 0.82-4.38 g/Kg db; and an average for 12 varieties of 5.68 g/Kg db. The differing values can be explained due to differences in the variety, or differences in the solubility in the extracting solvent. In the reduced model, there is a weak linear effect ($p = 0.2880$) of enzyme incubation time, quadratic effects of preheat time ($p = 0.1582$) and enzyme incubation time ($p = 0.2410$), and a significant interaction (0.02086) between enzyme incubation temperature and enzyme incubation time on the yield of total anthocyanins. There was no significant change in the yield of total anthocyanins with fresh and frozen berries as indicated in the Figure 3.2c. Also increased amount of enzyme didn't improve the yield by a significant amount.

3.3.3. Total antioxidant activity (FRAP and ORAC)

FRAP values of aqueous extracts of muscadine grape varied from 79.7 to 105.1 mmol TROLOX equivalents/Kg db (Table 3.2). Maximum FRAP values were obtained

when aqueous extracts were incubated at 60 C, however, time of incubation didn't affect them (Figure 3.1c). A response surface model with a coefficient of determination as R^2 = 0.780 was developed for FRAP. A reduced model was also developed as shown in Table 3.3. There is a significant linear effect (p = 0.0005) of incubation temperature, preheat time (p=0.1537), and enzyme incubation time (p=0.2575) on the FRAP. The reduced model was found to be significant (p=0.0060), with coefficient of determination as 0.78074. In this study, the correlation coefficient for FRAP vs. TPH was 0.62 and FRAP vs. ACY was 0.001979. The results suggested that the antioxidant activity of the fruit was mainly contributed by the total phenolics. Same results were reported for blueberries (Sellapan et al., 2002). ORAC values of aqueous extracts varied from 145 to 340.3 mmol TROLOX equivalents/Kg db (Table 3.2). Maximum values of ORAC were obtained when the aqueous muscadine grape extracts were incubated with pectinase for three hours at 60 C (Figure 3.1d). A significant response surface model (R^2 = 0.7350) was developed for ORAC. . A reduced model was also developed and was found to be significant (p=0.1279). There were significant linear and quadratic effects of incubation temperature, preheat time, and enzyme incubation time on ORAC as shown in Table 3.3. Correlation studies were conducted and the correlation coefficient for ORAC with TPH and ACY was found as 0.79 and 0.13 respectively. As in the case of the FRAP assay, this indicated that the antioxidant activity is mainly contributed by the total phenolics present in the aqueous extracts of muscadine grape.

The FRAP values of the aqueous extracts of Rabbiteye blueberry varied from 47.8 to 86.7 mmol TROLOX equivalents/Kg db (Table 3.4). Optimization studies were conducted and the optimum value of FRAP was found when incubated at 60 C for three

hours (Figure 3.2c). Response surface model was developed (R^2 = 0.678276). A reduced model was developed and significant linear effects of incubation temperature and preheat time on FRAP were found (Table 3.5). In the correlation studies, the correlation coefficient of TPH with FRAP was 0.504692 and of ACY with FRAP was -0.314765. The overwhelming effect of total phenolics on antioxidant activity is not surprising since the amount of total phenolics was found to be five times more than that of total anthocyanins. The ORAC values varied from 56.1 to 231.3 mmol TROLOX equivalents/Kg db. The ORAC values were comparable with that of the literature (Howard et al., 2003; Kalt et al., 2001; and Lee et al., 2004). Response surface model was developed (R^2 = 0.458408). Correlation studies were conducted and the correlation coefficient of TPH, ACY with ORAC was found as 0.49 and 0.06 respectively.

3.3.4. Model verification

Nine random experiments were performed corresponding to different preheat time, enzyme incubation temperature and enzyme incubation time. Four of them were chosen at the optimum, and five at non-optimum levels obtained from the corresponding response surface and contour plots. The observed and the predicted values of TPH, ACY, FRAP, and ORAC are shown in the Table 3.7 and Table 3.8 for aqueous extracts of muscadine grape and blueberries. The paired t-tests analysis showed that the observed values were not significantly different (at the 0.1 or 5% confidence levels) from predicted values of TPH, ACY, FRAP except ORAC ($p = 0.40$) for the aqueous extracts of muscadine grapes (Table 3.7). For the aqueous extracts of blueberries, the predicted values of ACY, FRAP, ORAC were not significantly different ($p<0.1\%$) from observed values. The TPH values of the aqueous extracts of blueberries were not successfully

predicted. (Table 3.8). These paired t-tests proved the validity and the robustness of the two models (muscadine grapes and blueberries).

3.3.5. Individual phenolics

Twelve different compounds were analyzed by high pressure liquid chromatography for the fifteen different aqueous extracts of muscadine grape. Peaks obtained by the HPLC for the standards and samples are given in Figure 3.5. All the acids were eluted earlier than the flavonoids in the current gradient. The maximum yield of these compounds was located at different points. Gallic acid ranged from 1.22 to 2.12 g/ 100 g fw. Maximum yield was attained at enzyme incubation temperature of 60 C, and a time of one hour and at 25 C at three hours time period. This indicates that the solubility of gallic acid is enhanced with rise in temperature. Our values were higher than previously reported (Pastrana-Bonilla et al., 2003; and Auw et al., 1996). Para -hydroxy benzoic acid ranged from 2.49 to 5.40 mg/100 g fw. Maximum yield of this compound was at incubation temperature of 60 C for time of two to three hours. High temperature assisted in improved extraction of hydroxy benzoic acid. Catechin, ferulic acid and resveratrol ranged from 19.6 to 44.6 g /100 g fw, 9.89 – 24.0 mg/100 g fw, and 0.9 – 5.05 mg/100 g fw respectively. Resveratrol contents were found comparable with the available literature (Ector et al., 1996). All of these compounds were extracted maximum at enzyme incubation temperature and time of 60 C and three hours. Caffeic acid ranged from 1.03 to 2.46 mg/100 g fw and maximum was extracted at 45 C, three hours. This indicated that the compound was not thermally stable. Epicatechin ranged from 29.1 to 107.6 mg/100 g fw which is similar / slightly higher than previously reported literature (Pastrana-Bonilla et al., 2003; Auw et al., 1996). The maximum yield of this compound

was found at 40 to 45 C. However, enzyme incubation time didn't affect the yield of this compound. Para-coumaric acid ranged from 0.3 to 3.40 mg/100 g fw. The maximum yield was found at 60 C, three hours. High temperatures and increased length of enzyme incubation time had improved the yield of this compound. Ellagic acid ranged from 1.57 to 4.30 mg/100 g fw. The maximum yield was attained after incubation at 45 C for two hours. Quercetin ranged from 0 to 8.78 mg/100 g fw. Optimal yield of quercetin in the extracts of muscadine grape was attained at 60 C, three hours. Kaempferol varied from 1.985 to 9.984 mg/100 g fw in the aqueous extracts of muscadine grapes. Our values were slightly higher than reported in the literature (Pastrana-Bonilla et al., 2003; Auw et al., 1996). Application of pectinase has helped in the increased yield of individual phenolic compounds by releasing them in the solvent by lossening the cells. The data indicated that most of the individual phenolic compounds were extracted maximum at 60 C, time of enzyme incubation varied from 1 to 3 hours.

Individual phenolic compounds were analyzed by high pressure liquid chromatography for the aqueous extracts of blueberries. Gallic acid ranged from 3.87 to 4.45 g/100 g fw in the enzyme assisted aqueous extracts of blueberry. There were two regions for gallic acid, at 25 C, three hours and at 60 C, one hour in the aqueous extracts of blueberry where the maximum amount was observed. This indicated that increasing temperature had improved the efficiency of extraction. Ferulic acid ranged from 0.016 to 0.019 g/100 g fw. Maximum yield was attained at enzyme incubation temperature of 60 C for three hours. Catechin ranged from 20.3 to 27.6 g/100 g fw. Maximum yield was attained around enzyme incubation temperature of 45 to 60 C, for a time period of 2 to 3 hours. Caffeic acid ranged from 0 to 0.056 g/100 g fw. Maximum yield was attained

around enzyme incubation temperature of 45 to 60 C, for a time period of 3 hours. Epicatechin ranged from 0.078 to 0.136 g/100 g fw in the aqueous extracts of blueberries. Optimum yield was attained around enzyme incubation temperature of 45 C. However, time of incubation didn't affect at all. Ellagic acid ranged from 0.007 to 0.024 g/100 g fw in the aqueous extracts of blueberries. The optimum yield was attained at enzyme incubation temperature ranging from 45 to 60 C, and time of two hours. Resveratrol ranged from 0.0006 to 0.0017 g/100g fw. Maximum yield was attained at enzyme incubation temperature of 60 C (three hours). Quercetin and Kaempferol ranged from 0.002 to 0.013 and 0.006 to 0.014 g/100 g fw respectively. All the compounds except catechin were less than previously reported in the literature (Sellapan et al., 2002). The study indicated that pectinase treatment didn't have a significant effect on the yield of individual phenolic compounds. The research leads to subsequent extraction of the pectinase treated extracts with solvents other than water. We plan to do our next study on the use of a different solvent other than water to improve the extraction process.

3.4. Significance

The study gave new information to the field of nutraceuticals. The treatment of the aqueous extracts of muscadine grapes and blueberries with pectinase enzyme and application of heat improved the yields of total phenolics, total anthocyanins and the total antioxidant potential indicating possible industrial processing conditions. We also learnt that freezing didn't facilitate the extraction of polyphenolic compounds from the fruits.

3.5. Acknowledgments

This research was supported by the Georgia Traditional Industries/Food Processing Advisory Council Program. We also thank Paulk Vinyards for providing us financial support, valuable advice, and raw materials.

Table 3.1.

Linear gradient of the solvents used in HPLC.

Time	Flow (mL/min)	%A	%B	%C
0	1	100	0	0
5	1	100	0	0
10	1	90	10	0
15	1	80	20	0
20	1	75	25	0
25	1	70	30	0
30	1	10	90	0
35	1	10	90	0
40	1	80	20	0
45	1	0	0	100
50	1	0	0	100
55	1	100	0	0
60	1	100	0	0

(A) 88% water, 10% HPLC grade methanol, 2% HPLC acetic acid;

(B) 50% HPLC grade acetonitrile, 20% HPLC grade methanol, 30% water, adjusted to1% in acetic acid; and

(C) 100% HPLC acetonitrile.

Table 3.2.

Total phenolics (TPH), total anthocyanins (ACY), antioxidant activity- FRAP and ORAC - in enzyme treated aqueous extracts of muscadine grapes.

Sample	Enzyme incubation temp (C)	Heating time (h)	Enzyme incubation time (h)	TPH (g/Kg db)	ACY (g/Kg db)	FRAP (mmol/Kg db)	ORAC (mmol/Kg db)
1	25	0.5	2	18.5	4.97	79.7	145.0
2	60	0.5	2	18.7	4.46	95.6	168.7
3	25	2	2	18.0	4.24	83.7	169.3
4	60	2	2	20.1	4.33	104.3	251.6
5	25	1	1	17.6	5.06	79.9	199.3
6	60	1	1	20.0	5.08	104.9	258.9
7	25	1	3	18.0	5.96	84.0	162.5
8	60	1	3	21.3	4.80	105.1	340.3
9	45	0.5	1	20.7	5.57	102.4	240.9
10	45	2	1	16.6	4.00	82.8	161.8
11	45	0.5	3	17.3	5.38	93.2	183.5
12	45	2	3	18.1	4.62	91.4	177.5
13	45	1	2	18.3	4.71	88.0	200.3
14	45	1	2	17.4	4.62	83.5	177.4
15	45	1	2	15.3	3.91	97.2	168.7

Table 3.3.

Regression coefficients of predicted quadratic polynomial models for the response total phenolics (TPH), total anthocyanins (ACY), total antioxidant activity (FRAP and ORAC) of muscadine grapes.

Coefficient	TPH		ACY		FRAP		ORAC	
		p		p		p		p
β_0	28.8179	0.0005	6.75184	0.0006	79.107	<0.0001	451.603	0.046
Linear								
β_1	-0.37346	0.1012	0.02245	0.3092	0.581	0.0005	-9.65584	0.1645
β_2	-5.2504	0.25	-1.04204	0.0583	12.1573	0.1537	127.808	0.2854
β_3	-1.55263	0.2001	-1.75969	0.0779	-5.57105	0.2575	199.736	0.0769
Quadratic								
β_{11}	0.00507	0.0661	-	-	-	-	0.10518	0.1688
β_{22}	-	-	-	-	-	-	-49.65	0.2754
β_{33}	0.85385	0.5744	0.5885	0.0106	-	-	32.6958	0.1542
Crossproduct					-	-		
β_{12}	-	-	-	-	-	-	1.58434	0.1993
β_{13}	-	-	-0.0174	0.1167	-	-	-	-
β_{23}	1.35789	0.1653	0. 25481	0.2851	5.36316	0.1791	18.5421	0.5664
F	1.8	0.2154	5.19	0.0183	6.99	0.006	2.47	0.1279
Rsquare	0.5751		0.7957		0.78074		0.7119	

The coefficients were related to the following variables,

$\beta_{1,}$ β_2, and β_3 : Regression Coefficients for linear Incubation Temperature, Preheat time, and Enzyme incubation time.

$\beta_{11,}$ β_{22}, and β_{33} : Regression Coefficients for quadratic Incubation Temperature, Preheat time, and Enzyme incubation time.

$\beta_{12,}$ β_{13}, and β_{23} : Regression Coefficients for interaction of Incubation Temperature and Preheat time, Incubation Temperature and Enzyme incubation time, PreheatTime and Enzyme incubation Time.

Table 3.4.

Total phenolics (TPH), total anthocyanins (ACY), antioxidant activity- FRAP and ORAC in enzyme treated aqueous extracts of Rabbiteye blueberries.

Sample	Enzyme incubation temp (C)	Heating time (h)	Enzyme incubation time (h)	TPH (g/Kg db)	ACY (g/Kg db)	FRAP (mmol/Kg db)	ORAC (mmol/Kg db)
1	25	0.5	2	11.4	1.70	63.5	124.0
2	60	0.5	2	18.1	0.30	86.7	184.5
3	25	2	2	11.0	1.51	57.2	156.0
4	60	2	2	12.0	2.28	57.2	88.2
5	25	1	1	9.8	1.56	47.8	141.1
6	60	1	1	16.1	2.53	54.4	129.4
7	25	1	3	13.1	1.58	53.4	74.0
8	60	1	3	17.6	3.22	64.1	231.3
9	45	0.5	1	16.7	1.79	53.7	213.4
10	45	2	1	8.5	1.62	54.8	59.7
11	45	0.5	3	9.4	1.81	65.8	56.1
12	45	2	3	16.6	1.94	86.1	76.9
13	45	1	2	14.0	1.78	57.4	343.8
14	45	1	2	14.5	1.80	65.9	144.2
15	45	1	2	9.2	1.80	58.2	85.0

Table 3.5.

Regression coefficients of predicted quadratic polynomial models for the response total phenolics (TPH), total anthocyanins (ACY), total antioxidant activity (FRAP) of rabbiteye blueberries.

Coefficient	TPH		ACY		FRAP	
		p		P		p
β_0	19.66852	0.0385	2.06858	0.2896	75.76889	0.0060
Linear						
β_1	-0.10406	0.7515	-0.01630	0.5493	0.29411	0.1312
β_2	0.14594	0.4422	1.45975	0.4476	61.77519	0.0490
β_3	-6.07402	0.2061	-1.35460	0.2880	-1.14737	0.8760
Quadratic					-	-
β_{11}	0.00414	0.2840	-	-		
β_{22}	-	-	-0.93616	0.1582	17.71639	0.0907
β_{33}	-	-	0.37146	0.2410	-	-
Crossproduct					-	-
β_{12}	-0.10303	0.2164	-	-	-	-
β_{13}	-	-	0.02680	0.02086	-	-
β_{23}	4.65263	0.0088	-	-	7.54211	0.2196
F	4.50	0.0274	1.42	0.3144	2.74	0.0896
Rsquare	0.7714		0.5159		0.6036	

The coefficients were related to the following variables,

β_1, β_2, and β_3 : Regression Coefficients for linear Incubation Temperature, Preheat time, and Enzyme incubation time.

β_{11}, β_{22}, and β_{33} : Regression Coefficients for quadratic Incubation Temperature, Preheat time, and Enzyme incubation time.

β_{12}, β_{13}, and β_{23} : Regression Coefficients for interaction of Incubation Temperature and Preheat time, Incubation Temperature and Enzyme incubation time, PreheatTime and Enzyme incubation Time.

Table 3.6.

Effect of different concentrations of pectinase on the yield of total phenolics from rabbiteye blueberries when incubate at 60 C for 1, 3, or 6 h.

Pectinase/ Fruit (μL/g)	Total Phenolics (mg/Kg db)		
	1 h	3 h	6 h
50	25.6	27.5	28.2
250	26.9	27.5	28.8

Table 3.7

Comparison of the observed (O) and predicted (P) values of Total Phenolics, Total anthocyanins, and Total antioxidant capacity (FRAP and ORAC) in aqueous extracts of muscadine grapes.

Preheat Time (h)	Temperature (°C)	Enzyme Incubation Time (h)	Total phenolics (mg/Kg db)		Anthocyanin (mg/Kg db)		FRAP (mmol/Kg db)		ORAC (mmol/Kg db)	
			O	P	O	P	O	P	O	P
1.6	28	16.9	17.6	7.6	2.08	4.77	31.9	36.7	122	125
1.1	30	64.3	17.5	10.9	1.7	4.58	33	53	153	180
0.6	35	43.2	18.1	11.9	1.58	4.84	35.2	61.8	170	165
0.7	37	31.5	18.1	13.5	1.37	5.29	34.4	54	164	167
1.4	41	27.8	20.2	10.3	1.54	4.42	38.8	48.5	194	158
0.9	44	20.5	17.6	13.3	1.36	4.9	34	53.7	164	176
1.7	48	39.3	18.6	8.9	1.27	4.67	38.1	42.1	121	67
0.8	52	51.4	19	14.9	1.42	4.6	38.8	63	193	197
1.3	56	55.6	20.9	13.7	2.49	4.5	43.4	53.3	182	161

Table 3.8.

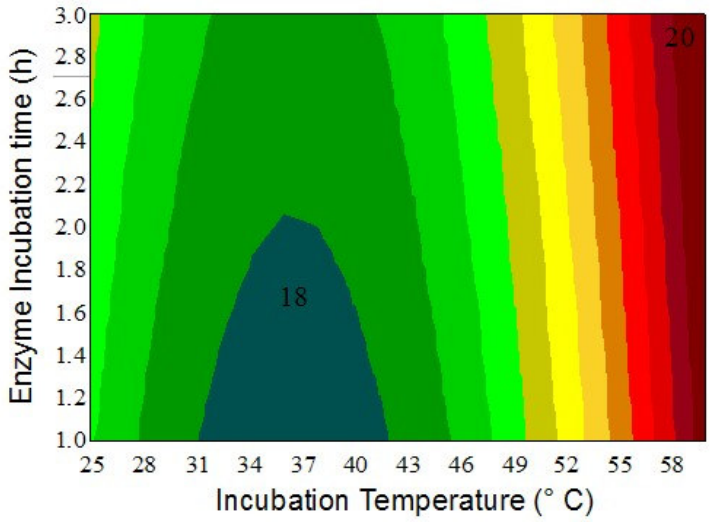
Comparison of the observed (O) and predicted (P) values of Total Phenolics, Total anthocyanins, and Total antioxidant capacity (FRAP) in aqueous extracts of rabbiteye blueberries.

Preheat Time (h)	Temperature (°C)	Enzyme Incubation Time (h)	Total phenolics (mg/Kg db)		Anthocyanin (mg/Kg db)		FRAP (mmol/Kg db)	
			O	P	O	P	O	P
1.6	28	16.9	9.7	16.4	1.21	2.23	20.6	23.4
1.1	30	64.3	11.1	15.3	1.32	1.71	21.6	18.6
0.6	35	43.2	10.6	13.4	1.33	1.37	22.2	13.5
0.7	37	31.5	11.1	16.4	1.44	1.87	22.2	14.2
1.4	41	27.8	11.7	17.2	1.33	2.01	21.5	22.0
0.9	44	20.5	12.1	17.1	1.53	1.9	24.4	16.4
1.7	48	39.3	12.5	17.5	1.52	2.27	25.1	25.5
0.8	52	51.4	13.5	17.5	1.41	1.65	22.5	15.9
1.3	56	55.6	14.2	19.5	1.68	2.18	26.1	21.4

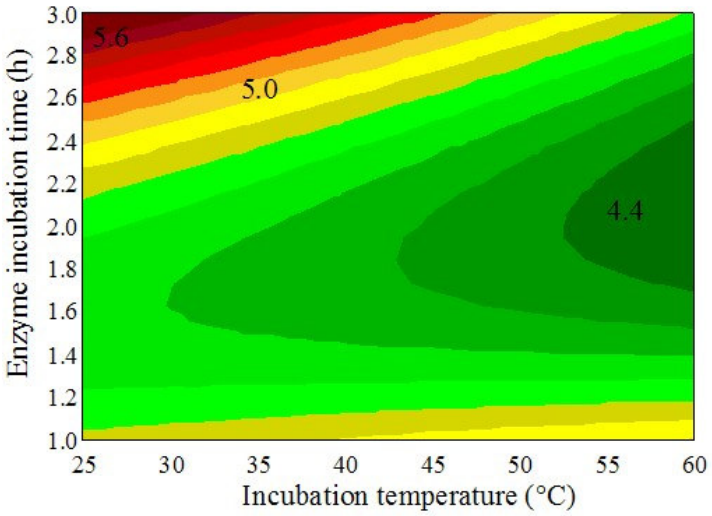
Table 3.9.

Paired t-tests analysis of the observed and predicted values of total phenolics (TPH), anthocyanins (ACY), antioxidant activity (FRAP, ORAC) in the aqueous extracts of muscadine grapes and rabbiteye blueberries.

Difference	DF	t-value	p	DF	t-value	p
	Muscadine			Blueberry		
TPH obs - TPHpre	8	8.61	<.0001	8	-0.26	0.8035
ACYobs - ACYpre	8	16.76	<.0001	8	5.04	0.001
FRAP obs – FRAP pre	8	-5.27	5E-04	8	-10.21	<0.0001
ORAC obs – ORAC pre	8	0.89	0.402	8	4.53	0.0019

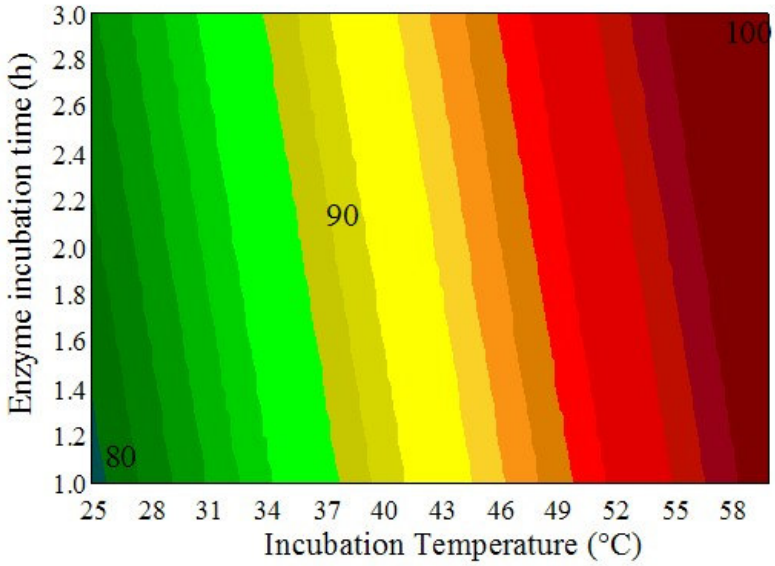


(a)

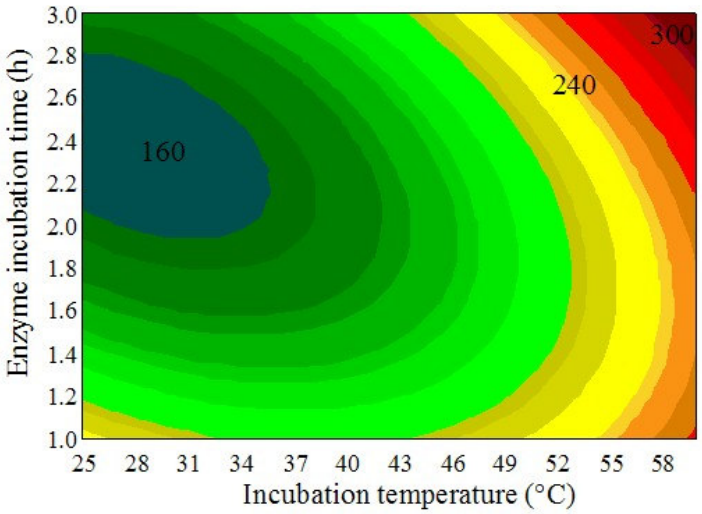


(b)

Fig. 3.1. Response surface for the effects of enzyme incubation temperature and time on the yield of (a) total phenolics expressed as gallic acid equivalents in g/Kg db, and (b) total anthocyanins expressed as cyanidin–3 glucoside equivalents in g/Kg db

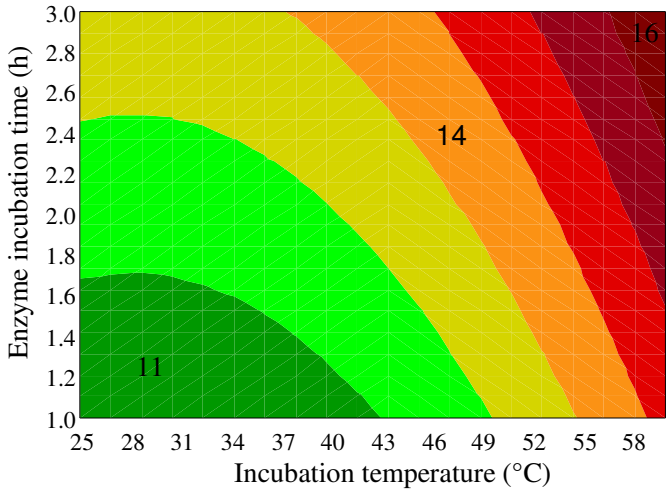


(c)

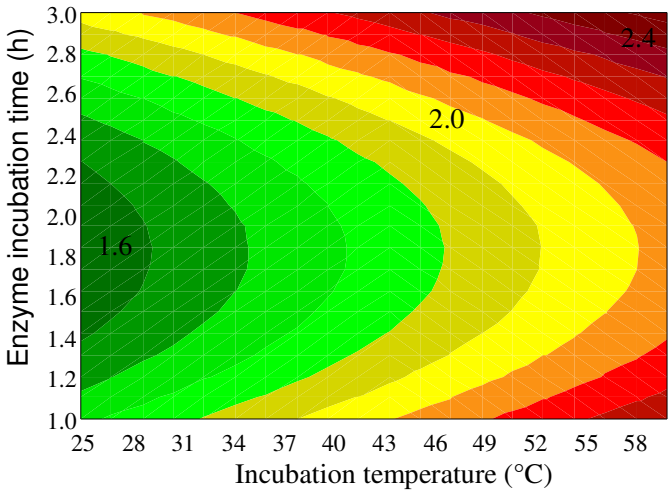


(d)

Fig. 3.1 (contd.). Response surface for the effects of enzyme incubation temperature and time on the yield of (c) total antioxidant activity (FRAP) expressed as trolox equivalents in mmol/Kg db, (d) total antioxidant activity (ORAC) expressed as trolox equivalents in mmol/Kg db in aqueous extracts of muscadine grapes.

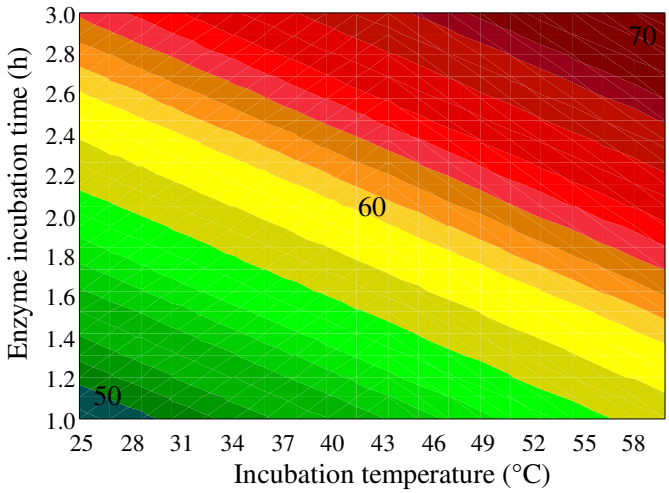


(a)



(b)

Fig. 3.2. Response surface for the effects of enzyme incubation temperature and time on the yield of (a) total phenolics expressed as gallic acid equivalents in g/Kg db, and (b) total anthocyanins expressed as cyanidin–3 glucoside equivalents in g/Kg db.



(c)

Fig. 3.2 (contd.). Response surface for the effects of enzyme incubation temperature and time on the yield of (c) total antioxidant activity (FRAP) expressed as trolox equivalents in mmol/Kg db in aqueous extracts of Rabbiteye blueberries.

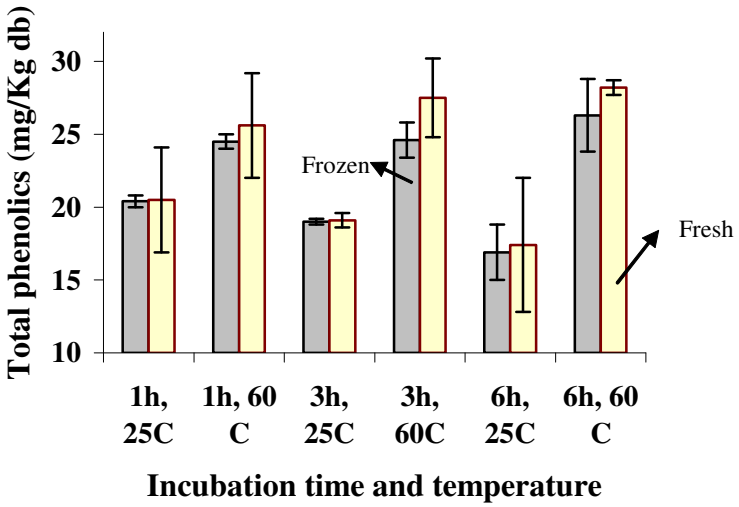


Fig. 3.3. Effect of freezing on the yield of total phenolics from rabbiteye blueberries.

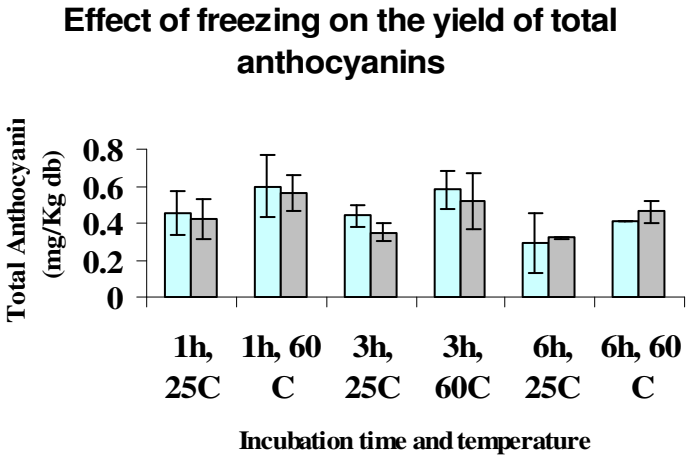


Fig. 3.4. Effect of freezing on the yield of total anthocyanins from rabbiteye blueberries

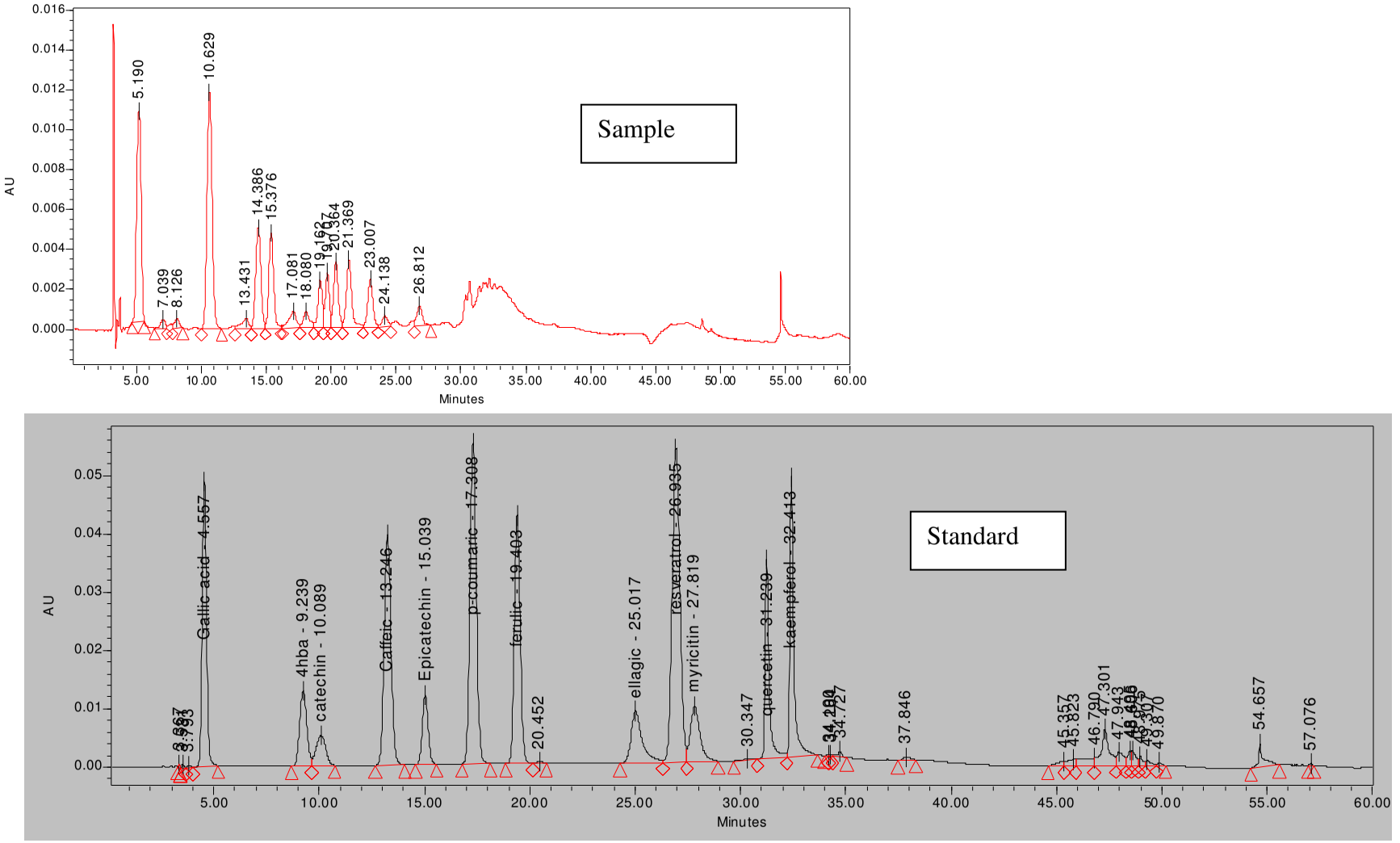


Fig. 3.5. HPLC chromatogram of twelve individual phenolic compounds (Sample and Standard).

References

Antolovich, M., Prenzler, P., Robards, K., & Ryan, D. (2000). Sample preparation in the determination of phenolic compounds in fruits. Analyst, 125, 989-1009.

Auroma, O.I., Bahorun, T., & Jen, L-S. (2003) Neuroprotection by bioactive components in medicinal and food plant extracts. Mutation research, 544, 203-215.

Auw, J.M., Blanco, V., O'Keefe, S.F., & Sims, C.A. (1996). Effect of processing on the phenolics and color of cabernet sauvignon, chambourcin, and noble wines and juices. American Journal Of Enology and Viticulture, 47 (3), 279 – 286.

Beckman, C.H. (2000). Phenolic storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? Physiological and molecular plant pathology, 57, 101-110.

Benzie, F.F.I., & Strain, J.J. (1996). The Ferric reducing ability of plasma as a measure of "antioxidant power" the FRAP assay. Analytical biochemistry, 239, 70 – 76.

California Rare Fruit Growers, Inc. (1999). http://www.crfg.org/pubs/ff/muscadinegrape.html.

Cao, G., & Prior, R. (1999). Measurement of oxygen radical absorbance capacity in biological samples. In Methods in enzymology, 299, 50-62.

Cao, G., Sofic, E., & Prior, R.L. (1996). Antioxidant capacity of tea and common vegetables. Journal of Agricultural and Food Chemistry, 44(11), 3426-3431.

Ector, B.J., Magee, J.B., Hegwood, C.P., & Coign, M.J. (1996). Resveratrol concentration in muscadine berries, juice, pomace, purees, seeds, and wines. American journal of Enology and Viticulture, 47 (1), 57-62.

Georgia crop Estimates, (2005). http://www.nass.usda.gov/ga/estpages/crops.htm.

Giusti, M.M., & R.E.Wrolstad. (2001). Characterization and measurement of anthocyanins by UV-visible spectroscopy. In current protocols in food analytical chemistry, Wrolstad, R.E., Acree, T.E., An, H., Decker, E.A., Penner, M. H., Reid, D.S., Schwartz, S.J., Shoemaker, C.F., Sporns, P., Eds., Wiley: New york, pp F1.2.1-F1.2.13.

Home gardening Rabbiteye blueberries (1998). http://www.aces.edu/pubs/docs/A/ANR-1078/ANR-1078.pdf.

Howard, L.R., Clark, J.R., & Brownmiller, C. (2003). Antioxidant capacity and phenolic content in blueberries as affected by genotype and growing season. Journal of the science of food and agriculture, 83, 1238-1247.

Howard, L.R., Clark, J.R., & Brownmiller, C. (2003). Antioxidant capacity and phenolic content in blueberries as affected by genotype and growing season. Journal of the science of food and agriculture, 83, 1238-1247.

Jayaprakash, G.K., Selvi, T., & Sakariah, K.K. (2003) Antibacterial and antioxidant activities of grape (Vitis vinifera) seed extracts. Food Research International, 36, 117-122.

Kalt, W., McDonald, J.E., & Donner, H. (2000). Anthocyanins, Phenolics, and Antioxidant capacity of processed lowbush blueberry products. Journal of Food Science, 65 (3), 390 – 393.

Kalt, W., Ryan, D.A.J., Duy, J.C., Prior, R.L., Ehlenfeldt, M.K., & Kloet, S.P.V. (2001). Interspecific variation in anthocyanins, phenolics, and antioxidant capacity among genotypes of highbush and lowbush blueberries (Vaccinium section cyanococcus spp.). Journal of agricultural and food chemistry, 49, 4761-4767.

Kammerer, D., Claus, A., Schieber, A., & Carle, R. (2005). A novel process for the recovery of polyphenols from grape (Vitis vinifera L.) pomace. Journal of Food Science, 70(2), C157-C163.

Kris-Etherton, P.M., hecker, K.D., Bonanome, A., Coval, S.M., Binkoski, A.E., Hilpert, K.F., Griel, A.E., & Etherton, T.D. (2002). Bioactive Compounds in Foods: Their Role in the prevention of cardiovascular disease and Cancer. The American Journal of Medicine, 113(9), 71-88.

Lee, J., & Wrolstad, R.L. (2004). Extraction of anthocyanins and polyphenolics from blueberry processing waste. Journal of food science, 69(7), C564-C573.

Lee, J.-H., & Talcott, S. T. (2004) Fruit Maturity and Juice Extraction Influences Ellagic Acid Derivatives and Other Antioxidant Polyphenolics in Muscadine Grapes Journal of agricultural and food chemistry, 52(2), 361-366

Meyer,A.S., Jepsen,S.M., & Sorensen,N.S. (1998). Enzymatic release of antioxidants for human low-density lipoprotein from grape pomace. Journal of Agricultural and Food Chemistry, 46(7), 2439-2446.

Naczk, M., & Shahidi, F. (2004). Extraction and analysis of phenolics in food. Journal of Chromatography A, 1054, 95-111.

Pastrana-Bonilla, E., Akoh, C. C., Sellappan, S., & Krewer, G. (2003). Phenolic Content and Antioxidant Capacity of Muscadine Grapes. Journal of Agricultural and Food Chemistry, 51(18), 5497-5503.

Prior, R.L., Lazarus, S.A., Cao, G., Muccitelli, H., & Hammerstone, J.F. (2001). Identification of procyanidins and anthocyanins in blueberries and cranberries

(Vaccinium Spp.) Using High-Performance Liquid Chromatography/Mass spectrometry. Journal of Agricultural and Food Chemistry, 49, 1270-1276.

Rice-Evans, C.A., Miller, N.J., & Paganga, G. (1996). Structure-Antioxidant activity relationships of flavonoids and phenolic acids. Free radical biology and medicine, 20(7), 933 – 956.

Rice-Evans, C.A., Miller, N.J., & Paganga, G. (1997). Antioxidant properties of phenolic compounds. Trends in plant science, 2(4), 152 – 159.

Schmidt, B.M., Erdman, Jr, J.W., & Lila, M.A. (2005) Effects of food processing on blueberry antiproliferation and antioxidant activity. Journal of food science, 70 (6), S389-S394.

Sellapan, S., Akoh, C.C., & Krewer, G. (2002). Phenolic compounds and antioxidant capacity of Georgia-Grown Blueberries and Blackberries. Journal of Agricultural and Food Chemistry, 50, 2432-2438.

Singleton, V.L., & J.A. Rossi. Jr. (1965). Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. American Journal of Enology and Viticulture, 16,144-158.

Su, M., & Silva, J.L. (2006)Antioxidant activity, anthocyanins, and phenolics of Rabbiteye blueberry (Vaccinium ashei) by-products as affected by fermentation. Food chemistry, 97 (3), 447 – 451.

Sun, A.Y., Simoni, A., Sun, G.Y. (2002). The “French paradox” and beyond: neuroprotective effects of polyphenols. Free radical biology and medicine, 32(4), 314-318.

Talcott, S. T., & Lee, J.-H. (2002). Ellagic Acid and Flavonoid Antioxidant Content of Muscadine Wine and Juice Journal of agricultural and food chemistry, 55(2), 255 – 260.

Talcott, S. T., Brenes, C. H., Pires, D. M., & Del Pozo-Insfran, D. (2003). Phytochemical Stability and Color Retention of Copigmented and Processed Muscadine Grape Juice Journal of agricultural and food chemistry, 51(4), 957-963

Taruscio, T.G., Barney, D.L., & Exon, J. (2004). Content and profile of flavonoid and phenolic acid compounds in conjunction with the antioxidant capacity for a variety of northwest Vaccinium berries. Journal of agricultural and food chemistry, 52, 3169-3176.

Yilmaz, Y., & Toledo, R. T. (2004). Major Flavonoids in Grape Seeds and Skins: Antioxidant Capacity of Catechin, Epicatechin, and Gallic Acid. Journal of Agricultural and Food Chemistry, 52(2), 255-260.

Youdim, K.A., Shukit-Hale, B., Martin, A., Wang, H., Denisova, N., Bickford, P. C., & Joseph, J. A. (2000). Short-term dietary supplementation of blueberry polyphenolics: Beneficial effects on aging brain performance and peripheral effects on aging brain performance and peripheral tissue function. Nutritional neuroscience, 3, 383-397.

Zheng, W., & Wang, S.Y. (2003). Oxyzen radical adsorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. Journal of agricultural and food chemistry, 51, 502-509.

CHAPTER 4

INFLUENCE OF ETHANOL CONCENTRATIONS, EXTRACTION TEMPERATURE, AND TIME ON THE YIELD OF TOTAL PHENOLICS, ANTHOCYANINS, ANTIOXIDANT CAPACITY, AND INDIVIDUAL COMPOUNDS IN ETHANOL EXTRACTS FROM MUSCADINE GRAPES AND RABBITEYE BLUEBERRIES[1].

[1]Biswas, R., Saalia, F.K., and Phillips, R.D. To be submitted to the *Food Research International*, 2007.

Abstract

The high content of phenolic antioxidants in muscadine grapes and blueberries makes them good sources of nutraceuticals. These compounds differ in their solubility in water, and their efficient extraction is a function of the matrix, and extracting solvent system. The objective of this work was to determine extraction conditions and ethanol/water ratio for maximum phenolics extraction from muscadine grapes and blueberries. Frozen blueberries and muscadine grapes were thawed under N_2 in reduced light, and macerated with ascorbic acid (0.2g/100g). The macerated pulp was incubated with Pectinex BE XXL (Novozyme®) for three hours at 60 C. It was then extracted with 10, 40, 70% alcohol by weight at 25, 45, 60 C for 1, 2, 3 H. The pulp was pressure filtered and the filtrate assayed for total phenolics (Folin-Ciocalteaux method), total anthocyanins (pH differential method), and antioxidant capacity (FRAP, ORAC). Regression models were developed to relate extraction parameters with phenolic content and antioxidant activity of extracts. Ethanol levels of 30-40% for muscadine grapes and 35-40% for blueberries at an extraction temperature of 60 C produced extracts with maximum phenolics concentration and antioxidant activity (FRAP, ORAC). Regression models for total phenolics and antioxidant activity (FRAP, ORAC) showed no lack of fit, and had high coefficients of determination (0.65 to 0.9). The models were adequate and robust, and can be used as a guide for extraction of phenolics from muscadine grapes and blueberries with ethanol solutions.

4.1. Introduction

The high content of phenolic compounds in berries makes them a good source of nutraceuticals (Lee, 2004; Pastrana-Bonilla, 2003; Sellapan, 2002; Talcott, 2003; Talcott, 2003; Yilmaz, 2004). The phenolic compounds are protective against many degenerative diseases like cancer and cardiovascular diseases (Auroma et al., 2003; Cao et al., 1996; Jayaprakash et al., 2003; Prior et al., 2001; Sun et al., 2002; Schmidt et al., 2005; and Youdim et al., 2000). However, these compounds differ in solubility in different types of solvent depending on their structure, so finding optimal extraction conditions remains a challenge.

Many researchers have used alcohols, including methanol, as well as other organic solvents to extract antioxidants from berries and grapes. Pastrana-Bonilla et al., (2003) used methanol to extract polyphenolic compounds from muscadine grapes and found the yield of polyphenolic compounds as 247.7 mg/100 g fresh weight. Prior et al., (2001) used a mixture of acetone, water, and acetic acid as extraction solvent to extract polyphenolic compounds from blueberries at an extraction temperature of 50 C. Sellapan et al., (2002) used methanol as an extracting solvent to extract the polphenolic compounds from blueberries.

Other researchers have used methanol (Guendez, 2005, Hulya, 2007, Giuseppe, 2007, Yusuf, 2006), ethanol (Giorgia, 2007; So-Young, 2006, Yusuf, 2006), ethyl acetate (Giorgia, 2007), and acetone (Yusuf, 2006) to extract polyphenolic compounds from other varieties of grapes. The solvents methanol (Hakkinen, 1999; Ayaz, 2005), and acetone (Skrede, 2000; Kalt et al., 2000; Lee, 2004) were used to extract polyphenolic compounds from blueberries. All the solvents except water and ethanol are toxic and are

associated with health problems. Producing extracts using food-grade solvents such as ethanol might minimize problems with perceived safety issues. The overall goal of our research is to produce edible concentrated nutraceutical powders from muscadine grapes and blueberries. So our objective is to develop an extraction protocol using ethanol water mixtures to produce extracts rich in polyphenolic compounds from muscadine grapes and blueberries. However, it is necessary to determine the optimum conditions for such an extraction procedure. The extraction of the compounds in the solvent is dependent on how fast the compound dissolves and reaches equilibrium. The rate of extraction can be improved by a large concentration gradient, high diffusion coefficient, and reduced particle sizes. Increase in extraction temperature also helps in high yield of total phenolics and anthocyanins. However, these compounds are not thermally stable, so too high temperature is not desirable (Cacace et al., 2003).

Several authors have done optimization studies on the extraction of polyphenolic compounds from different fruits. Liyana-Pathriana, (2005) conducted a study on the optimization of extraction of phenolic compounds from wheat by using response surface methodology. They investigated the effects of three independent variables, namely ethanol composition (%), extraction temperature (°C) and time (min) on the response, total antioxidant activity (TAA) and found that the optimum conditions were 54%, 61 C, 64 min and 49%, 64 C, 60 min, for whole grain and bran of soft wheat, respectively. Cacace et al., (2003) had conducted similar studies on the optimization of extraction of anthocyanins from black currants. They chose the independent variables as solvent concentration, temperature, and solvent to solid ratio and found the optimum conditions as 60 % ethanol at 30 C for anthocyanins and phenolics.

Our preliminary study (Chapter 3) showed that incubation of muscadine grape and blueberry extracts with pectinase BE ® enzyme at 60 C for a period of three hours had enhanced the leaching of the polyphenolic compounds into the solvent (water). In this study our objective was to optimize the extraction conditions for total phenolics and total anthocyanins from pectinase treated extracts of muscadine grapes and blueberries using aqueous ethanol as a solvent. We sought to investigate the effects of three independent variables (solvent composition (% ethanol), extraction temperature, and extraction time) on the yield of total phenolics and total anthocyanins for muscadine grapes and blueberries.

4.2. Materials and methods

4.2.1. Fruits

Muscadine grapes (Supreme variety) were obtained as a gift from Paulk vineyards. Blueberries (Brightwell variety) were obtained from a commercial grower in Florida.

4.2.2. Chemicals

Gallic acid, L-ascorbic acid, 6-hydroxy-2, 5, 7, 8-tetramethychroman-2-carboxylic acid (TROLOX); 2, 4, 6-tripyridyl-s-triazine (TPTZ), 6-carboxy fluorescein, and 2,2'-Azobis (2-methylpropionamidine) dihyrochloride (AAPH) were purchased from Sigma (St. Louis, MO). Moisture analysis of the berries was done by a vacuum oven method at 70°C and 25mm Hg for 8 H (AOAC 934.06).

4.2.3. Experimental design

Box-Behnken design (Box et al., 1960) was used to design the aqueous extraction. The factors were extraction temperature (25, 45, 60 C), alcohol concentration (10, 40, 70 % w/w) and extraction time (1, 2, 3 h).

4.2.4. Alcoholic extraction

Frozen berries were thawed under nitrogen, macerated with ascorbic acid (0.2g/100g fruit) and incubated with Pectinex BE ® enzyme for three hours at 60°C as was shown to be optimal in the preceding chapter. Amounts of 95% ethanol and deionized water were mixed such that a total of 250g of solvent (total water + ethanol) were present and ethanol concentrations were prepared as 10, 40, and 70 % respectively. The three different ethanolic solutions were then added to the enzyme treated macerated pulp and extracted for 1, 2, and 3 h. A wrist action shaker (Model 75, Burrell Corporation, Pitsburg, PA, USA) was used to shake the pulp in the bottles at 25 °C, and a reciprocating water bath, at 45, and 60 °C. The extracts were pressure filtered (50 psig, pre-filter RW03, TCLP filter) and the filtrates were collected and frozen before further analysis. The entire experiment was performed under subdued light.

4.2.5. Total phenolics

Total phenolics were estimated colorimetrically using the Folin-Ciocalteaux method (Singletonet al., 1965). A sample aliquot of 200 µL was added to 800 µL of deionized water, 5 mL of Folin-Ciocalteaux reagent, and 4 mL of saturated sodium carbonate solution (75g/L) and mixed on a vortex mixer. The absorbance was measured at 765 nm with a Hewlett Packard 8451A diode array spectrophotometer (Avondale, PA, USA) after

incubation for two hours at room temperature (~25 C). Quantification was based on the standard curve generated with 100, 200, 300, 400, and 500 mg/L of gallic acid. The final concentration of phenolics was calculated based on total volume of extract and initial weight of berries; and expressed as mg/Kg dry weight. The experiment was conducted under subdued light.

4.2.6. Total anthocyanins

Total anthocyanins were measured by the pH differential method (Giustiet al., 2001) using two buffer systems - potassium chloride buffer, pH 1.0 (0.025 M) and sodium acetate buffer, pH 4.5 (0.4 M). Extracts were diluted with water to give Absorbance in the range of 0.1 - 1.2 at 510 nm. Aliquots of 0.2 ml of diluted extracts were mixed with 1.8 mL of each buffer, vortexed and read against a blank at 510 and 700 nm with a Hewlett Packard 8451A diode array spectrophotometer (Avondale, PA, USA). Absorbance was calculated as

$A = (A_{570}\text{ nm} - A_{710}\text{ nm})\text{ pH 1.0} - (A_{570}\text{ nm} - A_{710}\text{ nm})\text{ pH 4.5}$

Monomeric anthocyanin pigment concentration in the extract was calculated as cyanidine-3-glucoside

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

Where A = absorbance, MW = molecular weight (449.2), DF = dilution factor, ε = molar absortivity (29,600), l = path length (1 cm).

The final concentration of anthocyanins was calculated based on total volume of extract and weight of starting fruit, and expressed as mg/Kg dry weight.

4.2.7. Assay of antioxidant capacity

The antioxidant capacity was assayed by Ferric reducing/antioxidant power assay (FRAP) and Oxygen radical-scavenging activity (ORAC).

Ferric reducing/antioxidant power assay (Benzie, and Strain, 1996). FRAP reagent was made by mixing 2.5 mL of 10 mM TPTZ in 40mM HCl , 25 mL of 300 mM acetate buffer, pH 3.6, and 2.5 ml of 20 mM $FeCl_3.6H_20$. One hundred mL of the diluted sample was added to 3 mL of the working FRAP reagent, vortexed, and allowed to stand for 10mins. Absorbance was measured at 593 nm with a Hewlett Packard 8451A diode array spectrophotometer (Avondale, PA, USA). Quantification was based on the standard curve generated with 100, 200, 300, 400, and 500 mM of TROLOX. The final concentration was expressed as umol of TROLOX equivalents per Kg dry basis. .

Oxygen radical absorbance capacity (Cao, and Prior, 1999). A stock solution of 6-carboxy fluorescein (1.5 × 10-3 M) was prepared in 75 mM phosphate buffer, pH 7.0. Working solution was prepared by diluting it with 75 mM phosphate buffer, pH 7.0, to a concentration of 1.5 × 10-4 M. AAPH (320 mM, held at 0 C) was prepared in 75 mM phosphate buffer. The reaction mixture containing, 2.625 mL 75 mM phosphate buffer, pH 7.0, 150 mL fluorescein and 150 mL diluted sample, was incubated at 37 °C for 15 mins. To initiate the reaction, 75 mL of AAPH was added to the reaction mixture, vortexed, transferred to thermostated cuvette (37 C) in a spectroflourometer (RF 5301PC Shimadzu scientific instruments Inc., Columbia, MD,USA),and decay of fluorescence measured. Optimal excitation (~325 nm) and emission (~520 nm) wavelengths of standard fluorescein solution (1.5 x 10-5 M) were selected daily using

RF-5301PC software. Quantification was based on the standard curve generated with 50, 100, 200, and 300mM of TROLOX. The final concentration was expressed as mmol of TROLOX equivalents per Kg dry basis. The experiment was conducted under subdued light.

4.2.8. Hydrolysis

The hydrolysis of the extracts was used to minimize the interferences in the chromatography and to simplify the chromatography data for cases where standards are unavailable. The hydrolysis method of filtered alcoholic extracts was modified from the method described by Hertog et al., (1992). Two milliliters of the extract was mixed with 10 mL of 6N HCl and 38 mL of methanol and the mixture was flushed with nitrogen. Then it was refluxed at 95 C for two hours. The extract was allowed to cool and was subsequently made up to 100 mL with methanol. Approximately 2 mL of the extract was filtered through 0.20 μm (Fisher Scientific, Pittsburg, PA)prior to injection.

4.2.9. High pressure liquid chromatography

Twelve individual phenolic compounds were analyzed by high pressure liquid chromatography. Analysis was done by using Waters (Waters Corporation, Milford, MA) HPLC system comprised of a Waters 717 sample injector, Waters 2695 separations module, and Waters 996 PDA set to monitor the UV spectrum from 240 to 400 nm. The analytical column was heated using a column heater module (Waters Corporation, Milford, MA) to 40 C equipped with a temperature control module (Waters Corporation, Milford, MA). The Waters Millenium^{32} software, version 3.05 (Waters Corporation, Milford, MA) was used to control the HPLC auto sampler, gradient settings, PDA and data acquisition. The column used was μBondapak^{TM} RP C18 (Waters Corporation,

Milford, MA) and the dimensions were 3.9 by 300 mm. The solvents used were (A) 88% water, 10% HPLC grade methanol, 2% HPLC acetic acid; (B) 50% HPLC grade acetonitrile, 20% HPLC grade methanol, 30% water, adjusted to1% in acetic acid; and (C) 100% HPLC acetonitrile. Water used was double deionized and filtered, by vacuum, through a 0.2 μm nylon filter (Millipore Corporation, Bedford, MA). A stock solution of all the standards was made in HPLC grade methanol. The carousel temperature was maintained at 20 C. The detection wavelengths used were 280 and 320 nm. The gradient used is shown in Table 4.1.

4.2.10. Data Analysis

The response surface regression (RSM) procedure was followed using Statistica ® software, Version 6.0 (Statsoft, Tulsa, OK). Experimental data was used to fit a quadratic polynomial equation to obtain the regression coefficients of the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X^2{}_i + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$$

where β_0, β_i, β_{ii}, β_{ij} are the regression coefficients for intercept, linear, quadratic, and interaction terms respectively. The independent variables and response variables are symbolically represented as Xi, Xj, and Y respectively. The independent variables were extraction temperature (X_1), solvent concentration (X_2), and extraction time (X_3) respectively. The dependent variables were total phenolics, total anthocyanins, total antioxidant activity (FRAP), and ORAC. . Statistica software was used to generate 3-D

surface and contour plots of dependent variables as function of two independent variables. Correlation studies were also conducted to correlate FRAP and ORAC with TPH and ACY respectively.

4.3. Results and discussion

4.3.1. Total phenolics

The total phenolics in alcoholic extracts of muscadine grapes varied from 14 –20.6 g gallic acid equivalents/Kg db (Table 4.2). These values were higher than previously reported in the literature as 11g/Kg db (Pastrana-Bonilla et al., 2003). The difference in the values can be attributed to the fact that the extracts were subjected to enzymatic hydrolysis at an enhanced temperature prior to ethanolic extraction. Enzymatic hydrolysis could have facilitated the loosening of the cells and high temperature had increased the solubility of the total phenolics in the solvent. Optimization of extraction parameters (extraction time, solvent concentration, and extraction temperature was achieved by using response surface methodology. The maximum yields of total phenolics were found at an extraction temperature of 60 C and alcohol concentration of 30 – 40 % w/w (Figure 4.1a). The response surface model for total phenolics was found to be significant at the 3.0 % level of significance with a R square value of 94.1 % and no significant lack of fit . A significant linear ($p = 0.004$) and a quadratic ($p = 0.003$) effect of ethanol concentration was found on the total phenolics. Temperature had a linear effect on the yield of total phenolics ($p = 0.29$). Extraction time did not effect the total phenolics at all which indicated that extraction could be conducted at 1 h with the maximum yield of total phenolics. The estimates of the parameters of the response surface equation are given in Table 4.3. The relationship between the independent and

the response variables were also studied by examining the plots generated by holding one of the variable of the second order polynomial equation as constant. The yield of total phenolics increased with increasing alcohol concentration starting from 30 % and then decreased after 40 %. The yield increases when the extraction temperature was raised to 60 C. Changes in the ethanol concentration altered the properties of the solvent as well as the properties of the solute. The energy required to break the configuration of water molecules is large for non-polar covalent molecules (flavonoids and complex phenolic acids). Due to which they were soluble in 30 – 40 % w/w ethanol (Cacace et al., 2003). Increase of temperature enhanced the extraction efficiency by increasing the solubility of phenolic compounds in the solvent.

The total phenolics in alcoholic extracts of blueberries varied from 15.1 – 28.4 mg gallic acid equivalents/Kg db (Table 4.4). Optimization experiments were conducted to study the response variable (total phenolics) as a function of extraction temperature, ethanol concentration, and extraction time. Response surface regression model for total phenolics as a function of extraction temperature, ethanol concentration and extraction time was developed. The model was significant at the 0.2 % level of signifiance with a R square value of 97.1 %. The model was found to have no significant lack of fit, which showed that model was adequate and robust. The coefficients of the regression parameters are given in Table 4.5. There was a significant linear ($p = 0.0002$) and quadratic effect ($p = 0.00009$) of ethanol concentration on the yield of total phenolics from the alcoholic extracts of blueberry. There was also a significant interaction effect of extraction temperature and concentration ($p = 0.004$) on the yield of total phenolics. There was a quadratic effect of extraction time and an interaction effect of ethanol

concentration and extraction time on the yield of total phenolics at 17 % and 15.6 % level of significance respectively. The response variable (total phenolics) was also studied from the contour plot generated by the model (Figure 4.1). The plot showed two optimum regions for total phenolics at alcohol concentration of 40 – 50 % w/w, extraction temperature of 25 C and alcohol concentration of 20 to 45 %w/w, extraction temperature of 60 C respectively. The latter region showed a wider area than the former region. We chose the latter region as our optimum region for total phenolics. Extraction with a higher temperature had improved the yield of total phenolics by increasing the solubility of the compounds in the solvent. Phenolic acids, with a carboxylic group and a glycosylated benzene ring may be considered as covalent polar molecules. Due to the covalent nature of the flavonoids, complex phenolic acids (ellagic acid and others), they were found to be more soluble as the concentration of ethanol was increased from 0 to 25 % till 50 %.

4.3.2. Total Anthocyanins

The total anthocyanins for the aqueous ethanolic extracts ranged from 6.6 – 7.9 g cyanidin 3-glucoside/Kg db (Table 4.2). Our results were comparable with previously reported (Pastrana-Bonilla et al., 2003).Response surface models were developed to find the optimum points of extraction. Contour surface plots generated by the predicted values of the response surface regression showed that the maximum anthocyanins extraction was achieved at 25 C instead of higher temperatures as that of total phenolics. Anthocyanins are less stable and may be destroyed with an increase in temperature. Additionally, the stability and the color intensity of the anthocyanins are influenced by a phenomenon called copigmentation. Copigment factors can be phenolic acids,

flavonoids, and anthocyanins and the copigment complexes are favored by low temperature (Brouillard, 2001). This explains the reason of instability of anthocyanins. Anthocyanins were found to be less soluble in higher concentration of alcohols because of their ionic nature. They are found to be more soluble in water than in ethanolic solutions (Biswas et al., 2007). In the previous study, the yield of anthocyanins from pectinase treated aqueous extracts of muscadine grape was determined as 5.57 g cyanidin 3 glucoside equivalents/Kg db. Response surface regression models were developed for anthocyanins with a R square value of 74.0 % (Table 4.3). The model was not found to be significant. There was a linear and a quadratic effect of temperature on the yield of anthocyanins at 27.7 and 35.2 % respectively. The concentration of ethanol did make any significant impact on the yield of total anthocyanins, however the yield was found to be maximum at 10 % w/w, ethanol concentration. The difference between the minimum and the maximum yields across the different levels of ethanol concentration was not substantial to exhibit a significant effect on the yield of the anthocyanins in the ethanolic extracts of muscadine grape.

Total anthocyanins in the ethanolic extracts of blueberries were found to vary from 8.0 – 18.6 g cyanidin 3 glucoside equivalents/Kg db (Table 4.3). Response surface regression model for anthocyanins was developed and the model was found to be significant at 1.5 % level of significance. The coefficients of the regression parameters are given in Table 4.5. There was a significant linear ($p = 0.001$) and a quadratic effect ($p = 0.001$) of ethanol concentration on the yield of anthocyanins. There was also a significant interaction effect of extraction temperature and ethanol concentration on the yield of anthocyanins ($p = 0.007$). The model also had a significant lack of fit at 3.8 %

level of significance. The significant lack of fit explains that the insignificant linear, quadratic effects of extraction temperature, time and interaction effect of extraction temperature and concentration with extraction time. The variation of anthocyanins with ethanol concentration and extraction temperature was also studied from the response contour plots generated by the model regression equation. Two optimal regions were found for anthocyanins at ethanol concentration of 40 – 70 %, extraction temperature of 25 C and at ethanol concentration of 20 – 50 %, extraction temperature of 60 C. Since anthocyanins were less stable at higher temperature we have a slightly wider area at the former optimum region. We chose the former region as the best optimum region for extraction of anthocyanins from blueberries. The anthocyanins stabilized themselves by forming copigment complexes either within themselves or with other molecules at the expense of hydroxyl group. These complexes were thermally unstable which explains the reason of lesser extractability at higher temperature as compared to total phenolics.

4.3.3. Total antioxidant capacity

The antioxidant activity is expressed in two ways namely FRAP and ORAC values. The FRAP values for alcoholic extracts of muscadine grapes ranged from 40 – 68.8 mmoles TROLOX equivalents /Kg db (Table 4.2). The response surface regression model was developed for FRAP values. The model was found significant at 4.3 % level of significance with R square value of 82.7 %. The model had no significant lack of fit . There was a significant linear ($p = 0.01$) and quadratic ($p = 0.01$) effect of ethanol concentration on the FRAP values. The interaction effect of extraction temperature and alcohol concentration on the FRAP values was found to be significant at 11.3 %. The correlation coeffecient between FRAP values and total phenolics values was found as

91.7 %, whereas the FRAP values were not well correlated with the total anthocyanins values. The correlation between the FRAP values and total phenolics values were also studied from the scatterplot (Figure 4.3). The variation of the FRAP values with respect to alcohol concentration and the extraction temperature was also studied from the contour plots generated by the predicted values of the model (Figure 4.4). The response surface plots (holding extraction time as constant) showed that maximum antioxidant potential was found at an alcohol concentration of 30 – 50 % and at an extraction temperature of 60 C. The results also showed that total phenolics were the main contributor to the total antioxidant potential. The ORAC values in alcoholic extracts of muscadine grape ranged from 313 – 641 mmol TROLOX equivalents / Kg db (Table 4.2). Optimization experiments were also conducted to investigate the maximum points of ORAC values. The response surface regression model was developed. The whole model was found to be significant at 1 % level of significance with a R square value of 95.7 %. The model had no significant lack of fit.. The ORAC values showed a different trend than the other values. There was a significant linear ($p = 0.0003$) and a quadratic effect ($p = 0.0001$) of temperature on the ORAC values. The alcohol concentration didn't have a significant linear and a quadratic effect on the ORAC values. The extraction time had a linear and quadratic effect on the ORAC values at 14.0 and 13.0 % respectively. Response surface plots were generated by the model equation (Figure 4.5). The plot showed that the ORAC value increased as the temperature was increased to 60 C and was optimal at an alcohol concentration of 25 to 70 %, w/w. The nature of the plots indicated that total phenolics was the major contributor to the total antioxidant potential represented by ORAC values.

The total antioxidant potential (FRAP) in the alcoholic extracts of blueberries varied from 64.7 – 138.1 mmol TROLOX equivalents /Kg db (Table 4.4). Response surface models were developed to study the effect of ethanol concentration, extraction temperature, and extraction time on the antioxidant potential. The response surface model was found to be significant at 1.4 % level of significance. The model was adequate and explained 99.3 % of the variation in the results. The coefficients of the regression parameters are given in Table 4.5. There was a significant linear effect of extraction temperature on the FRAP values at 0.5 % level of significance. A significant linear ($p = 0.000005$) and a quadratic effect ($p = 0.000003$) of ethanol concentration was found on the FRAP values. The interaction effect of extraction temperature and ethanol concentration and ethanol concentration and time was found to be significant at 0.01 % and 4 % level of significance respectively. There was no significant effect of extraction time on the FRAP values. The model did not have a significant lack of fit which showed that the model was robust and adequate. Contour plots generated by the model showed that the optimum region for the FRAP values was at ethanol concentration ranging from 20 to 70 %, w/w. This explains that the total phenolics and total anthocyanins were the major contributors to the antioxidant potential of the alcoholic extracts. A correlation study was performed to check the correlation of FRAP values with total phenolics an total anthocyanins. The correlation coefficients were found to be 0.91 with total phenolics and 0.90 with total anthocyanins respectively. The FRAP values were plotted against the total phenolics and total anthocyanins values (Figure 4.6). The values were found to stay close to the regression line which indicated that there was good correlation existed between the FRAP values and the TPH and the ACY values.

4.3.4. Individual phenolic compounds

Twelve indivudual phenolic compounds were analysed for the ethanolic extracts of both muscadine grape and rabbiteye blueberries by high pressure liquid chromatography. A chromatogram showing the retention time of each of the phenolic compounds is shown in Figure 4.7. The mean values of gallic acid, 4-hydroxy benzoic acid, caffeic acid, p-coumaric acid, ferulic acid, and ellagic acid were found as 0.86 ± 0.16, 0.007 ± 0.001, 0.002 ± 0.00009, 0.003 ± 0.0002, 0.003 ± 0.0005, and 0.009 ± 0.004 g/100 g fresh weight respectively in the ethanolic extracts of muscadine grapes.. Among the acids gallic acid was found to be the major acid followed by ellagic acid. Because of the polar nature of the molecule, gallic acid was the first to be eluted whereas ellagic acid was the last acid to be eluted (non-polar because of four benzene rings condensed to each other)from the column.. Catechin and epicatechin was found to be present in amounts of 8.2 ± 1.2, and 0.03 ± 0.01 g/100 g fresh weights respectively. The reason for unreasonably higher amounts of catechin could be due to coelution of other compounds with catechin. Myrecetin, quercetin, and kaempferol were present in amounts of 0.004 ± 0.002, 0.01 ± 0.001, and 0.005 ± 0.008 g/100 g fresh weight respectively. Resveratrol was estimated as 0.002 ± 0.00008 g/100 g fresh weight. In the ethanolic extracts of blueberries, gallic acid, 4-hydroxy benzoic acid, p-coumaric acid, ferulic acid, and ellagic acids were found as 1.2 ± 0.2, 0.002 ± 0.001, 0.009 ± 0.004, 0.001 ± 0.0002, 0.004 ± 0.0009, and 0.01 ± 0.003 g/100 g fresh weight respectively. Gallic acid was found to be the major acid present in the ethanolic extracts of blueberry. Ellagic acid was the second highest in the ethanolic extract of blueberry. Catechin, and epicatechin were present in amounts of 9.7 ± 0.1, and 0.2 ± 0.06 g/100 g fresh weights respectively. Myrecetin,

quercetin, and kaempferol were present in amounts of 0.008 ± 0.0008, 0.005 ± 0.0008, and 0.004 ± 0.001 g/100 g fresh weight respectively. Resveratrol was estimated as 0.002 ± 0.0001 g/100 g fresh weight respectively.

4.4. Significance

The findings of the research showed solvent had a prominent effect on the extraction of total phenolics, and anthocyanins from muscadine grapes and blueberries. Optimum results were found at an extraction temperature of 60 C and an ethanol concentration of 30 – 40 % for muscadine grapes and blueberries. The solvent concentration and the extraction temperature affected the solubility of these polyphenolic compounds significantly. These data provide important information about the use of optimal solvents to extract total phenolics efficiently.

Table 4.1.

Linear gradient of the solvents used in HPLC.

Time	Flow (mL/min)	%A	%B	%C
0	1	100	0	0
5	1	100	0	0
10	1	90	10	0
15	1	80	20	0
20	1	75	25	0
25	1	70	30	0
30	1	10	90	0
35	1	10	90	0
40	1	80	20	0
45	1	0	0	100
50	1	0	0	100
55	1	100	0	0
60	1	100	0	0

(A) 88% water, 10% HPLC grade methanol, 2% HPLC acetic acid;

(B) 50% HPLC grade acetonitrile, 20% HPLC grade methanol, 30% water, adjusted to1% in acetic acid; and

(C) 100% HPLC acetonitrile.

Table 4.2.

Influence of extraction temperature, ethanol concentration, and extraction time on the yield of total phenolics (TPH), and total anthocyanins (ACY) and total antioxidant activity (FRAP, ORAC) in alcoholic extracts of muscadine grapes.

Sample	Extraction Temperature (C)	Ethanol Concentration (% w/w)	Extraction Time (h)	TPH (g/Kg db)	ACY (g/Kg db)	FRAP (mmol/Kg db)	ORAC (mmol/Kg db)
1	25	10.1	2	14.7	7.1	40.7	578.7
2	60	10.1	2	18.9	7.4	58.6	543.7
3	25	70.5	2	18.7	7.6	59.5	641.2
4	60	70.5	2	19.3	7.3	60.2	589.3
5	25	40.3	1	21.3	7.9	68.8	508.4
6	60	40.3	1	20.0	6.9	62.5	580.3
7	25	40.3	3	21.1	7.3	64.4	559.8
8	60	40.3	3	22.4	7.2	65.5	631.2
9	45	10.1	1	17.0	7.4	57.5	313.4
10	45	70.5	1	18.5	7.2	59.8	358.0
11	45	10.1	3	16.5	7.1	54.5	361.5
12	45	70.5	3	16.3	6.6	54.2	404.7
13	45	40.3	2	20.3	7.6	67.1	394.9
14	45	40.3	2	20.6	6.8	63.8	420.6
15	45	40.3	2	21.2	7.1	65.6	421.0

Table 4.3.

Regression coefficients of predicted quadratic polynomial models for the response total phenolics (TPH), total anthocyanins (ACY), total antioxidant activity (FRAP and ORAC) of muscadine grapes.

Coefficient	TPH		ACY		FRAP		ORAC	
		p		p		p		P
β_0	14.49470	0.066976	9.056746	0.004237	32.81768	0.230313	1287.749	0.001018
Linear								
β_1	-0.17236	0.433616	-0.072513	0.277171	0.46140	0.581467	-54.270	0.000307
β_2	0.43819	0.004248	0.021640	0.442273	1.37843	0.009983	2.729	0.354627
β_3	-0.20158	0.951651	-0.465027	0.637884	-7.83355	0.550146	167.869	0.140101
Quadratic								
β_{11}	0.00253	0.295610	0.000654	0.352269	-0.00248	0.779409	0.648	0.000184
β_{22}	-0.00380	0.003065	-0.000032	0.882380	-0.01071	0.011367	-0.019	0.420293
β_{33}	-0.16087	0.813843	-0.017244	0.931335	0.79403	0.764243	-35.544	0.129973
Crossproduct					-	-		
β_{12}	-0.00179	0.186857	-0.000343	0.364240	-0.00867	0.113705	-0.009	0.818231
β_{13}	0.03167	0.411955	0.011392	0.323170	0.09748	0.508363	-0.014	0.990053
β_{23}	-0.01505	0.497857	-0.002482	0.698663	-0.02157	0.797454	-0.011	0.986634
F	29.10269	0.033651	0.57998	0.767685	23.11794	0.042136	79.97559	0.012409
Rsquare	0.888088		0.562459		0.827653		0.957550	

The coefficients were related to the following variables,

β_1, β_2, and β_3 : Regression Coefficients for linear Extraction Temperature, Ethanol Concentration, and Extraction Time.

β_{11}, β_{22}, and β_{33} : Regression Coefficients for quadratic Extraction Temperature, Ethanol Concentration, and Extraction Time.

β_{12}, β_{13}, and β_{23} : Regression Coefficients for interaction of Extraction Temperature and Ethanol Concentration, Extraction Temperature and Extraction time, Ethanol Concentration and Extraction Time.

Table 4.4.

Influence of extraction temperature, ethanol concentration, and extraction time on the yield of total phenolics (TPH), and total anthocyanins (ACY) and total antioxidant activity (FRAP) in alcoholic extracts of blueberry.

Sample	Extraction Temperature (C)	Ethanol Concentration (% w/w)	Extraction Time (h)	TPH (g/Kg db)	ACY (g/Kg db)	FRAP (mmol/Kg db)
1	25	10.1	2	17.2	8.1	64.7
2	60	10.1	2	22.7	18.4	105.4
3	25	70.5	2	21.9	18.2	116.7
4	60	70.5	2	15.1	12.7	84.2
5	25	40.3	1	24.7	17.6	119.5
6	60	40.3	1	27.8	18.2	132.2
7	25	40.3	3	26.4	19.4	132.4
8	60	40.3	3	28.4	19.1	138.1
9	45	10.1	1	19.2	8.6	88.7
10	45	70.5	1	16.0	13.0	95.3
11	45	10.1	3	19.1	8.3	86.0
12	45	70.5	3	20.1	16.1	110.1
13	45	40.3	2	24.6	17.7	130.1
14	45	40.3	2	26.0	18.6	136.4
15	45	40.3	2	26.1	18.3	135.4

Table 4.5.

Regression coefficients of predicted quadratic polynomial models for the response total phenolics (TPH), total anthocyanins (ACY), total antioxidant activity (FRAP) of rabbiteye blueberries.

Coefficient	TPH		ACY		FRAP	
		p		p		p
β_0	9.67029	0.178993	-3.53252	0.704303	-35.1385	0.083069
Linear						
β_1	-0.00855	0.967833	-0.12976	0.669530	2.4506	0.005715
β_2	0.80464	0.000262	0.80893	0.001310	4.8483	0.000005
β_3	-1.35350	0.685402	5.01230	0.313412	4.9407	0.576543
Quadratic						
β_{11}	0.00346	0.170810	0.00564	0.125410	-0.0079	0.222869
β_{22}	-0.00796	0.000096	-0.00598	0.001901	-0.0420	0.000003
β_{33}	0.32591	0.635195	-1.22929	0.237599	-0.5802	0.746208
Crossproduct					-	-
β_{12}	-0.00577	0.004302	-0.00717	0.007500	-0.0342	0.000101
β_{13}	-0.01246	0.738296	-0.01184	0.822463	-0.1052	0.307431
β_{23}	0.03425	0.156013	0.02777	0.384282	0.1450	0.043344
F	18.66402	0.002460	8.29654	0.015667	79.06077	0.000074
Rsquare	0.971094		0.937240		0.993022	

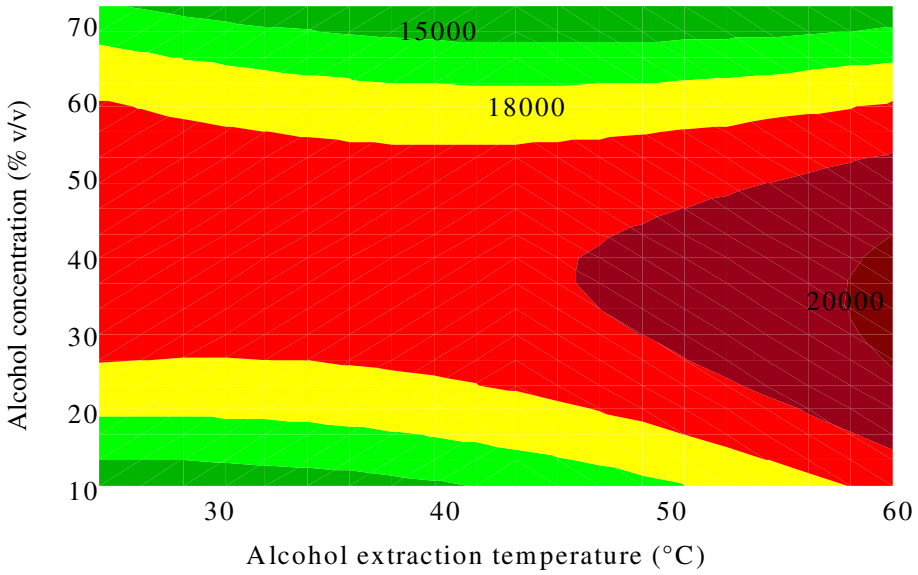
The coefficients were related to the following variables,

β_1, β_2, and β_3 : Regression Coefficients for linear Extraction Temperature, Ethanol Concentration, and Extraction Time.

β_{11}, β_{22}, and β_{33} : Regression Coefficients for quadratic Extraction Temperature, Ethanol Concentration, and Extraction Time.

β_{12}, β_{13}, and β_{23} : Regression Coefficients for interaction of Extraction Temperature and Ethanol Concentration, Extraction Temperature and Extraction time, Ethanol Concentration and Extraction Time.

A. Muscadines



B. Blueberries

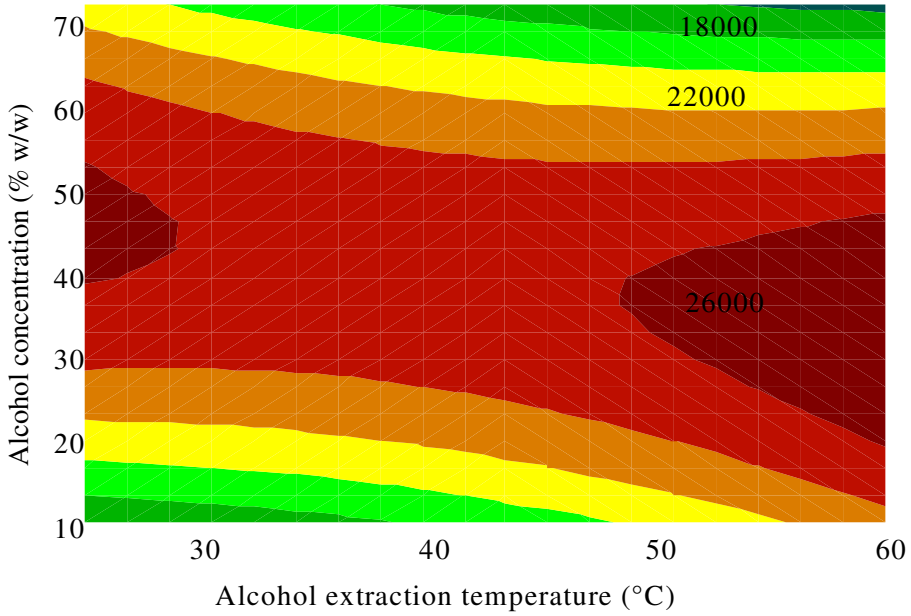
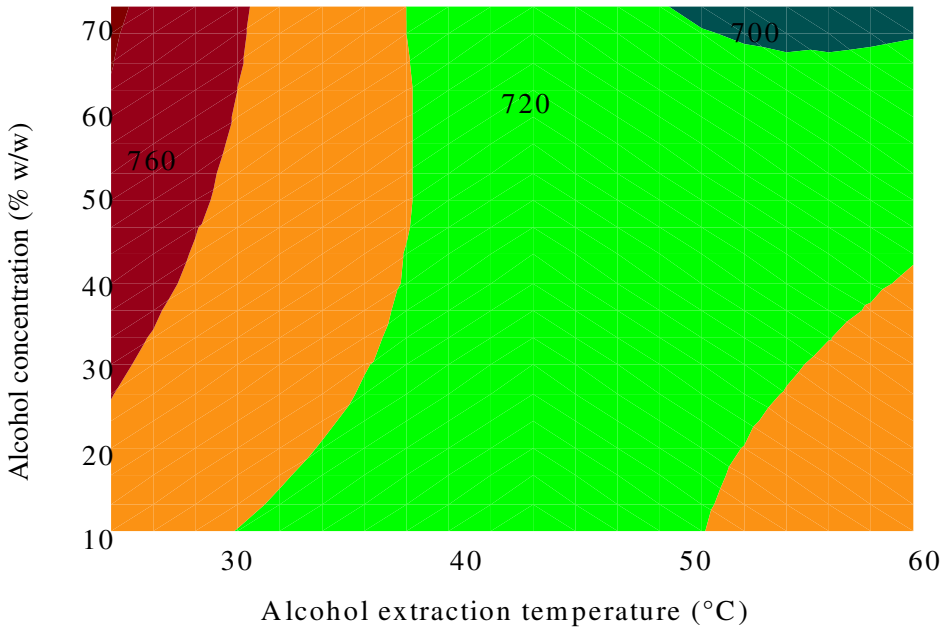


Figure 4.1. Effect of alcohol concentration and alcohol extraction temperature on the yield of total phenolics in alcoholic extracts of muscadine grape (A) and blueberries (B).

A. Muscadines



B. Blueberries

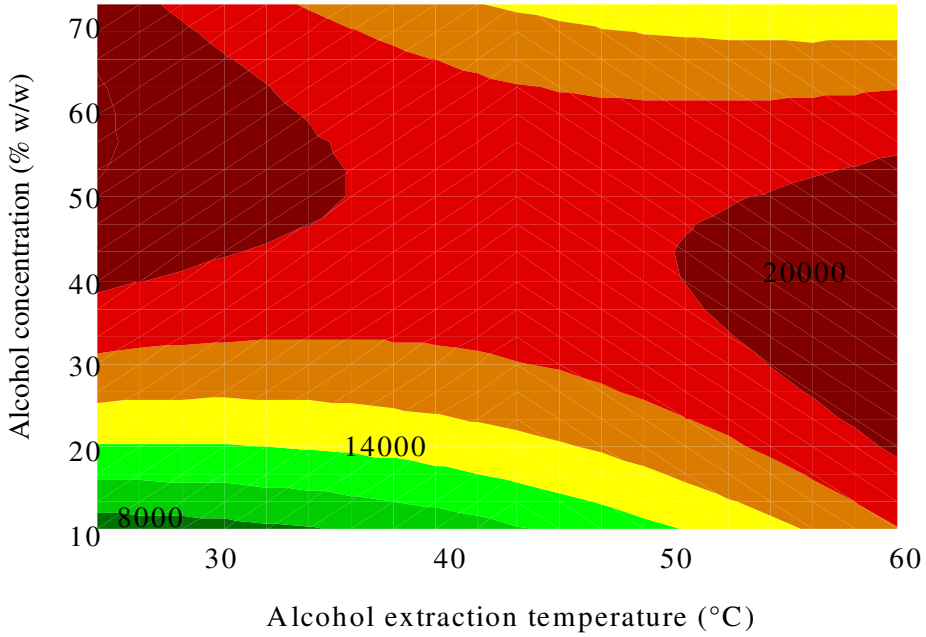


Figure 4.2. Efect of extraction temperature and alcohol concentration on the yield of total anthocyanins in alcoholic extracts of muscadine grapes (A) and blueberries (B).

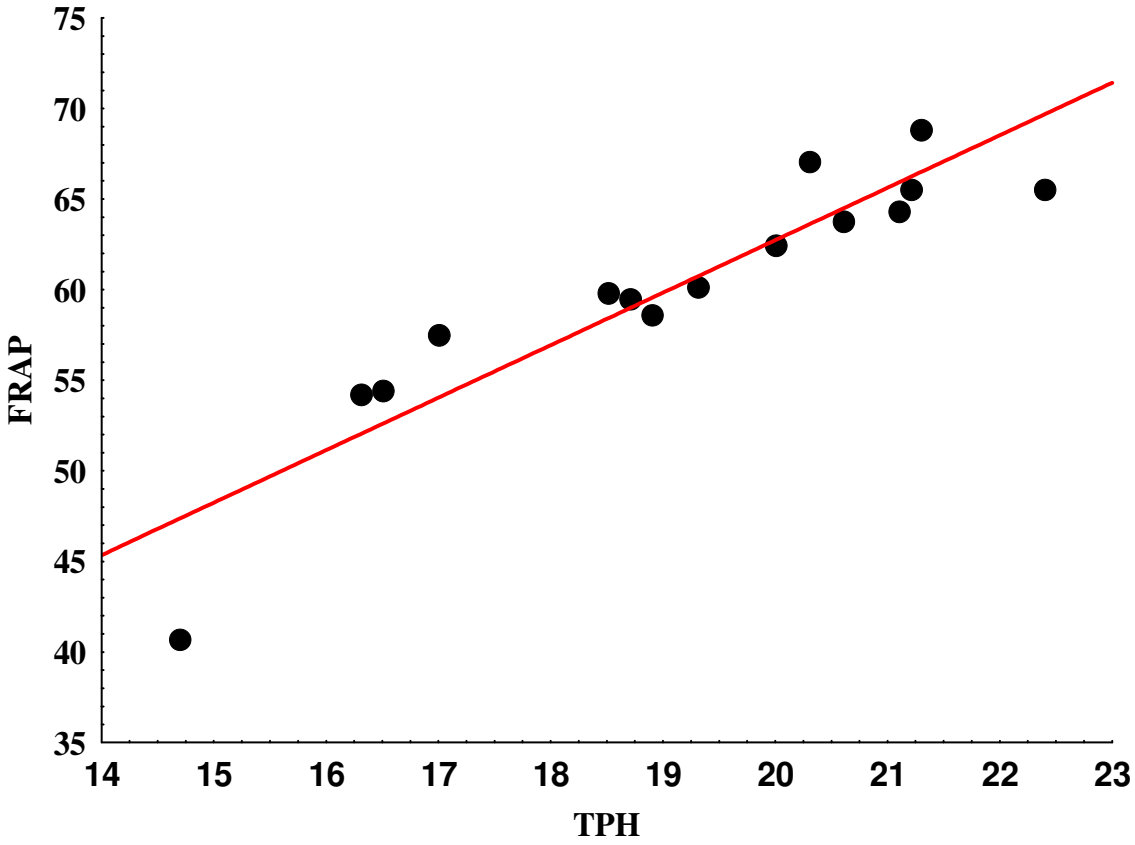
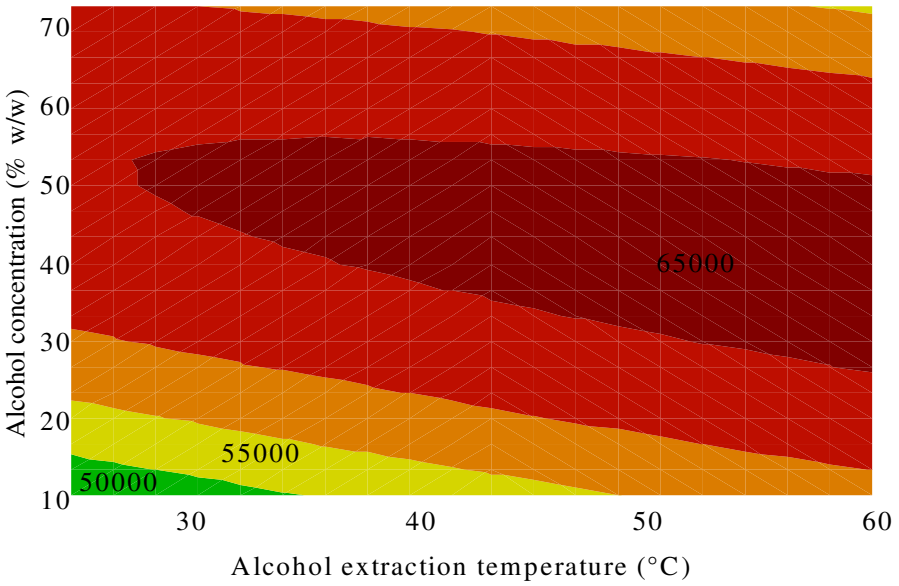


Figure 4.3. Total antioxidant (Ferric reducing/antioxidant power assay, FRAP) values versus total phenolics value (TPH) of alcoholic extracts of muscadine grape.

A. Muscadines



B. Blueberries

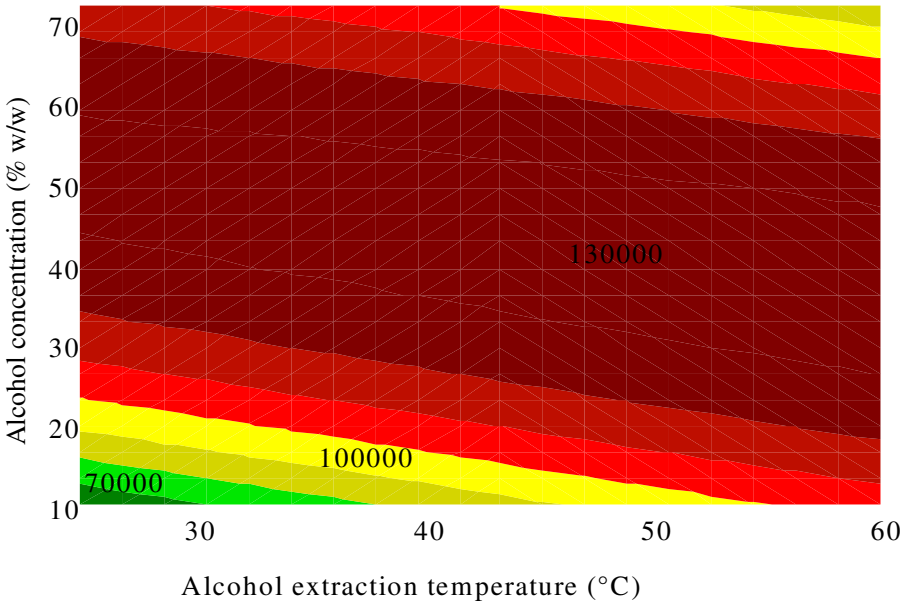


Figure 4.4. Effect of alcohol concentration and extraction temperature on the antioxidant values (FRAP) in alcoholic extracts of muscadine grapes (A) and blueberries (B).

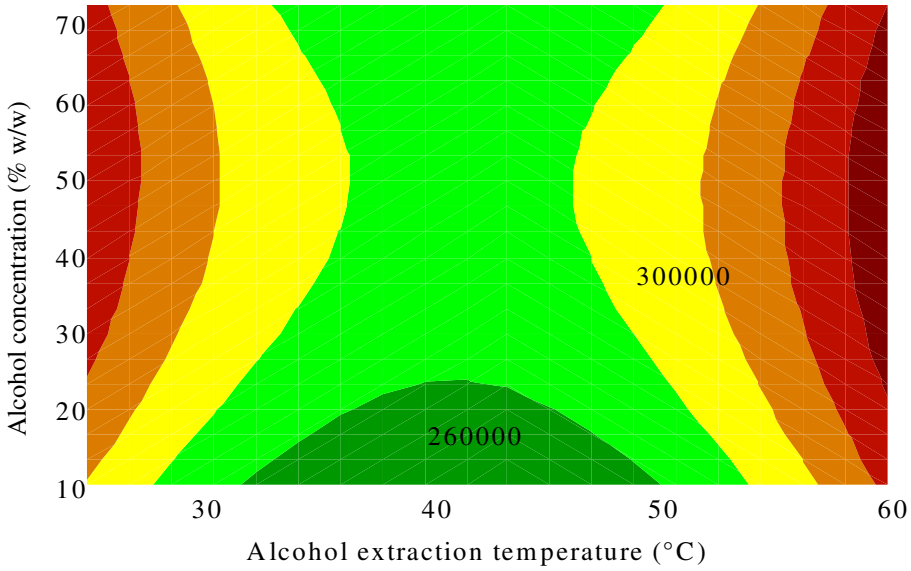


Figure 4.5. Effect of alcohol concentration and extraction temperature on the antioxidant potential (ORAC) in alcoholic extracts of muscadine grape.

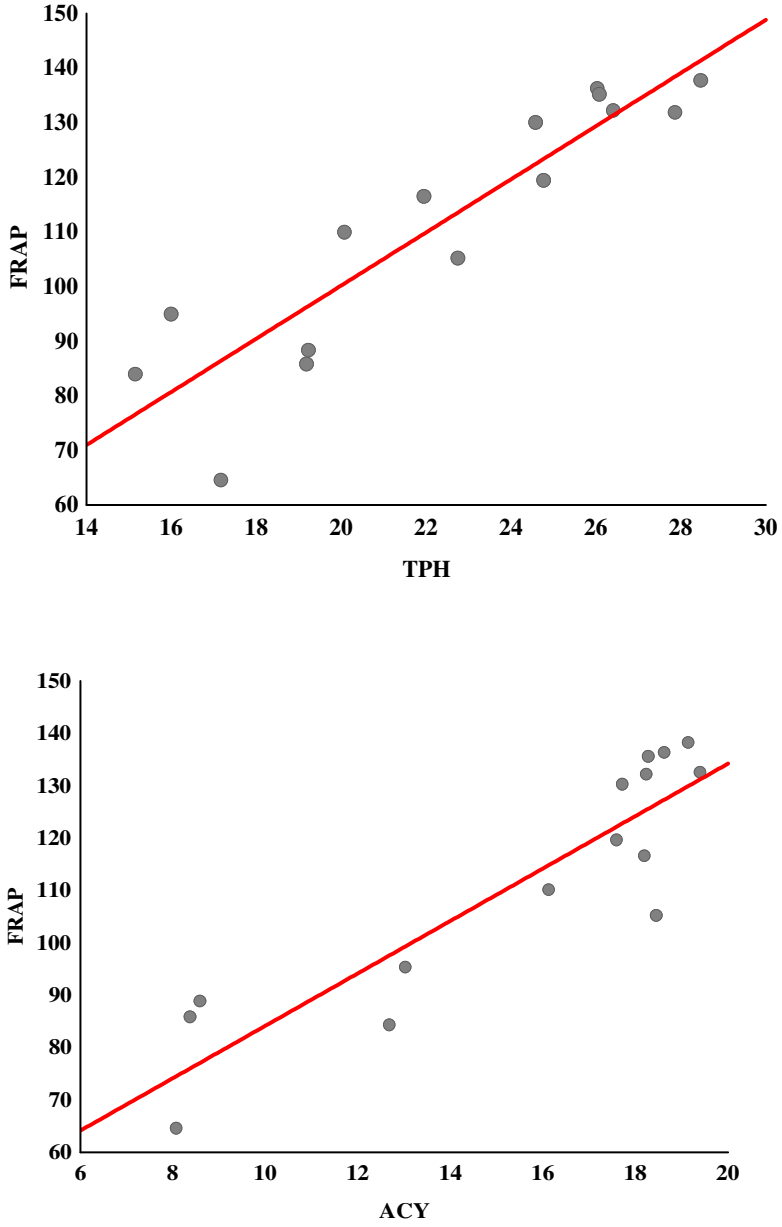


Figure 4.6. Plot of Total phenolics (TPH) and Total anthocyanins (ACY) against the total antioxidant potential (FRAP) values for ethanolic extracts of blueberries.

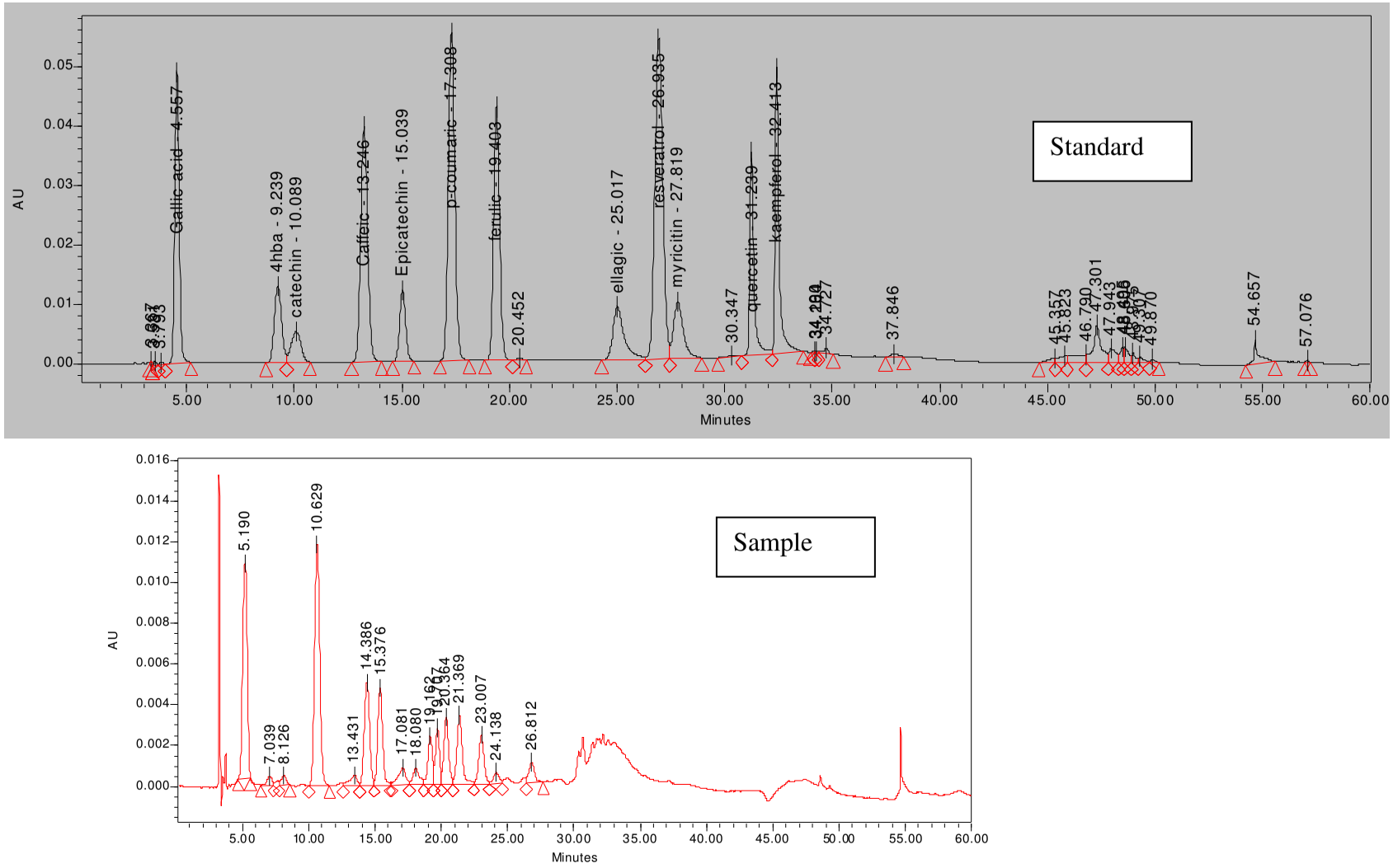


Figure 4.7. HPLC chromatogram of polyphenolic compounds (Standard and Sample) at 280 nm.

References

Box, G.E.P., & D.W Behnken. (1960). Some New Three Level Designs for the Study of Quantitative Variables.*Technometrics,* 2(4), 455-475.

Singleton, V.L., & J.A. Rossi Jr. (1965). Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. *American Journal Of Enology and Viticulture,* 16,144-158.

Giusti, M.M., & R.E. Wrolstad. (2001). Characterization and measurement of anthocyanins by UV-visible spectroscopy. **In** *current protocols in food analytical chemistry;* Wrolstad, R.E., Acree, T.E., An, H., Decker, E.A., Penner, M. H., Reid, D.S., Schwartz, S.J., Shoemaker, C.F., Sporns, P., Eds.; Wiley: New york, pp F1.2.1-F1.2.13.

Benzie, F.F.I., & J.J Strain. (1999). Ferric reducing/antioxidant power assay:direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. In *Methods in enzymology,* 299,15-27.

Cao, G., & R. Prior. (1999). Measurement of oxygen radical absorbance capacity in biological samples. In *Methods in enzymology,* 299,50-62.

Youdim, K.A., Shukit-Hale, B., Martin, A., Wang, H., Denisova, N., Bickford, P. C., & Joseph, J. A. (2000). Short-term dietary supplementation of blueberry polyphenolics: Beneficial effects on aging brain performance and peripheral effects on aging brain performance and peripheral tissue function. *Nutritional neuroscience*, 3, 383-397.

Sun, A.Y., Simoni, A., Sun, G.Y. (2002). The “French paradox” and beyond: neuroprotective effects of polyphenols. *Free radical biology and medicine*, 32(4), 314-318.

Schmidt, B.M., Erdman, Jr, J.W., & Lila, M.A. (2005) Effects of food processing on blueberry antiproliferation and antioxidant activity. *Journal of food science,* 70 (6), S389-S394.

Jayaprakash, G.K., Selvi, T., & Sakariah, K.K. (2003) Antibacterial and antioxidant activities of grape (*Vitis vinifera*) seed extracts. *Food Research International,* 36, 117-122.

Auroma, O.I., Bahorun, T., & Jen, L-S. (2003) Neuroprotection by bioactive components in medicinal and food plant extracts. *Mutation research,* 544, 203-215.

Prior, R.L., Lazarus, S.A., Cao, G., Muccitelli, H., & Hammerstone, J.F. (2001). Identification of procyanidins and anthocyanins in blueberries and cranberries (Vaccinium Spp.) Using High-Performance Liquid Chromatography/Mass spectrometry. *Journal of Agricultural and Food Chemistry*, 49, 1270-1276.

Cao, G., Sofic, E., & Prior, R.L. (1996). Antioxidant capacity of tea and common vegetables. *Journal of Agricultural and Food Chemistry*, 44(11), 3426-3431.

Pastrana-Bonilla, E., Akoh, C. C., Sellappan, S., & Krewer, G. (2003). Phenolic Content and Antioxidant Capacity of Muscadine Grapes. *Journal of Agricultural and Food Chemistry*, 51(18), 5497-5503.

Sellapan, S., Akoh, C.C., & Krewer, G. (2002). Phenolic compounds and antioxidant capacity of Georgia-Grown Blueberries and Blackberries. *Journal of Agricultural and Food Chemistry*, 50, 2432-2438.

Kalt, W., McDonald, J.E., & Donner, H. (2000). Anthocyanins, Phenolics, and Antioxidant capacity of processed lowbush blueberry products. *Journal of Food Science*, 65 (3), 390 – 393.

Lee, J., & Wrolstad, R.L. (2004). Extraction of anthocyanins and polyphenolics from blueberry processing waste. *Journal of food science*, 69(7), C564-C57.

Guendez, R., Kallithraka, S., Makris, D.P., & Kefalas, P. (2005). Determination of low molecular weight polyphenolic constituents in grape seed extracts: Correlation with antiradical activity. *Food Chemistry*, 89(1), 1-9.

Orak, H.H. (2007). Total antioxidant activities, phenolics, anthocyanins, polyphenoloxidase activities of selectd red grape cultivars and their correlations. *Scientia Horticulturae*, 111(3), 235-241.

Giuseppe, R., Renda, A., Amigo, V., Daquino, C., Tringali, C., Spatafora, C., & Tomnasi, N.D. (2007). Polyphenol constituents and antioxidant activity of grape pomace extracts from five Sicilian red grape cultivars. *Food Chemistry*, 100(1), 203-210.

Yilmaz, Y., & Toledo, R.T. (2006). Oxygen radical absorbance capacities of grape/wine industry byproducts and effect of solvent type on extraction of grape seed polyphenols. *Journal of Food Composition and Analysis*, 19(1), 41-48.

Giorgio, S., Tramelli, L., & Faveri, D.M.D. (2007). Effects of extraction time, temperature and solvent on concentration, and antioxidant activity of grape marc phenolics. *Journal Of Food Engineering*, 81(1), 200-208.

Kim, S-Y., Jeong, S-M., Park, W-P., Nam, K.C., Ahn, D.U., & Lee, S-C. (2006). Effect of heating conditions of grape seeds on the antioxidant activity of grape seed extracts. *Food Chemistry*, 97(3), 472-479.

Hakkinen, S., Heinonen, M., Karenlampi, S., Mykkanen, H., Ruuskanen, J., & Torronen, R. (1999). Screening of selected flavonoids and phenolic acids in 19 berries. *Food Research International*, 32(5), 345-353.

Ayaz, F.A., Hayirlioglu-Ayaz, S., Gruz, J., Novak, O., & Strnad, M. (2005). Separation, Characterization, and quantitation of phenolic acids in a little known blueberry (*Vaccinium arcstaphylos L.*) fruit by HPLC-MS. *Journal of Agricultural and Food Chemistry,* 53(21), 8116-8122.

Skrede, G., Wrolstad, R.E., & Durst, R.W. (2000). Changes in anthocyanins and polyphenolics during juice processing of highbush blueberries (*Vaccinium corymbosum L.*). *Journal of Food Science*, 65(2), 357-364.

Cacace, J.E., & Mazza, G. (2003). Optimization of extraction of anthocyanins from black currants with aqueous ethanol. *Journal Of Food Science*, 68(1), 240-248.

Liyana-Pathriana., & Shahidi, F. (2005). Optimizationof extraction of phenolic compounds from wheat using response surface methodology. *Food Chemistry*, 93, 47-56.

CHAPTER 5

INFLUENCE OF FERMENTATION ON THE YIELD OF TOTAL PHENOLICS, ANTHOCYANINS, AND ANTIOXIDANT CAPACITY FROM MUSCADINE GRAPES AND RABBITEYE BLUEBERRIES[1].

1 Biswas, R., Saalia, F.K., & Phillips, R.D. To be submitted in *Food Chemistry,* 2007.

Abstract

Muscadine grapes and Rabbiteye blueberries possess a wide range of phenolics and flavonoids, which have the potential to reduce the occurrence of degenerative diseases like cardiovascular, cancer, ageing, and Alzheimer's. This study optimized wine yeast fermentation conditions for extraction of phenolic compounds from muscadine grapes and Rabbiteye blueberrires. The study followed a Box-Behnken design in which the variables were solids/liquid ratio (100, 200, 300 g of muscadine grapes/200 ml H_2O); predicted alcohol content (7, 11, 15%); and fermentation temperature (20, 25, 30 C). Frozen muscadine grapes and Rabbiteye blueberries were thawed under N_2 in subdued light, macerated with ascorbic acid (0.2g/100g), and blended with water. Sucrose to yield the desired final alcohol content was added and the mixtures depectinized for 3 hours at 60 C with Pectinex BE (100µL/100 g fruit). Rehydrated yeast (0.25 g/L) and yeast nutrient (0.25g/L) were added, and fermentation allowed to proceed for three days in air, then anaerobically until complete. Ferments were centrifuged at 48,000 G for 15 min at 25 C, and supernatants analyzed for ethanol (GC), total phenolics (Folin-Ciocalteaux), total anthocyanins (pH differential), total antioxidant activity (FRAP, ORAC), and individual phenolics (HPLC-DAD). Extraction efficiency decreased as the ratio of berries to water increased. Maximum extraction of the compounds was at 25 C and 11% ethanol. Total phenolics, anthocyanins, antioxidant activity (FRAP, ORAC) were 16-25 (muscadine grapes) and 11.9 – 17.0 (blueberries) g/Kg db gallic acid equivalents, 0.2-1.6 (muscadine grapes) and 0.59-1.68 (blueberries) g/Kg db cyanidine-3 glucoside equivalents, 59-88 (muscadine grapes) and 47.9 – 81.4 (blueberries), 126-233 (muscadine grapes) and 123 – 197 (blueberries)mmoles TROLOX equivalents/Kg db respectively.

While compounds like gallic acid, resveratrol and, caffeic acid showed similar levels as was found in direct aqueous and ethanolic extraction, others like ellagic acid, quercetin, myrecetin, and p-coumaric acid were found to be lower. In addition to facilitating extraction of phenolic antioxidants, fermentation depleted sugars and facilitated drying to produce a dried nutraceutical extract. It would also generate ethanol as solvent and for other applications.

5.1. Introduction

Muscadine grapes and Rabbiteye blueberries, both indigenous to the Southeastern United states are known for their content of polyphenolic compounds (Sellapan et al., 2002; Pastrana-Bonilla et al., 2003;; Talcott et al., 2003; Lee et al., 2004; Yilmaz et al., 2004; Talcott et al., 2005). Polyphenolic compounds have aroused considerable interest among the consumers because of their potential health benefits as reviewed by many researchers (Dell'Agli et al., 2004; Erlund, 2004; Yilmaz et al., 2004; Oak et al., 2005; Ramasammy, 2006; Lotito et al., 2006). The development of a non-sticky, polyphenolic rich powder, which can be marketed, shipped, and ready for consumption either as capsules or as functional food containing the powder as an ingredient is of siginificant interest of research.

Fermentation of muscadine grapes and rabbiteye blueberries is one of the ways to achieve sugarless, polyphenolic extracts. Several researchers have studied on the effects of different fermentation parameters to maximize the extraction of polyphenolic compounds from muscadine grapes and Rabbiteye blueberries. Sims & Bates (1994) conducted a study on the effects of skin fermentation time on the phenols, anthocyanins of muscadine wine. They found that fermenting the grapes at 13 C with Prise de Mousse yeast to 6 % ethanol after six days gave the maximum total phenol content (1301 mg/L) regardless of the type of maturity of the grapes. According to Auw et al., (1996), increasing the fermentation time for the muscadine grapes enhanced the total phenol content from 162 to 1269 mg/L. They fermented muscadine grapes with Prise de Mousse (0.25 g/L) at 25 C for a total period of 14 days to complete dryness. They also found that

the total phenol content in hot pressed juice (60 C) was higher than immediate pressed juice but was lower than the fermented juice. Talcott & Lee (2002) also saw the same trend in total phenolics and total anthocyanins when they fermented muscadine grapes for a period of 7 days at 13 C. They saw an increase of 44 % and 86.5 % in total phenolics and total anthocyanins when compared to juice.

Su & Silva (2006) studied the effects of fermentation on rabbiteye blueberry pomace as shown by the changes in the total phenolic, total anthocyanins contents and total antioxidant capacity. They found that blueberry juice pomace had a higher total phenolic and total anthocyanins content than the wine and the vinegar pomace. Fermentation was done at 13 C to produce 5 – 6 % alcohol. The antiradical or the antioxidant activities were similar in all of these juices made from the three different pomaces. This provides an indirect explanation that the blueberry wine had higher polyphenolic content than blueberry juice. Similar findings were proved by Su & Chien (2007) by performing an experiment to study the effects of with and without skin contact fermentation on the total phenolic, total anthocyanins contents and total antioxidant capacity. They fermented rabbiteye blueberry must with *Saccharomyces cerevisiae* at 13 C to generate 5 – 6 % alcohol content and found that the wine produced by skin contact fermentation gave the highest total phenolic (1150 mg/L), total anthocyanins (99.6 mg/L) and had the highest antioxidant or antiradical activity than juice, without skin fermented juice, and vinegar.

Studies showed that fermentation of berries to 5 – 6 % alcohol content for a period of 7 – 14 days at 13 C had substantially increased total phenolic and total anthocyanins contents. However there were no studies on the effects of other alcohol

concentrations and fermentation temperatures on the total phenolics and total anthocyanins. Alcohol is produced from the grapes at the expense of sugar by *Saccharomyces cerevisiae*. The overall goal of our project is to produce non-sticky polyphenolic powder from muscadine grapes and rabbiteye blueberries. Since our objective is to eliminate the sugar and also to extract the compounds maximally, we investigated the effects of fermentation on the yield of total phenolics, total anthocyanins from muscadine grapes and Rabbiteye blueberries. The specific objectives of this study was to determine the effects of solid:solvent ratio, ethanol concentration (%, v/v), and fermentation temperature on the yield of total phenolics, total anthocyanins, and antioxidant potential from muscadine grapes and rabbiteye blueberries and to estimate the optimum conditions.

5.2. Materials and Methods

5.2.1. Fruits

Muscadine grapes (Supreme variety) were obtained as a gift from Paulk vineyards. Blueberries (Mixed variety) were obtained from a local grower.

5.2.2. Chemicals

Gallic acid, L-ascorbic acid, 6-hydroxy-2, 5, 7, 8-tetramethychroman-2-carboxylic acid (TROLOX); 2, 4, 6-tripyridyl-s-triazine (TPTZ), 6-carboxy fluorescein, and 2,2'-Azobis (2-methylpropionamidine) dihyrochloride (AAPH) were purchased from Sigma (St. Louis, MO). Moisture analysis of the berries was done by a vacumn oven method at 70°C and 25mm Hg for 8 H (AOAC 934.06).

5.2.3. Experimental design

A Box-Behnken design (Box et al., 1960) was used to design the fermentation. The factors were fermentation temperature (10, 20, 30 C), predicted ethanol concentration (7, 11, 15 % v/v) and solid-to-solvent ratio (0.5, 1.0, 1.5).

5.2.4. Fermentation

Frozen berries were thawed under nitrogen (Airgas South Inc. Marietta, GA, USA), macerated with ascorbic acid (0.2g/100g fruit) and incubated with Pectinex BE ® enzyme for three hours at 60°C as was shown to be optimal in previous work. Sugar to yield the desired final ethanol content was added and the mixtures depectinized for 3 hours at 60°C with Pectinex BE (100µL/100 g fruit). Active dry wine yeast, *Saccharomyces bayanus* (Red Star ® , Lesaffre Yeast Corp., Milwaukee, WI) in concentration of 0.25 g/L and yeast nutrient (Red Star ® , Lesaffre Yeast Corp., Milwaukee, WI) in concentration of 0.25g/L were added, and fermentation conducted for three days in air, then anaerobically until complete to dryness in nonagitated condition. The ferments were centrifuged at 48,000 G for 15 min at 25°C in Beckman model J2-21M Refrigerated Centrifuge (Beckmann Coulter, Inc. Fullerton, CA). The supernatants were collected and frozen before further analysis. The entire experiment was performed under subdued light.

5.2.5. Total phenolics

Total phenolics were estimated colorimetrically using the Folin-Ciocalteaux method (Singleton, and Rossi, 1965). A sample aliquot of 200 µL was added to 800 µL of deionized water, 5 mL of Folin-Ciocalteaux reagent, and 4 mL of saturated sodium carbonate solution (75g/L) and mixed on a vortex mixer. The absorbance was measured at 765 nm with a Hewlett Packard 8451A diode array spectrophotometer (Avondale, PA,

USA) after incubation for two hours at room temperature (~25 C). Quantification was based on the standard curve generated with 100, 200, 300, 400, and 500 mg/L of gallic acid. The final concentration of phenolics was calculated based on total volume of extract and initial weight of berries; and expressed as mg/Kg dry weight. The experiment was conducted under yellow light.

5.2.6. Total anthocyanins

Total anthocyanins were measured by the pH differential method (Giusti, and Wrolstad, 2001) using two buffer systems - potassium chloride buffer, pH 1.0 (0.025 M) and sodium acetate buffer, pH 4.5 (0.4 M). Extracts were diluted with water to give Absorbance in the range of 0.1 - 1.2 at 510 nm. Aliquots of 0.2 ml of diluted extracts were mixed with 1.8 mL of each buffer, vortexed and read against a blank at 510 and 700 nm with a Hewlett Packard 8451A diode array spectrophotometer (Avondale, PA, USA). Absorbance was calculated as

$$A = (A_{570}\text{ nm} - A_{710}\text{ nm})\text{ pH 1.0} - (A_{570}\text{ nm} - A_{710}\text{ nm})\text{ pH 4.5}$$

Monomeric anthocyanin pigment concentration in the extract was calculated as cyanidine-3-glucoside

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

Where A = absorbance, MW = molecular weight (449.2), DF = dilution factor, ε = molar absortivity (29,600), l = path length (1 cm).

The final concentration of anthocyanins was calculated based on total volume of extract and weight of starting fruit, and expressed as mg/Kg dry weight.

5.2.7. Assay of antioxidant capacity

The antioxidant capacity was assayed by Ferric reducing/antioxidant power assay (FRAP) and Oxygen radical-scavenging activity (ORAC).

Ferric reducing/antioxidant power assay (Benzie, and Strain, 1996). FRAP reagent was made by mixing 2.5 mL of 10 mM TPTZ in 40mM HCl , 25 mL of 300 mM acetate buffer, pH 3.6, and 2.5 ml of 20 mM FeCl3.6H20. One hundred mL of the diluted sample was added to 3 mL of the working FRAP reagent, vortexed, and allowed to stand for 10mins. Absorbance was measured at 593 nm with a Hewlett Packard 8451A diode array spectrophotometer (Avondale, PA, USA). Quantification was based on the standard curve generated with 100, 200, 300, 400, and 500 mM of TROLOX. The final concentration was expressed as umol of TROLOX equivalents per Kg dry basis. .

Oxygen radical absorbance capacity (Cao and Prior, 1999). A stock solution of 6-carboxy fluorescein (1.5 × 10-3 M) was prepared in 75 mM phosphate buffer, pH 7.0. Working solution was prepared by diluting it with 75 mM phosphate buffer, pH 7.0, to a concentration of 1.5 × 10-4 M. AAPH (320 mM, held at 0 C) was prepared in 75 mM phosphate buffer. The reaction mixture containing, 2.62 mL 75 mM phosphate buffer, pH 7.0, 150 mL fluorescein and 150 mL diluted sample, was incubated at 37 C for 15 mins. To initiate the reaction, 75 mL of AAPH was added to the reaction mixture, vortexed, transferred to thermostated cuvette (37 C) in a spectroflourometer (RF 5301PC Shimadzu scientific instruments Inc., Columbia, MD,USA),and decay of fluorescence measured. Optimal excitation (~325 nm) and emission (~520 nm) wavelengths of standard fluorescein solution (1.5 x 10-5 M) were selected daily using RF-5301PC software. Quantification was based on the standard curve generated with 50, 100, 200,

and 300mM of TROLOX. The final concentration was expressed as mmol of TROLOX equivalents per Kg dry basis. The experiment was conducted under subdued light.

5.2.8. Alcohol Determination

Concentration of Ethanol in the fermented extracts was determined by a gas chromatography method (Varian 3400 GC, Varian, Inc. Palo Alto, CA). The method of determination was adapted from Wang et al., (2003). . The injector and the detector was set at 225 and 285 C. A linear gradient of temperature was followed changing from 45 C to 225 C at the rate of 45 C for two minutes. The carrier gas was Helium (Airgas South Inc. Marietta, GA, USA) with a rate of 40cm/minute at 130 C. The GC column used was SuplecoWax10 30M x 0.25mm x 0.25um Film (Supelco, Bellefonte, PA). The injection syringe used was Hamilton 5ul syringe 7105 (Hamilton, Reno, NV). A standard curve was generated with 5, 50, 95 % ethanol with a internal standard (acetonitrile). The concentration of ethanol in the samples was determined from the standard curve.

5.2.9. Data Analysis

The response surface regression (RSM) procedure was followed using Statistica ® software, Version 6.0 (Statsoft, Tulsa, OK).. Experimental data was used to fit a quadratic polynomial equation to obtain the regression coefficients of the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X^2_i + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$$

where β_0, β_i, β_{ii}, β_{ij} are the regression coefficients for intercept, linear, quadratic, and interaction terms respectively. The independent variables and response variables are symbolically represented as Xi, Xj, and Y respectively. The independent variables were solid solvent ratio (R), solvent concentration (C), and fermentation temperature (T) subscripted as 1, 2, and 3 respectively. The dependent variables were total phenolics, total anthocyanins, and total antioxidant activity (FRAP, and ORAC). Reduced model were developed using SAS version 8. Statistica software was used to generate 3-D surface and contour plots of dependent variables as function of two independent variables. Correlation studies were also conducted to correlate FRAP and ORAC with TPH and ACY respectively.

5.3. Results and Discussions

5.3.1. Total phenolics

Total phenolics in the fermented extracts of muscadine grapes ranged from 16 – 24 g gallic acid equivalents/Kg db. Changes in the total phenolic content with change in the experimental conditions are given in Table 5.1. Response surface regression models were developed for total phenolics using the predicted ethanol concentrations. The model could explain 86.8 % of the variability in the response variable, total phenolics. The model was found to be significant at 8.3 % level of significance and did not have a significant lack of fit, which indicated that the model had a good fit. The coefficients of the regression parameters were given in the Table 5.2. There was a significant linear and quadratic effect of ethanol concentration on the yield of total phenolics at 4.6 % and 2.8 % level of significance. Fermentation temperature had significant linear ($p = 0.11$) and quadratic ($p = 0.10$) effects on the yield of total phenolics. There was no significant

linear and quadratic effect of solid to solvent ratio on the yield of total phenolics. None of the interaction effect made a significant impact on the total phenolics of the fermented extracts of muscadine grapes. Contour plots for total phenolics (TPH) were generated from the regression equation shown below were drawn holding one independent variable as constant.

$$TPH = -35.4647 - 4.7106R + 4.0646C + 3.3119T + 2.3868R^2 - 0.1548C^2 - 0.0639T^2 - 0.1820RC - 0.1024RT - 0.0153CT. \quad (5.1)$$

The plot of total phenolics as a function of solid to solvent ratio and ethanol concentration (Fig 5.1) showed an increasing trend in total phenolics when the solid to solvent ratio deceases to 0.5 and ethanol concentration increases to 11% for muscadine grapes and to 14 % for blueberries. This indicated that the extraction efficiency increased with increasing solvent. By decreasing the solid to solvent ratio, the concentration gradient of total phenolics between the fruit cells and the solvent increased which in turn increases the rate of diffusion of the solid to the solvent. The polyphenolic compounds were found to be more soluble in 11 – 14 % ethanol than in water because of polar and nonpolar characteristics of the molecules. Same results were reported by Cacace et al., (2003). In another plot for the response of total phenolics as a function of fermentation temperature and ethanol concentration (Fig 5.2), we observed the optimum region for total phenolics was at fermentation temperature ranging from 22 – 25 C and ethanol concentration ranging from 11 – 14 % respectively.

Total phenolics in the fermented extracts of blueberries were found ranging from 11.9 – 17.0 g gallic acid equivalents/Kg db. Response surface regression models were developed for total phenolics as a function of solid to solvent ratio, ethanol concentration,

and fermentation temperature. The model was found to be significant with a R square value of 86.3 % at 9.0 % level of significance. It did not have a significant lack of fit, which indicated the adequacy of the model. There was a significant linear and a quadratic effect of fermentation temperature on the yield of total phenolics at 3.5 and 2.5 % level of significance respectively. Both linear and quadratic effect of ethanol concentration did not have a significant effect on the yield of total phenolics. A linear effect of solid to solvent ratio on the yield of total phenolics was also found at 16.9 % level of significance. None of the interaction effect within the independent variables affected the total phenolics significantly. Contour plots were generated from the following regression equation.

$$TPH = -18.6849 - 10.8248R + 0.5664C + 2.9898T + 2.4924R^2 - 0.0164C^2 - 0.0621T^2 + 0.0252RC + 0.1141RT - 0.0043CT. \quad (5.2)$$

Contour plots were drawn to study the changes in the total phenolics with the change in the independent factors (Fig. 5.1, and 5.2). The plot showed that the yield of total phenolics decreased with increase in solid to solvent ratio irrespective of ethanol concentration. The maximum yield of total phenolics was found when the solid to solvent ratio was 0.5. Due to increase in concentration gradient as stated above, the extraction of total phenolics was facilitated. The optimum region for total phenolics was found at the ethanol concentration ranging from 11 to 15 %, v/v. This showed that the polyphenolic compounds present in blueberries were more non-polar than that present in the muscadine grapes. One of the reasons of their solubility in 15 % v/v ethanol concentration than in water could be due to their covalent nature and less energy to break

the water arrangement. The maximum yield of total phenolics was found at a fermentation temperature ranging from 23 to 26 C.

5.3.2. Total anthocyanins

The total anthocyanins (ACY) in the fermented extracts of muscadine grape varied from 3.2 – 7.8 g cyanidin 3 glucoside/Kg db (Table 5.1). Response surface regression model was developed for anthocyanins to predict the optimum regions. The overall model was found to be significant at 3.7 % level of significance. The model was able to explain 90. 8 % of the variability in the data. The model did not have a siginificant lack of fit, which indicated the robustness of the model. Response surface regression equation was developed and the coefficients of regression parameters were given in Table 5.2. There was a significant quadratic effect of ethanol concentration at 14.0 % level of significance. A significant interaction effect of solid to solvent ratio and ethanol concentration on the yield of anthocyanins was found at 18 % level of significance. Contour plots were generated from the following equation.

$$ACY = 4.65463 - 4.77200R + 0.46901C + 0.51914T - 0.41094R^2 - 0.04607C^2 - 0.02033T^2 - 0.31230RC - 0.04843RT - 0.00812CT. \quad (5.4)$$

Contour plots were drawn to see the variation in anthocyanins as a function of any two independent variables holding the third independent variable as constant (Figure 5.3, and 5.4). The plot of anthocyanins as a function of ethanol concentration and solid solvent ratio showed that the optimal region lied within an ethanol concentration of 7 – 12 % and solid to solvent ratio of 0.5 – 0.8. Due to ionic nature of the flavylium cation of anthocyanins, they were found to be more soluble in mixtures of higher amounts of water and lesser amounts of ethanol. Decreasing the solid to solvent ratio increased the yield of

anthocyanins because of the reason as noted above for total phenolics. The higher the solvent, the greater is concentration of the phenolics. However, we need to compromise on the solid solvent ratio depending on the application of final product such as extracts may require removal of solvent which involves cost and introduce complexity of removal process. Fermentation temperature was found to affect the yield of anthocyanins. The optimum region for anthocyanins was found at fermentation temperature ranging from 20 – 21 C. It appeared that anthocyanins were extracted maximum at a lower fermentation temperature than that of the total phenolic.

The total anthocyanins in the fermented extracts of blueberries ranged from 0.59 – 1.68 g cyanidin 3 glucoside equivalents/Kg db (Table 5.1). Response surface regression models were developed to study the linear, quadratic, and interaction effects of solid solvent ratio, ethanol concentration, and fermentation temperature on the yield of total anthocyanins. The model was found to be significant at 9.06 % level of significance and could explain 84.7 % of the variability among the results. It did not have any significant lack of it, which showed the appropriateness of the model. Estimate of regression coefficients were given in Table 5.4. A significant linear and quadratic effect of ethanol concentration on the yield of total anthocyanins was found at 7.7 and 5.2 % level of significance respectively. Solid to solvent ratio had a quadratic effect on the yield of total anthocyanins at 8.9 % level of significance. There was a quadratic effect of fermentation temperature at 16.0 % level of significance. There were no significant interaction effects found within the variables on the yield of total anthocyanins. Contour plots were drawn from the following regression equation.

$$ACY = - 4.99432 + 1.40060R + 0.40502C + 0.28805T - 0.81374R^2 - 0.01525C^2 - 0.00636T^2 - 0.01473RC + 0.00662RT - 0.00022CT. \quad (5.5)$$

Contour plots were drawn for total anthocyanins as a function of two independent variables holding one of the independent variable as constant (Figure 5.3, and 5.4). The optimum region for total anthocyanins was found at an ethanol concentration and solid to solvent ratio ranging from 12 – 14 %, v/v and 0.7 – 1.1 respectively. Increasing the solvent had increased the extraction efficiency of anthocyanins by increasing the rate of diffusion of the anthocyanins in the solvent. The anthocyanins were found to be extracted maximum at a fermentation temperature range of 20 – 26 C. This could be due to release of anthocyanins from the small pieces of blueberry left after fermentation by yeast. This fermentation temperature could be the optimum working temperature for yeast. Contrary to the anthocyanins in muscadine grapes, the anthocyanins in blueberries were extracted at a higher temperature, which showed that they were more thermally stable than that of the muscadine grapes.

5.3.3. Total antioxidant potential (FRAP, ORAC)

The antioxidant potential represented by FRAP values showed that they ranged from 58.8 to 88.2 mmol TROLOX equivalents/Kg db in the fermented extracts of muscadine grapes. The contour plots were generated from the following equation.

$$FRAP = - 108.015 + 24.633R + 16.139C + 6.542T + 17.546R^2 - 0.544C^2 - 0.082T^2 - 0.307RC - 1.906RT - 0.102CT. \quad (5.5)$$

Contour plots for FRAP as a function of ethanol concentration and solid to solvent ratio (Figure 5.1) showed that the antioxidant potential increased with increase in solid to solvent ratio. Antioxidant potential was measured maintaining extract concentration

differences. Higher solid to solvent ratio yielded concentrated extracts, which led to higher antioxidant values. Optimum region for antioxidant activity (FRAP) was found at ethanol concentration ranging from 10.5 – 13.5 %. Our results showed that the total phenolics and the total anthocyanins were extracted maximum at ethanol concentration of 11 – 14 % and 7 – 12 % respectively. We saw that the optimal region of antioxidant activity falls within these two regions. This indicated that both the total phenolics and the total anthocyanins had contributed to the antioxidant activity of the fermented extracts of muscadine grapes. The effect of fermentation temperature on the antioxidant potential (FRAP) was studied from the contour plots (Figure 5.6). The optimum region for the antioxidant potential was found at a fermentation temperature ranging from 20 to 24 C that again falls within the optimum regions of total phenolics and total anthocyanins. Response surface regression models were developed for FRAP. The model was found to be significant and could explain 73.5 % of the variability in the results. The model did not have a significant lack of fit, which revealed the adequacy of the model. Coefficients of regression parameters were determined (Table 5.2.). A significant linear ($p = 0.10$) and quadratic ($p = 0.096$) effect of the ethanol concentration was found. There were no significant linear and quadratic effects solid solvent ratio and fermentation temperature on the antioxidant potential (FRAP). None of the interaction effects made a significant impact on the antioxidant potential. The antioxidant potential of the fermented extracts of muscadine grape were also measured by ORAC values. The ORAC values ranged from 126.3 – 233.3 mmol TROLOX equivalents /Kg db. Response surface plots generated from the regression equation (5.6) were drawn (Figure5.7, and 5.8).

$$FRAP = 242.4393 + 68.5665R - 1.5368C - 2.1827T - 98.5871R^2 - 0.7081C^2 - 0.0624T^2 + 9.5865RC - 0.1728RT + 0.3467CT. \quad (5.6)$$

The antioxidant potential (ORAC) was found to increase with increasing solid to solvent ratio from 0.5 till 1.1 and then decreased irrespective of the ethanol concentration. The optimum region for ORAC was found at an ethanol concentration of 7 – 14 %. The maximum antioxidant potential in that region could be due to the contribution of both total phenolics and total anthocyanins. The antioxidant potential as represented by ORAC was found to be maximum at fermentation temperature ranging from 20 – 21 C. This showed that the major contribution in this region was mainly by anthocyanins. Reduction in the antioxidant activity with rise in temperature could be due to destruction of anthocyanins that might be compensated by the presence of other phenolic compounds, which were extracted more at high temperatures. Response surface regression models were developed for ORAC. The model was not found to be significant with a R square value of 57.7 %. The coefficients of regression parameters were determined and given in Table 5.2. A quadratic effect of solid to solvent ratio was found to have a significant impact on the ORAC values at 18.4 %. None of the other variables did affect the ORAC values significantly. There were no significant interaction effects of the variables on the ORAC value.

The antioxidants potential as represented by FRAP values ranged from 47.9 to 81.4 mmol TROLOX equivalents/Kg db in the fermented extracts of blueberries. Response surface regression model was developed for FRAP. The model was found to be significant at 13 % level of significance and had a R square value of 83.6 %. There was no significant lack of fit for the model. Regression coefficients were determined and

given in Table 5.4. A significant quadratic effect of solid solvent ratio on the antioxidant capacity was found at 9.1 % level of significance. Ethanol concentration had a significant linear effect on the antioxidant potential at 5.3 % level of significance. A linear effect of solid to solvent ratio and a quadratic effect of ethanol concentration on the antioxidant capacity were found at 12.0 and 15.1 % level of significance respectively. A significant interaction effect between solid to solvent ratio and ethanol concentration on the antioxidant potential was found at 15.5 % level of significance. Fermentation temperature was found to have linear ($p = 0.20$) and quadratic ($p = 0.23$) effects on the antioxidant potential. The other two interaction effects did not make a significant impact on the antioxidant potential. Contour plots generated from the regression equation (5.7)

$$FRAP = - 189.664 + 84.640R + 15.810C + 10.209T – 27.633R^2 – 0.350C^2 – 0.179T^2 – 2.666RC + 0.037RT - 0.133CT. \quad (5.7)$$

were drawn with FRAP as a function of solid solvent ratio and ethanol concentration (Figure 5.5) and fermentation temperature and ethanol concentration (Figure 6). Maximum antioxidant activity was shown at solid to solvent ratio range of 0.6 – 1.1. This region was also the optimum region for total anthocyanins which showed that the antioxidant activity was mainly due to total anthocyanins. However, the antioxidant activity was not maximum at solid to solvent ratio of 5.0. This could be because of the diluted extracts and hence did not correlate with the total phenolics. The optimum region for antioxidant activity was found at a ethanol concentration varying from 12.5 – 15 %, v/v and a fermentation temperature ranging from 20 – 25 C. The ORAC value for the fermented extracts of blueberry varied from 123.0 to 197.0 mmmol TROLOX equivalents/Kg db (Table 5.3). Response surface regression model was developed. The

model was found to be significant at 4.8 % level of significance with no significant lack of fit. The model could explain 89.7 % of the variability of the results. The model was also investigated to study the significant linear, quadratic, and interaction effects of solid solvent ratio, ethanol concentration, and fermentation temperature. The regression coefficients were given in the Table 4. A linear and quadratic effect of fermentation temperature on antioxidant capacity was found to be significant at 7.0 and 9.0 % level of significance respectively. None of the other factors had a significant effect on the antioxidant capacity. The interaction between the variables id not have a significant effect on the antioxidant capacity. Contour plots were drawn from the regression equation (5.8) to investigate the optimum region for antioxidant capacity (ORAC) with changes in the independent variables (Figure 7, and 8).

$$ORAC = -247.158 + 41.117R + 5.321C + 27.044T + 1.881R^2 + 0.378C^2 - 0.470T^2 - 2.618RC - 1.207RT - 0.224CT. \quad (5.8)$$

The optimum region for antioxidant potential was found at a ethanol concentration and fermentation temperature of 14 – 15 %, v/v and 20 – 28 C. The optimal region falls within the optimum region for total phenolics and total anthocyanins, which indicated that the antioxidant activity was mainly contributed by total phenolics and total anthocyanins.

5.4.Conclusions

The fermented extracts of both muscadine grapes and blueberries gave sugarless extract with optimum yield of total phenolics, and total anthocyanins. The optimal region of extraction of total phenolics and total anthocyanins for muscadine grapes was found to be at ethanol concentration of 11 – 12 %, v/v and fermentation temperature of 20 – 22 C.

The optimal region for extraction of total phenolics and total anthocyanins in the fermented extracts of blueberry was found at ethanol concentration ranging from 12 – 14 %, v/v and fermentation temperature ranging from 20 – 26 C. For both the fruits, maximum extraction was achieved with increasing amount of solvent with respect to the fixed amount of berries. The study gave valuable information of using fermentation as an approach to develop non-sticky polyphenolic rich powders from muscadine grapes and Rabbiteye blueberries.

Table 5.1. Influence of solid solvent ratio, predicted ethanol concentration, and fermentation temperature on the yield of total phenolics (TPH), and total anthocyanins (ACY) and total antioxidant activity (FRAP, ORAC) in the fermented extracts of muscadine grape.

Sample	Solid to solvent ratio	Predicted (Observed) Ethanol concentration (%, v/v)	Fermentation Temperature (C)	TPH (g/Kg db)	ACY (g/Kg db)	FRAP (mmol/Kg db)	ORAC (mmol/Kg db)
1	0.5	7(7.2)	25.0	21.4	14.0	63.1	212.1
2	1.5	7(6.6)	25.0	18.7	8.3	72.5	126.3
3	0.5	15(15.0)	25.0	22.8	10.6	78.6	188.9
4	1.5	15(15.5)	25.0	18.6	10.0	85.6	179.7
5	0.5	11(11.3)	20.0	23.6	15.7	69.5	178.3
6	1.5	11(11.1)	20.0	18.5	14.6	88.2	171.7
7	0.5	11(10.6)	30.0	24.5	7.3	84.5	202.2
8	1.5	11(10.9)	30.0	18.4	7.1	84.2	193.9
9	1.0	7(7.3)	20.0	19.2	13.5	71.6	233.3
10	1.0	15(15.9)	20.0	20.7	12.8	82.3	222.6
11	1.0	7(6.8)	30.0	16.3	6.4	58.8	163.2
12	1.0	15(12.3)	30.0	16.5	6.9	61.3	180.2
13	1.0	11(10.9)	25.0	21.8	13.8	85.1	232.9
14	1.0	11(10.9)	25.0	21.5	10.0	75.2	210.3
15	1.0	11(10.7)	25.0	23.5	13.4	77.4	195.1

Table 5.2. Regression coefficients of predicted quadratic polynomial models for the response total phenolics (TPH), total anthocyanins (ACY), total antioxidant activity (FRAP and ORAC) in fermented extracts of muscadine grapes.

Coefficient	TPH		ACY		FRAP		ORAC	
		P		p		p		p
β_0	-35.4647	0.230028	4.65463	0.742625	-108.015	0.462119	242.4393	0.654816
Linear								
β_1	-4.7106	0.689225	-4.77200	0.443699	24.633	0.689174	68.5665	0.766360
β_2	4.0646	0.046384	0.46901	0.582099	16.139	0.102022	-1.5368	0.961593
β_3	3.3119	0.111715	0.51914	0.583903	6.542	0.498923	-2.1827	0.950977
Quadratic								
β_{11}	2.3868	0.496722	-0.41094	0.816839	17.546	0.350235	-98.5871	0.184529
β_{22}	-0.1548	0.028716	-0.04607	0.140236	-0.544	0.096396	-0.7081	0.511005
β_{33}	-0.0639	0.106961	-0.02033	0.281066	-0.082	0.652240	-0.0624	0.926236
Crossproduct								
β_{12}	-0.1820	0.661474	0.31230	0.183062	-0.307	0.886563	9.5865	0.268053
β_{13}	-0.1024	0.756747	0.04843	0.776660	-1.906	0.296651	-0.1728	0.978687
β_{23}	-0.0153	0.711633	0.00812	0.704526	-0.102	0.638135	0.3467	0.671217
F	3.655958	0.083609	5.487214	0.037708	1.540779	0.330102	0.758510	0.662070
Rsquare	0.868086		0.908063		0.734987		0.577224	

The coefficients were related to the following variables,

β_1, β_2, and β_3 : Regression Coefficients for linear Solid to solvent ratio, Ethanol Concentration, and Fermentation Temperature.

β_{11}, β_{22}, and β_{33} : Regression Coefficients for quadratic Solid to solvent ratio, Ethanol Concentration, and Fermentation Temperature.

β_{12}, β_{13}, and β_{23} : Regression Coefficients for interaction of Solid to solvent ratio and Ethanol Concentration, Solid to solvent ratio and Fermentation Temperature, and Ethanol Concentration and Fermentation Temperature.

Table 5.3. Influence of solid solvent ratio, predicted ethanol concentration, and fermentation temperature on the yield of total phenolics (TPH), and total anthocyanins (ACY) and total antioxidant activity (FRAP, ORAC) in the fermented extracts of blueberries

Sample	Solid to solvent ratio	Predicted (Observed) Ethanol concentration (%, v/v)	Fermentation Temperature (C)	TPH (g/Kg db)	ACY (g/Kg db)	FRAP (mmol/Kg db)	ORAC (mmol/Kg db)
1	0.5	7(7.3)	25	16.5	0.80	47.9	157.9
2	1.5	7(7.0)	25	13.9	0.71	61.9	151.1
3	0.5	15(13.8)	25	17.0	1.20	77.8	196.9
4	1.5	15(16.3)	25	14.6	0.98	70.5	169.2
5	0.5	11(11.1)	20	15.6	1.28	64.2	154.1
6	1.5	11(11.3)	20	12.1	0.95	62.6	149.1
7	0.5	11(10.5)	30	15.7	1.03	68.5	158.9
8	1.5	11(11.4)	30	13.3	0.77	67.3	141.9
9	1	7(7.1)	20	13.3	0.89	61.1	123.4
10	1	15(14.7)	20	14.9	1.35	80.9	190.6
11	1	7(6.8)	30	11.9	0.59	58.4	131.5
12	1	15(11.1)	30	13.2	1.04	67.5	180.8
13	1	11(11.0)	25	14.0	1.14	68.9	169.5
14	1	11(11.4)	25	15.7	1.30	81.4	154.1
15	1	11(11.4)	25	15.6	1.68	80.9	163.2

Table 5.4.

Regression coefficients of predicted quadratic polynomial models for the response total phenolics (TPH), total anthocyanins (ACY), total antioxidant activity (FRAP and ORAC) in fermented extracts of blueberries.

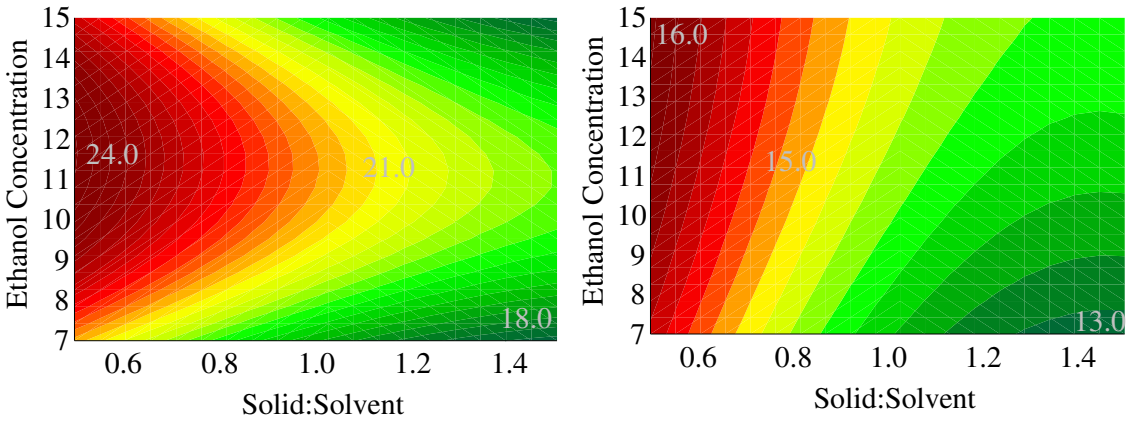
Coefficient	TPH		ACY		FRAP		ORAC	
		P		p		p		p
β_0	-18.6849	0.289168	-4.99432	0.165460	-189.664	0.132544	-247.158	0.225097
Linear								
β_1	-10.8248	0.169648	1.40060	0.336284	84.640	0.120197	41.117	0.613917
β_2	0.5664	0.572239	0.40502	0.077822	15.810	0.053452	5.321	0.637880
β_3	2.9898	0.035156	0.28805	0.216280	10.209	0.203997	27.044	0.070859
Quadratic								
β_{11}	2.4924	0.263492	-0.81374	0.088991	-27.633	0.091643	1.881	0.936414
β_{22}	-0.0164	0.618000	-0.01525	0.052786	-0.350	0.151806	0.378	0.329724
β_{33}	-0.0621	0.025793	-0.00636	0.160417	-0.179	0.234714	-0.470	0.090486
Crossproduct								
β_{12}	0.0252	0.919714	-0.01473	0.763699	-2.666	0.154994	-2.618	0.375691
β_{13}	0.1141	0.574549	0.00662	0.865494	0.037	0.978136	-1.207	0.599502
β_{23}	-0.0043	0.862980	-0.00022	0.964454	-0.133	0.440374	-0.224	0.444122
F	3.500213	0.090624	3.092271	0.113326	2.836607	0.131713	4.875627	0.047910
Rsquare	0.863021		0.847702		0.836224		0.897710	

The coefficients were related to the following variables,

β_1, β_2, and β_3 : Regression Coefficients for linear Solid to solvent ratio, Ethanol Concentration, and Fermentation Temperature.

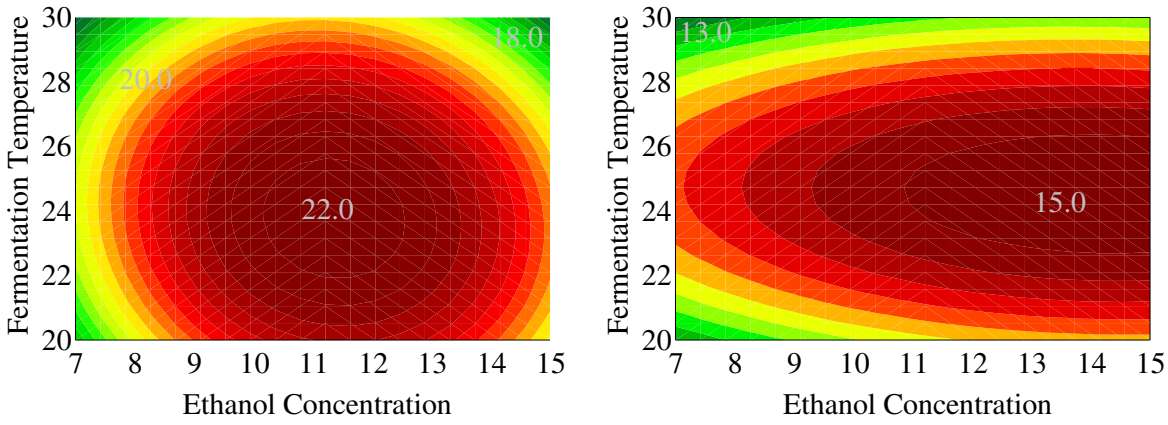
β_{11}, β_{22}, and β_{33} : Regression Coefficients for quadratic Solid to solvent ratio, Ethanol Concentration, and Fermentation Temperature.

β_{12}, β_{13}, and β_{23} : Regression Coefficients for interaction of Solid to solvent ratio and Ethanol Concentration, Solid to solvent ratio and Fermentation Temperature, and Ethanol Concentration and Fermentation Temperature.



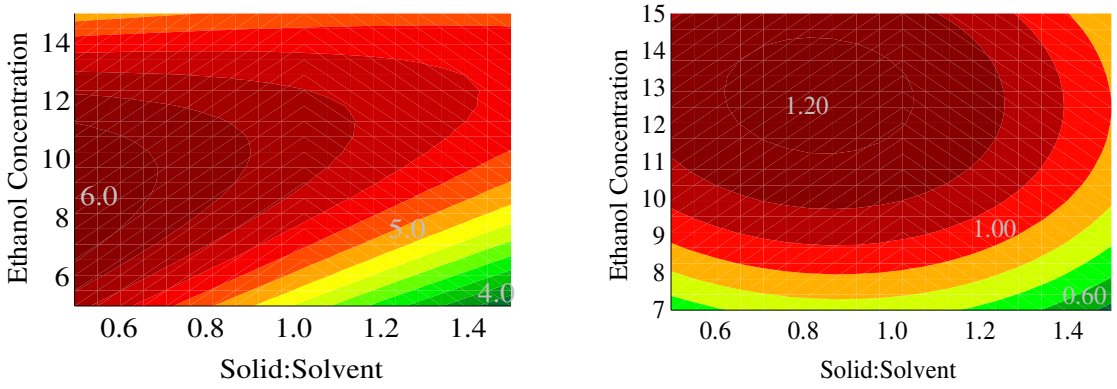
(a) (b)

Figure 5.1. Effects of ethanol concentration (%, v/v) and solid:solvent ratio on the yield of total phenolics in fermented extracts of muscadine grape (a) and blueberries (b)



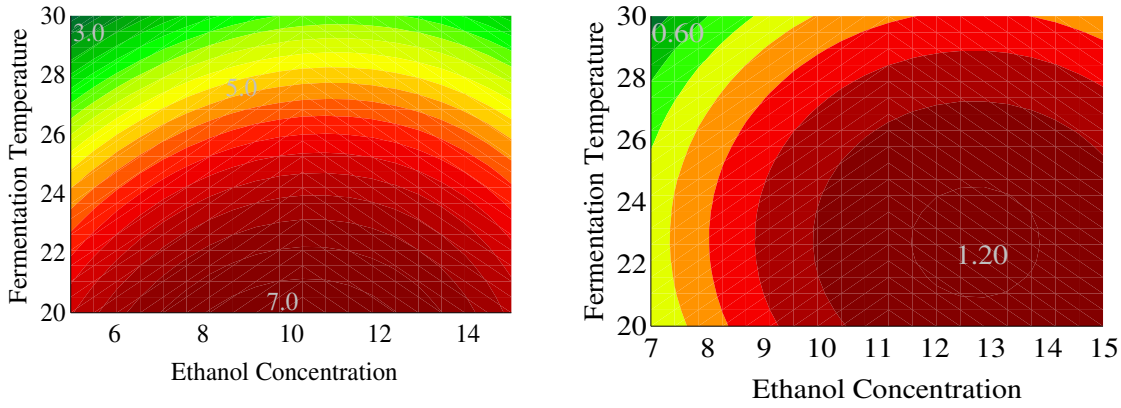
(a) (b)

Figure 5.2. Effects of fermentation temperature (C) and ethanol concentration (%, v/v) on the yield of total phenolics in fermented extracts of muscadine grape (a) and blueberries (b)



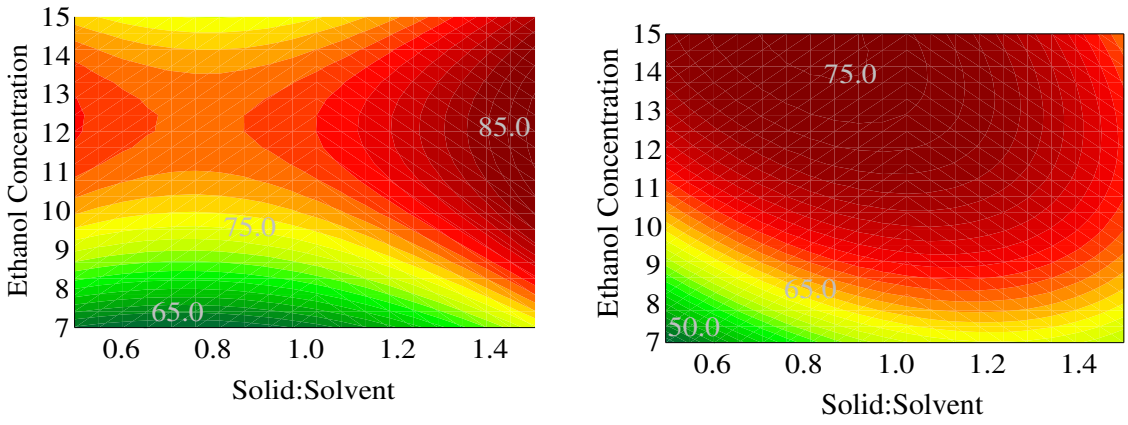
(a) (b)

Figure 5.3. Effects of ethanol concentration (%, v/v) and solid:solvent ratio on the yield of total anthocyanins in fermented extracts of muscadine grape (a) and blueberry (b).



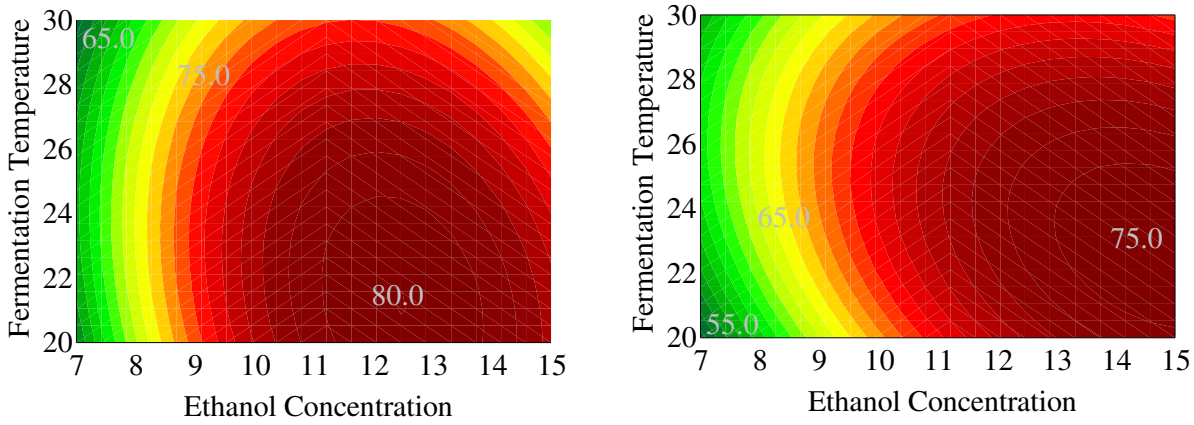
(a) (b)

Figure 5.4. Effects of fermentation temperature (C) and ethanol concentration (%, v/v) on the yield of total anthocyanins in fermented extracts of muscadine grape (a) and blueberry (b).



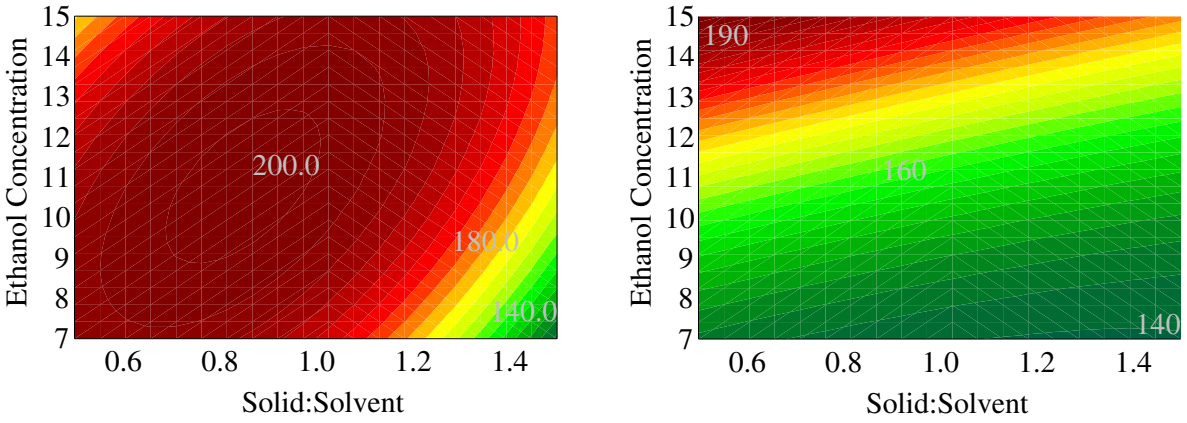
(a) (b)

Figure 5.5. Effects of ethanol concentration (%, v/v) and solid:solvent ratio on the antioxidant potential (FRAP) in fermented extracts of muscadine grape (a) and blueberry (b).



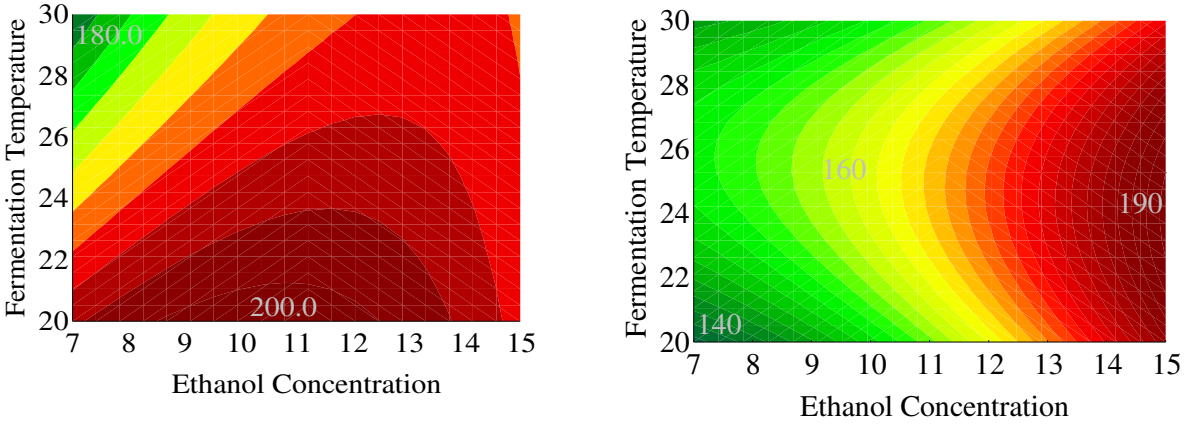
(a) (b)

Figure 5.6. Effects of fermentation temperature (C) and ethanol concentration (%, v/v) on the antioxidant potential (FRAP) in fermented extracts of muscadine grape (a) and blueberry (b).



(a) (b)

Figure 5.7. Effects of ethanol concentration (%, v/v) and solid:solvent ratio on the antioxidant potential (ORAC) in fermented extracts of muscadine grape (a) and blueberry (b).



(a) (b)

Figure 5.8. Effects of fermentation temperature (C) and ethanol concentration (%, v/v) on the antioxidant potential (ORAC) in fermented extracts of muscadine grape (a) and blueberry (b).

References

Auw, J.M., Blanco, V., O'Keefe, S.F., & Sims, C.A. (1996). Effect of processing on the phenolics and color of cabernet sauvignon, chambourcin, and noble wines and juices. *American Journal of Enology and Viticulture,* 47(3),279 – 286.

Benzie, F.F.I., & Strain, J.J. (1999). Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. **In** *Methods in Enzymology,* 299,15-27.

Bonilla-Pastrana, E., Akoh, C.C., Sellapan, S., & Krewer, G. (2003) Phenolic content and antioxidant capacity of muscadine grapes. *Journal of Agricultural and Food Chemistry,* 51,5497 – 5503.

Box, G.E.P., & D.W Behnken. (1960). Some New Three Level Designs for the Study of Quantitative Variables.*Technometrics,* 2(4), 455-475.

Cacace, J. E., & Mazza, G. (2003). Optimization of extraction of anthocyanins from black currants with aqueous ethanol. *Journal of Food Science*, 68(1),240-248.

Cao, G., & R. Prior. (1999). Measurement of oxygen radical absorbance capacity in biological samples. In *Methods in Enzymology,* 299,50-62.

Dell'Agli, M., Busciala, A., & Bosisio, E. (2004). Vascular effects of wine polyphenols. *Cardiovascular Research,* 63(4), 593-602.

Erlund, I. (2004). Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology. *Nutrition Research*, 24(10), 851-874.

Giusti, M.M & R.E.Wrolstad. (2001). Characterization and measurement of anthocyanins by UV-visible spectroscopy. **In** current protocols in food analytical chemistry. pp F1.2.1-F1.2.13. Wrolstad, R.E., Acree, T.E., An, H., Decker, E.A., Penner, M. H., Reid, D.S., Schwartz, S.J., Shoemaker, C.F., Sporns, P., Eds. Wiley: New york, NY.

Lee, J-H., Johnson, J.V., & Talcott, S.T. (2005). Identification of Ellagic Acid Conjugates and Other Polyphenolics in Muscadine Grapes by HPLC-ESI-MS. *Journal of Agricultural and Food Chemistry,* 53(15),6003 –6010.

Lee, J.-H. Talcott, S. T.(2004). Fruit Maturity and Juice Extraction Influences Ellagic Acid Derivatives and Other Antioxidant Polyphenolics in Muscadine Grapes. *Journal of Agricultural and Food Chemistry,* 52(2),361-366.

Lotito, S.B., & Frei, B. (2006). Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: Cause, consequence, or epiphenomenon? *Free Radical Biology and Medicine*, 41(12), 1727-1746.

Oak, M-H., Bedoui, J.E., & Schini-Kerth, V.B. (2005). Antiangiogenic properties of natural polyphenols from red wine and green tea. *The Journal of Nutritional Biochemistry*, 16(1), 1-8.

Ramassamy, C. (2006). Emerging role of polyphenolic compounds in the treatment of neurodegenerative diseases: A review of their intracellular targets *European Journal of Pharmacology, 545(1), 51-64.*

Sellapan, S., Akoh, C.C., & Krewer, G. (2002). Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. *Journal of Agricultural and Food Chemistry,* 50,2432 – 2438.

Sims, C. A., & Bates, R. P. (1994). Effects of skin fermentation time on the phenols, anthocyanins, ellagic acid sediment, and sensory characteristics of a red Vitis rotundifolia wine. *American Journal of Enology and Viticulture,* 45(1),56-62.

Singleton, V.L., & J.A. Rossi. Jr. (1965). Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. *American Journal of Enology and Viticulture,* 16,144-158.

Su, M-S., & Chien, P-J. (2007). Antioxidant activity, anthocyanins, and phenolics of rabbiteye blueberry (Vaccinium ashei) fluid products as affected by fermentation. *Food Chemistry,* (In press).

Su, M-S., & Silva, J.L. (2006). Antioxidant activity, anthocyanins, and phenolics of rabbiteye blueberry (Vaccinium ashei) by-products as affected by fermentation. *Food Chemistry,* 97(3), 447-451.

Talcott, S. T. Brenes, C. H. Pires, D. M. Del Pozo-Insfran, D. (2003). Phytochemical Stability and Color Retention of Copigmented and Processed Muscadine Grape Juice. *Journal of Agricultural and Food Chemistry,* 51(4), 957-963.

Talcott, S.T., & Lee, J-H. (2002). Ellagic acid and Flavonoid Antioxidant Content of Muscadine Wine and Juice. *Journal of Agricultural and Food Chemistry,* 50,3186 – 3192.

Wang, M-L., Choong, Y-M., Su, N-W., & Lee, M-H. (2003). A rapid method for determination of ethanol in alcoholic beverages using capillary gas chromatography. *Journal of Food and Drug Analysis*, 11(2), 133-140.

Yilmaz, Y., & Toledo, R.T. (2004). Health aspects of functional grape seed constituents. *Trends in Food Science & Technology,* 15(9), 422-433.

CHAPTER 6

EXTRACTION AND STABILITY OF TOTAL PHENOLICS AND TOTAL ANTHOCYANINS FROM A COMMERCIAL MUSCADINE SKIN/SEED BYPRODUCT[1]

1 Biswas, R., Adams, J.A., & Phillips, R.D. To be submitted in *Phytochemistry,* 2007.

Abstract

Muscadine grapes are known to be an excellent source of phenolic antioxidants, localized mainly in the skins and seeds. Industrial processing (cold pressing with or without pectinase treatment) results in a byproduct consisting of the skins and seeds. The overall goal of this research is to develop an integrated process for producing polyphenolic antioxidant powders suitable for marketing as nutraceuticals. This requires several operations, including extraction and drying. The objective of this study was (1) to determine optimal ethanol concentration for extracting antioxidants from a commercial muscadine skins/seeds byproduct, and (2) to examine the stability of TPH and ACY as affected by time, temperature and ethanol concentration as might be encountered during ethanol removal.

A muscadine skins/seeds byproduct from juice making was obtained from Paulk Vineyards, Wray, GA and kept frozen until used. Skins containing ascorbic acid (500:1 w/w) were ground in a Hobart food grinder. Twenty-five gram samples of the resulting paste were extracted under subdued light with 100ml of aqueous ethanol (0, 14.8, 35.4, 47.3, 59.9, or 78.0% w/w) for 1 h at 60 C, and solids removed by centrifugation at 48000xG. The resulting extracts were subjected to a stability study in which the other factors were temperature (35, 64, 78 C), and time of 0, 0.5, 1.0, 1.5, 2 h. Experiments were done in duplicate. Extracts were analyzed before and after heat treatment for TPH and ACY.

ACY and TPH concentrations of extracts ranged 1.75 to 2.40 g/kg and 7.5-13 g/kg (dry basis), respectively, with maximal values obtained in the 35-47% ethanol range. In the stability study, ACY declined slightly with increasing temp (35-74 C), TPH

appeared to increase with temp from 35 to 64C, then stabilize, and time had little effect on ACY or TPH.

This study shows that a significant amount of antioxidants can be isolated from muscadine skins/seeds remaining after previous juice extraction. Simulated distillation conditions revealed that removing ethanol under reduced pressure (equivalent to T<65C) resulted in higher retention of ACY and TPH, although cost effectiveness of vacuum distillation remains to be determined.

6.1. Introduction

Byproducts of grape fruits industries are of great importance because of its economic profitability. Residues are available in large quantities and may be expensive to dispose. There is a growing interest in minimizing wastes by utilization of the residues. The grape industry produces different processed products, such as wines, juices, jams, jellies, raisins, canned grapes; and byproducts as grape skins and seeds. The by products are used as cattle feed (Lawrence, 1991; Santiago et al., 1993), sources of dietary fiber (Valiente et al., 1995), fertilizers and sources of natural colors (Francis, 1992). The wine industry produces very large amounts of these byproducts, amounting to ~5-7 million tones per year as estimated from an annual use of 43 million tones of grapes for wine production (Jackson, 1994).

Recent reports showed that the byproducts of grape industry such as grape skins and seeds are a rich source of polyphenolic compounds (Kammerer et al., 2005; Lu et al., 1999; Meyer et al., 1998; Louli et al., 2004; Munoz et al., 2004; Selga et al., 2004; Yilmaz et al., 2004; Pastrana-Bonilla et al., 2003). These compounds are biosynthesized by plants as secondary metabolites through the phenyl propanoid pathway. They comprise of simple phenols, phenylpropanoids, benzoic acid derivatives, flavonoids, stilbenes, tannins, lignins, and lignans (Shahidi et al., 2004). Flavonoids and benzoic acid derivatives are reported to have health benefits (Cook et al., 1996; Rice-Evans et al., 1996). They have been demonstrated to exhibit a wide range of biological effects such as antibacterial, antiviral, anti-inflammatory, and antiallergic (Cook et al., 1996; Rice-Evans et al., 1996). In addition to that they also take part in inhibiting lipid peroxidation, platelet aggregation, and cyclooxygenase activity. Studies indicated an inverse

correlation between flavonoids and diseases like cancer, cardiovascular, Alzheimer (Hollman et al., 1997).

These protective effects are attributed to their antioxidant capacity and subsequently their ability to scavenge free radicals, the main cause of these diseases (Rice-Evans et al., 1996). The presence of o-dihydroxy structure in the B-ring, 2, 3 double bond in conjugation with a 4-oxo function in the C ring, or 3- and 5-OHgroups with 4 oxo function makes them a powerful free radical scavenger (marked as red and bold in Fig. 6.1). Flavonoids are more powerful radical scavenger than hydroxy benzoic acids. In case of hydroxy benzoic acids, the number of hydroxyl groups, presence of methyl group affects the radical scavenging power (Fig. 6.1). However, these compounds can act as prooxidants generating reactive free radicals (Rice-Evans et al., 1996; Galati et al., 2002).

These compounds occur as glycosides, which make them more water-soluble. Due to their water solubility they are stored in cell vacuole (Monagas et al., 2005). The maximum recovery of these bioactive compounds from the skins and seeds is a challenge to food scientists.

Many factors like solvent composition, particle size, extraction temperature, extraction time, solvent to solid ratio, solvent pressure affects the extraction process. Literature search of the past extraction method shows use of pectinase, and cellulase (Kammerer et al., 2005; Munoz et al., 2004), water and cysteamine hydrochloride (Selga et al., 2004), ethyl acetate followed by supercritical carbon-dioxide (Louli et al., 2004), different pomace particle size; enzymes followed by 70% acetone/ pure water (Meyer et

al., 1998), 80% methanol (Pastrana-Bonilla et al., 2003), 80 % ethanol (Lu et al., 1999), and 70 % methanol (Yilmaz et al., 2004).

The extracts have to be concentrated and dried to get a final product, which questions the stability of these compounds. The stability of these compounds is influenced by structure and concentration of the compounds, pH, temperature, light intensity and quality, presence of copigments, metallic ions, enzymes, oxygen, ascorbic acid, sugars, other degradation products, and sulphur dioxide (Cevallos-Casals et al., 2004). Removal of solvents from the extracts at specific temperature for specific time under reduced pressure can affect the integrity of the polyphenolic compounds.

Muscadine grapes are native to South eastern United States. Hot humid climates are favorable for the growth of muscadine grapes. The fruits are round with a thick tough skin surrounding it (California Rare Fruit Growers, Inc., 1999). Georgia is the largest producer of muscadine grape, covering 1100 acres each year (Georgia Agricultural Resources., 2002). Reports indicated that muscadine grape skins and seeds are rich sources of polyphenolic compounds (Yilmaz et al., 2004; Pastrana-Bonilla et al., 2003).

Optimization of parameters affecting the extraction and stability of polyphenolic compounds can be done efficiently using statistical procedures. Response surface methodology (RSM) (Liyana-Pathriana et al., 2005) helps us to develop profile of these compounds as affected by several independent factors. The main advantages of using response surface methodology is the decrease in the number of experimental trials needed to evaluate multiple parameters and their interactions, thus making it less labor intensive and time consuming. The basic principle of RSM is to develop a model, relating the dependent and the independent variables and generate the parameters of the model.

RSM has been used successfully to model and optimize extraction processes of phenolic compounds (Liyana-Pathriana et al., 2005; Cacace et al., 2003), β-carotene (Sanal et al., 2005), and Vitamin E (Lee et al., 2000) among other processes.

The current study aims at efficient extraction and stability of these polyphenolic compounds from muscadine skins and few seeds at different temperatures. Since ethanol is cheap and safe to use, it is chosen as extracting solvent. Ethanol can be evaporated from the extracts under reduced pressure at different temperatures. Optimization study relating the yield, stability of these compounds from muscadine grape pomace and ethanol to water ratio, distillation temperature, and time was conducted. Optimal conditions were achieved using response surface methodology.

6.2. Materials and methods

Chemicals. Folin-Ciocalteaux reagent, ethanol (95%), sodium carbonate, potassium chloride, hydrochloric acid, sodium acetate, acetic acid were purchased from Sigma (St. Louis, MO).

Samples. Grape skins with few seeds were provided by Paulk vineyards, Wray, GA, where they had been stored frozen since harvest and pressing. The samples were randomly selected from the bulk.

Extraction. The samples were thawed under nitrogen gas (Airgas South Inc. Marietta, GA, USA). Skins containing added ascorbic acid (500:1 w/w) were ground in a Hobart food grinder (Hobart Corp. Troy, OH). Twenty-five gram samples of the resulting paste were extracted under subdued light with 100ml of aqueous ethanol (0, 14.8, 35.4, 47.3, 59.9, or 78.0% w/w) for 1 h at 60C, and solids removed by centrifugation at 48000xG. The resulting extracts were subjected to a stability study in

which the other factors were temperature (35, 64, 78C), and time of 0, 0.5, 1.0, 1.5, 2 h. Experiments were conducted in duplicate. All the experiments were conducted in subdued light. Extracts were kept frozen under Nitrogen at –13 C before analysis for total phenolics and total anthocyanins.

Total Phenolics. Total phenolics were estimated colorimetrically using the Folin-Ciocalteaux method (Singleton et al., 1965). A sample aliquot of 200 µL was added to 800 µL of deionized water, 5 mL of Folin-Ciocalteaux reagent, and 4 mL of saturated sodium carbonate solution (75g/L) and mixed in vortex (Fisher vortex Genie 2, Fisher Scientific, Pittsburgh, PA, USA). The absorbance was measured at 765 nm with a Hewlett Packard 8451A diode array spectrophotometer (Avondale, PA, USA) after incubation for two hours at room temperature (~25 C). Quantification was based on the standard curve generated with 100, 200, 300, 400, and 500 mg/L of gallic acid. The final concentration of phenolics was calculated based on total volume of extract and weight and expressed as mg/Kg dry weight. The experiment was conducted under subdued light.

Total Anthocyanins. Total anthocyanins were measured by the pH differential method (Giusti et al., 2001) using two buffer systems – potassium chloride buffer, pH 1.0 (0.025 M) and sodium acetate buffer, pH 4.5 (0.4 M). A diluted sample of 0.2 mL (to give optical density in the range of 0.1 – 1.2 at 510 nm) was mixed with 1.8 mL of corresponding buffer in vortex (Fisher vortex Genie 2, Fisher Scientific, Pittsburgh, PA, USA) and read against a blank at 510 and 700 nm with a Hewlett Packard 8451A diode array spectrophotometer (Avondale, PA, USA).

Absorbance was calculated as

$$A = (A_{570\ nm} - A_{710\ nm})_{pH\ 1.0} - (A_{570\ nm} - A_{710\ nm})_{pH\ 4.5}$$

Monomeric anthocyanin pigment concentration in the extract was calculated as cyaniding-3-glucoside.

Monomeric anthocyanin pigment (mg/L) = $A \times MW \times DF \times 1000/(\varepsilon \times 1)$

Where A = absorbance, MW = molecular weight (449.2), DF = dilution factor, ε = molar absortivity (29,600). The final concentration of anthocyanins was calculated based on total volume of extract and weight and expressed as mg/Kg dry weight. The experiment was conducted under subdued light.

Selection of influencing variables. Three different ethanol distillation temperatures were chosen. Different times ranging from 0 to two hours were chosen expecting that the time required to evaporate off the ethanol will fall within that range.

Experimental design. Optimization of extraction and stability study was conducted by RSM. A full factorial design with three independent factors such as time (0, 0.5,1.0, 1.5, 2.0 H), temperature (35, 64, 78 C), and ethanol to water ratio (0, 14.8, 35.4, 47.3, 59.9, 78.0 % w/w) was employed to fit a quadratic polynomial equations. The experiment was conducted in duplicates. The order of the experiments was randomized to avoid the effects of unexplained variability due to external factors. The independent variables were time (X1, H), distillation temperature (X2, C), and extracting solvent composition (X3, % w/w, ethanol/water). The response variables were total phenolics (TPH) and total anthocyanins (ACY).

Data Analysis. The response surface regression procedure was followed using Statistica ® software, Version 6.0 (Statsoft, Tulsa, OK).Experimental data was used to fit a quadratic polynomial equation to obtain the regression coefficients of the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X^2_i + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$$

where β_0, β_i, β_{ii}, β_{ij} are the regression coefficients for intercept, linear, quadratic, and interaction terms respectively. The independent variables and response variables are symbolically represented as X_i, X_j, and Y respectively. The software was used to generate 3-D surface and contour plots of dependent variables as function of two independent variables.

Model verification. Optimal conditions were obtained from the predictive equations of RSM. Nine different combinations of the independent variables, which include points of optimum, yield of total phenolics, and total anthocyanins were chosen for the verification experiments. A paired t-test was done to compare the observed and predicted values using Statistica.

6.3. Results and discussion

Fitting the models. Optimization of the parameters influencing the extraction and stability of the polyphenolic compounds was tried using RSM. The quadratic polynomial equation was fitted to the experimental data (Table 6.1). The ANOVA results of TPH and ACY and the corresponding regression coefficients are given in Table 6.2. The model was robust and was self-explanatory. The analysis of variance table was shown in Table 6.3. The results indicate that the linear and quadratic terms of temperature, ethanol had a significant effect on the dependent variables at 5 and 0.1% level of significance respectively. Among the crossproduct terms, the product of time and temperature had a

significant effect on the dependent variables ($p < 0.05$). Thus ethanol concentration had a prominent effect on the dependent variables. Linear effect of time influence the TPH at 5% level of significance however didn't influence the ACY at all. In contrast, the quadratic effect of time was significant at 5 % level for the model of ACY, but not for TPH. It seems that ethanol and temperature were the important variables in the model. Both the models were found to be significant at 0.00 % level of significance and had a R square value of 88.38 % and 79.2 % for total phenolics and total anthocyanins respectively. Both the models of total phenolics and total anthocyanins did not have a significant lack of fit ($p = 0.99$), which indicated the adequacy of the models. Similarly ethanol concentrations had a significant effect on extraction of the polyphenolic compounds from natural sources (Liyana-Pathriana et al., 2005; Cacace et al., 2003).

Effect of ethanol on yield of TPH and ACY. The relationship between ethanol and temperature holding time constant for both TPH and ACY are illustrated in the response surfaces and two dimensional contour plots (Fig. 6.2, 6.4). It was shown that the maximum extraction of these compounds increased with increase in ethanol percentage followed by decrease with its further increase. Maximum extraction was achieved between 30 and 50 %, w/w and 15 and 50 %, w/w ethanol for total phenolics and anthocyanin respectively. This increase in the extraction of these compounds is attributed to the change in the polarity of the extraction solvents, which facilitated the extraction of these compounds from the pomace matrix. Research indicated that there is a maximum extraction of these polyphenolic compounds from wheat between 50 and 60 % ethanol (Liyana-Pathriana., 2005). There are other reports also stating the increase in the yield of total phenolics with increase in ethanol concentration to 60 % (Biswas et al.,

2007; Cacace et al. 2003). Changes in the ethanol concentrations alter the density, dynamic viscosity and dielectric constant of the solvent. Anthocyanins are considerably more polar molecules than phenolic acids than flavonoids. With increase in ethanol concentrations, there is a decrease in dielectric constant of the solvent. Thus the less polar flavonoids are more soluble in high ethanol concentrations (~ 45 %, w/w), whereas anthocyanins are more soluble at ~ 35 %, w/w ethanol concentrations respectively. Due to their insufiicient polar nature, they do not possess enough energy to break the water arrangement and are thus insoluble in water. The maximum yields of total phenolics and total anthocyanin was found to be 50 and 8.8 g/Kg db respectively. These yields from the muscadine skins were higher than reported (Pastrana-Bonilla et al., 2003).

Effect of temperature on stability of TPH and ACY in ethanolic extracts.

From the ANOVA (Table 6.3), we see that there is a significant linear effect of temperature on the stability of total phenolics and total anthocyanins at 4 % level of significance. A quadratic effect of temperature was found significant on total phenolics and total anthocyanins at 0.0002 % and 0.4 % level of significance respectively. A significant interaction between the ethanol concentration and temperature on the total phenolics and total anthocyanins was found at 10.0 % and 0.00 % level of significance respectively. As can be seen from Fig. 6.2 and 6.3, TPH increased with increase in temperature till ~ 74° C, and then it started decreasing. High temperatures inactivated these enzymes, which resulted in lesser degradation of these compounds. As a result an increase in the yield of TPH was observed at higher temperatures. However, too high temperature caused thermal degradation of these compounds and subsequently decreased the yield. Temperature seemed to have a prominent effect on the stability of ACY. The

anthocyanins were stable untill ~40° C, and then they started degrading. These findings tell us that these compounds are highly unstable at high temperatures. The stability of these compounds is enhanced by the degree of copigmentation. Various compounds in the extracts serve as copigments for intermolecular association with ACY. Sugars and their degradation products do not contribute to copigmentation. High temperature results in Maillard browning reactions with the production of furfural and others. Furfural inhibits copigmentation, which in turn destabilize ACY. The decrease in the stability of ACY from muscadine pomace extracts with rise in temperature was contributed to the degradation of copigmented forms. Past research also indicated the decrease in the stability of ACY with increase in temperature (Cevallos-Casals et al., 2004; Gomez-Plaza et al., 2006).

Effect of time on stability of TPH and ACY in ethanolic extracts. Time affected the stability of TPH and ACY in the ethanolic extracts of muscadine skins (Fig. 6.3, 6.5.). There was a decrease in the stability of these compounds with increase in time at different temperatures. The ethanolic extracts held for shorter time periods had stable TPH and ACY than those held for longer time periods at specific temperatures. Longer time periods did considerable thermal degradation which deteriorated the stability of TPH and ACY.

Model verification. Nine different random experiments were performed corresponding to different time, temperature and ethanol concentration. Two of them were chosen at the optimum levels obtained from the corresponding response surface and contour plots. The observed and the predicted values of TPH and ACY are shown in the Table 6.4. The paired t-tests analysis showed that the predicted values of TPH and ACY

were successfully achieved within 0.1 and 5 % confidence levels (Table 6.5.). These paired t-tests proved the validity and the robustness of the two models.

6.4. Conclusions

The significant level of these models indicates that they can be employed to achieve the optimum condition for extraction and stability of polyphenolic compounds from muscadine skins. Optimum conditions for maximum extraction and stability of these compounds were 1 h, 40 C, 40 %, w/w. The vacuum distillation of ethanol can be conducted at 40 C thereby ensuring the maximum stability of these compounds.

Table 6.1.

Experimental data for the dependent variables, Total Phenolics (TPH) and Total Anthocyanins (ACY) in aqueous ethanolic extracts of muscadine skins as affected by time, distillation temperature and ethanol/water ratio

Time (H)	Temp (°C)	% Ethanol (%, w/w)	TPH (g/Kg db)	ACY (g/Kg db)
0	35	78	35.609	6.655
0	35	78	33.003	6.719
0	35	59.9	45.253	7.852
0	35	59.9	40.31	7.105
0	35	47.3	48.101	8.117
0	35	47.3	47.16	8.35
0	35	35.4	50.304	8.68
0	35	35.4	47.617	8.608
0	35	14.8	36.576	7.635
0	35	14.8	39.128	8.342
0	35	0	28.275	6.984
0	35	0	26.636	7.056
0	64	78	34.455	6.389
0	64	78	33.713	6.285
0	64	59.9	43.515	8.013
0	64	59.9	38.008	6.976
0	64	47.3	47.995	8.182
0	64	47.3	45.858	7.844
0	64	35.4	47.689	8.68
0	64	35.4	47.992	8.278
0	64	14.8	36.185	7.74
0	64	14.8	37.325	8.069
0	64	0	26.463	6.783
0	64	0	24.753	6.759
0	78	78	34.753	6.542
0	78	78	35.414	6.522
0	78	59.9	44.526	7.571
0	78	59.9	38.138	7.165
0	78	47.3	48.47	8.358
0	78	47.3	44.653	7.736
0	78	35.4	48.114	8.238
0	78	35.4	43.635	7.129
0	78	14.8	37.12	7.639
0	78	14.8	36.611	7.788
0	78	0	27.652	6.96
0	78	0	24.903	6.59

Table 6.1 (contd.).

Experimental data for the dependent variables, Total Phenolics (TPH) and Total Anthocyanins (ACY) in aqueous ethanolic extracts of muscadine skins as affected by time, distillation temperature and ethanol/water ratio

Time (H)	Temp (°C)	% Ethanol (%, w/w)	TPH (g/Kg db)	ACY (g/Kg db)
0.5	35	78	27.687	6.787
0.5	35	78	36.361	6.823
0.5	35	59.9	37.69	7.643
0.5	35	59.9	40.471	7.466
0.5	35	47.3	40.29	7.856
0.5	35	47.3	46.381	8.013
0.5	35	35.4	41.483	8.226
0.5	35	35.4	46.838	8.463
0.5	35	14.8	30.603	7.703
0.5	35	14.8	38.644	8.366
0.5	35	0	22.292	7.048
0.5	35	0	26.851	7.008
0.5	64	78	35.015	6.655
0.5	64	78	32.973	5.979
0.5	64	59.9	44.05	7.715
0.5	64	59.9	40.084	6.558
0.5	64	47.3	48.453	7.957
0.5	64	47.3	45.46	7.306
0.5	64	35.4	49.14	8.294
0.5	64	35.4	48.162	7.796
0.5	64	14.8	35.702	7.434
0.5	64	14.8	37.439	7.691
0.5	64	0	26.514	6.647
0.5	64	0	26.829	6.325
0.5	78	78	34.6	5.819
0.5	78	78	32.971	5.65
0.5	78	59.9	43.889	7.024
0.5	78	59.9	38.723	6.337
0.5	78	47.3	48.267	7.635
0.5	78	47.3	43.482	7.105
0.5	78	35.4	50.634	7.547
0.5	78	35.4	44.144	7.093
0.5	78	14.8	36.611	7.06
0.5	78	14.8	36.254	7.257
0.5	78	0	27.601	6.393
0.5	78	0	23.987	6.152

Table 6.1 (contd.).

Experimental data for the dependent variables, Total Phenolics (TPH) and Total Anthocyanins (ACY) in aqueous ethanolic extracts of muscadine skins as affected by time, distillation temperature and ethanol/water ratio

Time (H)	Temp (°C)	% Ethanol (%, w/w)	TPH (g/Kg db)	ACY (g/Kg db)
1	35	78	27.793	6.55
1	35	78	35.26	7.024
1	35	59.9	39.761	7.836
1	35	59.9	40.471	7.129
1	35	47.3	40.471	8.121
1	35	47.3	46.005	8.35
1	35	35.4	41.725	8.37
1	35	35.4	46.972	8.093
1	35	14.8	30.225	7.663
1	35	14.8	38.591	8.399
1	35	0	22.247	7
1	35	0	26.824	6.639
1	64	78	35.473	6.446
1	64	78	35.362	5.867
1	64	59.9	43.948	7.611
1	64	59.9	39.288	6.462
1	64	47.3	47.944	7.924
1	64	47.3	45.233	7.322
1	64	35.4	48.631	8.278
1	64	35.4	48.39	7.724
1	64	14.8	36.745	7.378
1	64	14.8	37.638	7.531
1	64	0	26.947	6.574
1	64	0	25.947	6.285
1	78	78	35.542	5.988
1	78	78	32.745	5.497
1	78	59.9	44.653	6.936
1	78	59.9	38.188	6.112
1	78	47.3	47.554	7.217
1	78	47.3	43.457	6.586
1	78	35.4	47.911	7.45
1	78	35.4	43.711	6.936
1	78	14.8	36.89	6.755
1	78	14.8	36.127	7.093
1	78	0	26.659	6.265
1	78	0	24.14	5.421

Table 6.1 (contd.).

Experimental data for the dependent variables, Total Phenolics (TPH) and Total Anthocyanins (ACY) in aqueous ethanolic extracts of muscadine skins as affected by time, distillation temperature and ethanol/water ratio

Time (H)	Temp (°C)	% Ethanol (%, w/w)	TPH (g/Kg db)	ACY (g/Kg db)
1.5	35	78	28.155	6.63
1.5	35	78	34.749	6.566
1.5	35	59.9	37.645	7.808
1.5	35	59.9	43.346	7.482
1.5	35	47.3	40.199	8.061
1.5	35	47.3	46.757	8.085
1.5	35	35.4	41.786	8.266
1.5	35	35.4	47.187	8.479
1.5	35	14.8	29.697	7.86
1.5	35	14.8	39.182	8.358
1.5	35	0	22.413	7.089
1.5	35	0	27.442	6.976
1.5	64	78	34.633	5.939
1.5	64	78	34.537	5.923
1.5	64	59.9	44.228	7.748
1.5	64	59.9	38.434	6.116
1.5	64	47.3	48.071	8.053
1.5	64	47.3	44.806	7.201
1.5	64	35.4	48.86	8.407
1.5	64	35.4	49.755	7.74
1.5	64	14.8	36.185	7.41
1.5	64	14.8	26.829	5.232
1.5	64	0	27.66	6.518
1.5	64	0	25.293	6.092
1.5	78	78	34.702	6.526
1.5	78	78	33.149	5.545
1.5	78	59.9	44.22	6.912
1.5	78	59.9	39.308	5.73
1.5	78	47.3	47.809	7.189
1.5	78	47.3	41.446	6.325
1.5	78	35.4	48.369	7.374
1.5	78	35.4	43.737	6.377
1.5	78	14.8	35.72	6.634
1.5	78	14.8	36.916	6.61
1.5	78	0	26.888	5.927
1.5	78	0	23.758	5.324

Table 6.1. (contd.)

Experimental data for the dependent variables, Total Phenolics (TPH) and Total Anthocyanins (ACY) in aqueous ethanolic extracts of muscadine skins as affected by time, distillation temperature and ethanol/water ratio

Time (H)	Temp (°C)	% Ethanol (%, w/w)	TPH (mg/Kg db)	ACY (g/Kg db)
2	35	78	28.745	6.74
2	35	78	34.883	7
2	35	59.9	37.298	7.62
2	35	59.9	40.686	7
2	35	47.3	40.818	8.27
2	35	47.3	46.22	8.31
2	35	35.4	42.299	8.41
2	35	35.4	47.456	8.14
2	35	14.8	29.757	7.68
2	35	14.8	39.558	8.63
2	35	0	22.383	7.11
2	35	0	27.737	6.84
2	64	78	33.92	6.45
2	64	78	34.225	5.86
2	64	59.9	44.482	7.63
2	64	59.9	39.8	6.41
2	64	47.3	47.307	7.82
2	64	47.3	45.034	7.27
2	64	35.4	48.224	8.18
2	64	35.4	49.642	7.83
2	64	14.8	35.855	7.31
2	64	14.8	36.415	7.26
2	64	0	26.285	6.42
2	64	0	25.663	6.12
2	78	78	34.218	5.88
2	78	78	33.327	5.35
2	78	59.9	44.347	6.86
2	78	59.9	39.359	5.82
2	78	47.3	47.631	7.02
2	78	47.3	43.457	6.4
2	78	35.4	47.936	7.25
2	78	35.4	43.584	5.78
2	78	14.8	35.974	6.46
2	78	14.8	37.603	6.41
2	78	0	26.532	5.81
2	78	0	23.605	5.25

Table 6.2.

Regression coefficients of the predicted quadratic model for the response Total phenolics (TPH) and Total anthocyanins (ACY) in aqueous ethanolic extracts of muscadine skins

Coefficient	TPH	ACY
β_0	20487.17***	653.2223***
Linear		
β_1	-3528.61**	-0.966
β_2	254.59**	3.6579**
β_3	900.33***	6.1471***
Quadratic		
β_{11}	674.36	18.1214**
β_{22}	-2.38**	-0.0447**
β_{33}	-10.64***	-0.0923***
Crossproduct		
β_{12}	25.82	-1.18***
β_{13}	2.58	0.1981
β_{23}	0.39	0.0066
Rsquare	0.883855	0.792679

** Significant at 5 %

*** Significant at 0.1 %

The coefficients were related to the following variables,

β_1, β_2, and β_3 : Regression Coefficients for linear Distillation time, Distillation temperature, and Extracting ethanol concentration.

β_{11}, β_{22}, and β_{33} : Regression Coefficients for quadratic Distillation time, Distillation temperature, and Extracting ethanol concentration.

β_{12}, β_{13}, and β_{23} : Regression Coefficients for interaction of Distillation time and Distillation temperature, Distillation time and Extracting ethanol concentration, and Distillation temperature and Extracting ethanol concentration.

Table 6.3.

Analysis of variance for the extraction and stability variables as linear, quadratic and crossproduct on the response Total phenolics (TPH) and Total anthocyanins (ACY) in aqueous ethanolic extracts of muscadine skins

Regression	DF	SS	MS	F	p
TPH					
X_1	1	4.392495E+07	4.392495E+07	6.004	0.015288
X_2	1	3.191057E+07	3.191057E+07	1.958	0.163547
X_3	1	4.117480E+09	4.117480E+09	4.362	0.038246
X_{11}	1	1.432515E+07	1.432515E+07	4.899	0.028211
X_{22}	1	3.583827E+07	3.583827E+07	562.794	0.000000
X_{33}	1	7.788760E+09	7.788760E+09	1064.600	0.000000
X_{12}	1	1.923333E+07	1.923333E+07	2.629	0.106788
X_{13}	1	4.142125E+05	4.142125E+05	0.057	0.812213
X_{23}	1	6.201864E+06	6.201864E+06	0.848	0.358509
Model	9	9.464796E+09	1.051644E+09	110.8262	0.00
Residual	170	1.243743E+09	7.316137E+06		
Lack of fit	80	389721574	4871520	0.513379	0.998686
Pure Error	90	854021769	9489131		
Total	179	1.070854E+10			
ACY					
X_1	1	3.3	3.3	0.0021	0.963470
X_2	1	6587.4	6587.4	4.2096	0.041729
X_3	1	191942.8	191942.8	122.6588	0.000000
X_{11}	1	10344.1	10344.1	6.6103	0.010996
X_{22}	1	12678.6	12678.6	8.1021	0.004966
X_{33}	1	586497.3	586497.3	374.7943	0.000000
X_{12}	1	40185.9	40185.9	25.6804	0.000001
X_{13}	1	2441.3	2441.3	1.5601	0.213370
X_{23}	1	1754.8	1754.8	1.1214	0.291124
Model	9	1017132	113014.6	55.11121	0.00
Residual	170	266024.7	1564.851		
Lack of fit	80	81464.87	1018.311	0.496576	0.999197
Pure Error	90	184559.8	2050.665		
Total	179	1283156			

X_1: time(H),
X_2: temperature(°C)
X_3: ethanol(% w/w)

Table 6.4.

Comparison of the observed and predicted values of Total Phenolics and Total anthocyanins

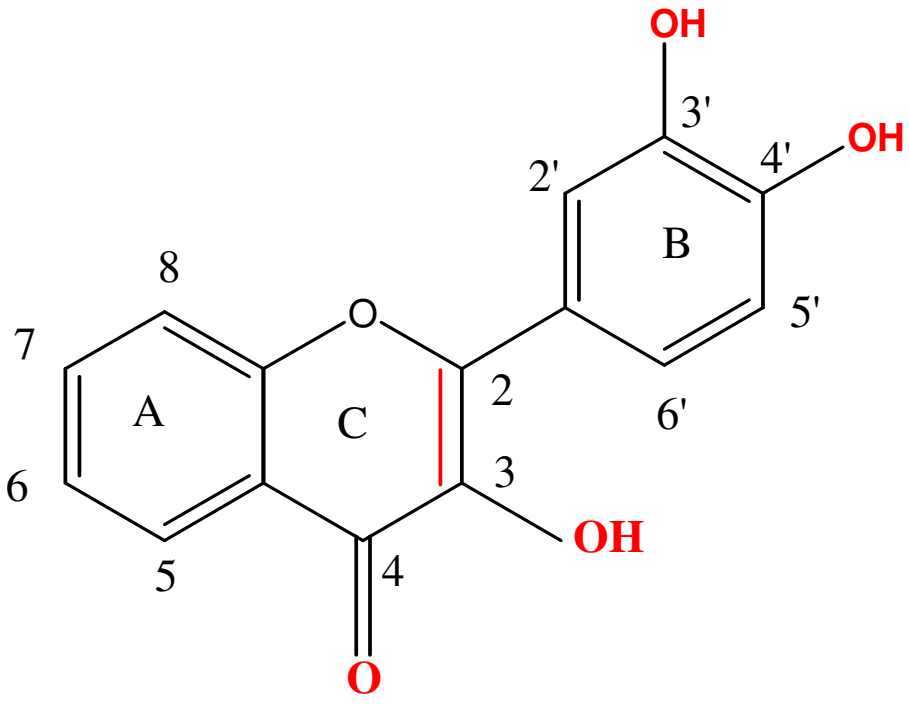
Time (H)	Temp (°C)	% Ethanol (%,w/w)	Total phenolics		Anthocyanin	
			Observed	Predicted	Observed	Predicted
1.4	35	16.9	41174	36602	753.9	788.9
1.9	40	64.3	43770	39784	612.4	756.6
0.5	44	43.2	50947	45898	765.5	815.8
1.8	48	31.5	47664	43837	737.4	804.0
1.3	51	27.8	48631	43007	791.2	790.7
0.6	55	20.5	45170	40734	793.6	783.2
0.7	58	39.3	50387	46080	777.2	794.7
0.9	61	51.4	48962	45476	742.6	760.9
1.6	68	55.6	48274	44576	699.6	710.6
0.5	72	24.1	44482	42056	748.6	754.7

Table 6.5.

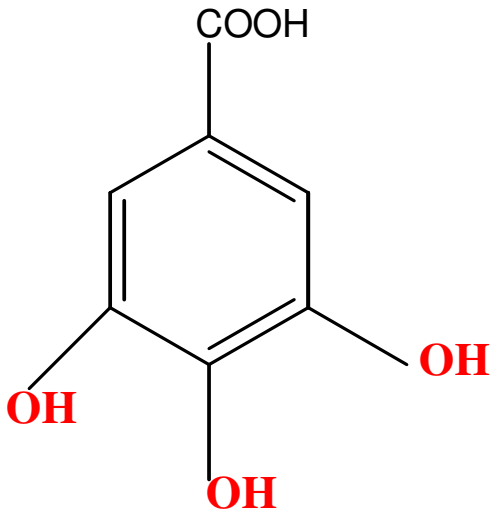
Paired t-tests analysis of the observed and predicted values of total phenolics and total anthocyanins in the ethanolic extracts of muscadine skins.

Difference	DF	t-value	p
TPHobs - TPHpre	9	14.84	<.0001
ACYobs - ACYpre	9	-2.36	0.0426

TPH: Total phenolics
ACY: Total anthocyanins



a



b

Fig. 6.1. Skeleton of flavan nucleus (a) and hydroxy benzoic acid (b).

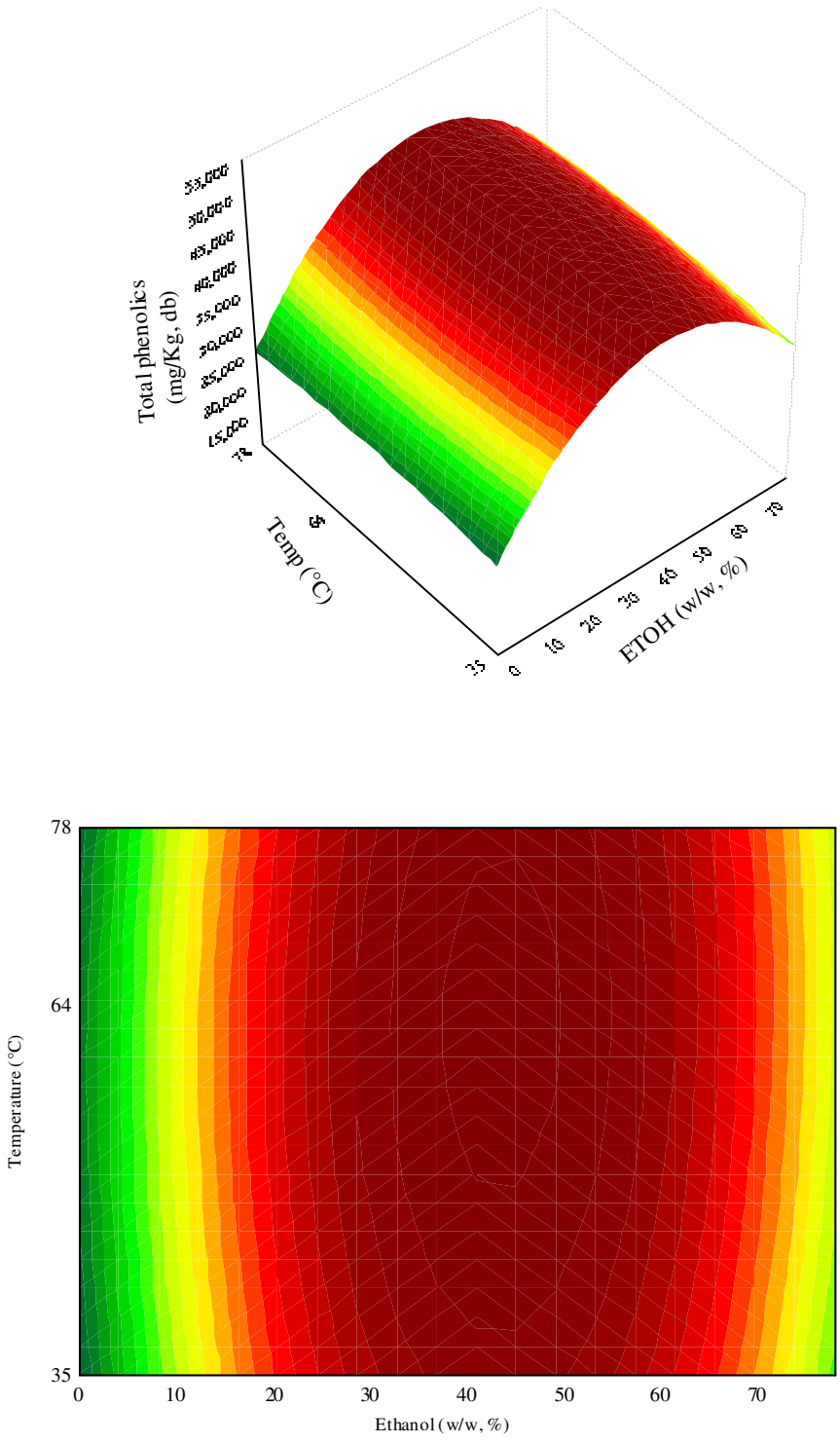


Fig. 6.2. Response surface and contour plots for the effects of temperature and ethanol concentration holding time constant on total phenolics in muscadine pomace.

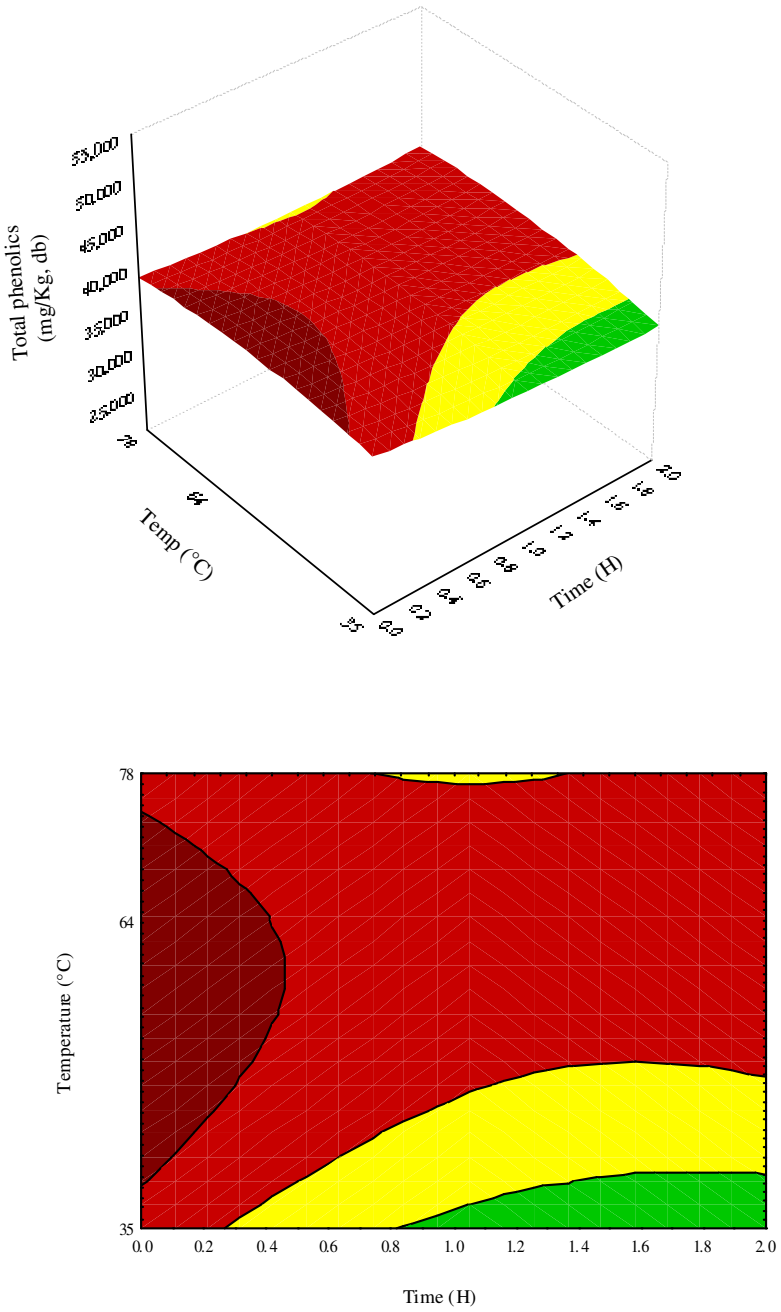


Fig. 6.3. Response surface and contour plots for the effects of simulated distillation temperature and time holding ethanol constant on total phenolics in muscadine pomace.

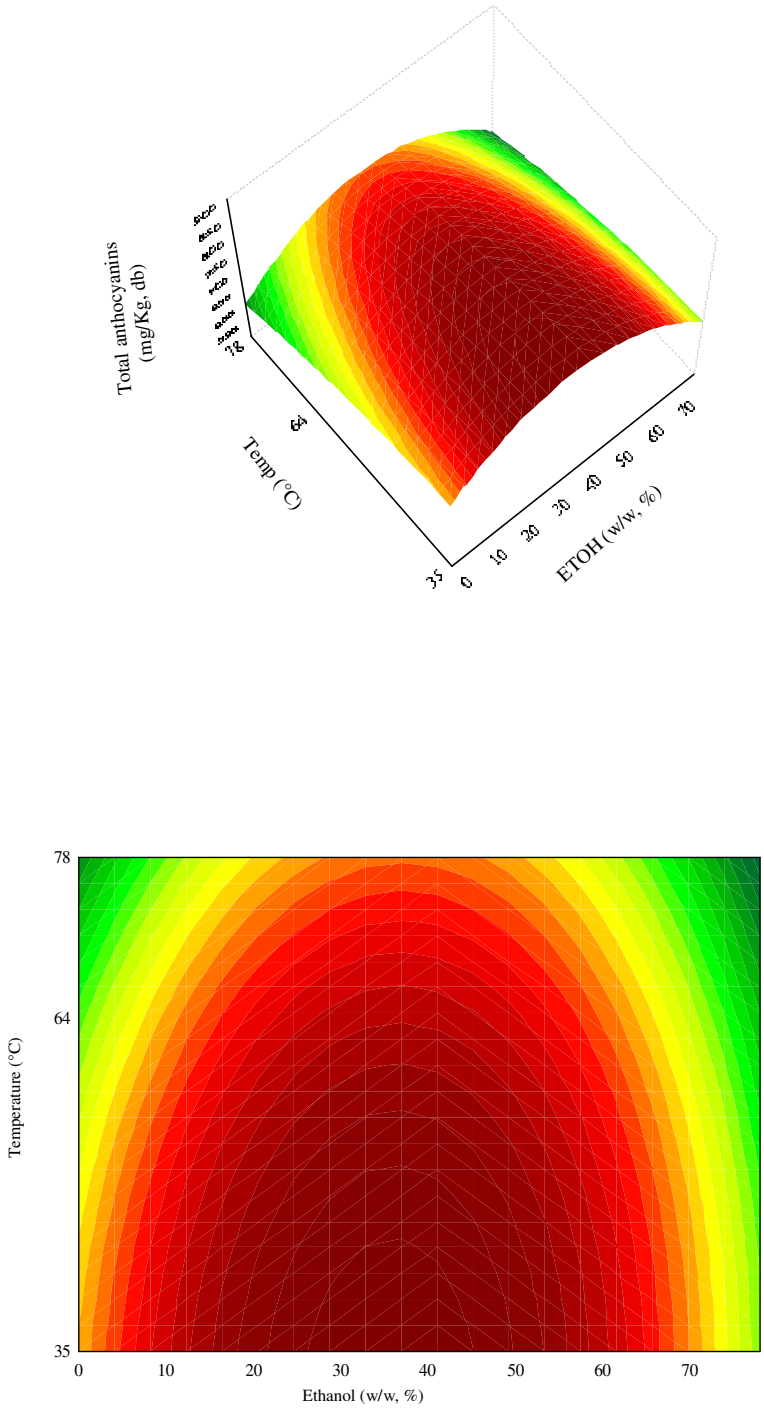


Fig. 6.4. Response surface and contour plots for the effects of temperature and ethanol holding time constant on anthocyanin in muscadine pomace.

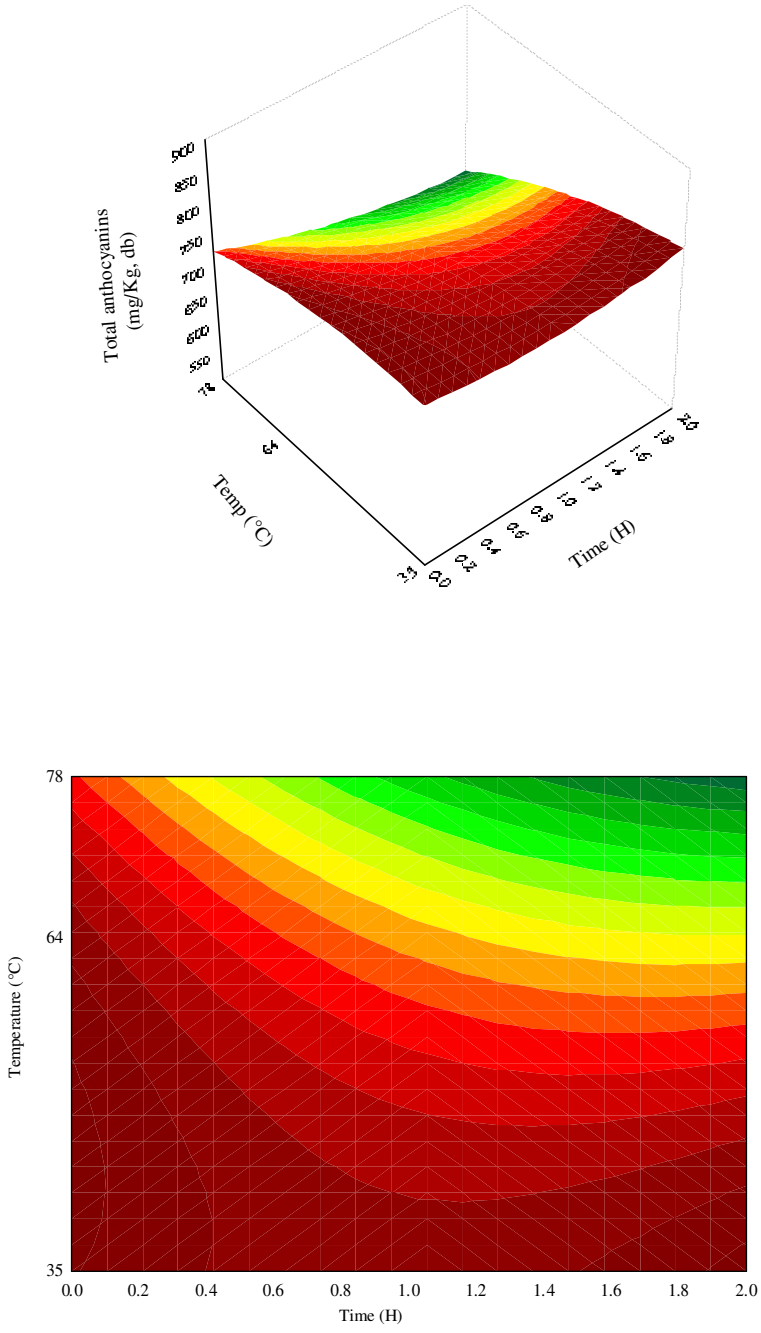


Fig. 6.5. Response surface and contour plots for the effects of simulated distillation temperature and time holding ethanol constant on anthocyanin in muscadine pomace.

References

Biswas, R., Saalia, F.K., Phillips, R.D. 2007Influence of ethanol concentrations, extraction temperature, and time on the yield of total phenolics, total anthocyanins, and antioxidant potential in aqueous extracts from Georgia-grown muscadine grapes and rabbiteye blueberries. Chapter 4 In Development of technologies for the production of polyphenolic neutraceuticals from Muscadine grapes and Rabbiteyeblueberries.Thesis.

Box, G.E.P., Wilson, K.B., 1951. On the experimental attainment of optimum conditions. Journal of the Royal Statistical Society. 50, 5939-5946.

Cacace, J.E., Mazza, G., 2003. Extraction of anthocyanins and other phenolics from black currants with aqueous ethanol. Journal of Food Science. 68, 240-248.

Galati, G., Sabzevari, O., Wilson, J.X., O'Brien, P. J., 2002. Prooxidant activity and cellular effects of the phenoxyl radicals of dietary flavonoids and other polyphenolics. Toxicology. 177(1), 91-104.

Gomez-Plaza, E., Minano, A., Lopez-Roca, J.M., 2006. Comparison of chromatic properties, stability and antioxidant capacity of anthocyanin-based aqueous extracts from grape pomace obtained from different vinification methods. Food Chemistry. 97(1), 87-94.

Lee, J., Ye, L., Landen, W.O., Eitenmiller, R.R., 2000. Optimization of an extraction procedure for the quantification of Vitamin E in tomato and broccoli using response surface methodology. Journal of Food Composition and Analysis. 13, 45-57.

Liyana-Pathriana, C., Shahidi, F., 2005. Optimization of extraction of phenolic compounds from wheat using response surface methodology. Food Chemistry. 93, 47 -56.

Monagas, M., Bartolome, B., Gomez-Cordoves, C., 2005. Updated knowledge about the presence of phenolic compounds in wine. Critical Reviews in Food Science and Nutrition. 45, 85-118.

California Rare Fruit Growers, Inc., 1999. http://www.crfg.org/pubs/ff/muscadinegrape.html.

Cevallos-Casals, B.A., zevallos, L.C., 2004. Stability of anthocyanin-based extracts of Andean purple corn and red-flashed sweet potato compared to synthetic and natural colorants. Food Chemistry. 86, 69-77.

Cook, N.C., Samman, S., 1996. Flavonoids--chemistry, metabolism, cardio protective effects, and dietary sources. Journal of Nutritional Biochemistry. 7, 66-76.

Francis, F.J., 1992. A new group of food colorants. Trends in Food Science and Technology. 3, 27–31.

Georgia Agricultural Resources., 2002. http://resources.caes.uga.edu/media/GAR/muscadines.htm.

Giusti, M.M., R.E.Wrolstad., 2001. Characterization and measurement of anthocyanins by UV-visible spectroscopy. In current protocols in food analytical chemistry., Wrolstad, R.E., Acree, T.E., An, H., Decker, E.A., Penner, M. H., Reid, D.S., Schwartz, S.J., Shoemaker, C.F., Sporns, P., Eds., Wiley: New york, pp F1.2.1-F1.2.13.

Hollman, P.C.H., Katan, M.B., 1997. Absorption, metabolism and health effects of dietary flavonoids in man. Biomedicine and Pharmacotherapy. 51:305-310.

Jackson, R.S., 1994.Wine Science Principles and Applications., Academic press: San Diego, CA, pp1 – 10.

Kammerer, D., Claus, A., Schieber, A., Carle, R., 2005. A novel process for the recovery of polyphenols from grape (Vitis vinifera L.) pomace. Journal of Food Science. 70(2), C157-C163.

Lawrence, A., 1991. Feed value of grape marc. VI. Extraction, fractionation, and quantification of condensed tannins. Chemical Abstracts. 20017c, 116.

Louli,V., Ragoussis,N., Magoulas,K., 2004. Recovery of phenolic antioxidants from wine industry by-products. Bioresource Technology. 92, 201-208.

Lu, Y., Foo, L. Y., 1999. The polyphenol constituents of grape pomace. Food Chemistry. 65, 1-8.

Meyer,A.S., Jepsen,S.M., Sorensen,N.S., 1998. Enzymatic release of antioxidants for human low-density lipoprotein from grape pomace. Journal of Agricultural and Food Chemistry. 46(7), 2439-2446.

Munoz,O., Sepulveda,M., Schwartz,M., 2004. Effects of enzymatic treatment on anthocyanic pigments from grapes skin from chilean wine. Food Chemistry. 87, 487-490.

Pastrana-Bonilla, E., Akoh, C. C., Sellappan, S., Krewer, G., 2003. Phenolic Content and Antioxidant Capacity of Muscadine Grapes. Journal of Agricultural and Food Chemistry. 51(18), 5497-5503

Rice-Evans, C.A., Miller, N.J., Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical biology and Medicine. 20(7), 933-956.

Şanal, I.S., Bayraktar, E., Mehmetoglu, U., Çalımlı, A., 2005. Determination of optimum conditions for SC-(CO2 + ethanol) extraction of β-carotene from apricot pomace using response surface methodology. The Journal of Supercritical Fluids. 34(3), 331-338.

Santiago, B., Ferrer, J., de Colmenares, N., Paez, G., 1993. Possible industrial uses for grape pomace. Chemical Abstracts. 159187a 122.

Selga,A., Sort,X., Bobet,R., Torres,J.L., 2004. Effecient one pot extraction and depolymerization of grape (Vitis vinifera) pomace procyanidins for the preparation of antioxidant thio-conjugates. Journal of Agricultural and Food Chemistry. 52(3), 467-473.

Shahidi, F., Naczk, M., 2004. Biosynthesis, Classification, and nomenclature of phenolics in food and nutraceuticals In Phenolics in food and nutraceuticals. CRC: Boca Raton, Florida. pp 1-14.

Singleton, V.L., J.A. Rossi. Jr., 1965. Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. American Journal of Enology and Viticulture. 16,144-158.

Valiente,C., Arrigoni,E., Esteban,R.M., Amado,R., 1995. Grape pomace as a potential food fiber. Journal of Food Science. 60(4), 818-820.

Yilmaz, Y., Toledo, R. T., 2004. Major Flavonoids in Grape Seeds and Skins: Antioxidant Capacity of Catechin, Epicatechin, and Gallic Acid. Journal of Agricultural and Food Chemistry. 52(2), 255-260.

CHAPTER 7

PURIFICATION AND CONCENTRATION OF POLYPHENOLIC RICH EXTRACTS FROM MUSCADINE POMACE BY USING STYRENE – DIVINYL RESIN[1].

[1]Biswas, R.; Adams, J.A.; & Phillips, R.D. To be submitted in *Journal of Agricultural and Food Chemistry,* 2007.

Abstract

Muscadine pomace is a rich source of polyphenolic compounds, known for the health benefits. Difficulty arises in drying the polyphenolic rich extracts derived from muscadine pomace due to the presence of sugar. Fermentation can eliminate the sugar, but it is time consuming. Purification and concentration of these extracts by highly porous, relative low cost, and easy regeneration styrene-divinyl benzene can generate a polyphenolic rich extract without sugar and pectin within few minutes/hours. This purified extract can be dried to give a non-sticky, stable powder. The objectives of the study were to compare the recovery of the polyphenolic compounds from muscadine pomace extracts using Sephabeads® 700 and Sephabeads® 70 and different solvents; to determine the recyclability and capacity of both the resins; to determine the effect of extract flow rate (loading) onto the column packed with resin; to determine the effect of column temperature on yield of polyphenolic compounds in the eluents; and to determine the amounts of polyphenolic compounds in reconstituted freeze dried powder.

About 12 Kg of thawed muscadine pomace (containing 24.0 g of ascorbic acid) were ground in a Hobart food grinder (Troy, OH), treated with 12.0 mL of pectinase and extracted with 16.0 L of water at 60 C for one hour. The extract was cooled to room temperature before loading onto columns columns (2.5 × 20 cm) of Sephabeads 700 and 70 resin (15g) which had been solvated by sequential soaking in 96.6 % w/w ethanol and DI water. For the recovery study 150 mL of extract was were loaded ontothe thermostatted column at a flow rate of 1.2 mL/min. The column was washed with 150 mL of water, and eluted with 150 mL each of 10 %, 20 %, 40 %, 60 %, and 95.6% w/w ethanol. The recyclability studies were carried out by repeating the above process

(except highest ethanol concentration was 80%) five times. For the capacity studies, 1500 mL of extract wereloaded onto the column, which was washed and eluted with 80 % ethanol. A (4 × 3) factorial design was followed to study the effect of temperature (5, 12, 27, and 60 C) and loading rate (1, 5, and 10 ml/min). The total phenolics and anthocyanins were analyzed by Folin-Ciocalteaux method and pH differential method. Eighty to ninety percent of total phenolics and 100% of anthocyanins were recovered from the resin. Both the resins gave the same recoveries for total phenolics and anthocyanins after 5 repetitions. The capacity of the Sephabeads 700 and Sephabeads 70 were found to be 54.5 (Total phenolics) and 10.1 (Anthocyanins) mg / g and 13.4 (Total Phenolics) and 2.26 (Anthocyanins) mg /g of wet resin respectively. The extract loading rate had a significant effect on the recovery of total phenolics ($p = 0.02$) and on anthocyanins ($p = 0.004$) , however there were no effects of temperature on their recovery. The resin-purified freeze dried extracts contained 71.7 % total phenolics and 71.4 % of anthocyanins and were non-hygroscopic in contrast to freeze dried extract which rapidly absorbed moisture from the air.

INRODUCTION

Muscadine grapes were found to be a potential source of polyphenolic compounds (*1, 2, 3, 4, 5, 6, 7,8*) which are protective against many degenerative diseases like cancer and cardiovascular diseases (*9, 10, 11, 12, 13, 14, 15*). The polyphenolic compounds have also found potential applications in food industries as natural colorants, or coloring foodstuffs, or as antioxidants protecting food components (e.g. unsaturated fatty acids) susceptible to oxidative changes (*16, 17*).

Purification of polyphenolic extracts from muscadine grapes pose a serious challenge to researchers because of many interfering substances such as sugars, pectins, acids, and others. The presence of sugar and pectins lead to serious problems during drying due to their hygroscopy which results in stickiness in the final product. Adsorption on to an active solid phase can be used as an alternative to fermentation for purification of these polyphenolic compounds from the extracts and is less time consuming than fermentation. Adsorption capacity of neutral styrene-divinyl benzene resins is a complex phenomenon and is dependent on several interaction forces between the sorbent and the aqueous phase containing the solute molecules. These interaction forces are mainly weak hydrophobic interactions due to van der Waals forces, and secondary stronger interactions due to hydrogen bonding and dipole dipole interactions (*18, 19*).

Several researchers have used this technology to purify polyphenolic extracts to eliminate sugars, citric acid and other undesirable compounds. Highly purified sugars concentrate was obtained after adsorption of flavonoids from citrus juices by XAD-116 resin (*20*). They used an intergrated approach consisting of subsequent treatments of the

pulp wash consisting of centrifugation, adsorption on neutral resin, adsorption on ion-exchange resin, ultrafiltration and reverse osmosis. The anthocyanins and other flavonoids were adsorbed completely by the neutral resin, and the treated column percolate was found to contain 96.1 % of total sugars which accpounted for a recovery of 80 % of the sugar present in the starting pulp wash.

Anthocyanins were purified and recovered by 96 – 100 % from the Cabernet Mitos grape pomace by adsorption/desorption to a styrene divinyl benzene copolymerisate, XAD 16HP (*21*). Theese authors used methanol, ethanol, and 2-propanol as eluting agents and found the best recovery of anthocyanins with methanol followed by ethanol, and 1-propanol. They used two different temperatures (25 and 50 C) during adsorption and did not find any significant differences. The same research group also investigated the adsorption of polyphenolic compounds from apple juice using a food grade ethylene glycol crosslinked polymethylmethacrylate adsorption resin (22). They optimized the adsorption process by varying the temperature and pH of the juice from 20 – 80 C and 2.0 – 3.9 respectively. They found that pH affected the adsorption process significantly.

Physical properties of resin (surface area, pore radius, and porosity) and chemical characteristics of solution (pH, temperature, and solvent) were found to play a significant role in the adsorption desorption process (*23, 24*). These authors tested thirteen commercial resins with different hydrophobicity, surface areas, and pore radius for adsorption of hesperidin and cyanidin3-glucoside at different pH value and temperatures. They found that there was no significant effect of pH on the adsorption phenomenon. They concluded styrene divinyl benzene (SDVB EXA 118, surface area 1200 m^2/g, pore

radii 90 Å) showed the maximum efficiency for adsorbing hesperidin and cyanidin3-glucoside from different aqueous solutions. They also found that increasing temperature had helped in increased adsorption of hesperidin.

Research conducted on the recovery of anthocyanins from pulp wash of pigmented oranges by adsorption to styrene divinyl benzene (*25*). They found that the resin materials, Sephabeads 70 and Relite EXA 90 were the efficient ones to recover 96 % of anthocyanins from the loaded pulp wash. The adsorption-desorption performances of commercial resins (two polystyrene-divinylbenzene copolymers and one methacrylic) resin were tested in column studies for the selective recovery of anthocyanins and hydroxycinnamates from pigmented pulp wash (*26*). They found that EXA-118, and a mixture of methanol/water 50:50 (v/v) to be the best resin and eluent to obtain highly concentrated extracts rich in anthocyanins and hydroxycinnametes.

The overall goal of the project was to produce non-sticky powder rich in polyphenolic compounds from muscadine grapes in a cost effective way. An essential step in the process would be to purify these extracts by adsorption/desorption through neutral styrene divinyl benzene resin materials. The use of resin materials may be less expensive than the expense incurred in removing large volumes of extracting solvent and less time consuming than removing sugars by fermentation. A systematic study on the purification and concentration of polyphenolic compounds from muscadine pomace extracts by the adsorption/desorption process using columns packed with resin materials was conducted. The specific objectives of this process were to perform recovery studies of anthocyanins and total phenolics, recyclability of the resin materials, capacity studies,

and influence of temperature and feed flow rate on the efficiency of resin materials (Sepabeads 700 and Sepabeads 70).

MATERIALS AND METHODS

Standards, solvents, and resins. Folin-Ciocalteaux reagent, ethanol (95%), sodium carbonate, potassium chloride, hydrochloric acid, sodium acetate, acetic acid were purchased from Sigma (St. Louis, MO). Commercial food grade resins Sepabeads SP-700 and SP-70 were purchased from Sorbent technologies, Atlanta, GA. They are macroporous styrene divinyl benzene with surface area 1200 and 800 m^2/g, respectively, and particle size greater than 250 μm.

Resin Activation. The resins were activated by overnight treatment with two bed volumes of 96.6 % w/w ethanol, followed by rinsing with two bed volumes of water before loading with extracts.

Samples. Grape skins with a few extraneous seeds were provided by Paulk vineyards, Wray, GA. The samples were randomly selected from the bulk.

Extracts. Aqueous extracts were prepared by grinding 12.0 Kg of muscadine pomace (24.0 g ascorbic acid added) in a Hobart grinder (Hobart Corp. Troy, OH), mixing with 16.0 L of deionized water and 12.0 mL of pectinase BE ®. The extracts were divided into 2.0 L each and were incubated in a water bath for an hour at 60 C. The extracts were then centrifuged at 30074 G, the supernatants were again refiltered through Whatman no. 40, and were divided into 6 aliquots (150 mL each), 1 aliquot (1500 mL), 2 aliqouts (each 500 mL), 4 aliquots (each 300 mL), 24 aliquots (each of 170 mL) and were frozen at –20 C before the analysis for the recovery, recylability, capacity and temperature-flowrate tudies of the resins.

Adsorption/desorption and Recovery Studies. Centrifuged extract (150 mL) was loaded onto the column (20 × 2.5 cm, Flex Column, Kontes Glass Company, Vineland, NJ) packed with 15 g of wet resin (Sepabeads 700) at an outflow of 0.8 mL /min. The column was then washed with double deionized water (100 mL). The adsorbed compounds were then eluted sequentially with 0 %, 10 %, 20 %, 40 %, 60 %, 80 %, and 95.6 % w/w ethanol and the eluants were analyzed for total phenolics and anthocyanins. The different eluents were passed through the column until the outflow was colorless. The volumes of the eluants varied from 140 – 160 mL. The entire experiment was performed under subdued light and at 27 C.

Recyclability studies. Centrifuged extract (150 mL) was loaded onto a column (packed with 15 g of wet activated resin, SP 700) at a outflow of 0.8 mL /min, washed with 100 mL of double deionized water and eluted with 150 mL of 80 % w/w ethanol. After elution, the packed column was kept in 96.6 % w/w ethanol overnight. The following day, it was washed with water untill all the ethanol had been driven off the resin material. The column was loaded with extracts, washed with water and finally eluted sequentially with 150 mL of 80 % w/w ethanol. Eluents were analysed for total phenolics and anthocyanins. The experiment was repeated five times to determine if performance declined with a few cycles. Another set of studies was conducted with the resin Sepabeads SP 70 following the same procedure as above except the loaded extract was 300 mL at a outflow rate of 6.1 mL/min. Then the resin was washed with 100 mL of deionized water and eluted with 100 mL of 80 % w/w ethanol. The resin was reused for four times. The entire set of experiments was performed at 27 C and under subdued light. Another extensive study was conducted to determine the number of recycle times for the

resin, Sepabeads SP 700. Ten milliliters of the muscadine pomace extact was pumped onto the column (1 × 6.5 cm, Becton, Dickinson and Company, Franklin Lakes, NJ), loaded with 1 g of resin, washed with 6 mL of deionized water, and eluted with 6 mL of 80 % w/w ethanol at an outflow rate of 0.25 mL/min. The resin was then rinsed with 8 mL of deionized water before loading the next 10 mL of the muscadine pomace extract. The study was done in a continuous fashion till the resin was no longer adsorbing the polyphenolic compounds.

Capacity Studies. The capacity of two resins SP 700 and SP 70 were tested with the extracts. The extracts were loaded onto the column packed with 15 g of activated wet resin at an outflow rate of 5.7 mL/min until the color of the outflow was similar to that of the inflow. The column was then washed with 215 mL of deionized water to remove residual compounds at the same outflow rate. Then the adsorbed compounds were eluted with 200 mL of 80 % w/w ethanol, followed by 100 mL of 95.6 % w/w ethanol at the same outflow rate. For the resin, Sepabeads SP 70 the loaded volume was lesser than that of SP 700, so the resin was washed with 100 mL of deionized water followed by eluting it with 100 mL of 80 % w/w ethanol and 50 mL of 95.6 % w/w ethanol at the same flow rate. The eluents were analyzed for total phenolics and anthocyanins, depending on their percentage of adsorption by the resin materials, the capacity of the resin were determined.

Influence of Temperature and Flow rate on the adsorption by the resin. A factorial design was followed to study the effects of temperature and flow rate on the adsorption/desorption studies of the resin (SP 700). Four different temperatures (5, 12, 27, and 60 C) and three different flow rates (1, 5, 10 mL/min) were studied. One hundred and fifty mL of extracts were loaded onto the column packed with 15 g of activated resin,

followed by 100 mL of water wash and elution by 100 mL of 80 % w/w ethanol, and 50 mL of 95.6 % w/w ethanol. The eluents were then analyzed for total phenolics and anthocyanins. Some of the eluents were freeze dried to study the recovery of total phenolics and anthocyanins in the freeze dried powder.

Statistical analysis. Factorial ANOVA analysis was done to investigate the significance of the model at 5 % level of significance and also to study the main effects (temperature, flow rate) and the interaction effects (temperature and flow rate) on total phenolics and anthocyanins. Contrasts analysis was done to study any linear, quadratic trends of temperature and flow rates and interaction effects on the dependent variables by using Statistica ® software, Version 6.0 (Statsoft, Tulsa, OK).

Total phenolics. Total phenolics were estimated colorimetrically using the Folin-Ciocalteaux method (*27*).A sample aliquot of 200 µL was added to 800 µL of deionized water, 5 mL of Folin-Ciocalteaux reagent, and 4 mL of saturated sodium carbonate solution (75g/L) and mixed in vortex (Fisher vortex Genie 2, Fisher Scientific, Pittsburgh, PA, USA). The absorbance was measured at 765 nm with a Hewlett Packard 8451A diode array spectrophotometer (Avondale, PA, USA) after incubation for two hours at room temperature (~25 C). Quantification was based on the standard curve generated with 100, 200, 300, 400, and 500 mg/L of gallic acid. The final concentration of phenolics was calculated based on total volume of extract and weight and expressed as mg/Kg dry weight. The experiment was conducted under subdued light.

Anthocyanins. Anthocyanins were measured by the pH differential method (*28*) using two buffer systems – potassium chloride buffer, pH 1.0 (0.025 M) and sodium acetate buffer, pH 4.5 (0.4 M). A diluted sample of 0.2 mL (to give optical density in the

range of 0.1 – 1.2 at 510 nm) was mixed with 1.8 mL of corresponding buffer in vortex (Fisher vortex Genie 2, Fisher Scientific, Pittsburgh, PA, USA) and read against a blank at 510 and 700 nm with a Hewlett Packard 8451A diode array spectrophotometer (Avondale, PA, USA).

Absorbance was calculated as

$A = (A_{570\ nm} - A_{710\ nm})_{pH\ 1.0} - (A_{570\ nm} - A_{710\ nm})_{pH\ 4.5}$

Monomeric anthocyanin pigment concentration in the extract was calculated as cyaniding-3-glucoside

Monomeric anthocyanin pigment (mg/L) = $A \times MW \times DF \times 1000 / (\varepsilon \times 1)$

Where A = absorbance, MW = molecular weight (449.2), DF = dilution factor, ε = molar absortivity (29,600). The final concentration of anthocyanins was calculated based on total volume of extract and weight and expressed as mg/Kg dry weight. The experiment was conducted under subdued light.

RESULTS AND DISCUSSION

Adsorption/desorption and recovery studies of Sepabeads 700. The concentration of total phenolics and anthocyanins in the different eluents collected at different time intervals were shown in the **Figure 7.1**. The plots showed that maximum concentration of total phenolics and anthocyanins in 40 % w/w ethanol and 20 % w/w ethanol. A pictorial representation of the color of the eluents in different concentrations of ethanol eluents was given in **Figure 7.2**. The color of the 40 % w/w eluents was found to be the darkest. The total phenolics and the anthocyanins of the combined eluents collected at different time intervals for the specific ethanol concentrations was analyzed and the recovery percent for total phenolics and anthocyanins was determined. The total

phenolics and the anthocyanins amounted to 310 and 5.81 mg, respectively in the muscadine pomace extracts. Two hundred and forty seven mg of total phenolics and 5.89 mg of anthocyanins were adsorbed which accounted to 80 % and >100 % recovery of total phenolics and anthocyanins respectively. The ethanol solution, rich in phenolics and anthocyanins could lead to several complex reactions such as copigmentation. The reason for > 100 % recovery of anthocyanins could be attributed to the copigmentation of anthocyanins and phenolic compounds through the formation of intermolecular hydrophobic complexes (*29, 30*). Sepabeads SP 700 resin was used to concentrate anthocyanins from pigmented orange juice by researchers and they reported a recovery of 80 % (*24*). The recoveries in this study were higher than previously reported as 61 – 88 % for anthocyanins from pulp wash of pigmented oranges by concentration on resins (*25*).

Recyclability studies. Both the resins, Sepabeads SP 700 and SP 70 were investigated for the number of recycle times. Both the resins were reused for five times with freshly thawed extracts loaded onto them. The results (**Table 7.1**) showed that the standard deviation of the recovery of the total phenolics and anthocyanins from muscadine pomace extract after concentration on resin reused for five times was 3.96 and 5.00 respectively. On the other hand, the food grade resin Sepabeads SP 70 did not give consistent results with the standard deviation of the recoveries of total phenolics as 7.40. The standard deviation for the recovery of anthocyanins was determined as 3.36. Even though the recoveries of total phenolics from the Sepabeads 70 were higher than that of Sepabeads 700, the recoveries of anthocyanins was much lesser than the former (**Table 7.2**). The results for the second study where the freshly thawed extracts through the same

bed of resin were run for 85 times were given in **Table 7.3**. The results indicated that the resin could be reused for 85 times with 68.8 - 63.4 % and 94.5 – 110.7 % recovery of total phenolics and anthocyanins from the original extract. The standard deviation of the recovery of total phenolic and anthocyanins from the Sepabeads 700 resin after reusing it for 85 times was determined as 2.49 and 5.60 for total phenolics and anthocyanins respectively. This suggested for a reduction in fresh material cost as we could use the same resin for 85 times with almost 100 % efficiency.

Capacity Studies. Three different volumes of extracts (1500, 2000, and 4000 mL) were thawed before loading onto the column packed with 15 g of activated wet resin (SP 700). The extract was loaded at an outflow rate of 5.7 mL/min. The color of the outflow after 450 mL of extract loaded onto the column was clear. Then the color changed to slightly pink to purple color as the original juice till loading of 1480 mL of extract. The ouflow was collected in aliquots of 50 mL each after the appearance of the slight pink color. Analysis of each of the outflow showed that amount of total phenolics and anthocyanins started to increase from 25.7 to 77.6 mg and 10.1 to 20.2 mg, respectively. The total amount of total phenolics and anthocyanins in the muscadine pomace extract (1480 mL) was 3153 mg and 590 mg respectively. After 450 mL of the extract went through the column packed with resin, which corresponded to 1051, and 179 mg of total phenolics and anthocyanins, respectively, the amounts of adsorbed total phenolics and anthocyanins in the resin was found to be as 1025.3 and 175.7 mg respectively. At that point the percent of total phenolics and anthocyanins adsorbed was determined as 97 %. Beyond that point with loading of more extracts onto the column the percent of total phenolics and anthocyanins adsorbed decreased from 97 % to 53.4 %

and 36.6 % respectively. This showed that the capacity of the resins was determined as 54.5 mg total phenolics and 10.1 mg of anthocyanins in muscadine pomace extact per gram of wet resin. Adsorption capacity for total phenolics and anthocyanins was determined as 54.5 mg/g of wet resin and 10.1 mg/g of wet resin respectively. Two plots of the amounts of total phenolics and anthocyanins were drawn representing the concentrations of phenolics and anthocyanins passing onto and out of the column (**Figure 7.3**). The capacity studies for the resin SP 700 were repeated two times with 400 mL of extracts loaded onto the column (**Table 7.4, 7.5**). The outflow from the column was collected in 50 mL aliquots and was analyzed for total phenolics and anthocyanins. The percentage of adsorbed total phenolics and anthocyanins varied from 97 % to 77 % and 99 % to 84 % respectively. These results confirmed that the capacity of the Sepabeads resin, SP 700 as 54.5 mg of total phenolics and 10.1 mg of anthocyanins in muscadine pomace extracts per g of wet resin. Both the total phenolics and the anthocyanins were concentrated by 25 times.

Capacity of the food grade resin, Sepabeads SP 70 was also investigated with by loading extracts onto the column. In this case a slight pink color outflow was observed after 100 mL of extract was loaded onto the column. The outflow from the column was collected in 50 mL aliquots and were analysed for total phenolics and anthocyanins. Loading of extracts was stopped at 300 mL since the color of the outflow was similar to the extract color. The changes in the amounts of total phenolics and anthocyanins in the collected fractions coming out of the column varied from 4.67 to 170 mg and 0.03 to 22.3 mg respectively till the end of the loading phase. The amount of total phenolics and anthocyanins in the muscadine pomace extract (300 mL) was 601 mg and 101 mg

respectively. The amount of total phenolics and anthocyanins adsorbed by the resin after loading of 100 mL of extract (Total phenolics: 201 mg, anthocyanins: 33.9 mg) was180 mg and 33.5 mg respectively (**Table 7.6, 7.7**). At that point the percent of total phenolics and anthocyanins adsorbed was determined as 89.6 and 98.8 % respectively. From the data it was concluded that the SP 700 was more efficient than the SP 70. After that point the adsorption of total phenolics and anthocyanins decreased from 84.6 – 71.6 % and 94 – 78 % respectively. It was concluded that the capacity of the resins as 13.4 mg of total phenolics and 2.26 mg of anthocyanins in muscadine pomace extact per gram of wet resin. Adsorption capacity for total phenolics and anthocyanins was determined as 13.4 mg/g of wet resin and 2.26 mg/g of wet resin respectively. **Figure 7.4**. showed the maximum adsorption capacity of the Sepabeads SP 70 for total phenolics and anthocyanins. The capacity studies for the resin SP 70 were repeated two times with 300 mL of extracts loaded onto the column. The outflow from the column was collected in 50 mL aliquots and was analyzed for total phenolics and anthocyanins. The percentage of adsorbed total phenolics and anthocyanins varied from 95 % to 72 % and 99 % to 77 % respectively. These results confirmed that the capacity of the Sepabeads resin, SP 700 as 6.67 mL of muscadine pomace extracts per g of wet resin. The study also tells us that Sephabeads 700 was more efficient than Sepabeads 70 as the former resin could absorb total phenolics and anthocyanins by 5.23 and 5.26 times more than the latter respectively. Adsorption/desorption on Sepabeads 700 would save time, cost than that by Sepabeads 70. .

Influence of temperature and feed flow rate on the recovery of total phenolics and anthocyanins by the adsorption/desorption process by SP 700 resin. The effects

of four different temperatures (5, 12, 27, 60 C) and three different feed flow rates (1,5, 12 mL/min) on the adsorption/desorption capacity of Sepabeads 700 for total phenolics and anthocyanins were investigated. The results (**Table 7.8**) showed that the recoveries of total phenolics and anthocyanins increased with increase in temperature to 27 C, but decreased when it was further increased to 60 C. Increase in temperature lowered the surface tension at the hydrophobic interface, which in turn increased the wetting of the resin material and henceforth facilitated the transfer of solute particles from the extract to the solid resin surface. Similar effects of temperature were reported (*23*) during adsorption of hesperidin by resin material, EXA-118. The recoveries of both total phenolics and anthocyanins remained similar with the flow rates of 1 and 5 mL/min but decreased with increasing flow rate to 10 mL/min. Factorial ANOVA analysis was conducted to investigate the significance of the model and the main, and the interaction effects of the model at 5 % level of significance. The whole model for total phenolics and anthocyanins was found to be significant at 0.3 % level of significance. The ANOVA table for the recovery of total phenolics and anthocyanins was given in **Table 7.9**. The main effects, temperature and feed flow rates were found to be significant at 0.04 and 0.3 % level of significance respectively. There were no significant interaction effects of temperature and feed flow rate on the recovery of total phenolics and anthocyanins. The least square means across different temperatures, feed flow rates, and interactions between temperatures and feed flow rates were computed for both the recoveries of total phenolics and anthocyanins and were shown in **Figures 7.3, 7.4**, and **7.5**. We also investigated linear, quadratic, and cubic effects of temperature and linear, and quadratic effects of feed flow rate by conducting linear, quadratic, and cubic

contrasts tests. **Table 7.10** showed the single components used for the contrasts tests. The estimates of the contrasts with their standard errors and p values were given in **Table 7.11**. Statistical analysis showed that there was a significant linear trend of the recovery of total phenolics ($p = 0.0003$) and anthocyanins ($p = 0.006$) across the temperature decreasing from an average of 78.6 % and 105.1 % at 27 C to an average of 70.3 % and 94.6 % at 60 C. A significant quadratic trend of the recovery of total phenolics and anthocyanins was found across the temperature at 0.007 % and 0.001 % level of significance. There was no significant cubic trend of both the recoveries of total phenolics and anthocyanins across the temperature. Significant linear trend of the recovery of total phenolics ($p = 0.006$) and anthocyanins ($p = 0.003$) was observed across the flow rates decreasing from an average of 78.1 % and 103.5 % at 1 mL/min to an average of 74.0 % and 97.8 % at 10 mL/min respectively. There was no significant quadratic trend of both the recoveries across the flow rates. Fisher's LSD tests were performed to compare the means across different temperatures and feed flow rates for the recoveries of total phenolics and anthocyanins (**Table 7.12**). Both the means of recoveries of total phenolics and anthocyanins at 60 C and flow rate of 10 mL/min was significantly different from the rest at 5 % level of significance.

Some of the concentrated eluents from the column were freeze dried and a recovery of 71.7 % total phenolics and 71.1 % anthocyanins was obtained in the freeze dried powder. This indicated that the activity of the compounds was retained in the powder, which provided us the possibility of selling the polypohenolic rich extracts in the form of powders to the consumers.

SIGNIFICANCE

The study provided useful information on optimal resin, feed flow rate, and temperature. This research concluded that the Sepabeads resin could be reused for 85 times without any significant changes in the recovery of total phenolics and anthocyanins. Both the loading of total phenolics and the anthocyanins were increased by 5.2 times when the extracts were loaded onto Sepabeads 700 than Sepabeads 70. This research demonstrated that Sepabeads 700 as the optimal resin to be used for concentration and purification of polyphenolic extracts from muscadine pomace in pilot scale studies. The capacity of the resin was determined as 70.1 mg total phenolics and 11.9 mg anthocyanins per g of wet resin. The entire process of adsorption and desoption onto the resin was optimal at 27 C. The total phenolics and the anthocyanins were concentrated by 25 times which reduces the cost of evaporation of ethanol to generate polyphenolic rich powders from the extracts.

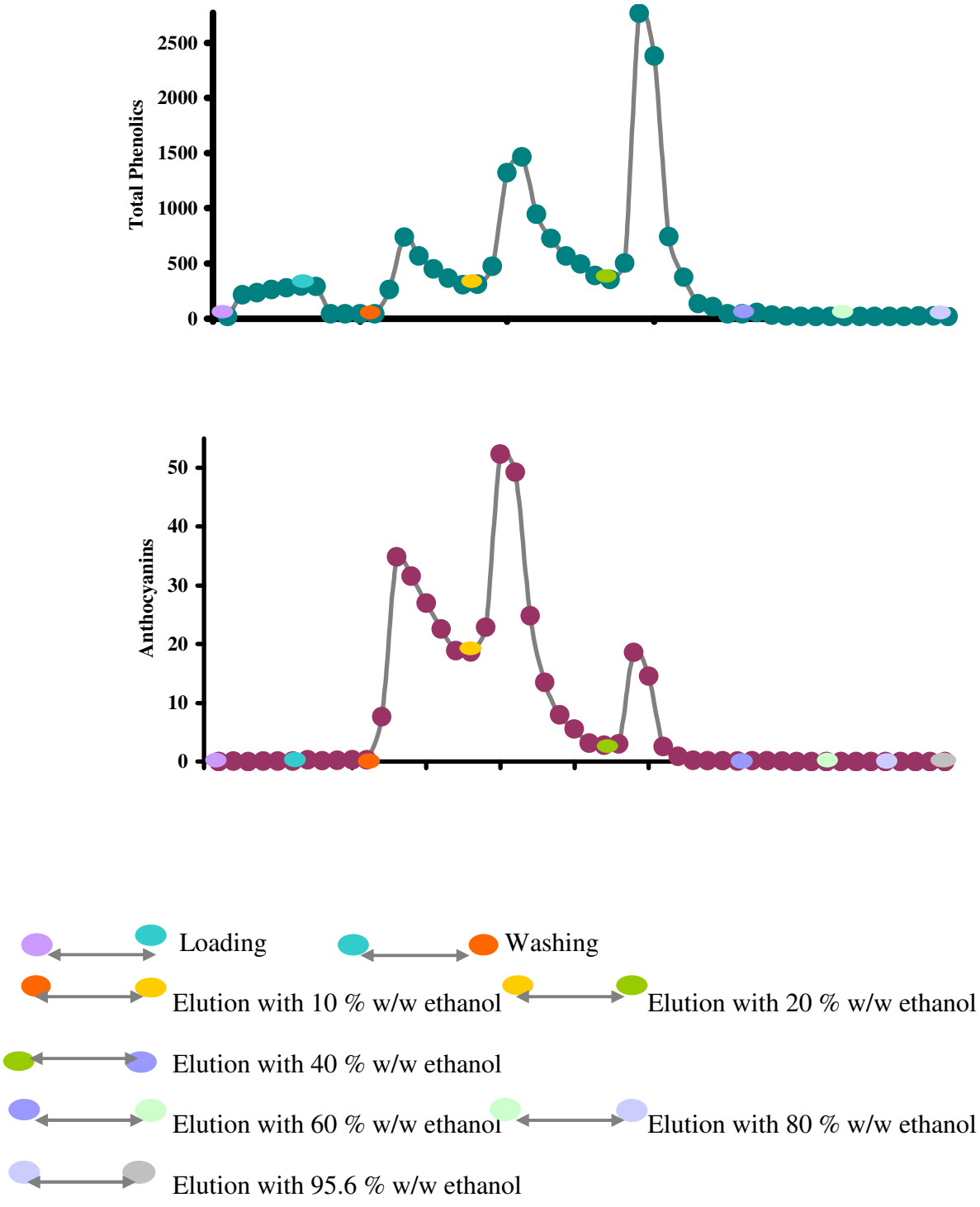


Figure 7.1. Concentration of total phenolics (mg/L) and anthocyanins (mg/L) in the fractions collected during loading, washing, and elution phases.

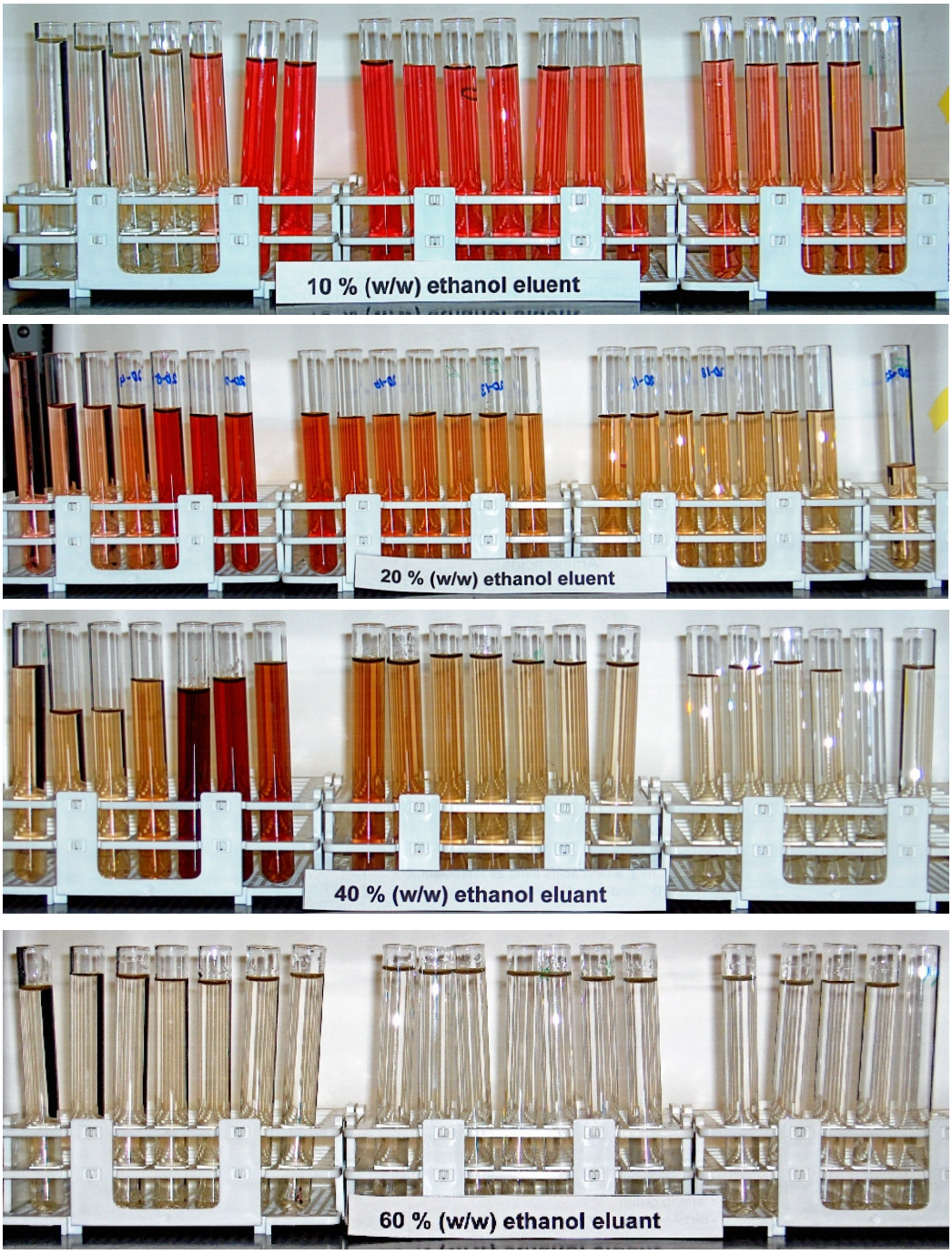


Figure 7.2. Pictorial representation of the color of the different ethanol concentration (%) eluents after passing through Sepabeads 700.

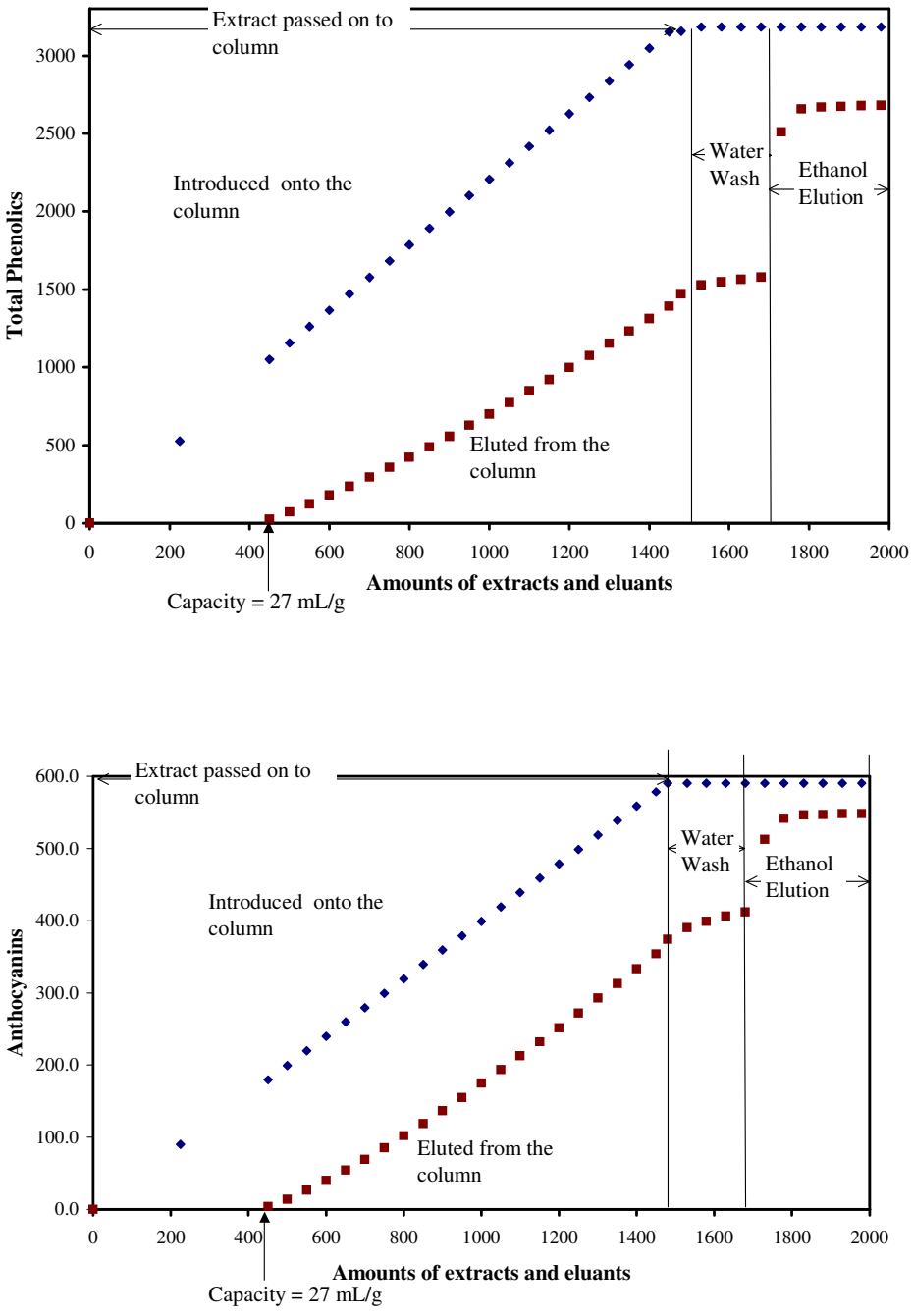
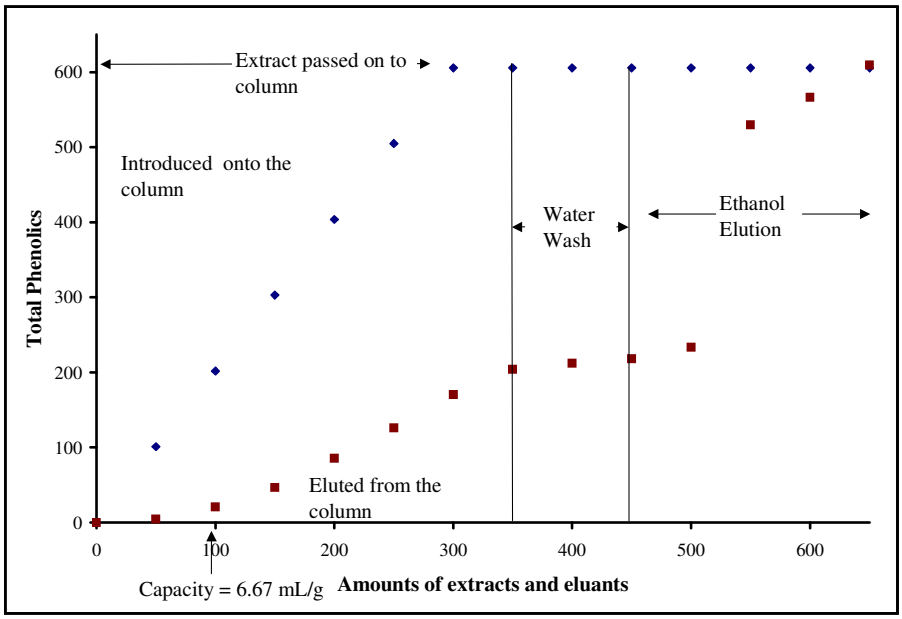


Figure 7.3. Binding capacities of anthocyanins and total phenolics to Sepabeads SP 700 upon adsorption from muscadine pomace extracts.



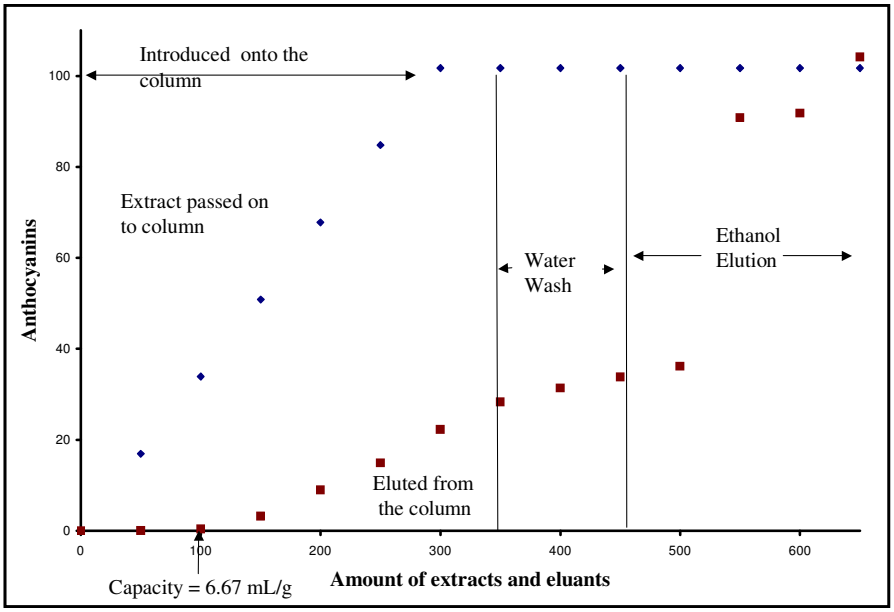


Figure 7.4. Binding capacities of total phenolics and anthocyanins to Sepabeads SP 70 upon adsorption from muscadine pomace extracts.

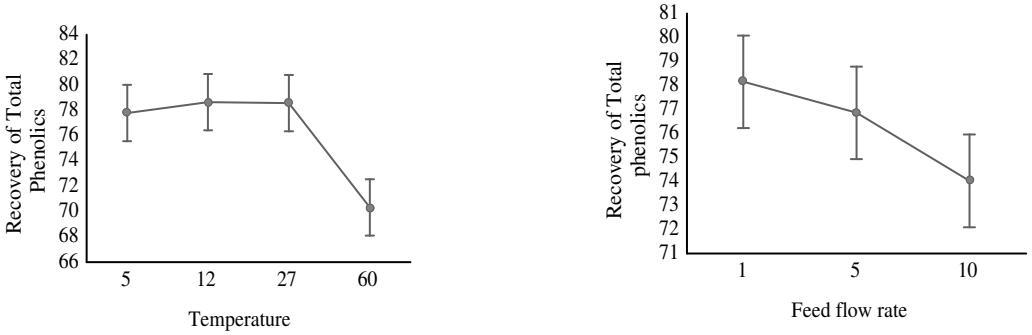


Figure 7.5. Least square means of recovery of total phenolics (%) after adsorption/desorption to Sepabeads 700 across different temperatures and feed flow rates.

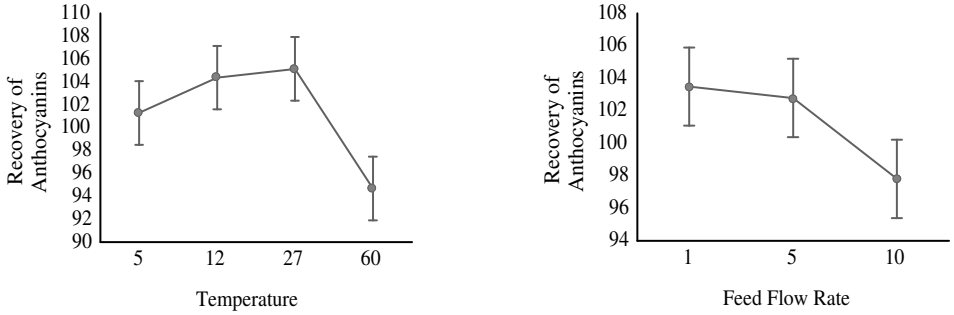


Figure 7.6. Least square means of the recovery of anthocyanins (%) after adsorption/desorption to Sepabeads 700 across different temperatures and feed flow rates

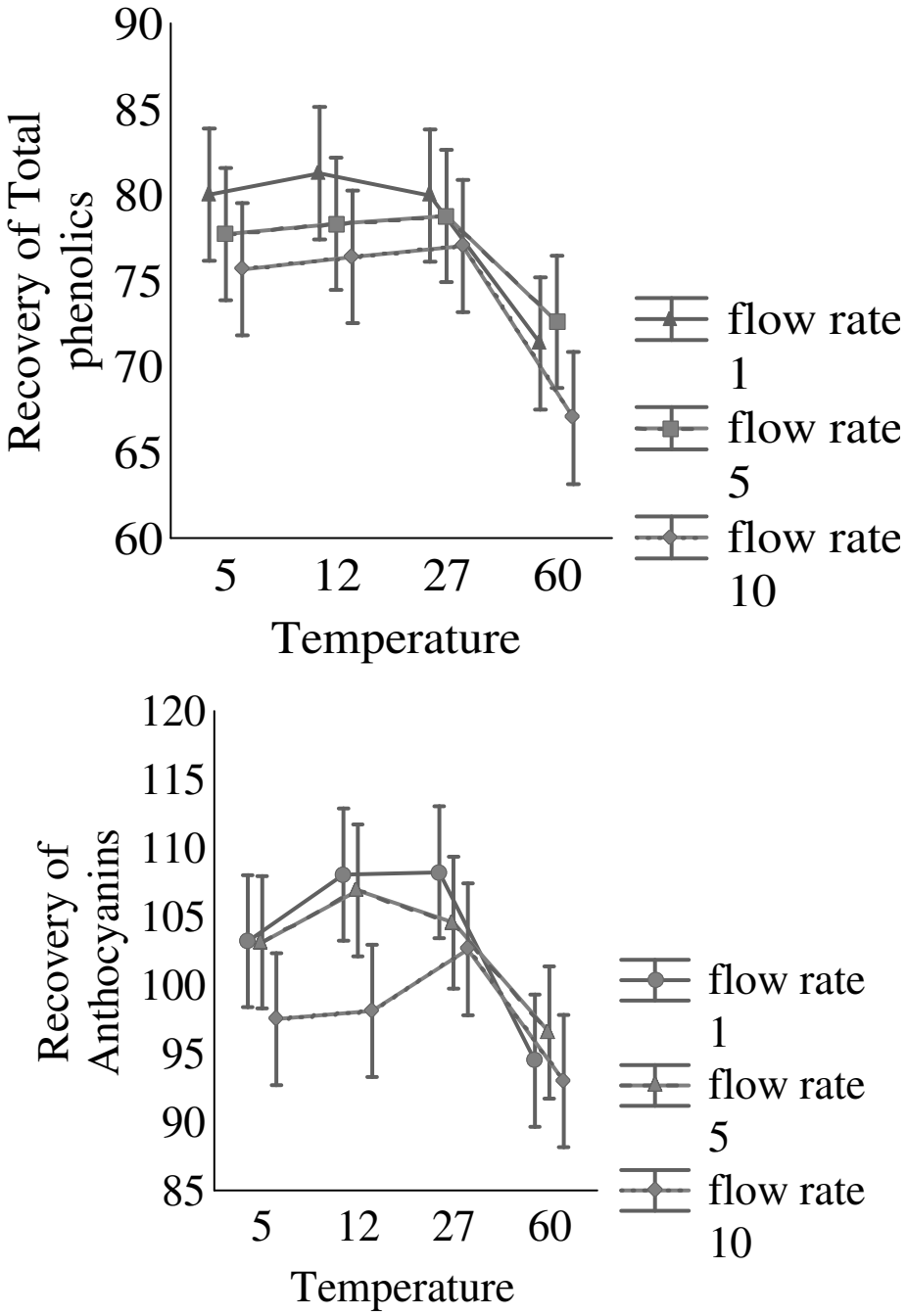


Figure 7.7. Recovery of total phenolics and anthocyanins after adsorption/desorption to Sepabeads 700 as affected by the interaction of temperature and feed flow rate.

Table 7.1. Amounts of total phenolics and anthocyanins in the loading, washing, and elution phases and recovery of total phenolics and anthocyanins in muscadine pomace extracts upon concentration by Sepabeads SP 700 resin reused for five times.

	Total Phenolics (mg)				
	1	2	3	4	5
Original Extract	310.4	378.2	378.5	360.7	375.3
Loading	27.7	34.5	37.6	38.6	37.4
Water wash	47	17	21.9	13.2	16.7
Eluents	247.7	259.5	280.5	265.7	279.6
Recovery (%)	79.8	68.6	74.1	73.7	74.5
	Anthocyanins (mg)				
	1	2	3	4	5
Original Extract	58.1	53.6	56.2	53.4	57.3
Loading	0.007	0.17	0.25	0.17	0.12
Water wash	0.3	0.4	0.7	0.1	0.1
Eluents	58.9	58.4	54.6	58.0	59.1
Recovery (%)	101.4	109.0	97.2	108.6	103.1

Table 7.2. Amounts of total phenolics and anthocyaninsin the loading, washing, and elution phases and recovery of total phenolics and anthocyanins in muscadine pomace extracts upon concentration by Sepabeads SP 70 resin reused for four times.

	Total Phenolics (mg)			
	1	2	3	4
Original Extract	605.7	618	615	610.5
Loading	171	155	154	157
Water wash	47	46	45	47
Eluents	593	529	562	626
Recovery (%)	97.8	85.5	91.4	102.5

	Anthocyanins (mg)			
	1	2	3	4
Original Extract	101.7	103.2	104.7	104.2
Loading	22.3	15.6	16.7	18.2
Water wash	11.6	11.9	12.5	12.5
Eluents	81.2	97.6	96.3	91.2
Recovery (%)	79.8	94.6	92.0	87.5

Table 7.3. Amounts of total phenolics and anthocyanins in the loading, washing, and elution phases and recovery of total phenolics and anthocyanins in muscadine pomace extracts (10 mL) upon concentration by 1 g of Sepabeads SP 700 resin reused for eighty five times.

	Total Phenolics (mg)					
Recycle times	1	20	40	60	75	85
Original Extract	16.72	16.72	16.72	16.72	16.72	16.72
Loading	2.33	2.19	2.57	2.84	3.09	2.80
Washing	1.78	1.71	2.01	2.17	2.22	2.14
Elution	11.38	11.50	11.02	10.65	10.61	10.54
Recovery (%)	68.1	68.8	65.9	63.7	63.4	63.0

	Anthocyanins (mg)					
Recycle times	1	20	40	60	75	85
Original Extract	2.10	2.10	2.10	2.10	2.10	2.10
Loading	0.10	0.07	0.11	0.17	0.21	0.15
Washing	0.16	0.13	0.18	0.24	0.25	0.21
Elution	2.33	2.17	2.20	2.11	2.07	1.99
Recovery (%)	110.7	103.3	104.5	100.1	98.3	94.5

Table 7.4. Replicates of binding capacity of Sepabeads resin, SP 700 for total phenolics from muscadine pomace extracts.

Loading Phase								
Cumulative Extract Volume (mL)	Total Phenolics (mg) Into the column Rep 1	Into the column Rep 2	Out of the column Rep 1	Out of the column Rep 2	Adsorbed in the resin Rep 1	Adsorbed in the resin Rep 2	Adsorbed (%) Rep 1	Adsorbed (%) Rep 2
0	0	0	0	0	0	0		
50	102.2	107.1	3.3	3.4	98.9	103.7	96.8	96.78
100	204.4	214.2	12.9	15.7	191.4	198.5	93.67	92.69
150	306.6	321.3	29	25.9	277.5	295.4	90.54	91.95
200	408.8	428.4	49.9	48.5	358.9	379.9	87.8	88.68
250	511	535.5	73.8	76	437.2	459.5	85.57	85.82
300	613.2	642.6	105.3	109.5	507.8	533.1	82.83	82.96
350	715.4	749.7	143.1	147.2	572.3	602.5	80	80.37
400	817.6	856.8	180.8	192.6	636.7	664.2	77.89	77.52

Washing Phase				
Cumulative Water Volume (mL)	Total Phenolics (mg) Into the column Rep 1	Into the column Rep 2	Out of the column Rep 1	Out of the column Rep 2
450	817.6	856.8	215.5	227.6
500	817.6	856.8	224.7	237.1

Elution Phase				
Cumulative 80 % Ethanol Volume (mL)	Total Phenolics (mg) Into the column Rep 1	Into the column Rep 2	Out of the column Rep 1	Out of the column Rep 2
550	817.6	856.8	664.8	720.6
600	817.6	856.8	724.4	787.8
650	817.6	856.8	728.8	793.3

Table 7.5. Replicates of binding capacity of Sepabeads resin, SP 700 for anthocyanins from muscadine pomace extracts.

Loading Phase								
Cumulative	Anthocyanins (mg)							
Extract	Into the column		Out of the column		Adsorbed		Adsorbed (%)	
Volume (mL)	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
0	0.0	0.0	0.0	0.0	0.0	0.0		
50	18.9	18.8	0.0	0.0	18.8	18.8	99.82	99.82
100	37.7	37.6	0.2	0.2	37.5	37.4	99.45	99.44
150	56.6	56.4	0.8	0.9	55.8	55.5	98.54	98.43
200	75.5	75.1	2.4	2.6	73.1	72.6	96.86	96.59
250	94.3	93.9	5.2	5.6	89.1	88.3	94.45	93.99
300	113.2	112.7	9.6	10.1	103.6	102.6	91.50	91.05
350	132.1	131.5	15.6	16.1	116.5	115.4	88.19	87.72
400	151.0	150.3	23.4	23.9	127.6	126.4	84.51	84.12

Washing Phase				
Cumulative	Anthocyanins (mg)			
Water	Into the column		Out of the column	
Volume (mL)	Rep 1	Rep 2	Rep 1	Rep 2
450	151.0	150.3	30.6	30.8
500	151.0	150.3	34.3	34.5

Elution Phase				
Cumulative	Anthocyanins (mg)			
80 % Ethanol	Into the column		Out of the column	
Volume (mL)	Rep 1	Rep 2	Rep 1	Rep 2
550	151.0	150.3	137.8	132.2
600	151.0	150.3	156.3	150.1
650	151.0	150.3	157.6	151.6

Table 7.6. Replicates of binding capacity of Sepabeads resin, SP 70 for total phenolics from muscadine pomace extracts.

Loading Phase								
Cumulative	Total Phenolics (mg)							
Extract	Into the column		Out of the column		Adsorbed		Adsorbed (%)	
Volume (mL)	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
0.0	0.0	0.0	0.0	0.0	0.0	0.0		
50.0	101.0	103.0	4.7	4.7	96.3	98.3	95.4	95.5
100.0	201.9	206.0	21.0	21.0	180.9	185.0	89.6	89.8
150.0	302.9	309.0	46.6	46.6	256.3	262.4	84.6	84.9
200.0	403.8	412.0	85.6	75.8	318.2	336.2	78.8	81.6
250.0	504.8	515.0	126.3	111.2	378.5	403.8	75.0	78.4
300.0	605.7	618.0	170.7	154.5	435.0	463.5	71.8	75.0

Washing Phase				
Cumulative	Total Phenolics (mg)			
Water	Into the column		Out of the column	
Volume (mL)	Rep 1	Rep 2	Rep 1	Rep 2
350.0	605.7	618.0	204.0	186.407
400.0	605.7	618.0	212.2	194.637
450.0	605.7	618.0	218.0	200.432

Elution Phase				
Cumulative	Total Phenolics (mg)			
80 % Ethanol	Into the column		Out of the column	
Volume (mL)	Rep 1	Rep 2	Rep 1	Rep 2
500.0	605.7	618.0	233.4	296
550.0	605.7	618.0	529.7	561
600.0	605.7	618.0	566.2	590
650.0	605.7	618.0	609.5	616

Table 7.7. Replicates of binding capacity of Sepabeads resin, SP 70 for anthocyanins from muscadine pomace extracts.

Loading Phase								
Cumulative	Anthocyanins (mg)							
Extract	Into the column		Out of the column		Adsorbed		Adsorbed (%)	
Volume (mL)	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
0	0	0	0	0	0	0		
50	16.9	17.2	0	0.3	16.9	16.7	99.8	97
100	33.9	34.4	0.4	1.5	33.5	32.4	98.8	94.3
150	50.8	51.6	3.2	4.3	47.7	46.5	93.7	90.2
200	67.8	68.8	9	8.9	58.8	58.9	86.7	85.6
250	84.7	86	14.9	15.6	69.8	69.2	82.4	80.4
300	101.7	103.2	22.3	21.5	79.4	80.2	78.1	77.7

Washing Phase				
Cumulative	Total Phenolics (mg)			
Water	Into the column		Out of the column	
Volume (mL)	Rep 1	Rep 2	Rep 1	Rep 2
350.0	101.7	103.2	28.3	21.5
400.0	101.7	103.2	31.4	24.8
450.0	101.7	103.2	33.8	27.5

Elution Phase				
Cumulative	Total Phenolics (mg)			
80 % Ethanol	Into the column		Out of the column	
Volume (mL)	Rep 1	Rep 2	Rep 1	Rep 2
500.0	101.7	103.2	36.2	41.1
550.0	101.7	103.2	90.9	96.6
600.0	101.7	103.2	91.8	97.3
650.0	101.7	103.2	104.1	98.1

Table 7.8. Influencce of temperature and flow rate on the recovery of total phenolics and anthocyanins after adsorption/desorption to Sephabeads 700 resin.

Temp (C)	Flow rate (mL/min)	Recovery (%) Total phenolics	Anthocyanins
5	10	76.7	101.2
5	10	74.6	93.8
5	5	79.7	104.5
5	5	75.8	101.8
5	1	79.1	102.8
5	1	80.9	103.6
12	5	77.5	104.2
12	5	79.1	109.6
12	10	76.7	95.8
12	10	76.1	100.4
12	1	79.9	104.9
12	1	82.6	111.2
27	1	77.5	105.8
27	1	82.5	110.6
27	5	82.6	108.1
27	5	74.9	101
27	10	76.7	101.3
27	10	77.4	103.9
60	1	73.5	94.8
60	1	69.2	94.2
60	5	74.1	97.1
60	5	71.1	96
60	10	65.8	92.4
60	10	68.2	93.5

Table 7.9. Analysis of Variance for the recovery of total phenolics and anthocyanins.

Source	SS	DF	MS	F value	p value
Recovery of Total Phenolics					
Model	379.1443	11	34.46767	5.504511	0.003264
Residual	75.1406	12	6.261713		
Total	454.2849	23			
R square	0.834596				
Recovery of Anthocyanins					
Model	614.6690	11	55.87900	5.710114	0.002776
Residual	117.4316	12	9.785968		
Total	732.1006	23			
R square	0.839596				

Table 7.10. Components of linear, quadratic, and cubic contrasts of temperature and feed flow rate.

Temperature (C)	No. of Observations	Contrasts Linear	Quadratic	Cubic
5	6	-3	1	-1
12	6	-1	-1	3
27	6	1	-1	-3
60	6	3	1	1

Feed Flow Rate (mL/min)	No. of Observations	Contrasts Linear	Quadratic
1	8	-1	1
5	8	0	-2
10	8	1	1

Table 7.11. Estimates of the linear, quadratic, cubic contrasts of temperature and linear, quadratic contrasts of feed flow rate.

Contrasts	Estimate	Standard Error	P value	Estimate	Standard Error	P value
	Total Phenolics			Anthocyanins		
Linear Temperature	-22.5056	4.568629	0.000350	-19.0812	5.711383	0.005878
Quadratic Temperature	-9.1281	2.043153	0.000769	-13.5512	2.554208	0.000187
Cubic Temperature	-7.2553	4.568629	0.138256	-8.9132	5.711383	0.144591
Linear Feed flow rate	-4.12824	1.251171	0.006347	-5.68590	1.564127	0.003418
Quadratic Feed flow rate	-1.54300	2.167091	0.490067	-4.26019	2.709147	0.141812

Table 7.12. Comparison of least square means of recovery of total phenolics and anthocyanins by Fisher's least significant difference tests.

Factors	Total phenolics	Anthocyanins
Temperature		
5	77.8a	101.2a
12	78.6a	104.3a
27	78.5a	105.1a
60	70.3b	94.6b
Feed Flow Rate		
1	78.1a	103.4a
5	76.8a	102.7a
10	74.0b	97.8b

The means of the recovery of total phenolics and anthocyanins with the same letter in a column are not significantly different from each other across different temperatures and feed flow rates at 5 % level of significance, n = 2.

REFERENCES

(1) Biswas, R.; Saalia, F.K.; Phillips, R.D. Effect of pectinase, heat treatment, and freezing on yield of total phenolics, total anthocyanins, and antioxidant potential in aqueous extracts from Georgia-grown Muscadine grapes and rabbiteye blueberries. Chapter 3 In *Development of technologies for the production of polyphenolic neutraceuticals from Muscadine grapes and Rabbiteyeblueberries.Thesis*. **2007.**

(2) Biswas, R.; Saalia, F.K.; Phillips, R.D. Influence of ethanol concentrations, extraction temperature, and time on the yield of total phenolics, total anthocyanins, and antioxidant potential in aqueous extracts from Georgia-grown Muscadine grapes and rabbiteye blueberries. Chapter 4 In *Development of technologies for the production of polyphenolic neutraceuticals from Muscadine grapes and Rabbiteyeblueberries.Thesis*. **2007.**

(3) Biswas, R.; Saalia, F.K.; Phillips, R.D. Influence of fermentation on the yield of total phenolics, total anthocyanins, and antioxidant potential in aqueous extracts from Georgia-grown Muscadine grapes and rabbiteye blueberries. Chapter 5 In *Development of technologies for the production of polyphenolic neutraceuticals from Muscadine grapes and Rabbiteyeblueberries.Thesis*. **2007.**

(4) Bonilla-Pastrana, E.; Akoh, C.C.; Sellapan, S.; Krewer, G. Phenolic content and antioxidant capacity of muscadine grapes. *J. Agric. Food Chem*. **2003**, 51:5497 – 5503.

(5) Yilmaz, Y.; Toledo, R.T. Major flavonoids in grape seeds and skins: Antioxidant capacity of catechin, epicatechin, and gallic acid. *J. Agric. Food Chem*. **2004**, 52 (2), 255 – 260.

(6) Talcott, S.T.; Brenes, C.H.; Pires, D.M.; Pozo-Insfran, D.D. Phytochemical stability and color retention of copigmented and processed muscadine grape juice. *J. Agric. Food. Chem*. **2003**, 51:957 – 963.

(7) Lee, J., and Wrolstad, R.E. 2004. Extraction of anthocyanins and polyphenolics from blueberry processing waste. *J. Food Sci*. **69**(7):C564-C573.

(8) Sellapan, S., Akoh, C.C., and Krewer, G. 2002. Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. *J. Agric. Food. Chem*. **50**:2432 – 2438.

(9) Youdin, K.; Martin, A.; Joseph, J. Incorporation of the elderberry anthocyanins by endothelial cells increases protection against oxidative stress. *Free Radic. Biol. Med*. **2000**, 29(1):51-60.

(10) Sun, A.Y.; Simonyi, A.; Sun, G.Y.. The "French Paradox" and beyond: neuroprotective effects of polyphenols. *Free Radic. Biol. Med*. **2002**, 32(4):314-318.

(11) Schmidt, B.M.; Erdman, Jr, J.W.; & Lila, M.A. (2005) Effects of food processing on blueberry antiproliferation and antioxidant activity. *Journal of food science,* 70 (6), S389-S394.

(12) Jayaprakash, G.K.; Selvi, T.; & Sakariah, K.K. (2003) Antibacterial and antioxidant activities of grape (*Vitis vinifera*) seed extracts. *Food Research International,* 36, 117-122.

(13) Auroma, O.I.; Bahorun, T.; & Jen, L-S. (2003) Neuroprotection by bioactive components in medicinal and food plant extracts. *Mutation research,* 544, 203-215.

(14) Prior, R.L.; Lazarus, S.A.; Cao, G.; Muccitelli, H.; & Hammerstone, J.F. (2001). Identification of procyanidins and anthocyanins in blueberries and cranberries (Vaccinium Spp.) Using High-Performance Liquid Chromatography/Mass spectrometry. *Journal of Agricultural and Food Chemistry*, 49, 1270-1276.

(15) Cao, G.; Sofic, E.; & Prior, R.L. (1996). Antioxidant capacity of tea and common vegetables. *Journal of Agricultural and Food Chemistry*, 44(11), 3426-3431.

(16) Bonilla, F.; Mayen, M.; Merida J.; Medina, M. Extraction of phenolic compounds from red grape marc for use as food lipid antioxidants. *Food Chem*. **1999**, 66, 209-215.

(17) Stintzing, F.C.; Carle, R. Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. *Trends Food Sci. Tech*. **2004**, 15(1), 19-38.

(18) Leon-Gonzalez, M.E.; Perez-Arribas, L.V. Chemically modified polymeric sorbents for sample preconcentration. *J. Chromatogr*. **2000**, 902, 3-16.

(19) Trochimczuk, A.W.; Streat, M.; Kolarz, B.N. Highly polar polymeric sorbents characterization and sorptive properties towards phenol and its derivatives. React. Funct. Polym. **2001**, 46, 259-271.

(20) Scordino, M.; Mauro, A.D.; Passerini, A.; Maccarone, E. Highly purified sugar concentrate from a residue of citrus pigments recovery process. *LWT-Food Sci. Technol.*. **2007**, 40(4), 713-721.

(21) Kammerer, D.; Kljusuric, J.G.; Carle, R.; Schieber, A. Recovery of anthocyanins from grape pomace extracts (*Vitis vinifera* L. cv. Cabernet Mitos) using a polymeric adsorber resin. *Eur. Food Res. Technol.* **2005**, 220,431–437.

(22) Kammerer, D.R.; Saleh, Z.S.; Carle, R.; Stanley, R.A. Adsorptive recovery of phenolic compounds from apple juice. *Eur. Food Res. Technol.* **2007**, 224, 605–613.

(23) Scordino, M.; Mauro, A.D.; Passerini, A.; Maccarone, E. Adsorption of flavonoids on resins: Hesperidin. *J. Agric. Food Chem*.**2003**, 52,1965–1972.

(24) Scordino, M.; Mauro, A.D.; Passerini, A.; Maccarone, E. Adsorption of flavonoids on resins: Cyanidin – 3 – gluoside. *J. Agric. Food Chem*. **2004**, 52,1965–1972.

(25) Mauro, A.D.; Arena, E.; Fallico, B.; Passerini, A.; Maccarone, E. Recovery of anthocyanins from pulp wash of pigmented oranges by concentration on resins. *J. Agric. Food Chem*. **2002**, 50, 5968–5974.

(26) Scordino, M.; Mauro, A.D.; Passerini, A.; Maccarone, E. Selective recovery of anthocyanins and hydroxycinnamates from a byproduct of citrus processing. *J. Agric. Food Chem.* **2005**, 53(3), 651-658.

(27) Singleton, V.L.; Rossi. Jr., J.A. Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. *Am. J. Enol. Vitic*. 1965, 16,144-158.

(28) Giusti, M.M.; Wrolstad, R.E.. Characterization and measurement of anthocyanins by UV-visible spectroscopy. In Current protocols in food analytical chemistry. **2001**, pp F1.2.1-F1.2.13. Wrolstad, R.E., Acree, T.E., An, H., Decker, E.A., Penner, M. H., Reid, D.S., Schwartz, S.J., Shoemaker, C.F., Sporns, P., Eds. Wiley: New york, NY.

(29) Boulton, R.. The copigmentation of anthocyanins and its role in the color of red wine: A critical review. *Am. J. Enol. Vitic*. **2001**, 52(2), 67–87.

(30) Brouillard, R.; Dangles, O. Anthocyanin molecular interactions: the first step in the formation of new pigments during wine ageing? *Food Chem*. **1994**, 51, 365–371.

CHAPTER 8

PILOT SCALE, PRE COMMERCIAL PRODUCTION OF SPRAY DRIED POLYPHENOLIC RICH POWDERS FROM MUSCADINE POMACE AND RABBITEYE BLUEBERRIES GROWN IN GEORGIA[1]

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Abstract

Excess production or byproducts of muscadine grapes and blueberries represent a promising source of isolated, nutraceutical grade antioxidants. The objective was to develop technologies to produce shelf-stable bioactive powders. Muscadine pomace and whole blueberries (28 Kg) were finely ground and depectinized (Pectinex BE®) then extracted with water or 35% ethanol at 60 C, 1h; or fermented by *Saccharomyces cerevisiae* at 25 C to ~15% ethanol. Crude extracts (~ 70L) were filtered by bladder press and plate and frame filter. Ethanol was removed from eluents and ethanolic extracts by thin-film vacuum evaporation. Phenolics from filtered, alcohol-free solutions were adsorbed/desorbed (80% ethanol) on Sephabeads 700 resin to purify and concentrate. Concentrated extracts were spray dried at inlet and outlet temperatures of 150 C and 90 C. Extracts and powders were analyzed for total phenolics, total anthocyanins and total antioxidant activity. Total phenolics in aqueous, ethanolic, and fermented extracts were 6.9, 12.2, and 4.6 and 7.8, 11.2, and 2.1 g gallic acid equivalents/ Kg db in muscadine pomace and blueberries respectively. Total anthocyanins were 1.6, 1.8, and 0.3 (muscadine pomaces) and 1.7, 2.6, and 0.07 (blueberries)g cyanidin-3-glucoside equivalents/Kg db. Antioxidant activity (FRAP) of the three extracts were 76, 160, and 62 mmol Trolox equivalents/Kg db for muscadine pomaces and 157, 240, 42 mmol Trolox equivalents/Kg db for blueberries. Spray-dried powders were flowable and non-hygroscopic. Muscadine pomace powders contained 9-11g total phenolics and 0.3-3.7g anthocyanins. Blueberry powders contained 1.6-11g total phenolics and 0.06-3.6g of anthocyanins. While this represents only a 22-52% recovery from original sources, it indicates the potential for commercial production of these materials.

8.1. Introduction

Muscadine grapes and rabbiteye blueberries are prominent, Georgia-grown products that have high levels of flavonoids (Flora, 1978, Boyle et al., 1990, Lamikanra et al., 1996, Auw et al., 1996, Ector et al., 1996, Prior et al., 1998, Prior et al., 2001, Musingo et al, 2001, Talcott et al., 2002, Sellapan et al., 2002, , Moyer et al., 2002,Pastrana-Bonilla et al., 2003, Talcott et al., 2003, Lee et al., 2004, Yilmaz et al., 2004, Lee et al.; 2005 Su et al., 2006, Stojanovich et al., 2007). In addition, Muscadine grapes are also a source of trans-resveratrol also another potent antioxidant (Magee et al., 2002). Both Muscadine grapes and Rabbiteye blueberries were found to contain total phenolics compounds ranging from 15 – 25 and 3.9 – 7.8 g/Kg db and 9 – 28 g/Kg and 0.5 – 19 g/Kg db resectively as determined by us in our previous studies and other researchers (Talcott et al., 2003, Pastrana-Bonilla et al., 2003, Yilmaz et al., 2004, Lee et al., 2004, Lee et al., 2005 , Biswas et al., 2007). Research done on the anti-inflammatory properties of muscadine grape extracts showed that there was 50 % less paw edema in rats fed with muscadine grape extracts compared with controls (Greenspan et al., 2005). Researchers had also demonstrated the potentiality of the polyphenolic compounds in their application as medicines either as powders or as liquids to cure diseases such as cardiovascular, cancer, Alzeihmer's disease, and others (Verlangieri et al., 1985, Joshipura et al., 1999, Joseph et al., 1999, 2005, Engelhardt et al., 2002, Rossi et al., 2006, Friedman et al., 2007,). This has led to the rapid growth of their inclusion as dietary supplements in the US market. The U.S. market for dietary supplements was estimated as $14 billion in 1999 (Lachance and Saba, 2002). Development of spray dried polyphenolic rich powder from the muscadine pomaces and rabbiteye blueberries

would strengthen the production of the commodities by providing new markets and/ or by adding value to byproducts and expand existing, or initiate new businesses to conduct such manufacturing and marketing. Finally, it will provide reliable nutraceuticals for consumers, while connecting these products to Georgia and its industries in the consciousness of consumers.

The use of pilot equipment allows an accurate evaluation of the feasibility of commercial scale production. The pilot scale production of neutraceutical powders from muscadine pomaces and rabbiteye blueberries consisted of grinding, extraction, filtration, purification by adsorption/desorption with resin, vacuum evaporation of ethanol and finally spray drying. Previous studies had already optimized the extraction, purification, and vacuumn evaporation. The optimum data points from lab scale studies were employed to perform pilot plant studies involving 20 gallons of extracts.

Liquid products have the disadvantages of limited shelf life, problems of transportation, and susceptiblity to oxidative damage as compared to powders. Powdered products have a longer shelf life when handled properly (Glicksmann, 1969 and Revie & Thomas, 1972). Freeze drying is one of the best ways to dry these compounds. However, spray drying is more practical and economical method of producing dry powders (processing cost is about 30 -50 times less than freeze drying) (Corke et al., 2000). Spray drying involves atomization of a feed slurry/solution in the form of spray in the drying medium with moisture removal caused by hot air in the chamber. It is difficult to spray dry sugar rich products like muscadine grape and blueberry extracts. The problem of stickiness is caused by very low glass transition temperatures (Tg) of glucose, fructose, tartaric acid, citric acid etc. It is necessary that the particle temperature should

be much below Tg to get a dry, non-sticky powder. As a consequence, high molecular weight compounds (having a high Tg) may be added to the spray drying feed to have a successful drying under practical conditions (Adhikari et al., 2005). There is also an added advantage of employing carrier materials, as they provide a protective wall around the feed particle preventing it from oxidation.

Cai et al., (2000) studied the production and properties of spray dried amaranthus betacyanin pigments. They used a range of M (10 – 25DE) and starch as coating agent to spray dry and found that inlet/outlet temp (165 – 180°C/ 92 – 96°C), 20 – 40 % solids feed content, M 25 and 10 DE (dextrose equivalent) gave highest product recovery and stability. Main et al., (1978) conducted experiments on production of spray dried anthocyanin powders using a carrier. They found outlet air temperature < 90°C gave the best yield. They used inlet air temperature of 175°C. Recommended solid concentration in the feed and particle size (produced by the atomizer) is 25%, and 5-30 μm respectively to ensure optimal drying. Common outlet drying temperature for sticky materials ranged from 65 – 90 C.

The other alternative to dry sugar rich extracts would be to remove sugar either by fermentation to dryness (Auw et al., 1996, Talcott & Lee 2002, Su & Silva 2006) or by purification/concentration by adsorption/desorption by polystyrene divinyl resin (Scordino, 2007; 2003; 2004, Kammerer, 2005).

In our laboratory experiments we had optimized the fermentation parameters and adsorption/desorption parameters for total phenolics and total anthocyanins from extracts of muscadine grapes and rabbiteye blueberries. The overall goal of this project was to conduct pilot plant production of polyphenolic powders from muscadine grapes and

rabbiteye blueberries. The specific objectives were to quantify the amount of powders from aqueous, ethanolic, and fermented extracts of muscadine pomaces and rabbiteye blueberries and determine the total phenolics, total anthocyanins, and the antioxidant activity of these powders. The proposed research will provide specific information needed by processors to set up a manufacturing facility to manufacture and market nutraceutical preparations from Georgia commodities, and to make valid claims as to the content and efficacy of their principal ingredients.

8.2. Materials and Methods

8.2.1. Fruits

Muscadine pomace (Supreme variety) were obtained as a gift from Paulk vineyards. Blueberries (mixed varieties) were obtained from a local grower near Griffin, GA.

8.2.2. Chemicals

Gallic acid, L-ascorbic acid, 6-hydroxy-2, 5, 7, 8-tetramethychroman-2-carboxylic acid (TROLOX); 2, 4, 6-tripyridyl-s-triazine (TPTZ), 6-carboxy fluorescein, and 2,2'-Azobis (2-methylpropionamidine) dihyrochloride (AAPH) were purchased from Sigma (St. Louis, MO). Moisture analysis of the berries was done by a vacuum oven method at 70°C and 25mm Hg for 8 H (AOAC 934.06).

8.2.3.Grinding

A quantity of 78.4 Kg of muscadine pomace and 89.2 Kg of whole blueberries were thawed and ground using a Urschel Mill (Urschel Comitrol Processor Model 1700, Urschel Laboratories Inc., Valparaiso, IN). The picture of Urschel mill was given in Fig. 8.2. While grinding, ~ 1 L of deionized water was added to reduce the heat produced

during grinding. The ground muscadine pomace and blueberries were mixed with ascorbic acid (0.2 %) to prevent oxidation and were divided into three parts for three different extraction, aqueous, ethanolic, and fermentation. Each of these parts was stored in 5 gallon buckets for a total of 10 buckets and was frozen at –20 C before the day of extraction.

8.2.4. Extraction

Three different extraction approaches were followed to extract polyphenolic compounds from muscadine pomace and rabbiteye blueberries at pilot scale using optimum points determined from our previous studies.

Aqueous Extraction

Twenty-six kilograms of ground muscadine pomace and ground blueberry were thawed at 25 C on consecutive days. Each lot was transferred to an extraction tank (Philipsburg, PA) of capacity 50 G, and 56 L of deionized water was added to it. The extraction tank was then covered with a lid coupled with a stirrer (Stir-pak Model 50002-02 Stirrer Controller Cole-Parmer Instrument Co., Vernon Hills, IL).

The tank was heated by two flexible drum heating bands of width 3" (Stir-pak Model 50002-02 Stirrer Controller Cole-Parmer Instrument Co., Vernon Hills, IL) set at 9 for an hour till the temperature reaches 60 C. The picture of the extraction tank was given in Fig. 8.2, and 8.3 Then the extraction was done at 60 C for 1 H with added Pectinex BE enzyme ® (28 mL). The extracts were then collected into19 L buckets before filtration. A flowchart of the extraction procedure is shown in Fig. 8.1.

Alcoholic extraction

Twenty-eight kilograms of ground muscadine pomace and ground blueberry were thawed at 25 C on consecutive days. They were then transferred to the extraction tank with a stirrer and incubated with Pectinex BE ® enzyme (28 mL) for one hour at 60 C as was shown to be optimal in the lab scale studied. Amounts of 95% ethanol (31 Kg) and deionized water (25 Kg) were mixed such that the ethanol concentration was 35 % w/w, and added to the pulp. This specific ethanol concentration was chosen for the pilot plant studies because this gave the maximum yield of total phenolics in the lab scale studies. The macerated fruits and the solvent mixture was extracted for an hour at 60 C. The extracts were then collected in 5 G jars and kept aside for filtration.

Fermentation

Twenty-three kilograms of ground muscadine pomace and 25 Kg of ground blueberry were thawed at 25 C on consecutive days., transferred to fermentation tanks and mixed with 56 L of deionized water and 14.5 Kg of sugar to yield the desired final ethanol content (15.0 % v/v). The mixtures were depectinized for 1 H at 60°C with Pectinex BE (100µL/100 g fruit), and rehydrated yeast (0.25 g/L) and yeast nutrient (0.25g/L) were added. Fermentation was conducted for three days in air, then anaerobically until complete dryness (no residual sugars) was acheived. The ferments were collected in 5 G jars before filtration. The picture of the fermentation tanks and muscadine pomace and blueberry fermentation was shown in Fig. 8.4.

8.2.5. Filtration

All the six extracts were divided into half. One half of it was frozen for a subsequent microfiltration study. The other half was loaded into a bladder press (RLS

Equipment Corp., Egg Harbor City, NJ) fitted with finely woven cloth bags, and was subjected to a pressure of 1 Kg/cm^2. The press cake was discarded and the filtrate was collected. The filtrate was passed through plate and frame filter (St. Patrick Of Texas, Austin, TX) consisting of 27 plates with inserted filter papers and was subjected to a pressure of 10 psig. The clear filtrate was collected. The pictures of both the bladder press and plate and frame filter in operation was shown in Figs. 8.5, and 8.6.

8.2.6. Adsorption/Desorption on Sepabeads 700 resin

The resins (600 g) were activated by overnight treatment with twice its volume of 96.6 % w/w ethanol, followed by rinsing with two bed volumes of water before loading with extracts. The extracts were loaded onto the column (4.8 × 60 cm, Kontes Glass Company, Vineland, N J) packed with 600 g of activated wet resin at a outflow rate of 15 mL/min. The column was then washed with 2 L of deionized water to remove sugars and other undesirable compounds at the same outflow rate. Then the adsorbed polyphenolic compounds were eluted with 2 – 9.0 L of 80 % w/w ethanol for the different extracts. After elution of each extract the column was run with 2.0 L of 95.6 % w/w ethanol at the same outflow rate. The picture of the columns during adsorption/desorption are shown in Figs. 8.7

8.2.7. Concentration

The ethanolic extracts of muscadine pomaces and blueberries and the 80 % w/w eluents of all the extracts were concentrated by thin film evaporator (LCI Corporation, Charlotte, NC). The fluids entered above the heated zone, which was set at 95 C and was uniformly distributed over the unit's internal surface by the rotor running at 70 % of its original speed (1800 RPM). Volatile components were rapidly evaporated and were

removed by vacuum. The temperature of the vapors was measured by thermocouple as 50 C, while the temperature of the concentrate was 27 C. The picture of the flash evaporator was given in Fig. 8.8.

8.2.8. Spray Drying

The concentrated eluents were spray dried in a pilot scale spry drier (Anhydro Inc., Olympia Fields, IL). The inlet temperature was set at 150 C. The feed flow rate into the spray dryer was adjusted to 20 mL/ min to get an outlet temperature of 90 C. The atomizer was running at 70 % of it maximum speed (50,000 RPM). The system was stabilized initially by deionized water, before the eluents were passed. The picture of the spray drier is given in Fig. 8.10. The entire set up of the pilot plant with the extraction tank and the filtration units was shown in Fig. 8.9.

8.2.9. Total phenolics

Total phenolics were estimated colorimetrically using the Folin-Ciocalteaux method (Singleton, and Rossi, 1965). A sample aliquot of 200 mL was added to 800 mL of deionized water, 5 mL of Folin-Ciocalteaux reagent, and 4 mL of saturated sodium carbonate solution (75g/L) and mixed on a vortex mixer. The absorbance was measured at 765 nm with a Hewlett Packard 8451A diode array spectrophotometer (Avondale, PA, USA) after incubation for two hours at room temperature (~25 C). Quantification was based on the standard curve generated with 100, 200, 300, 400, and 500 mg/L of gallic acid. The final concentration of phenolics was calculated based on total volume of extract and initial weight of berries; and expressed as mg/Kg dry weight. The experiment was conducted under subdued light.

8.2.10. Total anthocyanins

Total anthocyanins were measured by the pH differential method (Giusti, and Wrolstad, 2001) using two buffer systems - potassium chloride buffer, pH 1.0 (0.025 M) and sodium acetate buffer, pH 4.5 (0.4 M). Extracts were diluted with water to give Absorbance in the range of 0.1 - 1.2 at 510 nm. Aliquots of 0.2 ml of diluted extracts were mixed with 1.8 mL of each buffer, vortexed and read against a blank at 510 and 700 nm with a Hewlett Packard 8451A diode array spectrophotometer (Avondale, PA, USA). Absorbance was calculated as

$A = (A_{510}\ nm - A_{700}\ nm)\ pH\ 1.0 - (A_{510}\ nm - A_{700}\ nm)\ pH\ 4.5$

Monomeric anthocyanin pigment concentration in the extract was calculated as cyanidine-3-glucoside

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

Where A = absorbance, MW = molecular weight (449.2), DF = dilution factor, ε = molar absortivity (29,600), l = path length (1 cm).

The final concentration of anthocyanins was calculated based on total volume of extract and weight of starting fruit material, and expressed as mg/Kg dry weight.

8.2.11. Assay of antioxidant capacity

The antioxidant capacity was assayed by Ferric reducing/antioxidant power assay (FRAP) and Oxygen radical-scavenging activity (ORAC).

Ferric reducing/antioxidant power assay (Benzie, and Strain, 1996). FRAP reagent was made by mixing 2.5 mL of 10 mM TPTZ in 40mM HCl , 25 mL of 300 mM acetate buffer, pH 3.6, and 2.5 ml of 20 mM $FeCl_3.6H_20$. One hundred mL of the diluted sample was added to 3 mL of the working FRAP reagent, vortexed, and allowed to stand for

10mins. Absorbance was measured at 593 nm with a Hewlett Packard 8451A diode array spectrophotometer (Avondale, PA, USA). Quantification was based on the standard curve generated with 100, 200, 300, 400, and 500 mM of TROLOX. The final concentration was expressed as umol of TROLOX equivalents per Kg dry basis. .

8.3. Results and discussions

8.3.1. Total phenolics.

The total phenolics in aqueous extracts of muscadine pomace were estimated as 8 g gallic acid equivalents/Kg db or 40 g in 43 L of extracts (Table 8.1). After filtering the extracts through bladder press and plate and frame filter, the total phenolics decreased to 7 g gallic acid equivalents/Kg db or 35 g in 43 L of extracts. This could be due to the fact that there were some losses in the extracts due to handling operations, degradation of phenolic compounds on exposure to air, light. At pilot scale there was limited possibility of controlling the experiments precisely from being exposed to air and light. The filtered extracts were purified by adsorption to Sepabeads 700 and were water washed and finally eluted with 80 % w/w ethanol. The total phenolics in the eluants amounted to 12.7 g. Total volume of eluant was 6 L. The eluants were then and the total phenolics in the concentrate were estimated as 17.9 g. The total volume of concentrate collected was 2.28 L. Since the concentrate was less diluted than the eluant, the amount of total phenolics was found higher in the former. The concentrate was spray dried at a feed flow rate of 30 mL/min, inlet temperature of 150 C and outlet temperature of 90 C. The amount of spray dried powder collected was 40.7 g. The moisture content of the spray dried powder was determined as 4.88 %. The powder was very fluffy, non-sticky, amorphous and was easily soluble in deionized water at 25 C. The color of the powder was purple as shown

in the Fig. 8.11. The amount of total phenolics in the spray dried powder was estimated as 11.6 g. The recovery of total phenolics from aqueous extracts of muscadine pomace was 33.3 %. The recovery could be improved if we could control the losses due to transfer from equipment to another or initiate a continuous process instead of batch process. Total time taken for the entire operation was determined as 33 H.

Total phenolics in the ethanolic extracts of muscadine pomace were estimated as 14.8 g gallic acid equivalents/Kg db. After filtration through bladder press, followed by plate and frame filter the total phenolics was estimated as 12.2 g gallic acid equivalents/Kg db or 57 g gallic acid equivalents in 40 L of extracts. The ethanolic extracts had a higher total phenolic content than the aqueous extracts, which could be explained by the fact that the phenolic compounds were more soluble in 35 % w/w ethanol than in water. The extracts were then concentrated by thin film evaporator to eliminate ethanol before running through the column. The concentrate collected was not clear and the volume of the concentrate was 17.3 L. The reason could be due to formation of complexes between flavonoids and anthocyanins, or within anthocyanins (copigment complexes) as they were present in a very highly concentration solutions. The formation of copigment complexes are dependent on the pigment concentration, molar ratio of cofactor to pigment, pH, the extent of non-aqueous conditions, and the anions in the solution (Boulton, 2001, and Brouillard et al., 1994). The highly concentrated solution of the aqueous ethanolic extracts seemed to favor the formation of copigment complexes. However, formation of copigment complexes helps in stabilizing the anthocyanins fro degradation. So there would be an increase in the amount of anthocyanins, which will be discussed in the next section, at the expense of flavonoids, and phenolic acids. This was

confirmed by the amount of total phenolics, which was estimated as 27 g gallic acid equivalents. There was a reduction of 51 % in the total phenolics amount. Since the concentrated extract was so hazy, it was not possible to pass it through the column packed with Sepabeads 700. The existing filtration unit was not adequate to filter the concentrated extracts.

Our last approach was to ferment the ground muscadine pomace. The fermentation lasted for a month and was continued till dryness. The ethanol was determined as 14.9 %v/v in the fermented extract of muscadine pomace. The total phenolics in the fermented extracts were amounted to 10.5 g gallic acid equivalents/ Kg db or 43.9 g gallic acid equivalents in 36.5 L. The amount of total phenolics in the fermented extracts was in between the aqueous and the ethanolic extracts of muscadine pomace. After filtration through bladder press followed by plate and frame filter, the amount of total phenolics was estimated as 4.64 g gallic acid equivalents/ Kg db or 19.3 g gallic acid equivalents in 25.8 L. The amount of total phenolics in the filtered fermented extract was much less than reported for the aqueous and ethanolic extracts. We were optimizing the operating conditions for bladder press and plate and frame filters with the muscadine fermented pomace extracts. During the optimization process there were losses of extracts as well dilution of extracts with deionized water. It was not possible to control losses precisely at pilot scale operations due to technical problems. This could be the reason for the lower value of total phenolics in the filtered extracts. The filtered extract was adsorbed onto Sepabeads 700 and water washed and finally eluted with 80 % w/w ethanol. The amount of total phenolics in the eluents was estimated as 10.7 g gallic acid equivalents. During the loading phase of the fermented extracts of muscadine

pomace there was a fair amount of bleed through which indicated that the ethanol content in the extracts was so high that the phenolic compounds could not get adsorbed onto the resin. This suggested that a preconcentration step was necessary to remove ethanol or the extracts could be directly spray dried without passing thorugh the column. The main objective of the adsorption/desorption to Sepabeads 700 was to eliminate sugars. In the fermented extracts, sugar was already removed upon conversion to ethanol by *Saccharomyces cerevisiae*. So we could spray dry the fermented extracts directly. The ethanol was removed from the eluants by thin film evaporator. The total phenolic in the concentrated extracts amounted to 21.05 g in 0.98 L. The concentrated extract was then spray dried. The spray dried powder amounted to 25.5 g. The powder was dark purple in color, amorphous and was highly soluble in water (Fig. 8.11). The moisture content of the spray dried powder was determined as 2.08 %. The amount of total phenolics in the spray dried fermented muscadine pomace powder was estimated as 9.41 g gallic acid equivalents. The recovery of total phenolics amounted to 45 %, which was significantly good.

We followed the same three approaches for blueberries. The total phenolics in the aqueous extracts of blueberries amounted to 5.9 g gallic acid equivalents/Kg db or 29.6 g gallic acid equivalents in 43.4 L of the extracts (Table 8.2). After filtration through bladder press and plate and frame filter, the total phenolics in the aqueous extracts of blueberries amounted to 7.80 g gallic acid equivalents/Kg db or 28.3 g gallic acid equivalents in 43.4 L of the extracts. There was a loss of 4.53 % during the filtration step. The filtered extracts were adsorbed on the Sepabeads 700 resin, water washed, and finally eluted with 80 % w/w ethanol. The collected volume of 80 % ethanol eluents was

8 L. The total phenolics in the eluents amounted to 7.78 g gallic acid equivalents. The amount of total phenolics was found to be very less than the original amount (28.3 g). The reason for this could be attributed to the dilution factor or formation of copigment complexes with anthocyanins at the expense of phenolic acids, flavonoids and others. The eluents were then concentrated by evaporating ethanol by thin film evaporation. The total phenolics in the concentrated extract amounted to 19.8 g. The concentrated extract was spray dried. The amount of spray dried powder collected was determined as 39.9 g. The powder was deep red in color, non-sticky, amorphous, and was readily soluble in water (Fig. 8.12). The moisture content of the powder was estimated as 4.44 %. The amount of total phenolics in the spray dried powder of the aqueous extract of blueberry was determined as 11.3 g gallic acid equivalents. This amounts to a recovery of 40 % of total phenolics in the original extract.

Ethanolic extracts of blueberry gave a total phenolics yield of 15.7 g gallic acid equivalents/ Kg db or 56.8 g in 40.2 L of the extracts. The yield of total phenolics in the ethanolic extracts was almost twice than that of the aqueous extracts. This indicated that the solubility of the total phenolic compounds was increased in 35 % w/w ethanol than in pure water. After filtration through bladder press, followed by plate and frame filter, the amount of total phenolics estimated to 11.3 g gallic acid equivalents/ Kg db or 40.9 g in 40.2 L of the extracts. There was a loss of 28 % in the amount of total phenolics in the ethanolic extracts of blueberries after the filtration steps. The filtered extracts were concentrated by thin film evaporator to remove most of the ethanol before running through the column. The amount of total phenolics in the concentrated ethanolic extracts was estimated as 14.9 g gallic acid equivalents. The volume of the collected concentrate

was estimated as 22 L. The volume of the extract was reduced by 45.3 %. The removal of ethanol from the ethanolic extracts reduced the concentration of total phenolics by 33.2 %. The concentrated ethanolic exract of blueberries was not as cloudy as the muscadine pomaces. The reason was due to reduced formation of copigment complexes between phenolic acids, flavonoids and anthocyanins as compared to muscadine pomace extracts. The reduced formation of copigment complexes was attributed to the lesser amounts of total phenolic compounds in the ethanolic extracts of blueberries as compared to muscadine pomaces. Since the concentrated extracts were not hazy so it was purified by adsorption on the Sepabeads 700 resin. Then it was water washed and finally eluted with 80 % w/w ethanol. This purification step resulted in removal of sugar from the ethanolic extracts of blueberry. The amount of total phenolics in the eluents of the ethanolic extracts of blueberry was determined as 30.0 g gallic acid equivalents. The total volume of the eluents collected was 6.0 L. The eluents were further concentrated by removing the ethanol by thin film evaporation to a volume of 1.67 L. The total phenolics in the concentrated eluents of the ethanolic extracts of blueberries amounted to 16.9 g gallic acid equivalents. The concentrated eleuents were spray dried. The spray dried powder amounted to 41.8 g. The powder was deep red in color, amorphous, non-sticky (Fig. 8.12). The moisture content of the powder was determined as 4.68 %. The powder was sparingly soluble in water. The insolubility of the powder was due to the formation of the hydrophobic copigment complexes in the concentrated solutions. The amount of total phenolic in the spray dried powder was determined as 14.6 g gallic acid equivalents. This accounted for a recovery of 35.7 % in the amount of total phenolics in the ethanolic extracts of blueberry.

The blueberries were also fermented at pilot scale to examine the yield of total phenolics. Fermentation of blueberries lasted for one month. The ethanol produced by Saccharomyces cerevisiae at the expense of sugar present in the fruit and added sugar was determined as 10.9 % v/v. The total phenolics in the fermented extracts of blueberries was determined as 5.1 g gallic acid equivalents/Kg db or 18.6 g gallic acid equivalents in 31 L of fermented extracts. The yield of total phenolics in the fermented extracts was similar to that in the aqueous extracts. After filtration through bladder press, followed by plate and frame filter the total phenolics was amounted to 2.14 g gallic acid equivalents/Kg db or 7.79 g gallic acid equivalents in 20.8 L of fermented extracts of blueberry. There was a loss of 58.1 % in the amount of total phenolics after the filtration steps. The loss was mainly due to handling problems occurred while transferring from one instrument to the other. The filtered fermented extracts of blueberry were purified by adsorption on Sepabeads 700 resin, water washed and finally eluted with 80 % w/w ethanol. The volume of the eluents was determined as 2.0 L. The total phenolics in the eluents were determind as 2.24 g gallic acid equivalents. During the adsorption/desorption of phenolic compounds from the fermented extracts of blueberries on Sepabeads 700, there was a loss of 71.2 % in the amount of total phenolics. The reasons for this loss was attributed to the fact that there was a significant bleed through from the column during the loading phase of the fermented extracts of blueberries. The amount of total phenolics in the outflow during loading of the fermented extracts was determined as 4.32 g gallic acid equivalents. Due to the presence of 10.9 % v/v ethanol in the fermented extracts of blueberry, the phenolic compounds were not able to get adsorbed by the resin. The eluents were concentrated by removing ethanol by thin film

evaporator to a volume of 0.45 L. The total phenolics in the concentrated eluents of fermented blueberries were determined as 2.43 g gallic acid equivalents. The concentrated eluent were then spray dried to a dark red colored, non-sticky, amorphous powder (Fig. 8.12). The amount of spray dried powder was determined as 4.45 g. The moisture content of the powder was determined as 6.59 %. The total phenolics in the spray dried fermented blueberry powder was determined as 1.64 g gallic acid equivalents. Considering several losses at different stages of the process, there was a recovery of 21.0 % in the amount of total phenolics.

8.3.2. Total Anthocyanins

The total anthocyanins in the aqueous extracts of muscadine pomace were determined as 1.43 g cyandin 3 glucoside equivalents/ Kg db or 7.16 g cyandin 3 glucoside equivalents in 42.9 L. Upon filtration of the extracts through bladder press followed by plate and frame filter, the amount of anthocyanins remained almost the same. The filtered extracts were purified by adsorption on Sepabeads 700 resin, washed with water, and finally eluted with 80 % w/w ethanol. The total anthocyanins in the eluents of the aqueous extract of muscadine pomace amounted to 6.11 g. The total volume of the eluents was estimated as 6.0 L. There was a loss of 14.6 % during adsorption/desorption steps. The loss was mush less than compared to the total phenolics in the aqueous extracts of muscadine pomace. The reason for the reduced loss in the amount of total anthocyanins in the eluents was due to the increase in the stability of these compounds through the formation of copigment complexes either within themselves or with phenolic acids or flavonoids. The eluents were then subjected to concentration by removing ethanol by thin film evaporation technique. The concentrated eluents of aqueous extracts

of muscadine pomace amounted to 2.28 L with total anthocyanins amounting to 5.17 g. The concentrated eluents were then spray dried. The amount of anthocyanins in the spray dried aqueous extracts of muscadine pomace was determined as 3.80 g. This resulted in a recovery of total anthocyanins by 53.1 % in the powder from the aqueous extracts of muscadine pomaces.

For the ethanolic extracts, the anthocyanins was determined as 1.98 g cyanidin 3 glucoside equivalents/Kg db or 9.28 g cyanidin 3 glucoside equivalents in 40 L of the extracts. Upon filtration through bladder press followed by plate and frame filter, the total anthocyanins in the ethanolic extracts was determined as 1.80 g cyanidin 3 glucoside equivalents/Kg db or 8.40 g cyanidin 3 glucoside equivalents in 40 L of the extracts. So there was a loss of 9.09 % in the amount of total anthocyanins after the filtration steps. The filtered ethanolic extracts of muscadine pomace were concentrated by removing ethanol by thin film evaporation. The volume of the concentrated ethanolic extracts was determined as 17.3 L. The total anthocyanins in the concentrated ethanolic extracts of muscadine pomace were estimated as 6.94 g. As the concentrated extracts were cloudy and difficult to filter by the existing filtration units, so they were stored at –20 C before the set up of microfiltration unit. There was a recovery of 82.6 % in the yield of total anthocyanins after the concentration step.

The total anthocyanins in the fermented extracts of muscadine pomace was estimated as 0.6 g cyanidin 3 glucoside equivalents/Kg db or 2.50 g cyanidin 3 glucoside equivalents in 36.5 L of the extracts. The extracts were filtered by bladder press and subsequently by plate and frame filter. The total anthocyanins in the filtered extracts were determined as 0.35 cyanidin 3 glucoside equivalents/Kg db or 1.45 g cyanidin 3

glucoside equivalents in 36.5 L. There was a loss of 42 % in the amount of total anthocyanins after the filtration steps. The losses were mainly due to handling losses while transferring the extracts from the extraction tank to the filtration units. The other possible reasons were exposure to air and light. The filtered extracts were then purified by adsorption on Sepabeads 700 resin, water washed, and finally eluted with 80 % w/w ethanol. This adsorption/desorption process removes any pectin or other impurities from the extracts. The volume of eluents collected from the column packed with Sepabeads 700 resin was 3.30 L. The total anthocyanins in the eluents of fermented muscadine pomace extracts amounted to 1.10 g. The eluents were then concentrated by removing ethanol by thin film evaporation to a volume of 0.98 L. The total anthocyanins in the concentrated eluents of fermented extracts were estimated as 1.52 g. The concentrated eluents were spray dried to a non-sticky powder. The amount of total anthocyanins in the spray dried powder was estimatd as 0.71 g. This accounted for a recovery of 48.0 % in the amount of total anthocyanins in the spray dried powder from the fermented extracts. The recovery could be increased by skipping some steps in the whole process of poduction of spray dried powder from fermented extracts such as column adsorption/desorption, and concentration steps.

The total anthocyanins in the aqueous extracts of blueberries was determined as 1.76 g cyanidin 3 glucoside equivalents/Kg db or 6.40 g cyanidin 3 glucoside equivalents in 43.4 L of extracts. Filtration through bladder press followed by plate and frame filter did not change the amount of total anthocyanins in the aqueous extracts of blueberry as compared to the amount of total phenolics in blueberries. Since the amount of anthocyanins was much less than that of the total phenolics, a slight difference in the

concentration of anthocyanins did not change the amounts of anthocyanins by a substantial amount. The filtered aqueous extracts of blueberry was purified by adsorption on Sepabeads 700, water washed and finally eluted with 80 % w/w ethanol. The entire extract of blueberry was eluted in 8 L of 80 % w/w ethanol. The amount of anthocyanins in the eluents was determined as 5.07 g cyanidin 3 glucoside equivalents. There was a loss of 25.6 % in the amount of anthocyanins after the purification steps. This loss was much less than that of the total phenolics because of increase in the stability of anthocyanins through the formation of copigment complexes at the expense other phenolic compounds like phenolic acids, and flavonoids. Ethanol was removed from the eluents by thin film evaporation. The reason for ethanol removal was to ensure safety and prevent explosion while the extracts were spray dried. The amount of anthocyanins in the concentrated eluents of aqueous extracts of blueberry was determined as 6.49 g cyanidin 3 glucoside equivalents. The concentrated eluents were spray dried to a red colored, non-sticky, amorphous powder. The amount of anthocyanins in the spray dried powder was determined as 3.67 g, which accounted for a recovery of 57.6 %.

The total anthocyanins in the ethanolic extracts of blueberry was determined as 2.57 g cyanidin 3 glucoside equivalents/Kg db or 9.33 g cyanidin 3 glucoside equivalents in 40.2 L of the extracts. Filtration through bladder press, followed by plate and frame filter did not change the amount of anthocyanins substantially. The ethanolic extracts were concentrated by removing ethanol through thin film evaporation. The total concentrated volume was determined as 22 L, which accounted for a concentration of 54.7 % of the original extracts. The amount of total anthocyanins in the concentrated ethanolic extracts was determined as 9.18 g cyanidin 3 glucoside equivalents. There was

a loss of 1.61 % in the amount of total anthocyanins after the concentration steps. The concentrated extracts was purified by adsorption on Sepabeads 700, water washed, and finally eluted with 80 % w/w ethanol. The volume of eluents was determined as 6.0 L. the amount of anthocyanins in the eluents of concentrated ethanolic extracts was determined as 8.11g cyanidin 3 glucoside equivalents. There was a recovery of 86.9 % in the amount of anthocyanins after the purification steps. The recovery was high as compared to that of the total phenolics. The reason for the high recovery was the increased stability of anthocyanins through the formation of copigment complexes. The eluents was further concentrated by removing the ethanol by thin film evaporation. The volume of concentrated eluents was determined as 1.67 L. The total anthocyanins in the concentrated eluents were determined as 5.13 g cyanidin 3 glucoside equivalents. The concentrated eluents was then spray dried to a non-sticky powder. The amount of total anthocyanins in the spray dried ethanolic extracts of blueberries was determined as 3.69 g cyanidin 3 glucoside equivalents. This accounted for a recovery of 39.5 % in the amount of total anthocyanins in spray dried powder from the ethanolic extracts of blueberry.

The total anthocyanins in the fermented extracts of blueberry were determined as 0.13 g cyanidin 3 glucoside equivalents/Kg db or 0.46 g cyanidin 3 glucoside equivalents in 30.9 L of fermented extracts. After filtration through bladder press, followed by plate and frame filter, the total anthocyanins in the fermented extracts of blueberry decreased to 0.07 g cyanidin 3 glucoside equivalents/Kg db or 0.28 g cyanidin 3 glucoside equivalents in 30.9 L. This was due to handling losses, exposure to air and light. The filtered fermented extracts of blueberry was purified by adsorption on resin, water washed and finally eluted with 80 % w/w ethanol. The volume of eluent collected was

2.0 L and the amount of total anthocyanins was estimated as 0.27 g. The eluents were then concentrated by removing ethanol by thin film evaporation technique. The volume of the concentrated eluents was 0.45 L, which accounted for a concentration of 22.5 %. The total anthocyanins in the concentrated eluents were estimated as 0.12 g. There was a loss of 55.5 % in the amount of total anthocyanins after the concentration step. Upon spray drying, the amount of total anthocyanins in the spray dried powder was determined as 0.06 g, which accounted for a recovery of 22.2 %. This recovery of anthocyanins from the fermented blueberry extract was the lowest among others. The reason was due to handling losses, instrumental errors, and exposure to light and air.

8.3.3. Total antioxidant activity

The total antioxidant activity of the extracts of muscadine pomace and blueberry was determined by the FRAP method. For muscadine pomaces, in the aqueous and the fermented extracts the antioxidant activity was determined as 438867 and 577282 μmoles TROLOX equivalents in 42.9 and 36.5 L of the extracts respectively. Upon filtration through bladder press, followed by plate and frame filter, the antioxidant activity in the aqueous and fermented extracts decreased to 381729 and 261096 μmoles TROLOX equivalents in 42.9 and 36.5 L of the extracts respectively. After purification by adsorption on Sepabeads 700, the antioxidant activity of both the extracts decreased, then increased upon concentration by thin film evaporation technique, and then finally decreased upon spray drying. The changes in the antioxidant activiy of the aqueous and fermented extracts of muscadine pomace was represented in Fig. 8.13. The total antioxidant were very well correlated with the total phenolics and the total anthocyanins with correlation coefficient as 1.00 for both the aqueous and fermented extracts of

muscadine pomace. The correlation coefficient for the spray dried powder for the total antioxidant activity with total phenolics and total anthocyanins was also found as 1.00. The total antioxidant activity in ethanolic extracts of muscadine pomace was determined as 921600 µmoles TROLOX equivalents in 40 .0 L of the extract. The antioxidant activity decreased to 747600 µmoles TROLOX equivalents in 40 L of the extract after filtration through bladder press, followed by plate and frame filter. After concentration by thin film evaporation, the antioxidant activity was determined as 720718 µmoles TROLOX equivalents in 17.3 L of the extract. The antioxidant activity was found correlated with the total phenolics and the total anthocyanins. Since due to cloudy appearance of the extract, the extract was not further subjected to purification, concentration and spray drying steps.

The total antioxidant activity in the aqueous and fermented extracts of blueberry was estimated as 275764 and 178880 µmol TROLOX equivalents in 43.4 L and 30.9 L of the extracts respectively. After filtration through bladder press, followed by plate and frame filter the total antioxidant activity decreased to 76606 µmol TROLOX equivalents in 30.9 L of the fermented extracts. The total antioxidant activity in the aqueous extract remained the same. After adsorption on Sepabeads 700, water washed and elution by 80 % w/w ethanol, the antioxidant activity decreased to 224480 and 57400 µmol TROLOX equivalents in 8.0 L and 2.0 L of the aqueous and fermented extracts of blueberry. After concentration by thin film evaporation technique, the total antioxidant activity decreased slightly for the fermented extracts, whereas increased for the aqueous extracts and finally decreased in the spray dried powder for both the extracts shown in the Fig. 8.14. The

total antioxidant activity was found to be well correlated with the total phenolics and the total anthocyanins with correlation coefficient as 1.0.

The total antioxidant activity in the ethanolic extracts of blueberry was determined as 616266 µmol TROLOX equivalents in 40.2 L. After filtration through bladder press, followed by plate and frame filter, the total antioxidant activity was determined as 438582 µmol TROLOX equivalents. The ethanolic extracts were subjected to concentration by thin film evaporation technique to a volume of 22.0 L. The total antioxidant activity was determined as 605440 µmol TROLOX equivalents. After purification of the concentrated ethanolic extracts of blueberry by adsorption on Sepabeads 700, water washed, and elution by 80 % w/w ethanol, the antioxidant activity of the eluted extracts were estimated as 484860 µmol TROLOX equivalents in 6.0 L of the extracts. They were further concentrated to a volume of 1.67 L by removing ethanol by thin film evaporation technique. The total antioxidant activity in the concentrated eluents was determined as 307948 µmol TROLOX equivalents. The spray dried ethanolic extract exhibited an antioxidant activity of 174055 µmol TROLOX equivalents. The total antioxidant activity in the ethanolic extracts was found to correlate with the total phenolics and the total anthocyanins with the correlation coefficients as 1.0.

8.3.4.Comparison between lab scale and pilot scale studies

A comparative study was conducted between the lab scale and the pilot scale to see the efficiency of the scale up process. Despite all kind of handling, set up problems of pilot scale instruments the efficiency of extraction of total phenolics for muscadine pomace ranged from 37 – 67 %. The data was shown in Table 8.1. The efficiency of

extraction of total phenolic varied from 22 – 44 % in pilot plant studies for rabbiteye blueberry. The data was shown in Table 8.2.

Table 8.1

Comparison of total phenolics, total anthocyanins, and total antioxidnt activity of aqueous, ethanolic, and fermented extracts of muscadine pomace between lab scale and pilot scale studies.

	Aqueous Extract		Ethanolic Extract		Fermented Extract	
	Lab Scale	Pilot Scale	Lab Scale	Pilot Scale	Lab Scale	Pilot Scale
Total Phenolics (g/Kg db)	21.0	8.0	22.0	14.8	25.0	10.5
Total Anthocyanins (g/Kg db)	5.9	1.4	7.8	2.0	15.7	0.6
Total Antioxidant Activity (FRAP, mmol/Kg db)	105.0	87.0	69.0	197.0	88.0	138.0

Table 8.2

Comparison of total phenolics, total anthocyanins, and total antioxidant activity of aqueous, ethanolic, and fermented extracts of rabbiteye blueberry between lab scale and pilot scale studies

	Aqueous Extract		Ethanolic Extract		Fermented Extract	
	Lab Scale	Pilot Scale	Lab Scale	Pilot Scale	Lab Scale	Pilot Scale
Total Phenolics (g/Kg db)	18.0	5.9	28.0	12.2	17.0	3.8
Total Anthocyanins (g/Kg db)	3.2	0.6	19.0	1.9	2.5	0.1
Total Antioxidant Activity (FRAP, mmol/Kg db)	87.0	55.0	138.0	132.0	81.0	178.0

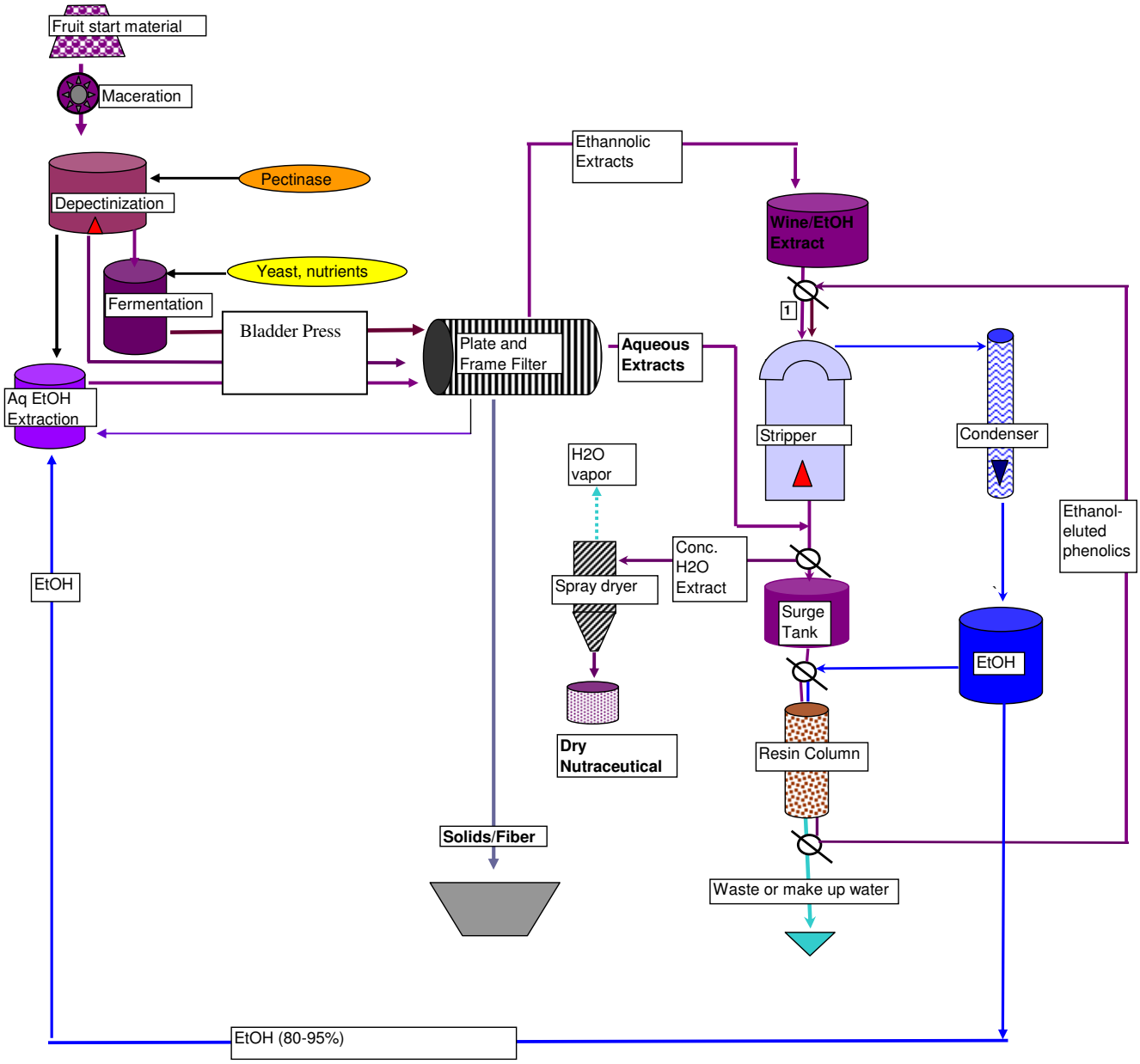
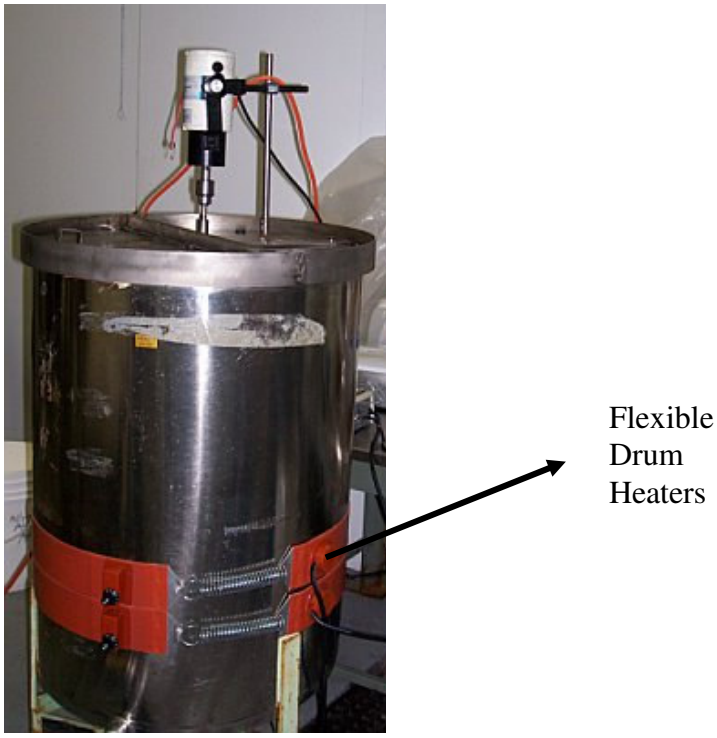
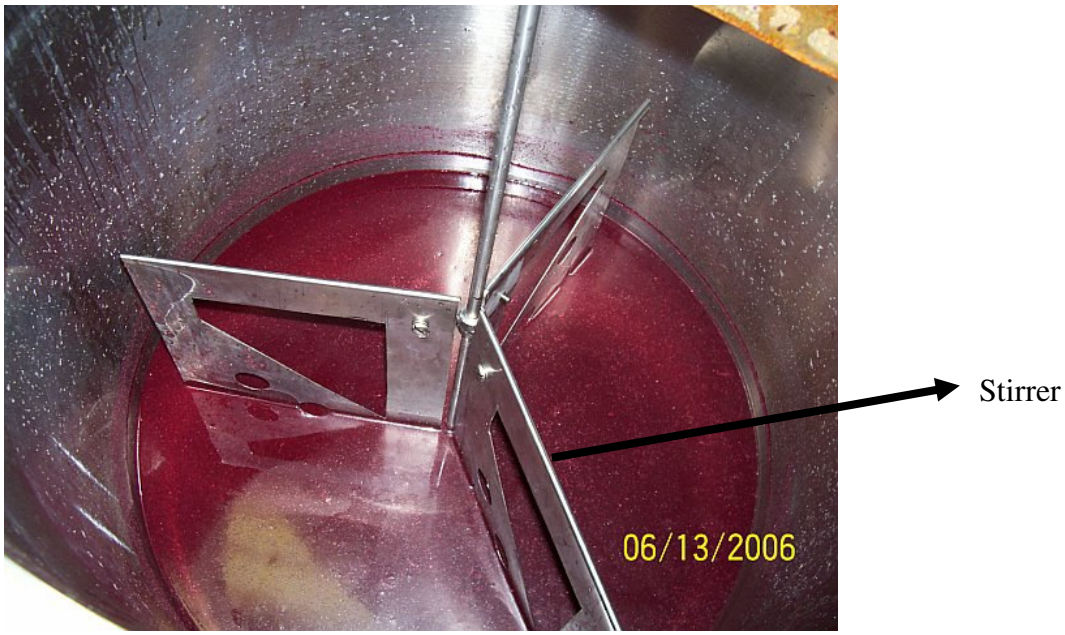


Fig. 8.1 Flow chart of pilot scale production of neutraceutical powder from muscadine pomaces and rabbiteye blueberries.

Fig. 8.2. Urschel Mill



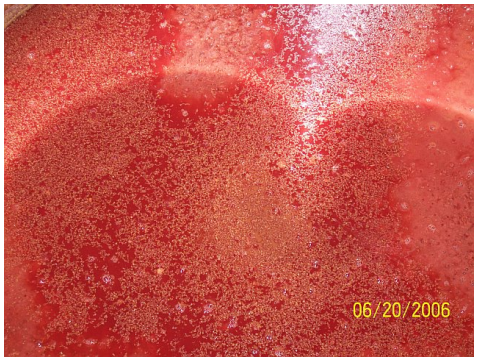
(a)



(b)

Fig. 8.3. Exrtaction tank of capacity 50 G (a) and Aqueous extracts of muscadine pomace in the extraction tank (b).

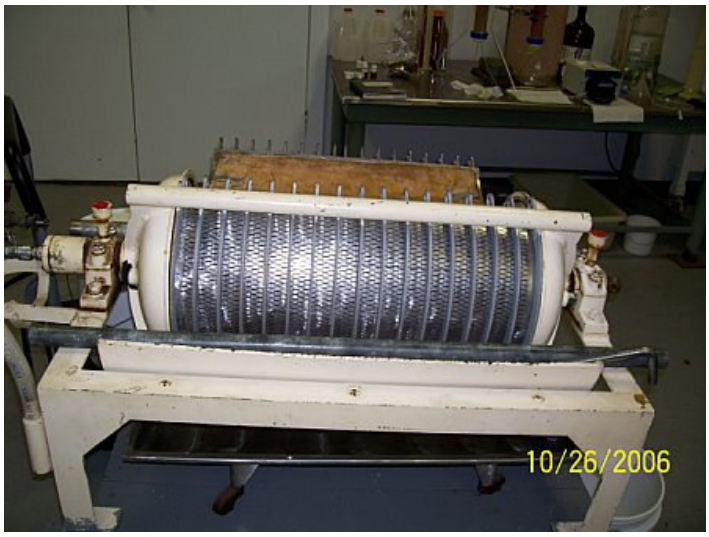
(a)



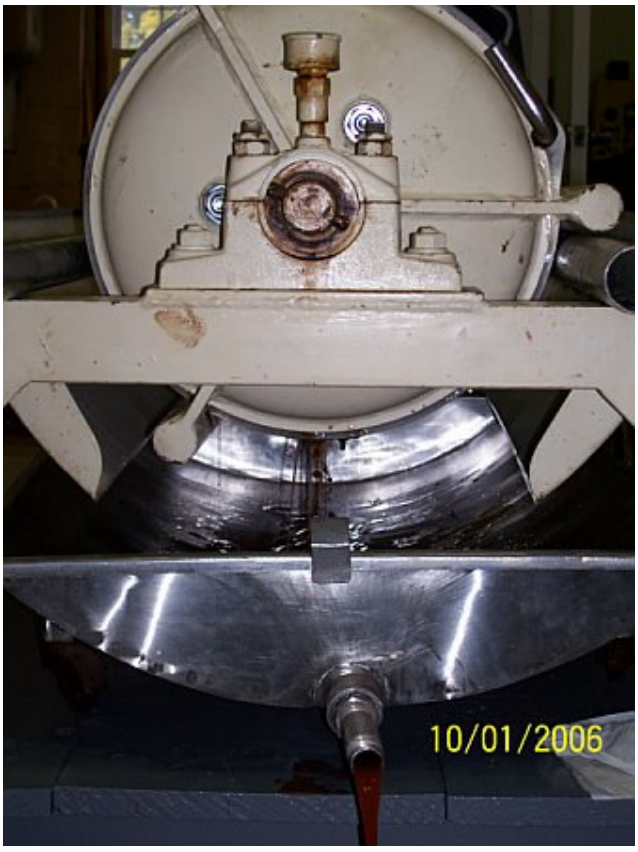
(b)

(c)

Fig. 8.4. Fermentation tank (a), fermentation of muscadine pomace (b) and fermentation of blueberries (c).



(a)



(b)

Fig. 8.5. Bladder press (a) and operation of bladder press during filtration of blueberry aqueous extracts (b).

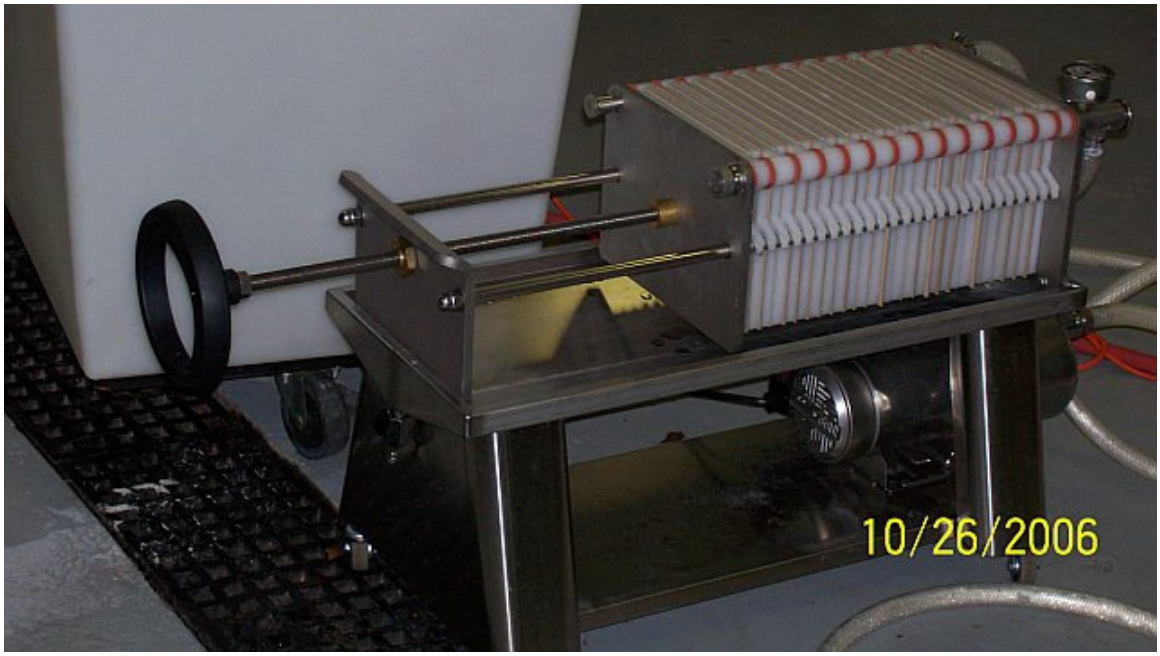


Fig. 8.6. Plate and frame filter

Fig. 8.7. Columns packed with Sepabeads 700 loaded with muscadine pomace extracts

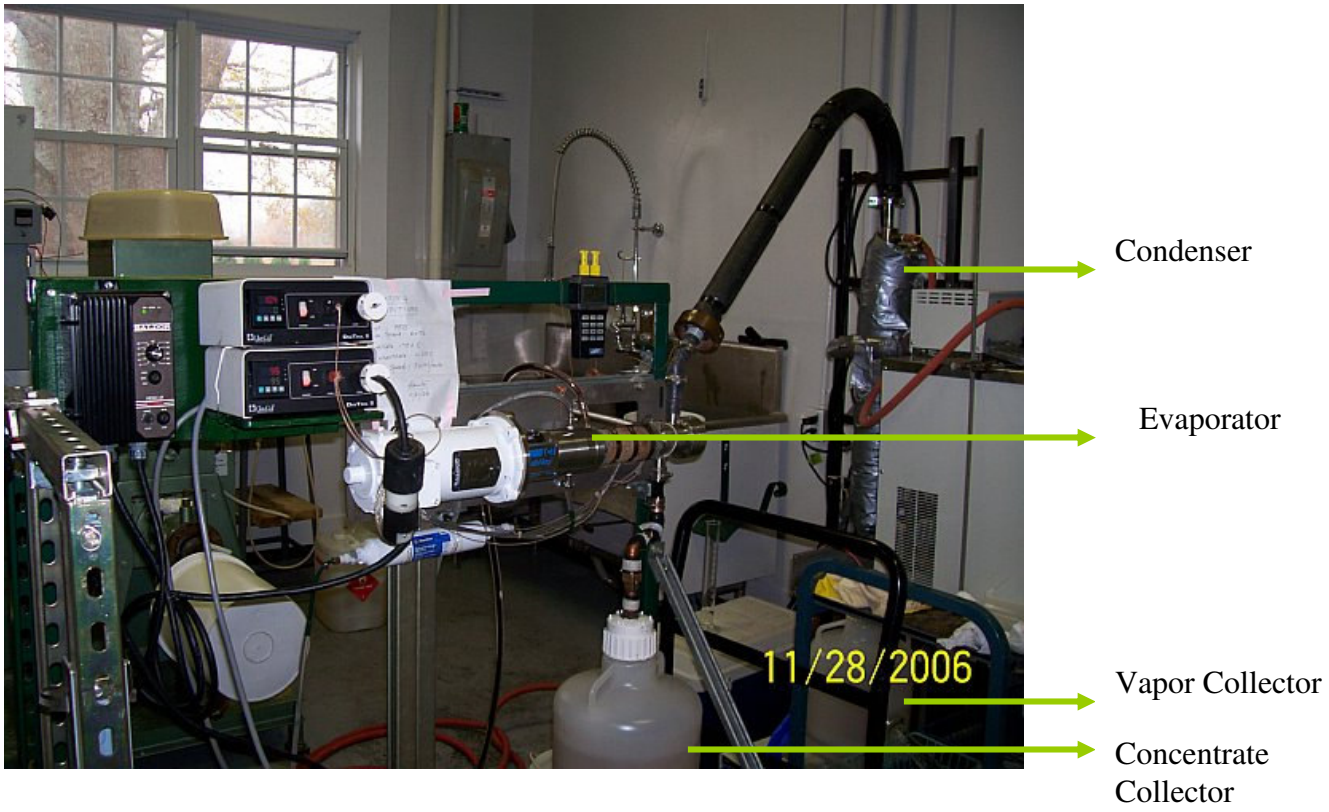


Fig. 8.8. Labvap Thin film evaporator in operation.

Fig. 8.9. Set up of the pilot plant with the extraction tank and the filtration units.

Fig. 8.10 Spray Dryer.

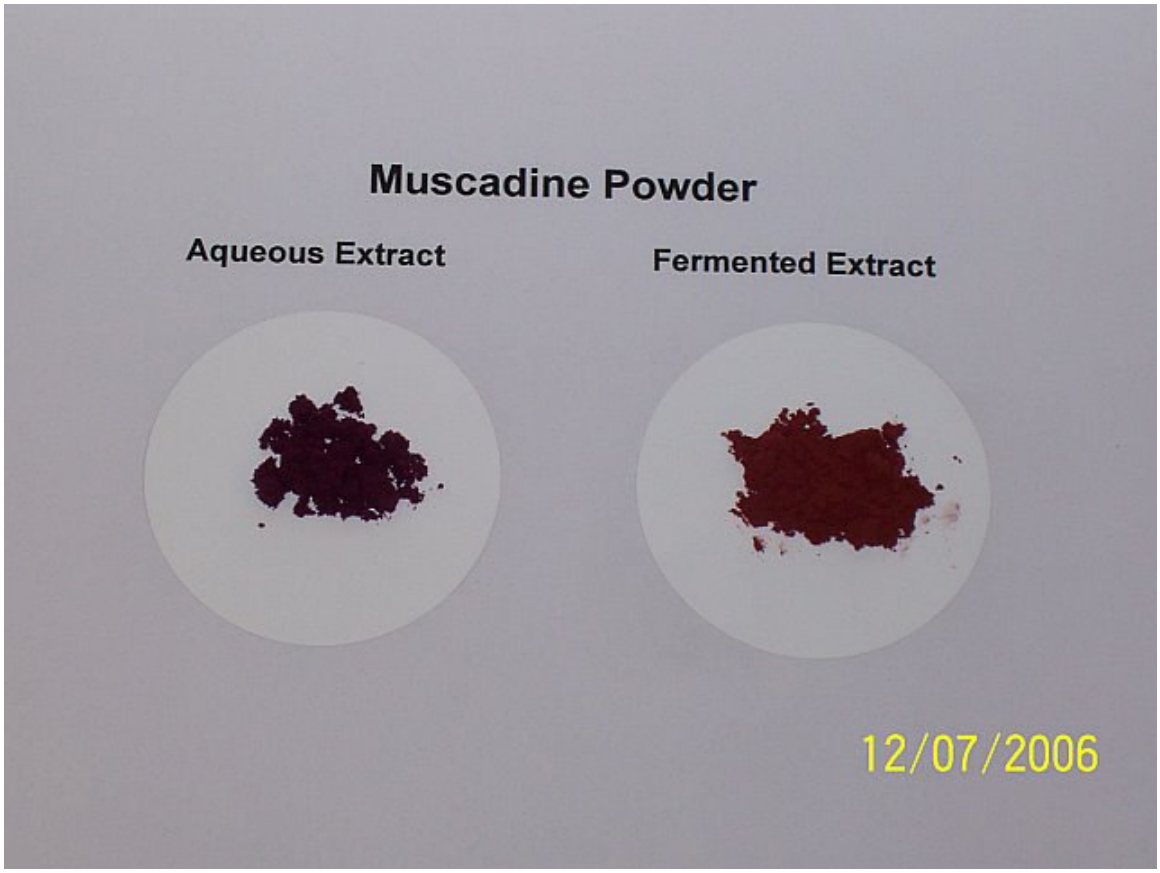


Fig. 8.11. Spray dried powder of aqueous and fermented extracts of muscadine pomace

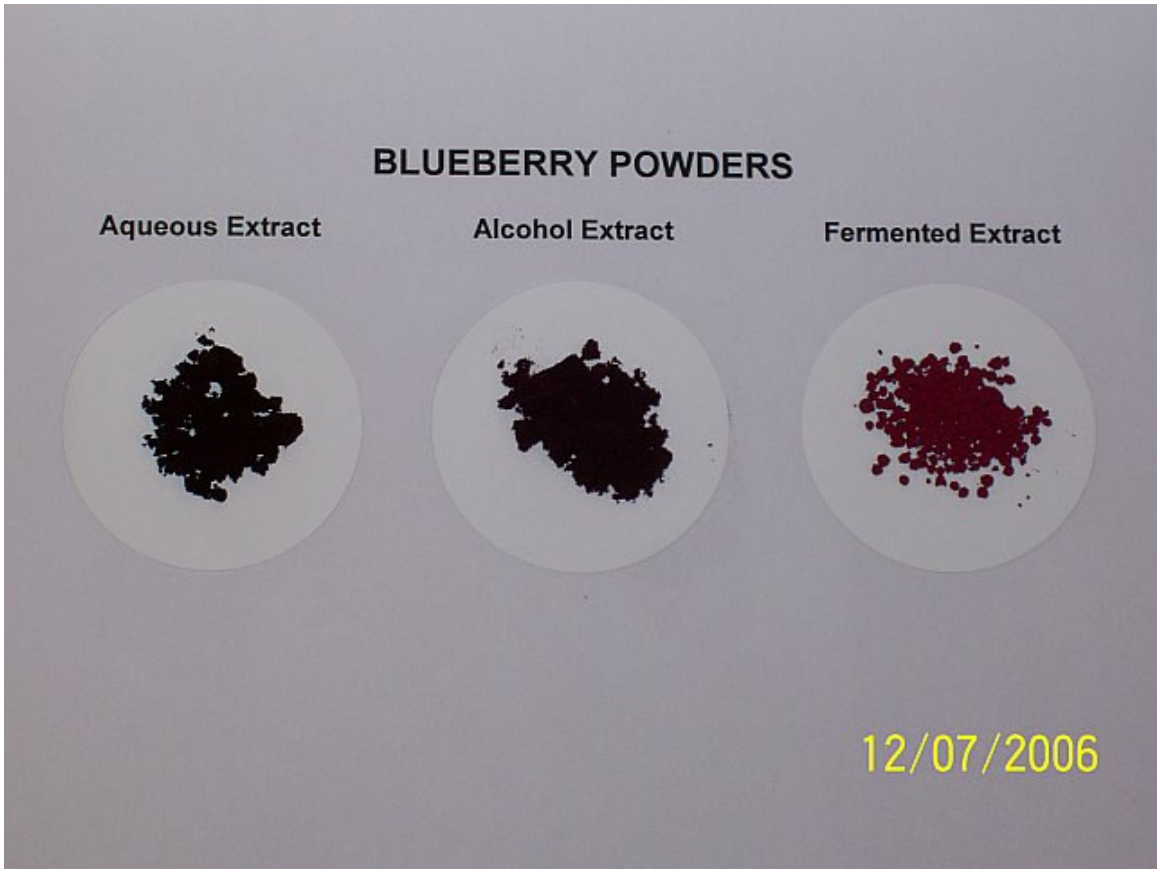


Fig. 8.12. Spray dried powder of aqueous, alcoholic, and fermented extracts of blueberries.

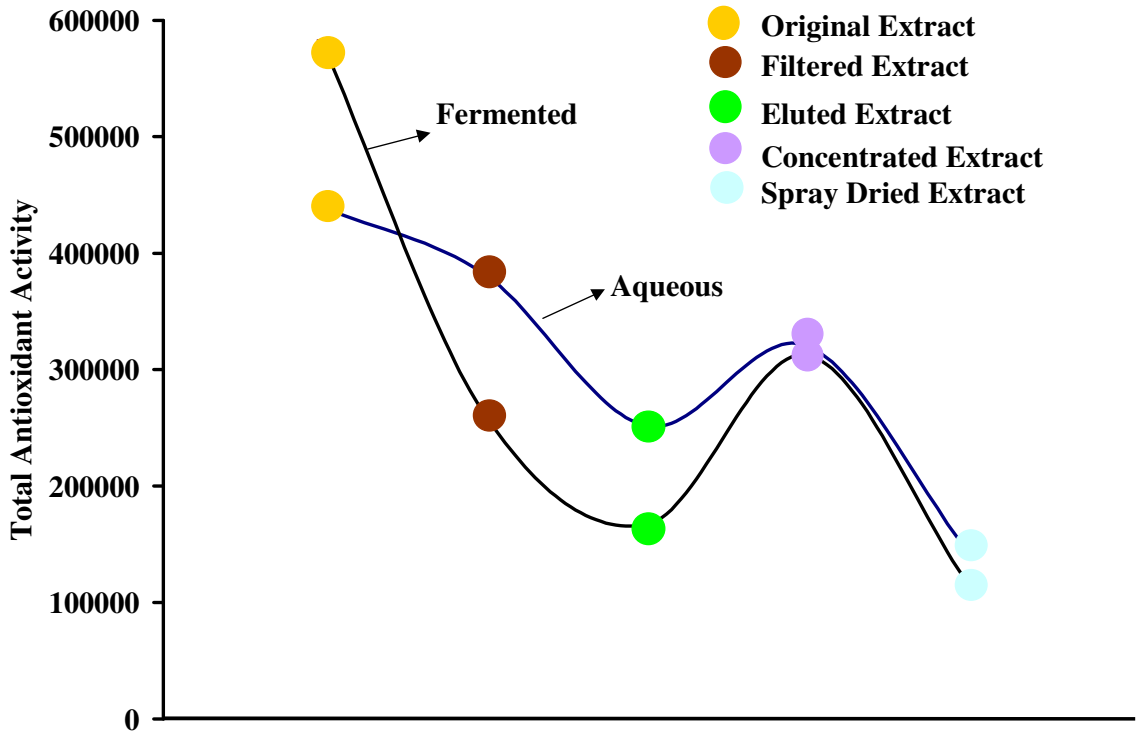


Fig. 8.13. Total antioxidant activity (FRAP, μmool) in original, filtered, eluted, concentrated and in spray dried aqueous and fermented extracts of muscadine pomace.

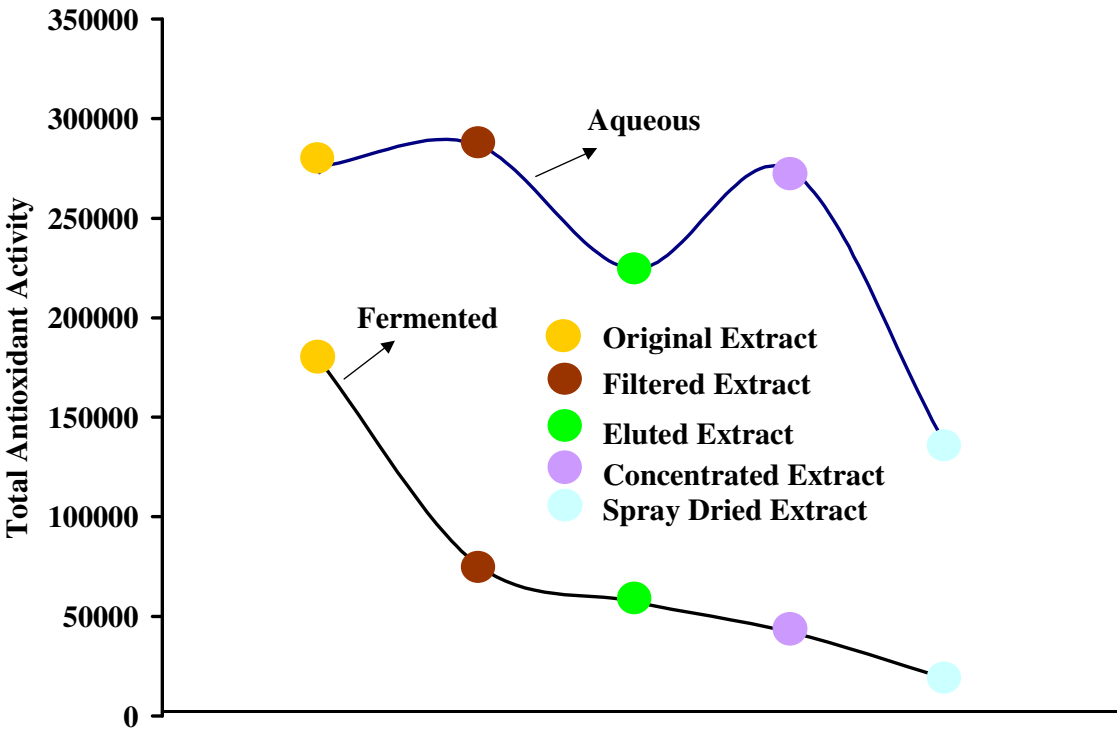


Figure 8.14. Total antioxidant activity (FRAP, µmole) in original, filtered, eluted, concentrated and in spray dried aqueous and fermented extracts of rabbiteye blueberry.

References

Adhikari, B.; Howes, T.; Lecomte, D.; and Bhandari, B. R. (2005). A glass transition temperature approach for the prediction of the surface stickiness of a drying droplet during spray drying. *Powder Technology*, 149, 168-179.

Auw, J.M., Blanco, V., O'Keefe, S.F., and Sims, C.A. (1996). Effect of processing on the phenolics and color of cabernet sauvignon, chambourcin, and noble wines and juices. *American Journal of Enology and Viticulture*, 47(3),279 – 286.

Benzie, F.F.I., and Strain, J.J. (1999). Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. In *Methods in Enzymology*, 299:15-27.

Bonilla-Pastrana, E.; Akoh, C.C.; Sellapan, S.; Krewer, G. (2003). Phenolic content and antioxidant capacity of muscadine grapes. *Journal of Agricultural and Food Chemistry*, 51, 5497 – 5503.

Boulton, R. (2001). The copigmentation of anthocyanins and its role in the color of red wine: A critical review. *American Journal of Enology and Viticulture,* 52(2), 67–87.

Boyle, J. A., and Hsu, L. (1990). Identification and quantification of ellagic acid in Muscadine grape juice. *American Journal of Enology and Viticulture,* 41, 43-47.

Brouillard, R., and Dangles, O. (1994). Anthocyanin molecular interactions: the first step in the formation of new pigments during wine ageing? *Food Chemistry*, 51, 365–371.

Cai, Y. Z. and Corke, H. (2000). Production and properties of spray-dried Amaranthus betacyanin pigments. *Journal of Food Science*, 65(6), 1248-1252.

Cao, G., and R. Prior. (1999). Measurement of oxygen radical absorbance capacity in biological samples. In *Methods in Enzymology,* 299, 50-62.

Ector, B.J., Magee, J.B., Hegwood, C.P., and Coign, M.J. (1996). Resveratrol concentration in muscadine berries, juice, pomace, purees, seeds, and wines. *American Journal of Enology and Viticulture,* 47(1), 57 – 62.

Engelhart, M.J., Geerlings, M.I., Ruitenberg, A., Van Swieten, J.C., Hofman, A., Witteman, J.C., and Breteler, M.M. (2002). Dietary intake of antioxidants and risk of Alzheimer disease. *The Journal of American Medical Association,* 287, 3223–3229.

Flora, L. F. (1978). Influence of heat, cultivar and maturity on the anthocyanin-3,5-diglucosides of muscadine grapes. *Journal of Food Science*, 43, 1819-1821.

Friedman, M., Mackey, B. E., Kim, H.-J., Lee, I.-S., Lee, K.-R., Lee, S.-U., Kozukue, E., and Kozukue, N. (2007). Structure-Activity Relationships of Tea Compounds against Human Cancer Cells. *Journal of Agricultural and Food Chemistry*, 55 (2),243 –253.

G.N. Revie and B.R. Thomas Jr. (1972). Powdered flavours offer many advantages, *Food Manufacture*, 37, p. 40.

Glicksmann, M. (1969).Gum technology in the food industry, Food Science and Technology, a series of monographs, Academic Press, Inc., New York, NY.

Greenspan, P.; Bauer, J. D.; Pollock, S. H.; Gangemi, J. D.; Mayer, E. P.; Ghaffar, A.; Hargrove, J. L.; Hartle, D. K. (2005). Antiinflammatory Properties of the

Muscadine Grape (Vitis rotundifolia). *Journal of Agricultural and Food Chemistry* , 53(22), 8481-8484.

Joseph, J.A., Shukitt-Hale, B., Denisova, N.A., Bielinski, D., Martin, A., McEwen, J.J., and Bickford, P.C. (1999). Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. *Journal of Neuroscience*, 19, 8114–8121.

Joseph, J.A., Shukitt-Hale, B., and Casadesus, G. (2005). Reversing the deleterious effects of aging on neuronal communication and behavior: beneficial properties of fruit polyphenolic compounds. *Americal Journal of Clinical Nutrition*, 81, 313S–316S.

Joshipura, K.J., Ascherio, A., Manson, J.E., Stampfer, M.J., Rimm, E.B., Speizer, F.E., Hennekens, C.H., Spiegelman, D., and Willett, W.C. (1999). Fruit and vegetable intake in relation to risk of ischemic stroke. *The Journal of American Medical Association,* 282, 1233–1239.

Kammerer, D.; Kljusuric, J.G.; Carle, R.; Schieber, A. (2005). Recovery of anthocyanins from grape pomace extracts (Vitis vinifera L. cv. Cabernet Mitos) using a polymeric adsorber resin. *European Food Research and Technology,* 220,431–437.

Lachance, A.L. and Saba, R.G. (2002). Quality management of Neutraceuticals: Intelligent product delivery system and safety through traceability. In: Quality management of neutraceuticals. pp 2-9. eds. Chi-Tang, H and Qun, Y.Z., American Chemical Society, Washington.

Lamikanra, O., Grimm, C.C., Rodin, J.B., and Inyang, D.I. (1996). Hydroxylated stilbenes in selected American wines. *Journal of Agricultural and Food Chemistry*, 44(4),1111 –1115.

Lee, J.-H. Talcott, S. T. (2004). Fruit Maturity and Juice Extraction Influences Ellagic Acid Derivatives and Other Antioxidant Polyphenolics in Muscadine Grapes. *Journal of Agricultural and Food Chemistry,* 52(2), 361-366

Lee, J-H., Johnson, J.V., and Talcott, S.T. (2005). Identification of Ellagic Acid Conjugates and Other Polyphenolics in Muscadine Grapes by HPLC-ESI-MS. *Journal of Agricultural and Food Chemistry,* 53(15), 6003 –6010.

Magee, J.B., and Smith, B.J. (2002). Resveratrol Content of muscadine berries is affected by disease control spray program., *HortScience*, 37(2), 358 – 361.

Moyer, A., Hummer, K.E., Finn, C.E., Frei, B., and Wrolstad, R.E. (2002). Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: Vaccinium, Rubus, and Ribes. *Journal of Agricultural and Food Chemistry*, 50, 519–525.

Musingo, M. N., Sims, C. A., Bates, R. P., O'Keefe, S. F., Lamikanra, O. (2001). Changes in ellagic acid and other phenols in muscadine grape (Vitis rotundifolia) juices and wines. *American Journal of Enology and Viticulture*, 52, 109-114.

Prior, R.L., Cao, G., Martin, A., Sofic, E., McEwen, J., O'Brien, C., Lischner, N., Ehlenfeldt, M., Kalt, W., Krewer, G., and Mainland, C.M. (1998). Antioxidant capacity as influenced by total phenolics and anthocyanins content, maturity, and variety of vaccinium species. *Journal of Agricultural and Food Chemistry,* 46, 2686–2693.

Prior, R.L., Lazarus, S.A., Cao, G., Muccitelli, H., and Hammerstone, J.F. (2001). Identification of procyanidins and anthocyanins in blueberries and cranberries (Vaccinium Spp.) using high performance liquid chromatography/mass spectrophotometry. *Journal of Agricultural and Food Chemistry,* 49,1270 – 1276.

Rossi, M., Garavello, W., Talamini, R., La Vecchia, C., Franceschi, S., Lagiou, P., Zambon, P., Maso, L.D., Bosetti, C., and Negri, E. (2006). Flavonoids and risk of squamous cell esophageal cancer. *International Journal of Cancer*, 120(7), 1560-1564.

Scordino, M.; Mauro, A.D.; Passerini, A.; Maccarone, E. Adsorption of flavonoids on resins: Cyanidin – 3 – gluoside. (2004). *Journal of Agricultural and Food Chemistry*, 52,1965–1972.

Scordino, M.; Mauro, A.D.; Passerini, A.; Maccarone, E. Adsorption of flavonoids on resins: Hesperidin.(2003). *Journal of Agricultural and Food Chemistry*, 52,1965–1972.

Scordino, M.; Mauro, A.D.; Passerini, A.; Maccarone, E. (2007). Highly purified sugar concentrate from a residue of citrus pigments recovery process. *LWT-Food Science and Technology*, 40(4), 713-721.

Sellapan, S., Akoh, C.C., and Krewer, G. (2002). Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. *Journal of Agricultural and Food Chemistry*, 50, 2432 – 2438.

Singleton, V.L., and J.A. Rossi. Jr. (1965). Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, 16, 144-158.

Stojanovich, J., and Silva, J.L. (2007). Influence of osmotic concentration, continuous high frequency ultrasound and dehydration on antioxidants, color, and chemical properties of rabbiteye blueberries. *Food Chemistry*, 101(3), 898 – 906.

Su, M-S., and Silva, J.L. (2006). Antioxidant activity, anthocyanins, and phenolics of rabbiteye blueberry (Vaccinium ashei) by-products as affected by fermentation. *Food Chemistry*, 97(3), 447 – 451.

Talcott, S.T., and Lee, J-H. (2002). Ellagic acid and Flavonoid Antioxidant Content of Muscadine Wine and Juice. *Journal of Agricultural and Food Chemistry*, 50, 3186 – 3192.

Talcott, S.T.; Brenes, C.H.; Pires, D.M.; Pozo-Insfran, D.D. (2003). Phytochemical stability and color retention of copigmented and processed muscadine grape juice. *Journal of Agricultural and Food Chemistry*, 51, 957 – 963.

Verlangieri, A.J., Kapeghian, J.C., el-Dean, S., and Bush, M. (1985). Fruit and vegetable consumption and cardiovascular mortality. *Medical Hypotheses*,16, 7–15.

Yilmaz, Y.; Toledo, R.T. (2004). Major flavonoids in grape seeds and skins: Antioxidant capacity of catechin, epicatechin, and gallic acid. *Journal of Agricultural and Food Chemistry*, 52 (2), 255 – 260.

CHAPTER 9

SUMMARY AND CONCLUSIONS

Different technologies including enzymatic aqueous extraction, ethanolic extraction, fermentation, filtration, purification, concentration and spray drying were developed to produce polyphenolic neutraceutical powders from Muscadine grapes and Rabbiteye blueberries. Incubation of aqueous extracts with heat stable pectinase enzyme improved the yield of total phenolics and anthocyanins for bothfruits. Pre-freezing of the fruits did not significantly improve the yield of total phenolics and anthocyanins over pectinase treatment.

Optimal aqueous extraction of polyphenolic compounds from Muscadine grapes and Rabbiteye blueberries, occurred at an extraction temperature of 60 C and time of 1 H. Optimal ethanolic extraction of polyphenolic compounds from pectinase treated extracts occurred at an extraction temperature of 60 C, a time of 1 H, and an ethanol concentration of 30 – 40 % w/w for Muscadine grapes and 35 – 40 % w/w for Rabbiteye blueberries. Optimal fermentation of Muscadine grapes and Rabbiteye blueberries, occurred at a fermentation temperature of 25 C, an ethanol concentration of 11 % v/v, and a solid to solvent ratio of 0.5. Response surface regression full and reduced models were developed for the three extraction processes and the yield of total phenolics and anthocyanins with respect to different extraction parameters were described.

A factorial experiment was performed to study the simulated distillation conditions for removal of ethanol from the ethanolic extracts. The stability of the extracts was determined maximum when ethanol was distilled at T<65 C under reduced pressure. Purification of the extracts of both Muscadine grapes and Rabbiteye blueberries is accomplishedby adsorption onto Sepabeads SP 700, and eluted by 80 % w/w ethanol at 25 C. Sepabeads SP 700 is recommended to reuse for at least 70 times.

A recovery of 80 % of total phenolics and 100 % of anthocyanins was observed upon adsorption/desorption onto the column.

Pilot plant studies were conducted with 20 G (76 L) of extracts of Muscadine grapes and Rabbiteye blueberries. A two step filtration step, consisting of an initial pass through a bladder press, followed by plate and frame filtration was effective. For ethanolic extracts, concentration of extracts by eliminating ethanol with flash evaporator is recommended before adsorption onto the column. Spray drying of the concentrated eluted extracts was done at an inlet temperature of 150 C and an outlet temperature of 90 C. A 22 – 52 % recovery of the polyphenolic compounds was achieved in the spray dried powder. The spray dried powder was non-sticky and amorphous. Proper handling care and precise control of pilot plant equipments is highly recommended to minimize losses in the recovery of polyphenolic compounds as these compounds are sensitive to light, heat and air.

This study suggests that a reasonably simple and cost-effective technology for producing highly concentrated polyphenolic powders, suitable for use in nutraceuticals and other applications, could be adopted for on-farm and small scale producer/processors. The use of byproducts and inferior grades of fruits would increase the feasibility of the process.