AN ABSTRACT OF THE DISSERTATION OF

Jungmin Lee for the degree of Doctor of Philosophy in Food Science and Technology presented January 13, 2004.

Title: The Blueberry: Composition, Anthocyanins, And Polyphenolics.

Abstract approved :

Signature redacted for privacy.

Ronald E. Wrolstad

Frozen blueberries were pilot-plant-processed into juice and concentrate by 2 treatments (heat and SO_2), and a control. Qualitative, and quantitative anthocyanin and polyphenolic content were monitored after each processing step. Major losses occurred after the initial processing steps. Juice processing by-product contained a substantial amount of anthocyanins and polyphenolics (>42% and >15%, respectively of the starting material), which indicated they remained a good source for further extraction. Commercial juice processing enzymes (n=9) and other extraction methods were evaluated for extraction of anthocyanins and dietary antioxidants from blueberry skins and wholeberries on a bench-top scale.

Blueberries were manually separated into peels, flesh, and seeds. The fractions were evaluated for (qualitative and quantitative) anthocyanin (ACY) and polyphenolic (TP) content, and antioxidant capacity. Blueberry skins had the greatest antioxidant capacity with the highest ACY and TP content, followed by flesh, and then seeds. The polyphenolic profiles were different in each of the three fractions. High performance liquid chromatography (HPLC), electrospray mass spectrometry (ES-MS), and liquid

chromatography mass spectrometry (LC-DAD-MS) were used in identifying the individual anthocyanins. LC-DAD-MS was an invaluable tool in identification of the individual anthocyanins.

Four Pacific Northwest native huckleberries (n>38 populations) were collected to compare their ACY and TP content to 'Rubel', which is an important criterion for breeding programs or commercialization. The ACY content of all huckleberry samples analyzed ranged from 101 to 563mg/100g (cyanidin-3-glucoside equivalents), while the TP varied from 367 to 1286mg/100g (gallic acid equivalents). The HPLC profile of the individual anthocyanins and phenolics of some of the huckleberries differed in the amounts of delphinidin, cyanidin, and malvidin glycosides.

A collaborative study following the guidelines of AOAC International was conducted to validate the pH differential method for determining total monomeric anthocyanin pigment, as an AOAC approved method. Eleven collaborators evaluated seven Youden pairs of test samples. The results (Horrat values ≤1.33) indicated that the method met the objective of determining ACY content of liquid samples, and was recommended to be adopted Official First Action.

The Blueberry: Composition, Anthocyanins, And Polyphenolics.

by

Jungmin Lee

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Presented January 13, 2004

Commencement June 2004

ACKNOWLEDGEMENTS

I definitely could not have made it without the support and encouragement surrounding me. I want to express my sincere gratitude to Ron Wrolstad. He has given me unconditional backing through the years I have been in the Food Science department. I could not have made it without him. I would like to thank Bob Durst for his unreserved assistance, encouragement, and friendship. My labmates, and visiting scientists, helped me with their advice, camaraderie, and just making time at work fun: Lucho Rodriguez-Saona, Monica Guisti, Xiaoling Dong, JoLynne Wightman, Matt Glaser, Ling Wen, Kjersti Aaby, Feryal Karadeniz, Florian Stintzing, Angela Stintzing, and Andreas Schieber.

I want to thank my committee members: Chad Finn, Jim Kennedy, Charles Boyer, and Cliff Pereira. Chad Finn and Jim Kennedy really went out of their way to help me.

I would also like to thank the Food Science department faculty, staff, and OSU / USDA friends for their endless help. Special thanks go to Anita Brown, Iola Williams, Christina Hull, Linda Dunn, Rosanna Leeson, Jan Carlson, Anne Adams, Dan Smith, Brian Yorgey, Boyd Wilcox, Jeff Clawson, James Batdorff, Seojin Chung, Mel Powell, Imin Tsai, Mona Nataprawira, Rhoda Sithole, Sandra Sandra, Chunran Han, Judy Briggs, Anne Plotto, Panida Pongviratchai, Keith Klesk, Heesup Kim, Jae Park, Ted Mackey, Kim Hummer, Don Griffin, and Rosie Hopkins.

I would like to thank the Northwest Center for Small Fruit Research, USDA competitive grant, and the Oregon Blueberry Commission for their support. I want to acknowledge my industry and AOAC collaborators: John Dysart, Ken Stewart, David Kuenzi, Bob Britto, Scott Brix, David Hammond, Sam Grubb, Jonathan Haché,

Francois Paquette, Dana Krueger, Alexandra Ryabkova, Steve Kupina, JoLynne Wightman, Grete Skrede, Berit Martinsen, Tom Eisele, Tom Miller, Hans Hofsommer, Susanne Koswig, Uwe Trenn, Susan Martin, Karen Barnes, Annie NG, Joanna Lynch, and many others who have contributed to this work.

I would like to thank firms and organizations that supported our research: Kerr Concentrate, Inc., Canandaigua Wine Co., Fall Creek farm and nursery, Inc., Van Cleave Farms, AB enzyme, DSM Food Specialties USA, Inc., Novozymes, Valley Research, Inc., The J. M. Smucker Co, and many others.

I especially want to thank Mark Redhead for his love, patience, and encouragement. And finally, I thank all my friends and family: Sherry Clark, Mona Nataprawira, Patrick Cornet, Minami Watanabe, Younhee Kim, Juhee Lee, Mel Powell, The Coopers, Seojin Chung, Scott Redhead, Kjersti Aaby, Joan Redhead, and the ODFW Fish Pathology gang for their support.

CONTRIBUTION OF AUTHORS

Ronald E. Wrolstad has contributed to the experimental design, data analyses, and writing of each chapter. Robert W. Durst was involved in juice and concentrate processing for chapter 3 and helped in data collection and analyses for chapter 7. Chad E. Finn of USDA-ARS (Corvallis, OR) was involved in the experimental design, cultivar selection, data interpretation, and writing of chapters 5 and 6. Cliff Pereira, Kerstine Carter, and Joseph Scherer of the department of Statistics (Oregon State University, Corvallis) were involved in the experimental design of extraction experiments in chapter 4. Donald A. Griffin of the Department of Environmental and Molecular Toxicology (Oregon State University, Corvallis) performed the ES-MS analysis in chapter 3. Deborah Hobbs of the Linus Pauling Institute (Oregon State University, Corvallis) conducted the antioxidant activity measurements for samples in chapters 4 and 6.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

LIST OF APPENDIX TABLES

To Mark Redhead,

who has been supportive through thick and thin

THE BLUEBERRY: COMPOSITION, ANTHOCYANINS, AND POLYPHENOLICS.

CHAPTER 1. INTRODUCTION

The Pacific Northwest is a major producer of blueberries that are marketed fresh, and processed into a wide variety of products, including fruit juices and concentrates. Blueberries have been reported to contain high levels of compounds that are beneficial to human health. These desirable traits have been attributed to anthocyanins and other phenolics (Prior, 1998; Prior et al., 1998; Kalt et al., 2000; Heinonen et al., 1998). Oregon ranked among the top five states in North America for blueberry production in 2002, 2001, and 2000 (Coba and Goodwin, 2003). The anthocyanins and other phenolics present in blueberries are within the flavonoid class of compounds. Flavonoids have been shown to be strong antioxidants, and because of this, may provide some protection against inflammation, atherosclerosis, cancer, and diabetes (Hollman, 2001).

This study investigated the effects of processing on the phytochemical content of blueberry juice and juice concentrate. Methods were examined to efficiently extract anthocyanins and phenolics from blueberries, and to improve processing unit operations. Blueberry processing waste (presscake) was evaluated as a source for further extraction of antioxidant compounds and natural colorants. Processors could utilize these findings to improve processing methods and minimize the loss of beneficial compounds in their products.

This study also surveyed the phytochemical composition of blueberries native to the Pacific Northwest, commonly called huckleberries (*Vaccinium* species). Previous

research has not extensively evaluated the anthocyanin and phenolic composition of these native *Vaccinium* species. High levels of anthocyanins and other phenolics in a cultivar could be used as a criterion for future breeding, or for specific products.

The final objective of this dissertation was to validate a simple method for determining the total monomeric anthocyanin content in samples containing anthocyanins. Currently, there is no validated method to ascertain total monomeric anthocyanin in a given sample. The interest in the healthful benefits of anthocyanins has fueled the nutraceutical market, and the need for a validated method to quantitate the levels of anthocyanin in a given product. The pH differential method was evaluated by a collaborative study for its performance as a potential AOAC approved method.

REFERENCES

Coba, K. and Goodwin, J.A. 2003. Oregon Agri-Facts. Oregon Agricultural Statistics Service. Oregon Department of Agriculture. Portland, OR.

Heinonen, I.M., Meyer, A.S., and Frankel, E.N. 1998. Antioxidant activity of berry phenolics on human low-density lipoprotein and liposome oxidation. J. Agri. Food Chem. 46:4107-4112.

Hollman, P.C. 2001. Evidence for health benefits of plant phenols: local or systemic effects? J. Sci. Food Agric. 81:842-852.

Kalt, W., McDonald, J.E., and Donner, H. 2000. Anthocyanins, phenolics, and antioxidant capacity of processed lowbush blueberry products. J. Food Sci. 65:390-393.

Prior, R.L. 1998. Antioxidant capacity and health benefits of fruits and vegetables: blueberries, the leader of the pack. Proc. 32nd Ann. Open House North Carolina Blueberry Council. 32:3-12.

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 phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. J Agric Food Chem 46:2686-2693.

CHAPTER 2. LITERATURE REVIEW
BLUEBERRY POLYPHENOLICS

HEALTH BENEFITS OF BLUEBERRIES

Historically, blueberries have been recognized for their possible health benefits. They have been widely used as folk medicine in North America and Europe. Fresh and dried berries from *Vaccinium* species were an important part of the Pacific Northwest aboriginal people's diet, providing ascorbic acid, calcium, iron, magnesium, and zinc (Keely et al., 1982). Bilberry (*V. myrtillus*, wild blueberries in Northern Europe) was used as folk medicine in Europe for inflammation and for the treatment of infections, prevention of scurvy, easing complications from diabetes, and for the treatment of urinary tract infections (Morazzoni and Bombardelli, 1996; Camire, 2002). Blueberry tea and syrup have also been used as a cough suppressant, a remedy for diarrhea, and as a treatment for "female illness" (Pellerro Society, 1974). There are also anecdotal reports of bilberry jam being consumed by British Royal Air Force pilots during World War II for improvement of night vision (Muth et al., 2000).

Epidemiological studies have demonstrated that a diet rich in fruits and vegetables reduces the risk of certain types of cancer, cardiovascular, and other chronic diseases. Accordingly, the National Cancer Institute and the National Research Council recommend at least five daily servings of fruits and vegetables (Criqui and Ringel, 1994; Steinmetz and Potter, 1991; American Institute of Cancer Research, 1997; Ness and Powles, 1997; Hertog et al., 1993; Hertog et al., 1994; Hertog et al., 1995).

Many of the health benefits that result from eating fruits and vegetables are attributed to phytochemicals that contribute dietary antioxidants (Wang et al., 1996). The natural antioxidants of fruits and vegetables (including anthocyanins,

polyphenolics, vitamin C, vitamin E, glutathione, and carotenoids) are thought to aid in the prevention of many chronic diseases (Hollman, 2001; Goodwin and Brodwick, 1995; Frankel et al., 1993; Steinberg, 1997). Antioxidants have been shown to retard the process of oxidative damage of free radicals (Wang and Jiao, 2000; Jacob and Burri, 1996). Though some free radicals are generated by normal body functions, such as respiration, and are sometimes beneficial, they can also enter the body through UV radiation, air pollution, consumption of charred food, and cigarette smoking (Halliwell and Gutteridge, 1999).

Healthful benefits from eating blueberries gained greater acceptance after Prior (1998) reported that blueberries had the highest antioxidant activity of the 42 fruits and vegetables evaluated. This finding sparked additional research into the health benefits of blueberries. Research continues to indicate blueberries contain a higher antioxidant capacity when compared to other fruits (Cao et al., 1996; Wang et al., 1996; Prior et al., 1998). In addition to high levels of dietary phenolics, blueberries are a good source of dietary fiber, calcium, iron, vitamin A, and vitamin C (Eck, 1988; Keely et al., 1982).

Animal studies have demonstrated some of the health benefits attributed to blueberry phenolics. Canadian researchers have demonstrated an improvement in rats with brain damage (due to ischemia) when fed lowbush blueberry enriched diets (Sweeney et al., 2002), while Youdim et al. (2000) found improvement in brain function of old rats that were given a blueberry extract supplement. Research by Joseph et al. (1999) claimed a blueberry extract to reverse symptoms of aging in rats. Cohen-Boulakia et al. (2000) reported that bilberry anthocyanins prevented the increase of capillary filtration in diabetic rats. Mice fed an anthocyanin fraction of *V. myrtillus*

(bilberry) showed inhibition of lipid peroxidation in their liver cells (Martín-Aragón et al., 1999).

Human investigations have also shown many positive results. Extracts of bilberry were found to be the most effective in inhibiting the growth of human leukemia and colon carcinoma cells of 10 berry extracts tested by Katsube et al. (2003). Blueberry and cranberry juices have been reported to contain compounds useful in treating urinary tract infection caused by *Escherichia coli* (Ofek et al., 1991). Blueberry anthocyanins and hydroxycinnamic acids have also been found to reduce inflammation in human microvascular endothelial cells (Youdim, et al., 2002).

There have been numerous reports of human studies that contradict one another. Research conducted in Europe demonstrated improvement of night vision by supplementation of bilberry in a variety of forms (Jayle and Aubert, 1964; Fiorini et al., 1965; Belleoud et al., 1966a; Belleoud et al., 1966b; Contestabile et al., 1991). Although, a study by Muth et al. (2000) examined the effects of a bilberry extract supplementation on night visual acuity, and night contrast sensitivity, and found no differences between subjects who were given the placebo and those who took active capsules. They further speculated that the form the supplement was delivered in, or the dose, recommended by the extract manufacturer, might not have been ideal.

Gao et al. (2002) detected the presence of blueberry anthocyanins in human blood serum (19 out of the 25 anthocyanins present in the freeze-dried blueberry powder), along with a corresponding increase in antioxidant capacity. Mazza et al. (2002) demonstrated a correlation between anthocyanins appearing in human blood serum, and an increase in antioxidant activity. They showed blueberry anthocyanins being absorbed in human subjects in their intact forms (Mazza et al., 2002).

Research by Wu et al. (2002) found elderberry anthocyanins in human plasma, along with low concentrations of their metabolites (peonidin monoglucuronide and cyanidin-3-glucoside monoglucuronide) in urine, four hours after consuming elderberry extracts (0.077% elderberry anthocyanins recovered). Though, they were unable to detect lowbush blueberry anthocyanins in blood plasma after blueberry anthocyanin consumption, but very low concentrations of blueberry anthocyanins were found in their urine (0.004% recovery). Other researchers have found unmetabolized blueberry anthocyanins in the urine of rats and humans (McGhie et al., 2003).

An increase in quercetin concentration was found in the blood of subjects in Finland who consumed of a combination of black currants (*Ribes nigrum*), lingonberries (*Vaccinium vitis-idaea*), and bilberries (*V. myrtillus*), which indicated the bioavailability of quercetin from berries (Erlund, 2003). Researchers from Denmark found no increase in plasma antioxidant activity, of healthy female subjects, after the consumption of blueberry juice, although they did report a 30% increase in plasma antioxidant activity when the subjects consumed the same amount of cranberry (*V. macrocarpon*) juice. They speculated a correlation between the quantities of vitamin C, not the phenolics, in the two juice samples as being responsible for the activity levels (Pedesen et al., 2000).

There is an ever increasing number of blueberry processed products available, according to the North American Blueberry Council. But, research conducted by Kalt et al. (2000) surveyed a wide range of commercially processed lowbush blueberry products (from blueberries in muffins to blueberry sorbet) and found that the least processed blueberry products had the highest antioxidant capacity. Skrede et al. (2000) showed the decrease in anthocyanins and polyphenolics in juice and concentrate when compared to the starting material. Häkkinen et al. (2000) demonstrated domestic

processing of bilberries (cooking bilberry soup) decreased the content of flavonols, and flavonols were undetectable after storing the soup at –20°C for 3 months. Sánchez-Moreno et al. (2003) compared four novel blueberry wines' antioxidant capacity to 15 red wines, and seven white wines, and found the antioxidant capacity to be comparable to red wines.

More research would better explain the inconsistencies reported and the potential health benefits of phenolic compounds in blueberries and blueberry products. Specifically, the absorption, distribution, metabolism, and excretion of these compounds have not been sufficiently understood.

BLUEBERRY STATISTICS

Blueberries (*Vaccinium* species, family *Ericaceae*) are extensively cultivated in North America. According to the Oregon Agricultural Statistic Service, Oregon produced 27,500,000 pounds of blueberries in the year 2002; 60% were processed, while 40% were sold in the fresh market (Coba and Goodwin, 2003). Processed blueberries are mostly found in frozen, canned, dried, puree, juice, or concentrate products. Selective breeding of wild (native) blueberry cultivars began in the early 1900's. Modern blueberry cultivars originate from three native selections: 'Rubel', 'Brooks', and 'Sooy' (Hancock and Siefker, 1982; Eck, 1988). Characteristics of the fruit and plant, its resistance to disease and insects, its cold hardiness, and its productivity are among the traits considered when breeding desirable blueberries (Eck, 1988).

Recently there has been intense interest in the possible health benefits of fruits and vegetables with high antioxidant capacity, correlated to their anthocyanin and total

phenolic content (Prior et al., 1998; Kalt et al., 2000; Heinonen et al., 1998). Horticulturists have become interested in breeding blueberries that yield fruit with even higher levels of anthocyanins (and phenolics in general), which would further increase antioxidant activity.

The genus *Vaccinium*, which includes 30 sections, is one of the major genera of the family Ericaceae (Stevens, 1969). Ten sections of the genus *Vaccinium* are found in North America including Vitis-idaea, Vaccinium, Oxyococcus, Polycodium, Batodendron, Herpothamnus, Hugeria, Pyxothamnus, Myrtillus, and Cyanococcus.

Highbush (*V. corymbosum*, L.), lowbush (*V. angustifolium*), bilberry (*V. myrtillus* L.), and rabbiteye (*V. ashei* Reade) blueberries are the four main taxa, all in the Cyanococcus, grown commercially in North America (Hancock, 1989; Hancock and Draper 1989). Highbush blueberry shrubs are usually more than 1.5 m tall, and lowbush blueberry shrubs are usually 0.09 to 0.27 m tall, while rabbiteye can grow as tall as 6 m (Vander Kloet, 1988; Hancock and Draper, 1989). Lowbush blueberries are mainly harvested in Maine and eastern Canada. Highbush blueberries are the major commercially grown blueberry crop in the Pacific Northwest. Highbush plants are perennials that thrive in acidic soil (pH 4.5 to 5.5). In the Pacific Northwest, they can have ripe fruit from mid-June to mid-September. Yield and fruit size are generally dependant upon cultivar and environmental conditions. Highbush blueberries are harvested either mechanically or by hand (Hancock and Draper, 1989; Doughty et al., 1981).

The pH of a blueberry fruit has been reported to range from 2.85 to 3.49, by Sapers et al. (1984), in the 11 cultivars examined. They reported titratable acidity to vary from 0.40 to 1.31% as citric acid. Sugars in blueberries have been found to be

chiefly (in decreasing order) fructose, glucose, and sucrose (Kalt and McDonald, 1996; Eck, 1988). The main organic acids found in blueberries are citric acid, malic acid, acetic acid, succinic acid, and shikimic acid; with citric acid being the most prevalent (Kalt and McDonald, 1996; Ehelnfeldt et al., 1994).

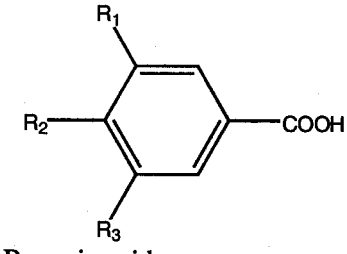
Phenolics are secondary metabolites produced by plants, and are a very diverse group of compounds (> 4000 flavonoids from plants have been identified) (Strack et al., 1994). These compounds have been implicated to have several functions within plants: protection from UV light, pigmentation, anti-fungal properties, attraction of pollinators and seed dispersers, and nodule production (Strack et al., 1994; Koes et al., 1994; Gould and Lee, 2002). The appearance and flavor of berries, and berry products, are influenced by phenolics. Factors that can affect the phenolic content of any fruit, and fruit products include: plant species, cultivar, ripeness, season, region, yield, field management practices, environmental factors, post-harvest storage, and processing practices (Eck, 1988; Moyer et al., 2002; Prior et al., 1998; Conner et al., 2002; Clark et al., 2002).

Currently, the term polyphenolics is used synonymously with phenolic compounds. Polyphenolics refer to the numerous phenolic compounds rather than one phenolic group. The term polyphenolics was first used in the early 1920s and has come into wide-spread usage since 1990s. Some speculate the term "polyphenolics" was intended to describe the complexity of compounds observed from a plant extract analyzed by liquid chromatography, monitored in the UV region (R. Wrolstad, pers. comm.).

Phenolic composition differs considerably among blueberry cultivars. Structures of the phenolics commonly found in blueberries are shown in Figure 2.1. Phenolic acids (benzoic acids and cinnamic acids), also known as non-flavonoids, and their derivatives

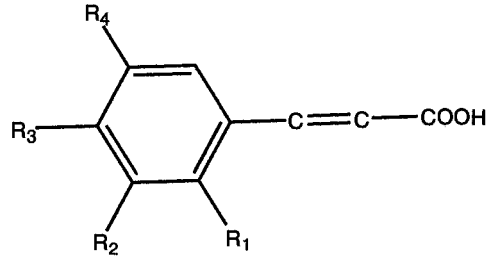
are found in blueberries. The benzoic acids reported to be in blueberries are vanillic acid, syringic acid, gallic acid, protocatechuic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, and ellagic acid (Azar et al., 1987; Sellappan et al., 2002; Häkkinen et al., 1999; Amakura et a., 2000). While the cinnamic acids found were chlorogenic acid, caffeic acid, ferulic acid, quinic acid, *p*-coumaric acid, *o*-coumaric, acid and *m*-coumaric acid (Häkkinen et al., 1999; Azar et al., 1987; Sellappan et al., 2002). Chlorogenic acid is the major phenolic present in the fruit (Kader et al., 1996; Lee et al., 2002; Skrede et al., 2000; Gao and Mazza, 1994; Kalt et al., 2000; Kalt and McDonald, 1996; Zheng and Wang, 2003).

Phenolic acids are rarely present as free acids, but rather are esterified with other phenolics, acids, or sugars (Herrmann, 1989). Resveratrol (stilbene class) has been reported in some blueberries (Lyons et al., 2003). Numerous flavonoids (anthocyanins, flavonols, flavones, flavanonols, and flavan-3-ols) have been reported to be present in blueberries. The presence of B_1, through B_8, procyanidin oligomers and monomers, which correspond to (+)-catechin and (-)-epicatechin, have been noted by researchers (Skrede et al., 2000; Prior et al 2001; Sellappan et al., 2002). Polymeric procyanidins (degree of polymerization found to range from 20 to 114) have been found in lowbush blueberries (Gu et al., 2002). Quercetin-, kampferol-, and myricetin- glycosides have been reported in blueberries (Häkkinen et al., 1999; Skrede et al., 2000; Lee et al., 2002; Zheng and Wang, 2003; Sellappan et al., 2002).



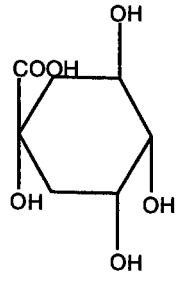
Benzoic acids

Gallic acid	$R_1=R_2=R_3=OH$
Protocatechuic acid	$R_1=H, R_2=R_3=OH$
Vanillic acid	$R_1=H, R_2=OH, R_3=OCH_3$
Syringic acid	$R_2=OH, R_1=R_3=OCH_3$

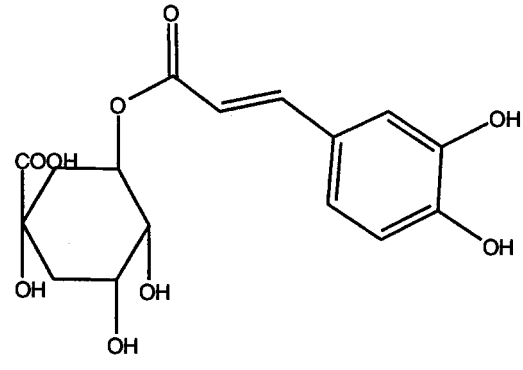


Cinnamic acids

Ferulic acid	$R_1=R_2=H, R_3=OH, R_4=OCH_3$
p-coumaric acid	$R_1=R_2=R_4=H, R_3=OH$
o-coumaric acid	$R_2=R_3=R_4=H, R_1=OH$
m-coumaric acid	$R_1=R_3=R_4=H, R_2=OH$
Caffeic acid	$R_1=R_4=H, R_2=R_3=OH$

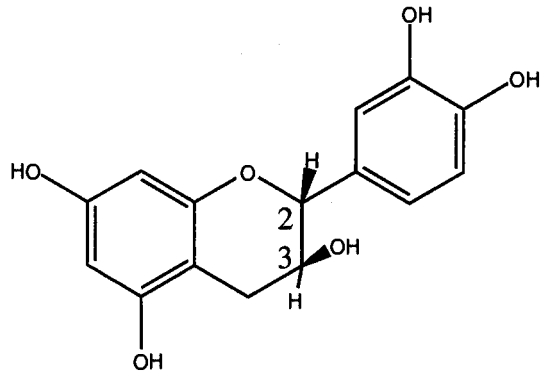


Quinic acid



Chlorogenic acid (5-caffeoylquinic acid)

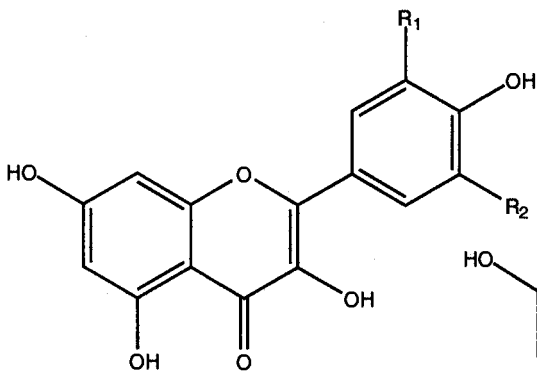
Figure 2.1. Structures of selected phenolic compounds reported to be present in blueberry (*Vaccinium* sp.).



Flavanols

(+)-Catechin (2R, 3S)

(-)-Epicatechin (2R, 3R)

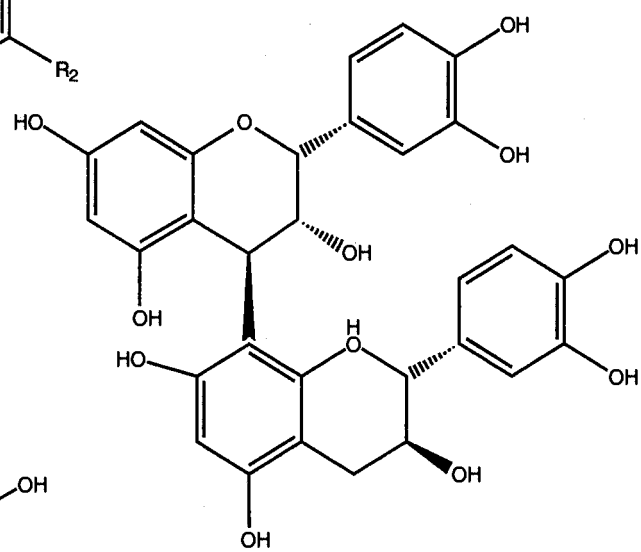


Flavonols

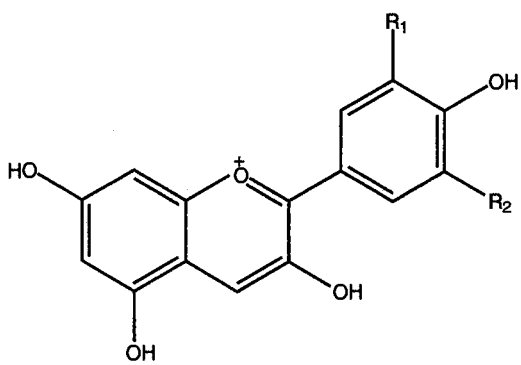
Kaempferol $R_1=R_2=H$

Quercetin $R_1=OH, R_2=H$

Myercetin $R_1=R_2=OH$



Procyanidin B1



Anthocyanidins

Cyanidin	$R_1=OH, R_2=H$	Petunidin	$R_1= OCH_3, R_2=OH$
Peonidin	$R_1=OCH_3, R_2=H$	Malvidin	$R_1=R_2= OCH_3$
Delphinidin	$R_1=R_2=OH$		

Figure 2.1. . .Continued.

Fifteen different anthocyanins are commonly present in blueberry fruit: galactosides, glucosides, and arabinosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin (Mazza and Miniati, 1993; Kalt and Defour, 1997). The proportions of individual anthocyanins differ among cultivars, and stage of fruit maturation, and can also be influenced by environmental conditions (Mazza and Miniati, 1993; Ballington et al., 1987; Prior et al., 1998). Acylated anthocyanins (mainly acylated with acetic acid) have also been reported in lowbush blueberry (Prior et al., 2001).

NATURAL ANTIOXIDANT EXTRACTION OF RESIDUAL BERRY SOURCES

While food scientists have been examining processing methods for improving antioxidant content of blueberry products, studies have been exploring techniques for utilizing processing wastes (chiefly skins and seeds) for additional recovery of antioxidant compounds. The portions remaining after traditional processing have the potential for further phytochemical extraction, while decreasing the volume of waste (Schieber et al., 2001).

Manufacturing a phytochemical-rich product requires the extraction of these compounds from source material using liquid solvents. Extraction depends on the dissolution rate of the phytochemical from the solid plant material into the solvent, and the rate of equilibrium concentration (Gertenbach, 2001). This can be maximized by increasing the concentration gradient (utilizing more solvents) and/ or decreasing the particle size of the solid plant material (Cacace and Mazza, 2002). Landbo and Meyer (2001) maximized extraction of black currant residue by decreasing the pomace residue particle size. Landbo and Meyer (2001) compared the treatments of four different commercial enzymes (industry recommended pectinase, macerase, and a protease), and

demonstrated the efficacy of enzymes in aiding solvent penetration into the pomace for extraction of phytochemicals. Cacace and Mazza (2003) recommended processing temperatures of 30 to 35°C, while demonstrating that solvent to solid ratio was the limiting factor when extracting anthocyanins from black currants, using aqueous ethanol.

Maximizing phytochemical extraction is dependant on several factors including solvent, solvent to solid ratio, native enzymes, processing enzymes, oxygen, pH, temperature, light and time of process as phenolics are highly reactive and can degrade rapidly (Cacace and Mazza, 2002; Skrede and Wrolstad, 2002).

QUANTITATIVE ANALYSIS OF ANTHOCYANINS

Anthocyanins are responsible for the red, purple, and blue hues present in fruits, vegetables, and grains. There are six common anthocyanidins (pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin) found in nature, whose structures can be varied by glycosidic substitution (glucose, galactose, rhamnose, xylose, or arabinose) at the 3 and 5 positions. Additional variations occur by acylation of the sugar groups with acids. Some acylating groups commonly found are acetic acid, *p*-coumaric acid, caffeic acid, malonic acid, sinapic acid, ferulic acid, oxalic acid, and succinic acid (Takeoka and Dao, 2002; Guisti et al., 1999).

Most research in the quantitation, purification, separation, and identification of anthocyanins has relied on expensive equipment, and/or requires lengthy sample preparation. These methods include paper chromatography, thin-layer chromatography, column chromatography, solid-phase extraction, counter current chromatography, UV-Visible absorption spectroscopy, high performance liquid chromatography, mass

spectrometry, and nuclear magnetic resonance spectroscopy (Takeoka and Dao, 2002; Skrede and Wrolstad, 2002).

Measurements of anthocyanin content, the major contributor to color, in berry samples have long been an important indicator of quality of fresh and processed berry products. The interest in beneficial effects of anthocyanins created the need for a simple method to quantify the total anthocyanin content of a sample, and allow the result to be compared to different samples and laboratories. Recent studies have shown a high correlation between anthocyanin content and antioxidant activity, which have increased the relevance of simple methods to determine the anthocyanin content within a sample (Heinonen et al., 1998; Kalt et al., 1999; Moyer et al., 2002).

Nonsubtractive and Differential method using UV-Visible Absorption Spectroscopy

A single pH method can be used when there are little or no interfering compounds in a sample. Anthocyanin content can be determined by measuring a sample's absorbance at its maximum wavelength. Anthocyanin absorbance is linear to the amount of pigment present (when absorption is within the linear region of the spectrophotometer). Anthocyanins typically absorb in the visible spectra of 490 to 550 nm region. This region is far from other interfering compounds; like phenolics with maximum absorption in the UV region (Fuleki and Francis, 1968a).

The presence of compounds, other than phenolics, can interfere with determination of the anthocyanin content of a sample. A differential method can be used with samples that contain interfering compounds such as anthocyanin degradation products (Guisti and Wrolstad, 2001). The method can be utilized for quantitative

determination of total monomeric anthocyanin content, based on the structural change of the anthocyanin chromophore between pH 1.0 and pH 4.5. The pH differential method has shown to be simple, quick, and accurate to measure the total monomeric anthocyanin of a sample (Wrolstad et al., 1995). The concept of measuring anthocyanins in a sample (strawberries or strawberry perserves) by utilizing the change in absorbance at two different pH values (3.4 and 2.0), was first introduced by Sondheimer and Kertesz in 1948. Since then, researchers have proposed the use of pH values of 1.0 and 4.5 (Wrolstad et al., 1995; Guisti and Wrolstad, 2001; Fuleki and Francis, 1968b; Wrolstad et al., 1982). Monomeric anthocyanins undergo a reversible structural transformation as a function of pH (colored oxonium form at pH 1.0 and colorless hemiketal form at pH 4.5). Thus the difference in absorbance at the pigments $\lambda_{vis\ max}$ ($\approx$ 520 nm) will be proportional to the pigment concentration. Degraded anthocyanins in the polymeric form are resistant to color change with pH. Hence, polymerized anthocyanin pigments will not be measured in this method as they absorb at pH 4.5, as well as pH 1.0.

High Performance Liquid Chromatography (HPLC) and Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)

Profiles of anthocyanins are distinctive in different fruits, and among different varieties of a given fruit. Many factors affect the total anthocyanin content in blueberry fruit, including species, cultivar, ripeness, season, growing region, yield, field management practices, and environmental factors. Total and individual anthocyanins can be monitored to study the influences of these factors (Mazza et al., 1993; Mazza, 1995). The composition of the different species and varieties of the *Vaccinium*

anthocyanins are distinct, and can be determined by High Performance Liquid Chromatography (HPLC) and/or Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS).

Reversed-phase HPLC coupled with photodiode array detection has been the most widely used tool for the identification, and quantification of anthocyanins. The different anthocyanins can be separated by their polarity, which elute at different times, and have different spectra. The anthocyanins can be quantitated with an external standard (cyanidin-3-glucoside or any purified anthocyanin standard).

Separation by HPLC is achieved by a variety of sorption mechanisms. Reversed-Phase HPLC bonded phase uses a nonpolar stationary phase (i.e. octadecyl, octyl, & dimethyl), chemically bonded, or organo-bonded to an insoluble matrix, and a polar mobile phase. Water is used as the base solvent, and the mobile phase strength is adjusted by using organic modifiers such as methanol or acetonitrile. Nonpolar and polar samples can be analyzed. The more nonpolar analytes are, the more they are attracted to the nonpolar bonded phase. This allows the more nonpolar compounds to increase their time associated with the bonded phase. So, more polar compounds will elute first. Retention is controlled by the partitioning (full embedding of the analyte between the stationary phase chains) process rather than by adsorption (hydrophobic interactions). Selectivity can be modified by changing solvents, solvent strength, or by altering the stationary phase. Retention times increase as the chain length of the bonded phase increases. Longer analyte retention provides enhanced resolution, and by varying chain length of bonded phase, resolution can be optimized. The degree of surface coverage by silica effects retention, selectivity, and pH stability of bonded phase. It is

best to use a column with high percentage coverage, or with an end capped residual silanol group (Braithwaite et al., 1996; Durst and Wrolstad, 2001).

MALDI-MS became available in 1987 and was initially used to analyze large biomolecules (Karas et al., 1987). MALDI is the initial instrumental step of MS analysis, which ionizes particles by laser light. MALDI is a soft-MS ionization technique that results in less fragmentation of molecular ions when compared to Electrospray (ES)-MS. The analyte is mixed with a matrix (a low mass organic compound). Ideally, the matrix should absorb strongly at laser wavelength, have stability in vacuum, and have little chemical reactivity. This mixture is condensed (air-dried) onto a sample probe, then placed in a high vacuum chamber. One matrix reported to be used in anthocyanin analysis by MALDI-MS, with a nitrogen laser is trihydroxyacetophenone (Wang et al., 2000). The probe is usually a metal appropriate for precision machining, with regular array of sites for sample application. Laser light (ultraviolet laser, nitrogen laser, or infrared laser) irradiates the condensed mixture on probe, resulting in particle being ejected from the condensed state. Ionized particles are resolved and detected according to their masses. For anthocyanins, positive ions are extracted from the ion source for TOF (time-of-flight) focusing. Delaying the extraction of ions, from the ion source results in improvement of mass accuracy and sensitivity. Once in the TOF tube, the extracted ions move toward the detector at a constant velocity characteristic to their mass/charge (m/z) ratios. Because of their positive charge, anthocyanins (mostly in the oxonium form at low pH) will generate a positive ion that can be measured by the mass spectrometer (MS). Anthocyanins' positive charge allows the mass/charge ratio to correspond directly to the molecular weight of the anthocyanin (Karas et al., 1996; Bruker Daltonics, 2002).

Sample preparation for HPLC analysis is simpler than that for MALDI-MS. Sample preparation methods are critical for MALDI-MS analysis, since the researcher has the greatest influence during this portion of the experiment (Durst and Wrolstad, 2001; Wang and Sporns, 1999; Wang et al., 2000).

Analysis by HPLC takes 55 minutes for each sample run to complete. A MALDI-MS run takes about 4 minutes, though poor reproducibility from spot to spot (due to crystal inhomogeneity) by MALDI-MS, a repetition of sample run is recommended. Five runs for one sample would take about 20 minutes, so even with replicate runs MALDI-MS analysis is much faster (Durst and Wrolstad, 2001; Wang and Sporns, 1999; Wang et al., 2000).

A major advantage of using HPLC, is that anthocyanin isomers can be differentiated. With MALDI-MS, anthocyanin isomers cannot be distinguished from one another as they have the same mass/charge ratio. For example: malvidin-3-glucoside and malvidin-3-galactoside could be separated by HPLC, but would have the same MALDI-MS m/z of 493.2 (Hong and Wrolstad, 1990; Wang and Sporns, 1999; Wang et al., 2000).

HPLC can result in an underestimation of the amount of anthocyanin present in a sample when using one standard for quantification. Quantification of anthocyanins in HPLC is determined by the peak area at a certain wavelength (520 nm), which is close to the maximum wavelengths (λ_{max}) of individual anthocyanins. Typically, cyanidin-3-glucoside is selected as the external standard. Numerous studies show that depending on the anthocyanin chromophores, the maximum wavelength shifts slightly. For example, the λ_{max} for malvidin-3-glucoside and cyanidin-3-glucoside are 534 nm and 523 nm,

respectively, with 0.01% HCl in methanol (Durst and Wrolstad, 2001; Hong and Wrolstad, 1990; Francis, 1982).

For anthocyanin quantification by MALDI-MS, intensity of monoglucoside anthocyanins is linearly correlated to their relative molar ratios. But, for diglucoside anthocyanins, the relative molar ratios were reduced (one-fourth of the monoglucoside anthocyanins for equal concentration) (Wang and Sporns, 1999).

Much promise has been shown by MALDI-MS as a new separation technique. However, it is still in a developmental state when compared to HPLC, which has been intensively used to analyze anthocyanins. MALDI-MS has shown capabilities for analyzing a wide variety of compounds, but only recently has the separation of anthocyanins been successful with MALDI-MS.

REFERENCES

Amakur, Y., Umino, Y., Tsuji, S., and Tonogai, Y. 2000. Influence of jam processing on the radical scavenging activity and phenolic content in berries. J. Agri. Food Chem. 48:6292-6297.

American Institute for Cancer Research. 1997. Food, nutrition, and the prevention of cancer: a global perspective. Amer. Inst. Cancer Res. Washington, D.C.

Azar, M., Verette, E., and Brun, S. 1987. Identification of some phenolic compounds in bilberry juice *Vaccinium myrtillus*. J. Food Sci. 52:1255-1257.

Ballington, J.R., Ballinger, W.E., and Maness, E.P. 1987. Interspecific differences in the percentage of anthocyanins, aglycones, and aglycone-sugars in the fruit of seven species of blueberries. J. Amer. Soc. Hort. Sci. 112:859-864.

Belleoud, L., Leluan, D., and Boyer, Y. 1966a. Study on the effects of anthocyanin glycosides on the night vision of flight personnel. Commun. Soc. Fr. Physiol. Med. Aeronaut Cosmonaut. 17.

Belleoud, L., Leluan, D., and Boyer, Y. 1966b. Study on the effects of anthocyanin glycosides on the nocturnal vision of air traffic controllers. Rev. Med. Aeronaut Spatiale. 18.

Bomser, J., Madhavi, D.L., Singletary, K., and Smith, M.A.L. 1996. In vitro anticancer activity of fruit extracts from *Vaccinium* species. Planta Medica. 62:212-216.

Braithwaite, A., and Smith, F. J. 1996. Chromatographic methods 5th Edition, Chapman & Hall., Bishopbriggs, Glasgow.

Bruker Daltonics. 2002. ReflexIV: the ultimate performance MALDI-TOF MS. Bruker Daltonics Product Brochure.1-7.

Cacace, J.E. and Mazza, G. 2002. Extraction of anthocyanins and other phenolics from black currants with sulfured water. J. Agric. Food Chem. 2002. 50:5939-5946.

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

Camire, M.E. 2002. Chapter 3: Phytochemicals in the *Vaccinium* family: Bilberries, blueberries, and cranberries. In Phytochemicals: their role in nutrition and health. Meskin M.S. Ed. CRC Press LLC, Boca Raton, FL. p19-40.

Cao, G., Sofic, E., and Prior, R.L. 1996. Antioxidant capacity of tea and common vegetables. J. Agric. Food Chem. 44:3426-3431.

Clark, J.R., Howard, L., and Talcott, S. 2002. Variation in phytochemical composition of blueberry cultivars and breeding seasons. Acta Hort. 574:203-207.

Coba, K. and Goodwin, J.A. 2003. Oregon Agri-Facts. Oregon Agricultural Statistics Service. Oregon Department of Agriculture. Portland, OR.

Cohen-Boulakia, F., Valensi, P.E., Boulahdour, H., Lestrade, R., Dufour-Lamartinie, J.F., Hort-Legrande, C., Behar, A. 2000. *In vivo* sequential study of skeletal muscle capillary permeability in diabetic rats: effect of anthocyanosides. Metabolism: Clin. Experi., 49:880-5.

Conner, A. M., Luby, J.J., Tong, C.B.S., Finn, C.E., and Hancock, J.F. 2002. Genotypic and environmental variation in antioxidant activity among blueberry cultivars. Acta Hort. 574:209-213.

Contestabile, M., Appolloni, R., and Suppressa, F. 1991. Prolonged treatment with blueberry anthocyanides administered in high doses: electrophysiological response in myopic patients. Boll. Ocul. 70:1157-1169.

Criqui, M.H. and Ringel, B.L. 1994. Does diet or alcohol explain the French paradox? Lancet. 344:1719-1723.

Doughty, C.C., Adams, E.B., Martin, L.W., Bristow, P., Byther, R., Antonelli, A., and Shanks, C.H. 1981. PNW 215: Highbush blueberry production. Oregon State University, Corvallis, OR.

Durst, R.W., and Wrolstad, R.E. 2001. Unit F1.3: Anthocyanins. Separation and characterization of anthocyanins by HPLC. In: Wrolstad RE, editor. Current Protocols in Food Analytical Chemistry. New York: John Wiley & Sons. p 1-13.

Eck, P. 1988. Blueberry Science. Rutgers University Press. London.

Ehlenfeldt, M.K., Meredith, F.I., and Ballington, J.P. 1994. Unique organic acid profile of rabbiteye vs. highbush blueberries. HortScience. 29:321-323.

Erlund, I., Marniemi, J., Hakala, P., Alfthan, G., Meririnne, E., and Aro, A. 2003. Consumption of black currants, lingonberries, and bilberries increases serum quercetin concentration. Eur. J. Clin. Nutr. 57:37-42.

Fiorini G., Biancacci, A., and Graziano, F. 1965. Perimetric and adaptometric changes following the administration of myrtillin associated with beta-carotene. Annali Di Ottalmologia E. Clinica Oculistica. 91:371-386.

Francis, F. 1982. Analysis of anthocyanins. In Anthocyanins as Food Colors., Markakis, P., Ed., Academic Press: New York.

Frankel, E.N., Kanner, J., German, J.B. Parks, E., and Kinsella, J.E. 1993. Inhibition of oxidation of human low-density protein by phenolic substances in red wine. The Lancet. 341:454-457.

Fuleki, T. and Francis, F.J. 1968a. Quantitative methods for anthocyanins. 1. Extraction and determination of total anthocyanins in cranberries. J. Food Sci. 33:72-78.

Fuleki, T. and Francis, F.J. 1968b. Quantitative methods for anthocyanins.2. Determination of total anthocyanin and degradation index for cranberry juice. J. Food Sci. 33, 78-82.

Gao, L. and Mazza, G. 1994. Quantitation and distribution of simple and acylated anthocyanins and other phenolics in blueberries. J Food Sci 59(5):1057-1059.

Gao, M., Kay, C.D., Cottrell, T., and Holub, B.J. 2002. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. J. Agric. Food Chem. 50:7731-7737.

Gertenbach, D.D. 2001. Solid-liquid extraction technologies for manufacturing nutraceuticals from botanicals. In: Functional Foods: Biochemical and Processing Aspects. Shi, J., Mazza, G., and Le Maguer. (Ed.). Vol2. Boca Raton, FL: CRC Press Inc, p331-366.

Giusti, M.M., Rodriguez-Saona, L.E., Griffin, D., and Wrolstad, R.E. 1999. Electrospray and tandem mass spectroscopy as tools for anthocyanin characterization. J. Agric. Food Chem. 47:4657-4664.

Giusti, M.M. and Wrolstad, R.E. 2001. Unit F1.2: Anthocyanins. Characterization and measurement with UV-visible spectroscopy. In Current Protocols in Food Analytical Chemistry. RE Wrolstad (Ed.) New York: John Wiley & Sons. p 1-13.

Goodwin, J.S. and Brodwick, M. 1995. Diet, aging, and cancer. Clinics in Geriatric Medicine. 11:577-589.

Gould, K.S. and Lee D.W. (Eds.). 2002. Anthocyanins and Leaves. The Function of Anthocyanins in Vegetative Organs (Vol. 37). Advances in Botanical Research. London: Academic Press.

Gu, L., Kelm, M., Hammerstone, J.F., Beecher, G., Cunningham, D., Vannozzi, S., and Prior, R.L. 2002. Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC-MS flourescent detection method. J. Agric. Food Chem. 50:4852-4860.

Halliwell, B. and Gutteridge, J..M.C. Ed. 1999. Free Radicals in Biology and Medicine. 3rd edition. Oxford University Press. Oxford, NY.

Hancock J.F. and J.H. Siefker. 1982. Levels of inbreeding in highbush blueberry cultivars. HortScience 17:363-366.

Hancock, J.F. 1989. Blueberry research in North America. Acta Hort. 241:19-30.

Hancock, J.F. and Draper, A.D. 1989. Blueberry culture in North America. HortScience. 24:551-556.

Häkkinen, S., Heinonen, M., Kärenlampi, S., Mykkänen, H., Ruuskanen, J., and Törrönen, R. 1999. Screening of selected flavonoids and phenolic acids in 19 berries. Food Res. Intl. 32:345-353.

Häkkinen, S.H., Kärenlampi S.O., Mykkänen, H.M., and Törrönen, A.R. 2000. Influence of domestic processing and storage on flavonol contents in berries. J Agric Food Chem 48:2960-2965.

Heinonen, I.M., Meyer, A.S., and Frankel, E.N. 1998. Antioxidant activity of berry phenolics on human low-density lipoprotein and liposome oxidation. J. Agri. Food chem. 46:4107-4112.

Herrmann, K. 1989. Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. Crit. Rev. Food Sci and Nutri. 28:315-347.

Hertog, M.G.L., Feskens, E.J.M., Hollman, P.C.H., Katan, M.R., and Kromhout, D. 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. The Lancet. 342:1007-1011.

Hertog, M.G.L., Feskens, E.J.M., Hollman, P.C.H., Katan, M.R., and Kromhout, D. 1994. Dietary antioxidant flavonoids and cancer risk in the Zutphen elderly study. Nutr. and Cancer. 22:175-184.

Hertog, M.G.L., Kronhout, D., Aravanis, C., Blackburn, H., Buzina, R., Fidanza, F., Giampaoli, S., Jansen, A., Menotti, A., Nedeljkovc, S., Pekkarinen, M., Simic, B.S., Toshima, H., Feskens, E.J.M., Hollman, P.C.H., and Katan, M.R. 1995. Flavonoid intake and long-term and risk of coronary heart disease and cancer risk in the seven countries study. Archives of International Medicine. 155:381-386.

Hollman, P.C. 2001. Evidence for health benefits of plant phenols: local or systemic effects? J. Sci. Food Agric. 81:842-852.

Hong, V. and Wrolstad, R. 1990. Use of HPLC separation/photodiode array detection for characterization of anthocyanins. J. Agric. Food Chem. 38:708-715.

Jacob, R.A. and Burri, B.J. 1996. Oxidative damage and defense. Amer. J. Clin. Nutri. 63:985S-990S.

Jayle, G.E. and Aubert, L. 1964. Action des glucosides d'anthocyanes sur la vision scotopique et mesopique du sujet normal. Theapie. 19:171-185.

Joseph, J.A., Shukitt-Hale, B., Denisova, N.A., Bielinski, D., Martin, A., McEwen, J.J., and Bickford, P. 1999. Reversal 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.

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

Kalt, W. and Dufour, D. 1997. Health functionality of blueberries. HortTechnology 7:216-221.

Kalt, W. and McDonald, J.E. 1996. Chemical composition of lowbush blueberry cultivars. J. Amer. Soc. Hort. Sci. 121:142-146.

Kalt, W., McDonald, J.E., and Donner, H. 2000. Anthocyanins, phenolics, and antioxidant capacity of processed lowbush blueberry products. J. Food Sci. 65:390-393.

Karas, M., Bachman, D., Bahr, U., and Hillenkamp, F. 1987. Matrix-assisted ultraviolet laser desorption of nonvolatile compounds. Int. J. Mass Spec. Ion Processes.78:53-68.

Karas, M. 1996. Matrix-assisted laser desorption ionization MS: a progress report. Biochem. Mass Spectrom. 24:897-900.

Katsube, N., Iwashita, K., Tsushida, T., Yamaki, K., and Kobori, M. 2003. Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. J. Agri. Food Chem. 51:68-75.

Keely, P.B., Martinsen, C.S., Hunn, E.S., and Norton, H.H. 1982. Composition of native American fruits in the Pacific Northwest. J. Amer. Diet. Assoc. 81:568-572.

Koes, R., F. Quattrocchio, and J. Mol. 1994. The flavonoid biosynthetic pathway in plants: Function and evolution. BioEssays. 16:123-132.

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

Lee, J., R.W. Durst, and R.E. Wrolstad. 2002. Impact of juice processing on blueberry anthocyanins and polyphenolics: comparison of two pretreatments. J. Food Sci. 67:1660-1667.

Luo, X.D., Basile, M.J., and Kennelly, E.J. 2002. Polyphenolic antioxidants from the fruits of *Chrysophyllum cainito* L. (star apple). J. Agri. Food Chem. 50:1379-1382.

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. 51:5867-5870.

Martín-Aragón, S., Basabe Belén, B., Benedí, J.M., and Villar, A.M. 1999. *In vitro* and *in vivo* antioxidant properties of *Vaccinium myrtillus*. Pharm Biol 37:109-113.

Mazza, G. 1995. Anthocyanins in grapes and grape products. Crit. Rev. Food Sci. Nutr. 35:341-371.

Mazza, G., and Miniati, E. 1993. Anthocyanins in fruits, vegetables, and grains. Boca Raton, FL.: CRC Press Inc. p 149-190.

Mazza, G., Kay, C.D., Cottrell, T., and Holub, B.J. 2002. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. J. Agric. Food Chem. 50:7731-7737.

McGhie, T.K., Ainge, G.D., Barnett, L.E., Cooney, J.M. and Jensen, D.J. 2003. Anthocyanin glycosides from berry fruit are absorbed and excreted unmetabolized by both humans and rats. J. Agri. Food Chem. 51:4539-4548.

Morazzoni, P. and Bombardelli, E. 1996. *Vaccinium myrtillus* L. Fitoterapia. 68:3-28.

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

Muth, E.R., Laurent, J.M., and Jasper, P. 2000. The effect of bilberry nutritional supplementation on night visual acuity and contrast sensitivity. Alter. Med. Rev. 5:164-173.

Ness, A.R. and Powles, J.W. 1997. Fruit and vegetables, and cardiovascular disease: a review. Int. J. Epidemiol. 26:1-13.

Ofek, I., Goldhar, D., Zafriri, H., Adar, R., and Sharon, N. 1991. Anti-*Escherichia coli* adhesion activity of cranberry and blueberry juices. New Engl. J. Med. 324:1599.

Pedersen, C.B., Kyle, J., Jenkinson, A.M., Gardner, P.T., McPhail, D.B., and Duthie, G.G. 2000. Effects of blueberry and cranberry juice consumption on the plasma antioxidant capacity of healthy female volunteers. Euro. J. Clin. Nutri. 54:405-408.

Pellerro Society. 1974. Nutritional comparison of Scandinavian and Canadian blueberries. Pellerro Soc., Helsinki, Finland.

Prior, R.L. 1998. Antioxidant capacity and health benefits of fruits and vegetables: blueberries, the leader of the pack. Proc. 32nd Ann. Open House North Carolina Blueberry Council. 32:3-12.

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 phenolic and anthocyanin 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 spectrometry. J. Agric. Food Chem. 49:1270-1276.

Sánchez-Moreno, C. Cao, G., Ou, B. and Prior, R.L. 2003. Anthocyanin and proanthocyanidin content in selected white and red wines. Oxygen radical absorbance capacity comparison with nontraditional wines obtained from highbush blueberry. J. Agric. Food Chem. 51:4889-4896.

Sapers, G.M., Burgher, A.M., Phillips, J.G., Jones, S.B., Stone, E.G. 1984. Color and composition of highbush blueberry cultivars. J. Amer. Soc. Hort. Sci. 109:105-111.

Schieber, A., Stintzing, F.A., and Carle, R. 2001. By-products of plant food processing as aa source of functional compounds- recent developments. Trends in Food Sci. & Tech. 12:401-403.

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

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

Skrede, G. and Wrolstad, R.E. 2002. Chapter 3: Flavonoids from berries and grapes. In: Shi, J., Mazza, G., and Maguer, M.L., editors. Functional Foods: Biochemical and Processing Aspects. New York, US: CRC press. p 71-133.

Sondheimer, E. and Kertesz, Z. I. 1948. Anthocyanin pigments. Colorimetric determination in strawberries and strawberry perserves. Anal. Chem. 20, 245-248.

Steinberg, D. 1997. A critical look at evidence for the oxidation of LDL in atherogenesis. Atherosclerosis. 131:S5-S7.

Steinmetz, K.A. and Potter, J.D. 1991. Vegetables, fruit, and cancer. I. Epidemiology. Cancer Causes Contr. 2:325-357.

Stevens, P.F. 1969. Taxonomic studies in the Ericaceae. PhD Diss., Univ. of Edinburgh, Edinburgh, U.K.

Strack, D. and V. Wray. 1994. The Anthocyanins. In: Harborne J.B. editor. The Flavonoids, London, UK: Chapman & Hall. p 1-22.

Sweeney, M.I., Kalt, W., Mackinnon, S.L., Ashby, J. and Gottschall-Pass, K.T. 2002. Feeding rats diet enriched in lowbush blueberries for six weeks decreases ischemia-induced brain damage. Nutri. Neurosci. 5:427-431.

Takeoka, G. and Dao, L. 2002. Anthocyanins. In. Methods of Analysis for Functional Foods and Nutraceuticals. Hurst, W.J. (Ed.) CRC Press LLC, Boca Raton FL. p 219-241.

Vander Kloet, S.P. 1988. The Genus *Vaccinium* in North America. Publication 1828, Research branch, Agriculture Canada.

Wang, H., Cao, G., and Prior, R.L. 1996. Total antioxidant capacity of fruits. J. Agri. Food Chem. 44:701-705.

Wang, J., and Sporns, P. 1999. Analysis of anthocyanins in red wine and fruit juice using MALDI-MS. J. Agric. Food Chem. 47:2009-2015.

Wang, J., Kalt, W., and Sporns, P. 2000. Comparison between HPLC and MALDI-TOF MS analysis of anthocyanins in highbush blueberries. J. Agric. Food Chem. 48:3330-3335.

Wang, H., Cao, G., Prior, R.L. 1996. Total antioxidant capacity of fruits. J. Agric. Food Chem. 44:701-705.

Wang, S.Y. and Jiao, H. 2000. Scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. J. Agric. Food Chem. 48:5677-5684.

Wrolstad, R.E., Hong, V., Boyles, M.J., and Durst, R.W. 1995. Use of the anthocyanin pigment analysis for detecting adulteration in fruit juices. In Vol. I, Methods to Detect Adulteration in Fruit Juice and Beverages. S. Nagy and R.L. Wade (Ed.). AgScience Inc., Auburndale, FL.

Wrolstad, R.E., Culbertson, J.D., Corwell, C.J., and Mattick, L.R. 1982. Detection of adulteration in blackberry juice concentrates and wines. J. Assoc. Off. Anal. Chem. 65, 1417-1423.

Wu, X., Cao, G., and Prior R.L. 2002. Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry. J. Nutr. 132: 1865-1871.

Youdim, K.A., McDonald, J., Kalt, W., and Joseph, J.A. 2002. Potential role of dietary flavnoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults. J. Nutri. Biochem. 13:282-288.

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

Zheng, W. and Wang, S.Y. 2003. Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. J. Agric. Food Chem. 51:502-509.

CHAPTER 3

IMPACT OF JUICE PROCESSING ON BLUEBERRY ANTHOCYANINS AND POLYPHENOLICS: COMPARISON OF TWO PRETREAMENTS.

Jungmin Lee, Robert W. Durst, and Ronald E. Wrolstad

Department of Food Science and Technology
Oregon State University, Corvallis, OR 97331-6602

Publication in Journal of Food Science
525 W. Van Buren, Ste 1000, Chicago, IL 60607
2002, 67:1660-1667

ABSTRACT

Frozen blueberries (*Vaccinium corymbosum* L. cv. Rubel) were pilot-plant-processed into juice and concentrate: 2 treatments (heat and SO_2) and a control. Pressed juice yield ranged from 74 to 83% (w/w), but only 13-23% of the anthocyanins and 36 - 39% of the polyphenolics were recovered in the pasteurized juice. A substantial amount of anthocyanins and polyphenolics (> 42% and > 15%, respectively of the starting material) were present in the presscakes. Measurements of total and individual flavonoids showed a great loss after the initial processing steps (thawing, crushing, and depectinization). Overall anthocyanin levels were higher in treated samples after each processing step, but polyphenolic levels remained similar to those in control.

INTRODUCTION

Anthocyanins and polyphenolics are secondary metabolites produced by the plant. They are a very diverse group of compounds (> 4000 flavonoids from plants have been identified). These compounds are important in the quality of food for their contribution to appearance, taste and health benefits (Strack and Wray, 1994).

There is intense interest in the possible health benefits of blueberries and blueberry products, because of their high antioxidant capacity, which is highly correlated to their anthocyanin and total phenolic content (Kalt and Dufour, 1997; Prior et al., 1998; Kalt et al., 2000). While there have been numerous investigations in measuring the antioxidant capacity of different *Vaccinium* species (Wang et al., 1996; Heinonen et al., 1998; Prior et al., 1998; Velioglu et al., 1998; Kalt et al., 1999b, 2000; Martín-Aragón et al., 1999; Miller et al., 2000; Smith et al., 2000; Wang and Jiao, 2000; Ehlenfeldt and Prior, 2001), less attention has been given to compositional changes

resulting from processing (Fuleki and Hope, 1964; Van Teeling et al., 1971; Amakura et al., 2000; Skrede et al., 2000).

Flavonoid degradation in blueberries and their processed products are serious problems. Along with the degradation of important micronutrients, color loss is also a significant concern. Studies indicate that native enzyme polyphenol oxidase (PPO, which is mainly located in the cytoplasm), anthocyanins and polyphenolics (predominantly located in the vacuole) undergo significant degradation when the fruit is processed. Researchers have proposed that native blueberry PPO oxidizes polyphenolics to quinones, which subsequently react with anthocyanins to produce brown pigments (Kader et al., 1997, 1998, 1999; Jiménez and García-Carmona, 1999).

Heating has been shown to inhibit native enzyme activity (Kader et al., 1997; Skrede et al., 2000). The addition of sulfur dioxide (SO_2) has also been effective in inhibiting the activity of PPO (Montgomery et al., 1982; Sayavedra-Soto and Montgomery, 1986; Sapers, 1993). Bakker et al., (1998) have shown an increase in anthocyanin extraction when making red table wine by increasing the amount of SO_2 added.

Previous work done by Skrede et al., (2000) demonstrated the pronounced deterioration of phenolic compounds in highbush blueberries when frozen blueberries were processed into juice and concentrate. The objective of this study was to determine the effectiveness of initial heat, and sulfur dioxide treatments, to improve blueberry juice color quality. Anthocyanin and polyphenolic substances were measured from samples taken after each step in the processing of berries into pasteurized juice and, finally, into concentrate.

MATERIALS AND METHODS

Plant material

Flow frozen highbush blueberries (*Vaccinium corymbosum* L. cv. Rubel) grown by Steve Erickson Farms (Salem, OR) were provided by the Oregon Blueberry Commission. The frozen berries were stored at –23 °C until juice processing. 'Rubel' is one of 3 wild highbush blueberries native to North America that have contributed to present-day cultivars (Hancock and Siefker, 1982).

Reagents and standards

Cyanidin-3-glucoside and quercetin-3-glucoside were obtained from Extrasynthèse (Genay, France). Potassium metabisulfite, caffeic acid, ferulic acid, *o*-coumaric acid, rutin (quercetin-3-rutinoside), chlorogenic acid, vanillic acid, syringic acid, and *p*-hydroxybenzoic acid were obtained from Sigma Chemical Co. (St. Louis, MO). The pectinase (Pectinex Smash Batch 1284210L) used in the juice processing was from Novo Nordisk Ferment Ltd (Dittingen, Switzerland). All solvents used in this investigation were HPLC grade.

Juice processing

The procedure for blueberry juice and concentrate processing is shown in Figure 3.1. Blueberries were processed into juice at the Oregon State University Department of Food Science and Technology pilot plant. There were 3 groups: initial heat treatment, SO_2 treatment, and no treatment (control). Control fruits were thawed, crushed, depectinized, pressed, clarified, pasteurized, and concentrated. The heat treatment procedure differed only in that the thawed blueberries were immediately heated to 95

°C for 2 min, and then cooled to 38 °C. SO_2 treatment differed from the control by the addition of potassium metabisulfite ($K_2S_2O_5$) during thawing and crushing (free SO_2 = 100 ppm). All 3 trials were replicated 2 times. Approximately 20 kg of frozen blueberries were used for each trial. Frozen blueberries were thawed in a steam kettle equipped with stirrer until room temperature was reached. Thawed berries were crushed with a Stephan Vertical Cutter Mixer, set at 30 s and slow speed. Pectinase (0.0827 mL/kg) was then added, as it is most active at 27 °C. Negative alcohol precipitation test was used as an indication of depectinization. After depectinization, rice hulls (1%) were added to aid in juice pressing. Then the depectinized crushed berries were pressed in a Willmes bag press (Type 60; Moffet Co., San Jose, CA). The maximum pressure applied was 5.0 bar. Pressed juice was clarified using a continuous centrifuge (model KA2 separator; Westfalia, San Leandro, CA). Due to the small amount of pressed juice, a filter unit was not used. Clarified juice was pasteurized (90 °C for 90 s) with a APV-Crepaco high temperature short-time (HTST) unit, type "junior", APV-Crepaco Inc. (Tonawanda, NY). Pasteurized juice was concentrated by a Centri-Therm centrifugal evaporator (model CT-1B; Alfa-Laval Inc., Newburyport, MA) operating at 40 °C. Pasteurized juice samples were concentrated to 65 - 70 °Brix. °Brix measurements were made with an Auto Abbe refractometer model 10500 (Reichert-Jung, Leica Inc., Buffalo, NY) in % solids and temperature-controlled mode. Samples were taken from each step of juice processing after crushing for analysis. Samples taken were stored at –23 °C until analysis.

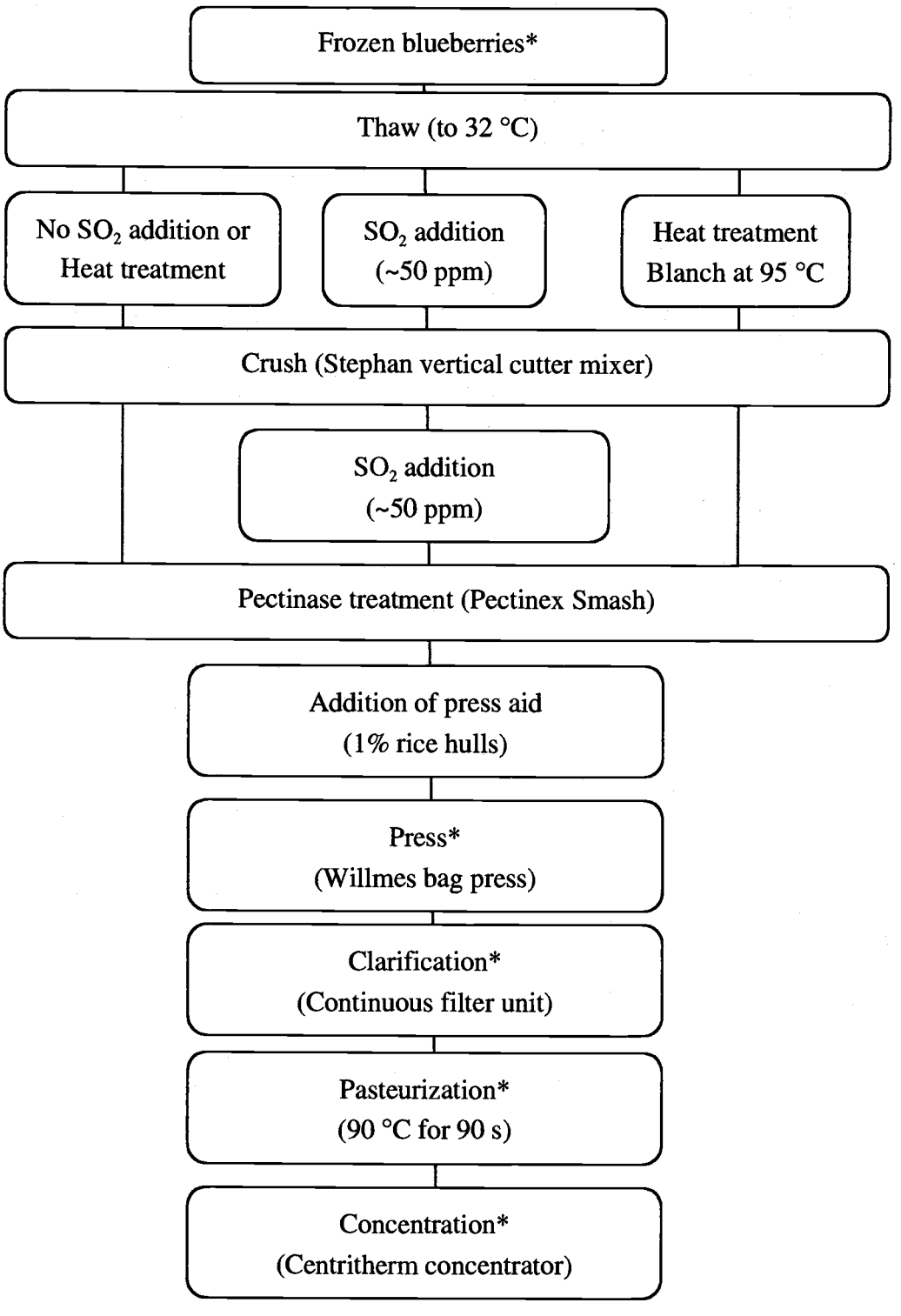


Figure 3.1. Blueberry juice and concentrate processing. (*: samples taken for analysis).

Pigment extraction

Frozen blueberry and presscake sample extractions were performed following the procedure described by Rodriguez-Saona and Wrolstad (2001). Samples were liquid nitrogen powdered using a stainless steel Waring Blender. Twenty-five g of powdered sample was blended with 50 mL of acetone and filtered with a Buchner funnel and Whatman #1 filter paper. The filter cake was re-extracted with 70% acetone until a clear filtrate was obtained. Filtrates were combined in a separatory funnel with chloroform (1:2 acetone:chloroform v/v) and stored overnight at 1 °C. The aqueous portion was collected, and residual acetone was evaporated by a Büchi rotovapor at 40 °C. The extract was dissolved to a final volume of 150 mL with distilled water. The samples for anthocyanin quantification by high-performance Liquid Chromatography (HPLC) were filtered through a 0.45-μm Millipore filter (Type HA for HPLC samples) prior HPLC injection. The extractions for the berries and presscakes were replicated 2 times.

Total monomeric anthocyanin and % polymeric color content of samples

Total monomeric anthocyanin pigment (ACY) contents of berries, pasteurized juice and concentrate were determined by using the pH differential method (Giusti and Wrolstad, 2001). Absorbance was measured at 510 and 700 nm. ACY was calculated using cyanidin-3-glucoside coefficients (molar extinction coefficient of 26,900 L cm^{-1} mol^{-1} and molecular weight of 449.2 g mol^{-1}).

Percent polymeric color contents of these samples were determined by the bisulfite bleaching method (Giusti and Wrolstad, 2001). Absorbance was measured at 420, 510, and 700 nm. A Shimadzu 300 UV-visible spectrophotometer and a 1-cm-

pathlength cell were used for both measurements. Determination of ACY and % polymeric color were replicated 2 times.

Anthocyanin and polyphenolic sample preparation

Anthocyanin and polyphenolic purification were performed as described in Giusti and Wrolstad (1996). The aqueous extract was passed through a C-18 Sep-Pak mini-column (Waters Associates, Milford, MA), rinsed with methanol, and activated with deionized water.

Anthocyanins. Anthocyanins were absorbed onto the column while other flavonoids, sugars, and acids were removed. Anthocyanins were collected with acidified (0.01% HCl) methanol, and samples for ES-MS analysis were then filtered through a 0.45-μm Millipore filter (type HV) before injection. For HPLC samples, the methanol was evaporated using a Büchi rotovapor at 40 °C. The pigments were re-dissolved in 2 mL acidified water (0.01% HCl) and filtered through a 0.45-μm Millipore filter (type HA) prior injection.

Polyphenolics. The polyphenolic fraction was obtained by eluting 2 mL of ethyl acetate. Ethyl acetate was removed from the fraction with the Büchi rotovapor at 40 °C, then the residue was re-dissolved in 2 mL deionized water. Again, samples were filtered through a 0.45-μm Millipore filter (type HA) before HPLC injection.

Alkaline hydrolysis

Anthocyanins. The methanolic anthocyanin fraction was obtained as described previous. The methanol was evaporated and the fraction was redissolved in 10 mL of 10% KOH. Saponification was carried out in a screw-cap test tube at room temperature

(23 °C) for 8 min. The solution was then neutralized with 2 N HCl. The hydrolysate was passed through a C-18 Sep-Pak mini-column and purified as described above for subsequent HPLC analysis.

Phenolics. Sugars and acids were removed using a C-18 Sep-Pak mini-column as described previous. Ethyl acetate was evaporated and the subsequent alkaline hydrolysis was performed as described for anthocyanins.

HPLC separation of anthocyanins and polyphenolics

Anthocyanins and polyphenolics were separated by reversed-phase HPLC using a Perkin-Elmer Series 400, equipped with a Hewlett-Packard 1040A photodiode array detector. Absorbance spectra were recorded for all peaks. Flow rate was 1 mL/min and injection volume was 50 μL.

Anthocyanins. A Prodigy 5 μm ODS (3) 100A (250 × 4.6 mm) column from Phenomenex was used (Torrance, CA). Solvent A was 100% acetonitrile and solvent B was 4% phosphoric acid. All solvents were HPLC grade. The program used a linear gradient from 6% to 25% solvent A, in 55 min with simultaneous detection at 280, 320, and 520 nm. Anthocyanins were quantified as cyanidin-3-glucoside by the external standard method (Durst and Wrolstad, 2001).

Polyphenolics. A Supelcosil column (5 μm) 250 × 4.6 mm i.d. (Supelco Inc., Bellefonte, PA), fitted with a 10 × 4.6 mm i.d. Spherisorb ODS-2 micro-guard column (Alltech, Deerfield, IL) was used. Solvent A was 100% acetonitrile, solvent B was 100% methanol, and solvent C was 0.07 M KH_2PO_4 (adjusted to pH 2.4 with concentrated H_3PO_4). The initial solvent composition was 10% solvent B and 90% solvent C; then a linear gradient of 10% to 22% solvent B, and 90% to 78% solvent C in

10 min; then a linear gradient of solvent A from 0% to 25%, solvent B constant at 22%, and solvent C from 78% to 53% in 25 min; then isocratic for 10 min. Detection occurred at 260, 280, 320, and 520 nm. Flavonol-glycosides were quantified as rutin (quercetin-3-rutinoside) and cinnamates were quantified as chlorogenic acid by the external standard method.

Electrospray mass spectroscopy (ES-MS) of anthocyanins

Analysis was performed using a Perkin-Elmer SCIEX API III+ mass spectrometer, equipped with an ion spray source (ISV = 4700, orifice voltage = 80) and positive ion mode with loop injection. Purified and filtered anthocyanins in acidified methanol (5 μL) were injected directly into the system.

Determination of total sulfur dioxide (SO_2)

Total SO_2 concentrations were measured in juice and concentrate samples, after dilution to single-strength, by the Ripper method with a redox electrode (Zoecklin et al., 1995). The redox electrode was from SensoreX (#S500-ORP BNC, Stanton, CA) used with a Corning pH meter 320 (Corning, New York, NY) in mV mode.

Color measurements

Measurements were made with a HunterLab CT1100 ColorQuest colorimeter (Hunter Associates Laboratories Inc., Reston, VA). The colorimeter was set to measure total transmittance with Illuminant C and 10° observer angle. Pasteurized juice and concentrate samples were diluted to 10 °Brix (standard single-strength) with distilled water. An optical glass cell (Hellma, Borough Hall Station, NY) with a pathlength of

0.25 cm was used to measure the samples. Three color parameters were recorded: Hunter CIE L* (lightness), chroma (saturation, C), and hue angle (color itself, h).

Statistical analysis

The significant difference among control, heat treatment and SO_2 treatment were determined at 95% level using the Tukey test of means. SAS statistical software (SAS systems for Windows, released 6.11, SAS Institute, Inc., Cary, NC 1996) was used.

RESULTS AND DISCUSSION

Characterization of blueberry anthocyanins

The total monomeric anthocyanin content of 'Rubel' blueberries was 192.4 mg/100 g determined by the pH differential method as cyanidin-3-glucoside (ε = 26,900 and MW = 449.2). This was lower than the values of 235.4 and 220 mg/100 g reported for 'Rubel' berries by Prior et al., (1998, 2001), as well as 325 mg/100 g reported by Ehlenfeldt and Prior (2001).

The separation of anthocyanins by HPLC are shown in Figure 3.2. Peak assignments were made according to their ultraviolet (UV)-visible spectra and retention time. Electrospray mass spectroscopy (ES-MS) was used to confirm peak identification. There were 13 peaks in 'Rubel' blueberries (in the HPLC elution order and the values in parentheses are the ES-MS mass/charge ratio) are as follows; delphinidin-3-galactoside (465.2), delphinidin-3-glucoside (465.2), cyanidin-3-galactoside (449.2), delphinidin-3-arabinoside (435.0), cyanidin-3-glucoside (449.2), petunidin-3-galactoside (479.2), cyanidin-3-arabinoside (419.0), petunidin-3-glucoside (479.2), peonidin-3-galactoside (463.2), petunidin-3-arabinoside (449.0), malvidin-3-galactoside (493.2), malvidin-3-

glucoside (493.2), and malvidin-3-arabinoside (463.0). There were no acylated anthocyanins (saponification did not eliminate peak 13, possibly a polymerized anthocyanin) detected by HPLC. Mass spectra data also showed no indication of acylated pigments, though Gao and Mazza (1994) reported the presence of acylated anthocyanins in highbush blueberries. Malvidin and delphinidin derivatives (77.2% of the constituents) were the major anthocyanins present in 'Rubel', which agrees with reported anthocyanin contents of *V. corymbosum* (Ballington et al., 1987; Kader et al., 1996; Kalt et al., 1999a).

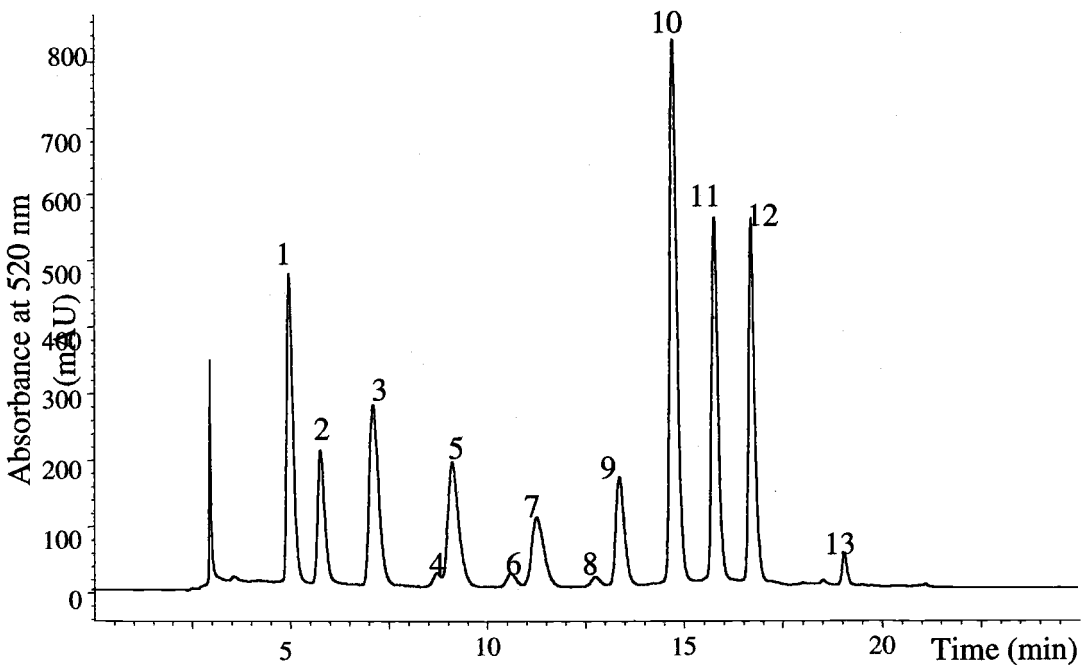


Figure 3.2. Anthocyanin HPLC profile of 'Rubel' blueberries. 1: delphinidin-3-galactoside, 2: delphinidin-3-glucoside, 3: cyanidin-3-galactoside & delphinidin-3-arabinoside, 4: cyanidin-3-glucoside, 5: petunidin-3-galactoside, 6: cyanidin-3-arabinoside, 7: petunidin-3-glucoside, 8: peonidin-3-galactoside, 9: petunidin-3-arabinoside, 10: malvidin-3-galactoside, 11: malvidin-3-glucoside, 12: malvidin-3-arabinoside, & 13:unknown.

Changes in anthocyanin composition during juice processing

Table 3.1 shows the change in total and individual anthocyanidin-glycosides of each sample from the 3 juice and concentrate processing trials. The starting material (frozen blueberries) had 130.8 mg anthocyanins/100 g of berries (from HPLC), which agrees with previous reports. Gao and Mazza (1994) reported that highbush blueberries had an average of 110 - 260 mg of anthocyanins/100 g of fresh berries; and Mazza and Miniati (1993) reported a wide range of total anthocyanins (25 - 495 mg/100 g) for highbush blueberries. Pigment contents of the samples taken from the processing steps were expressed as mg of anthocyanins/100 g of initial frozen fruit.

Pressed juice. The primary steps of processing (thawing, crushing, depectinization, and pressing) contributed to a large loss in total anthocyanins, which was also reported by Skrede et al., (2000). The crushed berries were visually brown. Pressed juice yields ranged from 75 - 83% (w/w). If no degradation occurred during juice processing, pressed juice samples would have in the order of 59.0 - 75.5 mg/100 g of the starting materials' anthocyanins, as 55.3 - 71.8 mg/100 g remained in the presscake. However, less than 22% (6.1 - 28.8 mg/100 g) of the frozen berries' anthocyanins were present in the pressed juices.

This low recovery of anthocyanins indicated degradation of the compounds. Control pressed juice had the lowest recovery, with significantly less (< 25%) anthocyanins than the heat-treated or SO_2-treated pressed juices ($p < 0.05$). Anthocyanins were not efficiently extracted into the pressed juice, which had a negative impact on juice quality.

Table 3.1. Total anthocyanin content in the different samples during the 3 juice processing trials (C: control, H: heat treatment, and S: SO_2 treatment)[1]

	Fruit	Pressed juice			Clarified juice		
		C	H	S	C	H	S
Delphinidin-glycosides	33.7	-	5.9	3.2	0.3	5.3	3.8
Cyanidin-glycosides	1.9	-	0.5	0.4	-	0.4	0.5
Petunidin-glycosides	25.7	0.2	4.9	3.4	0.1	4.5	3.8
Peonidin-glycosides	0.7	-	0.2	0.1	-	0.1	0.1
Malvidin-glycosides	67.3	5.6	16.9	17.2	5.5	15.7	17.6
Unknown	1.5	0.2	0.3	0.3	0.2	0.3	0.4
Total[2]	130.8	6.1[a]	28.8[b]	24.7[b]	6.1[a]	26.4[b]	26.2[b]
SD	7.4	2.4	1.7	1.6	3.5	0.6	2.2

	Pasteurized juice			Concentrate			Presscake		
	C	H	S	C	H	S	C	H	S
Delphinidin-glycosides	1.3	5.9	7.0	1.2	5.8	4.1	15.0	16.3	16.7
Cyanidin-glycosides	0.4	0.5	0.5	0.3	0.5	0.5	1.1	1.0	1.2
Petunidin-glycosides	1.9	5.0	4.1	1.9	5.0	4.1	13.7	11.3	14.5
Peonidin-glycosides	-	0.2	0.1	-	0.2	0.1	0.4	0.4	0.4
Malvidin-glycosides	12.7	17.9	18.2	12.8	17.4	17.9	40.6	25.8	34.4
Unknown	0.3	0.4	0.4	0.3	0.5	0.4	1.0	0.6	0.8
Total[2]	16.6[a]	29.8[a]	30.4[a]	16.5[a]	29.3[a]	27.2[a]	71.8[a]	55.3[a]	68.0[a]
SD	0.1	2.4	7.2	2.4	4.1	2.2	7.4	14.5	7.6

[1]:Units are mg of anthocyanin/100 g of berries (starting material).

[2]:Total with different lower case letters were significantly different ($p < 0.05$). To obtain single-strength 10 °Brix of juice samples, multiply by 0.618. To obtain 65 °Brix for concentrate, multiply value by 4.02.

Clarified juice. After clarification, heated and SO_2-treated juices had 4 times the anthocyanin content of control clarified juice ($p < 0.05$). There was approximately 8% loss of anthocyanins from pressed juices to clarified juices, less than 20% of the frozen fruit anthocyanins remained in any of the clarified juices.

Pasteurized juice. Heated and SO_2-treated pasteurized juices had 1.8 times the anthocyanin (29.8 and 30.4 mg/100 g, respectively) content of the control pasteurized juice (16.6 mg/100 g). There was greater than 76% loss of anthocyanins in the pasteurized juices compared to the frozen fruit. Control pasteurized juice had a higher amount of anthocyanins than the initial pressed juice. This may be due to enzymatic losses in the frozen samples during subsequent storage and thawing. Losses were observed with SO_2 and heat-treated samples, but not to the same extent as control samples. Twenty-five - 35% (32.4 - 45.7 mg/100 g) of the frozen berries' anthocyanins was degraded (or not accounted for in the final pasteurized juices and presscakes). Skrede et al., (2000) demonstrated that this degradation is mainly due to native enzymes present in the blueberry fruit and not due to the processing enzymes used.

Concentrate. There was less than a 10% loss of anthocyanins from the concentration step. Anthocyanin contents of heat-treated and SO_2-treated (29.3 mg/100 g and 27.2 mg/100 g, respectively) concentrate samples were greater than the control concentrate (16.5 mg/100 g). However, the values were not significantly different ($p < 0.05$).

Presscake. As mentioned earlier, the presscake residues contained a substantial amount of anthocyanins, 55.3 - 71.8 mg/100 g, or 42 - 55% of the frozen berries' anthocyanins. Control presscake contained the greatest amount of anthocyanins (71.8 mg/100 g), while heat treatment presscake had the least (55.3 mg/100 g). Heating the

berries may have contributed towards a breakdown of the skins, which could have increased extraction of the anthocyanins, but still a substantial amount of anthocyanins remained in the presscake. The actual anthocyanin content of the presscakes could feasibly have been higher; any residual enzyme activity of the presscake may have degraded anthocyanins prior to analysis. The presscakes were not treated before storage to stop the activity of the native enzymes or the processing enzymes.

The presscake could be a source of anthocyanins for natural pigments and nutraceuticals (natural antioxidant source). Re-extracting the presscakes or crushing the frozen berries into fine particles may aid in higher extraction of anthocyanins into the final product. As presscake is mainly composed of fruit skins, which are high in pigment, blueberry varieties with greater anthocyanin content in their flesh (such as *V. membranaceum*) may yield higher levels of anthocyanins in their processed juices. The importance of anthocyanins remaining within the presscakes is quite significant, as Kalt et al., (2000) contend that antioxidant capacity in blueberries is mainly due to anthocyanins, although other phenolics will also contribute to antioxidant activities. They also reported that the oxygen radical absorbing capacity (ORAC) can be predicted by simple colorimetric tests (pH differential test for anthocyanins and Folin-Ciocalteu test for phenolics).

Montgomery et al., (1982) have reported that heating crushed Concord grapes helped increase anthocyanin extraction, and reduced polymeric color in the final juice samples. Studies performed by Kalt et al., (2000) also indicate a high recovery of anthocyanins (and antioxidant capacity) in extracts of lowbush blueberry puréed at high temperatures (60 °C). They had a greater loss in these compounds, however, during storage at 20 °C, when compared to blueberries that were puréed at 25 °C.

Kader et al., (1997) demonstrated that PPO activity plays a dominant role in enzymatic browning of blueberry anthocyanins, and that the addition of chlorogenic acid (major phenolic acid present in blueberries) stimulated the degradation of anthocyanins. Cyanidin-3-glucoside did not degrade in the presence of PPO alone, but by adding chlorogenic acid the anthocyanin deteriorated (Kader et al., 1998). The addition of heat-inactivated PPO to the anthocyanins showed no degradation. Degradation of blueberry anthocyanins present in a juice system was shown by Skrede et al., (2000) by the addition of blueberry pulp (endogenous enzymes present in the fruit would be present in the pulp).

Anthocyanin profile changes from processing

The proportion of malvidin-glycosides increased with the initial pressing compared to the fresh fruit. Malvidin-glycosides in the berry fruit were 51% of total anthocyanins and increased to 60-77% in pasteurized juices and concentrates. There was a concomitant decrease in delphinidin and petunidin-glycosides. Skrede et al., (2000) reported a considerable decrease in the proportion of delphinidin-glycosides; in this study that was true with the control (8%) but not with heat-treated (20%) and SO_2–treated (23%) pasteurized juices (fruit was 26%). Only 1.3 - 7.0 mg/100 g of delphinidin-glycosides remained in the pasteurized juice, compared to the fresh fruit delphinidin-glycosides content (33.7 mg/100 g). The proportion of cyanidin-glycosides increased in the pasteurized juice (control - 2.4%, heat - 1.7%, and SO_2 - 1.6%) and concentrate (control - 1.8%, heat - 1.7%, and SO_2 - 1.8%) compared to the fruit (1.5%). The anthocyanin profile changed only slightly during the concentration step.

Total monomeric anthocyanin, % polymeric color, and color measurements

Control-pasteurized juice and concentrate were higher in polymeric anthocyanins than heated and SO_2-treated juice and concentrates (Table 3.2). Total anthocyanin contents of pasteurized juices and concentrates obtained by pH differential were higher (1.5 to 1.9 times higher) than the results from HPLC, but provided the same general trend. The difference in results may be partially explained by anthocyanins spectral characteristics being influenced by the differing solvent systems utilized for HPLC and pH differential. Additionally, determination of total anthocyanins by HPLC was a summation of individual peaks at 520 nm, while pH differential measured the difference in sample absorbance of pH 1.0 and pH 4.5 at 510 nm. It is also possible that the polymeric pigments present in the samples were retained in the HPLC column and not included in HPLC measurements, whereas they might have contributed to the pH differential results.

Table 3.2 shows the color measurements performed on the juice and concentrate samples. The L* values ranged from 23.3 to 36.4 for the pasteurized juices, and from 23.6 to 37.4 for concentrates. The heat-treated samples were darker (smaller L* values) than the control and SO_2-treated samples. Hue angles (h) ranged from 10.9 to 17.7 and chroma (C) ranged from 43.1 to 49.9. All pasteurized juices had a higher hue angle and chroma than their concentrates. Both treated pasteurized juice samples had a more intense color (higher chroma) than control pasteurized juice. The concentration step shifted the pasteurized juice samples from red towards red-purple (shift in hue angle) for the 3 different processes.

Table 3.2. Total monomeric anthocyanin content, % polymeric color, color measurements and actual °Brix of pasteurized juice and concentrate samples.

	Frozen fruit	Control		Heat treatment		SO_2 treatment	
		Pasteurized juice	Concentrate	Pasteurized juice	Concentrate	Pasteurized juice	Concentrate
Total monomeric anthocyanin[1] (mg /100 g)	192.4	31.9	31.8	50.3	46.6	46.7	46.5
% Polymeric color	37.6	50.4	54.3	40.7	40.9	42.1	38.8
Color measurements[2]							
L*		36.4	36.3	23.3	23.6	34.1	37.4
C		44.0	43.1	49.3	48.8	49.9	47.3
h		15.4	13.2	17.7	15.5	12.8	10.9
Actual °Brix		14.2	70.8	15.0	71.3	13.5	66.8

[1]Total monomeric anthocyanin content was expressed as cyd-3-glu, ε = 26,900 L cm^{-1} mol^{-1}, and MW = 449.2 g mol^{-1}. Units are mg /100 g of juice processing staring material. To obtain single-strength 10 °Brix standard for juice samples, multiply by 0.618. To obtain 65 °Brix for concentrate, multiply value by 4.02.

[2]All samples were diluted to 10 °Brix. Hunter colorimeter was used (Illuminant C, 10° observer angle, and 0.25 cm cell were used).

SO_2 levels and pH

There was 8.4 ppm of total SO_2 present in the single-strength SO_2-treated pasteurized juice, and 8.1 ppm present in the single-strength concentrate, which is below the maximum level of SO_2 (10 ppm) for labeling (Title 21, U.S. Code of Federal Regulations 101.100).

From all 3 treatments, pasteurized juice samples had a pH of 3.92 - 4.07. The diluted concentrate samples (diluted to 13.5 °Brix) had a pH of 3.84 - 4.09.

Characterization of blueberry polyphenolics

The HPLC chromatogram of the ethyl acetate fraction revealed chlorogenic acid (strong 320 nm absorber) and 3 flavonol-glycosides (quercetin-3-glucoside and 2 other flavonol-glycosides, strong 260 nm absorber). Chlorogenic acid was the major cinnamic acid present (65% of polyphenolics) in 'Rubel' blueberries.

Figure 3.3 shows the HPLC separation of the saponified ethyl acetate fraction monitored at 260, 280, and 320 nm. Alkaline hydrolysis of the ethyl acetate fraction revealed *p*-hydroxybenzoic acid, vanillic acid, chlorogenic acid, caffeic acid (released from chlorogenic acid), syringic acid, ferulic acid, *o*-coumaric acid, quercetin-3-glucoside, and 2 other flavonol-glycosides. Other researchers (Skrede et al., 2000; Prior et al., 2001) have reported the presence of catechin and epicatechin in highbush blueberries. Catechin and epicatechin were not detected in these 'Rubel' extracts.

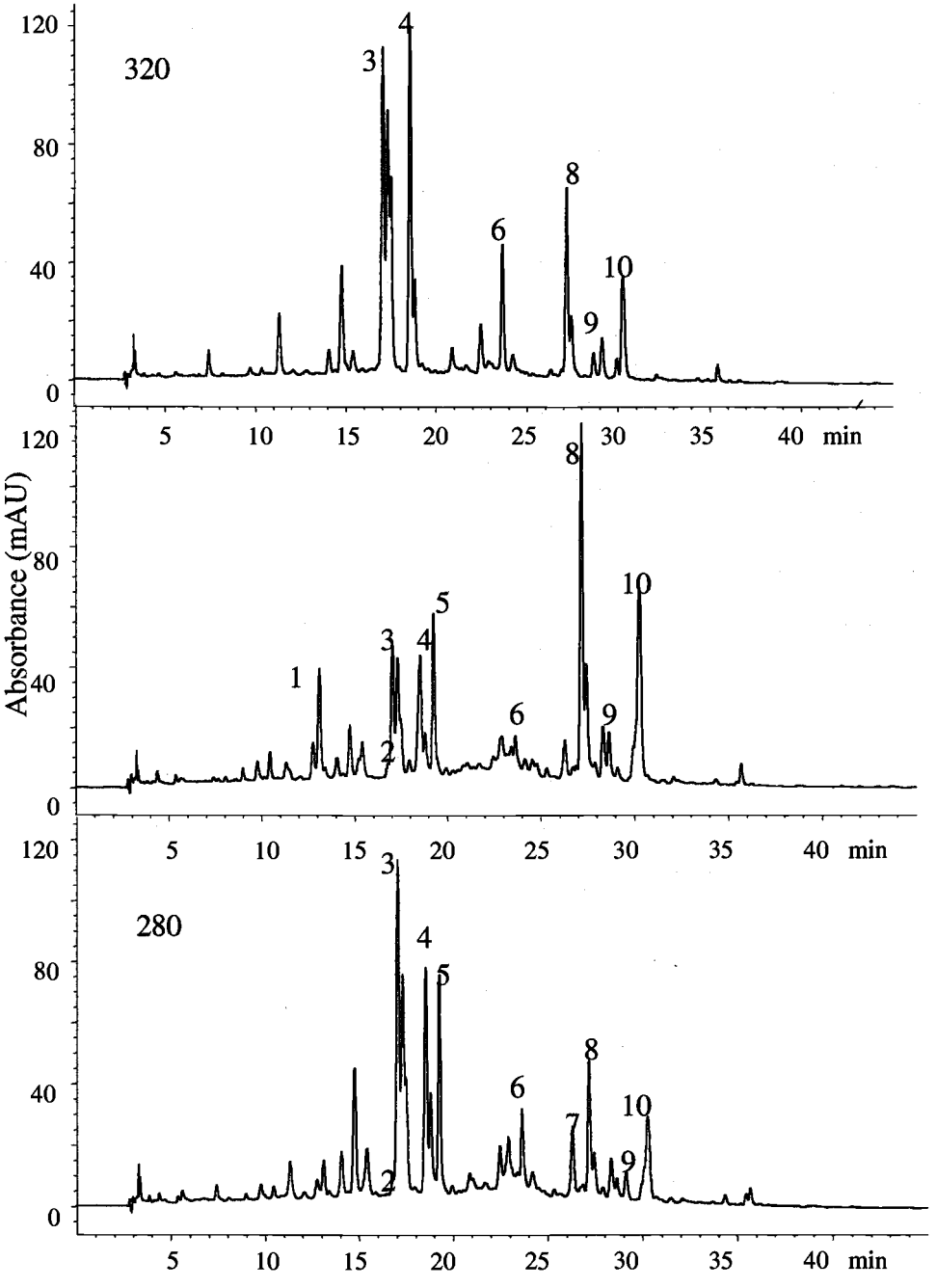


Figure 3.3. HPLC chromatogram of the saponified ethyl acetate fraction (polyphenolics). 1:*p*-hydroxybenzoic acid, 2:vanillic acid, 3:chlorogenic acid, 4:caffeic acid, 5:syringic acid, 6:ferulic acid, 7:*o*-coumaric acid, 8:quercetin-3-glucoside, 9:flavonol-glycoside, and 10:flavonol-glycoside.

Peak assignments were made according to the UV-visible spectra, retention time, and co-chromatography with authentic standards. Several workers have reported chlorogenic acid as the major cinnamic acid present, and also the greatest contributor of total polyphenolic content in highbush blueberries (Schuster and Hermann, 1985; Gao and Mazza, 1994; Kader et al., 1996; Kalt and McDonald, 1996; Häkkinen et al., 1999; Kalt et al., 2000; Skrede et al., 2000). Quercetin- and kaempferol-glycosides were also detected. Quercetin-3-glucoside was the major flavonol-glycoside present. Azar et al., (1987), Kader et al., (1996), and Häkkinen et al., (1999) reported quercetin-3-rhamnoside, quercetin-3-galactoside, quercetin-3-glucoside, kaempferol-3-glucoside, myricetin, caffeic acid and quinic acid (released from chlorogenic acid), chlorogenic acid, *p*-coumaric acid, ferulic acid, syringic acid, gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, *m*-hydroxybenzoic acid, vanillic acid, *m*-coumaric acid, and *o*-coumaric acid present in bilberries using HPLC, TLC, acid hydrolysis, and alkaline hydrolysis. Prior et al., (2001) noted the presence of B_1 through B_8 procyanidin oligomers and monomers corresponding to (+)-catechin and (-)-epicatechin.

The concentration of polyphenolics was found to be 175.8 mg/100 g of frozen berries. Prior et al., (1998) found 390.5 mg of polyphenolics/100 g of fresh berries (expressed as gallic acid equivalents) in 'Rubel' berries grown in Michigan, by the Folin- Ciocalteu method. Ehlenfeldt and Prior (2001) reported 'Rubel' to have 1.65 mg of total phenolics/g of fresh weight (expressed as gallic acid equivalents). Gao and Mazza (1994) reported that 50 - 100 mg/100 g of chlorogenic acid was present in lowbush and highbush blueberries, and Wang and Jiao (2000) found chlorogenic acid to be an active antioxidant. Prior et al., (2001) reported the total procyanidin content of

'Rubel' was 6 μg/g (dry weight basis), while Skrede et al., (2000) found 10 mg of procyanidins/100 g of frozen Bluecrop blueberries.

Changes in polyphenolics during juice processing

For simplicity, the individual compounds were summed as cinnamic acids and flavonol-glycosides. Table 3.3 shows the changes in the content of polyphenolics from each sample during the 3 juice processing trials. The starting material (frozen berries) had 175.8 mg/100 g of polyphenolics.

Table 3.3. Total polyphenolics content in the different samples during the 3 juice processing trials (C: control, H: heat treatment, and S: SO_2 treatment)[1]

		Cinnamic acids	Flavonol-glycosides	Total[2]	SD
Fruit		114.9	60.9	175.8	1.6
Pressed juice	C	37.5	23.2	60.7[a]	10.1
	H	40.8	29.0	69.7[a]	2.3
	S	38.1	26.0	64.1[a]	2.9
Clarified juice	C	40.1	26.9	66.9[a]	7.0
	H	38.5	30.0	68.5[a]	3.7
	S	38.6	26.2	64.8[a]	6.8
Pasteurized juice	C	39.5	23.7	63.2[a]	6.1
	H	39.4	28.2	67.6[a]	3.9
	S	40.4	28.4	68.8[a]	10.7
Concentrate	C	39.3	22.1	61.4[a]	6.5
	H	39.2	28.0	67.2[a]	2.7
	S	40.7	25.7	66.5[a]	12.6
Presscake	C	14.4	21.5	35.9[a]	5.8
	H	17.4	16.6	34.0[a]	1.2
	S	11.1	16.1	27.3[a]	3.0

[1]Units are mg of polyphenolics/100 g of berries (starting material).

[2]Total with different lower case letters were significantly different ($p < 0.05$). To obtain single-strength 10 °Brix for juice samples, multiply by 0.618. To obtain 65 °Brix for concentrate, multiply value by 4.02.

There was considerable loss from the initial steps of processing: 60 - 65% of the blueberries' polyphenolics was lost during thawing, crushing, and pressing. Similar results were also reported by Skrede et al., (2000). The polyphenolic content of samples taken at each step of heat and SO_2 treatments were not significantly different from the control ($p < 0.05$), which was an unexpected result. A possible explanation is that native enzymes (such as peroxidase), in addition to PPO, may also have degraded anthocyanins and, to a lesser extent, polyphenolics. Control pressed juice and SO_2-treated pressed juice had a lower value of polyphenolics than their pasteurized juices, but this was not the case for heat treatment. The presscakes held 15 - 20% of the frozen berries' polyphenolics, which is less than the proportion of anthocyanins left in the presscakes. Thirty-six to 39% of the polyphenolics in the berries was present in the pasteurized juice. Less than 3% of the polyphenolics in the pasteurized juices were lost during concentration. Forty-two to 45% (74.2 - 79.7 mg/100 g) of the frozen berries polyphenolics was lost during juice processing and not accounted in the final pasteurized juices and presscakes.

Cinnamic acids. The identified cinnamic acid's peaks were 65.4% of the total peak area in fresh fruit measured at 320 nm. Thirty-three to 36% of the frozen berries cinnamic acids was extracted into the pressed juice. There were only slight changes in the subsequent juice processing steps. The pasteurized juice contained only 34 to 35% of the frozen berries' cinnamic acid. The presscakes held 10 - 15% of the original cinnamic acid content.

Flavonol-glycoside. The identified flavonol-glycoside's peaks represented 25.7% of the total peak area in fresh fruit at 260 nm. Thirty-eight to 48% of the frozen

berries flavonol-glycosides was extracted into the pressed juice. There were minor changes in the flavonol-glycoside content of samples following the pressing step. Thirty-nine to 47% of the 'Rubel' flavonol-glycosides was in the pasteurized juice. Presscakes contained 26 - 35% of the starting material's flavonol-glycoside content.

Häkkinen et al. (2000) reported that black currant juices made with common domestic processing procedure resulted in a considerable loss of flavonols, and that cold-pressing was more efficient than steam-extraction in recovering flavonols. Processing aggravated the progressive enzymatic or chemical oxidation of phenolic compounds.

CONCLUSIONS

There was considerable loss of anthocyanins and polyphenolics in treatments and control during thawing, crushing, depectinization, and pressing steps of juice and concentrate processing. Heat and SO_2 treatments yielded a higher anthocyanin content in each processing step, and should offer attractive attributes to traditional blueberry juice processing methods for increasing anthocyanin recovery. There were no differences in polyphenolic contents among the treatments and control. These results suggest that antioxidant activity of blueberry juice and concentrate were considerably lower than the whole blueberry fruit. There were low losses of anthocyanins and polyphenolics during the clarification and concentration steps. The presscake contained a substantial amount of anthocyanins and polyphenolics, and would be a rich source for natural antioxidants and natural colorants. These findings will be useful to processors wishing to improve the final yield of flavonoids and the antioxidant capacity in their blueberry products.

ACKNOWLEDGEMENTS

This work was supported by a research grant from the Northwest Center for Small Fruits Research (Corvallis, OR). We thank the Oregon Blueberry Commission, Rod Cook and Steve Erickson of Van Cleave Farms at Salem, OR, for donating frozen blueberries for this research. Donald A. Griffin of the Department of Environmental and Molecular Toxicology (Oregon State University, Corvallis) performed the ES-MS analysis. This is technical paper no. 11774 from the Oregon Agricultural Experiment Station.

REFERENCES

Amakura, Y., Umino, Y., Tsuji, S., and Tonogai, Y. 2000. Influence of jam processing on the radical scavenging activity and phenolic content in berries. J. Agric. Food Chem. 48:6292-6297.

Azar, M., Verette, E., and Brun, S. 1987. Identification of some phenolic compounds in bilberry juice *Vaccinium myrtillus*. J. Food Sci. 52:1255-1257.

Bakker, J., Bridle, P., Bellworthy, S.J., Garcia-Viguera, C., Reader, H.P., and Watkins, S.J. 1998. Effect of sulphur dioxide and must extraction on color, phenolic composition and sensory quality of red table wine. J. Sci. Food Agric. 78:297-307.

Ballington, J.R., Ballinger, W.E., and Maness, E.P. 1987. Interspecific differences in the percentage of anthocyanins, aglycones, and aglycone-sugars in the fruit of seven species of blueberries. J. Amer. Soc. Hort. Sci. 112:859-864.

Durst, R.W. and Wrolstad, R.E. 2001. Unit F1.3: Anthocyanins. Separation and characterization of anthocyanins by HPLC. In: Wrolstad RE, editor. Current protocols in food analytical chemistry. New York: John Wiley & Sons. p 1-13.

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. 49:2222-2227.

Fuleki, T. and Hope, G.W. 1964. Effect of various treatments on yield and composition of blueberry juice. Food Technol. 18:166-168.

Gao, L. and Mazza, G. 1994. Quantitation and distribution of simple and acylated anthocyanins and other phenolics in blueberries. J. Food Sci. 59:1057-1059.

Giusti, M.M. and Wrolstad, R.E. 1996. Red radish anthocyanins as natural red colorant for maraschino cherries. J. Food Sci. 61:688-694.

Giusti, M.M. and Wrolstad, R.E. 2001. Unit F1.2: Anthocyanins. Characterization and measurement with UV-visible spectroscopy. In: Wrolstad RE, editor. Current Protocols in Food Analytical Chemistry. New York: John Wiley & Sons. p 1-13.

Hancock, J.F. and Siefker, J.H. 1982. Levels of inbreeding in highbush blueberry cultivars. HortScience 17:363-366.

Häkkinen, S., Heinonen, M., Kärenlampi, S., Mykkänen, H., Ruuskanen, J., and Törrönen, R. 1999. Screening of selected flavonoids and phenolic acids in 19 berries. Food Res. Intl. 32:345-353.

Häkkinen, S.H., Kärenlampi S.O., Mykkänen, H.M., and Törrönen, A.R. 2000. Influence of domestic processing and storage on flavonol contents in berries. J. Agric. Food Chem. 48:2960-2965.

Heinonen, I.M., Meyer, A.S., and Frankel, E.N. 1998. Antioxidant activity of berry phenolics on human low-density lipoprotein and liposome oxidation. J. Agric. Food Chem. 46:4107-4112.

Jiménez, M. and García-Carmona, F. 1999. Oxidation of the flavonol quercetin by polyphenol oxidase. J. Agric. Food Chem. 47:56-60.

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

Kader, F., Rovel, B., Girardin, M., and Metche, M. 1997. Mechanism of browning in fresh highbush blueberry fruit (*Vaccinium corymbosum* L). Role of blueberry polyphenol oxidase, chlorogenic acid and anthocyanins. J. Sci. Food Agric. 74:31-34.

Kader, F., Haluk , J., Nicolas, J., and Metche, M. 1998. Degradation of cyanidin-3-glucoside by blueberry polyphenol oxidase: kinetic studies and mechanisms. J. Agric. Food Chem. 46:3060-3065.

Kader, F., Nicolas, J., and Metche, M. 1999. Degradation of pelargonidin 3-glucoside in the presence of chlorogenic acid and blueberry polyphenol oxidase. J. Sci. Food. Agric. 79:517-522.

Kalt, W. and McDonald, J.E. 1996. Chemical composition of lowbush blueberry cultivars. J. Amer. Soc. Hort. Sci. 121:142-146.

Kalt, W. and Dufour, D. 1997. Health functionality of blueberries. HortTechnology 7:216-221.

Kalt, W., McDonald, J.E., Ricker, R.D., and Lu, X. 1999a. Anthocyanin content and profile within and among blueberry species. Can. J. Plant Sci. 79:617-623.

Kalt, W., Forney, C.F., Martin, A., and Prior, R.L. 1999b. Antioxidant capacity, vitamin C, phenolics, and anthocyanins after fresh storage of small fruits. J. Agric. Food Chem. 47:4638-4644.

Kalt, W., McDonald, J.E., and Donner, H. 2000. Anthocyanins, phenolics, and antioxidant capacity of processed lowbush blueberry products. J. Food Sci. 65:390-393.

Martín-Aragón, S., Basabe Belén, B., Benedí, J.M., and Villar, A.M. 1999. In vitro and in vivo antioxidant properties of *Vaccinium myrtillus*. Pharm. Biol. 37:109-113.

Mazza, G. and Miniati, E. 1993. Anthocyanins in fruits, vegetables, and grains. Boca Raton, FL.: CRC Press Inc. p 87-92.

Miller, H.E., Rigelhof ,F., Marquart, L., Prakash, A., and Kanter, M. 2000. Antioxidant content of whole grain breakfast cereals, fruits and vegetables. J. Am. Coll. Nutr. 19:312S-319S.

Montgomery, M.W., Reyes, F.G.R., Cornwell, C., and Beavers, D.V. 1982. Sugars and acid analysis and effect of heating on color stability of northwest Concord grape juice. J. Food Sci. 47:1883-1885, 1891.

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 phenolic and anthocyanin 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 Spectrometry. J. Agric. Food Chem. 49:1270-1276.

Rodriguez-Saona, L.E. and Wrolstad, R.E. 2001. Unit F1.1: Anthocyanins. Extraction, isolation and purification of anthocyanins. In: Wrolstad RE, editor. Current Protocols in Food Analytical Chemistry. New York: John Wiley & Sons. p 1-11.

Sapers, G.M. 1993. Browning of foods: control by sulfites, antioxidants, and other means. Food Tech. 47:75-84.

Sayavedra-Soto, L.A. and Montgomery, M.W. 1986. Inhibition of polyphenoloxidase by sulfite. J. Food Sci. 51:1531-1536.

Schuster, B. and Herrmann, K. 1985. Hydroxybenzoic and hydroxycinnamic acid derivatives in soft fruits. Phytochem. 24:2761-2764.

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

Smith, M.A.L., Marley, K.A., Seigler, D., Singletary, K.W., and Meline, B. 2000. Bioactive properties of wild blueberry fruits. J. Food Sci. 65:352-356.

Strack, D. and Wray, V. 1994. The Anthocyanins. In: Harborne JB, editor. The flavonoids, London, UK: Chapman & Hall. p 1-22.

Van Teeling, C.G., Cansfield, P.E., and Gallop, R.A. 1971. Anthocyanin complex isolated from the syrup of canned blueberries. J. Food Sci. 36: 1061-1063.

Velioglu, Y.S., Mazza, G., Gao, L., and Oomah, B.D. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J. Agric. Food Chem. 46:4113-4117.

Wang, H., Cao, G., and Prior, R.L. 1996. Total antioxidant capacity of fruits. J Agric Food Chem. 44:701-705.

Wang, S.Y. and Jiao, H. 2000. Scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. J. Agric. Food Chem. 48:5677-84.

Zoecklin, B.W., Fugelsang, K.C., Gump, B.H., and Nury, F.S. 1995. Ch. 20. Laboratory procedures. In: Zoecklin BW, Fugelsang KC, Gump BH, Nury FS, editors. Wine analysis and production. New York: Chapman & Hall. p 493-497.

CHAPTER 4

EXTRACTION OF ANTHOCYANINS AND POLYPHENOLICS FROM BLUEBERRY PROCESSING WASTE

Jungmin Lee and Ronald E. Wrolstad

Department of Food Science and Technology
Oregon State University, Corvallis, OR 97331-6602

To be submitted to Journal of Food Science.
525 W. Van Buren, Ste 1000, Chicago, IL 60607

ABSTRACT

The effectiveness of temperature, SO_2, citric acid, and industrial juice processing enzymes (n = 9) in producing extracts of blueberries, and blueberry skins, rich in anthocyanins and polyphenolics were evaluated individually and/or in combination. Enzyme treatment had little effect on total monomeric anthocyanins (ACY), and total phenolics (TP) recovery. Various combinations of heat, SO_2 and citric acid yielded extracts with higher concentrations of ACY and TP than the control. The distribution of anthocyanins and polyphenolics in blueberries was also investigated. Anthocyanins existed almost exclusively in the skins, and polyphenolics were mostly in the skins with lesser amounts in flesh and seeds. All portions contained the same individual anthocyanins, but in varying amounts. Cinnamic acid derivatives and flavonol-glycosides were found in the skins and seeds, while the flesh contained only cinnamic acids.

INTRODUCTION

The health benefits of blueberries (*Vaccinium* sp.) have become widely accepted since Prior (1998) reported that blueberries had the highest antioxidant activity of the 42 fruits and vegetables evaluated. This finding sparked numerous investigations into the health benefits of blueberries. Several studies have confirmed blueberries to contain high antioxidant activity when compared to other fruits (Cao et al., 1996; Wang et al., 1996; Prior et al., 1998). Epidemiological studies have demonstrated that a diet rich in fruits and vegetables reduces the risk of certain types of cancer, cardiovascular, and other chronic diseases (Criqui and Ringel, 1994; Steinmetz and Potter, 1991; American

Institute of Cancer Research, 1997; Ness and Powles, 1997; Hertog et al., 1993; Hertog et al., 1994; Hertog et al., 1995).

The Pacific Northwest is a major producer of berries, fruit juices, and fruit juice concentrates. In 2002, Oregon produced 27,500,000 pounds of blueberries. Of these, 40% were marketed as fresh fruit, while the remaining 60% were processed in some form (Coba and Goodwin, 2003). A substantial amount of blueberry fruit is processed into juice and juice concentrate, which is subsequently used in beverages, syrups, and other food products. Juice processing unavoidably generates waste by-products, consisting of seeds, stems, and skins that will be found in the press cake residue. Since blueberry presscake has high amounts of anthocyanins and polyphenolics (Lee et al., 2002), it is a potential source for natural colorants and nutraceuticals.

The objective of this study was to evaluate juice processing enzymes, and a number of processing parameters, for producing aqueous blueberry extract that was rich in anthocyanins and polyphenolics. The intent was to develop a process that would be suitable for manufacturing extracts from blueberry juice processing wastes for natural colorant and nutraceutical usage. Another approach for making an anthocyanin/polyphenolic rich extract would be to use only the skins for the starting material. For that reason, the anthocyanin and phenolic composition of the separate portions of blueberry fruit (skin, flesh, and seeds) were also determined.

MATERIALS AND METHODS

Plant material

Frozen blueberries (*V. corymbosum*, cv. Rubel; Grade A) were provided by the Oregon Blueberry Commission. Blueberry skins for the extraction experiments were

obtained by removing skins from partially thawed berries with the aid of a razor blade. The skins were immediately frozen in liquid nitrogen, and stored at -70°C. For analysis of blueberry fractions, skins were removed as described above and seeds were manually separated from thawed flesh. Samples were frozen in liquid nitrogen and stored at -70°C until analysis. Industrial blueberry processing waste was supplied by the J. M. Smucker, Co., Woodburn, OR in the forms of frozen puree waste, and frozen berries described as puree stock. These materials were stored at -23°C.

Reagents, standards, and enzymes

All solvents used in this investigation were HPLC grade. Potassium metabisulfite, citric acid, gallic acid, catechin, epicatechin, protocatechuic acid, ellagic acid, caffeic acid, ferulic acid, *m*-coumaric acid, *o*-coumaric acid, *p*-coumaric acid, chlorogenic acid, vanillic acid, syringic acid, 2,3-dihydroxybenzoic acid, quercetin, myricetin, hydrocinnamic acid and *p*-hydroxybenzoic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and TPTZ (tripyridyltriazine) were purchased from Fluka (Buchs, Switzerland). AAPH [2,2'-azobis(2-amidinopropane) dihydrochloride] was purchased from Wako (Richmond, VA).

Commercial juice processing enzymes were provided by AB Enzymes, Columbus, OH; DSM Food Specialties USA, Inc., Charlotte, NC; Novozymes North America. Inc, Franklinton, NC; and Valley Research, Inc., South Bend, IN. Substrate activity as described for the enzymes are listed in Table 4.1. Firms supplying the enzymes were assured that identity of the individual enzymes would be kept confidential.

Table 4.1. Characteristics of industrial juice-processing enzymes.

Enzymes	Production microorganism	Activity
E1	Information not provided	No information provided by the manufacturer, experimental stages.
E2	*Aspergillus niger*	Pectinase, Hemicellulase
E3	*Trichoderma reesei*	Cellulase
E4	*Aspergillus aculeatus / Trichoderma longibrachiatum / Aspergillus niger*	Pectinase, Cellulase, Cellobiase
E5	*Aspergillus aculeatus*	Pectinase
E6	Information not provided / *Trichoderma longibrachiatum / Aspergillus niger*	Experimental Pectinase, Cellulase, Cellobiase
E7	Fungal cultures (did not specify)	Pectinase, Cellulase
E8	*Aspergillus* species	Pectinase, Hemicellulase
E9	*Aspergillus niger*	Pectinase

Enzyme evaluation

Commercial enzyme companies supplied juice processing enzyme preparations (n = 9) for assessment. Frozen whole blueberries, or frozen skins, (250g each) were pureed with distilled water (1:2, berries or skins: distilled water) using a Waring blender and aliquoted to erlenmeyer flasks. The mixture was placed in a 50°C Orbit shaker water bath (Lab-Line Instruments Inc., Melrose Park, IL) for 10 minutes, then 1 mL of diluted enzyme solution (enzymes were diluted based on manufacturer recommendations) was added to the berry puree-water mixture, then incubated for 1 hour at 50°C. After the incubation period, samples were then placed in a boiling water bath (Precision 180 Series, Precision Scientific Inc., Chicago, IL) for 10 minutes to inactivate enzymes, and then immediately cooled in an ice bath. Samples were then

centrifuged using an International clinical centrifuge (International Equipment Co, Boston, MA) for 30 minutes. The supernatants were collected and kept frozen at -70°C until analysis.

Extraction experiments

Both whole blueberries and skins were used for extraction material. Experimental conditions evaluated included temperature (50°C vs. 80°C), citric acid addition (none vs. 1% citric acid), and SO_2 addition (0, 50, and 100 ppm free SO_2). These processing parameters were applied individually and in combination, representing a 2×3×2 randomized complete block design (RCBD). Details of the different parameters are summarized in Table 4.2. Blueberries were homogenized using a Polytron PT10-35 and PT-K Laboratory homogeniser (Kinematica Ag., Luzern, Switzerland), setting 3 for 30 seconds, in the presence of solutions containing the appropriate concentration of acid and/ or sulfur dioxide in distilled water (1:2, berries or skins:solution). Samples were then held at a constant temperature (either 50°C or 80°C, depending on the parameter) for 1 hour in an Orbit shaker water bath (Lab-Line Instruments, Inc., Melrose Park, IL). Samples were cooled in an ice bath after incubation, and then centrifuged. The supernatants were collected, frozen, and stored at -70°C until analysis. All trials were replicated.

Table 4.2. Experimental design for heat, SO_2, citric acid combinations for extraction experiments (12 different combinations).

	0 ppm of SO_2	50 ppm of SO_2	100 ppm of SO_2
50°C	0% citric acid-control (H50S0C0)	0% citric acid (H50S50C0)	0% citric acid (H50S100C0)
	1% citric acid (H50S0C1)	1% citric acid (H50S50C1)	1% citric acid (H50S100C1)
80°C	0% citric acid (H80S0C0)	0% citric acid (H80S50C0)	0% citric acid (H80S100C0)
	1% citric acid (H80S0C1)	1% citric acid (H80S50C1)	1% citric acid (H80S100C1)

Codes in parenthesis are abbreviations for the individual treatments.

Sample preparation

Sample materials were extracted following the procedure described by Rodriguez-Saona and Wrolstad (2001). Blueberries, blueberry fractions, and processing wastes were liquid nitrogen powdered using a mortar and pestle, and extracted with acetone. Five g of powdered sample were sonicated with 10mL of 100% acetone and re-extracted with 70% (v/v) aqueous acetone until the solution became colorless. It was then partitioned with chloroform (1:2 acetone: chloroform, v/v) to obtain the aqueous fraction. The aqueous portion was collected, and residual acetone was evaporated by a Büchi rotovapor (Westbury, NY) at 40°C. The aqueous extract was dissolved to a final volume of 25mL with distilled water. Samples were then stored at -70°C until analysis.

Determination of total anthocyanins, % polymeric color, and total phenolics

The pH differential method (spectrophotometric method) as described by Giusti and Wrolstad (2001) was used in determining total monomeric anthocyanins (ACY), and expressed as cyanidin-3-glucoside (molar extinction coefficient of 26,900 L·cm

$^{1}\cdot mol^{-1}$ and molecular weight of 449.2 $g\cdot mol^{-1}$). Absorbance measurements were conducted at 520 and 700 nm. The unit for ACY was mg of cyanidin-3-glucoside/100g. Samples' percent polymeric color was determined by resistance to bisulfite bleaching, as described by Giusti and Wrolstad (2001). The Folin-Ciocalteu (FC) method was used for measuring total phenolics (TP) (Waterhouse, 2002) and expressed as mg of gallic acid/100g. Absorbance measurements were conducted at 765 nm. A Shimadzu 300 UV-visible spectrophotometer (Shimadzu Inc., Kyoto, Japan) and 1-cm-pathlength cells were used for both measurements. Measurements of ACY, % polymeric color, and TP of sample extracts were replicated 2 times. ACY and TP were determined on whole berries, berry fractions (skin, flesh and seeds), and sample extracts. ACY and TP were expressed as mg/100g of berries, skin, flesh, or seeds.

Purification of anthocyanins and polyphenolics

Anthocyanin pigments and polyphenolics were isolated by solid-phase extraction as described by Rodriguez-Saona and Wrolstad (2001). The aqueous extract was applied to a C-18 Sep-Pak mini-column (Waters Associates, Milford, MA), rinsed with 10mL water, and the water eluant discarded. After drying with a nitrogen stream, polyphenolics were eluted with 10mL ethyl acetate. Ethyl acetate was removed from the fraction with a Büchi rotovapor at 40°C, then the residue was re-dissolved in 2mL deionized water. Samples were filtered through a 0.45-μm Millipore filter (type HA, Millipore Corp., Bedford, MA) before HPLC injection. For ACY, TP, and antioxidant activity measurements, the ethyl acetate was evaporated with a Büchi rotovapor at 40°C, and. the polyphenolics were re-dissolved in 2 mL acidified water (0.01% HCl).

Anthocyanins were collected with 10mL of acidified (0.01% HCl) methanol. The methanol was evaporated using a Büchi rotovapor at 40°C. For ACY, TP, and antioxidant activity measurements, the pigments were re-dissolved in 2mL acidified (0.01% HCl) water. Samples for electrospray ionization mass spectrometry (ES-MS), and liquid-chromatography equipped with a photodiode array detector and mass spectrometer (LC-DAD-MS) analysis were then filtered through a 0.45-μm Millipore filter (type HA, Millipore Corp., Bedford, MA) before injection.

Determination of antioxidant activity

Antioxidant activities of extracts were determined by ferric reducing antioxidant potential (FRAP), and oxygen radical absorbing capacity (ORAC) assays. FRAP assays were performed as described by Benzie and Strain (1996), utilizing a 96-well ThermoMax microplate spectrophotometer (Molecular Devices, Foster City, CA) to measure the formation of ferrous-TPTZ complex (λ_{max}=595 nm). FRAP measures the extract ability to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) in a solution of TPTZ prepared in sodium acetate at pH 3.6. Absorbance was measured at 595 nm. ORAC assays followed the method described by Cao et al (1993), with the alteration of utilizing a 96-well cytofluor 4000 microplate fluorometer (PerSeptive Biosystems, Framingham, MA), which recorded rate and duration of fluorescence. β-Phycoerythrin acted as target for the peroxyl radicals generated by AAPH (a peroxyl radical generator that destroys the fluorescence). Samples were monitored at 2-minute intervals, for 2 hours, at 485 nm (excitation wavelength) and 585 nm (emission wavelength). FRAP and ORAC values were expressed as μmol of Trolox (a water soluble tocopherol analogue) equivalents/g of sample.

Anthocyanin and polyphenolic analyses analysis by HPLC-DAD

Analytical HPLC system. Anthocyanins were separated by reversed-phase HPLC using a Hewlett-Packard 1090 (Agilent Technologies Inc, Wilmington, DE), equipped with a photodiode array detector (DAD). Absorbance spectra were recorded for all peaks. Flow rate was 1 mL/min.

Anthocyanins. A Prodigy 5μm ODS (3) 100Å (250×4.6 mm) column, fitted with 4.0×3.0 mm i.d. guard column, from Phenomenex (Torrance, CA) was used. Solvent A was 100% acetonitrile. Solvent B was 10% (v/v) acetic acid and 1% (v/v) phosphoric acid in water. The program used a linear gradient from 2 to 20% solvent A in 25 minutes; then a linear gradient of solvent A from 20 to 40% in 5 minutes, with simultaneous detection at 280, 320, and 520 nm (Durst and Wrolstad, 2001). Injection volume was 20 μL. Column temperature was maintained at 40°C. Samples were filtered before HPLC injection.

Polyphenolics. A Synergi 4μm Hydro-RP 80Å (250×4.6 mm) column, fitted with 4.0×3.0 mm i.d. guard column, from Phenomenex (Torrance, CA) was used. Solvent A was 100% acetonitrile. Solvent B was 1% (v/v) formic acid in water. All solvents used were HPLC grade. The program used a linear gradient from 5% to 25% solvent A in 50 minutes; then a linear gradient of solvent A from 25% to 50% in 5 minutes, then held for 5 minutes, with simultaneous detection at 260, 280, 320, and 520 nm. Injection volume was 100μL.

Electrospray mass spectroscopy (ES-MS) of anthocyanins

Analysis was performed using a Perkin-Elmer SCIEX API III+ mass spectrometer (Toronto, Canada), equipped with an Ion Spray source and loop injection. Purified and filtered blueberry anthocyanins (5μL), in acidified water, were injected directly into the system.

LC-DAD-MS of anthocyanins

A Hewlett-Packard 1090 HPLC (Agilent Technologies Inc, Wilmington, DE), equipped with a photodiode array detector (DAD) and mass spectrometer (MS) was used to confirm the identification of the blueberry anthocyanins. A Synergi 4μ Hydro-RP 80Å (250×2mm, 4μmicron) column, fitted with 4.0×3.0 mm i.d. guard column, from Phenomenex (Torrance, CA) was used. Absorbance spectra were collected for all peaks. Flow rate was 0.2mL/min and injection volume was 20μL. Solvent A was 5% formic acid and 80% acetonitrile (v/v), and solvent B was 5% formic acid. All solvents were HPLC grade. The initial solvent composition was 10% solvent A and 90% solvent B; then a linear gradient of 10% to 30% solvent A, and 90% to 70% solvent B in 30 minutes. Detection occurred simultaneously at 280, 320, and 520 nm. MS analysis was performed using a Perkin-Elmer SCIEX API III Plus triple-quadrupole mass spectrometer (Toronto, Canada), equipped with an ion spray source (ISV= 5500, orifice voltage= 50) and positive ion mode. LC-DAD-MS-MS was conducted on some of the peaks observed. Multiple reaction monitoring (MRM) mode was used. The mass of the molecular ions (anthocyanins) of interest were scanned in first quadrupole (Q1) and the daughter ions (also known as fragmented ions, anthocyanidins) were monitored in third quadrupole (Q3). Argon was used as the collision gas. The nebulizer gas and orfice

curtain gas were nitrogen. Mass spectra data were collected with Tune 2.5 software, while Mac Spec 3.3 software was used for data processing, both provided by SCIEX (Toronto, Canada).

Statistical analyses.

SPSS® (Chicago, IL) version 11.0 was used for the statistical analyses. Differences among enzyme treatment means were tested using one-way analysis of variances (ANOVA), and the Tukey Honest Significant Difference (HSD) at $\alpha = 0.05$ level. ANOVA was conducted on the results for the extraction treatments and Tukey HSD was also conducted.

RESULTS AND DISCUSSION

Anthocyanins, phenolics, and antioxidant properties of blueberries and their sub-fractions

The distribution of ACY, TP, FRAP, and ORAC of whole berries and in their skins, flesh and seeds are shown in Table 4.3. The skins percentage by weight (19.0%) would be expected to be higher for 'Rubel' blueberries than for other commercially grown highbush cultivars, since it has a smaller berry size (Moyer et al., 2002; Ehlenfeldt and Prior, 2001). Blueberry seeds are small, and represent a relatively small portion of the berry (1.5%). In determining the weight percentage of the three fractions, there was a 5.1% loss from conducting the manual separating operations. The high antioxidant capacity of whole blueberries has been highly correlated to their anthocyanin and total phenolic content (Kalt and Dufour, 1997; Prior et al., 1998; Kalt et al., 2000). The anthocyanins exist almost exclusively in the skin, while phenolics and

antioxidant properties are mostly in the skins. By extracting only from the skins, there is the potential for producing extracts with very high anthocyanin and polyphenolic concentrations.

Table 4.3. Percent weight distribution, total monomeric anthocyanin (ACY), total phenolics (TP), ferric reducing antioxidant power (FRAP), and oxygen radical absorbing capacity (ORAC) of whole 'Rubel' berries and their fractions.

	Weight distribution	Berry basis				Fraction basis			
	% weight	ACY[a]	TP[b]	FRAP[c]	ORAC[d]	ACY[a]	TP[b]	FRAP[c]	ORAC[d]
Whole berry	100.0	230.0	737.5	39.9	30.7	-	-	-	-
Skins	19.0	188.5	300.4	28.7	18.0	993.8	1583.8	151.3	94.9
Flesh	74.4	5.8	119.3	7.0	6.3	7.9	160.4	9.5	8.5
Seeds	1.5	0.1	4.3	0.3	0.1	7.8	285.6	20.5	9.8
Total	-	194.5	424.1	36.1	24.5	-	-	-	-
% loss	5.1	15.5	42.5	9.7	20.4	-	-	-	-

[a] ACY was expressed as cyanidin-3-glucoside equivalents (mg/100 g of berries, 100 g of skins, 100 g of flesh, or 100 g of seeds).

[b] TP was expressed as gallic acid equivalents (mg/100 g of berries, 100 g of skins, 100 g of flesh, or 100 g of seeds).

[c] FRAP was expressed as Trolox equivalents (μmol/1 g of berries, 1 g of skins, 1 g of flesh, or 1 g of seeds).

[d] ORAC was expressed as Trolox equivalents (μmol/1 g of berries, 1 g of skins, 1 g of flesh, or 1 g of seeds).

Results are expressed on a per 100 g or per g basis, and also arithmetically converted to show the amounts in skin, seeds and flesh for a 100 g berry sample. For example, the skins contained 994 ACY/100 g of skin, which represents 188 mg in a 100 g berry sample (994 x 19%). This permits comparison with whole berries and estimation of losses. There was a 16% loss in ACY, and a 43% loss in TP when comparing with values for whole berries. We suspect that the high loss of phenolics was due to enzymatic oxidation occurring during the manual separating and weighing operations. There were no reports in the literature on the composition of blueberry seeds, but the antioxidant properties were substantially lower than what has been reported for grape seeds (Pastrana-Bonilla et al., 2003).

Evaluation of juice processing enzymes

With the intent of using juice-processing enzymes to increase recovery of anthocyanins and polyphenolics from juice processing wastes, we developed a screening assay using either whole berries or skins as substrate. Requests were made to enzyme suppliers, and nine enzyme preparations were provided for evaluation. Two of the samples were experimental samples, but the others are available commercially. The characteristics of the enzymes regarding pectinase, cellulase, cellobiase, and hemicullase activity are shown in Table 4.1. The ACY, TP, and % polymeric results for the enzyme treated samples are summarized in Table 4.4.

Table 4.4. Results from the industry recommended enzymes (n=9) in extracting total anthocyanins (ACY), total phenolics (TP), and % polymeric color (%PC) from whole berries and skins (100 g of extract).

	Whole berries			Skins		
Enzymes	ACY[1]	TP[2]	%PC	ACY[1]	TP[2]	%PC
E1	27.1^{ab}	88.7^{abc}	40.9^{a}	128.2^{bc}	264.8^{abc}	28.5^{a}
E2	24.9^{ab}	87.6^{ab}	41.8^{ab}	109.0^{ab}	251.9^{abc}	32.2^{ab}
E3	27.2^{ab}	101.0^{cd}	67.6^{cd}	101.4^{a}	235.6^{ab}	35.8^{ab}
E4	23.7^{ab}	90.4^{abcd}	45.0^{ab}	109.9^{ab}	242.9^{abc}	33.7^{ab}
E5	23.5^{ab}	87.9^{ab}	45.5^{ab}	100.4^{a}	232.2^{a}	32.5^{ab}
E6	20.5^{a}	82.9^{a}	49.9^{b}	127.1^{bc}	268.3^{abc}	31.6^{ab}
E7	26.7^{ab}	91.7^{abcd}	43.9^{ab}	137.9^{c}	278.5^{c}	36.3^{b}
E8	28.1^{b}	98.3^{bcd}	61.6^{c}	106.5^{ab}	234.5^{a}	35.2^{ab}
E9	23.2^{ab}	85.9^{ab}	44.9^{ab}	136.7^{c}	276.1^{bc}	35.7^{ab}
Control	28.3^{b}	103.3^{d}	73.4^{d}	113.3^{abc}	248.6^{abc}	32.3^{ab}

[1] Total anthocyanin was expressed as cyanidin-3-glucoside equivalents.
[2] Total phenolics was expressed as gallic acid equivalents.
Values in each column sharing the same superscript are not significantly different from each other (Tukey's HSD, $p \leq 0.05$).

The amount of anthocyanin and phenolics extracted from the whole berries by enzymes ranged from 20.5 to 28.1 mg ACY, and 82.9 to 101.0 mg TP per 100 g of extract. The control (no enzyme addition) actually gave the highest yield of anthocyanin and phenolics. Enzyme 8 (pectinase and hemicellulase activities) performed as well as the control for extracting ACY. Enzyme 6 (pectinase, cellulase, and cellobiase) extracted the least ACY and TP from the berries. Although, control yielded the highest amount of TP, enzymes 3, 4, 7, and 8 (all containing cellulase or hemicellulase activity)

were equivalent in the extraction of TP. The percent polymeric color, which is an index for anthocyanin degradation, was highly variable and ranged from 40.9% (enzyme 1) to 73.4% (control). Surprisingly, while the control had the highest anthocyanin recovery, it also yielded the highest % polymeric color. Enzyme 1 produced the least amount of % polymeric color, and all enzymes, except enzyme 3 (only cellulase activity) yielded significantly less % polymeric color compared to the control. Depectinization with pectolytic enzymes remains an essential unit operation for producing blueberry juice and juice concentrates. So juice processing enzymes are required even if they do not give increased recovery of anthocyanins and polyphenolics.

ACY and TP for the different enzyme treatments to skins ranged from 100.4 to 137.9 mg of ACY, and 232.2 to 278.5 mg of TP per 100 g of extract. There were also differences between berries and skins when making comparisons to the control. Enzyme 7 (pectinase and cellulase activities) had the highest ACY, TP, and % polymeric color values. Enzymes 1, 6, 9, and control were equivalent to enzyme 7 in their ability to extract ACY. Enzymes 1, 2, 4, 6, 9, and control were equivalent to enzyme 7 in extracting TP from skins. The percent polymeric color yield ranged from 28.5% to 36.3%, which was not as variable as % polymeric values obtained from whole berries. Enzyme 1 (unknown experimental enzyme) produced the least amount of % polymeric color. Lower amounts of % polymeric color from skins processed with extracts might be explained by the absence of blueberry pulp (source of endogenous enzymes and chlorogenic acid). Skrede et al. (2000) demonstrated that adding unblanched blueberry pulp to pasteurized juice increased anthocyanin degradation significantly. Blueberry anthocyanins have been found to degrade rapidly in the presence of chlorogenic acid and PPO, but not by PPO alone (Kader et al., 1997 and 1998).

Delphinidin-glycosides (based on its structure, delphinidin is the most labile) were degraded the most and malvidin-glycosides appear to have degraded the least by processing enzyme treatments (Figure 4.1.B), especially when the starting material was whole berries, which implied that the native enzymes present in the blueberry destroyed anthocyanin.

Landbo and Meyers (2001) tested five different processing enzymes (the enzymes had pectinase, macerase, or protease activity) with varying degrees of extraction efficiency from black currant presscake residue. Enzyme assisted extractions yielded more polyphenolics, but were not found to increase anthocyanins, when compared to a control (no addition of enzyme). Particle size has shown to influence the amount of phytochemicals extractable from black currant pomace, where a reduction in particle size increased the amount extracted. Also, Stanley and Miller (2001) showed fine milling was more influential than the use of commercial enzymes, or heating in extraction of phytochemicals from blueberry juice processing waste. Meyer et al. (1998) tested pectinase and cellulase in phenolic extraction of grape pomace, and found that pectinase had positive effects, but that cellulase had a negative effect in the extraction of polyphenolics. The benefit of pectinase was correlated with plant cell wall breakdown from the enzymes (Meyer et al., 1998).

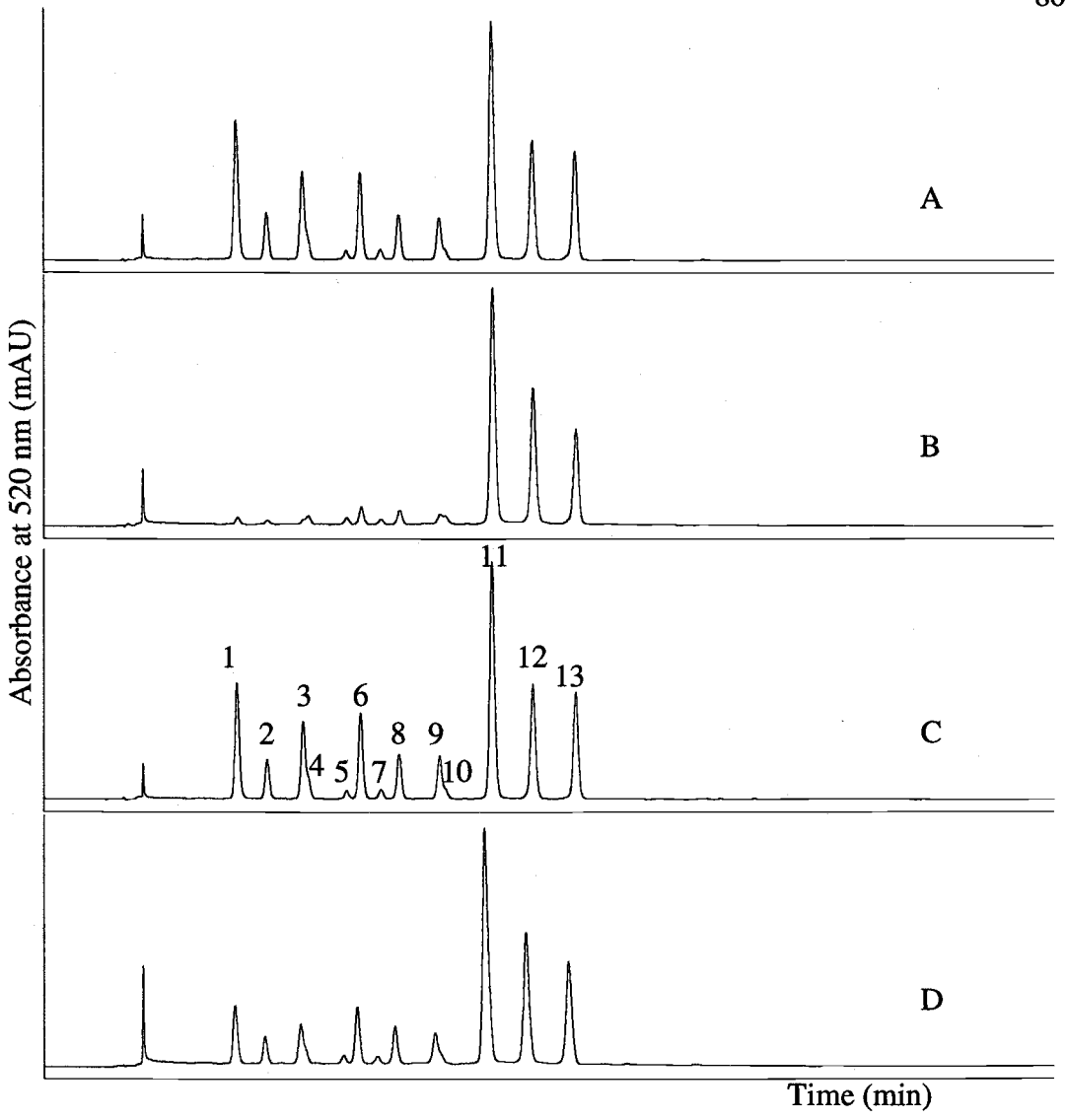


Figure 4.1. LC-DAD profiles of anthocyanin: A-whole berry, B-whole berry processed with enzyme 6, C-skin, and D-skin processed with enzyme 6. Corresponding anthocyanin peak assignments and % peak area for chromatogram C: Corresponding anthocyanin peak assignments are as follows: 1:delphinidin-3-galactoside (12.8), 2:delphinidin-3-glucoside (4.4), 3:delphinidin-3-arabinoside (8.4), 4:cyanidin-3-galactoside (2.0), 5:cyanidin-3-glucoside (0.9), 6:petunidin-3-galactoside (5.3), 7:cyanidin-3-arabinoside (1.1), 8:petunidin-3-glucoside (4.8), 9:petunidin-3-arabinoside (4.6), 10:peonidin-3-galactoside (1.0), 11:malvidin-3-galactoside (27.8), 12:malvidin-3-glucoside (13.9), and 13:malvidin-3-arabinoside (13.0).

Some commercial juice processing enzymes have shown negative effects on the anthocyanin content (color loss) of cranberry juice due to ß-galactosidase activity (Wrolstad et al., 1994; Wightman and Wrolstad, 1995). In this study, the enzymes used did not exhibit side activities, like ß-galactosidase activity and/or ß-glucosidase activity, since the enzyme side activities are specific (Wrolstad et al., 1994). There was no clear evidence of decrease in to ß-galactosides and to ß-glucosides from the proportions of individual anthocyanin profiles (Figure 4.1).

Extraction experiments

In the USA, the legal definition of fruit juices, and fruit juice concentrates, require that juice be extracted by physical methods and/or aqueous extraction (Title 21, U.S. Code of Federal Regulations 73). Thus, anthocyanin-based colorants under the classification of fruit and vegetable juices cannot be extracted with organic solvents. For this reason the use of ethanol (or other organic solvents) was not included in this investigation. Previously, we showed that High-temperature-short-time (HTST) treatment and SO_2 could increase anthocyanin recovery when blueberries were processed into juice and concentrate (Lee et al., 2002). Also, anthocyanins were more stable in acidic conditions (Wrolstad, 2000) and Kalt et al. (2000) showed that blueberry juice contained more anthocyanins, phenolics, and antioxidant activity at lower pH, so addition of citric acid might increase pigment recovery. In this experiment, the addition of 50 and 100 ppm SO_2, the addition of 1% citric acid, and extraction temperatures of 50°C vs. 80°C were tested individually and in combination (Table 4.5).

Table 4.5. Total anthocyanin content (ACY, expressed as cyanidin-3-glucoside equivalents) and total phenolic content (TP, expressed as gallic acid equivalents) of the extracts obtained from whole berries and skins from the individual and combination of the heat, SO_2, and citric acid treatments.

Treatment combinations				Berry[1]		Skins[1]	
Trt no.[2]	Heat levels (°C)	SO_2 levels (ppm)	Citric acid levels (%)	ACY	TP	ACY	TP
1	50	0	0	27.5	55.9	102.7	146.9
2	50	0	1	42.6	62.6	156.8	186.2
3	80	0	0	50.2	71.4	151.7	260.4
4	80	0	1	62.4	88.8	220.2	312.2
5	50	50	0	92.2	307.3	462.9	745.2
6	50	50	1	96.5	316.4	469.9	748.5
7	80	50	0	97.5	420.6	401.0	701.9
8	80	50	1	102.2	413.8	373.0	659.3
9	50	100	0	90.6	386.0	494.5	906.9
10	50	100	1	82.2	337.5	504.5	924.1
11	80	100	0	92.1	641.0	409.8	938.8
12	80	100	1	91.4	684.3	446.1	978.3

[1]100 g of extract basis.

[2]Trt no. : treatment number.

Values in each column sharing the same superscript are not significantly different from each other (Tukey's HSD, $p \leq 0.05$).

ACY and TP concentrations for the different treatments are shown in Table 4.5, and statistical results are shown in Tables 4.6 and 4.7. Abbreviations of the treatments are provided (Table 4.1).

Table 4.6. The results from ANOVA tables for the extraction experiments.

		Whole berry		Skins	
Effects	d.f.	ACY	TP	ACY	TP
Blocks	1	0.001	0.005	0.001	0.001
Heat levels (H)	1	0.001	0.001	0.001	0.006
SO_2 levels (S)	2	0.001	0.001	0.001	0.001
Citrate levels (C)	1	0.001	0.515	0.001	0.109
H×S	2	0.001	0.001	0.001	0.001
S×C	2	0.001	0.530	0.001	0.056
C×H	1	0.443	0.012	0.889	0.869
H×S×C	2	0.145	0.001	0.154	0.402

Total anthocyanin content (ACY, expressed as cyanidin-3-glucoside equivalents) and total phenolic content (TP, expressed as gallic acid equivalents).
d.f.= degrees of freedom

ACY extracted from whole berries ranged from 27.5 to 102.2mg/100g and TP from 55.9 to 684.3mg/100g of extract. The pH of the extracts ranged from 2.80 to 3.98. Addition of citric acid lowered the pH of the extract by 0.5 to 1.0 units. Extraction of both ACY and TP contents of samples cannot be explained by main effects alone; heat (H), SO_2 (S), or citric acid levels (C). There was no significant evidence for H×S×C effect (3-way interaction), or C×H effect (2-way interaction) for ACY content. The effect of heat changed with a change in SO_2 level. Higher levels of both heat and SO_2 increased the extraction of ACY from whole berries. Also, the effect of SO_2 addition changes with the level of citric acid. Addition of citric acid aided in extracting ACY with 0 and 50 ppm SO_2, but decreased the extraction at 100 ppm SO_2. The highest ACY extracts were with treatments H50S50, H80S50, and H80S100, also from S50C0, S50C1, and S100C0. For TP, there was a significant 3-way interaction among the factors. In general, an increase in SO_2 addition, and heat levels yielded higher amounts

of TP. A temperature of 80°C and 100 ppm of free SO_2 (H50S100C0 and H50S100C1) extracted the most TP. The addition of 1% citric acid aided in the extraction of TP at low temperature and when SO_2 was not added. The control had the lowest amount of ACY and TP.

ACY extracted from the skins ranged from 102.7 to 504.5 mg/100 g, and TP from 146.9 to 978.3 mg/100 g of extract. The pH of the extracts ranged from 2.58 to 3.77. Addition of citric acid lowered the extracts' pH in all cases. Again, the extraction of ACY and TP from skins cannot be explained by the main effects alone (Table 4.6), as there were interactions that were significantly different. There were no significantly different 3-way interactions and C×H effect for ACY content. From the H×S effect, combination of heat and addition of SO_2 assisted in the extraction of ACY from the skins when compared to low heat and no SO_2 addition. Low heat (50°C) and addition of 50 ppm or 100 ppm of SO_2 (H50S50C0, H50S50C1, H50S100C0, and H50S100C1) yielded the highest amount of ACY. Using high heat aided in extracting TP, when no SO_2 and citric acid was used. From the S×C effect, the use of SO_2 increased the extraction of ACY when used in combination with citric acid addition. Using 100 ppm of SO_2 and 1% citric acid yielded the highest ACY content from the skins.

Table 4.7. The results of the effect of heat, SO_2, and citric acid from 2-way or 3-way Tukey HDS.

		whole berry		skins	
	Levels	ACY	TP	ACY	TP
H×S effect	H50S0	35.0a	-	129.8a	166.6a
	H80S0	56.3b	-	185.9a	286.3b
	H50S50	93.3cd	-	466.4cd	746.9c
	H80S50	99.9d	-	387.0b	680.6c
	H50S100	86.4c	-	499.5d	915.6d
	H80S100	91.7cd	-	428.0bc	958.6d
S×C effect	S0C0	38.9a	-	127.2a	*
	S0C1	52.5b	-	188.5a	*
	S50C0	94.8cd	-	432.0b	*
	S50C1	99.4d	-	421.4b	*
	S100C0	94.8cd	-	452.1b	*
	S100C1	86.8c	-	475.3b	*
H×S× C effect	H50S0C0	*	55.9a	*	*
	H50S0C1	*	62.6a	*	*
	H80S0C0	*	71.4a	*	*
	H80S0C1	*	88.8a	*	*
	H50S50C0	*	307.3b	*	*
	H50S50C1	*	316.4b	*	*
	H80S50C0	*	420.6c	*	*
	H80S50C1	*	413.8c	*	*
	H50S100C0	*	386.0c	*	*
	H50S100C1	*	337.5b	*	*
	H80S100C0	*	641.0d	*	*
	H80S100C1	*	684.3d	*	*

Values in each column sharing the same letter are not significantly different from each other (Tukey's HSD, $p<0.05$).

* indicates not significantly different.

- indicates not applicable.

From the TP results, there was a significant interaction between heat and SO_2. TP of the skins treated with the 2 different levels of citric acid were not significantly different. Higher heat aided the extraction of TP at 0 ppm of SO_2, but not when 50 ppm or 100 ppm of SO_2 was added. High heat (80°C) and addition of citric acid (H80S0C1) was effective in extracting ACY when no SO_2 was added to the skins. High sulfured water (H50S100 and H80S100) extracted the highest amount of TP from the skins. Control had the lowest amount of ACY and TP.

While whole berries and blueberry skins were used in these experiments, the extraction conditions should also apply to juice processing waste products such as press-cake residue. Processing waste obtained by a local processor was analyzed for ACY and TP content. Puree waste was high in ACY (96.9 mg/100 g and TP (540.7 mg/100 g Puree stock contained 47.2 mg of ACY/100 g of stock, and 223.1 mg of TP/100 g. Puree waste appeared to be a good source for further extraction.

During the early 1960s, a combination of heat treatment (62°C) and pectinase before pressing had been reported to be effective in blueberry juice processing for improving yield, color, and soluble solids (Fuleki and Hope, 1964). Whole berries and skins were extracted a single time in this study. Since the residual appears to still retain pigment, multiple extractions of the residual might increase the final ACY and TP yield. Bakker et al. (1998) reported an increase in anthocyanin extraction with the increase in total SO_2 addition (0, 75, and 150 ppm) to must. They also reported no noticeable effect on the aroma and flavor of the final wine produced. Bocevska and Stevcevska (1997) have demonstrated boosting SO_2 addition (500-5000 ppm of total SO_2) increased the extract purity obtained from wine grape pomace. Cacace and Mazza (2002 and 2003) have conducted an intensive study of extracting phytochemicals, such as anthocyanins

and polyphenolics, from black currants utilizing sulfured water (28 to 1372 ppm of SO_2), aqueous ethanol (39 to 95% ethanol in water), and heat (6 to 74°C). They recommended that 19L of solvent per kg of milled frozen berries, 1000-1200 ppm of SO_2 (they did not indicate whether these values were total or free SO_2), and extraction temperature of 30-35°C. Sixty % aqueous ethanol yielded the most total phenolics, but had little effect on the anthocyanin content of the extract.

Free SO_2 concentrations of 0, 50, and100 ppm were selected for this study, since previous work (Lee et al, 2002) showed that after pasteurization the amount of total SO_2 left in the product was less than 10 ppm. The minimum level of total SO_2 for labeling is 10 ppm (Title 21, U.S. Code of Federal Regulations 101.100). Recently, researchers have demonstrated that steam blanching blueberries for 3 minutes increased the recovery of anthocyanins and cinnamic acids (Rossi et al., 2003). From this study, a combination of heating, with the addition of SO_2 will increase the yield of phytochemicals from whole blueberry fruit, and their process by-products.

'Rubel' anthocyanin profile

The anthocyanin pigment profile of 'Rubel' blueberry skins obtained from the LC-DAD is shown in Figure 4.1.C. Thirteen peaks were identified. They contained the glycosides of malvidin (54.7%), delphinidin (25.7%), petunidin (14.7%), cyanidin (3.9%), and peonidin (1.0%) based on % peak area. The major anthocyanin present in 'Rubel' was malvidin-galactoside (27.8%).

Peak assignments were based on their spectra and retention time. Peaks that were present in low quantities were difficult to identify based on their spectra alone. Utilizing tools like ES-MS and LC-DAD-MS helped in identification in peak

assignments. Anthocyanin's (mostly in the oxonium form at low pH) generate a positive ion that can be detected by MS. An anthocyanins single positive charge allows the mass-to-charge ratio (m/z) to correspond directly to the molecular weight of the anthocyanin. Based on the ES-MS results alone, it was difficult to rule out any of the 15 possible combinations of glycosides of delphinidin, cyanidin, peonidin, petunidin, and malvidin. The 13 peaks identified by HPLC can be assigned to the 8 ES-MS peaks (Figure 4.2). Anthocyanins with the same m/z were not distinguished by ES-MS. Malvidin-3-galactoside and malvidin-3-glucoside (both have the same m/z) had the largest peak in the ES-MS data (Figure 4.2, peak 8), and were also the largest peaks in HPLC (Figure 4.1.C, peaks 12 and 13).

Results for LC-DAD-MS analysis are shown in Figure 4.3. Resolution was not as good with the LC-DAD-MS system, probably due to the mobile phase contained formic acid, as phosphate buffer needed to be avoided. Despite this, LC-DAD-MS provided more information than LC-DAD alone. Blueberry anthocyanin peaks that co-eluted in the new LC system still differed in m/z, so the individual anthocyanins could be distinguished. For example: malvidin-3-glactoside (peak 12) and peonidin-3-glucoside (peak 11) co-eluted. The two compounds could be detected, however, with MS.

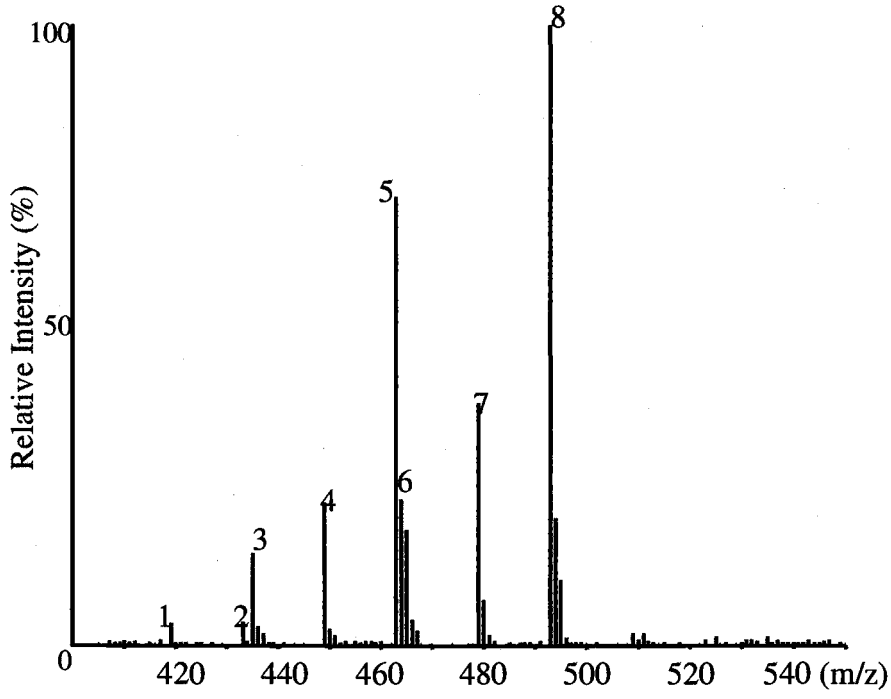


Figure 4.2. ES-MS separation of 'Rubel' blueberry skin anthocyanin. Corresponding presumptive anthocyanin peak assignments: 1-cyanidin-3-arabinoside (419), 2-peonidin-3-arabinoside (433), 3-delphinidin-3-arabinoside (435), 4-petunidin-3-arabinoside, cyanidin-3-galactoside, and cyanidin-3-glucoside (449), 5-peonidin-3-galactoside, peonidin-3-glucoside, and malvidin-3-arabinoside (463), 6-delphinidin-3-galactoside and delphinidin-3-glucoside (465), 7-petunidin-3-galactoside and petunidin-3-glucoside (479), and 8-malvidin-3-galactoside and malvidin-3-glucoside (493).

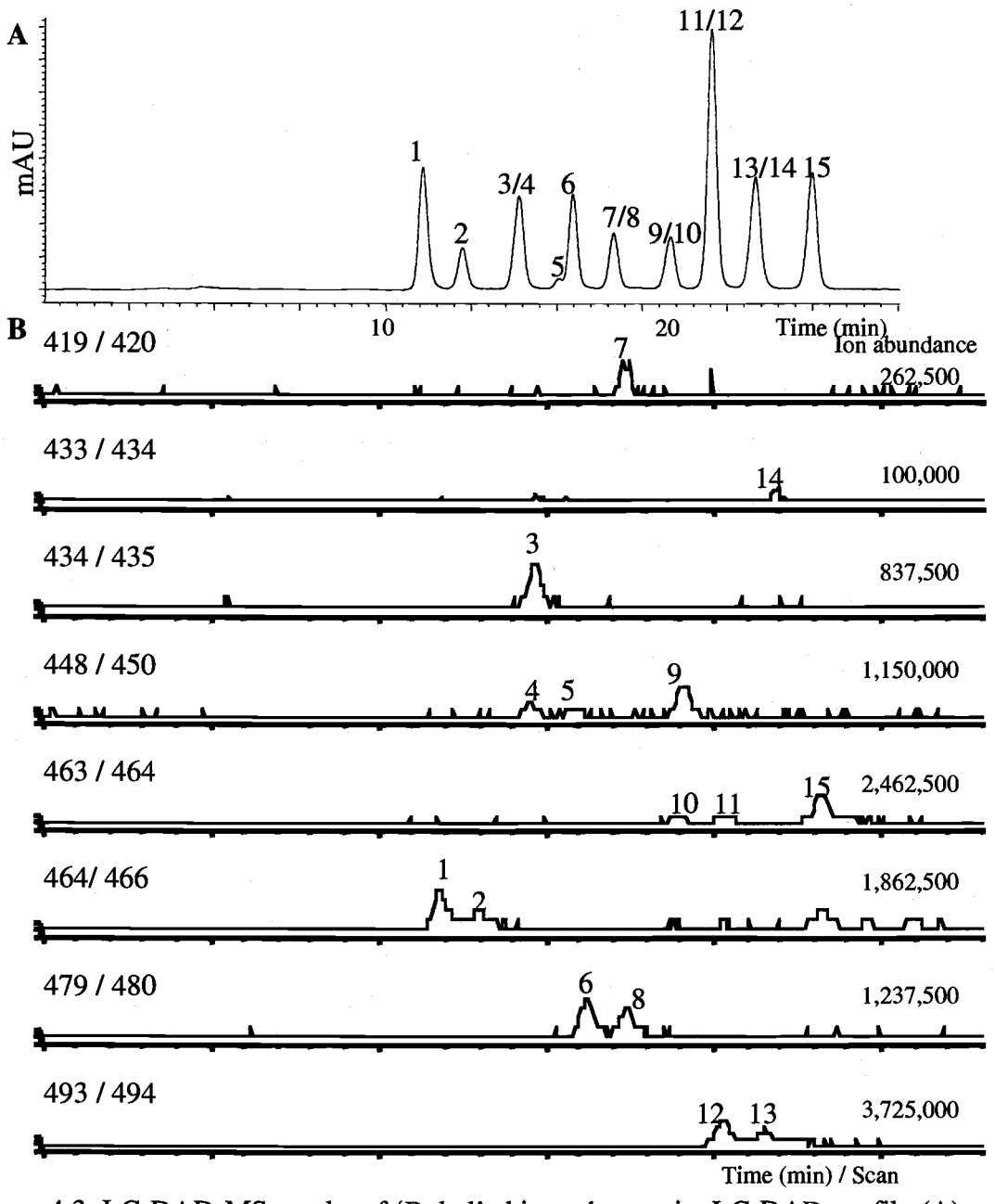


Figure 4.3. LC-DAD-MS results of 'Rubel' skin anthocyanin. LC-DAD profile (A) and extracted ion chromatograms (B). Corresponding anthocyanin peak assignments are as follows: 1:delphinidin-3-galactoside(465.2), 2:delphinidin-3-glucoside(465.2), 3:delphinidin-3-arabinoside(435.0), 4:cyanidin-3-galactoside(449.2), 5:cyanidin-3-glucoside(449.2), 6:petunidin-3-galactoside(479.2), 7:cyanidin-3-arabinoside(419.0), 8:petunidin-3-glucoside(479.2), 9:petunidin-3-arabinoside(449.0), 10:peonidin-3-galactoside(463.2), 11:peonidin-3-glucoside(463.2), 12:malvidin-3-galactoside(493.2), 13:malvidin-3-glucoside(493.2), 14:peonidin-3-arabinoside(433.0), and 15:malvidin-3-arabinoside(463.0).

From the LC-DAD-MS results there were a total of 15 peaks in 'Rubel' skins. There was a low concentration of peonidin-3-arabinoside in 'Rubel' skins, which made identification based on retention time and spectra difficult. LC-DAD-MS permitted positive identification of 'Rubel' skin anthocyanins. By using a combination of retention time, peak spectra, and m/z, identification of anthocyanins was simplified. LC-DAD-MS-MS was also performed on some peaks that co-eluted, and were present in low concentrations on the LC-DAD-MS system (peaks 3/4 and 11/12 in Figure 4.3). The m/z of the corresponding molecular ion ▸ fragmented ion were 435.0 ▸ 303.0 (delphinidin-arabinoside, peak 3 in Figure 4.3), 449.2 ▸ 287.0 (cyanidin-galactoside, peak 4 in Figure 4.3), 463.2 ▸ 301.0 (peonidin-glucoside, peak 11 in Figure 4.3), and 493.2 ▸ 331.0 (malvidin-galactoside, peak 12 in Figure 4.3).

The anthocyanin profile for whole berries was the same for skin alone (Figure 4.1.A and C), which was expected since the anthocyanins exist almost exclusively in the skins. The extracts of flesh and seeds were slightly different, having proportionately less delphinidin glycosides. We believe this difference can be attributed to differences in seed anthocyanins, since pigments in the flesh probably diffused from the skin.

Blueberry puree waste extracts had a similar anthocyanin HPLC profile to the stock, but the anthocyanin profiles for the industrial juice processing wastes differed slightly in the proportions of delphinidin, petunidin, and malvidin glucosides decreased when compared to the stock, which might be due to processing enzymes' side activity (i.e. glucosidase activity).

Polyphenolics profile

The HPLC separation for blueberry polyphenolics is shown in Figures 4.4, 4.5, and 4.6. Figure 4.4 represents a chromatogram of compounds that have been reported in blueberries. Most phenolics in the berry exist as glycosides or esters. In this study, hydrolysis was not conducted on the phenolic fractions, so only the native form was observed. Chlorogenic acid (5-caffeoylquinic acid), 58% of the total peak measured at 320 nm, was the major polyphenolic present in 'Rubel' (Figure 4.5), confirming previous reports (Lee et al., 2002).

The skin, flesh and seeds exhibited different polyphenolic profiles (Figure 4.6). The skins mainly consisted of cinnamic acids and flavonol-glycosides, with some minor peaks such as gallic acid and syringic acid. The flesh consisted of only cinnamic acids. Chlorogenic acid, 60% of the total peak area of 320 nm absorbers, was the main phenolic compound present in the flesh. The seed fraction also had cinnamic acids and flavonol-glycosides, with numerous unidentified peaks. The LC conditions used in this study were not ideal for analysis of procyanidins, but procyanidins in blueberry seeds have been reported (Prior et al., 2001; Gu et al., 2002). Prior et al. (2001) reported 6μg of total procyanidin/g of dry 'Rubel'. They also identified B-type procyanidin monomers through octamers by normal-phase HPLC-ESIMS. Gu et al. (2002) reported lowbush blueberries to contain 20mg of procyanidin/g of freeze-dried blueberries. Despite the efforts to identify polyphenolics based on their retention time and UV spectra, there were still numerous peaks unidentified that did not correspond to available standards (Figure 4.4).

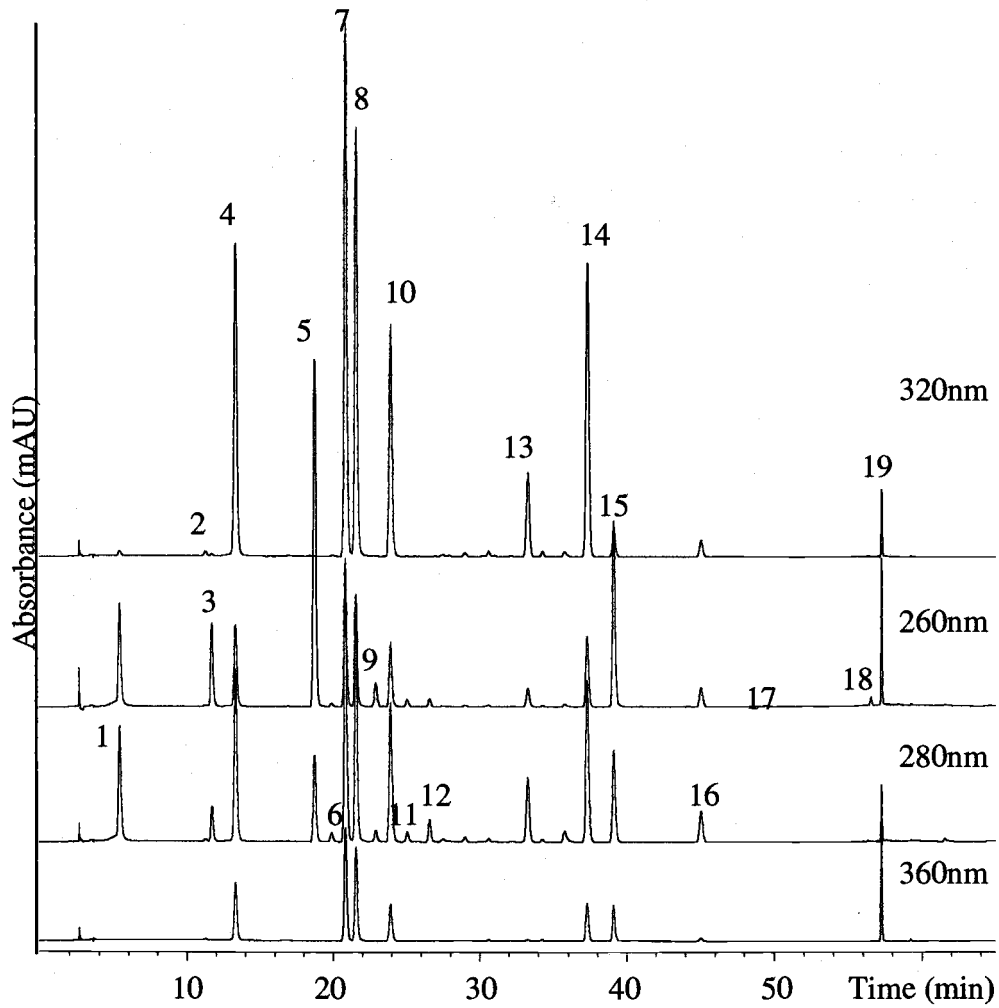


Figure 4.4. Polyphenolics separation of 20 standards. The peak assignments are as follows: 1: gallic acid, 2: 1-O-caffeoylquinic acid, 3: protocatechuic acid, 4: 3-O-caffeoylquinic acid (neochlorogenic acid), 5: *p*-hydroxybenzoic acid and 2,3-dihydroxybenzoic acid, 6: catechin, 7: 5-O-caffeoylquinic acid (chlorogenic acid), 8: 4-O-caffeoylquinic acid (crytochlorogenic acid), 9: vanillic acid, 10: caffeic acid, 11: syringic acid, 12: epicatechin, 13: *p*-coumaric acid, 14: hydroxycinnamic acid, 15: *m*-coumaric acid and ferulic acid coeluting, 16: *o*-coumaric acid, 17: myricetin, 18: ellagic acid, and 19: quercetin. Peak numbers are on the maximum detected absorbance.

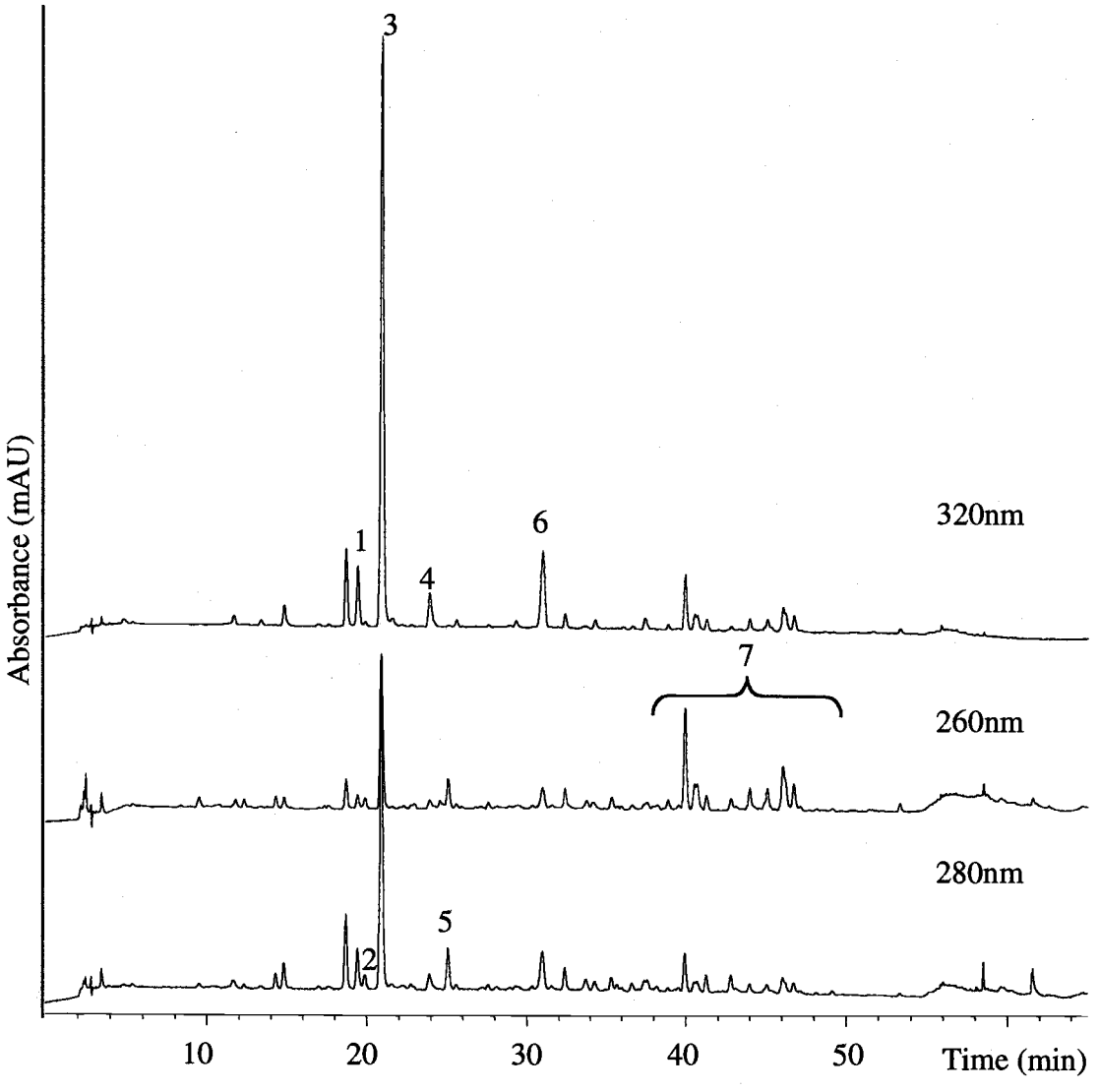


Figure 4.5. HPLC separation (detection at 320, 260, and 280 nm) of 'Rubel' whole berry polyphenolic fraction. Corresponding phenolics peak assignments: 1: cinnamic acid derivative, 2: catechin, 3: chlorogenic acid, 4: caffeic acid, 5: syringic acid, 6: cinnamic acid derivative, and 7: flavonol-glycosides.

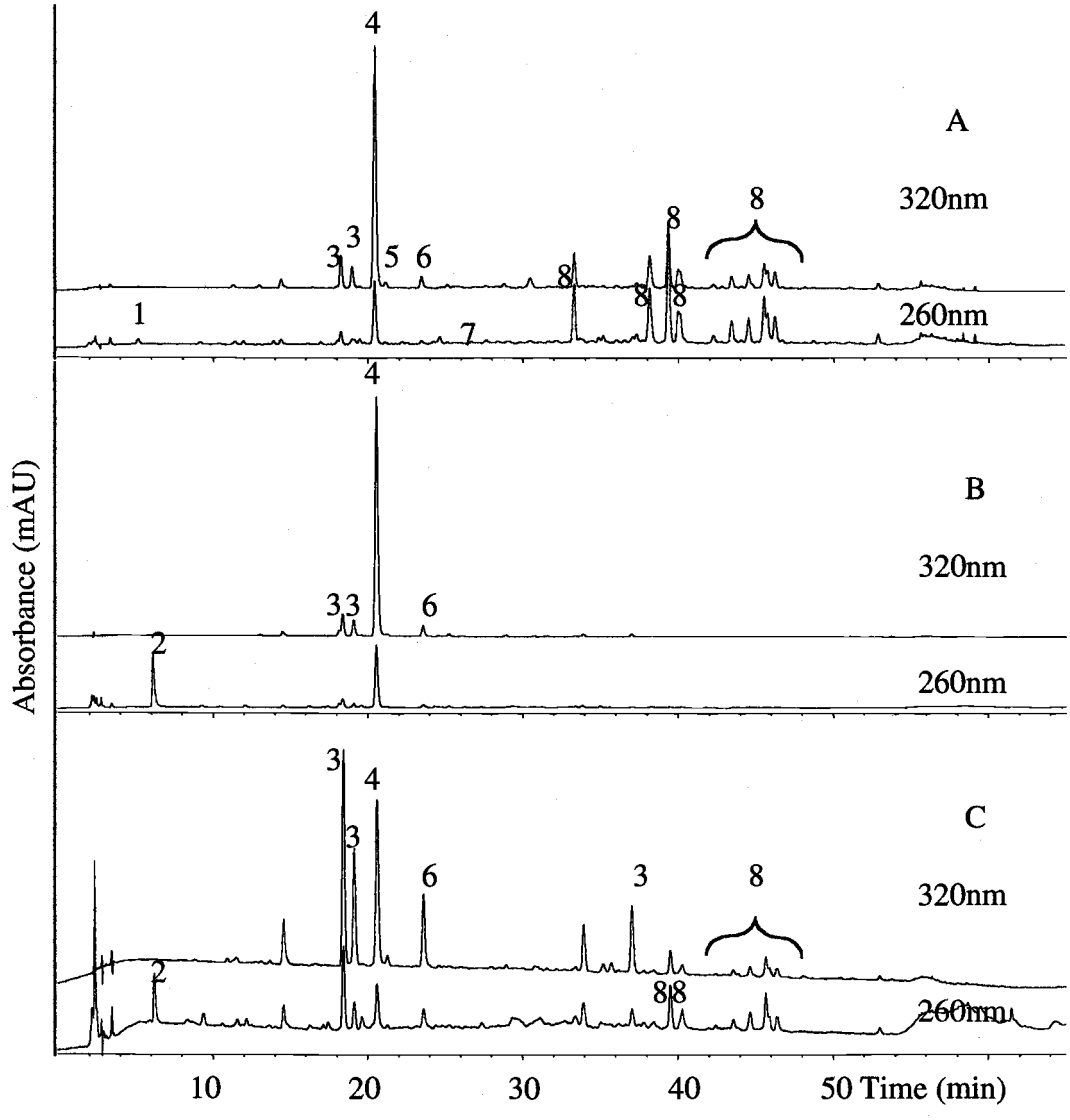


Figure 4.6. Polyphenolic profile of the three blueberry fractions (A: skins, B: flesh, and C: seeds) separated by LC (detection at 320 and 260 nm shown). Corresponding tentative peak assignments are as follows: 1: gallic acid, 2: residual acetone left from extraction, 3: cinnamic acid derivatives, 4: chlorogenic acid (5-caffeoylquinic acid), 5: 4-caffeoylquinic acid (chlorogenic isomer), 6: caffeic acid, 7: syringic acid, and 8: flavonol-glycosides.

The puree waste polyphenolic profile for the industrial blueberry juice processing wastes showed the puree stock to have higher amounts of cinnamic acids and flavonol-glycosides than the puree waste. Enzymatic destruction of polyphenolics in the puree is a possible explanation.

CONCLUSIONS

A combination of heat and SO_2 increased the extraction of anthocyanins and polyphenolics from whole berries and skins. Processing enzymes produced an extract from whole berries lower in % polymeric color but were not effective in extracting ACY and TP. However, enzymes were helpful in extraction of ACY and TP from skins. Blueberry skins were the highest in ACY, TP, and antioxidant activity compared to flesh and seed fractions. Blueberry processing waste is high in ACY and TP and has the potential to be a good source of natural colorants, and nutraceuticals. The findings will be useful to processors who would like to increase their recovery of anthocyanin and polyphenolic rich extracts, reduce by-product disposal, and enhance the value of their fruit commodities through more than one avenue.

ACKNOWLEDGEMENTS

We would like to thank Northwest Center for Small Fruit Research, Corvallis, OR for financial support, the Oregon Blueberry Commission for the berry samples, and the following companies for supplying the enzymes used in this study: AB enzyme, DSM Food Specialties USA, Inc., Novozyme, Valley Research, Inc. We would also like to thank The J.M. Smucker Co., Woodburn, OR for providing samples. Deborah Hobbs of the Linus Pauling Institute (Corvallis, OR) performed the ORAC and FRAP analysis.

Dr. Cliff Pereira, Kerstine Carter, and Joseph Scherer from the Statistics department of OSU assisted with the experimental design of the extraction experiment part of this study.

REFERENCES

American Institute for Cancer Research. 1997. Food, nutrition, and the prevention of cancer: a global perspective. Amer. Inst. Cancer Res. Washington, D.C.

Bakker, J., Bridle, P., Bellworthy, S.J., Garcia-Viguera, C., Reader, H.P., and Watkins, S.J. 1998. Effect of sulphur dioxide and must extraction on colour, phenolic composition and sensory quality of red table wine. J. Sci. Food Agric. 78:297-307.

Benzie, I.F.F. and Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239:70-76.

Bocevska, M. and Stevcevska, V. 1997. Quality evaluation of anthocyanin extract obtained from wine grape pomace. Food Technol. Bioltechnol. 35:139-143.

Cacae, J.E. and Mazza, G. 2002. Extraction of anthocyanins and other phenolics from black currants with sulfured water. J. Agric. Food Chem. 50:5939-5946.

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

Cao, G., Alessio, H.M., and Culter, R.G. 1993. Oxygen-radical absorbance capacity assay for antioxidants. Free Radical Biol. Med. 14:303-311.

Cao, G., Sofic, E., and Prior, R.L. 1996. Antioxidant capacity of tea and common vegetables. J. Agric. Food Chem. 44:3426-3431.

Coba, K. and Goodwin, J.A. 2003. Oregon Agri-Facts. Oregon Agricultural Statistics Service. Oregon Department of Agriculture. Portland, OR.

Criqui, M.H. and Ringel, B.L. 1994. Does diet or alcohol explain the French paradox? Lancet. 344:1719-1723.

Durst, R.W. and Wrolstad, R.E. 2001. Unit F1.3: Anthocyanins. Separation and characterization of anthocyanins by HPLC. In Current Protocols in Food Analytical Chemistry. RE Wrolstad (Ed.). New York: John Wiley & Sons, NY. p 1-13.

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. 49:2222-2227.

Fuleki, T. and Hope, G.W. 1964. Effect of various treatments on yield and composition of blueberry juice. Food Tech. 4:166-168.

Giusti, M.M. and Wrolstad, R.E. 2001. Unit F1.2: Anthocyanins. Characterization and measurement with UV-visible spectroscopy. In Current Protocols in Food Analytical Chemistry. RE Wrolstad (Ed.) New York: John Wiley & Sons. p 1-13.

Gu, L., Kelm, M., Hammerstone, J.F., Beecher, G., Cunningham, D., Vannozzi, S., and Prior, R.L. 2002. Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC-DAD-MS flourescent detection method. J. Agric. Food Chem. 50:4852-4860.

Hertog, M.G.L., Feskens, E.J.M., Hollman, P.C.H., Katan, M.R., and Kromhout, D. 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. The Lancet. 342:1007-1011.

Hertog, M.G.L., Feskens, E.J.M., Hollman, P.C.H., Katan, M.R., and Kromhout, D. 1994. Dietary antioxidant flavonoids and cancer risk in the Zutphen elderly study. Nutr. and Cancer. 22:175-184.

Hertog, M.G.L., Kronhout, D., Aravanis, C., Blackburn, H., Buzina, R., Fidanza, F., Giampaoli, S., Jansen, A., Menotti, A., Nedeljkovc, S., Pekkarinen, M., Simic, B.S., Toshima, H., Feskens, E.J.M., Hollman, P.C.H., and Katan, M.R. 1995. Flavonoid intake and long-term and risk of coronary heart disease and cancer risk in the seven countries study. Archives of International Medicine. 155:381-386.

Kader, F., Rovel, B., Girardin, M., and Metche, M. 1997. Mechanism of browning in fresh highbush blueberry fruit (*Vaccinium corymbosum* L). Role of blueberry polyphenol oxidase, chlorogenic acid and anthocyanins. J. Sci. Food Agric. 74:31-34.

Kader, F., Haluk , J., Nicolas, J., and Metche, M. 1998. Degradation of cyanidin-3-glucoside by blueberry polyphenol oxidase: kinetic studies and mechanisms. J. Agric. Food Chem. 46:3060-3065.

Kalt W. and Dufour, D. 1997. Health functionality of blueberries. HortTechnology 7:216-221.

Kalt W., McDonald, J.E., and Donner, H. 2000. Anthocyanins, phenolics, and antioxidant capacity of processed lowbush blueberry products. J. Food Sci. 65:390-393.

Landbo, A. 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, J., Durst, R.W. and Wrolstad, R.E. 2002. Impact of juice processing on blueberry anthocyanins and polyphenolics: comparison of two pretreatments. J. Food Sci. 67:1660-1667.

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, R.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.

Ness, A.R. and Powles, J.W. 1997. Fruit and vegetables, and cardiovascular disease: a review. Int. J. Epidemiol. 26:1-13.

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

Prior, R.L. 1998. Antioxidant capacity and health benefits of fruits and vegetables: blueberries, the leader of the pack. Proc. 32nd Ann. Open House North Carolina Blueberry Council. 32:3-12.

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 phenolic and anthocyanin 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 Spectrometry. J. Agric. Food Chem. 49:1270-1276.

Rodriguez-Saona, L.E. and Wrolstad, R.E. 2001. Unit F1.1: Anthocyanins. Extraction, Isolation and Purification of Anthocyanins. In Current Protocols in Food Analytical Chemistry. RE Wrolstad (Ed.) New York: John Wiley & Sons. p 1-11.

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

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

Stanley, R. and Miller, S. 2001. Enzymatic processing for the release of phenolic antioxidants from fruit waste. Biol. Act. Phyto. Food. 269:552-556.

Steinmetz, K.A. and Potter, J.D. 1991. Vegetables, fruit, and cancer. I. Epidemiology. Cancer Causes Contr. 2:325-357.

Wang, H., Cao, G., and Prior, R.L. 1996. Total antioxidant capacity of fruits. J. Agri. Food Chem. 44:701-705.

Waterhouse, A.L. 2002. Unit I1.1: Polyphenolics: Determination of Total Phenolics, p. 1-4. In: R.E. Wrolstad (ed.). Current Protocols in Food Analytical Chemistry. John Wiley & Sons, New York.

Wightman, J.D. and Wrolstad, R.E. 1995. Anthocyanin analysis as a measure of glycosidase activity in enzymes for juice processing. J. Food Sci. 60:862-867.

Wrolstad, R.E., Wightman, J.D., and Durst, R.W. 1994. Glycosidase activity of enzyme preparations used in fruit juice processing. Food Technol. 48:90-98.

Wrolstad, R.E. 2000. Anthocyanins, p.237-252. In: Natural Food Colorants. Science and Technology. Lauro, G.J. and Francis, F.J. Marcel Dekker, New York.

CHAPTER 5

ANTHOCYANIN PIGMENT AND TOTAL PHENOLIC CONTENT OF THREE *VACCINIUM* SPECIES NATIVE TO THE PACIFIC NORTHWEST OF NORTH AMERICA.

Jungmin Lee[1], Chad E. Finn[2], and Ronald E. Wrolstad[1]

[1]Department of Food Science and Technology
Oregon State University, Corvallis, OR 97331-6602
[2]USDA-ARS, HCRL, Northwest Center for Small Fruit Research
3420 NW Orchard Ave. Corvallis, OR 97330

Publication in HortScience
113 South West Street, Suite 200, Alexandria, VA 22314
In press

ABSTRACT

The total anthocyanin and total phenolic content of wild (samples from 4 populations) and cultivated (samples from 32 populations) Pacific Northwestern American *Vaccinium* species (*V. membranaceum*, *V. ovalifolium*, and *V. deliciosum*) were evaluated. The total monomeric anthocyanin content of all huckleberry samples analyzed ranged from 101 to 400 mg/100g (expressed as cyanidin-3-glucoside), and the total phenolics varied from 367 to 1286 mg/100g (expressed as gallic acid). Cluster analysis separated the samples into four different groups based on their anthocyanin and total phenolic content. Two groups had greater anthocyanin pigment and total phenolics; one consisted entirely of cultivated *V. ovalifolium* (LIG10, VAC485, VAC487, LIG33, LIG9, LIG2, and VAC349) and the other consisted of just cultivated *V. membranaceum* (LIG25). Significant variations in total anthocyanins, total phenolics, and the ratio of the total anthocyanins and total phenolics were observed among the different *V. membranaceum, V. ovalifolium,* and *V. deliciosum* populations cultivated in the Willamette Valley, OR. The profile of the individual anthocyanins of the wild *V. membranaceum*, wild *V. ovalifolium*, and *V. corymbosum* 'Rubel' were conducted by HPLC. The chromatograms of *V. membranaceum*, *V. ovalifolium*, and 'Rubel' were distinctly different in the amounts of delphinidin, cyanidin, and malvidin glycosides.

INTRODUCTION

The current interest in the possible health benefits of antioxidants has led many investigators to characterize, and determine the quantities of the antioxidants in many small fruit crops (Moyer et al., 2002; Kähkönen et al., 2001; Wada and Ou, 1992). Blueberries (*Vaccinium*) were one of the first berry crops to receive a great deal of

attention in part due to the longstanding recognition that consuming *V. myrtillus* L. (bilberry) fruit or extracts had health benefits (Kalt and Dufour, 1997). The high antioxidant capacity of blueberries has been highly correlated to their anthocyanin and total phenolic content (Kalt and Dufour, 1997; Prior et al., 1998; Kalt et al., 2000).

The Pacific Northwest of North America has 12-14 native species of *Vaccinium*, depending on taxonomic interpretation (Hitchcock and Cronquist, 1973; Vander Kloet, 1988). The fruits of these species are commonly referred to as "huckleberries" in the region, as are the fruits of a number of wild *Vaccinium* species in eastern North America. Both of these groups are distinct from the "true huckleberries", species of which belong to the genus *Gaylussacia* L. Three of the Pacific Northwest *Vaccinium* species, *V. membranaceum* Douglas ex Torrey, *V. ovalifolium* Smith, and *V. deliciosum* Piper, are commonly harvested from native stands for home consumption or commercial sale. In addition, in Pacific Northwest Native American cultures, "huckleberries" have medicinal and spiritual value beyond that of food (Moerman, 1998). These three species are in the *Vaccinium* section *Myrtillus* along with the bilberry, *V. myrtillus* L. (Vander Kloet, 1988). *Vaccinium deliciosum* and *V. membranaceum* are tetraploid, like cultivated highbush blueberry, while *V. ovalifolium* occurs in both diploid and tetraploid forms.

The cultivated blueberries in the section *Cyanococcus* produce several fruit in a cluster from each inflorescence bud. An abscission zone forms between each individual fruit and its pedicel (Vander Kloet, 1988). Nearly all of the anthocyanins are in the fruit skin. *Vaccinium* species in the section *Myrtillus* produce 1 to 2 fruit per bud, with no abscission zone between the individual fruit and pedicel, and the flesh and berry skin contains anthocyanins. The non-clustering fruiting habit of section *Myrtillus* species

makes harvesting labor-intensive and the tearing of the skin near the pedicel makes the fruit extremely perishable. The reliance on native stands for production means that the crop is subject to the vagaries of that year's environmental conditions. To have a more reliable crop, farmers are interested in commercializing Pacific Northwest huckleberry production in cultivated plantings. The potential of introgressing the fruit quality characteristics of the native species into commercially cultivated blueberries is also being pursued by the USDA-ARS in Corvallis, Oregon (C. Finn, pers. comm.) and by the University of Idaho in Sandpoint, Idaho (D. Barney, pers. comm.). Anthocyanin and phenolic content is an important criterion for identifying huckleberry populations suitable for breeding programs or commercialization.

Prior et al. (1998) studied 23 genotypes of four species (*V. corymbosum* L., *V. ashei* Reade, *V. angustifolium* Aiton, and *V. myrtillus*), Ehlenfeldt and Prior (2001) examined 87 *Vaccinium corymbosum* L. and hybrid genotypes, and Moyer et al. (2002) evaluated 30 genotypes of nine species (*V. angustifolium, V. constablaei* x *V. ashei, V. corymbosum, V. membranaceum, V. myrtilloides* Michaux, *V. ovalifolium, V. ovatum* Pursh, and *V. parvifolium* Smith) and reported their total anthocyanins and phenolics. This study sampled 36 populations *V. membranaceum*, *V. ovalifolium,* and *V. deliciosum* to survey and compare the total anthocyanins and total phenolic contents of huckleberry fruit. Reference comparison was made with 'Rubel' highbush blueberry. 'Rubel' was originally selected from native *V. corymbosum* growing in New Jersey and was a prominent parent in the development of present-day cultivars (Hancock and Siefker, 1982).

MATERIALS AND METHODS

Plant material

'Rubel', commercial grade A, frozen blueberry samples were obtained from the Oregon Blueberry Commission and represent machine-harvested berries for 2001, by a commercial grower in the Willamette Valley, OR. 'Rubel' fruit was used as a reference for this study. 'Rubel' fruit's new-found popularity with many processors is due to high pigment to volume content. The fruit for *V. membranaceum*, *V. ovalifolium*, and *V. deliciosum* were either harvested directly from wild stands ("wild huckleberry samples") or were harvested from plants grown in Corvallis (OR) from seed collected from wild stands ("cultivated huckleberry samples") (Finn and Young, 2002). The wild huckleberry samples (n = 4) from 1999 and 2001 were collected from one location in Oregon and two locations in Washington (Table 5.1). From each wild population, fully colored fruit were picked and stored on ice until they were frozen 1 to 3 days later. The cultivated huckleberries were harvested in 2002 from the U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS) plantings (Corvallis, OR). Cultivated huckleberries (n = 32 populations) were harvested in June and July 2002 from 19 populations of *V. membranaceum*, 12 populations of *V. ovalifolium*, and one population of *V. deliciosum*. These samples were collected as seeds directly from the wild in 1993 and 1994, or through the USDA-ARS National Clonal Germplasm Repository. Thirty-two genotypes from each population were planted in a randomized complete block design with eight genotypes in each replication. Details of the original collection locations are provided (Table 5.1). The huckleberries were established in 1995, with standard highbush blueberry management practices. Fruit were collected from all cultivated huckleberry plants that produced ripe berries and samples were

pooled by population. Berries representing a sample were collected from 1 to 10 genotypes per population (Table 5.1). Only one cultivated *V. deliciosum* produced fruit. The ripe berries were picked based on visual color and flavor. Samples were picked and placed in an icebox and immediately frozen upon arrival at the laboratory (within 2 hours of picking). All samples were kept frozen at –70°C until analysis.

Extraction

Anthocyanins and phenolics were extracted from frozen berries following the procedure described by Rodriguez-Saona and Wrolstad (2001). Samples were liquid nitrogen powdered using a mortar and pestle. Six g of powdered sample were sonicated with 12 mL of 100% acetone, followed by re-extraction with 70% (v/v) aqueous acetone until the solution became colorless. It was then partitioned with chloroform (1:2 acetone: chloroform v/v) to obtain the aqueous fraction. The aqueous portion was collected, and residual acetone present was evaporated by a Büchi rotovapor (Westbury, NY) at 40°C. The aqueous extract was dissolved to a final volume of 25 mL with distilled water. Samples were then stored at –70°C until analysis. Samples for anthocyanin separation by High Performance Liquid Chromatography (HPLC) were filtered through a 0.45 μm Millipore filter type HA (Millipore Corp., Bedford, MA) before HPLC injection. Hewlett-Packard HPLC-2D ChemStation software was used for data analysis.

Table 5.1. Locations, total monomeric anthocyanins (ACY), and total phenolic (TP) content of the samples.

	Location	State	Year[z]	ACY[y]	TP[x]	ACY/ TP	CA Abbrev.	Groups	No. of Genotypes/ sample[w]
Wild huckleberry samples (n=4)									
Vaccinium membranaceum[v]	Willamette NF ; Detroit Lake	OR	1999	223	489	0.46	99Vm	2	?
Vaccinium membranaceum[u]	Gifford Pinchot NF; Forlorn Lake	WA	2001	201	511	0.39	1Vm	2	?
Vaccinium ovalifolium[u]	Gifford Pinchot NF; Forlorn Lake	WA	2001	311	702	0.44	1Vo	4	?
Vaccinium delisciosum[u]	Gifford Pinchot NF; Cultus Creek	WA	2001	176	491	0.36	1Vd	2	?
			mean	*228*	*548*	*0.41*			
			S.D.	*59*	*103*	*0.05*			
	Original Location	State	Year	ACY	TP	ACY/ TP	CA Abbrev.		
Cultivated huckleberry samples (n=32)									
Vaccinium membranaceum (n=19)									
VAC427	Willamette NF ; Knutson Saddle	OR	2002	157	571	0.27	2Vm1	2	3
VAC370	Willamette NF ; Opal Lake	OR	2002	143	555	0.26	2Vm2	2	7
VAC382	Willamette NF ; Iron Mountain	OR	2002	144	541	0.27	2Vm3	2	4
VAC368	Deschutes NF; Corbette State Park	OR	2002	154	451	0.34	2Vm4	2	1
VAC432	Deschutes NF ; Link Lake	OR	2002	129	382	0.34	2Vm5	2	3
VAC426	Deschutes NF ; Cabot Lake	OR	2002	156	495	0.32	2Vm6	2	2
VAC425	Rogue River NF; Huckleberry Mountain	OR	2002	122	582	0.21	2Vm7	2	3
VAC429	Willamette NF	OR	2002	147	486	0.30	2Vm8	2	1
VAC423	Olympics NF; Mt. Washington	WA	2002	190	527	0.36	2Vm9	2	2

Table 5.1. . .Continued.

VAC416	Olympics NF; Buck Mountain	WA	2002	195	448	0.44	2Vm10	2	3
VAC398	Gifford Pinchot NF; La Wis Wis; Lily Lake	WA	2002	101	706	0.14	2Vm11	4	3
VAC385	Gifford Pinchot NF; Wind River	WA	2002	178	637	0.28	2Vm12	4	2
GP94-3-3	Mount Rainier NP; Paradise	WA	2002	212	530	0.40	2Vm13	2	3
GP94-4-2	Gifford Pinchot NF; Taklakh Lake	WA	2002	113	367	0.31	2Vm14	2	1
GP94-6-1	Gifford Pinchot NF; Killen Creek	WA	2002	146	512	0.29	2Vm15	2	1
LIG25	Wenatchee NF; E. of Stevens Pass	WA	2002	360	1286	0.28	2Vm16	1	2
LIG29	Wenatchee NF; between Steven Pass and Lake Wenatchee	WA	2002	274	716	0.38	2Vm17	4	1
LIG41	Gifford Pinchot NF; Mt. Adams Huck. Fields	WA	2002	127	371	0.34	2Vm18	2	1
VAC682	Lolo NF; Little Joe Creek	MT	2002	151	642	0.24	2Vm19	4	1
			mean	*168*	*569*	*0.30*			
			S.D.	*61*	*201*	*0.07*			
			Vaccinium ovalifolium (n=12)						
VAC392	Gifford Pinchot NF; Clear Creek	WA	2002	205	908	0.23	2Vo1	3	2
LIG12	Mt. Baker-Snoqualmie NF; Baker Lake	WA	2002	225	752	0.30	2Vo2	4	5
LIG10	Olympic NF; North Pt.	WA	2002	300	888	0.34	2Vo3	3	7
VAC485	Mt. Hood NF; Burnt Lake	OR	2002	354	1028	0.34	2Vo4	3	5
VAC487	Willamette NF; Cougar Reservoir	OR	2002	244	897	0.27	2Vo5	3	10

Table 5.1. . .Continued.

VAC344	Willamette NF; Salt Creek	OR	2002	185	1054	0.18	2Vo6	3	4
LIG33	Gifford Pinchot NF; Packwood	WA	2002	272	898	0.30	2Vo7	3	3
LIG9	Olympic NF; Mt. Muller	WA	2002	327	1015	0.32	2Vo8	3	5
LIG2	Olympic NF; SW Quilcene	WA	2002	400	1030	0.32	2Vo9	3	6
VAC349	Willamette NF; Quartz Creek	OR	2002	263	994	0.26	2Vo10	3	1
LIG3	Olympic NF; Lord's Lake Loop	WA	2002	201	693	0.29	2Vo11	4	1
LIG25	Wenatchee NF; E. of Stevens Pass	WA	2002	207	677	0.31	2Vo12	4	1
			mean	*265*	*903*	*0.29*			
			S.D.	*68*	*133*	*0.05*			
				Vaccinium deliciosum (n=1)					
VAC404	Olympic NF	WA	2002	160	413	0.39	2Vd1	2	1
				Highbush blueberry (n=1)					
V. corymbosum cv. 'Rubel'	Willamette Valley	OR	2001	230	738	0.31	R1	4	1

[z] Indicated the year of harvest.

[y] ACY was expressed as cyanidin-3-glucoside. Units were mg/100g of berries.

[x] TP was expressed as gallic acid equivalents. Units were mg/100g of berries.

[w] Number of individual genotypes that represent the population. For the samples collected from the wild, numerous bushes, often over 100 were sampled. However, it was impossible in the field to determine if these "bushes" were genetically distinct.

[v] Fruit collected by J. Lee and M. Redhead in 1999.

[u] Fruit collected by C. Finn, B. Fick, E. Finn, and I. Finn in 2001.

Abbreviations: NF= National Forest; NP= National Park; VAC= USDA-ARS National Clonal Germplasm Repository (Corvallis, OR) accessions; LIG= Collected by J. Luby, C. Finn, R. Harrison, and H. Hoover in 1993; GP= Collected by C. Finn or D. Barney in 1993; OR= Oregon; WA= Washington; MT= Montana; ACY= Total monomeric anthocyanin content; TP= Total phenolic content; ACY/TP= the ratio of total monomeric anthocyanin content versus total phenolics; CA Abbrev= Abbreviation of samples for cluster analysis; Groups assigned by cluster analysis.

Determination of total anthocyanins and total phenolics

Total monomeric anthocyanins (ACY) were determined using the pH differential method (Giusti and Wrolstad, 2001). Absorbance was measured at 520 and 700 nm. ACY was expressed as cyanidin-3-glucoside (molar extinction coefficient of 26,900 $L \cdot cm^{-1} \cdot mol^{-1}$ and molecular weight of 449.2 $g \cdot mol^{-1}$). The unit for ACY was mg of anthocyanin/100g of frozen berries. Total phenolics (TP) were measured by the Folin-Ciocalteu (FC) method (Waterhouse, 2002). Absorbance was measured at 765 nm. TP was expressed as gallic acid equivalents. The unit for TP was mg of gallic acid/100g of frozen berries. A Shimadzu 300 UV-visible spectrophotometer (Shimadzu Inc., Kyoto, Japan) and 1-cm-pathlength cells were used for both measurements. Measurements of ACY and TP on sample extracts were replicated 2 times.

HPLC separation of anthocyanins

Anthocyanins were separated by reversed-phase HPLC using a Hewlett-Packard 1090 (Agilent Technologies Inc, Wilmington, DE), equipped with a photodiode array detector. Absorbance spectra were recorded for all peaks. Flow rate was 1 mL/min and injection volume was 20 μL. Column temperature was maintained at 40°C. A Prodigy 5μm ODS (3) 100Å (250 × 4.6 mm) column, fitted with 4.0×3.0 mm i.d. guard column, from Phenomenex was used (Torrance, CA). Solvent A was 100% acetonitrile. Solvent B was 10% (v/v) acetic acid and 1% (v/v) phosphoric acid in water. All solvents were HPLC grade. The program used a linear gradient from 2 to 20% solvent A in 25 minutes; then a linear gradient of solvent A from 20 to 40% in 5 minutes, with simultaneous detection at 280, 320, and 520 nm (Durst and Wrolstad, 2001).

Statistical analyses

SPSS® (Chicago, IL) version 11.0 was used for the statistical analyses. Pearson correlation and cluster analysis were performed on the ACY and TP results. Initially, hierarchical cluster analysis was performed using squared Euclidean distance and Ward method, then a dendrogram was made regarding the individual samples. From the dendrogram (Figure 5.1), the decision was made to use four clusters. Four cluster solution was examined by K-means cluster (to create cluster membership) and discriminant analysis (to check proper clustering). Differences among group means were tested using the Tukey Honest Significant Difference (HSD) at $\alpha = 0.05$ level.

RESULTS AND DISCUSSION

Vaccinium membranaceum collected from a single location in the wild in 1999 had 223 mg of ACY/100g (Table 5.1). Fruit collected from single regions for each of the three species in 2001 had an ACY content from 176 to 311 mg/100g; *V. ovalifolium* had the highest ACY, *V. membranaceum* was next, and *V. deliciosum* had the least. The ACY for the wild-collected *V. ovalifolium* sample was the only one higher than 'Rubel' (230 mg ACY/100g, 738 mg TP/100g). All four of the huckleberry samples collected from the wild had lower TP than 'Rubel'. Samples collected in the wild could not be frozen immediately, so some degradation of ACY and TP could have occurred during the 1 to 3 days storage on ice before freezing.

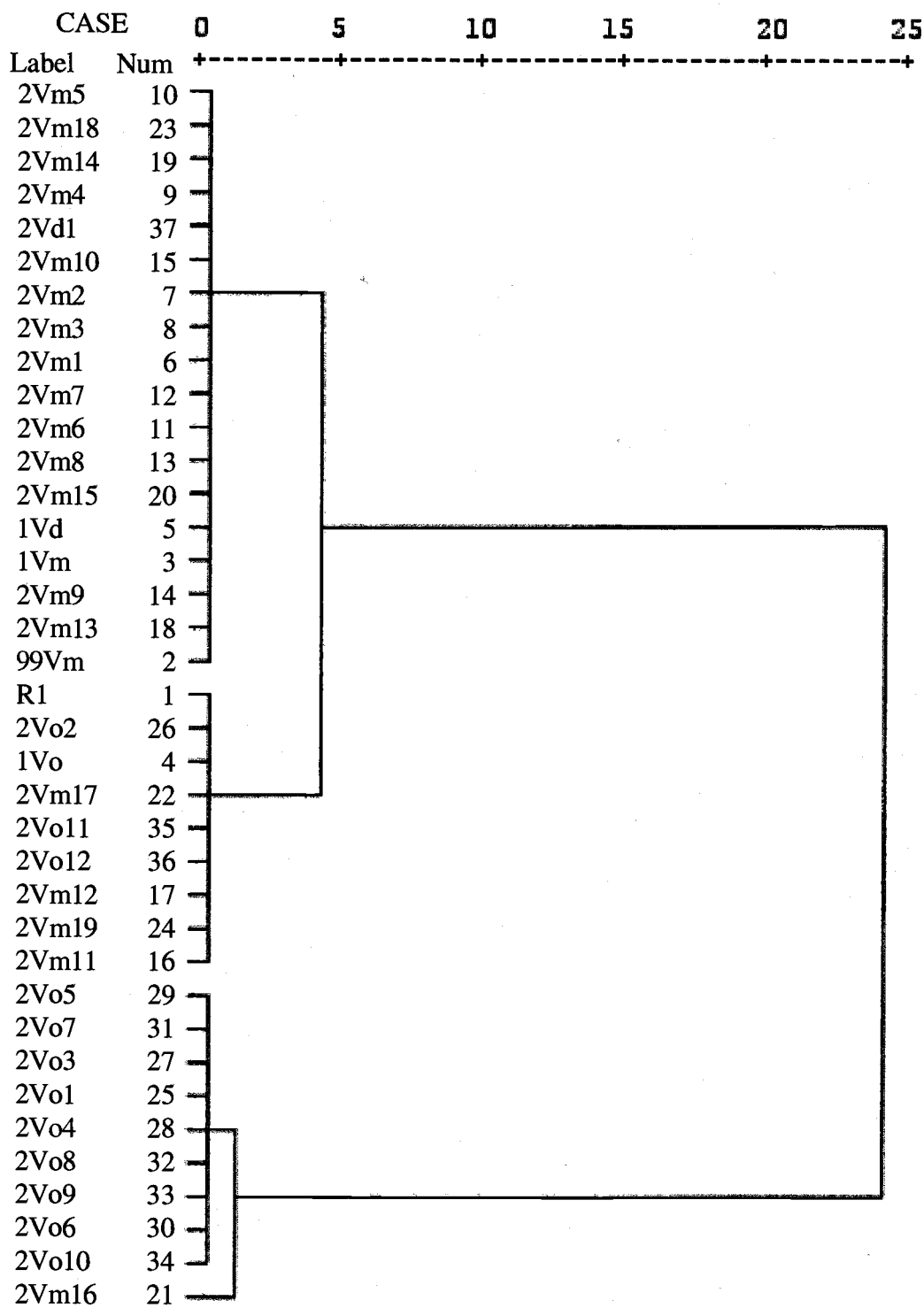


Figure 5.1. Dendrogram from cluster analysis of 37 fruit samples of *Vaccinium membranaceum*, *V. ovalifolium*, *V. deliciosum*, and 'Rubel' analyzed for total ACY and TP. The sample names for the abbreviations are listed in Table 5.1.

Samples from cultivated *V. membranaceum* had ACY contents ranging from 101 to 360 mg/100g (mean= 168 mg/100g). Samples from cultivated *V. ovalifolium* had ACY contents ranging from 185 to 354 mg/100g (mean= 265 mg/100g), which was similar to the values reported by Moyer et al. (2002). Only two samples (LIG25 and LIG29) of the 19 cultivated *V. membranaceum* samples had a higher ACY content than 'Rubel'. These two populations were originally collected from locations about 9 km apart. The majority (7 of 12) of the cultivated *V. ovalifolium* samples had greater ACY content than 'Rubel' (excluding VAC392, LIG12, VAC344, LIG3, and LIG25).

Total phenolic contents of cultivated *V. membranaceum* ranged from 367 to 1286 mg/100g (averaged 569 mg/100g). LIG25 was the only *V. membranaceum* sample that had more TP than 'Rubel' (43% more). Cultivated *V. ovalifolium* TP content ranged from 677 to 1054 mg/100g (averaged 903 mg/100g, which was higher than the value reported by Moyer et al. (2002)). The majority (10 of 12) of the cultivated *V. ovalifolium* samples had a higher TP than 'Rubel' (excluding LIG3 and LIG25). The single cultivated *V. deliciosum* sample had a TP of 413 mg/100g.

The average of ACY/TP of the wild huckleberry (0.41) samples were higher than the average ACY/TP of cultivated *V. membranaceum* (0.30), *V. ovalifolium* (0.29), and *V. deliciosum* (0.39) as well as 'Rubel' (0.31).

When ACY was plotted against TP (Figure 5.2), some generalizations within species could be made. Two of the three *V. membranaceum* samples originally from the Wenatchee National Forest (LIG25 and LIG29) had the highest ACY and TP contents of any samples. Samples from three (LIG10, LIG9, and LIG2) of the four *V. ovalifolium* populations from the Olympic National Forest were among the highest for ACY and TP. The fourth sample (LIG3) from this region was among the lowest of all *V.*

ovalifolium populations for these characters. The three *V. ovalifolium* samples (VAC487, VAC344, and VAC349) from the Willamette National Forest tended to have moderate ACY levels with fairly high TP levels. If variability for high ACY and TP content were targeted in germplasm collection or fruit harvest, the Wenatchee National Forest, particularly the region around Stephens Pass and Wenatchee Lake should be collected for *V. membranaceum*, and the Olympic National Forest, particularly in the mountain range above the Strait of Juan de Fuca should be similarly collected for *V. ovalifolium*.

The correlation between the ACY and TP was 0.774 ($P<0.01$). The huckleberry samples were divided into four distinct groups (or clusters) based on the cluster analysis (Figure 5.2 and Table 5.2). There was 0% misclassification of the samples. Group 1 had one sample (LIG25, cultivated *V. membranaceum*) with a distinctly high ACY and TP. The original collection notes for the location (Wenatchee NF; East of Stevens Pass) of LIG25 indicated that the population had what appeared to be hybrids between *V. ovalifolium* and *V. membranaceum*, which might explain the high ACY and TP content. The seeds might have been collected from plants that appeared to have vegetative characteristics of *V. membranaceum*, but fruit characteristics of *V. ovalifolium*. Also, the anthocyanin HPLC profile of LIG25 (Figure 5.3D), the cultivated *V. membranaceum*, was distinctly different from the wild *V. membranaceum* samples (Figure 5.3A) and other *V. membranaceum* (data not shown). LIG25's anthocyanin profile had elevated levels of the glucosides of delphinidin, cyanidin, petunidin, and malvidin (peaks 2, 5, 8, and 12) compared to the other *V. membranaceum* HPLC profiles. LIG25 had the anthocyanin profile that was more similar to *V. ovalifolium* (Figure 5.3B).

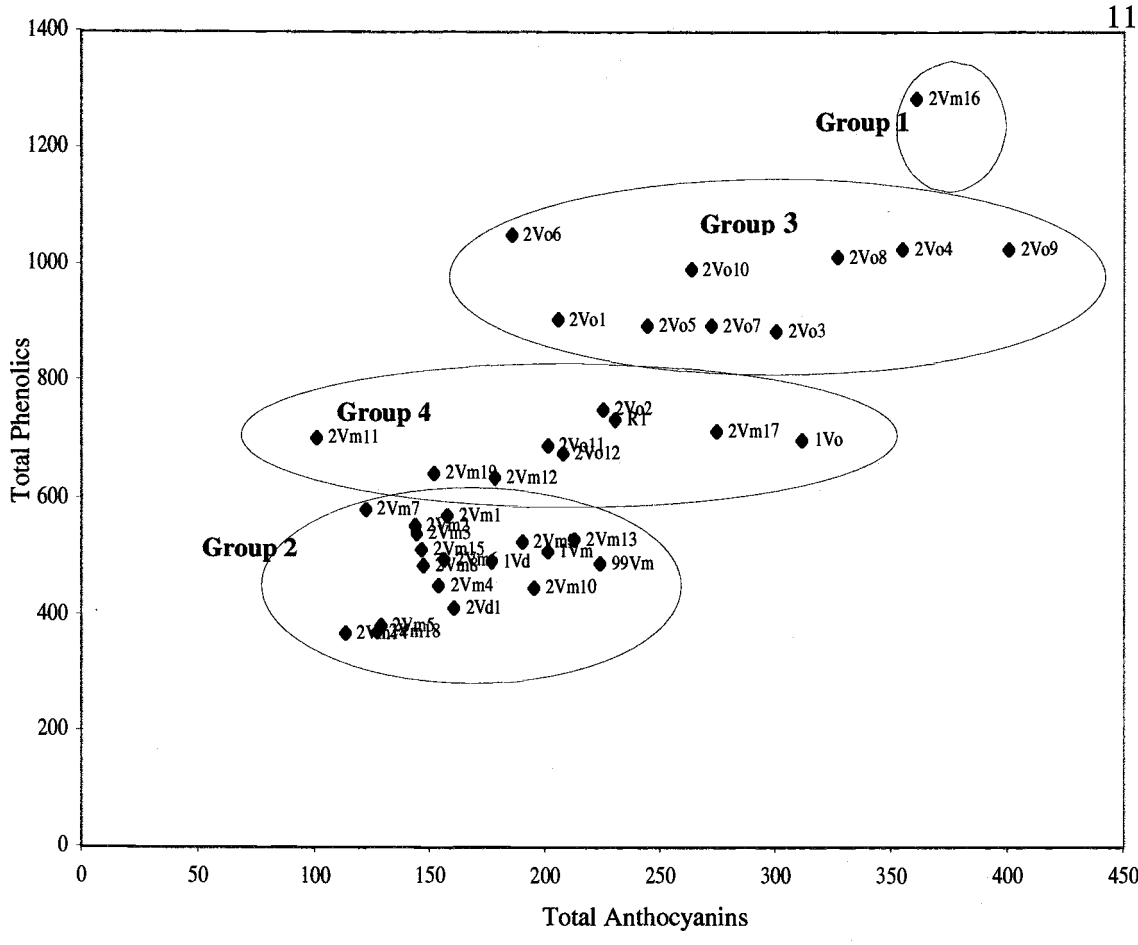


Figure 5.2. Discriminant analysis of all berry samples (n = 37) of *Vaccinium membranaceum*, *V. ovalifolium*, *V. deliciosum*, and 'Rubel', plotted TP versus ACY. Four clusters were obtained. The sample names corresponding to the abbreviations are listed in Table 5.1.

Table 5.2. The results of the Tukey HSD for the three groups obtained from the cluster analysis.

	Number of samples	ACY means[z]	TP means[z]	ACY/TP means[z]
Group 2	18	161[a]	485[A]	0.34[aa]
Group 3	9	284[b]	968[B]	0.28[aa]
Group 4	9	209[a]	696[C]	0.30[aa]

[z] Mean values with different superscript letters were significantly different at $P < 0.05$.

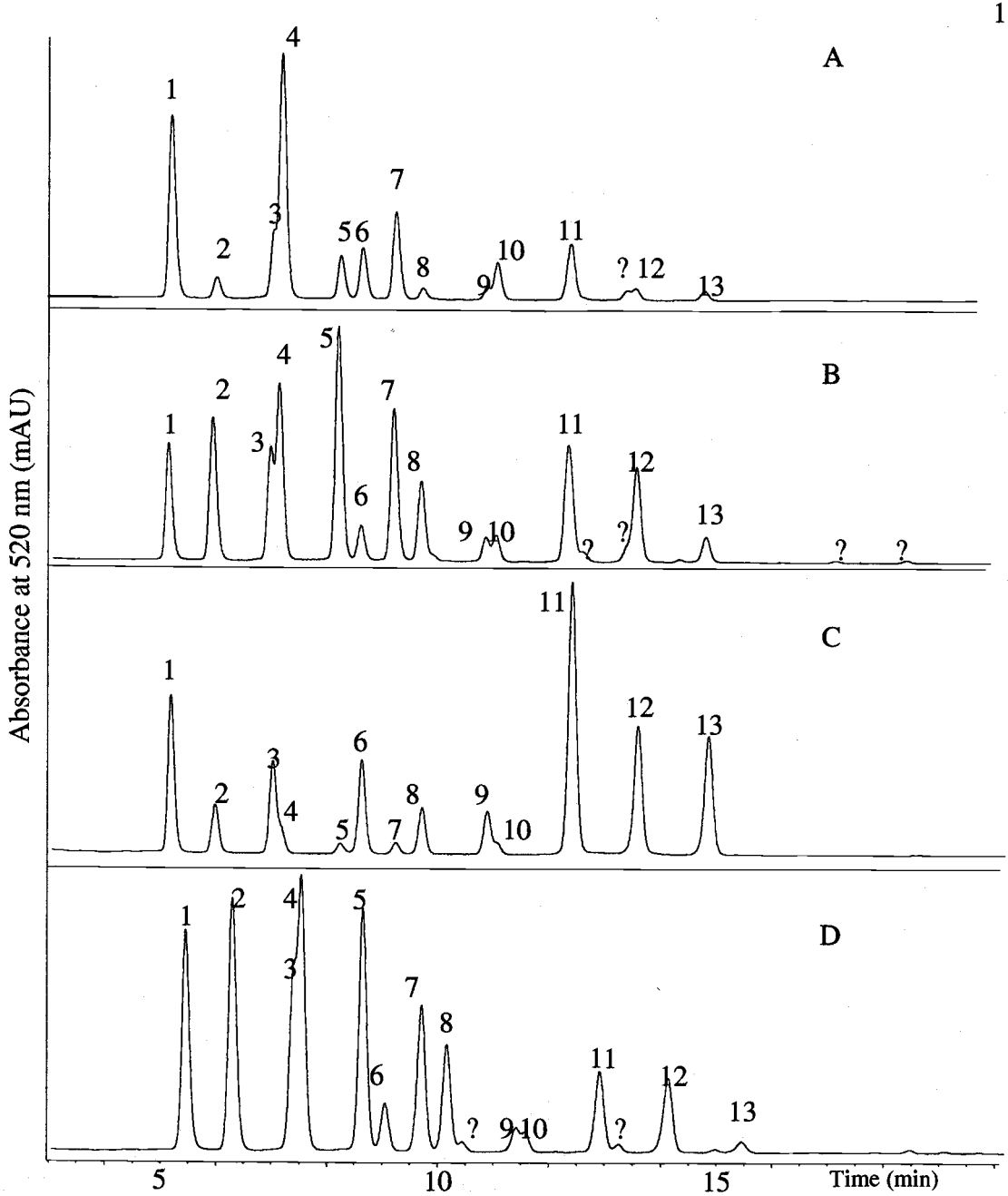


Figure 5.3. Anthocyanin HPLC profiles of (A) wild *V. membranaceum*, (B) wild *V. ovalifolium*, (C) 'Rubel' (*V. corymbosum*), and (D) LIG25 (cultivated *V. membranaceum*, the outlier). Tentative peak assignments: 1: delphinidin-3-galactoside, 2: delphinidin-3-glucoside, 3: delphinidin-3-arabinoside, 4: cyanidin-3-galactoside, 5: cyanidin-3-glucoside, 6: petunidin-3-galactoside, 7: cyanidin-3-arabinoside, 8: petunidin-3-glucoside, 9: petunidin-3-arabinoside, 10: peonidin-3-galactoside, 11: malvidin-3-galactoside, 12: malvidin-3-glucoside, 13: malvidin-3-arabinoside, and ?: unknowns.

Group 2 (n = 18) consisted of 14 cultivated *V. membranaceum*, two *V. deliciosum* samples (wild collected and cultivated VAC404), and both wild *V. membranaceum* samples. All of the cultivated *V. membranaceum* originally from Oregon were in group 2. Group 3 (n = 9) consisted only of cultivated *V. ovalifolium.* Group 4 (n = 9) contained 'Rubel', three cultivated *V. ovalifolium* (LIG12, LIG3, and LIG25), four cultivated *V. membranaceum* (VAC398, VAC385, LIG29, and VAC682), and the wild *V. ovalifolium.* Groups 1 and 3, which contains only cultivated huckleberries, appeared to have an overall higher ACY and TP than group 4, which contained 'Rubel'. All the huckleberries from the wild were either in group 2 or group 4. From the dendrogram, Group 1 (LIG 25, cultivated *V. membranaceum*) appeared to be an outlier and contained only one sample, so it was not included in the Tukey HSD analysis. Groups 2, 3, and 4 had significantly different TP averages. ACY of group 2 was significantly different from group 3 but not group 4. ACY/TP values of group 2, 3, and 4 were not significantly different.

In this study, bilberry (*Vaccinium myrtillus* L.) was not available. Prior et al. (1998) reported bilberry containing 330 mg of ACY/100g and 525 mg of TP/100g (using the same methods). Three of the cultivated huckleberries, LIG25 (*V. membranaceum*), VAC485 (*V. ovalifolium*), and LIG2 (*V. ovalifolium*) of this study had higher ACY and TP content than bilberry. All of the cultivated *V. ovalifolium* samples had a higher TP content than bilberry, as did 11 of the 19 cultivated *V. membranaceum* samples. High TP values are important, as the major non-anthocyanin flavonoid present in blueberries is chlorogenic acid (Lee et al., 2002), and it has been proven to possess a strong antioxidant activity (Zheng and Wang, 2003). Also, blueberry research by Prior et al. (1998) and Moyer et al. (2002) has shown a higher correlation between

antioxidant content and TP than between antioxidant content and ACY. Wild and cultivated samples of *V. deliciosum*, and 'Rubel' had lower ACY and TP content than Prior et al. (1998) reported for *V. myrtillus*. Care should be exercised when making comparisons of the ACY content of berries with other reported values, as researchers may use different standard coefficients and molecular weights to calculate the ACY. Kähkönen et al. (2001) reported the anthocyanin and TP values of bilberry obtained from three different growing locations. The anthocyanin content ranged from 2298 to 3090 mg /100g of dry matter (determined by HPLC) and the TP ranged from 3300 to 3820 mg /100g of dry matter (determined by FC method). Also, researchers have reported variation in anthocyanin and phenolic contents in blueberries due to different growing season and environmental factors (Conner et al., 2002; Clark et al., 2002; Prior et al., 1998). Cultivated huckleberry samples examined in this paper were from one growing season and a single location. In addition, the number of fruit per plant in the current study was, in general, too low, often zero, to allow a comparison of within and among population variation as has been done in blueberry (Conner et al., 2002). Based on these studies in blueberry, one would expect within population to be significant, but nonetheless, the best populations are likely sources of genotypes with the greatest ACY or TP levels.

The peak assignments of separation of anthocyanins by HPLC (Figure 5.3A-C) were made according to their Ultraviolet (UV)-visible spectra and retention time. All samples were analyzed by HPLC, but only two chromatograms that represented the general anthocyanin profile of *V. membranaceum* and *V. ovalifolium* samples are included in this paper. Although *V. membranaceum* samples had a lower ACY compared to *V. ovalifolium*, the individual anthocyanin present was distinctly different

when samples were examined by HPLC (Figure 5.3A and B). The major anthocyanins present in *V. membranaceum* samples were cyanidin glycosides (cyanidin-3-galactoside>>cyanidin-3-arabinoside>cyanidin-3-glucoside) and delphinidin glycosides (delphinidin-3-galactoside>>delphinidin-3-arabinoside>delphinidin-3-glucoside). *Vaccinium membranaceum* was low in malvidin glycosides. *Vaccinium ovalifolium* samples were high in delphinidin glycosides, cyanidin glycosides, and malvidin glycosides (galactoside, glucoside, and arabinoside of delphinidin, cyanidin, and malvidin were present in fairly even distribution). 'Rubel' was high in malvidin glycosides (galactoside, glucoside, and arabinoside of malvidin) and delphinidin glycosides (galactoside, glucoside, arabinoside of delphinidin), but low in the cyanidin glycosides. The qualitative analysis of anthocyanins agrees with identification work reported by Lee et al. (2002) and Ballington et al. (1987).

CONCLUSIONS

In conclusion, samples from *V. ovalifolium* and *V. membranaceum* populations grown in the Willamette Valley showed significant variation among samples and between the two species for ACY content, TP content, and the ACY/TP ratio. Fruit of *V. ovalifolium* generally had higher ACY and TP contents than *V. membranaceum* and TP content higher than 'Rubel'. *Vaccinium ovalifolium* fruit are smaller than *V. membranaceum,* which may explain some of the differences between the two species. A blueberry breeding program that is trying to develop selections with high ACY and TP could initially target genotypes within the best populations for more in-depth characterization to identify the best genotypes of *V. ovalifolium* and *V. membranaceum*

to use as a source of these traits although crossing barriers between sections *Cyanococcus* and *Myrtillus* are a continuing challenge.

ACKNOWLEDGEMENTS

We would like to thank the USDA- Forest Service for permission and advice in the process of collecting seed from National Forests in Oregon and Washington, and the USDA-Agricultural Research Service National Clonal Germplasm Repository for access to their seed collections for many of the populations grown in this study. We would also like to thank Mark Redhead for his help in numerous aspects of this project.

REFERENCES

Ballington, J.R., Ballinger, W.E., Maness, E.P., and Luby, J.J. 1988. Anthocyanin, aglycone and aglycone-sugar content of the fruits of five species of *Vaccinium* section *Myrtillus*. Can. J. Plant Sci. 68:241-246.

Clark, J.R., Howard, L., and Talcott, S. 2002. Variation in phytochemical composition of blueberry cultivars and breeding seasons. Acta Hort. 574:203-207.

Conner, A.M., Luby, J.J., Tong, C.B.S., Finn, C.E., and Hancock, J.F. 2002. Genotypic and environmental variation in antioxidant activity among blueberry cultivars. Acta Hort. 574:209-213.

Durst, R.W. and Wrolstad. R.E. 2001. Unit F1.2: Anthocyanins. Separation and characterization of anthocyanins by HPLC, p. 1-13. In: R.E. Wrolstad (ed.). Current Protocols in Food Analytical Chemistry. John Wiley & Sons, New York.

Ehlenfeldt, M.K. and Prior, R.L. 2001. Oxygen Radical Absorbance Capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf of highbush blueberry. J. Agric. Food Chem. 49:2222-2227.

Finn, C.E. and Young, A. 2002. Variability for reproductive and vegetative traits in *Vaccinium membranaceum.* Acta Hort. 574:181-187.

Giusti, M. and Wrolstad, R.E. 2001. Unit F1.2: Anthocyanins. Characterization and measurement with UV-visible spectroscopy, p. 1-13. In: R.E. Wrolstad (ed.). Current Protocols in Food Analytical Chemistry. John Wiley & Sons, New York.

Hancock J.F. and Siefker, J.H. 1982. Levels of inbreeding in highbush blueberry cultivars. HortScience 17:363-366.

Hitchcock, C.L. and Cronquist. A. 1973. Flora of the Pacific Northwest. University of Washington Press, Seattle, Wash.

Howard, L.R., Clark, J.R., and Brownmiller, C. 2003. Antioxidant capacity and phenolic content in blueberry as affected by genotype and growing season. J. Sci. Food and Agri. 83:1238-1247.

Kähkönen, M.P., Hopia, A.I., and Heinonen, M. 2001. Berry phenolics and their antioxidant activity. J. Agric. Food Chem. 49:4076-4082.

Kalt W. and Dufour, D. 1997. Health functionality of blueberries. HortTechnology 7:216-221.

Kalt W., McDonald, J.E., and Donner, H. 2000. Anthocyanins, phenolics, and antioxidant capacity of processed lowbush blueberry products. J. Food Sci. 65:390-393.

Lee, J., Durst, R.W. and Wrolstad, R.E. 2002. Impact of juice processing on blueberry anthocyanins and polyphenolics: comparison of two pretreatments. J. Food Sci. 67:1660-1667.

Moerman, D.E. 1998. Native American Ethnobotany. Timber Press, Portland, Ore.

Moyer, R.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.

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 phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. J. Agric. Food Chem. 46:2686-2693.

Rodriguez-Saona, L. and Wrolstad, R.E. 2001. Unit F1.1: Anthocyanins. Extraction, isolation and purification of anthocyanins, p. 1-11. In Current Protocols in Food Analytical Chemistry. R.E. Wrolstad (ed.). John Wiley & Sons, New York.

Vander Kloet, S.P. 1988. The Genus Vaccinium in North America. Publication 1828, Research branch, Agriculture Canada.

Wada, L. and Ou, B. 2002. Antioxidant activity and phenolic content of Oregon caneberries. J. Agric. Food Chem. 50:3495-3500.

Waterhouse, A.L. 2002. Unit I1.1: Polyphenolics: Determination of Total Phenolics, p. 1-4. In: R.E. Wrolstad (ed.). Current Protocols in Food Analytical Chemistry. John Wiley & Sons, New York.

Zheng, W. and Wang, S.Y. 2003. Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. J. Agric. Food Chem. 51:502-509.

CHAPTER 6

COMPARISON OF ANTHOCYANIN PIGMENT AND OTHER PHENOLIC COMPOUNDS OF *VACCINIUM MEMBRANACEUM* AND *V. OVATUM* NATIVE TO THE PACIFIC NORTHWEST OF NORTH AMERICA

Jungmin Lee[1], Chad E. Finn[2], and Ronald E. Wrolstad[1]

[1]Department of Food Science and Technology
Oregon State University, Corvallis, OR 97331-6602
[2]USDA-ARS, HCRL, Northwest Center for Small Fruit Research
3420 NW Orchard Ave. Corvallis, OR 97330

Submitted to HortScience
113 South West Street, Suite 200, Alexandria, VA 22314

ABSTRACT

Two huckleberry species, *Vaccinium membranaceum* and *V. ovatum*, native to Pacific Northwestern North America, were evaluated for their total, and individual, anthocyanin and polyphenolic compositions. *Vaccinium ovatum* had greater total anthocyanin (ACY), total phenolics (TP), and antioxidant activity (ORAC and FRAP values) than did *V. membranaceum*. The pH and °Brix were also higher in *V. ovatum.* Berry extracts from each species were separated into three different fractions: anthocyanin, polyphenolic, and sugar/acid by solid phase extraction. The anthocyanin fractions of each species had the highest amount of ACY, TP, and antioxidant activity. Each species contained 15 anthocyanins (galactoside, glucoside, and arabinoside of delphinidin, cyanidin, petunidin, peonidin, and malvidin), but in different proportions. Their anthocyanin profiles were similar by LC-DAD and LC-DAD-MS. Each species had a different polyphenolic profile. The polyphenolics of both species were mainly composed of cinnamic acid derivatives and flavonol-glycosides. The major polyphenolic compound in *V. membranaceum* was neochlorogenic acid; in *V. ovatum,* chlorogenic acid.

INTRODUCTION

Anthocyanins and polyphenolics are secondary metabolites of plants. They are a diverse group (> 4000 flavonoids have been identified). These compounds contribute to food quality through appearance, taste, and health benefits (Strack and Wray, 1994). The health benefits of blueberries have been linked to their anthocyanin and phenolic contents (Kalt and Dufour, 1997).

Research in blueberries has generated interest in other native *Vaccinium* sp., particularly the *Vaccinium* sp. called "huckleberries" and "bilberries", which have been shown to be high in anthocyanins and other phenolics compared to highbush blueberries (Lee et al., 2003). *Vaccinium myrtillus* L. (bilberry) fruit has one of the highest anthocyanin contents of berries examined (Prior et al., 1998). *Vaccinium ovatum* from section Pyxothamnus and *V. membranaceum* Douglas ex Torrey from section Myrtillus were examined in this study. *Vaccinium ovatum* (evergreen huckleberry) is the dominant huckleberry along the Pacific Coast of North America and is closely related to *V. consanguineum* Klotsch, native to Central America and *V. floribundum* Kunth, native to Andean S. America. *Vaccinium floribundum* is wild harvested in Andean countries and sold locally as fresh or processed fruit (Popenoe, 1924; C. Finn, pers. comm.). *Vaccinium membranaceum* (black huckleberry) grows in forested montane areas of Washington, Oregon, and Idaho (Vander Kloet, 1988).

Vaccinium membranaceum has been an important part of Native Americans' diet, and limited amounts of their fruits are commercially available as canned or frozen berries, and as preserves (Vander Kloet, 1988). The stems and leaves of *V. ovatum* have had more popularity in floral arrangements than its edible fruit (Camp, 1945; Vander Kloet, 1988). In Oregon, *V. ovatum* has recently been planted in commercial fields for their edible berries (C. Finn, pers. comm.). A small commercial huckleberry industry was based on established, managed fields in the early 1900's (Moll, 1933; Postman, 2004). Little is known about the chemical composition of *V. ovatum* fruit (Ballington et al., 1988; Moyer et al., 2002).

The objective of this study was to compare chemical composition, anthocyanin and polyphenolic profiles, and antioxidant properties of *V. ovatum* to *V. membranaceum*.

MATERIALS AND METHODS

Plant material

Berries of both species were harvested from plants grown at the U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS) Corvallis, OR from seed collected from wild stands. The huckleberries were established in 1995 using standard highbush blueberry practices. *Vaccinium membranaceum* berries were collected in June 2003 while *V. ovatum* berries were harvested during August 2003. Samples were picked and placed in an icebox and immediately frozen upon arrival at the laboratory (within 2 hours of picking). All samples were stored at –70°C until analysis. All visually ripe berries were picked and pooled by species (n > 9 genotypes for both species) for this study.

Reagents and standards

Chlorogenic acid, caffeic acid, protocatechuic acid, catechin, epicatechin, vanillic acid, gallic acid, and ß-phycoerythrin were obtained from Sigma Chemical Co. (St. Louis, MO). All solvents for this investigation were high performance liquid chromatography (HPLC) grade. Chlorogenic acid isomers were obtained by the method described by Nagels et al. (1980). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and TPTZ (tripyridyltriazine) were purchased from Fluka (Buchs,

Switzerland). AAPH [2,2'-azobis(2-amidinopropane)dihydrochloride] was purchased from Wako (Richmond, VA).

Extraction

Anthocyanins and polyphenolics were extracted from frozen berries following the procedure described by Rodriguez-Saona and Wrolstad (2001). Samples were liquid nitrogen powdered using a mortar and pestle. Six g of powdered sample were sonicated with 12 mL of 100% acetone, followed by re-extraction with 70% (v/v) aqueous acetone until the solution became colorless, it was then partitioned with chloroform (1:2 acetone: chloroform v/v) to obtain the aqueous fraction. The aqueous portion was collected, and residual acetone present was evaporated with a Büchi rotovapor (Westbury, N.Y.) at 40°C. The aqueous extract was dissolved to a final volume of 25 mL with distilled water. Samples were then stored at –70°C until analysis. Samples for anthocyanin separation by HPLC were filtered through a 0.45 μm Millipore filter type HA (Millipore Corp., Bedford, MA) before HPLC injection. Hewlett-Packard HPLC-2D ChemStation software was used for data analysis.

Berry size, titratable acidity (TA), pH, and °Brix

Berry size was determined by counting the number of berries per 100 g sample. Berry samples for determining TA, pH, and °Brix were initially homogenized by a mortar and pestle. TA was determined by titrating sample (5 g of homogenate + 45 mL of CO_2-free distilled water) with standardized 0.1N NaOH to pH 8.1 using a pH meter. TA was expressed as citric acid equivalents (g citric acid/100 g berries). The pH of the homogenate was determined using a Corning pH meter 340 (Corning Inc., Corning,

NY) equipped with a Corning electrode. The homogenates were centrifuged and the supernatants were used in determining percent soluble solids. An auto Abbe refractometer model 10500 (Reighert-Jung, Lecia Inc., Buffalo, NY) was used to measure °Brix in % solids and temperature-controlled mode.

Solid phase extraction (SPE)

Aqueous berry extracts were passed through a C-18 Sep-Pak mini-column (Waters Associates, Milford, MA), rinsed with methanol, and activated with deionized water. SPE was performed as described in Rodriguez-Saona and Wrolstad (2001).

Anthocyanins and polyphenolics were absorbed onto the column while sugars, acids, and other polar compounds that eluted with water (fraction 1) were removed and collected. The polyphenolic fraction (fraction 2) was obtained by eluting with 2 mL of ethyl acetate. Ethyl acetate was removed from the fraction with the Büchi rotovapor at 40 °C, and the residue was re-dissolved in 2 mL deionized water. This fraction was filtered and injected into the LC for polyphenolics separation. Anthocyanins (fraction 3) were collected with acidified (0.01% HCl) methanol. The methanol was evaporated using a Büchi rotovapor at 40 °C. Pigments (fraction 3) were re-dissolved in 2 mL acidified water (0.01% HCl). This fraction was also used for LC-MS analysis after filtering samples. All fractions were kept at -70°C until further analysis.

Determination of total anthocyanins and total phenolics

Total monomeric anthocyanins (ACY) were determined using the pH differential method (Giusti and Wrolstad, 2001). Absorbance was measured at 520 and 700 nm. ACY was expressed as cyanidin-3-glucoside (molar extinction coefficient of

26,900 $L \cdot cm^{-1} \cdot mol^{-1}$ and molecular weight of 449.2 $g \cdot mol^{-1}$). The unit for ACY was mg of anthocyanin/100 g of frozen berries. Total phenolics (TP) were measured by the Folin-Ciocalteu (FC) method (Waterhouse, 2002). Absorbance was measured at 765 nm. TP was expressed as mg of gallic acid/100 g of frozen berries. A Shimadzu 300 UV-visible spectrophotometer (Shimadzu Inc., Kyoto, Japan) and 1-cm-pathlength cells were used for both measurements. Measurements of ACY and TP on sample extracts were replicated 2 times.

Determination of antioxidant activity

Antioxidant activities of extracts were determined by ferric reducing antioxidant potential (FRAP) and oxygen radical absorbing capacity (ORAC) assays. FRAP assays were performed as described by Benzie and Strain (1996), utilizing a 96-well ThermoMax microplate spectrophotometer (Molecular Devices, Foster City, CA) to measure the formation of ferrous-TPTZ complex (λ_{max}=595 nm). FRAP measures the extract's ability to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) in a solution of TPTZ prepared in sodium acetate at pH 3.6. Absorbance was measured at 595 nm. ORAC assays followed the method described by Cao et al. (1993), with the alteration of utilizing a 96-well Cytofluor 4000 microplate fluorometer (PerSeptive Biosystems, Framingham, MA), which records rate and duration of fluorescence. β-Phycoerythrin acted as a target for the peroxyl radicals generated by AAPH (a peroxyl radical generator that destroys the fluorescence). Samples were monitored at 2 minute intervals, for 2 hours, at 485 nm (excitation wavelength) and 585 nm (emission wavelength). FRAP and ORAC values were expressed as μmol of Trolox (a water soluble tocopherol analogue) equivalents / g of frozen fruit.

LC-DAD of anthocyanins and polyphenolics

Anthocyanins and polyphenolics were separated by reversed-phase HPLC using a Hewlett-Packard 1090 (Agilent Technologies Inc, Wilmington, Del.), equipped with a photodiode array detector (DAD). Absorbance spectra were recorded for all peaks. Flow rate was 1 mL/min.

Anthocyanin. A Prodigy 5μm ODS (3) 100Å (250 × 4.6 mm) column, fitted with 4.0×3.0 mm i.d. guard column, from Phenomenex (Torrance, CA) was used. Solvent A was 100% acetonitrile. Solvent B was 10% (v/v) acetic acid and 1% (v/v) phosphoric acid in water. The program used a linear gradient from 2 to 20% solvent A in 25 minutes; then a linear gradient of solvent A from 20 to 40% in 5 minutes, with simultaneous detection at 280, 320, and 520 nm (Durst and Wrolstad, 2001). Injection volume was 20 μL. Column temperature was maintained at 40°C.

Polyphenolics. A Synergi 4μm Hydro-RP 80Å (250 × 4.60 mm) column, fitted with 4.0×3.0 mm i.d. guard column, from Phenomenex (Torrance, CA) was used. Solvent A was 100% acetonitrile. Solvent B was 1% (v/v) formic acid in water. The program used a linear gradient from 5% to 25% solvent A in 50 minutes; then a linear gradient of solvent A from 25% to 50% in 5 minutes, then held for 5 minutes, with simultaneous detection at 260, 280, 320, and 520 nm. Injection volume was 100 μL.

LC-MS of anthocyanins

Hewlett-Packard 1090 (Agilent Technologies Inc, Wilmington, DE), equipped with a photodiode array detector (DAD) and mass spectrometer (MS) was used to confirm the identification of the huckleberry anthocyanins. A Synergi 4μ Hydro-RP

80Å (250×2mm, 4μmicron) column, fitted with 4.0×3.0 mm i.d. guard column, from Phenomenex (Torrance, CA) was used. Absorbance spectra were collected for all peaks. Flow rate was 0.2mL/min and injection volume was 20μL. Solvent A was 5% formic acid and 80% acetonitrile (v/v), and solvent B was 5% formic acid. The initial solvent composition was 10% solvent A and 90% solvent B; then a linear gradient of 10% to 30% solvent A, and 90% to 70% solvent B in 30 minutes. Detection occurred simultaneously at 280, 320, and 520 nm. MS analysis was performed using a Perkin-Elmer SCIEX API III (Toronto, Canada), equipped with an ion spray source (ISV= 5500, orifice voltage= 50) in positive ion mode.

RESULTS AND DISCUSSION

The chemical composition of the two species is summarized (Table 6.1). *Vaccinium ovatum* (446 berries/100 g) had smaller fruits than did *V. membranaceum* (296 berries/100 g). This might account for the high ACY and TP content of the *V. ovatum* fruit, since smaller berries mean a greater proportion of skin, where the anthocyanins are predominately found, for equal masses of fruit. The total monomeric anthocyanin content of *V. ovatum* (563 mg of cyanidin-3-glucoside/100 g of frozen berries) was threefold greater than *V. membranaceum* (167 mg of cyanidin-3-glucoside/100 g of frozen berries). *Vaccinium ovatum* also had higher total phenolics content (1169 mg of gallic acid/100 g of frozen berries) than *V. membranaceum* (617 mg of gallic acid/100 g of frozen berries). Moyer et al. (2002) reported ACY content of two genotypes of *V. ovatum* to be 336 and 357 mg/100 g and 641 and 842 mg/100 g of TP. Also, Moyer et al. (2002) reported that *V. membranaceum* to contained 110 to 153 mg/100 g of ACY and 225 to 423 mg/100 g of TP using the same methodology.

Table 6.1. Chemical composition of fruit from *Vaccinium membranaceum* and *V. ovatum* grown in test plots in Corvallis, OR.

	V. membranaceum[a]	*V. ovatum*[b]
pH	2.6	2.8
TA [c]	1.73 (± 0.03)	1.63 (± 0.01)
°Brix	12.7	19.6
°Brix / TA (Sugar-acid ratio)	7.3	12.0
ACY[d]	167 (± 1)	563 (± 5)
TP[e]	617 (± 4)	1169 (± 10)
ACY / TP	0.271	0.482
Berry size (# berries/100 g)	296 (± 23)	446 (± 31)
FRAP values[f]	42.1	137.2
ORAC values[f]	26.2	103.4
Visual observation of berries	Pigment present in flesh and skin. Reddish-black berries.	Pigment mainly in the skin. Black berries.

Values in parentheses are standard deviations.

[a]: Fruit collected by T. Mackey in 2003.

[b]: Fruit collected by M. Redhead in 2003.

[c]: Titratable acidity (TA) expressed as g of citric acid/100 g of berries.

[d]: Total monomeric anthocyanin content (ACY) expressed as mg of cyanidin-3-glucoside (MW = 449.2 and extinction coefficient = 26,900)/100 g of berries.

[e]: Total phenolics (TP) expressed as mg of gallic acid/100 g of berries.

[f]: expressed as µmol of Trolox/ 1 g of berries.

Vaccinium ovatum fruits were high in ACY and TP when compared to those of the bilberry (*V. myrtillus*), which was not available for this study, but Prior et al. (1998) reported bilberry to contain 330 mg of ACY/100 g and 525 mg of TP/100 g. *Vaccinium ovatum* had a higher pH (2.8) and lower titratable acidity (1.63 g citric acid/100 g of berries) values compared to *V. membranaceum* (pH=2.6 and TA=1.73 g citric acid/100 g of berries). *Vaccinium ovatum* (19.6 °Brix) had higher percent soluble solids than *V. membranaceum* (12.7 °Brix). *Vaccinium membranaceum* (7.3) had a lower sugar-acid ratio, which might contribute towards better keeping qualities when compared to the *V. ovatum* (12.0), since low sugar-acid ratio has been shown to be a good indicator for keeping qualities (Kushman and Ballinger, 1968). The FRAP and ORAC values were three and four times higher in *V. ovatum* samples, which was expected, as *V. ovatum* fruit had higher ACY and TP than *V. membranaceum* fruit.

Cultivated huckleberry samples were taken from a single growing location and growing season. Numerous research groups have reported anthocyanin and phenolic contents in blueberry samples differing due to growing season and environmental factors (Conner et al., 2002; Clark et al., 2002; Prior et al., 1998).

Berry extracts from both species were further fractionated by solid phase extraction with a C_{18} Sep-Pak mini-column. The corresponding ACY, TP, FRAP, and ORAC values are shown (Table 6.2). While the majority of the anthocyanins were present in the anthocyanin fraction, as expected, there were some anthocyanins present in the polyphenolics and sugar/acid fractions. The anthocyanin fraction had the highest TP value, compared to the polyphenolic fraction and sugar/acid fraction. The antioxidant activity of the sugar/acid fraction could be due to water-soluble compounds (such as sugars, acids, ascorbic acid, and glutathione) that possess antioxidant activities

(Zheng and Wang, 2003; Cao and Prior, 1999). The anthocyanin fractions appeared to be the major contributor to FRAP and ORAC values, while the sugar/acid fractions contributed the least. In most cases, FRAP analyses indicated higher antioxidant measurements than did ORAC analyses.

Table 6.2. Anthocyanin, total phenolics, and antioxidant activity of fractions obtained from solid phase extraction of berries from two *Vaccinium* species.

	V. membranaceum[a]			*V. ovatum*[b]		
	Anthocyanin fraction	Polyphenolic fraction	Sugar/acid fraction	Anthocyanin fraction	Polyphenolic fraction	Sugar/acid fraction
ACY[c]	104 (± 1)	6 (± 1)	14 (± 7)	361 (± 1)	43 (± 1)	21 (± 0)
TP[d]	241 (± 2)	153 (± 5)	8 (± 0)	625 (± 8)	354 (± 2)	41 (± 19)
FRAP[e]	23.8	11.8	4.1	73.6	32.4	6.0
ORAC[f]	15.7	9.9	3.1	44.9	24.8	6.3

[a]: Fruit collected by T. Mackey in 2003.

[b]: Fruit collected by M. Redhead in 2003.

[c]: Total monomeric anthocyanin content (ACY) expressed as mg of cyanidin-3-glucoside (MW = 449.2 and extinction coefficient = 26,900)/100 g of berries.

[d]: Total phenolics content (TP) expressed as mg of gallic acid/100 g of berries.

[e]: Ferric Reducing Antioxidant Potential (FRAP) expressed as µmol of Trolox/ 1 g of berries.

[f]: Oxygen Radical Absorbing Capacity (ORAC) expressed as µmol of Trolox/ 1 g of berries.

The anthocyanin profiles obtained from the LC-DAD of the two species appear to differ slightly (Figure 6.1). Peak assignments were made based on their spectra and retention time. Both species had the same qualitative composition with different proportions of peak areas. From the LC-DAD results, *V. membranaceum* and *V. ovatum* had the same 13 different anthocyanins present. *Vaccinium membranaceum* had cyanidin-glycosides (50.0%), delphinidin-glycosides (27.4%), malvidin-glycosides (10.0%), petunidin-glycosides (7.9%), and peonidin-glycosides (4.7%), in the order of most abundance (based on % peak area). *Vaccinium ovatum* had cyanidin-glycosides (71.6%), delphinidin-glycosides (12.9%), peonidin-glycosides (6.9%), malvidin-glycosides (4.8%), and petunidin-glycosides (3.9%), in the order of most abundance (based on % peak area). The major anthocyanin present in both species was cyanindin-3-galactoside (peak 4, Figure 6.1), which confirms the findings of Ballington et al. (1988a; 1998). Ballington et al. reported that 15 galactosides, glucosides, and arabinosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin were present in both species. *Vaccinium membranaceum* had higher amounts of delphinidin-galactoside than did *V. ovatum*. The second largest peak for *V. membranaceum* was delphinidin-3-galactoside, whereas the second largest peak for *V. ovatum* was cyanidin-3-arabinoside. Both huckleberries had a smaller amount of malvidin-glycosides when compared to levels of the highbush blueberry *Vaccinium corymbosum* 'Rubel' (Lee et al., 2002). Cyanidin-glycosides have higher antioxidant activity than other anthocyanins (Zheng and Wang, 2003). In addition to the higher amounts of total anthocyanins and total phenolics in huckleberries, the presence of individual anthocyanins that are higher in antioxidant properties might also contribute to high antioxidant content of huckleberries in contrast with other blueberry relatives.

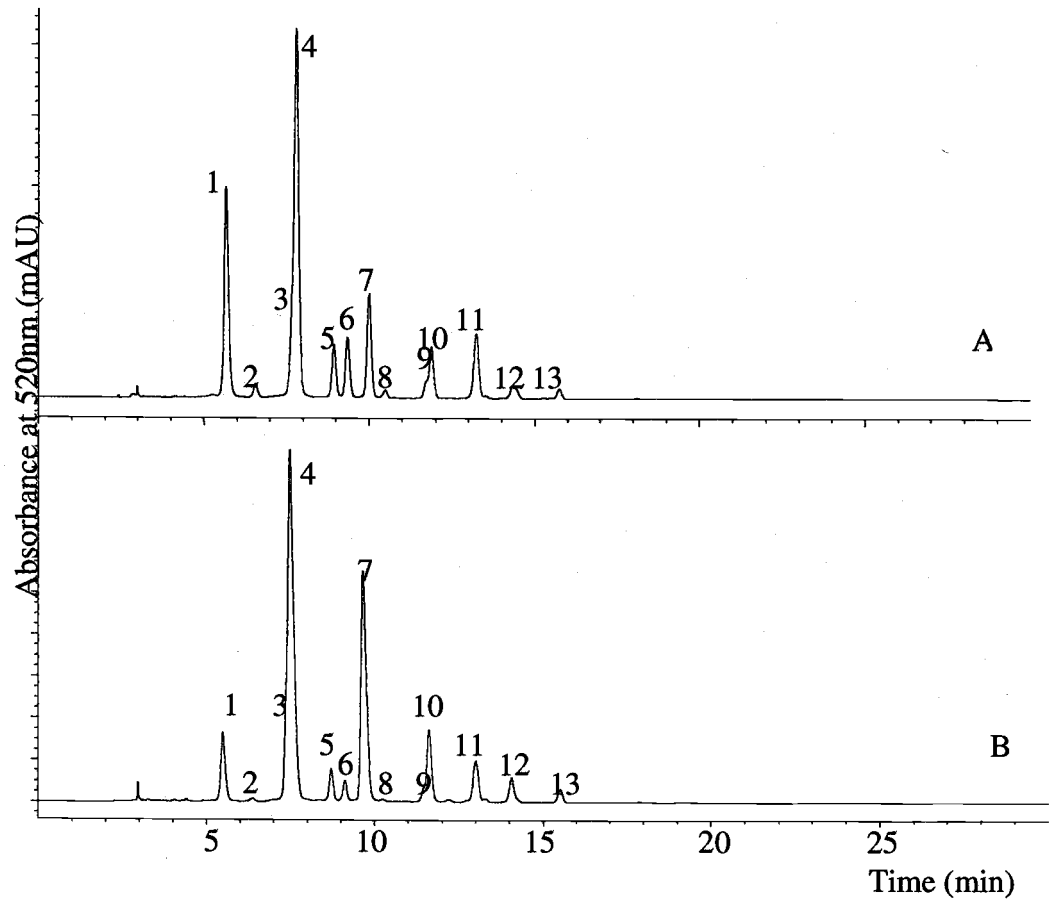


Figure 6.1. Anthocyanin HPLC profiles of *Vaccinium membranaceum* (A) and *V. ovatum* (B). Corresponding presumptive anthocyanin peak assignments and % peak area: 1:delphinidin-3-galactoside(A:20.3, B:6.9), 2:delphinidin-3-glucoside(A:1.3, B:0.4), 3:delphinidin-3-arabinoside(A:5.8, B:5.5), 4:cyanidin-3-galactoside(A:35.4, B:43.0), 5:cyanidin-3-glucoside(A:5.0, B:3.1), 6:petunidin-3-galactoside(A:5.7, B:2.2), 7:cyanidin-3-arabinoside(A:9.7, B:25.6), 8:petunidin-3-glucoside(A:0.8, B:0.3), 9:petunidin-3-arabinoside(A:1.4, B:1.4), 10:peonidin-3-galactoside(A:4.7, B:6.9), 11:malvidin-3-galactoside(A:7.1, B:2.6), 12:malvidin-3-glucoside(A:1.9, B:1.4), and 13:malvidin-3-arabinoside(A:1.0, B:0.8).

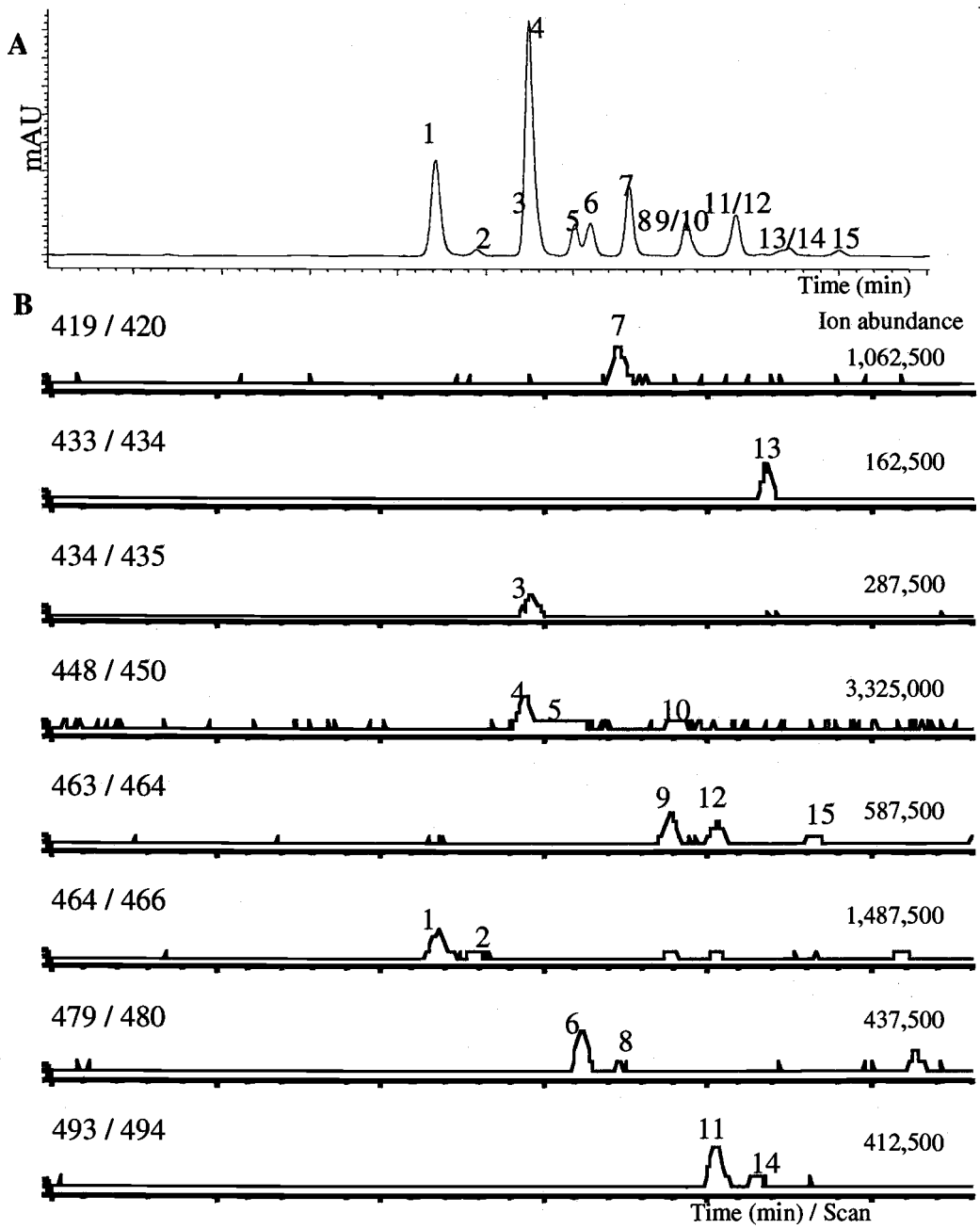


Figure 6.2. *Vaccinium membranaceum* results from LC-DAD-MS. LC-DAD profile (A) and extracted ion chromatograms (B). Corresponding anthocyanin peak assignments and masses: 1:delphinidin-3-galactoside(465.2), 2:delphinidin-3-glucoside(465.2), 3:delphinidin-3-arabinoside(435.0), 4:cyanidin-3-galactoside(449.2), 5:cyanidin-3-glucoside(449.2), 6:petunidin-3-galactoside(479.2), 7:cyanidin-3-arabinoside(419.0), 8:petunidin-3-glucoside(479.2), 9:peonidin-3-galactoside(463.2), 10:petunidin-3-arabinoside(449.0), 11:malvidin-3-galactoside(493.2), 12:peonidin-3-glucoside(463.2), 13:peonidin-3-arabinoside(433.0), 14:malvidin-3-glucoside(493.2), and 15:malvidin-3-arabinoside(463.0).

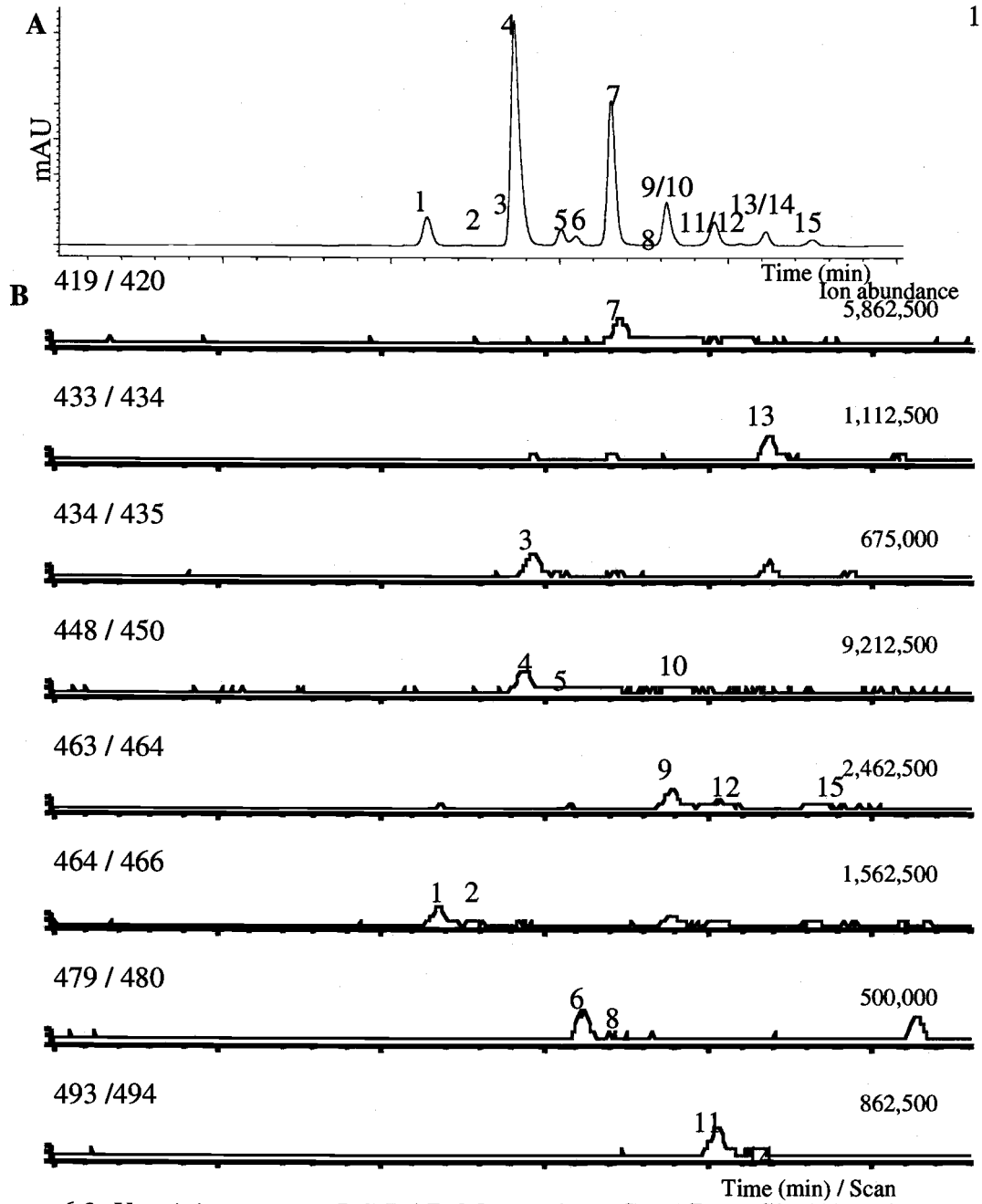


Figure 6.3. *Vaccinium ovatum* LC-DAD-MS results. LC-DAD profile (A) and extracted ion chromatograms (B). Corresponding anthocyanin peak assignments and masses: 1:delphinidin-3-galactoside(465.2), 2:delphinidin-3-glucoside(465.2), 3:delphinidin-3-arabinoside(435.0), 4:cyanidin-3-galactoside(449.2), 5:cyanidin-3-glucoside(449.2), 6:petunidin-3-galactoside(479.2), 7:cyanidin-3-arabinoside(419.0), 8:petunidin-3-glucoside(479.2), 9:peonidin-3-galactoside(463.2), 10:petunidin-3-arabinoside(449.0), 11:malvidin-3-galactoside(493.2), 12:peonidin-3-glucoside(463.2) 13:peonidin-3-arabinoside(433.0), 14:malvidin-3-glucoside(493.2), and 15:malvidin-3-arabinoside(463.0).

LC-DAD-MS was conducted on the huckleberry SPE extracts to firmly identify the individual anthocyanins. The LC-DAD-MS profiles, peak assignments, and masses of both species are provided (Figures 6.2 and 6.3). The individual peaks eluting at different times were analyzed by MS and the corresponding peaks' mass-to-charge ratio was obtained from the MS. Based on LC-DAD-MS there were 15 peaks present in both species. Peonidin-3-glucoside and peonidin-3-arabinoside, were not detected by LC-DAD using phosphoric acid and acetic acid mobile phase, but were found in low amounts with LC-DAD-MS (peaks 12 and 13). Separation was achieved by a different column and mobile phase (formic acid instead of a combination of acetic acid and phosphoric acid), with the LC-DAD-MS system, and the resolution was not as good as in the LC-DAD system. However, additional information was obtained with MS detection. Huckleberry anthocyanin peaks that co-eluted in the LC-DAD system, had different mass-to-charge ratios, and therefore could be distinguished by MS detection. For example, malvidin-3-glucoside and peonidin-3-glucoside co-eluted and peonidin-3-glucoside could not be detected with LC-DAD, but was detected by LC-DAD-MS. Since peonidin-3-glucoside and peonidin-3-arabinoside were present in low concentrations and co-eluted with other anthocyanins, they were difficult to identify based on LC-DAD alone. MS could also be helpful in identification of anthocyanins, due to the limited amount of authentic standards commercially available. Identification of anthocyanins was simplified by using a combination of retention time, peak spectra, and mass-to-charge ratio.

SPE was used to remove the anthocyanin fraction that interferes with the polyphenolic LC analysis. Further hydrolysis was performed. The polyphenolics in the two species were examined in their native forms. The LC chromatograms of the ethyl

acetate fractions of the two berries are shown (Figure 6.4). Only the absorbances at 320 and 260 nm are shown. Tentative peak assignments were made based on their retention time, UV-visible spectra, and standards (when available). The polyphenolic profiles of the two species were distinctly different. Neochlorogenic acid (3-O-caffeoylquinic acid, 26% of the total peak area measured at 320 nm) and a cinnamic acid derivative (peak 2, 21% of the total peak area measured at 320 nm) were the major polyphenolics present in *V. membranaceum*. Neochlorogenic acid was reported in large quantities in Hawaiian *V. reticulatum* Smith and *V. calycinum* Smith leaves (Bohm and Koupai-Abyazani, 1994), both of which are also in the Myrtillus section. Neochlorogenic acid has been reported in the green berries and leaves of whortleberry (*V. arctostaphylos* L.; Sect. Hemimyrtillus) (Mzhavanadze et al., 1972a; 1972b). Chlorogenic acid (5-O-caffeoylquinic acid, 43% of the total peak area measured at 320 nm) was the major polyphenolic present in *V. ovatum*. The *V. ovatum* polyphenolic profile was similar to 'Rubel' (data not shown). The major polyphenolic present in 'Rubel' is chlorogenic acid (Lee et al., 2002). Both species' polyphenolic profiles were mainly composed of cinnamic acids (strong 320 absorbers) and flavonol-glycosides (strong 260 nm absorbers). Minor peaks identified in the ethyl acetate fraction of *V. membranaceum* were gallic acid, protocatechuic acid, and epicatechin and *V. ovatum* had protocatechuic acid, catechin, and vanillic acid minor peaks, which might have hydrolyzed during extraction and/or SPE.

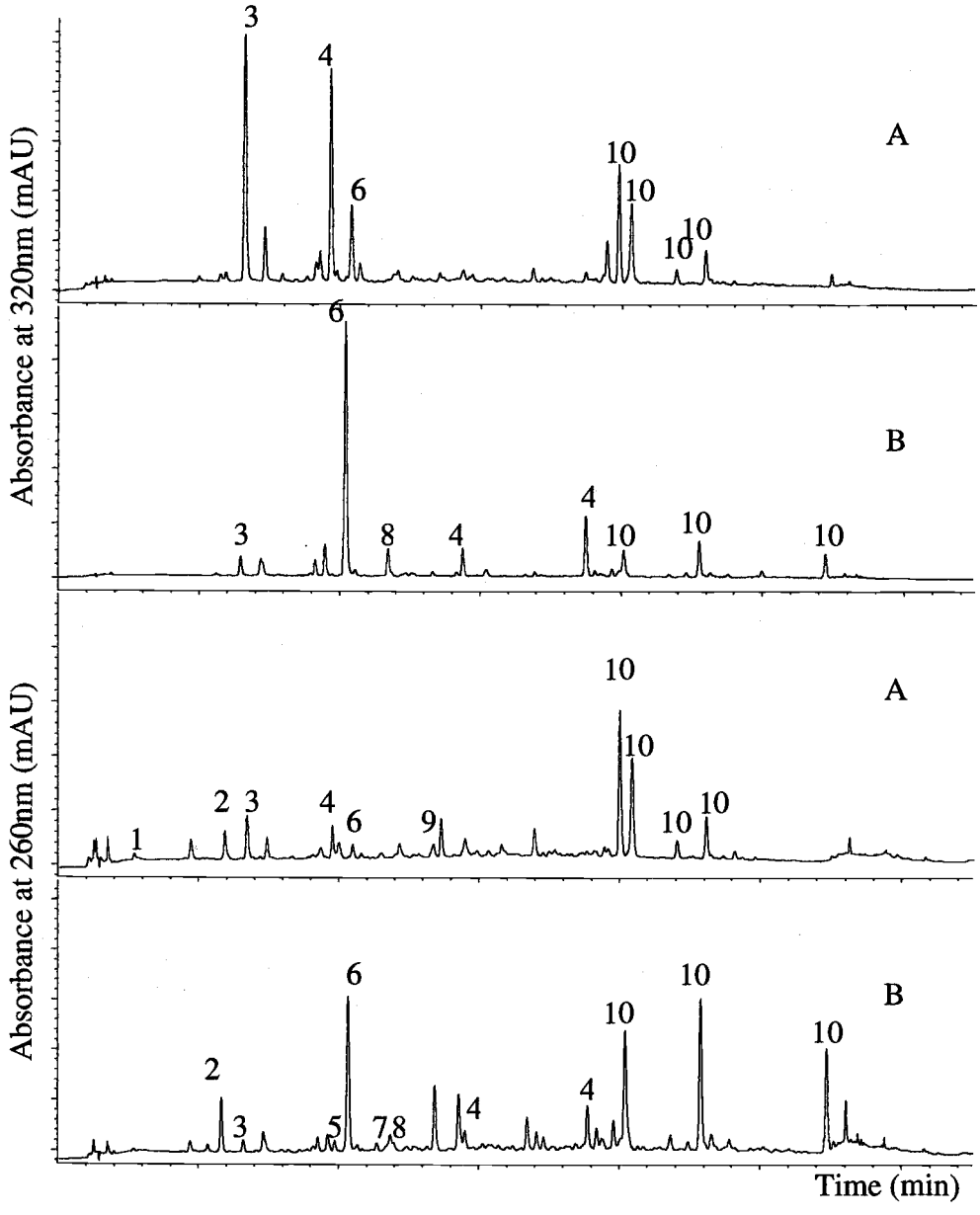


Figure 6.4. Polyphenolic HPLC separations of *Vaccinium membranaceum* (A) and *V. ovatum* (B) monitored at 320 and 260 nm. Corresponding tentative peak assignments: 1: gallic acid, 2: protocatechuic acid, 3: neochlorogenic acid, 4: cinnamic acid dervatives, 5: catechin, 6: chlorogenic acid, 7: vanillic acid, 8: caffeic acid, 9: epicatechin, and 10: flavonol-glycosides.

Vander Kloet (1988) provided three different phenograms to divide the 26 North American *Vaccinium* species into groups, which were dependant upon the mean character scores for quantitative and/or qualitative features of the species. None of these features included anthocyanin or polyphenolic data. Depending on the phenogram, *V. membranaceum* (Section: Myrtillus), *V. ovatum* (Section: Pyxothamnus), and *V. corymbosum* (Section: Cyanoccocus) links to one another were strengthened, or weakened. Data is insufficient and is highly variable to speculate on differences in the profiles of *Vaccinium* sections. The ranges of ACY, TP, and ACY/TP values of the various sections overlap.

CONCLUSIONS

The fruits of *V. ovatum* were higher in ACY, TP, antioxidant activities, and soluble solids than were those of *V. membranaceum*. Since *V. ovatum* ripens later than *V. membranaceum*, it could be an additional source to lengthen the huckleberry season for the fresh market and commercial processing.

ACKNOWLEDGEMENTS

We would like to thank Northwest Center for Small Fruit Research for their financial support. We would like to thank the USDA- Forest Service for permission and advice in the process of collecting seed from National Forests in Oregon and Washington, and the USDA-Agricultural Research Service National Clonal Germplasm Repository for access to their collections for many of the genotypes sampled in this study. We would like to thank Ted Mackey for the collecting of the cultivated *V. membranaceum* samples. We would also like to thank Mark Redhead for his help in

numerous aspects of this project. Deborah Hobbs of the Linus Pauling Institute (Corvallis, OR) performed the ORAC and FRAP analyses.

REFERENCES

Ballington, J.R., Kirkman, W.B., Ballinger, W.E., and Maness, E.P. 1988a. Anthocyanin, aglycone, and aglycone-sugar content in the fruits of temperate North American species of four sections in *Vaccinium*. J. Amer. Soc. Hort. Sci. 113:746-749.

Ballington, J.R., Ballinger, W.E., Maness, E.P., and Luby, J.J. 1988b. Anthocyanin, aglycone and aglycone-sugar content of the fruits of five species of *Vaccinium* section *Myrtillus*. Can. J. Plant Sci. 68:241-246.

Benzie, I.F.F. and Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239:70-76.

Bohm, B.A. and Koupai-Abyazani, M.R. Flavonoids and condensed tannins from leaves of Hawaiian *Vaccinium reticulatum* and *V. calycinum* (Ericaceae). 1994. Pacific Sci. 48:458-463.

Camp, W.H. 1945. The North American blueberries, with notes on other groups of Vacciniaceae. Brittonia. 5:203-275.

Cao, G., Alessio, H.M., and Culter, R.G. 1993. Oxygen-radical absorbance capacity assay for antioxidants. Free Radical Biol. Med. 14:303-311.

Cao, G. and Prior, R.L. 1999. The measurement of oxygen radical absorbance capacity in biological samples. Methods Enzymol. 299:50-62.

Clark, J.R., Howard, L., and Talcott, S. 2002. Variation in phytochemical composition of blueberry cultivars and breeding seasons. . Acta Hort. 574:203-207.

Conner, A.M., Luby, J.J., Tong, C.B.S., Finn, C.E., and Hancock, J.F. 2002. Genotypic and environmental variation in antioxidant activity among blueberry cultivars. Acta Hort. 574:209-213.

Durst, R.W. and Wrolstad, R.E. 2001. Unit F1.3: Anthocyanins. Separation and characterization of anthocyanins by HPLC. In: Current Protocols in Food Analytical Chemistry. R.E. Wrolstad (Ed.). New York: John Wiley & Sons, NY. p 1-13.

Giusti, M.M. and Wrolstad, R.E. 2001. Unit F1.2: Anthocyanins. Characterization and measurement with UV-visible spectroscopy. In: Current Protocols in Food Analytical Chemistry. R.E. Wrolstad (Ed.) New York: John Wiley & Sons. p 1-13.

Kalt, W. and Dufour, D. 1997. Health functionality of blueberries. HortTechnology 7:216-221.

Kushman, L.J. and Ballinger, W.E. 1968. Acid and sugar changes during ripening in Wolcott blueberries. Proc. Amer. Soc. Hort. Sci. 92:290-295.

Lee, J., Durst, R.W. and Wrolstad, R.E. 2002. Impact of juice processing on blueberry anthocyanins and polyphenolics: comparison of two pretreatments. J. Food Sci. 67:1660-1667.

Lee, J., Finn, C.E., and Wrolstad, R.E. 2003. Anthocyanin pigment and total phenolic content of three *Vaccinium* species native to the Pacific Northwest of North America. HortSci. In press.

Moll, F.O. 1933. Pacific coast huckleberry (*Vaccinium ovatum*), A thoroughbred in ornamental shrubs. Sunset Magazine (Pacific Edition), March 1933.

Moyer, R.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.

Mzhavanadze, V.V., Targamadze, I.L., and Dranik, L.I. 1972a. Phenolic compounds of unripe blueberry *Vaccinium arctostaphylos* fruits. Soobshcheniya Akademii Nauk Gruzinskoi SSR. 68:205-208.

Mzhavanadze, V.V., Targamadze, I.L., and Dranik, L.I. 1972b. Phenolic compounds from *Vaccinium arctostaphylos* leaves. Khimiya Prirodnykh Soedinenii. 8:124.

Nagels, L., Van Dongen, W., De Brucker, J., and De Pooter, H. 1980. High-Performance liquid chromatographic separation of naturally occurring esters of phenolic acids. J. Chrom. 187:181-187.

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 phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. J. Agric. Food Chem. 46:2686-2693.

Popenoe, W. 1924. Hunting new fruits in Ecuador. Nat. Hist. 24:455-466.

Postman, J.D. 2004. An evergreen huckleberry industry near the Oregon Coast early in the 20th Century. J. Am. Pom. Soc. Submitted.

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 phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. J. Agric. Food Chem. 46:2686-2693.

Rodriguez-Saona, L.E. and Wrolstad, R.E. 2001. Unit F1.1: Anthocyanins. Extraction, Isolation and Purification of Anthocyanins. In Current Protocols in Food Analytical Chemistry. RE Wrolstad (Ed.) New York: John Wiley & Sons. p 1-11.

Strack, D. and V. Wray. 1994. The Anthocyanins. In: Harborne J.B., editor. The flavonoids, London, UK: Chapman & Hall. p 1-22.

Vander Kloet, S.P. 1988. The Genus *Vaccinium* in North America. Publication 1828, Research branch, Agriculture Canada.

Waterhouse, A.L. 2002. Unit I1.1: Polyphenolics: Determination of Total Phenolics, p. 1-4. In: R.E. Wrolstad (ed.). Current Protocols in Food Analytical Chemistry. John Wiley & Sons, New York.

Zheng, W. and Wang, S.Y. 2003. Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. J. Agric. Food Chem. 51:502-509.

CHAPTER 7

DETERMINATION OF TOTAL MONOMERIC ANTHOCYANIN PIGMENT CONTENT OF FRUIT JUICES, BEVERAGES, NATURAL COLORANTS, AND WINES BY THE PH DIFFERENTIAL METHOD; A COLLABORATIVE STUDY FOR AOAC METHOD VALIDATION.

Jungmin Lee, Robert W. Durst, and Ronald E. Wrolstad

Department of Food Science and Technology
Oregon State University, Corvallis, OR 97331-6602

Submitted to Journal of AOAC International.
481 North Frederick Avenue Suite 500, Gaithersburg, MD 20877

ABSTRACT

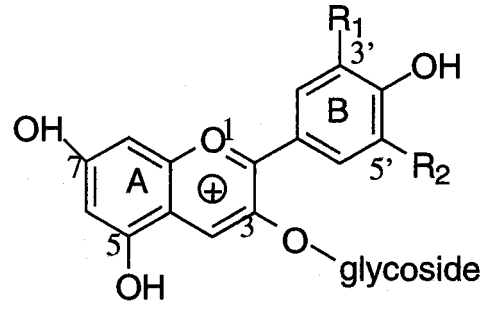
There is no approved AOAC method for determining total anthocyanin pigments, at present. This collaborative study was conducted to determine the total monomeric anthocyanin concentration by the pH differential method. The pH differential method is a rapid and simple spectrophotometric method based on the anthocyanin structural transformation that occurs with a change in pH (colored at pH 1.0 and colorless at pH 4.5). The eleven study collaborators represented commercial laboratories, academic institutions, and government laboratories. Seven Youden pairs of samples representing fruit juices, beverages, natural colorants, and wines were selected as test samples. The repeatability relative standard deviation (RSD_r) varied from 1.06 to 4.16%. The reproducibility relative standard deviation (RSD_R) ranged from 2.69 to 10.12%. The Horrat values were $\leq$ 1.33 for all test samples. The collaborative study results showed that the method meets the purpose of determining total monomeric anthocyanin content of liquid samples. The Study Director recommends that the method be adopted Official First Action.

INTRODUCTION

Anthocyanin pigments are important to food quality because of their contribution to color and appearance. There is increasing interest in the anthocyanin content of foods and nutraceuticals beccause of their possible health benefits. Anthocyanin pigment content can also be a useful criterion in quality control and purchase specifications of fruit juices, nutraceuticals, and natural colorants. There is need for an AOAC method to rapidly, and precisely, determine total monomeric

anthocyanin content. Currently, there is no approved AOAC method for measuring total anthocyanins. The goal of this study was to design, organize, and conduct a collaborative study to validate the pH differential method as an AOAC method and to determine between laboratory performance (precision of the method during use).

Anthocyanins are responsible for the red, purple, and blue hues present in fruits, vegetables, and grains. There are six common anthocyanidins (pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin), whose structures can be varied by glycosidic substitution at the 3 and 5 positions. Additional variations occur by acylation of the sugar groups with organic acids. Figure 7.1 shows the basic structure of an anthocyanin pigment.



R_1=R_2=H	Pelargonidin
R_1=OH, R_2=H	Cyanidin
R_1=OCH_3, R_2=H	Peonidin
R_1=R_2=OH	Delphinidin
R_1= OCH_3, R_2=OH	Petunidin
R_1=R_2= OCH_3	Malvidin

Figure 7.1. Basic structure of an anthocyanin pigment.

The pH differential method has been used extensively by food technologists and horticulturists in assessing the quality of fresh, and processed fruits and vegetables. The

method can be utilized for quantitative determination of total monomeric anthocyanin content, based on the structural change of the anthocyanin chromophore between pH 1.0 and pH 4.5 (Figure 7.2). The method's anticipated use is in research and quality control of anthocyanin containing fruit juices, wines, natural colorants, and other beverages.

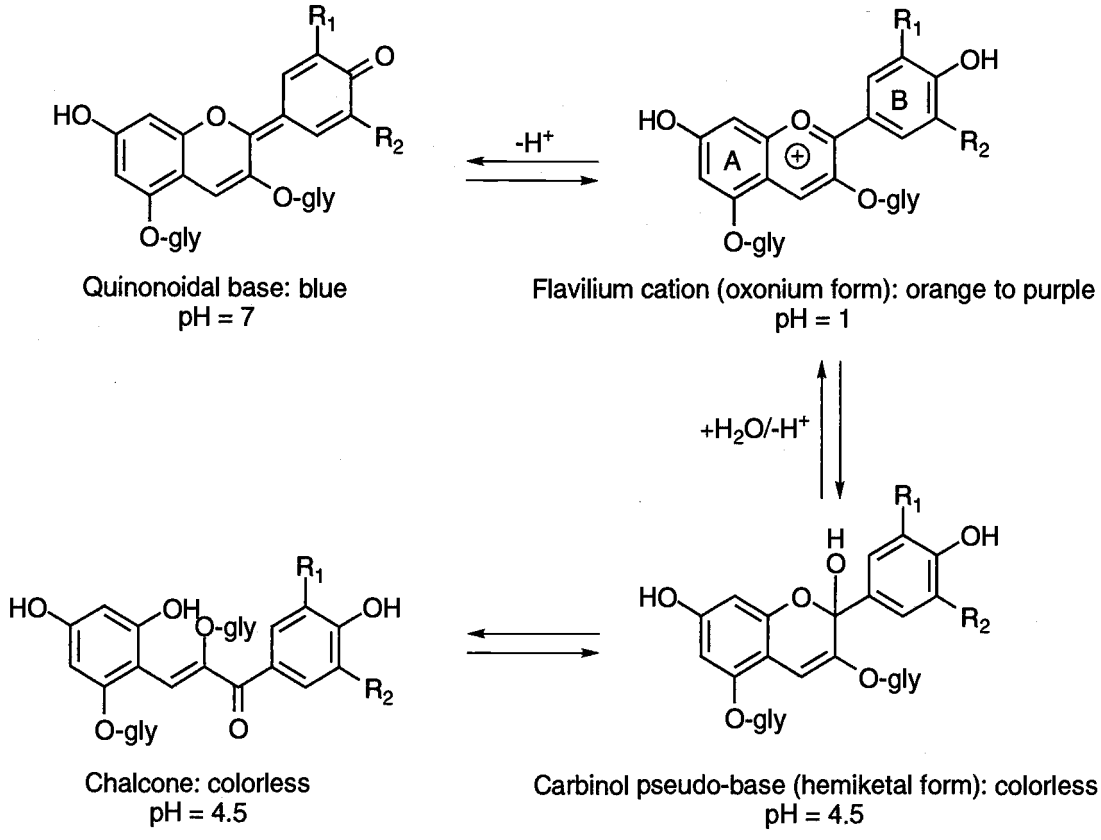


Figure 7.2. Predominant anthocyanin structural forms present at different pH levels.

The concept of measuring the amount of anthocyanin present in a sample (strawberries or strawberry preserves) by utilizing the change in absorbance at two different pH values (3.4 and 2.0) was first introduced by Sondhimer and Kertesz (1948). Since then, researchers have proposed using the pH values of 1.0 and 4.5 (Fuleki and

Francis, 1968; Wrolstad et al., 1995; Wrolstad et al., 1982; Giusti and Wrolstad, 2001). Monomeric anthocyanins undergo a reversible structural transformation as a function of pH (colored oxonium form at pH 1.0 and colorless hemiketal form at pH 4.5 (Figure 7.2). Thus the difference in absorbance at the pigments $\lambda_{vis\ max}$ ($\approx$ 520 nm) will be proportional to the pigment concentration. Figure 7.3 shows the spectra of huckleberry anthocyanins in pH 1.0 and pH 4.5 buffers. Degraded anthocyanins in the polymeric form are resistant to color change with pH. Hence, polymerized anthocyanin pigments will not be measured in this method as they absorb at pH 4.5, as well as at pH 1.0.

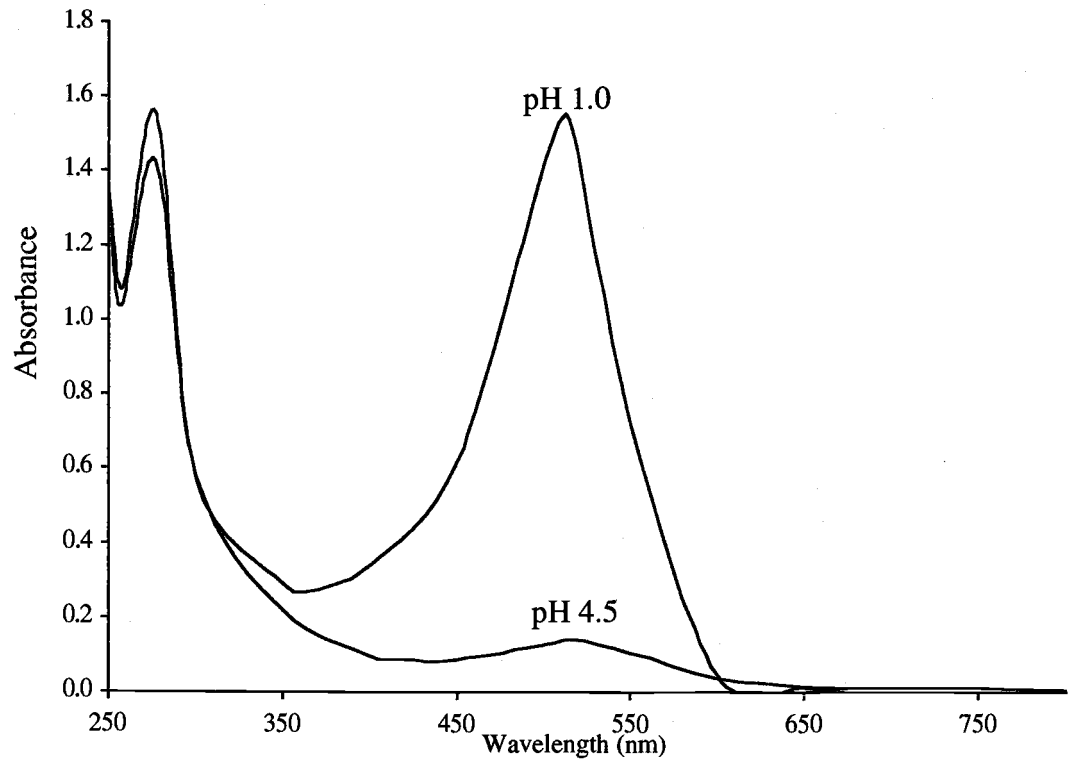


Figure 7.3. Spectral characteristics of huckleberry anthocyanin in pH 1.0 and pH 4.5 buffers.

MATERIALS AND METHODS

Collaborative Study

A precollaborative protocol was prepared following the guidelines of AOAC International, and was approved by the Methods Committee on Commodity Foods and Food Products.

Collaborating Laboratories. Fourteen laboratories were identified (including the study director's laboratory), 8 within the USA and 6 outside the USA, as potential collaborators for the study. All 14 laboratories agreed to participate. Characteristics of the laboratories are shown in Table 7.1. Participating laboratories (11 laboratories) represented commercial laboratories, academic institutions, and government laboratories. One analyst performed the analysis from each laboratory. Collaborators were not compensated for their participation.

Test Materials. There were 7 sample pairs to a sample set. Each sample pair (Youden pair) was prepared with one sample being neat (undiluted, X), and the other sample diluted with distilled water (Y) to make ≤5% difference based on the formula given in the Guidelines for Collaborative Studies. The formula used is as follows: $(x_c - y_c / x_c) \leq 0.05$, where x_c is the concentration of analyte in X and y_c is the concentration of analyte in Y. Details of test samples used in this study are listed in Table 7.2. Concentrate samples (strawberry, raspberry, and elderberry) were initially diluted to their reported single strength °Brix (8.0, 11.0, and 9.2, respectively) as in 21CFR parts 101, then diluted appropriately in a 1L volumetric flask. The authentic standard solution was prepared by weighing 82.2mg of cyanidin-3-glucoside chloride (76.2mg of

cyanidin-3-glucoside) on an analytical balance and dissolved in distilled water to a final volume of 1L in a volumetric flask.

Table 7.1. Characteristics of collaborating laboratories.

Lab No.	Type	Location
0	Academic institution (Study Director)	USA
1	Government laboratory	Outside USA
2	Commercial laboratory	USA
3	Commercial laboratory	USA
4	Commercial laboratory	USA
5	Academic institution	USA
6	Government laboratory	Outside USA
7	Commercial laboratory	USA
8	Commercial laboratory	USA
9	Government laboratory (dropped out)	Outside USA
10	Commercial laboratory (dropped out)	Outside USA
11	Commercial laboratory (dropped out)	Outside USA
12	Commercial laboratory	Outside USA
13	Commercial laboratory	USA

Table 7.2. Material used in the collaborative study, representing a typical range of samples analyzed by the pH differential method.

Material[a]	Source
Cranberry juice cocktail	Local grocery store (Corvallis, OR, USA)
Red Wine	Local grocery store (Corvallis, OR, USA)
Natural colorant[b]	Canandaigua West, Inc. (Madera, CA, USA)
Strawberry concentrate[c]	Kerr Concentrate Inc. (Salem, OR, USA)
Raspberry concentrate[c]	Kerr Concentrate Inc. (Salem, OR, USA)
Elderberry concentrate[c]	Kerr Concentrate Inc. (Salem, OR, USA)
Cyanidin-3-glucoside chloride[d]	Polyphenols Laboratories AS (Sandnes, Norway)

[a] Materials were prepared as Youden pairs.
[b] Natural colorant was a high colored grape juice concentrate.
[c] Samples were obtained as concentrate and diluted to the appropriate °Brix.
[d] Cyanidin-3-glucoside chloride was the authentic standard used in this study.

Two familiarization test samples were included with the sample sets. After accurately determining their values, collaborators were notified to continue with the actual test samples.

The anthocyanin content of the test samples ranged approximately from 20 to 3000 mg/L (expressed as cyanidin-3-glucoside). The method required the analyst to dilute the samples with buffers to an appropriate concentration for measurements.

The products used for the samples were homogenized by stirring, then aliquoted to samples vials (approximately 25 mL). Individual samples came from a single batch of homogenous product. All samples were labeled with a random 3-digit number. The

familiarization test samples (A and B) were also clearly labeled. The familiarization test samples were cranberry juice cocktail and an intensely pigmented grape juice concentrate diluted to appropriate strength.

Samples were placed in freezable containers and stored frozen (-23°C) until packaged for shipment to collaborators. Samples were packed with dry ice and sent overnight shipping via Federal Express. Shipping boxes were labeled to immediately store the samples frozen until future analysis.

The homogeneity and stability of the prepared samples were evaluated in-house. Homogeneity testing of prepared samples was performed by selecting two test tubes from the frozen samples at random, sampling 12 times from each tube, and measuring their °Brix by a digital refractometer (Auto Abbe refractometer model 10500, Reichert-Jung, Leica Inc., Buffalo, N.Y., USA). Stability testing was performed throughout the duration of the study. A thermal shock stability study and a long-term stability study were also conducted. Thermal shock (transportation) was performed by placing a test tube of frozen sample (stored at –23°C) at room temperature for 1 day, and then returning it to –23°C for 2 days (to stimulate temperature abuse during transportation), then pH differential was performed and replicated. Long-term stability testing was done by performing pH differential on random samples after 0 and 3 months of storage at –23°C. Total monomeric anthocyanin content was determined in the long-term stability test samples.

Statistical Analyses. The total monomeric anthocyanin contents of the individual test samples were used for the statistical analyses. AOAC Interlaboratory Statistical program 2001 for Youden pairs / split levels spreadsheet (Joanna M. Lynch,

Ithaca, NY, copyright 2001, version 1.1), provided by AOAC methods committee on commodity foods and food products, was used to calculate Horrat values and identify outliers. The statistical analyses were performed following the guidelines of AOAC International. The outliers were determined at an α= 0.025 and the t-statistics for equivalence of variance were tested at an α= 0.05. Repeatability standard deviation (S_r), reproducibility standard deviation (S_R), repeatability relative standard deviation (RSD_r), reproducibility relative standard deviation (RSD_R), and Horrat values were calculated. To assess the homogeneity of each material a one-way analysis of variance, using the PROC ANOVA software of the Statistical Analysis System (SAS), was used to determine if the mean °Brix values, for the two randomly selected tubes from each material, varied significantly between themselves. All comparisons were made using a significance level of α = 0.05. SAS statistical software was used (SAS systems for Windows, released version 8.2, SAS Institute, Inc., Cary, N.C., U.S.A., 2001).

Methods of Authentic Standard Analyses

To investigate recovery issues of the authentic standard, cyanidin-3-glucoside chloride was purchased from two different vendors, company A and company B. Ideally, further investigation would have been conducted on the sample utilized for the collaborative study, but only 18 mg remained after the collaborative study was concluded. Percent purity by high performance liquid chromatography (HPLC), percent purity by molar absorptivity, percent moisture content, and hygroscopicity at one relative humidity were determined for the authentic standard samples.

An analytical balance was used to measure samples, and containers, to the nearest 0.01 mg. Samples were weighed in a glass weigh bottle (with lid) that was cleaned, dried in an oven, and cooled in a desiccator over phosphorus pentoxide (P_2O_5) under vacuum overnight.

Due to the limitation in the accessibility of large quantities of purified samples, only moisture determination and hygroscopicity of samples were replicated for the newly purchased standards.

Determination of Molar Absorptivity and % purity. The purity of the "authentic standard" was investigated by determining its molar absorptivity at 520 nm (λ_{max} used in the collaborative study) and its true λ_{max} (510 nm) obtained in pH 1.0 buffer. This value was compared to the literature reported extinction coefficient for cyanidin-3-glucoside of 26,900 $L \cdot cm^{-1} \cdot mol^{-1}$ (Jurd and Asen, 1966), a molecular weight of 484.5 $g \cdot mol^{-1}$ for cyanidin-3-glucoside chloride, and 449.2 $g \cdot mol^{-1}$ for cyanidin-3-glucoside. Percent purity was measured on the same "authentic standard" material used in the collaborative study two months after the purchase date, and the two newly purchased standards (as received). Also, percent purity was determined by HPLC (monitored at 520 and 280 nm, respectively). Anthocyanins were separated by reversed-phase HPLC using a Hewlett-Packard 1090 (Agilent Technologies Inc, Wilmington, Del.), equipped with a photodiode array detector. Absorbance spectra were recorded for all peaks. Flow rate was 1 mL/min and injection volume was 20 μL. Column temperature was maintained at 40°C. A Prodigy 5μm ODS (3) 100Å (250 × 4.6 mm) column, fitted with 4.0×3.0 mm i.d. guard column, from Phenomenex was used (Torrance, Calif.). Solvent A was 100% acetonitrile. Solvent B was 10% (v/v) acetic

acid and 1% (v/v) phosphoric acid in water. All solvents were HPLC grade. The program used a linear gradient from 2 to 20% solvent A in 25 minutes; then a linear gradient of solvent A from 20 to 40% in 5 minutes, with simultaneous detection at 520 and 280 nm (Durst and Wrolstad, 2001). Peak areas were used in calculating percent purity based on HPLC, at both wavelengths.

Moisture Determination. Samples were weighed and dried in a desiccator in the presence of phosphorus pentoxide (P_2O_5) under vacuum, until a constant weight was reached. Weight loss after drying on phosphorus pentoxide was used to determine % moisture content (unbound water) in sample. Percent moisture content (truly, % mass loss) was reported as % moisture on a dry weight basis.

Determination of Hygroscopicity. Hygroscopicity (% EMC) was determined using a method reported by Callahan et al. (1982). Hygroscopicity of samples were evaluated by storing the samples in a desiccator containing a saturated potassium bromide (KBr) solution, and placing it in a 25°C precision incubator (Fisher Scientific Isotemp), which maintained a constant 83% relative humidity. Humidity of the chamber was measured using a Taylor hygrometer (Model 5522E, Fletcher, North Carolina). Samples were stored in the humidity chamber for several weeks, while the weight was monitored until a static weight was reached, and then reported as hygroscopicity (% equilibrium moisture content at 25 °C, %EMC).

Proposed AOAC Method

AOAC Official Method 2003.0X

Determination of Total Monomeric Anthocyanin Pigment Content by the pH Differential Method of Fruit Juices, Beverages, Natural Colorants, and Wines.

Proposed First Action 2003

(Applicable to the determination of monomeric anthocyanins in fruit juices, beverages, natural colorant, and wines within the range of 20-3000 mg/L cyanidin-3-glucoside equivalents.)

Cautions: Standard laboratory safety procedures must be followed.

A. Principle

Monomeric anthocyanin pigments reversibly change color with change in pH, the colored oxonium form existing at pH 1.0 and the colorless hemiketal form predominating at pH 4.5. The difference in absorbance of the pigments at 520 nm is proportional to the pigment concentration. Results are expressed on a cyanidin-3-glucoside basis. Degraded anthocyanins in the polymeric form are resistant to color change regardless of pH, hence they will not be included in the measurements as they absorb at pH 4.5 as well as pH 1.0.

B. Apparatus and Equipments

(a) *pH meter.* - Standardized with pH 4.0 and 7.0 buffer solutions

(b) *Vis spectrophotometer.*

(c) *Glass or disposable cuvettes for spectrophotometer.* – 1 cm pathlength

(d) Volumetric flasks – 50 mL

(e) Volumetric and serological pipettes – variety of volumes less than 10 mL.

C. Preparations of Reagents

(a) *pH 1.0 buffer (potassium chloride, 0.025 M).* -- Weigh 1.86 g KCl in a beaker, add distilled water to ca 980 mL. Measure the pH and adjust the pH to 1.0 with concentrated HCl (takes ca 6.3 mL). Transfer to a 1 L volumetric flask and fill to line with distilled water.

(b) *pH 4.5 buffer (sodium acetate, 0.4 M).* -- Weigh 54.43 g CH_3CO_2 Na·$3H_2O$ in a beaker, add distilled water to ca 960 mL. Measure the pH and adjust the pH to 4.5 with concentrated HCl (takes ca 20 mL). Transfer to a 1 L volumetric flask and fill to line with distilled water.

D. Preparations of Sample

Perform all dilutions in 50 mL volumetric flasks, B(d), using volumetric pipettes, B(e), to add sample. Maximum sample volume added should not be greater than 10 mL (1 part sample, 4 parts buffer) so as not to exceed the buffer capacity of the reagents.

Determine appropriate dilution factor for sample by diluting with pH 1.0 buffer, C(a), until absorbance of diluted sample at 520 nm is within the linear range of the spectrophotometer. (For most spectrophotometers, the absorbance should be between 0.2 and 1.4). Using the dilution factor determined previously, prepare two dilutions of the sample, one with pH 1.0 buffer and the other with pH 4.5 buffer.

E. Determination

Determine absorbance of sample diluted with pH 1.0, C(a), and 4.5, C(b), buffers, at both 520 nm and at 700 nm. The diluted samples are read against a blank cell filled with distilled water. Measure absorbance within 20 to 50 minutes of sample preparation.

Note: The reason for measuring the absorbance at 700 nm is to correct for haze. However, if the diluted sample is excessively turbid it should be clarified by centrifuging or filtering prior to measurement. A filter (i.e. Millipore™ membrane filters - ≤1.2μm pore size, Millipore Corporation, Bedford, MA) that will not absorb the anthocyanins should be used.

(1) Calculations

Calculate anthocyanin pigment concentration, expressed as cyanidin-3-glucoside equivalents, by the following formula:

Anthocyanin pigment (mg/L cyanidin-3-glucoside equivalents) = $\frac{A \times MW \times DF \times 10^3}{\varepsilon \times l}$

Where A = ($A_{520\ nm} - A_{700\ nm}$)pH 1.0 - ($A_{520\ nm} - A_{700\ nm}$)pH 4.5; MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu); DF = dilution factor established in (C); l = pathlength in cm.; ε = 26,900 molar extinction coefficient in $Lmol^{-1}cm^{-1}$, for cyanidin-3-glucoside (cyd-3-glu); 10^3 = conversion from g to mg.

Results should be reported as monomeric anthocyanins in mg/L cyanidin-3-glucoside equivalents.

(2) *Limitations and restrictions.* - The limitations of the method were found to be few. The presence of artificial colors or non-anthocyanin colorants can lead to confusion, but generally do not interfere adversely with the assay. Added colorants that might be encountered include Red No. 40, cochineal, and beet powder. Cochineal in high concentrations does lead to a reduction in measured anthocyanin content, but at low concentrations a quantitative total monomeric anthocyanin value can still be obtained. The presence of cochineal can be determined by ELISA technique. Beet powder can be detected by its much higher $\lambda_{vis\ max}$ (≈550 nm). FD&C Red #40 can be detected by its bright red color in the pH 4.5 treatment. Anthocyanins can be measured in the presence of these other colorants, if one measures at a wavelength of ≈520 nm. The presence of ethanol in samples does not interfere with the assay at the levels typically encountered in wine (10-14%).

It is customary to conduct spectral measurements at the wavelength of maximum absorbance and calculate pigment concentration using the molecular weight and molar extinction coefficient of the major anthocyanin present in the sample matrix. It is appropriate to modify the procedure by making measurements at the wavelength of maximum absorbance and calculating pigment concentration using the molecular weight of the major pigment in the sample matrix and its molar extinction coefficient, if known. Anthocyanin content of grape products, for example, should be determined as malvidin-3-glucoside.

F. References

Giusti, M.M. & Wrolstad, R.E. (2001) Unit F1.2: Anthocyanins. Characterization and measurement with UV-visible spectroscopy. In, Current Protocols in Food Analytical Chemistry. RE Wrolstad (Ed.) New York: John Wiley & Sons. p 1-13.

Giusti, M.M., Rodríguez-Saona, L.E., & Wrolstad, R.E. (1999) J. Agric. Food Chem. 47, 4631-4637.

Jurd, L. & Asen, S. (1966) The formation of metal and "co-pigment" complexes of cyanidin-3-glucoside. Phytochem. 5, 1263-1271

Niketic-Aleksic, G. and Hrazdina, G. (1972) Quantitative analysis of the anthocyanin content in grape juices and wines. Lebensm. Wiss U. Technol 5, 163-165.

Swain, T. (1965) in Chemistry and Biochemistry of Plant Pigments. Ch19. T. W. Goodwin (Ed). Academic Press, London, p-533-49.

McClure, J. W. (1967) Plant Physiol. 43, 193-200.

RESULTS AND DISCUSSION

The total monomeric anthocyanin results of each sample from the collaborative study are provided in Table 7.3. Table 7.2 provides a listing of the materials, and the source from which they were obtained, that were used in the collaborative study. The results from the analysis conducted in-house, which was conducted blind, were also included in the statistical analysis. The Horrat values for the samples ranged from 0.30 (authentic standard sample) to 1.33 (elderberry juice sample), which were well below the acceptable range (Horrat value of ≤ 2.0). Outliers (α=0.025) were removed by the Cochran's outlier test, and Grubbs outlier test. For each set of Youden pairs, there was not a significant difference ($\alpha = 0.05$) between the variances for the undiluted and diluted sample pair members using the t-test, except for the strawberry juice sample. This might be due to the high polymeric anthocyanin (high absorbance value from sample diluted with pH 4.5 buffer) present in strawberry concentrates.

The relative repeatability standard deviations (RSD_r) ranged from 1.06% for elderberry juice to 4.16% for cranberry juice cocktail, with 2.56% being the average for all analytes from all laboratories (Table 7.4). The relative reproducibility standard deviation (RSD_R) ranged from 2.69% for authentic standard, to 10.12% for strawberry juice, and 6.58% was the average for all analytes from all laboratories (Table 7.4).

Table 7.3. Total monomeric anthocyanin (mg/L, cyanidin-3-glucoside equivalents) levels of all Youden pair samples analyzed by collaborators. Samples were coded with a random-digit number.

Samples	Lab A	Lab B	Lab C	Lab D	Lab E	Lab F
Cranberry juice cocktail	15.0	14.2	16.3	12.5	13.0	13.2
Diluted cranberry juice cocktail	13.8	13.8	12.0	13.7	13.1	12.8
Red wine	218.8	225.0	220.0	184.0	211.7	209.7
Diluted red wine	210.9	215.8	225.0	192.0	195.2	200.6
Natural colorant	693.3	688.0	724.7	623.7	652.1	657.3
Diluted natural colorant	672.8	664.6	673.0	640.8	621.2	634.2
Strawberry juice	69.0	72.8	78.5	57.7	65.1	64.5
Diluted strawberry juice	66.1	70.3	69.3	56.5	62.6	64.5
Raspberry juice	364.2	356.1	367.4	321.9	345.7	353.2
Diluted raspberry juice	356.7	346.5	347.8	332.7	325.6	325.1
Elderberry juice	3113.5	3193.7	1527.9[a]	3181.1	3147.7	3208.3
Diluted elderberry juice	2948.2	3030.8	2187.6[a]	3093.5	2955.7	3076.8
Authentic standard	46.6	46.3	47.9	45.0	44.8	45.8
Diluted authentic standard	44.5	44.1	45.8	42.2	43.6	43.3

Samples	Lab G	Lab H	Lab I	Lab J	Lab K
Cranberry juice cocktail	14.4	13.4	12.4	13.4	15.0
Diluted cranberry juice cocktail	15.5	12.9	11.4	13.4	15.4
Red wine	173.9	199.6	202.7	182.9	213.7
Diluted red wine	172.1	200.2	200.7	180.5	200.8
Natural colorant	598.0	648.8	641.2	600.3	637.9
Diluted natural colorant	584.5	628.8	625.1	563.6	622.9
Strawberry juice	61.1	60.6	51.9	65.8	92.7
Diluted strawberry juice	58.8	58.0	56.7	62.3	66.8
Raspberry juice	359.7	326.5	308.1	331.5	350.7
Diluted raspberry juice	314.7	322.3	302.3	325.6	322.3
Elderberry juice	3101.5	3248.2	2941.5	3019.2	2615.0
Diluted elderberry juice	2931.4	3202.2	2782.5	2848.8	2496.5
Authentic standard	44.8	44.3	44.6	46.4	47.3
Diluted authentic standard	44.0	41.9	42.8	45.4	44.1

[a] Values from Lab C's elderberry juice pair was removed by the statistical analysis, due to the condition (cracked sample) it arrived at the collaborator's laboratory, therefore the sample does not represent the total monomeric anthocyanin content present and was an outlier based on the Cochran outlier test.

Table 7.4. Interlaboratory study results for the determination of total monomeric anthocyanin pigment content by the pH Differential Method.

Matrix	X (mg/L)	No. individuals[a] (b)	S_r	$\%RSD_r$	S_R	$\%RSD_R$	r	R	Horrat value
Cranberry juice cocktail	13.6	10 (1)	0.57	4.16	1.09	8.00	1.59	3.05	0.74
Red wine	201.6	11 (0)	5.29	2.62	15.99	7.93	14.81	44.76	1.10
Natural colorant	640.8	11 (0)	11.97	1.87	36.52	5.70	33.52	102.25	0.94
Strawberry juice	63.6	10 (1)	2.43	3.82	6.44	10.12	6.81	18.03	1.18
Raspberry juice	336.7	11 (0)	10.80	3.21	17.62	5.23	30.24	49.32	0.79
Elderberry juice	3006.8	10 (1)	31.78	1.06	191.84	6.38	88.97	537.15	1.33
Authentic standard	44.8	11 (0)	0.53	1.19	1.20	2.69	1.49	3.37	0.30

X = the mean in mg/L expressed as cyanidin-3-glucoside equivalents; a (b) a = numbers of individuals retained after eliminating outliers, (b)=number of individuals removed as outliers.; S_r = Repeatability Standard Deviation; S_R = Reproducibility Standard Deviation; RSD = Relative Standard Deviations; r = repeatability value; R = reproducibility value

For the homogeneity tests (see results in Table 7.5), the mean °Brix values, for the two randomly selected tubes from each material, did not vary significantly between themselves ($p>0.05$). The p-values ranged from 0.06 to 0.90. There was insufficient data to perform statistical analysis on the thermal shock and long-term study. However, the results for time 0 through 3 months indicate little change (Table 7.6 and Figure 7.4). In the precollaborative protocol, a 6- month period for the long-term stability was proposed, but the duration was shortened to 3 months as all participating collaborators returned their results within that time period.

The AOAC methods Committee on Commodity of Foods and Food Products advised that for this collaborative study, collaborators should use the same λ_{max} and a common molecular weight and extinction coefficient for all samples. Since the major objective of the collaborative study was to evaluate the performance of the method between laboratories. Cyanidin-3-glucoside was chosen as it is the most common anthocyanin in nature (Francis, 1989) and several investigators have determined, and reported, its extinction coefficient (Table 7.7). The method protocol had collaborators measure samples at 520 nm (typical $\lambda_{vis\text{-}max}$ of samples), although anthocyanins have a typical absorption band in the region of 490 to 550 nm. Typically, the λ_{max} of each sample should be determined with the sample diluted in the pH 1.0 buffer, and the extinction coefficient and molecular weight of the predominant anthocyanin present in the sample is used to calculate the final values. For example, a huckleberry (*Vaccinium membranaceum*) sample diluted in pH 1.0 buffer has a λ_{max} of 514 nm (see Figure 7.3) and the predominant anthocyanin present is cyanidin-3-galactoside (determined by HPLC, data not shown).

Table 7.5. °Brix values for the homogeneity of the test samples. Twelve measurements from 2 randomly selected vials.

Measurements	Cranberry juice cocktail		D Cranberry juice cocktail		Red wine		D Red wine		Natural colorant	
1	13.70	13.69	13.20	13.21	9.55	9.49	9.13	9.18	1.65	1.66
2	13.71	13.70	13.20	13.20	9.54	9.49	9.13	9.17	1.65	1.67
3	13.73	13.71	13.20	13.19	9.54	9.54	9.21	9.21	1.67	1.67
4	13.70	13.70	13.20	13.19	9.53	9.55	9.21	9.14	1.68	1.67
5	13.73	13.70	13.19	13.19	9.52	9.57	9.14	9.21	1.60	1.73
6	13.73	13.70	13.19	13.20	9.52	9.57	9.14	9.13	1.62	1.73
7	13.69	13.70	13.19	13.19	9.52	9.56	9.21	9.13	1.67	1.63
8	13.70	13.71	13.19	13.19	9.52	9.56	9.17	9.13	1.67	1.62
9	13.70	13.70	13.33	13.19	9.56	9.58	9.17	9.20	1.69	1.63
10	13.69	13.69	13.33	13.19	9.52	9.55	9.17	9.19	1.69	1.62
11	13.69	13.69	13.21	13.31	9.52	9.56	9.18	9.14	1.65	1.65
12	13.70	13.69	13.20	13.30	9.52	9.56	9.17	9.12	1.65	1.63
p-value	0.15	NSD	0.74	NSD	0.06	NSD	0.62	NSD	0.90	NSD
Mean(S.D.)	13.70 (0.01)		13.22 (0.05)		9.54 (0.02)		9.17 (0.03)		1.66 (0.03)	

Measurements	D Natural colorant		Strawberry juice		D Strawberry juice		Raspberry juice		D Raspberry juice	
1	1.56	1.56	7.88	7.87	8.82	8.82	9.17	9.17	8.82	8.82
2	1.56	1.59	7.87	7.87	8.82	8.82	9.17	9.16	8.82	8.82
3	1.59	1.59	7.86	7.87	8.82	8.82	9.18	9.18	8.82	8.82
4	1.59	1.56	7.86	7.87	8.80	8.82	9.18	9.18	8.80	8.82
5	1.57	1.56	7.86	7.87	8.81	8.82	9.17	9.16	8.81	8.82
6	1.56	1.57	7.86	7.87	8.82	8.81	9.17	9.16	8.82	8.81
7	1.58	1.59	7.85	7.86	8.81	8.81	9.16	9.16	8.81	8.81
8	1.59	1.60	7.86	7.85	8.81	8.81	9.17	9.16	8.81	8.81

Table 7.5. . .Continued.

9	1.60	1.63	7.86	7.87	8.82	8.82	9.16	9.17	8.82	8.82
10	1.62	1.62	7.87	7.87	8.81	8.82	9.16	9.16	8.81	8.82
11	1.63	1.63	7.86	7.86	8.82	8.82	9.17	9.17	8.82	8.82
12	1.62	1.65	7.86	7.86	8.82	8.82	9.17	9.17	8.82	8.82
p-value	0.57	NSD	0.26	NSD	0.26	NSD	0.41	NSD	0.30	NSD
Mean(S.D.)	1.59 (0.03)		8.18 (0.01)		7.86 (0.01)		9.17 (0.01)		8.82 (0.01)	

Measurements	Elderberry juice		D Elderberry juice		Authentic standard		D Authentic standard	
1	10.46	9.94	9.99	0.03	0.03	0.03	0.03	0.03
2	10.49	9.97	10.02	0.03	0.03	0.03	0.03	0.03
3	10.51	10.01	10.07	0.03	0.03	0.03	0.03	0.03
4	10.54	10.04	10.06	0.03	0.02	0.03	0.03	0.03
5	11.53	10.10	10.13	0.03	0.03	0.02	0.03	0.03
6	11.54	10.13	10.13	0.03	0.03	0.02	0.03	0.03
7	10.49	10.20	10.25	0.02	0.03	0.02	0.03	0.03
8	10.52	10.26	10.26	0.03	0.03	0.03	0.03	0.03
9	10.56	10.34	10.23	0.02	0.03	0.03	0.02	0.02
10	10.57	10.35	10.28	0.03	0.03	0.02	0.03	0.03
11	10.61	10.30	10.20	0.03	0.03	0.02	0.02	0.02
12	10.61	10.31	10.15	0.02	0.03	0.03	0.03	0.03
p-value	0.19	NSD	0.77	NSD	0.30	NSD	0.19	NSD
Mean(S.D.)	10.63 (0.28)		10.16 (0.12)		0.03 (0.00)		0.03 (0.00)	

S.D. = Standard Deviation; NSD = not significantly different at $\alpha = 0.05$; D = Diluted; Authentic standard = Cyanidin-3-glucoside chloride

Table 7.6. Total monomeric anthocyanin results of the test samples for the thermal shock and long term stability study.

Samples	Time 0	Thermal shock study (2 days later)	Long term study (3 months later)
Cranberry juice cocktail	15.0	15.4	15.5
Diluted cranberry juice cocktail	13.8	14.6	14.7
Red wine	218.8	222.2	216.9
Diluted red wine	210.9	213.9	211.0
Natural colorant	693.3	701.4	714.4
Diluted natural colorant	672.8	665.8	680.3
Strawberry juice	69.0	70.9	70.8
Diluted strawberry juice	66.1	69.0	68.6
Raspberry juice	364.2	389.3	388.8
Diluted raspberry juice	356.7	368.2	374.7
Elderberry juice	3113.5	3128.5	3113.5
Diluted elderberry juice	2948.2	3018.3	3028.3
Authentic standard	46.6	48.2	47.8
Diluted authentic standard	44.5	46.0	46.0

Units are mg/L, expressed as cyanidin-3-glucoside equivalents.

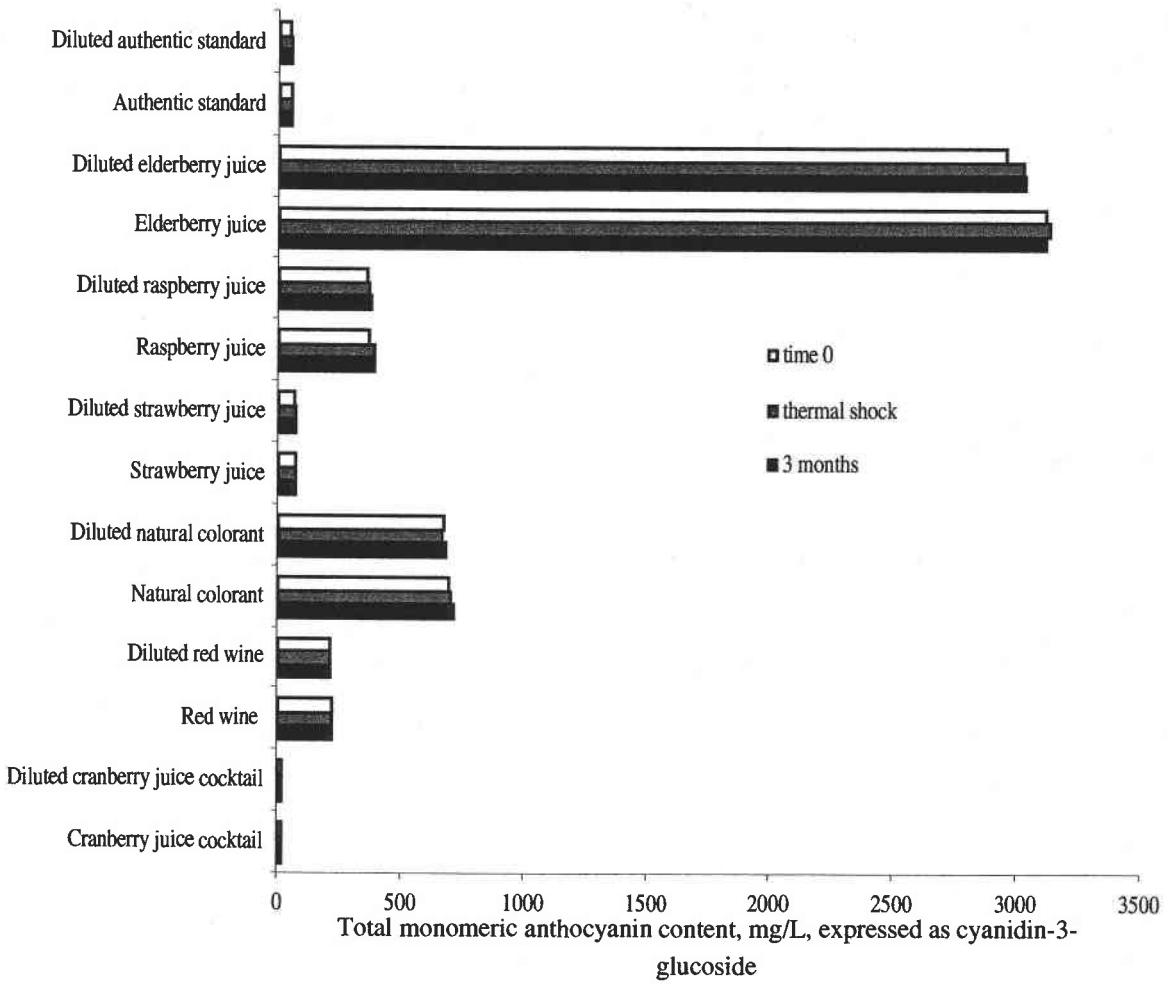


Figure 7.4. Total monomeric anthocyanin content of the thermal shock and long term stability study of the test samples.

Table 7.7. Literature reported molar absorptivity (ε) of cyanidin-3-glucoside.

Solvent system	$\lambda_{vis\text{-}max}$	Molar absorptivity (ε)	Publication year	Reference
pH 1.0 aqueous buffer	510	26,900	1966	(Jurd and Asen, 1966)
1% HCl in methanol	530	34,300	1958	(Siegelman and Hendricks, 1958)
0.1 N HCl	520	25,740	1967	(McClure, 1967)
pH 1.0 buffer	510	24,800	1994	(Rapisarda et al., 1994)
pH 1.5 10% ethanol	512	18,800	1998	(Heredia et al., 1998)
pH 1.0 buffer	510	20,000	1998	(Fossen et al., 1998)
0.1 M HCl in various concentration of aqueous ethanol	530	27,876 – 32,678	2000	(Rapisarda et al., 2000)
0.1 N HCl	510	26,300	2001	(Matsumoto et al., 2001)

The huckleberry sample could be expressed as cyanidin-3-galactoside using the literature reported extinction coefficient, that has been determined in a similar solvent system (acidic aqueous buffer). Literature reported cyanidin-3-galactoside extinction coefficients were determined in acidic alcohol system, so in this case cyanidin-3-glucoside extinction coefficient would be appropriate to use.

If the extinction coefficient of the major anthocyanin present in the sample is not available, or the major anthocyanin present in the sample is unknown, the total monomeric anthocyanin content of the sample can be calculated by using the extinction coefficient and molecular weight of cyanidin-3- glucoside (ε = 26,900 and MW = 449.2).

In the precollaborative protocol, the recommended detection limit was 20-1000 mg/L cyanidin-3-glucoside equivalents. From the collaborative study, the method has proven to be accurate even at higher concentrations (see values for elderberry juice samples). So, the method has been altered to recommend 20-3000 mg/L cyanidin-3-glucoside equivalents.

The pH differential method is based upon the assumption that monomeric, or "pure", anthocyanins have little or no absorbance in pH 4.5 buffer, but polymeric or degraded anthocyanins will absorb. While nearly all of monomeric anthocyanins are in the hemiketal form at pH 4.5, there will be a small proportion in the quinoidal or flavylium forms, which will make a small contribution to absorbance. The cyanidin-3-glucoside standard used in the collaborative study exhibited low absorbance in pH 4.5 buffer. If the anthocyanin content had been determined by a single pH method, where absorbance at pH 4.5 was ignored, the anthocyanin content of would have increased 2.8 mg/L (increased by 6%).

Presently, there is no certified reference material for cyanidin-3-glucoside. The authentic standard used in the collaborative study was purchased as a pure form of cyanidin-3-glucoside chloride (> 97% pure, as determined by HPLC, with UV/Vis detection at 520 and 280 nm, according to the datasheet provided by the company). Possible impurities listed on the datasheet were other anthocyanins, flavonoids, or polyphenols. When the total monomeric anthocyanin content of the authentic standard solution (82.2 mg of cyanidin-3-glucoside chloride/L = 76.2 mg of cyanidin-3-glucoside/L) was measured, the actual content was found to be much lower (mean from the 11 laboratories values was 45.8 mg/L, expressed as cyanidin-3-glucoside). This

gave an estimated "recovery" of only 60% (=45.8 / 76.2 * 100). While the sample was chromatographically pure, it may have contained bound and unbound water, and/or other materials not absorbing at 520 and 280 nm. Despite extreme care when purifying anthocyanins, researchers have reported the presence of crystal water (Matsumoto et al., 2001; Saito et al., 1964; Takeda and Hayashi, 1965), which would not be accounted for when determining purity by HPLC.

Percent purity of the same "authentic standard" material used in the collaborative study two months after the date of purchase was found to be 74.6% (using A_{510nm}) or 71.0% (using A_{520nm}) pure cyanidin-3-glucoside by molar absorptivity. Thus, measuring anthocyanin content at 520 nm rather than the true λ_{max} would contribute to the low recovery (or % purity) in the collaborative study.

Calculation of total monomeric anthocyanin is dependent on the extinction coefficient and molecular weight of the selected anthocyanin, which is usually the major pigment present in the sample. It is critical that the values used for the determination be reported along with the results. This permits the re-calculation of reported results when different extinction coefficients are used, so that a comparison can be made on an equal basis. Several investigators have determined the extinction coefficient for cyanidin-3-glucoside, with values ranging from 18,000 to 34,300, depending on the solvent, the wavelength of maximum absorbance, and "purity" (Table 7.7). The extinction coefficient value of 26,900 selected for this collaborative study, may not the "true" value, but, is reasonable in light of the values reported in the literature for cyanidin-3-glucoside in similar aqueous systems (Jurd and Asen, 1966; McClure, 1967; Rapisarda et al., 1994; Fossen et al., 1998; Matsumoto et al., 2001).

Efforts at determining the true molar absorptivity of a purified anthocyanin are hindered by the formidable problem of obtaining a pure crystalline anthocyanin in adequate quantities. Anthocyanins are highly hygroscopic (Lasagabaster et al., 1994; Grafe, 1912; Matsumoto et al., 1970) and difficult to obtain in a pure crystalline form.

Table 7.8 summarizes the properties of the cyanidin-3-glucoside standards. Moisture contents were 12.9% for the standard (sample 1) used in the collaborative study, 10.5% for the company A standard (sample 3), and 3.5% for company B standard (sample 2). When a portion of the standard used in the collaborative study (sample 1, Table 7.8) was analyzed by HPLC (regardless of sample size or dilution), the resulting chromatogram had 1 large peak (cyanidin-3-glucoside) and numerous small peaks (impurities detected by HPLC, that were 520 nm and 280 nm absorbers). Based on absorbance at 520 nm and 280 nm, sample 1 was 98.2% and 93.8% pure, respectively. Sample 2 was 99.6% (monitored at 520 nm) and 98.9% (monitored at 280 nm) pure. Sample 3 was 95.2% (monitored at 520 nm) and 94.1% (monitored at 280 nm) pure. Purity based on molar absorptivity were 99.1% for sample 2 and 80.3% for sample 3. Hygroscopicity for samples 1, 2, and 3 at 83% relative humidity were 22.4%, 10.0%, and 22.2%, respectively.

Table 7.8. Results from the investigation of cyanidin-3-glucoside.

	Sample 1[a]	Sample 2[b]	Sample 3[c]
% moisture[d]	12.9 %	3.5 %	10.5 %
Hygroscopicity [e] (%EMC)	22.4 %	10.0 %	22.2 %
% purity by molar absorptivity (as received) using A_{510nm}	74.6 %	99.1 %	80.3 %
Extinction coefficient determined using A_{510nm}[f]	20,072	26,672	21,606
% purity by molar absorptivity (as received) using A_{520nm}	71.0 %	93.2 %	76.3 %
Extinction coefficient determined using A_{520nm}[g]	19,103	25,076	20,526
% purity by HPLC reported in datasheet	> 97 %	99.3 %	> 97 %
% purity by HPLC conducted in-house monitored at 520 nm	98.2 %	99.6 %	95.2 %
% purity by HPLC conducted in-house monitored at 280 nm	93.8 %	98.9 %	94.1 %
Cost [h]	$ 290.00 for 100mg	$ 806.68 for 50mg	$ 416.08 for 100mg

[a] Authentic standard used in the Collaborative study. Purchased from Polyphenol As (Sandnes, Norway).

[b] Additional cyanidin-3-glucoside chloride purchased from Extrasynthese (Cedex, France).

[c] Additional cyanidin-3-glucoside chloride purchased from Polyphenol As (Sandnes, Norway).

[d] % moisture was determined by placing cyanidin-3-glucoside chloride over phosphorous pentoxide under vaccuum.

[e] Samples were placed in a 83 % relative humidity chamber (saturated potassium bromide solution placed in a 25°C incubator).

[f] Extinction coefficient determined by dissolving cyanidin-3-glucoside chloride in pH 1.0 buffer and measuring absorbance at 510 nm.

[g] Extinction coefficient determined by dissolving cyanidin-3-glucoside chloride in pH 1.0 buffer and measuring absorbance at 520 nm, which was the λ_{max} used in the collaborative study.

[h] Cost of standard did not include shipping and handling.

There was no uniformity in regards to company's instructions on how to store and handle pure anthocyanin standards. One datasheet advised storage in darkness at low temperatures (<-5°C) for no more than a few days. Another firm instructed storage of the sample at -20°C in a dry and dark place, and also to place the sample in a desiccator under vacuum for 48 hours before use. Neither provided an expiration date, but both recommended using the sample soon after reception, and to never store the product in solution. They further stated that the stability of the products were not always known and very difficult to measure.

The validity and basic principles for determining anthocyanin pigment concentration by the pH differential method have been widely accepted by natural product chemists for years. A combination of the following factors may account for the low recovery or purity of the standard: Moisture content, hygroscopicity of anthocyanins, presence of impurities (polyphenolics and/or polymeric anthocyanins), the possibility that the molar extinction coefficient of 26,900 is not the "true" value, measuring absorbance at 520 nm rather than 510 nm, and the minor contribution of quinoidal and flavylium forms to absorbance at pH 4.5. This collaborative study has demonstrated that total monomeric anthocyanin pigment content can be measured with excellent agreement between laboratories. A further advantage of the method is that that it does not require the purchase of costly standards, as is the case when anthocyanin content is measured by HPLC by the external standard method. The experiments concerning the moisture content, purity and hygroscopicity of anthocyanin standards in this investigation call attention to the importance of taking these properties into consideration when conducting experiments with anthocyanin standards.

Collaborators' Comments

Collaborators made the four following comments during the study:

- Several samples produced spectra whose maximum was not 520 nm.
- Sample absorbance was measured with a 2 mm cell.
- The use of 100 mL volumetric flasks or serial dilution for the dark juices should be allowed.
- Sample container arrived cracked.

The statement about samples having different maximum absorbance at different wavelength is well-taken and discussed above. We were advised to have all participating laboratories measure absorbance of all samples at the same wavelength. We feel that the statement "determine the appropriate dilution factor" is sufficient for the analyst to make the decision whether to use a 2 mm cell, 100 mL volumetric flasks, or serial dilutions. Collaborator's sample containers that arrived cracked were replaced (except in one case).

CONCLUSION

Based on the results from the collaborative study, the Study Director recommends that "Determination of Total Monomeric Anthocyanin Pigment Content by the pH Differential Method of Fruit Juices, Beverages, Natural Colorants, and Wines" be adopted as a First Action Official Method of AOAC International.

ACKNOWLEDGEMENTS

We would like to thank the Technical Committee for Juice and Juice Products (TCJJP) for their financial support, Canandaigua Wine Co. (Madera, CA) for donating MegaNatural Grape Red, and Kerr Concentrate Inc. (Salem, OR) for the numerous fruit concentrates. We would also like to thank all of our collaborators; Jonathan Haché and Francois Paquette (Canadian Food Inspection Agency), Dana A. Krueger and Alexandra Ryabkova (Krueger Food Laboratories Inc.), Steve Kupina (Canandaigua Wine Co.), JoLynne D. Wightman (Artemis International Inc.), M. Monica Giusti (University of Maryland), Grete Skrede and Berit K. Martinsen (Matforsk / Norwegian Food Research Institute), Tom Eisele (Tree Top Technical Center), Tom C. Miller (Alpha Laboratories / Division of Eurofins Scientific, Inc.), Hans Hofsommer, Susanne Koswig, and Uwe Trenn (GfL-Gesellschaft fur Lebensmittel-Forschung mbH), and Susan K. Martin and Karen W. Barnes (The Minute Maid Company).

REFERENCES

Callahan, J.C., Cleary, G.W., Elefant, M., Kaplan, G., Kensler, T., and Nash, R.A. 1982. Equilibrium moisture content of pharmaceutical excipients. Drug Dev. Ind. Pharm. 8: 355-369.

Durst, R.W. and R.E. Wrolstad. 2001. Unit F1.2: Anthocyanins. Separation and characterization of anthocyanins by HPLC, p. 1-13. In: R.E. Wrolstad (Ed.). Current Protocols in Food Analytical Chemistry. John Wiley & Sons, New York.

Fossen, T., Cabrita, L., and Andersen, Ø.M. 1998. Color and stability of pure anthocyanins influenced by pH including the alkaline region. Food Chem. 63: 435-440.

Francis, F.J. 1989. Food colorants: Anthocyanins. Crit. Rev. Food Sci. Nutri. 28:273-3114.

Fuleki, T. and Francis, F.J. 1968. Quantitative methods for anthocyanins. 2. Determination of total anthocyanin and degradation index for cranberry juice. J. Food Sci. 33:78-82.

Giusti, M.M. & Wrolstad, R.E. 2001. Unit F1.2: Anthocyanins. Characterization and measurement with UV-visible spectroscopy. In, Current Protocols in Food Analytical Chemistry. R.E. Wrolstad (Ed.) New York: John Wiley & Sons. p 1-13.

Grafe, V. Studies on Anthocyanin. III. 1912. Chemiker-Zeitung 35:768.

Heredia, F.J., Francia-Aricha, E.M., Rivas-Gonzalo, J.C., Vicario, I.M., and Santos-Buelga, C. 1998. Chromatic characterization of anthocyanins from red grapes I. pH effect. Food Chem. 63:491-498.

Jurd, L. & Asen, S. 1966. The formation of metal and "co-pigment" complexes of cyanidin-3-glucoside. Phytochem. 5:1263-1271.

Lasagabaster, A., Martin, C., and Goñi, M.M. 1994. Preparation of spherically agglomerated crystals of the 3,5-diglucoside of cyanidin (cyanin). J. Chem. Tech. Biotechnol. 60:397-403.

Matsumoto, H., Hanamura, S., Kawakami, T., Sato, Y., and Hirayama, M. 2001. Preparative-scale isolation of four anthocyanin components of black currant *(Ribes nigrum* L.) fruits. J. Agric. Food Chem. 49:1541-1545.

Matsumoto, T., Nishida, K., Noguchi, M., and E., Tamaki. 1970. Isolation and identification of anthocyanin from the cell suspension culture of Poplar. Agr. Biol. Chem. 34: 1110-1114.

McClure, J.W. 1967. Photocontrol of *Spirodela interdmedia* flavonoids. Plant Physiol. 43:193-200.

Rapisarda, P., Fallico, B., Izzo, R., and Maccarone, E. 1994. A simple and reliable method for determining anthocyanins in blood orange juices. Agrochemica. 38:157-164.

Rapisarda, P., Fanella, F., and Maccarone, E. 2000. Reliability of analytical methods for determining anthocyanins in blood orange juices. J. Agric. Food Chem. 48: 2249-2252.

Siegelman, H.W. and Hendricks, S.B. 1958. Photocontrol of alcohol, aldehyde and anthocyanin production in apple skin. Plant Physiol. 33: 409-413.

Saito, N., Hirata, K., Hotta, R., and Hayashi, K. 1964. Studies on anthocyanins XLIV. Isolation and crystallization of genuine red anthocyanins. Proc. Japan Acad. 40:516-521.

Siegelman, H.W. and Hendricks, S.B. 1958. Photocontrol of alcohol, aldehyde and anthocyanin production in apple skin. Plant Physiol. 33, 409-413.

Sondheimer, E. and Kertesz, Z. I. 1948. Anthocyanin pigments. Colorimetric determination in strawberries and strawberry perserves. Anal. Chem. 20:245-248.

Takeda, K. and Hayashi, K. 1965. Studies on Anthocyanins XLVIII. Crystallization and some properties of the genuine anthocyanin inherent to the deep violet color of pansy. Proc. Japan Acad. 39:449-454.

Wrolstad, R.E., Hong, V., Boyles, M.J., and Durst, R.W. 1995. Use of anthocyanin pigment analysis for detecting adulteration in fruit juices. In, Methods to detect adulteration in fruit juice and beverages, Vol. I. S. Nagy and R. L. Wade (Ed.). AgScience Inc., Auburndale, Fla. p 260-286.

Wrolstad, R.E., Culbertson, J.D., Corwell, C.J., and Mattick, L.R. 1982. Detection of adulteration in blackberry juice concentrates and wines. J. Assoc. Off. Anal. Chem. 65:1417-1423.

CHAPTER 8. SUMMARY

The heightened awareness of human nutrition has increased interest in foods high in dietary antioxidants. These beneficial compounds can be increased in food, and nutraceutical, products through crop selection, changes in crop management, choice of production region, breeding for improved genotypes, and improved processing methods.

Processing unit operations for manufacturing blueberry juice and concentrate were examined at pilot-plant scale for optimization of anthocyanin and polyphenolic extraction. Processing by-products (presscake) were found to contain significant amounts of these natural dietary antioxidants. Methods were evaluated to efficiently extract anthocyanins and polyphenolics from presscake. A combination of heat and SO_2 facilitated extraction of these compounds from presscakes.

Highbush blueberry (*Vaccinium corymbosum*) and huckleberries (*Vaccinium* sp.) contained cyanidin, peonidin, delphinidin, petunidin, and malvidin galactosides, glucosides, and arabinosides. Liquid chromatography linked to mass spectrometry (LC-DAD-MS) was an effective tool while identifying individual anthocyanins present in a complex sample. The limited availability of authentic anthocyanin standards further heightened the technique's value.

Four *Vaccinium* species native to the Pacific Northwest, commonly known as huckleberries, were evaluated for their qualitative, and quantitative anthocyanin and phenolic content. These plants were collected from the wild and cultivated at the U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS Corvallis, OR). Some huckleberry samples had a higher anthocyanin and phenolic content than commercially grown highbush blueberries.

The pH differential method, which measures total monomeric anthocyanin content of a sample, was evaluated for possible validation as an Association of Analytical Communities International (AOAC) official method. AOAC guidelines were followed, and eleven national and international laboratories collaborated in the study. The study results demonstrated good method performance (Horrat values ≤ 1.33) and was recommended to be adopted Official First Action.

These results will be useful to blueberry growers and processors who wish to improve the natural dietary antioxidant content of their fruit products. Processing practices and cultivar selection can have a major impact on the pigment content and antioxidant properties of the final products. Blueberry presscake was found to be a good source for natural pigments and nutraceuticals. As an AOAC official method, the pH differential method offers food and nutraceutical industries a simple, reliable, and economical means of monitoring anthocyanin content of products, from starting materials to marketable goods.

BIBLIOGRAPHY

Amakura, Y., Umino, Y., Tsuji, S., and Tonogai, Y. 2000. Influence of jam processing on the radical scavenging activity and phenolic content in berries. J. Agric. Food Chem. 48:6292-6297.

American Institute for Cancer Research. 1997. Food, nutrition, and the prevention of cancer: a global perspective. Amer. Inst. Cancer Res. Washington, D.C.

Azar, M., Verette, E., and Brun, S. 1987. Identification of some phenolic compounds in bilberry juice *Vaccinium myrtillus*. J. Food Sci. 52:1255-1257.

Bakker, J., Bridle, P., Bellworthy, S.J., Garcia-Viguera, C., Reader, H.P., and Watkins, S.J. 1998. Effect of sulphur dioxide and must extraction on color, phenolic composition and sensory quality of red table wine. J. Sci. Food Agric. 78:297-307.

Ballington, J.R., Ballinger, W.E., and Maness, E.P. 1987. Interspecific differences in the percentage of anthocyanins, aglycones, and aglycone-sugars in the fruit of seven species of blueberries. J. Amer. Soc. Hort. Sci. 112:859-864.

Ballington, J.R., Kirkman, W.B., Ballinger, W.E., and Maness, E.P. 1988a. Anthocyanin, aglycone, and aglycone-sugar content in the fruits of temperate North American species of four sections in *Vaccinium*. J. Amer. Soc. Hort. Sci. 113:746-749.

Ballington, J.R., Ballinger, W.E., Maness, E.P., and Luby, J.J. 1988b. Anthocyanin, aglycone and aglycone-sugar content of the fruits of five species of *Vaccinium* section *Myrtillus*. Can. J. Plant Sci. 68:241-246.

Belleoud, L., Leluan, D., and Boyer, Y. 1966a. Study on the effects of anthocyanin glycosides on the night vision of flight personnel. Commun. Soc. Fr. Physiol. Med. Aeronaut Cosmonaut. 17.

Belleoud, L., Leluan, D., and Boyer, Y. 1966b. Study on the effects of anthocyanin glycosides on the nocturnal vision of air traffic controllers. Rev. Med. Aeronaut Spatiale. 18.

Benzie, I.F.F. and Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239:70-76.

Bocevska, M. and Stevcevska, V. 1997. Quality evaluation of anthocyanin extract obtained from wine grape pomace. Food Technol. Bioltechnol. 35:139-143.

Bohm, B.A. and Koupai-Abyazani, M.R. Flavonoids and condensed tannins from leaves of Hawaiian *Vaccinium reticulatum* and *V. calycinum* (Ericaceae). 1994. Pacific Sci. 48:458-463.

Bomser, J., Madhavi, D.L., Singletary, K., and Smith, M.A.L. 1996. In vitro anticancer activity of fruit extracts from *Vaccinium* species. Planta Medica. 62:212-216.

Braithwaite, A., and Smith, F. J. 1996. Chromatographic methods 5th Edition, Chapman & Hall., Bishopbriggs, Glasgow.

Bruker Daltonics. 2002. ReflexIV: the ultimate performance MALDI-TOF MS. Bruker Daltonics Product Brochure.1-7.

Cacace, J.E. and Mazza, G. 2002. Extraction of anthocyanins and other phenolics from black currants with sulfured water. J. Agric. Food Chem. 2002. 50:5939-5946.

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

Callahan, J.C., Cleary, G.W., Elefant, M., Kaplan, G., Kensler, T., and Nash, R.A. 1982. Equilibrium moisture content of pharmaceutical excipients. Drug Dev. Ind. Pharm. 8: 355-369.

Camire, M.E. 2002. Chapter 3: Phytochemicals in the Vaccinium family: Bilberries, blueberries, and cranberries. In Phytochemicals: their role in nutrition and health. Meskin M.S. Ed. CRC Press LLC, Boca Raton, FL. p 19-40.

Cao, G., Alessio, H.M., and Culter, R.G. 1993. Oxygen-radical absorbance capacity assay for antioxidants. Free Radical Biol. Med. 14:303-311.

Cao, G., Sofic, E., and Prior, R.L. 1996. Antioxidant capacity of tea and common vegetables. J. Agric. Food Chem. 44:3426-3431.

Cao, G. and Prior, R.L. 1999. The measurement of oxygen radical absorbance capacity in biological samples. Methods Enzymol. 299:50-62.

Clark, J.R., Howard, L., and Talcott, S. 2002. Variation in phytochemical composition of blueberry cultivars and breeding seasons. Acta Hort. 574:203-207.

Coba, K. and Goodwin, J.A. 2003. Oregon Agri-Facts. Oregon Agricultural Statistics Service. Oregon Department of Agriculture. Portland, OR.

Cohen-Boulakia, F., Valensi, P.E., Boulahdour, H., Lestrade, R., Dufour-Lamartinie, J.F., Hort-Legrande, C., Behar, A. 2000. In vivo sequential study of skeletal muscle capillary permeability in diabetic rats: effect of anthocyanosides. Metabolism: Clin. Experi. 49:880-5.

Conner, A.M., Luby, J.J., Tong, C.B.S., Finn, C.E., and Hancock, J.F. 2002. Genotypic and environmental variation in antioxidant activity among blueberry cultivars. Acta Hort. 574:209-213.

Contestabile, M., Appolloni, R., Suppressa, F. 1991. Prolonged treatment with blueberry anthocyanides administered in high doses: electrophysiological response in myopic patients. Boll. Ocul. 70:1157-1169.

Criqui, M.H. and Ringel, B.L. 1994. Does diet or alcohol explain the French paradox? Lancet. 344:1719-1723.

Doughty, C.C., Adams, E.B., Martin, L.W., Bristow, P., Byther, R., Antonelli, A., and Shanks, C.H. 1981. PNW 215: Highbush blueberry production. Oregon State University, Corvallis, OR.

Durst, R.W. and Wrolstad, R.E. 2001. Unit F1.3: Anthocyanins. Separation and characterization of anthocyanins by HPLC. In: Wrolstad R.E., editor. Current Protocols in Food Analytical Chemistry. New York: John Wiley & Sons. p 1-13.

Eck, P. 1988. Blueberry Science. Rutgers University Press. London.

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. 49:2222-2227.

Erlund, I., Marniemi, J., Hakala, P., Alfthan, G., Meririnne, E., and Aro, A. 2003. Consumption of black currants, lingonberries, and bilberries increases serum quercetin concentration. Eur. J. Clin. Nutr. 57:37-42.

Finn, C.E. and Young, A. 2002. Variability for reproductive and vegetative traits in *Vaccinium membranaceum.* Acta Hort. 574:181-187.

Fiorini G., Biancacci, A., and Graziano, F. 1965. Perimetric and adaptometric changes following the administration of myrtillin associated with beta-carotene. Annali Di Ottalmologia E. Clinica Oculistica. 91:371-386.

Fossen, T., Cabrita, L., and Andersen, Ø.M. 1998. Color and stability of pure anthocyanins influenced by pH including the alkaline region. Food Chem. 63: 435-440.

Francis, F. 1982. Analysis of anthocyanins. In Anthocyanins as Food Colors., Markakis, P., Ed., Academic Press: New York.

Francis, F.J. 1989. Food colorants: Anthocyanins. Crit. Rev. Food Sci. Nutri. 28:273-3114.

Frankel, E.N., Kanner, J., German, J.B. Parks, E., and Kinsella, J.E. 1993. Inhibition of oxidation of human low-density protein by phenolic substances in red wine. The Lancet. 341:454-457.

Fuleki, T. and Hope, G.W. 1964. Effect of various treatments on yield and composition of blueberry juice. Food Technol. 18(4):166-168.

Fuleki, T. and Francis, F.J. 1968. Quantitative methods for anthocyanins. 2. Determination of total anthocyanin and degradation index for cranberry juice. J. Food Sci. 33:78-82.

Gao, L. and Mazza, G. 1994. Quantitation and distribution of simple and acylated anthocyanins and other phenolics in blueberries. J. Food Sci. 59(5):1057-1059.

Gao, M., Kay, C.D., Cottrell, T., and Holub, B.J. 2002. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. J. Agric. Food Chem. 50:7731-7737.

Gertenbach, D.D. 2001. Solid-liquid extraction technologies for manufacturing nutraceuticals from botanicals. In: Functional Foods: Biochemical and processing aspects. Shi, J., Mazza, G., and Le Maguer. (Ed.). Vol2. Boca Raton, FL: CRC Press Inc, p331-366.

Giusti, M.M. and Wrolstad, R.E. 1996. Red radish anthocyanins as natural red colorant for maraschino cherries. J. Food Sci. 61(4):688-694.

Giusti, M.M., Rodriguez-Saona, L.E., Griffin, D., and Wrolstad, R.E. 1999. Electrospray and tandem mass spectroscopy as tools for anthocyanin characterization. J. Agric. Food Chem. 47:4657-4664.

Giusti, M.M. and Wrolstad, R.E. 2001. Unit F1.2: Anthocyanins. Characterization and measurement with UV-visible spectroscopy. In: Wrolstad RE, editor. Current Protocols in Food Analytical Chemistry. New York: John Wiley & Sons. p 1-13.

Goodwin, J.S. and Brodwick, M. 1995. Diet, aging, and cancer. Clinics in Geriatric Medicine. 11:577-589.

Gould, K.S. and Lee D.W. (Eds.). 2002. Anthocyanins and leaves. The function of anthocyanins in vegetative organs (Vol. 37). Advances in botanical research. London: Academic Press.

Grafe, V. Studies on Anthocyanin. III. 1912. Chemiker-Zeitung 35:768.

Gu, L., Kelm, M., Hammerstone, J.F., Beecher, G., Cunningham, D., Vannozzi, S., and Prior, R.L. 2002. Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC-MS flourescent detection method. J. Agric. Food Chem. 50:4852-4860.

Halliwell, B. and Gutteridge, J..M.C. Ed. 1999. Free radicals in biology and medicine. 3rd edition. Oxford University Press. Oxford, NY.

Hancock, J.F. and Siefker, J.H. 1982. Levels of inbreeding in highbush blueberry cultivars. HortScience 17:363-366.

Hancock, J.F. 1989. Blueberry research in North America. Acta Hort. 241:19-30.

Hancock, J.F. and Draper, A.D. 1989. Blueberry culture in North America. HortScience. 24:551-556.

Häkkinen, S., Heinonen, M., Kärenlampi, S., Mykkänen, H., Ruuskanen, J., and Törrönen, R. 1999. Screening of selected flavonoids and phenolic acids in 19 berries. Food Res. Intl. 32:345-353.

Häkkinen, S.H., Kärenlampi S.O., Mykkänen, H.M., and Törrönen, A.R. 2000. Influence of domestic processing and storage on flavonol contents in berries. J. Agric. Food Chem. 48:2960-2965.

Heinonen, I.M., Meyer, A.S., and Frankel, E.N. 1998. Antioxidant activity of berry phenolics on human low-density lipoprotein and liposome oxidation. J. Agric. Food Chem. 46:4107-4112.

Heredia, F.J., Francia-Aricha, E.M., Rivas-Gonzalo, J.C., Vicario, I.M., and Santos-Buelga, C. 1998. Chromatic characterization of anthocyanins from red grapes I. pH effect. Food Chem. 63:491-498.

Herrmann, K. 1989. Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. Crit. Rev. Food Sci. and Nutri. 28:315-347.

Hertog, M.G.L., Feskens, E.J.M., Hollman, P.C.H., Katan, M.R., and Kromhout, D. 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. The Lancet. 342:1007-1011.

Hertog, M.G.L., Feskens, E.J.M., Hollman, P.C.H., Katan, M.R., and Kromhout, D. 1994. Dietary antioxidant flavonoids and cancer risk in the Zutphen elderly study. Nutr. and Cancer. 22:175-184.

Hertog, M.G.L., Kronhout, D., Aravanis, C., Blackburn, H., Buzina, R., Fidanza, F., Giampaoli, S., Jansen, A., Menotti, A., Nedeljkovc, S., Pekkarinen, M., Simic, B.S., Toshima, H., Feskens, E.J.M., Hollman, P.C.H., and Katan, M.R. 1995. Flavonoid intake and long-term and risk of coronary heart disease and cancer risk in the seven countries study. Archives of International Medicine. 155:381-386.

Hitchcock, C.L. and Cronquist. A. 1973. Flora of the Pacific Northwest. University of Washington Press, Seattle, Wash.

Hollman, P.C. 2001. Evidence for health benefits of plant phenols: local or systemic effects? J. Sci. Food Agric. 81:842-852.

Hong, V. and Wrolstad, R. 1990. Use of HPLC separation/photodiode array detection for characterization of anthocyanins. J. Agric. Food Chem. 38:708-715.

Howard, L.R., Clark, J.R., and Brownmiller, C. 2003. Antioxidant capacity and phenolic content in blueberry as affected by genotype and growing season. J. Sci. Food and Agri. 83:1238-1247.

Jacob, R.A. and Burri, B.J. 1996. Oxidative damage and defense. Amer. J. Clin. Nutri. 63:985S-990S.

Jayle, G.E. and Aubert, L. 1964. Action des glucosides d'anthocyanes sur la vision scotopique et mesopique du sujet normal. Theapie. 19:171-185.

Jiménez, M. and García-Carmona, F. 1999. Oxidation of the flavonol quercetin by polyphenol oxidase. J. Agric. Food Chem. 47:56-60.

Joseph, J.A., Shukitt-Hale, B., Denisova, N.A., Bielinski, D., Martin, A., McEwen, J.J., and Bickford, P. 1999. Reversal 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.

Jurd, L. & Asen, S. 1966. The formation of metal and "co-pigment" complexes of cyanidin-3-glucoside. Phytochem. 5:1263-1271.

Kähkönen, M.P., Hopia, A.I., and Heinonen, M. 2001. Berry phenolics and their antioxidant activity. J. Agric. Food Chem. 49:4076-4082.

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

Kader, F., Rovel, B., Girardin, M., and Metche, M. 1997. Mechanism of browning in fresh highbush blueberry fruit (*Vaccinium corymbosum* L). Role of blueberry polyphenol oxidase, chlorogenic acid and anthocyanins. J. Sci. Food Agric. 74:31-34.

Kader, F., Haluk, J., Nicolas, J., and Metche, M. 1998. Degradation of cyanidin-3-glucoside by blueberry polyphenol oxidase: kinetic studies and mechanisms. J. Agric. Food Chem. 46:3060-3065.

Kader, F., Nicolas, J., and Metche, M. 1999. Degradation of pelargonidin 3-glucoside in the presence of chlorogenic acid and blueberry polyphenol oxidase. J. Sci. Food Agric. 79:517-522.

Kalt, W. and McDonald, J.E. 1996. Chemical composition of lowbush blueberry cultivars. J. Amer. Soc. Hort. Sci. 121:142-146.

Kalt, W. and Dufour, D. 1997. Health functionality of blueberries. HortTechnology 7:216-221.

Kalt, W., McDonald, J.E., Ricker, R.D., and Lu, X. 1999a. Anthocyanin content and profile within and among blueberry species. Can. J. Plant Sci. 79:617-623.

Kalt, W., Forney, C.F., Martin, A., and Prior, R.L. 1999b. Antioxidant capacity, vitamin C, phenolics, and anthocyanins after fresh storage of small fruits. J. Agric. Food Chem. 47:4638-4644.

Kalt, W., McDonald, J.E., and Donner, H. 2000. Anthocyanins, phenolics, and antioxidant capacity of processed lowbush blueberry products. J. Food Sci. 65:390-393.

Karas, M., Bachman, D., Bahr, U., and Hillenkamp, F. 1987. Matrix-assisted ultraviolet laser desorption of nonvolatile compounds. Int. J. Mass Spec. Ion Processes. 78:53-68.

Karas, M. 1996. Matrix-assisted laser desorption ionization MS: a progress report. Biochem. Mass Spectrom. 24:897-900.

Katsube, N., Iwashita, K., Tsushida, T., Yamaki, K., and Kobori, M. 2003. Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. J. Agri. Food Chem. 51:68-75.

Keely, P.B., Martinsen, C.S., Hunn, E.S., and Norton, H.H. 1982. Composition of native American fruits in the Pacific Northwest. J. Amer. Diet. Assoc. 81:568-572.

Koes, R., F. Quattrocchio, and J. Mol. 1994. The flavonoid biosynthetic pathway in plants: Function and evolution. BioEssays. 16:123-132.

Kushman, L.J. and Ballinger, W.E. 1968. Acid and sugar changes during ripening in Wolcott blueberries. Proc. Amer. Soc. Hort. Sci. 92:290-295.

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

Lasagabaster, A., Martin, C., and Goñi, M.M. 1994. Preparation of spherically agglomerated crystals of the 3,5-diglucoside of cyanidin (cyanin). J. Chem. Tech. Biotechnol. 60:397-403.

Lee, J., R.W. Durst, and R.E. Wrolstad. 2002. Impact of juice processing on blueberry anthocyanins and polyphenolics: comparison of two pretreatments. J. Food Sci. 67:1660-1667.

Lee, J., Finn, C.E., and Wrolstad, R.E. 2003. Anthocyanin pigment and total phenolic content of three *Vaccinium* species native to the Pacific Northwest of North America. HortSci. In press.

Luo, X.D., Basile, M.J., and Kennelly, E.J. 2002. Polyphenolic antioxidants from the fruits of *Chrysophyllum cainito* L. (star apple). J. Agri. Food Chem. 50:1379-1382.

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. 51:5867-5870.

Martín-Aragón, S., Basabe Belén, B., Benedí, J.M., and Villar, A.M. 1999. In vitro and in vivo antioxidant properties of *Vaccinium myrtillus*. Pharm. Biol. 37:109-113.

Matsumoto, H., Hanamura, S., Kawakami, T., Sato, Y., and Hirayama, M. 2001. Preparative-scale isolation of four anthocyanin components of black currant *(Ribes nigrum* L.) fruits. J. Agric. Food Chem. 49:1541-1545.

Matsumoto, T., Nishida, K., Noguchi, M., and E., Tamaki. 1970. Isolation and identification of anthocyanin from the cell suspension culture of Poplar. Agr. Biol. Chem. 34: 1110-1114.

Mazza, G. 1995. Anthocyanins in grapes and grape products. Crit. Rev. Food Sci. Nutr. 35:341-371.

Mazza, G. and Miniati, E. 1993. Anthocyanins in Fruits, Vegetables, and Grains. Boca Raton, FL.: CRC Press Inc. p 87-92.

Mazza, G., Kay, C.D., Cottrell, T., and Holub, B.J. 2002. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. J. Agric. Food Chem. 50:7731-7737.

McClure, J.W. 1967. Photocontrol of Spirodela interdmedia flavonoids. Plant Physiol. 43:193-200.

McGhie, T.K., Ainge, G.D., Barnett, L.E., Cooney, J.M. and Jensen, D.J. 2003. Anthocyanin glycosides from berry fruit are absorbed and excreted unmetabolized by both humans and rats. J. Agri. Food Chem. 51:4539-4548.

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.

Miller, H.E., Rigelhof ,F., Marquart, L., Prakash, A., and Kanter, M. 2000. Antioxidant content of whole grain breakfast cereals, fruits and vegetables. J. Am. Coll. Nutr. 19:312S-319S.

Moerman, D.E. 1998. Native American Ethnobotany. Timber Press, Portland, Ore.

Moll, F.O. 1933. Pacific coast huckleberry (*Vaccinium ovatum*), A thoroughbred in ornamental shrubs. Sunset Magazine (Pacific Edition), March 1933.

Montgomery, M.W., Reyes, F.G.R., Cornwell, C., and Beavers, D.V. 1982. Sugars and acid analysis and effect of heating on color stability of northwest Concord grape juice. J. Food Sci. 47:1883-1885, 1891.

Morazzoni, P. and Bombardelli, E. 1996. *Vaccinium myrtillus* L. Fitoterapia. 68:3-28.

Moure, A., Cruz, J.M., Franco, D., Domínguez, J.M., Sineiro, J., Domínguez, H., Núñez, M.J., and Parajó, J.C. 2001. Natural antioxidant from residual sources. Food Chem. 72:145-171.

Moyer, R.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.

Muth, E.R., Laurent, J.M., and Jasper, P. 2000. The effect of bilberry nutritional supplementation on night visual acuity and contrast sensitivity. Alter. Med. Rev. 5:164-173.

Mzhavanadze, V.V., Targamadze, I.L., and Dranik, L.I. 1972a. Phenolic compounds of unripe blueberry *Vaccinium arctostaphylos* fruits. Soobshcheniya Akademii Nauk Gruzinskoi SSR. 68:205-208.

Mzhavanadze, V.V., Targamadze, I.L., and Dranik, L.I. 1972b. Phenolic compounds from *Vaccinium arctostaphylos* leaves. Khimiya Prirodnykh Soedinenii. 8:124.

Nagels, L., Van Dongen, W., De Brucker, J., and De Pooter, H. 1980. High-Performance liquid chromatographic separation of naturally occurring esters of phenolic acids. J. Chrom. 187:181-187.

Ness, A.R. and Powles, J.W. 1997. Fruit and vegetables, and cardiovascular disease: a review. Int. J. Epidemiol. 26:1-13.

Ofek, I., Goldhar, D., Zafriri, H., Adar, R., and Sharon, N. 1991. Anti-*Escherichia coli* adhesion activity of cranberry and blueberry juices. New Engl. J. Med. 324:1599.

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

Pedersen, C.B., Kyle, J., Jenkinson, A.M., Gardner, P.T., McPhail, D.B., and Duthie, G.G. 2000. Effects of blueberry and cranberry juice consumption on the plasma antioxidant capacity of healthy female volunteers. Euro. J. Clin. Nutri. 54:405-408.

Pellerro Society. 1974. Nutritional comparison of Scandinavian and Canadian blueberries. Pellerro Soc., Helsinki, Finland.

Popenoe, W. 1924. Hunting new fruits in Ecuador. Nat. Hist. 24:455-466.

Postman, J.D. 2004. An evergreen huckleberry industry near the Oregon Coast early in the 20th Century. J. Am. Pom. Soc. Submitted.

Prior, R.L. 1998. Antioxidant capacity and health benefits of fruits and vegetables: blueberries, the leader of the pack. Proc. 32nd Ann. Open House North Carolina Blueberry Council. 32:3-12.

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 phenolic and anthocyanin 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 Spectrometry. J. Agric. Food Chem. 49:1270-1276.

Rapisarda, P., Fallico, B., Izzo, R., and Maccarone, E. 1994. A simple and reliable method for determining anthocyanins in blood orange juices. Agrochemica. 38:157-164.

Rapisarda, P., Fanella, F., and Maccarone, E. 2000. Reliability of analytical methods for determining anthocyanins in blood orange juices. J. Agric. Food Chem. 48: 2249-2252.
Siegelman, H.W. and Hendricks, S.B. 1958. Photocontrol of alcohol, aldehyde and anthocyanin production in apple skin. Plant Physiol. 33:409-413.

Rodriguez-Saona, L.E. and Wrolstad, R.E. 2001. Unit F1.1: Anthocyanins. Extraction, isolation and purification of anthocyanins. In: Wrolstad RE, editor. In Current Protocols in Food Analytical Chemistry. R.E. Wrolstad (ed.). John Wiley & Sons, New York.

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

Saito, N., Hirata, K., Hotta, R., and Hayashi, K. 1964. Studies on anthocyanins XLIV. Isolation and crystallization of genuine red anthocyanins. Proc. Japan Acad. 40:516-521.

Sánchez-Moreno, C. Cao, G., Ou, B. and Prior, R.L. 2003. Anthocyanin and proanthocyanidin content in selected white and red wines. Oxygen radical absorbance capacity comparison with nontraditional wines obtained from highbush blueberry. J. Agric. Food Chem. 51:4889-4896.

Sapers, G.M. 1993. Browning of foods: control by sulfites, antioxidants, and other means. Food Tech. 47:75-84.

Sapers, G.M., Burgher, A.M., Phillips, J.G., Jones, S.B., Stone, E.G. 1984. Color and composition of highbush blueberry cultivars. J. Amer. Soc. Hort. Sci. 109:105-111.

Sayavedra-Soto, L.A. and Montgomery, M.W. 1986. Inhibition of polyphenoloxidase by sulfite. J. Food Sci. 51:1531-1536.

Schieber, A., Stintzing, F.A., and Carle, R. 2001. By-products of plant food processing as a source of functional compounds- recent developments. Trends in Food Sci. & Tech. 12:401-403.

Schuster, B. and Herrmann, K. 1985. Hydroxybenzoic and hydroxycinnamic acid derivatives in soft fruits. Phytochem 24:2761-2764.

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

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

Skrede, G. and Wrolstad, R.E. 2002. Chapter 3: Flavonoids from berries and grapes. In: Shi, J., Mazza, G., and Maguer, M.L., editors. Functional foods: biochemical and processing aspects. New York, US: CRC press. p 71-133.

Smith, M.A.L., Marley, K.A., Seigler, D., Singletary, K.W., and Meline, B. 2000. Bioactive properties of wild blueberry fruits. J. Food Sci. 65:352-356.

Sondheimer, E. and Kertesz, Z. I. 1948. Anthocyanin pigments. Colorimetric determination in strawberries and strawberry perserves. Anal. Chem. 20:245-248.

Stanley, R. and Miller, S. 2001. Enzymatic processing for the release of phenolic antioxidants from fruit waste. Biol. Act. Phyto. Food. 269:552-556.

Steinberg, D. 1997. A critical look at evidence for the oxidation of LDL in atherogenesis. Atherosclerosis. 131:S5-S7.

Steinmetz, K.A. and Potter, J.D. 1991. Vegetables, fruit, and cancer. I. Epidemiology. Cancer Causes Contr. 2:325-357.

Stevens, P.F. 1969. Taxonomic studies in the Ericaceae. PhD Diss., Univ. of Edinburgh, Edinburgh, U.K.

Strack, D. and Wray, V. 1994. The Anthocyanins. In: Harborne JB, editor. The Flavonoids, London, UK: Chapman & Hall. p 1-22.

Sweeney, M.I., Kalt, W., Mackinnon, S.L., Ashby, J. and Gottschall-Pass, K.T. 2002. Feeding rats diet enriched in lowbush blueberries for six weeks decreases ischemia-induced brain damage. Nutri. Neurosci. 5:427-431.

Takeda, K. and Hayashi, K. 1965. Studies on Anthocyanins XLVIII. Crystallization and some properties of the genuine anthocyanin inherent to the deep violet color of pansy. Proc. Japan Acad. 39:449-454.

Takeoka, G. and Dao, L. 2002. Anthocyanins. In. Methods of Analysis for Functional Foods and Nutraceuticals. Hurst, W.J. (Ed.) CRC Press LLC, Boca Raton FL. p 219-241.

Vander Kloet, S.P. 1988. The Genus Vaccinium in North America. Publication 1828, Research branch, Agriculture Canada.

Van Teeling, C.G., Cansfield, P.E., and Gallop, R.A. 1971. Anthocyanin complex isolated from the syrup of canned blueberries. J. Food Sci. 36:1061-1063.

Velioglu, Y.S., Mazza, G., Gao, L., and Oomah, B.D. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J. Agric. Food Chem. 46:4113-4117.

Wada, L. and Ou, B. 2002. Antioxidant activity and phenolic content of Oregon caneberries. J. Agric. Food Chem. 50:3495-3500.

Wang, H., Cao, G., and Prior, R.L. 1996. Total antioxidant capacity of fruits. J. Agric. Food Chem. 44:701-705.

Wang, J., and Sporns, P. 1999. Analysis of anthocyanins in red wine and fruit juice using MALDI-MS. J. Agric. Food Chem. 47:2009-2015.

Wang, J., Kalt, W., and Sporns, P. 2000. Comparison between HPLC and MALDI-TOF MS analysis of anthocyanins in highbush blueberries. J. Agric. Food Chem. 48:3330-3335.

Wang, S.Y. and Jiao, H. 2000. Scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. J. Agric. Food Chem. 48:5677-84.

Waterhouse, A.L. 2002. Unit I1.1: Polyphenolics: Determination of Total Phenolics, p. 1-4. In: R.E. Wrolstad (ed.). Current Protocols in Food Analytical Chemistry. John Wiley & Sons, New York.

Wightman, J.D. and Wrolstad, R.E. 1995. Anthocyanin analysis as a measure of glycosidase activity in enzymes for juice processing. J. Food Sci. 60:862-867.

Wrolstad, R.E., Wightman, J.D., and Durst, R.W. 1994. Glycosidase activity of enzyme preparations used in fruit juice processing. Food Technol. 48:90-98.

Wrolstad, R.E., Hong, V., Boyles, M.J., and Durst, R.W. 1995. Use of anthocyanin pigment analysis for detecting adulteration in fruit juices. In, Methods to Detect Adulteration in Fruit Juice and Beverages, Vol. I. S. Nagy and R. L. Wade (Ed.). AgScience Inc., Auburndale, Fla. p 260-286.

Wrolstad, R.E., Culbertson, J.D., Corwell, C.J., and Mattick, L.R. 1982. Detection of adulteration in blackberry juice concentrates and wines. J. Assoc. Off. Anal. Chem. 65:1417-1423.

Wrolstad, R.E. 2000. Anthocyanins, p.237-252. In Natural Food Colorants. Science and Technology. Lauro, G.J. and Francis, F.J. Marcel Dekker, New York.

Wu, X., Cao, G., and Prior R.L. 2002. Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry. J. Nutr. 132:1865-1871.

Youdim, K.A., McDonald, J., Kalt, W., and Joseph, J.A. 2002. Potential role of dietary flavnoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults. J. Nutri. Biochem. 13:282-288.

Youdim, K.A., Shukitt-Hale, B., Martin, A., Wang, H., Denisova, N., Bickford, P.C., and Joseph, J.A. 2000. Short-term dietary supplementation of blueberry polyphenolics:

beneficial effects on aging brain performance and peripheral tissue function. Nutritional Neuroscience. 3:383-397.

Zheng, W. and Wang, S.Y. 2003. Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. J. Agric. Food Chem. 51:502-509.

Zoecklin, B.W., Fugelsang, K.C., Gump, B.H., and Nury, F.S. 1995. Ch. 20. Laboratory procedures. In: Zoecklin BW, Fugelsang KC, Gump BH, Nury FS, editors. Wine analysis and production. New York: Chapman & Hall. p 493-497.

APPENDICES

APPENDIX A

APPROVED AOAC PRE-COLLABORATIVE STUDY PROTOCOL FOR THE DETERMINATION OF TOTAL MONOMERIC ANTHOCYANIN PIGMENT CONTENT BY THE PH DIFFERENTIAL METHOD OF FRUIT JUICES, BEVERAGES, NATURAL COLORANTS, AND WINES.

Jungmin Lee, Robert W. Durst, and Ronald E. Wrolstad

Department of Food Science and Technology

Oregon State University, Corvallis, OR 97331-6602

Accepted by AOAC International.

INTRODUCTION

Anthocyanin pigments are important in the quality of food for their contribution to appearance, taste, and health benefits. The importance of anthocyanin pigments in fruit juices, nutraceuticals, and natural colorants has led to a need for an AOAC method to rapidly, and precisely determine total monomeric anthocyanin content. Currently, there is no approved AOAC method that is quick, simple, and accurate to measure total monomeric anthocyanin content in beverage systems. The goal of this study is to design and organize a collaborative study to validate the pH differential method as an AOAC method.

The pH differential method is used extensively by food technologists and horticulturists in assessing the quality of fresh, and processed fruits and vegetables. The method can be utilized for quantitative determination of total monomeric anthocyanin content. It is intended for use in research (and quality control) with anthocyanin containing fruit juices, wines, and other beverages.

Monomeric anthocyanins undergo a structural transformation as a function of pH (colored oxonium form at pH 1.0 and colorless hemiketal form at pH 4.5). Thus the difference in absorbance at the pigments $\lambda_{vis\ max}$ ($\approx$ 520 nm) will be proportional to the pigment concentration. Degraded anthocyanins in the polymeric form are resistant to color change with pH. Hence, polymerized anthocyanin pigments will not be included in the measurement as they absorb at pH 4.5 as well as pH 1.0.

COLLABORATORS

We are members of Technical Committee of Juice and Juice Products (TCJJP) and at their request agreed to plan this Collaborative Study and submit it to AOAC for approval. Several members of TCJJP have shown interest in participating in this study. There will be a minimum of 8 collaborating laboratories. Currently, we have 13 collaborators that are interested in participating in this study, and if all agree to participate, we will use all 13 collaborators.

Table A.1. List of Collaborators*

Area of Specialization	Organization	Contact Name	Contact Information
Analytical Chemist	Ottawa Laboratory - (Carling) Canadian Food Inspection Agency 960 Carling Ave. Bldg 22 CEF Ottawa, Ontario, Canada K1A 0C6	Jonathan Hache Special Projects Chemist	Phone: 1-613-759-1218 Fax: 1-613-759-1260 E-mail: hachej@inspection.gc.ca
Analytical Chemist Fruit Juice Authenticiy	Krueger Food Laboratories Inc. 711 Concord Avenue Cambridge, MA 02139 U.S.A.	Dana A. Krueger President	Phone: 1-617-876-9118 Fax: 1-617-876-0572 E-mail: dkrueger@kfl.com
Beverages	Canandaigua Wine Co. 12667 Rd 24 Madera, CA 93639 U.S.A.	Steve Kupina Principal Chemist	Phone: 1-559-661-5548 Fax: 1-559-661-3430 E-mail: Steve.kupina@cwine.com
Analytical Chemist Nutraceuticals/ Colorants	Artemis International Inc. 9318 Airport Dr. Fort Wayne, IN 46809 U.S.A.	JoLynne D. Wightman Director of Technical Services	Phone: 1-219-436-6899 Fax: 1-219-459-1733 E-mail: jwightman@artemis-international.com
Food Chemist	3304 Marie Mount Hall Nutrition and Food Science Department, University of Maryland College Park, MD 20742	M. Monica Giusti Assistant Professor	Phone: 1-301-405-5421 Fax:1-301-314-9327 E-mail: mg237@umail.umd.edu
Food Chemist	Matforsk Norwegian Food Research Institute Osloveien 1, N-1430 Ås Norway	Grete Skrede Research Chemist	Phone: 1-47-64-97-0224 Fax: 1-47-64-97-0333 E-mail: grete.skrede@matforsk.no

Table A.1. List of Collaborators*(Continued)

Beverages	Tree Top Technical Center 111 S. Railroad Ave Selah, WA 98942 U.S.A.	Tom Eisele Manager Technical Services	Phone: 1-509-697-7251 Fax:1-509-697-0446 E-mail: teisele@treetop.com
Analytical Chemist & Nutrition	Alpha Laboratories Division of Eurofins Scientific, Inc. 1365 Redwood Way Petaluma, CA 94954 U.S.A.	Tom C. Miller Laboratory Director	Phone: 1-707-792-7300, Ext. 25 Fax:1-707-792-7309 E-mail: TomMiller@EurofinsUS.com
Analytical Chemist	Reading Scientific Services Ltd The Lord Zuckerman Research Centere The University, Whiteknights Reading, RG6 6LA UK	David Hammond	Phone: 1-44-118-9868-541 Fax: 1-44-118-9868-932 E-mail: David.A.Hammond@rssl.com
Technical Manager	Gerber Foods-Soft Drinks Limited 78 Wembdon Road Bridgewater, Somerset, U.K. TA6 7QR	Ian Howard / Annie Ng Technical Manager-Raw Materials	Phone: (01278) 441600 Fax: (01278) 441647 E-mail: ian.howard@gerberfoods.com
Analytical Chemist	Eurofins Scientific Analytics Rue Pierre Adolphe Bobierre Site de la Geraudiere BP 42301 44323 Nantes Cedex 3 France	Michele Lees	Phone: 33-2-51-83-27-07 Fax: 33-2-51-83-21-11 e-mail: Michelelees@eurofins.com
Analytical Chemist Fruit Juice Authenticity	Gfl Landgrafenstrasse 16 Berlin 10787 Germany	Susanne Koswig	Phone: 1-49-32-261-9075 Fax: 1-30-261-9076 E-mail: gfl.berlin@t-online.de
Analytical Chemist	The Minute Maid Co. 2631 Orange Ave Apopka, FL 32703 U.S.A.	Susan K. Martin Director, Ingredient Sciences	Phone: 1-407-814-2880 Fax: 1-407-814-9875 E-mail: sumartin@minutemaid.com

* Note: These laboratories have not been approved by AOAC International. These laboratories simply expressed interest in participating in the collaborative study.

STUDY DESIGN

The materials for the collaborative study will be from a variety of juice and fruit sources that are representative of the expected breadth of application of the method. These samples will all be grouped into Youden matched pairs (with one sample being neat, undiluted, and the other diluted to decrease the reported anthocyanin content by 1-5%). The following Table is a summary of the results from an in-house study.

Table A.2. Intralaboratory Study Results Table.

ID	Mean, mg/L	No. individuals[a] (b)	S_r	$\%RSD_r$	S_R	$\%RSD_R$	r	R	Horrat value
Cranberry	45.1	10(0)	0.08	1.74	0.21	4.72	0.22	0.60	0.52
Megared[1]	320.8	10(0)	1.08	3.38	1.25	3.89	3.04	3.49	0.58
Raspberry	594.8	10(0)	2.85	6.18	3.68	6.18	7.97	10.30	1.01
Wine	33.3	10(0)	0.08	2.38	0.16	4.82	0.22	0.45	0.51
Elderberry	814.3	10(0)	1.53	1.88	3.31	4.07	4.29	9.27	0.67
Strawberry	137.4	10(0)	0.48	3.53	1.46	10.60	1.36	4.08	1.39

[1]: Meganatural grape red is a high-colored grape juice concentrate from Canandaigua West, Inc (Madera, CA). a(b) a=numbers of individuals retained after eliminating outliers, (b)=number of individuals removed as outliers; S_r=Repeatability Standard Deviation; S_R= Reproducibility Standard Deviation; RSD=Relative Standard Deviations; r=repeatability value; R=reproducibility value.

TEST SAMPLE PREPARATION

The split levels (Youden pairs) will be prepared by one sample being neat (undiluted), and the other sample diluted with water to make a difference of 1-5 %. There will be a set of 7 samples. Replications will be obtained by the Youden pairs. The following is a list of proposed samples:

a. Cranberry juice cocktail (CJC), and CJC diluted by 1-5%.

b. Red wine and red wine diluted by 1-5%.

c. High Colored Grape juice concentrate (natural colorant, i.e. Meganatural grape red) diluted to appropriate strength (2 °Brix) and diluted by 1-5%.

d. Strawberry concentrate diluted to single strength (8.0 °Brix) juice and diluted by 1-5%.

e. Red raspberry concentrate diluted to single strength (9.2 °Brix) juice and diluted by 1-5%.

f. Elderberry concentrate diluted to single strength (11.0 °Brix) juice and diluted by 1-5%.

g. Cyanidin-3-glucoside made up to 80mg/L and diluted by 1-5%.

*Note: Samples a & b will be purchased from a local market, c will be obtained from Canandaigua West, Inc. (Madera, CA), while d, e, and f will be provided by Kerr Concentrate Inc. (Salem, OR). Sample g will be purchased from Polyphenols Laboratories (Sandnes, Norway).

Two familiarization test samples will also be included with the above samples. Collaborators will be asked to report values for the two familiarization test samples, and will be notified whether to continue with the actual test samples or not depending on how close their values are to the original.

The anthocyanin content of the provided samples will range approximately from 20.0 to 1000.0mg / L (expressed as cyanidin-3-glucoside). The method requires dilution of the samples with buffers by the analyst to an appropriate concentration.

The samples will be homogenized by stirring, then placed into individual containers (25 mL). The individual samples will come from a single batch of

homogenous product. All samples will be labeled with a random 3-digit number. The familiarization test samples will also be prepared and clearly labeled (cranberry concentrate diluted to single strength and high colored grape juice concentrate diluted to appropriate strength will be the practice samples).

Samples will be placed in a freezable container and frozen (-23°C) until shipped to participants. Samples will be shipped frozen (packed with dry ice and sent overnight shipping via Fed-ex). Shipping boxes will be marked to keep the samples frozen until future analysis. The exact amount of sample used in the assay will vary with commodity and be decided by each participating lab.

The stability and homogeneity of the prepared samples will be evaluated. The stability testing will be performed thoughout the duration of the study for each matrix. There will be a thermal shock stability study and a long term stability study. Thermal shock (transportation) will be performed by placing a test tube of frozen sample (stored at –23°C) at room temperature for 1 day and then returned to –23°C for 2 days (to stimulate temperature abuse during transportation), then pH differential will be performed and replicated. Long term stability testing will be done by analyzing random samples after 0, 3, and 6 months of storage at –23°C. The total monomeric anthocyanin content will be determined in the long term stability test samples. Homogeneity testing of prepared samples will be performed by selecting three test tubes at random and measuring their °Brix by a digital refractometer.

METHOD

AOAC Official Method 2001.0X

Determination of Total Monomeric Anthocyanin Pigment Content by the pH Differential Method of Fruit Juices, Beverages,, Natural Colorant, and Wines. Proposed First Action 2001

(Applicable to the determination of monomeric anthocyanins in fruit juices, beverages, natural colorant, and wines within the range of 20-1000 mg/L cyanidin-3 glucoside equivalents.)

Cautions: Standard laboratory safety procedures must be followed.

A. Principle

Monomeric anthocyanin pigments reversibly change color with pH, the colored oxonium form existing at pH 1.0 and the colorless hemiketal form predominating at pH 4.5. The difference in absorbance at the pigments 520 nm is proportional to the pigment concentration. Results are expressed on a cyanidin-3-glucoside basis. Degraded anthocyanins in the polymeric form are resistant to color change with pH, hence they will not be included in the measurements as they absorb at pH 4.5 as well as pH 1.0.

B. Apparatus and Equipments

(a) *pH meter.* - Standardized with pH 4.0 and 7.0 buffer solutions

(b) *Vis spectrophotometer.*

(c) *Glass or disposable cuvettes for spectrophotometer.* – 1 cm pathlength

(d) Volumetric flasks – 50 mL

(e) Volumetric and serological pipettes – variety of volumes less than 10 mL.

C. Preparations of Reagents

(a) *pH 1.0 buffer (potassium chloride, 0.025 M).* -- Weigh 1.86 g KCl in a beaker, add distilled water to ca 980 mL. Measure the pH and adjust the pH to 1.0 with concentrated HCl (takes ca 6.3 mL). Transfer to a 1 L volumetric flask and fill to line with distilled water.

(b) *pH 4.5 buffer (sodium acetate, 0.4 M).* -- Weigh 54.43 g CH_3CO_2 Na•$3H_2O$ in a beaker, add distilled water to ca 960 mL. Measure the pH and adjust the pH to 4.5 with concentrated HCl (takes ca 20 mL). Transfer to a 1 L volumetric flask and fill to line with distilled water.

D. Preparations of Sample

Perform all dilutions in 50 mL volumetric flasks, B(d), using volumetric pipettes, B(e), to add sample. Maximum sample volume added should not be greater than 10 mL (1 part sample, 4 parts buffer) so as not to exceed the buffer capacity of the reagents.

Determine appropriate dilution factor for sample by diluting with pH 1.0 buffer, C(a), until absorbance of diluted sample at 520 nm is within the linear range of the spectrophotometer. (For most spectrophotometers, the absorbance should be between 0.2 and 1.4). Using the dilution factor determined previously, prepare two dilutions of the sample, one with pH 1.0 buffer and the other with pH 4.5 buffer.

E. Determination

Determine absorbance of sample diluted with pH 1.0, C(a), and 4.5, C(b), buffers, at both 520 nm and at 700 nm. The diluted samples are read against a blank cell filled with distilled water. Measure absorbance within 20 and 50 minutes of sample preparation.

Note: The reason for measuring the absorbance at 700 nm is to correct for haze. However, if the diluted sample is excessively turbid it should be clarified by centrifuging or filtering prior to measurement. A filter (i.e. Millipore™ membrane filters - ≤1.2μm pore size, Millipore Corporation, Bedford, MA) that will not absorb the anthocyanins should be used.

Calculations

Calculate anthocyanin pigment concentration, expressed as cyanidin-3-glucoside equivalents, by the following formula:

$$\text{Anthocyanin pigment (mg/L cyanidin-3-glucoside equivalents)} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times l}$$

Where $A = (A_{520\ nm} - A_{700\ nm})pH\ 1.0 - (A_{520\ nm} - A_{700\ nm})pH\ 4.5$; MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu); DF = dilution factor established in (C); l = pathlength in cm.; ε = 26,900 molar extinction coefficient in $Lmol^{-1}cm^{-1}$, for cyanidin-3-glucoside (cyd-3-glu); 10^3 = conversion from g to mg.

Results should be reported as monomeric anthocyanins in mg/L cyanidin-3-glucoside equivalents.

(2) *Limitations and restrictions.* - We have found that the limitations of the method are very few. The presence of artificial colors or non-anthocyanin colorants can lead to confusion, but generally do not interfere adversely with the assay. Added colorants that might be encountered include Red No. 40, cochineal, and beet powder. Cochineal in high concentrations does lead to a reduction in measured anthocyanin content, but at low concentrations a quantitative total monomeric anthocyanin value can still be obtained. The presence of cochineal can be determined by ELISA technique. Beet powder can be detected by its much higher $\lambda_{vis\ max}$ (≈550 nm). Red #40 can be detected by its bright red color in the pH 4.5 treatment. Anthocyanins can be measured in the presence of these other colorants, if one measures at a wavelength of ≈520 nm. The presence of ethanol in samples does not interfere with the assay at the levels typically encountered in wine (10-14%).

F. References

Giusti, M.M. & Wrolstad, R.E. (2001) Unit F1.2: Anthocyanins. Characterization and measurement with UV-visible spectroscopy. In, Current Protocols in Food Analytical Chemistry. RE Wrolstad (Ed.) New York: John Wiley & Sons. p 1-13.

Giusti, M.M., Rodríguez-Saona, L.E., & Wrolstad, R.E. (1999) *J. Agric. Food Chem.* **47**, 4631-4637.

Jurd, L. & Asen, S. (1966) The formation of metal and "co-pigment" complexes of cyanidin-3-glucoside. *Phytochem.* **5**, 1263-1271

Niketic-Aleksic, G. and Hrazdina, G. (1972) Quantitative analysis of the anthocyanin content in grape juices and wines. *Lebensm. Wiss U. Technol* **5**, 163-165.

Swain, T. (1965) in *Chemistry and Biochemistry of Plant Pigments.* Ch19. T. W. Goodwin (Ed). Academic Press, London, p-533-49.

McClure, J. W. (1967) *Plant Physiol.* 43, 193-200

REPORTING RAW DATA

The following table will be given to the collaborators to report raw data.

Table A.3. Data Reporting Form.

Sample code	DF	A_{520}		A_{700}		Total monomeric anthocyanin (expressed as cyd-3-glu)
		pH 1.0	pH 4.5	pH 1.0	pH 4.5	

Comments:

ANALYZING RAW DATA

All statistical analysis will be performed using the statistical program provided by AOAC. Results from the collaborators will be collected and statistically analyzed for S_r=Repeatability Standard Deviation, S_R= Reproducibility Standard Deviation, RSD=Relative Standard Deviations, r=repeatability value, R=reproducibility value, and Horrat value. Outliers will be determined by the Cochran's test and Grubb's test and dealt with appropriately. Results will be reported as follows:

Table A.4. Example table of the Interlaboratory Study Results for the Determination of Total Monomeric Anthocyanin Pigment Content by the pH Differential Method.

Matrix	X mg/L	No. individuals[a] (b)	S_r	$\%RSD_r$	S_R	$\%RSD_R$	r	R	Horrat value

a(b) a=numbers of individuals retained after eliminating outliers, (b)=number of individuals removed as outliers; S_r=Repeatability Standard Deviation; S_R= Reproducibility Standard Deviation; RSD=Relative Standard Deviations; r=repeatability value; R=reproducibility value.

EXAMPLE OF THE INVITATION LETTER TO COLLABORATORS

08/08/01

Jungmin Lee
Oregon State University
100 Wiegand Hall
Corvallis, OR 97331
USA

[Collaborator's name, affiliation and address]

Dear [ ]:

I am working in Dr. Ronald E. Wrolstad's laboratory and have been appointed Study Director for an AOAC collaborative study, " Determination of total anthocyanin pigment in juice by the pH differential method". We have conducted an in-house study, and submitted a Collaborative Study Protocol. We are waiting approval to proceed with the collaborative study, which is part the next step towards validating the method as an AOAC(Official MethodSM. We are now seeking participants for this interlaboratory collaborative study.

This quantitative spectrophotometric method requires a Vis Spectrophotometer, along with standard laboratory glassware (50 mL volumetric flasks and a selection of ≤ 10mL volumetric and serological pipettes), and a pH meter. The method uses potassium chloride (pH 1.0) and sodium acetate (pH 4.5) buffers.

The study will consist of single analyses of 16 test samples (including 2 familiarization test samples). I estimate that 3-4 hours will be required to prepare buffers and samples, make calculations, and fill in the report forms. The total instrument use time for the analysis should be 0.5-1.5 hours. Therefore, the entire procedure, including instrumental time, should be a single working day.

Sufficient portions of the test samples, and a copy of the method will be provided to collaborators. AOAC, and I, would appreciate your participation in this program. All collaborators will be given credit in publication of the study results within the Journal of AOAC INTERNATIONAL. In addition, I will send you a copy of the study results (with your results annotated) as soon as they are compiled and analyzed.

Please reply by ?/?/02, using the postcard provided, or call me if you have any additional questions. Test samples will be forwarded to you shortly after the study protocol has been reviewed and accepted by AOAC. I will keep you informed of the schedule. My goal is to have all of the collaborators complete their analyses by no later than ?/?/02. Thank you for your consideration and support for development of reliable methods of analysis through AOAC INTERNATIONAL.

Regards,

Jungmin Lee
Study Director / Graduate Research Assistant
Phone #: 541-737-6490
Fax #: 541-737-1877
leeju@onid.orst.edu

EXAMPLE OF INVITATION POSTCARD FOR THE COLLABORATIVE STUDY

Title of study: Determination of Total Monomeric Anthocyanin Pigment Content by the pH Differential Method.

Applicability: Applicable to the quantitative determination of total monomeric anthocyanin pigment is chiefly used in research and quality control. The method is used for anthocyanin containing fruit juices, beverages, natural colorant, and wines. It can also be applied to other anthocyanin containing products such as anthocyanin based natural colorants and alcoholic and aqueous extracts of anthocyanin containing plant materials.

We will participate ☐

Sorry, we cannot participate ☐

We'd be interested in a future study ☐

_____
Name (please print)

_____
Phone # (please include area code) / Email address

_____
Affiliation

The front side of the postcard will be self addressed and stamped.

EXAMPLE LETTER NOTIFYING SHIPMENT OF MATERIALS

08/08/01

Jungmin Lee
Oregon State University
100 Wiegand Hall
Corvallis, OR 97331
USA

[Collaborator's name, affiliation and address]

Dear [ ]:

As the study director for the determination of anthocyanins in juice by the pH differential method, I wish to thank you for agreeing to participate in the collaborative study of the proposed AOAC® Official MethodSM. A copy of the test method, reporting forms, and a postage paid return envelope are enclosed for your use.

The samples should arrive by overnight delivery. Please check all the vials upon receipt for any signs of leakage, and report any to me. The test samples should be stored frozen until analysis. If all materials have arrived properly, without damage but omissions are found, please return the enclosed postage paid postcard to me.

Only a single determination of the total monomeric anthocyanin content in each sample is to be performed. Frozen samples should be thawed to room temperature and thoroughly mixed before analysis. Sample analysis should be started no later than an hour after thawing.

Please analyze the vials marked "Familiarization Test Samples" first (2 total) and send the results back to me. I will notify you when to continue with the actual samples. Please contact me to discuss any problem you are having with the analysis.

I estimate that approximately 3-4 hours are required to prepare reagents and solutions, calculations, and fill in report forms. Test sample analysis should require an additional 0.5-1.5 hours. Standard laboratory safety precautions are required for this study.

NOTE: This is a study of the method, not the laboratory. The method must be followed as closely as practical, and any deviations, no matter how trivial they may seem, must be noted on the report form. Your objective as an analyst should be to follow the procedure as closely as possible.

Please return the completed reporting forms in the enclosed envelope by ?/?/02.

Please do not hesitate to call me if you have any questions. My office hours are 9:00 – 17:00 (PST). I will give you a copy of the results of the collaborative study as soon as they are complied and analyzed. Again, Thank you for your cooperation as a collaborator on this study.

Regards,

Jungmin Lee
Study Director / Graduate Research Assistant
Phone #: 541-737-6490
Fax #: 541-737-1877
leeju@onid.orst.edu

EXAMPLE OF POSTCARD CONFIRMING RECEIPT OF TEST SAMPLES

AOAC INTERNATIONAL COLLABORATIVE STUDY

Title of study: Determination of Total Monomeric Anthocyanin Pigment Content by the pH Differential Method.

Applicability: Applicable to the quantitative determination of total monomeric anthocyanin pigment is chiefly used in research and quality control. The method is used for anthocyanin containing fruit juices, beverages, natural colorant, and wines. It can also be applied to other anthocyanin containing products such as anthocyanin based natural colorants and alcoholic and aqueous extracts of anthocyanin containing plant materials.

Study Director: Jungmin Lee **Contact:**leeju@onid.orst.edu (☎:541-737-6490)

The test samples arrived safely:_____

Name (please print)

_____

Affiliation

Comments:

_____

_____

_____

Please indicate if you need anything (test sample, information,etc.):

_____

_____

APPENDIX B

EXAMPLE LETTERS SENT TO THE COLLABORATORS ACCOMPANYING THE TEST SAMPLES

Jungmin Lee and Ronald E. Wrolstad

Department of Food Science and Technology

Oregon State University, Corvallis, OR 97331-6602

EXAMPLE OF AN ACTUAL LETTER INCLUDED IN THE MAILING BOXES

01/06/03

Dear Collaborator:

I would like to thank you for agreeing to participate in the collaborative study of the proposed AOAC® *Official Method*℠. A copy of the test method and reporting forms are enclosed (and will also be sent electronically).

Please examine each vial upon receipt for signs of leakage, and report any to me. The test samples should be stored frozen until analysis. If all materials have arrived properly, without damage but omissions are found, please let me know (via e-mail or call).

Only a **single determination** of the total monomeric anthocyanin content in each sample is to be performed. Frozen samples should be thawed to room temperature and thoroughly mixed before analysis. Sample analysis should begin no later than one hour after thawing.

Please analyze the vials marked "A & B" first (practice samples, 2 total), and e-mail the results back to me. I will, then notify you to continue with the actual samples (coded with 3-digit numbers, 14 total). Please contact me to discuss any problem you may have with the analysis.

I estimate 3-4 hours are required to prepare reagents and solutions, calculations, and fill in report forms. Test sample analysis should require an additional 0.5-1.5 hours. Standard laboratory safety precautions are required for this study.

NOTE: This is a study of the method, not the laboratory. The method must be followed as closely as practical, and any deviations, no matter how trivial they may seem, must be noted on the report form. Your objective as an analyst should be to follow the procedure as closely as possible.

Please e-mail me the completed report forms by 2/28/02.

Again, please do not hesitate to call me if you have any questions. My office hours are 9:00 – 17:00 (PST). I will forward you a copy of the results of the collaborative study as soon as they are complied and analyzed. Again, Thank you for your cooperation as a collaborator on this study.

Regards,

Jungmin Lee
Study Director / Graduate Research Assistant
Phone #: 541-737-6490
Fax #: 541-737-1877

EXAMPLE LETTER INDICATING THE RANDOMIZED THREE-DIGIT CODES LABELED ON SAMPLES.

The samples included in this shipment are as follows:

Practice samples: A & B

Actual test samples:

103	228
715	776
581	994
859	411
967	360
149	914
404	190

RANDOM THREE-DIGIT NUMBERS TABLE GENERATED FOR LABELING TEST SAMPLES

Samples	Lab A	Lab B	Lab C	Lab D	Lab E	Lab F	Lab G	Lab H	Lab I	Lab J	Lab K
Authentic standard	727	360	702	631	924	461	461	120	980	547	278
Diluted authentic standard	367	103	532	464	215	474	474	919	361	866	764
Natural colorant	780	994	280	053	420	106	106	699	357	672	065
Diluted natural colorant	098	859	673	328	300	425	425	329	152	407	009
Elderberry juice	412	581	061	809	192	097	097	475	808	676	537
Diluted elderberry juice	765	967	941	085	990	188	188	692	139	771	424
Natural colorant	057	404	522	025	567	438	438	100	857	113	728
Diluted natural colorant	989	715	533	007	62	304	304	160	308	826	524
Strawberry juice	804	914	185	202	306	129	129	196	443	906	709
Diluted strawberry juice	435	411	660	040	035	612	612	931	500	949	734
Cranberry juice cocktail	639	228	998	950	648	343	343	521	730	606	767
Diluted cranberry juice cocktail	340	190	114	738	483	471	471	281	868	434	588
Raspberry juice	005	149	988	310	259	845	845	929	946	686	602
Diluted raspberry juice	210	776	944	587	523	729	729	058	288	552	889

APPENDIX C

RAW DATA FROM THE AOAC COLLABORATIVE STUDY: DETERMINATION OF TOTAL MONOMERIC ANTHOCYANIN PIGMENT CONTENT OF FRUIT JUICES, BEVERAGES, NATURAL COLORANTS, AND WINES BY THE PH DIFFERENTIAL METHOD

Jungmin Lee and Ronald E. Wrolstad

Department of Food Science and Technology

Oregon State University, Corvallis, OR 97331-6602

DATA REPORTING FORM FROM LABORATORY A

Data Reporting Form for **Test** Samples

Name: -

Date: 1/9/03

Sample code	DF[1]	A_{520} [2]		A_{700} [3]		Total monomeric anthocyanin (mg/L expressed as cyd-3-glu)
		pH 1.0	pH 4.5	pH 1.0	pH 4.5	
727	5	0.603	0.049	0.000	0.046	46.6
367	5	0.578	0.047	0.001	0.045	44.5
780	60	0.746	0.059	0.002	0.052	693.3
098	60	0.724	0.055	0.003	0.050	672.8
412	300	0.738	0.121	0.006	0.111	3113.5
765	300	0.701	0.115	0.006	0.106	2948.2
057	15	0.384	0.118	0.004	0.105	69.0
989	15	0.368	0.115	0.003	0.102	66.1
804	10	0.156	0.068	0.008	0.058	15.0
435	10	0.147	0.065	0.008	0.057	13.8
639	15	0.926	0.053	0.003	0.049	218.8
340	15	0.892	0.051	0.002	0.048	210.9
005	60	0.444	0.084	0.001	0.079	364.2
210	60	0.437	0.084	0.001	0.080	356.7

[1]DF = Dilution Factor; [2]: A_{520} = Absorbance at 520 nm; [3]: A_{700} = Absorbance at 700 nm.

Comments:

DATA REPORTING FORM FROM LABORATORY B

Data Reporting Form for **Test** Samples

Name: -

Date: 1/17/03

Sample code	DF[1]	A_{520} [2]		A_{700} [3]		Total monomeric anthocyanin (mg/L expressed as cyd-3-glu)
		pH 1.0	pH 4.5	pH 1.0	pH 4.5	
360	5	0.603	0.049	0.000	0.001	46.3
103	5	0.576	0.049	0.000	0.001	44.1
994	50	0.891	0.068	0.002	0.003	688.0
859	50	0.859	0.065	0.002	0.004	664.6
581	250	0.910	0.147	0.006	0.008	3193.7
967	250	0.866	0.142	0.006	0.008	3030.8
404	10	0.606	0.178	0.004	0.012	72.8
715	10	0.583	0.170	0.005	0.013	70.3
914	5	0.307	0.137	0.012	0.012	14.2
411	5	0.296	0.132	0.010	0.011	13.8
228	25	0.569	0.031	0.000	0.001	225.0
190	25	0.548	0.031	0.001	0.001	215.8
149	25	1.056	0.205	0.002	0.004	356.1
776	25	1.025	0.197	0.002	0.004	346.5

[1]DF = Dilution Factor; [2]: A_{520} = Absorbance at 520 nm; [3]: A_{700} = Absorbance at 700 nm.

Comments:

DATA REPORTING FORM FROM LABORATORY C

Data Reporting Form for **Test** Samples

Name: -

Date: 2/20/03

Sample code	DF[1]	A_{520} [2]		A_{700} [3]		Total monomeric anthocyanin (mg/L expressed as cyd-3-glu)
		pH 1.0	pH 4.5	pH 1.0	pH 4.5	
702	10	0.709	0.423	0.505	0.506	47.9
532	10	0.696	0.423	0.505	0.506	45.8
280	100	0.865	0.432	0.506	0.507	724.7
673	50	1.271	0.468	0.507	0.510	673.0
061	100	1.481	0.569	0.508	0.511	1527.9
941	100	1.958	0.651	0.511	0.514	2187.6
522	25	0.650	0.465	0.507	0.510	78.5
533	25	0.622	0.459	0.506	0.509	69.3
185	25	0.464	0.426	0.508	0.509	16.3
660	16.7	0.474	0.432	0.508	0.509	12.0
998	12.5	1.511	0.457	0.508	0.508	220.0
114	25	0.968	0.429	0.507	0.507	225.0
988	50	0.887	0.447	0.507	0.507	367.4
944	25	1.430	0.600	0.507	0.510	347.8

[1]DF = Dilution Factor; [2]: A_{520} = Absorbance at 520 nm; [3]: A_{700} = Absorbance at 700 nm.

Comments: Sample 061 arrived with the tube cracked and leaking slightly.

Sample 061 was not resent.

DATA REPORTING FORM FROM LABORATORY D

Data Reporting Form for **Test** Samples

Name: -

Date: 2/28/03

Sample code	DF[1]	A_{520} [2]		A_{700} [3]		Total monomeric anthocyanin (mg/L expressed as cyd-3-glu)
		pH 1.0	pH 4.5	pH 1.0	pH 4.5	
631	5	0.601	0.062	0.003	0.003	45.0
464	5	0.568	0.062	0.004	0.003	42.2
053	50	0.819	0.072	0.000	0.000	623.7
328	25	1.681	0.150	0.005	0.009	640.8
809	500	0.456	0.075	0.000	0.000	3181.1
085	250	0.880	0.143	0.005	0.009	3093.5
025	5	1.060	0.397	0.011	0.039	57.7
007	5	1.013	0.361	0.009	0.034	56.5
202	10	0.135	0.060	0.000	0.000	12.5
040	5	0.307	0.147	0.012	0.016	13.7
950	10	1.185	0.083	0.000	0.000	184.0
738	25	0.497	0.040	0.000	0.003	192.0
310	25	1.003	0.239	0.003	0.010	321.9
587	25	1.014	0.225	0.003	0.011	332.7

[1]DF = Dilution Factor; [2]: A_{520} = Absorbance at 520 nm; [3]: A_{700} = Absorbance at 700 nm.

Comments: Code 085 and 809 were done with the 2mm cell.

Collaborator later indicated that the sample 053 arrived crack. A new sample 053 was mailed and was re-analyzed. Results shown were corrected for this error.

DATA REPORTING FORM FROM LABORATORY E

Data Reporting Form for **Test** Samples

Name: -

Date: 1/30/03

Sample code	DF[1]	A_{520} [2]		A_{700} [3]		Total monomeric anthocyanin (mg/L expressed as cyd-3-glu)
		pH 1.0	pH 4.5	pH 1.0	pH 4.5	
924	5	0.599	0.060	0.005	0.003	44.8
215	5	0.578	0.057	0.001	0.002	43.6
420	50	0.850	0.072	0.003	0.006	652.1
300	50	0.815	0.074	0.004	0.007	621.2
192	500	0.451	0.076	0.006	0.008	3147.7
990	500	0.429	0.079	0.006	0.010	2955.7
567	10	0.553	0.169	0.006	0.012	65.1
62	10	0.541	0.178	0.006	0.018	62.6
306	5	0.300	0.162	0.013	0.031	13.0
035	5	0.294	0.139	0.014	0.016	13.1
648	25	0.541	0.032	0.005	0.003	211.7
483	10	1.244	0.076	0.005	0.006	195.2
259	25	1.038	0.211	0.006	0.007	345.7
523	25	0.978	0.201	0.004	0.007	325.6

[1]DF = Dilution Factor; [2]: A_{520} = Absorbance at 520 nm; [3]: A_{700} = Absorbance at 700 nm.

Comments: I did not have a 1 mLvolumetric pipette so for samples 300 and 420 I used a 2mL volumetric pipette and a 100mL volumetric flask. For samples 192 and 990 I diluted (volumetrically) as follows: 2mL into 100mL and then 5mL of this solution into 50mL.

DATA REPORTING FORM FROM LABORATORY F

Data Reporting Form for **Test** Samples

Name: -

Date: 3/20/03

Sample code	DF[1]	A_{520} [2]		A_{700} [3]		Total monomeric anthocyanin (mg/L expressed as cyd-3-glu)
		pH 1.0	pH 4.5	pH 1.0	pH 4.5	
461	6	0.498	0.046	0.002	0.007	45.8
474	6	0.470	0.039	0.001	0.002	43.3
106	30	1.416	0.111	0.002	0.009	657.3
425	30	1.369	0.107	0.004	0.008	634.2
097	250	0.659	0.109	0.006	0.009	2308.6
188	250	0.878	0.145	0.008	0.012	3076.8
438	7.5	0.722	0.210	0.007	0.010	64.5
304	10	0.536	0.154	0.006	0.010	64.5
129	5	0.292	0.137	0.011	0.014	13.2
612	5	0.286	0.133	0.013	0.013	12.8
343	15	0.886	0.052	0.001	0.004	209.7
471	15	0.850	0.051	0.002	0.004	200.6
845	30	0.866	0.164	0.002	0.005	353.2
729	30	0.813	0.164	0.005	0.005	325.1

[1]DF = Dilution Factor; [2]: A_{520} = Absorbance at 520 nm; [3]: A_{700} = Absorbance at 700 nm.

Comments: Sample 097 seemed fine at first, but when thawing we realized the tude was broken. About 10% of total volume was lost during the early stages of the thawing process.

A new sample 097 was mailed and was re-analyzed. Results shown were corrected for this error.

DATA REPORTING FORM FROM LABORATORY G

Data Reporting Form for **Test** Samples

Name: -

Date: 3/17/03

Sample code	DF[1]	A_{520} [2]		A_{700} [3]		Total monomeric anthocyanin (mg/L expressed as cyd-3-glu)
		pH 1.0	pH 4.5	pH 1.0	pH 4.5	
141	5	0.5777	0.0417	-0.0074	-0.0067	44.8
199	5	0.5664	0.0387	-0.0071	-0.0077	44.0
661	50	0.7706	0.0591	-0.0070	-0.0023	598.0
046	100	0.3758	0.0238	-0.0039	-0.0058	584.5
892	200	1.0085	0.0798	0.0001	0.0000	3101.5
588	500	0.4075	0.0606	-0.0050	-0.0008	2931.4
897	10	0.5444	0.2109	-0.0018	0.0306	61.1
365	10	0.5195	0.1967	-0.0023	0.0273	58.8
930	10	0.1460	0.0600	-0.0007	-0.0006	14.4
825	10	0.1418	0.0460	0.0005	-0.0024	15.5
062	10	1.1053	0.0635	-0.0045	-0.0050	173.9
080	10	1.0919	0.0618	-0.0054	-0.0050	172.1
838	50	0.5106	0.0843	-0.0075	-0.0031	359.7
770	50	0.4650	0.0827	-0.0056	-0.0110	314.7

[1]DF = Dilution Factor; [2]: A_{520} = Absorbance at 520 nm; [3]: A_{700} = Absorbance at 700 nm.

Comments:

Sample 062 was resent to this collaborator.

DATA REPORTING FORM FROM LABORATORY H

Data Reporting Form for **Test** Samples

Name: -

Date: 1/21/03

Sample code	DF[1]	A_{520} [2]		A_{700} [3]		Total monomeric anthocyanin (mg/L expressed as cyd-3-glu)
		pH 1.0	pH 4.5	pH 1.0	pH 4.5	
120	5	0.592	0.062	0.001	0.001	44.3
919	5	0.563	0.065	0.004	0.008	41.9
699	50	0.860	0.087	0.006	0.010	648.8
329	50	0.830	0.080	0.003	0.006	628.7
475	250	0.975	0.200	0.012	0.015	3247.9
692	250	0.942	0.201	0.022	0.028	3118.5
100	10	0.542	0.187	0.010	0.018	60.6
160	10	0.516	0.176	0.008	0.015	57.9
196	10	0.159	0.080	0.009	0.010	13.4
931	10	0.157	0.080	0.011	0.011	12.9
521	10	1.284	0.090	0.002	0.003	199.6
281	10	1.287	0.090	0.005	0.007	200.2
929	50	0.509	0.119	0.005	0.006	326.5
058	50	0.491	0.108	0.001	0.004	322.3

[1]DF = Dilution Factor; [2]: A_{520} = Absorbance at 520 nm; [3]: A_{700} = Absorbance at 700 nm.

Comments:

DATA REPORTING FORM FROM LABORATORY I

Data Reporting Form for **Test** Samples

Name: -

Date: 3/20/03

Sample code	DF[1]	A_{520} [2]		A_{700} [3]		Total monomeric anthocyanin (mg/L expressed as cyd-3-glu)
		pH 1.0	pH 4.5	pH 1.0	pH 4.5	
980	6.25	0.475	0.045	0.005	0.002	44.6
361	6.25	0.455	0.042	0.003	0.000	42.8
357	50	0.844	0.085	0.005	0.013	641.2
152	50	0.819	0.075	0.004	0.008	625.1
808	250	0.864	0.168	0.011	0.018	2941.5
139	250	0.812	0.152	0.004	0.010	2782.5
857	6.25	0.805	0.347	0.010	0.050	51.9
308	25	0.210	0.081	0.006	0.012	56.7
443	7.14	0.205	0.102	0.007	0.009	12.4
500	6.25	0.218	0.111	0.009	0.012	11.4
730	12.5	1.042	0.068	0.003	0.000	202.7
868	25	0.520	0.041	0.004	0.006	200.7
946	50	0.479	0.117	0.000	0.007	308.1
288	50	0.471	0.115	0.004	0.011	302.3

[1]DF = Dilution Factor; [2]: A_{520} = Absorbance at 520 nm; [3]: A_{700} = Absorbance at 700 nm.

Comments: Several samples produced spectra were the maximum was not at 520 nm.

DATA REPORTING FORM FROM LABORATORY J

Data Reporting Form for **Test** Samples

Name: -

Date: 2/26/03

Sample code	DF[1]	A_{520} [2]		A_{700} [3]		Total monomeric anthocyanin (mg/L expressed as cyd-3-glu)
		pH 1.0	pH 4.5	pH 1.0	pH 4.5	
547	10	0.304	0.026	0.001	0.001	46.4
866	10	0.292	0.020	0.001	0.001	45.4
672	50	0.793	0.078	0.003	0.007	600.3
407	50	0.744	0.072	0.003	0.006	563.6
676	200	1.091	0.192	0.008	0.013	3019.2
771	200	1.031	0.182	0.009	0.013	2848.8
113	10	0.570	0.191	0.005	0.020	65.8
826	10	0.543	0.179	0.007	0.016	62.3
906	5	0.302	0.142	0.013	0.014	13.4
949	10	0.146	0.065	0.006	0.005	13.4
606	10	1.169	0.074	0.004	0.004	182.9
434	10	1.155	0.075	0.004	0.005	180.5
686	25	0.999	0.207	0.003	0.005	331.5
552	25	0.976	0.199	0.003	0.006	325.6

[1]DF = Dilution Factor; [2]: A_{520} = Absorbance at 520 nm; [3]: A_{700} = Absorbance at 700 nm.

Comments:

DATA REPORTING FORM FROM LABORATORY K

Data Reporting Form for **Test** Samples

Name: -

Date: 2/27/03

Sample code	DF[1]	A_{520} [2]		A_{700} [3]		Total monomeric anthocyanin (mg/L expressed as cyd-3-glu)
		pH 1.0	pH 4.5	pH 1.0	pH 4.5	
278	10	0.310	0.027	0.001	0.001	47.3
764	10	0.288	0.028	0.000	0.004	44.1
065	50	0.833	0.072	0.001	0.004	637.9
009	50	0.817	0.071	0.003	0.003	622.9
537	100	1.949	0.391	0.015	0.023	2615.0
424	100	1.862	0.374	0.015	0.022	2496.5
728	50	0.120	0.010	0.001	0.002	92.7
524	25	0.225	0.067	0.001	0.003	66.8
709	25	0.061	0.026	0.000	0.001	15.0
734	25	0.063	0.028	0.002	0.004	15.4
767	50	0.273	0.018	0.001	0.002	213.7
588	25	0.512	0.035	0.001	0.005	200.8
602	100	0.271	0.063	0.000	0.002	350.7
889	100	0.243	0.052	0.000	0.002	322.3

[1]DF = Dilution Factor; [2]: A_{520} = Absorbance at 520 nm; [3]: A_{700} = Absorbance at 700 nm.

Comments: The smallest class A volumetric pipette available in our lab was 0.5mL, thus 100 was the maximum dilution facto attainable using a 50mL volumetric flask. Since you requested that collaborators follow the protocol exactly, I did not use a larger flask or perform a serial dilution. For this reason, the absorbance values at 520 nm in pH 1 buffer are outside your desired range for samples 537 and 424. Allow the use of 100mL volumetric flasks or serial dilution for the dark juices.

Appropriate changes made to the method.

APPENDIX D
OVERVIEW OF AOAC OFFICIAL METHODS PROGRAM